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BREAST CARCINOMA CELLS PROMOTE THE DIFFERENTIATION OF $CD34^+$ PROGENITORS TOWARDS 2 DIFFERENT SUBPOPULATIONS OF DENDRITIC CELLS WITH $CD1a^{high}CD86^-$LANGERIN- AND $CD1a^+CD86^+$LANGERIN$^+$ PHENOTYPES

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Primary breast carcinoma are frequently infiltrated by dendritic cells (DC). The mechanisms involved in the localization and status of activation of DC within primary breast carcinoma were investigated. CCL20/MIP3α, a chemokine involved in immature DC and their precursors attraction, was detected by immunohistochemistry on cryopreserved tissue sections of primary breast tumors and by ELISA and biological assay in metastatic effusion fluids from breast cancer patients but not from other tumors. *In vitro*, irradiated breast carcinoma cell lines (BCC) as well as their conditioned media promoted $CD34^+$ cell differentiation into $CD1a^+$ Langerhans cells (LC) precursors as early as day 6, while at day 12, 2 different $CCR6^+$ subpopulations of DC with a Langerhans cell ($CD1a^+$Langerin$^+CD86^+$) and an immature DC ($CD1a^{high}$Langerin-$CD86^-HLA\text{-}DR^{low}CD40^{low}$) phenotype were observed. This phenomenon was partly driven by a TGFβ-dependent mechanism since a pan TGFβ polyclonal antibody completely blocks BCC-induced LC differentiation and partly reduces immature DC development. These DC failed to maturate in response to sCD40L or LPS stimuli and $CD1a^{high}$Langerin$^-CD86^-$ cells have a reduced T-cell stimulatory capacity in MLR experiments. The absolute number of T cells was reduced by 50% in both the $CD4^+$ or $CD8^+$ compartments, these T cells expressing lower levels of the CD25 Ag and producing less IFNγ. These results show that breast carcinoma cells produce soluble factors, which may attract DC and their precursors *in vivo*, and promote the differentiation of the latter into LC and immature DC with altered functional capacities. The infiltration of BCC by these altered DC may contribute to the impaired immune response against the tumor.

Key words: *Langerhans cells; immature dendritic cells; breast carcinoma; differentiation; cytokines; tumors*

Tumor cells have been reported to impair the function of immune system through various mechanisms, including hiding from the immune system in immune sanctuaries, immune-ignorance, production of immunomodulary cytokines or inhibitors, inhibition of the function of immune cells, protection against lytic activity of immune effectors and inhibition of DC differentiation *in vivo* (for review, see reference 1).

In vitro, renal cell carcinoma (RCC) tumor cells were found to produce cytokines, IL-6 and M-CSF, which block DC differentiation from $CD34^+$ progenitors and peripheral blood monocytes (PBM)[2,3] triggering the differentiation of DC progenitors towards monocyte/macrophage population exhibiting a poor antigen presenting cell (APC) capacity.[2] *In vivo*, RCC are rarely infiltrated by DC,[4,5] while the production of IL-6 has been correlated to a poor response to immunotherapy *in vivo*.[6]

In contrast, primary breast cell carcinoma (BCC) tumors[3] do not block DC differentiation in the same culture conditions. It has been recently reported that BCC are infiltrated by an immature DC subpopulation with a LC phenotype ($CD1a^+$Langerin$^+$), whereas mature DC are maintained at the periphery of the tumor.[7,8] The mechanisms by which breast carcinoma cells influence the localization of DC in primary breast tumors are not clear. In addition, the functional role of LC in tumor progression is not known, although their presence in overt primary malignant tumors indicate that they are unable both to promote an efficient immune antitumor response and to control tumor progression in breast carcinoma.

Our study was designed to understand the mechanisms that could explain the presence of DC in BCC. The results indicate that breast cancer tumor cells may recruit DC precursors through CCL20/MIP3α production *in vivo*, and promote in part through a TGFβ-dependent mechanism their differentiation into DC with impaired functions exhibiting 2 specific phenotypes ($CD1a^{high}$Langerin$^-CD8^-$, $CD1a^+$Langerin$^+CD86^+$).

METHODS

Hematopoïetic growth factors

Recombinant human (rh) granulocyte/monocyte-colony stimulating factor (GM-CSF) (specific activity: 2×10^6 U/mg; Schering Plough Research Institute, Kenilworth, NJ) was used at 100 ng/ml (200 U/ml); interleukin-4 (IL-4) (specific activity: 10^6 U/mg, Schering Plough Research institute, Kenilworth, NJ) at 50 IU/ml; rh Tumor Necrosis Factor (TNFα) (specific activity: 5×10^6 U/mg, Cetus, Amsterdam, Netherlands) at 2.5 ng/ml (50 U/ml); rh Stem cell Factor (SCF) (specific activity: 4×10^5 U/mg; R&D System, Abingdon, UK) at 25 ng/ml; rh Transforming growth factor ($rhTGF\beta_1$) (R&D Systems) at 10 ng/ml; and rhIL-1β (R&D Systems) was used at 10 ng/ml.

Production of breast cell carcinoma conditioned medium (BCC CM)

Breast Carcinoma cell lines obtained from ATCC (MCF-7 and T-47-D) or established in the laboratory (CLB-SAV) were plated in 100 mm-diameter dishes at a density of 5×10^5 cells/ml in RPMI-1640 or DMEM medium supplemented with 2 mM glutamine, 200 IU/ml penicillin, 200 μg/ml streptomycin (Gibco

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Laboratories, Grand Island, NY) and 10% fetal calf serum (FCS) (Biowittaker, Verviers, Belgium) further referred to as complete RPMI or DMEM medium. After 2 days of culture, supernatants were harvested, filtered, aliquoted and stored at −20°C for further use.

BCC cell lines activation

BCC cell lines (T47-D, CLB-SAV and MCF-7) were cultured, for 48 hr at 10^6 cells/3 ml in 6-well plates in complete RPMI medium alone (medium) or activated with rhIL-1β (10 ng/ml). At the end of the culture, supernatants were recovered and CCL20/MIP3α content was analyzed using a specific ELISA.

Purification of T lymphocytes

Total peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from healthy volunteers by Ficoll Hypaque density gradient centrifugation (Eurobio, Les Ullis, France). Lymphocytes were further purified on a multistep Percoll gradient (Amersham Biosciences, Saclay, France) as previously described[9] and total lymphocytes population was recovered in the pellet (less than 3% expressed CD14).

Naive T lymphocytes ($CD45R_A^+$) were purified from the total lymphocyte population by immunomagnetic depletion with a cocktail of MAbs.[9] After 2 rounds depletion with beads the purity of $CD45R_A^+$ was routinely higher than 95%. The T-lymphocyte population was also cryopreserved in 10% DMSO for further mixed lymphocyte reaction (MLR) assays.

Dendritic cell differentiation

Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen (Ag) were isolated from mononuclear fractions through positive selection by mini MACS (Miltenyi Biotec, Gmbh), using an anti-CD34 MAb (Immu 133.3, Immunotech, Marseille, France) and goat anti-mouse IgG-coated microbeads (Miltenyi Biotec, Gmbh)[9] and represented between 80 to 99% of isolated cells.

$CD34^+$ progenitors were seeded at 5×10^3 to 10^4 cells/ml in 24-well plates in complete medium in presence of GM-CSF, TNFα, SCF, and 2% human AB^+ serum (sAB^+) for 6 days as previously described.[9,10] Cells were then harvested, numbered, phenotypically characterized and seeded in the absence of sAB^+ but in the presence of GM-CSF and TNFα at 5×10^4 cells/ml for an additional 6 days expansion period. A last medium change was performed, if necessary, at day 10, and then DC were collected at day 12. Eventually, adherent cells were recovered using a 0.5 mM EDTA solution.

DC subpopulations selection

After 12 days of culture with medium or BCC CM conditions, cells were collected and labeled with FITC-conjugated CD1a (clone HI149) and phycoerythrin (PE)-conjugated CD86 [clone IT2.2 (Pharmingen, Becton Dickinson, Pont de Claix, France)]. Cells were separated into $CD1a^{high}CD86^-$ and $CD1a^+CD86^+$ using a FACStarplus® (laser setting power 250 mW, excitation wavelength 488 nm, Becton Dickinson). All the procedures of sorting were performed in the presence of 0.5 mM EDTA to avoid cell aggregation. Reanalysis of the sorted populations showed a purity >98%.

