

Acidification-dependent activation of CD1d-restricted natural killer T cells is intact in cystic fibrosis

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Summary

CD1d-restricted natural killer T (NKT) cells are emerging as critical regulators of the immune response to infectious agents, including *Pseudomonas aeruginosa*; and therapies to augment NKT-cell activation may represent a novel approach to treat chronic, antibiotic-resistant bacterial infections. We examined the capacity of dendritic cells (DCs) from people with cystic fibrosis (CF) to activate NKT cells. Our study was motivated by three lines of evidence: (i) NKT cells play a critical role in clearing *P. aeruginosa* infection; (ii) activation of NKT cells requires acidification-dependent processing of glycolipid antigens within the endolysosomal compartment; and (iii) endolysosomal acidification may be reduced in CF. We demonstrated that NKT-cell activation was dependent upon intact organelle acidification as inhibitors of the vacuolar (H⁺)-ATPases prevented DCs from activating NKT cells with two glycolipid antigens, α -galactosylceramide and galactose-galactosylceramide. In contrast, cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel dysfunction had no significant biological impact on the capacity of DCs to activate NKT cells. Dendritic cells from subjects with CF and DCs treated with the thiazolidinone CFTR_{inh}-172 inhibitor showed no reduction in their ability to activate NKT cells. Based on these data, we find no evidence for an inherent defect in glycolipid antigen presentation to NKT cells in CF subjects.

Keywords: acidification; cystic fibrosis; natural killer T cell; *Pseudomonas aeruginosa*

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP-regulated chloride channel. There is currently no cure for CF and the median life expectancy for CF patients is 37 years.^{1–3} Lung disease is the primary cause of morbidity and mortality in CF. Soon after birth, infection with bacterial pathogens begins, triggering an intense neutrophilic response localized to the peribronchial and endobronchial spaces. Intriguingly, only a limited number of bacterial pathogens colonize the airway in CF, with the most clinically relevant being *Pseudomonas aeruginosa*.⁴ Although a number of hypotheses have been proposed (reviewed in refs^{2,4}), there is still no clear consensus linking mutations in *CFTR* to development of chronic infection with

P. aeruginosa. In this study we explore the link between *CFTR* deficiency and lung infection with *P. aeruginosa* by examining the capacity of *CFTR*-deficient dendritic cells (DCs) to stimulate natural killer T (NKT) cells.

Natural killer T cells, a specialized type of T cell, are emerging as critical regulators of the immune response to infectious agents.^{5,6} The NKT cells may be particularly important in CF as evidence suggests that NKT cells play a central role in clearing *P. aeruginosa* from the lung⁷ and gastrointestinal tract.⁸ In contrast to conventional major histocompatibility complex-restricted T cells, NKT cells express a semi-invariant T-cell receptor (iTCR) that recognizes glycolipid antigens presented by CD1d molecules on the surface of antigen-presenting cells, such as DCs and macrophages.^{5,9} Current knowledge of the glycolipid antigens that activate NKT cells for antimicrobial defence is incomplete, although these glycolipids appear to include

both pathogen-derived and host-derived lipids.^{10–12} For experimental purposes most groups utilize α -galactosylceramide (α -GalCer) and galactose-galactosylceramide (GalGalCer), two well-characterized glycolipids that bind to CD1d and are recognized by the iNKT-cell TCR. Optimal lipid antigen presentation by CD1d, leading to activation of NKT cells, is dependent on endosomal acidification.^{13–16}

Natural killer T-cell biology has been the focus of much recent attention as therapeutic augmentation of NKT-cell activity through the administration of lipid antigens is being trialled as a cancer therapy and may also play a role in managing human infections, such as CF.^{17,18} Moreover, boosting innate immune responses is particularly attractive in CF as this may allow the eradication of pathogens that are resistant to multiple antibiotics.¹⁹

