

# Serpin B9 controls tumor cell killing by CAR T cells

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## ABSTRACT

**Background** Initial clinical responses with gene engineered chimeric antigen receptor (CAR) T cells in cancer patients are highly encouraging; however, primary resistance and also relapse may prevent durable remission in a substantial part of the patients. One of the underlying causes is the resistance mechanisms in cancer cells that limit effective killing by CAR T cells. CAR T cells exert their cytotoxic function through secretion of granzymes and perforin. Inhibition of granzyme B (GrB) can underlie resistance to T cell-mediated killing, and it has been shown that serine proteinase inhibitor serpin B9 can effectively inhibit GrB. We aimed to determine whether expression of serpin B9 by cancer cells can lead to resistance toward CAR T cells.

**Methods** Serpin B9 gene and protein expression were examined by R2 or DepMap database mining and by western blot or flow cytometric analysis, respectively. Coculture killing experiments were performed with melanoma cell line MeWo, diffuse large B cell lymphoma (DLBCL) cell line OCI-Ly7 or primary chronic lymphocytic leukemia (CLL) cells as target cells and natural killer cell line YT-Indy, CD20 CAR T cells or CD19 CAR T cells as effector cells and analyzed by flow cytometry.

**Results** Serpin B9 protein expression was previously shown to be associated with clinical outcome in melanoma patients and in line with these observations we demonstrate that enforced serpin B9 expression in melanoma cells reduces sensitivity to GrB-mediated killing. Next, we examined serpin B9 expression in a wide array of primary tumor tissues and human cell lines to find that serpin B9 is uniformly expressed in B-cell lymphomas and most prominently in DLBCL and CLL. Subsequently, using small interfering RNA, we silenced serpin B9 expression in DLBCL cells, which increased their sensitivity to CD20 CAR T cell-mediated killing. In addition, we showed that co-culture of primary CLL cells with CD20 CAR T cells results in selection of serpin B9-high CLL cells, suggesting these cells resist CAR T-cell killing.

**Conclusions** Overall, the data indicate that serpin B9 is a resistance mediator for CAR T cell-mediated tumor cell killing that should be inhibited or bypassed to improve CAR T-cell responses.

## BACKGROUND

Adoptive T-cell transfer is a form of personalized cancer therapy where a patient receives T cells that have antitumor activity.<sup>1</sup> After

isolation from a patient, ex vivo activation, selection and expansion allow for production of large numbers of tumor-reactive T cells. In addition, gene engineering strategies can be used to redirect T cells toward a specific antigen. Examples of gene engineered T cells that are currently being used for clinical use as anticancer therapy are T-cell receptor-modified T cells and chimeric antigen receptor (CAR) T cells.<sup>1,2</sup>

CAR T cells directed against CD19 have shown remarkable effectivity in B-cell lymphomas and leukemias, but noticeable differences between cancer types have been observed. Complete response rates (CRRs) in adults with B-cell acute lymphocytic leukemia varied between 83% and 93%, while CRR was significantly lower in patients with diffuse large B-cell lymphoma (DLBCL) (43%–54%) and patients with chronic lymphocytic leukemia (CLL) (21%–29%) treated with CD19 CAR T cells.<sup>3</sup> Since CRR rates on treatment with CD19 CAR T cells can be suboptimal, additional targets are currently being tested for CAR T-cell treatment of B-cell non-Hodgkin's lymphomas, including CD20.<sup>4</sup> Overall, the effectivity of CAR T-cell therapy is limited by the lack of highly tumor-specific markers and limited recruitment and persistence at the tumor site. Resistance of tumor cells to undergo apoptosis is another immune escape mechanism and can be mediated by downregulation of death receptors such as Fas (CD95) on cancer cells.<sup>5</sup> The main killing mechanism of T and natural killer (NK) cells, however, is not by death receptor activation but by releasing cytotoxic granules containing perforin and granzymes into the target cell.<sup>6</sup> There, granzymes cleave intracellular targets including two times a day, leading to intrinsic apoptosis pathway activation and caspase-mediated apoptosis.<sup>7</sup> Cytotoxic cells themselves are protected from endogenously produced granzymes by expression of certain serine protease inhibitors (serpins),



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such as serpin B9 (also named proteinase inhibitor 9), which bind and neutralize granzyme B (GrB).<sup>8</sup> Importantly, serpin B9 expression has also been reported in cancer cells, suggesting a mechanism of immune evasion. For example, in metastatic melanoma patients, clinical outcome was associated with elevated serpin B9 protein expression in tumor cells.<sup>9</sup> More recently, a computational model was developed to predict factors that are responsible for immune evasion of tumors and found serpin B9 expression to be a critical factor for immunotherapy resistance using immune checkpoint blockade.<sup>10</sup>

