**Aim 1: To determine the transcriptional immune landscape of ILC2s in viral myocarditis**

Our first aim involves characterization of cardiac innate lymphoid cells type 2 (ILC2) and their genomic level during acute viral (CVB3) myocarditis to unravel clues about their pathogenesis of this disease and more generally, myocarditis. The first subaim focused on single-cell RNA sequencing (scRNA Seq), while the second subaim involved spatial transcriptomic approaches.

These experiments are costly. Before obtaining original data, we searched for publicly available scRNA seq datasets to optimize our analysis strategy. To our knowledge, adequate (CD45-enriched) datasets do not exist for CVB3 myocarditis. Thus, we utilized a dataset from another murine myocarditis model, experimental autoimmune myocarditis (EAM), which included a naïve state (day 0) as well as 14-, 21- and 60-days post-induction timepoints. In each point of the EAM timeline, we were able to identify cardiomyocytes and the major cardiac immune cell types, such as T cells, B cells, macrophages, NK cells, dendritic cells and neutrophils. Although ILC2s are an expectedly rare population, we were able to identify them in each day (Fig 1). Compared to naïve state, by day 14 they shrink in frequency (due to expansion of other populations), but thereafter continue to accumulate throughout disease.

Next, we characterized these ILC2s. We identified 3 subclusters of cardiac ILC2s present in EAM (for now, named 0, 1 and 2; Fig 2). We were able to find expression of genes relevant for immune function and Th2 responses, including *Pdcd1* (encoding PD-1), *Il5, Il13, Klrg1, Nmur1* and *Areg.* Many of these have important function in other tissue-specific ILC2s and warrant further characterization in cardiac ILC2s. These were differentially expressed among the 3 ILC2 clusters, particularly in subcluster 1. Other differentially expressed genes are shown in Figure 3.

Finally, to obtain clues about the importance of ILC2s in myocarditis pathogenesis, we investigated their cytokine gene expression. Although EAM is a Th1 and Th17-driven disease, we found high levels of Th2 cytokine expression by ILC2s. These included *Il4, Il5, Il13* and *Areg.* ILC2s were the main source of these cytokines, even surpassing T cell contribution, at all points of EAM from the naïve state to day 60.

In conclusion, we were able to identify ILC2s in a murine model of myocarditis, and further characterize subclusters and cytokine expression, giving us a solid analysis framework to perform this experiment in our original CVB3 myocarditis data which we plan to obtain.

The second subaim involves identifying and characterizing ILC2s using spatial transcriptomics technology. As in the previous subaim, we optimized analysis strategies before performing these costly experiments. We analyzed human myocardial infarction spatial transcriptomic datasets (using the 10x Genomics Visium platform). Notwithstanding the scarcity of ILC2s and technical limitations of the technique, we were able to identify these cells in infarcted areas by finding gene expression spots that we defined as *CD3E- NCR1*(encoding NK-p46)*- IL1RL1*(encoding ST2)*+ Thy1*(encoding CD90)+ (Fig 5). ILC2s were very sparse in non-involved myocardial tissue but expanded after myocardial infarction peaking at day 5 post-MI.

**Aim 2: To determine the role of ILC2s in CVB3-myocarditis**

Our second aim is a flow cytometry-centered approach to characterizing ILC2 population changes, expansion and immunophenotyping. We developed and optimized a spectral flow cytometry gating strategy that allowed us to identify ILC2s, their subsets and also to characterize macrophage populations (since we hypothesized many of the effects of ILC2s in myocarditis were exerted indirectly by affecting macrophages by action of secreted cytokines). This gating strategy is shown in Fig 6. In brief, ILC2s were defined as live singlet CD45+ lineage negative (CD3- CD11c- NKp46- CD19- Ter119- CD31- FcERIa-) CD90+ CD127+ cells. From this, we further identified 3 subsets based on KLRG1 and ST2, namely double positives, double negatives and ST2+ KLRG1-. We hypothesize these subsets correspond to the level of ILC2 activation status, but further work is warranted to confirm this hypothesis.

Having optimized our flow panel and gating strategy, we proceeded to study the kinetics of these populations during CVB3 myocarditis. We infected 8-week-old male and female mice with heart-passaged CVB3 and sacrificed them at 10 days post-infection, a time point we have identified as the peak of myocardial inflammation. We harvested hearts and performed flow cytometry as described above. The first observation was that there was indeed an increase in ILC2s (Fig 7). The expansion of ILC2s was greater in females than males. When we dissected the expansion of the different ILC2 populations, we observed that all three increased in CVB3-infected mice compared to naïve, WT controls. However, we only noted sex discrepancies, both at the steady-state, naïve state as well as the myocarditis-associated expansion, in the KLRG1- ST2+ compartment. Thus, this subpopulation might be responsible for the sex differences observed in ILC2 expansion during CVB3-myocarditis.

Next, we were interested in determining the expression of surface markers associated with activation as reported in ILC2 literature from other tissues. We chose CD25, CD69 and ICOS. In general, no sex or disease differences were observed with CD25. Unexpectedly, the frequency of CD69+ ILC2s of the three subsets decreased during CVB3 myocarditis. Because CD69 has regulatory functions, it is conceivable that its downregulation leaves cardiac ILC2s more prone to activation during disease. ICOS had been reported to become upregulated in many tissue ILC2s, but we saw a decrease in our cardiac single ST2 positive and ST2/KLRG1 double negative populations. The implications of these findings require more study.

The next steps in this aim involve the use of transgenic mice that allow specific deletions of ILC2s. Breeder pairs were obtained from Dr Andrew McKenzie and we have been expanding and genotyping our colonies since. Figure 9 shows an excerpt of our advances in obtaining experimental mice. This model is Rora fl/fl x IL7RCre. As can be seen, the genotyping shown indicates we have obtained IL7RCre homozygous mice, with varying Rora flox genotypes, and are close to obtaining robust colonies of breeder, experimental and control mice. Similarly, we have also bred CD4Cre x ICOS-DTR mice (also original breeder pair obtained from Dr McKenzie).

In conclusion, the initial preparatory steps for this aim; flow panel and analysis optimization and transgenic mouse colony establishment, have been undertaken, obtaining some preliminary positive and negative data regarding cardiac ILC2s in CVB3 myocarditis. With this in place experiments can be performed to generate reliable data.

It must be said that this project (both aims) will be continued by other members of the Cihakova lab building on the preliminary data yielded during the first part of this fellowship.