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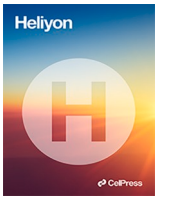

Research article

pHLIP-fused PD-L1 engages avelumab to elicit NK cytotoxicity under acidic conditions

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ABSTRACT

Natural killer (NK) cells represent key player in immune surveillance to eliminate transformed or malignant cells. One of mechanisms of action of NK cells is antibody-dependent cell-mediated cytotoxicity (ADCC) by recognizing tumor antigens on the surface of cancer cells. However, the heterogeneity of tumor antigens and the scarcity of membrane surface targets significantly restrict this strategy. Recently, we constructed a new cargo by tethering a low pH insertion peptide (pHLIP) to the C terminus of the ectodomain of programed death ligand-1 (PD-L1) and demonstrated its ability to modulate immune responses. Herein, the potential application of PD-L1-pHLIP in cancer therapy was determined. pHLIP tethering had no effect on the binding capacity of PD-L1 protein to an anti-PD-L1 antibody (i.e. avelumab). Association of pHLIP rendered PD-L1 segment display on the surface of cellular membrane in the acidic buffer instead of the neutral solution. Importantly, plate-coated or beads-coupled PD-L1-pHLIP enable robust activation and expression of cytotoxic mediators of NK cells via engaging avelumab. Overall, this work provides proof of concept that recombinant PD-L1 protein decorated on the cellular membrane driven by pHLIP in combination with appropriate monoclonal antibody has potentials to elicit NK cytotoxicity, which may represent a novel and promising therapeutic avenue in cancer.

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1. Introduction

The immune system consists of two arms, that is innate and adaptive immunity, playing vital roles in the recognition and eradication of invading microbes as well as tumors [1]. Recently, mounting evidence supports a key player of innate immunity in fighting against tumors, even at a very early stage of tumor progression. Natural killer (NK) cells are defined as a subset of innate lymphoid effector cells to recognize and kill malignant cells in primary and metastatic sites by natural cytotoxicity [2]. In addition, NK cells also release large amounts of cytokines, such as interferon-γ (IFN-γ), to fuel adaptive immune responses as well as remodel the microenvironment [3,4]. It is noteworthy that the efficacy of some monoclonal antibody (mAb) (e.g. trastuzumab, obinutuzumab, avelumab) used in clinic is proposed to be largely attributed to stimulation of NK cytotoxicity via the interaction between Fc fragment and FcγRIIIA on the surface of NK cells, named as antibody-dependent cell cytotoxicity (ADCC) [5–7]. Another prerequisite to provoke ADCC is the engagement with antigens presented on the membrane of tumor cells by the Fab fragment of antibodies. However, antigen heterogeneity and high mutation in solid tumors lead it difficult to screen specific tumor antigens that express constantly on multiple tumor types or different tumor cells from one individual [8,9].

Acidosis is well defined to be a hallmark of tumor as a consequence of aberrant glucose metabolism in cancer cells, a phenomenon named as the "Warburg effect" [10]. Although it is impossible to accurately titrate pH value in tumor microenvironment to date, bulk pH value in cancer tissues is estimated to be a range of 6.2–7.0 [11]. Of note, compared with the bulk extracellular pH, the pH on the surface of cancer cells is proposed to be lower due to weaker influence of tumor mass perfusion [12]. The cancer cell surface pH is roughly 0.3–0.7 pH units lower than the bulk pH in the extracellular surroundings [13]. pH low insertion peptides (pHLIP) is originally extracted from the C-helix of the protein bacteriorhodopsin, consisting of a flanking sequence and a transmembrane sequence, in which the flanking sequence harbors protonated amino acid residues and the transmembrane sequence contains hydrophobic residues [14]. The most outstanding trait of pHLIP is its capacity to respond the low pH in the vicinity of the lipid bilayer and to span the membrane by spontaneous formation of a helix when the extracellular niche is acidic [15,16]. Recently, we developed a new cargo by ligating the ectodomain of murine PD-L1 at the N terminus of pHLIP (termed PD-L1-pHLIP) and demonstrated its potency to actively inhibit T cell effector function by ligation of PD-1 on the surface of T lymphocytes in acidic buffer [17]. Given that avelumab is an afucosylated anti-PD-L1 mAb with ability to induce strong ADCC [7], we assume that the combination of PD-L1-pHLIP and avelumab may have potentials to trigger NK cytotoxicity and consequently destroy PD-L1-pHLIP-anchored cancer cells in acidic tumor microenvironment. To address this issue, we examine the binding of PD-L1-pHLIP to avelumab as well as the acid-driven exhibition of this construct on the cellular membrane of cancer cells. Furthermore, the ability of PD-L1-pHLIP plus avelumab to stimulate NK activation and mediator release is detected.

