

IDENTIFICATION OF HLA-A2 RESTRICTED T-CELL EPITOPES WITHIN THE CONSERVED REGION OF THE IMMUNOGLOBULIN G HEAVY- CHAIN IN PATIENTS WITH MULTIPLE MYELOMA

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1. ABSTRACT

Objective:

The aim of this study is the identification of HLA-A2 restricted T-cell epitopes in the conserved region of the immunoglobulin-G-heavy-chain (IgG_H) that can be used for immunotherapy in Multiple Myeloma (MM) patients.

Methods:

After the IgG_H gene sequence was scanned for HLA-A2 restricted T-cell epitopes with a high binding affinity to the MHC-I-complex, promising nona-peptides were synthesized. Peptide specific CD8⁺ T-cells were generated from peripheral blood mononuclear cells (PBMC) of healthy donors (HD) and patients with MM using peptide pulsed dendritic cells (DC) *in vitro*. The activation and cytotoxicity of CD8⁺ T-cells was analysed by IFN- γ ELISpot-assay and ⁵¹Chromium release-assay. HLA-A2 restriction was proven by blocking T-cell activation with anti-HLA-A2 antibodies..

Results:

Two HLA-A2 restricted T-cell epitopes - TLVTVSSAS derived from the IgG_H-framework-region 4 (FR4) and LMISRTPEV from the constant region (CR) - induced expansion of specific CD8⁺ T-cells from PBMC in 2 out of 3 (TLVTVSSAS) and 1 out of 3 (LMISRTPEV) HD respectively. Specific T-cells were induced from PBMC in 2 out of 6 (TLVTVSSAS) and 8 out of 19 (LMISRTPEV) patients with MM. Specific CD8⁺ T-cells also lysed peptide-pulsed target cells in ⁵¹Chromium release-assay. LMISRTPEV specific CD8⁺T-cells from MM patients lysed specifically the HLA-A2⁺ IgG myeloma cell line XG-6.

Conclusion:

We identified two HLA-A2 restricted T-cell epitopes – TLVTVSSAS and LMISRTPEV – which can yield an expansion of CD8⁺ T-cells with the ability to kill peptide-loaded target cells and HLA-A2⁺ IgG⁺ myeloma cells. We conclude that TLVTVSSAS and LMISRTPEV could be T-cell epitopes for immunotherapy in MM patients.

KEYWORDS

Multiple Myeloma, T-cell epitope, dendritic cells, immunoglobulin heavy-chain, immunotherapy, vaccination.

2. INTRODUCTION

High dose chemotherapy (HDC) and autologous stem cell transplantation (ASCT) is considered to be the gold standard in MM treatment. Complete remission with a prolonged disease-free survival can be achieved in 25-40 % of the patients (1), depending on the clinical trial. Immunotherapy might be a therapeutic option to improve these therapies.

In B-cell neoplasia the immunoglobulin (Ig) produced by tumor cells is a clonal marker containing tumor-specific idiotype epitopes that can function as targets for T-cell-mediated immune responses (2). Other putative antigens which can be used to develop immunotherapy in patients with MM are cancer testis antigens, Sperm-17, MUC1 core protein and HM1.24 (3-9).

The concept of idiotype vaccination as a treatment for murine plasmacytomas was first mentioned by Lynch et al. 30 years ago (10). An activation of idiotype-specific T-cells through vaccination with tumorprotein or autologous tumor protein-loaded DC was demonstrated *in vitro* and *in vivo* (11-17). It was possible to reduce circulating clonal tumor cells by idiotype vaccination in patients with MM stage I (16). In patients with malignant lymphomas, idiotype vaccination can induce a complete molecular remission (18). The activation of cytotoxic T-cells specific for idiotype peptides seems to be a promising tool for immunotherapeutic protocols. However, it is a time consuming and expensive process to generate an individual specific idiotype vaccine for each patient.

Trojan et al. (19) analysed T-cell epitopes within the Ig of 65 HLA-A2⁺ patients with B-cell malignancies and demonstrated that peptides derived from the Ig framework region (FR) could induce cytotoxic T-cells able to lyse malignant B-cells. Harig et al (20) showed that induction of cytotoxic T-cell responses against Ig FR-derived peptides was feasible in HD, as well as in patients with B-cell malignancies, and that an improved T-cell activation can be induced by heteroclitic peptides with a higher affinity to the MHC-I molecule.

In this study we describe the identification of two novel peptides (TLVTVSSAS and LMISRTPEV) within the FR4 and CR of the IgG_H that are recognized by CD8⁺ T-cells. It is important to emphasize, that it was possible to generate peptide-specific T-cells which were able to lyse specifically a HLA-A2⁺ IgG⁺ myeloma cell line, indicating that *in vivo* tolerance of tumor specific T-cells might be overcome under optimal conditions.

The data suggests that TLVTVSSAS and LMISRTPEV are broadly applicable target structures for specific T-cells in future immunotherapeutic trials in the treatment of MM.

