ORIGINAL RESEARCH

IL-25 dampens the growth of human germinal center-derived B-cell non Hodgkin Lymphoma by curtailing neoangiogenesis

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Interleukin (IL)-25, a member of the IL-17 cytokine superfamily, is produced by immune and non-immune cells and exerts type 2 pro-inflammatory effects in vitro and in vivo. The IL-25 receptor(R) is composed of the IL-17RA/IL-17RB subunits. Previous work showed that germinal centre (GC)-derived B-cell non Hodgkin lymphomas (B-NHL) expressed IL-17AR, formed by IL-17RA and IL-17RC subunits, and IL-17A/IL-17AR axis promoted B-NHL growth by stimulating neoangiogenesis. Here, we have investigated expression and function of IL-25/IL-25R axis in lymph nodes from human GC-derived B-NHL, i.e. Follicular Lymphoma (FL, 10 cases), Diffuse Large B Cell Lymphoma (6 cases) and Burkitt Lymphoma (3 cases). Tumor cells expressed IL-25R and IL-25 that was detected also in non-malignant cells by flow cytometry. Immunohistochemical studies confirmed expression of IL-25R and IL-25 in FL cells, and highlighted IL-25 expression in bystander elements of the FL microenvironment. IL-25 i) up-regulated phosphorylation of NFκBp65, STAT-1 and JNK in B-NHL cells; ii) inhibited in vitro proliferation of the latter cells; iii) exerted anti-tumor activity in two in vivo B-NHL models by dampening expression of pro-angiogenic molecules as VEGF-C, CXCL6 and ANGPT3.

In conclusion, IL-25, that is intrinsically pro-angiogenic, inhibits B-NHL growth by reprogramming the angiogenic phenotype of B-NHL cells.

Introduction

The lymphoma microenvironment plays a critical role in tumor initiation, progression, immune escape, and chemoresistance.1-4 In this vein, our group has recently demonstrated a role of IL-17A, the most renowned member of the IL-17 family, in B cell non-Hodgkin lymphoma (B-NHL) progression.5 IL-17A is produced by T helper-17 CD4+ T cells and plays pivotal roles in protection from bacterial and fungal infections and development of autoimmune diseases.6-8 IL-25, also known as IL-17E, diverges from the other members of the IL-17 cytokine family since it promotes Th2 immune responses.8-12 Thus, overexpression of IL-25 resulted in increased production of IL-4, IL-5, IL-9 and IL-13,13 increased antibody class switching to IgG1 and IgE, and recruitment of eosinophils, basophil, mast cells, along with CD4+Th2 cells.13,14 IL-25 is produced by immune cells, such as CD4+ T cells, macrophages, dendritic cells, mast cells, eosinophils, and non-immune cells, such as epithelial and endothelial cells.15 In murine models, IL-25 deficiency results in defective Th2 response and higher susceptibility to infections.16,17 IL-25 binds the heterodimeric receptor composed of IL-17RA and IL-17RB subunits.15 IL-17RA is expressed ubiquitously, with high levels in the hematopoietic cell compartment.7,18 IL-17RB is highly expressed in kidney, liver and brain.19 Both IL-17RA and IL-17RB are involved in IL-25 driven signal transduction.20 IL-25 is highly expressed in lungs of patients with idiopathic pulmonary fibrosis and induces collagen production by human fibroblasts.21 In a mouse model of allergen-induced airway response, blockade of IL-25 abolished airway remodelling.22 Intranasal challenge with IL-25 increased peribronchial endothelial vessels in the airways of mice with chronic asthma and strongly upregulated expression of various pro-angiogenic molecules.23 Endothelial cells express IL-25R,24 and IL-25 induced angiogenesis in vitro by increasing basic Fibroblast Growth Factor (bFGF) mRNA and protein expression in human vascular endothelial cells through IL-17RB signalling.25 IL-25 produced by synoviocytes antagonized the pro-inflammatory effects of IL-17A by acting as receptor antagonist.26 Conflicting results have been published on the role played by IL-25 in tumor growth. Thus, in breast cancer, IL-25 produced

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by normal epithelial cells was shown to bind IL-25R expressed on adjacent neoplastic cells and to induce selective apoptosis of the latter cells. Moreover, IL-25 treatment reduced tumor growth in xenograft models of melanoma, breast, lung, colon and pancreatic cancers. In contrast, IL-25 had a pro-oncogenic role in breast cancer and hepatocarcinoma, stimulating proliferation and survival of malignant cells, promoting metastasis, and contributing to drug resistance.

