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Abstract

CD26/dipeptidyl peptidase 4 (DPP4) is a transmembrane protein which is expressed by various malignant cells. We found that the expression of CD26/DPP4 was significantly higher in lung adenocarcinoma samples in our own patient cohort compared to normal lung tissue. We therefore hypothesize that the inhibition of CD26/DPP4 can potentially suppress lung cancer growth.

The CD26/DPP4 inhibitor vildagliptin was employed on Lewis Lung Carcinoma (LLC) cell line and a human lung adenocarcinoma (H460) cell line. Two weeks after subcutaneous injection of tumor cells into

C57BL/6 and CD1/nude mice, the size of LLC and H460 tumors was significantly reduced by vildagliptin. Immunohistochemically, the number of macrophages ($F4/80^{+}$) and NK cells ($NKp46^{+}$) was significantly increased in vildagliptin-treated tumor samples. Mechanistically, we found *in vitro* that lung cancer cell lines expressed increased levels of surfactant protein upon vildagliptin treatment thereby promoting the pro-inflammatory activity of macrophages. By the depletion of macrophages with clodronate and by using NK cell deficient ($IL\text{-}15^{-/-}$) mice, tumors reversed to the size of controls, suggesting that indeed macrophages and NK cells were responsible for the observed tumor-suppressing effect upon vildagliptin treatment. FACS analysis showed tumor-infiltrating NK cells to express TRAIL which induced the intracellular stress marker γH2AX. Accordingly, we found upregulated γH2AX in vildagliptin-treated tumors and TRAIL-treated cell lines. Moreover, the effect of vildagliptin-mediated enhanced NK cell cytotoxicity could be reversed by antagonizing the TRAIL receptor.

Our data provide evidence that the CD26/DPP4-inhibitor vildagliptin reduces lung cancer growth. We could demonstrate that this effect is exerted by surfactant-activated macrophages and NK cells that act against the tumor via a TRAIL-mediated cytotoxicity.

Key words: CD26/DPP4; lung cancer; surfactant protein; macrophage; NK cell; TRAIL.

1. Introduction

Lung cancer is the primary cause of death among all cancer-related deceases in both genders (1). Despite improved surgical techniques, radiation, chemo- and targeted therapy, the overall prognosis remains poor (2,3).

The microenvironment of a tumor plays a crucial role in tumor development, progression and the development of metastasis (4). By secreting chemokines, cytokines and growth factors, malignant cells interact with immune cells and the tumor microenvironment. Macrophages in particular can be found as the most abundant immune cells within the stroma of the tumor. These can be distinguished by their expression profile of surface markers and their production of cytokines transforming them into either pro-inflammatory or anti-inflammatory macrophages (5). However, this polarization is highly plastic and macrophages can easily convert into other phenotypes by changes in the microenvironment (6). It has been recently shown that the overall survival of patients suffering from lung adenocarcinoma was significantly correlated with the number of macrophages promoting antigen presentation on lung cancer cells within tumors (7). Also, there is evidence that macrophages recruit and activate NK cells, another key immune cell in the context of tumor defense (8). NK cells in turn are able to induce cell death towards target cells either by secreting perforin and granzyme or by the expression of tumor necrosis-related apoptosis inducing ligand (TRAIL) (9). The cytotoxicity of NK cell is mediated by the activation or suppression of activating and inhibiting receptors and depends on specific ligands, e.g. major histocompatibility class I (MHC-I) (10).

CD26/DPP4 is an ubiquitously expressed transmembrane exopeptidase on the cell surface of many hematopoietic cells but also on organs (11). Various CD26/DPP4-inhibitors, including vildagliptin, are in routine clinical use since years for the treatment of type II diabetes without showing serious side effects. During the last ten years, scientific interest focused on CD26/DPP4 describing its involvement in the field of tumor immunology (12). CD26/DPP4 is well recognized as a protein playing a significant role in tumor biology, e.g. in the pathogenesis and development of metastases in malignancies (13-17) as well as in the prognosis of cancer patients (18-21).

We previously showed in a murine model that vildagliptin decreased the growth of lung metastases from colorectal cancer by inhibiting epithelial-to-mesenchymal transition and autophagy resulting in increased apoptosis and an cell-cycle arrest (22). Extending our research on the tumor-suppressing function of CD26/DPP4-inhibitors and in the light of a high expression of CD26/DPP4 in patients having lung adenocarcinoma, we here hypothesize that CD26/DPP4 inhibition by vildagliptin can reduce the growth of primary lung cancer in mice.

2. Materials and Methods

2.1 Animal care

Male mice were used for all experiments. Breeders of CD26/DPP4$^{-/-}$ and IL-15$^{-/-}$ mouse strains (C57BL/6 background) were obtained from the European Mouse Mutant Archive (Orleans, France) and Taconic (Germany) respectively. Wild type mice (C57BL/6) were provided by Charles River (Germany). Nude mice (CD1-nude, Charles River, Germany) were purchased and used for human adenocarcinoma cell line injection. Animals were fed with a standard laboratory diet with water and food *ad libitum* and kept under constant environmental conditions in Biological Central Labor, University Hospital Zurich. All experimental procedures were approved by the Swiss animal welfare authorities and performed in accordance with the institutional animal care guidelines (License number: ZH83-2014).

2.2 Activity-based proteomic assay of CD26/DPP4 in lung adenocarcinoma patient samples

We have previously provided data on the activity of CD26/DPP4 from patients bearing lung adenocarcinoma including the methodology how to obtain these activities (23). In brief, we analyzed thirty-eight pairs of fresh-frozen human lung adenocarcinoma biopsies and corresponding normal lung tissues from patients who underwent surgical resection at the University Hospital Zurich between 2003 and 2006 (TNM stage: IA to IV). Proteins were labelled with the fluorophosphates derivative 6-N-biotinylaminohexyl isopropyl phosphorofluoridate (FP-1) serving as an activity probe. Labelled proteins enriched with streptavidin-beads were digested with trypsin and analyzed on an FT-ICR mass spectrometer (LTQ-FTMS, Thermo Finnigan). Tandem mass spectrometry data were searched in the recently updated human database (UniProtKB/Swiss-Prot) with Mascot 2.2 search engine. For relative quantification, MS data were analyzed with Progenesis LC-MS version 2.5 (Nonlinear Dynamics) with the three most intense peptides matching to a protein by The median ratio of the top three intense peptide ions matching to enzymes in malignant versus normal tissues was used. The specific activity of CD26/DPP4 was presented as the relative activity of mean activity of normal tissue.

2.3 Tumor cell lines

The mouse cell lines Lewis Lung Carcinoma (LLC) and Raw264.7 (macrophage) were purchased from American Type Culture Collection (Manassas, USA). The human NCI-60 cell line (NCI-H460) was obtained from Charles River (Boston, USA) under material transfer agreement with the National Cancer Institute (Bethesda, USA). Upon arrival, the cell lines were stored at early passages (<3) in liquid nitrogen and used for all experiments within 3 months. All cell lines were cultivated in DMEM containing 10 % FBS and penicillin/streptomycin within a 5 % CO_2 chamber.

