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# Cancer associated fibroblasts have phenotypic and functional characteristics similar to the fibrocytes that represent a novel MDSC subset

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**Keywords:** breast cancer, chemical carcinogenesis, fibroblast, T cell, tumor immunity

**Abbreviations:** CAF, cancer associated fibroblast; CFSE, carboxyfluorescein succinimidyl ester; DAB, diaminobenzidine; DC, dendritic cell; FAP- $\alpha$ , fibroblast activation protein- $\alpha$ ; FDR, false discovery rate; GEO, gene expression omnibus; GSEA, gene set enrichment analysis; IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid-derived suppressor cell; MFI, median fluorescence intensity; NES, normalized enrichment score; NF, normal tissue fibroblast; NMU, N-Nitroso-N-methylurea; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SEM, standard error of mean; w/, Coculture with the designated cells

Circulating fibrocytes were reported to represent a novel myeloid-derived suppressor cell (MDSC) subset and they were also proposed to be involved in the tumor immune escape. This novel fibrocyte subset had a surface phenotype resembling non-monocytic MDSCs (CD14<sup>-</sup>CD11c<sup>hi</sup>CD123<sup>-</sup>) and exhibited immunomodulatory roles. Most effector functions of fibrocytes (circulating fibroblast-progenitors) are accomplished as tissue fibroblasts, likewise in the tumor microenvironment. Therefore, fibroblasts at tumor tissues should be evaluated whether they display similar molecular/gene expression patterns and functional roles to the blood-borne fibrocytes. A chemically induced rat breast carcinogenesis model was utilized to obtain cancer associated fibroblasts (CAFs). CAFs and normal tissue fibroblasts (NFs) were isolated from cancerous and healthy breast tissues, respectively, using a previously described enzymatic protocol. Both CAFs and NFs were analyzed for cell surface phenotypes by flow cytometry and for gene expression profiles by gene set enrichment analysis (GSEA). PBMCs were cocultured with either NFs or CAFs and proliferations of PBMCs were assessed by CFSE assays. Morphological analyses were performed by immunocytochemistry stainings with vimentin. CAFs were spindle shaped cells unlike their blood-borne counterparts. They did not express CD80 and their MHC-II expression was lower than NFs. Although CAFs expressed the myeloid marker CD11b/c, its expression was lower than that on the circulating fibrocytes. CAFs did not express granulocytic/neutrophilic markers and they seemed to have developed in an environment containing T<sub>HELPER</sub>2-like cytokines. They also showed immunosuppressive effects similar to their blood-borne counterparts. In summary, CAFs showed similar phenotypic and functional characteristics to the circulating fibrocytes that were reported to represent a unique MDSC subset.

## Introduction

MDSCs are activated immature cells of myeloid origin that increase in number in various pathological conditions, including cancer.<sup>1,2</sup> This heterogeneous group was shown to express Gr-1 and CD11b in murine models,<sup>3</sup> and was described as monocytic (Ly6C<sup>+</sup>) and neutrophilic subsets (Ly6G<sup>+</sup>).<sup>4</sup> Although rat MDSCs were first described as CD11b/c<sup>+</sup> and HIS48<sup>+</sup> cells,<sup>5</sup> there is an ongoing debate concerning the specific markers for rat MDSCs. On the other hand, human MDSCs also seem to be more complex than their murine counterparts, and are most commonly defined as CD33<sup>+</sup>CD14<sup>-</sup>CD11b<sup>+</sup>HLADR<sup>-</sup> cells.<sup>6,7</sup>

The studies that have investigated the nature of the cells and molecules responsible for the functional insufficiency of the tumor infiltrating T cells usually focused on tumor cells

themselves, MDSCs and/or regulatory T cells.<sup>8-10</sup> However, the contribution of stromal cellular elements has not yet been well established. Fibroblasts, one of the most abundant cell types found in the stroma, become activated and differentiate into myofibroblasts during wound healing and fibrosis; or into CAFs in the tumor microenvironment. CAFs are the most prominent cell type within the tumor microenvironment of various cancers<sup>11,12</sup> and approximately 80% of stromal fibroblasts are thought to show an activated phenotype in breast carcinomas.<sup>13</sup> As “tumors are wounds that do not heal”,<sup>14</sup> CAFs are similar to myofibroblasts. The paracrine crosstalk between the epithelium and the stroma results in the activation of fibroblasts.<sup>15</sup> CAF derived factors, in turn, are able to promote a tumor permissive microenvironment and contribute to the metastatic properties of cancer cells.<sup>15-19</sup>

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Recently, the expansion of a special population of HLA-DR<sup>+</sup> cells that bear the morphological features of immature monocytes as well as the cell surface expression characteristics of neutrophilic MDSCs was reported in pediatric patients with metastatic sarcomas.<sup>20</sup> In contrast to a number of previous studies that focused on the monocytic or neutrophilic subsets of the MDSCs,<sup>1,4,21,22</sup> this study is of utmost importance as it redefines a player formerly known for its proinflammatory and profibrotic effects, the fibrocytes, that may also be involved in the tumor immune escape.<sup>23-26</sup> The results of Zhang et al. were obtained from a pediatric population and were not representative of other investigators' studies into MDSCs. They rather described a novel subset of cancer-induced MDSCs, which were CD11b<sup>+</sup>HLA-DR<sup>+</sup>.

