**Charles University in Prague**

**Faculty of Science**


**RNDr. Jakub Tomala**

# Immunocomplexes of IL-2 and anti-IL-2 mAbs as a novel class of selective and extremely potent immunostimulators

# Imunokomplexy IL-2 a anti-IL-2 monoklonálních protilátek jako nová třída selektivních a extrémně účinných imunostimulátorů

*PhD Thesis in Immunology*

Prague, 2012

Supervisor: RNDr. Marek Kovář, PhD.

**Prohlášení**

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Táto práce ani žádná její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 14.12.2012

Podpis

**Declaration**

I declare that I prepared this thesis solely on my own with all information sources and literature cited. I did not use either this thesis or its substantial part as a background to obtain another or equivalent academic degree.

Prague, 14.12.2012

Signature

*First of all, I would like to express my gratitude to Prof. RNDr. Blanka Říhová, DrSc. and RNDr. Marek Kovář, PhD., my supervisor and mentor, for all the support, help and advice. My thanks also belong to all members of Laboratory of Tumor Immunology at the Institute of Microbiology ASCR for their collaboration. Special thanks to Bc. Barbora Dvořáková for reading of the manuscript.*

*The work would not be possible without the cooperation of the team of Prof. Ing. Karel Ulbrich, DrSc. from the Laboratory of Biomedicinal Polymers at the Institute of Macromolecular chemistry ASCR. Special thanks to all the collaborators from this team for preparation of the polymer prodrugs and expertise.*

*Last, but not least, I would like to thank my family for endless support, encouragement and tolerance.*

## Table of contents

## List of abbreviations

| | |
|---|---|
| Ab | Antibody |
| AICD | Activation-induced cell death |
| AFP | Alpha-fetoprotein |
| APC | Antigen presenting cell |
| ART | Antiretroviral therapy |
| CA | Carcinoma antigen |
| CD | Cluster of differentiation |
| CEA | Carcinoembryonic antigen |
| CFA | Complete Freund´s adjuvant |
| CRP | C-reactive protein |
| CTLA-4 | Cytotoxic T lymphocyte antigen 4 |
| DC | Dendritic cell |
| DN | Double negative |
| DNAM-1 | DNAX Accessory Molecule-1, CD226 |
| Dox | Doxorubicin |
| DP | Double positive |
| EAE | Experimental autoimmune encephalomyelitis |
| EAMG | Experimental Myasthenia gravis |
| EPR | Enhanced permeability and retention |
| Fab | Fragment antigen-binding |
| Fc | Fc receptor |
| FcRn | Neonatal Fc receptor |
| FDA | Food and Drug Administration |
| Foxp3 | Forkhead box p3, scurfin |
| GFLG | Enzymatically cleavable tetrapeptide of Glycin-Phenylalanin-Leucin-Glycine |
| GITR | Glucocorticoid-induced TNFR-related protein |
| gp130 | Glycoprotein 130, CD130 |
| h | Human |
| HIV | Human immunodeficiency virus |
| HMW | High molecular weight |
| HPLC | High pressure liquid chromatography |
| HPMA | *N*-(2-hydroxypropyl)methacrylamide |
| i.p. | Intraperitoneally |
| i.v. | Intravenously |
| ICAM-1 | Intracellular adhesion molecule 1, CD54 |
| ICOS | Inducible T cell costimulator, CD278 |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-2R | Interleukin 2 receptor |
| IL-2Rα | α subunit of Interleukin 15 receptor, CD25 |
| IL-4R | Interleukin 4 receptor |
| IL-7R | Interleukin 7 receptor |
| IL-15R | Interleukin 15 receptor |
| IL-15Rα | α subunit of Interleukin 15 receptor, CD215 |
| JAK | Janus kinase |
| $K_d$ | Dissociation constant |

| | |
|---|---|
| KO | Knock-out |
| LAK | Lymphokine-activated killer |
| LMW | Low molecular weight |
| LN | Lymph nodes |
| m | Mouse |
| mAb | Monoclonal antibody |
| MDR | Multi-drug resistance |
| MG | Myasthenia gravis |
| MHC | Major histocompatibility comlex |
| MOG | Myelin oligodendrocyte glycoprotein |
| mRNA | Messenger ribonucleic acid |
| MS | Multiple sclerosis |
| MTD | Maximum tolerated dose |
| Mw | Molecular weight |
| NK | Natural killer |
| NKT | Natural killer T |
| $NO_2^-$ | Nitrite |
| NSCLC | Non-small cell lung carcinoma |
| NSE | Neuron Specific Enolase |
| PD-1 | Programmed cell death 1 |
| PDT | Photodynamic therapy |
| PEG | Polyethylene glycol |
| $PGE_2$ | Prostaglandin E2 |
| pHPMA | Polymeric HPMA |
| PNA | Peanut agglutinin |
| PR | Partial response |
| pSTAT | Phosporylated STAT |
| rh | Recombinant human |
| rm | Recombinant mouse |
| s | Soluble |
| s.c. | Subcutaneously |
| SAA | Serum amyloid A |
| SCID | Severe combined immunodeficiency disease |
| STAT | Signal transducer and activator of transcription |
| $T_c$ | T cytotoxic |
| TCGF | T cell growth factor, IL-2 |
| TCR | T cell receptor |
| TGF | Tumor growth factor |
| $T_h$ | T helper |
| $T_{reg}$ | T regulatory |
| VLS | Vascular leak syndrome |
| WGA | Wheat germ agglutinin |

# I. INTRODUCTION

IL-2 was the first interleukin discovered (1965) under the name blastogenic factor, followed by term describing his primary and the most apparent function - T cell growth factor (TCGF).

IL-2 is a small protein, approximately 15 kDa, and has wide variety of functions in the immune system, mostly as the prototypic autocrine growth factor produced by T cells after activation. This has led to exploiting IL-2 as a potential therapeutic agent. In 1984, recombinant IL-2 was used in cancer therapy for the first time, with novel roles in immune system slowly emerging after its FDA approval. IL-2 was tested not only for cancer immunotherapy, but also for other applications like treatment of chronic viral infections or as an adjuvant for vaccines.

Despite its positive effects, being 5-17% of complete responses in patients with malignant melanoma and renal cell carcinoma, treatment with IL-2 is rather difficult due to its severe side effects, ranging from flu-like symptoms to debilitating cytokine storm and vascular leak syndrome. These toxicities, associated with high-dose treatment necessary for IL-2 to function, have been found the most limiting factor for IL-2 applications.

There have been several efforts to manage or diminish those “limiting” toxicities. One approach was based on gene therapy, where tumor cells were transfected to produce IL-2, thus becoming more immunogenic. Other approaches came with the idea of a targeting strategy with immunocytokine (Ab-cytokine fusion protein) or alternatively with ligation of IL-2 to polymeric carrier (PEG) or serum protein (albumin) to increase its half-life in blood.

In 2006, it was found that particular anti-IL-2 monoclonal antibodies (mAb) can actually increase biological activity of IL-2 rather than block it. Binding of IL-2 to anti-IL-2 mAb creates a superagonistic immunocomplexes which have dramatically higher biological activity than IL-2 alone *in vivo.* Such approach may finally overcome the difficulties associated with administration of IL-2, thus opening brand new scopes for IL-2 and its application not only in the field of tumor therapy.

However, curative effect is hardly achievable by immunotherapy alone in case of tumors. To maximalize the benefits of treatment offered by immunotherapy, the best way is to combine immunotherapy with some conventional methods, particularly with chemotherapy.

Considering chemotherapy, radiotherapy or surgery, the classical trio in tumor treatment, chemotherapy is indispensable when the disease is systemic, i.e. in the case of metastatic spread. Unfortunately, conventional chemotherapy has considerable limitations due to the serious side-toxicities associated with the use of cytostatic drugs. Moreover, most cytostatic drugs are significantly immunosuppressive and thus they can damage immune system, leading to significant lowering of effectiveness of immunotherapy applied after chemotherapy.

One possible way how to overcome this obstacle is to covalently bind cytostatic drug to a polymeric carrier from which it can be released into its pharmacologically active form preferentially in tumor. Such macromolecular conjugates possess more favorable pharmacokinetic profile and considerably lower side-toxicities than parent free drug.

One of the most widely and successfully used polymeric carrier for cytostatic drugs is based on pHPMA, referring to the polymer consisting of repetitive *N*-(2-hydroxypropyl)methacrylamide (HPMA) units. Originally developed as a plasma expander "Duxon", it possesses almost ideal properties as a drug carrier. pHPMA is biocompatible, non-toxic and is excreted via kidney up to the size of about 40 kDa. When cytostatic drug, for example anthracycline antibiotic doxorubicin (Dox), is conjugated to HPMA copolymers, the conjugate has higher anti-tumor activity *in vivo* in comparison to free Dox. Such conjugates are able to completely cure mice with established syngeneic tumors and ensure establishment of protective and long-lasting anti-tumor immune memory, thus providing resistance to original tumor. The very first experience with application of this class of anti-cancer therapeutics in clinical trials also documented much lower hemato- and immunotoxicity and even activation of some parts of immune system was recorded.

Therefore, immunotherapy with IL-2/anti-IL-2 immunocomplexes combined with chemotherapy with HPMA-based drug conjugates could be a promising approach for tumor therapy.

## I.1. CYTOKINE/ANTI-CYTOKINE MAB IMMUNOCOMPLEXES

Increasing the biological activity of cytokines *in vivo* via binding to the corresponding anti-cytokine mAb is not a unique phenomenon restricted to IL-2. IL-3, IL-4, IL-6 and IL-7 complexed with relevant anti-cytokine antibody were shown to possess superior activity in comparison to free cytokine. IL-5 was also reported to fall into this category but very limited data is available [1].

Until now, all cytokines which clearly showed increased biological activity upon complexing with the respective anti-mAb share similar signaling molecules, as they all bind to the type I cytokine (hematopoetin) receptor family. These cytokine receptors are dimers (IL-3, IL-4, IL-6, IL-7) or trimers (IL-2) that typically consist of unique binding chains and one or more signal-transduction chains, which are often shared among other cytokine receptors. IL-2, IL-4 and IL-7 share a common $\gamma_c$ family subunit (CD132), while IL-3 and IL-6 belong to a subgroup sharing a common $\beta$ chain subunit (CD131) and common gp130 subunit, respectively. All type I cytokine receptors engage JAK-STAT signaling pathways.

Increase of biological activity mentioned above is only manifested *in vivo*. *In vitro*, free cytokine is equally or more potent then its mAb-complexed counterpart in all cases [2-3]. There are several reasons for this phenomenon - some important features of cytokines/anti-cytokine mAb complexes (e.g. prolonged circulation half-life) simply do not play a role *in vitro.*

### I.1.1. Biology of IL-2, its receptors and target populations

Interleukin-2 (IL-2) possesses a wide range of roles in modulating immune system. It is an autocrine (and also a paracrine) soluble factor produced mainly by activated T cells and it is critical factor for T cell proliferation, effector responses and establishment of T cell memory. It is vital for regulation of immune reactions in terms of generation, expansion and survival of regulatory T cells ($T_{reg}$) in thymus and periphery and also takes part in activation-induced cell death (AICD). IL-2 plays also a role in proliferation and cytolytic activity of natural killer (NK) cells [4].

Activity of IL-2 is mediated via binding to IL-2 receptor (IL-2R), which may exist in two distinct variants - dimeric and trimeric IL-2R. Dimeric receptor, which is also receptor for IL-15, is composed of β (CD122) and common $\gamma_c$ (CD132) subunit and binds IL-2 with intermediate affinity ($K_d=10^{-9}$). Together with "private" α subunit (CD25, IL-2Rα), complete trimeric high affinity IL-2R complex ($K_d=10^{-11}$) is formed. Complex of $\beta\gamma_c$ is responsible for downstream signaling in both types of IL-2 receptor. CD25, which does not take part on signal transduction, can also bind IL-2 but with low affinity ($K_d=10^{-8}$) [5]. Expression of CD25 is triggered by activation of T cell. As systemic level of IL-2 is too low to be utilized by dimeric IL-2 receptor, biological effects of IL-2 are mainly delivered by its binding to the high affinity trimeric receptor. Upon IL-2R triggering, T cells phosphorylate STAT 5, which is an important transcription factor in IL-2 signaling cascade.

## I.1.2. IL-2/anti-IL-2 mAb immunocomplexes

Describing IL-2/anti-IL-2 complexes in 2006 [2], sometimes referred to as IL-2 immunocomplexes [6], was important break-through event for use of cytokines in therapy and/or vaccination. Although there were previous studies showing that antibodies against rhIL-2 may act as a carrier protein and prolong its serum half-life and biological activity [7-8] and IL-2 immunocomplexes were definitely not the first ones described [9], the study of Boyman and his co-workers convincingly proved the idea of biological potentiation of cytokine via binding to respective anti-cytokine antibody and, moreover, this study demonstrated that IL-2 immunocomplexes possess not only higher but also selective stimulatory activity for various IL-2 responding cell subsets.

The study of Boyman *et al.* also resolved a long-time puzzle in T cell immunity, where administration of anti-IL-2 mAb S4B6 leads to very selective transient proliferation and expansion of memory $CD8^+$ T cells. This effect was explained by several different mechanisms [10-12], however, the study of Boyman *et al.* finally showed that all these explanations were completely incorrect [2].

Two basic types of IL-2 immunocomplexes and their distinctive features in terms of structure and effect on target cell subpopulations, based on the different mAb used for complexing IL-2, were described (Fig. 1).

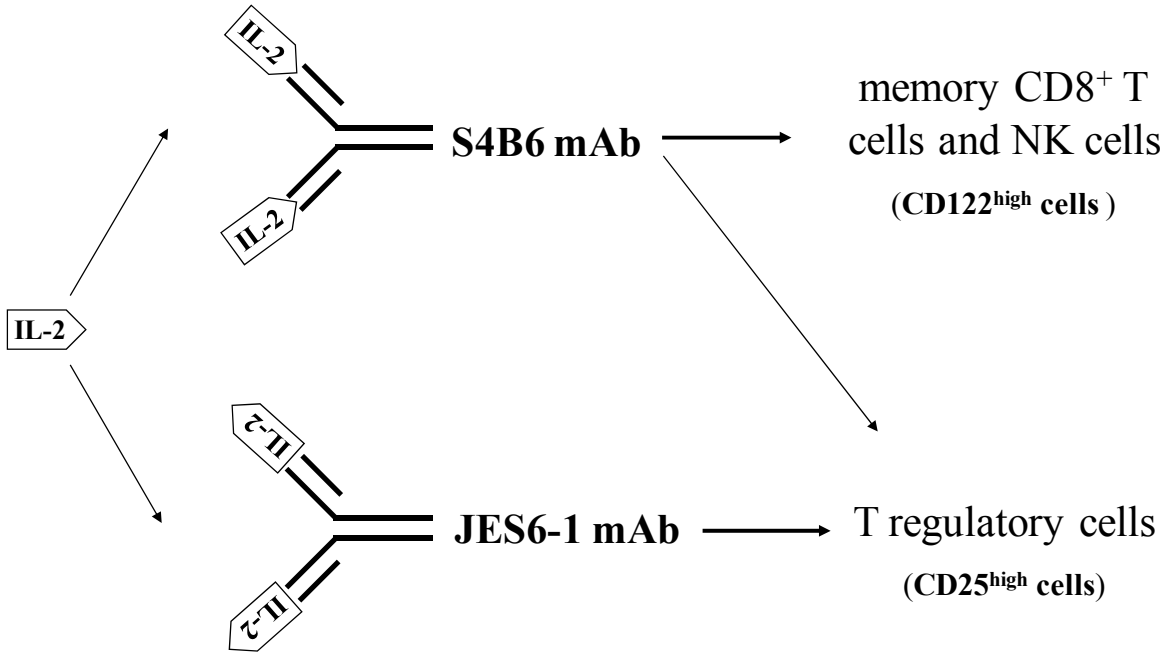



***Fig. 1****: Binding of IL-2 to two different anti-IL-2 mAbs leads to formation of two kinds of IL-2 immunocomplexes with different biological activity: selectivity of IL-2 immunocomplexes is governed by anti-IL-2 mAb used.*

IL-2 immunocomplexes based on the clone of anti-IL-2 mAb S4B6 (henceforth IL-2/S4B6), were found to be highly stimulatory for memory $CD8^+$ T cells and NK cells ($CD122^{high}$ populations). There was also moderate (<2-fold) relative increase of $T_{reg}$ cells ($CD4^+$ $CD25^+$ $Foxp3^+$). It was shown that this type of immunocomplexes require dimeric IL-2 receptor with low affinity. On the other hand, IL-2 immunocomplexes based on the clone of anti-IL-2 mAb JES6.1-A12 (henceforth IL-2/JES6.1) require $\alpha$ subunit (CD25) to be present on the surface of target cell together with $\beta$ subunit (CD122) and $\gamma_c$ subunit (CD132) (high affinity IL-2 receptor). $CD25^-$ cell populations are not able to utilize IL-2/JES6.1 immunocomplexes even if they express both CD122 and CD132. Thus, IL-2/JES6.1 immunocomplexes are shown to be highly selective and stimulatory for $T_{reg}$ cells [2].

There are several other mAbs binding IL-2 of human and murine origin and, interestingly, they fall into these two categories and exert similar effects. For example, MAB602 represents anti-human IL-2 mAb and its immunocomplexes strongly stimulate $CD122^{high}$ cells. JES6.5H4 binds murine IL-2 and resultant IL-2 immunocomplexes have identical biological activity as IL-2/S4B6. Finally, anti-human-IL-2 mAb 5344 has selective stimulatory activity for $CD25^{high}$ cells [13] and thus it resembles IL-2/JES6.1.

Boyman and his colleagues proved that the potentiation of cytokine biological activity through anti-cytokine mAbs works very well also in the case of IL-4 and IL-7, thus showing more general aspect of this finding. Further, rhIL-2/MAB602 immunocomplexes were demonstrated to work in mice at least as good as IL-2/S4B6 immunocomplexes. This implies that rhIL-2, and perhaps other already mentioned cytokines, might be potentiated with anti-cytokine antibodies even in humans, offering new perspective application. Several ideas how is the superior biological activity acquired by IL-2 immunocomplexes have been suggested, namely extension of half-life in circulation [2].

IL-2 immunocomplexes could be prepared simply by short incubation of IL-2 with anti-IL-2 mAb at molar ratio of 2:1 *in vitro* and then injected i.p. [14] or IL-2 and anti-IL-2 mAb could be applied separately via intraperitoneal injection within short time interval to manifest nearly the same effect as those prepared *in vitro* [2].

Both kinds of IL-2 immunocomplexes have been studied by several research groups, bringing new perspectives and new findings into view.

### I.1.2.1. IL-2/S4B6 immunocomplexes stimulate predominantly memory $CD8^+$ T cells and NK cells

IL-2/S4B6 stimulate predominantly NK and MP $CD8^+$ T cells [2, 14] as mentioned previously and they also expand $T_{reg}$, γδ T and NKT cells [15]. On the other hand, they have minimal effect on $CD4^+$ memory T cells ($CD44^{high}$) or B cells ($B220^+$) [2]. They are able to drive the expansion of activated naive $CD8^+$ T cells into functional memory-like $CD8^+$ T cells [3], even with minimal TCR stimulation by self-peptides/ligands [16]. These expanded cells have central memory phenotype ($CD44^{high}$ $CD122^{high}$ $CD62L^{high}$) and are able to protect against bacterial infection, but exhibit reduced cellular fitness in terms of lower homeostatic turnover rate and production of cytokines [16].

$CD8^+$ T cells in mice treated with IL-2/S4B6 are also able to significantly decrease viral load during persistent virus infections even without help of $CD4^+$ T cells. This is mediated via expansion of virus-specific but also virus-nonspecific $CD8^+$ T cells and about 8-fold increase in granzyme B production. Antiviral activity of IL-2/S4B6 is mediated via perforin/granzyme B and is IFN-γ independent [17].

NK cells ($DX5^+$ $NK1.1^+$) are dramatically expanded (20-fold or more, depending on the regiment) by IL-2/S4B6 immunocomplexes [2-3, 18]. Translated from work by Prlic *et al.* [18], these complexes stimulate immature NK cell precursors from bone marrow, as well as mature NK cells from spleen, however the latter to far greater extent. NK cells are even more sensitive to stimulation with IL-2/S4B6 than activated $CD8^+$ T cells, as they already expand moderately (2 to 3-fold) when mice are injected with 0,1 ug of IL-2 equivalent [3]. Splenocytes from IL-2/S4B6 treated mice also possess higher cytolytic activity when compared to the splenocytes from mice injected with free IL-2. This effect could be further increased by co-injection of IL-12 [15].

Interestingly, injecting S4B6 mAb alone results in decrease of $T_{reg}$ cells. In such situation, endogenous IL-2 bind to S4B6 mAb and is thus available for $CD122^{high}$ cells rather than $T_{reg}$ cells. Therefore, application of exogenous IL-2/S4B6 lead to the dramatic increase of the same population [2, 14].

### I.1.2.2. IL-2/JES6.1 immunocomplexes selectively stimulate $T_{reg}$ cells

IL-2/JES6.1, as stated before, require not only dimer of β and $\gamma_c$ subunit, but also CD25 to have effect on the target cells [2] and driving expansion of T cells [2-3]. It has negligible effect on other cell types, even though they express dimeric IL-2 receptor, including $CD4^+$ and non-activated $CD8^+$ T cells, memory phenotype (MP) $CD8^+$ T cells, NK and NKT cells [3, 19]. Our non-published data suggested that this might not be true in case of NKT cells, as these are able to upregulate CD25 and utilize IL-2/JES6.1 immunocomplexes to certain extent. However, this observation must yet to be verified.

Our work on BCL1 leukemia model [6] provided the first direct evidence that expansion of $T_{reg}$ cells can promote tumor growth. We were able to deliberately and highly selectively expand $T_{reg}$ cell population *in vivo* by the use of IL-2/JES6.1 and show that this increase of $T_{reg}$ numbers accelerate BCL1 leukemia progression.

The effect of IL-2/JES6.1 on $T_{reg}$ cells has been mostly studied in context of several experimental autoimmune model diseases. Study by Tang *et al.* [20] showed that continuous treatment of female non-obese diabetic mice with IL-2/JES6.1 for 10 weeks prevented the onset of diabetes.

The effect of IL-2/JES6.1 was also studied in experimental autoimmune encephalomyelitis (EAE) by Webster *et al.* [21] and this work convincingly proved that IL-2/JES6.1 may have therapeutical potential. The work showed that IL-2/JES6.1 are most effective (increase of $T_{reg}$ up to ~80% of $CD4^+$ T cell population) when prepared in 2:1 IL-2 to mAb molar ratio, with neither reagent in excess. Excess of mAb has mild inhibiting effect on expansion of $T_{reg}$ cells (increase of $T_{reg}$ up to ~50% of $CD4^+$ T cell population). This is in concordance with finding that JES6.1 mAb has decent neutralizing effect on IL-2 activity *in vivo*, as injection of JES6.1 alone leads to reduction of $T_{reg}$ cell counts [21]. Expansion of $T_{reg}$ cells after injection of IL-2/JES6.1 is seen to various extent in the whole immune system (spleen, thymus, bone marrow, lymph nodes, liver, lamina propria or Peyer´s patches), with special accent on lamina propria and liver. $T_{reg}$ cells undergoing expansion mediated by IL-2/JES6.1 reaches the peak around day 5 with immunocomplexes administered on day 0, 1 and 2 (1 μg of IL-2 equivalent/dose).

Phenotype of expanded $T_{reg}$ cells is characterized by considerable increase of molecules relevant for suppressive potential (CD25, ICOS, CTLA-4, GITR) and mild increase in some other markers (CD44, TGF-β, ICAM-1, PD-1) generally present on $T_{reg}$ cells. Nevertheless, this shift towards highly activated state is rather transient, as it is gone after several days (~5 days) without consecutive treatment. There is also considerable build-up of IL-10 mRNA production with respective increase in suppressive activity *in vitro* and with the same transient character as seen for the surface molecules [21]. Interestingly, despite the superior suppressive state of $T_{reg}$ cells, little change in TGF-β mRNA production could be found.

Short treatment of mice with IL-2/JES6.1 before induction of EAE, an experimental model of multiple sclerosis (MS) induced by immunogenic peptide of myelin oligodendrocyte glycoprotein ($MOG_{35-55}$) in complete Freund´s adjuvant (CFA), led only to the mild symptoms of the disease, suggesting the ability of expanded $T_{reg}$ cells to control autoreactive T cells responsible for the autoimmunity. Unfortunately, when IL-2/JES6.1 are given after the manifestation of the disease, which is more relevant to clinical situation during MS, IL-2/JES6.1 offer no beneficial effect. However, this is not the case when they are co-administered with rapamycin, which is reported to inhibit T effector cells and enhance activity of $T_{reg}$ cells, especially when administered early after immunization with MOG peptide. This approach resembles clinical intervention after the relapse of MS and results in delayed onset

of the disease with less intense symptoms, although it does not lead to the complete cure of EAE [21].

Further, Liu *et. al* [19] studied the effect of IL-2/JES6.1 on $T_{reg}$ cells in context of experimental Myasthenia gravis (EAMG) and clearly showed their potential in this application. Authors demonstrated, in concordance with above mentioned reports, that IL-2/JES6.1 immunocomplexes are able to dramatically expand $T_{reg}$ cell population (~10-fold increase) in spleen, lymph nodes or peripheral blood alike. Expanded $T_{reg}$ cells exhibit similar suppressive activity as naturally occurring $T_{reg}$ cells. TGF-β, an important cytokine for $T_{reg}$ cell developement, do not synergize with IL-2/JES6.1 immunocomplexes treatment, although it mildly increases expression of Foxp3.

IL-2/JES6.1 immunocomplexes were shown to be effective in treatment of experimental Myasthenia gravis, as their administration can hold clinical symptoms of this experimental muscle pathology at bay, be it in the early stage of the disease or later, following signs of emerging muscle weakness [19]. Moreover, adoptively transferred $T_{reg}$ cells expanded with IL-2/JES6.1 are also able to reduce the severity of this disease via specific suppression of acetyl choline receptor (AchR) reactive T cells. $CD4^+$ AchR specific T cells are critical in helping B cells to produce pathogenic anti-AChR IgG Abs [22]. These cells, if taken from IL-2/JES6.1-treated mice, showed reduced proliferative activity in comparison to non-treated controls. Furthermore, $CD4^+$ $T_h$ cells in mice treated with IL-2/JES6.1 gained significantly lowered $T_h1$ profile in terms of decreased IFN-γ and increased IL-4 production, pointing out the shift toward $T_h2$ response with corresponding differences in IgG isotypes against experimental Myasthenia gravis. Finally, elevated TGF-β was detected in the same mice, with no apparent effect seen, as no shift in IL-6 production, also necessary for expansion of Th17 cells, was found. Such skewing of immune responses might contribute to the capacity of IL-2/JES6.1 to successfully suppress the EAMG.

Report of Webster *et al.* [21], apart from other findings, followed very interesting and important role of IL-2/JES6.1 in transplantation immunology. It had been reported previously that expanded $T_{reg}$ cells *in vitro* can prevent graft rejection [23-24], while similar studies showed that prevention of graft rejection by $T_{reg}$ cells expanded *in vivo* required either lymphopenic conditions [24] or combination with immunosuppression [25]. Webster *et al.* utilized model of diabetic mouse with chemically mediated (streptozotocin) destruction of pancreatic insulin-producing β cells. Short pretreatment of diabetic C57BL/6 mice with IL-

2/JES6.1 immunocomplexes and grafting of MHC-mismatched BALB/c pancreatic islets resulted in low rejection rate (less than 18%) and seemingly indefinite survival of those grafts, that were not rejected until day 25 after transplantation. These findings were histologically confirmed, showing healthy islets with robust insulin production and negligible lymphocyte infiltration in case of long-term surviving grafts. Interestingly, despite long-term tolerance to MHC-mismatched BALB/c pancreatic islets in C57BL/6 mice, there was no tolerance to other BALB/c alloantigens. One possible explanation of such operational tolerance might be $T_{reg}$ cell infiltration in the grafts, as it was previously shown such $T_{reg}$ cells can mediate tolerance in various transplantational settings [26]. However, testing of the exact function of these $T_{reg}$ cells is difficult and other mechanisms remain elusive.

Interestingly, it is still not exactly known to the full extent, what is the mechanism of $T_{reg}$ cell expansion after treatment with IL-2/JES6.1. One report [21] describes that increase of $T_{reg}$ cells originates in existing $T_{reg}$ cells in extrathymical regions and clearly turns down the possibility of $CD4^+$ $Foxp3^-$ cell conversion into $T_{reg}$ cells. Other report [19], which is partly in contradiction with the previous one, suggests that IL-2 immunocomplexes expand $T_{reg}$ cells via proliferation of peripheral $CD4^+$ $CD25^+$ $Foxp3^+$ T cells and conversion of extrathymic $CD4^+$ $CD25^-$ $Foxp3^-$ T cells into $T_{reg}$ cells. However, contribution of thymic $T_{reg}$ cells on the expansion of $T_{reg}$ cells in periphery cannot be excluded in either report.

### I.1.2.3. Mechanisms of increased biological activity of IL-2 immunocomplexes

Increased biological activity of IL-2 immunocomplexes is probably governed by considerable prolonged half-life in circulation [3, 27]. The role of Fc receptors was also discussed [16, 27]. It was shown that $Fc_\gamma$ is dispensable for MP $CD8^+$ T cell expansion driven by IL-2/S4B6. On the other hand, neonatal Fc receptor (FcRn) plays a role in increasing the biological activity of the IL-2 immunocomplexes, since IL-2/S4B6 exhibited reduced stimulatory activity and cell recovery of $CD8^+$ T cells in $FcRn^{-/-}$ mice in comparison to WT mice. The role of FcRn was further supported by similar finding in β2-microglobulin deficient mice [28]. In contrast, IL-2/JES6.1 are almost completely dependent on FcRn function. FcRn significantly prolongs serum half-life of IgG [29-30] and thus extension of IL-2 half-life in circulation seems to be essential, but not the only one mechanism responsible for very high biological activity of IL-2 immunocomplexes *in vivo*. IL-2 immunocomplexes prepared from $F(ab')_2$ fragment of

MAB602 anti-IL-2 mAb previously showed reduced biological activity in comparison to IL-2 immunocomplexes prepared by the use of complete MAB602 [2]. Nevertheless, repeated administration of IL-2/F(ab´)$_2$MAB602 complexes can equalize or even surpass effect of single dose of IL-2/MAB602 complexes, which shows that specific binding of IL-2 to anti-IL-2 mAb is crucial for increased biological activity of IL-2 immunocomplexes *in vivo*. The blockade (anti-CD25 mAb application) or absence (CD25$^{-/-}$ mice) of CD25 in combination with repeated application of free IL-2 leads to slightly lower or similar proliferation of MP CD8$^+$ T cells as with IL-2/MAB602 complexes, respectively. This points out that impeding of interaction between IL-2 and CD25 is probably another important mechanism for IL-2/S4B6 biological activity and IL-2 in IL-2/S4B6 complexes indeed seems to be protected from interactions with CD25 [27]. Unfortunately, precise mechanisms of this effect remain unclear.

### I.1.2.4. Toxicity of IL-2 immunocomplexes

One of the most limiting factors in IL-2 application are severe toxicities associated with high-dosage, which is on the other hand necessary for its effectiveness. Well known toxicity associated with IL-2 treatment is vascular leak syndrome (VLS) [2], which is mediated mainly by proinflammatory cytokines produced mainly by NK cells. Krieg *et al.* [13] have compared toxicity and effect of free rhIL-2 and rhIL-2 immunocomplexes (rhIL-2/MAB602) on VLS. This study showed that free IL-2, besides notable expansion of CD8$^+$ T and NK cells, also induces significant increase of pulmonary wet weight, mediates histological changes of the lungs and a drop in $O_2$ saturation could be recorded. In comparison, rhIL-2 immunocomplexes specific for CD122$^{high}$ cells induce robust expansion of the same cell populations but with only mild pulmonary edema symptoms compared to the vigorous immune stimulation. Interestingly, rhIL-2 immunocomplexes with selectivity for CD25$^{high}$ cells showed high VLS in the near-absence of CD8$^+$ T and NK cells expansion. This finding, together with the data showing that severe VLS was also observed in immunodepleted environment and absence of CD25 blocked VLS formation, led to the finding that CD25 expressing lung endothelial cells (CD31$^+$) play important role in IL-2 induced VLS [13]. These cells (CD25$^{intermediate}$ CD132/CD122$^{low/intermediate}$ in comparison to immune cells) are able to increase their CD25 mRNA levels upon IL-2 stimulation, suggesting signaling via IL-2 receptor (pSTAT5 increase), and produce nitrite ($NO_2^-$) *in vitro*, which is reported to be elevated in VLS and is toxic for endothelial cells [31].

