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Novel IL-2-Poly(HPMA)Nanoconjugate Based Immunotherapy

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Interleukin-2 (IL-2) possesses a strong stimulatory activity for activated T and NK cells and it is an attractive molecule for immunotherapy. Nevertheless, extremely short half-life and severe toxicities associated with high-dose IL-2 treatment are serious and limiting drawbacks. In order to increase IL-2 half-life *in vivo*, we covalently conjugated synthetic semitelechelic polymeric carrier based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) to IL-2. Thus, we synthesized IL-2-poly(HPMA) conjugate containing 2–3 polymer chains per IL-2 molecule in average. Such conjugate has lower biologic activity in comparison to IL-2 *in vitro*. However, it exerts much higher activity than IL-2 *in vivo* as shown by expansion of memory $CD8^+$ T, NK, NKT, $\gamma\delta$T and Treg cells. Moreover, IL-2-poly(HPMA) extremely effectively potentiates $CD8^+$ T cell peptide-based vaccination. IL-2-poly(HPMA) shows also much longer half-time in circulation than IL-2 (~4 h versus ~5 min). Collectively, modification of IL-2 with poly(HPMA) chains dramatically improves its potency and pharmacologic features *in vivo*, which have implications for immunotherapy. To our knowledge, this is the first proof-of-concept report of the use of polymer/protein modification of IL-2 to obtain more pronounced biological activity.

KEYWORDS: *Interleukin-2, N-(2-hydroxypropyl)Methacrylamide, Poly(HPMA) Conjugate, $CD8^+$ T Cells, Vaccination, Immunostimulation.*

INTRODUCTION

Interleukin-2 (IL-2) is a small glycoprotein produced by activated T cells. IL-2 plays a crucial role in T cell-mediated immune responses as it promotes proliferation and survival of activated T cells and NK cells and it induces expression of effector functions in these cells. This cytokine further stimulates proliferation of memory $CD8^+$ T cells and, to a lesser extent, also activated B cells.[1] IL-2 is thus a very attractive molecule for immunotherapy and vaccination. However, due to its small molecular size, IL-2 is rapidly cleared through the kidneys, where it is metabolized completely.[2–4] This results into limited bioavailability and therefore low bioactivity of IL-2 when administered as a recombinant protein.[5] Attempts to administer IL-2 in high doses lead to many types of serious toxicities like vascular leak syndrome, severe pulmonary oedema[6] and many others.[7,8]

Increasing the molecular size of IL-2 may lead to reduced renal clearance, thereby achieving prolonged circulation.[9,10] IL-2 has been modified with synthetic polymeric carrier polyethylene glycol (PEG) for the first time more than 25 years ago. The IL-2-PEG conjugate was shown to have up to 20-fold increased plasma half-life in comparison to IL-2 and IL-2-PEG was thus considered as promising drug.[9] Although earlier studies in animals[9,11,12,15,16] and humans[13,14] suggested positive effects in the treatment of various cancers, more recent data, however, failed to clearly demonstrate an advantage for IL-2-PEG in comparison to IL-2.[17–20] Other alternative strategies such as fusion or conjugation of IL-2 to serum proteins like albumin (Albuleukin)[21] or IgG[22] were also employed and showed to be effective in terms of increasing the bioavailability and also antitumor activity of IL-2.

PEG is the gold standard in the emerging field of polymer-based drug delivery, although the situation regarding PEGylated proteins is rather complicated. Since a vast

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amount of clinical experience has been gained with PEGylated products up to this time, not only benefits but possible side effects and complications have also been found.[23] Among them, adverse effects in the body that can be provoked by the PEG itself or by side products formed during synthesis that lead to hypersensitivity are the most frequent ones.[25–28] Furthermore, PEG may possess antigenic and immunogenic properties as a haptene.[24] With all these potential drawbacks of PEGylated proteins in mind, we focused on another well characterized, but chemically significantly different synthetic polymeric carrier based on *N*-(2-hydroxypropyl)methacrylamide (HPMA). Poly(HPMA) is water-soluble and entirely biocompatible, i.e., non-immunogenic polymer which is easily eliminated from the body when chains up to 40 kDa are used.[30–32] Importantly, when comparing immunogenicity of protein-polymer conjugates, our laboratory showed that conjugates containing PEG possess higher ability to provoke hosts immunity than that ones with poly(HPMA).[33–35] Superoxide dismutase (SOD), bovine seminal ribonuclese (BSR) and α-chymotrypsin were conjugated to semitelechelic poly(HPMA). The conjugation decreased the immunogenicity of conjugates, increased the temperature stability of poly(HPMA)-SOD conjugates and proteolytic stability of poly(HPMA)-BSR conjugates.[36, 37]

In this study, we designed and synthesized an *in vivo* superagonistic form of IL-2 through its modification with well-defined polymeric carrier based on HPMA. By covalent conjugation of semitelechelic poly(HPMA) chains (MW∼20 kDa) to IL-2 through aminolysis of ε-amine group in lysine residues, we synthesized IL-2-poly(HPMA) conjugate containing in average 2–3 polymer chains per IL-2 molecule and with relatively narrow size distribution (∼Đ = 1.9). We determined the biological activity of IL-2-poly(HPMA) conjugate on activated $CD8^+$ T and NK cells *in vitro* and on $CD8^+$ T, NK, NKT, $\gamma\delta$T and Treg cells *in vivo* and we demonstrate here the benefits of this conjugate over unmodified IL-2. This study was not focused to develop new form of IL-2 for tumor immunotherapy, but rather much broadly to generally increase the biological activity and improve pharmacological features of IL-2 *in vivo* and thus potentially enable the use of this cytokine and, theoretically, also other cytokines in various biomedical applications like vaccination, immunodeficiency, and reconstitution of immune system after BMT, etc.

MATERIALS AND METHODS

Chemicals

Methacryloyl chloride, 1-aminopropan-2-ol, 4,4′-azobis (4-cyanopentanoic acid) 4,5-dihydro-thiazole-2-thiol, N,N′-dicyclohexylcarbodiimide (DCC) were purched from Sigma-Aldrich, Czech Republic. Mouse recombinant interleukin-2 (IL-2) was purchased from ProSpec-Tany TechnoGene Ltd. (Israel). All other chemicals and solvents were of analytical grade. The solvents were dried and purified by conventional procedures and distilled before use.

Synthesis of Monomer and Semitelechelic Poly(HPMA)

N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized by a modified reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane in the presence of sodium carbonate.[38] Azo initiator 3,3′-azobis (4-cyano-4-methyl-1-oxobutane-1.4-diyl)bis(thiazolidine-2-thione) (ABIC-TT) was prepared by reaction of 4,4′-azobis (4-cyanopentanoic acid) with thiazolidine-2 thione in the presence of DCC in THF.[39] Semitelechelic poly(HPMA) containing thiazolidine-2-thione reactive group (polyHPMA-TT) at the polymer end chain was prepared by radical solution polymerization of HPMA (0.5 g, 3.5 mmol) initiated with azo initiator ABIC-TT (0.228 g) in DMSO (3.5 mL) in a sealed ampule under nitrogen atmosphere at 60 °C for 6 h. Yield of polymerization was 0.299 g (59.8%) of polyHPMA-TT with molecular weight 24 kDa and polydispersity Đ = 1.9.

