

Comparative reactivity of human IgE to cynomolgus monkey and human effector cells and effects on IgE effector cell potency

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Abbreviations: MOv18 IgE, Chimeric MOv18 IgE; NIP IgE, Chimeric hapten 4-hydroxy- 3-nitro-phenacetyl specific NIP IgE; OD, optical density; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood leucocytes; ADCC, antibody-dependent cell-mediated cytotoxicity

Background: Due to genetic similarities with humans, primates of the macaque genus such as the cynomolgus monkey are often chosen as models for toxicology studies of antibody therapies. IgE therapeutics in development depend upon engagement with the FcεRI and FcεRII receptors on immune effector cells for their function. Only limited knowledge of the primate IgE immune system is available to inform the choice of models for mechanistic and safety evaluations.

Methods: The recognition of human IgE by peripheral blood lymphocytes from cynomolgus monkey and man was compared. We used effector cells from each species in ex vivo affinity, dose-response, antibody-receptor dissociation and potency assays.

Results: We report cross-reactivity of human IgE Fc with cynomolgus monkey cells, and comparable binding kinetics to peripheral blood lymphocytes from both species. In competition and dissociation assays, however, human IgE dissociated faster from cynomolgus monkey compared with human effector cells. Differences in association and dissociation kinetics were reflected in effector cell potency assays of IgE-mediated target cell killing, with higher concentrations of human IgE needed to elicit effector response in the cynomolgus monkey system. Additionally, human IgE binding on immune effector cells yielded significantly different cytokine release profiles in each species.

Conclusion: These data suggest that human IgE binds with different characteristics to human and cynomolgus monkey IgE effector cells. This is likely to affect the potency of IgE effector functions in these two species, and so has relevance for the selection of biologically-relevant model systems when designing pre-clinical toxicology and functional studies.

Introduction

The cynomolgus monkey is a laboratory primate commonly used as a model for the study of allergic diseases, in particular addressing symptoms, disease mechanisms and treatments for allergic asthma. It has also found use in preclinical toxicology studies of therapeutic agents prior to clinical trials. The choice of cynomolgus monkey as a species for allergic and toxicological evaluations is due to a perceived similarity of primate systems to human biology and to human immunity. However, limited information is available on the biological relevance of these models in relation to IgE immune responses and therapeutics.

Antibodies of the IgE class are known to contribute to the body's defense against parasites and toxins, but also to play critical roles in the pathogenesis and potentiation of allergic diseases.¹ IgE exerts its biological functions through engagement of its cognate receptors, the low affinity receptor CD23 ($K_a \sim 10^8\text{--}10^9\text{ M}^{-1}$), and the high affinity receptor FcεRI ($K_a \sim 10^{10}\text{ M}^{-1}$). The very slow dissociation from its high affinity receptor ($k_{\text{off}} \sim 10^5\text{ s}^{-1}$) is thought to play a crucial role in the biological functions of IgE: the high affinity for its receptors and resulting tenacious retention on effector cells, in the presence or absence of antigen, can potentiate immediate and strong activation by IgE of effector cells such as mast cells, basophils, monocytes/macrophages and eosinophils.

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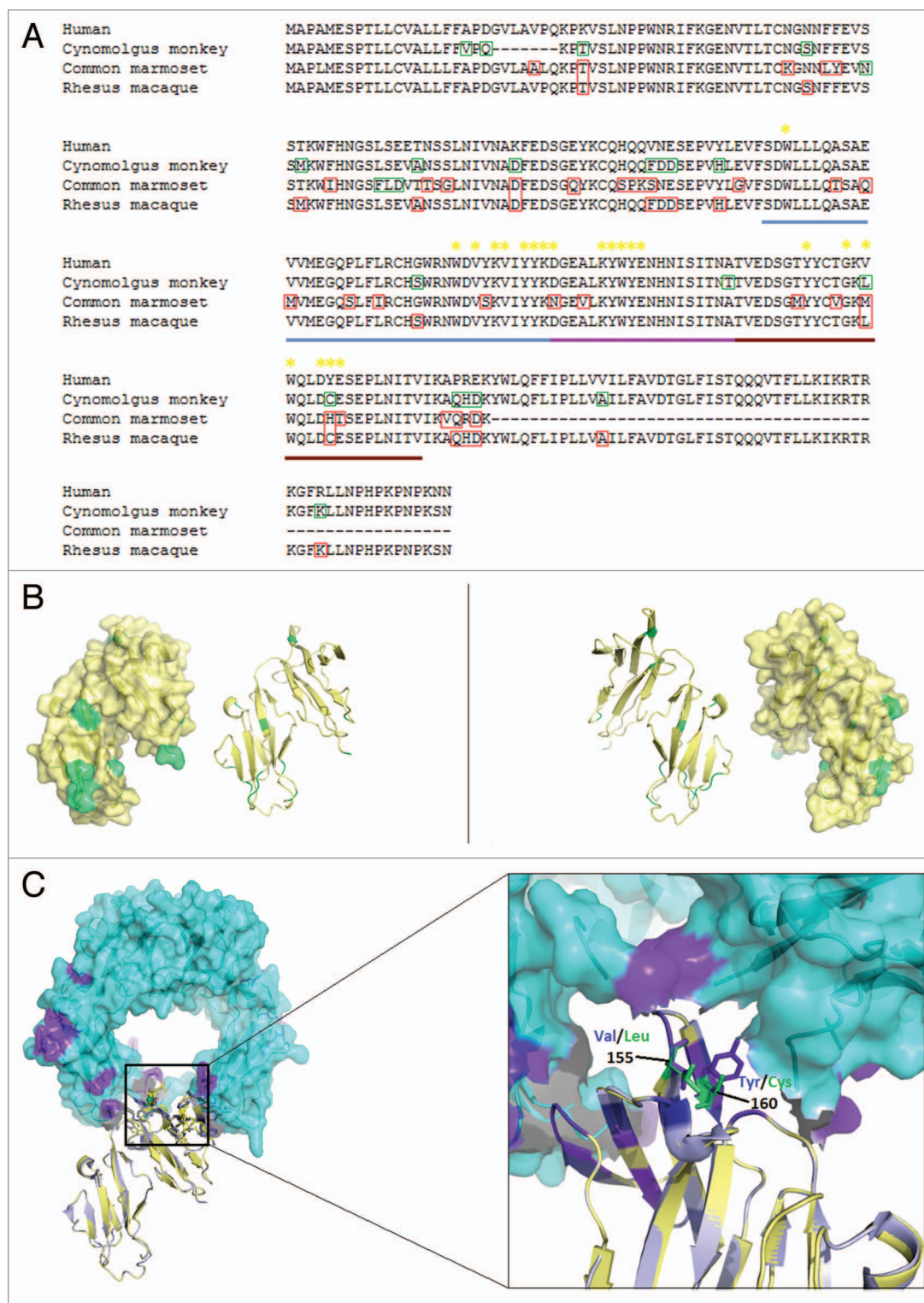


Figure 1. For figure legend, see page 511.

