Dendritic cells accumulate in the bone marrow of myeloma patients where they protect tumor plasma cells from CD8⁺ T cell killing

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Bone marrow dendritic cells play a dual, but opposing role in multiple myeloma

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ABSTRACT

Many researchers have speculated that the clinical progression from monoclonal gammopathy of undetermined significance (MGUS) to multiple myeloma (MM) is driven by defects in dendritic cell (DC) function. Evidence supporting this assumption is, however, controversial and no mechanism for the putative DC dysfunction has so far been demonstrated. We studied DC subsets from the bone marrow of MM patients, in comparison to those of MGUS patients and control subjects. We found that myeloid DC (mDC) and plasmacytoid DC (pDC) accumulate in the bone marrow during the MGUS-to-MM progression. After engulfment of apoptotic tumor plasma cells via CD91, bone marrow mDC and pDC mature and are able to activate tumor-specific CD8⁺ T cells. However, by interacting directly with CD28 on live (non-apoptotic) tumor plasma cells, bone marrow mDC downregulate the expression of proteasome subunits in these cells, thus enabling their evasion from HLA class I-restricted CD8⁺ T cell killing. These results suggest that DC play a dual, but opposing role in MM: on one hand DC activate CD8⁺ T cells against tumor plasma cells and, on the other hand, DC protect tumor plasma cells from CD8⁺ T cell killing. This information should be taken into account in designing immunotherapy approaches to enhance immune surveillance in MGUS and to break down immune tolerance in MM.
INTRODUCTION

Multiple myeloma (MM) is a lethal plasma cell malignancy that is often anticipated by a preneoplastic phase termed monoclonal gammopathy of undetermined significance (MGUS)\textsuperscript{1}. How MGUS progresses to MM is not exactly known, but it is thought to involve the generation of effective cytotoxic CD8\textsuperscript{+} T cell responses against tumor plasma cells. This generation relies on dendritic cells (DC), highly differentiated antigen-presenting cells with the unique capacity to internalize tumor antigens from the environment and present them as HLA class I-bound peptides (a process known as “cross-presentation”) \textsuperscript{2-4}.

In humans, DC circulating in the blood characteristically express high levels of HLA class II molecules and are proficient in antigen uptake and processing. However, they express low levels of HLA class I and co-stimulatory molecules (e.g. CD80, CD86) and lack common lineage markers such as CD3, CD14, CD16, CD19, CD20 and CD56. These lineage-negative (Lin\textsuperscript{−}) cells are subdivided into CD11c\textsuperscript{+} myeloid DC (mDC) and CD11c\textsuperscript{−}CD123\textsuperscript{+} plasmacytoid DC (pDC). Alternatively, BDCA-1, BDCA-2, and BDCA-3 may be used to distinguish between mDC (BDCA-1\textsuperscript{+} and BDCA-3\textsuperscript{+}) and pDC (BDCA-2\textsuperscript{+})\textsuperscript{5}.

DC detect and take up dead or dying tumor cells through the recognition of a variety of proteins, categorized as damage-associated molecular pattern molecules (DAMPs), expressed on the surface of such cells\textsuperscript{6}. One of these molecules is the endoplasmic reticulum protein calreticulin, which has been shown to be translocated to the plasma membrane of apoptotic cells where it acts as an “eat-me signal” for DC by binding with CD91 \textsuperscript{7-9}. Once DC engulf dead or dying tumor cells, they undergo a series of maturation events that reduce the antigen-capturing capacity, increase the expression of HLA class I and II and co-stimulatory molecules, develop an exceptional efficiency in processing tumor proteins and presenting tumor peptides to T cells, and augment the secretion of cytokines modulating T cell
activation. In particular, mDC are major producers of interleukin (IL)-12, while pDC are specialized in producing type I interferon (IFN)\textsuperscript{10}.

The process of degrading tumor proteins into peptides that will be presented by HLA class I molecules to CD8\textsuperscript{+} T cells occurs in the proteasome\textsuperscript{11}. In this cylindrical 26S proteolytic complex, some of the catalytic subunits can be exchanged in response to changing cellular environments. In particular, the β subunits Delta, Zeta, and MB1 can be replaced by the β\textsubscript{i} subunits LMP2, LMP7, and LMP10, respectively. In DC, a mixed population of proteasomes, some incorporating β subunits and others β\textsubscript{i} subunits, can be found.

