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Circulating galectins -2, -4 and -8 in cancer patients make important contributions to the increased circulation of several cytokines and chemokines that promote angiogenesis and metastasis

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Background: Circulating concentrations of the cytokines interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF) and chemokines monocyte chemotatic protein 1 (MCP-1)/CCL2 and growth-regulator oncogene α (GROα)/chemokine C-X-C motif ligand 1 are commonly increased in cancer patients and they are increasingly recognised as important promoters, via divergent mechanisms, of cancer progression and metastasis.

Methods: The effect of galectins-2, -4 and -8, whose circulating levels are highly increased in cancer patients, on endothelial secretion of cytokines was assessed *in vitro* by cytokine array and in mice. The relationship between serum levels of galectins and cytokines was analysed in colon and breast cancer patients.

Results: Galectins-2, -4 and -8 at pathological concentrations induce secretion of G-CSF, IL-6, MCP-1 and GROα from the blood vascular endothelial cells *in vitro* and in mice. Multiple regression analysis indicates that increased circulation of these galectins accounts for 41∼83% of the variance of these cytokines in the sera of colon and breast cancer patients. The galectin-induced secretion of these cytokines/chemokines is shown to enhance the expression of endothelial cell surface adhesion molecules, causing increased cancer-endothelial adhesion and increased endothelial tubule formation.

Conclusion: The increased circulation of galectins -2, -4 and -8 in cancer patients contributes substantially to the increased circulation of G-CSF, IL-6 and MCP-1 by interaction with the blood vascular endothelium. These cytokines and chemokines in turn enhance endothelial cell activities in angiogenesis and metastasis.

Galectins are a family of at least 15 galactoside-binding proteins that share similar carbohydrate recognition domains (CRD). They are widely expressed by various types of human cells. Altered expressions of galectins, usually increased, is commonly seen in various cancers (Liu and Rabinovich, 2005; Newlaczyl and Yu, 2011; Barrow *et al*, 2011b) and is increasingly shown to be involved in the regulation of various stages of cancer development, progression and metastasis (Liu and Rabinovich, 2005; Newlaczyl and Yu, 2011).

Research over the past few years has shown that the concentrations of several galectins, notably galectins -2, -3, -4 and -8 are markedly increased, typically 5- to 30-fold, in the bloodstream of various types of cancers including colorectal (Watanabe *et al*, 2011; Barrow *et al*, 2011a, 2013), breast

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(Iurisci *et al*, 2000), pancreatic (Senapati *et al*, 2011; Xie *et al*, 2012), melanoma (Vereecken *et al*, 2009), bladder (Sakaki *et al*, 2008), head and neck cancer (Saussez *et al*, 2008). Patients with metastasis have even higher levels of circulating galectins than those with only localised tumours (Barrow *et al*, 2011a). Our recent studies have revealed that increased circulation of galectin-3, a key member of the galectin family, is an important promoter of metastasis (Zhao *et al*, 2009). We have shown that part of the metastasis-promoting effect of circulating galectin-3 is related to its binding to the oncofetal Thomsen-Friedenreich carbohydrate Galβ1, 3GalNAc-(TF) antigen that commonly exists as an *O*-glycan on the extracellular domain of the cancer-associated mucin protein MUC1 (Yu *et al*, 2007). The galectin-3-MUC1 interaction induces MUC1 cell surface polarisation resulting in exposure of smaller cell surface adhesion molecules, leading to increased cancer cell adhesion to the vascular endothelium (Zhao *et al*, 2009) and increased cancer cell homotypic aggregation for the formation of tumour emboli that prolongs the survival of tumour cells in the circulation (Zhao *et al*, 2010). A similar effect on cancer cell adhesion and aggregation is also observed with circulating galectins -2, -4 and -8 (Barrow *et al*, 2011a).

More recently, we have revealed that the metastasis-promoting effects of circulating galectin-3 are also contributed to by its interaction with the blood vascular endothelium, inducing the endothelial cells to secret cytokines granulocyte colony-stimulating factor (G-CSF), GM-CSF, interleukin-6 (IL-6) and sICAM-1 (Chen *et al*, 2013). Pro-inflammatory cytokines such as IL-6 are well known for their roles in promoting cancer progression and metastasis via divergent mechanisms such as stimulation of cancer cell proliferation, angiogenesis, cell adhesion and by the local and systemic suppression of host anti-tumour immunity (Ara and Declerck, 2010; Tsujimoto *et al*, 2010). We have previously analysed the circulating levels of all the galectins (galectins -1, -2, -3, -4, -7 and -8) whose recombinant forms are commercially available and this revealed increase of circulating galectins -2, -3, -4 and -8 in cancer patients (Barrow *et al*, 2011a). As members of the galectin family share similar CRD, the effect of increased circulation of galectin-3 on endothelial secretion of the metastasis-promoting cytokines led us to speculate that the increased levels of circulating galectins -2, -4 and -8 in cancer might also, like that of galectin-3, induce secretion of cytokines from the vascular endothelium and thus contribute to cancer metastasis.

This study therefore investigated the effects of increased circulating galectins -2, -4 and -8 on cytokine secretion by the vascular endothelium *in vitro* and in mice and analysed the relationship between these circulating galectins and cytokine concentrations in the sera of colon and breast cancer patients.

MATERIALS AND METHODS

Materials. Recombinant human galectins -2, -4 and -8 (residual endotoxin levels $<1.0\,\mathrm{EU}\,\mu\mathrm{g}^{-1}$ protein), and human Cytokine Protein Array, antibodies against CD44, E-selectin, VCAM-1 and integrin$\alpha_v\beta_1$ were purchased from R&D Systems (Abingdon, UK). Calcein AM Cell Labeling Solution was from Invitrogen (Paisley, UK). Human recombinant cytokine IL-6, G-CSF, growth-regulator oncogene α (GROα) and monocyte chemotatic protein 1 (MCP-1), mouse recombinant IL-6, G-CSF, GROα, MCP-1 and mouse cytokine ELISA kits were from PeproTech (London, UK). Mouse cytokine GROα ELISA kit was from PromoKine (Heidelberg, Germany). *In Vitro* Angiogenesis Tube Formation kits were from AMS Biotechnology Ltd (Abingdon, UK). Non-Enzymatic Cell Dissociation Solution (NECDS) and all other chemicals were from Sigma (Dorset, UK).

Cell lines. The MUC1-negative HCT116 human colon cancer cells (Ren *et al*, 2004) were obtained from the European Cell Culture Collections via the Public Health Laboratory Services (Porton Down, Wiltshire, UK) and cultured in McCoy's 5a medium. The MUC1-negative ACA19$^-$ cells selected from human melanoma A375 cells (Wesseling *et al*, 1996) were kindly provided by Dr John Hilkens (The Netherland Cancer Institute) and cultured in Dulbecco's modified Eagle's medium (No authentication of the cell lines was done by the authors other than verification of appropriate presence/absence of MUC1). Human micro-vascular lung endothelial cells (HMVEC-Ls) and human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Wokingham, UK) and cultured in EGM-2 and EGM endothelial growth media (Lonza), respectively. Less than five passages of the endothelial cells were used in all experiments.

Cytokine array. Human micro-vascular lung endothelial cells (2×10^5 per well) were cultured in six-well plates at 37 °C for 24 h before introduction of $1.5\,\mu\mathrm{g\,ml}^{-1}$ recombinant galectins -2, -4, -8 or BSA for 24 h. The culture media were collected and the concentrations of cytokines in the culture media were analysed by the Human Cytokine Protein Arrays exactly as in the instruction of the kit. These arrays assayed 36 cytokines (C5/C5a, CD40 Ligand, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNF-α and sTREM-1), each in duplicate. The arrays were quantified with Bio-Rad Image Lab software (Image Lab 2.0, Hercules, CA, USA).

Cytokine determination. Human micro-vascular lung endothelial cells (1×10^5 per well) were cultured in 12-well plates at 37 °C for 24 h before introduction of control BSA or recombinant galectins -2, -4 or -8 for 24 h. The culture media were collected and the concentrations of G-CSF, IL-6, GROα and MCP-1 in the culture media were analysed by ELISA.

