Prediction of Cellular Immune Responses against CFTR in Patients with Cystic Fibrosis after Gene Therapy

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Different classes of mutations (class I–VI) of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene are responsible for lung/pancreatic disease. The most common mutation, $\Delta F508$, is characterized by expression of precursor forms of CFTR but no functional CFTR. Since only 5–10% of normal CFTR function is required to correct the electrophysiologic defect across the airway epithelium, gene therapy holds promise for treatment of patients with CF lung disease. However, efficient delivery and transgene expression are not the only parameters that may influence the success of gene therapy. Host-specific immune responses generated against the therapeutic CFTR protein may pose a problem, especially when the coding sequence between the normal CFTR and mutated CFTR differ. This phenomenon is more pertinent to class I mutations in which large fragments of the protein are not expressed. However, T cells directed against epitopes that span sequences containing class II–V mutations are also possible. We used MHC-binding prediction programs to predict the probability of cellular immune responses that may be generated against CFTR in $\Delta F508$ homozygote patients. Results obtained from running the prediction algorithms yielded a few high-scoring MHC-Class I binders within the specific sequences, suggesting that there is a possibility of the host to mount a cellular immune response against CFTR, even when the difference between therapeutic and host CFTR is a single amino acid (F) at position 508.

Keywords: MHC ligand; CFTR; gene therapy; $\Delta F508$

Cystic fibrosis (CF) is a debilitating human disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a protein that functions as a cAMP-regulated chloride channel. Defects of the CFTR protein result in abnormal chloride transport across the apical membranes of epithelial cells in the airways, pancreas, intestine, and *vas deferens*, leading to progressive lung disease, pancreatic dysfunction, elevated sweat electrolyte levels, and male infertility, respectively. More than 1,000 mutations have been identified in the CFTR gene. With reference to chloride transport dysfunction, CFTR mutations can be grouped into five 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, and (V) partially defective production or processing. While for classes I

CLINICAL RELEVANCE

These findings suggest that there is a strong possibility of a recipient of gene therapy to mount an immune response toward the therapeutic gene product. Immune modulation would then be required to improve the success rate of long-term gene therapy.

and II a major fraction of the CFTR protein does not reach the apical epithelial cell surface, it is present on the cellular surface in classes III, IV, or V with some residual function.

Correlation between genotype and phenotype has been shown for pancreatic status (1) and also, more recently, for airway disease (2). Patients with genotypes that include class I or II mutations on both chromosomes have more rapid deterioration in lung function and lower survival rates compared with the other genotypes with mutations that belong to class IV or V. The most common mutation among whites, $\Delta F508$, belongs to class II and consists of a 3-bp deletion in exon 10 causing a loss of phenylalanine at amino acid position 508 of the protein product (3). $\Delta F508$ results in misfolding of the CFTR protein and hence mislocation of the mature protein. According to the worldwide mutation survey conducted by the Cystic Fibrosis Genetic Analysis Consortium (CFGAC), the $\Delta F508$ allele accounted for 66% of 43,849 tested CF chromosomes (4). The occurrence of the $\Delta F508$ mutation varies considerably between different populations and geographical locations, with the lowest reported incidence in Tunisia (17.9%) (5) and the highest in Denmark (90%) (6). Most of the other CFTR mutations are rare, with only four mutations (G542X, N1303K, G551D, and W1282X) having overall frequencies above 1%. Gene therapy is the only curative option for treatment of patients with CF (7). However, potential obstacles to gene replacement therapy are the generation of host immune responses directed against vector-encoded CFTR product and the vector.