There is no agreement in the literature about the role of DC in MM pathogenesis. Different studies have shown that DC may promote myeloma-specific T cell responses\textsuperscript{12,13} while they may also support plasma cell proliferation and survival via engagement of their CD80/86 receptors by the ligand CD28 on plasma cells\textsuperscript{14-17}. Moreover, little information is available on DC in the bone marrow, which is the privileged site of clonal plasma cell proliferation and, therefore, the main site of tumor antigen expression.

In this study, we analyzed the \textit{ex vivo} distribution as well as the phenotypic and functional features of mDC and pDC of MM patients, in comparison to those of MGUS patients and control subjects, and explored the immunological effects of interactions among DC, T cells and plasma cells within the bone marrow milieu.
MATERIALS AND METHODS

Study subjects and biologic samples

Paired peripheral blood and bone marrow samples were obtained from 40 patients with newly diagnosed monoclonal gammapathies. Patients were classified as having MGUS (n = 20) or symptomatic MM (n = 20) according to the International Myeloma Working Group criteria.

Peripheral blood and bone marrow samples were also obtained from 10 healthy controls who were bone marrow donors for patients undergoing allogeneic transplantation. The study protocol was approved by the University of Bari Medical School Ethics Committee and conformed to the good clinical practice guidelines of the Italian Ministry of Health. Written informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

Cell preparations

Peripheral blood (PB) and bone marrow (BM) samples, from patients and controls, were used to isolate mononuclear cells (PBMC and BMMC, respectively) by Ficoll-Paque Plus (GE Healthcare Life Sciences) density gradient centrifugation. From the PBMC and BMMC preparations, CD8+ T cells, mDC and pDC were purified by automated magnetic sorting using, respectively, anti-CD8 microbeads and BDCA-1 and BDCA-4 Isolation Kits (Miltenyi Biotec). For certain experiments, mDC were alternatively purified by negative selection using Myeloid Dendritic Cell Isolation Kit (Miltenyi Biotec). Bone marrow from patients was also used to directly isolate tumor plasma cells by automated magnetic cell sorting with anti-CD138 microbeads (Miltenyi Biotec). All sorted cell populations exhibited >95% purity, as revealed by flow cytometry on immunostained cells.

Tumor plasma cells were made apoptotic by γ-irradiation (50 Gy) and then used immediately in phagocytosis assays. Tumor plasma cell lysates were prepared as described previously. K562 cells were purchased from ATCC.
Immunofluorescence staining and fluorescent labeling of cells

A set of commercial and in-house monoclonal antibodies (mAb) was used. Fluorescein isothiocyanate (FITC)-conjugated Lineage mAb mix, phycoerythrin (PE)-cyanine 5 (Cy5)-conjugated anti-CD123 mAb, PE-Cy5-conjugated anti-CD11c mAb were purchased from BD Biosciences; PE-Texas red (ECD)-conjugated anti-HLA-DR mAb, PE-conjugated anti-CD80 mAb, PE-conjugated anti-CD86 mAb, PE-conjugated anti-CD138 mAb, allophycocyanin (APC)-conjugated anti-CD28, FITC-conjugated anti-CD8 mAb were purchased from Beckman Coulter; APC-conjugated anti-BDCA-1 (CD1c), APC-conjugated anti-BDCA-3 (CD141) and APC-conjugated anti-BDCA-2 (CD303), PE-conjugated anti-IL-12 mAb and PE-conjugated anti-IFN-α mAb were purchased from Miltenyi Biotec. The delta mAb SY-5, MB1 mAb SJJ-3, zeta mAb NB1, LMP2 mAb SY-1, LMP7 mAb HB2 and LMP10 mAb TO-7, calreticulin mAb TO-11 were produced and characterized as described previously and labeled with FITC by the use of the Lightning-Link FITC Conjugation Kit (Innova Biosciences). The PE-conjugated NY-ESO-1 157-165-specific HLA-A*0201 pentamer was purchased from Proimmune.

Cells were incubated with mAb to surface antigens for 30 min at 4°C and then washed twice with cold PBS containing 0.1% BSA prior to flow cytometry. In some cases, surface-stained cells were fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences), washed with Perm/Wash solution (BD Biosciences), and stained with mAb to intracellular proteins at 4°C for 30 min. For mDC and pDC enumeration, whole PB and BM samples were incubated with the appropriate mAb, treated with FACS Lysing solution (BD Biosciences), washed with PBS, and mixed with Flow-Count fluorospheres (Beckman Coulter) at known concentrations. Staining with pentamers was performed according to the manufacturer’s instructions. For phagocytosis assays, DC and tumor plasma cells were labeled with PKH67 (green) and PKH26 (red) lipophilic dyes (Sigma-Aldrich), respectively, according to the
manufacturer’s instructions. For preparation of targets for cytotoxicity assays, tumor plasma cells were labeled with 0.5 μM 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies), as previously described 19, and K562 cells were labeled with 5 μM Far-Red dimethyldodecylamine oxide-succinimidyl ester (Life Technologies), according to the manufacturer’s instructions. For fluorescence microscopy, cells were incubated with 0.1 mg/mL DAPI (4,6 diamidino-2-phenylindole; Sigma-Aldrich).