In this study, we have investigated expression and function of IL-25 in the tumor microenvironment of different GC-derived B-NHL, i.e. Follicular Lymphoma (FL), Diffuse Large B-cell Lymphoma (DLBCL) and Burkitt Lymphoma (BL). FL presents a follicular growth pattern that is partially retained for a long time together with most of the microarchitectural characteristics of normal GCs. In contrast, BL and DLBCL are high-grade malignancies showing diffuse effacement of the lymphoid tissue architecture and displaying aggressive behavior and unfavorable outcome. Both DLBCL and FL occur commonly in adults and rarely in children or adolescents. DLBCL is the most frequent B-NHL subtype, with approximately one third of cases originating from the transformation of FL. BL affects predominantly children or young adults, with frequent intra-abdominal or extranodal involvement.

Here, we show that B-NHL cells expressed IL-25R and IL-25, and that the latter was expressed also in the non-malignant components of the tumor microenvironment. Moreover, IL-25 was found to exert anti-tumor activity in two different in vivo models of B-NHL lymphomas of GC origin, mainly through potent inhibition of neo-angiogenesis and consequent induction of ischemic necrosis.

Results

Expression of IL-25R/IL-25 in GC-derived B-NHL cells

We investigated by flow cytometry the expression of the heterodimeric IL-25R (composed of IL-17RA and IL-17RB chains) on tumor cells from lymph node biopsies of patients with FL (n = 10), DLBCL (n = 6) and BL (n = 3). Malignant cells were detected and gated according to the expression of monoclonal Ig κ or λ light chains. We showed IL-17RB expression and confirmed IL-17RA expression in B-NHL cells (Fig. 1A) (Mean of Mean Relative Fluorescence Intensity (MRFI) ± SD for IL-17RA: FL = 3.1 ± 1.4; DLBCL = 2.4 ± 1.2 and BL = 2.4 ± 0.8; MRFI ± SD for IL-17RB: FL = 3.4 ± 1.5; DLBCL = 2.9 ± 0.9 and BL = 1.8 ± 0.4). Accordingly, immunohistochemical analysis of five FL lymph nodes documented the expression of IL-17RB in neoplastic cells (Fig. 1B).

Next, we asked whether IL-25 was expressed in the B-NHL microenvironment. Cell suspensions from 5 FL, 3 DLBCL and 2 BL lymph node biopsies were stained with anti-Ig κ and anti λ mAbs on the cell surface and anti-IL-25 mAb in the

Figure 1. Expression of IL-25R and IL-25 in primary tumor cells from patients with FL, DLBCL or BL. (A) MNCs from B-NHL lymph nodes were double stained with anti-Ig κ or anti λ mAbs in combination with anti IL-17RA or anti IL-17RB mAbs, and analyzed by flow cytometry gating on the cell fraction expressing monoclonal κ or λ chains. Results for 10 FL, 6 DLBCL and 3 BL cases are shown in histograms, as mean % positive cells ± SD. (B) Immunohistochemical analysis of IL-17RB in representative lymph nodes from patients with FL (X200 left panel and X400 right panel). IL-17RB is expressed in the neoplastic GC of FL. (C) Immunohistochemical analysis of IL-25 in representative lymph nodes from patients with FL. IL-25 expression is diffusely distributed among FL cells and associated microenvironment components (X200 left panel and X400 right panel). (D) Cell suspensions from B-NHL lymph nodes were surface stained with anti-Ig κ and anti λ mAbs, permeabilized, stained intracellularly with anti-IL-25 mAb and analyzed by flow cytometry gating on the cell fraction expressing monoclonal κ or λ chains. Results are means % positive cells from 5 FL, 3 DLBCL and 2 BL cases pooled together ± SD.
cytoplasm, and analyzed by flow cytometry gating on cells expressing monoclonal $\kappa$ or $\lambda$ chains. Fig. 1C shows the results of all B-NHL cases pooled together. B-NHL cells (clonal Ig light chain positive) were the major source of IL-25 that was also detected in non-malignant cells (clonal Ig light chain negative) (Fig. 1C). Immunohistochemical analysis of five FL lymph nodes revealed diffuse expression of IL-25 both in neoplastic cells and bystander elements of the neoplastic GC microenvironment (Fig. 1D).

