A. SPECIFIC AIMS

Mutations in the Cystic Fibrosis (CFTR) gene result in viscous secretions as well as an excessive host inflammatory response. This proinflammatory response, even in the absence of infection, plays a critical pathogenic role not only in the classic form of CF, but also in adult onset diseases associated with “milder” or single allelic CFTR mutations such as chronic sinusitis, chronic pancreatitis, and Primary Sclerosing Cholangitis. To understand how CFTR mutations lead to this altered innate immune response, during the last grant period we determined that (i) CFTR dysfunction is tightly linked to defects in fatty acid metabolism in both transgenic mice as well as in humans and is characterized by increased arachidonic acid (AA) in the n-6 (omega 6) pathway and decreased docosahexaenoic acid (DHA) in the n-3 (omega 3) pathway; (ii) correction of this fatty acid defect with DHA decreases pseudomonas induced lung inflammation and corrects the pancreatic and intestinal pathology; (iii) the increased inflammatory response is due to innate immune defects in epithelial cells and monocytes/macrophages in both mice and humans with CF; (iv) the proinflammatory response and altered fatty acid metabolism in CF are linked to decreased expression of PPARγ in epithelial cells and PPARα in macrophages, the latter corrected with DHA; and (v) mutations in the CFTR gene are linked to bile duct inflammation in humans and in cftr⁻⁻⁻ mice following induction of colitis, with reversal of the inflammation by DHA, in part through activation of PPARα.

The underlying hypothesis for this proposal is that dysregulation of fatty acid metabolism leads to the excessive inflammatory response characteristic of CFTR dysfunction and represents a novel therapeutic target. We now have evidence that two pathways are involved in the dysregulation of fatty acid metabolism in CF. Our first hypothesis (Aim 1) will test whether primary changes in specific fatty acid biosynthetic enzymes occur with loss of normal CFTR function. The second hypothesis (Aim 2) will test whether the primary defect is altered phospholipid metabolism, in particular phosphatidylcholine formation through the PEMT pathway leading to secondary changes in fatty acids. The third hypothesis (Aim 3) will test whether these abnormalities in fatty acid metabolism directly lead to increased formation of downstream inflammatory mediators resulting in the characteristic CF pathology. The approach is as follows:

1. Determine in vitro the mechanism by which defective CFTR leads to abnormalities in the biosynthetic enzymes in the n-3 and n-6 pathway. Our prior studies focused on mouse tissues. To elucidate the mechanism by which CFTR dysfunction alters fatty acid metabolism, cell culture models are a critical requirement. We have now characterized two human airway epithelial cell culture models - 16HBE with sense and antisense CFTR RNA and IB3 cells which express ∆F508/W1282X mutations with control C38 cells transfected with wild type CFTR. Preliminary data indicate that the CF cells display similar defects in fatty acid metabolism as seen in vivo in CF mice and in humans with CF and is the result of increased ∆6 desaturase activity. We will determine the mechanism by which ∆6 desaturase activity and the formation of DHA is altered in CF cells.

2. Determine in vitro and in vivo whether defective phosphatidylcholine production via the PEMT pathway represents the primary defect leading to the fatty acid changes seen in CF. This aim will test whether loss of CFTR function results in decreased PEMT activity, whether RNAi knockout of PEMT reproduces the CF fatty acid defect in 16HBE sense (wildtype) cells, and whether restoration of PEMT activity with 5 methytetrahydrofolate corrects the fatty acid defect. This would explain the low DHA levels, increased ∆6 desaturase, and other associated abnormalities such as the reduced glutathione levels seen in CF.

