Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology
Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.13090

Immune cells regulate VEGF signalling via release of VEGF and antagonistic soluble VEGF receptor-1

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Accepted for publication 28 November 2017
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Summary

Vascular endothelial growth factor (VEGF) is an important regulator of physiological and pathological angiogenesis. Besides malignant and stromal cells, local immune cells shape VEGF signalling in the tumour microenvironment. Aminobisphosphonates such as zoledronic acid (Zol) are drugs known to inhibit osteoclast activity and bone resorption, but also have immunomodulatory and anti-tumour effects. These properties have been linked previously to the down-regulation of VEGF and interference with tumour neo-angiogenesis. It was therefore surprising to find that treatment with Zol in combination with low-dose interleukin (IL)-2 increased serum VEGF levels in cancer patients. In this study we aimed to characterize the effect of Zol and IL-2 on VEGF signalling of blood-derived immune cells *in vitro*. Upon stimulation with IL-2, T cells and natural killer (NK) cells increase production of VEGF consecutively to the release of proinflammatory interferon (IFN)-γ, and Zol accelerates this response specifically in γδ T cells. VEGF can, in turn, be antagonized by soluble VEGF receptor (sVEGFR)-1, which is released depending on stimulatory conditions and the presence of monocytes. Additionally, malignant cells represented by leukaemia and lymphoma cell lines produce VEGF and some release sVEGFR-1 simultaneously. Our findings indicate a mechanism by which the VEGF and the sVEGFR-1 production by immune cells regulates local VEGF signalling. Therefore, immunotherapeutic interventions may enable both pro- as well as anti-tumour effects via immune cell-mediated alterations of VEGF homeostasis.

Keywords: γδ, interleukin-2, monocyte, soluble VEGF receptor, VEGF

Introduction

Immune cells impact cancer growth via direct interaction with malignant cells and also indirectly by modulation of the tumour microenvironment [1]. It has been established that the density and composition of tissue infiltrating cells, for example enrichment with T cells or macrophages, are correlated to clinical outcome in many types of cancer [2]. Owing to the heterogeneity and complexity of immunological responses towards malignancies, the underlying local mechanisms are not understood completely.

Immune cells can be a double-edged sword in the context of cancer, as they are able to promote as well as counteract tumour growth [1]. In this regard it has been demonstrated that even though γδ T cell infiltration in the tumour tissue represents a positive prognostic marker in most types of cancer [2], specific subpopulations of γδ T cells have pro-tumour functions, such as the stimulation of tumour angiogenesis [3,4]. This is relevant, as the utilization of tumour-targeting γδ T cells is a promising concept in the field of cancer immunotherapy [5–7]: γδ T cells exhibit strong cytotoxic activity against a variety of cancer cell types [8,9] and can be activated safely *in vivo* using amino-bisphosphonates or synthetic phosphoantigens and low-dose interleukin (IL)-2 [9]. Bisphosphonates, such as zoledronic acid (Zol), have additional effects in cancer patients [10] and results reported by Santini *et al.* indicated a connection to the down-regulation of angiogenic growth factors such as vascular endothelial growth factor (VEGF) [11–13].

The VEGF family comprises several multi-functional signal molecules, of which VEGF-A is the best-characterized

member. VEGF-A (referred to henceforth as VEGF) is involved in many physiological and pathophysiological processes [14]: it is essential for embryonic development [15], stimulates angiogenesis, increases vascular permeability and enables tumour neovascularization [16]. Current clinically approved anti-VEGF therapies target pathological neo-angiogenesis and are now used frequently in cancer [17]. However, compared to the high expectations that emerged from experiments with mice, the current anti-VEGF drugs show only modest activity against human malignancies [14,17]. The reason for this discrepancy is unclear, but several reports indicate that human VEGF signalling in the context of cancer and inflammation is complex [18,19] and may be more heterogeneous than thought previously. For example, a retrospective analysis documented recently that in renal cell cancer only a subgroup of patients with certain inflammation patterns of tumour-infiltrating myeloid and T cell benefited from additional treatment with bevacizumab [20].

A possible linkage between the activity of T cells and VEGF signalling in cancer prompted us to assess the changes in VEGF serum levels during a recent clinical study that involved the use of Zol as an immunostimulatory drug. This study was designed primarily to evaluate the safety and anti-tumour efficacy of γδ T cells in adults with advanced solid and haematological malignancies [21]. Surprisingly, we found a substantial and fast increase in VEGF serum levels following treatment with Zol plus low-dose IL-2 in several patients [21], although we would have expected the contrary.

There are only few publications addressing VEGF production in human lymphocytes [22–25] or specifically in γδ T cells [26,27]. Due to the importance of VEGF signalling in human physiology and disease we therefore aimed to examine the VEGF production from primary immune cells as well as different leukaemia and lymphoma cell lines following treatment with Zol and IL-2.

Material and methods

Cell isolation and culture

All procedures were conducted in accordance with the 1975 Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) from healthy male and female volunteers, aged between 20 and 60 years and with normal peripheral blood counts, were isolated by density gradient centrifugation with Biocoll (Biochrom, Darmstadt, Germany); 2×10^5 PBMCs were cultured in 96-well microtitre plates using standard medium consisting of RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin + streptomycin (all from Biochrom). RPMI was 'low endotoxin' grade and fetal bovine serum contained less than 3 EU/ml endotoxin. One hundred U/ml IL-2 with or without 1 μM Zol (both Novartis, Nuremberg, Germany) were added on day 0 as indicated (+ IL-2 or Zol + IL-2). Cells were reseeded at 2×10^5 PBMCs per well on days 7 and 9. Additional IL-2 (100 U/ml) was added on days 7 and 9 to the previously Zol + IL-2- and IL-2-stimulated cultures. The leukaemia and lymphoma cell lines KG-1, THP-1, K562, HL-60, Daudi, U266 and Reh were obtained from the German collection of microorganisms and cell culture (DSMZ, Braunschweig, Germany) and cultured in standard medium. Cell counts and cell viability were established using a haemocytometer and the trypan blue exclusion method. Cell viability rate was $> 92\%$ in all experiments.

Quantification of soluble factors and interference

VEGF, soluble VEGF receptors 1 and 2 (sVEGFR-1 and sVEGFR-2) and interferon (IFN)-γ concentrations in the cell culture supernatants were determined using a Tecan Sunrise plate reader and enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The release rates in the indicated interval were normalized to 10^6 cells per 24 h in order to enhance comparability and to compensate for differences in cell proliferation as well as culture duration. Therefore, the concentration of the soluble factors were measured in the supernatants at the end of the respective period and related to the mean cell count, established by cell counting. For evaluation of cell culture supernatants, interference with VEGF detection, the supernatants and the calibrator diluent were each spiked with 400 pg/ml recombinant human VEGF (rhVEGF) (R&D Systems) and incubated for 30 min at room temperature before determination of VEGF concentration by ELISA. The level of interference is expressed as a percentage, based on the values measured in the calibrator diluent (VEGF concentration sample/VEGF concentration in calibrator diluent $\times$ 100).

Immunomagnetic selection

γδ T cells were enriched from Zol + IL-2-stimulated PBMCs on day 7 by positive immunomagnetic selection using the γδ T cell selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were enriched or depleted from PBMCs on day 0 using the CD14 monocyte kit (Miltenyi Biotec). All selections or depletions were performed using the MidiMACS system (Miltenyi Biotec), according to the manufacturer's instructions. The enrichment resulted in a purity of $> 99\%$ for enriched γδ T cells, $> 92\%$ $CD14^+$ cells in the monocyte-enriched and $< 0{\cdot}1\%$ $CD14^+$ cells in the monocyte-depleted cell fraction, as confirmed by flow cytometry.

