CCL17 and CCL22 determine Th2-dependent M2-macrophage polarization and associated CXCL13 secretion within breast tumor microenvironment
4.1. Introduction

The source of CXCL13 in breast tumor is still not well understood. Earlier literature has suggested that some breast cancer cell lines themselves secrete CXCL13, though the amount of such expression is very low [Panse J et al., 2008]. Immune cells constitute a major proportion of the tumor mass [Balkwill F and Mantovani A, 2001, DeNardo DG et al., 2011]. Therefore, we aimed to identify the immune cell population which may be a chief source of CXCL13 within the primary breast TME. There are few reports stating that human monocytes/macrophages could be a potent inducible source of CXCL13 and, in fact, they appear to be the main producers of this chemokine in inflammatory lesions where lymphoid neogenesis occurs [Carlsen HS et al., 2004; Stables MJ et al., 2011]. Within the breast TME, TAMs are often differentiated to M2 macrophages [Laoui D et al., 2011; Sousa S et al., 2015].

The two main subsets of CD4+ Th cells, Th1 and Th2 cells are the major cytokine producers within the TME. The cytokines that they produce are known as Th1-cytokines and Th2-cytokines, respectively. The balance between Th1 and Th2 cells in a tumor microenvironment (TME) is very important as these cells are the key factors for ensuing pro-inflammatory or anti-inflammatory responses [Ruffell B et al., 2010; Gobert M et al., 2009]. Th1-secreted cytokines induce protective pro-inflammatory responses against cancer [Gu-Trantien C et al., 2013]. IFN-gamma is a key factor in Th1-mediated inflammation [DeNardo DG and Coussens LM, 2007]. Excessive pro-inflammatory responses can lead to uncontrolled tissue damage, so there may be a mechanism which is actually counteracting this. The Th2-type cytokines which include IL-4, IL-5, IL-6, IL10 and IL-13, are associated with anti-inflammatory responses [DeNardo DG and Coussens LM, 2007].

Taken together, Th1 responses are thought to be beneficial towards anti-tumor immunity [Kacha AK et al, 2000; Fallarino F and Gajewski TF, 1999; Lowes MA et al, 1997; Tsung K et al., 1997], whereas, Th2 responses may inhibit the anti-tumor responses [Tsung K et al, 1997; Hu HM et al, 1998; Pellegrini P et al, 1996; Ostrand-Rosenberg S et al, 2000; Kobayashi M et al, 1998] and enhance pro-tumor humoral responses [Tan TT and Coussens LM, 2007; Johansson M et al, 2007]. CD294 is a Th2 cell specific marker, which was reported so far to distinguish between Th1 and Th2 cells [Mantovani A et al, 2007; Boin F et al., 2008].
Unlike Th1 cells, Th2 cells highly express the chemokine receptor CCR4 [Mantovani A et al., 2002]. CCL17 and CCL22 are the two main Th2-attracting chemokines. [Imai T et al., 1999]. Type of macrophage accumulation and/or polarization within the TME is also influenced by the type of cytokines produced by Th cells. Inside the TME, Th1-derived cytokines polarize monocytes and macrophages to M1 type, whereas, Th2-derived cytokines induce M2 polarization [Mantovani A et al., 2007; Mantovani A et al., 2002; Mantovani A et al., 2005; Baj-Krzyworzeka M et al., 2007]. These M2 type macrophages have key roles in subversion of adaptive immunity and they favor neoangiogenesis and tumor progression [Mantovani A et al., 2002]. HLA-DR and CD40 are specific M1 markers whereas, CD163 and CD206 are exclusive markers of M2 macrophages [Ma J et al., 2010; Satoh T et al., 2010].

Increasing reports from the western world have indicated the potential of these Th2-attracting chemokines to become useful prognosis markers for cancer [Mizukami Y et al., 2008; Wågsäter D et al., 2008]. However, we lack comprehensive understanding of these chemokines in breast cancer patients from eastern India. There are hardly any reports which correlate the expressions of Th2 attracting-chemokines (CCL17, CCL22) by the breast tumor and percentages of intra-tumoral M2 macrophage in Indian patients.

