IL-2 In Vivo Activities and Antitumor Efficacy Enhanced by an Anti-IL-2 mAb

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IL-2 In Vivo Activities and Antitumor Efficacy Enhanced by an Anti-IL-2 mAb

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IL-2 is a potent immunostimulant and has been tested for clinical use, including in immunotherapy for cancers and HIV infection. Here we show that a widely used neutralizing anti-murine IL-2 mAb (S4B6) exhibits unexpected activities that enhance the treatment effects of IL-2 in vivo. Coinjection of the anti-IL-2 mAb with a plasmid carrying murine IL-2 cDNA significantly increased the serum IL-2 levels and induced a substantial increase in the division of CD8+ T and NK1.1high cells in vivo. Injection of the mAb premixed with recombinant murine IL-2 showed the same enhanced effect. A 5-day treatment with the anti-IL-2 mAb alone gradually increased the CD44highCD8+ cell population, and the increased population was maintained for >300 days, suggesting that the mAb can gradually maintain and potentially enhance the bioactivity of endogenous IL-2 for extended periods. Furthermore, combined treatment with the anti-IL-2 mAb plus the IL-2 plasmid markedly enhanced Ag-specific CTL activity in vivo and partially protected mice from tumor metastasis to the lungs, compared with the anti-IL-2 mAb or IL-2 plasmid alone. These results demonstrated IL-2-enhancing effects of the anti-IL-2 mAb in vivo and suggest that combining a neutralizing anti-IL-2 Ab with IL-2 gene delivery might be used effectively to enhance IL-2 functions in clinical applications. The Journal of Immunology, 2006, 177: 306–314.

Interleukin 2 is a cytokine produced mainly by activated T cells and a subset of CD4+ T cells under normal conditions and is a potent growth factor for immune cells, including T cells and NK cells, in vitro (1, 2). Because treatment with exogenous IL-2 shows immunostimulatory effects in animals and humans, IL-2 immunotherapy has long been tested for clinical applications (1). However, the accumulated evidence suggests that the endogenous level of IL-2 has rather immunosuppressive and autoimmune-preventing activities in vivo (3). A recent study formally demonstrated, using foxp3gfp mice, that IL-2 is a critical survival factor for Foxp3+ regulatory CD4+ T cells (4), which explains the development of autoimmune disorders in IL-2 knockout (KO), CD25 KO, and CD122 (the common subunit for IL-2R and IL-15R) KO mice (5–7). The disease states associated with lymphoproliferation in these animals often hamper the acquisition of genetic evidence for the in vivo functions of IL-2 in various experimental systems. To overcome this, neutralizing and/or signal-blocking Abs against IL-2 and its receptor subunits have been used to investigate the in vivo IL-2 functions. One widely used neutralizing mAb is anti-murine IL-2 mAb clone S4B6 (8). This mAb is a rat IgG2a known to inhibit the activity of murine, but not human or rat, IL-2 (9). Because recombinant murine IL-2 (rmIL-2) derived from bacteria, yeast, monkey, and mouse cells are recognized by the mAb, the carbohydrate modification of IL-2 seems not to be important for the mAb binding (9). Extensive mutational studies revealed that an intact N-terminal portion of mIL-2 (Q26 to Y45) is required for its recognition by the anti-IL-2 mAb (9). This region encompasses an amino acid residue (D34) within the α helix A that is critical for the CD122 binding of IL-2 (10). In addition, the peptide encompassing α helix A of human IL-2, termed p1–30, acts as an agonist through CD122 (11). These lines of evidence establish the neutralizing potential of the anti-IL-2 mAb.

Using this anti-IL-2 mAb, we previously found that the deletion of memory CD8+ T cells increases significantly after IL-2 depletion in vivo (12–14). The increased cell divisions are associated with a reduction in the CD25+ CD4+ regulatory T cells, due to the IL-2 depletion (13), are independent of IL-15 but require CD122 signaling in memory CD8+ T cells (14). The CD122 dependency in IL-2-depleted IL-15 KO mice led us to hypothesize that a novel CD122-targeted cytokine mediates the increased division of the memory CD8+ T cells (14). To clone this factor, we developed an in vivo screening system using a hydrodynamic plasmid DNA delivery method (15). In the course of developing this assay, however, we noticed that the anti-IL-2 mAb S4B6 unexpectedly seemed to enhance the biological activities of IL-2 in vivo.

Previous studies showed that Abs against human IL-2 (16–19), human IFN-α (20), and murine IL-3, IL-4, IL-6, and IL-7 (21–23) act as carrier proteins for their respective cytokines, prolonging their serum half-life and in most cases enhancing their bioactivities.

Here we demonstrated that the widely used neutralizing anti-murine IL-2 mAb S4B6 also has IL-2 potentiating effects in vivo, especially when IL-2 is abundant. Furthermore, by exploiting this unexpected property, we found that concurrent treatment with the mAb and IL-2 gene delivery preferentially and significantly

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† Abbreviations used in this paper: Cy, carbocyanin; rh, recombinant human; rm, recombinant murine; IL-2-His, IL-2 tagged with 6× His at the C terminus; WT, wild type.

