A new platform for constructing antibody-cytokine fusion proteins (immunocytokines) with improved biological properties and adaptable cytokine activity

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A novel method for constructing immunocytokines has been developed that utilizes fusion of cytokines to the C-terminus of the Ig light chain, rather than fusing to the heavy chain. Such molecules are expressed well in transfected cells, are very stable in normal buffers and have biological properties that are superior to immunocytokines made by fusion to the heavy chain. These properties include longer circulating half-life, increased uptake following subcutaneous dosing and similar or improved antibody effector activities of antibody-dependent cytotoxic activity and complement-dependent cytotoxicity, respectively. Furthermore, the sequestering effect of this fusion junction allows one to adjust intermediate affinity (βγ) interleukin 2 receptor (IL2R) binding and activation by shortening the N-terminus of IL2 at the fusion point. This appears to limit access of the critical contact residue Asp20 of IL2 to the β-chain of βγ IL2R, while maintaining binding and activation of high-affinity (αβγ) IL2R-expressing cells. Several immunocytokine forms with varying degrees of IL2R specificity have been constructed, and some appear to regain their activity for the βγ IL2R when bound to antigen-coated beads. Such molecules may have reduced toxicity in the circulation and enhanced anti-tumor activity.

Keywords: immunocytokine/interleukin 2/receptor specificity

Introduction

The clinical use of interleukin 2 (IL2) for cancer immune therapy has been limited by insufficient activation of anti-tumor effector cells in the tumor microenvironment at doses that can be tolerated by the patient. These doses, in turn, are limited by several side effects including vascular toxicities of hypotension and vascular leak syndrome (Atkins et al., 1999). One way to increase the accumulation of IL2 at the tumor site is to genetically fuse it to the heavy (H) chains of a tumor specific antibody, and several such molecules (immunocytokines or ICs) have been shown to have improved anti-tumor activity compared with free IL2, the targeting antibody or the combination of both agents (Davis and Gillies, 2003). This is especially true of ICs with longer circulating half-lives that increase the amount of drug delivered to the tumor (Gillies et al., 2002). Unfortunately, this increases the vascular exposure and toxicity of the IL2 component that, again, limits the amount that can be dosed and the rate of infusion.

One way to reduce the toxicity of long-acting ICs is to dose them subcutaneously (SC) (greatly reducing Cmax levels in the blood) rather than intravenously (IV), which slowly releases the IC into the vascular compartment. The lower concentration favors activation of the high-affinity IL2 receptor (IL2R; containing the three receptor chains, α, β and γ, or αβγ IL2R) that is associated with T-effector cell activation, while preventing over-activation of the intermediate IL2R (composed of just the β- and γ-chains or βγ IL2R) that is associated with IL2 toxicities of hypotension and vascular leak syndrome (Atkins et al., 1999). Another way is to make an IC more selective for the high-affinity αβγ IL2R than the intermediate affinity βγ IL2R by mutagenesis of IL2 (Shanafelt et al., 2000). One such receptor-selective IC, NHS-IL2LT (also called Selectikine) containing a mutation in position 20 of IL2 from asparagines to threonine (D20T), has been shown to have dramatically lower IL2 toxicities in mouse tumor models and in monkeys (Gillies et al., 2011), as well as in human cancer patients (Gillessen et al., 2013). While NHS-IL2LT maintains some level of anti-tumor activity, its degree of receptor selectivity (~10 000-fold) may be too great, since some level of βγ IL2R activation may be needed to up-regulate the α-chain needed for αβγ IL2R expression on effector cells. In fact, ICs containing normal human IL2 have receptor specificity for the αβγ IL2R over the βγ IL2R on mouse immune cells of between 10- and 20-fold, relative to free rIL2 or to their activity on human immune cells but have shown potent anti-tumor activities at well-tolerated doses in mouse tumor models (Gillies et al., 2005). In other words, human IL2-containing ICs may have a better IL2R selectivity profile in mice than in humans that better balances the anti-tumor activities against potential vascular toxicity. The optimal IL2R selectivity remains to be determined but could be instrumental in optimizing this therapeutic approach. Maintaining effective binding to the αβγ IL2R may also be important to ICs, in which the antibody targeting component binds to the tumor cell surface (unlike other ICs targeting tumor necrosis, such as NHS-IL2LT, or the tumor vasculature), since it has recently been shown that whole antibody-IL2 ICs trigger re-directed tumor cell lysis, similar to anti-CD3-based bispecific antibodies (Gubbels et al., 2011). In addition to receptor selectivity (and its effect on toxicity and anti-tumor activities), several other properties of ICs would be desirable for their efficacy and clinical utility including high tumor targeting, long circulating half-life and high bioavailability by SC injection.