The fact that NKT-cell activation appears to be dependent on endosomal acidification, combined with the observation that NKT cells contribute to clearance of *P. aeruginosa* from the lung, provides an intriguing (and testable) link between CFTR deficiency and chronic airway infection with *P. aeruginosa*. For many years it has been suggested that endolysosomal acidification is defective in CF. In what has been coined the ‘defective organelle acidification’ hypothesis, it has been proposed that CFTR provides a conductive pathway for chloride transport across intracellular organelles promoting H⁺ influx via a counter-ion effect.^{20–23} Hence in CF, endosomal acidification may be inadequate as H⁺ pumping is electrically limited by defective CFTR-mediated chloride transport. Although we appreciate that evidence supporting an endosomal acidification defect in CF is far from unanimous,^{24–29} given that there is potential to develop therapies to boost NKT-cell activity and enhance clearance of infectious agents, in this study we examined endosomal acidification-dependent NKT-cell activation by DCs from patients with CF. Specifically, we hypothesized that DCs with dysfunctional CFTR would be less potent activators of CD1d-restricted NKT cells than DCs with intact CFTR-mediated chloride conductance.

Materials and methods

Dendritic cell preparation

Blood samples from healthy volunteers and patients diagnosed with CF were obtained at BC Children’s Hospital, Vancouver, Canada. The diagnosis of CF was established by classic clinical features including increased sweat chloride concentrations (> 60 mmol/l). The CF subjects enrolled in this study had the following *CFTR* mutations: $\Delta F508/\Delta F508 = 4$, $\Delta F508/621 + 1G > T = 1$, $\Delta F508/G85E = 1$, $\Delta F508/\text{unknown} = 1$, unknown/unknown = 1. Patients receiving systemic immunosuppressive medications such as oral corticosteroids and azithromycin were

excluded from the study. All samples were obtained with informed consent with the approval of the University of British Columbia Clinical Research Ethics Board. Peripheral whole blood was collected in sodium heparin Vacutainer[®] tubes (BD Biosciences, Mississauga, ON, Canada) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation as previously described.³⁰ Monocytes were purified from PBMCs by magnetic bead separation using a CD14-positive selection kit and an autoMACS[™] Pro Separator (Miltenyi Biotec, Auburn, CA) following the manufacturer’s protocols. The CD14-positive cells were resuspended in R10 consisting of RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (HyClone, Ottawa, ON, Canada), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U penicillin, 100 μ g streptomycin (Invitrogen, Burlington, ON, Canada), 100 ng/ml granulocyte–macrophage colony-stimulating factor and 100 ng/ml interleukin-4 (IL-4; Fitzgerald Industries, Acton, MA). The cells were incubated in six-well plates at 37° and 5% CO₂ for 96 hr to allow the cells to differentiate into immature DCs (iDCs).

Quantification of CFTR messenger RNA expression in human cells

Expression of *CFTR* messenger RNA (mRNA) in characterized cell populations was quantified by real-time polymerase chain reaction (PCR). The PBMCs from healthy volunteers were prepared as described above, stained with CD14-PECy7 antibody for monocyte isolation and sorted using a FACS Aria flow cytometer (BD Biosciences) into CD14-positive populations. Immature DCs were prepared from a fraction of sorted monocytes and differentiated for 96 hr as described above. A bronchial epithelial cell line (NuLi) was used as a positive control for *CFTR* expression. Messenger RNA was harvested from the cell populations using an RNeasy Plus kit (QIAGEN, Mississauga, ON, Canada) and transcribed to complementary DNA using a SuperScript cDNA synthesis kit (Invitrogen). Expression of *CFTR* was calculated relative to β -actin and was quantified by SYBR GreenER (Invitrogen) chemistry using a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The primers used were, *CFTR*-forward 5'-ACACGTTGAAAGCAGGTGGGATTC-3', *CFTR*-reverse 5'-ACTGCGACAACCTGCTATAGCTCCA-3', *ACTB*-forward 5'-GTTGCGTTACACCCTTTCTT-3' and *ACTB*-reverse 5'-ACCTTCACCGTTCCAGTTT-3' under standard cycling conditions. The expression of the *CFTR* gene was calculated relative to iDCs by the 2^{- $\Delta\Delta$ CT} method.

NKT-cell preparation

Human NKT-cell clone BM2a.3³¹ was thawed and mixed in a 1 : 2 ratio with irradiated PBMCs plus 5 μ g/ml phytohaemagglutinin (PHA) and allowed to proliferate for a

minimum of 14 days in T-cell medium [R10 supplemented with 1% non-essential amino acids (Invitrogen), 2% heat-inactivated human AB serum (Mediatech, Manassas, VA) and 200 U/ml IL-2] at 37° and 5% CO₂.

