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The two-faced T cell epitope

Examining the host-microbe interface with JanusMatrix

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Advances in the field of T cell immunology have contributed to the understanding that cross-reactivity is an intrinsic characteristic of the T cell receptor (TCR), and that each TCR can potentially interact with many different T cell epitopes. To better define the potential for TCR cross-reactivity between epitopes derived from the human genome, the human microbiome, and human pathogens, we developed a new immunoinformatics tool, JanusMatrix, that represents an extension of the validated T cell epitope mapping tool, EpiMatrix. Initial explorations, summarized in this synopsis, have uncovered what appear to be important differences in the TCR cross-reactivity of selected regulatory and effector T cell epitopes with other epitopes in the human genome, human microbiome, and selected human pathogens. In addition to exploring the T cell epitope relationships between human self, commensal and pathogen, JanusMatrix may also be useful to explore some aspects of heterologous immunity and to examine T cell epitope relatedness between pathogens to which humans are exposed (Dengue serotypes, or HCV and Influenza, for example). In Hand-Foot-Mouth disease (HFMD) for example, extensive enterovirus and human microbiome cross-reactivity (and limited cross-reactivity with the human genome) seemingly predicts immunodominance. In contrast, more extensive cross-reactivity with proteins contained in the human genome as compared to the human microbiome was observed for selected Treg epitopes. While it may be impossible to predict all immune response influences, the availability of sequence data from the human genome, the human microbiome, and an array of human pathogens and vaccines has made computationally-driven exploration of the effects of T cell epitope cross-reactivity now possible. This is the first description of JanusMatrix, an algorithm that assesses TCR cross-reactivity that may contribute to a means of predicting the phenotype of T cells responding to selected T cell epitopes. Whether used for explorations of T cell phenotype or for evaluating cross-conservation between related viral strains at the TCR face of viral epitopes, further JanusMatrix studies may contribute to developing safer, more effective vaccines.

Introduction

JanusMatrix is a new immunoinformatics tool that was developed to compare T cell epitope conservation between protein sequences from bacterial and viral organisms that make up the human gut microbiome (HM), autologous proteins from the human genome (HG), and human viral and bacterial pathogens (HP). We have used JanusMatrix to initiate the exploration of the relationships between conservation with self (and non-self), T cell phenotype (regulatory or effector), and immunodominance. For example, we postulated that *regulatory T cells* (Treg) might bear T cell receptors (TCRs) that are more likely to bind to T cell epitopes that are cross-conserved (at the TCR face) in many human (self) proteins, while, by comparison true *effector T cells* (Teff) might bear TCRs that recognize TCR-facing residues that are less cross-conserved with human genome proteins. We reasoned that such

cross-conserved epitopes might be important to remove from vaccines, because of the potential for cross-reactive T cells to the vaccine epitopes to suppress, or alter, the desired immune response. The tool may also be used to uncover pathogen epitopes that are associated with autoimmune responses, and epitopes that are conserved between two similar pathogens (Dengue serotypes) or slightly dissimilar pathogens (West Nile Virus and Yellow Fever Virus, for example). The purpose of this review is to describe the use of this tool, as it is currently being implemented in our research programs, and to illustrate several potential applications of JanusMatrix to vaccine research and development.

The mechanics of T cell receptor cross-reactivity. T cells recognize linear peptides or “T cell epitopes” that form a complex with human (HLA) or other species’ MHC molecules. The T cell bears a surface receptor (T cell receptor, or TCR) that binds to the T cell epitope’s amino acid side chains facing upwards, out of

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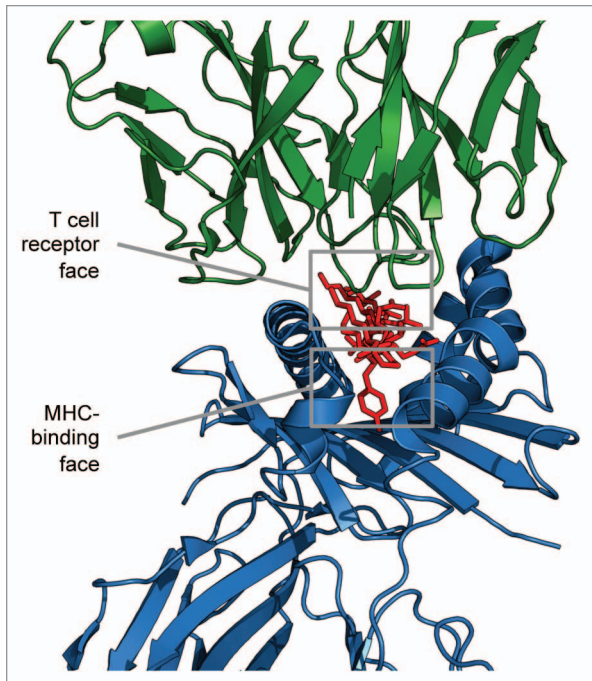


Figure 1. JanusMatrix separates the amino acid sequence of T cell epitopes into TCR-facing residues and HLA binding cleft-facing residues, and then compares the TCR face to other putative T cell epitopes. JanusMatrix defines cross-reactive T cell epitopes as those that have the same MHC allele restriction, the same or similar T cell-facing residues (epitope), and conserved binding of MHC-facing residues (agretope). The HLA-facing residues of the comparators are allowed to vary, as long as they still bind to the original HLA allele. Epitopes that are identical in terms of their TCR face and are equally able to bind to the identical HLA, but differ in sequence, are rapidly identified from a given database of genomic sequences. This enables large-scale comparisons between TCR-homologous T cell epitopes from the HG, the HM, and the HP.

the MHC binding cleft, while the MHC binds to the T cell epitope's amino acid side chains facing into "pockets" located on the sides and bottom of the binding cleft. The T cell epitope face that binds into the MHC-binding cleft is known as the "agretope", while "epitope" refers to either the whole linear peptide or to the TCR face of the peptide (Fig. 1).