With these objectives in mind, a novel method for constructing ICs has been developed that utilizes fusion of cytokines to the C-terminus of the Ig light (L) chain, rather than the traditional method of fusing to the H-chain. The fusion junction of IL2, in this case, is located adjacent to the antibody hinge...
region, where the L-chain forms a disulfide bond through its C-terminal cysteine residue to the H-chain. This constrains both the N-terminal region of IL2, as well as the hinge-CH2 region of the H-chain where both Fc receptors and the C1q subunit of the complement bind when they mediate antibody-dependent cytoxic activities (ADCC and CDC). Such ICs are expressed well in transfected cells, are very stable in normal buffers and have biological properties that are superior to ICs made by fusion to the H-chain. These properties include longer circulating half-life, increased uptake following SC dosing and similar or improved antibody effector activities of antibody-dependent cytoxic activity (ADCC) and complement-dependent cytoxicity (CDC), respectively. Furthermore, the sequestering effect of this fusion junction allows one to adjust βγ IL2R binding and activation by shortening the length of the N-terminus of IL2 at the fusion point. This appears to limit access of the critical contact residue Asp20 of IL2 to the β-chain of βγ IL2R, while maintaining binding and activation of αβγ IL2R-expressing cells, as well as antibody effector functions.

Materials and methods

Cell culture

Human 293F cell cultures were grown in suspension using the Freestyle System (Invitrogen, Carlsbad, CA, USA). Later experiments utilized attached 293T cells growing in Dulbecco’s modified Eagle’s medium (DMEM) medium containing 10% fetal bovine serum (FBS), 4 mM glutamine and penicillin-streptomycin. NS/0 mouse myeloma cells were grown in the same DMEM growth medium containing 2 mM glutamine. Human M21 melanoma cells, mouse CTLL-2T cells and the human TF-1β cell line (all provided by Dr Paul Sondel, University of Wisconsin, Madison) were grown in RPMI medium containing 10% FBS, 2 mM glutamine and penicillin-streptomycin. Recombinant human IL2 was added to a final concentration of 50 IU/ml for maintenance of the CTLL-2 and TF-1β cell lines. All of the media and supplements were purchased from Invitrogen.

Expression and purification of ICs

An IC expression vector, pDHLP, containing transcription units for expression of the immunoglobulin H- and L-chains on a single plasmid, as well as unique cloning sites for fusion of the IL2 coding sequence to the C-terminus of either the H- or L-chain, was constructed from overlapping synthetic oligonucleotides by Blue Sky Biotech (Worcester, MA). The vector also contained the V region coding sequences of the mouse anti-GD2 antibody, 14.18 (Mujoo et al., 1987), as well a dihydrofolate reductase gene for selection in medium containing methotrexate. For fusions to the H-chain, the coding sequence of human IL2 was fused directly to the coding sequence of the H-chain C-terminus, by deleting the terminal lysine. Several versions of human IL2 coding sequences were fused directly to the codon for the C-terminal cysteine residue of the L-chain (Table 1) including: direct fusion of native IL2; the addition of a four residue spacer (Gln–Arg–Val–Asp) derived from the C-terminus of canine C kappa; and deletions of residues 5–9 or residues 1–9. All of the IL2 coding sequences were purchased from Genscript as SacI to NotI fragments and cloned into the unique restriction sites engineered into the pDHLP vector. The SacI site is naturally present in the coding sequence of human C kappa gene and the NotI was engineered between the stop codon of the IL2 sequences and the polyA addition site derived from the SV40 virus. Transient expression was initially performed in suspension cultures of 293F cells using the Freestyle System (Invitrogen) and the 293Fectin reagent. After 96 h, the IC in the culture media was purified by binding to and elution from protein A Sepharose, followed by neutralization and dialfiltration into phosphate-buffered saline (PBS). Later, transient transfections were performed using adherent 293T cells grown in FBS-containing medium using Lipofectamine and Plus Reagent (Invitrogen). For stable transfection of NS/0 cells, electroporation was used as described previously (Gillies et al., 1998), followed by selection in growth medium containing 100 nM methotrexate. ICs were purified from spent medium when cell cultures reached ~30% viability using protein A Sepharose chromatography.

