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Novel anti-CD73-IL-2v bispecific fusion protein augments antitumor immunity by alleviating immunosuppressive adenosine pathways in CD8$^+$ T cells

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ABSTRACT

Background Adenosine accumulated in the tumor microenvironment functions as an immune-modulating factor, exerting immunosuppressive actions via adenosine A2A/A2B receptor (A2AR/A2BR) in various immune cell types. CD73, a key enzymatic regulator responsible for adenosine production, is frequently overexpressed in diverse cancers, and its overexpression is associated with reduced responsiveness to conventional anti-cancer drug treatments such as chemotherapy, radiation therapy, targeted therapy, or immunotherapy. Despite numerous therapeutic applications of IL-2 in cancer immunotherapy, the relationship between the CD73-adenosine axis and IL-2-based immunotherapy remains largely unexplored.

Methods To evaluate the effect of CD73 blockade on IL-2 signaling of CD8$^+$ T cells, we screened novel CD73 antibodies using human single-chain variable fragment phage library and immunized Alpaca phage library. To optimize targeting to CD73-expressing cells and reinvigorate the antitumor effect of IL-2 in adenosine-rich microenvironment, we engineered a novel bifunctional GI-αCD73/IL-2v fusion protein. Functionality of GI-αCD73/IL-2v fusion protein was assessed in the in vitro cell-based assays and the in vivo tumor-bearing mouse model or cynomolgus monkey.

Results IL-2-induced increase in proliferation of CD8$^+$ T cells was not observed under adenosine-rich microenvironment. We demonstrated that the functional impairment of IL-2 signaling in CD8$^+$ T cells in these conditions can be reversed by our anti-CD73 antibody (GI-αCD73). Furthermore, GI-αCD73/IL-2v fusion protein significantly restored the impaired proliferation of CD8$^+$ T cells and consequently enhanced tumor cell killing under adenosine-mediated immunosuppression, surpassing the combined treatment of GI-αCD73 and Fc-IL-2v. These synergistic effects were attributed to the enhanced delivery of the IL-2v component of GI-αCD73/IL-2v to IL-2Rβγ on CD73-expressing CD8$^+$ T cells through a cis-binding mechanism. GI-αCD73/IL-2v elicited a potent antitumor effect in both the human CD73 knock-in (hCD73 KI) mouse model and the humanized mouse model. In non-human primates, GI-αCD73/IL-2v exhibited excellent tolerability while inducing robust and durable expansions of cytotoxic lymphocytes.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ CD73 plays an important role as a key enzymatic regulator in the production of adenosine, which exerts immunosuppressive actions via A2ARs/A2BRs in multiple cell types. Overexpression of the CD73 gene is observed in various cancers, and this is associated with poor therapeutic response and worse prognosis.

⇒ Various IL-2-based immunotherapeutic applications have been developed to mitigate adverse effects and enhance their antitumor efficacy.

WHAT THIS STUDY ADDS

⇒ In this study, we observed that our novel CD73 antibody (GI-αCD73) effectively alleviated the functional deficiency of IL-2 signaling in CD8$^+$ T cells under adenosine-rich conditions. GI-αCD73/IL-2v bispecific fusion protein enhanced activation and proliferation of CD8$^+$ T cells, as well as their killing activity against tumor cells, compared with the combined treatment with GI-αCD73 and Fc-IL-2v under adenosine-rich conditions. The synergistic effect was attributed to the cis-binding action of GI-αCD73/IL-2v on CD73-expressing CD8$^+$ T cells. GI-αCD73/IL-2v induced robust antitumor activity through CD8$^+$ T cell activation, while maintaining a favorable safety profile.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ GI-αCD73/IL-2v fusion protein presents significant advantages not only in restoration of IL-2 signaling in CD8$^+$ T cells under adenosine-rich conditions but also in its targeting of CD73-expressing cells, particularly CD8$^+$ T cells. Consequently, our data propose novel approaches to an innovative IL-2-based agent that enhances antitumor immunity while overcoming the limitations associated with conventional IL-2 therapeutics.

Conclusions GI-αCD73/IL-2v bispecific protein is a novel and potent immunocytokine with significant antitumor immunity through cis-binding on $CD8^+$ T cells.

BACKGROUND

Tumor microenvironment (TME) consists of various cell types, such as tumor cell, immune cell, and stromal cell, as well as non-cellular components including growth factors, cytokines, and extracellular matrix proteins.[1] The complex crosstalk among these components within the TME significantly impacts tumor progression and therapeutic responses.[1] Cytotoxic lymphocytes, such as natural killer (NK) cells and $CD8^+$ T cells, exert antitumor effects against tumor cells through granule-mediated or death receptor-mediated cytotoxicity pathways within the TME.[2 3] Conversely, regulatory T (Treg) cells, myeloid-derived suppressor cells (MDSCs) or tumor-associated macrophages (TAMs) suppress the antigen presentation of dendritic cells (DCs) and inhibit the cytotoxic function of NK cells and $CD8^+$ T cells by inducing expression of immune checkpoint inhibitory molecules and releasing various immune suppressive factors, such as transforming growth factor β, interleukin-10 (IL-10), and indoleamine 2,3-dioxygenase.[4–6] Furthermore, the high concentration of adenosine within the TME imparts immunosuppression by acting through A2AR/A2BR on various immune cell populations such as $CD4^+$ and $CD8^+$ effector T (Teff) cells,[7 8] NK cells,[9 10] DCs,[11] Treg cells,[12] or MDSCs/TAMs.[13] In addition, adenosine contributes to tumor progression through direct actions on tumor cells[14] or cancer-associated fibroblasts (CAFs).[14 15]

Cluster of differentiation 73 (CD73) is a dimeric ecto-5'-nucleotidase (NT5E) which hydrolyzes AMP, derived from ATP by CD39, into adenosine.[16] CD73 overexpression has been reported in various tumor tissues,[17 18] and it is associated with poor prognosis in patients with pancreatic cancer, renal cell carcinoma (RCC), ovarian cancer, melanoma, colorectal cancer, triple-negative breast cancer (TNBC), non-small-cell lung cancer, or sarcoma. High expression of CD73 is also significantly associated with reduced therapeutic response to cancer treatments such as chemotherapy,[19] radiation therapy,[20] targeted therapy,[21] or immunotherapy.[22] Consistently, the gene expression signature analysis of tumor samples from patients treated with anti-programmed cell death protein-1 (PD-1) or anti-cytotoxic T-lymphocyte associated protein 4 revealed that the adenosine signaling pathway is more activated in tumors of patients with non-response to the immunotherapy compared with those with response.[23] Moreover, several recent studies suggested a correlation between high tumor expression of CD73 (or A2AR/A2BR) and reduced infiltration of $CD8^+$ T cells or NK cells, as well as an increased presence of exhausted T cells.[24 25] These findings imply that activation of the CD73-adenosine axis in tumors contributes to resistance against anticancer drugs by inducing functional impairment of antitumor immune cells, in addition to the previously reported $CD73^{high}$ or $A2BR^{high}$ tumor cell-intrinsic chemoresistance.[19 26] Notably, numerous studies suggested that CD73 is expressed not only in tumor cells but also in non-tumor cell types such as immune cells (T cell, NK cell, Treg, etc), non-hematopoietic cells, and stromal cells.[15 27–29] The CD73-adenosine axis in these cell populations has been reported to play a role in tumor immune escape and tumor metastasis in the TME.[15 27 28 30] Particularly, adenosine has been reported to inhibit lymphocyte-mediated cytolysis and T cell cytotoxicity via adenosine receptor.[31–33] Furthermore, it has been reported that CD73 is upregulated in tumor-infiltrating NK cells, and these $CD73^+$ NK cells suppress $CD4^+$ T cell proliferation and cytokine production.[28] In line with these findings, engineered anti-CD73 chimeric antigen receptor-NK cells have been reported to promote NK cell infiltration into $CD73^+$ tumors, leading to the suppression of tumor growth in mice.[34] In addition to the functional importance of CD73 in NK cells, growing evidence suggested that CD73 expression in $CD8^+$ T cells also influences the proliferation and cytokine production of $CD8^+$ T cells.[27 35] An adoptive transfer study of $CD8^+$ T cells revealed that CD73-deficient $CD8^+$ T cells are more effective in infiltrating into tumors and reducing tumor burden in B16 tumor-bearing mice compared with CD73-expressing $CD8^+$ T cells.[27] Immune profiling of head and neck cancer patient tumor samples revealed a strong inverse relationship between $CD73^+CD8^+$ T cells and overall T cell infiltration.[36] In parallel, patients with bladder cancers showing infiltration of $CD73^{high}CD8^{high}$ T cells exhibited a poor overall survival rate compared with those with infiltration of $CD73^{low}CD8^{high}$ T cells,[37] suggesting the importance of CD73 expression in $CD8^+$ T cells as a potential prognostic biomarker for bladder cancer. Taken together, these findings indicate that blockade of the CD73-adenosine axis in antitumor lymphocytes could be a promising therapeutic strategy to treat cancer.

IL-2 is a pleiotropic cytokine that plays crucial roles in anti-cancer immunotherapy as a potent inducer of $CD8^+$ T cells and NK cells.[38] High-dose IL-2 (Aldesleukin/Proleukin) was one of the first immunotherapy drugs to be approved for metastatic RCC in 1992 and melanoma in 1998.[39] Despite its moderate efficacy in achieving complete response in the approved indications, the widespread use of IL-2 in the clinic has been limited due to severe adverse effects, such as cytokine release syndrome and vascular leak syndrome, as well as the expansion of Treg cells which weaken the antitumor immune response.[40 41] IL-2 exerts functional action by binding to the IL-2 receptor (IL-2R) complex, which consists of IL-2Rα (CD25), IL-2Rβ (CD122) or IL-2Rγ (CD132).[42] IL-2 can bind to an intermediate affinity dimeric IL-2Rβγ which is expressed in NK cells and resting Teff cells.[38] In contrast, in Treg cells, which express IL-2Rα in addition to IL-2Rβγ, IL-2

binds to the trimeric IL-2Rαβγ complex with 10–100 fold higher affinity.[38] To reduce the toxicity and optimize antitumor efficacy, genetically engineered IL-2 variant (IL-2v) has been developed by lacking binding to IL-2Rα (predominantly expressed in lung endothelial cells and Treg cells)[43] and by enhancing binding to IL-2Rβ.[44] Restricted IL-2 actions within tumor or the TME could also be considered as strategies to reduce systemic toxicity. Representative examples include bispecific IL-2 molecules with antibodies against tumor antigens or tumor-associated antigens, like carcinoembryonic antigen (CEA),[45] and antibodies targeting CAFs, such as fibroblast activation protein α (FAP).[46] IL-2 pro-drug could be also conditionally activated within the tumors or the TME through the removal of masking peptides via a tumor-associated protease, such as matrix metalloproteinase 9.[47] Despite extensive studies aimed at reducing toxicity and optimizing efficacy, the clinical outcomes of IL-2-based immunotherapy indicate limited antitumor efficacy as a monotherapy.