Moreover, rhIL-2 immunocomplexes specific for CD122$^{high}$ cells were about 13-fold less toxic compared to free IL-2 when both agents were given in 5 daily doses. The same stimulatory effect was achieved with 40-fold lower dose of rhIL-2 immunocomplexes with selectivity for CD122$^{high}$ cells in comparison to free IL-2 [13].

### I.1.2.5. IL-2 immunocomplexes and their potential for immunotherapy of cancer

IL-2 immunocomplexes affect various cell populations which may play role in anti-tumor immunity. There are several reports dealing with the question, whether IL-2/S4B6 or IL-2/JES6.1 could be used to treat tumors. IL-2/S4B6 relatively selectively stimulate MP CD8$^+$ T and NK cells, known to be key players in anti-tumor immunity and tumor eradication. The first report investigating anti-cancer activity of these complexes [14] was using tumor model of metastatic B16 melanoma. B16 melanoma cells form nodules in the lungs when applied intravenously (i.v.). Authors did not use IL-2/S4B6 directly, they rather co-administered IL-2 producing plasmid and S4B6 mAb. Only application of both these moieties led to significant reduction in numbers of tumor nodules in lungs of experimental mice.

Our group published two papers [3, 15] focused on tumor immunity and therapy through the use of IL-2 immunocomplexes. In the earlier one [3], we addressed the question of therapeutic potential of IL-2/S4B6 in comparison to free IL-2 given at maximum tolerated dose (MTD) by employing two syngeneic tumor models, namely BCL1 leukemia and B16F10 melanoma. Based on different selectivity, we expected IL-2/S4B6 should be apparently more suitable for such task than IL-2/JES6.1. BCL1 leukemia (i.p. application) and B16F10 melanoma (s.c. application) were treated with IL-2/S4B6 relatively early after the inoculation of the tumor cells (day 4 and 8 or day 2 and 6, respectively), as immunotherapy alone may hardly show some significant effect on large established tumors [15]. This prophylactic regimens led to survival of one-third (BCL1) or up to two-thirds (B16F10) of tumor bearing mice, depending on the IL-2/S4B6 dosage [3, 15]. Further, combination of IL-2/S4B6 with anthracycline antibiotic doxorubicin was proved to be very potent, even if given late after the tumor cell inoculation (11 days). Latter report [15] follows the strategy of treatment of established tumors via combination of novel type of cytostatics bound to poly(*N*-(2-hydroxypropyl)metacrylamide) (HPMA) and IL-2/S4B6. Most interestingly, we reported that application of HPMA copolymer-bound doxorubicin conjugate followed by repeated IL-

2/S4B6 treatment prolongs survival or even completely cures mice with tumors in very late progression stages.

IL-2/JES6.1 have not been reported to be tested for any anti-tumor activity so far. The reason is most probably their dominant biological activity: they potently and very selectively expand $T_{reg}$ cells. This fact is likely discouraging enough to employ immunocomplexes of this type in immunotherapy of cancer. However, we know from our recent experiments that these immunocomplexes have surprisingly strong anti-tumor activity, which is even comparable to IL-2/S4B6 in some tumor models, since IL-2/JES6.1 also vigorously expand activated $CD8^+$ T cells [3]. This activity might probably override the effect of expanded $T_{reg}$ cells and lead to manifestation of anti-tumor immunity. Unfortunately, detailed mechanisms of action has to be elucidated.

### I.1.3. IL-3/anti-IL-3 mAb immunocomplexes

Interleukin 3 (IL-3) is one of the hematopoietic growth factors and it cannot be detected in circulation or bone marrow of healthy individuals. IL-3 is produced by activated T lymphocytes, mast cells, eosinophils and neutrophils. Its primary activity is to induce differentiation of pluripotent stem cells into myeloid progenitors. It also modulates the growth and effector functions of eosinophils, macrophages, lymphocytes and mast cells [32-33]. Detection of IL-3 in circulation was reported during graft-versus-host disease and it was suggested that IL-3 has a role in so-called "stress hematopoiesis" following infection [32]. It was postulated that IL-3 could be potentially promising for reconstitution of hematopoesis after myeloablation [34]. However, its short half-life is significant problem for such application.

Several reports addressed the problem of short half-life of IL-3 in circulation by complexing IL-3 with anti-IL-3 antibody [9, 35]. Murine IL-3 and neutralizing rat anti-mouse anti-IL-3 mAb (clone 8F8.1) were used in work of Finkelman *et al.* [9] to prepare IL-3 immunocomplexes, which were reported to considerably increase mast cells numbers in small intestine of BALB/c mice after two doses of IL-3 immunocomplexes (16-fold in comparison to free IL-3). Other report [35] on IL-3/anti-IL-3 antibody complexes utilized polyclonal antibody isolated from rabbits immunized with short peptide corresponding to the first N-

terminal 29 amino acids of IL-3. This polyclonal antibody formed complexes with IL-3 in a way that it did not interfere with IL-3 binding to IL-3 receptors.

As with other interleukin complexes, IL-3/anti-IL-3 mAb immunocomplexes manifested increased biological activity *in vivo*, whereas they had similar or slightly decreased biological activity in comparison to IL-3 *in vitro*. Thus, cross-linking of IL-3 receptors or antibody-induced conformational change in IL-3 resulting into "superagonist" is not involved as a mechanism in increasing the biological activity of IL-3 immunocomplexes *in vivo* [35].

IL-3 immunocomplexes were described to increase number of colony forming units cells (CFU-c) derived from spleen *ex vivo* (3 to 7-fold), to increase mast-cell precursor frequencies in spleen cell suspension *ex vivo* (3 to 5-fold increase) [35] and to significantly increase the number of mature mast cells in the spleen [9, 35], but not in other tissues. Free IL-3 or anti-IL-3 antibody alone did not manifest any of these effects, in contrast to IL-3 immunocomplexes [9, 35].

As a general rule, complexing IL-3 with anti-IL-3 antibody leads to extended half-life of IL-3 biological activity in serum. Clearance of IL-3 biological activity after administration of IL-3 immunocomplexes is 7 to 10-fold slower in comparison to free IL-3 [9, 35].

Application of IL-3 in human medicine has been tested in several clinical trials. They were oriented towards hematology, oncology and bone marrow transplantation but without gathering much success [36]. As mentioned above, short half-life in circulation limits its efficacy and must be compensated by higher dosage and/or more frequent administration. However, this is associated with severe side toxicities (grade III) common for hematopoietic growth factors like decrease in platelet counts, increase in basophilic counts, bone marrow fibrosis and pulmonary edema. Due to their prolonged stay in circulation and increased biological activity in comparison to free IL-3, IL-3 immunocomplexes thus might represent the key how to lower the dosage requirements which would markedly limit the severity of associated toxicities.

## I.1.4. IL-4/anti-IL-4 mAb immunocomplexes

Interleukin-4 (IL-4) plays a key role in humoral immunity and it is also involved in polarization of naive $CD4^+$ T cells into $T_h2$ cells. Furthermore, IL-4 induces immunoglobulin

class switch to IgE and IgG4 and upregulates expression of MHC class II glycoprotein (MHC II). It inhibits generation of $T_h1$ cells and M1 macrophages. IL-4 also decreases production of IFN-γ and IL-12 by dendritic cells.

IL-4/anti-IL-4 mAb immunocomplexes were in fact the first ones described to have beneficial effect of complexing cytokine to anti-cytokine monoclonal antibody by Finkelman and his co-workers [9].

The original idea probably sprout out of finding that naturally occurring anti-interleukin auto-antibodies or corresponding soluble receptors (e.g. sIL4R) might increase biological activity of their respective targets *in vivo* trough serving as large protein carrier [37].

Firstly, Finkelman *et al.* (1993) elegantly demonstrated that majority of IL-4 formed a complex with anti-IL-4 mAb (clone 1D11.2 and probably others). These IL-4 immunocomplexes, if formed by neutralizing anti-IL-4 mAbs (clone 1D11.2 or 11B11), were able to stimulate MHC II (I-A) expression in mice about 5-fold in comparison to both low or high dose of IL-4 [9, 28]. Interestingly, if non-neutralizing mAb (BVD6.24G2.3) was complexed with IL-4, the resultant immunocomplex did not increase expression of MHC II. Peak of MHC II expression was reached on day 3 after application of the first dose of complexes of IL-4 and neutralizing anti-IL-4 mAb 1D11.2 and this state could be sustained for more than 3 weeks with repeated injections. MHC II expression returned to normal level within 5 days after the last dose of IL-4 immunocomplexes. Moreover, IL-4 immunocomplexes doubled spleen cell count and induced more than 2-fold increase in expression of CD23, an IL-4 inducible molecule. About 20% increase in frequency of $I\text{-}A^+$ and $CD23^+$ splenocytes was also documented. This suggests that IL-4 immunocomplexes have an effect on mantle layer B cells, as these are the only cells in spleen expressing both molecules [9].

IL-4 immunocomplexes possess also stimulatory activity to MP $CD8^+$ T cells and this stimulatory activity is similar to that seen with IL-2/S4B6 [2, 28]. The explanation here is that memory, but not naive $CD8^+$ T cells, express CD124, an IL-4 receptor (IL-4R) α chain, thus rendering them sensitive to stimulatory activity of IL-4 immunocomplexes.

Unlike IL-2 immunocomplexes, IL-4 immunocomplexes are rendered biologically inactive with excess of antibody used for complexing, as IL-4 must dissociate from immunocomplex to bind to IL-4R and mediate its function. Excess of antibody leads to the state that

dissociated IL-4 binds rather to another anti-IL-4 mAb than to IL-4R, thus unable to activate downstream signaling. IL-4 immunocomplexes are thus not stimulatory *per se*, and dissociation of IL-4 and anti-IL-4 mAb is prerequisite for biological activity [9, 28].

As with other immunocomplexes, it was proven that IL-4 immunocomplexes exert prolonged half-life in circulation. While free IL-4 was undetectable in sera of mice 24 hours post injection, IL-4 immunocomplexes were detectable at least 72 hours after administration. This phenomenon was proposed as the only mechanism of enhanced biological activity *in vivo* [9]. However, latter report by Boyman *et al.* (2006) provided similar, but broader view and proved that this conclusion is rather incomplete, showing several different mechanisms of immunocomplexes´ function already mentioned for IL-2 immunocomplexes (see chapter I.1.2) and providing evidence that formation of immunocomplexes results in more than creating a carrier system for short-lived cytokines [2].

Concerning the application, IL-4 immunocomplexes were described to successfully cure or ameliorate experimental nematode infection (*Heligmosomoides polygyrus*, *Trichuris muris* or *Nippostrongylus brasiliensis*) in immunocompetent or even in SCID immunodeficient mice [38], as IL-4 is known to limit infections of such parasites [39-41].

## I.1.5. IL-6/anti-IL-6 mAb immunocomplexes

Interleukin 6 (IL-6), 26 kDa protein, is an important cytokine connected to inflammation, acute phase reaction, induction of fever, tissue healing, hematopoesis and oncogenesis. Apart from its immunological role, IL-6 has also biological function in CNS, as it can bypass hemato-encephalytic barrier to initiate synthesis of prostaglandin E2 ($PGE_2$) in the hypothalamus leading to the change of body´s temperature set point. It is also known as a "myokine" as IL-6 is elevated in response to muscle contraction [42]. IL-6 is produced and released in response to IL-1 and TNF-$\alpha$ by T cells and macrophages to stimulate the response of wide variety of immune cells to trauma or infection. Importantly, IL-6 orchestrates production of two most important acute phase proteins in liver - C-reactive protein (CRP) and serum amyloid A (SAA). Furthermore, IL-6 is indispensable in $T_h17$ cell polarization [43].

There are several older reports that IL-6 immunocomplexes are formed after application of various neutralizing anti-IL-6 mAbs and these immunocomplexes manifest increased

biological activity and retention in circulation. Two studies from group of Hubertine Heremans [44-45] showed endogenous IL-6 and rat anti-mouse IL-6 mAbs (6B4 and 20F3) formed immunocomplexes which prevented generalized Schwartzman reaction and affected cytokine production after LPS application in mice. These immunocomplexes were shown to be highly biologically active *in vitro* in comparison to free IL-6 [45]. Similar finding was made after application of anti-IL-6 mAb 20F3 into mice with EAE. Serum and cerebrospinal fluid of these mice exhibited increased IL-6 biological activity and symptoms of the disease were significantly reduced [46]. Further, mice infected with *Trypanosoma cruzi* given 6B4 anti-IL-6 mAb showed increased levels of IL-6 in sera and survived slightly longer in comparison to controls treated with control mAb [47]. Increase of biological activity of human IL-6 was also described after combined treatment of C3H/HeJ mice with hIL-6 and anti-hIL-6 mAb clone MH166 as an increase of half-life of IL-6 in serum led to increased antibody titers following vaccination [48].

Clinical application of IL-6 immunocomplexes is rather questionable as IL-6 alone acts as rather "damaging factor". On the other hand, studying IL-6 immunocomplexes might offer invaluable insight into situations when anti-IL-6 neutralizing mAbs would be used in patients. Clinicians should be aware that anti-IL-6 mAb might as well potentiate IL-6 and not the opposite. There is even a possibility that anti-inflammatory effects of IL-6 [49-50] might be enhanced via complexing with neutralizing anti-IL-6 mAb or completely different array of effects might be gathered, based on the pleiotropic nature of IL-6.

## I.1.6. IL-7/anti-IL-7 mAb immunocomplexes

Interleukin-7 (IL-7) is a key cytokine for B and T lymphocyte development. It is produced by non-lymphoid cells, such as stromal cells of primary and secondary lymphoid organs and cells of epithelia, reticular cells and antigen-presenting cells (APC). IL-7 stimulates the differentiation of pluripotent hematopoietic stem cells into lymphoid progenitor cells. It also stimulates proliferation of all lymphoid cells and is involved in homeostasis of naive T cell subsets.

IL-7/anti-IL-7 mAb immunocomplexes were described in 1993 by Finkelman *et al.*, together with IL-4 and IL-3 immunocomplexes [9]. Administration of IL-7 immunocomplexes is characterized by massive increase of B cells and B cell precursors in spleen and bone marrow.

Human rIL-7 (rhIL-7) and "neutralizing" anti-hIL-7 mAb clone m25 pre-mixed *in vitro* and applied 3 times in 2-day interval (5 μg of IL-7 equivalent per dose) into BALB/c mice showed more than 9-fold increase in spleen cellularity. Pre-B cells and isotype-switched B cells ($B220^+$ $IgM^-$ $IgD^-$) are major expanded populations (from 2% to 38% of relative count). Conversely, only moderate increase in cell count of $IgM^+/IgD^+$ B cells was seen. Similar situation was found in bone marrow, which appeared to be rather white than red with relative increase of $B220^+$ $IgM^-$ $IgD^-$ B cells from 30% to 70% and no relative change in $IgM^+/IgD^+$ B cells.

Study by Boyman *et al.* [51] using both human and mouse rIL-7 (1,5 μg of IL-7 equivalent per dose) in C57BL/6 mice identified difference in mature B cell expansion. However, these cells were described to expand only 2 to 3-times in spleen, resulting in considerable relative decrease in comparison to other expanding populations.

Contrary to the situation in spleen, a different picture was found in lymph nodes (LN) and thymus, as both these organs contain very few immature B cells. LN showed major expansion of T cells, most of which were $CD8^+$ $CD44^{high}$ T cells (4-fold increase of $CD8^+$ T cells versus 2-fold increase of $CD4^+$ T cells) with no change in expression of activation markers (CD25, CD69, CD62L). Moderate expansion of mature B cells in LN was also recorded. Thymus increased its cellularity by 20%, mostly due to the expansion of double-positive (DP) $CD8^+/CD4^+$ T cells [51].

Effect of IL-7 immunocomplexes on thymopoesis was studied in context of $IL\text{-}7^{-/-}$ knockout mouse, where it was possible to reach plato of thymus enlargement (50 to 100-fold) by 2 doses given in 7 days, with dose as low as 1 μg of IL-7 equivalent. Free IL-7 in similar dosage had only minor effect on enlargement of thymus. Comparable enlargement as with IL-7 immunocomplexes could be achieved with free IL-7, although in 100-fold higher dosage. Apart from great expansion of DP cells in thymus, dramatic increase of $CD44^-$ $CD25^+$ or $CD44^-$ $CD25^-$ double-negative (DN) cells was also detected. However, effect of IL-7 immunocomplexes has only transient character, as thymus of the $IL\text{-}7^{-/-}$ transgenic mouse returns to its normal size and hypocellular state in 3 weeks after the last dose of IL-7 immunocomplexes. Interestingly, effect of IL-7 immunocomplexes on a size of spleen in $IL\text{-}7^{-/-}$ transgenic mouse is limited just to 2 to 3-fold enlargement [51].

Interestingly, human rIL-7 (rhIL-7) and mouse rIL-7 (rmIL-7) have 81% sequence homology in coding regions and approximately 60-70% in non-coding regions [52]. rhIL-7 also exerts

slightly lower affinity to mouse IL-7 receptor (IL-7R) [53]. It is not clear whether this leads to lower biological activity of rhIL-7 in mice. However, what is known is that rhIL-7, when complexed with anti-IL-7 mAb, possesses lower biological activity in mice when compared with immunocomplexes formed by murine rmIL-7. Although the same anti-IL-7 mAb (clone m25) binds both rmIL-7 or rhIL-7, rmIL-7 immunocomplexes exert 2 to 3-fold higher biological activity in mice [51].

Apart from neutralizing mAb clone m25, there are already several other clones of anti-IL-7 mAb, neutralizing (MAB207) or not (m23, MAB407), which are able to bind IL-7 and possibly form complexes. Unfortunately, none of these is able to boost biological activity of IL-7 so far [51].

IL-7 immunocomplexes share at least two attributes with other cytokine immunocomplexes [51]. They have extremely extended IL-7 half-life in circulation and their biological activity can be thus detected even more than 24 hours after the application in comparison to ~20 minutes for free IL-7 [2, 14]. The second similarity is role of Fc-portion of mAb molecule in mechanisms responsible for very high biological activity of IL-7 immunocomplexes. Immunocomplexes prepared from Fab fragment of m25 anti-IL-7 mAb and rhIL-7 show strongly abrogated biological activity in comparison to immunocomplexes prepared by the use of intact mAb molecules.

The use of IL-7 immunocomplexes in mice might be successfully translated to human medicine, although the scope would be limited to T cells as a target cell population since B cells in humans express very low levels of IL-7R. Thus, IL-7 immunocomplexes could be used to restore T cell pool in cancer patients suffering from serious lymphodepletion after intensive chemotherapy or in patients after organ or bone marrow transplantation. Other possibility could be treatment of immunodeficient patients in case of functional IL-7R. Especially elderly patients often have dramatically lowered number of T cells, making them sensitive to some pathogens. Such patients might substantially benefit from IL-7 immunocomplexes administration. Similarly, IL-7 immunocomplexes might be very beneficial for patients with acute or chronic HIV infection, as IL-7 alone was shown to be very promising as an adjuvant therapy in combination with anti-retroviral therapy (ART) [54-56]. This is further supported by successful phase I/IIa clinical trial focused on T cell recovery and thymic output in patients receiving combinational therapy of ART and/or rhIL-7 [57].

## I.1.7. IL-15/ IL-15Rα-Fc complexes

Interleukin 15 (IL-15) belongs to family of $\gamma_c$ cytokines and although lacking sequence homology with IL-2, it shares very similar structure with it. IL-15 is membrane-associated cytokine acting through cell-to-cell contact as a part of an immunological synapse. The major way of IL-15 action *in vivo* is most probably the trans-presentation. It is produced by activated dendritic cells (DCs), monocytes, and stromal cells. Its role lies in being persistently needed *in vivo*, especially in the context of the IL-15-dependent survival of memory $CD8^+$ T cells, as well as in the developmental requirement for IL-15 in the ontogeny of NK cells and γδ T cells [58-59]. It should be also noted that IL-15 signals through the same dimeric receptor composed of $\beta\gamma_c$ subunits as IL-2 does [60]. Moreover, it shares the $\gamma_c$ subunit with several other cytokines as well, e.g. IL-4 and IL-7.

Complete IL-15 receptor (IL-15R) is composed of three subunits - α (CD215), an exquisite subunit for IL-15, β and common $\gamma_c$, two subunits also shared with IL-2R. Thus, the main difference in signal recognition and specificity for IL-2 and/or IL-15 lies in α subunits of the receptors. Similar to IL-2R, trimeric IL-15R have high affinity ($K_d=10^{-11}$) to IL-15, while $\beta\gamma_c$ dimer weakly recognizes IL-15. However, while CD25 alone has relatively low affinity to IL-2 ($K_d=10^{-8}$), α subunit of IL-15 receptor (IL-15Rα) binds IL-15 with the same intensity as complete IL-15 receptor, i.e. IL-15 has more than 100-times higher affinity to IL-15Rα than IL-2 to CD25 [61]. Moreover, IL-15Rα is *de facto* not a receptor, as it associates with IL-15 already in Endoplasmic reticulum/Golgi apparatus and is necessary for its transport to the cell surface. Thereby, IL-15Rα is crucial for providing of IL-15 signal, not for acquiring it. Thus, question was asked, whether IL-15Rα commercially available as recombinant mouse IL-15Rα-Fc chimera is able to bind IL-15 and provide the signal to the cells, even though switched from surface-associated to soluble form.

IL-15 complexes (IL-15/IL-15Rα-Fc) are composed of IL-15 and chimeric protein consisting of two α-subunits of IL-15 receptor linked to Fc portion of an IgG antibody (Fig. 2). IL-15 complexes were first described and tested by Rubinstein *et al.* [62]. Unlike IL-2/anti-IL-2 mAb immunocomplexes, IL-15 complexes possess higher biological activity than IL-15 also *in vitro*, being 6 to 9-times more stimulatory in comparison to free IL-15. IL-15 complexes had an effect on NK cells, MP $CD8^+$ T cells and, surprisingly, typical naive $CD8^+$ T cells ($CD44^{low}$, $CD122^{low}$) as well. MP $CD4^+$ T cells were not able to respond to IL-15 complexes.

Signaling of IL-15 complexes was solely mediated by $\beta\gamma_c$ (CD122, CD132, respectively) receptor and was readily abolished by addition of anti-CD122 mAb into the culture.

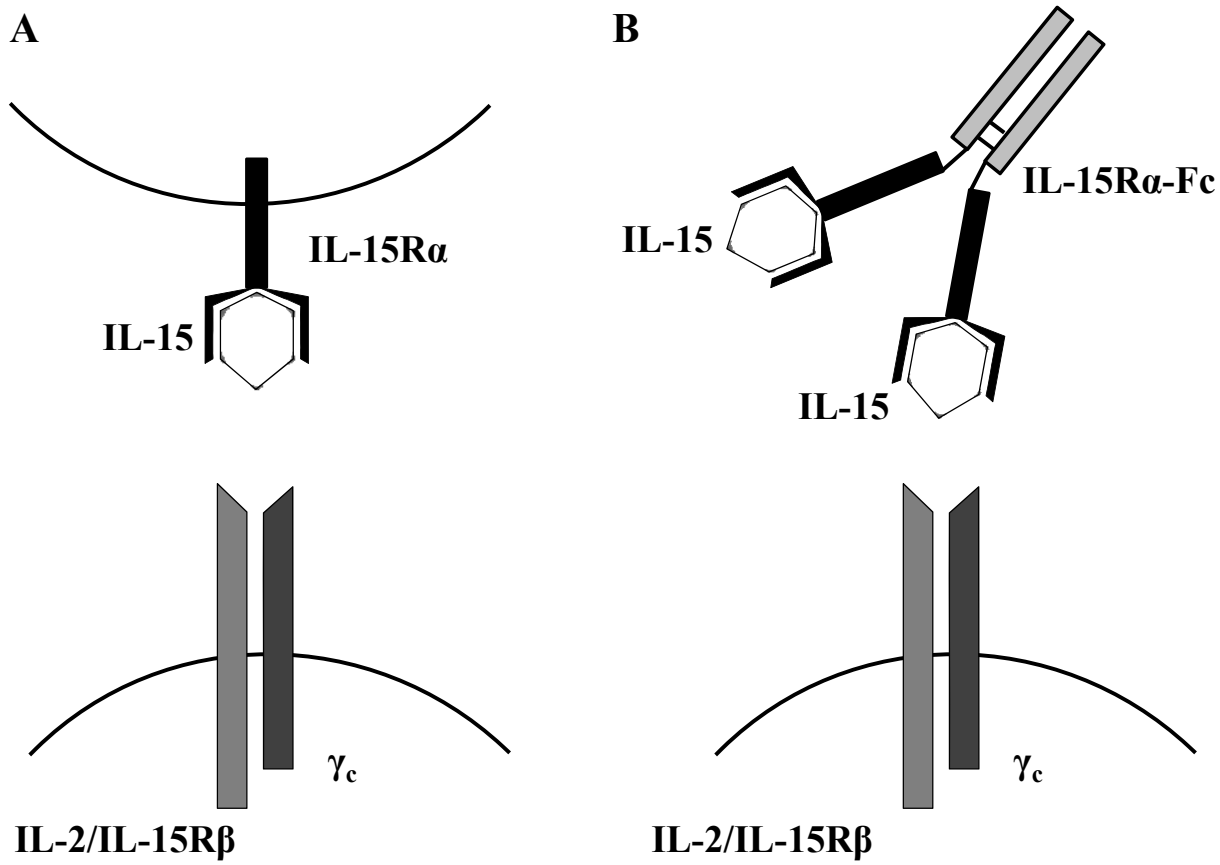



***Fig. 2:*** *IL-15 is presented in trans to the adjacent cell (A). Binding of soluble IL-15 to the chimeric protein consisting of α-subunit of IL-15 receptor linked to Fc portion of an IgG antibody leads to formation of IL-15 complexes, mimicking classic IL-15 presentation (B).*

*In vivo* data showed that MP $CD8^+$ T cells, NK cells [62-65] and NKT cells [64] proliferated and expanded vigorously in mice injected with of IL-15 complexes, marked by significant enlargement and increased cellularity of spleen (up to ~4-fold) [62, 65]. IL-15 alone had little effect on spleen and spleen cell counts. Role of $\beta\gamma_c$ in signal transduction was verified also *in vivo* through the use of anti-CD122 mAb and $CD122^{-/-}$ KO mice.

Naive $CD8^+$ T cells expanded with IL-15 complexes display activated effector phenotype of $CD69^{high}$, $CD127^{low}$ (IL-7R) [65] and $CD44^{high}$ [63] and leads to generation of stable and robust population of functional MP $CD8^+$ T cells in approximately 5-fold larger volume in comparison to untreated control [63, 65].

It was described that sustained stimulation (14-day/7 injections) led to very different phenotype of NK cells than the short-term one (2-day/1 injection). Short-term treatment with IL-15 complexes resulted in activated NK cell phenotype ($B220^{+}$ $CD11c^{+}$ $CD44^{high}$ $CD43^{high}$) with upregulated surface expression of NK activating molecules (NKG2D, CD84, 2B4, NKp46, and DNAM1). However, sustained application led to different phenotype closely resembling the one of unstimulated NK cells ($B220^{low}$ $CD11c^{low}$ $CD44^{+}$ $CD43^{+}$) with low presence of activating and increased level of inhibiting surface molecules (NKG2A and Ly49I/C/F), even in comparison to untreated controls [63].

IL-15 KO or IL-15Rα KO mice lack MP $CD8^{+}$ T cells and NK cells but these cells appear and repopulate the mice upon treatment with IL-15 complexes. IL-15 alone showed no such effect [62, 65]. This also demonstrates that IL-15Rα is dispensable and not involved in signal transduction, as it was already mentioned [65].

Similarly to cytokine-antibody immunocomplexes, IL-15 complexes exert significantly prolonged serum half-life of IL-15 in comparison to IL-15 alone. IL-15 has estimated half-life in serum about 1 h with peak of concentration at 30 min. IL-15 complexes show serum half-life of approximately 20 h with peak at around 2 h. Notably, concentration of IL-15 complexes at its serum peak was approximately 10-fold higher in comparison to IL-15 alone, when the same dose of IL-15, free or complexed, was injected. No doubt this is one of the key mechanisms involved in potentiation of biological activity of IL-15, apart from increasing its affinity for IL-15R [66] and providing a platform for trans-presentation [65].

Interestingly, monomeric fragments of IL-15Rα without Fc portion prepared by enzymatic cleavage of IL-15Rα-Fc and complexed with IL-15 possessed even higher stimulatory activity towards MP $CD8^{+}$ T cells than IL-15 complexes *in vitro*. Possible explanation might be steric reasons, although detailed explanation is unknown [62].

Potential use of IL-15 immunocomplexes for cancer treatment was suggested in several reports [64-65]. Stoklasek *et al.* [65] used B16F1 melanoma model of lung and liver tumor nodules, while Epardaud *et al.* [64] studied anti-tumor potential of IL-15 complexes in B16F10 melanoma growing in C57BL/6 mice and in spontaneous pancreatic tumors of RIP1-Tag2 mice. Significant reduction in tumor burden associated with IL-15 complexes application was seen while treatment with IL-15 showed negligible effect in all these settings.

## I.2. HPMA COPOLYMER-BOUND DRUG CONJUGATES

Chemotherapy plays an essential role in treatment of cancer. Unfortunately, it also exerts serious toxicities, which could be even life-threatening and thus requiring lowering the dosage. Increasing anti-tumor activity and eliminating toxicity of cytostatic drugs is a subject of massive research. There are many different systems to improve delivery of various chemotherapeutic agents to the tumor site, like dendrimers [67-68], liposomes [69], emulsions, solid lipid micelles or nanosphere [70-71] and synthetic polymers [72-73] or polymer-protein conjugates [74-76]. However, one of the most promising and the most extensively studied are water-soluble polymer-bound drug conjugates based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) (Fig. 3) [77-79].

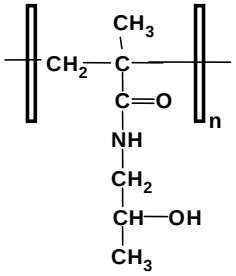



***Fig. 3:*** *HPMA monomer*

HPMA-based copolymer-bound drug conjugates are polymer-drug conjugates where a drug is attached as a pendant group to the polymer side chain. They are designed as prodrugs, which means that a drug is transported in an inactive form and it is released back into pharmacologically active form in the desired body compartment to manifest its biological activity. The conjugates consist of three distinct parts: water-soluble HPMA copolymer carrier (pHPMA), a linker and an anti-cancer drug. These conjugates have been designed to achieve: (i) improved drug accumulation at the target (tumor) site, (ii) reduction of unwanted toxicity by limiting access of the drug to the healthy tissues, (iii) induction of mechanisms overcoming tumor drug resistance, (iv) improved pharmacological profile of a cytostatic drug and (v) improved water solubility of extremely hydrophobic drugs.

Binding of a drug to pHPMA provides water-solubility to the drug, even if the drug has highly hydrophobic character, e.g. taxanes. Thus, no special solutions are necessary to dissolve such drugs for i.v. administration. Such solutions are not always well tolerated by

patients and often cause undesired effects. Furthermore, attachment of a drug to the pHPMA significantly affects its pharmacokinetics. Low molecular weight (LMW) cytostatic drugs administered i.v. reach relatively very high concentrations in circulation. However, such high concentrations are responsible for many side-toxicities. Drug concentration starts to decrease very rapidly shortly after its peak. The half-life of LMW drugs in the circulation is usually in order of hours or even less. On the contrary, HPMA copolymer-drug conjugates do not cause any severe toxicities when their concentration in circulation is high after i.v. administration since the drug bound to HPMA copolymer is pharmacologically inactive. The decrease in plasma concentration of HPMA copolymer-bound drug conjugates is much slower than that of LMW drugs - the half-life of these conjugates in circulation is in order of tens of hours or more [80].

Conjugation of the drug to polymer carrier also changes the fate of the drug at the cellular level. LMW drugs enter cells rapidly (within minutes) through the plasma membrane. Uptake of HPMA copolymer conjugates is much slower and is facilitated via endocytic route [81-83]. Endocytic internalization of conjugates has been verified in variety of cell lines, using several methods (radiolabelled probes, HPLC, confocal microscopy). This route of cell entry appears to enable drugs, at least partially, to bypass the P-glycoprotein or other ABC transporter-mediated drug efflux that is the main mechanism of multidrug resistance (MDR). As a result, HPMA copolymer conjugates are thought to be less sensitive to elimination of pharmacological activity mediated by ABC transporters than LMW drugs [84].

