Targeting CD96 in Cancer Immunotherapy Supplementary Material

Supplementary Methods

Biotinylation of antibody for immobilization onto Streptavidin Biosensors.

EZ-Link Sulfo-NHS-LC-LC Biotin from Thermo Fisher Scientific (Waltham) was added to the CD96 antibodies (clone 3.3 or 6A6) in a molar coupling ratio (MCR) of 1:1 and incubated for 30 minutes at room temperature and protected from light. The reaction was ended by removing excess of biotin reagent using the Zeba ^{TM} desalt spin columns (Thermo Fisher Scientific) following manufacturer's instructions for desalting procedures.

Affinity Measurements

The binding kinetics of anti-mouse CD96 antibodies (clone 3.3 or 6A6) to recombinant mouse CD96 (mCD96) (CSIRO) were quantified using a ForteBio Octet Red system (Forte Bio, Inc.). Affinity measurements were conducted in 96-well microtiter plates at 30°C in PBS buffer with 0.5 mg/ml BSA and 0.02% Tween 20. Sensor tips were submerged for 20 mins in buffer immediately prior to use, and the microplates were filled with 200 µl per well of diluted samples (mCD96) or buffer and mixed at 1000 rpm. The biotinylated CD96 antibody was immobilised on Streptavidin biosensors (Forte Bio, Inc.) at 25 µg/ml. The association and dissociation of recombinant mCD96 was measured using four descending two-fold dilutions ranging from 4.39 µg/ml to 0.54 µg/ml. The association and dissociation rates were measured for 5 mins and 30 mins, respectively. The Kinetics parameters (k*on* and k*off*) and affinities (K_d) were calculated by global fitting to a 1:1 interaction model using the Forte Bio Data Analysis Software V7.1 (ForteBio, Inc.) and exported as a

Microsoft Excel file for analysis and presentation in other software packages. Multiple independent measurements were performed with the same results.

Determining interactions between antibodies leading to reduced lung metastases

To examine the interactions between antibody combinations leading to reduced lung metastases, the number of experimental lung metastases in mice treated with cIg, antibody X, antibody Y or antibody X+Y were plotted out in a grouped table and plotted on a box plot. A Two-way ANOVA was performed to test the interaction of anti-CD96 on reducing metastases in anti-PD1 or anti-CTLA4 treated mice. Where the interaction was not significant, the effect of antibodies at reducing lung metastasis in combination was deemed as additive rather than synergistic. This method to analyse drug interactions was performed as previously described (1).

Organ histology

Mouse organs were fixed in 10% neutral buffered formalin for 24 hrs before being processed and embedded in paraffin. Four micron thick sections were cut and a hematoxylin and eosin (H&E) stain performed. H&E stained tissue sections were imaged by using Aperio Scanscope AT (Leica, Germany) and analyzed by Aperio ImageScope.

Assessment of mouse blood and serum

Blood was collected by retro-orbital bleed into EDTA containing tubes and ran on a Hemavet 950 analyzer (Drew Scientific). Alternatively, blood was collected and allowed to clot at room temperature before being centrifuged at 10000 rpm for 10 min and separated serum removed. Serum was diluted 1/4 in normal saline and ALT/AST

levels measured by Queensland Pathology using a Beckman Unicell DxC800 analyser or cytokines measured by CBA as per manufacturer's instructions (BD Biosciences).

Supplementary References.

1. Slinker BK. The statistics of synergism. J Mol Cell Cardiol. 1998;30:723-31.

Supplementary Figure Legends.

Supplementary Fig. 1. CD96 and CD226 have opposite roles in the control of experimental lung tumor metastases. C57BL/6 wild-type (WT) and indicated strains of C57BL/6 gene-targeted mice (*Cd96*$^{-/-}$ *and Cd226*$^{-/-}$) were injected i.v. with **(A)** 3LL lung carcinoma (1 x 10^5 cells), **(B)** RM-1 prostate carcinoma (1 x 10^4 cells), and **(C)** LWT1 melanoma (5 x 10^5 cells). The metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5-15 mice per group are shown. Significant differences between groups as indicated by crossbars were determined by a Mann-Whitney U test (*: $p<0.05$, **: $p<0.01$).

