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Cytokine therapy can activate potent, sustained antitumor responses, but collateral toxicity often limits dosages. Although antibody–cytokine fusions (immunocytokines) have been designed with the intent to localize cytokine activity, systemic dose-limiting side effects are not fully ameliorated by attempted tumor targeting. Using the s.c. B16F10 melanoma model, we found that a nontoxic dose of IL-2 immunocytokine synergized with tumor-specific antibody to significantly enhance therapeutic outcomes compared with immunocytokine monotherapy, concomitant with increased tumor saturation and intratumoral cytokine responses. Examination of cell subset biodistribution showed that the immunocytokine associated mainly with IL-2R–expressing innate immune cells, with more bound immunocytokine present in systemic organs than the tumor microenvironment. More surprisingly, immunocytokine antigen specificity and Fcγ receptor interactions did not seem necessary for therapeutic efficacy or biodistribution patterns because immunocytokines with irrelevant specificity and/or inactive mutant Fc domains behaved similarly to tumor-specific immunocytokine. IL-2–IL-2R interactions, rather than antibody–antigen targeting, dictated immunocytokine localization; however, the lack of tumor targeting did not preclude successful antibody combination therapy. Mathematical modeling revealed immunocytokine size as another driver of antigen targeting efficiency. This work presents a safe, straightforward strategy for augmenting immunocytokine efficacy by supplementary antibody dosing and explores underappreciated factors that can subvert efforts to purposefully alter cytokine biodistribution.

immunocytokine | biodistribution | immunotherapy | antibody | IL-2

Cytokines constitute a class of small immunomodulatory proteins, many of which possess tumoricidal properties useful for cancer immunotherapy (1, 2). One of the earliest successful cytokine therapies, IL-2, which has antitumor functions that include the activation of natural killer (NK) and cytotoxic T cells, can induce durable remissions in 5–10% of patients with metastatic melanoma and renal cell carcinoma, malignancies with poor prognoses (3). However, IL-2's potent immunostimulatory abilities often cause serious side effects, including life-threatening vascular leak syndrome, during systemic IL-2 administration. In theory, cytokine side effects might be mitigated by localizing cytokine activity to tumor tissues. One approach for tumor targeting has been to link cytokines to antibodies specific for tumor-associated antigens, generating immunocytokines (1, 2). Treatment with IL-2 immunocytokines has proven to be superior to treatment with equivalent antibody and cytokine given as separate agents (4–7). Nonetheless, systemic dose-limiting side effects have been observed after IL-2 immunocytokine administration, despite its expected tumor localization (8, 9).

Based on previous findings that antibody-dependent cell-mediated cytotoxicity (ADCC) plays an important role in immunocytokine efficacy (2), we hypothesized that IL-2 immunocytokine efficacy could be enhanced without introducing toxicity by the administration of additional tumor-specific antibody. We show in a syngeneic solid tumor model that IL-2 immunocytokine indeed synergized with antitumor antibody to significantly prolong

survival. Although the antibody component has generally been expected to mediate immunocytokine localization, we show instead that the IL-2 moiety entirely governed biodistribution, explaining our unexpected observation that immunocytokines recognizing irrelevant antigen performed equivalently to tumor-specific immunocytokines when combined with antibody. Mathematical modeling complemented our experimental results and highlights molecular size as another determinant of tumor-targeting outcome.

Results

Generation and Characterization of IL-2 Immunocytokines. Cytokine fusion to the IgG light-chain rather than the heavy-chain C terminus yields constructs that are more stable and possess greater effector functions (10, 11). To generate immunocytokines of this format, we fused murine IL-2 to full-length mouse IgG2a molecules with variable regions specific for either TRP1 antigen expressed by murine melanomas or CEA, a human tumor marker, to produce TA99-IL2 and sm3E-IL2, respectively (Fig. 1A and Dataset S1). The purified proteins were predominantly homogenous monomers (Fig. S1A) and showed the expected masses on SDS/PAGE (Fig. S1B). As confirmed by flow cytometry, both immunocytokines retained the ability to bind simultaneously to cell surface antigens and anti–IL-2 antibody (Fig. S1C). Assessment of simultaneous binding function also revealed excellent thermal stability and reasonable proteolytic stability for the two constructs (Fig. S1D). Moreover, the immunocytokines exhibited similar in vitro specific activity to WT IL-2 as measured by IL-2–dependent CTLL-2 cell proliferation (Fig. 1B). When injected into mice, TA99-IL2 and sm3E-IL2 displayed plasma β half-lives of 11–14 h (Fig. 1C and Table S1), considerably longer than that of WT IL-2, which is on the order of minutes (12). Both the

