

## Localization of Clonally Expanded CD8+ T Cells in Rejecting Kidney Allografts

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**Introduction:** Acute cellular rejection (ACR) of kidney allografts is mediated by CD8+ T cells. Previous work by our group used single-cell RNA-sequencing (scRNAseq) techniques and identified a limited number of clonally expanded CD8+ T cells in rejecting kidney allografts. These expanded CD8+ T cells expressed specific activation markers (GNLY, ITGAE, and KLRG1) which were not expressed in unexpanded CD8+ T cells. Here, we utilized multiplex immunofluorescence techniques to spatially localize these markers of CD8+ T cell activation in and around damaged kidney tubules in allograft biopsies of patients experiencing ACR.

**Methods:** FFPE tissue blocks and slides of kidney biopsies from ten patients with biopsy-proven ACR under belatacept (CTLA4-Ig, n=3), iscalimab (anti-CD40 mAb, n=3), or tacrolimus (CNI, n=4) maintenance immunosuppression, along with three patients with no rejection, were obtained from the University of Cincinnati Histopathology Core Laboratory (UCHCL) in accordance with the IRB (#2017-4696, 2019-0469). Slides were deparaffinization and sequentially stained over a period of 6 days for individual primary antibodies (CD8, GNLY, ITGAE, KLRG1, panCK/EpCAM, and DAPI) using the Opal 6-Plex Detection Kit. GNLY, ITGAE, and KLRG1 were selected based off previous scRNAseq data, while panCK and EpCAM identified kidney tubules. After overnight incubation at 4C, each primary antibody was followed by a secondary antibody and an opal tyramine fluorophore (Opal 520, 540, 570, 620, and 690). Images were taken using the CCHMC Confocal Imaging Core's Nikon AXR Inverted Confocal microscope. Numbers of CD8+ T cells and their activation markers in rejecting versus non-rejecting kidneys were compared using a student t-test and correlated with their Banff rejection score.

**Results:** Selected T cell activation markers were successfully identified in rejecting kidney allograft through multiplex immunofluorescence, confirming protein-level expression of these markers. We found greater numbers of CD8+ T cells present per region of interest (ROI) in rejecting allografts (94.66, 95%CI = 39.7-149) compared to non-rejecting kidneys (13.3, 95%CI = 1.64-28.3) (p=.003). Rejecting samples also showed greater numbers of KLRG1(33.6, 95%CI = 28.4-38.8) and ITGAE (10.6, 95%CI 4.41-16.9) compared to non-rejecting allografts (0.66, 95%CI = 0-3.52 & 2.67, 95%CI = 0-9.83) (p<.001, p=.02). GNLY, granules released by CD8+ T cells to cause damage, was almost exclusively present on kidney tubules in rejecting kidney samples and was largely absent in non-rejecting kidney samples, indicating the role of GNLY in causing tubule damage.

**Conclusion:** Multiplex immunofluorescence confirmed protein levels of T cell activation markers identified in the clonally expanded CD8+ T cells in our scRNAseq data. Compared to non-rejecting allografts, rejecting-allografts had significantly more graft-infiltrating CD8+ T cells. Further, intragraft activated CD8+ T cells were found localized to the kidney tubules, indicating their culpability in damaging donor kidney and driving ACR.

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