

ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

Ligelizumab impairs IgE-binding to plasmacytoid dendritic cells more potently than omalizumab and restores IFN- α production and FOXP3⁺ Treg generation

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Abstract

Background: Ligelizumab is an anti-IgE monoclonal antibody binding IgE with higher affinity than omalizumab that is under clinical investigation for several IgE-mediated diseases. We previously showed that omalizumab removes IgE bound to Fc ϵ RI on plasmacytoid dendritic cells (pDCs) and restores their ability to produce IFN- α and regulatory T cells (Tregs). The aim of this work is to investigate the capacity of ligelizumab to regulate functional properties of pDCs in comparison with omalizumab.

Methods: pDCs were isolated from atopic donors and IgE was detached from Fc ϵ RI on pDCs with designed ankyrin repeat protein (DARPin) bi53-79. pDCs were resensitized with IgE alone or in the presence of ligelizumab or omalizumab prior to IgE-Fc ϵ RI crosslinking and Toll-like receptor 9 (TLR9) stimulation. Flow cytometry, ELISA, co-culture experiments and intranuclear staining were performed to determine cytokine production and Treg generation. An antigen-specific model of resensitization and IgE-crosslinking was also performed.

Results: The levels of serum total free IgE show a non-linear positive correlation with the frequency of IgE⁺ pDCs displaying IgE bound to Fc ϵ RI within the 43 individual donors included in the study. Ligelizumab displays stronger capacity than omalizumab to block the binding of free IgE to Fc ϵ RI on human pDCs, resulting in a greater restoration of TLR9-L-induced IFN- α production. Ligelizumab also restores the ability of pDCs to generate FOXP3⁺ Tregs as previously reported for omalizumab.

Conclusions: The uncovered novel molecular mechanisms of ligelizumab to regulate functional properties of pDCs from atopic donors might have important clinical implications for anti-IgE treatments in different IgE-mediated diseases.

Abbreviations: BSA, bovine serum albumin; Ctrl, control; Fc ϵ RI, Fc ϵ receptor I; FOXP3, forkhead box P3; IFN, interferon; mAb, monoclonal antibody; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetyl; pDC, plasmacytoid dendritic cell; TLR9, Toll-like receptor 9 ligand; Treg, regulatory T cell.

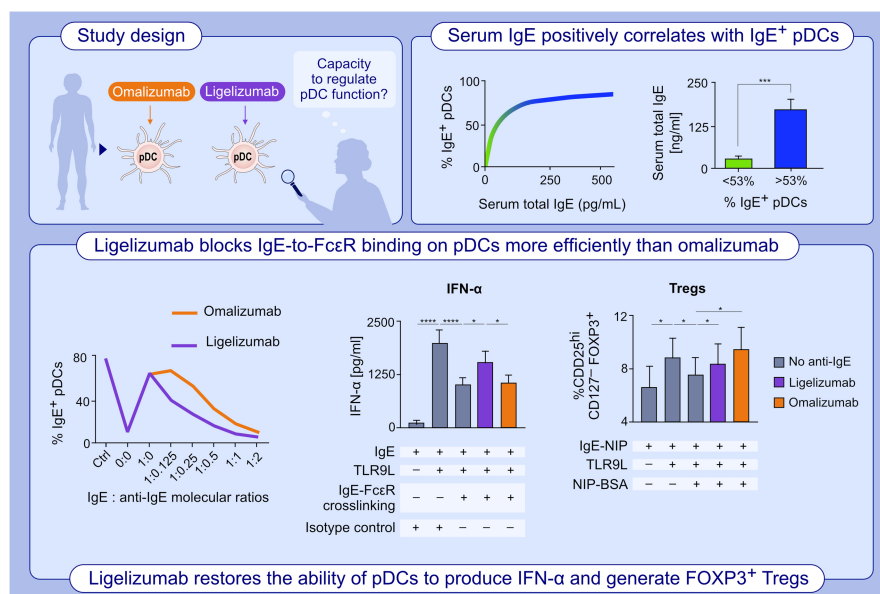
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KEYWORDS

allergy treatment, basic immunology, dendritic cells, IgE, T cells



GRAPHICAL ABSTRACT

Graphical Abstract This study investigates the capacity of ligelizumab to regulate functional properties of pDCs in comparison with omalizumab. The levels of serum total free IgE shows a non-linear positive correlation with the frequency of IgE⁺ pDCs. Donors with circulating IgE⁺ pDCs above 53% display significantly higher serum total IgE levels than those with percentages below 53%. Ligelizumab blocks the binding of free IgE to FcεR on pDCs much more efficiently than omalizumab, resulting in restore capacity of pDCs to produce high levels of IFN-α and to generate FOXP3⁺ Tregs.

Abbreviations: BSA, bovine serum albumin; Ctrl, control; FcεRI, Fcε receptor I; FOXP3, forkhead box P3; IFN, interferon; mAb, monoclonal antibody; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetyl; pDC, plasmacytoid dendritic cell; TLR9, Toll-like receptor 9 ligand; Treg, regulatory T cell

1 | INTRODUCTION

IgE plays a central role in the pathogenesis of different allergic and autoimmune diseases.^{1–4} Different biologicals targeting IgE have been developed and assayed as therapeutic strategies for such diseases.⁵ Up to date, omalizumab, an IgG1 humanized monoclonal antibody (mAb) that binds free IgE with an affinity of 6.8 nM, is the only one approved for allergic asthma,⁶ chronic spontaneous urticaria (CSU)^{7–9} and chronic rhinosinusitis with nasal polyps (CRSwNP).¹⁰ Omalizumab is a safe and effective treatment for many patients, but several limitations associated with its moderate affinity for IgE and its specific mode of action have been linked to the lack of efficacy reported for some patients and specific disease conditions.^{5,11} Alternative anti-IgE biologicals have been generated, which are being currently assessed in clinical trials at different stages. Among them, ligelizumab (QGE031), a humanized IgG1 anti-IgE mAb with higher affinity for free IgE (35 pM) than omalizumab (6.8 nM) represents a promising candidate.^{11–13} Detailed molecular and structural comparative studies showed that ligelizumab and omalizumab display different inhibition profiles for the high and low affinity IgE receptors (FcεRI and CD23, respectively), which is attributed to their different epitope recognition on IgE and to their diverse abilities to promote IgE conformational changes upon binding.^{5,11,14} It has been suggested that such differences might be connected to the varied clinical outcomes

reported for these mAbs in different IgE-mediated diseases.^{5,15} In line with its higher target affinity and potency to block IgE/FcεRI signaling in preclinical models, ligelizumab provided a greater and longer suppression of free serum IgE levels and IgE bound to circulating basophils, as well as improved skin prick wheal responses in atopic subjects.¹³ Ligelizumab demonstrated a greater efficacy than omalizumab in inhibiting allergen-induced early responses in patients with mild allergic asthma,¹⁶ but failed to demonstrate superiority over placebo or omalizumab in severe allergic asthma patients.¹⁵ Recently, ligelizumab showed significantly better symptom control than omalizumab in a phase IIb trial for CSU¹⁷, which has not been reproduced in larger phase III clinical trials (NCT03580369 and NCT03580356). This conundrum of clinical data suggests that apart from mast cells, basophils or B cells, additional FcεRI- and/or CD23-expressing cells may play important roles in asthma, CSU and other IgE-mediated diseases, which may have been underestimated so far.