Despite the discovery of the expansion of a novel circulating fibrocyte subset (CD45<sup>+</sup>CD34<sup>+</sup>HLA-DR<sup>+</sup>) which showed a surface molecular expression pattern similar to the non-monocytic MDSCs (CD14<sup>-</sup>CD11c<sup>hi</sup>CD123<sup>-</sup>) and at the same time possessed immunosuppressive properties,<sup>20</sup> whether fibrocytes at the tumor site exhibit similar characteristics to the circulating fibrocytes has not yet been clarified. Fibrocytes are circulating progenitors of fibroblasts.<sup>27,28</sup> These surface expression findings concerning the circulating fibrocytes<sup>20</sup> call for further investigations to determine if those results are also relevant for tissue fibroblasts. Therefore, we investigated whether the CAFs exhibited the molecular or gene expression profiles and functional effects similar to that of the blood-borne fibrocytes with MDSC characteristics.<sup>20</sup> In this study, we report that fibroblasts at tumor sites display a similar expression profile and functional characteristics, with some modifications, to the circulating fibrocytes which were shown to represent a novel MDSC subset. Furthermore, these fibroblasts that are located in the tumor microenvironment may take part in immune modulation.

## Results

### CAF and NFs were successfully isolated from cancerous and healthy tissues, respectively

The animals that were injected with N-Nitroso-N-methylurea (NMU) developed at least one palpable breast tumor after a period of 1–2 mo. Tumor excisions were performed after the tumors were  $\geq 1$  cm in diameter. NFs were obtained from healthy breast tissues of healthy control animals and CAFs were obtained from breast carcinomas, by enzymatic digestion with Collagenase I and Hyaluronidase.<sup>29</sup> These isolated fibroblasts were then cultured and stable primary cell cultures were achieved.

### CAF express MHC class II but do not provide costimulation through CD80

Fibrocytes were previously reported to express MHC class II (MHC-II) and CD80/86; thus functioning as antigen presenting cells.<sup>30</sup> Therefore, the expression of MHC-II on CAFs was analyzed and indeed, CAFs were found to express MHC-II (Fig. 1A, B). In order to compare the MHC-II expressions of NFs and CAFs, NFs were obtained from the breast tissues of healthy control rats. NFs were also shown to express MHC-II, even more

than CAFs did (Fig. 1A, B) (*p* value of MFI comparison was 0.024). Furthermore, the investigation of the costimulatory CD80 molecule expression on tumor-derived fibroblasts revealed that CAFs did not express CD80 (Fig. 1A).

In order to further investigate the distinction of CAFs from the NFs, we analyzed a publicly available human microarray study to compare gene expression in these two cell types (GSE21440). The experiment was analyzed by GSEA, as described previously.<sup>31</sup> REACTOMEMHC\_CLASS\_II\_ANTI-GEN\_PRESENTATION gene set, which comprises genes involved in MHC class II antigen presentation, was found to be differentially expressed. Furthermore, this gene set was under-expressed in CAFs compared with the NFs (NES: -1.27, FDR: 0.062). Fig. 3A shows the enrichment plot with 20 of the genes which contribute most to the enrichment result. This GSEA result is consistent with our findings concerning MHC Class II surface expression characteristics of CAF cells (Fig. 1A, B) and confirms that CAFs represent a cell population with reduced antigen presenting capacity compared with NFs.