T cell development within the thymus is heavily influenced by interaction of the $\alpha\beta$ T cell receptor (TCR) with the highly polymorphic products of the major histocompatibility complex (MHC) genes. Bone marrow-derived T cell precursors enter the thymus to be subjected to central tolerance, for further survival and differentiation along the appropriate lineage. The ligand specificity of the T cell receptor determines the ultimate fate of the T cell. Those precursors that express TCRs that bind to self-reactive antigenic peptides presented by MHC molecules, with high affinity/avidity, are eliminated via clonal deletion or negative selection, thereby preventing autoimmune disease, while T cells with TCRs that bind to self-reactive antigenic peptides in the context of MHC, with poor affinity/avidity, survive and

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enter the periphery by means of positive selection. Thus, positive and negative selections allow cells only with functional TCRs that will not be self-reactive in the periphery to pass the thymic checkpoints (8). The selected T cells emerge from the thymus as mature, self-MHC restricted and self-tolerant cells, bearing either CD4 if their T cell receptors are MHC class II restricted (so they will become helper cells), or CD8 if they are MHC class I restricted (and will become killer cells).

Recognition of an antigenic peptide bound to an MHC protein (peptide-MHC) by the $\alpha\beta$ TCR is necessary for the initiation and propagation of a cellular immune response, as well as the development and maintenance of the T cell repertoire. Antigens are processed intracellularly into short peptides, which are then presented by MHC molecules. These MHC molecules are highly polymorphic in nature. The polymorphic amino acids are generally clustered in the antigen peptide-binding groove, which is responsible for specificity of recognition by T cells, and for the variation of responsiveness to particular foreign antigens.

The type and magnitude of the immune response would depend primarily upon the underlying mutation in CFTR. Lack of expression of full length or a fragment of the endogenous protein would result in diminished central tolerance, thereby leading to the generation of an immune response toward epitopes in the missing fragments by nondeleted T cells specific for the full-length protein. A greater response toward CFTR would therefore be observed when class I mutations are involved, wherein the expression of endogenous full-length CFTR is absent. For class II mutations, the $\Delta F508$ mutation in particular, full-length CFTR is synthesized lacking a phenylalanine residue at position 508.

Those self-reactive T cells that were spared from thymic deletion enter into the periphery and are maintained in a quiescent state. Because the affinity threshold of TCR-peptide-MHC interaction that signals thymic deletion is lower than that for activation in the periphery, it is likely that some T cells with low avidity for self-antigens will not be activated in the periphery and, instead, remain "ignorant" of their cognate antigen. However, ignorance of the antigen cannot be relied upon to maintain peripheral tolerance because, given the proper stimulatory milieu, such antigens may no longer be ignored and could potentially initiate autoimmune responses, as observed after viral infection (9, 10). In addition to passive means of tolerance, peripheral tolerance has been shown to be maintained actively by the tolerance mechanisms exerted by regulatory T cells (11). Thus, another potential mechanism by which immune responses may be generated toward therapeutic CFTR would be due to perturbation of mechanisms that control peripheral tolerance.

To induce a cellular immune response, antigens have to be processed in intracellular compartments, transported, and presented by HLA molecules before recognition by specific T cells. T cell immune responses are driven by antigenic epitopes, and hence their identification is important for understanding disease

pathogenesis and etiology. There are two types of T cell epitopes, CD8 and CD4, which are recognized in the context of the MHC-I and MHC-II molecules, respectively, by the correspondent T cell types. Appropriate processing of antigen peptides must occur before their binding to the appropriate MHC molecules. Detailed understanding of antigen processing and MHC-peptide recognition and binding has led to the development of several prediction algorithms. These algorithms primarily predict MHC binding, and more recently also include proteasomal cleavage recognition, crucial for the identification of CD8-T cell epitopes. Some of the databases list published HLA ligands and T cell epitopes, while others offer prediction of T cell epitopes, for both Class I and Class II alleles.

In this study, we attempt to predict the likelihood of a host specific immune response mounted against the therapeutic CFTR protein in patients with the most frequent CFTR mutation, $\Delta F508$, after gene therapy.

MATERIALS AND METHODS

Epitope prediction was performed using free web-based MHC-binding prediction algorithms. The algorithms/programs that were used in this study are described in Table 1. Amino acid sequence (488–528) of human CFTR (fragment containing $\Delta F508$ and flanking amino acids) was queried against a series of HLA-Class I and II alleles using these algorithms. The criteria for selection of positive hits were set arbitrarily at lower to moderate stringency levels based on the cutoff limits as shown in Table 1 for each algorithm.

