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Self-Reactive CFTR T Cells in Humans: Implications for Gene Therapy

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Abstract

Cystic fibrosis (CF) is one of the most common autosomal recessive lethal disorders affecting white populations of northern European ancestry. To date there is no cure for CF. Life-long treatments for CF are being developed and include gene therapy and the use of small-molecule drugs designed to target specific cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations. Irrespective of the type of molecular therapy for CF, which may include gene replacement, exon skipping, nonsense suppression, or molecular correctors, because all of these modulate gene expression there is an inherent risk of activation of T cells against the wild-type version of CFTR. Here we report the validation of the human interferon-γ enzyme-linked immunospot assay and its application for the analysis of CFTR-specific T cell responses in patients with CF and in non-CF subjects. We found non-CF subjects with low levels of self-reactive CFTR-specific T cells in the United States and several patients with CF with low to high levels of self-reactive CFTR-specific T cells in both the United States and the United Kingdom.

Introduction

Cystic fibrosis (CF) is one of the most common autosomal recessive lethal disorders affecting white populations of Northern European ancestry. The main clinical sequelae associated with CF are progressively worsening lung disease, pancreatic dysfunction, elevated sweat electrolyte levels, and male infertility (as reviewed in Kreindler, 2010). CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, resulting in the absence of a functional Cl^- channel in epithelial cells. More than 1900 mutations have been identified in the CFTR gene (Ferec and Cutting, 2012). CFTR mutations can be grouped into six classes that reflect the associated biosynthetic or functional alterations in the CFTR protein: (I) CFTR not synthesized, (II) defective processing, (III) defective regulation, (IV) defective conductance, (V) partially defective production or processing, and (VI) altered membrane retention time. The most common mutation of the CFTR gene is ΔF508, a class II mutation caused by the deletion of phenylalanine at position 508. The frequency of the ΔF508 CFTR mutation in patients with CF of northern European descent is ~70% (as reviewed in Kreindler, 2010). Most other CFTR mutations are rare, with just three mutations (G542X, G551D, and W1282X) having overall frequencies of 1–5% (Figueredo *et al.*, 2007).

Although there is no effective cure for CF airway disease, successes in small-molecule-based drug design by Vertex Pharmaceuticals (San Diego, CA) have resulted in the U.S. Food and Drug Administration approval of Kalydeco (ivacaftor) that significantly improves lung function and has become integral to the day-to-day health management of patients with CF with the G551D mutation. Kalydeco works by binding to the CFTR protein and enhancing the channel activity of the mutant CFTR (Eckford *et al.*, 2012). VX809 and VX661, two other small-molecule drugs, also developed by Vertex Pharmaceuticals, are targeted to CF patients with the ΔF508 CFTR mutation. VX809 is currently in phase 3 clinical trials and VX661 is currently in phase 2 clinical trials (Clancy *et al.*, 2012). Both drugs allow translocation of the mutated ΔF508 CFTR from the endoplasmic reticulum (where the

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ΔF508 CFTR is degraded) to the apical side of the airway epithelial cell (Van Goor *et al.*, 2011). Gene therapy in which a normal copy of the *CFTR* gene is introduced to the defective airway cell is also being investigated as a life-long CF treatment (as reviewed in Kreindler, 2010). At present, the only active gene therapy clinical trial is being conducted by the United Kingdom CF Gene Therapy Consortium (UKCFGTC). The gene transfer agent used here is a complex of an optimized cationic-based lipid GL-67A and a plasmid encoding CpG-depleted and codon-optimized CFTR in patients with CF.

A report of gene replacement therapy for Duchenne's muscular dystrophy (DMD) demonstrated that some subjects developed cytotoxic T cell responses against the transgene-derived dystrophin protein because of activation of preexisting memory T cells (Mendell *et al.*, 2010; Bowles *et al.*, 2012). Typically, subjects should have circulating naive T cells to epitopes not expressed by the mutant gene, which in the case of the DMD study would be those located downstream of a series of internal deletions that prematurely terminate translation. One mechanism by which these naive T cells could be activated is somatic reversion of the dystrophin defect in muscle cells by mRNA splicing over the deleterious mutation, reconstituting the normal protein reading frame 3′ to the mutation. Mendell and colleagues concluded that primed T cells may contribute to DMD pathogenesis and enhance problematic T cell responses to molecular therapies that reconstitute normal dystrophin expression (Mendell *et al.*, 2010). The basis for the provocative conclusions of the DMD trial was the result of interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay analyses of peripheral blood mononuclear cells (PBMCs), which showed dystrophin-specific T cells at baseline in two of the six subjects, one of whom responded to gene therapy with a clear T cell response to the transgene product (Mendell *et al.*, 2010).