Considering that CD73 blockade has shown minimal or moderate effects on tumor regression in tumor-bearing mice or human patients across various cancer types,[48–51] researchers have investigated the concurrent use of CD73 blockade with immunotherapy or chemotherapy.[49 50] Synergistic antitumor effects have been observed in tumor-bearing mice subjected to combination treatment, resulting in superior outcomes compared with monotherapy. Based on these findings, multiple clinical trials evaluating the efficacy and safety of combined therapies, such as chemotherapy with CD73 blockade and immunotherapy with CD73 blockade in patients with different types of cancer, have been initiated and are currently ongoing.[52 53] Despite the notable antitumor effects of IL-2-based immunotherapy, comprehensive investigations into the combination of CD73 blockade and IL-2 therapy for cancer treatment have remained unexplored in both in vitro and in vivo studies. In the current study, we observed impaired IL-2 signaling in $CD8^+$ T cells under adenosine-rich conditions and demonstrated the restoration of this impaired signaling through our novel CD73 blockade. Expanding on this initial finding, we aimed to improve the safety and effectiveness of IL-2 against $CD73^{high}$ tumors by engineering a first-in-class bifunctional and aglycosylated immunoglobulin G4-Fc (IgG4-Fc) fusion protein comprising an anti-CD73 antibody (CSA0060) fused to IL-2v (herein, GI-αCD73/IL-2v). The fusion protein significantly exhibited greater effectiveness in reinvigorating impaired proliferation in $CD8^+$ T cells under adenosine-mediated immunosuppressive conditions compared with single treatment or a combination treatment with anti-CD73 and Fc-IL-2v. Therefore, our data highlight the potential of combined therapeutics targeting the CD73-adenosine axis and IL-2 as a promising strategy for cancer treatment, offering further insights into the development of a novel IL-2-based bispecific agent for cancer treatment.

MATERIALS AND METHODS

Cell lines and animals

MDA-MB-231 human TNBC cells were purchased from ATCC and were maintained in DMEM medium (ThermoFisher Scientific) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, ThermoFisher Scientific). CTLL-2 cells were purchased from ATCC and were maintained with RPMI-1640 medium (ThermoFisher Scientific), supplemented with 10% FBS, 0.2 mM sodium pyruvate, 0.2 mM L-glutamine and T-stim culture supplement with Con A (Lonza). HEK-Blue IL-2 reporter cells and human peripheral blood mononuclear cells (PBMCs) were obtained from InvivoGen and Zen-bio (or STEMCELL Technologies), respectively. MC38 and MC38-hCD73 cells were purchased from Applied StemCell and Biocytogen, respectively.

Female immune-deficient NOG-B2m (NOD.Cg-B-$2m^{tm1Unc}$Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice or NSG-MHC I/II DKO (NOD.Cg-Prkdc^{scid} H2-K1$^{b\text{-}tm1Bpe}$ H2-Ab1$^{g7\text{-}em1Mvw}$ H2-D1$^{b\text{-}tm1Bpe}$ Il2rg^{tm1Wjl}/SzJ) mice were acquired from The Jackson Laboratory and were maintained in individually ventilated cages placed in animal biosafety level 3 facility at 19°C–25°C and 30%–70% humidity. Human CD73/PD-1/PD-L1 (*NT5E/PDCD1/CD274*) triple KI C57BL/6 mice (herein, hCD73 KI mice) were purchased from Shanghai Model Organisms and were kept in individually ventilated cages within specific pathogen-free environment at 21°C± 3°C and 50%± 10% humidity.

Development and production of antibodies

Monoclonal CD73 antibodies were screened by immunotube panning using the human single-chain variable fragment (scFv) phage library (Y-Biologics). Single-domain antibodies (sdAbs) were screened by solid panning on human CD73-coated plates using phage library derived from Alpaca immunized four times at 21-day intervals with human CD73 protein (ACROBiosystems) as the immunogen; 0.5 mg for the first immunization and 0.25 mg for each subsequent immunization (Genscript Probio). A total of 64 purified monoclonal anti-CD73 antibodies and 10 purified CD73 sdAbs with diverse binding potentials against human CD73 were identified, and 1 monoclonal antibody (CSA0060/GI-αCD73) or 2 sdAbs (AHF10240 and AHP04167) were selected based on soluble CD73 and membrane-bound CD73 enzymatic inhibitory assays. To generate GI-αCD73/IL-2v or AHP04167/IL-2v fusion protein, GI-αCD73 or AHP04167 was fused to IL-2v at the C-terminus of aglycosylated IgG4-Fc. All immunocytokines and

antibodies were produced in transiently transfected Expi-Chinese hamster ovary (Expi-CHO) cells or stable CHO-ZN cells. For the manufacturing of drug substance for GI-αCD73/IL-2v, research working cell bank (RWCB) clone #13 was used. EX-CELL Advanced CHO Fed-batch Medium was used as a basal medium for the seed expansion and the main culture. Cell Boost 7 a/Cell Boost 7b were supplemented as feed media in the main culture. Briefly, the frozen RWCB clone #13 was thawed and seed-expanded in flasks (for five passages) and in a wave bioreactor (for two passages). It was then transferred to and cultured in a 200 L single-use bioreactor. To harvest, the media underwent processing through Millistak+ HC Pod depth filtration, followed by 0.2 µm microfiltration to remove cells and debris. For the downstream process, the flow through was further subjected to three chromatographies (protein A affinity chromatography, multimodal exchange chromatography, hydrophobic interaction chromatography), one virus inactivation, depth filtration I/II, virus filtration, ultrafiltration/diafiltration, and formulation/bulk fill process step. The resulting drug substance was passed through a 0.22 µm PVDF filter and then analyzed by various analytical methods, including reduced capillary electrophoresis-sodium dodecyl sulfate and size exclusion-high performance liquid chromatography.

Cis-trans-binding phosphorylated signal transducer and activator of transcription 5 (pSTAT5) assay of GI-αCD73/IL-2v in CD8+ T cells

Human CD8+ T cells were isolated from human PBMCs using an EasySep Human CD8+ T Cell Isolation Kit (STEMCELL technologies) according to the manufacturer's protocol. Half of the isolated CD8+ T cells were labeled with 0.5 µM CellTrace Violet (CTV) (ThermoFisher Scientific). The CTV-unlabeled cells were further divided into two groups, and only one of them was pretreated with saturating concentrations (1000 nM) of GI-αCD73 for 2 hours at 4°C to block all CD73 proteins on the cell surface, followed by a washing step to remove any unbound GI-αCD73. Then, GI-αCD73 pretreated or untreated CTV-unlabeled cells were co-cultured with CTV-labeled cells at a 1:1 ratio. Co-cultured cells were then treated with 0.5 nM of GI-αCD73/IL-2v for 20 min at 37°C. After incubation, cells were fixed using pre-warmed Transcription Factor Phospho Fix/Perm buffer (BD Biosciences) for 12 min and permeabilized using precooled Phosflow Perm buffer III (BD Biosciences) for 30 min. Then, cells were intracellularly stained with anti-pSTAT5-AF647 (Clone 47/Stat5, BD Biosciences). Flow cytometric data were acquired using Cytek Aurora (Cytek Biosciences) and analyzed using FlowJo software (BD Biosciences).

RESULTS

CD73 blockade attenuates AMP-induced suppression of IL-2 signaling in CD8+ T cells

Despite the considerable attention given to cytokine-based immunotherapy in cancer treatment, IL-2 signaling under CD73-mediated adenosine-rich conditions has not been extensively studied. To investigate the effect of IL-2 on immunosuppression of CD8+ T cells induced by CD73-mediated adenosine production, human PBMCs stimulated by anti-CD3/CD28 were exposed to AMP as a substrate for CD73. In the presence of AMP, human IL-2 (hIL-2, Proleukin) failed to restore the proliferation of CD8+ T cells from human PBMCs (figure 1A), indicating an impairment in IL-2 signaling under adenosine-rich conditions. To determine whether the blockade of CD73 could reverse the non-responsiveness to IL-2 on AMP-mediated suppression of CD8+ T cell proliferation, we screened novel CD73 antibodies using human scFv phage library and immunized Alpaca phage library (figure 1B). We identified 64 monoclonal antibodies and 10 sdAbs with diverse binding potentials against human CD73 (online supplemental figure S1A,B). From these, we selected one monoclonal antibody (CSA0060/GI-αCD73) and two sdAbs (AHF10240 and AHP04167) based on their maximum inhibitory efficacy on soluble CD73 enzymatic activity (figure 1C, online supplemental figures S1C,D and S2A). When the enzymatic blockade assay was conducted against membrane-bound human CD73 using MDA-MB-231 cells with high CD73 expression, the three antibodies also potently inhibited the enzymatic activity of membrane-bound CD73 (figure 1D, online supplemental figure S2B). Importantly, treatment with GI-αCD73 partially alleviated AMP-induced suppression on CD8+ T cell proliferation, while treatment with two sdAbs did not reverse AMP-mediated CD8+ T cell immunosuppression (figure 1E). Furthermore, the combination of GI-αCD73 and hIL-2 further enhanced the reversal of AMP-mediated suppression on CD8+ T cell proliferation, as compared with treatment with GI-αCD73 or hIL-2 alone (figure 1E), suggesting a synergistic effect between CD73 blockade and IL-2 stimulation. Indeed, we found that the counteracting effect of GI-αCD73 on AMP-mediated suppression of CD8+ T cell proliferation was due to the restoration of T cell division without impacting cell viability (online supplemental figure S3A,B). However, the same synergistic effect was not observed when each of the CD73 sdAbs was combined with hIL-2 (figure 1E). Together, these findings suggest that GI-αCD73-mediated CD73 enzymatic blockade can alleviate suppressed IL-2 signaling in CD8+ T cells under CD73-mediated adenosine-rich conditions.

