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Letters

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Chimera of IL-2 Linked to Light Chain of anti-IL-2 mAb Mimics IL-2/anti-IL-2 mAb Complexes Both Structurally and Functionally

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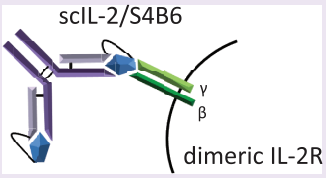
Supporting Information

ABSTRACT: IL-2/anti-IL-2 mAb immunocomplexes were described to have dramatically higher activity than free IL-2 *in vivo*. We designed protein chimera consisting of IL-2 linked to light chain of anti-IL-2 mAb S4B6 through flexible oligopeptide spacer $(Gly_4Ser)_3$. This protein chimera mimics the structure of IL-2/S4B6 mAb immunocomplexes but eliminates general disadvantages of immunocomplexes like possible excess of either IL-2 or anti-IL-2 mAb and their dissociation to antibody and IL-2 at low concentrations. This novel kind of protein chimera is characterized by an intramolecular interaction between IL-2 and binding site of S4B6 mAb similarly as in IL-2/S4B6 mAb immunocomplexes. Our protein chimera has biological activity comparable to IL-2/S4B6 mAb immunocomplexes *in vitro*, as shown by stimulation of proliferation of purified and activated OT-I $CD8^+$ T cells. The protein chimera exerts higher stimulatory activity to drive expansion of purified CFSE-labeled OT-I $CD8^+$ T cells activated by an injection of a low dose of SIINFEKL peptide than IL-2/S4B6 mAb immunocomplexes *in vivo*.



The *in vivo* biological activity of IL-2 can be dramatically increased by complexing IL-2 with certain anti-IL-2 mAb.[1] Moreover, these IL-2 immunocomplexes have selective stimulatory activity depending on the clone of anti-IL-2 mAb used (Supplementary Figure 1). IL-2/S4B6 mAb immunocomplexes (henceforth, IL-2ic) are highly stimulatory for memory $CD8^+$ T and NK cells ($CD122^{high}$ populations).[1,2] They have also moderate stimulatory activity for T_{reg} cells.[3] Conversely, IL-2/JES6.1 mAb immunocomplexes have no effect on $CD122^{high}$ cell populations, but they considerably expand T_{reg} cells ($CD25^{high}$ population).[1,4] Interestingly, both IL-2 immunocomplexes are very potent in expanding recently activated naïve $CD8^+$ T cells *in vivo*,[5] and IL-2ic possess significant antitumor activity.[2,3]

IL-2ic are prepared simply by mixing rmIL-2 and S4B6 mAb at molar ratio 2:1 (Figure 1A). In theory, this should lead to formation of IL-2/antibody complexes without either protein left (omitting the K_d). However, it is likely that one protein will be in small excess since it is very hard to exactly quantify and handle S4B6 mAb and IL-2, especially when small batches of IL-2ic are prepared (typically, 10−100 μg of IL-2 is complexed for laboratory experiments). In order to make a more defined structure, we designed chimeric protein of IL-2 and S4B6 mAb (henceforth, scIL-2/S4B6) where the C-terminus of IL-2 is linked via a flexible oligopeptide spacer to N-terminus of light chain of S4B6 mAb (Figure 1B). IL-2 could not dissociate far away from scIL-2/S4B6 like from IL-2ic where the interaction between IL-2 and binding site of S4B6 mAb is interrupted (Figure 1A,B). Instead, IL-2 stays in the vicinity of the binding site of S4B6 mAb (the length of the spacer defines maximal distance), and thereby, it is continuously available for reassociation (Figure 1B). Although this difference between scIL-2/S4B6 and IL-2ic does not have probably any measurable effect on biological activity at sufficiently high and stable concentrations, it might play a significant role at low and gradually decreasing concentrations, and thus, it could be the factor making scIL-2/S4B6 superior to IL-2ic in certain situations, for example, *in vivo*. The structure of scIL-2/S4B6 is a novel one since fusion protein of cytokine linked to N-terminus of light chain of respective anticytokine mAb has not been, to our best knowledge, described so far. Chimeras where IL-2 was linked to the C-terminus of the heavy chain of mAb (Figure 1C) recognizing tumor antigens were reported.[6−8] Nevertheless, these protein chimeras were designed to deliver IL-2 into tumor microenvironment and thereby induce antitumor immunity. IL-2 is linked to mAb molecule, but at very different sites, and there is not any binding interaction between IL-2 and antibody. These chimeras thus do not mimic cytokine/anticytokine mAb immunocomplexes at all. IL-2 was also linked to the Fc part of the antibody (Figure 1D), the structure known as immunocytokine, with the aim to prolong the half-life of the cytokine in circulation.[9,10]

