An Activated Immune and Inflammatory Response Targets the Pancreas of Newborn Pigs with Cystic Fibrosis

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Key Words
Cystic fibrosis · Pancreatitis · Flow cytometry · Inflammation · Lymphocytes · Neutrophils · Macrophages · NK cells

Abstract
Background/Aims: In cystic fibrosis (CF), pancreatic disease begins in utero and progresses over time to complete destruction of the organ. Although inflammatory cells have been detected in the pancreas of humans and pigs with CF, their subtypes have not been characterized. Methods: Using four-color flow cytometry, we analyzed the surface antigens of leukocytes in pancreas, blood, and mesenteric lymph nodes (MLN) of newborn pigs with CF (CFTR\(^{-/-}\) and CFTR\(^{F508/F508}\)) and in those without CF (CFTR\(^{+/+}\), CFTR\(^{+/F508}\), CFTR\(^{+/+}\)). Pancreatic histopathology was examined with HE stain. Results: CF pig pancreas had patchy distribution of inflammatory cells with neutrophils/macrophages in dilated acini, and lymphocytes in the interstitium compared to non-CF. B cells, effector (MHC-II\(^{+}\)) and cytotoxic (CD2\(^{+}\)CD8\(^{+}\)) \(\gamma\delta\) T cells, activated (MHC-II\(^{+}\) and/or CD25\(^{+}\)) and effector (CD4\(^{+}\)CD8\(^{+}\)) \(\alpha\beta\) T helper cells, effector natural killer cells (MHC-II\(^{+}\)CD3\(^{-}\)CD8\(^{+}\)), and monocytes/macrophages and neutrophils were increased in the CF pig pancreas compared to pigs without CF. Blood and MLN leukocyte populations were not different between CF and non-CF pigs. Conclusions: We discovered an activated immune response that was specific to the pancreas of newborn CF pigs; inflammation was not systemic. The presence of both innate and adaptive immune cells suggests that the disease process is complex and extensive.

Introduction
Cystic fibrosis (CF) is the most common life-threatening autosomal recessive disease in the US [1]. Caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, CF involves many organ systems, including the lungs, pancreas, liver, intestines, and vas deferens.

The pancreas is commonly involved in people with CF: 85% of CF patients have pancreatic insufficiency (PI), 50–65% have overt PI at birth, and 20–30% of pa-
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Pancreas has been activated. We discovered that there was activation of both the innate and adaptive immune systems in the pancreata of newborn pigs with CF. In contrast, the inflammatory cell populations in the blood and mesenteric lymph nodes (MLN) were not different between CF and non-CF pigs, suggesting that the pancreatic immune response was a localized rather than a systemic process.

Animals and Methods

Animals
All studies were approved by the University of Iowa Animal Care and Use Committee. CF (4 CFTR<sup>−/−</sup>, 1 CFTR<sup>−/−ΔF508</sup>), heterozygous (3 CFTR<sup>−/+</sup>, 1 CFTR<sup>−/+ΔF508</sup>) and wild-type (WT) (3 CFTR<sup>+/+</sup>) piglets were obtained from Exemplar Genetics (Sioux Center, Iowa, USA) and studied within 24 h after birth. Animals were euthanized with Euthasol (Virbac, Fort Worth, Tex., USA), followed by collection of intracardiac blood, pancreas and MLN.

Antibodies
The following antibodies were used to identify the cell surface markers of white blood cells: anti-CD2 (clone MSAa, mouse IgG2a and clone 1038H-5–37, mouse IgM); anti-CD3 (clone PPT3, mouse IgG1); anti-CD4 (clone 10.2H2, mouse IgG2b); anti-CD8 (clone 76–2–11, mouse IgG2a); anti-CD14 (clone MIL-2, mouse IgG2b); anti-CD25 (clone K231–3B2, mouse Ig1); anti-IgM (clone M160, mouse IgG1); anti-TCR γδ (clone PPT16, mouse IgG2b); anti-SWC1 (clone K263.3D7, mouse Ig1); anti-SWC8 (clone MIL3, mouse IgM); anti-MHC-II (clone 1038H-12–34, mouse IgG). Goat polyclonal antibodies specific for mouse immunoglobulin subclasses (Southern Biotechnologies Associates, Inc., Birmingham, Ala., USA) labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanine 5 (PE/Cy5, SpectralRed) or allophycocyanin (APC) were used as secondary immunoreagents.