Flow cytometry

A FC500 flow cytometer and CXP software (both Beckman Coulter, Brea, CA, USA) were used for multi-colour

immunofluorescence and for intracellular staining analysis. Intracellular staining was performed using the Inside Stain kit (Miltenyi Biotec) with fluorochrome-conjugated monoclonal antibodies: phycoerythrin (PE)-conjugated anti-VEGF (R&D Systems), anti-IFN-γ (Miltenyi Biotec) and isotype control antibody immunoglobulin (Ig)G1 (Miltenyi Biotec). Specific staining was expressed as delta mean fluorescence intensity (MFI) calculated by ΔMFI = MFI(target) – MFI(control). For identification of PBMC subpopulations cells were stained with combinations of anti-T cell receptor αβ-fluorescein isothiocyanate (FITC) (Miltenyi Biotec), CD14-FITC, anti-T cell receptor γδ-FITC, anti-CD3-extracellular domain (ECD) and anti-CD56-PE-cyanin 5.1 (PC5) monoclonal antibodies (all Beckman Coulter). PBMCs and cell lines were stained for expression of vascular endothelial growth factor receptors (VEGFR)-1, −2 and −3 using the PE-conjugated monoclonal antibodies: anti-VEGFR-1, −2 and −3 and isotype control antibody IgG1 (all from R&D Systems). The expression of VEGF receptors on cell lines was classified according to the MFIs quotient [MFI(VEGFRx)/MFI(isotype control)]: ≤ 2: −, > 2–3: (+), ≥ 3–10: +, > 10: ++.

Fluorescence microscopy

Washed cells were spun down on slides and fixed with 4% paraformaldehyde (Santa Cruz Biotechnology, Heidelberg, Germany). Cytospin specimens were rinsed with Tris-buffered saline (TBS) before being overlaid with blocking buffer (5% donkey serum diluted in TBS + 0·1% 1-TritonX100) for 20 min at room temperature. Incubation with primary antibodies [mouse anti-VEGF-PE, clone 23410 (R&D Systems), human anti-VEGF, bevacizumab (Roche, Basel, Switzerland)] or respective isotype antibodies (human IgG1, kappa purified myeloma protein (Sigma, Munich, Germany) and mouse IgG2A-PE, clone 20102 (R&D Systems), all diluted 1 : 10, was performed in a humid chamber overnight at 4°C. Slides were rinsed with TBS and incubated with donkey anti-mouse Alexa-488 or donkey anti-mouse-Cy3 (both from Jackson ImmunoResearch, West Grove, PA, USA) coupled secondary antibodies (diluted 1 : 200 in blocking buffer) for 1 h at room temperature. Cell nuclei were counterstained using 4′,6′-diamidino-2-phenylindole (DAPI) from Roche, diluted 1 : 100. Labelled cells were washed several times with TBS before mounting with fluoromount (Southern Biotech, Birmingham, AL, USA). The images were taken adopting a DMi8 confocal laser scanning microscope (Leica, Wetzlar, Germany).

Statistical analysis

Data were depicted as mean ± standard deviation (s.d.). Levels of significance are calculated by Kruskal–Wallis test and Dunn's test. Correlation is expressed with Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

VEGF release by PBMCs

First we evaluated the VEGF release kinetics of human PBMCs of healthy donors during unstimulated *in-vitro* culture and compared it to treatment with IL-2. In addition, Zol + IL-2 was used for specific activation of γδ T cells (Fig. 1a). VEGF release was decreased between days 0 and 2 in the Zol + IL-2-stimulated cultures and the release of VEGF until day 7 was low in any of the different types of stimulation. Zol + IL-2-treated PBMCs then depicted a significant increase in VEGF release compared to the unstimulated and IL-2-stimulated cells between days 7 and 9. Following day 9, both IL-2- and Zol + IL-2-stimulated cells produced more VEGF than unstimulated controls. Due to their sensitivity towards stimulation with Zol + IL-2, γδ T cells might secrete higher amounts of VEGF compared to other PBMCs. To test this hypothesis, we enriched γδ T cells from Zol + IL-2-treated PBMCs after 7 days. Results indicate that VEGF release from γδ T cells is similar to unselected PBMCs treated with Zol + IL-2. Secretion of VEGF was lower than the release of the classical proinflammatory cytokine IFN-γ, which peaks around day 2 when PBMCs are stimulated with Zol + IL-2 (Fig. 1b).

Intracytoplasmic VEGF in PBMCs

To analyse the production of VEGF by lymphocytes in detail, intracytoplasmic VEGF content of PBMCs subpopulations were compared between days 1 and 14 using identical treatment regimens (Fig. 1c,d). We found that IL-2 alone induces VEGF production after day 4 similarly in NK, αβ and γδ T cells. Additional stimulation with Zol leads to a more rapid and more pronounced VEGF production, predominantly in γδ T cells. Directly after isolation and before stimulation, monocytes depict the highest cytoplasmic VEGF signal of all PBMCs (Fig. 1e), but the amount of monocytes decreases very quickly during culture (data no shown).

However, VEGF release of PBMCs until day 7 was unexpectedly low when compared to their cytoplasmic VEGF content, depicted by flow cytometry and validated by fluorescence microscopy (Fig. 1f). Taken together, our results show that IL-2 stimulates VEGF release from PBMCs consecutively to the classical proinflammatory cytokine IFN-γ. Additional Zol accelerates this production of VEGF selectively in γδ T cells. However, the kinetic of VEGF release measured in the supernatant did not match the results of the intracytoplasmic VEGF measurement.

Expression of VEGF receptors by PBMCs

Due to the discrepancy between intracytoplasmic VEGF content and VEGF release, we screened for VEGFRs which may bind VEGF and thereby interfere with its detection in

the supernatant. CD14$^+$ monocytes depicted VEGFR-1 and VEGFR-3 but no VEGFR-2 expression, whereas NK, αβ and γδ T cells had no detectable VEGFRs on their cell surface following isolation (Fig. 2a). This was unaltered by culture and stimulation with IL-2 or Zol + IL-2 until day 14 (data not shown). Correspondingly, rhVEGF added to the cultures did not alter the proliferation (Fig. 2b) or composition of lymphocyte populations (Fig. 2c), even at very high concentrations.

Release of soluble VEGFR-1 by PBMCs

Even though the low expression of cell membrane-anchored VEGFRs on monocytes may contribute to the mismatch between intracytoplasmic VEGF and free VEGF in the supernatant, we suspected the additional release of a soluble factor that interferes with the detection of VEGF. To test this concept we added 400 pg/ml rhVEGF on cell culture supernatants of PBMCs harvested on day 7 and found an