3. MATERIALS AND METHODS

Cell Lines

The HLA-A2⁺ myeloma cell lines XG-6 (IgG), XG-19 (IgA) and the HLA-A2⁻ myeloma cell line XG-13 (IgG) were from B. Klein's laboratory (21-23). The HLA-A2⁺ breast adenocarcinoma cell line MCF-7, the LP-1 myeloma cell line, the IM9 B-cell line and the K562 hematopoietic cell line were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The HLA-A2⁺ hybrid T2 cell line which is deficient in transporter-of-antigenic-peptide (TAP) protein was obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7, IM-9, LP-1, K562 and T2 were maintained in RPMI-1640, supplemented with 10% heat-inactivated FCS, L-glutamine and penicillin / streptomycin (Gibco Life Technologies, Eggenstein, Germany). XG-6, XG-13 and XG-19 were maintained in RPMI-1640, 10% heat-inactivated FCS and 2 ng/ml of human recombinant IL-6 (AbCys SA, Paris, France).

Prediction of peptides with high binding affinity to the MHC-1 complex within the IgG heavy-chain

The sequence of the IgG_H gene was scanned for HLA-A2 binding peptides using the prediction software "SYFPEITHI" (Institute for Immunology, University of Tübingen, Germany) (24) and the "BIMAS" software (Section of Bioinformatics & Molecular Analysis, National Institutes of Health, USA) (25). Four nona-peptides which scored high in "SYFPEITHI" and "BIMAS" were selected for further evaluation. Although TLVTVSSAS was not a high ranking peptide in the prediction software it was also evaluated, because of the position in the IgG_H gene in the FR4 and the amino acid (aa) leucine (L) at the HLA-A2 anchor position 2 amino acid as a positive criteria for a good binding capacity to the MHC-I complex. The predicted peptides were synthesized using standard procedures at 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⁺ HD (Institute for Immunology, Blutbank, University of Heidelberg, Heidelberg, Germany) or MM patients were purified using ficoll-paque density centrifugation (Biochrom, Berlin, Germany). HLA-A typing was performed with flowcytometry using a flow cytometer (FACS Calibur, Becton Dickinson).

In vitro obtaining of DC

Immature DC were obtained through culturing plastic adherent PBMCs from HLA-A2⁺ HD for 5 days with GM-CSF (800 U/ml, Molgramostim, Essex Pharma, München, Germany), and IL-4 (500 U/ml, R&D), and subsequently 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 μ g/ml, Sigma-Aldrich, Deisenhofen, Germany) (26).

In vitro Expansion of Peptide-specific T-cells with autologous DC and PBMC

DC were pulsed with IgG_H peptides (10 μ g/ml) in a serum free RPMI1640 medium for two hours, and 5×10^5 peptide-loaded DC were cultured with $2,5 \times 10^6$ autologous PBMC in RPMI1640 and a 5 % human AB-serum for 7 days. T-cells were then restimulated with autologous peptide-loaded PBMC and IL-2 (50 U/ml) for 7 days, this restimulation was repeated 3 times. At the end of the third restimulation, T-cell function was studied.

IFN- γ ELISpot-Assay

The expanded T-cells were CD8 purified using immunomagnetic methods (MACS-system [Miltenyi Biotec, Bergisch Gladbach, Germany]). Their specificity was assayed with an IFN- γ ELISpot-assay. Expanded CD8⁺ T-cells were incubated with peptide-loaded T2 cells as targets in anti-IFN- γ -antibody [Mabtech AB, Nacka, Sweden] coated nitrocellulose-plates [Millipore, Eschborn, Germany] for 48 h. T2 cells were incubated with IgG_H peptides or control peptides for 2 h (10 μ g/ml).

To evaluate T-cell activation in ELISpot, we used the criteria established 2002 by Keilholz et al. The number of spots had to exceed the control by ten spots and the difference between the single values of the three wells containing the relevant peptide and the three control wells, had to be significant measured with the unpaired t-test ($P \leq 0,05$) (27). As irrelevant peptide we used HLA-A2 restricted nonamers. In addition, the differences in the number of IFN- γ ELISpot per well between T2 cells loaded with TLVTVSSAS and LMISRTPEV or T2 cells loaded with an irrelevant peptide, were analysed with the Wilcoxon's signed rank test for paired data.

MHC-I restriction was verified using blocking anti-HLA-A2 antibodies or isotype-matched control antibodies [One Lambda, Krefeld, Germany]. After detection with biotinylated anti-cytokine-antibodies [Mabtech AB, Nacka, Sweden] and conjugation with Avidin ALP [Sigma, Deisenhofen, Germany], the substrate BCIP / NBT was added [Sigma, Deisenhofen, Germany]. The assays were analysed with a computer controlled microscope [Zeiss-Vision, Eching, Germany].

⁵¹Chromium release-Assay

The functional characteristics of the IgG_H specific cytotoxic T-Lymphocytes (CTL) were analysed using the ⁵¹Chromium release-assay. MM cell lines and T2 cells were used as target cells. Briefly, target cells were labelled with ⁵¹Cr and incubated for 2 h. After washing, 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% CO₂), 75 µl supernatant were harvested. The activity was measured using a γ-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 %. In figure 3 a and b we used an effector: target ratio of 5:1. In figure 4a and b effector: target ratios ranging from 1:1 up to 90:1 were used.

