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IDENTIFICATION OF A NEW HLA-A2 RESTRICTED T CELL EPITOPE WITHIN HM1.24 AS IMMUNOTHERAPY TARGET FOR MULTIPLE MYELOMA

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1. ABSTRACT

Objective: Aim of this study was the identification of HLA-A2-restricted T-cell epitopes within the HM1.24-antigen as target for multiple myeloma (MM) directed specific peptide-based immunotherapy.

Methods: The HM1.24 sequence was scanned for immunogenic peptides using the HLA binding prediction softwares “SYFPEITHI” and “BIMAS”. Peripheral blood mononuclear cells (PBMC) from HLA-A2$^+$ normal donors (ND) were stimulated with autologous HM1.24-peptide loaded dendritic cells (DC), and expanded *in-vitro*. Activation of T-cells was assessed by ELISpot and cytotoxicity by ^{51}Cr-release-assays. T2-cells pulsed with irrelevant peptide, the HM1.24$^-$/HLA-A2$^+$ breast-carcinoma cell-line MCF-7 and the HM1.24$^+$/HLA-A2$^-$ myeloma cell-line RPMI-8226 were used as negative controls. Expression of the HM1.24-gene (BST2) was assessed using purified plasma cells and Affymetrix-U133A+B-microarrays.

Results: Of the 8 nona-peptides with the highest probability of binding to HLA-A2, the HM1.24-aa22-30-peptide (LLLGIGILV) showed the highest activation of T-cells. The antigen-recognition by the HM1.24-aa22-30-specific CD8$^+$ T-cells was HLA-A2 restricted (ELISpot with HLA-A2-blocking antibodies: median, 15; range, 14-18 spots/well; isotype-control-antibodies: median, 47; range, 44-48). HM1.24-aa22-30-specific CD8$^+$ T-cells lysed HLA-A2$^+$ myeloma-derived cell-lines (^{51}Cr-release-assay: XG-1 vs. MCF-7, 91% vs. 0%; U266 vs. MCF-7, 38% vs. 4.2%; IM-9 vs. RPMI-8226, 22% vs. 0%). The HM1.24-gene was expressed at comparable levels by plasma cells from 65 MM-patients, 7 patients with monoclonal gammopathy of undetermined significance (MGUS), and 7 ND.

Conclusion: HM1.24-aa22-30 is a new HLA-A2-restricted T-cell epitope processed and presented by MHC-I-complexes. Specific CD8$^+$ T-cells can lyse MM cell-lines. We conclude that HM1.24-aa22-30 is a suitable candidate target for a specific peptide-based immunotherapy of MM.

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2. INTRODUCTION

Despite advances in chemotherapy and hematopoetic stem cell transplantation, MM is still an incurable malignancy. With highdosed alkylating drugs followed by an autologous stem cell transplantation, a complete remission can be achieved in 40% of the patients (Bjorkstrand *et al*, 1995, Attal *et al,* 2003). Allogenic stem cell transplantation however is the only curative therapy, but is hampered by elevated treatment related mortality (Crawley *et al,* 2005). The cure of certain patients with allogenic transplantation can be explained in part by the killing of MM cells (MMC) by allogenic donor's T cells. This is evidenced by the profound anti-tumor effect of infusions of donor T lymphocytes in patients relapsing after allogenic stem cell transplantation (Tricot *et al.* 1996).

One of the targets for a MM-specific immunotherapy is the activation of idiotype-specific T-cells by vaccination with monoclonal immunoglobulin or autologous DC loaded with the monoclonal immunoglobulin (Bergenbrant *et al*, 1996, Osterborg *et al*, 1998, Massaia *et al*, 1999, Reichardt *et al*, 1999, Titzer *et al*, 2000, Rasmussen *et al,* 2003, Coscia *et al,* 2004). Other antigens for T-cell immunotherapy of MM are MAGE-antigens, Sperm-17 and the MUC1 core protein (Treon *et al*, 2000, van Baren *et al*, 1999, Pellat-Deceunynck *et al*, 2000b, Lim *et al*, 2001, Chiriva-internati *et al,* 2002, Choi *et al*. 2005).

Goto et al. described HM1.24 as a type II transmembrane-glycoprotein with a molecular weight of 29 – 33 kD, expressed on terminal differentiated human B-cells (Goto *et al*, 1994). This antibody detects human myeloma cell lines, primary myeloma cells and normal plasma cells. The cloning of the HM1.24-gene showed a homology to the previously described bone marrow stromal protein BST2 gene (Ohtomo *et al*, 1999). Some data indicate that BST2 plays a role in the early development of B cells (Ishikawa et al, 1995). No HM1.24+ cells are found in the peripheral blood, liver, spleen, kidney, or heart of normal individuals (Goto *et al,* 1994). HM1.24 is strongly expressed by normal plasma cells or MMC.

Ozaki *et al.* demonstrated the anti-myeloma activity of anti HM1.24 antibodies in a BALB/c mouse model (Ozaki *et al*, 1997). The lysis of MMC by HM1.24 antibodies depends on HM1.24 density on the cell surface (Ohtomo *et al*, 1999).

So far HM1.24 immunogenic epitopes that can be recognized by T cells remain to be determined. The aim of this study was the identification of HLA-A2 restricted T cell epitopes within the HM1.24 antigen for future clinical applications like vaccination or the adoptive transfer of T-cells against multiple myeloma.

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3. MATERIALS AND METHODS

Cell-lines

The myeloma cell lines RPMI-8226 and U266, the EBV transformed plasma-cell line IM-9 and the breast adenocarcinoma cell line MCF-7 were obtained from the German Collection of Microorganisms and Cell Cultures [Braunschweig, Germany]. The hybrid T2 cell line which is deficient in transporter for antigenic peptides (TAP) protein was obtained from American Type Culture Collection [Manassas, VA, USA]. The XG-1 was from Pr B. Klein laboratory (Zhang *et al*, 1994). Cell lines were maintained in RPMI 1640 and 10% heat inactivated fetal calf serum (FCS), with IL-6 (10 ng/ml, R&D systems, Abingdon, Oxon, United Kingdom) for the XG-1 cell line.

Prediction and synthesis of immunogenic peptides within the HM1.24 antigen

HM1.24 sequence was scanned for HLA-A2 binding peptides using the prediction software "Syfpeithi" [Institute for Immunology, University of Tübingen, Germany] (Rammensee *et al.* 1995) and the "BIMAS" software [Section of BioInformatics & Molecular Analysis, National Institutes of Health, USA] (Parker *et al*. 1994). The 8 nona-peptides that had the highest scores both by "Syfpeithi" and "Bimas" were selected for further evaluation.

