Antitumor efficacy and reduced toxicity using an anti-CD137 Probody therapeutic

Iñaki Etxeberria[a,b,c,d,1], Elixabet Bolaños[a,b,c,1], Alvaro Teijeira[a,b,c], Saray Garasa[a,b,c], Alba Yanguas[a,b,c], Arantza Azpilikueta[a,b,c], William M. Kavanaugh[e], Olga Vasiljeva[e], Marcia Belvin[e], Bruce Howng[e], Bryan Irving[e], Kimberly Tipton[e], James West[e], Li Mei[e], Alan J. Korman[f,g], Emanuela Sega[g], Irene Olivera[a,b,c], Assunta Cirella[a,b,c], Maria C. Ochoa[a,b,c], Maria E. Rodriguez[a,h], Ana Melero[a,h], Miguel F. Sanmamed[a,h], John J. Engelhardt[g,2], and Ignacio Melero[a,b,c,d,2,3]

[a]Program of Immunology and Immunotherapy, Center for Applied Medical Research (CIMA), Universidad de Navarra, 31008 Pamplona, Spain; [b]Centro de Investigación Biomédica en Red de Cáncer, 28029 Madrid, Spain; [c]Navarra Institute for Health Research, 31008 Pamplona, Spain; [d]Department of Immunology, Clinica Universidad de Navarra, 31008 Pamplona, Spain; [e]CytomX Therapeutics, Inc., South San Francisco, CA 94080; [f]Vir Biotechnology, San Francisco, CA 94158; [g]Immuno-Oncology Discovery, Bristol-Myers Squibb, Redwood City, CA 94063; and [h]Department of Oncology, Clinica Universidad de Navarra, 31008 Pamplona, Spain

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Costimulation via CD137 (4-1BB) enhances antitumor immunity mediated by cytotoxic T lymphocytes. Anti-CD137 agonist antibodies elicit mild liver inflammation in mice, and the maximum tolerated dose of Urelumab, an anti-human CD137 agonist monoclonal antibody, in the clinic was defined by liver inflammation–related side effects. A protease-activated prodrug form of the anti-mouse CD137 agonist antibody 1D8 (1D8 Probody therapeutic, Pb-Tx) was constructed and found to be selectively activated in the tumor microenvironment. This construct, which encompasses a protease-cleavable linker holding in place a peptide that masks the antigen binding site, exerted antitumor effects comparable to the unmodified antibody but did not result in liver inflammation. Moreover, it efficaciously synergized with both PD-1 blockade and adoptive T-cell therapy. Surprisingly, minimal active Pb-Tx reached tumor-draining lymph nodes, and regional lymphadenectomy did not abrogate antitumor efficacy. By contrast, S1P receptor–dependent recirculation of T cells was absolutely required for efficacy. The preferential cleavage of the anti-CD137 Pb-Tx by tumor proteases offers multiple therapeutic opportunities, including neoadjuvant therapy, as shown by experiments in which the Pb-Tx is given prior to surgery to avoid spontaneous metastases.

CD137 | cancer immunotherapy | 4-1BB | Probody

Immunotherapy based on blocking T-cell coinhibitory receptors has revolutionized cancer clinical management with the advent of anti–PD-(L)1 and anti–CTLA-4 checkpoint inhibitors (1). Immunostimulatory agonist antibodies targeting costimulatory targets such as CD137 (4-1BB), OX40, ICOS, GITR, or CD27 are lagging behind in clinical development for a variety of reasons (2, 3).

In the case of agonist anti-CD137 monoclonal antibodies (mAb), the immunotherapeutic effects in mouse models are impressive (4), especially when combined with anti–PD-(L)1 (5, 6), interleukin-12 (7), or adoptive T-cell therapy (ACT) (8, 9). The mechanism of action mainly relies on CD8 T-cell costimulation and reinvigoration of dysfunctional tumor-infiltrating lymphocytes (8, 10).

In the clinic, the strong CD137 agonist Urelumab underwent several clinical trials (11) but was limited to subtherapeutic doses by frequent and serious liver inflammation above 0.3 mg/kg (11). Another anti-CD137 mAb, Utomilumab (12), shows weak intrinsic agonist activity and could be safely dosed up to 10 mg/kg but without consistent evidence for clinical activity as a monotherapy (13).

Many attempts are ongoing in the clinic to safely target CD137 costimulation to the tumor while preserving liver safety (14, 15). These include bispecific antibodies (16) and tumor-targeted constructs containing trimeric natural ligand (CD137L) (17). Here, we report the immune and therapeutic effects of an agonist anti-CD137 antibody prodrug called a Probody therapeutic (Pb-Tx) (18, 19). The term Probody is a United States–registered trademark of CytomX Therapeutics to refer to a new class of recombinant, proteolytically activated antibody prodrugs. The anti-CD137 Pb-Tx is designed to be activated to exert agonist activity on CD137 only in the tumor when cleaved by tumor-associated proteases.

