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ORIGINAL ARTICLE

PD-L1, LAG3, and HLA-DR are increasingly expressed during smoldering myeloma progression

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Abstract

Symptomatic multiple myeloma (MM) is a plasma cell neoplasm that represents the final stage of a continuum of clinical conditions that start from monoclonal gammopathy of unknown significance (MGUS), then transits in the more advance, but still asymptomatic, smoldering MM (SMM), with a final evolution in symptomatic MM. To investigate SMM microenvironment modifications, we studied 16 patients diagnosed at our hospital. Eight of them (group A) developed MM within 2 years from diagnosis while the others (group B) had stable SMM. Samples were bone marrow biopsies at diagnosis and after 2 years (±4 months) and were analyzed by immunohistochemical analysis. Firstly, we found a significant increase in both CD4+ cells (11 vs 17%, $p < 0.01$) and CD8+ cells (15 vs 18%, $p < 0.01$) between diagnosis and at follow-up samples (whole cohort). This was associated to an increase in the CD4+/CD8+ ratio (0.74 vs 0.93, $p < 0.01$). Secondly, we discovered an increased expression of T cell inhibitory molecules during SMM evolution. In fact, plasma cell PD-L1 and microenvironment cell LAG3 expression increased from 1 to 12% ($p = 0.03$) and 4 to 10% ($p = 0.04$), respectively, from diagnosis to follow-up. Also, plasma cells and microenvironment cells HLA-DR expression augmented during SMM evolution from 7 to 10% ($p = 0.04$) and 29 to 39% ($p = 0.01$), respectively. When comparing group A vs group B, we found an increased CD68-KP1+ cell infiltration in favor of group B at diagnosis (23 vs 28%, $p = 0.01$) and a greater plasma cell infiltration at follow-up (50 vs 26%, $p < 0.01$). Our findings suggest how immune escape mechanisms appear earlier during multiple myeloma evolution, and that LAG3 could be a possible immunologic target in this setting.

Keywords Smoldering myeloma · Immune system · PD-L1 · LAG3 · HLA-DR

Introduction

The mechanisms of the transition from asymptomatic plasma cell dyscrasia to active MM have not been clearly elucidated so far [1]. From a clinical perspective, several prognostic models have been developed [2]. From a biological point of view, research was initially focused on sequentially acquired mutations in the plasma cell genome. However, this mechanism has not fully clarified this issue and other possible explanations need to be identified. Since MM has a strong dependence from bone marrow microenvironment [3], we hypothesized that SMM that progresses into active MM may have an enhanced ability in inducing immune tolerance, thus promoting disease progression.

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Methods

The study was approved by the institutional IRB and all procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

We retrospectively selected 16 patients diagnosed with SMM at our center (Table 1) from March 2009 to February 2015. From a total of 334 patients diagnosed with multiple

Table 1 Patient characteristics

	At diagnosis							
Pts#	Stable SMM	M-protein type	Serum M-protein (gr/dL)	Urine M-protein (gr/24 h)	Serum free light chain involved/uninvolved ratio	Plasma cell percentage at bone marrow biopsy	> 1 focal lesion at MRI	CRAB criteria
1	No	IgG lambda	2.02	Absent	10.0	40%	2 focal lesions at spine and pelvis MRI	–
2	No	IgG kappa	1.28	Absent	14.0	40%	Normal spine and pelvis MRI	–
3	No	IgG kappa	1.57	Absent	34.0	50%	"Salt-and-pepper" lesions at spine and pelvis MRI; absence of lytic lesions at X-ray survey	–
4	No	IgG lambda	2.33	Absent	NA	20%	Normal spine and pelvis MRI and X-ray survey	–
5	No	IgA lambda	1.26	Absent	NA	30%	Normal X-ray survey	–
6	No	IgA lambda	1.59	Absent	29.6	30%	Normal spine and pelvis MRI and X-ray survey	–
7	No	IgG lambda	2.16	Absent	NA	25%	Normal spine and pelvis MRI and X-ray survey	–
8	No	IgG lambda	3.95	Absent	20.0	50%	"Salt-and-pepper" lesions at spine and pelvis MRI	–
9	Yes	IgA kappa	1.31	Absent	1.4	50%	Normal whole body CT scan	–
10	Yes	IgM kappa	0.71	Absent	NA	30%	Normal spine and pelvis MRI and X-ray survey	–
11	Yes	IgG lambda	1.79	Absent	NA	15%	Normal spine and pelvis MRI and whole body CT scan	–
12	Yes	IgG kappa	1.96	Absent	3.7	20%	Normal spine and pelvis MRI and X-ray survey	–
13	Yes	IgG kappa	2.64	Absent	NA	27%	Normal spine and pelvis MRI and X-ray survey	–
14	Yes	IgG lambda	0.90	Absent	NA	15%	Normal spine and pelvis MRI	–
15	Yes	IgA lambda	0.42	Absent	1.2	30%	Normal whole-body MRI and X-ray survey	–
16	Yes	Micromolecular lambda	Absent	2.65	NA	40%	Normal whole body MRI	–