2. Results

The association of pHLIP has no effect on the recognition of PD-L1 by avelumab.

The potent engagement between PD-L1-pHLIP and avelumab is prerequisite for ensuing NK activation triggered by this cocktail. Therefore, firstly the binding of PD-L1-pHLIP to avelumab was measured by ELISA. The result showed that the binding was comparable to that of PD-L1 protein and avelumab (EC_{50} was 0.08 and 0.11 μg/mL for PD-L1-pHLIP and PD-L1 respectively) (Fig. 1), indicating that pHLIP association at the C-terminus of extracellular segment of PD-L1 did not influence the latter´s conformation, which was well recognized by avelumab. Furthermore, this recognition was independent of PD-L1 species used since the similar binding of murine and human PD-L1-loaded constructs to avelumab was observed (EC_{50} was 0.11 μg/mL for hPD-L1-pHLIP) (Fig. 1). In addition, we also generated a novel recombinant protein in which the N-terminus of pHLIP was fused with the fragment (T276-T652) of the human epidermal growth factor receptor 2 (HER2) that contains key residues to be recognized by trastuzumab [18]. pHLIP tethering had no

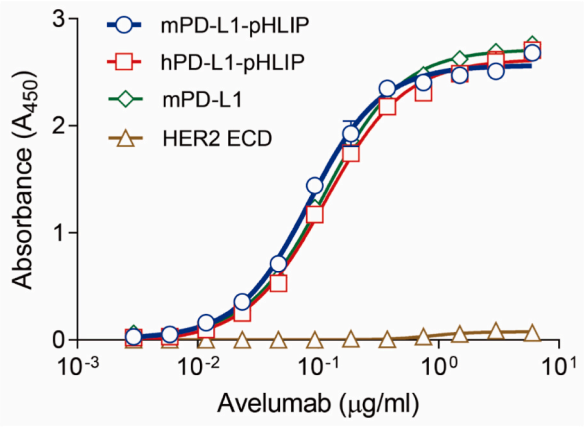

Fig. 1. The binding of PD-L1-pHLIP fusion protein to Avelumab. The binding ability of murine PD-L1-pHLIP/human PD-L1-pHLIP/PD-L1/HER2 ECD to Avelumab was determined by ELISA. The data were pooled from three independent experiments with similar results.

significant effects on the engagement between HER2 segment and trastuzumab (EC_{50} was 0.23 and 0.24 μg/mL for HER2T276-pHLIP and HER2T276 respectively) (Supplementary Figure 1). This established a paradigm to build a novel construct by fusion of appropriate antigenic protein/peptide to the N-terminus of pHLIP.

PD-L1-pHLIP is displayed on the surface of cancer cells in acidic solutions and can be recognized by avelumab.

It is well-established that pHLIP can insert across the membrane of cancer cells in response to the acidity at the surface of cancer cell [19]. Next, we determine whether PD-L1-pHLIP can display on the surface of cancer cells smoothly in response to low pH. To exclude the perturbation of PD-L1 constitutively expressed on cancer cells, endogenous PD-L1 in a murine colon cancer cell line (MC38) was deleted by CRISPR-Cas9 methods (Supplementary Figure 2). PD-L1-pHLIP was labeled by fluorescent dye and then incubated with PD-L1-KO MC38 cells in neutral and acidic buffer respectively. As shown in Fig. 2A and B, no fluorescence was seen in pH7.4 solutions detected by flow cytometry and microscopic imaging respectively. In sharp contrast, in pH5.8 buffer, fluorescence was readily observed on the cellular membrane. Furthermore, the intensity of fluorescence was increasing gradually as elevation of doses of PD-L1-pHLIP as well as decrease of pH value of the buffer (Fig. 2A and C). This effect was lost when PD-L1-pHLIP was utilized (Fig. 2B),