Effective T cell immunity requires that the host be able to generate a specific response to antigenic epitopes without any prior knowledge of their composition. In this regard, the immune system faces a fundamental dilemma: how to cover the vastly higher number of potential epitopes (estimated at $> 10^{12}$) with available TCRs (estimated maximum of $< 10^8$ in humans). One hypothesis for resolving this dilemma is that TCRs may recognize more than one peptide epitope. This theory has since been validated: indeed, each TCR has the potential to recognize as many as one million peptides. The possibility for cross-reactivity may seem high and even potentially dangerous, but given the number of potential T cell epitopes represented by modifying the 20 amino acids at each of the TCR-facing positions, it turns out that this reflects a minimum cross-specificity of 1:100,000 peptides.¹ Thus, there is a relatively limited probability of cross-reactivity with self as well as foreign antigens. While it is true

that altering MHC binding residues can modify the affinity of the peptide and change the shape of the TCR face, the TCR has been shown to adapt to minor changes, enabling a single TCR to bind to more than one epitope containing the same TCR-facing but different MHC-binding-facing residues.^{2,3}

Rules of contact enable the generation of TCR cross-reactivity predictions. Based on crystal structures of ternary MHC:peptide:TCR structures, general patterns of contact have been observed, with some peptide residues frequently in contact with the MHC and others frequently in contact with the TCR.⁴ These TCR-facing and MHC-facing amino acid residue rules can be adapted for use with T cell epitope prediction tools such as EpiMatrix⁵ to define cross-conserved TCR-facing residues across large sequence databases (see JanusMatrix algorithm, below). Once a single T cell epitope is defined, by holding its TCR-facing residues constant and allowing its MHC-binding residues to vary, it becomes possible to search protein sequence databases for epitopes that appear "homologous" to a given TCR, despite minor variations in MHC binding residues, that will still be predicted to bind to the same MHC. As a first approximation, this approach reduces the number of potential cross-reactive sequences identified and makes experimental validation feasible. In reality, however, TCRs are not required to recognize HLA-bound peptides by sequence similarity but rather by peptide side chain accessibility.^{6,7} Consequently, the set of potential cross-reactive sequences is very likely dramatically greater and would be unwieldy in initial validation experiments. We believe experimental confirmation in the simplest case (identical TCR-facing residues) is a reasonable first step to be followed by computational screening of an expanded sequence/structure space to capture additional cross-reactive epitopes.

TCR cross-reactivity between self and non-self. Cross-reactivity is an intrinsic characteristic of the TCR, i.e., each single TCR can potentially interact with different peptide-MHC complexes. In fact, T cell epitope cross-reactivity is critical to many aspects of T cell biology, including positive and negative selection. Cross-reactive immunity can have either advantageous or negative (e.g., leading to pathology) consequences. Deleterious autoimmune cross-reactivity can occur following infection, a response that has previously been described as molecular mimicry. For example, T cell epitope cross-reactive immune responses may contribute to the pathogenesis of systemic lupus erythematosus,⁸ tropical spastic paraparesis,⁹ and diabetes,¹⁰ among other conditions. While we have not yet used JanusMatrix to define cross-reactivity between human pathogens and T cell epitopes associated with auto-immune disease, this is an area of great interest that we are likely to pursue in the future.

Heterologous immunity: TCR cross-reactivity between pathogens. Heterologous immunity is a term coined by Welsh and Selin to describe the partial immunity (or altered immunopathology) that occurs in response to a pathogen if the host has been previously infected or immunized with a particular unrelated pathogen. Both human and murine immune responses to antigens can be modified as a consequence of T cell cross-reactivity.¹¹⁻¹³ In some cases, heterologous immunity is attributed to

T cells that cross-react with both pathogens. CD8⁺ T cell cross-reactivity has been documented between two epitopes on the same viral protein, between epitopes of different proteins from the same virus and, more importantly, between epitopes of proteins from different viruses.¹⁴

Heterologous immunity research focused initially on specific viral infections (e.g., vaccinia, EBV, influenza).^{15,16} It is now clear that T cells stimulated by epitopes from a given pathogen may subsequently respond differently to cross-reactive similar epitopes with later infection by a different virus.¹⁷ T cells that have been previously activated due to a cross-reactive infection are capable of restricting pathogen growth and reducing the severity of the second infection, which can be viewed as a positive effect. In the context of some infections caused by different viruses, heterologous immunity may contribute to the immunodominance of low-avidity memory responses over higher-avidity naïve responses. However, in other circumstances, the result of cross-reactive immunity is enhanced pathogen growth and/or immunopathology.¹⁸ Proposed mechanisms underlying these negative effects may include induction of Treg or, in contrast, increased production of inflammatory cytokines.^{11,19-21} JanusMatrix is currently being used to search for epitopes that may contribute to heterologous immunity.