Sequencing the Ig heavy-chain

The sequencing of the IgG_H gene CDR and FR regions were performed using bone marrow samples obtained from 8 patients with newly diagnosed MM (data not shown). Total RNA was isolated and amplified in a consensus PCR after reverse transcription. FR1C, FR2A with VH5, VH6 (28) and FR3A (29) were used as sense -primers, LJH (29) or a mixture of JH1245, JH3 and JH6 (28) were used as antisense-primers. After amplification with an annealing-temperature of 61° C, PCR-products were separated by agarose gel electrophoresis. PCR-products were purified using the EasyPure DNA Purification Kit [Biozym, Göttingen, Germany], subsequently cloned and sequenced. The V-D-J-rearrangement of the malignant clone was identified by its repeated appearance.

For the sequencing of the FR3A – IgG_H Region of the IM9 and LP-1 cell lines, RNA was isolated and amplified in a consensus PCR after reverse transcription. cFR3A was used as sense primer and clgG_H as antisense- primer. PCR-products were purified using the EasyPure DNA Purification Kit [Biozym, Göttingen, Germany], subsequently cloned and sequenced.

4. RESULTS

Prediction of peptides with a high affinity to the MHC-I complex within IgG_H

Five selected peptides with a high probability of binding and their respective “BIMAS” and “SYFPEITHI” score were listed in Table 1. The TLVTVSSAS sequence within FR4 is nearly conserved in the LP-1 myeloma cell line, the EBV transformed cell line IM9 (data not shown) and the sequence were previously described by crystallography in monoclonal IgG_H from patients with MM (30, 31). The LMISRTPEV peptide is located within the CR of the IgG_H and was also previously described by crystallographic investigations in IgG_H of patients with MM (30, 31).

In vitro Expansion of peptide-specific T-cells

Table 2 summarizes the results of the performed IFN- γ specific ELISpot assays in HD and MM patients using *in-vitro* expanded peptide specific CD8⁺ T-cells from peripheral blood.

We significantly generated TLVTVSSAS specific T-cells using peripheral blood from 3 out of 5 ELISpots of HD (10000 CD8⁺ T-cells / well, median IFN- γ spot number 70, range 63 - 150, $P= .0039$), and 2 out of 6 MM patients (10000 CD8⁺ T-cells / well, median IFN- γ spot number 109, range 5 - 190, $P= .0012$) (table 2). Specific T-cells to LMISRTPEV were generated using peripheral blood from 1 out of 4 ELISpot of HD (10000 CD8⁺ T-cells / well median IFN- γ spot number 102.5, range 41 - 179, $P= .0195$) and 8 out of 19 MM patients (10000 CD8⁺ T-cells / well median IFN- γ spot number 60, range 6 - 227, $P < .0001$) (table 2). No difference in activation of T-cells was found in IgG MM patients compared to MM patients with other Ig isotypes (table 3). The other three peptides from the FR were not further studied because there was no significant activation of T-cells in ELISpot analysed with the Wilcoxon's signed rank test for paired data (table 2).

Figure 1a shows the numbers of IFN- γ ELISpots obtained from 3 different HD and 6 MM patients after a 2 to 3 week stimulation of T-cells with the TLVTVSSAS (457) peptide. T2 cells pulsed with the TLVTVSSAS peptide, or an irrelevant peptide, were used as target cells. A significant amplification of peptide-specific T-cells was found for 2 out of 3 HD and 3 out of 6 patients with MM. In two experiments, we used the longer 15-mer QGTLVTVSSASTKGP peptide (538) to activate and expand T-cells and a similar activation by TLVTVSSAS pulsed T2 cells was found in 1 patient (figure 1a). The LMISRTPEV peptide (578) was also very efficient in activating T-cells in 1 out of 3 HD and 8 out of 19 MM patients (figure 2a,b). The 15-mer KDTLMISRTPEVTC peptide (577) was used in 4 patient and showed yielding an efficient activation of T-cells by LMISRTPEV peptide-pulsed T2 cells in 3 patients (figure 2a).

HLA-A2 restricted presentation of TLVTVSSAS and LMISRTPEV

The activation of CD8 T-cells by T2 cells pulsed with TLVTVSSAS or LMISRTPEV peptides was blocked by HLA-A2 antibodies, while the isotype-matched control antibodies did not block the activation (Figures 1b, 2b and 2c). This indicated the HLA-A2 restriction of TLVTVSSAS and LMISRTPEV presentation.

Cytotoxicity of TLVTVSSAS and LMISRTPEV specific CD8⁺ T-cells against peptide pulsed T2 cells or myeloma cells.