3. Determine whether the altered levels of fatty acids in CF lead to changes in downstream inflammatory mediators in vitro and in vivo. Our preliminary results have led us to hypothesize that dysregulation of linoleic acid metabolism in CF leads to increased AA, and in turn increases production of downstream inflammatory mediators. In contrast, in normal CFTR function, AA levels are tightly controlled as supported by our preliminary studies in wild type cells. We will test our hypothesis in three CF models: 1) Pseudomonas induced inflammatory responses in our cell culture model; 2) Pseudomonas endotoxin effects in the airways of cftr⁻⁻ mice; and 3) colitis induced bile duct inflammation in cftr⁻⁻ mice. In all cases, we will quantify cytokines/chemokines and prostaglandins as a function of linoleic acid levels in the media/diet. Since a high fat, high linoleic acid diet is currently recommended for CF patients, our experiments may indicate that this is harmful and leads to increased inflammatory responses.
D. RESEARCH DESIGN AND METHODS

It should be emphasized that the mechanism by which CFTR dysfunction directly causes the multitude of cellular abnormalities including the increased genesis of cellular inflammatory mediators, has not been elucidated despite intensive investigation by many investigators over the last couple of decades. However, our results over the past grant period as well as the preliminary data generated, have led to new insights into the mechanism by which CFTR dysfunction leads to alterations in fatty acid metabolism and its effects on the innate immune response. The new data that we have generated in both human airway epithelial cells in culture and in transgenic CF mice demonstrate that dysregulation of linoleic acid metabolism leads to increased AA formation that is directly connected to increased lung inflammation. These data also explain why levels of endogenous fatty acids may vary in different animal and cell culture models depending on the amount of linoleic acid in the diet or media, respectively. Our results may have direct relevance to the management of CF patients. A high linoleic acid diet is the current recommendation for CF patients in North America based on the thinking that low linoleic acid levels are due to insufficient uptake into cells resulting in a relative essential fatty acid deficiency state. However, the data we have obtained indicate that low linoleic acid levels are instead due to increased conversion to AA and that increasing linoleic acid in the diet can lead to increased inflammation.

This grant application addresses the next critical step in defining the mechanism by which this fatty acid abnormality occurs with loss of CFTR function. We hypothesize that there are two mechanisms which could explain the dysregulation of fatty acid metabolism in CF. The first (Aim 1) hypothesizes that defective CFTR leads to an abnormality in the biosynthetic enzymes in the n-3 and n-6 pathway (defective fatty acid metabolism). The second (Aim 2) hypothesizes that an additional mechanism which is not necessarily mutually exclusive of Aim 1, is related to a defect in phosphatidylcholine synthesis via the PEMT pathway (defective phospholipid metabolism) leading to the fatty acid defects seen in CF. The latter hypothesis is supported by three new key pieces of evidence. Aim 3 tests the hypothesis that loss of normal CFTR function through increased production of arachidonic acid directly leads to changes in downstream inflammatory mediators using cell culture and CF transgenic mouse models. Taken together these data will provide mechanistic insights into the role of fatty acids in normal cell function and CFTR related diseases and have direct implications on the development of treatment strategies.
RESUME AND SUMMARY OF DISCUSSION: In this application studies are proposed to investigate the mechanisms whereby CFTR mutation or deficiency leads to alterations in fatty acid metabolism and exacerbated inflammatory response. These studies are highly significant as results generated would likely provide mechanistic insights into the role of fatty acids in CFTR function, and to eventually develop new therapeutic strategies for the treatment of cystic fibrosis. During the discussion reviewers acknowledged that the PI responded to previous critique by deleting aim 3 (studies on human subjects) and revised the other two aims substantially and introduced a brand new aim 2 (PEM7 pathway and FA production). Reviewers noted that the revision improved the application but it was still viewed as ambitious. Several strengths were recognized including the expertise and track record of the PI in carrying out CFTR research; the strong scientific environment; the excellent progress made during the current funding cycle; the highly innovative nature of the proposed translational research; and the strong aim 3 which is adequately supported by preliminary data. However, there still remain significant weaknesses in aims 1 and 2. These include the broad scope of specific aim 1 with five different pathways being proposed to understand the mechanism by which defective CFTR leads to abnormalities in the biosynthetic enzymes; the problems in study design in aims 1 and 2 (detailed in critique 1 and 2); the lack of inclusion of certain experimental details; and the absence of crucial preliminary data to document the feasibility of testing multiple pathways in specific aims 1 and 2. As discussion ended, these weaknesses tempered the level of enthusiasm.