A novel GI-αCD73/IL-2v fusion protein alleviates CD73/adenosine-mediated suppression of IL-2 signaling in CD8+ T cells

Given that both CD73 and IL-2R complex are expressed in CD8+ T cells, and that fusion of IL-2 to anti-CD73 antibody contributes to increased targeting into CD73+

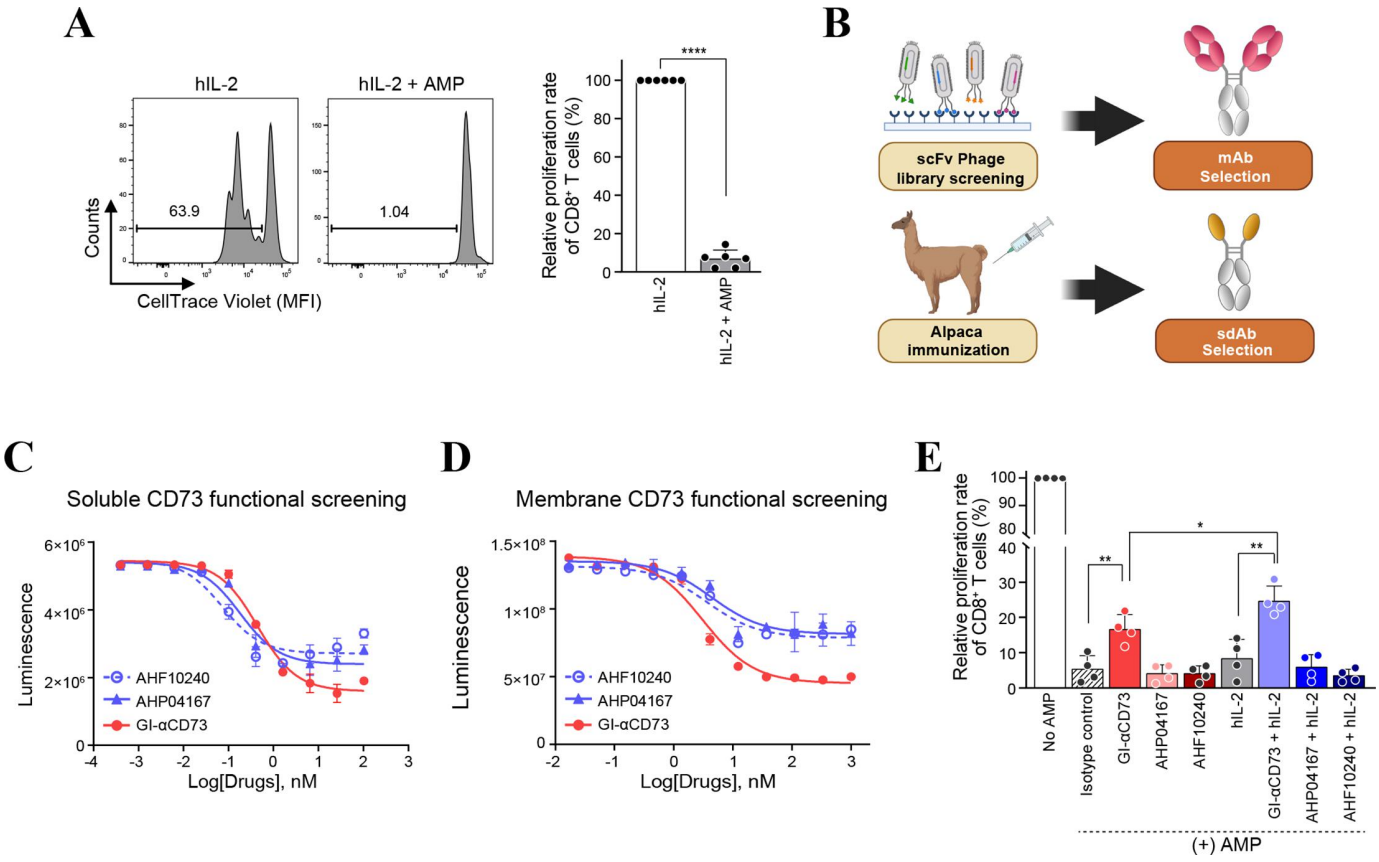
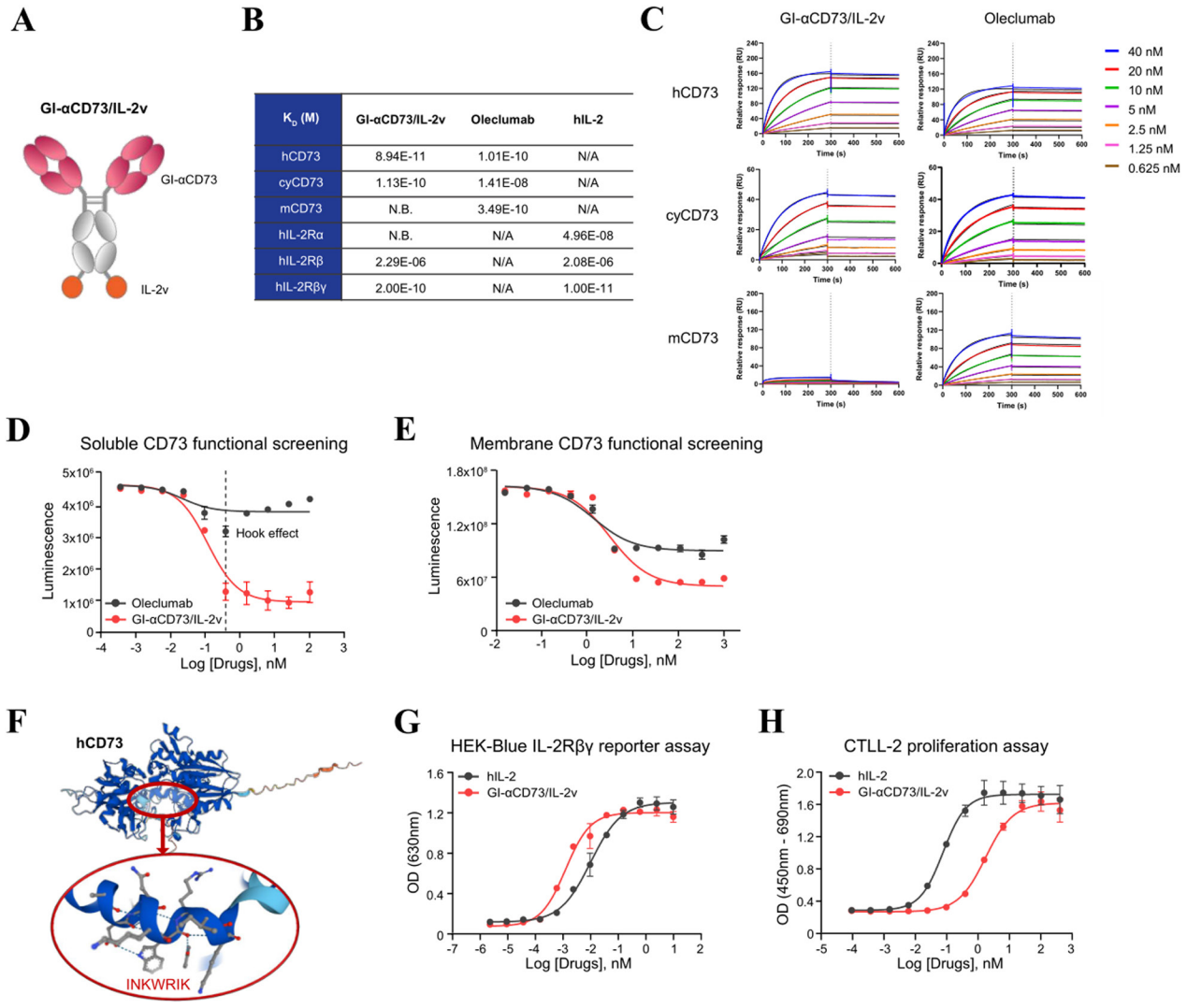


Figure 1 AMP-induced suppression of IL-2 signaling in CD8+ T cells is alleviated by treatment with GI-αCD73 antibody. (A) Flow cytometry analysis of CTV-labeled CD8+ T cells from human PBMCs incubated with hIL-2 (50 nM) in the presence of anti-CD3/CD28 beads at a cell-to-bead ratio of 1:1 and AMP at 0 or 500 µM for 3 days. The left panel shows a representative flow cytometry plot. The right panel presents data as mean±SEM of independent experiments using six healthy human PBMC donors. (B) Schematic representation of the development of anti-CD73 antibodies. (C, D) Enzymatic activity of soluble CD73 (C) and membrane-bound CD73 (D). Catalytic activity of soluble CD73 was measured using recombinant human CD73 protein incubated with serially diluted GI-αCD73, AHF10240, or AHP04167 by adding 10 µM AMP for 1 hour. Catalytic activity of membrane-bound CD73 was assessed using MDA-MB-231 cells incubated with serially diluted GI-αCD73, AHF10240, or AHP04167 by adding 200 µM AMP for 6 hours. Data are presented as mean±SEM of experiments performed in triplicate. (E) Frequency of CTV^{low}CD8+ T cells determined by flow cytometric analysis in human PBMCs incubated with indicated anti-CD73 antibodies (50 nM), hIL-2 (50 nM) or IgG4-Fc (isotype control, 50 nM) in the presence of anti-CD3/CD28 bead at a cell-to-bead ratio of 1:1 and AMP at 0 or 500 µM for 3 days. Data are shown as mean±SEM of experiments performed with four healthy human PBMC donors. *p<0.05, **p<0.01, ****p<0.0001 by unpaired t-test. PBMC, peripheral blood mononuclear cell.

tumors, we engineered a novel GI-αCD73/IL-2v bifunctional protein with GI-αCD73 fused to IL-2v (R38A/F42A/E61R) (with a lack in binding to IL-2Rα via three amino acid modification compared with the wild-type IL-2) (figure 2A). Surface plasmon resonance analysis revealed that GI-αCD73/IL-2v fusion protein had slightly greater binding affinity against human CD73 compared with Oleclumab (MEDI9447, most notable anti-CD73 antibody) (figure 2B,C). GI-αCD73/IL-2v also showed stronger binding affinity to cynomolgus monkey CD73, while it did not have binding potential against mouse CD73 (figure 2B,C). When we conducted enzymatic blockade assays against soluble human CD73 or membrane-bound human CD73 using MDA-MB-231 cells, GI-αCD73/IL-2v exhibited greater maximum inhibitory potential than Oleclumab (figure 2D,E, online supplemental figure S4A,B). Moreover, GI-αCD73/IL-2v did not display the hook effect which was observed in Oleclumab treatment due to the stoichiometry of antigen-antibody complexes (figure 2D). Additionally, epitope mapping analysis revealed that GI-αCD73 recognized a highly flexible α-helical linker (INKWRIK) between the N-terminal and the C-terminal domains of CD73 (figure 2F, online supplemental figure S5). Furthermore, competitive epitope binning assay showed that the binding epitope of GI-αCD73/IL-2v on human CD73 was different from that of Oleclumab (online supplemental figure S6). From these results, we speculated that the absence of the hook effect in GI-αCD73/IL-2v fusion protein is likely attributed to differences in the binding epitopes compared with Oleclumab. We then evaluated the activation of IL-2 signaling by GI-αCD73/IL-2v. In HEK293-IL-2 reporter cells expressing IL-2Rβγ, GI-αCD73/IL-2v induced IL-2 activation at a lower concentration than hIL-2 (figure 2G). In contrast, in CTLL-2 cells expressing