Results

A Tumor Protease–Cleavable Anti-CD137 Pb-Tx. To mediate tumor-associated activation of an anti-mouse CD137 mAb, a Pb-Tx construct was made based on an IgG1 murine version of the well-described agonist antibody 1D8 (4). The N terminus extension of the light chains of this antibody was engineered with a protease-sensitive linker (18, 19) designed to be cleaved by proteases active in the tumor microenvironment (*SI Appendix*, Fig. S1) (20, 21) and a specific mask that blocks the antigen binding site to create a Pb-Tx (*SI Appendix*, Fig. S2). The hypothesis was that such a masked antibody prodrug would be activated in the tumor microenvironment when tumor-associated proteases cleave the linker and release the masking peptide (Fig. 1*A*). Indeed, the protease-sensitive linker of CD137

Significance

CD137 (4-1BB) is a target for tumor immunotherapy, which has been pursued in clinical trials with agonist antibodies or the natural ligand (CD137L). Liver toxicity is a serious dose-limiting problem that may be circumvented by selective functional or physical targeting to the tumor microenvironment. A CD137 agonist antibody prodrug that is preferentially activated by tumor-associated proteases constitutes an appealing way to enhance safety while preserving efficacy.

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[1]I.E. and E.B. contributed equally to this work.

[2]J.J.E. and I.M. contributed equally to this work.

[3]To whom correspondence may be addressed. Email: imelero@unav.es.

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Pb-Tx has been designed to be cleaved by proteases known to be active in the tumor microenvironment (21), such as matriptase, urokinase plasminogen activator, and selected matrix metalloproteinases (MMPs) (*SI Appendix*, Fig. S1), that actually cleave CD137 Pb-Tx, as shown by SDS-PAGE and capillary electrophoresis following in vitro digestion with recombinant proteases (*SI Appendix*, Fig. S3 *A–C*). As a consequence of being masked (*SI Appendix*, Fig. S2), binding of the Pb-Tx to CD137 in an ELISA was impaired but was restored after activation by digestion of the Pb-Tx with matriptase, an example of a tumor-associated protease (Fig. 1*B*). In addition, Pb-Tx cleaved by recombinant matriptase bound CD137-expressing activated T cells (Fig. 1*C*). Moreover, overnight incubation of the Pb-Tx with cell suspensions of MC38-engrafted tumors leads to production of an antibody form enabled to bind CD137 on activated CD8 and CD4 T cells that are subsequently added to the cultures (Fig. 1*D*). This effect was not as prominent in the presence of protease inhibitors (Fig. 1*D*). Importantly, Pb-Tx remained masked in cultures with cell suspensions obtained from healthy spleen and livers from wild-type and tumor-bearing mice (*SI Appendix*, Fig. S4 *A–D*). In addition, in vivo antibody binding to liver-infiltrating T cells from wild-type and tumor-bearing mice was assessed by administering AF488-tagged native 1D8 or CD137 Pb-Tx and adoptively transferring CD137-expressing activated polyclonal CD45.1 T cells (*SI Appendix*, Fig. S4*E*). In this in vivo setting, a reduced binding of the Probody was observed in endogenous and transferred T cells from the livers of CD137 Pb-Tx–treated mice in comparison to the 1D8-treated mice (*SI Appendix*, Fig. S4*F*).

1D8 Anti-CD137 Pb-Tx Mediates Antitumor Effects with Reduced Liver Inflammation. In mouse models, potent antitumor immunotherapy based on systemic administration of anti-CD137 agonist mAb (4) is accompanied by augmented CD8 T-cell liver infiltration and increased transaminases (22–24). Intraperitoneal administration of both the murine IgG1 version 1D8 mAb or the Pb-Tx leads to consistent rejection of CT26-derived tumors engrafted for 6 d in BALB/c mice prior to therapy onset, which grew unaffected after treatment with control isotype–matched irrelevant IgG1 (Fig. 2*A*). Tumor rejection led to long-term survival with both forms of the antibody (Fig. 2*B*). While repeated administration of 1D8 elicited elevated circulating levels of alanine aminotransferases (ALT) denoting liver inflammation and resulted in liver infiltration by $CD8^+$ T cells as detected by flow cytometry on liver-derived cell suspensions, the Pb-Tx elicited a lesser liver inflammatory response, even if all CT26 tumors in these animals were rejected (Fig. 2 *C* and *D*).

We have previously reported exquisite synergy of anti-CD137 mAb therapy and ACT (25) against B16 OVA-expressing subcutaneous engrafted melanomas (B16OVA) (8). *SI Appendix*, Fig. S5 shows that such therapeutic synergy was preserved with 1D8 Pb-Tx (*SI Appendix*, Fig. S5 *A* and *D*), while liver T-cell infiltrates were markedly reduced as compared with the constitutively active 1D8 mAb (*SI Appendix*, Fig. S5 *E–H*). Importantly, the adoptively transferred anti-OVA OT1 T cells also patrolled the liver upon combination with the Pb-Tx (*SI Appendix*, Fig. S5 *D* and *H*).