DESCRIPTION (provided by applicant): Mutations in the Cystic Fibrosis (CFTR) gene result in viscous secretions as well as an excessive host inflammatory response. This proinflammatory response, even in the absence of infection, plays a critical pathogenic role not only in the classic form of CF, but also in adult onset, single organ diseases associated with CFTR mutations such as chronic sinusitis, chronic pancreatitis, and Primary Sclerosing Cholangitis. In an effort to understand how CFTR mutations lead to this altered innate immune response, during the last grant period we determined that CFTR dysfunction is tightly linked to defects in fatty acid metabolism in both transgenic mice as well as in humans and is characterized by increased arachidonic acid (AA) in the n-6 (omega 6) pathway and decreased docosahexaenoic acid (DHA) in the n-3 (omega 3) pathway. Correction of this fatty acid defect with high doses of DHA reverses the CF pathology in CF knockout mice. In addition, decreased PPAR expression and function is associated with the altered innate immune response and may explain the fatty acid defects.

The underlying hypothesis for this proposal is that dysregulation of fatty acid metabolism and alterations in PPAR/LXR nuclear transcription factors lead to the excessive inflammatory response characteristic of CFTR dysfunction and represent novel therapeutic targets. The first aim will determine whether the primary mechanism by which CFTR dysfunction leads to alterations in fatty acid metabolism is due to a primary defect in specific biosynthetic enzymes which affect the levels and function of n-6 and n-3 fatty acids. The second aim will determine whether instead the primary problem is a defect in phospholipid metabolism leading to secondary changes in fatty acid metabolism. The third aim will determine the effect of altered fatty acid levels on downstream inflammatory mediators in cell culture and in cfr +/- mice. These data will provide mechanistic insights into the role of fatty acids in normal cell function and CFTR related diseases and have direct implications on the development of treatment strategies.

CRITIQUE 1:

Significance: This is a resubmission of a competitive renewal aimed at establishing the role of the CFTR mutation on altered fatty acid metabolism and inflammatory response. Cystic fibrosis (CF) remains a devastating disease with high rate of morbidity and mortality secondary to pro-inflammatory responses and super-infections. During the previous funding cycle, the applicant generated data both in transgenic mice and in humans suggesting that CFTR dysfunction is linked to defects in fatty acid metabolism characterized by increased arachidonic acid (AA) in the n-6 (omega 6) pathway and
decreased docosahexaenoic acid (DHA) in the n-3 (omega 3) pathway. Since the last submission, the applicant generated additional preliminary data suggesting that rather than being the primary defect, changes in fatty acids can be secondary to altered phospholipid metabolism, in particular phosphatidylycerine formation through the PEMT pathway. Even more intriguing was the finding that the increased inflammatory response typical of CF was secondary to innate immune defects in epithelial cells and monocytes/macrophages in both mice and humans with CF and that the altered fatty acid metabolism in CF are linked to decreased expression of PPARγ in epithelial cells and PPARα in macrophages. Based on the combination of his preliminary data, the applicant formulated the overall hypothesis that dysregulation of fatty acid metabolism leads to the excessive inflammatory response characteristic of CFTR dysfunction. If this hypothesis will prove to be correct, the proposed studies may provide crucial information to develop new therapeutic strategies for the treatment of this devastating disease.

**Approach:** To address the previous criticisms and to capitalize on his additional preliminary data, the applicant made substantial changes in his proposed studies now focused on three revised specific aims. These aims will challenge the hypothesis that there are two mechanisms which could explain the dysregulation of fatty acid metabolism typical of CF. The first (that will be explored with Aim 1) hypothesizes that defective CFTR leads to defective fatty acid metabolism. The second (challenged with Aim 2) hypothesizes that an additional/alternative mechanism related to a defective phospholipid metabolism leading to the fatty acid defects seen in CF. The third and final aim will challenge the hypothesis that loss of normal CFTR function through increased production of arachidonic acid directly leads to changes in downstream inflammatory mediators.