Supplementary Fig. 2. Anti-metastatic activity of anti-CD96 is dependent on NK cells and IL-12p35, but independent of T cells. C57BL/6 wild-type (WT), *Cd96*$^{+/-}$, *Cd96*$^{-/-}$, *IL-12p35*$^{-/-}$, *Rag-1*$^{-/-}$, *Rag-2*$^{-/-}$, *Rag-2*$^{-/-}$*Il2rg*$^{-/-}$, *NKp46* cre/wt *Mcl1* wt/wt, *NKp46* cre/wt *Mcl1* fl/fl mice as indicated were injected i.v. with B16F10 melanoma **(A, B)** (2 x 10^5 cells) or **(C, D)** (5 x 10^4 cells). On day 0 and 3 after tumor inoculation, mice were treated with i.p. injections of cIg (250 μg) or anti-CD96 mAb (250 μg). The metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 4-10 mice per group are shown. Significant

differences between groups as indicated by crossbars were determined by a Mann-Whitney U test (*: $p<0.05$, **: $p<0.01$).

Supplementary Fig. 3. *Cd96*$^{-/-}$ mice are resistant to EO771 spontaneous lung metastases and treatment with anti-CD96 mAb does not increase anti-metastatic effect. In the same experiment shown in Fig. 3c, groups of female C57BL/6 (WT) or *Cd96*$^{-/-}$ mice were injected orthotopically into the fourth mammary gland EO771 mammary adenocarcinoma (2 x 10^5 cells). On day 16, the primary tumor was resected before mice received cIg (250 μg) or anti-CD96 (250 μg) on days 17, 21, 25, and 29 relative to tumor inoculation (day 0). Thirty-five days after tumor inoculation, the metastatic burden was quantified in the lungs by counting colonies on the lung surface. Means ± SEM of 10 mice per group are shown. Significant differences between groups as indicated by crossbars were determined by a Mann-Whitney U test (**: $p<0.01$, ****: $p<0.0001$). The data in WT + cIg and WT + anti-CD96 groups is the same as that in Figure 3c.

Supplementary Fig. 4. The effect of anti-CD96 combined with anti-PD1 or anti-CTLA4 in reducing experimental lung metastases is additive. To determine if interactions between anti-CD96 and anti-PD1 or anti-CTLA4 antibodies was additive or synergistic, the same experimental data of B16F10 and RM-1 lung metastases in mice treated with cIg, anti-PD1, anti-CTLA4 or anti-CD96 that is shown and plotted in Figure 4A and Figure 4B respectively was plotted out as a box plot, with whiskers indicating min and max values. **(A)** Number of B16F10 and **(B)** number of RM-1 lung metastases. A two-way ANOVA was performed to test the interaction of anti-CD96 on reducing metastases in anti-PD1 or anti-CTLA4 treated mice. Where the

interaction was not significant, the effect of antibodies at reducing lung metastasis in combination was deemed as additive rather than synergistic.

Supplementary Fig. 5. Anti-CD96 combines with anti-CTLA4 or anti-PD1 to enhance survival following challenge with experimental lung metastases. C57BL/6 wild-type (WT) mice were injected i.v. with **(A)** B16F10 melanoma (2 x 10^5 cells) or **(B)** RM-1 prostate carcinoma (1 x 10^4 cells). On day 0 and 3 after tumor inoculation, mice were treated with i.p. injections of control Ig (cIg)(250 μg), anti-CD96 mAb (250 μg), anti-CTLA4 (250 μg), anti-PD1 mAb (250 μg), anti-CD96/anti-CTLA4 mAbs (250 μg each), or anti-CD96/anti-PD1 mAbs (250 μg each). Mice were monitored for survival and the survival of groups of 10 mice are plotted. Statistically significant differences between groups as shown by crossbars was determined by a Log Rank test (**: $p<0.01$; ***:$p<0.001$; ****:$p<0.0001$).

