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VEGF-A promotes lymphoma tumour growth by activation of STAT proteins and inhibition of $p27^{KIP1}$ via paracrine mechanisms

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ABSTRACT

Increased levels of circulating VEGF-A have been demonstrated in patients with non-Hodgkin lymphoma (NHL) and are associated with progressive disease and poor clinical outcome. We investigated the role of VEGF-A in lymphoma tumour growth on a molecular level in order to identify the mechanism of VEGF-A-promoted tumour growth and to identify the potential targets for therapy. We used a model in which Daudi (human Burkitt lymphoma) tumour cells were transduced with VEGF-A165 or an empty vector (negative control) and subcutaneously injected in NOD/SCID mice. The weight of tumours overexpressing VEGF-A was increased 4-fold compared to that of control tumours ($p < 0.0001$), whereas no *in vitro* growth advantage was demonstrated upon VEGF-A overexpression. VEGF-A-tumours were associated with increased microvessel densities ($p = 0.004$) and increased tumour cell proliferation (Ki67; $p < 0.001$) compared to control tumours. VEGF-A-tumours were characterised by upregulation of phosphorylated STAT-4 and STAT-6 and downregulation of phospho-$p27^{KIP1}$, a crucial cell cycle inhibitor ($p < 0.05$). This was accompanied by increased levels of phosphorylated receptor tyrosine kinases, including EGFR (ErbB-2 and ErbB-4, $p < 0.05$), an upstream regulator of STAT proteins. We demonstrated that various mouse-derived cytokines produced by mouse-derived tumour stromal cells are upregulated in VEGF-A-tumours compared to control tumours ($p < 0.05$). These results indicate an important role for the tumour microenvironment in paracrine promotion of lymphoma tumour growth in response to tumour-derived VEGF-A. In conclusion, lymphoma-derived VEGF-A promoted lymphoma tumour growth in a paracrine loop by activation of tumour stromal cells. Our study reveals VEGF-A and STAT proteins as potential additional targets in the treatment of lymphoma.

1. Introduction

In non-Hodgkin lymphoma (NHL), vascular endothelial growth factor A (VEGF-A) expression has been identified in follicular B-cell lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), peripheral T-cell lymphoma[1,2] and mantle cell lymphoma.[3] Increased levels of circulating VEGF-A have been demonstrated in patients with NHL,[4–6] and have been associated with progressive disease and poor clinical outcomes.[6–8] VEGF-A is the most important pro-angiogenic factor involved

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in normal and pathologic angiogenesis. In the setting of NHL, increased vessel density has been found in the lymph node biopsies of patients with NHL compared to benign lymphadenopathies, and increased MVD has been related to the histological malignancy grade of the lymphoma sub-type.[9] Also, an association between high MVD and decreased overall survival has been found in diffuse large B-cell lymphoma (DLBCL).[10]

NHL cells have been shown to secrete VEGF-A, and a strong correlation has been found between the amount of VEGF-A produced *in vitro* by different types of human lymphoma cell lines and the onset of tumour growth *in vivo* in NOD/SCID mice. VEGF-A production of these lymphoma tumour cell lines correlated with the frequency of endothelial cells in the tumours and inversely correlated with the frequency of apoptotic tumour cells.[11] NHL cells have been shown to express VEGF-receptors (VEGFRs), suggesting the existence of autocrine and paracrine pathways in lymphoma tumour growth and progression.[12] These data suggest an important role for VEGF-A in lymphoma tumour growth and progression. However, the mechanism is still unclear. Understanding how VEGF-A influences lymphoma tumour growth on a molecular level could open new ways towards additional therapeutic strategies in the treatment of lymphoma.

In this study, we investigated the mechanism by which VEGF-A promotes lymphoma tumour growth. Using a xenograft mouse model with a human Burkitt lymphoma cell line transduced with VEGF-A165 or an empty vector, we demonstrate that VEGF-A significantly increased lymphoma tumour growth and tumour vessel formation. Our results demonstrate that lymphoma-derived VEGF-A stimulates tumour stromal cells to produce various cytokines resulting in paracrine activation of STAT proteins and inhibition of $p27^{KIP1}$. Our study reveals VEGF-A and STAT proteins as potential additional targets in the treatment of lymphoma and underlines the importance of targeting the tumour microenvironment in the treatment of lymphoma.

2. Materials and methods

2.1. Generation of the tumour cell lines

Daudi (human Burkitt lymphoma) cells[13] were cultured in Iscove's media supplemented with 2 mM L-glutamine, 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM ß-mercaptoethanol. Stably transduced Daudi-VEGF and Daudi-MOCK cell lines were generated using retroviral supernatants produced by 293T-cells after cotransfection of packaging plasmid pClAmpho and reporter plasmid pMSCV-VEGF-A-NGFR and pMSCV-NGFR, respectively. Retroviral supernatants were passed through a 0.45-μm filter before addition to the Daudi cells. Daudi cells were incubated with retroviral supernatant supplemented with 8 μg/ml polybrene for 16 h in two consecutive transduction rounds. NGFR-positive Daudi cells were sorted using MoFlo XPD Cell Sorter to obtain a purity of 99 ± 1% transduced cells. The functionality of secreted VEGF-A from transduced cells was assessed by adding its supernatant to HUVEC and to quantify the expression level of the VEGF-A responsive gene EGR-3 in HUVEC using real-time PCR as described in detail by Liu and colleagues.[14] Potential differences in cell growth between the generated cell lines were assessed by culturing Daudi-MOCK and Daudi-VEGF cell lines in duplicate at a density of 0.7×10^6 cells per ml for 20 consecutive days by passing and counting the cells every day. Cell viability was checked by Trypanblue exclusion. Cell growth was also checked by a WST assay (Roche, Woerden, The Netherlands) according to the protocol provided.

