Cancer Immunol Immunother (2010) 59:27–34
DOI 10.1007/s00262-009-0717-4

ORIGINAL ARTICLE

Tumor endothelial marker 8 expression levels in dendritic cell-based cancer vaccines are related to clinical outcome

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Received: 18 February 2009 / Accepted: 17 April 2009 / Published online: 7 May 2009

Abstract Previous studies have shown that tumor endothelial markers (TEMs 1-9) are up modulated in immunosuppressive, pro-angiogenic dendritic cells (DCs) found in tumor microenvironments. We recently reported that monocyte-derived DCs used for vaccination trials may accumulate high levels of TEM8 gene transcripts. Here, we investigate whether TEM8 expression in DC preparations represents a specific tumor-associated change of potential clinical relevance. TEM8 expression at the mRNA and protein level was evaluated by quantitative real-time RT-PCR and cytofluorimetric analysis in human clinical grade DCs utilized for the therapeutic vaccination of 17 advanced cancer patients (13 melanoma and 4 renal cell carcinoma). The analyses revealed that DCs from patients markedly differ in their ability to up-modulate TEM8. Indeed, mDCs from eight non-progressing patients [median overall survival (OS) = 32 months, all positive to the delayed-type hypersensitivity test (DTH)], had similar TEM8 mRNA expression levels [mDCs vs. immature iDCs; mean fold increase (mfi) = 1.97] to those found in healthy donors (mfi = 2.7). Conversely, mDCs from nine progressing patients (OS < 5 months, all but one with negative DTH) showed an increase in TEM8 mRNA levels (mfi = 12.88, $p = 0.0018$). The present observations suggest that TEM8 expression levels in DC-based therapeutic vaccines would allow the selection of a subgroup of patients who are most likely to benefit from therapeutic vaccination.

Keywords Tumor endothelial marker 8 · Dendritic cells · Immunotherapy · Clinical outcome

Abbreviations

iDCs	Immature dendritic cells
mDC	Mature dendritic cell
IL	Interleukin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
VEGF-A	Vascular endothelial growth factor A
PGE_2	Prostaglandin E_2
TNF-α	Tumor necrosis factor α
Poly I:C	Polyinosinic:polycytidylic acid
LPS	Lipopolysaccharide
DTH	Delayed-type hypersensibility

Introduction

Ex vivo generated DCs are currently used as autologous cell vaccines for advanced cancer patients [1]. However, in terms of clinical response, DC immunization only occasionally induces stable disease or regression of tumor metastases [2–4]. Thus, it is not known whether the suboptimal clinical responses observed have been caused by the vaccination itself, or whether they reflect patients with a better prognosis who are capable of an immunoresponse. The advanced stage (IV) of disease is one of the main challenges facing experimental trials. We are now aware that at this stage, tumors induce and/or expand heterogeneous

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populations of dysfunctional myeloid monocytes which may not only help tumor immune escape, but also stimulate or amplify tumor angiogenesis [5–7]. However, it remains unclear whether these monocytes act directly or as precursor cells.

Recent evidence has emerged for an important role of DCs in immune–vascular interactions that mediate tumor progression. Indeed, it has been shown that tumor microenvironmental factors induce the differentiation of monocytes into DCs that, in the presence of proangiogenic mediators (i.e. VEGF-A), transdifferentiate into endothelial-like cells [8]. The possibility that DC precursors with a mixed DCs endothelial-like phenotype could incorporate into tumor-blood vessels has also been reported [9]. Consistent with these observations, some tumor endothelial markers (TEMs), originally identified as genes specifically expressed or significantly upregulated in tumor versus normal blood vessels [10], have been shown to be expressed in vascular and perivascular DCs in the tumor microenvironment [9]. Tumor endothelial marker 8 (TEM8) is one of the few TEMs reported to date to be highly specific for tumor endothelium [10, 11]. Moreover, high TEM8 expression appears to be correlated with advanced tumor stage in breast and colorectal cancer [12, 13]. Interestingly, the TEM8 gene product has been identified as a receptor for anthrax toxin (ATRX1) [14]. A second TEM8-related gene, capillary morphogenesis gene 2 (CMG2) has also been identified as an anthrax toxin receptor (ATRX2) [15]. At present, three TEM8 and four CMG2 isoforms are known to result from alternative splicing [16]. Although TEM8 transcripts are only detectable at basal levels in the blood monocytes and iDCs of healthy individuals [17], there is evidence to suggest that mDCs from cancer patients, while acquiring proangiogenic (VEGF-A-releasing) potential, may markedly differ in their TEM8 gene expression profiles [18]. Here we report on the apparent correlation between TEM8 tumor-associated changes in DC therapeutic vaccines and clinical response.