Unlike IgG-IgG receptor complexes, IgE-FcεRI complexes constitute an anticipatory receptor for antigens. The interaction of antigen with IgE-FcεRI results in the immediate release of potent inflammatory mediators such as tryptase, histamine, lipid

mediators and cytokines such as interleukin (IL)-4. Antigen presentation and further IgE responses, often associated with the recruitment of antigen presenting cells by chemokines released by tissue mast cells during allergic inflammation, can

Figure 1 (See opposite page). Sequence comparison of human and cynomolgus monkey high affinity receptor for IgE, FcεRI. **(A)** Alignment of the α-chain of the high affinity IgE receptors (FcεRIα) from humans, cynomolgus monkey, the common marmoset, and the rhesus macaque. Differential residues from the human sequence are highlighted in green on the cynomolgus monkey sequence and in red on the rhesus and marmoset sequences. **(B)** Surface rendered and cartoon representation of the homology model of the cynomolgus monkey extracellular region of the high affinity IgE receptor, sFcεRIα (residues 1–172 of the mature construct), in yellow, based upon the human sFcεRIα (chain A from the X-ray crystallographic model, PDB ID: 1F6A), with the residues that differ in the cynomolgus monkey sequence from the human sequence highlighted in green. The representations on the left and right are rotated in 180° around the y-axis. **(C)** Left: homology model of the cynomolgus monkey sFcεRIα, in yellow, superimposed onto human sFcεRIα, in blue complexed with cε3-cε4 of the Fc region of human IgE, represented in cyan, showing the surface rendering of the Fc region (PDB ID: 1F6A). Those residues likely to be important in binding of IgE to FcεRI are highlighted in dark blue on the IgE-Fc and the FcεRIα. Right: a close up of the binding site between FcεRIα and IgE. The residues at the interface of the binding site that are important in interacting with the IgE-Fc are highlighted in dark blue, with the residues at the binding site that differ in the cynomolgus monkey sequence in green.

also be triggered.² The clinical efficacy of the anti-IgE antibody omalizumab emphasizes the importance of IgE-IgE receptor signaling for activation of immune effector cell cascades in response to IgE.³ Early efforts to derive non-human primate models of allergy were first reported in wild-caught monkeys pre-sensitized to *Ascaris suum*;⁴ however, this approach was disadvantaged by the lack of control over the level of sensitization to the allergen. Therefore, induced allergic asthma models against common human allergens, birch pollen and dust mites, were later introduced.^{5–7} In these models, significant similarities were shown between the human allergic asthma response and that of the cynomolgus monkey allergic asthma model. These included such physiological responses as a positive skin test to allergen, hyper-responsiveness to allergen, the infiltration of IgE+ cells into the site of inflammation, the presence of allergen-specific IgE in the circulation, hyper-reactivity to histamine and bronchoconstriction. More importantly, blocking Th2 associated cytokines results in prevention of an allergic response in sensitized macaques.^{8–10} This data showed that the macaque allergic response is physiologically similar to the human allergic response.

Cynomolgus monkey models continue to be used for target validation and assessment of therapeutic candidates designed to interfere with IgE biology, including IgE-IgE receptor interactions. Such agents include Fcε-Fcγ protein constructs¹¹ designed to reduce sensitization of mast cells and basophils, and to moderate allergic responses. Since Ishizaka et al. in 1970 reported that human IgE-rich serum cross-linked by multivalent antigen or anti-IgE could trigger histamine release in primate lungs in vitro,¹² to date little information has emerged about the cross-reactivity of human IgE Fc with non-human primate IgE effector cells, which has relevance for pre-clinical developmental therapeutics.

We designed this study to explore the recognition and binding properties of a human/chimeric IgE antibody to cynomolgus monkey peripheral blood lymphocytes. We performed comparative evaluations of the capacity of human IgE to elicit a functional response when added to effector cells from humans and from cynomolgus monkeys. We also assessed the cytokines produced when IgE effector cells from humans and cynomolgus monkeys engage and their IgE receptors undergo cross-linking with an IgE antibody. These studies will inform the use of cynomolgus monkey models in studies of IgE-mediated responses and therapeutic agents.

Results

Homology comparisons of human and cynomolgus monkey FcεRIα sequences

To assess the likelihood of human IgE recognizing the IgE high affinity receptors of non-human primates, we performed a sequence alignment of the mature constructs of the FcεRI α-chain (the subunit of the receptor responsible for binding IgE) for the human and three non-human primate species (cynomolgus monkey, rhesus macaque and common marmoset). We confirmed that the mature FcεRIα chain is highly conserved between these species, with a sequence similarity of 93.8% between humans, and the cynomolgus monkey and rhesus macaque mature sequences, while the human and marmoset mature sequences share 84.7% similarity (Fig. 1A). The homology model of the extracellular region of the mature cynomolgus monkey FcεRIα bears high similarity to the human sFcεRIα, with a QMEAN4 score of 0.69. We observed a large difference between the species in one region of the immature construct, where a short segment of the signaling peptide sequence seen in humans, rhesus macaques and common marmosets was absent in the cynomolgus monkey sequence (Fig. 1A).

We then aimed to identify the residues of the mature cynomolgus monkey sequence that are different to those of the human sequence and are potentially involved in, or could influence, binding to the Fc-region of human IgE. Differential residues in the cynomolgus monkey FcεRIα chain sequence were distributed throughout the two extracellular immunoglobulin domains that comprise the outer regions of the receptor recognizing the human IgE-Fc (Fig. 1B). Figure 1C shows where differences in the sequence of the mature human and cynomolgus monkey FcεRIα may affect binding of the cynomolgus monkey construct to human IgE (human and cynomolgus monkey sFcεRIα, residues 1–172 shown as a cartoon representation and the IgE-Fc is surface-rendered in PyMol). The homology model of the cynomolgus monkey sFcεRIα in yellow is superimposed onto the human FcεRIα; those residues that have been found by mutagenesis to be essential for binding of human IgE to the human FcεRIα have been highlighted on the human sFcεRIα and human IgE-Fc in dark blue.¹³ The residues associated with the binding site to IgE Fc that are different between the human and cynomolgus monkey sequences are residues 155 (human/cynomolgus monkey Val/Leu) and 160 (Tyr/Cys) (Fig. 1C, highlighted in green).