IL2 bioactivity assays

IL2 bioactivity was measured using the murine CTLL-2 cell line expressing the high-affinity IL2R (Gillis et al., 1978) or with the human TF-1β cell line expressing only the intermediate affinity IL2R (Hori et al., 1987). In both cases, cells were collected from IL2-containing growth medium by centrifugation and re-suspended in growth medium without IL2. Serial dilutions of IL2-containing ICs in 0.1 ml of growth medium were prepared in 96-well plates, after which 0.1 ml of responder cells were added to all wells (final number of 10⁴ cells per well). After incubation for 72 h, 20 μl of Presto Blue Cell Viability Reagent (Invitrogen) was added to all wells and the plates were incubated for an additional 2–3 h at 37°C. Cell proliferation was measured by fluorescence caused by increased dye reduction that is proportional to the number of metabolically active cells. Fluorescence was quantitated using a GENios Pro fluorescent plate reader (excitation at 535 nm and emission at 590 nm).

De-glycosylation of ICs

Purified ICs (6 μg at 0.6 mg/ml in PBS) were incubated for 2 h at 37°C with N-glycosidase F (EMD Millipore, Cat. #362185) in a volume of 20 μl and then diluted to 0.6 ml with growth medium containing 10% FBS. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) showed that these conditions completely shifted the migration of the H-chain to the de-glycosylated form (data not shown).

### Table 1. Immunocytokine variants

<table>
<thead>
<tr>
<th>L-chain sequence</th>
<th>IC variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSLSLSPG_aptstqkktqlqlehllld20</td>
<td>IC36</td>
</tr>
<tr>
<td>KSLSLSPG_aptstqkktqlqlehllld30</td>
<td>IC35, IC65</td>
</tr>
<tr>
<td>KSLSLSPG_aptstqkktqlqlehllld60</td>
<td>IC45</td>
</tr>
<tr>
<td>KSLSLSPG_aptstqkktqlqlehllld90</td>
<td>IC46</td>
</tr>
<tr>
<td>KSLSLSPG_aptstqkktqlqlehllld120</td>
<td>IC64</td>
</tr>
<tr>
<td>KSLSLSPG_aptstqkktqlqlehllld150</td>
<td>IC34</td>
</tr>
</tbody>
</table>

The last eight residues of the human Cκ domain are shown fused to the amino termini of the IC variants used in this study, extending to the Asp20 (d20) residue involved in IL2R β-chain binding. The italicized sequence in IC36 is derived from the canine Cκ. The last 8 residues of the H-chain (without the terminal lysine) are shown fused to the first 20 N-terminal residues of IL2 (IC34).
Treated preparations were stored at −80°C until tested for IL2 bioactivity.

Bioactivity of ICs bound to anti-idiotype antibody-coated beads

The bioactivity of the IL2 component of ICs bound to GD2-expressing tumor cells was simulated using magnetic beads coated with the 1A7 anti-idiotype antibody generated against the 14.18 antibody (Foon et al., 1998). Dynabeads (Invitrogen) were coupled according to the manufacturer’s instructions using 10 mg of beads and 100 μg of 1A7 antibody. The final bead preparation contained 50 ng of antibody per μl of beads. For comparing the bioactivity of the different constructs in their bound state, 8 μl of beads were mixed with 200 ng of IC and incubated for 1 h at 37°C. Non-bound ICs were removed by washing the beads with RPMI growth medium without IL2 and serial 2-fold dilutions were added in duplicate to wells of a 96-well plate. When using CTL-L2 cells as responders, the IC-coated beads were diluted 5-fold before making serial dilutions. Responder cells (0.1 ml) were added to a final concentration of 10^4 cells per well and the plates were incubated for 48 h at 37°C. Proliferation was measured using Presto Blue Cell Viability Reagent as described above.

