

**ASSOCIATION OF ANTIGEN-SPECIFIC T-CELL RESPONSES WITH ANTIGEN-EXPRESSION AND
IMMUNOPARALYSIS IN MULTIPLE MYELOMA**

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STATEMENT OF TRANSLATIONAL RELEVANCE

Despite a variety of therapeutical approaches including high dose chemotherapy with subsequent autologous stem cell transplantation, Multiple Myeloma remains an almost incurable disease. New treatment options, like T-cell based immunotherapy seem to be a promising approach. In this study, we evaluate the impact of immunosuppression and antigen-expression on the development of specific CD8⁺ T-cell responses against myeloma associated antigens in patients with different stages of plasmacell-dyscrasias.

Our analysis shows that antigen specific immunoresponses correlate with the expression of the antigen and an early stage of the disease. In conclusion, a T-cell based immunotherapy in patients with plasmacell-dyscrasias might be performed in an early stage of the disease, where the tumor associated immunosuppression is low and it might be possible to establish a prophylactic immune response against antigens that were not yet expressed.

Abstract

Cancer testis antigens are immunotherapeutical targets aberrantly expressed on multiple myeloma cells, especially at later stages, when a concomitant immunoparesis hampers vaccination approaches.

We assessed the expression of the multiple myeloma antigen HM1.24 (reported present in all malignant plasma-cells) and the cancer testis antigens MAGE-A2/A3 and NY-ESO-1 (aberrantly expressed in a subset of myeloma patients), in CD138-purified myeloma cells by qRT-PCR (n=149). In a next step, we analyzed the antigen-specific T-cell responses against these antigens by IFN- γ EliSpot-assay (n=145) and granzymeB ELISA (n=62) in relation to stage (tumor load) and expression of the respective antigen.

HM1.24 is expressed in all plasma-cell samples, whereas cancer testis antigens are significantly more frequent in later stages. HM1.24 specific T-cell responses, representing the immunological status, significantly decreased from healthy donors to advanced disease. For the cancer testis antigens, the probability of T-cell responses increased in early and advanced stages compared to healthy donors, paralleling increased probability of expression. In advanced stages, T-cell responses decreased due to immunoparesis.

In conclusion, specific T-cell responses in myeloma are triggered by antigen-expression but suppressed by tumor load. Future cancer testis antigen-based immunotherapeutical approaches might target early plasmacell-diseases to establish

prophylactically a specific T-cell response against late stage antigens in immunocompetent patients.

Introduction

Multiple myeloma (MM) is a rarely curable malignant disease of clonal plasma cells which accumulate in the bone marrow causing clinical signs and symptoms related to the displacement of normal hematopoiesis, formation of osteolytic bone lesions, and production of monoclonal protein (1, 2). MM cells harbor a high median number of chromosomal aberrations (3-5) and multiple changes in gene expression compared to normal bone marrow plasma cells (6-9). The corresponding myelomatous bone marrow is significantly altered due to factors aberrantly expressed by myeloma cells, those that are already expressed by normal plasma cells but present in higher abundance, and such expressed by a variety of cells of the (changing) bone marrow microenvironment (6-8, 10-12). One of the most prominent effects thereof is the induction of immunosuppression (13) visible in dysfunctional dendritic cells (DC) and diminished T-cell activation (13, 14).

In this paper, we evaluate the impact of immunosuppression and antigen-expression on the development of specific CD8⁺ T-cell responses in patients with different stages of plasmacell-dyscrasias. For the first question, we use an antigen constitutively expressed on normal and malignant plasma cells (HM1.24) to evaluate the influence of stage and tumour-load on immunosuppression. For the second, we investigate the association of the aberrantly expressed cancer testis antigens (CTA) MAGE-A2/A3 and NY-ESO-1 with specific T-cell responses.

HM1.24 is a type II transmembrane-glycoprotein with a molecular weight of 29 – 33 kDa, expressed on terminally differentiated human B-cells, in MM and infrequently on lung and renal cancer cells (11, 15). We identified the immunogenic oligo-peptide HM1.24_{aa22-30} (LLLGIGILV) derived from the HM1.24 antigen as a new HLA-A2 restricted T-cell epitope (16) and also showed a cross-reactivity of HM1.24_{aa22-30} (LLLGIGILV) with the Melan-A/MART-1 derived peptide Melan-A_{aa26-35}*A27L

(ELAGIGILTV) (17) indicated by their sequence conformity at the central peptide position (GIGIL). Emphasizing the immunogenic nature of the HM1.24 derived oligopeptides, Rew et al. showed the MHC-I restricted generation of HM1.24 specific cytotoxic T-cells by autologous peripheral blood mononuclear cells in patients with MM (18). Recently, Harada et al. found an upregulation of HM1.24 on the surface of MM cells induced by lenalidomide (19).

HM1.24 is thus a promising target for an immunotherapeutical approach in patients with plasmacell-dyscrasias.

CTAs are physiologically expressed in germ cells, but aberrantly in various cancer types, including MM (5, 20, 21). This expression pattern minimizes the likelihood of an immunological tolerance (22), and indeed, CTA show a strong immunogenicity *in vivo* in various cancers (23). As expected per definition, CTA are not expressed on normal bone marrow plasma cells and at varying frequencies in MM cells (20, 21). We also showed a correlation between the expression of CTA analysed by gene expression microarrays, RT-PCR and immunocytochemistry (21). The probability of expression increases with advanced stage and in relapsed MM (5). CTA have likely a pathophysiological role, as their expression is associated with adverse prognosis (8, 24, 25). Furthermore, in a functional silencing assay, MM cells expressing CTA show a survival benefit *in vitro* (7).

