Original article

# P-selectin glycoprotein ligand 1 promotes T cell lymphoma development and dissemination

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## ARTICLE INFO

## ABSTRACT

P-selectin glycoprotein ligand-1 (PSGL-1) is a membrane-bound glycoprotein expressed in lymphoid and myeloid cells. It is a ligand of P-, E- and L-selectin and is involved in T cell trafficking and homing to lymphoid tissues, among other functions. PSGL-1 expression has been implicated in different lymphoid malignancies, so here we aimed to evaluate the involvement of PSGL-1 in T cell lymphomagenesis and dissemination. PSGL-1 was highly expressed at the surface of human and mouse T cell leukemia and lymphoma cell lines. To assess its impact on T cell malignancies, we stably expressed human PSGL-1 (hPSGL-1) in a mouse thymic lymphoma cell line, which expresses low levels of endogenous PSGL-1 at the cell surface. hPSGL-1-expressing lymphoma cells developed subcutaneous tumors in athymic nude mice recipients faster than control empty vector or parental cells. Moreover, the kidneys, lungs and liver of tumor-bearing mice were infiltrated by hPSGL-1-expressing malignant T cells. To evaluate the role of PSGL-1 in lymphoma cell dissemination, we injected intravenously control and hPSGL-1-expressing lymphoma cells in athymic mice. Strikingly, PSGL-1 expression facilitated disease infiltration of the kidneys, as determined by histological analysis and anti-CD3 immunohistochemistry. Together, these results indicate that PSGL-1 expression promotes T cell lymphoma development and dissemination to different organs.

## 1. Introduction

P-selectin glycoprotein ligand-1 (PSGL-1) was identified on myeloid and lymphoid cells as a major calcium-dependent ligand for P-selectin [1,2,3]. PSGL-1 requires tyrosine sulfation and threonine O-glycosylation carrying sialyl Lewis X (sLeX) glycan structures to bind not only P-selectin but also E- and L-selectins [2,3,5,7,9]. These selectin-binding properties allow leukocytes to interact with each other and other cell types, and are crucial for leukocyte adhesion to and rolling on blood vessel walls and migration to specific tissues [8]. Through these mechanisms, PSGL-1 functions in lymphocyte homing to hematopoietic organs, such as thymus, bone marrow and secondary lymphoid organs [6,10]. Apart from selectins, it has been shown that PSGL-1 binds to CCL19 and CCL21 chemokines, which mediate immune cell trafficking to secondary lymphoid organs through the lymphatic system [11,12]. Recently, PSGL-1 was found to be an immune checkpoint protein neg-atively regulating T cell responses, promoting T cell exhaustion and inducing immune tolerance [13–15]. Furthermore, the V-domain im-munoglobulin suppressor of T cell activation (VISTA) was reported as a pH-selective ligand for PSGL-1 expressed in T cells, thereby suppressing T cell activation [16].

PSGL-1 has been implicated in lymphoid malignancies. In multiple myeloma, PSGL-1 was shown to mediate growth, dissemination and drug resistance of malignant cells in the bone marrow microenviron-ment context [17]. In a mouse model of BCR-ABL1 leukemia, selectins and their ligands were required for the homing and engraftment of leukemic stem cells to the bone marrow niche [18], while in murine lymphoma, PSGL-1 expression was associated with metastatic behavior of tumor cells [19]. Together, these studies indicate that PSGL-1 ex-pression in malignant cells promotes tumor development and disease progression.

Considering the reported functions of PSGL-1 in T cells and that PSGL-1 was found to be expressed in human malignant T cell lines [2,4], we set out to investigate the impact of PSGL-1 expression on T cell lymphoma development and progression. By overexpressing PSGL-1 in a mouse thymic lymphoma cell line, we found that PSGL-1 enhanced subcutaneous tumorigenesis and bloodborne infiltration of kidneys and lungs. These data support the notion that this pro-lymphomagenic glycoprotein could be a potential therapeutic target.

## 2. Materials and methods

### 2.1. Mice

Athymic Swiss nude (Crl:NU(Ico)-Foxn1$^{nu}$) outbred mice were purchased from Charles River Laboratories and bred in the CBMR/UAlg barrier animal facility in individually ventilated polysulfone H-Temp cages with positive pressure in a Sealsafe system (Techniplast, Italy), under 12 h light/dark cycles, controlled room temperature (22°C), and with autoclaved food (4RF25 diet; Mucedola, Settimo Milanese, Italy) and water *ad libitum*. The breeding and maintenance on a C57BL/6 background, monitoring and criteria for euthanasia of ETV6-JAK2 (also called TEL-JAK2) transgenic mice (B6.Cg-Tg(Emu-ETV6/JAK2)71Ghy) were performed as described [20]. Both female and male nude mice were used for subcutaneous (s.c.; interscapular region) or intravenous (i.v.; tail vein) injection experiments ($1 \times 10^6$ EL4.2 cells). S.c. tumor growth was monitored by regular caliper measurements of length (L) and width (W) and calculating volume (V) using the well-established formula [21]: $V (mm^3) = (LxW^2)/2$, being *L* the larger and *W* the smaller of two perpendicular tumor axes. Mice were euthanized by $CO_2$ inhalation when reaching predefined experimental or humane endpoints (s.c. tumors > 2000-3000 $mm^3$, signs of internal tumor growth, or low body condition). All experimental procedures followed recommendations for the care and use of laboratory animals from the European Commission (Directive 2010/63/UE) and the local Portuguese authorities (*Decreto-Lei* n°113/2013).

### 2.2. Primary human and mouse cells

Human cell experiments were conducted according to the principles expressed in the Declaration of Helsinki. T-cell acute lymphoblastic leukemia (T-ALL) peripheral blood leukocytes from a 25-year-old male patient (Centro Hospitalar Universitário São João [CHUSJ], Porto) were obtained with informed consent and ethical approval by the CHUSJ Ethics Committee (approval number 73-20), and isolated by density-gradient separation in a Vacutainer CPT tube (BD Biosciences). Approximately 85% of leukocytes were surface CD3$^+$CD5$^+$CD7$^+$ blasts, as determined by flow cytometry. Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy donors, provided by *Serviço de Imunohemoterapia*, CHUSJ (Ethics Committee approval number 398-20), by density-gradient separation using Lympholyte-H (Cedarlane). After centrifugation, PBMCs were isolated. A red blood cell lysis buffer was used to clear the pellet of remaining erythrocytes. Peripheral blood lymphocytes (PBLs) were isolated by exclusion of plastic-adherent monocytes. Primary ETV6-JAK2 transgenic leukemic cells and thymocytes and splenocytes from healthy C57BL/6 mice were obtained as single cell suspensions from gently pressed thymus or spleen against 70 $\mu$m nylon cell strainers (BD Biosciences) in cold phosphate-buffered saline (PBS).

