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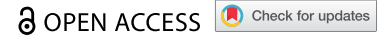


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BRIEF REPORT



## Ecto-calreticulin expression in multiple myeloma correlates with a failed anti-tumoral immune response and bad prognosis

Alfonso Serrano Del Valle <sup>a</sup>, Manuel Beltrán-Visiedo <sup>a</sup>, Victoria de Poo-Rodríguez <sup>b,c</sup>, Nelia Jiménez-Alduán<sup>a</sup>, Gemma Azaceta <sup>b,c</sup>, Rosana Díez<sup>a,d</sup>, Beatriz Martínez-Lázaro<sup>b,c</sup>, Isabel Izquierdo<sup>a,d</sup>, Luis Palomera <sup>b,c</sup>, Javier Naval <sup>a</sup>, Alberto Anel<sup>a</sup>, and Isabel Marzo <sup>a</sup>

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### ABSTRACT

Immunogenic cell death (ICD) has been proposed to be a crucial process for antitumor immunosurveillance. ICD is characterized by the exposure and emission of Damage Associated Molecular Patterns (DAMP), including calreticulin (CRT). A positive correlation between CRT exposure or total expression and improved anticancer immunosurveillance has been found in certain cancers, usually accompanied by favorable patient prognosis. In the present study, we sought to evaluate CRT levels in the plasma membrane of CD38<sup>+</sup> bone marrow mononuclear cells (BMMCs) isolated from 71 patients with varying degrees of multiple myeloma (MM) disease and examine the possible relationship between basal CRT exposure and the bone marrow immune microenvironment, as well as its connection with different clinical markers. Data show that increased levels of cell surface-CRT were associated with more aggressive clinical features and with worse clinical prognosis in MM. High CRT expression in MM cells was associated with increased infiltration of NK cells, CD8<sup>+</sup> T lymphocytes and dendritic cells (DC), indicative of an active anti-tumoral immune response, but also with a significantly higher presence of immunosuppressive Treg cells and increased expression of PD-L1 in myeloma cells.

### ARTICLE HISTORY

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### KEYWORDS

Calreticulin; multiple myeloma; immunogenic cell death


### Introduction

Some studies point to the role of Immunogenic Cell Death (ICD) in the clinical response to chemotherapy or chemioimmunotherapy combinations.<sup>1,2</sup> ICD is characterized by the emission of immunogenic molecules known as Damage-Associated Molecular Patterns (DAMP) that include calreticulin (CRT) surface expression and HMGB1 and ATP release, among others. These molecules are released by dying cells and bind to specific receptors in immune cells, like FPR1, TLR3, TLR4 and P2RX7, leading to antitumor immune response. Accumulating clinical evidence supports the notion that DAMPs, especially CRT, could be useful as prognostic biomarkers in cancer.<sup>3</sup> Also, dysregulation of CRT has been proposed to play a role in tumorigenesis and cancer progression.<sup>4–6</sup> However, clinical studies supporting cell death-associated immunogenicity are still limited and usually do not offer a clear-cut association.<sup>7,8</sup> Many studies investigating the role of CRT in ICD assume that CRT exposure is a consequence of the therapy itself and have not considered basal surface expression of CRT on cancer cells and its potential association with malignant transformation or tumor progression. Current data indicate that CRT expression is augmented in tumors compared to healthy tissue and that CRT levels may be associated with cancer aggressiveness and disease progression.<sup>3</sup> Different

studies have shown that CRT expression could behave as a positive<sup>9–13</sup> or negative<sup>14–16</sup> prognostic factor for cancer patients, depending on the cancer cell type.

Multiple myeloma (MM) is a hematological malignancy caused by the uncontrolled proliferation of abnormal plasma cells (PCs) in the bone marrow (BM). It accounts for 10–20% of all hematological neoplasms and 0.9% of all newly diagnosed cancer cases worldwide.<sup>17</sup> Despite radical improvements in treatment regimens in the last decades, MM remains incurable. Although MM patients usually hold a compromised immune system, immunotherapeutic interventions could have the potential to succeed, as suggested by the graft-vs-myeloma effect observed in Autologous Stem Cell Transplantation (ASCT), in donor lymphocyte infusions or by recent reports showing high efficacy of daratumumab.<sup>18,19</sup> Thus, ICD could contribute to the success of MM treatments, especially those based on the use of proteasome inhibitors. These agents have been described to induce the emission of DAMPs, especially if autophagy is inhibited.<sup>20–23</sup> However, data about the potential of ICD to boost the response of MM cells are very scarce. Recently, De Beck *et al.* showed that prophylactic vaccination with cells treated with epigenetic compounds delayed disease progression in the 5T33MM murine model.<sup>24</sup> In contrast, the only clinical study available indicates that the mutation of the P2X7 receptor is not a prognostic marker in MM.<sup>25</sup> However, as previously indicated, ectopic CRT (ecto-CRT) can be

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increased independently of treatment and have an impact on the prognosis of the disease. These elements have not been studied in depth in the context of MM disease. In the present study, we have determined the ecto-CRT expression in bone marrow mononuclear cells (BMMCs) from MM patients and interrogated its relationship with the clinical outcome and the BM immune landscape. Our data indicate that increased ecto-CRT exposure in MM plasma cells is associated with a poor prognosis of MM disease, since individuals with augmented expression of ecto-CRT exhibit lower time to progression rates and shorter overall survival and increased chances of developing extramedullary plasmacytomas, are heavily pretreated and harbor a high-risk cytogenetic signature. This negative correlation could be associated with an immunosuppressive environment, as suggested by higher Treg percentages and PD-L1 expression in samples with high ecto-CRT levels.

