The Journal of Immunology

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J Immunol 2008; 180:5118-5129; ;
doi: 10.4049/jimmunol.180.7.5118
http://www.jimmunol.org/content/180/7/5118

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IL-2/Anti-IL-2 Antibody Complex Enhances Vaccine-Mediated Antigen-Specific CD8$^+$ T Cell Responses and Increases the Ratio of Effector/Memory CD8$^+$ T Cells to Regulatory T Cells[1]

Sven Mostböck,* M. E. Christine Lutsiak,* Diane E. Milenic,† Kwamena Baidoo,† Jeffrey Schlom,[2]* and Helen Sabzevari*

IL-2 is well described as a cytokine with two markedly distinct functionalities: as a necessary signal during CD4$^+$ and CD8$^+$ T cell activation/expansion and as an essential cytokine for the maintenance of CD4$^+$CD25$^+$FoxP3$^+$ T cells (regulatory T (T_{REG}) cells) during homeostasis. In this study we demonstrate for the first time that, compared with the use of IL-2 alone, a complex of IL-2 and anti-IL-2 Ab (IL-2 complex) enhances the effectiveness of a viral vaccine in a mouse model with known Ag specificity. IL-2 complex led to an increase in the number of Ag-specific effector/memory CD8$^+$ T cells, cytokine production, and CTL lysis following Ag-specific restimulation in a vaccination setting. Our results further demonstrate that this effect is temporary and declines over the course of a few days after the IL-2 complex treatment cycle. Moreover, in contrast to the use of IL-2 alone, IL-2 complex greatly increased the ratio of effector/memory CD8$^+$ T cells to T_{REG} cells. This phenomenon can thus potentially be used in the enhancement of immune responses to vaccination. ***The Journal of Immunology,*** **2008, 180: 5118–5129.**

Cytokines play an important role in the life of T lymphocytes during all stages: development, homeostatic maintenance, activation, and effector activity. However, the role of a specific cytokine may vary between the various subclasses of T cells and their activation stage. IL-2 is well described as a cytokine with two markedly distinct functionalities. First, it is a necessary signal during T cell activation and expansion for both CD4$^+$ T cells and CD8$^+$ T cells. Second, it is an essential cytokine for the maintenance of CD4$^+$CD25$^+$FoxP3$^+$ T cells (regulatory T (T_{REG})[3] cells) during homeostasis (1). The presence of IL-2 during the primary response was shown to be important for the generation of fully functional memory CD8$^+$ T cells. Memory CD8$^+$ T cells generated without IL-2 were demonstrated to have reduced expansion in a later recall response (2), while the pool of memory CD8$^+$ T cells was shown to be increased by prolonged IL-2 receptor engagement (3). The timing of IL-2 presence during an immune reaction seems to be crucial for the generation of memory CD8$^+$ T cells. A previous study showed that low-dose IL-2 in the early phase of an immune response has a detrimental effect on the generation of memory CD8$^+$ T cells but has a positive effect on memory CD8$^+$ T cells in the end phase of the immune response (4).

The action of IL-2 has been attributed to the presence of CD25 on the cell surface of T_{REG} cells and activated T cells. CD25 is a part of the high affinity IL-2 receptor, which is composed of CD25, CD122, and the common γ-chain CD132. In addition, a low affinity IL-2 receptor has been described that is comprised of only CD122 and CD132. This low affinity IL-2 receptor is present on most memory CD8$^+$ T cells, memory CD4$^+$ T cells and NK cells. The receptor subunits are also part of the IL-15 receptor complex (comprised of the IL-15 receptor chain α and CD122 and CD132), which is necessary for the maintenance of memory T cells by IL-15 in vivo (1). This low affinity IL-2 receptor allows IL-2 action on memory T cells, but the amount of steady-state IL-2 in vivo is too low to affect memory T cells (5).

Previously it has been shown that enhanced levels of either IL-2 or anti-IL-2 Ab (6–11) can lead to increased numbers of memory T cells. Anti-IL-2 Ab has also been shown to reduce the numbers of T_{REG} cells (9–12), and this was suggested to cause increased homeostatic proliferation of memory T cells (9).

Complexation of cytokines with their respective anti-cytokine Ab has already been described for multiple cytokines. Increases in the stability of complexed IL-2, IL-3, IL-4, IFN-α, and IL-6 (13–21) and in the biological activity of the cytokine complex for IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IFN-γ, and TNF (2, 5, 16–29) have been demonstrated. In addition to these complexes formed with Ab, recent studies established the increase of biological activity of IL-15 complexed with the IL-15 receptor chain α (30, 31).

Coadministration of IL-2 and anti-IL-2 Ab in the form of a complex has been demonstrated to lead to strongly increased proliferation of CD122$^+$ memory T cells and CD122$^+$ NK cells, while the number of CD122^{low} T cells and T_{REG} cells hardly changed (5, 11, 27). It has been hypothesized that the anti-IL-2 Ab clone S4B6 covers the CD25 binding site of IL-2 while leaving the CD122 binding site of IL-2 free to bind to the low affinity receptor,

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Received for publication January 16, 2007. Accepted for publication January 26, 2008.

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[1] This research was supported by the Intramural Research Program of National Institutes of Health, National Cancer Institute, Center for Cancer Research.

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[3] Abbreviations used in this paper: T_{REG}, regulatory T (CD4$^+$CD25$^+$FoxP3$^+$) cell; CHX-A″-DTPA, isomer of 2-(*p*-isothiocyanatobenzyl)-cyclohexyl-diethylenetriaminepentaacetic acid; IL-2 complex, complex of IL-2 and anti-IL-2 Ab; NK cell, CD3e$^-$NK1.1$^+$DX5$^+$; NP, nucleoprotein; NP68, influenza $NP_{366-374}$ sequence ASNENMDAM; rV-NP-GFP/TRICOM, recombinant vaccinia vector expressing a fusion protein of influenza NP, enhanced GFP, and TRICOM; rF-GM-CSF, recombinant fowlpox vector expressing GM-CSF; TRICOM, triad of costimulatory molecules B7.1, LFA-3, and ICAM-1.

which can lead to increased homeostatic proliferation and accumulation of these memory cells (5). Recent studies demonstrated that naive $CD8^+$ T cells proliferate and differentiate into protective memory-like cells in the absence of TCR stimulus by foreign Ag after treatment with an IL-2/anti-IL-2 complex (referred to as "IL-2 complex" throughout) (28, 29).

Studies performed up until now on the effect of anti-IL-2 Ab on T lymphocytes in vivo focused solely on the maintenance of T cells in mice without an ongoing immune response. In this study, we demonstrate that IL-2 complex enhances the effector phase of an immune reaction after vaccination. Following vaccination with viral vectors and a 7-day treatment cycle with the IL-2 complex, the number of Ag-specific $CD8^+$ T cells increased dramatically compared with control animals treated with the isotype Ab, IL-2 alone, or anti-IL-2 Ab alone. Furthermore, we observed an increase in the number of cytokine-producing $CD8^+$ T cells and in the CTL activity of the $CD8^+$ T cells in the IL-2 complex-treated mice. This demonstrates for the first time the use of IL-2 complex as an immune enhancer of an ongoing immune response. We also demonstrate that the IL-2 complex formed with the anti-IL-2 Ab clone S4B6 binds strongly to effector/memory $CD8^+$ T cells and NK cells, but not to T_{REG} cells. These data further support the hypothesis that the IL-2 complex used in this study and previous studies is a specific stimulus for CD122-bearing cells in the immune system by binding exclusively to those cells.

Materials and Methods

Mice and peptide

F5 mice that are transgenic for an influenza nucleoprotein (NP) peptide ($NP_{366-374}$, designated NP68)-specific, H-$2D^b$-restricted TCR (32, 33) and C57BL/6 were obtained from Taconic Farms. All animal studies had been approved by the National Institutes of Health Animal Care and Use Committee before the experiments. The NP68 peptide (^{366}ASNENMDAM^{374}) was synthesized by the American Peptide Company.

Adoptive transfer of naive F5 $CD8^+$ T cells

Splenocytes from female F5 mice were incubated with biotin-labeled anti-Ter-119 Ab and biotin-labeled anti-CD44 Ab (both Abs are from BD Biosciences) for 15 min at 4°C, washed 1× in PBS with 1% BSA (PBS-BSA), and resuspended in PBS-BSA. Then, biotin-labeled cells were purified according to the manufacturer's instructions with either anti-biotin magnetic beads or streptavidin magnetic beads (Miltenyi Biotec). The purity of the $CD8^+CD44^{low}$ population was tested by flow cytometry and found to be ≥95% in all experiments. In one experiment, naive F5 $CD8^+CD44^{low}$ cells were labeled with CFSE before adoptive transfer into host mice. For this, cells were washed twice with PBS, incubated with 1 μM CFSE in PBS for 10 min at 37°C, and washed twice with PBS. Naive $CD8^+$ T cells (2×10^6) in 200 μl of PBS were adoptively transferred i.v. into female C57BL/6 mice and allowed to rest for 2–4 days.

Viral vectors and vaccination

rV-NP-GFP/B7-1/ICAM-1/LFA-3 (designated rV-NP-GFP/TRICOM) is a recombinant vaccinia virus containing a gene for a fusion protein of the influenza NP and enhanced GFP (NP-GFP) under the control of the 40k promoter and a triad of costimulatory molecules (TRICOM). NP-GFP carries the influenza-specific peptide sequence ASNENMDAM (NP68) and the plasmid is a generous gift from J. Yewdell (National Institutes of Health, Bethesda, MD) (34). TRICOM consists of three murine costimulatory molecules, B7.1, LFA-3 and ICAM-1, and its expression in vaccinia virus has been described previously (35). rF-GM-CSF, a recombinant fowlpox vector that carries the gene for murine GM-CSF, has been described previously (36). Animals received 10^8 PFU of rV-NP-GFP and 10^7 PFU of rF-GM-CSF in 100 μl of HBSS s.c. at the time of vaccination.