Plasmacytoid dendritic cells (pDCs) are the main producers of type-I interferons (IFNs), thus playing a key role in anti-viral responses.^{18,19} pDCs promote tolerance by different mechanisms including the generation of highly suppressive regulatory T cells (Tregs)^{20–22} or the suppression of Th2-mediated responses via type-I IFNs.^{23,24} Functional alterations in pDCs are recognized as a key element in the development of several IgE-mediated allergic and autoimmune diseases.^{25–27} The number and function of pDCs

are altered in asthma patients and low pDC levels during childhood represents an asthma risk factor.^{28–32} Defective IFN- α production by pDCs has been also reported in chronic idiopathic urticaria.³³ Recent studies show that this innate immune response appears to be heterogeneous among patients.^{34,35} Interestingly, mice and human data showed that pDCs play a key role for tolerance induction in food allergy, asthma and other allergic diseases.^{36–38} Mast cells and basophils express the classic $\alpha\beta\gamma 2$ Fc ϵ RI tetrameric form, whereas DCs, macrophages and eosinophils express a trimeric $\alpha 2$ variant of Fc ϵ RI.^{39,40} pDCs express high levels of Fc ϵ RI and crosslinking of the IgE-Fc ϵ RI complexes is associated with an impaired capacity of pDCs to produce IFN- α .^{41–43} We previously showed that omalizumab restores the ability of pDCs to produce IFN- α and Tregs by blocking Fc ϵ RI signaling due to removal of receptor-bound IgE.⁴¹ Ligelizumab, unlike omalizumab, does not detach IgE bound to Fc ϵ RI on mast cells and basophils.^{5,15} Therefore, the capacity of ligelizumab to regulate the functional properties of pDCs remains unexplored.

Herein, we show that ligelizumab blocks the binding of free IgE to Fc ϵ RI on pDCs much more efficiently than omalizumab, resulting in restore capacity of pDCs to produce high levels of IFN- α and to generate FOXP3⁺ Tregs. We uncover unprecedented mechanisms on the capacity of ligelizumab to regulate functional properties on pDCs from atopic donors, which might have clinical implications for anti-IgE treatments in different IgE-mediated diseases.

2 | METHODS

2.1 | Material, media and reagents

Buffy coats were obtained from anonymous healthy volunteers from Centro de transfusiones de la Comunidad Autónoma de Madrid (Valdebernardo, Madrid) and processed according to the standard procedures approved by the Complutense University of Madrid. For cell cultures, we used RPMI 1640 (Lonza, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum, 100 μ g/mL normocin (InvivoGen, CA, USA), 50 μ g/mL penicillin–streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mM sodium pyruvate (Life Technologies, CA, USA). Moreover, IL-3 (PeproTech, NJ, USA) at 10 ng/mL was always included to ensure the survival of pDCs in vitro. During our experiments, pDCs were incubated with designed ankyrin repeat protein (DARPin) bi53-79 recombinantly produced by us, human IgE (non-immune) with azide (BioPorto, Denmark), chimeric human IgE (JW8) protein (NBS-C, Austria), ligelizumab, omalizumab (Both from Novartis, Switzerland), rabbit anti-human IgE, purified rabbit IgG, purified human IgG (from Bethyl Laboratories, TX, USA), 4-hydroxy-3-iodo-5-nitrophenylacetyl-Bovine Serum Albumin (NIP-BSA) (NBS-C, Austria) and TLR9-ligand (TLR9-L) type B CpG ODN2006 (InvivoGen, CA, USA).

Flow cytometry monoclonal antibodies were purchased from BioLegend (CA, USA) unless otherwise notice: anti-human FOXP3-Alexa Fluor 488 (Reference: 320212, working dilution: 1:67), anti-Fc ϵ RI α -fluorescein isothiocyanate (FITC) (334,608, 1:50), anti-HLA-DR-FITC (307,604, 1:50), anti-CD123-FITC (306,014,

1:10), anti-CD23-phycoerythrin (PE) (338,508, 1:20), anti-CD127-PE (351,304, 1:200), anti-CD303-PE (Miltenyi Biotec, Germany, 130–113-755, 1:400), anti-CD4-peridinin chlorophyll protein complex (PerCP) (317,432, 1:50), anti-CD304-PerCP/Cy7 (354,508, 1:25), anti-IgE-allophycocyanin (APC) (385,508, 1:20), anti-CD25-APC (302,610, 1200), and Fc ϵ R blocking reagent (Miltenyi Biotec, Germany, 130–059-901, 1:100). The corresponding isotype controls were included in each staining (IgG1-A488 (400134), IgG2A-FITC (Miltenyi Biotec, Germany, 130–091-837), IgG1-PE (400112), IgG2A-PerCP (400256), or IgG1-APC (400122)).

2.2 | pDCs isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of atopic donors by Ficoll density gradient centrifugation (800 g, 20 min). pDCs were purified (85–90% purity) from these PBMCs by using “Plasmacytoid Dendritic Cell Isolation Kit II” in autoMACs Pro (Miltenyi Biotec, Germany).

Freshly purified pDCs were incubated for 1 h with DARPin bi53-79 at the indicated concentrations. After several washes to completely eliminate DARPin bi53-79 from media, pDCs were re-sensitized with the selected exogenous IgE alone or in combination with ligelizumab or omalizumab for another hour (IgE:ligelizumab and IgE:omalizumab mixes were previously incubated with continuous stirring for 1 h). Next, pDCs were washed again and bound-IgE was crosslinked with rabbit anti-human IgE (CL). In the conditions without CL, we always add purified rabbit IgG (IC) as control. After 2 h, pDCs were stimulated with 2 μ M TLR9-L for 18 h. Then, cell-free supernatants were used to quantify IFN- α , IL-6, and TNF- α production and glucose consumption and pDCs were cocultured with allogeneic naïve CD4⁺ T cells. In all the cases, viable cells were counted using trypan blue staining and an optical microscope.

2.3 | Co-cultures between pDCs and naïve CD4⁺ T cells

Naïve CD4⁺ T cells were purified from PBMCs using “Naïve CD4⁺ T cell Isolation Kit” in autoMACs Pro according to manufacturer's protocol (Miltenyi Biotec, Germany). Stimulated pDCs were cocultured with purified allogeneic naïve CD4⁺ T cells (1:5 pDCs:T-cell ratio) in complete RPMI supplemented with 10 ng/mL IL-3. After 5 days, IFN- γ , IL-5, IL-2, and IL-10 were quantified in cell-free supernatants and cells were harvested for FOXP3⁺ Tregs analysis.

2.4 | Cytokine quantification

Concentrations of IL-6, TNF- α , IL-10, IL-2, IFN- γ and IL-5 in cell-free supernatants were quantified by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using specific ELISA cytokine kits for each one (BD Biosciences, CA, USA). IFN- α levels were quantified by using Human IFN- α pan ELISA^{BASIC} kit (HRP) (Mabtech, Sweden).

In all cases, manufacturer's instructions were followed with minor modifications.

2.5 | Glucose consumption

Glucose (Go) Assay Kit (Sigma-Aldrich, MO, USA) was used to quantify glucose concentration in cell-free supernatants following manufacturer's instructions. To obtain the metabolic rate, glucose concentrations determined in each supernatant were subtracted from those of RPMI 1640 medium alone (glucose concentration in RPMI 1640 = 2 mg/mL). The resulting concentration, the consumed glucose by pDCs, was then expressed as a percentage of the glucose concentration of the medium alone.

2.6 | Flow cytometry

Cells were washed with PBS/EDTA 2 mM/0.5% BSA and stained for 15 min at room temperature in the darkness. For detection of the presence of membrane IgE in human pDCs, cells were subjected to surface staining with anti-human FcεRIα-FITC, CD303-PE and IgE-APC antibodies. For analysis of FOXP3 expression in human T cells primed with pDCs, cells were first subjected to surface staining with anti-human CD127-PE, CD4-PerCP and CD25-APC antibodies. After fixation and permeabilization, cells were stained with anti-human FOXP3-Alexa Fluor 488 according to manufacturer's recommendation.