### CAF express the myeloid marker CD11b/c

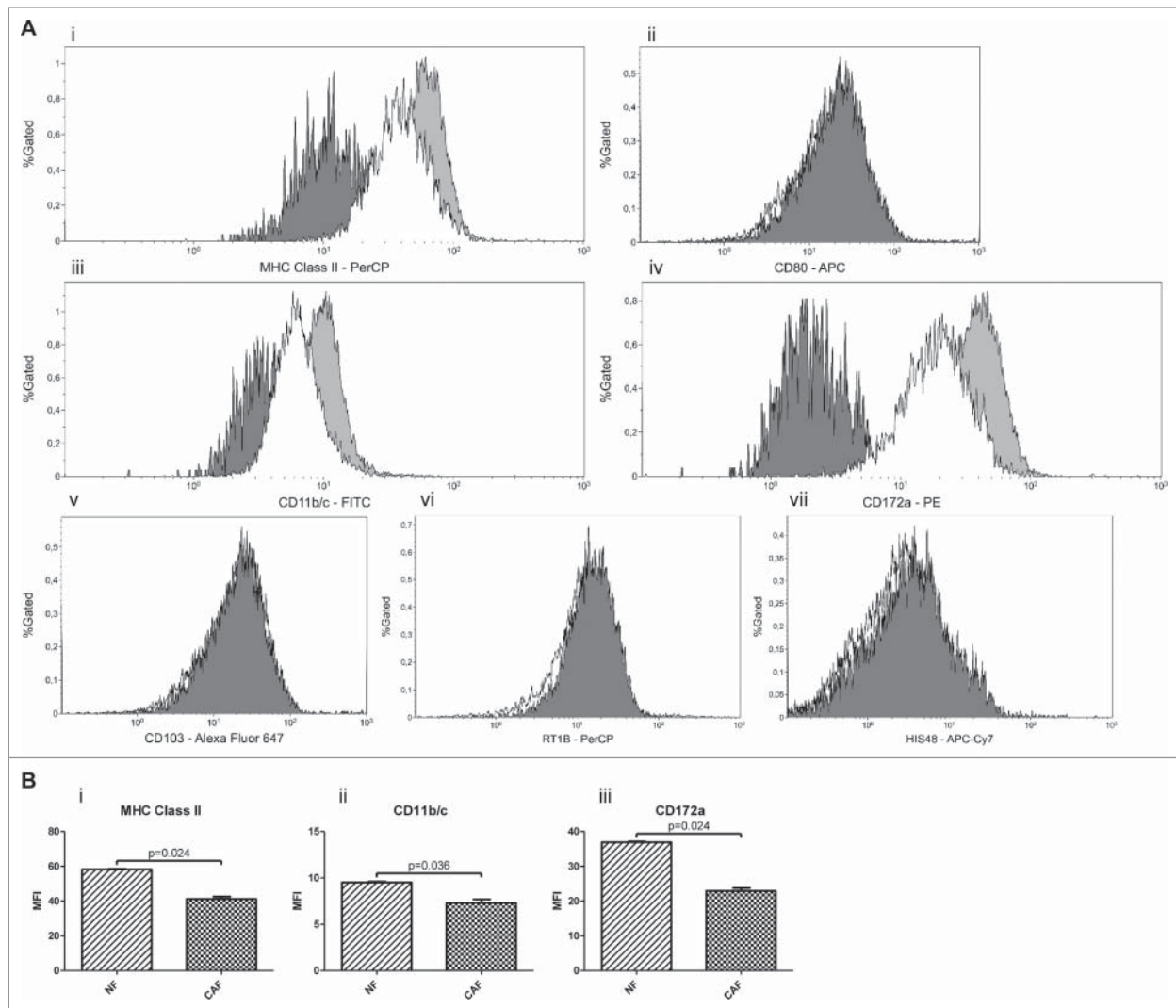
CD11b/c is a common myeloid marker. Both murine and human MDSCs have been reported to express CD11b. It was demonstrated that a group of circulating fibrocytes also expressed CD11b.<sup>20</sup> Accordingly, CAFs were observed to be mildly positive for CD11b/c (Fig. 1A, B) in the current study. Moreover, NFs displayed slightly higher expressions of CD11b/c in comparison to CAFs (Fig. 1A, B) (*p* value of MFI comparison was 0.036). Furthermore, considering the results from Zhang et al.,<sup>20</sup> CD11b/c expression in CAFs appears to be lower than that in the circulating fibrocytes.

### CAF do not express granulocytic / neutrophilic markers despite having myeloid characteristics

CAF were found to be mildly positive for CD11b/c (Fig. 1A, B). Based on the finding that fibrocytes displayed cell surface expression characteristics of neutrophilic cells,<sup>20</sup> the expressions of granulocytic/neutrophilic markers that are recognized by such antibodies as RP-1<sup>32</sup> and HIS48,<sup>33</sup> were investigated in CAFs. Neither of these markers was present on CAFs (one representative marker, HIS48, is shown in Fig. 1A).

### CAF seem to have developed in an environment containing T<sub>HELPER</sub>2-like cytokines

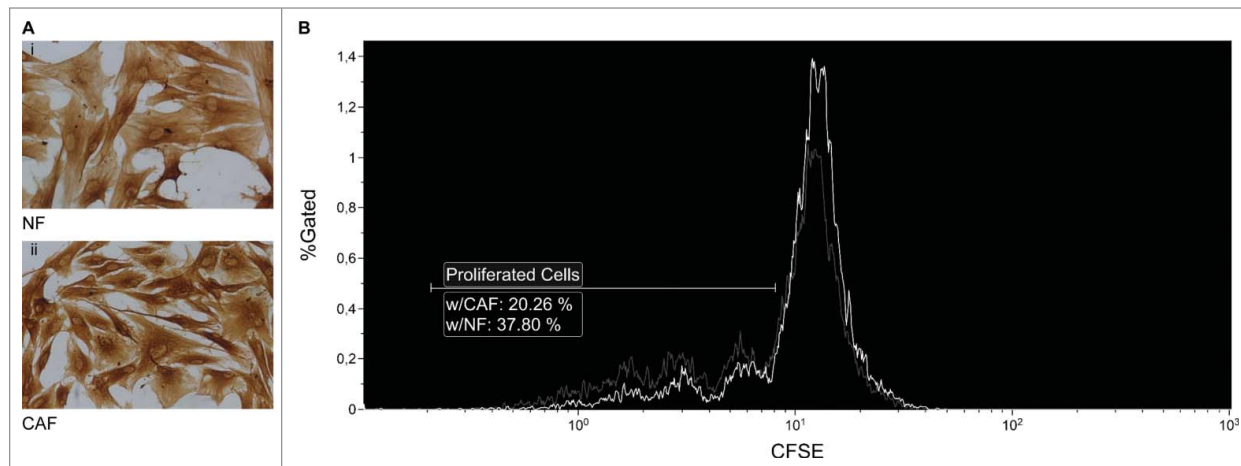
Pilling et al. previously proposed that CD172a can be used to demonstrate whether fibrocytes have been differentiated in a milieu that contains either profibrotic T<sub>HELPER</sub>2-like cytokines (IL-4 and/or IL-13) or proinflammatory T<sub>HELPER</sub>1-like cytokines (IL-12 and/or IFN $\gamma$ ).<sup>34</sup> Thus, CD172a (SIRP $\alpha$ ) expression on CAFs was analyzed in comparison to NFs, in an attempt to indirectly investigate whether those findings suggesting an IL-4 rich milieu for the blood-borne fibrocytes, also applied to CAFs. Indeed, CD172a was found to be expressed on both CAFs and NFs, the former with lower surface expressions (Fig. 1A, B) (*p* value of MFI comparison was 0.024), likely indicating for higher levels of IL-4 in the milieu of CAFs (see also Fig. S3).



