High-dose RHAMM-R3 peptide vaccination for patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma

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ABSTRACT

Background
Recently, we demonstrated immunological and clinical responses to a RHAMM-R3 peptide vaccine in patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma. To improve the outcome of the vaccine, a second cohort was vaccinated with a higher dose of 1,000 μg peptide.

Design and Methods
Nine patients received four vaccinations subcutaneously at a biweekly interval. Immunomonitoring of cytotoxic $CD8^+$ as well as regulatory $CD4^+$ T cells was performed by flow cytometry as well as by enzyme-linked immunospot (ELISpot) assays. Parameters of clinical response were assessed.

Results
In 4 of 9 patients (44%) we detected positive immunological responses. These patients showed an increase of $CD8^+$RHAMM-R3_tetramer$^+$/ $CD45RA^+$/$CCR7^-$/$CD27^-$/$CD28^-$ effector T cells and an increase of R3-specific CD8+ T cells. Two of these patients showed a significant decrease of regulatory T cells (Tregs). In one patient without response Tregs frequency increased from 5 to 16%. Three patients showed clinical effects: one patient with myelodysplastic syndrome RAEB-1 showed a reduction of leukemic blasts in the bone marrow, another myelodysplastic syndrome patient an improvement of peripheral blood counts and one patient with multiple myeloma a reduction of free light chains. Clinical and immunological reactions were lower in this cohort than in the 300 μg cohort.

Conclusions
High-dose RHAMM-R3 peptide vaccination induced immunological responses and positive clinical effects. Therefore, RHAMM constitutes a promising structure for further targeted immunotherapies in patients with different hematologic malignancies. However, higher doses of peptide did not improve the frequency and intensity of immune responses in this trial.
(This study is registered at http://ISRCTN.org as ISRCTN32763606)

Key words: acute myeloid leukemia, leukemia-associated antigens, receptor for hyaluronic acid mediated motility, RHAMM/CD168, epitope peptides, cancer vaccines.

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Introduction

In hematologic malignancies, several leukemia-associated antigens (LAAs) have been identified like BCL-2, OFA-iLRP, FLT3-ITD, G250, hTERT, PRAME, proteinase 3, RHAMM, survivin and WT-1. These LAAs have been demonstrated to induce specific T-cell responses and for some antigens also humoral immune responses.[1-3] LAAs are appropriate target structures in malignant hematologic diseases as these antigens are critically involved in different mechanisms for cell differentiation and proliferation like RHAMM, survivin or WT-1. Therefore, these antigens constitute appropriate target structures for anticancer treatment.[4] Among the LAAs RHAMM, proteinase 3 and WT-1 have been already tested in clinical peptide vaccination trials.[5-7]

The receptor for hyaluronic acid mediated motility (RHAMM) is an immunogenic antigen that is strongly expressed in several hematologic malignancies and induces humoral and cellular immune responses.[1,2,5,8,9] Specific T-cell responses against RHAMM showed a good correlation measured with different methods like ELISpot assays for IFN-γ and granzyme B, tetramer staining and also chromium release assays.[1,2] Therefore, a phase I/II R3 peptide vaccination for patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma overexpressing RHAMM was initiated. Patients were enrolled with RHAMM expression in their tumor cells but with a limited tumor load or a minimal residual disease. The first 10 patients were treated with 300 μg, showing clinical and immunological responses.[5] The study was therefore extended for further patients vaccinated with the increased dose of 1,000 μg R3 peptide. Moreover, patients with chronic lymphocytic leukemia were vaccinated with RHAMM-R3 peptide.[10] In the first 300 μg cohort, 70% of patients showed specific immune responses, but also positive clinical effects were detected in 5/10 of patients with myeloid disorders or multiple myeloma showing a reduction of blasts in the bone marrow or a reduction of free light chain serum levels, respectively. One patient with myelodysplastic syndrome did not need any further erythrocyte transfusions.[5]

In this study, patients with the diagnosis of acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma, as well as overexpression of RHAMM, were vaccinated four times subcutaneously with 1,000 μg RHAMM-R3 peptide emulsified in incomplete Freund's adjuvant. As a second adjuvant, granulocyte macrophage colony-stimulating factor (GM-CSF) was administrated.