IL-2 complex preparation and 7-day treatment cycle

IL-2 complex was prepared by incubating murine IL-2 (PeproTech) with anti-murine-IL-2 Ab (clone S4B6 from American Type Culture Collection, purified by Harlan Bioproducts for Science) for several minutes at room temperature. For one experiment analyzing the effect of IL-2 complex 1 day posttreatment cycle, 6 μg of IL-2 (1 μg/μl) was incubated with 120 μg of anti-IL-2 Ab (4.44 μg/μl) and 767 μl of PBS were added after the incubation period. Two hundred microliters of this solution were then injected i.p. into each mouse, resulting in IL-2 complexes formed by 1.5 μg of IL-2 with 30 μg of anti-IL-2 Ab per mouse. For all other experiments, 4.2 μg of IL-2 (1 μg/μl) were incubated with 22.4 μg of anti-IL-2 Ab (4.44 μg/μl) and 691 μl of PBS were added after the incubation period. HPLC analysis of the IL-2 complex solution confirmed that 40% of the IL-2 formed a complex with anti-IL-2 Ab. Two hundred microliters of this solution were then injected i.p. into each mouse, resulting in IL-2 complexes formed by 1.2 μg of IL-2 with 6.4 μg of anti-IL-2 Ab per mouse (a reduced dose of 80% IL-2 in complex). Animals treated with anti-IL-2 Ab received 200 μl of PBS i.p. with the same amount of Ab as the complex animals, either 30 or 6.4 μg of anti-IL-2 Ab. Animals treated with IL-2 received 200 μl of PBS i.p. with the same amount of IL-2 as the complex animals, either 1.5 or 1.2 μg. Control animals treated with isotype Ab received 200 μl of PBS i.p. with the same amount of isotype Ab as the complex animals, either 30 or 6.4 μg of isotype control Ab (rat IgG2a; BioLegend). This injection was repeated daily for 7 days starting on day 3 after vaccination.

Analysis of spleen cell populations

Splenocytes were harvested 1, 5, or 12 days posttreatment cycle and analyzed for various cell markers. In one repeat for each of these time points, single animals were analyzed for the isotype Ab- and the IL-2 complex-treated groups (2–4 mice for the isotype Ab-treated groups, 4–7 mice for IL-2 complex-treated groups). For those repeats, data are presented as average of the animals in the same group. For all other repeats, spleens were pooled per group (three mice for each group; in one repeat, two mice in the IL-2 complex group) before analysis and the data are values obtained from the pooled group splenocytes. Abs against CD19, CD4, CD8a, CD3e, NK1.1, DX5, CD44, CD25, GR-1, CD11b, CD11c, IA^b, CD11a, CD27, CD62L, Vβ11, and CD122 were obtained from BD Biosciences. The Ab against CCR7 was obtained from eBioscience. Surface marker staining was performed for 15 min on ice. Intracellular staining for FoxP3 was done with the FoxP3 staining kit from eBioscience following the manufacturer's instructions. Pentamer (ProImmune) staining for influenza NP68-specific $CD8^+$ T cells was performed by staining for 20 min at room temperature followed by CD8 surface marker staining as described above. Cells were analyzed by multicolor flow cytometry on a FACSCalibur apparatus (BD Biosciences).

BrdU treatment and detection

Animals received 0.8 mg of BrdU per milliliter of drinking water for the last 3 days of the 7-day treatment cycle. BrdU level in splenocytes was detected with the BrdU flow kit according to the manufacturer's instructions (BD Biosciences).

Detection of cytokines in serum

Serum of experimental animals was taken 1, 5, or 12 days posttreatment cycle and analyzed for IFN-γ, IL-2, IL-4, IL-6, IL-10, MCP-1, and TNF with the Cytometric Bead Array (CBA) Flex Set kit from BD Biosciences according to the manufacturer's instructions using a FACSCalibur flow cytometer. The usability of this kit to detect the IL-2 of the IL-2 complex despite the binding of the IL-2 to an anti-IL-2 Ab was tested by us on an IL-2 complex sample without any free IL-2. In that sample, 50% of the IL-2 was detectable. In the IL-2 complex preparation used in this study, 90% of the IL-2 was detectable. The amount of detected IL-2 is higher, most probably due to free IL-2 present in that preparation (see the paragraph above entitled *IL-2 complex preparation and 7-day treatment cycle*).

Detection of IL-2 complex binding on various cell populations

IL-2 complex was prepared as described above with FITC-labeled anti-IL-2 Ab (clone S4B6; BD Biosciences). A control isotype complex was prepared similarly with FITC-labeled rIgG2a (BD Biosciences). Splenocytes from untreated C57BL/6 were washed with PBS plus 1% FCS (PBS-FCS), incubated with FITC-IL-2 complex, FITC-isotype control complex, or FITC-anti-IL-2 Ab alone for 30 min on ice, washed once with PBS-FCS, and incubated with various cell surface markers on ice for 15 min. For blocking experiments, splenocytes were first incubated with 100 μg/ml unlabeled rIgG2b isotype control Ab, Ab against CD16/32, or Ab against CD122 (all Ab from BD Biosciences) for 15 min on ice. Then the staining was continued as described before by adding a FITC-IL-2 complex or a FITC-isotype control complex. After incubation, cells were washed with PBS-FCS and analyzed by flow cytometry on a FACSCalibur apparatus.

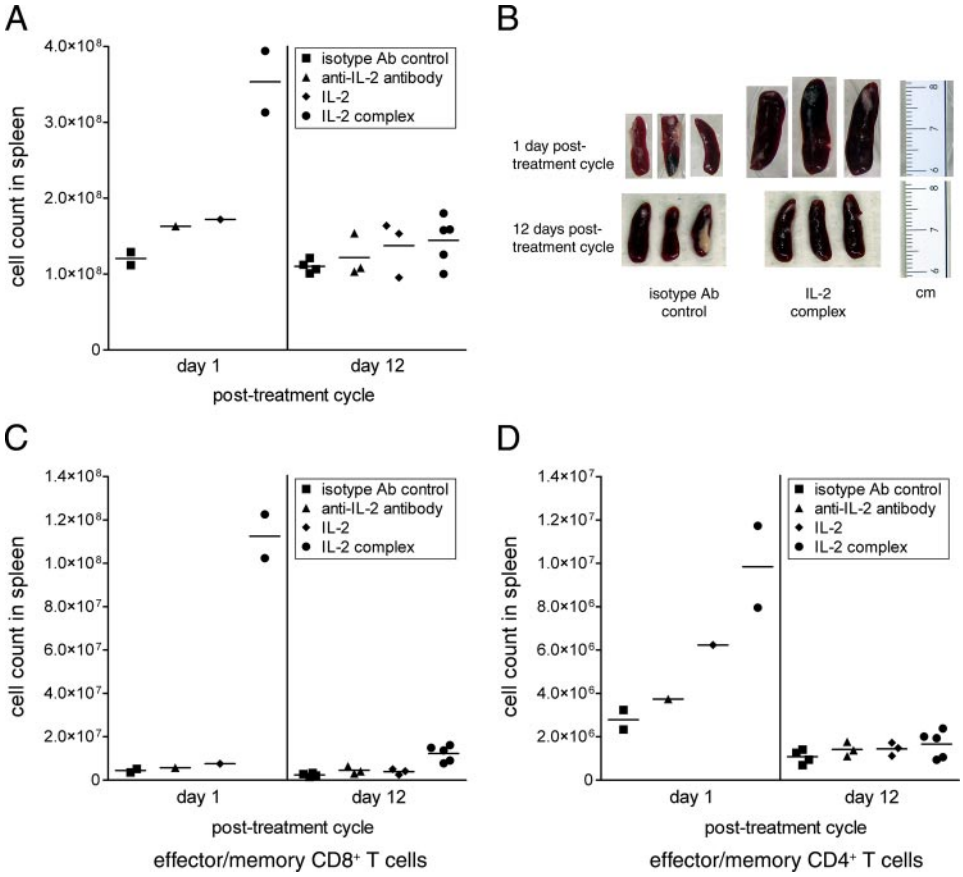

FIGURE 1. Increase in spleen size and effector/memory T cell population by IL-2 complex treatment. C57BL/6 mice received an adoptive transfer of naive CD8$^+$ T cells from F5 TCR-transgenic mice and were vaccinated with rV-NP-GFP/TRICOM (plus rF-GM-CSF) and treated with IL-2, anti-IL-2 Ab, isotype Ab, or IL-2 complex for 7 days. Animals were sacrificed and analyzed either 1 day or 12 days after the completed 7-day treatment cycle (1 day or 12 days posttreatment cycle). *A*, Spleen cell numbers from isotype Ab-treated mice (■), anti-IL-2 Ab-treated mice (▲), IL-2-treated mice (♦), and IL-2 complex-treated mice (●) were evaluated. Each symbol represents one group with multiple mice per group from multiple experimental repeats; the horizontal bar represents the average. Spleens were harvested 1 day (*left panel*) or 12 days posttreatment cycle (*right panel*). *B*, Representative picture of the spleen sizes 1 day (*upper panels*) and 12 days posttreatment cycle (*lower panels*), comparing the isotype Ab-treated control animals (*left panels*) with the IL-2 complex-treated animals (*right panels*). The analysis of 1 day posttreatment cycle was done in an experiment separate from the analysis of 12 days posttreatment cycle. *C*, Effector/memory (CD44^{high}CD122$^+$) CD8$^+$ T cell numbers in spleen from isotype Ab-treated mice (■), anti-IL-2 Ab-treated mice (▲), IL-2-treated mice (♦), and IL-2 complex-treated mice (●) were evaluated. Each symbol represents one group with multiple mice per group from multiple experimental repeats; the horizontal bar represents the average. Spleens were harvested 1 day (*left panel*) or 12 days posttreatment cycle (*right panel*). *D*, Effector/memory (CD44^{high}CD122$^+$) CD4$^+$ T cell numbers in spleen from isotype Ab-treated mice (■), anti-IL-2 Ab-treated mice (▲), IL-2-treated mice (♦), and IL-2 complex-treated mice (●) were evaluated. Each symbol represents one group with multiple mice per group from multiple experimental repeats; the horizontal bar represents the average. Spleens were harvested 1 day (*left panel*) or 12 days posttreatment cycle (*right panel*).

