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**Early detection of tumor cells by innate immune cells leads to Treg recruitment through CCL22 production by tumor cells**

**Running title:** innate immune recognition trigger CCL22 by breast tumor cells

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Abstract:
In breast carcinomas, patient survival appears to be negatively impacted by the recruitment of regulatory T cells (Treg) within lymphoid aggregates by CCL22. However, the mechanisms underpinning this process, which may be of broader significance in solid tumors have yet to be described.

In this study, we determined how CCL22 production is controlled in tumor cells. In human breast carcinoma cell lines, CCL22 was secreted at low basal levels that were strongly increased in response to inflammatory signals (TNFα, IFNγ, IL-1β), contrasting with CCL17. Primary breast tumors and CD45+ infiltrating immune cells appeared to cooperate in driving CCL22 secretion, as demonstrated clearly in co-cultures of breast tumor cell lines and peripheral blood mononuclear cells (PBMC) or their supernatants. We determined that monocyte-derived IL-1β and TNFα are key players as monocyte depletion or neutralization of these cytokines attenuated secretion of CCL22. However, when purified monocytes were used, exogenous human IFNγ was also required to generate this response suggesting a role for IFNγ-producing cells within PBMC. In this setting, we found that human IFNγ could be replaced by the addition of (i) IL-2 or K562-activated NK cells or (ii) resting NK cells in the presence of anti-MHC class I antibody.

Taken together, our results demonstrate a dialogue between NK and tumor cells leading to IFNγ secretion, which in turn associates with monocyte-derived IL-1β and TNFα to drive production of CCL22 by tumor cells and subsequent recruitment of Treg. As one validation of this conclusion in primary breast tumors, we demonstrated that NK and macrophages tend to colocalize within tumors. In summary, our findings suggest that at early times during tumorigenesis the detection of tumor cells by innate effectors (monocytes, NK) imposes a selection for CCL22 secretion that recruit Treg to evade this early anti-tumor immune response.
Introduction

Cancer immunosubversion is a process by which tumor cells escape destruction by the immune system through a variety of mechanisms including the production of immunosuppressive cytokines and the alteration of dendritic cells (DC) functions (1, 2). Several studies have shown that immune cells are present and functional in solid tumors and may promote both humoral and cellular anti-tumor immune responses. As an example, high levels of CD8⁺ T cells within the tumors have been associated with a better clinical prognosis in colorectal cancer (3). However, in most of the cases these T cells are unable to counteract tumor progression. In cancer patients, increased levels of CD4⁺CD25highFOXP3⁺ regulatory T cells (Treg), a lymphocyte subset with immuno-suppressive properties, are described in the peripheral blood, the primary tumor microenvironment and in the draining lymph nodes, supporting a role for Treg in cancer-induced immuno-suppression. However their effect on tumor progression varies according to the tumor type in humans. Treg have a negative impact on survival in lung, pancreatic, gastric, liver or ovarian carcinoma patients (4-7) whereas they may exert a beneficial role in B cell lymphoma, head and neck or colon carcinoma (8, 9) or have no impact in colon, prostate, renal or anal squamous cell carcinoma (10, 11) (for review (12)).

We recently obtained evidences, in breast carcinoma, that selectively activated Treg accumulation within lymphoid aggregates, but not in the tumor bed, has a negative impact on patients’ survival (13). Elucidating the mechanisms involved in Treg trafficking and accumulation in the breast tumor environment is thereby critical for innovative therapeutic development to fight tumor induced immuno-suppression.

Experiments in mice using Treg from CCR4⁻/⁻ or conditional CCR4 knockout in FOXP3⁺ Treg compartment have recently identified the critical role of CCR4 in Treg trafficking in secondary lymphoid organs or tissues (14, 15). Curiel’s group strongly suggested a role for CCR4/CCL22 axis in Treg recruitment in ovarian ascitis (4).

We recently demonstrated the selective loss of membrane CCR4 on tumor-associated Treg (TA-Treg) consecutive to an active recruitment through CCL22 and that breast tumors lacking CCL22 are not colonized by Treg independently of their CCL17 expression status (13) strongly suggesting the importance of CCL22 in TA-Treg recruitment within breast tumors.

CCL22, a CC chemokine produced by myeloid DC (mDC), B cells, macrophages, keratinocytes or epithelial cells (6, 16, 17) and CCL17 closely related to CCL22, produced by monocyte derived DC (MoDC) (18) and keratinocytes (19), are two ligands for CCR4 (6, 20) preferentially expressed on Th2 lymphocytes(21) and Treg (for review (12)). In PBMC,
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CCL22 is upregulated by IL-4 whereas it is downregulated by IFNγ treatment (22). In contrast, IFNγ favored CCL22 secretion by keratinocytes (16, 23) and intestinal epithelial cells (24).

