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[1]

Jakub Tomala, Helena Chmelova, Tomas Mrkvan, Blanka Rihova, and Marek Kovar[2]

IL-2 is potent imunostimulatory molecule that plays a key role in T and NK cell activation and expansion. IL-2 is approved by the FDA to treat metastatic renal cancer and melanoma, but its extremely short half-life and serious toxicities are significant limitations of its use. It was reported that in vivo biological activity of IL-2 can be increased by association of IL-2 with anti-IL-2 mAb (S4B6). IL-2/S4B6 mAb immunocomplexes were described to be highly stimulatory for NK and memory CD8^{+} T cells and intermediately also for regulatory T cells. IL-2/JES6-1 mAb immunocomplexes are stimulatory solely for regulatory T cells. In this study we show that although both mentioned IL-2 immunocomplexes are less potent than free IL-2 in vitro, they possess extremely high stimulatory activity to expand activated naive CD8^{+} T cells in vivo. IL-2 immunocomplexes expand activated naive CD8^{+} T cells several hundred-fold times after four doses and more than 1000-fold times after six doses (1.5 μg/dose of IL-2), whereas free IL-2 given at the same dosage shows negligible activity. IL-2/S4B6 mAb immunocomplexes also induce massive expansion of NK cells (40% of DX5^{+}NK1.1^{+} cells in spleen). Importantly, activated naive CD8^{+} T cells expanded by IL-2 immunocomplexes form robust population of functional memory cells. We also demonstrate in two distinct tumor models that IL-2/S4B6 mAb immunocomplexes possess considerable antitumor activity. Finally, by using radioactively labeled IL-2, we provide for first time direct evidence that IL-2 immunocomplexes have much longer half-life in circulation than free IL-2, being ~3 h vs <15 min, respectively. *The Journal of Immunology,* **2009, 183: 4904–4912.**

The cytokine IL-2 is produced mainly by Ag-activated T cells and promotes proliferation, differentiation, and survival of mature T and B cells as well as the cytolytic activity of NK cells in the innate immune defense (1–5). Effective T cell activation requires at least two distinct signals. One occurs when antigenic peptide bound to MHC molecule on APC is presented to the TCR. The second signal occurs following an interaction of costimulatory molecules expressed on APCs with counter-receptors expressed on T cells (6–8). CD80/86–CD28 interaction plays the most important role in delivering the second signal. TCR signaling induces expression of CD25, the α-chain of IL-2R, which is absent on naive resting T cells, except for T regulatory cells. Following stimulation by "signal two", activated T cells initiate expression and secretion of IL-2, which is subsequently used as autocrine growth factor as these cells already possess high-affinity IL-2R. IL-2 exerts its pleiotropic activities through binding to either dimeric receptor composed from β-chain IL-2R (CD122) and common cytokine receptor γ-chain (CD132) or trimeric receptor composed from IL-2Rα, IL-2Rβ, and common cytokine receptor γ-chain (9). CD25 has been termed the "low affinity" ($K_d \sim 10$ nM) IL-2R and it is not involved in signal transduction (10). A dimer of CD122 and CD132 binds IL-2 with intermediate affinity ($K_d \sim 1$ nM) and is present on CD122^{high} populations, namely memory CD8^{+} T cells (CD3^{+}CD8^{+}CD44^{high}CD122^{high}) and NK cells (CD3^{-}NK1.1^{+}DX5^{+}) (11, 12). A complex of CD25, CD122, and CD132 binds IL-2 with high affinity ($K_d \sim 10$ pM) and it is present on activated T cells and regulatory T cells (CD3^{+}CD4^{+}CD25^{+}Foxp3^{+}).

Strong stimulatory activity for activated T cells and NK cells makes IL-2 an attractive molecule for cancer immunotherapy. IL-2 was approved by the FDA to treat metastatic renal cancer and malignant melanoma, having induced a complete response in 5–17% of patients with these cancers (13). However, an extremely short half-life and serious toxicities associated with high-dose IL-2 treatment are the major limitations (14–18). One of the most dangerous toxicities accompanying high-dose IL-2 treatment is vascular leak syndrome, which affects (but is not limited to) lungs and liver thus leading to pulmonary edema and liver damage (19, 20). There are several ways to overcome the problems accompanying administration of high doses of IL-2. One approach is based on gene therapy when tumor cells are transfected to constantly produce IL-2 (21, 22). IL-2-producing tumor cells are supposed to be immunogenic and it was shown in several experimental tumor models that those cells are indeed able to elicit effective antitumor response. Another approach is to use targeted IL-2 therapy with immunocytokine (i.e., Ab-cytokine fusion proteins). In this study, H chain of mAb with specificity toward selected tumor Ag is linked via its C terminus to the N terminus of IL-2 (23, 24). Immunocytokines show longer half-life time than parental cytokine and target its activity specifically into the tumor microenvironment (25, 26). Alternatively, to increase the half-life of IL-2 in vivo,

Department of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic

Received for publication January 29, 2009. Accepted for publication August 11, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants IAA500200712 and KAN200200651 from Grant Agency of Academy of Sciences of the Czech Republic, Grant 310/08/H077 from Grant Agency of the Czech Republic, and by Grant AV0250200510 from the Institutional Research Concept.

[2] Address correspondence and reprint requests to Dr. Marek Kovar, Department of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, Prague 4-Krc 14220, Czech Republic. E-mail address: makovar@biomed.cas.cz

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900284

IL-2 was conjugated to a polymeric carrier of polyethylene glycol or to serum proteins like albumin and IgG (27–29).

Surprisingly, it was found that immunocomplexes of IL-2 with anti-IL-2 mAb strongly stimulate proliferation of specific population of immune cells (30), depending on the clone of anti-IL-2 mAb used. In this study, we decided to investigate whether IL-2 immunocomplexes are capable to expand activated naive $CD8^+$ T cells both in vitro and in vivo. We found that although IL-2 immunocomplexes are much less potent than free IL-2 in vitro, they possess extremely high stimulatory activity to expand activated naive $CD8^+$ T cells in vivo, being able to expand activated naive $CD8^+$ T cells more than three orders of magnitude within 1 wk. Furthermore, we compared the efficacy of IL-2/S4B6 mAb vs IL-2/JES6-1A12 mAb immunocomplexes to drive expansion of activated naive $CD8^+$ T cells and NK cells and we proved that activated naive $CD8^+$ T cells expanded by IL-2 immunocomplexes form a robust population of long-lived memory cells ($CD8^+$ $CD44^{high}CD122^{high}$), which are functional in terms of high IFN-γ expression after restimulation. We also showed that IL-2/S4B6 mAb immunocomplexes possess promising antitumor activity by using two distinct syngeneic tumor models. Finally, pharmacokinetics of IL-2 immunocomplexes vs free IL-2 in the blood was determined for the first time by using radioactively labeled IL-2, i.e., directly, which enabled approximation and comparison of their half-life in circulation. Considering these findings we concluded that IL-2 immunocomplexes are superior to free IL-2 in vivo and we hypothesized that IL-2 immunocomplexes could be useful in human medicine.

Materials and Methods

Mice

Female BALB/c and male C57BL/6 mice were obtained from a breeding colony at the Institute of Physiology (Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic). Transgenic OT-I mice and B6.SJL (Ly5.1) mice were bred and kept at the genetically modified organism facility of the Institute of Molecular Genetics (Academy of Sciences of the Czech Republic). The mice were used at 9–15 wk of age. All experiments were approved by the Animal Welfare Committee at the Institute of Microbiology (Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic).

Cell lines and mAbs

Murine B cell leukemia BCL1, B16F10 melanoma, and CTLL-2 cell lines were purchased from American Type Culture Collection. The following anti-mouse mAbs were used: CD8-A700, CD45.2-allophycocyanin, CD44-PE, DX5-PE, IFN-γ-PE mAb, CD3, CD25, CD122, IL-2 clone JES6-1A12 and IL-2 clone JES6.5H4, (eBioscience), CD122-PE (BD Pharmingen), and CD25-PE (Immunotech). Anti-human IL-2 clone MAB602 was from R&D Systems. CD25-allophycocyanin, NK1.1-allophycocyanin, and S4B6 mAb were provided by Dr. K. Drbal (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic).