Determination of cancer cell-endothelial adhesion. Human micro-vascular lung endothelial cells (4×10^4 per well) were cultured in 96-well plates for 24 h for the formation of endothelial cell monolayers. Recombinant galectins -2, -4 or -8 ($1.5\,\mu\mathrm{g\,ml}^{-1}$) or control BSA were then added with or without further addition of lactose (final concentration $200\,\mu\mathrm{g\,ml}^{-1}$) for 24 h. The endothelial cells were either washed and used for subsequent assessment of cancer cell adhesion, or the culture media (conditioned media) were collected and used for assessment of their effects on cancer cell adhesion to fresh, untreated HMVEC-Ls monolayers.

ACA19$^-$ and HCT116 cancer cells were detached from the culture plates with NECDS, washed and suspended at 5×10^6 cells per ml in serum-free DMEM. The cells were labelled with $10\,\mu\mathrm{l\,ml}^{-1}$ Calcein AM Cell Labeling Solution at 37 °C for 30 min. After two washes with PBS, the cells were resuspended into $5\times10^4\,\mathrm{ml}^{-1}$ with serum-free DMEM and applied (5×10^3 per well) to monolayers of fresh or galectin-treated HMVEC-Ls for 1 h at 37 °C. The HMVEC-Ls monolayer was washed twice with PBS and the endothelial cell-associated fluorescence was measured using a TECAN infinite F200 fluorescent microplate reader at 485 nm excitation/520 nm emission.

Analysis of endothelial cell surface adhesion molecule expression. Human micro-vascular lung endothelial cells were treated with galectins -2, -4, -8 or control BSA (all $1.5\,\mu\mathrm{g\,ml}^{-1}$) for 24 h before released by NECDS. The cells were washed with PBS and fixed with 2% paraformaldehyde for 20 min before incubation with 5% goat serum/PBS for 30 min. After removal of the supernatant, the cells were suspended in 1% goat serum/PBS ($10^6\,\mathrm{cells\,ml}^{-1}$) and introduced with antibodies against cell surface adhesion molecules (integrin$\alpha_v\beta_1$, CD44, E-selectin or VCAM, all at 1 : 400

dilutions in 1% BSA in PBS) for 1 h. The cells were washed twice with PBS before application of FITC-conjugated secondary antibodies (1 : 400 dilutions) for 1 h. After three washes, the cells were resuspended in PBS and analysed by flow cytometry.

Measurement of endothelial tubule formation. Human microvascular lung endothelial cells cultured in 12-well plates were incubated with 1.5 $\mu g\,ml^{-1}$ BSA, galectins -2, -4 or -8 for 24 h. The culture media were collected and used for assessment of their ability to stimulate HUVEC tubule formation assessed using the *in vitro* Angiogenesis kit with or without the addition of a combination of antibodies against G-CSF (5 $\mu g\,ml^{-1}$), IL-6 (3 $\mu g\,ml^{-1}$), GROα (20 $\mu g\,ml^{-1}$) or MCP-1(20 $\mu g\,ml^{-1}$). Endothelial tube formation was visualised by fluorescence microscopy. The tubule length and branch points were quantified using ImageJ.

***In vivo* measurement of the galectin effect on cytokine secretion in mice.** Twenty-seven 6–8 weeks old female C57BL/6 mice, obtained from Charles River Laboratories (Margate, Kent, UK) and maintained and used in accordance with the animal care protocol approved by University of Liverpool, were randomly divided into nine equal groups and 5 μg galectins -2, -4, -8 or a combination of 5 μg each galectins -2, -4 and 8 was injected by intravenous tail vein injection. The blood was obtained by cardiac puncture at 0, 24 and 48 h and the serum concentrations of G-CSF, GM-CSF, IL-6 and sICAM-1 were determined by ELISAs.

Human sera. Serum samples from 40 breast and 50 colorectal cancer patients were obtained from the CTBRC cancer tissue bank (Liverpool, UK) (Supplementary Table S1). The serum samples had been obtained by CTBRC from patients at the time of primary tumour resection at the Royal Liverpool University Hospital with informed consents from the patients. The study was approved by the Liverpool (Adult) Local Research Ethics Committee (REC number: 07/Q1505/14).

Serum galectin assays. The galectins -2, -4 and -8 concentrations in the serum of breast and colorectal cancer patients were determined by ELISA as described in our previous study (Barrow *et al*, 2011a).

Statistical analysis. Unpaired *t*-test was used for single comparison, one-way ANOVA followed by Bonferroni correction for multiple comparisons was used where appropriate. The associations between serum levels of galectins and cytokines in cancer patients were tested using both simple and multiple regression models. Multiple regression analysis was used to test whether there was evidence of an overall correlation between each cytokine outcome and a combination of galectins and the F-test results were reported. The beta estimates, which represent the change in cytokines outcomes (ng) as the galectin level is increased by 1 ng, were reported. R^2 values were also reported in the multiple regression analysis as a measure of the extent to which alterations in serum concentrations of galectins -2, -4 and -8 together account for the observed variance in cytokine concentration. $P<0.05$ from two-sided tests were considered significant in all the analyses.

RESULTS

Galectins -2, -4 and -8 each induce secretion of cytokines from HMVEC-Ls. To investigate the influence of galectins -2, -4 and -8 on endothelial secretion of cytokines, we first tested the cytokine secretion profile of HMVEC-Ls in their response to recombinant galectins -2, -4 or -8 at a concentration (1.5 $\mu g\,ml^{-1}$) typically found for each of these galectins in cancer sera. Treatment of HMVEC-Ls with galectins -2, -4 or -8 resulted in increased cell release of G-CSF, IL-6, GROα/chemokine C-X-C motif ligand 1 (CXCL1) and MCP-1/CCL2. Galectin-2 treatment resulted in a 3.1-fold increase of G-CSF, 1.5-fold IL-6 and 1.7-fold GROα. Galectin-4 treatment caused a 2.6-fold increase of G-CSF, 1.4-fold IL-6, 1.7-fold GROα and 3.0-fold MCP-1, whereas galectin-8 induced 2.4-fold increase of G-CSF, 1.5-fold IL-6, 1.7-fold GROα and 3.0-fold MCP-1 (Figure 1A). These effects of galectins were inhibited by the presence of lactose (Figure 1B) and showed to be time-dependent and occurred dose-dependently at various pathological galectin concentrations seen in cancer patients (Barrow *et al*, 2011a) (Figure 2).

It was found that the presence of each of these four cytokines/chemokines, either alone or in combination at concentrations (0.3 $ng\,ml^{-1}$ G-CSF, 0.2 $ng\,ml^{-1}$ IL-6, 1 $ng\,ml^{-1}$ GROα or 1 $ng\,ml^{-1}$ MCP-1) similar to that induced from HMVEC-Ls by 24-h treatment with 1.5 $\mu g\,ml^{-1}$ galectin, did not show any significant effect on the levels of galectins -2, -4 or -8 in the medium of HMVEC-Ls after 24 and 48 h culture (Data not shown). This indicates that the galectin-induced secretion of these cytokines does not form a feedback loop to enhance endothelial secretion of galectins.

