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Selective activation of interleukin-2/interleukin-15 receptor signaling in tumor microenvironment using paired bispecific antibodies

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ABSTRACT

Background Owing to their roles in promoting T cell and natural killer (NK) cell activation and proliferation, interleukins-2 (IL-2) and interleukins-15 (IL-15) have been pursued as promising pathways to target in cancer immunotherapy. Nonetheless, their wider therapeutic application has been hampered by severe dose-limiting toxicities including systemic cytokine release and organ edema for IL-2, and inconvenient intratumoral administration for IL-15. To address these safety issues, we generated IL-2R/IL-15R×TAA (tumor-associated antigen) bispecific antibody (bsAb) pairs to selectively activate IL-2R signaling in the tumor microenvironment.

Methods Each bsAb pair is composed of one bsAb targeting CD122 and a TAA epitope, and the other bsAb targeting CD132 and the same or a different TAA epitope. In vitro assays were performed to characterize the IL-2R/IL-15R agonistic activity of the bsAb pairs, as well as their capacity to enhance T-cell-mediated killing of TAA$^+$ malignant cells. Using a syngeneic mouse tumor model, in vivo biological activity and systemic toxicity of the bsAb pairs were assessed in comparison with IL-2. The in vivo antitumor activity was assessed in combination with an anti-mouse programmed cell death protein 1 (mPD-1) monoclonal antibody.

Results We demonstrated with two different TAAs (human epidermal growth factor receptor 2 (HER2) and mesothelin (MSLN)) that the CD122×TAA/CD132×TAA bsAb pairs mediate effective activation of immune cells exclusively in the presence of TAA$^+$ tumor cells. In syngeneic hMSLN-MC38 tumor-bearing mice, the CD122×MSLN-1/CD132×MSLN-2 bsAb pair promotes selective activation and expansion of NK cells and central memory CD8$^+$ T cells inside the tumor without inducing organ edema or systemic cytokine release, two well-known manifestations of IL-2 associated toxicity. In combination with checkpoint inhibitor anti-mPD-1, the bsAb pair boosts the accumulation of CD8$^+$ effector T cells and NK cells, leading to a favorable CD8$^+$ T cell to CD4$^+$ regulatory T cell ratio for a more robust inhibition of tumor growth.

Conclusions Overall, the findings suggest that this innovative therapeutic approach effectively leverages the antitumor activity of IL-2 and IL-15 pathways while minimizing their associated systemic toxicities. This dual bsAb format holds potential for broader application in other immune-activating pathways.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Interleukin (IL)-2 and IL-15 agonists have been approved as cancer therapies. However, their clinical application has been impeded by systemic toxicities.

WHAT THIS STUDY ADDS

⇒ We demonstrated that IL-2R/IL-15R signaling can be selectively activated within the tumor, thereby minimizing associated systemic toxicities, by using a bispecific antibody (bsAb) pair consisting of CD122×TAA and CD132×TAA bsAbs.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This strategy could pave the way for safer antitumor therapies targeting immune-activating cytokines.

INTRODUCTION

Interleukin (IL)-2 and IL-15 are two closely related T cell-stimulating and natural killer (NK) cell-stimulating cytokines that share two signaling receptor subunits, IL-2/IL-15 receptor beta (IL-2Rβ, IL-15Rβ, also known as CD122) and IL-2/IL-15 receptor gamma (the common gamma chain or CD132). Each of the two cytokines has a specific receptor subunit (IL-2Rα/CD25 for IL-2, IL-15Rα/CD215 for IL-15), which enhances the binding affinity of the cytokines to the signal transduction receptor subunits.[1] Owing to their role in promoting T cell and NK cell activation and proliferation, IL-2 and IL-15 have been pursued as promising therapeutic targets in cancer immunotherapy.[2] A high dose of recombinant human IL-2 (hIL-2/Proleukin) has been approved for the treatment of metastatic renal cell

carcinoma and metastatic melanoma. However, severe toxic effects due to systemic activation, the most severe being vascular leaking syndrome, culminating in multi-organ edema and damage, have limited its clinical use.[3 4] Another complication with IL-2, which is not a concern for IL-15, is that high-affinity trimeric IL-2R (comprising CD25, CD122 and CD132) is constitutively expressed on regulatory T cells (Tregs). A low dose of IL-2 preferentially stimulates Tregs and suppresses immune response. Thus, a high dose of IL-2 is required to stimulate immune activation, which also increases the risk of toxicity.

Both direct activation of high-affinity IL-2 trimeric receptor on endothelial cells and NK cell-mediated cytokine release have been shown to be involved in IL-2-induced systemic toxicity.[5 6] To mitigate endothelial cell-mediated toxicity and Treg activation, one approach is to reduce IL-2 binding to IL-2Rα, which is expressed on endothelial cells and renders them highly sensitive to IL-2. This so-called "not-alpha" strategy can be achieved by different approaches.[7–13] However, a pegylated IL-2, NKTR-214, although achieving favorable $CD8^+$ T cell/Treg ratio, showed comparable toxicity to non-modified IL-2 (Proleukin), which limited its dose escalation,[3] suggesting that the not-alpha strategy alone may not be enough to mitigate IL-2-associated toxicity in patients. As NK cells constitutively express only the intermediate-affinity IL-2 dimeric receptor composed of CD122 and CD132, efforts have been made to develop IL-2 variants with reduced binding to CD122 to mitigate NK-mediated toxicity. Unfortunately, one such molecule, BAY50-4798, showed a similar toxicity profile to that of non-modified IL-2, suggesting that reducing NK cell activation may also not be sufficient to mitigate IL-2-associated toxicity.[14]

Considering that reducing systemic activation of endothelial cells or NK cells appears insufficient to mitigate IL-2-associated toxicity, further avoidance of systemic IL-2 activation may be required to achieve this goal. Various approaches have been explored to guide IL-2 activation specifically in the tumor microenvironment (TME). A potential strategy involves engineering pro-IL-2 with an attached masking element to prevent systemic activation.[15 16] However, while cleavage of the mask occurs more in tumors, the cleavage has been observed in human serum, and cleaved IL-2 has been detected in the blood of treated mice.[15 17] IL-2 linked to a tumor-targeting antibody is also used to guide IL-2 activation preferentially in the TME.[18–20] As the IL-2 part of the molecule is not masked, the molecule is still active systemically. A further development of this strategy is to split an IL-2-mimetic molecule into two separate fragments. Each fragment is linked to a separate anti-tumor associated antigen (TAA) antibody. On binding of the two IL-2-mimetic fragment-anti-TAA conjugates to TAA on tumor cells, the two fragments of the IL-2-mimetic will be brought into proximity and induce receptor activation.[21] However, the two fragments of the IL-2-mimetic could bind to each other in the absence of TAA and potentially cause systemic activation.

We present a novel strategy for selectively activating IL-2/IL-15 receptors within the TME using a pair of bispecific antibodies (bsAbs). One bsAb has an arm that binds to CD122, while the other targets a TAA. The second bsAb binds to CD132 on one arm and either the same TAA-epitope or an alternate TAA-epitope (figure 1A). In circulation, these bsAbs cannot activate the CD122/CD132 receptor because TAAs are not expressed outside the tumor. However, within the tumor, binding to the TAA brings the two bsAbs into proximity, allowing their CD122 and CD132 binding arms to engage receptors on tumor-infiltrating T cells or NK cells, triggering receptor activation. Our findings from mouse models highlight the TME-specific activity and improved safety profile of this innovative approach.

METHODS

Reagents and cell lines

The reagents and cell lines used in this study were listed in the online supplemental tables 1-2.

IL-2 reporter cell assay

Reporter cell assays were performed using HEK-Blue IL-2 and/or HEK-Blue CD122/CD132 following the manufacturer's recommendation with bsAb combinations added onto 50,000 reporter cells and 150,000 TAA-coated streptavidin microspheres or 150,000 NCI-N87 cells. Absorbance at 640 nm was acquired using a SpectraMax-i3x (Molecular Devices).

Characterization of phosphorylated STAT5 induction

In 96-well plates, NK-92 cells, peripheral blood mononuclear cells (PBMCs) or purified human NK cells were stained with Fixable Viability Dye eFluor 506, washed and incubated with Fc Block. NK-92 and primary human NK cells were stained with anti-CD45 antibody while PBMCs cells were stained with anti-CD3, anti-CD45, anti-CD4, anti-CD8, anti-CD14, anti-CD19, and anti-CD56 antibodies. Cells were incubated with Proleukin or with TAA-expressing tumor cells and bsAbs at 37°C for 30 min. Cells were fixed, washed and permeabilized. NK-92 and primary human NK cells were stained with an anti-pStat5 antibody while PBMCs were stained with an anti-pStat5 and an anti-Foxp3 antibodies (antibodies are listed in online supplemental table 3).