	At diagnosis	2 years after diagnosis					
Pts#	Smoldering myeloma prognosis*	Serum M-protein (gr/dL)	Urine M-protein (gr/24 h)	Serum free light chain involved/uninvolved ratio	Plasma cell percentage at bone marrow biopsy	> 1 focal lesion at MRI	CRAB criteria
1	2	2.13	0.16	12.0	20%	4 focal lesions at spine and pelvis MRI + 1 lytic lesion at X-ray survey	Lytic lesions
2	2	1.74	Absent	19.2	55%	Multiple lytic lesions at whole body CT scan	Lytic lesions
3	2	2.35	Absent	NA	50%	Unchanged picture at spine and pelvis MRI and X-ray survey	Renal failure, anemia
4	2	3.83	1.67	208.0	70%	Multiple focal lesions at spine and pelvis MRI, normal X-ray survey	New focal lesions at MRI
5	2	1.02	Absent	10.8	35%	Normal X-ray survey	Splenic rupture due to isolated splenic amyloidosis
6	2	2.35	0.62	12.6	50%	Normal spine and pelvis MRI and X-ray survey	Anemia
7	2	2.74	Absent	4.7	60%	Pelvic plasmacytoma at spine and pelvis MRI	Plasmacytoma occurrence
8	2	4.0	Absent	23.1	70%	"Salt-and-pepper" lesions at spine and pelvis MRI, unchanged	Bone marrow plasmacytosis
9	2	1.52	Absent	1.8	25%	Normal whole body CT scan	–
10	2	0.85	Absent	10.4	25%	Normal spine and pelvis MRI	–

Table 1 (continued)

11	2	1.44	Absent	1.2	20%	Normal spine and pelvis MRI	–
12	2	1.78	Absent	8.0	25%	One focal lesion at spine and pelvis MRI, PET scan and CT negative	–
13	2	2.09	Absent	1.4	20%	Normal spine and pelvis MRI	–
14	2	0.62	Absent	3.3	20%	Normal spine and pelvis MRI	–
15	2	0.47	Absent	1.4	40%	Normal spine and pelvis MRI	–
16	2	0.03	5.15	NA	30%	Normal spine and pelvis MRI	–

SMM, smoldering multiple myeloma; *MRI*, magnetic resonance imaging; *NA*, not available

*From Kyle RA et al., *New England Journal of Medicine 2007 June 21, 356 (25): 2582–90:* group 1 (plasma cells, ≥ 10%; monoclonal protein level, ≥ 3 g per deciliter); group 2 (plasma cells, ≥ 10%; monoclonal protein level, < 3 g per deciliter); group 3 (plasma cells, < 10%; monoclonal protein level, ≥ 3 g per deciliter)

myeloma, 53 had smoldering myeloma at diagnosis. Of these 53 patients, only 16 had one bone marrow bioptic sample available at diagnosis and one at symptomatic disease progression or after a 2-year follow-up (± 4 months), whichever came first. We performed extensive immunohistochemical analysis of bone marrow samples of 16 patients affected by SMM at time 0 (16 samples) and at + 24 months (± 4 months, 16 samples). Half of these patients developed MM at 24 months (group A/progressed SMM), and the other half remained asymptomatic (group B/stable SMM). Analyses were performed on a total of 32 bone marrow specimens.

