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Dendritic-Cell (DC)-Based Immunotherapy: Tumor Endothelial Marker 8 (TEM8) Gene Expression of DC Vaccines Correlates with Clinical Outcome

PhD thesis in Biology

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ABSTRACT

Previous studies have shown that tumor-endothelial markers (TEMs) are up-regulated in immunosuppressive, pro-angiogenic dendritic cells (DCs) found in tumor microenvironments. We reported that pro-angiogenic monocyte-derived DCs (Mo-DCs), utilized for therapeutic vaccination of cancer patients upon maturation, markedly differ in their ability to up-regulate tumor-endothelial marker 8 (TEM8) gene expression. A DC vaccination trial of 17 advanced cancer patients (13 melanoma and 4 renal cell carcinoma), carried out at the Cancer Institute of Romagna (I.R.S.T.) in Meldola, highlighted a significant correlation between delayed-type hypersensitivity test (DTH) and overall survival (OS). In the study, relative TEM8 mRNA and protein expression levels (mature (m) vs. immature (i) DCs), in DCs obtained for therapeutic vaccines were evaluated by quantitative real-time RT-PCR and cytofluorimetric analysis, respectively. mDCs from six healthy donors were included for comparison purposes. Eight non-progressing patients, all DTH-positive, had a mean fold increase (mfi) of 1.97 in TEM8 expression. Similarly, a TEM8 mRNA mfi = 2.7 was found in healthy donor mDCs. Conversely, mDCs from nine progressing patients, all but one with negative DTH, had a TEM8 mRNA mfi of 12.88. Thus, mDC TEM8 expression levels would seem to identify ($p = 0.0018$) patients who could benefit from DC therapeutic vaccination.

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1. INTRODUCTION

Cancer is a complex disease initiated by a series of cumulative genetic and epigenetic changes that occur in normal cells. However in addition to the malignant cell itself, cancer is a disease of microenvironment and immunity. Although genetic and epigenetic alterations drive cellular transformation, genomic plasticity, and evolution, it has become increasingly apparent that multiple signals delivered within the tumor microenvironment by modifier genes, stromal, endothelial cells, and immune cells are critical factors in determining the progression vs. dormancy or destruction of an initiated lesion and also whether metastasis may occur.

Immune disregulation / immunosuppression in cancer patients is a composite event in which tumor-derived factor conditions not only peripheral immune niches but also the bone marrow and other hematopoietic organs (mouse spleen), leading to abnormal myelopoiesis and accumulation of immunosuppressive myelomonocytic cells at the tumor site. Disregulation / immunosuppression is therefore likely to occur at two separate sites: locally at tumor-host interface, where tumor directly conditions the tumor stroma, and systematically, where an expanded pool of immature and immunosuppressive myeloid cells are free to circulate and mediate suppression in lymphoid organs and in the blood.

1.1 CANCER IMMUNOTHERAPY

Cancer immunotherapy attempts to harness the exquisite power and specificity of the immune system for the treatment of malignancy. Although cancer cells are less immunogenic than pathogens, the immune system is clearly capable of recognizing and eliminating tumor cells. However, tumors frequently interfere with the development and function of immune responses. Thus, the challenge of the immunotherapy is to be in advance in the cellular and molecular immunology in order

to develop strategies that effectively and safely augment antitumor responses (Blattman J.N. el al. 2004).

The term immunotherapy refers to any approach that seeks to mobilize or manipulate the immune system of a patient for therapeutic benefit (Steinman R.M. et al. 2004). In this regard, there are numerous strategies to improve the patient's resistance to cancer. These include non-specific activation of the immune system with microbial components or cytokines, antigen-specific adoptive immunotherapy with antibodies and/or T cells, and antigen-specific active immunotherapy (that is, vaccination).

Therefore, such in vivo or direct vaccination approaches, although simple, cost effective, and broadly applicable, have not been effective in the setting of cancer (Livingston P.O. 1989). What is likely to contribute to such failures is that the vaccines against infectious agents are administered prophylactically to healthy individuals as a protective measure against future exposures, whereas the cancer vaccines are administered therapeutically in the cancer patient in the face of pre-existing antigenic load (the tumor). Other factors contributing to limit the efficacy of early cancer vaccination protocols include the need to stimulate the cellular arm of the immune response and the fact that immune responses are suppressed in cancer patients. Such failures underscore the need to develop increasingly more potent cancer vaccination strategies (Gilboa E. 2004).

1.2 ANTI-CANCER STRATEGIES

The immune system can respond to cancer cells in two ways: by reacting against tumor-specific antigens (molecules that are unique to cancer cells) or against tumor-associated antigens (molecules that are expressed differently by cancer cells and normal cells).

The identification of defined tumor antigens in humans (Boon T. et al. 1994; Rosenberg S.A. 1997) prompted the development of adoptive T-cell therapy. Yet, the most attractive strategy is vaccination, which is expected to induce both therapeutic

T-cell immunity (in the form of tumor-specific effectors T cells) and protective T-cell immunity (in the form of tumor-specific memory T cells that can control tumor relapse) (Pardol D.M. 1998; Gilboa E. 1999; Finn O.J. 2003).

Efficient antigen presentation and T-cell priming are essential components of effective antitumor immunity and the dendritic cells are critical to both of these functions.

One approach that is gaining increasing popularity among tumor immunologists, is to immunize cancer patients with autologous, patient derived DCs loaded with tumor antigens ex vivo. The underlying premise of this approach is that the efficiency and control provided by ex vivo manipulation of the DCs generates an optimally activated APC and a superior method for stimulating immunity in vivo if compared with more traditional vaccination methods.

This approach to the therapeutic vaccination of individuals who have cancer relies on random encounter of the vaccine with host DCs. A lack of encounter of the vaccine antigen with DCs might result in the absence of an immune response. Alternatively, an inappropriate encounter - for example, with inactivated DCs or with the 'wrong' subset of DCs - might lead to silencing of the immune response (Steinman R.M. et al. 2003).

We do not know how tumor antigens need to be delivered to DCs in vivo to elicit an appropriate immune response (Merad M., et al., 2002; Banchereau J. et al., 2005).

1.3 IMMUNOSUBVERSION: THE ACTIVE SUPPRESSION OF THE IMMUNE RESPONSE

In humans, tumors develop a series of strategies to evade immunosurveillance, and these strategies are presumably unrelated to the other characteristics of carcinogenesis and result from the selective pressure exerted by the immune system; these strategies are known as immunoselection.

Several tumor products that are dispensable for cell-intrinsic cancer-cell characteristics might be involved in immunosubversion: that is, the active suppression of the immune response. For example, tryptophan degradation by indoleamine 2,3-dioxigenase (IDO), which is constitutively expressed by human tumors (particularly by prostate, colon and pancreatic carcinomas, but also by interdigitating DCs), promotes resistance to immune mediated rejection of the tumor cells (Uyttenhove C. et al. 2003). Locally produced IDO can block the proliferation of $CD8^+$ T cells at the tumor site (Uyttenhove C. et al. 2003), as well as it can promote the apoptosis of $CD4^+$ T cells (Terness P. et al. 2002).

The exact molecular mechanisms by which tumors mediate immunosubversion are the subject of intense investigation. One possible explanation of how tumors subvert the immune response is to consider that the tumors are 'false' lymphoid organs; therefore, T-cell priming in the tumor microenvironment is defective as a result of the presence of dysfunctional or tolerogenic antigen-presenting cells (Zou W. 2005). Indeed, tumor beds contain various factors (such as VEGF, IL-6, IL-10, TGFβ, macrophage colony-stimulating factor (M-CSF), NOS2, arginase-1, IDO, PGE_2, COX2 and gangliosides) that can inhibit the differentiation, maturation and function of DCs (Zou W. 2005). Accordingly, local DCs tend to mediate immunosuppressive, rather than immunostimulatory, effects and to promote the T_{Reg}-cell differentiation (Ghiringhelli F. et al. 2005).
Another possible explanation for tumor-mediated immunosubversion is based on a quantitative argument. Tumor characteristics that are immunostimulatory in small tumors can become immunosuppressive in large tumors.

It seems that there are numerous ways by which tumor cells can evade or 'paralyze' immunosurveillance. However, it remains an open question which of these multiple mechanisms affects oncogenesis and cancer progression in humans.
Of note, in some cases, it is possible that, although an immune response to tumors is mounted, this response fails to eliminate the tumors or could even stimulate carcinogenesis and tumor progression, as a result of chronic inflammation.

1.3.1 IDO: enzyme indoleamine 2,3-dioxygenase that is upregulated in human DCs upon in vitro maturation

Immunological strategies to fight cancer have demonstrated less clinical efficacy than anticipated. Several factors contribute to this status quo, including immune escape mechanisms of tumors or the limited immunogenicity of the antigen delivery systems (Steinbrink K. et al. 1999).

One potential mechanism, leading to immunological tolerance, is the recently recognized immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) (Uyttenhove C. et al., 2003). IDO is a heme-containing enzyme which catalyses the initial, rate limiting-step in the degradation of the essential amino acid tryptophan into distinct kynurenine metabolites (Grohmann U., et al., 2003). The depletion of tryptophan renders T-cells more susceptible to apoptosis (Lee G.K., et al., 2002). Moreover, various tryptophan downstream metabolites, e.g. kynurenine and quinolinate, are by themselves directly toxic for T-cells (Terness P., et al., 2002). IDO-expressing, tolerizing antigen presenting cells (APCs) are furthermore supposed to induce regulatory T-cells (Tregs) (Faunce D.E., et al., 2004).

Clinically relevant immunoregulatory functions of IDO include the protection from intracellular pathogens (Gupta S.L., et al., 1994), the maintenance of maternal tolerance towards the fetus during pregnancy (Munn D.H., et al., 1998), the suppression of T-cell responses to MHC-mismatched allografts (Bauer DM., et al., 2005), the protection from autoimmune diseases (Sakurai K., et al., 2002), and – most important – the tumor resistance to cytotoxic $CD8^+$ T-lymphocytes. (Munn D.H., et al., 2004; Harlin H., et al., 2006). In this respect, IDO is expressed by two complementary constituents both in the tumor microenvironment as well as in the regional draining lymph nodes: the malignant cells themselves and the subset of APCs (Lee J.H., et al., 2005). Thereby, both local and systemic tolerance to neoplastic cells may be generated and maintained.

IDO-expression in APCs and its complex modulation by various cytokines and direct cellular interactions may become an issue for DC-based vaccine therapies. Indeed, previous reports have demonstrated that functional IDO is induced upon in vitro generation of mature DCs. (Braun D., et al., 2005; Bergwelt-Baildon MS., et al., 2006).

We analyzed IDO expression, its activity as well as its in vitro relevance in the patients receiving DC-based vaccinations.

1.4 DENDRITIC CELLS (DCs)

Dendritic cells (DCs) are professional antigen presenting cells (APCs) in the immune system, which are able to induce primary T cell responses. Because of their central role in the initiation of immune responses, DCs are an important tool for tumor antigen specific immunotherapy of cancer.

Immature DCs are present in peripheral tissues, where they possess the capacity to take up and process antigen into small peptides; in absence of inflammation, DCs remain in an immature state, and antigens are presented to T cells in the lymph node without co-stimulation, leading to either the deletion of T cells or the generation of inducible regulatory T cells.

The tissue inflammation induces the maturation of DCs and their migration to draining lymph nodes for presentation to resting lymphocytes. The mature DCs express high levels of cell-surface major histocompatibility complex (MHC) antigen complexes and co-stimulatory molecules. This allows the priming of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), the activation of B cells and the initiation of an adaptive immune response. It is now known that activated $CD4^+$ T-helper cells up regulate CD40 ligand, and that signaling through the CD40 receptor fully activates DCs.

Therefore DCs-based vaccines are now being explored in the clinic. So far, most DCs vaccines have been used to stimulate immune responses, in particular the ones which can combat the cancer. Recent findings indicate that whereas mature DCs induce immunity, immature or semi-mature DCs can cause immunological tolerance, opening up application in transplantation and autoimmunity (Figdor C.G., et al., 2004).