Synthesis and Characterization of IL-2-poly(HPMA) Conjugate

IL-2 (4 mg) was dissolved in PBS buffer pH 7.4 (0.8 mL), cooled to 0 °C and then polyHPMA-TT (12 mg) was added. Reaction mixture was stirred for 6 h and the pH = 8.2 was maintained by addition of sodium borate using pH-stat. Polymer conjugate IL-2-poly(HPMA) was separated from unbound polymer by size exclusion chromatography (SEC) on AKTAexplorer equipped with Superpose 6 column and UV detector set to 280 nm using 0.3 M sodium acetate buffer pH 6.5 as mobile phase. Fraction containing IL-2-poly(HPMA) conjugate was concentrated on Vivaspin with cut off 10 000 kDa, desalted using PD-10 column and lyophilized. The yield was 10 mg of IL-2-poly(HPMA) conjugate with molecular weight Mw 62 300 and polydispersity Đ = 1.9.

Number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity (Đ) of poly(HPMA)-TT and IL-2-poly(HPMA) conjugate were measured using SEC on a HPLC Shimadzu system equipped with UV, an Optilab rEX differential refractometer and multiangle light scattering DAWN 8 (Wyatt Technology, USA) detectors using Superose 6 column. The 0.3 M sodium acetate buffer pH 6.5 was used as mobile phase.

The content of IL-2 in the IL-2-poly(HPMA) conjugate (59 wt%) was determined by amino acid analysis of hydrolyzed conjugate (6 M HCl, 115 °C, 18 h in a sealed ampule) on a reverse-phase column Chromolith HighResulution RP-18e, 100 × 4.6 mm (Merck, Germany) using precolumn derivatization with phthalaldehyde (OPA) and 3-sulfanylpropanoic acid (excitation at 229 nm, emission at

450 nm). Gradient elution with 10–100% of solvent B for 35 min at a flow rate of 1.0 mL/min was used (solvent A, 0.05 M sodium acetate buffer, pH 6.5; solvent B, 300 mL of 0.17 M sodium acetate and 700 mL of methanol).

Mice

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

Cell Lines and mAbs

Murine YAC-1 cell line was purchased from American Type Culture Collection. The following anti-mouse mAbs were used: CD3-eF450, CD4-APC (/allophycocyanin), CD8-A700, CD8-PerCP-Cy5.5, CD44-APC, CD45.2-APC, Ly5.2-A700, DX5-PE, IFN-γ-PE mAb, IgG2b-PE, NK1.1-PerCP-Cy55, CD25-PE, Foxp3-PE, IL-2 clone JES6-1A12 and IL-2 clone JES6.5H4, CD122-PE (eBioscience), CTLA-4-IgG mAb (BD Pharmingen). CD25-APC and S4B6 mAb were provided by Drbal (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic). Functional grade purified blocking anti-mouse mAbs anti-CD25 (PC61.5) and anti-CD122 (Tm-b1) were purchased from eBioscience.

Proliferation Assay *In Vitro*

Purified $CD8^+$ T cells were seeded into Nunc 96-well flat-bottom plates in 0.2 ml volume and density of $2{,}5 \times 10^5$ cells/ml, cultured with 5 μg/ml soluble anti-CD3 and 1 μg/ml CTLA-4-IgG plus titrated amounts of free IL-2 or IL-2-poly(HPMA) conjugate. The plates were then cultured in 5% CO_2 for 72 h at 37 °C. A 18.5 kBq of [^{3}H]thymidine was added for the final 8 h of cultivation before harvesting. Purified NK cells were cultured at 1.25×10^6 cells/ml with IL-2 or IL-2-poly(HPMA) conjugate. The plates were cultured in 5% CO_2 for 72 h at 37 °C. A 18.5 kBq of [^{3}H]thymidine was added for the final 18 h of cultivation before harvesting.

Adoptive Transfer of OT-I Cells

Purified OT-I $CD8^+$ T cells (Ly5.2) were labeled with CFSE and injected i.v. into B6.SJL recipients (Ly5.1) at 1,2 x 10^6 cells per mouse. Next day, the mice were injected i.p. with PBS, SIINFEKL peptide (2 nmol, MBL International), SIINFEKL peptide plus polyinosinic-polycytidylic acid (poly(I:C), 75 μg), IL-2-poly(HPMA) conjugate plus SIINFEKL peptide, or free IL-2 plus SIINFEKL peptide. Detailed schedule of the treatment is indicated at each experiment.

Staining for Surface and Intracellular Markers

Cells were resuspended in FACS buffer (PBS with 2% FCS, 2 mmol EDTA and 0,05% sodium azide), blocked by 10% mouse serum for 30 min on ice and stained with fluorochrome labeled mAbs for 30 min on ice in the dark. Cells were washed twice after each step in FACS buffer and fixed in 4% paraformaldehyde before analysis. When intracellular markers were stained, cells were additionally incubated in Fixation Buffer (eBioscience) 30 min on ice in the dark. Then washed twice in 1× Permeabilisation Buffer (eBioscience) and stained with fluorochrome labeled mAbs for 30 min on ice in the dark. Cells were washed three times in 1× Permeabilisation Buffer and resuspended in FACS buffer before analysis. Labelling the cells with CFSE was conducted as described elsewhere.[40] Flow cytometric analysis was performed on LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Measuring of Intracellular IFN-γ Expression

Spleen cell suspensions from B6.SJL (Ly 5.1) mice were seeded into Nunc 12-well flat-bottom plates in 2 ml volume and restimulated with 50 nM SIINFEKL peptide. The plates were then cultured in 5% CO_2 for 6 h at 37 °C. Brefeldin A (Sigma-Aldrich) was added for last 4 h of cultivation at final concentration of 2,5 μg/ml. IFN-γ-PE or IgG2b-PE mAb (isotype control) were used in intracellular staining followed by flow cytometric analysis.

Detection of Antigen-Specific T-Cells with MHC-Dextramers

C57BL/6 mice were immunized i.p. with SIINFEKL peptide (40 μg) plus poly(I:C) (75 μg) with or without the IL-2-poly(HPMA) conjugate (4 μg of IL-2) administered i.p. on the same day and for next 3 consecutive days. Second and third round of immunization was performed in the same schedule and given with 10-day intervals between the courses. Splenocytes were isolated 10 days after the third immunisation and stained with the H-2Kb/SIINFEKL-PE dextramers (Immudex) at 2×10^6 cells per tube according to the manufacturer's protocol. All samples were then incubated for 30 min at 4 °C in the dark with CD3-FITC, CD4-PerCP, and CD8-Horizon V500 mAb in conditions recommended by the manufacturer (Immudex).