Design and Methods

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

Patients with positive HLA-A2 and RHAMM expression on malignant cells but with a limited tumor load were included. Patients were administered 1,000μg RHAMM-R3 peptide (ILSLELMKL, Merck Biosciences/Clinalpha, Laufingen, Switzerland) emulsified with 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 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 hematologic status before and after R3 peptide vaccination

Patients with a diagnosis of acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma were included who fulfilled the following criteria: AML, up to 25% blasts in the bone marrow (BM); MDS, up to 20% blasts in the bone marrow (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 bone marrow or peripheral blood were prerequisites for inclusion.

Bone marrow blood of all patients was analyzed before and after vaccination using microscopy and standard FACS analysis.[11] Patients with multiple myeloma were also examined for quantitative immunoglobulins and quantitative free light chains in serum and urine.[12] The frequency of erythrocyte and platelet transfusions, and the course of differential blood count were documented.

Clinical responses were assessed as described earlier[5] following the criteria by the World Health Organization (WHO)[13] and the International Working Group (IWG).[14]

Side effects were documented according to Common Terminology Criteria for Adverse Events v3.0 (CTCAE; 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, ultrasound of the abdomen and bone marrow aspiration was performed. For patients with MM additionally quantitative immunoglobulins 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.

mRNA was prepared from peripheral blood mononuclear cells or tumor samples by using mRNA QuickPrep Micro purification kits (Amersham Pharmacia Biotech, Little Chalfont, England, UK). From each mRNA sample, 2.0 μg was subjected to cDNA synthesis (Superscript II Gibco BRL, Frederick, Maryland). Polymerase chain reaction for RHAMM was performed as described[2] using the indicated conditions and materials.

The mRNA expression of RHAMM was quantified by realtime RT-PCR using the light cycler SYBR Green I technology according to the manufacturer's instructions and the primers described.[3] The amount of mRNA of RHAMM was normalized to the housekeeping gene TATA-box binding protein (TBP) and copy numbers were calculated.[3]

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.

Peripheral blood mononuclear cells from healthy volunteers or

acute myeloid leukemia 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 previously described;[1,2] however, the number of days of cell culture could be reduced to eight days producing similar results.[5]

ELISpots were performed *ex vivo* on cultured patient cells and the T2 cell line as antigen presenting cells (APCs). Briefly, the irradiated $CD8^-$ fraction of autologous patient peripheral blood mononuclear cells pulsed with peptide was used as antigen presenting cells (APCs) for the $CD8^+$ fraction in MLPC. After eight days of MLPC culture, the T2-cell line pulsed with peptide was used as APCs in the ELISpot. IFN-γ and granzyme B ELISpot assays were performed as previously described[1,2] 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 assays.[15]

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 as described earlier.[5] HLA-A2/R3 tetramer*PE was synthesized at the Lausanne Branch of the Ludwig Institute for Cancer Research. 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%)[5] during or after vaccination. An increase of $CD8^+$ T-cell response as demonstrated by two of three or one of two methods (tetramer staining, IFN-γ and granzyme B ELISpot assays) was defined as a positive immunological reaction of the patient.

Staining of patients' peripheral blood mononuclear cells before, during, and after vaccination was performed using the following fluorescence-labeled monoclonal antibodies: phycoerythrin (PE)-Cy7-conjugated anti-CD4 (BD Biosciences, Heidelberg, Germany), 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 min 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, Hamburg Germany). The cells were analyzed on a FACSAria™ flow cytometer (Becton Dickinson) using the CellQuest™ software (Becton Dickinson).

Results

Nine patients were included in the present study. All patients received 1,000 μg RHAMM-R3 peptide per vaccination and completed the course of peptide vaccination. The patients expressed both RHAMM and HLA-A2 as assessed by RT-PCR and flow cytometry. The clinical characteristics of these patients are listed in Table 1.