Supplementary Fig. 6. Anti-CD96 combines with anti-CTLA4, anti-PD1 or doxorubicin to increase survival and reduce metastases in mice bearing 4T1.2 spontaneous metastases. In the same experiment as shown in Fig. 4d, groups of female BALB/c WT mice were injected in the mammary fat pad with the mammary carcinoma cell line 4T1.2 (5 x 10^4 cells). On day 20, the primary tumor was resected and mice were treated i.p. with control (cIg) (250 μg), anti-PD1 (250 μg), anti-CTLA4 (250 μg), anti-CD96 mAb (250 μg) on days 20, 24, 28 and 32 after tumor inoculation. Some groups of mice as indicated received doxorubicin (DOX) (2 mg/kg i.v.) on day 20. Mice were monitored for survival and the survival of groups of 5-15 mice are plotted. The survival curves of cIg, anti-PD1, anti-CTLA4 and anti-CD96 treated mice are the same as those shown in Fig. 4d. **(B)** Groups of female BALB/c

mice were injected in the mammary fat pad with the mammary carcinoma cell line 4T1.2 (5 x 10^4 cells). On day 19, the primary tumor was resected and mice were treated i.p with cIg (250 μg), anti-PD1 (250 μg), anti-CTLA4 (250 μg), anti-CD96 mAb (250 μg), anti-CD96/anti-CTLA4 mAbs (250 μg each), or anti-CD96/anti-PD1 mAbs (250 μg each) on days 19, 23, and 27 after tumor inoculation. Metastatic burden was quantified in the lungs after 34 days by counting colonies on the lung surface. Means ± SEM of 6-8 mice per group are shown. One cIg-treated mouse had to be sacrificed at day 30 because of lung metastasis burden. **(C)** 4T1.2 cells in culture were trypsinised, and stained with anti-CD155 APC or anti-CD112 APC (shaded) or a respective rat IgG2a APC isotype control (clear) with representative histograms shown. Statistically significant differences between groups as shown by crossbars was determined by a Mann-Whitney U test for comparison of metastases or Log Rank test when comparing survival (*:$p<0.05$, **: $p<0.01$, ***:$p<0.001$).

Supplementary Fig. 7. Reduction in B16F10 lung metastases by anti-CD96/anti-PD1 is dependent on NK cells

C57BL/6 wild-type (WT) mice were injected i.v. with B16F10 melanoma (2 x 10^5 cells). On day 0 and 3 after tumor inoculation, mice were treated with i.p. injections of cIg (250 μg) or anti-CD96, anti-PD1 mAbs (250 μg each). Some groups of mice were treated i.p on days 0, 1, and 8 after tumor inoculation with cIg (100 μg), anti-CD4/anti-CD8β (100 μg each) or anti-asGM1 (100 μg). The metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5 mice per group are shown. Significant differences between groups as indicated by crossbars were determined by a Mann-Whitney U test (**: $p<0.01$, ns = not significant).

Supplementary Fig. 8. Anti-CD96 and anti-PD1 therapy does not induce significant toxicities in B16F10 tumor bearing mice. C57BL/6 wild-type mice were injected i.v. with B16F10 melanoma (2 x 10^5 cells). On day 0, 3, 6, 9, 12 after tumor inoculation, mice were treated with i.p. injections of control Ig (cIg) (250 μg), anti-CD96 mAb (250 μg), anti-PD1 mAb (250 μg) or anti-CD96/anti-PD1 mAbs (250 μg each). On day 14 mice were sacrificed, blood was collected by retro-orbital bleed, serum isolated and levels of **(A-C)** cytokines IFN-γ, IL-6 or TNF determined. Blood was also collected into heparinised tubes and numbers of **(D-F)** white blood cells (WBCs), red blood cells (RBCs) and platelets determined. Shown is mean ± SEM pooled from 2 independent experiments. Organs were also collected and representative H&E stained **(G)** skin, **(H)** liver and **(I)** proximal colon sections are shown. Not shown, no significant changes to ALT/AST, spleen/colon weight, or obvious pathology in mid colon, distal colon, kidney and spleen were observed. Statistically significant differences between groups as shown by crossbars were determined by a Mann-Whitney U test (*: $p<0.05$, **: $p<0.01$).