Here we present the development and clinical validation of a human IFN-γ-specific ELISPOT assay. Several parameters that can impact on the reproducibility of the assay were extensively evaluated before its use in the analysis of clinical samples. We describe the application of the human IFN-γ-specific ELISPOT assay in assessing the presence of preexisting CFTR-specific T cells in the PBMCs of patients with CF from both the United States and the United Kingdom. Furthermore, we characterized the nature of the CFTR-specific T cell response in two patients with CF.

Results and Discussion

Validation of the human CFTR peptide library

To evaluate IFN-γ T cell responses to CFTR we first validated the immunogenicity of the synthesized CFTR peptide library. Previously, we demonstrated that intramuscular injection of human adenovirus serotype 5 (HAdV5) vector expressing human CFTR (hCFTR) into CFTR knockout (KO) mice (Snouwaert *et al.*, 1992) induces a strong $CD8^+$ T cell-mediated immune response against hCFTR (Limberis *et al.*, 2007). T cell responses to hCFTR were evaluated in mice on day 8 postimmunization by subjecting isolated splenocytes to the CFTR-specific IFN-γ ELISPOT assay. Splenocytes were stimulated with six different peptide pools (A–F) of the hCFTR peptide library encompassing the full-length CFTR. As a positive control, splenocytes were stimulated with phorbol myristate acetate plus ionomycin (PMA + ION). As a negative control, splenocytes were evaluated without stimulation. CFTR-specific T cell responses were apparent in all six peptide pools for splenocytes harvested from CFTR KO mice immunized with HAdV5-CFTR vector (Supplementary Fig. S1; supplementary data are available online at http://www.liebertpub.com/humc). Pool D generated the highest response (715 spot-forming units [SFU]/10^6 cells), which was expected as this pool contained the immunodominant $CD8^+$ CFTR T cell epitope (Limberis *et al.*, 2007). Splenocytes isolated from mice injected with phosphate-buffered saline (PBS) did not yield a detectable T cell response to any of the six CFTR peptide pools (Supplementary Fig. S1). For both HAdV5-CFTR-treated mice and PBS-treated mice there was a comparable response to the positive control PMA + ION. These data demonstrate the immunogenicity of the hCFTR peptide library and validate its use in the IFN-γ ELISPOT assay designed to determine CFTR-specific T cell responses in human PBMCs.

Validation of the human IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay used to evaluate preexisting CFTR-specific T cell responses was first extensively validated before its use in clinical trial testing. The main objective of the validation was to estimate the cutoff value of a positive response and the reproducibility of this signal. This was accomplished by analyzing eight different human PBMC samples by two independent operators. Dimethyl sulfoxide (DMSO) was used as negative control and the CEF control peptide pool was used as positive control. CEF consists of 32 MHC class I-restricted T cell epitopes (8–12 amino acids in length), with sequences derived from the human cytomegalovirus (CMV), Epstein–Barr virus (EBV), and influenza virus (Mabtech, Nacka Strand, Sweden). A positive response was defined as a value greater than 55 SFU/10^6 PBMCs and three times the value generated in the presence of DMSO (negative control). Intraoperator variability of a positive response was $<14\%$ and interoperator variability of a positive response was $<25\%$.

To adhere to the quality assurance standards required for clinical trial testing and reporting, a rigorous validation of the IFN-γ ELISPOT assay was performed. The following parameters, in no particular order, were validated:

1. Value of the negative control (DMSO): Analysis of 24 human PBMC samples showed that the average SFU per 10^6 PBMC value is 9 with an accepted range of 0–54 SFU/10^6 PBMCs (Supplementary Table S1).
2. Value of the PMA + ION positive control: Analysis of 24 human PBMC samples showed that the average SFU per 10^3 PBMCs is 69 with an accepted range of 14–169 SFU/10^3 PBMCs (Supplementary Table S1).
3. Value of the human anti-CD3 positive control: Analysis of 24 human PBMC samples showed that the average value is 102 SFU/10^4 PBMCs with a range of 14–408 SFU/10^4 PBMCs (Supplementary Table S1).
4. Value of CEF pool positive control: Analysis of 24 human PBMC samples showed that the average value is 586 SFU/10^6 PBMCs with a range of 0 to >2000 SFU/10^6 PBMCs. Possible reasons for the variability include differences in the HLA haplotype combined with the

infection history of the subject. We also expect temporal differences in the immune response to the CEF pool as a result of natural infections (Supplementary Table S2).

