available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Systematic amino acid substitutions improved efficiency of GD_2-peptide mimotope vaccination against neuroblastoma

Matthias Bleeke[a,e], *Stefan Fest*[a,b,c,e], *Nicole Huebener*[a], *Christiane Landgraf*[d], *Burkhart Schraven*[c], *Gerhard Gaedicke*[d], *Rudolf Volkmer*[d,*], *Holger N. Lode*[a,*]

[a]*Charité – Universitätsmedizin Berlin, Department of Pediatrics, Experimental Oncology, Berlin, Germany*
[b]*Otto-von-Guericke University of Magdeburg, Faculty of Medicine Magdeburg, Department of Pediatrics, Magdeburg, Germany*
[c]*Otto-von-Guericke University of Magdeburg, Faculty of Medicine Magdeburg, Institute of Molecular and Clinical Immunology, Magdeburg, Germany*
[d]*Charité – Universitätsmedizin Berlin, Department of Medical Immunology, Berlin, Germany*

ARTICLE INFO

Article history:
Received 18 March 2009
Received in revised form 14 July 2009
Accepted 22 July 2009
Available online 18 August 2009

Keywords:
Immunotherapy
Neuroblastoma
Ganglioside
Mimotope
SPOT

ABSTRACT

The likelihood of identifying peptides of sufficient quality for the development of effective cancer vaccines by screening of phage display libraries is low. Here, we introduce the sequential application of systematic amino acid substitution by SPOT synthesis. After the substitution of two amino acids within the sequence of a phage display-derived mimotope of disialoganglioside GD_2 (mimotope MA), the novel mimotope C3 showed improved GD_2 mimicry *in vitro*. Peptide vaccination with the C3 mimotope induced an 18-fold increased anti-GD_2 serum response associated with reduction of primary tumour growth and spontaneous metastasis in contrast to MA mimotope controls in a syngeneic neuroblastoma model. In summary, SPOT provides an ideal optimisation tool for the development of phage display-derived cancer vaccines.

1. Introduction

The translation of carbohydrates or glycolipids into peptide surrogates by application of phage display libraries is an established tool to overcome the weak antigenicity of the nominal antigens. The generated peptides are mimotopes of the nominal antigen and their protective efficacy as vaccines against infective agents, including bacteria and viruses, as well as against cancer was demonstrated.[1–3] However, the application of phage display libraries is restricted by the technically limited number of actually expressed and screened peptide antigens and may therefore fail to identify the most efficient peptide mimotope.[4–6] We addressed the question if optimisation of a phage display-derived mimotope can be carried out by systematic alteration of the amino acid sequence resulting in improved antibody binding and vaccine efficacy. Here, we establish proof of concept that such an optimisation procedure is effective for a peptide mimotope of disialoganglioside GD_2 (mimotope MA) used as a vaccine against neuroblastoma.

Ganglioside GD_2 is an established target for immunotherapy against neuroblastoma, the most common extracranial solid tumour during early childhood. Infusions of monoclonal anti-GD_2 antibodies (mAbs), namely chimeric mAb ch14.18

* *Corresponding authors:* Address: Forum 4, R 2.0407, Augustenburger Platz 1, 13353 Berlin, Germany. Tel.: +49 (0) 30 450 666233; fax: +49 (0) 30 450 559917 (H.N. Lode). Address: Institut für Medizinische Immunologie, Abt. Molekulare Bibliotheken, Hessische Str. 3-4, 10115 Berlin, Germany. Tel.: +49 (0) 30 450 524267; fax: +49 (0) 30 450 524942 (R. Volkmer).
E-mail addresses: rve@charite.de (R. Volkmer), holger.lode@charite.de (H.N. Lode).
[e] Equally contributed to this paper.
0959-8049/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2009.07.026

and murine mAb 3F8, could improve the outcome of stage 4 patients.[7–10] Active vaccination with GD_2 encounters various obstacles including poor antigenicity and T-cell independency of glycolipid antigens.[11,12] In order to overcome this problem, GD_2-peptide mimotopes were identified by application of phage display libraries.[13–15] All these mimotopes were able to induce immune responses against GD_2-positive tumours, such as neuroblastoma and melanoma.[16–18]

In order to improve GD_2 mimicry of mimotopes phage display may be supported by sequential application of the SPOT technology,[19] which is based on synthesis of a defined peptide library onto a solid phase (reviewed in[20,21]). Therefore, our objective was to apply SPOT synthesis on a recently reported GD_2-peptide mimotope[16] by systematic replacement of each amino acid within the peptide mimotope sequence by all other genome-encoded amino acids.