DC activation

Day 12 DC, generated in the presence of either cytokines or BCC CM, were plated at 2.5×10^5 cells/0.5 ml in 24-well tissue culture plates for 48 hr in the presence of LPS (10 ng/ml) (Sigma Chemical Co., St. Quentin Fallavier, France) or sCD40-L (100 ng/ml) kindly provided by Immunex Corporation (Seattle, WA). GM-CSF (100 ng/ml) was added to favor DC survival. DC were stimulated in the presence or in absence of BCC CM. In all conditions, supernatants were tested for the production of IL-10 and IL-12p70 and cells were phenotyped as described in the results section.

MLR assay

After culture, $CD34^+$-DC were collected, irradiated (30 Gray) and then used as stimulator cells for allogeneic adult naive T lymphocytes. Ten to 10^4 stimulator cells were added to the T cells (2×10^4 cells/well) in 96-well round-bottomed culture plates (Nunc, Rockilde, Denmark). Cultures were performed in RPMI complete medium supplemented with 10% FCS. After 5 days of incubation, cells were pulsed with 0.5 μCi of ^{3}HTdR per well (specific activity 5 mCi/mMol) for the last 18 hr, harvested and counted. Tests were carried out in triplicates and results are expressed as mean counts per minute (cpm ± SD). The levels of ^{3}H-TdR uptake by stimulator cells alone were always below 100 cpm.

Cell surface phenotyping

Phenotype was performed at day 6 and day 12 for $CD34^+$-DC. Flow cytometry was carried out by incubating 5×10^4 to 10^5 cells for 20 min on ice under 50 μl with optimal concentrations of the appropriate antibodies coupled to PE (except when specified). Antibodies were purchased from Becton Dickinson (Pont de Claix, France) for CD14, HLA-DR, and CD80; Pharmingen (Becton Dickinson) for CCR6, CD86, and CD1a; Immunotech (Marseille, France) for CD83, CD40 and Langerin (CD207) (clone DCGM4). Analysis of the intracytoplasmic expression of DC-Lamp was performed, using indirect DC-Lamp MAb (clone 104G4) (Immunotech) followed with goat anti-mouse antibody coupled to PE (Dako, Trappes, France) in the presence of saponin (0.3 %). The staining was compared to that of a nonrelated control MAb used in the same conditions. Fluorescence analysis was performed on a FACScan flow cytometer after acquisition of 5,000 events (Becton Dickinson). Negative controls were performed with unrelated murine MAb or MAb-PE purchased from Pharmingen.

Cytokine detection

Cytokines were detected in conditioned media and culture supernatants using commercial quantitative sandwich immunoassay kits from Immunotech (Beckmann-Coulter, Marseille, France) (IFNγ, IL-10) and R&D System (Abingdon, UK) ($TGF\beta_1$, high sensitivity IL-12 p70). The detection limits of these immunoassays were 0.08 IU/ml, 5 pg/ml, 7 pg/ml, and 0.5 pg/ml, respectively.

Biological fluids samples collection

Effusion fluids (pleural or ascitic) were collected at the Centre Léon Bérard (Lyon, France) between 1995 and 2002 from patients with metastatic BCC ($n=25$) or other tumors (renal cell carcinoma, ovarian carcinoma and liver carcinoma) ($n=6$). Non tumoral ascitic fluids used as control were obtained from cirrhotic patients ($n=6$) (kindly provided by Dr. C. Lombard Bohas, Hopital Edouard Herriot, Lyon, France). Samples were centrifugated to eliminate cellular components and stored at −80°C until their use. Coupled sera were also collected simultaneously, when possible, immediately stored at −80°C after centrifugation, and their CCL20/MIP3α contents were compared to those of patients suffering from metastatic BCC ($n=55$) or from another neoplastic pathology (renal cell carcinoma) ($n=39$) without effusion component.

ELISA for CCL20/MIP3α

CCL20/MIP3α concentrations in effusion fluids and sera were tested using a sandwich immunoassay as previously described.[11] Maxisorp microplates (Merck Eurolab, Fontenay sous Bois, France) were coated with 3 μg/ml 319F6 antibody in carbonate buffer and CCL20/MIP3α content was revealed with 206D9 (1/3,000) antibody coupled to peroxydase conjugate (these antibodies were kindly provided by Schering Plough Corporation). The revelation was performed with the TMB reagent (Becton Dickinson). The assay proved to be specific for hCCL20/MIP3α with a sensitivity of 0.2 ng/ml.

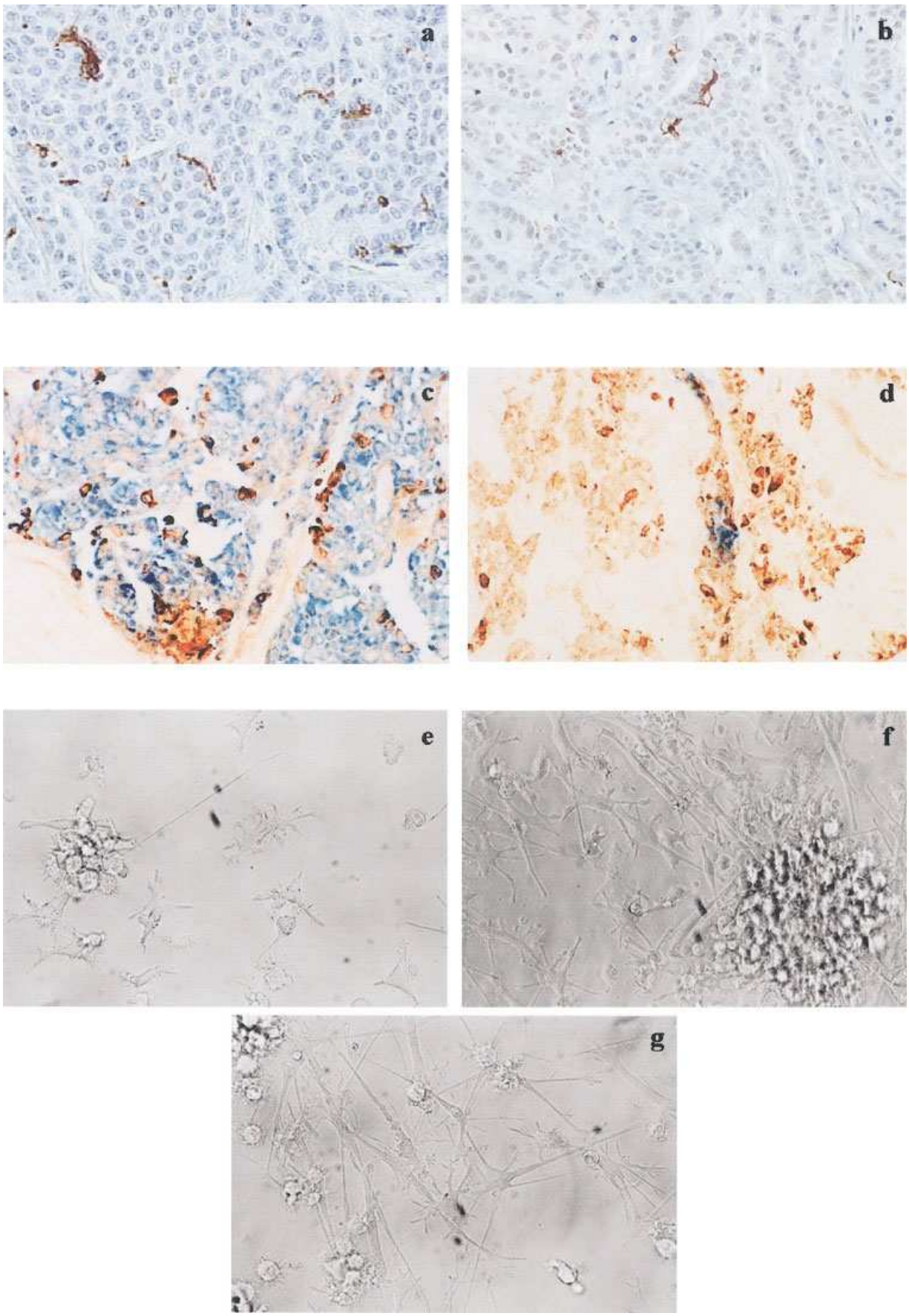

Figure 1.