**Functional Activity of IL-25 on B-NHL cells**

IL-25 signalling involves the canonical NF-kB pathway and the MAPK or JNK pathways.\(^{35}\) Signaling initiated by binding of hrIL-25 to IL-25R was investigated by flow cytometry in primary FL and DLBCL (assessing 3 cases each). Isolated tumor cells incubated from 0 to 60 minutes with 50ng/ml hrIL-25 up-regulated significantly phosphorylated (p)NF-kBp65 (with a peak at 5 minute) (Fig. 2A, left panel), but not unphosphorylated NF-kBp65 (similar levels of 91 ± 5% expression in treated and untreated cells). Furthermore, IL-25 enhanced pSTAT1 and pJNK, with peaks at 30 and 5 minutes, respectively (Fig. 2A, middle and right panels, respectively), while leaving unphosphorylated STAT-1 and JNK unaltered (similar levels of 94 ± 5% and 96 ± 2% expression, respectively, in treated and untreated cells).

Next, tumor cells purified from 5 FL and 3 DLBCL lymph nodes were incubated with 50 ng/ml hrIL-25 and tested for proliferation. IL-25 significantly dampened $^3$H-thymidine incorporation in tumor cells, whose proliferation was increased by hrCD40L tested as control (Fig. 2B).\(^{36}\) These results were confirmed by intracellular flow cytometric analysis of PCNA (Fig. 2C). The proportions of apoptotic cells, as assessed by AnnexinV/PI staining, were similar in IL-25 treated and

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**Figure 2.** IL-25 signaling and function in B-NHL cells. (A) Flow cytometric analysis of pNFkBp65, pSTAT1 and pJNK in purified lymphoma cells (3 FL and 3 DLBCL) treated for 0, 5 or 30 min with hrIL-25 (50 ng/ml). Results are shown as median MRFI, maximum, minimum and first and third quartile. (p = 0.0089 for pNF-kBp65, p = 0.0071 for pSTAT-1 at five minutes of treatment and p = 0.0024 for pJNK at 30 minutes of treatment). (B-C) Primary neoplastic cells from 5 FL and 3 DLBCL patients were cultured without (Med) or with hrIL-25 or hrCD40L and tested for proliferation by $^3$H-ThdR incorporation (B) or by intracellular staining for PCNA (C). Results are expressed in box plot, as median cpm (B) or % positive cells (C), maximum, minimum, first and third quartile. (D) CAM assay. Gelatin sponges were adsorbed with: vehicle alone-PBS (left panel), hrIL-25 (middle panel) or VEGF-A (right panel). Original magnification: x 50.
untreated lymphoma cells (Supplemental Fig. 1). Finally, IL-25 did not attract lymphoma cells (Supplemental Fig. 1) nor enhanced their migration to CXCL12 or CXCL13 (data not shown), as shown for normal GC B cells.37

We next performed CAM experiments to test the direct angiogenic activity of hrIL-25. At day 12, hrIL-25 induced a significant angiogenic response in the form of numerous allantoic neovessels developing radially towards the implant in a 'spoked-wheel' pattern (mean number of vessels = 24+/−4 for hrIL-25) (Fig. 2D, middle panel). The pro-angiogenic activity of hrIL-25 was comparable to that of Vascular Endothelial Growth Factor (VEGF)-A tested as positive control (Fig. 3B, panel g), as assessed by Ki-67 staining, compared with tumors from PBS treated mice (Table 1, andFig. 3B). Likewise, a scanty reactive cell infiltrate, mainly composed of monocytes/macrophages, was detected in tumors from both cohorts of mice (not shown). Decreased tumor cell proliferation in mice treated with IL-25 was confirmed flow cytometric analysis of Ki67 expression ex-vivo (Fig. 3C).

The in vivo anti-angiogenic activity of IL-25, that has a physiological pro-angiogenic effect,59 prompted further experiments. Small fragments of tumor masses explanted from hrIL-25 or PBS treated mice were tested in the CAM assay. CAMs treated with tumor xenografts from the latter group of mice showed numerous allantoic vessels (mean number of vessels = 25+/−7) radiating in a "spoked wheel" pattern toward the sponges, whereas CAMs treated with tumor xenografts from IL-25-treated mice formed a significantly lower number of vessels (mean number of vessels = 17+/−4, p<0.001 vs tumor xenograft alone) (Fig. 4A).

