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Original Article

Broad spectrum integrin inhibitor GLPG-0187 bypasses immune evasion in colorectal cancer by TGF-β signaling mediated downregulation of PD-L1

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Abstract: Integrin receptors have long posed as a potentially attractive target for disrupting cancer hallmarks. Promising preliminary findings with integrin inhibition as an adjuvant to chemotherapy have not translated to clinical success. However, the effect of integrin inhibition on tumor-immune cell interactions remains largely unexplored. Further investigation could shed light on a connection between integrin signaling and immune checkpoint expression, opening the path for using integrin inhibitors to sensitize otherwise resistant tumors to immunotherapy. Fluorescently labeled wild-type HCT-116 colorectal cancer cells and TALL-104 T-cells were co-cultured and treated with GLPG-0187, a small molecule integrin inhibitor, at various doses. This assay revealed dose dependent cancer cell killing, indicating that integrin inhibition may be sensitizing cancer cells to immune cells. The hypothesized mechanism involves TGF-β-mediated PD-L1 upregulation in cancer cells. To investigate this mechanism, both WT and p53-/- HCT-116 cells were pre-treated with GLPG-0187 and subsequently with latent-TGF-β. Western blot analysis demonstrated that the addition of latent-TGF-β increased the expression of PD-L1 in cancer cells. Additionally, a low dose of integrin inhibitor rescued these effects, returning PD-L1 expression back to control levels. This indicates that the immunostimulatory effects of integrin inhibition may be due to downregulation of immune checkpoint PD-L1 on cancer cells. It must be noted that the higher dose of the drug did not reduce PD-L1 expression. This could potentially be due to off-target effects conflicting with the proposed pathway; however, these findings are still under active investigation. Ongoing proteomic experiments will include a larger range of both drug and latent-TGF-β doses. Probing for additional downstream markers of TGF-β and up-stream markers of PD-L1 will help to further elucidate this mechanism. Further co-culture experiments will also include anti-PD-L1 and anti-PD-1 therapy to investigate the viability of integrin inhibition as an adjuvant to immune checkpoint blockade.

Keywords: TGF-β, GLPG-0187, PD-L1, T-cell killing, cancer, immunotherapy, mismatch repair-deficiency, immune checkpoint blockade

Introduction

Colorectal cancer (CRC) is the second most deadly, and third most common cancer globally, accounting for 10% of new cases and 9% of all cancer deaths [1]. Although the rates of incidence are declining, the number of CRC cases are projected to double by 2035 [2]. Additionally, the high lethality, especially of metastatic disease, is expected to continue for years to come due to cancer drug resistance and high rates of recurrence [3]. Screening of average-risk patients remains the most effective preventative measure against CRC considering that the disease is characterized by a slow progression from adenoma to carcinoma, potentially giving

ample time for pre-cancerous polyps to be detected and removed [4-6]. When the disease progresses to carcinoma, however, rapid development of chemotherapy resistance is common [7]. Therefore, malignant CRC treatment regimens consist of a broad array of chemotherapeutics including platin-based DNA cross-linkers (oxaliplatin), topoisomerase I inhibitors (irinotecan), and antimetabolites such as 5-FU (5-fluorouracyl) [8]. In addition, there are a number of targeted therapies that are approved for CRC. These include anti-angiogenic therapies such as anti-VEGF (Avastin). Due to the high occurrence of the *BRAF-V600E* mutation, as of 2019, anti-BRAF therapy has been approved and is employed in combination with anti-EGFR as a synthetic lethality therapy [9, 10]. These approaches are combined with ionizing radiation and surgical resection [8].

Even with all of these interventions, fewer than 20% of patients with metastatic disease survive past 5 years post diagnosis, highlighting the dire need for new and effective treatment options [11]. Immunotherapy shows much promise for treatment of CRC. Immune checkpoint blockade (ICB) therapies in particular have proven to be very effective in a certain cohort of cases [12]. Immune checkpoints are transmembrane proteins that suppress the adaptive immune system. Checkpoints and their ligands are found on both immune and cancer cells. Cancers develop checkpoint expression as an immune evasion strategy as it allows them to bind a complementing checkpoint on an T cell, thereby deactivating the cytotoxic function of that immune cell [13, 14]. ICB works by employing antibodies that preemptively block these sites, whether it be on the tumor or on the T cell, so that the T cells retain their cytotoxicity and ultimately destroy the cancer cell.

Unfortunately, these therapies only work in tumors that have high levels of neoantigens. This usually occurs when tumors have mutations in their mismatch repair mechanism, becoming mismatch repair deficient (MMRD), which leads to microsatellite instability [15]. This leads to an accumulation of genetic damage and a high degree of genetic foreignness which can be readily detected by the immune system. Approximately 15% of CRC cases are MMRD, making them candidates for ICB [16]. However, even among this subset of cases there are varying levels of response as some patients only experience short term benefit and become refractory, creating the need for novel strategies of sensitizing MMRD tumors to ICB [17, 18].