Similar to the 300 μg cohort of the first study, only mild side effects like CTC grade 1 erythema and induration of the skin at the site of injection were observed after peptide vaccination. No patient developed an elevated body temperature due to vaccination. We detected no therapy-related toxicity higher than CTC grade 1. One patient died due to infection in the progress of the underlying disease, acute myeloid leukemia. A patient with myelodysplastic syndrome suffered from a concomitant chronic coronary disease and experienced an episode of cardiac ischemia during the period of vaccination therapy.

We found a significant increase of specific $CD8^+$ T cells recognizing the RHAMM-R3 peptide in 4/9 patients by ELISpot analysis and/or by tetramer staining. The ELISpot data of these patients for the secretion of IFNγ and granzyme B, as well as tetramer staining results are summarized in Table 1. 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 of over 50% of HLA-A2/R3-tetramer$^+$/$CD8^+$ T lymphocytes below 0.1% prior to vaccination and with an increase of over 25% of HLA-A2/R3-tetramer$^+$/$CD8^+$ T lymphocytes over 0.1% prior to vaccination, during or after vaccination. An increase of $CD8^+$ T-cell response demonstrated by 2/3 or 1/2 assays (tetramer staining, IFN-γ and granzyme B ELISpot assays) was considered as a positive immunological reaction of the patient.

Figures 1 and 2 present an example of an increase in frequency of HLA-A2/R3-tetramer$^+$/$CD8^+$ T lymphocytes and a transient increase in R3-specific secretion of IFNγ and granzyme B during the course of vaccination of this patient with multiple myeloma.

Figure 2 shows an increase of R3-specific $CD8^+$ T cells and a decrease of regulatory T cells of a patient with multiple myeloma during vaccination with 1,000 μg RHAMM-R3 peptide. Of the 9 patients vaccinated with 1,000 μg peptide, 3 patients (2 of them with positive immune responses) showed a significant decrease of $CD4^+CD25hiFoxP3^+$ Treg cells; one patient without positive immune and clinical effects showed an increase of the frequency of regulatory T cells (5.03-15.9%) (Table 1 and Figure 3). All analyses were performed in an autologous system.

Clinical responses were assessed by the examination of peripheral blood and bone marrow samples before and after vaccination. For multiple myeloma patients, we additionally assessed their serum by immunofixation, serum free light chains and quantitative serum immunoglobulins. The results are summarized in Table 1. After vaccination, one patient with myelodysplastic syndrome RAEB 1 showed a reduction of leukemic blasts in the bone marrow to less than 5% of the nucleated cells in FACS analysis. One myelodysplastic syndrome patient had a normalization of peripheral blood (Figure 4) count and one patient with multiple myeloma showed a reduction of light chain in serum. Details of the 3 patients who showed these clinical effects are reported below.

Patient 1: a 65-year old male patient was diagnosed myelodysplastic syndrome RAEB 1 in March 2001 and received intermittently pegylated erythropoetin till he was vaccinated in September 2005. Under vaccination therapy, the percentage of blasts in the bone marrow decreased from 5-10% to less than 5% in the blast gate of FACS analysis. Under microscopy, the bone marrow showed a hypocellularity. The cell counts in peripheral blood as for leukocytes and platelets improved temporarily.

Patient 2: a 78-year old female patient was diagnosed myelodysplastic syndrome RAEB1 in December 2005 and did not receive any therapy until we initiated peptide vaccination in January 2006. Under peptide vaccination therapy, the peripheral blood counts improved to normal values for leukocytes and platelets (Figure 4), and the patient's blood transfusion needs were reduced. After cessation of vaccination therapy, the peripheral blood counts

dropped again.