**Figure 1.** Fibrocytic cells at the tumor site exhibit similar –but not the same– surface expression characteristics to the circulating fibrocytes. **(A)** Expression profiles of CAF cells for MHC Class II (i), CD80 (ii), CD11b/c (iii), CD172a (iv), CD103 (v), RT1B (vi), HIS48 (vii) and expression profiles of NF cells for MHC Class II (i), CD11b/c (iii), CD172a (iv) are shown. Fibroblast cells in cultures were evaluated for morphological features,<sup>76</sup> immunocytochemical characteristics; and microscopic confirmation was achieved before the experiments. Fibroblast cells were previously reported to show a wide range of variation in terms of size and granularity in flow cytometric evaluations.<sup>77,78</sup> Our studies also demonstrated that forward and side scatter characteristics of NF and CAF cells do not represent a single distinct population based on size and granularity; thus, cannot be used as a sensitive and specific gating strategy as in the case of the lymphocyte gate (**Fig. S1**). As reported previously,<sup>78</sup> fibroblast cells were gated on scatterplot of side light scatter vs. forward light scatter, allowing for gating of only the viable cell population, excluding cellular debris after doublet discrimination. Heavily shaded (dark gray) areas represent background fluorescence on the designated population as indicated by suitable isotype controls. Unshaded (white) areas represent breast tumor-derived CAF cells and lightly shaded (light gray) areas represent NF cells acquired from healthy donors. CAFs were shown not to express CD80, CD103, RT1B or HIS48. On the other hand, CAFs were shown to express MHC class II, CD11b/c and CD172a and their expressions of these molecules were lower than that of NF cells. Representative flow cytometry plots of more than three experiments are shown. **(B)** Comparison of MFI values of surface expressions of CAF vs. NF cells. There were statistically significant differences between CAF and NF expressions of MHC Class II ( $p = 0.024$ ) (i), CD11b/c ( $p = 0.036$ ) (ii) and CD172a ( $p=0.024$ ) (iii). Columns and error bars designate means and SEM, respectively. The results were derived from more than three experiments. CAF: Cancer Associated Fibroblast, NF: Normal Tissue Fibroblast, MFI: Median Fluorescence Intensity, SEM: Standard Error of Mean.

In order to more directly assess whether CAF cells were exposed to higher levels of IL-4 than NF cells, we investigated genes related to IL-4 mediated signaling by the “SIG\_IL4RECEPTOR\_IN\_B\_LYPHOCYTES” gene set with GSEA in a publicly available human microarray study (GSE21440), as described previously.<sup>31</sup> This *IL-4 signaling* gene set was found to be differentially expressed, and was upregulated

in CAFs compared with the NFs (NES: 1.21, FDR: 0.201). **Fig. 3B** shows the enrichment plot with 14 of the genes which contribute most to the enrichment result. The GSEA result suggests a higher IL-4 exposure for CAFs and thus is consistent with our findings of lower surface expressions of CD172a on CAFs than NFs (**Fig. 1A, B**), implying higher IL-4 levels in the micro-environment of CAFs.



**Figure 2.** Fibrocytic cells mediate immune suppression. (A) Vimentin stained NF (i) and CAF (ii) cells from representative samples (original magnification x200 under light microscope and visualized by DAB). CAF and NF cells were immunostained to show their surface expressions of vimentin, in order to clearly visualize their morphologies and expression characters. Both of these spindle shaped cell types stained uniformly for the mesenchymal marker vimentin, as expected.<sup>38</sup> (B) Suppression of PHA mediated PBMC proliferation by fibrocytic cells. 20.26% of PBMCs cocultured with CAFs (white line) proliferated in contrast to the proliferation of 37.80% of PBMCs cocultured with NFs (gray line). Thus, CFSE proliferation assays showed the immunosuppressive effect of CAFs compared to NFs. A representative histogram of three experiments performed using PBMC from three separate rats is shown (see Fig. S2 for positive and negative controls and Fig. S4 for the effect of the number of CAF cells). CAF: Cancer Associated Fibroblast, NF: Normal Tissue Fibroblast, w/: Coculture with the designated cells, DAB: Diaminobenzidine, PHA: Phytohemagglutinin, PBMC: Peripheral Blood Mononuclear Cell, CFSE: Carboxyfluorescein Succinimidyl Ester.