HPMA copolymer conjugates are passively accumulated in the solid tumors by Enhanced permeability and retention effect (EPR effect, Fig. 4). The concept of EPR effect was formulated by Matsumura and Maeda in 1986 [85] and it was validated for almost all types of solid tumors. The underlying mechanism of EPR effect is that solid tumors have poor or missing lymphatic drainage as well as large fenestration within tumor capillaries ("leaky endothelium").

Thus, leaky vasculature allows easy and rapid extravasation and decreased clearance than facilitates accumulation of macromolecules and nanoparticles in the tumor. The limit for EPR effect was evaluated to be about 40 kDa, molecules with lower Mw are cleared from tumors relatively rapidly. Moreover, targeting moiety that specifically binds to the target cells could be incorporated into the conjugate. In such a way, the conjugate could be also actively targeted to desired cells.

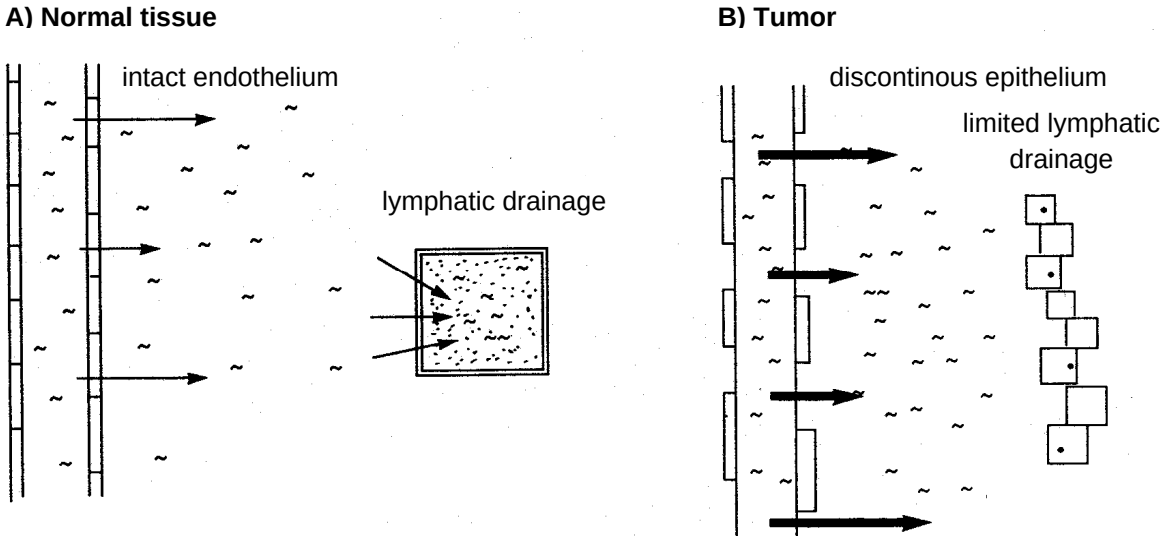



***Fig. 4:*** *EPR effect (Enhanced Permeability and Retention effect; adopted from Duncan et al., 1992).*

HPMA homopolymer was developed in 1965 at the Institute of Macromolecular Chemistry of Academy of Sciences of the Czechoslovak Republic. Kopeček *et al.* originally designed HPMA homopolymer as a plasma expander and named it "Duxon" [86]. HPMA homopolymer has no reactive groups and thus could not be used for conjugation with the drugs. This issue led to the synthesis of copolymers containing functional groups needed for binding of drug to the polymer backbone [87].

There is a large number of LMW anti-cancer drugs which have been successfully bound to HPMA copolymer. These are of very different structures and biological activity like: chlorin e6, dexamethasone, ellipticin derivative, puromycin, wortmanin, geldanamycin, camptothecin, methotrexate and its derivatives, taxane derivatives (paclitaxel, docetaxel), 5-flourouracil, TNP-470 (an anti-angiogenic agent), platinates and anthracyclines, in particular epirubicin, daunorubicin, and doxorubicin (Dox). The last mentioned drug is the most extensively studied in the field. Drugs might be even bound to the polymer backbone in combination for simultaneous delivery. Several such HPMA copolymer conjugates with dual activity have been reported. The first one carries Dox and mesochlorin e6 monoethylenediamine (representative drug for photodynamic therapy, PDT) bound to the same copolymer carrier [88]. Further tested combinations were aromatase inhibitor aminoglutethimid and Dox [89], combination of Dox and dexamethasone [90], Dox and gemcitabine [91], alendronate (bisphosfonate, commonly used for prevention and treatment of osteoporosis and bone metastases) and TNP-470 or alendronate and paclitaxel [92].

## I.2.1. Biocompatibility and immunocompatibility of pHPMA and HPMA copolymer conjugates

HPMA polymers were tested for many different factors important for biocompatibility. The homopolymer was found to be non-toxic up to the dose of 30 g/kg, not to bind plasma proteins and, with utmost importance, not to be immunogenic. Thus, the main limitation of HPMA-based carrier is just the lack of biodegradability. However, HPMA copolymer chains are excreted via kidney in case that their molecular weight (Mw) is below the renal excretion limit (40-45 kDa) [93].

Immunogenicity of both HPMA homopolymer and copolymers has been addressed already at the beginning of the research focused on HPMA in mouse system. Immunocompatibility is an essential parameter for any substance to be used in human medicine. Administration of immunogenic compound might be accompanied by various side-effects, which could be even life-threatening when administered repeatedly. Moreover, emerging antibodies specific for such drug would significantly limit its activity [75].

HPMA homopolymer (Mw<40 kDa) was shown to be completely non-immunogenic [94] and fully excretable with no long-term deposition in the organism. Its half-life in circulation is determined by the Mw [95]. HPMA homopolymer was found not to elicit any humoral or cellular immune response and it neither inhibited nor induced aberrant complement activation of classical or alternative pathway. The only exception was extremely high doses of HPMA homopolymer impossible to be used in treatment (20 mg/ml) [96]. HPMA copolymers with additional side chains terminated in various functional groups or drugs demonstrated some immunogenicity (almost exclusive induction of IgM, i.e. they are thymus-independent antigens). Such weak response is illustrated by four orders of magnitude lower antibody titers than reference immunogenic proteins (albumin, IgG). Composition of side chains or their content had little or no impact on antibody formation, while increasing Mw (5 kDa versus 200 kDa) of the copolymer was associated with stronger antibody-based immune response, simply explainable by prolonged circulation in blood with higher chance to meet B cells with appropriate specifity [97]. HPMA copolymer of various composition has no impact on immune response at all [93, 98]. Phagocytic and antigen-presenting capacity of macrophages and functional characteristics of bone marrow stem cells, B and T cells were not affected in mice treated with HPMA copolymers with enzymatically degradable tetrapeptide Gly-Phe-

Leu-Gly (GFLG) terminating in hydroxyl group. Both complement pathways were unaffected by the HPMA copolymers, similarly to homopolymer [99]. Finally, treatment of mice with large cumulative dose of HPMA copolymer with GFLG side chain (2 g/kg within two months) showed no toxicity and further proved that HPMA copolymers are truly biocompatible, also in case of long-term use [93, 99].

Of note, proteins conjugated to HPMA-based copolymers have significantly lowered immunogenicity in comparison to unmodified protein. Similar situation has been described for proteins which are modified by PEG, with decrease of immunogenicity also about two orders of magnitude in comparison to native protein. This effect was verified using large pool of proteins from different species (human serum albumin, bovine seminal RNAase, transferrin, chymotrypsin, superoxide dismutase, and mouse anti-Thy 1.2 monocloval antibody) [93, 100].

## I.2.2. Synthesis and structure

Two kinds of HPMA-based conjugates were developed according to the type of covalent bond between a drug and the polymer side chain terminus (Fig. 5).

The first one is represented by conjugates with oligopeptide spacer to which the drug is attached via amide bond. A variety of oligopeptides were tested, however, GFLG was used for most conjugates. Formerly, it was hypothesized that cleavage of the spacer and release of the drug from the polymer is a prerequisite for its cytotoxic effect [101-102], but later studies show certain doubts about strict requirement for drug release from its carrier to manifest its biological activity [103]. The second one is represented by conjugates with the drug bound to HPMA copolymer via hydrazone bond or *cis*-aconityl acid residue, i.e. pH-sensitive linkage. These conjugates are stable in the bloodstream (pH 7,4) but release a drug in acidic microenvironment, predominantly intracellularly in endosomes (pH 5,5-6) and lysosomes (pH 5-5,5) [104].

Basic HPMA copolymer conjugates consist of linear polymer chains of ~25 kDa to ensure that they can be easily excreted via renal filtration. HPMA copolymer conjugates with Mw higher than ~35 kDa could lead to deposition of the polymer in the organism. Unfortunately,

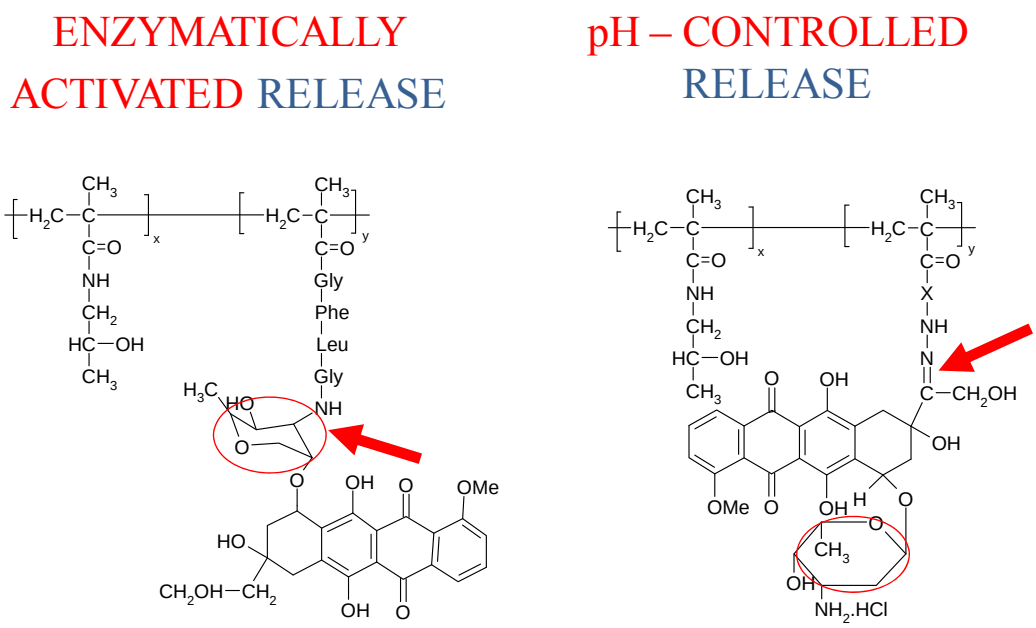



***Fig. 5:*** *Difference in structure of HPMA conjugates with oligopeptide spacer to which the drug is attached via amide bond and with the drug bound to HPMA copolymer via hydrazone bond.*

this is insufficient size for significant accumulation of the conjugate within a tumor via EPR effect. Thus, high molecular weight (HMW) HPMA copolymer carriers more suitable for such purpose have been developed. The first of these carriers was based on cross-linking short linear HPMA copolymers chains through degradable oligopeptides forming a branched structure of the carrier. This ensured the decomposition of large branched copolymer back into short linear portions by lysosomal enzymes and their swift renal clearance. Despite the improvement, several weaknesses of this system (difficult reproducibility of the synthesis, high polydispersity) led to the synthesis of more sophisticated structures containing biodegradable linkers, either with dendrimer core [73] or graft polymers with multivalent copolymer backbone and semitelechelic homopolymers as side chains [105]. These types of HMW HPMA-based carriers reported contained Dox bound via pH-sensitive hydrazone bond, while HPMA side chains were attached to the main polymer chain or dendrimer core by either enzymatically degradable tetrapeptide GFLG spacer, or reductively degradable disulfide bridges, or both.

There is also possibility to prepare a micellar structure from linear HPMA copolymers by linking of highly hydrophobic core (oleic acid, cholesterol) to the polymer chains, leading into formation of micelles in aqueous solution. These micelles have relatively narrow size

distribution, with Mw about 100-200 kDa and hydrodynamic radius of approximately 25 nm [71].

## I.2.3. HPMA copolymer-bound doxorubicin conjugates

HPMA copolymer-bound doxorubicin conjugates offer wide variety of options to manifest their primary function, i.e. eradication of tumor. There are two major groups which differ in mechanisms responsible for delivery of drug they carry to the target cells. These are either actively or non-actively targeted conjugates. While non-actively targeted HPMA copolymer conjugates rely solely on EPR effect to accumulate in the tumor, actively targeted ones bear various structures which specifically bind to target cells to facilitate site-specific drug delivery. Such conjugate is selectively taken up by the cells expressing the relevant receptor through receptor-mediated endocytosis. However, it is important to mention that conjugates carrying a targeting moiety do employ EPR effect as any other HMW prodrug. Resulting therapeutic outcome is thus additional effect of passive and active targeting in case of solid tumors. There is a completely different situation in non-solid tumors, e.g. leukemias. In such case, EPR effect has virtually no role and active targeting remains the only possibility how to selectively hit the tumor cells.

### I.2.3.1. Non-actively targeted HPMA copolymer-bound doxorubicin conjugates

$Dox^{AM}$-PHPMA (FCE 28068), also known as PK1 ("Prague-Kiel" - reflecting the two locations where the principal research work was done, Fig. 6) belong among the non-actively targeted conjugates. It was the first conjugate using linear HPMA copolymer carrier bearing Dox bound via GFLG spacer to the polymer backbone through amide bond.

$Dox^{AM}$-PHPMA possesses some of the key characteristics for polymer-bound prodrugs, i.e. prolonged circulation time in organism and reduced non-specific toxicity and improved therapeutic profile [106-107]. $Dox^{AM}$-PHPMA was shown to have anti-tumor effect both *in vitro* and *in vivo* in numerous tumor models of mouse, rat and human origin [80, 108-109].

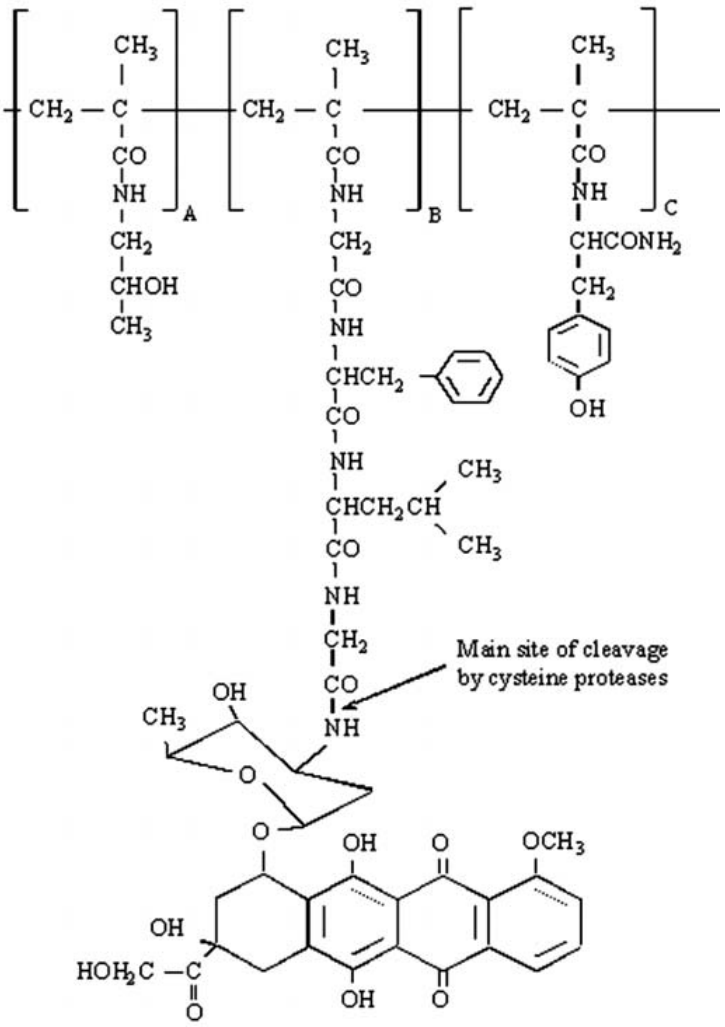



***Fig. 6:*** *Structure of PK1 (adopted from Seymour et al., 2009)*

Moreover, the accumulation of this conjugate was confirmed also in human solid tumors [110], thereby opening up its potential for clinical use. Other key representatives of non-targeted HPMA-based conjugates are linear conjugates of HPMA with pH-sensitive bond between copolymer side chain terminus and cytostatic drug. The first one had Dox coupled to HPMA polymer backbone via *cis*-aconityl linker and was shown to be active against human ovarian carcinoma cells [111]. Next, conjugates with hydrazone bond used for conjugation of the drug ($Dox^{HYD}$-PHPMA) were synthesized. These conjugates also demonstrated significant *in vitro* and *in vivo* activity in various experimental tumor models [112]. HMW conjugates either grafted or with dendrimer core showed significantly increased accumulation in tumor tissue in comparison to linear HPMA copolymer conjugates with Mw below renal excretion limit. They have also strong anti-tumor activity both *in vitro* and *in vivo* in several cell lines and murine tumor models [73, 105].

### I.2.3.2. Actively targeted HPMA copolymer-bound doxorubicin conjugates

Actively targeted HPMA copolymer-bound doxorubicin conjugates can utilize wide variety of structures as targeting moiety ranging from different receptors and lectins to monoclonal antibodies.

Targeting to receptors is represented by linear HPMA copolymer-bound conjugate bearing Dox and containing galactosamine (also named PK2 or FCE28069) [113] targeted to cells of hepatocellular carcinoma which overexpress asialoglycoprotein receptor (Fig. 7).

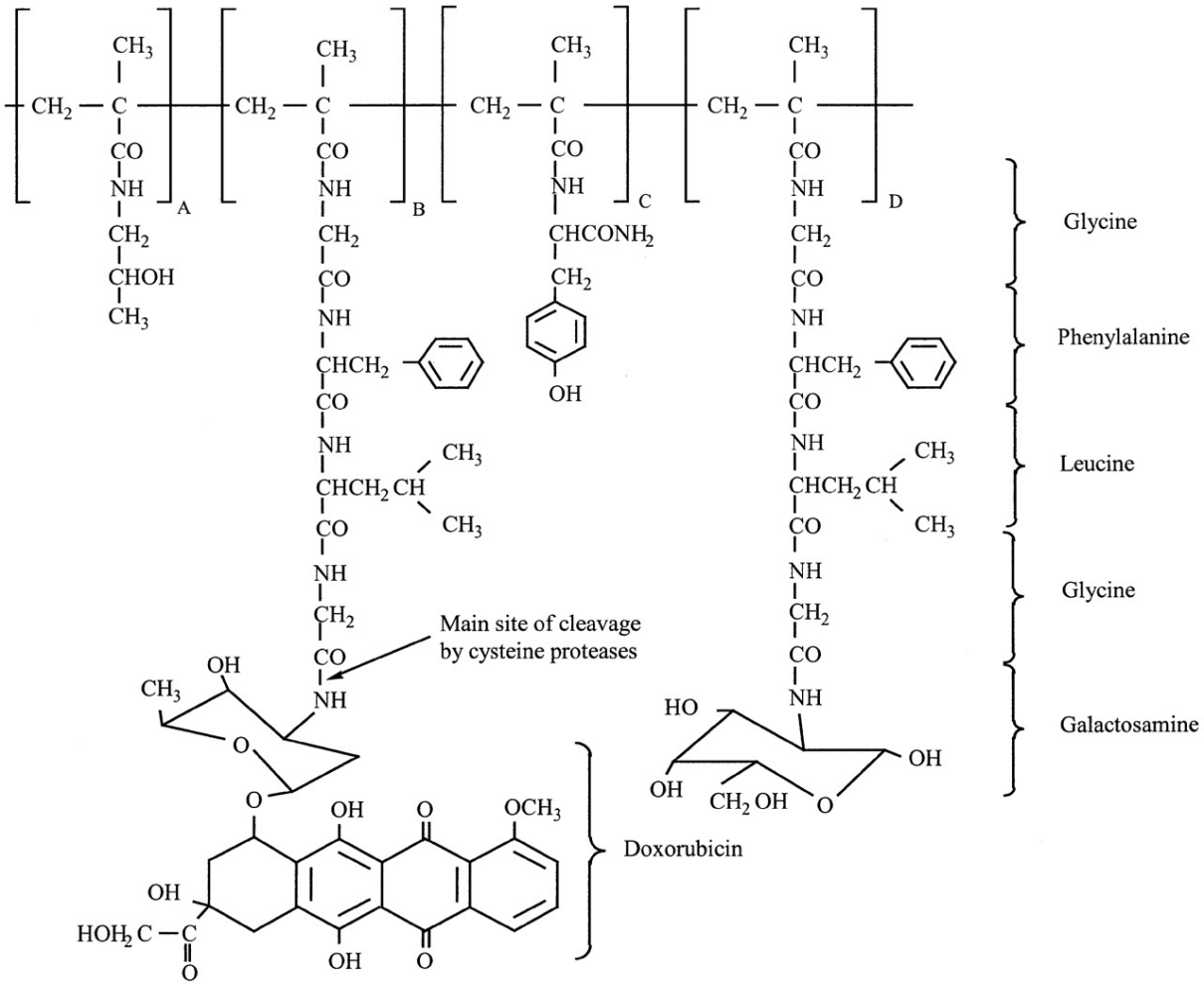



***Fig. 7:*** *Structure of PK2 (adopted from Seymour et al., 2002)*

The idea of targeting by lectins is based on the finding that cancer cells often express an altered glycosylation pattern. That led to a synthesis of conjugates, where wheat germ agglutinin (WGA) and peanut agglutinin (PNA) were bound to HPMA copolymer backbone and tested for binding to cells of colorectal cancer origin and to colon tissue. While WGA-bound HPMA conjugate showed the same binding in healthy and malignant tissue, similar

conjugate with PNA revealed higher binding activity to cancer cells, with targeting efficacy similar to the antibody [114-115].

The most straightforward targeting structures are represented by antibodies. Naturally, it is also the most often used structure for actively targeted polymer conjugates. The research of antibody-targeted HPMA copolymer conjugates led to the development of several types of conjugate structures. The original one consists of IgG molecule with its primary amino groups used to covalently bind polyvalent HPMA copolymer chains. It was called "classical structure", however, this method of synthesis had serious disadvantage that lied in the random character of polymer chain binding during synthesis. This approach could lead to significant decrease of binding activity of the antibody as binding site could be blocked by presence of copolymer chains directly linked into it or sterically hindered if copolymers were bound to the adjacent locations. Another serious problem was very broad Mw distribution of these HPMA conjugates which is mainly caused by branching of multivalent HPMA copolymer chains bound to the molecules of antibody. The problem with HPMA copolymer binding near or into the binding site of the antibody was solved by different concept, resulting initially in utilization of oxidized carbohydrate residues located in the Fc part of the antibody molecule for binding to the polymer [116]. Such approach involved relatively complicated system of synthesis and binding activity of most bound Abs were comparable to those in "classical" conjugates. The efforts to develop actively targeted HPMA copolymer conjugates with more defined structure culminated in synthesis of "star structure" (Fig. 8), where 30-40

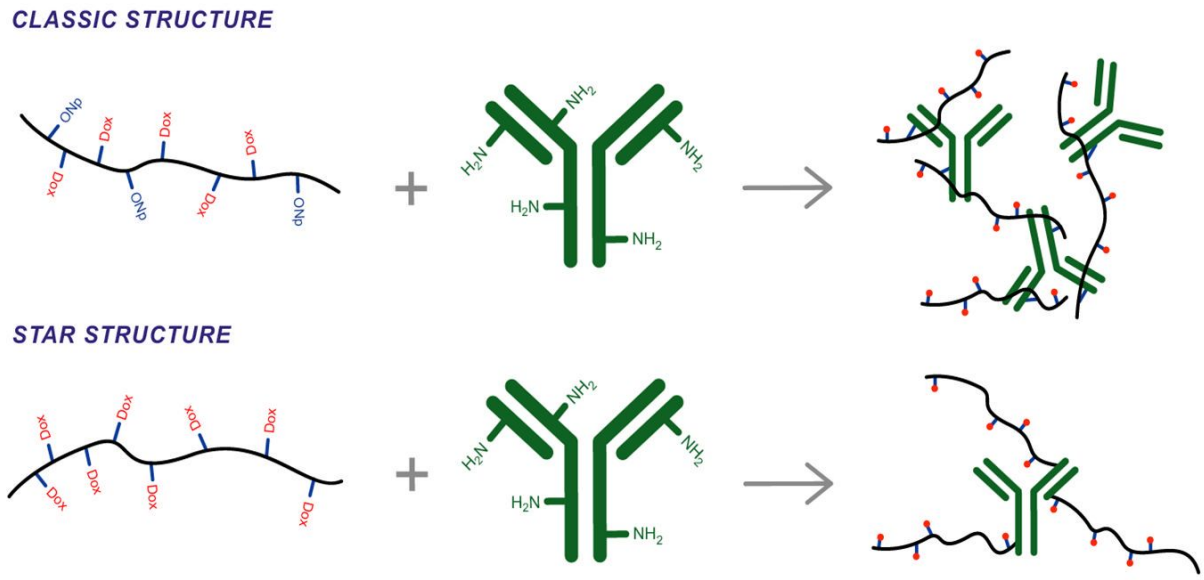



***Fig. 8:*** *Classical and star structure of HPMA copolymer-bound doxorubicin conjugate*

semitelechelic HPMA copolymer chains bearing Dox bound either via enzymatically cleavable amide bond or pH-sensitive hydrazone bond are linked to the central antibody molecule through their activated terminal carboxyl groups [117-119].

### I.2.3.3. HPMA copolymer-bound doxorubicin conjugates in clinical trials

Two HPMA copolymer-bound doxorubicin conjugates were tested as anti-cancer agents in regular clinical trials: $Dox^{AM}$-PHPMA (PK1, FCE28068) [110, 120-121] and $Dox^{AM}$-PHPMA-galactosamine (PK2, FC28069) [113, 122]. $Dox^{AM}$-PHPMA phase I included 36 patients with non-small cell lung carcinoma (NSCLC), colorectal or breast cancer. Prolonged half-life of the drug in the circulation, accumulation in the tumor, MTD of 320 mg/m$^2$ Dox equivalent i.v. and 5-fold decrease of Dox toxicity were found. Two partial and two minor clinical responses were also observed. Further, there was phase II clinical trial on 62 patients bearing NSCLC, breast, or colorectal carcinoma. These patients were given up to 8 courses of 280 mg/m$^2$ Dox equivalent i.v., which led to 6 partial responses (PR), 3 in breast cancer group and 3 in NSCLC group. All responding patients were Dox naive and no patient with colorectal carcinoma responded to the therapy. Tumor accumulation and tolerable toxicity reported from previous trial were proved. Taken together, the study confirmed that HPMA copolymer-bound Dox exert improved anti-cancer activity and could be further employed with NSCLC and breast carcinoma [80]. Unfortunately, with phase II clinical trial finished and no follow up until now, the possibilities of this conjugate to be incorporated into routine care are minimal.

$Dox^{AM}$-PHPMA-galactosamine (FCE28069) targeted to asialoglycoprotein receptor was tested in phase I/II and it showed MTD of 160 mg/m$^2$ Dox equivalent i.v. and higher accumulation in the liver in comparison to PK1 was seen. Several patients with hepatocellular carcinoma also responded with PR and/or stable disease. However, accumulation of the drug in the tumor and in the normal liver tissue was similar and led to disengagement in further clinical trials [122].

Pilot clinical study of a HPMA copolymer conjugate containing human immunoglobulin and Dox was performed in Prague. This study was done on four patients with generalized breast carcinoma after all available treatment failed [123]. In two patients, treatment with this type of conjugate was associated with continuous decrease in most of measured and previously

highly elevated tumor markers. Serum levels of some of these tumor markers returned to physiological level permanently (CRP, $\beta_2$-microglobulin, CA 72-4, ferritin), while others showed temporary normalization (CA 125 and CEA) or strong tendency towards it (CA 15-3, NSE and AFP). Further, NK and LAK cell activation was observed in peripheral blood of all patients 72 h after each of the first three treatments, as well as an increase in the numbers of $CD4^+$ and $CD16^+$ $56^+$ cells in the peripheral blood. All this was in concordance with data obtained in experimental animals pointing to a dual, i.e. cytostatic and immunomobilizing character of HPMA-based copolymer-bound Dox conjugates.

## II. AIMS OF THE THESIS

The general aim of this study was to contribute new findings to the recently established field of complexing cytokines and respective anti-cytokine mAbs, with IL-2 in particular. This main effort can be divided into more specific areas of interest:

1. Effects of IL-2/anti-IL-2 mAb immunocomplexes on various populations of immunocompetent cells, with special interest in expansion and biological activity of $CD8^+$ T cells, NK cells and $T_{reg}$ cells.
2. Half-life of IL-2/anti-IL-2 immunocomplexes in circulation in comparison to free IL-2.
3. Role of IL-2/anti-IL-2 mAb immunocomplexes in cancer immunotherapy.
4. Potential of combinatorial treatment of established progressively growing tumors with HPMA copolymer-bound Dox conjugate and IL-2/anti-IL-2 mAb immunocomplexes.

## III. PUBLICATIONS

The thesis was prepared on the basis of these publications:

Kovar M., Tomala J., Chmelova H., Kovar L., Mrkvan T., Joskova R., Zakostelska Z., Etrych T., Strohalm J., Ulbrich K., Sirova M. and Rihova B.: Overcoming immunoescape mechanisms of BCL1 leukemia and induction of $CD8^+$ T cell-mediated BCL1-specific resistance in mice cured by targeted polymer-bound doxorubicin. Cancer Res. 68 (23): 9875-83, 2008.

IF=7,856

Tomala J., Chmelova H., Mrkvan T., Rihova B., Kovar M.: In Vivo Expansion of Activated Naive $CD8^+$ T Cells and NK Cells Driven by Complexes of IL-2 and anti-IL-2 mAb as novel approach of cancer immunotherapy. J. Immunol. 183: 4904-12, 2009.

IF=5,788

Tomala J., Chmelova H., Strohalm J., Ulbrich K., Sirova M., Rihova B., Kovar M.: Antitumor activity of IL-2/anti-IL-2 mAb immunocomplexes synergizes with that of HPMA copolymer-bound doxorubicin conjugate due to its low immunosuppressive activity. Int J Cancer 129(8): 2002-12, 2011.

IF=5,444

# Overcoming Immunoescape Mechanisms of BCL1 Leukemia and Induction of CD8$^+$ T-Cell–Mediated BCL1-Specific Resistance in Mice Cured by Targeted Polymer-Bound Doxorubicin

Marek Kovar,[1] Jakub Tomala,[1] Helena Chmelova,[1] Lubomir Kovar,[1] Tomas Mrkvan,[1] Radka Joskova,[1] Zuzana Zakostelska,[1] Tomas Etrych,[2] Jiri Strohalm,[2] Karel Ulbrich,[2] Milada Sirova,[1] and Blanka Rihova[1]

[1]Department of Immunology and Gnotobiology, Institute of Microbiology and [2]Laboratory of Biomedical Polymers, Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic

## Abstract

**BALB/c mice bearing syngeneic BCL1 leukemia, a mouse model of human chronic lymphocytic leukemia, were treated with polymer-bound doxorubicin conjugate targeted with BCL1-specific monoclonal antibody. Such treatment can cure up to 100% of mice and the cured mice show long-lasting resistance to BCL1 leukemia. We show that both CD4$^+$ and CD8$^+$ T cells are required for establishment of the resistance, but only CD8$^+$ T cells are necessary for its maintenance. BCL1 cells express MHC class I and II and also costimulatory molecules CD80 and CD86, which can aid eliciting of antitumor response. On the other hand, BCL1 cells also use several immunoescape mechanisms, such as expression of PD-L1, PD-L2, and interleukin-10. BCL1 cells thus can be recognized by BCL1-specific T cells, but instead of effective priming, such T cells are anergized or deleted by apoptosis. Moreover, BCL1 leukemia progression is accompanied by robust expansion of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T ($T_{reg}$) cells. Although it has been shown that depletion of $T_{reg}$ cells in tumor-bearing mice can retard tumor growth, direct evidence that expansion of $T_{reg}$ cells can promote tumor growth was lacking. In this study, we provide first direct evidence that expanded $T_{reg}$ cells can indeed promote tumor progression by using mice with selectively expanded $T_{reg}$ cells before inoculation of BCL1 leukemia. Finally, we have also shown that elimination of some immunoescape mechanism (e.g., deletion of $T_{reg}$) can significantly improve the therapeutic outcome of chemotherapy.** [Cancer Res 2008;68(23):9875–83]

## Introduction

In this study, we used a conjugate of a synthetic, water-soluble, and biocompatible copolymer based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) with doxorubicin bound via a Gly-Phe(D,L)-Leu-Gly spacer (1, 2) and containing either human polyclonal (i.e., nonspecific) antibody or B1 monoclonal antibody (mAb). B1 mAb specifically binds with high affinity to an idiotype of surface IgM on BCL1 leukemia (3). Thus, we used a conjugate containing potent cytostatic drug specifically targeted to BCL1 cells (2), which enabled us to effectively treat mice bearing the leukemia while causing only minimal damage to the immune system (1, 4).