5. Cutoff value of a positive response: The average number of SFU per 10^6 PBMCs detected after DMSO stimulation was 9 (range, 0–54). The average number of SFU per 10^6 PBMCs detected after stimulation with a peptide pool of a self-antigen (α_1-antitrypsin, AAT or A1AT) which should be equal or similar to the DMSO response was 10 (range, 0–40). On the basis of these data the cutoff average value for a response to be positive is the highest value obtained in a sample stimulated with DMSO or the A1AT pool, which is 54 SFU/10^6 PBMCs. For a sample to be scored as positive the average number of SFU per 10^6 PBMCs must be above 55. As shown in Supplementary Table S1, the DMSO value varied greatly from sample to sample, illustrating the importance of assaying the background of each sample.
6. Reproducibility of a positive immune response:
   - Intraoperator variability: Four PBMC samples were run by two different operators (#1 and #2). Analysis of the positive controls CEF, anti-CD3, and PMA + ION showed that the percent coefficient of variation (%CV) was <25, independent of the operator who assayed the samples (Supplementary Table S2). Analysis of the negative controls DMSO and A1AT showed a large %CV, mainly due to the low number of SFU observed in these samples (<9 SFU/10^6 PBMCs). An exception was subject 20080312, who had a high SFU count in DMSO (49–54 SFU/10^6 PBMCs). The %CV for this subject was much lower (7) compared with those of the other three subjects (28, 35, and 141).
   - Interoperator variability: The average value obtained by operator #1 and operator #2 for each of the subjects stimulated with the positive control stimulants CEF, anti-CD3, and PMA + ION was comparable. The highest %CV was 21. Some of the stimulants had a %CV of 0% (Supplementary Table S2).

   The average value obtained by operator #1 and operator #2 for each of the subjects stimulated with the negative control DMSO and stimulant peptide A1AT varied compared with those obtained with the positive control stimulants. The reason for this was that the negative control values were low in three subjects (20060329M, 20060406I, and 20060524J). Subject 20080312, who had high values for DMSO and AAT, also had similar values (%CV 7 and 11, respectively).
7. Impact of 96-well plate lot numbers on the reproducibility of a positive and a negative response (Supplementary Table S3): The average value obtained by the operator for each of the subjects stimulated with positive controls CEF, anti-CD3, and PMA + ION and using plate lot A and plate lot B was comparable. The highest %CV obtained was 32 and was comparable to intraoperator %CV. The average value obtained by the operator for each of the subjects stimulated with the negative control DMSO and stimulant peptide A1AT and using lot A and lot B was also comparable although the %CV was high (141), due to the low number of SFU obtained.
8. Impact of different coating IFN-γ antibody lot numbers on the reproducibility of a negative and a positive immune response (Supplementary Table S4): The average value obtained by the operator for each of the subjects stimulated with the positive controls CEF, anti-CD3, and PMA + ION and using coating IFN-γ antibody lot A and lot B was comparable. The %CV was less than 25 and similar to intraoperator %CV (Supplementary Table S4). The average value obtained by the operator for each of the subjects stimulated with the negative control DMSO and stimulant peptide A1AT and using lot A and lot B was also comparable although the %CV was high because of the low number of SFU obtained.
9. Impact of a different IFN-γ detection antibody lot number on the reproducibility of a negative and a positive immune response (Supplementary Table S5): The average values obtained by the same operator for each of the subjects stimulated with the positive controls CEF, anti-CD3, and PMA + ION and using detection antibody lot A and lot B were comparable. The %CV was less than 30 and similar to intraoperator %CV. The average values obtained by the same operator for each of the subjects stimulated with the negative control DMSO and stimulant peptide A1AT and using lot A and lot B were comparable as well, although the %CV was high because of the low number of SFU obtained.

As a result of the extensive validation of the human IFN-γ ELISPOT assay the following parameters were defined for subsequent use in the analysis of clinical samples:

1. The value of the negative control (DMSO) is <55 SFU/10^6 PBMCs with an acceptable range of 0–54 SFU/10^6 PBMCs. A sample in which a value is higher than that will be labeled as "high background."
2. The value of the PMA + ION positive control is 69 SFU/10^3 PBMCs with an acceptable range of 14–169 SFU/10^3 PBMCs. A sample in which the value is (a) higher than that will be labeled as "high PMA + ION" or (b) lower than that will be labeled as "low PMA + ION."
3. The value of the human anti-CD3 positive control: 102 SFU/10^4 PBMCs with an acceptable range of 14–408 SFU/10^4 PBMCs. A sample in which the value is (a) higher than that will be labeled as "high CD3" or (b) lower than that will be labeled as "low CD3."

