RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma elicits immunological and clinical responses

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ABSTRACT

The receptor for hyaluronic acid mediated motility (RHAMM) is an antigen eliciting both humoral and cellular immune responses in patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and multiple myeloma (MM). We initiated a phase I clinical trial vaccinating ten patients four times at a biweekly interval with R3 (ILSLELMKL), a highly immunogenic CD8+ T cell epitope peptide derived from RHAMM. In 7/10 patients, we detected an increase of CD8+/HLA-A2/RHAMM R3 tetramer+/CD45RA+/CCR7-/CD27-/CD28- effector T cells in flow cytometry in accordance with an increase of R3-specific CD8+ T cells in enzyme linked immunospot (ELISpot) assays. In chromium release assays, a specific lysis of RHAMM-positive leukemic blasts was shown. 3/6 patients with myeloid disorders (1/3 AML, 2/3 MDS) achieved clinical responses: one patient with AML and one with MDS showed a significant reduction of blasts in the bone marrow three weeks after the last vaccination. One patient with MDS did not need any longer erythrocyte transfusions after four vaccinations. Two of four patients with MM showed a reduction of free light chain serum levels.

Taken together, RHAMM-R3 peptide vaccination induced both immunological and clinical responses, and therefore RHAMM constitutes a promising target for further immunotherapeutical approaches. This study is registered at http://ISRCTN.org as ISRCTN32763606 and is registered with EudraCT as 2005-001706-37.
INTRODUCTION

For patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and multiple myeloma (MM) different therapeutic options have been developed including risk-adapted polychemotherapy regimens, bortezomib, lenalidomide, 5-aza-2'-deoxycytidine and other novel drugs, as well as hematopoietic stem cell transplantation. Most of the patients with these diseases are not eligible for aggressive treatment options because of their age and co-morbidity. Moreover, a high percentage of them relapse after first-line therapy due to the persistence of residual tumor cells. Therefore, there is a fervent need for the development of novel treatment options. Targeted immunotherapies might constitute a synergistic therapeutic approach to eliminate minimal residual disease. Recent advances in the field of tumor immunology have resulted in the identification of a number of leukemia-associated antigens (LAAs): in leukemia patients, specific T cell responses of cytotoxic T-lymphocytes (CTLs) were detected against the Wilms’ tumor protein WT1, proteinase 3, the receptor for hyaluronic acid mediated motility (RHAMM) and further LAAs like PRAME, G250, BCL2, LAMR1, hTERT, survivin and FLT3-ITD. For two LAAs, i.e. WT1 and proteinase 3 clinical peptide vaccination trials have been initiated.

Earlier, we described the expression of RHAMM in more than 80% of AML/MDS, CML, CLL and MM patients. RHAMM is differentially expressed in tumor/leukemia cells, but not in peripheral blood mononuclear cells (PBMC) or CD34+ bone-marrow stem cells of healthy volunteers. RHAMM elicits both humoral and cellular immune responses in patients with different hematological malignancies and solid tumors. We defined a highly immunogenic CD8+ T cell epitope derived from RHAMM which was designated R3 (pos. 165-173: ILSLELMKL). R3-primed CD8+ T cells from AML patients were able to lyse autologous AML blasts expressing RHAMM. Because of these favorable characteristics of RHAMM, we initiated this phase I/II R3 peptide vaccination trial for HLA-A2 patients with AML, MDS or MM overexpressing RHAMM to assess the safety and feasibility of this therapeutic approach. All patients in the present study had a residual or a slowly progressive disease, received four subcutaneous R3 peptide vaccinations and were evaluated for the safety of the therapy as well as for their immunological and clinical response.
MATERIAL AND METHODS

Samples from patients with AML, MDS and MM

All samples were taken from patients treated in this clinical study approved by the Ethikkommission of the University of Ulm (EudraCT number: 2005-001706-37). Informed consent was obtained from all patients. Peripheral blood mononuclear cells (PBMC) from AML patients were prepared by Ficoll (Biochrom, Berlin, Germany) separation and stored for RNA-preparation at –80°C. For cellular assays, Ficoll separated PBMC were tested freshly or cryopreserved in AB serum (IKT, Ulm, Germany) containing 10% DMSO (Sigma Aldrich, Steinheim, Germany) and stored in liquid nitrogen.

Study design

Patients with positive HLA-A2 and RHAMM expression on malignant cells but with a limited tumor load were included. 300 mg RHAMM R3 peptide (ILSLELMKL, Merck Biosciences/Clinalpha, Laufingen, Switzerland) emulsified with the incomplete Freund’s adjuvant (ISA-51, Montanide (Seppic, Paris, France) on day 3 as well as GM-CSF (Leukine, Berlex, Richmond, CA) on days 1-5 was administrated four times subcutaneously at a biweekly interval. The primary aim of this phase I clinical trial was to test the safety and feasibility of this peptide vaccination, secondary aims were the evaluation of a specific T cell immune response to RHAMM R3 peptide and the assessment of the hematological status before and after R3 peptide vaccination.