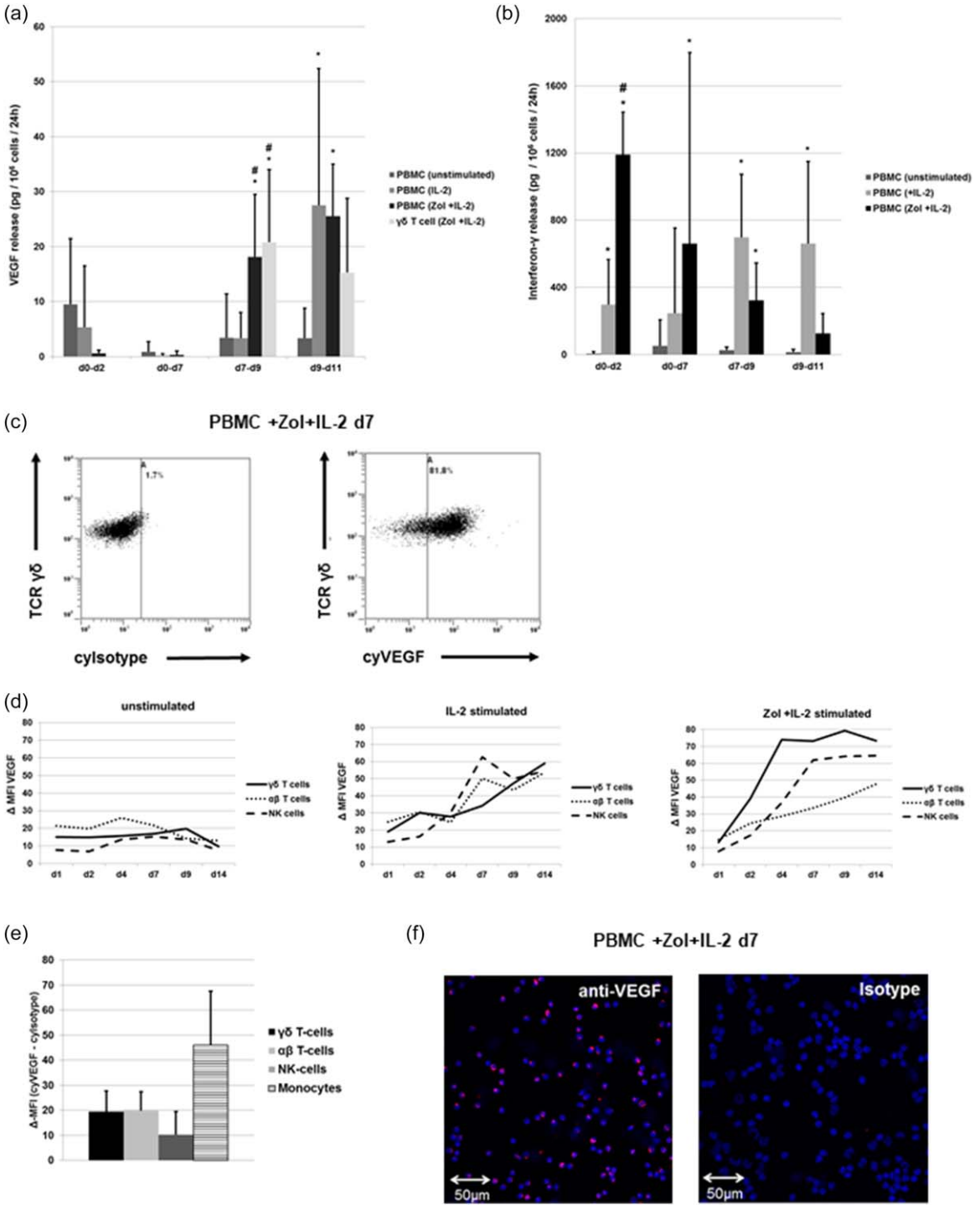

Fig. 1. Vascular endothelial growth factor (VEGF) and interferon (IFN)-γ production by peripheral blood mononuclear cells (PBMCs). (a) VEGF release/10^6 PBMCs or γδ T cells/24 h in the indicated intervals. Concentrations of VEGF were measured in the supernatants at the end of the respective interval using enzyme-linked immunosorbent assay (ELISA) and were normalized with regard to cell count and culture time. Shown are the results from four different treatment regimens: (1) unstimulated, (2) interleukin (IL)-2 (100 U/ml IL-2 added on days 0, 7 and 9), (3) zoledronic acid (Zol) + IL-2 (1 μM Zol added on day 0 and IL-2 100 U/ml added on days 0, 7 and 9) and (4) Zol + IL-2-stimulated γδ T cells (immune-magnetically selected on day 7 from previously Zol + IL-2 stimulated PBMCs with addition of IL-2 100 U/ml at days 7 and 9). (b) IFN-γ release/10^6 PBMCs/24 h in the indicated interval. Concentrations of IFN-γ were measured in the supernatants at the end of the respective period and normalized with regard to cell count and culture time. Shown are the results from three different treatment regimens: (1) unstimulated, (2) IL-2 (100 U/ml IL-2 added on days 0, 7 and 9, (3) Zol + IL-2 (1 μM Zol added on day 0 and IL-2 100 U/ml added on days 0, 7 and 9). (c) Flow cytometry analysis of either intracytoplasmic isotype or VEGF staining results from Zol + IL-2-stimulated PBMC measured on day 7 and gated on γδ T cells. T cell receptor (TCR)-δ, cytoplasmic Isotype (cyIsotype), cytoplasmic VEGF (cyVEGF). (d) Kinetic of intracytoplasmic VEGF content for three different treatment regimens (from left to right: unstimulated, IL-2 stimulated and Zol + IL-2-stimulated PBMCs), depicted as mean Δ-mean fluorescence intensity (MFI) [MFI(VEGF) – MFI(isotype control] in the respective populations (γδ T cells, αβ T cells and natural killer (NK) cells) between days 1 and 14 (d1 – d14). (e) Flow cytometry analysis showing Δ-MFI of intracytoplasmic VEGF content of freshly isolated PBMCs. Δ-MFI = MFI(VEGF) – MFI(isotype control). (f) Immunofluorescence analysis of the VEGF content in Zol + IL-2-stimulated PBMC on day 7 with anti-VEGF or isotype primary antibody and cyanin 3 (Cy3)-coupled secondary antibody. All data are presented either as a representative example of at least three independent experiments or depicted as mean ± standard deviation (s.d.) of at least three independent experiments. $^*P < 0{\cdot}05$ compared to unstimulated control, $^{\#}P < 0{\cdot}05$ compared to stimulation with IL-2. [Colour figure can be viewed at wileyonlinelibrary.com]

interference of 95·1% for unstimulated, an interference of 100% for IL-2-stimulated and an interference of 89·0% for Zol + IL-2-stimulated conditions (Fig. 2d).

Monocytes are a potential source of sVEGFR-1 which could be responsible for the observed interference. Therefore, we compared the release of sVEGFR-1 from CD14-depleted and non-depleted PBMCs cultured under unstimulated, IL-2- and Zol + IL-2-stimulated conditions (Fig. 2e). We found that monocytes are essential for the production of sVEGFR-1 under any of the tested conditions, as CD14 depletion strongly reduces sVEGFR-1 release. In summary, we found monocytes or monocyte-derived cells to be capable of (1) producing VEGF directly (Fig. 1e), but also (2) inhibiting its effects by facilitating the production of antagonistic sVEGFR-1 (Fig. 2e) under certain stimulatory conditions. These findings are complementary to earlier reports on the functions of monocytes using a different experimental set-up and stimulations [28,29]. In line with this, we found a significantly increased release of VEGF by CD14-depleted compared to undepleted PBMCs between days 0 and 7 (Fig. 3a). Additionally, conditioned medium harvested from IL-2-stimulated, monocyte-depleted PBMCs on day 7 supported sVEGFR-1 production dose-dependently by isolated monocytes (Fig. 3b).

Finally, we found that sVEGFR-1 in the supernatants of stimulated (IL-2, Zol + IL-2) and unstimulated PBMCs correlated positively with the ability to mask detection of spiked rhVEGF in these supernatants (Fig. 3c), supporting our interpretation that sVEGFR-1 is indeed responsible for the observed interference with VEGF. sVEGFR-2 could not be detected in the supernatants of stimulated or unstimulated PBMCs (data not shown). Together, our results show that sVEGFR-1 release from PBMCs is monocyte-dependent and stimulated by IL-2. As well as sVEGFR-1, membrane standing VEGFR-1 on monocytes might impact VEGF homeostasis by binding VEGF.