   For a sample to be acceptable, at least one positive control (PMA + ION or anti-CD3) must have an SFU value within the range described previously. If both the PMA + ION and anti-CD3 SFU values are out of range, the sample will be labeled as "positive controls failed" and the data generated will be considered invalid.
4. The value of CEF positive control is 564 SFU/10^4 PBMCs with an acceptable range of 0 to >2000 SFU/10^4 PBMCs. The reason for the apparently high variability is that the value is highly dependent on the specific subject HLA and the natural infection history concerning the three viruses (CMV, EBV, and influenza).
5. SFU values are consistently reproducible. Intra- and interoperator reproducibility of a positive response (to either CEF, CD3, or PMA + ION) was determined to be less than 26 (%CV). Intra- and interoperator reproducibility of a negative response (to DMSO and A1AT [irrelevant peptide pool]) was high because of the low SFU value obtained.

Table 1. Cystic Fibrosis Genotype Versus Level of Interferon-γ CFTR T Cells[a]

CF subjects					
Subject	*Genotype*	*Mutated amino acid location*	*Expected CFTR pool response*	*Observed CFTR pool response*	*SFU/10^6 PBMCs*
Scotland					
1	ΔF508/ΔF508	508	C	A, A	900, 1260[b]
2	ΔF508/3272-26 A > G	508/1087[c]	C/E-F	C, C	115, 53[d]
3	ΔF508/ΔF508	508	C	A	133
4	ΔF508/G551D	508/551	C/C	C	63
5	ΔF508/D1152H	508/1152	C/E	A, A	354, 200[b]
6	ΔF508/ΔF508	508	C	C	63
7	G551D/unknown	551	C	C	65
8	ΔF508/ΔF508	508	C	C	65
9	ΔF508/G551D	508/551	C/C	D	90
10	ΔF508/ΔF508	508	C	C, C	58, 55[d]
11	ΔF508/ΔF508	508	C	C	66
England					
12	ΔF508/ΔF508	508	C	E	117
13	ΔF508/L165S	508/165	C/A	A, A	510, 320[b]
14	ΔF508/2657 + 5G > A	508/886	C/D-E-F	F, F	205, 171[b]
15	W1282X/L206W	1282[c]/206	F/A	C	97
16	G542X/unknown	542[c]	C-D-E-F	D	63
17	ΔF508/ΔF508	508	C	E	100
18	ΔF508/ΔF508	508	C	D	131
19	ΔF508/unknown	508	C	C	75
20	ΔF508/G542X	508/542[c]	C	C	110
USA					
1	ΔF508/ΔF508	508	C	C, C	60, 60[d]
2	ΔF508/ΔF508	508	C	C	55
3	ΔF508/M1101R	508/1101	C/E	C	85
4	ΔF508/R560T	508/560	C/C	C, C	55, 73[b]

Non-CF subjects		
Subject	*Observed CFTR pool response*	*SFU/10^6 PBMCs*
USA		
1	C, C	208, 145[b]
2	D	68
3	F	65

Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; PBMCs, peripheral blood mononuclear cells; SFU, spot-forming unit.

[a]PBMCs were subjected to the IFN-γ ELISPOT assay and stimulated with the CFTR peptide library, which was divided into six pools (A-F). For those subjects who scored positive for a T cell response against CFTR, the number of SFU per 10^6 PBMCs, the type of CFTR mutation(s), and the expected and observed CFTR-specific T cell response are presented. The expected CFTR pool response was predicted on the basis of the region of the CFTR protein that was either mutated or missing. SFU values greater than $55/10^6$ PBMCs and three times greater than the background were considered positive.

[b]SFU value from a sample tested on two separate occasions.

[c]Premature stop codon.

[d]SFU values from samples obtained on two separate occasions.

6. Impact of assay materials: No significant effect on SFU values for positive controls (CEF, CD3, and PMA + ION) and negative controls (DMSO and A1AT) was observed when different lots of 96-well plates, IFN-γ coating antibody, and IFN-γ detection antibody were used.

A PBMC sample with an SFU/10^6 count between 55 and 100 is labeled as a low–medium responder and a PBMC sample with an SFU/10^6 count greater than 100 is labeled as a high responder. Validation of the IFN-γ ELISPOT assay was repeated with time as technology and antibody profiles improved.