The predicted peptides were synthesised using standard procedures by the peptide-synthesis-department of the German Cancer Research Center Heidelberg [DKFZ, Heidelberg, Germany].

Peripheral blood mononuclear cells (PBMC) for the in vitro expansion of peptide specific T-cells

PBMCs from HLA-A2$^+$ NDs [Institute for Immunology, Universität Heidelberg, Germany] were purified using ficoll-paque density centrifugation [Biochrom, Berlin, Germany]. HLA-A typing was performed by northern-blot analysis in the Institute for Immunology [Universität Heidelberg, Germany].

In vitro obtaining of DC

PBMCs from HLA-A2$^+$ ND were used. Immature DC were obtained culturing plastic adherent PBMCs for 5 days with GM-CSF (800 U/ml, Molgramostim, Essex Pharma, München, Germany) and IL-4 (500 U/ml, R&D) and then induced into mature DC for 2 days with TNF-α (10 ng/ml, Sigma-Aldrich, Deisenhofen, Germany), IL-6 (1000 U/ml, R&D systems, Abingdon, Oxon, United Kingdom) and prostaglandin E2 (1 µg/ml, Sigma-Aldrich, Deisenhofen, Germany) (Tarte *et al*. 2000).

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Expansion of CD40-Ligand activated B-cells

Peripheral blood B-cells were activated and expanded with irradiated (90 Gy) CD40-ligand transfected fibroblasts (Schultze *et al*, 1997). After 7 day, activated B cells were used for T cell restimulation. They were pulsed with the HM1.24 peptides (10 µg/ml) and irradiated (30 Gy) and incubated with T cells at a ratio of 1 / 5.

In vitro Expansion of Peptide-specific T-cells with autologous DC and CD40-Ligand activated B-cells

DC were pulsed with HM1.24 peptides (10 µg/ml) for 2 h in serum free RPMI1640 medium and 5 x 10^5 peptide-loaded DC were incubated with 2,5 x 10^6 autologous PBMC in RPMI1640 and 5 % human AB-serum for 7 days . T cells were restimulated with autologous peptide-loaded CD40-ligand activated B-cells with IL-2 (50 U/ml). T-cell restimulations were repeated 3 times and the function of T cells was studied.

IFN-γ ELISpot-Assay

The specificity of the $CD8^+$ cells was looked for with IFN-γ ELISpot-assays. Expanded $CD8^+$ cells were purified using immunomagnetic methods (MACS-system, Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated with MMC or peptide-loaded T2 cells as targets for 48 h in anti-IFN-γ-antibody [Mabtech AB, Nacka, Sweden] coated nitrocellulose-plates [Millipore, Eschborn, Germany]. After detection with biotinylated anti-cytokine-antibodies [Mabtech AB, Nacka, Sweden] and conjugation with Avidin ALP [Sigma, Deisenhofen, Germany], the BCIP / NBT substrate was added [Sigma, Deisenhofen, Germany]. ELISpots were counted with a computer controlled microscope [Zeiss-Vision, Eching, Germany]. T2 cells were loaded with with HM1.24 peptides or control peptides by 2-hour incubation with 10 µg/ml of peptide. For screening of HM1.24 aa22-30 specific $CD8^+$ cells, we used a high 4:1 effector:target ratio to detect weak T-cell responses. In further experiments, lower effector:target ratios ranging from 0.125:1 to 1:1 were used. MHC I restriction was verified with blocking anti-HLA-A2 antibodies and isotype IgM antibodies as a negative control [One Lambda, Krefeld, Germany]. An activation of $CD8^+$ cells by a HM1.24 peptide was significant if at least twofold more spots were found compared to an irrelevant peptide and if if the spot number was at least 15 or more. All experiments were assed in triplets.

^{51}Chromium release-Assay

The cytotoxicity of HM1.24 specific CD8 cells was analysed using the ^{51}Chromium release-Assay. MM cell lines and T2 cells were labeled with ^{51}Cr. Target cells were seeded out in a 96 well round bottom plate and effector cells were added. After an incubation period of 4 h (37° C, 5% CO_2) 75 µl supernatant were harvested. The activity was measured using a β

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plate-counter [Perkin Elmer, Boston USA]. The spontaneous and maximal release was determined in the presence of either medium or 2% Triton X-100, respectively. The specific lysis was calculated as follows: ((test – spontaneous release) / (maximal release - spontaneous release)) x 100 = specific lysis %. Effector:target ratio ranging from 1.25:1 up to 7.5:1 were used. All experiments were assed in triplets.

Preparation of complementary RNA (cRNA) and microarray

CD138$^+$ plasma cells were purified from bone marrow aspirates of 7 NDs, 7 patients with MGUS, and 65 patients with MM (median age: 59 years; 12 patients were in stage IA, 12 in stage IIA, 38 in stage IIIA, and 3 in stage IIIB) after informed consent was given. The bone marrow cells were separated by density gradient centrifugation over ficoll-hypaque [Biochrom, Berlin, Germany]. Mononuclear cells where incubated with anti-CD138 coated microbeads [Miltenyi Biotech, Bergisch Gladbach, Germany], and sorted using an automated magnetic cell sorter [autoMACS, Miltenyi Biotech]. The purity of the enriched CD138$^+$ plasma and myeloma cells was assessed by flowcytometry (median, 92%; range, 81% to 99.8%).
Furthermore, the following 20 human myeloma cell lines (HMCL) were included in the analysis: XG-1 to XG-7, XG-10 to XG-14, XG-16, XG-19, XG-20, LP-1, U266, OPM2, RPMI-8226, and SKMM. IL-6-dependant HMCLs were obtained in our laboratory and were routinely maintained in RPMI1640, 10% fetal calf serum, and 2 ng/ml of IL-6.
RNA was extracted using the RNeasy Kit (Quiagen, Hilden, Germany), the SV-total RNA extraction kit (Promega, Mannheim, Germany) and Trizol (Invitrogen, Karlsruhe, Germany) in accordance with the manufacturer's instructions. Biotinylated complementary RNA (cRNA) was amplified with a double in-vitro transcription, according to the Affymetrix small sample labeling protocol. The biotinylated cRNA was fragmented and hybridized to the human U133A+B GeneChip microarrays according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Fluorescence intensities were quantified and analyzed using the GECOS software (Affymetrix). Arrays were scaled to an average intensity of 100. A threshold of 1 was assigned to values under 1. Expression levels of the Affymetrix probe-set 201241_at for the HM1.24-gene (BST2) were compared between our data and data obtained in normal body tissues available from the group of Dr. Hogenesch (http://symatlas.gnf.org/SymAtlas/),

Statistical analysis

Differences in the number of spots per well in the IFN-γ ELISpot experiments between T2 cells loaded with HM1.24 aa22-30 peptide compared to T2 cells loaded with an irrelevant peptide were analysed by Wilcoxon's signed rank test for paired data using the Statistica for Windows software (StatSoft, Tulsa OK, USA).