Dendritic cell culture, loading, and stimulation

BMMC (6x10^5/well), mDC (16x10^4/well) and pDC (6x10^4/well) were cultured in 96-well round-bottom plates (BD Biosciences) in 200 μl of RPMI 1640 supplemented with 5% heat-inactivated human AB serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Sigma-Aldrich; culture medium). Cells were cultured alone or, when antigen loading was required, in the presence of apoptotic tumor plasma cells (from 0.60x10^4 to 6x10^4/well). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for different times after their isolation, depending on the assay. Prior to immunostaining of surface proteins, BMMC were cultured for 20 hours. Prior to intracellular cytokine staining, BMMC were incubated for a total of 6 hours, including 1 hour without followed by 5 hours with the secretion inhibitor Golgi Plug (1 μl/ml; BD Biosciences). For ELISA, mDC and pDC were cultured for 20 hours at which time aliquots of supernatant were removed and frozen at -80°C until batch analysis. Prior to antigen presentation assays, DC were incubated for 20 hours. To assess phagocytic activity, PKH67-labeled mDC and pDC from the same patient were pooled (to reach a suitable number of cells required for the experiments) and incubated for 20 hours with apoptotic PKH26-labeled plasma cells in the absence or presence of 10 μg/ml of a mouse IgG1 anti-human CD91 blocking mAb (GTX79843; Genetex) or the isotype control antibody (BD Biosciences).
Generation of epitope-specific and plasma cell-specific CD8⁺ T cell lines

PBMC (4×10⁵/well) were stimulated with 10 μg/ml NY-ESO-1₁₅₇-₁₆₅ peptide (Proimmune), 10 μg/ml influenza matrix₅₈-₆₆ peptide (Proimmune), or 100 μg/ml autologous plasma cell lysates in 200 μl culture medium in 96-well round-bottom plates. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced with fresh culture medium containing 10 U/ml rIL-2 (PeproTech) on days 4, 7, 11, 14 and 18; on day 7, the fresh culture medium also contained 10 ng/ml rIL-7 (PeproTech). Cultures were restimulated with 4×10⁵ irradiated autologous PBMC plus peptide or cell lysate on days 7 and 14. On day 21, cells from several wells were harvested, pooled and subjected to automated magnetic CD8⁺ T cell sorting.

CD8⁺ T cell epitope presentation assay

NY-ESO-₁₁₅₇-₁₆₅ or influenza matrix₅₈-₆₆ epitope-specific CD8⁺ T cells were cultured with autologous, pooled mDC and pDC, either unloaded or antigen-loaded, in 96-well round-bottom plates. Each well contained 2×10⁵ T cells and 2×10⁴ DC (T:DC ratio of 10:1). Cells were grown in 200 μl culture medium at 37°C in a humidified atmosphere containing 5% CO₂. Cells were harvested after 7 days and stained with epitope-specific pentamers.

Proteasome subunit expression analysis

Tumor plasma cells were incubated with or without autologous bone marrow mDC (plasma cell/DC ratio 2:1) in 96-well plates containing culture medium; some wells contained 50 μg/ml of a mouse anti-human CD28 blocking mAb (clone CD28.6, eBioscience). In other wells, tumor plasma cells were separated from mDC by a 1 μm pore size membrane (HTS Transwell-96 system, Corning). After 20 hours, cells were harvested, stained for CD138 and proteasome subunits, and analyzed by flow cytometry.
Cytotoxicity assay

To prepare target cells for cytotoxicity assays, CFSE-labeled tumor plasma cells were incubated alone or with bone marrow mDC, as described in the preceding section, and then either repurified by immunomagnetic selection or recovered from the Transwell insert. A second target cell population consisted of Far Red-labeled K562 cells (HLA-negative control) pulsed overnight with 10 μg/ml lysate from tumor plasma cells. Effector cells were tumor plasma cell-specific CD8+ T cells purified by magnetic cell sorting as described earlier.

