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A Pegylated Derivative of α-Galactosylceramide Exhibits Improved Biological Properties

Thomas Ebensen,[1] Claudia Link,[1] Peggy Riese, Kai Schulze, Michael Morr, and Carlos A. Guzmán[2]

The glycolipid α-galactosylceramide (αGalCer) has immunomodulatory properties, which have been exploited to combat cancer, chronic inflammatory diseases, and infections. However, its poor solubility makes αGalCer a suboptimal compound for in vivo applications. In this study, a pegylated derivative of αGalCer is characterized, which exhibits improved physical and biological properties. The new compound, αGalCerMPEG, is water-soluble and retains the specificity for the CD1d receptor of αGalCer. The in vitro stimulatory properties on immune cells (e.g., dendritic cells and splenocytes) are maintained intact, even when tested at a 33-fold lower concentration of the active moiety than αGalCer. NK cells isolated from mice treated with αGalCerMPEG also had stronger cytotoxic activity on YAC-1 cells than those obtained from animals receiving either αGalCer or CpG. Intranasal immunization studies performed in mice showed that αGalCerMPEG exerts stronger adjuvant activities than the parental compound αGalCer when tested at 0.35 vs 11.7 nM/dose. Coadministration of β-galactosidase with αGalCerMPEG resulted not only in high titers of Ag-specific Abs in serum (i.e., 1:512,000), but also in the stimulation of stronger Th2 and secretory IgA responses, both at local and remote mucosal effector sites (i.e., nose, lung, and vagina). The new synthetic derivative αGalCerMPEG represents a promising tool for the development of immune interventions against infectious and noninfectious diseases. *The Journal of Immunology,* **2007, 179: 2065–2073.**

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Most pathogens enter the host via the mucosal membranes. Therefore, the induction of systemic and mucosal immune responses following immunization represents a major goal in vaccine development. Vaccines delivered through mucosal surfaces induce not only systemic but also mucosal immune responses and trigger efficient immunological memory (1–6). In addition, this approach is associated with easier and less expensive administration logistics, being particularly suitable for mass vaccination. However, to implement this strategy, several hurdles should be overcome. The most important bottleneck is the poor immunogenicity of purified Ags administered by this route. This is in part due to their mechanical clearance, enzymatic degradation, and structural modification (e.g., by extreme pH), as well as to the fact that mucosal territories represent tolerance-prone niches.

To improve the immunogenicity of vaccine Ags, they can be coadministered with mucosal adjuvants. Unfortunately, the development of efficient and safe adjuvants still remains a challenge for the vaccine industry. Nevertheless, recent advances in our understanding of the immune system, in particular regarding early proinflammatory signals, have led to the identification of promising molecular targets for screening programs aimed at the discovery of compounds with immunomodulatory properties (7–12). Improved biochemical techniques also allow full synthesis of well-defined molecules.

The glycolipid α-galactosylceramide (αGalCer),[3] originally derived from the marine sponges *Agelas mauritianus*, exhibits potent antitumor activity (13). This compound also has immune modulatory properties, leading to the activation of various cell subsets of the innate and adaptive immune system. It was shown that αGalCer is presented by CD1d molecules on APCs, acting as a ligand for invariant $V\alpha 14^+$ NKT cells (14), which produce large amounts of IFN-γ and IL-4 upon αGalCer activation (15–20). The immune modulatory properties of αGalCer have been exploited to enhance responses against viral and parasitic Ags after vaccination (21–25). A recent study also suggested that αGalCer can act as mucosal adjuvant (1). However, there are major drawbacks preventing an efficient transfer of αGalCer into the clinical development pipeline, such as its poor solubility. To provide soluble formulations, nonorganic solvents or detergents are needed, which represent a safety concern and might affect the immunological properties of some Ags.

An efficient and safe method to improve the solubility of chemical compounds in aqueous solutions is their conjugation with polyethylene glycol (PEG). The process of pegylation can also improve their half-life by shielding, as well as by reduction of both metabolic degradation and receptor-mediated endocytosis (26–28). Of particular relevance for vaccine development is the fact that PEG is nontoxic and very poorly immunogenic (29–35). Therefore, in the present work, we evaluated whether conjugation to PEG can improve the immune modulatory properties of

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[3] Abbreviations used in this paper: αGalCer, α-galactosylceramide; PEG, polyethylene glycol; DC, dendritic cell; i.n., intranasal; β-Gal, β-galactosidase; αGalCerMPEG, pegylated derivative of αGalCer; ANS, I-anilino-8-naphthalenesulfonate; BAL, bronchial alveolar lavage; NL, nasal lavage; MFI, mean fluorescence intensity; sIgA, secretory IgA.

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αGalCer. The obtained results have demonstrated that the new PEGylated derivative of αGalCer (αGalCerMPEG) is able to activate in vitro primary cultures of murine dendritic cells (DC) and NKT cells more efficiently than αGalCer, even when tested at a 33-fold lower concentration of the active moiety. Intranasal (i.n.) coadministration of β-galactosidase (β-Gal) with αGalCerMPEG stimulated similar immune responses in mice to those observed using αGalCer, but using 33-times less compound (i.e., 0.35 and 11.7 nM/dose, respectively). Interestingly, αGalCerMPEG was a superior inducer of secretory (sIgA) and Th2 responses than the parental compound αGalCer.