In every case, effective migration of DCs to secondary lymphoid organs is essential for the DCs to exert their immune regulatory effect.

1.4.1 DCs-based vaccines

Multiple factors contribute to the failure of DCs in priming effective antitumor responses in tumor-bearing hosts: the low number of DCs available in the tumor site, poor access of DCs to tumor antigen, the limited capacity of tumor cells to activate intra-tumoral DCs, and secretion by the tumor cells of factors that inhibit DCs maturation.

Administration of DCs generated and loaded with tumor antigens ex vivo, can be used to circumvent tumor immunotolerance and thus as therapeutic cancer vaccines (Fong L. et al., 2000).

A variety of preparations of DCs can stimulate antitumor immunity, including DCs loaded with proteins, DCs fused with tumor cells, and DCs transduced with tumor-derived RNA or viral vectors. At present, all these approaches rely on ex vivo manipulation of isolated DCs to produce the vaccine.

DCs vaccination has been facilitated by the development of methods to generate DCs either from rare, but proliferating, $CD34^+$ precursors or from common, but mostly non-proliferating, $CD14^+$ monocytes (so-called monocyte-derived DCs) (Banchereau J., et al., 2001).

Although early clinical trials have indicated that DCs vaccines can induce anti-tumor immune responses in some cancer patients, there is still much to be learned regarding the subtype, maturation and activation status of DCs, antigen loading,

route of administration, the dose and timing interval, and migration of DCs-based vaccines.

1.4.2 DCs migration

The migration to secondary lymphoid organs is essential for the DCs to exert their immune regulatory effect.

DCs migration is a complex process that involves a coordinate activation of different classes of effectors molecules: chemokines, adhesion molecules, as well as matrix metalloproteinase's (MMPs) and lipid mediators.

DCs maturation results in the down regulation of chemokine receptors associated with tissue retention, whereas CCR7 and CD62L are up regulated (Dieu M.C. et al., 1998).

The expression of CCR7 on mature DCs and on naïve and central memory T cells is essential for their coordinate migration to the T-cell area of draining lymph nodes because this migration is guided by CCL19 and CCL21, the two ligands for CCR7. Both chemokines, are expressed by stromal cells in the T-cell area of secondary lymphoid organs (Scandella E. et al., 2004).

Recently, it was observed that the maturation-induced up-regulation of CCR7 expression on human monocyte-derived DCs was insufficient to allow MoDCs migration to CCL19 and CCL21 (Scandella E. et al., 2002; Luft T. et al., 2002). Human MoDCs matured either with soluble CD40L or with Poly I:C markedly enhanced surface expression of CCR7 but they were not at all or they were only poorly responsive to CCL19 and CCL21. Interestingly, MoDCs migration to CCL19 and CCL21 was readily observed on maturation in the presence of the inflammatory mediator prostaglandin E2 (PGE_2), though PGE_2 did not change the expression level of CCR7 on mature MoDCs, providing evidence for an alternative effect of PGE_2 (Scandella E. et al., 2004).

1.4.3 DCs maturation

Ex vivo expanded DCs are currently applied as autologous cellular vaccines for advanced cancer patients (Fong L. et al. 2000). Most commonly, patients monocytes-derived immature DCs (iDCs) are generated in the presence of granulocytes-macrophage colony stimulating factor (GM-CSF) and IL-4, loaded with tumor antigens, and exposed to inflammatory signals (i. e., LPS, CD40L, or double-stranded RNA and/or prostaglandins) to induce final maturation. This simple procedure yields a homogeneous population of DCs that resemble interstitial DCs.

It is not clear which maturation stimulus is best for the induction of tumor-specific T cells in vivo; however, the most commonly used maturation protocol for Mo-DCs consists of four reagents, TNFα, IL-1β, IL-6 (three inflammatory cytokines) and PGE_2, also known as monocyte-conditioned media mimic, or cytokine cocktail (CYC) (Jonuleit H. et al. 1997).

Pro- and anti-angiogenic molecules can emanate from cancer cells, endothelial cells, stromal cells, blood and the extracellular matrix. Their relative contribution is likely to change with type, site and tumor growth.
The balance between pro- and anti-inflammatory signals that are present in microenvironments determines the phenotype and the behavior of the immune cells at the site of inflammation. While DCs activated by pro-inflammatory signals (e.g. LPS and TNF-α) are characterized by pro inflammatory functions, the exposure of DCs to anti-inflammatory molecules such as IL-10, IL-4 and PGE_2 induces alternative programs of activation characterized by peculiar membrane phenotype and function.

At the same manner, it is the balance between pro- and anti-inflammatory molecules of the maturation cocktail to determine the angiogenic and migratory programs in activated MoDCs and the release of important cytokines subset that regulate the nature and the efficacy of immune responses.

1.4.4 PGE_2

Prostaglandin E_2 (PGE_2) is a common inflammatory mediator known to exert Th2-promoting and IL-12-antagonistic activity via several distinct mechanisms, affecting both APCs and Th cells.

The rational to include PGE_2 in the maturation cocktail is to endow the ex vivo-generated DCs with the capacity to migrate to draining lymph-nods and to enhance T cell priming (Scandella E. et al. 2002; Luft T. et al. 2002).

Therefore, in the context of the tumor microenvironment, PGE_2 can mediate Th2 polarization and promote the differentiation of DCs secreting the immunosoppressive cytokine IL-10 (Morelli A.E. et al. 2003). Several studies have shown that PGE_2 may induce mDCs with counterproductive immunosuppressive / proangiogenic features (Gilboa E. 2007; Sinha P. et al. 2007).

The key negative impact of PGE_2 on the function of ex vivo-generated DCs is probably that PGE_2 abolishes both the responsiveness of mature DCs to stimulation thought CD40 and their ability to synthesize IL-12 when they reach the lymph node and encounter cognate T cells (Luft T. et al. 2002). Additionally, cytokine cocktail-matured DCs were very effective, even more than immature DCs, at expanding a population of immunosoppressive Tregs expressing the forkhead box transcription factor FOXP3 (Banerjee D.K. et al. 2006).

Notwithstanding the limitation, the results from published trials (Morelli A.E. et al. 2003) showed that when treated with PGE_2-matured DC vaccines, subsets of patients developed clinical responses that occasionally correlated with antigen-specific immunoresponses.

Hence, on the basis of in vitro results, it is possible to construct arguments both for and against the inclusion of PGE_2 in the maturation cocktail, but it appears extremely difficult to predict whether the presence of PGE_2 during the DC maturation will increase or decrease the efficacy of the DC-based anticancer therapy in vivo.

1.5 PROANGIOGENIC PHENOTYPE OF DCs

The ability of tumors to compromise the phenotype and function of DC isolated from peripheral blood of patients has been described. In contrast, we known much less about tumor-associated changes in “in vitro” generated DCs. How DC vaccines are altered compared with similar DC from healthy donors still remains an open (often overlooked) question.

Indeed, one of the main problems in current experimental trials is the advanced stage (IV) of cancer patients. At this stage, progressing tumors often subvert physiological myelopoiesis, leading to the expansion of heterogeneous populations of dysfunctional monocytes which, may not only help tumors immune-escape, but also aid in the construction of new blood vessels for tumor growth (MacLean K. et al 2008; Curiel T.J. et al. 2004; Priebe A. et al. 2008). However, it remains unclear whether the cells are acting directly or represent precursor cells. Of interest, dendritic cell precursors in the tumor microenvironment are reported to assume a mixed DC - endothelial cell phenotype to promote angiogenesis (Albini A. et al., 2005). The possibility that these cells, named vascular DC (VDC), could incorporate into tumor-blood vessels has been also reported. Consistently with this observation, an increasing number of tumor endothelial markers (TEMs 1 - 9), originally uncovered as genes specifically expressed or significantly up-regulated in tumor versus normal blood vessels, (St. Croix B. et al., 2000) has been found to be expressed in vascular and perivascular leukocytes populations in tumor microenvironment (Gottgried E. et al. 2007).

1.6 TEM8: MARKER IN PROANGIOGENIC PROGRAMS

We recently reported that (Venanzi F.M. et al. 2006) PGE_2-matured DCs (PGE_2-mDCs) from cancer patients, utilized for autologous therapeutic vaccination, while acquiring pro-angiogenic (VEGF-releasing) potential, markedly differ in their ability to up-regulate tumor-endothelial marker 8 (TEM8) gene expression (Gabrielli F. 2007).

Tumor endothelial marker (TEM8) was uncovered as a gene expressed predominantly in the tumor endothelium. Elevated levels of TEM8 in tumor microvessels appear to correlate with disease progression in breast and colorectal cancer (Davies G. et al. 2004; Rmali K.A. et al. 2005). Of interest, the gene product of TEM8 was recently identified as the receptor of the anthrax toxin (ATRX1/TEM8). (Carson-Walter E.B., et al., 2001; Bradley K.A., et al., 2003; Whittaker C.A., et al., 2002).

The expression pattern of TEM8 was especially interesting in that it is the only human TEM characterized that shows undetectable mRNA expression in healing wounds and corpus luteum tissue, suggesting that this gene may be highly specific to tumor angiogenesis and not required for normal adult angiogenesis (St Croix et al. 2000).

However, TEM8 RNA is also expressed in a small proportion of the endothelial cells in normal brain, heart, intestines lung, skeletal muscle and pancreas, and at high levels in the endothelial cells of murine fetal liver and brain (St Croix et al. 2000, Carson-Walter E.B., et al. 2001).

TEM8 encodes a type I transmembrane protein, 564 amino acids in length. The intracellular domain is 220 amino acids in length and the extra cellular region (aa 1-318) contains a vWFA domain (aa 44-205) also known as an I-domain when it is present in integrins (Dickeson S.K., et al., 1998). Three different, apparently alternatively spliced, versions of the TEM8 gene have been described. The TEM8 variants share the same amino-terminal extra cellular part but differ in length and sequence in their putative cytosolic regions. Splice variant 1 (SV1) is the longest and is the original TEM8 cDNA that encodes a 564 amino acid protein with a long proline-rich cytoplasmic tail. Splice variant 2 (SV2) encodes a 368 amino acid protein with a short cytoplasmic tail. Splice variant 3 (SV3) encodes a protein that is identical to the other two throughout most of the extra cellular domain but diverges just before the

transmembrane region so that it does not contain a recognizable membrane anchoring sequence.

Interest in TEM8 variant 2 increased when it was identified as ATR, a cellular receptor for anthrax protective antigen (Bradley K.A. et al., 2001). ATR that is identical to TEM8 for the first 364 amino acids, includes the entire extracellular and transmembrane domains, and then it terminates after a 4 amino acid divergence from TEM8.

Anthrax toxin, the major virulence factor produced by *Bacillus anthracis*, consists of three polypeptides called protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa) (Leppla S.H. et al., 1999; Smith H. et al., 1962). The protective antigen mediates the binding of the complex to TEM8 variant 2 (Bradley K.A., et al., 2001) whereas lethal factor and edema factor are responsible for eliciting toxicity.

The identification of TEM8 as the anthrax receptor helped to clarify a potent antitumor response that had been previously obtained with anthrax toxin (Duesbery N.S. et al., 2001). Injection of the toxin into tumor-bearing mice led to a strong anti-tumor response, in some cases causing complete tumor regression. Although the mechanism responsible was unclear at the time, an unexpected anti-angiogenic effect was postulated to be involved owing to the small number of vessels observed in the treated tumors.

TEM8 interacts with the C5 domain of collagen $\alpha 3$ (VI), one of a limited number of transcripts preferentially expressed in tumor endothelium among the analyzed 32,500 total transcripts (St. Croix B., et al., 2000). The interaction of these proteins and their coordinate expression in tumor endothelial cells suggests a functional role in angiogenesis.

The large cytoplasmic tail of both the human and mouse TEM8 proteins share at least seven potential phosphorylation sites, supporting the hypothesis that TEM8 is involved in transmitting signals into the cells.

