Immunomodulatory glc/man-directed *Dolichos lablab* lectin (DLL) evokes anti-tumour response *in vivo* by counteracting angiogenic gene expressions

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Summary

Neovascularization and jeopardized immunity has been critically emphasized for the establishment of malignant progression. Lectins are the diverse class of carbohydrate interacting proteins, having great potential as immunopotentiating and anti-cancer agents. The present investigation sought to demonstrate the anti-proliferative activity of *Dolichos lablab* lectin (DLL) encompassing immunomodulatory attributes. DLL specific to glucose and mannose carbohydrate moieties has been purified to homogeneity from the common dietary legume *D. lablab*. Results elucidated that DLL agglutinated blood cells non-specifically and displayed striking mitogenicity to human and murine lymphocytes *in vitro* with interleukin (IL)-2 production. The DLL-conditioned medium exerted cytotoxicity towards malignant cells and neoangiogenesis *in vitro*. Similarly, *in-vivo* anti-tumour investigation of DLL elucidated the regressed proliferation of ascitic and solid tumour cells, which was paralleled with blockade of tumour neovasculature. DLL-treated mice showed an up-regulated immunoregulatory cytokine IL-2 in contrast to severely declined levels in control mice. Mechanistic validation revealed that DLL has abrogated the microvessel formation by weakening the proangiogenic signals, specifically nuclear factor kappa B (NF-κB), hypoxia inducible factor 1α (HIF-1 α), matrix metalloproteinase (MMP)-2 and 9 and vascular endothelial growth factor (VEGF) in malignant cells leading to tumour regression. In summary, it is evident that the dietary lectin DLL potentially dampens the malignant establishment by mitigating neoangiogenesis and immune shutdown. For the first time, to our knowledge, this study illustrates the critical role of DLL as an immunostimulatory and anti-angiogenic molecule in cancer therapeutics.

Keywords: anti-tumour, *Dolichos lablab* lectin, immunomodulation, mitogenecity, tumour neovasculature

Introduction

Tumours foster a tolerant microenvironment and the activation of plethoric immunosuppressive mechanisms, which may act in concert to counteract effective immune responses. The immunosuppressive tumour immune microenvironment evades T cell responses, either to avoid immune recognition or to disable effector T cells [1,2].

This strategy by malignant cells to breach the immune surveillance mechanism of the host has been linked most decisively to cancer progression [3]. Hence, dampening the tumour immunosuppressive microenvironment by stimulating the innate anti-tumour immunity may be an effective approach to cancer treatment. Immunotherapy aimed at harnessing endogenous anti-tumour immunity by

modifying immune regulatory mechanisms has shown promise in multiple tumour types [2]. In compliance with this, recent findings in 2014 indicated that a complete immunological manoeuvre through the manipulation of immune system has represented an effective and reproducible outcome successfully, leading to the regression of large tumours in humans [4]. These observations and experimental understanding has not only deviated from our fundamental view of cancer therapeutics but has also propelled forward the renaissance of immunomodulation in eliciting anti-tumour immune responses, thereby enabling immune-based therapies to join the mainstream of cancer treatment.

Tumour neovasculature, the blood vessels produced within malignant growth by chronically activated angiogenesis and an unbalanced mix of proangiogenic signals, are known to be highly indispensable, even at the microscopic premalignant phase of neoplastic progression [5–7]. Intensive basic research and clinical practice have accentuated the important correlation between immunomodulation and angiogenesis [8]. Substantial evidence indicates that immunological cells such as T cells and certain immunoregulatory cytokines have shown the capacity to enhance immune response sufficiently to induce cancer remissions and inhibit tumour angiogenesis [9–11]. This suggests that a combinatorial approach encompassing anti-angiogenic and immunostimulatory perspectives has significant implications in the design of dynamic immunotherapeutic strategies for treating malignancies [11].

Lectins are the ubiquitous and diverse class of haemagglutinating proteins of non-immune origin that bind stereospecifically to cell surface glycan moieties [12–14]. The lectin interactions on immune cell surfaces trigger signal transduction and the production of various beneficial cytokines, due to which these proteins are taking the forefront in biological research [14,15]. The presence of haemagglutinin in the dietary bean *Dolichos lablab* (common name: Hyacinth bean) has been known since 1964 [14,16]. *D. lablab* lectin (DLL), of the leguminoseae category, belongs to the tribe Phaseoleae, which is demonstrated to be unique in its specificity for the monosaccharides glucose and mannose, unlike the other members of its tribe. Although DLL shares many biological activities with other legume lectins, especially with a prototype legume lectin concanavalin A (Con A), the complete primary sequencing of DLL elucidated several distinguishing features from those of other legume lectins [12–14]. Indispensable evidence from as early as the 1980s reported the mitogenic properties of mannoside-specific DLL towards lymphocytes [17], but even after three decades there have been no significant translational investigations to date, in spite of its immunomodulatory and therapeutic possibilities. As legume lectins have received remarkable attention due to their potent anti-neoplastic activity [18], in this present study, for the first time to our knowledge, we sought to investigate the pharmacological potential of immunostimulatory *D. lablab* lectin directed to glucose and mannose in evoking the anti-tumour response in murine models targeting critical *in-vivo* tumour angiogenic parameters. This current study, envisaging a dietary and natural immunomodulator, promises a future scenario in overcoming immunosuppression and promoting tumour regression.

Materials and methods

Materials

The materials used were lung adenocarcinoma (A549), cervical carcinoma (SiHa, CaSki) and Ehrlich ascites carcinoma (EAC) from the National Centre for Cell Science (NCCS), Pune, India; squamous cell carcinoma (A388) from the National Centre for Biological Science (NCBS), Bengaluru, India and murine Dalton's ascites lymphoma (DLA) cells, a kind gift from Dr Sathees C. Raghavan, Indian Institute of Science (IISc), Bengaluru, India; and human umbilical vascular endothelial cells (HUVEC), endothelial cell growth media (EGM), sodium bicarbonate ($NaHCO_3$), cyanogen bromide (CNBr) activated Sepharose 4B, matrigel [extracellular matrix (ECM) gel], hydron polymer poly-hydroxyethyl-methacrylate (poly-HEMA), Ficoll histopaque, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), anti-vascular endothelial growth factor (VEGF) and anti-mouse/rabbit immunoglobulin (Ig)G antibodies, protease inhibitor cocktail and Freund's adjuvants were obtained from Sigma-Aldrich (St Louis, MO, USA), anti-IL-2, anti-hypoxia inducible factor 1α (HIF-1α); anti-MMP-2 and 9 antibodies from Santa Cruz Laboratories (Santa Cruz, CA, USA); anti-nuclear factor kappa B (NF-κB), anti-IκB and anti-β-actin from BD Biosciences (San Jose, CA, USA); RPMI medium, antibiotic–anti-micotic solution, fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA, USA); anti-CD-31 antibody and immunostaining kit from Leica Biosystems (Wetzlar, Germany). Schiff's fuchsin-sulphite reagent, ovalbumin, α-methyl mannoside (αMM) and other carbohydrates were from HiMedia Laboratories, India. Prestained protein marker was obtained from New England Biolabs (Ipswich, MA, USA); cell culture plastic wares were from Eppendorf (Hamburg, Germany); all the other chemicals used were of analytical grade. Fertilized hen's eggs were procured from a local farmhouse in Shivamogga, India. Photographs were taken using a Sony Steady Shot DSC-W610 camera. All the angiogenesis images were analysed by ImageJ software (NIH, Bethesda, MD, USA).

Purification and characterization of *D. lablab* lectin (DLL)

D. lablab lectin (DLL) was purified from the seeds of *D. lablab* by affinity chromatography, as described previously

by Mo *et al.*, with slight modifications [19]. Briefly, the dried bean flour of *D. lablab* was extracted at 4°C with 10 mM phosphate-buffered saline (PBS) pH 7·4. After subjecting to ammonium sulphate precipitation (20–60% saturation), the filtrate was dialysed and the clear dialysate was applied onto the ovalbumin-conjugated Sepharose 4B column. The affinity-adsorbed lectin was eluted specifically with 0·2 M α-methyl mannoside in PBS at a flow rate of 15 ml/h. The pure lectin fractions were pooled, dialyzed extensively against PBS and analysed by reducing 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were then subjected to Coomassie brilliant blue and periodic acid-Schiff (PAS) staining for protein band visualization and glycoprotein analysis, respectively [20]. The bands were documented using the Bio-Rad Gel Documentation^{TM} XR + imaging system. Polyclonal antibodies against purified DLL were raised in New Zealand White (NZW) rabbits in accordance with standardized protocols [21]. The immunoreactivity of rabbit polyclonal anti-serum was assessed by immunoblotting and double immunoprecipitation assays.