Chemotaxis assay

Migration assays were carried out using Transwell inserts (COSTAR, Dutscher, Brumath, France) with 5×10^5 cells/well as described.[12] $CD34^+$ HPC derived day 9 precursors were generated from day 0 to day 6 in GM-CSF^+ TNFα + SCF + 2% sAB^+ and GM-CSF + TNFα + 2% sAB^+ for 3 subsequent days. These cells were pre-incubated for 1 hr at 37°C and 5×10^5 cells were placed for 1 hr in 5 μm pore size inserts. Effusion fluids from BCC patients were diluted (1/3) and placed in the lower chamber. DC which have migrated in response to BCC effusion were revealed by CD1a/CD14 double staining. Positive control was performed using recombinant CCL20/MIP3α (0.5 μg/ml) and specificity of the migration was confirmed by the addition of a specific anti-CCL20/MIP3α blocking antibody (206D9) or a control isotype (10 μg/ml). Negative controls were performed using CLL20/MIP3α negative BCC effusion fluids.

Immunohistochemistry

Frozen tissues. Frozen 6 μm tissue sections from BCC primary tumors were fixed in acetone and then in 4% paraformaldehyde before the immunostaining. To block the nonspecific activities, sections were pretreated with avidin D and biotin solutions (Blocking kit; Vector Laboratories, AbCys Paris, France) for 10 min for each step and with 0.3% hydrogen peroxide (Sigma Chemical Co.) for 15 min at room temperature. After a brief washing in PBS, the sections were incubated with blocking serum (2% normal rabbit serum) for at least 30 min before adding both primary antibodies. Sections were immunostained between 2 of the following antibodies: anti-CCL20/MIP-3αgoat polyclonal antibody (IgG; R&D Systems), anti-Cytokeratin (IgG1, MNF116; Dako) and anti-CD1a (IgG2a, Leu-6; Becton Dickinson) mouse MAbs for 1 hr at room temperature in a wet atmosphere. The binding of goat IgG was detected by a biotinylated rabbit anti-goat IgG followed by streptavidin-peroxidase, both included in the Vectastain ABC kit (Goat IgG PK-4005; Vector Laboratories). The binding of mouse IgG1 or IgG2a was revealed by a rabbit alkaline phosphatase labeled anti-mouse IgG (DO-314, Dako) at room temperature in a wet atmosphere. The peroxidase and alkaline phosphatase activities were revealed using 3-amino-9-ethylcarbazole (AEC) substrate (SK-4200; Vector Laboratories) or diamino benzidine (DAB) substrate (Dako) and alkaline phosphatase substrate III (SK-5300; Vector Laboratories) for 1–10 min at room temperature, respectively. Negative controls were established by adding nonspecific isotype controls as primary antibody.

Paraffin embedded tissues. Four micrometer paraffin embedded primary breast tumors sections were used for the analysis. After deparaffinization and rehydratation, endogenous peroxidases were blocked by incubating the slides in 5% hydrogen peroxide in sterile water. Tissue sections were boiled in 10 nM citrate buffer pH 6 using a microwave for 15 min for heat-induced antigen retrieval. Nonspecific binding was blocked with a protein blocking reagent (Coulter-Immunotech) for 5 to 15 min.

The slides were then incubated for 1 hr with the primary antibodies (CD1a: mouse clone 10 (Coulter Immunotech); Langerin: mouse clone 310F7 (50 μg/ml) (Schering Plough)]. After rinsing in PBS, the slides were incubated with a biotinylated secondary antibody bound to streptavidin peroxydase conjugate (Ultratech HRP DAB kit, Coulter Immunotech). Bound antibodies were revealed by adding the substrate DAB and sections were couterstained with hematoxylin, dehydrated, and mounted. For the negative controls slides, the primary antibody was replaced by a nonimmune serum.

Statistical analysis

CCL20/MIP3α and IFNγ levels were compared using the Mann & Whitney U test according to the procedures of the SPSS 10.0 package. Lymphocyte numbers after MLR were compared using the procedure of the SPSS 10.0 package.

RESULTS

Production of CCL20/MIP3α in serum and metastatic effusions

$CD1a^+$ DC (Fig. 1*a*) and $Langerin^+$ LC populations (Fig. 1*b*) were detectable *in situ* within the tumor bed on paraffin embedded sections of primary breast carcinomas as previously described.[7] Staining performed on cryopreserved tissue sections from 3 primary breast carcinomas demonstrated the presence of CCL20/MIP3α in $cytokeratin^+$ cells (Fig. 1*c*). $CD1a^+$ DC could be detected in close contact with these CCL20/MIP3α-producing tumor cells (Fig. 1*d*).

CCL20/MIP3α levels were then measured in pleural/ascitic effusions and in sera of metastatic breast cancer patients ($n=25$) and compared to metastatic breast cancer patients without pleural nor peritoneal involvement ($n=55$), to patients with other metastatic tumors with ($n=6$) or without ($n=39$) peritoneal involvement, as well as to nontumoral (cirrhotic) ascitis ($n=6$) (Fig. 2*a*). CCL20/MIP3α levels in effusion fluids were significantly higher in BCC patients (14/25 with detectable levels in BCC patients *vs.* 1/6 in other tumors and 1/6 in nontumoral ascitis, $p=0.03$). The mean CCL20/MIP3α effusion levels were 0.69 ng/ml, 0.07 ng/ml and 0.08 ng/ml in these 3 cohorts respectively. Serum CCL20/MIP3α levels were also higher in patients with metastatic breast carcinoma as compared to other metastatic tumors (renal cell carcinoma) (mean 0.46 ng/ml *vs.* 0.13 ng/ml, $p=0.006$). In agreement with these observations, BCC cell lines produced detectable levels of CCL20/MIP3α spontaneously as well as under IL-1β stimulation (0.2 and 2.1 ng/ml, $<$ 0.2 and 0.6 ng/ml and 0.3 and 1.9 ng/ml for T47-D, CLB-SAV and MCF-7, respectively).

Biological activity of CCL20/MIP3α from effusion fluids was then analyzed in a chemotactic assay on day 9 $CD34^+$-DC expressing high CCR6 levels[12] as well as on mature DC. As shown in Figure 2*b*, CCL20/MIP3α containing effusion fluids (effusion 1:12.3 ng/ml; effusion 2:5.1 ng/ml) were selectively capable to attract day 9 $CD34^+$-DC in contrast to negative effusion fluids and this migration was specifically blocked by an anti-CCL20/MIP3α antibody. In contrast, mature DC did not migrate in response to these effusion fluids (data not shown).

These results suggest that CCL20/MIP3α produced within the BCC tumors may attract or trap immature DC or their precursors.

Breast carcinoma promotes the differentiation of DC precursors towards the LC pathway

Given the capacity of CCL20/MIP3α to attract subpopulations expressing CCR6 including immature DC as well as DC precursors, the capacity of BCC to modulate the differentiation of these precursors into DC and their maturation state was then analyzed. Irradiated BCC cell lines and BCC CM were found to significantly increase CD1a Ag expression on differentiating day 6 DC precursors (Fig. 3*a*). A simultaneous decrease of day 6 $CD14^+$ precursor subpopulation was also observed (Fig. 3*a*). Interestingly, the populations generated in the presence of BCC CM expressed CCR6 (Fig. 4). Importantly, addition of BCC CM all along the culture

FIGURE 1 – Detection of CCL20/MIP3α and DC subsets in primary breast carcinoma tumors and role of BCC CM on $CD34^+$-DC differentiation. (*a*,*b*) DC are detected *in situ* in primary breast carcinoma on paraffin embedded tissue sections using anti-CD1a (*a*) and Langerin (DCGM4) (*b*) antibodies. (*c–d*) CCL20/MIP3α expression was detected on tumoral cells on primary breast carcinoma frozen sections. Double staining with anti-CCL20/MIP3α polyclonal antibody (red) and anti-cytokeratin (blue) shows that CCL20/MIP3α is produced by tumoral cells (*c*). Moreover, double staining performed with an anti-CCL20/MIP3α polyclonal antibody (red) and anti-CD1a (blue) reveals that $CD1a^+$ DC colocalize with CCL20/MIP3α production within the tumoral area (*d*). (*e–g*) In the presence of BCC CM (*f*), $CD34^+$ progenitors differentiate into DC, which are morphologically distinct from those obtained in cytokines alone (*e*), with large aggregates and long, fine-shaped cells, as those generated in the presence of $rhTGF\beta_1$ (10 ng/ml) (*g*).

