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ORIGINAL RESEARCH

Human and murine IL2 receptors differentially respond to the human-IL2 component of immunocytokines

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ABSTRACT

The humanized immunocytokine, hu14.18-IL2 (ICp), leads to the immune cell-mediated destruction of GD2-expressing tumors in mouse models, resulting in potent antitumor effects with negligible IL2-related toxicity. In contrast, when ICp is used clinically, antitumor activity is accompanied by dose-limiting IL2-related toxicities. These species-specific differences in ICp toxicity may be linked to differential binding to mouse vs. human IL2 receptors (IL2Rs). We evaluated immunocytokines genetically engineered to preferentially bind either high-affinity $\alpha\beta\gamma$-IL2Rs or intermediate-affinity $\beta\gamma$-IL2Rs. These ICs have the IL2 fused to the C-terminus of the IgG light chains rather than the heavy chains. We found that IC35, containing intact huIL2, maintained activation of human and mouse $\alpha\beta\gamma$-IL2Rs but exhibited a 20-fold reduction in the ability to stimulate human $\beta\gamma$-IL2Rs, with no activation of mouse $\beta\gamma$-IL2Rs at the concentrations tested. The reduced ability of IC35 to stimulate human $\beta\gamma$-IL2Rs (associated with IL2-toxicities) makes it a potential candidate for clinical trials where higher clinical IC doses might enable better tumor targeting and increased antitumor effects with less toxicity. Contrastingly, ICSK (IC with an IL2 mutein that has enhanced binding to the IL2R β-chain) showed increased activation over ICp on mouse $\beta\gamma$-IL2Rs, with a dose-response curve similar to that seen with IC35 on human $\beta\gamma$-IL2Rs. Our data suggest that ICSK might be used in mouse models to simulate the anticipated effects of IC35 in clinical testing. Understanding the differences in species-dependent IL2R activation should facilitate the design of reagents and mouse models that better simulate the potential activity of IL2-based immunotherapy in patients.

Abbreviations: $\alpha\beta\gamma$-IL2R, high affinity IL2 receptor; $\beta\gamma$-IL2R, intermediate affinity IL2 receptor; GD2, disialoganglioside expressed on tumors of neuroectodermal origin; Hu, human; ICp, hu14.18-IL2 (humanized immunocytokine with IL2 fused to the heavy chains); IL2R, interleukin-2 receptor; IC, immunocytokine (fusion protein combining tumor reactive antibody with an immune activating cytokine); Mu, mouse; SK, superkine (mutated IL2 molecule with enhanced binding to the IL2R β-chain)

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Introduction

Therapy with an anti-disialoganglioside (GD2) reactive mAb, in combination with IL2 and GM-CSF, provides clinical benefit to some patients with GD2+ tumors.[1] The hu14.18-IL2 immunocytokine (ICp) is a fusion protein that combines a human IL2 molecule to each of the heavy chains of the intact humanized form of an anti-GD2 monoclonal antibody (hu14.18 mAb).[2] Throughout this manuscript, hu14.18-IL2 is referred to as the parent immunocytokine (ICp), since this is the original immunocytokine construct that has been modified to give rise to the novel immunocytokines studied in this report. ICp was created with two major goals: 1) to selectively target malignancies through the recognition of GD2 expressed on the surface of tumor cells, leading to the recruitment and activation of immune cells via engagement of Fc and/or IL2 receptors (IL2Rs); and 2) to reduce systemic IL2 effects by concentrating IL2 at the tumor site and better activating tumor-infiltrating immune cells.[3] When tested in mice, ICp demonstrated superior antitumor effects compared to equivalent amounts of the hu14.18 mAb and IL2 administered simultaneously as separate agents.[4-7] Interestingly, mice treated with ICp did not exhibit IL2-related toxicity, providing promising results for translation into clinical use. However, ICp therapy in patients was associated with dose-limiting effects related to IL2 toxicities, suggesting that there are differences in the ICp dose-related toxicity relationships with mouse vs. human IL2Rs.[8-11]

The IL2R family is made up of three separate subunits: α-, β-, and γ-chains, which non-covalently associate to form IL2Rs of

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varying affinity. When all three chains associate, they form the high-affinity receptor ($\alpha\beta\gamma$-IL2R); the intermediate affinity receptor ($\beta\gamma$-IL2R) is composed of just the β- and γ-chains.[12] Evidence suggests that the mechanisms of toxicity and efficacy achieved with IL2 can be therapeutically separated by selectively activating immune cells through their high-affinity IL2Rs, while reducing the activation of intermediate-affinity IL2Rs.[13-15] Engineered IL2 muteins with enhanced selectivity for cells expressing $\alpha\beta\gamma$-IL2Rs (vs. cells with $\beta\gamma$-IL2Rs) have been shown to induce comparable antitumor effects to wild-type human IL2 but with dramatically less IL2 toxicity in mice, monkeys, and chimpanzees.[16,17] A recent phase I dose-escalation study with an IC bearing an IL2 mutein with high specificity for cells with $\alpha\beta\gamma$-IL2Rs (initially developed to be combined with chemotherapy and/or radiation) demonstrated an improved tolerance and overall favorable safety profile in patients with advanced solid tumors.[18] Nevertheless, patients treated with this IC specific for $\alpha\beta\gamma$-IL2Rs showed long-term stable disease as best response but did not show any objective tumor responses by RECIST criteria,[18] suggesting that some level of $\beta\gamma$-IL2R activation may be required to mount an effective antitumor immune response using monotherapy.

In this study, we investigated the differences between human (hu) and mouse (mu) IL2R activation using novel ICs designed to exhibit varying degrees of selectivity for $\alpha\beta\gamma$-IL2R and $\beta\gamma$-IL2Rs. These new generation ICs have IL2 fused to the C-terminus of the light chains of the ch14.18 mAb rather than on the heavy chains.[19] These novel ICs were constructed using the ch14.18 (chimeric anti-GD2 mAb) rather than hu14.18 (humanized anti-GD2 mAb) due to patent limitations on the humanized mAb. Importantly, the chimeric mouse framework regions on ch14.18 do not change the function of the mAb and both the chimeric and humanized mAbs bind GD2 with the same affinity. Three new constructs were used in this study: IC35 with intact IL2 molecules, IC45 with a truncated form of IL2 (missing five amino acids), and ICSK with a mutated IL2 "superkine" (SK) protein[20] with enhanced affinity toward the IL2R β-chain (Fig. 1 and Table S1). In the three-dimensional conformation of these molecules (Fig. S1), the IL2 molecules within the ICp located at the end of the IgG heavy chains are exposed and available for interaction with IL2Rs. In contrast, for these new ICs with the IL2 molecules located at the end of the IgG light chains, the N-terminus of IL2 is adjacent to the C-terminal cysteine of the light chain which, in turn, is covalently linked to the hinge region of the IgG heavy chain. Therefore, the IL2 molecules are somewhat "tucked in" to the three-dimensional structure and thus are not easily accessible for interaction with IL2Rs. This modification creates a sequestering effect in IC35 and IC45 that results in less efficient binding of the IL2 to $\beta\gamma$-IL2Rs, limiting access to a critical contact residue of IL2 by the β-chain, while maintaining binding and activation of the $\alpha\beta\gamma$-IL2R.[19] On the other hand, ICSK, containing the mutated IL2-SK molecule,[20] which has enhanced binding to IL2Rβ, is able to overcome the sequestering effect and rescues the ability of ICSK to activate $\beta\gamma$-IL2Rs despite the IL2-SK being fused to the light chains of the mAb.

Previous work demonstrated that an anti-CD20-IL2 immunocytokine, which has IL2 located on the IgG heavy chains, activated both human $\alpha\beta\gamma$-IL2Rs and $\beta\gamma$-IL2Rs to the same extent as soluble IL2,[21] and had potent antitumor activity *in vivo* in T-cell deficient mice bearing human CD20+ tumor xenografts. However, this same anti-CD20-IL2 showed significantly less activation of mu$\beta\gamma$-IL2Rs when compared to soluble huIL2. A better understanding of the functional differences of the mouse and human IL2Rs may contribute to the development of IL2-based treatments in mouse models that will more accurately enable translation into the clinical setting.

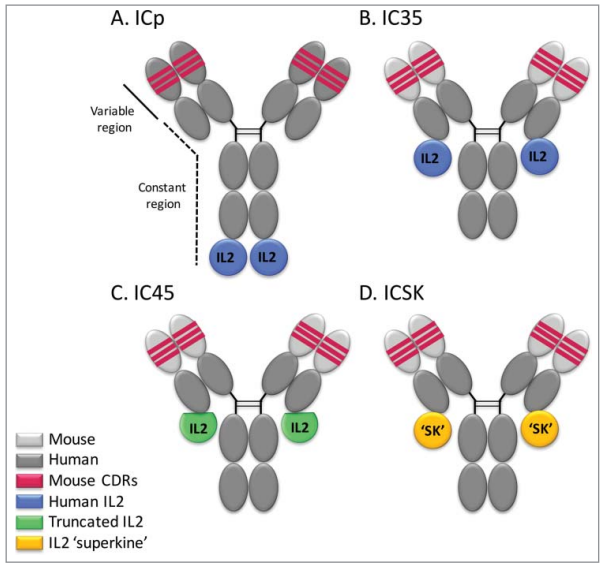

Figure 1. Schematic representation of hu14.18-IL2 immunocytokines. (A) Parental immunocytokine (ICp) with original intact hulL2 bound to the H-chains of the hu14.18 mAb. (B) IC35, with hulL2 bound to the L-chains of the ch14.18 mAb. (C) IC45, with a hulL2 variant missing 5 a.a. bound to the L-chains of the ch14.18 mAb. (D) ICSK, with the IL2 superkine mutein exhibiting enhanced binding to IL2Rβ bound to the L-chains of the ch14.18 mAb. Dark gray portion represents the 98% human portion of the hu14.18 mAb that recognizes GD2. Light gray portion represents the mouse framework in the chimeric mAb. Red portion represents the 2% mouse protein remaining in the antigen-binding region.