Staining for surface Ags

Cells were resuspended in FACS buffer (PBS with 2% FCS, 2 mmol EDTA and 0,05% sodium azide), blocked by 10% mouse serum for 30 min on ice and stained with fluorochrome labeled mAbs for 30 min on ice in dark. When biotinylated mAbs were used, cells were additionally incubated 10 min on ice with fluorochrome-conjugated streptavidin. Cells were washed twice after each step in FACS buffer and fixed in 4% paraformaldehyde before analysis. Labeling the cells with CFSE was conducted as described elsewhere (30). Flow cytometric analysis was performed on LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Adoptive transfer of OT-I cells

Purified OT-I $CD8^+$ T cells (Ly5.2) were labeled with CFSE and injected i.v. into B6.SJL recipients (Ly5.1) at 1.5×10^6 cells per mouse. Next day, the mice were injected i.p. with PBS, SIINFEKL peptide (MBL International), SIINFEKL peptide plus polyinosinic-polycytidylic acid (poly(I:C),[3] 75 μg), IL-2 immunocomplexes plus SIINFEKL peptide, or free IL-2 plus SIINFEKL peptide.

^{131}I labeling of IL-2

Recombinant human IL-2 (PeproTech) was labeled with ^{131}I using IODO-GEN Pre-Coated Iodination Tubes (Pierce) and the remaining free iodine was removed by column chromatography (PD-10; Pierce). IL-2 was injected i.v. into B6 mice either as a free labeled IL-2 or as IL-2 immunocomplex prepared by premixing the labeled IL-2 with mouse anti-human IL-2 mAb (clone MAB602) at a molar ratio 2:1.

Proliferation assay in vitro

$CD8^+$ population was depleted by i.p. injection of 200 μg of anti-CD8 mAb (clone 53-6.72). Purified OT-I $CD8^+$ T cells were seeded into Nunc 96-well flat-bottom plates in 0.2 ml volume and density of 5×10^4 cells/ml, cultured with 10 μg/ml soluble anti-CD3 mAb plus titrated amounts of IL-2 premixed with isotype control mAb, S4B6 mAb, JES6-1 mAb, or both JES6-5 and JES6-1 mAbs (eBioscience). The plates were then cultured in 5% CO_2 for 72 h at 37°C. A 18.5 kBq of [^{3}H]thymidine was added for the final 8 h of cultivation before harvesting.

Results

Stimulatory activity of IL-2/anti-IL-2 mAb immunocomplexes for activated naive $CD8^+$ T cells in vitro

Naive $CD8^+$ T cells do not response to physiologic levels of IL-2 unless they are activated via TCR, which leads to up-regulation of CD25 expression. TCR signal can be thus used to switch IL-2 nonresponsive naive $CD8^+$ T cells into IL-2 responsive cells. However, population of $CD8^+$ T cells from normal mice contains subset of memory cells (~10%), which are able to respond to IL-2 without any other stimuli. To use an experimental system in which purified $CD8^+$ T cells would contain solely naive cells and thus respond to IL-2 only after TCR signal, we used naive OT-I TCR transgenic mice because they lack memory $CD8^+$ T cells. Thus, $CD8^+$ T cells from OT-I mice activated with anti-CD3 mAb were used to determine stimulatory activity of IL-2/S4B6 mAb and IL-2/JES6-1 mAb immunocomplexes for activated naive $CD8^+$ T cells. Both immunocomplexes were able to stimulate proliferation of activated naive $CD8^+$ T cells, albeit at much higher concentrations (~30 times) than free IL-2 (Fig. 1*A*). Stimulatory activity of IL-2/JES6-1 immunocomplexes was totally abrogated by adding JES6-5 mAb, another clone of anti-mouse IL-2 recognizing distinct epitope than JES6-1 mAb.

To rule out the possibility that proliferative responses of activated naive $CD8^+$ T cells to high concentrations of IL-2 immunocomplexes are not caused by traces of unbound free IL-2 after mixing IL-2 and anti-IL-2 mAb at molar ratio 2:1, we performed a modified experiment. We coated wells with anti-IL-2 mAb, pulsed them with IL-2 and washed thoroughly before seeding purified OT-I $CD8^+$ T cells stimulated with anti-CD3 mAb. The presence of free IL-2 is thus excluded. Indeed, activated naive OT-I $CD8^+$ T cells proliferated in wells coated with S4B6 and JES6-1 mAb, but failed to proliferate in wells coated with isotype control mAb (Fig. 1*B*). Proliferation of activated naive OT-I $CD8^+$ T cells stimulated with immobilized IL-2 immunocomplexes was also shown to be inhibited by addition of anti-CD25 and anti-CD122 mAb (Fig. 1*C*).

IL-2 immunocomplexes expand activated naive $CD8^+$ T cells and NK cells in vivo

To examine in vivo stimulatory activity of IL-2/S4B6 mAb and IL-2/JES6-1 mAb immunocomplexes vs free IL-2, we adoptively transferred purified CFSE labeled OT-I $CD8^+$ T cells (Ly5.2) into B6.SJL mice (Ly5.1). OT-I $CD8^+$ T cells were then selectively

[3] Abbreviation used in this paper: poly(I:C), polyinosinic-polycytidylic acid.

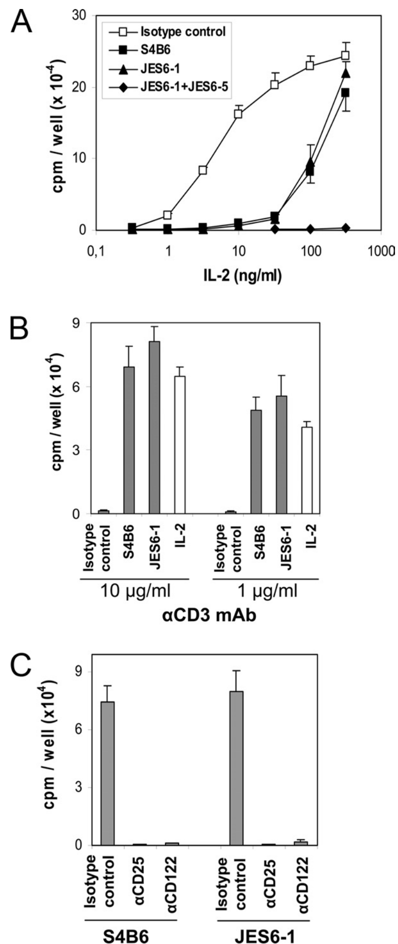

FIGURE 1. IL-2 in a complex with either S4B6 or JES6-1 anti-IL-2 mAb is capable to stimulate proliferation of activated naive CD8$^+$ T cells in vitro. *A*, Purified OT-I CD8$^+$ T cells were cultured at 5×10^4 cells per well with anti-CD3 mAb (10 μg/ml) and IL-2 premixed with isotype control mAb, S4B6 mAb, JES6-1 mAb, or both JES6-5 and JES6-1 mAbs. IL-2 was mixed with each mAb at molar ratio 2:1. *B*, Wells were coated with isotype control mAb, S4B6 mAb or JES6-1 mAb, washed, and pulsed with 100 ng/ml IL-2 for 1 h. After rigorous washing, purified OT-I CD8$^+$ T cells were cultured at 5×10^4 cells per well with either 10 or 1 μg/ml anti-CD3 mAb. Data show proliferation when 10 ng/ml IL-2 was added (○). *C*, The same cell conditions as in *B* plus 10 μg/ml anti-CD25 mAb, anti-CD122 mAb, or isotype control Ab. Data show mean levels ± SD of [^{3}H]thymidine incorporation for triplicate (*B* and *C*) or pentaplete (*A*) cultures on day 3. Data are representative of at least two independent experiments.