In the present study we demonstrated that breast tumor cells recognition by NK cells leads to their activation and IFNγ secretion that in turn triggers CCL22 production by tumor cells through cooperation with monocyte-derived IL-1β and TNFα.

Material and methods

Breast tumor cell lines culture: All tumor cell lines used in this study originated from ATCC except CLB-SAV generated in the laboratory. Cell lines were cultured in RPMI 1640 (invitrogen) completed with 10% foetal bovine serum (FBS) (Lonza, Vervier, Belgium), 100U/ml penicillin and 100µg/ml streptomycin (invitrogen)(complete medium) at 37°C in a 5% CO2 incubator.

Primary breast tumors: Breast tumor tissues collected at the Centre Léon Bérard after patient informed consent were mechanically dilacerated to obtain “mechanic tumor disaggregation supernatants” and then subjected to enzymatic digestion as previously described(13). Flow cytometry analyses (ADP Cyan, Beckman Coulter) were performed to assess the percentage of NK cells (CD3−NKp46+) and macrophages (CD4+CD68+CD163+) (all from Becton Dickinson except for CD163 from eBiosciences and CD68 from Dako Cytomation) within primary tumor cell suspension after gating on CD45+ cells and data were analyzed with FlowJo analysis software (Tree Star).

Immune cells (CD45+) or NK cells (CD3−CD56+) and macrophages (CD4lowCD163+) were purified on single cell suspension from breast primary tumor or ascitis respectively by cell sorting (FACS Aria, Becton Dickinson).

Breast tumor supernatants (TumSN): Single-cell suspensions from primary or metastatic (ascitis, pleural effusion) breast tumors were incubated at a final concentration of 1x5 10^6 cells/ml in complete medium in petri dishes. Culture supernatants were collected after 48h, filtrated on 0.22 µm, and frozen.

Immunohistochemistry (IHC): Expression of CCL22 on paraffin-embedded sections of breast tumor or peri-tumoral tissue was analyzed with a goat anti-CCL22 antibody (Ab) (Santa Cruz)) as previously described (13). Routinely used CD163 (mIgG1, Menarini Diagnostics) staining was performed according to the manufacturer. NKp46+ were detected as previously described (25) cells with a goat IgG Ab (R&D Systems). Hematoxylin counterstained sections
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were dehydrated, and mounted. For negative controls slides, primary antibodies were replaced by a non immune serum.

**Cytokines and antibodies:** Recombinant human rhGM-CSF (specific activity: \(2 \times 10^6\) U/mg, used at 100 ng/ml) and rhIL-4 (specific activity: \(10^6\) U/mg, used at 50 ng/ml) were from Schering Plough Research institute (Kenilworth, NJ, USA). rhTNF\(\alpha\) (specific activity: \(5 \times 10^6\) U/mg) at 10 ng/ml was provided by Cetus Corporation (Amsterdam, Netherlands). rhIL-1\(\beta\) (\(10^9\) UI/mg), rhIFN\(\gamma\) (2\( \times 10^7\) UI/mg) and IL-1RA were from Peprotech (Neuilly/Seine, France). rhCCL22, rhCCL17, rhCXCL12 and monoclonal Ab (mAb) against CXCL12 and CCL22 as well as isotype controls used for neutralisation experiments were from R&D Systems (Lille, France).

**Purification of cell subsets from peripheral blood:** Total peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from healthy volunteers by Ficoll Hypaque density gradient centrifugation (Dominique Dutscher, Brumath, France). Purified myeloid DC (mDC) and monocyte fractions, were obtained using positive selection kits whereas untouched NK cells were purified using negative selection kit (Miltenyi Biotech) and purity was confirmed by flow cytometry. For depletions experiments, different cell subsets (myeloid cells, mDC, plasmacytoid DC (pDC), monocytes, NK cells and T cells) were specifically depleted from PBMC using positive selection kits with magnetic beads. The absence of remaining positive cells in the depleted fraction was confirmed by magnetic beads. The absence of remaining positive cells in the depleted fraction was confirmed by flow cytometry.

**Culture conditions:** Tumor cell lines were cultured at \(2 \times 10^5\) cells/ml in complete medium in 48 well plates (Becton Dickinson) and incubated for 24h or 48h in medium condition or in the presence of rhIFN\(\gamma\) (0.1 to100ng/ml depending on the studies).

Coculture experiments were performed by incubating \(10^5\) tumor cells with \(10^6\) PBMC for 24 or 48h in the presence of 100ng/ml rhGM-CSF with or without rhIFN\(\gamma\).