K_D (M)	GI-αCD73/IL-2v	Oleclumab	hIL-2
hCD73	8.94E-11	1.01E-10	N/A
cyCD73	1.13E-10	1.41E-08	N/A
mCD73	N.B.	3.49E-10	N/A
hIL-2Rα	N.B.	N/A	4.96E-08
hIL-2Rβ	2.29E-06	N/A	2.08E-06
hIL-2Rβγ	2.00E-10	N/A	1.00E-11

Figure 2 GI-αCD73/IL-2v effectively inhibits CD73 enzymatic activity and activates IL-2 signaling. (A) Schematic diagram of GI-αCD73/IL-2v bifunctional protein. (B) Binding affinity (K_D) of GI-αCD73/IL-2v, Oleclumab or hIL-2 to human CD73 (hCD73), cynomolgus monkey CD73 (cyCD73), mouse CD73 (mCD73), human IL-2Rα (hIL-2Rα), human IL-2Rβ (hIL-2Rβ) or human IL-2Rβγ (hIL-2Rβγ), measured using SPR analysis. (C) SPR sensorgrams of binding experiments with GI-αCD73/IL-2v or Oleclumab to hCD73, cyCD73 or mCD73. (D, E) Enzymatic activity of soluble CD73 (D) and membrane-bound CD73 (E). CD73 catalytic activity in the presence of serially diluted GI-αCD73/IL-2v or Oleclumab was measured using recombinant human CD73 protein or MDA-MB-231 cells with the addition of 10 µM AMP for 1 hour or 200 µM AMP for 6 hours, respectively. The hook effect was observed with high concentrations of Oleclumab. Data are presented as mean±SEM of experiments performed in triplicate. (F) Overall predicted structure view of GI-αCD73 binding site in hCD73 protein. (G) Functional activity in HEK-Blue IL-2 reporter cells expressing IL-2Rβγ complex incubated with serially diluted GI-αCD73/IL-2v or hIL-2 for 24 hours. Data are presented as mean±SEM of experiments performed in triplicate. (H) Proliferation of CTLL-2 cells expressing IL-2Rαβγ complex incubated with serially diluted GI-αCD73/IL-2v or hIL-2 for 24 hours. Data are presented as mean±SEM of experiments performed in triplicate. N/A, not applicable; N.B., no binding; SPR, surface plasmon resonance.

IL-2Rαβγ, hIL-2 induced the proliferation of the CTLL-2 cells at a lower concentration than GI-αCD73/IL-2v (figure 2H), which is probably due to the lack of binding of GI-αCD73/IL-2v to IL-2Rα. These results implicate GI-αCD73/IL-2v's preferential interaction with IL-2Rβγ over IL-2Rαβγ.

We next investigated whether the GI-αCD73/IL-2v could reverse the adenosine-mediated suppression of $CD8^+$ T cells. The addition of AMP to a culture of human PBMCs stimulated with anti-CD3/CD28 reduced the percentage of $CD8^+$ T cell proliferation from 73.5% to 4.68%, however, the addition of GI-αCD73/IL-2v restored the $CD8^+$ T cell proliferation to 53.2% (figure 3A). Notably, the effect of GI-αCD73/IL-2v on $CD8^+$ T cell proliferation was significantly greater than that of GI-αCD73 monotherapy, Fc-IL-2v monotherapy, or their combined treatment (figure 3A,B), indicating the advantages of the bispecific nature of GI-αCD73/IL-2v, simultaneously blocking CD73 and activating IL-2 within a single molecule. In contrast, single treatment of Oleclumab did not increase the proliferation of $CD8^+$ T cells under AMP-induced suppression, and the effect

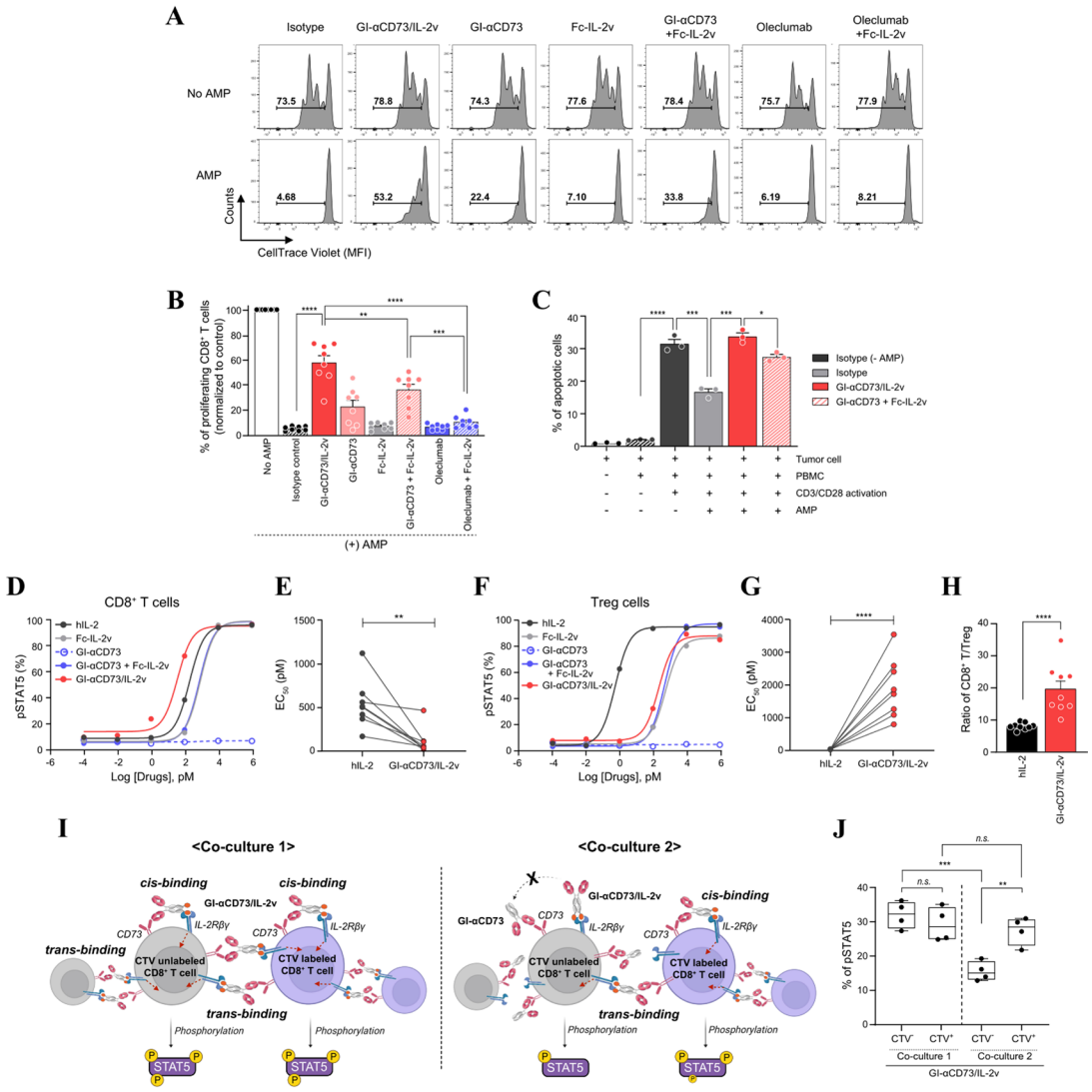


Figure 3 GI-αCD73/IL-2v reverses AMP-induced suppression on IL-2 signaling in CD8$^+$ T cells via *cis*-binding. (A, B) Flow cytometry analysis of CTV-labeled CD8$^+$ T cells from PBMCs incubated with 50 nM of either IgG4-Fc (isotype), GI-αCD73/IL-2v, GI-αCD73, Fc-IL-2v, combination of GI-αCD73 and Fc-IL-2v, Oleclumab, or combination of Oleclumab and Fc-IL-2v in the presence of αCD3/CD28 dynabeads at a cell-to-bead ratio of 1:1 and AMP at 500 µM for 3 days. Representative flow cytometric plots are presented from eight independent experiments conducted with human PBMCs from different healthy donors (A), and bar graph data are presented as mean±SEM (B). (C) Frequency of apoptotic CTV-labeled MDA-MB-231 that have undergone T cell-mediated apoptosis. PBMCs were preincubated with indicated immunocytokines or antibodies at 50 nM in the presence of αCD3/CD28 dynabeads at a cell-to-bead ratio of 1:1 or AMP at 0 or 500 µM for 2 days. After co-culturing for 24 hours at an effector-to-target ratio of 10:1, the percentage of apoptotic cells was determined by analyzing the annexin V positive and 7-AAD positive cells using flow cytometry. Data are shown as mean±SEM of experiments performed in triplicate. (D) Dose-response curves showing pSTAT5 levels in naïve CD8$^+$ T cells from human PBMCs following a 20 min stimulation with hIL-2, Fc-IL-2v, GI-αCD73, GI-αCD73/IL-2v or combination of GI-αCD73 and Fc-IL-2v. Data are representative of two independent experiments using human PBMCs from different healthy donors. (E) EC_{50} values of pSTAT5 in naïve CD8$^+$ T cells from eight healthy human PBMCs stimulated with hIL-2 or GI-αCD73/IL-2v for 20 min. (F) Dose-response curves showing pSTAT5 levels in Treg cells from human PBMCs stimulated for 20 min with hIL-2, Fc-IL-2v, GI-αCD73, GI-αCD73/IL-2v or combination of GI-αCD73 and Fc-IL-2v. Data are representative of two independent experiments using human PBMCs from different healthy donors. (G) EC_{50} values of pSTAT5 in Treg cells from eight healthy human PBMCs stimulated with hIL-2 or GI-αCD73/IL-2v for 20 min. (H) Ratio of CD8$^+$ T cells/Treg cells from human PBMCs incubated with 10 nM of hIL-2 or GI-αCD73/IL-2v in the presence of αCD3/CD28 dynabeads at a cell-to-bead ratio of 1:1 for 12 days. Three independent experiments were conducted using different healthy human PBMC donors. Representative data are presented as mean±SEM of nine measurements from one representative PBMC donor. (I) Schematic diagram illustrating cis-trans-binding-induced enhancement of pSTAT5 on purified CD8$^+$ T cells stimulated with GI-αCD73/IL-2v. GI-αCD73 is treated in CTV-unlabeled CD8$^+$ T cells under only the co-culture 2 condition but not the co-culture 1 condition. (J) Frequency of pSTAT5$^+$CD8$^+$ T cells following exposure of 0.5 nM GI-αCD73/IL-2v for 20 min in unblocked-CTV-unlabeled CD8$^+$ T cells or GI-αCD73-preblocked CTV-unlabeled CD8$^+$ T cells co-cultured with CTV-labeled CD8$^+$ T cells. Data are presented as mean±SEM of experiments performed with purified CD8$^+$ T cells from four healthy donors. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by unpaired t-test. n.s., not significant; PBMC, peripheral blood mononuclear cell.

of the combined treatment of Oleclumab and IL-2v on reversal of CD8+ T cell proliferation was significantly lower than that of the combined treatment of GI-αCD73 and Fc-IL-2v (figure 3A,B). These results indicate that Oleclumab cannot effectively restore CD8+ T cell proliferation under adenosine-mediated suppression. We also found that the counteracting effect of GI-αCD73/IL-2v on AMP-mediated suppression of CD8+ T cell proliferation was attributable to restored T cell division rather than cell viability (online supplemental figure S7A,B).