Therefore, we investigated whether expression of serpin B9 by cancer cells leads to resistance toward CAR T cells and found that serpin B9 overexpression impairs GrB-mediated tumor cell killing. Serpin B9 expression can be detected in the majority of malignant B cells and in a selection of other hematological and solid malignancies. Moreover, we reveal that knockdown of serpin B9 expression sensitizes DLBCL cells to CD20 CAR T cell-mediated killing and found that primary CLL cells with low serpin B9 expression were selectively enriched on coculture with CD20 CAR T cells. Combined, these results expose serpin B9 as a resistance factor against CAR T cell-mediated killing.

## METHODS

### Cell culture and chemicals

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, #12077549; Life Technologies) (MeWo), Iscove's Modified Dulbecco's Medium (#12539089, Life Technologies) (OCI-Ly7), or Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX HEPES culture medium (#12017599, Life Technologies) (YT-Indy and primary CLL cells), supplemented with 10%–20% fetal bovine serum (FBS, Sigma) and 100 µg/mL penicillin–streptomycin (p/s, #12017599; Gibco/Life Technologies). Human healthy donor peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin, Amsterdam, the Netherlands) using Ficoll-Paque according to the manufacturer's protocol. PBMCs were cultured in RPMI with 2.5% pooled AB+human serum (IPLA-CSER, Innovative Research), 50 µM β-mercaptoethanol (#11528926, Life Technologies) and 1% p/s.

### Immunoblotting

For western blot analysis, cells were lysed in buffer containing 1% Nonidet P-40, and proteins were separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-PROTEAN TGX Precast Gels, #4561093; Bio-Rad), transferred to low fluorescence polyvinylidene difluoride (PVDF) membranes (#1704274, Bio-Rad), blocked in phosphate-buffered saline (PBS) containing 2% non-fat dry milk, and stained using the following antibodies: mouse antiserpinB9 (#MA517648, Invitrogen) (7D8), mouse anti-α-tubulin (#CST3873S, Cell Signaling) (DM1A), and goat antimouse-680RD (#LI926-68070, LI-COR Biosciences). Infrared imaging

was used for detection (Odyssey Sa, LI-COR Biosciences). Analysis and quantification were performed using LI-COR Image Studio software.

### Generation of CD20 and CD19 CAR T cells

The CD20 CAR construct (pBu-CD20-CAR) was generated by cloning single-chain variable fragments from anti-CD20 antibody rituximab into a pBullet vector containing a D8α-41BB-CD3-ζ signaling cassette. Phoenix-Ampho packaging cells were transfected with gag-pol (pHit60), env (P-COLT-GALV) and pBu-CD20-CAR or pBu-CD19-CAR using FugeneHD transfection reagent (#E2312, Promega). Human PBMCs were preactivated with 30 ng/mL anti-CD3 (OKT3, #130-093-387; Miltenyi) and 50 IU/mL interleukin (IL)-2 (Sigma) and subsequently transduced two times with viral supernatant in the presence of 6 µg/mL polybrene (#H9268-5G, Sigma) and 50 U/mL IL-2. Transduced T cells were expanded using 50 U/mL IL-2 and anti CD3/CD28 dynabeads (#11161D, Thermo Fisher), and CD20-CAR-expressing cells were selected by treatment with 80 µg/mL neomycin. T cells were further expanded using rapid expansion protocol as described elsewhere.<sup>11</sup>

### Overexpression and knockdown of serpin B9

Generation of the retroviral vector pMSCV-serpinB9 is described elsewhere.<sup>12</sup> Virus production was performed as described for the CD20-CAR construct. Subsequently, MeWo cells were transduced two times with viral supernatant in the presence of 6 µg/mL polybrene, and stably overexpressing cells were selected using 1 µg/mL puromycin (#P8833-25MG, Sigma). In order to knock down serpin B9 expression, the ON-TARGETplus Human *SERPINB9* small interfering RNA (siRNA) SMARTpool (L-015400-00-0005, Dharmacon) was electroporated into OCI-Ly7 cells using a Neon transfection system 10 µL kit (#10124334, Thermo Fischer Scientific), at 1150 V, with 2×30 ms pulses.