2.4 Subcutaneous tumor model

Cell lines (LLC or H460) (1 x 10^6 cells/mouse) were subcutaneously injected in the upper back of mice with serum free DMEM. Vildagliptin was provided in drinking water (0.2 mg/ml) from the third day after the cell line injection until the end of experiment. Following exsanguination, tumors were isolated from skin and subsequently weighed (**Suppl. 1A**).

2.5 In vitro experiments

Both, mouse and human cell lines were maintained in 10% FBS, penicillin/streptomycin-containing DMEM. Cell lines were seeded into 10 cm plates to reach 70% confluence. Two days after cell seeding, media were exchanged with serum-free DMEM. Twelve hours later, cells were exposed according to the experimental conditions including vildagliptin (10 µM-1.3 mM), TRAIL (100 ng/ml), and surfactant (Curosurf (0.16 mg-1.28 mg), Chiesi Farmaceutici, Parma, Italy). These experiments were triplicated.

2.6 Protein expression analysis

Samples were homogenized in the lysis buffer pH 7.4 containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), phosphatase inhibitor cocktail 3, 50 mM Tris, 150 mM NaCl, 5 mM EDTA, and 0.5 % NP-40 (Sigma-Aldrich, Buchs, Switzerland). The protein concentration was determined using the Bradford protein assay (BioRad, Hercules, USA). Reducing SDS-PAGE was performed and samples were blotted onto a PVDF-membrane. Antibodies against surfactant protein (SP)-A (Millipore, CA, USA), SP-B, SP-C (Abcam, Cambridge, UK), H2AX, phospho-H2AX (Cell signaling) were used at a dilution of 1:2000. Secondary antibody binding and detection was performed according to standard protocols with the ECL detection reagent (BioRad). The expressions of TNF-α, and CD26/DPP4 were assessed by ELISA (R&D systems, Abingdon, UK), according to the manufacturer's instruction.

2.7 Flow cytometry

Single-cell suspensions from mouse tissues including tumor and spleen were prepared as previously described (24). Briefly, specimens were cut and incubated in collagenase II (2 mg/ml: PAN Biotech, Aidenbach, Germany) solution (10% fetal bovine serum (FBS), and 50 μg/ml gentamicin in RPMI1640) for 30 min at 37°C. The collagenase reaction was stopped by adding 1 ml of 1 M EDTA and cells were then filtered through a 70-μm cell strainer. Fixable Viability Dye (Thermofisher Sicentific, MA, USA) for dead cell stain and antibodies for CD3, CD11b, CD14, CD27, CD45, B220, NKp46, NKG2A, NKG2D, Ly49A, Ly49C/I, MHC-I (H2KD/DD), MHC-I (Qa1b), FasL, TRAIL (BD Pharmingen, Allschwil, Switzerland), MHC-I (ULBP1) (R&D systems, Abingdon, UK) were used and subjected to flow cytometry using a BD CANTO flow cytometer.

2.8 Histology and Immunohistochemistry

Samples stained with antibodies against Ki67, p-Histon3, CD4, CD8, F4/80, NKp46, and γH2AX were formalin-fixed, and paraffin-embedded. Two investigators conducted evaluation of each stain in a blinded and independent manner.

2.9 Macrophage and NK cell control

In order to confirm the mechanism of tumor suppression by vildagliptin treatment via macrophages and NK cells, we tested the anti-tumor effect of vildagliptin in our model of subcutaneous lung cancer using LLC cell line under the condition of macrophage ablation with clodronate liposome and by NK cell deficiency in IL-15 gene knockout mice (**Suppl. 1B and C**). NK cell agonizing antibody (anti-mouse NKp46 goat IgG: 1μg/mouse) and control (normal goat IgG: 1μg/mouse) (R&D systems, Abingdon, UK) were administered to LLC s.c. tumor-bearing mice by i.v. injection. Mice were treated with the antibodies at 6th and 10th day after LLC s.c. injection.

2.10 NK cell cytotoxicity test

Primary NK cells from mouse (C57BL/6) spleen were collected by a NK cell isolation kit (Miltenyi biotec, Bergisch Gladbach, Germany) according to the provider's instruction. Following exsanguination, the isolated spleen was mashed on a 30 μm filter. NK cells from spleens were negatively selected by the kit. Heparinized blood was taken from the inferior vena cava to receive peripheral blood mononuclear cells (PBMC) as controls. After lysis of red blood cells, PBMCs were isolated, and counted cells were cultured on the U-bottom non-adherent plate (FALCON, NY, USA) within RPMI1640 media containing 10% heated FBS, 50 μg/ml gentamicin, 50 μM beta-mercaptoethanol (Sigma-Aldrich, Buchs, Switzerland), 20 ng/ml IL-2, and 10 ng/ml IL-15 (PEPROTECH, NJ, USA). One day after seeding, FACS analysis confirmed the enrichment of NK cells with the specific marker CD49b (NK1.1) (**Suppl. 10A**). The cytotoxicity of NK cells (effector) against LLC (target) was assessed by testing different ratios of target to effector cells in 24 hours (**Suppl. 10B, C**). A Fixable Viability Dye (Thermofisher Sicentific, MA, USA) detected viable LLC cells. In order to block the cytotoxicity of NK cells, an antagonizing antibody against the TRAIL receptor (DR5) (1 μg/ml) (R&D systems, Abingdon, UK) was employed.

2.11 Statistical analysis

Data were presented as means ± SD. Groups were compared with the Student t-test for unpaired samples using Prism 5.0 (GraphPad Software, San Diego, CA, USA). A two-sided p-value <0.05 was considered to be statistically significant.

3. Results

3.1 CD26/DPP4 activity is high in human lung and mouse cancer

In order to evaluate the enzymatic activity of CD26/DPP4 in human lung cancer, we compared lung cancer tissue samples with normal lung tissue of the same patients (N=38). We found four times higher CD26/DPP4 levels in the lung cancer tissue samples compared to normal lung tissues (**Fig. 1A**). However, in this population, a wide spread could be observed in the tumor vs normal tissue. We further correlated these proteomic-based CD26/DPP4 activity data to the clinical stages in order to differentiate the activity according to the progression of disease. Interestingly, this evaluation revealed a significantly higher activity in patients with stage IA compared to patients in stage IB or IV (**Fig. 1B**). Using the fluorogenic substrate Gly-Pro-4-Me-β-NA, CD26/DPP4 activity was found along with the expression in lung adenocarcinoma cell lines from human (H460) and mouse (LLC) (**Suppl. 2A and B**).

3.2 Vildagliptin treatment reduces orthotopic tumor growth in vivo

Based on the clinical data of adenocarcinoma patients and the *in vitro* activity of CD26/DPP4 in lung cancer cell lines, we tested the CD26/DPP4-inhibitor vildagliptin *in vivo* by using models of lung cancer induced by subcutaneous (s.c.) injection of LLC (syngeneic tumor) and H460 (xenogeneic tumor) in C57BL/6 and CD1-nude mouse strains, respectively. In both models, the measured wet weights of tumors were significantly reduced by vildagliptin treatment. (**Fig. 2A and B**). This anti-tumor effect of vildagliptin could be observed for a longer experimental time period (**Fig. 2C**). Subcutaneously growing LLC induced tumor volume was significantly suppressed by vildagliptin treatment. Moreover, by using the orthotopic lung cancer model developed by intravenous (i.v.) injection of LLC-GFP cell line, we confirmed the effects by vildagliptin (**Suppl. 3**).