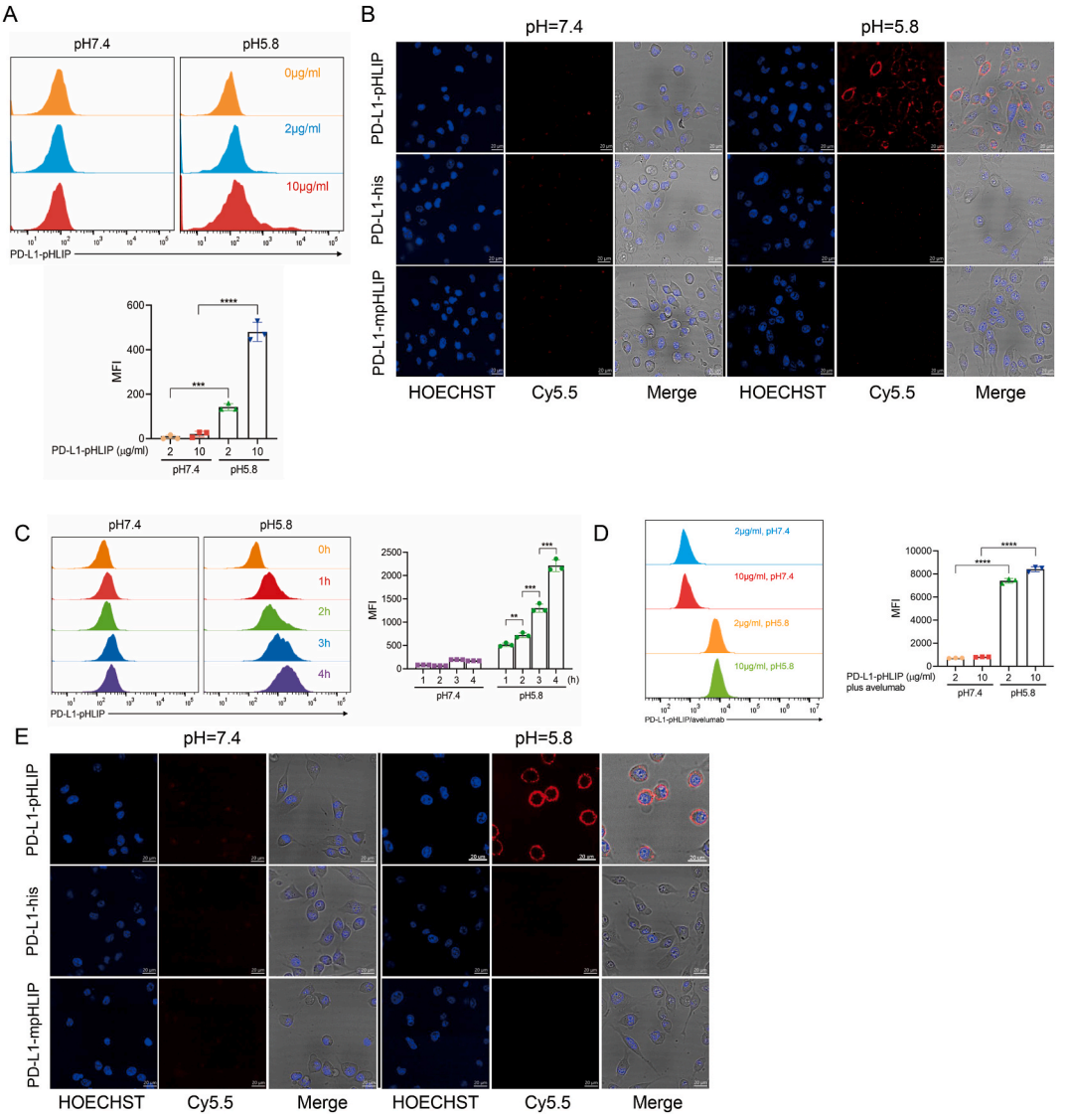

Fig. 2. The acid-responding ability of PD-L1-pHLIP to span cell membrane. (**A,B**) Cy5 or Cy5.5-labeled PD-L1-pHLIP (2, 10 μg/ml) was incubated with PD-L1-KO MC38 colon cancer cell line in pH 7.4 or 5.8 buffer for 1 h, respectively. The fluorescence was detected by flow cytometry (**A**) and microscopic imaging (**B**) respectively. (**C**) Cy5-labeled PD-L1-pHLIP (10 μg/ml) was incubated with PD-L1-KO MC38 in pH 7.4 or 5.8 buffer for 1–4h, respectively. The insertion was detected by flow cytometry. (**D,E**) Cy5 or Cy5.5-labeled Avelumab was mixed with PD-L1-pHLIP/PD-L1-his/PD-L1-mpHLIP (10 μg/ml) in pH 7.4 or 5.8 buffer for 30 min and the mixture was incubated with PD-L1-KO MC38 for 1 h, respectively. The fluorescence was detected by flow cytometry (**D**) and microscopic imaging (**E**) respectively. Scale bar: 20 μm. Representative plots and graphics were shown. The data were pooled from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

in which two Asp residues at 14 and 25 replaced by lysine respectively [17]. Notably, PD-L1-pHLIP was pre-mixed with dye-conjugated avelumab and then incubated with cancer cells. Consequently, the fluorescence was obviously seen and was comparable to that incubated with the construct alone (Fig. 2D and E), indicating that the ligation of avelumab did not affect the exhibition of PD-L1-pHLIP on the cell surface in acidic buffer. Taken together, we can make a conclusion that pHLIP tethering confers the competency of PD-L1-pHLIP to be decorated on the surface of cancer cells under acidic environment.