In this chapter, we intended for a better understanding of how the expression of Th2-attracting chemokines is associated with intra-tumoral M2 macrophages and CXCL13 expression in patients from eastern India. Further, we looked into potential of cancer cells to induce monocytes to secrete CXCL13 in vitro.

This study is novel because it highlights the prognostic value of intra-tumoral CCL17 and CCL22 expression. CCL17 and/or CCL22 expression was found to be positively associated with intra-tumoral M2 macrophage percentages that in turn correlated with CXCL13 expression within the TME. Moreover, we explored an insight of Th2 independent mechanism of M2 polarization from monocytes.

4.2. Materials and Methods

4.2.1. Clinical Samples

Post-operative primary tumor samples and associated healthy tissues were collected from 98 patients, diagnosed with IDC of breast. Permission was obtained from IEC of SGCC&RI [ECR/250/Inst/WB/2013] and informed consents were taken from patients. Tumors were
graded by RB – protocol [Bloom HJ and Richardson WW, 1957], and staged by UICC-TNM classification [Brierley JD et al, 2016] (Table 2.1 and Table 2.2). Parts of freshly operated tissues were immediately processed for RNA and protein extraction, and rest of tissues was used for flow-cytometry.

4.2.2. Cell culture

Human breast cancer cell lines MDA-MB-231 and T47D, and, human monocyte cell line THP1 were procured from National Centre for Cell Science, India. Cells were cultured in DMEM (Gibco, Invitrogen, CA, US), supplemented with 10% FBS (Gibco, Invitrogen, CA, US), 100 U ml⁻¹ penicillin and streptomycin (Gibco, Invitrogen, CA, US) and maintained in a cell culture incubator at 37°C with 5% CO₂. Conditioned medium of MDA-MB-231 and T47D cell lines were collected from 70-80% confluent cultures of 48 hr. THP1 cells were induced with either MDA-MB-231- or T47D-conditioned medium at 1:1 ratio with fresh medium and incubated for another 48 hr. Control THP1 cells were not treated with any of the conditioned medium of human breast cancer cell lines.

4.2.3. Reverse transcription and qPCR

Total RNA (5 µg) was reverse transcribed into c-DNA, using MMLV-RT (Invitrogen, CA, US), random hexamer (Thermo Scientific) and dNTPs (Promega). Quantitative real-time PCR were performed to analyze mRNA expressions of CXCL13, CCL17, CCL22, CD206, CD163 and CD294 using specific primers. 18s rRNA was used as internal control. Fold changes are represented as relative values normalized to control and quantified in the terms of $2^{-\Delta\Delta C_T}$. Experiments were performed thrice in triplicate.

4.2.4. Flow cytometry

Part of the tumor tissues and associated healthy breast tissues were digested using Collagenase/Hyaluronidase cocktail (Stemcell Technologies) in a water bath over night at 37°C. Single cell suspensions ($1 \times 10^6$) were incubated with fluorochrome-conjugated antibodies: anti-CD163 (Biolegend), anti-CD206 (Biolegend), anti-CD294 (Biolegend), anti-HLA-DR (Biolegend), anti-CD11b (Biolegend), anti-CD68 (Biolegend) and flow cytometry were performed in BD-AccuriC6. Fe blocker (Biolegend) was used to minimize non-specific antibody binding. Experiments were performed with taking appropriate isotype controls. Analyses were done using BD-AccuriC6 software.
4.2.5. ELISA

Quantitative determination of CXCL13 was performed using a commercially available kit (Ray Biotech) for sandwich ELISA that makes use of a capture antibody against human CXCL13 precoated onto a microplate. Human recombinant CXCL13 protein at serial concentrations was used to prepare a standard curve. Level of CXCL13 in THP1 cell lysates was estimated using the standard curve. A detection antibody conjugated with biotin specifically directed against CXCL13 protein was added to the wells. After further washings, HRP-conjugated streptavidin was added followed by addition of tetramethylbenzidine (TMB) substrate reagent. Finally stop solution was added to the wells and absorbance of the colored produced was measured using an ELISA reader (Multiskan™ GO, Thermo Scientific) at 450 nm. The sensitivity threshold of the test was 1.5 pg/ml.

