

Distinguishing benign from pathologic Th2-immunity in atopic children

Patrick G Holt DSc FAA^{1,2}, Deborah Strickland PhD¹, Anthony Bosco PhD¹, Danielle Belgrave MD PhD³, Belinda Hales BSc (Hons)¹, Angela Simpson MD PhD³, Elysia Hollams PhD¹, Barbara Holt BSc¹, Merci Kusel PhD¹, Staffan Ahlstedt PhD⁴, Peter D Sly FRCP DSc^{2*} and Adnan Custovic MD PhD^{3*}

¹Telethon Kids Institute, University of Western Australia, Perth, Western Australia

²Queensland Children's Medical Research Institute, The University of Queensland, Brisbane, Australia

³Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester, UK

⁴Centre for Allergy Research, Karolinska Institute, Stockholm, Sweden

*Equal contribution

Correspondence and requests for reprints:

Prof Patrick G Holt, Division of Cell Biology, Telethon Kids Institute, PO Box 855, West Perth, WA 6872, Australia; Tel: +61 8 9489 7838

Email: patrick.holt@telethonkids.org.au; marina.stubbs@telethonkids.org.au

Word count: 3589 ; Abstract word count: 250

Marina Stubbs 6/8/2015 2:43 PM

Formatted: Width: 21 cm, Height: 29.7 cm, Numbering: Continuous

Pat Holt 4/8/2015 11:19 AM

Deleted: 3,437

26 **ABSTRACT**

27 **Background:** Although most children with asthma and rhinitis are sensitized to aeroallergens, only
28 a minority of sensitized children are symptomatic, implying the underlying operation of efficient
29 anti-inflammatory control mechanisms.

30 **Objective:** To identify endogenous control mechanism(s) that attenuate expression of IgE-
31 associated responsiveness to aeroallergens in sensitized children.

32 **Methods:** In three independent population samples we analysed relationships between
33 aeroallergen specific (s)IgE and corresponding sIgG and associated immunophenotypes in atopic
34 children and susceptibility to asthma and rhinitis, focussing on responses to house-dust mite
35 (HDM) and grass.

36 **Results:** Amongst mite-sensitized children across all populations and at different ages, HDM-
37 specific IgG:IgE ratios (but not IgG4:IgE) were significantly lower in children with asthma compared
38 to those without, and lowest amongst the most severely symptomatic. This finding was mirrored by
39 relationships between rhinitis and antibody responses to grass. Depending on age/allergen-
40 specificity, 20-40% of children with sIgE ≥ 0.35 kU/L were skin test negative, and these also
41 expressed the "high sIgG:sIgE" immunophenotype. sIgG1 from these children inhibited allergen-
42 induced IgE-dependent basophil activation in a dose-dependent fashion. Profiling of aeroallergen-
43 specific CD4⁺ Th-memory responses revealed positive associations between sIgG:sIgE ratios and
44 IL-10-dependent gene signatures, and significantly higher IL-10/Th2-cytokine(protein) ratios
45 amongst non symptomatic children.

46 **Conclusion:** In addition to its role in blocking Th2-effector activation in the late phase allergic
47 response, IL-10 is a known IgG1 switch factor. We posit that its production [during allergen-induced](#)
48 [memory responses](#) contributes significantly to attenuation of inflammation via promoting IgG1-
49 mediated damping of the Fc ϵ R1-dependent acute phase reaction. sIgG1:sIgE "balance" may
50 represent a readily accessible therapeutic target for asthma/rhinitis control.

51

Pat Holt 15/7/2015 10:21 AM

Deleted: by allergen-responsive
regulatory cells

54 **Funding:** The Australian studies have been supported principally by a series of grants from the
55 National Health and Medical Research Council of Australia. The UK studies were funded by UK
56 Medical Research Council (MRC) Grants G0601361 and MR/K002449/1, and JP Moulton
57 Charitable Foundation.

58 **Acknowledgements:** We gratefully acknowledge the Raine Study participants and their
59 families, and the Raine Study Team, for cohort co-ordination and contribution to data
60 collection.

61 **Key Messages:**

- 62 • dissociation between allergic symptomatology and allergen specific (s)IgE status in children is
63 associated with high level co-production of sIgG1 viz. high sIgG1:sIgE ratios;
- 64 • an important exemplar is failure to respond to skin prick testing despite ≥ 0.35 kU/L sIgE in
65 serum, which is observed in up to 40% of children depending on age/allergen specificity;
- 66 • this atopic immunophenotype manifests *in vitro* as sIgG1-mediated inhibition of Fc ϵ R-
67 dependent basophil activation, and an accompanying strong IL-10-dependent gene signature
68 within corresponding aeroallergen-specific CD4⁺ Th2-memory responses from the serum
69 donors;
- 70 • IL-10 may have the dual function of attenuating activation of late phase-dependent Th2-
71 effector memory cells, and driving production of sIgG1 which modulates the acute phase
72 response

73 **Capsule Summary:**

74 In the majority of sensitized children endogenous humoral and cellular regulatory mechanisms
75 operative within allergen-specific memory responses act in concert to successfully maintain levels
76 of expression of Th2-associated inflammation below clinically relevant thresholds.

77

78

79

80 INTRODUCTION

81 Aeroallergen sensitization is a common feature in childhood asthma¹, and IgE-mediated
82 inflammation may play a causal role in the disease process². However, observations that only a
83 minority of atopic children have airway symptoms^{3,4} indicate that pathogenic effects of IgE are
84 usually attenuated by as yet poorly defined endogenous control mechanisms. A thorough
85 understanding of these mechanisms and how they attenuate the pathologic effects of atopy in
86 sensitized but asymptomatic subjects may point towards novel therapeutic targets.

87 Current concepts relating to immunoregulation in allergic diseases are derived principally by
88 extrapolation from studies of symptomatic atopic patients undergoing courses of high-dose allergen
89 immunotherapy, where clinical improvement occurs in the absence of significant reductions in
90 specific (s)IgE titres. A variety of evidence is consistent with the hypothesis that these repeated
91 high-dose treatments stimulate the generation of specialized cell populations expressing
92 “regulatory” phenotypes in particular those producing high levels of IL-10^{5,6} which target the
93 subsequent activation of allergen-specific Th-memory cells secreting Th2-effector cytokines
94 associated with the late-phase allergic response. These high-dose exposures also selectively boost
95 the production of sIgG4 antibodies⁷, resulting in large increases in sIgG4:sIgE ratios. Similar
96 findings have been reported in relation to the remission of occupational allergy in the context of
97 very high environmental exposure, which is also associated with high level allergen-specific IL-10
98 production⁸ and high sIgG4:sIgE ratios⁹. It has been hypothesized that such induced sIgG4 acts to
99 attenuate allergy via competing for binding of the allergen responsible for triggering IgE-loaded Fcε-
100 receptors on granulocytes¹⁰.

101 A crucial question that remains unanswered is the relevance of these mechanisms induced by
102 ultra-high exposure to those that control baseline allergic reactivity amongst sensitized subjects at
103 the community-wide level, where allergen exposures are within a log-scale lower range, and sIgG4
104 is generally only a minor component of allergen-specific IgG responses. The contribution of
105 humoral regulatory mechanisms in this context is generally considered minor. However, the
106 abundant evidence that “antigen dose selectivity” may be a key determinant of immune-phenotype
107 selection during immune induction (an archetypal example being the strikingly different dose-

Pat Holt 16/7/2015 10:38 AM
Deleted:

Pat Holt 16/7/2015 10:43 AM
Deleted: ures⁸
Marina Stubbs 16/7/2015 2:20 PM
Deleted: ⁸
Marina Stubbs 16/7/2015 2:19 PM
Deleted: ^{8A}.
Pat Holt 16/7/2015 10:43 AM
Deleted: .
Pat Holt 5/8/2015 1:46 PM
Deleted: Receptors

114 response curves for preferential triggering of Th1 *versus* Th2 immunity¹¹), argues that caution
115 should be exercised before extrapolation of findings relating to immunoregulatory mechanisms
116 between high and low dose exposure settings.

Pat Holt 16/7/2015 11:52 AM

Deleted: strongly against extrapolation between different allergen exposure settings.

