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Selective targeting of engineered T cells using orthogonal IL-2 cytokine-receptor complexes

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Interleukin-2 (IL-2) is a cytokine required for effector T cell expansion, survival, and function, especially for engineered T cells in adoptive cell immunotherapy, but its pleiotropic nature of IL-2, which induces both immune stimulatory and suppressive T cell responses as well as potentially severe toxicities (5). This is governed by the interaction between IL-2 and the IL-2 receptor (IL-2R), which consists of α, β, and γ subunits (6). IL-2Rβ and the common γ-chain (IL-2Rγ) together form the signaling dimer and bind IL-2 with moderate affinity, whereas IL-2Rα (CD25) does not signal but increases the affinity of IL-2 for the binary (βγ) IL-2 receptor to sensitize T cells to low concentrations of IL-2. The activity of IL-2 as an adjuvant to ACT is dependent on the balance between activation of transplanted and endogenous T cell subsets bearing natural IL-2 receptors, as well as host responses that can cause dose-limiting toxicities. Strategies to overcome these limitations could improve T cell immunotherapy (7, 8). Recognizing the need for new approaches that afford precise targeting of IL-2-dependent functions to a specific cell type of interest, we devised a strategy to redirect the specificity of IL-2 toward adoptively transferred T cells. This method, based on receptor-ligand orthogonalization, uses a mutant IL-2 cytokine and mutant IL-2 receptor that bind specifically to one another but not to their wild-type counterparts (Fig. 1A).

We focused on the murine IL-2/IL-2Rβ interaction to enable in vivo characterization in syngeneic mouse models. The IL-2Rβ chain was chosen as the mutant receptor because the β chain is required for signal transduction and can bind IL-2 independently. We devised a two-step approach to engineer orthogonal IL-2/IL-2Rβ pairs informed by the crystal structure of the IL-2 high-affinity receptor complex (6) (Fig. 1B). First, point mutations of the IL-2Rβ chain were identified from inspection of the interface between IL-2 and IL-2Rβ that abrogated binding to wild-type IL-2 (Fig. 1C to E). The IL-2Rβ hotspot residues His334 and Tyr335 make numerous contacts with IL-2 that contribute a majority of the binding free energy between IL-2 and IL-2Rβ (6) (Fig. 1E). A double mutant IL-2Rβ [H334→Asp (H334D) and Tyr335→Phe (Y335F)], referred to herein as orthoIL-2Rβ, lacked detectable binding to IL-2 (Fig. 1D), even in the presence of CD25 (fig. S1) (7, 9).

Next, we used yeast display-based evolution to mutate, and thus remodel, the wild-type IL-2 interface region that was opposing (or facing the site of) the IL-2Rβ mutations in the crystal structure, in order to create a molecule that bound to orthoIL-2Rβ but not to wild-type IL-2Rβ. IL-2 residues in proximity to the orthoIL-2Rβ binding interface were randomly mutated and were chosen on the basis of a homology model of the mouse IL-2/IL-2Rβ complex (Fig. 1E) derived from the crystal structure of the human IL-2 receptor complex (6). A library of ~106 unique IL-2 mutants was displayed on the surface of yeast (fig. S2) and subjected to multiple rounds of both positive (against orthoIL-2Rβ) and negative (against IL-2Rβ) selection (figs. S2 and S3). This collection of yeast-displayed IL-2 mutants bound the orthoIL-2Rβ, but not wild-type IL-2Rβ, and retained CD25 binding (Fig. 1D). Sequencing of yeast clones from the evolved IL-2 libraries revealed a consensus set of mutations at IL-2 positions in close structural proximity to the orthoIL-2Rβ mutations (fig. S4). Interestingly, a Glu30→Asn (Q30N) mutation was highly conserved across three independent mutant IL-2 yeast libraries, whereas all other IL-2 positions used a restricted and specific mutational signature. We found that IL-2 mutations Q30N, Met33→Val (M33V), and Asp34→Leu or Met (D34L/M) appear to form a small nonpolar pocket to compensate for the IL-2Rβ Y35F mutation, whereas Glu36→Thr, Ser, Lys, or Gln (Q36T/S/K/E) and Glu37→Tyr or His (E37Y/H) mutations present a polar or charged surface to compensate for the IL-2Rβ H34D mutation (fig. 1F).