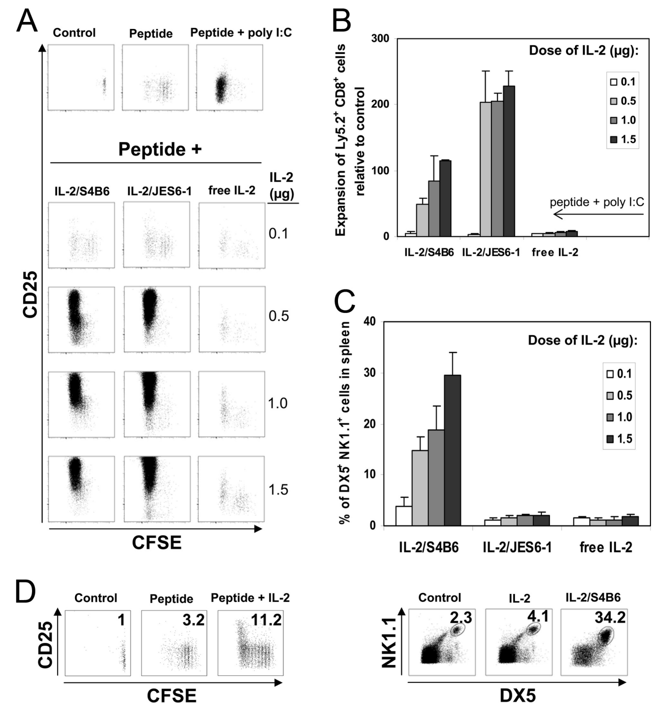

FIGURE 2. IL-2 immunocomplexes have a strong in vivo stimulatory activity for activated naive CD8$^+$ T cells and NK cells in comparison to free IL-2. Purified OT-I CD8$^+$ T cells (Ly5.2) were labeled with CFSE and injected i.v. into congeneic B6.SJL recipients (Ly5.1) at 1.5×10^6 cells per mouse (day 1). On day 2, the mice were injected i.p. with PBS (control), 2 nmol SIINFEKL peptide (peptide), SIINFEKL peptide plus poly(I:C) (75 μg), SIINFEKL peptide plus titrated amounts of different IL-2 immunocomplexes, or SIINFEKL peptide plus titrated amounts of free IL-2. IL-2 immunocomplexes were prepared as described in Fig. 1*A* and were injected i.p. also on days 3, 4, and 5. CFSE dilution and CD25 expression (*A*) and relative expansion (*B*) of Ly5.2$^+$ CD8$^+$ cells in spleen were analyzed 1 day after last injection. *C*, Relative expansion of NK cells after the same treatment as used above. *D*, Adoptive transfer was made as in *A*. Mice were injected i.p. with PBS (control), 2 nmol SIINFEKL peptide (peptide), or SIINFEKL peptide plus 25 μg of free IL-2 on day 2. The same amount of free IL-2 was injected i.p. also on days 3, 4, and 5. CFSE dilution, expression of CD25 and expansion relative to control (*top right corner*) of Ly5.2$^+$ CD8$^+$ cells in spleen were analyzed 1 day after last injection (*left*). Relative expansion of NK cells after the same treatment with free IL-2 as used in left part (25 μg/dose) or after treatment with IL-2/S4B6 immunocomplexes (1.5 μg of IL-2) (*right*). One representative mouse of two mice per each condition is shown. Data are representative of two independent experiments.

activated by injection of SIINFEKL peptide followed by treatment with IL-2 immunocomplexes or free IL-2. The dose of SIINFEKL peptide (2 nmol) was selected to cause only mild expansion of OT-I CD8$^+$ T cells when injected alone (3- to 5-fold expansion) but relatively high expansion when injected together with poly(I:C) (30- to 50-fold expansion). Both IL-2/S4B6 mAb and IL-2/JES6-1 mAb immunocomplexes exhibited very high efficacy in terms of stimulation of proliferation (Fig. 2*A*) as well as expansion (Fig. 2*B*) of activated OT-I CD8$^+$ T cells. Free IL-2 administered at the same doses showed negligible effect. Moreover, free IL-2 showed up to be much less stimulatory than IL-2 immunocomplexes also when the doses were escalated close to maximal tolerated dose (Fig. 2*D*, *left*), i.e., when the mice received four times 25 μg of IL-2. IL-2/JES6-1 mAb immunocomplexes were slightly more potent in terms of expansion than IL-2/S4B6 mAb immunocomplexes (2- to 4-fold difference). Both types of immunocomplexes showed only threshold activity at 0.1 μg of IL-2 (the lowest dose tested). OT-I CD8$^+$ T cells expanded by IL-2 immunocomplexes showed high expression of CD25 at day 5 after activation, whereas OT-I CD8$^+$ T cells expanded by SIINFEKL peptide plus poly(I:C) were CD25^{low}, although in both cases the cells vigorously proliferated and were significantly expanded. IL-2/S4B6 mAb immunocomplexes, but no IL-2/JES6-1 mAb immunocomplexes and free IL-2, caused also huge expansion of NK cells (Fig. 2, *C* and *D*, *right*). Notably, NK cells were moderately expanded (2–3 times) already at dose of IL-2/S4B6 mAb immunocomplexes corresponding to 0.1 μg of IL-2, showing that NK cells are more sensitive to stimulatory activity of this type of IL-2 immunocomplexes than activated CD8$^+$ T cells. In the next experiment we treated B6.SJL mice (Ly5.1) with adoptively transferred CFSE labeled OT-I CD8$^+$ T cells (Ly5.2) and activated by SIINFEKL peptide with two, four, or six doses of both types of IL-2 immunocomplexes (1.5 μg of IL-2). The mice were sacrificed

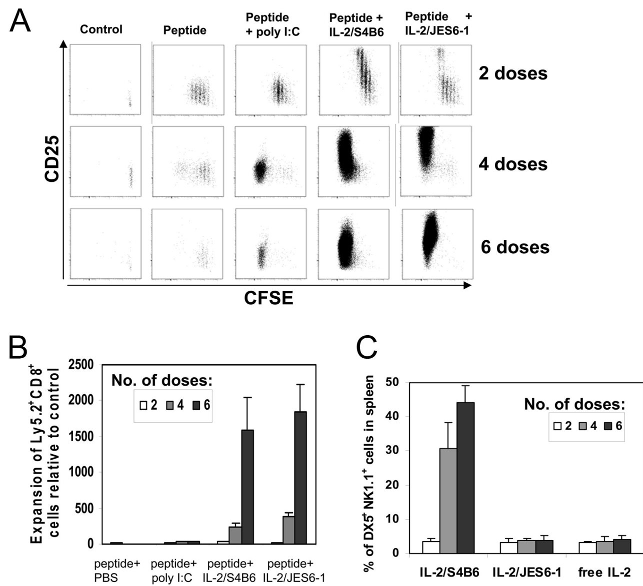

FIGURE 3. In vivo expansion of activated naive CD8$^+$ T cells and NK cells after treatment with two, four, or six doses of IL-2 immunocomplexes. Purified OT-I CD8$^+$ T cells (Ly5.2) were labeled with CFSE and adoptively transferred into B6.SJL recipients (Ly5.1) at 1.5 × 10^6 cells per mouse. One day later, the mice were injected i.p. with 2 nmol SIINFEKL peptide (peptide), SIINFEKL peptide plus poly(I:C) (75 μg), and SIINF EKL peptide plus IL-2/S4B6 mAb or IL-2/JES6-1 mAb immunocomplexes (1.5 μg IL-2 per dose i.p.). IL-2 immunocomplexes were then injected on a daily basis up to total of two, four, or six doses. The mice were euthanized 1 day after the last injection and CFSE dilution and CD25 expression (*A*), relative size (untreated control = 1) of Ly5.2$^+$ CD8$^+$ population (*B*), and relative counts of NK cells in spleen (*C*) were determined by flow cytometry.

and their spleens examined 1 day after the last dose. Expansion of OT-I CD8$^+$ T cells progressively correlated with number of doses, reaching more than 1- to 500-fold expansion after six doses (Fig. 3*B*). The enormous expansion of OT-I CD8$^+$ T cells after six doses of IL-2 immunocomplexes, i.e., only 1 wk after activation, is very convincing demonstration of high immunostimulatory potential of IL-2 immunocomplexes. The expression of CD25 on OT-I CD8$^+$ T cells was noticeably induced after two doses, peaked after four doses and then declined after six doses (Fig. 3*A*). Similar to CD8$^+$ T cells, almost no significant expansion of NK cells occurred after two doses. However, NK cells formed up to 31% and 44% of total spleen cells after four and six doses, respectively (Fig. 3*C*). Such a huge expansion of NK cells could explain why mice after six doses of IL-2/S4B6 mAb immunocomplexes develop an obvious splenomegaly.