Significance

Cytokines (potent immunostimulatory proteins) exert powerful antitumor effects but often cause severe whole-body inflammation when used as cancer therapies. Contrary to the current paradigm that fusion to antitumor antibodies can constrain cytokine activity to tumors, we have found that, for some immunocytokines incorporating the cytokine IL-2, the cytokine moiety overrides antibody-mediated targeting, localizing the fusion protein to IL-2 receptor-expressing cells rather than tumor cells. Although the IL-2 immunocytokines did not selectively home to tumors, they persisted longer in circulation than free IL-2, such that a nontoxic immunocytokine dose could synergize with tumor-specific antibody to cure mice with aggressive solid tumors.
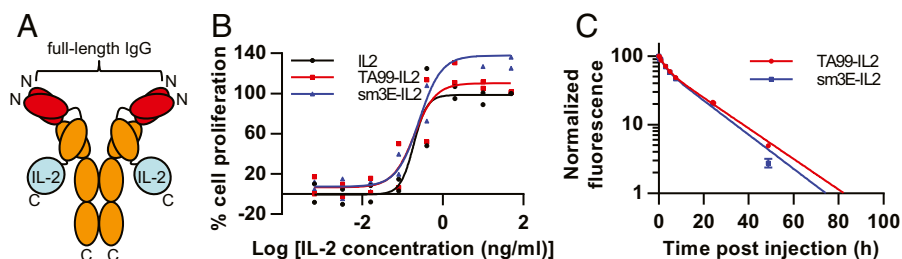
Fig. 1. Characterization of IL-2 immunocytokines. (*A*) IL-2 is fused to the light-chain C termini of an anti-TRP1 (TA99) or anti-CEA (sm3E) IgG molecule. (*B*) Immunocytokine bioactivity was measured by incubating CTLL-2 cells with varying concentrations of IL-2 species and detecting cell proliferation by WST-1 assay. Symbols indicate individual data points (normalized to control cells treated with 50 ng/mL IL-2); lines indicate fits to a four-parameter logistic curve. EC_{50} values were determined to be 0.1883 ng/mL (IL-2), 0.1943 ng/mL (TA99-IL2), and 0.2551 ng/mL (sm3E-IL2) from $n = 2$ independent experiments performed in triplicate. (*C*) Plasma clearance curves of Alexa Fluor 647-labeled immunocytokines (50 μg i.v.). Plasma mean fluorescence intensities (symbols) were normalized to the initial time point and fit to a biphasic exponential decay curve (lines). Error bars depict SEM ($n = 3$).

immunocytokines' larger size and the presence of the antibody Fc region, allowing recycling through the neonatal Fc receptor, likely account for this extended plasma persistence. Injection of immunocytokines but not antibody also resulted in splenomegaly, providing additional evidence of bioactivity (Fig. S1*E*).

Combination Therapy Using Immunocytokine and Tumor-Specific Antibody Provides Synergistic Antitumor Responses. We tested the antitumor efficacy of TA99-IL2 by administering it to C57BL/6 mice bearing established syngeneic B16F10 melanoma tumors, which express TRP1. To circumvent IL-2 toxicity, we used an immunocytokine dosing regimen that did not induce systemic toxicity as measured by weight loss (Fig. S2*A*). Despite significantly delaying tumor growth compared with PBS, TA99-IL2 was insufficient for prolonged tumor control (Fig. 2*A* and Fig. S2*B*). We hypothesized that the lack of durable remissions could be caused by insufficient tumor saturation with the immunocytokine, because antitumor activity has been correlated with a therapeutic antibody's ability to penetrate throughout tumor tissue (13). Because antitumor antibodies such as TA99 are generally far better tolerated than cytokines and can independently elicit tumoricidal immunity (14), we combined our original TA99-IL2 dose with additional TA99 murine IgG2a antibody to attain a dosage theoretically predicted to fully saturate the tumors (15). This combination therapy significantly improved the survival of tumor-bearing mice compared with treatment with TA99-IL2 alone, yielding long-term survivors (Fig. 2*A* and Fig. S2*B*). The additional antibody did not lead to systemic toxicity (Fig. S2*A*), although normal melanocytes also express TRP1. Interestingly, when mice were treated with equimolar WT murine IL-2 in lieu of TA99-IL2 concurrently with TA99, survival was similar to that of PBS-treated controls (Fig. 2*A* and Fig. S2*B*), indicating that some attribute of the immunocytokine [e.g., size, antigen specificity, or Fcγ receptor (FcγR) binding] was required for synergistic efficacy.