The aim of this study was to design and produce scIL-2/S4B6 as a recombinant protein. We wanted also to characterize the protein we produced and to verify whether it contains antibody and IL-2 within one protein molecule and possess predicted molecular weight both in reducing and nonreducing conditions. Further characterization of our protein chimera was focused on proving that IL-2 in this chimera interacts with the binding site of antibody in cis and thus showing that this

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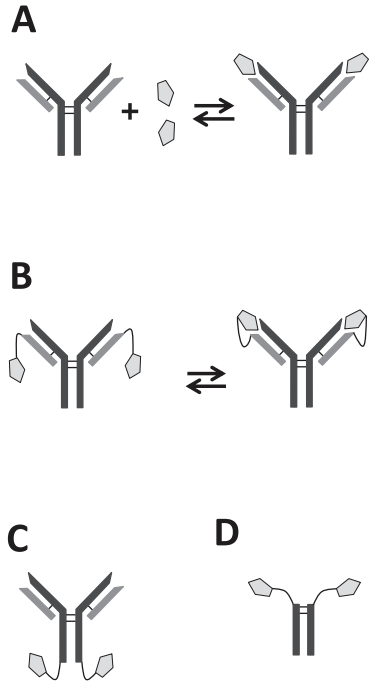

Figure 1. Schematic chart showing approaches of how to increase the biological activity of IL-2 *in vivo*. (A) IL-2 and anti-IL-2 mAb form immunocomplexes, but these can also dissociate back to free IL-2 and anti-IL-2 mAb. (B) Protein chimera mimicking IL-2 immunocomplexes. IL-2 can dissociate from its interaction with antibody; however, these two structures are linked through a short flexible peptide linker, and thus, it remains close to the binding site of the antibody, increasing the probability of reassociation. (C) IL-2 fused to the C-terminal part of heavy chains of selected mAb. (D) IL-2 linked to the Fc-part of the antibody, a structure also called immunocytokine.

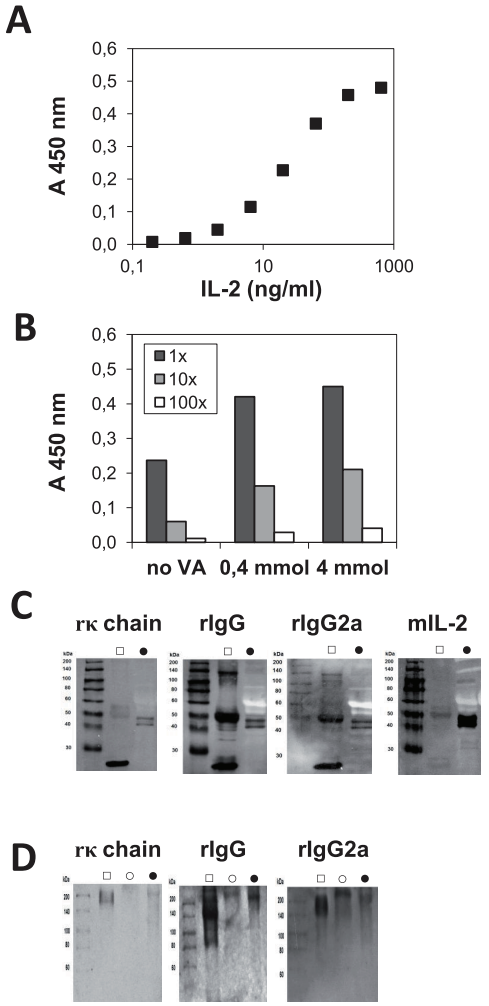

Figure 2. *In vitro* characterization of supernatant of CHO-S cells cotransfected with both expression vectors identifies a protein with biochemical features predicted for scIL-2/S4B6. (A) IL-2 was mixed with S4B6 mAb at a molar ratio 2:1, and resultant IL-2ic were used to calibrate sandwich ELISA for detection of scIL-2/S4B6. (B) Supernatant of CHO-S cells after cotransfection and 5 day cultivation either without (no VA) or with 0.4 or 4 mmol valproic acid (VA) were analyzed by ELISA calibrated as in panel A either undiluted or 10 times and 100 times diluted (black, gray, and empty bars, respectively). (C) Western blot of S4B6 mAb (●) and 50 times concentrated and extensively dialyzed supernatant of CHO-S cells cotransfected with both expression vectors (□). The specificity of each antibody used for detection is shown above each individual SDS-PAGE gel. Gels were run under reductive conditions. (D) Western blot of S4B6 mAb (●), 25 times concentrated and extensively dialyzed supernatant of CHO-S cells cotransfected with both expression vectors (○), and 50 times concentrated and extensively dialyzed supernatant of clone 104/53 (□). The specificity of each antibody used for detection is shown above each individual SDS-PAGE gel. Gels were run under nonreductive conditions.

