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Distinct immune characteristics distinguish hereditary and idiopathic chronic pancreatitis

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Abstract

Chronic pancreatitis (CP) is considered an irreversible fibroinflammatory pancreatic disease. Despite numerous animal model studies, questions remain about local immune characteristics in human CP. We profiled pancreatic immune cell characteristics in control organ donors and CP patients that included hereditary and idiopathic CP undergoing total pancreatectomy with islet auto-transplantation. Flow cytometric analysis revealed a significant increase in the frequency of CD68+ macrophages in idiopathic CP. In contrast, hereditary CP showed a significant increase in CD3+ T cell frequency, which prompted us to investigate the T cell receptor β (TCRβ) repertoire in CP and controls. TCRβ-sequencing revealed a significant increase in TCRβ repertoire diversity and reduced clonality in both CP groups versus controls. Interestingly, we observed differences in Vβ-Jβ gene family usage between hereditary and idiopathic CP and a positive correlation of TCRβ rearrangements with disease severity scores. Immunophenotyping analyses in hereditary and idiopathic CP pancreata indicate differences in innate and adaptive immune responses, which highlights differences in immunopathogenic mechanism of disease among subtypes of CP. TCR repertoire analysis further suggests a role for specific T cell responses in hereditary versus idiopathic CP pathogenesis providing new insights into immune responses associated with human CP.

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Introduction

Chronic pancreatitis (CP) is an inflammatory disease of the pancreas and remains a major source of morbidity in the US and Europe (1). CP is associated with an irreversible destruction of the pancreas parenchyma and fibrosis accompanied by severe abdominal pain, which leads to poor quality of life (2, 3). Alcohol and smoking are established as major etiological factors in adult CP. Still genetic variants and other idiopathic factors account for up to 20% of the cases, and especially genetic variants are a common risk factor in pediatric CP (4–7). Despite its prevalence, cost and societal impact, there is no active approved therapy or early diagnostic marker for CP. Inflammation is a known hallmark of CP and its pathogenesis, as demonstrated by studies of pancreas immune cell infiltrations in the human (8–10) and experimental models (11, 12). While many animal models have been used to study CP pathogenesis, questions remain regarding the translational accuracy of preclinical studies. Based on experimental model studies, the assumption in the field has been that the immune responses in CP are uniform or similar regardless of the etiology of CP, however this remains to be proven in human disease.

Human studies are hampered by lack of tissue access, and as a result many studies rely on the analysis of peripheral blood mononuclear cells, which might not necessarily reflect local immune responses. To address this gap in the field, we collected pancreas tissues from CP patients undergoing total pancreatectomy with islet auto-transplantation (TPIAT) (13–15) and deceased organ donors without known history of pancreatic diseases undergoing islet isolation. Unexpectedly, we found different types of CP had distinct immune cell compositions and T cell receptor β (TCRβ) repertoires such as, TCRβ gene usages and rearrangements. Interestingly, TCRβ rearrangement counts in CP patients had a positive correlation with disease severity scores. These findings implicate potential differences in immune mechanisms underlying hereditary versus idiopathic CP and their association with CP disease pathogenesis.

Results and Discussion
Pancreas tissues from CP patients (n=40) undergoing TPIAT and non-CP deceased organ donors (n=9) undergone similar islet extraction were collected consecutively from the University of Minnesota and University of California, San Francisco respectively. Subjects’ characteristics were comparable between the two groups except for age, weight and body mass index (Supplemental Table 1). Twenty-seven hereditary and thirteen idiopathic CP patients without known history of diabetes were included in the study. Significant differences were observed in age, gender, BMI, gross fibrosis severity score, and cigarette smoking history between two CP groups (Supplemental Table 2). Human pancreas tissues were used for different immune analyses including flow cytometry, Luminex assay and TCRβ-seq (Supplemental Figure 1, A-B).