In vitro generated TLVTVSSAS or LMISRTPEV specific CD8⁺ T-cells were able to efficiently kill T2 cells pulsed with the specific peptide, unlike T2 cells pulsed with an irrelevant peptide (lysis 31% vs 4,32% and 15% vs 1%; 15% vs 1% and 22,7% vs 1%) (Figures 3a and 3b) for an E:T-ratio of 5:1, while there was no lysis for an E:T-ratio of 1:1. In addition, we found that LMISRTPEV specific T-cells efficiently killed the HLA-A2⁺ IgG⁺ XG-6 myeloma cell line. As a control, these LMISRTPEV specific T-cells weakly killed the HLA-A2⁻ IgG⁺ XG13 myeloma cell line or the HLA-A2⁺ IgA⁺ XG-19 myeloma cell line in MM patients (figure 4).

5. DISCUSSION

The idiotype can be a highly specific tumor associated antigen (19, 32-34), but its use as a target for a peptide-based immunotherapy is compromised by the singularity of the idiotype antigen for each individual patient. In a cost and time consuming process one has to produce a patient-specific peptide. Peptides from conserved regions of the immunoglobulin like the FR-4 and CR are found on 100 % of primary IgG myeloma cells and can be used in a majority of the patients. Therefore, the identification of T-cell epitopes within these regions can provide a new tool for a common applicable immunotherapy against MM. To begin with, we scanned the amino acid sequence of the IgG_H gene for HLA-A2 restricted peptides with a high binding affinity to the MHC-I complex. We identified two peptides (TLVTVSSAS and LMISRTPEV) in the FR4 and CR of IgG_H. The TLVTVSSAS peptide was predicted by the BIMAS software with an average probability of binding to HLA-A2 molecules. In the literature TLVTVSSAS is described in crystallographic analysis to be a highly conserved part of the FR4 (30, 31). We found an aberration of one amino acid in LP-1 and two amino acids in IM-9 cell lines, compared to the sequence described in the literature. A Blast homology search showed that TLVTVSSAS is commonly expressed on plasma cells. LMISRTPEV has a high binding-score in the SYFPEITHI prediction software and because it is derived from the CR of the IgG_H (described in crystallographic analysis (30, 31) it was supposed to be presented by all the IgG myeloma cells.

We analyzed TLVTVSSAS / LMISRTPEV as potential new T-cell epitopes within FR-4 / CR in a gradual approach. In a first step we could show that peptide-specific T-cells could be generated in PBMC from HD. We then confirmed the HLA restricted specificity with antibody blocking experiments and could show the activation of TLVTVSSAS / LMISRTPEV-specific T-cells by DC pulsed with QGTLVTVSSASTKGP / KDTLMISRTPEVTCV the larger peptides. We could also show that the LMISRTPEV peptide is presented as an epitope on the XG-6 myeloma cell line, since LMISRTPEV-specific CD8⁺ T-cells could kill the HLA-A2⁺ IgG⁺ XG-6 myeloma cell line more efficiently than the HLA-A2⁺ IgG⁻ XG-13 myeloma cell line or the HLA-A2⁻ IgG⁺ XG-19 myeloma cell line.

In MM there are high amounts of immunoglobulin in the serum, so one could speculate that an anergy of the T-cells exists against epitopes from the immunoglobulin. We were able to generate and expand specific T-cells from patient with IgG myeloma and patients with other kinds of isotype, therefore we could show that specific T-cell can be generated, although these cells were exposed to high amounts of the targeted protein (Table 3).

So far there are only few reports published focusing on the T-cell response against FR of IgG. Recently Harig et al. described the effective generation of specific CTL using natural peptides and heteroclytic peptide from the FR of IgG (20). This indicates the absence of a T-

cell tolerance against the self peptides at least in an *in vitro* model. While Bogen described a negative correlation between the amount of monoclonal protein and the capacity of idiotype specific T-cells to protect against MM (35). Trojan (19) described a lower lysis of CLL-cells by specific T-cells directed against Ig-derived peptides, compared to normal B-cells, that may be due to the fact, that there is a lower expression of Ig-peptides on CLL cells. In MM, we expect an over expression of these peptides in case of IgG myeloma. Hansson et al. (33) found that the majority of the peptides with a high binding affinity to the MHC-I complex were found in the CDR rather than in the FR of patients with MM. Controversially, Trojan et al. (19) described the FR as location of peptides with a high binding affinity to the MHC-I complex.

A finding that needs to be discussed is that an immune response against FR4 / CR could result in a defect in the B-cell repertoire because normal B-cells express the FR4 peptides. From the clinical use of Rituximab, an antibody against CD20, we know that the treatment did not reveal severe side effects despite complete depletion of the CD20⁺ B-cell compartment.

Because TLVTVSSAS and LMISRTPEV are not found in IgA or IgD heavy-chain (36, 37), a clinical trial with the IgG_H peptides should be focused on patients with IgG myeloma which is the most common form of MM.