3.3 Stimulation of surfactant protein expression and macrophage activation in lung cancer by vildagliptin treatment in vitro and in vivo

To decipher a possible anti-tumor mechanism of the CD26/DPP4 inhibitor vildagliptin, we incubated LLC and H460 cell lines with or without vildagliptin (10 µM-1.3 mM) *in vitro* and measured the viability and pro-inflammatory surfactant protein (SP) expression. SP was shown to have activating immunologic properties against lung cancer (25). In the tested concentrations, vildagliptin showed no direct cytotoxicity on cell lines for 3 days (**Suppl. 4A**). After 3 hours of vildagliptin (10 and 20 µM) treatment, we harvested cells for the analysis of SP-A, B, and C by Western blotting. In both cell lines, we consistently found an increased expression of the SP-A, B and C upon vildagliptin treatment (**Fig. 3A, Suppl. 4C and D, Suppl. 9**). As surfactant protein overexpression is known to activate macrophages, we tested different concentrations of surfactant (0.16, 0.32, 0.64, 1.28 mg/ml) on the mouse macrophage cell line Raw264.7 without cytotoxicity upon the given doses of surfactant (**Suppl. 4B**). Upon surfactant treatment the secretion of pro-inflammatory cytokine TNF-α in the supernatant from the macrophages cell line was significantly enhanced in a dose dependent manner (**Fig. 3B**). In consistence with the *in vitro* experiment, the number (**Fig. 3C**) and the specific cytokine expression profile (**Suppl. 5A**) of tumor-infiltrating macrophages ($F4/80^{+}$) in LLC induced s.c. tumors indicated that vildagliptin enhances the pro-inflammatory status of the tumor environment. Although the gene expression of pro-inflammatory macrophage-derived cytokines (TNF-α, IL-12b, and IL-15) were significantly enhanced by vildagliptin treatment, anti-inflammatory cytokines (IL-10, Arginase, and TGF-β1) did not show a statistically significant difference between groups (**Suppl. 5A**). Interestingly, IL-12b and IL-15 were significantly elevated in the vildagliptin treated tumor *in vivo*. These genes translate into cytokines that are expressed in macrophages for the stimulation of NK cells for activation and proliferation.

3.4 Increased NK cell infiltration in LLC s.c. tumors by vildagliptin treatment

Next, we analyzed tumor-infiltrating NK cells as vildagliptin treated tumors expressed NK cell stimulating genes including IL-12b and IL-15 (**Suppl. 5A**). Beside the activation of macrophages by vildagliptin,

we found that NK cells changed within the tumor that was established by s.c. injection of the LLC cell line. Immunohistochemistry (IHC) showed a significant elevation of tumor-infiltrating NK cells (26) in vildagliptin treated tumors (**Fig. 4A**). The specific gene expression of NK cells, NKp46, NK1.1, IFN-γ, perforin, FasL and TRAIL were significantly upregulated within the tumor by vildagliptin treatment (**Suppl. 5B**), suggesting that the inhibition of CD26/DPP4 recruits activated and cytotoxic NK cells into the tumor. T- and B-cells did not show any difference (**Suppl. 6A- F**). Tumor-infiltrating NK cells expressed TRAIL and FasL (**Fig. 4B)**, the FACS gating strategy is represented in **Suppl. 7**). Intracellular IFN-γ intensity was differentiated among CD45+CD3-NKp46+ NK cells showing that CD11b+CD27+ NK cells are expressing higher levels of IFN-γ than others (**Fig. 4C**). Interestingly, vildagliptin increased the proportion of high IFN-γ expressing CD11b+CD27+ NK cells when compared to controls. This indicates that especially macrophages and NK cells are crucial in the pathway of tumor growth reduction, and an inhibition of CD26/DPP4 can induce changes in the microenvironment of the tumor.

3.5 NK-cell mediated TRAIL-induction delays tumor cell proliferation by inducing DNA damage

When further analyzing pathways of NK cell cytotoxicity, we found a higher number of phosphorylated H2AX (γH2AX) positive cells in LLC s.c. tumors when treated with vildagliptin (**Fig. 5A, Suppl. 6G**). TRAIL is able to induce phosphorylation of H2AX via the death receptors a histone with stabilizing functions of the DNA within cancer cells. γH2AX is a marker for DNA damage (27) and is required in the p53/p21- pathway of cell cycle arrest (28,29). In an *in vitro* experiment using the LLC cell line, we found an enhanced phosphorylation of H2AX one hour after treatment with TRAIL (100 ng/ml) (**Fig. 5B**). While phospho-Histone 3 marked cells undergoing mitosis, Ki-67 shows proliferating cells. By using the phosphorylated Histone 3/Ki-67 ratio, the amount of cells that are active in cell cycle progression can be calculated. We found a lower phospho-Histone 3/Ki-67 ratio of LLC s.c. induced tumor *in vivo* when treated with vildagliptin compared to the control groups which means that a higher amount of cells undergo a cell cycle arrest when treated with vildagliptin (**Fig. 5C**). This shows that NK cell-derived TRAIL induces DNA damage in cancer cells, arrests cell cycle, and thus reduces tumor growth.

3.6 Macrophage ablation and NK cell depletion reverse the size of LLC tumors

In order to prove the tumor-infiltrating immune cell dependent anti-tumor effect of vildagliptin, we tested vildagliptin on mouse models of either macrophage ablation or NK cell deletion. To ablate macrophages in mice, we used clodronate-liposome (20 mg/kg) in the LLC s.c. tumor bearing mice. Liposome was intraperitoneally administered on the 7^{th}, 10^{th}, and 12^{th} day after s.c. injection of LLC. We found a reversed tumor mass in macrophage-ablated and vildagliptin treated group compared to the group of mice that were only vildagliptin-treated (**Fig. 6A**). To investigate the effect of NK cells in CD26/DPP4 inhibition, we performed the same s.c. injection of tumor cells on IL-$15^{-/-}$ knockout mice. Consistently with the macrophage ablation experiment, the deletion of NK cells resulted in reversed tumor masses in vildagliptin-treated mice when compared to mice that were only treated by vildagliptin (**Fig. 6B**).