The exploration of integrin inhibitors for treating cancer has been a mostly fruitless endeavor. Trials of integrins inhibitors, concomitant to chemotherapy, have largely failed [19, 20]. However, the use of these drugs in an immunotherapy context remains largely unexplored. Besides tumor neoantigen signature and immune checkpoint expression, a further factor that modulates the immunogenicity of a tumor is the influence of immunosuppressive cytokines in the tumor microenvironment. The cytokine TGF-β is of particular interest as it has been shown to affect cancer immune evasion [21, 22]. However, due to the multitude of, partially still elusive, physiological processes that TGF-β is involved in, targeting this signaling molecule is difficult without incurring detrimental off-target effects. A more targeted approach than systemic TGF-β inhibition may offer a way of disrupting TGF-β signaling selectively in tumor cells with minimal side effects [14]. TGF-β is present exogenously in its latent form. Latent TGF-β then binds to one of the αv family integrin receptors and is thereby converted into the active form [23]. The active TGF-β can then interact with the TGF-β receptors which leads to induction of SMAD transcription factors within the cell. The induction of TGF-β signaling in cancer cells has been documented to reduce their immune profile, representing a resistance mechanism to cytotoxic immune cells [14].

The small molecule GLPG-0187 is a broad-spectrum integrin inhibitor that contains an RGD-motif which binds the αvβ1, αvβ3, αvβ5, αvβ6, and α5β1 integrin receptors [24]. The αvβ6 integrin receptor specifically is predominantly found on tumor cells, making it an excellent target for local TGF-β inhibition [25]. By utilizing GLPG-0187 to block the αvβ6 integrin receptors on cancer cells, the activation of TGF-β is prevented locally, thereby reducing TGF-β signaling and immune evasion of the cancer cell. Selective inhibition of integrins therefore may be a more targeted way of disrupting tumor TGF-β signaling. The present study investigates the hypothesis that integrin

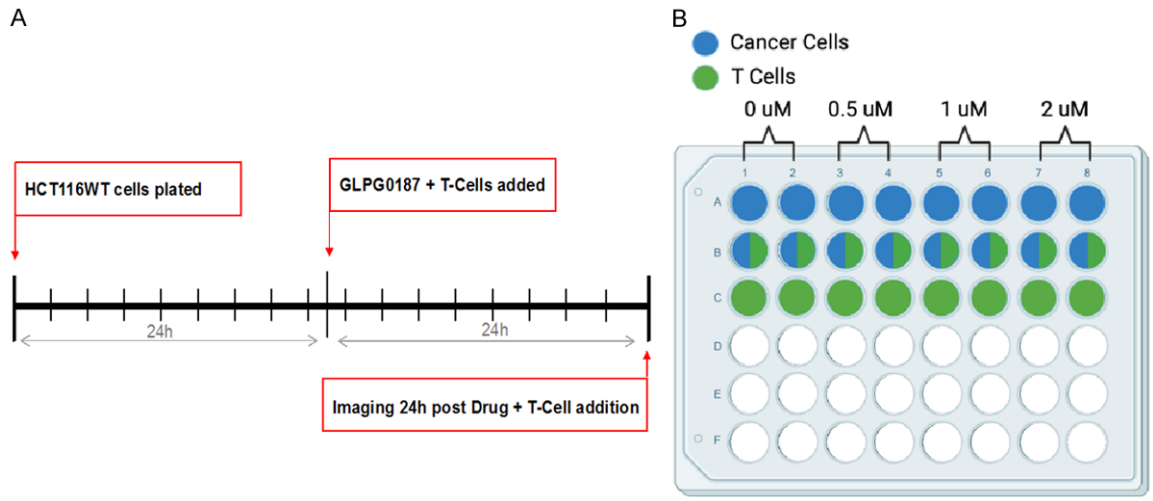

Figure 1. Co-culture experimental timeline and Plate Setup. A. Experimental timeline for co-cultures. B. Setup of 48-well plate used in co-culture experiments. Row A: HCT116WT cells, Row B: HCT116WT cells + TALL-104 cells, Row C: TALL-104 cells. Each cell line was plated at a density of 10,000 cells per well. Doses of GLPG-0187 are in duplicate and are indicated above.

inhibition in micro-satellite unstable CRC cells results in a reduction of their TGF-β mediated immune evasion. Our results indicate that integrin inhibition using GLPG-0187 reduces PD-L1 expression by CRC cells and increases their susceptibility to T cell killing.