Because genetic instability is a hallmark of all malignant cells (5), tumors accumulate enormous number of mutations, and although that only small proportion of these occur in open reading frames of genes expressed by tumor cells, it is inevitable that any given cancer cell will express at least few new antigenic determinants that could be recognized by the immune system (6). Numerous innate and adaptive immune effector cells participate in the recognition and destruction of cancer cells, a process that is known as cancer immunosurveillance (5–7). Cancer cells can escape innate and adaptive immune either by immunosubversion or by immunoselection (8–12). Immunosubversion is a process of active suppression of the immune response by tumor cells. Tumors use numerous different mechanisms (further referenced as immunoescape mechanisms) of immunosubversion (8, 13–16), including down-regulation of MHC class I expression (9, 10) and up-regulation of expression of CD95L (16), indoleamine-2,3-dioxygenase (14, 15), and arginase-1 or transforming growth factor-β and interleukin (IL)-10 (17). Moreover, a significant expansion of regulatory T ($T_{reg}$) cells, which are capable of inhibiting both CD4$^+$ and CD8$^+$ T-cell responses, was observed during progression of many types of tumors both in mice and humans (18).

It was reported previously that conjugates based on poly(HPMA) containing doxorubicin are capable to completely cure tumor-bearing mice and that some of these mice show tumor-specific resistance (1, 4). The goal of our study was to investigate this resistance; specifically, we aimed to determine which subset of immunocompetent cells is necessary for the establishment and which for the maintenance of the resistance. Another aim was to determine which immunoescape mechanisms can be used by BCL1 cells to avoid rejection by immune system. We identified several mechanisms used by BCL1 leukemia and we also showed that their abrogation can significantly improve the therapeutic outcome of chemotherapy. Moreover, we provided the first direct evidence that mice with expanded $T_{reg}$ cells show faster tumor progression than mice with normal counts of $T_{reg}$ cells.

## Materials and Methods

**Mice.** Female BALB/c mice were obtained from the breeding colony at the Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i. Mice were used at 9 to 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.

**Cell lines.** The murine B-cell leukemia BCL1, B16F10 melanoma, and RAW264.7 cell lines were purchased from the American Type Culture

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

**Requests for reprints:** Marek Kovar, Department of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, Prague 4-Krc 14220, Prague, Czech Republic. Phone: 420-241-062-365; Fax: 420-241-721-143; E-mail: makovar@biomed.cas.cz.



doi:10.1158/0008-5472.CAN-08-1979

Collection. DC2.4 dendritic cell line and J774A.1 were kindly provided by Professor Jonathan Sprent (Garvan Institute, Sydney, Australia).

**Monoclonal antibodies.** The following anti-mouse mAbs have been used for cell surface staining: CD80-APC (eBioscience), CD86-APC (Caltag-Invitrogen), CD4-PE, CD4-PerCP, H2Kd-PE, I-A/E-PE (BD Pharmingen), CD8-PB (Serotec), CD80-biotin, CD86-biotin (Southern Biotechnology Associates), and CD25-APC. Unconjugated anti-mIL-2 mAb JES6-1A12, anti-mouse Foxp3-PE, and IFN-γ-PE mAb were purchased from eBioscience. B1 mAb was prepared in our laboratory as described earlier (2).

**Staining for surface antigens.** Single-cell suspension was prepared from spleens. After RBC lysis, cells were resuspended in flow cytometry buffer (PBS/2% FCS/2 mmol EDTA/0.05% azide), blocked by 10% mouse serum for 30 min on ice, and stained with mAbs for 30 min on ice in the dark. When biotinylated mAbs were used, cells were additionally incubated for 10 min on ice with fluorochrome-conjugated streptavidin. Cells were washed twice after each step in flow cytometry buffer and fixed in 4% paraformaldehyde before analysis.

**Intracellular staining.** Foxp3 staining buffer set (eBioscience) was used for Foxp3 staining and IC Fixation buffer and Permeabilization buffer (eBioscience) were used for IFN-γ staining. Surface antigens were stained as described above. Cells were than resuspended in 100 μL of Fixation/Permeabilization working solution and incubated for 30 to 60 min on ice followed by washing twice with Permeabilization solution. Afterwards, cells were blocked by 2% mouse serum for 10 min on ice. Next, mAb-PE conjugate was added and cells were incubated for 30 min on ice in the dark. Finally, cells were washed twice in Permeabilization buffer and fixed in 4% paraformaldehyde.

**Flow cytometry.** Flow cytometric analysis was performed on LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

**Depletion of $CD4^+$, $CD8^+$, and $CD25^+$ populations.** $CD4^+$, $CD8^+$, and $CD25^+$ subpopulations were depleted by i.p. injection of the following mAbs: αCD4 mAb (clone GK 1.5), αCD8 mAb (clone 53-6.72), and αCD25 mAb (clone PC 61.5). Two hundred fifty micrograms per mice of all mAbs were injected.

**Reverse transcription-PCR.** Total RNA was isolated from $5 \times 10^6$ cells by using 1 mL of Trizol reagent (Life Technologies-Invitrogen) according to the manufacturer's protocol. RNA (1 μg) was reverse transcribed using $oligo(dT)_{12\text{-}18}$ primer and SuperScript II RNase $H^-$ Reverse Transcriptase (Invitrogen). Resulting cDNAs were used for PCR with specific primers for mIL-10 (Genbank accession no. NM_010548), PD-L1 (Genbank accession no. NM_021893), and PD-L2 (Genbank accession no. NM_021396).

**Cytokine detection.** IL-2, IL-10, IL-12, its subunits, and sTNFRI in supernatants were detected by RayBio Mouse Cytokine Antibody Array I (RayBiotech).

**ELISA.** Concentrations of IL-10, TNF-α, and IFN-γ in supernatants were determined by Mouse IL-10, TNF-α, and IFN-γ CytoSets (Biosource-Invitrogen).

**Polymer-bound doxorubicin conjugates.** All polymer conjugates were synthesized as described previously (19, 20).

**$T_{reg}$ cell expansion.** To expand $T_{reg}$ cells *in vivo*, BALB/c mice were treated for 7 consecutive days before BCL1 cell inoculation with IL-2/JES6-1A12 mAb immunocomplexes. These immunocomplexes (21) were prepared by adding rmIL-2 into solution of anti-IL-2 mAb JES6-1A12 (both reagents were in PBS) at a molar ratio of 2:1. After 15-min incubation at room temperature, the immunocomplexes were diluted with PBS into desired concentration and i.p. injected in 250 μL into BALB/c mice. Small sample (50–100 μL) of peripheral blood was taken from tail vein 24 h after last injection, and expansion of $T_{reg}$ cells ($CD4^+CD25^+Foxp3^+$) was checked by flow cytometry.

**Western blotting.** Cells ($1\text{–}5 \times 10^7$) were washed twice with an ice-cold TBS with 1 μmol/L $Na_3VO_4$ and centrifuged ($4{,}000 \times g$, 4°C). Then, cells were resuspended in Extract buffer composed of 1% NP40 (Pierce), 1 mmol/L $Na_3VO_4$, 1 mmol/L EDTA, 2 mmol/L EGTA, 10 mmol/L NaF, 1 mmol/L DTT, 5% protease mix (Sigma), 1 mmol/L phenylmethylsulfonyl fluoride, and TBS (pH 7.4) and passed 10 times through a needle (25–30 gauge). Cell lysates were centrifuged at $4{,}000 \times g$ (4°C) and protein concentration in aspirated supernatants was determined. Protein (20 μg) was loaded per lane and run on standard SDS-PAGE using 10% polyacrylamide gel. Semidry blotting procedure with nitrocellulose membrane was performed and CD86 was detected with goat anti-mouse CD86 (Santa Cruz Biotechnology) and anti-goat horseradish peroxidase–linked IgG (Santa Cruz Biotechnology).

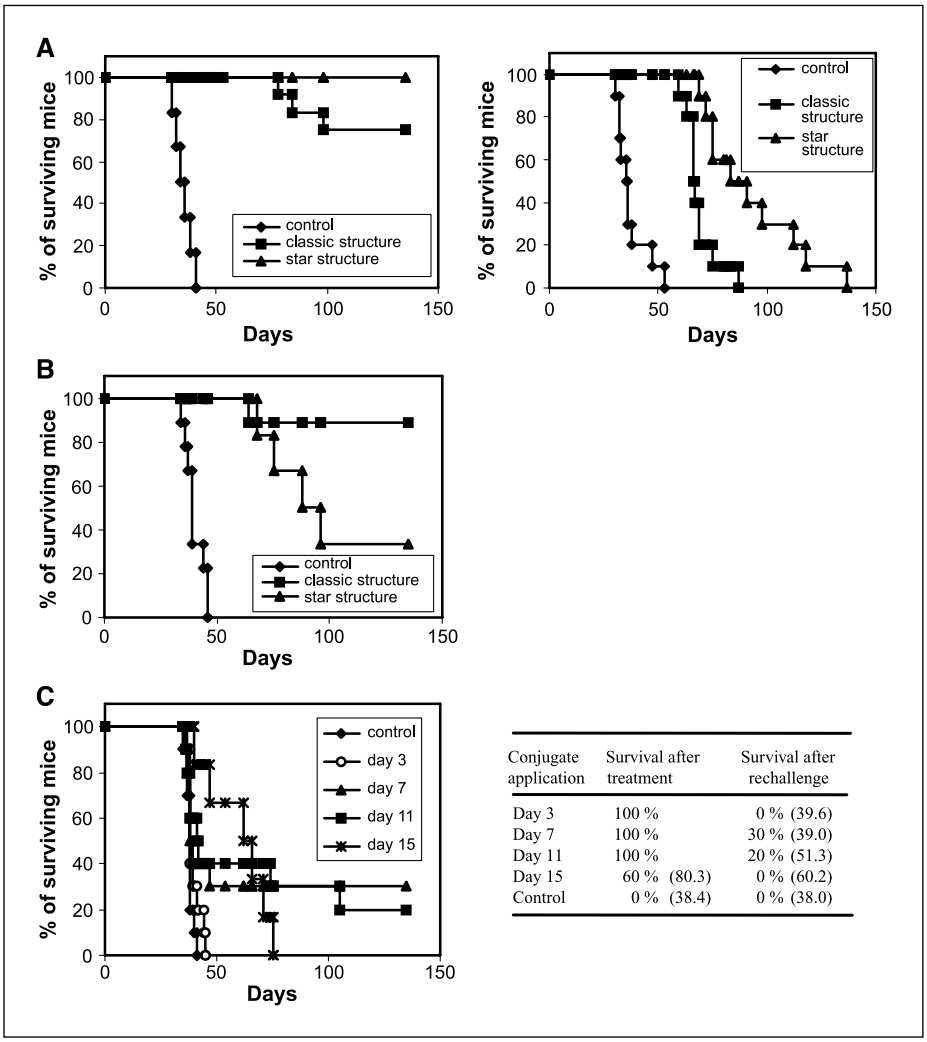
**Proliferation assay *in vitro*.** BCL1 cells were collected from an exponential growth phase culture. They were centrifuged for 5 min at $300 \times g$, washed, resuspended in fresh culture medium, and seeded into Nunc 96-well flat-bottom plates in 0.2 mL volume and $5 \times 10^4$ cells/mL density. Polymeric conjugates rmIFN-γ and rmTNF-α (R&D Systems) were then added to the wells to achieve desired concentrations. Five wells for each test condition were used. The plates were then cultured in 5% $CO_2$ for 72 h at 37°C. [$^3$H]thymidine (18.5 kBq) was added for final 16 h of cultivation before harvesting.

**Treatment of established BCL1 leukemia *in vivo*.** BCL1 cells were prepared as described for *in vitro* studies. BCL1 cells ($5 \times 10^5$) in 0.5 mL of culture medium without serum were i.p. inoculated on day 0 into BALB/c mice. HPMA copolymer-bound doxorubicin conjugates were injected i.v. on day 11 if not stated otherwise. Mice surviving day 90 without any signs of BCL1 leukemia were considered as long-term survivors (LTS). These mice were rechallenged with $10^4$ BCL1 and left without any additional treatment to determine the resistance of LTS to BCL1 leukemia.

**Statistical analysis.** The significance of any differences obtained among experimental groups was evaluated by the Student's $t$ test and significant difference was considered when $P < 0.05$.

## Results

**B1 mAb-targeted conjugates are able to cure BALB/c mice bearing BCL1 leukemia and induce BCL1-specific resistance.** B1 mAb recognizes idiotype of surface IgM on BCL1 cells and thus B1 mAb-targeted conjugates specifically kill BCL1 cells. Two structures of antibody-containing conjugates, called classic and star structure (20), were used (Supplementary Fig. S1). Cytostatic activity of B1 mAb-targeted conjugates against BCL1 cells *in vitro* was determined (Supplementary Table S1). To examine therapeutic efficacy of B1 mAb-targeted conjugates *in vivo*, BALB/c mice were i.p. inoculated with BCL1 cells and treated by i.v. injected conjugates. Two doses of star structure conjugate cured all experimental mice, whereas conjugate of classic structure cured 75% of BCL1 leukemia-bearing mice (Fig. 1*A, left*). Conjugates containing human polyclonal IgG instead of B1 mAb were not able to cure any mice, despite the fact that the treatment significantly prolonged survival (Fig. 1*A, right*). Neither free doxorubicin nor free B1 mAb was able to cure any BCL1 bearing mice even when doxorubicin was given at maximal tolerated dose (Supplementary Fig. S2). Interestingly, when the cured mice were rechallenged with a lethal dose of BCL1 cells, the mice were resistant to BCL1 leukemia (Fig. 1*B*). This resistance was observed even if the mice were reinoculated very long time after the treatment (4 months); thus, the phenomenon can be characterized as a long-lasting immune memory. The resistance is BCL1 leukemia specific because mice are not resistant to other BALB/c-derived tumors (RAW264.7 and J774A.1; data not shown). In the next step, we investigated whether the time when treatment is administered affects the strength of the induced resistance. Indeed, when the treatment is provided very early after BCL1 cell inoculation, the cured mice are not resistant to BCL1 leukemia at all (Fig. 1*C*). Similarly, treatment provided relatively late during the progression of BCL1 leukemia did not leave any mice able to survive rechallenge with BCL1 cells. The optimal time frame of treatment to establish effective resistance against BCL1 leukemia was between day 7 and 11 after BCL1 cell inoculation.




| Conjugate application | Survival after treatment | Survival after rechallenge |
|---|---|---|
| Day 3 | 100 % | 0 % (39.6) |
| Day 7 | 100 % | 30 % (39.0) |
| Day 11 | 100 % | 20 % (51.3) |
| Day 15 | 60 % (80.3) | 0 % (60.2) |
| Control | 0 % (38.4) | 0 % (38.0) |

**Figure 1.** B1 mAb-targeted conjugates are able to cure BALB/c mice bearing BCL1 leukemia and establish BCL1 resistance. *A,* BALB/c mice bearing BCL1 leukemia were treated either with B1 mAb-targeted conjugates (*left*) or conjugates containing irrelevant human polyclonal IgG (*right*) of either star or classic structure. Treatment was provided i.v. on days 11 and 14 after tumor cell inoculation ($5 \times 10^5$ i.p.) and one dose contained 5 mg/kg doxorubicin. Control mice were injected with the same volume of saline (300 μL). *B,* completely cured mice from *A* were reinoculated i.p. with lethal dose of BCL1 cells ($5 \times 10^4$) on day 110 and left without any treatment. *C,* BALB/c mice bearing BCL1 leukemia were treated with single dose of star structure of HPMA copolymer-bound doxorubicin conjugate targeted with B1 mAb. One dose containing 5 mg/kg doxorubicin was injected i.v. at selected intervals after i.p. inoculation of $5 \times 10^5$ BCL1 cells (day 3, 7, 11, or 15). The table on the right shows portion (%) of LTS (>90 d) after treatment as well as portion (%) of LTS after i.p. reinoculation of cured mice with $5 \times 10^4$ BCL1 cells (the numbers in brackets show mean survival time). All experiments were done at least twice with similar results.

**Role of different subsets of immune cells in conferring resistance to BCL1 leukemia following treatment with targeted polymeric drugs.** We hypothesized that immune system may contribute to efficient treatment of BCL1 leukemia by B1 mAb-targeted conjugates. Therefore, we compared the efficacy of the treatment in immunocompetent BALB/c mice versus immunocompromised *nu/nu* mice. *nu/nu* mice do not have thymus and thus they lack T cells, but they have B cells as well as natural killer cells. Treatment with B1 mAb-targeted conjugate at a dose that cured 50% of BALB/c mice did not cure any *nu/nu* mice (Fig. 2*A*), which implies that T cells augment the therapeutic effect. To elucidate which subset of T cells plays a key role in the resistance to BCL1 leukemia, we performed experiments on BALB/c mice with depleted $CD4^+$, $CD8^+$, or both subsets of T cells. Surprisingly, treatment with B1 mAb-targeted conjugate was more effective in $CD4^+$-depleted mice than in normal BALB/c mice (Fig. 2*B, left*). However, all mice that were $CD4^+$ depleted during treatment died on rechallenge with BCL1 cells, although significantly later than control mice (Fig. 2*B, right*). When $CD8^+$ or both $CD4^+$ and $CD8^+$ cells were depleted, establishment of BCL1-specific resistance was completely abrogated (Fig. 2*C*). To confirm that $CD8^+$ cells are essential for the resistance to BCL1 leukemia, we depleted $CD8^+$ or $CD4^+$ cells in mice that survived treatment of BCL1 leukemia with B1-targeted conjugate as well as subsequent rechallenge with BCL1 cells. Depletion of $CD8^+$ cells but not $CD4^+$ cells before second rechallenge with BCL1 cells caused abrogation of the resistance (Fig. 3*A*). $CD4^+$ cells are thus required for establishment of the resistance but $CD8^+$ cells are responsible for maintaining long-lasting BCL1-specific resistance. Next, we tried to elucidate whether BCL1-specific memory T cells can be found only in $CD8^+$ compartment or also in $CD4^+$ T-cell subset. As shown in Fig. 3*B*, both $CD8^+$ and $CD4^+$ T-cell subsets in spleen of BCL1-resistant mice contain ~1% of cells, which express IFN-γ on coculture with BCL1 cells. Almost all of these BCL1-specific $CD8^+$ and $CD4^+$ T cells are $CD44^{high}$, showing that they have phenotype of memory T cells. When splenocytes from BCL1-resistant but not from naive BALB/c mice were cocultured with BCL1 cells, very high concentrations of IFN-γ and also TNF-α were found in the supernatants (Fig. 3*C*). IFN-γ and TNF-α production was BCL1 specific, as these cytokines were not detected after coculture of splenocytes from BCL1-resistant mice with other BALB/c-derived tumor cells (J774A.1; data not shown). Once we realized that BCL1-specific T cells produce IFN-γ and TNF-α on contact with BCL1 cells, we decided to test the effect of these cytokines on proliferation of BCL1 cells. Figure 3*D* shows that proliferation of BCL1 cells is quite sensitive to IFN-γ (50% inhibition of BCL1 cell proliferation at ~1 ng/mL), whereas TNF-α has no effect on proliferation of BCL1 cells within the tested concentration range (10 pg/mL to 10 ng/mL).

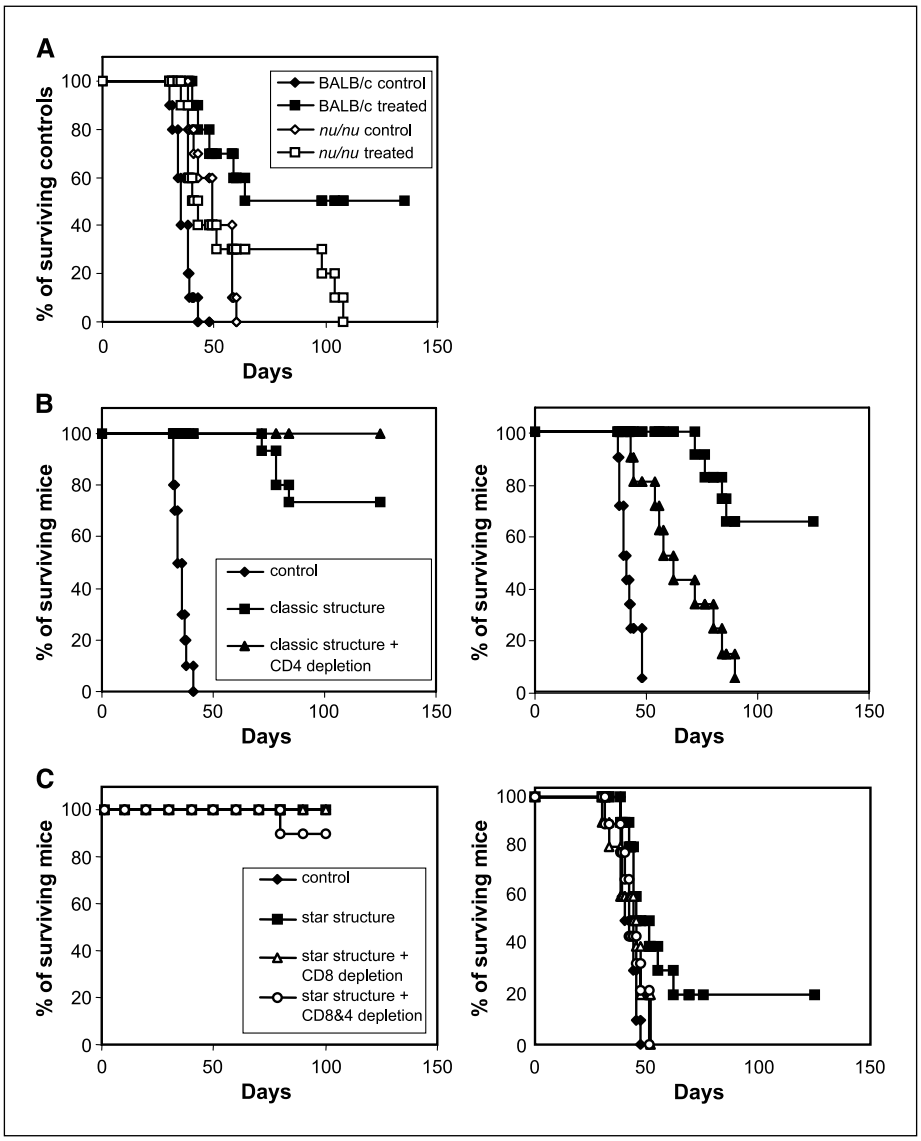



**Figure 2.** Involvement of immune system in complete eradication of BCL1 cells during treatment with B1 mAb-targeted conjugates; both $CD4^+$ and $CD8^+$ T cells are required for initiation of effective BCL1 resistance. *A, nu/nu* and BALB/c mice were i.p. inoculated with $5 \times 10^5$ BCL1 cells. Mice were i.v. injected at days 11 and 14 with classic structure of B1 mAb-targeted HPMA copolymer-bound doxorubicin conjugate (5 mg/kg doxorubicin per dose). BALB/c mice depleted of either $CD4^+$ cells (*B*), $CD8^+$ cells, or both (*C*) were inoculated as in *A. B* and *C, left,* treatment was given i.v. at day 11 (5 mg/kg doxorubicin) and day 14 (2.5 mg/kg doxorubicin). *Right,* cured mice were i.p. reinoculated with lethal dose of BCL1 cells ($5 \times 10^4$) and left without any treatment. The same symbol (*square, triangle*, and *circle*) is used for given experimental group in both right and left panels. Experiments were done twice with similar results.

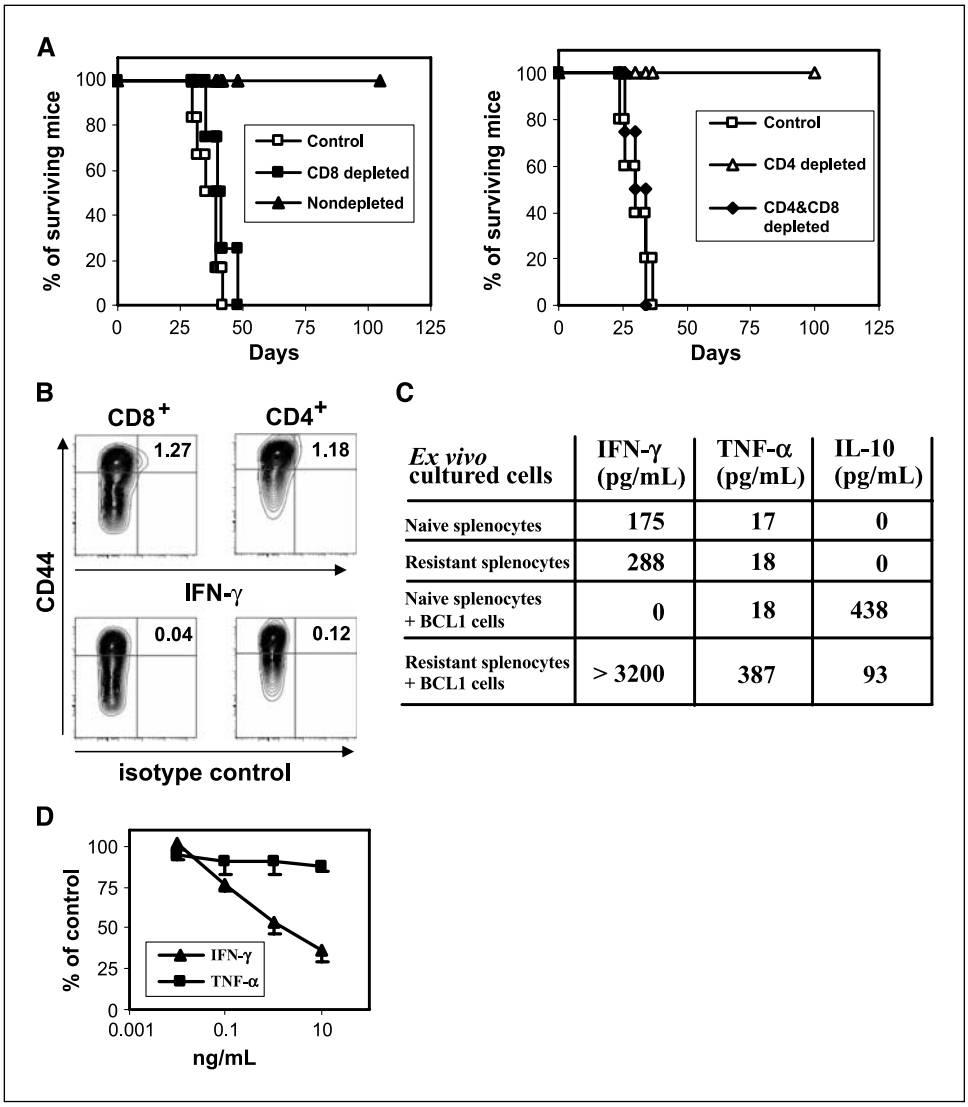
**BCL1 cells use several immunoescape mechanisms to abrogate immune response.** Because many types of cancer evade immune response by down-regulation of MHC I expression, we looked on the expression of this key molecule on BCL1 cells. Surprisingly, we found that BCL1 cells express MHC I at level comparable with normal B cells (data not shown). This applies also for MHC II and two costimulatory molecules, CD80 and CD86 (Fig. 4). CD80 expression was relatively high, whereas the expression of CD86 was intermediate. Strikingly, these data imply that BCL1 cells could work as antigen-presenting cells (APC) and thus could prime BCL1-specific $CD4^+$ and $CD8^+$ T cells. On the contrary, BCL1 cells also express PD-L1, PD-L2, and IL-10 (Fig. 4*B*). IL-10 was also detected in supernatant of *ex vivo*–cultured BCL1 cells but not in supernatant of *ex vivo*–cultured splenocytes from normal BALB/c mice (Fig. 4*B, right*). Furthermore, we found that level of IL-2 was significantly decreased in serum of mice bearing BCL1 leukemia (Fig. 4*C*). Serum of BCL1 leukemia-bearing mice contained the same level of IL-12 (detected as p70) as serum of healthy mice. Significant increase in serum level of IL-12 was observed when this cytokine was detected as p40/p70, but only in serum from mice bearing BCL1 leukemia. This implies that the serum contained p40 dimers, which are potent IL-12 antagonists. Next, we decided to examine whether BCL1 leukemia has any effect on population of $T_{reg}$ cells, which are known to hamper antitumor immunity by suppressing both $CD4^+$ and $CD8^+$ T-cell responses. As shown in Fig. 5*A*, relative number of $T_{reg}$ cells sharply increased during progression of BCL1 leukemia. Determination of $T_{reg}$ cells was based on $CD25^+Foxp3^+$ double positivity in $CD4^+$-gated cells. We showed that the increase of $T_{reg}$ cells is not only relative (Fig. 5*B, left* and *middle*) and that also absolute number of $T_{reg}$ cells increased (Fig. 5*B, right*). No significant increase of $CD4^+CD25^+Foxp3^-$ (i.e., activation of naive $CD4^+CD25^-$ cells) was observed. Then, we performed experiment when we either deliberately expanded or depleted population of $T_{reg}$ cells in BALB/c mice before inoculation of BCL1 cells. Mice with expanded $T_{reg}$ cell population ($\sim$40% of $CD4^+$ cells) died significantly earlier than control mice, whereas mice depleted from $T_{reg}$ cells died significantly later (Fig. 5*C* and *D*). This finding strongly suggests that BCL1 leukemia uses expansion of $T_{reg}$ cell population to protect itself from immune response.

**Elimination of $T_{reg}$ cells improves treatment with suboptimal dose of B1 mAb-targeted conjugate.** We examined whether abrogation of some immunoescape mechanisms of BCL1 leukemia (i.e., depletion of $T_{reg}$ cells) could increase therapeutic activity of B1 mAb-targeted conjugate. BCL1-bearing mice depleted from $T_{reg}$ cells were treated with suboptimal dose of B1 mAb-targeted conjugate. Indeed, treatment was more effective in $T_{reg}$ cell–depleted mice than in control mice containing normal numbers of $T_{reg}$ cells (Fig. 6*A*). This effect is rather modest than strong (three versus one cured mouse), but on the other hand, the experiment was done twice using relatively large experimental groups (eight mice), and thus we believe that elimination of $T_{reg}$ cells indeed improves treatment with suboptimal dose of B1 mAb-targeted conjugate. All mice cured in these two experiments were resistant to BCL1 leukemia, which was proved by rechallenge of these mice with lethal dose of BCL1 cells (data not shown).

## Discussion

The conjugates based on poly(HPMA) containing doxorubicin as cancerostatic drug and their antitumor activity were previously described in several different tumor models (1, 2, 4). We and others have previously highlighted the advantage of use of such conjugates in comparison with free drug, and now we show some interesting features and important immunologic mechanisms induced by the treatment with this novel class of anticancer drugs.