4.2.6. Statistical analyses

Association between gene expression and clinicopathological characteristics was analyzed by Fisher’s exact test. One-way analysis of variance (ANOVA) (Bonferroni correction) was performed to assess the level of significance among paired data sets. Statistical analyses were performed using SPSS Statistics 17.0 and OriginPro8. All data are presented as mean ± SD and $p$ value of ≤ 0.05 was considered statistically significant.

4.3 Results

4.3.1. Percentage of M2 macrophage in the primary breast tumor is significantly correlated with intra-tumoral CXCL13 expression

Initially, we have analyzed intra-tumoral M2 macrophage percentages by flow cytometry in representative tumor samples and associated healthy breast tissues. We have incubated single cell suspensions from primary tumor and autologous healthy tissues with fluorochrome-conjugated antibodies against M2 macrophage specific markers CD206 and CD163. Cells were gated as “R1” from SSC-A/ FSC-A according to the literature suggested usual position of macrophages, because of their relatively larger size (Fig.4.1). Our analyses have shown diverse percentages of CD206+ and CD163+ positive M2 macrophage in the R1-gated population in different samples. The sum of percentages of CD206+ cells and CD163+ cells were considered as M2 macrophage percentage within the primary tumor. In case of associated healthy tissues, the total percentage of CD206+ and CD163+ cells is usually very
low, below 1.0%. In tumor tissues, varied percentages of M2 were observed and sometimes it reaches 15% in the R1-gated population. Then, we have matched intra-tumoral expression scores of CXCL13 with the M2 macrophage percentages. Importantly, we have observed significant positive association ($p < 0.05$) between CXCL13 expression and M2 macrophage percentage within the primary tumor (Fig. 4.2).

![Flow cytometry analyses of M2 macrophages in representative primary tumor samples and in associated healthy tissues. FITC-conjugated anti-CD206 and PE-conjugated anti-CD163 were used for identifying M2 macrophages. Cells with a higher FSC-A is gated as R1 from the heterogeneous population of tumor cells because of relatively larger size of macrophages. Total percentages of CD206+ cells and CD163+ cells in R1-gated population were considered as M2 macrophage percentage. ‘HT’ is associated healthy tissue samples representative of non-tumor breast tissue. 87T, 2T, 42T, 15T, 31T, 76T and 84T are primary breast tumor samples of patients, bearing respective serial numbers. Summation of CD206+ and CD163+ cells show 0.9% in HT, 0.9% in 87T, 13.8% in 2T, 0.9% in 42T, 13.4% in 15T, 12.7% in 31T, 2.6% in 76T and 1.2% in 84T. Results are representative of three independent experiments.](image_url)

Primary tumors with increasing M2 percentage are associated with higher intra-tumoral CXCL13 level. For instances, in tumor samples from patients, bearing serial numbers 2, 15 and 31 have M2 macrophage 13.8%, 13.4% and 12.7%, respectively, within the R1-gated population (Fig. 4.1). All of these three samples have CXCL13 score 3+ (Table 2.2). Conversely, in tumor samples from patients, bearing serial numbers 42, 76 and 84 have CXCL13 scores 0, 1+, 1+, respectively (Table 2.2) and M2 percentages 0.9%, 2.6%, 1.2%, respectively (Figure 4.1). Moreover, percentage of M2 macrophage within the R1-gated
population in representative healthy tissues is comparable with that from tumor samples which express very low CXCL13 (score 0). Further, we have compared number of samples expressing high (3+/2+) or low (1+/0) CXCL13 with M2 macrophage percentage either >5% (n=24) or ≤5% (n=21) within the R1-gated population. Interestingly, 93.3% (p < 0.01) of tumors with ≤5% M2 cells have low CXCL13 whereas, 82.8% (p < 0.01) of tumors with >5% M2 cells have high CXCL13 scores (Fig.4.2). These results indicated that M2 macrophages could be a potential source of intra-tumor CXCL13 during breast cancer.