Galectin-induced cytokine secretion enhances cancer cell-endothelial adhesion. Our previous studies have shown that circulating galectin-3 induces endothelial secretion of G-CSF, IL-6, GM-CSF and ICAM-1 that enhances cancer cell adhesion to the vascular endothelium (Chen *et al*, 2013). As G-CSF and IL-6 were also seen to be induced from the vascular endothelium by galectins -2, -4 and -8, we then investigated the influence of the galectin-induced secretion of these cytokines on cancer cell adhesion to endothelium. Co-culture of HMVEC-Ls with 1.5 $\mu g\,ml^{-1}$ galectins -2, -4 or -8 for 24 h each increased 70 ~ 82% adhesion of human melanoma $ACA19^-$ (Figure 3A) and 39 ~ 60% adhesion of colon cancer HCT116 cells (Figure 3B). When the culture medium (conditioned medium) from 24 h galectin-treated HMVEC-Ls was used as culture medium to assess $ACA19^-$ and HCT116 cell adhesion to fresh HMVEC-Ls, similar increases of cell adhesion were observed. The increased cell adhesion induced by the galectins (Figure 3C and D) or the conditioned medium (Figure 3E) was largely prevented by the presence of lactose in the initial galectin-HMVEC-Ls culture. The presence of lactose alone did not have any effect on adhesion of $ACA19^-$ and HCT116 cells to HMVECs. Addition to the conditioned medium of neutralising antibodies against G-CSF, IL-6, GROα and MCP-1 almost completely negated the conditioned medium-induced cancer cell adhesion (Figure 3F). Furthermore, introduction of a combination of recombinant G-CSF, IL-6, GROα and MCP-1 or G-CSF, IL-6 and GROα at concentrations similar to that induced from HMVEC-Ls after 24 h galectin-treatment (Figure 2), to the conditioned medium from BSA-treated control HMVEC-Ls induced a similar increase of $ACA19^-$ cell adhesion as that from the conditioned medium from the galectin-treated HMVEC-Ls (Figure 3G). Collectively, these results indicate that the increased secretion of these cytokines by galectins -2, -4 or -8 enhances cancer cell adhesion to endothelium.

Increased expression of the cell surface adhesion molecules is responsible for galectin-induced-, cytokine-mediated cancer cell-endothelial adhesion. We next investigated whether the galectin-induced, cytokine-mediated increase of cancer cell adhesion was associated with change in the expression of endothelial cell surface adhesion molecules. Twenty-four hour treatment of HMVEC-Ls with each of these galectins enhanced the expression of several cell surface adhesion molecules in particular integrin$\alpha_v\beta_1$ (Figure 4A). Treatment with galectin-2 resulted in 34.8% increase of cell surface integrin$\alpha_v\beta_1$, a small increase of VCAM-1 (9.8%) but no effect on CD44 and E-selectin, whereas galectins -4 or -8 treatment induced 41.0% and 32.4%, respectively, increase of integrin$\alpha_v\beta_1$

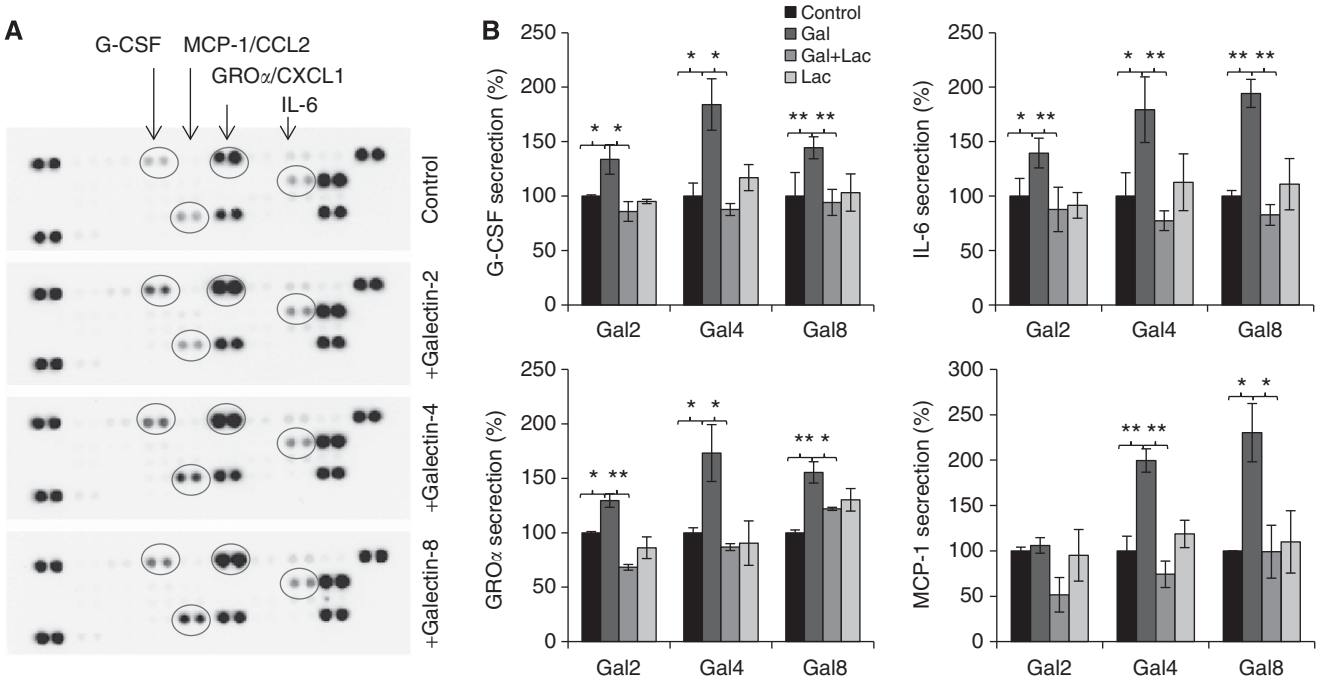

Figure 1. Galectins -2, -4 and -8 induce cytokine secretion from endothelial cells. Human micro-vascular lung endothelial cells were incubated with 1.5 $\mu g\,ml^{-1}$ galectins -2, -4, -8 or BSA for 24 h before the cytokine levels in the culture media were analysed by the cytokine array (**A**). In (**B**), HMVEC-Ls cells were treated with 1.5 $\mu g\,ml^{-1}$ galectins -2, -4, -8 or BSA in the presence or absence of 200 $\mu g\,ml^{-1}$ lactose for 24 h before the G-CSF, IL-6, GROα and MCP-1 levels in the cultured media were determined by ELISA. The data are expressed as percentage compared with BSA-treated controls from three independent experiments, each in triplicate. *$P<0.05$, **$P<0.01$ (ANOVA, Bonferroni).

and a small increase of CD44 (8.9 and 5.4%) but no effect on E-Selectin and VCAM-1 expressions.

To investigate whether the increased expression of these cell surface adhesion molecules was responsible for the galectin induced, cytokine-mediated cancer cell adhesion, adhesion of ACA19$^-$ cells to HMVEC-Ls was assessed with the conditioned medium from control- or galectin-treated HMVEC-Ls with and without addition of neutralising antibodies against integrin$\alpha_v\beta_1$, CD44, VCAM-1and E-selectin. The addition of neutralising antibodies against these cell adhesion molecules in the conditioned medium from galectin-treated HMVEC-Ls reduced the conditioned medium-mediated ACA19$^-$ cell adhesion (Figure 4B). Together, these results suggest that the increased expression of endothelial cell surface adhesion molecules is largely responsible for the galectin-induced, cytokine-mediated cancer cell adhesion.

Galectin-induced cytokine secretion promotes endothelial cell tube formation. As many pro-inflammatory cytokines and chemokines are angiogenesis promoters (Ara and Declerck, 2010), we then assessed the effect of galectin-induced secretion of these cytokines/chemokines on endothelial tubule formation, an important component of angiogenesis. Culture of HUVECs with the conditioned medium obtained from 24 h galectin-treated HMVEC-Ls increased the ability of HUVECs to form microtubule structures (67∼87% increase of branch points and 34∼78% increase of tubule length) in comparison with the HUVECs cultured in the conditioned medium from BSA-treated control HMVEC-Ls (Figure 5A–C). These effects of galectins were prevented by the addition to the conditioned medium of a combination of neutralising anti-cytokine antibodies. These results indicate that the galectin-induced secretion of cytokines from endothelium enhances endothelial angiogenesis.