As expected, deposits of CD45 (pan-leukocyte marker)-positive cells were significantly higher in CP compared to control pancreas tissues (Figure 1A). Given the increased leukocytes in CP, we sought to profile the immune subsets infiltrating the pancreas of CP compared to controls using flow cytometry. Innate and adaptive immune subset characterization included the identification of macrophage subsets, T cell subsets, mast cells, NK cells and NKT cells (Supplemental Figure 2). Interestingly, the frequency of CD3+ T cells was significantly increased in CP compared to controls. Even though the absolute count of CD68+ macrophages is increased in CP, their proportion within live CD45+ leukocytes was significantly reduced in CP compared to controls (Figure 1B) potentially due to the increased proportion of T cells. Among CD68+ cells, the percentage of CD68+CD11c- cells significantly increased in CP while the proportion of CD68+CD11c+ cells was significantly reduced in CP compared to controls (Supplemental Figure 3A). The CD68+CD11c+ population was enriched for cell expressing high levels of human leukocyte antigen-DR (HLA-DR) and CD11b, likely representing homeostatic functions such as antigen presentation and phagocytosis. Inversely, CP tissues appear to have expanded CD68+CD11c- cells that were negative or low for HLA-DR and CD11b expression (Supplemental Figure 3B).
Among the CP cases undergoing TPIAT in this study, a substantial proportion had hereditary/genetic mutations such as PRSS1, SPINK1, CFTR and CTRC, and the second most common etiology was classified as idiopathic CP without particular causes identified. When we compared these two cohorts, the frequency of CD68+ cells was significantly higher in idiopathic as compared to hereditary CP (Figure 1C, left). Among the CD68+ cell populations, the proportion of CD68+ CD11c- cells was significantly increased in idiopathic CP as compared to controls while CD68+ CD11c+ cell frequency was significantly lower in the both CP groups compared to controls (Figure 1C, middle and right). Our group previously reported anti-inflammatory macrophages (M2) are predominant in mouse CP and surgically resected pancreas specimens from human CP (11). Consistent with previous results, M2 macrophages were predominant in the CD68+CD11c- population of idiopathic and hereditary CP whereas no significant difference was observed between frequencies of M1 and M2 in the CD68+CD11c+ population of both CP groups (Supplemental Figure 4, A-B). Thus, even within the two CP subtypes, there are notable differences in immune cell composition with an expansion of CD68+ cells in idiopathic CP compared to hereditary CP. In addition to the cellular alterations, differential expression of cytokines and chemokines was prominent between hereditary and idiopathic CP by Luminex assay (Figure 1D). Consistent with the increased portion of CD68+ macrophages in idiopathic CP compared to hereditary CP, CCL7 (monocyte chemotactic protein 3), was the most significantly increased analyte in idiopathic versus hereditary CP (Figure 1E). Other significant differences included increased ratio of M2 (IL4,IL13) to M1 (TNFA) cytokine expression in idiopathic versus hereditary CP (Supplemental Figure 4C), higher innate (IL21, IL23) and Th2 (IL5, IL9, IL31) cytokines in idiopathic CP, whereas IL6 and LIF were higher in hereditary CP. Taken together with the macrophage profiles above, these cytokine profiles suggest idiopathic CP is locally enriched in innate immune cells, macrophages compared to hereditary CP.
Next, we compared the composition of the T cell population. Among CD3+ T cells, the percentage of CD4+ T cells was increased in CP while that of CD8+ T cells was significantly diminished in CP compared to controls (Figure 2A and Supplemental Figure 5A). Unlike the macrophage findings above, hereditary CP had a significantly higher proportion of T cells compared to controls or idiopathic CP (Figure 2B, left). Upon stratifying the T cells, there was a trend towards increased proportions of CD4+ T cells in both CP versus controls whereas CD8+ T cell frequency was significantly reduced in both CP compared to controls (Figure 2B, middle and right and Supplemental Figure 5B). Interestingly, CD4+ T cell subpopulations including T-BET+(Th1), GATA3+(Th2), RORγt+(Th17/22) and CD25+FOXP3+(Treg) were more expanded in hereditary CP than idiopathic CP as shown in average frequencies of different T cell subpopulations (Figure 2C) suggesting functionally active CD4+ helper T cell subsets may play critical roles in the pathogenesis of hereditary CP. Typically CP is characterized by a fibrotic condition with injury-driven inflammatory responses (16). Innate and adaptive immune cells contribute to pathologic fibrosis in different diseases (17, 18). We examined fibrosis of pancreatic tissues by trichrome staining. Both CP tissues showed a significantly higher proportion of fibrotic tissue area compared to controls (Supplemental Figure 6, A-B). Although there was a trend towards higher fibrosis in hereditary CP, this was not statistically significant between the two CP groups (Supplemental Figure 6C).