Materials and methods

Cell culture

Both HCT116WT and HCT116 p53-/- were cultured using McCoy's 5A medium with L-glutamine (Cytiva, cat # SH30200.FS) with 10% FBS and 1% penicillin/streptomycin added. TALL-104 cells were cultured with RPMI-1640 medium with L-glutamine (Cytiva, cat # SH30027.LS) with 20% FBS and 1% penicillin/streptomycin added. The cell lines were kept in a 5% CO_2 incubator at 37 degrees Celsius. Cell line authentication and mycoplasma infection testing were performed.

Cancer and immune cell co-culture

HCT116WT colorectal cancer cells were fluorescently dyed and plated into the first two rows of a cell culture coated 48-well plate at a density of 10,000 cells per well. After the cells had been allowed to adhere overnight, fluorescently labelled TALL-104 cells were plated into the second and third (as of yet empty) rows, also at 10,000 cells per well. This gave a row with only HCT116WT cells, one with HCT116WT and TALL-104, and one with only TALL-104 cells. At the same time as immune cell addition, all three rows were also treated with varying doses of GLPG-0187 (MedKoo, cat # 205842) in duplicates (**Figure 1A**, **1B**). These cohorts consist of vehicle control (DMSO), 0.5 μM, 1 μM, and 2 μM. Additionally, the cell death marker ethidium homodimer was added to all wells. The cells were imaged 24 h later at 10X magnification with a fluorescent microscope (**Figure 2**). Cell counts of cancer and immune cells were quantified using ImageJ software (**Figure 3**).

Cell adhesion assay

Both HCT116WT and HCT116 p53-/- were plated in a 12-well plate at a density of 100,000 cells per well. The cells were allowed to adhere for 24 hours in the incubator before being treated either with vehicle control (DMSO), 0.125 μM or 2.0 μM of GLPG-0187. Additionally, ethidium homodimer, a cell death marker, was added to all wells. 24 hours post treatment the cells were imaged using phase microscopy with the cell death signature being captured fluorescently. The fluorescent channel was digitally overlayed over the phase microscopy images (**Figure 4**).

Flow cytometry

HCT116WT were plated at a density of 50,000 cells per well in a 12-well plate and left in the

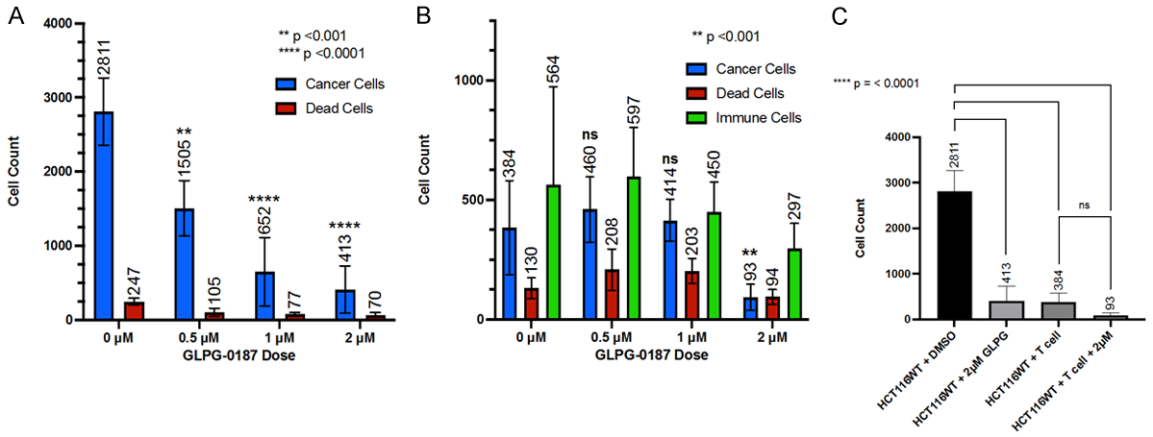

Figure 2. HCT116WT and TALL-104 co-culture. A. Cell counts of HCT116WT cells after 24 h treatment of vehicle (DMSO), 0.5 µM, 1 µM, 2 µM GLPG-0187. Blue indicates cancer cells and red indicates number of cancer cells that exhibited ethidium homodimer cell death marker. One-way ANOVA of cancer cell counts (blue) shown between control and treatment groups. B. Cell counts of co-culture experiment with green indicating TALL-104 cells. 1:1 cancer to immune cell ratio. One-way ANOVA of cancer cell counts is included. C. HCT116WT count after no treatment (control), 2 µM GLPG, TALL-104, and 2 µM + TALL-104. One-way ANOVA between various treatment arms is.