2.1. PD-L1-pHLIP plus avelumab potently trigger NK cell cytotoxicity

To test the ability of PD-L1-pHLIP in combination with avelumab to trigger NK cytotoxicity, two scenarios, namely plate-coated and mircobeads-coupled, were exploited. In addition, CD107a (a biomarker of NK degranulation following cytotoxicity was induced) and IFN-γ (a major proinflammatory mediator released by activated NK cells) as key parameters of antigen/mAb-triggered ADCC function [20–22], were detected in our system. As a result, plate-coated immobile PD-L1-pHLIP plus avelumab actively stimulated significantly increased expression of CD107a as well as IFN-γ in NK cells compared with control protein plus avelumab or PD-L1-pHLIP plus IgG1 isotype, in a dose-dependent manner (Fig. 3A and B). Of note, the NK-stimulating capacity of PD-L1-pHLIP was weaker than naked PD-L1 version, implying that the fixation of PD-L1-pHLIP on the plastic to some extent influences the engagement between avelumab and FcγRIIIA. Concurrently, we examined the capacity of microbeads-coupled PD-L1-pHLIP plus avelumab to activate NK cells. As well, PD-L1-pHLIP/avelumab potently triggered NK cells to highly express CD107a and IFN-γ, which was positively associated with the ratio of the number of beads and NK cells (Fig. 3C). Intriguingly, the NK-stimulating function of PD-L1-pHLIP was much stronger than PD-L1 protein (Fig. 3C). In addition, we also measured the ability of HER2T276-pHLIP plus trastuzumab in the system as described