### Apoptosis staining and flow cytometry

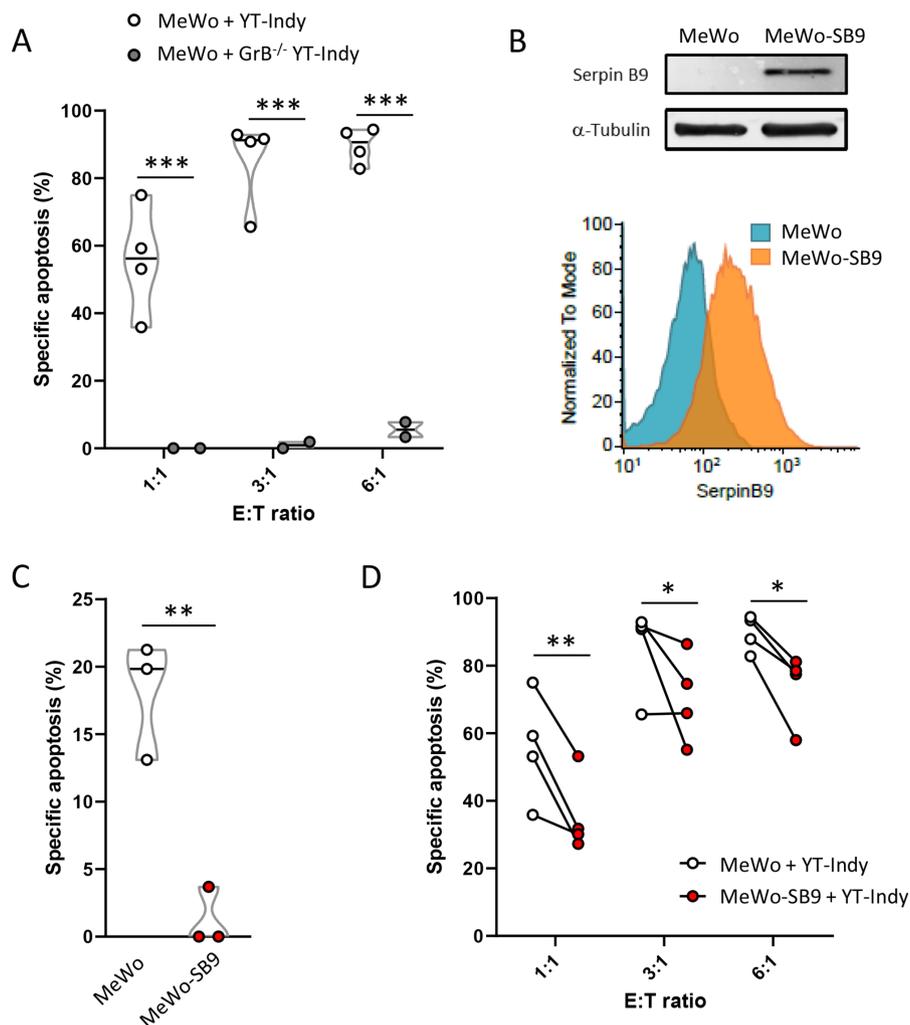
Assessment of cell viability took place by staining with 15 nM DiOC6 (#D273, Thermo Scientific) and 20 nM TO-PRO-3 (#T3605, Thermo Scientific) or with Fixable Viability Dy eFluor 780 (#15560607, eBioscience), followed by flow cytometric analysis (BD FACSCanto II or BD LSRFortessa, BD Biosciences). To determine the absolute amount of cells, Flow-Count Fluorospheres were used (#7547053, Beckman Coulter). In coculture experiments, target cells were identified by flow cytometric surface staining with CD19-BV421 (#2111170, Sony Biotechnology) (HIB19) (lymphoma cell lines) or by staining with Cell Trace Violet (#10220455, Invitrogen) (MeWo and pCLL) prior to adding effector cells. CD20 CAR T cells were characterized by staining with CD4-Pacific Blue (#300521, BioLegend) (RPA-T4), CD8-PE/Cy7 (#335822, BD Biosciences) (SK1), and biotinylated protein L (#M00097, Genscript) with streptavidin-PE (#554061, BD). Surface expression of

checkpoint molecules on CD20 CAR T cells was analyzed by staining with TIM-3 BV711 (#565566, BD Biosciences) (7D3), hLAG-3 PE (#FAB2319P, R&D Systems), and PD-1 FITC (#557860, BD) (MIH4). For intracellular staining of serpin B9, cells were fixed and permeabilized using BD Cytotfix/Cytoperm (#554714, BD Biosciences) and stained with mouse antiserpin B9 (Invitrogen) (7D8), or mouse anti-IgG Isotype (Southern Biotech) followed by goat antimouse IgG-AF647 (#A21235, Invitrogen). CAR T cells were activated by 20 ng/mL PMA (#P8139-1MG, Sigma-Aldrich), 1  $\mu$ g/mL ionomycin (#I0634-1MG, Sigma-Aldrich) in the presence of Golgistop (#554724, BD Biosciences) before intracellular cytokine production was analyzed by staining for interferon gamma (IFN- $\gamma$ )