Tissues and Pathology Examination
After euthanasia, pancreata were collected and immersed in fixative for 48–96 h, routinely processed, embedded, sectioned (4 μm), and stained with hematoxylin and eosin (HE). Histopathology examination was performed by a veterinary pathologist (D.K.M.), in a blinded fashion. Blood was collected with intracardiac puncture. MLN were isolated and placed in ice-cold phosphate-buffered saline (PBS) until it was processed for fluorescence-activated cell sorter (FACS).

Preparation of Cell Suspensions
Pancreas
Pancreas was minced and incubated in digestion media [RPMI-1640, 100 U/ml collagenase type IV (Sigma-Aldrich, St. Louis, Mo., USA), 2% fetal calf serum (FCS)] at 37 °C for 1 h. Digested tissue was filtered through a 70-μm cell strainer (BD Biosciences, San Jose, Calif., USA) and washed (400 g, 5 min, 4 °C) three times with PBS. Cells were pelleted by centrifugation at 400 g for 5 min and separated with 40%–80% Percoll gradient (GE Healthcare, Uppsala, Sweden) at 600 g for 20 min. Cell suspensions were then washed twice in cold PBS containing 0.1% sodium azide and 0.2% gelatin (PBS-GEL) prior to counting with hemacytometer.

Blood
Blood was collected in heparinized tubes. Red blood cells were lysed by hypotonic shock for 30 s by deionized water followed by addition of an equal volume of 2 × PBS and washed 3 times in PBS [18]. Cell suspensions were finally washed twice in PBS-GEL prior to counting with hemacytometer.
Far have shown that newborn flow cytometry, we grouped them as non-CF pigs. Our studies so differences between WT and heterozygous pigs (histology and test; \( p \)).

Samples were measured on a standard FACS Calibur flow cytometer (BDIS, Mountain View, Calif., USA). For each measurement, 200,000–700,000 events were collected. Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. The PCLysis software (BDIS, Mountain View, Calif., USA) was used for data processing. Lymphocyte and myeloid gate was set according to light scatter characteristics (forward versus light scatter). The approximate cellularity in individual tissues was determined by flow rate at flow cytometry acquisition (number of cells per second). In that case, all samples were handled by the same procedure starting with the same amount of initial tissue or blood, processed simultaneously, all samples were handled by the same procedure starting with the same amount of PBS-GEL and finally run at constant flow pressure through flow cytometry machine.

Statistics
Data were expressed as median with range. Differences between groups were analyzed using Mann-Whitney rank sum U test; \( p < 0.05 \) was considered as significant. Because there were no differences between WT and heterozygous pigs (histology and flow cytometry), we grouped them as non-CF pigs. Our studies so far have shown that newborn \( CFTR^{+/+} \) and \( CFTR^{F508/F508} \) pigs have similar pathology in the pancreas and other organs [21], therefore they were grouped as CF pigs.

Results

CF Pig Pancreas Has Increased Inflammatory Infiltrates

We observed no histological differences between WT and heterozygous pig pancreata. In contrast to non-CF pigs (fig. 1a, b), the CF pig pancreas contained a reduced number of acini and decreased cytoplasmic zymogen granule mass (fig. 1c, d). Neutrophils and macrophages were scattered within dilated acini. Lymphocyte aggregates were sometimes prominent in the interstitium, usually found adjacent to the dilated ducts. Acinar and ductal lumens were plugged and mucinous metaplasia were also detected; foci of duct proliferation were found in severely destroyed regions of the CF pig pancreas. These data are similar to that previously described in CF pigs [10, 12] and in humans with CF [15–17, 22].

Innate Immune Cells Are Increased in CF Pig Pancreas

To determine the abundance of neutrophils and macrophages in healthy vs. diseased animals, we performed flow cytometry of enzymatically dispersed CF and non-CF pig pancreata. Blood and MLN cell populations were similarly examined, using the SWC8 and CD14 antigens to identify monocytes, macrophages, and neutrophils. SWC8 is used to discriminate bone and blood marrow-derived granulocytes (SWC8+) from monocytes/macrophages (SWC8-) [23]. Swine CD14 is expressed at low levels in neutrophils, whereas monocytes/macrophages express higher levels of CD14 [24].

The CF pig pancreas exhibited a significantly increased proportion of monocytes/macrophages (SWC8–CD14+; fig. 2a) and neutrophils (SWC8–CD14+; fig. 2b) compared to non-CF pigs. Neutrophils were almost absent from the non-CF pig pancreas, a finding that clearly distinguished CF from non-CF pancreas (fig. 2b). There were no differences in the monocyte/macrophage/neutrophil populations in the blood or MLN of CF pigs, suggesting that the inflammatory response was not systemic.