Here, we present for the first time that amino acid substitution analysis carried out by SPOT synthesis for a phage display-derived mimotope of disialoganglioside GD_2 enhances the mimicry potential *in vitro* and improves anti-neuroblastoma efficacy of the optimised mimotope in a syngeneic murine model of neuroblastoma. Our results indicate that SPOT is a useful optimisation tool for vaccines generated from phage display-derived peptide mimotopes.

2. Materials and methods

2.1. Amino acid substitution analyses

Systematic alteration of the amino acid sequence and subsequent probing for GD_2 antibody binding were performed by SPOT synthesis and anti-GD_2 mAb ch14.18 binding assay on the SPOT membranes as described below.

2.2. SPOT synthesis

Peptide syntheses on a cellulose membrane were performed according to a standard SPOT synthesis protocol.[21] Briefly, peptides were synthesised on a β-alanine prepared membrane using the MultiPep SPOT-robot (INTAVIS Bioanalytical Instruments AG, Köln, Germany). Array design was performed with the aid of the in-house software LISA 1.82. Synthesis started with the definition of spots (ß-alanine, two cycles, double coupling, 15-min reaction each) followed by standard SPOT synthesis of the desired peptides. Solutions of Fmoc-amino acid-Opfp esters (Fmoc = fluoromethoxycarbonyl, Opfp = pentafluorophenyl) in N-methylpyrrolidone were used. After completion of peptide synthesis, cleavage of side chain-protecting groups was achieved through treatment with a mixture of trifluoroacetic acid (90% TFA w/v), tri-isobutylsilane (3%, TIBS w/v) and H_2O (2% v/v) in dichloromethane (DCM) for 1 h. Subsequent washing was done with DCM (3 × 3 min), dimethylacetamide (DMA 3 × 3 min), EtOH (3 × 3 min) and diethyl ether (2 × 3 min), followed by TFA (60% w/v), TIBS (3% w/v) and H_2O (2% v/v) in DCM for additional 2.5 h. Finally the membrane was washed with DCM (3 × 3 min), DMA (3 × 3 min), EtOH (2 × 3 min), phosphate buffer (pH 7.4, 0.1 m, 2 × 3 min), H_2O (2 × 3 min), EtOH (2 × 3 min) and diethyl ether (2 × 3 min) and dried.

2.3. Anti-GD2 mAb ch14.18 binding assay on the cellulose membrane

The membrane-bound GD_2 mimotope MA wild type and substitution variants were washed with ethanol and thrice (10 min, RT) with Tris-buffered saline (TBS, pH 8) containing 0.05% v/v Tween (T-TBS). Then, the membrane was blocked for 3 h with blocking buffer (10% blocking reagent v/v (CRB, Norwich, Great Britain) and 1% sucrose in 1: 10 TBS) and subsequently incubated with the chimeric anti-GD_2-mAb ch14.18 (1 μg/ml) in the same blocking buffer at 4 °C for 14 h. After washing (3×, T-TBS), the membrane was incubated with a peroxidase-conjugated anti-human IgG mAb (Sigma–Aldrich, Steinheim, Germany, 1 μg/ml). The membrane was washed again. Antibody binding was visualised using a chemiluminescence substrate (Pierce, Rockford, IL, USA) and the Lumi-Imager™ (Roche Diagnostics, Mannheim, Germany). Analysis and quantification of spot signal intensities were executed with the software Genespotter (MicroDiscovery GmbH, Berlin, Germany). Genespotter has a fully automatic grid finding routine resulting in reproducible signal intensities. The spot signal is calculated from a circular region around the spot centre detected on the image. The background signal for each spot is determined with a safety margin to this circular region.

2.4. Peptide synthesis

For further analyses selected peptides were synthesised by standard solid phase peptide synthesis on TentaGel S Ram resin (Rapp Polymere, Tübingen, Germany) using a multiple peptide synthesiser (Syro II, MultiSynTech, Witten, Germany). Synthesis was performed according to standard Fmoc-chemistry and PyBOP activation for all amino acids (2-fold coupling). Peptides were purified to >95% by preparative HPLC. Analytical HPLC analysis (Waters, Milford, MA, USA) was carried out using a linear gradient of solvents: A, 0.05% TFA (v/v) in water and B, 0.05% TFA (v/v) in acetonitrile; gradient 5–60% over 30 min. HPLC conditions: UV detector 214 nm, RP-18 column. Peptide identity was determined by ESI mass-spectrometry (Q-TOFmicro™, Waters, Milford, MA, USA). For *in vivo* experiments, peptides MA and C3 were synthesised and coupled to keyhole limpet haemocyanin (KLH) by Jerini Peptide Technologies (Berlin, Germany).