3.7 Tumor specific inhibition of CD26/DPP4 by vildagliptin in $CD26^{-/-}$ mice

To confirm that indeed vildagliptin exerts the observed anti-tumor effect via selective inhibition of CD26/DPP4, we employed a $CD26^{-/-}$ mouse strain and applied the LLC tumor model developed by s.c. injection. In this condition, we could focus on the mechanism of CD26/DPP4 inhibition via CD26-harbored LLC tumor in the absence of host CD26/DPP4. $CD26^{-/-}$ mice bore the same size of tumor compared to wild type control (**Fig. 6C**). The anti-tumor effect of vildagliptin remained in $CD26^{-/-}$ mice against LLC tumor, which decreased tumor size (**Fig. 6D**) and increased NK cell number (**Fig. 6E**). To confirm the anti-tumor effect of NK cell in wild type and $CD26^{-/-}$ mice, an NK cell agonizing antibody was applied to the LLC s.c. developed tumor. The treatment by the NK cell agonizing antibody significantly decreased the tumor size in both, wild type and $CD26^{-/-}$ mice (**Fig. 6F and G**).

3.8 Primary NK cell co-culture with LLC cells

In order to investigate a direct effect from NK cells on lung cancer cells with and without CD26/DPP4 inhibitor vildagliptin, we purified primary NK cells from the spleen of C57BL/6 mice. LLC cells were co-cultured with these NK cells (1:2 ratio) for 24 hours and were assessed by the expression levels of activation and inhibition receptors and ligands including the viability of NK cells. The treatment of vildagliptin showed a significant reduction of the viability of LLC cells (**Fig. 6H**). The antagonizing antibody against the TRAIL receptor (DR5) reversed this effect (**Fig. 6H**). At the same time, we found that the number of NK cells that were co-cultured with LLC was significantly higher under vildagliptin treatment (**Supple. 10D**). Although the expression of inhibitory receptors of NK cells including Ly49A, Ly49C/I, and NKG2A remained unchanged, the expression of inhibitory ligands on those co-cultured LLC cells increased significantly (**Suppl. 11A and B**). Moreover, the treatment with vildagliptin significantly reduced the inhibitory ligands Qa1b and programmed cell death ligand 1 (PD-L1) on co-cultured LLC cells. In contrast, the NKG2D ligand ULBP1 was unchanged among groups. However, the expression of NKG2D, the activation receptor of NK cell (10,30,31) was significantly decreased in LLC co-cultured NK cells (**Suppl. 11C**).

Discussion

We here provide the first evidence that the inhibition of CD26/DPP4 by vildagliptin reduces lung cancer growth. We show that the treatment with vildagliptin (I) increases surfactant protein production in lung cancer cells which (II) drives macrophages into a pro-inflammatory and anti-tumorigenic polarization that in turn (III) enhances the cytotoxicity of NK cells within the tumor. Furthermore, we show that the expression of TRAIL from NK cells leads to an intra-cellular damage resulting in phosphorylation of H2AX within the lung cancer cells thus decreasing tumor cell proliferation. These data provide mechanistic data for the anti-tumor effect of the CD26/DPP4-inhibitor vildagliptin thereby displaying this molecule to be a potential therapeutic strategy against lung cancer.

We found increased levels of surfactant protein within lung cancer cell lines LLC and H460 after vildagliptin treatment. Alveolar Type II cells are both the source of surfactant but these cells are also the origin of lung adenocarcinoma (32). These cells could thereby contribute to the regulation of tumor cell signaling in a surfactant-dependent manner (33). Although an exact mechanism is not yet proven, it was shown before that surfactant and surfactant protein (SP) secretion were enhanced by CD26/DPP4 inhibition in healthy rats (34). Moreover, an overexpression of SP-A gene in human adenocarcinoma cell lines decreased tumor growth in an experimental model (25).

Macrophage derived chemokines and growth factors play a crucial role in the tumor microenvironment (35). It is nowadays well recognized that tumor macrophages usually show a M2 polarization towards tissue remodeling and repair thus supporting the progression of tumor growth (5,25,36-38). We found an increase in the number of macrophages but also in the expression of macrophage-specific pro-inflammatory cytokines in tumors of vildagliptin-treated mice. As M1 macrophages are characterized by the production of pro-inflammatory cytokines, we conclude from our data that the treatment with vildagliptin drives macrophages towards a pro-inflammatory phenotype. Macrophage depletion experiments additionally supported our data obtained. In those macrophage-depleted mice, the weight of the vildagliptin treated tumor was significantly reversed compared to macrophage-competent mice suggesting that vildagliptin suppresses lung cancer growth via the activity of macrophages. Consistent with these data,

Jiang et al. found that the deletion of the SP-A gene in patients with early NSCLC is a predictor of poor outcome (39). Another study shows that the outcome in patients with lung adenocarcinoma is worsened when SP-A expression is proven to be low (40). Though these studies give a hint towards the relevance of surfactant protein in lung cancer and the modulation of the tumor milieu. , we were unable to find the exact mechanism why SP is elevated upon vildagliptin treatment. Two possible explanations are possible for this phenomenon: vildagliptin either elevated SP directly through an unknown mechanism, or vildagliptin preserved SP levels via a reduced SP degradation. For example, Stephan et al. found that significantly increased mRNA levels of SP in ovalbumin-induced allergy-like inflamed lung tissue of F334 rats after treatment with DPP4-inhibitors compared to a non-treated group, suggesting that vildagliptin may be involved in a different activation pattern of alveolar epithelial cells (41).

We found macrophages to be enhanced in their activity upon increasing SP levels. In order to show that macrophages were able to attack tumor cells, we found the inflammatory marker myeloperoxidase (MPO) in the tumor and circulation to be increased We therefore conclude that these macrophages do not have anti-tumorigenic abilities by themselves, but rather act as immune-modulatory cells by stimulating NK cells. We hypothesized that those activated macrophages stimulated NK cells which we could prove by confirming significantly elevated NK cell activating receptor within the treated tumor that are treated with vildagliptin. In support of these results, for example Mitsuhashi et al. found an increased number of NK cells and an upregulated expression of Granzyme B and Perforin 1 in a SP-A-expressing tumor (25). Authors proposed that tumor-derived SP-A recruits and activates macrophages which in turn activate NK cells against the tumor (25). In line with Mitsuhashis results, we found significantly higher tumor weights in vildagliptin-treated IL-15$^{-/-}$ -mice.

NK cells are able to kill aberrant cells in multiple ways, either by releasing perforin and granzyme by exocytosis, but also through the cell surface expression of the apoptosis-inducing proteins FasL or TRAIL (42). Both pathways induce apoptosis in the aberrant cell, either caspase-dependent or -independent. We found a significant elevated phosphorylation of H2AX in LLC cell lines *in vitro*, suggesting that TRAIL leads to intracellular stress, supposedly DNA damage, which in turn leads to the phosphorylation of H2AX. These results are supported by data from Solier, showing that TRAIL induced DNA damage

followed by phosphorylation of H2AX (43,44). As an early reaction to DNA damage, especially DNA double strand breaks, H2AX becomes phosphorylated.γH2AX than interacts with other DNA damage response proteins, to than finally to start apoptosis (45). It has been described that γH2AX is induced double strand breaks by TRAIL in HCT116 cells *in vitro (43)*. All this evidence supports our findings that TRAIL exposition leads to the elevated γH2AX, resulting in the suppression of proliferation of cancer cells by cell cycle arrest.