Pat Holt 16/7/2015 11:47 AM

Formatted: Font color: Red

117 In our previous population-based studies, we have demonstrated a strong relationship between
118 the risk of asthma and titres of aeroallergen-specific IgE^{3,12}, and obtained evidence that at
119 population-wide level cat-specific total IgG (but not corresponding sIgG4) could modify the
120 association between cat-specific IgE and asthma¹³. In the current study, we hypothesized that at
121 community level, aeroallergen sensitization may be benign or pathologic, and sought to
122 investigate whether the balance between allergen-specific (s)IgE and corresponding sIgG1 may
123 be a component of the regulatory process that determines the pathologic potential of IgE-
124 mediated sensitization. We addressed our hypothesis by bringing together comprehensive data
125 on aeroallergen-specific humoral immunity from a series of cohorts. We have focused exclusively
126 on atopic children, and included the assessment of sIgG levels to HDM and grass as archetypal
127 examples of allergens linked to expression of asthma and rhinitis. In a series of studies, we
128 examined the allergen-specific IgG:IgE balance amongst sensitized children in relation to (i) the
129 presence, severity and persistence of airway symptoms, (ii) their immediate hypersensitivity skin
130 test responses, (iii) the capacity of their sera to arm basophils *in vitro* for IgE-dependent allergen-
131 induced degranulation; and (iv) the activation profile of Th2-memory cell populations which
132 secrete cytokines that control both aeroallergen-specific antibody production and the late-phase
133 allergic response.

134 **METHODS**

135 **Study design, setting and participants**

136 We studied three population samples from Perth (Australia) and Manchester (UK): the Western
137 Australia Pregnancy Cohort (RAINE)^{3,13} and the Manchester Asthma and Allergy Study (MAAS)¹⁴
138 are population-based birth cohorts, and the Childhood Asthma Study (CAS; Perth)¹⁵ enrolled
139 children at high risk of atopy. We further investigated the observations relating to asthma severity in
140 a cross-sectional case-control study of school-age children with or without history of severe asthma
141 exacerbation who were equivalently highly HDM-sensitized¹⁶. All study populations are described in

Pat Holt 4/8/2015 12:52 PM

Deleted: Page Break

Marina Stubbs 6/8/2015 3:36 PM

Formatted: Right: 0 cm, Space After: 0 pt

147 | detail elsewhere^{3,4,13-16} and [studies](#) were approved by research ethics committees. Informed
148 | consent was obtained from parents.

149 | **Data sources**

150 | Children were followed prospectively, with clinical assessments and blood collection at ages six
151 | and 14 years (Yr6, Yr14) in RAINE, five and 11 (Yr5, Yr11) in MAAS, and three and five (Yr3, Yr5)
152 | in CAS. Comparable validated questionnaires were administered to collect information on
153 | parentally-reported symptoms, and children were skin-prick tested.

154 | **Antibody measurements:**

155 | Antibody assays in the Australian studies were (except where specified) performed in the authors'
156 | institution employing reagents supplied gratis by ThermoFisher (Uppsala, Sweden), whereas those
157 | for the UK studies were performed in the ThermoFisher [laboratories](#). In Australian birth cohorts, we
158 | measured sIgE against [whole](#) HDM and Rye allergen, and corresponding sIgG (total and IgG4)
159 | against major components Der p 1 and Phloem p 1 by ImmunoCAP™ (ThermoFisher, Uppsala,
160 | Sweden); levels were expressed in µg/L, from which we derived sIgG:sIgE ratios. We used the
161 | same methodology to measure HDM-specific antibodies in MAAS Yr5. In MAAS Yr11, we
162 | measured sIgE/sIgG to [major allergen components](#) Der p 1 [plus](#) Der p 2 and Phl p 1 using
163 | multiplex Solid-phase Allergen Chip (ImmunoCAP ISAC™, ThermoFisher)¹⁷. In a cross-sectional
164 | study on asthma hospitalizations, we employed dissociation-enhanced immunofluorescence assay
165 | [to measure IgE and IgG1 against the combined major mite components Der p 1 plus Der p 2](#)^{16,18}.
166 | [Both the latter assay platforms employ log\(s\)fold lower allergen coating concentrations than](#)
167 | [ImmunoCAP and accordingly select for higher affinity antibodies. Such differences militate against](#)
168 | [making direct quantitative comparisons of absolute antibody concentrations across all the cohorts,](#)
169 | [but the key analyses \(those relating IgG:IgE ratios to risk for expression of specific clinical](#)
170 | [phenotypes\) involve internal comparisons between subgroups within individual cohorts assayed](#)
171 | [with the same methodology.](#)

172 | **Definition of variable**

173 | [Current wheeze](#): Positive response to the question "Has your child had wheezing or whistling in the
174 | chest in the last 12 months?"

Pat Holt 15/7/2015 10:43 AM
Deleted: .
Pat Holt 5/8/2015 10:58 AM
Deleted: s

Pat Holt 5/8/2015 10:59 AM
Deleted: ,

Pat Holt 4/8/2015 12:52 PM
Deleted: [Page Break](#)
Pat Holt 4/8/2015 12:52 PM
Deleted: s

181 *Current asthma*: All three of the following: 1) Current wheeze; 2) Current use of asthma
 182 medication; 3) Physician-diagnosed asthma.

183 *Asthma severity*: We used asthma severity scale (mild/moderate/severe) developed from the
 184 Australian asthma management guidelines (www.nationalasthma.org.au).

185 *Rhinitis*: Positive response to "In the past 12 months, has your child had a problem with sneezing,
 186 or a runny nose, or a blocked nose when he/she did not have a cold or the flu?"

187 *Sensitization status (skin prick tests-SPT and slgE)*: We defined SPT as positive if the wheal
 188 diameter was ≥ 3 mm, and positive slgE if the titre was >0.35 kU/L). [SPTs employed whole mite](#)
 189 [extracts \(both sites\), mixed grass extract \(Manchester\) and whole rye grass extract \(Perth\).](#)

190 ***In vitro cell studies***

191 To investigate the potential inhibitory effect of slgG on slgE-mediated acute inflammatory
 192 responses, we employed the basophil activation assay¹⁹. [Briefly, donor basophils in PBMC were](#)
 193 [acid stripped to remove FcεR1-bound IgE, and passively re-sensitized by incubation for 1hr at](#)
 194 [37°C in buffer containing serum that had been pre-assayed to determine levels of Der p 1-slgG](#)
 195 [and HDM-slgE. Discrete sets of experiments utilized either a series of individual sera with](#)
 196 [comparable \(high level\) HDM-slgE but varying levels of corresponding slgG, or a series of pooled](#)
 197 [pre-assayed sera premixed to achieve a range of slgG:slgE ratios against a background of the](#)
 198 [same concentration of slgE.](#) The basophils were activated by addition of Der p 1 to a final
 199 concentration of 0.1ug/ml and incubated for a further 30 minutes, and the response stopped by
 200 addition of cold 20mM EDTA. Activation was assessed flow cytometrically via surface expression
 201 (above low background levels) of CD63 and/or CD203c on basophils²⁰ which were gated as CD3-,
 202 CD19-, CD14-, HLADR-, CD123hi, FcεR1hi, CD11c-.

203 As a follow up to [one series of experiments detailed in the text](#), the sera employed [were re-](#)
 204 [assayed for Der p 1-specific IgE](#) in order to [determine](#) Der p 1-IgG : Der p 1-IgE ratios, and
 205 [relevant basophil activation data were](#) re-expressed in these terms.

206 To investigate potential mechanisms which control slgG:slgE balance, we performed genome-wide
 207 expression profiling of HDM-induced CD4⁺ Th-memory responses in 45 [HDM-sensitized](#) RAINE

Pat Holt 5/8/2015 2:10 PM
 Formatted: Font:Not Italic

Marina Stubbs 6/8/2015 3:36 PM
 Formatted: Space After: 0 pt

Pat Holt 15/7/2015 4:12 PM
 Deleted: Briefly, donor basophils in PBMC were acid stripped to remove FcεR1-bound IgE, and passively re-sensitized by incubation in buffer containing serum pre-assayed for HDM-specific IgE and IgG for 1hr at 37°C. As detailed in the text, the experiments in Figure 3A utilized individual sera while those in Figure 3B used pooled sera from groups of donors, which were mixed to achieve the variable levels of specific IgG shown against a background of standardized specific IgE, spanning a range of Der p 1-specific IgG:HDM-specific IgE ratios.