2.5. Surface plasmon resonance measurement

The affinities of the mutated peptide mimotopes to the ch14.18 mAb were determined by surface plasmon resonance measurement in a BiacoreX-system (Biacore, Uppsala, Sweden) in HBS buffer (10 mmol/L HEPES [pH 7.4], 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005% (v/v) Surfactant P20). Analyses were carried out in two rounds. In the first round of measurements the ch14.18 mAb was immobilised on a CM5 sensor chip (measurement cell) via the amine coupling method as described by the producer. An irrelevant antibody (anti-GST-IgG) was used in the reference cell. Affinity of the peptide mimotopes was determined using a dilution series of cyclic and linear decapeptides from 2 mM to 4 μM. In the second round the peptide mimotope with the highest affinity to the ch14.18 mAb of round one (i.e. 'C3') and both of the original

peptide mimotopes MA and MD were immobilised on CM5 sensor chips (measurement cell). An irrelevant peptide (SATPWDLKTSL) was used in the reference cell. The affinity of the ch14.18 mAb was determined using a dilution series of antibodies from 75 μM to 4.5 nM. All binding experiments were executed at 25 °C with a flow rate of 5 μl/min. Data were analysed using the equilibrium method of the programme BIAevaluation 3.0.

2.6. Mimicry analysis

For mimicry analysis of the peptide mimotope KLH conjugates, 10 μg of the KLH-conjugated mimotopes MA and C3 were immobilised on a nitrocellulose membrane by vacuum blotting using the Delrin dot blotting apparatus (Schleicher and Schuell, Dassel, Germany) as described previously.[16] About 10 μg of unconjugated KLH and 100 ng of GD_2 (Sigma–Aldrich, Steinheim, Germany) served as negative and positive controls.

2.7. Cell lines and mice

NXS2 neuroblastoma cells were cultured in DMEM (PAA, Pasching, Austria) supplemented with 10% foetal calf serum (FCS) and 1% Penicillin/Streptomycin. Female A/J mice were purchased from Harlan-Winkelmann (Borchen, Germany) at 12 weeks of age and housed according to the German guide for the care and use of laboratory animals (i.e. 'Tierschutzgesetz').

2.8. Immunisation procedure, tumour growth and metastasis

Immunisation was carried out in a prophylactic setting as three subcutaneous injections of either 10 μg KLH, MA-KLH or C3-KLH adsorbed to aluminium hydroxide (Alu-Gel-S, Serva, Heidelberg, Germany) at two-week intervals. Attenuated *Salmonella typhimurium* (SL7207) transfected with the empty CpG-rich expression vector pSecTag2A (Invitrogen, Karlsruhe, Germany) were orally administered with every subcutaneous injection as an immunological adjuvant. Blood was collected at several time points through the experiment for determination of anti-GD_2 serum response. After completion of the prophylactic immunisation, each mouse was challenged by subcutaneous injection of 2×10^6 syngeneic NXS2 neuroblastoma cells. Primary tumour growth was measured with a digital calliper and tumour volume was calculated as follows: width × length × (length/2). Primary tumours were surgically removed 12 d after injection (Fig. 3A). Once metastatic disease was detected, mice were sacrificed and liver weights were measured as an indicator for liver metastasis.

2.9. Determination of anti-GD2 serum response

Anti-GD2 serum response was determined by standard ganglioside ELISA as described previously.[16] Total IgG and IgG subclasses were determined using HRP-labelled anti-mouse-IgG (Sigma, Steinheim, Germany), IgG1 and IgG2a (BD Pharmingen, Heidelberg, Germany). Fold increase was calculated as follows: absorbance of immune serum/absorbance of pre-immune serum.

2.10. Statistics

Differences in tumour growth and metastasis were analysed by Mann–Whitney *U*-tests, and ELISA data were analysed by two-tailed *t*-tests. Differences were considered to be statistically significant at $p < 0.05$ and indicated as '*' in the figures.

