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The αvβ3 integrin as a tumor homing ligand for lymphocytes

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Despite the presence of tumor-specific effector cells in the circulation of cancer patients, the immune response of the majority of these patients is not sufficient to prevent the growth and spread of their tumors. That tumor cells can be killed *in vitro* by tumor-reactive cytotoxic T cells is testimony to the fact that the tumors are not inherently resistant to T cell killing, but rather that there is a failure in immune recognition and effector cell activation. Many reasons for this failure of the body's defense system have been suggested, including the inability of tumor-reactive lymphocytes to migrate to tumor tissue. Here we designed a strategy to improve homing of primary lymphocytes into vascularized tumors. As a homing molecule we selected the integrin αvβ3 since it is expressed by angiogenic vascular endothelium in tumors. To promote lymphocyte adhesion to αvβ3 we "painted" primary lymphocytes with a recombinant, glycosylphosphatidylinositol-linked high-affinity ligand for αvβ3. These painted lymphocytes specifically bound to αvβ3 *in vitro* and homed to vascularized, solid tumors *in vivo*. This novel strategy may provide a significant advance in anti-tumor treatment such as adoptive immune therapy.

Key words: αvβ3 integrin / Tumor homing / Lymphocytes / Painting / Glycosylphosphatidylinositol-anchored proteins

1 Introduction

The homing of lymphocytes to organs and tissues requires the presence of adhesion molecules on the vascular endothelium adjacent to the tissue. This is true for both constitutive recirculation of naive lymphocytes and homing of tumor-reactive lymphocytes into tumors. However, it has been demonstrated that many melanoma and carcinoma actively down-regulate the expression of cell adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 [1], ICAM-1, and ICAM-2 [2] on the endothelium adjacent to the tumor. Furthermore, endothelial cells of tumor vasculature may be unresponsive to pro-inflammatory cytokines and fail to up-regulate the expression of cell adhesion molecules [3, 4]. This may provide one mechanism by which tumors escape the body's immune defense system [5]. This notion is further substantiated by the fact that while circulating tumor-specific CTL can be detected, these cells fail to invade the tumor [4, 6, 7].

Growth of solid tumors is dependent on the formation of new blood vessels, and this tumor-induced angiogenesis is initiated by the release of angiogenic peptides from tumor and stroma, the down-regulation of endogenous angiogenesis inhibitors, and modification of the extracellular matrix [8–10]. Importantly, angiogenesis is also characterized by migration, invasion, and proliferation of endothelial cells that express the integrin αvβ3 [11–16]. Accordingly, integrin αvβ3 expression is up-regulated in tumor-associated blood vessels in invasive carcinoma [17, 18] and αvβ3 was therefore proposed as a diagnostic marker for a variety of solid tumors [19–22].

While mice deficient in αvβ3 show normal tumor development [16], nevertheless αvβ3 is up-regulated during tumor angiogenesis, suggesting that this integrin could serve as a receptor to target leukocytes to growing tumors. In order to promote lymphocyte adhesion to αvβ3 and tumor homing, we previously transfected a recombinant ligand for αvβ3, termed KISS31, into immortal lymphocyte cell lines. In fact, these transfected

Abbreviations: DAF: Decay-accelerating factor **GPI:** Glycosylphosphatidylinositol

cells homed to vascularized tumors [23], thus demonstrating the potential of engineered ligands as homing receptors.

Nevertheless, adaptive immune therapy depends on the use of autologous primary lymphocytes that seed and reject the tumor. Therefore, we have applied a novel strategy to insert αvβ3 integrin ligands into primary lymphocytes without the need for genetic manipulation of the cells. This technology, termed cell surface 'painting', makes use of the unusual capacity of purified glycosylphosphatidylinositol (GPI)-anchored (glypiated) proteins to re-integrate into the plasma membrane of any target cell ([24–29], reviewed in [30]). Based on this biochemical property it is possible to express exogenous proteins at the cell surface without the need for transfection. KISS31 consists of the disintegrin kistrin (a viper venom peptide) fused to the N terminus of platelet-endothelial cell adhesion molecule (PECAM)-1/CD31 [23]. Kistrin contains an RGD motif that binds specifically and with high affinity to the integrins αvβ3 and αIIbβ3 [23, 31–33].

In this study we investigated whether GPI-linked KISS31 (KISS31-GPI)-painted primary human and mouse lymphocytes would mediate tight adhesion to recombinant soluble αvβ3 integrin *in vitro*, *i.e.* whether the cell surface painted with KISS31-GPI retains its native function. Furthermore, KISS31-GPI-painted primary lymphocytes were tested for their specific homing to vascularized, αvβ3 integrin-expressing Lewis lung carcinoma *in vivo*.