Prior *in vitro* and *in vivo* studies of ICs in mice suggest that antitumor efficacy with acceptable toxicity is obtained using an IC that has full activity in activating mu$\alpha\beta\gamma$-IL2Rs, with attenuated (but not absent) capability of activating mu$\beta\gamma$-IL2Rs, such as is the case with ICp. In contrast, ICp induces dose-dependent IL2-related toxicity *in vivo* in human patients after intravenous administration, due apparently to its more potent ability to activate hu$\beta\gamma$-IL2Rs than mu$\beta\gamma$-IL2Rs. Therefore, we are interested in creating and developing, for clinical use, an IC that retains signaling abilities through hu$\alpha\beta\gamma$-IL2Rs but has decreased ability to activate hu$\beta\gamma$-IL2Rs. We predict that the

Table 1. IL2 bioactivity in the presence of anti-IL2Rα and/or anti-IL2Rβ inhibitory mAbs.

	EC_{50} (IL2 ng/mL)			
Cells	IL2	IL2+ anti-IL2Rα	IL2+ anti-IL2Rβ	IL2+ anti-IL2Rα+ anti-IL2Rβ
Kit225	0.71	9.43	0.98	ND
Tf-1β	9.97	9.52	ND	NT
CTLL-2	1.35	3.61	1.69	ND
SCID splenocytes	35.74	34.79	ND	NT

EC_{50} values are the average of two–three separate experiments. ND: Not determined (proliferation curve did not reach plateau, leading to ambiguous EC_{50} values that were not considered in the calculation). NT: Not tested (no data were collected for these conditions).

activity in humans of an IC with these characteristics will resemble the functional behavior of anti-CD20-IL2 IC or ICp in mice (potent efficacy with little toxicity), potentially serving as a novel therapeutic strategy for treating patients and achieving antitumor benefits, while minimizing adverse side effects.

Here, we report that IC35 is a promising candidate for that purpose since it retains the activation of hu$\alpha\beta\gamma$-IL2Rs but exhibits significantly reduced ability to stimulate hu$\beta\gamma$-IL2Rs. Additionally, ICSK strongly activates mu$\alpha\beta\gamma$-IL2Rs and retains some level of attenuated activation of mu$\beta\gamma$-IL2Rs, showing that the use of the IL2-SK in creating this IC makes ICSK a potential surrogate molecule that will better represent in mice the level of hu$\alpha\beta\gamma$ and hu$\beta\gamma$-IL2R activation that we expect and desire to see in patients with an agent like IC35.

Materials and methods

Cell culture

The Kit225[22] human T-cell line was obtained from M. Tsudo in Kyoto University (Kyoto, Japan). The Tf-1β cell line was generated in our lab by transfecting the Tf-1 human myelomonocytic cell line (kindly provided by T. Kitamura, University of Tokyo, Tokyo, Japan) with a retroviral vector containing the IL2Rβ gene.[23] The CTLL-2[24] mouse T-cell line was obtained from the American Type Culture Collection (ATCC, Cat.TIB-214). Cell lines were grown in suspension using RPMI 1640 (Corning Cellgro, Cat.10-040-CV) complete medium supplemented with 10% FBS (GE Healthcare Hyclone, Cat.SH30910.03), 2 mM L-glutamine (Corning Cellgro, 25-005-CI), and 100 U/mL of penicillin/streptomycin (Corning Cellgro, 30-001-CI) at 37°C in a humidified 5% CO2 atmosphere. Recombinant human IL2 was added at a final concentration of 50 IU/mL for maintenance of Kit225 and CTLL-2, and at 100 IU/mL for maintenance of Tf-1β.

Recombinant IL2

Recombinant human interleukin-2 (rIL2, Hoffmann La Roche) was provided through the National Cancer Institute BRB Preclinical Repository (Rockville, MD). The NCI-BRMP standard for unit dosage was used, and the specific activity of the IL2 was 15×10^6 units/mg. This unit corresponds closely with the international standard of IL2 unitage.[25]

Immunocytokines (ICs)

Humanized hu14.18-IL2 (ICp) (APN301, Apeiron Biologics, Vienna, Austria) was supplied by the NCI Biologics Resources Branch (Frederick, MD) via a collaborative relationship with Merck KGaA (Darmstadt, Germany) and Apeiron Biologics (Vienna, Austria). ICp is an immunocytokine consisting of intact human IL2 genetically linked to the C-termini of each human IgG1 H chain of the hu14.18 mAb.[2] IC35, IC45, and ICSK were constructed by fusing a synthetic sequence coding for human or variant IL2 to the coding sequence of the human kappa light chain at the C-terminal cysteine. The constructs also contained the V regions of 14.18, the mouse anti-GD2 mAb.[19] IC35 contains the intact human IL2 molecule, whereas IC45 has a modified human IL2 molecule with a deletion of residues 5–9. ICSK contains a mutant IL2, also called "SK," with enhanced binding to the IL2R β-chain.[20] IC35, IC45, and ICSK were produced and provided by Provenance Biopharmaceuticals (Carlisle MA). To compare the IL2 activity in the soluble IL2 and different IC preparations, IL2 concentrations of IC solutions were based on weight/volume calculations of IL2 content. Since the ICs consist of 80% hu14.18 mAb and 20% IL2 by molecular weight, the concentrations were based on IL2 comprising 20% of the weight of the ICs. Thus, 50 ng/mL of IC would correspond to 10 ng/mL of IL2 within the IC.

Antibodies

MikB1, a mouse IgG2a inhibitory mAb directed against human CD122 (IL2Rβ), was kindly provided by M. Tsudo (Unitika Central Hospital, Kyoto, Japan).[26] GL439, a mouse IgG1 inhibitory mAb directed against human CD25 (IL2Rα), was provided by R. Robb.[27,28] Inhibitory antibodies against mouse IL2Rα [clone: PC61,[29] Rat IgG1 isotype, Cat.553864] and mouse IL2Rβ [clone:TM-β1,[30] Rat IgG2b isotype, Cat.553359] were purchased from BD Biosciences.

Splenocyte isolation from SCID mice

Female CB-17 SCID mice (7–8 week old) were obtained from Taconic Farms (Germantown, NY). Mice were housed in the University of Wisconsin-Madison animal facilities at the Wisconsin Institutes for Medical Research. All experimentations were performed in accordance to protocols approved by the National Institutes of Health and by the Animal Care and Use Committees of UW-Madison. For splenocyte isolation, spleens were removed from SCID mice immediately after death and placed in RPMI-1640 complete medium, supplemented with 1x MEM non-essential amino acids (Corning Cellgro, Cat.25-025-CI), 10 mM HEPES (Corning Cellgro, Cat.25-060-CI), 1 mM sodium pyruvate (Corning Cellgro, 25-000-CI), and 0.5 mM β−2-mercaptoethanol (Sigma Chemical, M-6250). The spleens were dissociated between the frosted ends of two microscope slides, and erythrocytes were lysed by brief hypotonic shock using cold water. Splenocytes were washed with media, resuspended, and counted using eosin dilution on a hemocytometer.

Proliferation assays

Cells were cultured for 72 h in round-bottom 96-well plates at a density of 5×10^3 cells/well for Kit225, CTLL-2, and Tf-1β or 5×10^4cells/well for SCID splenocytes in the presence of serial dilutions of IL2 or the different immunocytokines in a total volume of 200 μL. For the final 6 h, ^{3}H-thymidine (1 mCi/mL; Perkin Elmer, Cat.NET027A005MCI) was added to the wells in 50 μL of media at a final concentration of 1 μCi/well. ^{3}H-TdR-incorporation was determined using total cells harvested from the cell cultures onto glass fiber filters (Packard, Meriden, CT), using the Packard Matrix 9600 Direct β-counter (Packard, Meriden, CT). Results are presented as counts per 5 min for triplicate wells ± SD. For experiments in which mAbs were used to block the α- or β-chain of the IL2R, cells were incubated with the blocking mAbs at 10 μg/mL for 30–60 min at 4°C prior to plating with the IL2 sources. The median effective

dose (EC_{50}) value for each protein with respect to cell proliferation was obtained using GraphPad Prism by plotting the dose response curve and analyzing using nonlinear regression to identify the protein concentration that resulted in half-maximal response. For some experiments, the curves obtained for certain treatments did not exhibit a maximal response (top plateau), thereby preventing the program from fitting a unique curve through the data and leading to ambiguous EC_{50} values. Therefore, the EC_{50} values summarized in Tables 1–5 were calculated by averaging only the EC_{50} values obtained from properly fitted curves. The number of determinations used for this calculation is detailed under each table.

Flow cytometry detection of IC binding to IL2Rs

For the detection of IC binding to IL2Rs on the cell surface via the IL2 molecules of the IC, 2–5 × 10^5 Kit225, CTLL-2, SCID splenocytes, or Tf-1β cells were incubated with increasing concentrations of the ICs for 30–60 min at 4°C and the Alexa Fluor 647 goat anti-human IgG mAb (polyclonal, Invitrogen, Cat. A-21445) was used for secondary detection of IL2R-bound ICs. To reduce Fc receptor-mediated binding by the ICs, SCID splenocytes were pre-incubated (1 μg/1 × 10^6 cells) with anti-mouse CD16/CD32 (Mouse BD Fc block, clone: 2.4G2, BD PharMingen, Cat. 553142) for 15–30 min at 4°C. The ICs were tested in a range of concentrations similar to the concentrations used on those same cells for the proliferative assays. IC were added directly to pre-incubated cells without washing the Fc blocking mAb. Data [mean fluourescence intensity (MFI)] were acquired on the MACSQuant Analyzer 10 flow cytometer (Militenyi Biotec Inc. Auburn, CA) and analysis was performed using the FlowJo v10 software (FlowJo LLC., Ashland. OR).

Assessment of STAT5 phosphorylation by flow cytometry

IL2 responsive cells were starved by washing away IL2 used in culture and incubating at 37°C in complete media overnight. Cells were stimulated with increasing concentrations of IL2, ICp, IC35, or ICSK for 15 min at 37°C and then fixed with 1.5% paraformaldehyde (Polysciences, Inc., Cat.18814) for 10 min at room temperature. Cells were permeabilized with 100% ice-cold methanol (Fisher Scientific, Cat.A433P-4) for 30 min at 4°C and then washed and stained with FITC-labeled anti-STAT5-pY694 (Clone:SRBCZX, eBioscience, Cat.11-9010-42) for 30 min at room temperature. Intracellular staining was assessed by flow cytometry detection on the MACSQuant analyzer.

