A cytokine receptor-masked IL2 prodrug selectively activates tumor-infiltrating lymphocytes for potent antitumor therapy

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Article

Keywords: Tumor-associated Protease Substrate, CD8 T Cells, Cytokine Storm, Immune Checkpoint Blockade, Abscopal Effect

Posted Date: October 21st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-92075/v1>

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Version of Record: A version of this preprint was published at Nature Communications on May 13th, 2021. See the published version at <https://doi.org/10.1038/s41467-021-22980-w>.

A cytokine receptor-masked IL2 prodrug selectively activates tumor-infiltrating lymphocytes for potent antitumor therapy

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Conflict of interest statement

The authors have declared that no competing interest exists.

Summary

Interleukin-2 (IL-2) is an FDA approved treatment for multiple metastatic cancers. However, while IL-2 is a potent activator of CD8 and NK cells, its clinical use is limited by short in vivo half-life, low potency, and severe in vivo toxicity. Current strategies of reducing IL-2 receptor alpha binding and increasing IL-2 receptor beta binding reduce efficacy or increase toxicity. Here, we address these issues by engineering a novel IL-2 prodrug (ProIL2). We mask the activity of a CD8 T cell-preferred IL-2 mutein/Fc fusion protein with IL2 receptor beta linked to a tumor-associated protease substrate. ProIL2 restores activity after cleavage by tumor-associated enzymes, and preferentially activates inside tumors, where it is efficiently cleaved and expands CD8 T cells. This significantly reduces IL-2 associated cytokine storm, body weight loss, and mortality without compromising antitumor efficacy. ProIL2 also overcomes resistance of cancers to immune checkpoint blockade. Lastly, neoadjuvant ProIL2 treatment can effectively eliminate metastatic cancer by inducing an abscopal effect. Taken together, our approach presents an effective tumor targeting therapy with reduced toxicity.

Introduction

Interleukin-2 (IL-2) is a potent cytokine that activates cytotoxic T cells and NK cells¹. Because it so potently induces immune system activation, IL-2 was approved by the FDA as a treatment for renal cell carcinoma and metastatic melanoma². Unfortunately, IL-2 cancer immunotherapy encounters three major issues in the clinic. First, IL-2 does not specifically activate cytotoxic lymphocytes (CTLs) in the tumor and is used up by many IL-2 sinks in the periphery³. As a result, it has a short half-life⁴. Second, IL-2 binds with more affinity to the high affinity IL-2 receptor alpha (IL2R α), which is constitutively expressed on Tregs, than to the lower affinity IL-2 receptor beta (IL2R β), which is expressed on CD8 T cells, Tregs, and NK cells^{1,5-7}. Third, in part due to this high affinity for the trimeric high-affinity receptor complex on immunosuppressive Tregs, a higher dose of IL-2 is necessary to achieve CTL mediated immune killing of the tumor, which leads to severe toxicity in most patients².

Multiple strategies have been tested to address these three limitations. Synthesizing IL-2/Fc fusion proteins increases IL-2 half-life but increase toxicity. PEGylation of IL-2 can significantly improve IL-2 half-life but generation of consistent site-specific PEGylated IL-2 forms is a challenge to manufacturers. In addition, lack of tumor-targeted release of PEGylated IL-2 results in toxicity before reaching therapeutic doses, which limits repeatable results in clinical trials^{8,9}. Other groups have synthesized IL-2 muteins that increase IL-2 binding to IL2R β on CD8 T cells¹⁰⁻¹². Increased IL-2 selectivity of CD8 T cells may reduce its targeting and activation of Tregs but increases overall toxicity. There is an urgent need to target IL-2 binding to effector cells specifically in tumor tissues without causing peripheral toxicity.

53 In this study, we synthesized a cytokine receptor-masked IL-2 prodrug (ProIL2) that exhibits
54 minimal peripheral cytotoxicity. ProIL2 is cleaved and activated by tumor specific proteases into
55 its free form, which is an IL-2 mutein denoted as SumIL2-Fc. ProIL2 preferentially expands CTLs
56 in the tumor and has equivalent antitumor efficacy so SumIL2-Fc. Matrix metalloproteinases
57 (MMPs) have been observed to play a role in tumor angiogenesis and invasion by digesting cell-
58 matrix adhesions in cancer tissues, and are thus highly expressed on multiple tumor types¹³.
59 Because they are highly expressed in many tumors and on inflammatory cells, we choose to use
60 MMPs as our target tumor protease¹⁴. Overall, our effective and safe immunotherapy both presents
61 a novel option for combatting difficult clinical issues in the cancer field and serves as a platform
62 for developing novel potent cytokine and antibody therapies in the future.

Results

Engineering an optimal ProIL2 with high IL-2 blocking activity, MMP cleavage, and antitumor efficacy

To allow IL-2 to preferentially target tumor infiltrating lymphocytes (TILs) with long half-life, we designed ProIL2 to have four domains: human IgG1, an IL-2 variant, an IL-2 receptor, and a flexible MMP-cleavable linker. We modulate and engineer each of these four domains to maximize three criteria: blocking of IL-2 activity when uncleaved, MMP cleavability, and antitumor efficacy (Figure 1a). We initially tested a homodimeric format of ProIL2, which, in N to C terminus order, incorporates IL2R β , the MMP cleavable linker, and SumIL2 fused onto each “arm” of Fc. Intriguingly, this ProIL2 design exhibited no antitumor efficacy and could not be digested by MMPs, which raised the possibility that MMPs failed to access the cleavable linker (Supplementary Figure 1a). As a result, we engineered designs of ProIL2 to minimize potential steric clash that would prevent MMP access of the cleavable linker. These engineering strategies involved synthesizing hIgG1 heterodimers¹⁵, incorporating different or truncated IL-2 receptor subunits, and altering the orientation of domains on both the N and C termini of each Fc arm. We initially rejected the designs that exhibited minimal IL-2 blocking activity and recombinant MMP cleavage. For each ProIL2 that passed *in vitro* screening, we injected MC38 tumor bearing mice with PBS or ProIL2 and quantified the amount of tumor reduction by each ProIL2 treatment (Figure 1b). After determining which design exhibited the best antitumor efficacy, we tested modifications of the Fc region for this version of ProIL2. We observed that ProIL2 using wild-type hIgG1 Fc exhibited the same antitumor efficacy as using a mutant Fc that depletes antibody-dependent cell-mediated cytotoxicity¹⁶, but with reduced toxicity (Supplementary Figure 1b-c). Thus, we chose to use wild-type hIgG1 Fc.

