Switchable immune modulator for tumor-specific activation of anticancer immunity

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Immune stimulatory antibodies and cytokines elicit potent antitumor immunity. However, the dose-limiting systemic toxicity greatly hinders their clinical applications. Here, we demonstrate a chemical approach, termed “switchable” immune modulator (Sw-IM), to limit the systemic exposure and therefore ameliorate their toxicities. Sw-IM is a biomacromolecular therapeutic reversibly masked by biocompatible polymers through chemical linkers that are responsive to tumor-specific stimuli, such as high reducing potential and acidic pH. Sw-IMs stay inert (switch off) in the circulation and healthy tissues but get reactivated (switch on) selectively in tumor via responsive removal of the polymer masks, thus focusing the immune boosting activities in the tumor microenvironment. Sw-IMs applied to anti–4-1BB agonistic antibody and IL-15 cytokine led to equivalent antitumor efficacy to the parental IMs with markedly reduced toxicities. Sw-IM provides a highly modular and generic approach to improve the therapeutic window and clinical applicability of potent IMs in mono- and combinational immunotherapies.

INTRODUCTION

Immune stimulatory agonistic antibodies (e.g., anti–4-1BB) and cytokines [e.g., interleukin-15 (IL-15)] elicit potent anticancer immunity. However, their therapeutic potential is greatly hampered by the severe systemic toxicity deriving from excessive activation of immune and inflammatory responses in the circulation compartment and/or healthy tissues where the target receptors are widely present (1–4). In addition, combination therapies increase the patients’ response rate to immunotherapy at the cost of exacerbated toxicities. For instance, adoptive T cell transfer (ACT) adjuvanted by supporting cytokines enhances the efficacy against solid tumors (5, 6) but leads to severe, sometimes life-threatening, side effects (7, 8).

To enlarge the therapeutic window of biomacromolecular immune modulators (IMs) for mono- or combinational immunotherapies, there are two general means that could be applied. One is to physically confine the IM distribution in tumor through local (9–11) or targeted (12–14) delivery. The other is to achieve selective activation of IMs in the tumor microenvironment (TME) regardless of their biodistribution. The latter has been recently applied in developing antibody prodrugs that are masked by recombinant fusion peptides (8, 15–20) or protein domains (21–23) and unmasked specifically in the TME in response to tumor-associated proteases. These genetic engineering approaches have so far been exclusively limited on the design of protease responsiveness and applicable in a handful of antibodies or antibody fragments.

Here, we demonstrate a generalizable chemical approach, termed switchable IM (Sw-IM), which could be widely applied to a broad range of IMs including antibodies and cytokines with diverse responsiveness. IMs were reversibly blocked by biocompatible polymers through responsive covalent linkers that degraded in response to stimuli specific in the TME, such as the high reducing potential and acidic pH, to release the polymer blockades (Fig. 1). The prepared Sw-IMs remained at off status in peripheral blood and healthy tissues but rapidly switched on in the TME with regained immune stimulating activities (Fig. 1, A and B). Treatments with redox-responsive switchable anti–4-1BB antibody as a monotherapy, or a combination therapy of redox-responsive switchable IL-15 superagonist (IL-15SA) and ACT led to equivalent antitumor efficacy to the parental IMs with markedly enhanced safety profiles in mouse models of cancer. This facile and generally applicable approach allowed modular design using diverse protein therapeutics, masking polymers, and chemical linkers of different responsiveness (Fig. 1C). Analyses of a small library of Sw-IMs for their in vitro and in vivo performance enabled us to determine the favorable formulation parameters of Sw-IMs for the optimized antitumor efficacy and safety profile.

RESULTS

Design and preparation of Sw-IMs

To prove the concept, we first conjugated anti-trinitrophenol (TNP) antibody as a model drug with polyethylene glycol (PEG), a hydrophilic and biocompatible polymer with low immunogenicity (24), through a traceless redox-responsive linker bearing two amine-reactive N-hydroxysuccinimide (NHS) groups (fig. S1). These redox-responsive antibody-polymer conjugates, termed switchable anti-TNP, (SwredoxaTNP; Table 1, entry 1, and table S1, entries 1 to 7) were successfully prepared evidenced by the increased molecular weight (MW) characterized by SDS–polyacrylamide gel electrophoresis (PAGE) (fig. S2A) and ultrahigh-performance liquid chromatography (UHPLC) equipped with a size exclusion chromatography (SEC) column (fig. S2B), as well as the increased sizes measured by dynamic light scattering (DLS) (fig. S2C).

Next, we focused on anti–4-1BB, an agonistic antibody that stimulates tumor necrosis factor receptor superfamily member 9 (TNFRSF-9) and is a potent cancer immunotherapy, but its clinical applications have been greatly hindered by the hepatotoxicity (25, 26). We prepared a series of redox-responsive switchable anti–4-1BB antibodies (Swredoxa4-1BBs; Table 1, entries 3 to 8, and table S1, entries 8 to 13). To fine-tune the masking effects, we controlled the
degree of modification density and the MW of PEG (M_{PEG}) for conjugation. By varying the feeding mole ratios of PEG to antibody (R_{F}), the percentage of conjugated amino groups among all the detectable amino groups of the antibody molecule (termed conjugation ratio, R_C) can be tuned (Fig. 2A and Table 1, entries 3 to 8), which was determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBSA) assay (fig. S3) (27). A higher R_F generally resulted in a higher R_C; the maximal R_C is limited by certain number of reactive amino groups per antibody molecule (maximal R_C = 83.1% for Sw_{redox}a4-1BBs; table S1, entry 11). For example, anti–4-1BB antibody conjugated with PEG (M_{PEG} = 10 kDa) with an R_F of 50 (Sw_{redox}a4-1BB 10k-50) resulted in an R_C of 61.8%, while the R_C value of Sw_{redox}a4-1BB 10k-100 was 73.9%. In addition, a higher M_{PEG} led to a lower R_C because of reduced reactivity and increased steric hindrance during reaction (Fig. 2A and Table 1, entries 3, 5, and 7 and entries 4, 6, and 8). Sw_{redox}a4-1BBs showed larger MWs compared to the native anti–4-1BB antibody characterized by both SDS-PAGE and UHPLC (Fig. 2, B and C). Similarly, the hydrodynamic dimeter of Sw_{redox}a4-1BBs increased as measured by DLS (fig. S4A).

The syntheses of the chemically modified switchable antibodies can be readily extended to other immunoglobulin G (IgG) antibodies in addition to anti-TNP and anti–4-1BB antibodies, such as checkpoint blockade antibodies against programmed cell death protein 1 (anti–PD-1) (Sw_{redox}aPD-1 s; Table 1, entry 9, fig. S5A, and table S1, entries 14 to 21) and cytotoxic T lymphocyte–associated protein 4 (anti–CTLA-4) (Sw_{redox}aCTLA4s; Table 1, entry 10, fig. S5B, and

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**Fig. 1. Design of Sw-IMs.** (A) Chemical structures and responsive switch on of redox- and acidic pH–responsive Sw-IMs. Sw_{redox}IM, redox-responsive Sw-IM; Sw_{pH(D/S)}IM, acidic pH–responsive Sw-IM with dibenzocyclooctyne (DBCO) or thioether spacer. (B) Schematic illustration of the preparation and in vivo fate of Sw-IMs. Sw-IMs remain switched off in the circulation and healthy tissues and get switched on specifically in the TME to stimulate proliferation and effector functions of CD8+ T cells and NK cells for cancer immunotherapy. (C) Modular design of Sw-IMs by tuning the conjugation ratio (R_C) and MW (M_{PEG}) of masking polymers. R_C is defined as the percentage of conjugated amino groups among all the detectable amino groups of an IM.
Controlled switch off and on of Sw-IMs