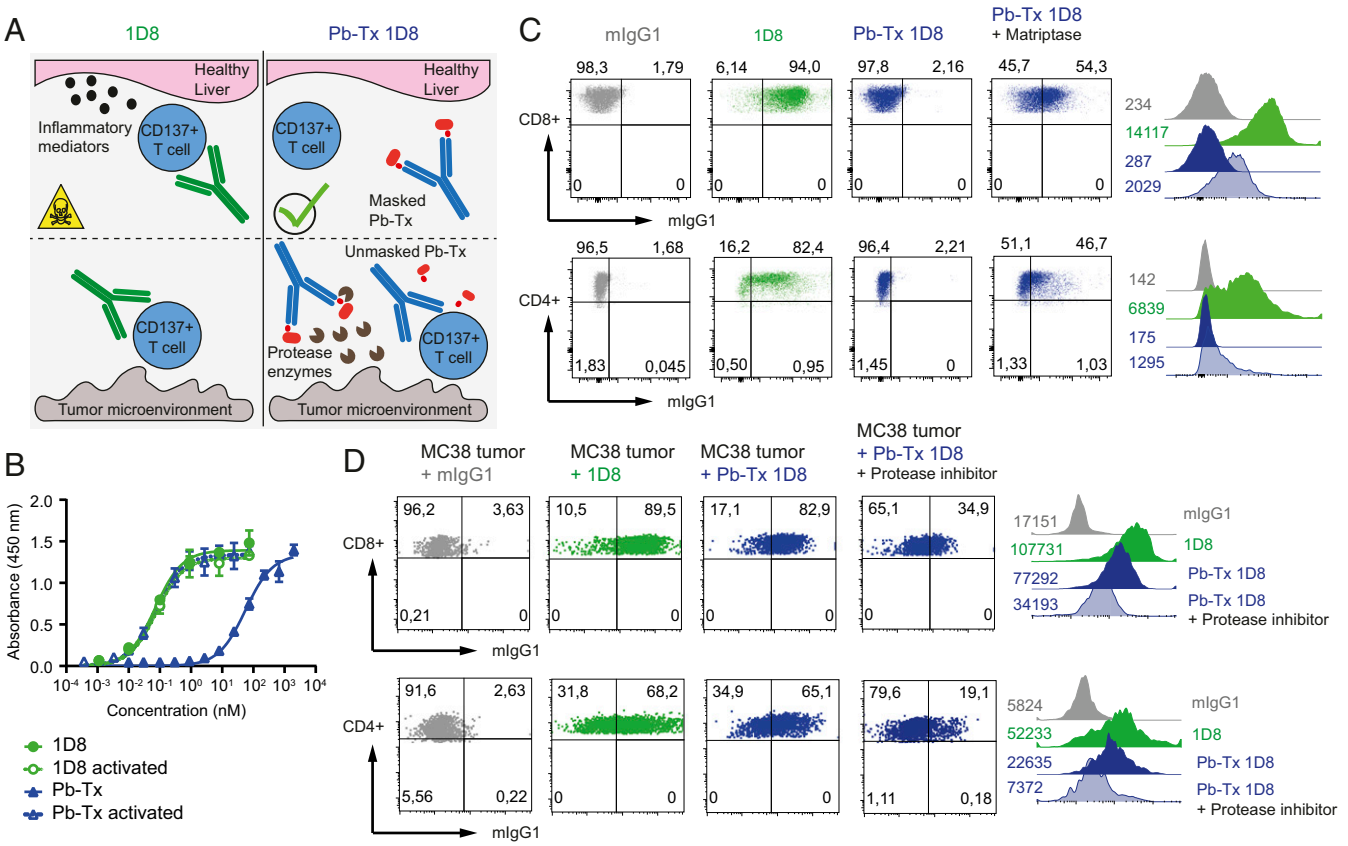

Fig. 1. An anti-mCD137 Pb-Tx that is activated by protease digestion in the tumor microenvironment. (*A*) Schematic representation of the strategy for functional tumor targeting by protease activation of an anti-mouse CD137 Pb-Tx in the tumor microenvironment. (*B*) Binding to immobilized mouse recombinant CD137 protein of matriptase-digested or undigested native 1D8 and 1D8 Pb-Tx determined by ELISA. (*C*) FACS analysis testing binding of the 1D8 Pb-Tx to anti-CD3 + CD28 activated mouse CD8 or CD4 T splenocytes following digestion with recombinant matriptase (blue). Unmasked 1D8 is used as a positive control as indicated (green). (*D*) Similar experiments as in *C*, in which $CD45.1^+$-activated splenocytes were stained with 1D8 Pb-Tx or native antibody, precultured overnight at 37 °C with cell suspensions obtained from MC38-engrafted tumors to mimic the tumor tissue microenvironment. Data in *C* and *D* are representative of at least three independent experiments in triplicate wells.