2 Results

2.1 KISS31-GPI: an αvβ3 integrin-binding molecule

Anti-cancer immune response requires homing of lymphocytes into the tumor. Depending on the tumor type, this process is inhibited by suppression of lymphocyte homing molecules on the tumor vasculature. To enable lymphocyte homing, we exploited the expression of the cell adhesion molecule αvβ3 integrin by angiogenic tumor vessels. The fusion protein KISS31 binds with high affinity to αvβ3 integrin and cell lines expressing KISS31 home to tumors [23]. Our aim was to direct primary lymphocytes into tumors using this ligand, without the need for constitutive expression of KISS31 and viral transfection, which could lead to detrimental effects *in vivo* in the long term. To achieve this, we equipped the plasma membrane of primary lymphocytes with KISS31-GPI. This strategy, called painting, is rapid, can be performed in standard medium and does not interfere with immune function [24, 26, 34–36]. KISS31-GPI was constructed using the GPI anchor derived from the decay-accelerating factor (DAF). KISS31 consists of αvβ3 integrin-binding kistrin, linked by the flexible CD8α hinge region to the six Ig domains of CD31 as spacer. An inserted Flag epitope enabled affinity purification of the construct (Fig. 1A, B). As a control we generated CD31-GPI without the kistrin domain.

HEK293 cells were stably transfected with KISS31-GPI and CD31-GPI and cell surface expression was deter-

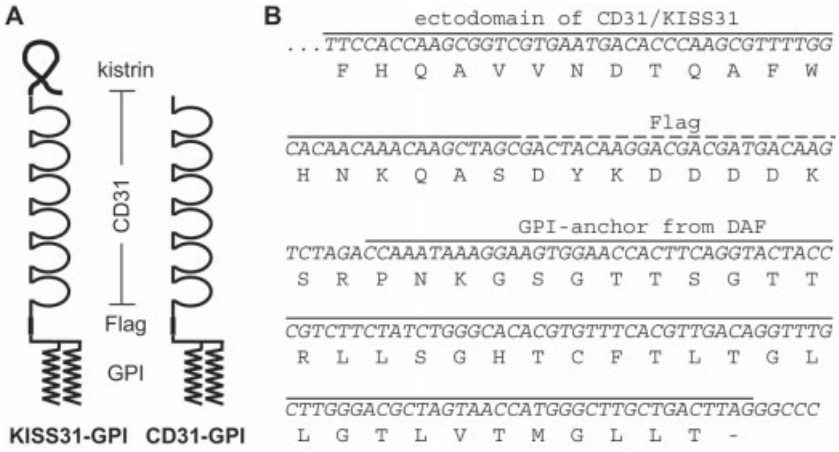

Fig. 1. Generation of glypiated KISS31 and CD31. (A) Schematic representation of KISS31-GPI and CD31-GPI. KISS31-GPI is a chimeric cell adhesion molecule, which consists of the soluble disintegrin kistrin that contains the high-affinity binding site specific for integrin αvβ3 linked by the CD8α hinge region to the N terminus of the ectodomain of CD31. KISS31 and CD31 are inserted into the plasma membrane via the GPI-anchor derived from DAF. The Flag tag was introduced for protein purification. (B) Nucleotide and deduced amino acid sequences from the C-terminal KISS31-GPI and CD31-GPI.