Galectin-3 induces cytokine secretion *in vivo*. Galectins from human and mouse origins all bind to galactoside-terminated glycans through their CRD. The galectin-mediated secretion of cytokines from human vascular endothelial cells was shown to be inhibited by lactose (Figure 3C and D), indicating the importance of galectin CRD in their actions on cytokine secretion from the vascular endothelium. As not all recombinant mouse galectins were commercially available, we used recombinant human galectins, which showed induced secretion of these cytokines from both human (Figures 1 and 2) and mouse vascular endothelial cells (Supplementary Figure S1) *in vitro*, in the subsequent animal experiments. When 5 μg per human galectins -2, -4 or -8, equating approximately to circulating galectin concentrations seen in cancer patients with metastasis (Barrow *et al*, 2011a), was injected intravenously into the animal tail vein, a 490.7 ± 68.6% and 683.7 ± 83.7% increase of serum G-CSF were seen at 24 and 48 h by galectin-4 (Figure 5D). A 375.9 ± 54.9% increase of MCP-1/CCL2 was also observed after 48 h by galectin-4. Galectin-8 injection caused a 543.3 ± 123.3% increase of serum IL-6 after 48 h. Interestingly, galectin-2 injection did not show any significant effect on serum levels of any of these cytokines/chemokines. Serum GROα/CXCL1 levels were not affected by injection of any of these galectins. Injection of a combination of 5 μg per mouse of each galectins -2, -4 and -8 increased serum G-CSF (448.8 ± 191.9%), IL-6 (691.7 ± 228.3%) and MCP-1 (373.3 ± 130.4%) after 48 h and the increase of each of these cytokines/chemokines was equivalent to that produced by the most influential galectin member (galectin-4) when injected individually. These results provide evidence of a direct impact of circulating galectins on secretion of these cytokines *in vivo*. They also indicate that these galectin members may share some of the endothelial receptors to exert their effects on endothelial secretion of these cytokines/chemokines, and thus showing lack of an additive effect when they are present in combination.

Relationship between serum levels of galectins and cytokines/chemokines in breast and colon cancer patients. To see whether the relationship between these galectins and cytokines/chemokines observed *in vitro* and in mice occurred in cancer patients, serum

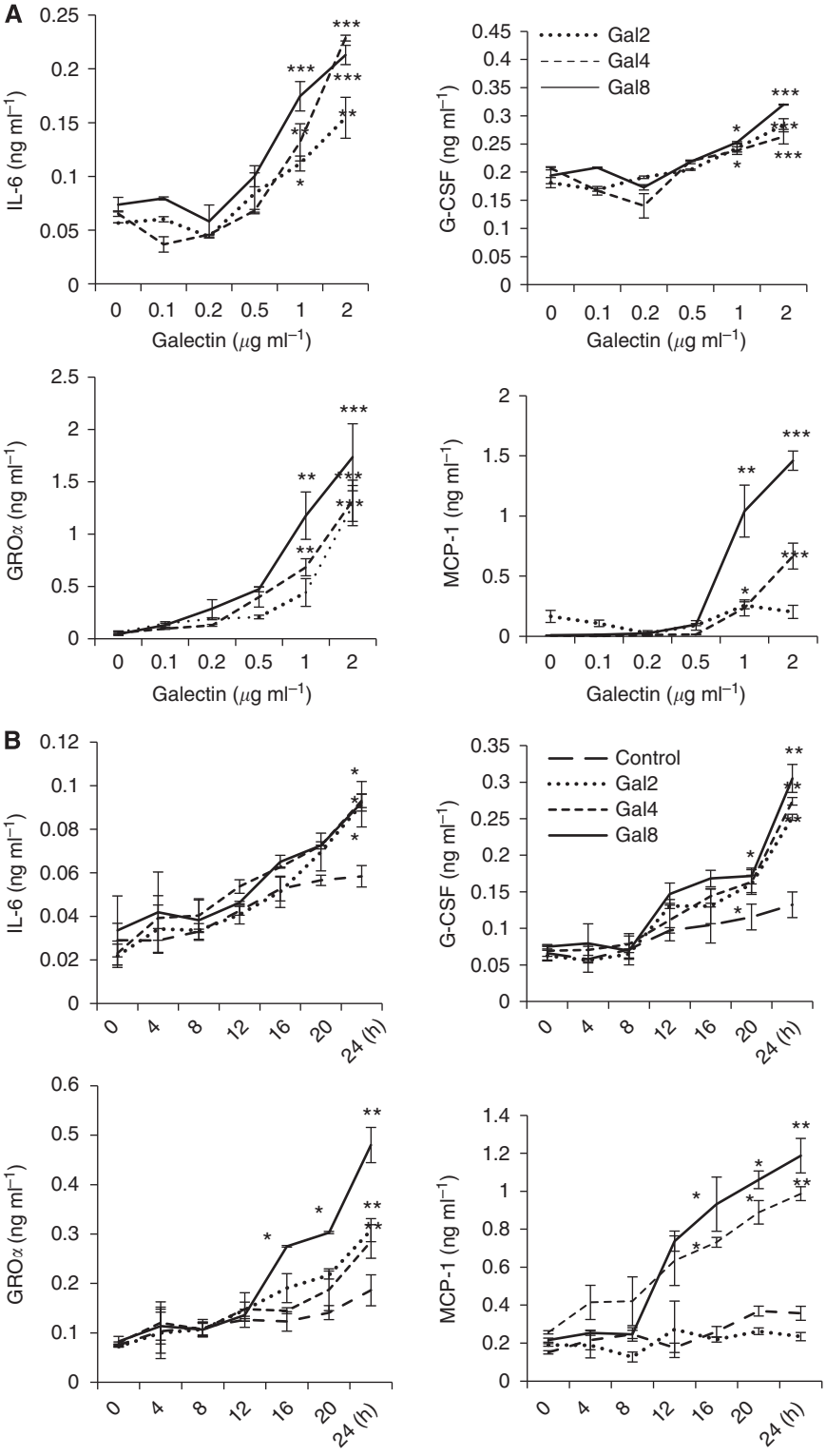

Figure 2. Galectins -2, -4 and -8-induced endothelial secretions of cytokines are time- and dose-dependent. Human micro-vascular lung endothelial cells were treated with various concentrations of galectins -2, -4 or -8 for 24 h (**A**), or with 1.5 μg ml^{-1} galectins -2, -4, -8 or BSA for various times (**B**), before the G-CSF, IL-6, GROα and MCP-1 levels in the cultured media were determined. The data are expressed as mean ± s.d. of triplicate determinations *$P<0.05$, **$P<0.01$, ***$P<0.001$ (ANOVA, Bonferroni).

levels of circulating galectins -2, -4 and -8 and G-CSF, IL-6, GROα/CXCL2, and MCP-1/CCL1 were analysed in breast and colon cancer patients (Supplementary Table S2). Simple regression analysis showed significant correlation of G-CSF and IL-6 levels, but not GROα and MCP-1, with each of the galectins in both breast and colon cancer patients (Table 1, Supplementary Figure 2). Multiple regression analysis demonstrated significant correlation of G-CSF ($P<0.001$) and IL-6 levels ($P<0.001$) with galectin-2 in breast and colon cancers. Granulocyte colony-stimulating factor and IL-6 levels were significantly ($P=0.048$) or nearly significantly ($P=0.056$) correlated with galectin-4 in colon cancer but not in breast cancer. Both G-CSF and IL-6 showed close to significant

