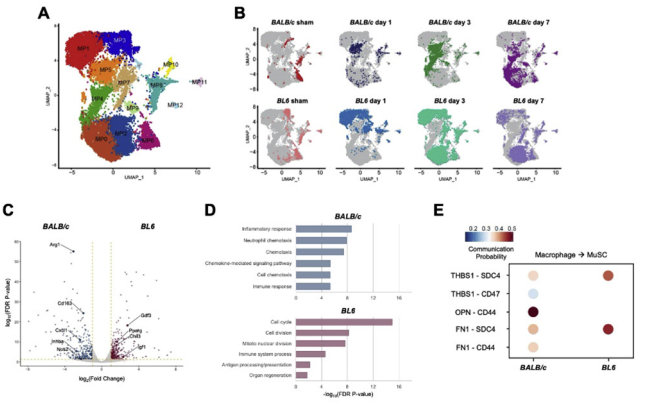
communication involving an array of cell types. These interactions are not well-understood, and the development of novel regenerative therapies for peripheral arterial disease will require new insight into the cellular heterogeneity and intercellular signaling that occurs in the ischemic limb. Macrophages play a role in orchestrating critical events in muscle regeneration; hence we sought to characterize their transcriptional signature and identify candidate signaling pathways in the context of limb ischemia.

Methods: We applied single-cell RNA sequencing to skeletal muscle obtained from regenerative (C57BL/6) and nonregenerative (BALB/c) mouse limbs at multiple time points following ligation of the femoral artery. We used CellChat, a computation tool, to predict signaling pathway activity between macrophages and muscle satellite cells (MuSC) based on our single-cell data.

Results: We identified 12 distinct macrophage populations in the regenerative and nonregenerative limb, including strain-specific macrophage clusters associated with the response to ischemia. Regenerative macrophages displayed a proliferative phenotype, while nonregenerative macrophages exhibited a persistent proinflammatory phenotype. Additionally, we identified several candidate signaling interactions between specific macrophage clusters and MuSCs. Notable potential interactions included enhanced FN1 to SDC4 and THBS1 to SDC4 signaling between regenerative macrophage and MuSCs, as well as OPN to CD44 signaling between nonregenerative macrophages and MuSCs.

Conclusions: Our study maps the dynamic macrophage response in the ischemic limb at single-cell resolution. This provides a valuable resource to investigate macrophage-mediated mechanisms of skeletal muscle regeneration in response to limb ischemia.



Macrophage response in murine model of limb ischemia.

A. Uniform Manifold Approximation Projection (UMAP) visualization of macrophage clusters identified by single-cell RNA sequencing (scRNA-seq) of *BALB/c* and *BL6* skeletal muscle following hindlimb ischemia (HLI) surgery.

B. UMAP visualization of macrophages at multiple time points following HLI surgery with cells colored by mouse strain and time point.

C. Differentially expressed genes in *BALB/c* vs. *BL6* macrophages isolated by fluorescence-activated cell sorting (FACS) on day 3 following HLI surgery. Gene expression determined by bulk RNA sequencing. Representative genes with known functions related to macrophage polarization or muscle regeneration are labeled.

D. Gene ontology (GO) enrichment analysis of day 3 scRNA-seq data highlighting pro-inflammatory gene expression profile in *BALB/c* macrophages and proliferative expression profile in *BL6* macrophages. Top 6 significantly enriched GO terms per mouse strain.

E. Predicted signaling interactions between macrophages and MuSCs based on CellChat analysis of day 3 scRNA-seq data. Shown are notable potential interactions distinguishing the regenerative response in *BALB/c* vs. *BL6* mice.

Fig.

Author Disclosures: D. Peters: Nothing to disclose; **Y. Xu:** Nothing to disclose; **X. Lin:** Nothing to disclose; **X. Wei:** Nothing to disclose; **Y. Diao:** Nothing to disclose; **K. Southerland:** Nothing to disclose

22-VIRC-478-AHA-VD

Mixed-lineage Leukemia 1 Regulates T-Cell Phenotype and Inflammation in Diabetic Wounds

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Background: Nonhealing wounds in patients with type 2 diabetes are a major cause of increasing morbidity and mortality. We and others have shown that a dampened local initial inflammatory response, followed by a prolonged proinflammatory state, is typical of diabetic wound healing. An imbalance between $CD4^+$ T_H17 and Treg cells has been shown in other diseases states to be responsible for pathologic proinflammatory states.