Recently, capillary morphogenesis protein 2 (CMG2), the closest homologue to TEM8, was identified as a second receptor for anthrax toxin (ATRX2) (Scobie H.M., et al., 2003). CMG2 sharing 51% amino acid identity with ATR/TEM8 in the I domain was identified originally in a screen for genes differentially regulated during capillary induction in vitro. The CMG2 transcript was found to be up regulated early during the process of capillary morphogenesis (Bell S.E., et al., 2001), and the CMG2 protein was shown to bind to at least two extra cellular matrix (ECM) components, collagen IV and laminin. The sub cellular localization of CMG2 was determined to be predominantly in the endoplasmic reticulum. This finding suggests that CMG2 may be involved in the assembly of the basement membrane matrix that is produced during new blood vessel formation.

The mRNA expression profile of ATR/TEM8 as well as mRNA expression and binding data of the highly similar protein, CMG2, suggest that one of the physiological roles of ATR/TEM8s may be in angiogenesis. However, the presence of ATR/TEM8 on the cell surface of most cell types tested in culture, on the tumor stroma as well as its presence on macrophages, indicates that there may be additional roles for this protein, perhaps in cellular adhesion.

Many of the molecular mechanisms that mediate the relationship between inflammation, innate immunity and cancer progression remain to be defined.
Tumor cells may usurp signaling molecules (i.e. integrins, chemokines and their receptors) by which innate immune system interfaces with cancers, for invasion, migration and metastasis (Muller A., et al., 2001).

Although TEM8 transcripts have been found to be selectively up regulated in tumor angiogenesis, the analyses of TEM8 expression profiles and bioinformatics suggest that this presumptive tumor-specific endothelial marker gene may be highly specific

in different cell types (like DCs and tumor cells) involved in extra cellular matrix-remodeling and migration processes, such those observed in inflammatory reactions and tumor progression (Novatchkova M. and Eisenhaber F., 2001).

2. AIM OF THE STUDY

Advanced tumors subvert expansion and differentiation of myelomonocytes cells leading to dysfunctional DCs. Previous studies have shown that tumor endothelial markers (TEMs) are up regulated in proangiogenic DCs found in tumor microenvironment.

Goal of this study is to provide evidence that monocytes-derived DCs from patients with advanced cancer, up-regulate TEM8 gene / protein expression. Specifically:

Aim 1. Compare the expression patterns of TEM8 and CMG2 (as judged by Quantitative real time RT-PCR and cytofluorimetric assays) of DCs obtained from both cancer patients and healthy individuals.

Aim 2. Evaluate whether and how TEM8 expression levels in DCs utilized for therapeutic vaccination of melanoma and renal cell cancer patients, are related to clinical outcome.

3. MATERIAL AND METHODS

3.1 Patients

The case series consisted of the 17 patients that had undergone DCs phase I/II vaccination trial (2001-2005) for advanced melanoma (n=13) and renal cell carcinoma (RCC; n = 4) (Ridolfi R. et al. 2006). **(See Tab. I).**

In this trial, 8 patients were scored as responders, and 9 as non-responders according to RACIST criteria, and it was observed a positive correlation between delayed-type hypersensitivity test (DTH) for tumor lysate (TL) and/or keyhole limpet hemocyanin (KLH) and overall survival **(see Tab. II)**. The clinical trial, was approved by the Italian Ministry of Health and by the Ethical Committee of Forlì Health and Social Services (Azienda ASL-Forlì, Italy). All patients gave the written informed consent.

3.2 Human cancer cell line

Different cancer cell lines were obtained from American Type Culture Collection (ATCC): HeLa (ATCC Catalog. Number: CCL-248); MDA-MB-231 (ATCC Catalog. Number: HTB-26); SkBr3 (ATCC Catalog. Number: HTB-30); ZR75-1 (ATCC Catalog. Number: CRL-1500). The cell lines were maintained at 37°C in a humidified 5% CO_2 environment in Dulbecco's modified Eagles medium (D-MEM) (Cambrex) with 1% L-glutamine, 1% penicillin/streptomycin, supplemented (growing condition) or not (starving condition) with 10% foetal bovine serum (FBS) (Cambrex).

The cells were trypsinized and used for the proteic lysate or the RNA extraction.

3.3 DCs generation

Mature DCs from each patient were regenerated from cryopreserved peripheral blood monocytes (PBMCs), previously obtained by leukapheresis (5 – 9 litres of blood were processed in each collection), without previous mobilization.

mDCs obtained from six unrelated healthy donors were included for comparison.
PBMC were purified on Ficoll-Plaque, and incubated in tissue culture flasks with CellGro DC Medium (Cell Genix, Freiburg, Germany) at 10 x 10^6 cells/ml for 2 h.
The non-adherent cells were discarded and the adherent cells were incubated in CellGro DC Medium containing 1000 IU/ml rhIL-4 (Cell Genix) and 1000 IU/ml rhGM-CSF (Shering Plough, Milan, Italy) for 7 days to generate a DCs-enriched cell population. On day 7, they were defined as iDCs. After eliminating the previous culture medium, iDCs were cultured for a further 2 days with a cocktail of cytokines (TNF-α, IL-1β, IL-6, Endogen, Pierce Biotechnology, Rockford, USA, PGE_2, Cayman Chemical, Ann Arbor, MI, USA). Alternatively, iDCs were cultured with the PGE_2-depleted cocktail or with TNF-α, IL-1β, IL-6 and Poly I:C (Amersham Biosciences).
On day 9 they were defined as mDCs.

3.4 Flow cytometry

iDCs and mDCs phenotypes were determined by single or two-color fluorescence analysis. 3–5·10^5 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 appropriate fluorescein isothiocyanate or phycoerythrin-labeled monoclonal antibodies (mAbs). The cells were then washed twice and resuspended in 500 μl of PBS. The fluorescence was analyzed by a FACS Vantage 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.
TEM8 protein expression was determined by flow cytometry analysis. Briefly, 5·10^5 cells were incubated for 2 hours at room temperature in the dark with 10 μl of the primary antibody, 0.5 μg Rabbit polyclonal to TEM8 (abCam, ab21270). The cells were then washed 3-times and resuspended in ice cold PBS. The fluorochrome-labeled secondary antibody (Goat polyclonal to Rabbit IgG-FITC (ab6717) was diluted at 1/50 in 3% BSA/PBS and added to cells that were incubated for 30 minutes at room temperature in the dark. After three washing the cells were analyzed by a FACS CANTO flow cytometer (Becton Dickinson, Milan, Italy).

3.5 ELISA assay

At each pre-set time (24h – 48h), the supernatants of iDCs and mDCs were collected and stored at -80°C until analysis was carried out using commercially available Endogen Human VEGF Elisa Kit (Pierce Biotechnology) to measure the production of $VEGF_{165}$ and $VEGF_{121}$ isoforms, according to the manufacture's protocols.

The quantitative measurement of Trombospondin-1 in culture supernatants was carried out using ChemiKine Human TSP-1 EIA kit (Product # CYT 168, Chemicon International.

3.6 RNA isolation and complementary DNA synthesis

Total RNA was isolated from monocytes, immature and mature (with standard cocktail, PGE_2-depleted cocktail or cocktail + Poly I:C) human MoDCs, obtained from patients, healthy donors, and breast cancer cells lines.

The cells ($5x10^5$) were lysed by incubation with a lyses buffer that immediately inactivates RNase and creates appropriate binding conditions which favour adsorption of RNA to the silica membrane. Contaminating DNA is removed by a DNase I solution which is directly applied onto the silica membrane during the preparation. Simple washing steps with two different buffers remove salts, metabolites and macromolecular cellular components. Pure RNA is eluted under low ionic strength conditions with RNase-free water.

The concentration of RNA was determined spectrophotometrically by measuring absorbance at 260 nm and RNA integrity was confirmed by electrophoresis on a 1.2% agarose gel.

We used 1 µg of total RNA for synthesis of first-strand complementary DNA (cDNA) by RevertAid H Minus First Strand cDNA Syntesis Kit (Fermentas, Life Sciences).

The RNA (1 µg) was incubated with H_2O and 1µl of Oligo dT Primer (0.5µg/µl) for 5 minutes at 70°C. At the reaction, 2 µl of 10x Reaction Buffer, the RNAsi inhibitor and

2µl of 10nM dNTPs mix were added. The reaction mixture was heated to 37°C for 5 minutes.

At the reaction was then added 1µl of the RevertAid H Minus M-MuLV Reverse Transcriptase (200u/µl) (final volume 20 µl) and incubated for 60 minutes at 42°C. The reaction was heated to 70°C for 10 minutes to inactivate Reverse Transcriptase.

The resulting cDNA was used for qualitative reverse-transcription polymerase chain reaction (RT-PCR) and for quantitative Real-Time PCR.

3.7 Qualitative RT-PCR

One µl of reverse-transcription reactions of iDCs and standard cocktail, PGE_2-depleted cocktail or cocktail + Poly I:C treated-mDCs was used as target in a PCR reaction to amplify the IDO.

Primers used:

IDO FW: CCAAgAACTTgCAgCTgAAg

IDO RV: TgggTCTATTCCgTTgTgTC

One µl of reverse-transcription reactions of the monocytes and dendritic cells was then used as target in a PCR reaction to amplify either a portion of extra cellular domain of human TEM8 (AF279145) (200 bp fragment) or a portion of the extra cellular domain of human capillary morphogenesis protein 2 (AY233452) (200 bp fragment).

The TEM8 and CMG2 transmembrane domains are indicated in blue.

Primers used:

TEM8:

FW: TgAAgATCTCTTTTTCTATTCAgAgAgggA

Rev: TTgATAATCACAgTgCAgCAgAgggg

CDS TEM8: 144...1838

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                        144 atggcca cggcggagcg gagagccctc ggcatcggct
  181 tccagtggct ctctttggcc actctggtgc tcatctgcgc cgggcaaggg ggacgcaggg
  241 aggatggggg tccagcctgc tacggcggat ttgacctgta cttcattttg gacaaatcag
  301 gaagtgtgct gcaccactgg aatgaaatct attactttgt ggaacagttg gctcacaaat
  361 tcatcagccc acagttgaga atgtccttta ttgttttctc cacccgagga acaaccttaa
  421 tgaaactgac agaagacaga gaacaaatcc gtcaaggcct agaagaactc cagaaagttc
  481 tgccaggagg agacacttac atgcatgaag gatttgaaag ggccagtgag cagatttatt
  541 atgaaaacag acaagggtac aggacagcca gcgtcatcat tgctttgact gatggagaac
  601 tccatgaaga tctctttttc tattcagaga gggaggctaa taggtctcga gatcttggtg
  661 caattgttta ctgtgttggt gtgaaagatt tcaatgagac acagctggcc cggattgcgg
  721 acagtaagga tcatgtgttt cccgtgaatg acggctttca ggctctgcaa ggcatcatcc
  781 actcaatttt gaagaagtcc tgcatcgaaa ttctagcagc tgaaccatcc accatatgtg
  841 caggagagtc atttcaagtt gtcgtgagag gaaacggctt ccgacatgcc cgcaacgtgg
  901 acagggtcct ctgcagcttc aagatcaatg actcggtcac actcaatgag aagccctttt
  961 ctgtggaaga tacttattta ctgtgtccag cgcctatctt aaaagaagtt ggcatgaaag
 1021 ctgcactcca ggtcagcatg aacgatggcc tctcttttat ctccagttct gtcatcatca
 1081 ccaccacaca ctgttctgac ggttccatcc tggccatcgc cctgctgatc ctgttcctgc
 1141 tcctagccct ggctctcctc tggtggttct ggcccctctg ctgcactgtg attatcaagg
 1201 aggtccctcc acccccctgcc gaggagagtg aggaagaaga tgatgatggt ctgcctaaga
 1261 aaaagtggcc aacggtagac gcctcttatt atggtgggag aggcgttgga ggcattaaaa
 1321 gaatggaggt tcgttgggga gaaaagggct ccacagaaga aggtgctaag ttggaaaagg
 1381 caaagaatgc aagagtcaag atgccggagc aggaatatga attccctgag ccgcgaaatc
 1441 tcaacaacaa tatgcgtcgg ccttcttccc cccggaagtg gtactctcca atcaagggaa
 1501 aactcgatgc cttgtgggtc ctactgagga aaggatatga tcgtgtgtct gtgatgcgtc
 1561 cacagccagg agacacgggg cgctgcatca acttcaccag ggtcaagaac aaccagccag
 1621 ccaagtaccc actcaacaac gcctaccaca cctcctcgcc gcctcctgcc cccatctaca
 1681 ctcccccacc tcctgcgccc cactgccctc ccccgccccc cagcgccccc acccctccca
 1741 tcccgtcccc accttccacc cttccccctc ctccccaggc tccacctccc aacagggcac
 1801 ctcctccctc ccgccctcct ccaaggcctt ctgtctag
```

Primers used:

CMG2:

FW: gTgTTTATTgTgTTggTgTCCTTg

Rev: gACAATCTgAAATTCCTCCCC

CDS CMG2: 4...1470

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    1 aggatggtgg cggagcggtc cccggcccgc agccccggga gctggctgtt ccccgggctg
   61 tggctgttgg tgctcagcgg tcccgggggg ctgctgcgcg cccaggagca gccctcctgc
  121 agaagagcct ttgatctcta cttcgtcctg gacaagtctg ggagtgtggc aaataactgg
  181 attgaaattt ataatttcgt acagcaactt gcggagagat ttgtgagccc tgaaatgaga
  241 ttatctttca ttgtgttttc ttctcaagca actattattt tgccattaac tggagacaga
  301 ggcaaaatca gtaaaggctt ggaggattta aaacgtgtta gtccagtagg agagacatat
  361 atccatgaag gactaaagct agcgaatgaa caaattcaga aagcaggagg cttgaaaacc
  421 tccagtatca taattgctct gacagatggc aagttggacg gtctggtgcc atcatatgca
  481 gagaaagagg caaagatatc caggtcactt ggggctagtg tttattgtgt tggtgtcctt
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 541 gattttgaac aagcacagct tgaaagaatt gctgattcca aggagcaagt tttccctgtc
 601 aaaggtggat ttcaggctct taaaggaata attaattcta tactagctca gtcatgtact
 661 gaaatcctag aattgcagcc ctcaagtgtc tgtgtggggg aggaatttca gattgtctta
 721 agtggaagag gattcatgct gggcagtcgg aatggcagtg ttctctgcac ttacactgta
 781 aatgaaacat atacaacgag tgtaaaacca gtaagtgtac agcttaattc tatgctttgt
 841 cctgcaccta tcctgaataa agctggagaa actcttgatg tttcagtgag ctttaatgga
 901 ggaaaatctg tcatttcagg atcattaatt gtcacagcca cagaatgttc taacgggatc
 961 gcagccatca ttgttatttt ggtgttactg ctactcctgg ggatcggtt tgatgtggtgg
1021 ttttggcccc tttgctgcaa agtggttatt aaggatcctc caccaccacc ccccccctgca
1081 ccaaaagagg aggaagaaga acctttgcct actaaaaagt ggccaactgt ggatgcttcc
1141 tattatggtg gtcgaggggt tggaggaatt aaaagaatgg aggttcgttg gggtgataaa
1201 ggatctactg aggaaggtgc aaggctagag aaagccaaaa atgctgtggt gaagattcct
1261 gaagaaacag aggaacccat caggcctaga ccacctcgac ccaaacccac acaccagcct
1321 cctcagacaa aatggtacac cccaattaag ggtcgtcttg atgctctctg ggctttgttg
1381 aggcggcagt atgaccgggt ttctttgatg cgacctcagg aaggagatga ggtttgtata
1441 tgggaatgta ttgagaaaga gctaactgct
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The PCR conditions were an initial denaturation at 95°C for 5 minutes, 35 cycles at 95°C for 1 minute, 63°C for 1 minute (to amplify extra cellular hTEM8), 60°C for 1 minute (to amplify hCMG2 and IDO), 72°C for 1 minute and then, after 35 cycles, 10 minutes of incubation at 72ºC. PCR reactions were performed using a thermal cycler (Biorad). Identical PCR condition were used for TEM8 expression in breast cancer cells except the selection of 24 cycles.

The PCR products were separated by horizontal gel electrophoresis in 1.2% agarose/ethidium bromide (10mg/ml). PCR products were run for approximately 60 minutes at 90 volts, visualised using a UV transilluminator (Biorad) and photographed.

As internal control to assess the integrity of the different RNAs and to confirm the success of the reverse-transcription reaction, primers for housekeeping gene GAPDH (glyceraldehyde phosphate dehydrogenase) were used in the reaction of amplification.

The primers used are:
Fw GAPDH: CAACAgCgACACCCACTCCT
Rev GAPDH: AggCCATgTgggCCATgA

3.8 DNA sequencing

To confirm the specific amplification of the extracellular and transmembrane domain of human TEM8 and the portion of the extra cellular domain of human capillary morphogenesis protein 2, DNA sequencing was performed on PCR products from each different sample monocytes, iDCs and mDCs. The PCR bands were excised from the agarose gel, purified with Macherey-Nagel gel extraction columns and sequenced in both orientations. The sequencing reactions were carried out by MWG Biotech/M-Medical (Germany).

3.9 Quantitative Real-Time PCR

One μl of reverse-transcription reactions of monocytes, iDCs and standard cocktail or cocktail + Poly I:C treated-mDCs was used as target in a Quantitative Real Time PCR.

Real-time RT-PCR was performed by means of the MX3000P Real-time PCR system (Stratagene) and the BRILLIANT SYB Green QPCR Master mix according to the protocol provided by the manufacturer. After initial denaturation for 10 minutes at 95°C, thermal cycling was performed for 40 cycles with steps of 94°C for 48 seconds, 60°C for TEM8, CMG2 and IDO, for 48 seconds, and 72°C for 48 seconds, with the fluorescence being read at the end of each cycle.