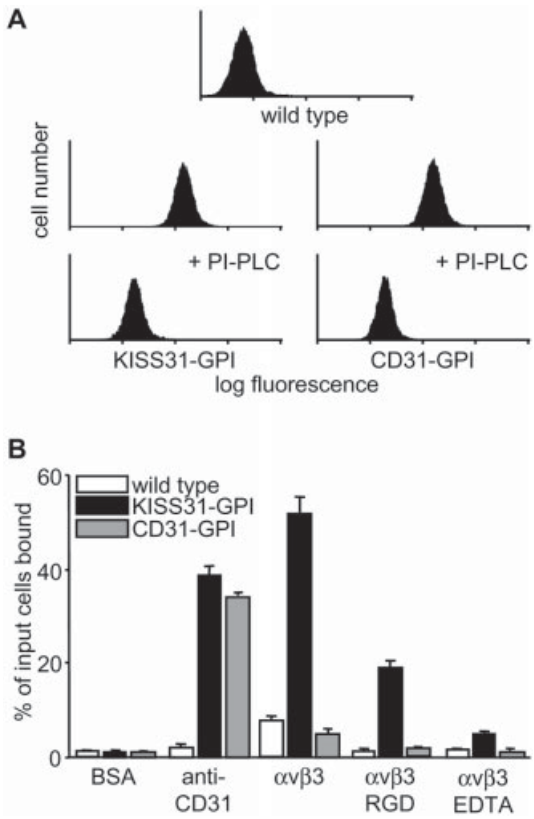

Fig. 2. Transfected KISS31-GPI is PI-PLC-sensitive and mediates adhesion to $\alpha v\beta 3$ integrin. (A) GPI-linked molecules expressed on HEK293 cells. KISS31-GPI and CD31-GPI were stably transfected into HEK293 producer cells. The GPI-anchored proteins were then cleaved by PI-PLC treatment for 2 h. Cell surface expression of the fusion proteins was examined by FACS staining using an anti-CD31 mAb. (B) Specific adhesion of KISS31-GPI-transfected cells to $\alpha v\beta 3$ integrin. KISS31-GPI-, CD31-GPI-expressing or non-transfected HEK293 cells were incubated in wells previously coated with 1 μg/ml recombinant soluble $\alpha v\beta 3$ or 3 μg/ml anti-CD31 mAb in the presence or absence of 10 μM cRGD peptide or 10 mM EDTA. Mean values and SD of triplicate values from a representative experiment are shown.

mined by FACS analysis (Fig. 2A). HEK293 clones showing high expression levels were selected for the purification of the glypiated proteins. To confirm that the newly generated molecules were indeed inserted into the plasma membrane of the cell via a GPI anchor, we incubated the cells with phosphatidylinositol-specific phospholipase C (Fig. 2A), which specifically cleaves most GPI-linked proteins [30, 37], including KISS31-GPI and CD31-GPI.

The KISS31-GPI-transfected cells avidly bound to recombinant soluble $\alpha v\beta 3$ integrin in a solid-phase adhesion assay (Fig. 2B), proving that the integrated kistrin retained its integrin-binding function. The adhesion was specific and could be blocked by EDTA and RGD peptides. Control CD31-GPI-transfected cells did not bind to $\alpha v\beta 3$. Binding to immobilized anti-CD31 mAb was comparable for both cell lines (Fig. 2B). Similar binding characteristics were obtained with transfected B300–19 cells expressing the transmembrane form of KISS31 and CD31, respectively (data not shown and [23]). These results demonstrate that the cells expressing a GPI-linked form of KISS31 specifically adhere to $\alpha v\beta 3$ integrin *in vitro*.

2.2 Painting of primary lymphocytes with KISS31-GPI

KISS31-GPI and CD31-GPI were purified by affinity chromatography from transfected HEK293 cell lysates. SDS-PAGE analysis of purified proteins revealed the predicted apparent molecular masses of 82 kDa and 72 kDa for KISS31-GPI and CD31-GPI, respectively (Fig. 3). For cell surface painting, affinity-purified glypiated proteins were incubated with cell lines and primary cells in culture

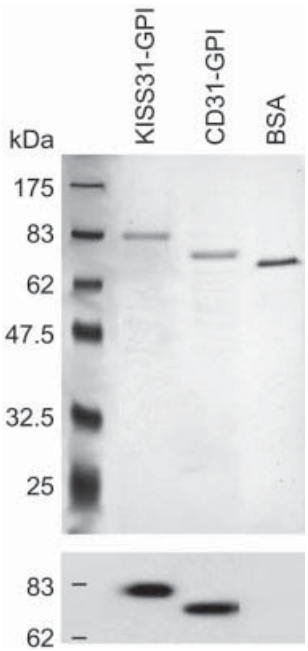

Fig. 3. Purification of glypiated KISS31 and CD31. GPI-linked proteins from transfected HEK293 cells lysates were affinity-purified using an anti-Flag mAb column. Purified proteins were analyzed by SDS-PAGE and silver staining (upper panel) or Western blotting using an anti-Flag mAb (lower panel). As a control for quantification 0.5 μg BSA was loaded. The molecular masses of standard proteins are indicated on the left.