## Materials and Methods

### Isolation of mononuclear cells from bone marrow samples

All samples were collected and obtained for research purposes with the informed consent from the subjects and the procedure was previously approved by CEICA (Comité Ético de Investigación Clínica de Aragón, PI16/0129). BMMCs were isolated by centrifugation on Ficoll-Paque<sup>TM</sup> Plus (GE Healthcare) and resuspended in 10 ml of RPMI1640. Patient characteristics and cytogenetics are summarized in Table 1.

### Analysis of ecto-calreticulin levels

Ecto-CRT analysis using flow cytometry was performed by direct immunostaining. BMMCs ( $5 \times 10^5$ ) were collected and incubated with 2  $\mu$ l of anti-calreticulin-Dylight 488 (Clone

FM75, Enzo) conjugated primary antibody (or the corresponding isotype control), 1  $\mu$ l of 7-AAD and 8  $\mu$ l of CD38-APC antibody in 100  $\mu$ l of PBS + 5% of FBS for 30 minutes at 4°C in the darkness. Cells were then washed and resuspended in PBS for flow cytometry analysis. Quantification of the percentage of ecto-CRT positive cells was conducted by gating on the CD38<sup>+</sup>/7-AAD negative population (Supplementary Figure S1). For some analyses, samples were segregated into two groups (CRT<sup>high</sup> and CRT<sup>low</sup>) based on the expression of CRT on the surface of CD38<sup>+</sup> cells. The stratification cutoff or threshold was the corresponding ecto-CRT median value from the total sample population, which corresponds to a 24% of positivity. The ROC analysis confirmed the suitability of this cutoff (AUC, 1; p-value < 0.0001).

### Cell surface protein expression by flow cytometry

Protein expression analysis by flow cytometry was performed by direct immunostaining. An appropriate number of cells, freshly isolated after sample collection, were incubated with primary fluorochrome-conjugated antibodies in PBS+5% FBS for 30 minutes at 4°C. Afterward, the cells were centrifuged 300xg for 5 minutes, washed with PBS+5%FBS, resuspended in PBS and analyzed in a BD FACScalibur cytometer.

The following antibodies were used for immunophenotyping of MM cells: CD38-FITC (Clone HIT2), CD138-FITC (Clone MI15), CD56-PE (Clone B159) and CD45-PE-Cy5 (Clone HI30), all from BD Pharmingen. Quantification of the T cell repertoire was performed through staining with the following antibodies: CD4-FITC (Clone RPA-T4, BD), CD8-PE (Clone REA734, Miltenyi) and CD3-APC (Clone UCHT-1, Immunotools). In case of NK cell population determination, CD45-PE-Cy5 (Clone HI30, BD Pharmingen) and CD56-APC (Clone REA196, Miltenyi) antibodies were used. Plasmacytoid

**Table 1.** Clinical characteristics and frequency of cytogenetic determinants of patients (n = 71). Cytogenetic analysis included karyotype, t(11;14), t(4;14), t(14;16), gain 1q, p53 alterations and IGH-FGFR3/IGH-MAF gene fusions. NA, not analyzed.

Characteristic	
Age	
Median	69.5
Range	46–88
Sex	
Male	39 (57%)
Female	30 (43%)
Diagnosis	
MGUS	13 (18.3%)
SMM	11 (15.5%)
MM	47 (66.2%)
PCs, %	
Median	22
Range	5–95
Immunoglobulin type	
IgG	70%
IgA	27%
Bence-Jones	3%
Light chain type	
k	67%
λ	33%
Cytogenetics	
Negative	25/56 (44.6%)
Altered	31/56 (55.4%)
NA	15

and myeloid DCs frequencies were determined by analysis of appropriate lineage markers with the following antibodies: CD14-FITC (Clone M5E2, BD Biosciences), CD19-FITC (Clone LT19, Miltenyi), CD20-FITC (Clone 2H7, BD Biosciences), CD3-FITC (Clone REA613, Miltenyi), CD56-FITC (Clone NCAM-1, BD Biosciences), CD16-FITC (Clone 3G8, BD Biosciences), CD123-PE (Clone 9F5, BD Biosciences), HLA-DR-PerCp-Cy5 (Clone G46-6, BD Biosciences) and CD11c-APC (Clone B-ly6, BD Biosciences). For the analysis of Treg frequencies, CD4-FITC (Clone RPA-T4), CD25-PE (Clone M-A251) and CD127-AlexaFluor647 (Clone HIL-7 R-M21), from BD Pharmingen, markers were analyzed. For PD-L1 expression on CD38<sup>+</sup> BMMCs, cells were labeled with anti-PD-L1-PE (Clone 29E.2A3, Biolegend) and anti-CD38-APC (Clone HIT2, BD Pharmingen) antibodies, or the corresponding isotype control. PD-1 expression on specific immune cells was also determined by incubating cells with anti-PD-1-AlexaFluor488 antibody (Clone EH12.2H7, Biolegend) or the corresponding isotype control and gating on the appropriate cell population of interest (NK cells, CD4 or CD8 T cells). Representative images of flow cytometry analyses are shown in Supplementary Figures S2-S6.