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4. RESULTS

Expression of HM1.24 on plasma cells, myeloma cell lines and primary myeloma cells

In figure 1 the expression of the HM1.24-gene assessed by U133A+B microarrays is shown. HM1.24-gene was expressed at comparable levels by myeloma cells from 65 MM-patients (median expression level according to U133A+B DNA microarrays, 222; range, 58 to 951), 7 MGUS-patients (median, 251; range,143 to 456), and plasma cells from 7 normal donors (median, 221; range, 88 to 339). The median expression level of HM1.24-gene was higher in the 20 human myeloma cell-lines analyzed compared to samples from ND, MM-, and MGUS-patients (median, 595; range, 108 to 1887) (see Figure 1).

For the comparison of our data with the expression level in a panel of normal tissues provided by the group of Dr. Hogenesch (http://symatlas.gnf.org/SymAtlas/), a threshold of 220 (representing the median value in purified plasma cell samples from MM patients) was defined. According to the data available from the group of Dr. Hogenesch (http://symatlas.gnf.org/SymAtlas/), HM1.24-gene was expressed at a level of 220 or higher in adrenal gland, liver, lung and ovary.

Prediction of immunogenic Peptides within the HM1.24 Antigen

Table 1 shows the 8 HM1.24 peptides with the highest propability of binding (predicted by “BIMAS” and “Syfpeithi”) and their respective score. While the exact ranking of peptides was different in the two binding prediction softwares, these 8 peptides with the maximum propability of binding were predicted by both the “Syfpeithi“ and the “BIMAS” software independently. The HM1.24 peptides listed in table 1 were synthesised and used for the subsequent T cell assays.

Immunogenic potential of HM 1.24-peptides

Table 2 summarizes IFN-γ specific ELISpot data with *in vitro* expanded HM1.24 peptide specific $CD8^+$ cells or influenza-matrix-protein specific T-cells (IMP aa58-66). TAP deficient T2 cells loaded with HM1.24 peptide, IMP peptide or irrelevant peptide were used as target cells. Only one out of the predicted HM1.24 peptides (HM1.24-aa22-30) can yield a reproducible (8 out of 11 experiments) and strong activation of CD8 T cells. For the seven remaining HM1.24 peptides, specific $CD8^+$ T-cells were generated at a lower frequency ranging from 1 out of 22 experiments (5 %) for HM1.24 aa31-39 to 6 out of 33 experiments (18 %) for HM1.24 aa126-134 (see table 2).

Figure 2 shows results of representative IFN-γ ELISpot experiments from three different donors after a 3 week stimulation of T-cells with the HM1.24 aa22-30 peptide. TAP deficient T2 cells pulsed with the HM1.24 peptide aa22-30 or HM1.24 aa126-134 as an irrelevant

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peptide were used as target cells. By using the Wilcoxon signed rank test, we found a significant activation of T-cells by HM1.24 aa22-30 peptide loaded T2 cells compared to T2 cells loaded with an irrelevant peptide (HM1.24 aa22-30 peptide loaded T2 cells: median: 40 spots/well range: 9-162 spots/well, negative control: median: 18 spots/well range: 1-61 spots/well $p = 0.007$). The possibility of an unspecific HM1.24 aa22-30 peptide binding to MHC-I molecules was analysed by a titration of the peptide concentration (data not shown).

HM1.24 aa22-30 specific CD8$^+$ T-cells against activated by HM1.24 aa22-30 pulsed T2 cells in various effector: target ratios

For the further analysis of HM1.24 aa22-30 specific CD8$^+$ T-cells, we conducted IFN-γ specific ELISpot assays using T2 cells pulsed with HM1.24 aa22-30 peptide as targets with various effector:target (ET) ratios (0.125:1 up to 1:1). As shown in figure 3, a maximum differential factor of 8.7 between T2 cells pulsed with HM1.24 aa22-30 compared to T2 cells pulsed with an irrelevant peptide was measured at an ET ratio of 0.25:1 (2.5×10^4 T2 cells incubated with 6.25×10^3 HM1.24 aa22-30 specific CD8$^+$ T-cells). Activation was observed up to an ET ratio of 0.125:1 (2.5×10^4 T2 cells incubated with 3.125×10^3 HM1.24 aa22-30 specific CD8$^+$ T-cells).

HLA-A2 restricted presentation of HM1.24 aa22-30

To analyse the HLA-A2 restriction of HM1.24 aa22-30, we set up an IFN-γ ELISpot assay with HM1.24 aa22-30 peptide specific CD8$^+$ T-cells and HLA-A2 blocking antibodies. In figure 5 a result of these experiments is shown. HM1.24 aa22-30 peptide specific CD8$^+$ T-cells were incubated with HM1.24 aa22-30 pulsed target cells (ET-Ratio: 0.25:1) in the presence of HLA-A2 blocking antibodies or isotype-matched control antibodies. The number of IFN-γ specific spots decreased from 46 per well in the test with the isotype-matched control antibody to 15 spots per well in the test with HLA-A2 blocking antibodies.

Cytotoxicity of HM1.24 aa22-30 specific CD8$^+$ T-cells against HM1.24 aa22-30 peptide loaded T2 cells

In figure 5 the result of the ^{51}Chromium release-assays is shown. 2×10^4 target cells / well were incubated with HM1.24 aa22-30 specific CD8$^+$ T-cells ranging from 1.5×10^5 T-cells / well down to 3.75×10^4 T-cells / well (effector:target ratio from 1.9:1 to 7.5:1). HM1.24 aa22-30 specific CD8$^+$ T-cells were able to lyse specifically peptide pulsed T2 cells corresponding to the results of the IFN-γ specific ELISpot-assays shown above, with a maximum specific lysis of 25.5% at an effector:target-ratio of 7.5:1.

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Activation of HM1.24 aa22-30 specific CD8$^+$ T-cells mediated by Myeloma Cell Lines

To evaluate the processing and presentation of the peptide HM1.24 aa22-30 by tumor cells to *in vitro* generated HM1.24 aa22-30 specific CD8$^+$ T-cells, we used HLA-A2$^+$ myeloma derived cell lines as targets. IFN-γ ELISpot-assays and ^{51}Chromium release-assays were performed.