Patient 8: a 57-year old patient with multiple myeloma and normal karyotype was treated with high-dose chemotherapy in the framework of the German Myeloma Study Group Trial V (DSMM V). Peptide vaccination was initiated 12 months after the last chemotherapy. The concentration of kappa free light chains in the serum of the patient decreased from 10.3 mg/L to 2.26 mg/L, the kappa/lambda ratio from 1.24 to 0.74. The subpopulation of kappa$^+$CD138$^+$CD38$^+$ plasma cells in the bone marrow as detected by FACS analysis decreased from 2.7% to 0.9%.

mRNA was quantitatively measured in 6 of the patients[1-3,7,8] before vaccination and three weeks after the last vaccination. Not enough material was available for this to be measured for the other patients. In patient 1, conventional RT-PCR was positive (one of the inclusion criteria) but the quantitative mRNA level was very low and remained stable. Interestingly, the other 2 patients with positive clinical effects (patients 2 and 8) showed a reduction of the RHAMM transcript that is concordant to the clinical course of these patients. Patient 7 showed a stable mRNA expression and patient 3 also a reduction of the transcript.

Discussion

Earlier, we described encouraging results of a peptide vaccination trial of patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma vaccinated with the RHAMM-derived peptide R3. The peptide as the core of the vaccine was emulsified in incomplete Freund's adjuvant (IFA), and concomitantly granulocyte macrophage colony-stimulating factor (GM-CSF) was administered subcutaneously. Seventy percent of the patients showed positive immunological results, and even positive clinical effects could be detected in several patients of this phase I/II clinical study.[5]

Therefore, in the present study, 9 patients with hematologic malignancies expressing both RHAMM and HLA-A2 were vaccinated with the RHAMM-derived peptide R3 subcutaneously at a high dose of 1,000 μg RHAMM-R3 peptide. We wondered whether the increase of the peptide dose would improve immunological and even clinical responses.

Similar to the first cohort of RHAMM-R3 peptide vaccination and also other trials for patients with solid tumors or hematologic malignancies, in this study we found no drug-induced adverse events higher than CTC grade 1 skin toxicity.[5,6,16]

The immunomonitoring of the patients in our study was performed using ELISA, six-color flow cytometry and ELISpot assays for the secretion of IFNγ and granzyme B. Four of 9 (44%) vaccinated patients showed an increase of RHAMM-R3-specific T cells in tetramer assays and also in ELISpot assays. This frequency is lower than in the 300 μg cohort of our study (7/10; 70%). Interestingly, similar to the 300 μg cohort a decrease of specific T-cell responses in longer follow-up could be observed in some patients after vaccination was stopped. This finding might be an important point which prompted us to extend the 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 Mailänder *et al.*[17] Administration of CD4$^+$ helper T-cell epitopes derived from RHAMM or other more unspecific CD4$^+$ T-cell stimulator keyhole limpet hemocyanine (KLH)[17,18] or CPG-rich oligodinu-

Table 1. Patients' characteristics and immunological responses and clinical effect: 8 of 9 patients completed the course of four vaccinations.

Age	Diagnosis	Karyotype	Therapy before vaccine	Vaccine data	% Tregs CD4$^+$ CD25 hi FoxP3$^+$ before/during/after	% CD8$^+$ tetra$^+$ before/during/after	ELISpot IFNg before/during/after	ELISpot GrB before/during/after	Immunological response	Clinical effect
65	MDS Mar. 01	RAEB 1 CN	PEG-EPO	09/05-10/05	9.6-5.4-8.6	0.15-0.26-0.26	33-45-7	2-22-1	positive	reduction of blasts in BM
78	MDS Dec. 05	RAEB 1 CN	None	01/06-03/06	4.38-6.47-6.05	0.12-0.13-0.05	28-53-12	7-1-2	NC	normal leukocyte and platelet count
61	MDS Jan. 05	RAEB 1/2 CN	None	01/06-03/06	5.03-10.9-15.9	0.12-0.33-0.07	0-4-1	0-0-1	NC	NC
69	MDS Nov. 05	5q- and deletion 12p	Valproic acid ATRA	05/06-06/06	1.3-3.5-1.9	0.07-0.01- 0.13	40-1-5	7-0-0	NC	NC
70	Sec. AML May 06	No metaphases	PEG-EPO	01/06-03/06	0.03 -0.01	0.02-0.04-0.05	69-2-2	7-0-0	NC	PD
70	MDS Oct. 06	RCMD CN	None	01/07-02/07	21.6-12.7-6.0	NA	16-21-28	0-0-1	positive	NC
65	MM Feb. 04	+11q25	DSMM II	02/07-03/07	23.9-11.2-10.9	0.7-0.56-2.33	17-45-16	1-6-0	positive	NC
57	MM Apr. 05	CN	DSMM V	02/07-03/07	9.8-11.9-12.7	0.61%-0.29-0.47	96-235-43	68-255-42	positive	reduction of free light chains
53	MM Apr. 05	Tetraploidy	DSMM V	07/07-08/07	11.9-10.4-9.3	0.62-0.4-0.64	118-95-37	NA	NC	PD