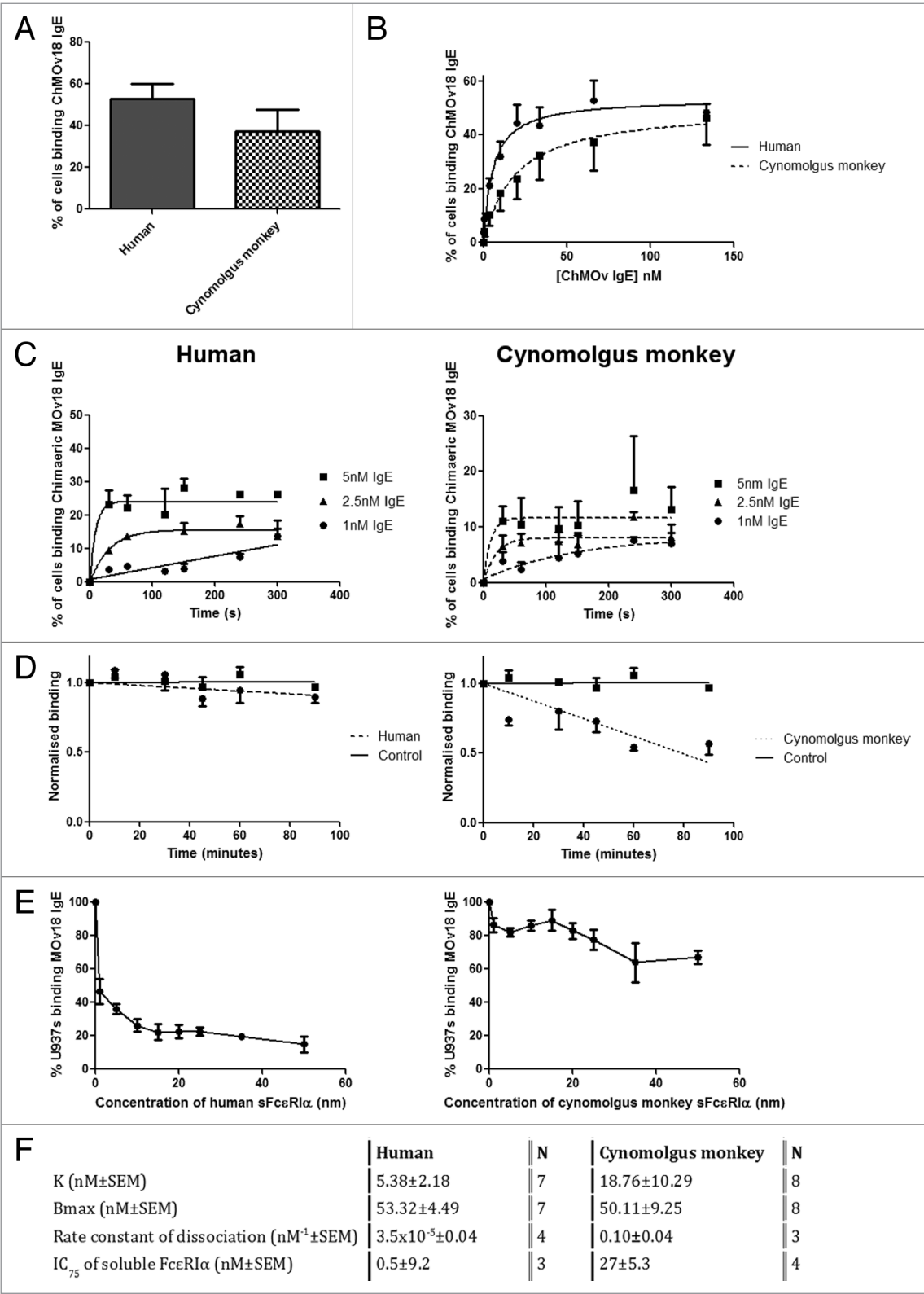


Figure 2. For figure legend, see page 513.

Based on the sequence similarities between humans and non-human primate FcεRIα and predictions of structural homology, it is possible that there is significant cross-reactivity between the human IgE and cynomolgus monkey FcεRIα. Therefore efforts were concentrated to first ascertain cross-reactivity using cynomolgus monkey and human effector

Figure 2 (See opposite page). Binding of human IgE to human and cynomolgus monkey effector cells. **(A)** The % of the total peripheral blood leucocyte population that showed specific binding to 66 nM (~equivalent 1 mg/kg dosing) MOv18 IgE (human samples in gray; cynomolgus monkey samples in black and white check). Binding of human and cynomolgus monkey cells at high concentrations of IgE is not significantly different. **(B)** Specific binding of increasing concentrations (range 0–133 nM) of IgE to human and cynomolgus monkey effector cells. **(C)** Time dependant increase of specific binding of IgE to human (left) and cynomolgus monkey (right) cells at different concentrations of IgE, from 1–5 nM over 5 min. **(D)** Dissociation of 5 nM PE-labeled IgE from human (left) and cynomolgus monkey (right) effector cells; at stated times, the cells were washed and fixed with 4% formaldehyde before acquiring for MFI to determine binding of labeled IgE (1 = total expected binding with PE-labeled MOv18 IgE in the absence of competition with unlabeled IgE). **(E)** Competition assay of binding of labeled IgE in the presence of recombinantly expressed extracellular regions of the human (left) and cynomolgus monkey (right) high affinity IgE receptors to compete binding of IgE to cells expressing the endogenous IgE receptors. **(F)** Table showing the quantifiable values determined from the experiments detailed above. N-numbers represent blood samples of individuals from each species.

cells and furthermore to determine if the cross-reactivity was quantifiably different between the two species.

Human IgE-Fc cross-reacts with cynomolgus monkey effector cells

To gain an insight into the ability of cynomolgus monkey FcεRIα to recognize human IgE-Fc, we performed comparative binding evaluations to ascertain cross-reactivity of a human/chimeric IgE antibody (MOv18 IgE) to cynomolgus monkey peripheral blood leucocytes (PBL) in comparison to its native reactivity with human peripheral blood leucocytes (PBL). At saturating concentrations of MOv18 IgE (66 nM), the proportions of PBL in both cynomolgus monkeys and humans that bound IgE were found to be similar (Fig. 2A). A dose-response experiment with IgE added to effector cells at a concentration range between 0 and 133 nM showed that human and cynomolgus monkey effector cells differ in their capacities to bind IgE at concentrations of IgE below 66 nM (the % of cells with bound IgE; $P = 0.026$; $n = 7$ for humans and $n = 8$ for cynomolgus monkeys), with binding only becoming comparable between the two species at higher IgE concentrations (Fig. 2B). We also observed comparable maximal binding as a function of time (Fig. 2C); the maximal % binding of IgE at a concentration of 2.5 nM to PBL after 5 min was $16.5 \pm 2.6\%$ of cells for human ($n = 3$) and $8.0 \pm 2.2\%$ for cynomolgus monkeys ($n = 4$) (mean $\pm$ standard error of mean (SEM)).

The well-described high affinity of IgE for FcεRI is due to extremely slow dissociation rate of the antibody from this receptor and this is responsible for the tenacious retention of IgE once bound to effector cells. To determine if human IgE retained slow dissociation properties in relation to the cynomolgus monkey effector cells, we conducted a dissociation assay and a competition assay. When retention of fluorescently-labeled MOv18 IgE binding to effector cells between the two species was compared as a function of time by competition with unlabeled IgE, we found that cynomolgus monkey cells had a reduced ability to retain labeled human IgE over a period of two hours. Consequently, an off-rate was calculated at $3.5 \times 10^5 \pm 0.04 \text{ nM}^{-1}$ ($n = 4$) for humans compared with $0.1 \pm 0.04 \text{ nM}^{-1}$ ($n = 3$) for cynomolgus monkeys (mean $\pm$ SEM) (Fig. 2D and 2F). We then sought to determine the capacity of recombinant sFcεRIα from each species to displace IgE binding to monocytic cells. Compared with the human receptor, the cynomolgus monkey sFcεRIα was inefficient in replacing human IgE binding to human FcεRI-expressing monocytic cells (Fig. 2E). The concentration required for cynomolgus monkey sFcεRIα to displace 25% of binding of nM concentrations of human IgE to human effector cells

was > 10-fold that required for the human sFcεRIα to displace human IgE (Fig. 2F). However, as nM concentrations of the recombinant human and cynomolgus sFcεRIα were required to inhibit binding of human IgE to U937 human monocytes, the overall affinity of IgE for the cynomolgus monkey FcεRIα appears to be in the nM range (IC_{75} : human $0.5 \pm 9.2 \text{ nM}$, $n = 3$; cynomolgus monkey $27 \pm 5.3 \text{ nM}$, $n = 4$).

In conclusion, we observed cross-reactivity for human IgE binding to cynomolgus monkey and human effector cells, but we found that IgE dissociates at a faster rate from the non-human primate compared with the human effector cells.

Comparisons between cynomolgus monkey and human serum IgE levels and IgE receptor-expressing cells

Evaluations of the IgE system in a potential model species should include assessment of endogenous IgE serum levels and the proportions of IgE effector cells capable of recognizing IgE. A serum IgE ELISA showed lower mean serum levels of IgE in cynomolgus monkey sera ($43.7 \pm 5.4 \text{ ng/ml}$, mean $\pm$ SEM, $n = 13$) compared with the levels of IgE in normal human sera ($110 \pm 25.5 \text{ ng/ml}$, mean $\pm$ SEM, $n = 10$) ($P = 0.008$) and a larger variability of serum IgE levels in the human cohort (Fig. 3A). Comparable proportions of cynomolgus monkey ($17.6 \pm 1.2\%$, mean $\pm$ SEM, $n = 3$) and human ($10.3 \pm 5.5\%$, mean $\pm$ SEM, $n = 4$) monocytes, as shown previously,¹⁴ express IgE receptors and are therefore capable of binding IgE ($P > 0.05$) (Fig. 3B, C, and D). Phenotypic analyses of cynomolgus monkey CD56-enriched cells suggest that, similarly to human monocytic cells, they express CD16 and can bind IgE (Fig. 3C and D; Table S1).^{14–16} These data suggest that cynomolgus monkeys and humans share similar proportions of Fcε receptor expressing cell compartments, but that the two species differ slightly in the normal levels of circulating IgE.