As a result of flow cytometry analyses with pancreatic immune cells, the ratio of CD3+ T cell frequency to CD68+ macrophage frequency in the pancreas was significantly higher in hereditary versus idiopathic CP (Figure 3A). Hereditary CP had a distinct immune cell distribution with greater T cell proportion, whereas idiopathic CP had expanded CD68+ macrophages suggesting unique immune mechanisms underlying the different etiology of CP groups. TCR clonality and diversity have been found to affect a wide variety of disease conditions including malignancy and autoimmune disorders (19–21). Given the differences in immune profiling among CP pancreata, we hypothesized there might be alterations of the TCR
repertoire in pancreas infiltrating T cells. To investigate this, genomic DNA (gDNA) was isolated from pancreas tissues of control donors and CP patients. The TCRβ repertoire was examined by sequencing the third complementarity determining region 3 (CDR3) loop of TCRβ and adjacent regions, which is typically the contact region for engaging antigenic peptides (Supplemental Table 3, Figure 3B). The number of total T cells and unique rearrangements were significantly increased in CP groups, especially in hereditary CP compared to controls (Figure 3C and Supplemental Figure 7A). TCR template diversity index was also significantly higher in both CP groups (Figure 3D and Supplemental Figure 7B left). However, productive clonality of CP groups, especially in idiopathic CP, was significantly lower than controls (Figure 3E and Supplemental Figure 7B right). These results implicate the increased number of T cells in CP was not due to the clonal expansion of infiltrating T cells but rather due to the increased variety of T cell clonotypes. Next, we compared CDR3β length distribution as an indicator of TCR repertoire change and T cell response to antigens (22, 23). Both CP had a tendency to be skewed toward shorter CDR3β length than controls although the mean length was not significantly different (Supplemental Figure 7C).

Next, we looked at the pattern of TCRβ V/J paired gene family usage among CP groups and identified shared V/J gene combinations among the groups. The pattern of V/J gene family usage was comparable across groups, and the dominant top 3 Vβ gene families were TRBV7, 6 and 5 in all groups (Figure 4A). The mean frequency of Vβ gene paired with Jβ2 showed differentially used Vβ gene families including TRBV10, 19 and 23 between hereditary and idiopathic CP (Figure 4B) whereas a comparison of the Vβ-Jβ1 gene family showed TRBV5 as a differentially used gene family between two CP groups (Supplemental Figure 7D). We next sought to further analyze Vβ/Jβ gene combinations in CP compared to controls and found 9 significantly differentially used Vβ/Jβ gene combinations (Figure 4C). Further, inter-repertoire homology between patient repertoires was examined by identifying shared CDR3βs

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representing identical amino acid composition and length (Figure 4D). We found 20 identical CDR3βs shared by at least 4 subjects, with some CDR3βs shared only among hereditary CP patients, or only CP patients, and others shared by controls and CP. It is noteworthy that the number of productive, functional TCR rearrangements positively correlates with the disease severity score in CP (Figure 4E). As the disease severity score is measured by considering multiple factors such as, calcification, cysts, parenchyma color, blood content, fat content, fibrosis and ductal destruction, a positive correlation between the disease severity score and the functional TCR clonotype counts implicates the importance of relationship between the TCRβ repertoire and CP disease progression. Overall, TCR-seq data presented an imprint of distinct antigenic repertoires in CP compared to controls as well as distinct mechanisms underlying disease-associated pancreas infiltrating T cells in different subtypes of CP.