To determine if treatment efficacy corresponded with tumor saturation by therapeutic agents, we generated a B16F10 cell line that stably expressed EGFP (Fig. S3). We then assessed tumor saturation by injecting Alexa Fluor 647-conjugated TA99-IL2 and/ or TA99 into mice bearing B16F10-GFP tumors and analyzing fluorescent protein binding to resected disaggregated tumor cells by flow cytometry. Although the combination of TA99-IL2 and TA99 resulted in nearly complete tumor saturation, TA99-IL2 alone labeled <2% of GFP⁺ tumor cells (Fig. 2*B*). Strikingly, a 25.4-μg TA99 dose equimolar to the TA99-IL2 dose yielded significantly higher saturation (Fig. 2*B*), although TA99-IL2 bound readily to B16F10 cells in vitro (Fig. S1*C*). Thus, the presence of IL-2 in the immunocytokine significantly reduced the degree of antibody saturation in the tumor. These results indicate that tumor saturation with antibody is necessary but not sufficient for effective tumor growth control in this model. When we

monitored the intratumoral immune response posttreatment using a 32-plex cytokine assay, we detected a general increase in cytokine expression after administration of TA99-IL2 + TA99 vs. IL-2 + TA99 or TA99-IL2 (Fig. 2*C*). Mice treated with TA99-IL2 + TA99 exhibited significantly higher expression of MIG and MIP-2
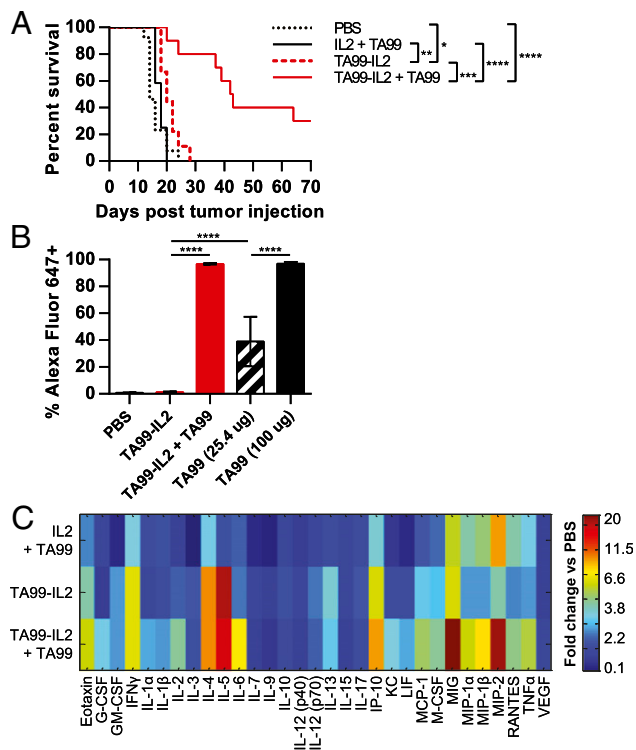

Fig. 2. Tumor-specific antibody synergizes with IL-2 immunocytokine for improved antitumor responses. (*A*) Kaplan–Meier survival curves for mice inoculated s.c. with 10^6 B16F10 tumor cells and then treated on days 6, 12, 18, 24, and 30 with i.v. PBS, 6 μg IL-2 + 100 μg TA99, 32 μg TA99-IL2, or 32 μg TA99-IL2 + 100 μg TA99 ($n = 9–13$). (*B*) Mice bearing established s.c. B16F10-GFP tumors were injected i.v. with Alexa Fluor 647-labeled TA99-IL2 (32 μg) and/or TA99 (100 μg unless otherwise noted), and tumors were harvested 24 h later for flow cytometry ($n = 5$). Percentages of Alexa Fluor 647⁺GFP⁺ cells are shown (mean percentage ± SEM). (*C*) Heat map depicts differential intratumoral cytokine responses in mice bearing established s.c. B16F10 tumors after treatment with IL-2 + TA99, TA99-IL2, or TA99-IL2 + TA99 compared with PBS-treated controls ($n = 5–6$). Mice were injected with the doses specified in *A*, and 24 h later, tumors were resected and homogenized for analysis using a bead-based multiplex assay. Color bar varies on a logarithmic scale. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

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($P < 0.0001$ vs. IL-2 + TA99 and TA99-IL2), chemokines known to recruit antitumor effector cells, including NK cells, T cells, and neutrophils (16, 17). The chemokine IP-10, a close relative of MIG, was also significantly up-regulated in mice treated with TA99-IL2 + TA99 compared with those treated with IL-2 + TA99 ($P < 0.05$). Overall, elevated intratumoral cytokine responses corresponded with better prognosis, linking immune activation with antitumor efficacy.

