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Tolerogenic Modulation of the Immune Response by Oligoglycerol–and Polyglycerol–Peptide Conjugates

Shilpi Gupta,[†,‡,#] Jennifer Pfeil,[§,#] Sumit Kumar,[†,∥] Christina Poulsen,[§] Uta Lauer,[§] Alf Hamann,*[,§] Ute Hoffmann,[§,#] and Rainer Haag*[,†,#]

[†]Organic and Macromolecular Chemistry, Department of Chemistry and Biochemistry, Free University Berlin, Takustrasse 3, 14195 Berlin, Germany

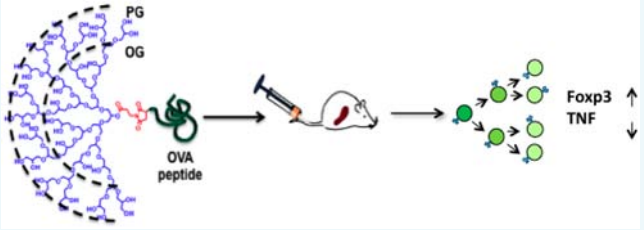
[‡]Department of Chemistry, Hindu College, Sonepat-131001, Haryana, India

[§]Deutsches Rheuma-Forschungszentrum and Charité Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

[∥]Department of Chemistry, Deenbandhu Chhotu Ram University of Science and Technology, Murthal, Sonepat-131039, Haryana, India

Supporting Information

ABSTRACT: Peptide-based therapy is a promising strategy for antigen-specific immunosuppression to treat or even heal autoimmune diseases with significantly reduced adverse effects compared to conventional therapies. However, there has been no major success due to the drawbacks of native peptides, i.e., limited bioavailability. Considering the importance and limitations of peptide-based therapies for treatment of autoimmune diseases, we designed and constructed oligoglycerol (OG)- and polyglycerol (PG)-based peptide conjugates. They were evaluated for their biological activity (in vitro and in vivo), bioavailability, and tolerogenic potential. Among the OG- and PG-peptide constructs, PG-peptide constructs exhibited an extended bioavailability compared to OG-peptide constructs and unconjugated peptide. Interestingly, size, structure, and linker chemistry played a critical role for the tolerogenic capacity of the constructs. The PG-peptide construct bound via an ester linkage was the most tolerogenic conjugate, while the PG-peptide construct bound via an amide induced stronger proliferation, but also higher TNF production and lower frequencies of Foxp3$^+$ regulatory T-cells. Therefore, we conclude that PG–peptide conjugates bound via an ester linkage are not only promising candidates for tolerogenic vaccination, but also open a new avenue toward the application of peptides for the treatment of autoimmune diseases.



INTRODUCTION

Pharmacological treatments for autoimmune diseases largely rely on nonspecific immunosuppressive drugs, which disrupt innate and induced immunity with significant, sometimes even dramatic, adverse effects. Lack or loss of tolerance to certain self-antigens in autoimmune diseases is a major feature of autoimmunity. Among the various strategies under development for the treatment of autoimmune diseases, there is renewed interest in the use of antigen-derived T-cell epitopes for tolerogenic peptide vaccines. Administration of antigens or peptides under appropriate conditions stimulates tolerogenic mechanisms and thereby establishes or re-establishes tolerance in animal models. Accordingly, delay or prevention of autoimmune diseases can be achieved. Peptide-based therapies currently under investigation are expected to be disease-specific and not associated with the side effects of conventional immunosuppression.[1,2] Peptide-based agents offer several advantages over protein drug candidates such as higher activity per unit mass, increased selectivity and specificity, greater stability in storage, weaker immunogenicity, and better organ or tumor penetration.[3] In spite of all these advantages and their good clinical potential, native peptides have seen limited use because of their short half-life in serum due to rapid excretion from the circulation and low stability caused by rapid enzymatic degradation in physiological conditions.[4] Furthermore, animal studies have demonstrated a significant risk of exacerbation of the disease or induction of anaphylactic responses by injection of certain soluble peptides,[5,6] which might be caused by aggregated material. However, recent murine studies suggested that tolerogenic vaccination might be made more effective by using peptides conjugated to biodegradable and biocompatible polymeric nano- or microparticles. Protective effects were found in autoimmune models without adverse effects such as anaphylaxis or exacerbation of the disease.[7] Thus, conjugation of immunodominant peptides to synthetic polymers is a promising approach.

Recent developments in chemical coupling strategies that permit efficient conjugation of synthetic polymers to peptides

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have been extensively reviewed in the literature.[8−11] The main focus has been on PEGylation of peptides/proteins, which has gathered widespread interest for therapeutic applications.[12−14] PEGylation masks the peptide's surface, which makes it unrecognizable to the plasma components, thereby improving the stability and solubility, increasing proteolytic resistance and circulation time, as well as decreasing antigenicity and immunogenicity.[15] PEGylation, however, has several drawbacks such as the limited end groups that restrict further functionalization, induction of PEG-directed hypersensitivity, unexpected changes in pharmacokinetic behavior, toxic side products, and its nonbiodegradability, as well as the resulting possible accumulation in the body.[16] Strategies to overcome these limitations to allow the development of protein−polymer conjugates with improved properties are therefore crucial for the next generation of peptidic drugs. Macromolecules such as dendritic architectures are attracting particular attention; they are multifunctional and can be efficiently conjugated to bioactive components.[17]

Dendritic polymers have shown many promising in vivo applications. They enhance cargo solubility and target specificity and prevent biodegradation by a masking effect, thus increasing bioavailability.[18] Only a few reports describe the synthesis of precisely defined protein−dendron conjugates which were explored for multivalent interactions with DNA,[19−22] while a number of studies have described the conjugation of polyamidoamine-type dendrimers to antibodies or proteins.[23−25] Dendrimer−peptide/protein conjugates have been studied for various biomedical and nanotechnology applications such as multiple antigenic peptide (MAP) carriers,[26] antimicrobials,[27−29] bone targeting,[30] immunochemistry,[31] and neutron capture therapy.[32]

For our studies we have chosen dendritic oligoglycerols (OG) and polyglycerols (PG) because they are an interesting class of bioinert polyether-based biomaterials.[33−37] PGs are attractive because they are characterized by tunable end group functionalities, have a defined topological 3D architecture, are inert to nonspecific interactions with biological environments, are nontoxic, and are equally biocompatible but thermally and oxidatively more stable than PEG.[38,39] Since dendritic PGs are synthesized in a controlled manner to obtain definite molecular weight and narrow molecular polydispersity, they have been extensively evaluated for a variety of biomedical applications.[33−37] Several studies have demonstrated the biocompatibility of dendritic PGs and classified them as a safe material for in vitro and in vivo applications.[33−37] We have previously reported on the use of the PG scaffold for conjugation to doxorubicin/methotrexate prodrugs and observed high cytotoxic activity in human tumor cell lines.[40−42] PG−doxorubicin conjugates have also shown optimal properties in vitro and in vivo, with improved antitumor efficacy over doxorubicin in an ovarian xenograft tumor model (A2780).[40−42] These applications demonstrated the potential use of PG for the development of an efficient multifunctional dendritic drug delivery system.