### 2.3. Cell lines and culture

Human T-ALL CEM, PF-382, DND41, P12, HPB-ALL and, SupT1 cell lines were provided by Hind Medyouf from Andrew Weng lab (BC Cancer Agency, Vancouver, Canada). The Jurkat T-ALL and Nalm6 B-cell acute lymphoblastic leukemia (B-ALL) cell lines were provided by João

T. Barata (IMM, Lisbon, Portugal) and the HUT-78 cutaneous T cell lymphoma was provided by Neil D. Perkins (Newcastle University, UK). The A20 B lymphoma cell line was acquired from American Tissue Culture Collection (ATCC; TIB-208). The EL4.2 cell line derives from the EL4 mouse thymic lymphoma cell line and was provided by Jose M. Almendral (CBM, Madrid, Spain), who obtained it in the 1980s from Bernhard Hirt (ISREC, Épalinges, Switzerland). Although short tandem repeat profiling of EL4.2 cells performed at ATCC, revealed significant allele divergence with the EL4 cell line (ATCC TIB-39) (Fig. S1A), EL4.2 cells expressed CD90.2 and CD44 (Fig. S1B), as reported for EL4 [22]. Also, EL4.2 cells were negative for surface CD3$\epsilon$ (sCD3), CD4 and CD8, and positive for CD24, CD25, and CD69 (Fig. S1B and data not shown), and carried X but not Y chromosomes (Fig. S1C), as reported for EL4 cells [23]. All cell lines were cultured in suspension in Falcon tissue culture flasks (cat. nos. 353136 and 353109; Corning) in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin-streptomycin (Gibco) and 1 mM L-Glutamine (Gibco), maintaining a density between $1 \times 10^5$ and $1 \times 10^6$ cells/ml, in a 5% $CO_2$ humidified incubator at 37°C. Absence of *Mycoplasma* was verified by Venor GeM Mycoplasma Detection Kit (Minerva Biolabs). For growth assays, cells were plated at $1 \times 10^5$ and counted (Neubauer hemocytometer) after 48 h. For Transwell migration assay, $2.5 \times 10^5$ cells suspended in 100 µl of RPMI medium without FBS were seeded into a 6.5 mm insert with a 5.0 µm pore size (Cat. no. 3421, Corning). The insert was placed in a well containing 600 µl of RPMI supplemented as above. After 24 h at 37°C, the lower chamber volume was collected and cell numbers counted using TC20 Automated Cell Counter (Bio-Rad). This experiment was performed twice with experimental triplicates.

### 2.4. Generation of stably transfected cell lines

The human *SELPLG* cDNA, variant 2 (NM_003006.4; encoding PSGL-1 isoform 2, of 402 a.a.) cloned in a pGEMT plasmid [24] was kindly provided by Roger McEver (Oklahoma Medical Research Foundation, Oklahoma City). The *SELPLG* cDNA was then cloned within the BgIII and XhoI restriction sites of the MigR1 (MSCV-IRES-eGFP) vector, kindly provided by Warren Pear (University of Pennsylvania, Philadelphia). The EL4.2 cell line was electroporated using Gene Pulser Xcell (Bio-Rad) with empty MigR1 or MigR1-PSGL-1 plasmids. Transfected cells expressing enhanced green fluorescent protein (GFP) were sorted in a FACS Aria cell sorter (BD Biosciences), cultured for 1 week, re-sorted, and the procedure repeated once more. EL4.2/EV and EL4.2/hPSGL-1 cell lines maintained GFP expression over more than 10 passages. To achieve stable clones, bulk cultures were submitted to serial dilution on 96-well plates and outgrowing cell clones collected for further expansion and analysis.

### 2.5. Flow cytometry

Mouse primary cells, EL4.2 cell lines and single-cell suspensions from dissociated s.c. tumors were washed with FACS buffer (PBS with 3% FBS and 10 mM NaN$_3$), centrifuged at 300 g, and resuspended in FACS buffer containing the following antibodies: R-phycoerythrin (PE)-conjugated human PSGL-1 (CD162; clone KPL-1, cat. no. 328805, Biolegend), mouse PSGL-1-AlexaFluor 647 (2PH1, cat. no. 562806, BD Pharmingen), and following Biolegend antibodies: CD3$\epsilon$-FITC (145-2C11, cat. no.100305) CD4-PE (GK1.5, cat. no. 100407), CD8a-PE/Cyanine5 (53-6.7, cat. no. 100709), CD24-APC (M1/69, cat. no. 101813), CD25-FITC (PC61, cat. no. 102005), CD44-APC (IM7, cat. no. 103011), CD69-FITC (H1.2F3, cat. no. 104505) and CD90.2-FITC (30-H12, cat. no. 105305). Human primary cells and cell lines were similarly prepared and stained with human PSGL-1-PE (KPL-1), and Biolegend antibodies CD3-PerCP (OKT3, cat. no. 317337), CD5-APC (L17F12, cat. no. 364015), CD7-PE (CD7-6B7, cat. no. 343105) and CD19-PE/Cyanine7 (HIB19, cat. no. 302215) antibodies. After incubation on ice for 30-45

min, cells were washed in FACS buffer. For intracellular staining, cells were fixed in 0.5% paraformaldehyde in PBS for 20 min at room temperature. Next, cells were washed twice, stained with antibodies on ice, and washed again, all with ice-cold FACS cell permeabilization buffer (PBS with 0.5% bovine serum albumin and 0.1% saponin). Detection of recombinant mouse P-selectin binding was performed as previously reported [25]. Briefly, 5 mM HEPES, 2 mM $CaCl_2$, 5% FBS in Dulbecco's PBS buffer was used for cell washes and reagent incubation. Cells were incubated on ice with 1 μg of recombinant mouse P-selectin (CD62P)-Fc chimeric (carrier-free) (cat. no. 755402, Biolegend). After two washes, cells were incubated with biotin-conjugated anti-human IgG Fc (1:200 dilution; HP6017, cat. no. 409307, Biolegend). Finally, cells were washed and incubated with PE/Cyanine5-conjugated streptavidin (1:500 dilution; cat. no. 405205, Biolegend). Samples were analyzed using BD Accuri C6 or BD FACSCanto II. Data was analyzed using FlowJo software.

## 2.6. Immunoblotting

Whole cell lysates were prepared from $1 \times 10^7$ cells after two rounds of ice-cold PBS washing and resuspension in ice-cold RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride) for 10 min. After 13,000 g centrifugation, the supernatants were denatured using SDS sample buffer (62.5 mM Tris pH 6.8, 20% glycerol, 2% SDS and 5% β-mercaptoethanol) and heating the samples for 5 min at 95°C. Samples were subjected to 8% SDS-polyacrylamide gel electrophoresis, together with PageRuler Plus prestained protein ladder (Thermo Fisher Scientific) and transferred to an immunoblot nitrocellulose membrane (GE Healthcare). Total protein loading was assessed by Ponceau staining (P7170, Sigma-Aldrich). Membranes were blocked with 5% nonfat milk in PBS/0.1% Tween 20 and then stained with primary antibodies: human PSGL-1 (1:1000 dilution; KPL1, cat. no. 328802, Biolegend), α-tubulin (1:5000 dilution; cat. no. T6199, Sigma-Aldrich). Diluted 1:5000 horseradish peroxidase-conjugated goat anti-mouse IgG (cat no. A00160; GenScript) was used as secondary antibody. The signal was acquired by incubating the membrane with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and captured by Amersham Hyperfilm (GE Healthcare).