Kinetics of IL-2-Poly(HPMA) in Circulation

C57BL/6 mice were injected i.p. with free IL-2 (2 μg) or IL-2-poly(HPMA) conjugate (2 μg of IL-2). Blood from the mice was collected 3 min, 15, and 75 min (for IL-2) and 3 min, 15 min, 1 h, 2, 4, 6, 8, 24

and 48 hours (for IL-2-poly(HPMA)) after the injection and kept on ice. Tubes with blood were left for 30 min at 5 °C, and then repeatedly centrifuged (14 000 rpm, 10 min, 4 °C) to obtain blood sera. Serum IL-2 concentrations were determined by standard sandwich ELISA with anti-mouse JES6.1A12 and anti-mouse JES6.5H4-biotin mAbs. Detection was performed using extravidin-HRP (Sigma) and 3,3′,5,5′-tetramethylbenzidine as a substrate (TMB, Sigma). Absorbance was read for each well with a microplate reader BIOLISA set to 450 nm.

Determination of the Maximum Tolerated DOSE (MTD) of IL-2-Poly(HPMA) Conjugate

B6.SJL mice were injected i.p. with PBS or IL-2-poly(HPMA) (1.25, 2.50 or 3.75 mg/kg of IL-2) on day 1. Weight of each mouse (3 mice/group) was recorded prior to injection (day 0) and each day for next 7 days. Survival was evaluated on day 9. Next, B6.SJL mice were injected i.p. with PBS or IL-2-poly(HPMA) daily for 5 consecutive days (one dose 0.2, 0.3 or 0.4 mg/kg of IL-2) starting on day 1. Weight of each mouse (5 mice/group) was recorded prior to injection (day 0) and each day for next 9 days. Survival was evaluated on day 13.

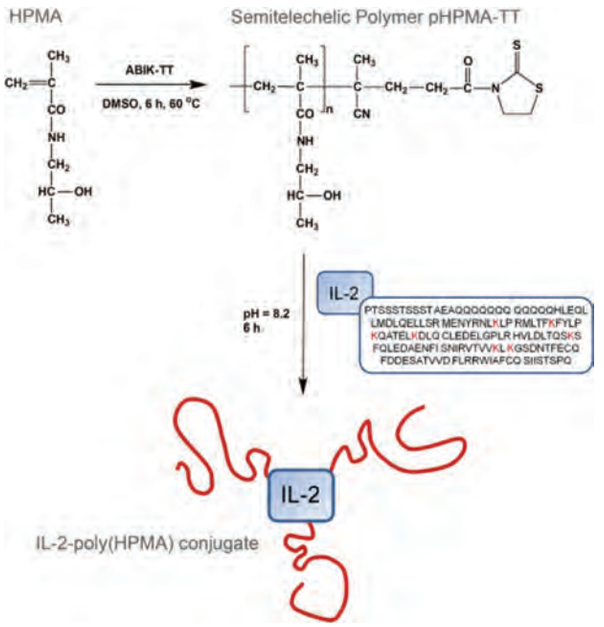
RESULTS

The semitelechelic polymer containing TT reactive groups at the end of polymer chain (polyHPMA-TT) was prepared by radical solution polymerization. The molecular weight of polyHPMA-TT was controlled by concentration of monomer, azo initiator ABIC-TT and polymerization temperature. The amino groups from lysine residues of IL-2 were reacted with semitelechelic polyHPMA-TT forming the IL-2-polyHPMA conjugate in which the polymer chains are attached to IL-2 via a single polymer end-chain amide bond (Scheme 1). The conjugation reaction was carried in buffer solution at pH = 8.2. At these conditions the difference between the rate of aminolysis and hydrolysis of polyHPMA-TT is significantly more pronounced and aminolysis is prevailing.

The IL-2-polyHPMA conjugate was synthesized with the aim to improve the biological activity and pharmacological features of IL-2 *in vivo*. However, we decided first to determine the biological activity of IL-2-poly(HPMA) *in vitro* and to compare it with unmodified IL-2. We used the same batch of IL-2 both for conjugation to poly(HPMA) and as the source of unmodified IL-2 just to make sure that we are starting with the protein of the identical biological activity.

Stimulatory Activity of IL-2-Poly(HPMA) for Activated CD8$^+$ *T* and NK Cells In Vitro

Since IL-2 potently stimulates proliferation of activated T cells and NK cells, we used purified CD8$^+$ T and NK cells from B6 mice as an experimental system. We activated



Scheme 1. Scheme showing a key steps in synthesis of IL-2-poly(HPMA) conjugate. The box at bottom right corner of IL-2 shows the primary amino acid sequence of this cytokine with lysines shown in red. ξ-amino groups of lysine residues are potentially modified with reactive semitelechelic poly(HPMA) precursor (pHPMA-TT).

CD8$^+$ T cells by anti-CD3 mAb and we also added CTLA-4-Ig to cultures to minimize the proliferation background, i.e., the level of proliferation without IL-2, since CD8$^+$ T cells upregulate expression of costimulatory molecule CD86 upon TCR signalling. NK cells do not need any stimulation to be responsive to IL-2. We found that IL-2-poly(HPMA) conjugate was able to stimulate proliferation of both activated CD8$^+$ T and NK cells, albeit at higher concentrations (~30–50 times) than IL-2 *in vitro* (Figs. 1(A) and (D)).

Such decrease of *in vitro* biological activity probably reflects the fact that modification of IL-2 molecule with several poly(HPMA) chains could impede the interaction with IL-2 receptor to some extent. To further investigate this hypothesis, we determined the effect of blocking anti-CD25 and anti-CD122 mAbs in cultures of activated CD8$^+$ T (CD25^{high}) and NK (CD122^{high}) cells stimulated with IL-2 and IL-2-poly(HPMA) conjugate. Both blocking mAbs inhibited stimulatory activity of IL-2-poly(HPMA) conjugate more severely than that of IL-2, except of anti-CD122 mAb in NK cells where complete inhibition was achieved both in IL-2 and IL-2-poly(HPMA) conjugate stimulated cells (Figs. 1(B), (C), (E) and (F)). Thus, modification of IL-2 with poly(HPMA) chains decreased the ability of IL-2 to interact with its receptor subunits, particularly with CD25, and leads to significantly lowered biological activity *in vitro*.