2.2. FACS analysis

VEGFR expression on Daudi tumour cells was evaluated using direct immuno-fluorescence. The cell suspensions (100 μl) were stained with 5 μl mouse-anti-human VEGFR-1 (clone 49560), mouse-anti-human VEGFR-3-APC (clone 54733) (R&D Systems, Abingdon, United Kingdom) and mouse-anti-human VEGFR-2 clone KDR-1 (Sigma, Munchen, Germany) in PBS/1% BSA w/v for 30 min at RT. For VEGFR-1- and VEGFR-2 staining a rabbit-IgG1-anti-mouse-PE secondary antibody (DAKO AS, Glostrup, Denmark) was used. For intracellular staining, the cells were fixed in 4% paraformaldehyde for 20 min and permeabilised in PBS/0.3%Triton × 100 for 10 min previous to adding the primary antibodies. After staining, the cells were washed with PBS/EDTA and resuspended in 300 μl PBS/EDTA before FACS analysis. The data were analysed by the Winlist software (Verity Software).

2.3. In vivo model

Non-obese diabetic–severe combined immunodeficient (NOD/SCID) mice (Charles River, Maastricht, The Netherlands) were bred and maintained in a pathogen-free environment at the Central Animal Facility, University of Groningen. All procedures involving animals were performed in accordance with local ethical animal laws and policies. During the experiment, the mice were kept under laminar flow conditions. Eight-week old mice were sublethally irradiated (2 Gy) and were subcutaneously injected with 10×10^6 Daudi tumour cell suspensions (100 μl in phosphate-buffered saline (PBS)) at their right flanks. The mice were injected with Daudi-MOCK ($n = 9$) or Daudi-VEGF-A cells ($n = 9$). The mice were evaluated for tumour growth every 2 d. The mice were sacrificed 11, 14 or 17 d after tumour inoculation. The tumours were removed, embedded in TissueTek (Sakura Finetek Europe, Zoeterwoude, The Netherlands), snapfrozen in melting isopentane and stored at –80 °C till later use. Blood samples were taken and the plasma was isolated after centrifugation of the blood and stored at –80 °C till later use. Plasma VEGF-A levels were determined using a commercially available ELISA (Quantikine human VEGF-A Immunoassay, R&D Systems, Abingdon, UK) according to the protocol provided.

2.4. (Q)RT-PCR

RNA was isolated from *ex vivo* tumours or tumour cell lines, using Machery Nagel RNA isolation kit (Machery Nagel, Düren, Germany), according to the protocol provided. cDNA was prepared and amplified using iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands) on a MyIQ thermocycler (Bio-Rad, Hercules, CA, United States of America) and quantified using MyIQ software (VEGF-A and β-actin) or by conventional PCR (EGF and IL-4R). Gene expression was assessed using the

following primer combinations (all from Invitrogen, Breda, The Netherlands): VEGF-A forward AAGGAGGAGGGCAGAATCAT, reverse CCAGGCCCTCGTCATTG; β-actin forward GCTG TGCTACGTCGCCCTG, reverse GGAGGAGCTGGAAGCAGCC; IL-4R forward ACACCTGGAGG AAGTAGAAC, reverse ACCGCATGTACAAACTCCTG; EGF forward GGACAGACAGAGCGAAATC, reverse GAGCTGGCTATAACCAGAC.

2.5. *Immunohistochemical staining for micro vessel density and proliferation*

Fresh serial tumour sections (4-μm) were thaw mounted on tissue-coated glass slides (Star Frost, Waldemar Knittel, Germany). Air-dried sections were fixed in acetone, blocked for endogenous peroxidase with TBS/0.25% H_2O_2 v/v and stained overnight with rat anti-mouse CD31 (1:100; Invitrogen, Gibco, Breda, The Netherlands) or goat polyclonal Ki67 (1:200; Santa Cruz, Heidelberg, Germany) diluted in PBS/1% BSA w/v. An appropriate biotin-labelled secondary antibody (DAKO AS, Glostrup, Denmark) was used, followed by amplification with streptavidin ABComplex/HRPO (DAKO AS, Glostrup, Denmark) and subsequent addition of 3-amino-9-ethylcarbazole substrate for peroxidase (Sigma, Munchen, Germany). Sections were counterstained with haematoxylin. Negative controls were incubated with non-specific IgG as the primary antibody. Vessel number was assessed using light microscopy in areas of the slide containing the highest numbers of CD31-positive small blood vessels (including sinusoids and capillaries), as previously described.[15] Proliferation was evaluated by determining the percentage of Ki67-positive cells by counting Ki67-positive cells in three random fields using light microscopy at 400× magnification.