## 2.7. Histological and immunohistochemical analyses

Mice organs were fixed in 10% formalin and embedded in paraffin using standard procedures. Four μm sections were then stained with hematoxylin and eosin using standard procedures. For immunohistochemistry, sections were deparaffinized. Antigen retrieval was performed with 10 $\mu M$ EDTA pH 8.0, 0.05% Tween 20 buffer (for CD3ε detection) or a 1:100 dilution of Antigen Unmasking Solution, Citrate-based (cat. no. H-3300, Vector Laboratories; for PSGL-1 detection) for 40 min under boiling temperature. Endogenous peroxidase was inactivated with 3% $H_2O_2$ in methanol at a 1:10 dilution. Nonspecific antibody binding was blocked using Ultravision Protein-block (Thermo Fisher Scientific) or AffiniPure Fab Fragment goat anti-mouse IgG (cat. no. 115-007-003, Jackson ImmunoResearch). Next, sections were incubated with rabbit anti-CD3ε (1:200; cat. no. ab5690, Abcam) or mouse anti-human PSGL-1 (1:200; KPL-1). Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (cat. no. K5007, Agilent) was used as secondary detection reagent. Images were obtained in Olympus DP 25 light microscope and Cell B imaging software. To evaluate organ infiltration, a blind analysis was performed. Five images were taken from different tissue section areas and the percentage of each area infiltrated with $CD3^+$ cells was calculated.

## 2.8. Statistics

Statistical analyses were performed with GraphPad Prism software. Two-tailed unpaired Student's t-test was used to compare the mean of two groups. For multiple group comparisons, two-way ANOVA test followed by Tukey's or Sidak's multiple comparisons test was performed. Log-rank (Mantel-Cox) tests were used to compare tumor onset curves. Statistical tests and sample numbers are indicated in figure legends. A $P$ value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. PSGL-1 expression in human and murine malignant T cells

By flow cytometry analysis, we found that PSGL-1 was expressed at the surface of all T-ALL cell lines analyzed, as well as the HUT-78 cutaneous T-cell lymphoma cell line, but not the Nalm6 B-ALL cell line (Fig. 1A). Immunoblotting of T-ALL cell lines corroborated the variable levels of PSGL-1 detected by cytometry and revealed both homodimeric (~240 kDa) and monomeric (~120 kDa) forms (Fig. 1B). Detection of homodimers by KPL1 monoclonal antibody is presumably due to incomplete disulfide bond reduction [5,26]. In addition, PSGL-1 was expressed at the surface of both patient primary T-ALL and healthy donor $CD3^+$ T cells (Fig. 1C). Because a single T-ALL patient sample was analyzed, no general conclusions can be drawn about PSGL-1 expression levels in this disease. We also assessed PSGL-1 surface expression in murine normal and malignant T cells. All thymocyte subsets expressed surface PSGL-1, with the highest levels in CD8 single-positive (SP) thymocytes (Fig. 2A). PSGL-1 expression in mature T cells from the spleen remained similar to SP thymocytes, and again higher in CD8 than CD4 T cells (Fig. 2A). Next, we assessed PSGL-1 expression in a mouse model of T cell leukemia/lymphoma, driven by transgenic expression of the ETV6-JAK2 fusion protein in lymphoid cells [27,28]. Leukemic cells, which typically display the sCD3+CD4±CD8+ immunophenotype, expressed surface PSGL-1 at similar or higher levels than those found in normal CD8 SP thymocytes (Fig. 2B). In contrast, PSGL-1 surface expression was lower in EL4.2 cells, a derivative of the EL4 murine thymic lymphoma cell line (see Materials and Methods), than in murine thymocytes, ETV6-JAK2 leukemic cells and the A20 mouse B lymphoma cell line (Fig. 2C). To assess total protein expression, we permeabilized fixed cells before cytometry detection. Thus, the total PSGL-1 protein levels in EL4.2 cells were high, as compared to A20 cells (Fig. 2C), indicating that, in the former, most PSGL-1 protein resides in intracellular compartments. In all, our data showed high PSGL-1 surface expression in most malignant T cells.