Materials and Methods

Synthesis of αGalCerMPEG

To render αGalCer soluble in aqueous solvents a pegylated derivative was generated which was prepared using a modification of the protocol from Zhou et al. (36). In brief, methyl-PEG-COOH was dissolved in dichloromethane, mixed with hydroxybenzotriazole and 1 di-isopropylcarbodiimide, and added to a solution of αGalCer (Fig. 1*A*) in dichloromethane. The resulting mixture was incubated under stirring in the absence of humidity for 15 h at room temperature to generate an intermediate compound (Fig. 1*B*), which was purified by silica gel chromatography using chloroform and chloroform/methanol, dissolved in ethyl acetate/methanol (1:1), and hydrogenated using palladium/charcoals as catalyst for 9 h at 40°C. The resulting hydrogenated compound (i.e., αGalCerMPEG, Fig. 1*C*) was finally purified by silica gel chromatography using a mixture of chloroform and methanol. The purity of αGalCerMPEG was analyzed by reverse-phase HPLC (Waters Alliance) using a LUNA column (Phenyl-Hexyl-phase; C18; 4.6 × 50 mm; 3 μm; Phenomenex) and evaporative light scattering detection (ELSD Waters; detection limit 0.01%). As shown in Fig. 1*D*, the HPLC analysis (37) demonstrated that the resulting compound exhibits a high degree of purity (96%). The structure of the αGalCerMPEG was confirmed by matrix-assisted laser desorption/ionization mass spectrometry (Table I). ^{1}H- and ^{13}C-spectra, which showed a shift referenced to the residual signal of $CHCl_3$ at 7.25 ppm and CD_3OD at 49 ppm, were recorded at 300°K on a Bruker AVANCE DMX600 NMR spectrometer locked to the major deuterium signal of the solvent. Samples were dissolved in $CDCl_3$ and a 4:1 mixture of $CDCl_3$ to CD_3OD, respectively.

Determination of the solubility in water of αGalCerMPEG

Comparative studies were performed to analyze the solubility in water of αGalCerMPEG with respect to the parenteral compound αGalCer. In addition to the conventional measurements, the fluorescence dye I-anilino-8-naphthalenesulfonate (ANS) magnesium salt (Sigma-Aldrich) was also used. To this end, probes were dissolved in water containing ANS, according to established protocols (38). In brief, changes in fluorescence parameters and induced circular dichroism spectra at 360 nm indicate conformational changes resulting from ANS binding to the soluble form of each compound (39).

Preparation and flow cytometric analysis of murine DC

Bone marrow-derived primary DC were prepared from BALB/c mice using recombinant murine GM-CSF (BD Pharmingen), as previously described (40). On day 5, DC were coincubated with αGalCerMPEG (1.7 pM), αGalCer (58 pM), or 56.3 pM MPEG (data not shown) for 40 h. Control cells were treated with LPS from *Salmonella enterica* serovar *typhimurium* (Sigma-Aldrich) at a final concentration of 1 μg/ml. For flow cytometry, cells were preblocked using anti-mouse CD32/16 Ab for 15 min. Then, DC were stained with FITC-labeled Abs against mouse MHC class I (SF1-1.1), MHC class II (AMS-32.1), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), CD54 (3E2) or CD1d (1B1), together with PE-labeled Ab against CD11c (HL3) (BD Pharmingen). As negative controls, FITC- or PE-conjugated isotype control Abs were used. The FACS analysis of 20,000 events was performed using a FACSort and the CellQuest software (BD Biosciences), gating on CD11c-positive cells. Results are expressed as percentages of the total number (i.e., 50,000) of viable gated CD11c$^+$ cells (%) and as geometric mean fluorescence intensity (MFI). Results correspond to one representative experiment of five independent tests.

Measurement of cellular proliferation

To analyze the in vitro activity of αGalCerMPEG on cellular proliferation, splenocytes (5 × 10^5 cells/well) of female BALB/c (H-2^d; Harlan Winkelmann) or CD1d$^{-/-}$ (The Jackson Laboratory) mice of 6 wk of age were incubated in triplicates with either αGalCerMPEG (0.35 nM), αGalCer (11.7 nM), DMSO, or sterile water (Ampuwa) for 48, 72, or 96 h. Then, cellular proliferation was determined by measuring the incorporation of [^{3}H]thymidine using a scintillation counter (Wallac 1450; MicroTrilux), as previously described (41).