Degradation profiles upon engagement and cross-linking of human IgE on cynomolgus monkey and human effector cells

To gain an insight into the functional implications of the differential binding kinetics of IgE for human and cynomolgus monkey effector cells, we performed a histamine release assay using PBL from each species. A slightly lower % histamine release from cynomolgus monkey cells was observed with lower concentrations of MOv18 IgE (3 nM) ($27.6 \pm 26.7\%$ for cynomolgus monkeys, $n = 3$) compared with humans ($57.1 \pm 26.3\%$, $n = 5$). Histamine release measured for the two species was similar when MOv18 IgE was added at concentrations $\geq 5.5 \text{ nM}$ ($66.4 \pm 33\%$ for cynomolgus monkeys and $75.2 \pm 12.2\%$ for humans at 5.5 nM). As previously reported for the human system, we observed a reduction in IgE effector cell activation,

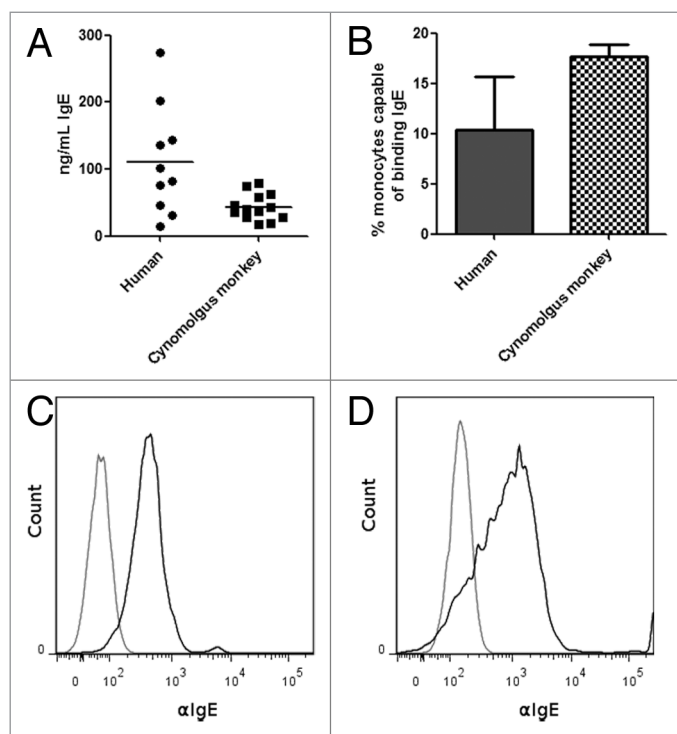


Figure 3. IgE levels in sera and the IgE receptor expression by humans and cynomolgus monkey monocytes. (A) Serum IgE levels were quantified by ELISA for 10 human and 13 cynomolgus monkey samples ($P = 0.032$, by Student's t test). (B) Using IgE and a polyclonal anti-human IgE, the % of human and cynomolgus monkey monocytes capable of binding IgE was determined by flow cytometry ($n = 4$ for humans and $n = 3$ for cynomolgus monkeys). (C and D) Flow cytometric histograms (black lines) representing the binding of saturating concentrations of human IgE and anti-human IgE to human (C) and to cynomolgus monkey (D) monocytes (secondary antibody alone, light gray lines).

measured by histamine release, at high concentrations of IgE for both species¹⁷ (Fig. 4A). These findings suggest that, while human IgE appears less efficient in activating cynomolgus monkey effector cells at lower concentrations, the antibody can trigger histamine release by cells from both species at higher concentrations. These data are in line with higher retention of IgE by human compared with cynomolgus monkey effector cells and lower dissociation kinetics of IgE from effector cells, which may have implications on cell activation and potency at suboptimal antibody concentrations.

Cynomolgus monkey PBMC display reduced capacity to kill antigen expressing cells in an IgE-mediated ADCC assay and a differential cytokine profile upon ADCC

We next investigated the effects of the interactions of MOv18 IgE with cynomolgus monkey effector cells with regards to their capacity to mediate a cytotoxic functional response. For this, an antibody-dependent cell-mediated cytotoxicity assay (ADCC) assay was employed to measure the ability of the tumor antigen-specific IgE antibody to trigger human and cynomolgus monkey effector cells (peripheral blood mononuclear cells, PBMC) to kill tumor cells in an antigen-dependent manner. MOv18 IgE mediated significant levels of ADCC above those triggered with the

non-specific NIP IgE antibody by effector cells from both species in a concentration-dependent manner (Fig. 4B). Cynomolgus monkey effector cells could mediate FR α + tumor cell death (% cytotoxicity above the isotype controls) of 12.5 ± 3.2 with 4 nM MOv18 IgE and 14.8 ± 2.9 with 6 nM MOv18 IgE ($n = 12$). For human cells, the % cytotoxicity above isotype controls were 16.8 ± 2.9 with 4 nM MOv18 IgE ($n = 12$) and 23.3 ± 3.7 with 6 nM MOv18 IgE ($n = 8$). However, there were no significant differences in ADCC when using 4 nM and 15 nM MOv18 IgE.

A significant difference in % of FR α + target cells killed by MOv18 IgE was measured between the two species at 6 nM MOv18 IgE, with human cells triggering higher levels of ADCC above controls ($23.3 \pm 3.7\%$ for human cells vs. $14.8 \pm 2.9\%$ for cynomolgus monkey cells) ($P = 0.044$) (Fig. 4B). At higher concentrations of IgE, FR α + cell killing (ADCC) capacity of cynomolgus monkey effectors was comparable to that of human cells (% cytotoxicity above isotype control: 23.7 ± 1.7 in humans; $n = 13$ vs. 28.8 ± 2.3 in cynomolgus monkeys; $n = 6$).

The cytokine profiles of human and cynomolgus monkey PBMC were next studied with unstimulated PBMC and from supernatants derived from ADCC assays in the presence of antibodies to elucidate any differences in effector cell activation triggered by human IgE cross-linked by antigen.

The background cytokine production profiles of unstimulated PBMC from each species were markedly different, with levels of the cytokine vascular endothelial growth factor (VEGF) significantly higher in cynomolgus monkeys compared with humans (p values of < 0.0001 ; $n = 6$ for humans; $n = 8$ for cynomolgus monkeys) (Fig. 5A). Resting human cells did not produce IL-13, while we detected IL-13 in the supernatants of cynomolgus monkey PBMC. Resting human cells produced low levels of interferon (IFN) γ , IL-4 and IL-5, but none of these cytokines were detected in the cynomolgus monkey setting.

When studying individual cytokine production in the presence of MOv18 IgE (corresponding to ADCC assays in Figure 4B), in the human system we found that production of IL-5 and VEGF significantly increased above isotype controls ($P = 0.0007$ for IL-5; $P = 0.0008$ for VEGF; $n = 6$), while levels of IFN γ , IL-1 β and tumor necrosis factor (TNF), showed a non-significant increase at 15 nM concentrations of IgE (Fig. 5B).

In cynomolgus monkeys, production of IL-4 and VEGF increased significantly with increasing concentrations of IgE (both $P < 0.0001$) and IL-5 showed a non-significant increase. The production of IL-13 did not increase significantly with increasing ADCC in the cynomolgus monkey setting. Additionally, the production of IL-10 ($P = 0.011$), IFN γ ($P = 0.0023$) and TNF (0.0006) decreased significantly in MOv18 IgE-treated cynomolgus monkey assays compared with non-specific IgE-treated samples (Fig. 5B).

When comparing cytokine levels between human and cynomolgus monkey cells stimulated with IgE, the commonly associated Th2-cytokines IL-4, IL-5, and IL-13 were higher in cynomolgus monkeys. In the human system, IFN γ levels were higher compared with cynomolgus monkeys.

