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Interleukin-17A promotes the growth of human germinal center derived non-Hodgkin B cell lymphoma

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Keywords: angiogenesis, B non-Hodgkin lymphoma, GC B cells, IL-17A, IL-17A receptor, tumor immunology

Abbreviations: Abs, antibodies; B-NHL, B cell non-Hodgkin lymphomas; BL, Burkitt lymphoma; CB, centroblasts; CC, centrocytes; CAM, Chorioallantoic membrane; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; FITC, Fluorescein 15 Isothiocyanate; GC, germinal center; HPSE, Heparan sulfate proteoglycan; ID1, inhibitor of differentiation/DNA binding 1; IGF-1, Insulin-like growth factor 1; IL, Interleukin; LAMA5, Laminin-a5; LECT1, leukocyte cell derived chemotaxin; MRFI, Mean Relative Fluorescence Intensity; m, monoclonal; NOD, Non Obese Diabetic; p, phosphorylated; PE, Phycoerythrin; PGE, prostaglandin; R, receptor; rh, recombinant human; SCID, Severe combined immunodeficiency; TGFb-R1, transforming growth factor b receptor 1; VEGF, vascular growth factor.

Interleukin (IL)-17A belongs to IL-17 superfamily and binds the heterodimeric IL-17 receptor (R)(IL-17RA/IL-17RC). IL-17A promotes germinal center (GC) formation in mouse models of autoimmune or infectious diseases, but the role of IL-17A/IL-17AR complex in human neoplastic GC is unknown. In this study, we investigated expression and function of IL-17A/IL-17AR in the microenvironments of 44 B cell non-Hodgkin lymphomas (B-NHL) of GC origin (15 follicular lymphomas, 17 diffuse large B cells lymphomas and 12 Burkitt lymphomas) and 12 human tonsil GC. Furthermore, we investigated the role of IL-17A in two *in vivo* models of GC B cell lymphoma, generated by s.c. injection of SU-DHL-4 and OCI-Ly8 cell lines in Severe combined immunodeficiency (SCID)/Non Obese Diabetic (NOD) mice. We found that: (i) B-NHL cell fractions and tonsil GC B cells expressed IL-17RA/IL-17RC, (ii) IL-17A signaled in both cell types through NF-kBp65, but not p38, ERK-1/2, Akt or NF-kBp50/105, phosphorylation, (iii) IL-17A was expressed in T cells and mast cells from neoplastic and normal GC microenvironments, (iv) IL-17A rendered tonsil GC B cells competent to migrate to CXCL12 and CXCL13 by downregulating RGS16 expression; (v) IL-17A stimulated *in vitro* proliferation of primary B-NHL cells; (vi) IL-17A (1 μg/mouse-per dose) stimulated B-NHL growth in two *in vivo* models by enhancing tumor cell proliferation and neo-angiogenesis. This latter effect depended on IL-17A-mediated induction of pro-angiogenic gene expression in tumor cells and direct stimulation of endothelial cells. These data define a previously unrecognized role of human IL-17A in promoting growth of GC-derived B-NHL and modulating normal GC B cell trafficking.

Introduction

IL-17A belongs to the IL-17 cytokine superfamily together with five additional structurally related cytokines, namely IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A is synthesized by Th17 cells, gamma-delta T cells, natural killer T cells, lymphoid tissue-inducer like cells, Paneth cells and neutrophils.[1]

The biological effects of IL-17 family cytokines are mediated by five different receptor (R) subunits (RA, RB, RC, RD, RE), that are single transmembrane domain-containing proteins which can heterodimerize or homodimerize in different combinations.[2] The heterodimer formed by IL-17RA and IL-17RC binds IL-17A, IL-17F and the heterodimeric cytokine IL-17A/IL-17F. [2,3] IL-17RA is expressed ubiquitously, with high levels in the hematopoietic cell compartment.[4,5] IL-17RC is highly expressed in epithelial cells of prostate, kidney and joints, and at lower levels in hematopoietic cells.[6]

The main physiological function of IL-17A is protection from bacterial and fungal infections through recruitment of

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neutrophils to the site of inflammation.[4,7] In addition, IL-17A stimulates the production of other cytokines and chemokines such as G-CSF, IL-6 and IL-8.[2] Th17 cell-derived IL-17A enhances isotype switching in mouse and human B cells [8] and is involved in the pathogenesis of several autoimmune and allergic diseases. [4] In this respect, IL-17A was found to promote GC formation in different mouse models of human autoimmune or infectious diseases [8-11] *via* specific signaling pathways.[12]

In this study, we have addressed IL-17AR expression and IL-17A activity on malignant B cells isolated from lymph node biopsies of patients with B-NHL of GC origin, namely follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL). In addition, we have investigated expression and function of IL-17AR on human tonsil GC B cells and of IL-17A in the GC microenvironment.

BL and DLBCL are tumors with predominant centroblastic morphology, while FL contains centrocytic and centroblastic components in different ratios depending on tumor grade.[13,14] BL and DLBCL are highly proliferating tumors that invade the GC and quickly replace the physiological microenvironment. In contrast, FL displays a follicular growth pattern that is partially retained for a long time over the natural history of the disease.[13,14] Both DLBCL and FL occur commonly in adults and rarely in children or adolescents.[15] DLBCL is the most frequent B-NHL subtype, with approximately one third of cases originating from the transformation of FL.[14] BL affects predominantly children or young adults, with frequent intra-abdominal or extranodal involvement.[15]

We show that IL-17A promotes the growth of B-NHL both *in vitro* and *in vivo* by stimulating tumor cell proliferation and neo-angiogenesis. In contrast, IL-17A does not affect proliferation or survival of freshly isolated normal GC B cells, but renders them competent to migrate to CXCL12 and CXCL13 through an NF-kBp65-dependent mechanism, thus contributing to regulate the trafficking of these cells within the GC.

Results

Expression of IL-17AR in human B-NHL lymph node and tonsil germinal center

Both IL-17RA and IL-17RC mRNAs were detected at comparable levels in FL, DLBCL and BL samples (**Fig. 1A**). Expression of IL-17RA and IL-17RC on the surface of primary neoplastic cells was detected by flow cytometry in 24 lymph node samples of GC-derived B cell lymphoma. In particular, **Fig. 1B** shows the results obtained with 9 FL, 11 DLCBL and 4 BL cases. The insets in **Fig. 1B** show a representative staining for IL-17RA and IL-17RC in a FL, BL and DLBCL case, respectively (Mean Relative Fluorescence Intensity (MRFI) ± SD for FL: IL17RA = 3.1 ± 1.5 and IL-17RC = 2.5 ± 0.5; MRFI ± SD for DLBCL: IL17RA = 2.5 ± 1.2 and IL-17RC = 2.2 ± 1.5; MRFI ± SD for BL: IL17RA = 2.8 ± 0.8 and IL-17RC = 2.3 ± 1.5).

IL-17RA and IL-17RC expression was next assessed by flow cytometry in different GC B cell subsets freshly isolated from six tonsils. GC B cells ($CD38^{high}$, $CD39^{-}$ IgD^{-}), were found to express IL-17RA and IL-17RC (**Fig. 1C**). The insets in **Fig. 1C** show a representative staining of GC B cells for IL-17RA and IL-17RC (MRFI ± SD for GC B cells: IL17RA = 3.7 ± 1.7 and IL-17RC=2.5 ± 0.9). Moreover, both centrocytes (CC, $CD38^{dim}$ $CD77^{-}$ B cells) and centroblasts (CB, $CD38^{high}$ $CD77^{+}$ B cells) were found to express IL-17RA and IL-17RC (MRFI ± SD for CB : IL-17RA= 3.2 ± 1.4 and IL-17RC= 2.4 ± 0.6; MRFI ± SD for CC: IL-17RA = 3.4 ± 1.6 and IL-17RC= 2.1 ± 0.7).