The same set of primers for TEM8 and CMG2 utilized in Qualitative RT-PCR reaction were used for the Quantitative PCR.

The analysis was performed with MxPro QPCR Software version 3.00 for MX3000P. The obtained values were within the linear range of a standard curve and were normalized to yield the same amount of glyceraldehyde phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) (Fw GAPDH: CAACAgCgACACCCACTCCT and Rev GAPDH: AggCCATgTgggCCATgA). All PCR products were analyzed by determination of melting profiles as well as by agarose gel electrophoresis.

4. RESULTS

4.1 PHENOTYPE ANALYSIS OF REGENERATED DCs

Two maturation cocktails, consisting of either TNF-α, IL-1β, IL-6 and PGE_2 or TNF-α, IL-1β, IL-6 and Poly I:C, were evaluated for the ability to induce phenotypic changes associated with maturation in regenerated DCs from melanoma and renal carcinoma patients. As detailed in Materials & Methods section, iDCs and mDCs were analyzed by single or two-color fluorescence analysis; the fluorescence was measured by a FACS Vantage flow cytometer, using a panel of monoclonal antibodies to detect CD1a, CD14, CD11c, CD33, CD80, CD86, DR, CD83, and CCR7 antigens. The data about iDCs and mDCs markers are reported in **Table III** and **Figure 1**.
Of note, similar expression patterns of DC antigens are obtained from healthy donors.

As expected the fraction of mDCs expressing $CD1a^+$ and $CD14^+$ antigens, markers for Langerhans cells and monocytes respectively, was low (3%), while 80% of mDCs showed a myeloid phenotype (either/both $CD11c^+$ or/and $CD33^+$ expression) (data not shown).

Although both TNF-α, IL-1β, IL-6 and PGE_2 and TNF-α, IL-1β, IL-6 and Poly I:C, maturation cocktails increased the fraction of cells expressing HLA-DR, CD80, CD86 and CD83 (molecules that are necessary to activate T cells in vivo), a considerable difference between the cocktails was observed. Indeed, while DCs treated with TNF-α, IL-1β, IL-6 + PGE_2 showed a significant expression of the chemokine receptor CCR-7, (**Table III**), while TNF-α, IL-1β, IL-6 + Poly I:C treatment was not (data not shown).

The finding suggests that the lack of CCR-7 up-regulation during maturation with Poly I:C might be the consequence of the fact that prostaglandin E_2 is a major inducer of this receptor (Scandella E. et al., 2002). It should be stressed that

regenerated mDCs did not differ substantially from those originally utilized for vaccination, in terms of phenotype cell yield, and viability (results not shown).

4.2 PROANGIOGENIC POTENTIAL OF REGENERATED DC VACCINES

Recently, human myeloid dendritic cells matured with LPS in the presence of anti-inflammatory molecules such as IL-10 or PGE_2 (LPS+IL-10 or LPS+PGE_2), have been reported as able to selectively secrete the potent angiogenic cytokine vascular endothelial growth factor (VEGF-A) in vitro and to possess proangiogenic activity in vivo (Riboldi E. et al., 2005).

Consequently, we evaluated the pro-angiogenic potential of regenerated CKT-PGE_2 matured DCs measuring (ELISA assay) the levels of VEGF-A in different culture media: IL-1β, IL-6, TNF-α and PGE_2; IL-1β, IL-6 and TNF-α; LPS and PGE_2; and IL-1β, IL-6, TNF-α and Poly I:C.

As shown in **Figure 2**, VEGF-A production (both VEGF-A isoforms 165 and 121 are recognized by antibodies) was selectively observed in the supernatants of DCs matured in presence of either CKT+PGE_2 or LPS+PGE_2. Conversely, VEGF-A production was strongly reduced following DCs maturation with PGE_2-depleted cocktail. Likewise, basal levels of VEGF-A were observed in DCs activated at the presence of Poly I:C. Although it has been reported that, IL-10 or PGE_2 treatments of monocytes-derived DCs may stimulate the production of the potent anti-angiogenetic factor Trombospondin I (TPS-I), we observed not production of TPS-I in the supernatant of CKT+PGE_2 matured DCs.

Collectively these results suggest that the balance between pro- and antiangiogenic activities of DCs may favor the angiogenesis when CKT+PGE_2 is used as a maturation stimulus. Thus, DCs seem to display an "alternative / type 2" state of activation which likely associated with a type 2-polarized immune response known to be responsible for the inhibition of inflammatory cytokines, the promotion of tissue remodeling and repair, the inhibition of Th1 responses (Mantovani A. et al., 2002).

4.3 TEM8 AND CMG2 GENE EXPRESSION IN MYELOID DCs

Possible modulation of TEM8 and CMG2 expression in the passage from immature to mature DCs, have not been previously reported. In order to address this question, we initially evaluated by conventional RT-PCR analysis the difference in relative mRNA expression levels of TEM8 and CMG2 in PEG_2-mDC versus iDC obtained from both cancer patients and healthy donors.

Some representative results are summarized in **Figure 3**. While CMG2 gene is widely expressed in immature DCs and in PEG_2 matured DCs obtained either from cancer patients (**Fig. 3.A,** n = 8) or healthy donors (**Fig. 3.B,** n = 3), TEM8 expression is much more restricted: undetectable or barely detectable in immature DCs generated from the same groups of subjects. However, we observed that TEM8 transcripts were clearly detected in some PGE_2-mDC from cancer patients but not in all examined PEG_2-mDC from healthy donors.

As control, TEM8 transcripts were detected in TEM8-positive HeLa (Premanandan C. et al. 2006) and MDA-MB-231 invasive breast cancer cells, but not in TEM8-negative SK-BR3 and ZR75-1 breast cancer cells lines (Venanzi F.M. et al. 2006) (**Fig. 3.C**).

To quantify the difference in relative expression levels during the process of DC differentiation, we determined the expression ration of both anthrax receptors from both cancer patients and healthy donors by Quantitative Real Time-PCR (Q-RT-PCR; SYBR-Green based) assays, by utilizing the same sets of primers that were used in Standard RT-PCR assay. Overall, the TEM8 and CMG2 real-time RT-PCR data parallel standard RT-PCR results.