NKT-cell activation assay

The iDCs and NKT cells were harvested by gentle pipetting, then washed and resuspended in R10. Both iDCs and NKT cells were mixed in a 1 : 1 ratio and incubated in a 96-well plate (5 × 10⁵ each cell type/well). In select experiments the iDCs were pre-treated with the vacuolar-type H⁺-adenosine triphosphatase (ATPase) inhibitors concanamycin A (2.5 or 12.5 nM) or bafilomycin (100 nM) or the thiazolidinone CFTR inhibitor CFTR_{inh}-172 (10 μM) (Sigma, Oakville, ON, Canada) diluted in dimethylsulphoxide (DMSO). The cells were pre-treated for 30 min at room temperature before addition of NKT cells and glycolipid antigens. CD1d glycolipid antigens α-GalCer or GalGalCer were prepared as described previously.³² Controls consisted of untreated NKT cells alone, NKT cells incubated with 5 μg/ml PHA and NKT cells treated

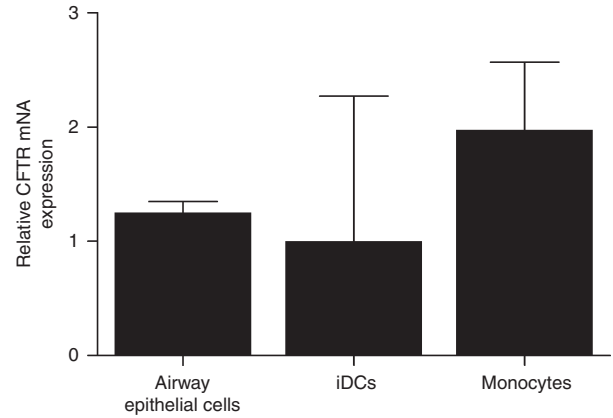


Figure 1. Quantification of *CFTR* messenger RNA (mRNA) expression by human dendritic cells (iDCs), monocytes and airway epithelial cells. Expression of *CFTR* mRNA was quantified by real-time polymerase chain reaction and the data were expressed relative to iDCs using the 2^{-ΔΔCT} method. Values represent mean ± SEM of iDCs and monocytes isolated from three healthy donors. The bronchial epithelial cell line (NuLi) was used as a positive control for *CFTR* expression (*n* = 3 separate experiments).