Agglutination and carbohydrate inhibition assay

Haemagglutination assay (HA) was carried out using trypsinized 2% chick, rabbit and human blood (types A, B, AB and O) erythrocyte suspension, as described previously [20]. Informed consent was obtained from all healthy human volunteers (age range = 20–30 years) for obtaining peripheral venous blood. Briefly, 100 μl of erythrocytes suspension was added to an equal volume of serially diluted protein solution in a concavity agglutination plate, mixed gently and incubated at 37°C for 1 h to visualize the agglutination. Carbohydrate-mediated HA inhibition was assessed by performing haemagglutination assay in the preincubated DLL with serially diluted sugars and glycoproteins [20].

Leucoagglutination assay was carried out using human peripheral blood lymphocytes (PBLs) that were separated using Ficoll-Hypaque and density gradient centrifugation, as described previously [21]. The separated lymphocytes (100 μl) in 10 mM PBS were then added to an equal volume of DLL in a concavity agglutination plate, mixed gently and incubated at 37°C for 1 h. Cell agglutinations were visualized using optical light microscopy and photographed.

Lymphocyte mitogenicity assay

Human PBLs, murine splenocytes and thymocytes were isolated and cultured in complete RPMI medium in a humidified environment at 37°C [21]. The human and murine lymphocytes were cultured separately in flat-bottomed 96-well microtitre plates (200 μl of 2·5 × 10^6 cells/ml) in a CO$_2$ incubator at 37°C with 5% CO$_2$. After 3 h incubation, the cells were treated with crude DLL extracts, DLL (0, 5 and 10 μg/ml of media) and reference mitogen Con A (0, 5 and 10 μg/ml) for 72 h and MTT assay was performed. Supernatants from 24 h DLL (10 μg/ml)-stimulated cultures were collected and used as conditioned media (DLL-CM) for further studies [17].

Cell culture and *in-vitro* treatment

The cells A549, A388, SiHa, CaSki, DLA and EAC were grown in RPMI medium supplemented with 10% FBS, antibiotic–micotics and NaHCO$_3$ (0·37%) and incubated at 37°C in 5% CO$_2$ with 98% humidity. The anti-proliferative effect of DLL (0–50 μg/ml) and DLL-CM (0–50%) for 48 h was evaluated by MTT, trypan blue and lactate dehydrogenase (LDH) release assays and IC$_{50}$ values were calculated. For every experiment 5-fluorouracil was used as a positive control. Each experiment was repeated a minimum of three times independently and analysed with an appropriate vehicle and positive control [22].

Chorioallontoic membrane (CAM) assay

DLL- and DLL-CM-mediated angiogenic responsiveness to the recombinant VEGF$_{165}$ (rVEGF$_{165}$) induced *in-vivo* CAM angiogenesis were studied as described previously [23]. Of note, DLL (10 μg/CAM) or DLL-CM (10 μg/CAM) were added to the growing fertilized eggs (5th day) and changes in the vascularization patterns were examined and photographed.

Rat aortic ring assay

The effect of DLL and DLL-CM on neovessel sprouting was studied by culturing aortic explants in matrigel as per the previously described methods, with slight modifications [24]. Briefly, transverse sectioned thoracic aortas with 1 mm diameter were cultured in matrigel immersed with RPMI media in a humidified environment at 37°C for 2 weeks. DLL (10 μg/ml) or DLL-CM (30%) was added directly to the culture media and allowed to diffuse into the gel. Cultures were investigated by inverted light microscopy (Olympus) at ×10 optical magnification.

Capillary tube formation assay

Functional analysis of DLL and DLL-CM on endothelial cells for vascular tube formation was performed following previous methods, with slight modifications [25]. Briefly, 100 μl of 2 × 10^4 endothelial cells in EGM were seeded onto matrigel-coated 96-well plates followed by treatment with or without rVEGF$_{165}$, DLL (10 μg/ml) and DLL-CM (30%). After 4–6 h of incubation at 37°C with 5% CO$_2$, the cells were assessed for formation of capillary-like structures using an inverted light microscope (Olympus) at ×10 optical magnification.

Transwell invasion assay

The cell invasion inhibitory effects of DLL and DLL-CM were investigated by Transwell invasion assay [26]. Briefly, the harvested A549 cells (3×10^4) in 200 µl of serum-free RPMI medium with or without DLL (10 µg/ml) and DLL-CM (30%), respectively, were added to the matrigel-coated upper chamber; 0·6 ml of RPMI with 20% FBS was added to the lower compartment and the Transwell-containing plates were incubated at 37°C and 5% CO_2 for 8 h. The invaded cells were stained with 0.1% crystal violet and were captured using a photomicroscope at ×40 resolution.

In-vivo studies

Animals. BALB/c mice, Swiss albino mice aged 6–7 weeks, weighing 25–30 g, and Swiss albino Wistar rats aged 7–8 weeks, weighing 125–150 g, were housed under standard laboratory conditions and fed with commercial rodent meal and water *ad libitum*. All the animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), National College of Pharmacy, Shimoga, India, in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for laboratory animal facility (NCP/IAEC/CL/101/05/2012-13).

Animal tumour development and treatment. DLA cells were cultured by intraperitoneal (i.p.) transplantation to develop ascites tumour. After the fourth day of tumour growth, the mice were administered with three doses of DLL [0, 20 and 30 mg/kg body weight (b.w.) i.p., $n = 6$] on every alternative day. Ascitic tumour parameters were noted. Solid lymphoma (DLS) was developed in mice by injecting the cells subcutaneously (s.c.) in the right hindlimb according to previously described methods [23]. After the visible development of the tumour, mice were administered with six doses of DLL (0, 20 and 30 mg/kg b.w. i.p., $n = 6$) on alternate days and tumour progression was monitored. The number of animals that survived and the duration after the treatment regimen were documented in a separate analysis. Toxicological parameters were examined by measuring the hepatic catalase and lipid peroxidation [27]. To assess the physiological effect of DLL in normal mice, the serum of DLL-treated animals were subjected to liver and kidney function tests by measuring the levels of alkaline phosphatase (ALP), creatinine and urea [23].

MVD evaluation in peritoneum. Microvessel density (MVD) was assessed in the peritoneal lining of control and treated ascitic lymphomas, as reported previously [28]. Briefly, after the treatment regimen as described above, the response of DLL to the tumour-induced neovessel formation in peritoneum lining was observed and documented. Further, peritoneal tissue sections were processed for haematoxylin and eosin (H&E) staining and the MVD/high-power field (HPF) was quantified manually.

ELISA. The *in-vitro* and *in-vivo* secretion of IL-2 levels and serum VEGF-A were quantified by enzyme-linked immunosorbent assay (ELISA). In brief, 100 µl of conditioned media or serum were coated in coating buffer at 4°C incubated with anti-IL-2 or anti-VEGF-A, followed by reincubation with ALP-conjugated secondary antibody. IL-2 and VEGF-A was quantified by measuring absorbance at 405 nm by using p-nitrophenyl phosphate (PNPP) as substrate.

Matrigel plug assay. BALB/c mice were implanted with 0·5 ml of matrigel + $rVEGF_{165}$ (10 ng/plug) mixture with or without DLL (10 µg/plug) in the right flank. After 7 days following the implantation procedure, matrigel implants were harvested and liquefied by incubation at 4°C overnight in 300 µl PBS. Further, the angiogenesis was quantified by estimating haemoglobin content in implants, as per previous reports [29].

Corneal neovascularization assay. The rat corneal micropocket assay was performed in Swiss albino Wistar rats, as described previously with slight modifications [30]. Briefly after anaesthetizing the animal, micropockets were created using the slit knife in cornea. Hydron pellets containing 10 ng/pellet of either $rVEGF_{165}$ plus or minus DLL (10 µg) were implanted into corneal pockets. After 6–8 days the eyes were examined for neovasculature and photographed. The corneas were then dissected and subjected to histopathological assessment.

Protein extracts and Western blotting

Protein extracts were obtained from DLA and DLS tumour tissues (*in vivo*) for Western blot analysis. The proteins (30 µg) were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. After blocking with 3% BSA in Tris-buffered saline Tween 20 (TBST) (150 mM NaCl, 10 mM Tris and 0.025% Tween 20 in dH_2O), immunoblot analysis was performed for HIF-1α, VEGF-A, MMP-2, MMP-9, NF-κB, inhibitory kappa B (I-κB) and β-actin proteins using appropriate primary/secondary antibodies in blocking buffer three times independently, as per the manufacturer's recommendations. Results were repeated to the control by using ImageJ software.