2.6. *Proteome profiler arrays*

Mouse-specific cytokine levels and human-specific phospho-kinase levels in Daudi-MOCK ($n = 4$) and Daudi-VEGF tumours ($n = 4$) were determined using a mouse cytokine array panel A kit (40 cytokines), a human phospho-RTK array kit (42 RTKs) and a human phospho-kinase array kit (46 kinases) (all from R&D Systems, Abingdon, UK) according to the protocols provided. About 400 μg of protein was applied on each array. Spot densities were quantified with Scanalyze software (http://rana.lbl.gov/EisenSoftware.htm) and exported to Microsoft Excel. The spot densities were corrected for the individual background to diminish interarray variances.

2.7. *Statistical analysis*

All values in the figures represent mean ± SD. The relevant data sets were compared by unpaired Student's *t*-test (tumour weight, MVD) or Mann–Whitney-*U* test (proteome profiler arrays, Ki67) using SPSS software. *p*-Values <0.05 were considered statistically significant.

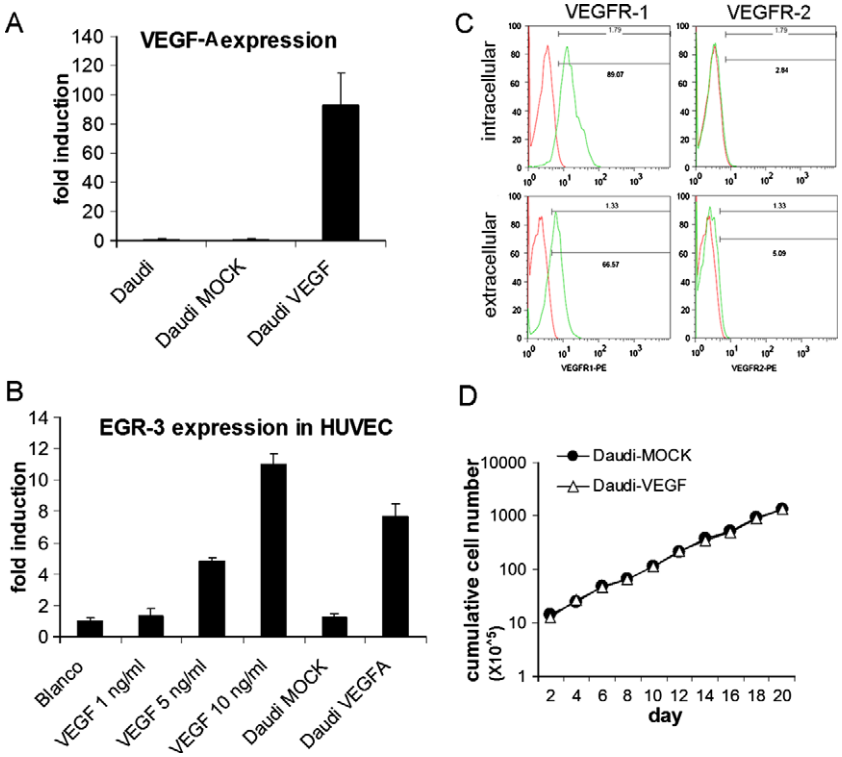

Fig. 1 – VEGF-A and VEGF-receptor expression in tumour cells. (A) Fold induction of VEGF-A mRNA-expression in VEGF-A-transduced and empty vector (MOCK)-transduced Daudi tumour cells compared to that of untransduced Daudi tumour cells. (B) Induction of mRNA-expression of the VEGF-A responsive gene EGR3 in HUVEC after incubation with conditioned medium from VEGF-A-transduced and empty vector (MOCK)-transduced Daudi tumour cells compared to that of control medium (blanco). (C) Extracellular and intracellular expression of VEGFR-1 and VEGFR-2 on Daudi tumour cells. (D) Growth curves of VEGF-A-transduced and empty vector (MOCK)-transduced Daudi tumour cells. The Y-axis represents cumulative cell numbers.

3. Results

3.1. Functional VEGF-A overexpression by the Daudi-VEGF cell line

A 92.6-fold induction of VEGF-A expression was achieved on mRNA level after stable transduction of Daudi tumour cells with the VEGF-A gene (Fig. 1A). Functionality of the produced VEGF-A was confirmed by the addition of supernatant of Daudi-VEGF-A or Daudi-MOCK to endothelial cells (HUVEC). Expression of the VEGF-A-responsive gene EGR-3 was 7.7-fold upregulated in HUVEC cells after incubation with DV-conditioned medium, whereas unconditioned control medium had no effect on EGR-3 expression in HUVEC cells (Fig. 1B). These results indicate that the Daudi-VEGF cell line produced and secreted functional VEGF-A-protein *in vitro*. FACS analysis showed that Daudi tumour cells do not express VEGFR-2, whereas the majority of cells expressed VEGFR-1 (65% extracellular and 87% intracellular; Fig. 1C) and VEGFR-3 (89% extracellular and 7% intracellular; data not shown). The results were similar for Daudi-MOCK and Daudi-VEGF cell lines. Growth and viability of Daudi-VEGF and Daudi-MOCK cells were similar (Fig. 1D), which were confirmed by a WST assay (data not shown), indicating that potential autocrine stimulation of tumour cell growth by VEGF-A is not detectable *in vitro*.