Determination of CFTR-specific T cell responses in PBMCs isolated from patients with CF and non-CF subjects

We used the human IFN-γ ELISPOT assay to detect CFTR-specific T cells in PBMCs isolated from subjects with CF. A large cohort of adult subjects with CF ($n = 74$) in the United States were assessed for the presence of preexisting CFTR-specific T cells. The subjects ranged in age from 18 to 55 years with varying lung function (FEV_1, 18–124% of predicted values from NHANESIII; Hankinson *et al.*, 1999). We also evaluated PBMCs from 24 non-CF subjects in the United States. Of the 74 subjects evaluated there were 5 sets (of 2

brothers/sisters) of siblings that shared the same mutation (three sets were ΔF508/ΔF508, one set was ΔF508/R1066C, and one was ΔF508/3849+10kbC>T); none of these subjects had CFTR T cell responses. Four of the 74 subjects were low–medium responders (65±12 SFU/10^6) with an overall frequency of 5.5% (Table 1 and Fig. 1A). Two of the four subjects were ΔF508 homozygous and reacted to peptide pool C and two were compound heterozygotes (ΔF508/560T and ΔF508/M1101R) who reacted to peptide pools C and C/E, respectively (Table 1). We were surprised to find a similar frequency of CFTR-specific T cells in three non-CF subjects (12%) ($p=0.74$, Fisher exact test; Fig. 1B).

We also evaluated patients with CF who were being assessed for participation in a lipid-based gene therapy trial for CF conducted by the UKCFGTC. The study was designed to validate biomarkers, collect pre–gene therapy baseline data, and help with selection of patients suitable for progression into a multidose clinical trial. A large cohort of subjects ($n=174$) with CF, ranging in age from 11.9 to 59.4 years with varying lung function (FEV_1, 39.79–104.04%), was assessed. We detected CFTR-specific T cells in 20 subjects with CF (11.5%; Fig. 1A) from three different clinical centers (one in London [England] and two in Edinburgh [Scotland]), many of which were found to be at low–moderate numbers (88±37 SFU/10^6, $n=16$; Table 1) and several subjects had high numbers (490±392 SFU/10^6 PBMCs, $n=4$; Fig. 1A and Table 1). An example of an IFN-γ response to CFTR, identified by the IFN-γ ELISPOT assay, is the low–medium responder (subject 1) and high responder (subject 8) depicted in Fig. 2. In each positive CF subject sample, reactivity was limited to only one of the six pools of the CFTR peptide library. The pools that stimulated the IFN-γ response varied between subjects, with the highest frequency found against pools A ($n=4$) and C ($n=10$) and with more limited numbers being observed against pools D ($n=3$), E ($n=2$), and F ($n=1$) (Table 1).

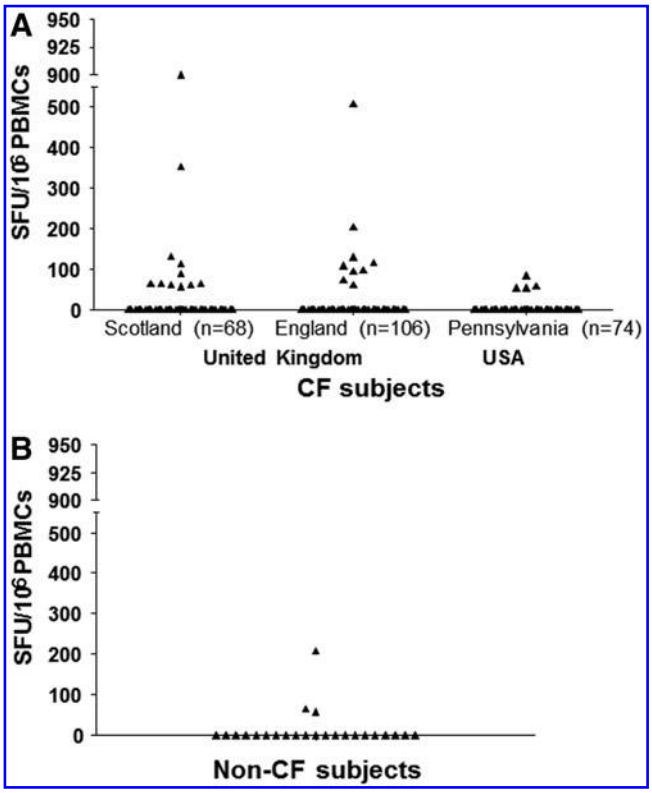

FIG. 1. Presence of IFN-γ CFTR T cells in PBMCs isolated from subjects with CF and non-CF subjects. **(A)** Frequency of CFTR-reactive T cells in 68 subjects with CF from Scotland (Edinburgh), 106 subjects with CF from England (London), and 74 subjects with CF from the United States (Philadelphia). **(B)** Frequency of CFTR-reactive T cells in 24 non-CF subjects. Each triangle represents one subject. SFU, spot-forming unit.