4.3.2. Th2-attracting chemokines CCL17 and CCL22 expression within the primary breast tumor is correlated with intra-tumoral M2 population as well as CXCL13 expression

Polarization of TAMs to M2 type is favored significantly by the cytokines secreted by Th2 cells. Two chemokines CCL17 and CCL22 are able to specifically attract CCR4+ Th2 cells and are known to be responsible for Th2 cell trafficking. Therefore, we have analyzed intra-tumoral mRNA expressions of CCL17 and CCL22 and compared with the percentages of M2 macrophages.

![Fig. 4.2. Bar graph showing comparison of relative CXCL13 expression in primary tumor samples with either >5% M2 macrophages (n=24) or ≤5% M2 macrophages (n=21) within R1-gated population. Data are represented as percentage of occurrence. CXCL13 score 3+ and 2+ were considered as high and 1+ and 0 were considered as low CXCL13 expression.](image)
Our initial experiments in representative samples clearly indicated an association between intra-tumoral percentage M2 macrophage and mRNA expressions of CCL17 and/or CCL22. For example, in the primary tumor of patient, bearing serial number 2, mRNA fold changes of CCL17 and CCL22 are 18.4 ($p < 0.001$) and 10.8 ($p < 0.01$), respectively, and the percentage of M2 macrophage within the R1-gated population is 13.8 (Fig. 4.3).

On the other hand, in the primary tumor of patient, bearing serial number 87, the percentage of M2 macrophage within the R1-gated population is 0.9 and mRNA fold changes of CCL17 and CCL22 are 1.8 ($p > 0.05$) and 1.2 ($p > 0.05$), respectively (Fig. 4.3). Further, analyses in larger sample size were performed. We aimed to compare mRNA fold changes of CCL17 and CCL22 in tumor samples with >10% (n=16) and <5% (n=16) of M2 macrophages within the R1-gated population. We have found that the median values of both CCL17 and CCL22 mRNA fold changes relative to adjacent healthy tissues is significantly ($p < 0.05$) higher in
primary tumors with >10% of M2 macrophages compared to that of primary tumors with <5% of M2 macrophages (Fig. 4.4). This finding is important as it supports the fact that higher CCL17 and/or CCL22 expression by the breast tumor may be associated with increased Th2 accumulation and thereby increased M2 polarization.

![Fig. 4.4](image)

**Fig. 4.4.** Statistical analyses comparing CCL17, CCL22 mRNA expression and intra-tumor M2 macrophage population. Box plot showing relative fold changes of intra–tumor CCL17 and CCL22 mRNA expression in samples (n=16) with more than 10% M2 macrophages in R1-gated population as well as in samples (n=16) with less than 5% M2 macrophages in R1-gated population in flow cytometry. Median values of CCL17 and CCL22 mRNA fold changes clearly indicates a significantly higher expression of both chemokines within primary tumor samples containing >10% M2 macrophage in R1-gated population. Macrophage is denoted as mϕ. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD.

### 4.3.3. Intra-tumoral mRNA levels of Th2 cell-specific marker CD294 is positively associated with M2 macrophage-specific markers CD206 and CD163

CD294 is an exclusive marker for Th2 cells [Mantovani A et al., 2007; Boin F et al., 2008]. Since we have found a significant association between intra-tumor CCL17 and CCL22 expression and percentage of M2 macrophage, we then aimed to study CD294 level in representative tumor samples.