chimera structurally mimics IL-2/S4B6 mAb immunocomplexes. Finally, we decided to determine the biological activity of our protein chimera and compare it with biological activity of IL-2/S4B6 mAb immunocomplexes both *in vitro* and *in vivo* and thus confirm that it mimics IL-2/S4B6 mAb immunocomplexes also functionally.

Flexible oligopeptide linker of 15 amino acids $(Gly_4Ser)_3$ has been used to link C-terminal amino acid of IL-2 to N-terminal amino acid of κ light chain of S4B6 mAb. Sequences encoding this fusion polypeptide and heavy chain of S4B6 mAb were cloned separately in two distinct expression vectors (Supplementary Figure 2) and could be seen in Supplementary Figure 3. Cotransfection of CHO-S cells with both prepared expression vectors should lead to production of scIL-2/S4B6. Indeed, we detected a protein in the supernatant of cotransfected CHO-S cells by our customized sandwich ELISA calibrated with IL-2ic (Figure 2A), which reacted both with antirat IgG2a mAb and anti-rκ light chain mAb. Thus, we decided to designate this produced protein as scIL-2/S4B6, to further characterize it and to confirm its structure and features. The production of scIL-2/S4B6 by transiently cotransfected CHO-S cells was considerably increased by valproic acid,[11] approximately from 25 to 80 ng/mL of IL-2 equivalent (Figure 2B). We also established stabile transfected CHO-S clone entitled 104/53 after two runs of single cell cloning and selection process, which continuously produce scIL-2/S4B6 (Supplementary Figure 4). However, the production of chimeric protein scIL-2/S4B6 by 104/53 clone was quite low (about 5−10 ng/mL), and we thus decided to use mainly transient transfection as the source of scIL-2/S4B6. Next, we analyzed our produced scIL-2/S4B6 by Western blot. Supernatants were concentrated 20−40 times on 100 kDa membranes, extensively dialyzed against PBS and ran together with S4B6 mAb on SDS-PAGE under reductive (Figure 2C) and nonreductive conditions (Figure 2D). Nonreduced S4B6 mAb and scIL-2/S4B6 gave bands of predicted M_w 150 and 190 kDa, respectively, when detected either with anti-rIgG2a mAb or anti-rIgG polyclonal antibody. The same antibodies under

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reductive condition detected S4B6 mAb as 50 and 25 kDa bands corresponding to heavy and light chains, respectively, and scIL-2/S4B6 as 50 and 45 kDa bands corresponding to heavy and IL-2-linker-light chains, respectively. Anti-rκ chain mAb under reductive condition detected S4B6 mAb as single band of 25 kDa and scIL-2/S4B6 as 45 kDa band. Unexpectedly, anti-rκ chain mAb also detected a 50 kDa band. Notably, anti-mIL-2 mAb under reductive conditions gave no signal in the case of S4B6 mAb, but a nice 45 kDa band was detected in the case of scIL-2/S4B6. All these data collectively show that the scIL-2/S4B6 we produce by cotransfection of CHO-S cells with both prepared expression plasmids has M_w of about 200 kDa and contains mIL-2, rκ light chain, and rIgG2a structures. Further, detection with anti-mIL-2 mAb and anti-rκ light chain mAb under reductive conditions provides the identical band with M_w of neither of these two proteins but with M_w corresponding to the sum of M_w of these two proteins. Since the produced scIL-2/S4B6 has all important features as theoretically predicted, we thus conclude that this protein is indeed scIL-2/S4B6 as we designed it.