3. Results

3.1. GD_2 mimotope optimisation by amino acid substitution analyses using SPOT technology

Our recently characterised GD_2 mimotope MA (C-GRLKMVPDLE-C) was subjected to systematic replacement of each amino acid within the mimotope sequence using the SPOT synthesis. All peptides had additional cysteine residues at N- and C-terminal ends for the formation of disulphide bridges important to keep a stabilised three-dimensional peptide structure on the membranes. Therefore, cysteine residues were omitted from systematic amino acid substitution. The first spot membrane revealed a peptide spot with a 23.5-fold higher signal intensity (2365 BLU) than the parental mimotope MA (112 BLU) when probed with the anti-GD_2 mAb ch14.18. This was attributed to a change from methionine to leucine at position (P) 5 in the amino acid sequence (C-GRLK**L**VPDLE-C) (Fig. 1A, upper panel). This novel mimotope was subjected to a second round of amino acid substitutions, leading to a further increase in binding affinity to ch14.18 (4184 BLU) (Fig. 1A, lower panel). This improvement was related to an exchange of lysine with histidine at P4. The new GD_2-peptide mimotope (C-GRL**HL**VPDLE-C) was named C3. This GD_2 mimotope was further characterised by determination of dissociation constants (K_D) in Biacore experiments. C3 revealed a 5.6-fold lower K_D compared to MA, indicating an increased binding affinity to anti-GD_2 mAbs (Fig. 2A). These findings clearly demonstrate that systematic amino acid substitution was effective in improving the binding characteristics of the phage display-derived GD_2 mimotope MA.

3.2. Mimicry analysis of mimotope-KLH conjugates

C3 was conjugated to KLH and its mimicry potential was compared to that of MA-KLH in dot blot experiments using the anti-GD_2 mAb 14G2a. The adjusted optical density (aOD) represents the intensity of binding between antigen and antibody. The dot blot showed 3.1-fold increased aOD of C3-KLH in contrast to MA-KLH demonstrating an improved GD_2 mimicry of the novel C3 vaccine over the MA vaccine (Fig. 2B). Moreover, conjugation of C3 to KLH did not adversely affect its mimicry potential. Unspecific anti-GD_2 antibody binding resulting from the carrier molecule KLH was not detected.

3.3. Anti-neuroblastoma effect of peptide mimotope C3 in vivo

The C3 mimotope vaccine was tested to suppress primary tumour growth and spontaneous metastasis in a syngeneic

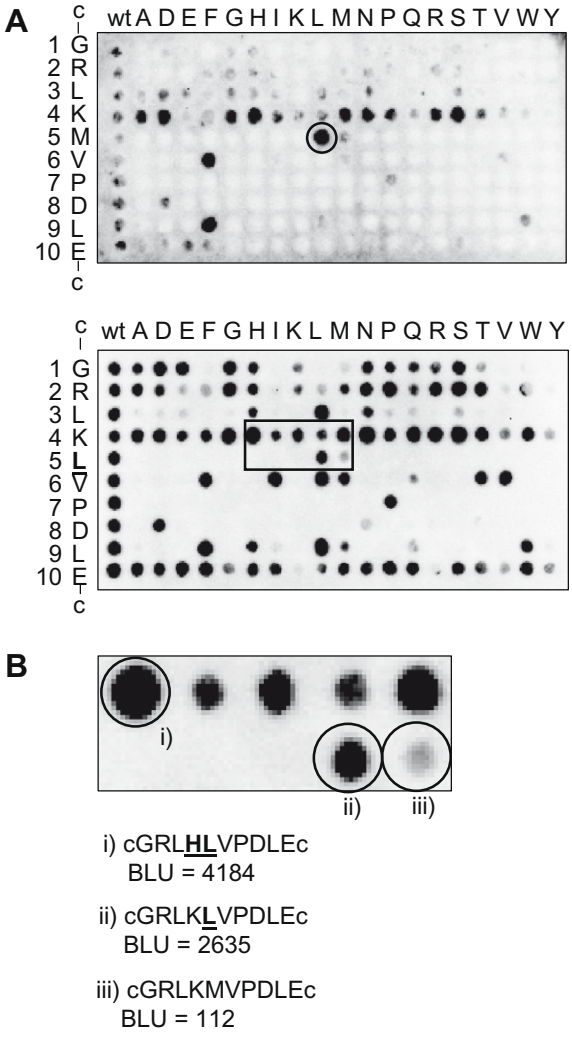

Fig. 1 – Intensity (Boehringer Light Units – BLU) of anti-GD2 binding to SPOT membranes after the first (A, top) and second (A, bottom) rounds of systematic amino acid substitution in the MA sequence (wt). Superior binding SPOTs are magnified (B).

model for neuroblastoma and the results were compared to those obtained from the parental mimotope MA (Fig. 3A). C3-KLH-vaccinated mice showed a significantly reduced growth of primary tumours of 77.1% compared to MA-KLH-vaccinated mice (Fig. 3B). Consequently, primary tumour weights after surgical removal were 61.6% lower after C3-KLH vaccination compared to MA-KLH vaccination (Fig. 3C). Furthermore, spontaneous liver metastasis was reduced in C3-vaccinated mice compared to MA-vaccinated mice as indicated by decreased liver weights to 41.6% only in C3-immunised mice (Fig. 3D). These findings indicate that optimisation of our peptide mimotope MA by amino acid substitution improved the anti-neuroblastoma response when administered as a prophylactic peptide conjugate vaccine.