IL-17AR function in human normal and malignant GC B cells

IL-17A activates the canonical NF-kB signaling pathway in different cell types.[12] As shown in **Fig. 2A** (upper panel), IL-17A upregulated significantly phosphorylated (p)NF-kBp65, but not NF-kBp65 (similar levels of about 95 ± 3% of expression in treated and untreated cells), in primary B-NHL cells upon incubation for 1 to 30 min with recombinant human (rh) IL-17A, with a peak at 1 min ($p = 0{,}003$ for three FL and three DLBCL samples). Likewise, a significant upregulation of pNF-kBp65, but not NF-kBp65 (similar levels of about 93 ± 2% of expression in treated and untreated cells), was observed in four tonsil GC B cell suspensions after 10 and 30 min culture with IL-17A ($p = 0{,}01$ and $= 0{,}002$, respectively), as assessed by flow cytometry (**Fig. 2A**, lower panels). The faster kinetics of NF-kBp65 phosphorylation in malignant B cells *vs.* their normal counterparts may depend on intrinsic characteristics of the former cells due to their transformed state. In contrast, IL-17A did not induce phosphorylation of NF-kB p50/105, [12] or of p-38, ERK1/2, AKT, JNK in either malignant or normal GC B cells.[12,16]

IL-17A, as well as CD40L tested as positive control, [17] significantly increased proliferation of tumor cells over background levels: $p = 0{,}01$ and $p = 0.009$ for five FL samples (**Fig. 2B** left panel) and $p = 0$, 009 and $p = 0.01$ for four DLBCL samples (**Fig. 2B** right panel), respectively. No direct effect of IL-17A on survival or migration of B-NHL cells was observed following co-stimulation with CD40L or anti-IgM/IgG tested alone or in combination (data not shown). For *in vitro* functional experiments, we tested FL and DLBCL as model of GC-derived B-NHL since the number of cases of these lymphomas was much higher than that of BL.

In contrast, IL-17A did not rescue normal GC B cells from apoptosis nor did it increase the rate of survival induced by rhCD40L stimulation at any time tested (**Fig. S1A**). Likewise, IL-17A did not affect proliferation of GC B cells either in the presence or absence of rhCD40L (**Fig. S1B**),[18] nor was chemotactic for the latter cells (**Fig. S1C**).

IL-17A confers migratory competence to CXCL12 and CXCL13 on tonsil GC B cells

We next asked whether IL-17A could modulate CXCR4 and CXCR5 expression and/or function in tonsil GC B cells, thus impacting on their migration within the GC.[19] The latter cells, freshly isolated from eight different tonsil samples were pre-incubated for 3 or 6 h with or without 20 ng/mL rhIL-17A, and

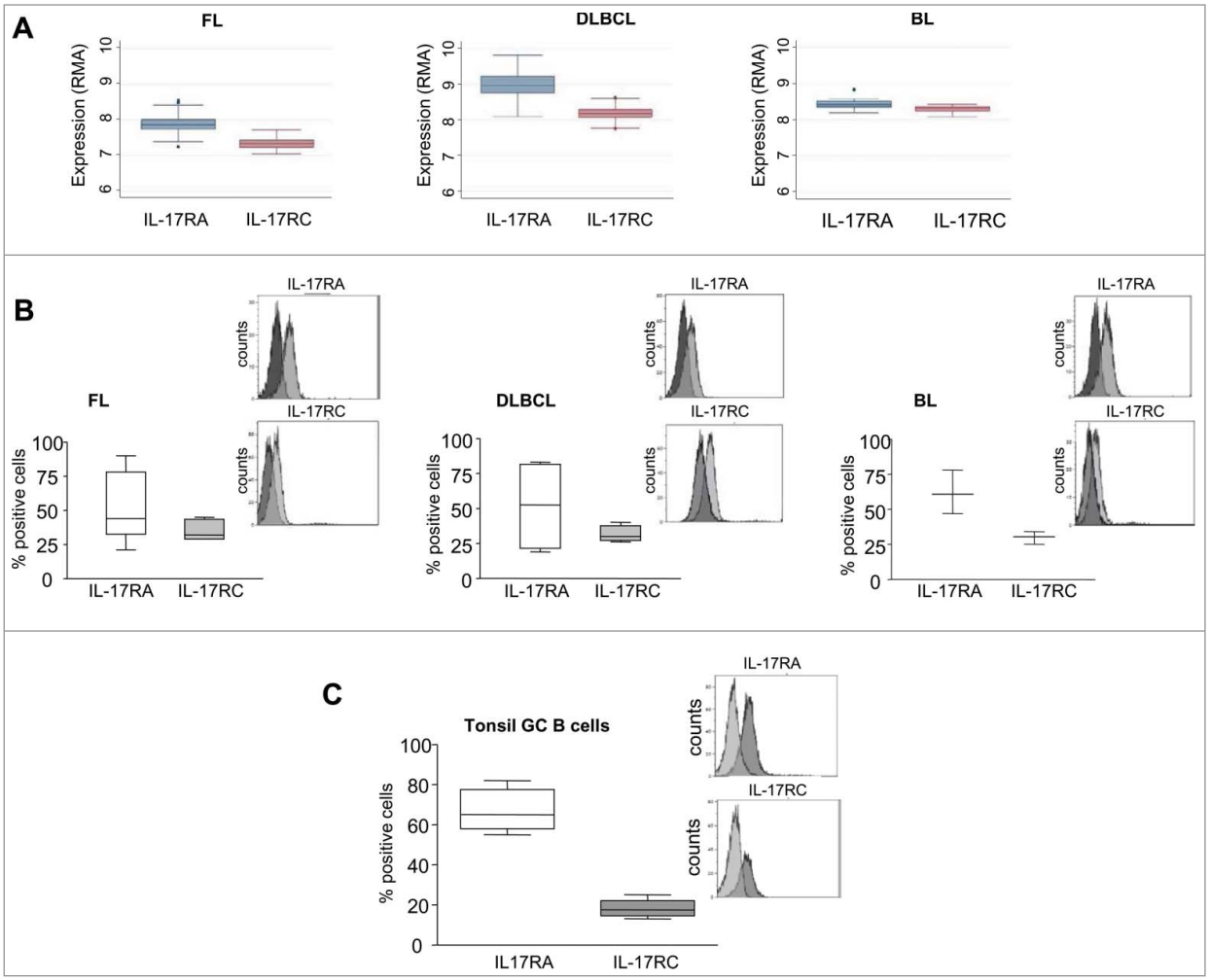

Figure 1. Expression of IL-17A receptor in primary tumor cells from patients with FL, DLBCL or BL and in their normal counterpart. (**A**) Expression levels of IL-17RA and IL-17RC in FL, DLBCL and BL, as measured using the Affymetrix GeneChip U133 array. Data obtained from the GSE16131 (FL) [48] and GSE4475 (DLBCL, BL) [47] datasets, both produced using the Affymetrix U133A. The line in the middle of the box-plot represents the median and the box extends from the 25th to the 75th percentile (interquartile range, IQ); the whiskers extend to the upper and lower adjacent values (i.e., ±1.5 IQ); outside values are individually plotted. Y-axis, expression values (RMA, Robust Multiarray Average). (**B**) Neoplastic B cells were stained with anti-κ or anti-λ mAbs in combination with anti-IL-17RA or anti-IL-17RC mAbs and analyzed by flow cytometry. Results for 9 FL, 11 DLCBL and 4 BL are shown in box plot, as median % positive cells, maximum, minimum and first and third quartile. Insets. A representative staining for IL-17RA and IL-17RC in FL, DLBCL and BL is shown, as assessed by flow cytometry. Dark gray histogram: isotype control. Gray histogram: receptor staining. In the plot mean of mean relative fluorescence intensity (MRFI) ± SD is reported. (**C**) Freshly isolated tonsil MNC were stained with CD38, CD39 or anti-IgD mAbs in combination with anti-IL-17RA or anti-IL-17RC mAbs and analyzed by flow cytometry. Results are mean % positive cells + SD in GC B cells subset ($N = 6$). Insets. A representative staining for IL-17RA and IL-17RC in tonsil GC is shown, as assessed by flow cytometry. Gray histogram: isotype control. Dark gray histogram: receptor staining. In the plot mean of MRFI ± SD is reported.

subjected to chemotaxis in response to CXCL12 or CXCL13. The proportions of apoptotic GC B cells after 3 and 6 h culture were on average 12 and 22%, respectively, as assessed by Annexin V staining, irrespective of they had been exposed or not to IL-17A. The poor viability of GC B cells is a well-known feature of this cell population.[20]