VEGF release by leukaemia and lymphoma cell lines

As VEGF is a signal molecule for monocytes and cells of the haematopoietic system, we asked whether secretion of VEGF and/or release of an antagonistic sVEGFR-1 is a more common characteristic, applicable to other haematopoietic cell types. Therefore, we evaluated the VEGF release of different leukaemia and lymphoma cell lines under the same culture conditions as PBMCs.

Six of seven tested cell lines released VEGF which was unaffected by treatment with IL-2 or Zol + IL-2 (Fig. 4a). However, measurement of intracytoplasmic VEGF depicted a strong discrepancy between intracytoplasmic VEGF content and VEGF release in one of the cell lines: the myeloid leukaemia cell line KG-1 released no detectable VEGF, but high amounts of VEGF were found in its cytoplasm using flow cytometry (Fig. 4b) and fluorescence microscopy (Fig. 4c). Similar results were obtained with a different primary VEGF antibody and retention of free VEGF inside the cells is unlikely, as cell lysate comprised virtually no detectable free VEGF (data not shown).

Expression of VEGF receptors by leukaemia and lymphoma cell lines

We next measured expression of VEGF-receptors on leukaemia and lymphoma cell lines: as well as Daudi cells, all tested cell lines expressed at least VEGFR-3 and some additionally expressed VEGFR-1 or VEGFR-2. KG-1 expressed all three VEGFRs and had the highest VEGFR-1 expression of all cell lines (Fig. 4d and Table 1). In summary, the examined cell lines are diverse regarding their expression of VEGF and VEGF receptors. KG-1 is of special interest, as it produces VEGF, expresses high amounts of different VEGFRs and releases soluble VEGF binding molecules.

Interference of culture supernatants with the detection of VEGF

Similarly to the results obtained with PBMCs, we suspected the release of sVEGFR-1 to interfere with the detection of VEGF. Therefore, we added 400 pg/ml rhVEGF to KG-1 supernatants, which were harvested after 48 h of culture, and we found a 98·5% interference with the detection of the added VEGF (Fig. 4e).

Release of soluble VEGFR-1 by leukaemia and lymphoma cell lines

Three of seven tested leukaemia and lymphoma cell lines released amounts of sVEGFR-1 comparable to PBMCs or higher (Fig. 4f). The highest quantities were produced by KG-1, which is in line with the observation that VEGF is detectable in the cytoplasm but masked completely in the cell culture supernatant. K562 also depicts a substantial production of sVEGFR-1, together with high amounts of free VEGF in its culture supernatant (Fig. 4a) and a high