T-cell-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity assays

1.5 million PBMCs were incubated with 1.5 million target tumor cells and a suboptimal dose of H4G5-trastuzumab for 72 hours in 6-well plates. PBMCs were distributed in 96-well plates with fresh target tumor cells (effector:target ratio 10:1) and bsAbs or Proleukin for 72 hours. 200,000 isolated NK cells were incubated with 10,000 NCI-N87 cells overnight at 37°C in the presence of 30 nM CD122×MSLN-1/CD132×MSLN-2 bsAb pair in 96-well plates. A suboptimal dose of trastuzumab is added and

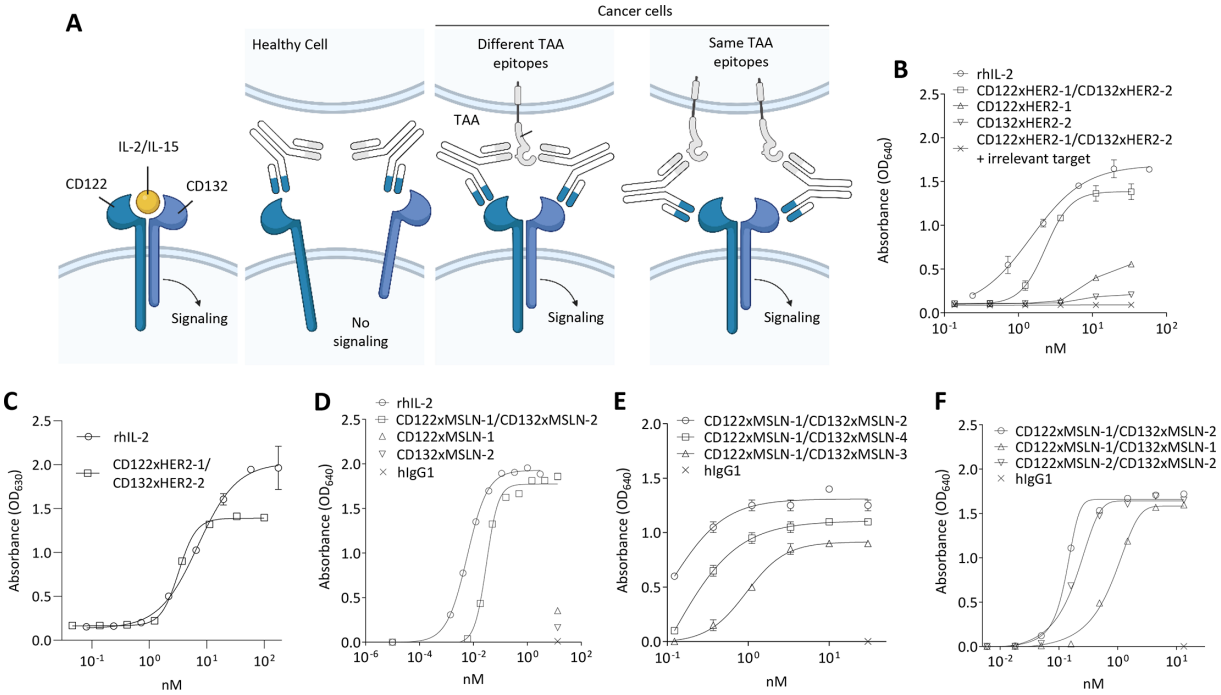


Figure 1 CD122×TAA/CD132×TAA bsAb pairs induce IL-2/IL-15 signaling in reporter cells. (A) Schematic representation showing the combination of CD122×TAA/CD132×TAA bsAbs selectively activating IL-2/IL-15 signaling on immune cells in the presence of TAA-expressing tumor cells by targeting different TAA epitopes (panel 3) or the same TAA epitope (panel 4). (B–F) In vitro biological activity of CD122×TAA/CD132×TAA bsAb pairs was explored using HEK Blue CD122/CD132 reporter cells and TAA-coated microspheres (B, D–F) or NCI-N87 tumor cells (C). rhIL-2 and hIgG1 were used as positive and negative controls. (B) Reporter activity induced by a dose range of the CD122×HER2-1/CD132×HER2-2 bsAb pair or individual bsAbs in presence of HER2-coated microspheres. (C) Reporter activity induced by a dose range of the CD122×HER2-1/CD132×HER2-2 bsAb pair in presence of HER2-expressing NCI-N87 cells. (D) Reporter activity induced by a dose range of the CD122×MSLN-1/ CD132×MSLN-2 bsAb pair or individual bsAbs tested at the maximal concentration in presence of MSLN-coated microspheres. (E) Reporter activity induced by a dose range of CD122×MSLN-1 bsAb paired with CD132×MSLN bsAbs targeting three different MSLN epitopes (MSLN-2, MSLN-3 and MSLN-4). (F) Reporter activity induced by a dose range of IL-2R×MSLN bsAb pairs targeting either two different MSLN epitopes (CD122×MSLN-1/CD132×MSLN-2) or the same MSLN epitope (CD122×MSLN-1/CD132×MSLN-1 or CD122×MSLN-2/CD132×MSLN-2) with MSLN coated microspheres. Graphs show mean±SEM. bsAb, bispecific antibody; HER2, human epidermal growth factor receptor 2; IL, interleukin; MSLN, mesothelin; rhIL-2, recombinant human IL-2; TAA, tumor associated antigen.

plates are incubated for 6 hours at 37°C. After incubation, specific killing was measured with CellTiter-Glo Luminescent Cell Viability Assay following manufacturer's recommendations (specific lysis = 100 − ((100 × sample mean) / effector:target mean)).

Tumor model in vivo

Female hCD122/hCD132 KI mice (Biocytogen, 6–8 weeks old) with established hMSLN-MC38 tumors were treated in a Specific Pathogen-Free room of the animal facility with vehicle (phosphate buffered saline (PBS)), Proleukin or CD122×MSLN-1/CD132×MSLN-2 bsAb pair. Proleukin was dosed intraperitoneally at 3 mg/kg daily for five consecutive days; bsAb combination was dosed intravenously at 10 mg/kg two times a week for 1 week. All treated animals were included in the analysis. To analyze the TME, tumors were harvested 5 days after treatment initiation for analysis by flow cytometry. Tumors, spleens and lungs were analyzed for immune cell infiltration and phenotype (antibodies listed in online supplemental table 4). Separately, to evaluate tumor growth control, hMSLN-MC38 tumor-bearing mice were treated intravenously two times a week for 2 weeks with vehicle (PBS), anti-mouse programmed cell death protein 1 (mPD-1) (5 mg/kg), CD122×MSLN-1/CD132×MSLN-2 bsAb pair (10 mg/kg) or both anti-mPD-1 and bsAb combination. To analyze the TME, tumors were harvested 9 days after treatment started for analysis by flow cytometry. For both experiments, tumor sizes were measured 3×/week using a caliper (tumor volume (mm^3) = length × $width^2$ × 0.5 and % of tumor growth inhibition (at time=t) = (1 − ((mean tumor volume (t) − mean initial tumor volume) / (mean control tumor volume (t) − mean initial control tumor volume)) × 100). To compare the probability of survival between treatment groups, Kaplan-Meier survival curves were generated. Mice with tumors >1500 mm^3 were euthanized.

IL-2–induced toxicity in vivo

hCD122/hCD132 KI mice engrafted with hMSLN-MC38 tumors, received vehicle (PBS), Proleukin (5 mg/kg daily intravenously for 5 days) or bsAb combination (10 mg/

kg biweekly intravenously for 1 week). Body weight was measured 3×/week. On sacrifice day, lungs and spleens were weighed and processed for immune profiling via flow cytometry. Tumor necrosis factor (TNF)-α and interferon (IFN)-γ levels in the serum were determined using MSD kits following manufacturer's instructions.

Human whole blood cytokine release assay

Cytokine secretion was detected using fresh human whole blood collected from healthy individuals (with Heparin-Lithium). 20× concentrated test articles were added to each well of a sterile 96-well round bottom plate: cetuximab (negative control, 300 nM), Proleukin (1000 nM), and CD122×MSLN-1/CD132×MSLN-2 bsAb pairs (300 nM each). Fresh human whole blood was incubated with test articles at 37°C overnight. Samples were centrifuged, and plasma was transferred to a 96-well microplate and kept frozen (−80°C) until MSD analysis of TNF-α and IL-6 levels.

Tissues immune profiling via flow cytometry

Single cell suspensions were stained for viability, washed with staining buffer and incubated with purified rat anti-mouse CD16/CD32. Fluorescent-conjugated antibody mix (extracellular markers online supplemental table 4) was added to the cell suspension. For bsAb tissue distribution anti-human Fc were used (online supplemental table 4). If no intracellular staining was needed, stained cells were washed and fixed. For intracellular staining, samples were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 4 hours, washed with staining buffer, fixed and permeabilized (online supplemental table 4). Cells were incubated first with rat anti-mouse CD16/CD32 and, later, with antibody mix (intracellular staining online supplemental table 4).