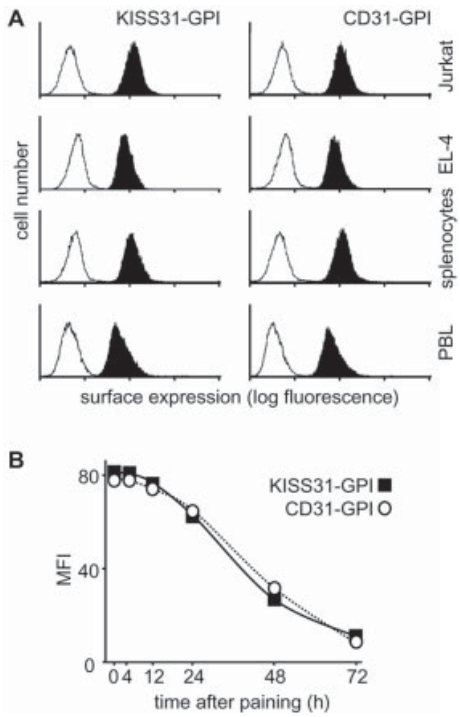

Fig. 4. Cell surface painting with purified KISS31-GPI and CD31-GPI. (A) Human Jurkat T cells and mouse T cell lymphoma EL-4 cells, as well as primary mouse splenocytes and human PBL were incubated for 90 min in the presence (filled histograms) or absence (open histograms) of purified KISS31-GPI (left panel) or CD31-GPI (right panel), respectively. After extensive washing of the cells, plasma membrane-inserted glypiated proteins were quantified by FACS analysis using either the anti-CD31 mAb GC51 (for Jurkat, EL-4 cells) or the anti-Flag mAb M2 (for primary cells). (B) Kinetics of the surface expression of painted GPI-anchored proteins. Human Jurkat T cells were painted with affinity-purified KISS31-GPI (filled squares) or CD31-GPI (open circles), washed and surface expression of the chimeric proteins was analyzed by flow cytometry at different time points after cell culture.

medium. Painting of KISS31-GPI and CD31-GPI was comparable on human Jurkat T cells, mouse EL-4 T lymphoma cells, as well as on primary mouse splenocytes and human PBL (Fig. 4A). This demonstrates that cell surface painting is applicable and efficient for many cell types. Insertion of the GPI-linked molecules into the plasma membrane was observed as early as 5 min following incubation at 37°C reaching a plateau at 90 min. Incubation for prolonged periods (up to 6 h) did not increase the painting efficiency (data not shown). Reduced cell surface expression was observed when the painting procedure was performed at either 4°C or room temperature (data not shown). The expression of cell surface-painted molecules remained stable at the cell surface for up to 12 h, and decreased thereafter relative to the rate of cell division (Fig. 4B). The expression level of painted KISS31-GPI was comparable to cell lines transfected with the transmembrane or GPI-linked form of KISS31 (Fig. 2, 4; [23]).

2.3 KISS31-GPI-painted lymphocytes adhere to αvβ3 integrin and migrate across monolayers of endothelioma cells

Primary human PBL painted with KISS31-GPI specifically adhere to coated αvβ3 integrin but not to BSA as a

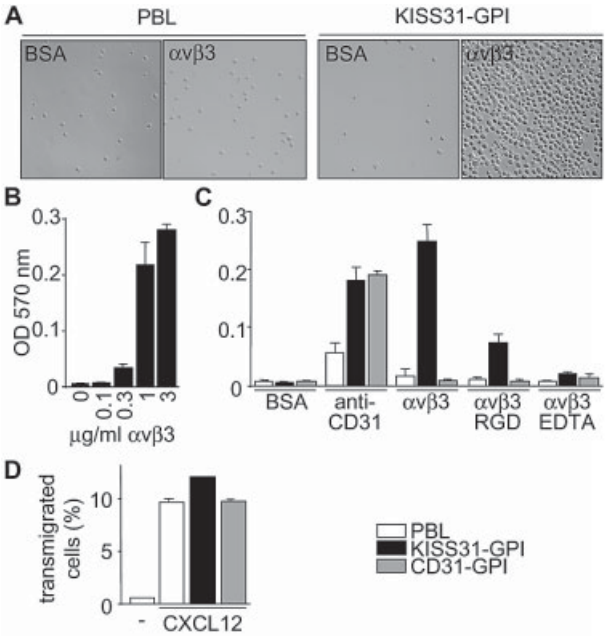

Fig. 5. Painted primary cells adhere to αvβ3 integrin and migrate across endothelioma cell monolayers. (A) Phase contrast image of adherent PBL. Lymphocytes were used untreated or painted with KISS31-GPI for 90 min and cells were allowed to adhere to αvβ3 or BSA coated at 1 μg/ml. (B) Adhesion assay with KISS31-GPI-painted PBL to αvβ3 coated at increasing concentrations. Adherent cells were fixed, stained with crystal violet and quantified by measuring the optical density at 570 nm. (C) Unpainted (white bars), KISS31-GPI- (black bars), or CD31-GPI-painted (gray bars) PBL were allowed to adhere to BSA, anti-CD31 mAb, or αvβ3 in the presence or absence of cRGD peptide or EDTA as described in Fig. 2B. Representative assays are shown with triplicate samples (mean values and SD). (D) Chemokine-mediated lymphocyte transmigration across endothelium. Human PBL were painted for 90 min with purified KISS31-GPI or CD31-GPI. Painted and unpainted cells were added to transwell filters coated with a monolayer of the endothelioma cell line tEnd.1. Lymphocytes that migrated towards 1 μg/ml CXCL12 across the endothelium were counted by FACS. Mean values and SD of two independent experiments with different donors are shown.