Lymphocyte Subsets in CF Pig Pancreas

To determine the subtypes of lymphocytes infiltrating the CF pig pancreas, we performed flow cytometry. Lymphocyte cellularity of the pancreas was 20–40 times lower in CF pigs compared to WT pigs, as determined by flow rate acquisition. We used anti-IgM to identify B cells, anti-TCR\( \gamma \delta \) for \( \gamma \delta \) T lymphocytes and the presence of anti-CD3 plus absence of anti-TCR\( \gamma \delta \) to identify \( \alpha \beta \) T lymphocytes. CD3+CD8+ phenotype was used for identification of the NK cells. Figure 3 shows the percentages of lymphocyte subtypes in blood, MLN, and pancreas of CF and non-CF pigs. The proportion of B cells was significantly increased in the pancreases of CF pigs compared to non-CF pigs (fig. 3a). The B cell populations were not increased in the blood or MLN of CF pigs, suggesting that this was not a global response.

There were no differences between blood and MLN lymphocyte populations of CF and non-CF pigs, except
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**Fig. 1.** CF pig pancreas has increased inflammatory infiltrates. The histology images show pancreas from WT (+/+) (a, b) and CF (−/−) pigs (c, d). Compared to WT pigs, CF pigs have acinar atrophy, plugging, decreased zymogen granules and increased loose connective tissue. Neutrophils and macrophages were found within dilated acini (arrows), lymphocyte aggregates were prominent in the interstitium (block arrows) (c, d). HE. a, c ×20, b, d ×40.

**Fig. 2.** Myelomonocytic cells are increased in CF pig pancreas. Macrophages (SWC8−CD14+) (a) and neutrophils (SWC8+CD14+) (b) were significantly elevated in CF pig pancreas compared to non-CF (*p < 0.01). There were no significant differences in MLN and blood of CF and non-CF pigs. Myelomonocytic gate used for the calculation of cell percentages.
that NK cells were increased in the MLN of CF pigs (fig. 3b). Interestingly, the percentage of NK cells was significantly decreased in the CF pig pancreas compared to non-CF (** p < 0.01). NK cells were slightly higher in MLN of CF pigs compared to non-CF (* p < 0.05). αβ T cells (c) and γδ T (d) cells were not different between CF and non-CF pig pancreas, MLN and blood.

**Fig. 3.** Lymphocyte subsets in blood, MLN and pancreas. The proportion of lymphocytes (B cells, αβ T cells, γδ T cells and NK cells) were examined in the blood, MLN and pancreas of CF and non-CF pigs. **a** B cells were significantly elevated in CF pig pancreas compared to non-CF (* p < 0.01). There were no significant differences in B cells population of MLN and blood between CF and non-CF. **b** NK cells were significantly lower in CF pig pancreas compared to non-CF (** p < 0.01). NK cells were not different between CF and non-CF pig pancreas, MLN and blood. **c** γδ T cells were not different between CF and non-CF pig pancreas, MLN and blood.

**Fig. 4.** Effector αβ T helper cells are increased in CF pig pancreas. **a** The proportion of CD4⁺CD8⁺ αβ T cells was increased in CF pig pancreas compared to non-CF (* p < 0.01). This cell population was not increased in the blood and MLN of pigs with or without CF. **b, c** There were no changes in CD4⁺CD8⁻ or CD4⁻CD8⁺ T cell population. Cells were gated to all αβ T cells.

**Effect αβ T Helper Cells Are Increased in CF Pig Pancreas**

Beyond conventional CD4⁺ T helper and CD8⁺ T cytotoxic αβ T cells, the peripheral lymphoid system of swine contains so-called ‘peripheral double-positive (DP) αβ T cells’ that become CD4⁺CD8⁺ by subsequent expression of CD8α on CD4⁺ T helper cells after activation [18, 25]. These cells can proliferate in response to stimulation with recall viral antigen consistent with the hypothesis that this population in swine includes memory/effector T cells [23].