### Statistical analysis

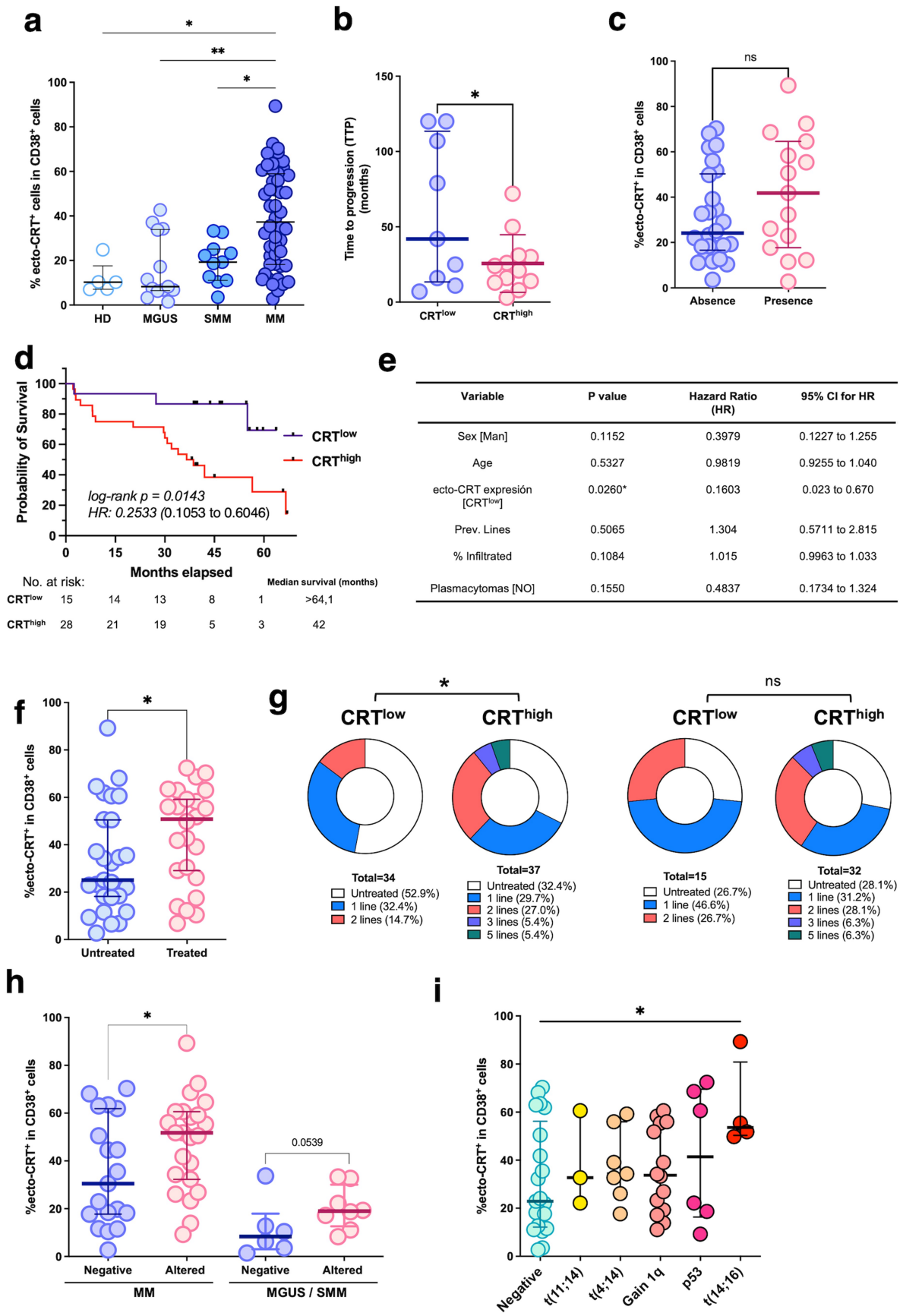
The statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc.). As indicated in each case, data were statistically analyzed using the following statistical tests: Mann-Whitney or unpaired t-test for the analysis of two groups of independent samples, Kruskal-Wallis test for multiple comparisons and Chi-square test for trend for comparing the proportions of two nominal variables with small sample sizes. Kaplan-Meier and Cox regression survival analyses were performed with overall survival from the time of sample collection and ecto-CRT analysis as the end point.

### Results and discussion

High CRT expression has been reported to be associated with favorable clinical outcomes in endometrial cancer, non-small cell lung cancer (NSCLC), neuroblastoma, glioblastoma, colorectal and ovarian cancer and endometrial cancer patients in which cancer cells expressed high levels of CRT.<sup>11,26-28</sup> In the opposite direction, overexpression of CRT could be an adverse prognostic factor in gastric,<sup>14</sup> oral squamous<sup>29</sup> and pancreatic<sup>15</sup> tumors. Also, increased CRT mRNA levels predicted worse clinical outcome in bladder cancer, neuroblastoma and mantle cell lymphoma.<sup>16</sup> In hematological malignancies, the predictive value of CRT in prognosis has been explored in acute myeloid leukemia (AML),<sup>12</sup> finding a positive correlation between ecto-CRT levels and overall survival.