Figure 6 shows the results of the IFN-γ ELISpot assay using HM1.24 aa22-30 specific CD8$^+$ T-cells as effector cells and the myeloma derived HLA-A2$^+$ cell lines IM-9 and U266 as target cells. 2.5 x 10^4 target cells / well were incubated with HM1.24 aa22-30 specific CD8$^+$ T-cells ranging from 2.5 x 10^4 T-cells / well down to 0.3125 T-cells / well (effector:target ratio from 0.125:1 to 1:1). The HLA-A2$^+$ mamma carcinoma cell line MCF7 was used as a negative control. In the case of the myeloma cell line IM-9 as target, we could detect a specific IFN-γ secretion for an effector:target ratio of 1:1 (308 spots, 8 spots with MCF7) down to an effector:target ratio of 0.125:1 (68 spots, 3 spots with MCF7). A reduced but still significant IFN-γ secretion was measured with the myeloma cell line U266 as target showing 100 spots for an ET of 1:1 down to 41 spots for an ET of 0.25:1 (8 and 3 spots, respectively, for the negative control with MCF7).

The cytotoxicity of HM1.24 aa22-30 specific CD8$^+$ T-cells against the HLA-A2$^+$ myeloma derived cell lines IM-9, U266 and XG-1 was analysed with a standard ^{51}Chromium release-assay using effector:target ratios ranging from 1.25:1 to 5:1. MCF7 and the HLA-A2$^-$ myeloma derived cell line RPMI 8226 were used as negative controls. In figure 7 the results are shown. Corresponding to the results obtained with the IFN-γ specific ELISpot assay a specific lysis was measured using IM-9 and U266 as target cells. Furthermore, a specific cytotoxicity was demonstrated for the myeloma derived cell line XG-1. In summary, these data indicate the processing and presentation of the HM1.24 aa22-30 peptide to specific CD8$^+$ T-cells by myeloma tumor cells.

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5. DISCUSSION

Several antigens for a specific immunotherapy against multiple myeloma have been described. The myelomal immunoglobulin might be an interesting tumor antigen. T cell epitopes within the variable region of the immunoglobulin were found by several groups (Fagerberg *et al*, 1999;Hansson *et al*, 2003;Trojan *et al*, 2000;Wen *et al*, 1998). However, several vaccination trials with the tumor Ig failed to demonstrate any benefit in patients with MM whereas anti-tumor Ig vaccination is a promising strategy in patients with B cell lymphoma. An explanation could be a deletion of the anti-tumor Ig T cell clones in patients with MM due to the high levels of circulating tumor Ig lasting for several years in the premalignat stage of the disease. Other antigens like MAGE, Sperm-17 or the MUC1 core protein are expressed on only a part of myeloma cells from a subset of patients (Lim *et al*, 2001;Paydas *et al*, 2001;Pellat-Deceunynck *et al*, 2000a;van Baren *et al*, 1999). In contrast HM1.24 is found on > 90 % of primary myeloma cells (Goto *et al*, 1994). Therefore the identification of T cell epitopes within the HM1.24 antigen may provide a new immunotherapy against multiple myeloma.

Unsing peptide predictor algorithm, IFN-γ ELISpot and cytotoxicity assay, we identified an HM1.24 peptide - aa22-30 – that is efficiently processed and presented by HLA-A2+ and HM1.24+ MMC. Of major interest, DC loaded with HM1.24 aa22-30 can efficiently activate and amplify HM1.24 aa22-30 specific $CD8^+$ cells in vitro.

We were able to expand HM1.24 aa22-30 specific $CD8^+$ cells from 8 out of 11 donors (73 %). Up to now there are few reports published focussing on the T cell response against HM1.24. Recently Chiriva-Internati et al. described the effective generation of HM1.24 specific CTL using recombinant adeno-associated virus-based antigen-loaded DC (Chiriva-Internati *et al*, 2003). Interestingly Chiriva-Internati et al. used donor T cells and myeloma targets matched for HLA-A1.

Rew et al. generated antitumor CTLs from patients with multiple myeloma *in vitro* by DC pulsed with HM 1.24 protein (Rew et al. 2005). The CTLs were able to lyse in a HLA-A2 restricted manner autologous myeloma cells but the immunogenic peptides were not identified. Our data show that HM1.24 aa22-30 is one of the HLA-A2 restricted immunogenic peptides.

HM1.24-aa126-134 peptide (sequence KLQDASAEV) was predicted by the BIMAS software with the highest probability of binding to HLA-A2 molecules. Our data showed that HM1.24 aa126-134 peptide specific T-cells were generated in 6 out of 33 experiments (18 %). Interestingly a further search with the syfpeithi program showed that Friede described HM1.24 aa125-139 as a HLA-DQA1*0301 / DQB1*0301 restricted T cell epitope (sequence HKLQDASAEVERLRR) (Friede, 2003). A possible explanation for the low frequency of HLA-

A2 restricted HM1.24 aa126-134 peptide specific $CD8^+$ T cell stimulations might be that for the stimulation of $CD8^+$ T-cells the assistance of $CD4^+$ T-cells is necessary. The simultaneous stimulation of CD8 and CD4 T-cells by overlapping epitopes was described by several authors (Carreno *et al*, 1992;van Binnendijk *et al*, 1993). Since HLA-DQA1*0301 / DQB1*0301 is a relatively rare HLA-type with a frequency below 30 % the low frequency of $CD8^+$ T cell stimulations could be correlated to the low coincidence of HLA-$A2^+$ / HLA-DQA1*0301^+ / DQB1*0301^+ donors. Our first results with HLA-$A2^+$ / HLA-DQA1*0301^+ / DQB1*0301^+ donors show a successful $CD8^+$ and $CD4^+$ T cell stimulation using the overlapping peptide HM1.24 aa125-139 in two out of two experiments. Further analyses concentrating on this aspect need to be done.

In conclusion, the current data demonstrate that HM1.24 aa22-30 is a good peptide candidate for vaccination of patients with MM with DC or the expansion of anti-MMC T cells in vitro.

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6. TABLES

Table 1 - predicted HM1.24 peptides

The table shows the eight peptides with the highest propability of binding to HLA-A2 predicted by "BIMAS" and "Syfpeithi".