Pat Holt 16/7/2015 11:57 AM
 Deleted: 1

Pat Holt 15/7/2015 4:16 PM
 Formatted: Font:

Pat Holt 15/7/2015 11:28 AM
 Deleted: these

Pat Holt 15/7/2015 11:30 AM
 Deleted: in Figure 3 experiments was re-assayed for Der p 1-specific IgE,

Pat Holt 15/7/2015 11:30 AM
 Deleted: generate

Pat Holt 15/7/2015 11:31 AM
 Deleted: the data in Figure 3

Pat Holt 15/7/2015 4:42 PM
 Deleted: highly

Pat Holt 15/7/2015 4:38 PM
 Deleted: sensitized

Yr14 subjects selected on the basis of cell availability and moderate-high level sensitization (IgE>24ug/L), using established methods. Cryobanked PBMC from cohort subjects were cultured for 24hrs with HDM allergen to activate Th-memory responses, and affinity purified CD4+ T-cells were harvested from the cultures for RNA extraction employing DYNAbeads, and subsequent expression profiling on Affymetrix microarrays, as detailed previously^{3,21,22}. We examined associations between HDM slgG:slgE ratios as continuous traits and differentially expressed genes activated during the *in vitro* HDM-specific recall responses.

Microarray data processing and analyses

The raw microarray data are available via the Gene Expression Omnibus repository (accession: GSE70760; <http://www.ncbi.nlm.nih.gov/geo/>). Raw microarray data was pre-processed in R employing the RMA algorithm and a custom mapping of probe sets to gene^{23,24}. Batch effects were identified using principal components analysis and removed using ComBat²⁵. Noisy probe sets were filtered out of the analysis employing the pvac algorithm²⁶. Quantitative associations between gene expression patterns and log:E ratios were identified employing the Significance Analysis of Microarrays (SAM) algorithm²⁷. SAM computes a test statistic derived from the linear regression coefficient of each gene on the outcome divided by the sum of the standard error and the square root of the residual error. Statistical significance is assessed by repeated permutations of the data. Quantitative associations between expression levels of the Th2 module and logGE ratios were assessed using Gene Set Analysis²². Upstream Regulator Analysis (URA) was employed to identify signalling molecules that have the capacity to drive the observed downstream gene expression changes²⁸. URA computes an overlap p-value based on enrichment of known target genes of each regulator in the data. An activation Z-score was also calculated to determine if the direction of the observed gene expression changes for each regulator is consistent with the predictable pattern based on prior studies.

Statistical analyses

We assessed differences in antibody titres and ratios thereof by Mann-Whitney *U* test or Kruskal-Wallis test, and categorical differences between groups using Chi-squared test (SPSS and STATA).

Pat Holt 15/7/2015 4:41 PM

Deleted:

Pat Holt 15/7/2015 4:35 PM

Deleted: s

Marina Stubbs 16/7/2015 4:34 PM

Deleted: {Hollams, 2009 #3}Bosco, 2006 #19;Bosco, 2009 #20}

Pat Holt 4/8/2015 11:49 AM

Formatted: Font:11 pt

Pat Holt 4/8/2015 11:49 AM

Formatted: Check spelling and grammar, Not Superscript/ Subscript

Pat Holt 15/7/2015 11:18 AM

Deleted:

Marina Stubbs 16/7/2015 2:22 PM

Deleted: ^{3,20,21}

Pat Holt 4/8/2015 11:49 AM

Formatted: Check spelling and grammar, Not Superscript/ Subscript

Pat Holt 4/8/2015 11:49 AM

Formatted: Check spelling and grammar, Not Superscript/ Subscript

Unknown

Field Code Changed

Pat Holt 4/8/2015 11:49 AM

Formatted: Font:11 pt

Pat Holt 5/8/2015 3:36 PM

Deleted: -

Pat Holt 5/8/2015 3:37 PM

Deleted: will be made

Pat Holt 4/8/2015 12:53 PM

Deleted: Page Break

Marina Stubbs 6/8/2015 3:36 PM

Formatted: Left, Right: 0 cm, Space After: 0 pt

Pat Holt 4/8/2015 12:53 PM

Deleted: s

270 **Role of funding sources**

271 Funding sources had no role in study design, data collection, analysis, interpretation, writing of the

272 report, or decision to submit the manuscript.

273

274 **RESULTS**

275

276 Table E1 summarizes the characteristics of cohort participants included in the current study.

277 **Association studies**

278 *Immune response phenotypes underlying symptomatic versus asymptomatic atopy*

279 We carried out initial analyses among RAINE Yr14 atopic children, and the findings were then

280 replicated in other populations and at different ages. Table 1 (top) focuses on asthma and their

281 responsiveness to HDM. Amongst the mite-sensitized children in the cohort, HDM sIgE titres

282 were significantly higher in those with asthma compared to non-asthmatics, and asthma

283 prevalence increased steeply across ascending HDM sIgE quartiles (11%, 16%, 24% and 41%

284 respectively, versus 5% in those not sensitized). The higher average sIgE titres in asthmatics

285 were mirrored by higher sIgG4 titres, whereas corresponding total sIgG (predominantly IgG1) was

286 not increased among asthmatics. A comparable relationship was observed amongst rye grass

287 sensitized children between grass sIgE titres and rhinitis prevalence, with corresponding sIgG4

288 titres again higher amongst symptomatic children (Table 1 bottom). In both the HDM and grass

289 antibody responses, the contribution of sIgG4 to overall sIgG titres was very low (in the order of

290 10%; Table 1), and we found no association between sIgG4:sIgE ratios with asthma and rhinitis;

291 all subsequent analyses involving IgG were thus focused on total sIgG or the dominant IgG1

292 isotype.

293

294 Expressing these data as sIgG:sIgE ratios revealed a sharp inverse relationship to clinical

295 symptoms, in that amongst mite-sensitized children the HDM sIgG:sIgE ratios were significantly

296 lower in RAINE 14Yr children with asthma compared to those without (Table 1 top), and

297 corresponding findings relating to grass sIgG:sIgE ratios and rhinitis susceptibility (Table 1

Pat Holt 4/8/2015 12:53 PM

Deleted: Page Break

Pat Holt 5/8/2015 9:33 AM

Deleted: children in the

Pat Holt 15/7/2015 1:30 PM

Deleted: /wheeze

Pat Holt 15/7/2015 1:32 PM

Deleted: children

Pat Holt 15/7/2015 1:32 PM

Deleted: RAINE Yr14:

Pat Holt 15/7/2015 1:32 PM

Deleted: ,

Pat Holt 4/8/2015 4:07 PM

Deleted: The

Pat Holt 4/8/2015 4:08 PM

Deleted: HDM-specific

Pat Holt 4/8/2015 4:08 PM

Deleted:

Pat Holt 4/8/2015 4:09 PM

Deleted: asthma

Pat Holt 4/8/2015 5:32 PM

Deleted: ,

Pat Holt 4/8/2015 4:12 PM

Deleted: -specific

Pat Holt 3/8/2015 1:36 PM

Deleted: strikingly and

Pat Holt 3/8/2015 1:36 PM

Deleted: wheeze

Pat Holt 17/7/2015 8:28 AM

Formatted: Font color: Red, Highlight

313 [bottom](#)) were comparable. This inverse relationship was replicated across all cohorts and at
314 different ages [for both wheezing-associated and rhinitis symptoms \(Figure 1 and Table E2\).](#)

315 [Asthma severity and persistence.](#)

316 The analysis amongst HDM-sensitized children with current asthma demonstrated a downward
317 trend of sIgG:sIgE ratios with increasing asthma severity, [against a background of high sIgE which](#)
318 [was significant in the RAINE cohort but not in MAAS](#) (Table [E3](#)).

319 In RAINE Yr14, in addition to subjects with current active asthma (physician-diagnosed asthma,
320 current asthma medication, current wheeze), we identified a subset of 49 children with well-
321 controlled asthma (physician-diagnosed asthma, current asthma medication, but no wheeze in the
322 previous year). Well-controlled asthmatics in whom symptoms had waned had significantly higher
323 sIgG:sIgE ratios, with minimal differences in sIgE (Table [E4](#)). There were insufficient numbers
324 expressing this phenotype for meaningful analyses in the younger age groups and other cohorts.