To validate the responsiveness, Sw_redox_a4-1BBs and Sw_redox_IL15s were treated with l-glutathione (GSH) mimicking the reducing environment in the TME (30). Compared to the nonreduced Sw-IMs (off status), the reduced Sw-IMs (on status) exhibited largely decreased R_C values, revealing liberation of free amino groups and thus successful responsive removal of the masking polymers (Fig. 2, A and D). The reduced Sw-IMs also had lowered MWs, which were close to that of the native IMs as characterized by the SDS-PAGE gels (Fig. 2, B and E). However, there were residual PEG polymers that were not completely removed in the set reducing condition. More potent reducing condition could not only potently remove fully the residue-conjugated polymers but also lead to cleavage of disulfide bonds in the hinge region and degradation of the proteins. Similarly to the redox responsive Sw-IMs, the PEG masks of pH-responsive Sw pH(D)_a4-1BBs and Sw pH(H)_a4-1BBs could be removed in response to the acidic pH (fig. S8, A and B).

Next, to assess the biological functions of Sw-IMs upon responsive reactivation, we first determined their binding capacity toward activated CD8^+ T cells, which expressed a variety of surface receptors including 4-1BB and IL-15R (Fig. 2G) (31–33). The binding capability of fluorescently labeled Sw-IMs toward activated CD8^+ T cell was determined by measuring the mean fluorescence intensities (MFIs) with flow cytometry. Sw-IMs at off status exhibited markedly reduced affinity compared to the native IMs, while the reactivated Sw-IMs (on status) showed completely or partially recovered binding capacity (Fig. 2, H to K). Sw_redox_a4-1BBs at off status exhibited

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### Table 1. Preparation and characterizations of Sw-IMs.

| Entry | Sw-IM | IM        | Linker* | M_PEG (Da) | RF | R_C (%) | Size (nm)† | EC50 off (nM)‡ | EC50 on (nM)‡ |
|-------|-------|-----------|---------|------------|----|---------|------------|---------------|--------------|
| 1     | Sw_redox_aTNP| Anti-TNP | SS      | 10k        | 100 | 59.5    | 8.2 ± 2.2  | 0.077         |              |
| 2     | a4-1BB | Anti–4-1BB| SS      | 5k         | 50  | 66.8    | 7.3 ± 2.1  | 1.3 ± 0.2     | 0.29         |
| 3     | Sw_redox_a4-1BB_10k-50 | Anti–4-1BB | SS      | 5k         | 100 | 78.9    | 8.7 ± 2.4  | 5.6 ± 0.4     | 0.41         |
| 4     | Sw_redox_a4-1BB_10k-100 | Anti–4-1BB | SS      | 5k         | 100 | 73.9    | 8.5 ± 2.3  | 1.2 ± 0.3     | 0.30         |
| 5     | Sw_redox_a4-1BB_10k-20k | Anti–4-1BB | SS      | 20k        | 50  | 54.6    | 7.6 ± 2.2  | 2.9 ± 0.7     | 0.77         |
| 6     | Sw_redox_a4-1BB_20k-50 | Anti–4-1BB | SS      | 20k        | 100 | 70.6    | 10.4 ± 3.0 | 1.3 ± 0.2     | 0.29         |
| 7     | Sw_redox_a4-1BB_20k-100 | Anti–4-1BB | SS      | 20k        | 100 | 62.3    | 8.3 ± 2.3  | 3.8 ± 1.7     | 1.7          |
| 8     | Sw_redox_a4-1BB_50k-50 | Anti–4-1BB | SS      | 50         | 100 | 66.8    | 7.3 ± 2.1  | 1.3 ± 0.2     | 0.29         |
| 9     | Sw_redox_a4-1BB_50k-100 | Anti–4-1BB | SS      | 100        | 100 | 64.2    | 13.8 ± 3.1 | 54.2 ± 10.5   | 7.8          |
| 10    | Sw_redox_a4-1BB_100k-100 | Anti–4-1BB | SS      | 50         | 100 | 56.5    | 14.9 ± 4.1 | 38.7 ± 11.7   | 11.7         |
| 11    | IL15   | IL-15SA   | S       | 50         | 100 | 35.5    | 12.5 ± 3.1 | 27.4 ± 10.5   | 10.5         |
| 12    | Sw_redox_a4-1BB_50k-50 | Anti–4-1BB | SS      | 50         | 100 | 81.2    | 10.3 ± 3.2 | 1.4 ± 0.4     | 0.40         |
| 13    | Sw_redox_a4-1BB_100k-100 | Anti–4-1BB | SS      | 100        | 100 | 54.3    | 8.0 ± 2.4  | 1.2 ± 0.082   |             |

*The chemical structure of the linkers is shown in fig. S1. †Hydrodynamic diameter measured by dynamic light scattering. ‡Half-maximal effective concentration values (EC50) of Sw-IMs at off and on status determined by CD8^+ T cell binding assay.
the half-maximal effective concentration (EC₅₀) values 16.2- to
73.4-fold higher than the native anti–4-1BB antibody, indicating
low/none biological activities; recovered Sw redox a4-1BBs (on status)
had comparable EC₅₀ values to that of the native anti–4-1BB
antibody (Fig. 2I). The partial recovery of the binding activities of
Sw redox a4-1BBs was possibly due to the incomplete removal of the
masking polymers in the set reducing condition and the degrada-
tion of part of the antibodies (34). EC₅₀ values of Sw redox IL15s
showed the similar trend, suggesting the inactivity of masked
IL-15SA and the almost fully regained affinity at on status (with

Fig. 2. Stimuli-responsive switch on of Sw-IMs. (A) Rₐ values of Sw redox a4-1BBs at off and on status. (B) Sw redox a4-1BBs at off and on status were analyzed by SDS-PAGE.
(C) Traces of Sw redox a4-1BBs measured by UHPLC equipped with a SEC column. (D) Rₐ values of Sw redox IL15s at off and on status. (E) Sw redox IL15s at off and on status were
analyzed by SDS-PAGE. (F) UHPLC-SEC traces of Sw redox IL15s. The dashed lines indicate the molecular (B and E) or elution time (C and F) of native IMs. (G) Schematic
illustration of CD8⁺ T cell binding assay. Activated CD8⁺ T cells (1 × 10⁵) were incubated with Alexa Fluor-647 (Alx-647)–labeled Sw-IMs at off or on status at series diluted
concentrations for 1 hour. Mean fluorescence intensity (MFI) of CD8⁺ T cells was measured by flow cytometry to represent the binding capacity. (H) Binding capacity of
Sw redox a4-1BBs at off and on status with CD8⁺ T cells. The plot is a representative of three independent experiments. (I) Half-maximal effective concentrations (EC₅₀) of
Sw redox a4-1BBs at off and on status. (J) Binding capacity of Sw redox IL15s at off and on status with CD8⁺ T cells. The plot is a representative of three independent experi-
ments. (K) EC₅₀ of Sw redox IL15s at off and on status.
EC_{50} values 1.3- to 1.5-fold higher than those of the native) (Fig. 2, J and K).