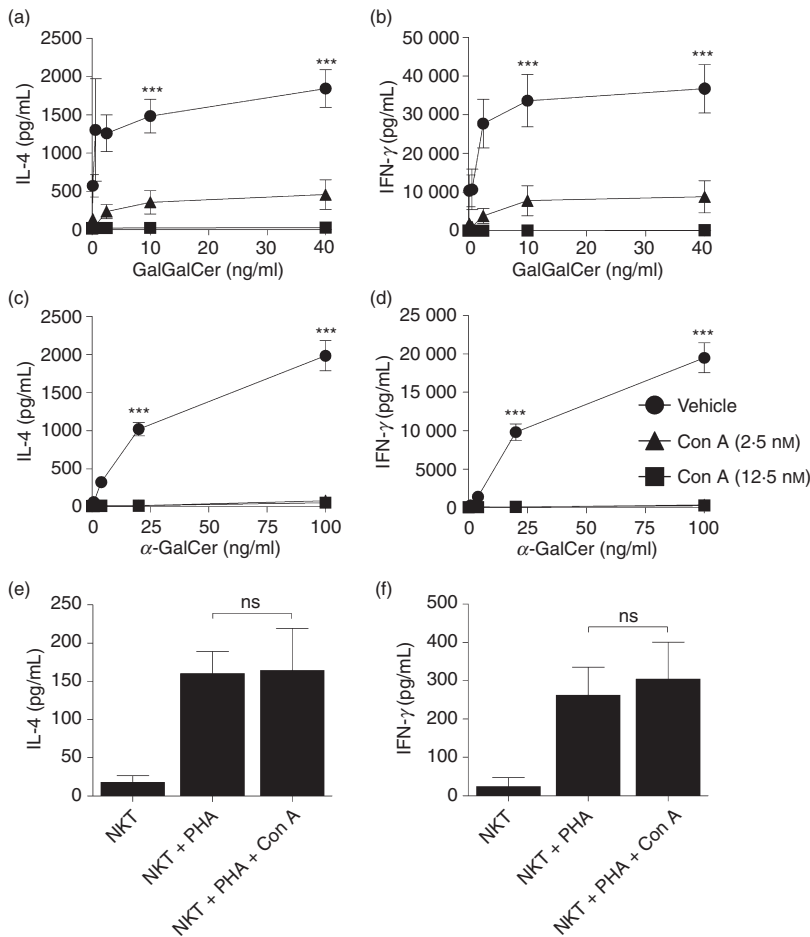


Figure 2. Endosomal acidification blockade using V-type H⁺ adenosine triphosphatase (ATPase) inhibitor concanamycin A prevents natural killer T (NKT) -cell activation. The human NKT-cell BM2a.3 clone was stimulated with GalGalCer (a, b) or α-GalCer (c, d) at varying concentrations presented by either untreated healthy adult dendritic cells (DCs) or healthy adult control DCs treated with concanamycin A (2.5 nM and 12.5 nM). Enzyme-linked immunosorbent assays were used to quantify NKT-cell secretion of the cytokines interleukin-4 (IL-4; a, c) or interferon-γ (IFN-γ; b, d). In control experiments, concanamycin A (12.5 nM) did not prevent the activation of NKT cells when stimulated with phytohaemagglutinin (PHA; e, f). Values represent mean ± SEM of 3–11 samples per group. *** signifies *P* < 0.001 and ns signifies no significant difference.

with PHA plus inhibitor or vehicle (DMSO). The cells were incubated at 37° and 5% CO₂ and the culture supernatants were harvested after 24 hr. Concentrations of IL-4 and interferon- γ (IFN- γ) were measured by enzyme-linked immunosorbent assay (eBioscience, San Diego, CA).

Statistical analysis

Cytokine production between groups was compared by analysis of variance with Bonferroni post-test for multiple comparisons. *P*-values < 0.05 were considered significant.

Results

CFTR mRNA is expressed by DCs and other peripheral blood cells

As a first step we examined *CFTR* mRNA expression in DCs, monocytes and airway epithelial cells. Using quantitative reverse transcription-PCR we demonstrated that immature DCs, monocytes and airway epithelial cells all expressed similar detectable levels of *CFTR* mRNA (Fig. 1).

Inhibiting DC organelle acidification prevents NKT-cell activation by glycolipid antigens

To validate the critical role that organelle acidification plays in the activation of NKT cells by DCs, we treated the DCs with inhibitors of the vacuolar (H⁺)-ATPases (V-ATPases). The pH within many intracellular compartments is regulated by V-ATPases and inhibition of V-ATPases prevents endosomal acidification.³³ For these studies, V-ATPases were blocked with the well-characterized and potent inhibitors concanamycin A and bafilomycin.³⁴

Blockade of V-ATPases with either concanamycin-A or bafilomycin abrogated the ability of the DCs to activate NKT cells with two prototypical glycolipid antigens, α -GalCer and GalGalCer (Figs 2 and 3). Specifically, pre-treatment of DCs with concanamycin A inhibited α -GalCer- and GalGalCer-triggered NKT-cell secretion of IL-4 and IFN- γ by > 97% (*P* < 0.001) (Fig. 2a–d). Similarly, bafilomycin pre-treatment of DCs inhibited glycolipid-dependent NKT-cell activation by > 73% (*P* < 0.001) (Fig. 3a–d). Importantly, control experiments demonstrated that V-ATPase inhibition did not directly influence the capacity of NKT cells to secrete IL-4 and