Regarding the extensive data from clinical vaccination trials with the MAGE-antigens and NY-ESO-1 in malignant melanoma it is obvious that peptide based vaccination schedules have the potential to induce remissions in some patients, while most of the patients so far, did not benefit from immunotherapeutical treatments for a longer time (26).

Like HM1.24, CTA are thus a promising target for an immunotherapeutical approach in MM, although to our knowledge, no completed CTA based clinical trial in MM is published up to now. Regarding the evolution of the malignant clone in MM from MGUS and smoldering myeloma to MM stage III a question arises: what is the best time point to vaccinate on the background of presumably stage dependent increase in likelihood of antigen-expression and concomitant onset of immunoparesis?

Materials and Methods

Patients and healthy donors

Patients presenting with previously untreated plasmacell-dyscrasias (n=149) at the University Hospital of Heidelberg as well as 37 healthy donors (HD) have been included in the study approved by the ethics committee (#229/2003, S-152/2010, S-369/2002) after written informed consent. Patients were diagnosed, staged and response to treatment assessed according to standard criteria (27, 28) and subdivided into early plasmacell diseases (EPD: MGUS, and MM stage I) and advanced plasmacell diseases (APD: MM stage II and III).

Samples

Normal bone marrow plasma cells and MM cells were purified as previously published (29-33). To analyze the generation of antigen-specific T-cells, 50 ml peripheral blood was drawn.

Mononuclear cells (MC) for *in vitro* expansion of peptide-specific T-cells

MCs from HLA-A2⁺ patients / HD were purified using density centrifugation (Biochrom, Berlin, Germany). HLA-A typing was performed by flow cytometry, as previously published (28).

Synthesis of peptides

The peptides MAGE-A2_{aa157-166} (YLQLVFGIEV), MAGE-A3_{aa271-279} (FLWGPRALV), NY-ESO-1_{aa155-167} (QLSLLMWITQCFL), Melan-A_{aa26-35}*A27L (ELAGIGILTV) and HLA-A2 restricted irrelevant control peptide (LLIIVILGV; as a control for unspecific, non-tumor antigen mediated T-cell activation) were synthesized by the peptide-synthesis-

department of the German Cancer Research Center Heidelberg (DKFZ, Heidelberg, Germany) using standard procedures.

In vitro generation of dendritic cells (DC)

MCs from HLA-A2⁺ patients / HD were used. Immature DC were obtained culturing plastic adherent MCs for 5 days with GM-CSF (800 U/ml, Molgramostim, Essex Pharma, München, Germany) and IL-4 (500 U/ml, R&D). Afterwards, differentiation into mature DC was induced by stimulation 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) for 2 days as previously published (28).

Antigen-specific T-cell response

To assess the antigen-specific T-cell response, we expanded antigen-specific T-cells from patients with plasmacell disease and HD by autologous DC pulsed with MAGE-A2_{aa157-166} (YLQLVFGIEV), MAGE-A3_{aa271-279} (FLWGPRALV), NY-ESO-1_{aa155-167} (QLSLLMWITQCFL) or Melan-A_{aa26-35}*A27L (ELAGIGILTV) peptides respectively and analyzed the IFN- γ secretion by EliSpot-assay and the granzymeB secretion of T-cells by ELISA-assay.

IFN- γ EliSpot-assay

The generation of antigen (MAGE-A2, MAGE-A3, NY-ESO-1, HM1.24) specific CD8⁺ T-cells was analyzed by IFN- γ EliSpot-assay. Expanded CD8⁺ cells were purified with immunomagnetic beads (MACS-system, Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated with peptide-loaded T2 cells as targets (effector / target-ratio 1:5) for 22h 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), 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 described peptides or a control peptide during 2-hour incubation with 10µg/ml of peptide.

GranzymeB ELISA

GranzymeB secretion of CD8⁺ T-cells was analyzed in the supernatant by ELISA as described elsewhere (34). Briefly, expanded CD8⁺ cells (2×10^4 / well) were purified with immunomagnetic beads (MACS-system, Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated with peptide-loaded T2 cells as targets (effector / target-ratio 1:5) for 22h according to the manufacturer's instructions (BD OptEIA™ ELISA Sets, BD Biosciences, USA) was used. The intensity of the colour was analyzed with an automated plate reader.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was extracted by using the RNeasy Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. After reverse transcription of 50 ng total RNA using the GeneAmp® RNA PCR Core Kit (Applied Biosystems, Weiterstadt, Germany), qRT-PCR was performed with TaqMan® Gene Expression Assays (Applied Biosystems, Weiterstadt, Germany). Amplification and fluorescence detection were carried out on the ABI Prism 7700 SDS analytical thermal cycler (Applied Biosystems, Weiterstadt, Germany). As reference, the human myeloma cell line (HMCL) OPM-2 expressing NY-ESO-1 and MAGEA2/3 was used (data not shown). Presence of expression of a respective gene was defined as reaching a

plateau in less than 38 qRT-PCR cycles. Relative gene expression was only assessed in case detectable expression as defined above. For each sample the level of expression of the respective gene was normalized to 18S RNA ($\delta\text{Ct} = \text{Ct gene} - \text{Ct 18S}$) and related to the values obtained for the HMCL OPM-2 using the following formula $100/2^{\delta\delta\text{Ct}}$ where $\delta\delta\text{Ct} = \delta\text{Ct patient sample} - \delta\text{Ct OPM-2}$.