The therapeutic potential of NK cells as natural killer cells against tumors has been widely shown through their spontaneous cytotoxicity, cytokine release, and antibody dependent cellular cytotoxicity (46,47). For example, the systemic administration of NK cell activating cytokine IL-2, were shown to be effective in the treatment of melanoma and in advanced renal cell carcinomas (48). Transfusions of *ex vivo* expanded autologous NK cells showed reasonably good clinical responses in patients with metastatic malignancies (49,50). A recent phase I study found a reduction of minimal residual disease in acute myeloblastic leukemia patients after transfer of umbilical cord blood $CD34^{+}$ hematopoietic stem and progenitor-derived NK cells, suggesting an application of engineered NK cell immunotherapy as an adjuvant or consolidating therapy following an intensive chemotherapy in the leukemia patients (51). Also activation receptors of NK cells have been shown to be a therapeutic targets against multiple cancers (52-54) and the inhibitory or activating involvement of NK cell receptors and ligands were shown to be important in terms of their cytotoxicity in lung cancer (10,55). For example, Shi et al. attributed the NK cell activating receptor NKG2D and the inhibitory receptor Ly49C/I a critical role in the LLC cell line tumor growth (55).

Similarly, we provide in our study experimental evidence that NK cells indeed killed tumor cells upon treatment with vildagliptin. When co-culturing NK cells with LLC tumor cells, we showed that NK cell activating receptors decreased and inhibitory ligands of LLC cells increased. However, inhibitory ligands such as Qa1b and PD-L1 were downregulated upon vildagliptin treatment, suggesting a higher NK cell viability and cytotoxicity. Of note, the increase of PD-L1 supports the notion that a possible combination therapy of a CD26-inhibitor with a checkpoint inhibitor might achieve a synergistic effect in the treatment of lung cancer.

We are able to conclude from these data that vildagliptin decreases tumor growth by enhancing surfactant protein within the tumor. Surfactant activates macrophages and NK cell cytotoxicity which in turn initiate cell cycle arrest by TRAIL-induced phosphorylation of H2AX.

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Figure legends

Figure 1. The activity of CD26/DPP4 in patient samples with lung adenocarcinoma. The measured activity is presented by the relative activity to the mean value of normal. The activity in tumors was significantly higher compared to normal tissue (A). Although no activity difference of CD26/DPP4 was found among normal tissues, the tumor-originated activity was significant higher in stage IA compared to stage IB and IV (B). n=38, *p<0.05, **p<0.01, ***p<0.001

Figure 2. Vildagliptin decreases the growth of lung cancer *in vivo*. Lung cancer models developed by subcutaneous injections of syngeneic (LLC) or xenogeneic (H460) lung cancer cell lines. Vildagliptin treatment significantly suppressed lung cancer growth in *in vivo* models. The kinetic growth of LLC cell line induced subcutaneous tumors was measured by caliper for 27 days (C). Vildagliptin significantly decreased tumor growth. LLC Control (n=11); LLC Vildagliptin (n=12); H460 Control (n=7); H460 Vildagliptin (n=9), *p<0.05, **p<0.01

Figure 3. The CD26/DPP4 inhibitor vildagliptin enhanced surfactant protein expression and macrophage activation. Surfactant protein A, B, and C were detected by Western blotting of LLC and H460 cell lysates with or without vildagliptin treatment *in vitro*. Treatment of vildagliptin increased the expressions of surfactant proteins in both cell lines (A). Quantifications of the bands are summarized in **Suppl.4 C and D** (LLC and H460 respectively). The pro-inflammatory cytokine TNF-α secretion in the culture media by surfactant treatment on macrophage cell line (Raw264.7) was significantly enhanced in a dose -dependent manner (B). The inflammatory activity of macrophages ($F4/80^{+}$) was significantly increased by vildagliptin treatment in the LLC s.c. developed tumor *in vivo* (C). **p<0.01, ***p<0.001

Figure 4. Increased tumor-infiltrating NK cells by CD26/DPP4 inhibitor treatment. Immunohistochemistry of NK cell marker (NKp46) showed significantly higher numbers of NK cells in vildagliptin treated tumors when compared to controls in the LLC s.c. induced tumor (A). Tumor infiltrated NK cells were

characterized by FACS analysis bein FasL- and TRAIL -positive (B) and CD11b+CD27+NKp46+IFNγ+ in the vildagliptin treatment group (C). *p<0.05, **p<0.01

Figure 5. Vildagliptin suppresses the proliferation of lung cancer via TRAIL-induced phosphorylation of H2AX. IHC of γH2AX in the s.c. LLC tumor showed that vildagliptin treatment increased the expression of γH2AX (**A**). The intensity of stainings was graded from 1 to 4. Western blotting of γH2AX showed an intracellular stress induced by TRAIL treatment (100 ng/ml) on LLC cell line *in vitro* (B). The expression of γH2AX was presented as relative expression of total amount of H2AX. β-actin served as a loading control. Proliferation rate (pH3/Ki-67) was reduced by vildagliptin treatment *in vivo* (C). *p<0.05, **p<0.01

Figure 6. Loss-of-function experiments showing the anti-tumor effect of vildagliptin in the absence of macrophages and NK cells. Macrophage ablating clodronate-liposome administration (100 µl intraperitoneal injections at 7, 10, and 12 days after LLC inoculation) significantly reversed the tumor size in vildagliptin-treated mice (A). The tumor size of NK cell deleted mice (IL-15$^{-/-}$) also significantly reversed the size of tumors (B). LLC s.c. developed tumors in CD26$^{-/-}$ mice (C) showed the same size as controls and was decreased by vildagliptin treatment (D). NK cells were also increased in CD26$^{-/-}$ mice by vildagliptin (E). Treatment with an NK cell agonistic antibodysignificantly decreased LLC s.c. induced tumor size in wild type (F) and CD26$^{-/-}$ mice (G) when compared to the controls. When testing for cytotoxicity, purified NK cell were co-cultured with LLC cells and revealed a significantly reduced viablility of LLC cells in the vildagliptin treated group. Furthermore, the TRAIL receptor (DR5) antagonizing antibody (1µg/ml) neutralized the vildagliptin-induced cytotoxicity of NK cells (H). Clodronate (n=7); Clodronate+Vildagliptin (n=10); Vildagliptin (n=11); IL-15$^{-/-}$ (n=7); IL-15$^{-/-}$+Vildagliptin (n=8); wt+Vildagliptin (n=7); NK agonist antibody (n=5); control antibody (n=4), NK cell cytotoxicity test (n=4), antibody (Ab) *p<0.05, **p<0.01

Supplement 1. Experimental models. Schematic display of cell line injection induced tumor models (A). Knocking out of NK cells was achieved in IL-$15^{-/-}$ mouse strain. Immunohistochemistry of NKp46 confirms the genetic deletion of IL-15 resulted in NK cell knockout (B). Spleens of wild type and $IL15^{-/-}$ mouse stain were used for the test. Clodronate liposome ablated macrophages in the mouse liver shown by F4/80 immunohistochemistry (C). x100