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increased the numbers of cells belonging to types believed to function in tumor immunosurveillance, including NK1.1highTCRβ+ classical NK cells and NK1.1+CD44highCD8+ T cells (24, 25), and elicited effective antitumor immunity in mice.

Materials and Methods

Mice

C57BL/6d (B6) mice were purchased from CLEA Japan. B6.SJL (CD45.1) mice were obtained from Taconic. B6.OT-1 TCR-transgenic mice were provided by Dr. W. R. Heath. All strains were kept under specific pathogen-free conditions in the animal facility of RIKEN RCAST. The animal experiments were approved by the RIKEN Yokohama Institute Safety Center.

Reagents and mAbs for flow cytometry

The mAbs used for flow cytometry were: PE-carboxyfluorescein (Cy7-) or allophycocyanin-labeled anti-CD4 mAb (RM4-5); PE- or PE-Cy7-labeled anti-CD8α mAb (53–67); PE-Cy7-labeled anti-CD19 mAb (BD5); FITC- or allophycocyanin-labeled anti-CD44 mAb (IM7); biotinylated anti-CD45.1 mAb (A20); PE-labeled anti-CD45R (B220) mAb (RA3-6B2); FITC-labeled anti-CD69 mAb (H1.2F3); PE-labeled anti-CD122 mAb (TM6); biotinylated anti-CD45R (B220) mAb; PE-labeled anti-CD44 conjugated streptavidin was used to detect the biotinylated mAbs. These mAbs and streptavidin reagents were obtained from BD Biosciences, eBioscience, BioLegend, and Caltag Laboratories. Foxp3+ regulatory T cells were detected using the allophycocyanin anti-mouse/rat Foxp3 Staining Set (eBioscience).

Preparation and injection of IL-2 plasmid DNA

The full-length cDNA for mIL-2 was obtained by PCR using cDNA from PMA-activated CD8+ T cells. The mIL-2 cDNA in the presence or absence of IL-2 targeted with 6x His at the C terminus (IL-2-His) was cloned into expression vectors: the pCAGGS vector (15); and Gateway pcDNA series destination vectors (Invitrogen). The controls were the Gateway vectors containing GFP or the pCAGGS vector without insert DNA. These plasmid DNAs were purified with the GeneElute endotoxin-free plasmid Mini Prep kit (Sigma-Aldrich) or EndoFree Plasmid Maxi Kit (Qiagen). For IL-2 protein expression in vivo, mice were given a bolus injection of 2 ml of lactate Ringer’s solution containing the IL-2 plasmid DNA. The pCAGGS plasmid vector induces very strong protein expression through a combination of the CMV enhancer and chicken β-actin promoter (15). In this study, this vector was used only for the serum IL-2 Western blot assay, because IL-2 from the pCAGGS vector, even when as little as 10 μg were given, was insufficient to detect mortality 4–5 days after the treatment, but up to 50 μg of pcDNA/mL-2L did not have this effect. The dose of 6.25 μg of the pcDNA was determined by dose-response experiments (data not shown). Some mice were also given i.p. injections of 1 mg of anti-IL-2 mAb (S4B6) or rat IgG (Sigma-Aldrich). Preparation of the protein G-purified anti-IL-2 mAb and anti-CD122 mAb (TMβ1) was performed as described previously (14). A sandwich ELISA system for IL-2 was obtained from BioSource International.

Preparation of the anti-IL-2 mAb/CIL-2 mixture

The anti-IL-2 mAb or rat IgG (1 mg) and mIL-2 or recombinant human IL-2 (rhIL-2; 2 μg/ml) with or without 2-ME) and heated for 10 min for the reducing condition. The resulted samples were run on 2–15% PAGE gel (Daiichikagakusha). Western blotting was performed by a standard method using anti-His-tag Ab.

In vivo cytotoxicity assay

The lymph nodes (inguinal, axillaries, cervical, and mesenteric) and spleen were harvested from OT-1 TCR transgenic mice, passed through a 100-μm pore size cell strainer (BD Falcon) with RPMI 1640, and washed. Erythrocytes were eliminated with 0.165 M NH4Cl. A T cell-enriched sample from B6.SJL mice were incubated for 30 min at 37°C with or without 10 μg/ml OVA257–264 peptide (SIINFEKL; Operon Biotechnologies). These peptide-pulsed target and nontarget populations were labeled with 5 and 0.25 μM CFSE (Invitrogen Life Technologies), respectively, mixed at a 1:1 ratio, and injected i.v. into the mice. After 12 h, the target cell-killing activity was evaluated using the ratio of CFSE-positive populations in the spleen. The CFSE-labeled populations were distinguished from host populations by CD45.1 expression.

B16 lung metastasis model

B16 melanoma cells were kindly provided by Dr. Shin-ichiro Fujita (RIKEN RCAST). B16 cells (2 × 10⁶/mouse) were injected i.v. on day 0. The tumor-bearing mice received a plasmid DNA injection (6.25 μg/mouse i.v.) with or without the anti-IL-2 mAb (1 mg/mouse i.p.) on day 2. The number of B16 nodules in the lungs was counted on day 14.

Statistical analysis

Statistical differences between groups were examined by unpaired Student’s t tests. A value of p < 0.05 was considered statistically significant.