We ultimately determined that the design in Figure 2a maximized our three screening criteria. On one Fc arm, we fused SumIL2 to the N terminus of wild-type Fc, linked together by a GGGGS linker. On the other Fc arm, we fused the full extracellular domain of IL2R β to the N terminus of Fc, linked together by a flexible linker that includes a synthetically determined 10 amino acid MMP protease substrate (Figure 2a). We purified and eluted this ProIL2 using Protein A chromatography and assessed its purity via SDS-PAGE, noting that we indeed created one single band of appropriate approximate size (Supplementary Figure 2a). Purification of this protein was high yield. This protein is also very stable, not degrading and still having antitumor activity even after 3 months of storage at 4°C. Overall, we tested numerous designs of ProIL2, modifying each domain of ProIL2 until we synthesized the most optimal form of ProIL2. From here on, the phrase “ProIL2” refers to our most optimal prodrug design in Figure 2a.

ProIL2 blocks IL-2 activity when uncleaved

We tested the ability of IL2R β to block both SumIL2 binding and functional activity when ProIL2 was uncleaved. First, we used flow cytometry to stain for ProIL2 binding to the surface of cells that express IL-2 receptor subunits. Compared to SumIL2-Fc (IC₅₀ = 1.1±0.2 nM), ProIL2 (IC₅₀= 14.4±1.4 nM) bound with 13-fold lower affinity to these cells (Figure 2b). We also used an HEK-Blue™ IL-2 reporter cell assay to compare functional activity of these two proteins. Similarly to the binding assay, ProIL2 (IC₅₀= 371.6±50.0 pM) also induced 8.5-fold less activity than SumIL2-Fc (IC₅₀= 43.9±4.5 pM) (Figure 2c).

We also assessed whether ProIL2 could cleave efficiently *in vitro*. We incubated ProIL2 with recombinant MMPs for multiple time points and then quantified the amounts of uncleaved and cleaved ProIL2 via SDS-PAGE (Supplementary Figure 2a). MMPs are able to cleave ProIL2

gradually over time, cleaving 70% of the initial amount of ProIL2 over 4 hours of incubation (Figure 2d). ProIL2 that has been sufficiently cleaved also has activity close to SumIL2-Fc, as demonstrated by IL-2 reporter cell assay (Figure 2e). Taken together, our design of ProIL2 effectively blocks SumIL2-Fc binding and stimulatory activity and is avidly cleaved by MMPs *in vitro*.

ProIL2 preferentially localizes to tumors and is cleaved intratumorally in vivo

Because ProIL2 cleaved well in response to MMPs, we assessed whether ProIL2 could also be cleaved *in vivo*, preferentially in the tumor. We first quantified the serum half-life of ProIL2. After injecting ProIL2 intraperitoneally into tumor bearing mice, we calculated its serum half-life to be 15.1 hours (Figure 3a). This is much longer than that of recombinant IL-2 (<15 minutes) and comparable to the 14.6 hours of SumIL2-Fc^{17,18}.

To determine how ProIL2 distributed into and cleaved in different tissues, we injected ProIL2 into CT26 tumor bearing BALB/c mice and extracted and homogenized tumors, livers, spleens, and lungs 24 hours after injection. We first quantified the amount of ProIL2 in each tissue homogenate. 2-4 times more ProIL2 per total amount of tissue localized into tumors compared to all of the other tissues (Figure 3b). We also determined the levels of multiple inflammatory cytokines in each tissue in response to ProIL2. Tumor tissue showed a high amount of IFN γ and TNF α (Figure 3c, Supplementary Figure 2b), which is consistent with a phenotype of TIL activation¹⁹⁻²¹.

We also aimed to quantify whether and how much ProIL2 would cleave in tumors *in vivo*. We used Protein A immunoprecipitation and Western Blot Analysis to quantify the amount and ratio of uncleaved and cleaved ProIL2 in each extracted tissue homogenate (Supplementary Figure

2c). On average, over 50% of ProIL2 in the tumor was cleaved, compared to less than 25% in the liver, lung, and spleen (Figure 3d). We also compared the amount of cleaved ProIL2 between tissues, and observed that cleaved ProIL2 product was staggeringly most prevalent in the tumor compared to any other tissue (Figure 3e). Similar results were observed in a B16 tumor bearing mouse model (Supplementary Figure 3). Altogether, these results demonstrate that ProIL2 preferentially localizes to and cleaves in tumors but not non-tumor tissues.

ProIL2 exhibits reduced toxicity compared to SumIL2-Fc without compromising anti-tumor efficacy

We have emphasized the necessity of reducing peripheral toxicity with our prodrug. However, its anti-tumor efficacy and ability to expand TILs should be comparable to that of SumIL2-Fc. We intraperitoneally injected equimolar doses of ProIL2 or SumIL2-Fc into MC38 tumor bearing mice and observed that while ProIL2 induced significantly less body weight loss compared to SumIL2-Fc, the two treatments exhibited equivalent efficacy in causing tumor regression (Figure 4a-b, Supplementary Figure 4a). At our lowest observed dose of maximum antitumor efficacy, we consistently observed this phenomenon in multiple tumor models (Supplementary Figure 4b-e).

We also examined cytokine related toxicity of ProIL2. We treated tumor bearing mice with PBS, SumIL2-Fc, or ProIL2, collected serum from these mice 24 hours post treatment, and quantified the level of serum inflammatory cytokines. We observed that compared to SumIL2-Fc, ProIL2 exhibits markedly reduced serum levels of inflammatory markers IFN γ and MCP-1 (Figure 4c-d). We also used flow cytometry to quantify the expansion of splenic CD8 T cells, which may also contribute to peripheral toxicity. We similarly observed that ProIL2 expands fewer CD8 T cells in the spleen relative to SumIL2-Fc (Figure 4e). Overall, ProIL2 exhibits decreased off-

target immune activation in non-tumor tissues compared to SumIL2-Fc and therefore provides a much wider therapeutic window than IL2-Fc.

While ProIL2 reduces immune activation outside of the tumor, we still want to ensure that ProIL2 potently activates intratumor CTLs. We extracted and digested tumors from PBS, SumIL2-Fc, or ProIL2 treated tumor bearing mice 72 hours after treatment. We then used flow cytometry to quantify immune cell levels in the tumor. We observed that both SumIL2-Fc and ProIL2 increased CD8 T cells in the tumor without increasing Tregs, thus inducing a high CD8:Treg ratio (Figure 4f-h). This suggests that ProIL2 induces a strong activating phenotype in the tumor microenvironment (TME). These *in vivo* experiments demonstrate that ProIL2 activates immunity in the TME and minimally stimulates toxic cytokine inflammatory responses in the periphery.