Inclusion criteria

Diagnosis of AML, MDS and MM. For AML: up to 25% blasts in the bone marrow (BM), MDS: up to 20% blasts in the BM (RA, RAEB 1, RAEB 2); MM: partial remission or near complete remission after high-dose chemotherapy with melphalan and autologous stem cell transplantation: immunofixation still positive, free light chains in serum and/or urine were detectable. HLA-A2 expression and expression of RHAMM-mRNA in BM or peripheral blood were prerequisites for inclusion. Patients with AML and MM should have received at least one standard therapy for this hematological malignancy before they were put on peptide vaccination. No administration of chemotherapy or radiotherapy within the last 12 weeks prior to RHAMM-R3 vaccination.
Detection of clinical responses

For all patients the BM and blood were analyzed before and after vaccination using microscopy and standard FACS analysis\textsuperscript{29}. Patients with MM were also examined for quantitative immunoglobulins and quantitative free light chains in serum and urine\textsuperscript{30}. The frequency of erythrocyte and platelet transfusions and the course of differential blood count were documented.

Response criteria were as following:

For patients with AML, the criteria by the World Health Organization (WHO)\textsuperscript{31} and the International Working Group (IWG)\textsuperscript{32} were followed as specified: CR: Reduction of blasts in the BM to less than 5%, in peripheral blood count: hemoglobin greater than 11 g/dl, neutrophils 1,500/mm\textsuperscript{3} or more, platelets 100,000/mm\textsuperscript{3} or more. PR: Reduction of blasts in the BM of more than 50%, in peripheral blood count: hemoglobin greater than 11 g/dl, neutrophils 1,500/mm\textsuperscript{3} or more, platelets 100,000/mm\textsuperscript{3} or more. SD: no CR or PR. PD (progressive disease): Increase of blasts in the BM by more than 50% or increase of WHO-classification or progress of transfusion requirements.

For patients with MDS, the criteria by the WHO\textsuperscript{31} and the IWG\textsuperscript{33} were followed as specified: CR: a complete response was defined as a normocellular BM with less than 5% blasts with normal maturation of all cell lines, with no evidence of dysplasia. In PB: hemoglobin greater than 11 g/dl, neutrophils 1,500/mm\textsuperscript{3} or more, platelets 100,000/mm\textsuperscript{3} or more. PR: blasts decreased by 50% or more over treatment or a less MDS WHO classification than pretreatment. HI (hematological improvement): an improvement was defined as a decrease of at least 50% transfusion requirements, together with at least an improvement of one to two cell lineages of the peripheral cell counts but not enough to qualify for a PR. SD: Failure to achieve at least a HI, but no evidence of progression for at least two months. PD: Increase of blasts in bone-marrow of more than 50% or increase of WHO-classification or progress of transfusion requirements.

For patients with MM, the International Uniform Response Criteria according to Durie et al. were applied\textsuperscript{34}: stringent complete remission, sCR: CR as defined below plus normal free light chain ratio and absence of clonal cells in the BM by immunohistochemistry or immunofluorescence. CR: negative immunofixation in the serum and urine. Very good partial response, VGPR: Serum and urine M-protein detectable by immunofixation but not on electrophoresis or 90% or greater reduction
in serum M-protein plus urine M-protein < 100 mg per 24 h. PR: >=50% reduction of serum M-protein and reduction in 24-h urinary M-protein by >=90% or to <200 mg per 24 h. SD: no CR or PR. PD: Increase of free light chains in serum or urine or of clonal plasma cells in bone-marrow of more than 25%.

**Assessment of toxicity of R3-peptide vaccination**

Side effects were documented according to Common Terminology Criteria for Adverse Events v3.0 (CTCAE; [http://ctep.cancer.gov](http://ctep.cancer.gov)). Before and three weeks after the fourth vaccination physical examination, body weight, ECOG performance score, laboratory tests (kidney and liver function tests, electrophoresis, electrolytes, CRP, LDH, and coagulation tests), chest x-ray, echocardiography, electrocardiography, urine analysis, abdominal sonography and bone-marrow aspiration was performed. For patients with MM additionally quantitative immunoglobulines and quantitative assessment of free light chains in serum and urine were tested. Before each vaccination physical examination, laboratory tests (WBC, differential blood count, kidney and liver function tests, electrolytes, CRP, LDH, coagulation tests) and urine analysis were performed. To detect autoimmune reactions, we measured TSH, fT3, fT4, MAK and TAK for autoimmune thyreoiditis, as well as ANCA, ANA and rheumatic factor for rheumatic disorders.

**Conventional RT-PCR**

mRNA was prepared from PBMC or tumor samples by using mRNA QuickPrep Micro purification kits (Amersham Pharmacia Biotech, Little Chalfont, England, UK). 2.0 µg of each mRNA sample was subjected to cDNA synthesis (Superscript II Gibco BRL, Frederick, Maryland). PCR for RHAMM was performed as described\(^\text{13}\) using the indicated conditions and ingredients.