### CAF do not resemble the DCs that are present in the gut-associated lymphoid tissue since they are CD103<sup>-</sup>RT1B<sup>-</sup>

Abnormal expansions of CD11b<sup>+</sup>HLA-DR<sup>+</sup> myeloid cells in peripheral blood of subjects with metastatic sarcomas were demonstrated.<sup>20</sup> It was also previously reported that a dendritic cell (DC) population is present at a high frequency in the gut-associated lymphoid tissue and non-T cell expression of a specific marker, CD103, is restricted primarily to these CD11c<sup>hi</sup> MHC-II<sup>hi</sup> DCs.<sup>35</sup> Given the resemblance of the phenotypic characteristics of those 2 cell types, CD103 expression of CAFs was analyzed. However, CAFs did not express CD103 (Fig. 1A). In addition, as those DCs were also reported to express another marker, RT1B,<sup>36</sup> the expression of RT1B on CAFs was also investigated. Similar to CD103, RT1B was not expressed on CAFs, either (Fig. 1A).

### CAF exhibit immunosuppressive characteristics

Blood-borne fibrocytes in cancer patients were reported to possess immunosuppressive properties.<sup>20</sup> Furthermore, Kraman et al. proposed a population of fibroblast activation protein- $\alpha$  (FAP- $\alpha$ ) expressing cells to be an immunosuppressive component of the tumor microenvironment.<sup>37</sup> Accordingly, fibroblasts at the tumor site were analyzed to find out whether they exhibit similar immunosuppressive characteristics like their circulating counterparts. Similarly, carboxyfluorescein succinimidyl ester (CFSE) proliferation assays in cocultures of CAFs and NFs with peripheral blood mononuclear cells (PBMCs) were utilized for functional assessment and the results revealed the immunosuppressive effects of CAFs compared to NFs (Fig. 2B), since PBMCs that were cocultured with CAF cells showed decreased proliferation.

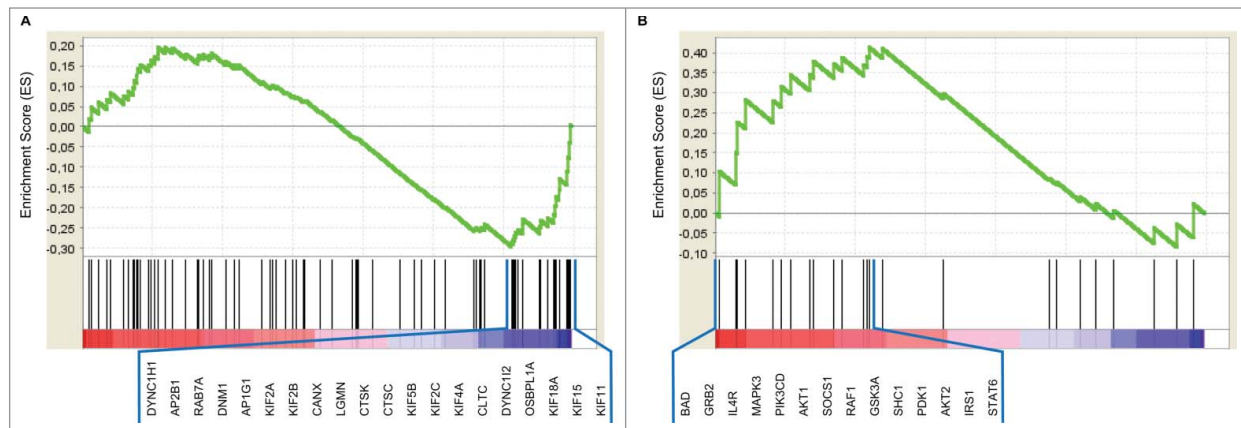
### CAF are spindle shaped, mesenchymally-derived cells unlike their blood-borne counterparts

Finally, both CAF and NF cells were immunostained to show the expression of the mesenchymal tissue marker vimentin, in order to clearly visualize their morphologies and expression characteristics. Both of these cell types were uniformly positive for vimentin, as expected<sup>38</sup> (Fig. 2A). Although it was reported that CD11c<sup>hi</sup>CD123<sup>-</sup>CD14<sup>-</sup> cells resembled immature monocytes,<sup>20</sup> their tissue counterparts (NFs and CAFs) were spindle shaped cells *ex vivo*.

## Discussion

Fibroblasts are one of the most abundant cell types found in the stroma. They mostly differentiate into CAFs within the tumor microenvironment. The straightforward perspective about the sole structural role of these cells has been contradicted by the findings of several independent studies. At present, they are thought to be involved in a dynamic and dense crosstalk with other cells of the tumor microenvironment.<sup>39</sup> CAFs were demonstrated to directly promote tumor progression by taking active part in invasion and metastasis processes.<sup>29,40,41</sup> Fibrocytes are fibroblast progenitor cells of hematopoietic lineage. The expansion of a circulating fibrocyte subset which showed a surface molecular expression pattern similar to the non-monocytic MDSCs and also exhibited immunosuppressive properties was recently reported.<sup>20</sup>

In the current study, we investigated whether these intriguing findings concerning the blood-borne fibrocytes which were proposed to represent an MDSC subset, were also relevant for the