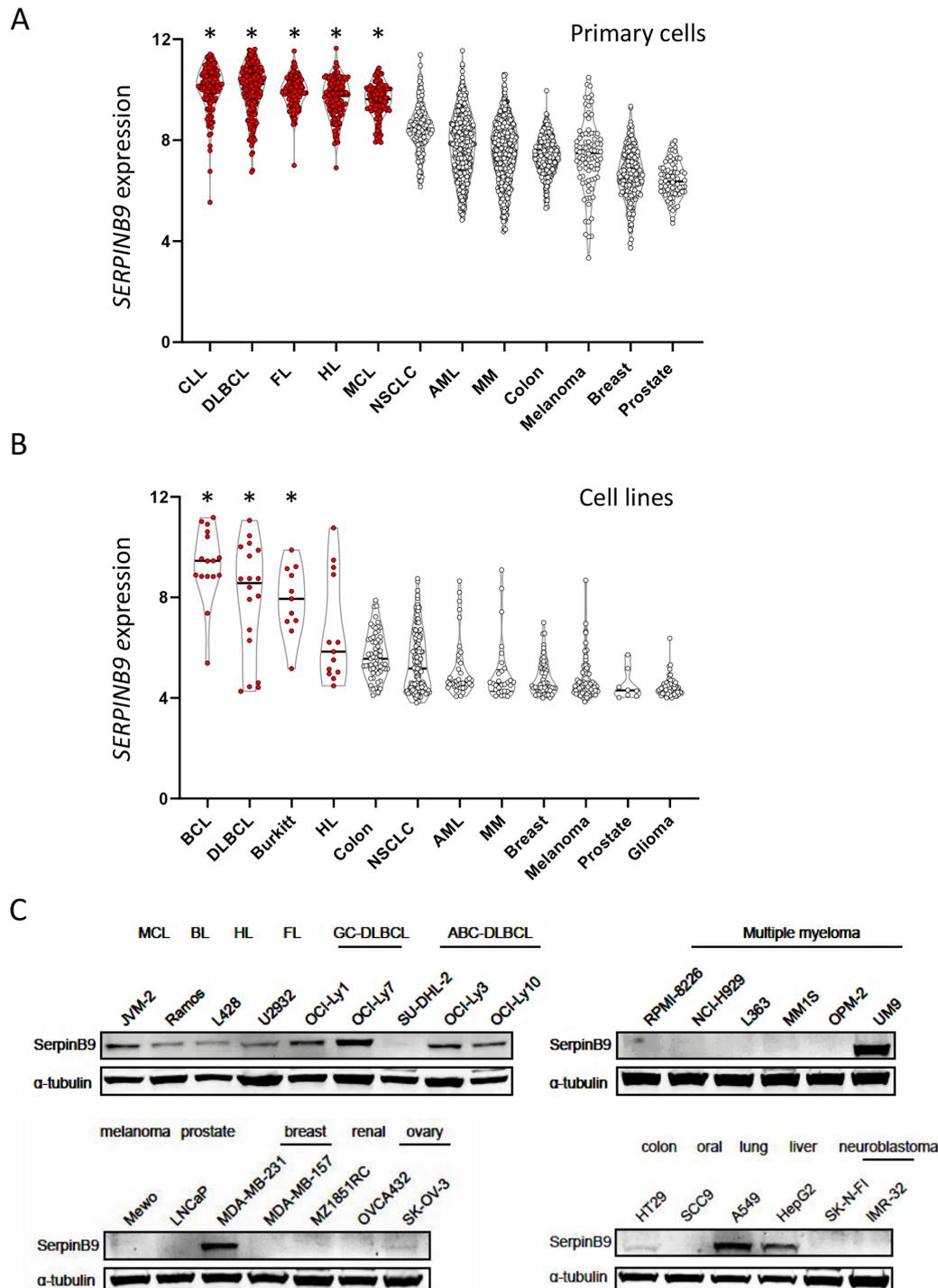
and tumor necrosis factor alpha (TNF- $\alpha$ ). Flow cytometry data analysis was performed using FlowJo.