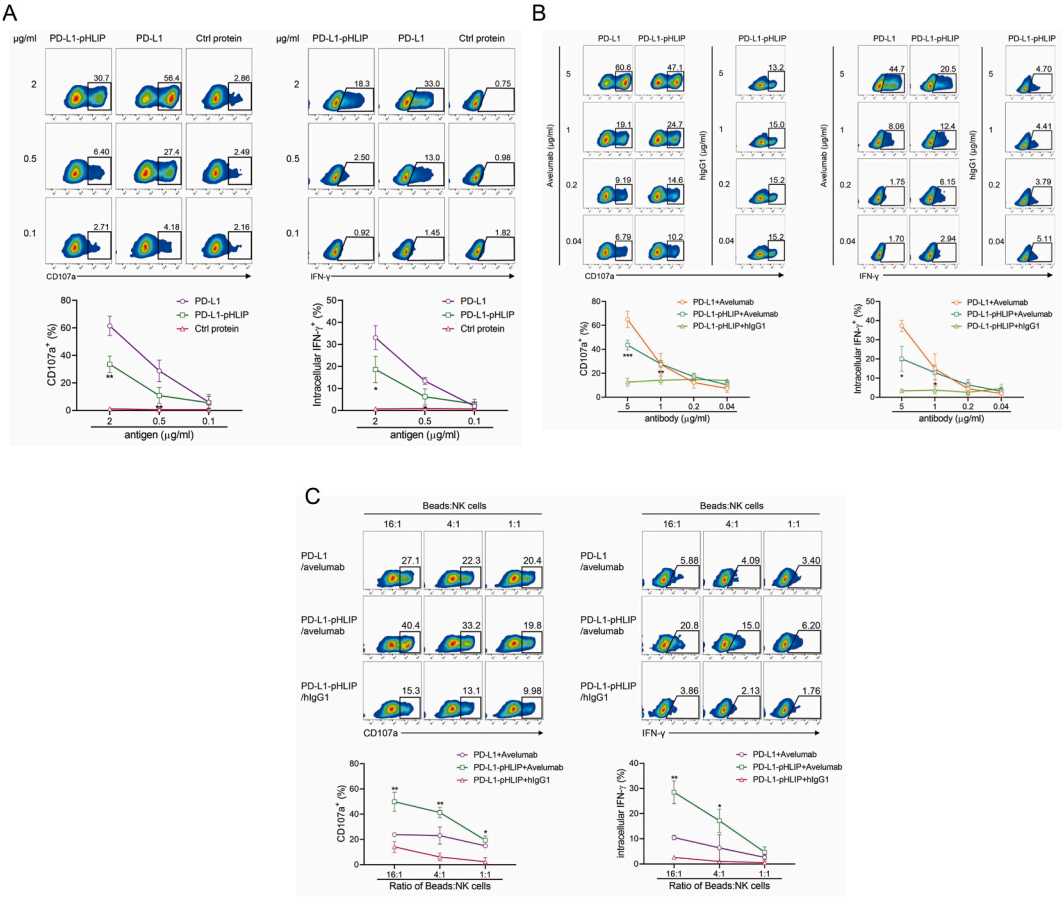

Fig. 3. The capacity of PD-L1-pHLIP plus avelumab to stimulate NK cells to highly express CD107a and IFN-γ. (**A**) PD-L1/PD-L1-pHLIP or control protein (2, 0.5, 0.1 μg/ml) were immobilized on plates overnight. Avelumab (5 μg/ml) was added and mixed for 1 h and were incubated with NK cells for 5 h. (**B**) PD-L1, PD-L1-pHLIP or control protein (2 μg/ml) were immobilized on plates overnight. Avelumab (5, 1, 0.2, 0.04 μg/ml) was added and mixed for 1 h and were incubated with NK cells for 5 h. The expression of CD107a and IFN-γ was determined by flow cytometry. (**C**) Microbeads were coupled to PD-L1/PD-L1-pHLIP overnight at 4 °C and mixed with avelumab or isotype hIgG1 (5 μg/ml) for 2 h. The mixture was incubated with NK cells according to the ratio indicated for 5 h. The production of CD107a and IFN-γ was determined by flow cytometry. Representative plots were shown. The data were pooled from five independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

above. Not only plate-coated but microbeads-coupled HER2T276-pHLIP plus trastuzumab enable augmented degranulation and expression of IFN-γ by NK cells (supplementary Fig. 3A and B). This suggests that cell-anchored PD-L1-pHLIP combined with avelumab may have potentials to evoke NK cytotoxicity.

3. Discussion

Antibody-targeted therapy has been widely leveraged to treat solid cancer currently. This avenue, however, is limited to tumor antigen heterogeneity and high mutation load of cancer cells. Herein, we propose one strategy that a tumor antigen is ligated to the N-terminus of the acid-responsive peptide, leading it to be displayed on the surface of cancer cells at the aid of pHLIP´s conformational change in response to low pH, which is a hallmark of tumor niches. Albeit immaturely, the results in this study suggest the potentials of this strategy to trigger NK cytotoxicity to fight against cancer in acidic tumor microenvironment (Fig. 4). This conception is in agreement with Thévenin´s work that used 2,4-dinitrophenyl (DNP) as antigen and IgG existing in human serum as effector to stimulate NK cytotoxicity [23]. The clinical application of this cargo in the future, however, may be some problematic because of no titration of total amounts of endogenous anti-DNP IgG in the serum as well as the difficulty to accurately titrate the doses of ADCC-inducing anti-DNP antibodies. Our strategy, at least in part, resolves this issue by exploiting antibodies that are commercially available and clearly harbor ADCC-inducing activity.