**Figure 3.** Genes involved in MHC class II antigen presentation were under-expressed and genes related to *IL-4* signaling were upregulated in CAFs compared with NFs. (A) Enrichment plots of GSEA of CAFs vs. NFs for the REACTOMEMHC\_CLASS\_II\_ANTIGEN\_PRESENTATION (NES: -1.27, FDR: 0.062) and (B) SIG\_IL4RECEPTOR\_IN\_B\_LYPHOCYTES (NES: 1.21, FDR: 0.201) gene sets are shown. Gene expression data was acquired from NCBI Gene Expression Omnibus (GSE21440) and GSEA was performed as described previously.<sup>31</sup> Genes that contribute to the leading-edge subsets within each gene set (20 and 14 genes, respectively) are listed below the figures. These GSEA results are consistent with our flow cytometry findings concerning MHC Class II and CD172a expressions of CAF and NF cells. CAF: Cancer Associated Fibroblast, NF: Normal Tissue Fibroblast, GSEA: Gene Set Enrichment Analysis, NES: Normalized Enrichment Score, FDR: False Discovery Rate.

fibroblasts at tumor sites. We utilized a murine chemical breast carcinogenesis model in order to obtain CAFs and conducted functional as well as phenotypical and genotypic assessments, *ex vivo*. The findings of this study rely on a well-characterized rat chemical breast carcinogenesis model, since cell lines are not representative for the heterogeneity of tumors and xenograft / transplantation type tumor models do not sufficiently represent the human tumors.<sup>42,43</sup> NMU induced breast tumor models display similar histopathological lesions with their human counterparts.<sup>44,45</sup> Furthermore, the ability of this model to simulate human breast cancer histopathology is probably better than similar models utilized in mice.<sup>46</sup>

In our study, we were able to demonstrate that CAFs expressed MHC-II. However, its expression was higher in NFs than in CAFs. Given the results stating that patients with metastatic cancer show an expansion of immunosuppressive fibrocytes,<sup>20</sup> the downregulation of both the MHC-II molecule and the genes involved in MHC class II antigen presentation on/in CAFs in comparison to NFs might constitute another mechanism for the immune escape of tumors, in addition to the pro-angiogenic and indoleamine 2,3-dioxygenase (IDO) mediated immune suppressive effects of fibrocytes.<sup>20</sup> Moreover, we demonstrated that CAFs did not express CD80. In contrast to the fibrocytes that were previously reported to express CD80,<sup>30</sup> no-to-low surface expression of CD80 on CAFs might be another immune escape mechanism, similar to what had been previously observed in colon carcinoma cells.<sup>47</sup>

Even though most studies with human and/or mouse derived cells use antibodies specific to CD11b or CD11c, CD11b/c is a marker commonly used in rat studies.<sup>48-53</sup> The OX-42 antibody, which was used in the study, reacts with the CR3 complement (C3bi) receptor and recognizes a common epitope shared between CD11b and CD11c (integrin  $\alpha_M$  and  $\alpha_X$  chains).<sup>54</sup>

This antibody immunoprecipitates three polypeptides with molecular weights of 160, 103 and 95 k<sub>D</sub>. OX-42 was also shown to inhibit complement mediated rosette formation.<sup>54</sup> The findings of the current study demonstrated that CAFs were mildly positive for CD11b/c. In addition, CD11b/c expression in CAFs was lower than that in the circulating fibrocytes. Such a decrease might be ascribed to the difference in tissue locations of the cells (blood vs. tumor). On the other hand, one must also bear in mind the fact that this might also be due to the biological differences between rats and humans. Furthermore, CAFs were previously shown not to express the granulocytic/neutrophilic markers (RP-1 and HIS48). Granulocytes, but not lymphocytes or monocytes, were reported to express the antigen recognized by RP-1 monoclonal antibody<sup>32,55</sup> and HIS48 is also a commonly used phenotypic marker for rat granulocytes.<sup>33,56,57</sup> Even though rat MDSCs were first described to express the neutrophil marker that is recognized by HIS48 antibody,<sup>5</sup> our results demonstrated that CAFs did not show the exact same surface expression characteristics of neutrophilic MDSCs. In fact, the previous description of rat MDSCs,<sup>5</sup> might prove to be insufficient to address various MDSC subsets.

Zhang et al. demonstrated that monocytes from healthy donors cultured with IL-4, differentiated into fibrocytes with similar phenotypic and immunosuppressive properties as those observed in patients with cancer.<sup>20</sup> This finding is suggestive for a milieu rich in T<sub>HELPER2</sub>-like cytokines (e.g., IL-4), in which those fibrocytes have differentiated.<sup>20,34</sup> It was also previously reported that fibrogenesis is linked with the development of a T<sub>HELPER2</sub> type response.<sup>58</sup> In another study, Pilling et al. reported that PBMCs cultured in serum-free medium containing IL-4 had increased numbers of fibrocytes and those fibrocytes had reduced expression of CD172a, in comparison to the fibrocytes cultured in serum-free medium alone.<sup>34</sup> They also suggested that CD172a can be used to determine