Statistical analysis

In an individual patient, the IFN- γ EliSpot-assay was considered as positive (EliSpot-pos) when the median number of IFN- γ spots in 3-5 wells of T-cells stimulated by relevant peptide-loaded T2 cells was at least twofold the median number of IFN- γ spots in 3 - 5 wells of T-cells activated by T2 cells loaded with control peptide according to Keilholz et al. (35). The frequency of EliSpot-pos and negative EliSpots (EliSpot-neg) in patients with EPD and with APD was compared by a Chi-square test. Statistical analysis of ELISA and gene expression by RT-PCR was performed by a Wilcoxon rang sum test. Statistical tests were significant if p-values are $\leq .05$. Non-significant p-values $> .05$ and $\leq .5$ are reported for descriptive reasons only.

Results

Expression of HM1.24 and cancer testis antigens

Expression of HM1.24 was detectable by RT-PCR in all CD138⁺ malignant plasma cell samples in EPD and APD (n=67). The CTAs MAGE-A2, MAGE-A3 and NY-ESO-1 were almost not expressed in samples from patients with EPD and in 4.7%, 38% and 29% of the APD samples (positive gene expression was defined by reaching a plateau in less than RT-PCR 38 cycles). Of these, MAGE-A3 and NY-ESO-1 are significantly more frequently expressed in APD vs. EPD ($p = .002$ and $p = .004$, figure 1).

T-cell responses against HM1.24

A strong T-cell response against the HM1.24 antigen assessed by IFN- γ EliSpot-assay could be found in HD and patients with EPD (figure 2A). The T-cell response is significantly decreased in patients with APD compared to HD or EPD ($p = .008$, $p = .03$, figure 3). Accordingly, the amount of granzymeB, a marker for activation of cytotoxic T-cells, in the supernatant of activated antigen-specific T-cells decreased from HD to APD ($p = .003$, figure 4). Both results indicate an immunosuppressive mechanism on T-cell responses in advanced stages of plasmacell-dyscrasias.

T-cell responses against CTA

The antigen-specific activation of T-cells displayed by their IFN- γ secretion as analyzed by EliSpot-assay (figure 2 and 3) showed almost no specific T-cell activation against the CTA in HD. We found a higher frequency of antigen-specific T-cells in patients with EPD against the MAGE-A3 and NY-ESO-1 antigen, although only a low frequency of gene expression was detected by RT-PCR (figure 1). While

the frequency of T-cell responses against MAGE-A2/3 increased in APD, the frequency against NY-ESO-1 decreased (figure 3). Regarding the granzymeB secretion of antigen-specific T-cells upon antigen-specific stimulation, only patients had measurable levels of granzymeB secretion, while almost no secretion was detected in HD (figure 4).

Discussion

In this study, we evaluate the impact of immunosuppression and antigen-expression on the development of specific CD8⁺ T-cell responses in patients with different stages of plasmacell-dyscrasias. Of the four CTA used, HM1.24 is expressed on all normal and malignant plasma cells (36). In contrast, MAGE-A2, MAGE-A3 and NY-ESO-1 are not expressed on normal bone marrow plasma cells but aberrantly in malignant plasma cells (21, 36).

In our study, the specific T-cell responses against HM1.24 was used to analyse the general stage dependent impact of myeloma-specific immunosuppression. The strong T-cell response against HM1.24 crossreactive peptide Melan-A_{aa26-35}*A27L (ELAGIGILTV) observed in HD is in agreement with previous reports (37). The occurrence of these T-cells in a high frequency in HD can be attributed to cross-reactivity against a broad panel of bacterial and viral antigens (38). The significant decrease in T-cell response from HD to EPD and concomitantly to APD indicates an increasing immunoparalysis, eventually due to a defective *in vitro* generation of DC and antigen-specific T-cells in APD. Pfeiffer et al. and Ratta et al. showed however, that *in vitro* culture conditions can circumvent the *in vivo* impaired DC-function of MM patients (39). As evidenced by the lack of T-cell responses against CTA in HD, no *de novo* T-cell responses are induced by *in vitro* activation of specific T-cells by DC, but rather a multiplication and expansion of already existing T-cell responses against expressed antigens. This makes our *in vitro* model a model for studying the *in vivo* events in MM patients and reflects the expansion of specific T-cells in the MM patient. The increasing immunological deficiency in APD is in line with recent reports that describe the abnormal immune function in advanced stages of the disease (40) especially regarding the T-cell compartment (41).

T-cell responses against CTA are influenced by two antidromic mechanisms: An increased probability of expression increasing-, and the immunosuppression evidenced by HM1.24 in later stages decreasing the probability of a T-cell response (Figure 2-4). These mechanisms can be manifest in different ways: An increase of expression outweighing immunosuppression (MAGE-A2/A3), or the other way round (NY-ESO-1).

It is interesting to denote that in a proportion of EPD an immune response against CTA is detected, although no expression of these antigens can be detected. One possible explanation is that CTA are expressed in these patients at a very low level, or in a subclone only. Another possibility is cross-reactivity between CTA (42). As we found almost no T-cell responses in HD, a false positive T-cell response in EPD is very unlikely.