Treatment of GC B cells with IL-17A for 3 or 6 h induced their *de novo* chemotaxis to 300 ng/mL CXCL12 or CXCL13 ($p = 0.0005$ and $p = 0.0007$, respectively at both times tested), whereas GC B cells pre-incubated with medium alone never migrated to either chemokine (**Fig. 3A**).[21] Likewise, GC B cells pre-incubated with rhCD40L were attracted by CXCL12[22] or CXCL13 (**Fig. 3A**). The ratio between CB and CC in migrated cells upon stimulation with IL-17A or CD40L was similar to that detected in the same cell fractions before being tested, indicating that CB and CC were equally attracted by both chemokines. Incubation of GC B cells with either rhIL-17A or rhCD40L did not change the MRFI (**Fig. 3B**) or the percentage of $CXCR4^+$ or $CXCR5^+$ GC B cells (median for CXCR4 of 89 ± 5 % and for CXCR5 of 92 ± 7 % with or without the treatment with rhIL-17A or rhCD40L). Therefore, we tested whether IL-17A driven acquisition of migratory competence to CXCL12

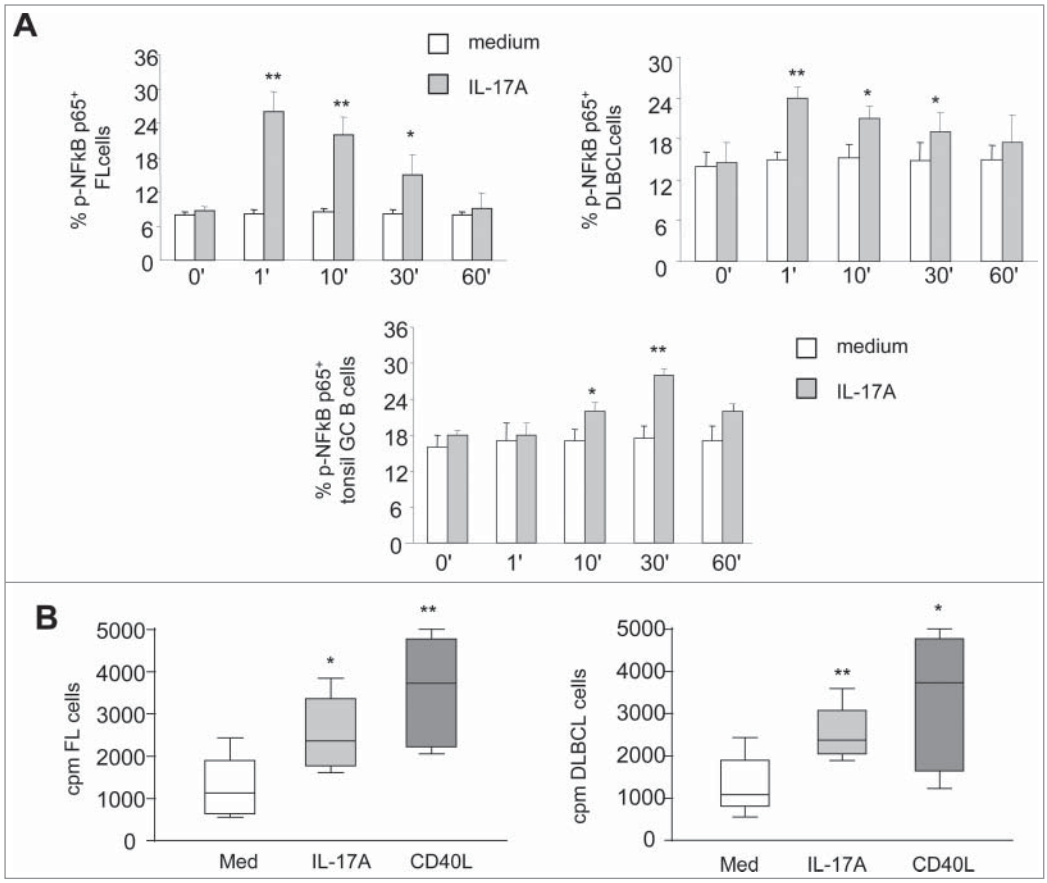

Figure 2. IL-17A signaling and function in tonsil and neoplastic GC (B)cells. (**A**) Upper panel: Flow cytometric analysis of pNF-kBp65 in neoplastic cells from three FL (left panel) or three DLBCL (right panel) treated for 0, 1, 10, 30 or 60 min with IL-17A. Results are shown as mean % positive cells + SD. White histograms: medium alone. Gray histograms: cells stimulated with rhIL-17A. Lower panel: analysis of pNF-kBp65 in isolated GC B cells stimulated for 0, 1, 10, 30 and 60 min with rhIL-17A, as assessed by flow cytometry. Results are mean % positive cells + SD of four sample tested. White histograms: medium alone. Gray histograms: cells stimulated with rhIL-17A. (**B**) Primary neoplastic cells from patients with five FL (left panel) and four DLBCL (right panel) were cultured without (Med) or with rhIL-17A or rhCD40L and tested for proliferation by ^{3}H-TdR incorporation. The results shown refer to experiments performed with 20 ng/mL IL-17A, but stimulation of lymphoma cell proliferation was significant also at 10 and 50 ng/mL IL-17A. Results are expressed in box plot, as median % positive cells, maximum, minimum and first and third quartile.

IL-17RA blocking abrogates IL-17-mediated signaling and related functional activities

We next performed functional experiments on neoplastic and normal GC B cells after IL-17RA blocking with a specific monoclonal antibody (mAb). NF-kBp65 phosphorylation was inhibited in both cell types following blocking with IL-17RA mAb, but not with control mAb (**Figs. 4A and 5A**). Accordingly, IL-17A induced proliferation of primary B-NHL cells (two FL and three DLCBL) was abrogated by pre-treatment of cells with the former, but not the latter, mAb (**Fig. 4B**). Finally, blocking of IL-17RA abolished IL-17A-induced chemotaxis of tonsil GC B cells to CXCL12 and CXCL13 (**Fig. 5B**) and downregulation of RGS16 expression in the same cells (**Fig. 5C**). In contrast, RGS16 downregulation induced by culture of tonsil GC B cells with CD40L was unaffected by IL-17AR blocking (**Fig. 5C**).

These results demonstrate unambiguously the involvement of IL-17A/IL-17AR in the functional activities of the cytokine in malignant and tonsil GC B cells here reported.

and CXCL13 by tonsil GC B cells was related to downregulation of RGS16, a negative controller of signaling through these chemokine receptors. [23] Indeed, treatment of GC B cells with rhIL-17A or rhCD40L, but not with IFNγ tested as negative control, caused significant downregulation of RGS16 expression ($p = 0$ .005 for rhIL-17A and $p = 0.002$ for rhCD40L) (**Fig. 3C**).

Consistent with previous studies, freshly isolated B-NHL cells of GC origin (five FL and four DLCBL samples) expressed CXCR4 and CXCR5 at levels comparable to those detected in normal GC B cells and migrated to CXCL12 and CXCL13.[22] Treatment of B-NHL cells with 20–50 ng/mL rhIL-17A in combination or not with CD40L and/or anti-IgM/IgG did not enhance chemotaxis to either chemokine or expression of the respective receptors CXCR4 and CXCR5 (not shown) nor modulated RGS16 expression (not shown).

IL-17A expression in normal and neoplastic germinal center microenvironments

Immunofluorescence analysis of tonsil tissue sections revealed that IL-17A was predominantly expressed within para-follicular T cell-rich areas where it marked scattered cells with a monocytoid morphology consistent with activated T cells and/or myeloid cells. Double-marker immunofluorescence analysis showed that most IL-17A-positive cells were CD3^{+} T cells admixed with rare Tryptase^{+} mast cells (**Fig. 6A**, B). No CD20^{+} B cells expressing IL-17A were detected. In B-NHL samples the expression of IL-17A was restricted to scattered reactive CD3^{+} T cells and occasional Tryptase^{+} mast cells (**Fig. 6C–H**).

