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Immunogenicity of coiled-coil based drug-free macromolecular therapeutics

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Abstract

A two-component CD20 (non-internalizing) receptor crosslinking system based on the biorecognition of complementary coiled-coil forming peptides was evaluated. Exposure of B cells to Fab'-peptide1 conjugate decorates the cell surface with peptide1; further exposure of the decorated cells to P-(peptide2)_x (P is the *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer backbone) results in the formation of coiled-coil heterodimers at the cell surface with concomitant induction of apoptosis. The aim of this study was to determine the potential immunogenicity of this therapeutic system that does not contain low molecular weight drugs. Enantiomeric peptides (L- and D-CCE and L- and D-CCK), HPMA copolymer-peptide conjugates, and Fab' fragment-peptide conjugates were synthesized and the immunological properties of peptide conjugates evaluated in vitro on RAW264.7 macrophages and in vivo on immunocompetent BALB/c mice. HPMA copolymer did not induce immune response in vitro and in vivo. Administration of P-peptide conjugates with strong adjuvant resulted in antibody response directed to the peptide. Fab' was responsible for macrophage activation of Fab'-peptide conjugates and a major factor in the antibody induction following i.v. administration of Fab' conjugates. There was no substantial difference in the ability of conjugates of D-peptides and conjugates of L-peptides to induce Ab response.

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Keywords

Peptide; coiled-coil; enantiomers; immunogenicity; HPMA copolymer; Fab' fragment; drug-free macromolecular therapeutics

1. Introduction

Self-assembled hybrid biomaterials composed from at least two distinct classes of macromolecules are major components of smart systems with a high translational potential [1]. One of the hybrid materials developed was based on graft copolymers composed from a synthetic polymer backbone and complementary peptide grafts that, when mixed, self-assemble through coiled-coil formation [2]. For example, a mixture of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers grafted with a pair of oppositely charged coiled-coil forming peptides, CCE and CCK (graft copolymers P-CCE and P-CCK), spontaneously self-assembled into a 3D hydrogel [3, 4].

Expansion of hydrogel design principles to a biological system led to the development of macromolecular therapeutics for the treatment of Non-Hodgkin lymphoma (NHL). It is well established that crosslinking of CD20 receptors at the B cell surface initiates apoptosis [5, 6]. A system composed of a conjugate of Fab' fragment of anti-CD20 antibody with CCE peptide and HPMA copolymer grafted with multiple copies of the complementary CCK peptide has been designed based on this rationale. Exposure of Raji B cells to anti-CD20 Fab'-CCE conjugate decorated the cell surface with CCE (CD20 is a non-internalizing receptor) through antigen (Ag)-antibody (Ab) fragment recognition. Further exposure of the decorated cells to P-(CCK)_x (P is the copolymer backbone grafted with multiple copies of CCK) resulted in the formation of CCE/CCK heterodimers at the cell surface. This second biorecognition between CCE and CCK induced the crosslinking of CD20 receptors and triggered the apoptosis of Raji B cells *in vitro* [7] and in a NHL animal model *in vivo* [8]. This is a new concept, where the biological activity of the therapeutic system is based on the biorecognition of complementary motifs. We coined the phrase “drug-free macromolecular therapeutics” for this system; no low molecular weight drug is involved, and the individual parts of the delivery system do not have apoptosis inducing activity.

For the ultimate translation of this system into the clinics, its biocompatibility and immunocompatibility are of utmost importance [9]. There is sufficient knowledge in the literature on the biocompatibility of antibodies and antibody fragments as well as on ways to manipulate their primary structure to enhance their biocompatibility [10]. HPMA homopolymer is non-immunogenic; it does not activate lymph node cells [11] and did not induce detectable levels of antibodies in five different strains of mice following intraperitoneal administration as an allum precipitate [12]. The presence of short oligopeptide side chains attached to polyHPMA results in a weak antibody (Ab) response. The intensity of Ab production depends on the structure of the short peptide side-chain, dose, and genetic background of the mice [12]. HPMA copolymers have been used as drug carriers for decades; the biocompatibility and non-immunogenicity of HPMA copolymer-

doxorubicin (adriamycin) conjugate containing a GFLG peptide spacer was determined on two inbred strains of mice [13] and validated in clinical trials (for reviews see [14-16]).

However, there is insufficient data on the potential immunogenicity of longer peptides and their conjugates with Fab' fragments and synthetic polymers. Peptides are commonly considered weak immunogens, and the production of antibodies against them requires the use of adjuvants [17]. In addition, attachment of peptides to non-immunogenic polymeric carriers results in a decrease in their immune response [18, 19]. However, the response may increase upon self-assembly [20] and result in the production of conformation-specific antibodies [21, 22]. Finally, the question of response to enantiomeric peptides needs to be addressed [23-25].

In this study, we have evaluated immunological properties of the drug-free macromolecular therapeutics system. To this end, we have synthesized enantiomeric peptides (L- and D-CCCE and L- and D-CCK), HPMA copolymer-peptide conjugates, Fab' fragment-peptide conjugates and evaluated their immunological properties *in vitro* on RAW264.7 macrophages and *in vivo* on immunocompetent BALB/c mice. Individual samples and complementary mixtures that form coiled-coil structures were evaluated. Both, B cell and T cell responses were assessed as well as the Ab response to another Ag (ovalbumin).

2. Materials and Methods

2.1 Materials

N- α -Fmoc protected amino acids were purchased from P3 Biosystems and AAPTEC. V-501 (4,4'-azobis(4-cyanopentanoic acid)) and V-65 (2,2'-azobis(2,4-dimethyl valeronitrile)) were purchased from Wako Chemicals (Richmond, VA). Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was purchased from Soltec Ventures (Beverly, MA). Chain transfer agent 4-cyanopentanoic acid dithiobenzoate (CPDB) [26] and *N*-(2-hydroxypropyl)methacrylamide (HPMA) [27] were synthesized as previously described. *N*-(3-Aminopropyl)methacrylamide (APMA) was purchased from Polysciences (Warrington, PA). 3,3',5,5'-Tetramethylbenzidine (TMB) and all solvents were purchased from Sigma-Aldrich.