In terms of the generalizability of our data, two additional lines of experiments would be desirable. First, in addition to CTL response against T2 cells, in terms of addressing T cell defects in MM, antigen specific T cell response against MM cells should be demonstrated in an autologous setting. Secondly, T cell response in principal needs to be confirmed by CTL-mediated cytotoxicity assays. These experiments were unfortunately, due to the limited number of primary MM cells obtainable in early plasma cell dyscrasias, not simultaneously possible with RT-PCR based antigen analysis. However, regarding HM1.24, we showed the cytotoxicity of Melan-A_{aa26-35}*A_{27L} specific T-cells with peptide loaded T2-cells and autologous MM cells by 51Chromium release-assays (17). For NY-ESO-1, Krönig et al. found that NY-ESO-1_{aa157-165} specific T-cells lysis MM cell lines, but they showed differences in the lysis capacity due to the avidity of the TCR(9). Cytotoxicity of MAGE specific T-cells against a variety of different MAGE-A3 positive tumor cell lines was described in vitro (2). One potential criticism remaining is that while cytotoxicity of these antigen-

specific T-cells was demonstrated in general, we cannot exclude that the activation of antigen-specific T-cells might not have resulted in the lysis of target cells in our experiments, e.g. due to TCRs with a low avidity or immune escape mechanism especially regarding cytotoxic T-cell effects vs. MM cells. So in our study, we can only give evidence about the antigen specific activation of T-cells.

What further mechanism could contribute to the observed immunoparalysis in advanced PD? First, augmented cellular regulatory elements like myeloid derived suppressor cells (MDSC), regulatory T-cells (Treg), or a T-helper cell imbalance (43) might have had an influence on our *in vitro* model. Secondly, unfunctional effector T-cells and thirdly the expression of immunosuppressive factors by the bone marrow micromilieu, described in MM patients with advanced disease (45). To this end, expression of the immunosuppressive costimulating factor PD-L1 on MM cells and its interaction with PD-1 on T-cells was recently described (46, 47).

On this background, an augmentation of cellular immunotherapeutical approaches by immunostimulating agents appears to be promising, especially in advanced disease. In multiple myeloma, immunomodulatory drugs like lenalidomide, having at the same time a direct anti-myeloma activity, seem the compounds of choice. Lenalidomide stimulates T-cells (48) and inhibits suppressor cells like MDSC (49) and Treg (50). In addition, blockage of PD-L1 / PD-1 interactions seems to be a promising goal in MM and might be combined with a cellular immunotherapy.

Another upcoming approach is the use of T-cells or NK cells with a chimeric antigen receptor "CAR" against targets preferentially expressed by MM cells, e.g. CD38 (51), CD138 (52), CS-1 (53).

Taken together, while current immunotherapeutical approaches in MM target broadly expressed antigens in patients with an advanced plasmacell disease, establishment

of an immune response against not yet expressed antigens in immunocompetent patients with an early stage plasmacell disease seems to be a promising option. Patients with MGUS and smoldering myeloma are optimal candidates for such a “prophylactic” vaccination, envisioned to prevent the evolution of either an aggressive myeloma clone or the bone marrow transformation to an advanced plasmacell disease. If patients with advanced plasma cell dyscrasias are to be treated by immunotherpeutical approaches, an augmentation of the potentially muted effect, e.g. by immunomodulatory compounds, can be considered.

Conclusion

In conclusion, specific T-cell responses in MM are triggered by antigen-expression but suppressed by tumor load. Future CTA-based immunotherpeutical approaches might target early plasmacell-diseases to establish prophylactically a specific T-cell response against late stage antigens in immunocompetent patients.

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Tables

Table 1. Patient data.

Stage		Number of patients/HD	Number of performed EliSpot-assays	Number of performed granzymeB ELISA`s	Number of performed TaqMan-PCR-analysis
HD :		37	27	24	0
EPD :		70	54	14	25
	monoclonal gammopathy	40	32	8	10
	MM stage I	30	22	6	15
APD :		79	64	24	42
	MM stage II	15	10	5	10
	MM stage III	64	54	19	32
		186	145	62	67

Table legend

Table 1. Patient data.

Shown is the number of HD and MM patients in (EPD/APD) analyzed by IFN- γ EliSpot-assay and granzymeB ELISA for T-cell immune responses and RT-PCR for antigen expression on CD138⁺ plasmacells.

Figure legends

Figure 1. Antigen expression analyzed by RT-PCR.

Expression of the antigens MAGE-A2, MAGE-A3, NY-ESO-1 and HM1.24 on CD138⁺ plasmacells from patients with EPD/APD is analyzed by RT-PCR and is displayed as relative expression (level of *HM1.24* was normalized to 18S RNA ($\delta\text{Ct} = \text{Ct HM1.24} - \text{Ct 18S}$) and compared with the values obtained for the HMCL OPM-2 using the following formula $100/2^{\delta\delta\text{ct}}$ where $\delta\delta\text{ct} = \delta\text{Ct patient sample} - \delta\text{Ct OPM-2}$). The p-value was determined by the percentage of positive versus negative gene expression, while the threshold for positive gene expression was less than 38 cycles RT-PCR.

Figure 2. Results of the EliSpot-assays

Results of IFN- γ EliSpot-assays after specific activation of CD8⁺ T-cells by peptide-pulsed T2 cells: shown are spots from T-cells activated by HM1.24 / Melan-A_{aa26-35}*A27L (ELAGIGILTV) (A) MAGE-A2_{aa157-166} (YLQLVFGIEV) (B), MAGE-A3_{aa271-279} (FLWGPRALV) (C) and NY-ESO-1_{aa155-167} (QLSLLMWITQCFL) (D). Black columns (■) represent T-cell responses triggered by HM1.24 pulsed T2 cells and grey columns (□) by T2 cells pulsed with a HLA-A2 restricted control peptide in HD, EPD and APD. A representative IFN- γ EliSpot-assay from a patient after specific activation of CD8⁺ T-cells by peptide-pulsed T2 cells is demonstrated in (E).