3.2. VEGF-A overexpression in lymphoma tumours promotes tumour growth and tumour vessel formation

Daudi-MOCK and Daudi-VEGF-A tumour cells were inoculated subcutaneously in mice using a NOD/SCID xenograft mouse model. The weight of tumours overexpressing VEGF-A (DV-tumours) was significantly increased compared to that of control tumours (DM-tumours) (tumour weight day 14; DV 807 ± 240 mg versus DM 205 ± 44 mg, $p < 0.0001$) (Fig. 2). Tumour micro vessel density (MVD) was significantly increased in DV-tumours compared to that of DM-tumours (day 14; DV 57.7 ± 15.5/HPF versus DM 14.7 ± 1.0/HPF, $p = 0.004$) (Fig. 3A). Tumours overexpressing VEGF-A were characterised by large nodules of extended networks of irregular, elongated blood vessels, while DM-tumours showed fewer blood vessels that were smaller and rounder than in VEGF-A-tumours (Fig. 3B). VEGF-A mRNA-expression quantified by QRT-PCR was upregulated 35.1-fold in DV-tumours compared to that in DM-tumours (data not shown). Moreover, VEGF-A-protein could be detected in plasma of mice bearing DV-tumours (mean $282.0 \times 10^{-12} \pm 147.1 \times 10^{-12}$ g/ml), whereas plasma VEGF-A in mice bearing DM-tumours was undetectable ($<10 \times 10^{-12}$ g/ml) (data not shown). Together, these data show that VEGF-A overexpression in Daudi lymphoma tumours is associated with increased tumour growth and vascularisation.

3.3. Activation of STAT-4 and 6 and inhibition of $p27^{KIP1}$ in VEGF-A-tumours

Ki67-staining on tumour sections showed an increased fraction of proliferative cells in tumours overexpressing VEGF-A (DV-tumours) compared to that in control tumours (DM-tumours) (Fig. 4A and B). Using proteome profiler arrays, we assessed whether the increased proliferative profile of DV-tumours would be reflected in altered intracellular signalling compared to DM-tumours. Levels of seven intracellular phosphorylated kinases were significantly increased in DV-tumours compared to those in DM-tumours, including STAT-4, STAT-6, eNOS, Pyk2, Paxillin, c-Jun and PLCγ-1 ($p < 0.05$) (Fig. 4C and D). In contrast, levels of phosphorylated $p27^{KIP1}$ (T198 and T157) were significantly decreased in VEGF-A overexpressing tumours compared to those in control tumours ($p < 0.05$). $p27^{KIP1}$ is an important cell cycle inhibitor,[16] and STAT proteins have been reported to regulate $p27^{KIP1}$ expression,[17] indicating an important role for STAT proteins and $p27^{KIP1}$ in promoting lymphoma tumour growth as a result of VEGF-A overexpression.

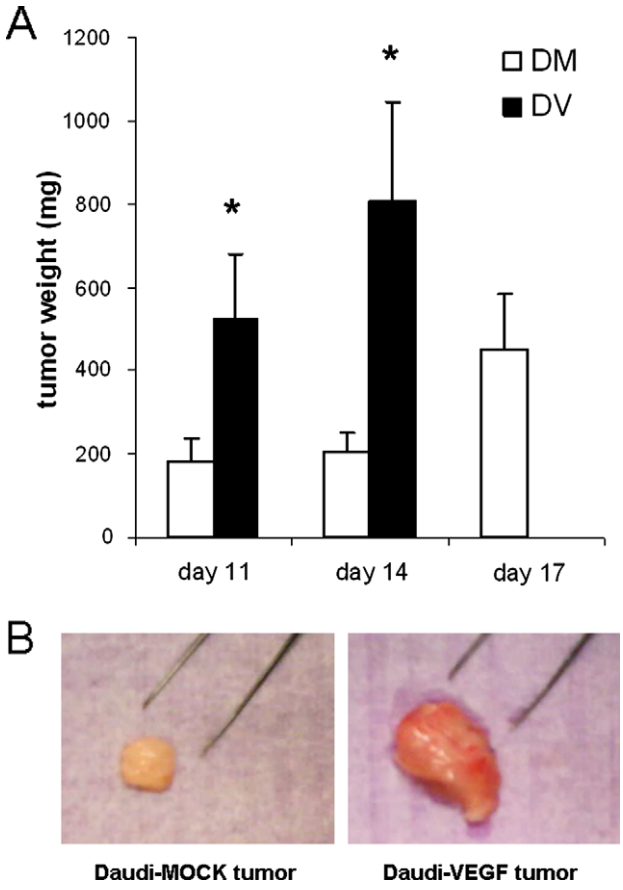

Fig. 2 – VEGF-A overexpression in lymphoma tumours increases tumour growth. (A) Tumour weight (mg) of Daudi-VEGF (DV) and Daudi-MOCK (DM) tumours 11, 14 and 17 d after *in vivo* inoculation. *$p < 0.05$. (B) Two representative pictures of a Daudi-MOCK tumour (left) and a Daudi-VEGF tumour (right) excised on day 14.