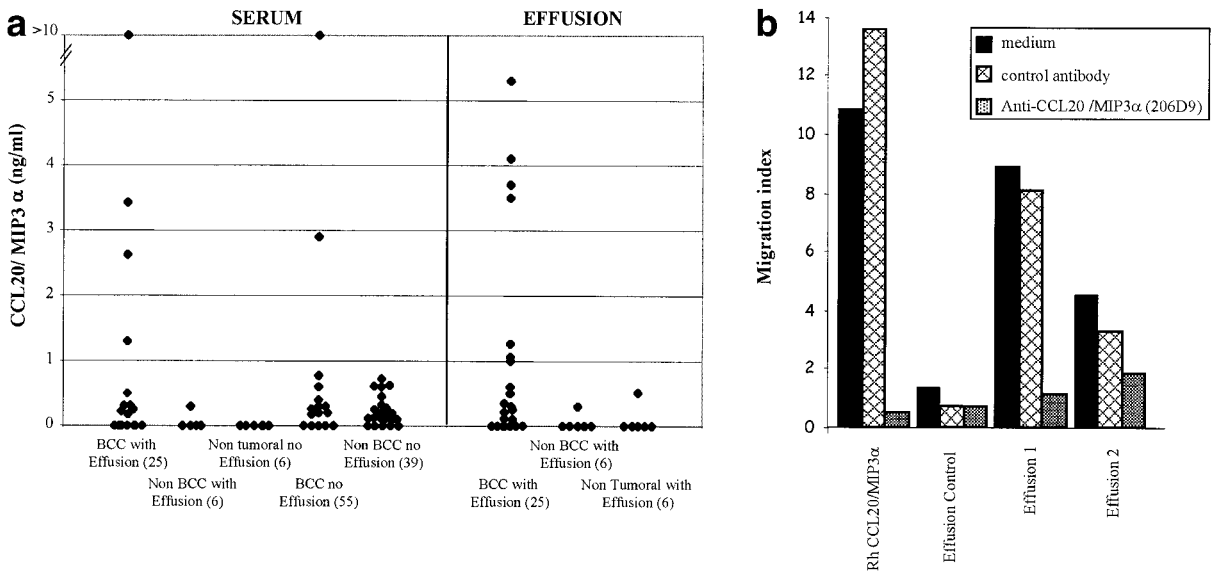

FIGURE 2 – Detection of biologically active CCL20/MIP3α in patients with metastatic breast carcinoma. (a) CCL20/MIP3α levels were analyzed by a specific double sandwich ELISA [capture antibody: 319F6 (3 μg/ml), revelation antibody: 206D9 (1/3,000)] on sera and effusion fluids from patients suffering from metastatic BCC with (25) or without effusion (55) and were found significantly higher than those observed in other tumors with (6) or without effusion component (39) or in patients with (6) or without (6) nontumoral effusion. (*b*) Functional activity of effusion fluids CCL20/MIP3α content was analyzed by chemotactic assay. Migration assays were carried out using 5 mm inserts with day 9 $CD34^+$ DC. After 1 hr preincubation at 37°C, 5×10^5 cells were placed for 1 hr in 5 μm pore size inserts. Effusion fluids from BCC patients were diluted (1/3) and placed in the lower chamber. Revelation of migrated DC was performed with CD1a-FITC/CD14-PE double staining. Rh CCL20/MIP3α (0.5 μg/ml) was used as positive control and specificity of the migration was confirmed by the addition of a specific anti-CCL20/MIP3α blocking antibody (206D9) or a control isotype (10 μg/ml). Negative controls were performed using CLL20/MIP3α negative BCC effusion fluids.

(Fig. 3) also increased CD1a and langerin expression on DC at day 12. Furthermore, the LC differentiation was also observed when BCC CM was present only during the differentiation period (day 6 to day 12) (23.3% $Langerin^+$ in medium condition *vs.* 54.1% $Langerin^+$ in BCC CM condition). DC cultured in BCC CM appeared either as large aggregates or as long, fine-shaped, loosely adherent cells (Fig. 1*f*), in contrast to those generated in medium, which appeared isolated or as small aggregates (Fig. 1*e*).

These results indicate that BCC cell lines produced soluble factors capable to favor the differentiation of DC progenitors towards the $CD1a^+$ LC pathway.

Role of TGFβ in the modulation of the differentiation of LC by BCC

Human $TGF\beta_1$ is known to play a key role in the generation of LC from both $CD34^+$ and monocyte progenitors.[13,14] DC generated in the presence of 10 ng/ml $rhTGF\beta_1$ were morphologically and phenotypically similar to that observed with BCC CM regarding loosely adherent cells morphology (Fig. 1*f*,*g*) and CD1a (Fig. 4) and Langerin Ag expression (data not shown).

The capacity of a polyclonal pan-TGFβ antibody to block the biological effect of BCC CM was therefore investigated. Whereas addition of control polyclonal antibody did not modify Langerin expression (Fig. 5), repeated additions of polyclonal pan-TGFβ antibody completely blocked the differentiation of progenitors into $Langerin^+$ cells (Fig. 5) induced by BCC CM. We demonstrate that BCC cell lines produced detectable levels of $TGF\beta_1$ (35 to 90 pg/ml/10^6 cells/48 hr) and $TGF\beta_2$ (265 to 430 pg/ml/10^6 cells/48 hr). This observation suggests that BCC CM-induced LC differentiation, from $CD34^+$ progenitors, is operated by TGFβ.

BCC CM promote the differentiation of an immature DC subpopulation

At day 12, 2 different subpopulations of DC could be distinguished after culture with BCC CM (Fig. 4): the first subpopulation expressed high levels of CD40 and HLA-DR and expressed CD86, while the second subpopulation expressed higher levels of CD1a but lower levels of CD40 and HLA-DR, with no detectable expression of the CD86 Ag (Fig. 4). In the presence of BCC CM, the $CD1a^{high}CD86^-$ subpopulation represented 26.4% of the whole DC population as compared to 7.7% for cells cultured without BCC CM. This population was also observed, but at lower level (12%), in the presence of $rhTGF\beta_1$ (Fig. 4). Using triple labeling, the $CD1a^{high}CD86^-$ DC subpopulation was found different from the $Langerin^+$ subpopulation (Fig. 6). Incubation in the presence of pan-TGFβ antibody reduced by 50% (46 to 54%) but did not completely block the emergence of this $CD1a^{high}CD86^-Langerin^-$ subpopulation (Fig. 7), suggesting that its development was only in part TGFβ dependent.

APC function of DC subpopulations generated in the presence of BCC CM

DC cultured with and without BCC CM, or with $rhTGF\beta_1$ in bulk condition, promoted similar levels of proliferation of naive $CD45R_A^+$ T cells in a MLR experiment (Fig. 8*a*). However, experiments performed with sorted $CD1a^+CD86^+$ and $CD1a^{high}CD86^-$ subpopulations emphasized some differences. Whereas $CD1a^+CD86^+$ DC selected from BCC CM condition, induced a 30% increased ability to stimulate naive T lymphocytes proliferation compared to those obtained with medium condition, $CD1a^{high}CD86^-$ DC subpopulation displayed a 20 to 30% decreased ability to stimulate T-cell proliferation (Fig. 9*a*).

With the sorted $CD1a^{high}CD86^-$ DC subpopulation obtained in the presence of BCC CM but not with the $CD1a^+CD86^+$ DC subpopulation, only 50% of viable T lymphocytes were recovered compared to medium condition, with significant reductions of both $CD4^+$ and $CD8^+$ subpopulations including their $CD25^+$ compartments (Fig. 9*b*) ($p<0.01$). Comparable results were also observed in bulk condition in spite of the similar thymidine incorporation

levels observed with medium and BCC CM culture conditions (Fig. 8*b*).

Moreover, DC cultured with BCC CM or with rhTGFβ_1 triggered lower levels of IFNγ production during the MLR, as compared to DC cultured with medium (280 pg/ml for rhTGFβ_1, 250 pg/ml for CLB-SAV compared to 500 pg/ml for medium condition, $p<0.05$), whereas neither IL-10 nor IL-12p70 production could be detected before or after culture with T cells (data not shown).