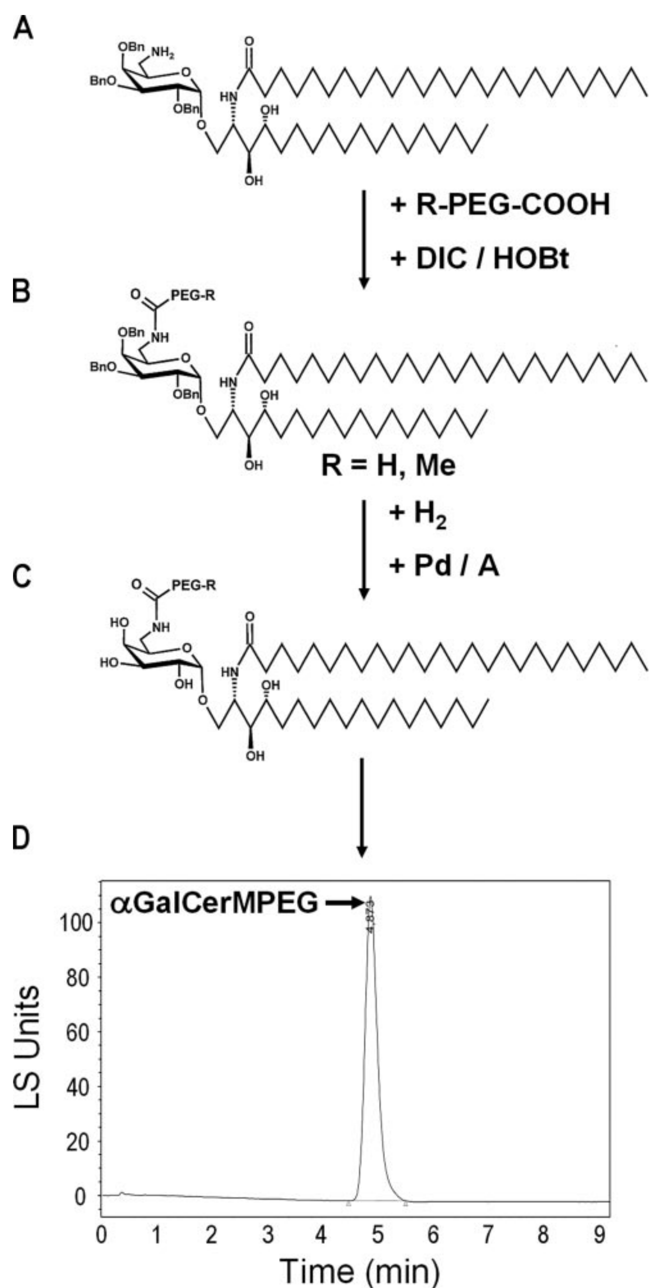

FIGURE 1. Synthesis of αGalCerMPEG. The parental compound αGalCer (*A*) was mixed with methyl-PEG-COOH (R-PEG-COOH) in the presence of hydroxybenzotriazole (HOBt) and 1 di-isopropylcarbodiimide (DIC). This led to an intermediate compound (*B*), which was purified and hydrogenated using palladium/active charcoals (Pd/A) as catalyst. The resulting αGalCerMPEG (*C*) was purified by silica gel chromatography, and its purity was analyzed by reverse-phase HPLC using a LUNA column and evaporative light scattering detection. The peak corresponding to the final product is indicated by an arrow (*D*).

Cytotoxicity assay

Mice received αGalCer (11.7 nM) or αGalCerMPEG (0.35 nM) by i.n. route, whereas control animals were injected by i.p. route with CpG (100 μg; i.e., 2.25 nM). After 2 days, animals were sacrificed and their splenocytes

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Table I. *αGalCerMPEG analysis in CDCl3/CD3OD (4/1 v/v) by ^1H and ^{13}C-NMR*

^1H-NMR			^{13}C-NMR	
Moiety	Shifts (56)	J (Hz)	Moiety	Shifts multiplicity
αGalactopyranosyl			αGalactopyranosyl	
H-1	4.68	(1–2) 3.8	C-1	100.0, d
H-2	3.59		C-2	[a]
H-3	3.53-3.40		C-3	[a]
H-4	3.53-3.40		C-4	[a]
H-5	3.59		C-5	69.2, d
H-6A	3.36		C-6	40.0, t
H-6B	3.05	(5-6B) 7.1	PhCH2 a/b/c	
6-NH		(6A-6B)13.8	PhCH2 i/o/m/p	
Ceramide			Ceramide	
H-1A	3.66	(1A-1B)10.6	C-1	67.6, t
H-1B	3.46	(1A-2) 4.9	C-2	50.7, d
H-2	4.00		C-3	75.1, d
2-NH			C-4	[a]
H-3	3.35		C-5	33.0, t
H-4	3.37		C-6	26.1, t
H-5A	1.47		C-1′	174.8, s
H-5B	1.19		C-2′	36.7, t
H-2′ AB	2.01		C-3′	26.1, t
H-3′	1.41		(CH2)n	30.1-29.6, t
(CH2)n	1.17		CH2	32.1, t
CH3	0.69		CH2	22.9, t
			CH3	14.1, q
MPEG			MPEG	
H-2	2.30		C-1	174.1, s
H-3	2.30		C-2	31.5, t
5-NH			C-3	31.5, t
H-6	3.20		C-4	173.3, s
H-7	3.36		C-6	39.5, t
OCH2CH2O	3.47		C-7	69.9, t
OCH3	3.20		(OCH2CH2)n	70.6, t
			OCH2CH2	70.3/72.0, t
			OCH3	59.0, t

[a] Signals at 69.2, 69.4, 70.3, and 72.4 ppm (4 × d).

were used as effector cells in a standard ^{51}Cr-release assay using YAC-1 cells as targets for NK cells. Effector cells were washed and their concentration was adjusted to 1×10^6/ml. In parallel, target cells were incubated in RPMI 1640 medium (Invitrogen Life Technologies) without FCS containing 100 μCi of ^{51}Cr for 2 h. Then, target cells were extensively washed with RPMI 1640 medium containing FCS and coincubated in triplicates with effector cells at different E:T ratios. After 4 h, cells were centrifuged and the radioactivity present in supernatants was measured by scintillation counting. Maximal lysis was determined after lysis with 5% Triton X-100, whereas spontaneous lysis was measured in supernatants of untreated target cells. Results are expressed as percentage of lysed cells, accordingly to the formula: (sample − spontaneous lysis)/(maximal lysis − spontaneous lysis) × 100.

Immunization protocols

Groups of female BALB/c (H-2^d) mice ($n = 5$) of 6–8 wk of age were immunized by i.n. route on days 0, 14, and 28 with 30 μg of the β-Gal protein (Roche) alone or coadministered with either αGalCer or αGalCerMPEG (11.7 and 0.35 nM active moiety/dose/animal, respectively). The optimal amount of the adjuvants used were experimentally determined in preliminary studies. Animals in the negative control group received only PBS. The animal permission was given by the local government of Lower Saxony (No. 509.42502/07-04.01).

Sample collection

Serum samples were collected on days −1, 13, 27, and 42. On day 42, mice were sacrificed, spleens were removed, and nasal (NL), bronchoalveolar (BAL), and vaginal (42) lavages were obtained by flushing the organs with PBS supplemented with 50 mM EDTA, 0.1% BSA, and 10 mM PMSF. For collecting the BAL, a catheter was inserted into the trachea after tracheotomy, whereas NL samples were obtained by gently flushing the nasal cavities from the posterior opening of the nose after removing the mandible. Lavages were then centrifuged to remove debris (10 min at 3000 × *g*) and supernatant fluids were stored at −20°C until processing. Abs were examined by investigating individual animals, whereas cellular responses were analyzed using pools of spleen cells, as previously described (43).

Detection of anti-β-Gal IgG in serum

The presence of β-Gal-specific serum Abs was determined by ELISA using microtiter plates coated with 100 μl/well of β-Gal (2 μg/ml in 0.05 M carbonate buffer (pH 9.6)), as previously described (43). β-Gal-specific IgG subclasses present in sera were measured using an isotype-specific ELISA. Endpoint titers were expressed as the reciprocal of the last dilution, which gave an OD at 405 nm of 0.1 U above the values of the negative controls after 15 min of incubation.