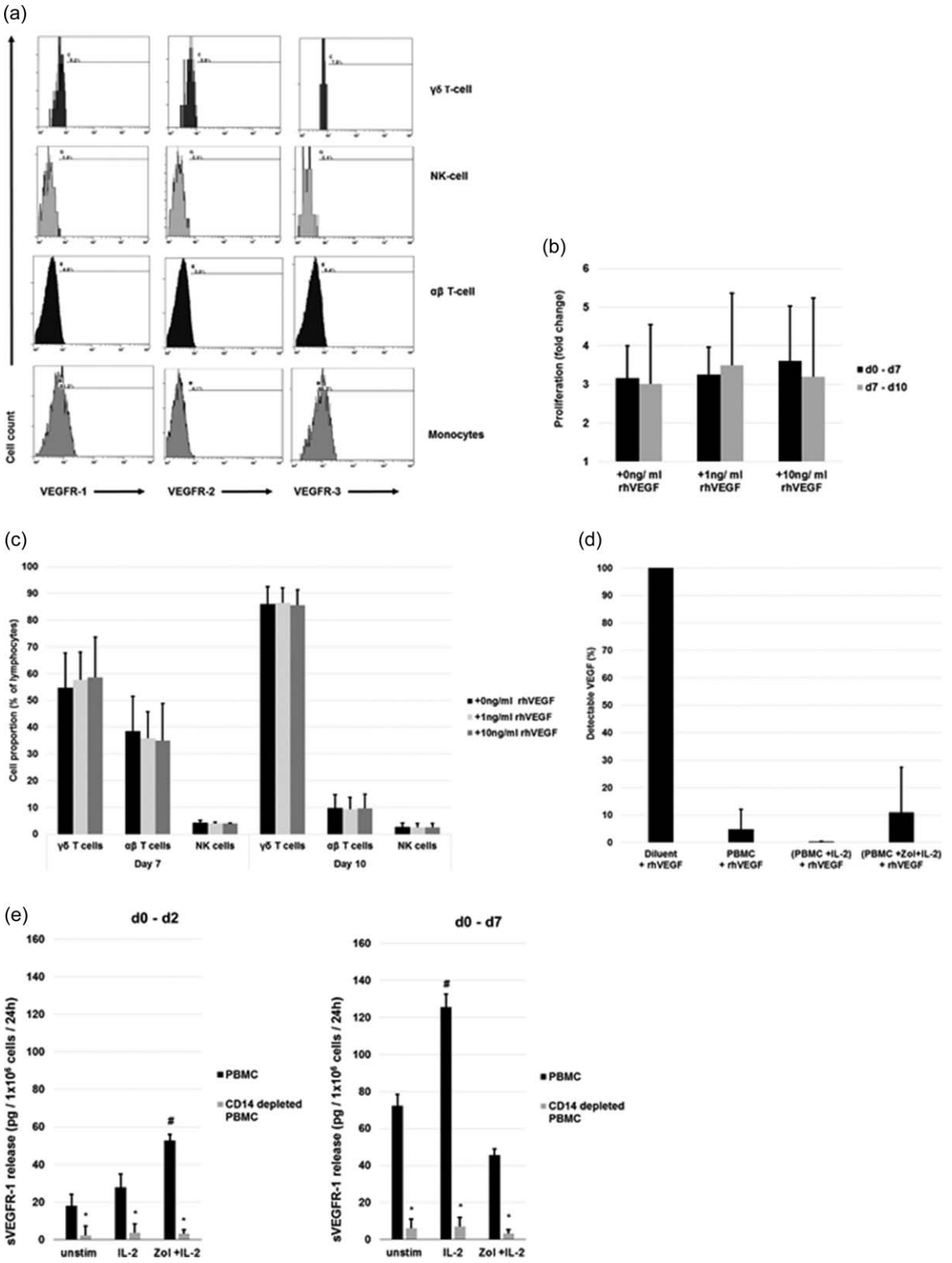

Fig. 2. Expression, function and interference of vascular endothelial growth factor receptors (VEGFRs) on peripheral blood mononuclear cells (PBMCs). (a) Flow cytometry analysis of VEGFR-1, −2 and −3 expression on freshly isolated, unstimulated PBMCs gated on different subpopulations. Positivity is defined according to the isotype antibody control staining. (b,c) Effect of recombinant human VEGF (rhVEGF) on proliferation of stimulated PBMCs. Different concentrations of rhVEGF were added to a culture of zoledronic acid (Zol) + interleukin (IL)-2 stimulated PBMCs on days 0 or 7. (b) Proliferation of cells between days 0–7 (rhVEGF added on day 0) and days 7–10 (rhVEGF added on day 7) as fold change from initial cell count. (c) γδ T cell, αβ T cells and natural killer (NK) cells as a fraction of the gated lymphocytes after 7 days (rhVEGF added on day 0) and 10 days (rhVEGF added on day 7) of culture. (d) Soluble factors interfere with detection of VEGF. Detectable free VEGF by enzyme-linked immunosorbent assay (ELISA) after spiking of 400 pg/ml rhVEGF into the supernatants of differently stimulated PBMCs compared to spiking the same amount into calibrator diluent. (e) Quantification of sVEGFR-1 in cell culture supernatants of differently stimulated PBMCs, with or without prior depletion of $CD14^+$ cells. Soluble VEGFR (sVEGFR)-1 release/10^6 cells/24 h between days 0–2 and 0–7 was measured at the end of the respective interval in the supernatants by ELISA and normalized with regard to cell count and culture time. Shown are the results from three different treatment regimens: (1) unstimulated, (2) IL-2 (IL-2 100 U/ml added on day 0), (3) Zol + IL-2 (1 μM Zol and IL-2 100 U/ml added on day 0). All data are either presented as a representative example of at least three independent experiments (Fig. 2a) or depicted as mean ± standard deviation (s.d.) of at least three independent experiments. $^*P < 0{\cdot}05$ compared to undepleted PBMCs, $^{\#}P < 0{\cdot}05$ compared to unstimulated control.

intracytoplasmic VEGF content (data not shown). sVEGFR-2 could not be detected in the supernatants of any of the leukaemia and lymphoma cell lines (data not shown).

Discussion

VEGF released by immune cells

It has been demonstrated previously that bisphosphonate monotherapy decreases VEGF levels in cancer patients [10–12] but, surprisingly, we found later that Zol combined with low-dose IL-2 can also induce a rapid increase in VEGF serum levels of such patients [21]. Due to the temporal sequence, our hypothesis was that substantial amounts of VEGF are released by IL-2- and Zol + IL-2-stimulated immune cells. To test this concept we assessed VEGF release kinetics by human PBMCs following different stimulations *in vitro* as a model for *in-vivo* immunotherapeutic treatment. We found that IL-2 and Zol + IL-2 treatment stimulates VEGF release by immune cells consecutively, but not immediately, to the classical proinflammatory cytokine IFN-γ. Zol + IL-2 stimulation compared to IL-2 alone accelerates but does not increase peak VEGF release rate. We next analysed the intracytoplasmic VEGF production kinetic for individual types of blood immune cells. Our results are in line with former reports showing that unstimulated monocytes produce VEGF [29,30]. Additionally, γδ, αβ T cells and NK cells have a low baseline VEGF content, but all up-regulate VEGF production when stimulated with IL-2, enabling these cells to play an opposing role upon activation. The combination of IL-2 with Zol, known to elicit a proinflammatory response from γδ T cells, also accelerates selectively the production of VEGF in these γδ T cells. As VEGF expression follows the proinflammatory cytokine release it suggests a distinct, probably more downstream role in the course of immune reactions. However, the low VEGF release into the culture supernatant of immune cells until day 7 and the increase thereafter did not match the results of the intracytoplasmic VEGF measurement.

It is already known that monocytes and macrophages from patients with rheumatoid arthritis can release high amounts of VEGF [31] and Freeman *et al.* described VEGF release by lymphocytes as early as 1995 [32]. Since then, others have also addressed VEGF production by lymphocytes, although the use of mouse models and the measurement of relative cytokine alterations but without absolute numbers limit direct comparability [27,33,34]. In addition, a commercial VEGF ELISA kit, which has been used in most studies, does not take into account the level of interfering factors such as sVEGFR-1 [35]. Moreover, serum VEGF levels are linked to thrombocyte counts, probably because platelets release VEGF during blood coagulation [36]. Without consideration of these factors, measurement of VEGF might not reflect the actual situation. We analysed our earlier clinical data and did not find a correlation between the changes in serum VEGF levels and changes in platelet count (data not shown). This would have been expected if an increased platelet count was indeed the cause for an increase in serum VEGF levels during immunotherapy.

VEGFRs on immune cells

VEGF can be released by various cell types, and its signals are mediated through the tyrosine kinase receptors, VEGFR-1 (or Flt-1) and VEGFR-2 (or KDR), which are expressed by endothelial cells, monocytes and many malignant tumours [17]. Furthermore, VEGF is a signal molecule for monocytes and haematopoietic progenitor cells [37]. Analysis of VEGFRs on PBMCs showed that monocytes expressed detectable but low amounts of VEGFR-1 and VEGFR-3 and no VEGFR-2. This is consistent with previous reports demonstrating a functional role for VEGF-signalling via VEGFR-1 in the attraction and activation of monocytes [15,37,38]. In contrast to monocytes, αβ, γδ T and NK cells were negative for VEGFRs, and neither stimulation with IL-2 nor Zol + IL-2 induced expression of VEGFRs in any of these populations. The lack of detectable receptors makes it unlikely that VEGF acts directly on these cells or that they are capable of interfering