In general, at off status, Sw-IMs with higher R_{C} (e.g., Sw_{redox}a4-1BB_{10k, 50} EC_{50} off = 1.2 nM versus Sw_{redox}a4-1BB_{10k, 100}, EC_{50} off = 2.9 nM) or higher M_{PEG} (e.g., Sw_{redox}IL15_{10k, 50} EC_{50} off = 22.0 nM versus Sw_{redox}IL15_{20k, 50}, EC_{50} off = 27.4 nM) showed higher EC_{50} values, implying that increased masking effects resulting in less activities of Sw-IMs (Table 1, entries 5, 6, 13, and 15). Exceptions were Sw_{redox}a4-1BB_{5k, 100} and Sw_{redox}IL15_{5k, 100} which had very high EC_{50} values at off status (73.4- and 7.0-fold higher than those of the native IMs, respectively), although they were conjugated with the shortest PEG polymer chain (M_{PEG} = 5 kDa) (Fig. 2, I and K). In addition, redox-responsive switchable anti–PD-1 antibodies (Sw_{redox}aPD1) and acid pH-responsive switchable anti–4-1BB antibodies (Sw_{ph(D)}a4-1BBs and Sw_{ph}a4-1BBs) can be controlled similarly at off and on status in response to the corresponding stimuli (Table 1, entries 9, 16, and 17, and fig. S9). We also examined the functional activities of Sw-IMs at off and on status in activating CD^{8+} T cell in vitro in addition to the binding assay (Fig. S10). Sw_{redox}a4-1BB and Sw_{redox}IL15 exhibited much higher EC_{50} values at off status but recovered the functional activities at on status. Together, the structural and functional assays provide evidence that Sw-IMs could regain the biological activities upon triggered removal of the masking polymers.

Selective switch on of Sw-IMs in the TME leading to antitumor immunity

Next, we examined whether the Sw-IMs were switched on in the TME by evaluating their immune stimulation and antitumor activities in vivo. C57BL/6 mice bearing subcutaneous MC38 murine colon adenocarcinoma, a model known to be responsive to costimulatory antibody therapy (35), received intraperitoneal injections of Sw_{redox}a4-1BBs of different formulations as compared to the native anti–4-1BB antibody (Fig. 3A). Sw_{redox}a4-1BBs showed equivalent tumor growth control compared to the native anti–4-1BB antibody except Sw_{redox}a4-1BB_{5k, 100} (Fig. 3, B and C, and fig. S11A). We further analyzed the immune cell infiltrates in tumor and their phenotypes to determine the activities of Sw-IMs for immune activation in the TME. Correlating with the induced tumor regression, all the Sw_{redox}a4-1BBs but Sw_{redox}a4-1BB_{5k, 100} enhanced the tumor infiltration of CD^{8+} T cells to a comparable degree to the native anti–4-1BB antibody (Fig. 3, D and E). Furthermore, Sw_{redox}a4-1BB_{10k, 50}, Sw_{redox}a4-1BB_{10k, 100}, and Sw_{redox}a4-1BB_{10k, 50} elicited similarly enhanced effector functions of intratumoral CD^{8+} lymphocytes to the native anti–4-1BB antibody evidenced by the frequency of interferon–γ (IFN–γ)–secreting CD^{8+} T cells (59.3, 78.1, and 73.8%, respectively, as compared to 75.3% of the native anti–4-1BB antibody) (Fig. 3F). Sw_{redox}a4-1BB_{5k, 100} showed the minimum immune activation and therapeutic efficacy likely due to the very dense PEG masking (R_{C} = 78.9%).

We next assessed the antitumor activity of Sw_{redox}IL15 in combination with ACT in C57BL/6 mice bearing subcutaneous B16F10 tumors, a poorly immunogenic murine melanoma model (Fig. 3G). We focused on Sw_{redox}IL15_{10k, 100} as Sw_{redox}a4-1BB of the same formulation (Sw_{redox}a4-1BB_{10k, 100}) exhibited the highest antitumor activity among the Sw_{redox}a4-1BBs tested. ACT of Pmel T cells alone through intravenous administration showed modest effect in tumor growth control (Fig. 3H and fig. S11B). Although the combination of Pmel T cells and the native IL-15SA enhanced the tumor suppression, severe toxicities were noticed with four of five treated mice sacrificed due to more than 10% body weight loss (Fig. 3, H and I, and fig. S11C). By contrast, the combination of Pmel T cells and Sw_{redox}IL15_{10k, 100} prominently enhanced the tumor regression compared to ACT alone without any overt toxicities, showing a 1.4-fold increase in median survival time relative to animals receiving Pmel T cells plus the native IL-15SA (Fig. 3I). Lymphodepletion before ACT is widely applied in the clinic to enhance the engraftment of adoptively transferred cells by eliminating the competition of endogenous lymphocytes (36). With lymphodepletion, Sw_{redox}IL15_{10k, 100} combined with ACT exhibited comparable tumor growth suppression as the combination of native IL-15SA and ACT, suggesting regained immune stimulation activities in the TME (fig. S12). Noticeably, Sw_{redox}IL15_{10k, 100} also improved the safety in this therapeutic regimen and led to prolonged survival of tumor-bearing mice (fig. S12, C and D).

Sw-IMs improve the safety of mono- and combinational immunotherapies

The prolonged survival of treated mice motivated us to investigate the safety profile of Sw-IMs as compared to that of the native IMs. Mice bearing tumors were treated following the dosing scheme as shown in Fig. 3A and sacrificed on day 16 for toxicity assessment. The treatment with native anti–4-1BB antibody induced evident splenomegaly, whereas the Sw_{redox}a4-1BB treatment mitigated this effect (Fig. 4A). Histopathological analyses of spleen tissues showed indistinct boundaries between the red and white pulps in the spleens of mice receiving native anti–4-1BB antibody. By contrast, architecture of spleen tissues remained intact in mice receiving Sw_{redox}a4-1BBs (Fig. 4B). Non-specific expansion of splenic effector immune cells, especially CD^{8+} T cells, leads to excessive production of IFN–γ that causes the splenomegaly accompanied by dissolution of typical histological architecture (37–39). We therefore analyzed the CD^{8+} T cells in spleen using flow cytometry. The treatment with native anti–4-1BB antibody notably expanded the effector memory CD^{8+} T cells (TEM; CD44^{high}CD62L^{low}) (Fig. 4, C and D), CD^{8+} T cells expressing the early activation marker CD69 (Fig. 4E), and CD^{8+} T cells secreting granzyme B (GrzmB) or IFN–γ (Fig. 4F and fig. S13A) compared to the phosphate-buffered saline (PBS) treatment. The agonistic antibodies administered in the form of Sw_{redox}a4-1BBs reduced the non-specific expansion of effector and cytotoxic T cells in spleen that was induced by the native anti–4-1BB antibody showing enhanced safety profile.

Immunotoxicity often leads to liver damage (40, 41). Upon systemic immune activation by the intraperitoneally administered native anti–4-1BB antibody, substantial mononuclear cells accumulated around the liver portal area, as shown in the images of histological analyses (Fig. 4B). By contrast, treatment with Sw_{redox}a4-1BBs resulted in minimum mononuclear cell accumulation in liver. The native anti–4-1BB antibody induced substantially increased frequency and counts of CD^{8+} T cells in liver compared to the PBS treatment, while the Sw_{redox}a4-1BB treatments elicited less liver-infiltrating CD^{8+} T cells as compared to the native antibody therapy showing alleviated toxicity (Fig. 4, G and H). Activated liver-infiltrating CD^{8+} T cells (CD69^{+} or CD44^{+}) were also expanded by native anti–4-1BB antibody but significantly less by Sw_{redox}a4-1BBs (Fig. 4I and fig. S13B). Further, treatment in the form of Sw_{redox}a4-1BBs, in particular, Sw_{redox}a4-1BB_{10k, 100} resulted in reduced cytotoxic and cytokine-secreting CD^{8+} T cells in liver, with 72 and 75% lower counts of liver-infiltrating GrzmB and IFN–γ–CD^{8+} T cells compared to the native anti–4-1BB antibody, respectively (Fig. 4J and fig. S13C). The
native anti–4-1BB antibody induced elevated serum levels of liver enzyme alanine transaminase (ALT) and systemic proinflammatory cytokine (IFN-γ), a side effect also reported in the clinic with typically higher severity in patients than mice (1). However, this liver toxicity was not observed in mice treated with Swredox4-1BBs, suggesting the alleviation of systemic immunotoxicity (Fig. 4, K and L).