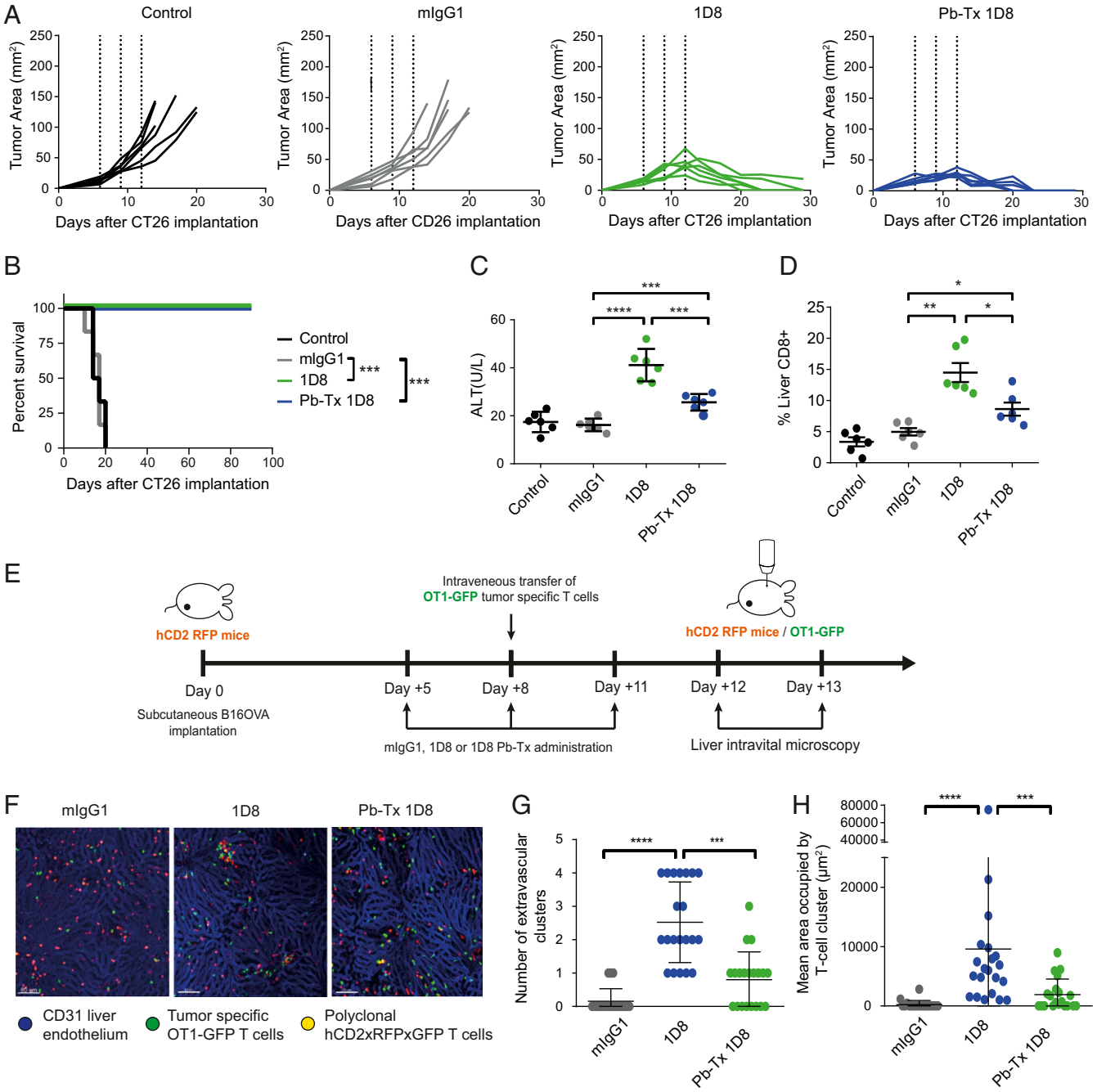

Fig. 2. 1D8 Pb-Tx exerts antitumor effects without liver inflammation. (*A*) Treatment of BALB/c mice bearing 6-d established subcutaneous tumors derived from the syngeneic CT26 colon cancer cell line, treated when indicated by dotted lines with mIgG1 control antibody, 1D8, or the 1D8 Pb-Tx. (*B*) Long-term survival of mice in *A*. Data in *A* and *B* are representative of at least two independent experiments with $n = 6$ mice per group. Survival differences were analyzed by log rank tests. $*P < 0.05$. (*C*) Circulating ALT in serum from treated mice on day +18 in experiments as in *A*. (*D*) $CD8^+$ T-cell content in the liver referred as total cells, assessed in the liver cell suspensions, 7 d following treatments as in *A*. (*C* and *D*) Data are representative of at least two independent experiments with $n = 6$ mice per group and expressed as mean ± SEM. (*E*) Representation of experimental setup for in vivo imaging of the surgically exposed livers of hCD2-RFP transgenic mice pretransferred with activated anti-OVA GFP^+ OT1 T cells and bearing established B16OVA tumors. (*F*) Intravital multiphoton representative frame images of the surgically exposed livers. Mice were treated with three doses of mIgG1 control antibody, 1D8, or Pb-Tx antibodies as indicated. Endogenous T cells are visualized in red, transferred OT1 lymphocytes in green, and sinusoid vessels in blue upon in vivo staining by perfusion of a fluorescence-labeled anti-CD31 mAb. (*G* and *H*) Quantification of extravascular T-cell clusters in multiple videos (Movie S1). Data in *F*, *G*, and *H* are representative of two independent experiments with $n = 3$ mice per group. Data are shown as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

To further analyze liver safety, intravital microscopy of surgically exposed livers was performed by multiphoton time-lapse confocal microscopy (8). Endogenous T cells were visualized because mice were transgenic for yellow fluorescent protein (YFP) under the CD2 promoter. In addition, these mice were engrafted with subcutaneous B16OVA and adoptively transferred with OVA-specific OT1 cells expressing green fluorescent protein (eGFP) (26). Upon systemic treatment with 1D8, prominent

T-cell extracellular clusters including OT1 cells and mostly endogenous T cells were clearly visualized (Fig. 2 *E–H* and Movie S1), while such clusters were rarely observed following systemic administration of the 1D8 Pb-Tx (Fig. 2 *E–H* and Movie S1). The extravascular nature of the clusters was distinguished by CD31 staining of liver sinusoidal endothelium in the living animal with a blue-fluorescent CD31-specific antibody given intravenously prior to intravital microscopy imaging. Together, these results indicate that the 1D8 Pb-Tx retains antitumor efficacy while liver inflammation is drastically reduced.