**HLA-A2-Typing**

Flow cytometry was performed using an HLA-A2 antibody (BD, Heidelberg, Germany). Patient cells, the T2 cell line as a positive control and the K562 cell line as negative control were stained with the HLA-A2 antibody. After incubation at 4°C for 20 min in the dark and washing twice, stained cells were analyzed by flow cytometry.
Enzyme linked immunosorbent assay (ELISA)

ELISAs were performed to assess the level of interleukin-2 (IL-2) and interleukin-10 (IL-10) in all patients’ sera samples before and three weeks after four vaccinations using ELISA kits according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA). All patients’ sera were also subjected to ELISA assays for the whole RHAMM protein and the R3 peptide following the method described earlier35,36.

Mixed lymphocyte peptide culture (MLPC)

PBMC from healthy volunteers or patients were separated by Ficoll and subsequently selected by magnetic beads through a MACS column (Miltenyi, Bergisch-Gladbach, Germany). More than 95% purity was reached in the CD8+ fraction as assessed by flow cytometry analysis (data not shown). MLPC was performed for IMP and R3 as described earlier13.

IFN-γ and Granzyme B ELISpot assays

IFN-γ and granzyme B ELISpot assays were performed as previously described13 to determine specific lysis of RHAMM (peptide) positive target cells according to the manufacturer’s instructions (BD, San Diego, USA). We participated in an inter-laboratory test for ELISpot assays37.

Tetramer staining

The frequency of R3 specific CD8+ T lymphocytes was determined after eight days’ MLPC by staining with anti-CD8 antibody and HLA-A2/R3 tetramer PE. HLA-A2/R3 tetramerPE was synthesized at the Lausanne Branch of the Ludwig Institute for Cancer Research. CD8+ T lymphocytes (0.5-1×10^6) stimulated with irradiated CD8-APCs in the presence of the R3 peptide were stained with HLA-A2/R3 tetramer PE 1 µg per test with respect to the peptide-MHC class I component in the dark and incubated for 40 minutes at room temperature. Thereafter, for four-color staining, 10 µl CD8 PerCP, 10 µl CD45RA FITC and 5 µl CCR7 APC (BD, Heidelberg, Germany) were added at 4°C for 20 minutes in the dark. As for six-color staining, the cells were stained with 1 µg HLA-A2/R3 tetramer PE and HLA-A2/WT1 tetramer PerCP per test and incubated for 40 minutes at room temperature in the dark. Thereafter 5 µl CD8 APC-Cy7, 5 µl CD45RA APC (Invitrogen, Caltag, CA, USA), 10 µl CCR7 PE-Cy7, 10
µl CD27 FITC or CD28 FITC (BD, Heidelberg, Germany) were added at 4°C for 20 minutes in the dark. After washing once with PBS, stained cells were fixed with 1% formaldehyde (Sigma, Germany) and then analyzed by flow cytometry. Whenever possible, at least 100,000 events were collected for analysis. Each sample was run with an appropriate isotype control to define the gate of positive cells. Analysis was performed on tightly gated lymphocytes to exclude dead cells and debris and on CD8+ T lymphocytes to evaluate responses to R3 peptide. Samples were defined as “tetramer positive” in case of an increase of specific R3-tetramer+/CD8+ T cells of more than 50% (if initial count was ≤ 0.1%), or 25% increase (if initial count was >0.1%). We participated in an inter-laboratory test for tetramer flow cytometry assays.

Chromium-51 release cytotoxicity assay

RHAMM+/HLA-A2+, RHAMM+/HLA-A2- and RHAMM-/HLA-A2+ AML blasts, T2 cells pulsed with 20 µg/ml RHAMM-derived peptide R3 and K562 cells were labelled with 51CrO4 (Cr-51) for 2 h as described earlier. After washing, labelled cells were incubated with CD8 positive T cells at effector-target ratios of 5:1 to 2.5:1 for 16 h at 37 °C. Radioactivity in the supernatant from all wells of round-bottom 96-well plates (Becton Dickinson, Heidelberg, Germany) was measured by a gamma counter (PerkinElmer, Boston, MA, USA). The percentage of specific lysis was calculated as (Cr-51 release in the test well minus spontaneous Cr-51 release) / (maximum Cr-51 release minus spontaneous Cr-51 release).