Fold changes of CD294, CD206 and CD163 mRNA in primary breast tumor relative to adjacent healthy tissues were analyzed. Correspondingly, we observed that higher the CD294 mRNA fold change, higher the mRNA fold changes of M2 macrophage markers CD206
and/or CD163 (Fig. 4.5). For example, in primary tumor of ‘patient-2’, mRNA fold changes of CD294, CD206 and CD163 are 5.2 ($p < 0.01$), 6.1 ($p < 0.01$) and 3.2 ($p < 0.05$), respectively. On contrary, in primary tumor of ‘patient-42’, mRNA fold changes of CD294, CD206 and CD163 are 1.2 ($p > 0.05$), 1.7 ($p > 0.05$) and 1.2 ($p > 0.05$), respectively (Fig.4.5).

Notably, M2 macrophage percentages of ‘patient-2’ and ‘patient-42’ within the primary tumor are 13.8% and 0.9%, respectively, among the R1-gated population (Fig.4.3). This results importantly validate the hypothesis of increasing Th2 accumulation is associated with higher M2 polarization.

Fig 4.5. Analyses of intra-tumoral mRNA levels CD294, CD206 and CD163 in representative samples. CD294 is a Th2 cell specific marker and CD206, CD163 are M2 macrophage marker. Quantitative real-time PCR was performed and fold changes ($2^{\Delta\Delta CT}$) are represented as relative values normalized with 18s r-RNA. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. One-way ANOVA (Bonferroni correction) was performed, where significance level stands for * $p \leq 0.05$, ** $p \leq 0.01$. 
4.3.4. Human breast cancer cell line-conditioned medium induces elevated CXCL13 expression by human monocyte/macrophage cell line THP1

Finally, we have performed in vitro studies with human monocyte cell line THP1 and human breast cancer cell lines MDA-MB-231 and T47D. We aimed to understand whether breast cancer cells can induce monocytes or macrophages to express elevated CXCL13. THP1 cells were treated with conditioned culture medium from MDA-MB-231 and T47D cells, and control cells were left untreated. Interestingly, significant increase in CXCL13 expression was observed in THP1 cells when treated with human breast cancer cell line-conditioned medium (Fig 4.6).

![Fig 4.6.](image)

**Fig.4.6.** CXCL13 mRNA and protein expression in control and induced THP1 cells. THP1 cells were induced with or without the MDA-MB-231 or T47D culture-conditioned medium for 48 hr. (a) Bar graphs showing CXCL13 mRNA fold changes in induced cells relative to the cells left untreated. (b) It shows quantitative CXCL13 protein level (pg/ml) in 100 µg of cell lysate. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. One-way ANOVA (Bonferroni correction) was performed, where significance level stands for * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Furthermore, the elevation was found more in cells treated with MDA-MB-231-conditioned medium compared to T47D-conditioned medium. The mRNA fold changes of CXCL13 were 8.2 ($p < 0.01$) and 3.5 ($p < 0.05$) in THP1 cells treated with MDA-MB-231-conditioned medium and T47D-conditioned medium, respectively (Fig. 4.6a). Intracellular CXCL13 protein concentration in THP1 cells was increased from 85 pg/ml to 464.2 pg/ml ($p < 0.001$) and 258.4 pg/ml ($p < 0.01$), when treated with MDA-MB-231-conditioned medium and T47D-conditioned medium, respectively, per 100ug of total protein (Fig. 4.6b). This finding
is utmost important because it highlights the ability of cancer cells to induce monocytes to secrete elevated amount of CXCL13.

4.3.5. **THP1 cells express CD206 and CD163 when induced with human breast cancer cell line-conditioned medium**

Breast cancer cell line-conditioned medium induces THP1 cells to produce significantly increased CXCL13. Our observation in clinical samples have shown that intra-tumoral CXCL13 level is positively correlated with percentage of M2 macrophages. Therefore, we intended to investigate whether conditioned medium treatment is inducing a phenotypic shift of THP1 cells to become M2 like cells.