2.2 Synthesis of conjugates

2.2.1 Polymer synthesis—A copolymer of HPMA and APMA was synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization as previously described [28]. Briefly, the monomers HPMA and APMA, chain transfer agent CPDB, and V-501 were placed in an ampule and sealed after bubbling the solution with nitrogen. The reaction proceeded at 70 °C for 18 h. After polymerization, the polymer was precipitated in acetone/ether. The copolymer end groups were modified using a 20x molar excess V-65 in methanol at 50 °C for 4 h. Pendant amine groups were converted to maleimide groups using the heterobifunctional linker SMCC [28]. Amine and maleimide contents were determined using ninhydrin and modified Ellman's assays, respectively [29, 30]. The polymer molecular weight and polydispersity were determined using an ÄKTA FPLC system (GE Healthcare, Piscataway, NJ) equipped with OptilabREX and miniDAWN detectors. Superose 6

HR10/30 column (GE Healthcare) was used with a mobile phase of sodium acetate buffer and 30% acetonitrile (v/v) (pH = 6.5).

2.2.2 Peptide synthesis—The peptides CCK (K VSALKEK VSALKEE VSANKEK VSALKEK VSALKE) and CCE (E VSALEKE VSALEKK NSALEKE VSALEKE VSALEK) [28] were synthesized (Fig. 1) using solid phase synthesis on a PS3TM peptide synthesizer (Protein Technologies, Tucson, AZ). The N terminus was optionally functionalized with a spacer of CYGG (denoted as “sh”) or SMCC capped YGG spacer (denoted as “mal”). In the case of L-CCEmal, the N terminus was functionalized with 3-maleimidopropionic acid instead of SMCC. Peptides of L- and D-chirality were synthesized. In total, eight different peptides were synthesized (L-CCKsh, D-CCKsh, LCCEsh, D-CCEsh, L-CCKmal, D-CCKmal, L-CCEmal, D-CCEmal). All peptides were purified using reverse phase high-performance liquid chromatography (RP-HPLC, Agilent Technologies 1100 series), and the molecular weights were confirmed using MALDI-TOF mass spectrometry (UltrafleXtreme, Bruker Daltonics). The mass spectra of peptides are in Supplementary Data Figs. S1-S8.

2.2.3 Polymer conjugate synthesis—Peptides were covalently attached to the copolymer by reaction of the thiol group on cysteine with the maleimide groups on the polymer backbone forming a stable thioether bond (Fig. 2). The polymer and peptide were dissolved in PBS with 10 mM tris(2-carboxyethyl)phosphine (TCEP). Attachment was allowed to proceed overnight. Unconjugated peptides were removed using ultrafiltration. The peptide content on the graft copolymers was determined using amino acid analysis. Four different graft copolymers were produced: P-L-CCK, P-D-CCK, P-L-CCE, P-D-CCE. The composition of the conjugates is in Table 1; a representative size exclusion chromatogram is on Fig. S9 and CD spectra on Figs. S10 and S11.

2.2.4 Fab' and Fab'-peptide conjugates preparation—Fab' fragment was prepared as previously described [28]. Briefly, 1F5 monoclonal Ab was prepared by culturing a hybridoma cell line in a CellMax bioreactor (Spectrum Laboratories, Rancho Dominguez, CA). Ab was harvested from the reactor and purified on a protein G column. Purified 1F5 Ab was then digested using pepsin (10 w%) in citric buffer (pH 4) for 2 h at 37 °C. F(ab')₂ was isolated using ultrafiltration to remove the digest products. F(ab')₂ was reduced using 10 mM TCEP in PBS for 1 h at 37 °C. TCEP was removed using ultrafiltration. Maleimide functionalized peptides were added to the Fab' solution in 20x molar excess. Unconjugated peptides were removed using ultrafiltration. The 1F5, F(ab')₂, Fab' and Fab'-peptide conjugates were analyzed using FPLC (Fig. S12) and SDS-PAGE (Fig. S13). The following Fab' conjugates were prepared: Fab'-L-CCE, Fab'-L-CCK, Fab'-D-CCE, Fab'-D-CCK.

2.3 Cells

Mouse macrophage cell line RAW264.7 was maintained in complete Dulbecco's modified Eagle's medium (D-MEM; Sigma-Aldrich) supplemented with 10 % fetal bovine serum (Hyclone, Thermo Fisher Scientific), 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 100 U/ml penicillin and 100 µg/mL streptomycin (Gibco/Life Technologies) at 37°C, 5% CO₂. The cells were seeded in 96 well plates at 4×10^5 /mL (200 µL/well) for 24 h. Then, the

supernatant was replaced with the selected components of therapeutics at 100 µg/mL in complete medium, in the presence of 10 µg/mL Polymyxin B. According to our preliminary experiments, this concentration of Polymyxin B does not significantly influence the cell viability (data not shown). Polymyxin B was used to inhibit LPS contamination in all wells except for positive controls containing 10 ng/mL *Escherichia coli* serotype O55:B5 (Sigma-Aldrich). The cells were then incubated for 24 h at 37°C, 5% CO₂. To analyze the dynamic of this reaction, selected samples were measured also after 1h and 72h. After the incubation, the supernatants were frozen at -20°C for subsequent analyses and the cells were collected for flow cytometry.