With the above-mentioned advantages in mind, we have designed a series of oligoglycerol/polyglycerol−peptide (OG/PG-peptide) conjugates for antigen-specific immunosuppression. We synthesized *N*-maleimido-functionalized OG and PG, followed by site-specific conjugation to the cysteine-modified model peptide via a Michael addition reaction. The resulting dendritic OG-peptide and PG-peptide conjugates were studied in the T-cell receptor (TCR) transgenic adoptive transfer model using DO11.10 mice. In these mice, $CD4^+$ T-cells express a transgenic T-cell receptor (TCR) recognizing the OVA-peptide 323−339 (pOVA: ISQAVHAAHAEINEAGR) presented via the major histocompatibility complex II (MHCII) molecule ($H\text{-}2^d$) by antigen-presenting cells (APC). pOVA-specific $CD4^+$ T-cells were transferred into syngeneic BALB/c mice to track the response of the antigen-specific $CD4^+$ T-cells, which could be identified by the KJ1.26 antibody detecting the pOVA-specific TCR. Depending on the dose, route, and immunogenic environment (e.g., adjuvants), pOVA treatment could trigger either immunity or tolerance.[43,44]

Tolerance can be induced by a high antigen dose and systemic or oral administration of antigen in the absence of immunogenic stimuli. This results in anergy (low pro-inflammatory cytokine expression) or deletion of $CD4^+$ T-cells.[43,45] A low antigen dose leads instead to induction of regulatory T-cells (Tregs).[44,46] Tregs expressing CD25 and the forkhead transcription factor Foxp3 are crucial for balancing immune responses and preventing autoimmunity. $Foxp3^+$ Tregs can be generated as a separate T-cell lineage in the thymus (thymic Tregs) or in the periphery (peripheral Tregs). Both subsets contribute to tolerance by inhibiting various types of effector cells and inflammatory mediators, involving a number of suppressive mechanisms including secretion of inhibitory cytokines, cell-contact-dependent inhibition, and metabolic cytokine competition.[47] Therefore, we analyzed the impact of the different dendritic OG-peptide and PG-peptide conjugates on proliferation of antigen-specific T-cells, cytokine production, and induction or expansion of $Foxp3^+$ Tregs in the DO11.10 adoptive transfer model. The results support the hypothesis that conjugation of peptides with polyglycerol leads to an increased bioavailability and improved efficacy of tolerogenic peptide vaccination strategies.

RESULTS AND DISCUSSION

Biological Activity of OG/PG Conjugates. T-cells from DO11.10 mice expressing a transgenic T-cell receptor (TCR) specific for the ovalbumin peptide 323−339 (pOVA) presented by MHCII molecules of antigen-presenting cells (APC) were used to study the potency of the OG/PG-conjugated pOVA to stimulate $CD4^+$ T-cells in vitro and in vivo.

Biological Activity of OG/PG Constructs in Vitro. To assess the biological activity of conjugates in comparison to the free peptide, CFSE-labeled pOVA-specific $CD4^+$ T-cells were cultured for four days with APCs and different concentrations of pOVA or equimolar amounts (based on peptide amount) of OG/PG-conjugated constructs. The loss of intensity of CFSE with every cell division allows tracking of the proliferative activity (Figure 1). All constructs were able to induce proliferation of $CD4^+$ T-cells. The linker chemistry did not seem to play a role in vitro, since PG-E-pOVA and PG-A-pOVA had a similar capacity to activate T-cells. However, 10-times higher concentrations of OG/PG-pOVA constructs were required to induce proliferation compared to unconjugated pOVA. [G2.0]-pOVA had the poorest activation capacity.

Previous experiments indicated a size-dependent cellular uptake of PG and demonstrated that higher-molecular-weight hyperbranched PG (40−870 kDa) accumulate better in the cytoplasm than their low-molecular-weight counterparts (2−20 kDa),[48] suggesting that the size of [G2.0]-pOVA (2.6 kDa) might influence its uptake by APCs. However, [G3.0]-pOVA (3.6 kDa) is only slightly larger, yet displays a higher stimulatory capacity compared to [G2.0]-pOVA. Thus, the

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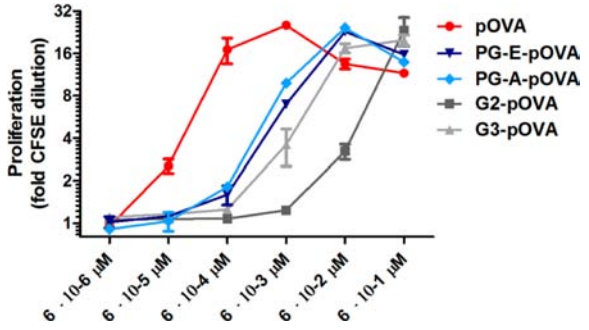

Figure 1. PG/OG-peptide conjugates stimulate antigen-specific CD4$^+$ T-cells in vitro. pOVA-specific CD4$^+$ cells were cultured with irradiated APCs (1:3) for four days. pOVA, PG-pOVA, or OG-pOVA conjugates were added as indicated. For the determination of cell proliferation, CFSE was used. This dye covalently binds intracellular proteins and becomes 2-fold diluted with every cell division. This allows identification of distinct cell generations based on fluorescence intensity using flow cytometry. To allow comparison of different experiments, the geometric mean fluorescence intensity values (GMFI) of CFSE were normalized using the undivided PBS (control) sample. Cells were gated on pOVA-specific CD4$^+$ cells and the GMFI of CFSE was estimated (fold CFSE dilution = GMFI control/GMFI sample; log 2-scale). Data represent means of triplicates ± SD. Data shown are representative for four independent experiments.

size and number of hydroxyl groups of the OG dendrons apparently have to exceed a critical threshold to allow efficient uptake and presentation to T-cells. We also would not exclude the possibility that the more rigid structure of the [G3.0] OG has an effect, by an unknown mechanism, on the uptake and stability of the conjugate.

All PG-peptide conjugates displayed a lower in vitro stimulatory capacity compared to pOVA, which is not surprising, as free peptide can associate directly with the binding pocket of MHC, which is likely to be impaired for bulky conjugates. In fact, the conjugates might even require intracellular or extracellular cleavage of the core peptide from the conjugate to become bound to MHC. However, unconjugated polymers are immunologically inert since they did not affect proliferation and cytokine production in vitro when added to pOVA-stimulated cultures (see Supporting Information, Figure SI 1 and SI 2).