Age and status of lung function of patients with CF in the United States and the United Kingdom did not appear to correlate with the absence or presence of CFTR-specific T cells. As expected, some patients with CF generated T cells against the region of the CFTR protein that was either mutated or missing. These were ΔF508 homozygous ($n=6$), ΔF508/G551D compound heterozygous ($n=1$), ΔF508/G542X compound heterozygous ($n=1$), and ΔF508/R560T compound heterozygous ($n=1$) patients who reacted to pool C (Table 1). Reconciling the CFTR genotype for each patient with the pool that stimulated T cells indicated that 12 patients with CF generated T cells against self-epitopes (Table 1). The observed CFTR-specific T cell response did not match the expected response, based on the location of the missing or mutated amino acid. We also identified compound heterozygotes in which the CFTR mutations from both alleles were located in different peptide pools; that is, ΔF508/L165S. In this instance the patient should not develop a T cell response to CFTR as the correct amino acid at position 508 is present on the L165S allele and likewise the correct amino acid at position 165 is present on the ΔF508 allele. However, this patient with CF developed a T cell response to pool A (which contains the L165S mutation), thus generating a T cell response against self-epitope. We also identified other patients with CF who had T cell responses against self-epitope. These were ΔF508 homozygous ($n=6$), ΔF508/3272-26 A>G compound heterozygous ($n=1$), ΔF508/G551D compound heterozygous ($n=1$), ΔF508/D1152H compound heterozygous ($n=1$), ΔF508/2657+5G>A compound heterozygous ($n=1$), W1282X/L206W compound heterozygous ($n=1$), and ΔF508/M1101R compound heterozygous (Table 1).

A positive CFTR-specific IFN-γ T cell response was confirmed in CF subjects 1, 5, 13, and 14 and also in non-CF subject 1 when PBMCs were tested on two separate occasions (Table 1). IFN-γ ELISPOT assay analysis of PBMCs from subject 10 harvested 2 years apart revealed similar CFTR-specific T cell responses (Table 1 and Fig. 3A). To further characterize the T cell response in this subject, we used polychromatic flow cytometry to identify the nature of the CFTR-specific T cell response. Intracellular cytokine staining demonstrated a $CD8^+$ T cell response dominated by degranulation factor CD107α in subject 10 (Fig. 3C). PBMCs from subject 2, who was a low–medium responder, harvested 2 years apart were also evaluated for CFTR-specific T cell responses (Fig. 3B). The PBMCs isolated on day 0 were analyzed by polychromatic flow cytometry. The CFTR-specific $CD8^+$ T cell response was found to be IFN-γ-dominated (Fig. 3D); however, when PBMCs were analyzed on day 886 by IFN-γ ELISPOT assay no CFTR-specific T cells were detected (Fig. 3B).

CF airway disease has become a target for therapies aimed at "correcting" the molecular defects of the most common

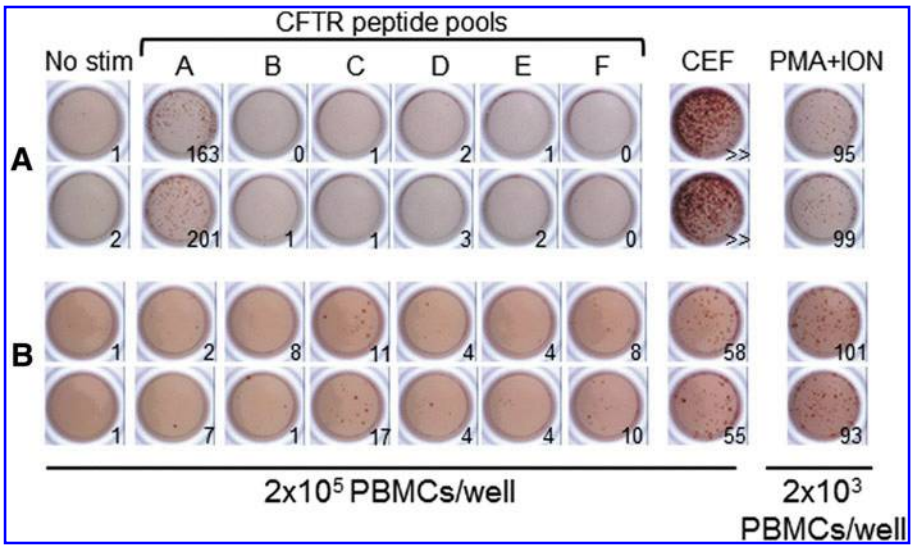

FIG. 2. IFN-γ ELISPOT assay analysis of **(A)** a representative high responder (subject 1) and **(B)** a representative low–medium responder (subject 8). PBMCs were stimulated in duplicate with individual pools of CFTR peptide library (A–F), with CEF or PMA + ION as positive control and without any stimulant (No stim). Number of SFU is indicated for each well. Color images available online at www.liebertpub.com/humc