Further, we decided to prove that IL-2 is bound to the binding site of S4B6 mAb in scIL-2/S4B6. Thus, we modified our sandwich ELISA by using anti-mIL-2 mAb instead of anti-rκ light chain mAb as the detection antibody. We used biotinylated S4B6, JES6.1, JES6.5 anti-mIL-2 mAbs, and control anti-IFNγ mAb as detection antibodies. Antirat IgG2a mAb was still used as catching antibody. When IL-2ic were analyzed in such ELISA, the only detection mAb that provided positive signal was JES6.1 (Figure 3A). It seems that IL-2 is significantly washing out from IL-2ic during numerous washing steps in the ELISA since only relatively high concentrations of IL-2ic (at least 100 ng/mL) provided reasonably strong signal. IL-2ic are not washed out from anti-IgG2a-coated wells since the same catching antibody is used for detection of produced scIL-2/S4B6 where we are able to detect much lower concentrations of IL-2ic (1 ng/mL being usually the detection limit). Next, scIL-2/S4B6 was bound to the wells coated with anti-rIgG2a mAb and detected by selected mAb (Figure 3B). JES6.1 mAb provided the highest signal, which is in concordance with the fact that this mAb recognizes the epitope located oppositely to the S4B6 mAb epitope. However, S4B6 mAb provided slightly higher signal than control anti-mIFN-γ mAb. This shows that probably a very little fraction of scIL-2/S4B6 is in conformation where IL-2 is available to bind S4B6 in trans. A slightly higher signal obtained with JES6.5H4 mAb reflects that JES6.5H4 mAb epitope is close, but not identical to, S4B6 mAb epitope, and thereby, these two mAbs significantly compete with each other in binding to IL-2. We also show that neither S4B6 mAb nor JES6.5 mAb compete with binding of JES6.1 mAb to IL-2 in IL-2ic (Figure 3C) and in scIL-2/S4B6 (Figure 3D).

Finally, we determined the biological activity of scIL-2/S4B6 *in vitro* and *in vivo*. We found that scIL-2/S4B6 added in titrated doses to naive $CD8^+$ T cells activated via TCR signal potently stimulated their proliferation *in vitro*. The maximal proliferation activity reached by scIL-2/S4B6 was the same as that reached by IL-2ic (Figure 4A,B). Interestingly, calculated concentrations of scIL-2/S4B6 in supernatants on the basis of this assay corresponded very well to those determined by sandwich ELISA calibrated with IL-2ic as shown in Figure 2A. Thus, it seems that scIL-2/S4B6 and IL-2ic possess very similar biological activity *in vitro* (Figure 4C). To assess the biological activity of scIL-2/S4B6 *in vivo*, we employed adoptive transfer of CFSE-labeled OT-I $CD8^+$ T cells into congeneic CD45.1

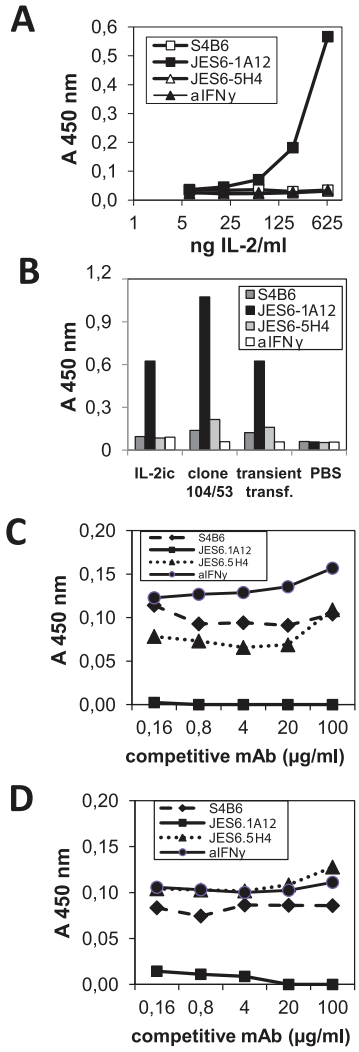

Figure 3. IL-2 moiety binds to the binding site of antibody moiety in scIL-2/S4B6 chimera, which structurally resembles IL-2/anti-IL-2 S4B6 mAb immunocomplexes. (A) IL-2ic were analyzed in a wide range of concentrations by ELISA with anti-rIgG2a mAb as coating antibody and S4B6, JES6.1, JES6.5H4, and anti-IFN-γ as detection mAbs. (B) IL-2ic, 50 times concentrated supernatant of clone 104/53 and 25 times concentrated supernatant from transient transfection were analyzed by ELISA with anti-rIgG2a mAb as coating antibody and with S4B6, JES6.1, JES6.5H4, and anti-IFN-γ as detection mAbs (dark gray, black, pale gray, and empty bars, respectively). The background of the assay is shown for each detection mAb by the use of PBS. (C) IL-2ic were analyzed by ELISA with anti-rIgG2a mAb as the coating antibody and with JES6.1 as the detection mAb. Detection with JES6.1 mAb was proceeded by incubation with S4B6, JES6.1, JES6.5H4, and anti-IFN-γ mAbs as competitive antibodies. (D) The same assay as that in panel C, but 25 times concentrated supernatant from transient transfection was used.

mice. Transferred T cells were activated by injection of a low dose of SIINFEKL peptide, and either IL-2ic or scIL-2/S4B6 were used to stimulate their proliferation and expansion (Figure 4D). The same supernatant as above was used as a source of scIL-2/S4B6, and the dose was determined by both ELISA and *in vitro* proliferation assay. Surprisingly, the results show that, unlike *in vitro*, biological activity of scIL-2/S4B6 is higher than IL-2ic *in vivo* (Figure 4D).