To characterize the cell subset responsible for CCL22 secretion, PBMC (PBMC SN) and tumor cells (Tumor SN) supernatants were generated by 24h incubation of either \(10^6\) PBMC or \(10^5\) tumor cells in 48 well plates (500\(\mu\)l).

**Cytokine detection:** CCL22, CCL17 and IL-1\(\beta\), TNF\(\alpha\) levels were quantified in cell supernatants using ELISA from R&D Systems and Bender MedSystems respectively.

**Migration in response to CCL22:** CCR4 expression on CCRF-CEM cell line was confirmed by flow cytometry (Figure S1). Migration assays were performed using transwell (6.5 mm diameter; COSTAR) with 5\( \times 10^5\) cells/well. After 2 hours pre-incubation at 37\(^\circ\)C, CCRF-CEM cells were placed in 3\(\mu\)m pore size inserts (100\(\mu\)l) and tested for their ability to migrate in
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response to rhCCL22 (1 to 50ng/ml) or culture supernatants (50%) added in the lower well. After 1h30 incubation at 37°C, cells were collected in cold PBS-EDTA and resuspended after centrifugation in 100µl. The number of migrated cells was analyzed by flow cytometry. In blockade experiments, anti CXCL12 or CCL22 mAb or their isotype controls were incubated for 30min with culture supernatants before CCRF CEM cells were added in the insert.

Results

1- High levels of CCL22 but not CCL17 are detectable in primary breast tumors
Analyses of breast TumSN demonstrated the production of high levels of CCL22 in TumSN from primary tumors (Figure 1A) that decreased in metastatic ones. CCL22 is also detectable in supernatants of mechanic tumor disaggregation from 27 primary tumors (mean = 1.02ng/ml range (0.13ng/ml-6.9ng/ml)). Low levels of CCL17 were detected in these TumSN. Moreover, SN from non tumor tissues (healthy breast tissue and fibro-adenoma) did not produce significant levels of CCL22 or CCL17.

We have previously shown that in breast tumors, expression of CCL22, but not that of CCL17 by tumor cells, correlate with TA-Treg infiltration (13).

In contrast to primary breast tumors where a strong CCL22 expression was observed by IHC (Figure 1C and Gobert 2009), CCL22 displayed a weak apical expression in peri-tumoral area by luminal breast epithelial cells within lobular acini (Figure 1B).

2- Breast tumor cell lines produce functional CCL22 in response to Th1/ innate immunity but not Th2 signals
Contrasting with primary tumors, spontaneous CCL22 secretion by breast tumor cell lines in vitro was low to undetectable (Figure 2A), suggesting mechanisms of regulation and a role of the microenvironment in CCL22 expression by tumor cells. Indeed, addition of rhIFNγ, a CCL22 inducer on keratinocytes (16, 23) and intestinal epithelial cells (24), induced strong CCL22 secretion on 5/7 tested cell lines (0. 28 - 1.1ng/ml for IFNγ) (Figure 2A). In contrast, in PBMC, CCL22 production was down-regulated by rhIFNγ but up-regulated by rhIL-4 (Figure 2B).

When bulk primary breast tumor disaggregation was used, the secretion of CCL22 was lost upon depletion of CD45+ immune cells. This CCL22 production by CD45 negative primary tumor was restored (9 fold increase) either by addition of associated CD45+ infiltrate (3x10⁴ CD45+ for 8x10⁴ tumor cells) or rhIFNγ(Figure 2C) strongly suggesting the cooperation between tumor cells and immune cells for specific CCL22 secretion.
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This observation was confirmed using breast tumor cell lines. The addition of allogeneic PBMC to breast tumor cell lines strongly enhanced the production of CCL22 but not of CCL17. We observed a 12.8, 18 and 121 fold increase for CLB-SAV, MDA-MB453 and MCF-7 cell lines respectively, in co-culture condition when compared to tumor cells alone. This secretion was further enhanced by rhIFN\(\gamma\) addition (17, and 5.5 fold increase CCL22 production for CLB-SAV and MDA-MB453 or MCF-7 respectively) (Figure 2D).

To test the functionality of the CCL22 secreted in [PBMC/Tumor cells] coculture supernatants, we used the CCRF-CEM T cell line that express CCR4 and migrate in response to rhCCL22 in a dose dependent manner (Figure S1A, S1B). The co-culture SN favored the CCRF-CEM cell migration in a transwell assay (five fold increase over background level) that was specifically blocked by pre-incubation of these supernatants with an anti-CCL22 neutralizing mAb but not with an anti-CXCL12 able to attract CXCR4+ CCRF-CEM (Figure S1C).