Detection of intracellular cytokines

CD8$^+$ T cells were purified from the splenocytes of experimental animals with CD8$^+$ magnetic DM beads from BD Biosciences according to the manufacturer's instructions and incubated with 10^{-4} μg/ml NP68 peptide and APC in DMEM medium with 10% FCS. The APC were splenocytes from C57BL/6 that were CD8$^+$ depleted with CD8 magnetic beads from Miltenyi Biotec according to the manufacturer's instructions and irradiated with 3,000 rad. Cells were incubated at 37°C in a CO_2 incubator for 18 h with 1 μg/ml GolgiPlug (BD Biosciences) added for the last 15 h. After incubation, cells were harvested and stained for CD8$^+$ surface marker and intracellular IFN-γ, TNF-α, and MIP-1β. Briefly, cells were washed with PBS-FCS, stained with PerCP-labeled anti-CD8a Ab for 15 min on ice, washed with PBS-FCS and fixed with Cytofix/Cytoperm (BD Biosciences) for 30 min at room temperature. After fixation, cells were washed with Perm/Wash buffer (BD Biosciences), stained with FITC-labeled anti-TNF-α, PE-labeled anti-IFN-γ (both Abs from BD Biosciences), and biotin-labeled anti-MIP-1β (R&D Systems) for 15 min on ice, washed with Perm/Wash Buffer, stained with allophycocyanin-labeled streptavidin (BD Biosciences) for 15 min on ice, washed with Perm/Wash Buffer, and analyzed by multicolor flow cytometry on a FACSCalibur apparatus.

Detection of CTL lysis activity

CD8$^+$ T cells were purified from the spleen of experimental animals with CD8$^+$ magnetic DM beads from BD Biosciences according to the manufacturer's instructions and incubated with ^{111}In-labeled target cells (the fibroblast cell line L929-D^b) at an E:T ratio of 100 to 1 at various peptide concentrations for 20 h. After the incubation, the release of ^{111}In into the cell culture supernatant was evaluated and the percentage of specific lysis was calculated.

For the evaluation of in vivo CTL activity, splenocytes from naive untreated C57BL/6 mice were treated with ammonium-chloride-potassium chloride (ACK) lysing buffer (Cambrex Bio Science) to remove RBC. Cells were then incubated with either no peptide or 10^{-3} μg/ml NP68 for 2 h, washed two times with PBS, and labeled with either 0.2 or 1 μM CFSE, respectively, in PBS for 10 min at 37°C. After incubation, cells were washed two times in PBS, counted, and equal numbers of the two populations were combined. This cell mixture (6.7×10^6 cells) was then adoptively transferred (i.v.) into untreated control C57BL/6 mice or C57BL/6 mice treated as described above. The next day, splenocytes from those mice were harvested, treated with ACK lysing buffer, and analyzed by FACS. The specific lysis was calculated as the percentage of reduction

Table I. *Increase in cell numbers by IL-2 complex*[a]

	Day 1 Posttreatment Cycle				Day 12 Posttreatment Cycle			
	Isotype Ab	Anti-IL-2 Ab	IL-2	IL-2 Complex	Isotype Ab	Anti-IL-2 Ab	IL-2	IL-2 Complex
Spleen cell count	121 ± 12	163	172	353 ± 57	110 ± 9	122 ± 28	138 ± 37	145 ± 31
$CD19^+$	41.5 ± 15.7 33.7 ± 10.0%	62.3 38.2%	56.8 32.9%	44.1 ± 6.1 12.5 ± 0.3%	36.3 ± 3.7 33.2 ± 5.5%	38.7 ± 5.0 32.2 ± 2.9%	44.1 ± 12.7 32.6 ± 6.7%	49.5 ± 8.3 34.7 ± 3.7%
$CD4^+$	39.5 ± 2.4 32.9 ± 1.5%	50.3 30.9%	52.5 30.4%	37.7 ± 17.1 10.4 ± 3.2%	34.0 ± 7.7 30.7 ± 5.4%	37.7 ± 8.8 30.9 ± 0.9%	44.4 ± 15.0 32.0 ± 3.5%	35.7 ± 13.2 23.9 ± 4.4%
$CD8^+$	28.2 ± 0.8 23.5 ± 1.8%	38.7 23.7%	43.6 25.3%	171 ± 0.1 49.4 ± 7.6%	23.8 ± 6.2 21.5 ± 4.3%	30.1 ± 10.2 24.3 ± 2.6%	34.3 ± 12.3 24.6 ± 3.5%	40.0 ± 11.7 27.3 ± 3.4%
NK cells	2.56 ± 0.70 2.2 ± 0.8%	4.31 2.6%	3.72 2.2%	44.4 ± 23.1 12.1 ± 4.4%	2.36 ± 0.99 2.1 ± 0.7%	3.78 ± 1.85 3.0 ± 1.1%	2.98 ± 1.57 2.0 ± 0.7%	1.87 ± 0.56 1.3 ± 0.4%
T_{REG} cells ($CD4^+CD25^+FoxP3^+$)	2.68 ± 1.01 2.3 ± 1.1%	1.30 0.80%	6.08 3.53%	6.97 ± 2.14 2.0 ± 0.3%	3.70 ± 0.89 3.3 ± 0.6%	3.58 ± 0.82 2.9 ± 0.1%	4.49 ± 1.14 3.4 ± 0.1%	3.94 ± 1.19 2.7 ± 0.4%

[a] Numbers on the top line for each cell type indicate total cell counts × 10^6; numbers on the lower line indicate the percentage of splenocytes. Values are given as average ± SD (day 1: for the anti-IL-2 Ab and IL-2 the experiment was repeated once with multiple mice per group; day 1: for isotype Ab and IL-2 complex and day 12: for all groups, average ± SD for 2–5 repeats with multiple mice per group.

of the CFSE population in relation to the corresponding CFSE population in the untreated control C57BL/6 mice.

Ab conjugation and radiolabeling

The synthesis, characterization, and purification of the bifunctional ligand CHX-A″-DTPA, an isomer of 2-(*p*-isothiocyanatobenzyl)-cyclohexyl-diethylenetriaminepentaacetic acid, has been previously described (37). The anti-IL-2 Ab was conjugated with the CHX-A″-DTPA by established methods using a 10-fold molar excess of ligand to Ab (38). The final concentration of the CHX-A″-DTPA-anti-IL-2 Ab was quantitated by the Lowry method (39). The number of CHX-A″ molecules linked to the Ab was determined using a spectrophotometric assay based on the titration of Y(III)-Arsenazo(III) complex (40).

Radiolabeling of CHX-A″-DTPA-anti-IL-2 Ab with indium-111 (100 μg in 0.15 M NH_4OAc buffer) was performed by adding 1 mCi (~2 μl) of indium-111 chloride (PerkinElmer). The reaction was quenched by the addition of 5 μl of 0.05 M EDTA to scavenge any free radiometal. The radiolabeled product was purified using a PD-10 desalting column (GE Healthcare). The specific activity of the final product was 6.6 mCi/mg.

In vivo studies with radiolabeled Ab

Four- to 6-wk-old female BALB/c mice (n = 4–5; Charles River Laboratories) were injected i.p. with either ^{111}In-CHX-A″-DTPA-anti-IL-2 Ab (6.2 μCi) alone or with ^{111}In- CHX-A″-DTPA-anti-IL-2 Ab complexed with IL-2. Complexation of the radiolabeled Ab was performed by incubating 130 μl of the ^{111}In-CHX-A″-DTPA-anti-IL-2 Ab (~9 μg) with 2 μl (2 μg) of IL-2 for 2 min at room temperature. The reaction mixture was diluted to 2 ml with PBS. The ^{111}In-CHX-A″-DTPA-anti-IL-2 Ab (130 μl) was also diluted to 2 ml for injection into the mice. Blood (10 μl) was drawn via tail nick by using a heparinized capillary tube (Drummond Scientific), expelled onto a cotton filter, and counted in a gamma scintillation counter (Wizard; PerkinElmer). The counts per minute were decay corrected and the percentage of injected dose per milliliter was calculated. The half-lives of the ^{111}In-CHX-A″-DTPA-anti-IL-2 Ab and the ^{111}In- CHX-A″-DTPA-anti-IL-2 Ab/IL-2 complex were calculated using SigmaPlot 2001, version 7.101 (SPSS).

Results

IL-2 complex increases the number of effector/memory $CD8^+$ T cells and NK cells 1 day after the completed treatment cycle

To investigate the effects of a complex formed from IL-2 and anti-IL-2 Ab (IL-2 complex) on various lymphocyte populations during an immune response, naive $CD8^+$ T cells from F5 TCR-transgenic mice were adoptively transferred into C57BL/6 mice. Four days after adoptive transfer, animals were vaccinated with rV-NP-GFP/TRICOM, a recombinant vaccinia virus that carries the influenza-NP68 sequence and additional costimulatory molecules (B7.1, ICAM-1 and LFA-3; TRICOM), and rF-GM-CSF, a recombinant fowlpox virus that carries the gene for murine GM-CSF. Three days postvaccination, different groups of animals received IL-2 alone, anti-IL-2 Ab, isotype Ab (isotype Ab) or IL-2 complex (1.2

Table II. *Increase in cell numbers by IL-2 complex*[a]

	Day 1 Posttreatment Cycle		Day 12 Posttreatment Cycle	
	Isotype Ab	IL-2 Complex	Isotype Ab	IL-2 Complex
Granulocytes ($CD3e^-GR1^+CD11b^-$)	1.27 ± 0.32 1.1% ± 0.5	12.8 ± 8.8 3.9% ± 2.5	1.17 ± 0.50 1.1% ± 0.4	1.28 ± 0.47 1.2% ± 0.3
Macrophages ($CD3e^-GR1^-CD11b^+$)	6.18 ± 1.35 5.3% ± 0.3	33.4 ± 11.1 10.2% ± 2.9	5.36 ± 1.32 5.2% ± 1.0	5.64 ± 1.40 5.5% ± 0.6
Dendritic cells ($CD3e^-IA^{b+}CD11c^+$)	3.17 ± 1.15 2.8% ± 1.4	6.42 ± 3.85 2.0% ± 1.1	3.42 ± 1.22 3.3% ± 1.0	2.43 ± 0.55 3.1% ± 1.3
Immature myeloid cells ($CD3e^-GR1^+CD11b^+$)	3.14 ± 0.03 2.7% ± 0.3	5.44 ± 2.0 1.7% ± 0.7	1.83 ± 1.19 1.7% ± 1.1	2.24 ± 1.73 1.8% ± 1.0

[a] Numbers on the top line for each cell type indicate total cell counts × 10^6, and numbers on the lower line indicate the percentage of splenocytes. Values are given as average ± SD for two repeats with multiple mice per group.