325 We have previously reported low HDM-sIgG1 in HDM-sensitized children with susceptibility to
326 hospitalization for severe asthma exacerbations in a case-control study¹⁷ re-analysis of these data
327 with addition of further subjects confirmed that low sIgG:sIgE ratio in serum collected at the time of
328 exacerbation is a marker of this clinical phenotype (Table [E5A](#)). Reduced sIgG:sIgE ratios were
329 also observed amongst HDM-sensitized MAAS Yr11 children who had been hospitalised with
330 asthma exacerbations (confirmed *via* transcription of medical records data)²⁹ (Table [E5B](#)).

331 **Mechanistic studies**

332 *Immediate hypersensitivity responses*

333 *(a) sIgG-mediated attenuation of skin prick test reactivity [in vivo](#)*

334 If sIgG attenuated sIgE-mediated responses in the airways, it was likely that SPT responses may
335 also be affected. To test this, we examined the relationship between sensitization determined by
336 sIgE *versus* SPT across the cohorts, which revealed a non-random disparity in a major subgroup of
337 atopics. For example, 27% of RAINE Yr14 children with HDM-sIgE>0.35kU/L (designated IgE⁺) and
338 44% of those who were IgE⁺ to grass, were skin test negative to the respective allergens (IgE⁺/SPT⁻
339). Of note, the frequencies of SPT⁺ children amongst the IgE⁻ groups were only 3% and 1% for

Pat Holt 4/8/2015 4:14 PM

Deleted: (RAINE Yr6, MAAS Yr5 and Yr11, CAS Yr3 and Yr5) (

Pat Holt 15/7/2015 1:35 PM

Deleted: 1

Pat Holt 4/8/2015 4:15 PM

Deleted: This finding was mirrored by the relationship between rhinitis and responsiveness to rye and timothy grass in RAINE (Yr14, Yr6) and MAAS (Yr11) respectively; amongst grass-sensitized children, grass-specific IgG:IgE ratio was significantly lower in children with rhinitis compared to those without (Table 2). Note that the Multiplex Allergen Chip¹⁶ which we used for MAAS Yr11 antibody measurements selects for higher affinity antibodies relative to ImmunoCAP.

Marina Stubbs 6/8/2015 2:46 PM

Deleted: -

Pat Holt 4/8/2015 5:32 PM

Deleted: To summarize, amongst aeroallergen sensitized children (mite and grass) we observed consistent inverse associations between allergen-specific IgG:IgE ratios and clinical disease (asthma and rhinitis respectively; Figure 1).

Pat Holt 3/8/2015 2:09 PM

Deleted: -

Pat Holt 15/7/2015 1:52 PM

Deleted: in RAINE and MAAS cohorts

Pat Holt 15/7/2015 1:51 PM

Deleted: consistent

Pat Holt 15/7/2015 1:52 PM

Deleted: ,

Pat Holt 15/7/2015 1:52 PM

Deleted: [relatively uniform](#)

Pat Holt 15/7/2015 1:38 PM

Deleted: E2

Pat Holt 15/7/2015 1:38 PM

Deleted: E3

Pat Holt 15/7/2015 1:38 PM

Deleted: E4A

Pat Holt 15/7/2015 1:39 PM

Deleted: E4B

372 HDM and grass respectively. A comparable dissociation between sIgE and SPT was observed
373 across all ages/cohorts (Figure 2A). Mean sIgE levels in the IgE⁺/SPT⁺ children were significantly
374 higher than in those who were IgE⁺/SPT⁻ (Table E6), but multiple IgE⁺/SPT⁻ children had sIgE titres
375 within the 20-30kU/L range. The IgE⁺/SPT⁻ phenotype was consistently associated with higher
376 sIgG:sIgE ratios (Figure 2B; Table E6). Note also the higher prevalence of asthma and rhinitis
377 amongst the IgE⁺/SPT⁺ relative to IgE⁺/SPT⁻ children (Figure 2C).

378 *(b) sIgG-mediated attenuation of sIgE-dependent basophil activation in vitro*

379 We next used the stripped basophil activation assay to test whether sIgG could attenuate allergen-
380 triggered basophil activation *in vitro*. We initially selected a series of individual sera from strongly
381 HDM-sensitised RAINE Yr14 participants who were within a narrow band of HDM-specific IgE
382 titres, but spanning a broad (~40-fold) range of Der p 1 sIgG titres. Single donor basophils were
383 pre-armed with these sera for 1 hour at 37°C prior to triggering by addition of Der p 1, and basophil
384 activation quantified 30 minute later. A strong trend towards IgG-dose-related inhibition of basophil
385 activation is evident in these data ([representative experiment in Figure 3A](#)).

386 We reasoned also that some of the noise in this plot was likely due to inter-subject variations in
387 antibody affinity, allergen component specificity, IgG subclass ratios etc. To reduce this variability,
388 we firstly repeated the titration employing pre-assayed serum pools, producing a spectrum of HDM-
389 specific IgE:IgG ratios against the background of precisely standardized HDM-specific IgE. Der p 1-
390 triggering of donor basophils pre-armed with these serum pools provided stronger evidence of IgG
391 dose-dependent inhibition of basophil activation: the titration in Figure 3B was performed in the
392 presence of Der p 1 at 0.1ug/ml, and comparable results were obtained in titrations employing Der
393 p 1 at 0.01, 1.0 and 10.0ug/ml (not shown). Secondly, we reassayed all these sera for IgE using a
394 modified ImmunoCAP conjugated with Der p 1 as opposed to whole HDM; we recomputed
395 sIgG:sIgE ratios as Der p 1-sIgG:sIgE and used these for reanalysis of the basophil activation data
396 from Figure 3A/B, with identical conclusions (Figure E1).

397 *Allergen-specific Th-memory responses and sIgE/sIgG "balance"*

398 Specific Th-memory cells play a major role in control of antibody production by B-cells, including
399 the balance between antibody isotypes. We proceeded to investigate whether the expression of

Pat Holt 15/7/2015 1:39 PM

Deleted: E5

Pat Holt 15/7/2015 1:39 PM

Deleted: E5

Pat Holt 15/7/2015 2:44 PM

Deleted: , and the low frequency of the IgE⁺/SPT⁻ phenotype amongst severe asthmatics (Table E6).

405 “high slgG:slgE” immunophenotype amongst atopics is reflected by characteristic pattern(s) of
406 gene expression in allergen-triggered Th-memory recall responses. Figure 4A is a q-q plot derived
407 from the Significance Analysis of Microarrays (SAM) algorithm²⁷, illustrating associations between
408 expression levels of individual HDM-induced genes and HDM-specific IgG:IgE ratios in the T-cell
409 donors; the differentially expressed genes falling outside the null distribution are negatively
410 associated with slgG:slgE ratios. The majority of these (listed in Table [E7](#)) are key components of
411 the atopy-associated Th2 module which we have previously characterized employing network
412 analysis of aeroallergen-specific Th-memory responses^{21,22}, and overall expression of this module
413 was inversely related to slgG:slgE ratios (fdr<0.001, Gene Set Analysis²²). The Upstream
414 Regulator Analysis²⁸ confirmed that increasing slgG:slgE ratios are associated with *reduced*
415 signalling of multiple pathways (particularly those denoting Th2 immunity/T-cell activation), coupled
416 with *increased* IL-10 signalling (Figure 4B; Table [E8](#)). Moreover, re-expressing our published HDM-
417 induced [PBMC](#) cytokine response data from [the](#) whole population of HDM-sensitized RAINE cohort
418 subjects³ [and stratification by clinical phenotype](#) demonstrated a reciprocal relationship between IL-
419 10 and Th2 cytokine production and asthma risk (Figure 4C). [Finally, across the same sensitized](#)
420 [population, Th2 cytokine:IL-10 response ratios also correlated negatively with HDM-slgG:slgE](#)
421 [ratios in serum from respective donors \(Spearman's Correlations, n=527 subjects: IL-5:IL-10, Rho=](#)
422 [-0.448, p=0.001; IL-13:IL-10, Rho=-0.390, p=0.001; IL-4:IL-10, Rho=-0.203, p=0.01\).](#)

423
424 **DISCUSSION**

425
426 Our findings indicate that a consistent feature of children who are moderately or highly sensitized,
427 but who do not develop asthma and rhinitis, is elevated aeroallergen-specific IgG:IgE antibody ratio
428 relative to equivalently sensitized but symptomatic subjects. This association is robust and
429 reproducible across different populations and at different ages, it holds for sensitisation-associated
430 risk for asthma and rhinitis in relation to sensitization against perennial and seasonal aeroallergens
431 (mite and grass respectively), and is demonstrable with [different assay platforms](#). This relationship
432 holds for asthma severity and exacerbations, and appears to hold in regard to asthma remission.