Single-dose treatment of BALB/c mice at various time points after BCL1 cell inoculation showed that the treatment must be given in optimal time frame (day 7–11) to establish BCL1-specific protective immunity in cured mice. As we show in Supplementary Table S2, a small mass of death tumor cells could be a reason explaining ineffective establishment of protective antitumor immunity in mice cured too early after tumor cell inoculation. Although this study was done with EL4 T-cell lymphoma, we believe that the results are also applicable to BCL1 leukemia as we have strong experience with both these cell lines and we know that the pattern of anti-EL4–specific and anti-BCL1–specific protective immunity is very identical. We also checked whether the failure to establish effective anti-BCL1 leukemia when the treatment is given too late could be caused by increased numbers of $T_{reg}$ cells. However, this explanation is not the right one, as $T_{reg}$ cell numbers remain constant at least until day 15 after i.p. inoculation of $5 \times 10^5$ BCL1 cells at day 0 (Supplementary Fig. S3).




| *Ex vivo* cultured cells | IFN-γ (pg/mL) | TNF-α (pg/mL) | IL-10 (pg/mL) |
|---|---|---|---|
| Naive splenocytes | 175 | 17 | 0 |
| Resistant splenocytes | 288 | 18 | 0 |
| Naive splenocytes + BCL1 cells | 0 | 18 | 438 |
| Resistant splenocytes + BCL1 cells | > 3200 | 387 | 93 |

**Figure 3.** $CD8^+$ T cells are essential for long-lasting resistance to BCL1 leukemia. *A,* BALB/c mice completely cured from BCL1 leukemia with B1 mAb-targeted conjugate were i.p. reinoculated with $5 \times 10^4$ BCL1 cells. *Left,* LTS (>90 d) were either depleted from $CD8^+$ cells or not and again i.p. reinoculated with $5 \times 10^4$ BCL1 cells; *right,* LTS that were depleted either from $CD4^+$ cells or from both $CD8^+$ and $CD4^+$ cells. *B,* spleen cells from LTS were cocultivated under standard conditions (37°C, 5% $CO_2$, humidified atmosphere) for 7 h with BCL1 cells. Brefeldin A was added for the last 5 h of incubation (1 μmol/L). Cells were then stained for extracellular markers, washed, and fixed. Next, cells were permeabilized and stained for mouse IFN-γ. *C,* splenocytes isolated from LTS or naive BALB/c mice were cultured either alone or with BCL1 cells. After 24 h, supernatants were collected and concentration of selected cytokines was measured by ELISA. *D,* BCL1 cells were isolated from spleens of BALB/c mice bearing BCL1 leukemia (>94% purity). rmTNF-α and rmIFN-γ were added at desired concentrations and cells were incubated under standard condition for 72 h. [$^3$H]thymidine (18.5 kBq) was added for the last 16 h. Cell proliferation for each experimental condition is expressed as % of [$^3$H]thymidine incorporation into control cells (higher than 20,000 cpm/well in all experiments). Each experiment was done twice with similar results.

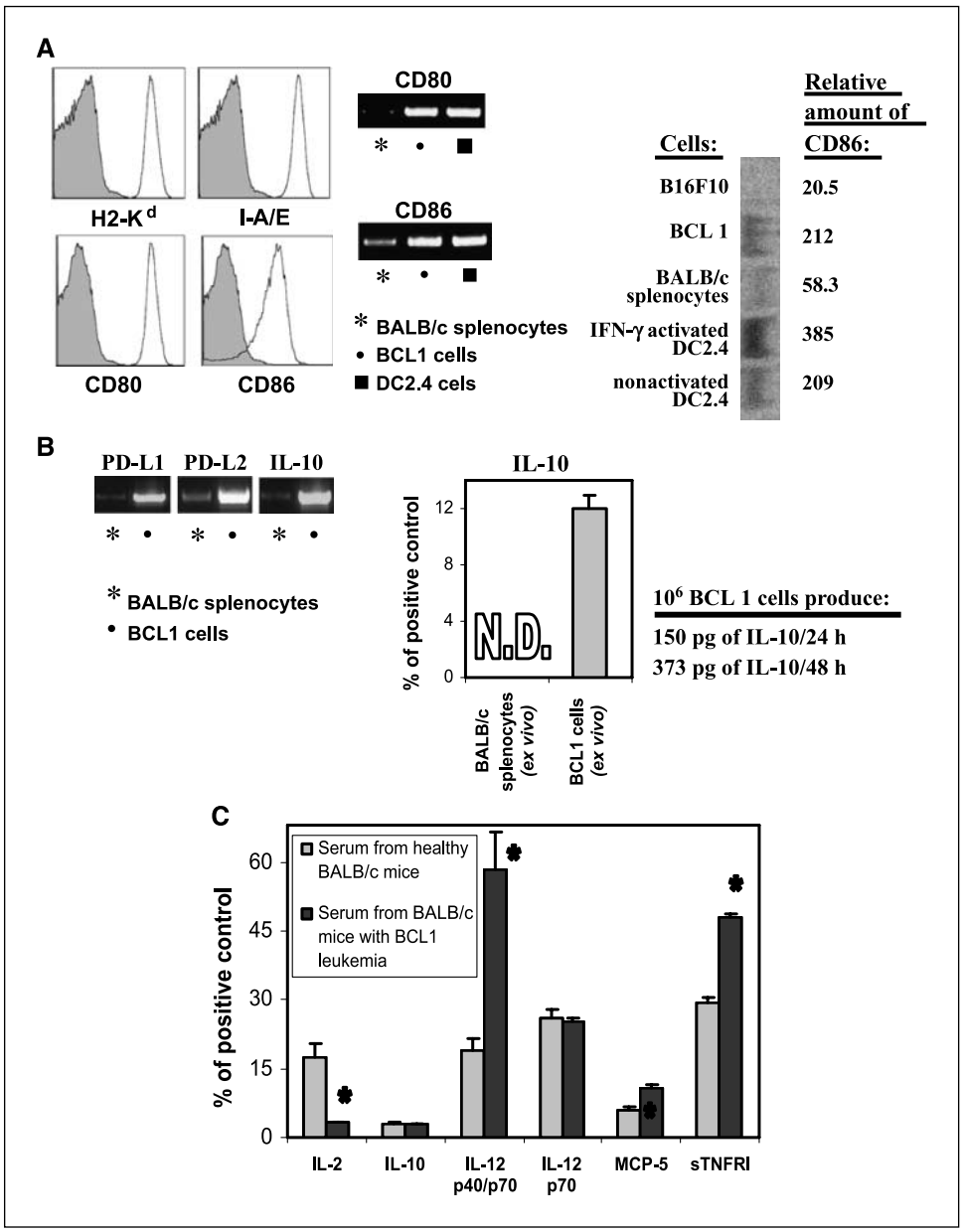



**Figure 4.** Phenotype of BCL1 cells resembles phenotype of APCs with tolerogenic activity. *A,* BCL1 cells express both MHC class I as well as MHC class II and also two major costimulatory molecules, CD80 and CD86 (*gray*, isotype control). Expression was detected by either flow cytometry (*left*) or RT-PCR (*middle*). *Right,* detection of CD86 molecule was carried out also by Western blotting of BCL1 cell lysates as well as in other different cell types. Membrane fraction was isolated from each type of cells and protein concentration was measured. The same amount of membrane fraction (in terms of protein content) from each sample was used for analysis. Relative amount of CD86 in analyzed samples was measured by densitometry. DC2.4 is an established cell line of dendritic cells with semimature phenotype, which can be maturated by IFN-γ. *B,* expression of selected molecules detected by RT-PCR in splenocytes from naive BALB/c mice (*) and BCL1 cells (•). RayBio Mouse Cytokine Antibody Array was used to detect various cytokines (see Materials and Methods) either in the supernatant of *ex vivo*–cultured BCL1 cells (*B, right*) or in the serum (*C*) of healthy BALB/c mice versus BALB/c mice bearing BCL1 leukemia. *, significant difference ($P < 0.05$) determined by Student's *t* test. *B, far right,* production of IL-10 by *ex vivo*–cultured BCL1 was measured by ELISA using rmIL-10 as a standard. Experiments were done twice with similar results.

The role of $CD8^+$ cells in effective antitumor response is well established, and particularly in BCL1 model, $CD8^+$ cells were shown to be vital for the allogeneic elimination of clonogenic leukemia cells (22). Once we confirmed that $CD8^+$ T cells are indeed the essential subset responsible for long-lasting BCL1-specific immune memory, we focused our interest on possible interactions of BCL1 cells with immune system and immunoescape mechanisms of this tumor. First, we found that BCL1 cells do not mask against immune system by down-regulation of MHC class I expression, which is a common mechanism of many tumors to evade immune system (9, 10). Moreover, BCL1 cells express not only MHC class I but also MHC class II and two main costimulatory molecules CD80 and CD86. Because coexpression of these molecules on tumor cells is rather striking, we confirmed this finding by three different methods [i.e., by flow cytometry, reverse transcription-PCR (RT-PCR), and Western blotting; Fig. 4*A*]. The BCL1 cells could therefore work as APCs and thus should be capable of priming BCL1-specific $CD4^+$ and $CD8^+$ T cells. To figure out how can BCL1 cells escape self-rejection while expressing molecules involved in T-cell priming, we intensively searched for molecules that are known to impair T-cell activation. We found that BCL1 cells express PD-L1, PD-L2, and also IL-10. It is well established that PD-L1 and/or PD-L2 expression on APCs renders those APCs strongly tolerogenic because engagement of PD-L1 or PD-L2 with their receptor PD on T cells causes either anergy or apoptosis of these T cells (23, 24). IL-10 has immunosuppressive activity on T cells, both indirectly via modulation of dendritic cell (25) and also in direct fashion (26, 27), particularly by inhibiting proliferation as well as cytokine synthesis by $CD4^+$ cells. In addition, IL-10 inhibits the monocytic production of IL-12, an essential mediator for the development of effector functions of $CD8^+$ T cells (28–31). Furthermore, the presence of IL-10 during the activation of $CD4^+$ cells results in the development of regulatory phenotype of these cells (32). Increased levels of IL-10 were not found in the serum of BALB/c mice bearing BCL1

leukemia, showing that IL-10 produced by BCL1 cells works only in paracrine fashion. However, we found that serum of BALB/c mice bearing BCL1 leukemia had significantly decreased IL-2 level, which is also an unfavorable condition for T-cell priming. More importantly, serum of BALB/c mice bearing BCL1 leukemia contained p40 dimers, strong IL-12 antagonists (33, 34), which can severely impair the effector functions of $CD8^+$ T cells (30). In conclusion, BCL1 cells show a phenotype of tolerogenic APCs (i.e., use rather unique immunoescape strategy while taking advantage of their ability to make cell-cell contact with T cells and anergize or delete those that are BCL1 specific).

Naturally arising $CD25^+$ $CD4^+$ $T_{reg}$ cells, which constitute 5% to 10% of peripheral $CD4^+$ T cells in normal rodents and humans, are produced in the thymus as a functionally mature and distinct subpopulation of T cells (18, 35). Constitutive high expression of CD25 plus forkhead winged-helix (Foxp3) transcription factor (36) is a typical feature of $T_{reg}$ cells distinguishing them from recently activated T cells, which also express CD25. They play key roles not only in the maintenance of immunologic self-tolerance (i.e., prevention of autoimmunity; refs. 37–39) but also in the control of aberrant or excessive immune responses to various invading infections (40, 41). A significant role for $T_{reg}$ cells has also been implicated in abrogating effective antitumor immunity (42). In this study, we showed that progression of BCL1 leukemia is accompanied by gradual increase of $T_{reg}$ cells (Fig. 5*A* and *B*). The increase in $T_{reg}$ cell numbers is relatively common for many other types of

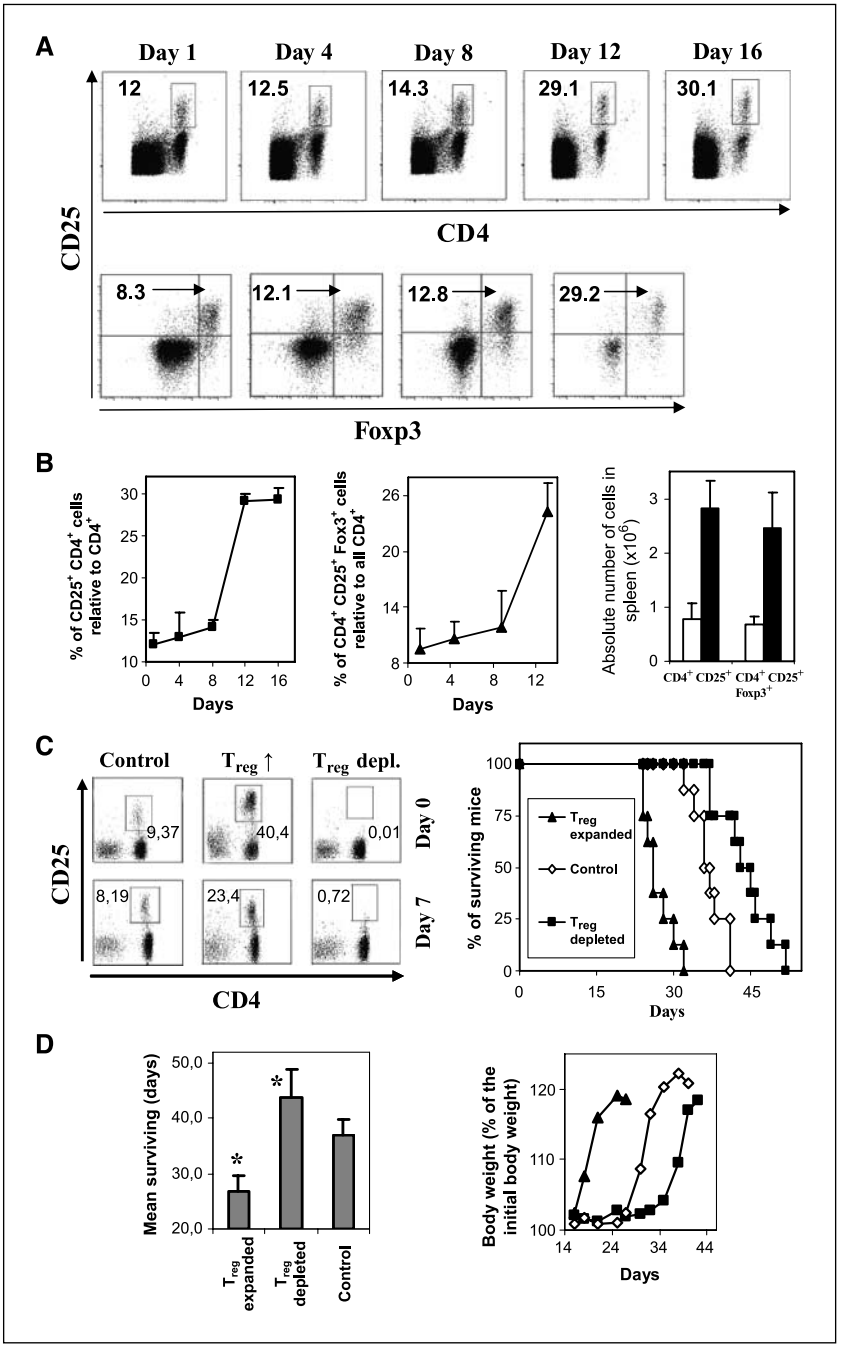



**Figure 5.** Robust expansion of $T_{reg}$ cells during progression of BCL1 leukemia: another immunoescape mechanism used by BCL1 leukemia. *A,* BALB/c mice were i.v. inoculated with $5 \times 10^6$ BCL1 cells. Mice were euthanized at different time points after BCL1 cell inoculation and $T_{reg}$ cells were detected either as $CD4^+CD25^+$ or $CD4^+CD25^+Foxp3^+$ cells (*top* or *bottom row*, respectively) by flow cytometry. Number in each dot plot shows their ratio (%) to all $CD4^+$ cells. Each dot plot shows one representative mouse of three experimental animals. *B,* kinetics of relative increase of $CD4^+CD25^+$ (*left*) and $CD4^+CD25^+Foxp3^+$ (*middle*) cell populations in BALB/c mice inoculated as in *A* (four mice per each experimental group) and absolute numbers of $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ cells (*black columns*) in spleen at day 12 (*white columns*). *White columns,* control mice. *C,* three groups of BALB/c mice, each with different size of $T_{reg}$ cell population, were i.p. inoculated with $5 \times 10^5$ BCL1 cells at day 0. Control group was not manipulated in $T_{reg}$ cell population. Next group was depleted from $T_{reg}$ cells (*$T_{reg}$ depleted*) and third group had strongly expanded $T_{reg}$ cell population (*$T_{reg}$ expanded*). *Left,* size of $T_{reg}$ cell populations in these three experimental groups at days 0 and 7; *right,* survival of mice in each experimental group. *D,* mean survival time (*left*) and an increase in body weight reflecting the progression of the disease (*right*) among the experimental groups described above in *C*. *, statistically significant difference ($P < 0.05$, Student's *t* test). Experiments were done twice with identical results.

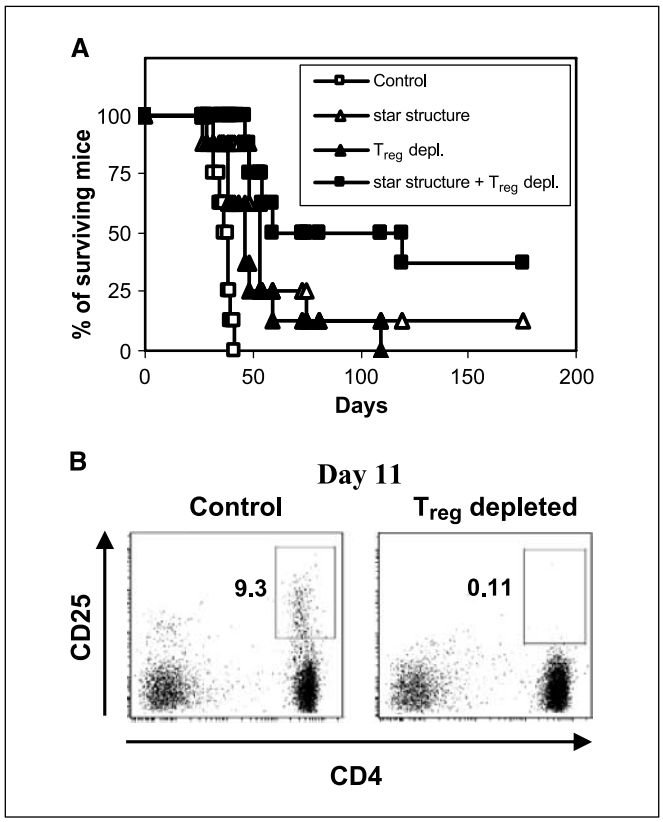



**Figure 6.** Poor therapeutic effect of suboptimal dose of B1 mAb-targeted conjugate can be increased by depletion of $T_{reg}$ cells. *A,* BALB/c mice were i.p. inoculated with $5 \times 10^5$ BCL1 cells at day 0 and distributed into four groups, each containing eight mice. Control group was left untreated, whereas mice in two other groups were i.p injected with anti-CD25 mAb (150 μg/mice) on day 8. *B,* efficiency of $T_{reg}$ cell depletion was checked by flow cytometry from small samples of peripheral blood taken at day 11. On the same day, one group with depleted $T_{reg}$ cells and one group with normal $T_{reg}$ cell population were i.v. treated with single suboptimal dose (3 mg/kg doxorubicin) of B1 mAb-targeted conjugate (star structure). Survival of mice in each experimental group was carefully recorded. Experiments were done twice with similar results.

tumors and can be seen both in humans and rodents (43, 44). Depletion of $T_{reg}$ cells by anti-CD25 mAb was shown to augment antitumor immunity in several models (44–47). Such depletion experiments were thus far the only evidence that expanded population of $T_{reg}$ cells impedes antitumor immunity and thus promotes tumor progression. Here, we report first direct evidence that an increase of $T_{reg}$ cells accelerates tumor progression (Fig. 5*C* and *D*). We could provide such evidence because we were able to deliberately expand $T_{reg}$ cell population *in vivo* by use of immunocomplexes of rmIL-2 and anti-mouse IL-2 mAb JES6-1A12, which were previously shown to stimulate the expansion of $T_{reg}$ cells (21, 48), before inoculation of BCL1 cells, which indeed exacerbated the disease. In addition, we also used mice depleted from $T_{reg}$ cells, which showed slower progression of BCL1 leukemia than control untreated mice as expected. Finally, we showed that the depletion of $T_{reg}$ cells significantly enhances the antitumor activity of our targeted conjugate (Fig. 6*A*). In sum, we showed that the increase of $T_{reg}$ cells accelerated the progression of BCL1 leukemia and thus could be considered as another immunoescape mechanism used by BCL1 leukemia. It is of note that the increased population of $T_{reg}$ cells may explain the significantly lower serum concentration of IL-2 in BCL1 leukemia-bearing mice compared with healthy mice (Fig. 4*C*). $T_{reg}$ cells are IL-2 dependent (49) and can use even very low concentration of IL-2. Higher number of $T_{reg}$ cells using IL-2 thus can cause a substantial decrease of IL-2 in the serum.

About the establishment of BCL1 resistance triggered by the therapy with targeted conjugates, we hypothesize that the treatment causes a massive death of BCL1 cells, whereas a considerable amount of cell fragments and other material is released and available for cross-presentation on dendritic cells. It is of special note that our targeted conjugate has negligible immunosuppressive activity (1, 4), and thus, nonimpaired immune system is ready to elicit BCL1-specific response. As we showed in this study, BCL1 cells are theoretically capable of antigen presentation to both $CD4^+$ and $CD8^+$ T cells but in tolerogenic/proapoptotic fashion. Thus, the cross-presentation of BCL1-specific antigens on host dendritic cells is particularly important to establish BCL1-specific resistance.

In conclusion, our results show that identifying the immunoescape mechanisms used by the particular tumor to evade immune response could be very important for the therapy. Elimination of these mechanisms most likely augments standard treatment procedure and can lead to complete remission with an establishment of long-lasting resistance to the tumor. Here, we showed that it could be possible to turn a tumor into its own cellular vaccine by combination of chemotherapy and immunomodulation.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 5/27/2008; revised 8/20/2008; accepted 9/2/2008.

**Grant support:** MEYS CR 1M 0505 (M. Kovar, J. Tomala, H. Chmelova, L. Kovar, M. Sirova, and B. Rihova), GACR 301/07/P192 (M. Kovar), GACR 310/08/H077 (J. Tomala, R. Joskova, and Z. Zakostelska), and IAAX00500803 (T. Etrych, J. Strohalm, and K. Ulbrich). The work was also supported by Institutional Research Concept AV 02 502 00 510.

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

## References

1. Sirova M, Strohalm J, Subr V, et al. Treatment with HPMA copolymer-based doxorubicin conjugate containing human immunoglobulin induces long-lasting systemic anti-tumour immunity in mice. Cancer Immunol Immunother 2007;56:35–47.
2. Kovar M, Mrkvan T, Strohalm J, et al. HPMA copolymer-bound doxorubicin targeted to tumor-specific antigen of BCL1 mouse B cell leukemia. J Control Release 2003;92:315–30.
3. Slavin S, Strober S. Spontaneous murine B-cell leukaemia. Nature 1978;272:624–6.
4. Rihova B, Strohalm J, Kovar M, et al. Induction of systemic antitumour resistance with targeted polymers. Scand J Immunol 2005;62 Suppl 1:100–5.
5. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
6. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Adv Immunol 2006;90:1–50.
7. Burnet FM. The concept of immunologic surveillance. Prog Exp Tumor Res 1970;13:1–27.
8. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329–60.
9. So T, Takenoyama M, Mizukami M, et al. Haplotype loss of HLA class I antigen as an escape mechanism from immune attack in lung cancer. Cancer Res 2005;65:5945–52.
10. Atkins D, Breuckmann A, Schmahl GE, et al. MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma. Int J Cancer 2004;109:265–73.

**11.** Medema JP, de Jong J, Peltenburg LT, et al. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci U S A 2001;98:11515–20.

**12.** Ochsenbein AF. Immunological ignorance of solid tumors. Springer Semin Immunopathol 2005;27:19–35.

**13.** Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 2005;5:641–54.

**14.** Uyttenhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003;9:1269–74.

**15.** Terness P, Bauer TM, Rose L, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. J Exp Med 2002;196:447–57.

**16.** Igney FH, Behrens CK, Krammer PH. CD95L mediates tumor counterattack *in vitro* but induces neutrophil-independent tumor rejection *in vivo*. Int J Cancer 2005;113:78–87.

**17.** Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;5:263–74.

**18.** Sakaguchi S. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531–62.

**19.** Ulbrich K, Subr V, Strohalm J, Plocova D, Jelinkova M, Rihova B. Polymeric drugs based on conjugates of synthetic and natural macromolecules. I. Synthesis and physico-chemical characterisation. J Control Release 2000;64:63–79.

**20.** Kovar M, Strohalm J, Etrych T, Ulbrich K, Rihova B. Star structure of antibody-targeted HPMA copolymer-bound doxorubicin: a novel type of polymeric conjugate for targeted drug delivery with potent antitumor effect. Bioconjug Chem 2002;13:206–15.

**21.** Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science 2006;311:1924–7.

**22.** Weiss L, Reich S, Slavin S. Allogeneic cell therapy in murine B-cell leukemia (BCL1). 2. The role of non-activated and rIL-2-activated $CD4^+$ and $CD8^+$ T cells in immunotherapy for leukemia. Cytokines Cell Mol Ther 1999;5:153–8.

**23.** Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. Curr Opin Immunol 2007;19:309–14.

**24.** Keir ME, Freeman GJ, Sharpe AH. PD-1 regulates self-reactive $CD8^+$ T cell responses to antigen in lymph nodes and tissues. J Immunol 2007;179:5064–70.

**25.** Bellinghausen I, Konig B, Bottcher I, Knop J, Saloga J. Inhibition of human allergic T-helper type 2 immune responses by induced regulatory T cells requires the combination of interleukin-10-treated dendritic cells and transforming growth factor-β for their induction. Clin Exp Allergy 2006;36:1546–55.

**26.** Joss A, Akdis M, Faith A, Blaser K, Akdis CA. IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. Eur J Immunol 2000;30:1683–90.

**27.** Akdis CA, Joss A, Akdis M, Blaser K. Mechanism of IL-10-induced T cell inactivation in allergic inflammation and normal response to allergens. Int Arch Allergy Immunol 2001;124:180–2.

**28.** Filatenkov AA, Jacovetty EL, Fischer UB, Curtsinger JM, Mescher MF, Ingulli E. CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. J Immunol 2005;174:6909–17.

**29.** Schmidt CS, Mescher MF. Peptide antigen priming of naive, but not memory, CD8 T cells requires a third signal that can be provided by IL-12. J Immunol 2002;168:5521–9.

**30.** Valenzuela J, Schmidt C, Mescher M. The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. J Immunol 2002;169:6842–9.

**31.** Valenzuela JO, Hammerbeck CD, Mescher MF. Cutting edge: Bcl-3 up-regulation by signal 3 cytokine (IL-12) prolongs survival of antigen-activated CD8 T cells. J Immunol 2005;174:600–4.

**32.** Horwitz DA, Zheng SG, Gray JD. The role of the combination of IL-2 and TGF-β or IL-10 in the generation and function of $CD4^+$ $CD25^+$ and $CD8^+$ regulatory T cell subsets. J Leukoc Biol 2003;74:471–8.

**33.** Martens E, Alloza I, Scott CJ, Billiau A, Vandenbroeck K. Protein disulfide isomerase-mediated cell-free assembly of recombinant interleukin-12 p40 homodimers. Eur J Biochem 2000;267:6679–83.

**34.** Stallmach A, Marth T, Weiss B, et al. An interleukin 12 p40-IgG2b fusion protein abrogates T cell mediated inflammation: anti-inflammatory activity in Crohn's disease and experimental colitis *in vivo*. Gut 2004;53:339–45.

**35.** Itoh M, Takahashi T, Sakaguchi N, et al. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol 1999;162:5317–26.

**36.** Ono M, Yaguchi H, Ohkura N, et al. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. Nature 2007;446:685–9.

**37.** Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 1996;184:387–96.

**38.** Sakaguchi S, Toda M, Asano M, Itoh M, Morse SS, Sakaguchi N. T cell-mediated maintenance of natural self-tolerance: its breakdown as a possible cause of various autoimmune diseases. J Autoimmun 1996;9:211–20.

**39.** Sakaguchi S, Ono M, Setoguchi R, et al. $Foxp3^+$ $CD25^+$ $CD4^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev 2006;212:8–27.

**40.** Rouse BT. Regulatory T cells in health and disease. J Intern Med 2007;262:78–95.

**41.** Taylor MD, Harris A, Babayan SA, et al. CTLA-4 and $CD4^+$ $CD25^+$ regulatory T cells inhibit protective immunity to filarial parasites *in vivo*. J Immunol 2007;179:4626–34.

**42.** Zhou G, Levitsky HI. Natural regulatory T cells and *de novo*-induced regulatory T cells contribute independently to tumor-specific tolerance. J Immunol 2007;178:2155–62.

**43.** Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 2003;9:606–12.

**44.** Chaput N, Darrasse-Jeze G, Bergot AS, et al. Regulatory T cells prevent CD8 T cell maturation by inhibiting CD4 Th cells at tumor sites. J Immunol 2007;179:4969–78.

**45.** Viehl CT, Moore TT, Liyanage UK, et al. Depletion of $CD4^+CD25^+$ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. Ann Surg Oncol 2006;13:1252–8.

**46.** El Andaloussi A, Han Y, Lesniak MS. Prolongation of survival following depletion of $CD4^+CD25^+$ regulatory T cells in mice with experimental brain tumors. J Neurosurg 2006;105:430–7.

**47.** Yoshimura K, Laird LS, Chia CY, et al. Live attenuated *Listeria monocytogenes* effectively treats hepatic colorectal cancer metastases and is strongly enhanced by depletion of regulatory T cells. Cancer Res 2007;67:10058–66.

**48.** Boyman O, Surh CD, Sprent J. Potential use of IL-2/anti-IL-2 antibody immune complexes for the treatment of cancer and autoimmune disease. Expert Opin Biol Ther 2006;6:1323–31.

**49.** de la Rosa M, Rutz S, Dorninger H, Scheffold A. Interleukin-2 is essential for $CD4^+CD25^+$ regulatory T cell function. Eur J Immunol 2004;34:2480–8.