ProIL2 can overcome ICB resistance in advanced cancers

ProIL2 may activate lymphocytes to produce IFN or other cytokines, thus upregulating PD-L1 on both host and tumor cells. We aimed to expose whether host or tumor cells respond to IL-2 mediated cytokines for PD-L1 expression. We observed that ProIL2 significantly increases the PDL1⁺ CD45⁺/CD45⁻ ratio (Figure 5a, Supplementary Figure 5a-b). We hypothesize that this particular expansion of cell populations would maximize the binding of anti-PDL1 to the PDL1⁺ host cells that are required for antitumor efficacy of PDL1 blockade^{22,23}. We also observe that in CD45⁺ populations, the MFI level of PDL1 increases more in ProIL2 treated tumors than in PBS treated tumors (Figure 5b, Supplementary Figure 5c). As a result, both the increased frequency and increased expression of PDL1 in host cells in the TME suggest that ProIL2 therapy may sufficiently stimulate immune cells the TME for synergy with anti-PDL1 treatment.

We inoculated mice with B16 tumors and treated these mice with PBS, anti-PDL1, ProIL2, or ProIL2/anti-PDL1. These tumors were resistant to either anti-PDL1 or ProIL2 single therapy, as neither treatment could significantly control these tumors. However, the combination of ProIL2 and anti-PDL1 controlled these established tumors much more effectively without causing body weight loss (Figure 5c-d). These data suggest that ProIL2 can effectively overcome resistance to ICB in unresponsive tumors, still without significant toxicity.

Preoperative ProIL2 treatment can eliminate metastatic cancers through an abscopal effect

Surgical removal of tumors has remained one of the most effective treatments of primary cancers. However, surgery is not able to cure metastases, and the resulting surgical wound inflammation may potentially even be a trigger for formation of metastatic disease²⁴. In order to prevent postoperative metastatic disease, many groups have proposed using neoadjuvant immunotherapy to generate a number of T cells that may be sufficient to patrol and outnumber metastatic tumor cells.

We subcutaneously inoculated mice with 4T1 mammary tumors, which spontaneously metastasize to the lung. We preoperatively treated these tumor-bearing mice with either PBS or ProIL2 and resected tumors from half of these mice. Approximately 3 weeks after tumor resection, we extracted lungs from these mice and quantified the number of metastatic nodules and colonies in each lung. We observed that while any single treatment group had many metastatic nodules and colonies, almost no mice in our preoperative ProIL2 and surgery group had any nodules or colonies (Figure 6a-b). We observed that that 80% of preoperative ProIL2 and surgery treated mice survived, whereas the majority of mice in any other treatment group died within the time course of this study (Figure 6c). We also rechallenged these surviving ProIL2/surgery-treated mice with 4T1 tumors at a secondary location and observed that 75% of

these mice exhibited no tumor relapse. These studies demonstrate that preoperative ProIL2 and surgery treatment is vastly superior to ProIL2 or surgery alone in eradicating metastases and can also prevent cancer recurrence.

We subsequently sought to determine how ProIL2 can prevent metastases. One potential model is that ProIL2 can flow into lung tissues and activate residual lymphocytes in the lung to control metastatic disease. Alternatively, TILs activated by ProIL2 are necessary to eradicate metastases. To investigate whether preoperative ProIL2 could generate T cells that migrate into distal sites, we additionally administered FTY720, which binds to sphingosine-1-phosphate receptors in lymph nodes to prevent lymphocyte egress, to preoperative control and ProIL2 treated mice. FTY720 prevents lymphocytes in different tissues (i.e. tumor and lymph nodes) from trafficking into other tissues (i.e. lung). We observed that while combined ProIL2 and surgery treated mice prevented metastatic nodule and colony growth, addition of FTY720 to combined ProIL2 and surgery treatment abrogated this effect (Figure 6a-b). As FTY720 prevents lymphocytes from migrating from treated tissues into the lung, these data suggest that residential lymphocytes in the lung are insufficient for metastatic control, and thus require TILs. These results suggest that neoadjuvant ProIL2 induces an abscopal effect that is effective and required for eradicating metastases.

Discussion

IL-2 was initially considered to be a promising potent antitumor therapy. However, its low half-life, low tolerability, and induction of cytokine release syndrome significantly limits its use in most human patients. We engineered and generated a unique IL-2 prodrug that combines a potent CD8 T cell preferential IL-2 mutein with a blocking IL-2 receptor that can be released by tumor specific matrix metalloproteinases. This next-generation pro-cytokine incorporates dual

advantages of having potent immunostimulatory activity in the tumor and side effect minimization through tumor specific targeting. Furthermore, our ProIL2 can effectively synergize with current standard of care cancer therapies to overcome ICB resistant and even metastatic cancers.

We proposed the concept of an IL-2 prodrug in order to address limitations of not only recombinant IL-2, but also of current next-generation IL-2 designs. One popular IL-2 variant (PEG-IL2) involves attaching PEG to regions of IL-2 that normally bind to IL-2 receptors, thus decreasing peripheral activity of IL-2⁹. However, production of consistently PEGylated regions of IL-2 is not reliable, and PEG may nonspecifically detach from IL-2. In contrast to PEG-IL2, for which PEGylation and additional conjugation processes may produce mixed products, ProIL2 can be produced at a high yield as a single molecule very quickly and efficiently. Therefore, synthesis of ProIL2 would comparatively ease any concerns manufacturers may have about the production and mixed nature of PEG-IL2. ProIL2 is also significantly preferentially active in the tumor.

A second IL-2 design fuses IL-2 to an antibody, such as epidermal growth factor receptor, that targets specific proteins on tumor cells. However, upon binding of antibodies to antigens on the tumor surface, bound proteins are endocytosed, potentially before IL-2 in this case can stimulate lymphocytes²⁵. Also, even when paired with antibodies, such high affinity IL-2 variants will still target and activate T cells in the periphery. On the other hand, ProIL2 targets MMPs that are secreted by tumor cells or are attached to inflammatory cells in the TME, thus decreasing the concern of endocytosis prior to IL-2 activation of CTLs. Furthermore, antibody/IL-2 fusions require tumors to specifically express the target antigen. Because so many tumors express

MMPs, ProIL2 is not as limited in terms of the expression of a targeted tumor antigen. It is possible that antibody/ProIL2 fusions may further increase tumor targeting.

A protease activated IL-2 fusion protein was designed to attempt to block wild-type IL-2 (WT-IL2) with IL2R α ²⁶. However, this fusion does not contain any modality to extend half-life, such as Fc. Furthermore, this fusion uses WT-IL2 and thus has higher affinity for Tregs when cleaved. To reduce the preferential targeting of IL-2 to Tregs, we designed IL-2 variants with mutations that lower IL2R α binding affinity. IL2R α failed to block this mutant IL-2, likely activating CTLs before reaching tumor tissues. Moreover, a design consisting solely of WT-IL2 and IL2R α may dimerize with other WT-IL2/IL2R α complexes, thus potentially decreasing access to the protease substrate linker due to protein structural hindrance²⁷. ProIL2 with Fc has a long half-life, high affinity for CD8 T cells, and consists of domains that prevent any evident dimerization. Overall, while many other next-generation IL-2 designs have been tested, they encounter limitations that we address with ProIL2.

