Human Immunology 80 (2019) 923–929

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

A survey of known immune epitopes in the enteroviruses strains associated with acute flaccid myelitis

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ARTICLE INFO

Keywords:
Enteroviruses
T cells
B cells
Epitopes
AFM

ABSTRACT

Enteroviruses are potentially linked to the emergence of Acute Flaccid Myelitis (AFM), a rare but very serious condition that affects the nervous system. AFM has been associated with coxsackievirus A16, enterovirus A71 (EVA71) and enterovirus D68 (EVD68). Little is known about host-pathogen interactions for these viruses, and whether immune responses may have a protective or immunopathological role in disease presentations. Towards addressing this issue, we used the Immune Epitope Database to assess the known inventory of B and T cell epitopes from enteroviruses, focusing on data related to human hosts. The extent of conservation in areas that are targets of B and T cell immune responses were examined. This analysis sheds light on regions of the enterovirus polypeptide that can be probed to induce a specific or cross-reactive B or T cell the immune response to enteroviruses, with a particular focus on coxsackievirus A16, EVA71 and EVD68. In addition, these analyses reveal the current gap-of-knowledge in the T and B cell immune responses that future studies should aim to address.

1. Introduction

Acute Flaccid Myelitis (AFM) is defined as a severe neurological condition affecting the gray matter area of the spinal cord, and is diagnosed by magnetic resonance imaging (MRI). Evidence suggests that AFM is an uncommon, sporadically occurring, complication of a common infection, but the specific virus responsible has been difficult to establish by direct detection [1]. Major concerns have been raised regarding the increased incidence of AFM. Recently, a polio-like syndrome in association with emerging non-polio enterovirus (EV) strains has been described and defined as AFM.

A recent characterization of the disease by CDC, based on surveillance following 2014, 2016 and 2018 outbreaks, has shed new light on the epidemiologic, clinical, and laboratory features of AFM. CDC defines a confirmed case of AFM when concomitance of acute flaccid limb weakness is observed with magnetic resonance imaging (MRI) evidence of a spinal cord lesion largely restricted to gray matter and spanning ≥1 spinal segments [2].

Treatment is primarily supportive, as there is no proven effective treatment for EV-associated AFM (particularly enterovirus D68), and the short-term prognosis for full recovery is poor [3]. AFM has been associated with coxsackievirus A16, enterovirus A71 (EVA71) and enterovirus D68 (EVD68), mainly based on epidemiological data correlating appearance of new viral lineages with AFM incidence, both in terms of geographical and temporal observations [4].

Enteroviruses (EVs) are non-enveloped, positive-sense, single-stranded RNA viruses of the Picornaviridae family. Enteroviruses encode a polyprotein as the primary translation product, which is initially processed by the viral proteases into three precursor proteins: P1, P2, and P3 (as schematically represented in Fig. 1). Precursor P1 is cleaved into the four structural proteins (VP1-VP4), while precursors P2 and P3 are processed into seven non-structural proteins (P2A-C, P3A-C and RdRp) that are responsible for viral replication and subsequent cell lysis necessary for virus release. EVs were originally distributed into four groups (polioviruses, coxsackie A, coxsackie B and the echoviruses). They are currently grouped based on the similarity of the VP1 protein, and now also include the rhinoviruses [5].

Human enterovirus isolates have been placed into 4 main groups (EV A-D) together with 3 main groups of rhinoviruses (RV A-C) [6]. The EV-A group includes coxsackievirus A6, coxsackievirus A16, and

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https://doi.org/10.1016/j.humimm.2019.08.004
Received 12 June 2019; Received in revised form 8 August 2019; Accepted 16 August 2019
Available online 23 August 2019
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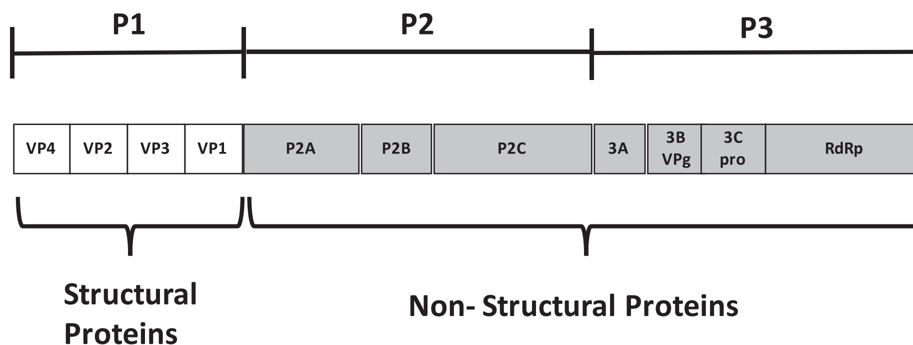