In conclusion this is the first report about the identification of the TLVTVSSAS T-cell epitope within the FR4 and the identification of the LMISRTPEV T-cell epitope within the CR. It was possible to generate specific T-cells against an antigen that is physiologically expressed on B-cells, as well as over expressed and secreted in the case of IgG myeloma. Therefore, we expect high amounts of this antigen in the serum. One could speculate, that there might be an anergy against these peptides, but we know from *in vitro* (14, 19, 38, 39) and *in vivo* data (11-15, 40, 41) from the investigation of idiotype-peptides, that this is not the case. Our data confirms these observations. Further *in vivo* studies need to evaluate the potency of TLVTVSSAS / LMISRTPEV as targets for a T-cell based immunotherapy against MM.

The fact that ultimately all patients with MM relapse even after HDC and ABSCT and the concomitant observation that even in the case of a “complete remission”, residual MM cells remain detectable, emphasize the interest of vaccination strategies that might allow eradicating residual MM cells. Vaccination could be initiated directly after HDC and graft of haematopoietic stem cells and also T cells (42) in order to use the “lymphodepletion window” associated with the effect of HDC and concomitant endogenous production of several cytokines, as reported in a murine melanoma model and patients with melanoma (43) (44).

This strategy is further promoted by the fact that in patients with MM, the efficacy of T-cells to pneumococcal antigens was stronger when they were injected 14 days after HDC rather than three months after HDC (45).

6. LEGENDS

Table 1. Predicted peptides.

The IgG_H gene-sequence of 8 patients (data not shown) was scanned for HLA-A2 restricted T-cell epitopes using the BIMAS and SYFPEITHI prediction software. The table shows 4 peptides derived from the framework region (FR) with the highest probability of binding to HLA-A2. The peptide from the constant region (CR) was identified in previous crystallographic investigations (30, 31).

Table 2. Detection of peptide-specific CD8 T cells with IFN- γ ELISpot-assay.

Experiments were done with PBMCs from HLA-A2⁺ HD (a) or HLA-A2⁺ MM patients (b). CD8⁺ T-cell activation was measured by IFN- γ ELISpot assay. To evaluate T-cell activation of each single T cell line in ELISpot, we used the criteria established 2002 by Keilholz et al. The number of spots had to exceed the control by ten spots and the difference between the single values of the three wells containing the relevant peptide and the three control wells, had to be significant measured with the unpaired t-test ($P \leq 0,05$) (27). All experiments were assed in triplets. We then analysed the statistical difference between the T2 cells loaded with TLVTVSSAS and LMISRTPEV or T2 cells loaded with an irrelevant peptide of all done T cell lines, with a Wilcoxon test for pairs.

Table 3. Patient characteristics.

26 patients with MM were tested for an *in vitro* generation of peptide-specific T-cells from peripheral blood by ELISpot or ⁵¹Chromium-release assay. Assays were conducted with peptide TLVTVSSAS (457) / QGTLVTVSSASTKGP (538) or LMISRTPEV (578) / KDTLMISRTPEVTCV (577). A plus (+) sign indicates a significant ELISpot-assay, cyt a ⁵¹Chromium-release assay. Status refers to the collection date of the patient PBMC.

Figure 1. HLA-A2 restricted presentation of TLVTSSAS peptide.

a. PBMC from HD or MM patients were stimulated three times with autologous PBMC pulsed with TLVTSSAS (457) or QGTLVTSSASTKGP (538). CD8⁺ T-cells were purified and stimulated with T2 cells pulsed with TLVTSSAS or an irrelevant peptide. The number of IFN- γ secreting T-cells was determined with an ELISpot-assay and compared with a Wilcoxon test for pairs. The error bars indicate the standard deviation. An asterisk (*) indicates a significant difference ($P \leq .05$).

b. TLVTSSAS (457) or QGTLVTSSASTKGP (538) peptide-specific CD8⁺ T-cells from 2 HLA-A2⁺ HD were incubated with TLVTSSAS pulsed T2 target cells and HLA-A2 blocking antibodies or isotype-matched control antibodies. T2 cells pulsed with an irrelevant peptide were used as negative control. The error bars indicate the standard deviation. Results were compared with a Wilcoxon test for pairs and an asterisk (*) indicates a significant difference ($P \leq .05$).

Figure 2. HLA-A2 restricted presentation of LMISRTPEV peptide.

a. PBMC from HD or MM patients were stimulated three times with autologous DC pulsed with LMISRTPEV (578) or KDTLMISRTPEVTCV (577). CD8 T-cells were purified and stimulated with T2 cells pulsed with LMISRTPEV or an irrelevant peptide. The number of IFN- γ secreting T-cells was counted by ELISpot-assay and compared with a Wilcoxon test for pairs. The error bars indicate the standard deviation. An asterisk (*) indicates a significant difference ($P \leq .05$).

b. LMISRTPEV (578) and KDTLMISRTPEVTCV (577) peptide specific CD8⁺ T-cells from one HLA-A2⁺ HD were incubated with LMISRTPEV pulsed T2 target cells together with HLA-A2 blocking antibodies or isotype-matched control antibodies. T2 cells pulsed with an irrelevant peptide were used as negative control. Results are the number of IFN- γ ELISpots and were compared with a Wilcoxon test for pairs. The error bars indicate the standard deviation. An asterisk (*) indicates a significant difference ($P \leq .05$).

c. LMISRTPEV (578) and KDTLMISRTPEVTCV (577) peptide specific CD8⁺ T-cells from two HLA-A2⁺ MM patients were incubated with LMISRTPEV pulsed T2 target cells together with HLA-A2 blocking antibodies or isotype-matched control antibodies. T2 cells pulsed with an irrelevant peptide were used as negative control. Results are the number of IFN- γ ELISpots and were compared with a Wilcoxon test for pairs. The error bars indicate the standard deviation. An asterisk (*) indicates a significant difference ($P \leq .05$).