BIMAS ranks potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced as previously described (12).

SYFPEITHI is an updated database with previous publications on T cell epitopes and MHC ligands (13) (access via: www.syfpeithi.de). The prediction is based on published motifs (pool sequencing, natural ligands) and takes into consideration the amino acids in the anchor and auxiliary anchor positions, as well as other frequent amino acids (13). Only those MHC class I alleles for which a large body of data is available are included in the "epitope prediction" section of SYFPEITHI. A reliability of at least 80% in retrieving the most apt epitope can be expected. Thus the naturally presented epitope should be among the top-scoring 2% of all peptides predicted in 80% of all predictions. For epitope predictions using MHC class II motifs, high reliabilities usually cannot be achieved due to the more variable pocket binding behavior. A reliability of only $\sim 50\%$ is estimated.

ProPred-I is an on-line service for identifying the MHC Class-I binding regions in antigens. This is a matrix-based method that allows the prediction of MHC-binding sites in an antigenic sequence for 47 MHC class-I alleles. The matrices used in ProPred-I have been obtained from both the BIMAS server and literature (14).

Propred web is an interface allow users to predict MHC Class II binding regions in antigen sequence. The server employs amino acid/position coefficient tables deduced from literature (15), in a linear prediction model (i.e., quantitative matrix-based prediction method).

Rankpep predicts peptide binders to MHC-I and MHC-II molecules from protein sequence/s or sequence alignments using position-specific

TABLE 1. ALGORITHMS/PROGRAMS USED TO PREDICT MAJOR HISTOCOMPATIBILITY COMPLEX BINDERS

Algorithm	Web Address	Criteria	Description
BIMAS: Bioinformatics and Molecular Analysis Section	http://bimas.dcrt.nih.gov/molbio/hla_bind/	Score > 50	Ranks potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules
ProPred-I	http://www.imtech.res.in/raghava/propred1/index.html	Threshold = 3%	Quantitative matrix for 47 MHC Class I alleles
ProPred	http://www.imtech.res.in/raghava/propred/	Threshold = 4%	Quantitative matrix based prediction method -51 MHC Class II alleles
RANKPEP	http://bio.dfci.harvard.edu/Tools/rankpep.html	Score > 30	Peptide binding to Class I and II alleles using Position specific scoring matrices (PSSM)
SYFPEITHI	http://www.syfpeithi.de/	Score ≥ 10	Database of published motifs and natural ligands

scoring matrices (PSSMs). In addition, it predicts those MHC-I ligands whose C-terminal end is likely to be the consequence of proteasomal cleavage (16).

RESULTS

The MHC prediction algorithms yielded several potential hits, restricted to both MHC-I and MHC-II, within the CFTR fragment at positions 488–528. Overall, 20 potential MHC-I and 8 potential MHC-II ligands were obtained, as shown in Tables 2 and 3, respectively. These MHC ligands are restricted by more or less frequent MHC alleles, as shown by phenotype frequency in the general U.S. white population in both Tables 2 and 3. Approximately 30% of the MHC-I ligands were hits obtained with a high score (SYFPEITHI score ≥ 20). In general, immunodominant epitopes are among the top ranked hits following a query. However, in addition to being a high-scoring MHC-I ligand, there are other factors that may influence the role of a potential MHC ligand in the formation of an immune response, such as (1) correct peptide processing, (2) stability of the MHC-peptide interaction, (3) ability of TCR to recognize peptide/MHC complex, and (4) precursor CTL frequencies.

While ligands predicted by BIMAS are selected on the basis of their binding affinity with the respective MHC-I allele, those predicted by SYFPEITHI are selected on motif-based matrices developed from naturally occurring ligands or T cell epitopes. ProPred-I uses quantitative matrices with the option of the proteasome cleavage filter to identify potential T cell epitopes. We used more than one algorithm to predict potential MHC ligands to take advantage of the features associated with each of these programs. Some of the peptides scored positive by more than one program. For example, peptide KENIIFGVSY, which is restricted by HLA-B*44, was identified by all four algorithms used. Rankpep employs position specific scoring matrices to predict binders and also predicts those MHC-I ligands whose C-terminal end is likely

to be the result of proteasomal cleavage, a classic feature of CD8-T cell epitopes. Thus, peptide(s) T/IKENIIFGV restricted for HLA-A*0201 and A*0204 represent potential CD8-T cell epitopes, by virtue of their C-terminal ends.

