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RHAMM/HMMR (CD168) is not an ideal target antigen for immunotherapy of acute myeloid leukemia

Running title: RHAMM in immunotherapy of AML

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Abstract

Background. Criteria for good candidate antigens for immunotherapy of acute myeloid leukemia are high expression on leukemic stem cells in the majority of acute myeloid leukemia patients and low or non-existing expression in vital tissues. It was shown in vaccination trials that Receptor for Hyaluronic Acid Mediated Motility (RHAMM/HMMR) generates cellular immune responses in patients with acute myeloid leukemia, which correlate with clinical benefit. It is not clear however whether this response actually targets the leukemic stem cell, especially since it was reported that RHAMM is expressed maximally during G2/M phase of the cell cycle. In addition, tumor specificity of RHAMM expression remains relatively unexplored.

Design and Methods. Blood, leucapheresis and bone marrow samples were collected from both acute myeloid leukemia patients and healthy controls. RHAMM expression was assessed at protein and mRNA level on various sorted populations, either fresh or after manipulation.

Results. High levels of RHAMM were expressed by CD34⁺CD38⁺ and CD34⁻ acute myeloid leukemia blasts. However, only baseline expression of RHAMM was measured in CD34⁺CD38⁻ leukemic stem cells, which was not different from that of CD34⁺CD38⁻ hematopoietic stem cells of healthy controls. RHAMM was significantly upregulated in CD34⁺ cells of healthy donors during in vitro expansion and during in vivo engraftment. Finally, we demonstrated an explicit increase in the expression level of RHAMM after in vitro activation of T cells.

Conclusions. RHAMM does not fulfill the criteria of an ideal target antigen for immunotherapy of acute myeloid leukemia. RHAMM expression in leukemic stem cells does not significantly differ from the expression in hematopoietic stem cells of healthy controls.
RHAMM expression in proliferating CD34\(^+\) cells of healthy donors and activated T cells further compromises RHAMM-specific T cell-mediated immunotherapy.

**Introduction**

A priority-ranked list of cancer vaccine target antigens was published in 2009, ranking the cancer-associated antigens based on predefined and preweighted objective criteria determining the likelihood for efficacy in cancer therapy (1). These criteria include therapeutic function in vaccine trials, immunogenicity, the number of patients with antigen-positive tumors, expression level, percentage of positive tumor cells and cellular location, as well as a role for the antigen in oncogenicity, a tumor-specific expression profile and expression in cancer stem cells. The latter criterium, the evidence for expression on putative cancer stem cells, is likely to be very important in the context of acute myeloid leukemia (AML). Although 65 to 75\% of the patients under 60 years of age with AML reach a complete hematological remission through the currently available standard therapy, the 5 year survival rate is less than 30\%, because a high percentage of the patients relapses (2). Accumulating evidence supports the role of leukemic stem cells (LSCs) in the high relapse rate of AML (2-6). These quiescent LSCs, possessing biological properties rendering them resistant to chemotherapy and radiotherapy, are probably responsible for the minimal residual disease (MRD) of AML and may eventually result in relapse. LSCs share with normal hematopoietic stem cells (HSCs) the properties of a low division rate, self-renewal ability and expression of some surface markers including the CD34\(^+\)CD38\(^-\) phenotype (3-6). It is generally believed that sensitive detection of MRD and targeted elimination of LSCs can be a very efficient way to achieve more durable remissions or even a cure for AML (3, 6, 7). Immunotherapy is expected to be successful in this setting of MRD, complementary to prior standard treatment, by the elimination of the residual blasts, containing the LSCs.

Receptor for Hyaluronic Acid Mediated Motility (RHAMM/HMMR/CD168), discovered by the SEREX (serologic screening of cDNA expression libraries) method, has been described as a
cancer-associated antigen and is involved in both tumorigenesis and progression or metastasis (8-13). Besides its expression in many solid tumors, RHAMM mRNA was detected in peripheral blood mononuclear cells (PBMCs) of 60-70% of newly diagnosed AML patients (10, 14). High expression of RHAMM has been correlated with a poor prognosis in patients with various types of solid tumors and hematological malignancies such as B cell chronic lymphocytic leukemia, multiple myeloma and AML (15-22). RHAMM-specific CD8+ T cells were detected in patients diagnosed with AML and CML (23-25). Using a lymphoma mouse model, anti-tumor activity mediated by CD4+ T cells was observed after vaccination with RHAMM mRNA-transfected dendritic cells (26). A recently published report demonstrated prolonged survival of immune deficient mice injected with an AML cell line after adoptive transfer of RHAMM-specific T cell receptor (TCR) transgenic lymphocytes (27). In addition, it was shown in clinical vaccination trials that RHAMM generates cellular immune responses and, importantly, clinical responses in some patients with AML, myelodysplastic syndrome and multiple myeloma (28, 29). Therefore RHAMM was identified as one of the most promising leukemia-associated antigens (LAAs) in AML.

