Uncovering the Role of Cancer Associated Fibroblasts in Tumor Immunosuppression

Kamal Amirneni[1], Ezekiel Bello[1], Omar Sarkar[1], Lewis Chew[1], and Kavitha Yaddanapudi[1,2]
[1]Department of Microbiology and Immunology, University of Louisville, KY
[2]Brown Cancer Center, University of Louisville, KY

Introduction

Immunotherapy is a relatively recent advent in the realm of cancer therapy that offers more targeted treatment in comparison to chemotherapy or radiation. The goal of immunotherapy is to employ the immune system, specifically T cells, to actively attack cancer cells while leaving healthy somatic cells unharmed. Cancer cells in tumors overexpress programmed death ligand 1 (PD-L1) which allows them to inactive the immune response via T cell suppression [1]. Countless mechanisms for immune-based therapies are being developed to reverse and prevent the inactivation of T cells including chimeric antigen receptor T-cell treatments, anti PD-1 therapy, and adoptive cell therapy. Although effective in nature, only around 20-50% of patients respond to treatment with immunotherapy depending on the type of cancer [2]. The low efficacy rate of current therapeutics suggests that alternative mechanisms exist in the tumor microenvironment (TME) to inactivate the immune system that current therapies do not take into account.

One such component of the TME is cancer-associated fibroblasts (CAFs), which have been implicated in serving an immunosuppressive role. Compared to normal human fibroblasts, CAFs have significantly greater proliferation, enhanced migratory capacity, and increased secretion of growth factors and chemokines. Cancers take advantage of these secreted factors to aid in their own personal growth [3]. Data suggests that chemokines released by CAFs, such as CCL2, attract circulating monocytes which are then converted into myeloid derived suppressor cells (MDSCs) [4]. MDSCs consequently suppress T-cells and natural killer cell proliferation up to 90% [5]. **However, the mechanism through which CAFs convert monocytes into MDSCs has yet to be confirmed.**

Prostaglandin E_2 (PGE_2) is a lipid released by CAFs into the TME that plays a role in mediating immunosuppression. CAFs are the primary source of PGE_2 in the TME, and studies have shown that removing PGE_2 from the TME inhibited fibroblast mediated dysfunction of natural killer cells and reduced the speed of neuroblastoma tumor growth [6][7]. Granulocyte-macrophage colony stimulating factor (GM-CSF) has also been shown to be involved with immunosuppression via the differentiation of monocytes into MDSCs in various pancreatic cancer models. GM-CSF alone was enough to convert human peripheral blood mononuclear cells (PBMC) into MDSCs [8]. **No causal link has yet been established between PGE_2 and GM-CSF released by CAFs and the differentiation of monocytes into MDSCs.**

Objectives

- Determine which factors produced by CAFs convert monocytes into MDSCs
- Determine the mechanism through which CAFs suppress the immune system

Hypothesis

Secreted PGE_2 and GM-CSF from CAFs are responsible for the conversion of monocytes into MDSCs which suppress the immune response in the TME.

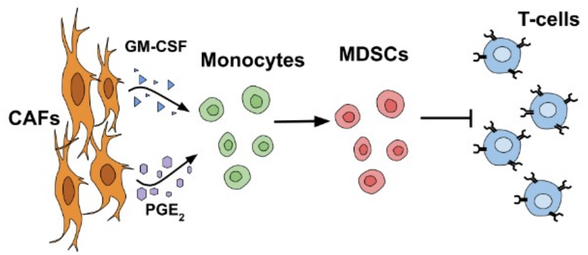

Figure 1. **Proposed Conceptual Diagram of CAF Immunosuppression.**

Methods

Cell Lines: Human primary Adenocarcinoma CAFs and human primary Squamous Cell Carcinoma CAFs isolated from human lung cancer patients were acquired from ATCC, WI-38 is a normal embryonic primary lung fibroblast cell line acquired from ATCC, MiaPaCa is an epithelial cell line derived from pancreatic tumor tissue acquired from ATCC, A375 is an epithelial cell line derived from malignant melanoma skin tissue acquired from ATCC.

Culture: Adenocarcinoma CAFs and Squamous Cell Carcinoma CAFs were cultured in Vitro Biopharma MSC-GRO complete media (low serum, 1% L-glutamine, 1% penicillin/streptomycin). WI-38, A375, and MiaPaCa were cultured in complete DMEM (10% Fetal Bovine Serum, 1% L-glutamine, 1% penicillin/ streptomycin) and incubated at 37°C.