Effector activities of H- and L-chain fusion proteins

The ability of the ICs to mediate the cytotoxicity of GD2-expressing tumor cells (ADCC) was performed as described before (Gillies et al., 2002) using ^51Cr-labeled human M21 melanoma cells as targets and human peripheral blood monocytes (PBMC) as effectors. CDC was measured initially with ^51Cr-labeled M21 target cells and human complement (Gillies et al., 2002) and later using a dye-based cell viability assay. In the latter case, serial dilutions of ICs in 0.1 ml RPMI growth medium containing 10% FBS were made in a 96-well plate. M21 target cells were trypsinized, re-suspended at a concentration of 4 × 10^5 cells/ml in growth medium and added to the wells (50 μl per well). After a short incubation, 50 μl of human complement (1/8 dilution of pooled human plasma) was added to all of the wells and the plate was incubated at 37°C for 1 h. Cell viability was measured using Presto Blue Cell Viability Reagent, as described above for IL2 bioactivity. CDC activity was measured as a function of the loss of cell viability (fluorescence) compared with wells containing no added IC.

Flow cytometry

The binding of ICs to intermediate affinity IL2R-expressing TF-Iβ cells was measured by flow cytometry using an anti-human IgG antibody labeled with Alexa 647 (Invitrogen, Cat. #A-21445). Cells growing in IL2-containing medium were collected by centrifugation, washed with PBS, re-suspended in RPMI containing FBS but no IL2 and incubated at 2 h at 37°C to internalize any receptors bound to IL2. Cells were collected and washed twice with cold PBS, re-suspended at 10^6 cells/ml and incubated on ice with either IC34 or IC45 at the indicated concentration and after washing with cold PBS, the cells were incubated for an additional hour with the anti-human IgG antibody (1 μg/ml). After washing, the stained cells were analyzed using a Becton Dickinson Flow Cytometer.

Pharmacokinetic analysis

Female CD-1 mice were injected with ICs either by IV tail injection (1 mg/kg) or SC (2.5 mg/kg) injection. At various time points, blood samples (50 μl) were taken and collected in heparin-coated tubes to prevent clotting. After centrifugation, the amount of intact IC was measured in plasma using an enzyme-linked immunosorbent assay (ELISA), in which an anti-human IgG anti-serum was used for capture (Pierce-Thermo, Cat. #PA1-29961), and a biotinylated monoclonal anti-human IL2 antibody (R&D Systems, Cat. #BAF 202) and a streptavidin–horseradish peroxidase conjugate (Thermo Fisher, Cat. #N100) were used for detection. Animal studies were performed under contract at Vivisource Laboratories (Waltham, MA).

Results

Transient expression studies

Two considerations guided the initial attempt at designing fusions of IL2 to the C-terminus of the IgG L-chain. The first was the possible interference of the unpaired cysteine (C125) of IL2 in the assembly of the H–L dimer and subsequent pairing of the HL–HL chains to form an intact IC. The second consideration was the effect of amino acid residues adjacent to the C-terminal L-chain Cys, and the potential for interfering with IC assembly. To deal with the first issue, IL2 coding regions were synthesized encoding either the wild-type C125 or the C125S mutation. To address the second issue, IL2 coding regions were prepared with the normal IL2 mature N-terminus (IC35), or with a small spacer of four additional residues that normally follow the Cys residue in canine Ck L-chain (IC36). The rationale was that these adjacent sequences would not interfere with the H–L chain assembly (since they do not in dog antibodies) and similarly, might improve the assembly of fusion proteins.