The relevance of the HM to adaptive T cell responses. Evidence linking gut microbes, adaptive immunity, metabolic syndrome, allergy, and autoimmune disorders has emerged.^{22,23} For example, linkages between immune responses to the HM and multiple sclerosis have been defined,²³ as have links between microbiota and autoimmune diseases of the gut.^{24,25} Exposure to a diverse array of gut microbes may reduce susceptibility to autoimmune diseases and allergy (the so-called “hygiene hypothesis”).^{22,23,26} This may be attributable to direct interactions between the microbes and the human immune system (due to not-yet-defined factors) or to modulation of immune responses to self and other potential pathogens.^{24,27-29}

In addition, there is a growing body of evidence that the gut microbiome shapes adaptive immune responses.^{27,30,31} Notably, post-thymic educated gut CD4⁺ T cells differentiate to become inducible Treg by training on commensal antigens.³² This finding is significant because it establishes the existence of T cells with specificities for commensals, which may cross-react with pathogen- or vaccine-derived sequences. Since the gut microbiome is both vast and highly variable, it is likely that commensal-shaped immune responses are highly diverse and vary from person to person due to individual variations in the microbiome (related to diet or geography) and to genetic differences in HLA. In published³³ and yet-to-be-published³⁴ work, Sztein and VerBerkmoes have established the uniqueness and commonalities between individual microbial and human proteomes.

The availability of fast and accurate tools to analyze a large number of sequences for common TCR-facing residues now makes it both feasible and exciting to examine the role that cross-reactive T cell epitopes play in the generation of autoimmune and the modulation of adaptive immune responses. In the next section, we provide a brief description of the JanusMatrix tool and its application to: (1) evaluations of common “positive control”

T cell epitopes for cross-reactivity between the HG and HM; (2) understanding the pathogenesis of hepatitis C virus (HCV); and (3) the discovery of T effector epitopes in EV71, the etiologic agent of Hand-Foot-Mouth disease (HFMD). For these initial studies, we applied JanusMatrix retrospectively to explore cross-reactive patterns of previously studied epitopes that induced various T cell responses, as well as prospectively to identify and experimentally evaluate new cross-reactive epitopes predicted to induce various responses.

Results

Developing epitope “silos”. Using EpiMatrix and JanusMatrix, we mapped groups of peptides that were experimentally shown to induce a defined T cell response (Teff, Treg, or no response) and searched for trends in the number of cross-reactive TCR-facing epitopes across the HG, HM, and HP databases supported by JanusMatrix. A set of 10,000 random nine-mers was also evaluated for comparison.

Based on cross-reactivity patterns, T cell epitopes examined thus far appear to fall into one of three categories, or “silos”: (1) patterns that are characteristic of Treg epitopes; (2) patterns that are characteristic of Teff epitopes; and (3) patterns associated with epitopes that are “null” (neither effector nor regulatory but are predicted to bind to HLA). We find these distinctive cross-reactivity patterns are generally correlated with the types of T cell epitopes defined in the literature. Specifically, T cell epitopes that appeared to be immunodominant were associated with Treg responses, as defined by upregulation of FoxP3 or expansion of CD4⁺CD25^{hi} Treg, and had higher levels of cross-reactivity with the HG.

Table 1 describes the emerging JanusMatrix patterns associated with T cell epitope categories (Treg, Teff) and provides an example for each category (a defined Treg epitope for HCV³⁵⁻³⁷ and a defined Teff epitope in influenza³⁸) in terms of their TCR cross-reactivity with the HG, HM, and selected human viral and bacterial pathogens (HP). “CEFT” epitopes that are commonly used as positive controls in human T cell assays to elicit a measurable antigen-specific memory response (see list of these epitopes in ref. 39) and Tregitopes (see ref. 40) were tested as representative sets of T effector and T regulatory epitopes respectively. Medians and ratios of cross-reactive hits are shown. We also describe cross-reactivity that could be expected in a random protein: 10,000 random nine-mers predicted to be HLA ligands show the lowest median of cross-reactive hits for each database analyzed. Number of genomes, proteins, and amino acids per database is also shown. Results of the statistical analysis (P-values) of the comparison between the distributions of ratios of cross-reactive hits between databases and types of epitopes are also shown in sub-tables. Comparisons between type of epitopes by database (e.g. CEFT vs. Treg for HG; see sub-table A) show that (1) Treg and CEFT are only different for HG; and (2) for the three databases, CEFT and Tregs are different from random; the only exception is the comparison of HP CEFT vs. Random. Comparisons between databases by type of epitope (e.g. HG vs. HM for CEFT; see sub-table B) we observe that (1) for CEFT,

Table 1. JanusMatrix TCR-cross reactivity frequencies for three types of epitopes

Database	Median cross-reactive hits (Ratio, 1 x 10 ⁶) ^a							
	T eff epitopes		Treg epitopes		Random	Per database, number of		
	CEFT	Influenza A	Tregs	HCV		Genomes	Proteins	Amino acids
Self (HG)	2 (0.18)	0 (0.00)	8.5 (0.75)	23.5 (2.08)	1 (0.09)	1	20,248	11,301,336
Microbiome (HM)	29 (0.13)	38 (0.17)	31 (0.14)	103 (0.47)	14 (0.06)	204	705,684	218,452,796
Pathogens (HP)	17 (0.12)	11 (0.08)	19 (0.13)	107.5 (0.73)	10 (0.07)	221	455,237	146,398,849