3.4. Paracrine mechanisms play important roles in VEGF-A-promoted tumour growth

Although Daudi-VEGF-A tumour cells had no growth advantage over Daudi-MOCK tumour cells *in vitro*, the *in vivo* growth benefit of tumours in response to VEGF-A overexpression was significant. Moreover, no VEGFR-2 expression was detected on Daudi-MOCK and Daudi-VEGF-A cells. The increased fraction of mouse-derived stromal cells, including endothelial cells, in

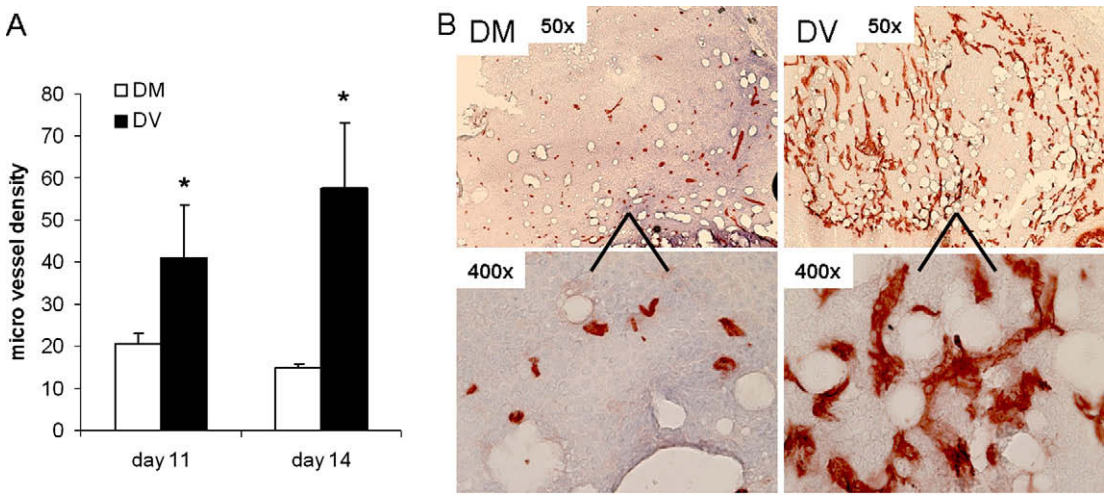

Fig. 3 – Micro vessel density. (A) Micro vessel densities (MVDs) scored in Daudi-MOCK (DM) and Daudi-VEGF (DV) tumours that were excised 11 and 14 d after *in vivo* inoculation. *$p < 0.05$. Graph represents the mean MVD ± SD. (B) Representative pictures of CD31-stained tumour sections of a DM-tumour and a DV-tumour at 50× and 400× magnification.

VEGF-A overexpressing tumours may be associated with increased production of growth factors and cytokines in these tumours. In turn, these growth factors may induce receptor signalling in tumour cells resulting in paracrine promotion of tumour cell proliferation. Indeed, we found that the levels of 27 mouse-derived cytokines were significantly increased in VEGF-A overexpressing tumours (DV-tumours) compared to those in control tumours (DM-tumours) ($p < 0.05$) (Fig. 5A and B). Upregulated cytokines were many interleukins (IL-1ra, IL-16, IL-1a, IL-23, IL-17, IL-27, IL-7, IL-1b, IL-4, IL-3, IL-2 and IL-10); chemokines (MIG/CXCL-9, IP-10/CXCL-10, RANTES/CCL-5, MIP-2, MIP-1b/CCL-4, I-309 (CCL-1/TCA-3), I-TAC/CXCL-11 and MIP-1a/CCL-3); growth factors (M-CSF, G-CSF and GM-CSF); and other cytokines (IFNγ, KC, TREM-1 and C5a). In accordance with the literature many of these cytokines can augment tumour cell proliferation by activation of

