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## Inconclusive Evidence for or against Positive Antigen Selection in the Shaping of Human Immunoglobulin E Repertoires: A Call for New Approaches.

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1 SHORT COMMUNICATION

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3 **Inconclusive evidence for or against positive antigen selection in the**  
4 **shaping of human IgE repertoires – a call for new approaches**

5  
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8  
9 **Short title:** Antigen selection shaping IgE

10  
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25

25 **Abstract**

26 **Background:** The mechanisms driving the development of IgE antibody repertoires are a  
27 matter of debate. Alternatives to the classical view on antibody development, involving  
28 somatic mutation and antigen driven selection of high-affinity variants in germinal centers,  
29 have been proposed.

30 **Methods:** We have re-analyzed the pattern of mutations in previously isolated and  
31 characterized human clonally unrelated IgE-encoding transcripts using the validated focused  
32 binomial methodology to find evidence in such genes of antigen-specific selection.

33 **Results:** As expected there is a selection against replacement mutations in IgE framework  
34 regions. In contrast, in all examined cases but one (assessing IgE repertoires of parasite-  
35 infected individuals) there was no evidence in favor of either positive or negative selection in  
36 complementarity determining regions. Importantly, however, the validated method also failed  
37 to detect selection for replacement mutations in two, non-IgE, hypermutated antibody  
38 population targeting tetanus toxoid and vaccinia virus, respectively.

39 **Conclusions:** Current methodology is unable to define with certainty, using commonly  
40 assessed IgE repertoire sizes, if antigen selection is or is not a major driving force in the  
41 establishment of human IgE. New approaches are needed to address this matter.

42

## 42 **Introduction**

43 Antibodies of the IgE isotype are well-described, key components of the defense against  
44 parasites but also of type 1-hypersensitivity reactions. The mechanisms behind the  
45 development of diversity in IgE antibody repertoires, however, are a matter of substantial  
46 debate. The classical view of development of hypermutated antibodies is that selection of B  
47 cells producing such antibodies occurs in germinal centers. To ensure development towards a  
48 high affinity antibody this process is believed to favor replacement mutations targeting the  
49 complementarity determining regions (CDR) rather than silent mutations. In contrast  
50 replacement mutations are counter-selected in the framework regions (FR), as they are likely  
51 to be accompanied by unacceptable structural effects on the folded protein. The end product  
52 of this antigen selection process should be a pool of B cells carrying genes encoding antigen-  
53 specific, high-affinity antibodies. In the case of IgE-repertoire development alternative  
54 mechanisms, not necessarily involving selection by antigen [1], have however been proposed.  
55 For instance, extensive polyclonal expansion of B-cells producing IgE has been suggested to  
56 be a major contributor to the establishment of such antibodies [1-2]. Microbial toxins might  
57 act as superantigens, exhibiting excessive polyclonal mitogenic activity on B-cell populations.  
58 Such repertoires may be dominated by sequences that carry a lower than expected number of  
59 substitutions in the CDR as there will be no selective advantage in creating an optimal  
60 binding site towards a conventional antigen. Indeed some [2-5] but not all [6] studies have  
61 indicated evidence of mutations in IgE not compatible with selection by antigens.

62 What is then the basis for the differences between different studies of the involvement of  
63 selection in the shaping of IgE repertoires? Firstly the studies have assessed IgE in different  
64 clinical conditions and it is not unreasonable to expect that IgE develop in different ways in  
65 different diseases. Secondly, the analytical approach is a source of error. Identification of  
66 antigen-directed selection in antibody evolution is problematic for a number of reasons  
67 including but not limited to high background of neutral mutations, different codon usage in  
68 different parts of antibody sequences and differences in the mutagenability of different  
69 codons [7-11]. Furthermore, unequivocal assignment of germline gene origin is difficult as  
70 there is generally no knowledge of the germline gene repertoire that generated the IgE  
71 repertoires. Consequently miss-assignment is likely occurring in several cases adding  
72 irrelevant noise to the data set.