Synergy Between Tumor-Specific Antibody and Immunocytokines Requires Neither Immunocytokine Antigen Specificity nor FcγR Interactions. Fusion of a full-length IgG to a cytokine allows not only targeting through the antibody variable region, but also ADCC and complement-dependent cytotoxicity effector functions and extended half-life through the Fc region. To examine which of these properties contributes to immunocytokine synergy with TA99, we compared the effectiveness of various immunocytokines. Surprisingly, treatment with sm3E-IL2, which recognizes an antigen absent in mice, had an almost identical effect on B16F10 tumor growth as TA99-IL2 therapy, acting synergistically with TA99 to promote long-term survival (Fig. 3A and Fig. S2). This unexpected observation shows that tumor targeting by the antibody variable region is dispensable for synergy with TA99. A single point mutation (D265A) in the antibody Fc region ablates interactions with FcγRs and complement, abolishing IgG effector functions (18). We introduced the D265A mutation to TA99-IL2 and sm3E-IL2 and found that the D265A immunocytokines did not perform significantly differently from their parent immunocytokines (Fig. 3A and Fig. S2), suggesting that immunocytokine Fc-mediated effector functions are also nonessential for effective combination therapy with TA99. Neither antigen specificity nor effector function competence affected immunocytokine saturation of established B16F10-GFP tumors, because there were no significant labeling differences among groups treated with fluorescently labeled immunocytokines (Fig. 3B). This observation contradicts predictions that tumor-targeted agents should display greater tumor accumulation than their nontargeted counterparts. After coinjection of fluorescently labeled TA99, ~100% of GFP⁺ cells were bound to fluorescent therapeutic agents (Fig. 3B). Because immunocytokine antigen specificity and Fc effector functions both were unnecessary for therapeutic synergy with TA99, IL-2 plasma persistence is likely the key factor in the combination therapy. Moreover, IL-2 with a short half-life did not produce durable remissions when coinjected with TA99 (Fig. 2A).

The IL-2 Moiety Governs Immunocytokine Localization. Our unanticipated findings that immunocytokine antigen specificity did not affect therapeutic efficacy or immunocytokine distribution to tumor cells (Figs. 2 and 3) prompted us to develop an assay for tracking immunocytokine and antibody localization at a cellular level. A similar approach has been successfully used in monitoring nanoparticle trafficking (19). We first examined the distribution of TA99 antibody and found that, as expected, TA99 binding was enriched intratumorally for immune populations [e.g., dendritic cells (DCs), macrophages, and NK cells] known to express FcγR (14, 20), which can interact with the antibody Fc region (Fig. 4). This enrichment presumably stems from tumor localization mediated by antibody recognition of the TRP1 antigen on the surface of tumor cells. In the blood and spleen, monocytes/macrophages exhibited the greatest percentage of binding to TA99 (Fig. 4), consistent with the high expression of FcγR by these cells (20). However, IL-2 fusion drastically altered the binding distribution pattern of its partner, and for TA99-IL2, NK and natural killer T (NKT) cells dominated binding. Nearly all NK cells and a high percentage of NKT cells in the analyzed organs were bound to TA99-IL2 (Fig. 4). In contrast, a lower percentage of monocytes/macrophages, particularly in the tumor,
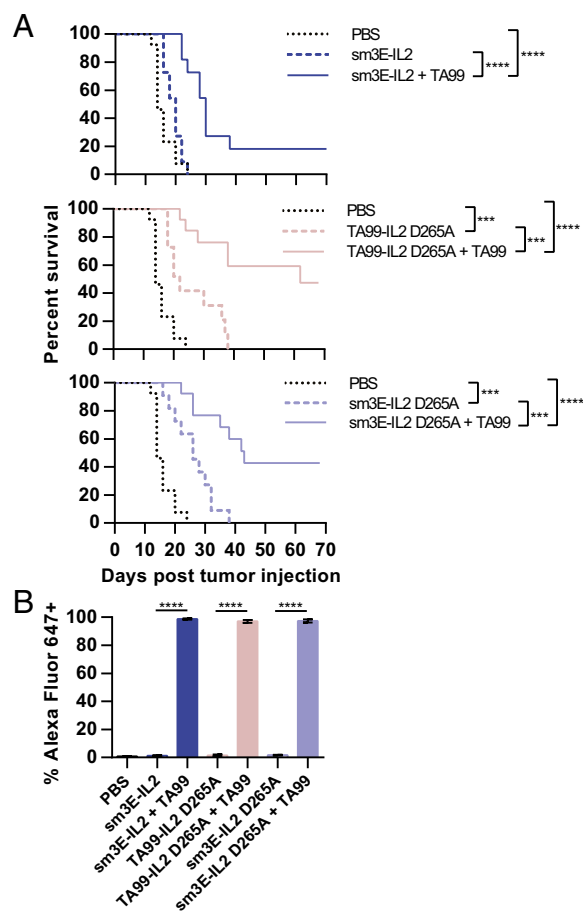

Fig. 3. IL-2 immunocytokine antigen specificity and Fc receptor interactions are dispensable for therapeutic synergy with tumor-specific antibody. (A) Kaplan–Meier survival curves for mice inoculated s.c. with 10⁶ B16F10 tumor cells and then treated on days 6, 12, 18, 24, and 30 with i.v. PBS, 32 μg sm3E-IL2, 32 μg sm3E-IL2 + 100 μg TA99, 32 μg TA99-IL2 D265A, 32 μg TA99-IL2 D265A + 100 μg TA99, 32 μg sm3E-IL2 D265A, or 32 μg sm3E-IL2 D265A + 100 μg TA99 (n = 10–13). (B) Mice bearing established s.c. B16F10-GFP tumors were injected i.v. with therapeutic dosages of Alexa Fluor 647-labeled versions of the indicated agents, and tumors were harvested 24 h later for flow cytometry (n = 5). Percentages of Alexa Fluor 647⁺GFP⁺ cells are shown (mean percentage ± SEM). ***P < 0.001; ****P < 0.0001.