Supplement 2. Activity of CD26/DPP4 *in vitro* and *in vivo*. Lung adenocarcinoma cell lines from human (H460) and mouse (LLC) present the activity of CD26/DPP4 *in vitro*. Cell lysis buffer served as control for the assay. Vildagliptin treatment inhibited plasma CD26/DPP4 activity of LLC s.c. tumor bearing mice (C). The total amount of CD26/DPP4 was not changed by vildagliptin treatment (D). Arbitrary fluorescent unit (AFU) *$p<0.05$, **$p<0.01$

Supplement 3. Anti-tumor effect of vildagliptin on the lung cancer models. The intensity of GFP representing LLC-GFP tumor load was significantly lower in the vildagliptin treated group. T (tumor), H (heart), L (Lung) *$p<0.05$

Supplement 4. Cytotoxicity test *in vitro*. Vildagliptin showed no changes of viability in LLC and H460 cell lines for three days within the tested dose ranges (A). Surfactant treatment on mouse macrophage cell line Raw264.7 has no toxic effect for three days (B). Quantification of western blotting of surfactant protein expression *in vitro* shows a significant upregulation by vildagliptin treatment in LLC (C) and H460 (D) consistently. *$p<0.05$, **$p<0.01$

Supplement 5. Gene expression levels of pro- and anti-inflammatory parameters in the tumor developed by subcutaneous LLC injection. RT-PCR was performed with extracted RNA from the tumors. The expression of macrophage specific genes (TNF-α, IL-12b, and IL-15) and NK cell specific genes (NK1.1, IFN-γ, Grenzyme A, Perforin1, FasL, and TRAIL) were significantly elevated compared to control while anti-inflammatory genes (IL-10, Arginase, and TGF-β1) showed no difference. *$p<0.05$, **$p<0.01$

Supplement 6. Histological analysis of LLC s.c. induced tumor. Immunohistochemistry of CD4, CD8, and B220 revealed that vildagliptin has no effect on T or B cell infiltration in LLC induced tumor (A, and B), which was confirmed by FACS analysis (D, E, and F). Tumor infiltrating T cells (CD3+) and B cells (B220+) were equally detected in control and vildagliptin treated tumors. Representative picture of FACS analysis (F). Vildagliptin treatment increased intracellular stress marker γH2AX (G).

Supplement 7. Gating strategy in the FACS analysis. After staining of markers, single cell suspension from LLC s.c. induced tumor ($1x10^6$ cells) was administered into BD CANTO. After gating singular cells, $CD45^+$ cells were selected from live cells to assess tumor-infiltrating NK cells.

Supplement 8. TRAIL and FasL expression in NK cells of normal mice. Splenocytes from wild type and IL-$15^{-/-}$ mice were analyzed by FACS to evaluate NK cell expressed TRAIL and FasL. The expression of TRAIL and FasL were assessed within the NK cell population ($NKp46^+CD45^+$: circle) which was absent in IL-$15^{-/-}$ mice after gating $CD3^-CD14^-$ cells. Only a limited number of NK cells expressed TRAIL and FasL in normal mice.

Supplement 9. Full length pictures of western blots. Representative western blotting pictures (Fig. 3A) of surfactant protein expression including SP-A, SP-B, and SP-C were selected from the blots of LLC and H460 cell line lysates (A and B respectively)

Supplement 10. Primary NK cell isolation and cytotoxicity. Negatively isolated NK cells from mouse spleen were evaluated by NK cell markers including CD49b and CD45 (A). Comparing with magnet bound non-NK cell fraction and PBMC, the NK cell fraction contained highly abundant CD49b positive cells (>70% of total). Cultured LLC cells and mixed LLC cells with primary NK cells were in U-bottom culture plate in order to test the cytotoxicity (B). Different cytotoxicity of target/effector ratio gradients

(C). Vildaglptin treatment significantly increased NK cell viability in co-culture with LLC cells when compared to no treatment control (D). *p<0.05

Supplement 11. The expression patterns of activation and inhibition receptors and ligands of NK cells. The inhibitory receptors of NK cells remain unchanged in co-culture with LLC cells (A). The inhibitory ligands of NK cells on LLC cells were significantly elevated during co-culture (B). Vildagliptin treatment significantly decreased the inhibitory ligands Qa1b and PD-L1. The NK cell activating receptor NKG2D was significantly suppressed in co-culture with LLC cells while its ligand ULBP1 showed no difference among groups (C). *p<0.05, **p<0.01

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Figure 1.

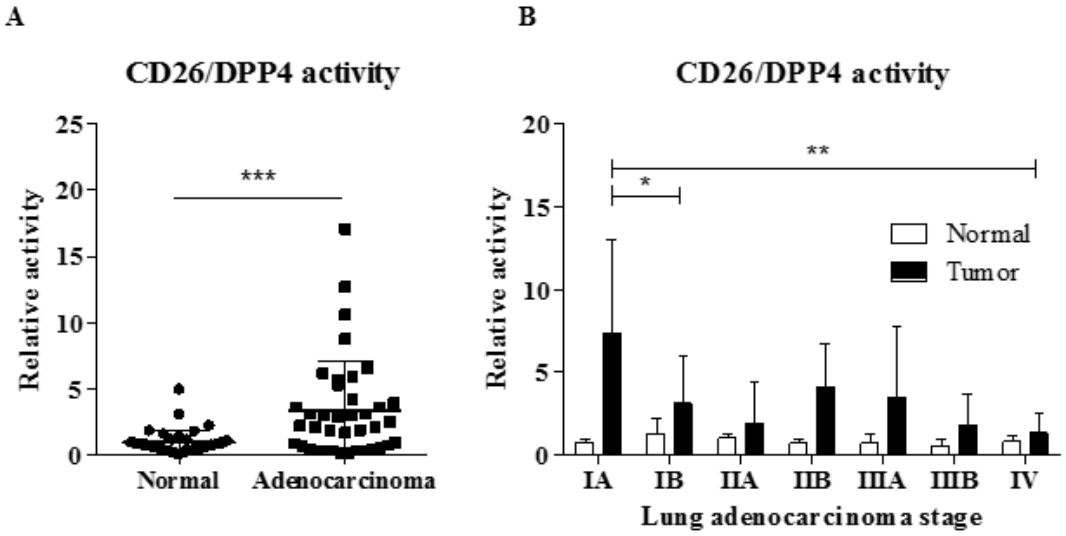
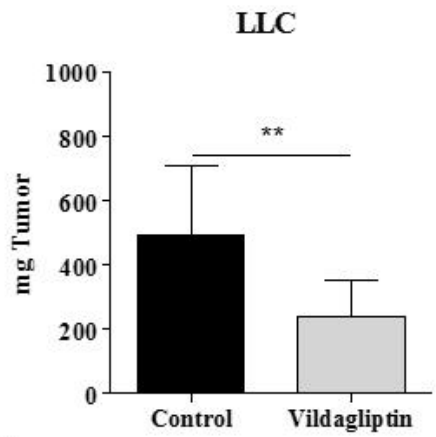
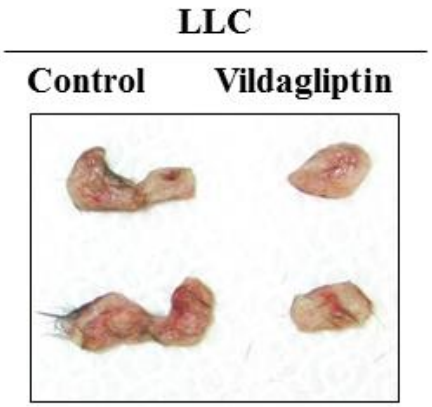


Figure 2.