Analysis of regulatory T cells (Tregs)

Staining of PMBC of the patients before, under and after vaccination was performed using the following fluorescence labeled monoclonal antibodies: phycoerythrin (PE)-Cy7-conjugated anti-CD4 (BD Biosciences), allophycocyanin (APC)-Cy7-conjugated anti-CD25 (BD Biosciences), and intracellular fluorescein isothiocyanate (FITC)-conjugated anti-Foxp3 (eBioscience, Kranenburg, Germany) with the appropriate normal isotype-matched control IgGs. For extracellular staining, cells were incubated for 30 minutes at 4°C with optimal dilution of each antibody. For intracellular staining, the cells were fixed with Reagent A and permeabilized with Reagent B (IntraStain, DakoCytomation, Germany). The cells were analyzed on a FACSARia™ flow cytometer (Becton Dickinson) using the CellQuest software (Becton Dickinson).
RESULTS

Patients’ characteristics

All ten patients included in the present study expressed both RHAMM and HLA-A2 as assessed by conventional PCR and flow cytometry. Cytogenetic and clinical characteristics of these patients are listed in Table 1a.

Toxicity of peptide vaccination

Ten patients (3 AML, 3 MDS and 4 MM) were enrolled and completed the course of four vaccinations. Only mild side effects like CTC I° erythema and induration of the skin at the site of injection were observed after peptide vaccination using the RHAMM-R3 peptide. One patient developed an increase in body temperature from 36.5°C to 38.0°C that persisted for several hours after the first vaccine administration, but this symptom was not observed after subsequent vaccinations. There was no other therapy-related toxicity. No clinical signs or laboratory results for vitiligo, autoimmune thyreoiditis or rheumatic disorders were observed.

Immunological Responses

To screen the balance of type 1 and type 2 T cell cytokines, we measured the levels of IL-2 and IL-10 in the sera of all patients before and three weeks after four vaccinations. IL-10 levels ranging from 10 to 20 pg/ml were detectable by high-resolution ELISA and remained stable in all patients during the period of vaccination, while IL-2 levels were rather dynamic as shown in Figure 1. High increases in IL-2 starting from levels at 50 pg/ml up to the five-fold was seen in patients #1,2,6 and 10, while IL-2 levels remained stable or even decreased in the rest of the patients.

As for cellular immune responses, we detected in the peripheral blood of the ten patients, a significant increase of specific CD8+ T cells recognizing the R3 peptide in 8/10 patients by ELISpot analysis as well as in 7/10 patients by tetramer staining. The ELISpot data of these 10 patients for IFNγ and granzyme B secretion are summarized in Table 1a and the tetramer staining results in Table 1b. The results of ELISpot assays were considered to be positive when an increase of more than 50% of spots was seen in the course of vaccination. A positive immunological response for tetramer staining was defined as an increase >50% of HLA-A2/R3-
tetramer+/CD8+ T lymphocytes <0.1% prior to vaccination and with an increase >25% of HLA-A2/R3-tetramer+/CD8+ T lymphocytes >0.1% prior to vaccination. FACS data combined with ELISpot results of two patients are exemplarily shown. Figure 2 presents an increase of the frequency of HLA-A2/R3-tetramer+/CD8+ T lymphocytes from 0.05% to 0.11% with up to 85% of these cells being CD8+/HLA-A2/R3 tetramer+/CCR7-/CD45RA+ effector T cells and a transient increase of the R3-specific secretion of IFNγ during the course of vaccination of this AML patient. Figure 3 displays a six-color staining for R3-specific CD8+ T lymphocytes with an increase of CD8+/HLA-A2/R3 tetramer+ T lymphocytes from 0.55% to 1.23% after three vaccinations and an increase of granzyme B secretion over the time of vaccination and decrease after cessation of vaccination of this patient with MM. In the course of vaccination the frequency of CD8+/HLA-A2/R3 tetramer+/WT1 tetramer-/CCR7-/CD45RA+/CD27- effector T cells rose up to almost 80% and the CD28- effector T cells increased up to 23%, decreased however three weeks thereafter.

Sera were collected from all patients before and after four vaccinations with the HLA class I peptide R3. Neither class G immunoglobulins (IgG) against the whole protein of RHAMM nor specific IgG against the vaccination peptide R3 could be detected.

**Generation of R3-specific cytotoxic CD8+ T lymphocytes**

By FACS analysis we detected an increase of CD8+ effector T cells (Table 1b, Figures 2 and 3) which secreted IFNγ as a marker of activation and released granzyme B (Table 1a, Figures 2 and 3) demonstrating their lytic potential. When we tested these CD8+ T cells in a standard chromium-51 release assay, they were able to lyse 80% of autologous AML blasts at an effector/target (E/T) ratio of 5:1 while allogeneic AML blasts lacking either RHAMM or HLA-A2 expression were not recognized beyond background level. T2 cells pulsed with the R3 peptide were used as a positive control for R3-specific lysis. Absence of lysis of RHAMM positive, but HLA-A2 negative K562 cells served as a negative control, and demonstrated that the lysis was not mediated through NK cells (Figure 4). Due to restrictions in T cells isolated from the peripheral blood and malignant cells of vaccinated patients, this assay could only performed for three patients, but with similar results. Figure 4 shows a representative result obtained in patient #1 with an AML.
Furthermore, we assessed changes in the frequency of CD4+/CD25hi/CD127dim/FoxP3+ regulatory T cells (Tregs) in the peripheral blood from four patients (#2, 5, 6 and 10) before, under and after R3 peptide vaccination. In all cases, the frequency of Tregs was rather moderate. Only in one patient (#2), we detected a decrease under vaccination followed by an increase after the end of vaccinations (Figure 5).