Biological Activity of OG/PG Constructs in Vivo. To investigate the effect of systemic PG-peptide conjugate administration in vivo on activation of antigen-specific CD4$^+$ T-cells, we used the DO11.10 transgenic adoptive transfer system. On day 0, 5 × 10^6 CFSE-labeled pOVA-specific CD4$^+$ T-cells were transferred into BALB/c mice. After 24 h, recipients received either PBS, 5 μg pOVA or equimolar amounts (based on peptide amount) of [G2.0]-pOVA, [G3.0]-pOVA, PG-A-pOVA, or PG-E-pOVA intravenously (i.v.). Six

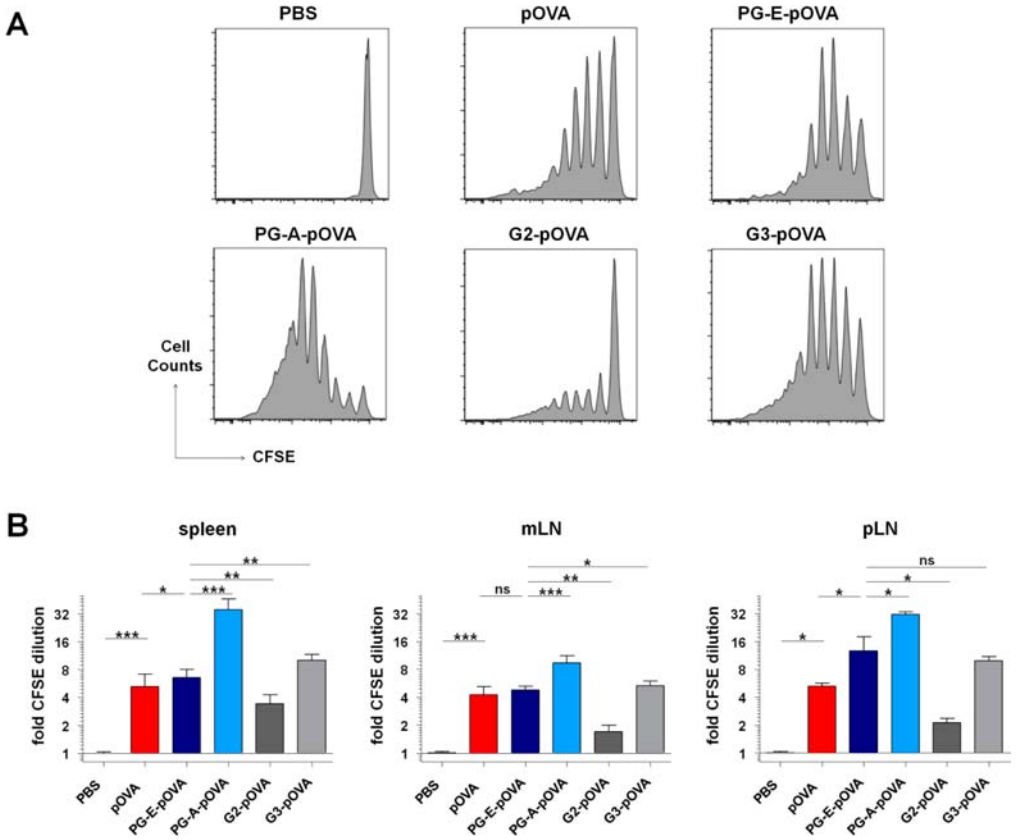

Figure 2. PG/OG-peptide conjugates efficiently activate antigen-specific CD4$^+$ T-cells in vivo. pOVA-specific CFSE-labeled CD4$^+$ cells were transferred into BALB/c mice (d0). On day 1, mice were treated i.v. with PBS (control), pOVA, PG-pOVA, or OG-pOVA conjugates. After six days, proliferation was assessed by flow cytometry. Splenocytes (spleen), mesenteric lymph node cells (mLN), and peripheral lymph node cells (pLN) were gated on pOVA-specific CD4$^+$ cells. (A) Representative histograms of CFSE fluorescence in pOVA-specific CD4$^+$ splenocytes. (B) Summarized data show fold CFSE dilution (GMFI control/GMFI sample; log 2-scale). Means ± SD from two to four independent experiments. spleen, n = 5–14; mLN, n = 5–14; pLN, n = 4–6; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

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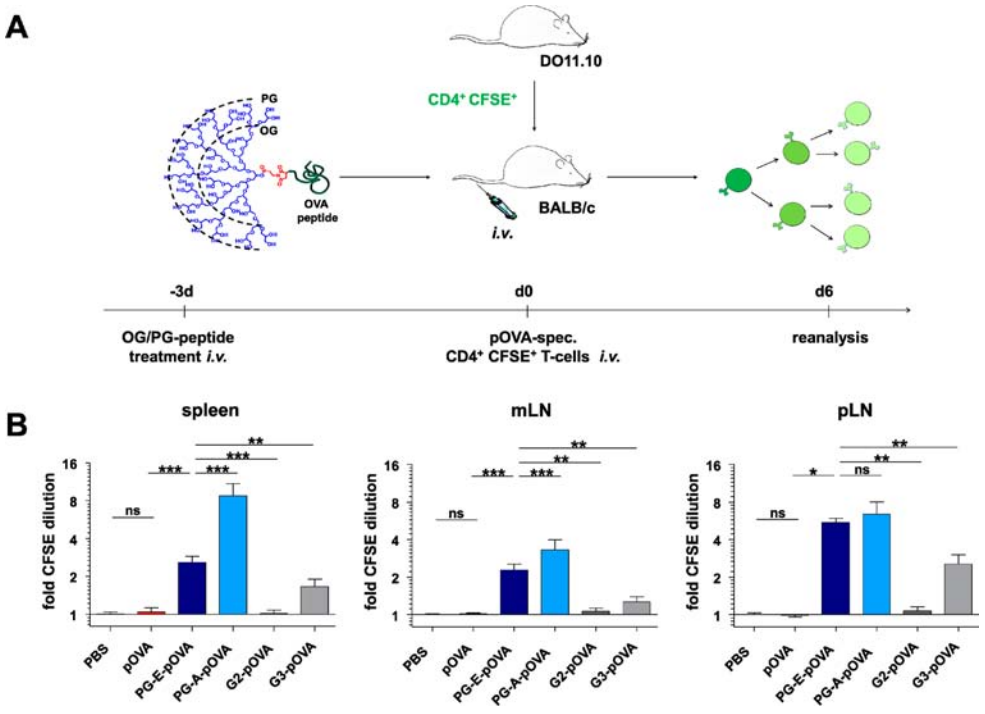

Figure 3. Conjugation of OVA-peptide to PG/OG extends peptide bioavailability. pOVA-specific CD4$^+$ CFSE-labeled cells were transferred into BALB/c mice three days after i.v. peptide vaccination with unconjugated pOVA or pOVA conjugated to PGs or OGs. Proliferation was analyzed on day 6. Splenocytes, mesenteric lymph node cells, and peripheral lymph node cells were gated on pOVA-specific CD4$^+$ cells. (A) Set-up for analysis of the bioavailability. (B) Summarized data (mean ± SD from two to four independent experiments). spleen, n = 5−11; mLN, n = 5−10; pLN, n = 5−6.

days later, pOVA-specific T-cells were reisolated from different lymphatic organs and analyzed by flow cytometry (Figure 2). In contrast to the diminished capacity of the conjugated peptides to activate pOVA-specific CD4$^+$ T-cells in vitro, T-cells strongly proliferated when the conjugates were tested in vivo. The proliferation rates of CD4$^+$ T-cells in mice treated with equimolar amounts (based on peptide amount) of either pOVA, PG-E-pOVA, or [G3.0]-pOVA were comparable. The highest activation, significantly higher compared to free pOVA, was found in mice treated with PG-A-pOVA.