### 3.2. Generation and characterization of a mouse lymphoma T cell line with human PSGL-1 expression

To study the role of PSGL-1 in T cell malignancy, we set out to enforce PSGL-1 expression in the EL4.2 mouse cell line, because it expresses reduced surface PSGL-1. We cloned human *SELPLG* cDNA in a vector containing GFP for expression in EL4.2 cells. Despite differences in amino acid sequence, human and mouse PSGL-1 are functionally conserved, as supported by reports showing PSGL-1 cross-species interactions with selectins [29–31]. After transfection of GFP-expressing empty and PSGL-1-expressing plasmids and serial sorting and culturing of GFP-positive cells (Fig. S2A), we obtained stably transduced EL4.2 cell lines expressing the human PSGL-1 protein (EL4.2/hPSGL-1) or carrying the empty vector (EL4.2/EV) (Fig. 3A). By flow cytometry, we observed that murine surface PSGL-1 remained low in transduced cell lines while the human isoform was highly expressed at the surface of EL4.2/hPSGL-1 cells (Fig. 3A). Moreover, the mono- and dimeric forms of human PSGL-1 were detected in the EL4.2/hPSGL-1 cells, similar to human leukemia/lymphoma cell lines (Fig. 3B). The surface expression of CD90.2 and CD44 remained high in all stably transduced EL4.2 cells
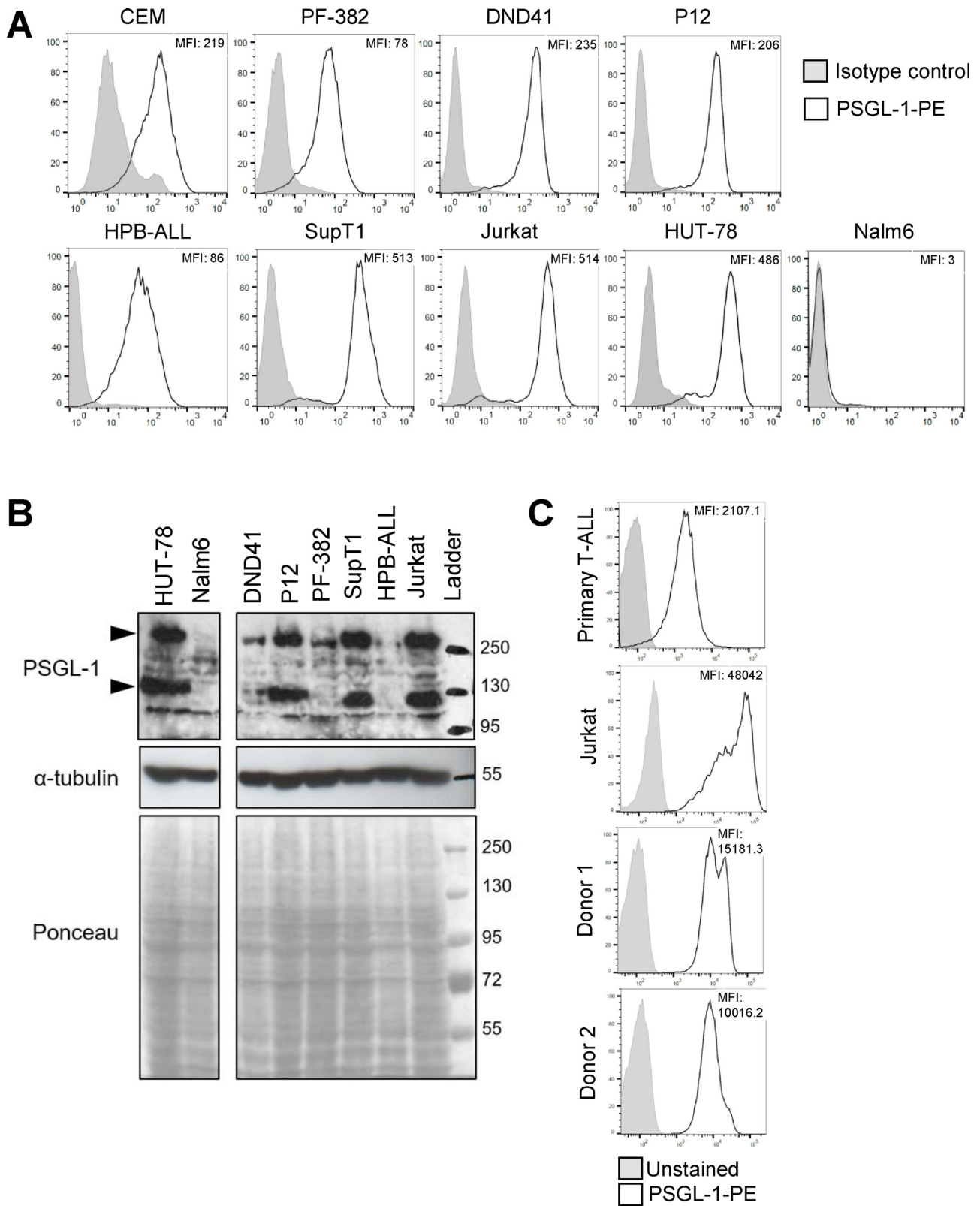
**Fig. 1.** PSGL-1 expression in malignant human T cells. A) PSGL-1 surface detection (using Accuri C6 cell analyzer) in T-ALL (CEM, PF-382, DND41, P12, HPB-ALL, SupT1, and Jurkat), cutaneous T cell lymphoma (HUT-78) and B-ALL (Nalm6) cell lines. B) Immunoblot detection of PSGL-1 protein (arrowheads mark homodimeric (~240 kDa) and monomeric (~120 kDa) forms) in the indicated cell lines. Total protein loading was assessed by Ponceau and $\alpha$-tubulin staining. C) PSGL-1 surface detection (using FACSCanto II cell analyzer) in primary T-ALL patient cells, Jurkat cell line, and normal T cells (CD3$^+$CD19$^-$ gating) from healthy donor PBLs. MFI, mean fluorescence index.
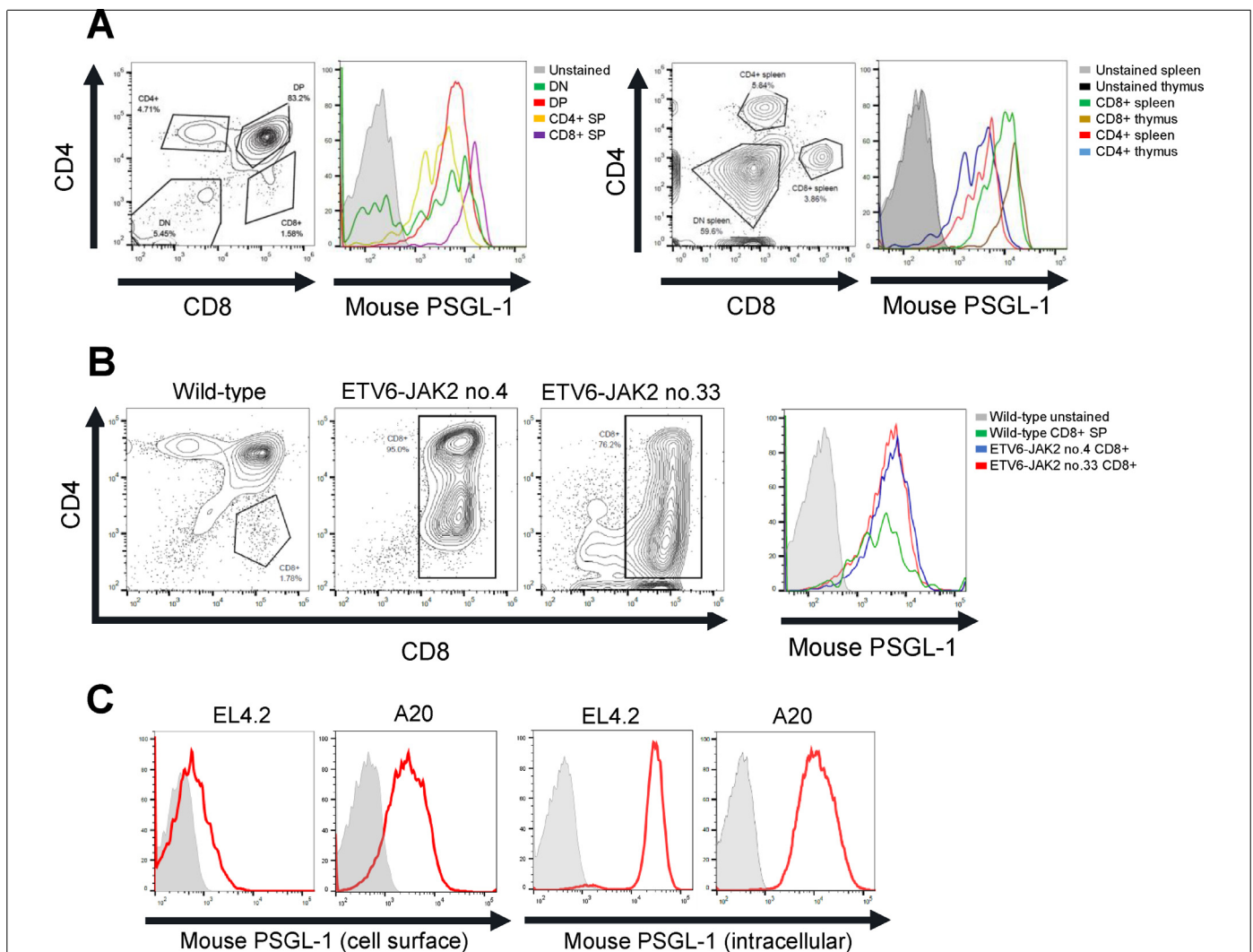
**Fig. 2.** PSGL-1 expression in normal and malignant murine T cells. A) PSGL-1 surface detection in thymus and spleen T cell subpopulations defined by CD4 and CD8 expression from a representative adult female C57BL/6 mouse. B) PSGL-1 surface detection in leukemic T cells from two representative E$\mu$-ETV6-JAK2 transgenic (male) mice (characterized by CD8 expression) and CD8 SP thymocytes from an adult male C57BL/6 mouse. C) PSGL-1 detection in viable (left panels) and permeabilized (right panels) EL4.2 and A20 cell lines.

(Fig. 3A), indicating that the plasmid transduction and selection did not alter differentiation marker expression. While EL4.2 cells tended to aggregate in cell culture, EL4.2/hPSGL-1 cells formed larger aggregates than the parental or EL4.2/EV cells (Fig. S2B). Moreover, EL4.2/hPSGL-1 cells proliferated about 1.5-fold faster than parental or EL4.2/EV cells (Fig. 3C), suggesting that PSGL-1 expression could increase cell division. To determine whether hPSGL-1 influenced cell motility, we performed Transwell migration assays and found no consistent changes in migration between PSGL-1-expressing and non-expressing EL4.2 cells (Fig. 3D). Furthermore, the ability of EL4.2 cells to bind to murine P-selectin under *in vitro* static conditions was not significantly increased by expression of exogenous PSGL-1 (Fig. 3E and F).