Immunohistochemical panel firstly described microenvironment cell composition (CD4+, CD8+, CD3+, CD45+, CD56+, CD68+) and plasma cell infiltration (CD138+). Then, loss of immunogenicity (PD-L1, PD-L2, PD1, LAG3, CTLA-4, IDO) and loss of antigenicity (HLA-DR) biomarker expression was reported. Immunogenicity and antigenicity biomarker expression was described as percentage on plasma cells and microenvironment separately. Immunostaining was performed on 3-μm-thick sections. They were unmasked with EDTA buffer at pH 8 or citrate buffer pH 6 with PTLink Dako to 96 °C for 15′, 30′, and 40′ (different for each antibody). The sections were then immunostained for CD4, CD8, CD68 KP1, CD68 PG-M1, CD56 (Dako, Glostrup, Denmark 1:2000); PD1 (Biocare Concord, CA, US); PDL1 (Spring/Roche 4300 Hacienda Drive Pleasanton, CA 94588); PDL2 (Proteinteck 5400 Pearl St., 60018 Rosemont, USA), IDO 1 (Novus Biologicals LLC 8100 Southpark Way, A-8 Littleton, CO 80120, USA), CTLA4 (Santa Cruz 2145 Delaware AvenueSanta Cruz, CA 95060 USA); LAG3 (Abcam 330 Cambridge Science Park CB4 0FL Cambridge); HLA-DR (Thermo 93–96 Chadwick Road, Astmoor Runcorn, Cheshire WA7 1PR, UK); FOX P3 (BD Pharmingen 10975 Torreyana Road San Diego, CA 92121-1106). Immunostaining was carried out using EnVision FLEX+ (Dako, Glostrup, Denmark) in an automated immunostainer (Dako Autostainer System) for development in peroxidase (DAB Chromogen) (Table 2). All examinations were revised by two hemopathologists. In case of discrepancy, the mean of the two values was reported. A first statistical analysis was performed between whole cohort samples at time 0 and + 24 months (32 samples, paired *t* test). A linear correlation was performed to study the expression of T cell inhibitory ligands on plasma cells and their respective receptors on microenvironment cells (HLA-DR with LAG3, PD-L1 with PD1). A second analysis compared at time 0- and + 24-month samples of stable SMM versus progressed SMM (16 samples each analysis, *t* test). *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed with STATA 13 (Statacorp LLC). International Myeloma Working Group Criteria were used to define smoldering and symptomatic smoldering myeloma [4]. Skeletal survey and spine and pelvis magnetic resonance imaging were used to assess bone lesions in all patients.

Table 2 List of monoclonal antibodies used for immunohistochemical analysis

Antibody	Species	Clone	Dilution	Ag retrieval	Company
FOX P3	Mouse	259D/C7	1:200	EDTA Ph 8, 15′ 96 °C	BD Pharmingen
PD1	Mouse	NAT105	1:50	Citrate Ph 6, 40′ 96 °C	Biocare
HLA-DR	Mouse	LN3	1:500	EDTA Ph 8, 15′ 96 °C	Thermo
LAG3	Rabbit mono	EPR4392	1:100	EDTA Ph 8, 30′ 98 °C	Abcam
CTLA-4	Mouse	F-8	1:100	EDTA Ph 8, 30′ 98 °C	Santa Cruz
IDO1	Mouse	2B5	1:200	atcl EDTA120°C 10’	Novus Biologicals
PDL1	Rabbit mono	SP142	1:100	EDTA Ph 8, 30′ 96 °C	Spring/Roche
PDL2	Rabbit	Policlonale	1:400	EDTA Ph 8, 30′ 98 °C	Proteintech
CD4	Mouse	4B12	1:300	EDTA Ph 8, 15′ 96 °C	Dako/Agilent
CD8	Mouse	C8/144B	1:20	EDTA Ph 8, 15′ 96 °C	Dako/Agilent
CD68	Mouse	KP1	1:3000	Citrate Ph 6, 15′ 96 °C	Dako/Agilent
CD68	Mouse	PG-M1	1:50	EDTA Ph 8, 30′ 96 °C	Dako/Agilent
CD56	Mouse	123C3	1:400	EDTA Ph 8, 30′ 98 °C	Dako/Agilent