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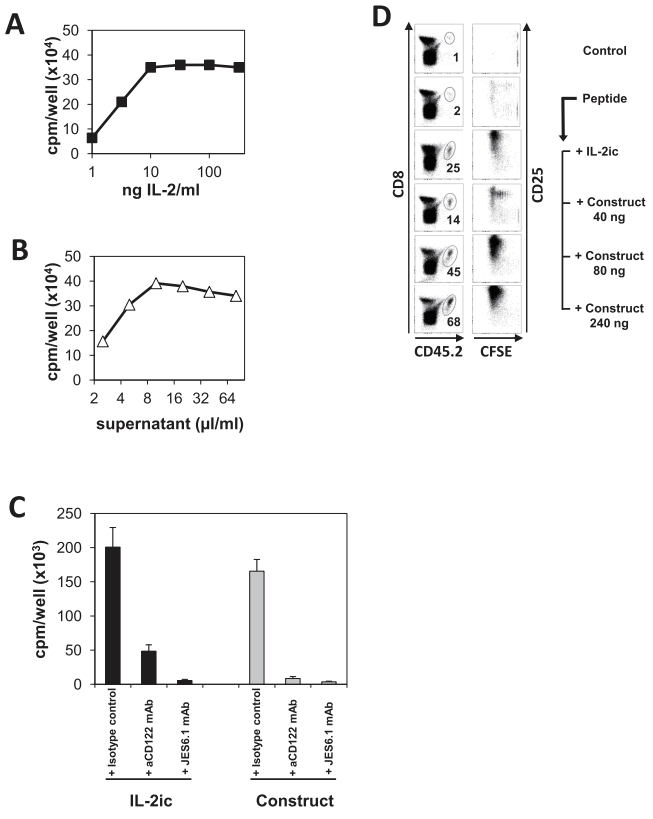

Figure 4. Biological activity of scIL-2/S4B6 mimicking IL-2/S4B6 immunocomplexes *in vitro* and *in vivo*. (A) $CD8^+$ T cells purified from the spleen of OT-I transgeneic mice were cultured with 50 nM SIINFEKL peptide and different concentrations of IL-2ic. CTLA-4-Ig protein chimera (2.5 μg/mL) was added to cultivated T cells to lower proliferation background. Proliferation was determined by $[^3H]$-thymidine incorporation assay. (B) The same assay as that in panel A, but 25 times concentrated supernatant from transient transfection was used. (C) $CD8^+$ T cells purified from the spleen of OT-I trangeneic mice were cultured with 50 nM SIINFEKL peptide and either with IL-2ic or with supernatant from transient transfection. Control, anti-CD122, and anti-IL-2 JES6.1 mAbs were added to cells on the beginning of cultivation. CTLA-4-Ig protein chimera (2.5 μg/mL) was added to cultivated T cells to lower proliferation background. Proliferation was determined by $[^3H]$-thymidine incorporation assay. (D) $CD8^+$ T cells purified from spleen of OT-I transgeneic mice were labeled with CFSE and adoptively transferred into Ly5.1 mice. Next day, all mice except controls were i.p. injected with 2 nmol SIINFEKL peptide. IL-2ic and scIL-2/S4B6 were injected i.p. the same day and then 24 and 48 h later. Spleen cells were analyzed by flow cytometry one day after the last dose. Numbers show expansion of transferred $CD8^+$ T cells relative to control.

METHODS

Animals and Cell Lines. Transgenic OT-I, congeneic B6.SJL (Ly5.1), and C57BL/6 mice were bred and kept at the GMO facility of the Institute of Molecular Genetics of ASCR, v.v.i. Mice were used at 9 to 15 weeks of age. All experiments were approved by the Animal Welfare Committee at the Institute of Microbiology of ASCR, v.v.i. CHO-S cell line was purchased from Invitrogen.