Because of the affinity-enhancing effects of CD25 expression on the interaction of IL-2 with the binary (βγ) IL-2 receptor (10), IL-2 mutants with negligible binding to IL-2Rβ alone may still form a functional signaling complex on cells that also express CD25 (8). Therefore, we used a yeast-based functional screen to further triage IL-2 mutants that bound specifically to orthoIL-2Rβ and signaled selectively on T cells that expressed the orthoIL-2Rβ (Fig. 1G and fig. S5), and produced recombinant forms of select IL-2 mutants (orthoIL-2) for characterization (figs. S6 to S8). We focused our efforts on two orthoIL-2 mutants, 1G12 and 3A10. OrthoIL-2 1G12 and 3A10 bound the orthoIL-2Rβ with an affinity comparable to that of the wild-type IL-2/IL-2Rβ interaction and displayed little to no detectable binding to wild-type IL-2Rβ (Fig. 1H and figs. S7 and S8) but differed in their ability to activate IL-2Rβ signaling in CD25-positive wild-type...
and orthoIL-2Rβ T cells. Stimulation of orthoIL-2Rβ T cells (fig. S3B) with orthoIL-2-1G12 resulted in dose-dependent phosphorylation of STAT5 (pSTAT5), a hallmark of IL-2R signaling, with potency similar to that of wild-type IL-2, but also induced pSTAT5 on wild-type T cells, albeit with significantly reduced potency relative to IL-2 (Fig. 1, G and I, and fig. S6). By comparison, orthoIL-2-3A10 was specific for orthoIL-2Rβ T cells, but with a weaker potency relative to IL-2 (Fig. 1, G and I, and fig. S6). We speculated that orthoIL-2-1G12 activity on wild-type T cells is a consequence of weak residual binding to wild-type IL-2Rβ (fig. S7). Low-affinity interactions with IL-2Rβ alone are enhanced in the presence of CD25 (8). Indeed, orthoIL-2-1G12 exhibited binding to wild-type IL-2Rβ when first captured by CD25, with limited binding in the absence of CD25 (figs. S1 and S8). OrthoIL-2-3A10 did not bind appreciably to IL-2Rβ even in the presence of CD25, in agreement with its negligible biological activity on CD25-positive T cells. Interaction of orthoIL-2-1G12 and 3A10 with orthoIL-2Rβ was significantly enhanced in the presence of CD25, with apparent binding affinities of the ternary CD25/orthoIL-2Rβ/orthoIL-2 complex that correlate with their respective potency on orthoIL-2Rβ T cells (fig. S1).

In clinical ACT regimens, patient-derived T cells for ACT are expanded in IL-2 before re-infusion in order to obtain sufficient numbers of therapeutic cells with the desired genotype/phenotype (2). We explored the in vitro activity of orthoIL-2 on activated primary mouse CD8+ T cells engineered to express the orthoIL-2Rβ and a yellow fluorescent protein (YFP) to distinguish modified (YFP+) and unmodified (YFP−) cells (Fig. 2A). The transcription factor STAT5 is phosphorylated upon IL-2 engagement with the IL-2R and translocates to the nucleus, where it promotes the proliferation and cell cycle progression of T cells (17). Wild-type IL-2 induced the phosphorylation of STAT5 (pSTAT5) in both wild-type and orthoIL-2Rβ CD8+ T cells with similar potency and signaling amplitude, indicating functional signal transduction through the wild-type receptor but not orthoIL-2Rβ (Fig. 2B). By comparison, orthoIL-2-1G12 potently activated STAT5 on orthoIL-2Rβ-transduced T cells, with a potency increase by a factor of ~5 relative to wild-type T cells. OrthoIL-2-3A10 induced somewhat weaker, albeit selective pSTAT5 on wild-type IL-2Rβ-expressing but not wild-type T cells (Fig. 2B and E). These results were consistent with the biased binding of the orthoIL-2Rβ to the orthoIL-2-3A10, which translated into the selective or specific expansion of orthoIL-2Rβ T cells cultured ex vivo in orthoIL-2-1G12 or 3A10, respectively (Fig. 2, C and D). The orthoIL-2Rβ-transduced T cells cultured in saturating concentrations of orthoIL-2-3A10 became enriched to near homogeneity after 3 to 5 days (Fig. 2F).

IL-2 is indispensable for the development and function of regulatory T cells (Treg) (22), which are sensitive to IL-2 as a result of constitutive expression of CD25 and require IL-2Rβ-dependent activation of STAT5 signaling for survival and function (23). Both orthoIL-2-1G12 and 3A10 re-
The selectivity of orthoIL-2 1G12 for orthoIL-2Rβ T cells was dose-dependent, with increased activity on wild-type cells at increased dose amounts and/or frequency of treatment (Fig. 3, B and C, and figs. S10 to S12). These results were consistent with the in vitro selectivity of orthoIL-2 1G12, although it remained possible that orthoIL-2 1G12 signaling through the orthoIL-2Rβ could trigger endogenous IL-2 production by the orthoIL-2Rβ T cells, leading to indirect signaling through the wild-type IL-2R in cis or trans.