Interestingly, the potential CD8-T cell epitope “TIKENIIFGV” is restricted by HLA-A*0201, one of the most frequent class I alleles in whites (Phenotype frequency = 38–50%). Since the $\Delta F508$ mutation is most common among whites, there exists a strong possibility that this epitope may be involved in the manifestation of a cellular immune response against therapeutic CFTR, in particular, in $\Delta F508$ homozygotes or compound heterozygotes comprising a class I mutation and the $\Delta F508$ mutation.

Prediction of MHC-II ligands is challenging because peptides binding to a single MHC-II molecule are extremely variable in length and share very limited sequence similarity (17–19). The RANKPEP algorithm, therefore, uses the motif discovery program MEME to create MHC-II profiles. On average, the sensitivity of these MHC-II-specific profiles is such that $\sim 60\%$ of known MHC-II restricted T cell epitopes are found among the top 2% scoring peptides from their protein sources using the RANKPEP algorithm (20). As shown in Table 3, we identified several potential CD4-specific MHC-II-binding peptides within the CFTR 488–528 fragment. Interestingly, three different peptides were identified as ligands for HLA-DRB1*0701, an MHC-II allele that is relatively frequent in the U.S. white population (allele phenotype frequency of 25.5%).

In silico T cell epitope mapping using bioinformatics, when combined with other *ex silico* means of evaluating MHC-peptide and T cell interaction such as tetramers and HLA transgenic mice, has proved very useful in the field of vaccinology (21). However, there are instances in which theoretical predictions cannot be supported with experimental evidence, which may be explained by sensitivity of the experimental procedures, and also low precursor frequencies of antigen-specific cells.

TABLE 2. PREDICTED MHC-CLASS I PEPTIDE BINDERS IN CFTR FRAGMENT 488–528

MHC-Class I	Peptide Ligand	Score (Algorithm)	Class I Phenotype Frequency (%) [*]
HLA-A*01	ENIIFGVSY	15 (S)	28.7
HLA-A*0201	KENIIFGVSY	15 (S), 101 (R)	47.8
	KENIIFGV	41 (R)	
	IKENIIFGV	16 (S)	
	TIKENIIFGV [†]	24 (S), 56 (R)	
HLA-A*0204	IKENIIFGV [†]	34 (R)	0.7 [‡]
HLA-A*03	TIKENIIFG	10 (S)	20.6
HLA-A*26	ENIIFGVSY	26 (S)	8, 5.8 [‡]
	GTIKENIIF	21 (S)	
	IIFGVSYDE	60 (R), 11 (S)	
HLA-A*6801	GTIKENIIFGV	102 (R), 10 (S)	3
HLA-B*08	TIKENIIF	22 (S)	22.5
HLA-B*2705	GTIKENIIF	20 (S)	8.6
HLA-B*44, B*4402, B*4403	ENIIFGVSY	75 (R), 16 (S)	27.1, 16.2, 11.6
	KENIIFGVSY	101 (R), 22 (S), 180 (B) (P)	
HLA-B*5801	GTIKENIIF	80 (B) (P)	0
HLA-Cw*0401	IIFGVSYDEY	(P)	23.5
HLA-Cw*0602	IKENIIFGV	(P)	13.6
HLA-Cw*0702	ENIIFGVSY, IIFGVSYDEY	(P)	31.1

Definition of abbreviations: B, BIMAS; CFTR, cystic fibrosis transmembrane conductance regulator; F, F508 residue highlighted in all peptides; MHC, major histocompatibility complex; P, ProPred-I; R, RANKPEP; S, SYFPEITHI.