Expression and function of IL-17AR in GC derived B-NHL cell lines

Five different malignant cell lines derived from GC B-NHL, i.e., SU-DHL-4, DoHH2, OCI-Ly8, Raji and Ramos, were next tested for IL-17AR expression and functionality. As shown in **Fig. S2A**, all cell lines expressed both IL-17AR subunits, as assessed by flow cytometry. Similarly to primary lymphoma cells, a significantly increased pNF-kBp65 was detected in all cell lines incubated with 20 ng/mL rhIL-17A compared to cells incubated with medium alone. **Fig. S2D** shows experiments performed with the SU-DHL-4 and OCI-Ly8 cell lines, in which pNF-kBp65 increased significantly over background levels at 1′ and remain significant at 60′. These cell lines were selected for the following *in vivo* experiments.

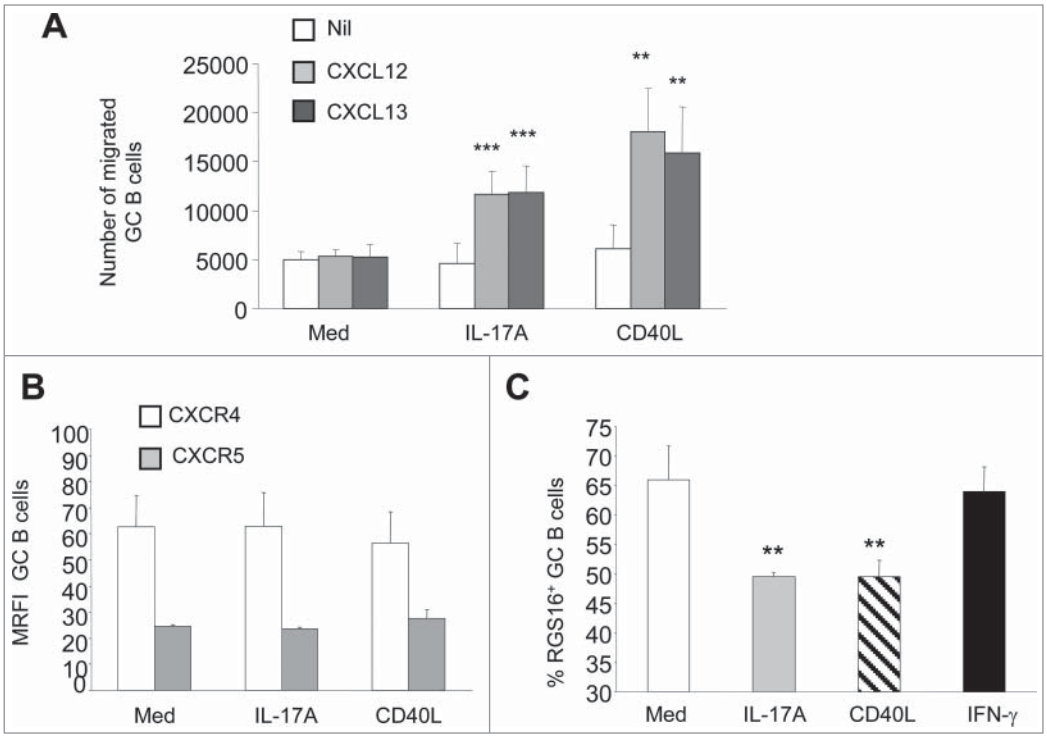

Figure 3. Effects of IL-17A receptor triggering in human freshly isolated tonsil (B)cells. (**A**) 5×10^6 freshly isolated GC B cells were treated for 6 h without (Med) or with rhIL-17A or rhCD40L and subjected to chemotaxis to 300 ng/mL CXCL12 or CXCL13. Results are shown as mean number of migrated cells + SD of eight sample analyzed. (**B**) Expression of CXCR4 and CXCR5 in GC B cells treated for 6 h without (Med) or with rhIL-17A or rhCD40L. Results are MRFI + SD of eight sample tested. (**C**) Intracellular expression of RGS-16 in freshly isolated GC B cells treated for 6 h with or without (Med) rhIL-17A, rhCD40L or IFNγ, as assessed by flow cytometry. Results are mean percent positive cells + SD of eight sample tested.

In vivo effects of IL-17A on B-NHL growth

The tumorigenicity of SU-DHL-4 and Oci-Ly8 cells injected in SCID-NOD mice by different routes of inoculation (i.p., i.v. and s.c.) was tested in order to develop a model suitable for the investigation of the *in vivo* effects of hrIL-17A. The best rate of growth was observed when neoplastic cells were injected s.c., allowing formation of masses easy to isolate and analyze. Thus, we inoculated 5×10^6 SU-DHL-4 or Oci-Ly8 cells s.c. into 30 NOD/SCID mice. The mice were separated in two groups and treated with 3 weekly doses of rhIL-17A (1 μg/doses), or PBS (controls) for 20 d

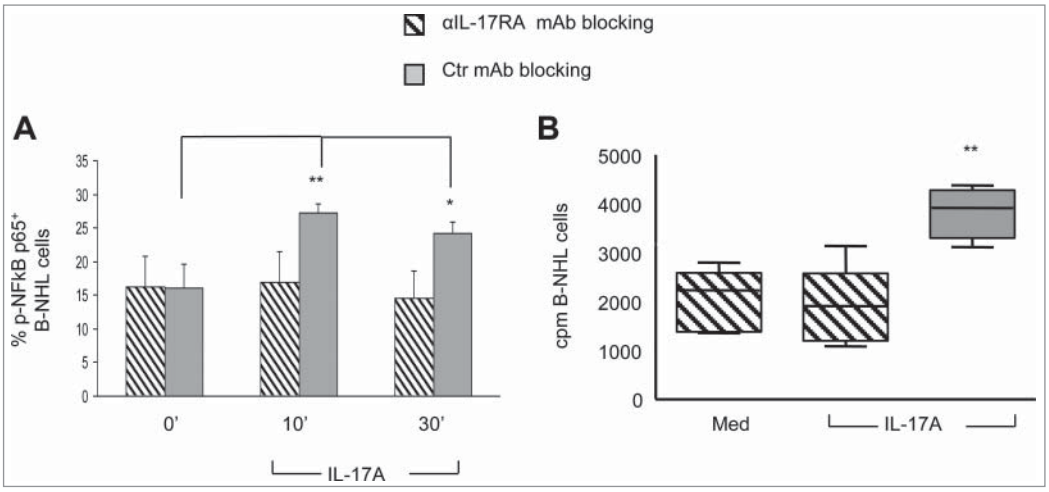

Figure 4. Effects of IL-17RA blocking on IL-17 mediated signaling in neoplastic cells and related functional activities. (**A**) Flow cytometric analysis of pNFkBp65 in neoplastic cells from neoplastic GC B cells (two FL and three DLCBL) pre-incubated with IL-17RA blocking mAb, or control mAb, and treated for 0, 10 or 30 min with rhIL-17A. Results are shown as mean % positive cells + SD. (**B**) Primary neoplastic cells (two FL and three DLCBL) were pre-incubated with IL-17RA blocking mAb, or control mAb, cultured without (Med) or with rhIL-17A and tested for proliferation by ^{3}H-TdR incorporation. Results are expressed in box plot, as median % positive cells, maximum, minimum and first and third quartile.