Fig. 1. Pictorial representation of Enterovirus viral proteins. The figure is adapted from ViralZone (https://viralzone.expasy.org/).

enterovirus A71 (EVA71), all of which are associated with Hand Foot and Mouth Disease (HFMD). EV-B is the largest enterovirus species, and includes the coxsackievirus B1-B6 serotypes responsible for myocarditis in newborns, as well as neurological disorders. EV-C includes the three polioviruses that are the main cause of poliomyelitis, which can range from a minor illness to a major illness involving the CNS and potential permanent paralysis. EV-D includes EV-D68, EV-D70, EV-D94, EV-D111, and EV-D120, and is mainly associated with lower respiratory illness [6]. Finally, rhinovirus groups (A, B and C) are responsible for more than one-half of upper respiratory tract infections, causing severe pneumonia in the elderly and immunocompromised patients, as well as exacerbations of chronic obstructive pulmonary disease and asthma, and share several characteristics with the EV-D group [7].

According to CDC, EV and RV viral load have been detected by pan-enterovirus RT-PCR strategies [8,9] in 44% of the respiratory specimens of confirmed AFM cases [2]. Among those, the major EV strains commonly detected were D68 and A71, also detected in the two cases isolated from cerebrospinal fluid (CSF) specimen [2].

Given the co-incidence of the AFM clusters and EV-D68 outbreaks in 2014 in the U.S., it has been estimated that ~10% of EV-D68 hospital cases with relatively severe respiratory disease are at risk of developing AFM [1,4].

This is likely to reflect the fact that by the time samples are typically drawn, the virus might already have been cleared. It is important to emphasize that this might create an opportunity for detection based on immunological methods, such as assaying memory antibody and T cell responses, which will however require the development of reagents of adequate sensitivity and specificity.

Protection from enteroviral infection is thought to rely largely on the efficiency of neutralizing antibodies [10]. However, many studies on the respiratory enteroviruses have shown that the phenotype of T helper cell responses is critical for the outcome of enteroviruses infection. Particularly, in the rhinovirus context, the recruitment of Th1 cells and the relevant production of IFN-γ have been linked to efficient viral clearance [11]. Overall, understanding T and B cell responses against enteroviruses is of critical importance for viral clearance and protection against severe disease.

Several antiviral agents with potential activity against EVs are at different stages of development, as well as development of vaccines for the emerging strains using the polio vaccination as a model. However, while encouraging progress has been observed in the context of the EV-A71 strain, EV-D68 vaccine development is still in the early stages [3]. Overall, further studies are needed to understand the causes of AFM pathogenesis, as well as the viral strains responsible for AFM. Further efforts are needed to investigate novel treatment strategies and immune correlates of protection. This knowledge is crucial for vaccine development, considering the efficacy shown by the polio vaccine in eradicating disease causing-agents [3].

In this work, the Immune Epitope Database (IEDB) [12] was utilized to assess the current literature related to enteroviruses. Specifically, the inventory of antibody and T cell data related to enteroviruses with emphasis on human data was reviewed. Conservation of the areas known to be targets of immune reactivity amongst the enterovirus proteomes, in particular coxsackievirus A16, EVA71 and EVD68, was examined. We further inspected, whether sequences unique to coxsackievirus A16, EVA71 and EVD68 are predicted to contain B and/or T cell epitopes and explored the feasibility of developing pools of peptides to probe cross-reactivity and specificity of the immune response to enteroviruses, with a particular focus on these three strains associated with AFM.