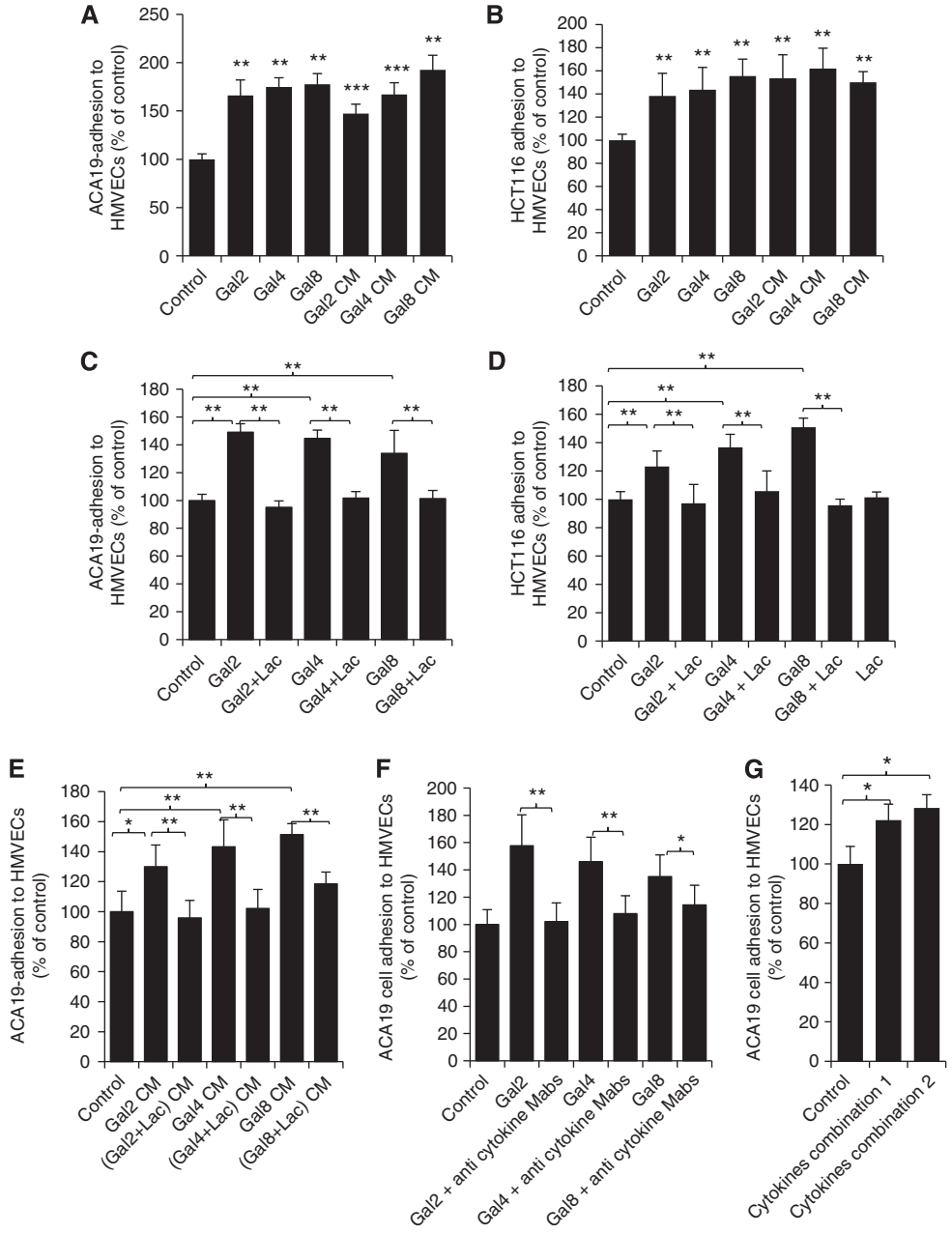

Figure 3. Galectin-induced cytokine secretion enhances cancer cell-endothelial adhesion. (**A** and **B**) The presence of galectins -2, -4 or -8 increase cancer cell adhesion to HMVEC-Ls. Human micro-vascular lung endothelial cells were treated with 1.5 μg ml^{-1} galectins -2, -4 or -8 for 24 h. The cells were either washed and used for subsequent assessment of ACA19$^-$ (**A**) or HCT116 (**B**) cell adhesion, or the culture medium (CM) were collected and used for subsequent assessment of ACA19$^-$ (**A**) or HCT116 (**B**) cell adhesion. (**C** and **D**) The galectin-induced cancer cell adhesion is inhibited by lactose. Human micro-vascular lung endothelial cells were treated with 1.5 μg ml^{-1} galectins -2, -4 or -8 in the presence or absence of 200 μg ml^{-1} lactose for 24 h. The HMVEC-Ls were then used for assessment of ACA19$^-$ (**C**) and HCT116 (**D**) cell adhesion or the culture medium were collected and used for assessment of ACA19$^-$ cell adhesion to fresh HMVEC-Ls (**E**). (**F**) Galectin-mediated cancer cell-endothelial adhesion is inhibited by neutralising anti-cytokine antibodies. Human micro-vascular lung endothelial cells were treated with or without 1.5 μg ml^{-1} galectins -2, -4 or -8 in the presence or absence of antibodies against G-CSF (5 ng ml^{-1}), IL-6 (3 ng ml^{-1}), GROα (20 ng ml^{-1}) and MCP-1(20 ng ml^{-1}) in combination for 24 h before ACA19$^-$ adhesion to the HMVEC-Ls was assessed. (**G**) Recombinant cytokines induce cancer cell adhesion to HMVEC-Ls. Human micro-vascular lung endothelial cells were treated without or with a combination of G-CSF (0.25 ng ml^{-1}), IL-6 (0.15 ng ml^{-1}) and GROα (1 ng ml^{-1}) (combination 1) or G-CSF (0.25 ng ml^{-1}), IL-6 (0.15 ng ml^{-1}), GROα (1 ng ml^{-1}) and MCP-1 (1 ng ml^{-1}) (combination 2) for 24 h before ACA19$^-$ adhesion to HMVEC-Ls was assessed. All the data are expressed as percentage compared with BSA-treated controls from at least three independent experiments, each in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (ANOVA, Bonferroni).

correlation with galectin-8 in breast ($P=0.06$ and 0.079) and colon cancers ($P=0.051$ and 0.069). In the fitted multiple regression model, galectins -2, -4 and -8 in combination was seen to account for 82 and 83% of the variance (R^2 values), respectively, of G-CSF and IL-6 in the sera of breast cancer patients and 69 and 51% in colon cancer (Table 1). They were also accountable for 41% variance of MCP-1 in the sera of colon cancer patients and 6% in breast cancer patients. A small (8%) variance in GROα in the breast cancer sera, but none in colon cancer sera, was seen to be accountable by these galectins. The combined impact of galectins