We next assessed the effect of GI-αCD73/IL-2v on T cell-mediated tumor cell killing using a co-culture system with MDA-MB-231 cells and healthy human PBMCs in the presence of anti-CD3/CD28 dynabead and AMP. When exposed to AMP, a substantial decrease in tumor cell apoptosis occurred (figure 3C). In comparison, GI-αCD73/IL-2v treatment significantly enhanced the tumor cell killing by PBMCs, surpassing the increase observed from the combined treatment of GI-αCD73 and Fc-IL-2v (figure 3C). This observation further supports the advantages of bispecific targeting of CD73 and IL-2. Taken together, these results demonstrate that the anti-CD73/IL-2v fusion protein effectively restores adenosine-mediated impairment of IL-2 signaling in CD8+ T cells.

Cis-binding of GI-αCD73/IL-2v enhances IL-2-mediated signaling in CD8+ T cells

To understand the molecular mechanism underlying the observed advantages of the bispecific targeting of CD73 and IL-2, we assessed the activation of STAT5, a well-known downstream target for IL-2 signaling. Specifically, the changes of pSTAT5 in CD8+ T cells and Treg cells from healthy human PBMCs were measured following treatment with GI-αCD73/IL-2v, GI-αCD73, Fc-IL-2v, or the combination of GI-αCD73 and Fc-IL-2v. The representative half maximal effective concentration (EC_{50}) value for STAT5 phosphorylation in CD8+ T cells was substantially lower following treatment with GI-αCD73/IL-2v compared with all other treatments (figure 3D), signifying the superior effect of GI-αCD73/IL-2v on STAT5 activation in CD8+ T cells. The superior efficacy of GI-αCD73/IL-2v in inducing STAT5 phosphorylation in CD8+ T cells was consistently observed even when compared with hIL-2 (figure 3D,E). In contrast, treatment with GI-αCD73/IL-2v resulted in lower levels of STAT5 activation in Treg cells than hIL-2 (figure 3F,G), probably due to the lack of binding of GI-αCD73/IL-2v to IL-2Rα. Consistent with the effects observed after short-term treatment with GI-αCD73/IL-2v on pSTAT5 (figure 3D–G), a 12-day long cultivation of PBMCs with GI-αCD73/IL-2v in the presence of anti-CD3/CD28 dynabeads also demonstrated that GI-αCD73/IL-2v displayed a preference for enhancing the proliferation of CD8+ T cells over Treg cell proliferation compared with hIL-2 (figure 3H).

Since a subset of CD8+ T cells coexpresses CD73 and IL-2R complex, we hypothesized that GI-αCD73/IL-2v binding to CD73 on CD8+ T cells enables the delivery of IL-2v to IL-2Rβγ on the same or neighboring cells. This hypothesis could explain the greater potency of GI-αCD73/IL-2v on activating IL-2 signaling in CD8+ T cells compared with the combination treatment of GI-αCD73 with Fc-IL-2v. To test this hypothesis, we conducted a “cis-trans-binding assay” using a mixture of CTV-labeled and unlabeled CD8+ T cells. First, CTV+ and CTV− CD8+ T cells were mixed without any prior exposure to anti-CD73 antibody and were subjected to GI-αCD73/IL-2v (Co-culture 1) (figure 3I, left panel). When STAT5 activation was quantified and normalized to the level observed from the CTV− cells, the levels of STAT5 phosphorylation in the CTV+ and CTV− CD8+ T cells were comparable, as expected (figure 3J). In the same experiment, another co-culture of CTV+ and CTV− CD8+ T cells was prepared, but this time, the CTV− CD8+ T cells were pre-exposed to a saturating concentration of parental anti-CD73 antibody (GI-αCD73) to block the effect of CD73 engagement by GI-αCD73/IL-2v on the IL-2 signaling (co-culture 2) (figure 3I, right panel). When STAT5 activation was measured after GI-αCD73/IL-2v treatment, a significant difference in STAT5 activation was observed between the CTV+ and CTV− CD8+ T cells (figure 3J). Specifically, the level of STAT5 phosphorylation was reduced by half in anti-CD73 pre-exposed CTV− cells compared with that in the CTV+ cells within the same co-culture, or in CTV− cells with no pre-exposure of anti-CD73. These results suggest that the cis-binding of GI-αCD73/IL-2v to CD73 and IL-2Rβγ on the same cells contributes more significantly to the enhancement of IL-2 signaling in CD8+ T cells than the trans-binding to neighboring cells.

GI-αCD73/IL-2v exerts in vivo antitumor activity primarily by targeting and activating CD8+ T cells

Since GI-αCD73, the N-terminus of GI-αCD73/IL-2v, had no binding affinity to murine CD73 (figure 2B,C), we evaluated the in vivo antitumor potency of GI-αCD73/IL-2v in human PBMC-engrafted humanized mice. These mice were orthotopically implanted with MDA-MB-231 showing high CD73 expression and were treated with IgG4-Fc control, GI-αCD73/IL-2v, AHP04167/IL-2v, or anti-PD-1. Treatment with GI-αCD73/IL-2v led to a significant reduction in tumor size and an increased frequency of mice with tumor growth inhibition (TGI) >80%, compared with IgG4-Fc or anti-PD-1 treatment (figure 4A). Importantly, no significant changes in body weight were observed in the mice before and after each treatment in all groups (data not shown). Interestingly, GI-αCD73/IL-2v appeared to have a slightly greater antitumor efficacy in terms of tumor size reduction and frequency of mice with TGI>80% compared with the IL-2v-fused bispecific protein with AHP04167,

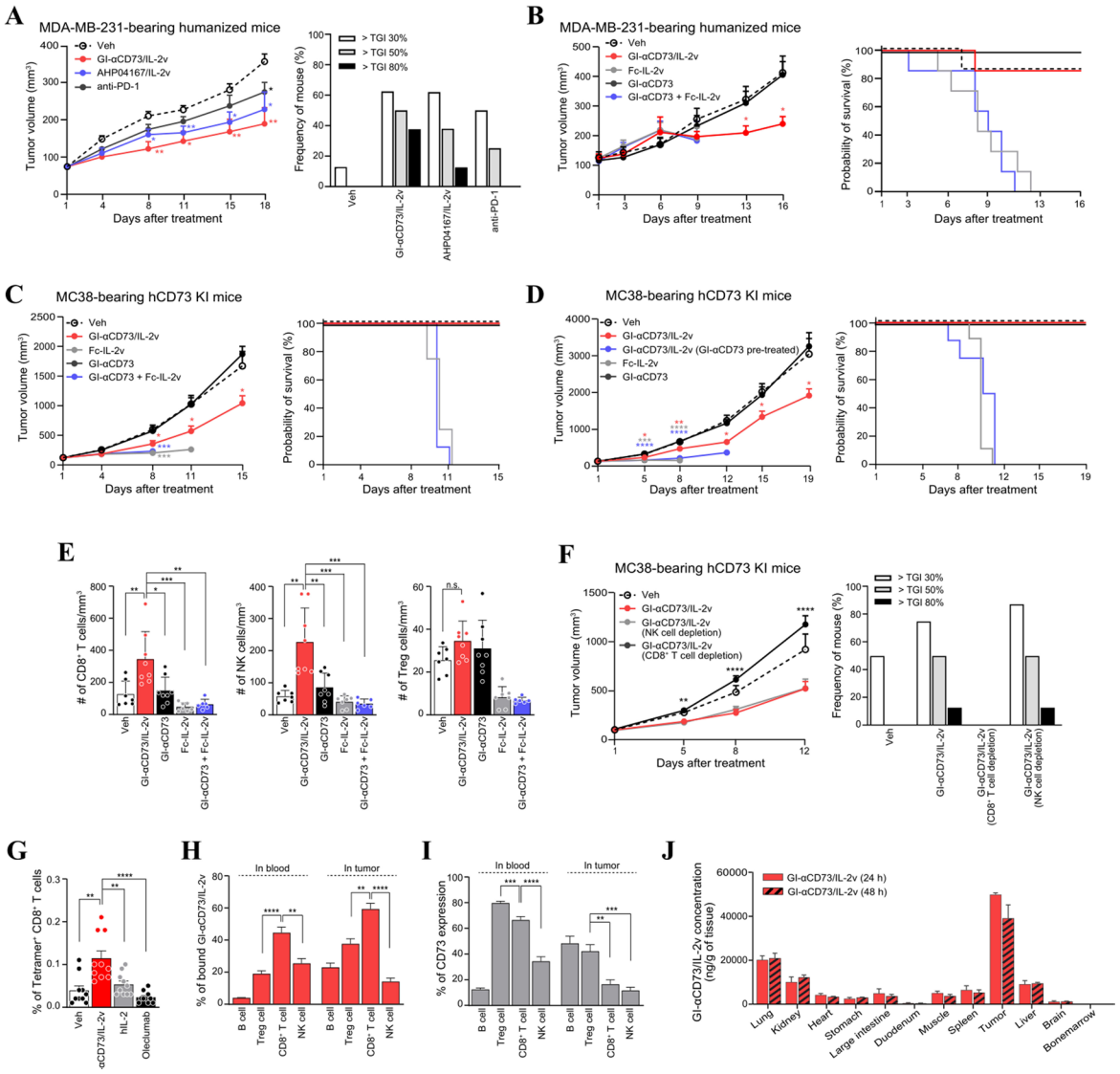
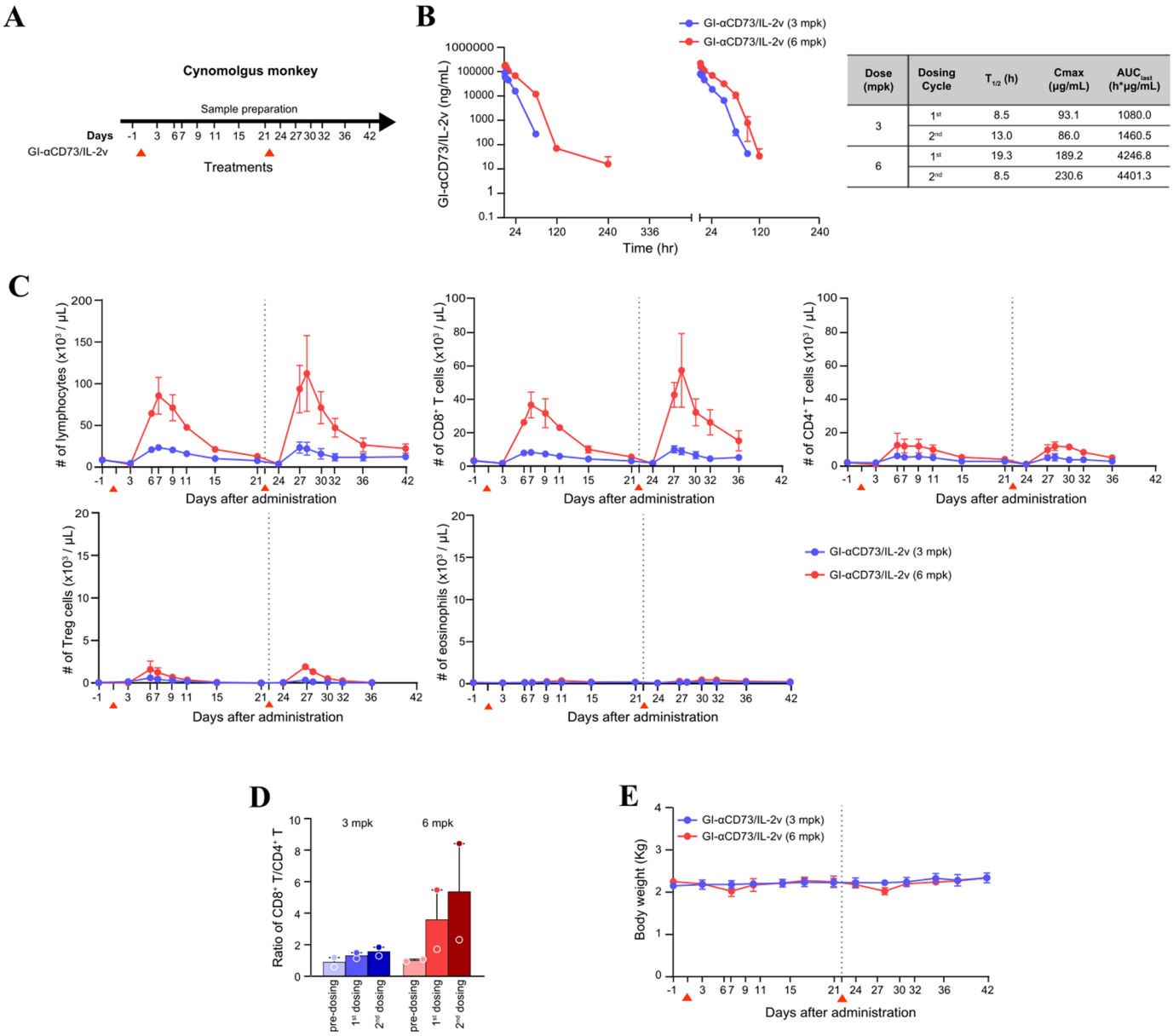