These data point to differential cytokine profiles between unstimulated human and cynomolgus monkey effector cells and to

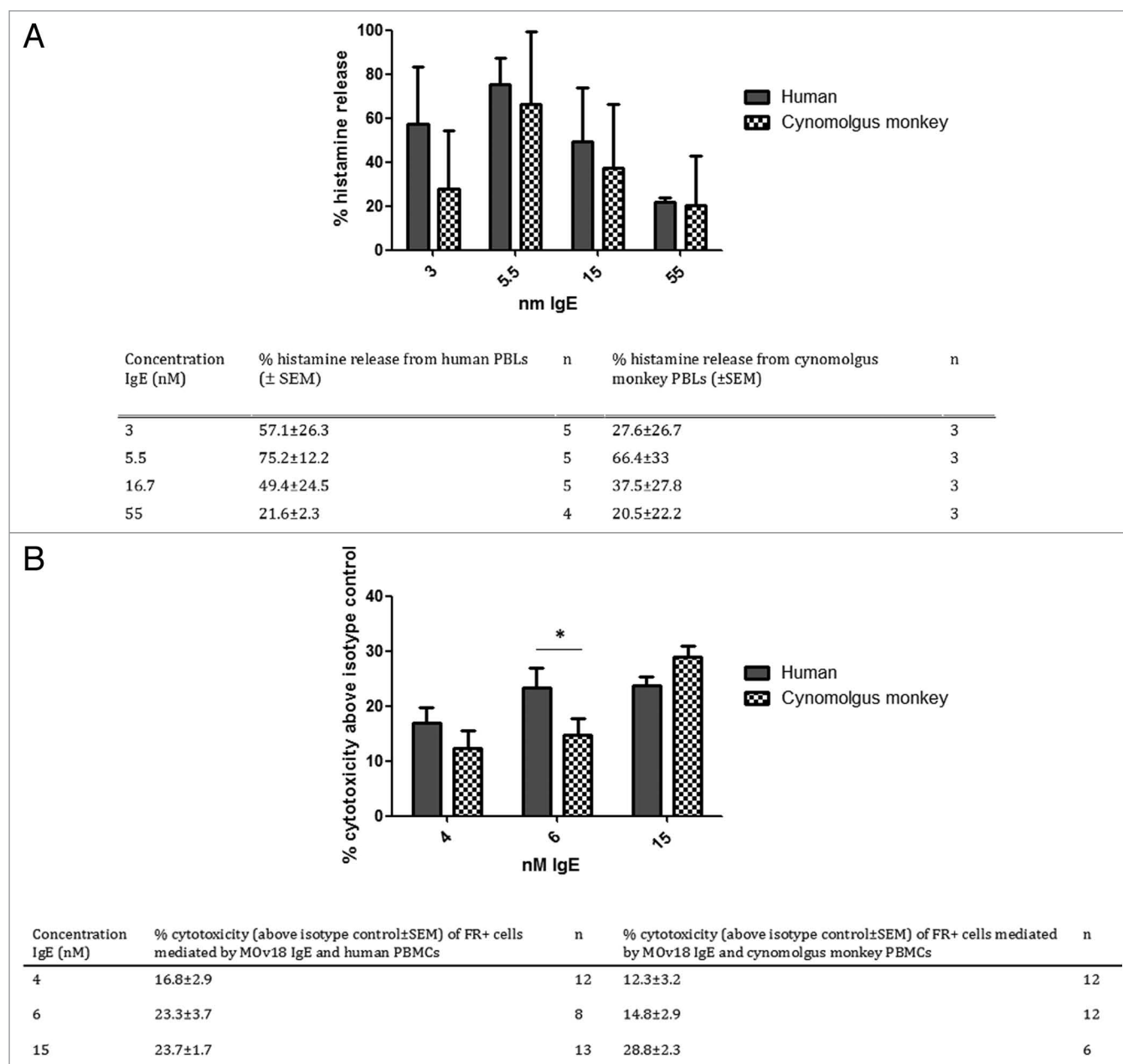


Figure 4. Human IgE mediates functional activation of IgE effector cells. **(A)** The % histamine release from human (gray) and cynomolgus monkey (checked) effector cells, mediated by cross-linking of IgE bound to Fc ϵ R1 with a polyclonal goat anti-human anti-IgE antibody (n = 5 for human and n = 3 for cynomolgus monkey). The % histamine released was quantified by ELISA (Abnova, Taiwan) and values shown on table (bottom panel). **(B)** ADCC of FR+ cells mediated by increasing concentrations of MOv18 IgE (4–15nM) with human and cynomolgus monkey PBMC. Data are represented as the % of tumor cells killed above the average ADCC values measured with the isotype control-treated samples, measured for each individual. N-numbers represent individuals assayed for each species; statistical significance calculated using an unpaired Student t test (GraphPad); % cytotoxicity $\pm$ SEM values are shown in the table below.

differences in human and cynomolgus monkey effector cell systems when effector cells are activated by antigen-specific human IgE.

Discussion

We investigated the cross-reactivity of human IgE to cynomolgus monkey and human immune effector cells, and examined whether human IgE could mediate cynomolgus

monkey and human effector cell activation in functional assays. Although there is significant cross-reactivity between human IgE and cynomolgus monkey effector cells, differences exist in the dissociation of human IgE from PBL between the two species. We also demonstrate that these differences may translate into divergent functional efficacy, possibly due to the differential interactions of human IgE with cynomolgus monkey compared with human effector cells.