It is not clear however whether this RHAMM-specific response actually targets the true LSC. Expression of RHAMM in CD34+CD38-LSCs has, to the best of our knowledge, never been specifically investigated. Promising results have been published concerning CD44, a hyaluronan receptor closely related to RHAMM, which has been described as a potential target to eliminate AML LSCs (6). Another relatively unexplored LAA criterium concerning RHAMM is its tumor specificity. Greiner et al. showed that RHAMM is not expressed in peripheral blood mononuclear cells (PBMCs) and CD34+ HSCs of healthy volunteers (10, 14). However, the specificity of RHAMM expression was shown to be not absolute, since testis, placenta and thymus showed significant RHAMM mRNA expression (10, 30). Immunohistochemistry of spermatocytes, the normal colonic mucosa and the normal gastric mucosa revealed a strong, weak and occasionally positive staining for RHAMM, respectively (16, 22). RHAMM immunostaining was also demonstrated throughout all layers of the cornea.
and suprabasal layers of the limbus (31). Furthermore, it was reported that RHAMM is differentially expressed during the cell cycle, with maximal RHAMM mRNA expression in the G2/M phase (32). As a consequence, RHAMM expression in actively dividing cells of physiological tissues might be upregulated.

In this paper, we investigated the expression pattern of RHAMM in various leukemic and non-leukemic hematopoietic cell populations that are relevant to the immunotherapeutic treatment of AML patients.
Design and Methods

Samples from healthy volunteers and patients with acute myeloid leukemia

All samples were taken from patients with AML treated at the Ghent University Hospital (Belgium) between 2009 and 2011. Samples were collected at the time of diagnosis or relapse as indicated in Table 1. AML samples (bone marrow, peripheral blood or leucapheresis) and cord blood, peripheral blood and leucapheresis samples (after HSC mobilization) of healthy donors were obtained and used following the guidelines of the Medical Ethical Committee of the Ghent University Hospital, after informed consent was obtained in accordance with the Declaration of Helsinki. Bone marrow, cord blood, peripheral blood and leucapheresis samples of patients or healthy donors were prepared by Ficoll separation (Lymphoprep, Nycomed Pharma, Brussels, Belgium) and cryopreserved in fetal calf serum (FCS, Gibco, Invitrogen, Merelbeke, Belgium) containing 10% dimethyl sulfoxide (DMSO, Serva, Heidelberg, Germany) in liquid nitrogen.

Culture of cell lines

The K562 cell line, a CML (chronic myeloid leukemia) blast crisis-derived cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and was cultured in standard medium consisting of IMDM (Gibco, Invitrogen), supplemented with 10% FCS (Gibco, invitrogen), 2 mM L-glutamine (Gibco, Invitrogen), 100 IU/ml penicillin (Gibco, Invitrogen) and 100 IU/ml streptomycin (Gibco, Invitrogen). K562 is often used in literature as a positive control for RHAMM expression (10, 14, 23, 33). The OP9-GFP bone marrow stromal cell line was obtained from Dr. J. C. Züñiga-Pflücker (University of Toronto, Toronto, Canada). This murine cell line constitutively expresses GFP, which can be flow cytometrically analyzed in the FITC channel. OP9-GFP was cultured in MEM α medium (Gibco, Invitrogen)
supplemented with 20% FCS (Gibco, Invitrogen), 2 mM L-glutamine (Gibco, Invitrogen), 100 IU/ml penicillin (Gibco, Invitrogen) and 100 IU/ml streptomycin (Gibco, Invitrogen). Cells were grown near confluence, harvested and split every 2 to 3 days.

**Isolation of subpopulations of acute myeloid leukemia and healthy donor cells**

After thawing, CD34 positive cells were first enriched by anti-CD34 magnetic activated cell sorting beads (MACS beads, Miltenyi, Leiden, The Netherlands) if the percentage of CD34⁺ cells in the sample was < 70%. Subsequently cells were labeled with CD38-PE (BD Biosciences, Erembodegem, Belgium) and CD34-FITC (Miltenyi) and sorted with the FACSAria II cell sorter (BD Biosciences) for the viable (assessed by lack of propidium iodide (Invitrogen) intake) CD34⁺CD38⁻ and CD34⁺CD38⁺ cells to a purity of > 99%, as determined by post sort analysis. If the percentage of CD34⁺ cells in the sample was > 70%, samples were immediately sorted as described above. From 5 AML samples, the CD34⁻ blasts were isolated too.

**In vitro culture of acute myeloid leukemia samples**

After Ficoll separation (Lymphoprep, Nycomed Pharma), bulk samples were cultured in standard medium (described above) supplemented with 50 ng/ml interleukin-3 (IL-3, R&D, Oxon, United Kingdom), 100 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF, Gentaur, Kampenhout, Belgium), 100 ng/ml granulocyte-colony stimulating factor (G-CSF, Gentaur), 25 ng/ml stem cell factor (SCF, Amgen, Brussels, Belgium), a cytokine mix which has already shown to allow in vitro expansion of AML samples (34). After 5 days of culture, cells were re-analyzed by flow cytometry and RT-qPCR.

**Isolation and culture of cord blood samples**

Cryopreserved cord blood of healthy individuals was thawed and the CD34⁺ cells were enriched by anti-CD34 MACS beads (Miltenyi), and subsequently cell sorted with the FACSAria II cell sorter (BD Biosciences) to obtain the viable (assessed by lack of propidium
iodide (Invitrogen) intake) CD34-APC (Miltenyi) positive cells, to a purity of > 99%, as
determined by post sort analysis. CD34⁺ cells were co-cultured with the OP9-GFP cell line in
MEM α medium (Gibco, Invitrogen) supplemented with 20% FCS (Gibco, Invitrogen), 2 mM
L-glutamine (Gibco, Invitrogen), 100 IU/ml penicillin (Gibco, Invitrogen) and 100 IU/ml
streptomycin (Gibco, Invitrogen) in the presence of 100 ng/ml SCF (Amgen), 20 ng/ml
thrombopoietin (TPO, R&D) and 100 ng/ml Flt-3 ligand (Flt3-L, R&D). The OP9 stromal cell
line, derived from bone marrow of the macrophage colony-stimulating factor-deficient
osteopetrotic mouse, has proven to be effective in supporting hematopoietic differentiation
from murine and human stem cells. Culture conditions above described are known to support
\textit{in vitro} expansion of human cord blood-derived CD34⁺ cells (35-37). After 4 days, cells were
harvested by forceful pipetting and transferred to a fresh confluent monolayer of OP9-GFP
until day 7. On day 7, the viable CD34-APC (Miltenyi) positive, CD45-PE (Miltenyi) positive,
GFP-negative cells were sorted again (FACSAria II cell sorter (BD Biosciences)) to a purity
of > 99%.