The first aim will determine in vitro the mechanism by which defective CFTR leads to abnormalities in the biosynthetic enzymes in the n-3 and n-6 pathway. To address this first aim, the applicant developed two human airway epithelial cell culture models (16HBE and IB3) in which knock-out of normal CFTR function (16HBE) as well as mutations in the CFTR gene (IB3) were engineered. Using these models, PI already generated some preliminary data suggesting that the CFTR antisense cells as well as those with the ΔF508/W1282X mutations display similar defects in fatty acid metabolism as seen in tissues from cftr-/- mice and in humans with CF.

To determine the mechanism by which loss of CFTR function leads to low levels of linoleic acid, the applicant proposed to study 5 different pathways. Pathway 1 examines whether increased Δ6 desaturase activity (that was detected by performing additional preliminary data) is due to altered function of PPAR, LXR, and/or SREBP-1c, nuclear transcription factors that regulate Δ6 desaturase expression. The applicant proposed to establish which factor(s) is altered in 16I1D and IB3 cell lines in the setting of loss of normal CFTR function by measuring PPARα and PPARγ expression at the RNA level by RT-PCR, protein level by western blot, and function by EMSA. With pathway 2, the applicant will establish whether linoleic acid is low as a result of increased phospholipase A2 cleavage of AA from the cell membrane with subsequent increased linoleic acid conversion to AA in order to replete AA levels. This hypothesis will be tested by using three different approaches by pharmacologically activating or inhibiting phospholipase A2 or by decreasing production of eicosanoids. The third pathway (linoleic acid levels are low as a result of being in the inappropriate lipid compartment affecting its metabolism to downstream fatty acids) will be tested by incubating wild type and CF cells with radiolabeled linoleic acid and determining linoleic acid distribution in isolated lipid fractions. The fourth pathway will explore the possibility that linoleic acid levels are low as a result of increased turnover of phosphatidylcholine (PC) in CF, a possibility that will be explored by incubating wild type and CF cells with radiolabeled choline, and radioactivity associated with PC, choline, phosphocholine, and lysophosphatidylcholine measured. With pathway 5, the applicant will establish whether linoleic acid and DHA levels are low as a result of decreased activity of fatty acyl-CoA synthetase and/or fatty acyltransferase activity in CF cells. This possibility will be challenged by analyzing wild type and CF cells without any prior treatment for fatty acyl-CoA synthetase and fatty acyltransferase activities. This list of 5 possible pathways, while logical in its conceptualization, seems a mere tentative to cover some of the many more potential mechanisms by which CFTR can influence linoleic acid metabolism. The
choice to focus on these specific pathways is only partially justified by the preliminary data presented, some of which have been recently generated since the previous submission, partially in response to the reviewers’ criticisms. The impression is that these are after-thoughts in which a web is cast to cover as many pathways as possible without a clear rationale to justify their choices.

The second specific aim is a new addition to the previous proposal to explore the possibility that a defect in phosphatidylcholine synthesis via the PEMT pathway (defective phospholipid metabolism) could represent an additional mechanism leading to the fatty acid defects seen in CF. The applicant proposed to use four approaches to test the hypothesis that a primary defect in PEMT derived PC leads to the alterations in fatty acid metabolism in the setting of loss of normal CFTR function. The first approach will test whether loss of normal CFTR function in cell lines and in cfr-/- mice leads to a selective decrease in PEMT activity compared with the de novo pathway enzymes; the second approach will determine if the fatty acid alterations in CF are directly related to decreased PEMT activity by compensating the defect by the addition of 5 methyltetrahydrofolate; the third approach will test in normal 16 HBE cells whether RNAi knock out of PEMT in WT cells reproduces the fatty acid defects seen in 16HBE antisense (CF) cells; the fourth approach will determine in cfr-/- mice whether correction of the PEMT defect through administration of 5 methyltetrahydrofolate reverses the CF pathology in ileum and pancreas and decreases inflammation in a model of pneumonia and bile duct injury. This aim suffers of the same study design drawbacks outlined for the previous aim. The reader is left with the impression of a list of alternative approaches dictated by the need to cover “as much as possible” rather than an evidence-based design with a primary approach integrated by alternative strategies in case that technical problem arise. Furthermore, as also acknowledged by the applicant, the defective fatty acid metabolism hypothesized in aim 1 and the defective phospholipid metabolism hypothesized in this aim are not necessarily mutually exclusive in causing dysregulation of fatty acid metabolism in CF. Therefore, the potential redundancy of these two pathways may affect the outcome of the studies proposed in this aim. Indeed, the interventions proposed in the in vivo experiments may be over-compensated or masked by the concomitant involvement of the defective fatty acid metabolism.

