Association of Antigen-Specific T-cell Responses with Antigen Expression and Immunoparalysis in Multiple Myeloma

Sabrina Fichtner, Dirk Hose, Melanie Engelhardt, Tobias Meißner, Brigitte Neuber, Fatime Krasniqi, Marc Raab, Stefan Schönland, Anthony D. Ho, Hartmut Goldschmidt, and Michael Hundemer

Abstract

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

Experimental Design: We assessed the expression of the multiple myeloma antigen HM1.24 (reported present in all malignant plasma cells) and the CTAs MAGE-A2/A3 and NY-ESO-1 (aberrantly expressed in a subset of patients with myeloma), 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 granzyme B ELISA (n = 62) in relation to stage (tumor load) and expression of the respective antigen.

Results: HM1.24 is expressed in all plasma-cell samples, whereas CTAs are significantly more frequent in later stages.

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

Introduction

Multiple myeloma 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). Multiple myeloma cells harbor a high median number of chromosomal aberrations (3–5) and multiple changes in gene expression compared with 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 article, we evaluate the impact of immunosuppression and antigen expression on the development of specific CD8+ T-cell responses in patients with different stages of plasma-cell 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 tumor 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 to 33 kDa, expressed on terminally differentiated human B cells, in multiple myeloma and infrequently on lung and renal cancer cells (11, 15). We identified the immunogenic oligopeptide HM1.24aa22–30 (ILLGISLILV) derived from the HM1.24 antigen as a new HLA-A2-restricted T-cell epitope (16), and also showed a cross-reactivity of HM1.24aa22–30 (ILLGISLILV) with the Melan-A/MART-1–derived peptide Melan-Aaa26–35 (ELAGIGILTV; ref. 17) indicated by their sequence conformity at the central peptide position (GILTV). Emphasizing the immunogenic nature of the HM1.24-derived oligo-peptides, Rev and colleagues (18) showed the MHC-I–restricted generation of HM1.24-specific cytotoxic T cells by autologous peripheral blood mononuclear cells in patients with multiple myeloma. Recently, Harada and colleagues (19) found an upregulation of HM1.24 on the surface of multiple myeloma cells induced by lenalidomide.
HM1.24 is, thus a promising target for an immunotherapeutical approach in patients with plasma-cell dyscrasias. CTAs are physiologically expressed in germ cells, but aberrantly in various cancer types, including multiple myeloma (5, 20, 21). This expression pattern minimizes the likelihood of an immunologic tolerance (22), and indeed, CTAs show a strong immunogenicity in vivo in various cancers (23). As expected per definition, CTAs are not expressed on normal bone marrow plasma cells and at varying frequencies in multiple myeloma cells (20, 21). We also showed a correlation between the expression of CTAs analyzed by gene-expression microarrays, RT-PCR, and immunocytochemistry (21). The probability of expression increases with advanced stage and in relapsed multiple myeloma (5). CTAs have likely a pathophysiologic role, as their expression is associated with adverse prognosis (8, 24, 25). Furthermore, in a functional silencing assay, multiple myeloma cells expressing CTAs 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, whereas most of the patients so far, did not benefit from immunotherapeutical treatments for a longer time (26).

Like HM1.24, CTAs are, thus, a promising target for an immunotherapeutical approach in multiple myeloma, although to our knowledge, no completed CTA-based clinical trial in multiple myeloma is published up to now. Regarding the evolution of the malignant clone in multiple myeloma from MGUS (monoclonal gammopathy of undetermined significance) and smoldering myeloma to multiple myeloma 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 plasma-cell 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, and 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 plasma-cell diseases (EPD: MGUS, and multiple myeloma stage I) and advanced plasma-cell diseases (APD: multiple myeloma stage II and III).

Samples
Normal bone marrow plasma cells and multiple myeloma cells were purified as previously published (29–33). To analyze the
Figure 2. (Continued.)
generation of antigen-specific T cells, 50 mL peripheral blood was drawn.

Mononuclear cells for in vitro expansion of peptide-specific T cells
Mononuclear cells (MC) from HLA-A2+ patients/HD were purified using density centrifugation (Biochrom). HLA-A typing was performed by flow cytometry, as previously published (28).