Ex vivo NK cell expansion

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from healthy donors by ficoll density gradient centrifugation. Freshly isolated PBMCs were co-incubated with (100 Gy) irradiated K562-mbIL15-41BBL cells (obtained from Dr. Dario Campana, formerly from St. Jude Children's Research Hospital, Memphis TN),[31] which were mixed at a 1:1 ratio (5 × 10^6cells/each) in 10 mL RPMI with 100 IU/mL IL2. Mixtures of PBMC and K562-mbIL15-41BBL cells were incubated at 37°C on a rocker at a constant speed. Fresh IL-2 supplemented RPMI was added every 2–3 d to the culture. All cells in the expansion cultures were harvested between expansion days 12–15 for CD3 and CD56 staining to determine NK cell percentage followed by functional analyses in the cytotoxicity assay.

Antibody-dependent cell-mediated cytotoxicity assay

M21, human melanoma cells expressing GD2, were used as target cells and labeled with ^{51}Cr (Perkin Elmer, Cat. NEZ030010MCI) for 1.5 h. PBMCs that were stimulated overnight with 100 IU/mL IL2 or expanded NK cells were plated in round bottom 96-well plates and ICp, IC35, or ICSK were added for a final concentration of 3000 IU/mL IL2 per well. ^{51}Cr-labeled M21 cells were added at various effectors to target (E:T) ratios and cells were co-incubated for 4 h at 37°C. Supernatants were harvested at the end of incubation using the Skatron harvesting system (Skatron, McLean, VA) and read by a gamma counter (Packard, Meriden, CT). Percent cytotoxicity was calculated as 100 × [(experimental release-spontaneous release)/(Maximum release – spontaneous release)].

Interferon-γ secretion by SCID splenocytes

Freshly isolated SCID splenocytes were plated at a density of 5 × 10^4 cells/well in round-bottom 96-well plates and incubated at 37°C for 72 h in the presence of serial dilutions of IL2 or the different immunocytokines in a total volume of 200 μL. On day 3, supernatants were harvested and secreted protein levels were measured using the BD™ CBA Mouse Inflammation Kit (BD Biosciences, Cat.552364). Data were acquired on the BD FACSCalibur and analysis was performed using the BD FACSArray software, both from BD Biosciences, San Jose, CA.

Statistical analyses

Cell proliferation counts and stimulation indices were evaluated for different treatment groups within various cell lines. Proliferation counts and stimulation indices were summarized using means and standard deviations (SD). Two-way ANOVA was used to compare proliferation counts between treatment groups within doses and to compare stimulation indices between treatment groups. The Bonferonni method was used to adjust for multiple treatment comparisons. *p*-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SAS v9.4 (SAS Institute, Cary, NC). Statistically significant differences of biological values are indicated in graphs as described in the figure legends.

Results

IL2-dependent cell lines and SCID splenocytes serve as an in vitro system to study high and intermediate affinity IL2Rs

To investigate the ability of the IL2 molecules within the new ICs to activate high ($\alpha\beta\gamma$) or intermediate affinity ($\beta\gamma$) IL2Rs, we established an *in vitro* system using mouse and human IL2-dependent cell lines expressing either $\alpha\beta\gamma$- or $\beta\gamma$-IL2Rs. For

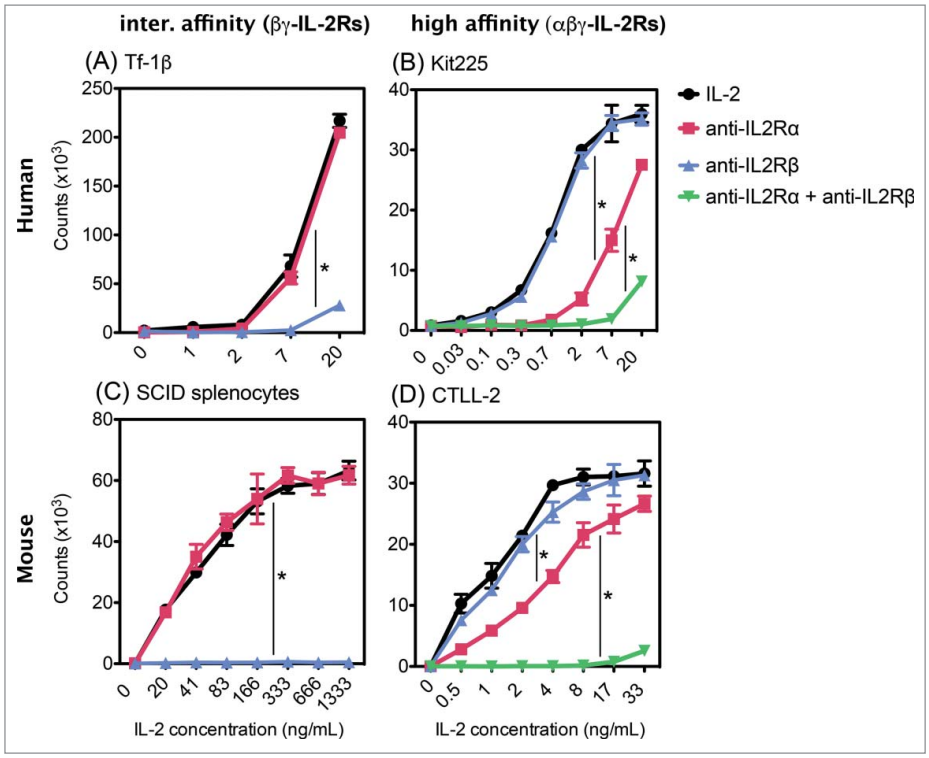

Figure 2. Effect of anti-IL2Rα and anti-IL2Rβ inhibitory mAbs on IL2 induced proliferation. (A) Tf-1β, (B) Kit225, (C) SCID splenocytes, or (D) CTLL 2 cells were incubated with inhibitory mAbs. MAbs used in (A) and (B) GL439 (anti-huIL2Rα), MikB1 (anti-huIL2Rβ) and in (C) and (D) PC61 (anti-muIL2Rα) and TM-β1 (anti-muIL2Rβ), each at 10 μg/mL for 30–60 min at 4C prior to addition of IL2. In (B) and (D), a combination of species-appropriate anti-IL2Rα and anti-IL2Rβ mAbs were also tested. Cells were cultured for 72 h at 37°C and were pulse labeled with 1 μCi of ^{3}H thymidine for 6 h. Error bars indicate SD of triplicate samples. Data are representative of 2–10 separate experiments. Statistical differences of biological significance are represented with an asterisk $^*p$-value = <0.001.

this purpose, we used Kit225,[22] a human T-cell line derived from a patient with T cell chronic lymphocytic leukemia, and CTLL-2,[24] a mouse T-cell line cloned from a leukemic mouse spleen, which have been reported to express high affinity IL2Rs. As a model of human intermediate affinity IL2Rs, we used Tf-1β,[23] a human myelomonocytic cell line transfected to express the human IL2Rβ subunit in order to form $\beta\gamma$ dimers. To our knowledge, there is no IL2-dependent mouse cell line available that only expresses mouse intermediate affinity IL2Rs. Therefore, to study mu$\beta\gamma$-IL2Rs, we isolated resting splenocytes from SCID mice.[32] For each cell line (Kit225, CTLL-2, Tf-1β) and SCID splenocytes, the surface expressions of both IL2Rα and IL2Rβ were verified via flow cytometry (Fig. S2). To confirm that the SCID splenocytes did not upregulate expression of endogenous muIL2Rα following IL2 stimulation, we verified the absent expression of IL2Rα. After a 72 h incubation with increasing concentrations of IL2, known to stimulate proliferation in these cells; even at the highest concentration of 1333 ng/mL, there was no IL2Rα detected (Fig. S3).

To validate the receptor specificity of the IL2-dependent cell lines and SCID splenocytes, we performed proliferation experiments in response to soluble human IL2 in the presence of blocking antibodies against IL2Rα and IL2Rβ (Fig. 2). As expected, addition of an inhibitory anti-IL2Rα mAb (10 μg/mL), which binds to the α-chain of the IL2R, did not interfere with the proliferation seen in Tf-1β or SCID splenocytes in response to IL2 (Fig. 2A and 2C). When the anti-IL2Rα mAb was added to Kit225 and CTLL-2 cells, IL2-induced proliferation was substantially inhibited; a 3–13-fold increase in IL2 was required to induce proliferation comparable to that seen in the absence of the anti-IL2Rα mAb (Fig. 2B and 2D, EC_{50} values summarized in Table 1). To determine if the decreased proliferation was due to incomplete blocking of $\alpha\beta\gamma$-IL2Rs, we increased the amount of anti-IL2Rα mAb and confirmed cell surface saturation by flow cytometry. Even with 10 times more blocking mAb (100 μg/mL), proliferation was not completely inhibited but continued to exhibit a ~15-fold decrease (Fig. S4), indicating that Kit225 and CTLL-2 are able to retain some lower-level proliferation in response to IL2 even when IL2Rα is unavailable, suggesting the presence of functional $\beta\gamma$-IL2Rs on these cells. The addition of anti-IL2Rβ mAb (10 μg/mL) did not affect proliferation of Kit225 and CTLL-2 when compared to IL2 alone, demonstrating that even in the presence of the anti-IL2Rβ mAb, these cells continue to utilize high affinity IL2Rs. However, when blocking the α- and β-chains simultaneously, by adding both anti-IL2Rα mAb and anti-IL2Rβ mAb, proliferation is virtually completely inhibited (Fig. 2B and D). Thus, the IL2-dependent growth observed in Kit225 and CTLL-2 cells when IL2Rα was blocked was mediated through IL2Rβ, most likely via activation of $\beta\gamma$-IL2Rs. This is evidenced by the increase in the effective concentrations (EC_{50}) of IL2 needed for Kit225 and CTLL-2 when blocked with anti-IL2Rα mAb (Table 1).