### Introduction of exogenous GrB into target cells

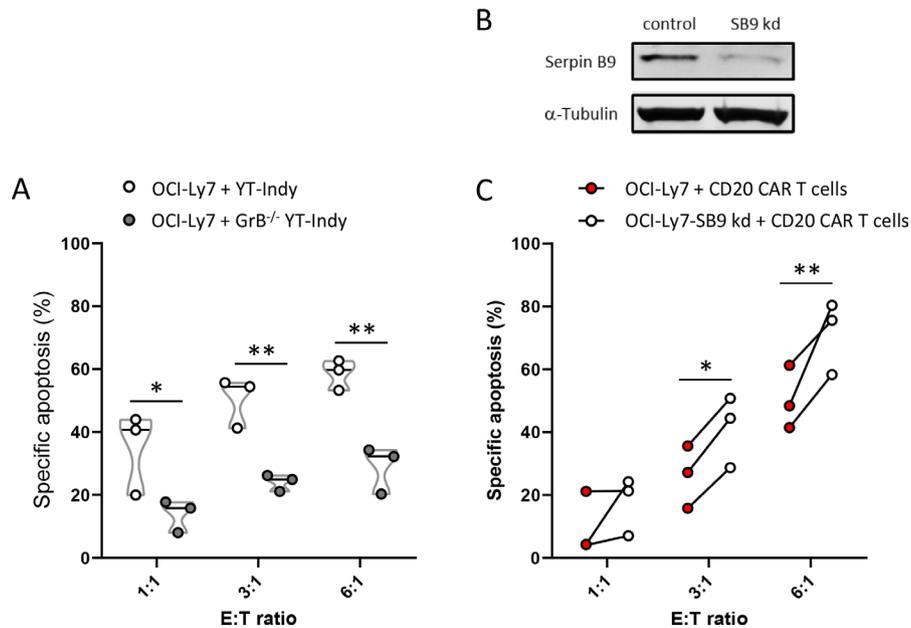
In order to determine the effect of serpin B9 overexpression or knockdown on GrB-mediated killing, the pore-forming streptolysin O (SLO) (#S5265-25KU, Sigma) was used to facilitate entry of exogenous GrB (Enzo) into target cells. SLO was activated with 10 mM dithiothreitol (DTT) for 20 min at room temperature, and subsequently diluted in serum-free DMEM to a final concentration of 4 U/mL. Target cells were incubated with SLO and 200 nM GrB for 30 min at 37°C, after which FBS-containing medium was added to inactivate SLO. After



**Figure 1** Serpin B9 expression constrains sensitivity to GrB-induced cell death in human melanoma cells. (A) Melanoma MeWo cells were cocultured with wild-type NK YT-Indy cells or GrB<sup>-/-</sup> YT-Indy cells at indicated E:T cell ratios. Specific apoptosis of target cells was measured after 24 hours of culture. Data represent two to four independent experiments. (B) Serpin B9 protein expression in mock-transduced (MeWo) and serpin B9-overexpressing (MeWo-SB9) cells, as determined by immunoblotting (top) and flow cytometry (bottom). (C) Specific apoptosis of MeWo or MeWo-SB9 cells after 24 hours of treatment with 200 nM recombinant GrB through SLO-mediated pores in the cell membrane. Data represent three independent experiments. (D) Specific apoptosis of MeWo or MeWo-SB9 cells after 24 hours of coculture with YT-Indy cells at the indicated E:T ratios. Data represent four independent experiments every circle (A,C), and connecting line (D) represents one individual experiment. Two-way analysis of variance with Sidak multiple testing correction (A) and (D) and unpaired Student's t-test (D) were performed to test statistical significance. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. E:T, effector-to-target; GrB, granzyme B; GrB<sup>-/-</sup>, granzyme B-deficient; NK, natural killer; SLO, streptolysin O.



**Figure 2** Serpin B9 is uniformly expressed in malignant B cells and in a subset of solid cancers. (A,B) Violin plots showing *SERPINB9* mRNA transcript expression in malignant cells. Samples are represented by individual data points, where red dots indicate B-cell malignancies. Data are retrieved from R2 databases (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>). (A) Expression in datasets representing primary cells from CLL (n=130), DLBCL (n=223), FL (n=149), classical HL (n=130), MCL (n=122), NSCLC (n=120), AML (n=460), MM (n=542), colon cancer (n=315), melanoma (n=87), breast cancer (n=204) and prostate cancer (n=72) patients. (B) expression in human cell lines representing mixed mature B cell lymphomas (Bcl, n=16), DLBCL (n=18), Burkitt's lymphoma (Burkitt, n=11), HL (n=13), colon cancer (n=61), NSCLC (n=134), AML (n=38), MM (n=29), breast cancer (n=59), melanoma (n=62), prostate cancer (n=8), and glioma (n=65). Data are retrieved from <https://depmap.org/portal/>. (C) Serpin B9 protein expression as measured by western blot in indicated human cancer cell lines and relative to expression of  $\alpha$ -tubulin as control. One-way analysis of variance with Tukey's multiple testing correction was performed in (A,B) to test statistical significance, where  $*p < 0.001$ . Indicated statistical differences are in comparison with each individual non-malignant B-cell group (white dots). AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HL, Hodgkin's lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; NSCLC, non-small-cell lung carcinoma.