Gelatin zymogram

DLA ascitic secretions were subjected to gelatin zymography to quantify gelatinase activity of MMP-2 and MMP-9. Briefly, proteins of the whole cell lysate were resolved in 8% SDS-PAGE gels containing 0·1% (w/v) gelatin and zymogram was developed, as reported previously [28,31]. Inhibition of gelatin lysis zones were documented using the Bio-Rad Gel Documentation™ XR+ Imaging System.
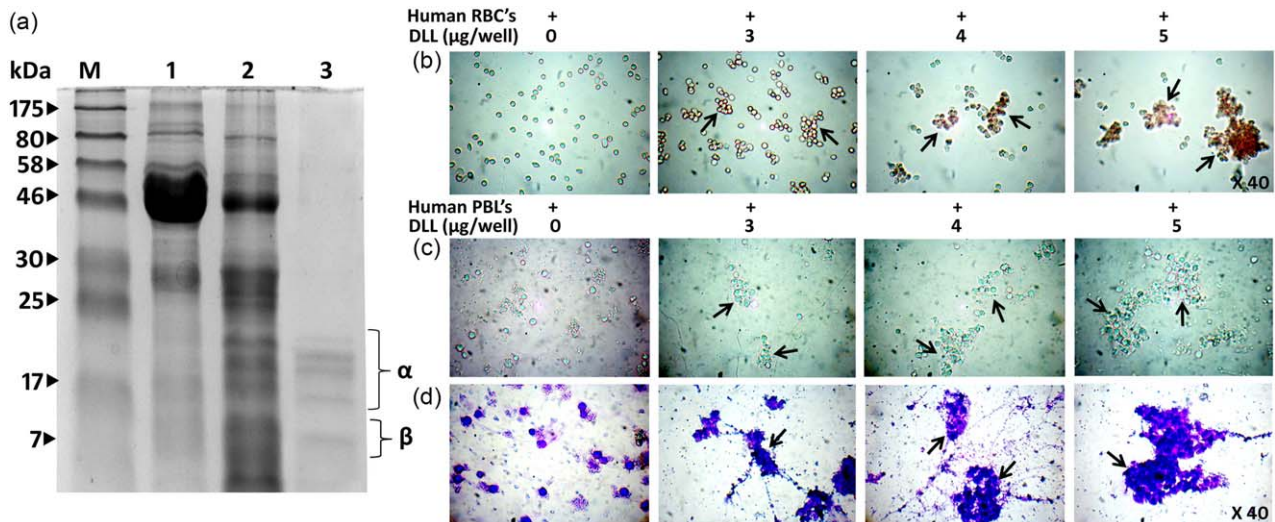
Fig. 1. Purified *Dolichos lablab* lectin (DLL) exerts potent erythro- and leucoagglutinating activity. (a) Molecular mass determination of purified *D. lablab* lectin by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Lane profile: M = marker, 1 = crude *D. lablab* extracts (3%), 2 = ammonium sulphate precipitated (20–60%) DLL extracts, 3 = purified Glc/Man-specific DLL. Lane 3 shows purified protein fractions with five subunits with apparent masses ranging from 10 to 22 kDa. The lectin activity of the purified DLL was examined by its cell agglutinating characteristics employing human red blood cells (RBCs) and leucocytes. (b) DLL potently agglutinates 2% trypsinized human RBCs in sharp contrast to the standard untreated with distinguished separate RBCs. (c) DLL displays leucocyte agglutinating behaviour in a concentration-dependent manner. The leucocytes were separated through density gradient centrifugation, as described in the Materials and methods section. The illustrations are true-colour microscopic photographs at ×40 optical resolution. (d) Leucocytes visualized post-DLL treatment and leucocyte staining at ×40 resolution exhibit significant agglutinating pattern that is conspicuously distinctive to that of control. The dark arrows indicate the agglutinated cells. The results are representative of three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com].

Immunohistochemical (IHC) analysis

Immunological detection of CD31, HIF-1α, MMP-2, MMP-9 and NF-κB in the formalin-fixed sections of the peritoneum and solid tumour tissues of the control and DLL-treated animals was performed using the specific antibodies, as per the manufacturer's instructions. Observations were made using the EVOS imaging system (Life Technologies, Paisley, UK) under ×20 brightfield objective and the changes in intensity of the antibody staining were evaluated [32].

Statistics

The data were analysed and graphs were made using MS Excel version 8.1. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Student's t-test. Values were expressed as mean ± standard error (s.e.m.). Values of $*P < 0.05$ and $**P < 0.01$ were considered statistically significant.

Results

Purified DLL specific to glucose/mannose agglutinates haemocytes and leucocytes

D. lablab lectin was purified to the electrophoretic homogeneity from the crude *D. lablab* bean extract (Supporting information, Fig. S1a). Molecular mass estimation by reducing SDS-PAGE revealed that purified DLL resolved into five bands comprising α (band 1–4) and β (band 5) subunits, with apparent molecular weight ranging from 10 to 22 kDa (Fig. 1a), similar to previous reports [19]. The chromatographic component DLL was tested for the haemagglutination activity which agglutinated chick, rabbit and human erythrocytes irrespective of blood type (Fig. 1b). Leucoagglutination assay deduced a clear agglutination of the cells mediated by DLL in the microscopic visualization (Fig. 1c,d). The minimal concentration for DLL to provide a visible haemagglutination was observed to be ~3 μg in human RBC suspension (Supporting information, Fig. S1b). DLL was found to be positive to PAS staining, confirming the glycoprotein nature of the lectin (Supporting information, Fig. S1c). Rabbit anti-DLL antibodies showed strong immunoreactivity to DLL as analysed by immunoblot and the double immunodiffusion technique (Supporting information, Fig. S1d,e). DLL haemagglutination was inhibited specifically by the monosaccharides D-mannose and D-glucose, with inhibitory concentration of ~6 mM (data not shown).

DLL induces potent mitogenicity in human and murine lymphocytes with IL-2 synthesis

Human PBLs, murine splenocytes and thymocytes were subjected to DLL stimulation to investigate the lectins'
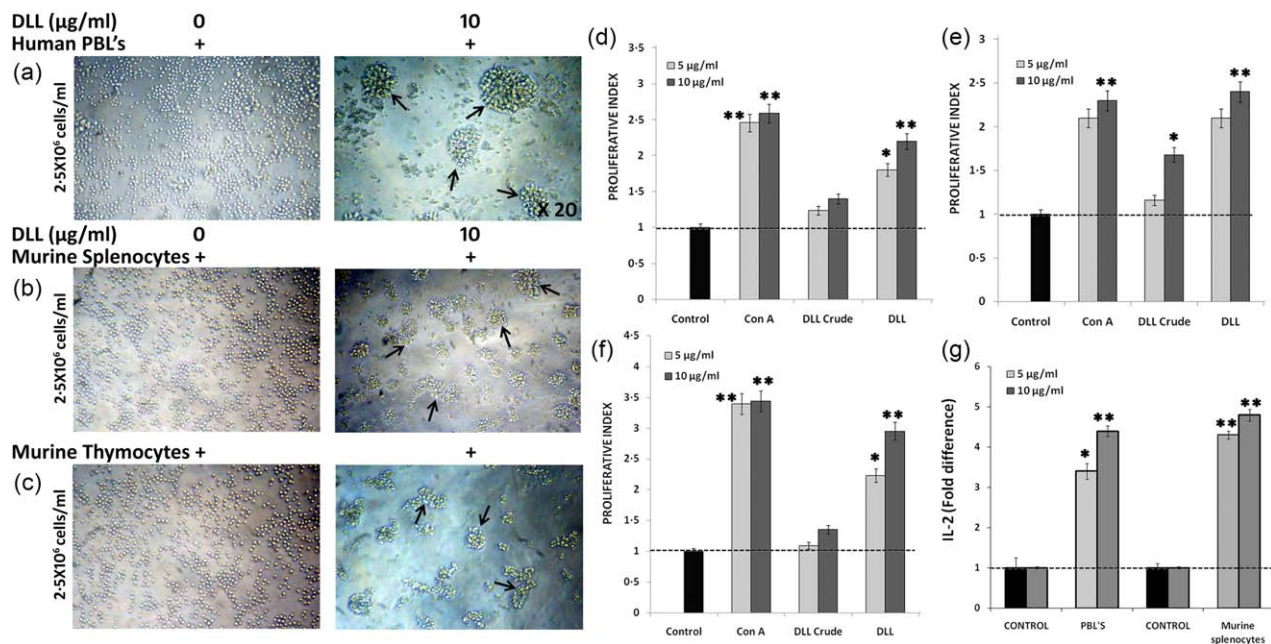
Fig. 2. Immunostimulatory activity of *Dolichos lablab* lectin (DLL) on human and murine lymphocytes *in vitro*. To investigate the mitogenic attribute of DLL, *in-vitro* cultured lymphocytes were treated with varying lectin concentrations and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) proliferation assay, as told in the Methodology section. (a) Representative visual depictions of the purified DLL-induced mitogenicity on human peripheral blood lymphocytes (2.5×10^6 cells/ml) at 10 μg/ml concentration. The cell cluster formation, a prerequisite event in the cellular proliferation, could be observed in DLL, whereas untreated cells appear distinctively separated. Similar cell clusters were also observed in (b) murine splenocytes (c) and thymocytes. The true-coloured and unstained images were observed through brightfield inverted microscope (Olympus) at ×20 optical magnification. The proliferative effect of DLL was expressed in terms of proliferative index. The index for control (non-stimulated) cells was taken as 1·0 and for others it is calculated as a ratio of absorbance at 570 nm of the test sample to that of the control. Graphs indicating the proliferative index of DLL on human peripheral blood lymphocytes (PBLs) (d), splenocytes (e) and thymocytes (f) *in vitro*. (g) Effect of DLL on *ex-vivo* interleukin (IL)-2 production by human PBL and murine splenocytes. Results are the means of three determinations, each conducted in triplicate. Statistically significant values are $^*P < 0.05$; $^{**}P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