Expression vectors containing these sequences and the V regions of an anti-GD2 antibody, 14.18, were used to transiently transfect human HEK 293 cells and the secreted products were purified using protein A and analyzed by SDS–PAGE before and after reduction. Results indicate that the mutation to C125S was not necessary, and actually detrimental to assembly and expression (not shown). Furthermore, the additional four residue spacer, derived from canine Ck was also unnecessary for efficient assembly, expression and protein stability, since construct IC35 was highly expressed and the same percentage was assembled into complete molecules as was seen with the H-chain fusion construct (Fig. 1). Based on these early results, the direct L-chain fusion construct (IC35) was selected for further study and compared with an IC constructed using the original H-chain fusion approach (IC34). Both of these proteins contained wild-type C125 in IL2 and no peptide spacers. IC34 is essentially the same as the original, extensively studied, ch14.18-IL2 molecule (reviewed in Davis and Gillies, 2003) except that it is missing the Lys residue between the end of the H-chain and the N-terminus of IL2 to improve its pharmacokinetic (PK) properties in vivo (Gillies et al., 2002).

Antigen binding and effector functions

The relative abilities of IC34 (H-chain fusion) and IC35 (L-chain fusion) to bind to antigen was first tested using an
anti-idiotype antibody (1A7) that mimics the GD2 antigen (Foon et al., 1998), and they were found to have the same binding activity (data not shown). A more relevant assay is binding to GD2 on tumor cells, and this is best tested by measuring the IC’s ability to mediate the effector functions of ADCC and CDC. These activities require binding to both the target antigen and subsequent engagement of either the Fc receptors (for ADCC) or the C1q complement protein (for CDC). When IC34 and IC35 were compared for their ability to mediate ADCC, again, they were found to have equivalent activity at comparable effector-to-target ratios and IC concentrations (Fig. 2A). This demonstrates that both bind equally well to cell-associated GD2 and that the juxtaposition of the IL2 molecule near the hinge-CH2 domain region (required for binding Fc receptors) did not reduce the ability of IC35 to mediate tumor cytotoxicity.

Surprisingly, the ability of IC35 to mediate CDC against melanoma target cells was significantly enhanced, compared with IC34, the hu14.18-IL2 IC and even the chimeric form of the parental antibody (Fig. 2B). Previous results with this and other ICs (with IL2 fused to the H-chain) routinely showed a 5-fold decrease in the ability to mediate CDC, relative to the parent antibody (Gillies et al., 2002, 2005). Like the FcR binding site, the critical site for C1q binding (and subsequent complement activation) is near the hinge-CH2 junction that would be in close proximity to the IL2 molecule in L-chain fusions.

Finally, when IC34 and IC35 were initially tested in the standard mouse CTLL-2 cell bioassay, they were found to have identical bioactivity (Fig. 2C), despite the very different locations of IL2 in the molecules—as an extension of the H-chain (IC34) or tucked into the cleft between the Fc region and the Fab arms (IC35). It should be noted that the response
to IL2 by this cell line is mediated by the high affinity form of the IL2R (αβγ IL2R).

**Pharmacokinetics in mice**

Groups of out-bred CD1 female mice were injected either IV or SC with IC34 or IC35 and the blood levels were compared over time using an ELISA that measures only intact fusion protein. IC34 had a significantly better PK profile following IV injection (Fig. 3A) due to the deletion of the Lys residue between the H-chain and IL2. The half-life of IC35 was significantly longer than IC34 (P = 0.015), with less IC cleared during the α-phase and a longer terminal half-life of ~16 h (vs. 6 h for IC34). The levels of intact IC in the blood of mice injected SC were also significantly higher (P = 0.02) in mice dosed with IC35 than with IC34 (Fig. 3B).