Monoclonal Antibodies and Other Reagents. The following antimouse mAbs were used for experiments: aCD8-PerCP-Cy5.5, aCD45.2-APC, and aCD25-PE (eBioscience) for flow cytometry analysis; funtional grade aIFN-γ, aCD122, anti-IL-2 mAb JES6.1-A12, anti-IL-2 mAb JES6.1.5H4 (eBioscience), anti-IL-2 mAb JES6.5H4-biotin, and aIFN-γ-biotin (eBioscience) for ELISA, proliferation assays, and Western blot. Unconjugated anti-IL-2 mAb S4B6 was purchased from Bioport. The following antirat mAbs were used for Western blot: anti-κ-biotin, anti-IgG2a (BD Biosciences), and polyclonal anti-IgG-biotin (eBioscience). CTLA-4Ig fusion protein chimera was purchased from BD Biosciences. Antibiotin-HRP and anti-IgG-HRP conjugates were purchased from Cell Signaling Tech. Extravidin–peroxidase conjugate was purchased from Sigma.

Biotinylation. Anti-IL-2 mAb JES6.1-A12 and S4B6 were biotinylated by EZ-Link Sulfo-NHS-LC-Biotin (Pierce) according to the standard protocol.

Preparation of Expression Vectors. Total RNA was isolated from *Mus musculus* C57BL/6 spleen cells stimulated for 24 h with aCD3 (5 μg/mL) and aCD28 (20 μg/mL) mAb and then from *Rattus rattus* S4B6 hybridoma cells using TRIZOL reagent (Gibco) according to the manufacturer protocol. DNaseI-treated total RNA (2 μg) were reverse-transcribed using oligo$(dT)_{12-18}$ and Superscript II Reverse Transcriptase (Invitrogen) to cDNA and then used in PCR. Mouse IL-2 cDNA was amplified by primers *F-IL2*/*R-IL2-SPACER*, and light rκ chain cDNA was amplified by primers *VKB1*-SPACER/*VKF MYS* using Platinum Taq DNA Polymerase (Invitrogen), resulting in specific PCR products (Table 1). Both products were digested by *Bam*HI (Fermentas) and ligated in one fused fragment linked by a 30bp spacer. The fragment was digested by *Kpn*I/XhoI and cloned into *Kpn*I/XhoI restriction site of pcDNA3.1(+)/HYGRO vector, which was then transformed into TOP 10F′cells. Rat heavy chain cDNA was amplified similarly to the light chain, yet by primers *VHB1NEW RF*/*VHFRAT2A*. The PCR product was digested with *Hin*dIII/*Kpn*I and then ligated into *Hin*dIII/*Kpn*I digested pSecTagA/ZEO, which was then transformed into TOP 10F′cells.

Transient Expression and Production of scIL-2/S4B6. Transient expression was carried out in CHO-S cells and mediated by FuGene transfecting reagent (Promega) according to the standard protocol in OptiMEM protein-free medium (Invitrogen). Cells were cotransfected by two expression vectors containing IL-2-κ light chain sequence and heavy chain, respectively. Transfectomas were grown in OptiMEM protein-free medium with desired concentration of valproic acid (R&D Systems), and supernatants were collected, dialyzed against PBS, concentrated, and stored in −70 °C.

Establishing of Stable Transfectant. Stable transfectant clone 104/53 was made using the same reagents, media, and expression vectors as in the transient transfection. CHO-S cells were first transfected by an expression vector containing IL-2-κ light chain sequence with Hygromycin B selection cassette, followed by cotransfection with an expression vector containg a heavy chain sequence with Zeocin selection cassette. Stable clone was obtained out of two subsequent single cell cloning and selection cycles with rising concentrations of Hygromycin B (10–75 μg/mL) and Zeocin (5–25

Table 1

primer	nucleotide sequence (5′–3′)	purpose	restriction site
F-IL2	GCAAGGTACCATGTACAGCATGCAGCTCGC	IL-2	*Kpn*I
R-IL2-*SPACER*	CA*GGATCCTCCTCCTCCAGAACCTCCGCCACC*TTGAGGGCTTGTTGAG		*Bam*HI
VKB1-*SPACER*	GT*GGATCCGGTGGCGGAGGTTCTG*ACATYCAGRTGACCCAGTCTC	light κ chain	*Bam*HI
VKF MYS	GCCACTCGAGctaACACTCATTCCTGTTG		XhoI
VHB1NEW RF	GTGTAAGCTTCRAGGTGCARCTGCAGGAGTCTG	heavy chain	*Hin*dIII
VHFRAT2A	GCAGGTACCTCATTTACCAGGAGAGTGGGAG		*Kpn*I

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μg/mL). Clones were screened for production of scIL-2/S4B6 by ELISA, cryopreserved, and stored in −152 °C.