# In Vivo Expansion of Activated Naive CD8$^+$ T Cells and NK Cells Driven by Complexes of IL-2 and Anti-IL-2 Monoclonal Antibody As Novel Approach of Cancer Immunotherapy[1]

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

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

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

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

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

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

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

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

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

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

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

## Materials and Methods

### *Mice*

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

### *Cell lines and mAbs*

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

### *Staining for surface Ags*

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

### *Adoptive transfer of OT-I cells*

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

### *$^{131}I$ labeling of IL-2*

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

### *Proliferation assay in vitro*

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

## Results

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

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

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

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

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

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

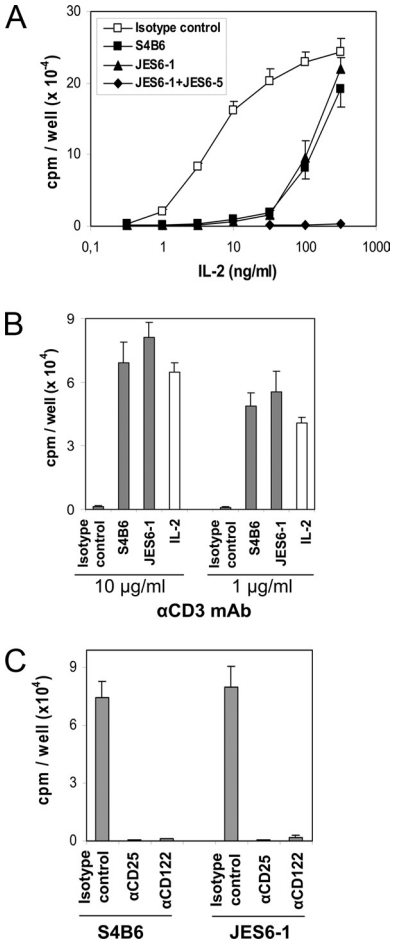



**FIGURE 1.** IL-2 in a complex with either S4B6 or JES6-1 anti-IL-2 mAb is capable to stimulate proliferation of activated naive CD8$^+$ T cells in vitro. *A*, Purified OT-I CD8$^+$ T cells were cultured at $5 \times 10^4$ cells per well with anti-CD3 mAb (10 $\mu$g/ml) and IL-2 premixed with isotype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

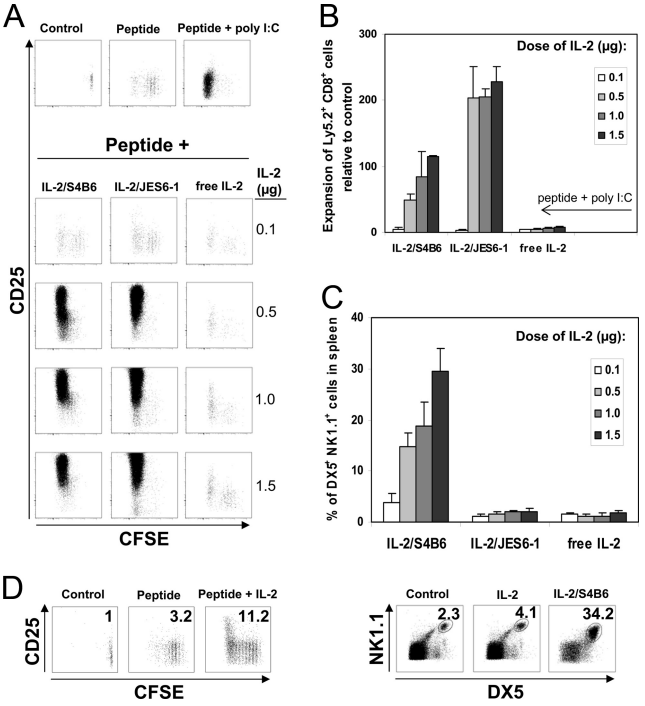



**FIGURE 2.** IL-2 immunocomplexes have a strong in vivo stimulatory activity for activated naive CD8$^+$ T cells and NK cells in comparison to free IL-2. Purified OT-I CD8$^+$ T cells (Ly5.2) were labeled with CFSE and injected i.v. into congeneic B6.SJL recipients (Ly5.1) at $1.5 \times 10^6$ cells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

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

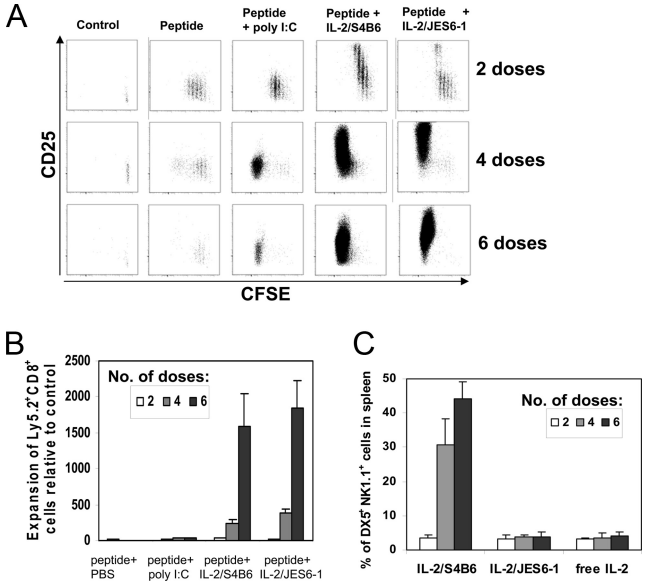



**FIGURE 3.** In vivo expansion of activated naive CD8$^+$ T cells and NK cells after treatment with two, four, or six doses of IL-2 immunocomplexes. Purified OT-I CD8$^+$ T cells (Ly5.2) were labeled with CFSE and adoptively transferred into B6.SJL recipients (Ly5.1) at $1.5 \times 10^6$ cells per mouse.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

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

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

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

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

### *IL-2/S4B6 mAb immunocomplexes possess significant antitumor activity*

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

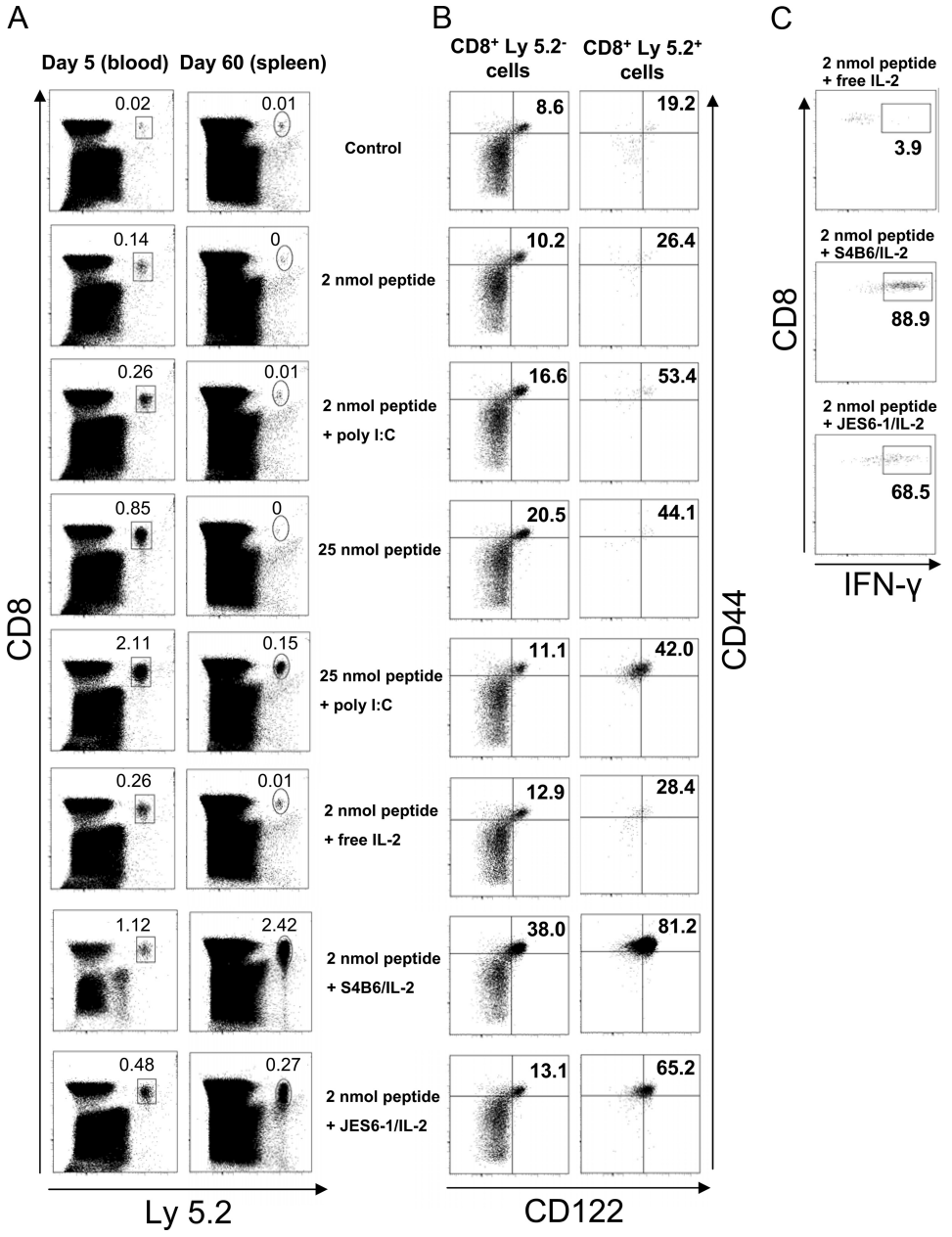



**FIGURE 4.** Activated naive CD8$^+$ T cells expanded by IL-2 immunocomplexes establish a robust population of functional memory cells. Purified OT-I CD8$^+$ T cells (Ly5.2) were injected i.v. into congeneic B6.SJL recipients (Ly5.1) at $1.5 \times 10^6$ cells per mouse (day 1). On day 2 the mice were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

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

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

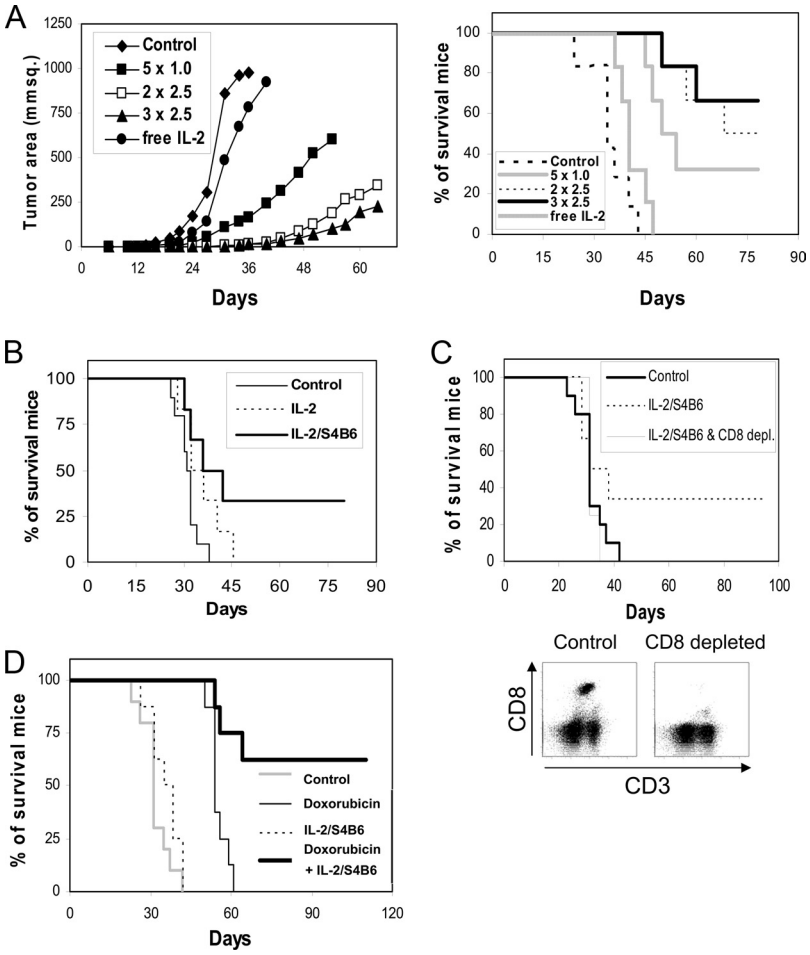



**FIGURE 5.** IL-2/S4B6 mAb immunocomplexes possess significant anti-tumor activity. *A*, B6 mice were injected s.c. with $1 \times 10^5$ of B16F10 melanoma cells on day 0. Mice were injected i.p. with PBS (control), with IL-2 (40 $\mu$g) on days 2 and 6 (free IL-2), with IL-2/S4B6 immunocomplexes (2.5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

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

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

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

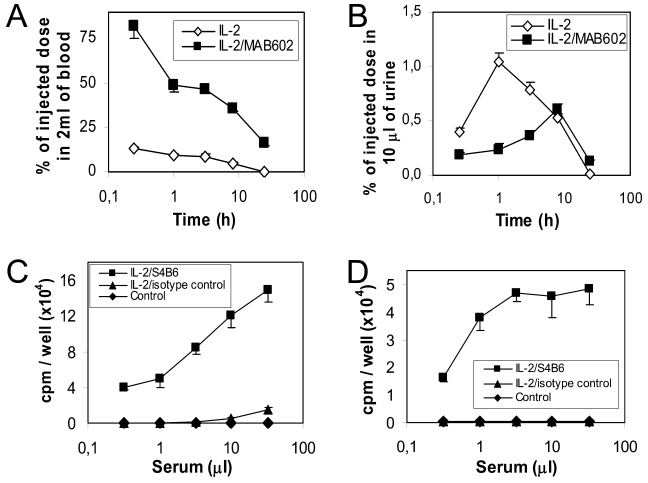



**FIGURE 6.** IL-2 immunocomplexes have much longer half-life than free IL-2. Recombinant human IL-2 was labeled with $^{131}I$ and injected i.v. into B6 mice either as a free labeled IL-2 or as IL-2 immunocomplexes. The injected dose contained 2 $\mu$g of labeled IL-2, which corresponded to $4 \times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

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

## Discussion

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

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

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

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

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

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

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

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

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

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

## Disclosures

Marek Kovar is listed as coinventor on patent entitled "Methods for Improving Immune Function and Methods for Prevention or Treatment of Disease" in a Mammalian subject, which was filed on February 16, 2007, and now bears International Application Number PCT/US2007/0623631. The remaining authors have no financial conflict of interest.

## References


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

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

uicc
global cancer control

IJC
International Journal of Cancer

# Antitumor activity of IL-2/anti-IL-2 mAb immunocomplexes exerts synergism with that of *N*-(2-hydroxypropyl)methacrylamide copolymer-bound doxorubicin conjugate due to its low immunosuppressive activity

Jakub Tomala[1], Helena Chmelova[1], Jiri Strohalm[2], Karel Ulbrich[2], Milada Sirova[1], Blanka Rihova[1] and Marek Kovar[1]

[1] Department of Immunology and Gnotobiology, Institute of Microbiology ASCR, v.v.i., Prague, Czech Republic
[2] Laboratory of Biomedical Polymers, Institute of Macromolecular Chemistry ASCR, v.v.i., Prague, Czech Republic

**Interleukin (IL)-2 has been approved for treatment of metastatic renal cancer and malignant melanoma. However, its unfavorable pharmacologic properties, severe side effects and the negative role of IL-2 in maintaining T regulatory cells are severe drawbacks. It has been shown that immunocomplexes of IL-2 and certain anti-IL-2 mAbs possess selective and high stimulatory activity *in vivo*. Here, we show that IL-2/S4B6 mAb immunocomplexes expand not only CD122$^{high}$ subsets and newly activated CD8$^+$ T cells but also natural killer T cells and γδ T cells. Further, we demonstrate that natural killer (NK) cells expanded by IL-2/S4B6 mAb immunocomplexes *in vivo* have high cytolytic activity, which can be further increased by coadministration of IL-12. We also demonstrate that IL-2/S4B6 mAb immunocomplexes possess noticeable antitumor activity in two syngeneic mouse tumor models, namely BCL1 leukemia and B16F10 melanoma, but only if administered early in tumor progression. To effectively treat established tumors, we administered the tumor-bearing mice first with *N*-(2-hydroxypropyl)methacrylamide copolymer-bound doxorubicin conjugate, and subsequently with IL-2/S4B6 mAb immunocomplexes alone or with IL-12 to induce an efficient antitumor immune response. Importantly, we show that the conjugate has significantly lower immunosuppressive activity than free doxorubicin when using dosage with comparable antitumor activity, thus eliminating the majority of tumor cells while leaving the immune system mostly unimpaired for stimulation with IL-2/S4B6 mAb immunocomplexes. Indeed, we demonstrate that the conjugate and IL-2/S4B6 mAb immunocomplexes together have synergistic antitumor activity.**

Chemotherapy is able to markedly reduce the number of tumor cells in many cases, but often fails to completely eradicate all of them resulting in so-called "minimal residual disease" and consequently in relapse of the disease.[1] On the other hand, one of the crucial limitations for effective tumor immunotherapy is the size of the tumor population, as antitumor immune responses are usually relatively weak and are able to control only a limited number of tumor cells. Thus, it would be desirable to investigate the therapeutic potential of chemoimmunotherapy by using a suitable form of chemotherapy with limited immunosuppressive effect in combination with some novel and potent immunotherapy approach.

In our study, we used a conjugate of a synthetic, water-soluble and biocompatible copolymer based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) to which doxorubicin is attached by an amide bond *via* a Gly-Phe(D,L)-Leu-Gly spacer[2] and containing also a human polyclonal nonspecific IgG (henceforth polymeric conjugate). For schematic structure of polymeric conjugate, see Supporting Information Figure 1. It has been shown previously that such a conjugate has considerable antitumor activity in various tumor models *in vivo*,[3,4] and it has been also successfully used in five patients with metastatic mammary carcinoma.[5,6] It was also demonstrated that this polymeric conjugate has a much longer half-life in circulation than free doxorubicin.[5] Moreover, macromolecular therapeutics are passively accumulated in solid tumors *via* enhanced permeability and retention effect, which is another crucial advantage over low-molecular free cytostatics.[7–9]

Interleukin (IL)-2 showed significant antitumor activity in many experimental tumor models,[10,11] and it was approved by the Food and Drug administration to treat metastatic renal

**Key words:** IL-2 immunocomplexes, CD8$^+$ T cells, NK cells, HPMA copolymer-bound doxorubicin, anticancer × immunosuppressive activity
Additional Supporting Information may be found in the online version of this article.
**Grant sponsor:** GAAS; **Grant number:** IAA500200712;
**Grant sponsor:** GACR; **Grant numbers:** 310/08/H077, 301/07/P192;
**Grant sponsor:** MEYSCR; **Grant number:** 1M 0505; **Grant sponsor:** Institutional Research Concept; **Grant number:** AV 0Z 502 00 510
**DOI:** 10.1002/ijc.25859
**History:** Received 10 Sep 2010; Revised 19 Oct 2010; Accepted 9 Nov 2010; Online 16 Dec 2010
**Correspondence to:** Marek Kovar, Department of Immunology and Gnotobiology, Institute of Microbiology ASCR, v.v.i., Videnska 1083, Prague, 14220, Czech Republic, Tel.: +420-241-062-362, Fax: +420-241-721-143, E-mail: makovar@biomed.cas.cz

cancer and malignant melanoma.[12,13] However, there are significant drawbacks in using IL-2; serious toxicities associated with high-dose IL-2 treatment and extremely short half-life being the most serious and limiting ones. Several different approaches, genetically engineered tumor cells producing IL-2,[14,15] fusion protein of IL-2 and albumin[16] or mAb,[17,18] were tested to improve the therapeutic potential of IL-2. Recently, it was described that the *in vivo* biological activity of IL-2 can be markedly increased by complexing IL-2 with anti-IL-2 mAb.[19] Moreover, it was shown that IL-2 immunocomplexes have selective stimulatory activity depending on the clone of anti-IL-2 mAb used. S4B6 mAb/IL-2 immunocomplexes (henceforth IL-2/S4B6) were found to be highly stimulatory for memory $CD8^+$ T cells and natural killer (NK) cells ($CD122^{high}$ populations), while only moderate stimulatory activity for T regulatory ($T_{reg}$) cells was found. Conversely, IL-2/JES6-1A12 mAb immunocomplexes have no effect on $CD122^{high}$ cell populations, but they vigorously induced expansion of $T_{reg}$ cells ($CD25^{high}$ population). Our group also demonstrated that both mentioned IL-2 immunocomplexes are very potent in expanding newly activated naïve $CD8^+$ T cells *in vivo*.[20]

In line with these findings, we decided to investigate the therapeutic activity of chemoimmunotherapy based on the use of polymeric conjugate in combination with IL-2/S4B6 immunocomplexes. We found that such designed chemoimmunotherapy possess high antitumor activity in BCL1 B cell leukemia and B16F10 melanoma, and it was able to completely cure BALB/c mice bearing BCL1 leukemia even when the treatment was given very late, up to 16 days after inoculation of tumor cells. Further, we demonstrated that the polymeric conjugate impairs the immune system to a considerably lower extent than free doxorubicin. We also showed that IL-2/S4B6 immunocomplexes noticeably increase not only the numbers, but also functional (cytolytic) activity of NK cells, which could be further increased by coinjection of IL-12. IL-12 also significantly increased antitumor activity of IL-2/S4B6 immunocomplexes in the BCL1 leukemia model. Considering these findings, we believe that the chemoimmunotherapy described in our study is an efficient antitumor treatment strategy.

## Material and methods

### Mice

Female BALB/c and male C57BL/6 mice were obtained from the animal facility of the Institute of Physiology (Academy of Sciences of the Czech Republic), v.v.i. Transgenic OT-I mice and B6.SJL (Ly5.1) mice were bred and kept at the GMO facility of the Institute of Molecular Genetics of ASCR, v.v.i. Mice were used at 9 to 15 weeks of age. All experiments were approved by the Animal Welfare Committee at the Institute of Microbiology of ASCR, v.v.i.

### Cell lines

The murine B cell leukemia BCL1 and B16F10 melanoma cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Both cell lines were used up to six passages, then discarded and a new vial was used. Both cell lines were checked regularly to determine if they kept their usual proliferation activity and morphology, as well as bimonthly for their capacity to form tumors *in vivo*. BCL1 cells were tested for surface markers MHC II, CD80, CD86 and surface IgM using BCL1-specific anti-idiotype B1 mAb.

### Monoclonal antibodies

The following anti-mouse mAbs were used for cell staining: CD3-PB, CD3-FITC, CD8-A700, CD8-PerCP-Cy5.5, CD45.1-APC, DX5-PE, Foxp3-PE (eBioscience, San Diego, CA), CD4-PerCP, ydTCR-PE (BD Pharmingen, San Diego, CA). CD161-APC and unconjugated anti-IL-2 mAb S4B6 were kindly provided by Dr. Karel Drbal (Institute of Molecular Genetics of ASCR, v.v.i., Prague, Czech Republic).

### Staining for surface antigens, intracellular staining and flow cytometry

All the preparations and subsequent analysis were done as described previously.[20]

### Polymer-bound doxorubicin conjugates

All polymer conjugates were synthesized as described previously.[2]

### IL-2/S4B6 immunocomplexes

These immunocomplexes[19] were prepared by adding rmIL-2 (Prospec, Israel) into solution of anti-IL-2 mAb S4B6 [both reagents were in phosphate buffered saline (PBS)] at molar ratio 2:1. After a 15 min incubation at room temperature, the immunocomplexes were diluted with PBS into desired concentration.

### IL-15/IL-15Rα-Fc complexes

These complexes[21] were prepared by adding rmIL-15 (Prospec, Israel) into solution of IL15Rα-Fc (R&D Systems, Minneapolis, MN) at molar ratio 2:1. After a 15-min incubation at room temperature, the immunocomplexes were diluted with PBS to the desired concentration.

### Adoptive transfer of OT-I cells

Purified OT-I $CD8^+$ T cells (Ly 5.2) were injected i.v. into B6.SJL recipients (Ly 5.1) at $1 \times 10^6$ cells per mouse. The next day, the mice were injected i.p. with PBS, SIINFEKL peptide (peptide; MBL International, Woburn, MA), peptide + poly I:C (75 μg) or peptide plus IL-2/S4B6.

### NK assay

B6 mice were injected i.p. with IL-2 or IL-2/S4B6 mAb immunocomplexes or/and IL12. After 5 days, mice were euthanized, and splenocytes were isolated using Histopaque 1083. YAC-1 cells, which are naturally sensitive to cytolytic activity of NK cells, were labeled for 20 hr with $^3$H-thymidine (3 μCi/ml), washed and seeded into 96-well plate with "U-shaped" wells ($1 \times 10^4$ cells/ml). Different numbers of

splenocytes were added to labeled YAC-1 cells and incubated in 5% $CO_2$ for 4 hr at 37°C.

### Proliferation assay *in vitro*

A single cell suspension was prepared from spleens. After red blood cells (RBC) lysis, cells were washed, centrifuged for 5 min at 300*g*, resuspended in fresh culture medium and seeded into Nunc 96-well flat-bottom plates in 0.2 ml volume and $2.5 \times 10^{-5}$ cells/ml density. Concanavalin A, IL-15/IL-15Rα-Fc, IL-2 or IL-12 was added to the wells to achieve desired concentrations; four wells for each test condition were used. The plates were then cultured in 5% $CO_2$ for 72 hr at 37°C and 18.5 kBq of [$^3$H]-thymidine was added for the final 16 hr of cultivation before harvesting.

### Treatment of BCL1 leukemia and B16F10 melanoma *in vivo*

BCL1 cells ($5 \times 10^5$) in 0.5 ml of culture medium without serum were i.p. inoculated on day 0 into BALB/c female mice. Polymeric conjugate was injected i.v. *via* a tail vein, and control mice were injected with the same volume (300 μl) of PBS. IL-2/S4B6 immunocomplexes and IL-12 were injected i.p., and control mice were injected with the same volume (250 μl) of PBS. Mice surviving day 90 without any signs of BCL1 leukemia were considered as long-term survivors. B16F10 cells ($1 \times 10^6$) in 0.1 ml of culture medium without serum were s.c. inoculated on day 0 into B6 male mice. Polymeric conjugate, IL-2/S4B6 and IL-12 were injected as described for BCL1 leukemia. Tumor size was measured every 2–4 days.

### Statistical analysis

The significance of any differences obtained among experimental groups (except tumor growth) was evaluated by the Student's *t*-test, and significant difference was considered when $p < 0.05$. Differences in tumor size of multiple groups were compared to the control group by one-way analysis of variance with Dunnett's multiple comparison test and significant difference was considered when $p < 0.05$.

## Results

### IL-2/S4B6 immunocomplexes expand newly activated CD8$^+$ T cells, NK cells as well as some other immunocompetent cells and possess noticeable antitumor activity if administered early in tumor progression

First, we determined the binding of IL-2/S4B6 immunocomplexes to naturally IL-2 responsive cells, namely memory CD8$^+$ T cells, NK cells and $T_{reg}$ cells. IL-2/S4B6 immunocomplexes bound to memory CD8$^+$ T cells and NK cells to considerably higher extent than to $T_{reg}$ cells. Binding of IL-2/S4B6 immunocomplexes to $T_{reg}$ cells at 1 μg/ml of IL-2 was almost undetectable, and even at 10 μg/ml of IL-2, it was about two times lower than the binding to NK cells at 1 μg/ml of IL-2 (Supporting Information Fig. 2). Next, we adoptively transferred purified CD8$^+$ T cells from OT-I mice into congeneic Ly5.1$^+$ mice, challenged the mice with SIINFEKL peptide and treated them with IL-2/S4B6 immunocomplexes. While challenge with SIINFEKL peptide alone caused only a weak expansion of transferred CD8$^+$ T cells, challenge with SIINFEKL peptide and treatment with IL-2/S4B6 immunocomplexes caused a robust expansion of these cells (Fig. 1*a*, left). Of note, treatment with IL-2/S4B6 immunocomplexes also caused a huge expansion of endogenous NK cells (Fig. 1*a*, right). Free IL-2 at the same dosage had no or minimal effect on expansion of activated CD8$^+$ T cells and NK cells (data not shown). IL-2/S4B6 immunocomplexes also increased the content of endogenous CD8$^+$ cells, natural killer T cells, γδ T cells and, although only slightly, $T_{reg}$ cells (Fig. 1*b*, top). The cellularity of spleens from mice treated with IL-2/S4B6 immunocomplexes was significantly increased in comparison to that from control (Fig. 1*b*, bottom). Moreover, NK cytolytic activity of splenocytes was increased considerably by administration of a single dose of IL-2/S4B6 immunocomplexes (Fig. 1*c*), and it was further increased by coinjection of IL-12 (Fig. 1*d*).

Next, we tested the antitumor activity of IL-2/S4B6 immunocomplexes, either alone or with IL-12, in BCL1 B cell leukemia and B16F10 melanoma mouse tumor models using two schedules of treatment. First, we treated mice early after inoculation of tumor cells (days 2 and 6, or 4 and 8 for B16F10 and BCL1 tumor bearing mice, respectively). We found that IL-2/S4B6 immunocomplexes in this schedule showed significant antitumor activity (Figs. 2*a* and 2*c*). IL-12 coinjected with IL-2/S4B6 immunocomplexes increased the antitumor activity of IL-2/S4B6 immunocomplexes in the BCL1 leukemia model (Fig. 2*a*) but had no effect in the B16F10 melanoma model (data not shown). Neither IL-2 nor IL-12 had any direct effect on proliferation of BCL1 cells (Supporting Information Fig. 3), which means that the antitumor effect seen was mediated by the immune system. On the other hand, IL-2/S4B6 immunocomplexes administered relatively late after inoculation of tumor cells (days 8 and 12, or 11 and 15 for B16F10 and BCL1 tumor bearing mice, respectively) had almost no therapeutic effect (Figs. 2*b* and 2*d*). Thus, immunotherapy with IL-2/S4B6 immunocomplexes was effective only when there was a limited number of tumor cells.

### Both polymeric conjugate and free doxorubicin prolong survival of, but did not cure, mice bearing established tumors

To investigate a possible method to significantly reduce the load of tumor cells before administration of IL-2/S4B6 immunocomplexes, we compared a classical chemotherapeutic drug, doxorubicin, either in free form or as polymeric conjugate in both tumor models. Free doxorubicin (5 mg/kg), administered either once or three times, prolonged survival of BALB/c mice bearing BCL1 leukemia 131% and 201%, respectively, as compared to controls (Fig. 3*a*). Of note, three doses of 5 mg/kg ($3 \times 5$ mg/kg) of doxorubicin is near the maximal tolerated dose. Polymeric conjugate administered once at 5 and 12.5 mg/kg of doxorubicin, prolonged survival

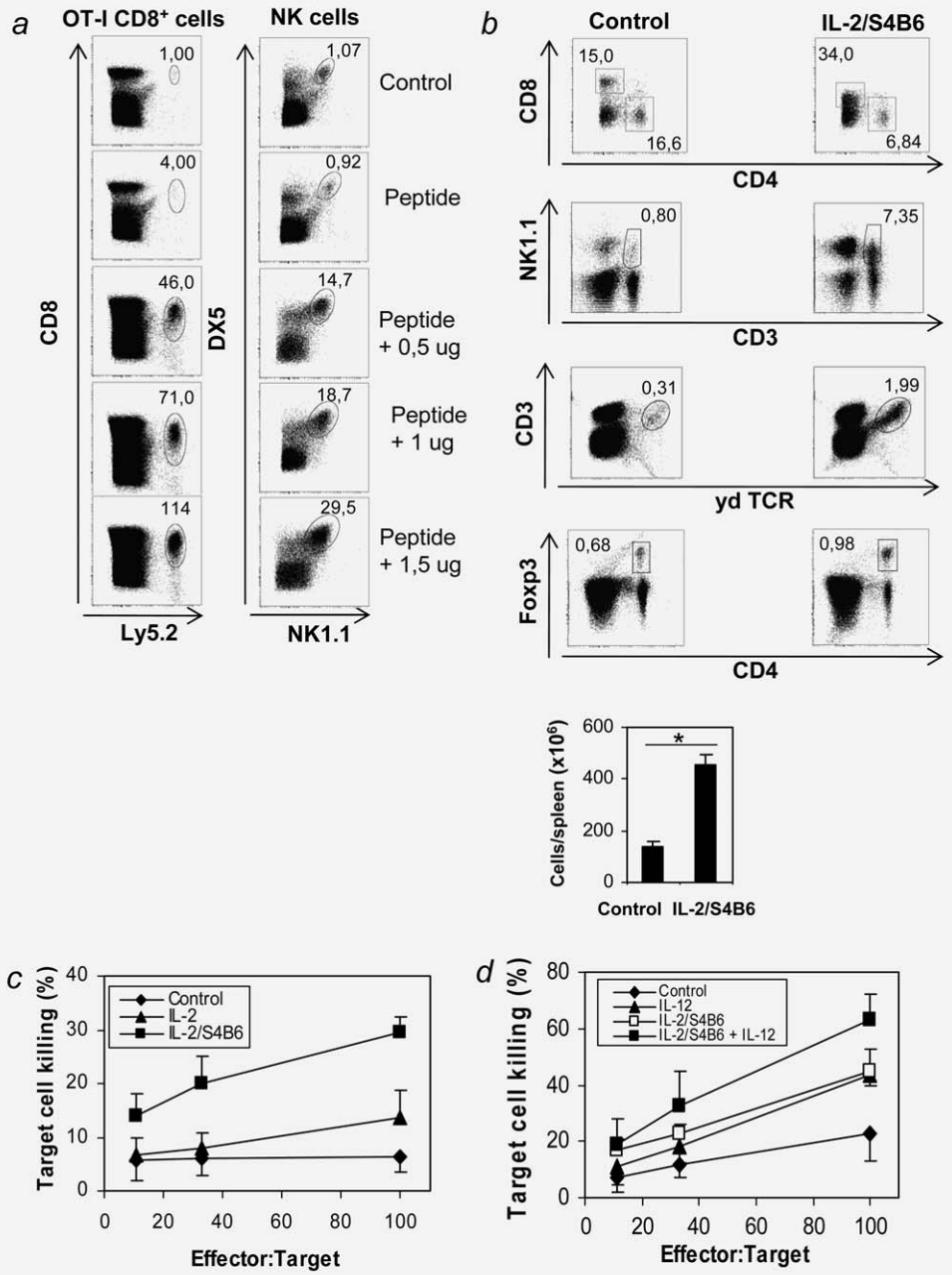



**Figure 1.** IL-2/S4B6 immunocomplexes have strong *in vivo* stimulatory activity for recently activated $CD8^+$ T cells, NK cells and some other immunocompetent cells. (*a*) Purified OT-I $CD8^+$ T cells (Ly 5.2) were injected i.v. into congeneic B6.SJL recipients (Ly 5.1) at $1 \times 10^6$ cells per mouse (day 1). On day 2, the mice were injected i.p. with PBS (control), 2 nmol of SIINFEKL peptide (peptide) and peptide + titrated amounts of IL-2/S4B6 immunocomplexes (0.5, 1.0 or 1.5 μg IL-2), which were injected also on days 3, 4 and 5. Expansion of Ly $5.2^+$ $CD8^+$ cells relative to control (numbers inside dot plots; control = 1) and percentage of $DX5^+NK1.1^+$ cells (numbers inside dot plots) in spleen was analyzed 1 day after the last injection. One representative mouse is shown. All groups $n = 2$. (*b*) B6 mice were injected i.p. with IL-2/S4B6 immunocomplexes (1.5 μg IL-2 per dose) or PBS (control) daily for 4 days and percentage (numbers inside dot plots) of various populations of immunocompetent cells (top; one representative mouse) and cellularity of the spleen (bottom) was analyzed 1 day after the last injection. Average ± SD is shown. Both groups $n = 4$. *, significant difference ($p < 0.05$) determined by Student's *t*-test. (*c*) B6 mice were i.p. injected with PBS (control), IL-2 (2.5 μg) and IL-2/S4B6 immunocomplexes (2.5 μg IL-2) or with (*d*) PBS, IL-2/S4B6 immunocomplexes, IL-12 (0.75 μg) and IL-2/S4B6 immunocomplexes + IL-12. IL-2 and IL-2/S4B6 immunocomplexes were injected on day 1. IL-12 was injected on days 3 and 4. NK cytolytic activity of spleen cells was analyzed on day 5. Average ± SD is shown. All groups in (*c*) and (*d*) $n = 2$. Experiments were done twice with similar results.