Flow cytometry analysis revealed that CD4⁺CD8⁺ DP T cell populations were significantly increased in CF pig pancreas (fig. 4a). There were no changes in CD4⁺CD8⁻ or CD4⁻CD8⁺ T cell population (fig. 4b, c). Blood and MLN T cell population were not different between CF and non-CF pigs. These findings suggest that the effector/memory αβ T cells are activated and possibly participate in the tissue damage of CF pig pancreas.
Cytotoxic αβ T Cells Are Increased in CF Pig Pancreas

CD25 is commonly used as an antigen to determine activation of T cells [26] while secondary MHC class II expression represents an activation status of effector T lymphocytes [27–30]. We asked whether cytotoxic T cells are activated in CF pig pancreas. FACS analysis for CD4, CD8, MHC-II and CD25 antigens revealed activation of CD4+CD8+MHC-II+CD25+ and CD4+CD8+MHC-II+CD25− αβ T cells in the pancreas of CF pigs (fig. 5a, b). CD4+CD8+MHC-II+CD25− and CD4+CD8+MHC-II−CD25− αβ T cells were not different between CF and non-CF pig pancreas (fig. 5c, d). There were no changes in the blood or MLN αβ T cell populations of CF pigs in comparison to non-CF pigs. These results show a shift among α/β helper cells in the direction of those that are activated/memory cells and suggest that these cells may contribute to organ damage in the CF pig pancreas.

CD2+CD8+ γδ T Cells Are Increased in CF Pig Pancreas

Pigs have large numbers of circulating γδ T cells that can proliferate following exposure to antigens [31]. All peripheral γδ T cells are negative for CD4 and can be divided into CD2+CD8−, CD2+CD8+ and CD2+CD8+ subsets. From these three subsets, only CD2+CD8+ γδ T cells are cytotoxic. It has been postulated that these cells develop from CD2+CD8− γδ T lymphocytes after activation [32].

To determine whether γδ T cell populations were different in CF pig pancreas, we performed flow cytometry studies using CD2 and CD8 antigens. The proportion of CD2+CD8+ γδ T cells was significantly increased in CF pig pancreas compared to non-CF (fig. 6a). The proportion of CD2+CD8+ γδ T cells was not different between CF and non-CF pig blood, MLN, and pancreas (fig. 6b). Blood and MLN CD2+CD8+ γδ T cell populations were not different between CF and non-CF pigs. These results show a shift towards activated CD8 (cytotoxic) T cells; these cells may contribute to damage in the CF pig pancreas.

Activated NK and γδ T Cells Are Increased in CF Pig Pancreas

CD2+CD8+ γδ T cells acquire MHC class II expression in the periphery indicating the full maturation and activation of this cell lineage [33]. Likewise, MHC class II positive NK cells represent activation status of these cells in pigs [27–30]. To determine whether γδ T cells and NK cells were activated in CF pig pancreas, we examined the MHC-II antigen expression of these cells. NK cells and γδ T cells were predominantly MHC-II antigen positive in CF pig pancreas compared to non-CF, indicating that they were activated and possibly causing cytotoxicity in this setting (fig. 7). MHC-II antigen expression was not different in the blood and MLN of CF and non-CF pigs.
Discussion

In this study, we found inflammatory infiltrates in the CF pig pancreas and characterized their subtypes using flow cytometry. Because flow cytometry studies do not provide anatomical origin of the cells in a tissue, we complemented our work with careful histopathological evaluations (fig. 1) [10, 12, 21]. We discovered that both the innate and the adaptive immune system cells were activated in the CF pig pancreas; the inflammatory response was localized to the pancreas, rather than a systemic reaction. This is the first study to characterize the subtypes of leukocytes in the CF pancreas using an animal model that closely resembles the human disease.

We found the infiltrating cells of the innate immune system (neutrophils, monocytes, macrophages, MHC-II+ effector NK cells) almost exclusively in the CF pig pancreas. Macrophages can be observed in the pancreas of humans with chronic pancreatitis [34], but the infiltration of the pancreas with neutrophils is unusual. Neutrophils can be seen in a rare form of chronic pancreatitis, idiopathic tumefactive chronic pancreatitis (TCP) that presents with pancreatic mass and obstructive symptoms [35]. The mechanisms that underlie the neutrophil infiltration in TCP are unknown and the histological findings are not entirely similar to those of CF.

We also observed the activation of adaptive immune system cells (B and T cells) in the pancreas of newborn CF pigs. An increased number of activated B cells seems to be unique to the CF pig pancreas, because T lymphocytes are the predominant cell types in humans with chronic pancreatitis [34]. The involvement of both B and T lymphocytes in the CF pig pancreas possibly represents a different immunological mechanism.