Pat Holt 15/7/2015 1:42 PM
Deleted: E7

Pat Holt 15/7/2015 1:42 PM
Deleted: E8

Pat Holt 4/8/2015 12:54 PM
Deleted: -

Pat Holt 4/8/2015 12:54 PM
Deleted: [Page Break](#)

Marina Stubbs 6/8/2015 3:36 PM
Formatted: Left, Right: 0 cm, Space
After: 0 pt

Pat Holt 15/7/2015 10:44 AM
Deleted: three

Pat Holt 15/7/2015 10:44 AM
Deleted: antibody

440 We identified a subset of children with positive sIgE, who were skin test negative; these expressed
 441 the same high sIgG:sIgE immuno-phenotype, and were also differentially resistant to asthma and
 442 rhinitis, despite high-level sIgE sensitization. Skin test reactivity results from allergen triggering of
 443 high-affinity IgE receptors on skin mast cells/basophils, and experimental evidence suggests that
 444 the presence of a sufficiently high sIgG:sIgE ratio during activation may interfere with this process
 445 via mechanisms which include activation of inhibitory Fc-gamma receptors on mast
 446 cells/basophils³⁰⁻³⁴. Our demonstration of the dose-dependent inhibitory effects of sIgG1 in sera
 447 from these children in the presence of saturating levels of mite allergen is consistent with such a
 448 mechanism. However, it is implausible that this alone could account for the overall effects observed
 449 in our studies in relation to symptoms. The development of persistent/severe aeroallergen-induced
 450 airway inflammation in sensitized children is considered to derive from a cascade involving
 451 sequential activation of IgE-dependent acute phase, and Th-memory-cell-dependent late-phase
 452 reactions⁽³⁵ and Figure 5). The late-phase reaction is driven by Th2 cytokines including IL-4 and IL-
 453 13, which also control IgE-B-cells maturation³⁶, and repeated cycles of production of these
 454 cytokines by aeroallergen-triggered Th-memory cells may account for the preferential expansion of
 455 sIgE relative to sIgG1 component of the humoral response in the symptomatic subgroup.

456 Our recent studies have established that aeroallergen-specific Th-memory responses in children
 457 involve activation of complex networks comprising multiple effector and regulatory genes^{21,22}, which
 458 we hypothesized may include those that can influence the balance between the IgE and IgG1
 459 antibody isotypes. In this regard, our Th-memory profiling studies in mite-sensitized children
 460 identified a distinctive pattern of gene expression by aeroallergen-specific CD4⁺ Th-memory cells
 461 associated with sIgG:sIgE ratios, which is characteristic of IL-10 exposure of these cells during
 462 allergen-induced reactivation. The most likely proximal sources of IL-10 in this context are IL-10-
 463 producing "regulatory" cells^{5,6}, which are co-activated to varying degrees in all Th-memory
 464 responses. The principal immunological function ascribed to IL-10 is control of T-cell-mediated
 465 inflammation; however, it has also been identified as a significant immunoglobulin switch factor
 466 driving IgG1 production³⁷. Its release in sufficient amounts during repeated aeroallergen-induced
 467 Th2-cell activation cycles may thus contribute to slowing the progressive decline in sIgG:sIgE ratios

Marina Stubbs 16/7/2015 2:26 PM

Deleted: ^{30A,30B}.

Marina Stubbs 16/7/2015 2:48 PM

Deleted: ^{5,6}

Pat Holt 16/7/2015 8:51 AM

Deleted: ^{5,Akdis, 2014 #6}

471 as the sIgE response expands over time in chronically exposed sensitized subjects, by provision of
472 an IgG1-trophic signal in parallel with those driving sIgE production. The presence of such sIgG
473 above a critical threshold provides a mechanism for attenuation of allergen-triggered FcεR1-
474 mediated acute-phase inflammation. It is important to note that the mediators released during these
475 acute reactions also play a crucial role in recruiting the myeloid and Th2-memory cells responsible
476 for the ensuing late-phase response³⁵, and it is plausible that both these components of the allergic
477 inflammatory cascade may be subject to co-regulation via complementary IL-10-dependent
478 mechanisms (proposed mechanism in Figure 5).

479 It is possible that the application of higher resolution technologies that could capture additional
480 measures of IgG antibody response maturation such as affinity and major/minor allergen
481 component specificity, may further add to its value as a risk assessment tool. The lack of such data
482 represents a limitation of our study that should be addressed in follow-up investigations.

483 It is of interest to note that children expressing the “asthma susceptible” phenotype also exhibit
484 deficient IgG1 antibody production against common respiratory pathogens^{18,38}. This suggests that
485 a generalized deficit in IgG response capacity at mucosal surfaces may be an integral component
486 of the high-risk phenotype in relation to inflammatory airway diseases³⁸, further emphasizing the
487 need for increased focus on immunological mechanisms beyond IgE in the host response to
488 aeroallergens.

489 Our findings may be relevant to designing improved desensitization strategies. In particular, our
490 observations on direct inhibitory effects of allergen-specific IgG on acute phase-associated basophil
491 activation point towards an alternative and testable approach: notably the use of short-course
492 allergen-containing vaccines appropriately adjuvanted to selectively promote allergen-specific
493 IgG1 synthesis and affinity maturation, conceptually similar to vaccines targeting IgG1-mediated
494 protection against microbial pathogens.

495

496

497 **REFERENCES**

- 498 |
- 499 1. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with
500 serum IgE levels and skin-test reactivity to allergens. *N Engl J Med.* 1989;**320**:271-7:
 - 501 2. Busse WW, Morgan WJ, Gergen PJ, Mitchell HE, Gern JE, Liu AH, et al. Randomized trial
502 of omalizumab (anti-IgE) for asthma in inner-city children. *N Engl J Med.* 2011;**364**:1005-15:
 - 503 3. Hollams EM, Devereill M, Serralha M, Suriyaarachchi D, Parsons F, Zhang G, et al.
504 Elucidation of asthma phenotypes in atopic teenagers through parallel immunophenotypic and
505 clinical profiling. *J Allergy Clin Immunol.* 2009;**124**:463-70, 70 e1-16:
 - 506 4. Simpson A, Tan VY, Winn J, Svensen M, Bishop CM, Heckerman DE, et al. Beyond atopy:
507 multiple patterns of sensitization in relation to asthma in a birth cohort study. *Am J Respir Crit Care*
508 *Med.* 2010;**181**:1200-6:
 - 509 5. Akdis M, Akdis CA. Therapeutic manipulation of immune tolerance in allergic disease. *Nat*
510 *Rev Drug Discov.* 2009;**8**:645-60:
 - 511 6. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor
512 factors at work in immune tolerance to allergens. *J Allergy Clin Immunol.* 2014;**133**:621-31:
 - 513 7. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, et al. Long-term
514 tolerance after allergen immunotherapy is accompanied by selective persistence of blocking
515 antibodies. *J Allergy Clin Immunol.* 2011;**127**:509-16 e1-5:
 - 516 8. Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-
517 secreting T regulatory cells in high dose allergen exposure. *J Exp Med.* 2008;**205**:2887-98:
 - 518 9. Carballido JM, Carballido-Perrig N, Kagi MK, Meloen RH, Wuthrich B, Heusser CH, et al. T
519 cell epitope specificity in human allergic and nonallergic subjects to bee venom phospholipase A2.
520 *J Immunol.* 1993;**150**:3582-91:
 - 521 10. Shamji MH, Durham SR. Mechanisms of immunotherapy to aeroallergens. *Clin Exp Allergy.*
522 2011;**41**:1235-46:
 - 523 11. Rogers PR, Croft M. Peptide dose, affinity, and time of differentiation can contribute to the
524 Th1/Th2 cytokine balance. *J Immunol.* 1999;**163**:1205-13:

525 12. Simpson A, Soderstrom L, Ahlstedt S, Murray CS, Woodcock A, Custovic A. IgE antibody
526 quantification and the probability of wheeze in preschool children. *J Allergy Clin Immunol.*
527 2005;**116**:744-9:

528 13. Custovic A, Soderstrom L, Ahlstedt S, Sly PD, Simpson A, Holt PG. Allergen-specific IgG
529 antibody levels modify the relationship between allergen-specific IgE and wheezing in childhood. *J*
530 *Allergy Clin Immunol.* 2011;**127**:1480-5:

531 14. Custovic A, Simpson BM, Murray CS, Lowe L, Woodcock A, Asthma NACM, et al. The
532 National Asthma Campaign Manchester Asthma and Allergy Study. *Pediatr Allergy Immunol.*
533 2002;**13 Suppl 15**:32-7:

534 15. Holt PG, Rowe J, Kusel M, Parsons F, Hollams EM, Bosco A, et al. Toward improved
535 prediction of risk for atopy and asthma among preschoolers: a prospective cohort study. *J Allergy*
536 *Clin Immunol.* 2010;**125**:653-9, 9 e1-9 e7:

537 16. Hales BJ, Martin AC, Pearce LJ, Rueter K, Zhang G, Khoo SK, et al. Anti-bacterial IgE in
538 the antibody responses of house dust mite allergic children convalescent from asthma
539 exacerbation. *Clin Exp Allergy.* 2009;**39**:1170-8:

540 17. Prosperi MC, Belgrave D, Buchan I, Simpson A, Custovic A. Challenges in interpreting
541 allergen microarrays in relation to clinical symptoms: a machine learning approach. *Pediatr Allergy*
542 *Immunol.* 2014;**25**:71-9:

543 18. Hales BJ, Chai LY, Elliot CE, Pearce LJ, Zhang G, Heinrich TK, et al. Antibacterial antibody
544 responses associated with the development of asthma in house dust mite-sensitised and non-
545 sensitised children. *Thorax.* 2012;**67**:321-7:

546 19. Pruzansky JJ, Grammer LC, Patterson R, Roberts M. Dissociation of IgE from receptors on
547 human basophils. I. Enhanced passive sensitization for histamine release. *J Immunol.*
548 1983;**131**:1949-53:

549 20. MacGlashan D, Jr. Marked differences in the signaling requirements for expression of
550 CD203c and CD11b versus CD63 expression and histamine release in human basophils. *Int Arch*
551 *Allergy Immunol.* 2012;**159**:243-52:

552 21. Bosco A, McKenna KL, Devitt CJ, Firth MJ, Sly PD, Holt PG. Identification of novel Th2-
553 associated genes in T memory responses to allergens. *J Immunol.* 2006;**176**:4766-77:

554 22. Bosco A, McKenna KL, Firth MJ, Sly PD, Holt PG. A network modeling approach to
555 analysis of the Th2 memory responses underlying human atopic disease. *J Immunol.*
556 2009;**182**:6011-21:

557 23. Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, et al. Evolving gene/transcript
558 definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res.* 2005;**33**:e175:

559 24. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al.
560 Exploration, normalization, and summaries of high density oligonucleotide array probe level data.
561 *Biostatistics.* 2003;**4**:249-64:

562 25. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data
563 using empirical Bayes methods. *Biostatistics.* 2007;**8**:118-27:

564 26. Lu J, Kerns RT, Peddada SD, Bushel PR. Principal component analysis-based filtering
565 improves detection for Affymetrix gene expression arrays. *Nucleic Acids Res.* 2011;**39**:e86:

566 27. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the
567 ionizing radiation response. *Proc Natl Acad Sci U S A.* 2001;**98**:5116-21:

568 28. Kramer A, Green J, Pollard J, Jr., Tugendreich S. Causal analysis approaches in Ingenuity
569 Pathway Analysis. *Bioinformatics.* 2014;**30**:523-30:

570 29. Belgrave DC, Simpson A, Semic-Jusufagic A, Murray CS, Buchan I, Pickles A, et al. Joint
571 modeling of parentally reported and physician-confirmed wheeze identifies children with persistent
572 troublesome wheezing. *J Allergy Clin Immunol.* 2013;**132**:575-83 e12:

573 30. Cady CT, Powell MS, Harbeck RJ, Giclas PC, Murphy JR, Katial RK, et al. IgG antibodies
574 produced during subcutaneous allergen immunotherapy mediate inhibition of basophil activation
575 via a mechanism involving both FcγRIIA and FcγRIIB. *Immunol Lett.* 2010;**130**:57-65:

576 31. Flicker S, Linhart B, Wild C, Wiedermann U, Valenta R. Passive immunization with
577 allergen-specific IgG antibodies for treatment and prevention of allergy. *Immunobiology.*
578 2013;**218**:884-91:

579 32. Holm J, Willumsen N, Wurtzen PA, Christensen LH, Lund K. Facilitated antigen
580 presentation and its inhibition by blocking IgG antibodies depends on IgE repertoire complexity. *J*
581 *Allergy Clin Immunol.* 2011;**127**:1029-37:

582 33. Cassard L, Jonsson F, Arnaud S, Daeron M. Fcγ receptors inhibit mouse and human
583 basophil activation. *J Immunol.* 2012;**189**:2995-3006:

584 34. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-mediated
585 anaphylaxis in vivo through both antigen interception and FcγRIIb cross-linking. *J Clin*
586 *Invest.* 2006;**116**:833-41:

587 35. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature.*
588 2008;**454**:445-54:

589 36. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch
590 recombination. *Nat Rev Immunol.* 2003;**3**:721-32:

591 37. Briere F, Servet-Delprat C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin
592 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. *J Exp Med.*
593 1994;**179**:757-62:

594 38. Holt PG, Strickland DH, Hales BJ, Sly PD. Defective respiratory tract immune surveillance
595 in asthma: a primary causal factor in disease onset and progression. *Chest.* 2014;**145**:370-8:
596
597
598

599 | **LEGEND FOR TABLE**

600

601 | **Table 1:** Allergen specific antibody response profiles and respiratory symptoms amongst HDM and
602 | rye grass sensitized children from the 14yr follow-up of the RAINE community cohort

603 | **Legend:** The study population comprised 521 14yr olds sensitized to HDM (**top panel**) and 543
604 | sensitized to grass (bottom panel), as defined by sIgE \geq 0.35kU/L. Data shown are group
605 | mean/standard errors of HDM-specific IgE, IgG (total) and IgG4 levels assayed via ImmunoCAP
606 | and re-expressed in a common unit (ug/L); IgG:IgE ratios were initially computed for individual
607 | children, and group mean ratios were then calculated from these. P values derived from Mann
608 | Whitney U test comparing the two clinical phenotypes.

609

610 | **LEGENDS FOR FIGURES**

611

612 | **Figure 1:** Relationship between HDM and grass-specific IgG:IgE ratios amongst sensitized children
613 | and expression of asthma/wheezing or rhinitis
614 | Children with HDM-specific or grass-specific serum IgE>0.35 kU/L were recruited in the Australian
615 | RAINE and CAS birth cohorts, and the UK MAAS cohort as specified.

616 | *** p<0.001; ** p<0.01; * p<0.05; ϕ p=0.08

617 | **Figure 2:** Dissociation between sensitization status determined by sIgE versus skin prick tests (A)
618 | and its relationship to sIgG:sIgE ratios (B) and clinical symptoms of asthma and rhinitis (C) in three
619 | cohorts at different ages.

620 | Children were selected on the basis of HDM-IgE or grass-IgE titres>0.35 kU/L ("IgE⁺") and then
621 | stratified on the basis of positive or negative responses to relevant allergen into dichotomous IgE⁺
622 | SPT⁺ and IgE⁺ SPT⁻ groups.