3.4. *Determination of anti-GD2 serum response*

In order to test the efficacy of the C3 peptide mimotope vaccination the induction of an anti-GD_2 serum response was analysed by GD_2 ELISA in serum samples collected before and after the immunisation period. We observed an 18.4-fold increase of anti-GD_2 IgG 7 days after the third immunisation in mice vaccinated with C3-KLH with further increase over time (Fig. 4A). This finding was in contrast to that in MA-KLH-vaccinated controls. Determination of IgG subclasses revealed that C3-KLH primarily induced a GD_2-specific serum response of IgG class 1 which favours humoral anti-tumour immune responses (Fig. 4B). In summary, the C3-KLH vaccine induced an anti-neuroblastoma response characterised by production and systemic circulation of anti-GD_2 IgGs of IgG subclass I.

4. Discussion

The dismal outcome of stage 4 neuroblastoma demands the development of novel adjuvant therapeutics supporting the currently available treatment protocols. Here, we addressed the concept of anti-tumour vaccination and optimised our recently generated phage display-derived GD_2 mimotope MA by systematic amino acid substitution analyses.

Peptide mimotopes generated by phage display might not be the optimal antigens for cancer vaccine development. This is due to practical limitations since commonly used biopanning methods usually screen between 10^7 and 10^8 unique phage clones.[22] In contrast, the theoretic variety of 20×10^{10} peptide sequences for phages expressing decapeptides exceeds this figure by 3–4 logs. Additionally, not all phage clones which bound during biopanning are able to subsequently transfect *Escherichia coli* strains and are therefore not available for amplification and sequencing.[4] These limitations considerably reduce the efficiency of phage display experiments for the identification of peptide mimotopes for the development of effective GD_2-based neuroblastoma vaccines.

We faced these limitations by the application of SPOT technology to our phage display-derived GD_2 mimotope MA. After only two rounds of systematic amino acid alterations the resulting peptide C3 showed clearly improved GD_2 mimicry as indicated by increased binding affinity to anti-GD_2 antibodies. Interestingly, exchange of the first 3 amino acids and last amino acid (position (P) 1 to P3 and P10) of the wild type (wt) sequence of each optimisation round did not change the binding characteristics suggesting that these specific amino acids are not essential for paratope binding at these positions. On the other hand, substitution of lysine with histidine at P4 and methionine with leucine at P5 strongly enhanced ch14.18 mAb binding of the resulting mimotope. These changes seem to be independent of polarity or side chain charges. Changes in the amino acids at remaining positions (P6 to P9) abrogated antibody binding indicating that these amino acids are essential for antibody binding and therefore sufficiently mimic one part of the nominal GD_2 glycolipid epitope.

Up to date the combination of phage display and subsequent optimisation by SPOT was only applied to generate more effective anti-microbial drugs, such as inhibitors of β-lactamase[23] or chitinase, important for proliferation of fungi, insects and crustaceans.[24] Here, we introduced this combinatorial approach into the field of cancer vaccine development and optimised our phage display-derived mimotope MA into

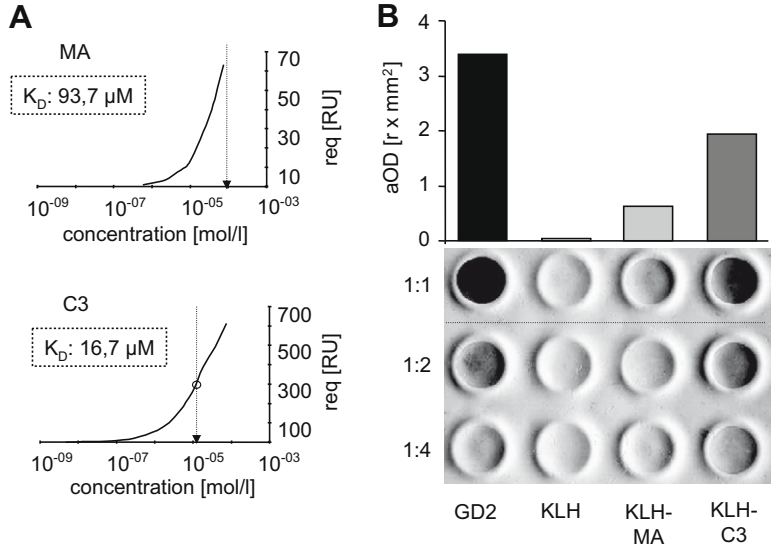

Fig. 2 – Determination of C3 and MA dissociation constants (K_D) with ch14.18 in Biacore experiments (A) and adjusted OD values in dot blot assays of corresponding KLH-peptide conjugates (B).