*3.3. PSGL-1 expression promotes T cell lymphoma tumorigenesis*

To study the impact of PSGL-1 expression on EL4.2 tumorigenesis, EL4.2 cell lines were injected s.c. in athymic nude mice. The use of immunodeficient mice avoided potential confounding effects from adaptive immunity responses towards exogenous human PSGL-1 or GFP proteins. Although EL4.2/EV cells could form tumors in most recipient mice, EL4.2/hPSGL-1 tumors appeared earlier and grew faster (Fig. 4A

and B). Since the transduced EL4.2/hPSGL-1 population included cells with different levels of surface PSGL-1, we derived cell clones. These clones displayed PSGL-1 surface expression and maintained expression of the CD90.2 and CD44 surface markers (Fig. 4C and data not shown). EL4.2/hPSGL-1 clones 3 and 5, with different levels of PSGL-1 expression, formed tumors as rapidly as the non-clonal transduced cells (Fig. 4D and E, and S3A and S3B). Despite showing different levels of surface PSGL-1 expression, clones 3 and 5 tumors grew at similar rates. Furthermore, EL4.2/hPSGL-1 cells growing in s.c. tumors maintained human PSGL-1 expression, as detected by flow cytometry and IHC (Fig. 5A and B), thus confirming its stability *in vivo*. Interestingly, kidneys from tumor-bearing mice were generally enlarged and displayed conspicuous white foci, often more frequently in EL4.2/hPSGL-1 recipients (Fig. 5C). Histological and IHC analyses revealed extensive lymphocytic kidney infiltrates marked by conspicuous CD3 labeling (Fig. 5D and S4). Histological and CD3 IHC analyses also revealed EL4.2 infiltration in livers and lungs (Fig. S5 and S6). Infiltrates were found in both EL4.2 and EL4.2/hPSGL-1 tumor-bearing mice, but more markedly in the latter (Table S1). Confirming that CD3 labels specifically EL4.2 cells, no CD3 staining was observed in kidney, liver and lung from non-injected nude controls (Fig. S4B, S5B, and S6B). These data indicate that increased
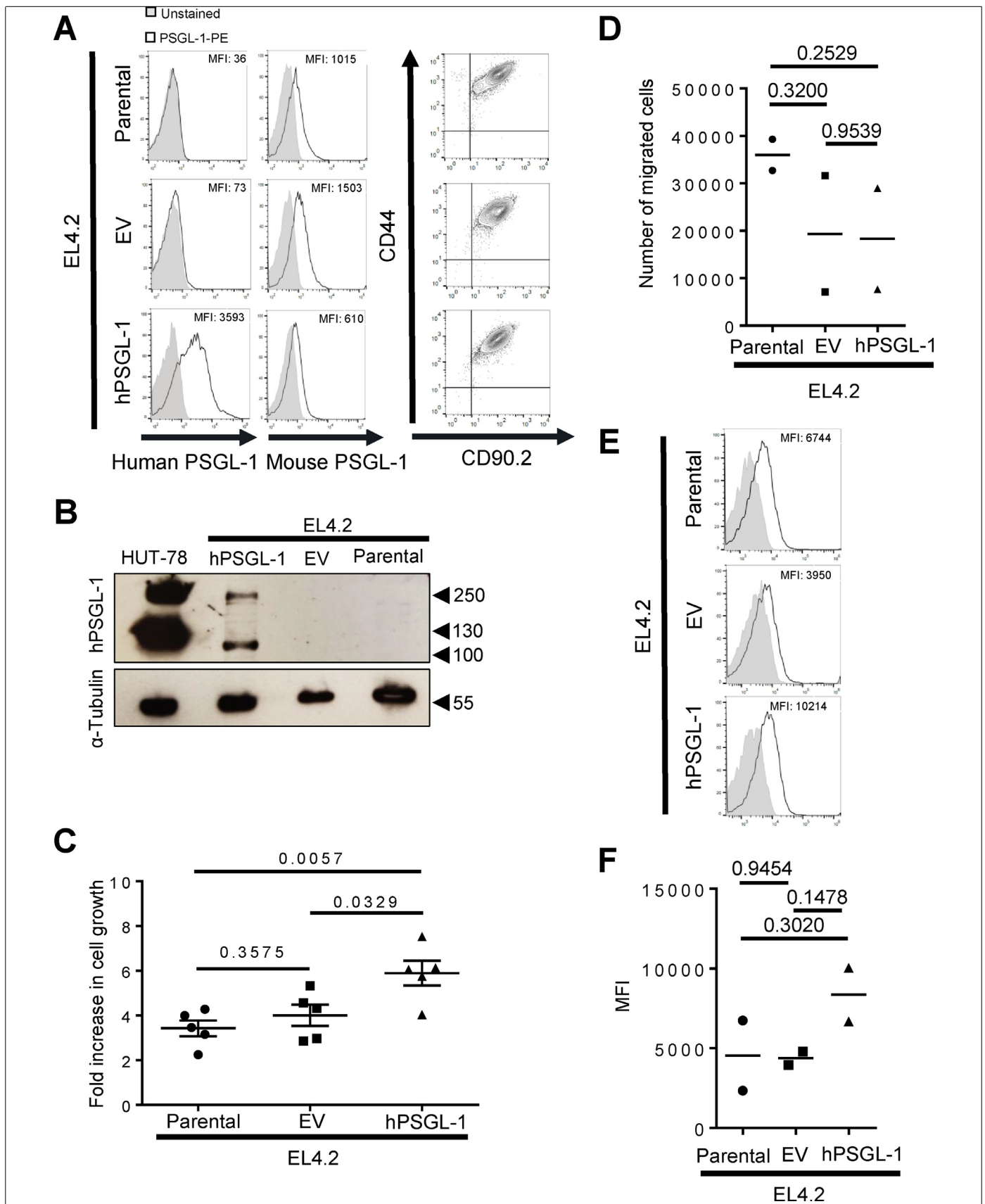
**Fig. 3.** Stable expression of human PSGL-1 in the murine EL4.2 lymphoma cell line. A) Surface detection of human and mouse PSGL-1 (left and middle panels) and CD44 and CD90.2 (right panels) in parental EL4.2 and cell lines carrying stably hPSGL-1 or empty vector (EV). MFI, mean fluorescence index. B) Immunoblot detection of human PSGL-1 in parental EL4.2, EL4.2/EV (clone 6), EL4.2/hPSGL-1 (clone 3) and HUT-78 cell lines. C) *In vitro* growth of cultured parental and stably transduced EL4.2 cells after 