All flow cytometry analyses were performed with a FACSCalibur cytometer (Becton Dickinson, CA, USA) in the Cytometry and Fluorescence Microscopy unit at Complutense University of Madrid.

2.7 | Serum IgE quantification

Microtiter plates (Corning Inc, NY, USA) were coated with 100 μl of anti-human IgE (clone G7-18 from BD Pharmingen) overnight at 4°C. Plates were washed 4 times with PBS and 0.1% vol/vol Tween-20 and blocked for 2 h with PBS and 10% wt/vol fetal bovine serum. Then, plates were incubated overnight at 4°C with individual serum from patients or standards. After 4 washes, bound IgE antibodies were detected by incubating for 2 h with goat anti-human IgE-biotin (1:2000 diluted) (ThermoFisher, MA, USA), followed by 1 h with horseradish peroxidase-coupled streptavidin (diluted 1:500). The peroxidase reaction was developed by using fresh enzyme substrate (0.03% H₂O₂ and 0.63 mg/mL o-phenylenediamine in 0.1 M sodium citrate, pH 5.0), and the reaction was stopped with 3 N H₂SO₄. OD was measured at 492 nm in an ELISA reader.

2.8 | Data analysis and statistics

All data and statistical analyses were performed using GraphPad Prism (GraphPad Software, CA, USA). Data represent the

mean ± SEM of the indicated parameters. Statistical differences were determined with the paired or unpaired Student t test when data follow normal distribution and Wilcoxon matched-pairs test when they not. Correlation analysis was performed by Spearman's correlation test. P values are denoted through the text as follows: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001; *p* < 0.05 or lower values were considered significantly different.

3 | RESULTS

3.1 | Serum total IgE levels positively correlate with the frequency of circulating pDCs displaying FcεRI-bound IgE

To assess potential correlations between circulating free IgE and IgE⁺ pDCs, we compared the levels of serum total IgE as determined by ELISA with the percentage of circulating pDCs displaying surface-bound IgE as determined by flow cytometry within the 43 individual donors included in the study. The levels of serum total free IgE showed a non-linear positive correlation with the frequency of IgE⁺ pDCs (Figure 1A, *n* = 43). As shown in this figure, the percentage of IgE⁺ pDCs increased at a constant rate with the levels of serum total IgE, reaching a plateau (at around 80% of IgE⁺ pDCs) from 100 pg/mL of total IgE onwards. The mean value of the percentage of IgE⁺ pDCs when considering all the assayed donors was 53% ± 5 (mean ± SEM). When stratifying donors according to this cut-off value, those with percentages of circulating IgE⁺ pDCs above 53% displayed significantly higher serum total IgE levels than those with percentages below 53% (170 ± 28 vs 26 ± 6 ng/mL; mean ± SEM, *n* = 24) (Figure 1B). Representative flow cytometry dot plots are displayed (Figure 1C). For further experiments, we established these values as the cut-off to classify donors as non-atopic vs atopic and to include atopic donors displaying circulating IgE⁺ pDCs above 53%. To further characterize IgE⁺ pDCs from atopic donors, we isolated them from PBMCs as previously described.⁴¹ The purity of isolated pDCs ranged between 85–95% (Figure 1D). Purified human pDCs expressed high levels of the high affinity IgE receptor subunit FcεRIα, which was occupied by IgE, with negligible expression levels of the low affinity receptor CD23 detected (Figures 1E, F). Collectively, these data show that the levels of serum total IgE positivity correlate with the percentage of circulating pDCs carrying IgE bound to FcεRI.

3.2 | Ligelizumab does not detach IgE bound to FcεRI on pDCs from atopic donors

We previously showed that omalizumab is able to detach IgE already bound to FcεRI on human pDCs.⁴¹ To determine the capacity of ligelizumab, in comparison with omalizumab, to remove the IgE bound to pDCs, purified pDCs from an atopic donor were treated for 18 h with increasing doses of ligelizumab or omalizumab (0.25, 1.25, and

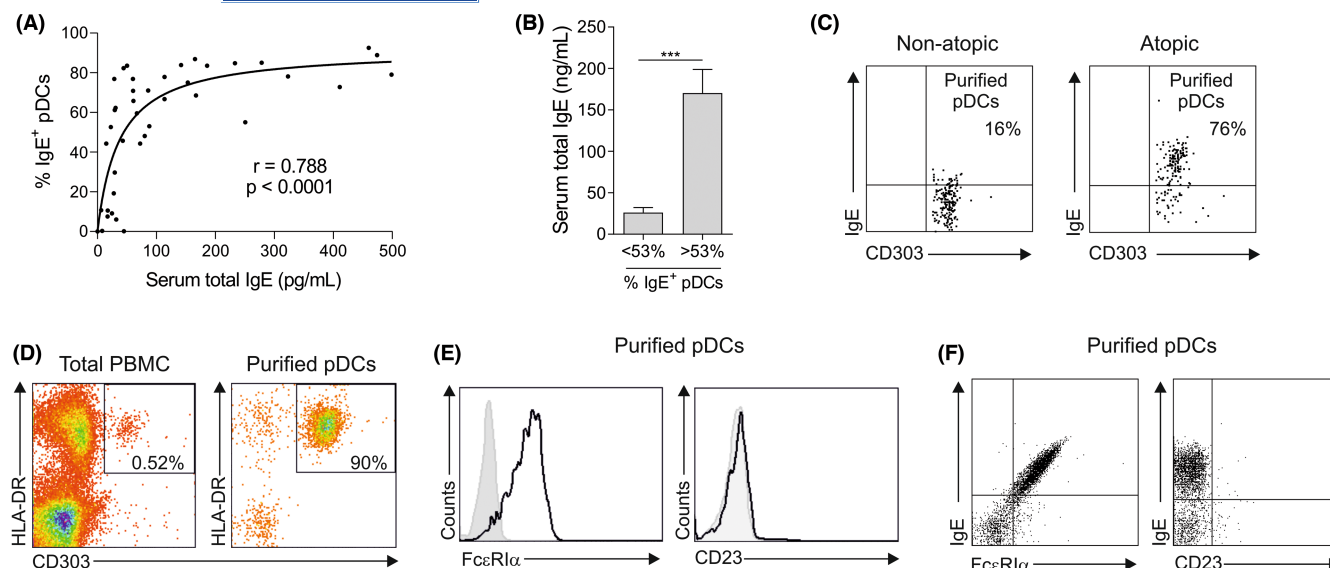


FIGURE 1 Frequency of pDCs expressing IgE correlates with serum total IgE levels in blood donors. A, Non-linear correlation between the frequency of pDCs expressing FcεRI-bound IgE (% IgE⁺ pDCs) and serum total IgE levels in blood donors ($n = 43$). "r" Spearman correlation coefficient. B, Serum total IgE levels detected in <53% IgE⁺ pDCs and >53% IgE⁺ pDCs donors ($n = 24$). Unpaired Student *t* test, *** $p < 0.001$. C, Dot plots showing representative frequency of IgE⁺ pDCs displayed by <53% IgE⁺ pDCs (non-atopic) and >53% IgE⁺ pDCs (atopic) donors. D, Representative dot plots showing the purity of pDCs before and after their isolation from PBMCs from an atopic donor. Purified pDCs were HLA-DR⁺CD303⁺CD304⁺CD123⁺. E, Representative histograms of FcεRIα and CD23 expression in freshly purified pDCs from an atopic donor. The isotype control is displayed in grey. F, Representative dot plots showing IgE⁺FcεRIα⁺ and IgE⁺CD23⁺ pDCs displayed by an atopic donor.

