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

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Online Supplementary Design and Methods

Preparation and cryopreservation of samples from healthy volunteers and patients with acute myeloid leukemia

Bone marrow, cord blood, peripheral blood and leukapheresis samples from 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

K562 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and were 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). The OP9-GFP cell line was obtained from Dr. J. C. Zúñiga-Pflücker (University of Toronto, Toronto, Canada) and 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 to near confluence, harvested and split every 2 to 3 days.

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, UK), 100 ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF, Gentaur, Kampenhout, Belgium), 100 ng/mL granulocyte-colony stimulating factor (G-CSF, Gentaur), and 25 ng/mL stem cell factor (SCF, Amgen, Brussels, Belgium). After 5 days of culture, cells were re-analyzed by flow cytometry and real-time quantatitive polymerase chain reaction (RT-qPCR).

Isolation and culture of cord blood samples

Cryopreserved cord blood from healthy individuals was thawed and the $CD34^+$ cells were enriched by anti-CD34 MACS beads (Miltenyi, Leiden, The Netherlands), and subsequently cell sorted with the FACSAria II cell sorter (BD Biosciences, Erembodegem, Belgium) to obtain the viable [assessed by lack of propidium iodide (Invitrogen) intake] $CD34^+$ cells, to a purity of >99%, as determined by analysis after sorting. $CD34^+$ 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 *in vitro* expansion of human cord blood-derived $CD34^+$ cells. 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 $CD34^+CD45^+$ GFP-negative cells were sorted again [FACSAria II cell sorter (BD Biosciences)] to a purity of >99%.

T-cell activation

Bulk peripheral blood mononuclear cells from healthy donors were cultured in standard medium (described earlier) supplemented with 10 μg/mL phytohemagglutinin (PHA, Sigma-Aldrich, Bornem, Belgium) and 40 IU/mL interleukin-2 (IL-2, Roche, Anderlecht, Belgium) for the indicated period. Peripheral blood mononuclear cells were also sorted at day 0 with the FACSAria II cell sorter (BD Biosciences) to obtain the $CD3^+TCR\alpha\beta^+$ and the $CD3^+TCR\alpha\beta^+CD8^+$ 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 μg/mL, on an anti-CD3 antibody [OKT3, ATCC; 10 μg/mL in PBS (Lonza, Verviers, Belgium)] pre-coated 96-well plate (BD Falcon, Erembodegem, Belgium).

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: CD34 (Miltenyi), CD38 (Miltenyi), CD3 (BD biosciences), CD8β (BD biosciences); non-conjugated: RHAMM/HMMR/CD168 [Neuromics (Edina, Minnesota, USA)], 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 75% (Normapur, VWR, Heverlee, Belgium) 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 min 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^{scid}/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). Within 24 h 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 used immediately for injection without previous *in vitro* culture. A sample from each cord blood donor was retained for RT-qPCR. Mice were injected with 2.5x10^5 viable (assessed by lack of propidium iodide uptake) CD34$^+$ cells. After 2 weeks, mice were sacrificed and human CD34$^+$, human CD45$^+$, mouse 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-sorting analysis. Human and mouse FcR blocking reagents were used to block non-specific binding of antibodies.

Real-time quantitative polymerase chain reaction analysis

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 was performed with SYBR Green I technology 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 manufacturers' protocols. The primers (Operon and Biolegio) used were: RHAMM forward (fw) 5'-CAGGTCACCCAAAGGAGTCTCG-3'; RHAMM reverse (rv) 5'-CCACTTGATCTGAAGCACAACTAA-3'; GAPDH fw 5'-TGCACCACCAACTGCTTAGC-3'; GAPDH rv 5'-GGCATGGACTGTGGTCATGAG-3'; Ribosomal S18 protein (R18S) fw 5'- ATACATGCCGACGGGCGCTG -3'; R18S rv 5'- AGGGGCTGACCGGGTTGGTT-3'; YWHAZ fw 5'- ACTTTTGGTACATTGTGGCTTCAA -3'; YWHAZ rv 5'- CCGCCAGGACAAACCAGTAT -3'. Results were analyzed using the delta-delta Ct method.