\textbf{T cell activation}

Bulk PBMCs of healthy donors were cultured in standard medium (described earlier)
supplemented with 10 \(\mu\)g/ml phytohemagglutinin (PHA, Sigma-Aldrich, Bornem, Belgium)
and 40 IU/ml interleukin-2 (IL-2, Roche, Anderlecht, Belgium) for the indicated period.
PBMCs were also sorted at day 0 with the FACSAria II cell sorter (BD Biosciences) to obtain
the CD3⁺ TCRαβ⁺ and the CD3⁺ TCRαβ⁺ CD8⁺ subpopulations to a purity of > 99%. The sorted
populations were cultured for the indicated period in standard medium supplemented with
anti-CD28 antibody (BD biosciences) and anti-CD49d/VLA-4 antibody (BD biosciences), both
at a final concentration of 2 \(\mu\)g/ml, on an anti-CD3 antibody (OKT3, ATCC; 10 \(\mu\)g/ml in PBS
(Lonza, Verviers, Belgium) precoated 96 well plate (BD Falcon, Erembodegem, Belgium).

\textbf{Flow cytometry and antibodies}
Staining of surface markers was performed in DPBS (Lonza), supplemented with 1% FCS (Gibco, Invitrogen) by adding labeled antibody concentrations as recommended by the supplier. Intracellular staining for RHAMM was performed after the initial cell surface staining using Cytofix/Cytoperm reagents (Becton Dickinson, Erembodegem, Belgium), according to the instructions of the supplier. Flow cytometric analysis was performed using a LSR II Cytometer (BD biosciences). The following anti-human monoclonal antibodies were used: FITC-conjugated: CD34 (Miltenyi), CD8 (BD biosciences); PE-conjugated: CD38 (BD Biosciences), CD45 (Miltenyi), TCRαβ (Miltenyi); APC-conjugated: CD38 (Miltenyi), CD3 (BD biosciences), CD8β (BD biosciences); non-conjugated: RHAMM/HMMR/CD168 (Neuromics (Edina, Minnesota), mouse IgG1κ, clone 2D6). Anti-mouse antibodies used were: PE-conjugated: Rat-anti-mouse IgG1 (BD biosciences); PE-Cy7-conjugated: anti-mouse CD45 (eBioscience). Relevant isotypes were used where mentioned. Viable human cells were gated based on forward and side scatter and, for simple surface staining, on lack of propidium iodide (Invitrogen) uptake. For combined surface and intracellular staining, aqua fluorescent reactive dye (amcyan) (Invitrogen) was used to exclude the non-viable cells.

**Cell cycle analysis**

Cells were fixed in ethanol (Normapur, VWR, Heverlee, Belgium) 75% in PBS (Lonza). Samples were stored at 4°C until analysis. On the day of analysis RNAse A (Qiagen, Venlo, The Netherlands) was added to a final concentration of 100 µg/ml and samples were incubated for 15 minutes at 37°C. Subsequently, propidium iodide (Invitrogen) was added to a final concentration of 10 µg/ml. Flow cytometric analysis was performed using a LSR II Cytometer (BD biosciences). Data were analyzed according to the Dean-Jett-Fox model in FlowJo.

**In vivo transplantation experiment**

NOD.CB17-Prkdc<sup>scid</sup>/J (NOD/SCID) mice were obtained from Charles River Laboratories (L’Arbresle, France) and provided with a sterile diet and sterile water. Eight mice aged 8
weeks were given a sublethal dose of whole-body irradiation (linear particle accelerator, 3.5 Gy) and injected intraperitoneally with 200 µg of a rat monoclonal antibody against the murine IL2-Rβ chain, purified from supernatant of the hybridoma cell line TM-β1, kindly provided by Dr. T. Tanaka (Tokyo, Japan) (38, 39). Within 24 hours after irradiation, mice were injected intravenously with CD34+ cells, derived from cryopreserved human cord blood of four different healthy individuals and isolated as previously described. CD34+ sorted populations (FACSaria II cell sorter (BD Biosciences), post-sort purity > 99%) were immediately used for injection without previous in vitro culture. An aliquot of each cord blood donor was retained for RT-qPCR. Mice were injected with 2.5 x 10^5 viable (assessed by lack of propidium iodide (Invitrogen) uptake) CD34+ cells. After 2 weeks, mice were sacrificed and human (h)CD34+, hCD45+, mouse (m)CD45- cells, engrafted in the bone marrow (tibiae, femora and humeri, bilateral), were sorted with the FACSaria II cell sorter (BD Biosciences) to a purity of > 99%, as determined by post-sort analysis. Human and mouse FcR blocking reagents were used to block non-specific binding of antibodies.