Results

Our first analysis’ aim was to describe microenvironment changes occurring during SMM progression in the whole cohort of patients. We found a significant increase between time 0- and + 24-month samples of CD4+ cell infiltration (11 vs 17%, $p < 0.01$), CD8+ cell infiltration (15 vs 18%, $p < 0.01$), and CD4+/CD8+ ratio (0.75 vs 0.94, $p < 0.01$).

Eventually, we observed that SMM evolution is associated with an increased expression of T cell inhibitory molecules such as PD-L1 expression on plasma cells (1 vs 12%, $p = 0.03$) and LAG3 expression on microenvironment cells (4 vs 10%, $p = 0.04$, Table 3, Fig. 1). Also, HLA-DR expression was increased on plasma cells (7 vs 10%, $p = 0.04$) and microenvironment cells (29 vs 39%, $p = 0.01$). As expected, a correlation between LAG3 microenvironment cells expression and its ligand HLA-DR on plasma cells ($r = 0.47$, $p = <0.01$) (Fig. 2) was found.

In the second analysis, we compared progressed SMM versus stable SMM (group A vs group B) at time 0 and + 24 months. The aim of this comparison was to find potential immune biomarkers able to discriminate between SMM at high risk of evolution versus stable SMM. At time 0, we only found an increased CD68-KP1+ cells (macrophage differentiation cell marker) infiltration in favor of group B (stable SMM, 28 vs 23%, $p = 0.01$). At time + 24 months, no differences were observed but an increased plasma cell marrow infiltration in group A (progressed SMM group, 50 vs 26%, $p < 0.01$) (Table 3).

Discussion

Our first analysis studying the whole cohort of patients at diagnosis and at follow-up, showed a progressive inflamed marrow during SMM evolution. In fact, a significantly increased infiltration of CD4+ and CD8+ T cells associated with an increased HLA-DR expression on both plasma cells and microenvironment cells is a sign of a proinflammatory microenvironment. Of interest, generation of CD4+CD25+FoxP3+ T-regulatory cells (T-regs) has been described when CD4+ T cells were cocoltured with MM cells [5]. In consideration of this, we analyzed FOX P3 expression on microenvironment cells but we did not find a significant increase between diagnosis and follow-up samples. However, since T-regs are a subset of CD4+ lymphocytes, we compared CD4+/CD8+ T cell ratio. We observed a significant increase in CD4+/CD8+ ratio between SMM diagnosis and follow-up samples.

Regarding T cell inhibitory molecule expression, it is well known that MM expresses markers of immune system impairment at different stages of disease. We found an increased expression of PD-L1 on plasma cells and of LAG3 on microenvironment cells during myeloma evolution. Ray and colleagues showed that PD-L1 on this protein is highly expressed on plasma cells and plasmacytoid dendritic cells of MM patients. Moreover, the use of anti-PD-L1 antibodies could enhance NK cell-mediated plasma cell killing and generate MM-specific CD8+ cytotoxic lymhpocytes [6]. In clinical studies, a higher expression of PD-L1 on plasma cells of patients affected by MGUS or SMM has been associated to a higher risk of progression to symptomatic myeloma [7]. Moreover, a significant increase in PD-L1 expression on plasma cells was observed between patients with MGUS and SMM (11 vs 44%, $p = 0.015$). In another study, Tamura and colleagues reported a positive correlation between PD-L1 expression on MM cells and plasma cell concentration at marrow level. Of note, PD-L1 level was higher in patients with relapsed or refractory disease and its expression correlated with drug resistance [8]. More recently, a low serum level