2.3.1 Cytokine quantification by sandwich ELISA—The concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 in cell culture supernatants were detected using mouse ELISA antibody pair sets (Invitrogen, Carlsbad, CA) according to the manufacturers' recommendations with minor modifications. Briefly, flat bottom 96-well ELISA plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 100 µL/well of capture antibody diluted in coating buffer (50 mM NaHCO₃, 50 mM Na₂CO₃; pH to 9.4) and incubated overnight at 4°C. After washing one time with PBS containing 0.05% Tween 20 (Sigma-Aldrich) (PBS-T), the plates were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 2 h at room temperature (RT). Then, 100 µL/well of samples diluted either 2x (IL-10 and IL-1β), 4x (TNF-α) or 10x (IL-6) in 1% BSA or relevant standards were added, together with 50 µL/well of horseradish peroxidase (HRP)-labeled detection antibody diluted to working concentration in 1% BSA, and incubated for 2 h at RT. The TMB substrate solution was prepared just before the detection by mixing even amounts of 1.66 mM 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich) dissolved in 27% (4 M) dimethylformamide and citrate buffer (pH=4.2), and supplemented with 0.006% of H₂O₂. After washing four times with PBS-T, 100 µL/well substrate solution was added and reacted for 5-15 min at RT in the dark. The reaction was stopped by the addition of 2 M sulfuric acid (50 µL/well) and the absorbance was detected photometrically at 450 nm with correction at 650 nm (Bio-Rad ELISA plate reader, Hercules, CA).

2.3.2 Nitrite production by Griess assay—Nitrite (NO⁻²) production was measured by a microplate adaptation of the Griess assay. Briefly, 50 µL of each supernatant sample or sodium nitrite standard dissolved in cultivation medium was incubated with 50 µL of Griess reagent (40 mg/mL; Sigma-Aldrich) for 15 min at room temperature and the absorbance at 540 nm was measured with an ELISA plate reader (Bio-Rad).

2.3.3 Flow cytometry—After the supernatants were collected for measuring cytokine and nitrite levels, the cells were transferred to the 96 well plate (Becton Dickinson, San Jose, CA) and washed with PBS. After blocking with 10% normal mouse serum in PBS for 20 min at 4°C, the cells were stained for 30 min with PE-Cyannine7 conjugated CD11b, APC conjugated CD40, and APC-eFluor[®] 780 conjugated CD127 (IL-7R) (all rat anti-mouse, eBioscience, San Diego, CA). After two washings with PBS, the dead cells were stained by 1 µg/mL of Hoechst 33258 (Life Technologies) for 10 min and measured on a FACS Canto II with high-throughput sampler (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Ashland, OR). At each experiment, the unstained cells and single-stain controls

were used for fine voltage adjustment using BD FACSDiva™ acquisition software (Becton Dickinson) and for fluorescence compensation using FlowJo. The cell viability was defined as percentage of Hoechst 33258⁻ cells out of singlets (FSC-H vs. FSC A), the degree of cell activation was defined as the percentages of CD40⁺, and polarization towards classical (M1-type) phenotype was defined as the percentages of CD127⁺ cells out of live and CD11b⁺ cells, respectively.

2.4 Mice and immunizations

BALB/c mice (6 weeks, female) purchased from Charles River Laboratories (Wilmington, MA) were used in all experiments. To induce the hyperimmune serum and cells, the mice were immunized subcutaneously with 50 µg of one of the major components (Fab'-L-CCE, Fab'-D CCE, P-L-CCK or P-D-CCK) in complete Freund's adjuvant (CFA) at day 1 and boosted 3 times at the day 7, 17 and 21 with 25 µg of the same material in incomplete Freund's adjuvant (IFA). The mice were sacrificed at day 35, and their serum, spleens and inguinal lymph nodes (ILN) were harvested for further analyses. Pool of the sera from mice immunized by Fab'-conjugates or by HPMA copolymer-conjugates was used as positive control for ELISA.

To analyze the immune response against therapeutics *in vivo*, the mice were immunized subcutaneously with 50 µg of ovalbumin (OVA) in complete Freund's adjuvant (CFA) and boosted 2 times at the day 7 and 17 with 25 µg of OVA in incomplete Freund's adjuvant (IFA). At day 24, 26 and 28, the mice were treated intravenously with therapeutics based either on L-peptides (Fab'-L-CCE/P-L-CCK (MIX L)) or D-peptides (Fab'-D-CCE/P-D-CCK (MIX D)). To analyze the dose response, 3 different doses of each therapeutic were used; high (250 µg of Fab'-CCE with 1630 µg of P-CCK), medium (50 µg of Fab'-CCE with 324 µg of P-CCK) or low (10 µg of Fab'-CCE with 64.8 µg of P-CCK). Negative control mice received injections of PBS only. This regime, three doses of medium dose premixture, showed high therapeutic potential in animal model of NHL [8]. To monitor the formation of specific antibodies (Ab), small amount of blood was collected at day 1, 24, 31 and 38, by cheek puncture method. The mice were sacrificed at day 45, and their blood and spleens were harvested for further analyses. The blood samples were allowed to clot and the serum was removed by centrifugation at 1,500 g for 20 min at 4°C and stored at -20°C before analysis. In all animal work, institutional guidelines for the care and use of laboratory animals were strictly followed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah.

2.4.1 Antibody responses (ELISA)—To analyze the Ab response, we developed an indirect ELISA protocol. Briefly, flat bottom 96-well ELISA plates (MaxiSorp; Nunc) were coated with antigens (50 µl/well at 5 µg/mL in PBS) and incubated overnight at 4°C. After washing one time with PBS-T, the plates were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 2 h at room temperature. After washing one time with PBS-T, the plates were incubated with serum samples diluted 1:100, unless stated otherwise. One percent BSA was used as a blank, and a pool of the hyper-immune mouse sera as a positive control. After washing three times with PBS-T, secondary antibodies (50 µL/well) were added and incubated for 1 h at room temperature. Horseradish peroxidase (HRP)-

labeled Fc-specific goat F(ab')₂, anti-mouse IgM or IgG (both Abcam, Cambridge, UK) diluted 1:12000 in 1% BSA were used. After a washing step, freshly prepared TMB substrate (Sigma-Aldrich) was added to each well; then the reaction was stopped with 50 μ L/well of 2 M H₂SO₄, and absorbance at 450 nm with correction at 650 nm was measured. The optical density (OD) of the background (1% BSA) was subtracted, and samples on each plate were normalized to the OD of positive control prepared from hyperimmune sera (100%), to allow comparison between multiple plates.