Maturation of DC generated in presence of BCC CM

While both LPS or sCD40L induced DC maturation in medium condition as demonstrated by increased DC-Lamp and CD40 expression as well as decreased membrane Langerin expression (Table I), these agents were unable to induce full maturation of DC generated in the presence of BCC CM. Indeed, most of the CD1a^{high}CD86^{-} cells failed to mature *in vitro*: the number of CD1a^{high}CD86^{-} cells remains unchanged after activation (18.8–20% of CD1a^{high}CD86^{-} after maturation *vs*. 25.5% before) and only low levels of DC-Lamp Ag expression (low MFI) were observed after exposure to LPS or CD40L. Moreover, Langerin expression was lower but still detectable on cell membrane (Table I). Of note, the presence of BCC CM during the maturation period further reduced their phenotypic maturation (data not shown).

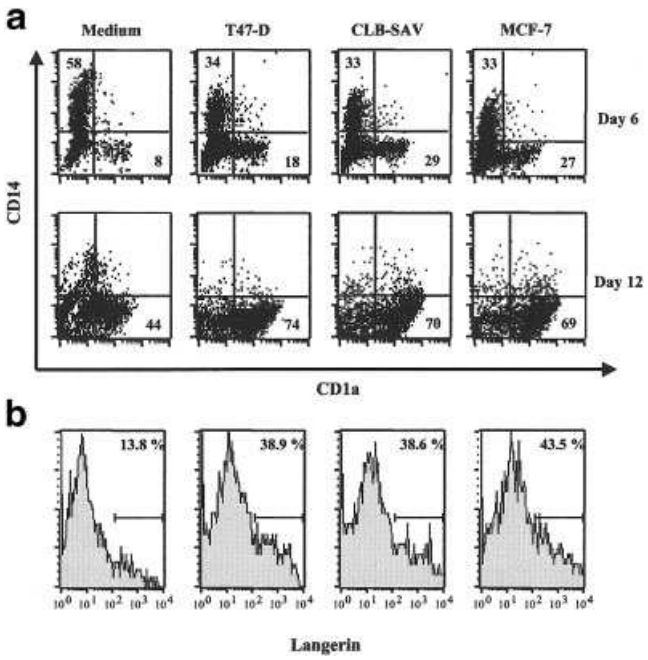

FIGURE 3 – Breast carcinoma cells promote Langerin^{+} DC differentiation from CD34^{+} progenitors. CD34^{+} progenitors were cultured for 12 days, either in the presence of cytokines alone (medium) or BCC CM (20%) (T47-D, CLB-SAV, MCF-7). (*a*) At day 6 and day 12, cells were recovered and stained with anti-CD14-PE and anti-CD1a-FITC antibodies to determine their phenotype. The fluorescence was analyzed on a FACScan. (*b*) Surface Langerin expression was analyzed with Langerin(DCGM4)-PE antibody. These results are representative of 10 experiments.

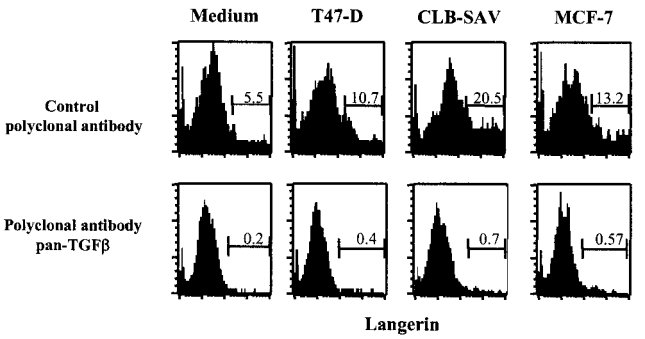

FIGURE 5 – Anti-TGFβ polyclonal antibody inhibits Langerin^{+} DC differentiation induced by breast carcinoma cells. A polyclonal anti-TGFβ antibody (10 μg/ml) or a rabbit control antibody (10 μg/ml) was added from day 6 to day 12 to cultures of CD34^{+} progenitors in the presence of BCC CM (20%) or cytokines alone (medium) to assess the role of TGFβ in the emergence of Langerin^{+} DC. At day 12, cells were collected and stained with Langerin (DCGM4)-PE antibody. The % of Langerin^{+} cells was analyzed on a flow cytometer (5,000 events). These results are representative of 3 independent experiments.

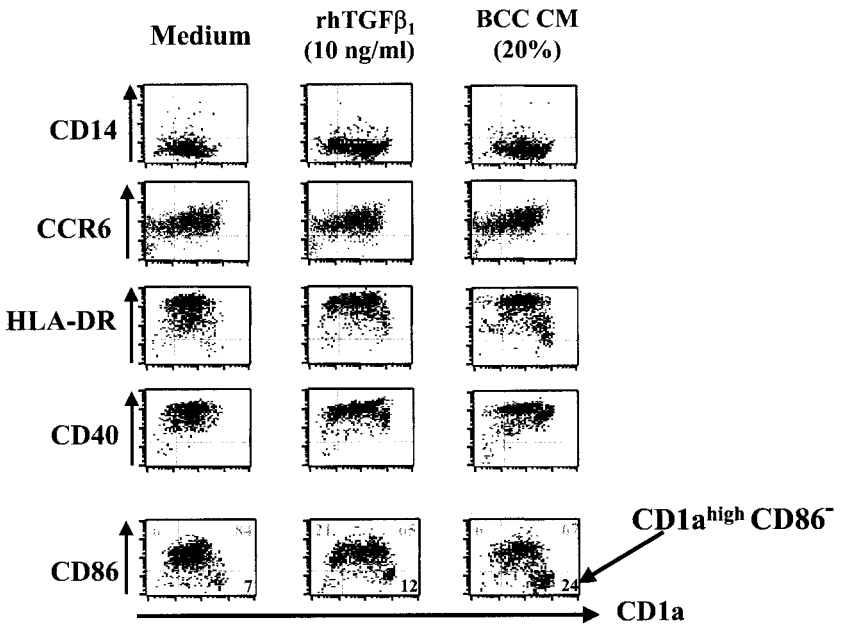

FIGURE 4 – Breast carcinoma cells promote the differentiation of 2 distinct DC subpopulations. BCC CM (20%) (results shown for CLB-SAV) was added to CD34^{+}-DC progenitors culture during all the culture period and was compared to those generated in cytokines alone (medium) or rhTGFβ_1 (10 ng/ml). At day 12, cells were collected and a double staining was performed to analyze the expression of HLA-DR (PE), CD40 (PE), CCR6 (PE) and CD86 (PE) on CD1a^{+} DC (FITC). Five thousand events were analyzed by flow cytometry and results are representative of 10 independent experiments.

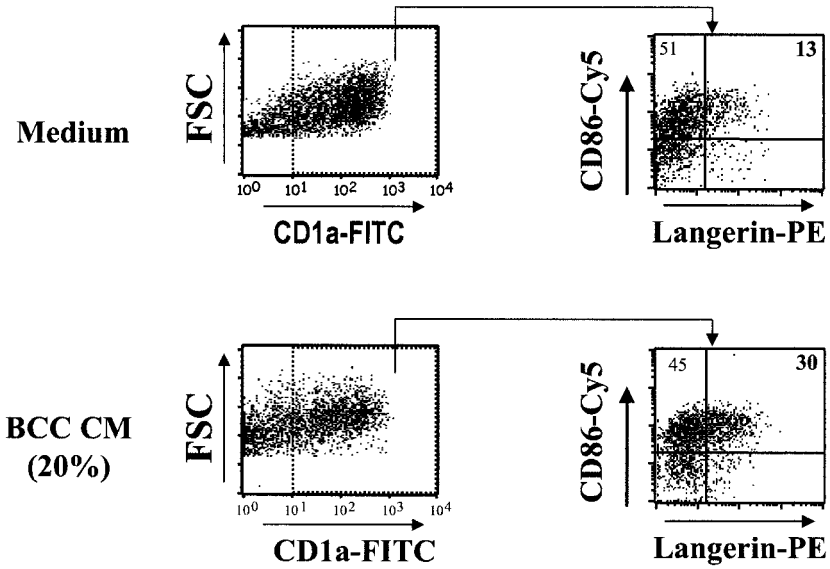

FIGURE 6 – CD1a^{high}CD86^{-} and CD1a^{+} Langerin^{+} DC subpopulations are distinct. To assess that Langerin^{+} and CD86^{-} DC are different subpopulations, a triple staining was performed using fluorochrome-labeled antibodies. Analysis of Langerin (DCGM4)-PE and CD86-Cy5 expression was performed on the gated CD1a^{+} population (CD1a-FITC). This experiment is representative of 3 experiments.