Statistics

Statistical analyses were performed using GraphPad Prism Software, V.10. Differences between multiple groups were determined using a one-way analysis of variance with multiple comparison test. P values are the following *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistical difference between survival curves was calculated using the log-rank (Mantel-Cox) test with the Bonferroni correction comparison.

More details on methods could be found in the online supplemental methods (online supplemental file 4).

RESULTS

CD122/CD132×TAA bsAb pairs induce IL-2/IL-15 signaling in reporter cells

We hypothesize that, in the presence of TAA-expressing cells, a bsAb pair of CD122×TAA and CD132×TAA could bring CD122 and CD132 into proximity on immune cells and induce CD122/CD132 dimeric receptor signaling by trans-engaging TAAs on tumor cells (figure 1A). To this end, we generated bsAbs, with one arm targeting either human CD122 or human CD132 and the other arm targeting distinct epitopes on human epidermal growth factor receptor 2 (HER2), referred to as HER2-1 and HER2-2 or mesothelin (MSLN), referred to as MSLN-1 and MSLN-2, using antibody arms pre-existing or generated in-house with phage display (online supplemental figure S1A-C). In the presence of HER2-coated microspheres to mimic HER2-expressing cancer cells, CD122×HER2-1/CD132×HER2-2 bsAb pair, but not an individual bsAb, activated IL-2/IL-15 signaling in reporter cells to a level similar to that induced by rhIL-2 (figure 1B). Importantly, the activation is reliant on TAA as the bsAb pair did not activate cells when combined with irrelevant TAA-coated microspheres (figure 1B). IL-2R agonistic activity was confirmed using $HER2^{+}$ NCI-N87 cells instead of HER2-coated beads (figure 1C). The approach is not limited to HER2 since the CD122×MSLN-1/CD132×MSLN-2 bsAb pair, but not an individual bsAb, also activated reporter cells in the presence of MSLN-coated microspheres (figure 1D). When testing bsAb pairs targeting different MSLN epitopes, different reporter activation profiles were observed with bsAb pairs targeting different TAA epitope combinations (figure 1E and online supplemental figure S2A). The CD122×MSLN-1/CD132×MSLN-2 pair, with MSLN-1 arm targeting a membrane-proximal epitope and MSLN-2 arm targeting a membrane-distal one, showed the highest potential to induce reporter cell activation and thus was selected for further study. A similar epitope-dependent phenomenon was also observed with HER2-targeting bsAb pairs (online supplemental figure S2B). We also evaluated bsAb pairs targeting the same TAA epitope and demonstrated that this approach can activate signaling for both MSLN-targeting and HER2-targeting bsAbs. While there was some loss of potency when targeting the same epitope for MSLN, this reduction was not observed for HER2 (figure 1F and online supplemental figure S2C). Altogether, we demonstrated with two different TAAs that IL-2/IL-15 signaling can be activated through combinations of bsAbs that bring CD122 and CD132 into proximity in a TAA-dependent manner.

CD122/CD132×TAA bsAb pairs induce STAT5 phosphorylation in NK cell line and human peripheral blood mononuclear cells

In a more physiologically relevant system, we tested the concept with the human NK cell line, NK-92 cells, in the presence of cancer cells expressing different levels of target TAAs. IL-2/IL-15 signaling was evaluated by measuring the level of phosphorylated signal transducer and activator of transcription 5 (pSTAT5), a transcription factor downstream of the IL-2/IL-15 R signaling. The CD122/CD132×HER2 bsAb pair induced a substantial increase of pSTAT5 in the presence of NCI-N87 cells, the tested tumor cell line with~400 k HER2/cell, while it induced a lower level of pSTAT5 in the presence of JIMT-1 cells, a cell line with~69 k HER2/cell. When incubated with MC38-hHER2, an engineered cell line with~44 k HER2/cell, we observed only marginal levels

of pSTAT5 (figure 2A, left panel, online supplemental table 2). The TAA-density-dependent pSTAT5 increase was also observed with the MSLN-targeting bsAb pair, with the difference that the MSLN bsAb pair was more potent at inducing pSTAT5 towards cell lines expressing lower levels of MSLN compared with the HER2 counterparts (figure 2A, online supplemental table 2). As shown in figure 2A, although JIMT-1 cells express a lower level of MSLN than HER2 (9 k MSLN/cell vs 69 k HER2/cell), HER2 bsAb pair and MSLN bsAb pair stimulated similar profiles of pSTAT5 in NK-92 when combined with this cell line.

As T cells play a pivotal role in IL-2-mediated antitumor activity,[22] we tested whether the CD122/132×MSLN bsAb pair could stimulate primary T cells from human PBMCs in the presence of NCI-N87 cells. In contrast to rhIL-2, which stimulated IL-2Rα-positive Tregs at lower concentrations than those required to stimulate IL-2Rα-negative $CD8^+$ T cells (figure 2B, left panel), the CD122×MSLN-1/CD132×MSLN-2 combination led to very similar activation of Tregs and $CD8^+$ T cells (figure 2B, right panel), as expected for a not-alpha strategy of IL-2 activation.

CD122/CD132×TAA bsAb pairs induce TAA-dependent killing of cancer cells in vitro

IL-2/IL-15 signaling plays a supportive role in TCR-mediated antigen-specific target cell killing but is not expected to induce non-activated T cells to kill tumor cells alone. To assess the tumoricidal potential of our bsAb pairs, we preactivated human PBMCs for 3 days using NCI-N87 cells and H4G5×trastuzumab, a T-cell engager that binds to CD3 and HER2. These preactivated PBMCs were then used to evaluate the specific killing of MSLN-expressing cancer cells, induced by the CD122×MSLN-1/CD132×MSLN-2 bsAb pair. As a positive control, Proleukin induced killing by preactivated PBMCs of all tested cancer cell lines, including MSLN-negative MDA-MB-468 and SH-SY5Y cells (online supplemental figure S3A-D). In contrast, the CD122×MSLN-1/CD132×MSLN-2 bsAb pair only induced killing of the MSLN-positive cell lines, with significant killing of MSLN-high NCI-N87 cells (~20 k MSLN/cell) as well as HPAC cells (~14 k MSLN/cell), and detectable but not significant killing of MSLN-low JIMT-1 cells (~9 k MSLN/cell) (online supplemental figure 2C and online supplemental figure S3B-D). Killing of MSLN-negative cells, MDA-MB-468 and SH-SY5Y, was not induced by the bsAb pair. Complementary experiments were also conducted using human primary NK cells. The overnight incubation of NK cells with CD122×MSLN-1/CD132×MSLN-2 bsAb pair and NCI-N87 cells enhanced NK-mediated antibody-dependent cellular cytotoxicity of NCI-N87 cells induced by the Fc-active anti-HER2 antibody trastuzumab (figure 2D). The increased killing correlates with a detectable, although variable, increase in pSTAT5 in primary NK cells when co-cultured with MSLN-positive NCI-N87 cells in the presence of the CD122×MSLN-1/CD132×MSLN-2 bsAb pair (online supplemental figure S3E). Altogether, we showed that our bsAbs induced killing of tumor cells by pre-activated T cells and NK cells in a TAA-dependent manner. The reliance on signal 1 pre-stimulation or the presence of Fc-active TAA antibody and TAA expression by target tumor cells supports the superior safety profile of this novel approach.

TAA-dependent tumor engagement of CD122/CD132×TAA bsAb pairs induces robust accumulation and activation of tumor-infiltrating $CD8^+$ T cells and NK cells

To evaluate the in vivo activity of the CD122/CD132×TAA bsAb pairs, we tested the CD122×MSLN-1/CD132×MSLN-2 bsAb pair in a syngeneic and fully immunocompetent mouse model. As the targeting arms of the bsAb pair are not mouse cross-reactive (online supplemental figure S1C), hCD122/hCD132 transgenic mice (Tg-mice) were engrafted subcutaneously with hMSLN-expressing MC38 colon cancer cells (figure 3A). Tumor immunophenotyping by flow cytometry at day 5 post-treatment initiation showed that the CD122/CD132×MSLN bsAb pair enhanced $CD45^+$ leukocyte infiltration and significantly increased the number of infiltrating $CD8^+$ tumor-infiltrating T lymphocytes (TILs) (figure 3B, online supplemental figure S4). A non-significant increase of $CD8^+$ TILs was observed in Proleukin-treated mice (figure 3B). The reported low expression of CD122/CD132 on $CD4^+$ T cells[23] may account for the observed only marginal effect on $CD4^+$ TILs as reported by other not-alpha strategies.[24 25] Importantly, while Proleukin had no effect, the bsAb pair drastically increased the proportion of central memory $CD8^+$ TILs ($CD44^+$ $CD62L^+$) (figure 3C) and enhanced significantly $CD8^+$ TILs proliferation (measured by Ki67) (figure 3D). However, quantitative PCR analysis demonstrated that the bsAb pair did not increase intratumoral proinflammatory cytokines (IFN-γ, IL-6, and TNF-α) 5 days post-treatment initiation (online supplemental figure S5). The number, proliferation and cytotoxic potential of tumor-infiltrating NK cells were also significantly increased by the bsAb pair (figure 3E). The CD122/CD132×MSLN bsAb pair did not alter the Treg population within the tumor or the spleen. In contrast, Proleukin decreased intratumoral Tregs, likely through their redistribution to the spleen (online supplemental figure S6A). In another set of experiments, we compared two bsAb pairs with high or medium affinity for CD122 and CD132 (referred to CD122-H/CD132-H vs CD122-M/CD132-M, online supplemental figure S1). In vitro tests revealed greater IL-2R agonistic potency for the high-affinity pair compared with the medium-affinity pair (online supplemental figure S6B). Interestingly, the medium affinity CD122/CD132 arms increased the intratumoral NK cell infiltration more substantially compared with the higher affinity CD122/CD132 arms (online supplemental figure S6C), likely due to less binding to $CD122^+$ and/or $CD132^+$ immune cells in circulation leading to better tumor infiltration by the medium affinity bsAb pair. The high and medium affinity bsAb pairs induced similar levels of central memory $CD8^+$ T