A

Epitope type	HG	HM	HP
Treg vs. CEFT	<0.05	0.63	0.62
CEFT vs. Random	<0.05	0.05	0.11
Treg vs. Random	<0.05	<0.05	<0.05

B

Database	CEFT	Treg	Random
HG vs. HM	0.10	<0.05	<0.05
HG vs. HP	0.24	<0.05	<0.05
HM vs. HP	0.50	0.15	<0.05

^aRatio of cross-reactive hits per number of amino acids in the comparison database. ^bNine-mer predicted to be an epitope. **(A)** P-values of comparisons between ratios across three types of epitopes by database. **(B)** P-value of comparisons between ratios across databases by type of epitope. Median of cross-reactive hits for T effector epitopes, T regulatory epitopes, and Random 9-mers are shown. CEFT and Tregs are tested as representative sets of T effector and T regulatory epitopes. Examples for both categories are also included; a defined Teff epitope in influenza A and a Treg epitope for HCV. TCR cross-reactivity with HG, HM, and selected human viral and bacterial pathogens (HP) was evaluated. Ratios of cross-reactive hits by number of amino acids in the comparison database are shown in parenthesis. Number of genomes, proteins, and amino acids per database is also shown. Analyses were performed with a 95% confidence level.

the distributions of the ratios of cross-reactive hits of the three databases are not different; (2) for Tregs, HG is different from both HM and HP and HM is not different from HP; and (3) for random peptides, HG, HM, and HP are all different.

In summary, these results indicate that validated Treg epitopes have statistically greater TCR cross-reactivity with the human genome, based on JanusMatrix. Teff and Treg cross-reactivities with HG, HM, and HP are different from random nine-mers (Teff vs. Random for HP is the exception). Teff distributions of the ratios of cross-reactive hits are not different between databases. Some of the overlap between HP and HM and HG might be due to co-opting of HG cross-reactivity by human pathogens to escape immune response. Cytoscape-based TCR-epitope network patterns for the HCV Treg epitope and influenza Teff epitope are shown in **Figure 2**.

Illustration of JanusMatrix patterns. *Example 1. "Positive control" T cell epitopes (Table 2).* We had previously evaluated these 15 CEFT epitopes for MHC binding affinity, using EpiMatrix and found that their reported MHC restriction was highly correlated with EpiMatrix predictions, and that most epitopes were also predicted to bind to a number of additional MHC class II alleles with high affinity.³⁹ Although the median number of cross-reactive hits between these T cell epitopes and the HM (Median = 29, SD = 68.03) is different from the median number of hits with the HG (Median = 2, SD = 5.96), no significant difference is found when the ratios of cross-reactive hits

are compared ($p = 0.1$; **Table 1**). However, the HLA allele for which the predicted nine-mer ligand with the highest number of cross-reactive hits with HG, HM, and HP is not the same allele for which immunogenic response to CEFT 09 is reported. This may suggest that the peptide contained within the larger epitope induces a null (tolerant) or Treg response for individuals possessing the predicted cross-reactive HLA.

Example 2. Viral HP. We recently mapped T cell epitopes for EV71, the enterovirus that has been responsible for several outbreaks of HFMD in Asia.⁴¹ We found that extensive cross-reactivity with HM seemed to be associated with immunodominance (**Fig. 3**). Shown in the figure are the T cell epitopes that were tested in HFMD patients. One of the two immunodominant epitopes (NTAYIIALAAAQKNFTMKL) was highly cross-conserved with enteroviruses such as polio virus (not shown) and with the human microbiome and pathogen sequences (**Fig. 3**). As is described in greater detail in the original paper, cross-reactivity between EV71 and poliovirus, appeared to be linked to protection from severe HFMD and thus may be an example of beneficial cross-reactive T cell responses.

Example 3. Human Treg epitopes from Hepatitis C. For a set of Treg epitopes that were previously defined in HCV disease, HG cross-reactivity appears to be much more extensive than HM. Overall, greater cross-reactivity with HG seems to distinguish published Treg and Teff epitopes from the same viral pathogen (**Fig. 4**).