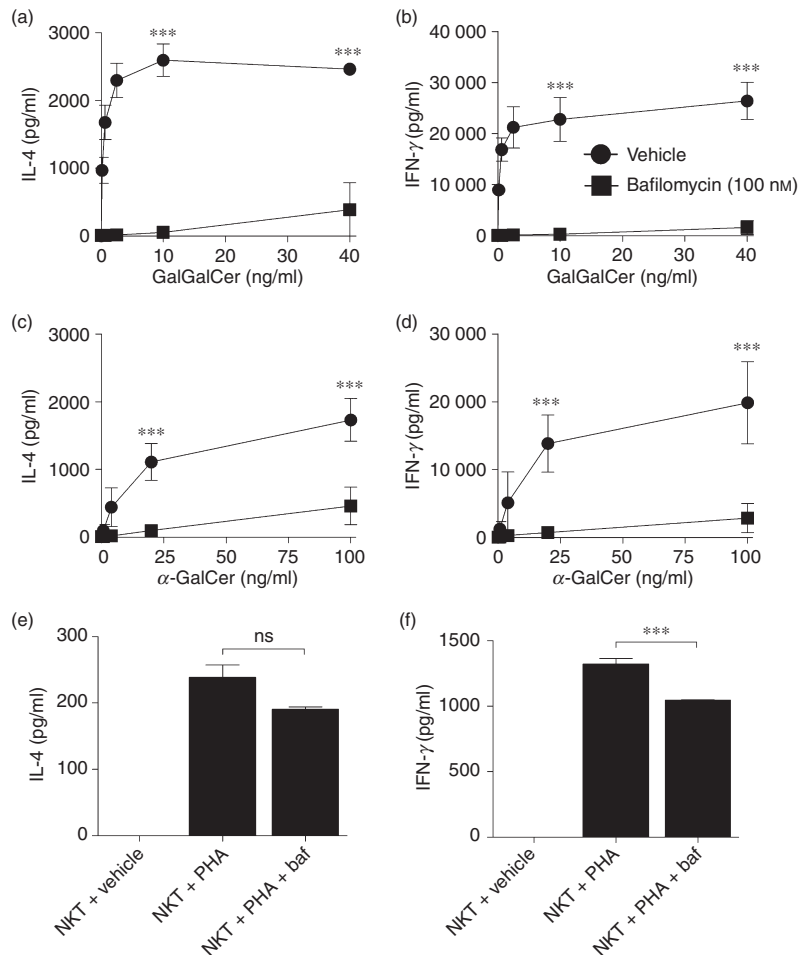


Figure 3. Endosomal acidification blockade using V-type H⁺ adenosine triphosphatase (ATPase) inhibitor bafilomycin prevents natural killer T (NKT) -cell activation. The human NKT-cell BM2a.3 clone was stimulated with GalGalCer (a, b) or α -GalCer (c, d) at varying concentrations presented by either untreated healthy adult control dendritic cells (DCs) or healthy adult control DCs treated with bafilomycin (100 nM). Enzyme-linked immunosorbent assays were used to quantify NKT-cell secretion of the cytokines interleukin-4 (IL-4; a, c) or interferon- γ (IFN- γ ; b, d). In control experiments, bafilomycin (100 nM) had a minimal biological effect on the activation of NKT cells when stimulated with phytohaemagglutinin (PHA; e, f). Values represent mean \pm SEM of three samples per group. *** signifies *P* < 0.001 and ns signifies no significant difference.

IFN- γ . The NKT-cell responses to PHA were not altered significantly by the addition of concanamycin A whereas the addition of bafilomycin only decreased the cytokine output by approximately 20% (Figs 2e,f and 3e,f).

CFTR inhibition does not alter glycolipid-dependent activation of NKT cells by DCs

Having established that activation of NKT cells by DCs in our experimental system was sensitive to blockade of organelle acidification, we next examined if NKT-cell activation was influenced by inhibition of CFTR function. The well-characterized thiazolidinone CFTR_{inh}-172 inhibitor was used to block CFTR chloride conductance with high affinity and selectivity.³⁵ Importantly, two independent groups have demonstrated that 10 μ M CFTR_{inh}-172 prevented complete endosomal acidification^{21,22}, although a third group failed to reproduce these data.²⁵ Therefore, we hypothesized that CFTR blockade by CFTR_{inh}-172 would decrease the ability of DCs to activate NKT cells following stimulation with glycolipid antigens. However, pre-treatment of DCs from healthy donors with CFTR_{inh}-172 did not alter the NKT-cell responses triggered by GalGalCer (Fig. 4a,b) whereas α -GalCer stimulated cytokine

output was statistically significantly reduced, although this biological effect was small compared with the V-ATPase inhibitors (Fig. 4c,d). Importantly, the responsiveness of NKT cells alone to PHA stimulation was unaltered by CFTR_{inh}-172 (Fig. 4e,f).