Indeed, the quantization of the relative mRNA expression levels between CMG2 and TEM8 indicated that the CMG2 transcripts were always preferentially expressed over TEM8 transcripts in all DCs and monocyte precursors that had been examined (**Figure 4**). The most obvious difference in the expression ratio of the two genes is seen for iDCs.

As shown in **Figure 5**, moderate to comparable increased levels of CMG2 mRNA were observed following iDC maturation in both groups of subjects (mean fold increase [mfi] = 2.88 vs. 1.95 for patients and healthy donors, respectively). On the other hand, a significant difference was found between patients and healthy donors ([mfi] = 8.4 vs 2.7, respectively; p = 0.015), when evaluating the effect of PGE_2 on TEM8 mRNA expression. In agreement with findings by other authors (Xu Q. et al., 2007), mRNA levels of CMG2 transcripts always exceeded those of TEM8 in all types of examined cells (**Fig. 5**). The most important difference in the expression ratio of the two genes was seen for iDCs.

We also proceed to investigate the regulation of TEM8 mRNA expression by different stimuli. As in the case of VEGF-A production, TEM8 expression was strongly down regulated either in mDC matured with the PGE_2-depleted cocktail, or by replacement of PGE_2 with Poly I:C in the cocktail. However, at variance of VEGF–A, the LPS+PGE_2 maturation of patients DCs did not influence TEM8 or CMG2 basal expression (**Figure 6**). From these data we can deduce that VEGF-A production and TEM8 expression might be not subject to the same mechanism of regulation. Thus, TEM8 up regulation is not simply a direct consequence of PGE_2 in the maturation cocktail, but depends on the presence of inflammatory cytokines.

4.4 CORRELATION OF TEM8 GENE EXPRESSION WITH CLINICAL RESPONSES

As mDCs from cancer patients displayed high inter-individual variability in TEM8 expression, we retrospectively checked over a possible correlation between TEM8 gene expression and the clinical course of the disease. In order to minimize bias due to differences in follow-up times, clinical outcome (as detailed in **Table II**), was only divided in two categories: progressive (PD) and not progressive (NP) disease (including complete response *CR*, partial response *PR*, or stable disease *SD* (> 6 months), as defined in previous studies) (Ridolfi R. et al. 2006). An inverse relationship was observed between TEM8 mRNA levels and both clinical and

immunological responses (**Figure 7**). Indeed, eight NP patients (including 2 *CR*, 1 *PR* and 5 *SD*), all DTH-positive (5 for both TL and KLH, 3 for KLH only; median OS = 32 months) showed low levels of TEM 8 mRNA expression ([mfi] = 1.97) similar to healthy donors ([mfi] = 2.7). Conversely, mDC from nine PD patients, all but one negative DTH (median OS = 5 months), showed high TEM8 mRNA expression levels ([mfi] = 12.88). Additionally, mDCs from both PD and NP patients displayed similar (moderate) levels of CMG" expression ([mfi] = 2.88 vs. 3.2, respectively) close to those of healthy donors ([mfi] = 1.95).

Finally, cytofluorimetric data for TEM8 protein expression in mDCs from PD and NP patients paralleled TEM8 mRNA results. As an example, **Figure 8** shows that, more than 97% of mDC and 30.5% iDC from PD patients (TEM8 mRNA mDC vs. iDC; mfi = 30) expressed TEM8 protein, wherease TEM8 protein was expressed in 41% of iDC and 23% of mDC from NP patients (TEM8 mRNA mDC vs. iDC; mfi = 2.70).

We conclude that in our study, high TEM8 expression levels in DC vaccine 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 reported (>10 fold) in a study on serial gene expression analysis (SAGE) of purified endothelial cells from tumor-associated versus normal blood vessels (St. Croix B. et al., 2000).

4.5 INDOLEAMINE 2,3-DIOXYGENASE (IDO) GENE EXPRESSION IN mDC

A recent report (Wobser M. et al., 2007) demonstrated that enzyme indoleamine 2,3-dioxygenase (IDO) is strongly up-regulated in human dendritic cells (DCs) from cancer patients upon in vitro maturation with IL-1, IL-6, TNF-α and PGE_2 cocktail. IDO is supported to convey immunosoppressive effects by degrading the essential amino acid tryptophan, thereby down-regulating T-cell functions. Moreover, IDO expression in DC-based therapeutic vaccines in vivo, seem to attract or induce

FoxP3^{+} T cells (Wobser M. et al., 2007). Indeed, IDO expression was detected both by standard RT-PCR and by real-time-PCR in our series of DCs from cancer patients and healthy donors. Although these analysis revealed marked intra- and interpersonal variation in IDO mRNA levels, all analyzed specimens, DC showed a strong mRNA up-regulation in upon PGE_2 in vitro maturation over a time course of 48h, (**Fig. 9**). However, our data do not significantly differ between DCs from PD and NP patients.

5. DISCUSSION

The experimental therapeutic DC vaccination represents so far one of the best-documented treatments for metastatic melanoma and renal cell cancer (RCC). The results from published trials phase I-II show that it is possible to induce antigen-specific immunoresponses and obtain tumor regression in a subset of treated patients. However, despite occasional correlation between immunological and clinical responses, we don't know whether the modest clinical responses we observed were caused by the vaccination or whether reflect patients with better prognosis capable of mounting immunoresponses.

Although most efforts are dedicated to generate optimal DC vaccine by improving maturation stimuli, administration routes, and immunomonitoring, no study has been published to date to find predictive biological markers that select the patients with increased or decreased likelihood of responding to DC vaccine.
This selection is important, because testing treatments that benefit only a subset of patients in an unselected population might obscure clinical important results.