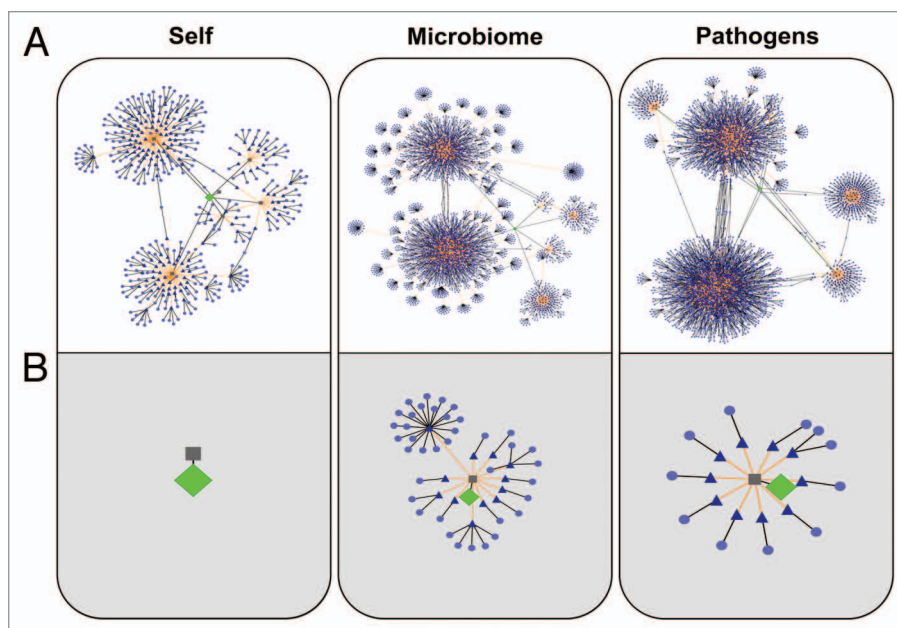


Figure 2. TCR-Epitope Networks (developed using Cytoscape) for regulatory T cell epitopes (A) and effector T cell epitopes (B). Epitopes with TCR-facing residues similar to each of the test epitopes were identified in protein sequences from the human genome (HG; left), human microbiome (HM; center), and human viral and bacterial genomes (HP; right) databases. Green diamonds represent source peptides; gray squares are predicted nine-mer epitopes derived from the source peptide (predicted using EpiMatrix); blue triangles are nine-mers that are 100% identical to the TCR face of the source epitope and that are predicted to bind to the identical HLA; and light purple circles are proteins containing the cross-reactive epitope.

Discussion

The future: two-faced epitopes. Using the newly developed algorithm JanusMatrix, we have begun to expand the information available on the role of T cell epitopes conserved between the HM, HG, and HP, and to understand how these epitopes may influence the immune response. We are applying the tool to examine whether exposure to human commensals alters subsequent T cell responses to common human pathogens and to re-examine prevailing paradigms related to autoreactive T cells. This approach, in which we define a T cell epitope based on a well-established T cell epitope-mapping algorithm,^{42,43} is significantly different from previous analyses.⁴⁴ JanusMatrix compares putative T cell epitopes and their TCR-facing residues across genome sequences rather than linear peptide fragments.

JanusMatrix may enable the definition of microbial T cell epitopes that contribute to host-microbial homeostasis. As disruption of microbial homeostasis may contribute to autoimmunity, and other pathological processes, the availability of the JanusMatrix tool could significantly advance the understanding of and treatment for autoimmune and other disorders. The extensive cross-reactivity of TCR-facing residues across genomic sequences and the distinct patterns of cross-reactivity associated with T cell phenotype may have important implications for the development of vaccines and for vaccine safety and efficacy, as T cells activated by cross-reactive HM epitopes can exhibit differential ability(ies) to respond to vaccine antigens.

Cross-reactive T cells such as those described for HFMD and other enteroviruses may enhance immune responses following

exposure to a new pathogen or to a vaccine containing the conserved T cell epitope. In contrast, cross-reactivity between pathogens (or commensals) might redirect the immune response towards suboptimal epitopes or enhance cytokine responses to vaccine antigens, contributing to vaccine-related adverse events. T cells activated by vaccination can also have cross-reactivity with self-antigens.^{45,46} This can lead to the breakdown of tolerance mechanisms and subsequent autoimmune reactions. Autoimmune reactions such as myocarditis, Guillain-Barré syndrome, and vasculitis have been described after administration of several vaccines,⁴⁷⁻⁵² but their cause is not fully understood. The JanusMatrix tool may be particularly valuable for future explorations of immune-mediated adverse responses to vaccination.

JanusMatrix may also be useful for defining homologous T cell epitopes among variant strains or serotypes of the same pathogen. For example, use of these defined rules to search for conserved TCR-facing residues in large Dengue sequence databases may contribute to understanding the immunopathogenesis of Dengue Hemorrhagic Fever, a severe manifestation of Dengue infection. In addition, the use of the tool may uncover important similarities between HIV and the human genome, that have long been surmised but not yet validated.

Aspects of JanusMatrix analysis that may lead to inaccurate predictions. The current analysis relies on the accuracy of the EpiMatrix epitope prediction tool, as well as the careful curation of protein sequences in each of the analyzed databases. While each of these sources is most certainly subject to some error, the large deviations in the frequencies (see Table 1) that we are observing seem to suggest that cross-reactivity at the

