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 biogel chromatography 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⁻) phenotype were observed. These 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 mature 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.

Hematopoietic growth factors

Recombinant human (rh) granulocyte/macrophage colony-stimulating factor (GM-CSF) (specific activity: 2 x 10⁶ U/mg; Schering Plough Research Institute, Kenilworth, NJ) was used at 100 ng/ml (200 U/ml); interleukin-4 (IL-4) (specific activity: 10⁸ U/mg, Schering Plough Research institute, Kenilworth, NJ) at 10 ng/ml. rh Tumor Necrosis Factor (TNFα) (specific activity: 5 x 10⁶ U/mg, Cetus, Amsterdam, Netherlands) at 2.5 ng/ml (50 U/ml); rh Stem cell Factor (SCF) (specific activity: 4 x 10⁶ U/mg; R&D System, Abingdon, UK) at 25 ng/ml; rh Transforming growth factor (rhTGFβ1) (R&D Systems) at 10 ng/ml; and rhIL-1β (R&D System) 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 x 10⁵ cells/ml in RPMI-1640 or DMEM medium supplemented with 2 mM glutamine, 200 IU/ml penicillin, 200 μg/ml streptomycin (Gibco

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 immunomodulatory 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 (PBMs) triggering the differentiation of DC progenitors towards monocyte/macrophage population exhibiting a poor antigen presenting cell (APC) capacity. In vivo, RCC are rarely infiltrated by DC, while the production of IL-6 has been correlated to a poor response to immunotherapy in vivo.

In contrast, primary breast cell carcinoma (BCC) tumors 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. 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⁺ CD86⁺, CD1a⁺ Langerin⁻ CD86⁻).

**METHODS**

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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/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 Ulis, France). Lymphocytes were further purified on a multistep Percoll gradient (Amersham Biosciences, Saclay, France) as previously described and total lymphocyte population was recovered in the pellet (less than 3% expressed CD14). Naïve T lymphocytes (CD45RA−, CD62L−) were purified from the total lymphocyte population by immunomagnetic depletion with a cocktail of MAbs. After 2 rounds of depletion with beads the purity of CD45RA−, CD62L− was routinely higher than 95%. The T-lymphocyte population was 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 MAbs (Immu 133.3, Immunotech, Marseille, France) and goat anti-mouse IgG-coated microbeads (Miltenyi Biotec, GmbH) 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. 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^+CD86^− and CD1a^+CD86^+ using a FACSComp instrument (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.

**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) (TGFβ1, 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 centrifuged 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. 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 206DY (1/3,000) antibody coupled to peroxidase 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 × 10³ cells/well as described. 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 plated at 1 × 10⁶ cells/1 ml at 37°C and 5 × 10⁵ cells were plated 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 by CD1a/CD14 double staining. Positive control was performed using recombinant CCL20/MIP3α by CD1a/CD14 double staining. Positive control was performed using recombinant CCL20/MIP3α by CD1a/CD14 double staining. Positive control was performed using recombinant CCL20/MIP3α by CD1a/CD14 double staining.

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-3α goat polyclonal antibody (IgG; R&D Systems), anti-Cytokeratin (IgG1, MNF116; Dako) and anti-CD1a (IgG2a, Leu-6; Becton Dickinson) mouse MAb's 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 goat polyclonal antibody (red) and anti-CD1a (blue) reveals 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 CCL20/MIP3α negative BCC effusion fluids.

Results

CCL20/MIP3α and IFNγ levels were measured 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.

Production of CCL20/MIP3α in serum and metastatic effusions

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

CCL20/MIP3α levels were then measured in pleural/asitic effusions and in sera of metastatic breast cancer patients (n = 25) and compared to metastatic breast cancer patients without pleural or peritoneal involvement (n = 55), to patients with other metastatic tumors with (n = 6) or without (n = 39) peritoneal involvement, as well as to non-tumoral (cirrhotic) ascities (n = 6) (Fig. 2a). 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 non tumoral ascities, 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 as well as on mature DC. As shown in Figure 2b, 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).

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 peroxidase conjugate (UltraStar HRP DAB kit, Coulter Immunotech). Bound antibodies were revealed by adding the substrate DAB and sections were counterstained 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.
guished after culture with BCC CM (Fig. 4): the first subpopula-
BCC CM promote the differentiation of an immature DC
that BCC cell lines produced detectable levels of TGF
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H9252
LC from both CD34
H11001
entiation, from CD34
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expression (Fig. 5), repeated additions of polyclonal pan-TGF
added (1/3) and placed in the lower chamber. Revelation of migrated DC was performed with CD1a-FITC/CD14-PE double staining. Rh
cells (Fig. 5) induced by BCC CM. We demonstrate
in BCC CM condition). DC cultured in BCC CM
appeared either as large aggregates or as long, fine-shaped, loosely
adherent cells (Fig. 1f), in contrast to those generated in medium,
which appeared isolated or as small aggregates (Fig. 1e).
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 TGFβ1 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 rTGFβ1 were morphologically and phenotypically similar to that observed with BCC CM regarding loosely adherent cells morphology (Fig. 1f,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 TGFβ1 (35 to 90 pg/ml/10^6 cells/48 hr) and TGFβ2 (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 distinguis-
hed after culture with BCC CM (Fig. 4): the first subpopula-
tion 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 rTGFβ1 (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 rTGFβ1 in bulk condition, promoted similar levels of proliferation of naive CD45RA+ T cells in a MLR experiment (Fig. 8a). However, experiments performed with sorted CD1a^{high}CD86+ and CD1a^{low}CD86− subpopulations emphasized some differences. Whereas CD1a^{high}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^{low}CD86− DC subpopulation displayed a 20 to 30% decreased ability to stimulate T-cell proliferation (Fig. 9a).