Age means age at time of vaccination therapy. CN: cytogenetically normal/normal karyotype; DSMM II/V (protocols II and V of the German Study Group for Multiple Myeloma); positive: increase; NA: not available, NC: no change, RCMD: refractory cytopenia with multilineage dysplasia, PEG-EPO: pegylated erythropoietin, PD: progressive disease. 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.

cleotides[19] might help to induce more long-lasting vaccination results. Of the 9 patients vaccinated with 1,000 μg peptide, 3 patients showed a significant decrease of $CD4^+CD25hiFoxP3^+$ regulatory T cells (Tregs). Of these 3 patients, 2 showed positive immunological responses and one patient also a reduction of bone marrow blasts. Therefore, downregulation of Tregs (which inhibit LAA-specific $CD8^+$ T cells) might constitute an important step towards an enhanced response to peptide vaccines. Downregulation of Tregs might be induced by different ways of action. Cytokines released by the specific RHAMM-R3 peptide vaccination might result in a reduction of Tregs, or the reduction of Treg frequencies is caused by unspecific bystander effects of the adjuvant and/or GM-CSF.[20,21] One patient without positive immune and clinical effects showed a relevant increase in the frequency of Tregs (5.03% to 15.9%; patient #3 in Table 1 and Figure 3). However, due to a small patient number in this phase I trial no statistical analysis with sufficient power could be performed. We, therefore, present the data in a descriptive manner.

Three of the 9 patients of the present cohort showed positive clinical effects. This confirms the results observed in our first cohort[5] and also other clinical peptide vaccination trials.[6,7,22] In this high-dose peptide trial, an association between immunological and clinical responses was observed similar to the first cohort receiving 300 μg RHAMM-R3 peptide per vaccination.[5] The increase in peptide dosage did unfortunately not result in a higher frequency of immunological and clinical responses, somewhat reflecting the *in vitro* finding that dose/response curves of experiments with peptide stimulation of T cells are bell-shaped, i.e. the optimum dose is less than the maximum dose.[2] Nevertheless, a robust statistical analysis to compare the 300 μg cohort of patients with the patients vaccinated with 1000 μg is not possible as the patient cohorts are heterogenic including different diseases and clinical situations.

Patient #2 described in Figure 4 showed an increase of peripheral blood cells but not of immunological responses. The increase of peripheral blood cells might be induced by a combination of the peptide RHAMM-R3, the adjuvant, but also by the application of GM-SCF and not due to spe-