Table 2. JanusMatrix analysis of the CEFT peptide pool

Source	Reported allele	Peptide sequence	Nine-mer	Alleles	Number of cross-reactive hits		
					HG	HM	HP
CEFT 01	DR4	FVFTLTVPSER	FVFTLTVPS	6	4	29	54
			VFTLTVPSE	1	0	8	6
			FTLTVPSER	4	6	31	40
CEFT 02	DR1	SGPLKAEIAQRLEDV	LKAEIAQRL	4	5	65	43
CEFT 03	DR1	YDVPDYASLRSLVASS	YASLRSLVA	7	7	49	68
CEFT 04	DR1	PYYTGEHAKAIGN	YTGEHAKAI	3	0	5	6
CEFT 05	DR3	GQIGNDPNRDIL	IGNDPNRDI	1	0	1	0
CEFT 06/07	DR1, DR4	PKYVKQNTLKLAT	YVKQNTLKL	8	5	180	96
			VKQNTLKLA	2	2	23	18
CEFT 09	DR15	AGLTLSLLVICSYLFISRG	AGLTLSLLV	1*	33	336	364
			TLSLLVICS	1	2	18	11
			LLVICSYLF	5	0	14	7
			VICSYLFIS	1	1	3	2
CEFT 10/11	DR8, DR11, DR13, DR15	QYIKANSKFIGITEL	YIKANSKFI	8	2	104	76
			IKANSKFIG	5	4	102	64
CEFT 12	DR7, DR11	FNNFTVSFWLRVPKVSASHLE	FNNFTVSFW	1	0	2	2
			FTVSFWLRV	2	2	8	14
			FWLRVPKVS	4	0	5	3
			WLRVPKVSA	4	5	64	44
			LRVPKVSAS	2	2	30	12
CEFT 13	DR1	TSLYNLRRGTALA	LYNLRRGTA	4	0	39	9
			YNLRRGTAL	5	4	31	34
CEFT 15	DR11	VSIDKFRIFCKALNPK	VSIDKFRIF	1	0	12	9
			FRIFCKALN	4	6	25	23
CEFT 17	DR8	DKREMWMACIKELH	MWMACIKEL	1	0	0	1
			WMACIKELH	1	1	47	17
CEFT 19	DR3	KELKRQYEKKLRQ	LKRQYEKKL	5	3	67	17
			KRQYEKKLR	2	5	71	33
CEFT 22	DR4	AEGLRALLARSHVER	LRALLARSH	5	12	167	220
			LLARSHVER	2	3	41	43
CEFT 23	DR7	PGPLRESIVCYFMVFLQTHI	LRESIVCYF	1	0	6	2
			YFMVFLQTH	1	0	4	1

*Immunogenicity for this peptide is not reported for the predicted allele (DR7), suggesting that the nine-mer peptide contained within the larger epitope may induce a null (tolerant) or Treg response for individuals possessing this HLA.

TCR level may indeed influence T cell phenotype. We also note that the sheer volume of genomic data that is available in the HP and HM databases, compared to that of the HG may obscure important pattern differences, and that redundancies in epitopes between pathogens may further obscure differences; future iterations of this tool will allow a slight discount or 'weighting' of redundant epitopes. We also plan to normalize the comparisons of cross-reactive hits for each genome by defining the

median number of cross-reactive hits for 1 million random nine-mer sequences. This would create a norm and standard deviations against which all other epitopes could be compared, and also make it possible to report deviations from the norm for each genome as a Z-score.

Population vs. individual specificity. We also note that the JanusMatrix predictions are performed at the population-wide level and are not based on any individual HLA type. Even if

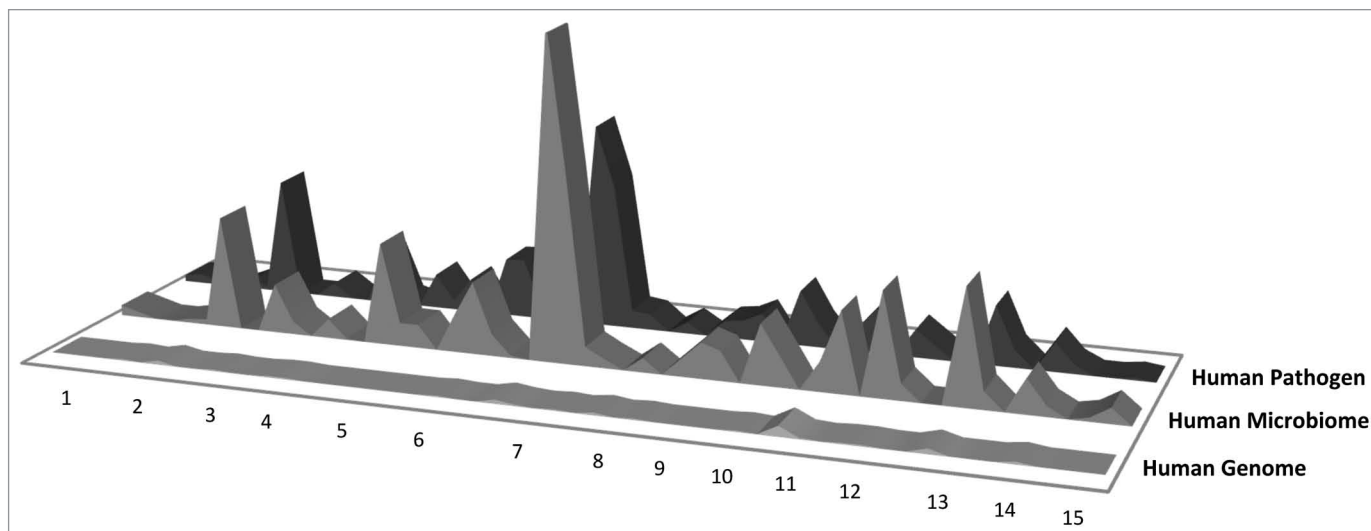


Figure 3. Hand-Foot-Mouth Disease (HFMD) epitope conservation in human microbiome/human pathogen genome sequence and immunodominance. The figure shows the potential importance of cross-reactivity between microbial genomes to the immunodominance of a particular epitope. In HFMD, extensive cross-reactivity with the HM seems to predict immunodominance. Y axis: number of cross-reactive hits in the database; x axis, individual epitopes (and nine-mers within those epitopes).

the general observation of the relationship between cross-reactivity and T cell phenotype holds true, these findings may vary from individual to individual based on their HLA, their history of exposures to vaccines and human pathogens, and their gut microbiome.