Effector cells were incubated with both target cell populations together in round-bottom 96-well plates in culture medium at 37°C in a humidified atmosphere containing 5% CO2. Each well contained 5×10^4 CFSE-labeled plasma cells, 5×10^4 Far Red-labeled K562 cells and a serial dilution of effectors (from 50 to 5×10^4 cells). Wells containing target cells only served as a control. In some wells, the HLA-ABC–specific mAb TP25.99.8.425 was added at 10 μg/ml. The assay was performed in triplicate. After 6 hours, all cells were harvested and immediately analyzed by flow cytometry.

ELISA

Quantikine HS Human IL-12 kit (R&D Systems) and Verikine Human IFN-α kit (PBL Biomedical Laboratories) were used. The assays were read using a Bio-Rad 3550 plate reader.

Fluorescence microscopy and flow cytometry

Stained cells were examined under a Nikon TE2000 inverted microscope. Protein expression in these cells was expressed as the percentage of cells staining positively. Stained cells were also analyzed without delay on a FC500 (Beckman Coulter) flow cytometer using CXP software (Beckman Coulter) and FlowJo software (Tree Star). Levels of protein expression here were quantified in units of molecular equivalents of soluble fluorochrome (MESF) 13.
For cells stained with pentamers, the level of nonspecific binding was calculated from the background signal observed in cells from four HLA-A*0201-negative patients. In particular, the cutoff for pentamer-positive signals was set as the average background signal plus 3 SD.

For the assessment of cytotoxicity, the entire target cell population was defined by a live gate in a forward scatter/side scatter dot plot. Specific target cells were denoted by regions in Fl-1 (CFSE)/Fl-4 (Far Red) dot plots and detected and enumerated as specific target cells as CFSE⁺ (R2) and control target cells as Far Red⁺ (R3). Percent specific lysis was calculated from the ratio (R2/R3) in cultures containing defined numbers (n) of effector T cells (R2/R3)n in comparison to control (co) wells without T cells (R2/R3)co using the formula: 100% − [(R2/R3)n /(R2/R3)co]×100%.

**Statistical analysis**

Statistical analyses were performed using Prism (GraphPad Software). Nonparametric statistics were used because much of the data was not normally distributed. Tests included the Kruskal-Wallis analysis of variance, the Mann-Whitney test for comparisons of groups, the Wilcoxon signed rank test for comparison of matched samples, and Spearman’s rank test for correlations.
RESULTS

To understand the role that bone marrow DC play in the MGUS-to-MM progression, we explored a variety of features and functions of these cells from 20 patients with MGUS and 20 patients with newly diagnosed, symptomatic MM. For comparisons, we used bone marrow DC from 10 healthy controls as well as DC from peripheral blood of all three groups. First, we determined the frequencies of mDC and pDC in the peripheral blood and bone marrow of the three groups. To this aim, freshly drawn whole blood and marrow samples were stained with two different antibody cocktails (1 and 2) and analyzed by flow cytometry (Figure 1). Frequencies were determined as the proportion of live cells that were Lin⁻, HLA-DR⁺, and either CD11c⁺ (mDC) or CD123⁺ (pDC) after staining with cocktail 1. Additionally, they were determined as the proportion of live cells that were Lin⁻, HLA-DR⁺, and either BDCA-1⁺ BDCA-3⁺ (mDC) or BDCA-2⁺ (pDC) after staining with cocktail 2.

When cell frequencies were determined according to CD11c or CD123 immunoreactivity (cocktail 1, Figure 2A), the mean percentages of mDC and pDC in peripheral blood were significantly greater in control samples than in MM samples, with intermediate values in MGUS samples. In bone marrow, in contrast, the mean frequencies of mDC and pDC in the MM group were significantly elevated to approximately twice that in MM peripheral blood, reaching levels comparable to those of the bone marrow in the other groups. Similar patterns emerged when the analysis was based on BDCA immunoreactivity (cocktail 2, Figure 2B). Absolute numbers of mDC and pDC showed similar trends, with peripheral blood values that progressively decrease moving from control to MM patients and bone marrow values that progressively decrease moving from MM to controls, with intergroup differences not reaching statistical significance in the latter case (Figure 2C-D). The absolute numbers of bone marrow mDC and pDC were found to correlate positively with the numbers of bone marrow plasma cells (CD138⁺) in both MGUS and MM patients (Figure 2E). Altogether, these results
suggest that mDC and pDC accumulate in the bone marrow during the MGUS-to-MM progression and that this accumulation is proportional to the extent of tumor burden. These results also document that the two different antibody cocktails are able to identify the same cell populations; thus, for the rest of our study, we used the cocktail 1 to label mDC and pDC.