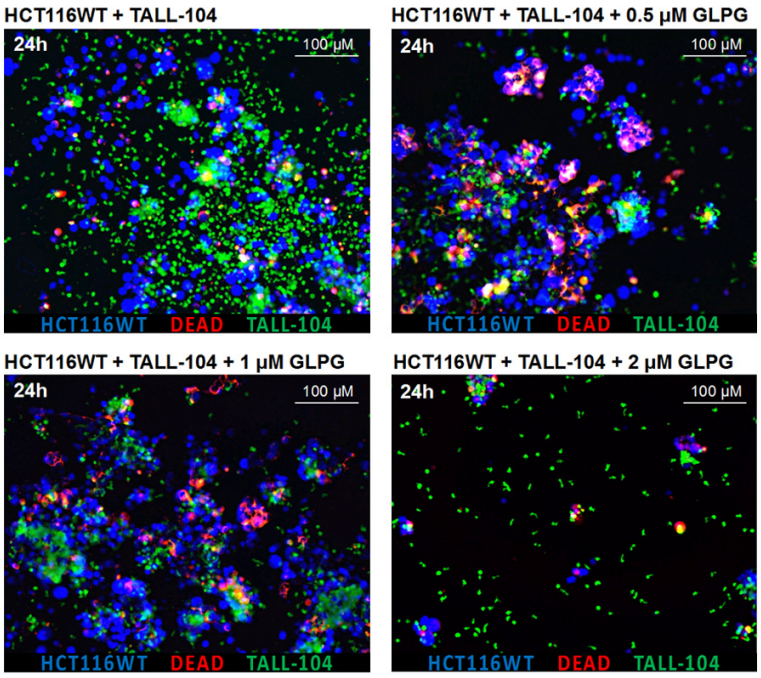

Figure 3. HCT116WT and TALL-104 co-culture. Corresponding images to co-culture from **Figure 2C**.

incubator for 24 hours to adhere. The cells were dyed with Green CMFDA (Cayman, cat # 19583) and washed with PBS. Subsequently, the cells were treated with either vehicle (DMSO) or 0.125 µM GLPG-0187 and ± 100,000 TALL-104 cells pre well. The cohorts, plated in triplicate, were as follows: HCT116WT + DMSO, HCT116WT + GLPG-0187, HCT116WT + DMSO + TALL-104, HCT116WT + GLPG-0187 + TALL-104 (**Figure 5**). After another 24 hours, the cells were trypsinized and harvested. After a PBS wash and centrifugation, all conditions were stained with Sytox Blue nucleic acid stain (Thermo Fisher, cat # S11348) as a cell death marker. After a final washing step, the samples were evaluated using flow cytometry to quantify the number of dead cancer cells (**Figure 6**). The CMFDA stain of the cancer cells and the nucleic acid stain allow for making the distinction between cancer and immune cells and whether they are alive or dead. In addition to the experimental cohorts, single wells of both undyed TALL-104 and HCT-116WT as well as Sytox Blue fluorescing HCT116WT cells and CMFDA dyed HCT116WT were plated and utilized to establish gating for the flow cytometry experiment.

Western blots

HCT116WT and HCT116 p53-/- were plated in a tissue culture coated 6-well plate at a density of 500,000 cells per well in 2 mL of media. The cells adhered over a 24 h period and were then