We next evaluated the toxicity of SwredoxIL15s in a combination therapy with ACT. Mice bearing subcutaneous B16F10 tumors were treated with intravenous administration of Pmel T cells alone, or Pmel T cells adjuvanted by the native IL-15SA or SwredoxIL1510k-100. Mice were sacrificed on day 14 for toxicity assessment (Fig. 5A). Treatment of ACT plus the native IL-15SA induced pronounced splenomegaly, which were greatly alleviated in mice receiving ACT plus SwredoxIL1510k-100 (Fig. 5, B and C). The observed splenomegaly was likely due to the massive expansion of both CD8+ T cells and natural killer (NK) cells in spleen by the native IL-15SA (Fig. 5, D, K, and L, and fig. S14A) (3). Among the splenic CD8+ T cells, activated CD69+CD8+ T cells, effector memory CD8+ T cells (TEM), and CD8+ T cells secreting GrzmB and IFN-γ were all prominently expended by the native IL-15SA (Fig. 5, E and F, and fig. S14, B and C). On the contrary, ACT adjuvanted by SwredoxIL1510k-100 elicited no significant change of counts of splenic CD8+ T cells, NK cells, or any of these CD8+ T cell subsets as compared to ACT alone. In addition, treatment of ACT supported by the native IL-15SA caused liver injury evidenced by the elevated serum level of ALT (Fig. 5G). This combination therapy nonspecifically expanded the liver-infiltrating CD8+ T cells, activating CD69+CD8+ T cells, effector memory CD8+ T cells (TEM), and CD8+ T cells secreting GrzmB and IFN-γ, and NK cells (Fig. 5, H to K and M, and fig. S14, D to F). When ACT was combined with SwredoxIL1510k-100, no overt toxicity was observed in liver; the counts of total liver-infiltrating lymphocytes...
Fig. 4. Sw-IM reduced the toxicity of anti–4-1BB agonistic antibody therapy. C57BL/6 mice bearing MC38 tumor were treated as shown in Fig. 3A. Mice were euthanized on day 16, and the tissues were processed for histological and flow cytometry analyses (n = 5 mice). (A) Average spleen weight. (B) Histopathological analyses of spleen and liver tissues. Red boxes and yellow arrows show the tissue damage. Scale bars, 100 μm. (C) Representative flow cytometry plots of CD8+ T cells in spleen. The frequencies of effector memory CD8+ T cells (TEM; CD44highCD62Llow) among CD8+ T cells are shown. (D) Counts of TEM in spleen. (E) Counts of CD69+CD8+ T cells in spleen. (F) Counts of GrzmB-secreting CD8+ T cells (GrzmB+CD8+) in spleen. (G) Representative flow cytometry plots of liver-infiltrating CD8+ T cells. The frequencies of CD8+ T cells among liver-infiltrating lymphocytes (CD45.2+) are shown. (H) Counts of liver-infiltrating CD8+ T cells. (I) Counts of liver-infiltrating GrzmB-secreting CD8+ T cells. (J) Serum activity of alanine aminotransferase (ALT). (K) Serum concentration of IFN-γ. All data represent the mean ± SEM and are analyzed by one-way ANOVA.
Investigating the optimum formulation of Sw-IMs

The formulation parameters of Sw-IMs play a crucial role in their antitumor activity and safety profile. To quickly determine the optimum formulation for a given IM, we used the in vitro T cell activation assay for screening various formulations. The MFI of CD69 was used as a representative indicator for antitumor activity (on status) and toxicity (off status). We prepared a small library of 15 different formulations of Sw_redoxIL15s (Fig. S16A). As expected, both R_C and M_PEG influenced the CD69 MFI for Sw_redoxIL15s at on or off status (Fig. S16, B and C).

To better compare the overall performance of Sw-IMs of various formulations, we defined a comprehensive performance index, E_S, which was the ratio of CD69 MFI for Sw_redoxIL15s at on status to that at off status (Fig. 6A). The higher E_S value suggests enhanced overall performance of Sw-IM with potentially increased antitumor activity as well as reduced toxicity in healthy tissues. The native (CD8+ T cells and NK cells) as well as the activated and cytotoxic CD8+ T cell subsets were maintained at the same level as that in the ACT alone group. In the therapeutic setting with prior lymphodepletion, the treatment with Sw_redoxIL15s (table S1, entry 35) at equivalent or even fourfold higher dose of the native IL-15SA induced greatly reduced spleen and liver toxicities (Fig. S15). Together, Sw-IMs markedly improved the safety of mono- and combinational immunotherapies.

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To better compare the overall performance of Sw-IMs of various formulations, we defined a comprehensive performance index, E_S, which was the ratio of CD69 MFI for Sw_redoxIL15s at on status to that at off status (Fig. 6A). The higher E_S value suggests enhanced overall performance of Sw-IM with potentially increased antitumor activity as well as reduced toxicity in healthy tissues. The native
IL-15SA has an $E_S$ value of 1 as there is no difference of its activities in various tissues. In addition, we defined a comprehensive formulation index, $R_C \times M_{PEG}$, representing the overall masking effect of conjugated polymers. The smooth fit to CD69 MFI values at off status as a function of $R_C \times M_{PEG}$ via penalized cubic regression splines showed a concave curve with higher slope at low $R_C \times M_{PEG}$, while the plot of CD69 MFI at on status showed a near-linear curve (Fig. 6, B and C). As a result, the plot of $E_S$ as a function of $R_C \times M_{PEG}$ shows a convex curve with a peak in the range of $R_C \times M_{PEG} = 6.7$ to 10.1 kDa, suggesting an optimal range of the formulation index (Fig. 6D). These observations correlated well with the in vivo results of Sw-IMs. Among the Swredoxa4-1BBs tested in vivo (Fig. 6E), Swredoxa4-1BB10k-100 with an $R_C \times M_{PEG}$ value of 7.4 kDa showed the highest antitumor efficacy and the least toxicity; Swredoxa4-1BB10k-50 and Swredoxa4-1BB20k-50 with $R_C \times M_{PEG}$ values of 6.2 and 10.9 kDa, respectively, also exhibited high antitumor efficacy but slightly increased toxicity compared to Swredoxa4-1BB10k-100.

**DISCUSSION**

IM therapeutics that stimulate anticancer immunity may result in systemic toxicities because of nonspecific immune activation in the circulation or healthy tissues, greatly limiting their therapeutic potential in the clinic. Here, we demonstrated a responsive chemical masking strategy, termed Sw-IM, to achieve tumor-specific activation of the IMs. Sw-IMs applied to antibody- or cytokine-based immunotherapeutics markedly reduced the immunotoxicity while retaining their therapeutic potency, leading to prolonged survival of tumor-bearing mice.