73 To minimize the influence of methodological differences we have now re-assessed the  
74 pattern of mutations in several IgE-encoding gene populations using a single, extensively

75 validated methodology [10-12] to define whether or not it is possible to pin-point antigen  
76 selection as a major driving force shaping the development of IgE repertoires as they are  
77 described in today's literature.

78

79

## 80 **Materials and Methods**

### 81 **Study material**

82 Antibody heavy chain variable domain-encoding sequences (Table 1, Supplementary Table 1)  
83 were derived from IgE-producing cells found in peripheral blood of patients diagnosed with  
84 allergic rhinitis [13-14] and in sinus mucosa of patients diagnosed with chronic rhinosinusitis  
85 or non-allergic fungal eosinophilic sinusitis [15], the latter being a condition devoid of  
86 systemic allergy but with signs of local reactions similar to allergic inflammation. We also  
87 included sequences encoding IgE derived from children with allergic asthma [6] and  
88 parasitized subjects [5] in this study. In addition, two repertoires of antibodies, likely mostly  
89 IgG, specific for tetanus toxoid and vaccinia virus of recently boosted individuals were  
90 included for comparison [16-17].

91

### 92 **Analysis**

93 To avoid repeated analysis of frequently occurring rearrangements, only one randomly  
94 picked sequence of the different clonotypes (a clonotype is defined as described by the  
95 authors or as a set of clones with an origin in a given rearrangement (with an origin in a  
96 common IGHV gene and a nucleotide identity within the heavy chain CDR3 of >80%) that  
97 has occurred in a single individual) was used in the study. To be included, a sequence from  
98 each clonotype must also be accessible from GenBank. The sequence must furthermore be  
99 complete across the investigated sequence, must not show evidence (as assessed by the V-  
100 QUEST algorithm [18]) of mutations involving insertions or deletions, and must not contain  
101 unspecified bases. The IGHV gene/allele that had been used in the process that generated a  
102 rearranged heavy-chain encoding sequence was defined using the IMGT/V-QUEST online  
103 tool [18]. As PCR had been used to amplify sequences bases comprising the first codons were  
104 not included in the analysis in order to avoid an influence of primer design on the assignment  
105 of germline gene origin and mutation. Consequently, codons 1 to 8 were always excluded and  
106 in the case of sequences described by Kerzel *et al.* [6] codons 1-26 were excluded. All these  
107 gene populations represent diverse sets of sequences in terms of V gene subgroup and gene

108 usage (Table 1). All sets also carry high frequencies of mutations at the nucleotide level  
109 (Table 1).

110 The analysis of selection was performed using the extensively validated focused binomial  
111 test (<http://clip.med.yale.edu/sel/>) as described by Hershberg *et al.* [11] and as modified by  
112 Uduman *et al.* [12]. We used the default setting of the online tool, i.e. CDR were defined as  
113 codons 27-38 and 56-65 in accordance with the unique IMGT CDR definition rules and the  
114 IMGT numbering system [19]. These residues create a surface directed towards an antigen  
115 sitting in a binding site and avoids analysis of residues in the C''  $\beta$ -strand of the folded heavy  
116 chain variable domain as included in the Kabat definition of the second heavy chain CDR.  
117 Codons beyond residue 104 were not included in the analysis to eliminate confounding effects  
118 caused by sequence alterations associated with VDJ rearrangement process, i.e. modifications  
119 that are unrelated to the somatic hypermutation and the selection process. The Benjamini–  
120 Hochberg false discover method was employed, using an on-line tool  
121 (<http://sdmproject.com/utilities/?show=FDR>), to correct for the fact that multiple statistical  
122 tests were performed.