immunoproliferative properties (Fig. 2a–c). The purified DLL (5 and 10 μg/ml) triggered a potent and significant mitogenic response in both human (Fig. 2d) and murine lymphocytes (Fig. 2e,f) in a dose-dependent manner, which is more strikingly equivalent to Con A. Upon treatment with DLL, formation of aberrant lymphocyte clusters were observed, which is reported to be one of the prerequisite events in the lymphocyte stimulation (Fig. 2a). As can be seen in Fig. 2a–c, microscopic evaluation reveals a varied and distinctive pattern of cell cluster formation in human and murine lymphocytes by DLL. The proliferative index for control was taken as 1·0 and others are represented as fold increase or decrease over the control. Supernatants from the DLL-stimulated cell cultures were subjected to the estimation of IL-2 secretions. Similar to the previous report [17], DLL-stimulated lymphocytes showed increased IL-2 synthesis in optimal concentrations (Fig. 2g).

DLL-CM displays cytotoxic and anti-angiogenic attribute *in vitro*

Compelling evidence from various experiments has indicated that T cells are essential for an anti-proliferative and anti-angiogenic immune response. As it is known that glucose/mannose-specific DLL is a potent T cell mitogen [17], we attempted to understand the anti-proliferative and neovessel inhibitory effect of DLL-CM and DLL in standard *in-vitro* assay systems. *In-vitro* cytotoxicity activity of DLL-CM, as verified by cytotoxic assays, elucidated a decreased cell viability of A549, A388, SiHa, CaSki, DLA and EAC cells, where DLL alone did not show significant cytotoxicity or sensitivity, as shown in Table 1. With respect to the effect of DLL and DLL-CM on angiogenesis, results inferred that MVD counts in *ex-ovo* CAM and rat aortic angiogenesis influenced by rVEGF$_{165}$ was regressed markedly by the conditioned medium (68·8 and 77·1%, respectively) (Fig. 3a,b). Treatment with DLL displayed 28·4 and 22·7% inhibition of CAM vessel density and aortic sprouting, respectively, which is apparently 2·4- and 3·5-fold less than the effect of conditioned medium (Fig. 3d,e). To further affirm the anti-angiogenic activity, DLL-CM was verified by tube formation assay, which indicated a decreased tube-forming efficiency by 71·3%, whereas DLL alone showed very negligible inhibition (Fig. 3c,f).

Table 1. IC_{50} value of *Dolichos lablab* lectin (DLL) and DLL-conditioned medium (CM) against various human and mouse cancer cell lines

Cell line	DLL (µg) IC_{50} value	DLL-CM (µg) IC_{50} value	5-Fluorouracil (µg) IC_{50} value
A549	34·7 ± 2·9	4·2 ± 0·6*	3·1 ± 0·5*
A388	36·1 ± 3·7	5·3 ± 0·4**	4·7 ± 0·8
SiHa	38·3 ± 3·3	5·7 ± 0·3**	3·7 ± 0·6*
CaSki	37·7 ± 3·1	6·1 ± 0·5*	4·4 ± 0·4**
DLA	38·6 ± 1·5	6·4 ± 0·6*	2·3 ± 0·3**
EAC	44·3 ± 3·5	7·3 ± 0·5*	4·0 ± 0·2**

5-Fluorouracil is used a positive control. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Trypan blue and lactate dehydrogenase (LDH) release assays against each cell line in three independent ($n = 3$) and values represent IC_{50} by MTT. Dimethylsulphoxide (DMSO) is used as a vehicle control, which showed very negligible cytotoxicity. Values are indicated by mean ± standard error (s.e.) and statistically significant values are expressed as *$P < 0.05$ and **$P < 0.01$.

DLL impedes proliferation of murine ascitic and solid lymphoma tumour *in vivo* through immunomodulation

IL-2 is an immunoregulatory cytokine possessing anti-tumour activity [4]. Our observations and previous report indicate [17] that DLL stimulates the immune system through the secretion of IL-2 (Fig. 2g). This has prompted us to investigate the effect of lectins in mitigating the progression of malignant cells in *in-vivo* murine ascitic and solid lymphoma tumour models. Results inferred that, in ascites tumour, administration of DLL resulted in significant diminution of tumour growth by 71·8 and 82·6% in varying concentrations (Supporting information, Fig. S2a). Decisive evidence has indicated that DLL treatment has impacted negatively the various tumorigenic parameters, such as cell count and ascites secretion, in a dose-dependent manner (Supporting information, Fig. S2b,c). Moreover, survival analysis after the treatment regimen showed an approximate 2·1–2·5-fold prolonged lifespan, although a moderate recurrence (∼10%) was observed, as analysed by body weight index (Supporting information, Fig. S2d).

The tumour inhibitory potential of DLL was re-evaluated further in Dalton's lymphoma solid tumour in mice. Following six consecutive DLL administrations on alternate days, there was considerable dose-dependent growth inhibition. The dissected tumour in the control weighed 17 g, whereas in treated mice it was 2·61–3·12-fold less than that of control at varying concentrations, thereby providing a clear perspective on the regression of tumour growth (Fig. 4a–e). As in the ascites model study, DLL was able to extend the animals' lifespan from 45 to 98 and > 120 days (Fig. 4f). However, oral administration of DLL after tumour onset did not produce the same tumour inhibitory results (data not shown). As DLL-CM exerted cytotoxicity *in vitro* (Table 1), the immunomodulatory implications of DLL in endogenous systems were evaluated. Interestingly, concurrent to the *in-vitro* conditions, DLL up-regulated the serum IL-2 by twofold in tumour mice to that of control (Fig. 4i). Furthermore, histopathological and gross examinations of internal organs showed no toxicity after DLL treatment (Supporting information, Fig. S3a–c). Biochemical evaluation of liver enzymes revealed a decreased lipid peroxidation and elevated catalase activity upon DLL treatment (Fig. 4g,h). Serological and haematological examination produced normal values after DLL treatment in normal non-tumour-bearing mice, indicating limited secondary complications (data not shown).

DLL deters tumour-reliant and independent neovascularization *in rodents*

Tumour development induces an early angiogenesis response, which is a critical and rate-limiting event in tumour progression [5,6]. Hence, modalities blocking neovascular sprout are of particular interest in oncolytic strategies. Because we observed DLL-CM inhibiting angiogenesis *in vitro* (Fig. 3), DLL was investigated for its deterrent effect on tumour-induced angiogenesis in murine ascites and solid lymphoma. Visible observation demonstrated that DLL decreased the development of ascitic lymphoma-induced peritoneal angiogenesis considerably by 1··21–1·64-fold compared to the control (Fig. 5a). Measurement of microvascular density (MVD) by H&E and anti-CD31 immunostained histological sections again validated that DLL restricted the MVD/HPF to 28·8 ± 1·2 and 24·32 ± 2·6 in 20 and 30 mg/kg concentrations, respectively, compared to the untreated mice (57 ± 6·2, Fig. 5b,c). Similarly, the implication of DLL in blocking the tumour vessel density was studied further by employing Dalton's lymphoma solid tumours, which were subjected to histological and IHC studies. The results show that DLL lowered the MVD/HPF drastically, by ∼56 and ∼72% at 20 mg/kg b.w. and 30 mg/kg b.w., respectively, to that of control (Fig. 5d,e). It should be noted that the decreased tumour MVD concurred with the regressed tumour volume and prolonged survival in the DLL group (Fig. 5f).