showed binding to TA99-IL2 vs. TA99 (Fig. 4 and Fig. S4A), although no differences in absolute cell numbers were observed (Fig. S4B). Notably, ~80% of circulating DCs were TA99-IL2–bound (Fig. 4). Greater percentages of intratumoral CD8⁺ T cells and Tregs bound to the immunocytokine compared with corresponding populations from other organs, which is attributable to the IL-2 moiety given that both cell types do not express FcγR and did not bind to naked TA99 (Fig. 4). Remarkably, the binding distribution of sm3E-IL2 closely mirrored that of TA99-IL2 (Fig. 4), showing that the antigen specificity of the parent antibody had negligible effects on immunocytokine localization.

To determine the contribution of Fc–FcγR interactions to immunocytokine localization, we monitored the binding distribution of D265A mutant immunocytokines. In most cases, ablation of Fc–FcγR interactions did not change the distribution patterns. However, for all analyzed compartments, a higher percentage of Tregs bound to the D265A than to the corresponding parent immunocytokine (Fig. S4A). Intratumoral CD8⁺ T cells also showed greater binding to sm3E-IL2 D265A than to sm3E-IL2 (Fig. S4A). Although the parent immunocytokines could conceivably mediate immune cell depletion through their intact
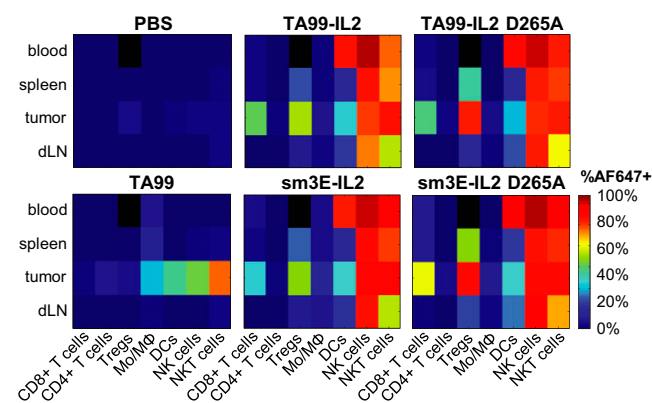
Fig. 4. The IL-2 moiety targets immunocytokines to IL-2R–expressing immune cells both systemically and tumor proximally, regardless of antigen specificity or Fc receptor interactions. Mice bearing established s.c. B16F10 tumors were injected i.v. with equimolar doses of Alexa Fluor 647-labeled proteins (n = 5); 24 h later, organs were collected, rendered into single-cell suspensions, and stained with immune cell lineage markers before flow cytometric analysis. Heat maps depict Alexa Fluor 647$^+$ percentages of indicated cell lineages in the blood, spleen, tumor, or draining lymph node (dLN).

effector activity, there were no significant differences in Treg or CD8$^+$ T-cell numbers between mice treated with D265A and parent immunocytokines (Fig. S4B). Overall, the four examined immunocytokines had only the IL-2 component in common but displayed distribution patterns strikingly similar to one another and distinct from TA99 (Fig. 4 and Fig. S4A). In addition, the immune cell lineages exhibiting the highest percentage binding to immunocytokines have all been documented to express IL-2R but not necessarily FcγR at appreciable levels in previous studies (14, 21) and our own experience (Fig. S5). Ex vivo blood binding analysis further corroborated the existence of distinct FcγR- and IL-2R-expressing cell populations that act as sinks for therapeutic proteins containing intact Fc or IL-2 domains, respectively, in a dose- and affinity-dependent manner (Fig. S4C). These data strongly suggest that the IL-2–IL-2R interaction was the main determinant of these immunocytokine distributions, with Fc–FcγR interactions playing, if anything, a minor role.