Basal ecto-CRT expression in MM patients was very heterogeneous, with a great interindividual variability. We questioned then whether CRT exposure may vary with the different stages of MM disease. Thereby, we compared ecto-CRT expression in CD38<sup>+</sup> plasma cells isolated from control BM (HD), monoclonal gammopathy of undetermined significance (MGUS), smoldering MM (SMM) and MM BM biopsies. Control BM samples corresponded to individuals that may

have an underlying pathological state that required a BM aspiration to discard any hematological pathology, but did not harbor any pathological or morphological alteration of the BM. As shown in **Figure 1a**, CRT exposure clearly increased with disease progression. Mean ecto-CRT expression was significantly higher in MM patients, compared to MGUS, smoldering or control samples. Although slightly increased, differences in CRT exposure between MGUS and SMM were not statistically significant. These results suggest that ecto-CRT may be associated with malignant transformation. Finally, CRT<sup>high</sup> individuals, including MGUS and SMM patients that evolved to MM, significantly exhibited a reduced time to progression (TTP) compared to patients displaying low levels of ecto-CRT (median, 22.5 vs 42 months; intervals, 3 to 72 vs 7 to 120), as shown in **Figure 1b**. Development of soft-tissue plasmacytomas is a sign of the presence of clonal plasma cells growing outside the BM that can appear either in newly diagnosed individuals or during disease progression and patients bearing extramedullary plasmacytomas often exhibit poor clinical outcomes.<sup>30,31</sup> Our data show that extramedullary plasmacytomas were more frequent in MM patients with higher levels of ecto-CRT (**Figure 1c**), although the difference did not reach statistical significance. Kaplan-Meier survival curves of the CRT<sup>high</sup> and CRT<sup>low</sup> groups indicate that CRT<sup>high</sup> group have significant shorter OS (**Figure 1d**). A significant difference was observed in probability of survival between CRT<sup>high</sup> and CRT<sup>low</sup> groups in the univariate Mantel-Cox analysis (HR = 0.2533, log-rank p = 0.0143). Multivariate Cox analysis including sex, age, CRT stratification, previous lines of therapy and presence of plasmacytomas also indicated that the CRT<sup>low</sup> displayed a lower risk of death (MV HR = 0.1603, p-value = 0.0260, 95% CI 0.023 to 0.670) (**Figure 1e**). Additionally, we tested whether chemotherapeutic treatment could have any impact over CRT expression on the plasma membrane of myeloma cells. As shown in **Figure 1f**, samples from patients previously treated with chemotherapy displayed higher levels of ecto-CRT. We also found a significant association between higher expression of surface-CRT and a higher number of lines of therapy when we analyzed all the samples, including MGUS, SMM and MM (**Figure 1g**, left panel). However, we did not find a significant difference when exclusively considering MM samples (**Figure 1g**, right panel). Altogether, these results suggest that the levels of ecto-CRT could correlate mainly with disease progression, more than with exposure to chemotherapy. We also interrogated whether a linkage between the expression of ecto-CRT and the presence of cytogenetic abnormalities in MM could also exist. As depicted in **Figure 1h**, cells from MM patients with an altered cytogenetic signature, significantly revealed augmented ecto-CRT levels compared with patients in which frequent alterations routinely tested were not detected. Higher median ecto-CRT levels were also detected in MGUS and SMM samples with cytogenetic alterations, although in this case the differences were not statistically significant. We dissected the different specific cytogenetic abnormalities harbored by these patients and the exposure of CRT that exhibited in each case (**Figure 1i**). No significant differences were found in ecto-CRT levels between patients bearing a t(11;14) or t(4;14) translocation and patients with no detected cytogenetic abnormalities.



**Figure 1.** Analysis of CRT exposure in patients with plasma cell dyscrasias. (a) Ecto-CRT levels were determined in CD38<sup>+</sup> BMMCs isolated from clinically diagnosed patients with varying degrees of MM disease (MGUS, SMM, MM). Control BM (HD) was obtained from individuals with other pathologies that held an uncompromised BM evidenced by histological or flow cytometry examination. Statistical analysis was performed using one-way ANOVA test with Tukey post test, where \*p < .05, \*\*p < .01, \*\*\*p < .001, ns, non-significant. (b) Time to progression (TTP) was compared between CRT<sup>high</sup> and CRT<sup>low</sup> subgroups. Data are shown as median with interquartile range. Statistical analysis was performed using unpaired t test with Welch's correction (\*p < .05). (c) The expression of ecto-CRT in patients showing or not

Finally, the high-risk cytogenetic t(14;16), associated with an adverse clinical outcome,<sup>32,33</sup> showed significant increased levels of surface-CRT compared to the group without detected cytogenetic abnormalities. Other cytogenetic abnormalities also showed a tendency to higher ecto-CRT levels.