**Figure 3** Serpin B9 controls CAR T-cell killing of OCI-Ly7 DLBCL cells. (A) Specific apoptosis of OCI-Ly7 cells after 24 hours of coculture with WT NK YT-Indy cells or GrB<sup>-/-</sup> YT-Indy cells at indicated effector (E) to target (T) cell ratios. data represent three independent experiments. (B) representative western blot staining of serpin B9 protein expression in OCI-Ly7 cells after siRNA-mediated SB9 kd or cells treated with non-targeting siRNA (control). (C) Specific apoptosis of WT or SB9 kd OCI-Ly7 cells, 4 hours after coculture with anti-CD20 CAR expressing primary T cells. Connected data points show three independent experiments with primary T cells isolated from different healthy donors per experiment at indicated E:T cell ratios. Two-way analysis of variance with Sidak post hoc test was performed to test statistical significance. \*P<0.05, \*\*P<0.01. CAR, chimeric antigen receptor; DLBCL, diffuse large B-cell lymphoma; E:T, effector-to-target; GrB<sup>-/-</sup>, granzyme B-deficient; NK, natural killer; SB9 kd, knockdown of serpin B9; WT, wild type.

24 hours, apoptosis staining was performed to measure target cell viability using flow cytometry.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism V.8.3. Unpaired groups were compared with Student's t-test. For comparison of more than two groups, one-way or two-way analysis of variance tests were used. P values of <0.05 were considered statistically significant.

## RESULTS

### Serpin B9 overexpression impairs sensitivity to GrB-induced cell death

Since serpin B9 expression in patients with melanoma was shown to be associated with clinical outcome, we first examined the dependence of melanoma cells on GrB-mediated killing.<sup>9</sup> Human melanoma cell line MeWo was cocultured with NK cell line proficient or deficient in GrB. Coculture of MeWo cells with the GrB-deficient NK cell line completely abrogated apoptosis induction of MeWo cells, indicating that MeWo cell killing was fully dependent on GrB (figure 1A). Next, serpin B9 was stably overexpressed in MeWo cells that lack endogenous serpin B9 expression (figure 1B). Sensitivity of wild type (WT) and serpin B9-overexpressing MeWo cells to GrB was first assessed by introducing recombinant GrB using SLO, a perforin-like bacterial pore-forming exotoxin. In this experimental setting, serpin B9 overexpression rendered

the cell line resistant to GrB-mediated killing (figure 1C). Co-culture of NK cell line YT-Indy with serpin B9-overexpressing MeWo cells revealed significantly reduced apoptosis induction compared with WT MeWo cells (figure 1D). Combined, these experiments show that serpin B9 expression abrogates GrB-mediated killing of melanoma cells.

### B-cell lymphomas uniformly express serpin B9

Serpin B9 is normally expressed in CTL, NK cells, antigen-presenting cells, endothelial and mesothelial tissues, and immune privileged tissues.<sup>9–13</sup> In order to determine *SERPINB9* gene expression in malignant cells, we analyzed publicly available datasets comprising primary patient material (figure 2A) and human cell lines (figure 2B). This revealed that *SERPINB9* was uniformly expressed across different B-cell lymphoma and leukemia subtypes, and average expression was significantly higher compared with other cancer types. In addition, we measured serpin B9 protein expression in panels of human cancer cell lines, again revealing consistent serpin B9 expression in B-cell lymphoma cell lines as expected based on gene expression analysis (figure 2C). These experiments establish that, although incidentally expressed in various cancer types, serpin B9 is expressed in the majority of B-cell lymphomas.