Immunocytokines Predominantly Remain Systemic and Associate with Innate Immune Cells Regardless of Antigen Specificity or FcγR Interactions. Quantifying the percentages of immunocytokine-bound (IC$^+$) cells highlights the immune cell lineages showing the greatest relative binding to immunocytokines but does not allow comparisons of immunocytokine abundances between different organs or cell types. For each immune lineage of interest, we approximated the amounts of immunocytokine associated with each organ by multiplying the total number of IC$^+$ cells from that organ by the median fluorescence intensity, which is proportional to the number of fluorescent molecules binding to the cells. For all four immunocytokines, the partitioning patterns among organs were remarkably similar (Fig. 5A). Immunocytokines bound to the DC, NK cell, and NKT cell populations were mostly found in the spleen, with a smaller percentage located in the blood. Meanwhile, more than 80% of immunocytokines bound to CD8$^+$ T cells and Tregs were found in the spleen, with very little in the blood. In contrast, only a small proportion of immunocytokines localized in the tumor, and even less was found in the draining lymph node. Because these immunocytokines nonetheless showed therapeutic efficacy when combined with TA99 antibody (Figs. 2A and 3A), high tumor uptake is not necessary for effective antitumor therapy. Ablation of Fc–FcγR interactions did not appreciably alter organ distribution (Fig. 5A). Although the tumor-specific TA99-IL2 had been expected to accumulate to

a greater extent in tumors than the nontumor-specific sm3E-IL2, the two immunocytokines actually displayed similar tumor uptake proportions (Fig. 5A). These results indicate that neither antigen specificity nor Fc mutations greatly impact immunocytokine distribution at the organ level and that the majority of immunocytokine associates with systemic rather than tumor-proximal immune cells. Next, we estimated the fraction of immunocytokine or antibody bound to each immune cell lineage by summing the products of IC$^+$ lineage cells and median fluorescence intensity for the analyzed organs. The largest proportion of all four immunocytokines was associated with NK cells, with DCs and NKT cells harboring the second and third largest proportions, respectively (Fig. 5B). In contrast, NK cells bound a smaller percentage of TA99 antibody, and monocytes/macrophages and NKT cells played far greater roles in interacting with TA99 than with the immunocytokines (Fig. 5B). Once again, the distribution patterns of TA99-IL2 were distinct from those of TA99 and closely resembled those of sm3E-IL2, TA99-IL2 D265A, and sm3E-IL2 D265A, suggesting that for these immunocytokines, antibody variable and Fc region-mediated interactions were largely superseded by IL-2–mediated interactions.

Mathematical Modeling Reveals Size Dependence of Immunocytokine Targeting. To further explore additional parameters for immunocytokine targeting effectiveness and the generalizability of our observations, we developed a simple ordinary differential equation (ODE) model (Fig. 6A and Fig. S6). We modeled the two most common formats for immunocytokines currently in clinical trials (1, 2): large (full-length IgG) and small (diabody consisting of dimeric single-chain variable fragment). Moreover, we
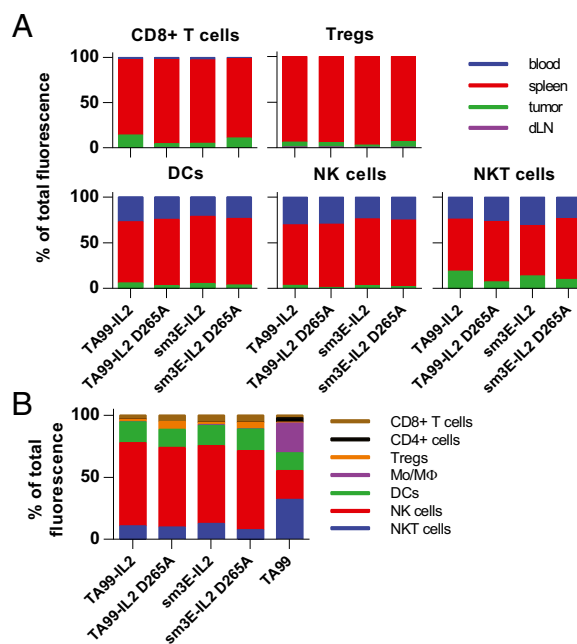
Fig. 5. Most immunocytokine localizes outside the tumor and associates mainly with innate immune cells, regardless of antigen specificity or Fc receptor interactions. Mice bearing established s.c. B16F10 tumors were injected i.v. with equimolar doses of Alexa Fluor 647-labeled proteins, and organs were collected and processed as in Fig. 4 (n = 5). (A) For each indicated immune cell lineage, the total fluorescence resulting from cell-bound immunocytokines was calculated by summing the following quantity for the examined organs: total IC$^+$ cell number × median fluorescence intensity. (B) The total fluorescence resulting from immunocytokines bound to all examined immune cell lineages was calculated by summing the total fluorescence values for each cell lineage calculated in A. dLN, draining lymph node.
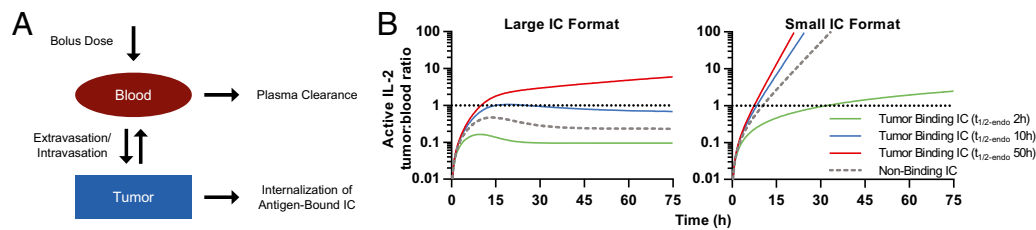
Fig. 6. A simple ODE-based mathematical model of immunocytokine distribution shows that immunocytokine targeting depends highly on construct size. (*A*) A simplified model schematic. In this two-compartment model, the immunocytokine can be cleared from the blood or the tumor or undergo inter-compartmental transport by extravasation or intravasation. (*B*) Tumor-to-blood ratios of active immunocytokine exposure for (*Left*) large, full-length IgG and (*Right*) small diabody-based formats. The dotted black line indicates equal exposure in tumor and blood. IC, immunocytokine.