Determination of total and anti-β-Gal IgA

The amount of total and β-Gal-specific IgA present in the lavages was determined by ELISA, as previously described (41). To compensate for variations in the efficiency of recovery of secretory Abs among animals, the results were normalized and expressed as endpoint titers of Ag-specific IgA per microgram of total IgA present in the sample.

ELISPOT assay

To determine the amount of IFN-γ-, IL-2-, and IL-4-secreting cells, ELISPOT kits for the detection of murine IFN-γ, IL-2, and IL-4 (BD Pharmingen) were used. Spleen cells (1×10^6 and 5×10^5/well) were incubated for 24 h (IFN-γ) or 48 h (IL-2 and IL-4) in the absence or presence of a β-Gal peptide (TPHARIGL) encompassing a MHC class I-restricted epitope (for IFN-γ) or the β-Gal protein (for IL-2 and IL-4), at a concentration of 10 μM. Then, cells were removed and the plates processed according to the manufacturer's instructions. Colored spots were counted with a CTL ELISPOT reader and analyzed using the ImmunoSpot image analyzer software version 3.2.

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Cytometric bead array

For the characterization of the cytokines secreted by splenocytes of vaccinated animals restimulated in vitro with the β-Gal protein, supernatants were collected on days 2 and 4, and stored at −70°C until the content of IFN-γ, TNF-α, IL-2, and IL-10 was determined using the cytometric bead array (BD Pharmingen) by flow cytometry, according to the manufacturer's instructions.

Statistic analysis

The statistic significance of the differences observed between the different experimental groups was analyzed using the Student unpaired *t* test and the nonparametric Mann-Whitney *U* test. Differences were considered significant at $p < 0.05$.

Results

αGalCerMPEG exhibits stronger stimulatory activity on bone marrow-derived DC and splenocytes than αGalCer

To characterize the functional properties of the water soluble derivative αGalCerMPEG, a side-by-side comparative analysis of its biological activities with respect to those of the parental compound αGalCer was conducted. First, we compared the solubility in water of αGalCerMPEG with respect to the parenteral compound αGalCer. As expected, the hydrophobic parental compound αGalCer was completely insoluble in water, being essential the addition of DMSO to render it soluble. In contrast, the pegylated derivative was soluble in water up to a concentration of at least 100 mg/ml. Additional studies were performed to evaluate the fluorescence of the αGalCer/ANS complexes. The hydrophobic parental compound αGalCer was insoluble in water (i.e., no changes in the spectra due to the lack of binding to ANS), whereas an enhanced fluorescence resulting from the generation of αGalCerMPEG/ANS complexes was observed in the aqueous phase when the pegylated derivative was tested (data not shown).

Then, the effect of αGalCerMPEG on the activation and maturation of bone marrow-derived murine DC was assessed. As shown in Fig. 2 and Table II, αGalCerMPEG (1.7 pM active moiety/well) promotes an efficient activation and maturation of DC in vitro, as demonstrated by the up-regulated expression of MHC class II, costimulatory (CD80, CD86) and adhesion (CD40, CD54) molecules. The expression of the surface receptor for the αGalCer moiety, CD1d, was also enhanced on αGalCerMPEG-treated DC. In contrast, there was only a weak stimulation of the activation markers when DCs were stimulated using a 33-fold higher concentration of the parental compound αGalCer (58 pM/well), both in terms of MFI and percentage of positive cells (Fig. 2).

The in vitro capacity of αGalCerMPEG to stimulate the proliferation of splenocytes and its dependency on the expression of the CD1d receptor were then investigated. To this end, splenocytes of naive BALB/c and CD1d$^{-/-}$ mice were stimulated with either αGalCer or αGalCerMPEG. A time-dependent stimulation of cellular proliferation was observed in cells treated with αGalCerMPEG (Fig. 3*A*). The response was significantly stronger than the one observed when splenocytes were treated with a 33-fold excess of αGalCer ($p < 0.05$). As expected, the stimulation was dependent on the expression of the CD1d molecule, as demonstrated by the lack of any effect when spleen cells from CD1d$^{-/-}$ mice were used.

Next, the effect of αGalCerMPEG on the cytotoxic activity of NK cells was investigated. Similar responses were observed using control cells from mice stimulated in vivo with αGalCer (11.7 nM) or CpG (2.25 nM), namely 37-36% and 34-27% at E:T ratios of 100:1 and 50:1, respectively. In contrast, when animals received a 33-fold lower dose of the αGalCerMPEG active moiety (0.35 nM), an even stronger response was observed (46–56% lysis; see Fig. 3*B*). αGalCerMPEG was stable and active for at least 2 mo at room

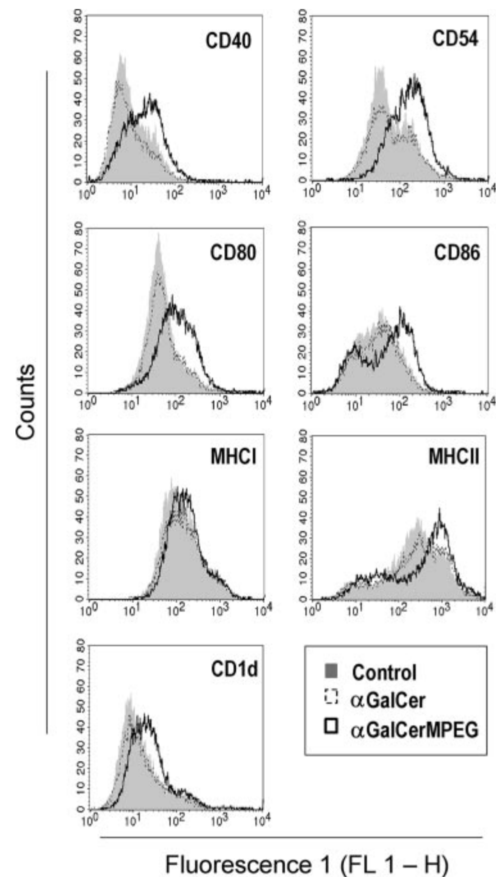

FIGURE 2. Activation and maturation of murine DC by αGalCerMPEG. The expression of surface markers was investigated by flow cytometry on CD11c^{+}-gated bone marrow-derived murine DC before (shaded area) and after stimulation with either 1.7 pM αGalCerMPEG (continuous line) or 58 pM αGalCer (broken line). One representative experiment of three is shown.

temperature and 4°C, as shown by the intact ability to stimulate the proliferation of spleen cells (data not shown).