To examine the angiopreventive effect of DLL in non-tumour systems, lectin was subjected to rVEGF$_{165}$-induced angiogenesis *in vivo*. The results indicated that DLL arrested the corneal angiogenesis substantially in rats (Fig. 5f), with the MVD count reduced by 1·43-fold compared to the rVEGF$_{165}$-treated control, and this was confirmed further by histopathological examination of corneal sections (Fig. 5g). Matrigel plug assay also displayed a similar 1·35-fold reduction in rVEGF$_{165}$-mediated vasculature in the DLL group compared to the control, as verified visibly and by haemoglobin estimation (Fig. 5h).

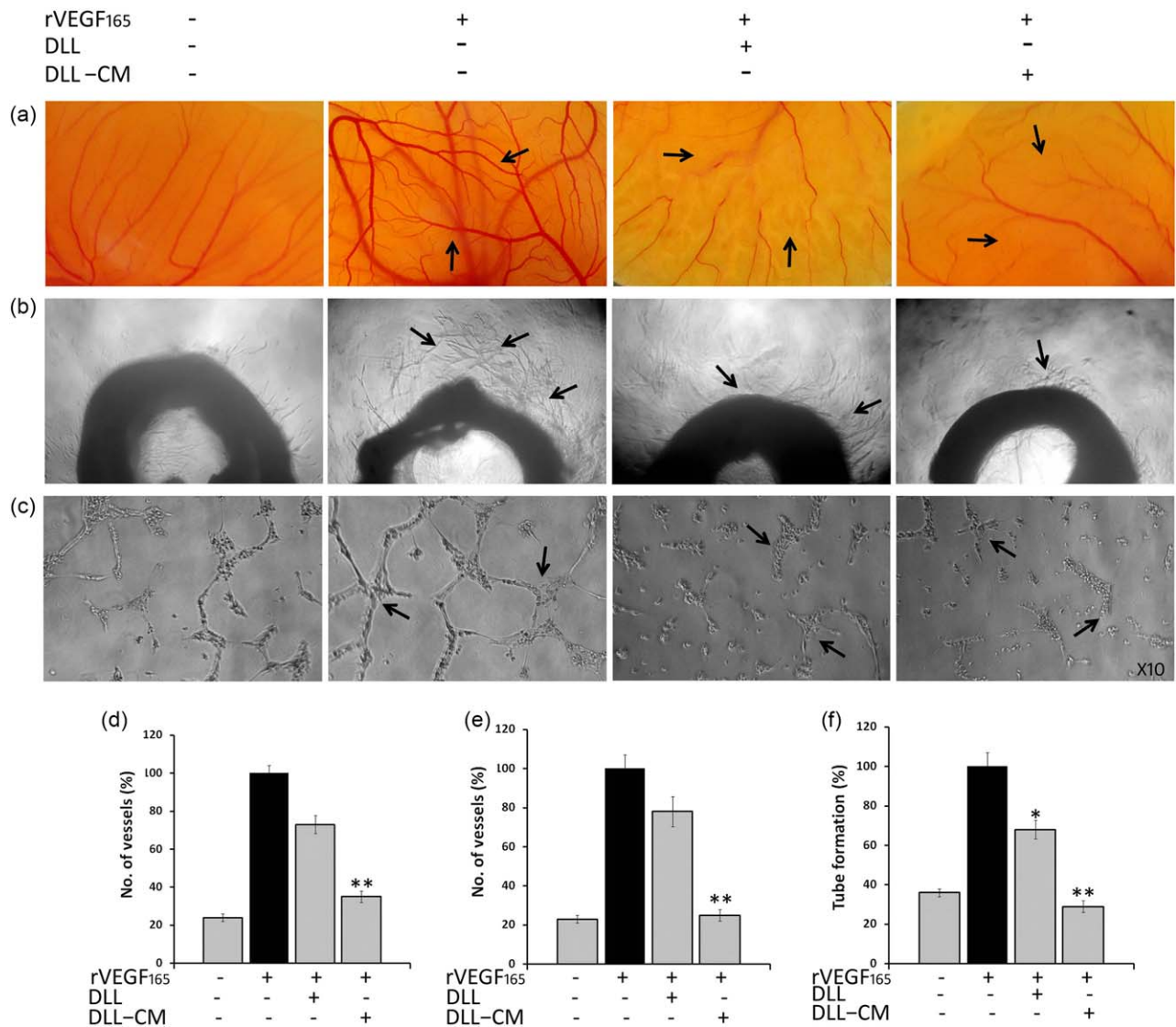|			
rVEGF₁₆₅	−	+	+	+
DLL	−	−	+	−
DLL −CM	−	−	−	+

Fig. 3. Anti-angiogenic activity of *Dolichos lablab* lectin-conditioned medium (DLL-CM) in *in-vitro* angiogenic assay models. Indirect angioinhibitory effect of DLL on various angiogenic systems *in vitro*. DLL-CM was the supernatant of DLL exposed to murine splenocytes after 24 h incubation. DLL-CM exhibits regressed angiogenesis in (a) *ex-vivo* chorioallontoic membrane (CAM) and (b) rat aortic angiogenesis assay. DLL directly exhibited only a minimal angiolytic effect. (c) DLL-CM exposed to human umbilical vein endothelial cells (HUVEC) cells inhibits the tube-forming efficiency. Pictographical representation of (d) *ex-vivo* CAM, (e) aortic ring angiogenesis and (f) tube formation assay. Data were represented as mean ± standard error of the mean (s.e.m.) of three independent experiments. Statistically significant values are expressed as $*P < 0.05$ and $**P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

DLL abrogates neovasculogenesis through altering the angiogenic signalling cascade in malignant cells

As the angiogenic capacity of tumour cells is largely attributable to the intrinsic genetic alterations that contribute to the establishment of the malignant phenotype [33], DLL-treated cells were subjected to specific gene expression studies. DLL down-regulated various pro-angiogenic gene expressions, such as HIF-1α, VEGF-A, NF-κB, I-κB, MMP-2 and MMP-9 in both *in-vivo* ascitic and solid tumours compared to that of untreated tumours, as validated by