The present results confirm previous findings showing that total CRT, measured by flow cytometry or RT-PCR methods, is upregulated in MM, compared to MGUS or control samples.<sup>34</sup> Our data demonstrate that myeloma cells also display elevated levels of ecto-CRT on their surface that seem to increase with disease progression. This observation, together with the fact that CRT exposure is chemotherapy-independent and patients with an altered cytogenetic signature display increased levels of ecto-CRT, may point toward malignant transformation as the instigator of this molecular event. Other authors have proposed that cancer cells may experience chemotherapy-independent cellular stress that drives DAMP signaling and trafficking. For instance, similar to our data in MM, AML<sup>12,22</sup> and NSCLC<sup>10</sup> cancer cells expose increased levels of CRT in their surface, irrespective of the chemotherapeutic regimen administered. Most probably, this could be a consequence of cellular stress caused by the malignant transformation process, which already impinges a prominent burden in many cellular processes, including proteostasis.<sup>12</sup> Overexpression of CRT in MM could also be associated with hyperploidy or copy number variants (CNVs) involving chromosome 19 gain, two common events in MM,<sup>33</sup> where the CALR gene is located (19p13.3). However, we have observed ecto-CRT high levels in patients with a normal karyotype, indicating that, although trisomy of the chromosome 19 could contribute to increased levels of ecto-CRT, other mechanisms can also be involved.

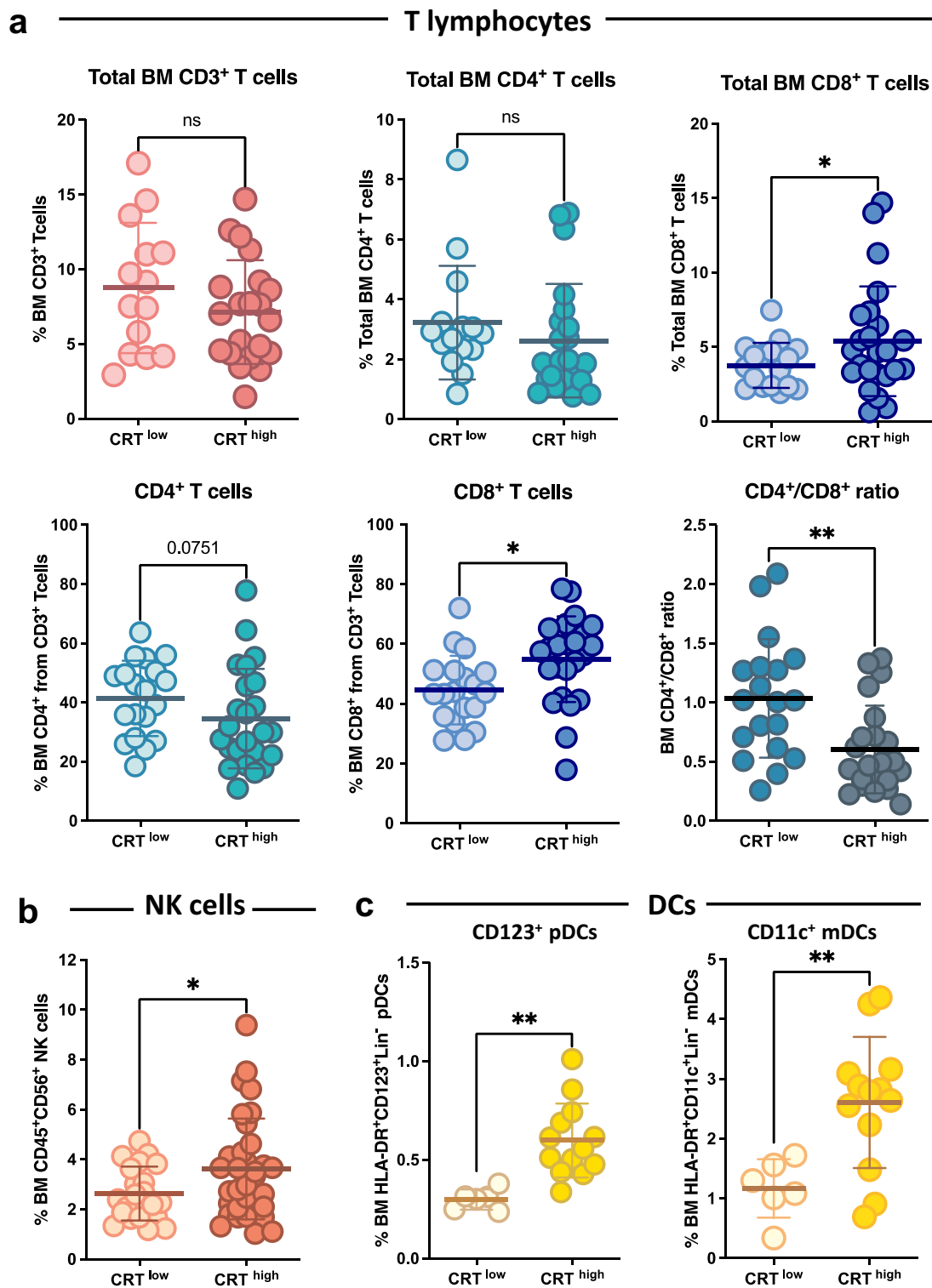
Our results suggest that higher ecto-CRT expression in MM correlates with presence of more cytogenetic alterations and worse outcome. On the other hand, increased basal ecto-CRT was associated with improved immunosurveillance in AML,<sup>35</sup> NSCLC<sup>9,10</sup> and ovarian cancer.<sup>10</sup> Thus, we wondered whether immune infiltration could be impaired in MM with high ecto-CRT levels. Hence, we evaluated the infiltration of different immune populations in bone marrow samples from MM patients in the CRT<sup>high</sup> and CRT<sup>low</sup> groups. Regarding the total number of CD3<sup>+</sup> T lymphocytes, a strong tendency toward lower total BM CD3<sup>+</sup> cells, which did not reach statistical significance ( $p = 0.07$ ), was encountered in CRT<sup>high</sup> samples (Figure 2a, upper left panel). The CRT<sup>high</sup> samples exhibited lower total CD4<sup>+</sup>CD3<sup>+</sup> T cells compared to CRT<sup>low</sup> samples (Figure 2a, upper middle panel) although they did not arrive to be statistically significant. In contrast, a slight tendency toward higher total CD8<sup>+</sup>CD3<sup>+</sup> T cell frequencies could