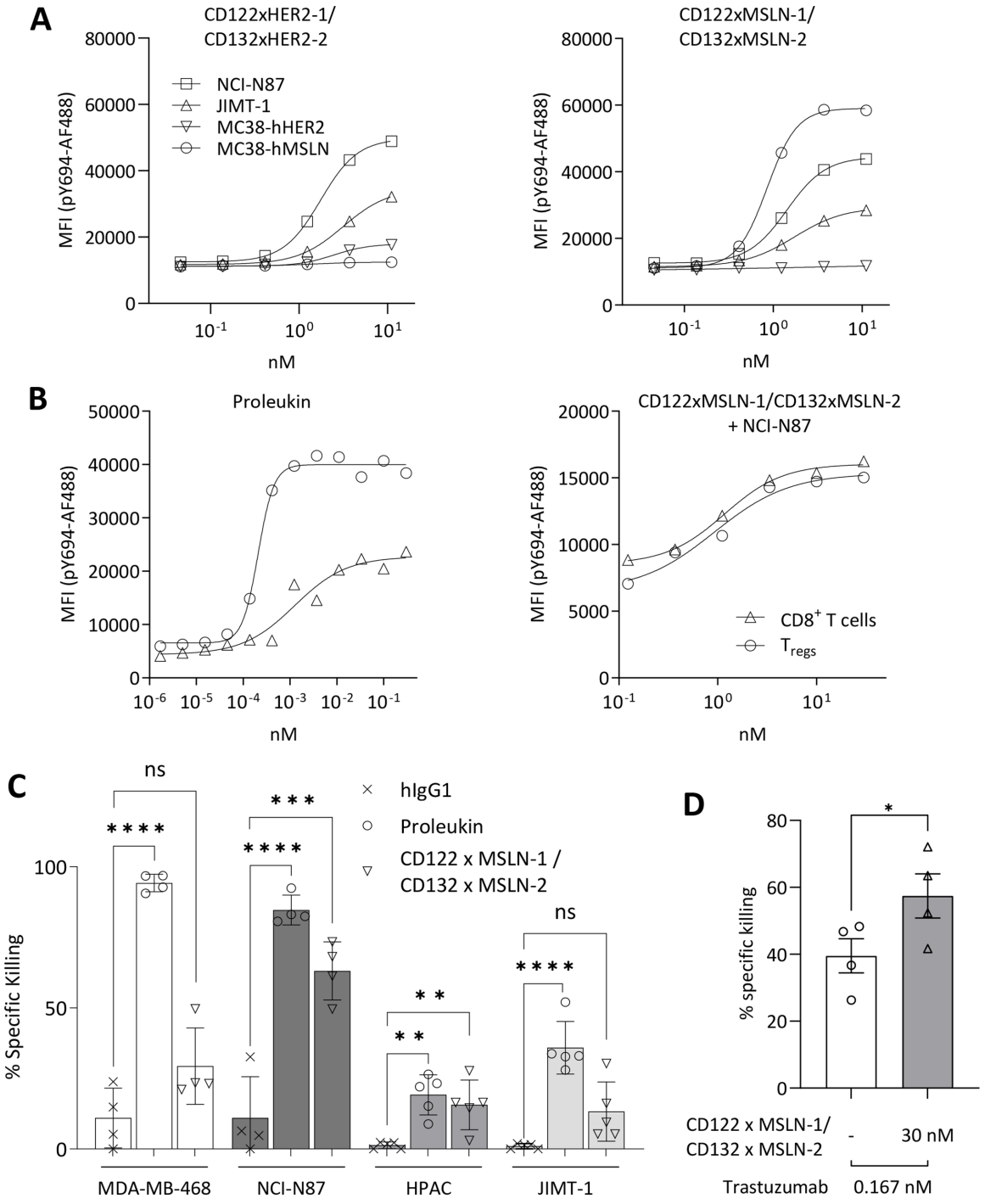


Figure 2 CD122×TAA/CD132×TAA bsAb pairs induce STAT5 phosphorylation (pSTAT5) in human NK cells and T cells and enhance T-cell-dependent cytotoxicity (TDCC) of tumor cells as well as NK-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) in a TAA-dependent manner. (A) Dose-dependent induction of pSTAT5 in NK-92 cells by CD122×HER2-1/CD132×HER2-2 (left panel) or CD122×MSLN-1/CD132×MSLN-2 (right panel) following incubation with cancer cells expressing different levels of the corresponding TAA. (B) Dose-dependent induction of pSTAT5 in human $CD8^+$ T cells and Tregs by Proleukin (left panel) or CD122×MSLN-1/CD132×MSLN-2 in the presence of NCI-N87 cells (right panel). (C) TDCC of cancer cell lines expressing different levels of MSLN induced by a hIgG1 isotype control, CD122×MSLN-1/CD132×MSLN-2 or Proleukin. Specific killing was calculated by subtracting unspecific killing measured in control wells with peripheral blood mononuclear cell (n=4/5) and cancer cell lines only. Graphs show mean±SEM and statistical significance between groups was measured using one-way analysis of variance for each cancer cell line independently. (D) NK cell-mediated ADCC of NCI-N87 cancer cells induced by overnight incubation with CD122×MSLN-1/CD132×MSLN-2 followed by 6 hours incubation with trastuzumab. Specific killing was calculated by subtracting unspecific killing measured in control wells with NK cells and cancer cell lines only. Graphs show mean±SEM and statistical significance between groups was measured using paired t-test. bsAb, bispecific antibody; HER2, human epidermal growth factor receptor 2; MFI, mean fluorescence intensity; MSLN, mesothelin; NK, natural killer; STAT5, signal transducer and activator of transcription 5; TAA, tumor associated antigen; Treg, regulatory T cell.