Western blot (Fig. 6a,b). Serological determination of secreted VEGF showed abridged levels in contrast to control (Fig. 6c). In corroboration with these observations, gelatin zymography revealed a decreased level of MMP-2 and MMP-9 in DLL-treated ascitic secretions (Fig. 6d). Counteraction of MMP-2 and MMP-9 expression resulted in the inhibition of invasive potency of A549 cells (Fig. 6e). Immunohistochemical examination for the detection of HIF-1α, NF-κB, MMP-2 and MMP-9 corroborated the immunoblot, gelatin zymogram and invasion assay results further (Fig. 6f–j).
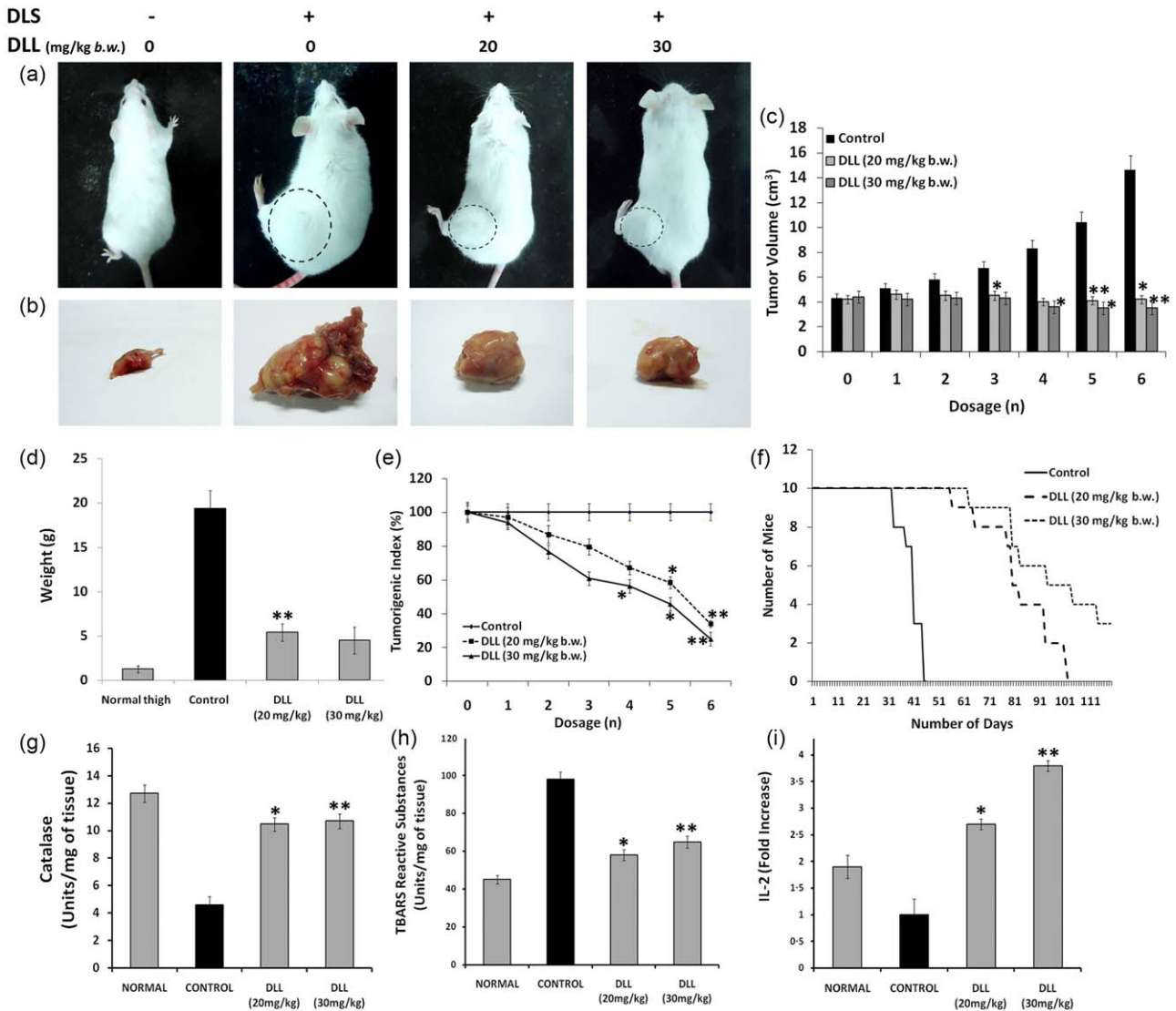
Fig. 4. *Dolichos lablab* lectin (DLL) mitigates the proliferation of murine Dalton's lymphoma solid tumour *in-vivo* with up-regulation of IL-2 levels. Solid tumour was induced by injecting Dalton's lymphoma cells (1×10^6 cells/mouse) in hindlimbs of Swiss albino mice. After the palpable development of tumour, six doses of DLL (20 and 30 mg/kg body weight b.w.) were administered intraperitoneally (i.p.) to tumour-bearing mice on every alternate day. (a) Tumour morphology of the DLS-induced mice with and without DLL treatment. The circular dotted lines represent the extent of tumour growth. (b) Gross anatomical appearance of the excised DLS tumours. Note the extensive vascularization surrounding the tumour tissue of the untreated control. (c) The effect of DLL on DLS tumour progression at different time-points. (d) Weight of the excised normal thigh, control and DLL-treated tumours (g). (e) Tumorigenic index elucidating the dose-dependent decrease of tumour progression post-DLL regimen. (f) Kaplan–Meier survival curve of the animals treated with DLL. Effect of DLL in the endogenous hepatic anti-oxidant system (d) pronounced catalase enzymes levels in DLL-treated mice. Note control with severely declined enzyme content. (e) Decreased lipid peroxidation in DLL-treated mice, as assessed by the thiobarbituric reactive substances (TBARS) method. (f) Fold difference in the serum interleukin (IL)-2 levels of DLL-treated animals compared to control. The control animal shows declined levels of IL-2, whereas DLL at 20 and 30 mg/kg b.w. via i.p. administration shows pronounced IL-2 levels. Results are the means of three determinations, each conducted in triplicate. Statistically significant values are $^*P < 0.05$; $^{**}P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

Discussion

The legume lectin family is well known for its far-ranging biological functions [18]. The unique glycan-interacting potential of lectins on the immune cell surfaces ensues positive amplification of the immune system, thereby rendering them invaluable as tools in oncological and bio-medical research [14,15]. In line with previous reports [19], we have purified a well-known glucose/mannose-specific *D. lablab* (DLL) lectin from the common dietary bean *D. lablab* of the leguminoseae family. The complete
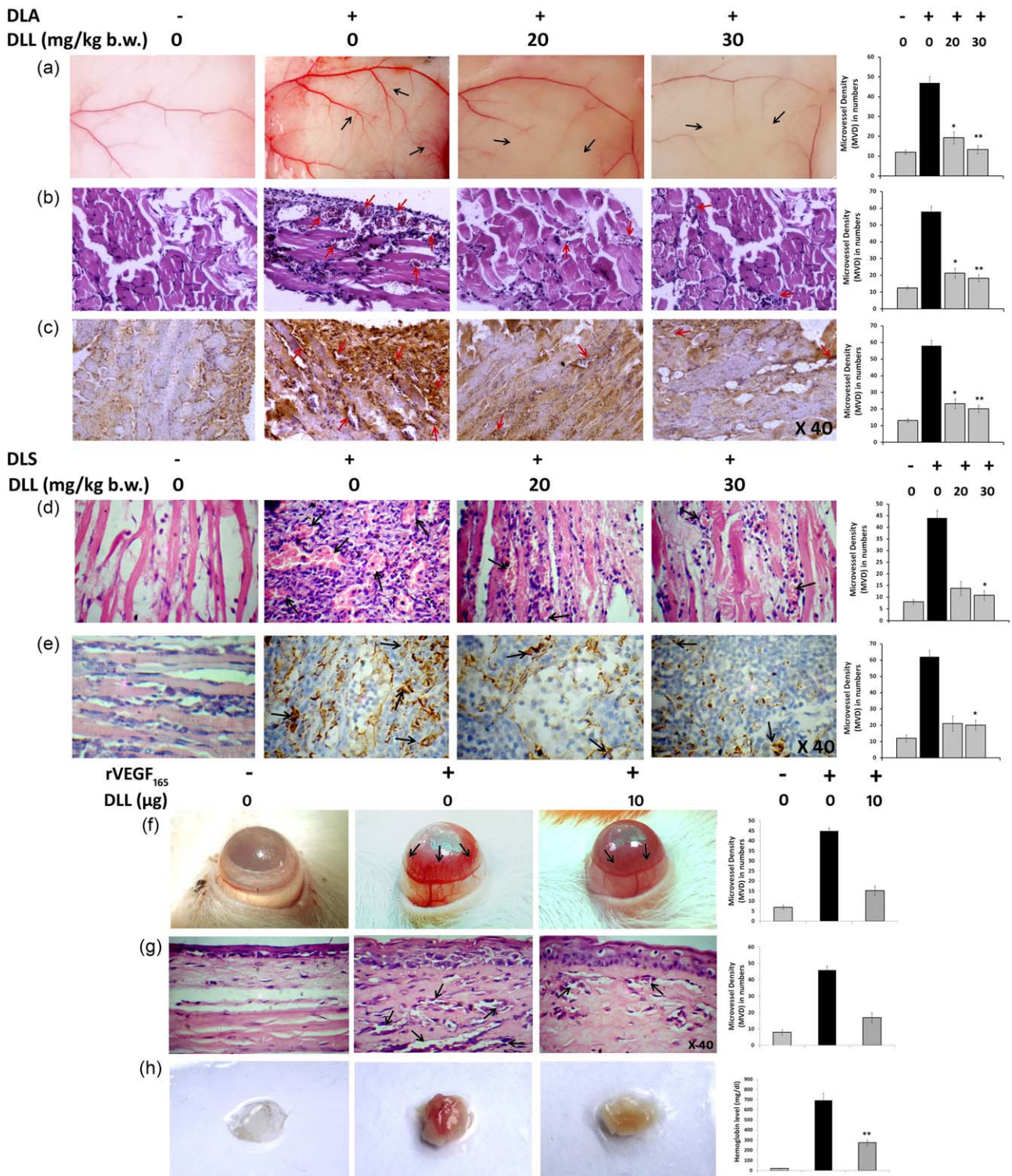
amino acid sequence of the lectin and its mitogenic attribute was reported long ago [14] but, despite its well-characterized and immunostimulatory properties there were only negligible reports promulgating its pharmacological implications. It is apparent that this immunomodulatory drug class has salience in oncotherapeutics, due to the notion that survival of malignant cells is dependent upon the microenvironment and evasion of the host's anti-tumour immune response [34]. In our study, screening against multiple cancer cells revealed that DLL