We next investigated by PCR array the expression of a panel of 84 genes involved in angiogenesis in four tumors from hrIL-25 treated mice and 4 tumors from control mice. Treatment with IL-25 downregulated the expression of different angiogenesis-related genes, i.e. VEGF-C, VEGF-A, angiopoietin like (ANGPTL)-3, alanylaminopeptidase (ANPEP), chemokine C-X-C motif ligand (CXCL)10 and 6, ephrin-B2 (EFNB2), IFN-γ, IL-6, Neurilpin-2 (NPR2), transforming growth factor (TGF)β1, TGFβR1 and TNF-α induced protein (TNFAIP)2 (Fig. 4B). Genes downregulated by IL-25 in vivo included the anti-angiogenic IFN-γ38,39 and CXCL10 (an IFN-γ inducible chemokine)40, perhaps in an effort of the tumor to counteract the massive dampening of pro-angiogenic genes operated by IL-25.

Immunohistochemical analyses of tumor masses from hrIL-25 vs PBS treated mice confirmed the down-regulation of VEGF-C, CXCL6 and ANGPT3 at protein level (Fig. 4C and Table 1). TNFα-IP2 protein was barely expressed in tumors from PBS treated mice, thus making it difficult to assess its down-regulation in tumors from hrIL-25 treated mice (not shown).

In summary, inhibition of angiogenesis, a pivotal component of the in vivo anti-tumor activity of IL-25, was driven by the ability of the cytokine to downregulate the expression of different pro-angiogenic molecules in B-NHL cells.

**Discussion**

FL, DLBCL and BL originate from the malignant transformation of GC-B cells. The definitive cure for these malignancies is still to be achieved, and a deeper insight into the microenvironment of GC-derived B-NHL may help develop new therapeutic strategies.

We have demonstrated that normal and neoplastic GC B cells expressed IL-17AR, composed of the IL-17RA and IL-17RC chains.5 IL-17AR signaled in both cell types by inducing phosphorylation of NF-kBp65. IL-17A expressed by T cells and mast cells in B-NHL microenvironment promoted growth of lymphoma cells in vitro and in vivo through stimulation of tumor cell proliferation and neoangiogenesis. Thus, the IL-17A/IL-17AR axis may represent a potential therapeutic target in B-NHL.5
Normal GC B cells isolated from tonsil expressed IL-17RB, that heterodimerizes with IL-17RA to form the IL-25R. Both IL-17A and IL-25 induced de novo chemotaxis of GC B cells to CXCL12 and CXCL13, two crucial chemokines for B cell trafficking in the GC, by down-regulating RGS-16 protein expression and NFkBp65 phosphorylation. IL-25 is a Th2-type cytokine endowed with pro-angiogenic activity related to induction of pro-angiogenic molecules as bFGF and VEGF in human endothelial cells and fibroblasts. Furthermore, we show that hrIL-25 exerts a direct pro-angiogenic activity in the CAM assay. Although these features would be predictive of a tumor-promoting role of IL-25, the literature on this topic is controversial.

B-NHL cells expressed IL-25R while IL-25 was abundantly expressed by the tumor cell themselves and, at a lower extent, non-neoplastic cells infiltrating the B-NHL microenvironment. IL-25 signaled in B-NHL cells through multiple pathways, as witnessed by the upregulation of pNFkB-p65, pSTAT-1 and pJNK, and inhibited the in vitro proliferation of tumor cells. We next investigated the in vivo effects of IL-25 on the growth of human SU-DHL-4 and Oci-Ly8 DLBCL cells in immunodeficient SCID/NOD mice. Tumors formed in mice treated with hrIL-25 vs PBS were significantly smaller and displayed necrotic-hemorrhagic areas associated with defective microvascular supply and reduced neoplastic cell proliferation. Expression of different pro-angiogenic genes was found to be down-modulated by IL-25 treatment. These genes included: VEGF-C, that promotes angiogenesis and lymphangiogenesis by stimulating endothelial cell growth and migration; VEGF-A, a primary molecule driving expansion of the tumor vascular bed; ANGPTL-3, a member of the angiopoietin-like family of secreted factors expressed predominantly in the liver during development; ANPEP, responsible for post-secretory processing, involved in intracellular cell signaling and invasion/metastasis of various malignancies, and endowed with a relevant role in neoangiogenesis; CXCL6, a pro-angiogenic gene induced in microvascular endothelial cells after stimulation with pro-inflammatory stimuli; IL-6, a gene coding for...
a cytokine with potent pro-angiogenic activity, in part through VEGF induction, and TNFAIP2, a TNFα-regulated gene expressed in endothelial cells and peripheral blood monocytes that stimulates endothelial capillary tube formation in vitro.