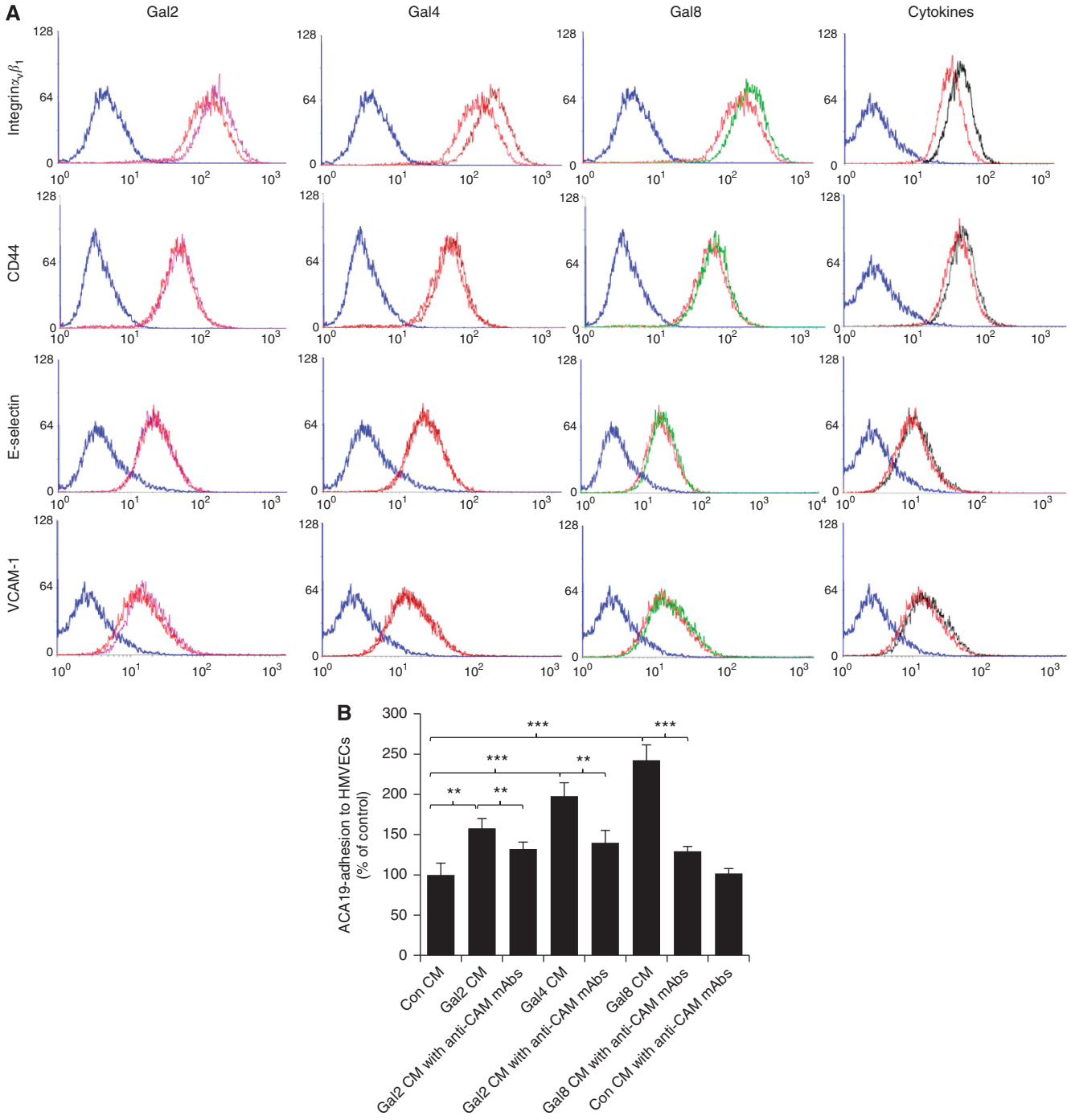

Figure 4. Galectin-induced cytokine secretion enhances expression of the endothelial cell surface adhesion molecules, which are responsible for galectin-mediated cancer cell-endothelial adhesion. (**A**) The presence of galectins induces expressions of cell surface adhesion molecules. Human micro-vascular lung endothelial cells were treated with control 1.5 μg ml^{-1} BSA (red colour) or 1.5 μg ml^{-1} galectins -2 (purple), -4 (brown), -8 (green), a combination of G-CSF (0.25 ng ml^{-1}), IL-6 (0.15 ng ml^{-1}), GROα (1 ng ml^{-1}), MCP-1 (1 ng ml^{-1}) (black) for 24 h before the expressions of the HMVEC surface integrin$\alpha_v\beta_1$, VCAM-1, CD44 and E-selectin were analysed by flow cytometry. (**B**) The presence of neutralising antibodies against cell surface adhesion molecules inhibits galectins -2, -4 or -8-mediated cancer cell adhesion. Human micro-vascular lung endothelial cells were treated without or with 1.5 μg ml^{-1} galectins -2, -4 or -8 for 24 h. The culture media were collected and used to assess ACA19$^-$ cell adhesion to fresh HMVEC-Ls without or with addition of a combination of neutralising antibodies against integrin$\alpha_v\beta_1$ (10 μg ml^{-1}), CD44 (10 μg ml^{-1}), VCAM-1(10 μg ml^{-1}) and E-selectin (10 μg ml^{-1}). $^{**}P<0.01$, $^{***}P<0.001$ (ANOVA, Bonferroni).

-2, 4- and -8 on these cytokines in the sera is large for most of these cytokines as manifested by the high coefficient of multiple correlation (R) values (0.91 for G-CSF, 0.91 for IL-6 in breast cancer and 0.78 for G-CSF, 0.71 for IL-6 and 0.64 for MCP-1/CCL2 in colon cancer patients). These observations provide further support to an important role of these galectins on secretion of these cytokines in the presence of cancer. It should be mentioned that as circulating galectin levels are correlated with each other in the presence of cancer, the multiple regression analysis is likely to identify only the significance of the strongest association and may mask weaker associations between the galectins and cytokines/chemokines.

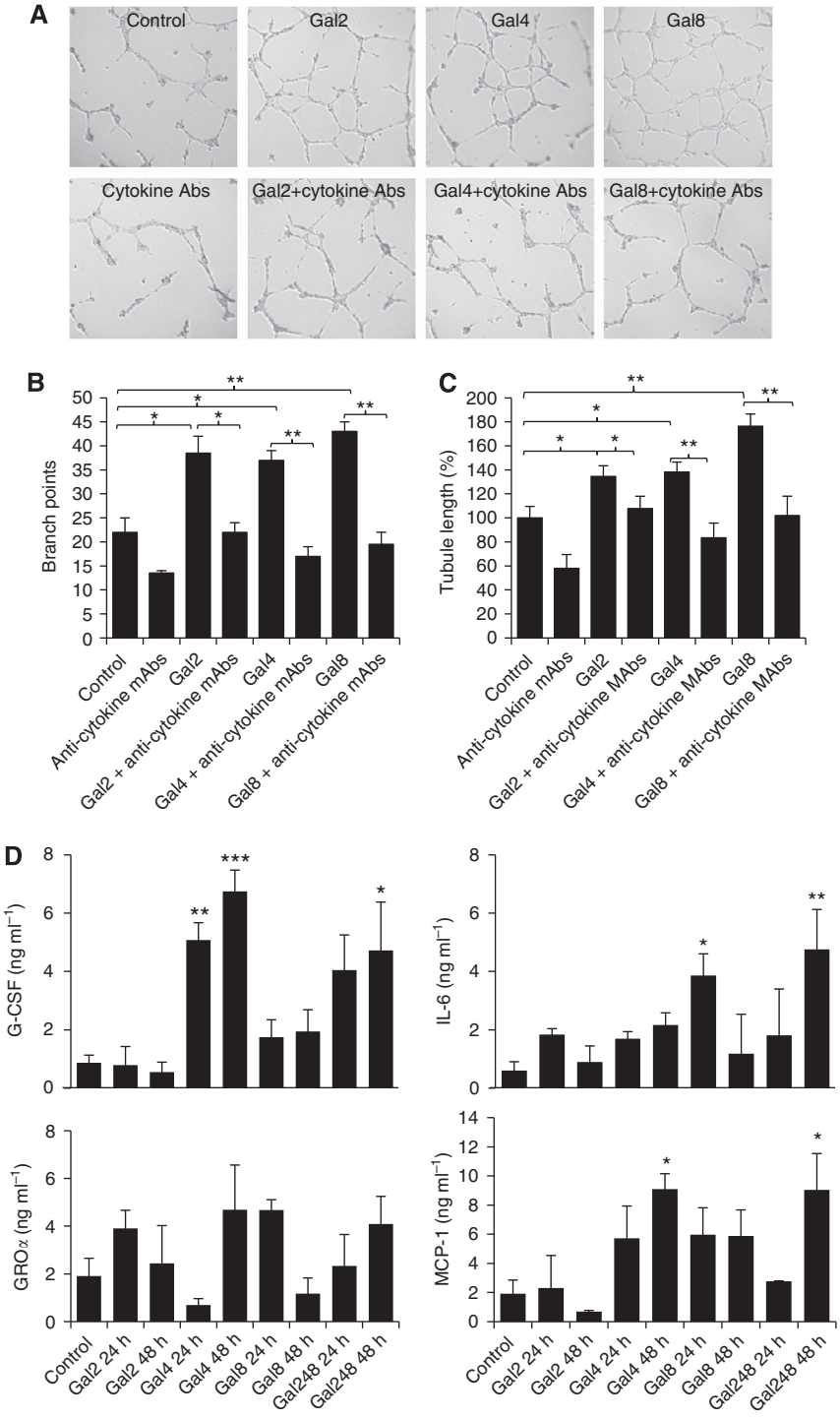

Figure 5. Galectins induce cytokine secretion *in vivo* and the galectin-induced cytokine secretion enhances endothelial tubule formation. Human umbilical vein endothelial cells cultured on matrix proteins were incubated with conditioned medium (CM) obtained from HMVEC-Ls treated with BSA, galectins -2, -4 or -8 (1.5 μg ml^{-1}) for 24 h, with or without introduction to the CM of a combination of neutralising antibodies against G-CSF (5 ng ml^{-1}), IL-6(3 ng ml^{-1}), GROα (20 ng ml^{-1}) and MCP-1(20 ng ml^{-1}) for 24 h at 37 °C. Representative images are shown in (**A**). The tubule length (**B**) and branch points (**C**) were quantified. Data are expressed from three independent experiments, each in triplicate. (**D**) Shows *in vivo* effect of tail vein injection of 5 μg galectins -2, -4, -8, individually, or in combination (5 μg each) on serum levels of the cytokines/chemokines at 0, 24 and 48 h in mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (ANOVA, Bonferroni).