CFTR gene mutations, namely ΔF508 and G551D. One such molecular therapy is Kalydeco for patients with the G551D CFTR mutation. In addition, the CFTR correctors VX809 and VX661 have been met with excitement as results from phase 2 clinical trials in patients with CF appear promising (Clancy *et al.*, 2012). Regardless of the type of therapy used to treat the underlying defect of CF, an issue that needs to be addressed with both gene therapy and molecular therapies is the likely consequence of CFTR-specific T cell activation in response to the "correct" version of CFTR. In a gene therapy

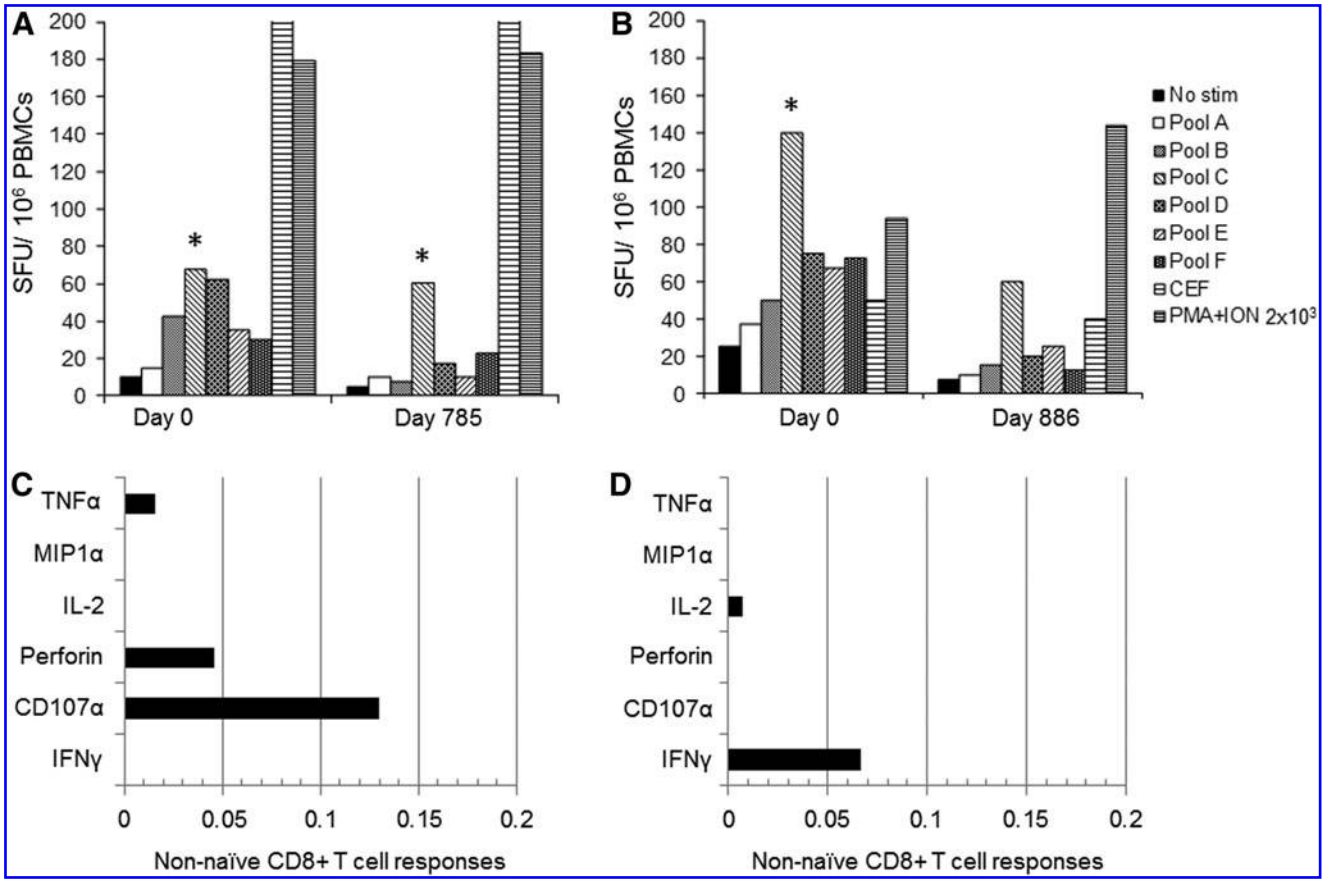

FIG. 3. Quantification and phenotype analysis of the T cell response to CFTR. PBMCs isolated from blood obtained on two different occasions (as indicated) from **(A)** subject 10 and **(B)** subject 2 were analyzed by IFN-γ ELISPOT assay. Solid columns indicate unstimulated (No stim) PBMCs. Horizontal striped columns indicate PBMCs stimulated with CEF or PMA + ION (positive controls). The remaining columns indicate PBMCs stimulated with individual CFTR peptide pools A–F. **(C** and **D)** Characterization of the T cell response to CFTR peptide pool C by polychromatic flow cytometry of PBMCs isolated from **(C)** subject 10 and **(D)** subject 2. *Positive response to CFTR.

clinical trial for Duchenne muscular dystrophy two subjects were reported to have preexisting dystrophin-specific T cells at baseline (Mendell *et al.*, 2010). The investigators monitored these subjects closely after gene therapy and noted that one of them went on to respond to gene therapy with a clear T cell response to the transgene product (Mendell *et al.*, 2010).

The surprising aspect of our study is the abundance and breadth of T cells specific to portions of CFTR that should be viewed as self in 5–11.5% of subjects with CF, with 16% of these patients scoring as high responders. Of interest, however, was the finding of almost 12% of low–medium responders among normal non-CF subjects. It is well established that negative selection in the thymus does not eliminate all self-reactive T cells, especially those with low affinity (Kappler *et al.*, 1987), allowing them to escape to the periphery. Here they are suppressed by the natural regulatory T cells, also originated in the thymus, protecting the host against autoimmunity. Unless stimulated with antigen, the frequency of circulating CFTR-specific T cells should be low and undetectable by the IFN-γ ELISPOT assay. In the case of the ΔF508 CFTR mutation the stimulation of circulating CFTR-specific T cells may be caused by the misfolding of the CFTR protein, which may lead to abnormal processing of the protein by the immune-proteasome and a change in repertoire of CFTR peptides loaded in the major histocompatibility complex (MHC) class I (Kappler *et al.