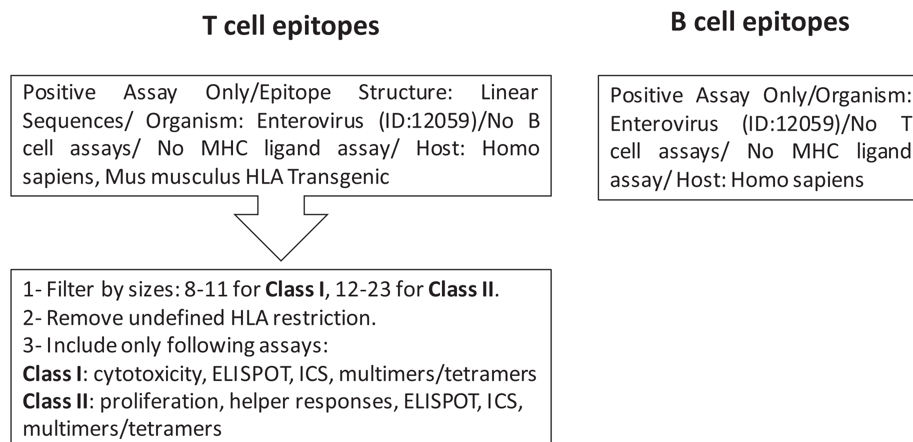

Fig. 2. Pipeline for Enterovirus-specific retrieval using IEDB (www.IEDB.org) based on queries performed on April 1st 2019.

2. Materials and methods

2.1. B and T cell epitope data retrieval

The IEDB was used to extract the B and T cell epitopes related to enteroviruses. A flowchart indicating all the steps in retrieval of epitopes is shown in Fig. 2.

For the analysis, an epitope was defined as a distinct and unique molecular structure; hence, two largely overlapping epitopes were counted as distinct. The NCBI taxonomy ID 12059 was utilized for the enterovirus genus, which encompasses enterovirus (A-L; coxsackieviruses) and rhinovirus (A-C) species. All the enteroviral epitopes were grouped based on their host organisms. To inventory enterovirus and rhinovirus T cell epitopes in the published literature, and curated by the IEDB as of April 1st 2019, searches targeted linear epitopes recognized in humans or HLA transgenic mice and associated with positive responses in T cell assays.

Identified epitopes were mapped to the coxsackievirus B4 polypeptide (UniProtKB ID: P08292) [13] using the IEDB hosted ImmunomeBrowser tool [14]. Each epitope was assigned to a specific protein in the enteroviral polypeptide based on ImmunoneBrowser mapping, and assigned corresponding protein start and end positions from coxsackievirus B4 polypeptide.

2.2. Refining T cell epitope query

The query for T cell epitopes was additionally filtered to consider only those of canonical size (8–11 residues for class I, and 12–23 residues for class II), as previously described [15] for selecting sets, or "megapools", of well-validated epitopes for use in multiple approaches towards characterizing pathogen specific responses. Epitopes with undefined restrictions, or restrictions associated with non-classical or mutant alleles were eliminated. Finally, the set was trimmed to include on those with restrictions defined using assays meeting standards of stringency and reliability, as described previously [15]. Thus, for class I, only data from assays based on cytotoxicity, ELISPOT, intracellular staining (ICS) and multimer/tetramer platforms, and for class II assays based on proliferation, helper responses, ELISPOT, ICS and multimer/tetramer platforms were included in the analysis.

2.3. Sequence analysis of enteroviral proteins

The polyprotein sequences of enteroviruses groups A, B, C, and D isolated in humans only were extracted from ViPR database [16]. Partial protein sequences were excluded in this analysis, to avoid bias due to the larger number of sequences for the VP1 region being compared to the other EV proteins. These sequences were aligned using the MUSCLE algorithm [17]. Polyprotein sequences from each EV group were aligned, for each EV group a consensus sequence was derived. Sequences of EVA71, EVD68 and coxsackievirus A16 were excluded from their respective EV groups and considered separately to generate consensus sequences. Consensus sequences were used to identify a representative reference sequence for each group searching for the closest homologous polypeptide sequence using BLAST [18], as previously described [19]. The polyprotein sequences were then parsed into the four structural (VP1-VP4) and seven non-structural proteins (P2A-C, P3A-C and RdRp) based on the specific ViPR-generated CoDing Sequence (CDS) start and end positions for each reference strain. Percent homology was derived using BLAST [18] comparing EVA71, EVD68 and coxsackievirus A16 strain-specific proteins with the representative reference sequence for each EV group.