Surprisingly, PG-A-pOVA also induced a stronger proliferation than PG-E-pOVA. Thus, linker chemistry appears to play an important role in vivo while being irrelevant for in vitro responses, as shown above. As size determination by GPC gave a value much higher than expected as per NMR-analysis (see Supporting Information), we would not exclude that the PG-A-pOVA might be prone to oligomerization under the given conditions, which would be compatible with higher stimulatory capacity if also true for in vivo conditions. Consistent with its low stimulatory capacity in vitro, [G2.0]-pOVA also had the lowest in vivo capacity to activate CD4$^+$ T-cells.

Bioavailability of OG/PG Constructs in Vivo. Rapid renal clearance of native peptides as well as enzymatic degradation results in a short time window where the peptide is available in the organism for recognition by T-cells.[15] Accordingly, hardly any T-cell activation was observed when pOVA was injected three days before the adoptive transfer of pOVA-reactive T-cells to host animals (Figure 3B). It has been reported that PEGylation can reduce the renal clearance and protect peptides from protease degradation, thereby increasing the serum half-life of biomolecules.[49] We therefore examined whether conjugation of pOVA to the different OG/PG constructs leads to an extended bioavailability of our model peptide and whether this depends on linker chemistry and structure.

To test whether conjugates are retained and presented to T-cells for a longer period in the organism, BALB/c mice were treated on day 3 with either PBS, 5 μg pOVA or equimolar amounts (based on peptide amount) of [G2.0]-pOVA, [G3.0]-pOVA, PG-A-pOVA, or PG-E-pOVA. CFSE-labeled pOVA-specific CD4$^+$ T-cells were transferred into the mice on day 0. After six days, secondary lymphatic organs were removed and analyzed (Figure 3A). No detectable proliferation of CD4$^+$ T-cells in pOVA or [G2.0]-pOVA-treated mice was observed (Figure 3B), suggesting a complete systemic clearance within three days. In contrast, significant proliferation was noticed in mice treated with [G3.0]-pOVA, PG-A-pOVA, or PG-E-pOVA.

PG-A-pOVA and PG-E-pOVA induced the strongest proliferation in all secondary lymphatic organs analyzed. As found under the conditions used before, PG-A-pOVA induced significantly stronger proliferation in spleen and mLN than PG-E-pOVA (Figure 3B). Also, the rather small [G3.0] conjugate significantly increased the bioavailability of pOVA compared to unconjugated pOVA and [G2.0]-pOVA. Thus, the size and structure of the conjugate have an important impact on prolongation of the bioavailability. Since [G3.0]-pOVA is only slightly larger than [G2.0]-pOVA (3.2 kDa versus 2.6 kDa), it is again tempting to assume a critical size threshold or structural properties for uptake/storage of the conjugates.

It is questionable whether the increased serum half-life of conjugates alone is sufficient to explain their bioavailability for >72 h in vivo. Previous studies indicated a strongly increased plasma half-life for PG from 32 to 57 h for only the higher molecular weights of 106 and 540 kDa, respectively.[33] The

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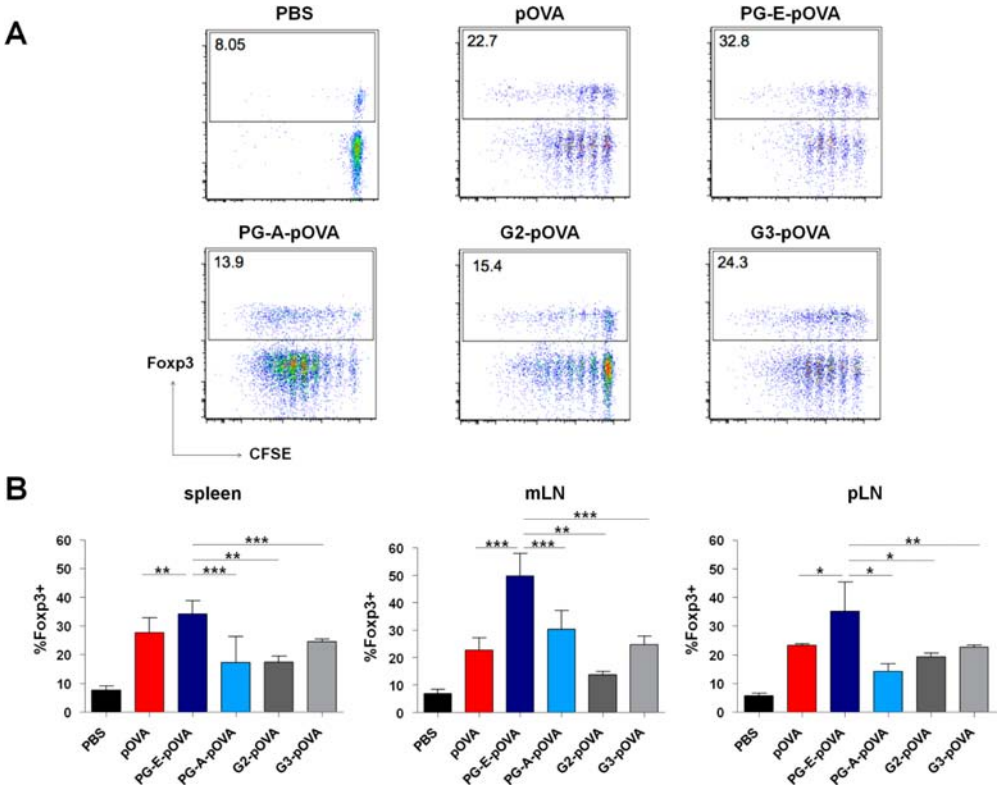

Figure 4. Increased frequency of regulatory Foxp3$^+$ CD4$^+$ T-cells upon PG-E-pOVA treatment. pOVA-specific CFSE-labeled CD4$^+$ cells were transferred into BALB/c mice (d0). On day 1, mice were treated i.v. with PBS (control), pOVA, PG-pOVA, or OG-pOVA conjugates. After six days, Foxp3 expression was assessed by flow cytometry. Cells were gated on pOVA-specific CD4$^+$ cells. (A) Representative dot plots of CFSE and Foxp3 in pOVA-specific CD4$^+$ splenocytes. (B) Summarized data (mean ± SD of % Foxp3$^+$ cells from two to four independent experiments). spleen, n = 5−14; mLN, n = 5−14; pLN, n = 4−6.

smaller [G3.0]-pOVA and the 10 kDa hyperbranched PGs allowed recognition of the conjugated peptide by T-cells beyond 72 h. Thus, improved uptake, intracellular storage, or stability of the conjugates is likely contributing to their prolonged activity in vivo.