Immunohistochemical staining of tumors explanted from IL-25 vs PBS treated mice confirmed at protein level the strong down regulation of some of the genes enlisted above, the most prominent being VEGF-C. Moreover, tumors explanted from IL-25-treated mice displayed a strongly reduced pro-angiogenic activity in the CAM assay compared to tumors from PBS-treated mice, reinforcing the evidence for the in vivo anti-angiogenic effects of IL-25.

IL-25 inhibited in vivo tumor cell proliferation, consistently with the results obtained in vitro with B-NHL cells isolated from invaded lymph nodes. The possibility that part of the anti-proliferative effect of IL-25 in vivo was secondary to the anti-angiogenic activity of the cytokine cannot be excluded.

These results demonstrate for the first time the ability of IL-25 to inhibit neoangiogenesis in two different xenograft models of B-NHL by reprogramming the angiogenic phenotype of these malignancies. The finding that the intrinsic pro-angiogenic activity of IL-25 was overwhelmed by the effects of the cytokine on B-NHL cells may appear surprising since IL-25 was mainly produced by malignant cells that also expressed IL-25R. Thus the question arises why should B-NHL cells be involved in an autocrine/paracrine loop that dampens their growth. A few considerations deserve mention. First, the possibility that human IL-25 did not stimulate efficiently mouse angiogenesis is disproved by the evidence that human and mouse IL-25 display species cross-reactivity. Second, the xenograft models tested in this study highlight the direct effects of IL-25 on B-NHL cells, but do not allow to investigate the activity of the cytokine on other cell types present in the tumor microenvironment. Third, tumor cells can upregulate the expression of some anti-angiogenic genes in the course of a prominent pro-angiogenic response. For example, we have previously demonstrated that, in a xenograft model of human neuroblastoma, resistance to anti-angiogenic immunotherapy was characterized by an impressive wave of neo-angiogenesis with thousands fold upregulated expression of numerous pro-angiogenic and few anti-angiogenic genes, such as CXCL9 and CXCL10. This latter phenomenon, that is not easily explained, may represent an attempt of cancer cells to modulate their exuberant pro-angiogenic activity. In conclusion, further studies are needed to gain more insight into the functional impact of IL-25 on tumor neoangiogenesis in the frame of the effects of the cytokine on non-malignant cells present in the B-NHL microenvironment.

Table 1. Immunohistochemical assessment of microvessels, apoptotic cells and pro-angiogenic molecules in tumors developed after SU-DHL-4 cell injection in PBS or hrIL-25 treated mice.

|                      | PBS   | rhIL-25 |
|----------------------|-------|---------|
| **Microvessels**     | 9.6 ± 2.8 | 3.5 ± 1.7* |
| **Proliferation index %** | 87.5 ± 6.8 | 67.4 ± 8.5* |
| **Apoptotic index %** | 14.5 ± 4.2 | 15.8 ± 5.0 |
| **VEGF-C**           | 85.3 ± 8.3 | 20.7 ± 6.1* |
| **CXCL6**            | 74.4 ± 7.2 | 13.2 ± 4.6* |
| **ANGPT3**           | 78.3 ± 9.0 | 26.5 ± 5.5* |

The count of apoptotic and proliferating cells and microvessels was performed as reported in Methods. Eight fields were examined for each tumour section and three sections per tumour (three rhIL-25 treated versus three PBS treated) were evaluated. Results are expressed as mean ± SD of TUNEL or Ki-67 positive cells/number of total cells (X600) or, CD31 positive vessels per field (X400) evaluated on formalin-fixed sections by immunohistochemistry.

*Values significantly different (p < 0.05) from corresponding values in tumors developed in PBS treated mice.

1Pro-angiogenic molecule expression values were represented as the mean percentage of positively stained areas/total area of the examined field (85431.59 μm²).

2p < 0.05, Mann-Whitney U test from values in tumors from PBS-treated mice.