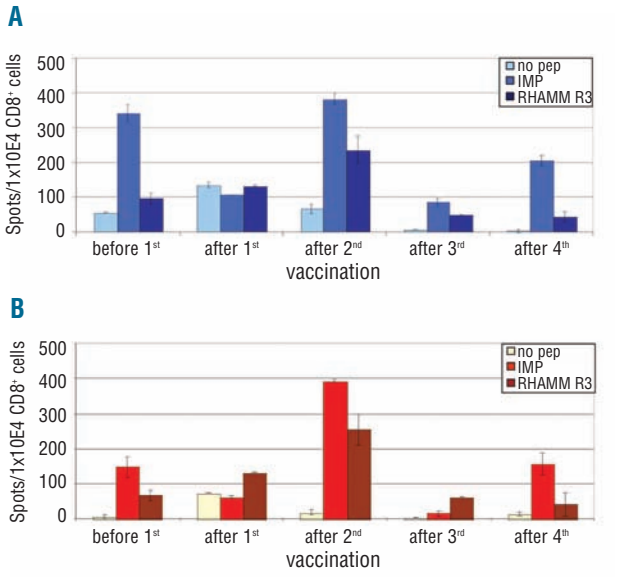


Figure 1. Immunological responses of patient #8 (see Table 1) with multiple myeloma during the course of RHAMM-R3 peptide vaccination. ELISpot assays for the release of interferon gamma (Panel A) and granzyme B (Panel B) were performed after stimulation with RHAMM-R3 peptide. Stimulation with an influenza matrix protein (IMP)-derived peptide served as a positive control, no peptide stimulation as a negative control. Peptide-specific T-cell activity was measured by interferon release, whereas granzyme B secretion indicated the lytic potential of the T-cells. An increase of RHAMM-R3 specific T-cell activity could be noted.

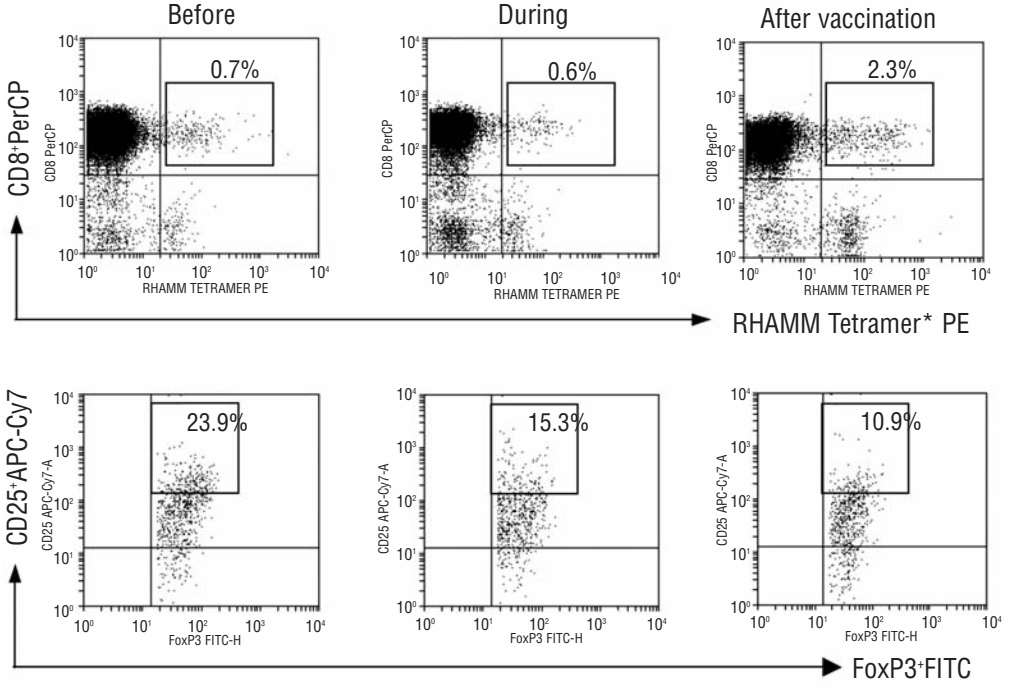


Figure 2. Change in frequency of RHAMM specific $CD8^+$ T cells and $CD4^+$ $CD25hiFoxP3^+$ regulatory T cells in the peripheral blood from patient #7 (see Table 1) with multiple myeloma during the course of RHAMM-R3 peptide vaccination. While the frequency of $CD8^+$ T lymphocytes specifically recognizing RHAMM-R3 as demonstrated by tetramer binding increased, the frequency of regulatory T cells decreased.