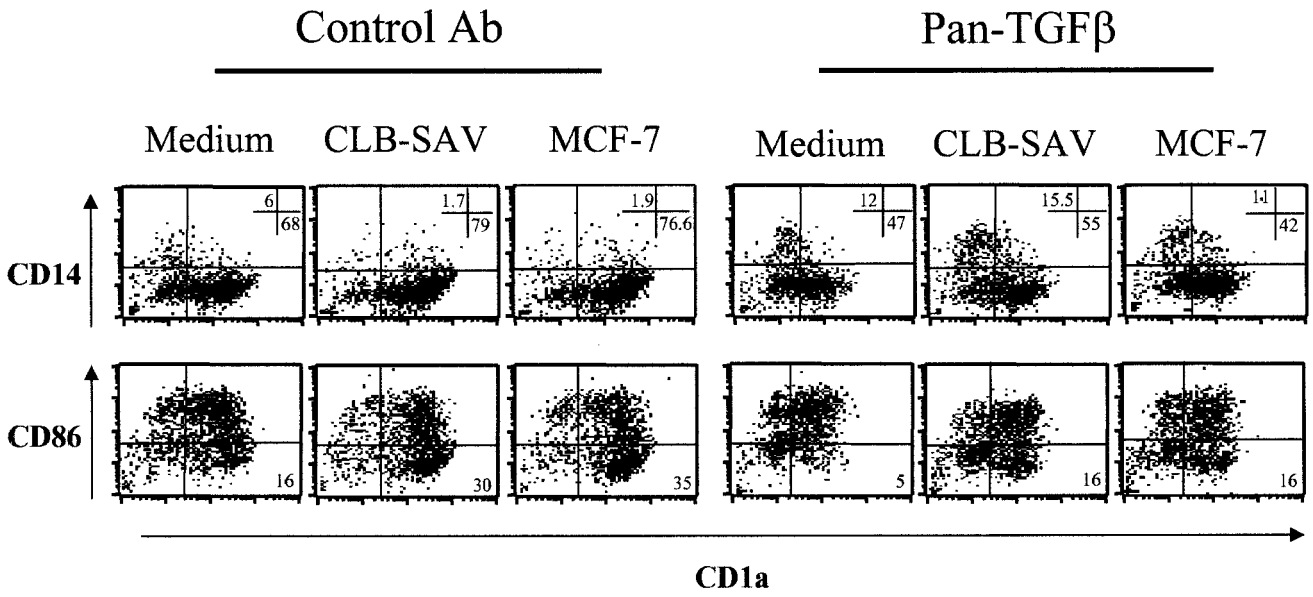

FIGURE 7 – Breast carcinoma cell-induced CD1a^{high}CD86^{-} DC subpopulation is partly TGFβ-dependent. Polyclonal anti-TGFβ antibody (10 μg/ml) or rabbit control antibody (10 μg/ml) was added to CD34^{+}-DC cultures generated in the presence of cytokines (medium) or 20% BCC CM. At day 12, cells were collected and double staining were performed (CD1a-FITC/CD14-PE, CD1a-FITC/CD86-PE). Fluorescence of 5,000 events was analyzed on a FACScan and results presented as dot plots. Percentages of the different populations are added on the dot plots. This experiment is representative of 3 experiments.

Activity of the effusion fluids of metastatic breast carcinoma patients on DC differentiation

To assess the clinical relevance of these *in vitro* observations, the effects of BCC-containing effusion fluids obtained from 3 metastatic breast cancer patients on DC phenotype were also evaluated. Interestingly, when CD34^{+} progenitors were cultured in the presence of effusion fluids (10% final concentration) and compared to culture with normal human serum (medium) (Fig. 10), we observed 1) the increase of the CD1a^{+}Langerin^{+} LC subpopulation (22% in medium condition *vs.* 54% in the presence of effusion fluids) and 2) the increase of the CD1a^{high}CD86^{-} immature subpopulation (31.7% of whole CD1a^{+} DC in medium condition *vs.* 44.4% of whole CD1a^{+} DC in the presence of effusion fluids). Effusion fluids were therefore capable to induce the differentiation of the 2 subpopulations as described above indicating the relevance of these observations *in vivo*.

DISCUSSION

The role of the immune system in the control of breast carcinoma progression remains largely unknown. BCC incidence does not augment or only minimally in immunocompromized patients[15] and immunostimulating therapies have no established activity in this tumor.[16] However, the presence of immature DC and infiltrating T cells within primary breast carcinoma has been recently reported, suggesting that breast carcinoma may attract or trap immunocompetent cells.[7,17,18] Although the clinical relevance of these observations remains largely unknown, it is clear that im-

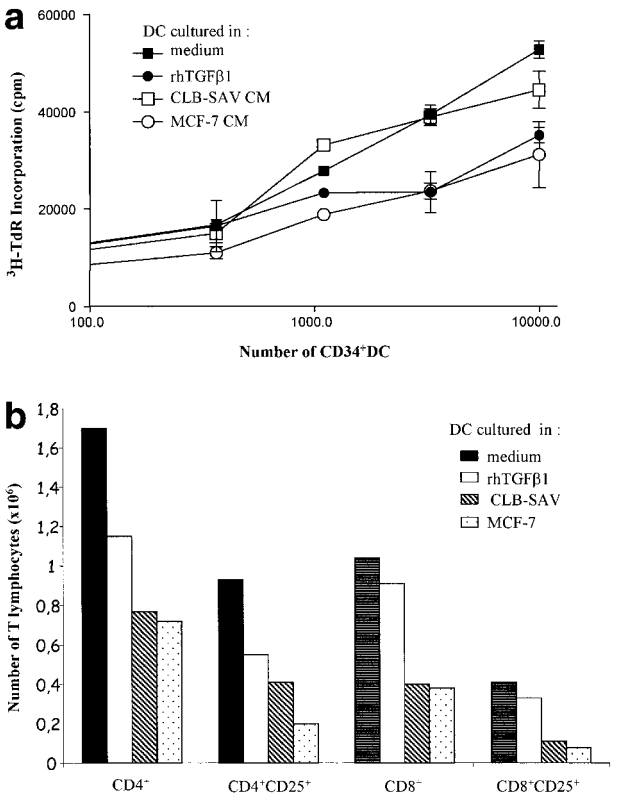

FIGURE 8 – Altered T-cell number and phenotype induced by DC cultured with breast carcinoma cells conditioned media. $CD34^+$ progenitors were differentiated into DC in the presence of cytokines alone (medium) (closed square) or supplemented with $rhTGF\beta_1$ (10 ng/ml) (closed circle) or 20% CLB-SAV CM (open square) or MCF-7 CM (open circle). At day 12, cells were collected and their capacity to induce the proliferation of naive T lymphocytes was tested in MLR. Briefly, 10 to 10^4 DC previously irradiated (30 Gray) were cultured in triplicate in round-bottomed tissue culture plates in the presence of 2×10^4 $CD45R_A^+$ T lymphocytes under 200 μl complete medium. (*a*) After 5 days, T-cell proliferation was evaluated by ^{3}HTdR uptake and presented as mean ± SD. (*b*) After the MLR period, T lymphocytes were collected from each culture (medium, ■; $rhTGF\beta_1$, ▨; CLB-SAV, ▩; MCF-7, ▭) counted and double staining were performed with CD4-FITC or CD8-FITC and CD25-PE to analyze the activation stage of the T cells recovered. Phenotype was analyzed by flow cytometry on 5,000 events. Results are expressed as number of each T-cell population obtained and are representative of 3 experiments.

mune cells infiltrating the tumors are not capable to prevent breast carcinoma cell growth *in vivo*.

In vivo, tumor cells could affect immune response using different mechanisms: loss of tumor Ag or expression of Ag variants, production of immunomodulatory cytokines or inhibitors, protection against the lytic activity of immune effectors (for review, see reference 1) or inhibition of DC differentiation and functions through VEGF-, IL-6-, and/or MCSF-dependent mechanisms.[2,19–21]

In our study, 2 distinct phenomenon were identified, which could explain the presence of immature DC in primary breast tumors: 1) tumor cells produced CCL20/MIP3α allowing them to attract $CCR6^+$ DC precursors and 2) production of soluble factors promoting DC differentiation yet altering DC maturation and function.