**Real-time quantitative PCR (RT-qPCR)**

Total RNA was prepared from the indicated (FACS-sorted) samples, using the miRNeasy minikit of Qiagen. cDNA synthesis was performed by the Superscript First Strand Synthesis System for RT-PCR kit (Invitrogen). RT-qPCR with the SYBR Green I technology was performed using the qPCR Core Kit for SYBR Green I (Eurogentec, Seraing, Belgium) on an ABI PRISM 7300 RT-PCR System (Applied Biosystems, Ghent, Belgium) or using the LightCycler 480 SYBR Green I Master kit (Roche) on a LightCycler 480 II (Roche), both according to the manufacturer’s protocol. Used primers (Operon and Biolegio) were: RHAMM fw 5'-CAGGTCACCCAAAGGAGTCTCG-3'; RHAMM rv 5'-CCACTTGATCTGAAGCACAACTAA-3'; GAPDH fw 5'-TGCACCACCAACTGCTTAGC-3'; GAPDH rv 5'-GGCATGGACTGTGGTCATGAG-3'; Ribosomal S18 protein (R18S) fw 5'-ATACATGCCGACGGCGCCTG -3'; R18S rv 5'- AGGGGCTGACC GGTTGGTT-3'; YWHAZ fw 5'-ACTTTTTGCTACATTGGGGCTTCAA -3'; YWHAZ rv 5'-CGGCCAGGACAAACCAGTAT
-3’. RHAMM primers were designed to amplify the 4 described isoforms of RHAMM indifferentially (19). Results were analyzed using the delta-delta Ct method and normalized to GAPDH expression and shown relative to the transcript level of K562. If pre- and post-proliferation samples were compared, normalization was performed to the geometric mean of GAPDH, YWHAZ and R18S expression (40).

**Statistical analysis**

Statistical analysis was performed in SPSS statistics version 19. The non-parametric Related Samples Wilcoxon Signed Rank Test and Independent Samples Mann-Whitney U Test were used where indicated. The significance level is $p = 0.05$. 

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Results

**RHAMM is not expressed in acute myeloid leukemic stem cells**

CD34⁺CD38⁻ AML blasts and CD34⁺CD38⁺ LSCs were isolated from bone marrow, peripheral blood or leucapheresis samples of 13 AML patients. The clinical, immunophenotypic and genetic features of the AML patients cover a range of subtypes of AML and are presented in Table 1. Samples were taken at diagnosis or relapse as indicated. Two cord blood samples and 2 leucapheresis samples were selected as controls from healthy donors. Figure 1A shows only background expression of RHAMM in CD34⁺CD38⁻ HSCs of healthy donors. This population is considered to be RHAMM negative (10). No significant difference in RHAMM expression level could be demonstrated between the CD34⁺CD38⁻ and the CD34⁺CD38⁺ subpopulations of healthy donors (p= 0.273). Expression of RHAMM in the CD34⁺CD38⁻ AML subpopulation was divergent, with relative expression levels varying from undetectable to 0.74, calculated relative to RHAMM expression in K562. RHAMM expression in CD34⁻ blasts was investigated in 5 patients and was not statistically different from the expression in the CD34⁺CD38⁺ blast population (p=0.893). Strikingly, only baseline RHAMM expression was observed in the CD34⁺CD38⁻ LSCs, which was not statistically different from the expression measured in healthy CD34⁺CD38⁻ HSCs (p=0.296). These RT-qPCR data were confirmed by flow cytometry. In Figure 1B, intracellular staining for RHAMM expression within the CD34⁺ AML cells is shown for a representative AML sample (AML12): a subset of the CD34⁺CD38⁺ population clearly expressed RHAMM, whereas the CD34⁺CD38⁻ cells were virtually all negative for RHAMM. This result provides
evidence that, in addition to the differential RHAMM transcript level, a differential RHAMM protein expression profile can be seen in the LSCs compared with the CD34⁺CD38⁺ AML control population.

**RHAMM expression in acute myeloid leukemia is cell cycle-dependent**

It was previously shown that RHAMM expression in human primary fibroblasts is differentially regulated during the cell cycle with maximal RHAMM mRNA expression in the G2/M phase (32). This has been linked to its important role in mitosis, where RHAMM binds to the mitotic spindle to regulate spindle integrity and stability (41). Therefore we hypothesized that, also in AML, RHAMM expression may not be static during cell cycle progression. In Figure 2, cell cycle analysis and RHAMM expression are shown prior to and after *in vitro* expansion of AML samples. Freshly isolated AML cells were mainly situated in G0/G1 phase of the cell cycle, while 5 days of *in vitro* culture allowed a subpopulation to be pushed towards S phase (20.1%) and G2/M phase (11.5%) of the cell cycle in this representative sample (Figure 2A). As hypothesized, in parallel with this shift in the distribution of the AML cells in the different phases of the cell cycle, an approximately 8-fold increase in RHAMM mRNA expression was observed after 5 days of *in vitro* culture of 2 representative AML samples (Figure 2B). These RT-qPCR results were confirmed by flow cytometry showing a clear upregulation of RHAMM protein expression post-culture in the bulk of AML cells (Figure 2C). Interestingly, after 5 days of culture, although most cells have differentiated to CD34⁻ cells, a small but distinct CD34⁺CD38⁻ LSC subpopulation was sustained, of which the RHAMM protein expression remained undetectable (Figure 2D).