control protein (Fig. 5A). Unpainted PBL were unable to bind to either substrate. The adhesion of KISS31-GPI-painted human PBL gradually augmented with increasing $\alpha v\beta 3$ integrin concentration (Fig. 5B). Maximal binding was reached at 1 μg/ml of coated integrin. PBL painted with KISS31-GPI, but not CD31-GPI, specifically bound to $\alpha v\beta 3$ in a calcium- and RGD-dependent manner (Fig. 5C). Painted cells also bound to immobilized anti-CD31 mAb, demonstrating that plasma membrane insertion of the chimeric molecules was efficient (Fig. 5C). Untreated PBL adhered weakly to immobilized anti-CD31 mAb due to endogenous expression of CD31 on certain cell subpopulations (Fig. 5C). Similar results were obtained for painted Jurkat and EL-4 cells (data not shown). To investigate whether KISS31-GPI-painted lymphocytes would retain the ability to transmigrate an endothelial monolayer, we performed transendothelial migration assays. Indeed, KISS31-GPI- and CD31-GPI-painted human PBL, as well as unpainted cells, migrated towards the chemokine CXCL12 across monolayers of the $\alpha v\beta 3$-expressing endothelioma cell line tEnd.1 (Fig. 5D). In conclusion, cell surface painting is an efficient method to insert KISS31-GPI into the plasma membrane of various cell types, including primary cells.

2.4 *In vivo* homing of primary lymphocytes to tumors

The Lewis lung carcinoma line LLC-1 develops well-vascularized solid tumors after s.c. injection in mice. Primary splenocytes from syngeneic mice were labeled with PKH67green and PKH26red fluorescent dyes, respectively, and recovered from labeling by overnight culture. The green-labeled lymphocytes were then painted with KISS31-GPI, red-labeled lymphocytes with CD31-GPI. A 1:1 mixture of green and red lymphocytes was injected i.v. into mice with s.c. tumors. After 4 h the mice were sacrificed, and tumor homing of painted lymphocytes evaluated by fluorescence microscopy of tissue sections (Fig. 6A). The relative number of lymphocytes that homed to the tumor was compared with that of other organs of the same mouse.

In tumors, more than five times as many KISS31-GPI- than CD31-GPI-painted control lymphocytes were found (Fig. 6B), demonstrating that cell surface expression of KISS31-GPI mediated the specific homing of painted primary lymphocytes to the tumor. By contrast, the number of KISS31-GPI-painted lymphocytes found in spleen, liver, and kidney did not significantly differ from that of CD31-GPI-painted lymphocytes (Fig. 6B). Inverting the fluorescent labeling revealed identical results (data not shown). For our analysis we could not distinguish between KISS31-GPI-painted lymphocytes that

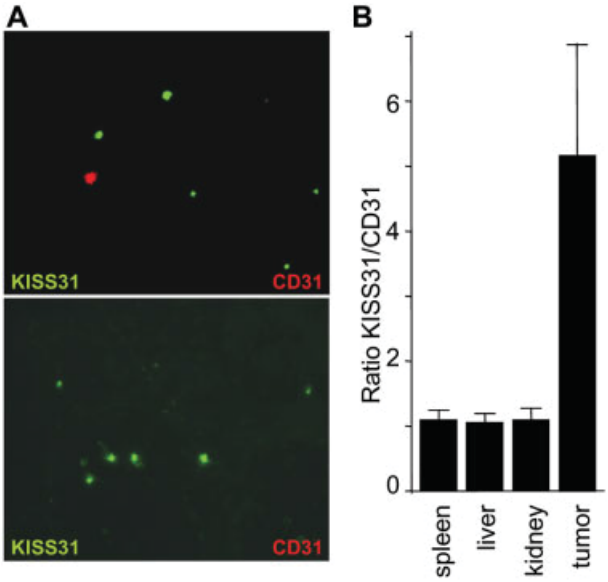

Fig. 6. Tumor homing of painted primary lymphocytes. Mouse splenocytes were labeled with PKH67green or PKH26red, cell surface-painted with either KISS31-GPI or CD31-GPI, mixed in equal numbers, and injected i.v. into tumor-bearing mice. At 4 h post injection, the mice were sacrificed and frozen sections from tumors and control organs prepared. (A) Tumor homing of KISS31-GPI- (green) and CD31-GPI-painted (red) lymphocytes were visualized by fluorescent microscopy. Two examples of tumor tissue sections are shown. (B) Ratio of trapped KISS31-GPI versus CD31-GPI lymphocytes in tumor, spleen, liver, and kidney. Mean values and SD of the ratio of KISS31-GPI- and CD31-GPI-painted cells in 15–30 frozen sections/organ/mouse (n=10, two independent experiments) are shown.

remained stuck to the blood vessels via tight adhesion to the integrin $\alpha v\beta 3$ expressed on the endothelium and cells that had migrated into the tumor. Nevertheless, the ability of KISS31-GPI-painted cells to transmigrate monolayers of endothelioma cells *in vitro* (Fig. 5D) suggests that KISS31-GPI-painted cells may efficiently emigrate from the blood vessel into the tumor. In conclusion, painting with KISS31-GPI is an efficient strategy to send primary lymphocytes into tumors.