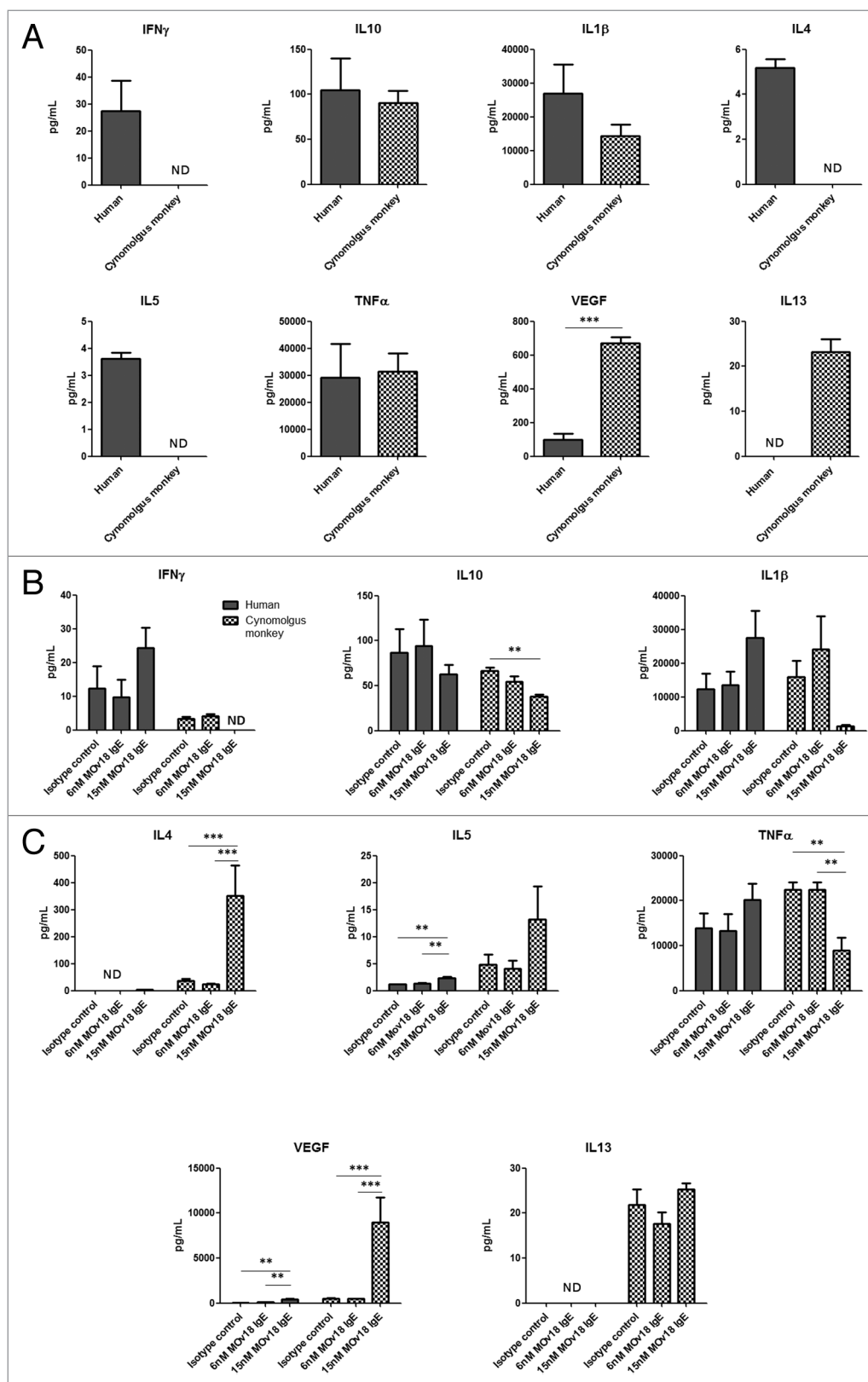


Figure 5. For figure legend, see page 517.

Initiating the study with in silico modeling, sequence alignment of the IgE Fc-binding Fc ϵ RI α chains (Fig. 1A) confirmed a high

degree of sequence conservation. This led us to hypothesize that human IgE may have significant cross-reactivity for cynomolgus

Figure 5 (See opposite page). Cytokines produced by cross linking IgE on effector cells of human and cynomolgus monkey PBMC. **(A)** The background levels of key cytokines produced by human and cynomolgus monkey PBMC were quantified by collecting culture supernatants and quantifying the cytokines by Luminex (n = 6 for humans, and n = 8 for cynomolgus monkey). The quantified human cytokines are shown by gray bars and the cynomolgus monkey cytokines are shown by black and white checked bars. **(B)** The cytokines produced by human (gray bars) and cynomolgus monkey (black and white checked) PBMC during ADCC assays by an isotype control and at two concentrations of MOv18 IgE, 6 and 15nM, that cross-links the high affinity IgE receptors in the presence of FR+ cells, were quantified (n = 6 for humans and n = 8 for cynomolgus monkeys). Statistical significance was measured by one way ANOVA using GraphPad. ND (not detected) indicates that levels of cytokines were below the detection limit of the assays.

FcεRIα. However, sequence comparison also suggested possible differences at the binding site; of particular note is the amino acid divergence between human and cynomolgus monkey at residue 160. Here, the tyrosine in the human sequence is replaced with a cysteine, which could potentially have a notable effect on binding or dissociation kinetics. In addition, the species differ in the secretion peptide of FcεRIα, a portion of which is absent in the cynomolgus monkey sequence (Fig. 1A). This signaling peptide might be important for trafficking of the receptor to the cell surface, but also for negative regulation of intracellular tyrosine-based activation motif (ITAM) domains in other subunits of the IgE receptor.¹⁸ As ITAMs are partially responsible for the propagation of phosphorylation signals, these differences could potentially underlie differences in FcεRI receptor function and surface expression between cynomolgus monkeys and humans.

Based on the sequence similarities and predictions of structural homology, we wished to examine whether human and cynomolgus monkey FcεRIα might differ in their affinities for the human IgE Fc domain. As predicted from the high sequence homology between human and cynomolgus monkey FcεRIα, we confirmed that human IgE cross-reacts with cynomolgus monkey effector cells. However, higher doses of human IgE are required with cynomolgus monkey PBL to obtain a similar level of binding compared with human PBL (Fig. 2A and B). The total binding of human IgE to cynomolgus monkey and human effector cells was comparable, but, in line with the differences in critical amino acids involved in the IgE binding site (Fig. 1C), we demonstrate that human IgE dissociates at a faster rate from the cynomolgus than from the human PBL (Fig. 2D). In an in vivo setting in the cynomolgus monkey, this would result in human IgE engaging less effectively with effector cells, reducing the potency for an IgE-FcεRI or IgE-FcεRII complex against the target antigen. These findings are supported by the results of our competition experiment. The efficacy of cynomolgus monkey sFcεRIα is impaired, compared with human, in its inhibition of binding of human IgE to a human monocytic cell line. This results in lower overall affinity of the cynomolgus monkey FcεRI receptor for human IgE-Fc (Fig. 2F), and implies that this non-human primate species may represent an imperfect model for pre-clinical toxicity testing of novel IgE therapeutics.

When studying effector cell populations from each species, any similarities or differences in leucocyte population between species should be considered. Of the circulating blood cell populations, monocytes, basophils, eosinophils and B cells express receptors for binding IgE, basophils and eosinophils express only FcεRI, and a subset of B cells express only CD23. Immunophenotyping of cynomolgus lymphocytes demonstrated that populations of B cells, CD4+ and CD8+ T cells, and monocytes were similar to those in humans.¹⁹ However, the eosinophil cell count may

differ between the species,²⁰ so the possibility that the differential binding we observe may be due to differences in heterologous cell populations, rather than a result of differing binding affinities, cannot be excluded. Nevertheless, we found that cynomolgus and human PBL share comparable proportions of Fcε receptor-expressing cell compartments (Fig. 3B). This supports differences in association or dissociation kinetics rather than effector cell populations as the reason for the differential binding properties observed between the two species.

We observed lower levels of serum IgE in the cynomolgus monkey than in human (Fig. 3), as well as a larger inter-individual variation in the human. This variability in human IgE levels has been observed previously,²¹⁻²³ and may be due to genetic heterogeneity, allergic conditions and to differential allergen exposure in the human population, compared with the cynomolgus monkey cohort bred and maintained in captivity. Furthermore, laboratory cynomolgus monkeys of Mauritian origin have been shown to have less genetic diversity than cynomolgus monkeys from across other areas of Asia.²⁴ Based on these observations, it may be concluded that an IgE antibody therapy introduced into a cynomolgus monkey may not engage in the breadth of binding and functional response seen in the human setting.

The binding kinetics of human IgE to human and cynomolgus monkey effector cells were partly reflected in their capacity to activate effector cells, in turn assessed by measuring histamine release and ADCC by the effector cells from each species (Fig. 4). At low IgE concentrations, we observed small but not significant differences in the potency of human IgE to trigger histamine release by cynomolgus monkey compared with human effector cells. At higher concentrations, human IgE triggered similar levels of histamine release in both species. At concentrations lower than 66 nM, the relative association of human IgE for effector cells of the two species was markedly different (Fig. 2A), but this was not reflected in the levels of IgE-mediated histamine release in each species, and in concordance with previous findings.¹² One reason for this may be due to the high density of IgE receptors on the cell surface of human basophils,²⁵ the cells responsible for histamine release in blood. By contrast, the ADCC assay demonstrated, at low IgE concentrations, the capacity of the human antibody to activate cynomolgus monkey effector cells to kill tumor was impaired compared with its effect on human effector cells. These differences in ADCC potency were not observed at higher antibody concentrations.

We also found differences in cytokine release profiles between the two species, both by resting cells, and by cells activated by IgE antibody in the presence of antigen-expressing target cells. Resting human cells produce low levels of IFNγ, IL-4 and IL-5, whereas cynomolgus monkey cells produce IL-13 and VEGF. The

cytokines secreted at higher levels in the cynomolgus monkey in response to IgE, were IL-4 and IL-5, both of which are commonly associated with stimulation of IgE production.²⁶ In the human system, there are instead small increases in the production of the Th1-biased cytokines IFN γ , IL1 β and TNF, previously described to increase with ADCC.^{27,28} VEGF production, a response normally triggered in hypoxic environments,²⁹ was also higher in cynomolgus monkey than human cells in response to IgE, suggesting the relative promotion of inflammatory and hypoxic conditions in the monkey. These distinctive cytokine profiles, consistent with reports of differences in responses between human and non-human primates,^{30,31} suggest differences in effector cell activation upon engagement with IgE.