We next examined the ability of bone marrow mDC and pDC to phagocytize apoptotic tumor plasma cells. For this purpose, both DC subsets from bone marrow were pooled (to have enough cells for the experiment) and labeled with the green membrane dye PKH67, while bone marrow CD138⁺ cells were labeled with the red dye PKH26 and γ-irradiated. The cell preparations were incubated together for 20 hours, after which the uptake of apoptotic cells by DC was visualized by fluorescence microscopy (Figure 3A) and measured by flow cytometry as the percentage of doubly stained cells (Figure 3B-C). DC from both MGUS and MM patients showed strong and comparable phagocytic activity. This activity was negligible in control samples incubated on ice (data not shown) and was drastically reduced when incubation was performed in the presence of a CD91-blocking mAb but not in the presence of an unrelated isotype control antibody (Figure 3B-C). These results indicate that CD91 is crucial for the recognition and uptake of apoptotic tumor plasma cells by DC.

We therefore checked whether the natural CD91 ligand, i.e. calreticulin, was exposed on the surface of tumor plasma cells. To this aim, bone marrow CD138⁺ cells were surface-stained for calreticulin and analyzed by fluorescence microscopy (Figure 3D) and by flow cytometry (Figure 3E-F). Calreticulin was found on the surface of tumor plasma cells from both MGUS and MM patients, and the percentages of stained cells were similar between the groups. These results indicate that pre-apoptotic tumor plasma cells, susceptible of being recognized and engulfed (via CD91) by DC, are present in the bone marrow of these patients.

We then assessed the co-stimulatory molecule expression and cytokine production by mDC and pDC in response to stimulation by apoptotic tumor plasma cells. To this aim, mDC and
pDC were cultured in the absence or presence of γ-irradiated CD138+ cells prior to staining and analysis. Flow cytometry revealed that the stimulation by plasma cells significantly upregulated CD80 and CD86 expression in mDC (Figure 4A-B) and in pDC (Figure 4C-D), for both MGUS and MM patients without significant differences between the two groups. It also revealed that plasma cell stimulation significantly increased the intracellular expression levels (reported as MESF units) of IL-12 in mDC and IFN-α in pDC (Figure 4E-F), in both groups. A similar increase was found when cytokine concentration was measured in cell culture supernatants by ELISA (Figure 4G-H). Overall these results demonstrate that the ability of mDC and pDC from MM patients to mature in response to apoptotic plasma cells is equivalent to that of MGUS patients.

We then analyzed the ability of bone marrow DC (that have engulfed apoptotic tumor plasma cells) to present tumor plasma cell antigens to CD8+ T cells. To this aim, we selected from the study population four HLA-A*0201-positive MM patients. CD8+ T cells from their bone marrow were expanded in vitro in the presence of autologous mDC and pDC pooled and loaded with allogeneic apoptotic plasma cells from HLA-A*0201-negative MM patients. DC loading had been performed in the absence or presence of a CD91-blocking mAb. The percentage of CD8+ T cells specific for the HLA-A*0201-restricted NY-ESO-1_157-165 epitope was detected by pentamer staining and flow cytometry after 21 days (two re-stimulations). A sizeable population of NY-ESO-1_157-165-specific CD8+ T cells was found in all four patients and became significantly smaller when CD91 blockade was performed (Figure 5). No NY-ESO-1_157-165 pentamer-binding cells were observed when the same experiment was performed with a control influenza matrix58-66 epitope-specific CD8+ T cell line (data not shown). These results suggest that bone marrow DC are able to efficiently activate anti-tumor CD8+ T cells via cross-presentation and that this process is dependent on CD91.
We then examined the expression of CD28, a receptor for CD80/CD86, on live tumor plasma cells. To this end, BMMC were stained for CD28 and CD138 and analyzed by flow cytometry. CD28 expression in plasma cells (CD138⁺) was significantly higher in MM patients than in MGUS patients (Figure 6A-C), indicating that CD28 associates with disease status (progression). Based on this result, we investigated the effect of the CD28-mediated interaction between plasma cells and bone marrow mDC on the expression of proteasome subunits in live tumor cells. To this aim, we incubated mDC with CD138⁺ cells prior to intracellular staining with a set of in-house mAb specific for proteasome subunits. Flow cytometric analysis of protein levels, expressed in molecular equivalents of soluble fluorochrome (MESF) units, revealed a significant downregulation of proteasome subunits in tumor plasma cells after culture with mDC (Figure 6D-E). This downregulation was not observed in tumor plasma cells cultured with mDC in the presence of a CD28-blocking mAb (Figure 6E) or in cultures where plasma cells were separated from mDC by a Transwell membrane (Figure 6D). Of note, when the experiment was repeated using tumor plasma cells additionally stained for CD28, no downregulation of proteasome subunits was observed in the CD28⁻ fraction of CD138⁺ cells (Figure 6F-G). These results indicate that bone marrow mDC modulate the expression of proteasome subunits in tumor plasma cells in a contact-dependent manner involving CD28.