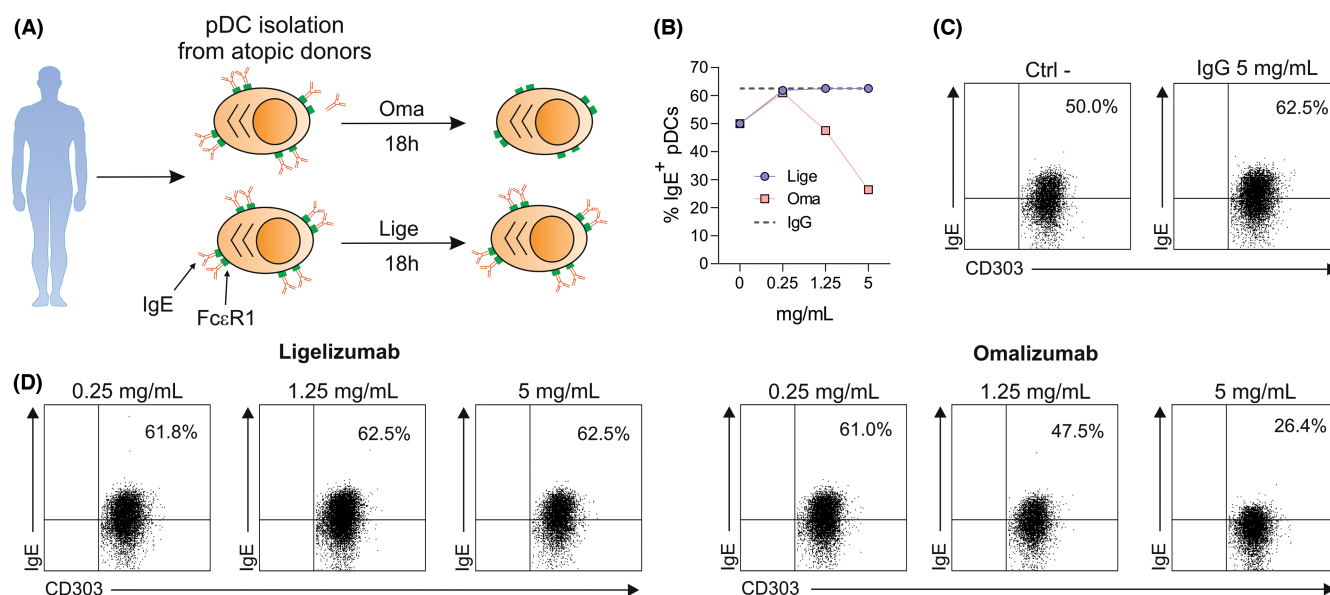


FIGURE 2 Ligelizumab is unable to remove IgE from the surface of pDCs from atopic donors. A, Experimental outline. pDCs isolated from an atopic donor were incubated with ligelizumab or omalizumab for 18h. Human IgG was used as a negative control. B, Graph showing the capacity of ligelizumab and omalizumab to detach IgE from isolated pDCs. C and D, Representative dot plots showing the effect of human IgG (C) and ligelizumab or omalizumab (D) on pDCs expressing IgE levels.

5 mg/mL), and the levels of IgE on pDCs analyzed by flow cytometry (Figure 2A). The treatment of IgE⁺ pDCs with an unrelated IgG control at the highest dose tested (5 mg/mL) did not reduce the levels of IgE on the surface of the cells (Figure 2B). Representative dot plots for freshly purified untreated IgE⁺ pDCs and cells treated with the

highest dose of the unrelated IgG control are displayed (Figure 2C). As expected, omalizumab efficiently removed IgE from already formed IgE:FcεRI complexes on pDCs in a dose-dependent manner (Figure 2B). In contrast, incubation with ligelizumab did not detach IgE from IgE:FcεRI preformed complexes on pDCs at any of the

doses tested (Figure 2B). Representative dot plots for ligelizumab and omalizumab treatments at all the assayed doses are shown in Figure 2D. Collectively, these data demonstrate that ligelizumab, unlike omalizumab, is not able to remove IgE already bound to FcεRI on pDCs, which is in accordance with previously reported data for mast cells and basophils.^{5,15}

3.3 | Ligelizumab blocks the binding of free IgE to FcεRI on pDCs and restores IFN-α production more efficiently than omalizumab

To assess the capacity of ligelizumab, in comparison with omalizumab, to block the binding of free IgE to FcεRI on pDCs from atopic donors and the potential functional consequences, we first developed an in vitro model in which IgE was initially removed from IgE:FcεRI complexes on purified pDCs and, then reloaded with exogenous IgE. We used the bispecific designed ankyrin repeat protein (DARPin) bi53-79, an alternative binding scaffold protein that recognizes FcεRI-bound IgE on the surface of the allergic effector cells and is able to disrupt IgE-FcεRI complexes through a facilitated dissociation mechanism.^{44–46} Dose- and time-response experiments showed that when freshly isolated pDCs from an atopic donor displaying IgE bound to FcεRI (Figure S1A) were incubated for 1 h with 1 μM of DARPin bi53-79, the IgE-FcεRI complexes were almost completely dissociated (Figure S1B, upper line, right side, left dot plot). The stripped pDCs were able to bind exogenous IgE to a similar percentage to that observed before DARPin treatment (Figure S1B, upper line, right side, right dot plot).

IgE-stripped pDCs were incubated for 1 h with exogenous IgE alone or in combination with ligelizumab or omalizumab at different molar ratios and the percentage of IgE⁺ pDCs quantified by flow cytometry (Figure 3A). At all the tested ratios of IgE:Anti-IgE, ligelizumab displayed a significantly higher capacity to block the binding of free IgE to FcεRI on pDCs than omalizumab (Figure 3B, $n = 40$). For the next experiments, we selected 1:0.25 and 1:0.5 ratios, which displayed the highest differences between ligelizumab and omalizumab to block IgE binding to FcεRI on pDCs while maintaining high expression levels of FcεRIα (Figure 3C, $n = 36$). Representative flow cytometry dot plots of the IgE stripping, and the addition of exogenous IgE alone or mixed with ligelizumab or omalizumab (at 1:0.5 IgE:Anti-IgE ratio) are shown (Figure 3D).

To determine how the blocking of IgE binding to FcεRI on pDCs affects their functional properties, after the IgE-sensitization step under the different assayed conditions, IgE-FcεRI complexes were crosslinked with an anti-human IgE antibody (CL) and stimulated with TLR9-L (Figure 4A). TLR9-L-stimulated pDCs produced high levels of IFN-α, IL-6, and TNF-α (Figure 4B, $n = 22–34$) without production of IL-10 detected (data not shown). IgE-FcεRI crosslinking on pDCs significantly impaired TLR9-L-induced IFN-α production and increased IL-6 production without affecting TNF-α (Figure 4B). Remarkably, ligelizumab but not omalizumab restored IFN-α

production on TLR9-L-stimulated pDCs (Figure 4B). Both anti-IgE antibodies avoided the increase in IL-6 and reduced TNF-α production upon IgE-FcεRI crosslinking on TLR9-stimulated pDCs (Figure 4B). The metabolic rate (glucose consumption) significantly increased in pDCs stimulated with TLR9-L, being even higher upon IgE-FcεRI crosslinking (Figure 4C, $n = 7$). Ligelizumab and omalizumab treatment, significantly reduced the metabolic rate in pDCs, suggesting that both anti-IgE mAbs specifically impairs IgE-FcεRI crosslinking on pDCs (Figure 4C).