3- Soluble factors produced by activated PBMC induce CCL22 secretion by tumor cells

Whereas PBMC alone are devoid in IFN\(\gamma\) secretion (Figure S3 lower panel), coculture with tumor cells increased this production favoring CCL22. Furthermore, as previously mentioned, addition of rhIFN\(\gamma\) strongly enhanced CCL22 production in [PBMC /Tumor cells] coculture while decreasing that of PBMC (Figure 2D). We therefore wished to decipher the relative role of PBMC and tumor cells in CCL22 secretion in the [PBMC/tumor cells] coculture in presence of rhIFN\(\gamma\). We compared the impact of rhIFN\(\gamma\)-activated PBMC supernatant [rhIFN\(\gamma\)-PBMC SN] on CCL22 production by tumor cell lines with that of rhIFN\(\gamma\)-activated tumor cells supernatants [rhIFN\(\gamma\)-Tumor cells SN] on PBMC. As shown in Figure 3, CCL22 levels secreted by rhIFN\(\gamma\)-treated tumor cells were strongly enhanced in presence of [rhIFN\(\gamma\)-PBMC SN] (2.75, 19.36 and 16.46 fold respectively for MDA-MB453, MCF-7 and CLB-SAV respectively). In contrast, [rhIFN\(\gamma\)-Tumor cells SN] addition did not affect the low CCL22 levels detected in rhIFN\(\gamma\)–treated PBMC cultures. Altogether those data strongly suggest that rhIFN\(\gamma\) treated PBMC produce soluble factors capable of inducing CCL22 production by tumor cells.

Interestingly the effects observed were specific for CCL22 as CXCL8 that was produced by rhIFN\(\gamma\) activated PBMC was down regulated in the presence of tumor cells and not induced when tumor cells were cultured with PBMC SN (data not shown).
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4- Monocytes and IFNγare both required for the secretion of CCL22 by tumor cells

In order to determine the major cell fraction within PBMC responsible for the effects observed on tumor cells, specific depletions of myeloid cells (CD33+), monocytes (CD14+), mDC (BDCA1+BDCA3+), pDC (BDCA2+), NK cells (CD56+) or T cells (CD3+) were performed using magnetic beads. Each depleted fraction was added on tumor cell lines in the presence of rhIFNγ to assess the CCL22 production. As shown in Figure 4A, whereas addition of PBMC induced a strong CCL22 production (2.95 ± 0.1ng/ml), we observed a drop in this secretion when monocytes (CD14+) or myeloid cells (CD33+)-depleted fractions were used (86% and 75% inhibition respectively). In contrast, the depletion of NK cells or T cells did not decrease the CCL22 secretion. The increase observed with T cells depletion likely results from increased monocytes percentage in the culture. The depletion of mDC reduced the basal level of CCL22 produced by PBMC alone as shown in Figure S2 (26) but did not impact on the CCL22 production by tumor cells. These results suggest that monocytes are the main actors in CCL22 production by tumor cells within the coculture.

To confirm their role, purified monocytes were added to tumor cells in presence or not of rhIFNγ (Figure 4B). Whereas purified monocytes were not able to mimic PBMC action on tumor cells, further addition of rhIFNγ induced CCL22 levels comparable to those obtained with PBMC. This effect was specific to monocytes as mDC, even in presence of IFNγ, did not reconstitute PBMC effect. Of most importance, these results suggest that monocytes act in cooperation with other cell subsets (i.e NK cells, NKT cells or T cells) capable of IFNγ secretion to increase CCL22 secretion by tumor cells.

5- Involvement of rhIL-1β and TNFα in inducing CCL22 secretion by tumor cells

As demonstrated above (Figure 4A), depletion of monocytes strongly reduced the ability of tumor cells to produce CCL22 in coculture. Monocytes are strong producers of IL-1β and TNFα, previously described to cooperate with IFNγ in CCL22 production on epithelial cells or keratinocytes (24, 27, 28). As shown in Figure S3, whereas PBMC produced low IL-1β and TNFα levels (6.5 ± 0.2 pg/ml and 99 ± 1 pg/ml respectively), the addition of rhIFNγ increased their secretion (IL-1β:133 ± 12 pg/ml and TNFα: 879 ± 53 pg/ml). These two cytokines are also detected in [tumor cell lines /PBMC] coculture in presence of rhIFNγ. Moreover the loss of CCL22 production in monocyte-depleted fraction was associated with the absence of IL-1β and TNFα secretion in the coculture (data not shown).
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Whereas CCL22 production by MDA-MB453 cells was mostly dependent on IFN\(\gamma\) (Figure S4A, S4C), the culture of MCF-7 (Figure S4B and S4D) or CLB-SAV (data not shown) with a cross range of recombinant cytokines demonstrated an important impact of low doses of IL-1\(\beta\) (100pg/ml) or TNF\(\alpha\) (10ng/ml) on CCL22 production with an additive effect of IFN\(\gamma\) (Figure S4B and S4D).