623 | **Panel A:** The relative frequency of each of the phenotypes within each cohort (analyses in the 5yr
624 | olds restricted to HDM as the frequency of grass responders was very low).

625 | **Panel B:** sIgG:sIgE ratios in each group

Pat Holt 5/8/2015 10:20 AM
Deleted: S

Pat Holt 3/8/2015 2:35 PM
Deleted: Table 1: House dust mite allergen-specific antibody response profiles amongst asthmatic versus non-asthmatic children sensitized to dust mite allergen. ... [1]

Pat Holt 5/8/2015 10:21 AM
Deleted:

Pat Holt 5/8/2015 1:53 PM
Deleted: Grass

635 **Panel C:** Relative frequency of subjects expressing symptoms of asthma or rhinitis respectively in
636 the HDM and grass-sensitized groups.

637 *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ϕ $p = 0.078$

638

639 **Figure 3:** HDM-induced basophil activation following basophil arming with HDM-IgE-rich sera
640 against a background of increasingly high levels of HDM Der p 1-specific IgG.

641 **Panel A:** Individual sera from 10 Yr14 RAINE participants strongly sensitized to HDM (HDM-IgE
642 titres 41-61 ug/L as shown) were used to arm stripped donor basophils prior to activation by
643 incubation with HDM-derived Der p 1; resultant basophil activation levels achieved with each serum
644 were plotted in rank order as determined by individual specific IgG:IgE ratios.

645 **Panel B:** Preassayed sera from HDM-sensitized Yr14 RAINE participants were used to generate a
646 series of pools standardized for HDM-specific IgE level (50ug/L) but with corresponding Der p 1-
647 specific IgG varying across a logfold range. Data illustrated show levels of basophil activation
648 achieved at each specific IgG:IgE ratio.

649

650 **Figure 4:** Variations in HDM-specific Th-memory programming as determinants of specific IgG:IgE
651 ratios and asthma susceptibility amongst RAINE Yr14 participants

652

653 **Panel A:** HDM-IgG:IgE ratios in 45 sensitized RAINE Yr14 participants were tested as a continuous
654 trait against corresponding HDM-induced CD4⁺ T-cell gene expression profiles, employing the SAM
655 algorithm. Data are illustrated as a q-q plot; differentially expressed genes inversely associated
656 with the trait (negative scores) are shown in green (see corresponding TableE7).

657 **Panel B:** Candidate upstream regulators driving HDM-induced gene expression patterns
658 associated with IgG:IgE ratios were identified utilizing the Ingenuity Pathway URA algorithm, as
659 detailed in online methods. Activation Z-scores illustrated for candidate regulators were calculated
660 based on the pattern match between observed gene expression patterns and predicted patterns
661 based on prior studies. The p-value for IL-10 is based on enrichment of known IL-10 target genes
662 identified in the analysis (see corresponding TableE8 for p-values for candidate negative
663 regulators).

666 **Panel C:** Banked data on HDM-induced cytokine secretion by PBMC from 521 HDM-sensitized
667 children (92 asthmatics/429 non asthmatics) was reanalyzed to derive Th2 cytokine:IL-10 ratios in
668 the two groups.

669

670 **Figure 5:** Complementary IL-10-dependent pathways for regulation of allergic inflammation

671 As detailed in text, IL-10 may play a dual role in attenuation of the allergic inflammatory cascade:

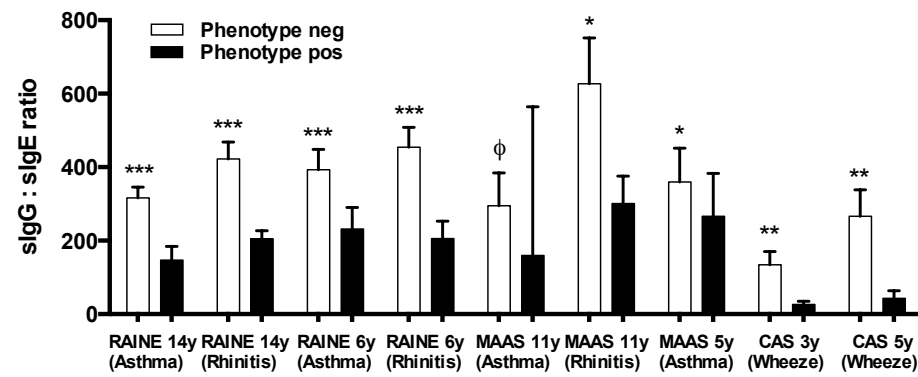
672 (i) acting indirectly via promotion of sIgG1 production which modulates the FcεR1-dependent acute
673 phase, and (ii) acting directly via modulation of Th2-memory cell activation in the late phase
674 response.

675

RAINE Yr14 (HDM-sensitized)	Asthma	N	Mean ± S.E	P
HDM-IgE	No	429	70.8 ± 7.9	<0.001
	Yes	92	187.5 ± 34.4	
HDM - IgG	No	429	1850.9 ± 81.4	0.160
	Yes	92	2126.6 ± 187.4	
HDM - IgG4	No	429	195.3 ± 14.4	<0.001
	Yes	42	332.8 ± 37.7	
HDM – IgG:IgE ratio	No	429	316.4 ± 29.2	<0.001
	Yes	92	147.1 ± 37.4	
HDM - IgG4:IgE ratio	No	429	21.0 ± 3.4	0.90
	Yes	92	20.0 ± 8.1	
RAINE Yr14 (Grass-sensitized)	Rhinitis	N	Mean ± S.E	P
Grass-IgE	No	243	36.7 ± 6.3	<0.001
	Yes	300	88.2 ± 10.1	
Grass – IgG	No	243	1465.2 ± 103.6	0.13
	Yes	300	1518.6 ± 78.8	
Grass – IgG4	No	243	83.3 ± 10.8	0.001
	Yes	300	148.0 ± 14.5	
Grass - IgG4:IgE ratio	No	243	32.0 ± 9.5	0.11
	Yes	300	17.5 ± 3.76	
Grass - IgG:IgE ratio	No	243	422.6 ± 45.7	<0.001
	Yes	300	204.9 ± 22.4	

679
680
681
682

Figure 1



683
684
685

Marina Stubbs 6/8/2015 2:42 PM

Deleted: [Section Break \(Next Page\)](#)