Results

Neutralizing anti-murine IL-2 mAb (S4B6) enhances the in vivo effect of IL-2

We have reported that in vivo treatment with the neutralizing anti-IL-2 mAb (clone S4B6) promotes the division of memory CD8+ T cells in mice, by an IL-15-independent but CD122-dependent mechanism (12, 14). To further investigate the in vivo roles of IL-2 and IL-15 and to screen for a putative novel CD122-targeted cytokine (14), we used an in vivo hydrodynamic delivery system for plasmid DNA, by which proteins of interest can be transiently expressed and their functions examined (15, 26). In the course of this assay, however, we unexpectedly found that the anti-IL-2 mAb potentiates the in vivo effects of IL-2.

After an i.v. injection of 6.25 μg of pcDNA expression vectors encoding murine IL-2 cDNA, serum IL-2 was transiently detected by ELISA; it returned to baseline by 3 days posttreatment (see Fig. 1A, ○). As expected, overexpression of IL-2 by the IL-2 plasmid (plus rat IgG) significantly increased the BrdU uptake in CD8+ T cells in vivo, as compared with the vehicle injection (Fig. 2A). Total splenocyte counts also increased 5 days after the IL-2 plasmid DNA injection (Fig. 2B, hatched columns).

To check whether this effect was really due to IL-2 overexpression, the anti-IL-2 mAb (S4B6), which has been used as a neutralizing mAb, was injected i.p. at the same time as the IL-2 plasmid DNA. As reported previously, the anti-IL-2 mAb (plus control plasmid DNA) increased the BrdU+CD44highCD8+ T cell population, compared with rat IgG and vehicle treatment (14). After the coinjection of the anti-IL-2 mAb and IL-2 plasmid DNA, however, the biological effects of IL-2 were markedly enhanced: almost all the CD8+ T cells became BrdU positive (Fig. 2A), and the number
treatment with rat IgG and the control plasmid.

IL-2 mAb and clearly detected by Western blotting after coinjection of the anti-

signal intensity for serum IL-2 between the anti-IL-2 mAb plus

obtained from the treated mice. No detectable signal corresponding to mIL-2 was

ELISA results by a Western blotting analysis using sera from plasmid combination treatment. Therefore, we sought to verify the with the ELISA system, thereby leading to an underestimation of

plex of the anti-IL-2 mAb and rmIL-2 was not detected by the IL-2

ELISA (data not shown). This suggests that the mAb interfered

with the ELISA system. As shown in Fig. 1

increased and sustained lev-

A,

4 –5).

Serum IL-2 levels after the injection

plasmid DNAs (6.25 μg) with or without the anti-IL-2 mAb, measured

by ELISA. Data are given as the mean ± SEM (n = 4–5). * p < 0.01 vs
treatment with rat IgG and the control plasmid. B, Serum IL-2 (top) and

and L-chain of IgG as the loading control (bottom) 1 day after treatment

were detected by Western blotting. Lane 1, rat IgG plus pCAGGS; lane 2, anti-

IL-2 mAb plus pCAGGS; lane 3, rat IgG plus pCAGGS/mIL-2; lane 4, anti-IL-2 mAb plus pCAGGS/mIL-2. The doses of Abs and plasmid DNA

were 1 mg and 50 μg, respectively.

of splenocytes substantially increased compared with their number

after treatment with rat IgG and IL-2 plasmid DNA (Fig. 2B). The

preferential increase of CD8+ T cells and TCRβ+ NK1.1high NK

cells accounted for most of the cell population expansion follow-

ing the coinjection of the anti-IL-2 mAb and IL-2 plasmid DNA

(Fig. 2B). These results suggest that the neutralizing anti-IL-2

mAb, clone S4B6, unexpectedly enhances the biological activities

of IL-2 when it is overexpressed by injected plasmid DNA.

Anti-IL-2 mAb increases the amount of IL-2 in the serum

To investigate the effect of the anti-IL-2 mAb on serum IL-2 levels

after IL-2 plasmid DNA injection, we first attempted to use an

ELISA system. As shown in Fig. 1A, increased and sustained lev-

els of serum IL-2 were detected after the anti-IL-2 mAb plus IL-2

plasmid DNA treatment compared with the rat IgG plus IL-2 plas-

mid DNA treatment. However, we found that a preformed com-

plex of the anti-IL-2 mAb and rmIL-2 was not detected by the IL-2

ELISA (data not shown). This suggests that the mAb interfered

with the ELISA system, thereby leading to an underestimation of

the serum IL-2 levels attained after the anti-IL-2 mAb plus IL-2

plasmid combination treatment. Therefore, we sought to verify the

ELISA results by a Western blotting analysis using sera from
treated mice. No detectable signal corresponding to mIL-2 was

obtained from the IL-2 plasmid DNA alone, when 0.1 μl of the

serum was assayed (Fig. 1B, lane 3). In contrast, serum IL-2 was

clearly detected by Western blotting after coinjection of the anti-

IL-2 mAb and IL-2 plasmid (Fig. 1B, lane 4). The difference in

signal intensity for serum IL-2 between the anti-IL-2 mAb plus

IL-2 plasmid DNA and rat IgG plus IL-2 plasmid DNA on the

Western blot was much greater than the ELISA results indicated

(Fig. 1A and data not shown), suggesting that both free IL-2 and a

relatively large amount of mAb-bound IL-2, which was not de-
tectable by ELISA, were present in the circulation after the anti-

IL-2 mAb plus IL-2 plasmid DNA injections.