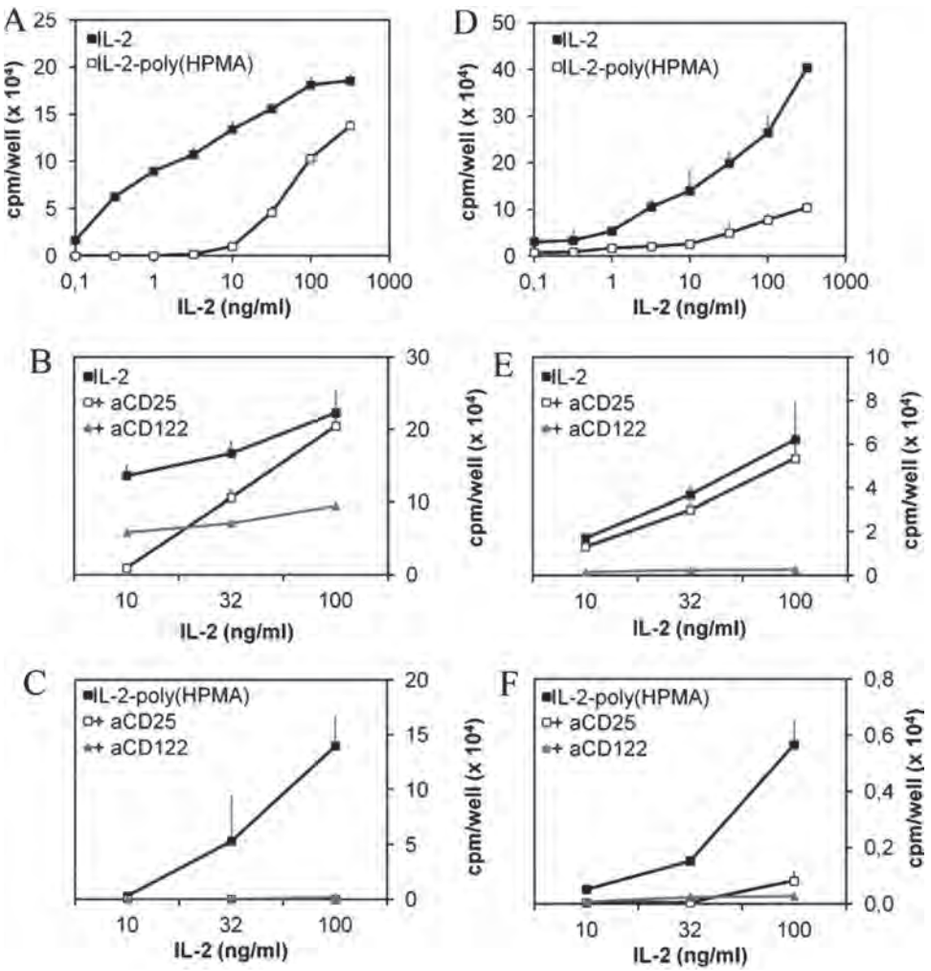


Figure 1. **IL-2 covalently modified by poly(HPMA) is capable of stimulating proliferation of activated CD8$^+$ T cells and NK cells *in vitro*. (A), Purified CD8$^+$ T cells were cultured at 5×10^4 cells per well with anti-CD3 mAb (5 μg/ml), CTLA-4-Ig (1 μg/ml) and IL-2 or IL-2-poly(HPMA) conjugate. (B) and (C), Purified CD8$^+$ T cells were cultured at 6.6×10^4 cells per well under the same cell conditions as in A plus 10 μg/ml anti-CD25 mAb or anti-CD122 mAb. (D), Purified NK cells were cultured at 2.5×10^5 cells per well with IL-2 or IL-2-poly(HPMA) conjugate. (E) and (F), Purified NK cells were cultured at 2.5×10^4 cells per well with IL-2 or IL-2-poly(HPMA) plus 10 μg/ml anti-CD25 mAb or anti-CD122 mAb. Data show mean levels ± SD of [^{3}H]thymidine incorporation for triplicate cultures on day 3. Data are representative of at least two independent experiments with similar results.**

IL-2-Poly(HPMA) Acts as an IL-2 Superagonist *In Vivo*

Next, we asked whether IL-2-poly(HPMA) conjugate also possess a biological activity *in vivo*. We decided to investigate the effect of IL-2-poly(HPMA) in comparison to IL-2 on several subsets of immunocompetent cells which are naturally responsive to IL-2 per se. Thus, we injected naive B6 mice with IL-2 and equivalent dose of IL-2-poly(HPMA) daily for four consecutive days and analyzed spleen cells by flow cytometry one day after the last injection. IL-2-poly(HPMA) conjugate was found to be far more potent than IL-2 (Fig. 2(A)) in terms of expansion of memory CD8$^+$ T cells (CD3$^+$CD8$^+$CD44^{high}CD122^{high}), NK cells (CD3$^-$NK1.1$^+$DX5$^+$), NKT cells (CD3$^+$NK1.1$^+$), $\gamma\delta$ T cells (CD3$^+$$\gamma\delta$TCR$^+$) and T regulatory cells (CD4$^+$Foxp3$^+$; henceforth Treg).

Memory CD8$^+$ T cells and NK cells had very high relative counts in spleen of mice treated with IL-2-poly(HPMA) conjugate, being about 25% and 15% of all splenocytes, respectively (Fig. 2(B)). Dramatic expansion of IL-2 responsive cell subsets was also reflected by considerably increased spleen cellularity in mice treated with IL-2-poly(HPMA) conjugate while spleen cellularity in mice treated with IL-2 remained comparable to PBS treated controls (Fig. 2(C)). Memory CD8$^+$ T, NK and NKT cells increased their absolute numbers in mice treated with IL-2-poly(HPMA) about 90–120 times relatively to controls and 50–90 times relatively to IL-2 treated mice (Fig. 2(D)). Absolute numbers of $\gamma\delta$ T and Treg cells in mice treated with IL-2-poly(HPMA) were increased about