**IL2R specificity of L-chain fusions**

The increased circulating half-life of IC35 would increase vascular exposure to IL2 following IV injection and potentially increase toxicity such as hypotension and vascular leak syndrome. One way to decrease these toxicities is to create ICs (by mutating IL2) with reduced activation through the intermediate βγ IL2R (Gillies et al., 2011), and to compare their bioactivity using cells expressing the high-affinity αβγ IL2R (e.g. mouse CTLL-2 cells) or just the βγ IL2R (e.g. human mouse CTLL-2 cells).
Surprisingly, when the IC34 and IC35 constructs (both containing normal human IL2) were compared by using these two cell lines, they were found to differ by at least 8-10-fold, with IC35 being less able to activate the bg IL2R on TF-1β cells, but maintaining the ability to activate abg IL2R on CTLL-2 cells (Fig. 4). A possible explanation for this reduced activity might be restricted binding of IL2 Asp residue 20 (D20) to the IL2R β-chain when it is juxtaposed to the hinge region of the IC by fusion of IL2 to the terminal Cys of the L-chain.

To test this hypothesis, additional L-chain fusion ICs were constructed with differing polypeptide lengths between the Cys contact point (where the L-chain Cys forms a disulfide bond to the Cys in the H-chain hinge) and the first IL2 α-helix containing D20 (Table I). One construct has an increased polypeptide length (IC36) while two others have progressively shorter lengths of -5 residues (IC45) and -9 residues (IC46). As summarized in Table II, there was a direct relationship between the length of the polypeptide extending from the Cys disulfide bond to the D20 residue in helix A of
IL2, and the ability to activate the \( \beta \gamma \) IL2R expressed on TF-1β cells. The data were compiled from several individual bioassays, two examples of which are shown in Fig. 4A and B for cells expressing the \( \beta \gamma \) and \( \alpha \beta \gamma \) IL2Rs, respectively. Apparently, the decrease in polypeptide length had no effect on the ability to activate the \( \alpha \beta \gamma \) high-affinity IL2R, thus making these ICs progressively more specific (from 8- to >1000-fold) for the high-affinity receptor expressed on effect-, or T cells, while reducing the activation of receptors on NK and other immune cells associated with IL2-mediated toxicities. To assess whether the lack of receptor activation was due to a lack of receptor binding, the two ICs with the greatest difference in bioactivity (IC34 and IC45) were compared for binding with TF-1β cells and analyzed by flow cytometry. The results show a dramatic difference between them for binding to \( \beta \gamma \) IL2R on TF-1β cells (Fig. 4C). The effect of reducing the length of the N-terminus of IL2 was also tested in the context of antibody effector activity. The deletion of five residues (IC45) had little effect on the high CDC activity of IC35, while a deletion of nine residues appeared to have a more pronounced effect (Fig. 4D) that was statistically significant (\( P = 0.003 \)).

**Effect of de-glycosylation on IL2 bioactivity**

Since it is well known that the structural integrity of the CH2 domain of antibodies depends in part on the N-linked glycan, it might be possible that its removal could have an effect on IL2R selectivity by increasing the binding to the \( \beta \gamma \) IL2R. Two of the receptor-selective molecules, IC35 and IC45, were treated with N-glycosidase F to see if it affected the bioactivity of TF-1β cells. Results in Fig. 5 shows that the de-glycosylated forms of these ICs were less active by \( \sim 2 \)-fold, rather than more active, as a consequence of the less structured CH2 domain (and its ability to block \( \beta \)-chain binding to D20). Since de-glycosylation of ICs increases their circulating half-life, these results suggest that this approach can be used with L-IL2 ICs without an increased concern of \( \beta \gamma \) IL2R-related toxicity.