Arguably, the benefit of DCs vaccination is likely to differ in patients according to their tumor loading. Tumor takes the advance of several different strategies to interfere with DCs maturation and functions. Tumor-derived factors not only condition peripheral immune niches, but also the bone marrow and other hematopoietic organs. Indeed, recent studies suggest that advanced tumor might subvert expansion and differentiation of myelo-monocytes cells leading to abnormal myelopoiesis, with tumor-altered myeloid cells playing a critical role in tumor progression by promoting both tumor evasion from immune attack, and stimulation or amplification of tumor angiogenesis (McLean K., et al., 2008; Curiel T.J., et al., 2004; Priebe A., et al., 2008; Melani C., et al. 2003). Having said that, it should be pointed out that most studies have focused on alterations in phenotype and function of DCs isolated directly from peripheral blood, while only few studies have described tumor-

associated changes in monocyte-derived DCs. Thus, whether and how Mo-DCs generated from cancer patients differ from Mo-DCs from healthy individuals remain an open issue.

A previous study (Pedersen A.E., et al. 2005), compared TNF-α + IL-1β + IL-6, and PGE_2 matured DCs from breast cancer patients to similar DC from healthy donors. Patient-derived DC exhibited a more mature phenotype when compared with DC from healthy controls, particularly when comparing levels of CD40 and CD54 expression, confirming the findings for other kinds of DC preparations in cancer patients (Della Bella S., et al. 2003; Kiertscher S.M., et al. 2000). Moreover DCs from breast cancer patients showed a significantly decreased allostimulatory capacity compared with DC of healthy controls. Thus, enhanced IL-10 production, IL-12 down regulation, and the low capacity for allogeneic stimulation are factors pointing towards a reduced functionality of the patient-derived DC (Pedersen A.E., et al. 2005). The predictive value of these factors for the clinical applicability of the DC preparations is, however, uncertain.

In the process of studying DCs vaccines, we become aware that TNF-α + IL-1β + IL-6 + PGE_2-maturated DCs either from cancer patients or healthy donors represent alternative activated DCs (M2-polarized) known to have tolerogenic properties and proangiogenic potential because of their overexpression of IDO mRNA and secretion of VEGF-A (Mantovani A., et al., 2002). In other words TNF-α + IL-1β + IL-6 + PGE_2-maturated DCs utilized as cancer vaccine might paradoxally, mimic tumor resident DCs suspected to promote neovascularization and tumor growth (Priebe A. et al., 2008; Gottgried E., et al., 2007; St. Croix B., et al., 2000). Of interest, these DCs might display a mixed DC-endothelial cell phenotype and up modulate a number of tumor endothelial markers (TEMs1-9) (see above).

Bearing this in mind, we set out to explore the possibility that monocyte-derived DCs utilized for therapeutic vaccination of cancer patients, could upregulate tumor-endothelial marker 8 (TEM8) expression. Focusing on TEM8, its expression pattern is especially intriguing in that it is the only TEM characterized so far that shows no

detectable expression in either corpus luteum or wound healing, suggesting that this gene may be highly specific to tumor angiogenesis and not required for normal adult angiogenesis. Accordingly, although the biological function(s) of TEM8 remain essentially unknown, this anthrax receptor has been proposed as a marker of tumor progression and a potential therapeutic target for a variety of tumors (Xu Q., et al., 2007; Duan H.F., et al., 2007).

Here we report that TEM8 gene-protein expression levels in mDCs (evaluated by means of quantitative real-time RT-PCR and cytofluorimetric analysis, respectively) were clearly related to clinical outcome and immunoresponses in a case series of 17 cancer patients who had taken part in DC phase I/II vaccination trials (2001-2005) for advanced melanoma (n = 13) and renal cell carcinoma (RCC; n = 4) (Ridolfi R., et al., 2006). In the study, increased TEM8 mRNA and protein expression levels (mature (m) vs. immature (i) DCs) were observed in monocyte-cultured DCs generated from treatment-nonresponsive patients all but one with negative DTH. Conversely, DCs obtained from eight treatment-responsive patients, all DTH-positive, had TEM8 expression values, not different from that found in healthy donor mDCs. Thus, mDC TEM8 expression levels, seems to identify ($p = 0.0018$) patients who could benefit from DC therapeutic vaccination.
Of interest, neither VEGF-A production levels nor IDO gene overexpression in DCs had a clinical impact in this case series of patients.

Are TEM8 expressing DCs “tolerogenic”, and therefore responsible for lack of therapeutic impact? or, Are the patients highly immunologically compromised, and TEM8 upregulation in their monocyte-derived DC is a correlate but not cause?

We provide evidence that TEM8 up regulation is a direct consequence of PGE_2 in the maturation cocktail. Moreover, when the response rates in clinical trials were evaluated, TNF-α + IL-1β + IL-6 + PGE_2-maturated DCs did not seem to be less effective compared with DC maturated otherwise (McIlroy D., et al., 2003) Thus, is unlikely that TEM8 negative DCs induce better clinical responses.

It must be underlined that DC vaccines are customized in vitro artifacts (Nanda A., et al., 2004) developed from high variable mixtures of inflammatory monocytes of uncertain cell-lineage (i. e., $CD14^{+}CD16^{+}$ monocytes) (Nestle F.O., et al., 2005; Nagaraj S., et al. 2008), and immature (immunosuppressive) myeloid precursors (Arroyo J.C. et al. 2004; Nagaraj S. et al., 2008; Serafini P. et al. 2006).
It is therefore conceivable that overexpression of TEM8 in mDCs from progressive patients is related to an overload of tumor "educated" myeloid-DC precursors. The fact that TEM8 overexpression is not detectable at the stage of iDCs may indicate that "education" entails increased sensitivity to one or more components of the maturative cocktail. This would still held even if we had just compared TEM8 mDC of the responder vs. non-responder patients. Of interest, preliminary results from our laboratory, indicate that TNF-α + IL-1β + IL-6 + PGE_2-maturated DCs from 3 out of 3 patients with Policytemia Vera, with myeloprliferative disorders (e. g. Policytemia Vera) display TEM8 up modulation (results not shown).