Figure 3. Cumulative results of EliSpot-assays

Results of IFN- γ EliSpot-assays after specific activation of CD8⁺ T-cells by peptide-pulsed T2 cells: shown are % of HD/EPD/APD with antigen-specific T-cell responses (a T-cell response is considered as positive, when the median number of IFN- γ spots (EliSpot-assay) in 3-5 wells of T-cells stimulated by relevant peptide-loaded T2 cells was at least twofold the median number of IFN- γ spots in 3 - 5 wells of T-cells activated by T2 cells loaded with control peptide) against the antigens HM1.24, MAGE-A2/3 and NY-ESO-1.

Figure 4. Results of the granzymeB ELISA

Results of granzymeB ELISA's after specific activation of CD8⁺ T-cells by peptide-pulsed T2 cells. Shown is the amount of antigen-specifically released granzymeB (pg / ml) in HD, EPD and APD, calculated as follows: granzymeB from T-cells activated by MAGE-A2_{aa157-166} (YLQLVFGIEV), MAGE-A3_{aa271-279} (FLWGPRALV), NY-ESO-1_{aa155-167} (QLSLLMWITQCFL) or Melan-A_{aa26-35}*A27L (ELAGIGILTV) pulsed T2 cells minus granzymeB from T-cells activated by control peptide pulsed T2 cells as amount of pg granzymeB / ml in HD, EPD and APD.