Materials and methods

Patient characteristics

The case series consisted of 17 patients who had taken part in phase I/II DC vaccination trials (2001–2005) for advanced melanoma ($n = 13$) and renal cell carcinoma (RCC; $n = 4$). The study details have been described previously [19]; patients received autologous tumor-lysate (LYS) and/or keyhole limpet hemocyanin (KLH) pulsed monocyte-derived DCs. Patient characteristics are summarized in Table 1. Phenotypic characterization of immature and mature DCs from patients is reported in Table 2.

Table 1 Patient demographics, disease status, and pretreatment characteristics ($n = 17$)

Patient ID	Sex	Age	PS (ECOG)	Site of evaluable disease	Pretreatments
35 G.D.	M	46	0	ln	NT
38 B.A.	F	59	2	lv, st	BIOCT, Locoreg CT
39 C.P.	F	39	0	kd, st	Locoreg CT
40 O.M.	M	56	1	lg, st	BIOCT
44 Z.S.	*M*	*62*	*1*	*ln*	*BIO*
46 R.P.	*M*	*56*	*0*	*lg, st*	*CT*
51 D.P.	*M*	*56*	*0*	*lg, st*	*CT*
52 L.B.	F	39	2	pv, ln	BIOCT
53 D.U.	*M*	*68*	*0*	*ln*	*BIOCT*
54 M.G.L.	F	37	0	lg, kd, ln, st	BIOCT
55 O.G.	M	65	2	ag, ln, st	BIOCT
56 R.M.	M	48	0	ln, lg, lv	BIO
57 M.R.	F	38	0	ln	BIOCT
58 De.G.	M	28	2	sk, lg, ln	BIOCT
60 T.M.	M	26	1 (2)	sk, ln	BIOCT
61 D.G.	M	34	0	ln, sk	NT
62 B.F.	M	64	0	ln, sk	NT

Male/female 12/5. Median age 48 years (34–68). Italicized entries represent renal cancer cell patients. *PS (ECOG)* performance status according to ECOG. Site of evaluable disease: *ln* lymph node, *lv* liver, *st* soft tissue, *kd* kidney, *lg* lung, *pv* pelvis, *ag* adrenal glands, *sk* skin. Pretreatments: *NT* no treatment, *CT* chemotherapy, *BIO* immunotherapy (interferon, IL-2), *BIOCT* chemotherapy + immunotherapy

Table 2 Surface expression of DC markers

Marker	iDC median % (range)	mDC median % (range)
CD1A	27 (4.8–53)	18 (1–77)
CD14	2 (0–33)	20 (0–56)
CD80	6 (1–23)	66 (8–88)
CD83	2 (0.1–13)	53 (2–95)
CD86	29 (5.4–75)	61 (5–93)
HLA-DR	55 (8.2–76)	80 (48–97)
CCR7	4 (2–5)	31 (1–91)

iDC immature dendritic cells, *mDC* mature dendritic cells. Data represent the percentage of positive cells out of the total number of DC analyzed

Vaccine composition and clinical and immunological outcome are detailed in Table 3. mDCs obtained from six healthy donors were also included for comparison.