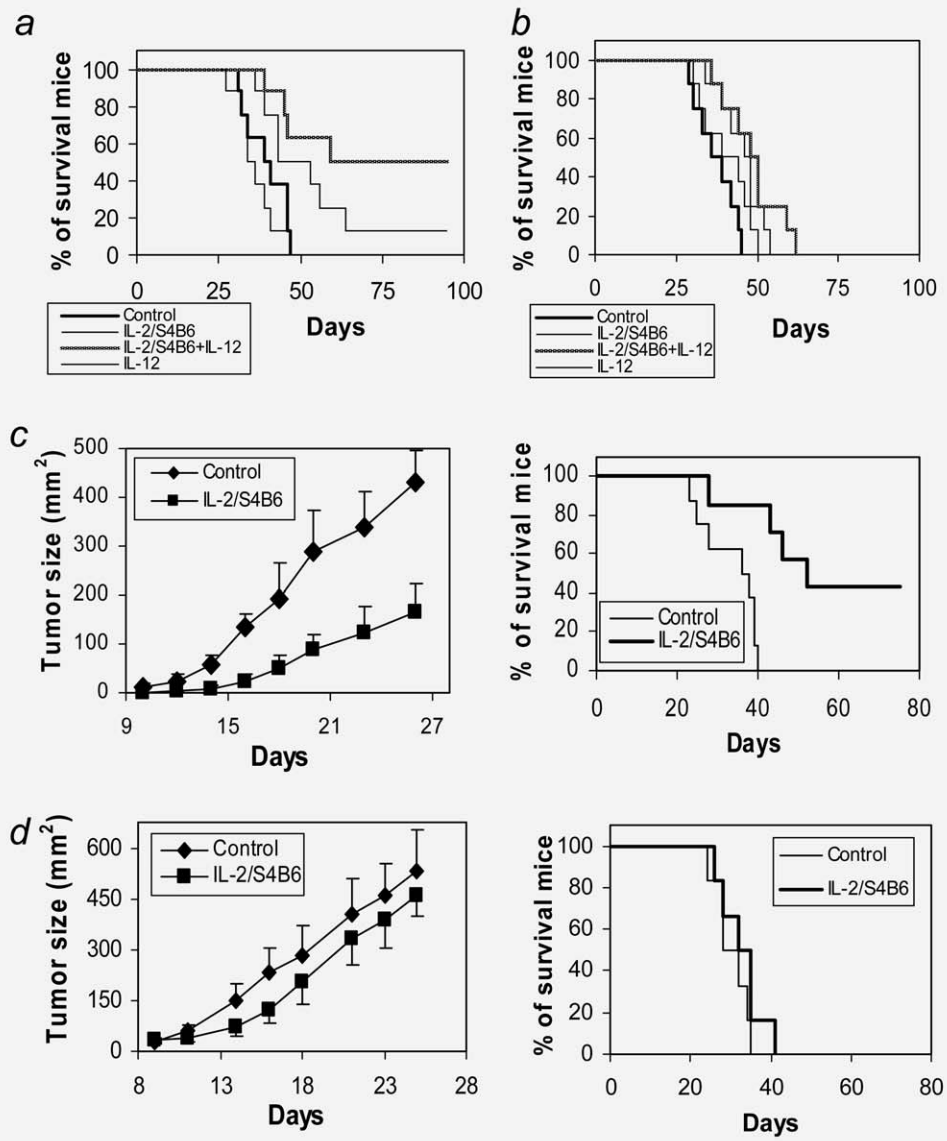



**Figure 2.** IL-2/S4B6 immunocomplexes possess considerable antitumor activity only when injected early after tumor cell inoculation. BALB/c mice were injected i.p. with $5 \times 10^5$ of BCL1 leukemia cells on day 0 and i.p. with PBS (control), IL-2/S4B6 immunocomplexes (2.5 μg IL-2 per dose), IL-12 (0.75 μg per dose) and IL-2/S4B6 immunocomplexes + IL-12 on days 4 and 8 (*a*) or on days 11 and 15 (*b*). Survival of mice was recorded. All groups in (*a*) and (*b*) $n = 8$. B6 mice were injected s.c. with $1 \times 10^6$ of B16F10 melanoma cells on day 0. Mice were injected i.p. with PBS (control) and IL-2/S4B6 immunocomplexes (2.5 μg IL-2 per dose) on days 2 and 6 (*c*) or on days 8 and 12 (*d*). Tumor size was measured (left; average ± SD is shown), and survival was monitored (right). Both groups in (*c*) $n = 8$, both groups in (*d*) $n = 6$ (*d*). Experiments were done twice with similar results.

of mice 138 and 153%, respectively, as compared to controls, and 222% when administered three times at 12.5 mg/kg (Fig. 3*b*). As we have shown previously,[22,23] polymeric conjugate containing BCL1-specific B1 mAb instead of irrelevant antibody, has a very high therapeutic potential even at a low dosage (Supporting Information Fig. 4). In the B16F10 melanoma model, polymeric conjugate (12.5 mg/kg of doxorubicin) suppressed the tumor growth better than free doxorubicin (5 mg/kg) when both were administered three times (Fig. 3*c*). Survival was also prolonged significantly by polymeric conjugate as compared to free doxorubicin (150% versus 116% of respective controls, Fig. 3*d*). Taken together, polymeric conjugate had slightly higher therapeutic activity than free doxorubicin in BCL1 leukemia and considerably higher in B16F10 melanoma.

### Polymeric conjugate impairs the immune system to a significantly lower extent than free doxorubicin

Free doxorubicin ($3 \times 5$ mg/kg) and polymeric conjugate ($3 \times 12.5$ mg/kg of doxorubicin) caused a 15% and 13.5%

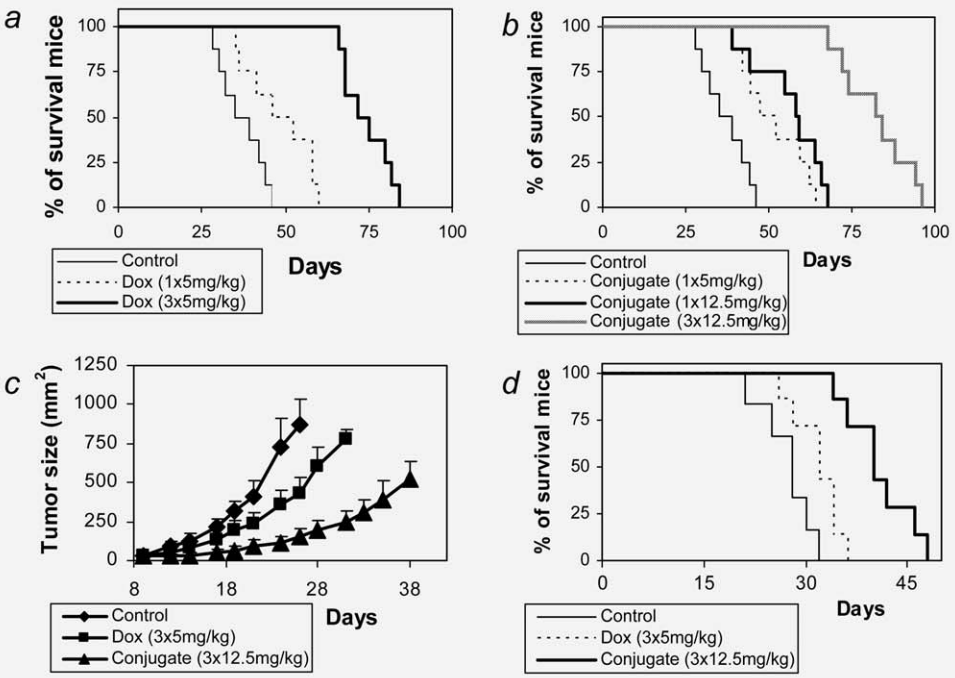



**Figure 3.** Antitumor activity of polymeric conjugate and free doxorubicin. BALB/c mice were injected i.p. with $5 \times 10^5$ of BCL1 leukemia cells on day 0 and i.v. with (*a*) doxorubicin (Dox) either on day 11 ($1 \times 5$ mg/kg) or on days 11, 14 and 17 ($3 \times 5$ mg/kg) or with (*b*) polymeric conjugate (conjugate) on day 11 ($1 \times 5$ or 12.5 mg Dox/kg) or on days 11, 14 and 17 ($3 \times 12.5$ mg Dox/kg). Survival of mice is shown. All groups in (*a*) and (*b*) $n = 8$. B6 mice were injected s.c. with $1 \times 10^6$ of B16F10 melanoma cells on day 0. Mice were injected i.v. with Dox at 5 mg/kg or with conjugate at 12.5 mg Dox/kg on days 8, 11 and 14. Tumor size was measured [(*c*); average ± SD is shown] and survival was monitored (*d*). For (*c*) and (*d*): control group $n = 6$, treated groups $n = 7$. Experiments were done twice with similar results.

weight loss, respectively, in BALB/c mice (Fig. 4*a*). However, free doxorubicin significantly reduced the number of splenocytes while polymeric conjugate had almost no effect (Fig. 4*b*). Splenocytes from BALB/c mice injected with free doxorubicin showed a significantly lower proliferation response *in vitro* to suboptimal concentrations of concanavalin A than splenocytes from BALB/c mice injected with polymeric conjugate (Fig. 4*c*). Splenocytes from BALB/c mice injected with free doxorubicin also showed a significantly lower proliferation response *in vitro* to IL-15/IL-15Rα-Fc complexes, which are highly stimulatory for memory $CD8^+$ T cells and NK cells,[21] than splenocytes from BALB/c mice injected with polymeric conjugate (Fig. 4*d*).

The data above well document that free doxorubicin impaired the immune system in terms of lowering the number and proliferative potential of immunocompetent cells to a greater extent than the polymeric conjugate. Nevertheless, it does not provide any information about the impact on the ongoing immune response. Thus, we adoptively transferred purified $CD8^+$ T cells from OT-I mice into congeneic $Ly5.1^+$ mice, challenged the mice with SIINFEKL peptide and treated them with IL-2/S4B6 immunocomplexes to promote expansion of transferred $CD8^+$ T cells and endogenous NK cells. These mice were also injected either with free doxorubicin or polymeric conjugate. Free doxorubicin (either $1 \times 8$ mg/kg or $3 \times 5$ mg/kg) suppressed expansion of transferred $CD8^+$ T cells considerably more than the polymeric conjugate (either $1 \times 20$ mg/kg or $3 \times 12.5$ mg/kg of doxorubicin), which showed only moderate suppressive activity (Figs. 5*a* left and 5*b*). The difference in the suppressive effect of free doxorubicin and polymeric conjugate was still present but less pronounced in NK cells (Figs. 5*a* right and 5*c*). These results thus show that polymeric conjugate is a more suitable chemotherapeutic modality than free doxorubicin, especially when considering the subsequent use of immunotherapy.

### Chemoimmunotherapy with polymeric conjugate and IL-2/S4B6 immunocomplexes alone or with IL-12 has synergistic antitumor activity and is able to completely cure mice with established tumors

To evaluate the therapeutic effect of polymeric conjugate in combination with IL-2/S4B6 immunocomplexes, we injected BALB/c mice bearing BCL1 leukemia with polymeric conjugate (7.5 mg/kg of doxorubicin) either on days 11 and 23 (Fig. 6*a*) or on days 16 and 28 (Fig. 6*b*). Subsequently, IL-2/S4B6 immunocomplexes and IL-12 were injected daily for 3 days. Polymeric conjugate with IL-2/S4B6 immunocomplexes cured three and two out of eight mice when the

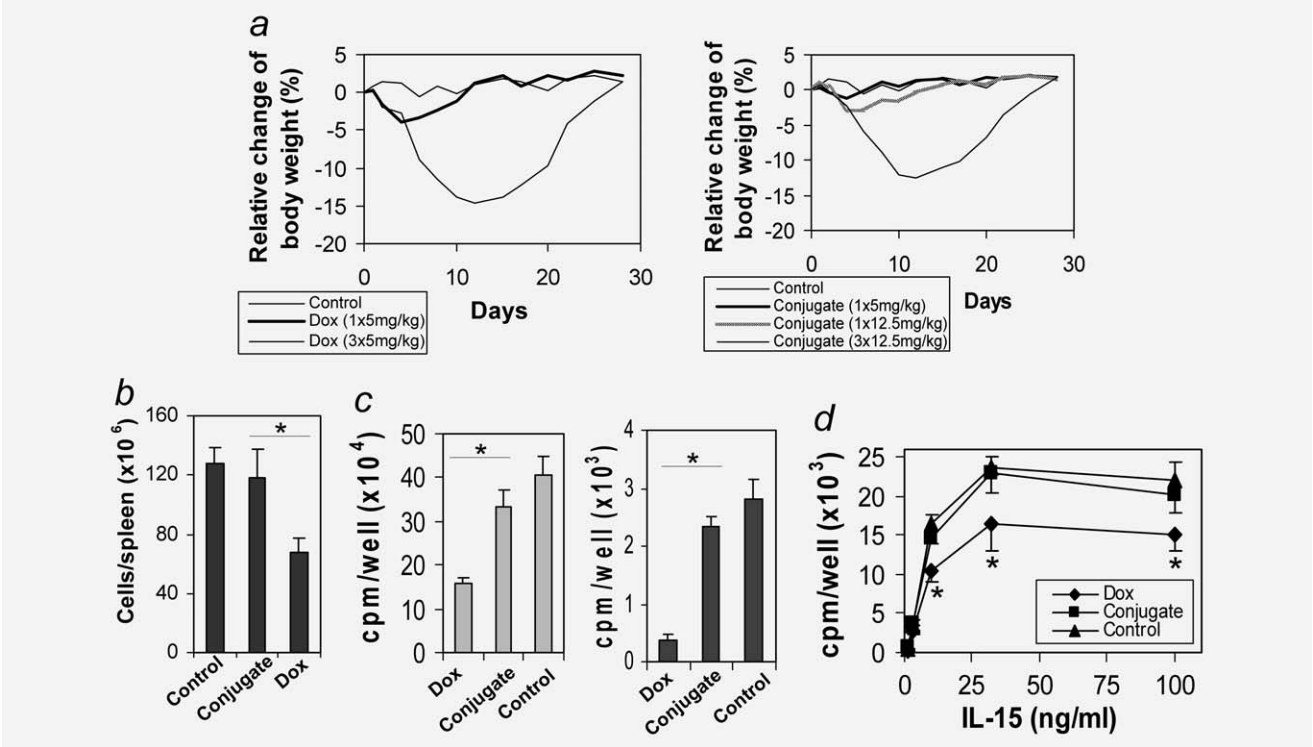



**Figure 4.** Polymeric conjugate possesses significantly lower immunotoxicity than free doxorubicin. (*a*) BALB/c mice were injected i.v. with (left) doxorubicin (Dox; 1 or 3 × 5 mg/kg) or with (right) polymeric conjugate (conjugate; 1 × 5 or 12.5 mg Dox/kg or 3 × 12.5 mg Dox/kg) every third day starting on day 0. Body weight of experimental mice and mice injected with PBS (control) was monitored for 4 weeks. All groups $n = 5$. (*b*) BALB/c mice were injected i.v. with doxorubicin (Dox; 5 mg/kg), polymeric conjugate (conjugate; 12.5 mg Dox/kg) or PBS (control), and cellularity of the spleen was analyzed after 24 hr. Average ± SD is shown. All groups $n = 5$. Spleen cells from each individual mouse from (*b*) were pooled for each experimental condition (Dox, conjugate, control). Spleen cells were cultured at $2.5 \times 10^5$ cells/well with concanavalin A [(*c*) left ~ 1 μg/ml; (*c*) right ~ 0.32 μg/ml) or with titrated concentrations of IL-15/IL-15Rα-Fc complexes (*d*). The data show mean levels of [$^3$H]-thymidine incorporation ± SD for quatriplete cultures on day 3. *, significant difference ($p < 0.05$) determined by Student's *t* test. Experiments were done twice with similar results.



treatment started on day 11 and 16, respectively. IL-12 further increased the therapeutic effect of this treatment as seven and four out of eight mice were cured when the treatment started on day 11 and 16, respectively. In B16F10 melanoma model, polymeric conjugate with IL-2/S4B6 immunocomplexes was not able to cure any mice, but it significantly inhibited the tumor growth (Fig. 6*c*; $p < 0.05$ on day 11 and up) and markedly prolonged survival (Fig. 6*d*). Although we have shown that free doxorubicin is much less convenient than polymeric conjugate for combination with IL-2/S4B6 immunocomplexes, we nevertheless decided to test and to compare the therapeutic activity of free doxorubicin and polymeric conjugate in combination with IL-2/S4B6 immunocomplexes and IL-12. Thus, BALB/c mice bearing BCL1 leukemia were injected either on day 11 or on day 20 with free doxorubicin (5 mg/kg) and polymeric conjugate (12.5 mg/kg of doxorubicin). Subsequently, IL-2/S4B6 immunocomplexes with IL-12 were injected daily for 3 days. Polymeric conjugate and free doxorubicin with IL-2/S4B6 immunocomplexes plus IL-12 cured five and one out of eight mice, respectively, when treatment started on day 11 (Supporting Information Figs. 5*a* and *b*). Polymeric conjugate, but not free doxorubicin, with IL-2/S4B6 immunocomplexes and IL-12 were able to cure three out of eight mice when treatment started on day 20 (Supporting Information Fig. 5*c* and *d*).

## Discussion

Immunotherapy is an attractive approach to treat cancer due to the superior specificity of the immune system to selectively eliminate tumor cells in comparison to any other available method. Although many cytokines possess very high biological activity *in vitro*, their use as immunostimulators *in vivo* is often limited because of poor *in vivo* activity of injected cytokines.[19] Complexing cytokine with anti-cytokine mAb seems to be a straightforward and effective approach to bring a new class of *in vivo* highly potent immunostimulators/immunomodulators.[19,21,24,25] Immunocomplexes of IL-3, IL-4, IL-6 and IL-7 with respective mAbs have been successfully used in various models of antitumor responses, autoimmune disease,

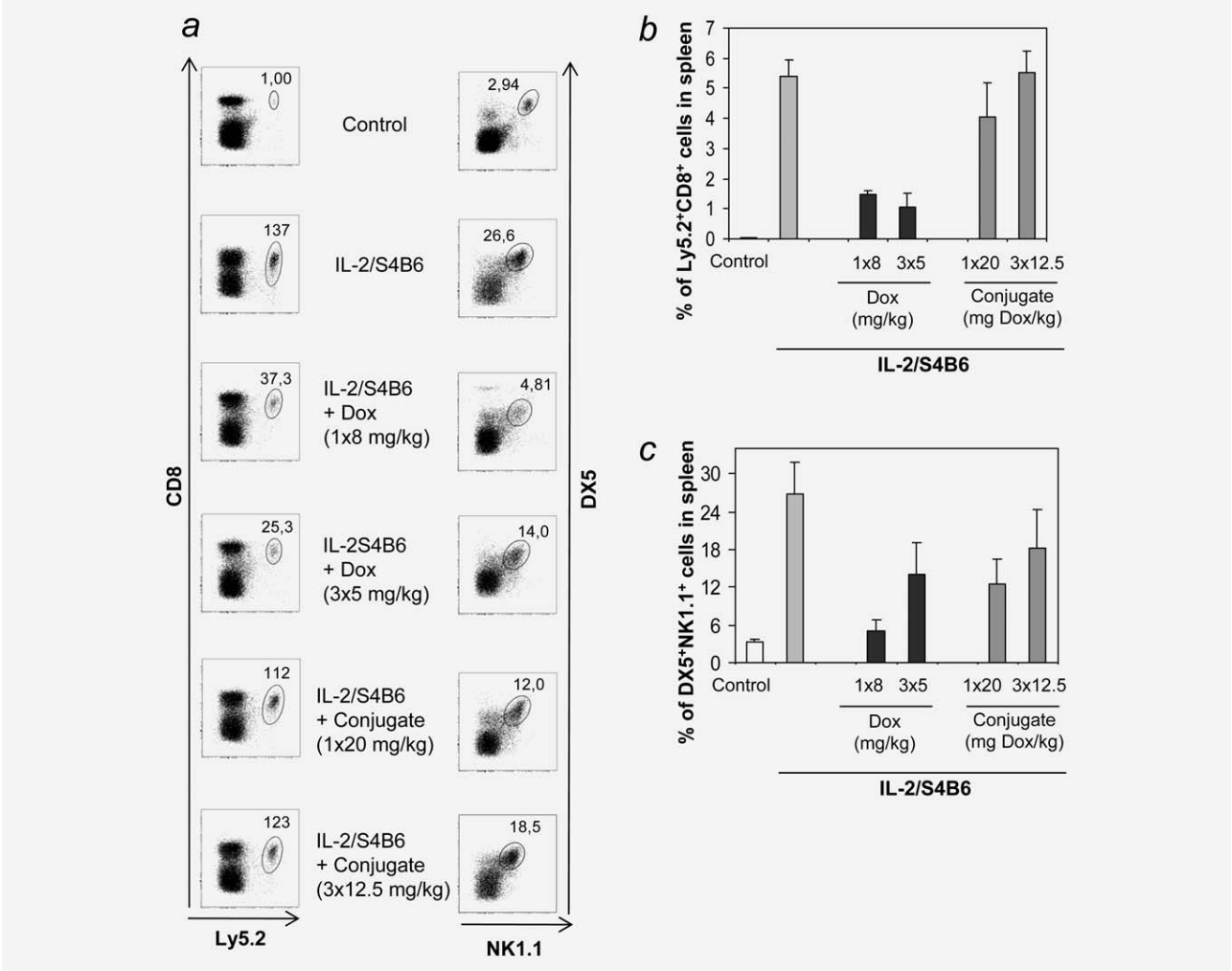



**Figure 5.** Free doxorubicin suppresses expansion of $CD8^+$ T cells and NK cells to higher extent than polymeric conjugate. Purified OT-I $CD8^+$ T cells (Ly 5.2) were injected i.v. into congeneic B6.SJL recipients (Ly 5.1) at $1 \times 10^6$ cells per mouse (day 1). Control mice were injected i.p. with PBS while all other mice were injected i.p. with IL-2/S4B6 immunocomplexes (1.5 μg IL-2 per dose) on days 2, 3, 4 and 5. Mice were further injected i.v. with doxorubicin (Dox) or polymeric conjugate (conjugate) either on day 3 only ($1 \times 8$ and $1 \times 20$ mg/kg for Dox and conjugate, respectively) or on days 3, 4 and 5 ($3 \times 5$ and $3 \times 12.5$ mg/kg for Dox and conjugate, respectively). Mice were euthanized and their spleens were analyzed on day 6. One representative mouse per each condition is shown in (*a*). Expansion of Ly $5.2^+$ $CD8^+$ cells relative to control (left column; control = 1) and percentage of $DX5^+NK1.1^+$ cells in spleen (right column) is shown inside dot plots. Average percentage ± SD of Ly $5.2^+$ $CD8^+$ cells and $DX5^+NK1.1^+$ cells in spleen is shown in (*b*) and (*c*), respectively. All groups $n = 2$. Experiments were done twice with similar results.

graft tolerance or viral infections.[24–26] In our study, we employed IL-2/S4B6 immunocomplexes and showed that although they have considerable antitumor activity when injected soon after tumor cell inoculation, they are almost ineffective when used for treatment of advanced tumors. However, when the mice bearing advanced tumors were first treated with polymeric conjugate, which impairs the immune system to lower extent than free doxorubicin and possesses at least comparable antitumor activity, IL-2/S4B6 immunocomplexes significantly improved the therapeutic outcome.

It was previously demonstrated that some cytostatic drugs, including anthracyclines, induce so-called "immunogeneic cancer cell death" characterized by translocation of calreticulin on cell surface[27] and by release of HMGB1.[28] Such process can lead to the induction of an effective anticancer immune response.[29] Our polymeric conjugate induced neither translocation of calreticulin nor release of HMGB1; however, it caused a significant increase of Hsp110, 90, 70 and 60 on the cell surface (our unpublished data). The polymeric conjugate thus alters the immunogenicity of tumor cells which could contribute to induction of an antitumor immune response.[30] Such antitumor immune response could be significantly amplified by IL-2/S4B6 immunocomplexes similarly as OT-I $CD8^+$ T cell response to a low dose of antigenic

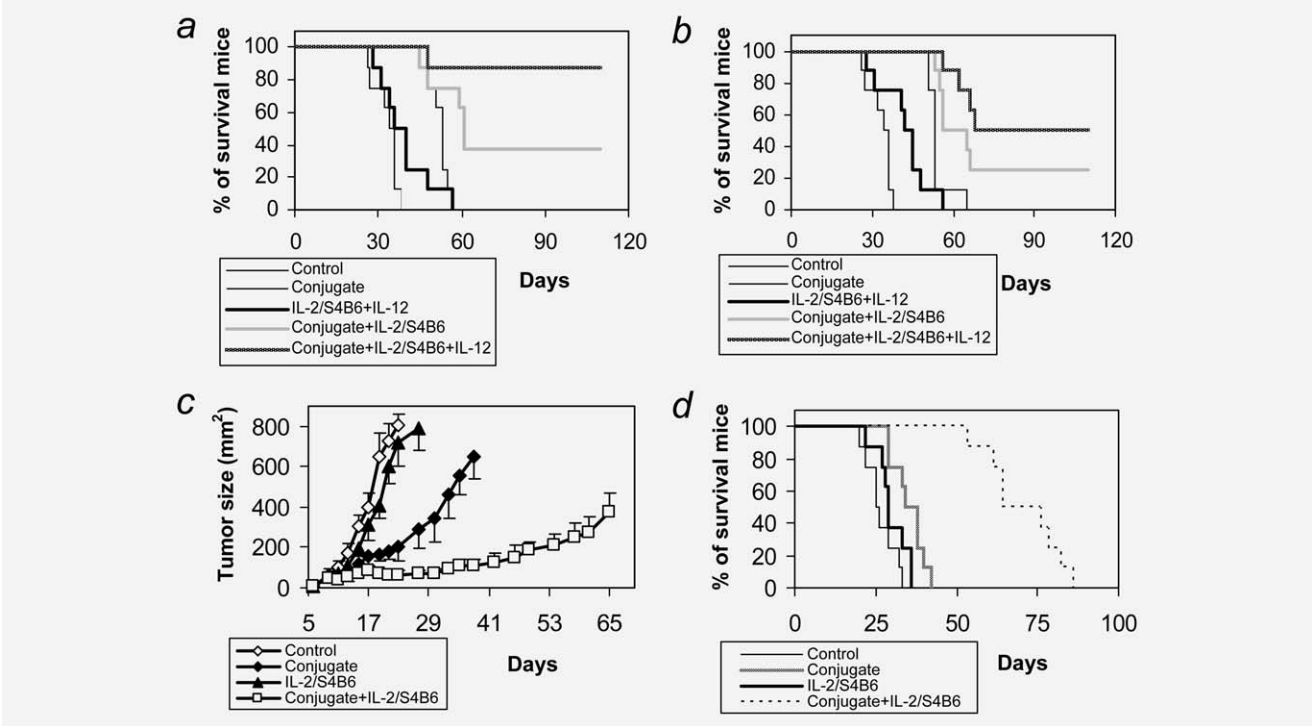



**Figure 6.** Antitumor activity of polymeric conjugate synergize with that of IL-2/S4B6 immunocomplexes. (*a*) BALB/c mice were injected i.p. with $5 \times 10^5$ of BCL1 leukemia cells on day 0. Mice were injected with PBS (control), polymeric conjugate (conjugate), conjugate and IL-2/S4B6 immunocomplexes (conjugate + IL-2/S4B6), conjugate + IL-2/S4B6 and IL-12 (conjugate + IL-2/S4B6 + IL-12) and IL-2/S4B6 immunocomplexes with IL-12 (IL-2/S4B6 + IL-12). Conjugate was injected i.v. on days 11 and 23 (each dose 7.5 mg/kg of doxorubicin). IL-2/S4B6 immunocomplexes (each dose 1 μg IL-2) and IL-12 (each dose 0.5 μg IL-2) were injected i.p. on days 12, 13, 14, 24, 25 and 26. Survival of mice was recorded. (*b*) Experiment was done as in (*a*) but conjugate was injected on days 16 and 28, and IL-2/S4B6 immunocomplexes and IL-12 were injected on days 17, 18, 19, 29, 30 and 31. B6 mice were injected s.c. with $1 \times 10^6$ of B16F10 melanoma cells on day 0. Mice were injected with PBS (control), polymeric conjugate (conjugate), IL-2/S4B6 immunocomplexes (IL-2/S4B6) and conjugate and IL-2/S4B6 (conjugate + IL-2/S4B6). Conjugate was injected i.v. on days 8 and 17 (each dose 12.5 mg/kg of doxorubicin). IL-2/S4B6 immunocomplexes (each dose 1.5 μg IL-2) were injected i.p. on days 9, 10, 11, 18, 19 and 20. Tumor size was measured [(*c*); average ± SD is shown] and survival was monitored (*d*). All groups $n = 8$. Experiments were done twice with similar results.

peptide. Hence, complete cure of tumor-bearing mice could be achieved by combination of polymeric conjugate with IL-2/S4B6 immunocomplexes. Moreover, we confirmed that six out of seven BALB/c mice cured from BCL1 leukemia in Figure 6*a* were resistant to rechallenge with lethal dose ($5 \times 10^4$ i.p.) of BCL1 cells (data not shown).

IL-12 was employed because we found that it further augmented the NK cytolytic activity increased by IL-2/S4B6 immunocomplexes. We hypothesize that there could be at least three additional mechanisms responsible for beneficial effect of IL-12 seen in BCL1 leukemia. First, IL-12 increases production of interferon-γ,[31] which has a direct inhibitory activity for BCL1 cells.[23] Second, BCL1 cells produce p40 dimers, antagonists of IL-12, as one of their immuno-escape mechanisms[23] and administration of IL-12 can thus override this. Third, IL-12 inhibits expansion of $T_{reg}$ cells during progression of BCL1 leukemia (our unpublished data), which is another important immuno-escape mechanism of BCL1 leukemia.[23]

Although powerful stimulatory activity of IL-2 immunocomplexes was described previously[19] and IL-2 immunocomplexes are an emerging class of immunostimulants,[32] there are a limited number of reports investigating their potential in cancer immunotherapy. Kamimura *et al.*[26] showed that coinjection of S4B6 mAb with a plasmid carrying murine *il2* cDNA is capable to reduce the number of B16 melanoma lung metastasis to half that seen with injection of the plasmid alone. Other studies have described the antitumor activity of IL-2 immunocomplexes, which was especially prominent when coadministrated with poly I:C.[33] However, the tumor elimination described in our work was achieved by adoptive transfer of tumor-specific T cells followed by treatment with IL-2 immunocomplexes and was thus a rather artificial model of tumor treatment. The potential of IL-2 immunocomplexes for rapid generation of a functional NK cell compartment after bone marrow transplantation was highlighted by Prlic *et al.*,[34] and the implications for preventing the tumor relapse was discussed. There is so far only one report which

marginally mentions the use of IL-2 immunocomplexes in combination with doxorubicin,[20] but no report is available on combination of IL-2 immunocomplexes with some advanced chemotherapy. Theoretically, IL-2 immunocomplexes and polymeric conjugate used in our study could be seen as structural/functional analog: biologically active compound (doxorubicin) is covalently attached to synthetic macromolecular carrier (HPMA copolymer) in polymeric conjugate, while in IL-2 immunocomplexes the biologically active compound (IL-2) is noncovalently, but still tightly, attached to IgG which can be viewed as a macromolecular carrier of natural origin. Consequences are very similar in both cases: significant improvement of pharmacological properties leading to enhanced therapeutic activity. Furthermore, IL-2 immunocomplexes can revive the enthusiasm for using IL-2 in cancer immunotherapy not just because they have superior pharmacologic properties and much higher biological activity *in vivo* than free IL-2, but they moreover possess selectivity for the subsets of immune cells with positive role in tumor immunotherapy when appropriate anti-IL-2 mAb is used. This is especially important because it was shown that tolerance, not immunity, crucially depends on IL-2.[35]

## Acknowledgements

Marek Kovar is listed as co-inventor on patent entitled "Methods for Improving Immune Function and Methods for Prevention or Treatment of Disease in a Mammalian subject," which was filed on February 16, 2007, and now bears International Application Number PCT/US2007/0623631.