On the other hand, blocking Tf-1β and SCID splenocytes with anti-IL2Rβ mAb alone completely abrogated their proliferation in response to IL2 (Fig. 2A and C). Since the absence of proliferation in the presence of this anti-IL2Rβ mAb could be due to non-specific inhibition, we confirmed the specificity of

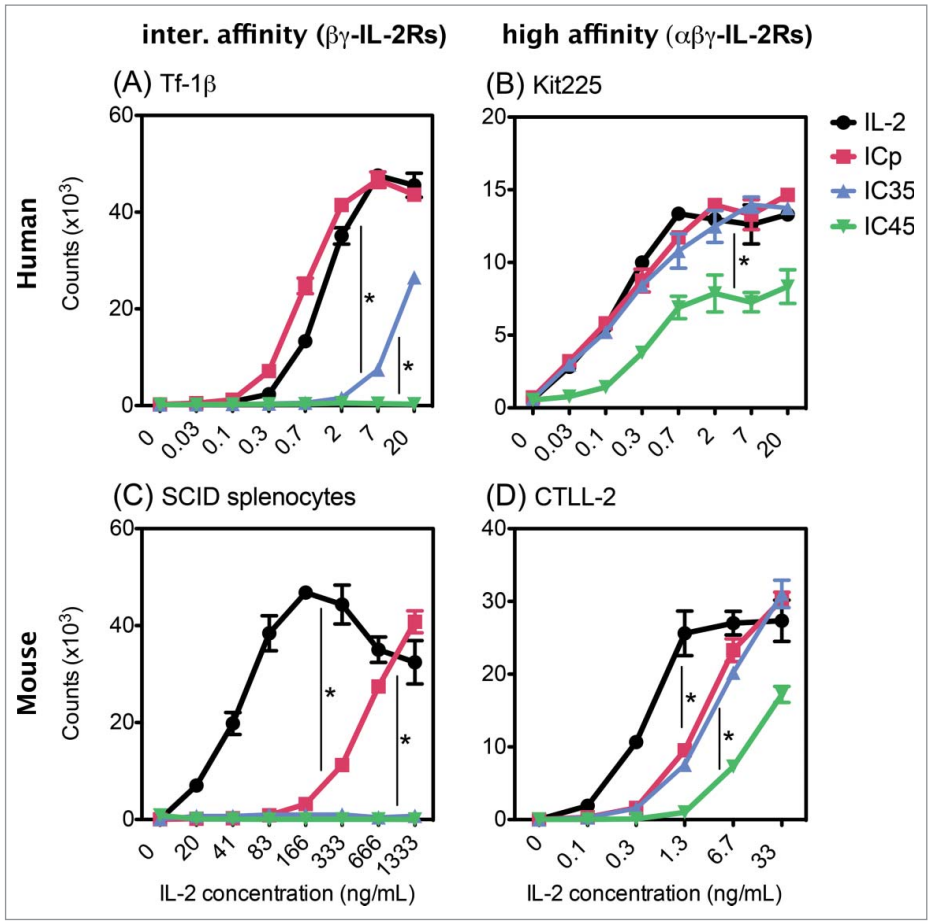

Figure 3. Novel ICs exhibit reduced ability to induce proliferation of cells expressing $\beta\gamma$-IL2Rs. Comparison of IC35 and IC45 induced proliferation on: (A) Tf-1β, (B) Kit225, (C) CTLL-2, and (D) SCID splenocytes. Cells were stimulated with increasing concentrations of IL2, ICp, IC35, or IC45 and cultured for 72 h at 37°C. The counts were determined by [^{3}H]TdR incorporation by proliferating cells. Error bars indicate SD of triplicate samples. Data are representative of two–six separate experiments. Statistical differences of biological significance are represented with an asterisk $^*p$-value = <0.001.

the anti-IL2Rβ blocking antibody by verifying that it did not interfere with GM-CSF-induced proliferation of these same Tf-1β cells. Even at the highest concentrations, IL2-induced proliferation was virtually abrogated when Tf-1β cells were first blocked with anti-IL2Rβ. In contrast, blocking IL2Rβ did not interfere with the ability of GM-CSF to induce proliferation of these same Tf-1β cells (Fig. S5). Taken together, these results indicate that Tf-1β and SCID splenocytes proliferate in response to IL2 solely through $\beta\gamma$-IL2Rs; thus, these two cell types serve as suitable *in vitro* models to study activation of human and mouse intermediate affinity IL2Rs, respectively.

Novel ICs with IL2 on the light chains exhibit reduced ability to stimulate $\beta\gamma$-IL2Rs

IC35 and IC45, with human IL2 fused to the light chains of the anti-GD2 mAb (Fig. 1), were designed to reduce activation of $\beta\gamma$-IL2Rs. We evaluated their bioactivity in comparison to the parent ICp (which has IL2 fused to the heavy chains of the mAb) and to soluble IL2 in our established *in vitro* systems using cells that express human and mouse $\alpha\beta\gamma$- or $\beta\gamma$-IL2Rs (Fig. 3). In our human cell models, ICp-induced proliferation to the same extent as IL2 (Fig. 3A and B), demonstrating that placing the IL2 molecules on the heavy chains of the mAb does not interfere with the ability of human $\alpha\beta\gamma$ or $\beta\gamma$-IL2Rs to bind and respond to the IL2 component of ICp. In contrast to the results with human cells, ICp was less potent than soluble IL2 at inducing proliferation of cells expressing muIL2Rs, illustrated by a 10–12-fold increase (Table 2) in the amount of ICp needed to induce proliferation comparable to that by soluble IL2 for CTLL-2 and SCID splenocytes, respectively (Fig. 3C and D).

When exposed to IC35, both human and mouse cells expressing $\alpha\beta\gamma$-IL2Rs responded similarly to ICp (Fig. 3B and D), with superimposed proliferation curves for both IC35 and ICp on Kit225 cells and on CTLL-2 cells. These results suggest that changing the location of IL2 from the heavy chains to the light chains does not interfere with the ability of IC35 to activate human or mouse $\alpha\beta\gamma$-IL2Rs. However, placing the IL2 molecules on the light chains interfered with the bioactivity of

Table 2. IL2 bioactivity of IL2, ICp, IC35 and IC45.

	EC_{50} (IL2 ng/mL)			
Cells	IL2	ICp	IC35	IC45
Kit225	0.11	0.18	0.15	0.26
Tf-1β	1.76	1.91	17.73	ND
CTLL-2	0.35	3.51	3.95	11.75
SCID splenocytes	44.10	538.43	ND	ND

EC_{50} values are the average of three separate experiments. ND: Not determined.

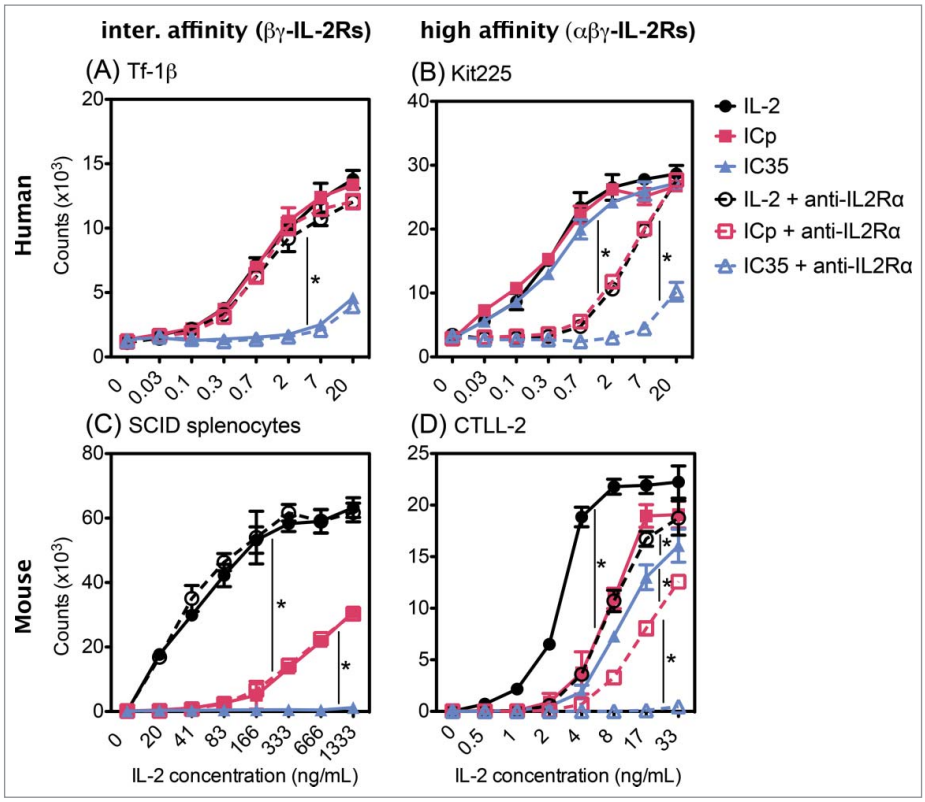

Figure 4. Blocking IL2Rα allows Kit225 and CTLL-2 to respond through βγ-IL2Rs. Inhibitory antibody against the α-chain of the IL2R effects proliferation of cells expressing high affinity IL2Rs (B) and (D) but not cells expressing intermediate affinity IL2Rs (A) and (C). (A) Tf-1β, (B) Kit225, (C) CTLL-2, and (D) SCID splenocytes were incubated with inhibitory mAbs at 10 μg/mL for 30–60 min at 4C prior to addition of IL2. Cells were cultured for 72 h at 37°C and were pulse labeled with 1 μCi of ^{3}H thymidine for 6 h. Error bars indicate SD of triplicate samples. Data are representative of 2–10 separate experiments. Statistical differences of biological significance are represented with an asterisk *p-value = <0.001.

IC35 on human and mouse βγ-IL2Rs. Tf-1β cells required 15–20 times more IC35 to induce proliferation comparable to that induced by ICp (Fig. 3A). The sequestering effect achieved by having IL2 on the light chains was even more evident when evaluating muβγ-IL2Rs; IC35 was completely unable to induce proliferation of SCID splenocytes at the concentrations tested (Fig. 3C). These data comparing proliferation induced by soluble IL2, ICp, and IC35 demonstrate the decreased capability of muIL2Rs (vs. huIL2Rs) to respond to huIL2 when fused to heavy or light chains of a mAb (EC_{50} values summarized in Table 2).