DC generation

Mature DCs from each patient were generated from cryopreserved peripheral blood monocytes (PBMCs) collected

Table 3 Patient vaccination and clinical–immunological outcome

Pt. ID	No. Vacc	Administered DC $n \times 10^6$ (range)	DTH response LYS/KLH	Clinical response	Response duration	OS (months)
35 G.D.	16	12.6 (2.8–20.8)	++/+++	CR	8	34
38 B.A.	4	5.9 (3.7–12)	−/−	PD	–	7
39 C.P.	6	7.8 (1.6–15)	−/++	PD	–	20
40 O.M.	4	11.5 (10–21)	−/−	PD	–	5
44 Z.S.	*10*	*10 (6.6–17)*	−/+	*SD*	*6*	*12*
46 R.P.	*26*	*10 (8.2–11.6)*	+/++	*PR*	*30*	*36*
51 D.P.	*7*	*10 (9.6–10.8)*	−/−	*PD*	–	*10*
52 L.B.	4	12.5 (10–15.5)	−/−	PD	–	3
53 D.U.	*9*	*10 (5.3–10)*	+/+	*SD*	*10*	*36*
54 M.G.L.	32	9.1 (2.2–11)	+/+++	PR	22	39+
55 O.G.	5	10 (8.8–12.3)	−/−	PD	–	3
56 R.M.		NA	−/−	PD	–	5
57 M.R.	4	9.2 (8–10)	+/++	SD	4	6
58 De.G.		NA	−/−	PD	–	3
60 T.M.		NA	−/−	PD	–	1
61 D.G.	10	10 (10–10.7)	+/++	CR	30+	30+
62 B.F.	18	10 (10–10)	++/++	PR	24+	27+

Italicized entries represent renal cancer cell patients. DTH response: *LYS* autologous tumor cell lysate, *KLH* keyhole limpet hemocyanin, *DTH* delayed-type hypersensitivity test (best response after four or more vaccinations). Clinical response: *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *OS* overall survival, *OS+* patient still alive

at the beginning of treatment (from the first leukapheresis bag). Briefly, frozen PBMCs were thawed and incubated in tissue culture flasks with CellGro DC Medium (Cell Genix, Freiburg, Germany) at 1×10^7 cells/ml for 2 h. The non-adherent cells were discarded and the adherent cells were incubated in CellGro DC Medium containing 1,000 IU/ml rhIL-4 and 1,000 IU/ml rhGM-CSF (Cell Genix, Freiburg, Germany) for 7 days to generate immature DCs. After the removal of the culture medium, iDCs were incubated for a further 2 days with a standard maturation cocktail containing TNFα, IL-1β, IL-6 and PGE_2 (Cell Genix, Freiburg, Germany; PGE2, Pfizer, Puurs, Belgium) [19]. Where indicated, iDCs were matured in a cocktail either PGE_2-depleted, PGE_2 replaced with PolyI:C (GE Healthcare, Milano, Italy) (20 μg/ml), or with LPS (100 ng/ml; *Escherichia coli* 055:B5; Sigma-Aldrich, Milano, Italy), plus TNFα (20 ng/ml).

Cytofluorimetric analysis

iDC and mDC phenotypes were determined by single or two-color fluorescence analysis as previously reported [19]. Briefly, 3–5 $\times 10^5$ cells were suspended in 100 μl of buffer (PBS, 2% FCS, 1% sodium azide) and incubated for 30 min at 4°C with 10 μl of fluorescein isothiocyanate or phycoerythrin-labeled monoclonal antibodies (mAbs). The cells were then washed twice and resuspended in 500 μl of assay buffer. The fluorescence was analyzed by a FACSCalibur and Canto flow cytometer (Becton Dickinson, Milan, Italy). mAbs specific for human CD1a, CD14, CD80, CD86, CD11c, CD33, DR (Becton Dickinson), CD83 (Immunotech, Marseille, France), and CCR7 (BD Pharmingen, Milan, Italy) were used. Anti-TEM8 rabbit polyclonal antibody (abCam, Cambridge, UK; cod. ab21270) and goat anti-rabbit IgG-FITC (abCam, Cambridge, UK; cod. ab6717) were utilized for TEM8 protein detection.