Figure 4 GI-αCD73/IL-2v elicits the in vivo antitumor activity primarily by targeting and activating CD8$^+$ T cells. (A) Average tumor volume (left panel) and frequency of mice with indicated TGI percentage (right panel) in treatment groups of MDA-MB-231-bearing PBMC-humanized mice. Mice were randomly divided into four treatment groups (n=8/group) and were administered with GI-αCD73/IL-2v (6 mg/kg, once a week), AHP04167/IL-2v (6 mg/kg, once a week), control IgG4-Fc (Veh, 6 mg/kg, once a week) or anti-PD-1 (Pembrolizumab, 5 mg/kg, twice a week) for indicated periods. Data are presented as mean±SEM. (B, C) Average tumor volume (left panel) and survival curve (right panel) in treatment groups of MDA-MB-231-bearing PBMC-humanized mice (B, n=7/group) and MC38-bearing hCD73 KI mice (C, n=8/group). Both mice were administered with GI-αCD73/IL-2v (5 mg/kg, once a week), GI-αCD73 (4.2 mg/kg, once a week), Fc-IL-2v (2.5 mg/kg, once a week), combination of GI-αCD73 and Fc-IL-2v (4.2 mg/kg and 2.5 mg/kg, once a week), or control IgG4-Fc (Veh, 1.6 mg/kg, once a week) for indicated periods. Data are presented as mean±SEM. (D) Average tumor volume (left panel) and survival curve (right panel) in MC38-bearing hCD73 KI mice (n=8/group) with 5 mg/kg GI-αCD73/IL-2v administration in the presence or absence of pretreatment with GI-αCD73 (50 mg/kg, once a week). Data are presented as mean±SEM. (E) Tumor-infiltrating lymphocyte analysis in tumors from treatment groups of MC38-bearing hCD73 KI mice. The numbers of CD8$^+$ T cells, NK cells, or Treg cells were quantified in each treatment group (n=6–8/group). (F) Average tumor volume (left panel) and frequency of mice with indicated TGI percentage (right panel) in MC38-bearing hCD73 KI mice (n=8/group) pretreated with anti-CD8 or anti-NK1.1 following the administration of GI-αCD73/IL-2v (5 mg/kg, once a week) or IgG4-Fc (Veh, 1.6 mg/kg, once a week). Data are presented as mean±SEM. (G) The number of adpgk tetramer-specific CD8$^+$ T cells in the peripheral blood of MC38-bearing hCD73 KI mice (n=10/group) treated with GI-αCD73/IL-2v (5 mg/kg, once), Oleclumab (5 mg/kg, once), IgG4-Fc (1.6 mg/kg, once) or hIL-2 (0.85 mg/kg, once daily for 5 days). (H) Proportion of GI-αCD73/IL-2v-bound immune cells in the blood and tumors from MC38-bearing hCD73 KI mice (n=8/group) treated with GI-αCD73/IL-2v (3 mg/kg). (I) Proportion of CD73-expressing immune cells in the blood and tumors from MC38-bearing hCD73 KI mice (n=8/group). (J) Tissue distribution analysis of GI-αCD73/IL-2v after 24 hours or 48 hours of administration of GI-αCD73/IL-2v (15.2 mg/kg) into MC38^{hCD73}-bearing hCD73 KI mice (n=3/group). In the in vivo tumor volume analysis, the p value was evaluated by comparing the Veh group to the indicated treatment group, except for the p value in F comparing GI-αCD73/IL-2v to the combination of GI-αCD73/IL-2v and CD8$^+$ T cell depletion. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by unpaired t-test. PBMC, peripheral blood mononuclear cell.



Dose (mpk)	Dosing Cycle	$T_{1/2}$ (h)	Cmax (μg/mL)	AUC_{last} (h*μg/mL)
3	1st	8.5	93.1	1080.0
	2nd	13.0	86.0	1460.5
6	1st	19.3	189.2	4246.8
	2nd	8.5	230.6	4401.3

Figure 5 GI-αCD73/IL-2v is well tolerated and elicits durable immune responses in non-human primates. (A) Study design for evaluating the tolerance and immune response to GI-αCD73/IL-2v in cynomolgus monkeys. Cynomolgus monkeys were intravenously administered with 3 or 6 mg/kg of GI-αCD73/IL-2v every 3 weeks for 2 cycles (n=2/group). (B) Serum concentration-time profiles (left panel) and pharmacokinetic parameters (right panel) of GI-αCD73/IL-2v in cynomolgus monkeys after intravenous administration of GI-αCD73/IL-2v (3 or 6 mg/kg). Pharmacokinetic parameters including terminal half-life ($T_{1/2}$), maximum serum concentration (C_{max}) or area under the concentration-time curve from 0 hour to the last measurable time (AUC_{last}) were determined based on serum concentration-time profiles of GI-αCD73/IL-2v. Data in the left panel are presented as mean±SEM, and the right panel is presented as mean value. (C) Pharmacodynamic profiles in PBMCs of cynomolgus monkeys after intravenous administration of GI-αCD73/IL-2v (3 or 6 mg/kg). The number of total lymphocytes, $CD8^+$ T cells, $CD4^+$ T cells, Treg cells, or eosinophils was measured using flow cytometry. Data are presented as mean±SEM. (D) The ratio of $CD8^+$ T/$CD4^+$ T in PBMCs of cynomolgus monkeys before and after administration of GI-αCD73/IL-2v (3 or 6 mg/kg dose). Data are presented as mean±SEM. (E) Average body weight change in cynomolgus monkeys after intravenous administration of GI-αCD73/IL-2v (3 or 6 mg/kg). Data are presented as mean±SEM. Red arrowheads indicate time points of GI-αCD73/IL-2v administration. PBMC, peripheral blood mononuclear cell.

which retains binding potential to CD73 but lacks the capacity to counteract AMP-induced suppression of $CD8^+$ T cell proliferation (figure 4A), suggesting the importance of the αCD73 component's functional activity in the antitumor effect of GI-αCD73/IL-2v. We next evaluated the advantages of the bispecific nature of GI-αCD73/IL-2v on its in vivo antitumor effect in MDA-MB-231-bearing PBMC humanized mice. Notably, while mice receiving monotherapy with Fc-IL-2v or combination therapy with GI-αCD73 and Fc-IL-2v succumbed after the second injection, those

treated with GI-αCD73/IL-2v survived with a significant reduction in tumor size (figure 4B), supporting the potential of this bispecific fusion protein as a therapeutic strategy. Consistent with these findings in humanized mice, we also observed improved survival and enhanced antitumor efficacy with GI-αCD73/IL-2v fusion protein in MC38-bearing hCD73 KI mice compared with monotherapy or combination therapy (figure 4C). Additionally, when these mice were pretreated with GI-αCD73 prior to GI-αCD73/IL-2v administration, all mice succumbed within 1–2 weeks, showing a survival pattern similar to that of mice treated with Fc-IL-2v monotherapy or combination therapy (figure 4D), further supporting the functional importance of the αCD73 component in GI-αCD73/IL-2v's in vivo antitumor effect in MC38-bearing hCD73 KI mice.