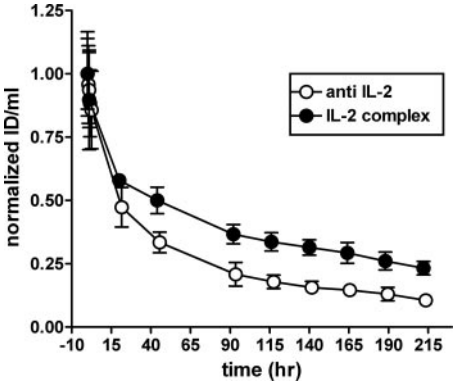

FIGURE 2. Persistence of IL-2 complex in blood after i.p. injection. Radiolabeled IL-2 complex (●) or radiolabeled anti-IL-2 Ab (○) was injected into mice i.p. and the amount of radioactivity in the blood was assessed at multiple time points. Data represent the average ± SD of four to five mice, normalized to the peak value for the respective compounds reached in the blood. The time line starts at peak value for the respective compounds.

or 1.5 μg of IL-2 in IL-2 complex) for a treatment cycle of 7 days. Mice were sacrificed at three time points, either 1, 5, or 12 days after the completed treatment cycle (1 day posttreatment cycle).

After vaccination of mice and treatment with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex, splenocytes were analyzed by multicolor flow cytometry for various cell markers. There was a 3-fold increase in the number of total splenocytes in the animals treated with IL-2 complex compared with the isotype Ab control (Fig. 1*A*), and their spleens were enlarged (Fig. 1*B*) 1 day posttreatment cycle. Animals that received either anti-IL-2 Ab or IL-2 alone had a 1.3- to 1.4-fold increase in spleen cell numbers (Fig. 1*A*). This increase in splenocyte numbers corresponded to a 6-fold increase in the total CD8$^+$ T cell number and a 17-fold increase in the CD3e$^-$NK1.1$^+$DX5$^+$ (NK cell) number in the IL-2 complex group; in the anti-IL-2 Ab group and the IL-2 group the effects were reduced. There was a 1.4- to 1.5-fold increase in CD8$^+$ T cell numbers and a 1.5- to 1.7-fold increase in NK cell numbers (Table I). In contrast to the CD8$^+$ T cells, the total CD4$^+$ T cell number was not affected by IL-2 complex; the total number of CD4$^+$ T cells remained unchanged in the IL-2 complex-treated mice. CD4$^+$ T cell numbers were increased 1.3-fold for both IL-2-treated mice and anti-IL-2 Ab-treated mice (Table I). The total numbers of T_{REG} cells showed similar increases in both IL-2 complex-treated mice and IL-2-treated mice (~2-fold increases). In contrast, the population size of T_{REG} cells as a percentage of total splenocytes was increased in IL-2-treated mice (1.5-fold) as compared with the mice that received IL-2 complex or isotype Ab (Table I). Anti-IL-2 Ab-treated mice showed a marked difference in the T_{REG} cell population compared with the other groups in this experiment: the absolute number of T_{REG} cells was reduced 2-fold and the percentage of T_{REG} cells in the spleen was reduced 2.9-fold. Lastly, the number of CD19$^+$ cells in the spleen was minimally affected (1.1-fold increase) in the IL-2 complex-treated mice compared with the isotype Ab-treated control mice. The number of CD19$^+$ cells in IL-2-treated mice and anti-IL-2 Ab-treated mice was increased 1.4- to 1.5-fold (Table I).

A more detailed analysis of CD8$^+$ and CD4$^+$ T cells revealed a 26-fold increase in the CD8$^+$CD44^{high}CD122$^+$ cell (effector/memory CD8$^+$ T cell) number (Fig. 1*C*) and a 3.5-fold increase in the CD4$^+$CD44^{high}CD122$^+$ cell (effector/memory CD4$^+$ T cell) number (Fig. 1*D*) in IL-2 complex-treated mice compared with the isotype Ab-treated control mice. These cells were defined as memory T cells based on their expression of high levels of CD44 and CD122, markers commonly used to identify memory T cells (5). We also stained other memory markers and found the CD8$^+$ T cells to be CD11a^{high}, CD62L$^-$, and partly CCR7$^+$ and CD27$^+$. However, in our in vivo experiments all mice received a vaccination, which can also lead to the generation of effector CD8$^+$ T cells and early memory CD8$^+$ T cells with a similar marker profile. Because of this, the CD44^{high}CD122$^+$CD8$^+$ T cells might be a mixture of effector CD8$^+$ T cells and memory CD8$^+$ T cells. Therefore, we refer to these cells as effector/memory cells in the vaccination experiments in this study. The effector/memory CD8$^+$ T cells and effector/memory CD4$^+$ T cell populations were also slightly elevated after treatment with anti-IL-2 Ab or IL-2 (Fig. 1, *C* and *D*). Similar effects on NK cells, CD4$^+$ T cells, CD8$^+$ T cells, and CD19$^+$ cells, as well as the CD44^{high}CD122$^+$ populations of CD4$^+$ T cells and CD8$^+$ T cells and the T_{REG} cell population, were observed in the lymph node 1 day after the treatment cycle (data from a representative experiment; not shown).

To address the mechanism for the increase in CD8$^+$ T cells in IL-2 complex-treated mice, animals were given BrdU in drinking water for the last 3 days of the regular 7-day treatment cycle with IL-2 complex in one representative experiment. A higher percentage of CD8$^+$ T cells had incorporated BrdU in IL-2 complex-treated mice compared with isotype Ab-treated mice 1 day after the completed treatment cycle (26.5% compared with 17.5%, respectively). Treatment with IL-2 or anti-IL-2 Ab increased the percentage of proliferating (BrdU-incorporating) CD8$^+$ T cells only slightly compared with isotype Ab-treated control mice (21.5 and 19.9%, respectively, compared with 17.5%).

In addition, the number of granulocytes (CD3e$^-$GR1$^+$CD11b$^-$) was 10-fold higher in IL-2 complex-treated animals compared with the isotype Ab-treated mice 1 day posttreatment cycle (Table II). The number of macrophages (CD3e$^-$GR1$^-$CD11b$^+$) were increased ~5.4-fold, and the numbers of dendritic cells (CD3e$^-$IA^{b+} CD11c$^+$) and immature myeloid cells (CD3e$^-$GR1$^+$CD11b$^+$) were roughly doubled (2- and 1.7-fold increase, respectively) 1 day posttreatment cycle (Table II).

Table III. *Cytokine levels in the serum of IL-2 complex-treated animals*[a]

	Day 1 Posttreatment Cycle		Day 5 Posttreatment Cycle		Day 12 Posttreatment Cycle			
	Isotype Ab	IL-2 Complex	Isotype Ab	IL-2 Complex	Isotype Ab	Anti-IL-2 Ab	IL-2	IL-2 Complex
IFN-γ	6.6 ± 5.9	6.5 ± 1.0	3.0 ± 4.2	3.2 ± 3.7	3.2 ± 3.1	5.2 ± 2.9	2.6 ± 3.1	4.1 ± 2.9
IL-6	15.4 ± 5.7	10.3 ± 2.3	2.7 ± 3.7	11.1 ± 4.3	7.4 ± 4.6	11.0 ± 3.1	6.3 ± 5.0	10.2 ± 3.9
IL-4	3.7 ± 6.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 1.4
MCP-1	203.2 ± 93.6	98.0 ± 19.5	54.8 ± 77.5	112.2 ± 78.9	100.4 ± 38.2	110.7 ± 39.2	64.2 ± 41.4	84.7 ± 56.4
IL-10	37.9 ± 24.9	8.9 ± 12.0	0.0 ± 0.0	11.1 ± 13.0	8.5 ± 13.4	16.5 ± 11.3	1.8 ± 5.4	6.7 ± 12.6
TNF	93.1 ± 63.4	35.3 ± 17.8	40.9 ± 11.3	65.2 ± 27.4	32.4 ± 33.1	43.1 ± 24.2	40.5 ± 25.6	49.9 ± 22.7
IL-2	5.3 ± 4.7	17.2 ± 3.5	5.3 ± 0.0	8.5 ± 1.1	1.9 ± 3.0	11.3 ± 1.3	1.9 ± 2.9	7.0 ± 0.8

[a] Values are given as picograms per milliliter (pg/ml) and average ± SD of single mouse data from multiple experiments.