2.4. Homology mapping of B cell epitopes

Known B cell epitopes from enteroviruses in the IEDB from all available hosts were mapped to EVA71, EVD68 and coxsackievirus A16 polyproteins on the basis of homology. B cell epitopes from these 3 strains were excluded for mapping to their own polypeptides. The ImmunomeBrowser tool was used to map all the known B cell epitopes from enteroviruses to the representative reference polyproteins of EVA71, EVD68 and coxsackievirus A16 strains identified in Section 2.3, above. Epitopes were mapped to the respective polyproteins of these 3 strains if (a) their source antigen shared at least 60% sequence identity, (b) the source antigen had 70% or higher coverage in the alignment with polyprotein, and (c) mapped peptide to known epitope sequence identity was 80% or higher.

3. Results and conclusions

3.1. T Cell epitopes related to enteroviruses

We queried the IEDB to inventory T cell epitopes derived from enterovirus (including coxsackievirus and rhinovirus species) to identify points of strength in the available data, but also potential gaps in knowledge. The results of the IEDB query identified 265 T cell epitopes associated with 534 different assays, as reported in 13 published articles. Epitopes were derived from enterovirus B (coxsackievirus B3 and B4 serotypes), enterovirus C (poliovirus 1 and 3 serotypes), rhinovirus A (A16, A34 and A39 serotypes) and rhinovirus C (C3 serotype). No human data was found for coxsackievirus A16 (enterovirus A), enterovirus A71 and enterovirus D68, enterovirus serotypes of particular current interest.

The T cell epitopes identified were then filtered based on HLA restriction, epitope length and assay types (see Section 2.2). This analysis identified 5 unique HLA class I, and 252 HLA class II, epitopes (Table 1). The paucity of CTL (Cytotoxic T Lymphocyte) epitopes restricted by HLA class I was surprising, with the few identified only associated with enterovirus B. At the same time, the over 200 HTL (Helper T Lymphocyte) epitopes reported include several restricted by multiple common HLA DRB1 alleles. These epitopes are derived from enterovirus B and C serotypes, and rhinovirus A and C serotypes. No human data was found for coxsackievirus A16, EVA71 and EVD68, all viruses of particular current interest.

Table 1
Number of T cell epitopes in the literature associated with enterovirus and rhinovirus species recognized in humans.

Species	Species ID	Acc. No.	Serotype	Class I epitopes	Class II epitopes
Enterovirus B	138,949	P08292	Coxsackievirus B3	4	2
			Coxsackievirus B4	1	91
Enterovirus C	138,950	P03300	Human poliovirus 1 Mahoney	0	8
			Poliovirus type 3	0	15
Rhinovirus A	147,711	P07210	Human rhinovirus A16	0	30
			Human rhinovirus A34	0	53
			Human rhinovirus A39	0	1
Rhinovirus C	463,676	A4UHT9	Human rhinovirus C3	0	52
Total				**5**	**252**

Table 2
CTL epitopes with defined HLA class I restriction.

EV-B serotype	Epitope	Length	Start[a]	Donors +/tested	% Respond	HLA restriction	Tetramer
Cox. B3	GIIYIIYKL	9	1503	4/89	4.5	A*02:01	
	ILMNDQEVGV	10	1585	31/107	29.0	A*02:01	Yes
	MLDGHLIAFDY	11	1945	3/89	3.4	A*01:01	
	YGDDVIASY	9	2048	2/89	2.2	A*01:01	
Cox. B4	EVKEKHEFL	9	1137	5/8	62.5	A*02:01	

[a] Epitopes were aligned to the enterovirus B reference sequence (UniProtKB: P08292).

3.1.1. T Cell responses in the context of their HLA restriction

As noted above, all 5 class I epitopes were associated with enterovirus B. Two have been reported as restricted by HLA-A*01:01 and 3 by HLA A*02:01 (Table 2). However, reported responses were relatively low (< 5% of donors responding) for 3 of the epitopes, including both of those restricted by A*01:01. However, the A*02:01 restricted coxsackievirus B4 1137-45 and B3 1585-94 epitopes were found to have elicited responses in 63% and 29% of donors tested, respectively; the HLA A*02:01 restriction for the latter epitope was defined using tetramers.