DCs from CF patients are able to activate NKT cells

To model the human situation, we isolated DCs from patients with CF and healthy age-matched controls and assessed the capacity of these DCs to activate NKT cells following exposure to glycolipid antigens. Consistent with the lack of impact of CFTR_{inh}-172 on NKT-cell activation (Fig. 4), DCs from CF patients and controls were indistinguishable in their ability to activate NKT cells following stimulation with GalGalCer and α -GalCer (Fig. 5a–d). We also examined the capacity of CF and control DCs to present self-antigens to NKT cells. It has been demonstrated previously that NKT cells will generate weak responses to CD1d-presented endogenous self-antigens in the absence of added antigen or microbial products.³¹ However, the baseline cytokine secretion of NKT cells co-cultured with DCs without glycolipid was not significantly altered if DCs were from CF patients or healthy controls

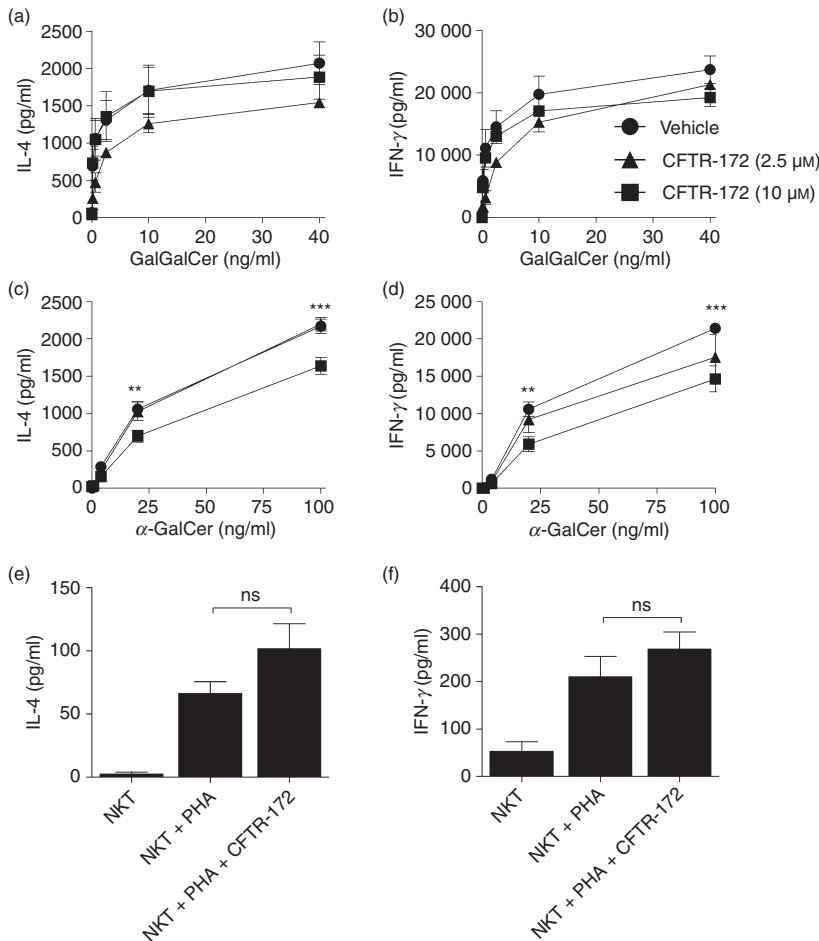


Figure 4. Cystic fibrosis transmembrane conductance regulator (CFTR) inhibition does not alter natural killer T (NKT) -cell activation. The human NKT cell BM2a.3 clone was stimulated with GalGalCer (a, b) or α -GalCer (c, d) at varying concentrations presented by either untreated healthy adult control DCs or healthy adult control DCs treated with CFTR-172_{inh} inhibitor (2.5 μ M and 10 μ M). Enzyme-linked immunosorbent assays were used to quantify NKT-cell secretion of the cytokines interleukin-4 (IL-4; a, c) or interferon- γ (IFN- γ ; b, d). In control experiments, CFTR-172_{inh} (10 μ M) had no impact on the activation of NKT cells when stimulated with phytohaemagglutinin (PHA; e, f). Values represent mean \pm SEM of three to five samples per group. $**P < 0.01$, $***P < 0.001$ and ns signifies no significant difference.