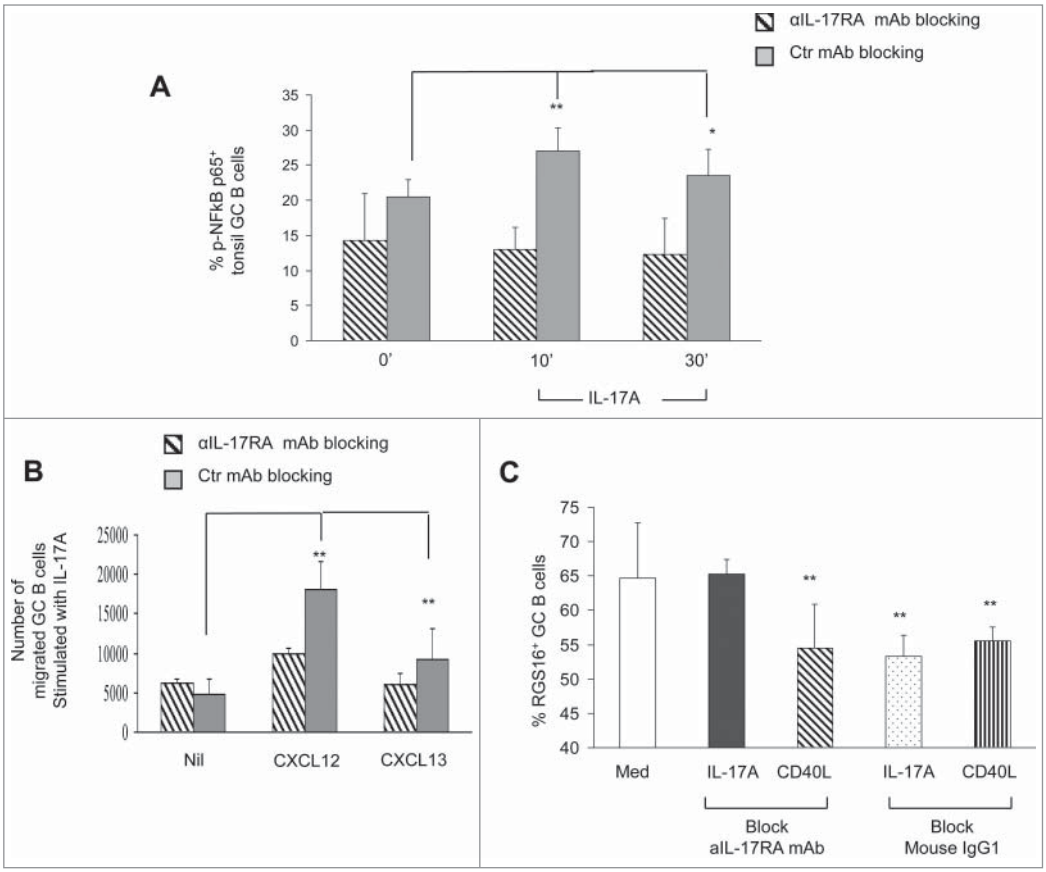

Figure 5. Effects of IL-17RA blocking on IL-17 mediated signaling in normal GC (B)cells and related functional activities. (**A**) Flow cytometric analysis of pNFkBp65 in tonsil GC B cells ($n = 5$) pre-incubated with IL-17RA blocking mAb, or control mAb, and treated for 0, 10 or 30 min with rhIL-17A. Results are shown as mean % positive cells + SD. (**B**) Freshly isolated tonsil GC B cells were pre-incubated with IL-17RA blocking mAb, or control mAb, and then treated for 6 h without (Med) or with rhIL-17A and subjected to chemotaxis to 300 ng/mL CXCL12 or CXCL13. Results are shown as mean number of migrated cells + SD. (**C**) Intracellular expression of RGS-16 in freshly isolated tonsil GC B cells pre-incubated with IL-17RA blocking mAb and treated for 6 h with or without (Med) rhIL-17A or rhCD40L, as assessed by flow cytometry. Results are mean percent positive cells + SD.

The size of tumors formed by SU-DHL-4 cells (**Fig. 7A**) or Oci-Ly8 cells (**Fig. S3**) was significantly increased in IL-17A treated *vs.* untreated mice ($p = 0$, 0131 and 0.04, respectively), indicating that the cytokine stimulated B-NHL growth. The animals did not show any evidence for increased size of internal organs (spleen, liver, lymph nodes, lung, kidney, brain), irrespective of whether they had received IL-17A or PBS, making it unlikely that IL-17A could promote tumor metastasis.

Histological and immunohistochemical studies carried out on lymphoid tumor masses formed by SU-DHL-4 (**Fig. 7B**) or Oci-Ly8 cells (not shown) provided similar results. Tumors from PBS treated SCID/NOD mice showed a few, but clearly detectable, apoptotic events (as assessed by TUNEL assay) and a distinct microvascular supply (as assessed by laminin and CD31 staining) (**Fig. 7B,** panels **A–D**, and **Table 1**). Treatment with rhIL-17A reduced the frequency of apoptotic events and clearly increased microvascular network development (**Fig. 7B**, panels **E–H**). Flow cytometric analysis of cell suspensions from explanted tumors revealed significantly ($p = 0,0087$) increased proportions of Ki-67^+ proliferating cells in mice treated with IL-17A *vs.* PBS (**Fig. 7C**).

Human angiogenesis PCR array experiments performed with SU-DHL-4 tumors from 4 rhIL-17A treated mice in comparison to tumors from four PBS-treated mice showed that 17A treatment upregulated mRNA expression of the pro-angiogenic genes Heparan sulfate proteoglycan (HPSE), inhibitor of differentiation/DNA binding 1 (ID1), leukocyte cell derived chemotaxin (LECT1), IL-1B, and Laminin-α 5 (LAMA5) and downregulated expression of two additional pro-angiogenic genes, namely Insulin-like growth factor 1 (IGF-1) and transforming growth factor β receptor 1 (TGFβ-R1) (**Fig. 7D**).

Chorioallantoic membrane (CAM) incubation with rhIL-17A for 12 d resulted in a significant angiogenic response in the form of numerous allantoic neovessels developing radially toward the implant in a 'spoked-wheel' pattern (mean number of vessels = 26 ± 2 for hrIL-17A) (**Fig. 7E**, panel B). Such response was comparable to that induced by vascular growth factor (VEGF)-A tested as positive control (mean number of vessels = 24 ± 3) (**Fig. 7E**, panel C). Negative control incubated with PBS was devoid of any pro-angiogenic activity (mean number of vessels = 7 ± 1) (**Fig. 7E**, panel A). These results provide demonstration of the direct pro-angiogenic activity of IL-17A.

Discussion

The GC is a specialized microenvironment formed by proliferating B cells in the follicles of secondary lymphoid organs during T cell-dependent antibody responses to exogenous antigens.[24] The GC dark zone is enriched in proliferating CB that undergo somatic hypermutation of the Ig V region genes resulting into diversification of the IgV repertoire. Thereafter, CB migrate to the light zone where they transform into CC, a minority of which is selected to survive, undergoes Ig isotype switch and