The Sw-IM approach is highly versatile and modular. We have applied the syntheses to the preparation of 58 different Sw-IMs in total (Table 1 and table S1). Each Sw-IM consists of three modular parts: a protein-based therapeutic, a responsive linker, and a bio-compatible polymer for reversible masking. We focused on a costimulatory agonist antibody, anti–4-1BB antibody, and a cytokine superagonist, IL-15SA, to prove the concept in mouse tumor models, because these two IMs have been shown to not only have potent antitumor activities but also exhibit severe immune-related adverse effects in preclinical and clinical studies (1, 3, 42, 43). This approach was also successfully extended to checkpoint blockade antibodies including anti–PD-1 and anti–CTLA-4 antibodies. Any biomacromolecule agents bearing multiple functional groups (e.g., amino groups) could potentially be formulated into Sw-IM. Sw-IMs can be
rendered with different responsiveness using diverse trigger-degradable linkers. The TME is characterized as being reducing, acidic, hypoxic, and overexpressing various proteases among other signatures (44). Implementation of responsive chemistry that has been developed for drug delivery applications in addition to the redox and acidic pH–responsive linkers could further expand the library of Sw-IMs. Other natural or synthetic polymers besides PEG that are hydrophilic, biocompatible, and with low immunogenicity could also be used as masking polymers for Sw-IMs.

The high modularity of the Sw-IM enabled us to fine-tune the formulations for the optimized antitumor efficacy and safety profile. An optimal Sw-IM should exhibit the minimum activities in blood circulation and healthy tissues (off status) but the maximum activities in the TME (on status). Given a biomacromolecule therapeutic and a responsive linker, the performance of Sw-IM is determined by the overall masking effect of the conjugated polymers. We thus defined a comprehensive formulation index, $R_C \times M_{PEG}$, which is proportional to the average total mass of polymer mask. The activity of Sw-IM at off status drops rapidly as $R_C \times M_{PEG}$ increases (especially, when $R_C \times M_{PEG} < 6 \text{ kDa}$) because the steric hindrance on binding between Sw-IM and its corresponding cellular receptor is augmented with increasing masking effect (Fig. 6B). One can speculate that the curve would eventually reach a constant minimum as the slope continuously declines, implying that the maximum hindrance would be achieved when the masking effect reaches saturation (Fig. 6B). Increasing masking effect also decreases the accessibility of reducing agents to the responsive linker, resulting in almost linearly declined activities of Sw-IM at on status (Fig. 6C). The peak in the plot of $E_S$ as a function of $R_C \times M_{PEG}$ implies that the optimal range of masking effect indeed exists. When the $R_C \times M_{PEG}$ value is too low (<6.7 kDa), Sw-IM exhibits relatively high toxicity profile because of insufficient masking (Fig. 6D). On the other hand, when the $R_C \times M_{PEG}$ value is too high (>10.1 kDa), Sw-IM may hardly regain any biological activities because of the inaccessibility of the reducing agent to the linker and thus may have low efficacy and low toxicity. Therefore, we need to optimize the formulation parameters for each Sw-IM with a suitable degree of masking for the balanced potency and safety.

This chemical strategy complements the recombinant approaches to target the IM activities specifically to the TME. Strategies using customized masking peptides (19), proteins (22, 23), or a generalizable coiled-coil domain (20) for the preparation of recombinant antibody produg, termed "probodys," are being actively pursued. Probodys in immunotherapy targeting ligand of programmed death 1 (PD-L1) is currently tested in the clinic (ClinicalTrials.gov, identifier: NCT03013491) (45, 46). However, this probody design relies exclusively on the overexpressed proteases in the TME for triggered removal of masking peptides. The chemical modification with masking polymers presented here may allow much more diverse choices of responsiveness, therapeutics, and masking polymers. Besides the redox and acidic pH–responsive chemistry described above, other environment responsive chemistries, such as those responsive to matrix metalloproteinases (47), reactive oxygen species (48), or even external signals (49, 50), can be potentially adapted to the syntheses of Sw-IMs. Recent reports of a hypoxia-activated conditional aptamer-PEG conjugate (51) and an antibody against hemagglutinin masked by a bivalent peptide-DNA ligand (52) provide the hint that the chemical approach can be further extended to nonprotein therapeutics or imaging probes.

### MATERIALS AND METHODS

#### Materials

The monoclonal antibodies including anti–4-1BB (anti-CD137, clone 3H3), anti–PD-1 (clone 29F.1A12), anti–CTLA-4 (anti-CD152, clone 9H10), rat IgG2a isotype control (anti-TNP, clone 2A3), and anti-CD3 (clone 17A2) were purchased from Bio X Cell (West Lebanon, NH, USA). Methoxy PEG amine (mPEG-NH$_2$) with various MWs, methoxy PEG thiol (mPEG-SH), and methoxy PEG–DBCO (mPEG–DBCO) were purchased from JenKem Technology (Plano, TX, USA). Alexa Fluor 647 succinimidyl ester was purchased from Invitrogen (now Thermo Fisher Scientific, Waltham, MA, USA). Benzoyl peroxide [BPO; containing 25% (w/w) water as stabilizer, CAS 94-36-0] was purchased from Alfa Aesar (Ward Hill, MA, USA) and recrystallized twice with chloroform and methanol before use. 2-Hydroxyethyl disulfide (technical grade, CAS 1892-29-1), phosgene [15% (w/w) in toluene, CAS 75-44-5], trimethylamine (TEA; 99.5%, CAS 121-44-8), NHS (98%, CAS 6066-82-6), dimethylmaleic anhydride (DMMA; 98%, CAS 766-39-2), N-bromosuccinimide (NBS; 99%, CAS 128-08-5), TNBSA [5% (w/v) in H$_2$O, CAS 2508-19-2], SDS (20% in H$_2$O, CAS 151-21-3), reduced l-GSH (98%, CAS 70-18-8), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise noted, all chemicals and reagents were used as received.

#### Mice

Six- to 8-week-old female Thy1.2$^+$ C57BL/6 mice were purchased from Charles River Laboratories (Lyon, France). T cell receptor (TCR)–transgenic Thy1.1$^+$ pmel-1 (Pmel) mice [B6.Cg-Thy1.1$^+$/Cy Tg[TcraTcrb]8Rest/J] were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in École Polytechnique Fédérale de Lausanne (EPFL) animal core facility [Center of Phenogenomics (CPG)].

#### Cell lines

The MC38 murine colon adenocarcinoma cell line was provided by D. J. Irvine’s laboratory (MIT, MA, USA). B16F10 murine melanoma cells were originally acquired from the American Type Culture Collection (Manassas, VA, USA). The tumor cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco/Thermo Fisher Scientific) supplemented with fetal bovine serum [FBS; 10% (v/v), Gibco/Thermo Fisher Scientific] and penicillin/streptomycin [1% (v/v), Gibco/Thermo Fisher Scientific].

#### Production of mouse IL-15SA

The engineered IL-15SA construct (gWIZ-mIL-15SA) was a gift from D. J. Irvine (MIT). IL-15SA contains a mouse IL-15 fused at the C terminus of Sushi domain of a mouse IL-15R$\alpha$ (which is the C terminus of Sushi domain of a mouse IL-15R$\alpha$) from D. J. Irvine (MIT). IL-15SA contains a mouse IL-15 fused at the C terminus of Sushi domain of a mouse IL-15R$\alpha$ from D. J. Irvine (MIT).