Table 3 Comparison of immune markers median values between A) Evolved and Stable SMM at diagnosis and at follow-up control, B) Evolved SMM vs Stable SMM at diagnosis, C) Evolved SMM vs Stable SMM at follow-up. *Abbreviations: SMM = Smoldering Multiple Myeloma*

Variables	A) Diagnosis samples vs follow-up sample: whole cohort [mean value (CI95%:range)]	*p* value	B) Diagnosis samples: evolved SMM vs stable SMM [mean value (CI95%:range)]	*p* value	C) Follow-up samples: evolved SMM vs stable SMM [mean value (CI95%:range)]	*p* value
Plasma cell concentration	32% (26–38) vs 38% (29–47)	0.23	36% (26–45) vs 29% (18–39)	0.23	50% (37–63) vs 26% (20–31)	< 0.01
CD4+ cell concentration	11% (CI95%:9–13) vs 17% (15–18)	<0.01	10% [6–14] vs 12% [8–15]	0.42	16% (13–20) vs 17% (15–19)	0.88
FOXP3+ cell concentration	3% (0–6) vs 4% (2–6)	0.59	1% (0–2) vs 5% (0–11)	0.10	3% (0–7) vs 4% [1–7]	0.72
CD8+ cell concentration	15% (13–17) vs 18% (16vs20)	<0.01	15% (11–19) vs 15% (12–17)	0.95	18% (15–22) vs 18% (16–20)	0.88
CD4/CD8 ratio	0.74 (0.60–0.89) vs 0.93 (0.81–1.07)	<0.01	1% (0–1) vs 1% (0–1)	0.43	1% (0–1) vs 1% (0–1)	0.90
CD68 PGM1+ cell concentration	24% (23–26) vs 24% (23–36)	0.88	24% (22–25) vs 25% (23–26)	0.23	24% (22–26) vs 25% (22–27)	0.58
CD68 KP1+ cell concentration	26% (23–28) vs 28% (27–29)	0.07	23% (19–26) vs 28% (25–31)	0.01	27% (26–29) vs 29% (26–31)	0.26
CD56+ cell concentration	59% (38–80) vs 59% (36–81)	0.98	57% (20–94) vs 60% (28–91)	0.90	64% (30–98) vs 53% (15–92)	0.63
PDL1+ plasma cell concentration	1 (0–3) vs 12 (3–22) (1 outlier excluded from analysis)	0.03	12% (0–39) vs 2% (0–4)	0.36	16% (0–33) vs 13% (0–28)	0.81
PD1+ cell concentration	4% [2–6] vs 6% [3–10]	0.13	3% (0–7) vs 5% [2–8]	0.46	9% [2–16] vs 4% [2–7]	0.17
HLA-DR+ plasma cell concentration	7% [5–8] vs 9% [7–12]	0.04	6% [3–8] vs 8% [6–9]	0.14	10% [6–14] vs 9% [5–12]	0.41
HLA-DR+ cell concentration	29% (24–34) vs 39% (32–45)	0.01	28% (18–38) vs 30% (24–36)	0.67	44% (33–55) vs 33% (25–42)	0.10
LAG3+ plasma cell concentration	8% [4–13] vs 15% (7–23)	0.16	12% (4–20) vs 5% [1–9]	0.07	13% (4–22) vs 18% (1–34)	0.55
LAG3+ cell concentration	4% [2–7] vs 10% [5–15]	0.04	4% [1–6] vs 5% [1–10]	0.47	8% [2–15] vs 12% (3–21)	0.42
CTLA4+ cell concentration	4% [2–5] vs 3% [2–4]	0.27	4% [1–8] vs 3% [1–5]	0.44	2% (0–4) vs 4% [2–5]	0.27
IDO+ cell concentration	8% [2–13] vs 5% [2–7]	0.23	6% [1–12] vs 9%(0–20)	0.55	4% [1–6] vs 6% [2–10]	0.27
PDL2+ plasma cell concentration	11% [8–15] vs 11% [7–14]	0.74	11% (4–17) vs 12% (7–17)	0.73	9% [5–13] vs 12% (6–18)	0.42