be noticed in the CRT<sup>high</sup> cohort (Figure 2a, upper right panel). Additionally, the proportion of CD4<sup>+</sup> cells within the CD3<sup>+</sup> subset was reduced in patients with increased ecto-CRT levels, while the percentage of CD8<sup>+</sup> T lymphocytes was significantly increased (Figure 2a, lower panels). These differences resulted in a significant reduction of the CD4<sup>+</sup>/CD8<sup>+</sup> ratio in the CRT<sup>high</sup> group. The NK frequencies were significantly increased in the CRT<sup>high</sup> group (Figure 2b). Total frequencies of different subsets of dendritic cells were also analyzed. Our results indicate that both CD123<sup>+</sup>HLA-DR<sup>+</sup> plasmacytoids (pDCs) and CD11c<sup>+</sup>HLA-DR<sup>+</sup> myeloid DCs (mDCs) subpopulations, were increased in patients with elevated ecto-CRT expression levels (Figure 2c). High CD8<sup>+</sup> T cells, NK and DC infiltration could reflect an attempt of the patient's immune system to fight against MM development. Our present results are in accordance with reports showing that a reduced CD4<sup>+</sup>/CD8<sup>+</sup> ratio in MM is associated with shorter survival rates,<sup>36</sup> while increased baseline CD4<sup>+</sup> T cell levels have been associated with longer survival rates.<sup>36,37</sup> Similarly, marrow-infiltrated CD4<sup>+</sup> lymphocytes progressively decreased, while CD8<sup>+</sup> T cells gradually increased with advancing disease stages.<sup>38</sup> We also found that high ecto-CRT correlated with increased percentages of NK cells and DCs. Alone, the high densities of these two immune subsets within the tumor milieu are indicative of good prognosis in a wide variety of human cancers, especially in solid tumors,<sup>9,39,40</sup> but it seems not to be the case in MM. We next investigated the presence of Tregs in MM samples, and we found that samples from CRT<sup>high</sup> patients displayed higher Tregs infiltration (Figure 3a), suggesting that immunosurveillance could be impaired in MM patients with high ecto-CRT levels. Conflicting data have been reported on the number and functionality of Tregs in the context of MM disease. High percentages of functional CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Tregs have been reported in peripheral blood of MM patients,<sup>41–44</sup> but some authors have found no changes,<sup>45</sup> reduced peripheral blood frequencies<sup>46</sup> or dysfunctional Tregs in MM.<sup>47</sup> Finally, some studies revealed that the median survival rate and TTP of patients with lower Tregs were significantly longer than those with increased Treg numbers,<sup>43,44</sup> a fact that would be in agreement with our results.