IL-2/S4B6 Immunocomplexes. These immunocomplexes[1,5] were prepared by adding rmIL-2 (Prospec) into a solution of anti-IL-2 mAb S4B6 (both reagents in PBS) at a molar ratio 2:1. After a 15 min incubation at RT, the immunocomplexes were diluted with PBS to the desired concentration.

Surface Staining and Flow Cytometry Analysis. A single cell suspension was prepared from harvested spleens of OT-I mice by GentleMACS Dissociator (Miltenyi Biotech). After RBC lysis, cells were resuspended in FACS buffer (PBS, 2% FCS, and 2 mmol EDTA), blocked by 10% mouse serum for 30 min on ice, and stained with fluorochrome-labeled mAbs for 30 min on ice in dark. Cells were washed twice after each step in FACS buffer and fixed in 4% paraformaldehyde prior to analysis. Flow cytometric analysis was performed on LSRII (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

ELISA. NUNC MaxiSorp 96-well flat-bottom plates were plated with 5 μg/mL anti-IgG2a mAb and incubated overnight in 4 °C. Wells were rinsed and then blocked with 1% gelatin in PBS (2 h, RT), and samples were plated in a desired dilution or concentration, alone or in the presence of competitive mAbs, along with blank (PBS) and titrated IL-2/S4B6 as standard (2 h, RT, agitated). After rinsing, detection antibodies were added in dilution buffer (PBS, 0.5% gelatin, 3% PEG 6000, and 0.1% Tween 20) in a concentration of 0.05 μg/mL (1.5 h, RT), followed by rinsing and incubation with extravidin−peroxidase conjugate (1 h, RT). After the last rinse, a plate was developed using 3,3′,5,5′-tetramethylbenzidine (Sigma) for 10 min maximum, stopped by 2 M H_2SO_4, and analyzed on Tecan Rainbow ELISA reader and BIOLISA software (Tecan).

Proliferation Assay *in Vitro*. A single cell suspension was prepared from the spleen of OT-I mice by GentleMACS Dissociator (Miltenyi Biotech). After RBC lysis, cells were washed, centrifuged for 5 min at 300*g*, resuspended in fresh culture medium, and seeded into Nunc 96-well flat-bottom plates in 0.2 mL volume and 5×10^4 cells/mL density. SIINFEKL peptide (50 nM) (MBL international), CTLA-4-Ig protein chimera (2.5 μg/mL), and either IL-2ic or supernatant from transient transfection was added, together with control, anti-CD122, and anti-IL-2 JES6.1 mAbs on the beginning of cultivation. The plates were then cultured in 5% CO_2 for 72 h at 37 °C, and 18.5 kBq of [^{3}H]-thymidine was added for the final 8 h of cultivation before harvesting.

Western Blot. Cells ($1-5 \times 10^7$) were washed twice with an ice-cold Tris-buffered saline (TBS) with 1 μM Na_3VO_4 and centrifuged (4000*g*, 4 °C). Cells were then resuspended in extract buffer composed of 1% Nonidet P-40 (Pierce), 1 mM Na_3VO_4, 1 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM DTT, 5% Protease mix (Sigma), 1 mM PMSF, and TBS at pH 7.4 and passed ten times through a needle (25−30G). Cell lysates were centrifuged at 4000*g* (4 °C) and protein concentration in aspirated supernatants was determined. 20 μg of protein was loaded per lane and run on SDS-PAGE either under standard or denaturating conditions using 10% polyacrylamide gel. Semidry blotting procedure with nitrocellulose membrane was performed, and scIL-2/S4B6 was detected by one of the primary antibodies (anti-rκ-biotin, anti-rIgG-biotin, anti-rIgG2a, or anti-mIL-2 JES6.5H4-biotin) together with a secondary antibody (antibiotin-HRP or anti-mIgG-HRP).

Adoptive Transfer. Purified OT-I CD8$^+$ T cells (Ly5.2) were labeled with CFSE and injected i.v. into B6.SJL recipients (Ly5.1) at 1×10^6 cells per mouse. The next day, the mice were injected i.p. with PBS, 4 nmol SIINFEKL peptide (MBL International), 4 nmol SIINFEKL peptide plus IL-2ic, or 4 nmol SIINFEKL peptide plus scIL-2/S4B6 (40, 80, or 240 ng of IL-2 equiv). IL-2ic and scIL-2/S4B6 were injected 24 and 48 h later (total of 3 doses). Mice were sacrificed 24 h after the last dose, and spleens were harvested.