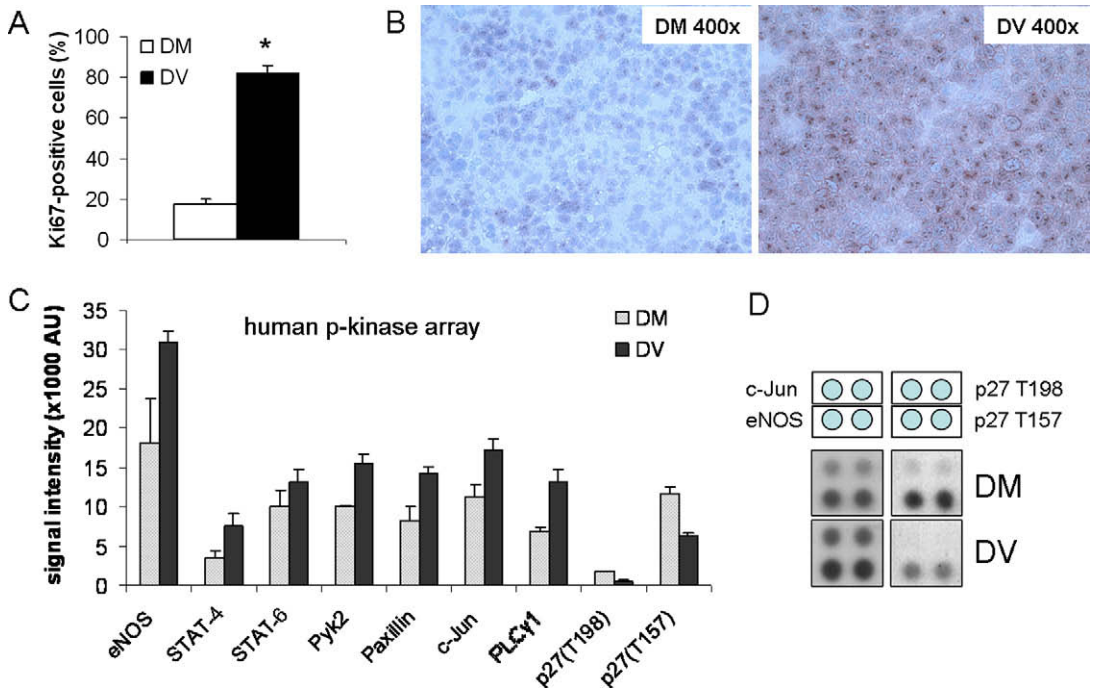

Fig. 4 – Proliferation index and phospho-kinase arrays. (A) Graph represents the mean percentage of Ki67-positive cells ± SD in 3 DM-tumours and DV-tumours (day 14) *$p < 0.001$. (B) Representative pictures of Ki67-stained sections of a DM-tumour and a DV-tumour at 400x magnification. (C) Human-specific phospho-intracellular kinase array. Graph represents the mean signal intensity (AU) ± SD of significantly upregulated or downregulated phospho-kinases ($p < 0.05$) in DV-tumours and DM-tumours (day 14). (D) Close-up of array expression of c-Jun, eNOS and p27 in a representative example of a DV-tumour and a DM-tumour.

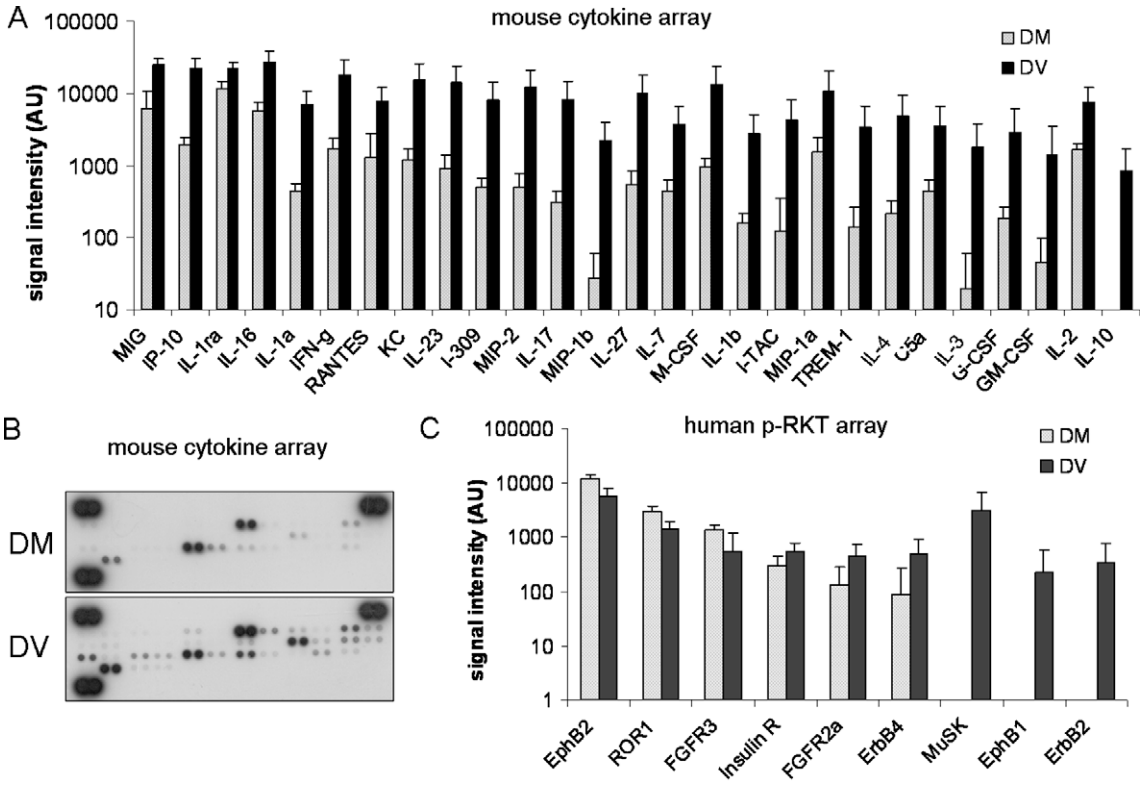

Fig. 5 – Proteome profiler arrays: (A) Mouse-specific cytokine arrays and (C) Human-specific phospho-receptor tyrosine kinase (RTK) arrays. Graphs represent the mean signal intensity (AU) ± SD of significantly upregulated or downregulated cytokines (A), or phospho-RTKs (C) ($p < 0.05$) in DV-tumours and DM-tumours (day 14). (B) Representative example of a cytokine array of a DV-tumour and a DM-tumour.