MMP expression has been also observed to increase as tumors become more invasive or metastatic, and is expressed in a wide range of cancers^{14,28-31}. Therefore, we chose to use MMPs based on its specificity and abundance in cancers. Our lab then used mutational screening to synthesize a 10 amino acid peptide substrate that would most effectively cleave in the presence of murine and human tumor fragments. As observed, significant levels of ProIL2 are cleaved in response to MMPs within 1 hour *in vitro* and inside tumors within 24 hours *in vivo* (Figure 2d, 3d). This suggests that ProIL2 can favorably become an active cytokine in the tumor quite shortly after injection. Strikingly, very little cleaved ProIL2 was in the liver. The liver can express many proteases such as cathepsins, plasminogen activators, and metalloproteases³²⁻³⁴. The liver is heavily involved in clearance of different proteins in the host, and proteinases may

potentially ultimately end up in the liver. The lack of cleaved ProIL2 in the liver suggests that our MMP substrate is minimally affected by these hepatic proteases and is thus more specific to MMP cleavage.

Despite the “release of brakes” by ICB, only a small fraction of patients responds to ICB. For anti-PDL1 in particular, we propose that these patients exhibit resistance to anti-PDL1 for two major reasons. First, some patients may have lower expression of PDL1 in the TME^{35,36}. Second, even after anti-PDL1 treatment, limited T cell activating and expanding cytokines in the TME may prevent effector lymphocytes from being fully stimulated. Therefore, T cell growth and activating cytokines are required for rapid and effective T cell activation and expansion, especially after anti-PDL1 treatment. We have observed that ProIL2 can “fuel” immune cells and overcome their dysfunctional state. In particular, anti-PDL1 treatment is dependent on host cell PDL1 expression^{37,38}, and ProIL2 is able to both expand the number of host PDL1⁺ cells and increase the expression level of PDL1 on host cells (Figure 5a-b). Furthermore, after anti-PDL1 treatment, ProIL2 also provides the follow-up stimulation to effector CD8 T cells in the TME to induce tumor regression (Figure 4f, 5c). Therefore, ProIL2 overcomes anti-PDL1 resistant tumors, thus providing a safe option for synergistic, powerful combination therapy against tumors that may be difficult to treat.

Tumor resection is an effective modality for curing patients of primary tumors. However, most of these patients still relapse from metastasis. Immunotherapy can potently prime TILs to egress from tumors and combat metastatic cancer cells, but can often fail to significantly overcome large tumor burdens, especially those that continue to metastasize and disseminate into peripheral tissue³⁹. Therefore, the hypothesis that combining neoadjuvant immunotherapy, which would activate and egress a sufficient number of tumor-specific T cells, with surgery can

289 eradicate a relatively small number of metastatic tumors at distal sites has been proposed.
290 Neoadjuvant immunotherapy, including preoperative IL-2, has improved patient responses to
291 surgery, likely due to improved tumor-specific CTL expansion⁴⁰⁻⁴². We also proposed that
292 ProIL2, as a potent tumor targeting immunotherapy, would effectively activate TILs which
293 would be essential for control of metastatic disease. We initially demonstrate that neoadjuvant
294 ProIL2 can indeed eliminate metastatic cancer very effectively (Figure 6). However, when we
295 also inhibited intratumor CTLs from leaving the tumor with FTY720, we observed that ProIL2
296 could no longer control distal lung metastases (Figure 6a-b). These results are consistent with our
297 hypothesis that neoadjuvant immunotherapy can prime and activate tumor specific CTLs which
298 can exit the tumor to eradicate distant metastases.

299 Taken together, our work has designed and synthesized a novel immunocytokine therapy that is
300 safe but still exhibits potent antitumor efficacy. In particular, ProIL2 preferentially targets
301 intratumor CTLs tumor without substantially inducing Tregs or peripheral lymphocytes, thus
302 overcoming a large hurdle for IL-2 therapy. Administration of ProIL2 also overcomes advanced
303 and metastatic tumor resistance to current standard of care therapies. Overall, our study has
304 provided a low toxicity but effective immunotherapy that can overcome clinically relevant
305 advanced cancers and metastasis.

306

Acknowledgments

We thank the Institutional Animal Care and Use Committee Animal Resources Center, and Animal Research Center for assistance with welfare and caretaking of all animals. This work was supported by Cancer Prevention and Research Institute of Texas (CPRIT) grant RR150072 given to Yang-Xin Fu, the T32 Integrative Immunology Training Grant, and the T32 Medical Scientist Training Program Training Grant. We also thank Jian Qiao, Zhenhua Ren, Longchao Liu, and Chunbo Dong for providing experiment materials and helpful discussions.

Author contributions

Conceptualization, E.H., X.C., and Y.-X.F.; Methodology, E.H. and Y.-X.F.; Investigation, E.H., X.C., B.M., J.B. and Z.L.; Writing - Original Draft, E.H.; Writing-Review & Editing, E.H. and Y.-X.F.; Funding Acquisition, Y.-X.F.; Resources, X.C. and Z.S.; Supervision, Y.-X.F.

Figure Legends

Figure 1. Engineering ProIL2 Design. **a** Pipeline for designing and screening the most optimal ProIL2 designs. ProIL2 designs were screened using HEK-BlueTM IL-2 reporter cell assay, MMP cleavage assay for 4 hours, and tumor inoculation and growth measurement as described in the methods. **b** Multiple designs of ProIL2 that passed the *in vitro* screening stage were tested. The Fc arms, listed from N terminus to C terminus in each entry, of each proposed design are shown. HEKBlueTM IL-2 Reporter cell assay was used to determine the IC₅₀ in nM of functional activity of each ProIL2 design. Fold block of IL2 activity was measured by dividing the IC₅₀ of each ProIL2 design to that of SumIL2-Fc. Each ProIL2 design was cleaved by MMPs and run on SDS-PAGE, where the amount of cleaved vs uncleaved ProIL2 product was quantified. MC38 s.c. tumor bearing mice were injected i.p. with PBS and an equimolar dose of the listed ProIL2 design 9 days after tumor inoculation. 12 days after treatment, tumor volumes of PBS treated and ProIL2 treated mice were measured, and the decrease in tumor volume in ProIL2 treated mice on this day was quantified. Abbreviations: IL2R β = IL-2 Receptor Beta, IL2R β * = Truncated version of IL-2 Receptor Beta, MMP = 10-mer cleavable MMP substrate, IL2R α = IL2 Receptor Alpha, WTIL2 = Wild-type IL-2. Data represent the mean $\pm$ s.e.m of replicates.