Although IC35 retained the same stimulatory ability as ICp on human and mouse cells with αβγ-IL2Rs (Fig. 3B and D), IC45 showed substantially less ability to stimulate these cells than IC35 or ICp. IC45 exhibited a 3-fold decrease in bioactivity when compared to ICp on Kit225 (Fig. 3B and Table 2), and this effect was more pronounced on muαβγ-IL2Rs with a 30-fold decrease on CTLL-2 (Fig. 3D). Moreover, IC45 completely lost the ability to activate βγ-IL2Rs with no proliferation seen on Tf-1β or SCID splenocytes (Fig. 3A and C). These data indicate that the deletion of residues within the IL2 molecules of IC45 significantly reduced its ability to interact with both αβγ- and βγ-IL2Rs (human and mouse).

To further evaluate the selectivity of IC35 for αβγ-IL2Rs, we investigated its bioactivity in the presence of the anti-IL2Rα mAb, which blocks αβγ-IL2Rs, thus driving Kit225 and CTLL-2 cells to respond only through βγ-IL2Rs. As observed previously in Fig. 2, anti-IL2Rα did not alter the proliferation of Tf-1β or SCID splenocytes; both responded to IL2, ICp, and IC35 to the same extent in the presence or absence of the blocking mAb (Fig. 4A and C). In contrast, Fig. 4B shows that though Kit225 cells displayed similar proliferation curves in response to IL2, ICp, and IC35 in the absence of the blocking mAb, there was a significant decrease in proliferation when the α-chain was blocked. For both IL2 and ICp, 10 times higher concentrations were required in order to achieve proliferation of Kit225 in the presence of anti-IL2Rα that was comparable to that seen in the absence of the blocking antibody (EC_{50} values summarized in Table 3). This is consistent with these Kit225 cells treated with the blocking mAb responding to proliferative signals through βγ-IL2Rs. Importantly, in the presence of the blocking antibody, IC35 showed a further significant reduction in Kit225 bioactivity, resembling the proliferation curves obtained on Tf-1β cells, with 30-fold decrease when compared to ICp (Table 3).

Table 3. IC bioactivity in the presence of anti-IL2Rα.

	EC_{50} (IL2 ng/mL)					
Cells	IL2	IL2 + anti-IL2Rα	ICp	ICp + anti-IL2Rα	IC35	IC35 + anti-IL2Rα
Kit225	0.23	2.40	0.66	6.39	0.44	14.41
Tf-1β	1.41	1.67	1.77	1.06	24.86	22.54
CTLL-2	1.30	2.45	3.46	5.37	4.34	ND
SCID splenocytes	33.87	34.16	975.27	695.33	ND	ND

EC_{50} values are the average of three–four separate experiments. ND: Not determined.

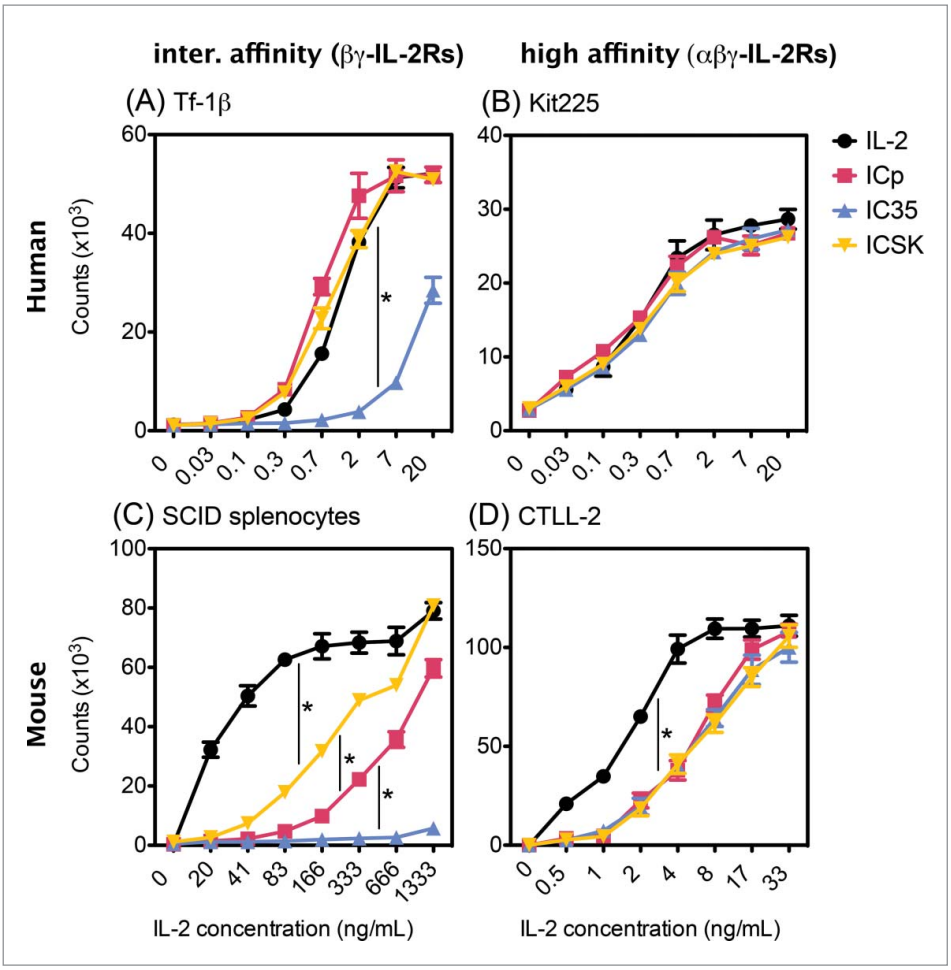

Figure 5. The "superkine" molecules within ICSK rescue the ability to elicit proliferation through $\beta\gamma$-IL2Rs. Comparison of IC35 and ICSK induced proliferation on: (A) Tf-1β, (B) Kit225, (C) SCID splenocytes, and (D) CTLL-2. Cells were stimulated with increasing concentrations of IL2, ICp, ICSK, or IC35 and cultured for 72 h at 37°C. The counts were determined by [^{3}H]TdR incorporation by proliferating cells. Error bars indicate the SD of triplicate samples. Data are representative of six–eight separate experiments. Statistical differences of biological significance are represented with an asterisk $^*p$-value = <0.001. Bold font: These values are similar when compared to one another.

Furthermore, blocking the α-chain on CTLL-2 cells (Fig. 4D) produced similar proliferation curves as those observed on SCID splenocytes (in the absence of blocking mAb), where more ICp is required to induce proliferation comparable to soluble IL2 and IC35-induced proliferation is completely inhibited. Together, these results demonstrate that IC35 and IC45 have a significantly reduced ability to activate $\beta\gamma$-IL2Rs, with this effect being more pronounced on mu$\beta\gamma$-IL2Rs where bioactivity has been completely abrogated.

The IL2 "superkine" molecules within ICSK restore activation of $\beta\gamma$-IL2Rs

Proliferation curves from Fig. 3 and Fig. 4 show that IC35 modestly activates hu$\beta\gamma$-IL2Rs, whereas activation of mu$\beta\gamma$-IL2Rs is completely lost. This disparity in bioactivity suggests important differences in IL2Rs between species that must be taken into account when designing *in vivo* experiments. In order to more accurately simulate in mice the level of IL2R activation anticipated in patients treated with IC35, we sought to find a separate IC, capable of modest activation of mu$\beta\gamma$-IL2Rs (comparable to the ability of IC35 to activate hu$\beta\gamma$-IL2Rs), to be used as a surrogate in our mouse models for the activity of IC35 in the stimulation of human cells. ICSK was created using the same IC construction platform as IC35, with fusion of an intact IL2 molecule, in this case the IL2-SK, to each of the light chains of the 14.18 mAb. The soluble IL2-SK molecule harbors mutations that increase binding affinity to the β-chain of the IL2R.[20] Fig. 5 shows that ICSK can activate both mouse and human $\alpha\beta\gamma$-IL2Rs, with identical proliferation curves for ICp, IC35, and ICSK on both Kit225 and CTLL-2 (Fig. 5B and D, Table 4). Furthermore, ICSK induced proliferation to the same extent as soluble IL2 and ICp on Tf-1β cells (Fig. 5A), demonstrating that incorporating the IL2-SK molecules into ICSK overcomes the sequestering effects of IL2 placement on the light chains (as seen with IC35) and renders ICSK capable of uninhibited activation of human $\beta\gamma$-IL2Rs. Importantly, ICSK stimulates proliferation of SCID splenocytes five times better than ICp, achieving a proliferation curve similar to that observed with IC35 on Tf-1β cells (Fig. 5C), suggesting that ICSK might better recapitulate in mice the extent of IC35 induced activation of $\beta\gamma$-IL2Rs in humans (EC_{50} values summarized in Table 4).

We hypothesized that these differences in IL2R activation were due to differences in the ability of these ICs to bind $\alpha\beta\gamma$-IL2Rs and $\beta\gamma$-IL2Rs. Therefore, we performed flow cytometry-based binding titrations of the ICs on the mouse

Table 4. IL2 bioactivity of IL2, ICp, IC35, and ICSK.

	EC_{50} (IL2 ng/mL)			
Cells	IL2	ICp	IC35	ICSK
Kit225	0.31	0.42	0.39	0.47
Tf-1β	2.40	0.89	46.16	0.61
CTLL-2	1.25	5.51	6.83	6.30
SCID splenocytes	36.52	907.00	ND	614.65

EC_{50} values are the average of four–seven separate experiments. ND: Not determined.

and human IL2R-expressing cells used in these proliferation experiments to assess differences in the kinetics of receptor engagement between the ICs. In Fig. 6B and D, cells expressing high affinity IL2Rs show that ICp, IC35 and ICSK all exhibited similar binding capabilities on Kit225 cells, and all three of these ICs also exhibited similar binding capabilities on the CTLL-2 cells[AQ]. In addition, these ICs appear to bind slightly better to Kit225 than to CTLL-2 (plateau level binding is seen at 40 ng/mL for Kit225, whereas 200 ng/mL are required on CTLL-2), suggesting that mu$\alpha\beta\gamma$-IL2Rs have less affinity for the IL2 molecules within the ICs than hu$\alpha\beta\gamma$-IL2Rs. These binding patterns are very similar to the proliferation data presented for these ICs on these same two cell lines (Fig. 5B and D). Similarly, binding on intermediate affinity IL2Rs (Fig. 6A and C) reflected the proliferation results obtained in Fig. 5A and 5, namely IC35 exhibited less binding on Tf-1β (Fig. 6A) and on SCID splenocytes (Fig. 6C) when compared to either ICp or ICSK. This difference was significant for Tf-1β cells at the lower concentrations of 8–40 ng/mL, the range in concentrations used for the proliferative studies. On SCID splenocytes, ICSK bound significantly better than ICp at the higher concentrations, which is consistent with the proliferation results that show that ICSK better activates mu$\beta\gamma$-IL2Rs than ICp does.