Class II epitopes were identified for 8 serotypes, with at least 23 associated with each species. For 208 of the 252 HTL epitopes, restriction was only defined broadly as "HLA class II". The remaining 44 epitopes, however, were associated with defined restrictions, representative of 14 different HLA class II serological or allelic specificities (Table 3), including 31 epitopes with restrictions defined using HLA class II tetramers. Notably, all restrictions defined with resolution at the 4-digit allelic level were determined using tetramers. Further, with respect to promiscuity (i.e., restriction by multiple HLAs), it was noted that 10 epitopes have been reported as being restricted by more than one HLA class II specificity (Table 4); all but one of these are derived from rhinovirus A16.

3.1.2. Source proteins of known enterovirus CTL and HTL epitopes

To understand the specific targets of anti-enterovirus responses, the epitopes identified above were aligned to a reference sequence (see Section 2.3) to identify the specific protein of provenance.

The CD4+T cell response has been more thoroughly studied, with 252 known HTL epitopes present in the IEDB. The data available reveals HLA restrictions associated with more than 14 different class II alleles, suggesting promising worldwide population coverage. The structural protein VP1 was found to be the main target of CD4+T cell responses, followed by remaining structural proteins VP2, VP3 and VP4 (Table 5). Reactivity to non-structural proteins was largely limited to P2C and was overall minor by comparison.

Conversely, the targets of CD8+T cell responses were ascribed to the Non-structural proteins (Table 5). However, because of the paucity of CD8+T cell epitope data available in the IEDB, it is not possible to reach a meaningful conclusion in regards of the main target of CD8+T cell responses.

Table 3
Number of epitopes restricted by common HLA class II specificities.

Restriction	No. epitopes	Strains (# of epitopes per strain)
HLA-DPw4	1	Coxsackievirus B3
HLA-DQ3	1	Poliovirus 1 Mahoney
HLA-DR1	3	Coxsackievirus B4
HLA-DR2	1	Coxsackievirus B4
HLA-DR4	6	Coxsackievirus B3 (2), B4 (4)
HLA-DR9	2	Coxsackievirus B4
HLA-DRB1*01:01	7	Rhinovirus A16
HLA-DRB1*03:01	2	Rhinovirus A16
HLA-DRB1*04:01	6	Rhinovirus A16(5), A39 (1)
HLA-DRB1*04:04	6	Rhinovirus A16
HLA-DRB1*07:01	6	Rhinovirus A16
HLA-DRB1*11:01	7	Rhinovirus A16
HLA-DRB1*15:01	7	Rhinovirus A16
HLA-DRB5*01:01	5	Rhinovirus A16

3.2. Inventory of B cell epitope data related to enteroviruses

As of April 1st, 2019, the IEDB contained 584 different B cell epitopes from enteroviruses (EV), described in 129 research articles. For 221 of these B cell epitopes, responses were reported from experiments involving humans as host (Table 6). EVA71 and coxsackievirus A16 were associated with 100 and 2 epitopes, respectively. These results are striking when compared to those obtained in the case of T cell epitopes. Particularly striking is the good coverage of EVA71, for which no T cell epitopes were defined.

Over 90% of all the known B cell epitopes were from structural proteins (Table 7). The known B cell response was biased towards the VP1 antigen (59%). Several studies have shown neutralizing B cell epitopes in structural proteins (VP1-4) of EVA71 [20]. The majority of the known B cell response to non-structural antigens was reported by genome-wide linear peptide qualitative binding assays. For example, genome-wide experiments reported 42 linear B cell epitopes in non-structural proteins of EVA71 [21,22]. However, these studies also reported linear epitopes derived from structural proteins.

3.2.1. Homology mapping of B cell epitopes

To date, no known EVD68 B cell epitopes identified in human hosts have been reported. Due to the limited number of known epitopes from the EVD68 and coxsackievirus A16 strains, known enterovirus B cell epitopes were mapped to their polyproteins on the basis of homology. The 100 known B cell epitopes from EVA71, and 2 epitopes from coxsackievirus A16, were excluded for mapping to the EVA71 and coxsackievirus A16 polyproteins, respectively. The ImmunomeBrowser tool was used to map 584 enteroviral B cell epitopes from all known hosts to the representative reference polyproteins of EVA71, EVD68 and coxsackievirus A16 strains (see Section 2.4 for more details). A total of 50, 66 and 13 epitopes were mapped to EVA71, coxsackievirus A16 and EVD68 strains by homology (Table S1).