RNA isolation and real-time RT-PCR

DNase-treated total RNA was isolated from monocytes and DCs (5×10^5 cells) using an RNeasy Micro Kit (Stratagene, La Jolla, CA, USA) and stored at −70°C in RNase-free water. RNA integrity was determined by the presence of 18S and 28S ribosomal RNA bands on a formaldehyde gel. RNA quantization was performed by spectrophotometry. Isolated RNA was converted to cDNA using a reverse transcription kit (Promega, Madison, WI, USA). The reaction was performed in a 20 μL volume containing 5 mM $MgCl_2$, 1× reverse transcription buffer, 1 mM dNTPs, 15 U of RNasin ribonuclease inhibitor, 15 U of AMV RT and 0.5 μg of oligo (dT)15 primers. Prior to starting the reverse transcription reaction, 1 μg of RNA was incubated at 70°C

for 10 min, and then cooled on ice. The reverse transcription reaction steps were performed as follows: 42°C for 60 min, 95°C for 5 min, and 5°C for 5 min.

For quantitative real-time RT-PCR, universal primer sets of oligonucleotides that recognize all the human TEM8 and CMG2 splice variants, and GAPDH were designed and utilized as described previously [16]. Real-time quantitative PCR was performed using the MX3000P Real-time PCR system (MxPro QPCR Software version 3.00, Stratagene) and the BRILLIANT SYB Green QPCR Master mix according to the manufacturer's instructions. PCR reactions were performed after an initial denaturation step of 10 min at 95°C; thermal cycling was performed for 40 cycles with steps of 94°C for 48 s, 60°C for 48 s, and 72°C for 48 s, with fluorescence reading at the end of each cycle. Purity of the amplified PCR products was verified by DNA melting profiles. In addition, TEM8 and CMG2 amplicons were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Product bands were excised and purified with Macherey-Nagel gel extraction columns (NucleoSpin, M-Medical, Milano Italy) for DNA sequencing. Double-oriented sequencing reactions were carried out by MGW Biotech/M-Medical (Martinsried, Germany). The relative mRNA expression of TEM8 and CMG2 in monocyte precursors, and DCs was calculated in relation to the housekeeping gene, GADPH. Measurements were performed in triplicate.

Statistical analysis

Statistical analyses were performed using the non-paired Student's *t* test.

Results

TEM8 expression in DC therapeutic vaccines

Recent observations have shown that iDCs matured in the presence of anti-inflammatory molecules such as PGE_2, may acquire pro-angiogenic/immunosuppressive features [20–24]. Accordingly, we reported that iDCs from melanoma patients, upon in vitro maturation by TNFα, IL-1β, IL-6 and PGE_2, while releasing VEGF-A, may differ markedly in TEM8 gene expression levels [18]. To asses whether TEM8 up-modulation in mDCs represents a specific tumor-associated change, we analyzed the differences in TEM8 and CMG2 mRNA expression in mDCS and iDCs (see "Materials and methods") of melanoma and renal cell cancer patients ($n = 17$, Table 1), and healthy donors ($n = 6$). Table 2 details patients DCs phenotypes. Expression of DC markers was similar to that described in literature [19], and there were no significant differences in DCs