Figure 2. ProIL2 effectively masks IL-2 activity and is cleaved by MMPs *in vitro*. **a** Schematic of ProIL2 depicting that IL2RB binds and blocks IL-2 activity prior to cleavage (left) and activates into SumIL2-Fc upon MMP cleavage (right). **b** Binding of SumIL2-Fc and ProIL2 at different doses to HEK-BlueTM IL-2 Reporter cells was assessed via flow cytometry (n=3 experiments, 3 replicates each). **c** Functional activity of SumIL2-Fc and ProIL2 was assessed by using HEK-BlueTM IL-2 Reporter cell assay (n=3 experiments, 3 replicates each). **d** Protease activation of ProIL2. ProIL2 was incubated with human MMP2, MMP9, and MMP14 for 1 or 4 hours, then run

on non-reducing SDS-PAGE, where gel band intensity was subsequently quantified (n=3 experiments). **e** Functional activity of SumIL2-Fc, ProIL2, and ProIL2 that was incubated with human MMP2, MMP9, and MMP14 for 4 hours was assessed by using HEK-BlueTM IL-2 Reporter cell assay (n=1 of 2 experiments, 3 replicates each). Data represent the mean $\pm$ s.e.m of replicates.

Figure 3. ProIL2 preferentially localizes to and cleaves effectively in tumors. **a** Equimolar doses of SumIL2-Fc (20 μ g) or ProIL2 (30 μ g) was injected i.p. into CT26 s.c. tumor bearing mice, and serum was collected and isolated at multiple time points. hIgG ELISA was used to quantify the amount of SumIL2-Fc or ProIL2 in the serum (n=2 experiments, 4 replicates each). **b-e** 20 μ g ProIL2 was injected i.p. into CT26 s.c. tumor bearing mice, and the labeled tissues were collected and homogenized 24 hours after treatment (n=2 experiments, 4 replicates each). **b** hIgG ELISA was used to quantify the amount ProIL2 in each homogenate, normalized by total tissue weight. The quantity within each mouse were then normalized to the amount of ProIL2 in that mouse's tumor. **c** Cytometric Bead Array was used to quantify the amount of IFN γ in each tissue homogenate. **d-e** hIgG immunoprecipitation of equivalent weights of tissue homogenate was performed with Protein A binding beads, and then run on Western Blot with hIgG binding antibody. **d** Relative gel band intensity per lane was quantified. **e** Western Blot relative gel band intensities were quantified, and within each mouse, the total percentage of cleaved ProIL2 between each tissue was compared and quantified. Data represent the mean $\pm$ s.e.m of replicates.

Figure 4. *In vivo* assessment of ProIL2. **a-b** MC38 s.c. tumor bearing mice were injected i.p. with one dose of the labeled treatment on day 9 post tumor inoculation; tumor growth and body weight were measured (n=2 experiments, 3-4 replicates each). **c-e** B16 s.c. tumor bearing mice were injected i.p. with PBS, SumIL2-Fc or ProIL2 (120 pmol) one time on day 9 post tumor inoculation (n=2 experiments, 3-4 replicates each). Serum was collected and isolated from mice

24 hours post treatment, and Cytometric Bead Array was used to quantify the amount of serum IFN γ or MCP-1. **e** 72 hours after treatment, mice were euthanized and spleens were extracted and resuspended as single cells. Flow cytometry was used to quantify the amount of CD8 T cells in each respective group. **f-h** B16 s.c. tumor bearing mice were injected i.p. with PBS, SumIL2-Fc or ProIL2 (300 pmol) one time on day 9 post tumor inoculation (n=4 experiments, 4 replicates each). 72 hours after treatment, mice were euthanized and tumors were extracted, digested in collagenase/DNAse, and resuspended as single cells. Flow cytometry was used to quantify the amount of CD8 T cells, amount of Tregs, and CD8:Treg ratio in each respective group. Data represent the mean $\pm$ s.e.m. of replicates.

Figure 5. ProIL2 can overcome advanced tumor resistance to ICB. **a-b** B16 s.c. tumor bearing mice were injected i.p. with PBS or ProIL2 (300 pmol) one time on day 9 post tumor inoculation. 72 hours after treatment, mice were euthanized and tumors were extracted, digested in collagenase/DNAse, and resuspended as single cells. Flow cytometry was used to quantify the amount of PDL1⁺ and CD45⁺ cells. In CD45⁺ cells, the MFI of PDL1⁺ and PDL1⁻ cells were compared (n=3 experiments, 4 replicates each). **c-d** B16 s.c. tumor bearing mice were injected i.p. with PBS, anti-PDL1 (200 μ g each), ProIL2 (300 pmol), or anti-PDL1 and ProIL2 one time on day 9 post tumor inoculation; tumor growth and body weight were measured (n=1 of 2 experiments, 3-4 replicates each). Data represent the mean $\pm$ s.e.m. of replicates.

Figure 6. Preoperative ProIL2 combined with surgery can eliminate metastases through an abscopal effect. 4T1 s.c. tumor bearing mice were injected i.p. with PBS or ProIL2 (300 pmol) on day 9 and 12 post tumor inoculation. Mice treated with FTY720 were i.p. treated with 10 μ g every other day for 14 days starting on day 8 post tumor inoculation. On day 14 post tumor inoculation, the listed mice had their tumors surgically removed. **a** 25 days post initial treatment, mice were

388 euthanized and metastatic nodules in the thoracic cavity were counted. **b** 25 days post initial
389 treatment, mice were euthanized and lungs were extracted, digested in collagenase/DNAse, and
390 resuspended as single cells. Resuspended cells were plated at a 1:400 ratio with 6-thioguanine
391 selection medium, and the number of colonies were counted 6 days post seeding (n=2 experiments,
392 2-5 replicates each). **c** Mice were analyzed for survival starting the day after the first i.p. injection
393 treatment (n=2 experiments, 5 replicates each). Data represent the mean $\pm$ s.e.m. of replicates.

394

395 **Methods**

396 **Mice**

397 Female C57BL/6J and BALB/c mice were purchased from The Jackson Laboratory. All mice were
398 maintained under specific pathogen-free conditions. Animal care and experiments were carried out
399 under institutional and National Institutes of Health protocol and guidelines. This study has been
400 approved by the Institutional Animal Care and Use Committee of the University of Texas
401 Southwestern Medical Center.

402 **Cell lines and reagents**

403 B16, MC38, CT26, and 4T1 cell lines were purchased from American Type Culture Collection
404 (ATCC). All cell lines were routinely tested using mycoplasma contamination kit (R&D) and
405 cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal
406 bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin under 5% CO₂ at 37 °C. Anti-
407 PDL1 (Atezolizumab) mAb was purchased from Genentech. FTY720 was purchased from
408 Selleckchem

409 **Flow Cytometry Analysis**

410 Single cell suspensions from either spleen, tumor or *in vitro* co-cultured cells were incubated with
411 anti-FcγIII/II receptor (clone 2.4G2) for 15 minutes to block non-specific binding before staining
412 with the conjugated antibodies. Fixable Viability Dye eFluorTM 506 was used to exclude dead cells.
413 Foxp3 was stained intracellularly by using True-Nuclear transcription factor buffer set (BioLegend)
414 following the manufacturer's instructions.