48 h. Pooled data of five independent experiments are plotted as mean ± standard error of the mean (SEM). Indicated *P* values were obtained from two-tailed unpaired *t*-tests. D) Migration Transwell assays were performed for 24 h. Data from two pooled experiments performed each with three technical replicates are plotted as mean. Indicated *P* values were obtained from two-tailed unpaired *t*-tests. E) Flow cytometry detection of recombinant mouse P-selectin bound *in vitro* to parental EL4.2, EL4.2/EV and EL4.2/hPSGL1 cells under static conditions. Negative control (grey histograms) corresponds to cell incubation with secondary reagents without recombinant selectin. F) Mean fluorescence index (MFI) levels of mouse P-selectin bound to each cell line. Data are expressed as means of two pooled independent experiments. Indicated *P* values were obtained from two-tailed unpaired *t*-tests.
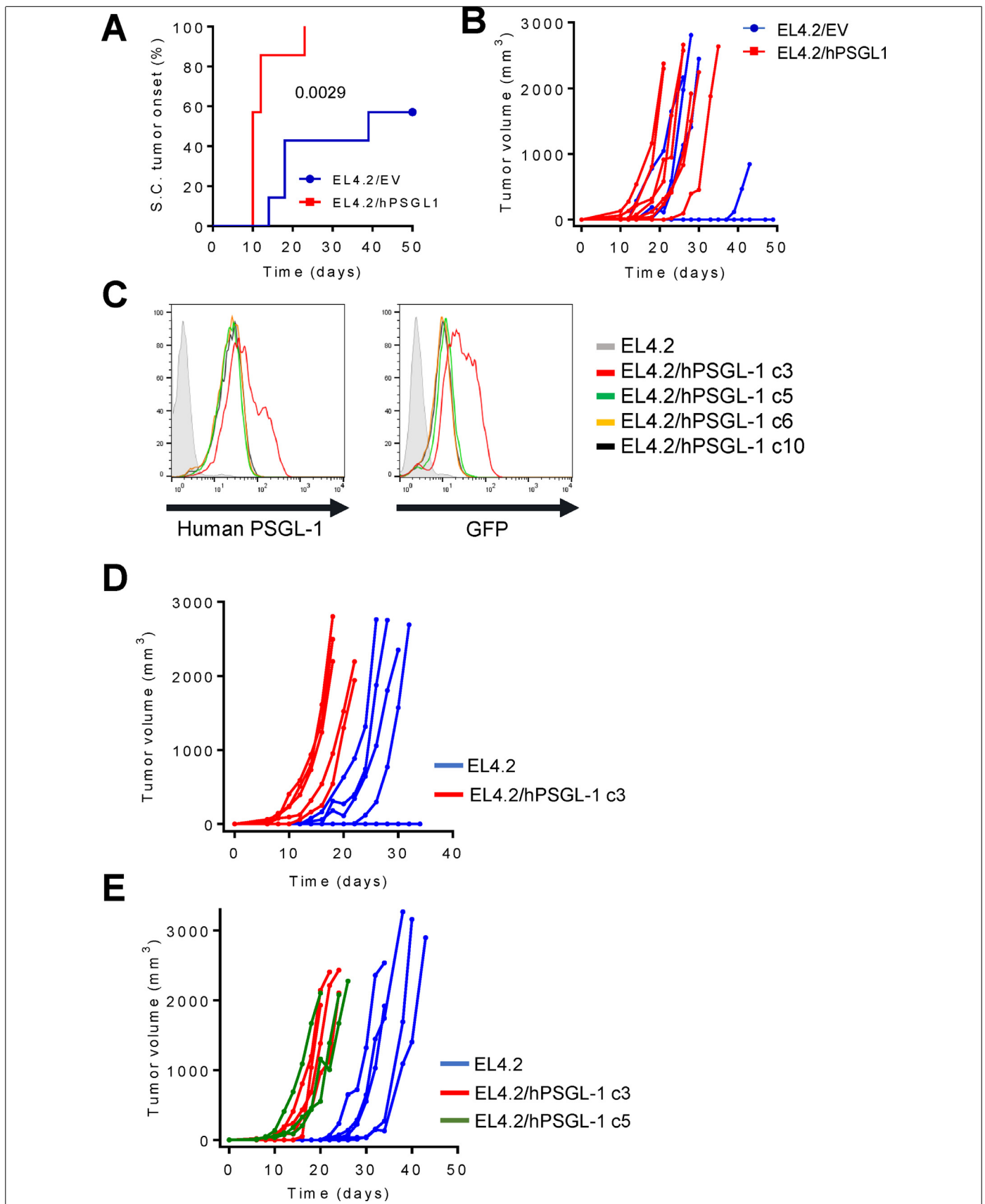
**Fig. 4.** Human PSGL-1 expression accelerates EL4.2 subcutaneous tumor growth. A) Representative independent experiment, of five performed, showing time of tumor detection in female nude mice injected with EL4.2/hPSGL-1 (n=7) and EL4.2/EV (n=7) cell lines. All EL4.2/hPSGL-1 mice developed tumors by day 23 whereas only 57% (4 of 7) of EL4.2/EV mice developed tumors by day 50. Indicated *P* value was obtained from log-rank (Mantel-Cox) test. B) S.c. tumor growth of individual mice depicted in A). Two-way ANOVA was performed (F = 3.447; *P* = 0.0881) and Sidak's multiple comparisons test showed significant differences at day 26 (*P* = 0.0011). C) Human PSGL-1 and GFP expression levels in representative clones isolated from the bulk EL4.2/hPSGL1 population. D) S.c. tumor growth in female nude mice inoculated with EL4.2/hPSGL-1 clone 3 (n=5) or parental EL4.2 (n=5) cell lines. Two-way ANOVA was performed (F = 12.90; *P* = 0.0071) and Sidak's multiple comparisons test showed significant differences at day 14 (*P* = 0̄.0002) and 16 (*P* < 0.0001). E) S.c. tumor growth in male nude mice inoculated with EL4.2/hPSGL-1 clone 3 (n=4), clone 5 (n=3) or parental EL4.2 (n=5) cell lines. Two-way ANOVA was performed (F = 8.427; *P* = 0.0087) and Tukey's multiple comparisons test showed significant differences at day 16 (EL4.2/hPSGL-1 c3 vs EL4.2, *P* = 0.0354 and EL4.2/hPSGL-1 c5 vs EL4.2, *P* = 0.0073), 18 and 20
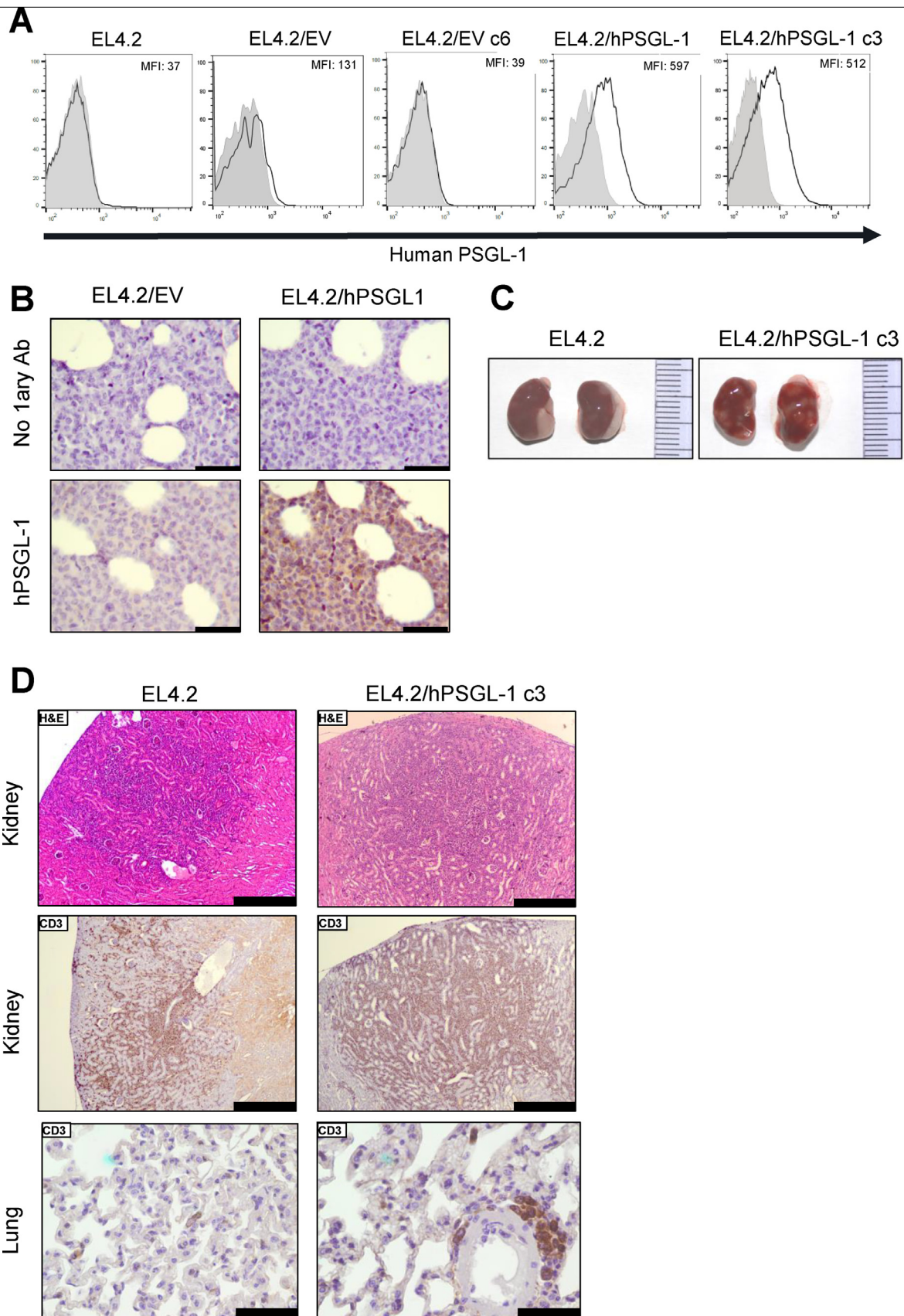(EL4.2/hPSGL-1 c3 vs EL4.2, *P* < 0.0001 and EL4.2/hPSGL-1 c5 vs EL4.2, *P* < 0.0001).