Tumor-Draining Lymph Nodes Are Dispensable for the Antitumor Effects. We hypothesized that once activated in the tumor, lymphatic drainage should carry the activated Pb-Tx to tumor-draining lymph nodes (tdLNs). An AF488-tagged Pb-Tx did stain a fraction of tumor-infiltrating T cells similar to the native AF488-tagged 1D8 mAb (Fig. 3*A* and *SI Appendix*, Fig. S6 *A* and *B*). However, contrary to our expectations, while 1D8 stained some lymphocytes in the tdLNs and in the spleen, the labeled Pb-Tx failed to do so (Fig. 3*A*). To ensure CD137 expression in the system, we adoptively transferred CD45.1 CD8 OT1 cells activated with anti-CD3 + anti-CD28 to induce bright CD137 surface expression (Fig. 3*B*). Such cells were markedly bound by the Pb-Tx in vivo when collected from the tumor microenvironment but very poorly so when found in tdLNs or the spleen (Fig. 3*C*). In contrast, in these conditions, native fluorescent 1D8 did stain activated CD45.1 OT1 cells both in the tumor and the lymphoid organs (Fig. 3*C*).

Since lymphatic drainage could be insufficiently developed in transiently engrafted tumors, we performed similar experiments in spontaneous breast carcinomas developing in MMTV-NeuT transgenic female mice. In this setting, the AF488-labeled Pb-Tx stained tumor-infiltrating lymphocytes (TILs) but not CD8 T cells in the tdLNs, which were stained in vivo by native 1D8 mAb that was similarly AF488 labeled (Fig. 3*D*). Furthermore, such tumor-bearing MMTV-NeuT transgenic mice were adoptively transferred with anti-CD3 + anti-CD28 activated violet-labeled splenocytes from BALB/c mice that intensely expressed surface CD137. In these conditions, no staining by the Pb-Tx occurred in the tdLNs, contrary to the staining achieved by the constitutively active 1D8 mAb (Fig. 3*E*).

Given these results, we interrogated if treatment with 1D8 or the Pb-Tx could be efficacious against CT26 tumors in the absence of tdLNs. To study this, lymphadenectomy of the tdLN or mock surgery (*SI Appendix*, Fig. S6*C*) were performed prior to treatment with 1D8 mAb or the 1D8 Pb-Tx given intraperitoneally. Fig. 3*F* shows that tumor rejections occurred in both instances following lymphadenectomy, although with a tendency to slower complete rejections in the case of lymphadenectomized mice.

A possible explanation was that all T cells that mattered for the antitumor effects were already present and primed in the tumor tissue microenvironment, and therefore tdLNs were dispensable. However, similar therapy experiments on CT26-established tumors showed that inhibition of S1P-dependent T-cell egress and circulation was critical (*SI Appendix*, Fig. S6*D*) since treatment with FTY720 completely abolished efficacy (Fig. 3*G*). Hence, tdLNs were dispensable, but the entrance of new T cells into the tumor microenvironment as previously reported (8, 27) was required.

Activity of Anti-CD137 Pb-Tx Synergizes with Anti–PD-1 mAb, Especially in Neoadjuvant Immunotherapy Strategies. Given the synergistic effect obtained by the combination of anti-CD137 mAb and anti-PD1 mAb therapies in mouse models (6), we explored whether Pb-Tx activity could be further potentiated by its combination with systemic antagonist anti–PD-1 mAb. *SI Appendix*, Fig. S7 shows that such therapeutic synergy was preserved with 1D8 Pb-Tx against the MC38 tumor model. Importantly, in this anti–PD-1 resistant setting, anti-CD137 or CD137 Pb-Tx in combination with anti–PD-1 resulted in delayed tumor progression and increased survival as compared to single agent treatments (*SI Appendix*, Fig. S7*B*).

Among the most powerful ways to use immunotherapy preclinically and clinically is the presurgical neoadjuvant setting (28–31) in which immunotherapy is used while the to-be-removed primary tumor is in place in order to prevent metastasis progression. These treatment strategies using anti-CD137 and PD-1 mAbs as the neoadjuvant therapy were discovered by Liu et al., utilizing the 4T1 breast cancer model that spontaneously metastasizes to the lungs (28). To study if a combination of the 1D8 Pb-Tx and PD-1 could also be efficacious in the neoadjuvant setting, experiments were performed combining PD-1 blockade and 1D8 or the Pb-Tx 2 d prior to surgery of 12-d orthotopically established 4T1 tumors transfected to express mCherry (Fig. 4*A*). The analysis of excised lungs of the treated mice showed that while control mice developed abundant spontaneous lung metastasis, administration of anti–PD-1, 1D8, or Pb-Tx 1D8 as single agents reduced the number of lung metastases (Fig. 4 *B* and *C*). Importantly, mice presurgically treated with anti–PD-1 in combination with either 1D8 Pb-Tx or unmasked 1D8 mAb had no detectable metastases in most instances (Fig. 4 *B* and *C*). Additionally, the superiority of the neoadjuvant regimen over the adjuvant treatment in terms of preventing lung metastases in this experimental setting is shown in Fig. 4 *B* and *C*. Moreover, Fig. 4 *D* and *E* show in separate experiments that neoadjuvant 1D8 Pb-Tx was at least as efficacious in terms of survival as the constitutively active 1D8 in the described (Fig. 4*D*) neoadjuvant regimen (28).