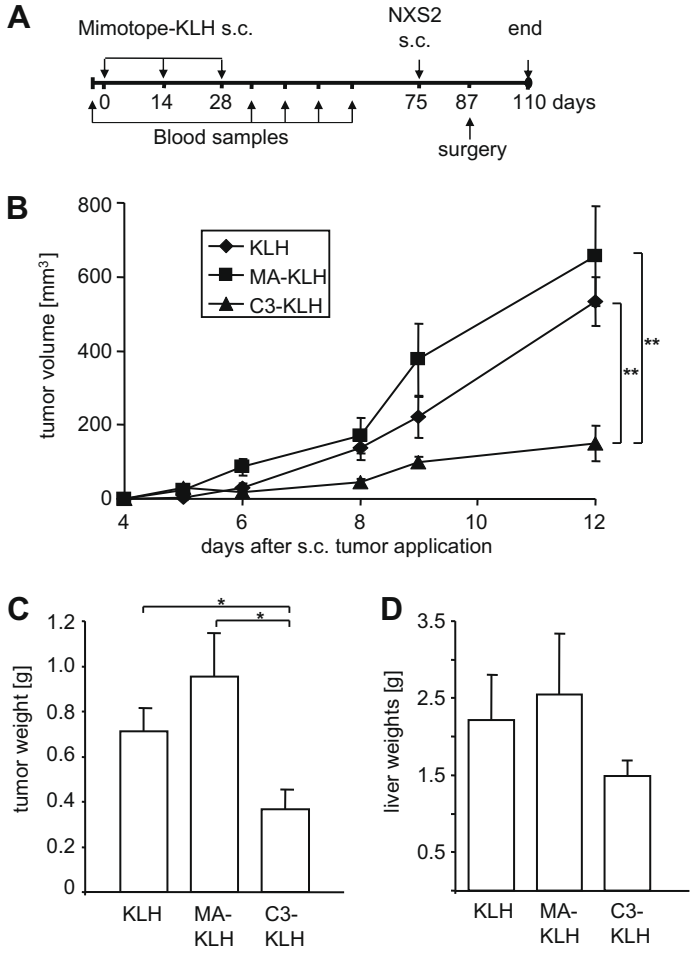

Fig. 3 – Schematic of the prophylactic vaccination experiment (A). Anti-tumour response of C3-KLH vaccination is presented by reduced growth indicated as volume (B) and weight (C) of primary tumours and reduced spontaneous liver metastasis (D). Data are shown as mean, ±SEM, $^{**}p < 0.01$.

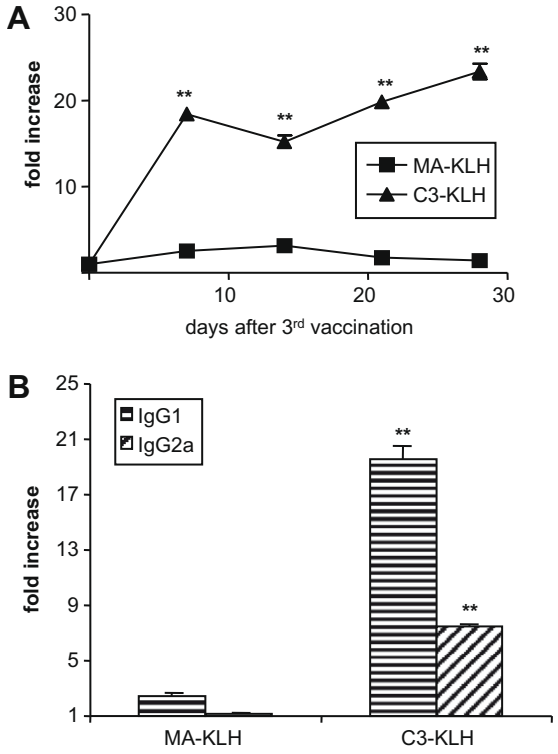

Fig. 4 – GD_2-specific immune response was normalised with pre-immune serum and is shown as fold increase in anti-GD_2 IgG (A) and IgG subclasses 1 and 2a (B). Data are shown as mean fold increase, ±SEM, $^{**}p < 0.01$.