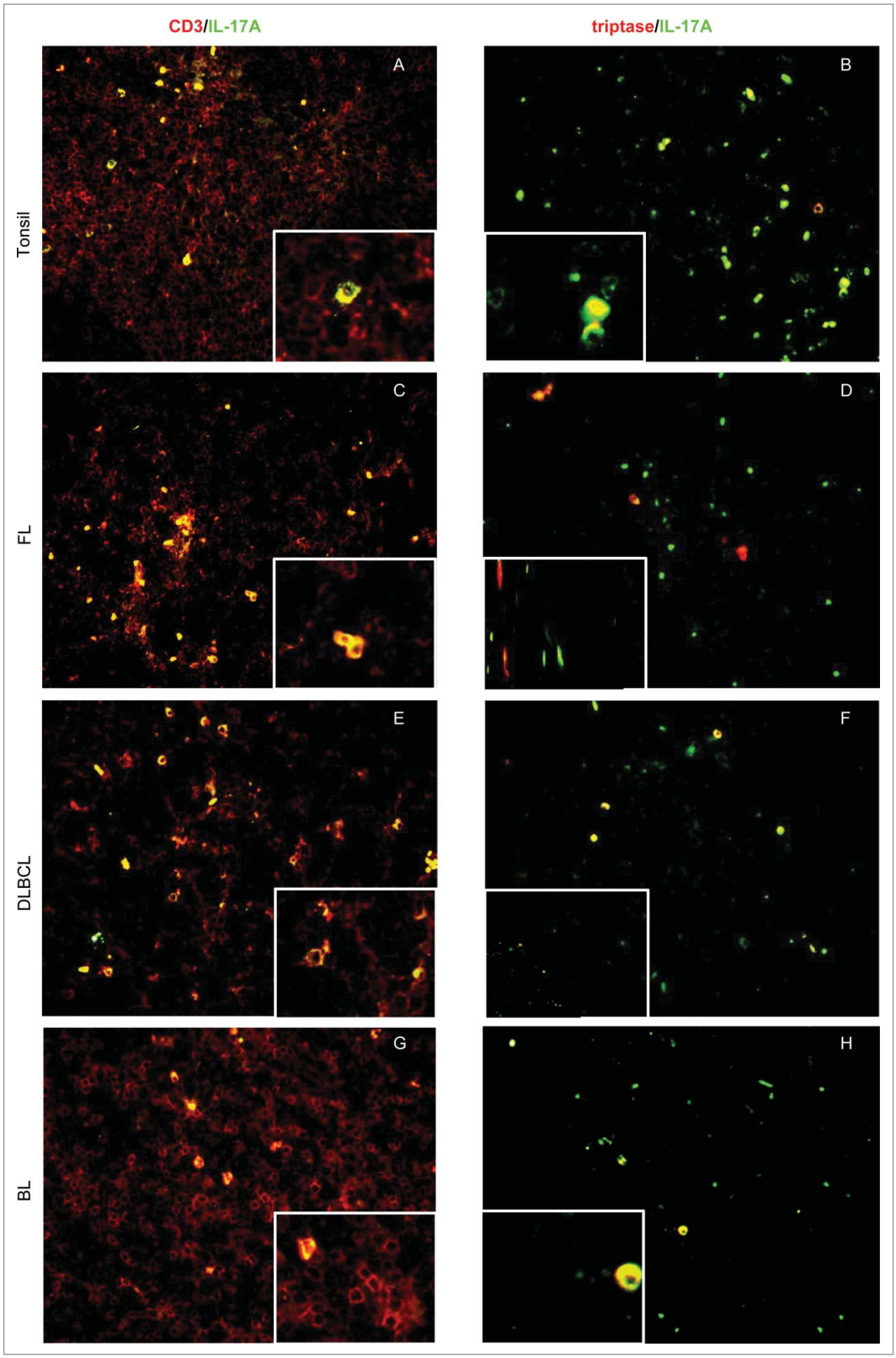

Figure 6. Expression of IL-17A in normal and neoplastic GC microenvironment. (**A–H**) Double-marker immunofluorescence analysis of IL-17A^{+} cells within reactive tonsil tissue, and in the neoplastic infiltrates of FL, DLBCL, and BL samples. Most of the IL-17A^{+} (green) cells co-express (yellow) the T-cell marker CD3 (red) (panel **A–C–E–G**), which indicates their T-cell nature. In the same cases, scanty IL-17A-expressing (green) tryptase^{+} (red) mast cells are also found (yellow, panel **B–D–F–H**). Pictures are relative to full sections of 4 representative cases out of the 26 analyzed. Original magnification, ×400. Double-staining yellow cells are highlighted in an inset present in all panels (**A–H**) of the Figure. Magnification, ×630. Data were analyzed under a Leica DM3000 optical microscope and captions were collected using a Leica DFC320 digital camera (Leica).

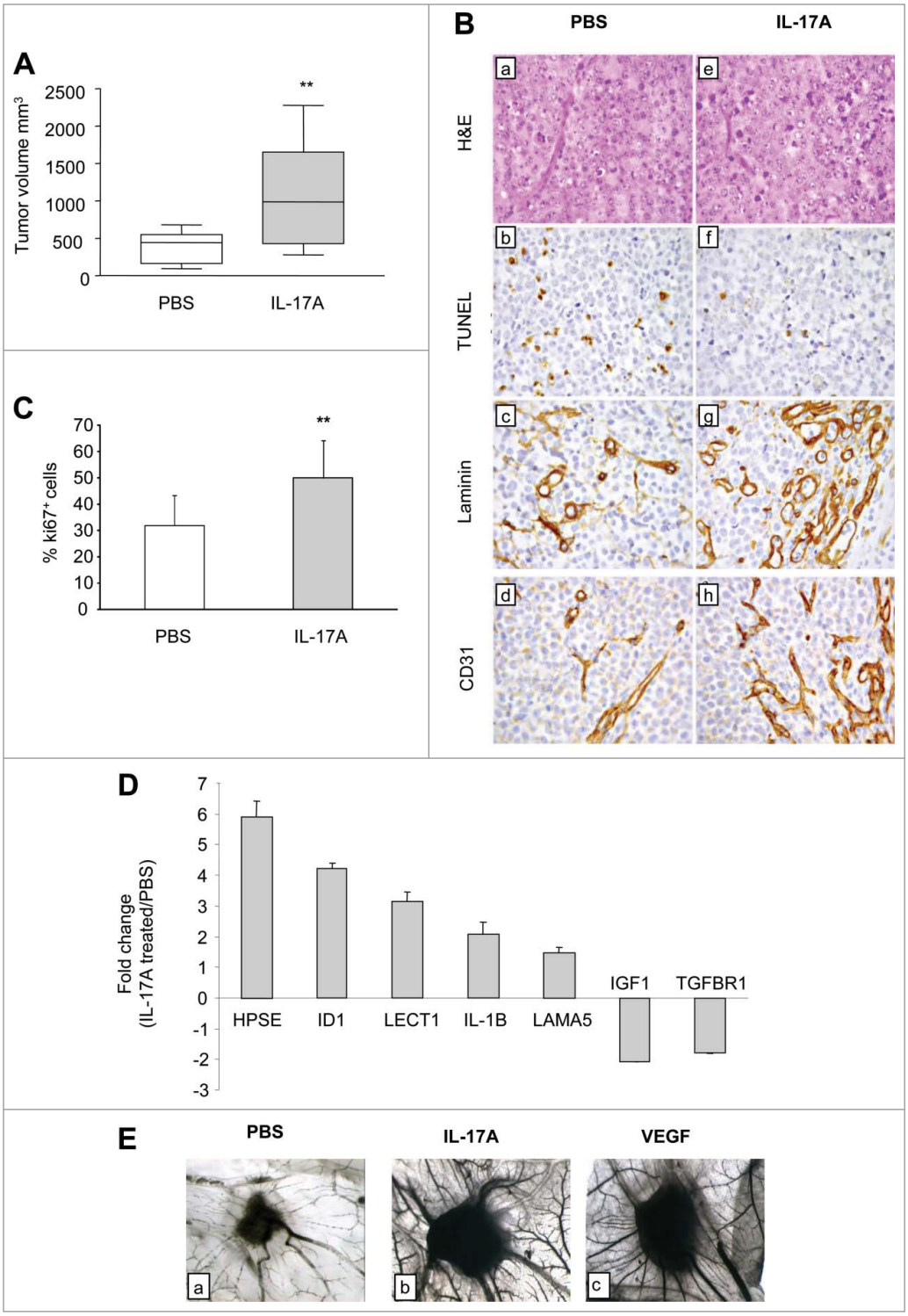

Figure 7. Role of IL-17A on *in vivo* tumor growth and angiogenesis. (**A**) Volume of tumors grown s.c. in PBS or IL-17A treated mice 20 d after SU-DHL-4 cell injection. Results are expressed in box plot, as median % positive cells, maximum, minimum and first and third quartile ($N = 30$). (**B**) Tumors developed after s.c. injection of SU-DHL-4 cells in PBS-treated SCID/NOD mice are formed by a mixture of small and large lymphoid cells with centrocytes and centroblasts morphology (**A**). These tumors display some apoptotic events (tunel assay (**B**)) and a distinct microvascular network (laminin immunostaining (**C**) and CD31 staining (**D**)). The histologic features of SU-DHL-4 tumors are not substantially altered by rhIL-17-A treatment (**E**), while apoptotic events appears less frequent (**F**) and the microvascular supply is clearly more developed (**G–H**), particularly at the tumor edge, in comparison with control tumors. (**C**) Flow cytometric analysis of cell proliferation in cell suspensions from explanted SU-DHL-4 cell tumors, as assessed by Ki67 staining. Results for eight PBS and eight IL-17A-treated mice are expressed as mean % positive cells + SD. (**D**) 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 IL-17A or PBS treated mice. Pooled results from four different experiments are shown. (**E**) Angiogenic activity of IL-17A (**B**), as assessed CAM assay. PBS (**A**) and VEGF (**C**) were used as negative or positive control, respectively.

differentiates extrafollicularly into memory B cells or long-lived plasma cells.[24] CB express CXCR4 and are attracted to the dark zone by CXCL12 produced by stromal cells, CC express CXCR5 and are recruited to the light zone along a gradient of CXCL13 produced by FDC.[19] Therefore, CXCL12 and CXCL13 play crucial roles in the regulation of GC B cell trafficking.