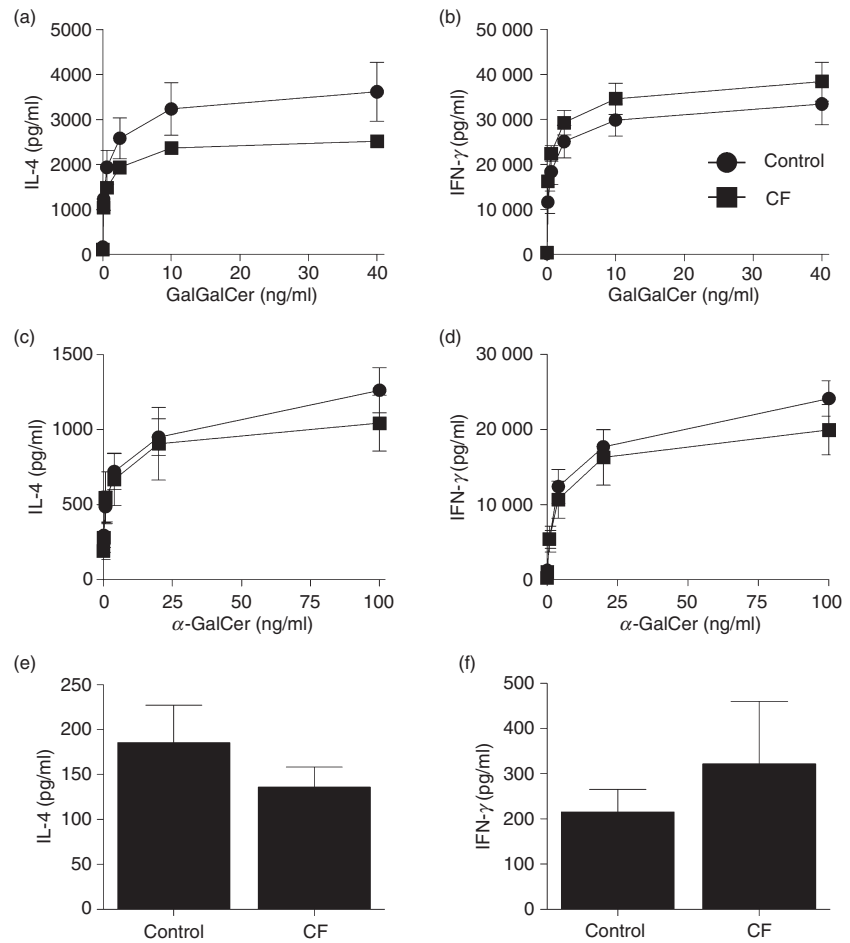


Figure 5. Cystic fibrosis dendritic cells (CF DCs) are able to activate natural killer T (NKT) cells. The human NKT cell BM2a.3 clone was stimulated with GalGalCer (a, b) or α -GalCer (c, d) at varying concentrations presented by either DCs derived from CF patients ($n = 5$) or healthy child controls ($n = 4-5$). Enzyme-linked immunosorbent assays were used to quantify NKT-cell secretion of the cytokines interleukin-4 (IL-4; a, c) or interferon- γ (IFN- γ ; b, d). Values represent mean \pm SEM of four or five samples per group.

(IL-4: control = 185 ± 41 pg/ml versus CF = 135 ± 22 pg/ml; IFN- γ : control = 215 ± 49 pg/ml versus CF = 321 ± 138 pg/ml).