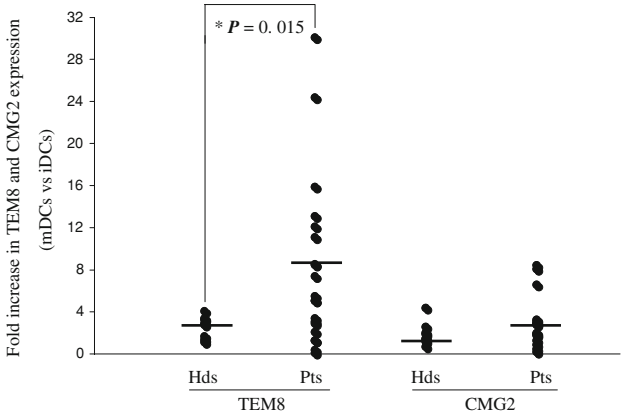

Fig. 1 Scatter plot of the fold increase (mDCs vs. iDCs) in TEM8 and CMG2 mRNA expression in cancer patients (Pts) and healthy donors (Hds). Each *dot* represents a separate measurement for each subject. The results reported are the mean from three independent real-time RT-PCR experiments. *Horizontal bars* mfi

obtained from donors and cancer patients (results not shown).

As shown in Fig. 1, although a modest increase in CMG2 mRNA levels was observed following iDC maturation, the patients and healthy donors were comparable [mean fold increase (mfi) = 2.88 (0.12–8.34) vs. 1.95 (0.6–4.3), respectively]. In contrast, there was a significant difference in mDC TEM8 mRNA expression between patients and healthy individuals (mfi = 8.4 vs. 2.7, respectively; $p = 0.015$), with patients showing strong interpersonal variation compared to healthy donors [(0.30–30) vs. (1.3–4), respectively]. No correlation between TEM8 expression with other DC markers was observed. Notably, an explorative analysis performed on a limited number of patients ($n = 6$),[1] indicated that increased TEM8 mRNA levels in mDCs fairly correlated with enhanced protein expression as determined by cytofluorimetric analysis using anti-TEM8 antibody (Fig. 2).

In agreement with findings by other authors [25], the levels of CMG2 transcripts always far exceeded those of TEM8 in all the cell types examined [monocytes (MOs), 143 (65–230); iDCs, 230 (50–461); mDCs 150 (60–278)] (Fig. 3). The most important difference in the expression ratio of the two genes was seen for iDCs ($p < 0.05$).

[1] In order to avoid strong interpersonal variations that usually associate with different DC preparations, all DCs were generated from PBMCs contained in the first leukaphereses bags. Because of the limited amount of cells, and because reliable anti-human TEM8 antibody were not commercially available when this study begun, we focused on DCs' TEM8 transcriptional activity. Unscheduled TEM8 protein expression analysis was therefore performed only in a minority of patients with enough clinical material left. A positive correlation between TEM8 mRNA and protein expression has been confirmed in additional analysis performed with mDCs from different melanoma patients study.

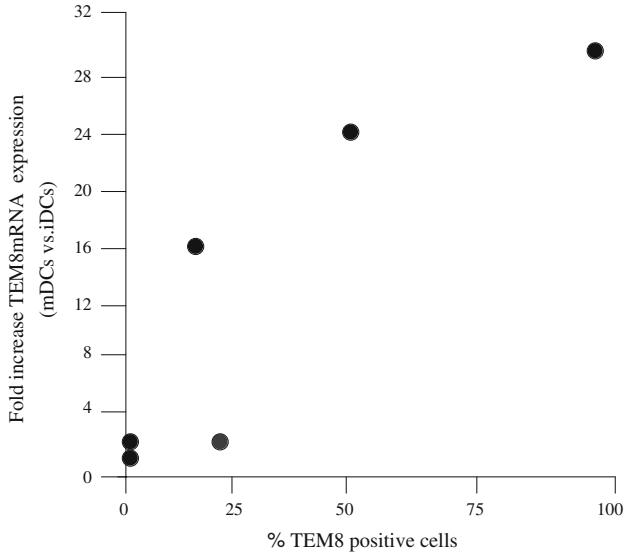

Fig. 2 TEM8 transcription levels and protein expression in Pts mDCs. TEM8 expression was detected by flow cytometry using rabbit anti-TEM8 Abs. TEM8 protein level is shown as a function of % TEM8 positive cells out of the total number of DC analyzed