3.4 | Ligelizumab restores the capacity of pDCs to generate Tregs in a similar manner than omalizumab

Next, we studied whether ligelizumab ability to block IgE binding on pDCs from atopic donors translated into pDCs-driven restoration of Treg generation. We cocultured pDCs stimulated under the different assay conditions with allogeneic naïve CD4⁺ T cells for 5 days (Figure 5A). TLR9-L-activated pDCs induced significantly higher numbers of CD4⁺CD25^{high}CD127⁺FOXP3⁺ Tregs than unstimulated pDCs. The IgE-FcεRI crosslinking significantly impaired the capacity of TLR9-L-activated pDCs to induce the generation of Tregs (Figure 5B, $n = 17$). As shown in this figure, ligelizumab restored the capacity of pDCs to induce the generation of Tregs in a similar manner than omalizumab. Supporting these data, TLR9-L-stimulated pDCs generated T cells produced significantly higher levels of IL-10, IL-2 and IFN-γ than unstimulated pDCs, which was significantly reduced upon IgE-FcεRI crosslinking without significant changes observed for IL-5 in any case (Figure 5C, $n = 18–29$). Ligelizumab and omalizumab restored the production of IL-10, IL-2, and IFN-γ in the T cells generated by TLR9-L-stimulated pDCs in the presence of IgE crosslinker (Figure 5C). The IFN-γ/IL-5 ratio was significantly lower when T cells were primed by TLR9-L-activated pDCs after IgE-FcεRI crosslinking than with TLR9-L-stimulation alone, which was also significantly increased by ligelizumab and omalizumab (Figure 5D, $n = 16$).

Finally, we wanted to verify in an antigen-specific setting the ability of ligelizumab to restore the capacity of pDCs to induce Treg, as this response was ultimately the main outcome of our study. Given the singularity of the binding of an IgE with its specific antigen, an antigen-specific approach allows to model in a more physiological way the IgE-FcεRI crosslinking upon allergen encounter and how it affects the capacity of pDCs to induce Treg in allergic patients. For this purpose, coculture experiments were repeated using 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP)-specific IgE to resensitize pDCs followed by IgE/FcεRI crosslinking with NIP-BSA (Figure 6A). As shown in Figure 6, the crosslinking of chimeric NIP-specific IgE with NIP-BSA significantly decreased the capacity of TLR9-L-activated pDCs to induce the generation of CD4⁺CD25^{high}CD127⁺FOXP3⁺ Tregs. Interestingly, ligelizumab and omalizumab also restored the generation of FOXP3⁺ Tregs (Figure 6B, $n = 7$), thus verifying our results in an antigen-specific model.

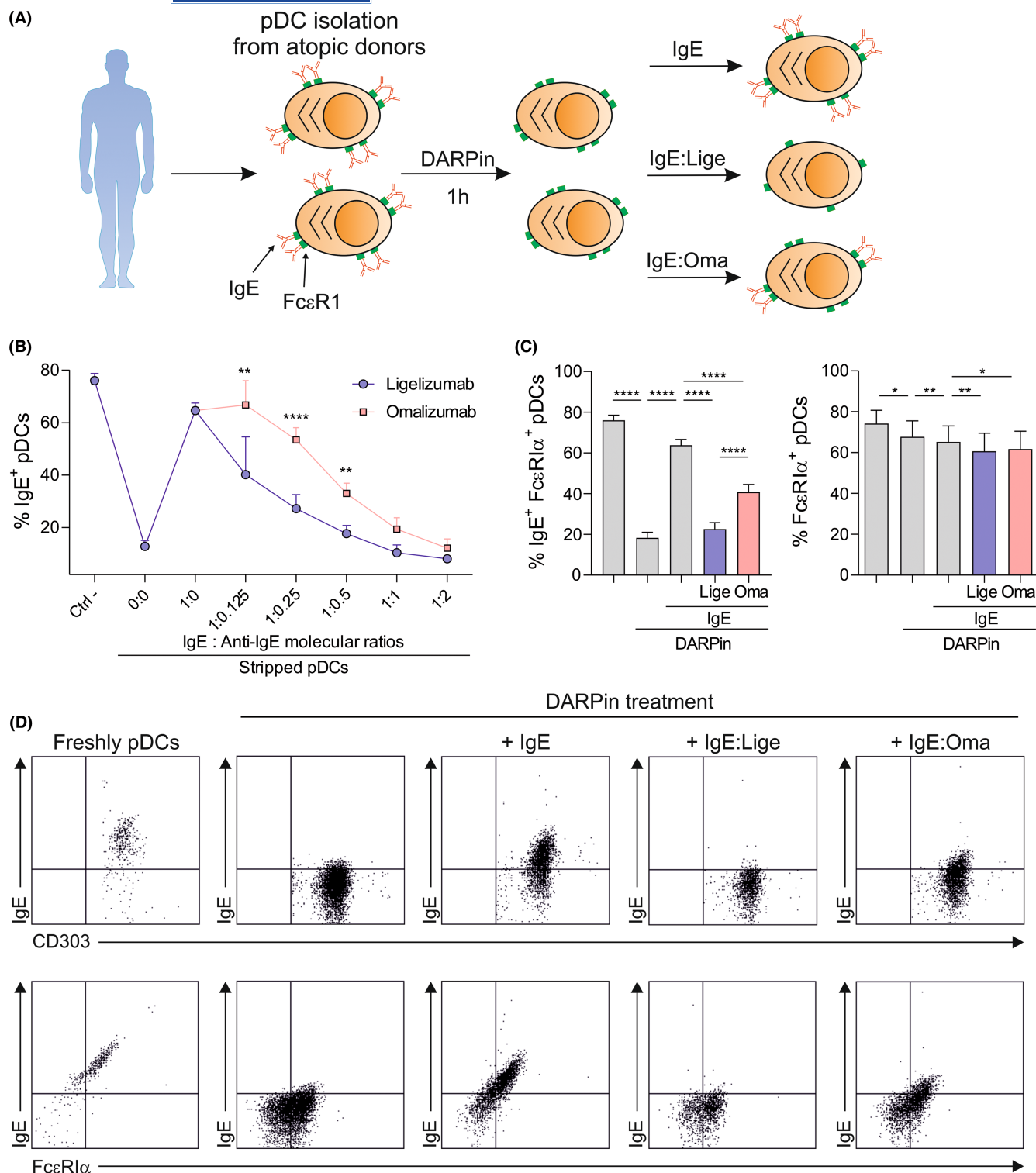


FIGURE 3 Capacity of ligelizumab to prevent IgE-binding to pDCs. A, Experimental outline. pDCs isolated from atopic donors were treated with DARPin bi53-79 1 μ M for 1 h. After several washes to remove DARPin bi53-79, pDCs were incubated for 1 h with IgE 5.26 nM alone or in combination with ligelizumab or omalizumab at the indicated IgE:Anti-IgE molecular ratios. B, Percentage of IgE-containing pDCs after incubation ($n = 40$). C, Percentage of IgE⁺FcεRIα⁺ and FcεRIα⁺ pDCs after incubation. Graphs represent pooled data from experiments performed at IgE:Anti-IgE ratios of 1:0.25 and 1:0.5 ($n = 36$). Paired Student *t* test or Wilcoxon test, * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$. D, Representative dot plots showing the capacity of ligelizumab to impair IgE-binding to pDCs at IgE:Anti-IgE ratio of 1:0.5. Upper line, percentage of IgE-containing pDCs. Lower line, percentage of IgE-FcεRIα-expressing pDCs. DARPin, DARPin bi53-79; Lige, ligelizumab; Oma, omalizumab.