Preformed complex of anti-IL-2 mAb and rmIL-2 also shows the agonist effect

We were concerned that the enhancement of the IL-2 bioactivity

by the anti-IL-2 mAb might be observed only when the plasmid

DNA infusion system was used. To exclude this possibility, 2 μg

of rmIL-2 or rhIL-2, as a nonbinding control, was incubated in

vitro with excess anti-IL-2 mAb (1 mg) to allow the formation of

the immune complex (in the case of rmIL-2). After the in vitro

incubation, the murine IL-2 ELISA failed to detect signals in the

anti-IL-2 mAb and rmIL-2 mixture, suggesting that the immune

complex had formed and was interfering with the ELISA system

(data not shown). These mixtures were then injected into mice. As

shown in Fig. 3, BrdU uptake in the CD44highCD8+ (Fig. 3, top)

and NK1.1high (Fig. 3, bottom) populations was slightly increased

by the coinjection of rat IgG and 2 μg of rmIL-2 or rhIL-2. The

anti-IL-2 mAb treatment without IL-2 (medium) enhanced BrdU

uptake, as reported (14). The combination of the anti-IL-2 mAb

plus rhIL-2 showed only an additive effect, particularly on the

CD44highCD8+ population. In contrast, a synergistic effect was

observed after treatment with the preformed complex of the anti-

IL-2 mAb plus rmIL-2, and almost all the CD44highCD8+ and

NK1.1high cells became BrdU positive. This synergistic effect

was diminished when an anti-CD122 mAb was injected to inhibit IL-2

signaling (Fig. 3). This result, together with the those shown in

Fig. 2, demonstrate that the anti-IL-2 mAb enhances the biological

activities of IL-2 in vivo, that this effect is mediated at least in part

through IL-2Rβ molecules (CD122), and that proper mAb/Ag

recognition is required, given that the mAb plus rhIL-2 had only an

additive and no synergistic effect.

The anti-IL-2 mAb reduces the Foxp3+-regulatory CD4+ T cell

population in vivo

In contrast to the results shown in Figs. 2 and 3, i.p. injections of

the anti-IL-2 mAb alone decreased the population of Foxp3+-regu-

latory CD4+ T cells after 7 days, by about one-half (Fig. 4),

which is consistent with other reports using the same clone (2, 13).

Moreover, the population of Foxp3+-regulatory CD4+ T cells in

the periphery is about one-half that of WT in IL-2 KO and CD25

KO mice (4). Our result suggests that the anti-IL-2 mAb exhibits

an in vivo neutralizing activity, at least for Foxp3+-regulatory

CD4+ T cells, unless IL-2 is overexpressed or the protein level of

IL-2 exceeds the physiological concentration.

Treatment with the anti-IL-2 mAb alone causes a long term increase

in the CD44highCD8+ T cell population

Given our results thus far and the reported evidence that IL-2 is

produced by a subset of CD4+ T cells under normal conditions (2),

we were attracted to the idea that the anti-IL-2 mAb injected in

vivo could increase IL-2 biological activity by capturing and re-

taining endogenous IL-2, which would then accumulate and even-

tually show increased activity. To investigate this possibility, WT

mice were treated with the anti-IL-2 mAb alone for the first 5 days,

and the CD44highCD8+ T cell population in the peripheral blood

was checked periodically for >300 days. As shown in Fig. 5, the

CD44highCD8+ T cell population in the anti-IL-2 mAb-treated

mice gradually increased for ~50 days, and this increased

CD44highCD8+ population could be detected for >300 days (Fig.
At this point, the control mice also showed a large proportion of CD44<sup>high</sup>CD8<sup>+</sup> T cells due to the age-associated increase of these cells (12, 27). Similar results were obtained in a second set of experiments, in which rat IgG was used as a control (Fig. 5, experiment 2). These results indicate that the anti-IL-2 mAb may indeed capture the endogenous IL-2 and increase the IL-2 levels to exhibit enhanced biological activity in long term assays.

**Concurrent treatment with the anti-IL-2 mAb and IL-2 plasmid increases phenotypically activated NK1.1<sup>high</sup> NK cells and CD8<sup>+</sup> T cells**

The immunostimulatory action of IL-2 has been tested for clinical use to target several types of cancer and HIV infection (1). One obstacle to the clinical use of IL-2 is its short half-life in the circulation (28, 29). We hypothesized that the ability of the anti-IL-2 mAb to retain IL-2 in vivo would be beneficial for IL-2 immunotherapy, especially when treatment with the mAb was combined with delivery of the IL-2 plasmid DNA. Therefore, we investigated the possibility of using concurrent treatment with the anti-IL-2 mAb and IL-2 plasmid DNA to generate a sustained immune activation.