To test the Ab avidity, the Ag-Ab binding was disrupted with increasing concentrations (0.16-2 M) of chaotropic agent (sodium thiocyanate [NaSCN]). In preliminary experiments, we found that concentrations of NaSCN up to 2M do not affect the binding of Ag to the plate.

To test which part of the therapeutic raises the Ab formation, we coated the ELISA plates with all four conjugates, Fab', and HPMA copolymer and then analyzed the same serum samples from mice treated with medium dose of MIX L (n=3) or MIX D (n=3). The OD of anti-Fab' or anti-HPMA antibodies were compared with the OD of relevant anti-conjugate antibodies in the same sample. Moreover, to support these data, one set of blocking experiments was performed with the samples from mice after i.v. injection of MIX D, where the samples were incubated with increasing amounts of either Fab' or D-CCKsh for 30 min at RT before they were analyzed for anti-Fab'-D-CCE or anti-P-D-CCK Ab. To determine the role of Ab directed against the structure of coiled coils, the plates were coated either with D-CCE, D-CCK or their premixture.

2.4.2 T cell responses—Spleens were mechanically disrupted, and passed through a 70 μ m cell strainer (Becton Dickinson), washed and red blood cells were lysed by incubating in RBC lysing buffer (1 mM EDTA, 150 mM NH₄Cl, 10 mM KHCO₃). For intracellular staining, 1×10^6 splenocytes were resuspended in complete RPMI 1640 medium (containing 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin) and stimulated *ex vivo* with 100 μ g/mL of therapeutics, their major components, or Cell stimulation cocktail (eBioscience, San Diego, CA) containing phorbol 12-myristate 13-acetate with Ionomycin (PMA/I) as positive control.

Early T cell response was measured by intracellular production of interferon (IFN)- γ after 12 h of incubation with therapeutics. The IFN- γ trafficking and secretion were inhibited by addition of Brefeldin A (3 μ g/mL; eBioscience) for the last 4 h of the cultivation. Then the cells were transferred to a 96 well plate (Becton Dickinson) and washed in PBS. The cells were kept in the PBS with Brefeldin A on ice and incubated in the dark at 4°C. The centrifugations were done at 300 g, 5 min, 4°C, unless stated otherwise. After blocking with 10% normal mouse serum in PBS for 20 minutes, the cells were stained for 30 min with Fixable Viability Dye eFluor® 450 and PE-Cyannine7 conjugated hamster anti-mouse CD3 (both eBioscience). After washing, the cells were fixed and permeabilized with Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) for 45 min. After two washings with Permeabilization buffer (eBioscience), intracellular staining was performed using PE-conjugated rat anti-mouse IFN- γ . After two washings with Permeabilization buffer, cells

were resuspended in PBS and measured on a FACS Canto II as described above. The percentage of IFN- γ ⁺ CD3⁺ cells out of singlets/live cells was used for statistical analysis.

Late T cell response was measured by T cell proliferation. Splenocytes were labeled with 4 μ M carboxy-fluorescein succinimidyl ester (CFSE; Invitrogen) and incubated for 72 h at 37°C, 5% CO₂ with therapeutics or PMA/I. Then the cells were transferred to the 96U well plate (Becton Dickinson) and washed in PBS. After blocking with 10% normal mouse serum in PBS for 20 min, the cells were stained for 30 min with PE-Cyannine7 conjugated hamster anti-mouse CD3 (both eBioscience). After two washings with PBS, the cells were stained by 1 μ g/mL of Hoechst 33258 (Life Technologies) for 10 min and analyzed as described above. For each sample, the mean fluorescence intensity (MFI) in CFSE of singlets/cells/live CD3⁺ cells was obtained, and its decrease after treatment with different stimuli was analyzed. The more the cells proliferate the lower the MFI. For easier comparison and to avoid the imprecisions of initial CFSE staining, the MFI of untreated cells were considered 100% and the treated samples are depicted as percentage of untreated MFI.

2.5 Statistical analysis

The bar graphs show mean and standard deviation (SD). The differences between treated groups and control groups (stimulation of RAW264.7 cells *in vitro*, T cell response), were analyzed by one-way ANOVA with Dunnett's post hoc test, and the differences between the treated groups and untreated group in time were analyzed by repeated-measure two-way ANOVA with Bonferonni post-hoc test (Ab response in mice). The differences in the amount of antibodies produced between i.v. and s.c. treated mice were analyzed by unpaired Student's t-test. GraphPad Prism statistical software (GraphPad Software, La Jolla, CA) was used for analyses, and p-values < 0.05 were considered significant.

3. Results

3.1 Synthesis and characterization of conjugates

The *HPMA copolymer-peptide conjugates* were prepared in several steps (Fig. 2). First, a copolymer of HPMA and APMA (P-NH₂) was prepared by RAFT copolymerization. The Mw was 100 kDa and polydispersity (PDI) = 1.07. The NH₂ content was 372 nmol/mg copolymer. The size exclusion chromatogram is shown in Fig. S9.

In the second step the amino groups at side chain termini were converted into maleimido groups by reaction with SMCC (copolymer P-mal). This polymer precursor was used for attachment of four peptides, L-CCKsh, D-CCKsh, L-CCEsh, D-CCEsh, via thioether bonds to produce HPMA copolymer-peptide conjugates, P-L-CCK, P-D-CCK, P-L-CCE, P-D-CCE. They are characterized in Table 1.