With regard to the immediate response in vivo and reactivity after preinjection of the conjugates, the PG-A-pOVA was superior to PG-E-pOVA, indicating the important impact of the linker chemistry. Amide bonds are more stable to hydrolysis and display a lower release rate than ester linkages.[50] However, we would not exclude the possibility that the linker structure has a more specific impact on uptake, storage, or intracellular processing pathways or leads to oligomerization of the conjugate.

Tolerogenic Potential of OG/PG Constructs in Vivo. Systemic exposure to antigen in the absence of immunogenic stimuli can lead to induction of tolerance. Underlying mechanisms include the induction of anergy in antigen-specific T-cells, deletion of effector cells, or induction of Foxp3$^+$ regulatory T-cells (Tregs). We again used the DO11.10 transgenic mouse model to test if our constructs modulate the quality of the immune response by measuring the induction/expansion of Foxp3$^+$ cells and the frequency of the T-cells producing inflammatory cytokines.

Induction/Expansion of Foxp3$^+$ Tregs by Treatment with OG/PG Constructs. CD4$^+$ Foxp3$^+$ Tregs are powerful regulators of immune responses in autoimmunity, allergic responses, and allograft rejection.[51] The transcription factor Foxp3 is the most specific marker identifying Tregs generated either as a distinct lineage in the thymus[52] or by conversion of conventional naive CD4$^+$ Foxp3$^-$ T-cells in the periphery.[46] As mentioned before, it has been shown that vaccination with low-dose peptides leads to expansion or induction of Foxp3$^+$ Tregs.[44,46] To investigate whether conjugation of pOVA peptide to OG/PG carriers enhances this effect, 5 × 10^6 transgenic pOVA-specific CD4$^+$ T-cells were transferred into BALB/c recipients and mice treated with 5 μg pOVA or equimolar amounts (based on peptide amount) of OG/PG-pOVA conjugates 24 h later. After six days, CD4$^+$ T-cells were re-isolated from different secondary lymphatic organs and Foxp3 expression was analyzed by flow cytometry (Figure 4). A significant increase in the frequency of pOVA-specific Foxp3$^+$ regulatory T-cells was found after intravenous administration of free peptide. As expected, the effect was antigen-specific since the frequency of Foxp3$^+$ Tregs was not altered among endogenous CD4$^+$ T-cells, which do not express the transgenic TCR (data not shown). An increase in Foxp3$^+$ frequencies among pOVA-specific cells was also found upon treatment with PG-A-pOVA, [G3.0]-pOVA, or [G2.0]-pOVA. Surprisingly, administration of PG-E-pOVA significantly increased the frequency of pOVA-specific Foxp3$^+$ Tregs compared to pOVA and other types of conjugates (Figure 4).

Previous studies demonstrated an inverse relationship between antigen dose and resulting proliferation and Foxp3 expression.[44,46] However, the differential effects of the constructs on proliferation and Treg frequencies cannot be explained by the phenomenon that PG-E-pOVA induced a stronger T-cell response compared to pOVA, although the frequencies of Foxp3$^+$ Tregs were significantly elevated (Figure 4). PG-A-pOVA induced the strongest proliferation, but

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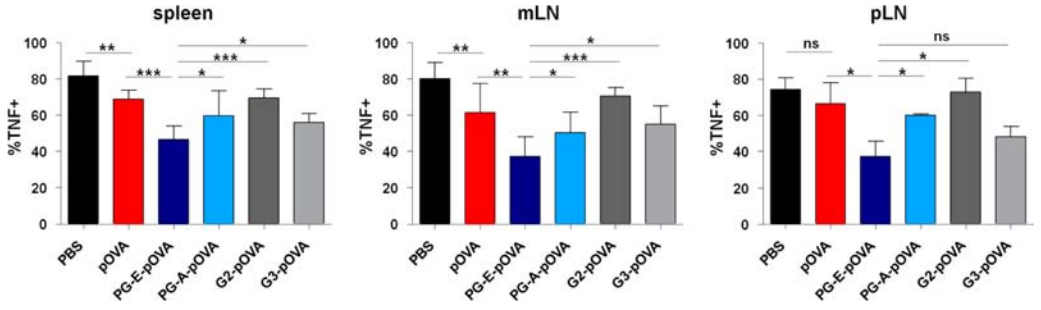

Figure 5. Reduced frequency of TNF$^+$ CD4$^+$ T-cells upon PG-E-pOVA treatment. pOVA-specific CFSE-labeled CD4$^+$ cells were transferred into BALB/c mice (d0). On day 1, mice were treated i.v. with PBS (control), pOVA, PG-pOVA, or OG-pOVA conjugates. After six days TNF production was assessed by intracellular staining and flow cytometry. Cells were gated on pOVA-specific CD4$^+$ cells. Data represent means ± SD of % TNF$^+$ cells from two to four independent experiments. spleen, n = 5−10; mLN, n = 5−10; pLN, n = 4−6.

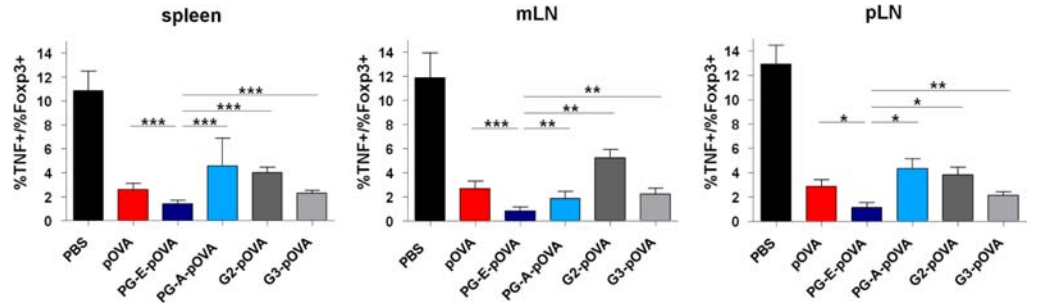

Figure 6. Reduced TNF/Foxp3 ratio upon PG-E-pOVA treatment. pOVA-specific CFSE-labeled CD4$^+$ cells were transferred into BALB/c mice (d0). On day 1, mice were treated i.v. with PBS (control), pOVA, PG-pOVA, or OG-pOVA conjugates. After six days, TNF production as well as Foxp3 expression was assessed by intracellular staining and flow cytometry. The TNF/Foxp3 ratio was calculated, reflecting the effector T-cell/Treg ratio of responding cells. Cells were gated on pOVA-specific CD4$^+$ cells. Data represent means ± SD of % TNF$^+$ cells/% Foxp3$^+$ cells from two to four independent experiments. spleen, n = 5−10; mLN, n = 5−10; pLN, n = 4−6.