After the anti-IL-2 mAb plus IL-2 plasmid DNA treatment, a time course analysis revealed that the percentage of CD8<sup>+</sup> and NK1.1<sup>high</sup> (mostly CD4<sup>+</sup> CD8<sup>+</sup>) populations in the total PBL substantially increased, reaching ~50% and 30%, respectively, on day 5 (Fig. 6A). The increase in the NK1.1<sup>high</sup> population declined thereafter, but the CD8<sup>+</sup> population remained at >40% of the PBL until at least day 12. Treatment with the IL-2 plasmid DNA alone (plus rat IgG) did not significantly affect the CD8<sup>+</sup> population and slightly but significantly increased the NK1.1<sup>+</sup> population (Fig. 6A). After the IL-2 plasmid DNA treatment, the activation marker CD69 was clearly up-regulated on the NK1.1<sup>high</sup> population, and this up-regulation was more prolonged when the anti-IL-2 mAb was coinjected with the plasmid (Fig. 6B, bottom).

Interestingly, the anti-IL-2 mAb plus IL-2 plasmid DNA treatment also selectively induced CD44<sup>high</sup>CD8<sup>+</sup> T cells expressing NK1.1 molecules (Fig. 6C). The NK1.1 level on the CD44<sup>high</sup>CD8<sup>+</sup> T cells was lower than on the TCR<sup>+</sup> NK1.1<sup>high</sup> cells (data not shown). It is reported that IL-2-activated splenocytes from NKT cell-deficient mice acquire the expression of NK cell-associated molecules including NK1.1 on CD8<sup>+</sup> T cells (30). In addition, IL-2-activated CD44<sup>high</sup>CD8<sup>+</sup> T cells with NK receptors can kill syngeneic tumor cells in vitro (25). We examined the existence of anti-IL-2 Ab in serum and observed the Ab existed at least 25 days after the final injection of the Ab (1 mg in 5 i.p. injections) by Western blotting (data not shown). We also showed that the expression of the complex between IL-2-His and anti-IL-2 Ab (around 200 K<sub>d</sub> in a nonreducing condition) as well as IL-2-His alone (between 28 K<sub>d</sub> and 21 K<sub>d</sub> in nonreducing and reducing conditions) in serum at least 3 days after the treatment of a expression vector carrying IL-2-His plus the anti-IL-2 Ab in vivo under nonreducing conditions (Fig. 6D). These results suggest that the combined treatment of the anti-IL-2 mAb plus the IL-2 plasmid induces a prolonged activation state in NK cells and CD8<sup>+</sup> T cells in vivo.

**Concurrent treatment with the anti-IL-2 mAb and IL-2 plasmid enhances Ag-specific CTL activity in vivo**

To investigate whether the expansion of activated CD8<sup>+</sup> T cells after treatment with the anti-IL-2 mAb plus IL-2 plasmid was functional, Ag-specific CTL activity was evaluated in vivo. Mice that had received an injection of OVA-specific CD8<sup>+</sup> T cells (OT-1 T cells) on day −2 were treated with combinations of Abs plus plasmid DNAs on day 0. Equal numbers of target cells, which were pulsed with the OVA<sub>257-264</sub> peptide that was recognized by the OT-1 T cells and labeled with a high concentration of CFSE, and nontarget cells with low CFSE labeling and without the peptide pulse were injected on day 6 after the treatment. After 12 h, the
Combined use of the anti-IL-2 mAb and the anti-IL-2 plasmid alone (plus rat IgG) had some killing effect, the tumor population was almost completely eliminated by the coinjection of the anti-IL-2 mAb and the IL-2 plasmid (Fig. 7). This result indicates that concurrent treatment with the anti-IL-2 mAb and IL-2 plasmid substantially augmented the Ag-specific CD8\(^+\) T cell responses in vivo.

**Concurrent treatment with the anti-IL-2 mAb and IL-2 plasmid after tumor infection elicits an effective antitumor immune response**

The simultaneous administration of the anti-IL-2 mAb and the IL-2 plasmid generated sustained activation of CD8\(^+\) T cells and NK cells. Both of these populations are known to be involved in tumor immunity (24, 25). Therefore, we investigated whether the combined use of the anti-IL-2 mAb and IL-2 plasmid would prevent tumor progression in a murine B16 lung metastasis model. In this experiment, a single simultaneous treatment with the anti-IL-2 mAb and IL-2 plasmid was performed 2 days after the mice were inoculated with B16 melanoma cells by i.v. injection. On day 14 (i.e., 12 days posttreatment), tumor metastasis was evaluated by counting the B16 nodules in the lungs. We found that the combined use of the anti-IL-2 mAb and the IL-2 plasmid was effective and significantly reduced the number of B16 nodules in the lung, whereas the IL-2 plasmid alone (plus rat IgG) only slightly decreased the number of nodules (Fig. 8). These results clearly demonstrated that therapeutic treatment consisting of the concurrent administration of the anti-IL-2 mAb and the IL-2 plasmid effectively protects mice from tumor metastasis in this tumor inoculation model.