123

124

## 125 **Results and Discussion**

126 In agreement with prior studies of mutated immunoglobulin sequences [5,7], the vast  
127 majority of sequences did not show statistical significant evidence of positive or negative  
128 selection on their own (data not shown). All populations showed strong negative selection  
129 against mutations in immunoglobulin FR (Table 1), in agreement with expectations (assuming  
130 that many mutations in FR will detrimentally influence the stability of the V domains) and  
131 past experience [10]. Furthermore there was little evidence of positive selection in the CDR of  
132 IgE-encoding sequence populations. Only IgE derived from parasitized individuals [5]  
133 showed statistically significant selection in CDR, in this case negative selection. This is in  
134 agreement with past studies of this population of sequences that showed less evidence of  
135 antigen selection as compared to IgG [5]. The study by Kerzel *et al.* [6], a study that involved  
136 sequencing of more than 1000 IgE-encoding genes from 13 lymphocyte donors, has, however,  
137 previously reported evidence of antigen selection in IgE. That repertoire, however, did not  
138 show evidence of positive selection as assessed using the focused binomial test. Importantly,  
139 though, the tetanus toxoid-specific and the vaccinia virus-specific antibody population also  
140 did not show evidence of positive selection in CDR using the focused binomial test system.

141 This finding suggests that an extensively validated analytical approach like the one employed  
142 in this study cannot with ease detect positive selection in human antibody populations highly  
143 suspected to have undergone positive selection. This points to the limitation of the approach  
144 itself. It has previously even been suggested that none of the frequently used tests, approaches  
145 like the binomial test [8], the multinomial test [20] or the focused binomial test [11], are able  
146 to detect selection by antigen in the CDR of immunoglobulin-encoding transcripts [10]. This  
147 highlights the complexity of designing such algorithms with high enough specificity without  
148 reducing sensitivity to levels where selection is very difficult to detect. The lack of selection  
149 was not accompanied by a low level of mutation in the sequences used for analysis as the  
150 frequency of mutation was 5.6-8.4 % (Table 1). Thus, sample sizes like those used in this  
151 study, a size common to most studies of IgE-encoding gene sequences, are likely insufficient  
152 to establish evidence of positive selection in CDR using those tests available today.

153 The failure to detect evidence for positive selection in sets of antibody-encoding genes of  
154 sizes common to many studies of IgE repertoires suggest that new approaches need to be  
155 taken to address this issue. Firstly one has to realize that it by no means is certain that antigen  
156 selection translates into a mathematically detectable enhancement of substitutions in CDR.  
157 Indeed many affinity-enhancing mutations do not reside in CDR [21], a fact that confounds  
158 this analytical approach. It has also been argued that false-positive results are frequent in  
159 particular when not applying an analytical strategy based on a focused binomial methodology  
160 and, even more damaging, that one should not even expect to find mathematically detectable  
161 signs of selection in the CDR of antigen-binding site of antibodies [10].

162 A problem if we ever are going to be able to detect subtle increases in substitution in CDR  
163 as a consequence of antigen selection is that even rather extensive conventional sequencing  
164 efforts (exemplified by Kerzel *et al.* [6]) generates a relatively small number of independent  
165 clones due to the oligoclonal nature of IgE repertoires [13]. It is expected that larger, carefully  
166 controlled studies to clarify the existence, or not, of positive and negative selective pressure  
167 during the development of the different human IgE repertoires will be required to resolve this  
168 matter. It is anticipated that high throughput sequencing of genes derived from large numbers  
169 of donors may aid in this endeavor. Firstly such methodology provides larger data sets that  
170 may be required to resolve this matter with statistical significance. Secondly the methodology  
171 can be used to deduce the germline repertoire of each individual [22] thereby facilitating  
172 correct germline gene assignments eliminating background noise in the data set. It is  
173 anticipated that such efforts may eventually permit us to address this matter.