At high doses and twice-daily administration, orthoIL-2 3A10 resulted in the substantial expansion of orthoIL-2Rβ T cells with high specificity and no wild-type T cell expansion (Fig. 3, B and C, and figs. S11 and S12). This finding suggests that the effects of high-dose orthoIL-2 1G12 treatment were due not to induction of endogenous IL-2 by orthoIL-2Rβ T cells, but rather to low-level cross-reactivity with the wild-type IL-2Rβ by this molecule. The orthoIL-2 variants also promoted the in vivo expansion of orthoIL-2Rβ CD4+ effector T cell (Teff) (Fig. 3I and fig. S12) and orthoIL-2Rβ CD4+ Teff (fig. S9, C and D) cell subsets with specificity similar to that in CD8+ T cells.

The two different orthoIL-2 variants exhibited specificities in vivo that mirrored their relative specificities in vitro. Despite its ability to activate wild-type IL-2Rβ signaling, albeit with about one order of magnitude less potency than orthoIL-2Rβ signaling, orthoIL-2 1G12 administration was relatively specific for orthoIL-2Rβ T cells in vivo (Fig. 3, B to H, and figs. S10 to S12). In mice treated twice daily with orthoIL-2 1G12 only, CD4+ T cells were elevated to a substantially lower degree than observed in IL-2–treated mice (Fig. 3F). However, the orthoIL-2 3A10 variant, consistent with the lack of wild-type IL-2Rβ signaling, had no detectable activity on host cell subset numbers (fig. S11) or expression of CD25, PD-1, and TIM-3, which are up-regulated by early or late IL-2R signaling (fig. S13).

To improve in vivo half-life and enable more convenient dosing, we fused IL-2 and orthoIL-2 to mouse serum albumin (17) (MA), which has been shown to extend the half-life of mouse IL-2 from 5 hours to 50 hours (18). Fusion to MA had little to no impact on IL-2– or orthoIL-2–dependent T cell proliferation in vitro (fig. S14); however, the in vivo activity was greatly enhanced. Fusion of
**Fig. 3. OrthoIL-2 promotes the specific expansion of orthoIL-2Rβ-modified T cells in mice with negligible toxicity.** (A) Schematic of the adoptive CD8+ T cell transplant mouse model. (B) Quantification of donor wild-type and ortho CD8+ T cells in the spleen of recipient mice treated twice daily with phosphate-buffered saline (PBS), IL-2 (250,000 IU/dose), orthoIL-2 1G12 (250,000 IU/dose), or orthoIL-2 3A10 (2,500,000 IU/dose). (C) Representative flow cytometry data quantified in (B) depicting donor (Thy1.1’) wild-type (YFP+) and orthoIL-2Rβ (YFP+) CD8+ T cells in the spleen of recipient mice. (D) Spleen weight of mice treated in (B) normalized to total body weight on day of killing. (E to G) Quantification of exogenous cytokine administration on host (E) CD8+ memory phenotype T cell (MP, CD44+CD62L+), (F) CD4+ Treg (CD25Foxp3+), and (G) natural killer (NK) cell (CD3-NK1.1’CD49b’) numbers in the spleen of mice treated in (A). (H) Representative flow cytometry data as quantified in (F) and (G). Data in (B) to (H) are means ± SD (n = 5 mice per group). *P < 0.05, ****P < 0.0001 [analysis of variance (ANOVA)]; ns, not significant. (I) Quantification of donor wild-type and orthoIL-2Rβ CD4+ T cells in the spleen of recipient mice treated once daily with PBS, IL-2 (250,000 IU/dose), or orthoIL-2 1G12 (1,000,000 IU/dose). Data are means ± SD and are representative of two independent experiments (n = 4 mice per group). *P < 0.05, ****P < 0.0001 (ANOVA). (J) Survival of mice that received a mixture of wild-type and orthoIL-2Rβ CD8+ T cells following daily administration of IL-2 or orthoIL-2 fused to MSA. All mice received a total of 250,000 IU/day of the respective MSA fusion protein on an IL-2 basis for 5 days. (K) Mouse body weight over time normalized to the group average on day 0 as treated in (J). (L) Platelet counts in peripheral blood on day 4 as treated in (J). Data in (J) to (L) are means ± SD (n = 5 mice per group). ****P < 0.0001 (ANOVA). (M to O) Quantification of cytokine administration on host (M) CD8+ and (N) CD4+ T cell production of IFN-γ upon ex vivo restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. (O) Representative flow cytometry data as quantified in (M) and (N). (P and Q) Serum (P) IFN-γ and (Q) IL-5 concentrations on day 7 in mice treated daily with PBS or with MSA-IL-2, MSA-1G12, or MSA-3A10 (each 25,000 IU/dose) for 7 days. Data are means ± SD (n = 5 mice per group). ****P < 0.0001 (ANOVA).

