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

Maisam Abu-El-Haija a, Marek Sinkora f, David K. Meyerholz b, Michael J. Welsh d, e, Paul B. McCray, Jr. a, John Butler c, Aliye Uc a

Departments of a Pediatrics, b Pathology, c Microbiology, and d Internal Medicine, and e Howard Hughes Medical Institute, University of Iowa Carver College of Medicine, Iowa City, Iowa, USA; f Department of Immunology and Gnotogiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Novy Hradek, Czech Republic

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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 F508del/F508del) and in those without CF (CFTR+/−, CFTR+/F508del, 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+) γδ T cells, activated (MHC-II+ and/or CD25+) and effector (CD4+CD8+) αβ 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-
tients with pancreatic sufficiency (PS) become insufficient during the first few months and years of life [2–4]. CF is by far the most common form of PI in children [5]. Whilst sufficient exocrine pancreatic function is present and pancreatic enzyme supplementation is not needed, the pancreas of CF patients with PS is never normal. The pancreas is involved in CF irrespective of PS or PI status.

Pancreatic enzyme replacement therapy has been the mainstay for patients with CF and PI, but the current therapy of PI with exogenous pancreatic enzymes is far from perfect. These enzymes do not effectively treat the malabsorption, mal-digestion, growth delay, and gastrointestinal symptoms of CF patients with PI [6, 7]. The mechanisms that lead to pancreatic destruction in CF are not well understood and currently there are no treatments to prevent disease progression.

Because the pancreas is not easily accessible in humans, CFTR gene knock-out mouse models were developed to study the disease mechanisms in CF [8, 9]. However, pathological changes in the pancreas are either mild or absent in CF mouse models. In contrast, pancreatic histopathology is very similar between humans and pigs with CF [10–12]. Therefore, the porcine model may be useful for investigating the pancreatic disease mechanisms in CF and that knowledge might enable the development of innovative therapies.

Two findings in human CF pancreas suggest that inflammation may be contributing to the disease process. First, pancreatitis is a well-known complication of CF in humans with the PS phenotype and it is an important factor leading to PI in these patients [13, 14]. Second, inflammatory cell infiltrates (mixed cell type) are well-known features of human CF pancreatic pathology, as described in the early autopsy studies [15–17]. However, the subtypes of inflammatory cells infiltrating the pancreas have not been identified in humans with CF. As a result, it is not known whether the innate and/or adaptive immune systems have been activated.

In this study, we studied the subtypes of inflammatory cells in the pancreas of newborn pigs with CF to investigate whether a specific inflammatory pathway was 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−/−, 1 CFTR−/AF508/AF508), heterozygous (3 CFTR−/+, 1 CFTR−/AF508) and wild-type (WT) (3 CFTR+/+) 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 MSA4, mouse IgG1), 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 IgG1); anti-IGM (clone M160, mouse IgG1); anti-TCR γδ (clone PPT16, mouse IgG2b); anti-SWC1 (clone K263.3D7, mouse IgG1); anti-SWC8 (clone MIL3, mouse IgM); anti-MHC-II (clone 1038H-12–34, mouse IgM). 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.
Mesenteric Lymph Nodes

Cell suspensions from MLN were prepared by careful teasing of tissue with two forceps in PBS. Final suspension was filtered through a 70-μm cell strainer (BD Biosciences, San Jose, Calif., USA) and washed three times with PBS. Cell suspensions were then washed twice in PBS-GEL prior to counting with hemacytometer.

Staining of Cells

Staining of cells for flow cytometry was performed as described previously by indirect sub-isotype staining [19, 20]. Briefly, multi-color staining was done using 2 × 10^6 cells that had been incubated with a combination of three (three-color staining) or four (four-color staining) primary mouse mAbs of different sub-isotypes. Cells were incubated for 30 min and subsequently washed twice in PBS-GEL. Mixtures of goat secondary pAbs specific for mouse immunoglobulin subclasses that had been labeled with FITC, PE, PE/Cy7 and APC conjugate were then added to the cell pellets in appropriate combinations. After 30 min, cells were washed three times in PBS-GEL and analyzed by flow cytometry.

Flow Cytometry

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. Emission 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 enzymatic dispersal. We used anti-IgM to identify B cells, anti-CD3 for T lymphocytes and the presence of anti-TCR for CD3+CD8−CD4+ 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 pancreas 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...
Inflammation in the Pancreas of Newborn Pigs with CF

**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.

**Effector 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 cell populations were not different between CF and non-CF pig pancreas. 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.

Fig. 5. Cytotoxic αβ T cells are increased in CF pig pancreas. The proportions of (a) CD4+CD8+MHC-II–CD25+ αβ T cells and (b) CD4+CD8+MHC-II–CD25+ αβ T cells were higher in CF pig pancreas compared to non-CF (* p < 0.01), proportions were not significantly different in blood and MLN of CF and non-CF pigs. c, d CD4+CD8+MHC-II–CD25+ and CD4+CD8+MHC-II–CD25+ αβ T cells were not different between CF and non-CF pig pancreas. Cells were gated to all αβ T cells.
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.

**Fig. 6.** CD2+CD8+ γδ T cells are increased in CF pig pancreas. a The percentage of CD2+CD8+ γδ T cells was increased in CF pig pancreas compared to non-CF (* p < 0.01). Blood and MLN cell populations were not different between CF and non-CF pigs. b The proportion of CD2+CD8+ cells was not different between CF and non-CF pig blood, MLN, and pancreas. Cells were gated to all γδ T cells.

**Fig. 7.** Activated NK and γδ T cells are increased in CF pig pancreas. Effector MHC-II+ NK cells (gated to all NK cells) (a) and MHC-II+ CD2+CD8+ γδ T cells (gated to all γδ T cells) (b) were significantly elevated in CF pig pancreas compared to non-CF (* p < 0.01). These cells were not significantly different in MLN and blood 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.

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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 auto-reactive. 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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