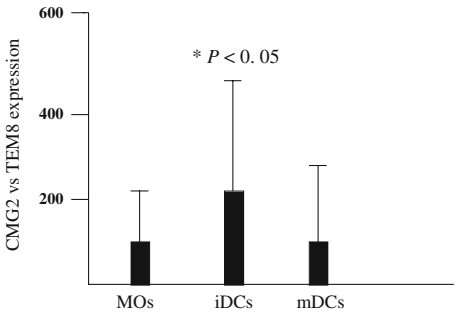

Fig. 3 CMG2 vs. TEM8 mRNA expression in MOs, iDCs, and mDCs in relation to GADPH (calibrator)

TEM8 expression depends on the type of media to which iDCs of cancer patients are exposed

Since the balance between pro- and anti-inflammatory signals influences the phenotype and the behavior of iDCs [24], in some experiments we investigated the effect of different DC activation stimuli, with "classic" proinflammatory and cytotoxic properties, on TEM8 expression. As depicted in Fig. 4, TEM8 expression in mDCs of cancer patients (39 C.P. and 56 R.M. Pts, mfi = 8.8 and 30, respectively) was suppressed if iDCs, generated from the same leukapheresis samples, were matured in a cytokine cocktail either PGE_2-depleted (mfi: 39 C.P. = 1.3; 56 R.M. = 3), PGE_2 replaced with PolyI:C (mfi: 39 C.P. = 1.3; 56 R.M. = 2.57), or with LPS plus TNFα (mfi: 39 C.P. = 2.2; 56 R.M. = 2.3).

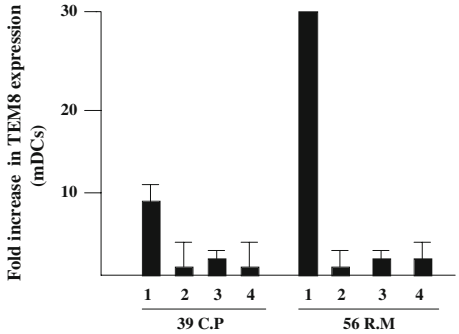

Fig. 4 Fold increase in TEM8 mRNA expression following different DC activation stimuli. iDCs from 39 C·P. and 56 R.M. Pts (Table 1) matured with: TNFα + IL-1β + IL-6 + PGE_2 cocktail (*1*); PGE_2-depleted cocktail (*2*); TNFα + IL-1β + IL-6 + Poly I:C (*3*); LPS + TNFα (*4*)

TEM8 expression in DC vaccines and clinical outcomes

As mDCs from cancer patients displayed high inter-individual variability in TEM8 expression, we retrospectively checked for a possible correlation between TEM8 gene expression and the clinical course of the disease. In order to minimize the possibility of any bias due to differences in follow-up times, clinical outcome was divided into only two categories: progressive (PD) and non-progressive (NP) [including complete response (CR), partial response (PR), or stable disease (SD; >6 months), as defined in a previous study [19]] (Table 3). An inverse relationship was observed between TEM8 mRNA levels and both clinical and immunological (i.e. DTH) responses. Indeed as shown in Fig. 5a, eight NP patients (comprising two CR, one PR, and five SD) showed low levels of TEM8 mRNA expression [mfi = 1.97 (0.3–3.3)], similar to those of healthy donors [mfi = 2.7]. Conversely, mDCs from nine PD patients showed high TEM8 mRNA expression [mfi = 12.88 (5–30)]. Notably, mDCs from both PD and NP patients displayed similar (moderate) levels of CMG2 expression [mfi = 2.88 (0.12–8.43) vs. 3.2 (0.4–8), respectively] to those of healthy donors (mfi = 1.95). In Fig. 5b is shown the inverse correlation between TEM8 expression with the DTH response in NP patients [all DTH-positives (five for both tumor lysate and KLH, three for KLH only; median OS = 32 months)], and in PD patients [all but one DTH negative DTH (median OS = 5 months)].