Our findings are relevant to the design and application of IgE antibodies as potential therapeutic agents for cancer, an area of oncology called AllergoOncology.³² AllergoOncology therapies are designed to direct the potent responses to IgE against cancer, and include monoclonal antibodies engineered with Fc regions of the IgE class.³³⁻³⁶ For translation of this concept, preclinical toxicology models are required in which the interactions of human therapeutic IgE with effector cells of a potential model species are understood. It is possible that lower doses of a human IgE may be required to induce a given response in humans than required in the cynomolgus monkey, and this may be because of inter-species differences in dissociation and IgE-mediated effector functions. Although differences in responses to antibody administration cannot be completely modeled *in vitro*, our data showing differential cytokine profiles *ex vivo* (Fig. 5) suggest clear differences in downstream activation of effector cells in the presence of cell-bound IgE between humans and cynomolgus monkeys. This may bear relevance in the selection of model species for the preclinical application of IgE antibodies as potential therapeutics.

Since adverse effects were seen in the first-in-man study of the CD28 superagonist monoclonal antibody TGN1412, there have been calls for more reasoned approaches toward toxicology models.³⁷⁻³⁹ The Expert Scientific Group (ESG) meeting held to discuss the problems with the TGN1412 trial concluded that the downstream biological relevance, both cross-reactivity and effects of any drug need to be assessed in the candidate species before moving into human trials.

We conclude that differential kinetics of human IgE Fc binding to cynomolgus monkey and human effector cells affect the activation and potency of these cells. These inter-species differences mandate careful selection of *in vivo* models of Allergy and AllergoOncology.

Materials and Methods

Antibodies and reagents

The human/chimeric monoclonal antibody MOv18 IgE, recognizing the tumor-associated antigen folate receptor α (FR α), and the hapten 4-hydroxy-3-nitro-phenacetyl specific NIP IgE, were prepared as before.^{14,33,40,41} Soluble Fc ϵ RI α -chain (sFc ϵ RI α) constructs of humans and cynomolgus monkeys were produced as previously described⁴² and mature constructs of residues 1–176

from each species were used. The cynomolgus monkey construct was cloned into the mammalian expression vector pEXPR-IBA42 to retain the secretion peptide in the vector, expressed in the HEK293F FreeStyle expression system (Invitrogen Life Sciences) and purified using a streptavidin-conjugate affinity column (IBA Life Sciences). Human CFSE dye, tissue culture medium, and reagents were obtained from Invitrogen Life Technologies. IgE was labeled with PE using the Lightning Link antibody-labeling kit (Innova Biosciences) according to the manufacturer's instructions.

Sequence alignment and homology modeling of cynomolgus monkey sFc ϵ RI α

The amino acid sequences for the cynomolgus monkey (US patent ID 60/824, 029) the rhesus macaque amino acid sequences (accession number XP_001117218) and the common marmoset mRNA sequence (accession number XM_002760222.1, translated into protein sequence using ExPASy Translate) were aligned with the human Fc ϵ RI α sequence using clustalW.⁴³ SWISS-MODEL software was used for homology modeling of the cynomolgus monkey sFc ϵ RI α , using chain A of the PDB ID 1F6A as a template and the resultant model was superimposed onto the human model in PyMol.^{13,44}

Isolation of human and cynomolgus monkey PBL, PBMC and monocytes

Peripheral venous blood was collected in vacutainers with K2 EDTA (BD Biosciences) from human healthy volunteers after obtaining informed consent in accordance with the Helsinki Declaration (approved by the Guy's Research Ethics Committee, Guy's and St. Thomas' NHS Foundation Trust), and from a cynomolgus monkey colony of predominantly Mauritian origin that has been stable in the UK for 30 y. Animals were housed in social groups according to the Home Office (UK) Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989). Peripheral blood leucocytes (PBL) were isolated from whole blood by the lysis of red blood cells using a 5 $\times$ volume of filter-sterilized red cell lysis buffer (155mM NH $_4$ Cl, 12mM NaHCO $_3$, 0.1mM EDTA) and washed in PBS before use. PBMC were isolated by mixing with an equal volume of PBS, 2mM EDTA and by layering onto Ficoll-Paque Premium (GE Healthcare) and centrifuged at 1200 $\times$ g to isolate human PBMC and 800 $\times$ g to isolate cynomolgus monkey PBMC. The PBMC were removed from the interface and any remaining red blood cells were lysed using filter-sterilized red cell lysis buffer as above.

Human monocytes were isolated using RosetteSep monocyte enrichment cocktail (Stem Cell Technologies) as before according to the manufacturer's instructions.⁴⁴ Cynomolgus monkey monocytes were isolated by selecting for the cell surface marker CD56, previously described to identify rhesus macaque monocytes.¹⁵ Anti-NHP CD56 magnetic beads (Miltenyi Biotechnology) were incubated with PBMC isolated using Ficoll-Paque as described above, then used with an LS magnetic column and MidiMACS magnetic separator to positively select monocytes, as per the manufacturer's instructions (Miltenyi Biotechnology). Phenotypic analysis (expression of CD16 and binding of IgE) was used to corroborate that CD56+ cells might be monocytes in cynomolgus monkeys as in rhesus macaques.

Dissociation of IgE from human and cynomolgus monkey cells

PBMC isolated from human and cynomolgus monkey blood were seeded in complete medium [CM, RPMI 1640 medium, 10% FCS, 2 mM l-glutamine, penicillin (5 000 U/mL) and streptomycin (100 µg/mL)] (GIBCO, Invitrogen) at a concentration of 2×10^6 cells/mL and incubated with 5 nM PE-labeled ChMOv18IgE for 15 min before a wash in PBS. The equivalent volume of complete medium was added to the washed cells, cells were resuspended in CM and 200 µL was aliquoted and incubated with 50 nM unlabeled MOv18 IgE at 4 °C to compete PE-labeled IgE binding. At different time-points, reactions were stopped by a wash in PBS at 400xg and cells were fixed in 4% paraformaldehyde. The mean fluorescence intensity (MFI) of the cells was determined by analysis on a FACS Canto II (BD Biosciences). The MFI at each time point was normalized to determine the total loss of IgE binding as follows: the MFI for cells incubated with PE-labeled MOv18 IgE at time = 0 was taken to be 1; and the unlabeled cells were equal to no IgE binding. Therefore, an equation to determine the normalized binding is:

$$\text{IgE binding at } x(s) = \frac{(\text{MFI of PBMCs at } t=0) - (\text{MFI of PBMCs at } t=x)}{(\text{MFI of PBMCs at } t=0) - (\text{MFI of unlabelled PBMCs})} \quad (1)$$

Inhibition of binding of IgE to U937 monocytes by human and cynomolgus monkey sFcεRIα

The human monocytic cell line U937, which expresses the native IgE receptor FcεRI, was maintained in CM (GIBCO, Life Sciences) and used for this assay. A range of concentrations of recombinant human or cynomolgus monkey sFcεRIα (1–50 nM) were added to 150 µL aliquots of PBS/2% FCS with PE-conjugated IgE to a concentration of 5 nM. After 15 min of incubation, allowing the IgE to mix with the human and cynomolgus monkey FcεRIα at 18 °C, monocytic cells were added to these samples at a final cell density of 1×10^6 /mL and incubated for a further 15 min to allow binding of IgE to the native human cell-bound FcεRI receptors on the cell surface. The cells were then washed with PBS and fixed in 4% paraformaldehyde before analysis on a FACSCanto II (BD Biosciences). The % binding of IgE in the presence of sFcεRIα was calculated as follows: 100% binding was determined as the MFI of U937s with bound PE-conjugated IgE with no sFcεRIα present; 0% binding was determined as the MFI of the U937s with no PE-conjugated IgE bound; and the 0% binding MFI was subtracted from the MFI measured for each concentration of sFcεRIα used:

$$\text{Binding of IgE at [sFcεRIα]} = \frac{(\text{MFI measured at [sFcεRIα]}) - (\text{MFI of unlabelled cells})}{\text{MFI of cells with PE labelled IgE alone}} \quad (2)$$