One limitation of our study is the lack of the assays for PD-L1-pHLIP/avelumab-triggered ADCC-mediated cell lysis. In fact, we try to perform the co-culture assay with PD-L1-pHLIP/avelumab, MC38 and NK cells in acidic buffer. However, the results showed no cytotoxic effects on cancer cells assayed by LDH release (data not shown), which may be ascribed to the fact that lactate treatment led to the impairment of NK activity [24–27]. Additionally, it should be noted that only one cancer cell line was utilized for ADCC assay in our study, making us not conclude that the lack of ADCC activity is due to the assay because no additional cancer cell lines were tested. In this case, the lack of ADCC activity could be due to an intrinsic resistance of MC38 cell line to the ADCC mediated by PD-L1-pHLIP/avelumab. One resolution is to utilize acid-resistant/lysis-sensitive cancer cell lines and NK cells, which is being investigated. In addition, one may argue that the virtual pH in microenvironment is not as low as 5.8, used in our study. This discrepancy may be resolved by leveraging a pHLIP variant with a pH_{50} of 6.79 and improved pH response [28]. Another issue to be addressed is that upon PD-L1-pHLIP application into a tumor model, if not 100 % successfully bound by avelumab, PD-L1-pHLIP will bind to PD-1 on immune cells and reduce the efficacy of this strategy. One approach is combined administration of PD-L1-pHLIP/avelumab and PD-1 antagonist, which will not only block the interaction between PD-L1-pHLIP and PD-1 but also improve the efficacy through synergistically evoking activation of NK and T cells.

In summary, our study provides a novel regimen with potentials to combat tumor antigen-null solid cancers. The concept is infant and need to be further studied. Considering that NK cells from tumor mass is proposed to be dysfunctional [29,30], the agents to retrieve NK fitness or ex vivo pre-expanded NK cells will potentiate this therapeutic avenue.

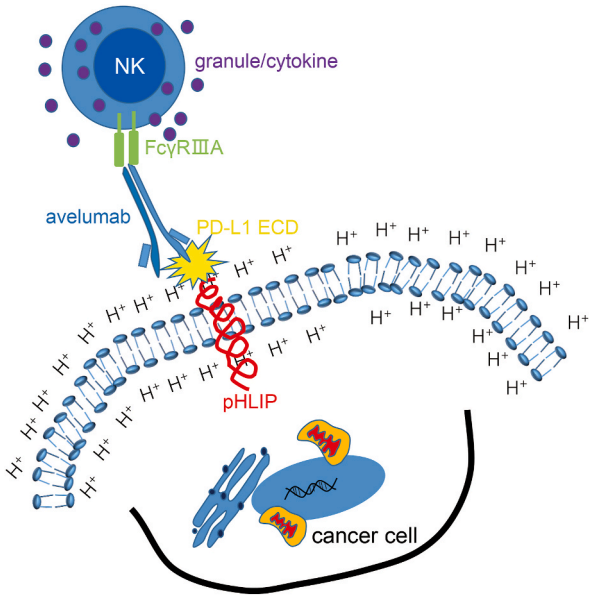

Fig. 4. A schematic illustration on the mode of action of PD-L1-pHLIP plus avelumab. pHLIP-conjugated PD-L1 extracellular domains were specifically assembled onto the membrane of tumor cells by responding to the acidic microenvironment via the conformational transformation of pHLIP. PD-L1 protein was subsequently recognized by avelumab, leading to NK degranulation and cytokine release through the interaction between Fc fragment and FcγRIIIA on the surface of NK cells.

4. Materials and methods

4.1. Cell culture

Murine colon adenocarcinoma cell line (MC38) was purchased from ATCC and PD-L1 knockout was operated by Cyagen Biosciences Inc. and the details was described in supplementary methods. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10 % fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C.

4.2. Recombinant protein expression and purification

PD-L1 ECD, PD-L1-pHLIP, PD-L1-mpHLIP, HER2 ECD, HER2T276-T652 (276aa-652aa of HER2, termed as HER2T276), pHLIP-HER2T276 (TGEDADVLLALDLLLLPTTFLWDAYRAWYIPNQEA-(G4S)3-T276-T652) were prepared according to the protocol described previously [17].

4.3. ELISA

96-well plates were coated with the indicated proteins at 2 μg/ml overnight, washed three times with PBS-T, and blocked with 4 % nonfat dried milk for 1 h at room temperature (RT). Twofold serial dilution of Avelumab (Merck) or trastuzumab (Roche) from 6 μg/ml and incubated for 1 h at RT. The wells were washed three times and HRP-conjugated goat anti-human IgG Fc Secondary Antibody (1:6000) was added and incubated for 30min at RT. TMB substrate (Invitrogen) was used for detection at 450 nm.