HM1.24 Peptide	***Sequence***	***BIMAS score*** [#]	***BIMAS Ranking***	***Syfpeithi score*** [#]	***Syfpeithi Ranking***
aa21-29	KLLLGIGIL	33.914	7	29	2
aa22-30	LLLGIGILV	437.482	2	26	6
aa23-31	LLGIGILVL	34.246	6	29	3
aa27-35	GILVLLIIV	33.368	8	26	7
aa31-39	LLIIVILGV	271.948	3	30	1
aa126-134	KLQDASAEV	998.071	1	26	8
aa167-175	QLLIVLLGL	181.794	4	28	4
aa171-179	VLLGLSALL	134.369	5	28	5

[#] estimate of half time of disassociation of a molecule containing this subsequence

Table 2 - T cell activation

Table 2 shows an overview about the *in vitro* experiments using the predicted and synthesised HM1.24 peptides. The $CD8^+$ T cell activation was measured by IFN-γ ELISpot assays. The experiments were done with PBMCs from healthy $HLA\text{-}A2^+$ donors.

HM1.24 Peptide	***Sequence***	***Specific $CD8^+$ T cell activation*** ***n / number of experiments (%)***		***Range/ Media+*** ***spots/well***	***Range/ Median-*** ***spots/well***	***p-value***
aa21-29	KLLLGIGIL (520)	1/9	(11 %)	Range 3-85 Median: 22.5	Range: 4-78 Median: 16	0.17
aa22-30	LLLGIGILV (516)	8/11	(73 %)	Range: 9-162 Median: 40	Range 3-61 Median 18	0.007
aa23-31	LLGIGILVL (519)	1/6	(17 %)	Range: 2-100 Median: 13	Range: 2-102 Median: 7	0.68
aa27-35	GILVLLIIV (521)	1/6	(17 %)	Range 4-35 Median: 18.5	Range: 0-52 Median: 12	0.60
aa31-39	LLIIVILGV (416)	1/20	(5 %)	Range: 0-114 Median: 17	Range: 0-101 Median: 16.5	0.97
aa126-134	KLQDASAEV (415)	6/32	(19 %)	Range: 0-134 Median: 9	Range: 0-86 Median: 6.5	0.05
aa167-175	QLLIVLLGL (517)	1/7	(14 %)	Range: 1-82 Median: 15	Range: 1-73 Median: 16	0.24
aa171-179	VLLGLSALL (518)	1/6	(17 %)	Range: 2-105 Median: 15.5	Range: 2-59 Median: 13	0.46

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7. FIGURES

Figure 1 – Relative expression levels of the HM1.24-gene (BST2) according to the probe-set Affymetrix 201241_at

The HM1.24-gene expression of CD138$^+$ enriched plasma cells from normal donors (ND, n=7), donors with monoclonal gammopathy of undetermined significance (MGUS, n=7), patients with multiple myeloma (MM, n=65) and human myeloma cell lines (HMCL, n=20) was analysed by Microarray-analysis. The median of the relative expression level of each group is denoted as grey bar.

Figure 2 – T cell activation with HM1.24 aa22-30

ELISpot-assays with HM1.24 aa22-30 pulsed T2 cells as target cells were performed with an effector:target ratio of 4:1. T2 cells pulsed with HM1.24 aa126-134, T2 cells alone pulsed with HM1.24 aa22-30, and T-cells alone were used as negative controls. Shown are representative results (mean of triplets) from 3 of 8 different donors.

Figure 3 – HM1.24 aa22-30 specific CD8$^+$ T-cells against peptide pulsed T2 cells with various effector: target ratios

HM1.24 aa22-30 peptide specific CD8$^+$ T-cells were incubated with T2 target cells pulsed with HM1.24 aa22-30 (■) or with an irrelevant peptide (▲) using various effector:target ratios ranging from 1:1 to 0.125:1. The figure shows the results of the IFN-γ specific ELISpot assays (mean of triplets).

Figure 4 – HLA-A2 restricted presentation of HM1.24 aa22-30

HM1.24 aa22-30 peptide specific CD8$^+$ T-cells were incubated with HM1.24 aa22-30 pulsed T2 target cells together with HLA-A2 blocking antibodies or isotype-matched control antibodies. The figure shows the results of the IFN-γ ELISpot assays (mean of triplets).

Figure 5 – Cytotoxicity assay with HM1.24 aa22-30 specific CD8$^+$ T-cells

HM1.24 aa22-30 peptide specific CD8$^+$ T-cells were incubated with HM1.24 aa22-30 (■) or HM1.24 aa171-179 (▲) pulsed T2 cells as target cells in varying effector:target ratios ranging from 7.5:1 (0.5 x 10^4 HM1.24 aa22-30 pulsed T2 cells incubated with 3.75 x 10^4 HM1.24 aa22-30 specific CD8$^+$ T-cells) to 1.875:1. The figure shows results of the ^{51}Chromium release-assay with the specific lysis in %.

Figure 6 – Activation of HM1.24 aa22-30 specific CD8$^+$ T-cells using the Myeloma Cell Lines IM-9 and U266 as targets

HM1.24 aa22-30 peptide specific CD8$^+$ T-cells were incubated with the myeloma derived HLA-A2$^+$ cell lines IM-9 (■) and U266 (●) in effector:target ratios ranging from 0.125:1 to 1:1. The HLA-A2$^+$ mamma carcinoma cell line MCF7 (▲) was used as a negative control. The figure shows the results of the IFN-γ ELISpot assays.

Figure 7 – Lysis of Myeloma Cell Lines mediated by HM1.24 aa22-30 specific CD8$^+$ T-cells

HM1.24 aa22-30 peptide specific CD8$^+$ T-cells from 3 different donors were incubated with the myeloma derived HLA-A2$^+$ cell lines XG-1, U266 and IM-9 in varying effector:target ratios ranging from 1.25:1 to 5:1. The mamma carcinoma cell line MCF7 (HLA-A2$^+$, HM1.24$^-$) and the myeloma cell line RPMI8226 (HLA-A2$^-$, HM1.24$^+$) were used as negative controls. The figure shows results from ^{51}Chromium release-assays with the specific lysis in %.