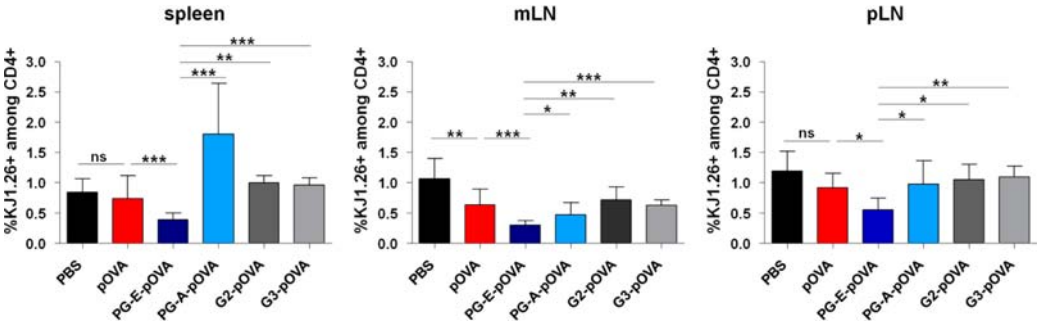

Figure 7. Reduced frequency of pOVA-specific CD4$^+$ T-cells upon PG-E-pOVA treatment. pOVA-specific CFSE-labeled CD4$^+$ cells were transferred into BALB/c mice (d0). On day 1, mice were treated i.v. with PBS (control), pOVA, PG-pOVA, or OG-pOVA conjugates. After six days, the frequency of pOVA-specific CD4$^+$ cells among total CD4$^+$ T-cells was assessed by flow cytometry. Cells were gated on pOVA-specific CD4$^+$ cells. Data represent means ± SD of % KJ1.26$^+$ cells (pOVA-specific T-cells) among total CD4$^+$ cells from two to four independent experiments. spleen, n = 5−10; mLN, n = 5−10; pLN, n = 4−6.

frequencies of Foxp3$^+$ Tregs were significantly increased in mLN compared to pOVA-treated mice ($p \leq 0.01$) (Figure 4). This supports the notion that the difference in size and structure of the conjugates and, interestingly, the linker chemistry as well, affects both quality and quantity of the immune response.

Induction of Anergy and Deletion of Antigen-Specific Cells by Treatment with OG/PG Constructs. Another important indication of tolerance induction is a diminished pro-inflammatory cytokine response, reflecting anergy of the cells.[44] Pro-inflammatory cytokines such as TNF are involved in many autoimmune diseases and TNF is a major target for treatment of, e.g., rheumatoid arthritis.[53] To investigate the impact of OG/PG-peptide conjugates on the capacity of antigen-specific T-cells to produce inflammatory cytokines, we studied the expression of TNF after stimulation in the pOVA transgenic adoptive transfer model. Six days after tolerogenic vaccination with OG/PG-pOVA CD4$^+$ T-cells were isolated from lymphatic organs and re-stimulated with PMA/ionomycin in vitro (Figure 5).

In mice treated with unconjugated pOVA, the frequency of TNF-producing pOVA-specific CD4$^+$ T-cells was significantly reduced compared to PBS control (except in pLN). A significant reduction was also found for OG/PG-peptide constructs (except [G2.0]-pOVA in pLN and mLN). Interestingly, PG-E-pOVA again was superior to pOVA and

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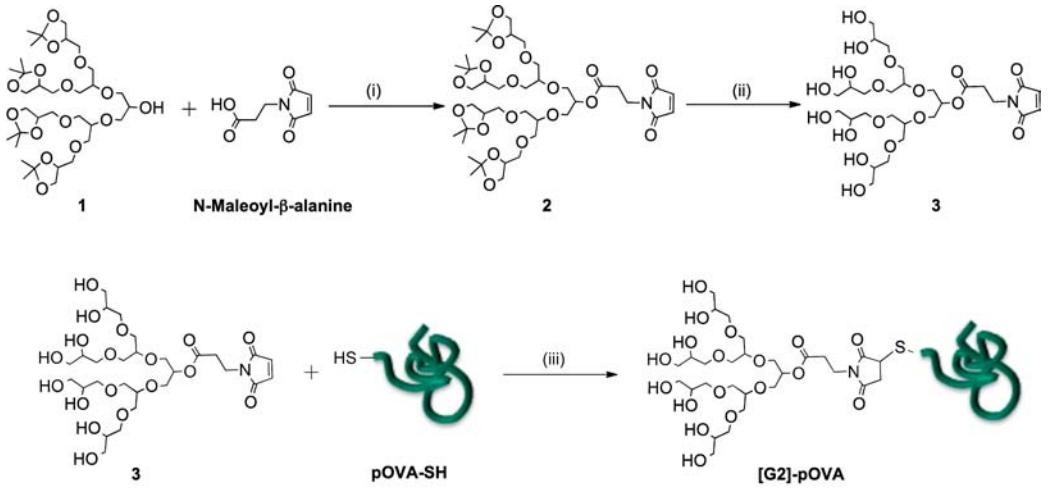
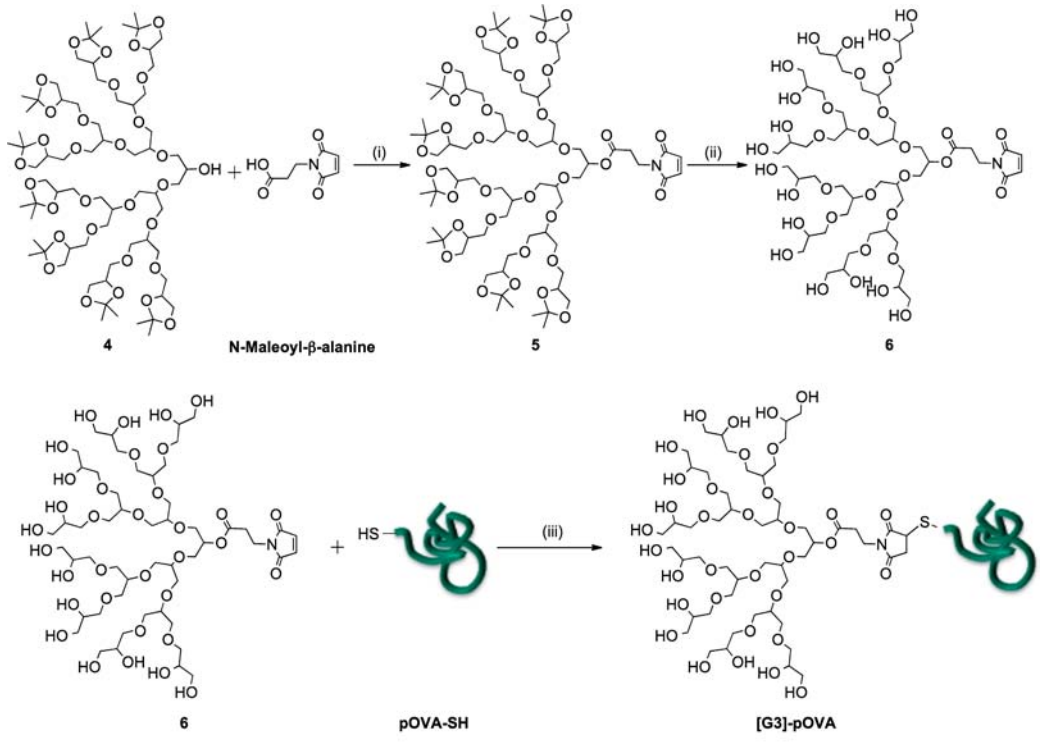
Scheme 1. Synthesis of [G2] Generation of Oligoglycerol Dendron–Peptide Conjugate [G2]-pOVA