DISCUSSION

This study shows that galectins -2, -4 and -8, at concentrations found in the bloodstream of cancer patients, induce secretion of G-CSF, IL-6, MCP-1/CCL2 and GROα/CXCL1 from the vascular endothelium *in vitro* and in mice. Such a relationship between circulating galectin-2, -4, -8 and G-CSF, IL-6 and MCP-1/CCL2 was further confirmed in the sera of breast and colon cancer patients. Multiple regression analysis shows that these galectins in combination account for 51–83% of the changes in G-CSF and IL-6 in the sera of breast and colon cancer patients and 41% of the

Table 1. Simple and multiple regression analysis of serum galectins -2, -4, and -8 and GCS-F, IL-6, GROα and MCP-1 levels in human breast and colon cancer patients

	Simple regression								Multiple regression							
	G-CSF		IL-6		GROα/CXCL-1		MCP1/CCL-2		G-CSF		IL-6		GROα/CXCL-1		MCP1/CCL-2	
	Beta (95%CI)	*P*	Beta (95%CI)	*P*	Beta (95%CI)	*P*	Beta (95%CI)	*P*	Beta (95%CI)	*P*	Beta (95%CI)	*P*	Beta (95%CI)	*P*	Beta (95%CI)	*P*
Breast cancer ($n = 40$)																
Gal-2	16.02 (13.42–18.62	<0.001	4.56 (3.84–5.27)	<0.001	−0.002 (−0.007–0.004)	0.59	0.000 (−0.001–0.001)	0.71	15.97 (12.56–19.39)	<0.001	4.56 (3.62–5.51)	<0.001	−0.003 (−0.01–0.005)	0.51	−0.0002 (−0.002–0.001)	0.75
Gal-4	16.28 (7.93–24.62)	<0.001	4.63 (2.28–6.99)	<0.001	−0.002 (−0.012–0.007)	0.61	0.001 (−0.002–0.003)	0.60	−2.87 (−8.53–2.79)	0.31	−0.79 (−2.36–0.78)	0.32	−0.003 (−0.015–0.001)	0.64	0.0002 (−0.002–0.003)	0.88
Gal-8	19.96 (8.32–31.60)	0.001	5.55 (2.24–8.86)	0.002	0.008 (−0.005–0.020)	0.22	0.002 (−0.001–0.005)	0.13	6.162 (−0.269–12.59)	0.06	1.59 (−0.19–3.37)	0.079	0.012 (−0.003–0.026)	0.11	0.002 (−0.001–0.005)	0.16
F-test		NA		NA		NA		NA		<0.001		<0.001		0.40		0.50
R^2	NA		NA		NA		NA		0.82		0.83		0.08		0.06	
Colon cancer ($n = 50$)																
Gal-2	1.64 (1.28–2.01)	<0.001	0.13 (0.09–0.17)	<0.001	0.00 (−0.011–0.11)	0.96	0.001 (0.003–0.008)	<0.001	1.45 (1.08–1.82)	<0.001	0.11 (0.064–0.15)	<0.001	−0.000 (−0.012–0.12)	0.99	0.005 (0.0024–0.007)	<0.001
Gal-4	1.34 (0.50–2.18)	0.002	0.14 (0.04–0.2)	0.003	−0.002 (−0.019–0.015)	0.82	0.003 (−0.001–0.008)	0.15	0.56 (0.006–1.12)	0.048	0.064 (−0.002–0.13)	0.056	−0.002 (−0.02–0.16)	0.82	0.0002 (−0.003–0.004)	0.91
Gal-8	2.07 (0.39–3.76)	0.017	0.20 (0.04–0.36)	0.017	0.002 (−0.031–0.035)	0.90	0.13 (0.005–0.20)	0.002	1.04 (0.005–2.10)	0.050	0.11 (−0.01–0.24)	0.069	0.002 (−0.031–0.037)	0.88	0.0095 (0.002–0.16)	<0.001
F-test		NA		NA		NA		NA		<0.001		<0.001		0.99		<0.001
R^2	NA		NA		NA		NA		0.69		0.51		0.00		0.41	

Abbreviations: CI = confidence interval; CXCL-1 = chemokine C-X-C motif ligand 1; G-CSF = granulocyte colony-stimulating factor; GRO-α = growth-regulator oncogene α; IL-6 = interleukin-6; MCP-1 = monocyte chemotatic protein 1.

changes in MCP1/CCL2 in colon cancer sera. The galectin-induced secretion of these cytokines/chemokines was shown to enhance the expression of endothelial cell surface adhesion molecules, which causes increased cancer cell-endothelial adhesion, and also to increase endothelial tubule formation, a component of angiogenesis. Thus, the increased circulation of galectins -2, -4 and -8 found in cancer makes important contribution to the elevated circulating concentrations of these cytokines/chemokines that are frequently observed in cancer patients.

Interleukin-6 and G-CSF are pro-inflammatory cytokines and GROα, also known as CXCL1, NAP-3 (neutrophil-activating protein 3) or MSGAα (melanoma growth stimulating activity, alpha) and MCP-1, also known as CCL2 (chemokine c-c motif ligand 2) or small inducible cytokine A2 are chemoattractant cytokines, or chemokines. All these cytokines/chemokines are known to have very important roles in cancer progression and metastasis by regulating divergent tumour cell behaviours and the tumour microenvironment. Interleukin-6 interaction with its receptor IL-6Rα on cancer cells induces activation of cell survival signalling pathways such as JAK/STAT and Ras/ERK leading to increased cell proliferation and growth (Ara *et al*, 2009; Ara and Declerck, 2010). Interleukin-6 also induces the release of angiogenesis-promoting factors such as VEGF and bFGF (Wei *et al*, 2003). Interleukin-6 can activate STAT-3 signalling in regulator T cells and help tumour cells to escape immune surveillance (Mantovani *et al*, 2008). Interleukin-6 also promotes recruitment of circulating tumour cells back into their primary tumour sites, a process called 'tumour self-seeding' that accelerates tumour growth and angiogenesis (Kim *et al*, 2009a). Elevated concentrations of circulating IL-6 are common in cancer patients and correlate with metastasis and poor prognosis (Salgado *et al*, 2003; Michalaki *et al*, 2004; Ikeguchi *et al*, 2009; Knupfer and Preiss, 2010).

Interaction of G-CSF with its receptor G-CSFR increases cancer cell proliferation, invasion and migration (Wang *et al*, 2012). Higher serum concentrations of G-CSF are associated with poor prognosis in urothelial cancer (Mizutani *et al*, 1995). The presence of G-CSF modifies the distant microenvironment by mobilising Ly6G + Ly6C + granulocytes to create the so called 'pre-metastatic niche' before the arrival of metastatic tumour cells and facilitates subsequent tumour cell homing, migration, angiogenesis and metastasis at this newly created pre-metastatic environment (Kowanetz *et al*, 2010). Direct injections of G-CSF into the tail vein of nude mice increases lung metastasis of animals injected not only with metastatic but also with otherwise non-metastatic breast cancer cells (Kowanetz *et al*, 2010).