CFSE Labeling. Labeling of the cells with CFSE was carried out as described elsewhere.[1]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Scheme of two kinds of IL-2 immunocomplexes; scheme showing the cloning of genes; protein sequences cloned into expression vectors; identification of stabile transfected CHO-S clone 104/53. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Marek Kovar is listed as co-inventor on patent entitled Methods for Improving Immune Function and Methods for Prevention or Treatment of Disease in a Mammalian subject, which was filed on February 16, 2007, and now bears International Application Number PCT/US2007/0623631.

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■ REFERENCES

(1) Boyman, O., Kovar, M., Rubinstein, M. P., Surh, C. D., and Sprent, J. (2006) Selective stimulation of T cell subsets with antibody−cytokine immune complexes. *Science 311*, 1924−1927.

(2) Kamimura, D., Sawa, Y., Sato, M., Agung, E., Hirano, T., and Murakami, M. (2006) IL-2 *in vivo* activities and antitumor efficacy enhanced by an anti-IL-2 mAb. *J. Immunol. 177*, 306−314.

(3) Tomala, J., Chmelova, H., Strohalm, J., Ulbrich, K., Sirova, M., Rihova, B., and Kovar, M. (2011) Antitumor activity of IL-2/anti-IL-2 mAb immunocomplexes exerts synergism with that of *N*-(2-hydroxypropyl)methacrylamide copolymer-bound doxorubicin conjugate due to its low immunosuppressive activity. *Int. J. Cancer 129*, 2002−2012.

(4) Webster, K. E., Walters, S., Kohler, R. E., Mrkvan, T., Boyman, O., Surh, C. D., Grey, S. T., and Sprent, J. (2009) *In vivo* expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J. Exp. Med. 206*, 751−760.

(5) Tomala, J., Chmelova, H., Mrkvan, T., Rihova, B., and Kovar, M. (2009) *In vivo* expansion of activated naive CD8+ T cells and NK cells driven by complexes of IL-2 and anti-IL-2 monoclonal antibody as novel approach of cancer immunotherapy. *J. Immunol. 183*, 4904−4912.

(6) Hornick, J. L., Khawli, L. A., Hu, P., Lynch, M., Anderson, P. M., and Epstein, A. L. (1997) Chimeric CLL-1 antibody fusion proteins containing granulocyte-macrophage colony-stimulating factor or interleukin-2 with specificity for B-cell malignancies exhibit enhanced effector functions while retaining tumor targeting properties. *Blood 89*, 4437−4447.

(7) Hornick, J. L., Khawli, L. A., Hu, P., Sharifi, J., Khanna, C., and Epstein, A. L. (1999) Pretreatment with a monoclonal antibody/interleukin-2 fusion protein directed against DNA enhances the delivery of therapeutic molecules to solid tumors. *Clin. Cancer Res. 5*, 51−60.

(8) Xiang, R., Lode, H. N., Dolman, C. S., Dreier, T., Varki, N. M., Qian, X., Lo, K. M., Lan, Y., Super, M., Gillies, S. D., and Reisfeld, R. A. (1997) Elimination of established murine colon carcinoma metastases by antibody-interleukin 2 fusion protein therapy. *Cancer Res. 57*, 4948−4955.

(9) Craiu, A., Barouch, D. H., Zheng, X. X., Kuroda, M. J., Schmitz, J. E., Lifton, M. A., Steenbeke, T. D., Nickerson, C. E., Beaudry, K., Frost, J. D., Reimann, K. A., Strom, T. B., and Letvin, N. L. (2001) An IL-2/Ig fusion protein influences CD4+ T lymphocytes in naive and simian immunodeficiency virus-infected Rhesus monkeys. *AIDS Res. Hum. Retroviruses 17*, 873−886.

(10) Budagian, V., Nanni, P., Lollini, P. L., Musiani, P., Di Carlo, E., Bulanova, E., Paus, R., and Bulfone-Paus, S. (2002) Enhanced inhibition of tumour growth and metastasis, and induction of antitumour immunity by IL-2-IgG2b fusion protein. *Scand. J. Immunol. 55*, 484−492.

(11) Fan, S., Maguire, C. A., Ramirez, S. H., Bradel-Tretheway, B., Sapinoro, R., Sui, Z., Chakraborty-Sett, S., and Dewhurst, S. (2005) Valproic acid enhances gene expression from viral gene transfer vectors. *J. Virol. Methods 125*, 23−33.