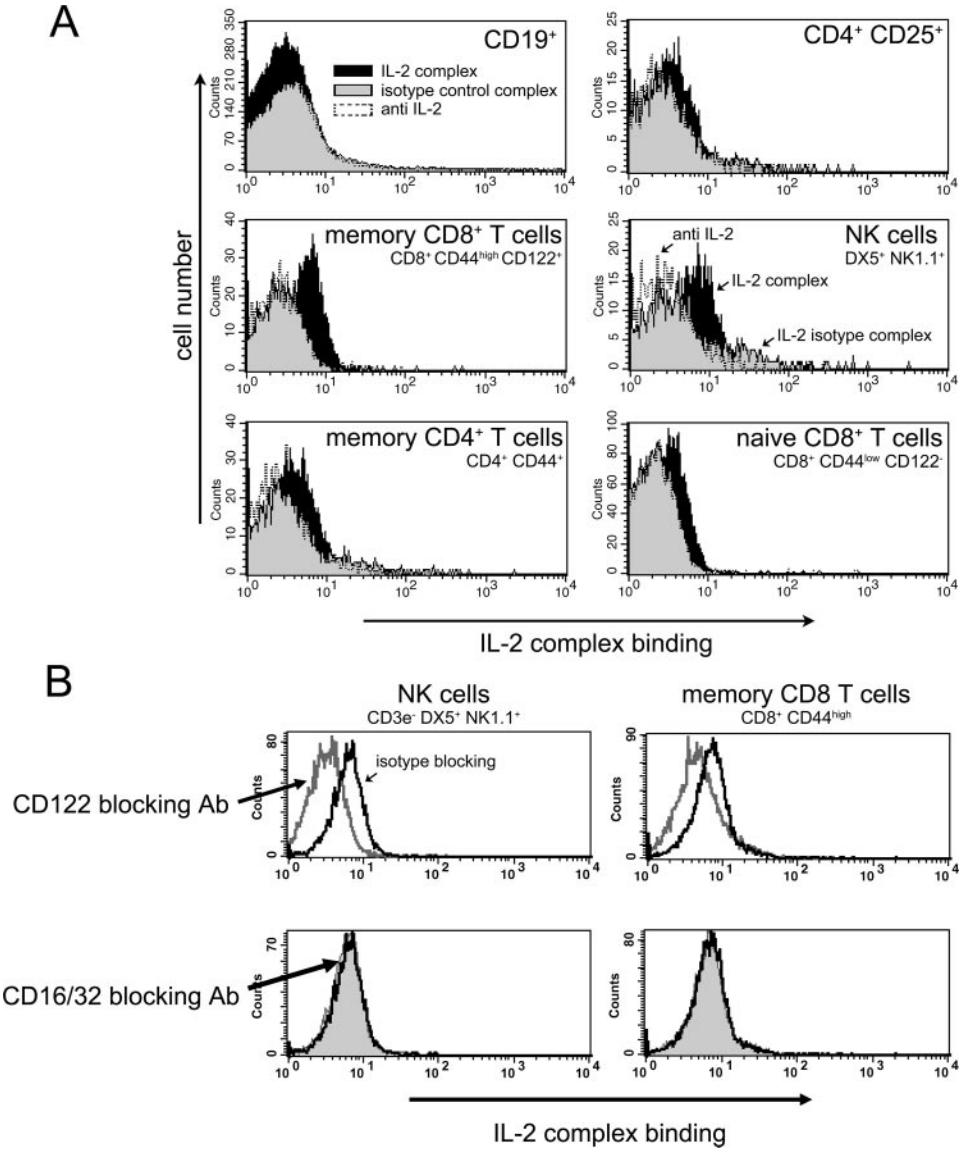

FIGURE 3. IL-2 complex binds to memory CD8$^+$ T cells and NK cells. *A*, Splenocytes from control mice were stained for several surface markers to identify various cell populations. IL-2 complex, formed from IL-2 and FITC-labeled anti-IL-2 Ab (black filled histogram), isotype control complex, formed from IL-2 and FITC-labeled isotype Ab (gray filled histogram), and FITC anti-IL-2 Ab (dotted line histogram) were used to stain these cells. Samples were evaluated by multicolor FACS analysis. *B*, Splenocytes from control mice were first blocked with Ab against CD122 (gray line histogram; *upper panels*), Ab against CD16/32 (gray filled histogram; *lower panels*), or isotype Ab (black line histogram), stained with IL-2 complex (formed from IL-2 and FITC-labeled anti-IL-2 antibody), and then stained for several surface markers to identify various cell populations. Samples were evaluated by multicolor FACS analysis.

IL-2 complex exhibits a transient effect on effector/memory T cells

To evaluate the kinetics of the IL-2 complex effects, we analyzed the spleen cell populations 5 days after the completed 7-day treatment cycle. At that time, the spleen cell number was reduced from a 3-fold increase to a 1.3-fold increase in IL-2 complex-treated mice compared with the isotype Ab-treated control mice. Similarly, the number of CD8$^+$ T cells was reduced from a 6-fold increase to a 1.7-fold increase, the number of effector/memory CD8$^+$ T cells from a 26-fold increase to a 5-fold increase, and the number of NK cells from a 17-fold increase to a 1.6-fold increase in IL-2 complex-treated mice compared with control mice (data not shown).

Twelve days after the completed 7-day treatment cycle, the various cell populations were largely returned to the same levels in IL-2 complex-treated mice compared with the control animals. Although there was still a 1.3-fold increase in the spleen cell number and a 1.7-fold increase in the total CD8$^+$ T cell numbers, these populations were strongly reduced compared with 1 day posttreatment cycle (Table I). However, in the groups that received the IL-2 complex, the NK cell number was decreased to 0.8-fold compared with the NK cell number in the control groups. The total CD4$^+$ T cell number was 1.1-fold higher in the IL-2 complex-treated group compared with the control group (Table I). We still observed a strong effect on the effector/memory T cell populations at this time point: effector/memory CD8$^+$ T cells were 5-fold increased (see Fig. 3*A*) and effector/memory CD4$^+$ T cells were 1.6-fold increased (Fig. 1*D*) in IL-2 complex-treated mice compared with control mice treated with the isotype Ab. There were only minor differences in the numbers of CD4$^+$, CD8$^+$, CD19$^+$, NK, and T_{REG} cell populations in the animals that were treated with either IL-2 or anti-IL-2 Ab compared with animals treated with isotype Ab on day 12 posttreatment cycle (Table I). The numbers of macrophages and granulocytes were similar in IL-2 complex-treated mice and isotype Ab-treated mice 12 days posttreatment cycle. However, the numbers of immature myeloid cells were increased 1.2-fold in the IL-2 complex-treated mice compared with isotype Ab-treated mice. The number of dendritic cells in the IL-2 complex-treated mice was slightly lower than the number of dendritic cells in the isotype Ab-treated mice 12 days posttreatment cycle (Table II). This analysis demonstrates that the effects of IL-2 complex on spleen enlargement, effector/memory T cells, T_{REG} cells, and NK cells had largely disappeared 12 days after the treatment ended, with the exception of a marked increase in the number of effector/memory CD8$^+$ T cells.

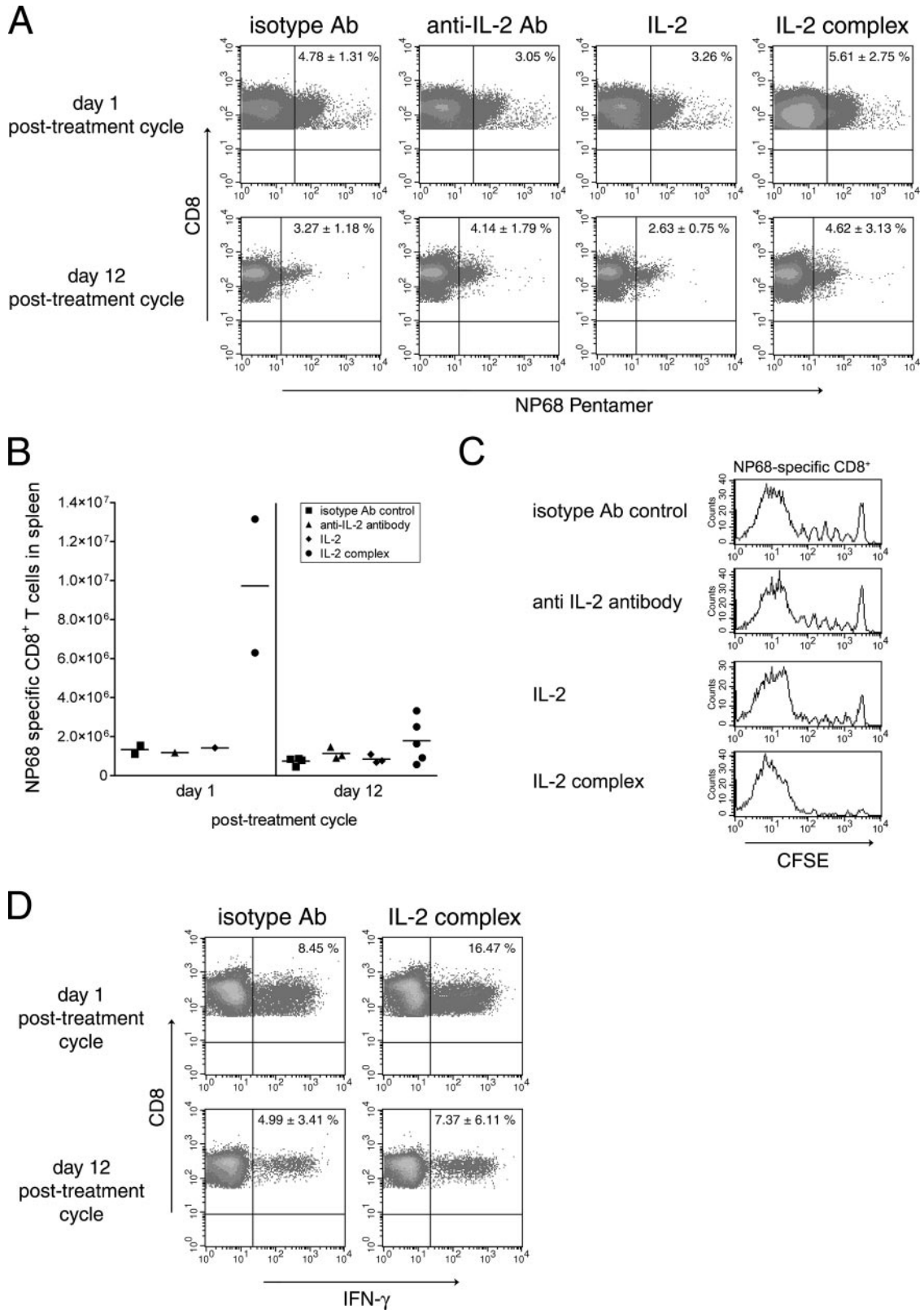

FIGURE 4. Increase in Ag-specific CD8$^+$ T cell numbers and functionality by IL-2 complex treatment. C57BL/6 mice received an adoptive transfer of naive CD8$^+$ T cells from F5 TCR-transgenic mice and were vaccinated with rV-NP-GFP/TRICOM (plus rF-GM-CSF) and treated with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex for 7 days. Animals were sacrificed and analyzed either 1 day or 12 days after the completed 7-day treatment cycle (1 day or 12 days posttreatment cycle). *A*, The NP68-specific CD8$^+$ T cell populations in spleen of isotype Ab-treated mice, anti-IL-2 Ab-treated mice, IL-2-treated mice, or IL-2 complex-treated mice were evaluated 1 day (*upper panels*) or 12 days (*lower panels*) posttreatment cycle. Data are representative dot plots (gated on live CD8$^+$ T cells) with captions displaying the percentage of NP68-specific cells in the CD8$^+$ T cell population (day 1: for anti-IL-2 Ab and IL-2, experiment was repeated once with multiple mice per group; day 1: for isotype Ab and IL-2 complex and day 12: for all groups, average ± SD for 2–5 repeats with multiple mice per group. *B*, NP68-specific CD8$^+$ T cell numbers in spleen from isotype Ab-treated mice (■), anti-IL-2 Ab-treated mice (▲), IL-2-treated mice (♦), and IL-2 complex-treated mice (●) were evaluated. Each symbol represents one group

IL-2 complex has a high persistence in blood of treated mice

To investigate the mechanism of IL-2 complex action, we assessed the clearance of IL-2 complex in vivo. Radiolabeled anti-IL-2 Ab or IL-2 complex formed with radiolabeled anti-IL-2 Ab was injected into mice and the amount of radioactivity in the blood was assessed over the course of several days. As shown in Fig. 2, the half-life of IL-2 complex was found to be higher than the half-life of the anti-IL-2 Ab in blood. The half-life for the early phase ($t_{1/2}$ α) of the clearance from blood was 23.2 h for the IL-2 complex compared with 8.2 h for anti-IL-2 Ab. Similarly, the half-life for the late phase ($t_{1/2}$ β) was 143.2 h for the IL-2 complex compared with 100.6 h for the anti-IL-2 Ab. These data demonstrated that the complex of IL-2 and anti-IL-2 Ab remained in the blood for extended periods of time, with a slower clearance than free anti-IL-2 Ab and a markedly slower clearance than the previously published clearance time of ~2 h for i.p.-administered IL-2 (41). It should be mentioned that these data were obtained by the use of a radiolabeled anti-IL-2 Ab. Therefore, it is possible that the IL-2 dissociated from the Ab at an early time point and the measured half-life is solely the half-life of the free Ab, not that of the IL-2 complex. However, in that case the kinetics of the IL-2 complex and the anti-IL-2 Ab should be the same. The different clearance rates we demonstrate here point to the continued binding of IL-2 to the anti-IL-2 Ab in the IL-2 complex-treated mice.