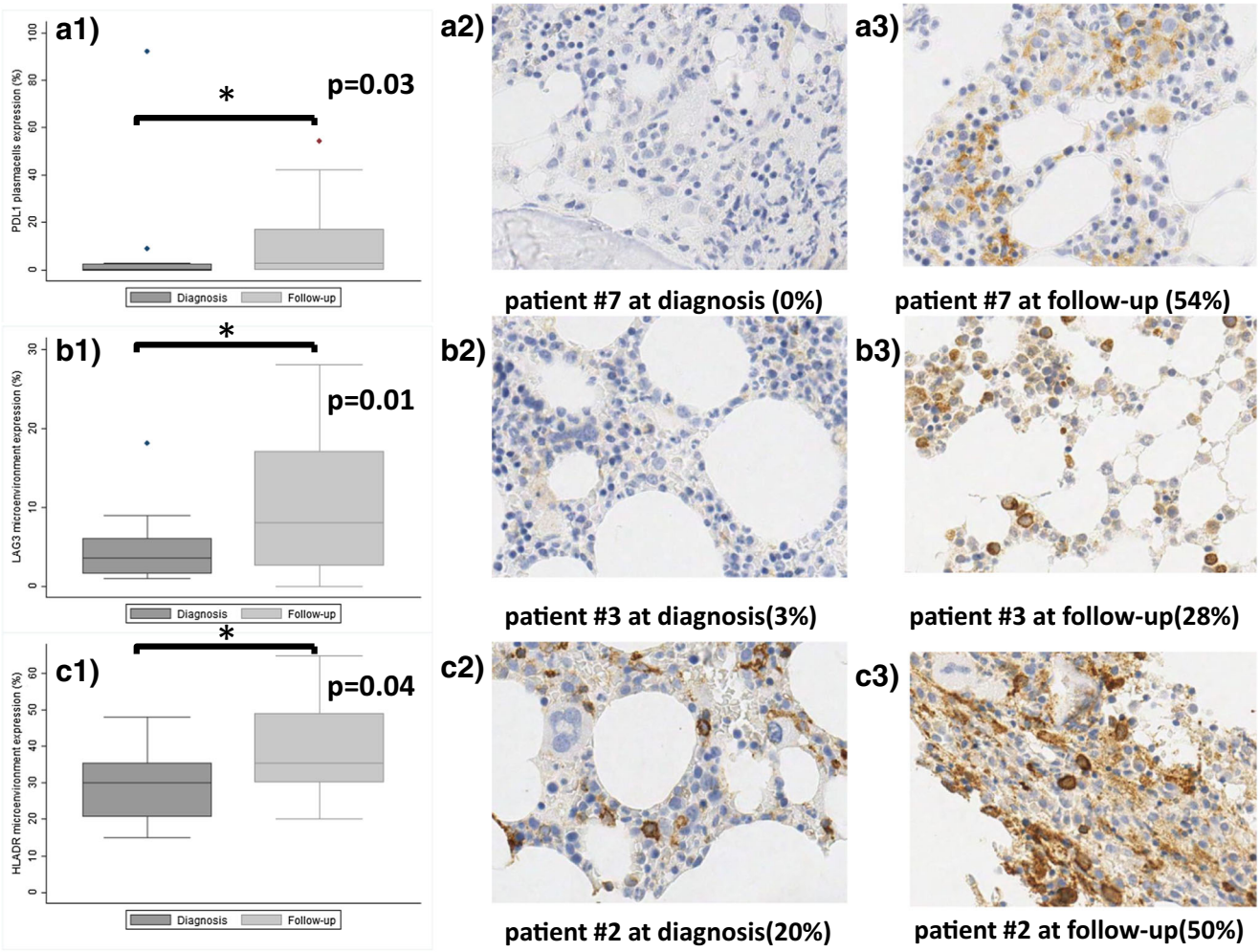

Fig. 1 Overexpression of PDL1 on plasma cells (A panels), LAG3 on microenvironment cells (B panels), and HLA-DR on microenvironment cells (C panels) is shown for the whole cohort in bar charts (A1, B1, C1) and on immunohistochemical analysis for most significant cases (A2, A3, B2, B3, C2, C3)

of soluble PD-L1 was an independent factor of a prolonged progression-free survival in newly diagnosed