3 Discussion

Extravasation of leukocytes from the bloodstream occurs during constitutive lymphocyte recirculation into lymph nodes, and during immune responses at sites of inflammation. These processes involve a multi-step adhesion cascade, which is driven by a co-ordinated expression of adhesion molecules and their ligands [38–41]. The homing of T cells to tumors is similarly mediated by tissue-specific adhesion molecules,

although the failure of the immune system to effectively eradicate a growing tumor is testimony in part to the fact that tumor homing is inefficient. Interestingly, vascularized tumors down-regulate certain adhesion molecules, such as VCAM-1, ICAM-1, and ICAM-2, thereby preventing T lymphocytes from adhering to the vessels and subsequently migrating into the tumor [1, 2, 4]. In contrast, $\alpha v\beta 3$ integrin is highly expressed by angiogenic vascular endothelium. Since angiogenesis is a pre-requisite for tumor growth, antigens expressed by angiogenic vasculature are an attractive target for anti-tumor therapy. Thus, $\alpha v\beta 3$ integrin potentially is an ideal target adhesion molecule for inducing lymphocyte homing to tumors. To exploit this potential, however, lymphocytes would need to express a ligand for $\alpha v\beta 3$. To this end we generated a chimeric high-affinity cell adhesion molecule for (mouse and human) $\alpha v\beta 3$ integrin, termed KISS31, which can mediate homing of transfected cell lines to tumors [23].

The ultimate goal for anti-tumor immune therapy is the homing of primary effector cells into tumors. The successful transfection of primary cells remains a challenging goal since using available methods, transfection frequencies are extremely low. Furthermore, constitutive expression may not be necessary for the desired function of the exogenously expressed protein, such as a homing molecule. As an alternative method for expressing KISS31 in primary cells, we painted cells with a GPI-anchored form of KISS31. Studies in humans with parasitic infections or mice transgenic for DAF had already provided evidence that insertion of dissociated GPI-anchored proteins into cell membranes occurs *in vivo* [42, 43]. The addition of a glypiation signal to cDNA permits expression of the protein as a GPI-anchored form [30]. In our hands, production of the GPI fusion protein was optimal in fast-growing HEK293 cells enabling purification of large quantities of KISS31-GPI from cell lysates. A unique property of purified GPI-anchored proteins is their ability to re-insert into the plasma membrane when added to living cells [27]. For example, GPI-anchored MHC was loaded with hepatitis B virus peptide and painted to lymphoma target cells. These cells were then efficiently killed by peptide-specific CTL [26]. In a separate study, immunization of mice with CD80-GPI-painted tumor cell membranes induced the proliferation of T lymphocytes and protected mice from parental tumor challenge [24].

Painting offers several advantages over conventional gene transfection or viral infection methods: It is applicable to any type of cell, including primary cells that are difficult to transfect. The efficiency of painting is high and reveals homogenous insertion of the glypiated protein into the plasma membrane. Painting is rapid and maximal insertion is reached after 90 min of incubation of the GPI-linked protein with the cells and protein expression on the surface is immediate. Painting is performed under normal culture conditions and does not require special treatment of cells. Painting is not restricted to a minimal number of cells. The introduced proteins remain stable on the cell surface for several hours, thus enabling them to mediate biological functions. Indeed, cell surface-painted molecules have been shown to retain full biological function [26, 34–36, 44]. Furthermore, the painted proteins are completely removed from the cell surface in the course of several cycles of cell division.

Does improved tumor homing represent a real hope for anti-tumor therapy? Interestingly, recent progress in anti-tumor immune therapy has been made employing adoptive transfer of *in vitro* expanded effector T lymphocytes. These cells could be isolated from lymph nodes draining a tumor by sorting for L-selectin^{low} (activated) cells [45], or IFN-γ-expressing cells (also a marker of activation) using IFN-γ capture [46]. The rationale was that such activated cells would contain a significant proportion of tumor-reactive T cells. Surprisingly, a high proportion of T cells isolated in this way comprised naive L-selectin^{hi} or IFN-γ^- cells, showing no reactivity against the tumor [45]. However, upon sorting and enrichment of activated cells using the above criteria, it was possible to generate high numbers of tumor-reactive T cells *in vitro*. The drawback to these studies is the high number of transferred effector T cells (4×10^7) needed to achieve tumor clearance in mice. This is probably due to inefficient homing of the T cells into the tumor. Using painting with KISS31-GPI we propose a method to deliver cells far more efficiently into tumor tissue, hence the number of T lymphocytes needed should be dramatically reduced.