STAT proteins. For example, IL-4-induced proliferation is mediated by STAT-6.[17] IL-4 binds to the IL-4-receptor (IL-4R), which is known to be expressed by Daudi tumour cells.[18] We confirmed IL-4R expression on Daudi tumour cells by conventional PCR (data not shown). Together, these data show that stroma-derived cytokines are upregulated in tumours overexpressing VEGF-A, suggesting that VEGF-A exerts its effect on lymphoma tumour growth via a paracrine loop.

Next, we validated this hypothesis by investigating whether the upregulation of cytokines in DV-tumours is associated with alterations in the activity of receptor tyrosine kinase signalling. In DV-tumours, the levels of phosphorylated ErbB-4 (EGFR), ErbB-2 (EGFR), FGFR-2a, EphB-1, MuSK and InsulinR were significantly increased compared to those in control tumours ($p < 0.05$) (Fig. 5C). Ligand-binding to EGFR is known to activate STAT proteins.[19] Using conventional PCR we confirmed the expression of EGF (EGFR-ligand) in Daudi tumour cells (data not shown). The levels of other phosphorylated receptors were significantly decreased in DV-tumour compared to those in DM-tumours (EphB-2, ROR-1 and FGFR-3) (Fig. 5C). The levels of phosphorylated VEGF-receptors were not different in DV-tumours compared to those in control tumours (data not shown).

4. Discussion

In this study, we showed that VEGF-A overexpression by Daudi lymphoma tumours significantly increased lymphoma tumour growth *in vivo*. This was accompanied by increased tumour angiogenesis and an increased fraction of proliferating tumour cells. VEGF-A-tumours were characterised by upregulation of phosphorylated STAT-4 and STAT-6 and downregulation of phosphorylated $p27^{KIP1}$, a crucial cell cycle inhibitor. This was accompanied by increased levels of phosphorylated receptor tyrosine kinases, including EGFR, a known upstream regulator of STAT proteins. In addition, we demonstrated that tumour-derived VEGF-A activates mouse-derived stromal cells in a way that mouse-derived cytokines are upregulated in VEGF-A-tumours compared to those in control tumours. All together, our results indicate an important role for the tumour microenvironment in paracrine promotion of lymphoma tumour growth in response to tumour-derived VEGF-A, which is mediated by STAT proteins and $p27^{KIP1}$. Fig. 6 shows a provisional scheme of the pathway of VEGF-A-promoted tumour growth.

In patients with NHL, increased levels of circulating VEGF-A have been associated with progressive disease.[6] In our model, VEGF-A overexpression by the tumour resulted in detectable levels of plasma VEGF-A, and was associated with increased tumour growth, underlining the feasibility of our model to study human lymphoma growth. In this study, tumours overexpressing VEGF-A were associated with increased levels of phosphorylated STAT proteins and decreased levels of phosphorylated $p27^{KIP1}$. $p27^{KIP1}$ is a target gene of STAT proteins, and the expression of $p27^{KIP1}$ in lymphomas is inversely related with proliferation, except for mantle cell-type

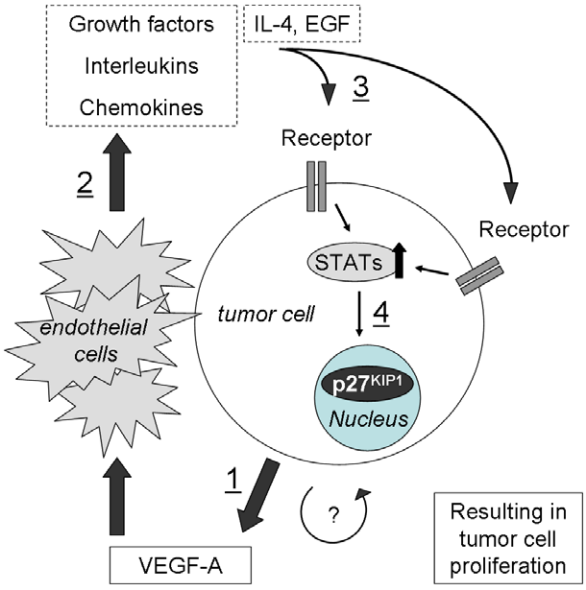

Fig. 6 – Provisional scheme on the pathways involved in VEGF-A-promoted tumour growth. VEGF-A production in the tumour results in an increased fraction of endothelial cells in the tumour microenvironment (1). These tumour endothelial cells produce a variety of factors (growth factors, interleukins and chemokines) (2) that act on tumour cells by binding to receptors that are present on the tumour cell membrane (3). Activated receptors induce signal transduction pathways (4), which results in activation of STAT proteins and inhibition of $p27^{KIP1}$. This, in turn, results in increased tumour cell proliferation, which may explain the increased growth of VEGF-A overexpressing tumours in our model.