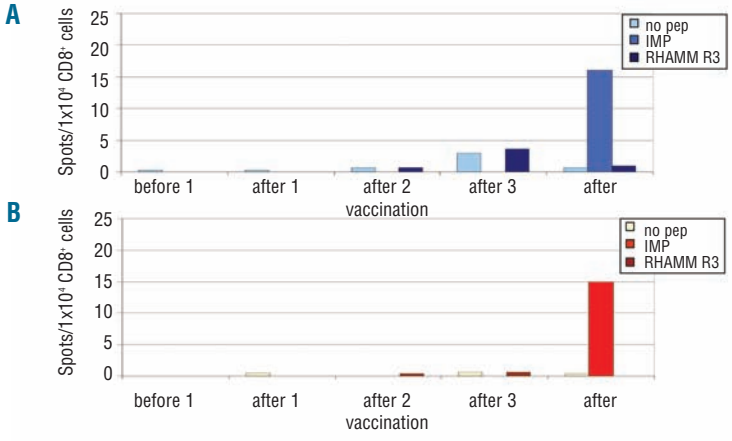
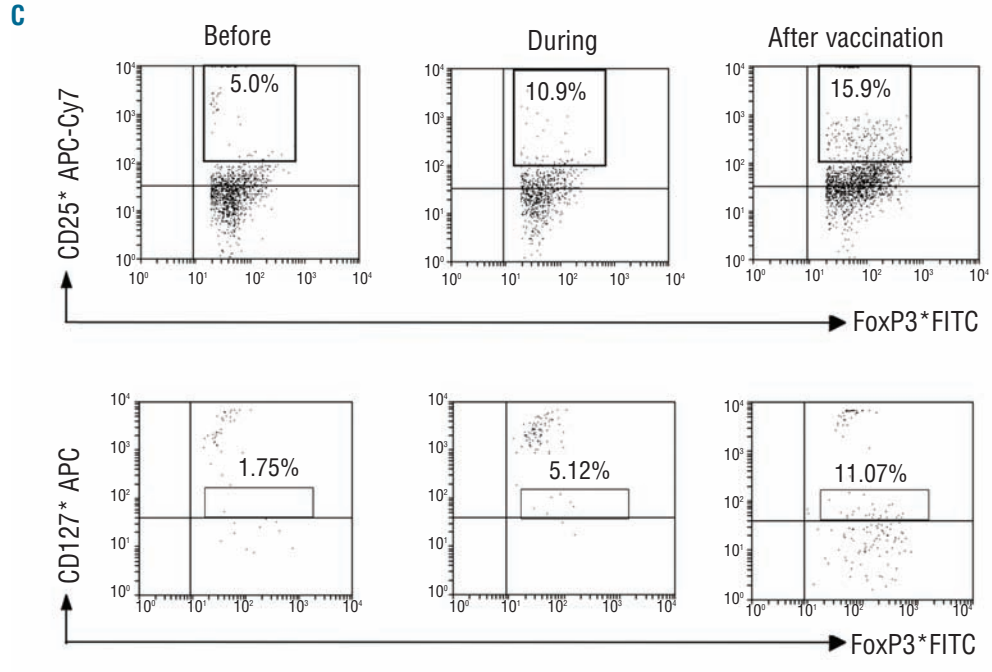


Figure 3. Immune responses by RHAMM-R3 specific CD8⁺ T cells and regulatory T cells of patient # 3 (see Table 1) with MDS in the course of RHAMM-R3 peptide vaccination. ELISpot assays for the release of interferon gamma (Panel A) and granzyme B (Panel B) were performed after stimulation with RHAMM-R3 peptide. Stimulation with an influenza matrix protein (IMP)-derived peptide served as a positive control, no peptide stimulation as a negative control. Peptide-specific T-cell activity was measured by interferon release, whereas granzyme B secretion indicated the lytic potential of the T cells. In this particular patient, no increase of RHAMM-R3 specific T-cell activity could be noted. In contrast, the frequency of regulatory T cells as measured by flow cytometry (Panel C) for this patient showed a relevant increase.



cific T-cell responses against RHAMM-R3. Patient #2 is the only patient in both peptide dose groups with clinical changes without similar immunological responses. As the peripheral blood counts in this patient were seen to improve only during the vaccination therapy and then fall after vaccination these findings might be induced by such bystander effects of the vaccine and underlines the importance of an induction of enduring immune responses for more robust effects.