**Clinical responses to R3 peptide vaccination**

Clinical responses were assessed by the examination of peripheral blood and bone-marrow samples before and after vaccination. For MM patients, we additionally assessed immunofixation, serum free light chains and quantitative serum immunoglobulins. Three out of six patients with myeloid disorders (1 AML, 2 MDS) showed a reduction of CD33+/HLA-DR+ cells in FACS analysis of the bone-marrow after four vaccinations. One patient with MDS did not need any further erythrocyte substitution after four vaccinations. Two patients with MM showed a reduction of plasma cells and of β2-microglobulin in the bone-marrow as by FACS analysis and of free light chains in the serum and/or urine. One patient with AML and one patient with MM developed a progress of their disease.

The data on immunological and hematological responses are summarized for all patients in Tables 1a and 1b.
DISCUSSION

In the present study, ten patients with hematological malignancies expressing both RHAMM and HLA-A2 were vaccinated with the RHAMM-derived peptide R3 subcutaneously four times at a biweekly interval. All patients concluded the course of four vaccinations. No adverse events greater than CTC I° skin toxicity could be observed which is in accordance with the results in numerous peptide vaccination trials performed in patients with solid tumors following the concept of emulsification of the peptide as the core of the vaccine in incomplete Freund’s adjuvant (IFA) and concomitant administration of granulocyte-macrophage colony stimulating factor (GM-CSF). The emulsification in IFA seems to be essential to prevent the peptide from rapid degradation in the subcutis. GM-CSF has been shown to be a potent enhancer of a T cell response to the vaccination peptide and might enhance the maturation of hematopoietic cells. We saw a transient increase in WBC count and platelet count in most of our patients during the time of vaccination. One might speculate that GM-CSF contributes to the maturation of blasts as hematopoietic precursor cells. However, long-lasting effects were seen in the bone marrow while peripheral blood counts went back to the previous level.

The immunomonitoring of the patients in our study was performed using ELISA, six-colour flow cytometry and two-parametric ELISpot assays for the secretion of IFNγ and granzyme B. A good concordance of these three read-out systems was noted (Table 1a+b). To screen type 1 and type 2 T cell responses after vaccination, we measured IL-2 and IL-10 levels in the sera of the patients before and three weeks after four vaccinations. While IL-10 levels remained at a rather low level over the time of vaccination, we detected an increase of IL-2 up to the five-fold of the initial levels in four of ten patients. Interestingly, these four patients showed also a clinical response. R3-specific CD8-T cells were defined by positive staining with a R3*tetramer and the failure of counterstaining with WT1*tetramer. Seven of ten vaccinated patients (70%) showed an increase of RHAMM-R3-specific T cells in tetramer assays, 8/10 (80%) in ELISpot assays. The number of effectors cells characterized to be CD8+/R3 tetramer+/WT1 tetramer-/CCR7-/CD27-/CD28-/CD45RA+ T cells increased over the time of vaccination (Figures 2 and 3). Interestingly, a decrease in this subpopulation could be observed in three patients after vaccination was stopped (Table 1b, Figure 3). Moreover, for
CD4+/CD25hi/CD127dim/FoxP3+ regulatory T cells (Tregs) also a countercurrent fluctuation could be observed (Figure 5, pat. #2). This finding might be an important point to extend the initial series of four vaccinations in our present trial to additional boost vaccinations at a longer interval in future trials as described in the case report by Mailander et al.\textsuperscript{21}. Administration of CD4+ helper T cells epitopes derived from RHAMM or the rather unspecific CD4+ T cell stimulator keyhole limpet hemocyanine (KLH) used by the same group\textsuperscript{21} or other adjuvants such as CPG-rich oligodinucleotides\textsuperscript{40} might help to induce more long-lasting vaccination results. In chromium-51 release cytotoxicity assays, the lysis of 40% of AML blasts at an E/T ratio of 10:1 could validate the functionality of the effector T cells characterized in flow cytometry and ELISpot analysis in the clinically responding patient. As monitored by ELISA, no increase of RHAMM-specific immunoglobulin class G antibodies could be detected during or after the vaccination. Vaccination with a HLA class I restricted peptide does not typically result in the induction of humoral immune responses\textsuperscript{38,39}. Vaccination with the whole protein or an additional class II epitope peptide might overcome that problem.

In this trial, we vaccinated patients with a residual or controlled disease as in this setting specific T cells were present but not affected by chemotherapy. Such T cells might better cope with a limited tumor load than in an AML patient with a high blast count. Five of ten patients in this stage showed a blast reduction in the BM (AML/MDS) or a decrease of the serum or urine level of free light chains (MM) three weeks after the last peptide vaccination. These positive clinical results are in accordance with observations in other clinical peptide vaccination trials\textsuperscript{21-23}. Overall, a good correlation between immunological and clinical responses was observed which suggests RHAMM to constitute an important factor in the proliferative process of leukemia and myeloma. Involvement of RHAMM in the formation of the mitotic spindle apparatus, the ras-raf signal transduction and metastasis have been described\textsuperscript{41}.