Fab'-peptide conjugates. The Fab' fragment of the 1F5 Ab (see 2.2.4) was conjugated to maleimide terminated peptides, L-CCKmal, D-CCKmal, L-CCEmal, D-CCEmal, via thioether bonds to produce Fab'-peptide conjugates, Fab'-L-CCE, Fab'-L-CCK, Fab'-D-CCE, Fab'-D-CCK. Size exclusion chromatograms of 1F5, its fragments and Fab'-D-CCE conjugate are shown in Fig. S12 and SDS-PAGE results in Fig. S13.

3.2 Stimulation of mouse macrophage cell line RAW264.7

To determine the biocompatibility of therapeutics and their major components *in vitro*, we analyzed viability, polarization towards M1-type of macrophages and activation of the RAW264.7 cells, by Hoechst 33258 exclusion, surface expression of CD127 or CD40, and by production of TNF- α , IL-6, IL-1 β , IL-10 or NO $^-_2$ by flow cytometry, ELISA or Griess assay, respectively (Fig. 3 and Supplementary Data Fig. S14). In all samples, the concentrations of IL-10 and IL-1 β were below the limit of detection. While synthetic peptides or the HPMA copolymer did not activate the macrophages, most compounds prepared from the original antibody reduced cell viability (Fig. 3A) and activated the cells (Fig. 3 B-F). The pre-mixtures of Fab'-L-CCK or Fab'-D-CCK with relevant HPMA copolymer-peptide conjugates (P-L-CCE or P-D-CCE, respectively) were the exceptions. There were no statistically significant differences between L- and D-based peptides and their conjugates.

The cells treated with pre-mixtures of Fab'-L-CCE/P-L-CCK (MIX L) and Fab'-D-CCE/P-D-CCK (MIX D) showed similar decrease in viability, CD40 expression and NO $^-_2$ production. The MIX L induced expression of CD127 on 3 times more cells and induced almost three times higher production of TNF- α than MIX D. On the other hand, the MIX D treated cells produced approximately 100 times more IL-6 than the MIX L treated cells.

3.3 Ab response

The occurrence of an Ab response after i.v. therapy may be influenced by the dose of therapeutics or the presence of D-amino acids [23-25]. To explore the Ab response against the i.v. treatment with therapeutics at various doses on an already established immune response, we immunized mice with ovalbumin (OVA) s.c. and then treated them i.v. with high (5x), medium (1x) or low (0.2x) dose of either MIX L or MIX D (Fig. 4). Both MIX L and MIX D induced an Ab response, but did not change Ab response to unrelated Ag (OVA). There was only a minor difference between Ab response to MIX L and MIX D. The anti-Fab' IgG response to high dose of MIX L (high and medium in case of MIX D) of therapeutics shows a spike 7 days after the first dose and in case of anti-Fab'-D-CCE have a tendency to disappear till the end of the experiment. The low dose of premixtures elicited a lower IgG response against Fab'-conjugates and a higher response against HPMA copolymer conjugates. Intravenously administered premixtures induced anti-Fab' Ab of both IgG and IgM isotypes, but anti-HPMA copolymer conjugate Ab were present mainly in IgG isotype. There were no substantial differences in the ability of conjugates with D- and L-amino acid based peptides to induce the Ab response. The IgG Ab response against MIX D was slightly lower than against MIX L (at day 31, the high doses differ by 20%). There was no transient increase in anti-P-D-CCK IgM Ab at day 31 as in case of anti-P-L-CCK.

Figure 5 shows the amount of Ab produced (Fig. 5A) and their avidity (Fig. 5B). Avidity was determined using increasing concentrations of NaSCN. The IgG Ab elicited by intravenous injections of therapeutics were of low titer and possessed a low avidity when compared to antibodies elicited with subcutaneous injections with adjuvant (Fig. 5 and Fig. S15). One exception was the Fab'-D-CCE, which elicited high titers of low avidity Ab when administered subcutaneously (Fig. 5B). The IgM Ab responses to intravenous injections

induced anti-Fab' Ab of high avidity in the case of both Fab'-L-CCE and Fab'-D-CCE (Fig. 5B).

To determine which part of the conjugated therapeutics is responsible for the Ab response, we coated the ELISA plates with conjugates or their components (HPMA copolymer, CCK, CCE, and Fab'), and analyzed the sera. We found that peptide, but not HPMA copolymer, caused the induction of IgG Ab response after either s.c. immunization (Fig. S15) or i.v. treatments (Fig. 6A). There were, however, some IgM anti-HPMA antibodies present at similar levels even before the start of the experiment (Fig. 4). The rapid decrease in OD after the serum was incubated with increasing amounts of free peptide prior the ELISA shows that the anti-HPMA copolymer-peptide conjugates are directed against the peptide in i.v. treated mice (Fig. 6B). In general, therapeutics based on D-peptides produced antibodies with lower avidity than those based on L-peptides, even after subcutaneous immunization with strong adjuvant (Fig. 5 and Fig. S16).

To analyze if there is response to the conformational epitopes [22] formed by coiled-coil superhelix, we coated plates with individual peptides or with their premixture. The treatment with medium dose of MIX D induces significantly higher response to D-CCK in IgG and significantly higher response to D-CCE in IgM (Fig. 7A). The IgG response against irrelevant (L) peptides is very low, slightly higher in D-CCK and D-CCE/D-CCK, and the IgM response is similar for all three tested antigens and it is magnitude, and it is not different from the one in mice treated with relevant (D) peptides (Fig. 7B).

3.4 T cell response

The T cells from spleen of naive mouse did not respond to the tested therapeutics neither by the production of IFN- γ nor by proliferation (Fig. 8). There was a tendency (difference is not statistically significant) to produce IFN- γ if the cells were taken from mice previously immunized by Fab'-L-CCE or Fab'-D-CCE. There was no fundamental difference in response between Fab'-L-CCE and Fab'-D-CCE or between P-L-CCK and P-D-CCK. The cells isolated from local lymph nodes responded in a similar fashion (Fig. S17). Interestingly, the T cells from mice treated intravenously with MIX L or MIX D (Fig 8D) did not proliferate even when stimulated with non-specific stimulator PMA/I. These results suggest that the pathway leading to T cell proliferation may be influenced by the therapeutics. Interestingly, the ability of the cells to produce IFN- γ after stimulation with PMA/I was not changed (Fig 8C).