*In vitro* expansion of cord blood-derived CD34⁺ cells causes upregulation of RHAMM
Taking into account that the RHAMM protein and mRNA levels increased upon in vitro expansion in AML cells, we investigated whether this was also the case for non-malignant (both CD38+ and CD38-) CD34+ HSCs. To explore the RHAMM expression profile of expanding HSCs, human cord blood-derived CD34+ cells of five different donors were analyzed prior to and after in vitro culture. RT-qPCR showed a significant upregulation of RHAMM mRNA in the expanded CD34+ cells compared with freshly isolated CD34+ cells (Figure 3A). The expression levels in expanding healthy CD34+ cells were of comparable magnitude as those in AML samples under expansion conditions. These results suggest that not only malignant AML cells, but also CD34+ cells from healthy donors show cell cycle-dependent changes in the level of RHAMM mRNA. In addition to higher RHAMM mRNA expression, a strong upregulation of cytoplasmic RHAMM protein levels was observed in the CD34+ cells after expansion (Figure 3B). This result could be linked again to the distribution of the cells in the different phases of the cell cycle prior to and after in vitro expansion, as shown in Figure 3C. Cell cycle analysis of a representative sample shows that freshly isolated CD34+ cells were virtually all in G0/G1 phase, in contrast to the distribution of cells post-expansion where a subpopulation shifted towards S phase (29.3%) and G2/M phase (7.3%) of the cell cycle.

**Cord blood-derived CD34+ cells upregulate RHAMM during engraftment in a NOD/SCID mouse model**

A NOD.CB17-Prkdcscid/J (NOD/SCID) mouse model was used to address the question whether the in vitro observed upregulation of RHAMM during expansion of cord blood-derived CD34+ cells can be observed in vivo too, and therefore whether it is clinically relevant. Two weeks after injection of cord blood-derived CD34+ cells, mice were sacrificed and bone marrow was collected. Engrafting hCD45+ hCD34+ mCD45- cells made up in average 1.88% (representative sample shown: 5.79%) of the total viable bone marrow cells and were sorted to high purity (Figure 4A). RHAMM expression in the CD34+ cells prior to injection and after 2 weeks of engraftment was determined by RT-qPCR. As shown earlier,
RHAMM expression level was very low in freshly isolated healthy CD34⁺ cord blood samples (Figure 4B). Two weeks after transplantation, a significant upregulation in RHAMM expression ($p = 0.012$) was seen in the CD34⁺ human cord blood-derived cells: there was a mean fold increase of 75.3 after 2 weeks. RHAMM primers were tested on whole mouse bone marrow of a non-injected mouse, and no RHAMM expression could be observed, indicating the specificity of the human RHAMM primers (data not shown). Because the monoclonal antibody against RHAMM is non-conjugated, it was technically not feasible to confirm these RT-qPCR results flow cytometrically, combining intracellular RHAMM staining with surface staining of all required markers. In conclusion, these data suggest that, also in vivo, RHAMM is clearly expressed on the engrafting non-malignant CD34⁺ cells in the bone marrow, during the expansion phase after HSC transplantation.

**In vitro activated T cells overexpress RHAMM**

Immunotherapeutic strategies are based on 2 major principles. On one hand, cytotoxic T cells can be isolated from the patient and selected or genetically engineered to express a TCR of interest to be subsequently expanded *in vitro* and re-transfused to the patient. On the other hand, cytotoxic T cells can be activated *in vivo* by vaccination with peptides, DNA or dendritic cells (28, 29, 42-47). In both cases activated cytotoxic T cells are indispensable in order to obtain an immunological and clinical response in the AML patient. As we observed upregulation of RHAMM in cycling CD34⁺ cells of healthy donors, we also studied the change in RHAMM expression upon T cell activation. No RHAMM mRNA expression could be detected in fresh PBMCs, freshly isolated TCRαβ⁺ T cells and freshly isolated CD8⁺ TCRαβ⁺ cytotoxic T cells (Figure 5A), in accordance to previous reports (10, 30). Upon activation, a clear increase in RHAMM mRNA levels was observed. This upregulation was confirmed by intracellular flow cytometric analysis (Figure 5B). Taken together, these data indicate that *in vitro* activation induces RHAMM expression in cytotoxic CD8⁺ T cells.
Discussion

Herein, we show that expression of RHAMM, a cancer-associated antigen currently explored as a target for immunotherapy and especially for vaccination trials, is low to undetectable in the CD34⁺CD38⁻ LSCs of AML patients and not significantly different from CD34⁺CD38⁻ HSCs of healthy donors. These results are in accordance to the putative role assigned earlier to RHAMM as a negative marker of the stem cell-containing population of human limbal epithelial cells. Ahmad et al. could locate RHAMM in all layers of corneal epithelium and suprabasal layers of the limbal epithelium. In contrast, RHAMM was completely absent in the basal layer of the limbus where the stem cells are located (31). In view of the fact that the beneficial effects of immunotherapeutic strategies for AML are expected to be mainly situated in targeting MRD after prior standard therapy, targeting the chemoresistant LSCs is essential for a durable effect (48). However, the data presented here suggest that RHAMM-directed immunotherapeutic strategies will not target LSCs but only the rapidly proliferating progeny. Therefore, such therapies may offer little added value to standard therapy (e.g. poly-chemotherapy). Of course, we cannot exclude that the background level of RHAMM mRNA, detected in LSCs, may be sufficient for recognition by high-avidity T cells. Still, the conclusion remains that, as RHAMM mRNA expression levels in HSCs and LSCs are not significantly different, both LSCs and HSCs may be attacked similarly by RHAMM-directed high-avidity T cell-based therapies.
We observed that RHAMM expression in AML blasts is not static but cell cycle dependent, in accordance to a previous paper indicating that RHAMM is not constitutively expressed by all AML blasts, but only by a subpopulation (17). These findings are in line with earlier reports demonstrating that RHAMM expression in human primary fibroblasts and the HeLa cell line varies during cell cycle, as low expression was measured in G0/G1 cells and a peak in mRNA level was observed during the G2/M phase (32, 49). This expression pattern can be related to the intracellular function of RHAMM: it decorates the mitotic spindles and seems to be necessary for stable mitotic spindle formation and progression through the G2/M phase (8, 32, 41, 50-52). In addition, other studies demonstrated a direct or indirect correlation between RHAMM and proliferation, both in malignant cells and in physiological non-homeostatic settings such as wound healing and regeneration (18, 49, 51, 53-56). Therefore it is not surprising that in a variety of hematological and solid malignancies, RHAMM overexpression was described to be correlated with a poor prognosis (15-22). We strongly believe that this correlation is not only due to the function of RHAMM in cell motility and consequently invasiveness of tumor cells (41), but can also be attributed to RHAMM being a genuine ‘proliferation marker’. The major drawback of the proliferation-dependent expression of RHAMM is that non-malignant, actively dividing cells tend to express high levels of RHAMM too.