Activated naive CD8$^+$ T cells expanded by IL-2 immunocomplexes are able to establish a robust population of functional memory cells

A key question is whether activated naive CD8$^+$ T cells expanded by IL-2 immunocomplexes are able to establish long-lasting population of memory cells. Thus, we adoptively transferred purified OT-I CD8$^+$ T cells (Ly5.2) into B6.SJL mice (Ly5.1) and mice were then injected with low or high (2 or 25 nmol, respectively) dose of SIINFEKL peptide either with or without poly(I:C) or with IL-2 immunocomplexes for four consecutive days. The expansion of the transferred cells was analyzed in peripheral blood 5 days after activation (Fig. 4*A*, *left column*), i.e., close to the peak of expansion, and in spleen of the same mice at day 60 (Fig. 4*A*, *right column*), i.e., late enough to be sure that only real memory cells are remaining. Fig. 4*B* shows transferred CD8$^+$ cells (Fig. 4*B*, *right column*) and host CD8$^+$ cells (Fig. 4*B*, *left column*) from the same mice as shown in Fig. 4*A* stained for CD44 and CD122 to allow determination of memory CD8$^+$ T cell phenotype. The magnitude of primary expansion of transferred cells in mice injected with peptide alone reflected whether high or low dose of peptide was given. However, the transferred cells disappeared in both groups during retraction phase and none were left at day 60. When the high dose of peptide was injected together with poly(I:C), a significant population of the transferred cells could be found in spleen at day 60 and almost all these cells were CD44^{high} and nearly half of them were also CD122^{high}, i.e., showed memory cell phenotype. Mice injected with the low dose of peptide and treated with free IL-2 showed a modest primary expansion of transferred cells, whereas only negligible population of memory cells was formed; an observation corresponding with mice injected with a low dose of peptide together with poly(I:C). Mice injected with a low dose of peptide and treated with IL-2/JES6-1 mAb immunocomplexes had almost two times more of transferred cells in spleen at day 60 than mice treated with high dose of peptide plus poly(I:C). The population of transferred cells was even far bigger in mice injected with low dose of peptide and treated with IL-2/S4B6 mAb immunocomplexes than that found in mice treated with a high dose of peptide plus poly(I:C) (more than 16 times). Almost two thirds and more than 80% of transferred cells had CD44^{high}CD122^{high} phenotype in mice treated with IL-2/JES6-1 mAb and IL-2/S4B6 mAb immunocomplexes, respectively.

Finally, we asked whether CD8$^+$ cells with memory phenotype established from activated naive CD8$^+$ T cells expanded by IL-2 immunocomplexes are functional, i.e., develop effector functions upon encounter with appropriate peptide/MHC class I complex. To address this question, we harvested splenocytes on day 60 from the mice with population of OT-I memory cells established by injection of low dose of peptide plus IL-2 or IL-2 immunocomplexes as described. Splenocytes were ex vivo stimulated with SIINFEKL peptide and analyzed for expression of IFN-γ. As shown in Fig. 4*C*, OT-I cells from mice treated with free IL-2 did not express IFN-γ upon re-activation, whereas OT-I cells from mice treated with IL-2 immunocomplexes did. Nearly 90% and 70% of OT-I cells from mice treated with IL-2/S4B6 mAb and IL-2/JES6-1 mAb expressed IFN-γ, respectively. Similar results were seen when we detected the expression of granzyme B (data not shown), although the difference between treatment with free IL-2 and with IL-2 immunocomplexes was less striking. Thus, IL-2 immunocomplexes are able both to strongly expand activated naive CD8$^+$ T cells and to establish population of long-lived cells with memory phenotype, which are able to express effector functions upon re-activation.

IL-2/S4B6 mAb immunocomplexes possess significant antitumor activity

IL-2 has been used in treatment of cancer for years. Prospectively, IL-2 immunocomplexes could become promising alternative therapeutic agents to free IL-2 due to their superior biological activity in vivo. It is obvious that in terms of cancer immunotherapy IL-2/S4B6 mAb immunocomplexes are more suitable than IL-2/JES6-1 mAb immunocomplexes as they are able to expand both activated naive CD8$^+$ and NK cells. Moreover, IL-2/S4B6 mAb immunocomplexes show considerably lower stimulatory activity for regulatory T cells, which are known to dampen antitumor responses, than IL-2/JES6-1 mAb immunocomplexes

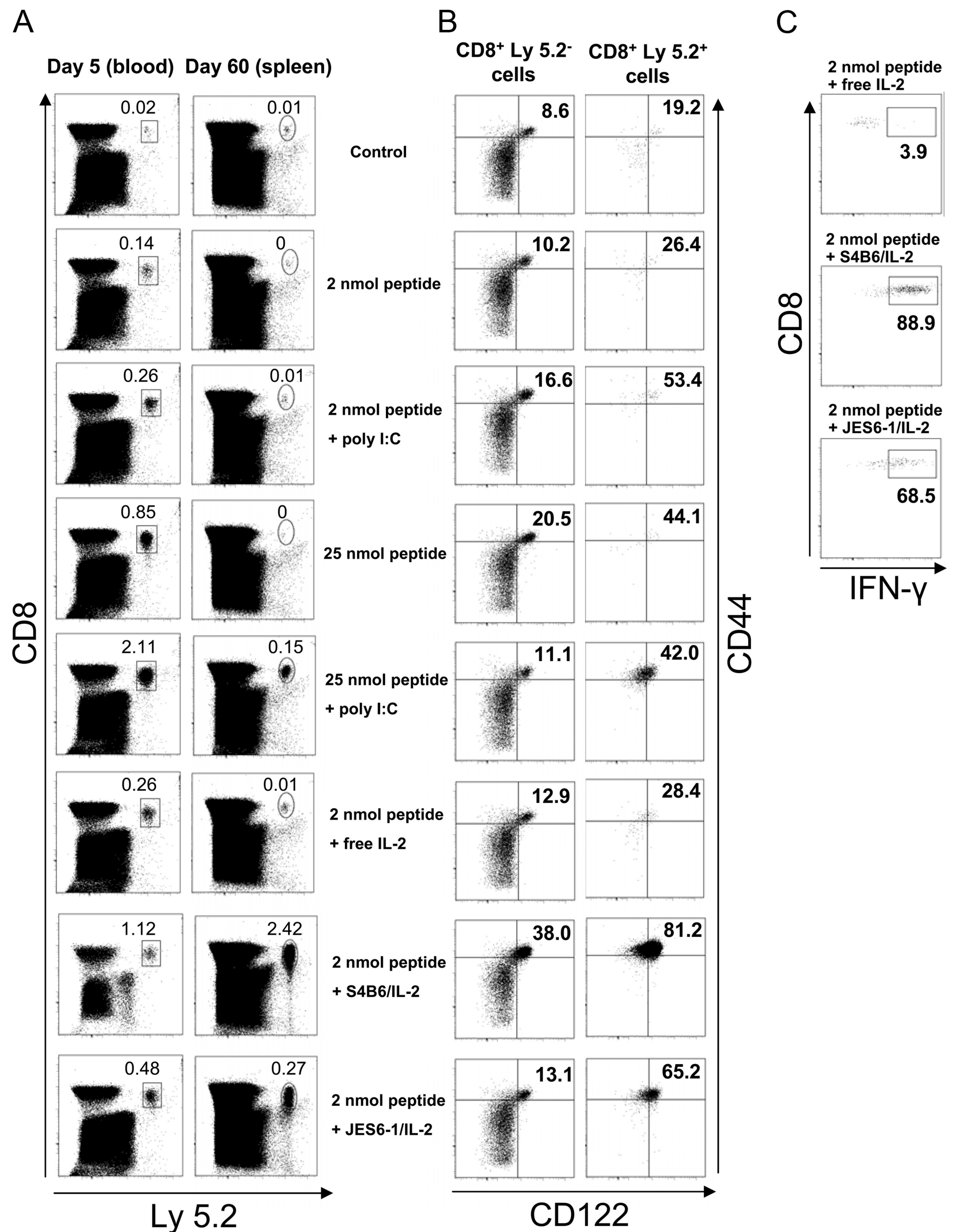

FIGURE 4. Activated naive $CD8^+$ T cells expanded by IL-2 immunocomplexes establish a robust population of functional memory cells. Purified OT-I $CD8^+$ T cells (Ly5.2) were injected i.v. into congeneic B6.SJL recipients (Ly5.1) at 1.5×10^6 cells per mouse (day 1). On day 2 the mice were injected i.p. with PBS (control), 2 nmol SIINFEKL peptide (peptide) with or without poly(I:C) (75 μg), 25 nmol SIINFEKL peptide with or without poly(I:C) (75 μg), 2 nmol SIINFEKL peptide plus free IL-2 (1.5 μg), 2 nmol SIINFEKL peptide plus IL-2/S4B6 or JES6-1 mAb immunocomplexes (1.5 μg of IL-2). IL-2 and IL-2 immunocomplexes were also injected on days 3, 4, and 5. *A*, On day 5, counts of $Ly5.2^+$ $CD8^+$ cells relative to all lymphocytes were assessed in peripheral blood (*left column*). On day 60, counts of $Ly5.2^+$ $CD8^+$ cells relative to all splenocytes were assessed in spleens of the same mice (*right column*). *B*, Host $CD8^+$ cells (*left column*) and transferred $CD8^+$ cells (*right column*) were stained for CD44 and CD122. *C*, On day 60, spleen cells were stimulated by SIINFEKL peptide for 6 h ex vivo with brefeldin A for last 4 h, and expression of IFN-γ was determined in $Ly5.2^+$ $CD8^+$ cells. One representative mouse of two mice per each condition is shown. Data are representative of two independent experiments.