Synthesis of peptides
The peptides MAGE-A2 aa157-166 (YLQLVFQGIEV), MAGE-A3 aa271-279 (FLWGPRALV), NY-ESO-1 aa155-167 (QSLILMWITQCF1L), Melan-A aa26-35 (ELAGIGILTV), and HLA-A2 restricted irrelevant control peptide (LIIVILVCV, 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 DCs
MCs from HLA-A2+ patients/HD were used. Immature DCs were obtained culturing plastic adherent MCs for 5 days with GM-CSF (800 U/mL, Molgramostim; Essex Pharma) and IL4 (500 U/mL; R&D Systems). Afterwards, differentiation into mature DC was induced by stimulation with TNFa (10 ng/mL, Sigma-Aldrich), IL6 (1,000 U/mL; R&D Systems), and prostaglandin E2 (1 μg/mL; Sigma-Aldrich) 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 plasma-cell disease and HD by autologous DC pulsed with MAGE-A2 aa157-166 (YLQLVFQGIEV), MAGE-A3 aa271-279 (FLWGPRALV), NY-ESO-1 aa155-167 (QSLILMWITQCF1L), or Melan-A aa26-35 (ELAGIGILTV) peptides, respectively, and analyzed the IFNγ secretion by EliSpot assay and the granzymeB secretion of T cells by ELISA assay.

IFNγ EliSpot assay
The generation of antigen (MAGE-A2, MAGE-A3, NY-ESO-1, and HM1.24)–specific CD8+ T cells was analyzed by IFNγ EliSpot assay. Expanded CD8+ cells were purified with immunomagnetic beads (MACS-system; Miltenyi Biotec) and incubated with peptide-loaded T2 cells as targets (effector/target ratio 1:5) for 22 hours in anti–IFNγ-antibody (Mabtech AB) coated nitrocellulose plates (Millipore). After detection with biotinylated anti–cyto-kine-antibodies (Mabtech AB) and conjugation with Avidin ALP (Sigma), BCIP/NBT substrate was added (Sigma). EliSpots were counted with a computer-controlled microscope (Zeiss-Vision). T2 cells were loaded with described peptides or a control peptide during a 2-hour incubation with 10 μg/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 × 106/well) were purified with immunomagnetic beads (MACS-system; Miltenyi Biotec) and incubated with peptide-loaded T2 cells as targets (effector/target ratio 1:5) for 22 hours according to the manufacturer’s instructions (BD OptEIA ELISA Sets; BD Biosciences) was used. The intensity of the color was analyzed with an automated plate reader.

RNA extraction and quantitative real-time PCR
RNA was extracted by using the RNeasy Kit (Qiagen) in accordance with the manufacturer’s instructions. After reverse transcription of 50 ng total RNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems), quantitative real-time PCR (qRT-PCR) was performed with TaqMan Gene Expression Assays (Applied Biosystems). Amplification and fluorescence detection were carried out on the ABI Prism 7700 SDS analytic thermal cycler (Applied Biosystems). 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 (8Cg = C gene – C 18S) and related to the values obtained for the HMCL OPM-2 using the following formula 100/2ΔCt, where 8Cg = 8Ct patient sample – 8Ct OPM-2.

Statistical analysis
In an individual patient, the IFNγ EliSpot assay was considered as positive (EliSpot-pos) when the median number of IFNγ spots in 3 to 5 wells of T cells stimulated by relevant peptide-loaded T2 cells was at least 2-fold the median number of IFNγ spots in 3 to 5 wells of T cells activated by T2 cells loaded with control peptide according to Keilholz and colleagues (35). The frequency of EliSpot-pos and negative EliSpots (EliSpot-neg) in patients with EDP and with APD was compared by a χ2 test. Statistical analysis of ELISA and gene expression by RT-PCR was performed by a Wilcoxon rank sum test. Statistical tests were significant if P values are ≤0.05. Nonsignificant P values >0.05 and ≤0.5 are reported for descriptive reasons only.

Results
Expression of HM1.24 and CTAs
Expression of HM1.24 was detectable by RT-PCR in all CD138+ malignant plasma-cell samples in EDP and APD (n = 67). The CTAs MAGE-A2, MAGE-A3, and NY-ESO-1 were almost not expressed in samples from patients with EDP 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 versus EDP (P = 0.002 and P = 0.004, Fig. 1).

T-cell responses against HM1.24
A strong T-cell response against the HM1.24 antigen assessed by IFNγ EliSpot assay could be found in HD and patients with EDP (Fig. 2A). The T-cell response is significantly decreased in patients with APD compared with HD or EDP (P = 0.008, P = 0.03, Fig. 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 = 0.003, Fig. 4). Both results indicate an immunosuppressive mechanism on T-cell responses in advanced stages of plasma-cell dyscrasias.