Discussion

Pb-Txs constitute inactive antibody prodrug molecules that are rendered active by proteases, which are preferentially active in the tumor microenvironment compared to healthy tissues (18, 19). This feature provides functional targeting to the tumor microenvironment, which is highly desirable for CD137 agonists to avoid liver inflammation as a side effect following systemic administration (11).

Our results indicate that efficacy with the anti-CD137 Pb-Tx form is preserved while liver inflammation is reduced. Similar Pb-Tx strategies are in the clinic for protease-cleavable forms of CTLA-4 and PD-L1 checkpoint inhibitors (18). Early clinical trials evaluating anti–PD-L1 Pb-Tx (CX-072) showed 1) a favorable toxicity profile alone or in combination (32); 2) a stable pharmacological profile with a predominantly intact circulating form of the Pb-Tx in human plasma following administration of up to 30 mg/kg (33–35); 3) a tumor-localized activation of the active Pb-Tx with a >98% of PD-L1 receptor occupancy at doses of ≥3 mg/kg (34); and 4) evidence of antitumor activity as a monotherapy (36). Hence, taking into consideration the compromised safety profile of the clinically developed anti-CD137 (Urelumab) (15), the Pb-Tx approach is especially attractive for this anti-CD137 immunotherapy target.

With the same safety goals of our anti-CD137 Pb-Tx studies, a monoclonal antibody selectively binding to human CD137 only in tissues with high extracellular concentrations of ATP, such as in tumors, has been made. This agent also exerts antitumor efficacy in hCD137 knock-in mice while showing remarkably good liver safety (37). Another similar approach to preferentially activate mAbs in the tumor microenvironment would include pH-sensitive antibodies taking advantage of the commonly relatively acidic tumor milieu (38).

In our hands, the 1D8 Pb-Tx synergized with a PD-1 blocking agent and ACT, thereby providing excellent opportunities for clinical development in combination with checkpoint blockade and/or TIL therapy (39). The fact that the Pb-Tx synergized with ACT indicates that CD137 costimulation is not required systemically and is sufficient when adoptively transferred cells reach the tumor tissue (9) where the Pb-Tx is activated.