Figure 3. Ig-peptide specific cytotoxic T cells

- a. TLVTVSSAS (457) and QGTLVTVSSASTKGP (538) peptide specific CD8⁺ T-cells from HD were incubated with TLVTVSSAS or irrelevant peptide pulsed T2 cells as target cells. T2 cells pulsed with an irrelevant peptide were used as negative control. The figure shows results from a ⁵¹Chromium release-assay with the specific lysis in %.
- b. LMISRTPEV (578) peptide specific CD8⁺ T-cells from two different MM patients were incubated with LMISRTPEV pulsed T2 cells as target cells. T2 cells pulsed with an irrelevant peptide were used as negative control. The figure shows results from a ⁵¹Chromium release-assay with the specific lysis in %.

Figure 4. Killing of HLA-A2+ IgG+ myeloma cells by Ig peptide specific T cells.

LMISRTPEV peptide specific CD8⁺ T-cells from a HLA-A2⁺ MM patient with a IgG monoclonal protein (patient 13) were incubated with the HLA-2⁺ IgG+ myeloma cell line XG-6, the HLA-A2⁻ IgG+ myeloma cell line XG-13, the HLA-A2⁺ IgA+ myeloma cell line XG-19 and the cell lines MCF-7 and K562. The figure shows results from a ⁵¹Chromium release-assay with the specific lysis in %.

Tables

Table 1 Predicted peptides

<u>sequence</u>	<u>region</u>	<u>HLA type</u>	<u>BIMAS score</u>	<u>SYFPEITHI</u>
			T _{1/2} min #	<u>score</u>
QLVESGAEV	FR 1	A2	285.163	26
SLRASDTAI	FR 3	A2	0.793	21
GLVKPGGSL	FR 1	A2	2.777	24
TLVTVSSAS	FR 4	A2	27.324	14
LMISRTPEV	CR	A2	85.394	27

Estimate of half time of disassociation of a molecule containing this subsequence

Table 2 Detection of peptide-specific CD8 T cells with IFN- γ ELISpot-assay

a.

Epitope	IFN-γ ELISPOT-assay positive [n]	Wilcoxon signed rank test p-value
QLVESGAEV	1 [7]	0,1284
SLRASDTAI	3 [10]	0,3222
GLVKPGGSL	2 [11]	0,219
TLVTVSSAS	3 [5]	0,0039
LMISRTPEV	1 [4]	0,0195

b.

Epitope	IFN-γ ELISPOT-assay positive patients [n]	Wilcoxon signed rank test
TLVTVSSAS	2 [6]	0,0012
LMISRTPEV	8 [19]	<0.0001

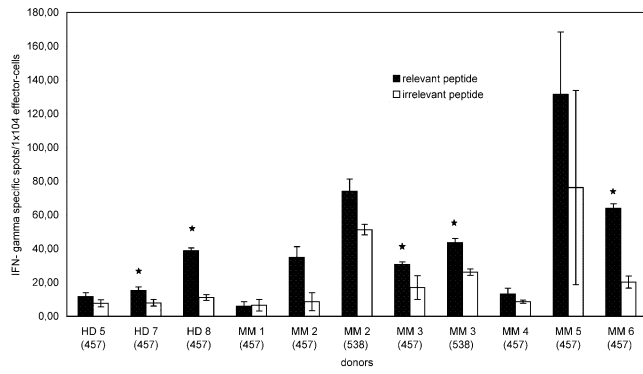
Table 3. Patient characteristics.