Here we uncovered distinct local immune characteristics in different subtypes of human CP, hereditary and idiopathic CP. Our study did not include alcohol etiology of CP, due to the insufficient number of these patients that undergo TPIAT (24). Remarkably, an increased T cell frequency was found in hereditary versus idiopathic CP whereas a higher proportion of macrophage population was observed in idiopathic CP compared to hereditary CP. This finding indicates distinct immune subpopulation-mediated mechanisms may exist in different etiology-driven CP pathogenesis. Since we could only access and analyze the tissue at the time of TPIAT, it is possible that differences in disease duration and extent of histological changes may influence distinct immune responses in the different CP groups. Although we find no significant difference in tissue fibrosis between the two CP groups that we utilized for our immune analysis, it would be worth to examine immune responses over time during CP progression if repeat tissue biopsies become safe and available in CP patients in the future. Our data also brings into light the translational aspect of animal model studies. Most if not all CP models are not genetically driven, and interestingly the immune profile of these models appears to resemble
that of idiopathic CP. It will be interesting in the future to compare the immune characteristics of genetically driven models with our findings in hereditary CP. Given the average disease severity score (pre-TPIAT clinical measurements) was significantly higher in hereditary CP compared to idiopathic CP and hereditary CP has a substantially increased risk of pancreatic cancer (6), our results further support potential roles of T cell-driven immune landscape in CP pathogenesis as well as its progression to malignancy.

To our knowledge, this is the first report of high-throughput TCRβ-seq with pancreatic T cells from CP patients. Our results unveil the TCR signatures of pancreas infiltrating T cells in hereditary and idiopathic CP by using gDNA isolation method, which has the least in vitro experimental manipulation. Our TCRβ-seq data showed the increased T cell infiltrates in CP with increased functional TCR rearrangements and clonotype diversity suggesting multiple antigenic or polyclonal T cell infiltrates in CP, which might be a unique inflammatory feature of CP due to the exposure to a variety of insults over disease progression. We also identified CDR3β motifs uniquely shared among hereditary CP or both CP groups implicating locally infiltrating T cells respond to disease-specific antigen targets although further analyses will be necessary to prove this notion. A positive correlation between TCR rearrangement counts and the disease severity score found in this study suggests TCR repertoire might serve as a prognostic predictor for CP progression and severity. Since the higher severity score is associated with a decline in islet yield and lower insulin independence rate in CP patients who received TPIAT (25), the number of TCR rearrangements might be an additional predictor and/or pathologic indicator for poor outcome of islet transplantation.

Overall, our studies show distinct immune characteristics in the pancreata of hereditary and idiopathic CP patients highlighting potential roles of identified immune subpopulations as key regulators of CP pathogenesis. Further in-depth single cell level analysis with antigen/epitope screening is likely to advance our understanding of disease pathogenesis.
mechanisms for the different etiology-driven CP syndrome. Such information will contribute to developing the cellular and animal models needed to enhance our understanding of how the specific immune responses drive the pathogenesis of these subtypes of CP and developing CP subtype specific therapies.
Methods

Detailed methods are provided in the Supplemental Methods.

*TCRβ sequencing data.* TCRβ sequencing data have been deposited in Adaptive Biotechnologies’ immuneACCESS database (doi:10.21417/BL2020JCI; clients.adaptivebiotech.com/pub/lee-2020-jci).

*Study approval.* For the use of human samples in research, the protocol was reviewed and approved by the University of Minnesota and Stanford University Institutional Review Board.
**Author contributions**

B.L. and A.H. designed experiments and wrote manuscript. A.H. provided overall guide and supervision. A.H., S.J.P., M.D.B. and J.W. arranged collaborations and/or provided CP patients’ tissues. G.S. provided control pancreatic tissues. B.L. performed all experiments and analyzed data. H.N. contributed to flow cytometry panel design and data analysis. J.Z.A. and D.M.L. contributed to TCR-seq data analysis. M.M.D., S.J.P., M.D.B., and J.W. reviewed manuscript and participated in the interpretation of data.
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References