Finally, we assessed the effects that lower levels of proteasome subunits might have on the recognition of tumor plasma cells by cytotoxic T lymphocytes. To this aim, we performed flow cytometry-based cytotoxicity assays (Figure 7). In the assays, effectors were CD8⁺ T cells that had been expanded in vitro in the presence of autologous DC loaded with autologous apoptotic plasma cells; targets were autologous CFSE-labeled CD138⁺ cells that had been pre-incubated alone or in the presence of autologous bone marrow mDC, in either normal or Transwell culture plates, with or without a CD28-blocking mAb. The tumor plasma
cell-specific CD8⁺ T cells were cytotoxic to CD138⁺ (plasma) cells not exposed to DC, with the percent specific lysis increasing with the effector:target ratio (Figure 7, first row). Minimal specific lysis was instead observed when the plasma cells had been pre-incubated with bone marrow mDC in normal culture plates (second row). However, the cytotoxic effect was retained when the pre-incubation had been done across a Transwell insert (third row) or in normal culture plates containing a CD28-blocking mAb (fourth row). These results suggest that DC protect tumor plasma cells from CD8⁺ T cell killing.
DISCUSSION

There is no agreement in the literature about the role of DC in MM pathogenesis. Frequency, phenotype and function of these cells have been described to be altered by some authors and normal by others. Moreover, different protocols of DC purification and maturation and different ways of measuring DC activity have produced data that cannot be compared and that do not often include MGUS patients.

Our paper provides a sharper picture of the distribution and functioning of DC during the MGUS-to-MM progression. It shows that a considerable pool of mDC and pDC is "stockpiled" in bone marrow in MGUS patients and even more so in MM patients. Marrow-resident mDC and pDC are fully capable of maturing and activating tumor-specific CD8+ T cells upon engulfment of apoptotic tumor plasma cells that present calreticulin on their surface. mDC are also able to interact directly with live tumor plasma cells, downregulate the expression of proteasome subunits in these cells, and make them resistant to CD8+ T cell killing.

Beyond the general interest in knowing the exact number of differentiated DC within the bone marrow, the finding that DC are abundant where tumor plasma cells are located is extremely important, for two reasons: bone marrow is an autonomous priming site for T cell responses and DC at the site of antigen expression are the cells most directly involved in determining T cell immunity against the tumor. It can be supposed that DC in bone marrow are derived from peripheral blood and that bone marrow recruitment is chemokine-mediated, tumor antigen-dependent, or both. The finding that recognition and uptake of apoptotic tumor plasma cells by DC depends on the binding of surface calreticulin by its receptor CD91 extends the growing evidence that calreticulin acts as a crucial "eat me signal" for DC that are equipped to perform antigen cross-presentation. Importantly, we show that this mechanism is inhibited when CD91 is blocked, thus demonstrating for the first time that the
calreticulin–CD91 pathway is critical for the establishment of antitumor T cell immunity in MM.