A





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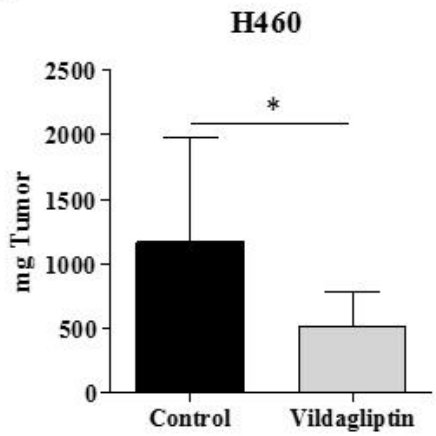

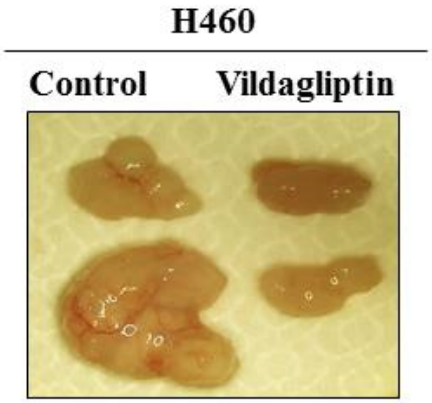

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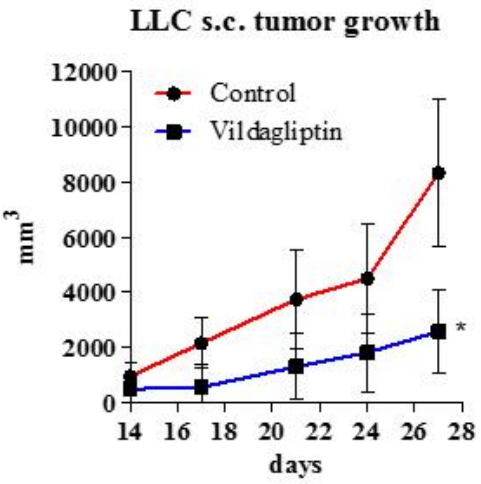

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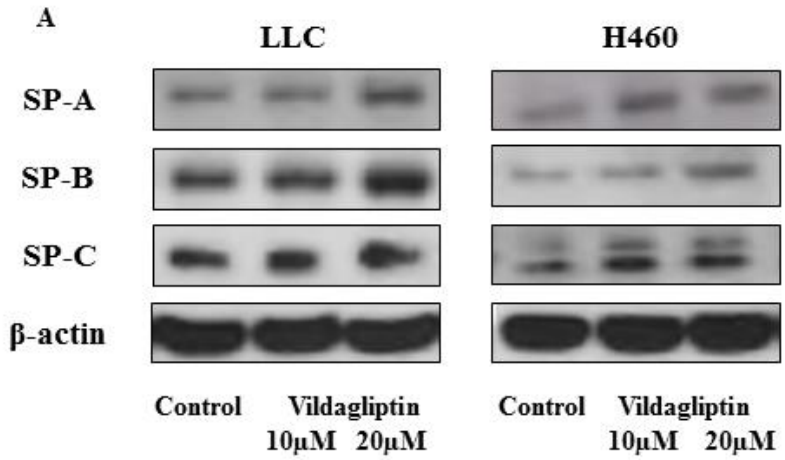