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Figure 1

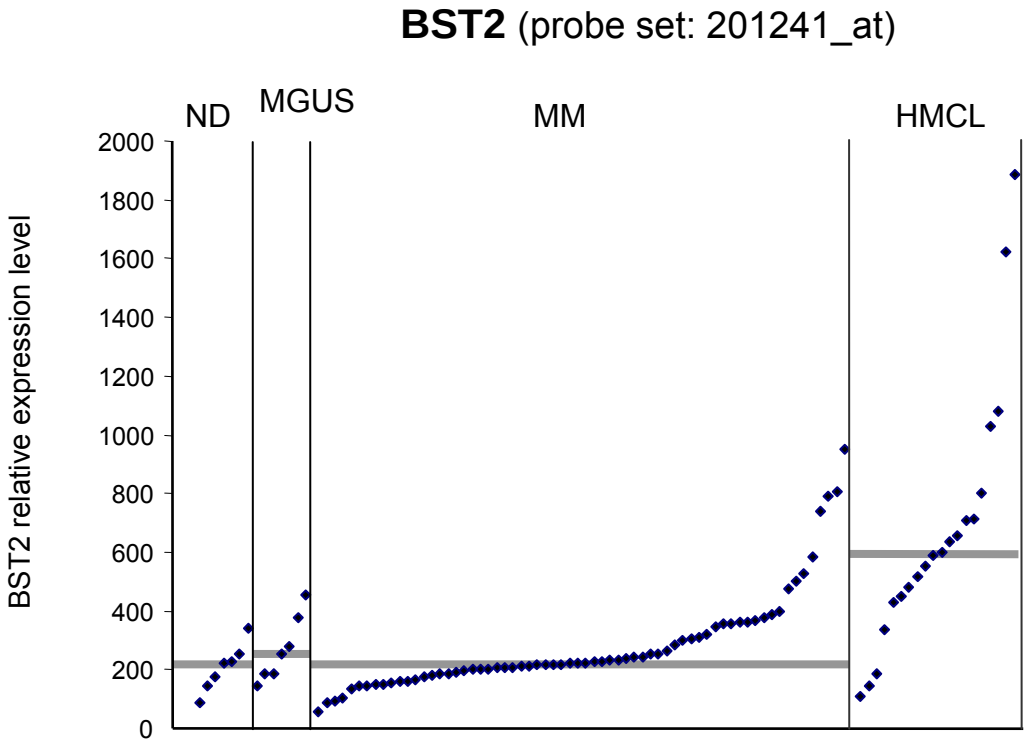

Figure 2

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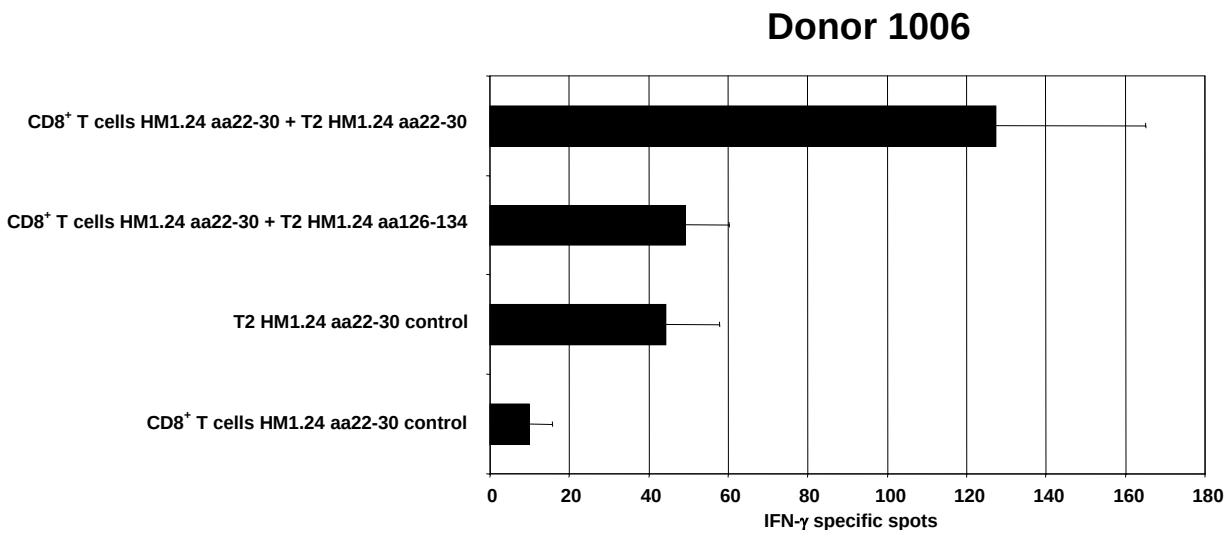