Furthermore, it is likely that cross-reactive immune responses are due to a diverse set of TCR-peptide-MHC interactions. Rather than being defined by a single cross-reactive T cell clone, it is likely that many different T cells, each with a unique TCR, respond to cross-reactive epitopes. Each clonal TCR that interacts with a set of peptide-MHC complexes (pMHC) may respond with a different set of effector functions. Consequently, specific patterns of cross-reactive immunity may occur only in certain people due to individual variation in the pMHC-specific T cell repertoire (“private specificity”).⁵³ Nonetheless, detailed understanding of these cross-reactive immune responses to prior exposures, and the relatedness of these responses to HLA, may lead to better vaccine designs and significant improvements in vaccine efficacy.

The future. Due to advances in computational power, the availability of vast genome sequences from commensals and pathogens to which humans are exposed, and the development of algorithms such as JanusMatrix, it is now possible to begin exploring the HM-HG and HP-HG intersections at the level of the T cell epitope. No comprehensive effort to uncover the conservation of HM epitopes across different pathogens and self proteins has been published to date, nor has the available literature defined a set of generalizable principles underlying T cell cross-reactivity that would permit predictions to be experimentally tested in a prospective manner. We expect that the exploration of the two-faced T cell epitope using JanusMatrix will significantly advance human immunology in this respect.

Methods

JanusMatrix tool development. As implied by its name,^a JanusMatrix examines predicted T cell epitopes from both the HLA-binding and TCR-facing perspectives, identifying as potentially *cross-reactive* those that are predicted to bind the same HLA (though perhaps with different amino acid composition on the HLA-facing side) while presenting the same amino acids to the TCR (Fig. 1). Given a starting protein sequence, putative T cell epitopes are first identified using the EpiMatrix epitope-prediction tool. The JanusMatrix tool parses the epitopes into nine-mer frames and divides each nine-mer into T cell receptor-facing residues and MHC-binding residues, based on defined rules from the literature.⁵⁴ Specifically, for Class I: TCR facing residues are considered to be residues 4, 5, 6, 7, 8, and for Class II: 2, 3, 5, 7, 8. JanusMatrix then searches for potentially cross-reactive TCR-facing epitopes across any number of large sequence databases that have been pre-loaded into the tool, including the HM, the HG, and HP. JanusMatrix focuses in 9-mer searches because although peptides of different lengths interact with the MHC, most T cell epitopes can be mapped to a minimum of nine or 10 amino acids in any given peptide, even if the peptide is longer.^{55,56} Future versions of the algorithm will include 10-mer as well as 9-mer searches.

Sequence databases. We have integrated available sequence datasets into our JanusMatrix toolkit so as to uncover potentially cross-reactive epitopes between self and microbes, including the HG,⁵⁷ HM,⁵⁸ and bacterial⁵⁹ and viral⁶⁰ proteomes that we have compiled into one HP database. The number of genomes, proteins, and amino acids per database was determined. In addition, we have access to unpublished databases comprising “frequently expressed proteins” of the HM, courtesy of Szein