considered different rates of tumor antigen endocytosis to account for antigen targets with varying turnover rates. As validation, we input pharmacokinetic parameters from several prior studies of therapeutic protein distribution into our model and found that the predicted biodistributions closely matched experimental data (Fig. S7). Unlike experiments using radio- or fluorescently labeled immunocytokines, this model distinguishes when immunocytokines are active (extracellular and capable of signaling through cytokine receptors). To assess the effects of tumor targeting, we computed expected tumor-to-blood exposure ratios for large and small immunocytokines. Consistent with our experimental results, the predicted tumor-to-blood ratios for active large immunocytokines are poor, even with a very slow rate of antigen endocytosis (Fig. 6*B*). Notably, large immunocytokines recognizing rapidly endocytosed antigens have tumor-to-blood ratios <1, localizing to tumors to an even lesser extent than immunocytokines with irrelevant antigen specificity, because fast turnover translates to a depletion effect. In contrast, small immunocytokines have much better predicted tumor-to-blood exposure ratios at varying endocytosis rates (Fig. 6*B*). These results indicate that another major determinant of immunocytokine targeting efficiency is the balance between systemic and tumor clearance. Small immunocytokines have much faster blood clearance than large ones, minimizing systemic exposure, whereas tumor clearance mostly depends on antigen turnover rates, which are unaffected by immunocytokine size. Hence, for the same antigen target, small immunocytokines can achieve higher tumor-to-blood ratios than large ones. Another way to view the benefits of immunocytokine tumor targeting is to determine relative cytokine activities in different compartments based on the EC_{50} of IL-2. Although large immunocytokines maintain constant activities ~100% in both the blood and tumor, small immunocytokines can achieve a window in which the cytokine is active in the tumor but not in the blood (Fig. S8). Importantly, in the model, we did not account for immunocytokine uptake into extratumoral cellular sinks to parse out the effects of size independent of this process. However, as we have shown (Figs. 2*B* and 5), immunocytokine consumption outside the tumor can dominate overall distribution.

Discussion

Immunocytokines are designed to localize cytokine therapy to tumors but still suffer from systemic dose-limiting toxicities (5, 9). We have shown that tumor-specific antibody synergizes with IL-2 immunocytokines to produce long-term remissions in the poorly immunogenic B16F10 tumor model. Similarly, cotherapy with a tumor vasculature-directed IL-2 immunocytokine and an anti-CD20 antibody significantly enhanced survival in a lymphoma xenograft model in immunocompromised mice lacking T cells (6); we report here for the first time, to our knowledge, an immunocytokine/antibody combination strategy for the treatment of established syngeneic solid tumors in mice with an intact immune system. Surprisingly, it is the IL-2 moiety rather than

immunocytokine antigen specificity that almost completely determines cellular and organ biodistribution. Neither immunocytokine tumor targeting nor effector functions are required for or even seem to contribute to effective combination therapy. Using a mathematical model, we show that, in general, slow systemic clearance may further impair antigen targeting efficiency by IgG-based immunocytokines, such as the ones tested here.

This study shows that the only essential contribution of antibody fusion to IL-2 efficacy in this model is prolonging IL-2 plasma half-life from minutes to roughly one-half of a day. This observation is consistent with previous hypotheses that IL-2's rapid clearance hampers its therapeutic use (21, 22). In the clinic, IL-2 therapy requires frequent infusions (3), and in a preclinical trial using the hu14.18-IL2 immunocytokine, which has a half-life of 3–4 h, complete eradication of murine neuroblastoma required combination with continuous IL-2 administration (23). Saturation of tumor cells by ADCC-activating molecules and a robust intratumoral cytokine response likely account for the impressive antitumor activity exhibited by the immunocytokine/TA99 antibody combination therapies (Figs. 2 and 3). Additional work is necessary to definitively identify key immune cell types and mechanisms underlying the synergistic tumor control observed for IL-2 immunocytokines and TA99.