Figure 1

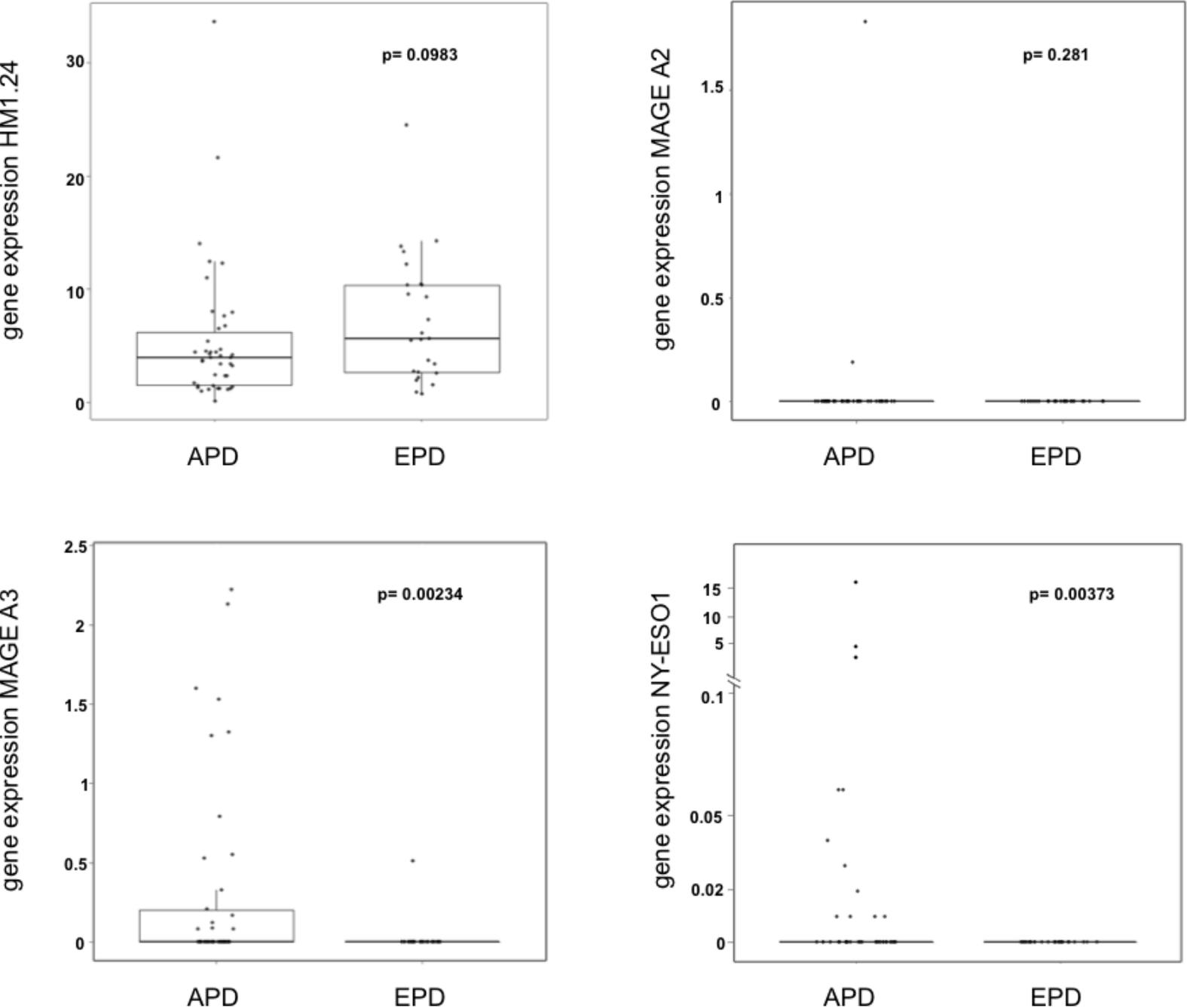


Figure 2

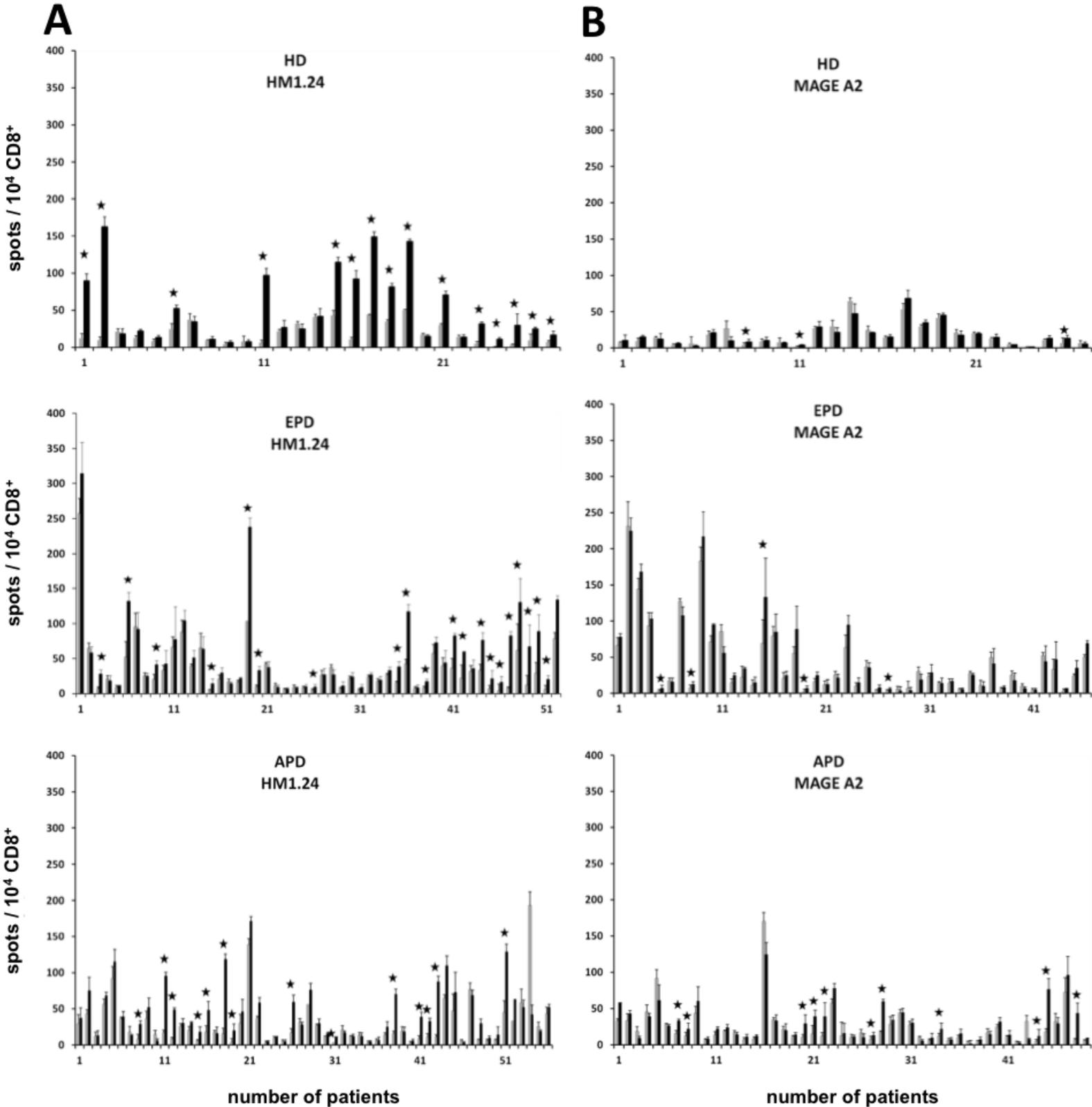