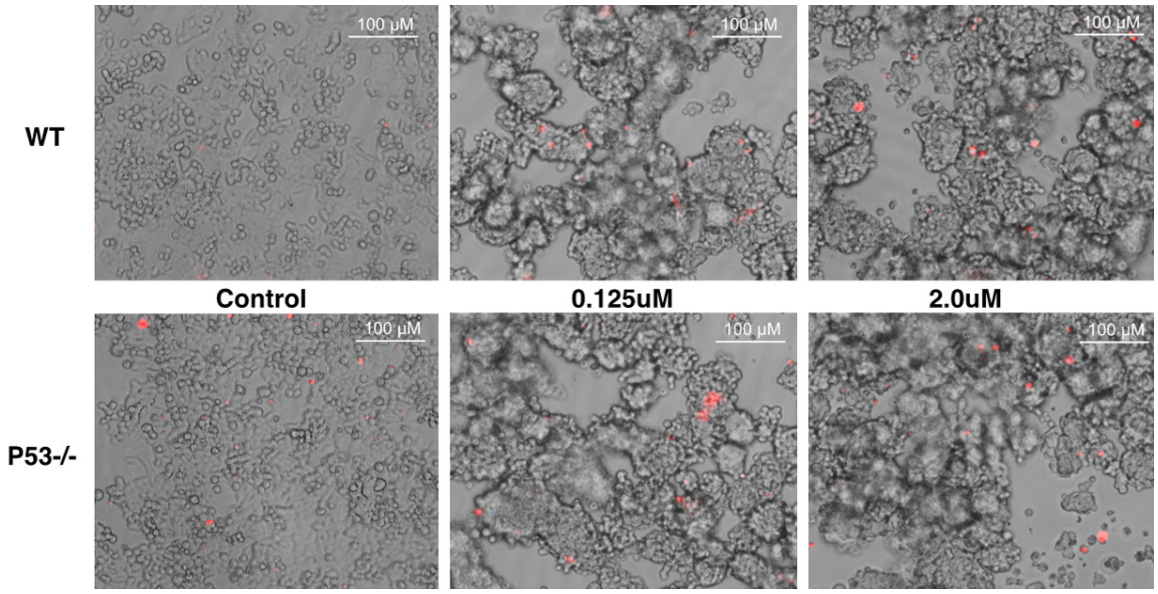

Figure 4. HCT116WT and HCT116 p53-/- adhesion assay. 100,000 cells plated per well and treated after 24 hours with Control (DMSO), 0.125 µM, or 2.0 µM GLPG-0187. Representative images shown were taken 24 hours post treatment. The overlayed red fluorescent channel corresponds to ethidium homodimer cell death marker.

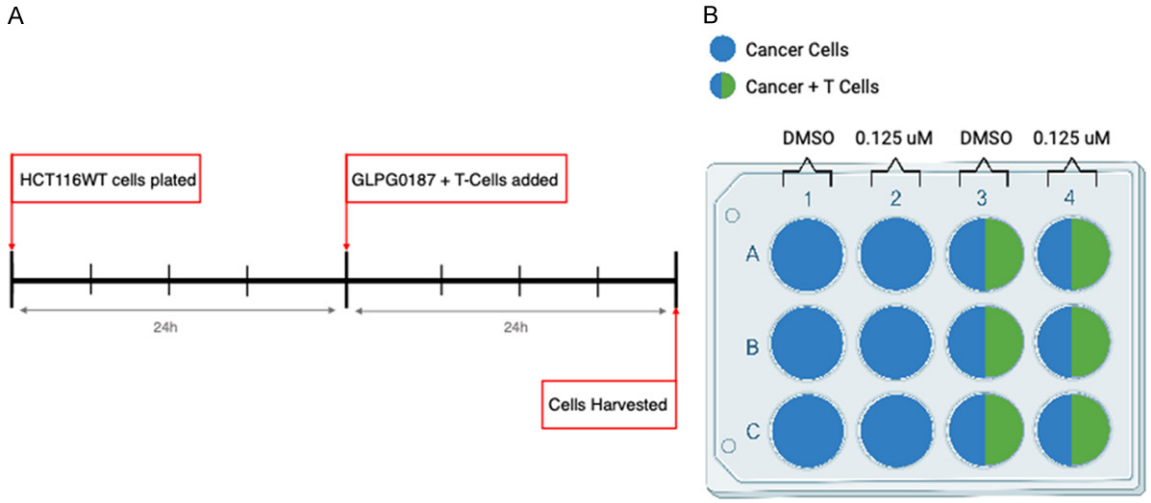

Figure 5. Flow cytometry co-culture timeline and plate setup. A. Experimental timeline for co-cultures. B. Setup of 12-well plate used in co-culture experiments. Column 1: HCT116WT cells + DMSO, Column 2: HCT116WT cells + 0.125 µM GLPG-0187, Column 3: HCT116WT cells + DMSO + TALL-104, Column 4: HCT116WT cells + 0.125 µM GLPG-0187 + TALL-104. Each cell line was plated at a density of 100,000 cells per well. Conditions were plated in triplicate.

pretreated with vehicle control, 0.125 µM, or 2 µM GLPG-0187. After another 24 h period the cells were treated with 10 ng/ml of Latent-TGF-β (R&D Systems, cat # 299-LT-005/CF) or vehicle control (DMSO) (**Figure 7A**). After yet another 24 h, the cells were harvested, and the lysates were analyzed via SDS-Page Western blot (**Figure 7B**). The samples were probed using PD-L1 antibodies (Cell Signaling Technologies, cat # 13684S).