Figure 4. Role of IL-25 on in vivo tumor angiogenesis. (A) CAM assay performed ex vivo with tumor masses explanted from PBS (left panel) or hrIL-25 (right panel) treated mice. (B) Gene expression profiling of human angiogenesis related genes in SU-DHL-4 tumors explanted from SCID/NOD mice as assessed by PCR Array. Results represent fold differences in individual mRNA expression between PBS or hrIL-25 treated mice. Pooled results from 4 different experiments are shown. (C) Immunohistochemical analysis of the expression of selected pro-angiogenic molecules in SU-DHL-4 tumors from PBS or hrIL-25 treated mice. In comparison with tumors developed in PBS treated mice (a-c), the small tumor masses from hrIL-25 treated mice showed very low expression of VEGF-C (d), CXCL6 (e) and ANGPT3 (f).
Material and methods

Patients, cell isolation and cell lines

The present study was approved by the Institutional Review Board of Istituto Gianna Gaslini, Genova, Italy on October 27, 2005. Nineteen infiltrated lymph nodes from patients with FL (n = 10, 6 males and 4 females, age range: 43–68), DLBCL (n = 6, 3 males and 3 females, age range: 56–69) and BL (n = 3, 2 males and 1 female, age range: 6–16) were obtained after informed consent in accordance with the Declaration of Helsinki. Diagnosis of FL, DLBCL (GC-type), and BL was established according to the criteria of the Revised European-American Classification of Lymphoid Neoplasms.54 From now onwards DLBCL (GC-type) will be referred to as DLBCL. All patients were studied at diagnosis and were untreated.

Lymph node mononuclear cells (MNCs) were isolated with standard procedure,54 and cryopreserved in a freezing solution composed of 50% RPMI 1640 (Sigma Chemical Co., St. Louis, MO), 40% fetal bovine serum (FBS) (Sigma), and 10% DMSO (Sigma). Cells were kept in liquid nitrogen until tested. Then, neoplastic B-NHL cells, expressing either κ or λ immunoglobulin (Ig) light chains, were enriched (>95%) by immunomagnetic positive selection for the clonotypic light chain, as reported.5

The human SU-DHL-4 (DLBCL origin),55 DoHH2 (FL origin),56 Raji and Ramos (BL origin),57 and Oci-Ly8 (considered of DLCBL origin) cell lines were provided 6 months ago by Interlab Cell Line Collection (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy), that certifies their origin by multiplex short tandem repeat profiling. These cell lines were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS (Sigma).

Antibodies and flow cytometry

The following antibodies were used for flow cytometry experiments: Phycoerythrin (PE)-conjugated anti IL-17RA, PE anti IL-17RB (R&D System Inc.); PE or Fluorescein Isothiocyanate (FITC)-conjugated anti-κ and anti-λ Ig light chains (R&D System); FITC-anti human Ki67 (DAKO), anti-PCNA, anti-IL-25 (R&D System Inc). Cells were stained with fluorochrome conjugated antibodies or with isotype and fluorochrome matched control antibodies. For intracellular experiments cells were fixed and permeabilized using cytofix/cytoperm kit (Becton-Dickinson), in accordance to manufacturer’s instructions. Cells were run on a Gallios Instrument (Beckman Coulter) and data were analyzed using the Kaluza software (Beckman Coulter). Data were expressed as percentage of positive cells.

Immunohistochemistry on human tissue samples

For immunohistochemical analysis tissue samples from 5 lymph nodes involved by FL were collected from the archives of the Human Pathology Section, University of Palermo. IL-25 and IL-17RB protein expression was evaluated using the primary polyclonal antibody goat anti-human IL-25 (Millipore, Massachusetts, USA) and the primary polyclonal antibody rabbit anti-human IL-17RB (Sigma). The formalin-fixed and paraffin embedded tissue sections of four-micrometer-thick were deparaffinized and rehydrated to water. High temperature antigen unmasking was performed using Tris-HCl/EDTA pH 9.0 or pH6 citrate buffer (Novocastra, Newcastle, UK) in a PT Link pre-treatment module (Dako, Denmark) at 98°C for 30 minutes.

Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase activity using 3% H2O2 for 10 min, the sections were incubated with protein block (Novocastra) for 10 minutes. The slides were then incubated overnight with the primary polyclonal antibody goat anti-human IL-25 (dilution 1:50) and primary polyclonal antibody rabbit anti-Human IL-17RB (dilution 1:20) at the temperature of 4°C. Staining was revealed by LSAB kit and DAB (3,3’-Diaminobenzidine, Novocastra) substrate-chromogen. After counterstaining with Harris hematoxylin (Novocastra), all the sections were analyzed under an AXIO Scope A1 optical microscope (ZEISS, Germany) and microphotographs were collected through an Axioacam 503 Color digital camera (Zeiss) using the Zen2 software.

Cell signaling

The phosphorylation of NF-κBp65 (Ser536), STAT-1 and JNK (Cell Signaling Technology, Danvers, MA USA) was investigated by flow cytometry as reported.5 Briefly, cells were treated for 0–1.5–10–30–60 min with or without (medium) 50ng/ml rhIL-125 (R and D System, Minneapolis, USA) and stained with an Alexa 488-conjugated anti-phosphorylated and unphosphorylated NF-κBp65, STAT-1 or JNK mAbs (Cell Signaling Technology), according to the instructions of the manufacturer. Cells were washed and run on Gallios cytometer. Data were analyzed using the Kaluza software.

Cell proliferation, apoptosis and chemotaxis

Lymphoma B cells were cultured in RPMI for 24–72 h with or without different concentrations of rhIL-25 (50ng/ml) and tested for proliferation. Tumor cell proliferation was investigated: i) by intracellular flow cytometric detection of PCNA; ii) by overnight pulse with tritiated thymidine (3H-TdR) of cells. 100 ng/ml rhCD40L (Immunotools, Friesoythe, Germany) was tested as positive control. The results of the latter assay were obtained by comparing cpm of cytokine-treated vs cpm of untreated cells. Apoptosis was assessed by the rhAnnexin V/FITC kit (Bender Medsystems) and analyzed by flow cytometry. Apoptotic cells were identified as Annexin V+ cells. Chemotaxis was investigated using 5 μm pore-size transwell plates (Costar) as reported.58 Five × 105 normal or neoplastic cells were dispensed in the upper chamber, and increasing concentrations of rhIL-25 (100–300–600ng/ml) or medium were added to the lower chamber.58 Plates were incubated 2 h at 37°C. Migrated cells were collected and counted.

In vivo studies

All procedures involving animals were performed in accordance with national and international current regulations (D.l.vo 27/01/1992, n.116, European Economic Community
Morphologic and immunohistochemical analyses ex-vivo

Morphological and immunohistochemical analyses were performed on tumors explanted from mice, as described. Briefly, formalin-fixed, paraffin-embedded sections were incubated for 30 min with anti-Mac-1 (Abcam, Cambridge, UK), Gr-1 (Ly-6G/Ly-6C, clone RB6/8C5, BioLegend, San Diego, CA, anti-TNFα (clone 55B7, R&D Systems, Minneapolis, MN, USA), anti-VEGF-A (R & Systems, Abington, UK) as positive control; 1 μl of PBS containing hrIL-25. For experiments with explanted tumor masses, on day 8 of incubation, the cover-slips were removed and the growing CAMs (10 eggs per group) were treated with either 1–2-mm³ fragments of tumor xenografts alone or with rhIL-25. The coverslips were replaced after these treatments, and the CAMs were examined daily until day 12 of incubation, when they were photographed in ovo using a stereomicroscope equipped with a camera and image analysis system (Olympus Italia, Milano, Italy). Blood vessels entering the sponges within the focal plane of the CAM were counted by two observers in a doubleblind fashion at a magnification of 50x.

Statistical analysis

For CAM assays, means ± Standard Deviation (SD) were evaluated for all the parameters and the statistical significance of the differences between the counts of vessels number was determined by Student’s t-test for unpaired data. Differences in tumor volume, proliferation and apoptotic index, pro-angiogenic molecule expression and cell signaling were calculated using Mann-Whitney test comparing two independent samples, with 99% confidence interval (GraphPad Prism 3). All statistical tests were two tailed. A P value lower than 0.05 was considered statistically significant.

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Abbreviations

B-NHL  B cell non-Hodgkin lymphomas
BL  Burkitt lymphoma
DLBCL  diffuse large B-cell lymphoma
FL  follicular lymphoma
GC germinal center
IL Interleukin
MRFI Mean Relative Fluorescence Intensity
P phosphorylated
R receptor

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