4.4. Fluorescent linking and confocal imaging

Recombinant proteins or antibody were labeled by fluorescence dye (Cy5 or Cy5.5) with labeling Kit, according to manufacturer's protocol (Abcam). The PD-L1-KO MC38 cells were cultured in a laser confocal dish (NEST, Wuxi, China) overnight. Cy5.5-linking proteins were added at 10 μg/ml and cultured at 37 °C for 1 h. Cells were washed twice with PBS with lactate-titrated pH7.4 or 5.8, and then the fluorescence was observed under confocal laser scanning microscope (Andor, Dragonfly 200).

4.5. Flow cytometry

The cells were incubated with Cy5-labeled PD-L1-pHLIP at 2 or 10 μg/mL for 1–4 h in PBS with pH 7.4 or 5.8. In some settings, cells were incubated with PD-L1-pHLIP at 10 μg/mL for 1 h in PBS (pH 7.4 and 5.8). Cy5-labeled avelumab (10 μg/mL) was added and incubated for another 30 min. Cells were washed twice with PBS with corresponding pH, and then detected by flow cytometry. In addition, PE-conjugated anti-murine PD-L1 (Biolegend, 124307), FITC-conjugated anti-human CD3 (Biolegend, 300306), APC-conjugated anti-human CD56 antibodies (Biolegend, 304610) were used.

4.6. NK activation assays

Peripheral blood mononuclear cells (PBMCs) were pooled from healthy donors within 4 h of collection using Ficoll–Hypaque centrifugation. NK cells were then enriched by negative isolation using a commercial kit (Miltenyi Biotec). The isolated NK cells ($CD3^-CD56^+$) were 95 % pure as depicted in Supplementary Figure 4. Two strategies to assay NK cell activation were employed.

Plate-coated method: PD-L1 ECD/PD-L1-pHLIP/HER2 ECD/HER2T276/pHLIP-HER2T276 (0.1–2 μg/mL, 100μL/well) was coated overnight at 4 °C. Wells were then washed three times with PBS and blocked with 20 % BSA for 2h at 37 °C. After washing, avelumab or trastuzumab at the indicated doses was added and incubated for 1 h at 37 °C. Unbound antibodies were removed by washing three times. Human NK cells were cultured (3×10^5 cells/well) in the presence of 4 μg/mL brefeldin A (BFA, Sigma-Aldrich) and 5 μg/mL GolgiStop solution (Life Technologies) for CD107a assay or in the presence of only 4 μg/mL BFA for detection of IFN-γ. Cells were harvested after 5 h incubation at 37 °C and stained with PE-conjugated anti-CD107a antibody (Biolegend, 328608). For IFN-γ assay, cells were fixed and permeabilized according to the manufacturer's instructions (Invitrogen, 00-8222-49, 00-8333-56). PE-conjugated anti–IFN–γ antibody (Biolegend, 506507) was then added. Cells were washed and subjected to FACS Aria II (BD Biosciences). Data analysis was performed using the FlowJo software.

MicroBeads-coupled method: Beads with diameter of 4.5 μm (Thermo Fisher, 14013) were used to stimulate NK cells at a ratio indicated. To couple beads with saturating amounts of proteins, 4×10^7 beads were incubated with 3 μg premixed proteins in PBS containing 2 % FBS for 1 h at 4 °C. Beads were washed three times in PBS containing 2 % FBS to remove excess proteins, incubated with 3 μg antibodies for 1 h at 4 °C and washed three times. For cell stimulation, 4×10^6 beads were incubated with resting NK cells at the indicated ratio in 500 μL IMDM supplemented with 10 % human serum for 5 h at 37 °C. The cultures were rotated end-over-end during the stimulation. CD107a and IFN-γ expression was assayed as described above.

4.7. Statistics

Data were analyzed and presented as the mean ± standard error. The graphs were plotted using Prism software (GraphPad Prism 8,

San Diego, USA). Statistical differences were calculated using unpaired t-tests or ANOVA. The $P < 0.05$ was considered statistically significant.

Data availability statement

Data are included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Junjuan Feng: Writing – original draft.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30551.

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