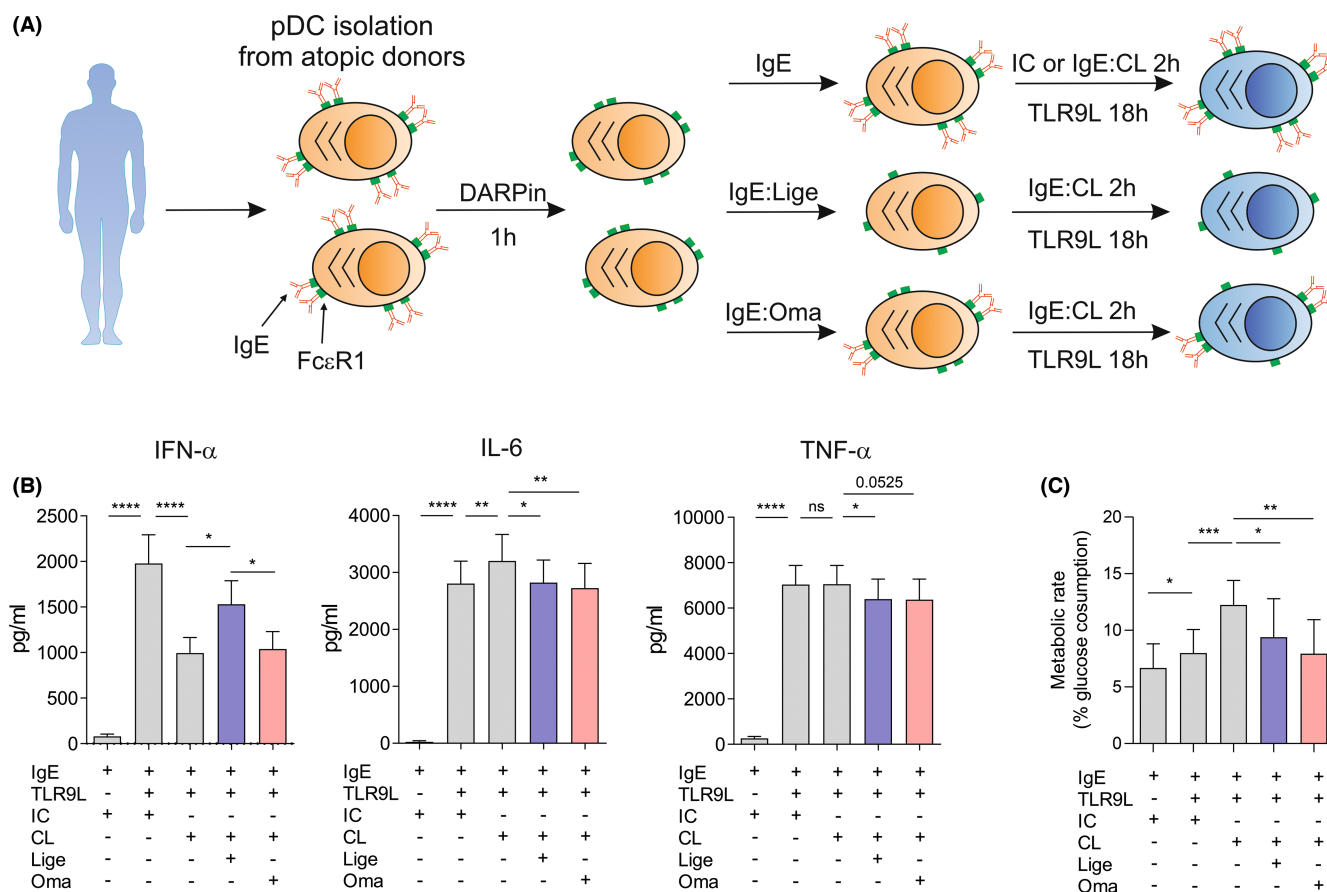


FIGURE 4 Ligelizumab prevents IgE-FcεRI crosslinker from altering the cytokine production and metabolic rate displayed by pDCs. A, Experimental outline. pDCs isolated from atopic donors were treated with DARPin bi53-79 1 μM for 1 h. Once DARPin bi53-79 was removed, pDCs were resensitized with IgE 5.26 nM alone or mixed with ligelizumab or omalizumab for 1 h at IgE:Anti-IgE ratios of 1:0.25 and 1:0.5. After several washes, pDCs were incubated for 2 h with IgE-crosslinker or its isotype control at 10 μg/mL. Then, pDCs were stimulated for 18 h with TLR9-L 2 μM before analysis. B, Cytokine production ($n = 22-34$). Paired Student *t* test or Wilcoxon test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$. C, Metabolic rate ($n = 7$). Paired Student *t* test or Wilcoxon test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. IC, isotype control; CL, IgE-crosslinker; Lige, ligelizumab; Oma, omalizumab.

4 | DISCUSSION

In the present work, we show for the first time that the next-generation high affinity anti-IgE mAb ligelizumab displays stronger capacity than omalizumab to block binding of free IgE to FcεRI on pDCs, resulting in a greater ability than omalizumab to restore TLR9-L-induced IFN-α production on pDCs. In addition, ligelizumab is able to restore the capacity of pDCs to generate FOXP3⁺ Tregs, as previously reported for omalizumab. Overall, we provide novel insights into the molecular mechanisms by which ligelizumab could restore the functional properties of pDCs from atopic donors.

Herein, we show that purified pDCs from atopic donors express high levels of FcεRI (occupied by IgE) but not CD23, thus representing a suitable model to study the functional effects of anti-IgE mAbs on IgE:FcεRI complexes on these cells without potential interferences due to CD23. By using purified IgE⁺ pDCs from atopic donors, we have established a new reliable in vitro model mimicking the decrease in FcεRI-bound IgE previously reported in pDCs from omalizumab-treated patients,⁴⁷ which allows the analysis and

comparison of the functional effects of ligelizumab and omalizumab on pDCs. Our data showed that ligelizumab prevents IgE binding to FcεRI on pDCs much more efficiently than omalizumab, thus reducing IgE levels to those shown by non-sensitized pDCs. Among the different ratios of IgE:anti-IgE mAbs tested, the ratios 1:0.25 and 1:0.5 showed the highest significant differences between ligelizumab and omalizumab in terms of IgE sensitization. This superior capacity is consistent with the higher affinity against IgE displayed by ligelizumab (35 pM vs 6.8 nM), which allows the neutralization of free-IgE more efficiently than omalizumab.^{12,13} In addition, the epitope of ligelizumab on IgE is different from the one of omalizumab and overlaps strongly with the FcεRI binding site, thereby explaining the superior capacity to inhibit the IgE:FcεRI interaction on basophils and mast cells. Moreover, the binding of ligelizumab to IgE traps Cε3 domains into a conformation incompatible with FcεRI binding.^{5,11,14} Our data revealed, for the first time, that this is also the case for the trimeric α2 variant of FcεRI expressed on pDCs. Our data demonstrated that, in contrast to omalizumab, ligelizumab did not reduce the percentage of IgE⁺ pDCs, in accordance with previous data for