The capacity of bone marrow-resident DC to mature and successfully activate tumor-specific CD8\(^+\) T cells adds to our and others’ previous observations that tumor plasma cell-specific CD8\(^+\) T cells can be readily isolated from the blood and bone marrow of patients with MGUS and MM\(^{12,13,31}\). Moreover, it strengthens the idea that T cell priming by DC is not abolished or impaired, indicating, in other words, that there is no tolerance to plasma cell tumors. These considerations call into question the other novel aspect of our study that pertains to the consequences of the interaction between DC and live tumor plasma cells. If there is no failure of priming or activation of T cells, we must suppose that progression of MGUS to MM is promoted by the ability of tumor plasma cells to evade T cell recognition and killing. We previously demonstrated that tumor plasma cells downregulate the expression of proteasome subunits, whose levels correlate with the extent of the tumor plasma cells’ specific lysis by CD8\(^+\) T cells and predict the clinical progression of MGUS to MM\(^{13}\). Now we provide evidence for a mechanism responsible for this downregulation that surprisingly involves the binding of CD28 expressed on tumor plasma cells with its ligands CD80/CD86 expressed on bone marrow mDC. The mechanism intensifies during the transition from MGUS to MM, with the progressive upregulation of CD28 on tumor plasma cells and the progressive accumulation of DC in the bone marrow. This finding also adds a new piece of information to the role that CD28, best characterized as the prototypic costimulatory receptor in T cells, plays in plasma cell biology. CD28 expression in MM has been found to associate with poor prognosis, disease progression, and resistance to chemotherapy\(^{15-17}\). Thus, its upregulation during the MGUS-to-MM transition may indeed reflect an escalation in the intrinsic plasma cell malignancy, meaning that oncogenic events accumulate in a clone that gains proliferative advantages.
The molecular link between CD28 activation and proteasome subunit downregulation remains to be determined, but it is likely to involve the PI3K/AKT pathway for several reasons: i) binding of CD28 on MM cells triggers PI3K/AKT signaling \(^{15}\); ii) PI3K/AKT signaling induces epigenetic silencing in tumors \(^{32,33}\); and iii) treatment of tumor plasma cells with decitabine, a potent DNA methyltransferase inhibitor, restores proteasome subunit expression, as we have already reported \(^{13}\). Additional studies are also required to understand whether the reduced killing, by CD8\(^+\) T cells, of tumor plasma cells with lower levels of proteasome subunits is due to their decreased efficiency of epitope generation or their altered antigen repertoire.

In conclusion, we propose that DC double-cross T cells: on one hand DC activate CD8\(^+\) T cells against tumor plasma cells but, on the other hand, they nullify this activation by protecting tumor plasma cells. This knowledge has implications for the design of immunotherapy approaches that can enhance immune surveillance in MGUS and break down immune tolerance in MM. In particular, blocking CD28 interactions in vivo would prevent immune escape of tumor plasma cells and resensitize them to lysis by cytotoxic T cells. In this respect, abatacept (CTLA4-Ig), a synthetic antagonist of CD28 used for the treatment of rheumatoid arthritis \(^{34}\), could readily be moved to MM treatment. CD28 blockade would also improve the so far limited efficacy of DC-based vaccines \(^{35}\). Our in vitro findings encourage the development of these new immunotherapies.
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AUTHOR CONTRIBUTIONS

P.L. and V.R. designed the research, analyzed data and wrote the paper. P.L., S.B., M.A.F., and R.R. performed research; V.D.R., S.C., S.B., P.D. provided reagents, materials and analysis tools; F.D. and A.V. read and made comments on manuscript.

DISCLOSURES

The authors have no financial conflict of interest.
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LEGENDS TO FIGURES

Figure 1. Strategy of flow cytometry analysis of immunostained whole blood and marrow samples (from MGUS and MM patients): gating was done sequentially on live cells, on lineage-negative cells, and then on CD11c+HLA-DRhi cells or BDCA-1/BDCA-3+HLA-DRhi cells to identify mDC and on CD123+HLA-DR+ cells or BDCA-2+HLA-DR+ to identify pDC. Representative plots from one patient for each group.

Figure 2. Frequency of DC in whole blood and marrow samples (from 10 control subjects, 20 MGUS and 20 MM patients). (A-D) Bar graphs show mean and standard deviation of percentages (A-B) and absolute numbers (C-D) of mDC and pDC identified with CD11c and CD123 mAb (A,C) or with BDCA-1/BDCA-3 and BDCA-2 mAb (B,D) in differently gated cells (see Figure 1). Kruskal-Wallis analysis of variance and Mann-Whitney test for comparisons of groups. (E) Correlation between absolute numbers of bone marrow plasma cells and absolute numbers of both mDC and pDC in MGUS and MM patients. Spearman’s rank test for correlations. P-values are shown only for statistically significant comparisons.

Figure 3. Uptake of apoptotic tumor plasma cells by bone marrow DC and role of calreticulin in this process. (A) Representative micrographs of green fluorescent, immunomagnetically purified DC (from MGUS and MM patients) that have engulfed, in vitro, γ-irradiated red fluorescent fragments of apoptotic plasma cells. (B-C) Changes in phagocytic activity of DC in vitro, upon treatment with a CD91-blocking mAb, determined by flow cytometry. Representative plots (B) and percentages (C) of green DC (from 9 MGUS and 9 MM patients) containing red apoptotic plasma cell fragments. Mann-Whitney test. (D) Representative micrographs of immunomagnetically purified plasma cells (from MGUS and MM patients) showing membrane-associated fluorescence after surface staining with FITC-
conjugated mAb to calreticulin. **(E-F)** Representative plots (**E**) and percentages (**F**) of tumor plasma cells (from 20 MGUS and 20 MM patients) that stained positively for surface calreticulin. Mann-Whitney test. P-values are shown only for statistically significant comparisons.