the C3 peptide. This optimisation translated into an improved anti-neuroblastoma immune response *in vivo* since a KLH-coupled peptide vaccine with this novel GD_2 mimotope was able to further decrease primary tumour growth and spontaneous metastasis compared to the parental peptide mimotope MA. C3-KLH vaccination was also associated with an anti-GD_2 serum response in favour of IgG subclass 1. Increased levels of IgG1 are typically observed as a humoral immune response against protein antigens, in contrast to IgG2a associated with a response against T-cell-independent antigens, e.g. glycolipids.[25,26] The IgG1-biased anti-GD2 serum response observed in the present study indicates the induction of glycolipid-reactive antibodies by GD2-peptide mimotope vaccines. In summary, these findings clearly demonstrate that mimotope optimisation performed by systematic amino acid alterations and SPOT synthesis could improve the efficacy of a peptide mimotope vaccine against neuroblastoma.

However, the efficacy of peptide vaccination also depends on the co-administration of adjuvants, since the absence of a second immunostimulatory signal may result in the induction of tolerance rather than immunity. Oral application of attenuated *S. typhimurium* SL7207 carrying plasmid DNA provides a 'danger signal' induced by lipopolysaccharides and CpG motifs.[27] Therefore, we used this adjuvant in combination with peptide vaccination as reported previously.[16]

Another interesting advantage of our approach is the possibility of including non-natural amino acids into the screening process further increasing the diversity of peptidomimetics. Since we plan to use DNA vaccination as immunisation tool we only screened peptides composed of natural amino acids.

In summary, we describe the optimisation of a phage display-derived GD_2 mimotope performed by systematic amino acid alteration and demonstrate the improved efficacy of the novel GD_2 mimotope to induce a humoral immune response against neuroblastoma. SPOT synthesis provides an ideal tool for optimisation of phage display-derived mimotopes to develop immunogenic and effective mimotope vaccines targeting tumour-associated glycolipid antigens.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by DFG (Lo 635), Fördergesellschaft Kinderkrebs-Neuroblastom Forschung e.V and the NGFNplus Program (Bundesministerum für Bildung und Forschung, ENGINE) (HNL). SF was supported by DFG (Fe 914/1-1; Fe 914/2-1). MB was supported by trainee fellow grant from Kind-Philipp-Foundation (T020/15797/2006/sm).

REFERENCES

1. Agadjanyan M, Luo P, Westerink MA, Carey LA, Hutchins W, Steplewski Z, et al. Peptide mimicry of carbohydrate epitopes on human immunodeficiency virus. *Nat Biotechnol* 1997;**15**(6):547–51.
2. Bramswig KH, Knittelfelder R, Gruber S, Untersmayr E, Riemer AB, Szalai K, et al. Immunization with mimotopes prevents growth of carcinoembryonic antigen positive tumors in BALB/c mice. *Clin Cancer Res* 2007;**13**(21):6501–8.
3. Legutki JB, Nelson M, Titball R, Galloway DR, Mateczun A, Baillie LW. Analysis of peptide mimotopes of Burkholderia pseudomallei exopolysaccharide. *Vaccine* 2007;**25**(45):7796–805.
4. Lesinski GB, Westerink MA. Novel vaccine strategies to T-independent antigens. *J Microbiol Methods* 2001;**47**(2):135–49.
5. Mazzucchelli L, Burritt JB, Jesaitis AJ, Nusrat A, Liang TW, Gewirtz AT, et al. Cell-specific peptide binding by human neutrophils. *Blood* 1999;**93**(5):1738–48.
6. Scott JK, Smith GP. Searching for peptide ligands with an epitope library. *Science* 1990;**249**(4967):386–90.
7. Cheung NK, Kushner BH, Cheung IY, Kramer K, Canete A, Gerald W, et al. Anti-G(D2) antibody treatment of minimal residual stage 4 neuroblastoma diagnosed at more than 1 year of age. *J Clin Oncol* 1998;**16**(9):3053–60.
8. Handgretinger R, Anderson K, Lang P, Dopfer R, Klingebiel T, Schrappe M, et al. A phase I study of human/mouse chimeric antiganglioside GD2 antibody ch14.18 in patients with neuroblastoma. *Eur J Cancer* 1995;**31A**(2):261–7.
9. Simon T, Hero B, Faldum A, Handgretinger R, Schrappe M, Niethammer D, et al. Consolidation treatment with chimeric anti-GD2-antibody ch14.18 in children older than 1 year with metastatic neuroblastoma. *J Clin Oncol* 2004;**22**(17):3549–57.
10. Zeng Y, Fest S, Kunert R, Katinger H, Pistoia V, Michon J, et al. Anti-neuroblastoma effect of ch14.18 antibody produced in CHO cells is mediated by NK-cells in mice. *Mol Immunol* 2005;**42**(11):1311–9.