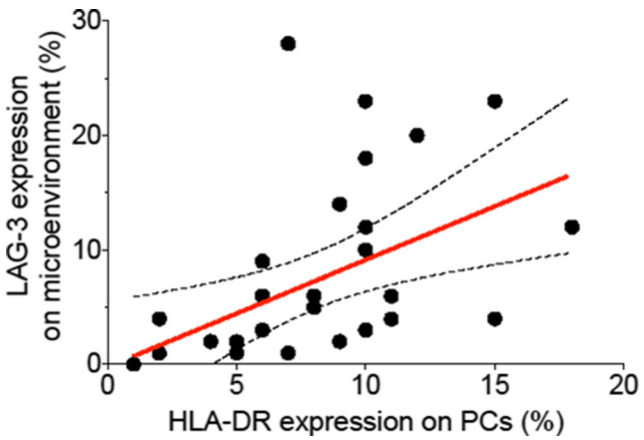

Fig. 2 Regression line analyzing the relation between LAG3 expression on microenvironment cells and HLA-DR on plasma cells on all bone marrow samples

MM patients [9]. Finally, another important observation with a relevant clinical implication is the reduced PD-L1 expression on MM cells after lenalidomide treatment [10]. Also, PD-1 has been found overexpressed on T cells of myeloma patients, representing another feature of the MM microenvironment [11]. Despite a possible prognostic role, the therapeutic implications of PD1/PD-L1 overexpression in MM are generally limited. In the setting of SMM to symptomatic MM progression, a pilot study of PD-1 inhibitor pembrolizumab showed a response rate of only 8% (1/12 patients with high-risk SMM). Interestingly, 83% (10/12) of patients had durable stable disease suggesting a possible disease-control effect [12]. In the setting of symptomatic MM, the use of PD-1 inhibitors pembrolizumab (KEYNOTE 183, KEYNOTE 185) or nivolumab (Checkmate-602) in association with IMIDs and dexamethasone was not associated with better survival in randomized trials. On the contrary, higher lethal toxicity was observed compared to control arms [13]. These

observations highlight that checkpoint inhibitors may not be an effective strategy for MM. More research is needed to improve such results. Similar considerations could be applied to LAG3, a more recently discovered T cell inhibitory molecule. In vitro studies showed an increased expression of this molecule on murine myeloma models [14]. In a recent study, its expression was elevated on T cells of MM patients before and post autotransplant [15]. Again, its altered expression in the setting of SMM has never been reported so far. Interestingly, the combination of PD-L1 inhibitors with LAG3 inhibitors increased survival in myeloma murine models [14].

In the second analysis, we could not find any differences between group A and group B but an increased macrophage infiltration in favor of stable SMM group (28 vs 23%, $p = 0.01$). This is in contrast with the observation that tumor-associated macrophages (TAM) contributes to MM pathogenesis by means of increased secretion of VEGFA (angiogenesis), IL-10 (immune tolerance), IL-6/TNF-alpha/IL-1beta (promoting survival) [16]. However, there are no data regarding a role of TAM in the setting of SMM.

Our study confirms that microenvironment modifications are already present during the pre-symptomatic stage of disease and not only in symptomatic MM. However, due to study limitations, results should be interpreted with caution and possibly confirmed in the context of a prospective study. In particular, the low number of patients could have affected the power of the study. Moreover, only an immunohistochemical analysis was used as a study technique due to the only availability of marrow specimens. Finally, the retrospective nature of the study did not allow us to recollect or perform all tests which could have played a role in smoldering MM classification and stratification.

In conclusion, we reported a comprehensive analysis of microenvironment modifications in the setting of SMM with a paired-samples analysis. Features of an inflamed but exhausted immune microenvironment were observed. In particular, increased in PD-L1 and LAG3 expression could be of clinical interest due to the current availability of checkpoint inhibitors drugs targeting these molecules.

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Compliance with ethical standards

The study was approved by the institutional IRB and all procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Conflict of interest The authors declare that they have no conflict of interest.

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