3.3. Enterovirus variability and known immune responses

The variability amongst all enteroviruses (EV) genome sequences, including coxsackieviruses, polio and rhinoviruses, is rather significant. This genetic plasticity allows for generation of emerging EV strains causing widespread epidemics and sporadic outbreaks [6]. Additionally, the genetic variability can also cause gain-of-function phenotypes in emerging strains, such as the neurotropism observed in recent cases of EVD68 [23].

The VP1 protein is currently used to classify picornaviruses into different genotypes [5], and more recently to identify genetic changes within the different EV clades, as shown in the case of the 2014 enterovirus D68 outbreak [24]. As shown above, querying T cell epitopes in the IEDB revealed that VP1 is the main target of CD4+T cell responses, followed by VP2, VP3 and VP4. Likewise, for antibody responses, VP1 and VP2 were found to be the most reported targets.

Here, we performed analysis of the homology across EV groups A, B,

Table 4
HTL epitopes with promiscuous HLA class II restriction.

Species (serotype)	Epitope	Length	Start [a]	Donors +/tested	% Respond	HLA restriction	Tetramer
EV-B (Cox. B3)	WLKVKILPEVKEKHEFLNRL	20	1129	2/4	50.0	DPw4, DR4	
Rhino.A (A16)	TSSNRFYTLDSKMWNSTSKG	20	127	2/2	100.0	DRB1*04:04, DRB1*11:01	Yes
	GIFGENMFYHFLGRSGYTVH	20	159	2/2	100.0	DRB1*01:01, DRB1*11:01	Yes
	ASKFHQGTLLVVMIPEHQLA	20	183	2/2	100.0	DRB1*04:04, DRB1*15:01	Yes
	NEKQPSDDNWLNFDGTLLGN	20	233	5/5	100.0	DRB1*03:01, DRB1*04:01, DRB1*04:04	Yes
	NWLNFDGTLLGNLLIFPHQF	20	241	8/8	100.0	DRB1*01:01, DRB1*03:01, DRB1*04:01, DRB1*11:01	Yes
	PHQFINLRSNNSATLIVPYV	20	257	3/3	100.0	DRB1*01:01, DRB5*01:01	Yes
	SNNSATLIVPYVNAVPMDSM	20	265	3/3	100.0	DRB1*04:01, DRB1*15:01	Yes
	HIVMQYMYVPPGAPIPTTRN	20	716	4/4	100.0	DRB1*01:01, DRB1*04:01, DRB1*04:04	Yes
	PRFSLPFLSIASAYYMFYDG	20	756	6/6	100.0	DRB1*01:01, DRB1*07:01, DRB1*15:01, DRB5*01:01	Yes

[a] Epitopes were aligned to the respective reference sequences (acc. no.) in Table 1.

Table 5
Protein of provenance of enterovirus HLA class I and II epitopes in Human T Cells.

EV proteins	Total	CD4	CD8
VP4	6	6	0
VP2	42	42	0
VP3	24	24	0
VP1	149	149	0
P2A	0	0	0
P2B	2	2	0
P2C	30	29	1
P3A	1	0	1
P3B	0	0	0
P3C	1	0	1
RdRp	2	0	2
Total	**257**	**252**	**5**

Table 6
Number of B cell epitopes in the literature associated with enterovirus and rhinovirus species recognized in humans.

Species	Species ID	Serotype	B cell epitopes
Enterovirus A	138,948	Enterovirus A71	100
	39,054	Coxsackievirus A16	2
	31,704	Other	3
Enterovirus B	138,949	All	55
Enterovirus C	138,950	All	59
Rhinovirus A	147,711	All	2
Total			**221**

Table 7
B cell response against enterovirus antigens from the literature.