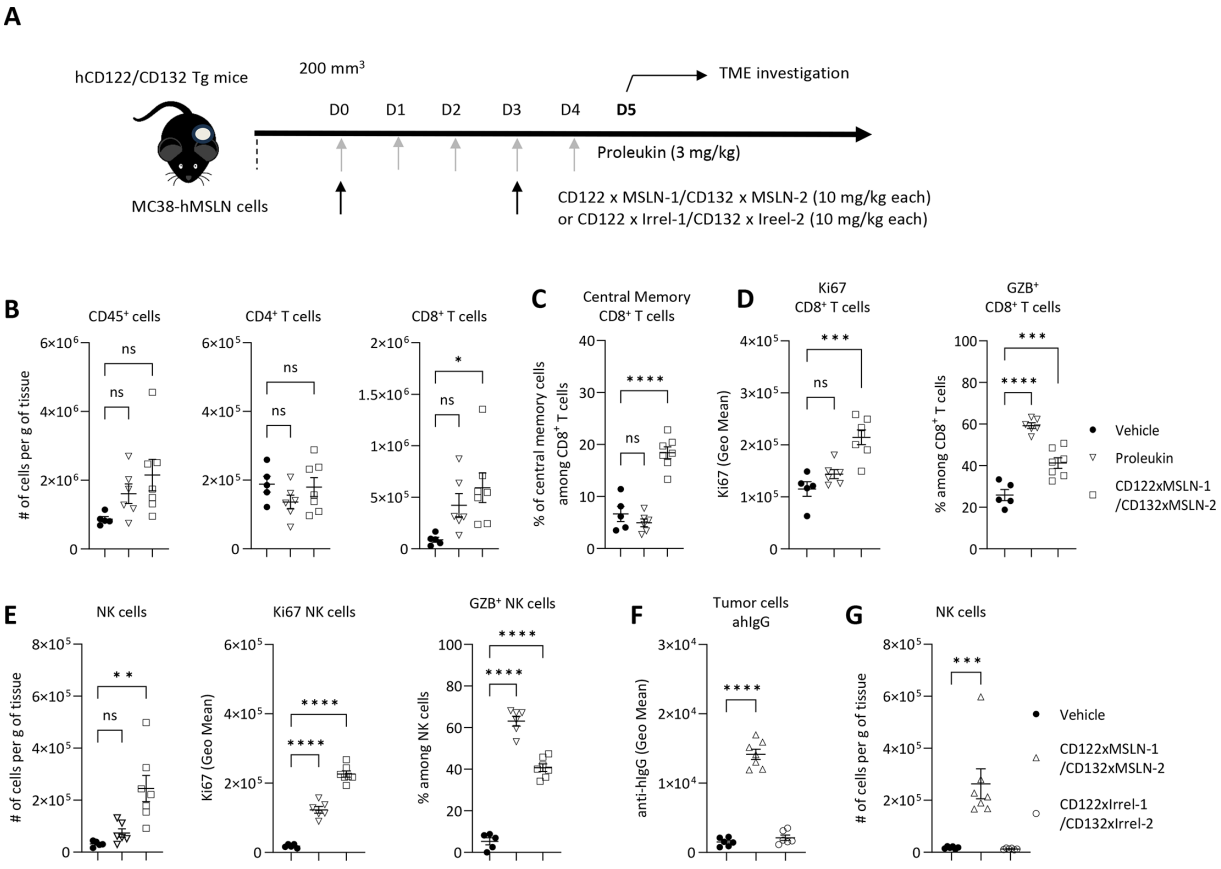


Figure 3 Treatment with CD122/CD132×MSLN bsAb pairs induces MSLN-dependent expansion and activation of intratumoral CD8$^+$ T cells and NK cells. In vivo biological activity was explored in hCD122/hCD132 transgenic mice engrafted with hMSLN-expressing MC38 colon cancer cells. (A–E) Mice were treated with vehicle control (n=5), Proleukin (intraperitoneally 3 mg/kg for five consecutive days, n=6) or CD122×MSLN-1/CD132×MSLN-2 bsAb pair (intravenously 10 mg/kg, twice (d0 and d3), n=7). Tumor-infiltrating immune cells were assessed 5 days post-treatment initiation. Shown are (B) absolute counts of CD45$^+$ leukocytes, CD4$^+$ or CD8$^+$ T cells, (C) proportion of central memory T cells among CD8$^+$ T cells, (D) Ki67 mean fluorescence intensity of CD8$^+$ T cells and proportion of GZB$^+$ cells among CD8$^+$ T cells, (E) absolute count of NK cells, Ki67 MFI of NK cells and proportion of GZB$^+$ cells among NK cells. (F–G) Mice were treated with vehicle control (n=7), CD122-M×MSLN-1/CD132-M×MSLN-2 (n=7) or bsAb pair targeting an irrelevant TAA (CD122×irrel-1/CD132×irrel-2 (n=6)), twice (d0 and d3) at 10 mg/kg (intravenously) and tumors were analyzed 5 days post treatment initiation. Shown in the panels are (F) specific detection of human antibodies binding to MSLN-positive tumor cells, (G) absolute numbers of intratumoral NK cells. Graphs show mean values±SEM and statistical significance was determined using a one-way analysis of variance with multiple comparison test. bsAb, bispecific antibody; MSLN, mesothelin; NK, natural killer; TAA, tumor-associated antigen; TME, tumor microenvironment.

cells (online supplemental figure S6D). As such, the next experiments were performed using the bsAb pair carrying the medium affinity CD122/CD132 binding arms.

To confirm that the CD122/CD132×MSLN bsAb pair-induced TME modulation is TAA-dependent, we compared it with a bsAb pair targeting an irrelevant TAA (CD122×irrel-1/CD132×irrel-2). Flow cytometry analysis demonstrated that the CD122/CD132×MSLN bsAbs accumulated on the cancer cell surface (gated as CD45$^-$ cells) in hMSLN-expressing MC38 tumors of mice treated with the CD122/CD132×MSLN bsAb pair, whereas no human antibody binding to the cancer cells was detected in tumors of mice treated with the CD122×irrel-1/CD132×irrel-2 bsAb pair (figure 3F). As expected, CD122/CD132×MSLN bsAb pair but not CD122×irrel-1/CD132×irrel-2 bsAb pair increased infiltration of NK cells and T-cell subpopulations into the TME (figure 3G and online supplemental figure S6E). In summary, these data demonstrate that combining CD122/CD132×TAA bsAbs increases the numbers of CD8$^+$ TILs similarly to Proleukin. In contrast to Proleukin, the bsAb pair also increases the number of tumor-infiltrating central memory CD8$^+$ T and NK cells as well as enhancing NK cell activation in a TAA-dependent manner.

Improved safety profile of the CD122/CD132×TAA bsAb pair relative to Proleukin

To test whether the targeted activation of immune cells with the CD122/CD132×TAA bsAb pairs could avoid the adverse effects associated with high-dose Proleukin, hMSLN-MC38 tumor-bearing Tg-mice were dosed with either Proleukin or the CD122×MSLN-1/CD132×MSLN-2 bsAb pair and mouse body weights were monitored. After five consecutive daily injections of Proleukin, significant body weight loss was observed and mice showed reduced activity as well as piloerection. In contrast, mice treated

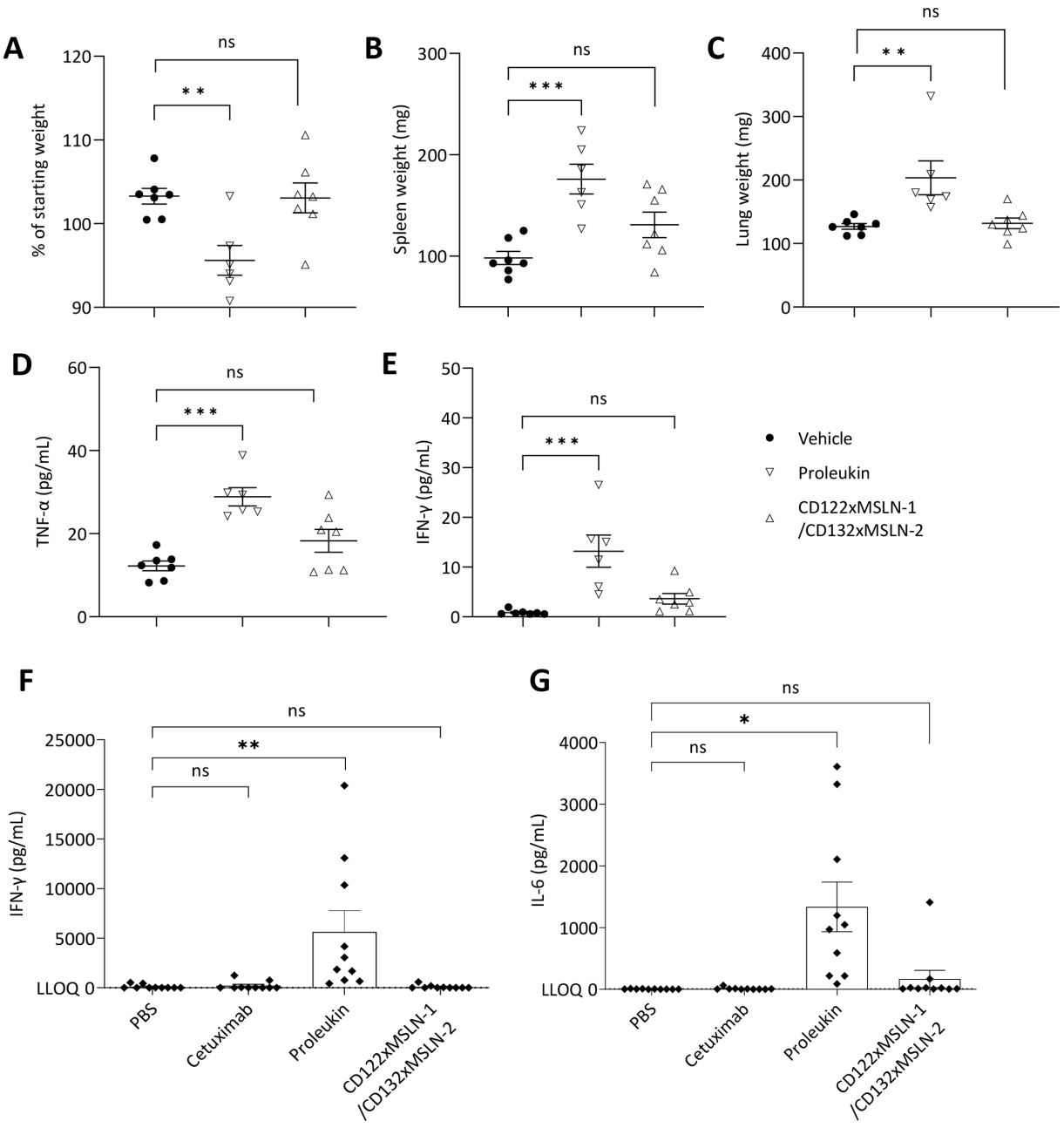


Figure 4 Improved safety profile of CD122/CD132×MSLN bsAb pairs in comparison to Proleukin. (A to E) hCD122/hCD132 transgenic mice were engrafted with hMSLN-expressing MC38 colon cancer cells. Mice were treated with vehicle (PBS) (n=7), Proleukin (n=6) or CD122×MSLN-1/CD132×MSLN-2 bsAbs (n=7). Mice were euthanized 5 days post treatment initiation. Shown in the panels are body weight (A), spleen weight (B), lung weight (C), serum TNF-α (D) and serum IFN-γ (E) of the treated mice. (F–G) Induction of IFN-γ (F) and IL-6 (G) release in human whole blood (10 healthy donors) by cetuximab, Proleukin or CD122/CD132×MSLN bsAb pair. Graphs show mean values±SEM and statistical significance between treatment groups was determined using a one-way analysis of variance with multiple comparison test. bsAb, bispecific antibody; IFN, interferon; IL, interleukin; MSLN, mesothelin; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