Growth-regulator oncogene α/CXCL1 and MCP-1/CCL2 are chemoattractant cytokines and, like many other chemokines, promote cancer progression and metastasis (Verbeke *et al*, 2011; Acharyya *et al*, 2012; Viola *et al*, 2012) by several mechanisms such as increased angiogenesis, activation of tumour-specific immune responses to weaken the host anti-tumour immunity, stimulation of tumour cell proliferation and metastasis (Payne and Cornelius, 2002; Verbeke *et al*, 2011). Interaction of GROα/CXCL1 with its receptor CXCR2 promotes cancer cell invasion and migration by activation of cellular AKT/NF-κB signalling (Kuo *et al*, 2012). Administration of anti- GROα/CXCL1 antibodies inhibits tumour formation and angiogenesis *in vitro* and in mice (Luan *et al*, 1997). Suppression of GROα/CXCL1 expression in human colon cancer LS174T cells by shRNA before cell inoculation into the spleens of nude mice almost completely prevents liver metastasis in comparison with those inoculated with parental LS174T cells (Bandapalli *et al*, 2012). Higher serum concentrations of GRO/CXCL1 are seen in cancer and, it, like IL-6, promotes colonisation of circulating tumour cells to their tumour of origins (self-seeding), thus accelerating tumour growth and angiogenesis of human breast, colon and melanoma tumours (Kim *et al*, 2009b).

Recently, GROα/CXCL1 has been reported to be at the centre of chemo-resistance triggered by chemotherapeutic agents of breast cancer by helping the tumour cells to recruit pro-survival factor S100A8/9 (Acharyya *et al*, 2012).

Monocyte chemotatic protein 1/CCL2, through interaction with its receptor CCR2 on cancer cells, increases cancer cell invasion and migration by activation of protein kinase C and protein tyrosine phosphorylation (Monti *et al*, 2003; Chiu *et al*, 2012). Inhibition of MCP-1/CCL2 biosynthesis by MCP-1/CCL2 inhibitor bindarit inhibits cancer cell proliferation and migration *in vitro* and significantly impairs metastasis of prostate cancer in mouse xenografts (Zollo *et al*, 2012). More lung metastases were formed in MCP-1(−/−) mice than in wild-type mice when 4T1 breast cancer cells were transplanted into the mammary pads (Yoshimura *et al*, 2013). Monocyte chemotatic protein 1/CCL2 concentrations are frequently elevated in cancer patients and it, like IL-6, is involved in driving the 'stemness' of tumour-initiating cells (Chin and Wang, 2013).

Thus, IL-6, G-CSF, GROα/CXCL1 and MCP-1/CCL2 are all critical regulators of cancer progression and metastasis via divergent mechanisms that act locally or remotely. The galectin-mediated increase of these cytokines/chemokines in cancers therefore likely makes an important contribution to the impact of these cytokines/chemokines on cancer progression and metastasis.

It is noted that, although galectins-2, -4 and -8 have each been shown to induce secretion of IL-6, G-CSF, GROα/CXCL1 and MCP-1/CCL2 from vascular endothelial cells *in vitro*, direct correlation of serum galectin concentrations in cancer patients was observed predominately with G-CSF and IL-6 and less with MCP-1/CCL2 and no correlation with GROα. It is possible that the galectin-mediated secretion of MCP-1/CCL2 and GROα/CXCL1 may be a secondary effect consequent to galectin stimulation of IL-6 and G-CSF secretion. Both GROα/CXCL1 and MCP-1/CCL2 have been shown previously to be inducible by pro-inflammatory cytokines. For example, IL-1 or TNFα can induce the secretion of GROα/CXCL1 from cancer as well as cancer stromal cells (Son *et al*, 2007; Kogan-Sakin *et al*, 2009), and IL-6 can induce the secretion of MCP-1/CCL2 from tumour cells (Lederle *et al*, 2011). The observation that these galectins had no significant effect on GROα/CXCL1 levels when injected directly into the animal tail vein is in keeping with this possibility. Further investigation to determine the identity and nature of the galectin-binding ligands on endothelial cells that are responsible for the galectin-mediated secretion of these cytokines should help to understand the actions of these galectins. It will also help to understand whether the galectin-induced increase in cytokine concentration shown in mice were entirely from the galectin-endothelium interaction or also contributed to by galectin interaction with non-endothelial cells.

The source of the increased circulation of these galectins in cancer patients is not yet known. Earlier studies have reported reduction of serum levels of galectin-3 (Iurisci *et al*, 2000), -1 and -4 (Watanabe *et al*, 2011) following surgical removal of the primary tumours in colorectal cancer patients, indicating that tumour cells may make significant contributions to the increased circulation of those galectin members. On the other hand, cellular expressions of galectin-8 and -4 have been reported to be lower in colorectal cancer than in healthy colonic epithelium (Rechreche *et al*, 1997; Nagy *et al*, 2002), although their circulating levels are both higher in cancer. This suggests that non-cancer cells likely make important contribution to the increased circulation of these galectins. Some galectins, e.g., galectin-1 are shown to be expressed more strongly in the peri-tumour stromal cells than in the tumours (Sanjuan *et al*, 1997), indicating the possible contribution of the peri-tumour stromal cells to the increased circulation of these galectins in cancer. All immune cells express galectins and such expression is known to be influenced by inflammatory regulators (Nangia-Makker *et al*, 1993). Many pro-inflammatory cytokines, such as TNF-α, IL-1, IL-8 and GM-CSF, are upregulated in cancerous conditions, and their presence may cause the immune cells to secrete more galectins into the bloodstream. Thus, the increased circulation of the members of galectins in cancer patients likely comes from the peri-tumour stromal tissues, the immune cells as well as the tumour cells themselves. Simultaneous determination of serum galectin-3 and -4 levels has shown to provide good sensitivity and specificity in predicting metastasis in colorectal cancer patients (Barrow *et al*, 2013).

The half-life of circulating galectins in the body is unknown. An earlier study has shown that galectin-3C, an N-terminally truncated form of galectin-3, had a 3 h half-life in the serum when injected intramuscularly into nude mice (John *et al*, 2003). This suggests that the half-life of circulating galectins in the body is likely to be a few hours.

It should be mentioned that the presence of serum glycoproteins (or specific antibodies) is likely, by competitive binding, to influence the biological availability of these galectins in the circulation. Members of the galectin family bind serum glycoproteins with very different affinities. A systemic analysis of the galectin-binding proteins in human serum has shown that galectin-3 and -8 both recognised a broad range of serum proteins, whereas galectin-2 and -4 showed binding to only trace or no serum ligands (Cederfur *et al*, 2008). For example, IgGA1 is shown to bind strongly to galectin-1 (Sangeetha and Appukuttan, 2005), whereas a haptoglobin-related serum glycoprotein (Bresalier *et al*, 2004) and the Mac-2-binding protein (Iacovazzi *et al*, 2010) are serum ligands of galectin-3. Our own study has shown that, although circulating galectin-2, -3, -4 and -8 levels were all elevated in the circulation of cancer patients and all were shown to induce cancer cell adhesion by interaction with the TF disaccharide on the cancer-associated MUC1 *in vitro*, only serum galectin-2 level was found to be directly associated with a significantly increased mortality in patients with colorectal cancer (Barrow *et al*, 2011a). Moreover, the galectin-2-associated reduction in patients' survival was found to be significantly reduced by the presence of autoantibodies against the TF epitope of MUC1 in the serum (Barrow *et al*, 2011a). Thus, the biological impact of the galectin-induced cytokine secretion and consequently cytokine-mediated metastasis and angiogenesis will be significantly influenced by the presence of galectin-binding glycoproteins and autoantibodies in the circulation and a net influence is likely to be contributed more by those galectins that have minimal binding competitors in the circulation (e.g., galectins -2 and -4). No significant correlation between cancer stages and the levels of circulating galectins -2, -4, and -8 was observed in the serum of breast and colorectal cancer patients (Barrow *et al*, 2011a).

Thus, the increased circulation of galectins -2, -4 and -8 in cancer enhances endothelial secretion of G-CSF, IL-6, GROα/CXCL1 and MCP-1/CCL2 into the blood circulation and makes important contribution to the increased circulation of these cytokines/chemokines frequently seen in cancer patients. As these cytokines/chemokines are important promoters in cancer progression and metastasis via divergent mechanisms locally and remotely, the galectin-mediated increase of these cytokines/chemokines in cancer patients likely has a profound influence on cancer progression and metastasis and represents a good target for cancer therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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