Reaction conditions: (i) EDC, DMAP, DMF; (ii) dowex, methanol, 60 °C; (iii) DMF, sodium phosphate buffer pH = 7.4, room temperature

Scheme 2. Synthesis of [G3] Generation of Oligoglycerol Dendron–Peptide Conjugate [G3]-pOVA



Reaction conditions: (i) EDC, DMAP, DMF; (ii) dowex, methanol, 60 °C; (iii) DMF, sodium phosphate buffer pH = 7.4, room temperature

other constructs in suppressing the TNF response, with a highly significant reduction in spleen and significantly reduced frequencies in mLN and pLN compared to pOVA. Other cytokines, including IFNγ or IL-10, could not be detected under the conditions used (see Supporting Information, Figures SI 3 and SI 4).

The combined impact of tolerogenic treatment on both induction/expansion of Foxp3$^+$ Tregs and induction of anergy is best visualized by the ratio of TNF-producing to Foxp3$^+$ cells, representing the effector/regulatory T-cell ratio in vivo (Figure 6). The lowest effector cell/Treg ratio was found in all analyzed lymphatic organs in mice treated with PG-E-pOVA compared to pOVA-injected mice. All other OG/PG constructs significantly reduced the TNF/Foxp3 ratio compared to PBS control, but not compared to pOVA (except [G3.0]-pOVA in pLN, $p \leq 0.05$).

Beside induction of anergy, deletion of antigen-specific T-cells by activation-induced cell death also limits the immune

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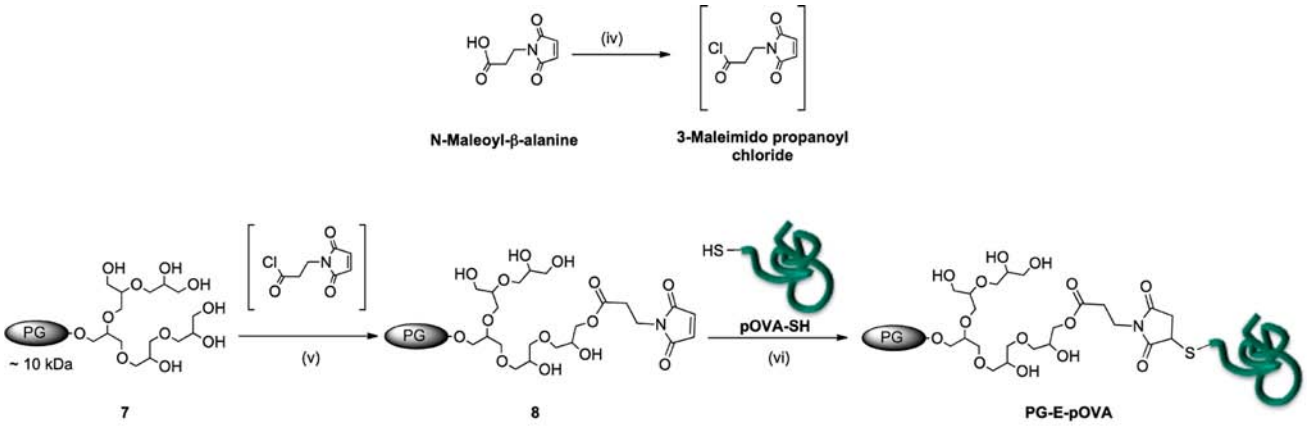
Scheme 3. Synthesis of Polyglycerol Dendrimer–Peptide Conjugate (via Ester Linkage) PG-E-pOVA



response.[54] In our study, we observed a significantly reduced frequency of pOVA-specific $CD4^+$ cells among total $CD4^+$ cells after PG-E-pOVA treatment compared to unconjugated pOVA (Figure 7), although PG-E-pOVA induced a stronger proliferation than pOVA in pLN or equivalent proliferation in mLN and spleen. This suggests an increased apoptosis rate and deletion of pOVA-specific effector $CD4^+$ T-cells as a further mechanism of suppression of antigen-specific effector response by the tolerogenic vaccination.

CONCLUSION

Based on findings, we conclude that the conjugation of peptides to synthetic carriers such as oligo- and polyglycerols can increase the efficacy of tolerogenic peptide vaccination compared to free peptide. However, size, structure, and linker chemistry are critical for the tolerogenic capacity of the constructs. Here we identified the hyperbranched PG (10 kDa) bound via an ester linkage to pOVA (PG-E-pOVA), as the most tolerogenic conjugate. In contrast, the PG-A-pOVA conjugate differing only in the linker chemistry (having an amide linkage) induced stronger proliferation, but also higher TNF production and lower frequencies of $Foxp3^+$ Tregs.

Future studies have to elucidate the underlying mechanisms responsible for the peculiar properties of the ester-linked conjugate. It is tempting to speculate that the linker structure not only generally affects the conjugate uptake, storage, and stability, as discussed above, but might additionally target them into specific subsets of APC such as immature or $CD103^+$ dendritic cells that favor a tolerogenic T-cell response.[46,55] Alternatively, a functional modulation of APC by the conjugate structure has to be taken into consideration.

These results suggest that auto antigen-derived peptides coupled to OG/PG carriers are promising novel candidates for tolerogenic vaccines that can be used for the treatment of autoimmune diseases and allergy.