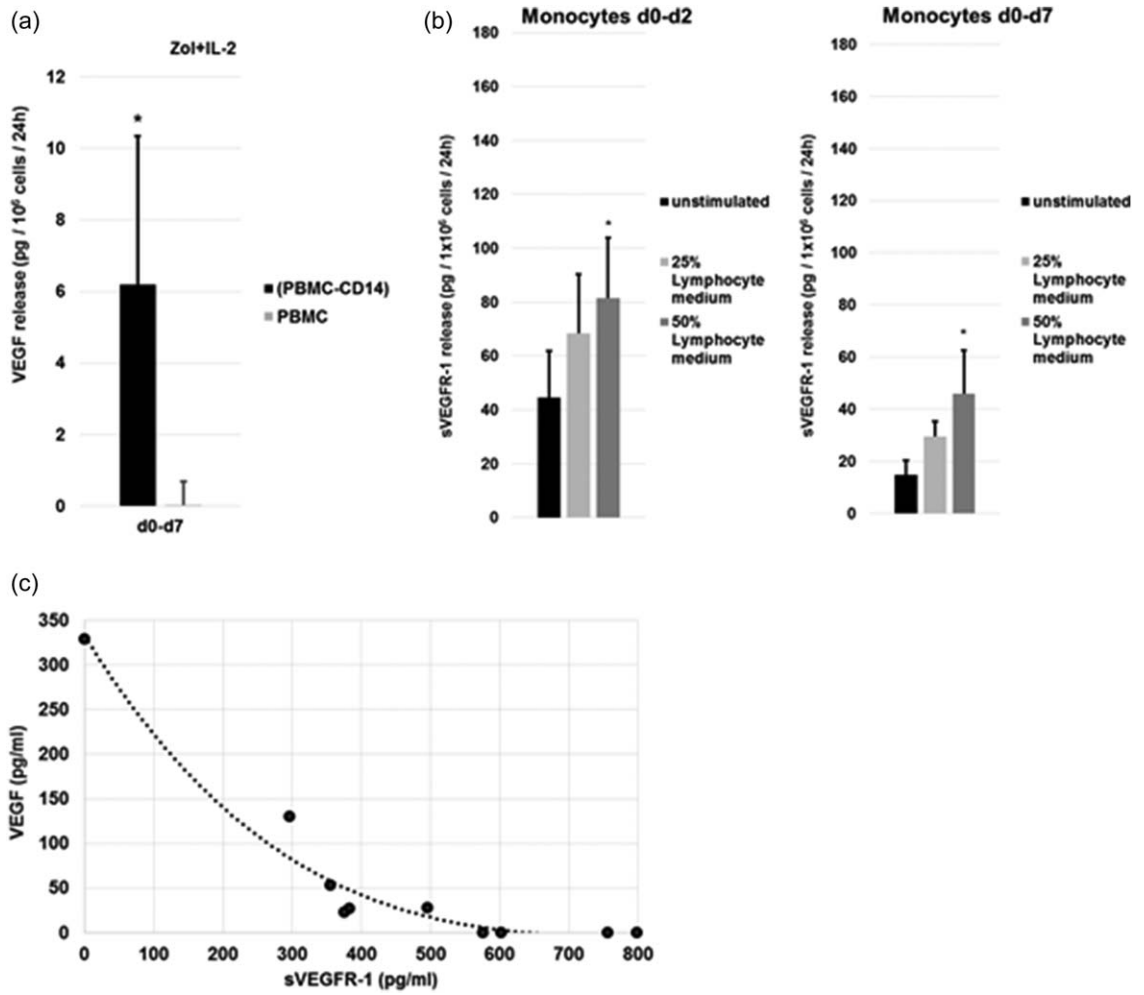

Fig. 3. Monocytes and lymphocytes influence vascular endothelial growth factor (VEGF) signalling. (a) Both CD14-depleted peripheral blood mononuclear cells (PBMCs) and undepleted PBMCs were stimulated with 1 μM zoledronic acid (Zol) and 100 U/ml interleukin (IL)-2 on day 0 and the concentrations of VEGF were measured in the supernatants on day 7. Release rates were normalized to 1×10^6cells/24 h. Data from three independent experiments are depicted as mean ± standard deviation (s.d.); $^*P < 0.05$ compared to undepleted control. (b) Effect of lymphocyte conditioned medium on the release of Soluble VEGFR (sVEGFR)-1 by enriched monocytes. Monocytes were enriched from blood PBMCs by immunomagnetic selection for CD14 on day 0 and cultured at 1×10^6 cells/ml. Shown are the results from three different treatment regimens: (1) unstimulated monocytes, (2) monocytes stimulated with 25% lymphocyte conditioned medium (addition of 25% cell-free medium which was harvested on day 7 of IL-2-stimulated, monocyte-depleted PBMC culture and (3) monocytes stimulated with 50% lymphocyte-conditioned medium (addition of 50% cell-free medium which was harvested on day 7 of IL-2-stimulated, monocyte-depleted PBMC culture). Concentrations of VEGF were measured in the supernatants at the end of the respective period, the concentration of soluble VEGFR (sVEGFR)-1 in the added medium was subtracted from this value and results normalized with regard to cell count and culture time. Data from three independent experiments are depicted as mean ± standard deviation (s.d.). $^*P < 0{\cdot}05$ compared to unstimulated control. (c) Correlation between the sVEGFR-1 concentration in supernatants of differently stimulated (unstimulated, IL-2 and Zol + IL-2) PBMCs after 7 days and the detectable free VEGF concentration by enzyme-linked immunosorbent assay (ELISA) after spiking of 400 pg/ml recombinant human VEGF (rhVEGF) into these supernatants. The reference point at 0 pg/ml sVEGFR1 is assigned to the VEGF concentration measured in the calibrator diluent of the assay. Each point represents an individual experiment. Spearman's rank correlation coefficient is $r = -0{\cdot}89$ and $P < 0{\cdot}05$.

with detection of VEGF via sequestration of VEGF by membrane-bound receptors. Accordingly, we could not find any change in proliferation of stimulated PBMCs when rhVEGF was added to PBMCs cultures. However, we did not examine subpopulations, and there are indeed reports describing expression and functional implications for either VEGFR-2, VEGFR-1 or both receptors on certain T cell subpopulations, such as an impact on cell proliferation, cytotoxicity and IFN-γ production [22–25]. The heterogeneous results may also be due to differences in the methodology or studied species [33,39,40]. In line with previous publications, we suspect that while VEGFR-2 is very important for endothelial cell functions, it is probably not for most immune cells [24,25,39,41].

VEGF signalling in leukaemia and lymphoma cell lines

Testing of leukaemia and lymphoma cell lines regarding their VEGF release *in vitro* showed that VEGF production varies widely between different haematological cell types. VEGF release was observed in similar quantities in promyeloblast/pro-myelocytic leukaemia (HL-60), THP-1 (monocytic cell/acute monocytic leukaemia), U266 (B-lymphocyte/multiple myeloma), lymphoblast/non-T, non-