Based on these findings, we conclude that high TEM8 expression levels in DC vaccines are significantly ($p = 0.0018$) correlated with vaccination failure (i.e. PD). It is noteworthy that the mfi values for TEM8 mRNA in PD patients were in line with those (>10-fold) measured by serial gene expression analysis of purified endothelial cells from tumor-associated versus normal blood vessels [10].

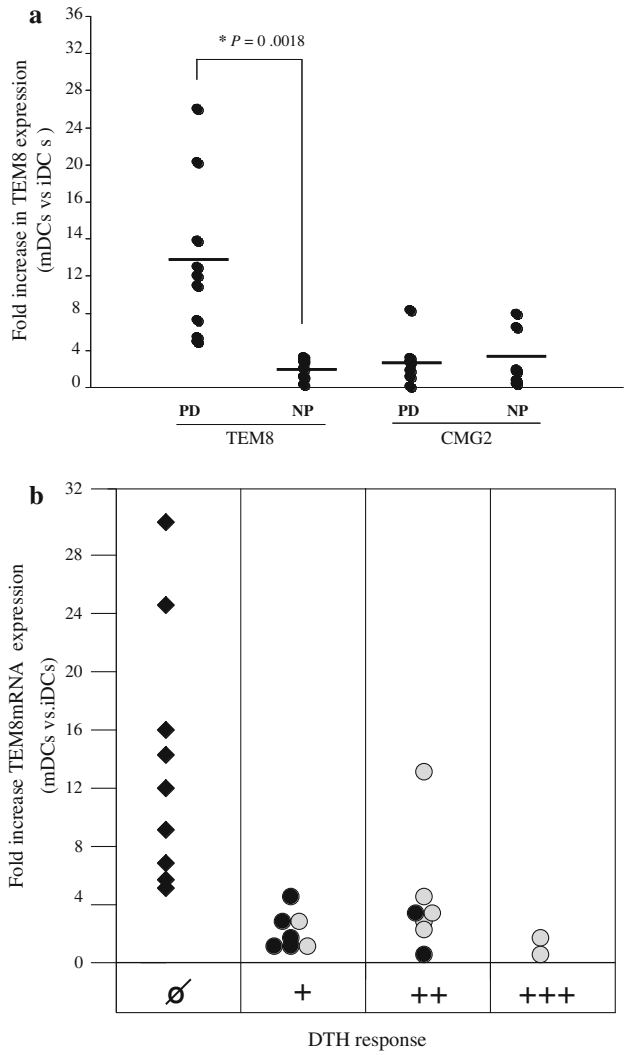

Fig. 5 **a** Scatter plot of the fold increase in TEM8 and CMG2 mRNA (mDCs vs. iDCs) in cancer Pts with different clinical outcomes. *PD* progressive disease, *NP* non-progressive disease. *Horizontal bars* mfi. **b** Graph showing the correlation between TEM8 expression and DTH response after four vaccinations cycles. *Diamond* PD DTH-negative patients (Ø); *circles* NP patients showing low (+), medium (++), and high (+++) DTH response. *Black circles* LYS; *white circles* KLH

Discussion

Dendritic cell-based vaccination for advanced cancer has so far demonstrated high variability and low clinical impact. Therefore, a central challenge to improve DC vaccination, as well as other immunotherapeutic approaches, is to find predictive biological markers enabling the selection of subgroups of patients who are most likely to benefit from treatment.

Tumor endothelial markers 8 mRNA has been originally found to be selectively up regulated in tumor-associated endothelial cells [10]. However, analyses of TEM8 expression profiles and bioinformatics suggest that this presumptive tumor-specific endothelial marker gene may be highly expressed in different cell types (like DCs and tumor cells) involved in extra cellular matrix (ECM) remodeling and migration processes, such those observed in inflammatory reactions and tumor progression [26]. Consistent with this hypothesis several studies indicate TEM8 as a new adhesion molecule coupling ECM ligands (e.g. collagens) to actin cytoskeleton [27–29].