Results

GLPG-0187 increases killing of HCT116WT CRC cells by TALL-104 T cells

The co-cultures of HCT116WT and TALL-104 were evaluated under fluorescence microscopy at varying doses of GLPG-0187. The goal of this assay was to determine if there was a GLPG-0187 mediated increase in immune killing of

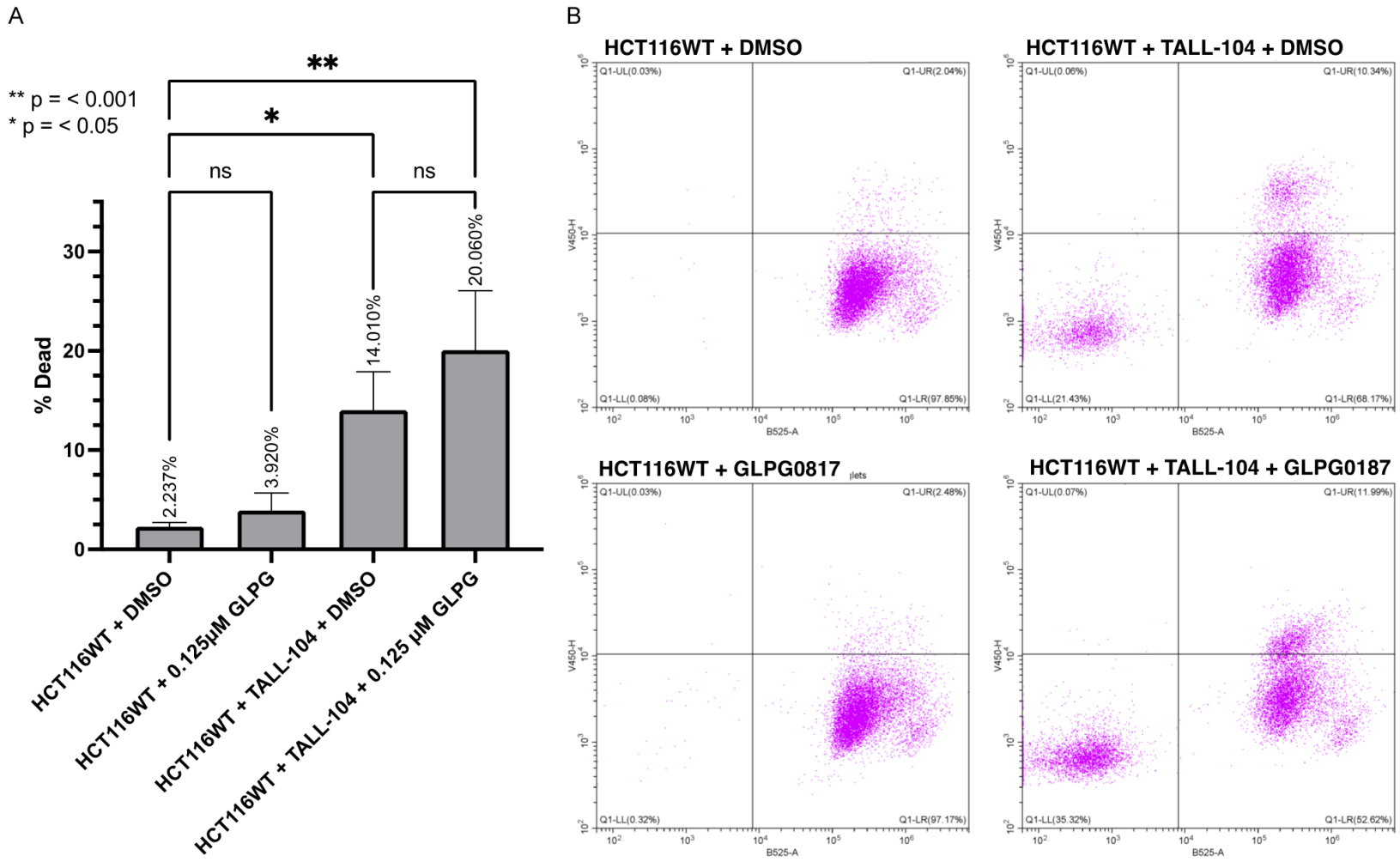

Figure 6. Flow cytometry co-culture results. A. Graph showing percentage of cancer cells that are dead (Sytox Blue). One-way ANOVA is shown between various treatment arms. B. Scatter plots showing events recorded during flow cytometry. X-axis corresponds to the CMFDA dye emission, the marker for cancer cells. Y-axis corresponds to Sytox Blue emission, cell death marker. Using these two axis the various cell populations were characterized.