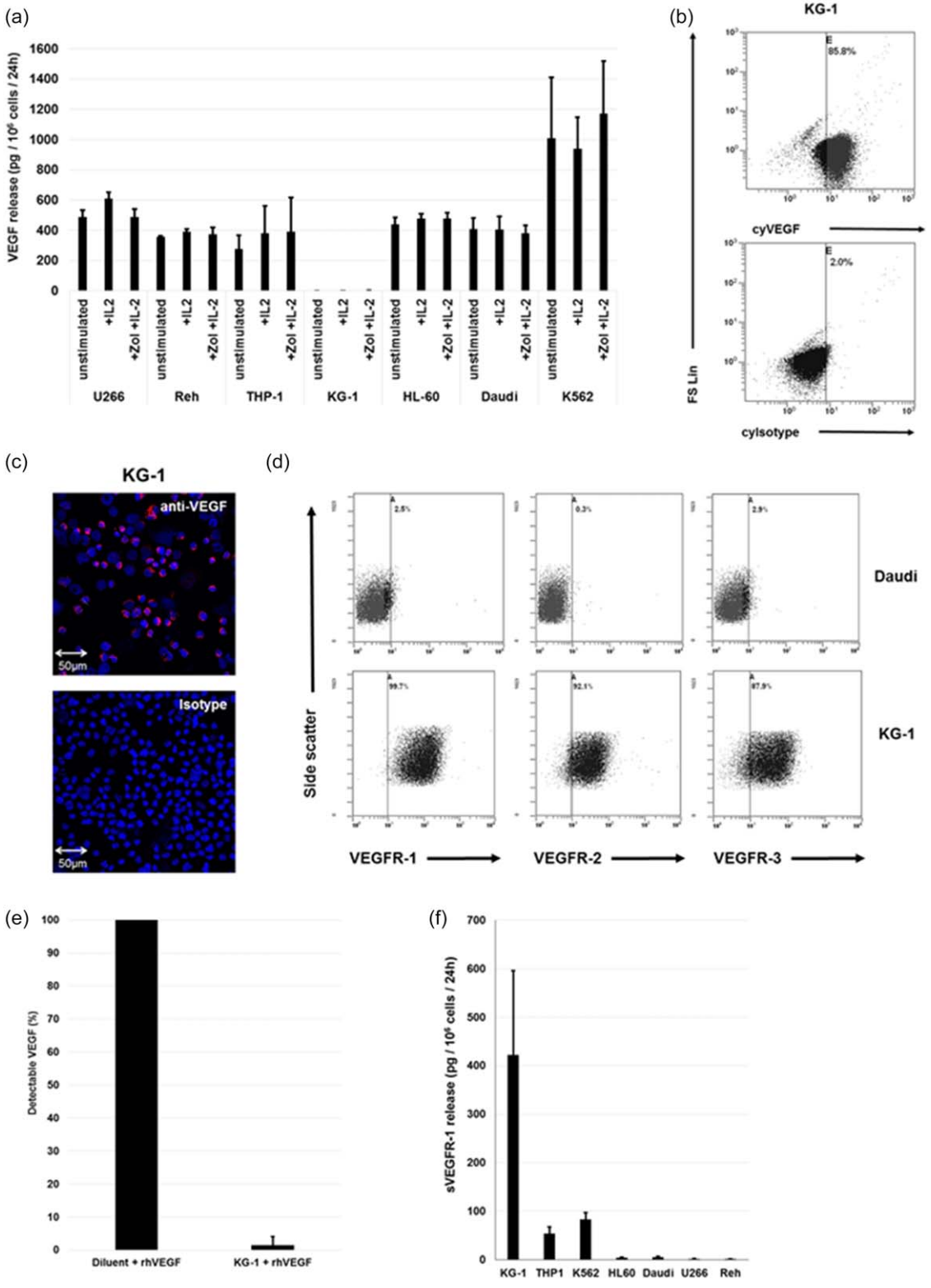

Fig. 4. Vascular endothelial growth factor (VEGF) production and VEGF receptor (VEGFR) expression by leukaemia and lymphoma cell lines. (a) VEGF release/10^6 unstimulated leukaemia or lymphoma cells/24 h and effect of interleukin (IL)-2 and zoledronic acid (Zol) + IL-2 treatment. Shown are the results from three different treatment regimens: (1) unstimulated, (2). IL-2 (100 U/ml IL-2), (3) Zol + IL-2 (1 μM Zol and IL-2 100 U/ml). Concentrations of VEGF were measured in the supernatants using enzyme-linked immunosorbent assay (ELISA) and normalized with regard to cell count and culture time. Differences in VEGF production comparing treatments were not statistically significant (b) Measurement of VEGF content in the KG-1 cell line by flow cytometry. Cytoplasmic Isotype (cyIsotype), cytoplasmic VEGF (cyVEGF), forward-scatter linear (FS Lin). (c) Immunofluorescence analysis of the VEGF content in KG-1 cell line with anti-VEGF or isotype primary antibody and cyanin 3 (Cy3)-coupled secondary antibody. (d) Flow cytometry analysis of VEGFR-1, −2 and −3 expression of two of seven cell lines: Daudi and KG-1. (e) Soluble factors interfere with detection of VEGF. Detectable free VEGF by ELISA after spiking of 400 pg/ml recombinant human VEGF (rhVEGF) into supernatants of KG-1 compared to spiking the same amount into calibrator diluent. (f) Soluble VEGF (sVEGFR)-1 release/10^6 leukaemia or lymphoma cells/24 h. Concentrations of sVEGFR-1 were measured in the supernatants by ELISA and normalized with regard to cell count. All data are either presented as a representative example of at least three independent experiments or depicted as mean ± standard deviation (s.d.) from at least three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

B acute lymphocytic leukaemia (Reh) and B-lymphoblast/Burkitt lymphoma (Daudi). Particularly high amounts of VEGF were released by K562 (erythroleukaemia/CML). Cells from KG-1 (macrophage/acute myeloid leukaemia) did not release detectable VEGF. The VEGF release rate was not altered significantly in any of the cell lines by treatment with IL + 2 or Zol + IL-2. This argues against the interpretation that Zol or IL-2 had interfered directly with the VEGF release of leukaemia and lymphoma cells and caused the changes in VEGF serum levels observed in our earlier clinical study [21].

Similarly to PBMCs, it was interesting to find a marked discrepancy between VEGF release into the culture supernatant and the semiquantitative results of the intracytoplasmic VEGF measurement in the KG-1 cell line. We therefore analysed expression of leukaemia cells VEGFRs under the assumption that the differences between intracytoplasmic VEGF levels and detectable VEGF in culture supernatants are caused by sequestration of free VEGF. The strong expression of VEGFRs found on KG-1 underlies this hypothesis, at least for that cell line.

We did not investigate the functional relevance of VEGFRs on leukaemia or lymphoma cell lines ourselves, but there is evidence that VEGFRs located on or inside VEGF-producing cells are part of an autocrine or intracrine VEGF-feedback loop that impacts cell growth and survival in cancer cells [42,43].

sVEGFR-1

Binding by membrane resident VEGFRs was not the only reason for the discrepancies between the amount of intracytoplasmic VEGF and those detectable in the culture medium, as the cell-free supernatants of KG-1 and PBMCs cultures effectively interfered with the detection of added rhVEGF.

In this regard, it has been demonstrated previously that VEGFR-1 has a physiological relevant splice variant, encoding sVEGFR-1. This soluble receptor is expressed by endothelial cells [44] and also by cells of the monocyte lineage [45], and is capable of interfering with the detection and functioning of VEGF [46]. We therefore assumed that this molecule contributes to the observed discrepancy in our experiments. In line with a possible interference we found high amounts of sVEGFR-1 in the supernatants of the leukaemia cell lines KG-1, K562 and THP-1, as well as in the supernatants of PBMC cultures. By contrast, sVEGFR-2 was not detectable in any of the cell culture supernatants and is most probably not a physiologically relevant antagonist of VEGF [35,47]. It was interesting to find that certain cell lines and PBMCs concomitantly produce contradictory VEGF signals, and VEGF production can either exceed or fall below the binding capacity of produced sVEGFR-1. The release of sVEGFR-1 from PBMCs was highly variable, depending on the applied stimulus. When PBMCs were depleted of $CD14^+$ monocytes, the sVEGFR-1 production rate decreased substantially and the detectable free VEGF between days 2 and 7 increased. Even though the VEGF output was still low when compared to cell lines (Fig. 4a) or macrophage colony-stimulatory factor (M-CSF) stimulated monocytes [29], this indicates that monocytes are essential for the release of sVEGFR-1 and may have an important role as regulators of VEGF signalling in collaboration with lymphocytes.

Pro- and anti-tumour roles of VEGF

VEGF has multiple biological effects, but in the context of malignancies it is recognized primarily as a pro-tumour factor that facilitates neo-angiogenesis and thereby cancer progression. Less focused upon are the immunomodulatory effects of VEGF; for example, the promotion of leucocyte trafficking into tissues [22,48]. In addition, predominantly immunosuppressive functions have been attributed to VEGF, such as the inhibition of dendritic cell maturation [49], induction of an immunosuppressive microenvironment and activation of regulatory T cells [40,50]. In the context of cancer immunotherapy it is interesting that high pretherapeutic blood levels of VEGF are an independent predictor of a lack in clinical response and a decreased overall survival of patients treated with high-doses of IL-2 [51]. However, this observation cannot be

Table 1. Vascular endothelial growth factor (VEGF) receptor expression on leukaemia and lymphoma cell lines

Cell line	VEGF R1	VEGF R2	VEGF R3
KG-1	++	+	+
K562	+	−	+
HL-60	(+)	−	+
THP-1	−	−	+
U266	−	−	(+)
Reh	−	−	(+)
Daudi	−	−	−

The expression of VEGF receptors is classified according to the respective mean fluorescence intensities (MFIs) quotient [MFI(-VEGFRx)/MFI(isotype control)]: $\leq$ 2: −, > 2–3: (+), $\geq$ 3–10: +, > 10: ++.

generalized or transferred to all types of cancer or other treatment modalities.