**Discussion**

In this study, we demonstrated 1) that a neutralizing anti-murine IL-2 mAb (clone S4B6) sustained IL-2 in the circulation in vivo and thereby enhanced its biological functions, and 2) that the combined treatment with the anti-IL-2 mAb and the IL-2 plasmid strongly provoked antitumor immunity in mice. These unexpected functions of the anti-IL-2 mAb became more evident when IL-2 was overexpressed (Figs. 1, 2, and 6–8) or injected as a mAb/ rmIL-2 immune complex (Fig. 3). In addition, the anti-IL-2 mAb potentially effects the accumulation of endogenous IL-2 so that it exerts biological activity on CD8\(^+\) T cells for extended periods (Fig. 5).

We have investigated T cell homeostasis in vivo and reported that memory CD8\(^+\) T cells undergo increased cell divisions in vivo when IL-2 is depleted by the anti-IL-2 mAb (12–14). The increased division of memory CD8\(^+\) T cells after anti-IL-2 mAb treatment is associated with a reduction in CD25\(^+\)/CD4\(^+\)-regulatory T cells (13), is independent of IL-15, and requires CD122 expression on memory CD8\(^+\) T cells (14). From these observations, we concluded that a novel putative CD122-targeted cytokine was probably involved in this process (14). The findings in the present study revealed an IL-2-enhancing activity of the anti-IL-2 mAb and may challenge the conclusion of our previous report. However, our experimental protocol of anti-IL-2 mAb injections that led to increased divisions of memory CD8\(^+\) T cells actually reduced the Foxp3\(^+\)/CD4\(^+\) and CD25\(^+\)/CD4\(^+\)-regulatory T cells in vivo (Fig. 4 and Ref. 13). A recent report by Fontenot et al. (4) using foxp3\(^{gfp}\) mice demonstrated genetically that IL-2 is critical for the peripheral maintenance of regulatory T cells, suggesting that although an increased proliferation of memory CD8\(^+\) T cells was induced by the anti-IL-2 mAb treatment, the in vivo IL-2 level is reduced by this treatment to levels that are insufficient to support the survival of regulatory T cells. In addition, a negative effect of IL-2 on the maintenance of memory CD8\(^+\) T cells in IL-2 KO mice is also reported (31). Furthermore, there is experimental evidence that human amniotic fluid lacks IL-2 and IL-15 but can interact with CD122 (32). These observations support the conclusions from our previous studies. However, a more careful re-examination of the in vivo relationship between IL-2 and the maintenance of memory CD8\(^+\) T cells is necessary. In any case, we originally were looking for another CD122-binding cytokine to explain early work with this Ab (14), and it is not clear from the results here whether that question was answered or explained entirely with the data presented here. Additional studies to answer this question would be warranted.

Similar to the anti-IL-2 mAb, some other Abs against cytokines are reported to increase in vivo levels of their target cytokine, thereby enhancing their biological activities in most cases. These include Abs against human IL-2 (16–19), human IFN-α (20), and murine IL-3, IL-4, IL-6, and IL-7 (21–23). The precise mechanisms underlying the agonist action of these Abs are still unclear, but the following possibilities can be proposed. First, the anti-IL-2 mAb could reduce the clearance of IL-2, which is reported to take place in the kidney rather than in the liver in rodents (28, 33). Because small proteins (<50 kDa) are filtered in the kidney (34), the binding of IL-2 molecules to a macromolecule such as IgG would prevent their renal clearance, as reported for polymer-attached IL-2 (35). Second, the mAb could act as a carrier protein of IL-2. The in vivo half-lives of IgGs are dependent on the subtype. Rat IgG2a, the subtype of the anti-IL-2 mAb S4B6, has a half-life of ~10 days (β phase) in mice (36), which is much longer than that of IL-2 (28, 29, 35). Although we did not precisely determine the half-life of the IL-2/mAb complex, this relatively long half-life of rat IgG2a could contribute to sustaining the IL-2/mAb complex in vivo.

**FIGURE 3.** Preformed complex of the anti-IL-2 mAb and rmIL-2 is more effective than rmIL-2 alone. Anti-IL-2 mAb or rat IgG (1 mg) was incubated overnight in vitro with 2 μg of rmIL-2 or rhIL-2. Mice were given an i.p. injection of the mixture and BrdU in drinking water for 4 days. One treatment group also received an i.p. injection of anti-CD122 mAb (200 μg/mouse). BrdU levels in the spleen were assessed on day 5. Histograms are gated on the CD44\(^{high}\)/CD8\(^{high}\) (top) or the NK1.1\(^{high}\) (bottom) population. The limit bars show percentage of BrdU\(^+\) cells.