αGalCerMPEG promotes the elicitation of efficient humoral immune responses when coadministered with an Ag by i.n. route

To evaluate the adjuvant properties of the pegylated derivative of αGalCer, mice were immunized by i.n. route with β-Gal (30 μg/dose) alone or coadministered with either αGalCer (11.7 nM/dose) or αGalCerMPEG (0.35 nmol/dose). Similar humoral responses were observed in sera from animals vaccinated with αGalCerMPEG and αGalCer as adjuvant, with high Ab titers even after a single boost (Fig. 4*A*). In contrast, very weak responses were detected in control animals receiving β-Gal alone. This demonstrated that the pegylated derivative is able to stimulate strong humoral responses also when used at a 33-fold lower concentration than αGalCer.

Then, the capacity of the two compounds to stimulate mucosal immune responses was investigated. To this end, β-Gal-specific sIgA was measured in NL, BAL, and VL from vaccinated animals (Fig. 4*B*). Immunization with αGalCerMPEG by the i.n. route resulted in the induction of significantly stronger β-Gal-specific sIgA responses in all tested mucosal territories than those observed in mice receiving β-Gal alone ($p < 0.05$). In contrast, in mice receiving β-Gal and αGalCer at a 33-fold higher concentration, the

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Table II. *Results are expressed in percentages of the total number (i.e., 50,000) of viable gated CD11c$^+$ cells (%) and as geometric MFI*[a]

Marker	Control		αGalCer (58 pM/Well Active Moiety)		αGalCerMPEG (1.7 pM/Well Active Moiety)	
	%	MFI	%	MFI	%	MFI
CD40	22	9.5	18	8.6	47	17.5
CD54	37	54.1	41	60.1	78	129.5
CD80	14	42.3	15	44.9	41	83.4
CD80	21	27.4	21	29.3	42	41.1
MHC class I	18	116.8	21	129.7	22	139.1
MHC class II	29	162.6	34	168	49	199.4
CD1d	17	13.9	18	15.1	22	21.7

[a] One representative experiment of three independent tests is shown.

differences were statistically significant with respect to the β-Gal-vaccinated control group only in BAL.

The use of αGalCerMPEG as mucosal adjuvant results in the stimulation of a dominant Th2 response

First, the subclass distribution of β-Gal-specific serum IgG was determined to evaluate the major Th response pattern stimulated in vaccinated mice. A significant increment on β-Gal-specific IgG1 was observed in mice receiving either αGalCerMPEG or αGalCer as adjuvants, whereas IgG2a was increased to a significant minor extent (Fig. 5*A*). This demonstrates that αGalCerMPEG promotes a Th2-type response, suggesting also that pegylation does not affect the immune modulatory properties of the active αGalCer moiety.

To further characterize the Th responses, the number of β-Gal-specific IFN-γ-, IL-2-, and IL-4-secreting cells present in spleens of vaccinated mice was determined by ELISPOT. In agreement to what was observed for the IgG isotypes, high numbers of IL-4-secreting cells were detected in mice receiving αGalCerMPEG or αGalCer (Fig. 5*B*). In contrast, the number of IFN-γ- and IL-2-secreting cells was increased to a significant minor extent in response to stimulation with the MHC class I-restricted peptide and the β-Gal protein, respectively. Thus, cytometric bead array studies were performed with supernatants from restimulated splenocytes to confirm the secretion of Th1 cytokines. The obtained results showed that IFN-γ and IL-2 were indeed secreted by spleen cells from vaccinated mice in which αGalCer or αGalCerMPEG were coadministered ($p < 0.05$) in comparison to those from

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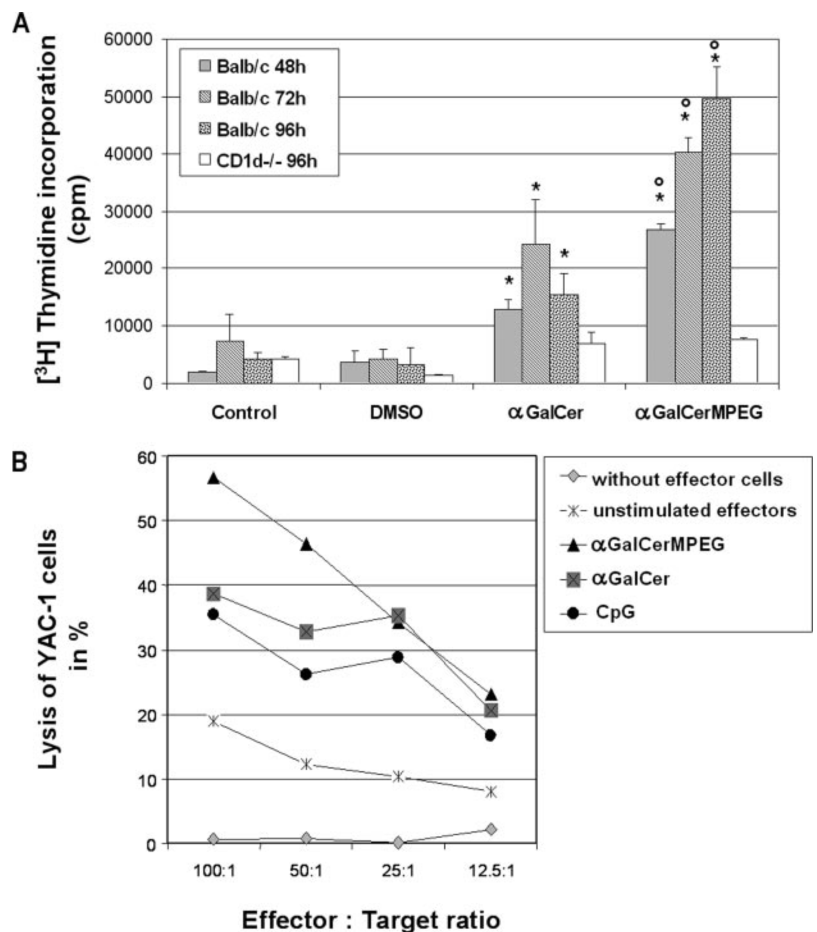