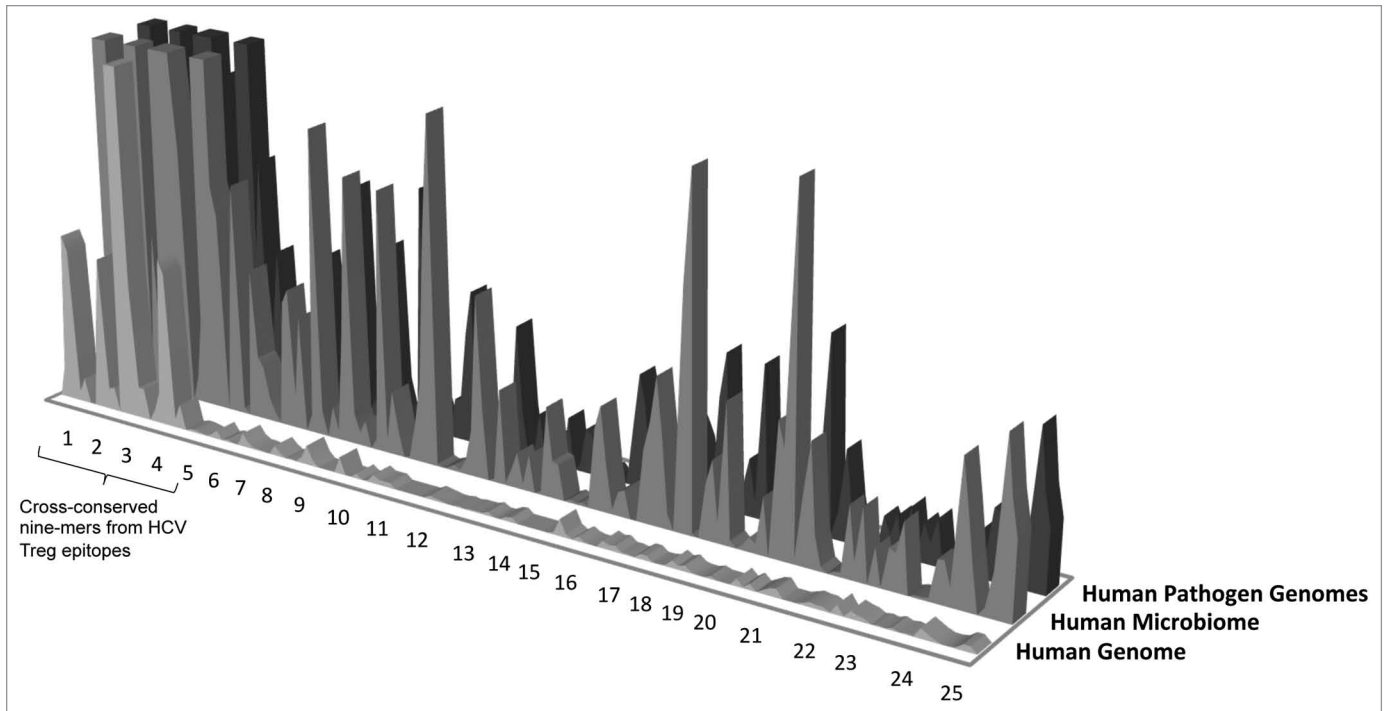


Figure 4. Hepatitis C virus (HCV) epitope conservation in human microbiome/human pathogen genome sequence and immunodominance. For regulatory T cell epitopes defined in HCV disease, HG cross-reactivity is more extensive. Overall, greater cross-reactivity with HG, HM, and HP seems to distinguish published Treg and T effector epitopes. The exact parameters defining “greater” and “lesser” cross reactivity remain to be defined following the evaluation of a number of well-defined Treg and T effector epitope examples. Y axis: number of cross-reactive hits in the database; x axis, individual epitopes (and nine-mers within those epitopes).

and VerBerkmoes (method described in ref. 24). The HM database of expressed peptide sequences was created from the most extensive healthy human-microbial distal gut metaproteome available to date.³³ In order to obtain the metaproteome, cells are enriched from fecal samples via differential centrifugation. The samples are then enriched in microbial cells and thus had a higher abundance of microbial proteins while still containing large amounts of interacting human proteins. The mixed human-microbial proteome was lysed, denatured, and reduced with 6 M Guanidine/10 mM DTT, and subsequently digested into peptides with sequencing grade trypsin (Sigma). The complex peptide mixture was analyzed via shotgun proteomics on a 2d-LC-MS/MS system (Linear Ion trap Orbitrap). The resultant MS/MS spectra were searched against a combined human (human ref seq) and microbial isolates database (JGI-IMG HM database). Proteins were computationally assembled from identified peptides (strictly filtered at -1% false positive rate). The entire dataset from both subjects was extracted for all identified peptides and proteins and was provided as an annotated database for upload into Janus Matrix.

Computational methods. JanusMatrix builds upon the EpiMatrix epitope predictor, processing each genome to identify TCR-facing cross-conserved HLA-binding nine-mers.^{37,41,43} In addition to identifying cross-reactive epitopes and describing these in tabular format, we have found it useful to visualize the patterns with “epitope networks” using Cytoscape. An epitope network has nodes for the epitopes and for the proteins that

contain them, with edges between cross-reactive pairs of epitopes and between the proteins and their constituent epitopes. We have incorporated Cytoscape⁶¹ into the JanusMatrix toolkit so as to interactively visualize relatedness between selected T cell epitopes and target genomes (HG, HM, HP) and to describe the resulting epitope networks. In the examples provided in Results, we used JanusMatrix, EpiMatrix v1.2,⁶² and the genome databases described above. Ratios of the cross-reactive hits per amino acids in the comparison database (HG, HM, or HP) were calculated for comparison purposes. Since the distributions of the ratios of cross-reactive hits were not normally distributed, non-parametric tests (Wilcoxon-Mann-Whitney test and Wilcoxon rank sum test) were used to evaluate differences in the distributions of ratios of cross-reactive hits between databases. Two type of comparisons were performed: (1) Comparisons between ratios across types of epitopes (Teff vs. Treg, Teff vs. Random, and Treg vs. Random) by database, and (2) comparisons between ratios across databases (HG vs. HM, HG vs. HP, and HM vs. HP) by type of epitope. All the analyses were performed with a 95% confidence level.

Disclosure of Potential Conflicts of Interest

A.DG. and W.D.M. are senior officers and majority shareholders at EpiVax, Inc., a privately owned immunoinformatics and vaccine design company located in Providence, RI USA. L.M. and F.T. are employees at EpiVax, in which L.M. holds stock options. These authors acknowledge that there is a potential conflict of interest related to their relationship with EpiVax and attest that

the work contained in this research report is free of any bias that might be associated with the commercial goals of the company.

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draft manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

Endnote

¹In Roman mythology, Janus is “god of beginnings and transitions”, of gates, doors, doorways, endings and time. He is usually portrayed as a two-faced god since he looks to the future and the past.

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