![Image](image.png)

**Fig. 4.7.** Flow cytometry analyses of THP1 cells for surface expression of CD206 and CD163 using FITC-conjugated anti-CD206 and PE-conjugated anti-CD163 antibodies. Black colored histograms are showing fluorescence intensities of isotype controls. (a) Histograms of fluorescence intensities of CD206 and CD163 showing higher MFI in cells treated with either MDA-MB-231 or T47D-conditioned medium. (b) Bar graphs showing relative MFI of FITC and PE in treated samples compared to untreated cells. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. One-way ANOVA (Bonferroni correction) was performed, where significance level stands for *p ≤ 0.05.

We have analyzed surface expressions of CD206 and CD163 by flow cytometry in control and induced THP1 cells using FITC-conjugated anti-CD206 and PE-conjugated anti-CD163 antibodies. We have found increased MFI for both CD206 and CD163 in induced cells (Fig. 4.7). MFI values of CD206 in untreated, MDA-MB-231-conditioned medium-treated and T47D-conditioned medium-treated cells were 1286.77, 1971.47 and 1799.26, respectively. MFI values of CD163 in untreated, MDA-MB-231-conditioned medium-treated and T47D-conditioned medium-treated cells were 2468.56, 4266.18 and 3573.80, respectively (Fig. 4.7).
4.7a). Relative MFI measurements were done. Significant increase ($p < 0.05$) of relative MFI for FITC and PE was found in induced THP1 cells treated with cell line-conditioned medium (Fig.4.7b). This data is important not only because it has validated our hypothesis of M2 cells as probable producer of CXCL13 but also it indicates the potential of breast cancer cells to polarize monocytes to M2 macrophages independent of Th2 cells.

### 4.4. Discussion

Death due to breast cancer is still an issue of major concern and breast cancer is fatal when the disease becomes metastatic [Assi HA et al, 2013; Bhoo-Pathy N et al, 2015]. Considerable percentage of diagnosed breast cancer cases attains metastatic state [Assi HA et al, 2013; Bhoo-Pathy N et al, 2015]. With recent understanding of the biology of breast TME, it is widely documented that cross-talk between tumor infiltrating immune cells and cancer cells regulate the process of metastasis [Cimino-Mathews A et al, 2015]. Trafficking of T-lymphocytes in breast TME is currently under intense investigation as its mechanism remains largely unknown. Some breast tumors have substantial T-lymphocytic infiltration and their interaction with breast tumor cells appears to be associated with disease progression [Slaney CY et al, 2014]. Th1 cells are associated with favorable clinical outcomes, whereas Th2 cells have been reported to be associated with pro-tumor anti-inflammatory responses [Ruffell B et al, 2010; Gobert M et al, 2009].

M2 macrophages have gained prime importance in recent years for understanding breast cancer metastasis [Valastyan S and Weinberg RA, 2011; Condeelis J and Pollard JW, 2006; Mantovani A et al, 2002; Santoni M et al, 2013; Allavena P et al, 2008; Sica A et al, 2008; Mantovani A et al, 2005; Eriksson F et al, 2009]. Infiltrated Th2 cells directly influence the differentiation of monocytes/macrophages to M2 type [Van Ginderachter JA et al, 2006]. Our finding that higher intra-tumoral CCL17 and/or CCL22 is positively correlated with M2 macrophage percentage in the eastern India population is certainly very significant, while the novelty of the study lies in the fact that CCL17-CCL22 can be considered as a poor prognosis marker of breast cancer for patients with eastern Indian origin.

The most significant finding which have been unfolded in this study is that, not all macrophages secrete CXCL13, rather, only the M2 macrophages is positively correlated with intra-tumoral CXCL13 and cancer cell line-conditioned medium is able to induce monocyte
polarization to M2 type macrophages and also direct CXCL13 secretion by induced human monocyte cell line in vitro.