(see supplemental Figs. 1 and 2).[4] Thus, we tested antitumor activity of IL-2/S4B6 mAb immunocomplexes in two different syngeneic tumor models, B16F10 melanoma and BCL1 B cell leukemia. B6 mice were injected s.c. with 1×10^5 of B16F10 cells and treated with free IL-2 (40 μg/dose) on days 2 and 6 or with IL-2/S4B6 mAb immunocomplexes (2.5 μg/dose) either on days 2 and 6 or on days 2, 6, and 10. Alternatively, IL-2/S4B6 mAb immunocomplexes were injected on days 2–6 (1.0 μg/dose). Free IL-2 had very limited effect on the tumor growth and on the survival of tumor bearing mice, although it was used at relatively high dosage. On the contrary, the growth of the tumors was significantly retarded in mice treated with IL-2/S4B6 mAb immunocomplexes (Fig. 5*A*, *left*) and survival of these mice was dramatically affected (Fig. 5*A*, *right*). Three doses of IL-2 immunocomplexes (2.5 μg/dose) showed up to most effective; four of six total mice were completely cured. In the other model, BALB/c mice were injected i.p. with 5×10^5 of BCL1 cells and treated with free IL-2 (40 μg/dose) or with IL-2/S4B6 mAb immunocomplexes (2.5 μg/dose) on days 4 and 8. Similarly to B16F10 melanoma model, free IL-2 only slightly prolonged the survival, but did not completely cure any of BCL1 leukemia bearing mice. IL-2/S4B6 immunocomplexes were shown also in this tumor model to be much more therapeutically effective than free IL-2 as they completely cured two of six mice (Fig. 5*B*). Antitumor activity of IL-2/S4B6 mAb immunocomplexes was totally abolished in BALB/c mice with depleted $CD8^+$ T cells (Fig. 5*C*) showing that these cells are essential for the antitumor activity of IL-2/S4B6 mAb immunocomplexes in BCL1 leukemia model and that NK cells are not involved. However, the situation is different in the model of B16F10 melanoma in which we have data showing that both NK cells and $CD8^+$ cells are necessary for maximal antitumor effect of IL-2/S4B6 immunocomplexes (see supplemental Fig. 3).

[4] The online version of this article contains supplemental material.

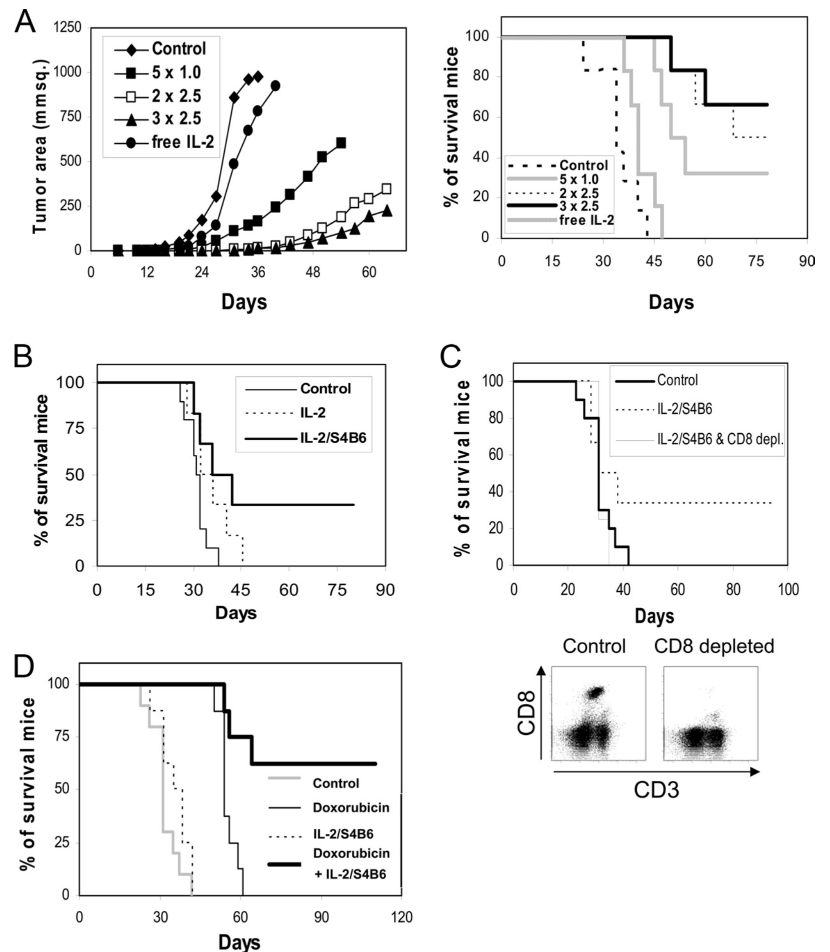

FIGURE 5. IL-2/S4B6 mAb immunocomplexes possess significant anti-tumor activity. *A*, B6 mice were injected s.c. with 1×10^5 of B16F10 melanoma cells on day 0. Mice were injected i.p. with PBS (control), with IL-2 (40 μg) on days 2 and 6 (free IL-2), with IL-2/S4B6 immunocomplexes (2.5 μg of IL-2) on days 2 and 6 (2×2.5) or on days 2, 6, and 10 (3×2.5) and with IL-2/S4B6 immunocomplexes (1 μg of IL-2) on days 2–6 (5×1.0). Tumor size was measured (*left*) and survival was monitored (*right*) for $n = 6$ mice per group. *B*, BALB/c mice were injected i.p. with 5×10^5 of BCL1 leukemia cells on day 0 and i.p. with free IL-2 (40 μg), IL-2/S4B6 mAb immunocomplexes (1.5 μg of IL-2) or PBS (control) on days 4 and 8. Survival is shown (treated and control mice) for $n = 6$ and 10 mice per group, respectively. *C*, BALB/c mice were injected i.p. with 5×10^5 of BCL1 leukemia cells on day 0 and i.p. with IL-2/S4B6 mAb immunocomplexes (1.5 μg of IL-2) or PBS (control) on days 4 and 8. One group treated with IL-2/S4B6 mAb immunocomplexes was injected i.p. with 200 μg of anti-CD8 mAb on days 2 and 6. Depletion of $CD8^+$ T cells was checked in spleen on day 4 (bottom) for two extra mice. Survival is shown (*top*) for treated and control mice in $n = 6$ and 10 mice per group, respectively. *D*, BALB/c mice were injected i.p. with 5×10^5 of BCL1 leukemia cells on day 0 and injected i.p. with doxorubicin (5 mg/kg) on day 11. Mice were further treated with IL-2/S4B6 immunocomplexes (1.5 μg of IL-2) on days 12, 13, and 14 (doxorubicin plus IL-2/S4B6). Some mice were treated by either the first modality (doxorubicin) or the second (IL-2/S4B6). Control mice were left without any treatment. Survival is shown in treated and control mice ($n = 8$ and 10 mice per group, respectively). Data are representative of at least two independent experiments.