To confirm a role for these two cytokines within rhIFN\(\gamma\)-PBMC SN, in CCL22 secretion by tumor cells, we tested the impact of IL-1 receptor antagonist (IL-1RA) or an anti TNF\(\alpha\) blocking antibody, previously validated (Figure S5A), on tumor cell lines cultures. As shown in Figure 4C, treatment with either IL-1RA or anti TNF\(\alpha\) mAb was able to block 40% of CCL22 secretion induced by rhIFN\(\gamma\)-PBMC SN. The simultaneous blockade of IL-1\(\beta\) and TNF\(\alpha\) decreased CCL22 secretion up to 80% demonstrating the role of IL-1\(\beta\) and TNF\(\alpha\) contained in rhIFN\(\gamma\)-PBMC SN on CCL22 secretion by tumor cells.

**6- NK cells and monocytes cooperate to induce CCL22 production by tumor cells**

To better understand the mechanisms involved in IFN\(\gamma\) secretion within [PBMC/tumor cells] coculture (Figure S3) that act in synergy with IL-1\(\beta\) and TNF\(\alpha\), we hypothesized that NK cells could be activated and secrete IFN\(\gamma\) after interaction with tumor cells.

To mimic NK activation, NK cells were pre-treated with IL-2 for 16h. We tested their impact on CCL22 production by tumor cells in presence of purified monocytes or mDC-depleted PBMC fraction. Whereas activated NK cells, mDC-depleted fraction or purified monocytes each alone (Figure 5A and 5B) did not trigger CCL22 production by tumor cells, a combination of activated NK cells with either mDC-depleted fraction (Figure 5A) or purified monocytes (Figure 5B) induced CCL22 levels comparable to those obtained in presence of PBMC or exogenous rhIFN\(\gamma\) (Figure 5A). This suggests that IFN\(\gamma\) released by activated NK cooperate with monocytes to promote CCL22 release by tumor cells.

Interaction of K562 tumor cell line with NK cells also favors their activation (29). The addition of K562 (1:1 K562:NK ratio) to resting NK cells in presence of purified monocytes and tumor cells increased CCL22 secretion by tumor cells that was dependent on IFN\(\gamma\) as anti IFN\(\gamma\)R1 blocking mAb reversed this effect (Figure S6).

As tumor cell lines upregulate MHC class I in response to IFN\(\gamma\) or TNF\(\alpha\) (Figure S7), we neutralized MHC class I expression on tumor cells as an alternative approach to revert blockade of NK activation through MHC class I/KIR (killer inhibitory receptor) interactions. The pre-incubation of tumor cells with blocking anti MHC class I mAb (W6/32) before the
addition of resting NK and monocytes, significantly increased the CCL22 production. This increase was strictly dependent on the presence of NK and monocytes (Figure 6). In these experimental conditions addition of IL-1RA, anti-IFNγR1 and anti TNFα mAb used alone have all moderate to strong effect depending on the cell line, but when combined they completely blocked anti Class I impact, demonstrating the involvement of IFNγ, TNFα and IL-1β (Figure 6).

7- NK cells and macrophages colocalize with tumor cells in situ

As demonstrated in Figure 7A and 7B, NK cells (CD3−NKp46+) as well as macrophages (CD163+CD68+) were detected within the primary tumor cell suspensions by flow cytometry (mean=3.72% (0.15%-8.2%), for NKp46+ and mean=11.7% (0.58%-37.1%) for CD163+). As shown by IHC on paraffin embedded tumor sections NK cells (NKp46+) (Figure 7C-7D), as well as macrophages (CD163+) (Figure 7E, 7F) are localized in the vicinity of tumor cells. Moreover, purified in situ activated ascite derived macrophages are able to cooperate with NK cells to favor a strong CCL22 production by breast tumor cell line (Figure 7G). All together, this suggests the potential recognition of tumor cells by NK cells favoring in combination with macrophages, the initiation of CCL22 secretion by these tumor cells.

DISCUSSION

In the present study, we demonstrated that recognition of transformed mammary epithelial cells favors NK cells activation and subsequent IFNγ secretion associated with the release of monocyte-derived IL-1β and TNFα that triggers CCL22 production by tumor cells. This tumor cell associated CCL22 secretion favors blood CCR4+ Treg recruitment leading to the development of a tolerogenic environment conducive to the tumor immunosubversion and development.