**Bioactivity of antigen-bound ICs**

The standard IL2 bioactivity assay tests the ability of free IL2 or IC molecules to engage and activate IL2R on responder cells. Since it is possible that L-chain fused IL2 is constrained by the Fab arms of the antibody component, an assay using antigen-coated beads (a model for IC targeted tumor cells) was designed to test whether the IL2 molecules in the bound form of the ICs were less constrained and thus, had an increased ability to activate the \( \beta \gamma \) IL2R on TF-1β cells. The antigen used in these studies was the 1A7 anti-idiotypic antibody that is reactive with 14.18V regions and mimics the ganglioside GD2 (Foon et al., 1998). 1A7-coated beads were mixed first with two H-IL2 ICs, one a negative control IC that does not contain the 14.18V regions, and the other IC34, that does. After binding for 1 h, the beads were washed and mixed with responder cells. The beads mixed with the negative control IC were able to induce proliferation of TF-1β (Fig. 6A), while beads mixed with IC34 induced proliferation in a dose-dependent manner. After demonstrating that proliferation was dependent on antigen-bound IC, this assay was used to compare IC34 with two L-chain fused molecules, IC35 and IC45. In solution, these molecules are roughly 10- and 500-fold less active than IC34 in their ability to stimulate the proliferation of TF-1β cells. In contrast, IC35 in its antigen-bound state was better able to activate \( \beta \gamma \) IL2R on TF-1β cells, with only a 2-fold difference from IC34 (Fig. 6B). IC45, with dramatically lower binding to \( \beta \gamma \) IL2R in solution, maintained its receptor selectivity in the antigen-bound state, i.e. it could not bind and activate the \( \beta \gamma \) IL2R.

**Table II. IL2R selectivity of IC variants**

<table>
<thead>
<tr>
<th>Immunocytokine</th>
<th>CTLLL-2 (high affinity) ED50 (ng/ml)</th>
<th>TF-1β (intermediate affinity) ED50 (ng/ml)</th>
<th>Relative activity high affinity/intermediate affinity</th>
<th>Selectivity for high-affinity IL2R</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.18-H-IL2 (34)</td>
<td>0.9–2.0</td>
<td>7.0–9.0</td>
<td>1.0/1.0</td>
<td>1</td>
</tr>
<tr>
<td>14.18-L-IL2 (35)</td>
<td>1.0–2.0</td>
<td>65–70</td>
<td>1.0/0.12</td>
<td>8.3</td>
</tr>
<tr>
<td>14.18-L-IL2 (36)</td>
<td>1.5–3.0</td>
<td>10–20</td>
<td>0.65/0.53</td>
<td>1.23</td>
</tr>
<tr>
<td>14.18-L-IL2 (45)</td>
<td>0.8–2.0</td>
<td>3000–4000</td>
<td>1.00/0.002</td>
<td>500</td>
</tr>
<tr>
<td>14.18-L-IL2 (46)</td>
<td>0.7–2.0</td>
<td>&gt;10 000</td>
<td>1.00/0.001</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>14.18-L-IL2 (64)</td>
<td>1.0–2.0</td>
<td>700</td>
<td>1.00/0.011</td>
<td>91</td>
</tr>
</tbody>
</table>

ED50 values were determined from an average of four separate assays. Relative activity is based on a comparison with the values obtained using IC34 which has IL2 fused to the H-chain.
When bound to the 1A7-coated beads, IC64 had the same high level activity as IC35 and IC45 using CTLL-2 cells (Fig. 6C), but in contrast to IC45, it showed high activity using TF-1 cells as well (Fig. 6D).

Discussion

IL2-based ICs in various forms have been studied in the clinic for more than a decade. For the most part, the maximum tolerated doses are based on side effects due to the IL2 component since, even when targeted by an antibody, these molecules transit through the vascular compartment for extended lengths of times before reaching the tumor microenvironment (King et al., 2004; Ko et al., 2004). The number and intensity of these side effects is proportional to their concentration in the blood ($C_{\text{max}}$) and occur shortly following injection. These observations are consistent with the activation of the intermediate $\beta\gamma$ IL2R on circulating immune cells causing these side effects, as proposed by multiple groups (Shanafelt et al., 2000; Gillies et al., 2011). Clinical studies using an IL2-based IC that is highly selective for the high-affinity $\alpha\beta\gamma$ IL2R, and only minimal activation of $\beta\gamma$ IL2R (Selectikine), have demonstrated a greatly reduced amount of IL2-related side effects at much higher IV doses given at increased infusion rates (Gillessen et al., 2013).