![Fig.4.8. Schematic representation of the hypothesis. CCL17 and CCL22 regulate Th2 infiltration within the breast TME, which in turn facilitates M2 polarization. Finally, M2 macrophages secrete CXCL13.](image)

It has been already established that CXCL13 is associated with progression of breast cancer [Panse J et al., 2008; Biswas et al., 2014]. Thus, understanding the source of CXCL13 within the primary breast tumor will definitely amount additional information. Most notably, our results linked CCL17 and CCL22 with CXCL13. Expression of CCL17 and/or CCL22 induces Th2 cell infiltration and accumulation within the primary breast TME. This is followed by secretion of Th2 cytokines such as IL4, IL10 etc. These cytokines induce polarization of infiltrated and tumor resident macrophages to M2 phenotype. Apart from other pro-tumor functions, we report that within the breast TME, M2 macrophages secrete CXCL13 (Fig. 4.8). CXCL13 finally promotes EMT, migration and metastasis of breast cancer cells.
5. Summary of Ph.D. Research
The aim of my Ph.D. research is identification of prognostic marker of human breast cancer and associated cellular signaling. We have screened expression of different chemokine ligand and receptors within primary breast tumor and autologous healthy tissues, collected from surgically operated breasts, and correlated with clinicopathological parameters. We have found that co-expression of a chemokine ligand receptor pair CXCL13-CXCR5 is positively associated with metastasis to lymph nodes. Moreover, we have shown that CXCL13 induces EMT of breast cancer cells and activates MMP9. From our study, we have indicated that RANKL is taking part in CXCL13-CXCR5-mediated signaling pathway, upstream of both PI3Kp110α and Src.

Further, transcription regulation of cxcl13 and cxcr5 has been studied. RelA has multiple binding sites in the promoter regions of cxcl13 and cxcr5 both. Significantly, increased expression of CXCL13 was observed in RelA-overexpressed breast cancer cell lines. Furthermore, increased cxcl13 promoter activity was also observed in RelA-induced cells. Additionally, we have identified two functional RelA binding sites within the cxcl13 promoter. Interestingly, Nrf2, which has potential binding sites in the promoter of cxcl13, was found to negatively regulate cxcl13 transcription and we observed a significantly decreased CXCL13 mRNA and protein expression in breast cancer cells induced with RelA-Nrf2 co-overexpression. However, Nrf2 did not show any effect in cxcr5 transcription.

RelA is also found to regulate transcription of cxcr5. We observed significantly increased CXCR5 expression in RelA-induced breast cancer cells. Importantly, increased surface CXCR5 level was detected and quantified in breast cancer cell lines, when induced for RelA overexpression.

With our surprise, we observed in a considerable number of primary tumors that substantial RelA expression is not always associated with considerable CXCR5 expression. After detailed analysis of cxcr5 promoter, we have found that cxcr5 promoter encompasses a CpG methylation island, which lies over the RelA binding sites. Methylation-bisulfate conversion assay indicated that RelA may bind to cxcr5 promoter only when it is not methylated.

M2 macrophage has gained immense importance in recent years for the understanding of breast cancer metastasis. We have identified that among the different immune cell populations within the breast TME, intra-tumor M2 macrophage percentage is positively associated with level of CXCL13 within the primary tumor. Our in vitro analyses indicated that breast cancer cell line-conditioned medium is able to induce human monocytes to
express significantly higher CXCL13 and increased M2-specific surface markers, CD206 and CD163. Additionally, we have confirmed that M2 macrophage percentage is sensitive to expression of Th2-attracting chemokines CCL17 and/or CCL22. Th2 marker CD294 was found significantly higher in primary tumor samples expressing elevated CCL17 and/or CCL22.

In summary, we have identified co-expression of CXCR5-CXCL13 ligand-receptor pair as a prognosis marker for breast cancer metastasis for patients from eastern India. This axis may be considered as a potential target for future therapeutics. We were able to penetrate vertically into the intracellular signaling pathway of CXCR5-CXCL13 and spotted out RANKL in the signaling axis. Negative effect of Nrf2 in cxcl13 transcription supports the fact that Nrf2 may act as tumor suppressor during breast cancer progression.

CCL17 and CCL22 expression within the primary breast tumor could also be considered as a marker for poorer prognosis as it is positively associated with intra-tumor M2 macrophage percentage.