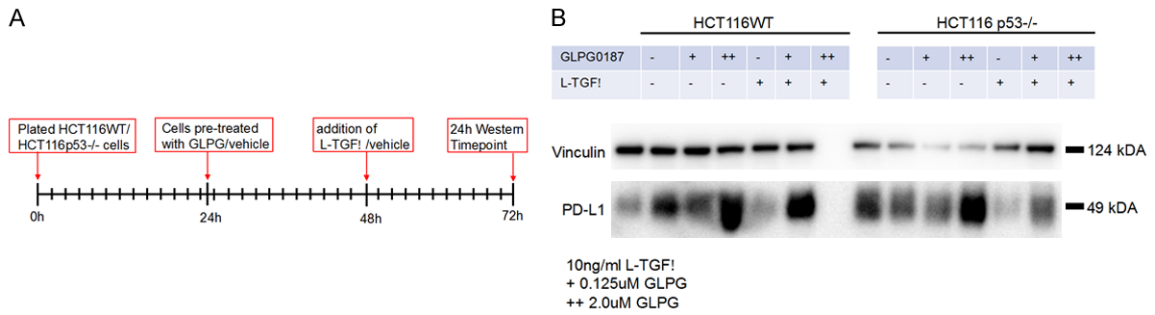

Figure 7. Modulation of PD-L1 expression with GLPG-0187 and Latent TGF-β. A. Experimental timeline for Western Blot experiments of HCT116WT and HCT116 p53-/- cell lines. B. Western Blots probing for vinculin (loading control) and PD-L1 in both cell lines. Treatments are shown in table above immunoblots.

HCT116WT cells by TALL-104 cells. Although GLPG-0187 alone showed a reduction in the number of microscopically captured cancer cells, the cytotoxic effect of the drug on HCT116WT cells is minimal when measured by the cell death marker (**Figure 2A**). However, in the co-culture experiments, GLPG-0187 in the presence of T cells led to a dose dependent increase in cancer cell killing (**Figure 2B**). At highest concentration of GLPG-0187 (2 μM) there was toxicity to TALL-104 cells. Although the co-culture condition did not show synergy (more than the sum of the parts), the results indicate that the maximal dose of GLPG-0187 in combination with T cells is causing considerably more cancer cell death than drug or T cells alone (**Figure 2C**). However, it must be considered that the cell adhesion experiments described in the following section elucidate a limitation of this model. Representative Images of co-culture conditions show drastic dose dependent HCT116WT death (**Figure 3**).

Integrin inhibition leads to inhibition of cell adhesion in HCT116WT cancer cells

Loss of in vitro cell adhesion in the presence of RGD-integrin antagonists has been demonstrated in the literature [26]. The adhesion assay revealed that GLPG-0187, also an RGD based integrin inhibitor, also induces this phenomenon in both HCT116WT and HCT116 p53-/- cells. Separation of cancer cells from the wells they were plated in was observed to an equal degree between a dose of 0.125 μM and 2.0 μM GLPG-0187, with the control cells remaining adhered (**Figure 4**). Additionally, there is only limited death of cancer cells uniformly across both treated and untreated conditions. This indicates that GLPG-0187 alone has no appreciable direct cytotoxic on the cancer cell lines tested, at 24 hours. The loss of adherence in cancer cells treated with GLPG-0187 alluded to the fact that a limitation of the co-culture microscopy assay is that not all of the non-adherent cells can be captured in one focal plane. Therefore, a flow cytometry-based assay was devised to validate the results from the previous experiments.

Flow cytometry reveals GLPG-0187 modulated TALL-104 killing of HCT116WT cells

As in the previous microscopy experiments, HCT116WT and TALL-104 were co-cultured with either GLPG-0187 or vehicle control (**Figure 5**). Flow cytometry was utilized to alleviate the issue of not being able to capture all of the cells in one microscopic focal plane due to GLPG-0187 mediated loss of cell adhesion. The experiment revealed that even a low dose of GLPG-0187 was able to induce increased T cell killing of HCT116WT, corroborating the results from the initial co-culture experiments (**Figure 6A**). Additionally, the flow cytometry data showed that GLPG-0187 had no significant cytotoxic effect on cancer cells in the absence of immune cells at 0.125 μM (**Figure 6B**). It was also demonstrated that, at the dose tested, the drug has no toxic effect on TALL-104 cells.

Exogenous latent-TGF-β upregulates PD-L1 in HCT116 wild type and p53-/- cells

Western blots of both HCT116WT and HCT116 p53-/- investigated the expression levels of the immune checkpoint PD-L1 in response to latent-TGF-β and GLPG-0187. Both WT and