**OrthoIL-2Rβ to MSA substantially increased its activity on cells that express the wild-type IL-2R relative to native orthoIL-2 R1G12, leading to increased off-target effects and toxicity (fig. S15). However, the MSA-orthoIL-2 3A10 fusion protein retained unique specificity for orthoIL-2Rβ T cells (fig. S16). One of the major limitations of IL-2 in the clinic is that IL-2 toxicity limits the use of high-dose IL-2 therapy for metastatic cancer and as an adjuvant to adoptive T cell therapy (12). IL-2 administered as a MSA fusion resulted in a number of dose-dependent and dose-accumulating toxicities that led to weight loss, restricted mobility, hypothermia, ruffled fur, hunched posture, splenomegaly, lymphomegaly, and death (Fig. 3, J to L, and figs. S15 to S18). In contrast, MSA-orthoIL-2 3A10 was nontoxic at all doses evaluated. MSA-orthoIL-2 3A10 activity was negligible on all IL-2-responsive host cell subsets evaluated. In addition to its role as a proliferative cytokine, IL-2 is a potent effector cytokine capable of activating cytotoxic T cell functions and T cell inflammatory pathways (19). We determined the capacity of adoptively transferred orthoIL-2Rβ CD8+ T cells to produce interferon-γ (IFN-γ) and cell surface levels of the immune inhibitory receptors PD-1 and TIM-3 after expansion in vivo with orthoIL-2. TIM-3 expression correlates with a highly dysfunctional CD8+ T cell state, whereas PD-1 expression is associated with both T cell activation and exhaustion (20). OrthoIL-2Rβ T cells expanded in orthoIL-2 produced significantly more...**
OrthoIL-2−expanded T cells retain effector function and promote an antitumor response against syngeneic B16-F10 tumors in mice. (A) Quantification of total number of IFN-γ-positive wild-type or orthoIL-2Rβ CD8+ T cells recovered from the spleen as treated in Fig. 3 (left) and representative flow cytometry data (right). (B) Cell surface expression levels of PD-1 (left) and TIM-3 (right) on wild-type and orthoIL-2Rβ CD8+ T cells in the spleen after administration of the indicated cytokines. Data are means ± SD (n = 5 mice per group). *P < 0.05, ****P < 0.0001 (ANOVA). (C) gp100 pMHC tetramer staining of orthoIL-2Rβ-transduced pmel-1 transgenic CD8+ T cells. (D) In vitro cytotoxicity of orthoIL-2Rβ pmel-1 transgenic T cells against antigen-positive (B16-F10) but not antigen-negative (MC38) tumor cells at a 2:1 (ET) ratio. Data are means ± SD (n = 3 biological replicates). **P < 0.01 (Student’s t test). (E and F) Tumor growth (E) and survival (F) of C57BL/6J mice bearing subcutaneous B16-F10 tumors treated with wild-type (wt T) or orthoIL-2Rβ pmel-1 transgenic CD8+ T cells (ortho T) and IL-2 or orthoIL-2 1G12. Data are means ± SEM (n = 5 mice per group). ****P < 0.0001 (two-way ANOVA). (E): **P < 0.01 (log-rank test) (F). (G and H) Tumor growth (G) and survival (H) of C57BL/6J mice bearing subcutaneous B16-F10 tumors treated with wild-type (wt T) or orthoIL-2Rβ pmel-1 transgenic CD8+ T cells (ortho T) and IL-2 or orthoIL-2 3A10 fused to MSA. Data are means ± SEM (n = 4 mice per group). ****P < 0.0001 (two-way ANOVA) (G); **P < 0.01 (log-rank test) (H).
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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S18

References (29–35)

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Selective targeting of engineered T cells using orthogonal IL-2 cytokine-receptor complexes


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Engineering cytokine-receptor pairs

Interleukin-2 (IL-2) is an important cytokine that helps T cells destroy tumors and virus-infected cells. IL-2 has great therapeutic promise but is limited by toxic side effects and its capacity to both activate and repress immune responses. Sockolosky et al. set out to improve IL-2-based immunotherapy by engineering synthetic IL-2–receptor pairs (i.e., IL-2 and its receptor, IL-2R) (see the Perspective by Mackall). Engineered complexes transmitted IL-2 signals but only interacted with each other and not with endogenous IL-2/IL-2R. Treatment of mice with IL-2 improved the ability of engineered T cells to reject tumors with no obvious side effects. This type of approach may provide a way to mitigate toxicities associated with some cytokine-based immunotherapies.

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