Many molecules are involved in GC formation.[13,19] IL-17A represents a recent addition to such list since it promoted GC formation in a few mouse models of human autoimmune or infectious diseases.[8–12] Furthermore, IL-17A was found to induce Ig class switching *in vitro* in mouse and human B cells.[25,26] So far, little is known on IL-17A in relation to human normal or malignant GC B cell microenvironments.[27,28]

Variable IL-17A expression was previously detected in lymph nodes and spleen from CLL patients, where IL-17A producing cells were identified as Th17 cells, mastocytes and immature

Table 1. Immunohistochemical assessment of microvessels and apoptotic cells in tumors developed after SU-DHL-4 cell injection in PBS or hrIL-17A treated mice

	SU-DHL-4	
	PBS	hrIL-17A
Microvessels	8.4 ± 2.5	14.9 ± 3.0*
Apoptotic index %	15.7 ± 5.0	6.2 ± 3.2*

Apoptotic cell and microvessel count was performed as reported in Methods. Eight fields were examined for each tumor section and three sections per tumor (three cytokine treated *vs.* three PBS treated) were evaluated.
Results are expressed as mean ± SD of TUNEL positive cells/number of total cells (×600) or, CD31 positive vessels per field (×400) evaluated on formalin-fixed sections by immunohistochemistry.
*Values significantly different ($p < 0.05$) from corresponding values in tumors developed in PBS-treated mice.

myeloid cells.[27] In another study, *in vitro* expanded T cells from lymph nodes of B-NHL patients were found to contain scanty Th17 cells.[27,28]

The role of IL-17A and of Th17 cells in tumor growth is controversial. IL-17A is released in the tumor microenvironment of human cervical malignancies, non-small cell lung carcinoma, hepatocellular carcinoma and multiple myeloma, for which the cytokine was found to enhance tumor growth.[29-33] In contrast, IL-17A was shown to inhibit indirectly the growth of mouse mastocytoma and plasmocytoma through T-cell-dependent mechanisms.[34] Th17 related IL-21 may counteract tumor progression in primary B-NHL with ocular localization.[35] Moreover, elevated levels of circulating Th17 cells in CLL correlated with better prognosis and longer survival. [27]

In this study, we have investigated the effects of IL-17A on B cells from B-NHL of GC origin and their normal counterparts, i.e., GC B cells. We demonstrated that IL-17RA and IL-17RC were expressed in normal and neoplastic GC B cells, and the heterodimeric IL-17AR signaled exclusively through the canonical NF-kB pathway by phosphorylating NF-kBp65 in both cell types, as previously shown in other cellular models.[12,16,36] Neoplastic B cells from FL, DLBCL and BL proliferated *in vitro* in response to IL-17A, suggesting a tumor promoting function of the cytokine. IL-17A-driven cell signaling and proliferation of B-NHL lymphoma cells were abrogated by blocking of IL-17AR with a specific mAb.

Since IL-17A expression was detected in $CD3^+$ T cells and occasional tryptase-positive mast cells present in B-NHL microenvironment, [27] we next investigated the *in vivo* effects of IL-17A on the growth of human SU-DHL-4 and Oci-Ly8 DLBCL cells, tested as models of B-NHL cells. IL-17A increased tumor growth in SCID/NOD mice by enhancing significantly malignant cell proliferation and neo-angiogenesis. Human angiogenesis PCR array analyses revealed upregulation of different pro-angiogenic genes in explanted tumors from IL-17A *vs.* PBS-treated mice, including: HPSE, associated with metastatic cancer;[37] ID-1, promoting angiogenesis in normal and cancer cells;[38] IL-1B, encoding a pro-inflammatory cytokine broadly involved in the pathogenesis of cancers;[39] LECT1 and LAMA-5, two pro-angiogenic genes with a poorly understood role in cancer. In contrast IGF1 and TGFBR1, two additional pro-angiogenic genes, were downregulated in IL-17A-treated mice, possibly witnessing an attempt of malignant cells to limit up-regulation of pro-angiogenic molecule expression. We also performed experiments (not shown) in which tumors explanted from mice were subjected to mouse angiogenesis PCR array analysis. In these experiments, no modulation of gene expression was detected in tumors from IL-17A treated *vs.* control mice, indicating that the stimulation of tumor angiogenesis observed in the former mice depended on the direct activity of the cytokine on human lymphoma cells.

Different studies have demonstrated the importance of neo-angiogenesis among the pro-tumoral effects of IL-17A,[29,30] highlighting the ability of the cytokine to promote neo-vessel formation through direct and indirect mechanisms. Thus, IL-17A stimulated migration of vascular endothelial cells [29] and induced fibroblasts and tumor cells to produce pro-angiogenic growth factors and cytokines, such as prostaglandin (PGE)1, PGE2, VEGF, keratinocyte-derived chemokine or macrophage inflammatory protein-2.[29] In a human non-small cell lung cancer model, IL-17A increased *in vivo* tumor growth and angiogenesis *via* a CXCR2-dependent mechanism.[30]

Here we show that hrIL-17A had an intrinsic direct pro-angiogenic activity, as assessed by the ability of the cytokine to stimulate new vessel formation in the CAM assay. It is conceivable that in our experiments the intrinsic pro-angiogenic activity of IL-17A was a major player in the enhancement of tumor angiogenesis since expression of a relatively limited number of pro-angiogenic genes was disclosed in tumor masses by PCR-array experiments.

At variance with that observed with B-NHL cells, IL-17A did not influence proliferation of normal tonsil GC B cells, possibly due to intrinsic proneness of these cells to apoptosis and the inability of IL-17A to counteract this latter process.

We have previously shown that freshly isolated human GC B cells do not migrate to CXCL12 although they express CXCR4. [40] Nonetheless, incubation of the latter cells with CD40L was found to render them competent to migrate to CXCL12.[40] In the present study, pre-incubation of human GC B cells with IL-17A conferred on the latter cells the capacity to migrate to CXCL12 and CXCL13 in the absence of CD40L stimulation. The mechanism underlying such effects was found to be related to downregulation of RGS16 expression operated by both IL-17A or CD40L, highlighting a common mechanism for these molecules.

Detection of IL-17A expressing cells with a predominant localization in the para-follicular T cell-rich areas of tonsil GC, expression of IL-17A receptor by GC B cells, and IL-17A-mediated induction of migratory competence to CXCL12 and CXCL13 in GC B cells all point to IL-17A as a previously unknown regulator of B cell trafficking in the GC of secondary lymphoid organs. Our results are consistent with the demonstration of a role of IL-17A in the development of GC in an autoimmune mouse model.[10,12] Although RGS16 was found also in the latter studies to be target of IL-17A activity, the final result of this molecular interaction was an increased spontaneous GC formation mediated by abolished B cell chemotaxis to CXCL12 and enhanced retention of GC B cells in the GC.[10,12] The reasons for the discrepancy between the latter results and our findings may be related to the different experimental models (man *vs.* mouse

and *in vitro vs. in vivo* studies) and to the strong inflammatory environment associated with mouse autoimmunity compared that of human tonsil.

In conclusion, this study delineates previously unrecognized roles of IL-17A in neoplastic and normal GC. In the latter context, IL-17A unambiguously promotes the growth of human B-NHL by enhancing tumor cell proliferation and neo-angiogenesis, paving the way to future studies addressing the IL-17A/IL-17AR complex as potential therapeutic target. On the other hand, IL-17A appears to participate in the regulation of GC B cell chemotaxis in the normal GC microenvironment.

Materials and Methods

Patients and controls

Heavily infiltrated lymph nodes from patients with FL (n = 15; 9 males and 6 females, age range: 46–61), DLBCL (n=17, 9 males and 8 females, age range: 49–65) and BL (n = 12, 8 males and 4 female, age range: 7–18), biopsied for diagnostic purposes, were obtained after informed consent in accordance with the Declaration of Helsinki. Diagnosis of FL, DLBCL and BL was established according to the criteria of the Revised European–American Classification of Lymphoid Neoplasms.[15,34] All patients were studied at diagnosis and were untreated. Twelve tonsils, surgically removed due to localized inflammation, were obtained after informed consent. The study was approved by the Institutional Review Board of Istituto Giannina Gaslini, Genova, Italy on October 27th, 2005.