Figure 2

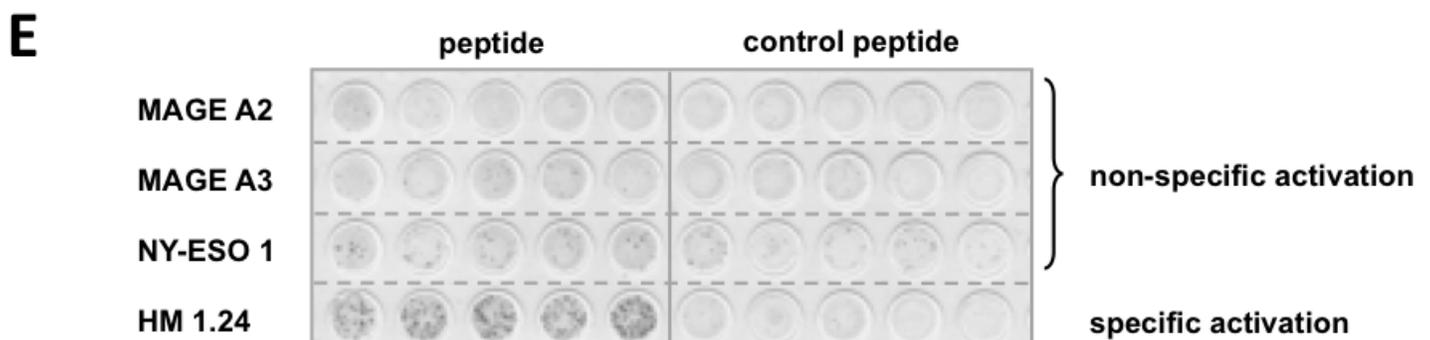
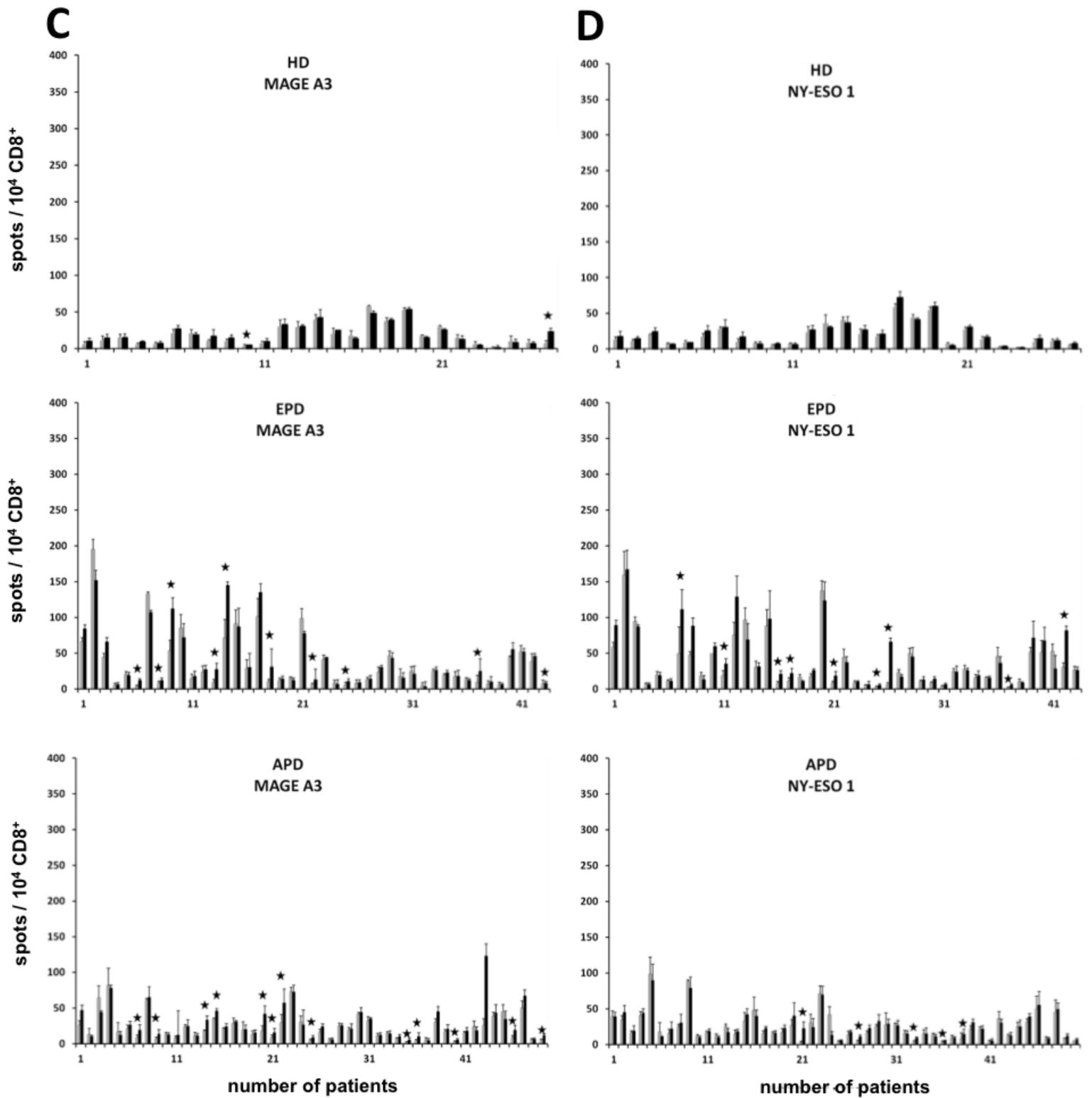