**Figures and Figure legends**

**A** CD45 IHC

**B** CD45+ cells (%)  
CD3+ T cells (%)  
CD68+ cells (%)

**C** CD68+ cells (%)  
CD68+ CD11c+ cells (%)  
CD68+ CD11c- cells (%)

**D** Luminex data (Hereditary vs Idiopathic)

**E** CCL7 (MFI)

**Figure 1.** CD68+ macrophages are predominant in idiopathic CP compared to hereditary CP.  
(A) Immunohistochemistry staining using pan-leukocyte marker, CD45 (400x). Scale bars: 100μm. The percentage of CD45+ cell counts in total nuclei is presented as dot plot. (Mean ± SD, Unpaired two-tailed t-test) (B) The frequency of CD3+ T cells and CD68+ cells in live CD45+ cells from control(n=8) and CP(n=24). (Mean ±SD, Unpaired two-tailed t-test was used) (C) Bar graphs show frequencies of CD68+ cells and their subsets in live CD45+ cells from control(n=8), hereditary(n=15) and idiopathic(n=9) CP. (Mean ±SD, One-way ANOVA with Tukey’s multiple comparisons test). (D) Heatmap represents expression levels of analytes with mean fluorescence intensity (MFI) values by the human 62 multiplex Luminex assay (T-test p<0.05, FDR<0.25). Fold change of the average expression in idiopathic versus hereditary CP for each analyte. (E) Comparison of MFI values of most differentially regulated chemokine (CCL7) between hereditary(n=17) and idiopathic(n=8) CP (Mean ± SD, Unpaired two-tailed t-test was used.).  
*p<0.05, **p<0.01, ***p<0.001.
Figure 2. CD3+ T cells are more frequent in hereditary CP compared to idiopathic CP. (A) Representative plots of flow cytometry analyses of CD3+ T cells based on CD4 and CD8 expression in control(n=8) and CP(n=24) (Mean ± SD). Bar graphs show frequency of CD4+ or CD8+ T cells in control and CP (Mean ± SD, Unpaired two-tailed t-test). (B) Bar graphs represent frequencies of total CD3+, CD4+, and CD8+ T cells among live CD45+ cells from control(n=8), hereditary(n=15) and idiopathic(n=9) CP. (Mean ± SD was used. One-way ANOVA with Tukey’s multiple comparisons test) (C) Pie charts represent the average frequencies of T-BET+, GATA3+, RORγt+ and FOXP3/CD25+ T cell subsets in CD4, CD8 or DN (double negative) T cells from hereditary(n=15) and idiopathic(n=9) CP. *p<0.05, **p<0.01, ***p<0.001.
**Figure 3. TCRβ repertoire of pancreas T cells in control and CP.**

(A) Waterfall and dot plots show the ratio of CD3+ T cells to CD68+ macrophage frequency in control, hereditary and idiopathic CP. Identified gene mutations are indicated in individual hereditary CP patient. (Mean ±SD was used. One-way ANOVA with Tukey’s multiple comparisons test) (B) Top 100 most frequent rearrangements in each sample are ranked from bottom (#1 most frequent clone) to top (#100 most frequent clone), and samples are listed by their clonality order from left to right. (C) The number of productive rearrangements, (D) TCR clonotype diversity (Mean normalized Shannon-Wiener diversity index), and (E) Productive clonality are shown. (C-E) Comparison between control(n=5) and CP(n=13). (Non-parametric Mann-Whitney U-test) *p<0.05, **p<0.01, ***p<0.001.
Figure 4. Differences in TCRβ repertoire of pancreas T cells between hereditary and idiopathic CP. (A) Circos plots indicate frequencies of Vβ-Jβ productive gene usage in controls (n=5), hereditary (n=7) and idiopathic CP (n=6). The width of the Vβ-Jβ pair band is proportional to the frequency in each group. (B) The comparison of Vβ-Jβ2 gene family usage among groups is displayed by mean frequencies. (Mean ± SEM, One-way ANOVA with Kruskal-Wallis test, comparison between hereditary and idiopathic CP, *p<0.05) (C) Heatmap representing frequencies of Vβ-Jβ gene pairs which are significantly different (Significance Analysis of Microarray T-test, 90th percentile FDR=0) between control and CP. (D) Full length CDR3 amino acid sequences shared among at least 4 subjects. Numbers in squares represent the count of unique clonotypes in a subject’s repertoire with the CDR3β sequence indicated. (E) Correlation of the number of functional TCR rearrangements with CP disease severity score (n=13, Non-parametric Spearman correlation r=0.8361, p<0.001).