Patient number	Stage	Sex	Isotype/lightchain	Tested Peptide	Status	Monoclonal protein serum/urine-level at diagnosis
1	IIA	M	IgG kappa	- 457/-538	remission	68.0 g/l
2	IIIA	M	BJ kappa	- 457/- 538	during chemotherapy	n.e.
3	IIA	M	IgA lambda	+ 457/+538	remission	n.e.
4	IIIA	M	IgG lambda	-457/-538/-578	during chemotherapy	37.1 g/l
5	IA	M	IgG kappa	+ 457	remission	27.9 g/l
6	IIIA	F	IgG kappa	+457	remission	56.0 g/l
7	IIIA	M	IgG lambda	- 578/ -577	remission	100.0 g/l
8	IIA	F	IgG kappa	+ 578	remission	44.8 g/l
9	IIIB	M	IgD kappa	+578/ +577	remission	n.e.
10	IIIA	M	BJ lambda	-578/+577	prior chemotherapy	2320 mg/d
11	IA	F	IgA kappa	-578	during chemotherapy	16.3 g/l
12	IIIA	M	BJ kappa	+ 578	progressive disease	665 mg/d
13	IIA	M	IgG kappa	- 578/+578 cyt	prior chemotherapy	62.1 g/l
14	IIIA	F	BJ lambda	- 578	remission	87 mg/d
15	IA	M	IgG kappa	-578	prior chemotherapy	41.5 g/l.
16	IIIA	M	BJ kappa	+ 578	remission	n.e.
17	IIIA	F	IgA kappa	+ 578/ cyt	remission	39.2 g/l
18	IIIA	M	IgG kappa	- 578/+577	remission	33.1 g/l
19	IIIA	M	IgG kappa	- 578	during chemotherapy	n.e.
20	IIIA	M	IgG kappa	+ 578	remission	n.e.
21	IIIA	M	BJ kappa	-578	remission	n.e.
22	IIIA	F	BJ kappa	- 578/-577	remission	8960 mg/l
23	IIIA	M	IgA lambda	Cyt	remission	53.3 g/l
24	IIB	F	IgG kappa	Cyt	remission	12.0 g/l
25	IIIA	F	IgG kappa	+578/+577	remission	45.0 g/l
26	MGUS	M	IgG kappa	+578/+577	prior chemotherapy	15.5 g/l

Figures

Figure 1. HLA-A2 restricted presentation of TLVTSSAS peptide.

a



b

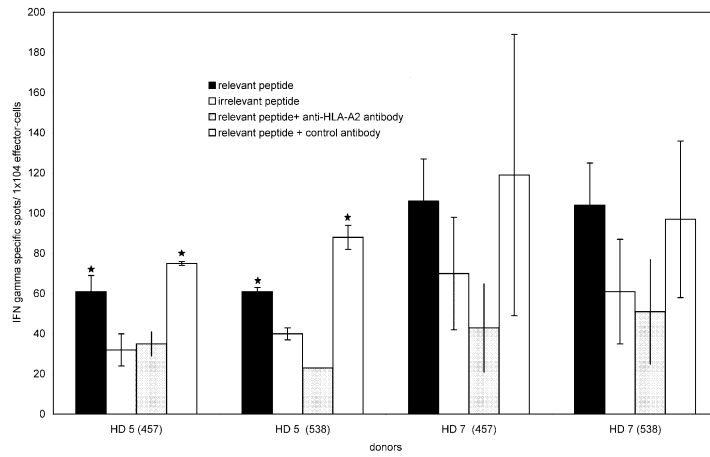
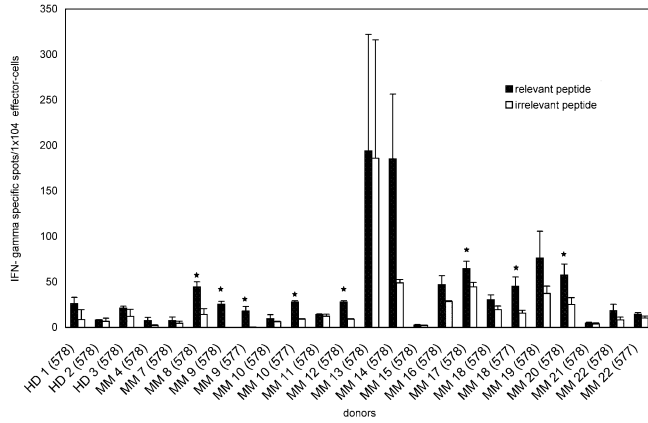
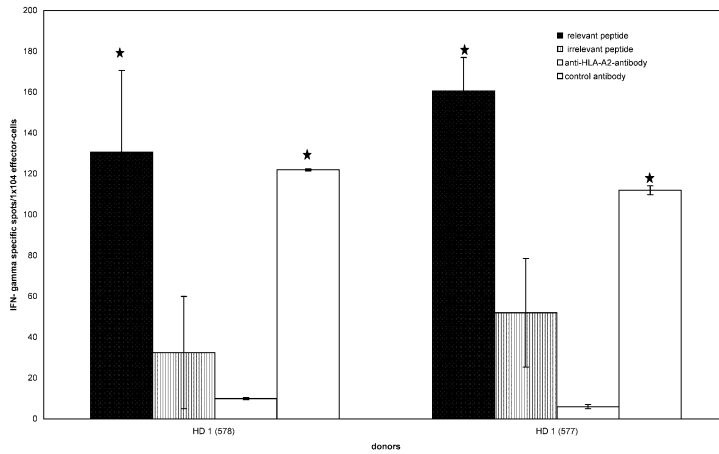


Figure 2. HLA-A2 restricted presentation of LMISRTPEV peptide.