To use the 'appropriate' LAA that is indeed clinically effective might be one of the most important steps in designing a vaccination study. Several LAAs that are able to induce specific T-cell responses are involved in different critical mechanisms for cell differentiation and proliferation like BCL-2, FLT3-ITD, G250, hTERT, RHAMM, survivin and WT-1 and therefore these antigens are appropriate target structures.[4] LAAs are critically involved in mechanisms responsible for *in vitro* tumor growth such as proliferation, inhibition of apoptosis, differentiation and demethylation and therefore constitute exquisite target structures for targeted immunotherapies. Recently,

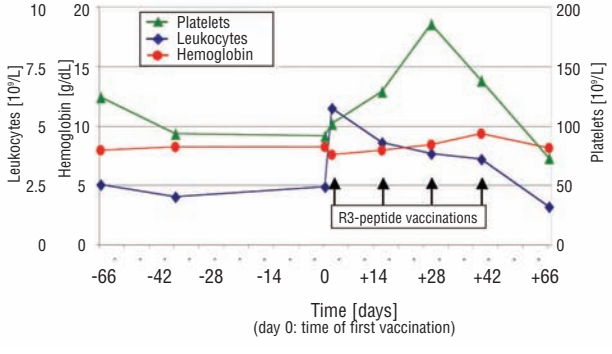


Figure 4. Changes in the peripheral blood count of patient # 2 (see Table 1) with MDS during the course of RHAMM-R3 peptide vaccination. The three different lineages are marked by different symbols as indicated in the inlay box; please note also the three different scales for the y-axis. During RHAMM-R3 peptide vaccination the peripheral blood counts normalized. However, a certain decrease could be noted after cessation of vaccination therapy.

we demonstrated the impact of the LAA RHAMM on the survival of acute myeloid leukemia patients.[4,5] Therefore, immune therapies targeting RHAMM might not only target an immunogenic antigen, but also a gene critically involved in cell cycle, differentiation and proliferation. By RHAMM-R3 peptide vaccination, 2 of 3 patients with positive clinical effects showed a reduction of RHAMM mRNA. In the third patient, RHAMM expression persisted at very low level. The important role of RHAMM in tumor cell growth might be the reason for these encouraging results detected by RHAMM-R3 peptide vaccination.

In summary, we demonstrated in the present phase I/II clinical trial the safety and feasibility of a high-dose (1,000 μg) RHAMM-R3 peptide vaccination in patients with hematologic malignancies. RHAMM-R3 peptide vaccination strategies in patients with malignant myeloid diseases were shown to be safe and specific immune responses could be detected at a high frequency. However, the increase in the peptide dose did not improve the immunological and clinical results in comparison to lower peptide doses like 300 μg. Scheduling and vaccine adjuvants have to be further optimized to improve these positive results of RHAMM-R3 peptide vaccination, and might be more important than the peptide dosage. Depletion of $CD25^+$ regulatory T cells by specific monoclonal antibodies like danileukine diftitoxin (Ontak™;[23]) or addition of toll-like receptor stimulation oligodinucleotides might pave the way for new approaches in the field of peptide vaccination.[24] Moreover, advances are being made in the combination of peptide vaccination with allogeneic stem cell transplantation.[6]

Authorship and Disclosures

JG was the principal investigator and takes primary responsibility for the paper; AS, KG, MR and DB co-ordinated the research; MR and MG performed the laboratory work for this study; IF participated in the statistical analysis; SH co-ordinated the research, participated in the statistical analysis; GR contributed important materials; HD discussed the paper; MS discussed and wrote the paper.

The authors reported no potential conflicts of interest.

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