EV antigens	All hosts	Human
VP4	36	24
VP2	98	32
VP3	57	23
VP1	362	168
P2A	6	3
P2B	2	2
P2C	19	14
P3A	4	4
P3B	0	0
P3C	7	7
RdRp	20	16
Total epitopes	**611**	**293**
Total distinct epitopes[a]	**584**	**221**

[a] In several cases, an epitope straddles multiple proteins. In these cases, the epitope has been tabulated for each protein accordingly.

C, and D, with a particular focus on the three enterovirus strains associated with AFM, namely EVA71, EVD68 and coxsackievirus A16. A total of 298, 276 and 567 polyprotein sequences for the enterovirus A, B, and C groups, respectively were extracted from ViPR database [16], while 221, 753, 367 polyprotein sequences were extracted specifically for the EVA71, EVD68 and coxsackievirus A16 strains. The EVD68 strain was the main representative of the enterovirus D group, and as such the same consensus sequence was used to investigate variability across EV groups.

The percent homology of each protein of the EVA71, EVD68 and coxsackievirus A16 strains associated with AFM with those of the various EV groups is shown in Table 8. A similar analysis comparing these three strains with consensus sequences generated from the rhinoviruses A, B and C groups is summarized in Table 9. Fewer polyprotein sequences are available for rhinovirus groups, and only 42, 11 and 36 sequences have been considered for the rhinoviruses A, B, and C groups, respectively.

As expected, the highest homology was observed across strains belonging to the same EV group, with higher conservancy in the non-structural, as compared to structural, proteins. In general, the highest homologies were observed in the RdRp protein, followed by P2C and P2A for non-structural proteins. The highest homology among structural proteins was typically observed with VP4, while VP1 was the most variable. Reduced homology is observed when A16, A71 and D68 were compared with rhinoviruses, suggesting that despite similarity in symptomatology, studying only the enterovirus group might be more beneficial in the context of AFM.

4. Discussion

Acute Flaccid Myelitis (AFM) cases reported since 2014 have increased exponentially every two years, with 230 cases in the US confirmed by the CDC [25], including 7 confirmed cases so far (March) in 2019 (https://www.cdc.gov/acute-flaccid-myelitis/afm-cases.html). Despite the epidemiological co-localization of AFM cases and outbreaks of particular emerging EV strains (such as D68), the absence of EV in the spinal fluid samples of most of the AFM patients make it more difficult to establish a direct causality. However, in most of the AFM cases, neurological symptoms are preceded by symptoms consistent with a viral illness [25]. Additionally, 44% of the confirmed AFM cases in the 2014–2018 outbreaks were also positive for EV strains in respiratory specimens [2].

Altogether, we could speculate that the virus might still be the main cause, but it would be already cleared by the time neurological symptoms appear in these patients. That would also suggest that adaptive memory responses specific to EV may be useful for diagnostic purposes.

In this study, we summarized what is known in terms of EV B and T cell immune responses by assessing the current literature, with a focus on human host data. These analyses will allow to identify potential diagnostic candidates, and at the same time to efficiently allow focusing future studies on areas associated with a lack of data.

In the context of CD8+T cell responses, there is still debate on whether they are protective or not against enteroviruses [26,27].

Table 8
Percentage homology between A16, A71 and D68 strains and enterovirus A, B and C groups.

Classification	EV proteins	Average size	A16/A71			D68			
			A	B	C	D68	A	B	C
Structural	VP4	69	70/67	59/56	60/59	58/55	58	51	59
	VP2	257	73/73	55/55	53/51	57/55	57	55	52
	VP3	239	71/72	46/46	44/43	50/52	51	49	43
	VP1	299	61/59	41/39	38/36	42/41	44	45	42
Non Structural	P2A	149	93/95	74/74	60/59	49/49	48	53	54
	P2B	99	95/100	54/54	47/47	54/56	56	61	58
	P2C	329	98/98	65/64	64/65	62/62	62	65	62
	P3A	87	92/95	52/52	47/46	57/56	56	63	57
	P3B	22	91/95	59/52	46/46	52/54	54	77	62
	P3C	183	94/98	55/57	55/57	52/55	54	68	66
	RdRp	461	93/97	68/66	69/68	63/63	63	73	73
Polyprotein		**2195**	**84/85**	**57/56**	**55/54**	**54/54**	**56**	**59**	**54**