**FIGURE 4.** The anti-IL-2 mAb reduces the Foxp3\(^+\)/CD4\(^+\) T cell population in vivo. Mice received an i.p. injection of 1 mg of anti-IL-2 mAb or rat IgG on days 0, 1, 2, 3, and 5. The Foxp3\(^+\) population in the spleen was examined on day 6. Data are from two mice for each treatment; the histograms are gated on the TCRβ\(^+\)/CD4\(^+\) population. The limit bars show percentage of Foxp3\(^+\) cells.
vivo. The stability of the IgG also explains the long term effect of the mAb on the CD44<sup>high</sup>CD8<sup>+</sup> population (Fig. 5). Consistent with this, we observed the Ab against IL-2 was existed at least 25 days after final injection of the Ab (1 mg in 5 i.p. injections) by Western blotting (data not shown). Because we also showed that the complex between IL-2-His and anti-IL-2 Ab was observed in serum at least 3 days after treatment of an expression vector carrying IL-2-His plus the anti-IL-2 Ab in vivo using Western blotting under nonreducing conditions (Fig. 6D), we hypothesize that there are certain amounts of SDS-stable complex between IL-2 and anti-IL-2 Ab in vivo using Western blotting under nonreducing conditions (Fig. 6D), we hypothesize that there are certain amounts of SDS-stable complex between IL-2 and anti-IL-2 Ab in serum after injection of anti-IL-2 Ab for a certain time period. Because CD44<sup>high</sup> memory CD8<sup>+</sup> T cells have a long life span and show slow homeostatic proliferation (12, 37), it is possible that the anti-IL-2 mAb was present and effective in vivo for at least 50 days, whereas the CD44<sup>high</sup>CD8<sup>+</sup> population increased (Fig. 5). The reported finding that the level of CD25<sup>+</sup>CD4<sup>+</sup>-regulatory T cells is reduced for 2 mo after a single i.v. injection of the same anti-IL-2 mAb clone also supports our observation of a long term effect of the mAb (2). Third, the mAb could protect IL-2 from physiological inactivation, such as via proteolysis. Finkelman et al. (22) reported that neutralizing mAbs for IL-4 (11B11 and BVD4.1D11.2), but not a nonneutralizing anti-IL-4 mAb (BVD6.24G2.3), increased IL-4 bioactivity in vivo, suggesting that the masking of an important region of the cytokine, such as a receptor contact site, plays a key role in this effect.

T regulatory cell percentage has been recovered within 2 mo, but the memory/activated phenotype of the conventional T cells has not been recovered at this time point after anti-IL-2 Ab treatment (Fig. 5 and data not shown). Thus, we hypothesize that the Ab/IL-2 complexes cannot delete functional T regulatory cells but can stimulate the conventional T cells. It is possible that there might be two modes of effect of the Ab: 1) a soluble Ab efficiently neutralizes IL-2 to reduce the T regulatory cells; and 2) the antibody-IL2 complexes can stimulate CD8 and NK cells but not neutralize extra IL-2 in circumstances, which is critical for T regulatory cell survival. Therefore, we hypothesize that the described effect is the results of both receptor agonism (on CD8 and NK cells) and antagonism (on T regulatory cells) of the Ab. We actually spent much time for the experiments to identify the mechanism of the Ab-mediated proliferation/activation of T cells. These experiments include the stimulation of CD44<sup>high</sup>CD8<sup>+</sup>, CD4<sup>+</sup>, and CTLL2 cells in the combination of rmIL-2, anti-IL-2, anti-CD3 and/or splenic CD11c<sup>+</sup> dendritic cells. However, we have always detected that addition of excess amount of the anti-IL-2 Ab did not enhance but suppressed IL-2-induced survival/proliferation of the T cells tested in vitro (data not shown), although the complex formed in vitro.

**FIGURE 5.** Treatment with the anti-IL-2 mAb alone increases the CD44<sup>high</sup>CD8<sup>+</sup> T cell population over time. Mice were given an i.p. injection of 1 mg of anti-IL-2 mAb or rat IgG for the first 5 days. Control groups had no treatment (experiment (Exp.) 1) or were given 1 mg of rat IgG on the same days (experiment 2). The PBL were examined periodically for CD44 levels on the CD8<sup>+</sup> T cells. Data are from two mice for each treatment; histograms are gated on the CD8<sup>+</sup> population. The limit bars show percentages of CD44<sup>high</sup> cells.
stimulated CTLL2 cells just like IL-2 plus control IgG (data not shown) and induced proliferation of CD8 T cells in vivo (Fig. 3). Our data indicate that formation of IL-2 and anti-IL-2 mAb complex in advance of injection is not necessary for the effect of the Ab to show the effect of the Ab in vivo, because we confirmed this point by using a different route of injections (i.e., conditioned medium containing IL-2 i.p. and anti-IL-2 mAb i.v.; data not shown).

In vitro, the S4B6 is well known to have neutralizing activity to CD4 T cells. We have similar in vitro results using CD8 T cells, CD4 T cells, and CTLL2, although an excess amount of anti-IL-2 mAb relative to IL-2 is required for significant inhibition of IL-2 activity in vitro (data not shown). Because we did not observe the enhancing effect of IL-2 by anti-IL-2 mAb in vitro but observed that the preformed complex between the Ab and IL-2 stimulated CTLL2 cells just like IL-2 plus control IgG (data not shown), we speculate that it is due to metabolic advantages of the IL-2/anti-IL-2 mAb complex in vivo, although we do not completely exclude the other possibilities.