Specific Aim 3 seeks to determine the effect of altered fatty acid levels on downstream inflammatory mediators in CF cell culture models and in cfr-/- mice. The applicant generated preliminary data indicating that dysregulation of linoleic acid metabolism in CF causes increased AA levels and subsequent increased production of downstream inflammatory mediators. To capitalize on this preliminary data, the applicant will initially perform in vitro studies in which 16 HBE cfr sense and antisense cells will be incubated in the absence or presence of Pseudomonas PA01 and with different concentrations of linoleic acid in order to modulate AA levels. These studies will be integrated with additional experiments using cultured cells expressingAF508 in order to establish the effect of CFTR mutation rather than its absence. Cultured cells media will be analyzed for their content of cytokines, chemokines, and eicosanoids known to be altered in CF known products of AA metabolism and known to be implicated in CF inflammation. Furthermore, the effect of CFTR dysfunction on Nfkb activity will be measured by co-transfecting 16HBE cells with luciferase tagged Nfkb. Additional nuclear transcription factors, including PPAR/LXR/VRXR, will also be studied, since PPAR/LXR pathways seem to play critical roles in diverse aspects of lipid metabolism and inflammatory signaling in response to infection and their expression is affected in CF (as shown by the applicant’s preliminary data).

The studies described above will be complemented by in vivo studies in which the involvement of linoleic acid metabolism dysregulation in the increased production of downstream inflammatory mediators will be tested in cfr-/- mice in vivo. The PI will apply the same two models of inflammation that he developed in cfr-/- mice. These models will assist in establishing whether defective CF-driven dysregulation of linoleic acid metabolism leads to increased chemokines and/or AA-derived eicosanoids. Linoleic acid levels in cfr-/- and wild type mice will be modulated by maintaining the animals on either Peptamen supplemented with 100 mg oleic acid as a control lipid which does not directly modulate the n-3 or n-6 pathways or Peptamen supplemented with 100 mg of linoleic acid/day. Changes in all fatty acids including linoleic acid and AA levels in plasma and tissue as a result of the
different yet isocaloric diets will be correlated with changes in the same inflammatory mediators analyzed for the above cell culture model. The experiments proposed in this aim seem to be more logical and justified by the applicant’s preliminary data. The study design is straightforward and should not pose any major challenge for their execution.

**Innovation:** The nutritional recommendations currently in place for CF patients can result not only inadequate, but even deleterious if the overall hypothesis of the applicant will be confirmed. Therefore, the data possibly generated by this application may provide mechanistic insights into the role of fatty acids under both physiological and pathological (CF) circumstances. Therefore, they may have direct implications on the development of novel treatment strategies, making this application quite novel.

**Overall Evaluation:** This is a revised application aimed at studying the role of fatty acid metabolism dysfunction typical of CF on the innate immune function of patients with CF. Strengths of the application include the translational aspect of the proposal, the qualifications of the applicant and his team to perform the proposed project, the strong environment and collaborative team that will assist the P.I. with his project. Despite the honest attempt to address the criticisms of previous reviewers, the application still suffers of several weaknesses, particularly concerning the first two aims that are still approximate in their study design that was poorly outlined and justified. A more hypothesis-driven study algorithm that capitalizes on preliminary data and provides a clear rationale to propose focused experiments challenging well-described hypothesis will significantly improve the quality of the application.