T-cell responses against CTA
The antigen-specific activation of T cells displayed by their IFNγ secretion as analyzed by EliSpot assay (Figs. 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 EDP against the MAGE-A3 and NY-ESO-1 antigen, although...
only a low frequency of gene expression was detected by RT-PCR (Fig. 1). Although the frequency of T-cell responses against MAGE-A2/3 increased in APD, the frequency against NY-ESO-1 decreased (Fig. 3). Regarding the granzymeB secretion of antigen-specific T cells upon antigen-specific stimulation, only patients had measurable levels of granzymeB secretion, whereas almost no secretion was detected in HD (Fig. 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 plasma-cell 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 were used to analyze the general stage-dependent impact of myeloma-specific immunosuppression. The strong T-cell response against HM1.24 cross-reactive peptide Melan-A aa26-35/C3A27L (ELAGIGLTV) 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. Ratta and colleagues (39) showed, however, that in vitro culture conditions can circumvent the in vivo impaired DC function of patients with multiple myeloma. 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 patients with multiple myeloma and reflects the expansion of specific T cells in the patient with multiple myeloma. The increasing immunologic 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 antitumoral 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 (Fig. 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 multiple myeloma, antigen-specific T-cell response against multiple myeloma cells should be demonstrated in an autologous setting. Second, 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 multiple myeloma 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-Aaa26–35/C3A27L–specific T cells with peptide-loaded T2 cells and autologous multiple myeloma cells by 51Chromium release assays (17). For NY-ESO-1, Kronig and colleagues found that NY-ESO-1aa157-165 specific T cells lysis multiple myeloma 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, although 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, for example, due to TCRs with a low avidity or immune escape mechanism especially regarding cytotoxic T-cell effects versus multiple myeloma 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

### Table 1. Patient data

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of patients/HD</th>
<th>Number of performed EliSpot assays</th>
<th>Number of performed granzymeB ELISA's</th>
<th>Number of performed TaqMan–PCR analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>37</td>
<td>27</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>EPD</td>
<td>70</td>
<td>54</td>
<td>34</td>
<td>25</td>
</tr>
<tr>
<td>Monoclonal gammopathy</td>
<td>40</td>
<td>32</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>MM stage I</td>
<td>30</td>
<td>22</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>APD</td>
<td>79</td>
<td>64</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>MM stage II</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>MM stage III</td>
<td>64</td>
<td>54</td>
<td>19</td>
<td>32</td>
</tr>
</tbody>
</table>

NOTE: Shown is the number of HD and patients with multiple myeloma in (EPD/APD) analyzed by IFNy EliSpot assay and granzymeB ELISA for T-cell immune responses and RT-PCR for antigen expression on CD138⁺ plasma cells.

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-A2aa157-166 (YQLVNGIEY), MAGE-A3aa271-279 (FLWRGPRALV), NY-ESO-1aa155-167 (QLSLLMWITQCL), or Melan-Aaa26-35/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.
regulatory elements like myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), or a T-helper cell imbalance might have had an influence on our in vitro model. Second, unfunctional or T cells and third, the expression of immunosuppressive factors by the bone marrow micromilieu, described in patients with multiple myeloma with advanced disease. To this end, expression of the immunosuppressive costimulating factor PD-L1 on multiple myeloma 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 and inhibits suppressor cells like MDSC (49) and Treg (50). In addition, blocking of PD-L1–PD-1 interactions seems to be a promising goal in multiple myeloma and might be combined with a cellular immunotherapy.

Another upcoming approach is the use of T cells or natural killer cells with a chimeric antigen receptor 'CAR' against targets preferentially expressed by multiple myeloma cells, for example, CD38, CD138, and CS-1.

Taken together, although current immunotherapeutical approaches in multiple myeloma target broadly expressed antigens in patients with an advanced plasma-cell disease, establishment of an immune response against not yet expressed antigens in immunocompetent patients with an early-stage plasma-cell disease seems to be a promising option. Patients with MGLIS 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 plasma-cell disease. If patients with advanced plasma-cell dyscrasias are to be treated by immunotherapeutical approaches, an augmentation of the potentially muted effect, for example, by immunomodulatory compounds, can be considered.

References

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

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: M. Hundemer

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