Unknown

Formatted: Font:(Default) Arial, 11 pt

Marina Stubbs 6/8/2015 2:42 PM

Formatted: Left

Marina Stubbs 6/8/2015 2:41 PM

Formatted: Centered

Marina Stubbs 6/8/2015 2:41 PM

Deleted: <sp>

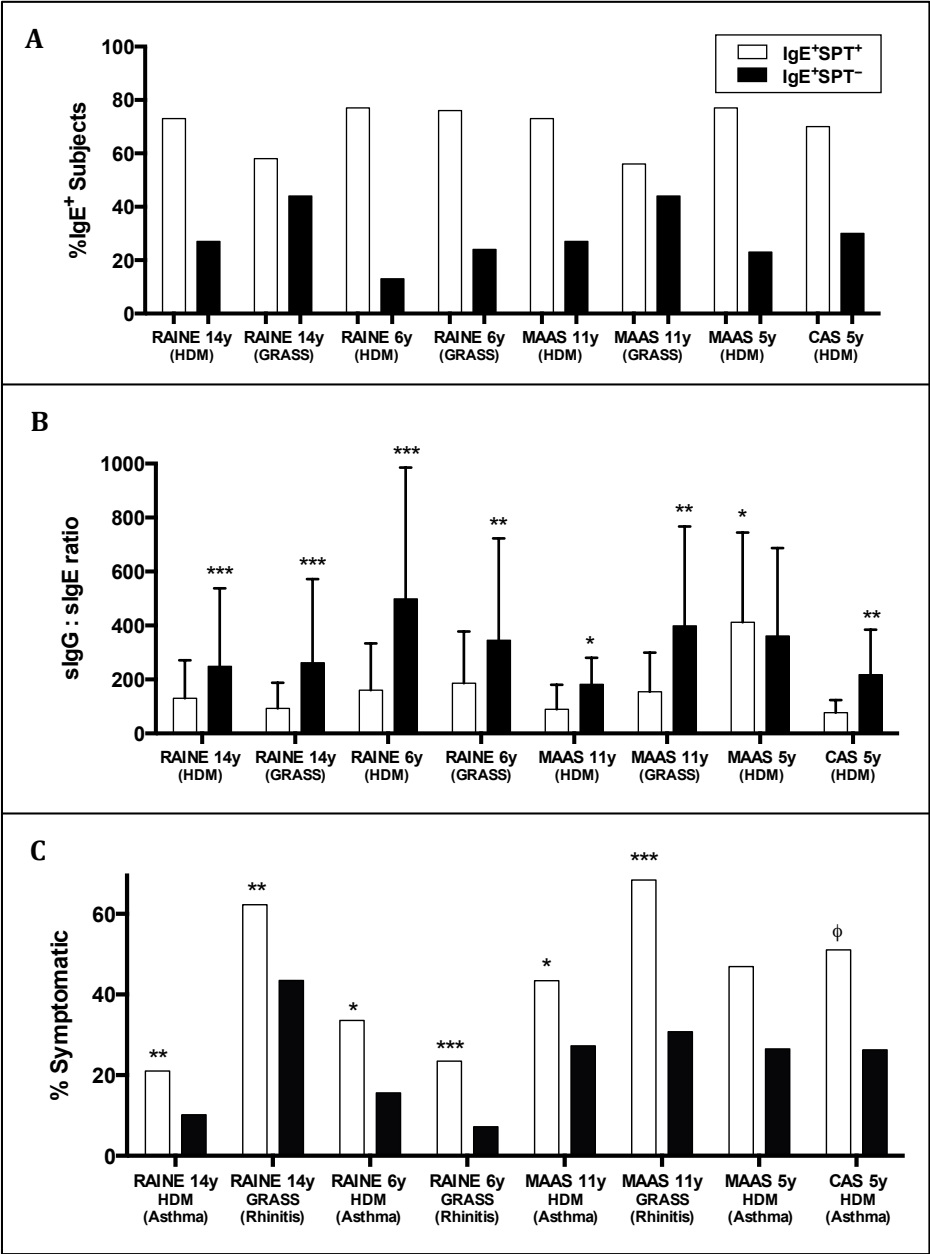


Figure 3

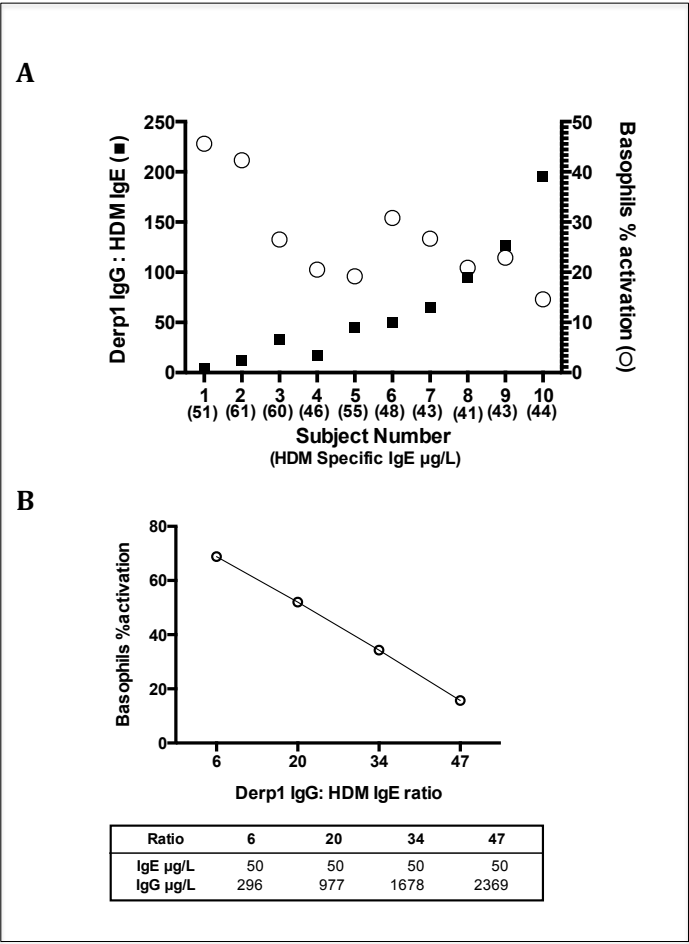
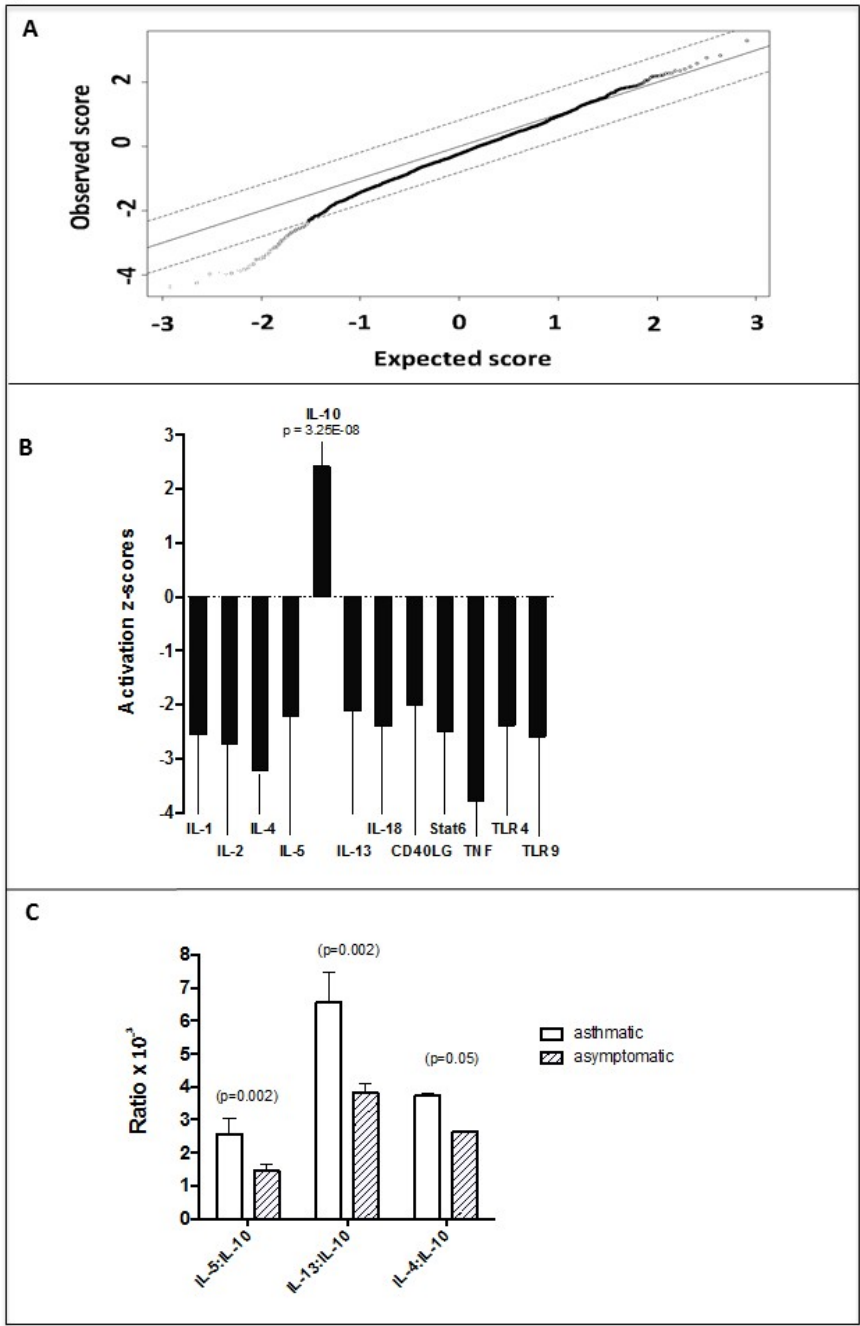


Figure 4



Marina Stubbs 6/8/2015 3:08 PM

Deleted:

Marina Stubbs 6/8/2015 3:08 PM

Deleted: Figure 4

Unknown

Formatted: Font:(Default) Arial, 11 pt, Bold

731
732