Figure 3

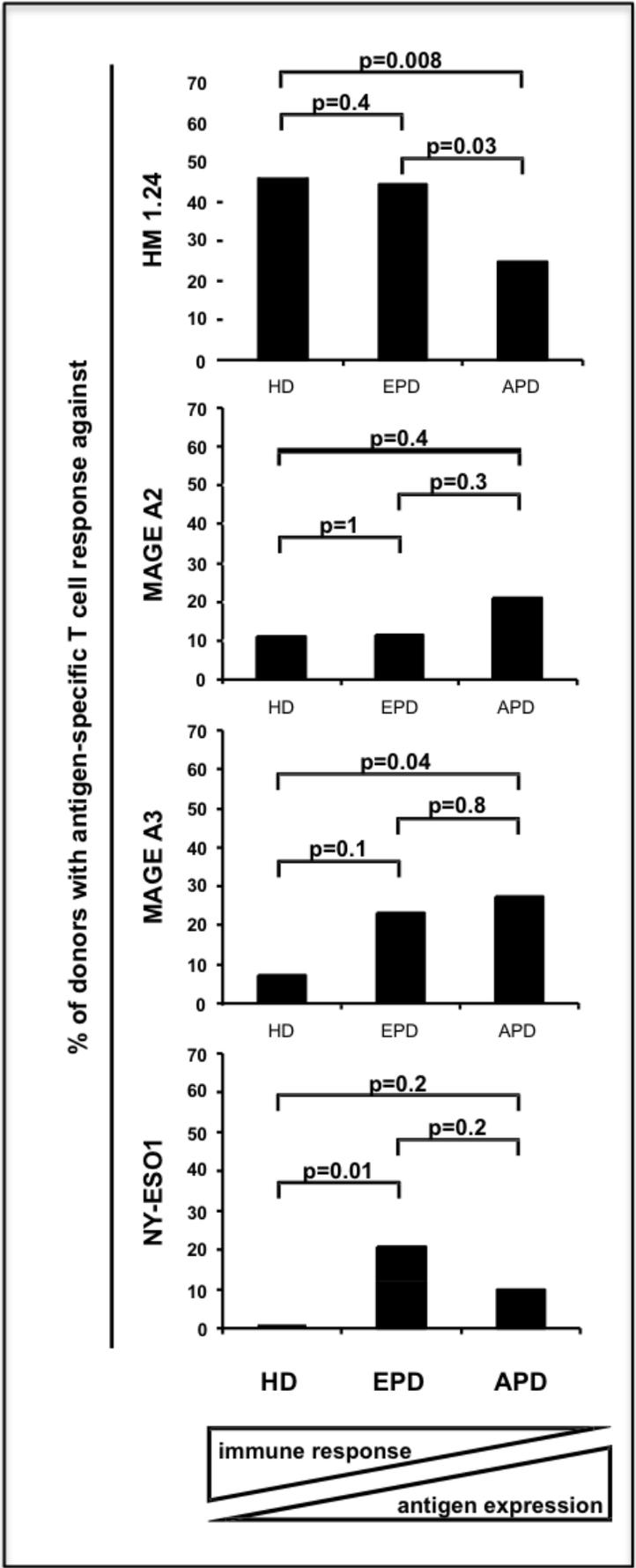
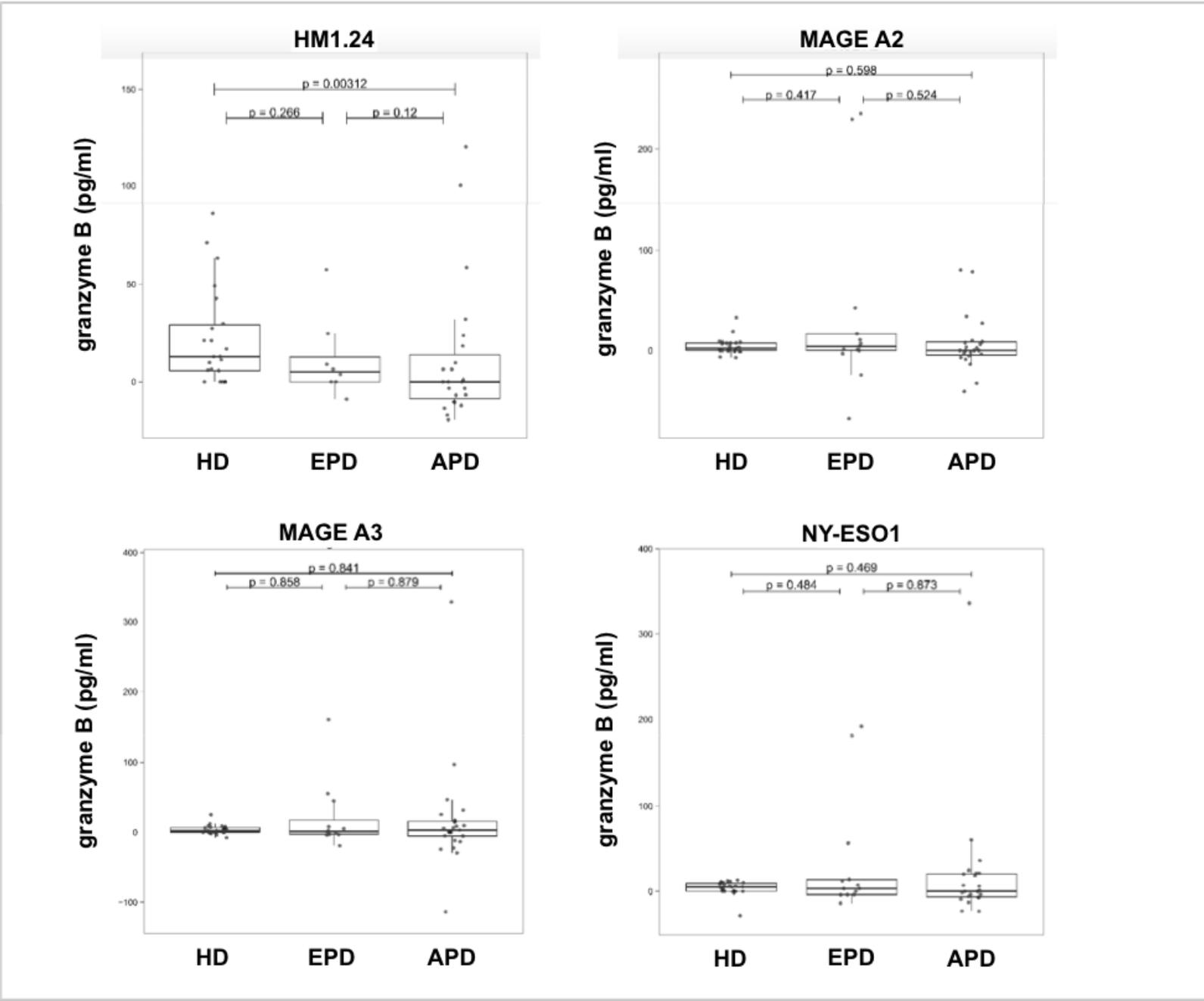


Figure 4



Clinical Cancer Research

ASSOCIATION OF ANTIGEN-SPECIFIC T-CELL RESPONSES WITH ANTIGEN-EXPRESSION AND IMMUNOPARALYSIS IN MULTIPLE MYELOMA

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