## References

1. Mikyskova R, Indrova M, Simova J, Jandlova T, Bieblova J, Jinoch P, Bubenik J, Vonka V. Treatment of minimal residual disease after surgery or chemotherapy in mice carrying HPV16-associated tumours: cytokine and gene therapy with IL-2 and GM-CSF. *Int J Oncol* 2004;24:161–7.
2. Ulbrich K, Subr V, Strohalm J, Plocova D, Jelinkova M, Rihova B. Polymeric drugs based on conjugates of synthetic and natural macromolecules. I. Synthesis and physico-chemical characterisation. *J Control Release* 2000;64:63–79.
3. Sirova M, Strohalm J, Subr V, Plocova D, Rossmann P, Mrkvan T, Ulbrich K, Rihova B. Treatment with HPMA copolymer-based doxorubicin conjugate containing human immunoglobulin induces long-lasting systemic anti-tumour immunity in mice. *Cancer Immunol Immunother* 2007; 56:35–47.
4. Rihova B, Strohalm J, Kubackova K, Jelinkova M, Hovorka O, Kovar M, Plocova D, Sirova M, St'astny M, Rozprimova L, Ulbrich K. Acquired and specific immunological mechanisms co-responsible for efficacy of polymer-bound drugs. *J Control Release* 2002;78:97–114.
5. Rihova B, Strohalm J, Kovar M, Mrkvan T, Subr V, Hovorka O, Sirova M, Rozprimova L, Kubackova K, Ulbrich K. Induction of systemic antitumour resistance with targeted polymers. *Scand J Immunol* 2005; 62 (Suppl 1):100–5.
6. Rihova B. Clinical experience with anthracycline antibiotics-HPMA copolymer-human immunoglobulin conjugates. *Adv Drug Deliv Rev* 2009;61: 1149–58.
7. Satchi-Fainaro R, Puder M, Davies JW, Tran HT, Sampson DA, Greene AK, Corfas G, Folkman J. Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470. *Nat Med* 2004; 10:255–61.
8. Vetvicka D, Hruby M, Hovorka O, Etrych T, Vetrik M, Kovar L, Kovar M, Ulbrich K, Rihova B. Biological evaluation of polymeric micelles with covalently bound doxorubicin. *Bioconjug Chem* 2009;20: 2090–7.
9. Etrych T, Chytil P, Mrkvan T, Sirova M, Rihova B, Ulbrich K. Conjugates of doxorubicin with graft HPMA copolymers for passive tumor targeting. *J Control Release* 2008;132:184–92.
10. Oppenheim MH, Lotze MT. Interleukin-2: solid-tumor therapy. *Oncology* 1994;51: 154–69.
11. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 2006;6:595–601.
12. Rosenberg SA, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA* 1994;271:907–13.
13. Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. *N Engl J Med* 2004;351:998–1012.
14. Bui LA, Butterfield LH, Kim JY, Ribas A, Seu P, Lau R, Glaspy JA, McBride WH, Economou JS. In vivo therapy of hepatocellular carcinoma with a tumor-specific adenoviral vector expressing interleukin-2. *Hum Gene Ther* 1997;8: 2173–82.
15. He P, Tang ZY, Ye SL, Liu BB, Liu YK. The targeted expression of interleukin-2 in human hepatocellular carcinoma cells. *J Exp Clin Cancer Res* 2000;19:183–7.
16. Melder RJ, Osborn BL, Riccobene T, Kanakaraj P, Wei P, Chen G, Stolow D, Halpern WG, Migone TS, Wang Q, Grzegorzewski KJ, Gallant G. Pharmacokinetics and in vitro and in vivo anti-tumor response of an interleukin-2-human serum albumin fusion protein in mice. *Cancer Immunol Immunother* 2005; 54:535–47.
17. Becker JC, Pancook JD, Gillies SD, Furukawa K, Reisfeld RA. T cell-mediated eradication of murine metastatic melanoma induced by targeted interleukin 2 therapy. *J Exp Med* 1996;183:2361–6.
18. Pancook JD, Becker JC, Gillies SD, Reisfeld RA. Eradication of established hepatic human neuroblastoma metastases in mice with severe combined immunodeficiency by antibody-targeted interleukin-2. *Cancer Immunol Immunother* 1996;42:88–92.
19. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody–cytokine immune complexes. *Science* 2006;311:1924–7.
20. Tomala J, Chmelova H, Mrkvan T, Rihova B, Kovar M. In vivo expansion of activated naive CD8+ T cells and NK cells driven by complexes of IL-2 and anti-IL-2 monoclonal antibody as novel approach of cancer immunotherapy. *J Immunol* 2009; 183:4904–12.
21. Rubinstein MP, Kovar M, Purton JF, Cho JH, Boyman O, Surh CD, Sprent J. Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}. *Proc Natl Acad Sci USA* 2006;103:9166–71.
22. Kovar M, Mrkvan T, Strohalm J, Etrych T, Ulbrich K, Stastny M, Rihova B. HPMA copolymer-bound doxorubicin targeted to tumor-specific antigen of BCL1 mouse B cell leukemia. *J Control Release* 2003;92: 315–30.
23. Kovar M, Tomala J, Chmelova H, Kovar L, Mrkvan T, Joskova R, Zakostelska Z, Etrych T, Strohalm J, Ulbrich K, Sirova M, Rihova B. Overcoming immunoescape mechanisms of BCL1 leukemia and induction of CD8+ T-cell-mediated BCL1-specific resistance in mice cured by targeted polymer-bound doxorubicin. *Cancer Res* 2008;68:9875–83.

24. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, Grey ST, Sprent J. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J Exp Med* 2009;206: 751–60.
25. Boyman O, Ramsey C, Kim DM, Sprent J, Surh CD. IL-7/anti-IL-7 mAb complexes restore T cell development and induce homeostatic T Cell expansion without lymphopenia. *J Immunol* 2008;180: 7265–75.
26. Kamimura D, Sawa Y, Sato M, Agung E, Hirano T, Murakami M. IL-2 in vivo activities and antitumor efficacy enhanced by an anti-IL-2 mAb. *J Immunol* 2006;177: 306–14.
27. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 2007;13:54–61.
28. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 2007;13:1050–9.
29. Zitvogel L, Kepp O, Senovilla L, Menger L, Chaput N, Kroemer G. Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway. *Clin Cancer Res* 2010;16:3100–4.
30. Rihova B, Kovar L, Kovar M, Hovorka O. Cytotoxicity and immunostimulation: double attack on cancer cells with polymeric therapeutics. *Trends Biotechnol* 2009;27:11–7.
31. Wilson DC, Matthews S, Yap GS. IL-12 signaling drives CD8+ T cell IFN-gamma production and differentiation of KLRG1+ effector subpopulations during *Toxoplasma gondii* Infection. *J Immunol* 2008;180: 5935–45.
32. Mostbock S. Cytokine/antibody complexes: an emerging class of immunostimulants. *Curr Pharm Des* 2009;15:809–25.
33. Verdeil G, Marquardt K, Surh CD, Sherman LA. Adjuvants targeting innate and adaptive immunity synergize to enhance tumor immunotherapy. *Proc Natl Acad Sci USA* 2008;105:16683–8.
34. Prlic M, Kamimura D, Bevan MJ. Rapid generation of a functional NK-cell compartment. *Blood* 2007;110: 2024–6.
35. Malek TR, Bayer AL. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol* 2004;4:665–74.

# IV. CONCLUSION

- IL-2/S4B6 and IL-2/JES6.1 are able to potently drive the expansion of activated naive $CD8^+$ T cells into functional memory-like $CD8^+$ T cells.
- NK cells are even more sensitive to stimulation with IL-2/S4B6 than activated $CD8^+$ T cells.
- NK cells are dramatically expanded by IL-2/S4B6 and splenocytes from IL-2/S4B6 treated mice also possess higher cytolytic activity when compared to the splenocytes from mice injected with free IL-2. This effect could be further increased by co-injection of IL-12.
- IL-2/S4B6 immunocomplexes also expand $T_{reg}$, γδ T and NKT cells.
- IL-2/JES6.1 highly selectively expand $T_{reg}$ cell population *in vivo* and such increase of $T_{reg}$ cell numbers accelerate BCL1 leukemia progression.
- Increased biological activity of IL-2 immunocomplexes is probably governed by considerable prolonged half-life in circulation.
- IL-2/S4B6 treated mice bearing BCL1 leukemia and B16F10 melanoma only if injected relatively early after the inoculation of the tumor cells. This prophylactic regimens led to survival of one-third (BCL1) or up to two-thirds (B16F10) of tumor bearing mice, depending on the IL-2/S4B6 dosage.
- Combination of IL-2/S4B6 with doxorubicin was proved to be effective, even if given relatively late after the tumor cell inoculation (11 days).
- Treatment of established tumors via combination HPMA copolymer-bound doxorubicin conjugate followed by repeated injections of IL-2/S4B6 treatment prolongs survival or even completely cures mice with established and progressively growing tumors.

# V. REFERENCES


1. Zabeau, L., et al., *Neutralizing monoclonal antibodies can potentiate IL-5 signaling.* Eur J Immunol, 2001. **31**(4): p. 1087-97.
2. Boyman, O., et al., *Selective stimulation of T cell subsets with antibody-cytokine immune complexes.* Science, 2006. **311**(5769): p. 1924-7.
3. Tomala, J., et al., *In vivo expansion of activated naive CD8+ T cells and NK cells driven by complexes of IL-2 and anti-IL-2 monoclonal antibody as novel approach of cancer immunotherapy.* J Immunol, 2009. **183**(8): p. 4904-12.
4. Henney, C.S., et al., *Interleukin-2 augments natural killer cell activity.* Nature, 1981. **291**(5813): p. 335-8.
5. Rickert, M., et al., *The structure of interleukin-2 complexed with its alpha receptor.* Science, 2005. **308**(5727): p. 1477-80.
6. Kovar, M., et al., *Overcoming immunoescape mechanisms of BCL1 leukemia and induction of CD8+ T-cell-mediated BCL1-specific resistance in mice cured by targeted polymer-bound doxorubicin.* Cancer Res, 2008. **68**(23): p. 9875-83.
7. Sato, J., et al., *Enhancement of anti-tumor activity of recombinant interleukin-2 (rIL-2) by immunocomplexing with a monoclonal antibody against rIL-2.* Biotherapy, 1993. **6**(3): p. 225-31.
8. Sato, J., et al., *Pharmacokinetic alteration in rats of recombinant interleukin-2 (rIL-2) by immunocomplexing with a monoclonal antibody against rIL-2.* Biol Pharm Bull, 1994. **17**(4): p. 535-8.
9. Finkelman, F.D., et al., *Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes.* J Immunol, 1993. **151**(3): p. 1235-44.
10. Ku, C.C., et al., *Control of homeostasis of CD8+ memory T cells by opposing cytokines.* Science, 2000. **288**(5466): p. 675-8.
11. Murakami, M., et al., *CD25+CD4+ T cells contribute to the control of memory CD8+ T cells.* Proc Natl Acad Sci U S A, 2002. **99**(13): p. 8832-7.
12. Kamimura, D., et al., *Evidence of a novel IL-2/15R beta-targeted cytokine involved in homeostatic proliferation of memory CD8+ T cells.* J Immunol, 2004. **173**(10): p. 6041-9.

13. Krieg, C., et al., *Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells.* Proc Natl Acad Sci U S A, 2010. **107**(26): p. 11906-11.
14. Kamimura, D., et al., *IL-2 in vivo activities and antitumor efficacy enhanced by an anti-IL-2 mAb.* J Immunol, 2006. **177**(1): p. 306-14.
15. Tomala, J., et al., *Antitumor activity of IL-2/anti-IL-2 mAb immunocomplexes exerts synergism with that of N-(2-hydroxypropyl)methacrylamide copolymer-bound doxorubicin conjugate due to its low immunosuppressive activity.* Int J Cancer, 2011. **129**(8): p. 2002-12.
16. Kamimura, D. and M.J. Bevan, *Naive CD8+ T cells differentiate into protective memory-like cells after IL-2 anti IL-2 complex treatment in vivo.* J Exp Med, 2007. **204**(8): p. 1803-12.
17. Molloy, M.J., W. Zhang, and E.J. Usherwood, *Cutting edge: IL-2 immune complexes as a therapy for persistent virus infection.* J Immunol, 2009. **182**(8): p. 4512-5.
18. Prlic, M., D. Kamimura, and M.J. Bevan, *Rapid generation of a functional NK-cell compartment.* Blood, 2007. **110**(6): p. 2024-6.
19. Liu, R., et al., *Expansion of regulatory T cells via IL-2/anti-IL-2 mAb complexes suppresses experimental myasthenia.* Eur J Immunol, 2010. **40**(6): p. 1577-89.
20. Tang, Q., et al., *Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction.* Immunity, 2008. **28**(5): p. 687-97.
21. Webster, K.E., et al., *In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression.* J Exp Med, 2009. **206**(4): p. 751-60.
22. Fujii, Y. and J. Lindstrom, *Regulation of antibody production by helper T cell clones in experimental autoimmune myasthenia gravis.* J Immunol, 1988. **141**(10): p. 3361-9.
23. Joffre, O., et al., *Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes.* Nat Med, 2008. **14**(1): p. 88-92.
24. Nishimura, E., et al., *Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ regulatory T cells.* Int Immunol, 2004. **16**(8): p. 1189-201.
25. Tao, R., et al., *Deacetylase inhibition promotes the generation and function of regulatory T cells.* Nat Med, 2007. **13**(11): p. 1299-307.
26. Graca, L., S.P. Cobbold, and H. Waldmann, *Identification of regulatory T cells in tolerated allografts.* J Exp Med, 2002. **195**(12): p. 1641-6.

27. Letourneau, S., et al., *IL-2/anti-IL-2 antibody complexes show strong biological activity by avoiding interaction with IL-2 receptor alpha subunit CD25.* Proc Natl Acad Sci U S A, 2010. **107**(5): p. 2171-6.
28. Phelan, J.D., T. Orekov, and F.D. Finkelman, *Cutting edge: mechanism of enhancement of in vivo cytokine effects by anti-cytokine monoclonal antibodies.* J Immunol, 2008. **180**(1): p. 44-8.
29. Roopenian, D.C. and S. Akilesh, *FcRn: the neonatal Fc receptor comes of age.* Nat Rev Immunol, 2007. **7**(9): p. 715-25.
30. Roopenian, D.C., et al., *The MHC class I-like IgG receptor controls perinatal IgG transport, IgG homeostasis, and fate of IgG-Fc-coupled drugs.* J Immunol, 2003. **170**(7): p. 3528-33.
31. Epstein, A.L., et al., *Identification of a protein fragment of interleukin 2 responsible for vasopermeability.* J Natl Cancer Inst, 2003. **95**(10): p. 741-9.
32. Schrader, J.W., *The panspecific hemopoietin of activated T lymphocytes (interleukin-3).* Annu Rev Immunol, 1986. **4**: p. 205-30.
33. Yang, Y.C. and S.C. Clark, *Interleukin-3: molecular biology and biologic activities.* Hematol Oncol Clin North Am, 1989. **3**(3): p. 441-52.
34. Oster, W., et al., *Interleukin-3. Biologic effects and clinical impact.* Cancer, 1991. **67**(10 Suppl): p. 2712-7.
35. Jones, A.T. and H.J. Ziltener, *Enhancement of the biologic effects of interleukin-3 in vivo by anti-interleukin-3 antibodies.* Blood, 1993. **82**(4): p. 1133-41.
36. Eder, M., G. Geissler, and A. Ganser, *IL-3 in the clinic.* Stem Cells, 1997. **15**(5): p. 327-33.
37. Sato, T.A., et al., *Recombinant soluble murine IL-4 receptor can inhibit or enhance IgE responses in vivo.* J Immunol, 1993. **150**(7): p. 2717-23.
38. Urban, J.F., Jr., et al., *IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice.* J Immunol, 1995. **154**(9): p. 4675-84.
39. Else, K.J., et al., *Cytokine-mediated regulation of chronic intestinal helminth infection.* J Exp Med, 1994. **179**(1): p. 347-51.
40. Urban, J.F., Jr., et al., *Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5513-7.

41. Spencer, L., L. Shultz, and T.V. Rajan, *Interleukin-4 receptor-Stat6 signaling in murine infections with a tissue-dwelling nematode parasite.* Infect Immun, 2001. **69**(12): p. 7743-52.
42. Febbraio, M.A. and B.K. Pedersen, *Muscle-derived interleukin-6: mechanisms for activation and possible biological roles.* FASEB J, 2002. **16**(11): p. 1335-47.
43. Korn, T., et al., *IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells.* Proc Natl Acad Sci U S A, 2008. **105**(47): p. 18460-5.
44. Heremans, H., et al., *Protective effect of anti-interleukin (IL)-6 antibody against endotoxin, associated with paradoxically increased IL-6 levels.* Eur J Immunol, 1992. **22**(9): p. 2395-401.
45. Martens, E., et al., *Increased circulating interleukin-6 (IL-6) activity in endotoxin-challenged mice pretreated with anti-IL-6 antibody is due to IL-6 accumulated in antigen-antibody complexes.* Eur J Immunol, 1993. **23**(8): p. 2026-9.
46. Gijbels, K., et al., *Administration of neutralizing antibodies to interleukin-6 (IL-6) reduces experimental autoimmune encephalomyelitis and is associated with elevated levels of IL-6 bioactivity in central nervous system and circulation.* Mol Med, 1995. **1**(7): p. 795-805.
47. Truyens, C., et al., *Interleukin-6 (IL-6) production in mice infected with Trypanosoma cruzi: effect of its paradoxical increase by anti-IL-6 monoclonal antibody treatment on infection and acute-phase and humoral immune responses.* Infect Immun, 1994. **62**(2): p. 692-6.
48. Mihara, M., et al., *Murine anti-human IL-6 monoclonal antibody prolongs the half-life in circulating blood and thus prolongs the bioactivity of human IL-6 in mice.* Immunology, 1991. **74**(1): p. 55-9.
49. Xing, Z., et al., *IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses.* J Clin Invest, 1998. **101**(2): p. 311-20.
50. Tilg, H., et al., *Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55.* Blood, 1994. **83**(1): p. 113-8.
51. Boyman, O., et al., *IL-7/anti-IL-7 mAb complexes restore T cell development and induce homeostatic T Cell expansion without lymphopenia.* J Immunol, 2008. **180**(11): p. 7265-75.

52. Lupton, S.D., et al., *Characterization of the human and murine IL-7 genes.* J Immunol, 1990. **144**(9): p. 3592-601.

53. Goodwin, R.G., et al., *Human interleukin 7: molecular cloning and growth factor activity on human and murine B-lineage cells.* Proc Natl Acad Sci U S A, 1989. **86**(1): p. 302-6.

54. Levy, Y., et al., *Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment.* J Clin Invest, 2009. **119**(4): p. 997-1007.

55. Pellegrini, M., et al., *IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology.* Cell, 2011. **144**(4): p. 601-13.

56. Vassena, L., et al., *Treatment with IL-7 prevents the decline of circulating CD4+ T cells during the acute phase of SIV infection in rhesus macaques.* PLoS Pathog, 2012. **8**(4): p. e1002636.

57. Levy, Y., et al., *Effects of recombinant human interleukin 7 on T-cell recovery and thymic output in HIV-infected patients receiving antiretroviral therapy: results of a phase I/IIa randomized, placebo-controlled, multicenter study.* Clin Infect Dis, 2012. **55**(2): p. 291-300.

58. Waldmann, T.A., S. Dubois, and Y. Tagaya, *Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy.* Immunity, 2001. **14**(2): p. 105-10.

59. Steel, J.C., T.A. Waldmann, and J.C. Morris, *Interleukin-15 biology and its therapeutic implications in cancer.* Trends Pharmacol Sci, 2012. **33**(1): p. 35-41.

60. Bamford, R.N., et al., *The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells.* Proc Natl Acad Sci U S A, 1994. **91**(11): p. 4940-4.

61. Vamosi, G., et al., *IL-2 and IL-15 receptor alpha-subunits are coexpressed in a supramolecular receptor cluster in lipid rafts of T cells.* Proc Natl Acad Sci U S A, 2004. **101**(30): p. 11082-7.

62. Rubinstein, M.P., et al., *Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 9166-71.

63. Elpek, K.G., et al., *Mature natural killer cells with phenotypic and functional alterations accumulate upon sustained stimulation with IL-15/IL-15Ralpha complexes.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21647-52.

64. Epardaud, M., et al., *Interleukin-15/interleukin-15R alpha complexes promote destruction of established tumors by reviving tumor-resident CD8+ T cells.* Cancer Res, 2008. **68**(8): p. 2972-83.

65. Stoklasek, T.A., K.S. Schluns, and L. Lefrancois, *Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo.* J Immunol, 2006. **177**(9): p. 6072-80.

66. Mortier, E., et al., *Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma. Hyperagonist IL-15 x IL-15R alpha fusion proteins.* J Biol Chem, 2006. **281**(3): p. 1612-9.

67. Li, Y., et al., *A dual-targeting nanocarrier based on poly(amidoamine) dendrimers conjugated with transferrin and tamoxifen for treating brain gliomas.* Biomaterials, 2012. **33**(15): p. 3899-908.

68. Tang, Y., et al., *Efficient in vitro siRNA delivery and intramuscular gene silencing using PEG-modified PAMAM dendrimers.* Mol Pharm, 2012. **9**(6): p. 1812-21.

69. Whiteman, K.R., et al., *Poly(Hpma)-coated liposomes demonstrate prolonged circulation in mice.* J Liposome Res, 2001. **11**(2-3): p. 153-64.

70. Kataoka, K., et al., *Doxorubicin-loaded poly(ethylene glycol)-poly(beta-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance.* J Control Release, 2000. **64**(1-3): p. 143-53.

71. Chytil, P., et al., *New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting.* J Control Release, 2008. **127**(2): p. 121-30.

72. Pechar, M., et al., *Poly(ethylene glycol) multiblock copolymer as a carrier of anti-cancer drug doxorubicin.* Bioconjug Chem, 2000. **11**(2): p. 131-9.

73. Etrych, T., et al., *Biodegradable star HPMA polymer-drug conjugates: Biodegradability, distribution and anti-tumor efficacy.* J Control Release, 2011. **154**(3): p. 241-8.

74. Kovar, M., et al., *HPMA copolymer-bound doxorubicin targeted to tumor-specific antigen of BCL1 mouse B cell leukemia.* J Control Release, 2003. **92**(3): p. 315-30.

75. Rihova, B., et al., *Drug-HPMA-HuIg conjugates effective against human solid cancer.* Adv Exp Med Biol, 2003. **519**: p. 125-43.

76. Chu, T.W., J. Yang, and J. Kopecek, *Anti-CD20 multivalent HPMA copolymer-Fab' conjugates for the direct induction of apoptosis.* Biomaterials, 2012. **33**(29): p. 7174-81.

77. Lammers, T. and K. Ulbrich, *HPMA copolymers: 30 years of advances.* Adv Drug Deliv Rev, 2010. **62**(2): p. 119-21.

78. Ulbrich, K. and V. Subr, *Structural and chemical aspects of HPMA copolymers as drug carriers.* Adv Drug Deliv Rev, 2010. **62**(2): p. 150-66.

79. Rihova, B. and M. Kovar, *Immunogenicity and immunomodulatory properties of HPMA-based polymers.* Adv Drug Deliv Rev, 2010. **62**(2): p. 184-91.

80. Seymour, L.W., et al., *Phase II studies of polymer-doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer.* Int J Oncol, 2009. **34**(6): p. 1629-36.

81. Hovorka, O., et al., *HPMA based macromolecular therapeutics: internalization, intracellular pathway and cell death depend on the character of covalent bond between the drug and the peptidic spacer and also on spacer composition.* J Drug Target, 2006. **14**(6): p. 391-403.

82. Liu, J., et al., *Endocytic uptake of a large array of HPMA copolymers: Elucidation into the dependence on the physicochemical characteristics.* J Control Release, 2010. **143**(1): p. 71-9.

83. Omelyanenko, V., et al., *Targetable HPMA copolymer-adriamycin conjugates. Recognition, internalization, and subcellular fate.* J Control Release, 1998. **53**(1-3): p. 25-37.

84. Minko, T., et al., *HPMA copolymer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line.* J Control Release, 1998. **54**(2): p. 223-33.

85. Matsumura, Y. and H. Maeda, *A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs.* Cancer Res, 1986. **46**(12 Pt 1): p. 6387-92.

86. Kopecek, J., L. Sprincl, and D. Lim, *New types of synthetic infusion solutions. I. Investigation of the effect of solutions of some hydrophilic polymers on blood.* J Biomed Mater Res, 1973. **7**(2): p. 179-91.

87. Duncan, R., J.B. Lloyd, and J. Kopecek, *Degradation of side chains of N-(2-hydroxypropyl) methacrylamide copolymers by lysosomal enzymes.* Biochem Biophys Res Commun, 1980. **94**(1): p. 284-90.

88. Krinick, N.L., et al., *A polymeric drug delivery system for the simultaneous delivery of drugs activatable by enzymes and/or light.* J Biomater Sci Polym Ed, 1994. **5**(4): p. 303-24.

89. Greco, F., et al., *Investigating the mechanism of enhanced cytotoxicity of HPMA copolymer-Dox-AGM in breast cancer cells.* J Control Release, 2007. **117**(1): p. 28-39.

90. Krakovicova, H., T. Etrych, and K. Ulbrich, *HPMA-based polymer conjugates with drug combination.* Eur J Pharm Sci, 2009. **37**(3-4): p. 405-12.

91. Lammers, T., et al., *Simultaneous delivery of doxorubicin and gemcitabine to tumors in vivo using prototypic polymeric drug carriers.* Biomaterials, 2009. **30**(20): p. 3466-75.

92. Miller, K., et al., *Antiangiogenic antitumor activity of HPMA copolymer-paclitaxel-alendronate conjugate on breast cancer bone metastasis mouse model.* Mol Pharm, 2011. **8**(4): p. 1052-62.

93. Rihova, B., *Biocompatibility and immunocompatibility of water-soluble polymers based on HPMA.* Composites: Part B, 2007. **38** p. 386–397.

94. Korcakova, L., et al., *Asimple test for immunogenicity of colloidal infusion solutions-the draining lymph node activation.* Z Immunitatsforsch Exp Klin Immunol, 1976. **151**(3): p. 219-23.

95. Sprincl, L., et al., *New types of synthetic infusion solutions. III. Elimination and retention of poly-[N-(2-hydroxypropyl)methacrylamide] in a test organism.* J Biomed Mater Res, 1976. **10**(6): p. 953-63.

96. Simeckova, B., et al., *Activity of complement in the presence of N-(2-hydroxypropyl)methacrylamide copolymers.* J Bioact Comp Polym, 1986. **1**: p. 20–31.

97. Rihova, B., et al., *Effect of the chemical structure of N-(2-hydroxypropyl)methacrylamide copolymers on their ability to induce antibody formation in inbred strains of mice.* Biomaterials, 1984. **5**(3): p. 143-8.

98. Rihova, B., et al., *Biocompatibility of N-(2-hydroxypropyl) methacrylamide copolymers containing adriamycin. Immunogenicity, and effect on haematopoietic stem cells in bone marrow in vivo and mouse splenocytes and human peripheral blood lymphocytes in vitro.* Biomaterials, 1989. **10**(5): p. 335-42.

99. Volfova, I., et al., *Biocompatibility of biopolymers* J Bioact Compat Polym, 1992. **7**: p. 175–90.

100. Flanagan, P.A., et al., *Immunogenicity of protein-N-(2-hydroxypropyl)methacrylamide copolymer conjugates in A/J and B10 mice.* J Bioact Compat Polym, 1990. **5**(2): p. 151-165.

101. Duncan, R. and F. Spreafico, *Polymer conjugates. Pharmacokinetic considerations for design and development.* Clin Pharmacokinet, 1994. **27**(4): p. 290-306.

102. Kopecek, J., *Controlled biodegradability of polymers--a key to drug delivery systems.* Biomaterials, 1984. **5**(1): p. 19-25.

103. Rihova, B., et al., *Doxorubicin release is not a prerequisite for the in vitro cytotoxicity of HPMA-based pharmaceuticals: in vitro effect of extra drug-free GlyPheLeuGly sequences.* J Control Release, 2008. **127**(2): p. 110-20.

104. Ulbrich, K., et al., *HPMA copolymers with pH-controlled release of doxorubicin: in vitro cytotoxicity and in vivo antitumor activity.* J Control Release, 2003. **87**(1-3): p. 33-47.

105. Etrych, T., et al., *Conjugates of doxorubicin with graft HPMA copolymers for passive tumor targeting.* J Control Release, 2008. **132**(3): p. 184-92.

106. Lammers, T., et al., *Effect of physicochemical modification on the biodistribution and tumor accumulation of HPMA copolymers.* J Control Release, 2005. **110**(1): p. 103-18.

107. Seymour, L.W., et al., *Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier.* Eur J Cancer, 1995. **31A**(5): p. 766-70.

108. Lammers, T., et al., *Effect of intratumoral injection on the biodistribution and the therapeutic potential of HPMA copolymer-based drug delivery systems.* Neoplasia, 2006. **8**(10): p. 788-95.

109. Sirova, M., et al., *Treatment with HPMA copolymer-based doxorubicin conjugate containing human immunoglobulin induces long-lasting systemic anti-tumour immunity in mice.* Cancer Immunol Immunother, 2007. **56**(1): p. 35-47.

110. Vasey, P.A., et al., *Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents-drug-polymer conjugates. Cancer Research Campaign Phase I/II Committee.* Clin Cancer Res, 1999. **5**(1): p. 83-94.

111. Choi, W.M., et al., *Synthesis of HPMA copolymer containing adriamycin bound via an acid-labile spacer and its activity toward human ovarian carcinoma cells.* J Bioact Compat Polym, 1999. **14**: p. 447-556.

112. Etrych, T., et al., *New HPMA copolymers containing doxorubicin bound via pH-sensitive linkage: synthesis and preliminary in vitro and in vivo biological properties.* J Control Release, 2001. **73**(1): p. 89-102.

113. Julyan, P.J., et al., *Preliminary clinical study of the distribution of HPMA copolymers bearing doxorubicin and galactosamine.* J Control Release, 1999. **57**(3): p. 281-90.

114. Rihova, B., et al., *Antiproliferative effect of a lectin- and anti-Thy-1.2 antibody-targeted HPMA copolymer-bound doxorubicin on primary and metastatic human colorectal carcinoma and on human colorectal carcinoma transfected with the mouse Thy-1.2 gene.* Bioconjug Chem, 2000. **11**(5): p. 664-73.

115. Wroblewski, S., et al., *Biorecognition of HPMA copolymer-lectin conjugates as an indicator of differentiation of cell-surface glycoproteins in development, maturation, and diseases of human and rodent gastrointestinal tissues.* J Biomed Mater Res, 2000. **51**(3): p. 329-42.

116. Rihova, B., N.L. Krinick, and J. Kopecek, *Targetable photoactivatable drugs. 3. In vitro efficacy of polymer bound chlorin e 6 toward human hepatocarcinoma cell line (PLC/PRF/5) targeted with galactosamine and to mouse splenocytes targeted with anti-Thy 1.2 antibodies.* J Contr Rel 1993. **25**: p. 71–87.

117. Kovar, M., et al., *Star structure of antibody-targeted HPMA copolymer-bound doxorubicin: a novel type of polymeric conjugate for targeted drug delivery with potent antitumor effect.* Bioconjug Chem, 2002. **13**(2): p. 206-15.

118. Jelinkova, M., et al., *Starlike vs. classic macromolecular prodrugs: two different antibody-targeted HPMA copolymers of doxorubicin studied in vitro and in vivo as potential anticancer drugs.* Pharm Res, 2003. **20**(10): p. 1558-64.

119. Etrych, T., et al., *Star-shaped immunoglobulin-containing HPMA-based conjugates with doxorubicin for cancer therapy.* J Control Release, 2007. **122**(1): p. 31-8.

120. Thomson, A.H., et al., *Population pharmacokinetics in phase I drug development: a phase I study of PK1 in patients with solid tumours.* Br J Cancer, 1999. **81**(1): p. 99-107.

121. Bilim, V., *Technology evaluation: PK1, Pfizer/Cancer Research UK.* Curr Opin Mol Ther, 2003. **5**(3): p. 326-30.

122. Seymour, L.W., et al., *Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin.* J Clin Oncol, 2002. **20**(6): p. 1668-76.

123. Rihova, B., et al., *Cytostatic and immunomobilizing activities of polymer-bound drugs: experimental and first clinical data.* J Control Release, 2003. **91**(1-2): p. 1-16.