CAF Induction: WI-38s were cocultured with cancer cells (A375, MiaPaCa) using transwell plates. WI-38s were plated at the bottom at a concentration of 500,000 cells, while the cancer cells were plated on top of the membrane.

Supernatant Collection: Cancer educated fibroblasts were cultured for 72 hours for supernatant collection. Supernatant from primary fibroblasts were collected after a 72 hour culture and supernatant was collected.

PGE_2 and GM-CSF ELISA: Supernatants were collected as described above. Manufacturer protocol was followed to measure total PGE_2 and GM-CSF concentrations in samples.

Human Cytokine Array: Supernatants were collected as described above. Manufacturer protocol was followed to obtain inflammatory cytokine profiles for each sample.

Monocyte Coculture and Flow Cytometry: Monocytes were isolated from frozen leukopak PBMC utilizing the auto MACS Pro Separator. Adenocarcinoma cells were plated in a 6-well plate at a concentration of 500,000 cells/well and allowed to adhere for 4-6 hours prior to the addition of 1 million monocytes directly on top. Following a 96-hour incubation time, flow cytometry was ran to assess the resulting monocyte population.

Results

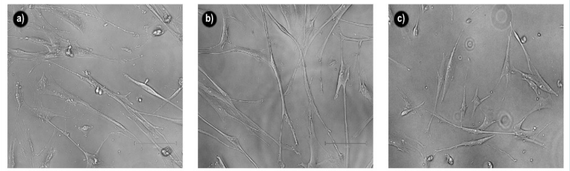

Figure 2. **Fluorescent Light Microscopy Images (20x).** a) WI-38, b) Squamous Cell Carcinoma CAFs and c) Adenocarcinoma CAFs

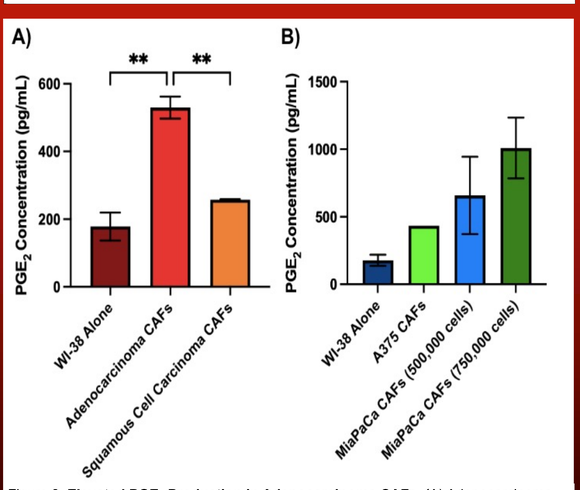

Figure 3. **Elevated PGE_2 Production in Adenocarcinoma CAFs. (**A) Adenocarcinoma CAFs produced significantly higher PGE_2 than both WI-38 and Squamous Cell Carcinoma after 72 hours as determined through a one way ANOVA and Tukey Test (n=2, p<0.01). (B) The graph displays PGE_2 concentrations for *in vitro* induced CAFs cocultured with cancer cells. Although the results of a one-way ANOVA were not statistically significant (n=2 for all groups except A375 where n=1), an upward trend is evident for all cocultured WI-38 vs WI-38 alone.

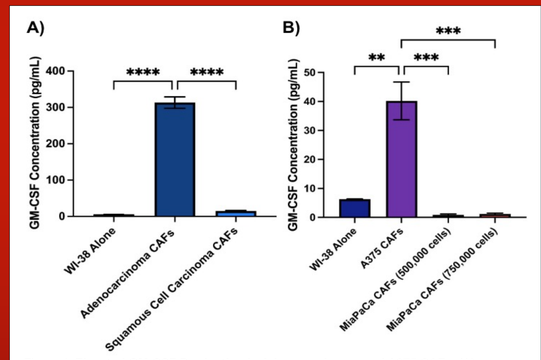

Figure 4. **Elevated GM-CSF Production in Adenocarcinoma and A375 CAFs.** (A) Adenocarcinoma CAFs (n=8) produced significantly higher GM-CSF than both WI-38 (n=2) and Squamous Cell Carcinoma (n=4) after 72 hours as determined through a one way ANOVA and Tukey Test (n=2, p<0.0001). (B) A375 induced CAF supernatant displayed significantly greater GM-CSF concentrations than WI-38 (n=2, p<0.01) and MiaPaCa CAFs plated at a concentration of 500,000 cells/well and 750,000 cells/well (n=2, p<0.001) as determined through a one-way ANOVA and Tukey Test.