Numerous reports have characterized immunocytokine biodistribution at the organ level (1), but to our knowledge, this is the first to examine the cellular-level distribution of multispecific proteins, allowing us to identify unexpected trends in localization patterns. Although naked TA99 was driven by antigen recognition to accumulate intratumorally, fusion to IL-2 introduced a competing IL-2–IL-2R interaction that overrode both antigen specificity and FcγR interactions as the key mechanism for localization (Figs. 4 and 5). In retrospect, IL-2R binding probably explains the significantly reduced association of TA99-IL2 with tumor cells compared with that of equimolar TA99 (Fig. 2*B*). Similar instances of cytokine–cytokine receptor-dominated biodistribution for tumor vasculature-targeted IFN-γ immunocytokines have been observed (24, 25). Moreover, the markedly faster plasma clearance of immunocytokines relative to their parent antibodies as reported by several groups (26, 27) may reflect extensive binding of immunocytokines to IL-2R–expressing cells. Circulating immune cells expressing CD122, which may correspond to the immunocytokine-bound NK cells that we detected, have been linked to IL-2 toxicity (28) but may also contribute to therapeutic efficacy. Overall, the relationship between immunocytokine binding and efficacy or toxicity requires additional investigation.

It may seem paradoxical that NK and NKT cells, which express the intermediate-affinity IL-2R, bind a greater proportion of immunocytokine than Tregs and activated CD8^{+} T cells, which express the high-affinity IL-2R. However, NK and NKT cells are generally more abundant than Tregs and activated CD8^{+} T cells, especially in the blood (29), the first point of contact for i.v.-administered immunocytokines. On entering the blood, immunocytokines first encounter and bind to NK and NKT cells,

which may preclude efficient distribution to other sites that contain Tregs and activated CD8$^+$ T cells. Analogously, although the affinities of TA99 for TRP1 and IL-2 for intermediate-affinity IL-2R are both ~10^{-9} M (21, 30), i.v.-injected immunocytokines encounter and associate with circulating IL-2R–expressing immune cells well before they can reach TRP1$^+$ tumor cells, resulting in predominant immunocytokine accumulation in systemic rather than tumor-proximal compartments.

Our results suggest that in targeted therapy development, the biodistribution of any construct with multiple potential targeting interactions should be directly assessed rather than assumed. If the desired antigen targeting is not achieved, several general strategies may be used to promote selective localization, with the caveat that such targeting may not be required for therapeutic efficacy, which was the case for this study. For accessible solid tumors, intratumoral administration may exhibit superior antitumor activity and reduced systemic toxicity compared with the i.v. route (31–33), consistent with our findings that i.v.-injected immunocytokine resided predominantly in the blood and spleen rather than the tumor (Fig. 5A). Antigen choice also impacts targeting efficacy: in contrast to our results, several studies have shown that immunocytokines specific for antigens expressed by tumor vasculature or liquid/disseminated tumors perform better than irrelevant immunocytokines (4, 7, 34), likely because these antigens are in closer contact with the blood and thus, more accessible than solid tumor antigens. Alternatively, the affinity of the cytokine for its receptor can be weakened, so that cytokine function is potentially restored only on high-avidity binding to tumor cells (35). Finally, our mathematical model suggests that small-format immunocytokines may intrinsically exhibit better antigen-targeting specificity than large-format immunocytokines because of size-dependent transport properties (rapid systemic clearance and diffusion by smaller molecules).

In conclusion, we have explored a facile strategy for improving immunocytokine antitumor efficacy and uncovered limitations for selective targeting by multicomponent constructs. Addition of tumor-specific antibody to existing immunocytokine therapies may be a promising option for enhancing clinical outcomes without introducing incremental toxicity. Because extended plasma persistence seems to be far more important than tumor localization for effective IL-2 synergy with naked antibody, simply prolonging IL-2 circulation by fusion to serum albumin or antibody Fc region may allow equivalent efficacy when combined with tumor-specific antibody. This nontargeted, persistent cytokine could be readily coadministered with clinically approved antibodies, circumventing the need to develop a new immunocytokine for each tumor antigen. Future studies will be needed to validate these experimental and theoretical concepts in additional tumor models using a variety of cytokine moieties, antigen targets, and immunocytokine formats.

Materials and Methods

Detailed methods are provided in *SI Materials and Methods*. In summary, IL-2 immunocytokines were generated by PCR-based cloning, produced by transient HEK293-F cell transfection, purified using protein A beads, and characterized in vitro and in vivo. TA99 antibody was produced by a stable HEK293-F cell line and purified using protein A beads. Fluorescently labeled proteins were generated by reaction with Alexa Fluor 647 NHS ester. C57BL/6 mice were inoculated s.c. with 10^6 B16F10 or B16F10-GFP tumor cells, and tumors were allowed to establish before treatment with i.v. IL-2, immunocytokine, and/or TA99 for tumor control assessment, intratumoral cytokine measurement, or flow cytometry analysis. Mathematical modeling was performed using MATLAB.

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