Effect of IL-2 complex treatment on cytokine levels in serum

To evaluate the effect of IL-2 complex on the cytokine levels in vivo, we analyzed the serum of mice after vaccination and treatment with either the IL-2 complex or the isotype Ab (as described above). As shown in Table III, there were very small amounts of IFN-γ, IL-4, IL-5, IL-10, and TNF found in the serum of the mice treated with either IL-2 complex or control isotype Ab at any given time point. MCP-1 was the only cytokine that was found in higher levels in the serum and its level dropped posttreatment in either group.

Even though the IL-2 levels in serum shows a consistent pattern of slight elevation in the IL-2 complex-treated animals as compared with the isotype Ab-treated animals at all three time points (3.2-fold increase on day 1, 1.6-fold on day 5, and 3.7-fold on day 12), the detected cytokine levels were very low. Animals treated with either IL-2 or anti-IL-2 Ab showed no alterations in the serum cytokine levels at day 12 posttreatment cycle as compared with animals treated with isotype Ab (Table III).

IL-2 complex binds selectively to memory CD8$^+$ T cells and NK cells in vitro

To further elucidate the mechanism underlying the stimulation of memory CD8$^+$ T cells and NK cells, we stained splenocytes from untreated C57BL/6 mice in vitro with FITC fluorochrome-labeled anti-IL-2 Ab, IL-2 complex created from IL-2 and FITC anti-IL-2 Ab, or an isotype control complex created from IL-2 and FITC-labeled isotype control Ab. This isotype control complex should in fact not form any complex at all, because the IL-2 should not bind to the FITC isotype Ab. Hence, this is a control for an unspecific interaction and binding of Ab and cytokine in this staining experiment. These splenocytes were then further stained with Abs to identify memory CD8$^+$ T cells (CD8$^+$CD44^{high}CD122$^+$), memory CD4$^+$ T cells (CD4$^+$CD44^{high}), CD19$^+$, DX5$^+$NK1.1$^+$ (to identify NK cells), and CD4$^+$CD25$^+$ T cells. The cells were then analyzed by multicolor flow cytometry. As shown in Fig. 3*A*, anti-IL-2 Ab by itself (dotted line histogram) did not bind to any analyzed cell type as compared with the isotype control complex (gray filled histogram); in all graphs the two histograms are practically superimposed over each other. The IL-2 complex (black filled histogram) did not bind to CD19$^+$ cells or to CD4$^+$CD25$^+$ T cells any more strongly than the isotype control complex or anti-IL-2 Ab. In contrast, IL-2 complex bound to memory CD8$^+$ T cells and also to NK cells, both of which express CD122. Blocking experiments on NK cells and memory CD8$^+$ T cells confirmed the specific binding of IL-2 complex to CD122 (Fig. 3*B*). The binding of IL-2 complex was reduced to background level by pretreatment with purified anti-CD122 Ab (gray line histogram, *two upper panels*) compared with cells blocked with isotype Ab (black line histogram). This experiment demonstrated that the blocking of CD122 completely demolished the specific binding of IL-2 complex to NK cells and memory CD8$^+$ T cells in vitro. In contrast, blocking of the Fc receptor CD16/32 by pretreatment of NK cells or memory CD8$^+$ T cells with purified anti-CD16/32 Ab did not have any impact on the binding of IL-2 complex on treated cells (gray filled histogram, *two lower panels*) compared with cells that were pretreated with isotype Ab (black line histogram, *two lower panels*). The binding of IL-2 complex to NK cells and memory CD8$^+$ T cells was not different between cells that were pretreated with isotype Ab and cells that were not treated before staining (data not shown).

These results support the hypothesis that IL-2 complexed to anti-IL-2 Ab (clone S4B6) can still bind to the low affinity receptor CD122 but not to the high affinity receptor CD25.

IL-2 complex increases the number of Ag-specific CD8$^+$ T cells

After adoptive transfer of naive F5 CD8$^+$ T cells, vaccination of mice, and treatment with IL-2 complex, anti-IL-2 Ab, IL-2, or isotype Ab as described above, splenocytes from pooled spleens were analyzed for influenza NP68-specific CD8$^+$ T cells by pentamer staining. It should be mentioned that all adoptively transferred NP68-specific F5 CD8$^+$ T cells express the TCR chain Vβ11. In a representative experiment we stained the CD8$^+$ T cells not only with a pentamer to identify NP68-specific CD8$^+$ T cells but also with Vβ11. The NP68-specific CD8$^+$ T cells were Vβ11$^+$ compared with other CD8$^+$ T cells, suggesting that the NP68-specific CD8$^+$ T cells descended from the adoptively transferred naive F5 CD8$^+$ T cells.

As shown in Fig. 4*A*, the percentage of NP68-positive CD8$^+$ T cells barely increased in IL-2-treated animals, anti-IL-2 Ab-treated animals, or IL-2 complex-treated animals compared with the isotype Ab-treated control animals for both time points, 1 day posttreatment cycle and 12 days posttreatment cycle. However, as

with multiple mice per group from multiple experimental repeats; the horizontal bar represents the average. Spleens were harvested 1 day (*left panel*) or 12 days posttreatment cycle (*right panel*). *C*, C57BL/6 mice received an adoptive transfer of naive CFSE-labeled CD8$^+$ T cells from F5 TCR-transgenic mice and were vaccinated with rV-NP-GFP/TRICOM (plus rF-GM-CSF). Mice were then treated with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex for 7 days. Animals were sacrificed and the pooled spleens of two to three mice per group were analyzed 1 day posttreatment. Data are the CFSE profile of NP68-specific CD8$^+$ T cells of two to three pooled animals per group. *D*, The amount of IFN-γ-producing cells in the CD8$^+$ T cell population in spleen of isotype Ab-treated mice or IL-2 complex-treated mice were evaluated 1 day (*upper panels*) or 12 days (*lower panels*) posttreatment cycle. CD8$^+$ T cells were isolated from the spleen and incubated for 18 h with irradiated APC (CD8$^+$ depleted) and Ag (NP68 peptide). Data are representative dot plots (gated on live CD8$^+$ T cells) with captions displaying the percentage of IFN-γ-producing cells in the CD8$^+$ T cell population (day 1, one repeat with multiple mice per group; day 12, average ± SD for 3–5 repeats with multiple mice per group).