with CD122/CD132×MSLN bsAb pair did not lose body weight (figure 4A, online supplemental figure S7A) nor display any clinical signs of toxicity. Monitoring body weight over an extended period in a separate experiment did not reveal any weight loss, further supporting the safety profile of the bsAb pair (online supplemental figure S7B). Proleukin treatment also increased spleen and lung weights (figure 4B–C), two common manifestations of IL-2-induced organ edema. In contrast, no change in spleen or lung weight was observed in mice treated with the CD122/CD132×MSLN bsAb pair (figure 4B–C). Furthermore, Proleukin induced a significant upregulation of serum TNF-α and IFN-γ while the CD122/CD132×MSLN bsAb pair did not stimulate cytokine release in tumor-bearing mice (figure 4D–E). In agreement with these observations, Proleukin injection resulted in increased numbers of $CD45^+$, $CD3^+$ and $CD8^+$ T cells and effector $CD8^+$ T cells ($CD44^+$ $CD62L^-$) in the

lungs relative to the vehicle group. Although to a lower level, the same cellular change was observed in the spleen as well. In contrast, most of these immune cell subpopulations were not altered by the CD122/CD132×MSLN bsAb pair treatment, with the exception of a significant increase in the NK cells in both lung and spleen (online supplemental figure S8A-B), which may be attributed to immune cell recirculation from the tumor. The presence of soluble MSLN (sMSLN) detectable in the blood of tumor-engrafted mice (online supplemental figure S9A) could be another factor driving the increase of NK cells in the spleen and lung of mice treated with the bsAb pair, as the bsAb pair mediated low level of IL-2R signaling in the presence of similar sMSLN concentrations in an in vitro assay (online supplemental figure S9B). Proleukin treatment reduced the number of circulating lymphocytes on day 5 post-treatment initiation. The bsAb pair did not affect the number of circulating lymphocytes at either day 5 or day 9 post-treatment initiation (online supplemental figure S9C-D).

Additionally, we performed a human whole blood assay to assess potential cytokine release induction in the presence of CD122/CD132×TAA bsAb pairs. Results showed that in contrast to Proleukin, which significantly induced IFN-γ and IL-6 production in human blood as expected, the CD122/CD132×MSLN bsAb pair did not induce any cytokine production with levels of IFN-γ and IL-6 close to those obtained with a negative control antibody (cetuximab) or PBS (figure 4F–G). It is worth mentioning that even in the presence of a large amount of sMSLN (400 nM), the CD122/CD132×MSLN bsAb pair did not upregulate any of the proinflammatory cytokines tested (online supplemental figures S9E-F). These data further corroborate our in vivo findings, where the bsAb pair did not induce cytokine release even in the presence of circulating sMSLN (figure 4D–E).

CD122/CD132×TAA bsAb pairs drive potent antitumor activity in combination with anti-PD-1

Preclinical evidence suggests that combining IL-2 pathway activation with PD-1 checkpoint blockade represents a promising immunotherapy strategy.[20 25–27] To address the potential benefit of combining the CD122/CD132×TAA bsAb pairs and PD-1 blockade, in vivo efficacy studies were performed. Treatments were initiated when tumors reached a volume of 100–150 mm^3 (figure 5A). As expected, anti-mPD-1 monotherapy resulted in only partial control of tumor growth. Although the CD122/CD132×MSLN bsAb pair alone did not control tumor growth, combining the bsAb pair with anti-mPD-1 therapy resulted in a significantly stronger inhibition of tumor growth compared with anti-mPD-1 alone (at day 13, tumor growth inhibition=45% for anti-mPD-1 alone vs 75% for the combination), with 2/7 animals achieving nearly complete tumor clearance (figure 5B and online supplemental figure S10A). Combined treatment also led to an increased survival probability relative to the vehicle group (figure 5C). Immunophenotyping of TILs at day 9 post-treatment initiation showed that in vivo efficacy correlates with immunophenotypic changes in the TME. Anti-mPD-1 monotherapy or combined with CD122/CD132×MSLN bsAb pair induced a significant increase in tumor infiltration of $CD45^+$ and $CD8^+$ T cells as well as cytotoxic GZB^+ $CD8^+$ T cells over vehicle-treated mice (figure 5D–F). In contrast to anti-mPD-1 monotherapy, the combined treatment with anti-mPD-1 and the bsAb pair resulted in a significant increase of NK, proliferating NK and a more favorable $CD8^+$ T cell/Treg ratio compared with the vehicle control (figure 5G–I, online supplemental figures S10B-D). The treatments did not change the proportion of tumor-specific $CD8^+$ T cells in circulation or in the tumor, however, the absolute number showed a trend of increase in the tumor of mice from the anti-PD-1 and the combination treatment groups (online supplemental figure S11). The results suggest that the combination therapy enhances tumor clearance by increasing the presence of cytotoxic T cells and NK cells relative to Tregs. In summary, anti-mPD-1/bsAb pair combination therapy integrates immunological outcomes observed with anti-mPD-1 monotherapy, characterized by an increase in effector and cytotoxic T cells, with those observed with CD122/CD132×MSLN bsAb pair therapy, which promotes expansion of NK cells and central memory $CD8^+$ T cells, resulting in a favorable TME for better tumor control.

Distinct TAAs can be combined to further improve specificity

TAA expression on normal cells is a common cause of toxicity in TAA targeted therapies. Targeting two distinct TAAs simultaneously may further restrict the beneficial effects to tumor cells co-expressing the two TAAs while sparing normal cells expressing only one TAA. We thus tested whether the bsAb pair strategy also works by combining bsAbs targeting two different TAAs (HER2 and MSLN). First, we tested the concept with CD122×HER2-1/CD132×MSLN-2 bsAb pair using HEK Blue CD122/CD132 reporter cell line and microspheres co-coated with various levels of MSLN and HER2. Results demonstrated that reporter cells were activated by the combination of CD122×HER2-1/CD132×MSLN-2 in a TAA density-dependent manner (figure 6A). It was interesting to observe that higher TAA densities were required to support the bsAb pair targeting two distinct TAAs than those targeting a single TAA (figure 6A, online supplemental figure S12A). Next, we sought to repeat this compelling observation using cancer cells expressing both TAAs (NCI-N87 cells) and immune cells expressing the dimeric receptor (NK-92 cells). Our data showed that the bsAb pair targeting both HER2 and MSLN induced an increase in pSTAT5 level, although to a level slightly lower than those induced by bsAb pairs targeting a single TAA (figure 6B). We also tested the concept with a cell line expressing lower levels of both TAAs (JIMT-1) than NCI-N87. With this cell line, the bsAb pair targeting both TAAs also induced detectable pSTAT5 in NK-92 cells, which was substantially lower than that induced by the