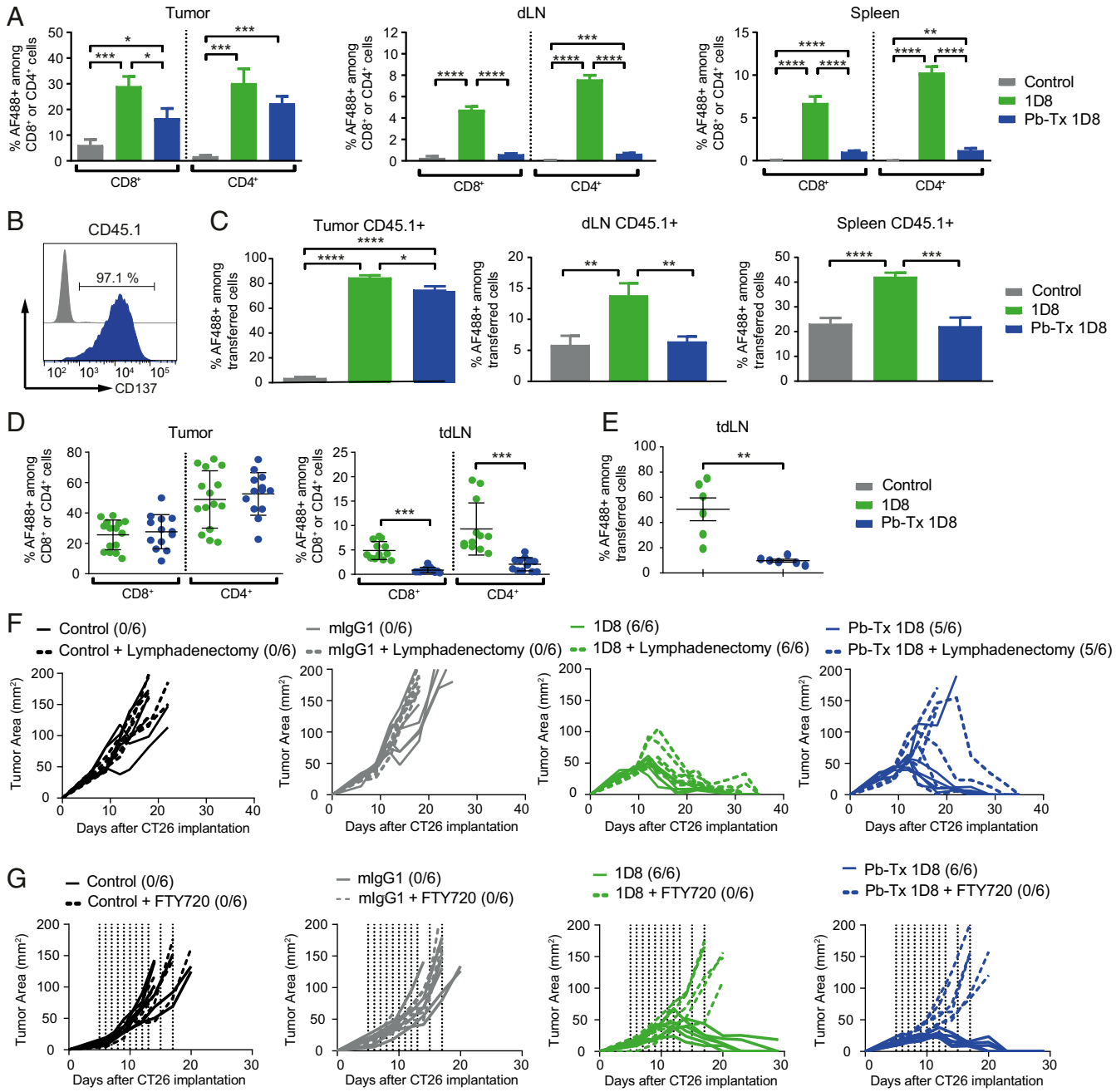

Fig. 3. The 1D8 anti-CD137 Pb-Tx is activated in the tumor and fails to functionally reach tdLNs, which are not necessary for efficacy. (*A*) An AF488 conjugated version of the 1D8 antibody or the Pb-Tx were given to mice bearing B16OVA tumors. Tumor, spleen, and tdLNs cell suspensions were studied by FACS to comparatively detect binding to CD4 and CD8 T lymphocytes. (*B*) OT-1 CD45.1$^+$ T cells activated with CD3 + CD28 to express high-surface CD137 levels. (*C*) OT1 T cells as in *B* were injected to B16OVA tumor-bearing mice that were intraperitoneally given AF488-labeled versions of 1D8 antibody or the Pb-Tx as indicated. Cell suspensions from tumor, tdLNs, and spleen were analyzed by FACS for CD137 staining on CD45.1$^+$ gated cells. (*A*–*C*) Data are representative of at least two independent experiments with $n = 6$ mice per group and expressed as mean ± SEM (*D*) Similar experiments as in *A* performed in Her2/NeuT female transgenic mice bearing spontaneously developed breast carcinomas. Data are shown as mean ± SEM with $n = 15$ mice per group. (*E*) Similar experiments as in *C*, where Violet track–marked CD3 + CD28 activated polyclonal T cells were adoptively transferred to Her2/NeuT female mice bearing spontaneously developed breast tumors to provide T cells readily expressing CD137. Data are expressed as mean ± SEM with $n = 6$ mice per group. (*F*) Treatment with control antibody, 1D8, or 1D8 Pb-Tx of locally lymphadenectomized mice on day +6 after engraftment with the CT26 cell line. Mice undergoing mock surgery were used as a control. (*G*) Treatment of 6-d established subcutaneous tumors derived from CT26 cell line treated with S1P inhibitor FTY720 given daily from day +5. Control antibody, 1D8, or the Pb-Tx treatment days are indicated by dotted lines. (*F* and *G*) Data are represented as individual tumor growth with $n = 6$ mice per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Interestingly, tdLNs are dispensable for antitumor activity, and it remains to be fully determined how and when T-cell trafficking and priming are necessary for CD137-based immunotherapy to be successful. Previous reports had suggested the S1P-dependent recirculation was not needed for anti-CD137 + anti–CTLA-4 or anti-CD137 + anti–PD-L1 combinations (40). In our case, some level of recirculation was required since FTY720 treatment spoiled efficacy. This is consistent with our previous reports of