### Serpin B9 restricts DLBCL cell killing by CAR T cells

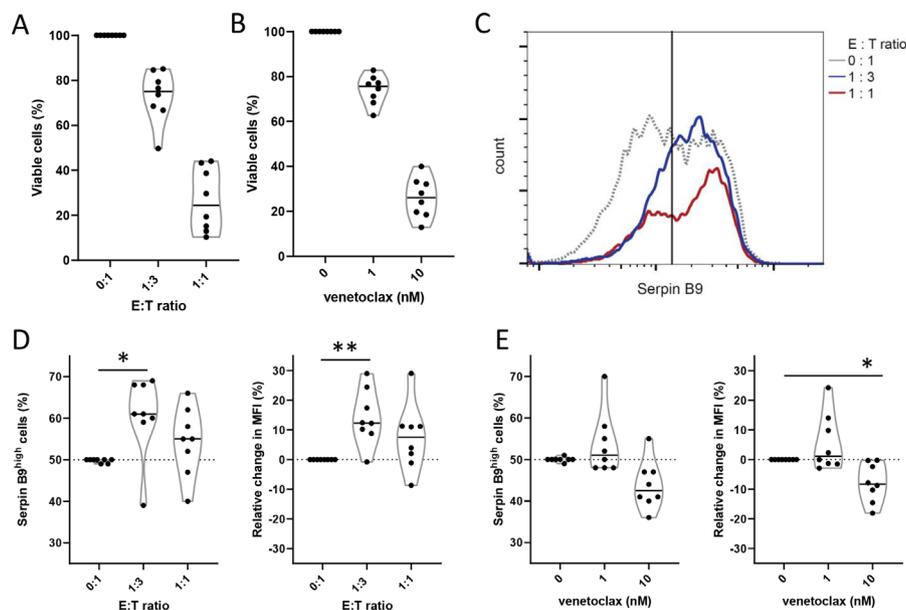
Since serpin B9 was uniformly expressed in DLBCL cells (figure 2A–C), we examined its role in CAR T cell-mediated

cell killing of this B-cell lymphoma subtype. First, DLBCL cell line OCI-Ly7 was examined for dependence on GrB. Specific apoptosis of OCI-Ly7 cells was significantly lower on coculture with GrB-deficient YT-Indy cells compared with coculture with WT YT-Indy cells but was not fully abrogated as observed with MeWo cells (figure 3A). This suggests that, in addition to GrB other cell death pathways, such as death receptor signaling, are effectively engaged in OCI-Ly7 cells in the absence of GrB. Next, we performed siRNA-mediated knockdown of serpin B9 in OCI-Ly7 cells (figure 3B). Like CD19, CD20 is highly expressed on non-Hodgkin's B-cell lymphomas, including DLBCL and CLL, and used as a target for CAR T-cell treatment.<sup>4</sup> Coculture of CD20 CAR T cells and OCI-Ly7 cells with serpin B9 knockdown revealed significantly improved tumor cell killing as compared with unmodified OCI-Ly7 cells (figure 3C). This difference in tumor cell killing using CD20 CAR T cells was comparable when using CD19 CAR T cells (online supplemental figure 1). Although serpin B9 specifically inhibits GrB,<sup>8</sup> it is conceivable that serpin B9 expression in target cells indirectly affects the CAR T-cell phenotype and effector functions during coculture, thereby modifying the T-cell response. However, we did not detect any change in expression of checkpoint molecules PD-1, TIM-3 or LAG-3, or in the expression of effector cytokines IFN- $\gamma$  or TNF- $\alpha$  by CD20 CAR T cells during coculture, suggesting a direct effect of serpin B9 on T-cell killing

(online supplemental figure 2). Combined, these data highlight serpin B9 as a resistance factor for CAR T-cell killing of DLBCL cells.

### Sensitivity of primary CLL cells to CAR T-cell killing is mediated by serpin B9

In addition to DLBCL cells, serpin B9 was uniformly expressed in primary CLL cells (figure 2A). Therefore, we examined a potential contribution of serpin B9 to CAR T cell-mediated killing of CLL cells. CLL cells isolated from the blood of patients were cocultured with CD20 CAR T cells for 24 (online supplemental figure 3A) or 48 (figure 4A and online supplemental figure 3B) hours, and serpin B9 protein expression was measured in these cells by flow cytometry. As control, the same patient samples were incubated with BCL-2-inhibitor venetoclax that induces GrB-independent cell death in CLL cells (figure 4B). CLL cells that survived CAR T cell coculture expressed significantly more serpin B9 compared with CLL cells cultured without CAR T cells (figure 4C,D). The selection for serpin B9-high CLL cells during coculture could only be observed in conditions where significant CAR T cell-mediated killing took place, making it independent of other culture conditions (online supplemental figure 1A,B). In line with these findings, CLL cells that died during CAR T-cell coculture showed a reduced expression of serpin B9, indicating that serpin B9-low CLL cells were more susceptible to CAR T cell-mediated killing (online supplemental figure 4A). Selection of serpin B9-high expressing CLL cells