**Figure 4.** Co-stimulatory molecule expression and cytokine production by bone marrow DC (from 20 MGUS and 20 MM patients). (**A-D**) Changes in percentages of mDC (**A-B**) and pDC (**C-D**) expressing CD80 (**A,C**) and CD86 (**B,D**) upon stimulation with apoptotic tumor plasma cells. Wilcoxon signed rank test. (**E-H**) IL-12 (**E,G**) and IFN-α (**F,H**) production by mDC and pDC, respectively, upon stimulation with apoptotic tumor plasma cells, assessed by flow cytometry (**E-F**) and ELISA (**G-H**). Mann-Whitney test. IL-12 and IFN-α protein levels in positive cells analyzed with flow cytometry are reported in MESF units. The MESF rate was calculated as the MESF of the stimulated sample divided by that of the unstimulated sample for the same subject. P-values are shown only for statistically significant comparisons.

**Figure 5.** Bone marrow HLA-A2 tumor epitope-specific CD8+ T cells activated in vitro by bone marrow DC through cross-presentation. Representative plots and percentages (from 4 MM patients) of pentamer-binding populations upon stimulation with DC loaded with tumor antigen in the absence (top) and presence of a CD91-blocking mAb (bottom).

**Figure 6.** CD28 expression and CD28-mediated interaction of tumor plasma cells with bone marrow DC (effect on proteasome subunit expression by tumor plasma cells). Representative plots (**A**) and percentages (**B-C**) of tumor plasma cells expressing CD28 (from 20 MGUS and 20 MM patients). Mann-Whitney test. (**D**) Representative histograms for the expression of selected proteasome subunits by tumor plasma cells after incubation with bone marrow mDC.
in different conditions. (E) Effect of CD28 blockade on proteasome subunit expression in tumor plasma cells. Expression levels in positive cells are reported in MESF units. Wilcoxon signed rank test. (F-G) Representative histograms (F) and levels (MESF units) (G) of expression of proteasome subunits by CD28⁻ tumor plasma cells after incubation with bone marrow mDC in different conditions. P-values are shown only for statistically significant comparisons.

**Figure 7.** Effect of bone marrow DC on tumor plasma cell susceptibility to CD8⁺ T cell-mediated killing in vitro. Representative plots and percentages (mean and standard deviation) of lysis of CFSE-labeled tumor plasma cells by bone marrow CD8⁺ T cells from 15 MM patients. Immunomagnetically purified CFSE-labeled plasma cells were cultured with autologous bone marrow mDC in different conditions. After a pre-incubation period, Far Red-labeled K562 cells and autologous bone marrow CD8⁺ T cells were added to each well at the indicated ratios. Far Red-labeled-K562 cells were used as negative control targets.
Fig. 1
Fig. 2
Fig. 4
Fig. 5
**A** Gated on live bone marrow mononuclear cells

**B**

\[
\frac{\text{% CD138}^+\text{CD28}^+\text{cells within bone marrow mononuclear cells}}{\text{MGUS}}<\frac{\text{% CD138}^+\text{CD28}^+\text{cells within bone marrow mononuclear cells}}{\text{MM}}
\]

\[p=0.0063\]

**C**

\[
\frac{\text{% CD28}}{\text{of CD138}}\text{cells within bone marrow mononuclear cells}
\]

\[\text{MGUS} < \text{MM}\]

\[p=0.0013\]

**D**

- **Delta**
- **LMP2**

Plasma cells incubated with mDC in a transwell system

Plasma cells incubated with mDC and a CD28 blocking mAb

Plasma cells incubated with mDC

Plasma cells alone

**E**

- **Delta**
- **MB1**
- **Zeta**
- **LMP2**
- **LMP7**
- **LMP10**

\[p=0.0001\]

**F**

- **Delta**
- **LMP2**

CD28+ plasma cells incubated with mDC in a transwell system

CD28+ plasma cells incubated with mDC

CD28+ plasma cells alone

**G**

- **Delta**
- **MB1**
- **Zeta**
- **LMP2**
- **LMP7**
- **LMP10**

\[\text{MESF U x 10}^4\]

mDC - +

CD28 blockade - +

**Fig. 6**
Dendritic cells accumulate in the bone marrow of myeloma patients where they protect tumor plasma cells from CD8+ T cell killing

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