**Fig. 5.** Dissemination of EL4.2 T cells to the kidneys and lungs of subcutaneous tumor-bearing mice. A) Detection of surface human PSGL-1 in freshly collected cells derived from EL4.2, EL4.2/EV (bulk population and clone 6), and EL4.2/hPSGL-1 (bulk population and clone 3) subcutaneous tumors. MFI, mean fluorescence index. B) Human PSGL-1 IHC staining of EL4.2/hPSGL-1 and EL4.2/EV subcutaneous tumors. Staining with secondary reagent without primary antibody (no 1ary Ab) was performed as negative control. Scale bars, 50 μm. C) Kidneys from representative nude mice (no. 43, left, and no.49, right) from experiment presented in Fig. 4D. Ruler lines mark 1 mm. D) H&E staining and CD3 IHC staining of kidneys and lungs from nude mice shown in (C). Scale bars, 500 μm for kidneys (top and middle panels) and 50 μm for lungs (bottom panels).
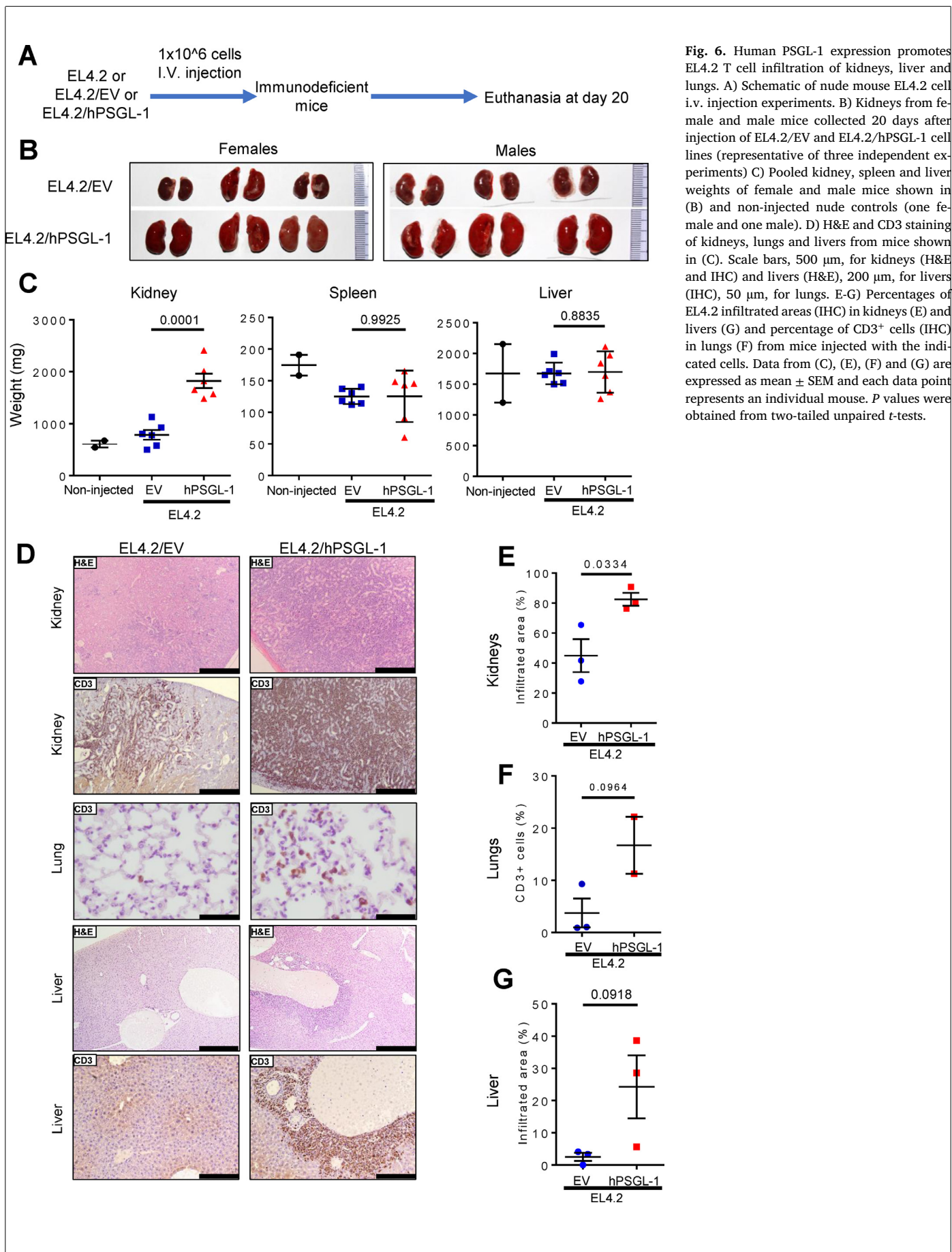
**Fig. 6.** Human PSGL-1 expression promotes EL4.2 T cell infiltration of kidneys, liver and lungs. A) Schematic of nude mouse EL4.2 cell i.v. injection experiments. B) Kidneys from female and male mice collected 20 days after injection of EL4.2/EV and EL4.2/hPSGL-1 cell lines (representative of three independent experiments) C) Pooled kidney, spleen and liver weights of female and male mice shown in (B) and non-injected nude controls (one female and one male). D) H&E and CD3 staining of kidneys, lungs and livers from mice shown in (C). Scale bars, 500 μm, for kidneys (H&E and IHC) and livers (H&E), 200 μm, for livers (IHC), 50 μm, for lungs. E-G) Percentages of EL4.2 infiltrated areas (IHC) in kidneys (E) and livers (G) and percentage of CD3$^+$ cells (IHC) in lungs (F) from mice injected with the indicated cells. Data from (C), (E), (F) and (G) are expressed as mean ± SEM and each data point represents an individual mouse. *P* values were obtained from two-tailed unpaired *t*-tests.

PSGL-1 expression promotes EL4.2 lymphoma growth, accompanied by dissemination to kidneys and other organs.