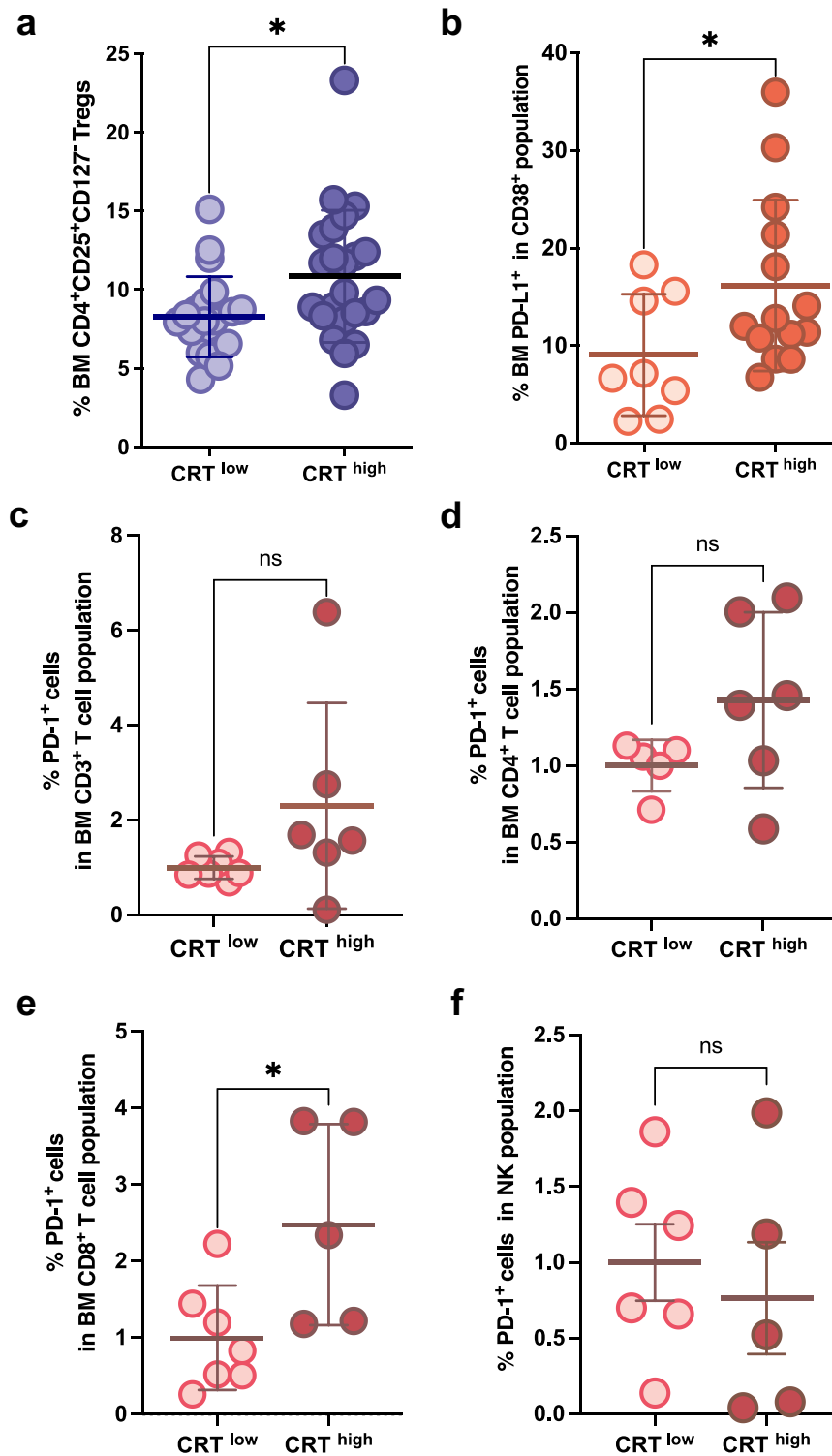
Not only cell numbers but also altered functionality of immune cells could be responsible for the immunosuppressive environment found in MM. For instance, CD8<sup>+</sup> T cells from MGUS patients have been shown to be more efficient in targeting autologous transformed plasma cells compared to their MM counterparts.<sup>34</sup> Several studies have shown increased expression of inhibitory checkpoint proteins like PD-1, TIM-3, LAG-3 and

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extramedullary plasmacytomas was analyzed. Statistical analysis was performed using Fisher's exact test, where \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ , ns, non-significant. (d) Kaplan–Meier curve comparing the survival of patients in the CRT<sup>high</sup> and CRT<sup>low</sup> groups. Overall survival of patients with overt MM was calculated from the time of sample retrieval and CRT analysis. HR was obtained by the univariate log-rank (Mantel-Cox) test. (e) Multivariate Cox regression analysis of the indicated variables. (f) Patients were stratified in two groups (treated and untreated) depending on whether they have previously received any chemotherapeutic regimen or not at the moment when BM sample was collected. Ecto-CRT levels were compared between these two groups. Statistical analysis was performed using two-way t test, where \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ , ns, non-significant. (g) Samples were stratified in two groups based on their median ecto-CRT levels and number of total lines of therapy patients received was compared between CRT<sup>high</sup> and CRT<sup>low</sup> subgroups. Statistical analysis was performed using Chi-square test for trend (\* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ ) Left: global analysis; Right: samples from overt MM patients. (h) Individuals with MM or MGUS/SMM diagnosis were stratified depending on their cytogenetic profile and ecto-CRT levels were compared between these two groups. Bars indicated median with interquartile range. Statistical analysis was performed using Mann-Whitney test. (i) Relationship between CRT exposure and the presence of different cytogenetic abnormalities with varying degrees of risk. Statistical analysis was performed by comparing each of the different groups harboring cytogenetic abnormalities with the normal group, using Kruskal-Wallis test where \* $p < .05$ .



**Figure 2.** CRT exposure and the BM immune landscape in MM. Patients were stratified in two different cohorts based on their median ecto-CRT levels. Individuals with an equal or inferior value to the median CRT exposure cutoff of the entire population were deemed CRT<sup>low</sup>, while individuals with an ecto-CRT expression level over the median cutoff were classified under CRT<sup>high</sup> group. (a) BM frequencies of CD4<sup>+</sup>CD3<sup>+</sup> T cells, CD8<sup>+</sup>CD3<sup>+</sup> T cells, CD3<sup>+</sup> T lymphocytes, as well as the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells within CD3<sup>+</sup> population and the corresponding CD4<sup>+</sup>/CD8<sup>+</sup> ratios were analyzed by flow cytometry. (b) The percentage of CD56<sup>+</sup>CD45<sup>+</sup> NK cells in BM was determined by flow cytometry in the CRT<sup>high</sup> and CRT<sup>low</sup> groups. (c) Plasmacytoid lineage-negative/HLA-DR<sup>+</sup>/CD123<sup>+</sup> DCs (pDCs) and myeloid lineage-negative/HLA-DR<sup>+</sup>/CD11c<sup>+</sup> DCs (mDCs) subpopulations were also analyzed. Lineage-negative cells (Lin<sup>-</sup>) were determined by staining with FITC-conjugated antibodies against the following markers: CD3, CD14, CD16, CD19, CD20 and CD56. Statistical analysis was performed using two-way t test (\*p < .05, \*\*p < .01, \*\*\*p < .001). Data plotted in the figure represent the frequency of each cell type in individual samples. The mean and SD values in each patient cohort are also illustrated.



