Recapitulating Lymph Node Stromal Cell Reticula in Type 1 Diabetes Using Gelatin Scaffolds

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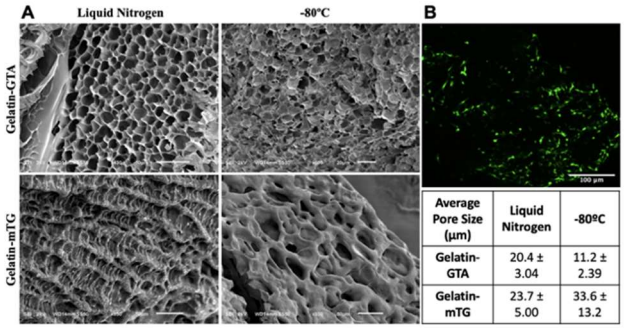
Statement of purpose: Type 1 Diabetes (T1D) is an autoimmune disease precipitated by the T-cell mediated destruction of insulin-producing pancreatic β-cells. T1D results from impaired central and peripheral tolerance and exploiting tolerance mechanisms therapeutically can protect against this disease. Peripheral tolerance in lymph nodes (LNs) is in part maintained by fibroblastic reticular cells (FRCs), which are non-hematopoietic ($CD45^-$) stromal cells that express podoplanin (gp38) and lack CD31 expression. FRCs express and present peripheral tissue antigens, including β-cell antigens, to autoreactive T-cells as non-professional antigen-presenting cells. FRCs also create interconnected reticular networks that confer structural support to LN. During adaptive immune responses, FRCs allow the LN to expand to accommodate T-cell proliferation by relaxing their actin cytoskeleton. In the context of T1D, FRC frequency and FRC expression of T1D-related self-antigens are decreased in LNs, likely affecting the capability of FRCs to interact with autoreactive T cells and promote tolerogenic engagement to reduce their diabetogenic potential. Our lab has previously shown that FRC reticular properties are also altered in T1D and developed a tissue-engineered 3D *in vitro* model of FRC reticula that recapitulates these reticular alterations to quantify autoreactive T-cell engagement in our 3D model. We showed that 3D culture of FRCs improves interactions with T-cells, resulting in more productive engagement of antigen-specific T-cells. We have assessed the reticular properties of FRC networks in LNs from murine models of T1D (NOD) and diabetes-resistant controls (NOR) and of 3D models with NOD and NOR-derived FRCs and shown that NOD FRC reticula have looser reticular pores than NOR controls. However, the reticular pores of engineered 3D FRC reticula using commercially available SpongeCol® collagen scaffolds with interconnected pores with diameters ranging from 100 to 200 µm are larger than the FRC reticular pores in LNs ranging from 15 to 20 µm in diameter. The goal of this work is to create gelatin scaffolds with smaller pore diameters that recapitulate the LN FRC reticula more closely. Creating these tunable scaffolds will allow us to form an engineered FRC reticula with LN-relevant pore sizes to study FRCs in a physiologically relevant environment and understand their role in T1D pathogenesis to develop novel therapies for peripheral tolerance induction based on FRCs.

Methods: 2% Glutaraldehyde (GTA, Sigma) solution was added to a 5% sterilized gelatin (type B, Sigma) solution to achieve a final 0.1% (v/v) GTA concentration. The mixed solution was allowed to gelate overnight (ON). The cross-linked scaffolds were placed in 100 mM glycine solution (Sigma) at room temperature (RT) for 1 hour to discard unreacted GTA aldehyde groups. The scaffolds were placed in Water for Injections (Gibco Thermofisher) for three consecutive 24 hour-washes. Scaffolds were frozen at -80ºC ON or via rapid freezing using liquid nitrogen (LN2) and lyophilized ON. Alternatively, gelatin (type B, Sigma) was dissolved in PBS at 50ºC to make a 4% solution and filtered through a 0.22 µm sterile filter. Microbial transglutaminase powder (mTG, ACTIVA®) was dissolved in PBS at RT to obtain a 10% solution. mTG and gelatin were then mixed in a 1:3 ratio and allowed to gelate at 37ºC ON. The hydrogels underwent the same freezing and freeze-drying conditions as above. A JSM-6010PLUS/LA analytical scanning electron microscope (JEOL) was used to image the morphology of the dry scaffolds after gold sputtering (Desk V, Denton Vacuum) and quantify pore sizes. For cell viability studies, female NOD 12-wk old mice-derived peripheral LN FRCs were transduced with lentivectors to express GFP. Scaffolds were sterilized under ultraviolet light for 1 hour, and 200,000 FRCs were seeded onto each scaffold and allowed to attach ON and then imaged with a confocal microscope.

Results: Gelatin-GTA-LN2 scaffolds showed the smallest, most interconnected and homogenously sized pores with pore diameters in the 20 µm range, which recapitulate the LN reticula pore size (**Fig. 1A**). FRCs attached to the scaffolds and were viable after ON culture as assessed by GFP expression and confocal microscopy (**Fig. 1B**).



Average Pore Size (µm)	Liquid Nitrogen	-80ºC
Gelatin-GTA	20.4 ± 3.04	11.2 ± 2.39
Gelatin-mTG	23.7 ± 5.00	33.6 ± 13.2

Fig. 1. (A) SEM micrographs of produced scaffolds. Gelatin-GTA crosslinked scaffolds were compared to gelatin-mTG crosslinked scaffolds; Rapid freezing in LN2 was compared to -80ºC ON freezing. (**B**) Viability of GFP+ FRCs in gelatin-GTA-LN2 scaffolds by confocal and average pore sizes of scaffolds.

Conclusion: We optimized gelatin scaffold fabrication by freeze-drying and obtained scaffolds with pore sizes in the desired range of LN FRC reticula. Next, we will use these scaffolds for tissue-engineering 3D FRC reticula and quantify interactions with autoreactive T cells by flow cytometry and T cell tracking *in vitro* and *in vivo*.