Serum IgE ELISA

An ELISA to detect human and cynomolgus monkey IgE in sera was conducted as previously described.⁴⁵ Human FcεRIα at a concentration of 3 µg/mL in carbonate buffer was added to wells of NUNC 96-well flat bottom plates (Thermo Scientific) before incubating for 18 h at 4 °C. The next day, plates were washed in PBS-T, then blocked with SuperBlock (Pierce) diluted (1:2) in PBS for 1 h at room temperature. IgE standards (IgE standard, NIBSC) were added in the range 200–1.2 ng/mL and 100 µL of serum samples were added to the plates either neat or diluted 1:4; standards and samples were all added in duplicate. To detect

serum IgE antibodies, a number of secondary antibodies were tested to select one with the best cross-reactivity to cynomolgus monkey IgE. A polyclonal goat anti-human IgE was determined to give the best cross-reactivity and was added to plates at 0.2 µg/mL for 2 h at room temperature (Bethyl laboratories), followed by detection with a donkey anti-goat IgG antibody conjugated to HRP (Vector laboratories, diluted 1:10,000). A color reaction was developed for 30 min with o-phenylenediamine dihydrochloride (OPD, 10 mg was added to peroxide substrate (Pierce) diluted 1:10 in dH₂O) and 1 M HCL was added to quench the reaction. The optical density (OD) was measured on an ELISA plate reader (BMG Labtech) at 450 nm. The standard data was fitted using a 4-parameter fit in the omega software with MARS data analysis software (BMG Labtech), which also calculated the values of the samples in ng/mL.

IgE receptor expression by human and cynomolgus monkey monocytes

Monocytes were isolated as described above and seeded at a concentration of 2×10^6 /mL in PBS/5% FCS. Two hundred µL aliquots per individual blood donor were prepared and incubated for 20 min at 4 °C as follows: one sample was treated with IgE, followed by goat anti-IgE polyclonal antibody (Bethyl laboratories) and finally with donkey anti-goat polyclonal antibody conjugated to APC (R&D Systems) to detect the percentage of cells bearing IgE receptors. Phenotypic characterization of the total cell population enriched for possible monocyte markers from human and cynomolgus monkeys were also determined with antibodies for the cell surface markers (CD56, CD89, CD16) to ascertain the % positive cells in the enriched population. Following incubations, cells were fixed in 4% paraformaldehyde and analyzed using a FACSCantoII (BD Biosciences). Receptor expression for individual samples was calculated by gating for monocyte populations on FSC/SSC dot plots.

Histamine release functional assay

The experimental design was based upon the principles of a basophil activation test previously published.¹⁷ Briefly, peripheral blood lymphocytes from human and cynomolgus monkey blood were obtained by lysing the red blood cells in whole blood using red cell lysis buffer at 5× the blood volume at 18 °C for 15 min. The cells were washed with PBS and resuspended in PBS at 2×10^6 cells/mL, and 100 µL of the cell suspension were added to Eppendorf tubes. IgE was added to the cells at different concentrations (range of 0–10 µg/mL, equivalent to 0–55 nM IgE), followed by 100 µL of a polyclonal goat anti-human IgE antibody (Bethyl laboratories) at 1 µg/mL in PBS, and samples were incubated at 37 °C for 20 min. The negative control sample consisted of human cells with no added IgE or anti-IgE, and the positive control was human cells incubated with an anti-FcεRI antibody at 2 µg/mL to cross-link IgE receptors. Following incubations, supernatants were harvested by centrifugation at 500 × g and snap-frozen in liquid nitrogen until use. The relative amounts of histamine released were determined using a Histamine ELISA kit (Abnova): 8-well strips coated with a histamine-binding reagent were incubated with the samples diluted 1:10 in PBS for 3 h at room temperature. The ELISA proceeded with

an HRP-conjugated anti-histamine secondary antibody. Color reactions were developed with OPD substrate and 1M HCL was added to quench the reactions, before the OD was measured on an ELISA plate reader (BMG Labtech) at 450nm and 625nm (reference wavelength). The % histamine release was measured by subtracting the OD₄₅₀₋₆₂₅ of the negative control measurement from the OD₄₅₀₋₆₂₅ measured for each IgE-stimulated sample used. Maximum (100%) histamine release was determined to be the mean of the increase in OD₄₅₀₋₆₂₅, above the human negative controls mediated by the addition of anti-FcεRI. The % histamine release was calculated using the following equation and applied for both human and cynomolgus monkey samples:

$$\text{Histamine release} = \frac{\text{OD}_{450-625} \text{ of sample} - \text{OD}_{450-625} \text{ of negative}}{\text{Mean of Positive} - \text{negative OD}_{450-625} \text{ across human samples}} \quad (3)$$

Tumor cell killing assay

An in vitro cell killing ADCC assay was conducted as described previously.^{16,40,46,47} In brief, cells of an ovarian carcinoma cell line (IGROV1) expressing the tumor-associated antigen FRα were labeled with the cell tracker dye CFSE 24 h prior to experiments. Human and cynomolgus monkey PBMC were isolated as described earlier, resuspended in RPMI 1640 CM, and mixed with the FRα+ cells at a effector cell:tumor cell ratio of 5:1, with a final number of 5.5x10⁵ cells per tube. Mixed cell suspensions were incubated with the anti-FRα IgE (MOv18 IgE) at different concentrations (4 nM-15 nM). Cells incubated with no antibody and cells given 15 nM of the chimeric hapten-specific NIP IgE antibody, which did not bind to FRα+ cells, were used as background and isotype controls, respectively. FRα+ cell killing (ADCC) was allowed to take place at 37 °C, in 5% CO₂ for 3 h, after which time the supernatants were removed. The cells were washed with PBS and resuspended in PBS containing DAPI to label dead cells. The total cell cytotoxicity was measured by determining the decrease in the number of CFSE+ cells and the increase in DAPI+/CFSE+ dead cells, in both instances in comparison to the no antibody control, when analyzed on a FACSCanto II (BD Biosciences) as previously described.

Quantification of cytokine release by effector cells

The supernatants produced from the killing assay for the controls (no antibody (background reading) and isotype (NIP) antibody controls) and the assay samples treated with 6 nM and 15 nM MOv18 IgE were retained and spun at 500 × g to remove any contaminating cells. Specific human and non-human primate reactive Milliplex magnetic bead assays were used according to the manufacturer's instructions (HCYTOMAG-40K and PRCYTOMAG-40K, Merck Millipore), selecting for the following analytes: IL-1β, IL-4, IL-5, IL-10, IFNγ, TNF,

and VEGF. The samples were loaded in duplicate and diluted 1:2 in serum-free RPMI 1640 media for acquisition using a FlexMap 3D (Luminex Corporation) with integrated software for analysis.

Statistical methods

A two-tailed Student *t* test was used to compare binding kinetic evaluations, serum IgE and ADCC assays (values *P* < 0.05 were considered statistically significant). Analyses of cytokine profiles determined using Milliplex magnetic bead assays were analyzed using the FlexMap software and GraphPad Prism to quantify the amounts of each analyte present under each experimental condition; measured background readings of analytes were compared using a Student *t* test and the difference in analytes with increasing cell cytotoxicity were compared using one-way ANOVA with Bonferroni post-test. All statistical analyses were performed using GraphPad Prism software (version 5.03, GraphPad). Error bars in all ex vivo and in vitro analyses represent standard error of mean (SEM).

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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