Short half-life of IL-2 is greatly prolonged by complexing IL-2 with anti-IL-2 mAb

The superior biological activity of IL-2 immunocomplexes to free IL-2 in vivo could be at least partially caused by their prolonged half-life in circulation, i.e., bioavailability. To address this possibility, we radiolabeled recombinant human IL-2 and measured the radioactivity in the blood and urine at various time points after i.v. injection of free radiolabeled IL-2 or radiolabeled IL-2 complexed with anti-human IL-2 mAb (clone MAB602). IL-2/MAB602 mAb immunocomplexes have comparable biological activity in mouse as IL-2/S4B6 mAb immunocomplexes (30). At 15 min after the injection, less than 15% of injected activity was found in mice injected with free IL-2, but almost 80% of injected activity was detected in mice injected with IL-2 immunocomplexes (Fig. 6*A*). At 24 h after the injection (the longest period tested), no activity was detected in mice treated with free IL-2, but almost 20% of injected activity was still detectable in mice treated with IL-2 mAb immunocomplexes. The activity in urine reflecting the elimination of IL-2 showed a profile that peaked 1 h after administration in mice injected with free IL-2 and 8 h after administration in mice injected with IL-2 mAb immunocomplexes (Fig. 6*B*). These data collectively show that free IL-2 has a very short half-life (less than 15 min) in the blood and that it is eliminated from the body much faster than IL-2 immunocomplexes. The half-life of IL-2 immunocomplexes in the circulation was determined to be ~3 h.

To further confirm that IL-2 complexed with anti-IL-2 mAb remains in the circulation for much longer time than free IL-2 and moreover that it retains its biological activity, we injected i.v. B6 mice with IL-2 mixed with S4B6 mAb (IL-2/S4B6 mAb immu-

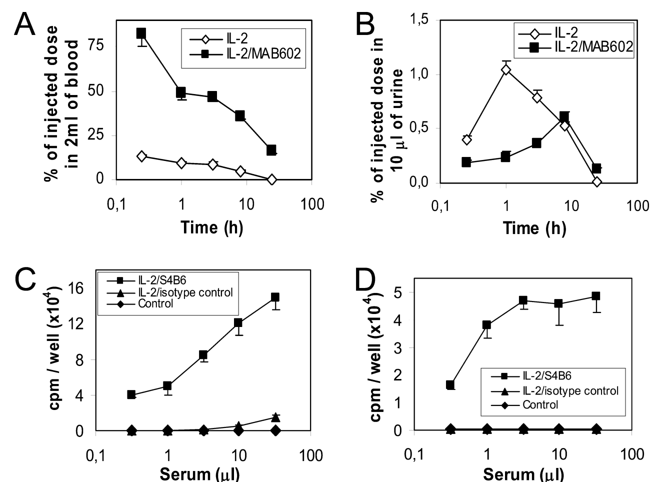

FIGURE 6. IL-2 immunocomplexes have much longer half-life than free IL-2. Recombinant human IL-2 was labeled with ^{131}I and injected i.v. into B6 mice either as a free labeled IL-2 or as IL-2 immunocomplexes. The injected dose contained 2 μg of labeled IL-2, which corresponded to 4×10^6 cpm at the time of application. The radioactivity in blood (*A*) and urine (*B*) was measured at 15 min and 1, 3, 8, and 24 h after injection. Data presented are mean ± SD of $n = 3$ mice per each condition. *C*, CTLL-2 cells were cultured at 5×10^4 cells per well with serially diluted sera from B6 mice injected i.v. with PBS (control), recombinant mouse IL-2/isotype control mixture or IL-2/S4B6 mAb immunocomplexes (1.5 μg of IL-2). Sera were harvested 1 h after the injection. *D*, Wells were coated with mouse anti-rat IgG mAb, washed and pulsed for 1 h with the sera used in *C*. After rigorous washing, CTLL-2 cells were seeded into the wells at 5×10^4 cells per well. Data show mean levels ± SD of [^{3}H]thymidine incorporation (*A* and *B*) for triplicate cultures on day 3.

nocomplexes), IL-2 mixed with an isotype control mAb and PBS. At 1 h after the injection, their sera were harvested and used for stimulation of CTLL-2 cell proliferation in vitro. An extensive and dose-dependent proliferation of CTLL-2 cells was achieved when sera from mice injected with IL-2/S4B6 mAb immunocomplexes were used (Fig. 6*C*). On the contrary, sera from the mice injected with IL-2 mixed with isotype control mAb induced very poor proliferation only when a very high amount was added (32 μl/well). In the next experiment, we coated the wells with anti-rat IgG mAb, then we added the same sera that were used in previous experiment, washed the wells, and finally seeded CTLL-2 cells into the wells. Hence, only IL-2 bound to S4B6 mAb (rat IgG), but not free IL-2, bound to anti-rat IgG mAb-coated wells and provide IL-2 signal to CTLL-2 cells (Fig. 6*D*). This finding implies that proliferation of CTLL-2 cells seen in Fig. 6*C* was stimulated by IL-2/S4B6 mAb immunocomplexes and not by free IL-2, which could have been theoretically released from the immunocomplexes under in vivo conditions.

Discussion

It is interesting that complexing cytokine with anti-cytokine mAb as novel approach to increase or modify cytokine activities in vivo seems to be not limited to IL-2 only but it is valid for at least several other cytokines. The study by Boyman et al. (30), which demonstrated strong and selective stimulatory activity of IL-2 immunocomplexes, also showed that IL-4 complexed with MAB404 or 11B11 anti-IL-4 mAb was very potent in stimulating the proliferation of memory-phenotype CD8^{+} cells, whereas IL-4 alone had almost no effect. It was also reported that IL-4 immunocomplexes strongly increases spleen cell Ia expression, increase number of CD23^{+} cells in the spleen and potently stimulate mucosal mastocytosis, which was demonstrated also for IL-3 immunocomplexes (31). IL-7 immunocomplexes were shown to induce enlargement of the spleen and lymph nodes 5- to 10-fold in cellularity, which was caused mainly by enormous expansion (200- to 400-fold) of immature (B220^{+} IgM^{-}) B cells (32). The same report also demonstrates that IL-7 immunocomplexes induce homeostatic proliferation of naive and memory T cells and they are able to enhance and restore thymopoiesis and augment primary CD8^{+} cell responses to foreign Ags (32). It was also mentioned that under some conditions anti-IL-6 mAb can act as protective carrier protein and thus work as agonist (33).

In this study we showed that IL-2 immunocomplexes can be used to strongly expand activated naive CD8^{+} T cells and that such expanded cells are able to form a robust population of memory cells which are functional in terms of effective IFN-γ production upon re-activation. Notably, this report is the first describing the powerful stimulatory activity of IL-2 immunocomplexes for activated naive CD8^{+} T cells. These results suggest that IL-2 immunocomplexes could be used as a strong enhancer of CD8^{+} T cell responses and thus they could significantly improve vaccination protocols aimed to trigger CTL responses. Especially low immunogenic vaccines, which are known to cause weak immunostimulation and thus provide only low or short-lasting protection should benefit from being coadministered with IL-2 immunocomplexes. We hypothesize that IL-2 immunocomplexes would improve vaccination also in the case in which CD4^{+} T cells or B cells play the main role. However, this question remains to be verified in appropriate experiments.

We demonstrated that IL-2/S4B6 immunocomplexes are also very potent in expanding NK cells. Actually, NK cells showed up to be even more sensitive to IL-2/S4B6 immunocomplexes than activated naive CD8^{+} T cells. High expansion of NK cells could be useful in cancer immunotherapy, especially in case of tumors that are known to express low levels of MHC class I. However, such expanded NK cells must retain their cytolytic activity. In preliminary experiments, we found that NK cell activity (detected as killing of YAC-1 cells by JAM assay) of spleen cells isolated from mice injected with IL-2/S4B6 immunocomplexes is significantly higher than those isolated from control mice or from mice inhjected with the same dose of free IL-2. Even after recalculation taking into account a higher number of NK cells in mice injected with IL-2/S4B6 immunocomplexes it seems that NK cells from such treated mice possess higher cytolytic activity than in controls.

We have shown in three distinct models that IL-2 immunocomplexes prepared in vitro under well-defined conditions could be useful in cancer immunotherapy. Coinjection of the S4B6 mAb with a plasmid carrying murine *il2* cDNA was used by Kamimura et al. (34) as an experimental method to show the enhancing effect of S4B6 mAb on IL-2 activity in vivo. This experimental design, however, prevents making any conclusions regarding the quantification of efficacy of IL-2/S4B6 mAb complexes on the increase of CD8^{+} T cells and NK cells (the observed parameters) because it was not possible to either determine the total amount of IL-2 expressed or to resolve the kinetics of this expression. Kamimura et al. (34) also showed that coinjection of S4B6 mAb reduced the number of lung metastasis of B16 melanoma to half that seen with injection of plasmid carrying murine *il2* cDNA alone. There are a significant number of reports showing that both NK cells (35, 36) and CD8^{+} T cells (35, 37) are involved in rejection of B16 melanoma cells and some authors even show that NK cells and CD8^{+} T cells work in cooperative manner (38, 39). Our results show that IL-2 immunocomplexes can promote their antitumor effects both via CD8^{+} T cells and NK cells implying that IL-2 immunocomplexes can be useful for cancer immunotherapy no matter whether the particular tumor is CD8^{+} T cell sensitive or NK cell sensitive.