^{*} MHC Class I phenotype frequency obtained from U.S. white populations: Bethesda, $n = 307$, white pop 1, $n = 61,655$; <http://allelefrequencies.net/>.

[†] Predicted binder with proteasome cleavage site at C terminus.

[‡] MHC-I phenotype freq. from USA Caucasian pop 1, $n = 61655$.

TABLE 3. PREDICTED MHC-CLASS II PEPTIDE BINDERS IN CFTR FRAGMENT 488–528

MHC-Class II	Peptide Ligand	Score (Algorithm)	MHC II Phenotype Frequency (%)
HLA-DRB1*0401 (DR4Dw4)	KENIIFGVSYDEYRY	14 (S), (P)	13.8
HLA-DRB1*0301 (DR17)	ENIIFGVSYDEYRYR	19 (S)	18.6
	IIFGVSYDEYRYSV	16 (S) 14.27 (R)	
HLA-DRB1*0701	SWIMPGTIKENIIFG	24 (S)	25.5
	NIIFGVSYDEYRYS	22 (S)	
	IMPGTIKENIIFGV	12 (S), 13 (R)	
HLA-DRB1*1101	FGVSYDEYRYSVIK	16 (S)	10.4
HLA-DRB1*1501 (DR2b)	PGTIKENIIFGVSYD	20 (S)	15.9

For definition of abbreviations, see Table 2.

MHC Class II phenotype frequency obtained from U.S. white population: Bethesda, $n = 307$, <http://allele-frequencies.net/>.

Our results suggest that there exists a number of potential CD8- and CD4-specific T cell epitopes within the CFTR fragment encompassing the “F” residue at position 508. The possibility of the CFTR gene therapy recipient mounting an immune response to the therapeutic CFTR would depend upon activation of T cells specific for epitopes within normal CFTR containing F508 residue (1) following an escape from central tolerance due to the underlying CFTR mutation, and/or (2) due to disturbance of peripheral tolerance toward CFTR.

Antigens are processed and presented differentially depending on their entry into the cell. Endogenously produced antigens are processed via the proteasomal pathway followed by transporter associated with antigen processing (TAP)-mediated transport of the peptides to the endoplasmic reticulum, where they are loaded onto class I MHC molecules and then presented on the cell surface in context of MHC-I. Exogenously derived antigens are processed via the lysosomal endocytic pathway, after which the peptides are loaded onto MHC-II for presentation on the cell surface. Occasionally, antigens may enter the cell through an exogenous pathway and get shunted toward proteasomal cleavage followed by peptide loading on MHC-I, leading to presentation in the context of MHC-I. This alternative pathway is termed “cross presentation.” In patients who are homozygous for $\Delta F508$, full-length CFTR protein is synthesized; however, due to misfolding, the protein is rapidly degraded. This rapid misfolding may lead to increased class I-mediated processing and presentation or cross-priming, thereby resulting in the perturbation of peripheral tolerance toward CFTR. This has been demonstrated for full-length misfolded tyrosinase in a melanoma cell line, wherein the increased misfolding led to increased proteasomal availability, followed by increased class I presentation (22). Although for patients homozygous for the $\Delta F508$ CFTR mutation, central tolerance may be in place by thymic deletion, the constant degradation may result in increased antigen processing and presentation, and activation of antigen-presenting cells (APCs), thereby leading to antigen perturbation in the periphery. Excess levels of therapeutic CFTR expression may lead to cross-presentation of the antigen or trigger activation of quiescent CFTR-specific T cells in the periphery. The combined environment in which endogenous CFTR is being rapidly degraded (resulting in the activation of APCs, as well as increased cross presentation of the CFTR transgene-specific epitopes via common MHC alleles along with appropriate co-stimulation and activation of CFTR-specific T cells) may all be conducive toward breaking peripheral tolerance, thereby leading to an onslaught of a cellular response toward the therapeutic CFTR. We suggest that T cells to CFTR be evaluated in preclinical and clinical studies of CF gene therapy.

Conflict of Interest Statement: J.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.L. is

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