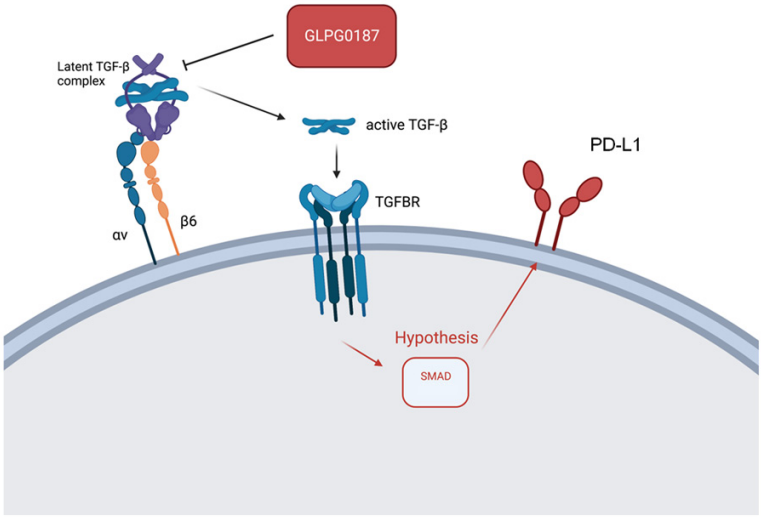

Figure 8. Proposed mechanism for TGF-β mediated PD-L1 expression via GLPG-0187 integrin inhibition.

p53-/- cells that were treated with latent-TGF-β 48 h after plating (**Figure 7A**). These cells were then harvested 24 h later and probed for PD-L1. This revealed that the addition of 10 ng/ml of latent-TGF-β into the media led to a substantial increase in PD-L1 expression in both cell lines (**Figure 7B**).

Modulation of PD-L1 expression of in CRC cells with GLPG-0187

The other cohorts of the western blot experiments were pretreated with varying doses of GLPG-0187 24 h prior to being treated with latent-TGF-β (**Figure 7A**). After probing for PD-L1, it was revealed that the increases in PD-L1 via exogenous latent-TGF-β could be rescued and brought back down to control levels with a low dose (0.125 μM) of GLPG-0187 (**Figure 7B**). In the HCT116 p53-/- cells the level of PD-L1 was even reduced below control conditions. However, the results also indicate that GLPG-0187 may also increase PD-L1 in certain conditions. In the wild type cell line, a large dose (2 μM) of GLPG-0187 did not reduce PD-L1 levels as the small dose did. In fact, in this cell line, both the small and large doses of GLPG-0187 even caused a slight increase in PD-L1 when latent-TGF-β was not present. This PD-L1 upregulating effect was not as pronounced in the p53 deficient cell line, however, even in this case the large dose was still less effective at reducing PD-L1 expression than the low dose was.

Discussion

The co-culture experiments indicate that GLPG-0187 is sensitizing CRC cells to immune killing. However, GLPG-0187 also shows cytotoxicity towards T cells at high doses (2 μM), thereby compromising the T cells ability to kill cancer cells (**Figure 2C**). The western blot experiments demonstrated that very low amounts of GLPG-0187 are capable of downregulating PD-L1 in the tested cell lines (**Figure 7B**). Therefore, going forward it may be possible to achieve GLPG-0187 and T cell synergy by optimizing dosing and timing of drug delivery to maintain the immuno-stimulatory effect of the drug while staying under a threshold of reasonable T cell toxicity. Additionally, the flow cytometry co-culture assay that was devised allows for accurate cell viability quantification with non-adherent cells.

αvβ6 Integrin inhibition modulated sensitization to T cell killing has been demonstrated previously in CRC cells [14]. The present western blot experiments demonstrate down regulation of immune checkpoint PD-L1 on CRC cells through GLPG-0187, an inhibitor of αvβ6 (**Figure 7B**). Therefore, the present data, combined with the existing literature, indicate that αvβ6 integrin inhibition induced downregulation of PD-L1 is a hypothetical mechanistic explanation for the anti-tumor immune activity seen in the presence of integrin inhibitors. The proposed mechanism involves blocking of the αvβ6 integrin by an inhibitor (GLPG-0187), thereby preventing activation of TGF-β. This prevents active TGF-β from binding to the TGF-β receptor and prevents induction of intracellular SMAD transcription factors (**Figure 8**). SMAD transcription has been associated with increased PD-L1 expression [27].

The paradoxical increase of PD-L1 seen at high doses of GLPG-0187 could potentially be due to off-target effects conflicting with the proposed pathway as GLPG-0187 is also an inhibitor of avb1, avb3, avb5, and a5b1 integrin

receptors which may be implicated in counteracting pathways.

These findings are still under active investigation with proteomic experiments that include a larger range of both drug and latent-TGF-β doses. Probing for additional downstream markers of TGF-β and up-stream markers of PD-L1 will help to further elucidate the mechanism. Future co-culture experiments will include anti-PD-L1 and anti-PD-1 therapies, to investigate the viability of integrin inhibition as an adjuvant to immune checkpoint blockade, potentially devising novel therapeutic combinations. Additionally, we aim to expand our studies to CRC cell lines that are mismatch repair proficient in order to assess whether integrin inhibition can sensitize these otherwise resistant cell lines to immune checkpoint blockade.

Disclosure of conflict of interest

None.

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