We confirm previous findings that freshly isolated CD34+ HSCs of healthy donors do not express RHAMM at mRNA level (10, 14). However, we observed a clear upregulation of RHAMM after *in vitro* expansion of CD34+ HSCs of healthy donors. These findings were confirmed *in vivo* during the process of CD34+ cell expansion in a mouse model for human stem cell transplantation. Because of ethical and practical reasons, we were unable to study CD34+ cells during the engraftment phase after hematopoietic stem cell transplantation (HSCT) in patients. However, we and others have shown that human CD34+ cells transplanted in irradiated immune deficient mice engraft in the bone marrow and expand dramatically during the first weeks after infusion (38, 57). As this mouse model mimics *in vivo*
engraftment of autologous (post-chemotherapy) or donor (allogeneic HSCT) hematopoietic stem cells, our data suggest a major limitation for immunotherapy targeting RHAMM: RHAMM-specific cytotoxic T cells might not be able to discriminate engrafting non-leukemic HSCs from leukemic cells, and therefore hamper recovery post-chemotherapy or engraftment of allogeneic HSCs in the context of allogeneic HSCT. A recent paper showed a reduction of colony-forming units of all lineages when freshly isolated HLA-A2+ CD34+ HSCs of healthy donors were co-incubated with TCR-transgenic lymphocytes specific for RHAMM in the context of HLA-A2, suggesting that freshly isolated CD34+ cells may express enough RHAMM to be killed by these T cells (27). However, it is more likely that CD34+ HSCs upregulated RHAMM expression in response to the growth factors added during the assay. Alternatively, since an allo-HLA-A2-restricted RHAMM-specific TCR was used, it is also possible that CD34+ cells were killed due to off-target promiscuity (58). Although, until today, no major toxicity was reported in RHAMM vaccination trials (28, 29), more potent immunotherapeutic strategies such as adoptive transfer of RHAMM-specific T cells might induce important hematological and non-hematological side effects.

We could not only show RHAMM expression in HSCs during hematological recovery but also in cytotoxic T cells proliferating upon immune activation. Our results are consistent with those of Leisegang et al. (59), who showed that survivin-specific T cells underwent fratricide when activated, because of the target antigen, survivin, being also expressed in activated T cells. In a recently published paper by the same group on RHAMM-specific T cells, this issue was not addressed in depth as it was not reported whether HLA-A2 negative or positive effector T cells were used for treatment and whether differential T cell toxicity was observed (27). Nevertheless, our data suggest that cellular therapy targeting RHAMM might be limited by in vivo fratricide of the RHAMM-specific T cells activated by the AML cells, and by collateral damage to neighboring activated T cells with unknown specificity. Similarly, vaccination strategies able to induce autologous RHAMM-specific T cells, could be of limited efficacy because of apoptosis of the responding cytotoxic T cells.
Although RHAMM is a well-known and abundant cancer-associated antigen, overexpressed in several hematological and solid malignancies, our data raise the question whether it should be used at all as a target for immunotherapy. We have not directly addressed whether certain splice variants may be uniquely associated with AML or malignancy in general. It is known that some truncated isoforms of the RHAMM protein have transforming properties when overexpressed in cell lines (50). In multiple myeloma, overexpression of mRNA of a splice variant without exon 4 was seen (19). If these truncated protein forms result in unique newly composed peptides specifically expressed in malignant cells, and one of those peptides binds with a high affinity to a major histocompatibility complex (MHC) class I allotype, new immunotherapeutic strategies could emerge that are more specific and therefore potentially safer and more efficient. However, our RT-qPCR results, using primers that were designed to amplify all four isoforms of RHAMM described until now, showed no expression in the LSCs, which excludes expression of known alternatively spliced mRNA isoforms. The dual function of RHAMM, involving both an extracellular and intracellular localization, makes it more complicated to address the RHAMM protein expression level. Extracellular expression of RHAMM results from a redistribution of intracellular pools by non-conventional export to the extracellular compartment, which is not necessarily associated with increased synthesis or improved stability of RHAMM mRNA or protein (41). Surface staining for RHAMM of AML cells with a monoclonal antibody could not demonstrate clear extracellular RHAMM expression on AML blasts in our hands (data not shown), in accordance with the predominant intracellular localization of RHAMM in AML blasts described earlier in immunohistochemical stainings (17). Furthermore, it is expected that epitopes processed from intracellular RHAMM by the target cells and presented on MHC class I complexes, will be by far the most important targets for recognition by cytotoxic T cells.