4. Discussion

Drug free macromolecular therapeutics have a high translational potential to treat blood cancers such as NHL. This class of therapeutics is especially innovative due to its versatility and applicability to other diseases such as multiple sclerosis and rheumatoid arthritis. The therapeutic system is composed of self-assembled hybrid biomaterials forming two distinct conjugates, Fab'-peptide and Polymer-peptide, which have several important advantages over the classical one-component systems. First, this system is more amenable to incorporation of different ligands so it may recognize different targets. Then it may be used to treat different diseases and, in theory, it may be even used in basic research for the receptor

signaling analysis. Second, this system is more versatile, because by modifying its backbone the same target may be visualized, which may be useful in diagnostics. Moreover, “pre-targeting” can decrease toxicity by timing for most favorable biodistribution of each component. Third, this system should have higher efficacy than treatment with similar monoclonal antibodies, because it engages several receptors at once. Fourth, it has much easier synthesis, e.g. due to the lower steric hindrance, and consequently superior quality, and cheaper production.

Inflammation is a complex biological response mediated by activated inflammatory cells such as macrophages. Inflammation’s primary role is to both directly combat the infection and to govern the magnitude and type of the subsequent adaptive immune response. When macrophages encounter an infection they release toxic molecules such as nitrite, produce a broad array of pro-inflammatory and immuno-modulatory molecules (cytokines), and express various costimulatory molecules. The pattern of all these actions governs the subsequent immune response. Since there is no specific type of immune response to macromolecular therapeutics, this anti-infection immunity is the basis for immune-mediated adverse events.

We studied the ability of the therapeutics and its components to activate a well established mouse macrophage cell line RAW264.7 by measuring cell viability, surface expression of costimulatory molecule CD40, and M1-type macrophage marker CD127 (IL-7R). Since the peptides made of L-amino acids may elicit a different immune response than those made of D amino acids [23-25], we tested components based on either all-L-peptides or all-D-peptides. The L- and D- components were tested for their ability to stimulate an inflammatory reaction and by which mechanism the reaction was initiated. Recognition of lipopolysaccharide (LPS) of Gram negative bacteria by Toll-like receptor (TLR)4 induces activation of the canonical Nuclear factor (NF)- κ B signaling pathway [31]. In response to this activation, macrophages produce TNF- α and other pro-inflammatory cytokines, that shape subsequent immune response. Similar triggers lead to increase in inducible NO synthase and release of small molecule with strong microbicidal activity - NO $^-_2$ [32]. Microbial products, but also some other cytokines, can induce production of the pro-inflammatory cytokine IL-6. This multifunctional cytokine binds to the plasma membrane receptor complexes containing gp(glycoprotein)130, which leads to the activation of the JAK/STAT, MAPK, and PI-3K cascades [33]. IL-6 is the major activator of acute-phase protein expression in the liver, is a chemotactic factor for monocytes, and as an inducer of the Th17 response, it is crucial in the anti-infectious adaptive immune response [34, 35]. The IL-1 β is mediator of acute inflammation, but its production is not usually induced via a surface pattern recognition receptor (PRR), but by intracellular PRR and conveyed by the inflammasome pathway, thus reacting to a slightly different inflammatory stimulus than TNF- α . IL-1 β is important for T cell activation and the subsequent induction of adaptive immune response [36]. There are more types of macrophages, characterized by specific phenotype and by specific biological function. The typical activated macrophages (so called M1 type) express CD127 on its surface and induce the pro-inflammatory immune response. In specific environments, e.g. inside the tumor, the macrophages may switch to M2 type and start to dampen the immune response by production of anti-inflammatory cytokine IL-10

[37]. Since there are many ways that macrophages can respond to stimuli, we decided to measure several parameters after their cultivation with the therapeutics.

We found that unconjugated peptides and HPMA copolymer did not induce any response in RAW264.7 cells, but two of their conjugates (P-L-CCK and P-D-CCK) slightly increased the nitrite production. None of the conjugates significantly increased the proportion of CD40⁺ cells, CD127⁺ cells or production of cytokines. Our experiments showed that the Ab (anti-human CD20; 1F5) and its derivatives (Fab' fragments and Fab'-peptide conjugates) are responsible for macrophage activation by Fab'-peptide conjugates, and that this response is fast (as measured by TNF- α production at 1 h). This effect is present also in the whole Ab and in the unconjugated Fab'. This activation is also present in both Fab'-CCK conjugates (L and D), yet the absence of IL-6 production suggests a different type of activation. The activation observed for the Ab and its derivatives was not caused by LPS contamination of the original sample. Interestingly, when the Fab'-CCK and P-CCE conjugates were used in premixtures, their activating potential was much smaller than in case of the Fab'-CCE and P-CCK conjugate premixture. The observed decrease in macrophage activation of the Fab'-CCK/P-CCE premixture may be caused by P-CCE providing a 'shielding' effect of the Fab'-CCK. However, since this 'shielding' effect is not observed in the Fab'-CCE/P-CCK premixture suggesting that other mechanisms are involved in the decrease of macrophage activation for the Fab'-CCK/P-CCE mix. The different charge on the two conjugates P-CCE (overall negative charge) and P-CCK (overall positive charge) could explain the difference in macrophage activation. The P-CCK conjugates stimulated nitrite production while the P-CCE conjugates did not.