To further investigate the impact of GI-αCD73/IL-2v on immune cell population within the tumors, we isolated and analyzed tumor-infiltrating lymphocytes from tumors of MC38-bearing hCD73 KI mice. $CD8^+$ T cell and NK cell infiltration was increased in tumors from GI-αCD73/IL-2v-treated mice compared with tumors treated with IgG4-Fc control, monotherapy, or combination therapy, while Treg cell infiltration showed no significant difference between GI-αCD73/IL-2v and IgG4-Fc control (figure 4E). These findings suggest that increased infiltration of $CD8^+$ T cells and NK cells contributes to the antitumor efficacy induced by GI-αCD73/IL-2v. To determine which of the two cell types is responsible for the in vivo therapeutic effects of GI-αCD73/IL-2v, we treated MC38-bearing hCD73 KI mice with anti-CD8 (to deplete $CD8^+$ T cells) or anti-NK1.1 (to deplete NK cells) prior to GI-αCD73/IL-2v administration and confirmed the successful depletion of the respective immune cells in treated mice (online supplemental figure S8A,B). GI-αCD73/IL-2v-induced tumor size reduction was remarkably less pronounced in mice treated with anti-CD8 but not in those treated with anti-NK1.1 (figure 4F), indicating the primary contribution of $CD8^+$ T cells to the in vivo antitumor efficacy of GI-αCD73/IL-2v. We then analyzed tumor-specific $CD8^+$ T cell response in MC38-bearing hCD73 KI mice treated with GI-αCD73/IL-2v using an MHC-I $H\text{-}2D^b$-restricted Adpgk neoantigen tetramer peptide, which is widely reported and used in the MC38 syngeneic model.[54] Tumor-specific $CD8^+$ T cell populations were increased in the peripheral blood of MC38-bearing hCD73 KI mice following GI-αCD73/IL-2v administration, but not after treatment with hIL-2 or anti-CD73 antibody, Oleclumab (figure 4G), suggesting that GI-αCD73/IL-2v induces endogenous $CD8^+$ T cell responses against tumor neoantigens, resulting in superior antitumor efficacy.

Next, we evaluated in vivo delivery of GI-αCD73/IL-2v to immune cells, specifically $CD8^+$ T cells, in MC38-bearing hCD73 KI mice following GI-αCD73/IL-2v treatment. Since CD73 has been reported to be expressed in various immune cells, including $CD8^+$ T cells, Treg cells, NK cells, and B cells,[27 28] we also investigated CD73 expression levels in these immune cells from the blood and tumors of MC38-bearing hCD73 KI mice, as well as the in vivo distribution of GI-αCD73/IL-2v among these cell types from the blood and tumors of MC38-bearing hCD73 KI mice treated with GI-αCD73/IL-2v. Notably, in vivo binding proportion of GI-αCD73/IL-2v was substantially higher in $CD8^+$ T cells than in Treg cells, even though the proportion of CD73-expressing $CD8^+$ T cells was moderately lower than that of CD73-expressing Treg cells in both blood and tumors (figure 4H,I). Additionally, the in vivo binding proportion of GI-αCD73/IL-2v in $CD8^+$ T cells was also higher than in NK cells (figure 4H). These results suggest that $CD8^+$ T cells are a primary in vivo target of GI-αCD73/IL-2v. In addition to targeting CD73-expressing immune cells, we investigated the potential distribution of GI-αCD73/IL-2v to CD73-expressing tumor cells using hCD73-expressing MC38 ($MC38^{hCD73}$)-bearing hCD73 KI mice. Tissue distribution analysis revealed that GI-αCD73/IL-2v was predominantly accumulated in $MC38^{hCD73}$ tumors rather than in other organs (figure 4J), suggesting targeted delivery of GI-αCD73/IL-2v to tumors with high CD73 expression. Furthermore, the direct trans-binding potential of GI-αCD73/IL-2v to tumor cells was observed under co-culture conditions with MDA-MB-231 (expressing only CD73, not IL-2Rβγ) and HEK293-IL-2Rβγ (expressing IL-2Rβγ, not CD73) (online supplemental figure S9). Taken together, all these findings indicate that GI-αCD73/IL-2v preferentially, though not exclusively, targets $CD8^+$ T cells, driving its antitumor effect primarily through $CD8^+$ T cell activation.

GI-αCD73/IL-2v is well tolerated and induces durable immune responses in non-human primates

Since the αCD73 portion of GI-αCD73/IL-2v was cross-reactive to cynomolgus CD73 (figure 2B,C), we investigated the pharmacokinetics, pharmacodynamics, and safety of GI-αCD73/IL-2v in cynomolgus monkeys. The drug substance of GI-αCD73/IL-2v was produced in a CHO-ZN stable clone, purified, and characterized under non-GMP conditions (online supplemental figure S10). For the repeated dose study, cynomolgus monkeys received intravenous administration of 3 or 6 mg/kg of GI-αCD73/IL-2v once every 3 weeks for two cycles (figure 5A). Pharmacokinetic analysis revealed that the serum concentration of GI-αCD73/IL-2v declined in a multiexponential manner with a mean terminal half-life ($T_{1/2}$) of around 19.3 hours after the first dosing and 8.5 hours after the second dosing at 6 mg/kg of GI-αCD73/IL-2v (figure 5B). We next evaluated the pharmacodynamic effects of GI-αCD73/IL-2v on various immune cell types including $CD4^+$ T cells and $CD8^+$ T cells. GI-αCD73/IL-2v led to substantial expansions of total lymphocytes and $CD8^+$ T cells with peak levels at 6–7 days after

the first administration in monkeys with doses of 3 or 6 mg/kg (figure 5C). Similar patterns of expansions were observed after the second dosing (figure 5C), indicating durable immune responses of GI-αCD73/IL-2v in cynomolgus monkeys. $CD4^+$ T cells and Treg cells were also increased but to a lesser extent compared with $CD8^+$ T cells following GI-αCD73/IL-2v administration (figure 5C). Consequently, the ratio of $CD8^+$ T cells/$CD4^+$ T cells was higher after GI-αCD73/IL-2v dosing compared with pre-dosing levels in monkeys (figure 5D). However, there were no significant changes in eosinophil numbers in cynomolgus monkeys before and after administration of 3 or 6 mg/kg of GI-αCD73/IL-2v (figure 5C), and hematotoxicity or clinical signs, except diarrhea, were not observed (data not shown). Furthermore, histopathological and plasma biomarker analyses revealed no significant tissue damage in monkeys following GI-αCD73/IL-2v administration, despite minimal to mild mononuclear cell infiltration, which is a known pharmacological effect of the IL-2 cytokine molecule (online supplemental figure S11). While baseline values of body weight were slightly decreased at 7 days after the first and second administration of 6 mg/kg GI-αCD73/IL-2v in monkeys, the decreased body weight completely recovered after 9–11 days following each administration (figure 5E), indicating tolerability and safety of GI-αCD73/IL-2v. Furthermore, when we conducted a cytokine release assay using human PBMCs, GI-αCD73/IL-2v showed little to no effects on the production of cytokines, including tumor necrosis factor α (TNFα), IL-1β, interferon γ (IFNγ), IL-4, IL-6 or IL-8 in the absence of anti-CD3 treatment, which contrasted with those of hIL-2 at high doses (figure 6A). Under conditions with anti-CD3 stimulus, GI-αCD73/IL-2v exposure also led to lower levels of cytokine production compared with hIL-2 (figure 6A). Notably, the frequency of receptor occupancy of GI-αCD73/IL-2v against surface CD73 on $CD8^+$ T cells from human PBMCs ranged from 62% at 1 nM to 98% at 10 nM (figure 6B). In concentrations of 1–10 nM, GI-αCD73/IL-2v had remarkably low effect on the production of cytokines associated with cytokine release syndrome (figure 6A), suggesting that GI-αCD73/IL-2v may elicit both CD73-targeting efficacy and IL-2 functionality without inducing systemic toxicity. Finally, we investigated the "on-target, off-tumor" effect of GI-αCD73/IL-2v on normal cells expressing CD73. Despite the elevated expression of CD73 on human endothelial cells, we did not observe vascular leak in human umbilical vein endothelial cells (HUVECs) treated with GI-αCD73/IL-2v (figure 6C), indicating a low potential of GI-αCD73/IL-2v on "on-target, off-tumor" toxicity. Altogether, these results suggest that GI-αCD73/IL-2v is well tolerated and induces significant expansion of cytotoxic immune cells in cynomolgus monkeys.

DISCUSSION

The CD73-adenosine axis contributes to tumor progression by inducing multilayered immunosuppression through its influence on various target cells, including immune cells, tumor cells, and stromal cells within the TME.[30] Especially, recent numerous studies have suggested that the overexpression of CD73 in cytotoxic lymphocytes weakens their antitumor activity,[28] whereas deficiency of the CD73-adenosine axis in cytotoxic lymphocytes enhances antitumor immune responses.[10 27] Furthermore, an elevated infiltration of $CD73^{high}CD8^{high}$ T cells has been reported to be a potent prognostic biomarker for overall survival in patients with bladder cancer.[37] In addition, the antitumor effect of IL-2 immunocytokine, achieved through the activation of cytotoxic lymphocytes such as $CD8^+$ T cells and NK cells, has been widely recognized.[38] Despite this evidence implicating the role of IL-2 signaling and the CD73-adenosine axis in cancer therapeutic strategies, a comprehensive understanding of the relationship between these mechanisms has not been elucidated. In the current study, we observed that CD73 blockade restored the defective IL-2 signaling in $CD8^+$ T cells. Furthermore, CD73-targeted immunocytokine, consisting of anti-CD73 and IL-2v, demonstrated strong potential for tumor regression by activating $CD8^+$ T cells.

While IL-2 is effective in expanding cytotoxic lymphocytes, high-dose wild-type hIL-2-based immunotherapy has limited clinical applications due to the induction of systemic toxicities, including vascular leak syndrome and pulmonary edema. Moreover, the high binding affinity of wild-type IL-2 for IL-2Rα leads to Treg activation, which diminishes its antitumor efficacy. To enhance the antitumor effects and reduce adverse effects of wild-type IL-2, several strategies have been developed. These strategies involve preventing IL-2 binding to IL-2Rα, enhancing IL-2 binding to IL-2Rβ, and conditionally activating IL-2 within tumors.[43 44 47] The strategy of tumor-targeted IL-2 such as αCEA/IL-2v and αFAP/IL-2v is also an appealing approach to enhance its antitumor activity while reducing systemic toxicity by confining IL-2's action within the tumor or TME.[45 46] However, the anti-CEA or anti-FAP component of IL-2-based bispecific fusion proteins contributes to only a simple increase in delivery to CEA^+ tumors or FAP^+ tumors. In addition, immune cell-targeted IL-2 therapeutics are emerging as a promising trend in the development strategies of IL-2-based treatments. Fusion proteins such as αPD-1/IL-2v,[55 56] αTCRβ/IL-2v,[57] and αCD8-IL-2v[58] elicit antitumor effects via the preferential cis-action of IL-2v on specific subpopulation of $CD8^+$ T cells, including PD-1-expressing exhausted $CD8^+$ T cells, Vβ6/Vβ10-expressing $CD8^+$ T cells, and CD8-expressing T cells, respectively. In the present study, we engineered a novel GI-αCD73/IL-2v bifunctional fusion protein, comprizing an anti-CD73 component fused to IL-2v that lacks binding affinity to IL-2Rα. GI-αCD73/