In summary, painting primary cells with a high-affinity ligand for tumor vasculature offers an exciting potential for tumor therapy. In addition to the delivery of tumor-reactive T cells to sites of tumor growth, it is envisaged that such an approach could be adapted for the delivery of cytotoxic drugs to the growing tumor. The results of immune tumor therapy have so far yielded modest clinical benefits, although exciting new methods for adaptive T cell therapy are on the horizon. These, combined with advances such as described in the present study, should herald a new dawn for tumor immunotherapy.

4 Materials and methods

4.1 Cells

Human PBL were isolated from donor blood buffy coats (Swiss Red Cross, Lausanne, Switzerland) by separation on Ficoll-Paque followed by two steps of plastic adherence to

deplete monocytes. Human PBL, splenocytes derived from C57/BL6 mice, EL-4 and Jurkat cell lines were cultured in RPMI 1640 supplemented with 10% FCS, 5 μg/ml penicillin, 5 μg/ml streptomycin, and 10 μg/ml neomycin (Invitrogen, San Diego, CA). The human embryonic kidney cell line, HEK293, and the mouse thymic endothelioma cell line, tEnd.1, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 5 μg/ml penicillin, 5 μg/ml streptomycin, and 10 μg/ml neomycin.

4.2 Cloning and production of αvβ3 integrin

The cloning and expression of the Flag-tagged mouse αvβ3 have been described previously [23, 33]. Recombinant soluble αvβ3 was purified from the supernatant of HEK293 transfectants using an anti-Flag affinity column (Eastman Kodak Company, New Haven, CT). Bound integrin was eluted with a Flag-peptide and purified by fast protein liquid chromatography as described [33].

4.3 Cloning of KISS31-GPI and CD31-GPI

The cloning of KISS31 and CD31 has been described previously [23, 33]. Briefly, KISS31 consists of the ectodomain of mouse adhesion molecule CD31 fused to the disintegrin kistrin that contains the high-affinity binding site for αvβ3. To generate the Flag-GPI vector, two oligonucleotides encoding the Flag epitope (sense 5'-CTCGAGTGCTAGCGACTACAAGGACGACGATGACAAGT and antisense 5'-TCTAGACTTGTCATCGTCGTCCTTGTAGTCGCTAGCAC) were annealed, digested (restriction sites are underlined), and cloned into the XhoI/XbaI sites of the mammalian expression vector pcDNA3 (Invitrogen), revealing pcDNA3-Flag. The cDNA encoding for the GPI anchor from DAF was generated by PCR using human umbilical vein endothelial cell cDNA as template, the Pfu polymerase (Stratagene AG, Basel, Switzerland), the sense primer 5'-ATTATTCTAGACCAAATAAAGGAAGTGGAACC, and the antisense primer 5'-TAATTGGGCCCTAAGTCAGCAAGCCCATG. The amplified DNA was purified, digested, and subcloned into the XbaI/ApaI sites of pcDNA3-Flag (pcDNA3-Flag-GPI). Finally, the ectodomains of KISS31 and CD31, respectively, were cloned in frame into the HindIII/NheI site of pcDNA3-Flag-GPI.

4.4 Transfection of GPI-anchored proteins into HEK293 producer cells

HEK293 cells were stably transfected by the calcium phosphate procedure [47] and G-418-resistant HEK293 clones were established. The expression of the GPI-linked (glypiated) constructs was analyzed by flow cytometry in a FACStar (Becton Dickinson, Erembodegen, Belgium) using either the mouse-anti-Flag mAb M2 (Sigma, Buchs, Switzerland) or the rat-anti-mouse CD31 mAb GC51 and FITC-conjugated goat anti-mouse or goat anti-rat IgG mAb (Milan Analytica AG, La Roche, Switzerland).

4.5 Purification of GPI-anchored proteins

Twenty 15-cm dishes of confluent HEK293 transfectants expressing KISS31-GPI or CD31-GPI were washed twice with PBS. The cells were resuspended in 15 ml of ice-cold homogenization buffer (20 mM sucrose, 1 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4, 20 μg/ml benzamidine, 4 μg/ml antipain, 0.25 μg/ml leupeptin). The cells were kept on ice for 10 min and then homogenized using a tight-fitting glass dounce homogenizer (25 strokes). The homogenate was spun for 5 min at 500×g and the supernatant was retained. To maximize the yield, the pelleted nuclei were further washed twice with homogenization buffer and the supernatants were added to the first harvest. The final volume of the supernatants was adjusted to 50 ml and Brij78 (Fluka, Buchs, Switzerland) was added to a final concentration of 0.5%.