Cell isolation and culture

Tonsil B cells (>95 % $CD19^+$ cells) were isolated as reported [41] and enriched for GC B cells (> 95% $CD19^+$, $CD38^{high}$, $CD10^+$, $CD44^-$ cells) by centrifugation on a discontinuous (60–30%) Percoll (Pharmacia) density gradient [41] followed by depletion of $CD39^+$ naïve and memory B cells. For *in vitro* experiments, we used either freshly isolated or frozen tonsil B cells obtaining superimposable results. The latter cells were cryopreserved in freezing solution containing 50% RPMI 1640 (Sigma Chemical Company), 40% FBS (Sigma), and 10% DMSO (Sigma).

Malignant B cell suspensions (>80–85% $CD19^+$ cells) were first isolated from lymph nodes as reported,[22] then cryopreserved in freezing solution and further enriched (at least 96%) after thawing by removing (i) residual normal B cells according to the expression of the Immunoglobulin light chain not expressed by the malignant clone and (ii) other contaminant cell types (i.e., $CD3^+$, $CD56^+$ and $CD14^+$ cells), through immunomagnetic bead manipulation (Miltenyi, Miltenyi Biotec). The human SU-DHL-4 (of DLBCL origin),[42] DoHH2 (of FL origin)[43] and Raji and Ramos (of BL origin)[44] were used, together with the Oci-Ly8 cell line that is considered with a DLCBL origin. All cell lines were provided 2 yr 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, that were revalidated 4 months ago by Interlab using the same assay, were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS (Sigma).

Antibodies and flow cytometry

The following mAbs were used: Phycoerythrin (PE)-conjugated anti-IL-17RA, Alexa Fluor 488 anti-IL-17RC (R&D System Inc.); PE or Fluorescein Isothiocyanate (FITC) anti-κ and anti-λ Ig light chains, PE-CD10 (R&D System); unconjugated-CD39 (Immunotech); PC7-CD19, PC5-CD38, FITC-CD44, PC5 and FITC-CD3 (Beckman Coulter); FITC-anti human Ki67 (DAKO); PE or FITC anti-IgD (Biolegend). PeCy7-CD39 and FITC-CD68 were from eBiosciences (eBiosciences). Anti-human RGS-16 unconjugated mAb was from Abcam (Abcam). For intracellular flow cytometry detection, cells were stained with anti-Ki67 or anti-RGS16 using cytofix/cytoperm kit (Becton-Dickinson). Cells were stained with fluorochrome-conjugated mAbs, or unconjugated mAbs followed by fluorochrome-conjugated secondary reagents, or with isotype- and fluorochrome-matched control antibodies. 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 or MRFI, calculated as follows: fluorescence intensity obtained with specific mAb/fluorescence intensity obtained with irrelevant isotype-matched mAb. For cytofluorimetric analysis of tonsil mononuclear cells, we first gated the lymphocyte population based upon physical parameters and then gated lymphocytes stained with a specific mAb, e.g., CD19.

Cell signaling

NF-kBp65 (Ser536) phosphorylation was investigated by flow cytometry.[45] Cells were treated for 0–1–5–10–30–60 min with or without (medium) 20 ng/ml rhIL-17A and stained with an Alexa 488-conjugated anti-pNF-kBp65 mAb (Cell Signaling Technology) and an unconjugated anti-NF-kBp65 polyclonal Ab (Cell Signaling Technology) according to the instructions of the manufacturer. In brief, cultured cells were collected, fixed with 2% paraformaldeheyde for 10 min at 37°C, incubated with 90% ice-cold methanol for 30 min to permeabilize the cells, and subsequently stained with the specific Ab for 1h at room temperature. The same protocol was used to investigate the phosphorylation of NF-kBp105, ERK1/2, Akt, JNK (Cell Signaling Technology). Cells were washed and run on Gallios cytometer. Data were analyzed using the Kaluza software.

Cell proliferation, apoptosis and chemotaxis

Normal and neoplastic cells were analyzed for proliferation and apoptosis in presence or absence of 10–20–50-ng/mL rhIL-17A (see the Supplemental Methods). Chemotaxis was investigated using 5 μm pore-size transwell plates (Costar) as reported [41] (see the Supplemental Methods).

Blocking experiments

Normal and neoplastic B cells were pre-incubated for 20 min with 2 μg/mL of an IgG1 blocking mouse mAb to human IL-17RA (R&D System, clone#133617) or of mouse IgG1 control mAb (R&D System) at 37°C. Thereafter, cells were cultured

with 50 ng/mL rhIL-17A for different times and subjected to functional tests.

Immunofluorescence

Tissue samples selected for immunofluorescent analyses were retrieved from the archives of the Human Pathology Section, Department of Health Sciences, University of Palermo. Formalin-fixed and paraffin-embedded tissue sections from six FL, six DLBCL and eight BL lymph nodes, as well as from six tonsils with reactive follicular hyperplasia were tested. The immunofluorescence techniques are detailed in the Supplemental Methods online.

Gene expression profiling

Raw expression profiles (CEL files) were downloaded from three publicly available data-sets (GSE2350,[46] GSE4475,[47] GSE16131)[48] and individually imported and normalized using RMA (Robust Multiarray Average) algorithm in Partek Genomics Suite 6.4 (Partek, St. Louis, MO, USA). Box-plots were created with Stata/SE v.12.1 (StataCorp, College Station, TX, USA) and differential expression among groups was evaluated with the Wilcoxon rank-sum test. A p value lower than 0.05 was considered statistically significant.

In vivo studies

Four–six week old SCID-NOD mice (Harlan Laboratories) were housed under specific pathogen-free conditions. 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 Council Directive 86/609, OJL 358, December 1, 1987). Thirty animals were injected s.c. in the left flank with 5×10^6 SU-DHL-4 cells and separated in two groups of 15 mice each. The same protocol was applied to 30 SCID/NOD mice injected s.c. in the left flank with 2.5×10^6 Oci-Ly8 cells. The first group of mice was treated with 3 weekly doses of rhIL-17A (1 μg/mouse per dose) starting one day after injection of tumor cells. The second group was treated with PBS (controls) following the same time schedule. The concentration of IL-17A to be injected *in vivo* was chosen on the basis of previous studies.[1] Twenty days after tumor cell inoculation, mice were killed since signs of poor health such as enlarging tumor masses and presence of ruffled fur, became evident and autopsies were carried out. Tumor masses were explanted, measured as described [49] and used for following *ex vivo* analyses.

Morphologic and immunohistochemical analyses on mouse tissue

Tumor masses formed by SU-DHL-4 cells were subjected to morphological and immunohistochemical analyses, as described.[49,50] Formalin-fixed, paraffin-embedded sections were incubated for 30 min with rabbit anti-human/mouse laminin (Biogenex) Abs or with rat anti-mouse CD31 (clone SZ31; Dianova). CD31 positive vessels were counted in eight randomly chosen fields under a microscope ×400 field (×40 objective and ×10 ocular lens; 0.180 mm^2 per field). The rates of apoptotic cells were determined by counting the number of TUNEL positive cells/number of total cells in the viable neoplastic tissue under a microscope ×600 field (×60 objective and ×10 ocular lens; 0.120 mm^2 per field).

Immunocomplexes were detected using the Bond™ Polymer Refine Detection Kit according to the manufacturer's protocol (Leica), then sections were counterstained with hematoxylin and eosin. DNA fragmentation was detected in 4 μm tissue sections by TUNEL staining with the ApopTag® Plus Peroxidase In Situ Apoptosis Kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol.

Angiogenesis PCR-Array

RNA was extracted from mouse tumors using Qiagen (Hilden) kit, reverse transcribed and processed using human or mouse Angiogenesis RT^2 PCR Array (SABioscience), as described. [49] PCR was performed on ABI Prism 7700 Sequence Detector (Applied Biosystems).

CAM assay

CAM assay was carried out as previously reported [49] to investigate the angiogenic activity of IL-17A. The technique is detailed in the Supplemental Methods online.

Statistical analysis

Differences in tumor volume, chemotaxis, RGS16 expression, apoptotic and proliferating cells and NF-kBp65 phosphorylation 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. Quantitative studies of stained sections were performed independently by two pathologists in a blind fashion. Differences in the number of apoptotic cells and tumor microvessels in immunohistological studies were evaluated by Student's t test. All statistical tests were two-tailed and a p value lower than 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

The samples used for this study were obtained from patients after informed consent in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of Istituto Giannina Gaslini, Genova, Italy on October 27th, 2005.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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