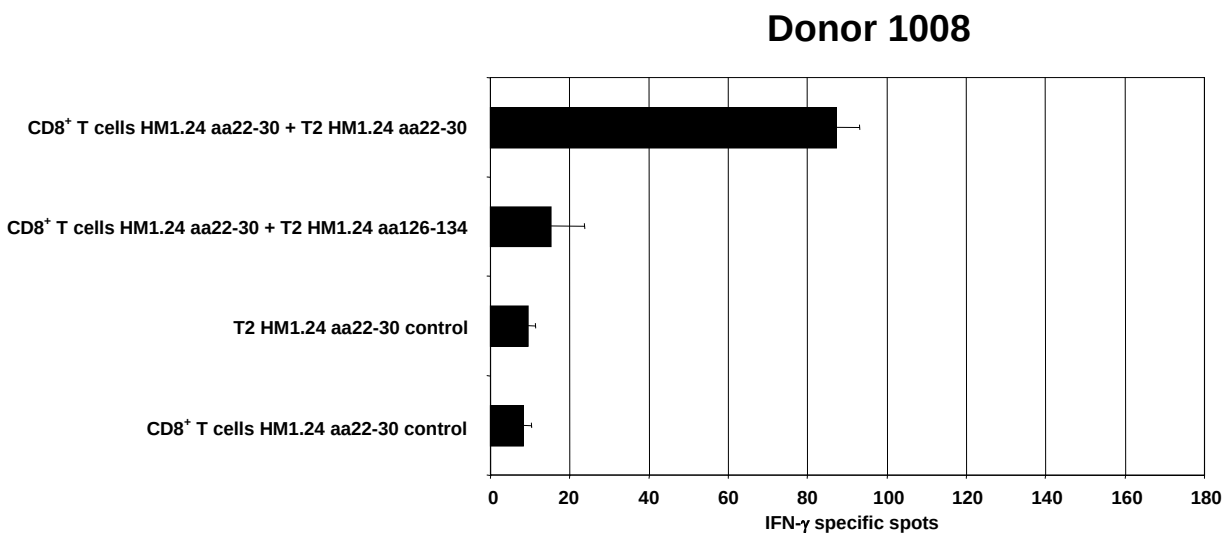

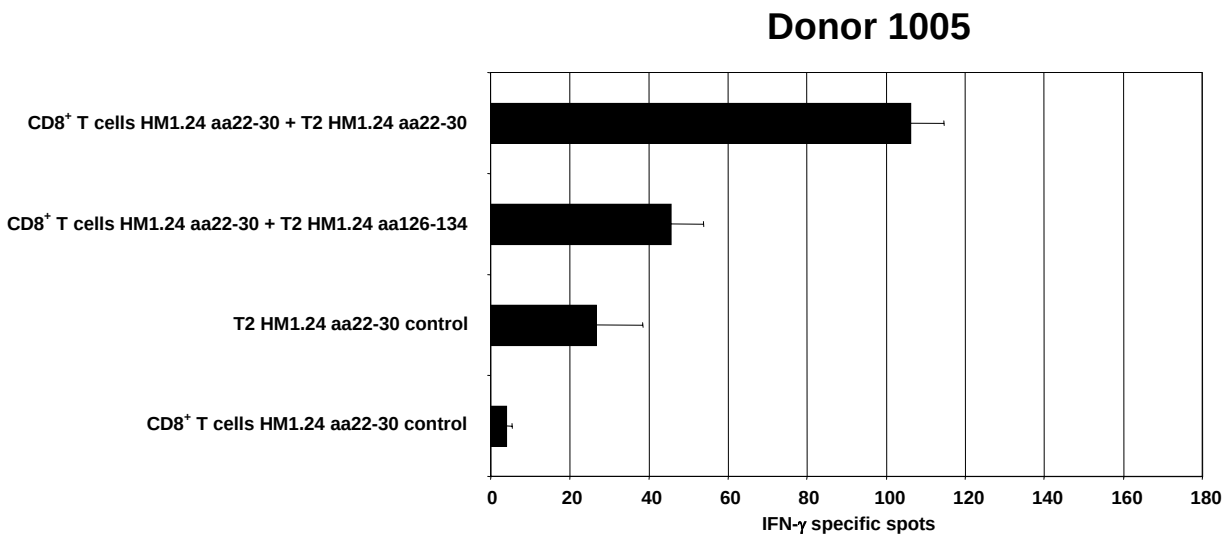

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Figure 3

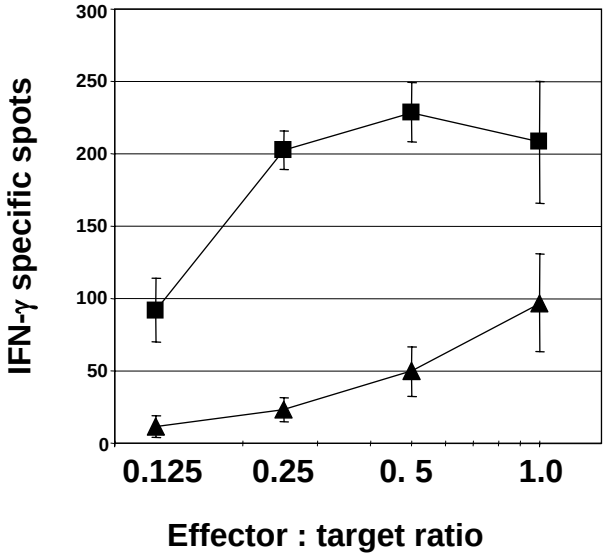

Figure 4

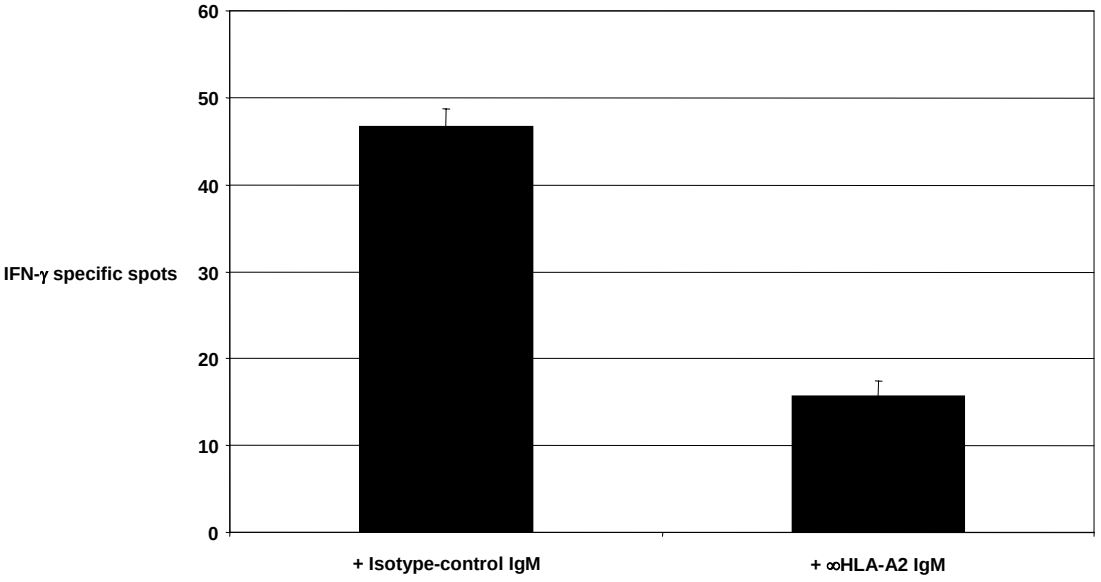

Figure 5

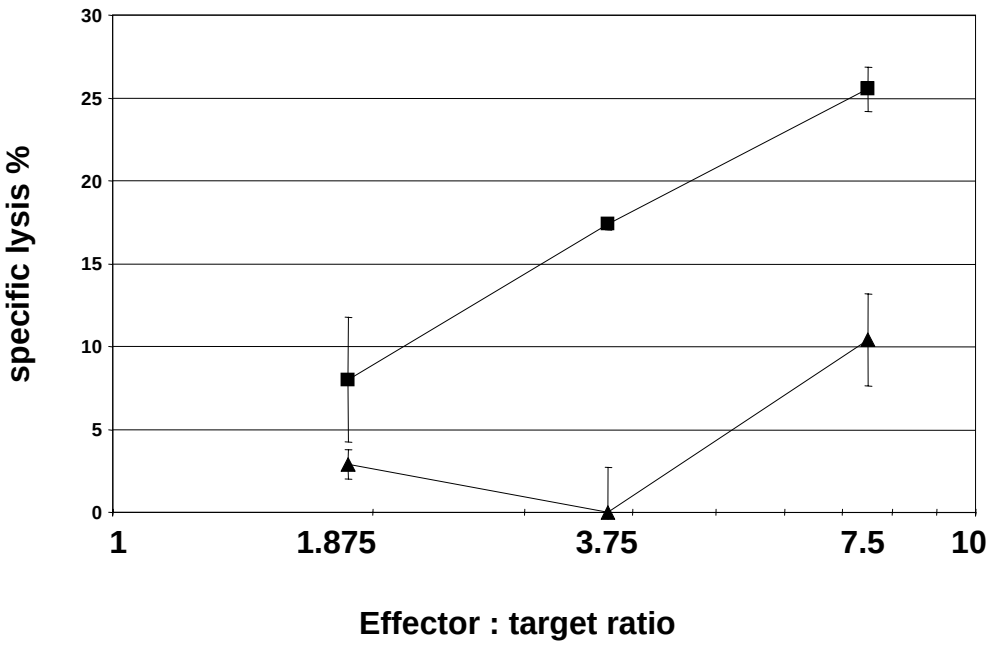