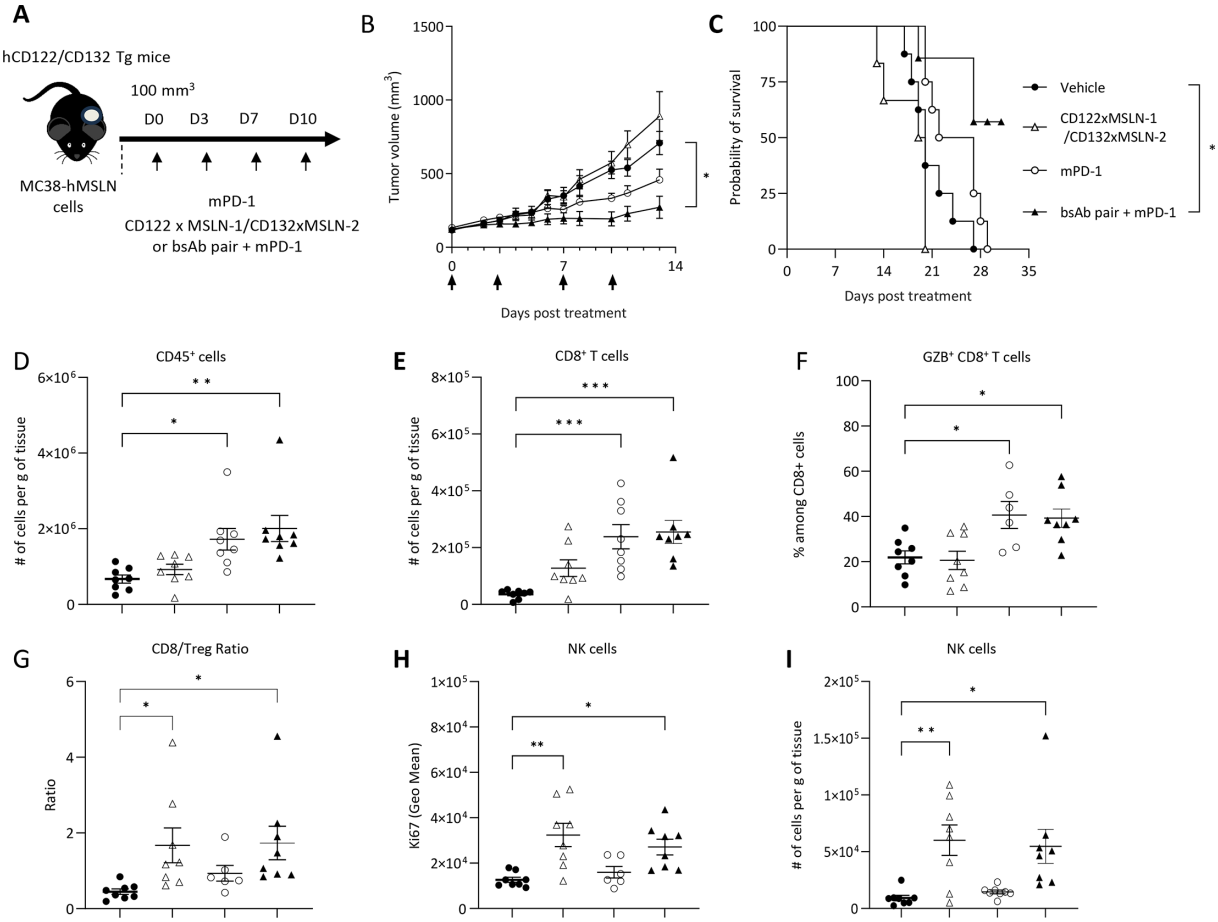


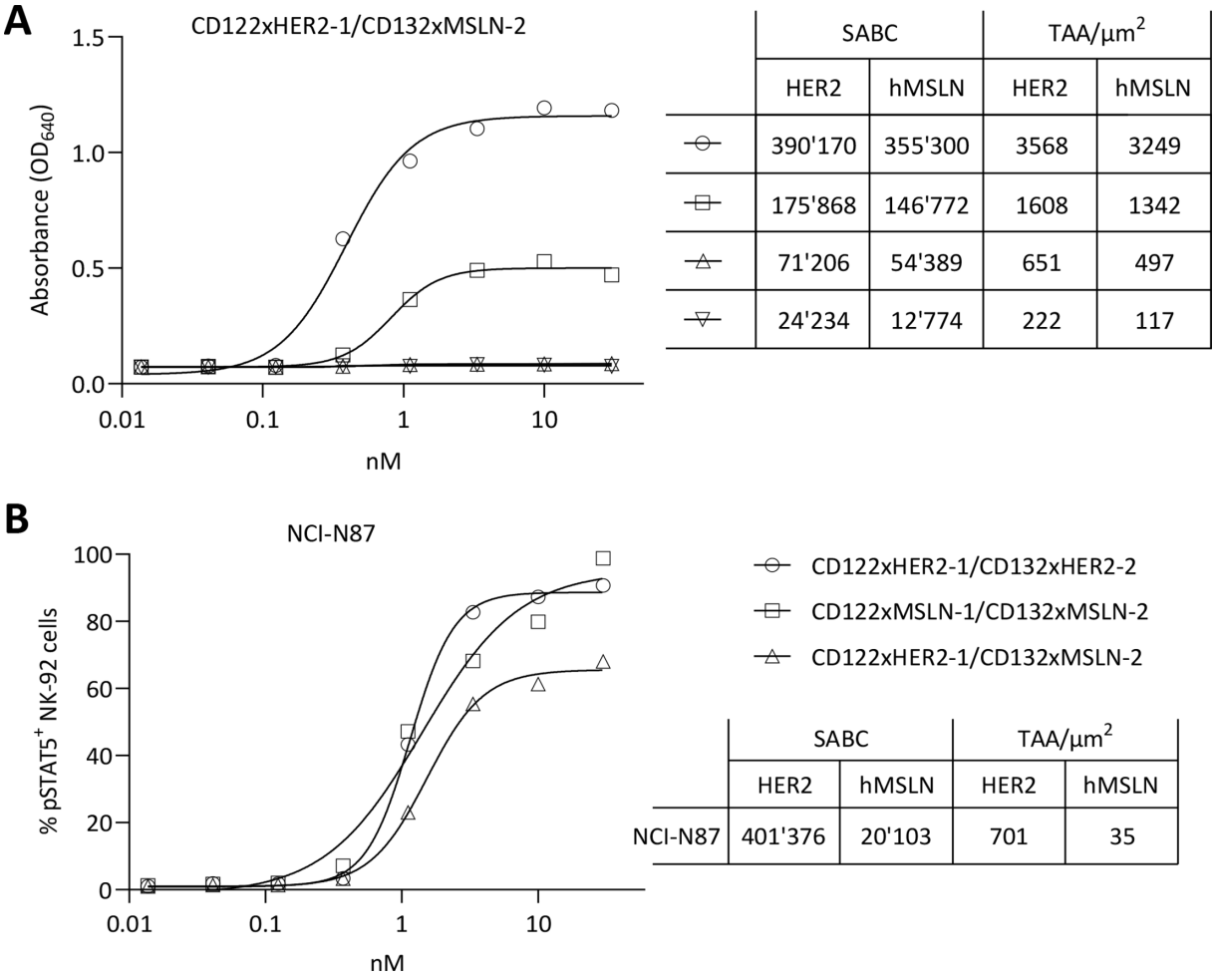
Figure 5 CD122/CD132×MSLN bsAb pair potently inhibits tumor growth in combination with anti-PD-1. Therapeutic efficacy was tested in hCD122/hCD132 transgenic mice engrafted with hMSLN-expressing MC38 cancer cells. (A–C) Mice were treated with vehicle (phosphate-buffered saline) (n=8), CD122×MSLN-1/CD132×MSLN-2 bsAb pair, anti-mPD-1 (n=7) or both anti-mPD-1 (n=6) and bsAb combination (n=8). Shown are average tumor volume (B) and Kaplan-Meier survival curves (C). Arrows indicate dosing of antibodies. Statistical analysis was performed using the log-rank (Mantel-Cox) test with Bonferroni correction comparison (*p<0.0125). (D–I) Analysis of tumor-infiltrating immune cells 9 days post-treatment initiation. Shown in the panels are absolute counts of $CD45^+$ immune cells (D), $CD8^+$ T cells (E), proportion of GZB^+ cells among $CD8^+$ T cells (F), $CD8^+$ T cells/regulatory T cells ratio (G), Ki67 MFI within NK cells (H) and absolute counts of NK cells (I) in tumors of treated mice. Data are represented as mean±SEM. Statistical analysis was performed using a one-way analysis of variance. bsAb, bispecific antibody; mPD-1, mouse programmed cell death protein 1; MSLN, mesothelin; NK, natural killer.

bsAb pairs targeting a single TAA (online supplemental figure S12 B). As expected, no activation was observed when using TAA-negative cancer cells (online supplemental figure S12C).

DISCUSSION

In this study, we described a strategy to restrict IL-2/IL-15 activation to the TME by using a pair of bsAbs, with one bsAb targeting CD122 and a TAA, the other bsAb targeting CD132 and the same TAA or a different TAA expressed on the same tumor cell. This approach allows a broad T-cell activation regardless of their T-cell receptor (TCR) specificity by stimulating tumor-specific and bystander infiltrating T cells, both of which may contribute to tumor cell killing when combining the bsAb pair with a T-cell engager bsAb. We demonstrated with HER2 and MSLN that this strategy activates IL-2/IL-15 signaling in a TAA-dependent manner. In contrast to Proleukin, the bsAb pair did not induce cytokine release in human blood nor lung edema in mouse models, confirming it as a safe strategy to harness IL-2/IL-15 activation inside the tumor. The strategy is likely to be expandable to other TAAs.