CD2+CD8+ γδ T cells were increased in CF pig pancreas and they were predominantly MHC-II+, indicating activation. The role of γδ T cells in the pig immune system is poorly understood. γδ T cells can downregulate inflammatory responses [36, 37] and constitute a first line of defense at mucosal surfaces, representing a link between innate and adaptive immunity [38, 39]. It is possible that γδ T cells are activated in CF pig pancreas as a reaction to the inflammatory response and provide cross-talk between the innate and adaptive immune systems.
Our results contrast with a well-known inflammatory disease process of the pancreas, chronic pancreatitis. Studies in humans with chronic pancreatitis (mainly alcohol related) suggest that the cell-mediated cytotoxicity and T cell responses (CD4+ and CD8+) are involved in the disease pathogenesis [34, 40, 41]. The activation of both the innate and adaptive immune systems in the CF pig pancreas suggests that the disease process is complex and extensive and involves multiple leukocyte subsets. The factor(s) that initiated the activation of these inflammatory cascades are currently unknown. Our findings in the pancreas of newborn CF pigs likely represent advanced disease stage manifestations. Analysis of earlier time points in utero may be helpful in identifying the initiating mechanisms and help us tease out pathways that are activated during early phases.

Although inflammatory cells (a mixture of neutrophils, lymphocytes, plasma cells) have been observed in the pancreas of humans [15] and pigs [10, 12] with CF, leukocyte subtypes have not been investigated. Previous studies used limited fetal tissues or samples from children that died at young ages with CF [15–17] and were performed when techniques to identify leukocyte subsets were unavailable. Today most children with CF are diagnosed by newborn screening and the median survival has increased to 37 years of age [42]. There is no practical way to obtain pancreas tissue from infants and young children at early stages of the disease. CF mice are not suitable for study because their pancreas is very mildly involved and does not contain inflammatory cells. With a pancreatic involvement similar to human pancreatic disease, the CF pig model allows access to tissues and opens the door for new mechanistic studies of pancreatic disease pathogenesis. If inflammatory pathways are shown to play an important role in the CF pancreatic disease pathogenesis, the CF pig model may be instrumental in the identification of inflammatory biomarkers that could be useful for the diagnosis and treatment of other pancreatic inflammatory disorders [43, 44].

The current study does not identify the cause of pancreatic disease in CF, when it begins during development or whether inflammation contributes to the pathogenesis. Studies in CF mice have shown a heightened inflammatory response to cerulein hyperstimulation, suggesting that the inflammation may be playing a role in the pancreatic disease pathogenesis [45]. What our study shows is that the pancreatic lesion at birth in the piglet model is characterized by an inflammatory infiltrate comprised of activated lymphocytes, NK cells, as well as macrophages and neutrophils. Whether the lesion represents an aftermath of a much earlier event cannot be addressed by the current experimental design. It will be necessary to obtain a variety of fetal samples at various stages of gestation to identify the onset of the lesion. Characterization of the cellular constituency of the initial lesion can then provide additional clues as to the mechanism that initiates the disease. With an immune system distinctly similar to humans, pigs are an excellent model in which to study diseases of immune system that also affect humans [30, 46, 47].

The initial source of the inflammatory lesions remains uncertain. However, we can rule out certain factors. The porcine fetus is a bacteria and bacterial product-free environment. Unlike the human fetus, the pig fetus is devoid of maternal antibodies that could be autoreactive. Certain viruses can cross the placenta in swine and can activate the immune system, but the breeding stock used in this research was free of all commonly known viruses. We cannot exclude the possibility that the CFTR defect could activate the pre-immune system of the fetus resulting in auto-reactive cells and antibodies that target the pancreas. This would be consistent with the different types of activated T and B cells that comprise the lesion at birth. It is also possible that abnormal innate and/or adaptive immune responses [48–50] play an important role in the CF pancreatic disease pathogenesis. We currently have no evidence of abnormal innate and/or adaptive immune responses in the CF pig pancreas, but those could be studied in the future. We have not observed any pancreatic inflammation in the heterozygous pig pancreata using histology and flow cytometry. However, it is not known whether the heterozygous pigs will respond to a challenge (alcohol, bile acids, cerulein, etc.) differently than the WT pigs.

In summary, we discovered an activated inflammatory response in newborn pigs with CF that targeted the pancreas; the inflammation was not systemic. The presence of both innate and adaptive immune cells at this early time point suggests that the disease process is complex and extensive. Future studies in fetal pigs will be helpful to better understand the onset and contributions of inflammation in CF pancreatic disease.

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