To assess ProIL2 and SumIL2-Fc binding affinity, HEK-BlueTM IL-2 cells (InvivoGen) were first stained with various dilutions of ProIL2 or SumIL2-Fc, then PE conjugated donkey anti-human IgG was used as a secondary antibody. All staining steps were conducted at 4 °C in the dark. BDTM Cytometric Bead Array (CBA) Mouse Inflammation Kit was used to measure the cytokines in the supernatants from mouse serum or tissue according to the manufacturer's protocol (BD Biosciences). Data were collected on CytoFLEX flow cytometer (Beckman Coulter, Inc) and analyzed by using CytExpert (Beckman Coulter, Inc) or FlowJo (Tree Star Inc., Ashland, OR) software.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Microtiter plates (Corning Costar) were coated with 2 µg/mL (100 µL/well) capture antibody (AffiniPure Goat Anti-Human IgG, Fcγ fragment specific) overnight at 4 °C. After washing and blocking, diluted tissue lysate or serum from treated mice were added and incubated at 37 °C for 1 hr. After washing, Alkaline Phosphatase (AP) conjugated Goat Anti-Human IgG (Fcγ fragment specific) was added and incubated at 37 °C for 30 minutes. Finally, the plates were visualized by adding 100 µL KPL Diethanolamine + pNPP substrate (Seracare Life Sciences) and read at 405 nm using the SPECTROstar Nano (BMG LABTECH).

Tumor growth and treatment

A total of 1×10^6 MC38, 3×10^5 B16, 5×10^5 CT26, or 5×10^5 4T1 cells were inoculated subcutaneously into right dorsal flanks of the mice in 100 µL phosphate buffered saline (PBS). Tumor-bearing mice were randomly grouped into treatment groups when tumors grew to around 70-100mm³. For SumIL2-Fc treatment in MC38/B16/CT26 tumor bearing mice, one dose of 1 mg/kg (300 pmol or 20 µg) was intraperitoneally given on day 9. For ProIL2 treatment in

MC38/B16/CT26 tumor bearing mice, one dose of 1.5 mg/kg (300 pmol or 30 µg) was intraperitoneally given on day 9. For ProIL2 treatment in 4T1 tumor bearing mice, two doses of 1.5 mg/kg (300 pmol or 30 µg) was intraperitoneally given on days 9 and 12. For surgical treatment, tumors and surrounding skin were removed on day 14. For FTY720 treatment, 10 µg FTY720 was intraperitoneally administrated one day before ProIL2 treatment initiation and then 10 µg every other day for 2 weeks or until experiment termination. 200 µg anti-PDL1 was administered concurrently with ProIL2 treatment. For rechallenge experiments, 2.5×10^6 4T1 cells were inoculated subcutaneously into left dorsal flanks of mice in 100 µL PBS. Tumor volumes were measured by the length (a), width (b) and height (h) and calculated as tumor volume = $abh/2$.

Production of Fusion Proteins

Based on the heterodimeric Fc variant KiHss-AkKh platform as previously described, sumIL2 was fused with knob variant Fc region, and the IL2Rβ/MMP linker was fused with hole variant Fc region. ProIL2 and SumIL2-Fc were generated by transient co-transfection of two arms of plasmids into FreeStyle™ 293-F cells. The supernatant containing fusion proteins was purified using Protein A affinity chromatography according to the manufacturer's protocol. The heterogeneity and purity were confirmed by SDS-PAGE.

Tissue Homogenate Preparation

Spleen, lung, liver and tumor were excised one day after ProIL2 treatment and homogenized in the Cell Lysis Kit (Bio-Rad Laboratories) with the FastPrep-24 5G Homogenizer. The samples were centrifuged for 10 minutes at 13000 rpm, and the supernatant was collected.

Tumor Digestion

Tumor tissues were excised and digested with 1 mg/mL Collagenase I (Sigma) and 0.5 mg/mL DNase I (Roche) in the 37°C for 30mins, tumor was then passed through a 70 µm cell strainer to remove large pieces of undigested tumor. Tumor infiltrating cells were washed twice with PBS containing 2 mM EDTA.

IL-2 Reporter Cell Culture Assay

Various dilutions of IL-2 fusion protein variants were incubated with 5x10⁴ HEK-Blue™ IL-2 cells (InvivoGen) for 24 hours as per manufacturer's protocol. Afterwards, 25 µL cell supernatant was collected and incubated with 75 µL Quanti-Blue reagent (InvivoGen) for 1 hour. The mixture was read at 650 nm using the SPECTROstar Nano (BMG LABTECH).

MMP cleavage of ProIL2 and SDS-PAGE

Recombinant human MMP2 and MMP9 (BioLegend) were activated with 1mM 4-Aminophenylmercuric acetate for 1 hour as per manufacturer's instructions. Recombinant human MMP14 (R&D Sciences) was activated with 0.1 µg/mL rhTrypsin (R&D Sciences) for 1 hour and then incubated with 1 ng/µL AEBSF (R&D Sciences) for 15 minutes as per manufacturer's instructions. Cleavage Assay Buffer consisted of 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, and 0.05% (w/v) Brij 35 at pH 7.5. 50 ng MMP2, 50 ng MMP9, and 100 ng MMP14 were incubated with 1.5 µg ProIL2 in Cleavage Assay Buffer at 37°C for 1 or 4 hours. Proteins were then run on SDS-PAGE and relative gel band intensities were analyzed to quantify the amount of cleavage in each ProIL2/MMP mixture.

Tissue Homogenate Immunoprecipitation and Western Blot Assay

Tumor, liver, lung, and spleen tissues were homogenized as described above. Equivalent amounts of tissue homogenate by weight were incubated with Protein A binding beads at a 100:1

ratio for 12 hours at 4°C. The beads were then isolated and run on a non-reducing SDS-PAGE. Proteins were transferred onto membrane using Trans-Blot Turbo manufacturer instructions. The membrane was incubated and rotated on shaker with 5% milk in Phosphate Buffered Saline with 0.1% Tween 20 Detergent for 1 hour. After washing, Horseradish Peroxidase (HRP) conjugated Goat Anti-Human IgG (H+L) was added at a 1:2000 dilution and rotated on shaker for 1 hour. The membrane was developed using SuperSignal West Dura Extended Duration Substrate and imaged with ChemiDoc Touch Imaging System (BioRad).