We demonstrated by immunohistochemistry the presence of CCL20/MIP3α in the tumor cell area in primary breast carcinomas, which suggests that CCL20/MIP3α is produced at the tumor site, in agreement with previous data reported by Bell *et al.*[7] Consistent with these observations, detectable levels of biologically active CCL20/MIP3α were observed in a large proportion of BCC effusion fluids compared to effusion fluids from other neoplastic and nonneoplastic processes. *In vitro*, CCL20/MIP3α was also secreted by all the BCC cell lines spontaneously or upon inflammatory stimulation (IL-1β). CCL20/MIP3α is one of the chemokines produced at the site of inflammation, involved in the migration of immature DC necessary for the initiation of immune reaction.[22,23] This overproduction of CCL20/MIP3α *in vivo* and *in vitro* by tumor cells strongly suggests that this chemokine is a major contributing factor for the accumulation of immature DC and DC precursors, expressing CCR6,[12,24] in tumor tissues. Inadequate CCL20/MIP3α secretion in ascitis, pleural effusion or serum could alter the function of the immune system by affecting the normal migration of immature circulating DC toward the inflammation place. The loss of CCL20/MIP3α gradient *in vivo* could induce an inadequate routing of immune cells, in particular DC and effector T lymphocytes[25,26] and therefore may hamper the induction of a normal immune response. The contribution of this phenomenon to the immune escape of breast carcinomas is under investigation.

Breast carcinomas were found not only to produce chemoattractants for DC and their precursors but also to modulate DC phenotype and function. In contrast to RCC,[2] BCC as well as their conditioned media do not block DC differentiation but instead promote their differentiation towards 2 distinct DC subpopulations with specific phenotypes and functions: LC ($CD1a^+CD40^{high}HLA^-DR^{high}Langerin^+CD86^+$) and a distinct immature DC subpopulation with a $CD1a^{high}CD86^-$ $HLA\text{-}DR^{low}CD40^{low}Langerin^-$ phenotype.

BCC CM promoted a commitment of $CD34^+$ into LC precursors of the $CD14^-CD1a^+$ phenotype[9] as early as day 6. At day 12, this LC population ($Langerin^+$) further increased when DC progenitors were continuously cultured with BCC CM. These results are consistent with *in vivo* observations in metastatic breast cancer patients: 1) ascitis or pleural metastatic fluids obtained from metastatic breast cancer patients were found capable to induce the generation of this population *in vitro* (our study) and 2) $Langerin^+$ DC are detectable by immunohistochemistry in situ within tumoral area in primary BCC tumor specimens (our study and reference 7). Taken together, these results show that BCC produce soluble factors promoting the differentiation of DC progenitors into LC, both *in vitro* and *ex vivo*.

In vivo, LC represent a specific DC subpopulation that resides in epithelia where they have a role of sentinel of the immune system. This function is associated with an immature stage with high processing efficiency of native Ag and a poor capacity to present Ag and then activate T cells (for review, see reference 27). In a recent study on 256 primary breast cancers, LC were found localized within the tumor area and distant from the T-cell infiltrates.[8]

The second subpopulation of DC induced by BCC CM expressed at day 12 an immature phenotype with high expression of CD1a Ag, low levels of CD40 and HLA-DR and the absence of CD86 Ag expression. As we demonstrated by triple labeling this population is distinct from the LC population described above. This $CD1a^{high}CD86^-CD40^{low}HLA^-DR^{low}$ DC was found to arise from the $CD14^+CD1a^-$ progenitor subpopulation at day 6 (not shown).

Importantly, these 2 subpopulations were unable to fully mature even in the presence of sCD40L or LPS for 48 hr as shown by their low upregulation of DC-Lamp and CD40 expression. This was associated with an only slight decrease of the immature $CD1a^{high}CD86^-$ subpopulation and the persistent membrane expression of Langerin, previously described to be downregulated during maturation process in the LC subpopulation.[28] The presence of BCC CM during the maturation period amplified this phenomenon. These *in vitro* results suggest that BCC tumors are capable to impair the capacity of DC to mature in response to appropriate signals.

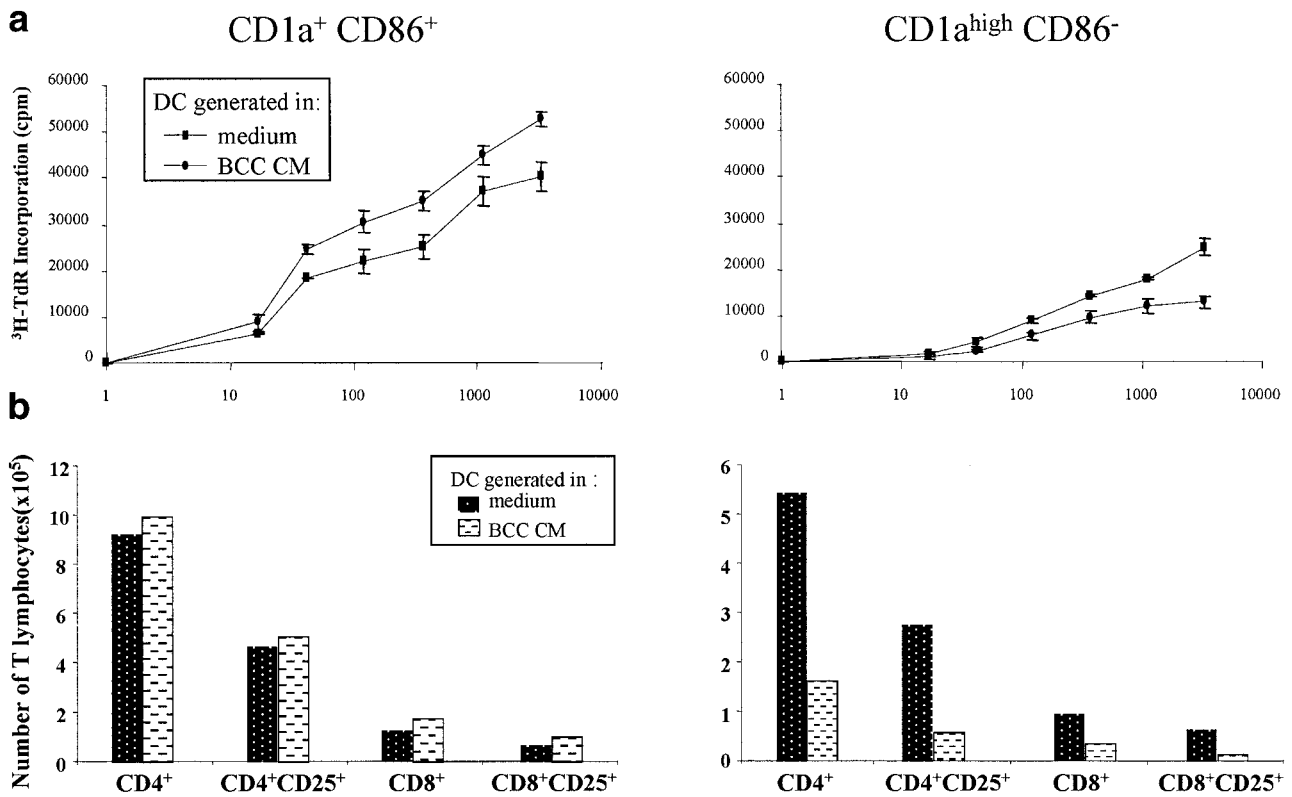

FIGURE 9 – APC capacities of CD1a^{high}CD86$^-$ and CD1a$^+$CD86$^+$ sorted populations. CD34$^+$ progenitors were differentiated into DC in the presence of cytokines alone (medium) or 20% BCC CM. At day 12, cells were collected, sorted according to their phenotype CD1a^{high}CD86$^-$ and CD1a$^+$CD86$^+$ and their capacity to induce the proliferation of naive T lymphocytes was tested in MLR. (*a*) After 5 days, T-cell proliferation was evaluated by ^{3}HTdR uptake and presented as mean ± SD [medium (closed square), BCC CM (closed circle)]. (*a*) After the MLR period, T lymphocytes were collected from each culture (medium, ■; MCF-7, ▨) counted and double staining were performed with CD4-FITC or CD8-FITC and CD25-PE to analyze the activation status of the T cells recovered. Phenotype was analyzed by flow cytometry on 5,000 events. Results are expressed as number of each T-cell population obtained and are representative of 2 experiments.