lymphoma.[20] Lack of $p27^{KIP1}$ in $p27^{KIP1}$-knockout mice has been associated with lymphoproliferative dysregulation.[21,22] Moreover, loss of $p27^{KIP1}$ is indicative of poor outcome (shorter survival) in non-hodgkin lymphoma (NHL) patients.[23] Recently, Zhao and colleagues reported a negative relation between $p27^{KIP1}$ and Ki67 in NHL patients; low levels of $p27^{KIP1}$ were associated with high levels of Ki67 after immunohistochemical staining of tumour sections.[24] Interestingly, we also found increased Ki67-staining in tumours overexpressing VEGF-A. To our knowledge, our study is the first to show that the effect of VEGF-A on lymphoma growth is mediated by tumour STAT proteins and $p27^{KIP1}$.

VEGF-A is known to be the most important mediator of angiogenesis. In our study, we observed that VEGF-A overexpression in the tumour indeed was associated with significantly increased micro vessel density (MVD). In general, blood vessels not only supply oxygen and nutrients to the tumour cells but also provide various endothelium-derived growth factors that are of potential benefit for tumour cells. In our study, we found many stromal (mouse) derived chemokines, cytokines (such as G-CSF and GM-CSF) and many interleukins (such as IL-4 and IL-6) to be upregulated in response to VEGF-A overexpression in the tumour. Exposure of human endothelial cells to VEGF-A has been shown to result in increased production of cytokines, including IL6, G-CSF, GM-CSF and M-CSF.[25,26] Besides endothelial cells other tumour stromal cells also can contribute to cytokine, interleukin and growth factor production upon VEGF-A stimulation. These stroma-derived cytokines, in turn, can act as growth factors for lymphoid malignant cells, suggesting paracrine interactions between haematopoietic malignant cells and the newly generated endothelium.[25,26] In addition, interleukins are mainly described in relation to inflammation, a process that promotes progressive tumour growth and angiogenesis.[27] Together, these results indicate an important role for the tumour stroma in promoting lymphoma tumour growth via paracrine mechanisms. Besides paracrine mechanisms, VEGF-A has been shown to contribute to leukaemia and lymphoma tumour growth by autocrine stimulation.[12,28,29] In our study, however, despite a ~90-fold induction of VEGF-A in the Daudi cell line overexpressing VEGF-A, *in vitro* growth of these cells was similar to that of control Daudi cells. Moreover, Daudi cells lacked expression of VEGFR-2, suggesting that the VEGF-A-promoted tumour growth observed in our *in vivo* model is not the result of autocrine mechanisms.

Despite improvement of treatment strategies in the last decades, about 20.3% of children and about 31% of adults with NHL still die from their diseases within 5 years after diagnosis,[1] underlining the relevance of additional research to improve lymphoma treatment strategies. Our study reveals VEGF-A, STAT proteins and $p27^{KIP1}$ as potential additional targets in the treatment of Burkitt lymphoma. Interestingly, the tyrosine kinase inhibitor AGL2592 has been shown to induce persistent growth arrest and apoptosis in human NHL cell lines by inhibition of STAT3 phosphorylation.[30] Moreover, inhibition of STAT3 phosphorylation sensitised the NHL cell line 2F7 to a wide range of chemotherapeutic drugs, demonstrating the potential of inhibiting STAT pathways to overcome drug resistance in NHL.[31] Recently, single and multicentre phase II clinical studies showed that the proteasome inhibitor bortezomib (which allows preventing, amongst others, the degradation of $p27^{KIP1}$, thereby inhibiting cell cycle progression) can be used as additional drug in the treatment of mantle cell and follicular lymphoma.[32–35] Inhibition of VEGF-A/VEGFR signalling in lymphoma has shown to be promising in preclinical studies.[12,36,37] Moreover, in humans, the results of a phase II clinical trial with bevacizumab, a monoclonal antibody against VEGF-A, has already shown well-tolerated and prolonged stabilisation of disease in patients with relapsed, aggressive NHL.[38] Combining conventional chemo- or immunotherapy regimens with drugs targeting the tumour microenvironment may be of benefit for the treatment of lymphoma.

In conclusion, we demonstrated that VEGF-A significantly increased Burkitt lymphoma tumour growth and tumour vessel formation. Our results indicate an important role for the tumour microenvironment in paracrine promotion of lymphoma tumour growth in response to tumour-derived VEGF-A, by activation of STAT proteins and inhibition of $p27^{KIP1}$ in tumour cells. Our study reveals VEGF-A and STAT proteins as potential additional targets in the treatment of lymphoma.

Conflict of interest statement

None declared.

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