FIGURE 3. Comparative analysis of the stimulatory activities of αGalCerMPEG and αGalCer. *A*, Spleen cells from CD1d$^{-/-}$ and BALB/c mice were stimulated in vitro with αGalCer (11.7 nM active moiety) and αGalCerMPEG (0.35 nM active moiety) for 48, 72, and 96 h. Cellular proliferation was assessed by determination of the [^{3}H]thymidine incorporated into the DNA of replicating cells. Results are averages of triplicates and they are expressed as cpm. SEM is indicated by the vertical lines. One representative of four independent experiments is shown. *B*, In vivo stimulation of lytic activity by αGalCerMPEG. Spleen cells from mice injected with αGalCer (11.7 nM), αGalCerMPEG (0.35 nM), or CpG (2.25 nM) were recovered after 48 h and used as effectors in a ^{51}Cr-release assay with YAC-1 cell targets. The results are expressed as percentage of lysis and they are average of triplicates. One representative of three independent experiments is shown. ∗, The values were significantly ($p < 0.05$) different with respect to those from control cells (untreated and DMSO treated). ○, Significantly ($p < 0.05$) different values with respect to results obtained with αGalCer-treated cells.

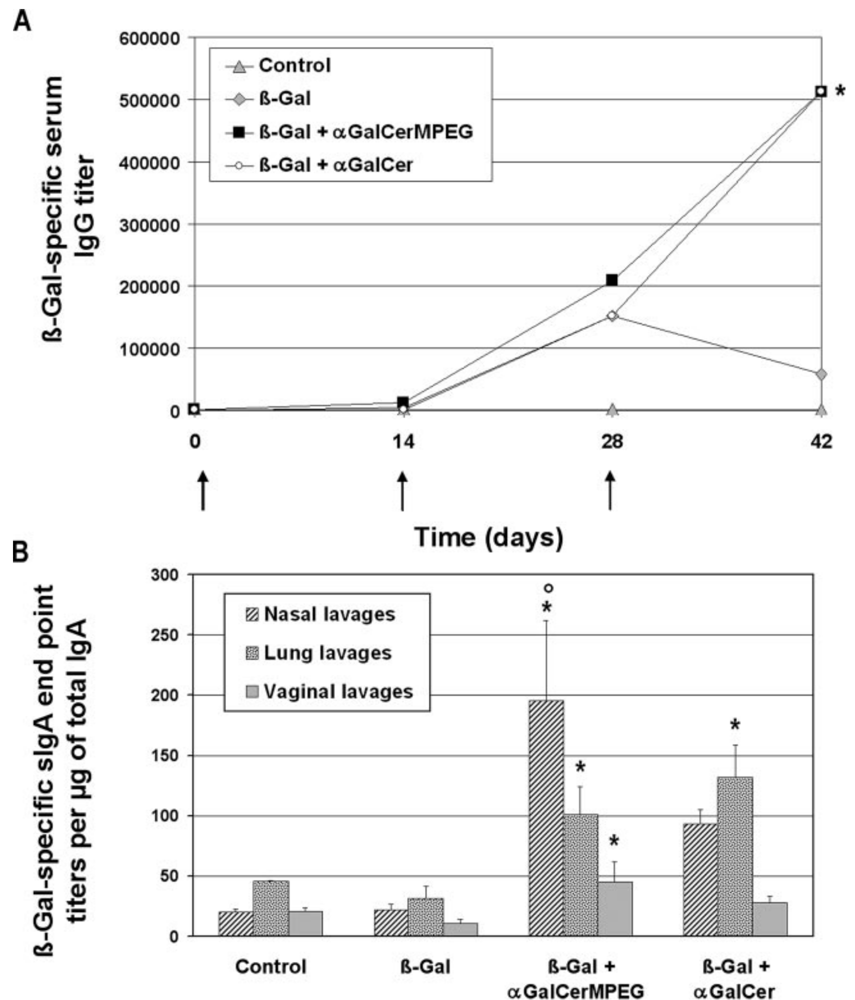

FIGURE 4. Humoral immune responses stimulated in mice vaccinated using αGalCerMPEG as adjuvant. *A*, Kinetic analysis of anti-β-Gal IgG responses in sera from mice ($n = 5$) immunized on days 1, 14, and 28 (indicated by arrows) with PBS (controls), β-Gal (30 μg/dose), β-Gal + αGalCer (11.7 nM/dose), or β-Gal + αGalCerMPEG (0.35 nM/dose) by the i.n. route. Results are expressed as the mean end point titers; SEM is indicated by vertical lines. One representative of three independent experiments is shown. *B*, Stimulation of sIgA responses in mice immunized using αGalCerMPEG as adjuvant. The presence of β-Gal-specific sIgA was investigated in NL, BAL, and VL by ELISA. Results are expressed as mean β-Gal-specific sIgA titers with respect to 1 μg of total sIgA. SEM is indicated by vertical lines. One representative of three independent experiments is shown. *, The obtained results were significantly different ($p < 0.05$) with respect to those from animals immunized with PBS or β-Gal alone. ○, Significantly ($p < 0.05$) different values with respect to results obtained in mice immunized with β-Gal + αGalCer.