The information gained from the present study could contribute substantially to increasing response rates to DC immunotherapy by narrowing this high-cost and labour-intensive treatment to cancer patients whose *in vitro* mDCs display low levels of TEM8 expression. However, because of the relatively small number of patients involved and the retrospective nature of the study, the present results need to be confirmed in a prospective case series.

7. FIGURES

Patient ID	Sex	AGE	P.S. (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.J.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 C.G.	M	28	2	sk, lg, ln	BIOCT
60 T.M.	M	26	1 (2)	sk, ln	BIOCT
61 Di I.	M	34	0	ln, sk	NT
62 B.F.	M	64	0	ln, sk	NT

Table I. Patient demographic, disease status, and pre-treatment characteristics (n = 17). Male/Female 12/5. Median age, 48 years (36-68). Yellow shading highlights renal cancer cell patients. PS (ECOG), performance status according to ECOG; Abbreviations: ln, lymph-node; lv, liver; st, soft tissues; kd, kidney; lg, lung; pv, pelvis; ag, adrenal glands; sk, skin; NT, no treatment; CT, chemotherapy; BIO, immunotherapy (Interferon, Interleukin 2); BIOCT, chemotherapy + immunotherapy.

Pt. ID	N° VACC.	ADMINISTERED CELLS N° X 106 (range)	DTH Response LIS / KLH		CLINICAL RESPONSE	RESPONSE and 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.J.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
58De C.G.		na	-	-	PD	-	3
60 T.M.		na	-	-	PD	-	1
61 Di I.	10	10 (10 - 10.7)	+	++	CR	30+	30+
62 B.F.	18	10 (10 - 10)	++	++	PR	24	27+

Table II. Patients vaccination and clinical-immunological outcome.

Yellow shading shows renal cancer cell patients. Abbreviation: LIS, autologous tumor cells lysate; KLH, keyhole limpet hemocyanin (KLH); DTH, delayed-type hypersensitivity test (best response after 4 or more vaccinations); na, not assessed; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; OS, overall survival; OS+, patients still alive.

	iDC median value (range)	mDC median value (range)
CD1a	27 (4.8-53) 35 (8.3-66)	3 (0-14) 39.3 (0-54)
CD14	2.6 (0-33) 3.6 (2-6)	3 (0-25) 1.3 (1-2)
CD80	6 (1-23) 21.6 (8-39)	43 (14.2-76) 86.6 (80-91)
CD86	29 (5.4-75) 13.6 (6-21)	80 (21.56-94) 95.3 (94-97)
DR	55 (8.2-76) 73.6 (64-83)	70.7 (20.18-92) 75.6 (64-84)
CD83	2.08 (01-13) 1 (0-3)	46 (4.72-80) 95 (91-98)
CCR7	4 (2-5) 7 (3-9)	86.5 (48-92) 79.6 (62-92)
CD33	37.6 (9-63) 38.3 (22-49)	11.6 (2-55) 2 (1-3)
CD11c	95.6 (43-98)	98.6 (96-99)

Table III. DCs phenotype analysis.

The table shows the percentage of dendritic cells with a particular surface marker. 3-5 x 10^5 immature (iDCs), cytokine-cocktail (TNF-a, IL-1b, IL-6, PGE_2) or matured (mDCs) dendritic cells from both melanoma and renal cell carcinoma patients (in black) and healthy donors (in blue) were incubated with appropriate fluorescein isothiocyanate or phycoerythrin-labeled monoclonal antibodies. iDCs and mDCs phenotypes were determined by single or two-color fluorescence analysis by a FACS Vantage flow cytometer.

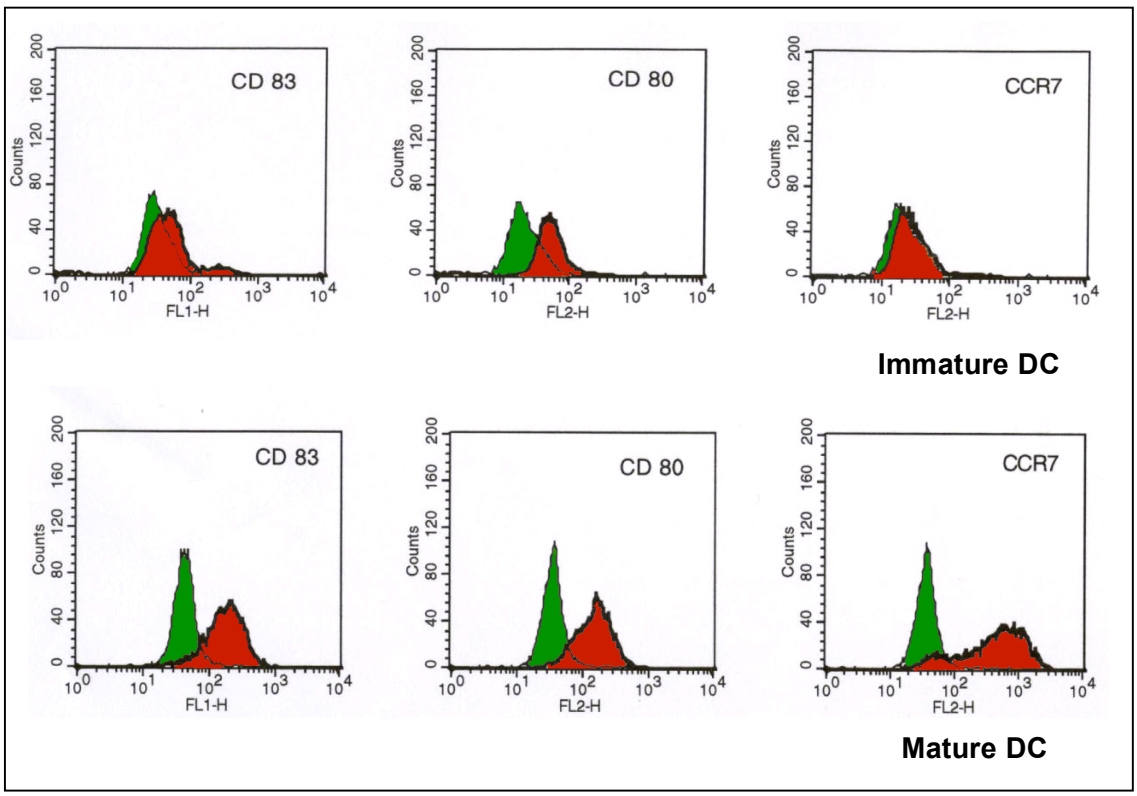

Figure 1. DCs migration phenotype: CD83, CD80 and CCR7 expression (% positive cells).

FACS analysis of CD83, CD80 and CCR7 expression on human immature and cytokine cocktail matured dendritic cells.

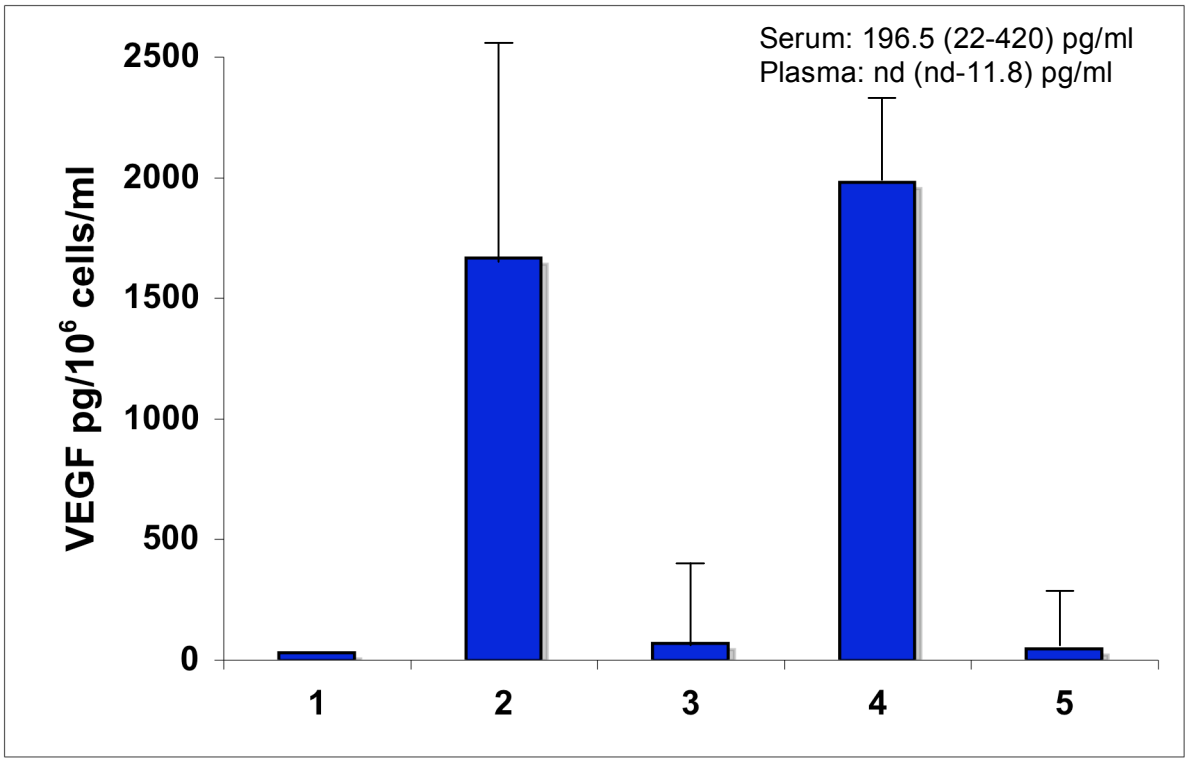

Figure 2. DCs angiogenic phenotype.

ELISA test (Pierce Biotechnology): median values (pg/ml) of VEGF-A (165 and 121 splicing forms) in supernatants of iDCs (1), DCs matured with the standard cocktail (2), with the PGE_2-depleted cocktail (3), with LPS + PGE_2 (4) or with TNF-a, IL-1b, IL-6 and Poly I:C (5).

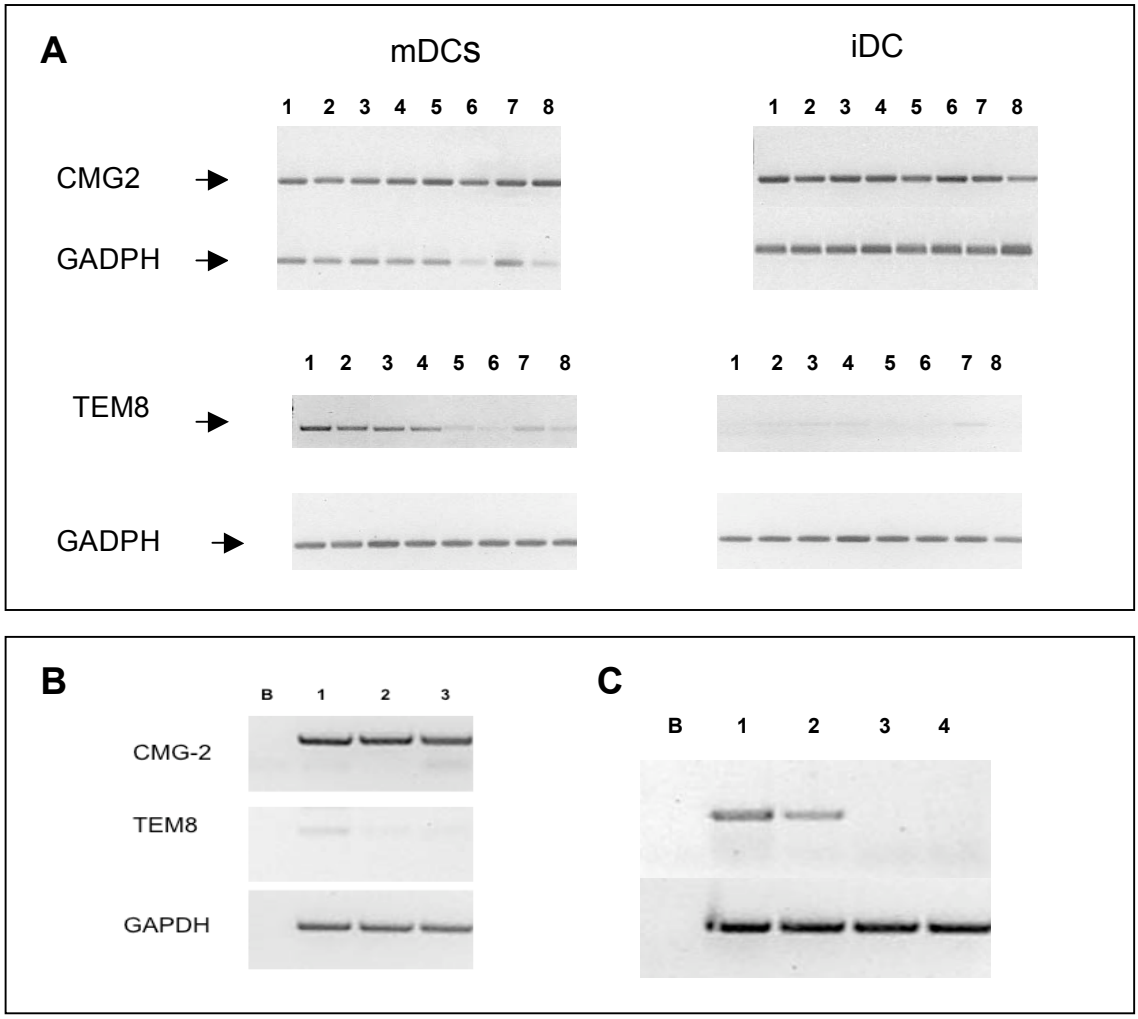

Figure 3. TEM8 and CMG2 gene expression in myeloid DC

(**A**) CMG2 and TEM8 expression in immature DC (iDCs) and PGE_2-matured DC (mDCs) from cancer patients (n = 8). (**B**) CMG2 and TEM8 expression in mDCs from healthy donors (n = 3). (**C**) TEM8 expression in tumor cell lines: HeLa (1), MDA-MB-231 (2), SK-BR3 (3), and ZR75-1 (4). B, blank. GAPDH expression was measured by RT-PCR as a positive amplification control in each experiment.

Purified PCR products were visualized by agarose gel electrophoresis.

CELLS	mRNA ratio	medium value (range)
Mo	CMG2 vs TEM8	143 (65 - 230)
iDCs	CMG2 vs TEM8	230 (50 - 461)
mDCs	CMG2 vs TEM8	150 (60 - 278)

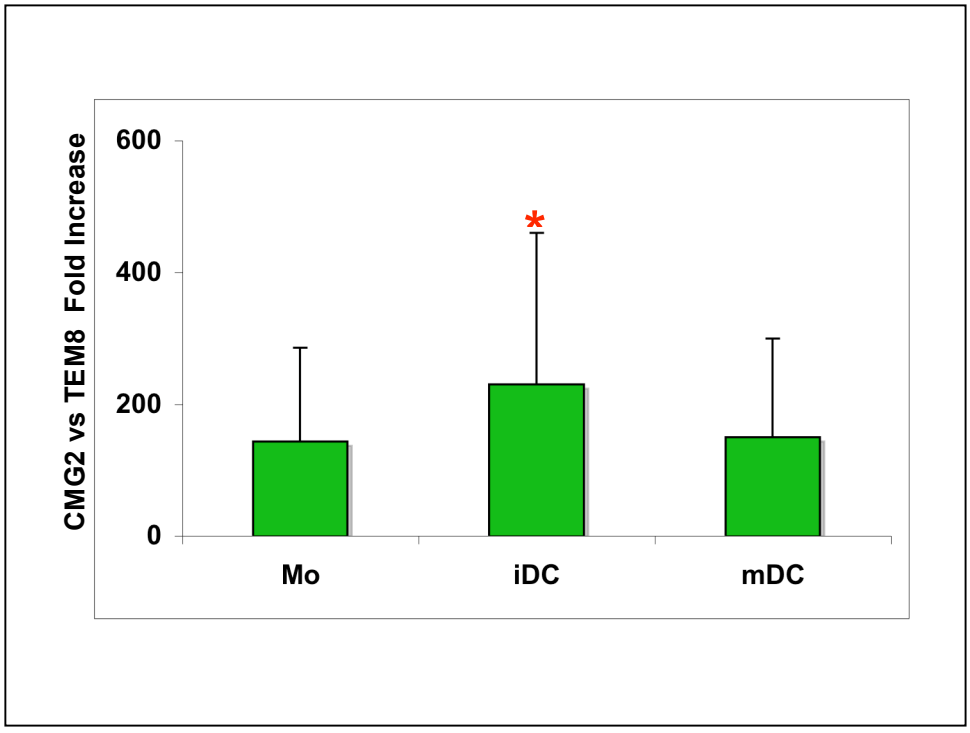

Figure 4. Quantitative-RT-PCR.

Relative quantization of CMG2 to TEM8 gene transcripts in monocytes (Mo), immature DCs (iDC) and PGE_2-matured DCs (mDC), evaluated as relative abundance of TEM8 and CMG2 compared to GAPDH. *$p < 0.05$.

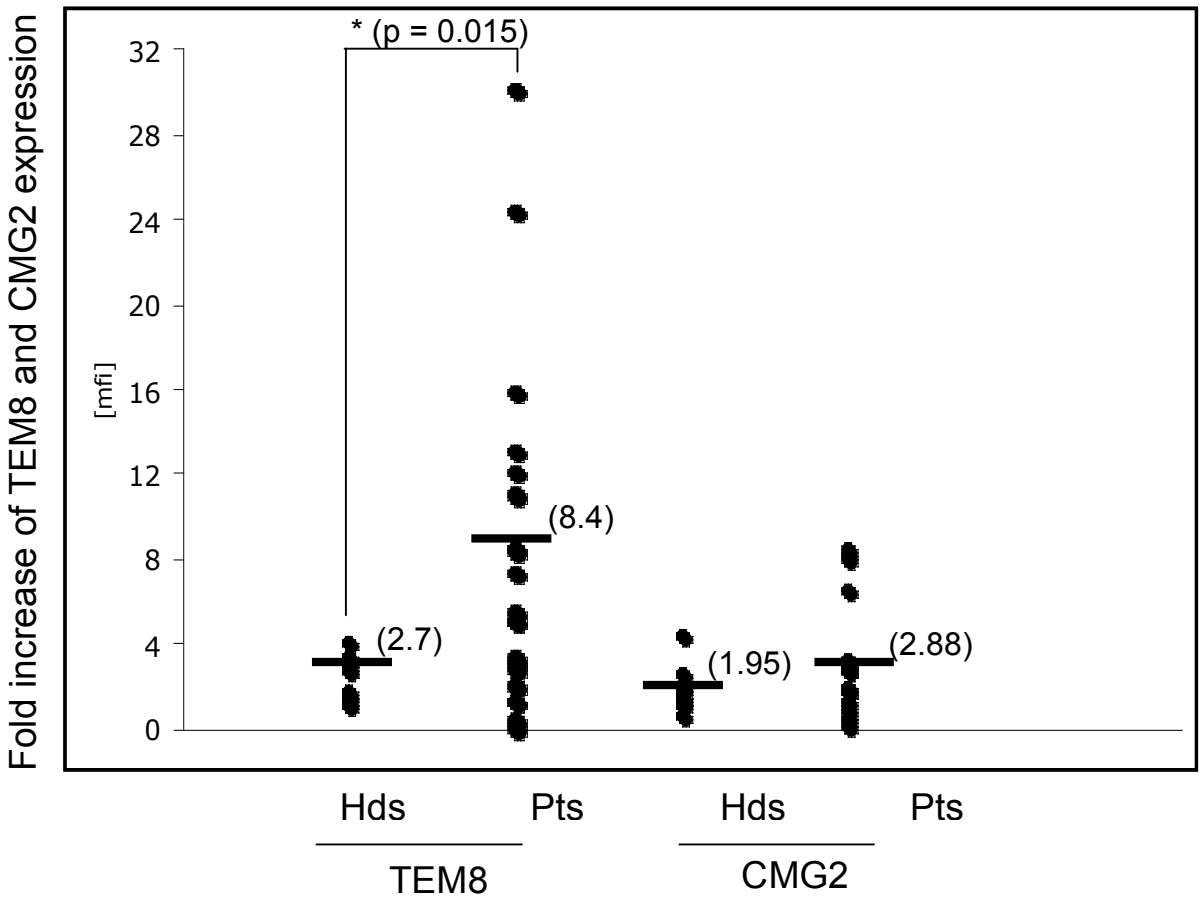

Figure 5. Scatter plot of relative increase (mDCs vs. iDCs) of TEM8 and CMG2 mRNA expression in cancer patients and healthy donors. Each dot represents a measurement for each subject: cancer patients (Pts. n = 17), healthy donors (Hds. n = 6). The results indicate the mean from three independent real-time RT-PCR reactions. Bars, mfi (range): TEM8 Hds = 2.7 (1.3 - 4); TEM8 Pts = 8.4 (0.3 - 30); CMG2 Hds = 1.95 (0.6 - 4.3); CMG2 Pts = 2.88 (0.12 - 8.34).

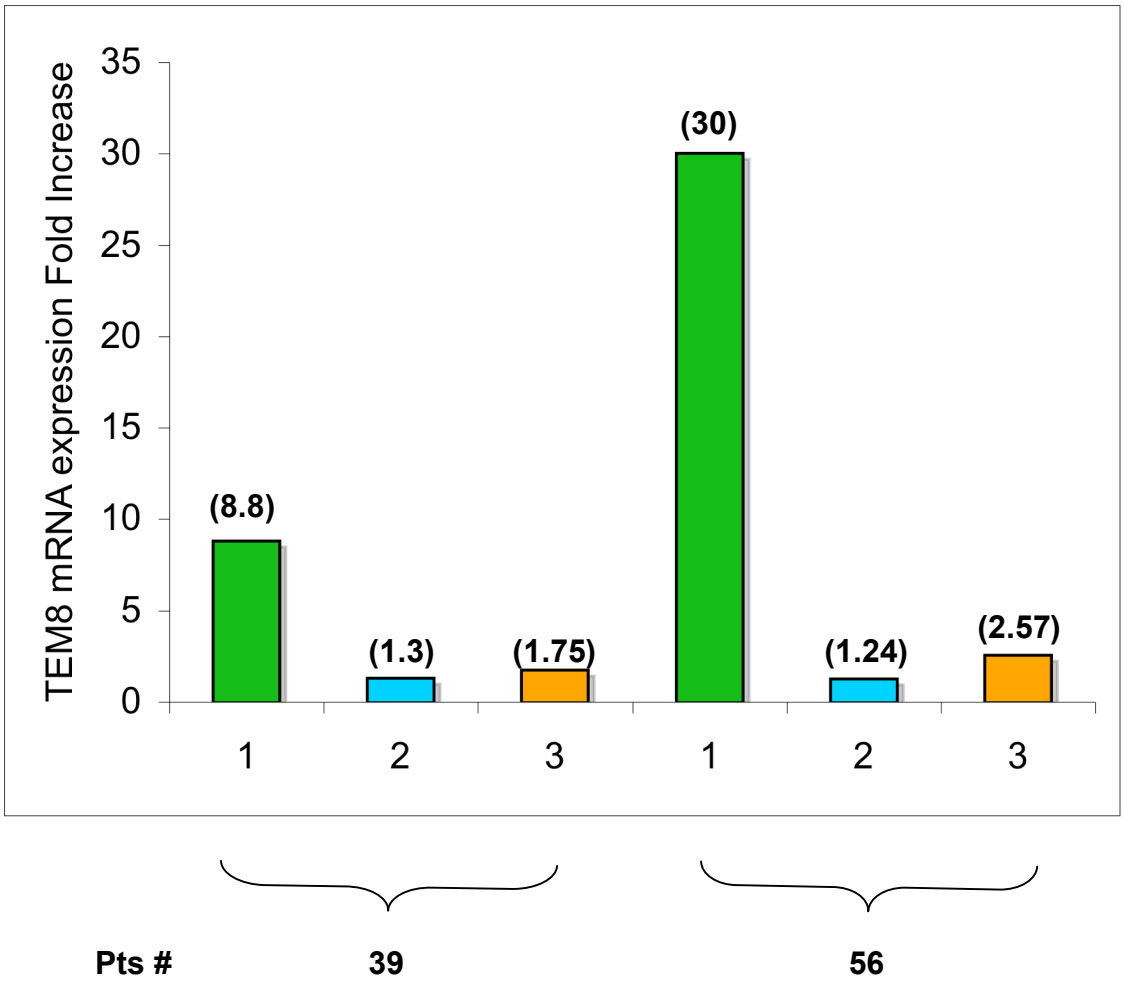

Figure 6. TEM8 mRNA expression by DCs activated by different maturative stimuli.

(1) CKT + PGE_2; (2) LPS+ TNFα; (3) LPS + PGE_2. PGE_2-depleted cocktail does not up-regulate TEM8 (results not shown). Pts, melanoma patients (ID; DC 39 and DC 56).

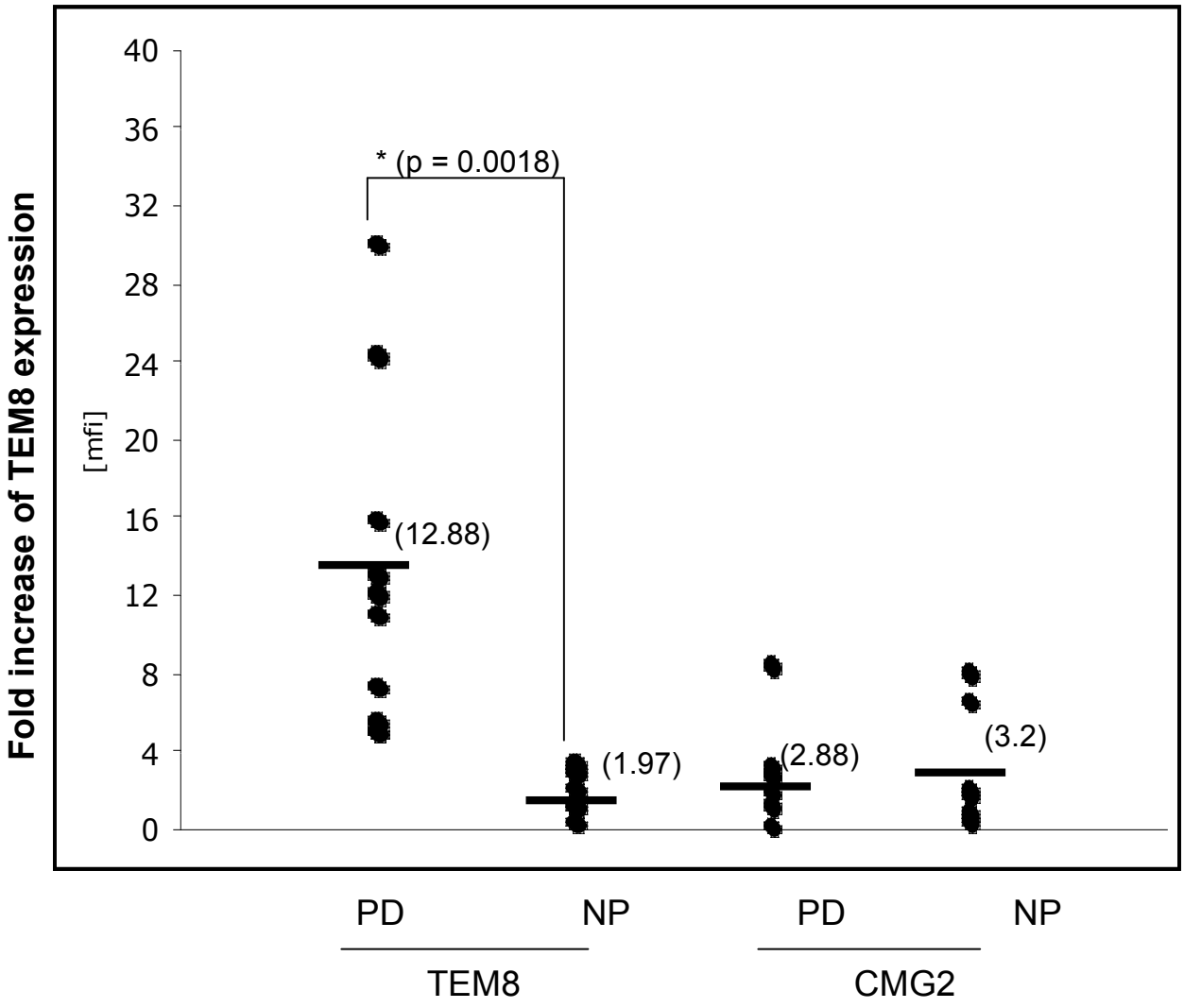

Figure 7. Scatter plot of relative abundance of TEM8 and CMG2 mRNA (mDC vs. iDC) in cancer patients with different clinical outcome.

PD, progressive disease; NP, non progressive patients. Bars, medium values (range): TEM8 PD = 12.88 (5 - 30); TEM8 NP = 1.97 (0.3 - 3.30); CMG2 PD = 2.88 (0.12 - 8.34); CMG2 NP = 3.2 (0.4 - 8).

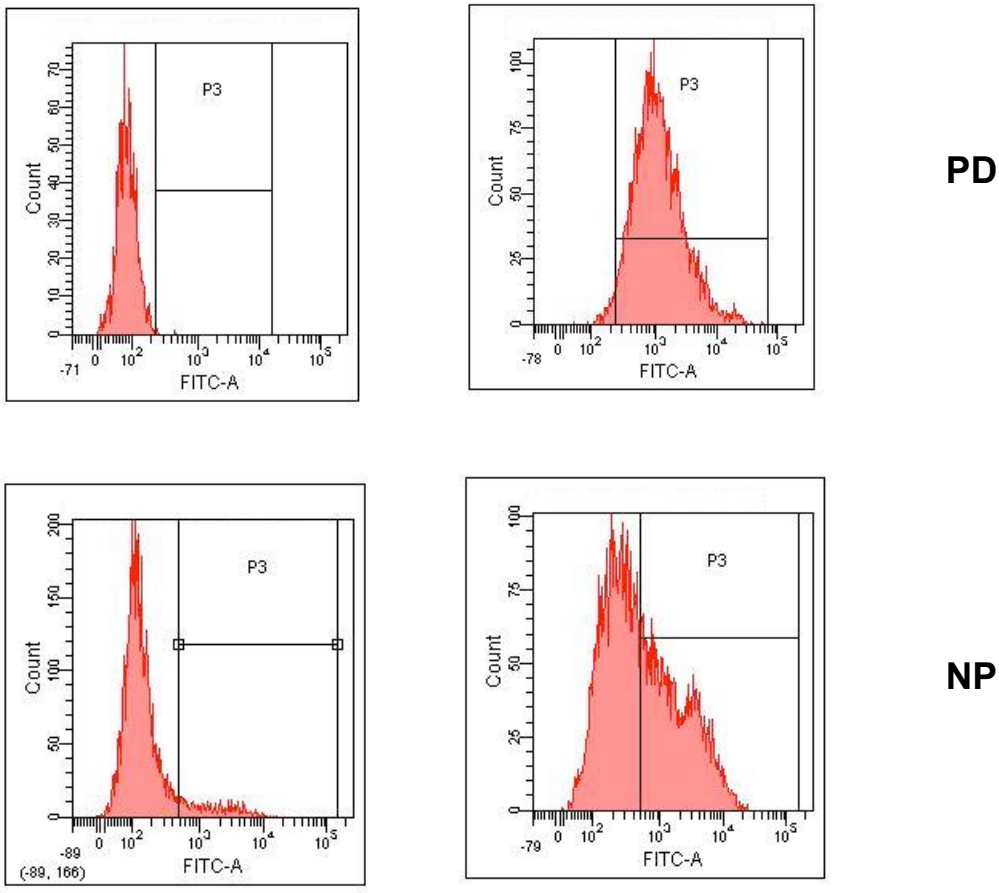

Figure 8. Cytofluorimetric analysis of TEM8 protein expression.

PD patient (ID. 51; TEM8 mRNA mDC vs. iDC; mfi = 30); NP patient (ID. 61; TEM8 mRNA mDC vs. iDC; mfi = 2.7). PD= 97% of mDC TEM8 positive cells; NP= 23% of mDC TEM8 positive cells.

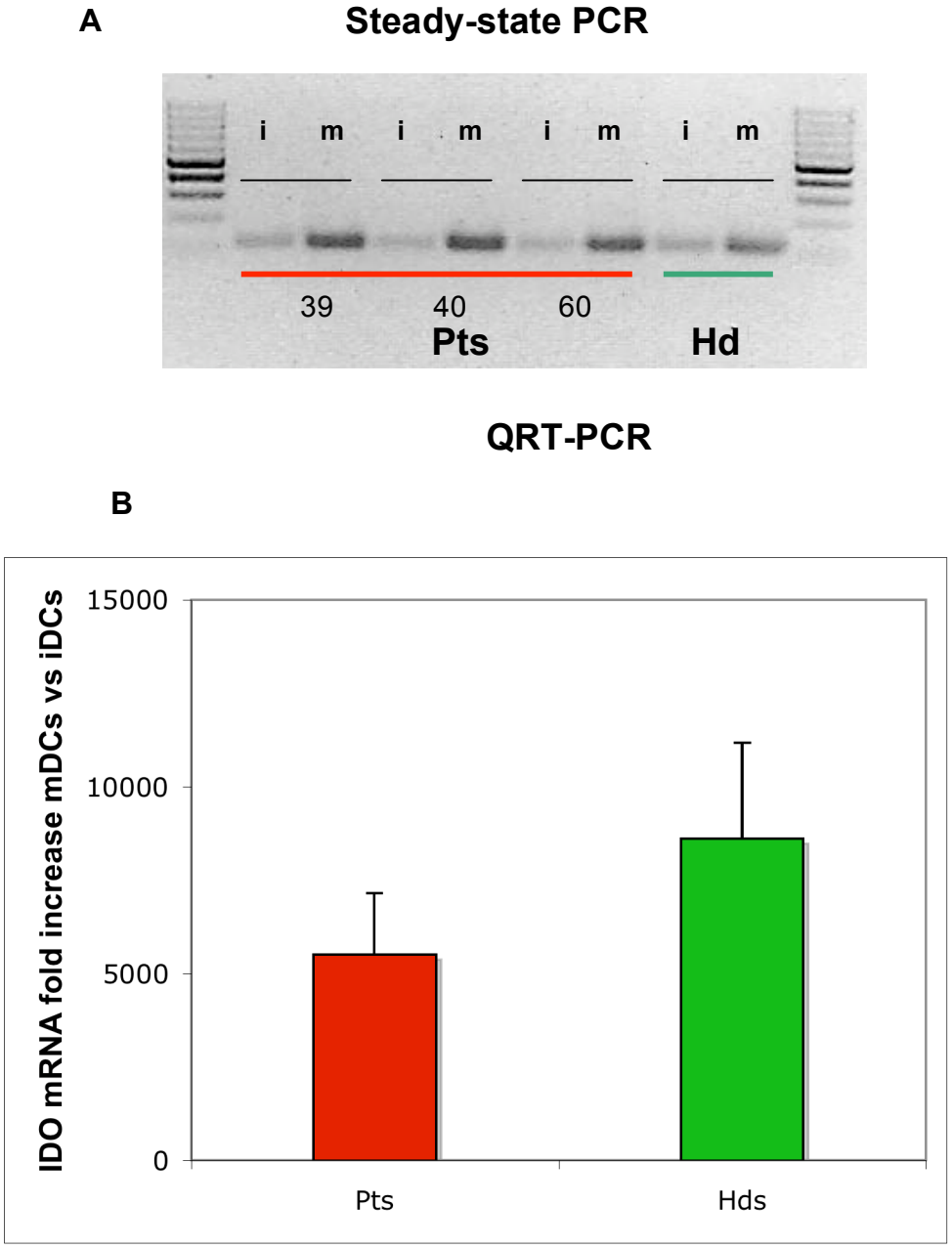

Figure 9. IDO mRNA expression. (**A**) RT PCR-Steady state by DCs from different subjects. Pts, melanoma patients (ID; DC 39, DC 40 and DC 60); Hd; healthy donors; i, iDCs; m, mDCs. (**B**) Quantitative-RT-PCR.

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