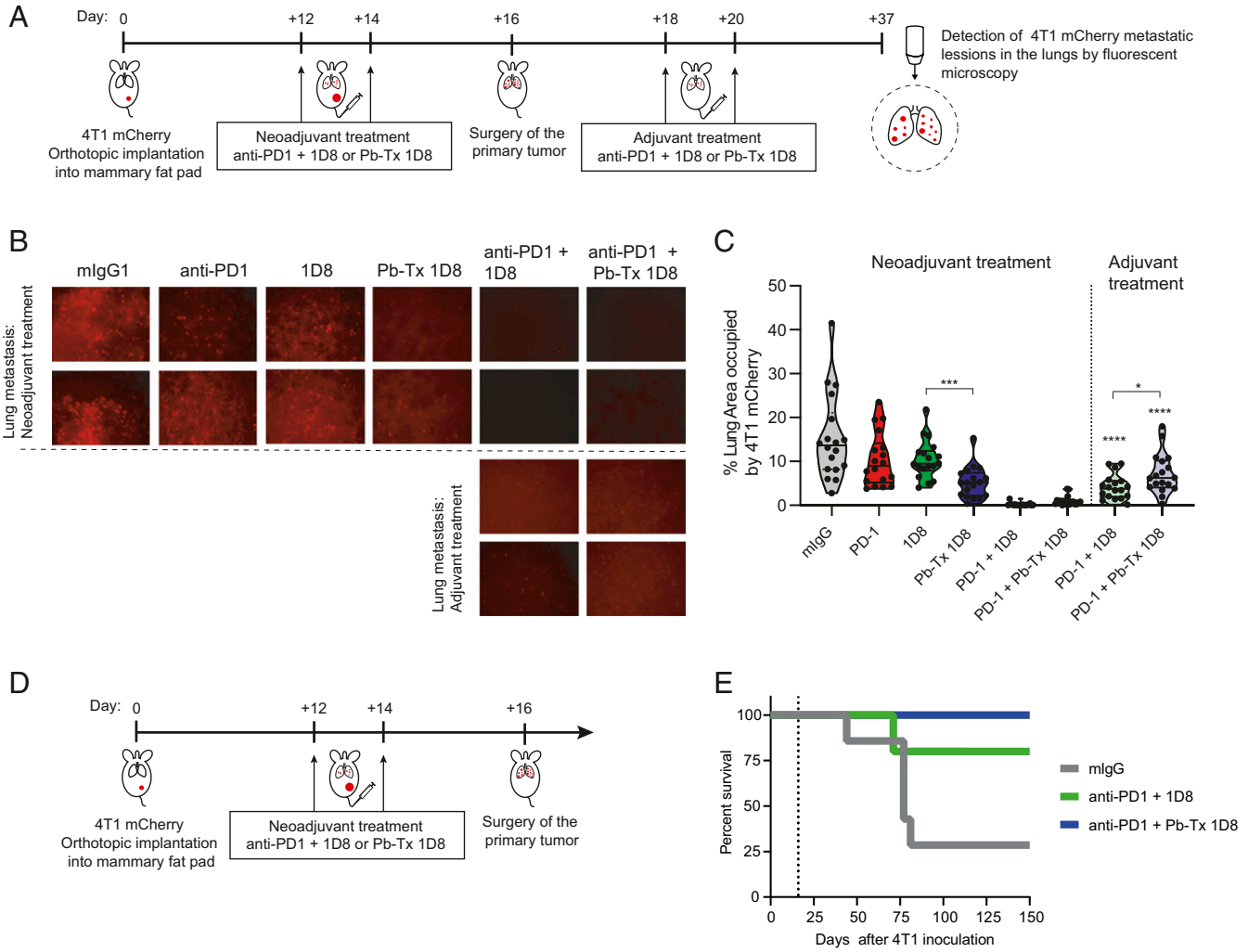

Fig. 4. The 1D8 anti-CD137 Pb-Tx in combination with anti-PD1 blockade effectively protects against 4T1 lung metastasis in a neoadjuvant treatment setting. (*A*) Schematic representation of the in vivo experiments in 4T1 mCherry breast cancer model treated with anti–PD-1 combined with 1D8 or 1D8 Pb-Tx antibody in adjuvant or neoadjuvant regimens with respect to surgery as outlined in the scheme. (*B*) Representative low-magnification fluorescent microscopy images of the lungs to assess metastases present in treated mice euthanized 37 d after 4T1 mCherry orthotopic implantation and subsequent surgical removal of the primary tumors on day +16. (*C*) Violin plot showing the quantification of the fluorescent microscopy images of the lungs from experiment in *B*. Results are shown as the percentage of the area occupied by mCherry fluorescence. Dots represent three distinct lung area quantifications per mouse, with $n = 6$ mice per experimental group. (*D*) Schematic representation of the survival in vivo experiments in 4T1 mCherry breast cancer model treated with anti–PD-1 combined with 1D8 or 1D8 Pb-Tx antibody in a neoadjuvant setting. (*E*) Survival following neoadjuvant treatment in the orthotopic 4T1 breast cancer model performed as indicated in "d". Primary tumor surgery day is indicated by a dashed line. Survival percentage over time with $n = 9$ mice per group is represented. Survival differences were analyzed by log rank tests. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

T-cell infiltration promotion assessed by intravital microscopy experiments (8).

The efficacy found in the neoadjuvant setting with 1D8 or its Pb-Tx form require some level of egress of costimulated lymphocytes from the primary tumor to prevent metastatic progression. Indeed, a better tolerated form of anti-CD137 mAb may find efficacious use in the clinic when given before radical surgery in neoadjuvant strategies.

In patients who are potentially curable with surgery, the liver safety advantage of a CD137 Pb-Tx is particularly important, and our results also suggest that lymphadenectomy could be undertaken if needed. The Pb-Tx platform is currently being investigated clinically in multiple studies with both checkpoint inhibitor targets as well as antibody drug conjugates. Safer and efficacious forms of CD137 agonists may clearly benefit from this biotechnology approach and open interesting clinical opportunities.

Materials and Methods

A detailed description of the materials and methods is included in *SI Appendix, Supplementary Material and Methods*. Procedures and methods are provided for cell lines and tumor engraftment; construction, production, and purification of the antibodies and probodies; flow cytometry; assays of activation of the Probody by proteases; intravital microscopy; treatments and surgeries performed to tumor-bearing mice; neoadjuvant immunotherapy experiments; and bioinformatics and statistical analyses.

Data Availability. All study data are included in the article and/or supporting information.

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