By targeting a single leukemia-associated antigen by peptide vaccination, one might cover only a part of the total patient cohort. In a recent study\textsuperscript{14}, we found a better survival in AML patients expressing at least one of the three antigens G250, PRAME and RHAMM, when compared with AML patients without expression of any of these antigens. Based on our encouraging results reported here using monovalent
vaccination, we plan now a phase I/II-clinical polyvalent vaccination trial targeting the three antigens G250, PRAME and RHAMM.

In summary, we demonstrated in the present phase I clinical trial the safety and feasibility of a RHAMM-R3 peptide vaccination in patients with hematological malignancies. Both immunological and to some extent clinical responses could be observed characterizing RHAMM as a promising target for further immunotherapies.
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Author contributions
This study was designed by MS and JG. All research was performed by AS, MR, JC, KG, FF, YY and MG. The vaccine preparation was developed by DS. The manuscript was written by MS, AS and JG. MR, RS, PL and MH supplied patient material and discussed the manuscript. PG synthesized the tetrameric complexes. GR, DS, SG, DB, HS and HD discussed the manuscript.

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The authors declare no competing financial interests.
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TABLE LEGENDS

Table 1a: Synopsis of patients' characteristics, clinical and immunological responses. Ten patients (3 AML, 3 MDS, and 4 MM) have completed the course of four vaccinations. Tetramer staining data of these patients are further specified in Table 1b.

Table 1b: RHAMM-R3-tetramer CD8+ T cells in the peripheral blood of the patients during the course of vaccination. The percentage of HLA-A2/R3-tetramer*PE positive CD8+ T lymphocytes of each patient during the course of vaccination is indicated. Peripheral blood was taken one to two weeks before the first vaccination and is indicated two weeks after each vaccination respectively. n.t.: not tested.
<table>
<thead>
<tr>
<th>#</th>
<th>Diagnosis</th>
<th>Age (yrs)</th>
<th>WHO classification; Karyotype</th>
<th>Therapy before vaccination</th>
<th>Status at vacc.</th>
<th>Period of vacc.</th>
<th>Level of IL-2 in ELISA</th>
<th>CD8+ tetra+ (§)</th>
<th>ELISpot IFNγ Before / After</th>
<th>ELISpot GrB Before / After</th>
<th>Malignant cells (FACS)</th>
<th>Clinical findings</th>
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<td>12/04-01/05</td>
<td>Up</td>
<td>Pos 0 77</td>
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<td>Reduction of blasts by 93% in the BM*; relapse at +23 months</td>
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<tr>
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<td>RAEB I; normal karyotype IPSS Int-1</td>
<td>VACI 05/2004, PR</td>
<td>PR</td>
<td>02/05-03/05</td>
<td>Up</td>
<td>Pos 5 16</td>
<td>11 19 8</td>
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<tr>
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<td>03/05-04/05</td>
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<td>5</td>
<td>MDS</td>
<td>80</td>
<td>RCMD; not done IPSS Int-1</td>
<td>Weekly erythrocyte and platelet transfusions</td>
<td>RCMD</td>
<td>05/05-06/05</td>
<td>Down</td>
<td>n.t. 32</td>
<td>35 2 1</td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>MDS</td>
<td>81</td>
<td>RA; 45.X.-Y IPSS Low risk</td>
<td>Erythrocyte transfusions</td>
<td>RA</td>
<td>05/05-06/05</td>
<td>Up</td>
<td>Pos 0 49</td>
<td>n.t. n.t. 1.5 0.5</td>
<td>Cessation of transfusions (+22 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>MM</td>
<td>67</td>
<td>IgG kappa, stage IIIA del13q14 β2-MG 4.5 mg/l</td>
<td>DSMM II, auto TX 07+09/2004</td>
<td>PD β2MG:</td>
<td>06/05-07/05</td>
<td>Stable</td>
<td>NC</td>
<td>0 20</td>
<td>10 44 n.t. n.t.</td>
<td>PD</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>MM</td>
<td>51</td>
<td>IgG kappa stage IIIA; no del13q14 β2-MG 1.27 mg/l</td>
<td>DSMM V, auto TX</td>
<td>VGPR</td>
<td>06/05-07/05</td>
<td>Stable</td>
<td>Pos 0 5</td>
<td>29 56 1.5</td>
<td>1</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AML</td>
<td>44</td>
<td>AML without maturation; complex karyotype; FLT3-ITD negative, NPM1 wildtype</td>
<td>2xICE, 1xHAM, auto TX</td>
<td>PR</td>
<td>06/05-08/05</td>
<td>Down</td>
<td>Pos 3 0</td>
<td>0 14 6 7</td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>MM</td>
<td>70</td>
<td>IgG kappa, stage IIIA; no del13q14 β2-MG 4.4 mg/l</td>
<td>DSMM II, auto TX</td>
<td>VGPR β2MG:</td>
<td>07/05-08/05</td>
<td>Up</td>
<td>Pos 0 84</td>
<td>0 7</td>
<td>0.15 0.02</td>
<td>Reduction of kappa-LC*; but PD at +4 months</td>
<td></td>
</tr>
</tbody>
</table>
**Table legend/Abbreviations used:**