174       Ultimately the resolution of the matter of whether or not selection for higher affinity  
175 occurs in human IgE may have to await a very laborious analysis of mutated, allergen-specific  
176 IgE clones in comparison to their unmutated germline counterparts. Current technology has,  
177 as far as we are aware, not been able to identify gene sequences encoding native combinations  
178 of heavy and light chain variable domains of IgE with known allergen specificity, for instance  
179 through sorting of cells of the B cell lineage by flow cytometry in combination with cloning  
180 of the corresponding variable domain-encoding genes. Such technology in combination with  
181 determination of the affinity for the immunizing agent has in the past been used to decipher  
182 the extent of affinity maturation in the much more commonly occurring cells of the B cell  
183 lineage that encode isotypes other than IgE [16]. However, even when access to such human  
184 IgE-producing clones can be ensured in the future, many IgE responses pose a very specific  
185 problem in the context of analysis of affinity maturation. Many allergens have cross-reacting  
186 counterparts in other species or are even represented by a multitude of more or less similar  
187 isoallergens and isoforms within a given species [23]. In most cases it will likely be  
188 impossible to know the allergen form(s) that originally induced the response and that  
189 was/were the driver in the affinity maturation process that eventually resulted in the  
190 population of IgE-producing B cells observed in a given allergic individual. Consequently it  
191 will likely be very difficult to define with certainty the extent of affinity maturation that has  
192 occurred in vivo unless one is able to assess a response involving few cross-reacting  
193 allergens/allergen isoforms or a response focused on a conserved epitope. Until these  
194 conceptual matters have been resolved and given the inability of even a highly validated  
195 statistical methodology to detect positive selection in IgG-encoding antibody populations, we  
196 suggest that claims of the presence or lack of selection in IgE responses, and its relation to the  
197 mechanism of IgE-mediated disease, have to be treated with caution.

198

199

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203



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**Table 1. Characteristics of investigated antibody repertoires.**

Donor condition	Lymphocyte source	Isotype	Number of donors	Number of analyzed IGHV sequences	Number of IGHV germline <sup>a</sup>		Frequency of mutations in IGHV gene (%)	Focused binomial test P-value <sup>b</sup>		Corrected focused binomial test P-value <sup>b,c</sup>		Reference
					gene subgroups	genes		CDR	FR	CDR	FR	
Chronic rhinosinusitis	Sinus mucosa	IgE	4	24	4	14	6.1	0.16	-2.4 x 10 <sup>-7</sup>	0.19	-4.8 x 10 <sup>-7</sup>	15
Non-allergic fungal eosinophilic sinusitis	Sinus mucosa	IgE	4	34	4	19	6.6	0.37	-1.2 x 10 <sup>-14</sup>	0.40	-2.8 x 10 <sup>-14</sup>	
Seasonal allergic rhinitis	Peripheral blood	IgE	2	70	5	20	5.6	0.056	-6.4 x 10 <sup>-20</sup>	0.087	-1.8 x 10 <sup>-19</sup>	13
Allergic asthma (children)	Peripheral blood	IgE	13	102	5	28	6.8	-0.11	-7.4 x 10 <sup>-25</sup>	-0.14	-5.2 x 10 <sup>-24</sup>	6
Parasitized	Peripheral blood	IgE	14	53	6	28	8.4	-0.015	-8.3 x 10 <sup>-29</sup>	-0.026	-1.2 x 10 <sup>-27</sup>	5
Immunized with tetanus toxoid	Peripheral blood	IgG <sup>d</sup>	2	57	5	23	8.2	-0.42	-4.4 x 10 <sup>-20</sup>	-0.42	-1.5 x 10 <sup>-19</sup>	16
Immunized with vaccinia virus	Peripheral blood	IgG <sup>d</sup>	5	85	7	27	7.6	0.081	-1.1 x 10 <sup>-20</sup>	0.11	-5.1 x 10 <sup>-20</sup>	17

<sup>a</sup> In addition, several different allelic variants of these germline genes were used in some repertoires

<sup>b</sup> Test was performed as described by Uduman et al [12]. Positive and negative P-values suggest positive and negative selection, respectively. Repertoires showing significant evidence for selection are shaded in grey.

<sup>c</sup> Corrected for the influence of multiple comparisons using the Benjamini–Hochberg false discover method.

<sup>d</sup> The isotype of this repertoire was not described in detail. However, the 3' -primer used for amplification of the heavy chain variable domain-encoding genes shows perfect match with sequences encoding IgG but 11, 8, 7 and 3 mismatches with sequences encoding IgM, IgA, IgD and IgE, respectively. Furthermore, transcripts encoding IgE are likely to be very rare in this material. Altogether this suggests that the majority of isolated genes encoded IgG.