In conclusion, we have evaluated RHAMM as an immunotherapeutic target in the context of AML, guided by the internationally accepted criteria for prioritization of cancer-associated
antigens (1). The expression in LSCs is low to undetectable and RHAMM is not AML-specific, since it is also expressed in expanding healthy hematopoietic stem cells and activated T cells, two clinically relevant populations in the context of a future integrated AML treatment, consisting of the standard induction chemotherapy followed by immunotherapy as a consolidation treatment or an allogeneic HSCT followed by AML-directed donor lymphocyte infusions (DLI). Therefore, the two major benefits of immunotherapy over chemotherapy, the elimination of the LSC and reduction of the side effects, are not likely to be achieved by RHAMM-directed immunotherapy.

Authorship and Disclosures

SS performed and designed research, analyzed and interpreted data, wrote the manuscript. TK and BV designed and supervised research, interpreted data, wrote the manuscript. SV provided critical reagents and helped with the design of the research and the analysis of data. GG performed research, analyzed and interpreted data. J Philippé provided the immunophenotypic data of the AML samples. GV, YVC, TT, KT, GL, J Plum and IV provided critical reagents. ZNB provided ideas and reviewed the manuscript. The authors reported no potential conflicts of interest.
References


Table 1. Clinical, immunophenotypic and genetic characteristics of AML patients.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gender</th>
<th>Age (years)</th>
<th>De novo / relapse</th>
<th>Cytogenetics (karyotype and FISH)</th>
<th>CD34 (%)</th>
<th>FAB</th>
<th>WHO</th>
<th>FLT3-ITD mutation</th>
<th>NPM1-mutation</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>AML1</td>
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<td>34</td>
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<td>normal</td>
<td>80</td>
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<td>negative</td>
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<tr>
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<td>normal</td>
<td>30</td>
<td>M2</td>
<td>AML-NOS with maturation</td>
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<td>negative</td>
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<tr>
<td>AML3</td>
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<td>de novo</td>
<td>normal</td>
<td>29</td>
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<td>AML-NOS without maturation</td>
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<td>negative</td>
<td>bone marrow</td>
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<tr>
<td>AML4</td>
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<td>de novo</td>
<td>normal</td>
<td>50</td>
<td>M1</td>
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<td>negative</td>
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<tr>
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<td>52</td>
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<td>normal</td>
<td>&lt;1</td>
<td>M2</td>
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<td>normal</td>
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<tr>
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<td>Male</td>
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<td>de novo</td>
<td>inv(16)(p13;q22)</td>
<td>70</td>
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<td>AML with inv16 (type D)</td>
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<td>46XX,inv(7)(q11.2;q21) (constitutional)</td>
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<td>inv(16)(p13;q22)</td>
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<td>AML with inv16</td>
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<td>t(15;17)(q22;q21)</td>
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<td>ND</td>
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<tr>
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<td>de novo</td>
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<td>70</td>
<td>M4</td>
<td>AML with t(10;11), MLL-AF10</td>
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<td>negative</td>
<td>leucapheresis</td>
</tr>
<tr>
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<td>relapse</td>
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<td>71</td>
<td>M2</td>
<td>AML-NOS with maturation</td>
<td>negative</td>
<td>negative</td>
<td>bone marrow</td>
</tr>
</tbody>
</table>

Table 1. Clinical, immunophenotypic and genetic characteristics of AML patients. The age of the patients varies between 24 and 77. All AMLs are primary and cover a range of subtypes of AML according to both the World Health Organization (WHO) classification and the French-American-British classification (FAB) classification. CD34 (%) represents the percentage of CD34+ cells in the investigated samples. Abbreviations: ND: not determined; NOS: not otherwise specified; u.s.: unknown significance; NPM1 mutation: mutation of the nucleophosmin gene at chromosome 5q35; inv16: inversion 16; MLL-AF10: mixed-lineage leukemia gene at chromosome 11q23 fused with the AF10 transcription factor at chromosomal locus 10p12; FLT3-ITD: internal tandem duplication in the fms-like tyrosine kinase-3 gene; FISH: fluorescence in situ hybridization.
Figure legends

Figure 1. RHAMM expression profile in LSCs versus HSCs (A) From 13 AML samples and 4 samples of healthy donors 2 subpopulations were isolated by FACS sorting: CD34+/CD38– LSCs and the CD34+/CD38– control population from AML patients, and the CD34+/CD38– HSCs and CD34+/CD38+ subpopulation of healthy donor samples. From 5 AML patients the CD34– blast population was isolated too. Boxplot shows RHAMM expression levels measured by RT-qPCR on mRNA. Results were normalized to GAPDH expression and shown relative to the RHAMM transcript level in K562. The ○ symbol represents an outlier, with a relative expression of RHAMM of 0.74. There is no significant difference in RHAMM expression level between the CD34+/CD38– HSC samples and the CD34+/CD38+ healthy subpopulation (p=0.273 calculated by Wilcoxon test). Supplementary analysis showed no significant difference in RHAMM expression between the CD34– and the CD34+/CD38+ AML blasts for 5 investigated samples (p=0.893 calculated by Wilcoxon test). The CD34+/CD38– LSC samples have a significant lower RHAMM expression level compared with the CD34+/CD38+ AML control population (p= 0.001 calculated by Wilcoxon test). No statistically significant difference could be shown between the LSCs and the HSCs (p=0.296 calculated by Mann Whitney U test). (B) Intracellular staining for RHAMM on a representative AML sample (AML12). Plots are gated on the viable cells and show further gating strategy. Gated on the CD34+ AML cells (left dot plot), the right dot plot shows that intracellular RHAMM expression can be mainly found in the CD34+/CD38+ subpopulation. Gated on isotype control (not shown).