Because extremely short half-life of IL-2 in circulation is notoriously known, it is obvious that prolonged half-life was suggested as one of the possible mechanisms explaining increased biological activity of IL-2 immunocomplexes in vivo. Two reports providing evidence regarding this hypothesis (30, 40) were published; however, neither of them provides data that would allow direct comparison of pharmacokinetics of IL-2 immunocomplexes vs free IL-2. In the first report (30), the pharmacokinetics was determined by use of an assay in which IL-2 immunocomplexes were injected at various time points before adoptive transfer of CFSE-labeled memory-phenotype CD8^{+} T cells and the subsequent analysis of proliferation of transferred cells by flow cytometry. Thus, rather a lifespan of biological activity of IL-2 immunocomplexes than lifespan of IL-2 immunocomplexes per se is determined by using this assay. In the second report (40), the pharmacokinetics of IL-2 immunocomplexes was not determined and only concentration of IL-2 in blood and urine measured by ELISA at a single time point (1 day) after injection was explored. Unfortunately, any conclusions based on determination of IL-2 concentration in urine at a single time point 1 day after the injection (i.e., very late) can be misleading.

Another originally suggested mechanism for the enhanced activity of the complexes was that IL-2 immunocomplexes are captured and presented by Fcγ receptor-bearing cells. This hypothesis seemed to be in concord with an observation that IL-2 complexed with F(ab$'$)$_2$ fragments of S4B6 mAb was less stimulatory than those with intact S4B6 mAb (30). However, the role of FcγR was ruled out by using FcγR$^{-/-}$ FcγRII$^{-/-}$ double knockout mice (41) and by using FcγR$^{-/-}$ mice treated with anti-FcγRII/RIII mAb (40). Nevertheless, a possible accumulation of IL-2 immunocomplexes in secondary lymphoid organs via mechanism independent to FcR could not be excluded. We found that radioactivity in the

spleen of mice injected with radioactively labeled IL-2 immunocomplexes was similar to that found in the liver and kidneys (except for 15 min time point, data not shown) thus ruling out the suggested possibility.

IL-2 immunocomplexes were found to induce activation and proliferation of naive CD8$^+$ T cells with subsequent differentiation into effector cells and later on also into central memory phenotype cells even in the absence of TCR stimulation. IL-2 immunocomplex-driven memory-like CD8$^+$ T cells were able to protect against lethal bacterial infection but showed incomplete cellular fitness compared with Ag-driven memory cells in terms of homeostatic turnover and cytokine production (41). We have shown in this study that providing TCR signal before treatment with IL-2 immunocomplexes produced memory CD8$^+$ T cells capable of strong IFN-γ production and that these cells could be found in the mice even very late (>120 days) after treatment (data not shown). These data suggest that homeostatic turnover of memory cells developed by TCR activation and providing strong IL-2 signals is not impaired.

Kamimura et al. (34) observed that IL-2/S4B6 immunocomplexes are equally potent as IL-2 plus isotype control Ab in stimulation of CTLL-2 cells in vitro. This finding is rather surprising to us since we have an extensive experience in testing stimulatory activity of IL-2 immunocomplexes in vitro using different responders including CTLL-2 cell line and we have never seen that IL-2/S4B6 immunocomplexes would be equal to IL-2. Instead, 5- to 10-fold higher activity of IL-2 than IL-2/S4B6 immunocomplexes was always recorded in CTLL-2 cells. We as well as other investigators (34) noticed that S4B6 mAb acts as truly IL-2 neutralizing Ab in vitro because increasing molar excess of S4B6 mAb over IL-2 is accompanied by decreasing proliferation of CTLL-2 cells (data not shown). We proved that this is true also for other anti-IL-2 mAb available, JES6-1A12 and JES6-5H4 clones and it is not limited to CTLL-2 cells only but it was also seen in memory-phenotype CD8$^+$ T cells (data not shown). Such observations suggest that IL-2 needs to dissociate from anti-IL-2 mAb to acquire biological activity (32) because the excess of anti-IL-2 mAb should be irrelevant if whole immunocomplexes are stimulatory per se. This would be further corroborated if the affinity of the mAb to the cytokine is lower than the affinity of the cytokine to its receptor. Thus, an excess of mAb would be required to block the activity of the cytokine (32, 34). It implicates the need to determine the affinity of each clone of anti-IL-2 mAb in the future.

The discussed phenomenon seems to be even more complicated under in vivo conditions, as administering a huge excess (>100 times) of S4B6 mAb over IL-2 into mice was shown to stimulate CD8$^+$ T cell proliferation to the same extent as administering only a small excess (~4.5 times) of S4B6 mAb over IL-2 (40). M25 clone of anti-IL-7 mAb augments the biological activity of IL-7 ~50–100 times in vivo, but contrary to S4B6 mAb and IL-2, injecting the excess of M25 mAb over IL-7 inhibits the in vivo activity of IL-7/M25 mAb immunocomplexes (32). Furthermore, three clones of anti-IL-7 were described to have no augmenting activity for IL-7 in vivo (32). Thus, elucidation of the mechanisms involved in the high activity of cytokine/mAb immunocomplexes, except for the prolongation of the half-life, remains a significant challenge. We hypothesize that these additional mechanisms could be 1) protection of IL-2 by anti-IL-2 mAb from proteolysis by masking the most proteolysis-sensitive sequences of IL-2 and 2) IL-2 immunocomplexes probably extravasate much slower than free IL-2 and, moreover, free IL-2 could bind to components of extracellular matrix and thus be unavailable for stimulation of immune cells as it was described that extracellular matrix shows a binding activity for various cytokines/growth factors (42–44). The binding to extracellular matrix as well as other nonspecific interactions of IL-2 might be prevented when IL-2 is complexed with anti-IL-2 mAb.

Disclosures

Marek Kovar is listed as coinventor 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. The remaining authors have no financial conflict of interest.