The bsAb pair does not induce IL-2R signaling without the presence of TAA, as the two bsAbs are unable to bring the IL-2R subunits together in the absence of TAA-expressing cells. This is different from the immunocytokine strategy where an active IL-2 molecule is ligated to a tumor targeting antibody,[18 19] which still induces systemic IL-2R activation although with preferred tumor accumulation. As antibodies have a much longer half-life than IL-2, the strategy also addresses the inconvenient dosing schedule issues facing the high-dose IL-2 therapy. In addition, the two bsAbs do not naturally associate with each other in the absence of TAA-expressing cells, they thus can be administered simultaneously in a clinical setting, which may not be possible for the split IL-2 strategy.[21]



	SABC		TAA/µm²	
	HER2	hMSLN	HER2	hMSLN
○	390'170	355'300	3568	3249
□	175'868	146'772	1608	1342
△	71'206	54'389	651	497
▽	24'234	12'774	222	117

	SABC		TAA/µm²	
	HER2	hMSLN	HER2	hMSLN
NCI-N87	401'376	20'103	701	35

Figure 6 BsAbs targeting different TAAs can be combined to further improve specificity. (A) Induction of reporter activity of HEK Blue CD122/CD132 reporter cells by a dose range of CD122×HER2-1/CD132×MSLN-2 bsAb pair in presence of microspheres coated with different quantities of both MSLN and HER2. (B) Dose-dependent induction of pSTAT5 in NK-92 cells by CD122×HER2-1/CD132×HER2-2, CD122×MSLN-1/CD132×MSLN-2 or CD122×HER2-1/CD132×MSLN-2 bsAb pairs following incubation with HER2 and MSLN expressing NCI-N87 cells. bsAb, bispecific antibody; HER2, human epidermal growth factor receptor 2; MSLN, mesothelin; NK, natural killer; pSTAT5, phosphorylated signal transducer and activator of transcription 5; SABC, specific antibody binding capacity; TAA, tumor associated antigen.

When pairing bsAbs targeting different epitopes on the same TAA, bsAbs targeting certain epitope combinations showed higher potency than targeting other combinations. It has been reported that targeting a membrane proximal TAA epitope by a bsAb bridging immune cells and tumor cells induced higher levels of immune cell activation than targeting a membrane distal epitope.[28–30] Although the activity of the bsAb pair is also affected by the TAA epitope, we did not see a clear correlation with the membrane proximity of the TAA epitopes, suggesting that CD45 exclusion may not be the underlying mechanism here. As binding by CD122×CD132 bsAbs induces alternative dimeric receptor geometries compared with IL-2,[13] it is of interest to investigate whether targeting different TAAs or different TAA epitopes by our strategy also induces different geometries of the CD122/CD132 dimer, which is out of scope of this study. The affinity of the different TAA binding arms could be another factor in addition to epitope position affecting the activity of the bsAb pairs.

Treatment with CD122/CD132×MSLN bsAb pair, but not Proleukin, induced a significant increase of central memory CD8⁺ T cells and NK cells in the TME of an engineered mouse tumor model. One possible explanation for the observed difference in central memory T cells is that the weaker signaling induced by the bsAb pair favors memory T-cell proliferation, as reported previously.[13 31] Alternatively, the in vivo biological effects of the CD122/CD132×MSLN bsAb pair could mimic those of IL-15, which is well-established for a role in promoting memory T cell and NK cell proliferation.[2] IL-15 is usually[25 27 32] trans-presented to CD122/CD132 dimer-expressing immune cells by IL-15Rα-expressing cells while IL-2 engages its receptor subunits through cis-binding.[2] As such, the trans-presentation of IL-15 could be geometrically similar to the trans-presentation of the CD122/CD132×TAA bsAb pair by TAA-expressing tumor cells to CD122/CD132

dimer-expressing immune cells. This geometrical similarity may be one factor underlying the similar biological effects of the CD122/CD132×TAA bsAb pair and IL-15. Upon IL-2-induced activation, CD122 and CD132 are known to be internalized as a negative feedback mechanism to downregulate signaling.[33] The trans-presentation mechanism may decrease the rate of CD122/CD132 internalization by ligating them to another cell and thus prolong signal activation induced by IL-15 and the bsAb pair, in contrast to IL-2. It could be interesting to assess whether the receptor dynamics and downstream signaling kinetics induced by the bsAb pair are similar to those induced by IL-15 instead of IL-2 in a separate study. Due to technical limitations, IL-2 signaling in vivo was not measured directly with pSTAT5, but with surrogate readouts such as T cell and NK cell activation/proliferation in this study, following a common practice in the field.[21 25] Further technical innovation allowing in vivo pSTAT5 tracking in real time will provide more insights into this field. Cis-binding to IL-2Rα, or other engineered strategies to guide IL-2 signaling to antigen-specific T cells have been demonstrated to be important to the in vivo efficacy of IL-2 targeting strategies,[25 27 32] which, together with the clinical failure of bempegaldesleukin,[34] casts doubts on the validity of the non-targeted not-alpha IL-2 strategy. However, the clinical success and subsequent approval by the Food and Drug Administration of N803,[35 36] an IL-15 superagonist, suggests that mimicking IL-15 biological effects locally in the tumor, although being not-alpha by nature, could be a valid antitumor therapeutic strategy. Similar to other not-alpha strategies, the bsAb pair also avoids preferential activation of Tregs versus $CD8^+$ T cells, in contrast to IL-2.

In addition to inducing immune activation in the TME, treatment with the CD122/CD132×MSLN bsAb pair also showed some signs of immune activation in mouse spleens and lungs. This could be due to recirculation of activated immune cells from the tumor to the circulation, or be mediated by sMSLN in circulation, which is reported in sera of patients with MSLN-expressing cancers,[37] and detected in sera of the tumor-bearing mice from this study. Nonetheless, the combination of sMSLN up to 400 nM (more than 20-fold higher than the levels detected in the tumor model) with the CD122/CD132×MSLN bsAb pair does not induce cytokine release in human whole blood samples from healthy donors. In agreement, the CD122/CD132×MSLN bsAb pair does not induce systemic cytokine release and does not induce lung edema in the tested mouse tumor model, in contrast to Proleukin. The results suggest that CD122/CD132×TAA bsAb pairs are safe even with soluble TAA in circulation.

We demonstrated that the bsAb strategy synergizes with checkpoint inhibitors in a mouse model, potentially by combining the immunological effects of anti-mPD-1 monotherapy, which increases effector and cytotoxic T cells, with the effects of the CD122/CD132×MSLN bsAb pair therapy, which promotes the expansion of NK cells and memory $CD8^+$ T cells. Our data showed that the bsAb combination can control tumor growth only when combined with PD-1 blockade. While our CD122/CD132×TAA bsAb pair activates IL2R signaling, it is less potent than WT IL-2 possibly due to its not-alpha nature and may be insufficient to control tumor growth on its own. The addition of PD-1 blockade likely enhances this effect by synergistically promoting T-cell activation and functionality within the TME. Several other published not-alpha IL-2 variants have also shown only modest or no efficacy in directly controlling tumor growth, but efficacy was achieved when combined with additional treatment modalities.[25 38] Due to observed effects on memory T cells and NK cells, the strategy may also be combined with other T-mediated and NK cell-mediated immunotherapies such as T/NK cell engagers, chimeric antigen receptor (CAR) T cells and antitumor mAbs. Combination with immune-stimulating strategies may be required to treat cold tumors lacking immune infiltration. The absence of mouse cross-reactivity of our targeting bsAbs and the usage of hCD122/hCD132 transgenic mice are a limitation of our study. Additional safety studies will be necessary before advancing to clinical trials.

Importantly, this strategy also proved effective when each bsAb in the pair targeted a different TAA. Since many TAAs are also expressed on healthy cells, although at lower levels, targeting two distinct TAAs with the bsAb pair could further restrict IL-2 activation to tumors co-expressing both TAAs, while sparing healthy cells that express only one. Looking ahead, this dual-targeting approach holds significant potential to enhance the specificity and safety of IL-2-based immunotherapies, offering a more precise way to target tumors while minimizing off-target effects on healthy tissues.

With both bsAb pairs targeting a single TAA and those targeting two distinct TAAs, we noticed that lower TAA densities on tumor cells than that on TAA-coated beads were required to activate IL-2R signaling. This could be explained by the better TAA mobility on cell membranes than on TAA-coated beads. In addition, we noticed that the bsAb pairs targeting two TAAs required higher TAA density than those targeting a single TAA, which could be due to the potential clustering of a single TAA to increase its local density and the possibility of engaging both bsAbs with a single TAA molecule. On top of TAA density, other factors, such as the membrane distribution and targeted geometry of TAAs on cancer cell surface, may also influence the overall agonistic activity of our CD122/CD132×TAA bsAb combination strategy. Addressing these pertinent questions would require additional specific experiments that fall outside the scope of the present study. We recognize the regulatory challenges of developing a combination of two bsAbs but believe

that the potential clinical benefits justify pursuing this approach.

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Patient consent for publication Not applicable.

Ethics approval In vivo experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and approved by the Cantonal Veterinary Office (protocol: #GE319).

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