Diabetic Complications Consortium

Application Title: Single cell transcriptome sequencing of diabetic foot skin

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1. Project Accomplishments:

We have collected a total of 18 discarded patient specimens from lower extremity surgical procedures, which we have processed for single cell suspension generation and cryostorage in liquid nitrogen. We have proceeded to capture single cells and analyze 12 of these samples (4 from healthy skin, 4 from diabetic skin and 4 from diabetic foot ulcer skin) for single cell RNA sequencing with the 10x genomics platform. Moving forward we plan to analyze the remaining 6 samples (2 healthy, 1 diabetic and 3 from foot ulcer), while also collecting an average of 2 specimens per week to further expand our sample size.

In addition we have processed 26 patient samples for bulk RNA sequencing. At the foot level, these include 4 samples from diabetic foot ulcer skin and 3 samples from healthy foot skin. Moreover, we have processed 19 forearm biopsies from the following groups of patients: 4 from healthy controls, 5 from diabetics without ulcer, 5 from diabetics with ulcers that healed and 5 from diabetics with ulcers that did not heal.

2. Specific Aims:

Aim: Evaluate changes in immune, endothelial and stromal cell subpopulations by scRNA-seq in foot-ulcers and fore-arm of diabetic patients with impaired wound healing.

Results:

We performed systems biology analysis on the bulk RNAseq samples on commonly dysregulated genes using a master regulator (MR) approach that assists in identifying the key transcriptional regulators that may be responsible for wound healing. In master regulator analysis, after developing an interactive network of genes that converge as highly connected, upstream regulators were selected as key regulators. The regulators were ranked on the basis of multiple interactive network analysis parameters for assessing importance of the regulator in network stability and the degree of dysregulation (fold change) samples. The analysis identified activation of highly connected cohesive network of inflammatory genes (e.g. CD40, NFkB, IL6, IL1, IL1A) as MRs driving wound healing. Furthermore pathways enrichment analysis of these MRs shows significant over presentation in TREM1 signaling and dendritic cell maturation pathways. This clearly depicts the role of these pathways in wound healing via impacting highest number of MRs. We hypothesize that activation of these key inflammation related molecules leads to the conversion of chronic to acute inflammation resulting in improved wound healing. Further, this shift in inflammation from chronic to acute phase may be able to mobilize significant number of T cells and macrophages to wounds resulting in improved healing. The analysis also depicted significant

role of WNT signaling in wound healing that is involved in regulating inflammatory response, cell death as well as mobilization of stem cells at site of injury.

Singe cell analysis on DFU, DM and non-DM Human skin samples: Single cell suspensions were generated from discarded surgical specimens using standard 10X approach and samples with >80% viable cells were further selected for single cell transcriptome analysis. The single cells were subjected to scRNASeq analysis that involved preparation of single cell droplets, RNA amplification and library preparation and deep sequencing. In this study, we successfully generated the single cell transcriptome profiles of 3,766 and 3,469 cells from non-DM and DM patients respectively. The analysis also generated transcriptome profile of 1,300 cells from patients with active diabetic foot ulcers. Further, bioinformatics analysis based on transcriptome correlation identified clusters of cells with similar transcriptome states. Using established cell markers these clusters were annotated into different cell-types including macrophages (M), adipocytes (ADP), fibroblasts (FB), endothelial cells (EC) and different immune cells. Interestingly clustering analysis indicated Non-DM, DM, and DFU groups have transcriptionally distinct cell clusters of adipocytes, fibroblasts, ECs and immune cells. Fibroblasts and adipocytes are the most abundant cell populations characterized in scRNASeq analysis. These adipocytes/fibroblasts were spread over more than one transcriptionally distinct clusters demonstrating heterogeneity of expression that can only be characterized using single cell analysis. Heterogeneous forms of each cell type likely point towards the existence of subtypes or different states (activated or repressed) of each cell type. DFU group had significantly lower number of adipocytes as compared to DM and healthy control groups. The analysis also showed that DM patients have significantly higher number of mast cells as compared to non-DM controls. DFU group also have lower number of CD34+ vasculature cells that are considered important marker for predicting wound healing as compared to DM and non-DM groups. As a next step, we also performed supervised analysis among clusters of single cells to identify significantly differentially expressed genes associated with each cell type cluster. The pathways level analysis of adipocytes clusters associated genes depicted consensus dysregulation of multiple pathways including ILK, IL8, CXCR4 across DM, non-DM and DFU groups. On the other hand, one cluster of adipocytes from DFU patients depicted unique activation of genes regulated by MAP3K8, KLF4, IL13 pointing towards existence of specific population of adipocytes uniquely in DFU patients.

Since macrophages and their polarization is considered critical for healing of diabetic foot ulcers, we performed an analysis to estimate the abundance of total macrophages as well as their M1-M2 polarization on data single cell data from DM, non-DM and DFU groups. The analysis depicted that approximately ~9-10% of cells are macrophages in each group (i.e. Non-DM, DM, DFU). Further analysis of macrophage polarization toward pro-inflammatory/classically activated M1 and pro-healing/alternatively activated M2 phenotypes indicated that the DFU group has significantly more M1 as compared to M2 (Ratio (M1/M2=2.4 times) whereas the rest of groups have similar proportion of M1 and M2. To characterize the transcriptome landscape of non-DM, DM and DFU macrophages, we performed supervised analysis to identify the genes significantly associated with them. The analysis identified a set of 66 and 105 genes that are significantly differentially expressed in macrophages of DFU and Diabetes patients respectively as compared to non-DM controls. We performed systems biology analysis on dysregulated genes using a MR approach. In DFU patients MR analysis identified activation of highly connected cohesive network of inflammatory and angiogenesis related master regulators (e.g., STAT3, IL15, HIF1a). Similar analysis in DM patients identified activation of master regulators (e.g. SMAD3, CD44, TGFb).

3. Publications:

A manuscript is in preparation.