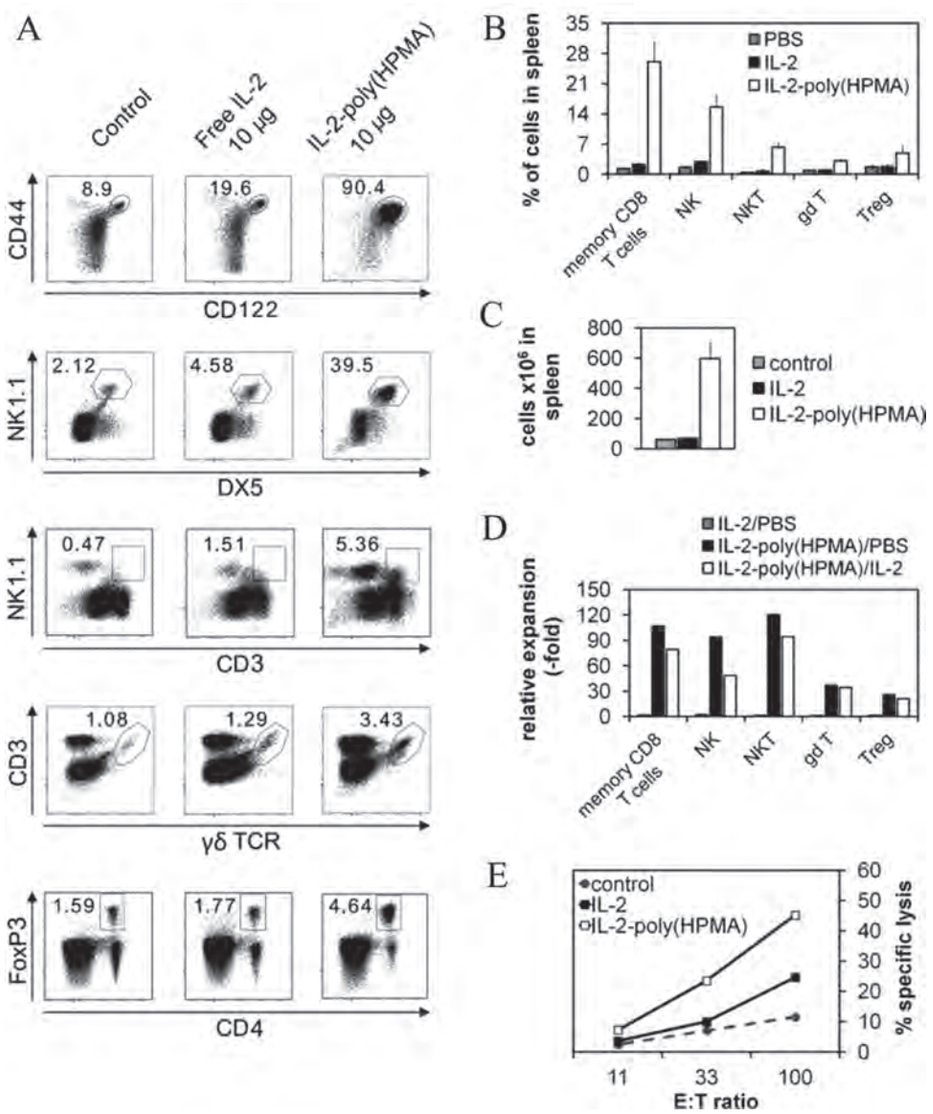


Figure 2. **IL-2-poly(HPMA) conjugate potently drives expansion of memory CD8$^+$ T, NK, NKT, $\gamma\delta$ T, and Treg cells. B6.SJL mice were injected i.p. with PBS (control), IL-2 (10 μg) or IL-2-poly(HPMA) (10 μg of IL-2) daily for 4 days. Mice were euthanized 1 day after last injection and their spleen cells were analyzed. (A), Relative expansion of CD44$^+$CD122$^+$ cell population within CD3$^+$CD8$^+$ cells (memory CD8$^+$ T cells; upper row), NK1.1$^+$DX5$^+$ cell population within CD3$^-$ cells (NK cells; second row from top), CD3$^+$NK1.1$^+$ cell population within all splenocytes (NKT cells; middle row), CD3$^+$$\gamma\delta$TCR$^+$ cell population within all splenocytes ($\gamma\delta$ T cells; second row from bottom) and CD4$^+$Foxp3$^+$ cell population within all splenocytes (Treg cells; bottom row). One representative mouse of 2 mice per each condition is shown. (B), Relative size of cell populations described above in spleen. Average of 2 mice per condition ± SD is shown. (C), Spleen cellularity of mice treated as in (A). Average of 4 mice per condition ± SD is shown. (D), Total numbers of memory CD8$^+$ T, NK, NKT, $\gamma\delta$ T and Treg cells in spleen were calculated. Relative expansion of these cell populations is given as a ratio calculated from their total numbers in each condition (PBS, IL-2 and IL-2-poly(HPMA) treatment). (E), B6.SJL mice were injected i.p. with PBS (control), IL-2 (2 μg) or IL-2-poly(HPMA) (2 μg of IL-2) on day 1. Mononuclear cells (effectors; E) were isolated from spleens on day 6 and incubated with [^{3}H]thymidine-labeled target (*T*) YAC-1 cells at various E:T ratios for 4 h. Lysis of target YAC-1 cells by cytolytic effector cells was determined as loss of radioactivity in the harvested cells relative to appropriate controls. Data are representative of two independent experiments with similar results.**

20–35 times relatively to controls as well as IL-2 treated mice (Fig. 2(D)). We also wanted to address the question whether IL-2-poly(HPMA) is capable of increasing the NK cytolytic activity in spleen cells of treated mice. We injected B6 mice with single dose of IL-2-poly(HPMA) or IL-2 and analyzed cytolytic activity of mononuclear cells isolated from spleens of these mice by JAM assay on day 6. Indeed, cytolytic activity of mononuclear cells from spleen was markedly increased by treatment with IL-2-poly(HPMA) but only slight increase was observed

after the treatment with IL-2 (Fig. 2(E)). Thus, based on much higher stimulatory activity to expand naturally IL-2 responsive immunocompetent cell populations, we can conclude that IL-2-poly(HPMA) acts as IL-2 superagonist *in vivo*.

IL-2-Poly(HPMA) Enormously Potentiates CD8+ *T* Cell Response

We demonstrated above that IL-2-poly(HPMA) conjugate possesses much higher stimulatory activity than IL-2 to expand naturally IL-2 responsive immune cell subsets *in vivo*. We further asked whether IL-2-poly(HPMA) has also such a potent stimulatory activity for activated T cells. Thus, we employed adoptive transfer of purified and CFSE-labeled OT-I $CD8^+$ T cells from $Ly5.2^+$ OT-I mice into congeneic $Ly5.1^+$ mice. OT-I $CD8^+$ T cells were then selectively activated by injection of SIINFEKL peptide one day post transfer followed by daily treatment with IL-2-poly(HPMA) conjugate or IL-2 for four days. The IL-2-poly(HPMA) conjugate exhibited extremely high efficacy to stimulate the proliferation (Fig. 3(A)) and expansion (Fig. 3(B)) of activated OT-I $CD8^+$ T cells, while IL-2 showed practically no activity even at 10 μg IL-2/dose. Moreover, OT-I $CD8^+$ T cells expanded by injection of SIINFEKL peptide plus IL-2-poly(HPMA) conjugate showed high expression of CD25 as long as 5 days after activation, whereas OT-I $CD8^+$ T cells expanded by injection of SIINFEKL peptide plus poly (I:C) were $CD25^{low}$ (Fig. 3(A)).

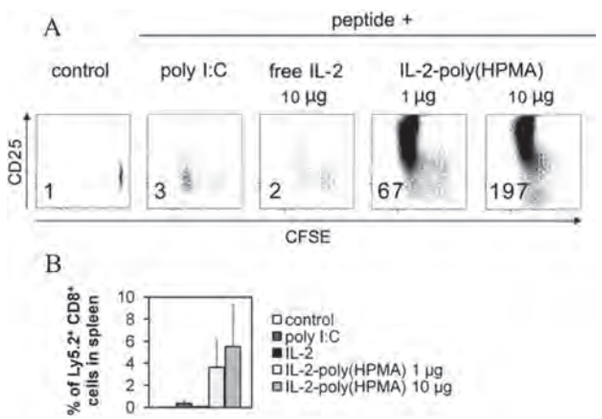


Figure 3. IL-2-poly(HPMA) conjugate has dramatically increased potential to expand activated $CD8^+$ T cells in comparison to IL-2 *in vivo*. Purified OT-I $CD8^+$ T cells (Ly5.2) were labeled with CFSE and injected i.v. into congeneic B6.SJL recipients (Ly5.1) at 1.2×10^6 cells per mouse on day 1. On day 2, the mice were injected i.p. with PBS (control), SIINFEKL peptide (2 nmol) plus poly (I:C) (75 μg), SIINFEKL peptide plus IL-2 (10 μg) or SIINFEKL peptide plus IL-2-poly(HPMA) (1 μg or 10 μg of IL-2). IL-2 and IL-2-poly(HPMA) were injected i.p. also on days 3, 4, and 5. (A), CFSE dilution, CD25 expression and expansion of $Ly5.2^+CD8^+$ T cells relative to control (bottom left corner of each dot plot) in spleen were analyzed 1 day after last injection. One representative mouse of 2 mice per each condition is shown. (B), Relative counts of $Ly5.2^+CD8^+$ cells in spleens of experimental mice. Average of 2 mice per condition ± SD is shown. Data are representative of at least two independent experiments with similar results.