Age means age at the time of vaccination therapy. DSMM II/V (protocols II and V of the German Study Group for Multiple Myeloma): ID, IEV, Mel, double-auto/auto-allo-stem cell transplantation; ID: Idarubicin/Dexamethason; IEV: Ifosfamid/Epirubicin/Etoposide; Mel: Melphalan; LC: Serum free light chains; bef. vacc.: before vaccination; Pos: increase; n.t.: not tested; response criteria according to ref. 36-39: CR: complete remission, PR: partial remission, NC: no change, PD: progressive disease; RCMD: refractory cytopenia with multilineage dysplasia; HiDAC: high dose arabinoside C; VACI: valproic acid, arabinosids C, idarubicin; TX: stem cell transplantation; w/o: without; BM: bone marrow; *: assessed three weeks after the last vaccination; RA: refractory anemia; RAEB: with excess of blasts; IPSS: International Prognostic Scoring System; VGPR: very good partial response; ß2MG: beta-2 microglobulin.

The ELISpot numbers indicated in the table were calculated as follows: Before: R3-specific spots minus background spots before vaccination, Max: maximum difference of R3-specific spots minus background spots during or after vaccination, w/o: without, § see also extra table for tetramer assay results in detail.
Table 1b: Tetramer staining of the ten vaccinated patients in this trial

<table>
<thead>
<tr>
<th>Patient</th>
<th>Before 1. vaccination</th>
<th>After 1. vaccination</th>
<th>After 2. vaccination</th>
<th>After 3. vaccination</th>
<th>After 4. vaccination</th>
<th>Immune response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>n.t.</td>
<td>0.03</td>
<td>0.06</td>
<td>0.11</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>0.54</td>
<td>0.46</td>
<td>0.51</td>
<td>0.73</td>
<td>0.63</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>0.46</td>
<td>0.65</td>
<td>0.42</td>
<td>0.7</td>
<td>n.t.</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>0.48</td>
<td>n.t.</td>
<td>0.48</td>
<td>0.52</td>
<td>n.t.</td>
<td>No change</td>
</tr>
<tr>
<td>5</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>6</td>
<td>0.33</td>
<td>0.28</td>
<td>0.29</td>
<td>0.42</td>
<td>0.31</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>0.16</td>
<td>0.13</td>
<td>0.16</td>
<td>0.15</td>
<td>0.13</td>
<td>No change</td>
</tr>
<tr>
<td>8</td>
<td>0.55</td>
<td>0.41</td>
<td>0.40</td>
<td>1.23</td>
<td>0.46</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>0.06</td>
<td>0.14</td>
<td>0.13</td>
<td>0.09</td>
<td>0.11</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>0.28</td>
<td>0.53</td>
<td>0.47</td>
<td>0.38</td>
<td>0.56</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Immunological response:** initial value <0.1%: judged positive, if consecutive increase >50%,
initial value >0.1%: judged positive, if consecutive increase >25%.
n.t. = not tested, i.e. not sufficient cells for tetramer staining (apart from ELISpot assays)
FIGURE LEGENDS

Figure 1:
Changes in the cytokine milieu of the peripheral blood from patients before and after vaccination. To screen type 1 and type 2 T cell activation, we measured the levels of interleukin-2 and -10 (IL-2 and IL-10) in the sera of all ten patients before and three weeks after the last of four RHAMM-R3 peptide vaccinations. While IL-10 levels remained rather low, an up to five-fold increase in IL-2 levels as well as stable or decreasing levels for the cytokine were observed. Patient numbers indicated below the columns refer to the patient numbers in Table 1.

Figure 2:
Immunological and clinical responses of patient #1 with acute myeloid leukemia to the course of RHAMM-R3 peptide vaccination. (a) The FACS dot plots show the percentage of HLA-A2/R3-tetramer+/CD8+ T lymphocytes. The frequency of CD8+/HLA-A2/R3-tetramer+ T lymphocytes increased after four vaccinations (A – before, B – after second, C – after third, D – two weeks after fourth / last vaccination). The HLA-A2/R3-tetramer*PE positive CD8+ T cells were further analyzed for their expression of CCR7 and CD45RA. Most of the cells (70-85%) revealed to be CD8+/HLA-A2/R3 tetramer+/CCR7-/CD45RA+ effector T cells. (b) The frequency of R3-specific CD8+ T lymphocytes increased during the course of vaccination as assessed by ELISpot assays for IFN-γ and granzyme B release. All assays were performed in triplicate. Error bars indicate the SD. (c) Monitoring of malignant cells correlated with the other findings showing a decrease of CD33+/HLA-DR+ malignant cells during vaccination.