Discussion

Pseudomonas aeruginosa is the predominant and most clinically relevant respiratory pathogen infecting patients with CF.³⁶ The unique tropism of *P. aeruginosa* for the CF respiratory tract has not been adequately explained and although a variety of hypotheses have been proposed, there is still no widely accepted unifying explanation for the peculiar propensity of *P. aeruginosa* to infect the CF airway (reviewed in refs^{2,4}). Understanding the immune mechanisms that facilitate chronic *P. aeruginosa* infection of the CF lung may lead to the development of novel therapies. The ability to boost dysfunctional immune responses may be particularly effective in combating *P. aeruginosa* as this organism frequently develops resistance to multiple antimicrobial agents.

The aim of this study was to assess a possible link between CFTR dysfunction and *P. aeruginosa* infection through an examination of acidification-dependent NKT-cell immune function in patients with CF. Our

study was motivated by three lines of evidence: (i) NKT cells play a critical role in clearing *P. aeruginosa* infection;^{7,8} (ii) activation of NKT cells by antigen-presenting cells requires acidification-dependent processing of glycolipid antigens within the endolysosomal compartment;¹³⁻¹⁶ and (iii) endolysosomal acidification may be reduced in CF.²⁰⁻²³ Combined, these previous studies raise the testable possibility that acidification-dependent NKT-cell activation might be deficient in patients with CF, helping to explain the tropism of *P. aeruginosa* for the CF airway. Testing this hypothesis was particularly timely because therapeutic activation of NKT cells with the exogenous glycolipids is currently being tested as a cancer treatment; and if NKT-cell activation were found to be deficient in patients with CF, a similar immunotherapy approach might be useful to help clear *P. aeruginosa* from the CF airway.¹⁷

We have specifically documented CFTR expression in human monocytes and DCs (Fig. 1), consistent with previous reports from studies in humans and mice that some monocytes/macrophages express CFTR at the mRNA and protein level.^{21,37} In addition, recent work by Xu and colleagues confirmed CFTR mRNA and protein expression in mouse DCs.³⁸

In our model system we confirmed that glycolipid-mediated activation of NKT cells by DCs was dependent on organelle acidification. Pre-treatment of DCs with the well-characterized V-ATPase inhibitors, concanamycin A and bafilomycin, almost completely abolished NKT-cell responses to glycolipid antigens (Figs 2 and 3), as reported by other groups.^{13–16} In contrast, CFTR chloride channel dysfunction had no significant biological effect on the capacity of DCs to activate NKT cells following exposure to the classic glycolipid antigens, α -GalCer and GalGalCer (Figs 4 and 5). Although we found that the highest concentrations of CFTR_{inh}-172 decreased NKT-cell activation by 10–30% (Fig. 4), these small changes are unlikely to be immunologically significant *in vivo*. Most importantly, we validated these findings in the human system and showed that DCs from patients with CF had no defects in their ability to activate NKT cells.

Why did we fail to support our original hypothesis? Why did functional CFTR deficiency fail to reduce the capacity of DCs to activate NKT cells following exposure to glycolipid antigens? The most likely answer is that CF is not associated with an immunologically relevant defect in organelle acidification. There is long-standing interest concerning the possibility of defective organellar acidification in CF cells (as recently reviewed in ref.³⁹). Defective acidification of the trans-Golgi, endosomes and pre-lysosomes in CF was initially reported in 1991 by al-Awqati's group.²⁰ Since this initial provocative observation, multiple groups using an array of experimental approaches have both supported^{20–23} and refuted^{24–29} the observation that organelle acidification is defective in CF, and the debate continues. Rather than join in this divided biochemical debate, we investigated a clinically relevant immunological function that is dependent on endolysosomal acidification – namely, glycolipid-dependent activation of NKT cells by DCs. Our data demonstrate that the ability of DCs to activate NKT cells with glycolipid antigens was not dependent on CFTR function. Importantly, the results were similar regardless of whether CFTR dysfunction was achieved pharmacologically, using CFTR_{inh}-172, or naturally, using DCs from CF patients with pathological CFTR mutations.

In conclusion, we have shown that DCs from patients with CF have no defect in their ability to activate NKT cells in response to classic glycolipid antigens. Therefore, it is unlikely that a failure to fully activate NKT cells contributes to the susceptibility of CF patients to develop chronic pulmonary infection with *P. aeruginosa*, and people living with CF are unlikely to benefit from therapies designed to augment NKT-cell activation.

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Disclosure

The authors declare no competing interests related to this manuscript.

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