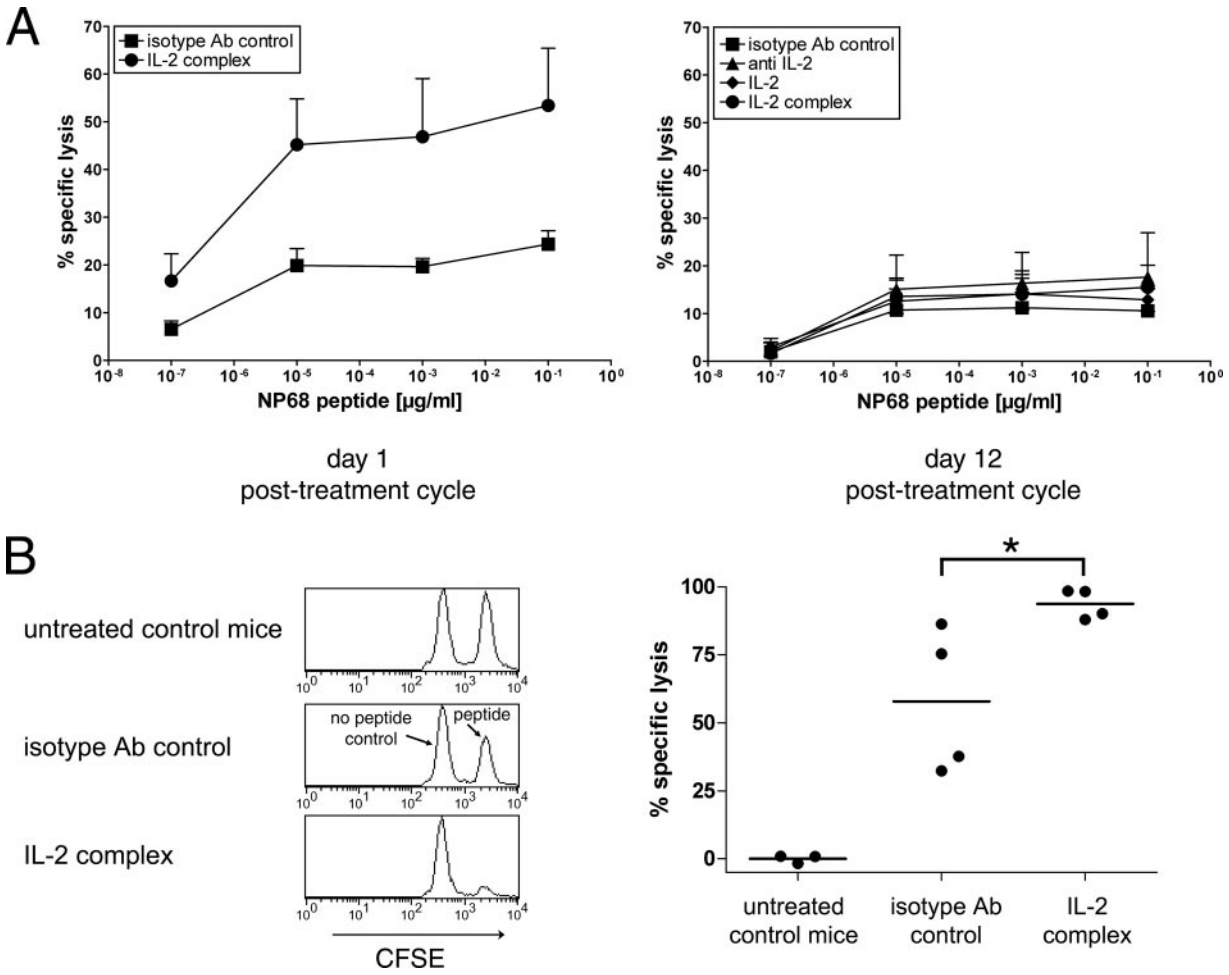

FIGURE 5. Increase in specific CTL activity by IL-2 complex treatment. *A*, Naive CD8$^+$ T cells from F5 TCR-transgenic mice were adoptively transferred into C57BL/6 mice. Mice were then vaccinated with rV-NP-GFP/TRICOM (plus rF-GM-CSF) and treated with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex for 7 days. Animals were sacrificed and analyzed either 1 day or 12 days after the completed 7-day treatment cycle (1 day or 12 days posttreatment cycle). CTL lysis activity of the CD8$^+$ T cell population in spleen from isotype Ab-treated mice (■), anti-IL-2 Ab-treated mice (▲), IL-2-treated mice (♦), and IL-2 complex-treated mice (●) was evaluated 1 day posttreatment cycle (*left panel*) and 12 days posttreatment cycle (*right panel*). Data for 1 day posttreatment cycle (*left panel*) are from one experiment and the average ± SD from three animals for the isotype Ab control and seven animals for the IL-2 complex-treated group. Data for 12 days posttreatment cycle (*right panel*) are average and SD from groups with multiple mice per group from 3–5 repeats. *B*, Naive CD8$^+$ T cells from F5 TCR-transgenic mice were adoptively transferred into C57BL/6 mice. Mice were then vaccinated with rV-NP-GFP/TRICOM (plus rF-GM-CSF) and treated with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex for 7 days. One day after the last treatment, these mice and untreated C57BL/6 control mice received a transfer of CFSE^{high} target cells loaded with NP68 peptide and unloaded CFSE^{low} target cells. Animals were sacrificed the next day and the killing of NP68-loaded CFSE^{high} cells was assessed. The *left panel* shows representative histograms of the CFSE-labeled target cell populations. Each symbol in the *right panel* represents one animal; the horizontal bar represents the average.

shown in Fig. 4*B*, the increase in spleen cell numbers and especially the increase in CD8$^+$ T cells led to a 7-fold increase in the number of influenza NP68-specific CD8$^+$ T cells 1 day after the completed 7-day treatment cycle in IL-2 complex-treated animals. Interestingly, even 12 days after the completed 7-day treatment cycle the number of influenza NP68-specific cells in the IL-2 complex-treated animals remained elevated (2.4-fold the number in isotype Ab-treated control mice; Fig. 4*B*). The expansion of Ag-specific cells in IL-2 complex-treated mice depended on the presence of Ag during the vaccination; control animals that were treated with IL-2 complex but vaccinated with control virus that expressed TRICOM but not NP68 showed a similar expansion in total CD8$^+$ T cells (data not shown) but a markedly lower percentage of NP68-specific CD8$^+$ T cells compared with animals vaccinated with NP68-expressing virus 1 day and 12 days after the treatment cycle (~0.5% compared with ~5%, respectively).

To investigate the effect of IL-2 complex treatment on the expansion of the adoptively transferred naive F5 CD8$^+$ T cells, naive F5 CD8$^+$ T cells were labeled with CFSE before the adoptive transfer. As shown in Fig. 4*C*, CFSE-labeled NP68-specific CD8$^+$ T cells in all groups proliferated at 1 day after the completed 7-day treatment cycle. However, the IL-2 complex treatment led to increased cycling of the adoptively transferred cells, leading to the expansion of the adoptively transferred cells in the IL-2 complex-treated mice (Fig. 4*C*).

IL-2 complex increases the number of IFN-γ-producing CD8$^+$ T cells after Ag-specific restimulation

After vaccination of mice and treatment with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex as described above, purified CD8$^+$ T cells from pooled spleens were cultured with NP68 peptide and irradiated splenocytes (CD8$^+$ depleted) as APC. After 20 h of culture, cells were harvested and CD8$^+$ T cells were analyzed for intracellular IFN-γ production. As shown in Fig. 4*D*, treatment with IL-2 complex led to a 2-fold increase of IFN-γ-producing CD8$^+$ T cells as compared with the isotype Ab control

group 1 day after the completed 7-day treatment cycle. The percentage of IFN-γ-producing $CD8^+$ T cells is reduced in both groups, IL-2 complex-treated mice and isotype Ab-treated control mice, 12 days after the completed 7-day treatment cycle as compared with 1 day posttreatment cycle. There was also an increased level of IFN-γ-producing cells in IL-2 complex-treated mice compared with isotype Ab-treated mice at 12 days posttreatment cycle (Fig. 4*D*). We did not observe any increase in the percentages of IFN-γ-producing cells in either IL-2-treated mice or anti-IL-2 Ab-treated mice compared with isotype Ab-treated mice (data not shown). At both time points, treatment with IL-2 complex also led to enhanced numbers of cells producing intracellular TNF-α and MIP-1β (data not shown).

Enhanced CTL functionality in vaccinated mice treated with IL-2 complex

To investigate the functionality of $CD8^+$ T cells that are generated in the presence of IL-2 complex, purified $CD8^+$ T cells from the pooled spleens of animals vaccinated and treated with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex as described above were used as effector cells in a CTL assay with ^{111}In-labeled target cells and NP68 peptide at various concentrations. As shown in Fig. 5*A*, the CTL activity of $CD8^+$ T cells from mice treated with IL-2 complex was enhanced compared with control mice treated with the isotype Ab 1 day after the completed 7-day treatment cycle. The CTL functionality of both groups was reduced 12 days after the completed 7-day treatment cycle compared with 1 day posttreatment cycle and was similar in the IL-2 complex-treated group, the anti-IL-2 Ab-treated group, the IL-2-treated group, and the isotype Ab-treated control group (Fig. 5*A*).

To determine whether treatment with IL-2 complex leads to improved immune responses in vivo, we also tested the in vivo CTL functionality of the mice treated with IL-2 complex during a vaccination and compared it with mice that were treated with isotype Ab. Mice were vaccinated and treated with either IL-2 complex or isotype Ab as described above. One day after the end of the treatment cycle, all treated mice and untreated control mice received an adoptive transfer of CFSE-labeled target cells. These target cells consisted of a 1:1 mixture of two different populations: one population was labeled with a high concentration of CFSE and loaded with 10^{-3} μg/ml NP68-peptide, and the other population was labeled with a low concentration of CFSE and remained unloaded. One day after the adoptive transfer the mice were sacrificed and the CFSE populations in the spleen were assayed. The NP68-loaded $CFSE^{high}$ target cells were markedly reduced in experimental animals compared with nontreated control mice, demonstrating the specific lysis of the NP68-loaded target cells. Among the experimental animals, the animals that were treated with IL-2 complex showed a stronger reduction of NP68-loaded $CFSE^{high}$ target cells compared with the control $CFSE^{low}$ target cells (Fig. 5*B*, *left panel*). This experiment demonstrated a statistically significant increase of specific lysis in IL-2 complex-treated mice (93.7% specific lysis) compared with isotype Ab-treated mice (57.9% specific lysis; $p < 0.05$) (Fig. 5*B*, *right panel*).

IL-2 complex increases the ratio of Ag-specific T cells compared with T_{REG} cells

After vaccination of mice and treatment with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex as described above, splenocytes from pooled spleens were analyzed for effector/memory $CD8^+$ T cells, influenza NP68-specific $CD8^+$ T cells, and T_{REG} cells. Our data demonstrate that IL-2 complex led to a shift in balance toward the NP68-specific $CD8^+$ T cells, increasing the ratio of NP68-specific $CD8^+$ T cells to T_{REG} cells from 0.5 in the isotype Ab control group to 1.4 in the IL-2 complex-treated group 1 day after the completed 7-day treatment cycle (Fig. 6*A*). There was a slight increase of the ratio of NP68-specific $CD8^+$ T cells to T_{REG} cells in the anti-IL-2 Ab-treated group compared with the isotype Ab-treated group (0.9 vs 0.5) (Fig. 6*A*). This change in the ratio reflects the reduction of the T_{REG} cell population instead of an increase in the NP68-specific $CD8^+$ T cells (Table I and Fig. 6*B*). Interestingly, the animals that were treated with IL-2 alone demonstrated a decreased ratio of NP68-specific $CD8^+$ T cells to T_{REG} cells as compared with the isotype Ab-treated mice (0.5 vs 0.2)