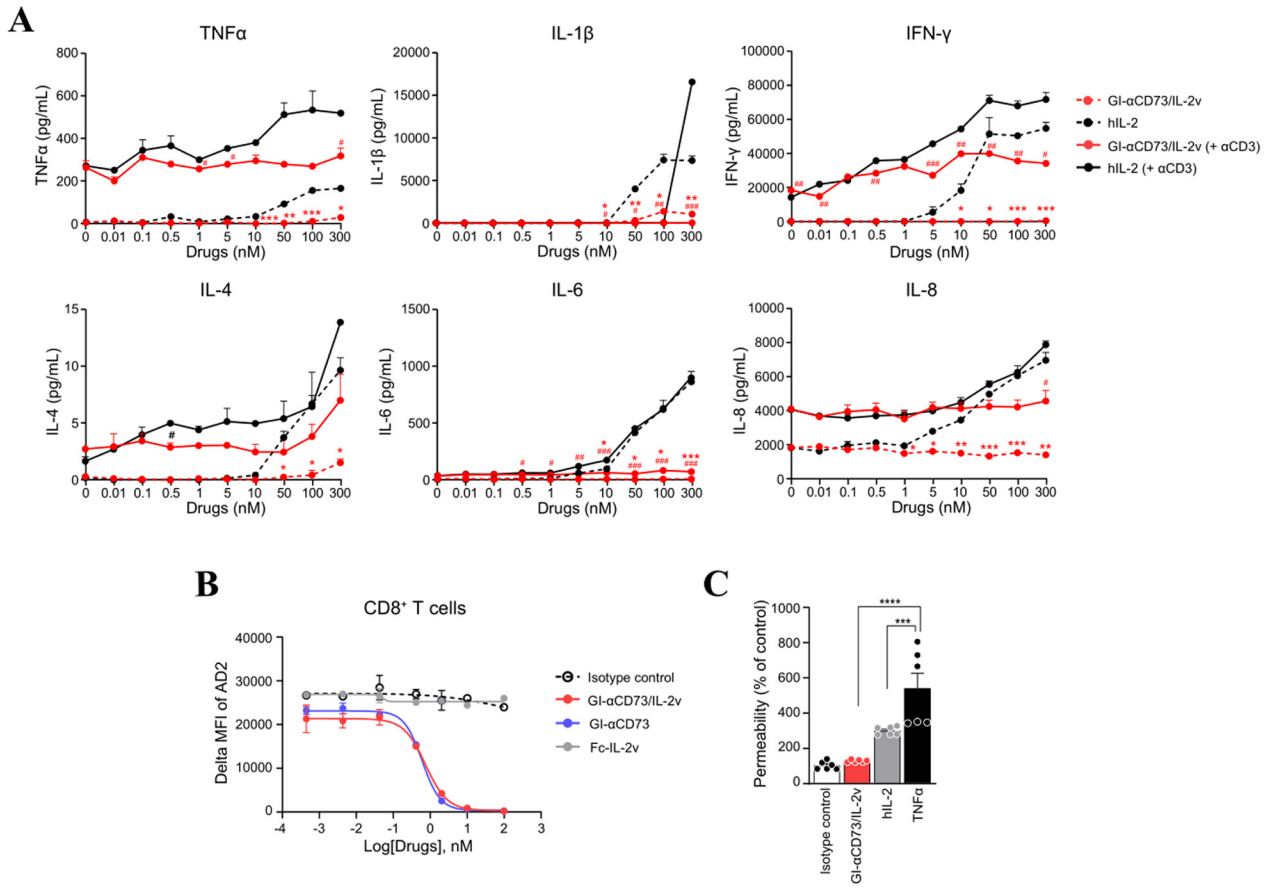


Figure 6 GI-αCD73/IL-2v has negligible risk of cytokine release syndrome and endothelial vascular leakage. (A) Cytokine release assay in human PBMCs treated with 0.01–300 nM concentration of hIL-2 or GI-αCD73/IL-2v in the absence or presence of anti-CD3 treatment at 1 µg/ml for 24 hours. Two independent experiments were performed using human PBMCs from different healthy donors. Data are presented as mean±SEM of duplicate from one representative PBMC donor. (*p<0.05, **p<0.01, ***p<0.001 for hIL-2 vs GI-αCD73/IL-2v under no anti-CD3 stimulus; #p<0.05, ##p<0.01, ###p<0.001 for hIL-2 vs GI-αCD73/IL-2v under anti-CD3 stimulus). (B) Receptor occupancy of GI-αCD73/IL-2v measured against surface CD73 on CD8+ T cells from human PBMCs using flow cytometric analysis. Human PBMCs were preincubated with indicated concentrations of IgG4-Fc (isotype control), GI-αCD73, Fc-IL-2v or GI-αCD73/IL-2v at 4℃ for 30 min followed by exposure to 250 nM of anti-CD73-BV421 (AD2 clone, a competitive antibody with GI-αCD73 against human CD73) at 4℃ for 30 min. Data are representative of two independent experiments using human PBMCs from different healthy donors. (C) Changes in permeability of HUVECs after 24 hours incubation with supernatants from human PBMCs treated with 10 nM of IgG4-Fc (isotype control), GI-αCD73/IL-2v, or hIL-2 for 24 hours. TNFα (100 ng/mL) was directly treated in HUVECs for 24 hours as a positive control. ***p<0.001, ****p<0.0001 by unpaired t-test. PBMC, peripheral blood mononuclear cell.

IL-2v distinguishes itself from other tumor cell-targeted or immune cell-targeted IL-2 fusion proteins through its functional N-terminus antibody domain; the anti-CD73 component effectively targets CD73-expressing CD8+ T cells and mitigates AMP-induced CD8+ T cell suppression, while also targeting CD73-expressing tumors to a certain extent. Consequently, GI-αCD73/IL-2v is an attractive immunocytokine with a distinct mechanism of action compared with other IL-2-based bispecific fusion proteins.

In hCD73 KI mice bearing MC38 tumors, GI-αCD73/IL-2v exerted antitumor effect by inducing the accumulation of CD8+ T cells and NK cells, but not Treg cells, within tumors. Since NK cells and Treg cells have been reported to express CD73 and IL-2Rαβγ (or IL-2Rβγ), we assessed the potent in vivo delivery of GI-αCD73/IL-2v to CD8+ T cells compared with NK cells or Treg cells and evaluated its expression levels in the three immune cell types. In the blood and tumors, the proportion of CD8+ T cells with in vivo binding of GI-αCD73/IL-2v was significantly higher than that of Treg cells, even though the proportion of CD8+ T cells expressing CD73 was moderately lower than that of CD73-expressing Treg cells. Given that GI-αCD73/IL-2v does not bind to IL-2Rα, which is predominantly expressed in Treg cells, we speculate that GI-αCD73/IL-2v could preferentially target CD8+ T cells over Tregs, despite the moderate CD73 expression in CD8+ T cells. Additionally, the in vivo binding proportion of GI-αCD73/IL-2v in NK cells is lower than in CD8+ T cells, which may be attributable to their low CD73 expression, regardless of the expected strong binding of the IL-2v portion of GI-αCD73/IL-2v to NK cells.

Considering our findings regarding the complete elimination of GI-αCD73/IL-2v-induced antitumor effect by $CD8^+$ T cell depletion but not NK cell depletion, we conclude that GI-αCD73/IL-2v preferentially, though not exclusively, targets $CD8^+$ T cells, driving its antitumor effect through $CD8^+$ T cell activation, while NK cells act as bystanders in the tumor-suppressive action of GI-αCD73/IL-2v. Despite the cis-binding potential significance of GI-αCD73/IL-2v-induced in vivo antitumor effect, we cannot exclude the potential contribution of trans-binding of GI-αCD73/IL-2v between neighboring CD73-expressing $CD8^+$ T cells or other adjacent CD73-expressing immune cells.

In the current study, GI-αCD73/IL-2v outperformed both GI-αCD73 and Fc-IL-2v when used individually or in combination in assays measuring $CD8^+$ T cell proliferation after treatment. This result was attributed to the cis-binding activity of GI-αCD73/IL-2v, which allows the targeted delivery of IL-2 to CD73-expressing $CD8^+$ T cells, as evident from the enhanced STAT5 activation within the population. In previous reports examining the effect of IL-2-IL-2Rα fusion protein (Nemvaleukine/ALKS-4230) and pegylated IL-2 (SAR444245/THOR-707) on STAT5 activation of $CD8^+$ T cells,[59 60] ALKS-4230 or THOR-707 exhibited similar or slightly higher EC_{50} values than hIL-2, respectively. On the contrary, GI-αCD73/IL-2v exhibited significantly lower EC_{50} values than hIL-2 in the same assays, suggesting that GI-αCD73/IL-2v is more effective at activating $CD8^+$ T cells than the other non-bispecific IL-2 drug candidates, possibly due to its cis-binding in CD73-expressing $CD8^+$ T cells. However, we cannot exclude the potential contribution of trans-binding of GI-αCD73/IL-2v between neighboring CD73-expressing $CD8^+$ T cells in enhancing IL-2 signaling mediated by GI-αCD73/IL-2v.

In conclusion, our results indicate that IL-2 alone is unable to enhance the antitumor response of $CD8^+$ T cells under adenosine-rich conditions. However, when combined with anti-CD73 antibody, IL-2 effectively counteracts CD73/adenosine axis-induced suppression of IL-2 signaling for $CD8^+$ T cell function. These results underscore that CD73 blockade is required to enhance the antitumor response of IL-2 in the TME and in tumors with CD73 high expression. Furthermore, our novel GI-αCD73/IL-2v fusion protein exhibits an enhanced ability to target and activate $CD8^+$ T cells, surpassing the combined effects of GI-αCD73 and Fc-IL-2v treatment and achieving potent in vivo antitumor efficacy without systemic toxicity. Overall, our GI-αCD73/IL-2v fusion protein offers competitive advantages attributed to its IL-2Rα bias, minimized systemic effects, sustained IL-2 activity in adenosine-rich environment, cis-acting signaling on CD73-expressing $CD8^+$ T cells, and targeted delivery to $CD73^{high}$ tumors. Therefore, these findings provide valuable insights into the importance of the CD73-adenosine axis in IL-2-based cancer immunotherapy and propose an innovative strategy for developing next-generation IL-2-based immunocytokines.

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Correction notice This article has been corrected since it was first published. Figure 4 was displaying the incorrect image and has now been replaced. 14th March 2025.

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Contributors YJK, KHK and MHJ supervised and designed the study. KS, MP, SK, HL, YL, JoK, SP, JiK, KL, CWP, J-HK, E-JL, HM, S-MO, SL and Y-GC performed experiments or analyzed data. YMO and WL produced GI-αCD73, AHF10240, AHP04167, Fc-IL-2v, AHP04167/IL-2v, or GI-αCD73/IL-2v. JP and J-YL analyzed and interpreted data. KS, YAS and KHK wrote the manuscript. MHJ is the guarantor.

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Competing interests KS, MP, SK, HL, YL, JoK, SP, JiK, KL, CWP, J-HK, E-JL, HM, S-MO, SL, YMO, WL, YAS, Y-GC, YJK, KHK and MHJ are employees of GI Innovation, and hold stock/stock options of GI Innovation.

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