There were only minor differences between L and D therapeutics and their components. One difference between MIX L and MIX D was that MIX L induced significantly lower IL-6 production, but higher CD40 and CD127 expression (M1-polarization) and TNF- α production. The reduction in viability and NO⁻² production was similar in both treatments.

LPS induces both TNF- α and IL-6 (as well as NO⁻²) via TLR4 - NF- κ B pathway. This should also lead to IL-1 β production, but RAW264.7 may be defective in the secretory part of the pathway [38], although others found sufficient amounts of IL-1 β after 8 h of RAW264.7 cultivation [39]. This may be, however, caused by different antibodies and methods used for the IL-1 β detection. Fascin is one factor that plays a role in both cytokine response to LPS, but not in NO⁻² response to LPS in RAW264.7 cells [40]. There is also cross-regulation among pro-inflammatory cytokines. In some cases, IL-6 acts as an anti-inflammatory cytokine by blocking the TNF- α production. The cross regulation goes even deeper, because the TNF- α induces the phosphorylation and internalization of IL-6 receptor gp130, thus blocking the IL-6 signaling [41]. If the therapeutics are able to leave the phagosome the peptides may interfere with intracellular MAP kinase pathways, thus giving different signature of pro-inflammatory mediators [42]. Furthermore, the cells may release different mediators once the concentration of a particular stimulus reaches a certain dose, so the pattern of released mediators may be different for different concentrations of a particular stimulus [43]. In our experiments, using 10, 1 or 0.1 ng of LPS, the RAW264.7 reached maximal production of TNF- α at 0.1 ng/ml, IL-6 at about 1 ng/ml and the NO⁻² production was not at its maximum levels even at 10 ng/ml (Fig. S14).

Although free peptides of 20-30 amino acids are capable of eliciting an Ab response *in vivo*, they need either an adjuvant or immunogenic carrier to raise effective anti-peptide antibodies. If a strong adjuvant is used, such as CFA, even short peptides composed of either L- or D- amino acids elicit a strong Ab response [24, 44]. The difference in response between the enantiomeric peptides was of considerable interest. To compare the ability of premixtures based on L-peptides with D-peptides, we injected either of these therapeutics i.v. in three different doses. To produce the hyper-immune sera and cells and to compare the strength of the immune response with primary immunogenic protocol, we immunized mice subcutaneously with one of the four conjugates emulsified in strong adjuvant (CFA/IFA). We found that there were no major differences between L- and D-peptides or their conjugates *in vivo*, but there are significant differences in the Ab response elicited to s.c. or i.v. treatments.

Similarly as *in vitro*, HPMA copolymer did not induce an immune response *in vivo*. When the HPMA copolymer-conjugates are administered with strong adjuvant (CFA/IFA), the Ab response against P-CCK is entirely directed against the peptide or the neoepitope formed by peptide-HPMA conjugation. All these antibodies have moderate or high avidity (Fig. 5). There are no anti-HPMA copolymer IgG antibodies in mice and it is not possible to produce them even with s.c. immunization, suggesting that there is no epitope spreading to the HPMA copolymer backbone. The anti-P-CCK antibodies we observed in both intravenously and subcutaneously treated mice were directed toward the CCK peptide. This suggests, that HPMA copolymer may act as a carrier that increases immunogenicity of the peptide or the sum of all peptides bound to the HPMA backbone may give stronger signal to activate Ab production due to the “repetitiveness” of the peptide motif [45].

There was a low amount of anti-P-CCK IgM antibodies in the mouse serum even before immunization, and the amount was not changed by the immunization (Fig. 4). These antibodies had very low avidity (Fig. 5) and probably belong to the polyreactive naturally occurring antibodies [46]. Our inability to block these Ab with the peptide (Fig. 6B) supports this conclusion.

As showed by the experiments *in vivo*, low i.v. doses of premixtures induced lower anti-Fab'-conjugate response than the high or medium doses (Fig. 4). The Fab' is the major (90%) immunogenic portion of the conjugate responsible for an IgG response to s.c. immunization with Fab'-L-CCE, but it is responsible for only 20-50% of the reactivity in mice treated with the Fab'-D-CCE conjugate (Fig. S15). The immunogenicity of Fab' may be responsible for the high immune response against the peptide by bystander activation [47]. To achieve this effect, the immunogenic and non-immunogenic component does not need to be connected as a hapten-carrier system. It is interesting to note that previously four NHL patients were treated with 1F5 Ab therapy [48, 49]. They received continuous i.v. infusion of 52 to 2,380 mg of 1F5 over five to ten days. The “treatment toxicities were minimal, with low-grade fevers and mild transient cytopenias observed during the antibody infusions” [48].

The magnitude of Ab response in mice was roughly the same against Fab'- and HPMA copolymer-conjugates. As mentioned earlier, almost all antibodies of IgG isotype against

HPMA copolymer-conjugates were against the peptide, but a significant amount of antibodies were against Fab' (50-90%) among the antibodies against Fab'-conjugates (Fig. 6A). Interestingly, when different doses of MIX D or MIX L were administered, the overall IgG response stayed almost constant; in low doses, the response was more against P-CCK and less against Fab'-CCE and the opposite is true for high doses (Fig. 4). The biggest difference is in the ability to mount the IgM Ab response, which is significant against Fab'-conjugates, but not against HPMA copolymer-conjugates. Ab against D-peptide conjugates had a lower avidity than those against the L-peptide conjugates (Fig. 5 and Fig. S15). There is one limitation of these measurements, because the homogeneity of the antibodies present in the serum sample may influence the measurement of the Ab avidity [50].

We measured the Ab response to the conformational epitopes of the coiled-coil superhelix by comparing the Ab response against individual peptides with the response to their premixture. We found that there is marked difference in the anti-peptide response between IgG and IgM. After i.v. injection of MIX D, the anti-peptide Ab response in IgG is mainly against D-CCK, and the IgM response is mainly against D-CCE. The response against the pre-mixture was usually slightly lower, which suggests that one peptide masked the epitopes recognized on the second peptide.