Methods: Using human diabetic wound single cell sequencing and murine wound healing models, we have identified that diabetic wounds express fewer regulatory T cells (Tregs) ($CD4^+CD25^+FoxP3^+$) compared with their nondiabetic counterparts. Previous literature has shown Notch signaling drives naïve $CD4^+$ differentiation into Tregs; thus, we hypothesized that loss of Notch signaling in diabetic T cells decreased wound Tregs.

Results: We found that amount of Notch1 and Notch2 receptor was decreased in in vivo diabetic wound T cells compared with nondiabetic $CD4^+$ cells as measured by flow cytometry and quantitative polymerase chain reaction. We also found decreased Notch intracellular cleaved domain in in vivo diabetic T cells. We also found that mice with $CD4^+$ specific deficiency in Notch signaling ($CD4^{f/f}DNMAML^{cre+}$) demonstrated worse wound healing and decreased wound Tregs compared with their $CD4^{f/f}DNMAML^{cre-}$ littermate controls. We performed chromatin immunoprecipitation analysis of the promoters of *Notch1* and *Notch2* and found decreased tri-methylated lysine 4 on histone 3 (H3K4me3) in in vivo diabetic T cells from wounded *C57BL/6* mice. We then found there was a decrease in MLL1 in in vivo diabetic T cells. Furthermore, in vivo T cells from wounded $MLL^{f/f}CD4^{cre+}$ mice demonstrated decrease of H3K4me3 at promoters of *Notch1* and *Notch2*, decreased Notch intracellular cleaved domain, decreased Notch gene expression (*Hey1, Hey2, Hes1*), decreased wound Tregs, and impaired wound healing compared with their $MLL^{f/f}CD4^{cre-}$ littermate controls.

Conclusions: In diabetic wound $CD4^+$ cells, a decrease of MLL1 regulates Notch signaling and subsequently decreases Tregs, contributing to pathologic prolonged inflammation and impaired healing in diabetic wounds.

Author Disclosures: W. J. Melvin: Nothing to disclose; **K. Gallagher:** Nothing to disclose; **C. Audu:** Nothing to disclose; **E. Barrett:** Nothing to disclose; **A. Joshi:** Nothing to disclose; **K. Mangum:** Nothing to disclose; **S. Wolf:** Nothing to disclose; **S. Sharma:** Nothing to disclose; **A. T. Obi:** Nothing to disclose; **B. Moore:** Nothing to disclose

22-VIRC-498-AHA-VD

Distinct Satellite Cell Trajectories Characterize Regenerative and Nonregenerative Responses in the Ischemic Limb

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Background: Skeletal muscle regeneration is a critical determinant of outcomes in the ischemic limb. The precise mechanisms of skeletal muscle recovery in the ischemic limb remain unknown. A major hindrance to fully understanding the regenerative process in limb ischemia is determining the heterogeneity and cell fate decisions of muscle satellite cells (MuSCs).

Methods: We analyzed muscle regeneration in C57BL/6 (regenerative phenotype) and BALB/c (nonregenerative phenotype) mice undergoing limb ischemia using single-cell RNA sequencing spanning four distinct time points (no injury, days 1, 3, and 7). We also performed trajectory inference to observe stage-specific regulatory programs during this dynamic process.

Results: We identified nine distinct MuSC populations in both strains (Figure, *A*). Pseudotime analysis presented an organized regeneration trajectory of cells from quiescent to proliferative to differentiated MuSCs (Figure, *B, C*). Further analysis demonstrated disparate MuSC fate decisions between C57BL/6 and BALB/c mice (Figure, *D, E*). C57BL/6 mice displayed an organized progression of cells from a quiescent sate to proliferating and committed myoblast. In contrast, BALB/c mice demonstrated aberrant MuSC regeneration, highlighted by precocious differentiation (Figure, *E*). Furthermore, gene set enrichment analysis confirmed impaired MuSC proliferation in BALB/c mice (Figure *F*).

Conclusions: These findings advance our understanding of MuSC fate decisions in the ischemic limb and provide a potential mechanism for myopathy observed in patients with peripheral artery disease.