In order to extend these early results, and to try to optimize both tolerability as well as increased efficacy, a new IC platform utilizing cytokine fusion to the L-chain has been developed with features that appear favorable to this objective. Unlike Selectikine (which targets DNA in necrotic tissue), the current model system utilizes an antibody target (ganglioside GD2) on the tumor cell surface so that several other antibody and IC-specific anti-tumor activities can contribute to tumor cell killing (ADCC, CDC and IFCC) and in this way contribute to the anti-tumor response. Similar to Selectikine, the IL2R specificity has been altered to reduce the activation of $\beta\gamma$ IL2R (although not necessarily to the same extent) and maintain the binding to and activation of $\alpha\beta\gamma$ IL2R. This receptor specificity is especially important since it was shown that ICs based on L-chain fusion have much longer circulating half-lives in blood following either IV or SC administration. If such molecules had the same receptor specificity as IL2 or the original H-chain fusion constructs, this might only lead to increased toxicity. The ability to tune in the degree of receptor specificity, based simply on altering the length of the N-terminus of IL2, will make it possible to identify the optimal receptor selectivity and its effect on the balance between tolerability and anti-tumor efficacy. For example, an IC with a selectivity of 50–100 in favor of the high-affinity IL2R may be dosed at levels at least 10-fold higher than the MTD of the currently tested ICs. These dose levels would be consistent with those used for naked antibody therapy, in which the antibody itself mediates direct antibody killing through ADCC, CDC or even growth or survival signal blockade.
Such higher levels would also be expected to greatly increase the level of IC accumulating in the tumor. Previous studies with anti-GD2 ICs (as well as anti-EpCAM ICs) have demonstrated that increased circulating half-life (Gillies et al., 2002) or intra-tumoral injection (Yang et al., 2012) results in increased uptake as well as increased anti-tumor effects based on both innate and adaptive immune responses. With the use of L-chain-based IL2 fusions, one can gain from the longer circulating half-life after either IV or SC dosing methods, while at the same time preventing unwanted side effects that would otherwise be caused by the higher blood C\text{max}. Based on the current study using antigen-coated beads (to mimic targeted cancer cells), there is evidence that some of these constructs (e.g. IC35 and IC64) may increase their ability to activate βγ ILR once bound to cell surface antigen. This pro-drug type of effect could minimize uptake by and activation of systemic immune cells following injection and localize the full cytokine activity to the local tumor environment. The locally increased activation of both receptor forms could improve CD8+ T-cell proliferation, NK cell activation and prevent overstimulation of regulatory T cells, as seen in clinical studies with Selectikine (Gillessen et al., 2013).

Another interesting finding of the current study was that, rather than blocking the ability of the antibody component to bind to FcR (for ADCC activity) or complement C1q (for CDC activity), the L-IL2 ICs maintained a high level of ADCC activity and had greatly increased CDC activity against GD2-expressing tumor cells. This may be the result of bivalent binding of the Fab arms to the antigen-coated cell surface, which causes a conformational change allowing access to the hinge-CH2 region; however, it does not explain why CDC activity is at least 10-fold higher than the H-IL2 IC. In any case, the ability to induce direct anti-tumor killing through multiple mechanisms would be expected to increase both innate and adaptive immune responses by providing more tumor antigens to presenting cells, while at the same time activating them with IL2 and the additional cytokines and chemokines induced by the IC.

Finally, it will be important to study these new IL2R-selective forms of IC in tumor models that closely mimic the selectivity pattern intended for clinical development. As mentioned earlier, the original IC forms, based on H-chain fusion of human IL2, already have selectivity in mice for the αβγ IL2R between 10- and 20-fold, much like IC35 has shown for human immune cells. Therefore, if one intends on clinically developing an IC35-like molecule, the same receptor selectivity needs to be demonstrated using mouse immune cells. Preliminary data (P. Sondel, personal communication) indicates that IC35 has very low ability to activate mouse splenocytes and, as a result, we are currently designing and testing modified versions that have increased activity for the βγ IL2R in order to match the pattern using human immune cells). Such an approach has important implications not only for anti-tumor activity, but also for effects on receptor-mediated clearance, tumor targeting and drug tolerability.

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