Figure 2. RHAMM expression before and after in vitro culture of AML cells (A) Cell cycle analysis of a representative bulk AML sample (AML8) before (day 0) and after in vitro culture (day 5). Freshly isolated AML samples are mainly in G0/G1 phase. Dean/Jett/Fox analysis after 5 days of in vitro culture shows 67% of cells in G0/G1 phase, 20.1% in S phase and 11.5% in G2/M phase. (B) RT-qPCR analysis on mRNA is shown for the expression of RHAMM in freshly isolated bulk AML samples (day 0) and in AML samples after 5 days of in vitro culture (day 5). Results were normalized to the geometric mean of the expression of GAPDH, R18S and YWHAZ, and are shown relative to the RHAMM transcript level in K562. Data shown are the mean values and standard deviations (SD) calculated from duplicate PCRs of the same sample. Results are shown for 2 representative AML
samples (AML8 and AML12). (C) Histogram demonstrating flow cytometric results of intracellular RHAMM expression on day 0 and day 5 after *in vitro* culture of a representative bulk AML sample (AML8). Compared with isotype staining (day0 = day5) (solid gray) and expression on day 0 (full line), clear upregulation can be seen after 5 days of culture (dotted line). (D) Dot plots are gated on the viable cells and show further gating strategy. Gated on the CD34+ AML cells (AML8; left dot plot), the right dot plot shows intracellular RHAMM expression after 5 days of *in vitro* culture. The expression in the CD34+CD38- subpopulation remains low, compared with the CD34+CD38+ population. Gated on isotype control (not shown).

**Figure 3. RHAMM expression in human cord blood-derived CD34+ cells pre and post *in vitro* expansion** (A) Boxplot showing RHAMM mRNA expression in freshly isolated CD34+ cells (day 0) and in CD34+ cells after 7 days of *in vitro* culture (day 7) of five different cord blood donors. RHAMM expression was measured by RT-qPCR on mRNA and results were normalized to the geometric mean of the expression of GAPDH, R18S and YWHAZ and shown relative to the RHAMM transcript level in K562. There is a significant difference in RHAMM expression level between day 0 and day 7 (p= 0.043 calculated by Wilcoxon test). (B) Histogram showing flow cytometric results of intracellular RHAMM expression on day 0 and after 7 days of *in vitro* culture of CD34+ cells. Gated on the viable CD34+ GFP- cells. Compared with isotype staining (day0 = day7) (solid gray) and with RHAMM expression on day 0 (full line), clear upregulation can be seen after 7 days of culture (dotted line). Results shown are representative of three distinct cord blood donors analyzed. (C) Cell cycle analysis of a representative CD34+ cord blood sample before (day 0) and after *in vitro* culture (day 7). Freshly isolated CD34+ cells are virtually all in G0/G1 phase. Dean/Jett/Fox analysis after 7 days of *in vitro* culture shows 63% of cells in G0/G1 phase, 29.3% in S phase and 7.3% in G2/M phase.
Figure 4. RHAMM expression pre- and post-transplantation of human cord blood-derived CD34+ cells in NOD/SCID mice

Eight adult NOD/SCID mice were intravenously injected with 2.5 x 10^5 cord blood-derived CD34+ cells. (A) Plots are gated on the viable cells, and show sorting strategy. Dot plots illustrate a representative mouse 2 weeks after transplantation of hCD34+ cord blood-derived cells. The percentage of hCD45+ mCD45- hCD34+ cells within the viable bone marrow cells is 5.79%. (B) Boxplot showing the results of RT-qPCR analysis for RHAMM mRNA in freshly isolated CD34+ cells (pre-injection = pre) and in CD34+ cells isolated from the bone marrow 2 weeks after intravenous injection (post). Results were normalized to the geometric mean of the expression of GAPDH, R18S and YWHAZ, and are shown relative to the RHAMM transcript level in K562. There is a significant difference in RHAMM expression level between the pre- and post- samples (p= 0.012 calculated by Wilcoxon test), with firm upregulation of RHAMM mRNA 2 weeks after transplantation.

Figure 5. RHAMM expression by in vitro activated T cells

(A) RT-qPCR on mRNA from T cell subpopulations (peripheral blood) of 2 different healthy donors. Bulk PBMCs were analyzed prior to and 1 respectively 5 days after in vitro activation by PHA. From the same donors the CD3+ TCRαβ+ and the CD3+ TCRαβ+CD8+ fractions were sorted and analyzed unstimulated, on day 1 and on day 5 of CD3/CD28/CD49d activation. The results were normalized to the geometric mean of the expression of GAPDH, R18S and YWHAZ, and are shown relative to the expression level in K562. Data shown are the mean values and SD calculated from two donors. (B) Histograms demonstrating flow cytometric results of intracellular RHAMM expression on day 0 and 5 days after in vitro activation by PHA of a representative bulk PBMC sample (left) and gated on the CD8+ T cell subpopulation (right). Compared with isotype staining (day0 = day 5; solid gray) and expression on day 0 (full line), clear upregulation can be seen after 5 days of stimulation (dotted line).
Figure 1
Figure 3
Figure 5