4T1 Metastatic Tumor Colonogenic Assay

Lung tissues from 4T1 bearing or tumor resected mice were excised and digested with 1 mg/mL Collagenase I (Sigma) and 0.5 mg/mL DNase I (Roche) at 37°C for 30mins, and then passed through a 70 µm cell strainer to remove large pieces of undigested tumor. Lungs cells were resuspended in 3 mL Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 10 µg/mL 6-thioguanine. The cells were plated in 6 well plates at a 1:400 dilution. After 6 days, cells were stained with crystal violet staining solution and colonies were counted.

Statistical analysis

All the data analyses were performed with GraphPad Prism statistical software and shown as mean ± SEM. P value was determined by two-way ANOVA for tumor growth, Log-rank test for survival, or unpaired two-tailed t-tests for other analysis. A value of $p < 0.05$ was considered statistically significant. For notation on figures, * corresponds with $p < 0.05$, ** with $p < 0.005$, and *** with $p < 0.0005$.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-PDL1 Atezolizumab	Genentech	N/A
Anti-CD45 (Flow cytometry, 30-F11)	BioLegend	Cat# 103126
Anti-CD8 (Flow cytometry, 53-6.7)	BioLegend	Cat# 100730
Anti-CD4 (Flow cytometry, RM4-5)	BD Biosciences	Cat# 550954
Anti-Foxp3 (Flow cytometry, MF-14)	BioLegend	Cat# 126408
Anti-PD-L1 (Flow cytometry, 10F.9G2)	BioLegend	Cat# 124308
Fixable Viability Dye eFluor™ 506	Thermo Fisher	Cat# 65-0866-18
Anti-FcγIII/II receptor (clone 2.4G2)	BD Biosciences	Cat# 553141
Goat Anti-Human IgG-HRP	Santa Cruz Biotech	Cat# sc-2453
AffiniPure Goat Anti-Human IgG, Fcγ fragment specific	Jackson ImmunoResearch	Cat# 109-005-098
Alkaline Phosphatase-AffiniPure Goat Anti-Human IgG, Fcγ fragment specific	Jackson ImmunoResearch	Cat# 109-055-098
Donkey Anti-Human IgG (H+L)	Jackson ImmunoResearch	Cat# 709-116-149
Bacterial and Virus Strains		
N/A		
Biological Samples		
N/A		
Chemicals, Peptides, and Recombinant Proteins		
FTY720 (hydrochloride)	Selleckchem	Cat# S5002
Dulbecco's Modified Eagle's Medium	Sigma- Aldrich	Cat# D6429
Collagenase type I	Sigma	Cat# C0130
DNase I	Roche	Cat# 11284932001
6-Thioguanine, 98%, Alfa Aesar	Thermo Fisher	Cat# AAB2128003
Recombinant Human MMP-2 (carrier-free)	BioLegend	Cat# 554302
Recombinant Human MMP-9 (carrier-free)	BioLegend	Cat# 550502
4-Aminophenylmercuric acetate	Sigma- Aldrich	Cat# A9563
Recombinant Human MMP-14/MT1-MMP (NS0-expressed) Protein, CF	R&D Systems	Cat# 918-MPN-010
Recombinant Human Active Trypsin 3/PRSS3 Protein, CF	R&D Systems	Cat# 3714-SE-010
AEBSF, CF	R&D Systems	Cat# EI001
QUANTI-Blue™	InvivoGen	Cat# rep-qbs
Critical Commercial Assays		
BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit	BD Biosciences	Cat# 552364
True-Nuclear™ Transcription Factor Buffer Set	BioLegend	Cat# 424401
pNPP Microwell Substrate System	Seracare Life Sciences	Cat# 50-80-00
Deposited Data		
N/A		
Experimental Models: Cell Lines		

B16	ATCC	Cat# CRL-6322
MC38	ATCC	N/A
CT26	ATCC	Cat# CRL-2638
4T1	ATCC	Cat# CRL-2539
FreeStyle™ 293-F	Thermo Fisher	Cat# R79007
HEK-Blue™ IL-2 Cells	InvivoGen	Cat# hkb-il2
Experimental Models: Organisms/Strains		
C57BL/6J	Jackson Laboratory	Cat# 000664
BALB/c	Jackson Laboratory	Cat# 000651
Oligonucleotides		
N/A		
Recombinant DNA		
Plasmid: pEE6.4-IL2R β -MMP-Fc9	This paper	N/A
Plasmid: pEE6.4-SumIL2-Fc6	This paper	N/A
Plasmid: pEE6.4-Fc9	This paper	N/A
Software and Algorithms		
GraphPad Prism software 7.0	GraphPad Software, Inc.	https://graphpad.com/scientific-software/prism/
CytExpert	Beckman Coulter, Inc	https://www.beckman.com/coulter-flow-cytometers/cytoflex/cytextpert
FlowJo	Tree Star Inc.	https://www.flowjo.com/solutions/flowjo
Image Lab™ Software	Bio-Rad	http://www.bio-rad.com/en-us/category/image-analysis-software
Other		
N/A		

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607 Figure 1

b

Arm 1	Arm 2	IC50	Fold Block of IL2 Activity	% Cleavage	% Tumor Reduction
SumIL2-Fc	Fc	43.9 ± 4.5	N/A	N/A	93.8 ± 2.8
IL2Rβ-Fc	SumIL2-Fc	1072.6 ± 115.8	24.4	0	4.5 ± 17.0
IL2Rβ-MMP-Fc	SumIL2-Fc	371.6 ± 50.0	8.5	66.4	89.8 ± 3.0
IL2Rβ-MMP-IL2Rα-Fc	WTIL2-Fc	360.7 ± 24.2	8.2	52.8	75.5 ± 11.5
IL2Rβ*-MMP-SumIL2-Fc	Fc	347.4 ± 17.9	7.9	29.1	56.6 ± 10.2
IL2Rβ-MMP-SumIL2-Fc	Fc	846.1 ± 40.0	19.3	14	54.3 ± 11.7
Fc-SumIL2-MMP-IL2Rβ	Fc	1301.0 ± 229.5	29.5	15.8	24.5 ± 19.8
IL2Rβ-MMP-IL2Rα-WTIL2-Fc	Fc	4837.7 ± 1032.6	110.0	28.6	21.8 ± 25.1

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610 Figure 2

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613 Figure 3

616 Figure 4

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Figures

Figure 1

Engineering ProIL2 Design. a Pipeline for designing and screening the most optimal ProIL2 designs. ProIL2 designs were screened using HEK-Blue™ IL-2 reporter cell assay, MMP cleavage assay for 4 hours, and tumor inoculation and growth measurement as described in the methods. b Multiple designs of ProIL2 that passed the in vitro screening stage were tested. The Fc arms, listed from N terminus to C terminus in each entry, of each proposed design are shown. HEKBlue™ IL-2 Reporter cell assay was used to determine the IC50 in nM of functional activity of each ProIL2 design. Fold block of IL2 activity was measured by dividing the IC50 of each ProIL2 design to that of SumIL2-Fc. Each ProIL2 design was cleaved by MMPs and run on SDS-PAGE, where the amount of cleaved vs uncleaved ProIL2 product was quantified. MC38 s.c. tumor bearing mice were injected i.p. with PBS and an equimolar dose of the listed ProIL2 design 9 days after tumor inoculation. 12 days after treatment, tumor volumes of PBS treated and ProIL2 treated mice were measured, and the decrease in tumor volume in ProIL2 treated mice on this day was quantified. Abbreviations: IL2Rβ = IL-2 Receptor Beta, IL2Rβ* = Truncated version of IL-2 Receptor

Beta, MMP = 10-mer cleavable MMP substrate, IL2R α = IL2 Receptor Alpha, WTIL2 = Wild-type IL-2. Data represent the mean $\pm$ s.e.m of replicates.