We previously reported a strong correlation between CCL22 expression by tumor cells and the presence of Treg within breast tumor environment. Breast tumors lacking CCL22 are not colonized by Treg independently of their CCL17 expression status (13). Moreover, Treg recruitment in tumor environment induces a loss of CCR4 expression, a phenomenon observed when Treg are cultured in vitro with CCL22 but not with CCL17 (30). Similar observations were made for CCR7 expression that was down regulated on T cells after interaction with CCL19 but not with CCL21 (31, 32). In agreement with our observation, CCR4 and CCL22 requirement for Treg recruitment was also reported in the mouse model of
Inflammatory bowel disease where the inability of CCR4−/− Treg to migrate within the colon tissue leads to disease exacerbation (15).

Our IHC analyses demonstrate, in peri-tumoral breast tissues samples, polarized apical CCL22 secretion by healthy luminal epithelial cells within lobular acini as described for other chemokines (CXCL8, GROβ, GROγ, GROα, ENA78, MIG, IP10, RANTES) detected in the milk or the colostrum (33, 34). Moreover, non hematopoietic cells such as keratinocytes and epithelial cells can secrete CCL22 (16, 24, 35). Polarized CCL22 secretion toward the lumen has also been described in colon epithelium (24). Moreover, the cyclic hormonal modulation may also impact Treg recruitment within the mammary gland via CCL22 secretion by epithelial cells. Indeed, treatment of women with progesterone favors in the endometrium a high CCL22 production by stromal cells and glandular epithelial cells at the end of the hormonal cycle (35). Taken together these results suggest that CCL22 secretion within the breast tissue may be part of the mammary gland physiology controlling the local inflammation associated with tissue remodeling either at the end of menstrual cycle or breastfeeding.

In accordance to the structural disorganization characteristic of primary breast tumor tissue, we observed that CCL22 secretion is no more polarized favoring its diffusion within the tumor environment that may favor recruitment of macrophages, NK, Th2 cells (6) and Treg (for review (12)) expressing CCR4. Moreover CCL22 production is strongly enhanced when compared to healthy tissue. This is consistent with the levels of CCL22 found either in primary breast tumor mechanical disaggregation supernatants (median=−) or in 48h culture primary breast TumSN with more than 40 fold increase in CCL22 levels (median=2.91ng/ml; range: 0.53 - 12.4ng/ml) in comparison with non tumor SN (median=0.07ng/ml; range: 0.03 - 0.23ng/ml p=0.004). It is also important to notice that CCL22 content is tenfold higher than that of CCL17 (median=0.3ng/ml; range: 0 - 4.4ng/ml). Importantly we show using either primary breast tumor or tumor cell lines the cooperation between tumor cells and immune infiltrate to induce high quantities of CCL22 whereas CCL17 secretion remains barely detectable. Interestingly, although healthy bronchial epithelial cells secrete CCL17 (36) their tumor counterpart in lung carcinoma pleural effusion produce CCL22 (37). Taken together, these results suggest the capacity of the tumor environment to modulate the chemokine arsenal of epithelial cells to favor the migration of specific cell subsets. CCL17 via the recruitment of Th2 CCR4+ cells will favor a Th2 response as described in atopic dermatitis (38) whereas CCL22 is more specialized in the recruitment of Treg as observed in tumors (for review (12)).
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IL-4 and IL-13, critically involved in the development of cutaneous pathologies like atopic dermatitis, have been largely shown to induce CCL22 secretion by cells of myeloid origin (monocytes, mDC) (22, 39) and to favor CCL17 production by fibroblasts (40). In contrast, we demonstrated in this study that IL-4 reduces the CCL22 production in breast tumor epithelial cell lines as previously described for immortalized keratinocytes (16, 23), colon epithelial cells (24) and glioma cell lines (41).

In the present study we deciphered the mechanisms involved in the increased secretion of CCL22 within the tumor environment. We demonstrated the existence of a dialogue between tumor cells and circulating immune cells leading to CCL22 production by tumor cells and to Treg recruitment. We reported that breast tumor cell lines produced CCL22 in response to rhIFNγ, as previously described for keratinocytes (16, 23). This secretion is strongly enhanced in coculture with PBMC but is lost after myeloid cells (CD33⁺) or monocytes (CD14⁺) depletion demonstrating the major role of monocytes in this CCL22 secretion although they do not secrete CCL22 by themselves.

NK cells constitute a unique component of the innate immune system able, without specific sensitization, to recognize autologous cells undergoing various forms of stress, such as malignant transformation (42). Target recognition occurs via the integration of negative and positive signals mediated by inhibitory (KIR) or activating (KAR) receptors expressed at the surface of NK. Breast tumor cells expressing ULBP or MICA/MICB markers that bind NKG2D on NK cells will stimulate their IFNγ secretion (Figure S5, and (43, 44). However expression of MHC class I (Figure S7), a KIR ligand, by breast tumor cells reduced this IFNγ secretion. In coculture of breast tumor cell lines with purified monocytes, rhIFNγ could be omitted upon addition of NK cells in conditions leading to their activation, i.e in presence of i) IL2, ii) K562 NK target cell line or iii) anti MHC class I antibody. All these culture conditions lead to IFNγ secretion required for CCL22 production as shown by the use of blocking anti IFNγR antibody.