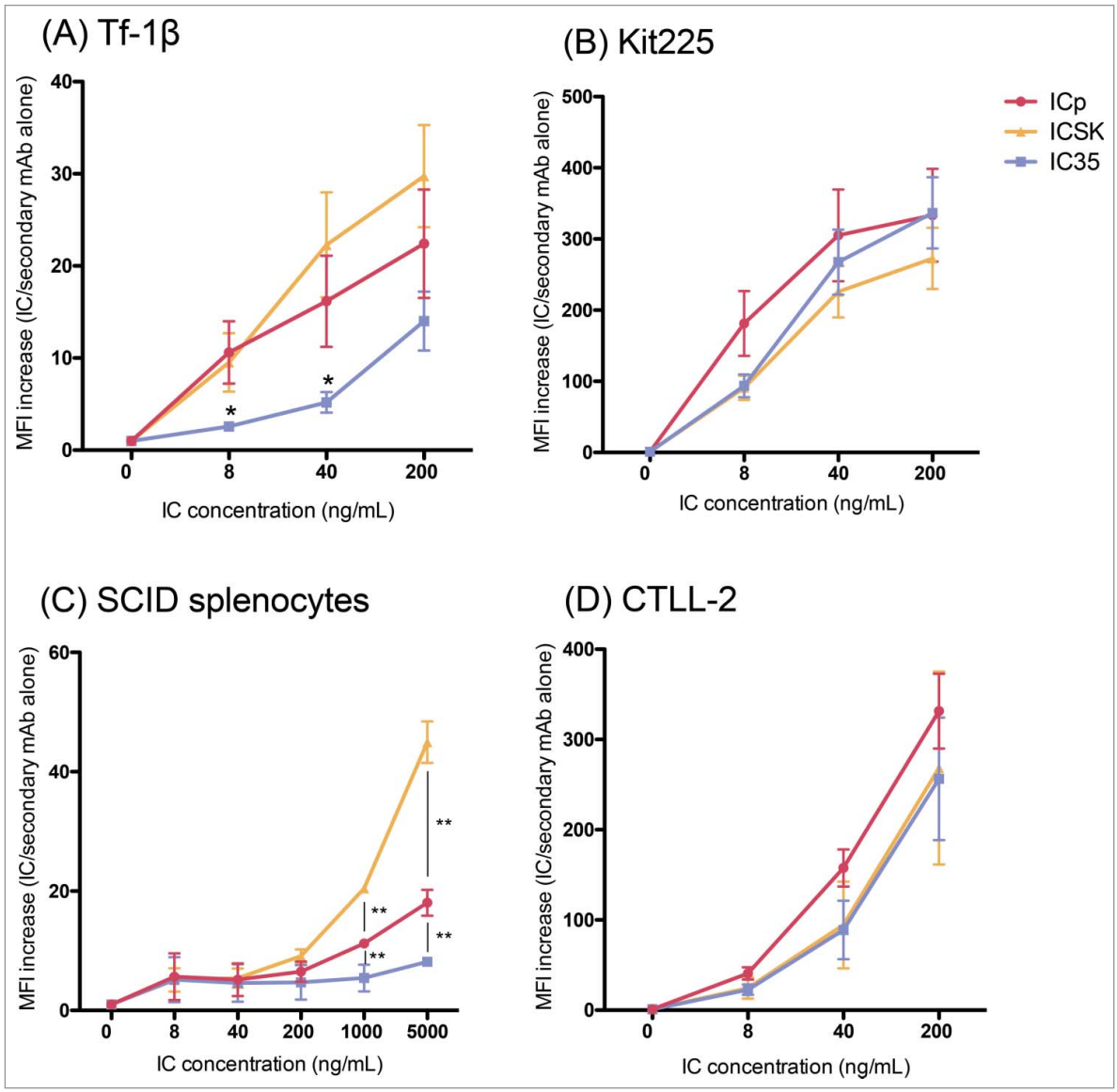

Figure 6. IC binding to cells expressing high or intermediate affinity IL2Rs. Flow cytometry analysis of IC binding on IL2R-expressing cells. (A) Tf-1β, (B) Kit225, (C) SCID splenocytes, and (D) CTLL-2. Cells (0.2×10^6 cells/sample) were incubated with increasing amounts of the different ICs (ICp, IC35, or ICSK) in 0.2 mL of staining buffer for 30–60 min at 4°C. SCID splenocytes were pre-incubated with Mouse Fc block to minimize Fc receptor mediated binding of the ICs. For detection an Alexa fluor 647-conjugated goat anti-hulgG secondary mAb was used. MFI fold increase = (MFI of IC-stained sample/MFI of secondary mAb alone). Data represent the mean ± SD from three to five separate experiments. Statistical differences of significance are represented with an asterisk $^{*}p$-value = <0.05 and $^{**}p$-value = <0.01.

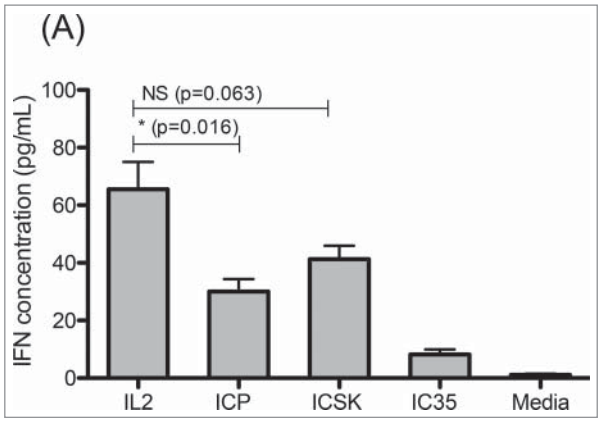

Figure 7. ICSK leads to increased IFNγ secretion on SCID splenocytes when compared to ICp. Comparison of IL2, ICp and ICSK induced secretion of interferon-γ on SCID splenocytes. Freshly isolated SCID splenocytes were stimulated with 7 μg/mL (equivalent to 1.3 $\times$ 10^3 ng/mL IL2) ICp, IC35, or ICSK for 72 h at 37°C. Supernatants were harvested and secreted IFNγ was measured via flow cytometry using a colorimetric bead array. Error bars indicate SD of five samples. Data are representative of three separate experiments. Statistical differences of biological significance are represented with an asterisk $^*p$-value = <0.05.

These binding studies provide evidence to support the hypothesis that moving the location of the IL2 molecules from the heavy to the light chains (compare data with ICp to data with IC35) does not influence binding to $\alpha\beta\gamma$-IL2Rs but does interfere with the interaction with $\beta\gamma$-IL2Rs. Furthermore, replacing these IL2 molecules on the light chains with the SK IL2 muteins overcomes this effect and restores the interaction with $\beta\gamma$-IL2Rs back to (or above) the original configuration (compare data with ICSK to data with ICp).

One downstream effect of IL2 stimulation on NK cells is the secretion of secondary cytokines such as IFNγ. Therefore, to further evaluate the ability of ICSK to activate mouse $\beta\gamma$-IL2Rs, we measured IFNγ secretion of SCID splenocytes after they had been stimulated for 72 h with IL2, ICp, and ICSK. Our results show that IL2 stimulation led to the highest accumulation of IFNγ in culture, with a median concentration of 71.8 pg/mL, whereas ICp-stimulated cells had the lowest IFNγ with 31.6 pg/mL. On the other hand, ICSK stimulated cells to produce 44.0 pg/mL IFNγ (Fig. 7). Blocking IL2Rβ on these cells inhibited IFNγ secretion for all treatments demonstrating that IFNγ is being produced as a result of $\beta\gamma$-IL2R activation (not shown). These results provide additional evidence to show that ICSK is able to activate mouse $\beta\gamma$-IL2Rs to a greater degree than ICp.

Phosphorylation of STAT5 as a direct measure of IL2R activation

One of the earliest cell signaling events that are triggered upon IL2 binding to the IL2R is the phosphorylation of STAT5 proteins. In order to confirm the selectivity of the novel ICs with a direct measure of IL2R engagement and activation, we measured phosphorylated STAT5 (pSTAT5) levels in cells expressing high or intermediate affinity IL2Rs. Using intracellular flow cytometry staining, we assessed the levels of pSTAT5 in Kit225 and Tf1b cells after 15 min incubation with IL2, ICp, IC35, and ICSK (Fig. 8). Similar to the proliferation results, there was no difference in pSTAT5 levels for all the treatments on Kit225 cells. This is further evidence to confirm that neither the location of the IL2 moiety nor the mutations within the SK molecules interfere with the ability of these ICs to interact and activate $\alpha\beta\gamma$-IL2Rs. On Tf1b cells, IC35 was the only IC unable to induce the phosphorylation of STAT5, confirming that having the IL2 on the light chains significantly hinders that ability of IC35 to engage $\beta\gamma$-IL2Rs.