animals receiving β-Gal alone (Fig. 5*C*). The concentrations of the Th1 cytokines, such as IFN-γ or IL-2, secreted by cells recovered from mice vaccinated with αGalCerMPEG were significantly ($p < 0.05$) lower than those observed in animals receiving αGalCer (Fig. 5*C*). This suggested the induction of more strongly polarized Th2-like response when the pegylated derivative of αGalCer was used. The secretion of the proinflammatory cytokine IL-6 was similar when splenocytes recovered from mice immunized with either αGalCerMPEG or the parental compound were tested ($p > 0.05$). In contrast, the secretion of TNF-α was significant higher in cells from animals immunized with β-Gal plus αGalCer with respect to cells from mice receiving β-Gal plus αGalCerMPEG ($p < 0.05$). Interestingly, significantly ($p < 0.05$) higher levels of the anti-inflammatory cytokine IL-10 were secreted by cells derived from mice receiving αGalCerMPEG (Fig. 5*C*). This might hint to a better pharmacological profile for the pegylated derivative.

Discussion

Experimental studies have shown that αGalCer has strong immunomodulatory properties, which can be exploited to prevent tumor metastases, modulate autoimmunity, and improve the clearance of microbial pathogens (44). Additional work has demonstrated that αGalCer exhibits adjuvant properties that can be used for vaccine development (23, 25). More recently, it was established that the adjuvant properties of αGalCer are also exerted after mucosal administration (45). In fact, mice vaccinated by the i.n. route using αGalCer as adjuvant were protected against a viral infection or a challenge with tumor cells in experimental models. Encouraging results are also emerging from clinical trials performed in cancer patients, in which αGalCer has been used as immune therapeutic (42, 46–48).

Despite these promising results, the physicochemical properties of αGalCer are suboptimal for in vivo use. The chemical structure renders αGalCer completely insoluble in aqueous solutions, making necessary the preparation of stocks in nonorganic solvents or in the presence of detergents. This not only represents a safety concern, but it might in turn affect the immunological properties of some Ags. Recent studies have also showed that derivatization of αGalCer can lead to compounds with novel biological properties. For example, α-C-GalCer shows a more stable binding to DC (15). Previous pharmacological studies have shown that pegylation cannot only render a molecule soluble in water, but also increase its half-life by reducing metabolic degradation and clearance (26–28). In contrast, the poor immunogenicity of PEG renders it an ideal conjugation partner, particularly for a compound to be used as immunomodulator such as αGalCer (29–35). Thus, we decided to evaluate whether pegylation might indeed improve the physical and/or biological properties of αGalCer. To this end, a pegylated derivative of αGalCer was generated and characterized both in vitro and in vivo.

The obtained results demonstrated that the pegylated derivative of αGalCer is completely soluble in water. The new compound, αGalCerMPEG, also exhibits an enhanced capacity to activate bone marrow-derived murine DC with respect to αGalCer, even at a 33-fold lower concentration (see Fig. 2 and Table I). This is a

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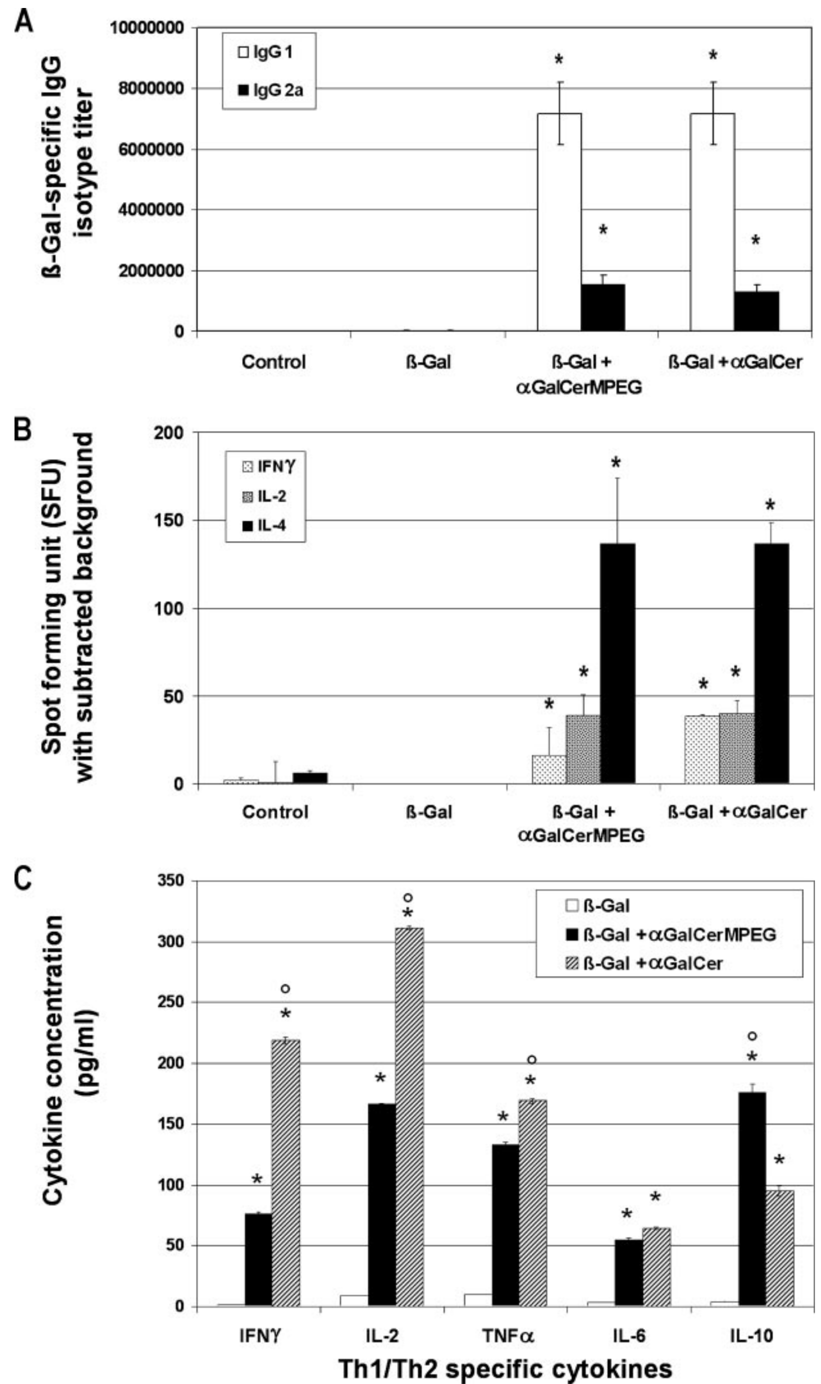