*, 1987). Moreover, the abnormal expression of CFTR protein, in addition to the underlying disease pathogenesis, may also trigger robust inflammatory signals that activate low-affinity circulating CFTR-specific T cells (Chmiel *et al.*, 2002; Koehler *et al.*, 2004). We identified compound heterozygous patients with CF in whom the CFTR mutations from both alleles were located in distinctly separate amino acid positions. In these subjects we did not expect a CFTR T cell response as the correct amino acid for each of the respective mutations was expressed by the opposing allele. However, the presence of self-reactive CFTR-specific T cells suggests abnormal processing and/or presentation of the mutated CFTR.

We were surprised by the unexpected finding of CFTR-specific T cells in normal healthy subjects. In comparison with the patients with CF the magnitude of the T cell response was lower. A reason for this difference is that normal healthy subjects lack the chronic expression of inflammatory stimuli present in patients with CF, which exacerbate the CFTR-specific T cell responses. The non-CF subjects were white donors and, based on the carrier frequency in the United States of 1 in 29 (www.cff.org), it is likely that one of the low–medium responders is a CF carrier. In future studies we plan also to screen CF carriers for the presence of preexisting CFTR-specific T cells.

Although it is unclear what primes these CFTR-specific T cells, they do not appear to cause extensive immunopathology as would be expected in the case of an autoimmune disease (Goebels *et al.*, 2000). Until more compelling data emerge, one should be cautious in ascribing these self-reactive T cells to disease pathogenesis or confounding host responses to molecular therapies.

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Author Disclosure Statement

Roberto Calcedo, no competing financial interests; Uta Griesenbach, no competing financial interests; Daniel J. Dorgan, no competing financial interests; Samia Soussi, no competing financial interests; A. Christopher Boyd, no competing financial interests; Jane C. Davies, no competing financial interests; Tracy E. Higgins, no competing financial interests; Stephen C. Hyde, no competing financial interests; Deborah R. Gill, no competing financial interests; J. Alastair Innes, no competing financial interests; David J. Porteous, no competing financial interests; Eric W. Alton, no competing financial interests; James M. Wilson is a consultant to ReGenX Holdings, and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, relevant to this work, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings; Maria P. Limberis, no competing financial interests.

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