TABLE I – BCC CM GENERATED DC HAVE IMPAIRED CAPACITIES TO MATURATE

	Medium			BCC CM		
	Day 12	LPS	CD40L	Day 12	LPS	CD40L
CD1a^{high} CD86$^-$	10.8	6.4	3.2	25.5	20.4	18.8
Langerin$^+$	21.6 (1198)	14	9.3	44 (2192)	24.8	17.6
CD40$^+$	88.3 (348)	83.8 (1007)	81.5 (1259)	82.3 (236)	85.6 (765)	82.6 (982)
DC-Lamp$^+$	58 (1055)	96 (1900)	94 (2996)	36 (835)	75.9 (1049)	79.3 (1346)

[1]Percentage of positive cells (MFI of positive cells).

In our report, we showed that DC cultured in BCC CM were still capable in bulk condition to promote naive T-lymphocyte proliferation in an MLR. However, modulations in their APC capacity could be observed on day 12 sorted subpopulations: whereas sorted CD1a$^+$CD86$^+$Lang$^+$ subpopulation presented an increased capacity to induce T-cell proliferation, the sorted CD1a^{high}CD86$^-$ subpopulation displayed a reduced capacity to stimulate T-cell proliferation.

In addition, the nonsorted BCC CM-DC yielded a significantly decreased absolute T-cell number, with reduced CD4$^+$ as well as CD8$^+$ populations (58 to 63% reduction, respectively, compared to medium DC condition). Interestingly, a reduction of CD25$^+$ expressing T cells was also observed both among the CD8$^+$ (75% reduction) and the CD4$^+$ (55% reduction) subsets. Finally, the resulting T-cell population produced lower levels of IFNγ. This reduction in T-cell total number was also obtained when sorted CD1a^{high}CD86$^-$ subpopulation was used in the MLR experiments, whereas no modulation was observed with the CD1a$^+$CD86$^+$-Lang$^+$ subpopulation, strongly suggesting that this effect resulted from an incomplete costimulation pathway inducing the anergy of T cells. However, we cannot rule out other hypotheses: 1) different proliferation kinetics in subtypes of T lymphocytes in response to BCC CM-treated DC or 2) an increased CD25$^+$ T cells apoptosis during this culture period. Although both hypotheses remain to be explored, the present results show that the expanded T-cell populations obtained upon culture with BCC-cultured DC have an altered phenotype and function.

The lack of costimulatory molecules expression in tumor-associated DC and a phenotype of immature nonactivated DC are associated with *in vivo* defective functions of tumor DC.[17,29–31] In BCC patients, Gabrilovich *et al.*[32] have demonstrated an increased percentage of circulating immature DC (lower levels of HLA-DR and costimulatory molecules) with a lower capacity to stimulate Ag-specific T-cell response. Enk *et al.*[33] demonstrated, in patients with metastatic melanoma, the presence of CD86$^-$ DC in progressive lesions in contrast to CD86$^+$ DC in regressive ones, suggest-

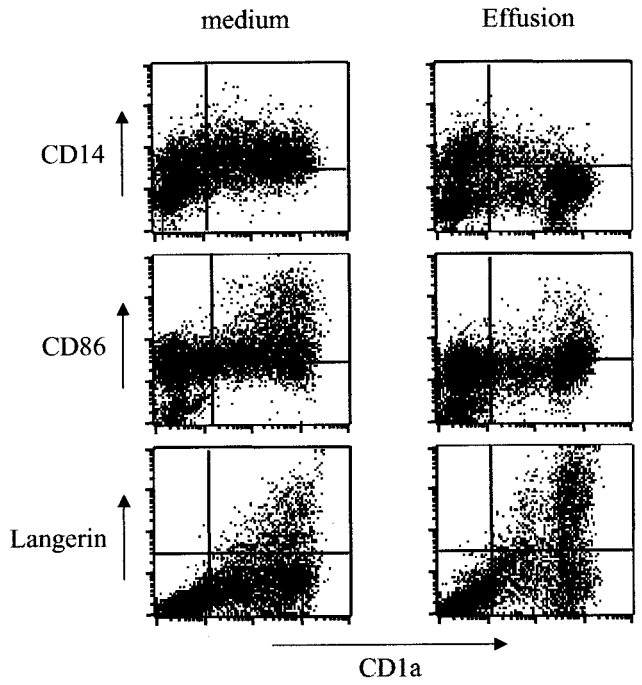

FIGURE 10 – Effusion fluids from metastatic breast carcinoma promote the differentiation of the 2 distinct DC subpopulations. $CD34^+$ progenitors were cultured either in cytokines alone (medium) supplemented with 10% human serum or in the presence of 10% effusion fluids from 3 patients suffering from metastatic breast carcinoma from day 0 to day 10. Next, cells were washed and cultured in fresh medium supplemented with cytokines for 2 additional days. At day 12, cells were recovered and double stained to analyze the different subpopulations (CD1a-FITC coupled with CD14-PE, CD86-PE or Langerin-PE). Fluorescence was analyzed by flow cytometry on 5,000 events. Results are representative of 4 independent experiments.

ing the role of maturation state of DC in the evolution of the disease. Almand *et al.*[34] observed that this immature DC population was strongly reduced after the tumor mass resection, supporting the hypothesis that soluble factors produced by tumor cells were involved in the impaired capacity of peripheral blood DC to mature. Moreover, in several epithelial tumors (colon carcinoma, RCC and BCC), immature DC expressing intracellular class II MHC Ag[7] or low levels of costimulatory molecules[30] have been described in the tumor bed, whereas mature DC could also be observed in the peritumoral area within lymphoid infiltrates,[4,7,18] suggesting that tumors are able to sequestrate the immature DC generated.

The present results showed that TGFβ is the major soluble factor contributing to the promotion of the $CD1a^+Langerin^+$ subpopulation, and an important factor that promotes the emergence of the immature $CD1a^{high}HLA\text{-}DR^{low}CD40^{low}CD86^-$ DC population. Polyclonal neutralizing pan-TGFβ antibody, which antagonizes all the TGFβ isoforms (isoforms 1 to 5), completely inhibited the effects of BCC CM on LC generation. The relatively low levels of $TGF\beta_1$ and $TGF\beta_2$ detected in BCC CM suggest however that TGFβ might also be produced during the DC differentiation process under the influence of other soluble factors present in BCC CM. Rh TGFβ was capable to mimic most phenotypic and functional modulation of LC induced by BCC CM, in particular regarding IFNγ secretion and reduction of the number of $CD4^+$ and $CD4^+CD25^+$ lymphocytes at the end of MLR. It must be noted however that rhTGFβ had no effect on the resulting number of $CD8^+$ and $CD8^+CD25^+$ lymphocyte subsets at the end of the MLR, suggesting that BCC produce additional soluble factors affecting the function of non LC immature DC, in particular their capacity to promote the expansion of these lymphocyte subsets.

The involvement of TGFβ in our observations is consistent with the reported role of $TGF\beta_1$ in the generation of LC from $CD34^+$ or peripheral blood monocyte precursors in human and mice.[35–39] In contrast to its key role in LC differentiation, the results presented here showed that $TGF\beta_1$ was not the sole soluble factor responsible for the differentiation of the $CD1a^{high}CD40^{low}HLA\text{-}DR^{low}$ $CD86^-$ immature DC population.

Some factors have been characterized in the literature to interfere with the ability of DC to mature; Ishida *et al.*[40] demonstrated, in a murine model of MethA induced sarcoma, a reduction of the APC capacity of DC, which was associated with the presence of VEGF. However, BCC CM contain only low levels of VEGF,[3] which do not account for the appearance of this $CD1a^{high}CD86^-$ subpopulation. IL-10 has also been characterized to downregulate the APC function of DC by decreasing their expression of CD80 and CD86,[41,42] blocking their maturation process and favoring the generation of tolerogenic DC,[43] which could induce the generation of T_{reg} lymphocytes capable to block conventional $CD4^+$ helper activation.[44] However, whereas IL-10 production has been detected in some cases of BCC tumors by immunohistochemistry and PCR on cryo-preserved BCC primary tumors specimens,[17] the absence of IL-10 production by the tumor cell lines used in our study, as assessed by a specific ELISA (data not shown), ruled out a direct effect of IL-10 in our observations.

In conclusion, the results presented here suggest that breast carcinoma tumor cells through CCL20/MIP3α production recruit DC or DC precursors and promote, in part through a TGFβ−dependent mechanism, their differentiation into LC ($CD1a^+Langerin^+CD86^+$) as well as into a more immature DC subpopulation ($CD1a^{high}Langerin^-CD86^-$) with altered maturation and activation capacities towards T lymphocytes.

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