Pro- and anti-tumour roles of tumour-associated immune cells

In contrast to the observations that suggest that γδ T cell infiltration into the tumour is a positive prognostic marker [2], studies have shown that the amount of macrophages in tumour tissues is often correlated with a poor prognosis in numerous types of cancer and treatment modalities [52]. This is especially recognized for the immunosuppressive M2-like macrophages, which are capable of producing VEGF [53]. In this regard, it has been proposed that Zol decreases VEGF levels in tumour patients by reducing the amount of tumour-infiltrating macrophages [54]. However, under certain circumstances macrophage infiltration is also associated with an improved prognosis [55,56], suggesting a variable role of immune cells in the course of malignant diseases. There is evidence that proinflammatory signals such as IFN-γ- or Fc-receptor-γ-I-dependent activation of monocytes and macrophages promote anti-angiogenic functions of immune cells by inhibiting VEGF [57,58]. It has been demonstrated earlier that sVEGFR-1 is a potent anti-angiogenic molecule which blocks cellular proliferation and migration induced by VEGF [46]. Monocyte-derived dendritic cells stimulated with tumour necrosis factor (TNF)-α or soluble CD40 ligand plus IFN-γ can produce very high amounts of sVEGFR-1 and can have an anti-angiogenic effect *in vivo* [59]. However, not only proinflammatory but also anti-inflammatory cytokines such as IL-4 or IL-10 can inhibit VEGF signalling [60]. It seems plausible that some of these apparently contradictory findings are the result of the time-dependent regulation of immune responses and the diversity of macrophage phenotypes [61].

Depending on the type of stimulation with either M-CSF or granulocyte–macrophage colony-stimulating factor (GM-CSF), monocytes themselves produce high amounts of VEGF (response to M-CSF) or antagonistic sVEGFR-1 (response to GM-CSF) [28,29]. This observation adds to the plasticity of monocytes functioning as pro- or anti-tumour factors and further connects them to T cell functions and the effects of IL-2 treatment. In this regard, it has also been demonstrated in a melanoma mouse model that IL-2 can have both pro- and anti-tumour effects and changes the infiltration of tumour tissue and lymph nodes by T and NK cells, depending on the applied dose [62]. We know that upon stimulation with IL-2, both unstimulated and pre-activated T cells as well as NK cells are able to produce GM-CSF [63,64]. Furthermore, an interesting gene expression study in melanoma patients demonstrated that high doses of IL-2 change the expression patterns of lymphocyte and monocyte cytokines, including GM-CSF and VEGF, within a few hours *in vitro*, in the blood and in the tumour microenvironment [65]. However, the physiological role for GM-CSF *in vivo* remains unclear, because very high concentrations were needed to induce the release of anti-angiogenic sVEGFR-1 *in vitro* [28]. A model for lymphocyte–monocyte interaction in control of VEGF signalling and tumour angiogenesis is given in Fig. 5.

Our results suggest that lymphocytes are a potential source of VEGF, which may act as a chemoattractant for monocytes initially and helps to establish lymphocyte–monocyte co-operation *in vivo*. We suggest that the local interaction of immune cells will result in pro- or anti-tumour reactions depending on the milieu, cell composition and priming. γδ T cells might have predominant anti-tumour functions when activated in the tumour environment, but in the following course of immune reactions they could also develop pro-tumour characteristics.

Conclusions

In this study we characterize VEGF production by blood-derived human immune cells, as well as leukaemia and lymphoma cell lines, and identify sVEGFR-1 as a potent regulatory factor in VEGF signalling. We provide evidence that IL-2 induces VEGF production and also the release of sVEGFR-1 by immune cells, which may sequester free VEGF and inhibit angiogenesis. Depending on the process of lymphocyte–monocyte interaction, VEGF signalling is influenced predominantly in either way. It is plausible that these immune cells regulate VEGF homeostasis locally in a time-dependent manner, influenced by the type and strength of primary stimuli and co-stimulation (Fig. 5).

When changes in VEGF signalling *in vivo* are assessed by measuring blood levels it should be considered that this may not reflect local VEGF signalling, which is compartmentalized. Various cells produce VEGF and blood levels are influenced by breakdown, the abundance of VEGF binding proteins and by local differences in tissue permeability [66,67]. Our current study shows that

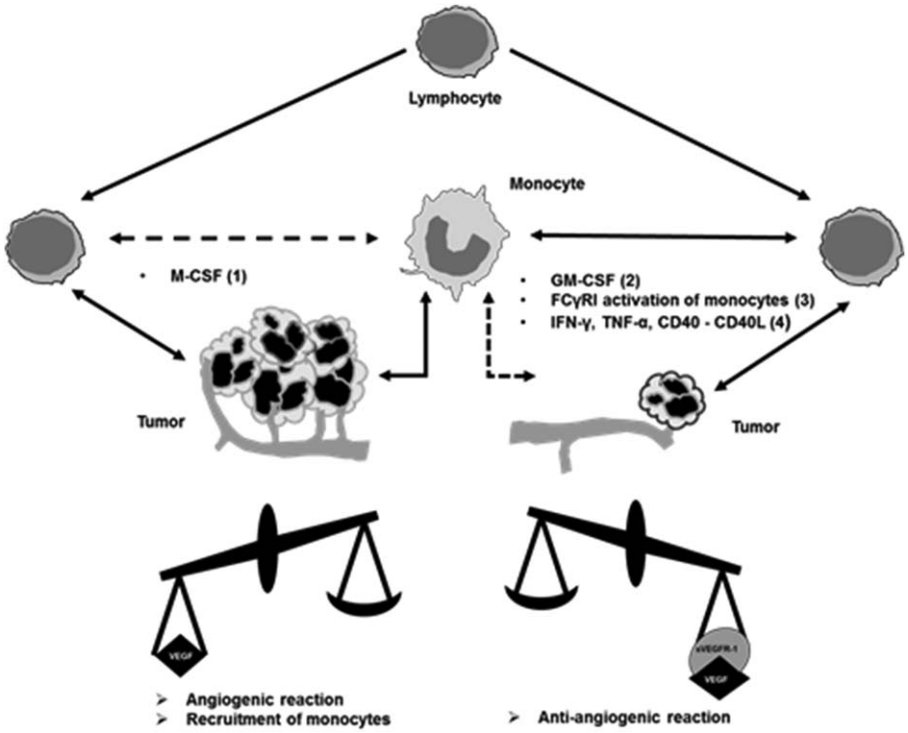

Fig. 5. Model of lymphocytes and monocytes effects on vascular endothelial growth factor (VEGF) signalling. The figure is developed from the models by Eubank *et al.* [28] and incorporates results from (1, 2) Eubank *et al.* [28,29] (3) Justiniano *et al.* [58], (4) Kishuku *et al.* [59] and our own observations. M-CSF = macrophage colony-stimulating factor; GM-CSF = granulocyte–macrophage colony-stimulating factor; FcγRI = Fcγ-receptor I; TNF-α = tumour necrosis factor α; IFN-γ = interferon-γ.

manipulation of immune cell functions with IL-2 and Zol can change VEGF signalling and therefore impact tumour angiogenesis, the local vascular permeability and may induce secondary immunomodulatory effects via VEGF and antagonistic sVEGFR-1. In our opinion, the modest and variable clinical response to anti-VEGF therapies in cancer demands additional effort in the detailed analysis of VEGF signalling in the tumour microenvironment and the contribution of different immune cells. Future research should apply more elaborate models for the time-dependent study of cell interactions and cytokine patterns which regulate VEGF signalling. This may lead to more effective immunotherapeutic approaches with less negative effects.

Acknowledgements

This study was supported by Verein Hilfe für Krebskranke e.V. and a scholarship for Timm Hoeres from W. Lutz Stiftung.

Disclosure

The authors declare no competing financial interests.

Author contributions

T. H. designed the experiments, performed research, analysed and interpreted the data and wrote the paper; M. W. contributed to conception and design, analysed and interpreted the data and edited the paper; M. S. contributed to conception and design, analysed and interpreted the data; E. H. analysed the data and performed research; G. S. analysed the data, performed research and edited the paper; and J. B. contributed to conception and design, supervised research and edited the paper.

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