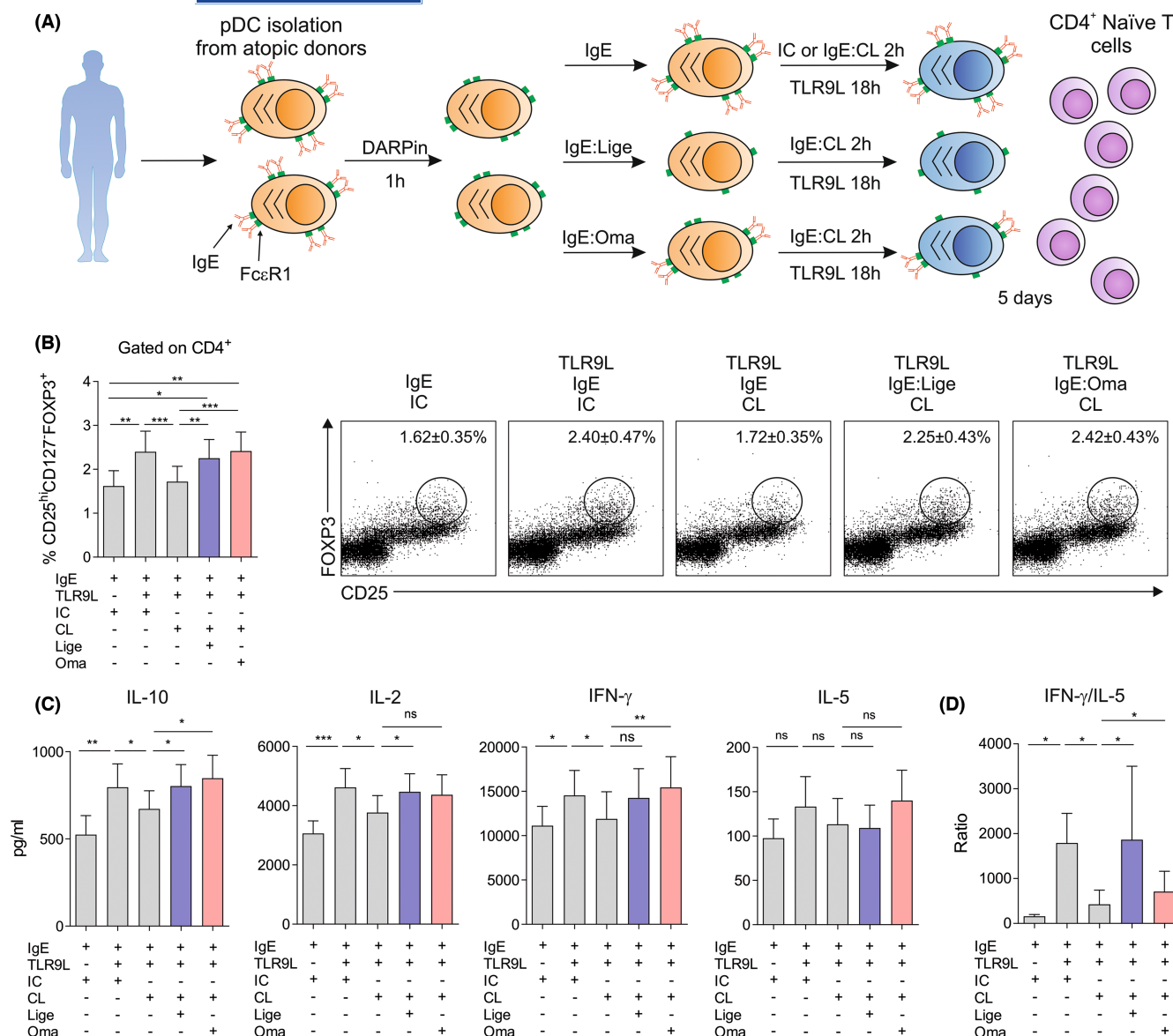


FIGURE 5 Ligelizumab prevents IgE-Fc ϵ R1 crosslinker from impairing the ability of pDCs to induce Tregs. **A**, Experimental outline. pDCs isolated from atopic donors were treated with DARPin bi53-79 1 μ M for 1 h. Once DARPin bi53-79 was removed, pDCs were resensitized with IgE 5.26 nM alone or mixed with ligelizumab or omalizumab for 1 h at IgE:Anti-IgE ratios of 1:0.25 and 1:0.5. After several washes, pDCs were incubated for 2 h with IgE-crosslinker or its isotype control at 10 μ g/mL. Then, pDCs were stimulated for 18 h with TLR9-L 2 μ M. Finally, pDCs were cocultured for 5 d with allogeneic naïve CD4⁺ lymphocytes before analysis. **B**, Percentages of CD4⁺CD25^{high}CD127⁺FOXP3⁺ Tregs induced by allogeneic pDCs (gating in lymphocytes) ($n = 17$). Paired Student t test or Wilcoxon test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. **C** and **D**, Cytokines (**C**) or cytokine ratio (**D**) produced by allogeneic naïve CD4⁺ T cells primed by pDCs. Results are shown as means $\pm$ SEMs of 18–29 (**B**), and 16 (**C**) independent experiments. Paired Student t test or Wilcoxon test, * $p < 0.05$, and ** $p < 0.01$ and *** $p < 0.001$. IC, isotype control; CL, IgE-crosslinker; Lige, ligelizumab; Oma, omalizumab.

human basophils and mast cells.^{11,15} The inability of ligelizumab to remove IgE from Fc ϵ R1 can be explained by the strong overlap of its epitope with the Fc ϵ R1 binding site as discussed above. In contrast, the binding site of omalizumab shows only partial overlap with the Fc ϵ R1-binding residues on IgE, which allows disruption of IgE-Fc ϵ R1 complexes through a facilitated dissociation mechanism.^{45,46,48} The ability of omalizumab to prevent IgE binding to Fc ϵ R1 is enough to restore the capacity of pDCs to generate Tregs. In contrast, for IFN- α

production, a full depletion of IgE on pDCs seems to be needed, which is only achieved with ligelizumab. Our data suggest that not only IFN- α , but other additional molecular mechanisms contribute to Treg generation by pDCs.

The concentrations of the anti-IgE antibodies used in our in vitro experimental setting will not be achieved in treated patients. However, the effects shown in these assays will still be of relevance in vivo. In patients treated with ligelizumab (or omalizumab), the