a



b



C

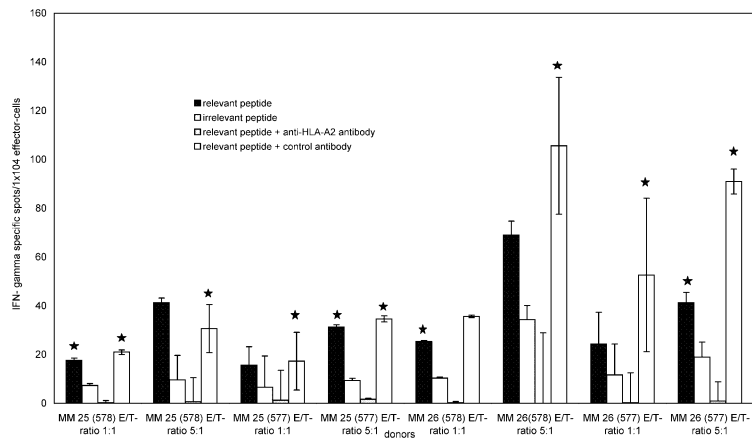
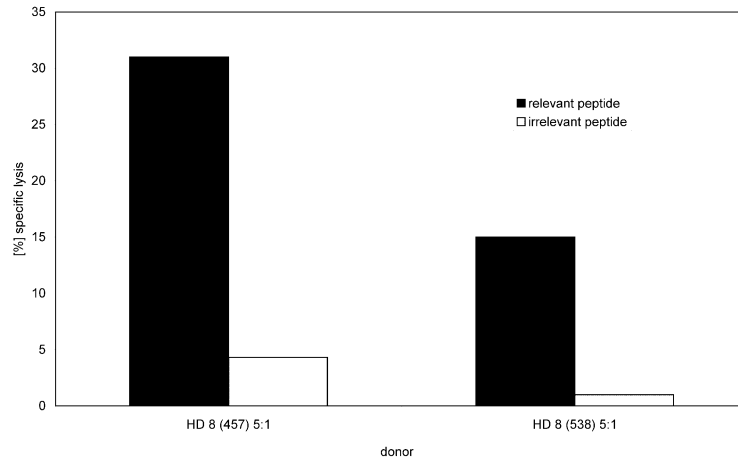


Figure 3. Ig-peptide specific cytotoxic T cells

a



b

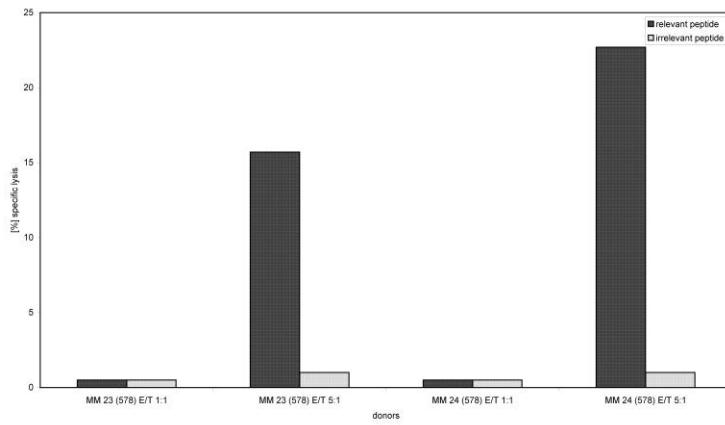
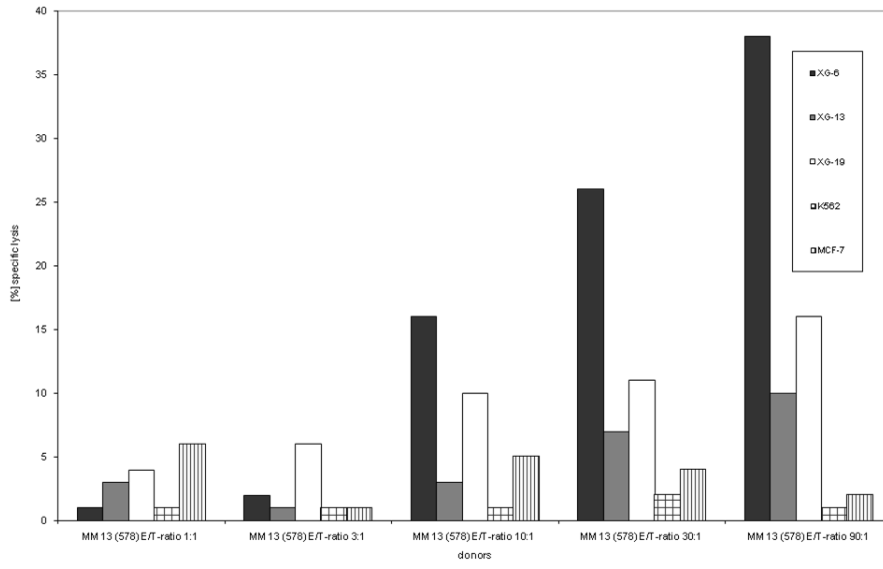


Figure 4. Killing of HLA-A2+ IgG+ myeloma cells by Ig peptide specific T cells.



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