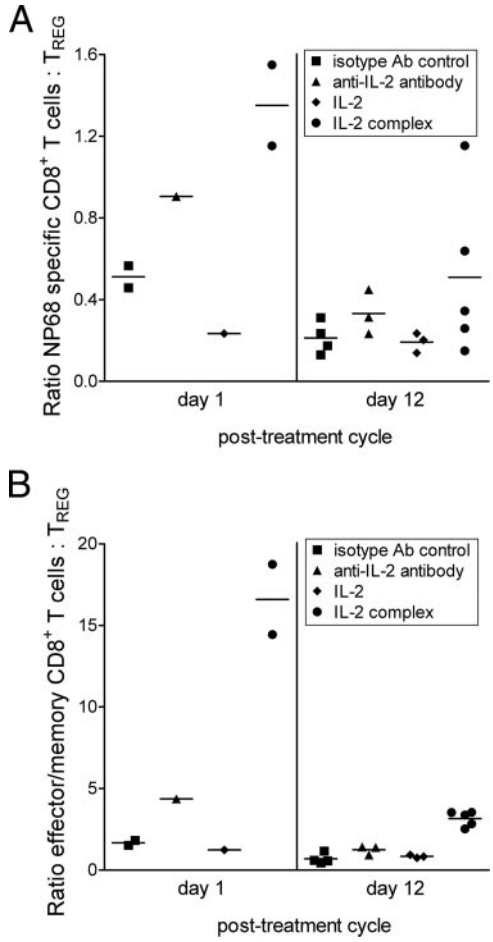

FIGURE 6. Increase in the ratio of effector/memory $CD8^+$ T cells and Ag-specific $CD8^+$ T cells vs T_{REG} cells by IL-2 complex. C57BL/6 mice received an adoptive transfer of naive $CD8^+$ T cells from F5 TCR-transgenic mice and were vaccinated with rV-NP-GFP/TRICOM (plus rF-GM-CSF) and treated with IL-2 alone, anti-IL-2 Ab, isotype Ab, or IL-2 complex for 7 days. Animals were sacrificed and their spleens were analyzed either 1 day or 12 days after the completed 7-day treatment cycle (1 day or 12 days posttreatment cycle). *A*, NP68-specific $CD8^+$ T cell numbers were evaluated and divided by the numbers of T_{REG} cells for isotype Ab-treated mice (■), anti-IL-2 Ab-treated mice (▲), IL-2-treated mice (♦), and IL-2 complex-treated mice (●). Each symbol represents one group with multiple mice per group from multiple experimental repeats; the horizontal bar represents the average. Spleens were harvested 1 day (*left panel*) or 12 days (*right panel*) posttreatment cycle. *B*, Effector/memory ($CD44^{high}CD122^+$) $CD8^+$ T cell numbers were evaluated and divided by the numbers of T_{REG} cells for isotype Ab-treated mice (■), anti-IL-2 Ab-treated mice (▲), IL-2-treated mice (♦), and IL-2 complex-treated mice (●). Each symbol represents one group with multiple mice per group from multiple experimental repeats; the horizontal bar represents the average. Spleens were harvested 1 day (*left panel*) or 12 days (*right panel*) posttreatment cycle.

(Fig. 6*A*), which reflects the increase in T_{REG} cells in IL-2-treated mice (Table I). Twelve days after the completed 7-day treatment cycle, the ratio of Ag-specific CD8$^+$ T cells to T_{REG} cells was reduced compared with 1 day posttreatment cycle. However, the IL-2 complex group and the anti-IL-2 Ab group still showed a higher ratio of NP68-specific CD8$^+$ T cells compared with the isotype Ab control group (0.5 and 0.3, respectively, vs 0.2) (Fig. 6*A*). A similar pattern is seen in the ratio of effector/memory CD8$^+$ T cells to T_{REG} cells in IL-2 complex-treated mice (Fig. 6*B*). Our data demonstrate that IL-2 complex led to a strong increase in the number of effector/memory CD8$^+$ T cells in relation to T_{REG} cells.

Discussion

This study demonstrates for the first time that an immune complex formed from IL-2 and anti-IL-2 Ab strongly enhances the immune reaction against an Ag during vaccination. This is shown by the increased number of Ag-specific CD8$^+$ T cells as well as increased cytokine production and CTL lysis activity of CD8$^+$ T cells following Ag-specific stimulation. Treatment of vaccinated mice with either IL-2 alone or anti-IL-2 Ab also led to some effects on the immune system. However, these effects were much weaker and, in the case of IL-2 treatment, included the potentially undesirable effect of an increased T_{REG} cell percentage in spleen. It should be specified that the effect we observed upon treatment with IL-2 complex is temporary; a strong effect was seen 1 day after the completed 7-day treatment cycle, but had largely disappeared 12 days after the completed 7-day treatment cycle. When the IL-2 complex was used at the dose of 1.5 μg of IL-2 with 30 μg of anti-IL-2 Ab, the animals demonstrated a bloated abdomen, a hunched posture, and two of six animals died. Therefore, a reduced dose (80%) was used in all other repeats in this study with no sign of toxicity. It should be mentioned that our HPLC studies demonstrated that the IL-2 complex formation was not complete; therefore the IL-2 complex solution used in this study contained free IL-2. However, because we had both an IL-2-treated control group and an anti-IL-2 Ab-treated control group in our experiments, one may conclude that the strong effects seen in the IL-2 complex-treated mice can be attributed to the actual complex of IL-2 and anti-IL-2 Ab.

We further investigated the mechanism of action of IL-2 complex and demonstrated that the IL-2 complex binds to NK cells and memory CD4$^+$ and CD8$^+$ T cells, but not to T_{REG} cells. Our data also demonstrated that the Fc receptor does not contribute to the binding of IL-2 complex. This is in agreement with a previously published study showing that Fc receptors are dispensable for IL-2 complex action (28). This finding supports the hypothesis that the IL-2 complex formed by the anti-IL-2 Ab clone S4B6 blocks binding of the IL-2 to the high affinity IL-2 receptor CD25 but allows binding to the low affinity IL-2 receptor CD122 (5). We also observed a weak binding of IL-2 complex to CD122$^-$CD8$^+$ T cells, most probably due to the presence of a very low level of CD122 (5). It has previously been suggested that complexation with anti-IL-2 Ab increases the half-life of IL-2. An additional factor is the increase in biological half-life of IL-2 in the system by complexation with anti-IL-2 Ab (5, 13, 19–21). We now demonstrate a half-life of 143 h for the IL-2 complex in blood after i.p. delivery, which is markedly longer than the time that free IL-2 is present in blood after i.p. delivery (~2 h) (41). Together, this allows a strong and long-lasting stimulus to all CD122-bearing cells in the immune system (NK and memory T cells) while not affecting other cells in the immune system, notably T_{REG} cells. Due to the resulting increase in size of the T cell population, the ratio of effector/memory T cells and Ag-specific CD8$^+$ T cells to T_{REG} cells actually increases, and this might reduce the efficiency of the T_{REG} cells to suppress T cell-mediated immune responses and further enhance an ongoing immune reaction. However, the number of T_{REG} cells in fact increases, which demonstrates that the homeostasis of the T_{REG} cells is not negatively affected by IL-2 complex. This is a noticeable difference from treatment with anti-IL-2 Ab alone. Our results demonstrate that treatment with anti-IL-2 Ab leads to a decrease in the number of T_{REG} cells. A previous study has shown similar results and demonstrated that the removal of IL-2 in the system by using anti-IL-2 Ab disturbs the homeostasis of the T_{REG} cells and leads to the induction of autoimmunity (12).

IL-2 complex has been demonstrated previously to affect the homeostasis of various cell types. It was shown to lead to strong increases in memory CD8$^+$ T cells and NK cell numbers and to small increases of T_{REG} cells and memory CD4$^+$ T cells (5, 11). Moreover, it has been demonstrated that treatment with IL-2 complex can increase the NK cell lysis activity of splenocytes and lead to tumor reduction (19–21, 42). A previous study also demonstrated that combining a neutralizing anti-IL-2 Ab with a plasmid encoding IL-2 effectively attenuated the specific CD8$^+$ T cell response in the absence of vaccination in mice (11). Our study demonstrates for the first time that IL-2 complex treatment can lead to the expansion of Ag-specific CD8$^+$ T cells during a vaccination strategy using recombinant viruses. The expansion of total effector/memory CD8$^+$ T cells induced by IL-2 complex in this study is in the same range as previously published in a study by Boyman et al.: 1.1×10^8 cells at day 1 posttreatment cycle compared with $\sim 1 \times 10^8$ to 1.5×10^8 cells (5). However, the Ag-specific CD8$^+$ T cells in our study were derived from naive CD8$^+$ T cells, which had to be activated before they started to expand and express CD122. This explains the 7-fold increase in the number of Ag-specific CD8$^+$ T cells in IL-2 complex-treated mice compared with isotype Ab-treated mice. It should be emphasized that IL-2 complex treatment not only increased the number of Ag-specific CD8$^+$ T cells and effector/memory CD8$^+$ T cells, but also resulted in an increase in specific CTL lysis of Ag-presenting target cells in vitro and in vivo. The increase in Ag-specific CD8$^+$ T cells and the resulting increases in cytokine production and CTL lysis following Ag-specific restimulation demonstrate the usability of IL-2 complex for enhancing the effectiveness of immune therapies based on vaccination strategies. The strong effect of IL-2 complex, especially on memory CD8$^+$ T cells, emphasizes the possibility of using the IL-2 complex instead of IL-2 to improve immune therapeutic strategies for enhancement of immune responses.

It has previously been established that treatment with IL-2 alone during an immune response can have positive effects on CD8$^+$ T cell responses (2–4). Our study similarly demonstrated that IL-2 treatment leads to increased numbers of T cells in the spleen. However, the number of T cells in the IL-2-treated mice was much lower compared with IL-2-complex-treated mice. Interestingly, the numbers of Ag-specific CD8$^+$ T cells and NK cells were hardly affected in IL-2-treated mice compared with the control mice. The use of IL-2 alone in conjunction with vaccination in our study led to an increased percentage of T_{REG} cells in the spleen, whereas treatment with IL-2 complex did not alter the percentage of T_{REG} cells in spleen of vaccinated mice. This resulted in a markedly different ratio of Ag-specific CD8$^+$ T cells vs T_{REG} cells, which might result in a stronger suppression of Ag-specific CD8$^+$ T cells in IL-2-treated mice. The application of IL-2 complex specifically increases CD8$^+$ T cells (including Ag-specific cells) and NK cells, and thus can create a window of opportunity for the expansion of Ag-specific CD8$^+$ T cells upon vaccination in the context of cancer therapy. In light of our observations along with previously published results stating that IL-2 treatment not only increases the T_{REG} cell population in humans (43, 44) but also leads to toxicities (45), treatment with IL-2 complex may be a suitable substitute for IL-2.

It is important to stress the temporary effects of IL-2 complex on the immune system. Our studies demonstrate for the first time that the influence of IL-2 complex on the organism has largely disappeared 12 days posttreatment cycle. This might allow the use of IL-2 complex as a temporary immune adjuvant during an ongoing immune reaction, with a reduced risk of long lasting alterations in the immune system that might lead to immunopathology in later life stages.

Acknowledgments

We thank Judith DiPietro for technical assistance and Debra Weingarten and Bonnie L. Casey for editorial assistance in the preparation of this manuscript.

Disclosures

The authors have no financial conflict of interest.

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