Changing the location of the IL2 molecules does not interfere with ADCC

The ability of the novel ICs to induce effector functions and mediate tumor cell killing via antibody-dependent cell-mediated cytotoxicity (ADCC) was assessed in a 4 h ^{51}Cr release assay. Expanded NK cells and peripheral blood mononuclear cells (PBMCs) from healthy donors were incubated with GD2+ M21 human tumor cells in the presence of ICp, IC35, or ICSK. IL2R expression was verified by flow cytometry; both effector cell populations showed the expression of IL2Rβ and the absence of detectible IL2Rα, confirming the presence of human $\beta\gamma$-IL2Rs on these cells (data not shown). Our data show that all three ICs had equivalent ADCC activity at

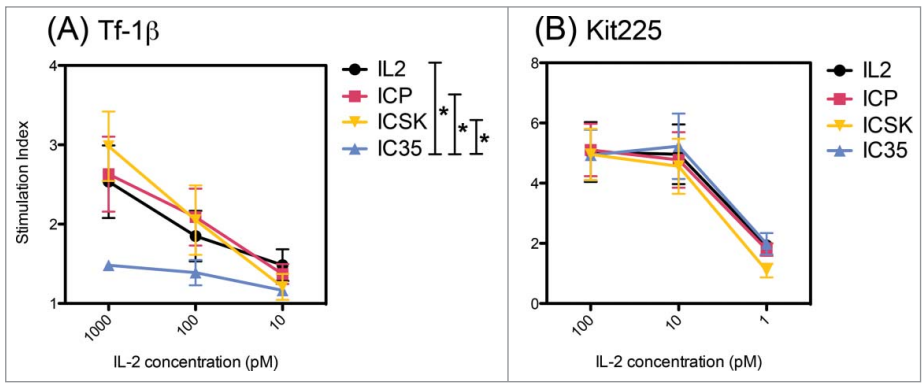

Figure 8. IC35 exhibits reduced phosphorylation of STAT5 via intermediate affinity IL2Rs. Comparison of IL2, ICp, IC35, and ICSK induced phosphorylation of STAT5 on: (A) Tf-1β and (B) Kit225. Cells were stimulated with increasing concentrations of IL2, ICp, ICSK, or IC35 and incubated for 15 min at 37°C. pSTAT5 levels were determined by intracellular flow cytometry staining using anti-pSTAT5(pY694). SI = (MFI stimulated cells/MFI unstimulated cells). Error bars indicate SD of triplicate samples. Data are representative of three separate experiments. Statistical differences of biological significance are represented with an asterisk $^*p$-value = <0.05. SI, stimulation index; MFI, mean fluorescence intensity.

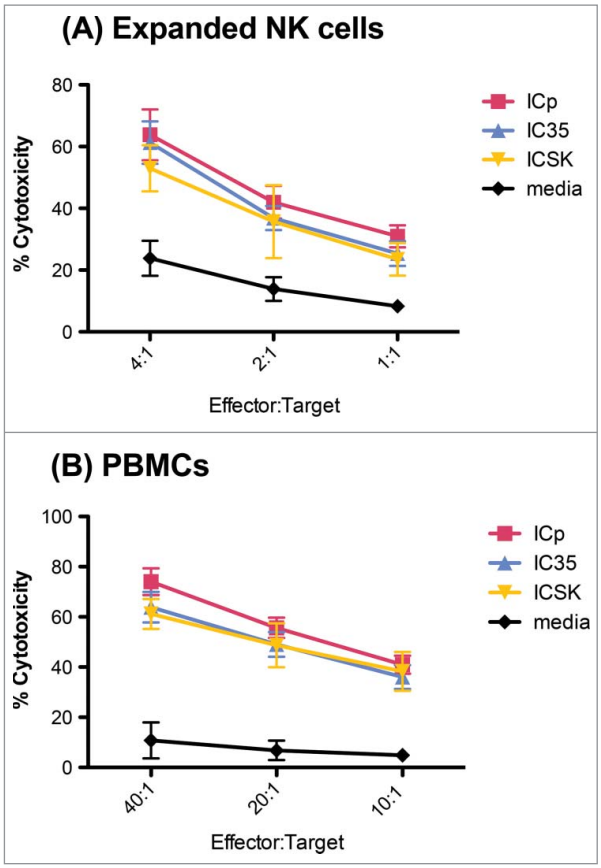

Figure 9. ADCC activity is not affected by moving the IL2 molecules to the light chains of the mAb. Comparison of ICp, IC35, and ICSK-mediated ADCC on: (A) Expanded NK cells and (B) PBMCs. Effector cells were co-incubated with ^{51}Cr-loaded M21 tumor targets in the presence of 1 μg/mL (equivalent to 195 ng/mL IL2) ICp, IC35, or ICSK for 4 h at 37°C. Supernatants were harvested and the released ^{51}Cr from lysed target cells was measured using a gamma counter. Percentage Cytotoxicity = [(experimental release − spontaneous release)/(maximum release − spontaneous Release)]×100. Error bars indicate SD of quadruplicate samples. Data are representative of three separate experiments.

comparable effector-to-target ratios and IC concentration (Fig. 9). These results demonstrate that all ICs are able to bind GD2+ tumor cells and mediate ADCC to the same extent irrespectively of the position or nature of the IL2 moiety on the antibody.

Discussion

In this report, we sought to dissect the differences in IL2R activation between the human and murine IL2R systems using novel ICs designed to selectively activate either high or intermediate affinity IL2Rs.[19] Previous studies investigated the IL2 bioactivity of these novel ICs compared only between mu$\alpha\beta\gamma$-IL2Rs and hu$\beta\gamma$-IL2Rs.[19] Here, we studied both human and murine $\alpha\beta\gamma$-IL2Rs and $\beta\gamma$-IL2Rs in parallel. We found that unlike huIL2Rs, muIL2Rs are less able to respond to IL2 when presented on the ICp, and this reduced activation of mu$\beta\gamma$-IL2Rs likely accounts for the relative absence of IL2-induced toxicity observed when ICp and related ICs are used to treat tumor-bearing mice.[4,6,7,33,34]

Murine and human IL2 and IL2R subunits share 60–70% homology;[35-38] thus, it is not surprising that mouse and human IL2Rs are both responsive to human IL2 at somewhat similar levels.[39,40] Consequently, human IL2 has been widely used to study the biology of IL2Rs interchangeably in models that use human and murine IL2Rs.[41,42] However, preclinical and clinical studies with hu14.18-IL2 (ICp) have revealed somewhat distinct *in vivo* effects when comparing mice to humans. Although *in vivo* studies in mouse tumor models have demonstrated promising antitumor effects without IL2-related side effects,[4-7] ICp immunotherapy in patients (at doses that achieve relatively similar peak blood levels, and similar half-lives[8,9,43] to those in mice that show antitumor benefit) is limited by dose-dependent IL2 toxicity.[8-11] This toxicity appears to be the result of overactivation of immune cells bearing intermediate affinity $\beta\gamma$-IL2Rs, leading to inflammatory cytokine release.[13,16,17] Therefore, the relative absence of IL2-induced toxicity in mice treated with ICp suggests that the IL2 component of this IC elicits less activation of mu$\beta\gamma$-IL2Rs than of hu$\beta\gamma$-IL2Rs.

We evaluated IL2R function using human and mouse cells that expressed either high or intermediate affinity IL2Rs and proliferated in response to IL2. Kit225 and CTLL-2 expressed both IL2Rα and IL2Rβ and proliferated in response to low doses of IL2, consistent with the activation of human and mouse $\alpha\beta\gamma$-IL2Rs, respectively. Blocking IL2Rα on these cells substantially inhibited the IL2 induced proliferation, shifting the IL2 response curve toward the concentrations needed to stimulate $\beta\gamma$-IL2Rs. This suggests that, in the presence of an anti-IL2Rα mAb, both Kit225 and CTLL-2 retained some lower levels of IL2 responsiveness via IL2R with intermediate affinity, thereby providing an additional tool to study the activation of $\beta\gamma$-IL2Rs. Surprisingly, even though IL2 interaction with the β-chain component of high affinity IL2R is required for IL2-induced signaling,[12] blocking IL2Rβ alone was not enough to inhibit proliferation of Kit225 or CTLL-2 cells at any of the IL2 concentrations tested. The absence of a significant shift in the dose-dependent proliferation curves demonstrates that even in the presence of the IL2Rβ blocking antibody, these cells continue to have fully functional $\alpha\beta\gamma$-IL2Rs mediating IL2 signaling. This retained function of high affinity IL2Rs, in the presence of mAb against the IL2Rβ, seen by us and others,[26,30,44] likely reflects a tight association between the IL2/IL2Rα complex and the β-chain capable of overcoming the interaction of the blocking mAb with IL2Rβ, resulting in its detachment from the trimeric IL2R $\alpha\beta\gamma$ complex.[45] When mAbs against both IL2Rα and IL2Rβ are added, the proliferative response by Kit225 cells is abrogated, due to complete blockade of both high and intermediate affinity receptors.

Flow cytometry analyses showed expression of IL2Rβ, but no detectible IL2Rα on the surfaces of Tf-1β cells and SCID splenocytes was seen, suggesting their ability to respond through $\beta\gamma$-IL2Rs[AQ]. Adding the blocking mAb that specifically recognizes the IL2 binding site on IL2Rβ was sufficient to abolish the proliferative response of Tf-1β and SCID splenocytes at the range of IL2 doses tested, confirming the absence of $\alpha\beta\gamma$-IL2Rs on these cells and demonstrating that they respond solely through $\beta\gamma$-IL2Rs. The IL2Rβ-dependent proliferation observed with SCID splenocytes is particularly important to illustrate the existence of functional mouse intermediate affinity IL2Rs, since earlier work proposed that contrary to the human $\beta\gamma$ heterodimer, mu$\beta\gamma$-IL2Rs are unable to bind IL2 or induce intracellular signaling.[38,46,47] In those prior studies, various cell

lines were transfected with muIL2Rβ and it was concluded that expression of IL2Rα is required for the formation of functional IL2Rs in mice. However, our data are in accordance with other reports, wherein splenocytes from various mouse strains with endogenous expression of mu$\beta\gamma$-IL2Rs exhibited IL2 induced proliferation consistent with the activation of intermediate affinity IL2Rs.[30,48]

The evaluations of IL2R expression and specificity in these different cell lines and SCID splenocytes allowed us to establish a useful *in vitro* model system to analyze the behavior of the novel IC constructs on human and murine IL2Rs. We acknowledge that these cells are not perfect controls for one another, exhibiting significant biological differences, including their difference in immune cell type (T cells vs. monocytes vs. NK cells) and their IL2R expression levels. These limitations can be evidenced in the concentrations needed to achieve maximal response. For example, SCID splenocytes require ~40 times more IL2 to achieve proliferation likely due to a combination of factors including the fact that these are primary cells that have not been sensitized to growing in culture. However, for the purpose of our study, this model allows us to draw conclusions of the behavior of these ICs by focusing on the relative patterns of response of these distinct ICs compared to each other and to soluble IL2 within each particular cell line, rather than by comparing the response of distinct cell lines quantitatively to each other.