FIGURE 5. Analysis of the Th responses stimulated in mice vaccinated using αGalCerMPEG as adjuvant. *A*, Determination of the β-Gal-specific IgG isotypes present in sera. Results are expressed as mean end point titers. One representative of four independent experiments is shown. *B*, Detection of IFN-γ-, IL-2-, and IL-4-secreting cells in immunized mice. Spleen cells (1×10^6 and 5×10^5 cells/well) recovered from vaccinated animals were incubated in the presence of either the β-Gal protein (for IL-2 and IL-4) or a peptide (TPH PARIGL) encompassing its immunodominant MHC class I-restricted epitope (for IFN-γ). Then, the numbers of IFN-γ-, IL-2-, and IL-4-producing cells was determined by ELISPOT. Results are presented as spot forming units per 10^6 cells, which were subtracted from the values obtained from nonstimulated cells. One representative of three independent experiments is shown. The SEM of triplicates is indicated by vertical lines. *C*, Determination of the cytokines secreted by spleen cells recovered from vaccinated animals after in vitro restimulation with the β-Gal Ag. The amount of cytokines present in supernatant fluids of stimulated cells was measured by FACS using a cytometric bead array (BD Pharmingen). One representative of three independent experiments is shown. *, The obtained results were significantly different ($p < 0.05$) with respect to those from animals immunized with PBS and/or β-Gal alone. ○, Significantly ($p < 0.05$) different values with respect to results obtained in mice immunized with β-Gal + α-GalCer.

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critical feature for a compound aimed at the development of immune interventions, because the activation of DC maturation is recognized as a key event in the stimulation of adaptive immune responses (49). Splenocytes (Fig. 3) and purified NK cells (data not shown) were also efficiently stimulated in vitro by αGalCerMPEG. These activities of the pegylated derivative were still dependent on the expression of the CD1d molecule, suggesting that conjugation does not affect the binding features of the active moiety. Additional work also demonstrated that NK cells isolated from mice treated with αGalCerMPEG have stronger cytotoxic activity than those obtained from animals receiving either higher doses of αGalCer or CpG (Fig. 3). It is important to highlight that the stimulatory capacities of αGalCerMPEG on immune cells were maintained intact for at least 2 mo after incubation of a stock solution (10 μg/ml in water) at either 4 or 25°C (data not shown).

The excellent performance showed by αGalCerMPEG when tested in vitro encouraged us to perform an in vivo side-by-side comparison of its adjuvant properties with respect to those of the parental compound αGalCer. The obtained results proved that αGalCerMPEG is a more potent adjuvant than αGalCer when administered by i.n. route, even at a 33-fold lower concentration of the active moiety. Coadministration of αGalCerMPEG with the β-Gal protein resulted not only in high titers of β-Gal-specific Abs in serum (i.e., 1:512,000; Fig. 4), but also in the stimulation of more efficient sIgA responses, both at local and remote mucosal effector sites (i.e., nose, lung, and vagina). Significantly increased levels of Ag-specific serum IgG were detected after a single boost

in mice receiving αGalCerMPEG (Fig. 4). The analysis of the IgG subclasses present in sera (i.e., IgG1:IgG2a ratio of 4.6), together with the profile of the cytokines secreted by the splenocytes from vaccinated animals demonstrated that αGalCerMPEG promotes a dominant Th2 response (Fig. 5*C*). In this regard, BALB/c mice have been described as more prone to mount Th2 responses, whereas stronger Th1 responses are usually observed on C57BL6 mice (50–53). This feature seems to correlate with the TLR expression pattern on DC and a higher number of $CD25^+$ regulatory T cells. However, immunization studies performed using OVA as Ag showed that αGalCerMPEG also promotes Th2 dominant responses in C57BL/6 mice (data not shown).

Interestingly, the use of αGalCerMPEG resulted in a weaker stimulation of Th1-specific and proinflammatory cytokines (i.e., IFN-γ, IL-2, TNF-α, and IL-6) with respect to what was observed in mice receiving αGalCer. The secretion of the anti-inflammatory cytokine IL-10 was also significantly increased in mice receiving αGalCerMPEG. In this context, there are currently attempts to develop agents able to promote endogenous IL-10 production for the treatment of allergies and inflammatory diseases (54, 55). This suggests that αGalCerMPEG might also found an application for the development of immune therapies in this field.

In conclusion, our studies have led to a practical approach for engineering a pegylated derivative of αGalCer, which exhibits improved physical and biological properties. The new compound is water-soluble and retains intact both the specificity for the CD1d receptor and the immune stimulatory properties on immune cells (e.g., DC and NK cells). The αGalCerMPEG also exhibits stronger adjuvant properties than αGalCer, being a superior inducer of sIgA and Th2 responses. The inexpensive nature of the pegylation process, together with the fact that the new derivative is biologically active at 33-fold lower concentrations suggests that its use would be associated with considerable economic benefits. Therefore, the new synthetic derivative αGalCerMPEG represents a promising tool for the development of immune interventions against both infectious and noninfectious diseases.

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Disclosures

Thomas Ebensen, Michael Morr, Carlos A. Guzman, and the Helmholtz Centre for infection research have a pending patent for the usage of αGalCerMPEG (European community Patent-No.: 05022771.9-2402).

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