**Figure 4** Serpin B9-high primary CLL cells are selected on coculture with CD20 CAR T cells. (A,B) Viable primary CLL cells were measured after 48 hours of coculture with CD20 CAR T cells at indicated E:T cell ratios (A) or after treatment with indicated concentration of BCL-2-inhibitor venetoclax (B). (C) Serpin B9 protein expression was measured by intracellular flow cytometry in CLL cells after 48 hours of coculture (E:T of 1:3 for the blue line and 1:1 for the red line). The black line demarcates the median in serpin B9 expression of control CLL cells (dotted line). (D) Percentage of primary CLL cells above the median of control cells (C) (left panel) or the change in MFI (right panel), after 48 hours of coculture with CD20 CAR T cells. (E) Percentage of primary CLL cells above the median of control cells (C) (left panel) or the change in MFI (right panel), after 48 hours of treatment with venetoclax. Every dot represents one patient sample. Two-way analysis of variance with Sidak post hoc tests were performed to test statistical significance. \* $P < 0.05$ , \*\* $P < 0.01$ . CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukemia; E:T, effector-to-target; MFI, mean fluorescence intensity.

was not observed on culture with venetoclax (figure 4E and online supplemental figure 4B). Combined, these data reveal a selection for high serpin B9 expression and indicate that these CLL cells are consequently more resistant to CD20 CAR T-cell killing.

## DISCUSSION

In this study, we show that overexpression of serpin B9 can be a resistance mechanism of cancer cells toward GrB-mediated killing by CAR T cells. Serpin B9 expression can be found across hematological and solid cancers, with relatively high and ubiquitous expression in B-cell malignancies that include DLBCL and CLL (figure 2). By siRNA-mediated knockdown of serpin B9 in DLBCL cell line OCI-Ly7, and subsequent coculture with anti-CD20 CAR T cells, we show that serpin B9 expression promotes resistance to killing by CAR T cells (figure 3). In addition, we found that primary CLL cells with high serpin B9 expression were positively selected on CD20 CAR T-cell coculture, thereby confirming our finding that serpin B9 controls CAR T cell-mediated tumor cell killing (figure 4).

In immune cells, serpin B9 acts in a cytoprotective manner by preventing proteolysis of cytotoxic cells by their endogenously produced GrB.<sup>14</sup> Additionally, serpin B9 expression has been reported in a wide range of other healthy cell types, including endothelial and mesothelial cells as well as cells in immune-privileged tissues such as the testis.<sup>9,13</sup> In cases where serpin B9 and GrB expression do not overlap, the likely function of serpin B9 expression is to protect bystander cells from damage caused by cytotoxic cells.<sup>15</sup> Thus, serpin B9 expression can be induced in many different cell types and may therefore be a widespread mechanism of immune evasion.

Cytotoxic T cells, including CAR T cells, have the potential to act as serial killers. It has been reported that each cytotoxic T cell is capable of eliminating multiple target cells using in vitro and in vivo models.<sup>16,17</sup> However, such efficient serial killing has not been observed in patients with cancer who received CAR T cells.<sup>18</sup> In fact, recent literature revealed that ‘additive cytotoxicity’ is required for cytotoxic T cells to kill target cells, where the number of hits delivered in a set time span determines cancer cell death or survival after recovery.<sup>19</sup> Whether a target cell is killed could theoretically depend on the amount of GrB released by CAR T cells or by the presence of resistance mechanisms, such as serpin B9 expression in cancer cells.

Serpin B9 expression has previously been reported to protect cancer cells from T cell-mediated or NK cell-mediated killing<sup>20–22</sup> and to predict clinical outcome.<sup>9,23,24</sup> In clinical cohorts of immune checkpoint blockade, serpin B9 gene expression was found to be higher in non-responders than in responders, and high serpin B9 expression correlated with worse overall survival.<sup>10</sup> These results suggest that serpin B9 is a resistance factor to effective cancer treatment and that its inhibition could improve clinical responses to CAR T-cell treatment.

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**Contributors** TK, AS, ZS, and VP designed the research; TK, AS, AM, SG, SL EvD, JM, and TK performed the experiments; TK, AS, SG, and VP analyzed the results; TK, AS, SG, JK, MCM, EE, NB, ZS, and VP contributed to interpretation and discussion; TK, AS, and VP wrote and revised the manuscript; JK, MCM, EE, NB, ZS, and VP supervised the study. All authors reviewed and approved the final manuscript.

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**Competing interests** VP received royalty payments related to venetoclax. VP, ZS, and TK are inventors on a patent for improving cytotoxicity of gene engineered T and NK cells. MCM received honoraria from Medscape, Jansen Cilag, and BMS. ZS and JK are inventors on different patents for  $\gamma\delta$  T-cell receptor sequences, recognition mechanisms, and isolation strategies. JK is scientific cofounder and shareholder of Gadeta. The remaining authors declare no competing interests.

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**Ethics approval** All primary chronic lymphocytic leukemia samples were obtained after written informed consent, and protocols were approved by the local ethics committee of the Academic Medical Center, Amsterdam.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data availability statement** The datasets used in the current study are available from the corresponding author on reasonable request.

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