Fig. 5. *Dolichos lablab* lectin (DLL) abrogates tumoral and non-tumoral angiogenesis *in vivo*. The effect of DLL in the tumour-induced angiogenesis was evaluated by murine ascites and solid tumour models *in vivo*, treated with DLL 20 and 30 mg/kg body weight intraperitoneally (i.p.). (a) The peritoneal lining of mice showing the visible suppression of peritoneal microvessel in DLL-treated mice. (b) Haematoxylin and eosin (H&E)-stained peritoneum sections showing the decreased vascular count (c). CD31 immunohistochemistry (IHC) of the peritoneal sections depicting hypervascularization (intensive brownish staining) in the control section in sharp contrast to the DLL. The reticence of tumour angiogenesis was confirmed by (d) H&E and (e) IHC (CD31) photomicrographs of solid tumour sections depicting a significant reduction in vessel density. The effect of DLL on corneal angiogenesis and matrigel plug model systems was studied by inducing angiogenesis with recombinant vascular endothelial factor $(rVEGF)_{165}$ and treatment with DLL. (f) DLL regresses rat corneal angiogenesis. (g) Micrographs of H&E-stained rat corneal sections confirming the reduced microvascular density/high-power field (MVD/HPF) upon DLL treatment. The black arrows indicate the extent of vascularization. The representative MVD counts were provided adjacent to every corresponding image. (h) Matrigel plug assay depicting control with aberrant vascularization in contrast to the DLL-treated mice. Pictograph depicting the level of haemoglobin content in matrigel implants is provided adjacently. Results are the means of three independent experiments. Statistically significant values are $*P < 0.05$; $**P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

failed to produce a significant inhibitory activity. Interestingly, DLL-CM, which expresses significant IL-2 levels, exhibited an anti-proliferative effect (Table 1). The immunomodulatory implication of DLL prompted us to expand our investigation into Dalton's lymphoma, a reliable *in-vivo* tumour model which induces potential lethal effects in mice. Results demonstrated that DLL effectively regressed pivotal tumour parameters of DLA/DLS *in vivo* with an extended life span (Supporting information, Figs S2 and S4).

There is a well-recognized relationship between anti-tumour activity and immunomodulation [35]. T cells are a central component of an adaptive immune system that is indispensable for immune system maintenance and immune surveillance to eliminate transformed cells [36]. However, chronic malignancy results in T cell exhaustion, with a major decline in IL-2 synthesis that leads to debilitating consequences regarding anti-tumour immunity [36–38]. As DLL is a potent T cell mitogen [17], tumour inhibitory activity of the lectin (Supporting information, Figs S2 and S4) in our study suggests that the protein abrogates immunosuppression and T cell exhaustion in tumour mice. Our experimental evidence has indicated that DLL up-regulated IL-2 levels significantly in tumour mice compared to that of severely decreased levels in untreated mice (Fig. 4i). This corresponds clearly with the regressed tumour growth in ascitic (Supporting information, Fig. S2) and solid tumour (Fig. 4), thereby indicating that DLL has probably reversed the T cell exhaustion, consequentially breaking the IL-2 shutdown and eliciting an anti-tumour response.

Transformed cells do not become tumorigenic unless they acquire angiogenic potential [39]. Tumour neovasculaturization, one of the integral operations in malignant expansion, is marked by precocious capillary sprouting with convoluted, distorted and excessive vascular branching [5,6,40]. It is well known that Dalton's lymphoma induces massive peritoneal microvessel and vascular pathologies leading to invasiveness. Our observations showed that DL ascitic tumours and DL solid tumours showed enormous blood vessels which appeared to be highly disorganized and chaotic (Fig. 5a–e). Convincing observations

from our study have clarified that the DLL regimen potently regressed the tumour-induced neovasculature, which correlates positively with a reduced tumorigenic index (Fig. 4e). Furthermore, earlier findings have provided evidence that among diverse roles, IL-2 displays anti-angiogenic and tumour-regressive properties [41]. Collectively, our results demonstrate that elevated IL-2 cytokine has been attributed to the DLL-mediated anti-angiogenesis (Fig. 5) and tumour regression *in vivo* (Supporting information, Figs S2 and S4). Relative evidence could be observed from the angiopreventive and anti-proliferative effect of DLL-CM, with high IL-2 levels in *in-vitro* angiogenesis (Fig. 3a–f) and cytotoxicity assay systems (Table 1), respectively. These observations explain that DLL has abrogated the tumour progression possibly through altering the immunological and angiogenic parameters.

Compelling reports provide a potential link between NF-κB activation and oncogenesis, which functions as a transcriptional regulator in a variety of malignancies as evidenced through the identification of cancer-specific, cancer-relevant gene targets controlled by NF-κB [1,42]. In line with this, our experiments have demonstrated that both DL ascitic and solid tumours have shown elevated NF-κB levels with aberrant cellular proliferation and invasiveness (Fig. 6a,b), but administration with DLL was able to reduce NF-κB expression substantially in tumour mice, which explains the potential role of lectin in altering the pro-oncogenic transcription factor, thus showing visible impairment in tumour progression (Fig. 4a,b). One of the prominent features of the tumour microenvironment is the low oxygen tension or hypoxia which promptly induces HIF-1-mediated vascular switch, a prerequisite event for most malignancies and solid tumours to develop from a quiescent tumour into a more aggressive form [42–45]. Our studies suggest that DLL treatment lowered the expression of the oxygen sensor HIF-1, a key regulatory factor involved in oncogenic conversion, and promoting neoangiogenesis in mice (Fig. 6a,b). Reports show that constitutive HIF-1α and NF-κB activation in cancer cells has been shown to trigger autocrine of angiogenic chemokines, mainly VEGF, a pivotal factor for mitogenesis and capillary
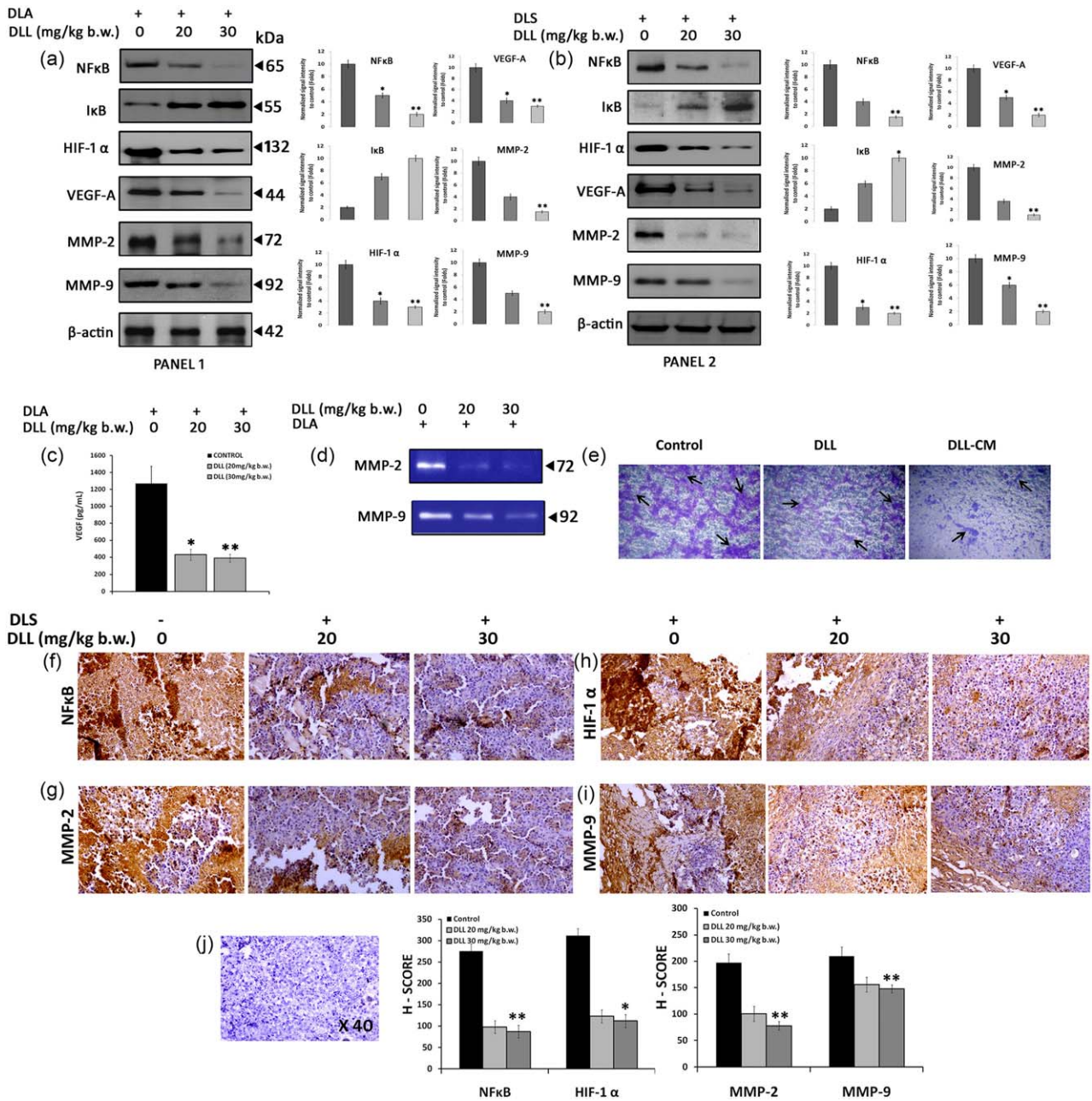
Fig. 6. Translational down-regulation of tumoral angiogenic gene expression by *Dolichos lablab* lectin (DLL). Immunoblots, gelatin zymography and immunohistochemistry (IHC) were carried out using *in-vivo* tumour cells of both Dalton's ascites lymphoma (DLA) and Dalton's lymphoma solid tumour (DLS) with or without DLL treatment in two different concentrations. (a,b) Immunoblots showing altered translational expression of proangiogenic genes such as nuclear factor kappa B (NF-κB), inhibitory kappa B (I-κB), hypoxia inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF)-A, matrix metalloproteinase (MMP)-2 and 9 in ascitic (panel 1) and solid tumour (panel 2). The graphs indicate the densitometric values of corresponding Western blot data. (c) Reduction of secreted serum VEGF levels in DLL-treated DLA mice *in vivo*. (d) Gelatin zymography showing reduced gelatinolytic activity in DLL-treated mice. (e) DLL-conditioned medium (DLL-CM) inhibits the migration of A549 cell *in vitro*. IHV should be replaced with IHC detection of DLL-induced altered gene expression. (f,i) IHC detection of proangiogenic gene expression in solid tumours *in vivo* after DLL treatment. The intense brown staining indicates the respective protein expression. Representative graphs demonstrating the gene expression corresponding to the IHC data are given below. (j) Representative counter haematoxylin-stained section. Data are represented as mean ± standard error of the mean (s.e.m.) of three independent experiments. Statistically significant values are expressed as *$P < 0.05$ and **$P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].
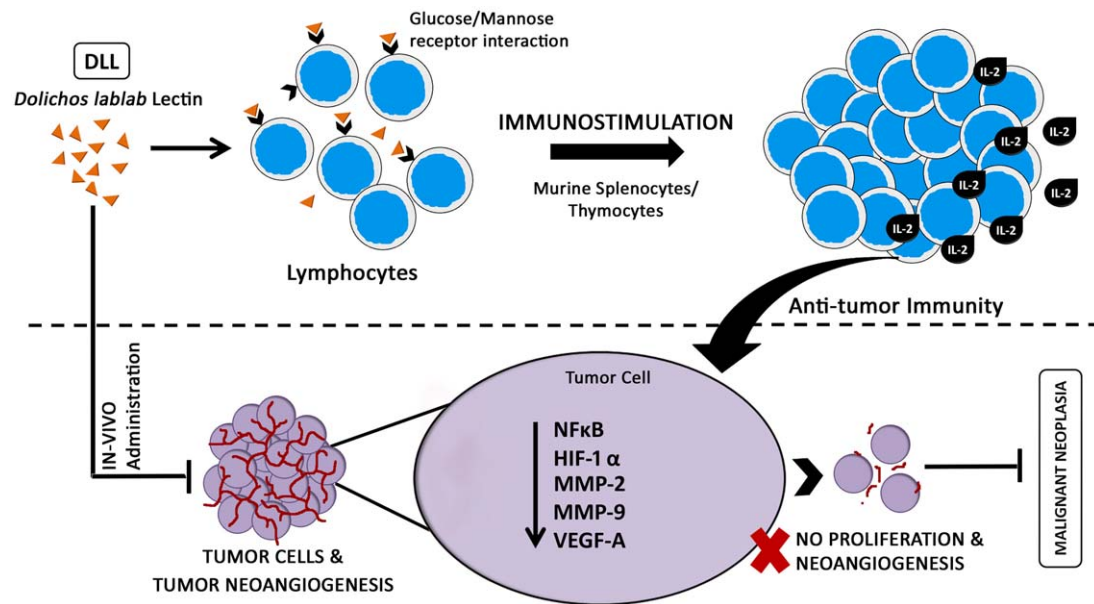
Fig. 7. Schematic demonstration of glc/man interacting *Dolichos lablab* lectin (DLL) induced anti-tumour effect. Hypothetical model diagram depicting the possible mechanism of action of immunostimulatory DLL in eliciting the potent anti-neoplastic and anti-angiogenic response in *in-vivo* murine Dalton's lymphoma tumour. [Colour figure can be viewed at wileyonlinelibrary.com].