Expansion of activated T cells is a key process for primary T cell response; however, the complete T cell response should to be finalized by the establishment of long-lived functional memory T cells which provide rapid and efficient immune response in the case of second antigen encounter. Thus, we asked whether activated $CD8^+$ T cells massively expanded by IL-2-poly(HPMA) conjugate are able to establish memory $CD8^+$ T cells which would persist in the organism for long time. We used the same experimental system as above, i.e., adoptive transfer of purified OT-I $CD8^+$ T cells from $Ly5.2^+$ OT-I mice into congeneic $Ly5.1^+$ mice and activation of transferred cells by injection of SIINFEKL peptide. IL-2-poly(HPMA) conjugate or IL-2 was injected only once together with SIINFEKL peptide, but at significantly higher dose (50 μg IL-2) than in previous experiments. The reason was that such schedule is much more convenient for the potential use of IL-2-poly(HPMA) as adjuvant for boosting T cell-based vaccination and we thus wanted to explore whether this schedule of administration is effective. OT-I $CD8^+$ T cells expanded only slightly in mice treated with SIINFEKL peptide plus IL-2 in comparison to mice treated with peptide alone (~3 times) on day 4 post treatment (Fig. 4(A)).

Nevertheless, OT-I $CD8^+$ T cells were able to form long-lived cell population in mice treated with SIINFEKL peptide plus IL-2 albeit the population was very tiny on day 50. On the other hand, OT-I $CD8^+$ T cells dramatically expanded in mice treated with SIINFEKL peptide plus IL-2-poly(HPMA) in comparison to mice treated with peptide alone (more than 100 times) on day 4 post treatment. Almost 10% of all splenocytes were OT-I $CD8^+$ T cells on day 4 and these cells formed very distinguishable population of long-lived cells with phenotype of memory $CD8^+$ T cells on day 50, i.e., most of them were $CD44^{high}CD122^{high}$ (Figs. 4(A) and (B)).

To elucidate whether memory OT-I $CD8^+$ T cells formed in mice injected with SIINFEKL peptide plus IL-2-poly(HPMA) conjugate are also functional in terms of rapid expression of effector functions upon TCR signal, we isolated spleen cells from these mice on day 50, cultivated them with SIINFEKL peptide *in vitro* and analyzed their intracellular expression of IFN-γ by flow cytometry. Indeed, nearly all memory OT-I $CD8^+$ T cells isolated from mice treated with SIINFEKL peptide plus IL-2-poly(HPMA) conjugate expressed high levels of IFN-γ upon stimulation with SIINFEKL peptide *ex vivo* (Fig. 4(C)). Thus, we have shown that IL-2-poly(HPMA) conjugate is extremely potent in driving the expansion of antigen-activated $CD8^+$ T cells even if administered only in one dose together with the antigen and that such expanded $CD8^+$ T cells form robust population of functional memory CD8+ T cells.

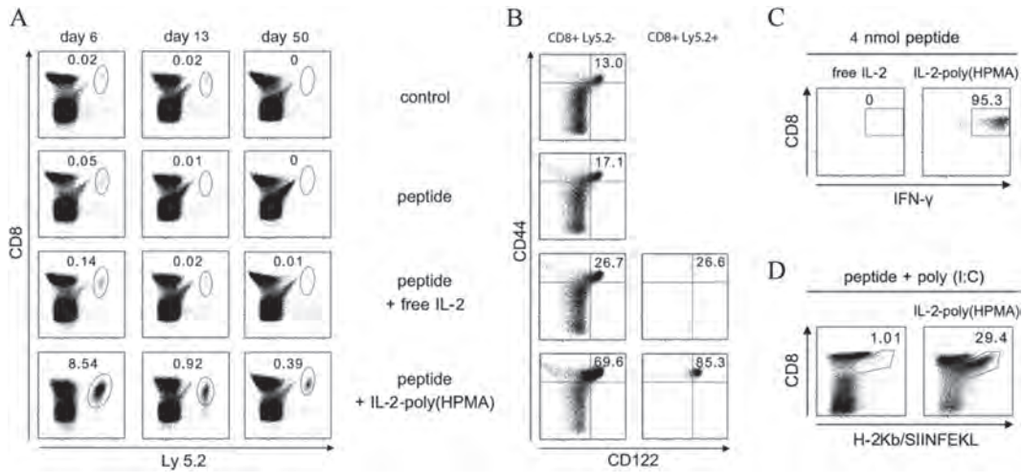


Figure 4. **Activated CD8$^+$ T cells expanded by the IL-2-poly(HPMA) conjugate establish a robust population of functional memory cells. Purified OT-I CD8$^+$ T cells (Ly5.2) were injected i.v. into B6.SJL recipients (Ly5.1) on day 1. Mice were injected i.p. with PBS (control), 4 nmol SIINFEKL peptide (peptide), SIINFEKL peptide plus IL-2 (50 μg), or SIINFEKL peptide plus IL-2-poly(HPMA) conjugate (50 μg of IL-2) on day 2. (A), Counts of Ly5.2$^+$ CD8$^+$ T cells relative to all splenocytes were assessed on days 6, 13 and 50 (upper part of each dot plot, in %). (B), Host and transferred CD8$^+$ T cells (left and right column, respectively) were stained for CD44 and CD122 on day 50. (C), On day 50, spleen cells were stimulated by SIINFEKL peptide for 6 h *ex vivo.* Brefeldin A was added for last 4 h, and expression of IFN-γ was determined in Ly5.2$^+$CD8$^+$ cells. (D), C57BL/6 mice were immunized i.p. with SIINFEKL peptide (40 μg) plus poly(I:C) (75 μg) with or without the IL-2-poly(HPMA) conjugate (4 μg of IL-2) administered i.p. on the same day and for next 3 consecutive days. Second and third round of immunization was performed in the same schedule and given with 10-day intervals between the courses. Splenocytes were isolated 10 days after the third immunization and stained with the H-2Kb/SIINFEKL-PE dextramers. Live CD3$^+$ cells are plotted. One representative mouse of 2 mice per each condition is shown. Data are representative of at least two independent experiments with similar results.**

Since previous experiments on stimulatory activity of IL-2-poly(HPMA) conjugate were done using OT-I transgenic system, i.e., by using monoclonal CD8$^+$ T cells, we decided to prove the potential of IL-2-poly(HPMA) to dramatically enhance CD8$^+$ T cell responses also in polyclonal model, i.e., in B6 mice. We injected B6 mice with SIINFEKL peptide plus poly I:C and either treated mice with IL-2-poly(HPMA) for four days or not. This immunization was repeated twice with 10 day gap. Spleen cells isolated from the mice 10 days after the last immunization were analyzed by flow cytometry and SIINFEKL-specific CD8$^+$ T cells were identified by H-2Kb/SIINFEKL-PE dextramers. Almost one third of CD8$^+$ T cells were SIINFEKL-specific in mice where immunization with SIINFEKL peptide plus poly I:C was boosted by IL-2-poly(HPMA) while very low counts of SIINFEKL-specific CD8$^+$ T cells were found in mice immunized with SIINFEKL peptide plus poly I:C only (Fig. 4(D)). Thus, we clearly showed that very high potential of IL-2-poly(HPMA) conjugate to boost CD8$^+$ T cell immune response is not limited to monoclonal OT-I experimental model but it works also in wild type B6 mice.