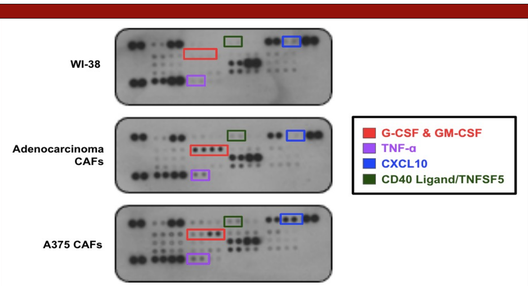

Figure 5. **Human Cytokine Array Highlighting Differential Gene Expression Between Samples.** G-CSF, GM-CSF, TNF-α, and CD40 Ligand/TNFSF5 are highly expressed in Adenocarcinoma CAFs and A375 CAFs but not in WI-38. Additionally, CXCL10 is expressed in A375 CAFs but not in WI-38 or Adenocarcinoma CAFs.

Results

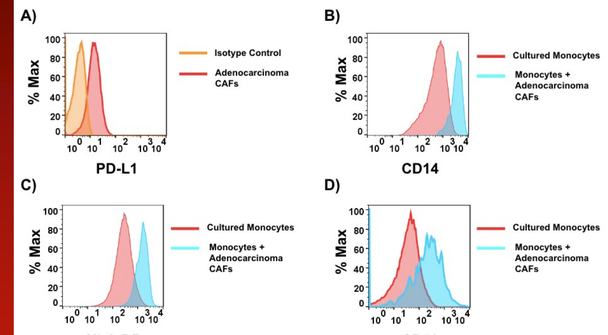

Figure 6. **Flow Cytometry of MDSCs.** (A) Adenocarcinoma CAFs cultured alone had elevated expression of immunosuppressive PD-L1 as compared to the isotype control. (B) Monocytes co-cultured with Adenocarcinoma exhibited high levels of CD14, signifying monocytes are becoming activated. (C) They also had elevated HLA-DR compared to cultured monocytes alone. HLA-DR low populations is a typical marker for MDSCs. (D) Co-cultured monocytes had increased CD73 levels when compared to cultured monocytes, which is an immunosuppressive marker.

Conclusions and Future Directions

•It is evident that both PGE_2 and GM-CSF are upregulated in Adenocarcinoma patient CAFs and A375-induced CAFs when compared to WI-38 alone. While MiaPaCa-induced CAFs did exhibit higher concentrations of PGE_2 than WI-38 and A375 CAFs, they also expressed less GM-CSF than both. This data suggests that there may also be other factors involved in the tumor, CAFs, and MDSC setting.

•TNF-α, CXCL10, and CD40 Ligand appear to be distinguishing markers of CAFs and may play a potential role in the conversion of monocytes into MDSCs in conjunction with PGE_2 and GM-CSF. However, many immunosuppressive factors are also expressed in non educated WI-38 so they may not serve as the ideal control. This could be due to the fact that WI-38 fibroblasts were isolated from embryonic lung tissue, so future directions involve the use of adult human primary lung fibroblasts as a control for experiments moving forward.

•Monocytes cocultured with Adenocarcinoma CAFs upregulated HLA-DR, whereas the typical sign of MDSCs is HLA-DR suppression. Regardless, we are confident that the monocyte population is immunosuppressive and differentiated into MDSCs based on high levels of CD73, which suggests that a dichotomy exists between monocytes differentiated from *in vitro* conditioned CAFs vs patient samples. This finding is further corroborated by evidence that suggests MDSCs don't exhibit low HLA-DR levels in the tumor microenvironment itself.

•Future directions involve confirming the immunosuppressive nature of MDSCs beyond surface markers by running a proliferation assay with T-cells. We also aim to study the interaction of CAFs with T-cells directly to investigate whether or not alternate pathways of immunosuppression are present beyond MDSCs.

•Gaining a clearer understanding of other immunosuppressive mechanisms at work in the TME is essential for improving current immunotherapy protocols. If the role of CAFs in immunosuppression is determined, novel therapeutics can target various interactions such as PGE_2 and GM-CSF as supplements to standard immunotherapy to provide cancer patients with more effective treatments.

Acknowledgments and References

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