permeability of endothelial cells [42–47]. Our experimental observations showed that DLL reduced VEGF expression, along with MMP-2 and MMP-9, thereby confirming the down-regulated proangiogenic signalling cascade *in vivo* (Fig. 6). In support of this, decreased MMP expression is reflected in the retarded invasiveness of cancer cells (Fig. 6e). Significantly, DLL retarded the tube formation of functional endothelial cells (HUVECs) and rVEGF$_{165}$-induced neovessel formation *in vitro* (Fig. 3). Moreover, DLL potentially reduced the peroxidized lipids in tumour mice (Fig. 4h), which have been highly implicated in tumour angiogenesis and release of HIF-1α into the tumour microenvironment [48,49]. Together, this evidence highlights DLL's potential in neovascularization inhibition.

Taken together, decisive observations demonstrate that dietary lectin DLL exerts potent anti-proliferative activity towards malignant cells. Immunopotentiating DLL impedes tumour progression specifically by antagonizing the neoangiogenesis formation in murine Dalton's lymphoma ascitic and solid tumours. This study highlights the critical role of dietary protein as an immunostimulatory and anti-angiogenic molecule targeting cancer cell progression (Fig. 7). More in-depth investigations expediting molecular insights into tumour immunomodulation of this leguminous lectin could prove vital in making breakthrough improvements in cancer therapeutics.

Acknowledgements

Disclosure

The authors declare that there are no conflicts of interest.

Author contributions

V. V. performed experiments and wrote the manuscript. P. T. and V. B. R. provided experimental assistance and V. K. provided research advice. B. T. P. and S. N. P assisted in the experimental design and data analysis.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Purification of *Dolichos lablab* lectin (DLL) by ovalbumin-Sepharose 4B affinity chromatography. (a) Chromatograph of DLL purification on an ovalbumin-Sepharose 4B column. DLL specifically eluted by α-methyl mannoside, protein elutions monitored at 280 nm. The arrows indicate the point of elution. (b) Haemagglutination assay employing the serially diluted purified DLL starting with 400 μg concentration in concavity assay plate. The titre value of DLL is ∼3 μg, which represents the minimal concentration of the extracts required to produce visible agglutination at the highest dilution. (c) Periodic acid-Schiff (PAS) staining of the purified DLL resolved on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to detect the glycoprotein nature of the proteins. Lane profile:

M = marker, 1 = purified DLL, 2 = ovalbumin, 3 = bovine serum albumin. (d) Immunoblot analysis of rabbit anti-DLL polyclonal antibodies. DLL resolved on 12% SDS-PAGE was transferred to polyvinylidene difluoride (PVDF) membrane and probed with anti-serum (1 : 500). Load concentration for all the gels: 20 μg/well. (e) Double immunodiffusion to analyse the immunoprecipitation reaction of anti-DLL antibodies towards crude DLL extract and purified DLL. Antigen/anti-sera load volume: 10 μl/well. 1 = anti-DLL antibody, 2 = purified DLL, 3 = crude DLL. A clear 'v'-shaped precipitin line indicating the pattern of immunologically identical proteins from the crude and purified DLL.

Fig. S2. *Dolichos lablab* lectin (DLL) exerts anti-tumour potential on murine Dalton's lymphoma ascites tumour *in vivo*. Anti-neoplasmic property of DLL was investigated in Dalton's lymphoma ascites (DLA) *in-vivo* tumour model. Ascites tumour was induced in Swiss albino mice by injecting DLA cells (5×10^6 cells/mouse) intraperitoneally (i.p.). Treatment regimen included three doses of DLL on alternative days, administered in the concentration range of 20 and 30 mg/kg body weight (i.p.) followed by the evaluation of tumour parameters. (a) Regressed tumorigenic index in a dose- and concentration-dependent manner indicative of the percentage reduction in the tumour growth, (b) contracted-tumour cell population (c) and decrease in ascites secretion. All the parameters were evaluated in comparison to the respective control tumoral-bearing animal. (d) Kaplan–Meier graph showing the prolonged lifespan of DLL-treated animals. Results are the means of three determinations, each conducted in triplicate. Statistically significant values are $^*P < 0.05$; $^{**}P < 0.01$.

Fig. S3. *Dolichos lablab* lectin (DLL) exhibits minimal adverse secondary complication on internal organs. To investigate the toxicological profiles of DLL in the internal organs, paraffin-embedded liver and spleen sections of the normal and experimental animals were stained with haematoxylin and eosin (H&E) followed by evaluation under light microscopy. Images depicting the representative histological sections of liver (a) and spleen (b) at ×40 optical magnification. (c) Weight of liver and spleen.