IL-2-Poly(HPMA) Conjugate has much Longer Half-Life in Circulation than IL-2

IL-2 is reported to be rapidly eliminated from the circulation upon i.v. injection with elimination half-life of 3 to 13 min.[2,5] Since IL-2-poly(HPMA) conjugate has significantly higher molecular weight than IL-2, we wanted to determine the half-life of IL-2-poly(HPMA) in circulation upon i.v. administration. Thus, we established ELISA for detection of IL-2 and IL-2-poly(HPMA) in mouse serum with detection limit 0,004 ng IL-2/ml and 0,006 ng IL-2/ml, respectively (see Material and Methods).

As expected, IL-2 was rapidly eliminated from the circulation after i.v. administration (Fig. 5). We found just very low concentration of IL-2 (60 ng/ml) in mouse serum as early as 3 min after i.v. injection of 2 μg IL-2 and IL-2 was undetectable in serum 75 min after the administration. IL-2-poly(HPMA) conjugate was detected in serum even 48 h after i.v. injection of 2 μg IL-2 equivalent and with concentration actually higher than that determined 15 min after injection of IL-2. Moreover, there is a remarkably high concentration of IL-2-poly(HPMA) in serum (almost 400 ng IL-2/ml) which is stable for at least 1 h post administration. We determined the half-life of IL-2-poly(HPMA) in circulation to be approximately 4 h. In other words, modification of IL-2 with poly(HPMA) chains prolonged the half-life of IL-2 in circulation nearly 50 times.

Maximal Tolerated DOSE of IL-2-Poly(HPMA) Conjugate

Next, we decided to estimate the maximal tolerated dose (MTD) of IL-2-poly(HPMA) to further characterize the pharmacological features of this conjugate. We defined the MTD as highest possible dose which causes no mortality

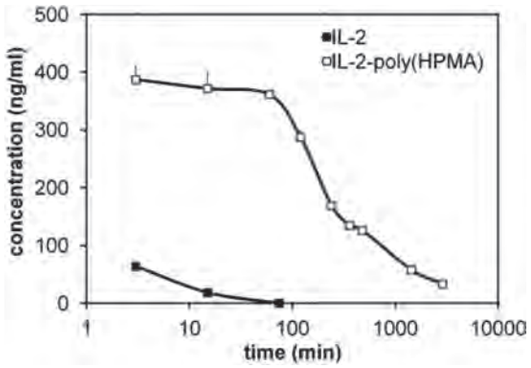


Figure 5. **Short half-life of IL-2 in circulation upon i.v. administration is enormously extended when IL-2 is modified with poly(HPMA). C57BL/6 mice were i.v. injected with IL-2 (2 μg) or IL-2-poly(HPMA) conjugate (2 μg of IL-2). IL-2 concentrations were determined in sera isolated 3, 15, and 75 min after IL-2 administration and 3 min, 15 min, 1, 2, 4, 6, 8, 24 and 48 h after IL-2-poly(HPMA) administration. Concentration of IL-2 in serum was determined by sandwich ELISA with anti-mouse IL-2 mAb JES6.1A12 as capture antibody and anti-mouse IL-2 mAb JES6.5H4 labeled with biotin as detecting mAb. Data presented are average of 2 mice per each condition ± SD. Data are representative of three independent experiments with similar results.**

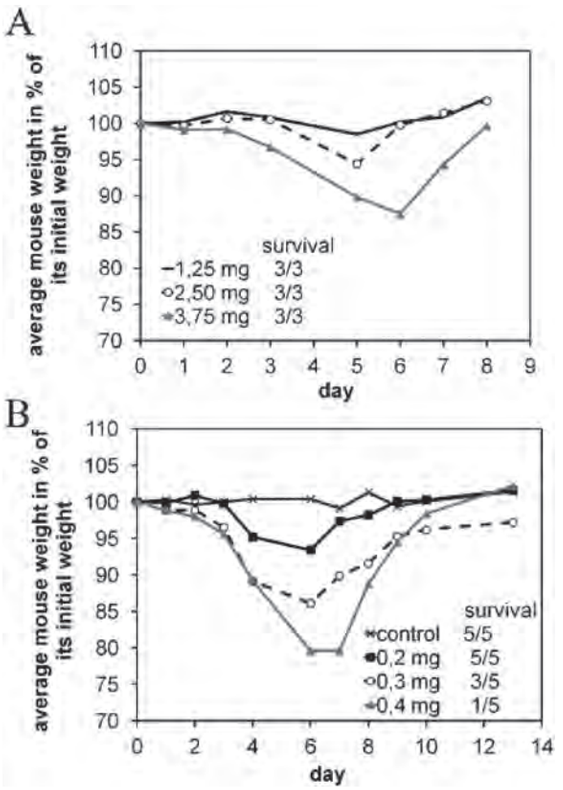


Figure 6. **Maximum tolerated dose of IL-2-poly(HPMA) after i.p. administration of single dose or five daily doses. A, B6.SJL mice were injected i.p. with IL-2-poly(HPMA) conjugate (1.25, 2.50 or 3.75 mg/kg of IL-2) on day 1. Body weight of each mouse (3 mice per group) was recorded prior to injection (day 0) and then daily up to day 8. B, B6.SJL mice were injected i.p. with PBS (control) or IL-2-poly(HPMA) (one dose 0.2, 0.3 or 0.4 mg/kg of IL-2) on days 1, 2, 3, 4, and 5. Weight of each mouse (5 mice per group) was recorded prior to injection (day 0) and then daily up to day 13. Survival of mice in each group is indicated in graph legend.**

and weight loss not higher than 15%. Titrated doses of IL-2-poly(HPMA) administered as single i.p. injection showed that MTD of the conjugate is approximately 3.75 mg IL-2/kg (Fig. 6(A)). Such dose corresponds to about 75 μg IL-2 per mouse showing that we used IL-2-poly(HPMA) still bellow MTD in experiments analyzing the potential of the conjugate to boost $CD8^+$ T cell immune response.

Finally, we determined the MTD of IL-2-poly(HPMA) when given i.p. daily for 5 consecutive days. Of note, IL-2-poly(HPMA) showed to be much more toxic when using this administration schedule in comparison to single bolus injection. MTD was found to be 0.2 mg IL-2/kg per dose, i.e., cumulative total dose 1 mg IL-2/kg (Fig. 6(B)). Even slightly higher dosage 0.3 mg IL-2/kg per dose, i.e., cumulative total dose 1.5 mg IL-2/kg was lethal for 2 out of 5 experimental mice. This finding further supports the use of IL-2-poly(HPMA) in single high dose as an adjuvant for potentiation of T cell-based vaccines.