11. Becker R, Eichler MK, Jennemann R, Bertalanffy H. Phase I clinical trial on adjuvant active immunotherapy of human gliomas with GD2-conjugate. *Br J Neurosurg* 2002;**16**(3):269–75.
12. Chapman PB, Morrisey D, Panageas KS, Williams L, Lewis JJ, Israel RJ, et al. Vaccination with a bivalent G(M2) and G(D2) ganglioside conjugate vaccine: a trial comparing doses of G(D2)-keyhole limpet hemocyanin. *Clin Cancer Res* 2000;**6**(12):4658–62.
13. Bolesta E, Kowalczyk A, Wierzbicki A, Rotkiewicz P, Bambach B, Tsao CY, et al. DNA vaccine expressing the mimotope of GD2 ganglioside induces protective GD2 cross-reactive antibody responses. *Cancer Res* 2005;**65**(8):3410–8.
14. Forster-Waldl E, Riemer AB, Dehof AK, Neumann D, Bramswig K, Boltz-Nitulescu G, et al. Isolation and structural analysis of peptide mimotopes for the disialoganglioside GD2, a neuroblastoma tumor antigen. *Mol Immunol* 2005;**42**(3):319–25.
15. Kieber-Emmons T, Luo P, Qiu J, Chang TY OI, Blaszczyk-Thurin M, et al. Vaccination with carbohydrate peptide mimotopes promotes anti-tumor responses. *Nat Biotechnol* 1999;**17**(7):660–5.
16. Fest S, Huebener N, Weixler S, Bleeke M, Zeng Y, Strandsby A, et al. Characterization of GD2 peptide mimotope DNA vaccines effective against spontaneous neuroblastoma metastases. *Cancer Res* 2006;**66**(21):10567–75.
17. Riemer AB, Forster-Waldl E, Bramswig KH, Pollak A, Zielinski CC, Pehamberger H, et al. Induction of IgG antibodies against the GD2 carbohydrate tumor antigen by vaccination with peptide mimotopes. *Eur J Immunol* 2006;**36**(5):1267–74.
18. Wondimu A, Zhang T, Kieber-Emmons T, Gimotty P, Sproesser K, Somasundaram R, et al. Peptides mimicking GD2 ganglioside elicit cellular, humoral and tumor-protective immune responses in mice. *Cancer Immunol Immunother* 2008;**57**(7):1079–89.
19. Landgraf C, Panni S, Montecchi-Palazzi L, Castagnoli L, Schneider-Mergener J, Volkmer-Engert R, et al. Protein interaction networks by proteome peptide scanning. *PLoS Biol* 2004;**2**(1):E14.
20. Frank R. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports – principles and applications. *J Immunol Methods* 2002;**267**(1):13–26.
21. Wenschuh H, Volkmer-Engert R, Schmidt M, Schulz M, Schneider-Mergener J, Reineke U. Coherent membrane supports for parallel microsynthesis and screening of bioactive peptides. *Biopolymers* 2000;**55**(3):188–206.
22. Clackson T, Wells JA. In vitro selection from protein and peptide libraries. *Trends Biotechnol* 1994;**12**(5):173–84.
23. Huang W, Beharry Z, Zhang Z, Palzkill T. A broad-spectrum peptide inhibitor of beta-lactamase identified using phage display and peptide arrays. *Protein Eng* 2003;**16**(11):853–60.
24. Petter C, Scholz C, Wessner H, Hansen G, Henklein P, Watanabe T, et al. Phage display screening for peptidic chitinase inhibitors. *J Mol Recognit* 2008(8).
25. Hammarstrom L, Smith CI. IgG subclass changes in response to vaccination. *Monogr Allergy* 1986;**19**:241–52.
26. Ullenhag GJ, Frodin JE, Strigard K, Mellstedt H, Magnusson CG. Induction of IgG subclass responses in colorectal carcinoma patients vaccinated with recombinant carcinoembryonic antigen. *Cancer Res* 2002;**62**(5):1364–9.
27. Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. *Nature* 2005;**438**(7066):364–8.