EXPERIMENTAL PROCEDURES

Synthesis of OG-Peptide and PG-Peptide Conjugates. The development of carrier-conjugated peptide prodrugs for the treatment of various autoimmune and inflammatory diseases increases the need for simple, effective, and controlled approaches for the synthesis of biocompatible nanocarriers. Herein, we have strategically designed and explored a simple, effective, and controlled approach for the synthesis of *N*-maleimide-functionalized oligoglycerols and polyglycerols, which are highly biocompatible. These maleimide-functionalized dendritic polyglycerols were coupled with free thiol-functionalized peptides via a Michael reaction to give OG-peptide and PG-peptide conjugates.

Acetal-protected [G2.0] and [G3.0] generation OG dendrons (**1** and **4**) and hyperbranched PG (**7**) and PG-amines (**9**) were synthesized according to published procedures.[56−59] The synthetic protocol for the OG-peptide conjugates consisted of three steps. Initially, *N*-maleoyl-*β*-alanine was coupled to the core hydroxyl group of the acetal-protected OG dendrons via the EDC-mediated standard esterification protocol (see the Supporting Information (SI)), and in the next step they were deprotected using Dowex 50W ion-exchange resin to obtain the target dendrons (**3** and **6**) for peptide coupling (Schemes 1 and 2).

The purified compounds for both the steps were characterized by ^{1}H NMR and HRMS, respectively. The respective maleimido-functionalized OG dendrons (**3** and **6**) were then conjugated in the final step to the free sulfhydryl group of the pOVA (C-*β*A-ISQAVHAAHAEINEAGR) via a Michael reaction in PBS of pH 7.4 (Schemes 1 and Scheme 2). The thiol group was added to the double bond of the maleimide group in a fast and selective reaction at room temperature, forming a stable thioether bond, yielding OG-peptide conjugates, [G2]-pOVA and [G3]-pOVA. The conjugates were purified by dialysis using 500 Da MWCO dialysis tubing in Millipore water, and finally the conjugates were lyophilized at −60 °C for 16 h. The conjugation at this stage was followed by ^{1}H NMR and MALDI spectra. The characteristic disappearance of the peak at δ 6.84−6.85, for the maleimide olefinic protons and appearance of peptide (pOVA) peaks in the ^{1}H NMR confirmed the conjugate formation (cf. SI). Additionally, the MALDI data clearly indicated the molecular ion peaks corresponding to the conjugate (cf. SI).

In the case of hyperbranched PGs, esterification was performed via the in situ generated acid chloride of *N*-maleoyl-*β*-alanine, while amidation was performed via the in situ generated NHS ester of *N*-maleoyl-*β*-alanine with PG-amine (cf. SI). The ester and amide linked PG-maleimide conjugates (PG-E-pOVA and PG-A-pOVA) were purified by dialysis using 2000 Da MWCO dialysis tubing in Millipore

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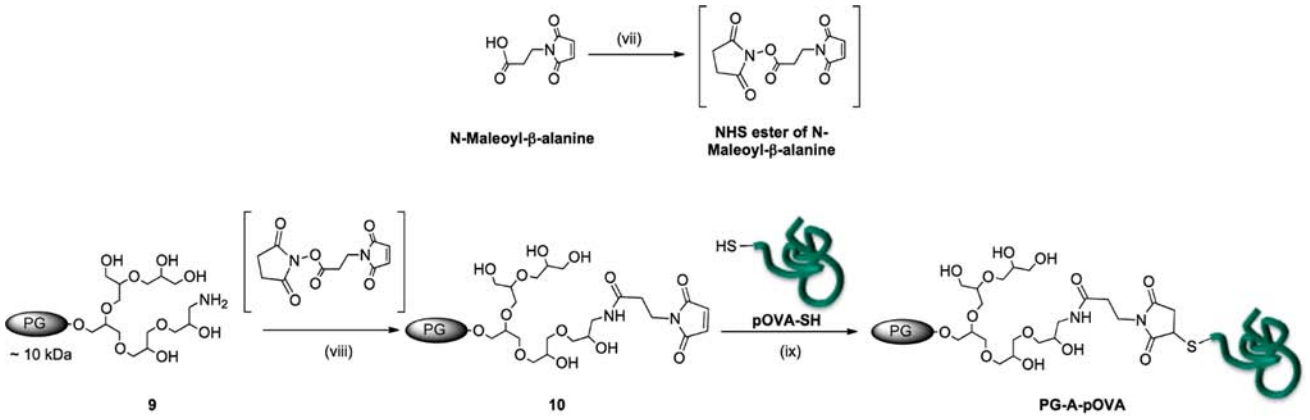
Scheme 4. Synthesis of Polyglycerol Dendrimer–Peptide Conjugate (via Amide Linkage) PG-A-pOVA



Reaction conditions: (vii) N-hydroxy succinimide, EDC, DMAP, DMF, rt, 8h; (viii) triethyl amine, DMF, 0° - rt, 24h; (ix) DMF, sodium phosphate buffer pH = 7.4, rt, 24h

water, and lyophilized at −60 °C for 16 h. The maleimido-functionalized PGs (**8** and **10**) were then conjugated to the free sulfhydryl group of the pOVA (C-*β*A-ISQAVHAAHAEIN-EAGR) via a Michael reaction in PBS at pH 7.4 (Schemes 3 and 4). The thiol group was conjugated to the double bond of the maleimide group via a stable thioether bond.

The PG-pOVA conjugates were purified by dialysis using 2000 Da MWCO dialysis tubing in Millipore water, and finally the conjugates were lyophilized at −60 °C for 16 h. The conjugation at this stage was again followed by ^{1}H NMR spectroscopy, where the characteristic appearance of peptide (pOVA) peaks confirmed the conjugate formation (cf. SI).

Highly water-soluble conjugates, [G2.0]-pOVA, [G3.0]-pOVA, PG-E-pOVA, and PG-A-pOVA, were obtained with the above-mentioned protocols, respectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, instrumentation; details of the biological materials and procedures (mice, antibodies, staining and sorting reagents, preparation of pOVA-specific cells, determination of cell proliferation in vitro and in vivo, adoptive T-cell transfer assay, analysis of bioavailability, flow cytometry, statistics), synthesis and characterization of OG- and PG-peptide constructs (impact of unconjugated OG/PG polymers on immune reaction under Th0 and Th1 conditions, and analysis of IL-10 production and IFNγ production). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: hamann@drfz.de. Tel: (+49) 30 28460 655. Fax: (+49) 30 28460 656.

*E-mail: haag@chemie.fu-berlin.de. Tel: (+49) 30 838 52633. Fax: (+49) 30 838 53357.

Author Contributions

[#]Shilpi Gupta and Jennifer Pfeil, and Ute Hoffmann and Rainer Haag, respectively, contributed equally to this work.

Notes

The authors declare no competing financial interest.

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