Currently, only 5 CTL epitopes from enteroviruses recognized in humans are known, and none are associated with coxsackievirus A16, EVA71 and EVD68. However, of the five epitopes identified, all were associated with non-structural proteins. By contrast, the CD4 + T cell response has been more thoroughly studied. Additionally, an immunodominance of structural proteins (particularly VP1) is observed in EV-specific CD4 T cell responses, in line with previous studies on rhinoviruses [28,29]. However, as VP1 sequences are the basis for group classification, we cannot exclude that the majority of CD4 + T cell epitopes were identified in this protein, not because of lack of reactivity to other proteins, but because other targets were not investigated in the same detail. This is supported by the evidence that EV-specific CD4 + T cell responses non-VP1 specific are identified in 30% of healthy donors exposed to enteroviruses and is mostly directed against RdRp [30]. In addition, large screen analyses on healthy donors based only on structural proteins has shown immunodominance of VP2 protein over VP1 [31].

Overall, paucity of CTL epitopes and VP1-only biased studies suggest that there is a gap-of-knowledge in regards of the EV-specific T cell response and that VP1 might not be the right target to elicit efficient T cell immune responses. Future studies are needed to address the contribution of T cell immune response in protecting against the EV infection as well as the immunodominance of those responses.

Majority of the known B cell response is against structural proteins. The immunodominance of VP1 was confirmed by analyzing IgM and IgG antibodies in patients with EV-A71 associated disease, but epitopes against non-structural proteins were also detected in these genomic-scale studies [22]. Neutralizing antibody responses to EVA71 associated with HFMD are well studied [10,21] but there is lack of B cell epitope data for EVD68 and coxsackievirus A16. In this study, the epitopes mapped based on homology to EVD68 and coxsackievirus A16 polyproteins from other enteroviruses may provide insights into the regions of interest to elicit B cell responses across the different EVs associated with AFM.

Finally, due to the extreme variability across Enterovirus groups, and the fact that multiple strains are thought to be in association with AFM, identifying regions conserved across different EVs can be determinant in the development of efficient vaccines able to cross-protect against different stains. Conversely, unique protein regions are fundamental for diagnostic purposes, to allow specifically identifying one EV strain, as opposed to cross-reacting with others.

Overall, the homology analyses we performed herein show that P2C (non-structural) and VP4 (structural) proteins are conserved across different EVs. Based on the available P2C and VP4 T cell epitope data in IEDB, we hypothesize that those proteins might be interesting candidates to detect conserved T cell responses across the different EV groups. Conversely, due to the lower homology observed in VP1 and VP2 proteins it is most likely that structural proteins might offer the best opportunity to identify AFM specific T cell and B cell epitopes and derive specific reagents, as already implied by the use of VP1 for serological classification.

In conclusion, future studies should address the immunodominance of adaptive responses in order to have a better picture of the EV-specific T and B cell response. Based on the current knowledge, VP1-protein appear to be the best candidate for diagnostic purposes, vice versa antigen-specific responses should be investigated on the non-structural counterpart to identify epitopes within protein region conserved across the different EV groups.

Table 9
Percentage homology between A16, A71 and D68 strains and rhinovirus A, B and C groups.

Classification	RV proteins	Average size	A16/A71			D68		
			RV-A	RV-B	RV-C	RV-A	RV-B	RV-C
Structural	VP4	68	53/48	57/55	56/48	54	53	60
	VP2	264	52/52	55/53	51/49	52	53	53
	VP3	237	42/42	37/43	40/39	46	45	41
	VP1	289	39/37	40/38	34/39	41	45	39
Non Structural	P2A	141	38	37	51	44	48	39
	P2B	97	40/46	46/46	41/41	44	58	50
	P2C	326	48/46	55/55	46/49	48	55	46
	P3A	79	49/48	47/48	32/32	42	44	32
	P3B	22	75/75	56/52	52/52	71	48	63
	P3C	183	43/44	48/48	44/46	47	41	48
	RdRp	460	54/55	55/60	60/55	57	63	56
Polyprotein		**2166**	**46/46**	**49/50**	**45/45**	**47**	**51**	**47**

Funding

This work was supported by the National Institutes of Health [75N93019C00001].

Declaration of Competing Interest

None.

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