The supernatants were solubilized for 5 h at 4°C. After centrifugation (30 min, 5,000×g) the two GPI-linked molecules were then separately affinity-purified on an anti-Flag-coupled agarose column (Sigma), extensively washed with 20 mM Tris-HCl pH 7.0 containing 0.5% Brij78 followed by PBS. The purified proteins were eluted with 0.1 M glycine pH 2.8. Fractions of 500 μl were collected and neutralized with 60 μl 1 M Tris-HCl pH 8.0. Purified proteins were analyzed by SDS-PAGE followed by silver staining and Western blotting using anti-Flag mAb. Aliquots were stored at –80°C and sequential thawing and freezing was avoided.

4.6 Phosphatidylinositol-specific phospholipase C cleavage of KISS31-GPI and CD31-GPI

HEK293 cells (2×10^5) expressing KISS31-GPI or CD31-GPI in 100 μl of complete medium were incubated for 2 h at 37°C with 0.05 U of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (Glyco Inc., Navato, CA) prior to FACS analysis.

4.7 Cell surface painting with KISS31-GPI and CD31-GPI

For cell surface painting with KISS31-GPI or CD31-GPI, target cells were washed twice, resuspended in culture medium to up to 10^7 cells/ml and incubated with 0.5–5 μg/ml purified GPI-anchored proteins for 90 min at 37°C with occasional mixing. The cells were washed extensively and the efficiency of painting was measured by FACS analysis.

4.8 Cell adhesion assay

Purified, recombinant soluble αvβ3 integrin was coated overnight onto microtiter plates (Nunc Maxisorb; Polylabo, Strasbourg, France) at 0.1–3 μg/ml in PBS at 4°C. The wells were then blocked with 0.5% BSA (A7511; Sigma) for 1 h at room temperature and unbound proteins washed off. Cells (2×10^5) in 100 μl of adhesion buffer (RPMI, 1% BSA,

BSA, 20 mM Hepes) were added per well in the presence or absence of blocking agents [10 μM cyclic RGD peptide (Bachem AG, Bubendorf, Switzerland) or 10 mM EDTA]. After 30 min at 37°C, unbound cells were removed by four to six washes with pre-warmed adhesion buffer. Bound cells were fixed with methanol for 10 min and stained with 20% methanol containing 0.1% crystal violet for 20 min. After four washes with water, 100 μl methanol was added and the adherent stained cells were quantified using an ELISA reader at 570 nm. Alternatively, the cells were labeled with 10 μM calcein-AM (Molecular Probes, Eugene, OR) for 30 min at 37°C prior to the adhesion assay. Bound cells were then quantified using a Cytofluor II fluorescence reader (Stehlin AG, Basel, Switzerland) as described [23, 33]. In addition, adherent cells were visualized by light microscopy (DC200; Leica, Heerbrugg, Switzerland).

4.9 Transendothelial migration assay

A total of 10^5 tEnd.1 cells were cultured in Transwell culture inserts in 24-well plates (6.5 mm diameter, 5 μm pore size; Corning Inc, Corning, NY) for 48 h. Inserts with tEnd.1 monolayers were placed on 600 μl of medium containing 1 μg/ml human CXCL12 (PromoCell, Heidelberg, Germany). KISS31-GPI- or CD31-GPI-painted and unpainted lymphocytes (2×10^5 in 100 μl) were added to each filter and incubated for 3 h at 37°C. Transmigrated lymphocytes were counted by FACS.

4.10 *In vivo* tumor homing of painted cells

Splenocytes from C57/BL6 mice (Iffa Credo, L'Arbresle, France) were labeled separately with the PKH67green- and PKH26red-fluorescent linker kit (Sigma), respectively, according to the manufacturer's protocol. After labeling, cells were cultured overnight in RPMI containing 10% FCS to allow full recovery. After cell surface painting with KISS31-GPI or CD31-GPI, the cells were washed twice in PBS, mixed in a ratio of 1:1, and injected into tumor-bearing mice as described [23]. Briefly, C57/BL6 mice were injected s.c. with 5×10^5 Lewis lung carcinoma LLC-1 cells (European Collection of Cell Cultures, Salisbury, GB). Two weeks later, mice bearing tumors of around 1 cm in diameter were injected via their tail vein with a total of 5×10^6 labeled KISS31-GPI/CD31-GPI-painted cells in a 1:1 ratio. The animals were sacrificed 4 h later with CO_2. Animal experiments were carried out in accordance with the Swiss veterinary office regulations (accreditation number 31.1.1005/1911/II). Tumors and control organs were embedded in OCT Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, Netherlands) and frozen on dry ice. Cryosections of 7 μm thickness were cut, air-dried, and fluorescent cells were counted using an AxioCam microscope (Carl Zeiss, Jena, Germany) equipped with a 20× magnification objective lens.

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