Although the pathophysiological role of TEM8 remains essentially unknown, this anthrax receptor has been proposed as a suitable marker of tumor progression as well as a potential therapeutic target for a variety of tumors [30, 31]. In this context, our data indicate that TEM8 expression levels in mDCs may represent a marker of treatment response in tumor-vaccinated patients. However, due to the explorative nature of this study, caution should be taken when interpreting the result.

Whether TEM8-expressing DCs are responsible for the vaccination failure remain to be seen. Future studies will have to evaluate if mDCs maintain TEM8 expression at the vaccination sites, and whether the modulation of TEM8 expression by different maturation cocktails (see above) may help to improve the clinical outcome.

Another possibility is that high TEM8 expression in mDCs may have nothing to do with vaccine success or failure. It may simply be part of a gene expression program(s) found in more advanced disease. In this regard, it would be of interest to investigate about the correlation between TEM8 expression and levels of well-established serological melanoma markers such as LDH and S100β [32]. Intriguingly, TEM8 has been reported to interact with tumor type M2 pyruvate kinase (TuM2-PK) [30], a novel plasma marker for tumor load, whose combination with serum S100β improves the prediction of progression-free and overall survival in metastasized melanoma patients [33]. It has been speculated that the released TuM2-PK might stimulate angiogenesis by binding to TEM8, and thus improve the hypoxia status [30].

Lastly, it must be underlined that DC vaccines are customized in vitro artifacts [34] developed from highly variable mixtures of inflammatory monocytes and immature proangiogenic myeloid precursors [35, 36]. It is therefore conceivable that upregulation of TEM8 in mDCs is related to an outflow of tumor "educated" myeloid-DC precursors. The fact that TEM8 overexpression is not detectable in the monocyte or iDC stages may indicate that "education" entails increased sensitivity to one or more components of the standard maturation cocktail.

On a final note, among the different possible mechanisms linking TEM8 to immune suppression, the involvement of regulatory cell pathways, such those mediated by myeloid-derived suppressor cells (MDSC) can be

hypothesized. Indeed, immunosuppression in cancer is associated with expansion/mobilization of bone marrow MDSC that reduce activated T-cell number and inhibit their function by multiple mechanisms, including depletion of L-arginine by arginase-1 (ARG1) production of nitric oxide, reactive oxygen species, and reactive nitrogen oxide species by inducible nitric oxide synthase (NOS) [37, 38]. Whether MDCS express TEM8, or whether TEM8 expression in DCs correlate with the enrichment of MDCS subset in the peripheral blood of metastatic patients, remain to be seen. Vice versa the possibility that TEM8-positive DCs express suppressive molecule utilized by MDSC (i.e. ARG1, NOS or TGF-β) could also be investigated.

Findings from the present study could contribute towards a significant increase in the response rates of DC immunotherapy by limiting this high-cost and labor-intensive treatment to cancer patients whose in vitro mDCs display low (healthy donor-like) levels of TEM8 expression. However, because of the relatively small number of patients involved, and the retrospective nature of this study, our results need to be confirmed in a larger prospective case series. Hopefully, future studies will contribute relevant information about the involvement of TEM8 in vaccination failure, immune suppression and/or worse cancer prognosis.

Acknowledgments This work was supported by Biosooft Srl, Castelraimondo (MC), Italy, Fondazione CARIMA, Macerata, Italy, and the Italian Ministry of Health (grant nos. R.F. 2000.352, R.F. 2002.234, R.F.00.184, and R.F. 2003.116). The authors wish to thank Prof. E. Gilboa (University of Miami, USA), Prof. L. Matera (University of Turin, Italy) and Dr. J. Wolchok (Memorial-Sloan Kettering Cancer Center, New York, USA) for their critical reading of the manuscript. They also thank Dr. Ian Seymour for editing the manuscript.

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