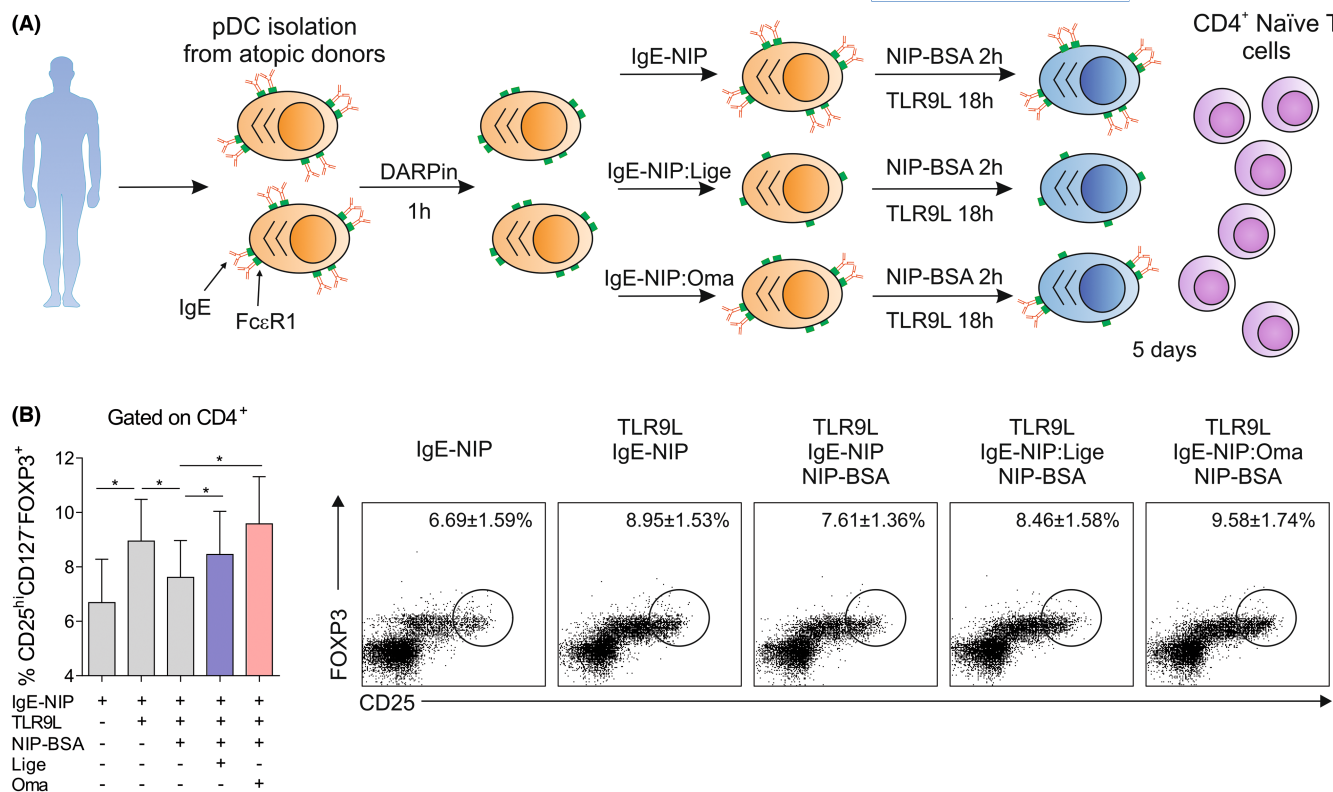


FIGURE 6 Ligelizumab restores the tolerogenic capacity of pDCs in an antigen-specific model of resensitization and IgE-crosslinking. A, Experimental outline. pDCs isolated from atopic donors were treated with DARPin bi53-79 1 μM for 1 h. Once DARPin bi53-79 was removed, pDCs were resensitized with NIP-specific IgE 10.52 nM alone or mixed with ligelizumab or omalizumab for 1 h. After several washes, pDCs were incubated for 2 h with NIP-BSA at 10 ng/mL. Then, pDCs were stimulated for 18 h with TLR9L 2 μM. Finally, pDCs were cocultured for 5 days with allogeneic naïve CD4⁺ lymphocytes before analysis. B, Percentages of CD4⁺CD25^{high}CD127⁻FOXP3⁺ Tregs induced by allogeneic pDCs (gating in lymphocytes) are shown ($n = 7$). Paired Student *t* test or Wilcoxon test, * $p < 0.05$. NIP, 4-hydroxy-3-iodo-5-nitrophenylacetyl; Lige, ligelizumab; Oma, omalizumab.

restoration of pDC functions with regards to IFN- α secretion and Treg generation may be linked to the half-life of pDCs in blood and in tissues. Upon ligelizumab treatment and therefore absence of free IgE, any new pDC generated from hematopoietic progenitors would be devoid of IgE:Fc ϵ RI complexes and therefore unable to carry out unwanted signaling via Fc ϵ RI. Atopy and viral respiratory tract infections promote asthma exacerbation,⁴⁹ and the inhibition of the capacity of pDCs to mount IFN- α responses can be deleterious for allergic asthma patients. Previous clinical studies have demonstrated that omalizumab significantly reduce the annual rate of viral-induced exacerbations in inner-city severe asthma children by mechanisms partially depending on the resaturation of pDC's ability to mount IFN- α responses upon depletion of free IgE and IgE-Fc ϵ RI-mediated signaling pathways.^{41,50-53} Therefore, it is tempting to speculate that the stronger capacity of ligelizumab to restore the ability of pDCs to produce IFN- α might well also play a major role in mediating early anti-viral responses. In this regard, ligelizumab did not show clinical efficacy in a phase II study in severe allergic asthma patients, which could be attributed to the short duration of the trial (16 weeks) to address exacerbations as an outcome.¹⁵ Based on our data, we hypothesize that 16 weeks treatment was not long enough to fully replace IgE⁺ pDCs from lung tissues with new cells lacking IgE on their

surface due to their extended tissue half-life. This new pDC population would be able to properly mount an inhibitory IFN- α response against a viral exacerbation in these patients.

Nowadays, there are no ideal biomarkers for allergy diagnosis and treatment.⁵⁴ It has recently been suggested the use of the ratio between serum specific IgE and total IgE values,⁵⁵ which does not consider the importance of the IgE bound to functional receptors on functional cells. Our data suggest that the quantification of peripheral IgE⁺ pDCs could be a useful potential biomarker to monitor ligelizumab or omalizumab treatment efficacy by indirectly tracking circulating free IgE clearance in treated patients. Remarkably, ligelizumab also restores the capacity of pDCs to generate FOXP3⁺ Tregs, as previously reported for omalizumab, which might have important clinical implications for different allergic diseases.⁴¹ Tregs numbers inversely correlate with the severity of type 2 inflammation,⁵⁶ and it is widely accepted that the induction and maintenance of Tregs is essential for healthy immune responses to allergens.^{27,57} In this regard, whether monitoring the restoration of Tregs could be correlated with anti-IgE treatment efficacy needs to be further investigated in future clinical trials. Ligelizumab has shown efficacy improving sleep interference and disease burden in CSU,⁵⁸ with robust and sustained clinical responses and safety profile.⁵⁹ Although ligelizumab initial

phase II trials in CSU demonstrated greater efficacy than omalizumab,¹⁷ this has not been reproduced in larger phase III clinical trials (NCT03580369 and NCT03580356; Novartis Pharmaceuticals). The potential contribution of pDCs and Tregs to the mode of action of ligelizumab in CSU as well as in other diseases currently under investigation such as food allergy (NCT04984876) needs to be investigated. The importance of IgE neutralization in food allergy and the crucial role of pDCs in the induction of oral tolerance via Tregs generation may hint towards some disease modifying potential of ligelizumab in this indication.^{60,61} Therapeutic approaches inhibiting IgE are currently explored in multiples indications. Recently, UB-221, another anti-IgE mAb that recognizes CD23-bound IgE more potently than ligelizumab has also shown promising results in a phase I CSU clinical trial.⁶²

In summary, our findings enhance our knowledge about the mode of action of ligelizumab and the potential implications in restoring proper anti-viral responses. We uncover novel molecular mechanisms by which this anti-IgE mAb might contribute to restore tolerance to allergens and autoantigens and therefore improve the control of symptoms of different IgE-mediated diseases. Our findings also suggest pDCs and Tregs as potential novel surrogate markers to monitor and predict such effects. Validation of our results in patients treated with ligelizumab remains a priority. The better understanding of the molecular mechanisms underlying the mode of action of different anti-IgE mAbs on the functional properties of specific cell subsets involved in IgE-mediated diseases might help to pave the way for a better interpretation of clinical outcomes and to the designing of future clinical trials in different IgE-mediated diseases.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: O.P. Performed the experiments: C. B.-V., A. R.-M. and J. L.-A. Provided reagents: A.E., I.B., T.S., M.W. and O.P. Analyzed and discussed the data: C. B.-V., A. R.-M., J. L.-A., A.E., I.B., T.S., M.W. and O.P. Wrote the paper: O.P., C. B.-V. and A.R.-M. All the authors read, provided comments and approved the final version of the manuscript.

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CONFLICT OF INTERESTS

O.P. has received payment for lectures and participation in Advisory Boards from Allergy Therapeutics, Amgen, AstraZeneca, GSK, Pfizer,

Inmunotek SL, Novartis, Sanofi-Genezyme and Stallergenes. OP has received research grants from Novartis SL, Immunotek SL, MINECO, MICINNIN and CAM. A.E. is a co-founder of ATANIS Biotech and Excellergy, received grant support from Novartis and did consulting work for Pfizer, GSK, Novartis, ATANIS Biotech and Excellergy. I.B., T.S. and M.W. are Novartis employees. The rest of authors declare no competing financial interests.

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SUPPORTING INFORMATION

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