None of the therapeutics introduced i.v. or the conjugates introduced s.c. induced a T cell response in the spleen or in ILN, as measured by T cell proliferation or IFN- γ production. Moreover, there was no difference in T cell response among all tested groups (comparison of L and D Fab'-conjugates, different doses or MIX L vs. MIX D). A similar response was observed both in spleen cells or in ILN. The ability of i.v. administration of MIX L or MIX D to prevent PMA/I induced cell proliferation, without affecting the ability to produce IFN- γ , needs to be addressed with further analysis.

5. Conclusions

Here, we tested the biocompatibility and immunogenicity of a novel type of self-assembled hybrid nanomedicines composed from two distinct conjugates, Fab'-peptide and Polymer-peptide. We found that use of D- instead of L-amino acids in synthetic peptides induces a different type of macrophage response *in vitro*. Neither HPMA copolymer nor any peptide, either L or D, induced any response in murine macrophages. The major component responsible for macrophage activation was the Ab and its derivatives. Using the model *in vivo*, the therapeutics based on L-peptides (MIX L, Fab'-L-CCE/P-L-CCK) did not induce substantially different Ab response than those based on D peptides (MIX D; Fab'-D-CCE/P-D-CCK). The titer and avidity of Ab induced by i.v. treatment with MIX L or MIX D were generally low, slightly lower in case of MIX D, except for anti-Fab'-CCE IgM Ab. In general, there was detectable Ab, but no cellular response to the therapeutics administered i.v. The Ab response was predominantly directed against the Fab' part of the therapeutics. Therefore, we suggest to use fully human Ab, prepared in a manner similar to MIX D with D-CCK conjugated to the Fab' and D-CCE conjugated to the copolymer (Fab'-D-CCK/P-D-CCE). Further analysis of immunogenicity and biocompatibility should be performed using human cells. Then, we believe that this type of hybrid therapeutic would have good translational potential into the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Helical wheel diagram of the anti-parallel heterodimer of CCE/CCK [4]. The heptad repeats are labeled a-f for CCE and a'-f' for CCK. All peptides were modified with a YGG spacer and were functionalized with either cysteine or a heterobifunctional linker bearing a reactive maleimide group.

Figure 2.

B>. Synthesis of HPMa copolymer-peptide conjugates.

Figure 3.

RAW264.7 cell (A) viability, surface expression of (B) CD40 and (C) CD127 was analyzed by flow cytometry, production of (D) TNF-α and (E) IL-6 was analyzed by ELISA and (F) production of NO₂⁻ was analyzed by Griess assay after 24 h incubation with 100 μg/ml of selected component. Values represent the mean ± SD of n= 5-10 measurements. *p<0.05; **p<0.01; ***p<0.001 one-way ANOVA with Dunnett's post-hoc test vs. control (medium only) cells.

Figure 4.

Ab response following administration of a premixture of Fab'-L-CCE/P-L-CCK (MIX L) or premixture of Fab'-D-CCE/P-D-CCK (MIX D) to BALB/c mice. The mice were first immunized against ovalbumin (OVA) and on days 24, 26 and 28 the treated mice received either high (250 µg of Fab'-CCE with 1630 µg of P-CCK), medium (50 µg of Fab'-CCE with 324 µg of P-CCK) or low (10 µg of Fab'-CCE with 64.8 µg of P-CCK) dose of the premixtures. The optical density (OD) values on each plate were normalized for the optical density values obtained with serum standard serum sample diluted 1:100 (100%). The values obtained from sera of treated mice were compared with those of saline by repeated-measure 2-way ANOVA with Bonferonni post-hoc test. The significant differences from control mice ($p < 0.05$) are marked with h (high dose), m (medium dose) or l (low dose).

Figure 5.

Amounts (A) and avidity (B) of anti-conjugate Ab. After incubation with 3 samples collected at the end of the experiments, the Ag-Ab binding was disrupted with increasing concentrations of chaotropic agent (sodium thiocyanate [NaSCN]). All data are given as mean $\pm$ SD (n = 3); *p < 0.05 **p < 0.01. The OD ratio is OD sample with NaSCN/OD sample without NaSCN.

Figure 6.

Reactivity of antibodies raised by i.v. injections against the various parts of the therapeutics in both IgG and IgM isotypes. The data are presented as OD against coated antigens (A) or as specific Ab blocking with either Fab' or CCKsh (B). Each bar represent serum from one mouse (n=3) (A) and line graphs represent mean $\pm$ SD (B).

Figure 7.

Reactivity of antibodies raised by i.v. injections against the D amino acid based peptides or their premixtures in both IgG and IgM isotypes. The reactivity was measured either in sera from mice (n = 6) treated i.v. with MIX D (A) or MIX L (n = 3) (B) to compare the crossreactivity. Each line represent individual mouse and grey line represent mean.

Figure 8.

The response of T cells from spleen to conjugates. All analyses were performed on singlets (FSC-A vs. FSC-H), live (Viability Dye eFluor[®] 450⁻ or Hoechst 33258⁻) and CD3⁺ cells. The graphs show either percentage of IFN- γ^+ cells from subcutaneously (A) and intravenously (C) treated mice, or percentage of CFSE MFI (carboxyfluorescein succinimidyl ester mean fluorescence intensity) decrease of splenocytes from subcutaneously (B) and intravenously (D) treated mice. The values are mean $\pm$ SD of $n = 3-5$ measurements from one representative experiment out of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ one-way ANOVA with Dunnett's post-hoc test vs. control cells from the same mouse. Cells from the same group of mice are grouped together; control mice (PBS), s.c. immunized mice (Fab'-L-CCE, Fab'-D-CCE, P-L-CCK, P-D-CCK), and mice treated i.v. (MIX L or MIX D) with high, medium and low dose of therapeutics; and substances used for re-stimulation *in vitro* are on the x axis.