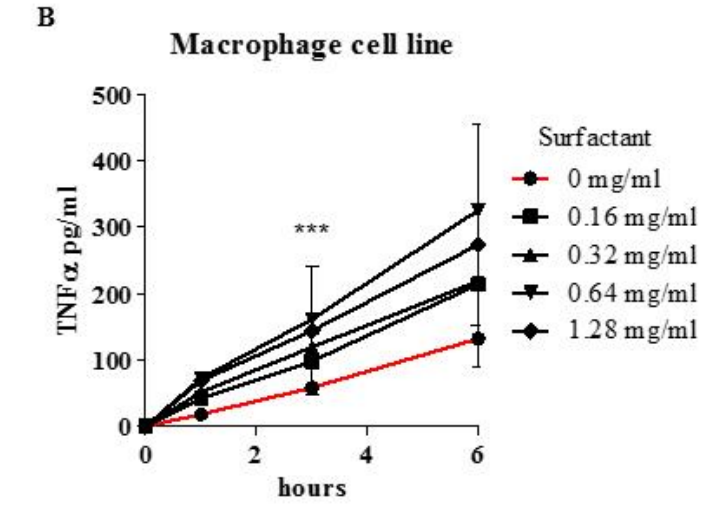

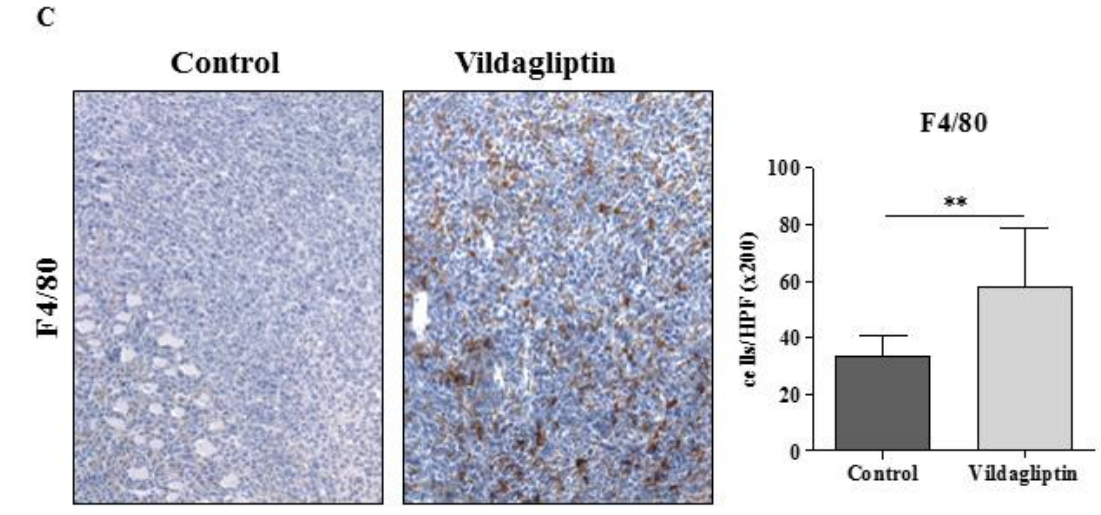

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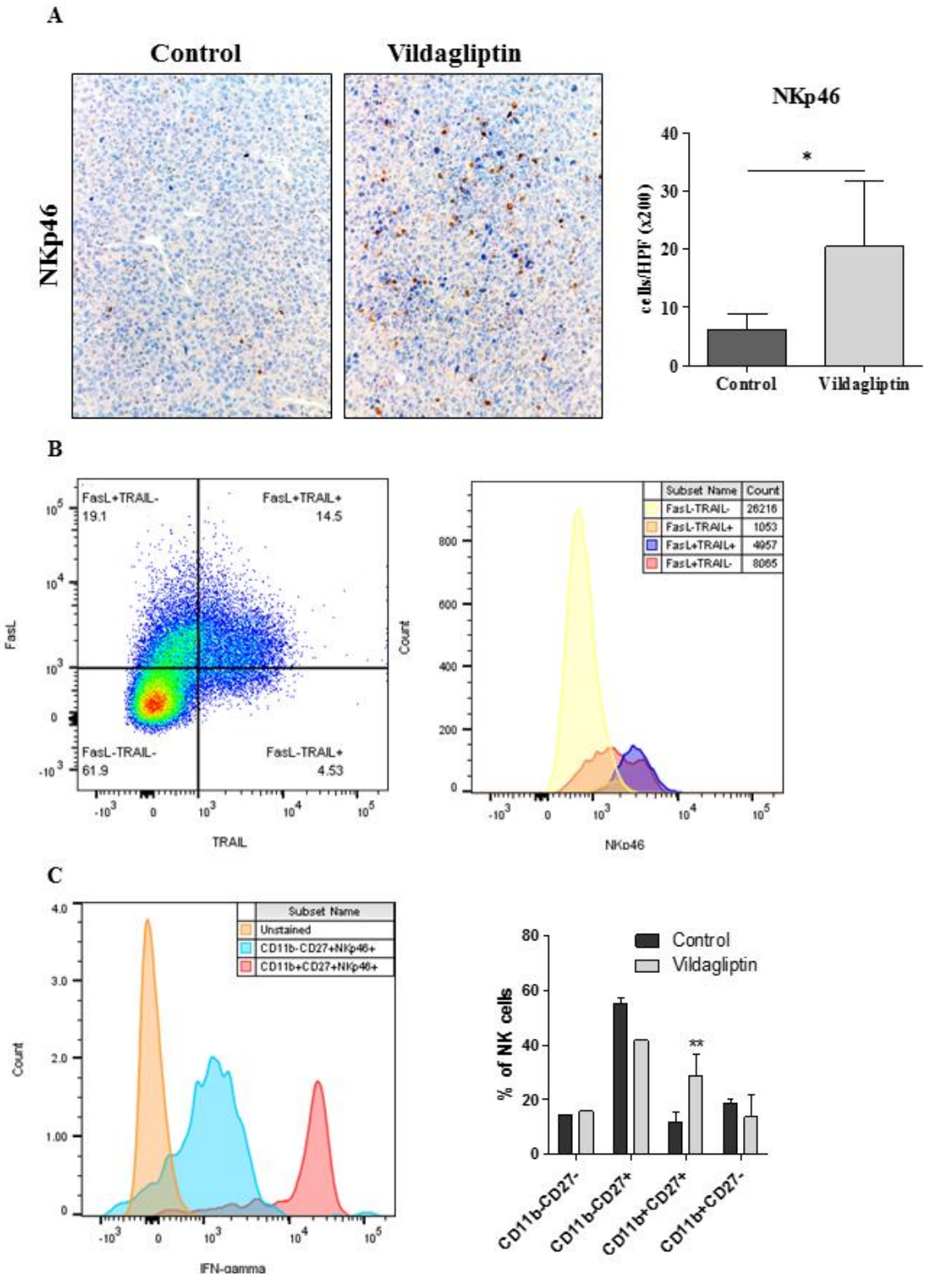

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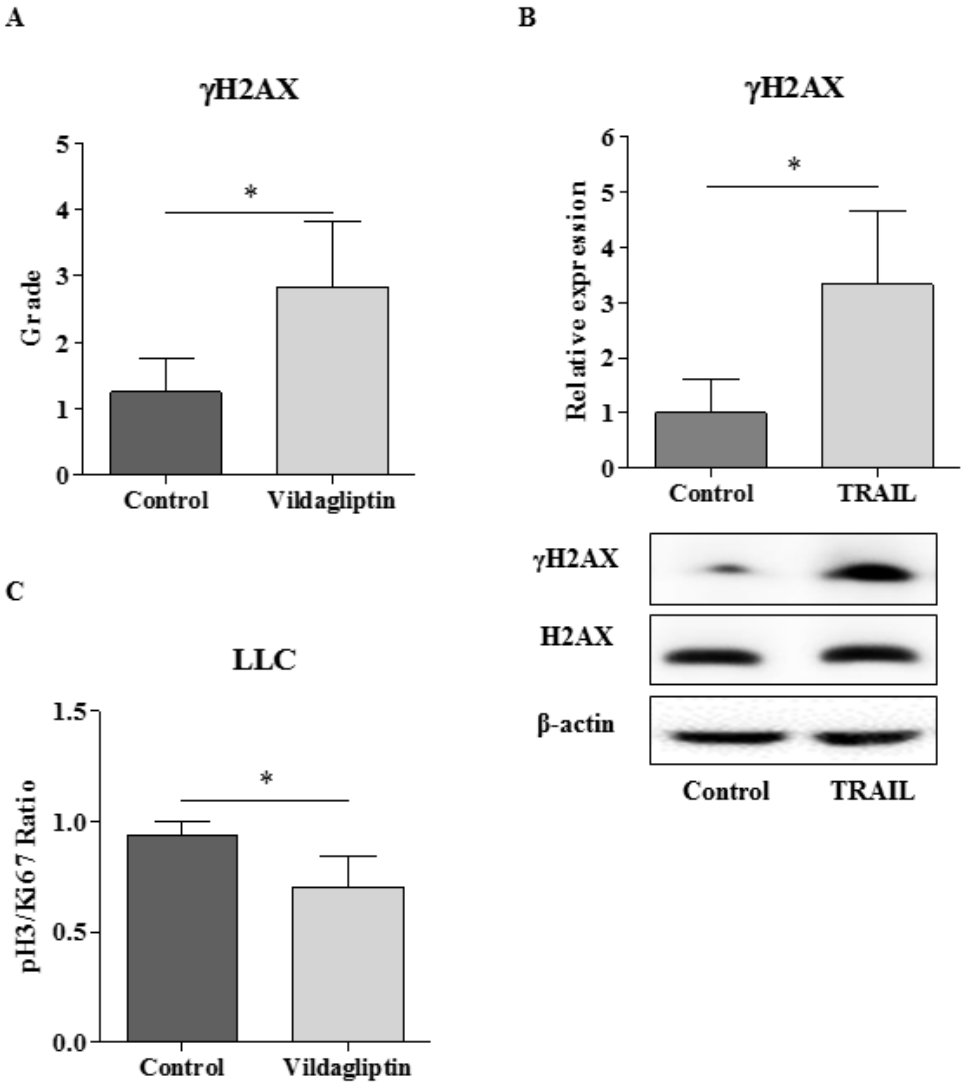

Figure 6.