Figure 2

ProlL2 effectively masks IL-2 activity and is cleaved by MMPs in vitro. **a** Schematic of ProlL2 depicting that IL2RB binds and blocks IL-2 activity prior to cleavage (left) and activates into SumIL2-Fc upon MMP cleavage (right). **b** Binding of SumIL2-Fc and ProlL2 at different doses to HEK-BlueTM IL-2 Reporter cells was assessed via flow cytometry (n=3 experiments, 3 replicates each). **c** Functional activity of SumIL2-Fc and ProlL2 was assessed by using HEK-BlueTM IL-2 Reporter cell assay (n=3 experiments, 3 replicates each). **d** Protease activation of ProlL2. ProlL2 was incubated with human MMP2, MMP9, and MMP14 for 1 or 4 hours, then run on non-reducing SDS-PAGE, where gel band intensity was subsequently quantified (n=3 experiments). **e** Functional activity of SumIL2-Fc, ProlL2, and ProlL2 that was incubated with human MMP2, MMP9, and MMP14 for 4 hours was assessed by using HEK-BlueTM IL-2 Reporter cell assay (n=1 of 2 experiments, 3 replicates each). Data represent the mean $\pm$ s.e.m of replicates.

Figure 3

ProlL2 preferentially localizes to and cleaves effectively in tumors. **a** Equimolar doses of SumIL2-Fc (20 μ g) or ProlL2 (30 μ g) was injected i.p. into CT26 s.c. tumor bearing mice, and serum was collected and isolated at multiple time points. hIgG ELISA was used to quantify the amount of SumIL2-Fc or ProlL2 in the serum ($n=2$ experiments, 4 replicates each). **b-e** 20 μ g ProlL2 was injected i.p. into CT26 s.c. tumor bearing mice, and the labeled tissues were collected and homogenized 24 hours after treatment ($n=2$ experiments, 4 replicates each). **b** hIgG ELISA was used to quantify the amount ProlL2 in each homogenate, normalized by total tissue weight. The quantity within each mouse were then normalized to the amount of ProlL2 in that mouse's tumor. **c** Cytometric Bead Array was used to quantify the amount of IFN γ in each tissue homogenate. **d-e** hIgG immunoprecipitation of equivalent weights of tissue homogenate was performed with Protein A binding beads, and then run on Western Blot with hIgG binding antibody. **d** Relative gel band intensity per lane was quantified. **e** Western Blot relative gel band intensities were quantified, and within each mouse, the total percentage of cleaved ProlL2 between each tissue was compared and quantified. Data represent the mean $\pm$ s.e.m of replicates.

Figure 4

In vivo assessment of ProlL2. a-b MC38 s.c. tumor bearing mice were injected i.p. with one dose of the labeled treatment on day 9 post tumor inoculation; tumor growth and body weight were measured (n=2 experiments, 3-4 replicates each). c-e B16 s.c. tumor bearing mice were injected i.p. with PBS, SumIL2-Fc or ProlL2 (120 pmol) one time on day 9 post tumor inoculation (n=2 experiments, 3-4 replicates each). Serum was collected and isolated from mice 24 hours post treatment, and Cytometric Bead Array was used to quantify the amount of serum IFN γ or MCP-1. e 72 hours after treatment, mice were euthanized and spleens were extracted and resuspended as single cells. Flow cytometry was used to quantify the

amount of CD8 T cells in each respective group. f-h B16 s.c. tumor bearing mice were injected i.p. with PBS, SumIL2-Fc or ProIL2 (300 pmol) one time on day 9 post tumor inoculation (n=4 experiments, 4 replicates each). 72 hours after treatment, mice were euthanized and tumors were extracted, digested in collagenase/DNAse, and resuspended as single cells. Flow cytometry was used to quantify the amount of CD8 T cells, amount of Tregs, and CD8:Treg ratio in each respective group. Data represent the mean $\pm$ s.e.m. of replicates.

Figure 5

ProIL2 can overcome advanced tumor resistance to ICB. a-b B16 s.c. tumor bearing mice were injected i.p. with PBS or ProIL2 (300 pmol) one time on day 9 post tumor inoculation. 72 hours after treatment, mice were euthanized and tumors were extracted, digested in collagenase/DNAse, and resuspended as single

cells. Flow cytometry was used to quantify the amount of PDL1+ and CD45+ cells. In CD45+ cells, the MFI of PDL1+ and PDL1- cells were compared (n=3 experiments, 4 replicates each). c-d B16 s.c. tumor bearing mice were injected i.p. with PBS, anti-PDL1 (200 µg each), ProlL2 (300 pmol), or anti-PDL1 and ProlL2 one time on day 9 post tumor inoculation; tumor growth and body weight were measured (n=1 of 2 experiments, 3-4 replicates each). Data represent the mean ± s.e.m. of replicates.

Figure 6

Preoperative ProlL2 combined with surgery can eliminate metastases through an abscopal effect. 4T1 s.c. tumor bearing mice were injected i.p. with PBS or ProlL2 (300 pmol) on day 9 and 12 post tumor inoculation. Mice treated with FTY720 were i.p. treated with 10 µg every other day for 14 days starting on day 8 post tumor inoculation. On day 14 post tumor inoculation, the listed mice had their tumors surgically removed. a 25 days post initial treatment, mice were euthanized and metastatic nodules in the thoracic cavity were counted. b 25 days post initial treatment, mice were euthanized and lungs were extracted, digested in collagenase/DNase, and resuspended as single cells. Resuspended cells were plated at a 1:400 ratio with 6-thioguanine selection medium, and the number of colonies were counted 6 days post seeding (n=2 experiments, 2-5 replicates each). c Mice were analyzed for survival starting the day after the first i.p. injection treatment (n=2 experiments, 5 replicates each). Data represent the mean ± s.e.m. of replicates.

Supplementary Files

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