whether fibrocytes have been differentiated in an environment containing profibrotic T<sub>HELPER</sub>2-like cytokines (IL-4 and/or IL-13) or proinflammatory T<sub>HELPER</sub>1-like cytokines (IL-12 and/or IFN $\gamma$ ). The direct assessment of IL-4 levels in the tumor microenvironment would only demonstrate whether mature fibroblasts reside in a milieu containing this cytokine and it would reveal limited information about the extent of fibroblast exposure to IL-4 during their differentiation. On the other hand, CD172a molecule and the genes related to IL-4 mediated signaling may give hints about the presence of IL-4 in the milieu where the fibrocytes have been differentiated. We found that CD172a was expressed on both CAFs and NFs, the latter displaying higher surface expressions. Furthermore, *IL-4 signaling* gene set was shown to be upregulated in CAFs compared with the NFs. Our results are consistent with the findings of Zhang et al.,<sup>20</sup> suggesting a role for IL-4 in the immunosuppressive effects of fibrocytes/fibroblasts in cancer bearing hosts. Furthermore, Haniffa et al. previously discovered a marked induction of T<sub>HELPER</sub>2 cytokines in T cells exposed to stromal cells, but no increase in T<sub>HELPER</sub>1 cytokines.<sup>59</sup> Taken together, all of these findings might suggest a positive feedback loop between stromal cells and the immune system, resulting in a more tumor permissive milieu. In another study, Dugast et al. identified plastic-adherent cells that expressed CD172a, as well as other myeloid markers.<sup>60</sup> These cells were able to inhibit proliferation of effector T cells and their suppressive action was under the control of inducible nitric oxide synthase. They proposed that these cells can be defined as MDSCs.<sup>60</sup> Our findings demonstrated that CAFs, too, expressed CD172a.

Blood-borne fibrocytes in cancer patients have immunosuppressive properties.<sup>20</sup> Accordingly, in our *ex vivo* model, CAFs also exhibited similar inhibitory effects since PBMC proliferation was significantly decreased in CAF cocultures. NFs were previously reported to have immunoinhibitory effects on T cells.<sup>61,62</sup> Pinchuk et al. reported that human colonic myofibroblasts also suppressed the proliferation of peripheral blood lymphocytes.<sup>63</sup> However, co-implantation tumor xenograft model experiments revealed that CAFs were able to promote the growth of breast carcinoma cells significantly more than mammary NFs did.<sup>29</sup> In accordance with those findings, our results also demonstrated that the negative impact of CAFs on PBMC proliferation was prominent in comparison to NFs.

Although their blood-borne counterparts resembled immature monocytes,<sup>20</sup> CAFs were spindle shaped cells that were strongly positive for vimentin. In addition, the phenotypic resemblance between the DCs that are present in the gut-associated lymphoid tissue and CAFs, led us to investigate their similarities further. However, CAFs did not express CD103 and RT1B, which were reported to be expressed by those DCs.

Even though the murine chemical carcinogenesis models are widely used in cancer studies and they were shown to display histopathological similarities with their human counterparts,<sup>44,45</sup> differences between rat and human biologies should also be taken into consideration while interpreting the findings of the current study. Hence, we believe it would be useful to confirm the results of the current study by further studies in humans. Moreover, the relationship of fibrocytes and MDSCs, which was pointed out by

our findings, might be assessed in more detail by labeling the blood-borne fibrocytes and tracking their localizations in the tumor microenvironment *in vivo*. CAFs may also be labeled in a co-implantation model in order to investigate whether tissue fibroblasts enter the bloodstream, thus allowing for better understanding of their association with MDSCs.

In summary, this study investigated the phenotype, genotype and functional characteristics of CAFs in contrast to NFs. Although circulating fibrocytes were not directly studied in this project, our findings suggest that fibrocytes at tumor sites (CAF) show similar expression and functional characteristics to the circulating fibrocytes which represent an MDSC subset, with some modifications; based on the findings of Zhang et al.<sup>20</sup> CAFs expressed MHC-II, but the expression was lower than that in NFs. The expression of lower levels of MHC-II molecule and the genes involved in MHC class II antigen presentation, and also the lack of the expression of CD80 together may decrease the antigen presenting capacity and contribute to the mechanisms of tumor immune escape. Although CAFs were positive for CD11b/c, its expression was lower compared to their circulating counterparts. CAFs also showed immunosuppressive effects similar to the blood-borne fibrocytes that represent an MDSC subset. Our findings support a role for IL-4 in the immunosuppressive effects of fibrocytes, too. However, tissue fibrocytes did not express granulocytic/neutrophilic markers and were spindle shaped, contrary to their circulating counterparts.

## Methods

### Animals and cell isolations

In order to acquire tumor tissue associated fibroblasts, NMU induced experimental breast carcinogenesis model in Sprague Dawley rats was utilized, as previously described.<sup>44,64</sup> 21 d old female Sprague Dawley rats were injected ( $n = 20$ ) once a week for 4 weeks with i.p. 50 mg/kg NMU (Sigma-Aldrich, St. Louis, MO, USA). When the animals were about 3 mo old, developed breast tumors were surgically harvested under sterile conditions for CAF isolation. The tissues were selected to be minimally necrotic regions of the tumor mass. CAFs were isolated from breast tumor tissues using a previously described protocol that utilizes Collagenase I (1 mg/mL) and Hyaluronidase (125 U/mL) enzymes (Sigma-Aldrich, St. Louis, MO, USA) followed by differential sedimentation and plating.<sup>29</sup> Enzymatically digested tissues were then cultured in high serum media conditions which select for fibroblast growth (DMEM supplemented with 10% fetal calf serum at 37°C, 5% CO<sub>2</sub>).<sup>29</sup> The same protocol was also used to isolate NFs from healthy breast tissues ( $n = 7$ ). We used fibroblasts passaged for up to five population doublings for the experiments, in order to minimize culture stress and clonal selection. All animal studies were performed in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and in accordance with the Helsinki Declaration of 1975. Animal studies were reviewed and approved by "Hacettepe University Institutional Animal

Care and Use Committee, Ankara” before the commencement of the experiments (Approval number: 2011/28–3).