Production of mouse IL-15SA

The engineered IL-15SA construct (gWIZ-mIL-15SA) was a gift from D. J. Irvine (MIT). IL-15SA contains a mouse IL-15 fused at the C terminus of Sushi domain of a mouse IL-15R$\alpha$, which is next fused at the C terminus with a mouse IgG2c Fc. IL-15SA was expressed by human embryonic kidney (HEK) 293-E cells in Freestyle medium (Gibco) at the EPFL Protein Expression Core Facility (PECF). The supernatant of culture medium containing IL-15SA was harvested by centrifugation after a 7-day culture and was filtered through a filter membrane (0.22 $\mu$m) to obtain a clear solution. IL-15SA was first captured with a HitTrap Protein A affinity chromatography column on an ÄKTA pure 25 system (GE Healthcare, Chicago, IL, USA) and eluted with an elution buffer (0.05 M sodium citrate, 0.3 M sodium chloride, pH 3.0). The eluted protein was next collected immediately in a neutralization buffer (1 M tris-HCl, pH 10.0) followed by concentration with membrane ultrafiltration.

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Synthesis of redox-responsive linker NHS-SS-NHS

NHS-SS-NHS was prepared following a previous report (53). Briefly, in a 100-ml round-bottom flask, 2-hydroxethyl disulfide (1.329 g, 8.616 mmol) in anhydrous tetrahydrofuran (20 ml) solution was added dropwise to a phosgene solution [12.5 ml, 15% (w/w), 18.95 mmol] in toluene. The reaction mixture was protected with N2 and stirred under room temperature for 2 hours. The obtained solution was concentrated under vacuum, and the residue was dissolved with anhydrous dichloromethane (DCM; 10 ml) followed by addition of NHS (2.182 g, 18.95 mmol) and anhydrous TEA (1.918 g, 18.95 mmol) in DCM solution (45 ml). The reaction mixture was protected with N2 and stirred at room temperature overnight and then concentrated under vacuum. The crude product was purified with column chromatography (DCM:methanol, 10:1) and recrystallized with icy petroleum ether. The acicular crystal (2.14 g, yield: 57%) was dried under vacuum and characterized by 1H nuclear magnetic resonance (NMR) (Bruker AVANCE NEO 400 MHz spectrometer, Billerica, MA, USA). 1H NMR (400 MHz, CDCl3): δ (ppm) = 4.61 (t, J = 4 Hz, 4H, CCH2), 2.19 (s, 3H, CH3).

Preparation of redox-responsive Sw-IMs

In the preparation of Swredox-a4-1BB, for example, mPEG-NH2 in anhydrous dimethyl sulfoxide (DMSO) solution (133.3 nmol, 20 mg/ml, 1.0 equiv) was mixed with an NHS-SS-NHS solution (133.3 nmol, 40 mg/ml in anhydrous DMSO, 1.0 equiv). The reaction mixture was shaken in ThermoMixer (Eppendorf, Hamburg, Germany) at room temperature for 4 hours (800 rpm). The intermediate product was next added into an IM solution (1.33 nmol, 1 mg/ml in 0.1 M NaHCO3, pH 8.5) for another hour of shaking. For other formulations, different feeding mole ratios (RF) of PEG to IM were chosen. The crude product was purified by ultrafiltration (7500 rpm, 5 min × 5) in an Amicon centrifugal filter (MW cutoff, 50 kDa; Merck Millipore, Burlington, MA, USA). The concentration of the purified Sw-IM was determined with a NanoDrop microvolume ultraviolet-visible (UV-vis) spectrophotometer (Thermo Fisher Scientific). For the preparation of fluorescently labeled Sw-IMs, Alexa Fluor 647 succinimidyl ester (2.67 nmol, 10 mg/ml in anhydrous DMSO, 2.0 equiv) was added to a solution of IM (1.33 nmol, 1 mg/ml in 0.1 M NaHCO3 buffer, pH 8.5, 1.0 equiv) followed by shaking in ThermoMixer for 20 min before the addition of the PEG intermediate product.

Switch on of redox-responsive Sw-IMs

A solution of Sw-IM in PBS (pH 7.4, 1 mg/ml, 50 μl) was mixed with a solution of m-PEG-SH in PBS (10 mM, pH 7.4, 50 μl). The mixed solution was incubated at room temperature for 1 hour and purified by ultrafiltration (MW cutoff, 50 kDa; 7500 rpm; 5 min × 2) in an Amicon centrifugal filter to get rid of any small molecules and the released mPEG-NH2. The concentration of recovered IMs was determined by a NanoDrop UV-vis spectrophotometer. Released IMs were analyzed by SDS-PAGE, TNBSA, and flow cytometry assays.

Synthesis of (bromomethyl)methylmaleic anhydride

BrMMMA [(bromomethyl)methylmaleic anhydride] was prepared following a previous report (54). Briefly, recrystallized BPO (200 mg, 0.83 mmol, 0.017 equiv) was added into the mixture of DMMA (5.04 g, 50 mmol, 1.0 equiv) and NBS (14.24 g, 100 mmol, 2.0 equiv) in carbon tetrachloride in a 500-ml round-bottom flask. The suspension was refluxed for 5 hours. After cooling down to room temperature, a second portion of BPO (200 mg, 0.83 mmol, 0.017 equiv) was added to the reaction mixture followed by another 5-hour reflux. The reaction was then cooled down to room temperature and kept stirring overnight. The suspension was filtered to get rid of any insoluble impurities and washed with water (100 ml × 2) and brine (100 ml × 2). The organic layer was then dried with anhydrous Na2SO4 and concentrated under vacuum. The obtained crude product as sticky yellow oil was purified by column chromatography (EtOAc:petroleum ether, 4:1) and further distilled with a Kugelrohr apparatus (90°C, 0.005 mbar). The second fraction as light yellow oil was collected as pure product (4.0 g, yield 60%). 1H NMR (400 MHz, CDCl3): δ (ppm) = 4.20 (2H, CH2Br), 2.19 (3H, CH3).

Synthesis of AzMMMA

AzMMMA was prepared following a previous report (55). Briefly, sodium azide (130 mg, 2.0 mmol, 1.0 equiv) was added rapidly to an acetone solution (20 ml) of BrMMMA (410 mg, 2.0 mmol, 1.0 equiv) in a 50-ml round-bottom flask. The mixture was vigorously stirred at room temperature overnight to generate a purple suspension. The reaction mixture was then filtered to get rid of solid residues and then concentrated under vacuum. The obtained sticky oil was diluted with ethyl acetate (50 ml) and washed with water (50 ml × 2) and brine (50 ml × 2). The organic layer was then collected and dried with anhydrous Na2SO4. After being concentrated under vacuum, the crude product was purified by column chromatography (ethyl acetate:petroleum ether, 2:1) to provide the pure product as colorless oil (270 mg, yield 80%). 1H NMR (400 MHz, CDCl3): δ (ppm) = 4.29 (d, J < 1.0 Hz, 2H, CH2N3), 2.23 (t, J = 1.0 Hz, 3H, CH3).

Preparation of SwpH(D)-a4-1BB

In the preparation of SwpH(D)-a4-1BB, for example, to a basic buffer solution (0.1 M NaHCO3, pH 8.5) of anti–4-1BB (200 μg, 1.33 nmol, 2 mg/ml), AzMMMA (20 mg/ml in anhydrous DMSO, 4.45 μl, 0.53 μmol, 400 equiv) was added. The reaction mixture was shaken in ThermoMixer at room temperature for 2 hours. The mixture was washed via ultrafiltration (MW cutoff, 50 kDa; 7500 rpm; 5 min × 5) in an Amicon centrifugal filter to remove the unreacted AzMMMA. The purified azido-functionalized antibodies were then diluted with basic buffer to a concentration of 1 mg/ml and added to a solution of mPEG-DBCO (MW = 5 kDa, 25 mg/ml in basic buffer, 400 equiv). The reaction mixture was shaken for another 2 hours (800 rpm, room temperature) and purified by ultrafiltration similarly.