**Figure 3.** CRT exposure and immunosuppressive markers in MM. Patients were stratified in two different cohorts based on their median ecto-CRT levels. (a) The BM frequencies of Tregs were analyzed in CRT<sup>high</sup> and CRT<sup>low</sup> samples. (b) PD-L1 expression in CD38<sup>+</sup> BM cells was analyzed and compared between CRT<sup>high</sup> and CRT<sup>low</sup> groups. (c-f) PD-1 expression was analyzed in the indicated immune subpopulations and compared in patient's subgroups based on their ecto-CRT expression levels. Statistical analysis was performed using two-tail t test (\*p < .05, \*\*p < .01, \*\*\*p < .001). Data plotted in the figure represent the frequency of each cell type in individual samples. Global mean and SD in each cohort are also illustrated.



TIGIT on the surface of T cells (CD4<sup>+</sup> or CD8<sup>+</sup>) in peripheral blood or BM of MM patients.<sup>48–51</sup> Additionally, CD8<sup>+</sup> T cells in an MM environment can downregulate the costimulatory molecule CD28, upregulate CD57 (indicating low proliferative capacity) and exhibit increased PD-1 expression.<sup>52</sup> Moreover, patients bearing T cells with this exhausted or senescent phenotype at baseline or after therapy are the ones with more advanced disease stage,<sup>51,53</sup> minimal residual disease (MRD) positivity<sup>54</sup> or with higher odds of relapse after ASCT.<sup>51,52</sup> Also, increased PD-L1 expression in MM cells that has been associated with a more aggressive phenotype and with more advanced disease stages.<sup>55–58</sup> On the other hand, downregulation of activating receptors such as NKG2D, NKp30 or DNAM-1 (CD226),<sup>59–62</sup> together with the overexpression of inhibitory molecules like PD-1,<sup>63</sup> has been found in NK cells from MM patients, potentially contributing to dampening NK effector functions and anti-myeloma activities. Importantly, PD-1 expression on the surface of NK cells seems to correlate with an exhausted phenotype that does not improve with PD-1 blockade by specific checkpoint inhibitors.<sup>56,64</sup> Thus, we analyzed the expression of PD-L1 in myeloma cells and PD-1 NK and T cells. CD38<sup>+</sup> plasma cells from MM CRT<sup>high</sup> samples exhibited significantly increased PD-L1 expression compared to the CRT<sup>low</sup> group (Figure 3b). In agreement with these findings, immune effector cells from patients expressing high levels of ecto-CRT revealed an increased expression of the exhaustion marker PD-1, although it was only statistically significant in the CD8<sup>+</sup> T cell subset and a small percentage of cells (Figure 3c-e). Further studies would be needed to clarify the role of the PD-1/PD-L1 axis in the anti-tumor immunosurveillance in MM and its relationship with DAMP exposure.

## Conclusions

In summary, our data showed that patients with elevated ecto-CRT levels were associated with increased BM infiltration of immune effectors like CD8<sup>+</sup> T cells, NK cells and DCs. Also, the CRT<sup>high</sup> cohort harbored more cytogenetic abnormalities, some of which have been correlated with worse prognosis. Our data suggest that CRT<sup>high</sup> patients show hints of anticancer immunosurveillance with increased infiltration of NK cells, CD8<sup>+</sup> T cells and DCs, reminiscent of a T<sub>H</sub>1 anticancer immune response. However, CRT<sup>high</sup> correlated with expanded immunosuppressive hallmarks like Tregs and PD-L1 expression in myeloma cells. Therefore, attending to the data presented in this study and the panorama discussed above, it can be suggested that CRT<sup>high</sup> patients probably carry immune features associated with an undermined and subverted immune microenvironment, which could also be translated into a poor clinical outcome. Other factors might also contribute to explain the negative relationship between CRT exposure and the clinical outcome in MM and should be analyzed in the future to fully depict the immune landscape and the potential of immunotherapies in MM.

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## Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Instituto Aragonés de Ciencias de la Salud (CEICA, protocol code PI16/0129 and date of approval 05/25/2016). Informed consent was obtained from all subjects involved in the study.

## Authorship

Contribution: A.S.V., A.A., J.N. and I.M. designed the study; A.S.V., M. B-V. and N.J-A. performed sample analysis; A.S.V., MB-V and I. M. interpreted and analyzed data; V.P.R, G.A., R.D., B.M-L, I.I. and L. P. collected samples, reviewed patient records and collected data; A.S. V. and I.M. wrote the manuscript. All authors had full access to all data, carefully reviewed the manuscript, and approved the final version.



## Disclosure statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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## Data availability statement

The data that support the findings of this study are available from the corresponding author, IM, upon reasonable request.

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