DISCUSSION

Cytokines are key immunomodulatory agents that shape immune responses. Our growing understanding of the biological basis of immunity and of ways in which this can potentially be affected ultimately led to the use of recombinant cytokines as a new approach to immunotherapy. IL-2 has potent stimulatory effect on T and NK cells and also some other cells of the immune system and IL-2 immunotherapy has been shown to be beneficial in a variety of clinical trials.[41–44] However, the major problem of this immunotherapeutic approach is the toxicity of high dosage IL-2 therapy and the resultant decrease in curative effect with low doses of IL-2.[7, 45, 46]

Conjugation of IL-2 to carrier molecules such as PEG or albumin and thereby increasing the hydrodynamic radius of IL-2, which leads to a decrease in renal elimination, and thus increasing the half-life of IL-2 upon parenteral administration, has revealed as a promising approach.[9–14, 21] We have explored an alternative strategy of using poly(HPMA) as polymeric carrier for IL-2 to create a new drug with improved pharmacological properties. HPMA homopolymers and copolymers have been proposed for a wide variety of biomedical applications. Initially they were explored as biomaterials and then as drug delivery systems; both hydrogels and polymer-drug conjugates have been explored. The latter became a particular focus of attention.[47–49] HPMA homopolymers and copolymers are being currently explored in a diverse range of applications including their proposed use as coatings for

viruses[50], promotion of sub-cellular organelle (mitochondrial) targeting,[51–51] and as second-generation anticancer conjugates containing combination therapy.[53, 54] Our decision to choose poly(HPMA) was based mainly on our data indicating that proteins modified with HPMA polymer are less immunogenic than proteins modified with PEG polymer[33–35] and that the activity of enzymes is not dampened after their conjugation to HPMA copolymers.[36]

The *in vitro* results showed that IL-2-poly(HPMA) conjugate promotes the proliferation of activated $CD8^+$ T cells and NK cells and it competes with blocking anti-CD25 and anti-CD122 mAbs for binding to IL-2 receptor. *In vitro*, the biological activity of IL-2-poly(HPMA) conjugate was about 30–50 times lower than that of IL-2 which is, however, consistent with results obtained with PEG or albumin protein conjugates.[21, 55] This probably reflects the fact that modification of IL-2 molecule with several poly(HPMA) chains could impede the interaction with IL-2 receptor to some extent. However, the decreased *in vitro* potency of IL-2-poly(HPMA) was far outweighed by the significant increase in the half-life of circulating conjugate *in vivo*, which yielded a greater biological activity *in vivo*.

The striking differences in the pharmacokinetic characteristics of IL-2-poly(HPMA) and IL-2 is approximately 50-fold difference in the elimination half-life following i.v. injection. As expected[2, 5], IL-2 was rapidly cleared from the blood upon i.v. administration. In contrast, IL-2-poly(HPMA) had a much more prolonged circulation in blood, with a remarkably high concentration which was stable for at least 1 h post administration. This new approach for developing long-acting IL-2 thus showed to be more successful than that creating IL-2-PEG or human IL-2-albumin fusion protein (Albuleukin).[9, 11, 21]

So far, other studies have solely described the pharmacokinetics of PEG- or albumin-modified IL-2 and efficacy of the treatment in various tumor models *in vivo*.[9, 11, 12, 16, 17, 19, 21, 56, 57] We are, to our knowledge, the first group showing the data concerning the changes in biological attributes of IL-2 after modification with polymer carrier. First of all, we found that the IL-2-poly(HPMA) conjugate possesses extremely high stimulatory activity *in vivo* as shown by strong expansion of memory $CD8^+$ T cells, NK, NKT, $\gamma\delta$ T cells and T regulatory cells, i.e., cell subsets naturally responsive to IL-2. Actually, memory $CD8^+$ T cells and NK cells ($CD122^{high}$ cells) showed up to be even more sensitive to IL-2-poly(HPMA) conjugate than other cells which was reflected by their very high relative counts in spleen of mice treated with IL-2-poly(HPMA) conjugate. Such a high expansion of NK cells could be useful in cancer immunotherapy, especially in case of tumors that are known to express low levels of MHC class I. However, these expanded NK cells must retain their cytolytic activity. Indeed, we found that NK cell activity of spleen cells isolated from mice injected with IL-2-poly(HPMA) conjugate, detected as killing of YAC-1 cells by JAM assay, was significantly higher than those isolated from control mice or from mice injected with the same dose of IL-2.

The above mentioned ability of IL-2-poly(HPMA) conjugate to induce superior expansion of IL-2-responsive cells could be beneficial in various immunotherapeutic interventions. First of all, IL-2 administered as consolidating immunotherapeutic agent early after allogenic HSCT at a time of minimal residual disease might reduce the relapse rate and increase the immunocompetence status of these patients. Exogenous IL-2 might thus lead to an enhancement of the autologous GVL effect.[58] There are currently several clinical trials just completed or still ongoing in the allogeneic HSCT setting and it is believed that these will define the use of IL-2 (e.g., http://ClinicalTrials.gov identifiers NCT00003962, and NCT00941928). In addition to that, studies to identify antigen-nonspecific strategies for enhancing immune reconstitution in individuals with HIV infection include those focusing on the use of IL-2.[59]

We further showed that IL-2-poly(HPMA) conjugate can be used *in vivo* to strongly expand activated naive $CD8^+$ T cells and that such expanded cells are able to form a robust population of long-lived memory cells which are functional in terms of effective IFN-γ production upon re-activation. Notably, this report is the first describing the powerful stimulatory activity of a modified form of IL-2, the IL-2-poly(HPMA) conjugate in this case, for activated naive $CD8^+$ T cells. These results suggest that IL-2-poly(HPMA) conjugate could be used to potently boost $CD8^+$ T cell responses and thus it could significantly improve vaccination protocols aimed to trigger responses mediated by cytotoxic T lymphocytes (CTL). Indeed, we further demonstrated that three immunizations with antigen given together with IL-2-poly(HPMA) conjugate resulted in markedly increased number of antigen-specific $CD8^+$ T cells. Therefore, especially low immunogenic vaccines, which are known to cause weak immunostimulation and thus provide only low or short-lasting protection should benefit from being coadministered with IL-2-poly(HPMA) conjugate. Based on the above mentioned findings, we assume that the use of IL-2-poly(HPMA) as an adjuvant should receive a considerable attention. We also hypothesize that IL-2-poly(HPMA) conjugate would improve vaccination also in the case in which $CD4^+$ T cells or B cells play the main role. However, this question remains to be verified in appropriate experiments.

CONCLUSION

Our results show improved pharmacological features of IL-2 covalently conjugated to poly(HPMA) carrier and they also provide a detailed view of the superior biologic activity of the IL-2-poly(HPMA) conjugate. To our knowledge, this is the first proof-of-concept report of the use of polymer/protein modification of IL-2 to obtain more pronounced biological activity. We believe that our results

provide a rationale for the use of IL-2-poly(HPMA) in various immunological interventions.

General Disclosure Statement

The authors have nothing to disclose.

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