Figure 3:
Immunomonitoring for R3-specific CD8+ T lymphocytes in patient #8 with multiple myeloma. (a) The dot plots show the percentage of HLA-A2/R3-tetramer+/CD8+ T lymphocytes. Six-color staining for R3-specific CD8+ T lymphocytes revealed an increase of CD8+/ HLA-A2/R3-tetramer+ T lymphocytes over four vaccinations and a decrease thereafter (A – before, B – after first, C – after second, D – after third, E – three weeks after fourth / last vaccination). The HLA-A2/R3-tetramer*PE positive CD8+ T lymphocytes could not be counterstained by
WT1-tetramers demonstrating their specificity for the peptide R3. The staining for CCR7 and CD45RA demonstrated an increase of CD8+/HLA-A2/R3 tetramer+/WT1 tetramer-/CCR7-/CD45RA+ effector T cells from 47% to 81%. The frequency of CD8+/HLA-A2/R3 tetramer+/WT1 tetramer-/CCR7-/CD45RA+/CD27- effector T cells rose from 63% up to 78%. The CD8+/HLA-A2/R3 tetramer+/WT1 tetramer-/CCR7-/CD45RA+/CD28- effector T cells increased over four vaccinations, but decreased three weeks thereafter. (b) The results of the ELISpot assay for granzyme B correlated with the FACS data showing an increase and subsequent decrease of R3-specific CD8+ T lymphocyte function during the course of vaccination. All assays were performed in triplicate. Error bars indicate the SD.

**Figure 4:**

Chromium release assay for the verification of R3-specific cytotoxic activity of CD8+ T lymphocytes in patient #1 with acute myeloid leukemia after the course of vaccination. A 80% specific lysis of HLA-A2+/R3+ AML blasts by cytotoxic CD8+ T lymphocytes was achieved at a ratio of 5:1 (effector:target cell ratio), 46% of HLA-A2+ T2 cells pulsed with R3 peptide were lysed at this E/T ratio. Allogeneic HLA-A2neg/R3+ AML blasts, HLA-A2+/R3neg AML blasts, as well as HLA-A2neg/R3+ K562 cells were only lysed at background level.

**Figure 5:**

Changes of regulatory T cells in the peripheral blood.

Flow cytometry was performed on PBMC. For details see the Material and Methods section. Regulatory T cells (Tregs) were defined as CD4, CD25hi and FoxP3 co-expressing cells. The graph gives the frequency of Tregs as percent of all CD4+ T cells in the respective sample at different time points before, during and after R3 peptide vaccination in four patients.
Figure 1

ELISA for IL-2

IL-2 (pg/ml)

Before vaccination
After vaccination

ELISA for IL-10

IL-10 (pg/ml)

Before vaccination
After vaccination
Figure 2

(a) Flow cytometry histograms showing the percentage of CD8+ T-cells expressing CD45RA, CCR7, and the R3 tetramer. The histograms are labeled A, B, C, and D.

(b) Bar graphs showing the number of spots / 10,000 CD8+ T-cells for Interferon γ and Granzyme B with bars for R3 peptide in blue and irrelevant peptide in light blue.

(c) Flow cytometry plots before and after vaccination for CD10, CD33, and HLA-DR with percentages indicated for each quadrant.
Figure 3

(a) Flow cytometry dot plots showing the percentage of CD8+ T-cells expressing R3 tetramer and CD45RA, CD27, and CD28. The plots are labeled A, B, C, D, and E, respectively.

(b) Bar graph showing the number of spots per 10,000 CD8+ T-cells for No pep, IMP, R3, and Irr. pep conditions. The graph includes data for samples 1 to 5.
Figure 4

Chromium release assay

% specific lysis

AML RHAMM+ HLA-A2+
T2 + RHAMM/R3 peptide
K562(RHAMM+ HLA-A2-)
AML RHAMM- HLA-A2+
AML RHAMM+ HLA-A2-
Figure 5

% CD4+CD25hiCD127dimFoxP3+ cells

- Patient # 2
- Patient # 5
- Patient # 6
- Patient # 10

(before) after 3rd vaccination | 3 weeks after 4th vaccination | 3 months after 4th vaccination

0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0
RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma elicits immunological and clinical responses

Michael Schmitt, Anita Schmitt, Markus T Rojewski, Jinfei Chen, Krzysztof Giannopoulos, Fei Fei, Yingzhe Yu, Marlies Gotz, Marta Heyduk, Gerd Ritter, Daniel E Speiser, Sacha Gnjatic, Philippe Guillaume, Mark Ringhoffer, Richard F Schlenk, Peter Liebisch, Donald Bunjes, Hiroshi Shiku, Hartmut Dohner and Jochen Greiner