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Figure 6

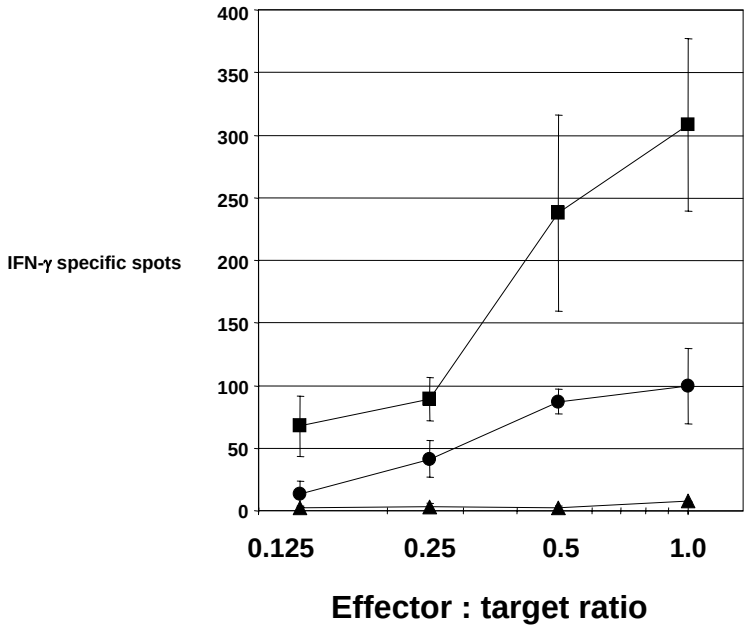

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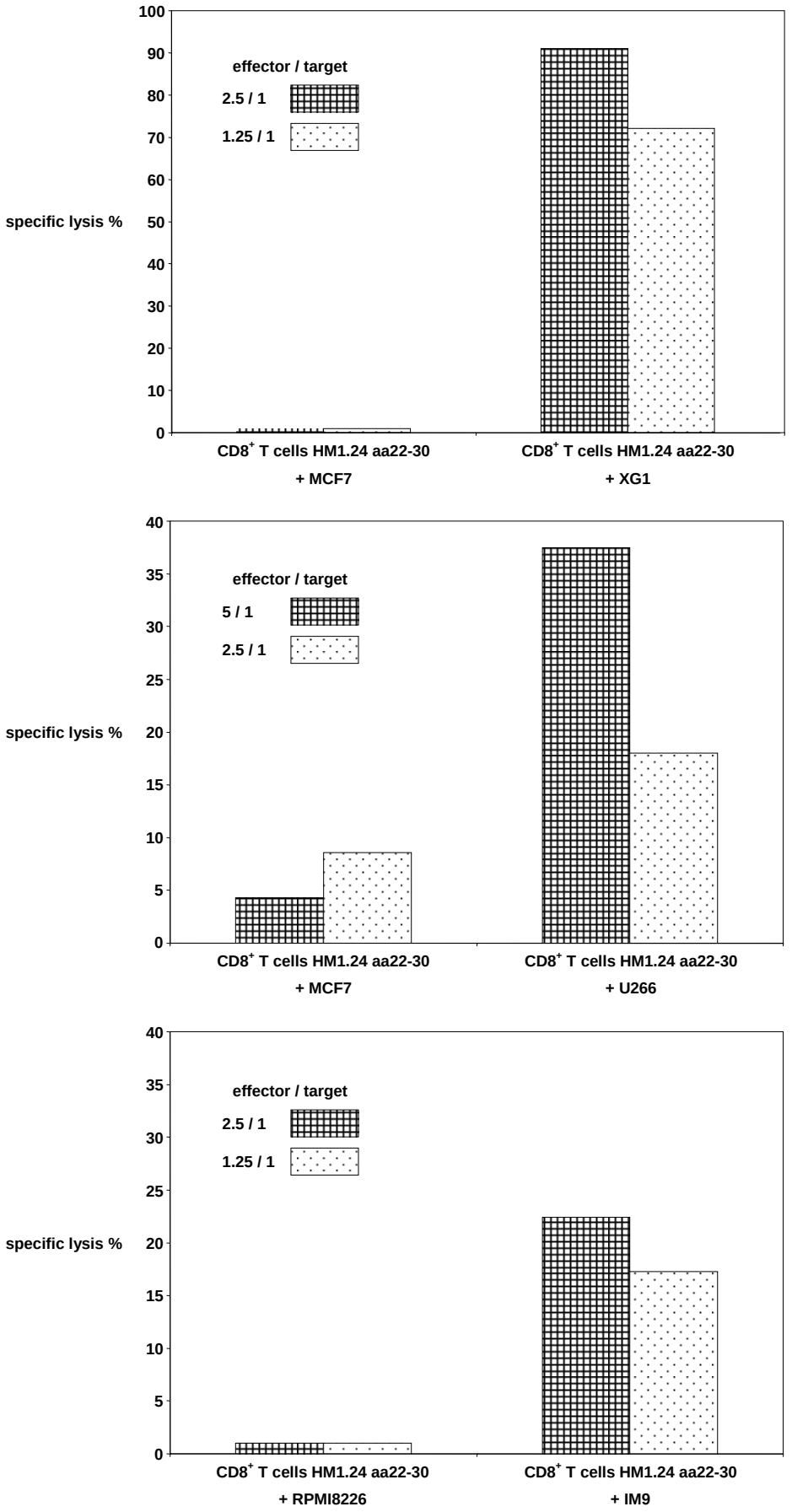
Figure 7



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