### Flow cytometry analyses

CAF and NF cells (which were acquired enzymatically from breast tumors and healthy donors, respectively) were analyzed for cell surface phenotypes. Cultures were incubated at most two weeks before the flow cytometry analyses. Fibroblast cells were stained with appropriate monoclonal antibodies in FACS staining solution and were analyzed by FACS Aria II (equipped with 488 nm and 635 nm lasers) using FACS Diva software. Doublets were gated out by plotting voltage pulse width vs. area. Mouse anti-rat MHC Class II (PerCP, OX-6), mouse anti-rat CD11b/c (FITC, OX-42), mouse anti-rat RT1B (PerCP, OX-6) and mouse anti-rat granulocytes (PE, RP-1) were obtained from BD Biosciences, San Jose, CA, USA. Mouse anti-rat CD172a (PE, OX-41), mouse anti-rat CD103 (Alexa Fluor 647, OX-62) and APC-Cy7 labeled streptavidin were obtained from BioLegend, San Diego, CA, USA. Mouse anti-rat CD80 (APC, 3H5) was obtained from Invitrogen, Camarillo, CA, USA. Mouse anti-rat granulocyte (Biotinylated, HIS48) was obtained from eBioscience, San Diego, CA, USA. Non-specific isotype-matched antibodies were used as controls.

### PBMCs, cocultures and CFSE proliferation assay

PBMCs were obtained from healthy adult Sprague Dawley rats’ peripheral blood (by venipuncture) using density gradient separation with Histopaque-1077 (Sigma–Aldrich, St. Louis, MO, USA), as previously described.<sup>65</sup> PBMCs were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2.1 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C, 5% CO<sub>2</sub>. Cell counts and viability were assessed using the trypan blue dye exclusion method with a hemocytometer. Proliferation of fresh PBMCs was evaluated by CFSE (Life Technologies, Carlsbad, CA, USA) proliferation assay,<sup>66</sup> according to the manufacturer’s instructions.  $1 \times 10^6$  PBMCs were cocultured with  $5 \times 10^4$  of either NF or CAF cells in a 24-well plate with the presence of 1 µg/mL of phytohemagglutinin (PHA) (Biochrom, Berlin, Germany). CFSE (5 µM) proliferation assay was performed for four d and the percentage of proliferated PBMCs was evaluated with FACS Aria II according to CFSE fluorescent signal intensities.

### Gene expression profiling by gene set enrichment analysis

We performed analyses on a publicly available gene expression study. The raw data was extracted from a major public repository of microarray experiments, NCBI Gene Expression Omnibus (GEO).<sup>67</sup> Walter et al., 2010, performed microarray experiments in pancreatic CAFs and control fibroblasts investigating signaling pathways important in tumor-stromal cell interactions (data accessible at NCBI GEO database,<sup>68</sup> accession GSE21440). This microarray study, titled “*Gene expression analysis of pancreatic cancer associated fibroblasts*,” included both CAF and NF samples (platform: *Affymetrix Human Exon 1.0 ST*).

In addition to manual curation and customized scripts, we used methods available from GEOquery,<sup>69</sup> oligo,<sup>70</sup> limma,<sup>71</sup>

packages for R/Bioconductor<sup>72,73</sup> to retrieve the data and get the “ExpressionSet.” After preparing the expression set; we performed GSEA as described previously,<sup>31</sup> in order to investigate pathways and biological processes associated with antigen presentation and IL-4 receptor signaling. Gene lists were obtained from Molecular Signatures Database (MSigDB).<sup>31</sup> REACTOMEMHC\_CLASS\_II\_ANTIGEN\_PRESENTATION” gene set includes genes involved in MHC class II antigen presentation (Reactome DOI: 10.3180/REACT\_121399.1). “SIG\_IL4RECEPTOR\_IN\_B\_LYPHOCYTES” gene set comprises genes related to IL-4 mediated signaling<sup>74</sup> (Signaling Gateway – UCSD Molecule Pages<sup>75</sup>).

### Morphological analyses - immunocytochemistry

Morphological analyses and characterizations of CAF and NF cells were performed by immunocytochemistry stainings with vimentin (Clone V9; ScyTek Laboratories, West Logan, UT, USA), according to the manufacturer’s instructions. Cells ( $6 \times 10^4$  cells / 400 µL in each well) were cultured in eight well chamber slides (BD Biosciences, San Jose, CA, USA) and immunocytochemical/morphological assessments were performed when the cells reached a surface confluency of >70 %. These CAFs and NFs were immunostained to show their surface expressions of vimentin, in order to clearly visualize their morphologies and expression characters. A biotin/streptavidin/horseradish peroxidase detection system was utilized and binding of the antibody was demonstrated with diaminobenzidine (DAB) substrate. The images were captured by Olympus BX50 microscope (Tokyo, Japan).

### Statistical analyses

Statistical analyses (except for GSEA) were performed with Mann–Whitney *U*-test using IBM SPSS Statistics 22, with  $p < 0.05$  taken as statistically significant. Gene set enrichment analyses were performed by the software provided by the Broad Institute.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.



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