### 3.4. PSGL-1 enhances kidney infiltration by EL4.2 lymphoma cells

To elucidate a potential role for PSGL-1 in organ colonization by EL4.2 cells, cell lines were infused i.v. in nude mice and organ infiltration analyzed 20 days post-injection (Fig. 6A). Strikingly, kidneys from mice infused with EL4.2/hPSGL-1 bulk population or clones were significantly larger and had more prominent white foci as compared to mice infused with EL4.2/EV or EL4.2 cells (Fig. 6B and C, and S7). Histological analysis and CD3 IHC staining revealed more extensive kidney infiltration by EL4.2/hPSGL-1 cells than by EL4.2/EV cells (Fig. 6D and E). Although no significant differences in liver and spleen weight or bone marrow infiltration between mice infused with EL4.2/hPSGL-1 and control EL4.2/EV were observed (Fig. 6C, and S7), CD3 IHC staining revealed more extensive lung and liver infiltration in the former than the latter mice (Fig. 6D, F and G). Together, these results show that human PSGL-1 not only promotes EL4.2 tumorigenesis but also enhances dissemination and infiltration of kidneys and other organs.

## 4. Discussion

In this report, we demonstrate that PSGL-1 surface expression is not only frequent in malignant T cells but also enhances the tumorigenic and disseminative potential of a T-cell lymphoma cell line. The PSGL-1 glycoprotein is expressed in several hematopoietic lineages and has been earlier implicated in leukemia and lymphoma, of myeloid and lymphoid lineage [17,32–35]. We found high PSGL-1 surface expression in all malignant T cell lines analyzed, thus confirming and extending previous reports based on three or four cell lines [2,4]. Furthermore, PSGL-1 mRNA expression in human T leukemia and lymphoma cell lines and patient samples was found to be frequent in the Oncomine database (not shown). Therefore, the high prevalence of PSGL-1 expression in malignant T-cells prompts the study of its role in disease development.

Since the murine EL4.2 T lymphoma cell line displayed low PSGL-1 surface expression, we enforced human PSGL-1 expression in these cells. Human PSGL-1 became stably expressed at high levels in transduced cells, while endogenous murine PSGL-1 surface expression remained low. Although the human and mouse PSGL-1 peptide sequences are not identical, not even in the extracellular domain, several lines of evidence support the notion that human PSGL-1 expressed in EL4.2 cells can interact with murine ligands. First, the PSGL-1 function is evolutionary conserved within mammals, as demonstrated by the ability of human selectins to bind PSGL-1 from different species [29]. Moreover, a recent report showed that human myeloid leukemia cell lines expressing PSGL-1 and sLeX could bind both murine and human E- and P-selectins in static and dynamic conditions, albeit with subtle interspecies differences [30]. The present *in vivo* data showed that increased PSGL-1 surface expression was linked to accelerated EL4.2 s.c. tumorigenesis. Indeed, EL4.2/hPSGL-1 recipient mice reached the experimental endpoint about 10 days earlier than EL4.2 or EL4.2/EV recipient mice. This phenomenon was observed using both bulk transduced cells and clones expressing PSGL-1 or empty vector, indicating that it was linked to PSGL-1 expression and not potential cell culture artifacts. Increased PSGL-1 expression promoted EL4.2 proliferation *in vitro*, as compared to parental or empty vector-transduced EL4.2 cells. This finding that PSGL-1 expression induces malignant T cell proliferation is in agreement with the reported observation that PSGL-1 knockdown in multiple myeloma cells resulted in reduced proliferation [17]. This contrasts, however, with previous studies showing that the constitutional knockout of the murine *Selplg* gene (encoding PSGL-1) led to increased proliferation of CD8+ T cells [13,36]. This raises the issue whether PSGL-1 may regulate cell proliferation differently depending on the cellular context. Although unlikely, we cannot exclude the possibility that the cellular effects associated with surface overexpression of human PSGL1

arise from suppression of endogenous PSGL-1 expression and function rather than from a gain of function.

In addition to increased tumorigenesis, our data showed that increased PSGL-1 surface expression favored EL4.2 cell dissemination. The kidneys of EL4.2/hPSGL-1 tumor-bearing mice were often enlarged and infiltrated with CD3-positive EL4.2 cellular foci. Although EL4.2 tumor-bearing mice also showed kidney infiltration, this was less frequent and less widespread than in EL4.2/hPSGL-1 recipient mice, even considering that the former developed equally large tumors at experimental endpoint. In fact, several mice with EL4.2 tumors reaching similar size as EL4.2/hPSGL-1 tumors did not present significant infiltration of kidneys or other organs analyzed. This result suggests that increased PSGL-1 surface expression increased the ability of EL4.2 cells to disseminate from the primary tumor to other organs. To know if PSGL-1 could increase the EL4.2 organ colonization ability, nude mice were infused intravenously with the different cell lines. By doing so, we found that EL4.2/hPSGL-1 recipient mice presented widespread kidney infiltration, as well as lung and liver infiltration. An earlier study showed that PSGL-1 is required for lymphoma metastases and colonization of liver and kidney [19]. Thus, our data reinforces the notion that PSGL-1 promotes T cell lymphoma dissemination and metastatic ability to infiltrate organs such as kidneys, lungs and liver.

The mechanism by which PSGL-1 promotes EL4.2 proliferation and tumorigenic potential was not determined in this study, but interaction with selectins has been pinpointed in other reports. In a BCR-ABL1 chronic myeloid leukemia mouse model, E-selectin and PSGL-1 were involved in leukemic stem cell bone marrow homing and engraftment [18]. Moreover, blockade of PSGL-1 interaction with L-selectin *in vitro* prevented myeloid leukemia cell line adhesion and rolling [37]. Similarly, Spertini and co-workers demonstrated that primary acute myeloid leukemia and acute lymphoblastic leukemia blasts adhered and rolled on selectins *in vitro* through PSGL-1 and other selectin ligands [33]. In multiple myeloma, PSGL-1 expression by myeloma cells was shown to be required for extravasation and bone marrow homing through P-selectin interaction [17]. Although not directly demonstrating a role for PSGL-1, Bélanger and St-Pierre demonstrated that selectins were important for mouse T cell lymphoma dissemination to peripheral organs [38]. Together these studies suggest that selectins are important mediators for PSGL-1 function in hematological malignancies. Taking this into consideration, we assessed the ability of EL4.2 cells expressing human PSGL-1 to bind murine selectins *in vitro*, as compared to controls. No major differences in P-selectin binding under static conditions were observed among cell lines, although we cannot exclude the possibility that selectin binding *in vivo* was increased by human PSGL-1 expression. Whether the tumorigenic role of PSGL-1 depends on glycosylation and binding to selectins or binding to the VISTA protein under acidic pH, as recently demonstrated [16], remains to be investigated.

In summary, our results show that human PSGL-1 glycoprotein, promotes the development, progression and dissemination of T cell lymphoma. Further studies to unveil the pro-oncogenic properties of PSGL-1 and targeting mechanisms are warranted.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**João L. Pereira:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Patrícia Cavaco:** Methodology, Validation, Investigation. **Ricardo C. da Silva:** Methodology, Investigation. **Ivette Pacheco-Leyva:** Methodology, Writing – review & editing. **Stefan Mereiter:** Conceptualization, Methodology, Resources. **Ricardo Pinto:** Methodology. **Celso A. Reis:** Conceptualiza-

tion, Writing – review & editing. **Nuno R. dos Santos:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Supervision, Funding acquisition.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101125.

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