Extensive mutational studies of IL-2 revealed that an intact N-terminal portion (Q26–Y45) is required for its recognition by the anti-IL-2 mAb (9). This region contains an amino acid residue (D34) within α helix A that is critical for the CD122 binding of IL-2 (10). In addition, the peptide encompassing α helix A of human IL-2, termed p1–30, has an agonist effect through CD122 (11). Although we do not have direct evidence that the anti-IL-2 mAb blocks the binding of IL-2 to CD122, these lines of evidence suggest that the mAb can mask the important region of IL-2, showing a neutralizing potential at least in vitro (Ref. 9; data not shown) and for Foxp3+ CD4+ regulatory T cells in vivo (Fig. 4 and Refs. 2 and 13). In addition, in vivo treatment with a protease inhibitor, pepstatin, increases the in vivo half-life of IL-2 (33). Therefore, we speculate that the anti-IL-2 mAb also protects the receptor-contact site of IL-2 in vivo, which may curtail its physiological inactivation. The ELISA-detectable free form of serum IL-2 was greater after the anti-IL-2 mAb plus IL-2 plasmid DNA treatment than after the rat IgG plus IL-2 plasmid treatment (Fig. 1A), suggesting the dissociation of bioactive IL-2 from the IL-2/mAb complex. The on-off cycles between IL-2 and the mAb may contribute to the IL-2 enhancing effects of the mAb.

We noticed that after treatment with the anti-IL-2 mAb plus IL-2 plasmid DNA, CD4+ T cells did not expand as vigorously as did NK cells and CD8+ T cells (Fig. 2B). It is reported that NK cells and CD44highCD8+ T cells express higher levels of CD122 than do CD44highCD4+ T cells (38), and CD8+ T cells proliferate in response to IL-2 more sensitively than do CD4+ T cells (39). Therefore, the selective expansions of NK cells and CD44highCD8+ T cells may simply be due to the difference in sensitivity of these cell populations to IL-2 and competition between them. However, treatment with the IL-2 plasmid alone increased the levels of CD4+ T cells as well as of NK cells and CD8+ T cells.

![Figure 6](http://www.jimmunol.org/Downloadedfrom/figure6.png)
CD8$^+$ T cells, compared with the control plasmid (Fig. 2B). Because the anti-IL-2 mAb and IL-2 plasmid treatment activated CD8$^+$ T cells and NK cells (Fig. 6, B and C), it would be intriguing to learn whether the activated effector cells actively suppress the proliferation and/or survival of CD4$^+$ T cells.

IL-2 has been tested for clinical use targeting various types of cancer and HIV infection, due to its potent immunostimulatory properties (1). Because the half-lives of rIL-2 in human and mouse are on the order of minutes (28, 29, 35), a high dose and frequent injections are required to obtain sufficient therapeutic efficacy. Several strategies have been used to increase the bioavailability of IL-2. These include changing the injection route (40), using polyethylene glycol-IL-2 (41), an IL-2-IgG fusion protein (42), proteolysis-resistant IL-2 (43), an IL-2-albumin fusion protein (44, 45), and gene therapy (46). The present study adds an alternative means to maintain and enhance IL-2 efficacy in vivo by combined treatment with the anti-IL-2 mAb and IL-2 plasmid DNA. One great advantage of this combined treatment is its potent effects with only a single administration. Most interestingly, vigorous and preferential expansion of NK1.1$^+$CD44$^+$CD8$^+$ T cells, which are known to have antitumor effects (24, 25, 47), was observed after the combined treatment, which probably explains the efficient prevention of lung metastasis by this therapeutic regimen (Fig. 8). Moreover, critical roles of cooperation between NK cells and CD8 T cells for the rejection of B16 tumor cells in vivo has been reported. Markovic et al. (48) have shown that CD8 T cells regulate IFN-activated NK cell activity against B16 melanoma cells in vivo. Another study demonstrated that NK cells play a crucial role in generation of the CTL response against B16 melanoma cells (49). Recently, Xu et al. (50) have shown a synergistic interaction between NK cells and CD8 T cells in rejecting B16 melanoma in response to IL-12 treatment. All these studies indicate that interactions between NK cells and CD8 T cells play an important role in the rejection of cancer cells. We here showed that both NK cells and CD8 T cells significantly increased after the combined treatment of anti-IL-2 Ab plus IL-2 expression in vivo. Therefore, it is reasonable that both NK cells and CD8 T cells, which increased after the treatment, are critical for the rejection of B16 tumor cells. Consistent with this, we observed there were clustering of CD3$^+$ and CD8$^+$ T cells as well as CD3$^-$ and NK1.1$^+$ NK cells in the nodules of B16 tumors in lung after the treatment of anti-IL-2 Ab plus IL-2 expression (data not shown). Therefore, we hypothesize that the cooperation between CD8 T cells and NK cells that are increased by the combined treatment of IL-2-plasmid plus anti-IL-2 Ab plays a critical role for the rejection.

In summary, we have described unexpected actions of the anti-IL-2 mAb S4B6 that augment IL-2 bioactivities in vivo, especially when IL-2 is abundant. Furthermore, we demonstrated that combined immunotherapy using the anti-IL-2 mAb and plasmid DNA delivery of IL-2 effectively attenuates lung metastasis in mice. Our results suggest the concurrent use of neutralizing anti-IL-2 Ab with gene delivery as an alternative strategy for enhancing and maintaining cytokine functions for therapeutic purposes.

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Disclosures
The authors have no financial conflict of interest.

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