Synthesis of MMMA functionalized PEG

mPEG-SH (MW = 5 kDa, 300 mg, 0.06 mmol) was dissolved in methanol (2 ml) in a 5-ml round-bottom flask. The solution was purged with N2 for 10 min to eliminate O2. KOH (6.7 mg, 0.12 mmol) in methanol solution (1 ml) was then added dropwise over 10 min to the mPEG-SH solution with vigorous stirring. The mixed solution was cooled down to 0°C in an ice-salt bath and added with...
BrMMMA (25 mg) in methanol solution (2 ml) dropwise over 10 min. The reaction mixture was then stirred at 0°C for 30 min followed by concentration under vacuum. The residues were redissolved in dilute hydrogen chloride (HCl) solution (1 M, 5 ml). After stirring at room temperature for another 2 hours, the aqueous solution was saturated with NaCl and extracted with DCM. The organic layer was combined and dried with anhydrous Na2SO4, and concentrated under vacuum to provide the product as white powder (230 mg, yield 77%). 1H NMR (400 MHz, CDCl3): δ (ppm) = 3.67 (s, CH2-CH2), 2.19 (s, CH3), 2.78 (s, CH2).

Preparation of SwpH(S)4-1BBs
In the preparation of SwpH(S) 4-1BB 5k-400, for example, powder of mPEG-MMMA (MW = 5 kDa, 2.67 mg, 0.53 μmol, 400 equiv) was added to the native anti–4-1BB (200 μg, 1.33 nmol, 1 mg/ml) in a basic buffer (PBS + Na2CO3, pH 9.5). The reaction mixture was shaken in ThermoMixer at room temperature for 4 hours and then purified by ultrafiltration (MW cutoff, 50 kDa; 7500 rpm; 5 min × 5) with an Amicon centrifugal filter.

Switch on of acidic pH–responsive Sw-IMs
Solution of acidic pH–responsive Sw-IMs was transferred to an acidic phosphate buffer (pH 6.5) by ultrafiltration (MW cutoff, 50 kDa) with an Amicon centrifugal filter and diluted to the concentration of 1 mg/ml. The solution was incubated at 37°C overnight. The recovered antibodies were then transferred back to neutral PBS (pH 7.4) via ultrafiltration (MW cutoff, 50 kDa) for SDS-PAGE, TNBSA, and flow cytometry assays.

SDS-PAGE gel electrophoresis
The PBS solution of native IMs and Sw-IMs at off and on status (0.5 mg/ml, 15 μl, pH 7.4) was mixed with Bolt LDS sample buffer (4×, 5 μl; Life Technologies, Carlsbad, CA, USA) and added into the well of a NuPAGE 4 to 12% bis-tris gel (1.0 mm × 12 wells; Novex/Thermo Fisher Scientific). The samples were separated in Mops SDS running buffer (Novex/Thermo Fisher Scientific) at 110 V for 1 hour. The collected gel was then stained with Coomassie Brilliant Blue staining buffer [0.25% (w/v) in 45% MeOH, 45% H2O, and 10% acetic acid] for 1 hour followed by washing with destaining solution (10% acetic acid, 30% EtOH, and 60% H2O). The gels were recorded with an E-Gel Imager system (Thermo Fisher Scientific).

UHPLC-SEC characterization
Native IMs and Sw-IMs were diluted by filtered PBS (pH 7.4) to 1 mg/ml. A mobile phase of PBS (containing 200 mM NaCl, pH 7.4) was applied on an UltiMate 3000 UHPLC system equipped with a BioBasic SEC 300 LC column (Thermo Fisher Scientific) with a flow rate of 0.5 ml/min. The traces were monitored with a diode array detector (UV, Dionex UltiMate 3000, Thermo Fisher Scientific) and a fluorescence detector (FLD, Dionex UltiMate 3000, Thermo Fisher Scientific).

DLS characterization
Native IMs and Sw-IMs were diluted with filtered PBS (pH 7.4) to 0.1 mg/ml and transferred into PS semi-micro cuvettes (Brand, Wertheim am Main, Germany). The diameter of the product was measured with a NanoZS (Marven, Worcester, UK) DLS instrument at room temperature. The measurement was repeated three times independently for each sample.

Conjugation ratio determined by TNBSA assay
Solutions of native IMs and Sw-IM at off or on status (100 μl, 200 μg/ml in NaHCO3 buffer, pH 8.5) or a small-molecule standard, 5-amino-1-pentanol (100 μl, gradient diluted from 20 μg/ml in NaHCO3 buffer, pH 8.5), were mixed with a solution of TNBSA [50 μl, 0.1% (w/v) in NaHCO3 buffer, pH 8.5] in a flat-bottom 96-well plate. The reaction mixture was incubated at 37°C for 2 hours. A solution of SDS [50 μl, 10% (w/v) in H2O] was added to each well followed by addition of HCl solution (25 μl, 1 N in H2O) to stop the reaction. The UV absorbance at the wavelength of 335 nm was measured with a Varioskan LUX microplate reader (Thermo Fisher Scientific). The concentration of primary amines was calculated using the calibration curve of 5-amino-1-pentanol standard.

Preparation of activated Pmel CD8+ T cells
Spleens from Pmel mice were mechanically disrupted into individual cells by smashing on a 70-μm strainer (Fisher Scientific, Pittsburgh, PA, USA) and then lysed with ACK lysing buffer (2 ml per spleen, Gibco/Thermo Fisher Scientific) for 5 min to get rid of the red blood cells. The cells were then washed with PBS and resuspended to a cell density of around 1.0 × 106/ml with complete RPMI 1640 culture medium (Gibco/Thermo Fisher Scientific) containing FBS (10%, v/v), Hepes (1%, v/v), penicillin/streptomycin (1%, v/v), and 10% (v/v) in H2O]. The collected gel was then stained with Coomassie Brilliant Blue staining buffer [0.25% (w/v) in 45% MeOH, 45% H2O, and 10% acetic acid] for 1 hour followed by washing with destaining solution (10% acetic acid, 30% EtOH, and 60% H2O). The gels were recorded with an E-Gel Imager system (Thermo Fisher Scientific).

In vitro T cell binding assay
Activated Pmel CD8+ T cells (1 × 106) in each well of a U-bottom 96-well plate were blocked with anti-CD16/32 antibodies and stained with anti-CD8β. After washing twice with flow cytometry buffer [PBS containing bovine serum albumin, 0.2% (w/v)], the cells were resuspended with series diluted fluorescently labeled IM solutions (133.3 to 1.7 pM for anti–4-1BB and anti–PD-1, and 222.2 to 2.8 pM for IL-15SA, 100 μl for each well) or fluorescently labeled Sw-IM solutions (equivalent mole concentration to native IM) in flow cytometry buffer and incubated at 4°C for 1 hour. After washing twice with flow cytometry buffer (200 μl), the cells were stained with 4’,6-diamidino-2-phenylindole (DAPl; Sigma-Aldrich) for flow cytometry analysis.