In this line, MICA (NKG2D-L) expression in breast tumors has been associated with a poor prognosis (43). This could result either from the production of soluble MICA that block the killing function of NK cells or the impact of IFNγ secretion by NK cells on Treg recruitment through CCL22 secretion by tumor cells. In the Lewis Lung carcinoma mouse model, depletion of NK cells blocked CCL22 production in the tumor environment however NK cells were proposed as the major source of CCL22 (45). In contrast, we never detected, in our experimental set-up, CCL22 secretion by resting as well as activated NK cells.
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The replacement of purified monocytes/macrophages by the combination of rhIL-1β and rhTNFα in the culture of breast tumor cells with rhIFNγ or NK cells, strongly enhanced the CCL22 production in agreement with previous publications on keratinocytes and colon epithelial cell lines (16, 24) whereas blockade of IL-1β and TNFα abrogate this secretion in [PBMC/Tumor cells] coculture. Interestingly IFNγ increased IL-1R1 and TNFR on tumor cell lines (data not shown), as previously described (46), suggesting a potential amplification loop of CCL22 production. Taken together, these results suggest the importance of inflammation in the high CCL22 levels in breast tumors environment that will favor Treg recruitment leading to reduced specific antitumor immune response. This is in agreement with studies in colon tissue reporting the involvement of intestinal flora mediated chronic inflammation in the increased recruitment of Treg (for review (47)). This suggests that inflammation in the mammary gland may participate in the tumor development. In favor of this, TNFα secretion by leucocytes infiltrating tumors strongly contributes to mammary carcinogenesis in murine mammary models (48). Importantly the in situ analyses on primary breast tumors allow us to demonstrate the presence of NK cells and macrophages in the vicinity of tumor cells.

Treg have been described to reduce NK cell cytotoxicity (for review (49)) suggesting that CCL22 production by tumor cells inducing Treg recruitment represents one of the mechanisms elaborated by tumors to avoid its destruction through NK cell cytotoxicity. Taken together, our results allow us to propose a model in which mammary epithelial cells transformation processes favored activation of NK cells present in the breast tissue, by reducing KIR and inducing KAR ligands expression and their subsequent IFNγ secretion leading to the production of TNFα and IL-1β by resident monocytes/macrophages. Acting together these three cytokines will favor CCL22 overproduction by tumor cells allowing the recruitment of CCR4+ blood Treg that favor the development of a tolerogenic environment.

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LEGENDS TO FIGURES

Figure 1: CCL22 and CCL17 are differently produced within primary breast tumors and breast tumor supernatants.
A) Quantification of CCL22 and CCL17 levels within healthy or tumor breast tissue supernatants by ELISA; Primary breast tumors (C) as well as peri-tumoral area (B) frozen sections were stained with CCL22 antibody in green (x10).

Figure 2: CCL22 secretion by breast epithelial cell lines after activation.
Breast epithelial cell lines (10^5)(A) or healthy PBMC (10^6)(B) secreted CCL22 after 24h culture in response to rhIL-4 (50ng/ml) or rhIFNγ (100ng/ml). (C) Quantification of CCL22 secreted by primary tumor disaggregation (8x10^5/200µl) and cell-sorted tumor cells (CD45^-)(8x10^4/200µl) with either recombinant cytokines (IFNγ) or CD45^+ immune cells (3x10^4/200µl). (D) Analysis of CCL22 and CCL17 production, by breast epithelial cell lines alone or in coculture with PBMC with or without rhIFNγ(100ng/ml).

Figure 3: Soluble factors secreted by PBMC favor the production of CCL22 by breast tumor cells
Forty eight hours culture period of 10^5 breast tumor cells (with rhIFNγ(100ng/ml)) alone or in co-culture with PBMC (10^6) or 24h PBMC-SN obtained in rhIFNγ(100ng/ml) medium induced the secretion of CCL22 whereas culture of PBMC with 24h breast tumor cells-SN did not induce CCL22 secretion.