With the sorted CD1a^{low}CD86− DC subpopulation obtained in the presence of BCC CM but not with the CD1a^{high}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. 9b) (p<0.01). Comparable results were also observed in bulk condition in spite of the similar thymidine incorporation

**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.

(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. 1f), in contrast to those generated in medium, which appeared isolated or as small aggregates (Fig. 1e).

This observation suggests that BCC CM-induced 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. 1f), in contrast to those generated in medium, which appeared isolated or as small aggregates (Fig. 1e).

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

Moreover, DC cultured with BCC CM or with rhTGFβ1 triggered lower levels of IFNγ production during the MLR, as compared to DC cultured with medium (280 pg/ml for rhTGFβ1, 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 1), these agents were unable to induce full maturation of DC generated in the presence of BCC CM. Indeed, most of the CD1a<sup>high</sup>CD86<sup>−</sup> cells failed to mature in vitro: the number of CD1a<sup>high</sup>CD86<sup>−</sup> cells remains unchanged after activation (18.8–20% of CD1a<sup>high</sup>CD86<sup>−</sup> cells 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<sup>+</sup> DC differentiation from CD34<sup>+</sup> progenitors. CD34<sup>+</sup> 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 FACSscan. (b) Surface Langerin expression was analyzed with Langerin(DCGM4)-PE antibody. These results are representative of 10 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<sup>+</sup>-DC progenitors culture during all the culture period and was compared to those generated in cytokines alone (medium) or rhTGFβ1 (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<sup>+</sup> DC (FITC). Five thousand events were analyzed by flow cytometry and results are representative of 10 independent experiments.

**FIGURE 5** – Anti-TGFβ polyclonal antibody inhibits Langerin<sup>+</sup> 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<sup>+</sup> progenitors in the presence of BCC CM (20%) or cytokines alone (medium) to assess the role of TGFβ in the emergence of Langerin<sup>+</sup> DC. At day 12, cells were collected and stained with Langerin (DCGM4)-PE antibody. The % of Langerin<sup>+</sup> cells was analyzed on a flow cytometer (5,000 events). These results are representative of 3 independent 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+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 and immunostimulating therapies have no established activity in this tumor. 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. Although the clinical relevance of these observations remains largely unknown, it is clear that im-

FIGURE 6 – CD1a+CD86+ DC 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+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 FACSscan and results presented as dot plots. Percentages of the different populations are added on the dot plots. This experiment is representative of 3 experiments.

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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 and immunostimulating therapies have no established activity in this tumor. 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. Although the clinical relevance of these observations remains largely unknown, it is clear that im-
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 loss of tumor Ag or expression of Ag variants, associated with an only slight decrease of the immature phenotype9 as early as day 6. At day 12, this LC population (Langerin+™) further increased when DC progenitors were continuously cultured with BC 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 could induce an inadequate routing of immune cells, in particular DC and effector T lymphocytes25,26 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+™CD40hiHLAGDRloLangerin±CD86+) and a distinct immature DC subpopulation with a CD1ahiCD86+HLA-DRloCD40hiLangerin™ phenotype.

BCCM promoted a commitment of CD34+™ into LC precursors of the CD14+™CD1a+™ phenotype as early as day 6. At day 12, this LC population (Langerin+™) further increased when DC progenitors were continuously cultured with BC 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 could induce an inadequate routing of immune cells, in particular DC and effector T lymphocytes25,26 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.

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 BC CM promoted a commitment of CD34+™ into LC precursors of the CD14+™CD1a+™ phenotype as early as day 12 and at day 12 an immature phenotype with high expression of CD1a Ag, low levels of CD40 and HLA-DR and the absence of 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

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+™CD86+ population and the persistent membrane expression of Langerin, previously described to be downregulated during maturation process in the LC subpopulation.28 The presence of BC 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.
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<sup>+</sup>CD86<sup>+</sup>/Langerin<sup>+</sup> subpopulation presented an increased capacity to induce T-cell proliferation, the sorted CD1a<sup>high</sup>CD86<sup>+</sup> 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<sup>+</sup> as well as CD8<sup>+</sup> populations (58 to 63% reduction, respectively, compared to medium DC condition). Interestingly, a reduction of CD25<sup>+</sup> expressing T cells was also observed both among the CD8<sup>+</sup> (75% reduction) and the CD4<sup>+</sup> (55% reduction) subsets. Finally, the resulting T-cell population produced lower levels of IFN<sub>γ</sub>. This reduction in T-cell total number was also obtained when sorted CD1a<sup>high</sup>CD86<sup>+</sup>-Lang<sup>+</sup> subpopulation was used in the MLR experiments, whereas no modulation was observed with the CD1a<sup>+</sup>CD86<sup>+</sup>-Lang<sup>+</sup> 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<sup>+</sup> 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. In BCC patients, Gabrilovich et al. 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. demonstrated, in patients with metastatic melanoma, the presence of CD86<sup>−</sup> DC in progressive lesions in contrast to CD86<sup>+</sup> DC in regressive ones, suggest-
population, and an important factor that promotes the emergence of the immature CD1a^{low}HLA-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 TGFβ1, TGFβ2, and TGFβ3, 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 TGFβ 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 TGFβ1 was not the sole soluble factor responsible for the differentiation of the CD1a^{low}CD40^{low}HLA-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. 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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