In vitro T cell activation assay for Sw redox IL15
Activated Pmel CD8+ T cells were washed twice with PBS (200 μl) to remove the trace cytokines in the culture medium. The cells were resuspended with the solutions of native IL-15SA (2.2 nM to 0.18 pM, 100 μl for each well) or SwredoxIL15s (equivalent mole concentration to native IL-15SA, at either off or on status) in complete RPMI 1640 culture medium in a U-bottom 96-well plate (1 × 105 cells for each well). The cells were cultured at 37°C for 48 hours and then stained for flow cytometry analysis. For the investigation on optimal formulations, the applied concentration is 0.55 nM for native IL-15SA and equivalent mole concentration for SwredoxIL15s.
In vitro T cell activation assay for Swredox a4-1BB

Spleens from Pmel mice were mechanically disrupted and lysed with ACK lysing buffer as described above. After washing with PBS, naïve CD8+ T cells were isolated using the CD8+ T Cell Isolation Kit (Miltenyi Biotec, North Rhine-Westphalia, Germany) following the manufacturer’s protocol. Briefly, cells were resuspended in PBS (200 μl) and added with CD8 (TIL) MicroBeads (20 μl). After incubating at 2° to 8°C for 15 min, the cell suspension was passed over a MACS Column using the MACS Separator for positive selection. The fraction of purified CD8+ T cells was resuspended in complete RPMI 1640 culture medium containing FBS (10% v/v), Heps (1% v/v), penicillin/streptomycin (1% v/v), and β-mercaptoethanol (0.1% v/v) at cell density of 2.0 × 10^6/ml.

U-bottom 96-well plates were first coated with anti-CD3 antibody by incubating with the PBS solution of anti-CD3 (0.1 μg/ml, 100 μl) overnight at 4°C. The supernatant was then discarded, and each well was loaded with naïve CD8+ T cells (2 × 10^5 per well) suspended in complete RPMI culture medium. Next, the solution of native anti–4-1BB (133.3 to 0.013 nM, 10-fold gradient dilution) and Swredox a4-1BBs (equivalent mole concentration with native anti–4-1BB) in complete RPMI culture medium (100 μl) was added on top of each well. The cells were incubated at 37°C for 48 hours and stained for flow cytometry analysis.

Flow cytometry analyses

For intracellular cytokine staining, the cells were first stimulated with the Cell Stimulation Cocktail (protein transport inhibitors included, Invitrogen/Thermo Fisher Scientific) at 37°C for 6 hours. The surface marker staining and LIVE/DEAD staining with DAPI or Zombie Aqua Fixable Dye (BioLegend, San Diego, CA, USA). After washing with flow cytometry buffer (200 μl), the cells were resuspended with the same buffer (200 μl) for flow cytometry analysis.

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All flow cytometry data were collected with an Attune NxT flow cytometer (Invitrogen/Thermo Fisher Scientific) and analyzed by FlowJo 10.6.1 (Tree Star, Ashland, OR, USA). Gate margins were determined by isotype controls and fluorescence-minus-one (FMO) controls.

Antibodies for flow cytometry

The antibodies for flow cytometry analysis including anti-CD16/32 (clone 93), anti-CD3e (clone 17A2), anti-CD8β (clone 53-6.7), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-CD19 (clone 6D5), anti-CD45.2 (clone 104), anti-CD4 (clone RM4-5), anti–Thy1.1 (clone OX-7), anti-NK1.1 (clone PK136), anti–GranzB (clone GB11), anti–IFN-γ (clone XMG1.2), anti–TNF-α (clone MP6-XT22), and anti–IL-2 (clone JES6-5H4) were purchased from BioLegend.

Therapeutic and toxicity studies

In evaluating Swredox a4-1BBs as monotherapies, C57BL/6 mice were inoculated subcutaneously with B16F10 murine melanoma cells (5 × 10^5) and received adoptive transfer of Thy1.1+ CD8+ T cells (1 × 10^7) on day 7 followed by intravenous injection of IL-15SA (5 μg for each injection), Swredox IL15 10k-100 (equivalent dose of IL-15SA), or PBS every other day starting from day 7 until day 21. The tumor volume and body weight were measured every other day. In a toxicity study, mice were treated similarly and euthanized on day 14 and the tissues (tumor, liver, and spleen) were processed for flow cytometry analyses. To evaluate the combination therapy with lymphodepletion, the tumor-bearing mice were irradiated under 4 Gy 1 day before adoptive transfer of Thy1.1+ CD8+ T cells on day 7 and intravenous injection of native IL-15SA (10 μg for each injection), Swredox IL15 10k-100 (equivalent dose of IL-15SA), or PBS every other day from days 7 to 21. In toxicity study, the tumor-bearing mice were irradiated under 4 Gy 1 day before adoptive transfer of Thy1.1+ CD8+ T cells on day 7 and intravenous injection of native IL-15SA, Swredox IL15 10k-75 (equivalent or four times dose of IL-15SA), or PBS on days 7, 9, and 11. The mice were euthanized on day 12, and the tissues were processed for flow cytometry analyses. In all studies, mice were euthanized when the body weight loss was >10% of the predosing weight or the tumor area reached 1000 mm^3 (as a predetermined end point).

Tissue processing for flow cytometry analyses

After euthanasia, the spleen was collected and disrupted into single-cell suspension with a 70-μm cell strainer. Red blood cells were lysed with ACK lysis buffer at room temperature for 5 min. For liver- and tumor-infiltrating lymphocytes, one lobe of liver and the tumor was first digested with tumor digestion buffer [RPMI 1640 medium with collagenase type IV (1 mg/ml; Gibco/Thermo Fisher Scientific), dispase II (100 μg/ml, Sigma-Aldrich), hyaluronidase (100 μg/ml; Sigma-Aldrich), and deoxyribonuclease I (100 μg/ml; Sigma–Aldrich)] at 37°C for 1 hour. The remaining residue of liver and tumor tissue was next ground through a 70-μm cell strainer. After ACK lysis, live cells were enriched by density gradient centrifugation with a Percoll solution (40 and 80%, v/v) and then washed (10 ml) and resuspended in flow cytometry buffer (200 μl) for flow cytometry analyses.

Histological study

Half of the spleen, one lobe of the liver, or part of the tumor was fixed in 4% paraformaldehyde overnight followed by embedding in paraffin blocks. Paraffin slides were sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (H&E) for pathological analysis at the EPFL Histology Core Facility (HCF). H&E images were captured with the DM5000 Upright Microscope (Leica, Wetzlar, Germany) at the EPFL Bioimaging & Optics Platform (BIOP).

Measurement of liver enzymes and cytokine concentration in serum

The whole blood (200 to 300 μl) was collected from inferior vena cava of the mice immediately after the euthanasia. The serum
samples were then prepared by centrifuging the whole blood in a Microutkit 500 Z tube (Sarlstedt, Nümbrecht, Germany). The activity of ALT in serum was measured using Stanbio Chemistry Reagents (Sanbio, Boerne, TX, USA) per the manufacturer’s instructions. The concentration of IFN-γ was measured with an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences).

**Scatterplot smoothing**

In analyzing the results of comprehensive performance index \( E_S \) of various formulations (Fig. 6, B to D), we used the penalized cubic regression splines as implemented in the mgcv R package (version 1.8-33) to fit smooth curves through scatterplots (56). To obtain the plot of in vitro comprehensive performance index \( E_S \)

\[
E_S = \frac{\text{CD69 MFI on CD69 MFI off}}{\text{off}}
\]

as a function of \( R_c \) or comprehensive formulation index \( R_c \times M_{PEG} \), we separately fitted smooth curves for CD69 MFI at off and on status. We took the ratio of predicted fits to compute \( E_S \) and used the chain rule to propagate the prediction intervals obtained for the numerator and the denominator to the \( E_S \) ratio

\[
\text{if } z = c \frac{x}{y} \text{ then } \frac{\partial z}{x} = \left[ \left( \frac{\partial x}{x} \right)^2 + \left( \frac{\partial y}{y} \right)^2 \right]^{0.5}
\]

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Unless otherwise noted, the data are presented as mean ± SEM. Comparisons of survival curves were performed by the log-rank test. Comparisons of multiple groups at first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. J. Clin. Oncol. 33, 74—82 (2015).

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