Figure 4: Within PBMC, monocytes are essential to favor CCL22 production by breast tumor cells but required the presence of rhIFNγ and CCL22 production in culture of tumor cells with PBMC-SN is dependent on IL-1β and TNFα
(A) 10^5 tumor cells (MCF-7 and CLB-SAV) were cultured in the presence of rhIFNγ for 48h with medium or 10^6 PBMC or fractions depleted in myeloid cells (CD33^-)(10^6), in CD14^- (CD14^-)(9x10^5), in mDC (mDC^-) (10^5), in NK cells (NK^-)(9x10^5) or in T cells (Tcells^-) (5x10^5) for analysis of CCL22 production. (B) CCL22 production after culture of 10^5 breast tumor cells (CLB-SAV, MCF-7) with purified monocytes (CD14^-) (10^5), or mDC (mDC^-) (10^5) for 48h in medium or in the presence of rhIFNγ (100ng/ml). (C) Twenty four hours rhIFNγ pre-activated MCF-7 tumor cells were incubated for 2h with IL-1RA or not. In parallel, supernatant of a 24h PBMC culture in the presence of 100ng/ml rhIFNγ (PBMC-SN) were pre-incubated with 10µg/ml Ctrl mAb or anti TNFα mAb and then added on tumor cells for 24h. CCL22 production was quantified at the end of the culture.

Figure 5: Cooperation of monocytes and activated NK cells to promote CCL22 secretion by tumor cells
(A) mDC depleted fraction (mDC^-) (10^6), IL-2 activated NK cells (IL2-NK) (10^5) or their combination were added on 24h medium pre-treated breast tumor cells (MCF-7, MDA-MB453)(10^5) and CCL22 secretion was analysed after a 48h culture period. The control was performed by addition of mDC depleted fraction (10^6) on rhIFNγ pre-treated tumor cells. (B) Purified monocytes (CD14^-) (10^5), IL-2 activated NK cells (IL2-NK) (10^5) or their combination were added on 24h medium pre-treated breast tumor cells (MCF-7, MDA-MB453)(10^5) and CCL22 secretion was analysed after 48h of culture.
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Figure 6: Blockade of IL-1β, TNFα and IFNγ reverse tumor cells CCL22 secretion induced after culture with purified monocytes and NK cells in the presence of anti MHC class I blocking Ab
24h medium cultured breast tumor cells (MCF-7, MDA-MB453) (10⁵) were treated with anti Class I mAb, Ctrl mAb, anti IFNγR mAb (10µg/ml), IL-1RA (100ng/ml), and their combination for 2h before the addition of purified monocytes (10⁵) and NK cells (5x10⁵) with or without anti TNFα mAb (10µg/ml). CCL22 secretion was analysed after 48h culture period.

Figure 7: NK cells and macrophages are detectable within breast tumors and are functional
(A) NK cells (CD3NKp46⁺) and macrophages (CD68⁺CD163⁺) detection after gating on CD45⁺ cells within primary breast tumor enzymatic disaggregation or in associated peripheral blood. (B) Summary data of NK and macrophages percentages in seven tumor dilacerations by flow cytometry (C-F) localization of NK (NKp46) (C, D) and macrophages (CD163) (E, F) by IHC on paraffin embedded primary breast tumor tissue sections (magnification x 20 (C, E) or x 40 (D, F)). (G) CCL22 production after culture of 10⁵ breast tumor cells (MCF-7) with breast ascite purified macrophages (10⁵) and NK cells (5x10⁵) or their combination for 48h.
Figure 1

A

CCL22 (ng/ml)

Primary | Metastasis | Healthy

Breast Tumor | Mammary Gland

CCL17 (ng/ml)

Primary | Metastasis | Healthy

Breast Tumor | Mammary Gland

B

C

Research.
Figure 3

The figure illustrates the levels of CCL2 (ng/ml) for different cell lines and treatments. The y-axis represents the concentration of CCL2, and the x-axis lists various conditions such as Tumor cells, Tumor cells + PBMC, PBMC SN + Tumor cells, and Tumor Cell SN + PBMC. The bars indicate the mean concentration with error bars representing the standard deviation.
Figure 4

A

B

C

CCL22 (ng/ml)

0 0.5 1 1.5 2 2.5

medium

Ctrl mAb

IL1RA

anti TNFα

IL1RA + Ctrl mAb

IL1RA + anti TNFα

medium

PBMC SN

MCF-7 +

PBMC SN

Tumor cells

mDC -

Tcell -

PBMC

CD33 -

NK -

PBMC

CD14 +

mDC +

medium

IFNγ

MCF-7

CLB-SAV

MCF-7

CLB-SAV

medium

IFNγ

PBMC SN

anti TNFα

IL1RA + Ctrl mAb

IL1RA + anti TNFα

IL1RA
Figure 5

A

- medium
- mDC-
- IL2-NK
- mDC- + IL2-NK
- rhIFNγ
- mDC+ rhIFNγ

B

- medium
- CD14+
- IL2-NK
- CD14+ + IL2-NK
Early detection of tumor cells by innate immune cells leads to Treg recruitment through CCL22 production by tumor cells

Julien Faget, Cathy Biota, Thomas Bachelot, et al.

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