References

1. Gillis, S. 1983. Interleukin 2: biology and biochemistry. *J. Clin. Immunol.* 3: 1–13.
2. Herberman, R. B. 1986. Natural killer cells. *Annu. Rev. Med.* 37: 347–352.
3. Choi, Y. S. 1997. Differentiation and apoptosis of human germinal center B-lymphocytes. *Immunol. Res.* 16: 161–174.
4. Oppenheim, M. H., and M. T. Lotze. 1994. Interleukin-2: solid-tumor therapy. *Oncology* 51: 154–169.
5. Somersalo, K. 1996. Migratory functions of natural killer cells. *Nat. Immun.* 15: 117–133.
6. Johnson, J. G., and M. K. Jenkins. 1992. Co-stimulatory functions of antigen-presenting cells. *J. Invest. Dermatol.* 99: 62S–65S.
7. Rothenberg, E. V., and S. B. Ward. 1996. A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation. *Proc. Natl. Acad. Sci. USA* 93: 9358–9365.
8. Schwartz, R. H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* 71: 1065–1068.
9. Stauber, D. J., E. W. Debler, P. A. Horton, K. A. Smith, and I. A. Wilson. 2006. Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor. *Proc. Natl. Acad. Sci. USA* 103: 2788–2793.
10. Wang, H. M., and K. A. Smith. 1987. The interleukin 2 receptor: functional consequences of its bimolecular structure. *J. Exp. Med.* 166: 1055–1069.
11. Nelson, B. H., and D. M. Willerford. 1998. Biology of the interleukin-2 receptor. *Adv. Immunol.* 70: 1–81.
12. Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype CD8$^+$ T cells in vivo by IL-15. *Immunity* 8: 591–599.
13. Rosenberg, S. A., J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, J. S. Weber, D. R. Parkinson, C. A. Seipp, J. H. Einhorn, and D. E. White. 1994. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *J. Am. Med. Assoc.* 271: 907–913.
14. Dutcher, J. P., E. R. Gaynor, D. H. Boldt, J. H. Doroshow, M. H. Bar, M. Sznol, J. Mier, J. Sparano, R. I. Fisher, G. Weiss, *et al*. 1991. A phase II study of high-dose continuous infusion interleukin-2 with lymphokine-activated killer cells in patients with metastatic melanoma. *J. Clin. Oncol.* 9: 641–648.
15. Chang, A. E., C. L. Hyatt, and S. A. Rosenberg. 1984. Systemic administration of recombinant human interleukin-2 in mice. *J. Biol. Response Mod.* 3: 561–572.
16. Kruit, W. H., C. J. Punt, S. H. Goey, P. H. de Mulder, J. W. Gratama, A. M. Eggermont, R. L. Bolhuis, and G. Stoter. 1996. Dose efficacy study of two schedules of high-dose bolus administration of interleukin 2 and interferon α in metastatic melanoma. *Br. J. Cancer* 74: 951–955.
17. Kruit, W. H., K. J. Punt, S. H. Goey, P. H. de Mulder, D. C. van Hoogenhuyze, S. C. Henzen-Logmans, and G. Stoter. 1994. Cardiotoxicity as a dose-limiting factor in a schedule of high dose bolus therapy with interleukin-2 and α-interferon. An unexpectedly frequent complication. *Cancer* 74: 2850–2856.
18. Lotze, M. T., Y. L. Matory, S. E. Ettinghausen, A. A. Rayner, S. O. Sharrow, C. A. Seipp, M. C. Custer, and S. A. Rosenberg. 1985. In vivo administration of purified human interleukin 2. II. Half life, immunologic effects, and expansion of peripheral lymphoid cells in vivo with recombinant IL 2. *J. Immunol.* 135: 2865–2875.
19. Assier, E., V. Jullien, J. Lefort, J. L. Moreau, J. P. Di Santo, B. B. Vargaftig, J. R. Lapa e Silva, and J. Theze. 2004. NK cells and polymorphonuclear neutrophils are both critical for IL-2-induced pulmonary vascular leak syndrome. *J. Immunol.* 172: 7661–7668.
20. Kondapaneni, M., J. R. McGregor, D. Salvemini, V. E. Laubach, and W. E. Samlowski. 2008. Inducible nitric oxide synthase (iNOS) is not required for IL-2-induced hypotension and vascular leak syndrome in mice. *J. Immunother.* 31: 325–333.
21. Bui, L. A., L. H. Butterfield, J. Y. Kim, A. Ribas, P. Seu, R. Lau, J. A. Glaspy, W. H. McBride, and J. S. Economou. 1997. In vivo therapy of hepatocellular carcinoma with a tumor-specific adenoviral vector expressing interleukin-2. *Hum. Gene Ther.* 8: 2173–2182.
22. He, P., Z. Y. Tang, S. L. Ye, B. B. Liu, and Y. K. Liu. 2000. The targeted expression of interleukin-2 in human hepatocellular carcinoma cells. *J. Exp. Clin. Cancer Res.* 19: 183–187.
23. Lode, H. N., R. Xiang, J. C. Becker, S. D. Gillies, and R. A. Reisfeld. 1998. Immunocytokines: a promising approach to cancer immunotherapy. *Pharmacol. Ther.* 80: 277–292.
24. Reisfeld, R. A., J. C. Becker, and S. D. Gillies. 1997. Immunocytokines: a new approach to immunotherapy of melanoma. *Melonoma Res.* 7(Suppl. 2): S99–S106.

25. Becker, J. C., J. D. Pancook, S. D. Gillies, K. Furukawa, and R. A. Reisfeld. 1996. T cell-mediated eradication of murine metastatic melanoma induced by targeted interleukin 2 therapy. *J. Exp. Med.* 183: 2361–2366.
26. Pancook, J. D., J. C. Becker, S. D. Gillies, and R. A. Reisfeld. 1996. Eradication of established hepatic human neuroblastoma metastases in mice with severe combined immunodeficiency by antibody-targeted interleukin-2. *Cancer Immunol. Immunother.* 42: 88–92.
27. Katre, N. V., M. J. Knauf, and W. J. Laird. 1987. Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. USA* 84: 1487–1491.
28. Melder, R. J., B. L. Osborn, T. Riccobene, P. Kanakaraj, P. Wei, G. Chen, D. Stolow, W. G. Halpern, T. S. Migone, Q. Wang, et al. 2005. Pharmacokinetics and in vitro and in vivo anti-tumor response of an interleukin-2-human serum albumin fusion protein in mice. *Cancer Immunol. Immunother.* 54: 535–547.
29. Rao, B. M., I. Driver, D. A. Lauffenburger, and K. D. Wittrup. 2005. High-affinity CD25-binding IL-2 mutants potently stimulate persistent T cell growth. *Biochemistry* 44: 10696–10701.
30. Boyman, O., M. Kovar, M. P. Rubinstein, C. D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science* 311: 1924–1927.
31. Finkelman, F. D., K. B. Madden, S. C. Morris, J. M. Holmes, N. Boiani, I. M. Katona, and C. R. Maliszewski. 1993. Anti-cytokine antibodies as carrier proteins: prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. *J. Immunol.* 151: 1235–1244.
32. Boyman, O., C. Ramsey, D. M. Kim, J. Sprent, and C. D. Surh. 2008. IL-7/anti-IL-7 mAb complexes restore T cell development and induce homeostatic T cell expansion without lymphopenia. *J. Immunol.* 180: 7265–7275.
33. Klein, B., and H. Brailly. 1995. Cytokine-binding proteins: stimulating antagonists. *Immunol. Today* 16: 216–220.
34. Kamimura, D., Y. Sawa, M. Sato, E. Agung, T. Hirano, and M. Murakami. 2006. IL-2 in vivo activities and antitumor efficacy enhanced by an anti-IL-2 mAb. *J. Immunol.* 177: 306–314.
35. Kawarada, Y., R. Ganss, N. Garbi, T. Sacher, B. Arnold, and G. J. Hammerling. 2001. NK- and CD8$^+$ T cell-mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides. *J. Immunol.* 167: 5247–5253.
36. Kumar, V., and M. E. McNerney. 2005. A new self: MHC-class-I-independent natural-killer-cell self-tolerance. *Nat. Rev. Immunol.* 5: 363–374.
37. Dhanji, S., and H. S. Teh. 2003. IL-2-activated CD8+CD44high cells express both adaptive and innate immune system receptors and demonstrate specificity for syngeneic tumor cells. *J. Immunol.* 171: 3442–3450.
38. Kurosawa, S., M. Harada, G. Matsuzaki, Y. Shinomiya, H. Terao, N. Kobayashi, and K. Nomoto. 1995. Early-appearing tumour-infiltrating natural killer cells play a crucial role in the generation of anti-tumour T lymphocytes. *Immunology* 85: 338–346.
39. Xu, D., P. Gu, P. Y. Pan, Q. Li, A. I. Sato, and S. H. Chen. 2004. NK and CD8+ T cell-mediated eradication of poorly immunogenic B16–F10 melanoma by the combined action of IL-12 gene therapy and 4-1BB costimulation. *Int. J. Cancer* 109: 499–506.
40. Phelan, J. D., T. Orekov, and F. D. Finkelman. 2008. Cutting edge: mechanism of enhancement of in vivo cytokine effects by anti-cytokine monoclonal antibodies. *J. Immunol.* 180: 44–48.
41. Kamimura, D., and M. J. Bevan. 2007. Naive CD8$^+$ T cells differentiate into protective memory-like cells after IL-2 anti IL-2 complex treatment in vivo. *J. Exp. Med.* 204: 1803–1812.
42. Mazzieri, R., V. Jurukovski, H. Obata, J. Sung, A. Platt, E. Annes, N. Karaman-Jurukovska, P. E. Gleizes, and D. B. Rifkin. 2005. Expression of truncated latent TGF-β-binding protein modulates TGF-β signaling. *J. Cell Sci.* 118: 2177–2187.
43. Tatti, O., P. Vehvilainen, K. Lehti, and J. Keski-Oja. 2008. MT1-MMP releases latent TGF-β1 from endothelial cell extracellular matrix via proteolytic processing of LTBP-1. *Exp. Cell Res.* 314: 2501–2514.
44. ten Dijke, P., and H. M. Arthur. 2007. Extracellular control of TGFβ signalling in vascular development and disease. *Nat. Rev. Mol. Cell Biol.* 8: 857–869.