Our results show that both ICp and soluble huIL2 have comparable IL2-weight-based IL2 activity on human cells bearing $\alpha\beta\gamma$-IL2Rs and $\beta\gamma$-IL2Rs. This illustrates that huIL2Rs are able to recognize and be activated by huIL2, even when IL2 is presented on the IgG heavy chains as part of the fusion protein. In contrast, ICp shows decreased ability to activate murine cells expressing muIL2Rs when compared to soluble huIL2. These data are in agreement with a separate study evaluating the IL2 bioactivity of different ICs containing IL2 on the heavy chains, wherein muIL2Rs were less responsive than huIL2Rs.[21] Fusing IL2 to the heavy chain of ICp partially disrupts the ability of muIL2Rs to respond to the IL2 component of the IC, as shown by the decreased proliferation elicited by ICp when compared to IL2 on CTLL-2 but not on Kit225 cells. This suggests that muIL2Rs are less efficient at binding huIL2 than huIL2Rs, making them more susceptible to subtle structural changes within the huIL2 molecules that might occur when fused to the hu14.18 mAb. These conformational differences seem to be most pronounced when evaluating stimulation of mu$\beta\gamma$-IL2Rs as evidenced by the striking decrease in proliferation of SCID splenocytes in response to ICp compared to IL2. Therefore, this difference in the IC selectivity profile for mu$\beta\gamma$-IL2Rs may account for the successful retention of the antitumor effect and reduced IL2 toxicity observed in mice treated with ICs such as ICp.[4,6,7,33,34]

In order to reduce the ability of the IC to interact and activate hu$\beta\gamma$-IL2Rs, a new platform for constructing ICs was developed by utilizing cytokine fusion to the light chain of the mAb.[19] In these ICs, the fusion junction of the IL2 molecules is located adjacent to the antibody hinge region, creating a sequestering effect that restricts IL2Rβ binding. Our binding data demonstrate that IC35 is able to bind $\alpha\beta\gamma$-IL2Rs with similar affinity to ICp and ICSK but has significantly reduced ability to bind $\beta\gamma$-IL2Rs, particularly at the lower concentrations tested. Furthermore, ICSK showed similar affinity to ICp on both $\alpha\beta\gamma$-IL2Rs and $\beta\gamma$-IL2Rs, with higher activity on SCID splenocytes, demonstrating that the enhanced SK molecules used in this construct restore the binding ability of this IC for intermediate affinity IL2Rs. However, this new placement of IL2 does not interfere with antigen binding as confirmed with flow cytometry staining of GD2+ M21 human tumor cells (not shown). Furthermore, when compared for their ability to mediate ADCC, these novel ICs were found to have equivalent activity to ICp, demonstrating that the ability of these ICs to bind Fc receptors and mediate tumor cytotoxicity was unaltered. Therefore, fusing the IL2 molecules to the light chains of the mAb allows fine-tuning the degree of $\beta\gamma$-IL2R binding specificity without affecting the other functions of the IC, such as antigen and FcR binding. IC35 has one huIL2 molecule fused directly to the C-terminal cysteine residue of each L-chain. The current experiments demonstrate that IC35 retains intact bioactivity (comparable to ICp) on human and mouse $\alpha\beta\gamma$-IL2Rs, whereas it shows decreased or no activation of human or mouse $\beta\gamma$-IL2Rs, respectively. Therefore, IC35 achieves a low-level activation on hu$\beta\gamma$-IL2Rs comparable to that which ICp has on mu$\beta\gamma$-IL2Rs.

To further modulate the degree of receptor specificity, the length of the N-terminus of the huIL2 was adjusted on IC45 by removing five residues.[19] We showed that the ability of IC45 to activate mouse and human $\alpha\beta\gamma$-IL2Rs was decreased; therefore, shortening the IL2 molecules on IC45 resulted in a reduction of high affinity activation. Furthermore, IC45 was unable to stimulate proliferation of mouse and human $\beta\gamma$-IL2Rs in the range of concentrations tested, although it does stimulate proliferation at higher concentrations (SG unpublished results). This high level of IL2R selectivity with IC45 is likely unfavorable for effective clinical immune cell activation at the low doses normally used for IC therapy, since it has been shown that initial activation of $\beta\gamma$-IL2Rs is needed for the upregulation of IL2Rα.[49] It remains to be determined whether higher doses, typically used for antibody therapy, might benefit from this reduced activity form of IL2. In addition, clinical studies that utilized a fusion protein containing a genetically modified IL2 protein (Selectikine), which was designed to be highly selective for $\alpha\beta\gamma$-IL2R, found that although it did not exhibit any IL2-related toxicity, it also did not show any objective antitumor responses as a single agent in end-stage patients with bulky disease (although several patients had long-term stable disease >4 y). These findings suggest that some minimal activation of $\beta\gamma$-IL2Rs is needed to mount an effective $\alpha\beta\gamma$-IL2R-based therapeutic immune response.[18]

It is important to note that in addition to toxicity mediated via the activation of immune cells expressing $\beta\gamma$-IL2Rs, the direct response of endothelial cells to IL2 via $\alpha\beta\gamma$-IL2Rs on their surface has also been shown to contribute to IL2 toxicity.[50] Recent studies using IL2 muteins or IL2/anti-IL2 mAb complexes directed toward $\beta\gamma$-IL2Rs over $\alpha\beta\gamma$-IL2Rs have demonstrated an expansion of T and NK cells accompanied with a reduction in endothelial cell activation resulting in antitumor effects with acceptable toxicity.[51,52] Although these results may seem contradictory, the mechanisms mediating IL2 toxicity are complex and remain to be completely understood. It is possible

that in these previously reported model systems, the contribution of endothelial cells to IL2 toxicity is more prominent due to the systemic administration and exposure to $\alpha\beta\gamma$-IL2Rs on blood vessels for these IL2 formulations; in this setting, it may be possible that using IL2 formulations that reduce contact with $\alpha\beta\gamma$-IL2Rs on endothelial cells may reduce this component of IL2-related toxicity. In contrast, in our model, the novel immunocytokines target IL2 to malignant cells via antigen binding, increasing the concentration of IL2 within the tumor microenvironment, and resulting in the activation of tumor-infiltrating immune cells. In this scenario, over-stimulation of NK cells expressing intermediate affinity IL2Rs might contribute more significantly to IL2-induced toxicity and therefore reducing these $\beta\gamma$-IL2R interactions would allow safer administration of the ICs to doses that achieve clinical efficacy.

Our data suggest that IC35 administration to patients bearing GD2+ tumors might achieve the desired balance between antitumor efficacy and tolerability that we observed in mice treated with ICp. However, due to its inability to activate mu$\beta\gamma$-IL2Rs, preclinical *in vivo* studies designed to study the antitumor effect and safety of this IC35 molecule have limited translatability to the clinical setting. In order to overcome this limitation and better represent in our mouse model the level of IC35-induced $\beta\gamma$IL2R activation expected in humans using an IC with IL2 on the immunoglobulin light chains, ICSK was developed. This IC contains two IL2 "SK" molecules that have been mutated to exhibit high affinity binding to IL2Rβ, and thus overcome the sequestering effect of being placed on the light chains. Our results show that indeed, ICSK has regained the ability to stimulate hu$\beta\gamma$-IL2Rs completely (comparable to IL2). On mu$\beta\gamma$-IL2Rs, ICSK retains a modest level of IL2 bioactivity, exhibiting a ~20-fold difference when compared to huIL2, similar to the ~20-fold difference observed with IC35 on hu$\beta\gamma$-IL2Rs (Table 5). Furthermore, when compared to ICp, ICSK stimulated mu$\beta\gamma$-IL2Rs to secrete higher levels of IFNγ that is an integral part of the NK effector machinery.[53] The ability to induce increased production of this pro-inflammatory cytokine is a functional consequence of IL2R binding, showing that ICSK can better activate mouse NK cells than ICp. These results suggest that ICSK might be used in the future as a surrogate in mouse model systems to better predict the effects that we hypothesize IC35 will have in humans. In this way, we could improve our understanding of IL2-induced dose-dependent toxicity vs. antitumor efficacy in mice, and subsequently advance the design of therapeutics such as IC35 for assessment in clinical trials.

In summary, our findings demonstrate that human and mouse IL2R have quantitative differences in their ability to respond to human IL2. These differences are magnified when IL2 is presented as part of a fusion protein, such as the ICs evaluated here. In order to use mouse models to simulate the results expected in clinical testing with an IL2-based clinical reagent, the IL2-based compound evaluated in mice should simulate with murine IL2Rs, the biology of the clinical reagent seen with human IL2Rs. Further work is warranted to investigate the functional capabilities of the new ICs presented here, and similar IC constructs, to elicit immune effector functions *in vitro*, and their stability, safety, and antitumor efficacy *in vivo*. These, together with a detailed *in vivo* analysis of the stimulatory effects of these ICs on different immune cell populations expressing $\alpha\beta\gamma$- vs. $\beta\gamma$-IL2Rs will enhance our understanding of murine models and the mechanism of action of these molecules. As ours[54,55] and other preclinical data[56,57] are now demonstrating the potential for augmented antitumor activity by ICs when combined with other modalities, it will be important to use ICs in the clinic that simulate the IL2R biology shown to be effective in the murine models.

Table 5. Fold difference of the bioactivity of the ICs when compared to IL2.

	Ratio of EC_{50}		
Cells	ICp/IL2	IC35/IL2	ICSK/IL2
Kit225	1.36	1.27	1.52
Tf-1β	0.37	**19.24**	0.25
CTLL-2	4.42	5.48	5.06
SCID splenocytes	24.84	ND	**17.52**

Ratio of EC_{50} were calculated by using the EC_{50} values shown in Table 5. Ratio of EC_{50} = IC EC_{50} / IL2 EC_{50}. ND: Not determined. Bold font: These values are similar when compared to one another.

Disclosure of potential conflicts of interest

Dr. Steve Gillies discloses equity ownership in Provenance Biopharmaceuticals of Carlisle MA and ownership of intellectual property related to the light chain fusion proteins. All other authors declare no competing financial interests.

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