Omalizumab restores the ability of human plasmacytoid dendritic cells to induce Foxp3^{+}Tregs

To the Editor:

Allergic sensitisation and viral respiratory tract infections represent the main risk factors for asthma development and severity. IgE plays a key role in the pathophysiology of allergic asthma and allergic multimorbidities [1, 2]. Omalizumab, a recombinant humanised monoclonal antibody against IgE, has been used to treat allergic asthma in children and adults for many years [3–5]. Omalizumab restores the capacity of human plasmacytoid dendritic cells (pDCs) to produce interferon (IFN)-α, increasing their antiviral activity and reducing viral-induced asthma exacerbations [6, 7]. pDCs prime T-helper cell (Th)1 or Th2 responses depending on the encountered antigens. In both cases, they are able to generate functional regulatory T-cells (Tregs), suggesting that pDCs display intrinsic tolerogenicity [8, 9]. pDCs and Tregs numbers are increased and decreased, respectively, during asthma exacerbations and correlate with the severity of type 2 inflammation [10, 11]. Omalizumab treatment increases the frequency of Tregs in asthmatic children, which correlates with asthma control [12]. The aim of this work is to study the molecular mechanisms by which IgE-mediated signalling in human pDCs from atopic donors could impair their capacity to generate Tregs and how Omalizumab could restore this ability.

We purified pDCs to homogeneity (purity higher than 90% in all the cases without basophil contamination) from peripheral blood of adult atopic donors by magnetic cell sorting (pDC isolation kit II, Miltenyi Biotec) in autoMACS Pro (Miltenyi Biotec). Purified pDCs expressed the high-affinity (FcεR1) but not the low-affinity (CD23) IgE receptor (data not shown). *In vitro* treatment of purified pDCs with Omalizumab (Xolair, Novartis-Pharma-AG) but not with an unrelated human IgG (Privigen, CSL-Behring) significantly reduced the levels of IgE bound to FcεR1 in a specific, dose–dependent manner (figure 1a), without affecting cell viability. This allowed us to mimic *in vitro* the decrease in FcεR1-bound IgE on pDCs demonstrated in Omalizumab-treated patients [13]. Next, we assessed whether IgE-FcεR1 cross-linking (IgE-CL) with an anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX, USA) on human pDCs could influence their ability to prime allogeneic naïve CD4^{+} T-cells (purified from adult blood samples using the Naïve CD4^{+} T-cell isolation kit, Miltenyi Biotec) into Foxp3^{+} Tregs and how Omalizumab could impact this capacity. Purified pDCs stimulated with the Toll-like receptor 9-ligand type B CpG ODN2006 (TLR9-L, Invitrogen) induced higher numbers of CD4^{+}CD127^{low}CD25^{+}Foxp3^{+} Tregs than unstimulated pDCs, which was impaired by IgE-CL in TLR9-L-activated pDCs (figure 1b). Pretreatment of pDCs with Omalizumab restored the capacity of TLR9-L-activated pDCs under IgE-CL to generate CD4^{+}CD127^{low}CD25^{+}Foxp3^{+} Tregs (figure 1b). Cell viability was not affected in any of the assayed pDCs or coculture conditions (data not shown). Supporting these data, the T-cells generated by TLR9-L-activated pDCs under IgE-CL conditions produced lower IL-10 and IL-2 levels than those T-cells generated by TLR9-L-activated pDCs without IgE-CL. The levels of IL-10 and IL-2 produced by the generated T-cells were restored by pretreating pDCs with Omalizumab (figure 1c). Remarkably, the IFN-γ/IL-5 ratio associated with T-cell secretion was lower when pDCs were activated with TLR9-L in the presence of IgE-CL, which was reversed by Omalizumab (figure 1c). Our data show that IgE-CL in TLR9-L-activated pDCs not only impairs the generation of Tregs but also favours Th2 allergic profiles, which is restored by pretreatment of pDCs with Omalizumab. The high dose

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IgE-FcεR1 cross-linking on human pDCs impairs their capacity to generate regulatory T-cells and *in vitro* omalizumab restores this ability. These findings may pave the way for novel biomarkers to assess omalizumab clinical efficacy and responder patients. https://bit.ly/2ZQbJ9t

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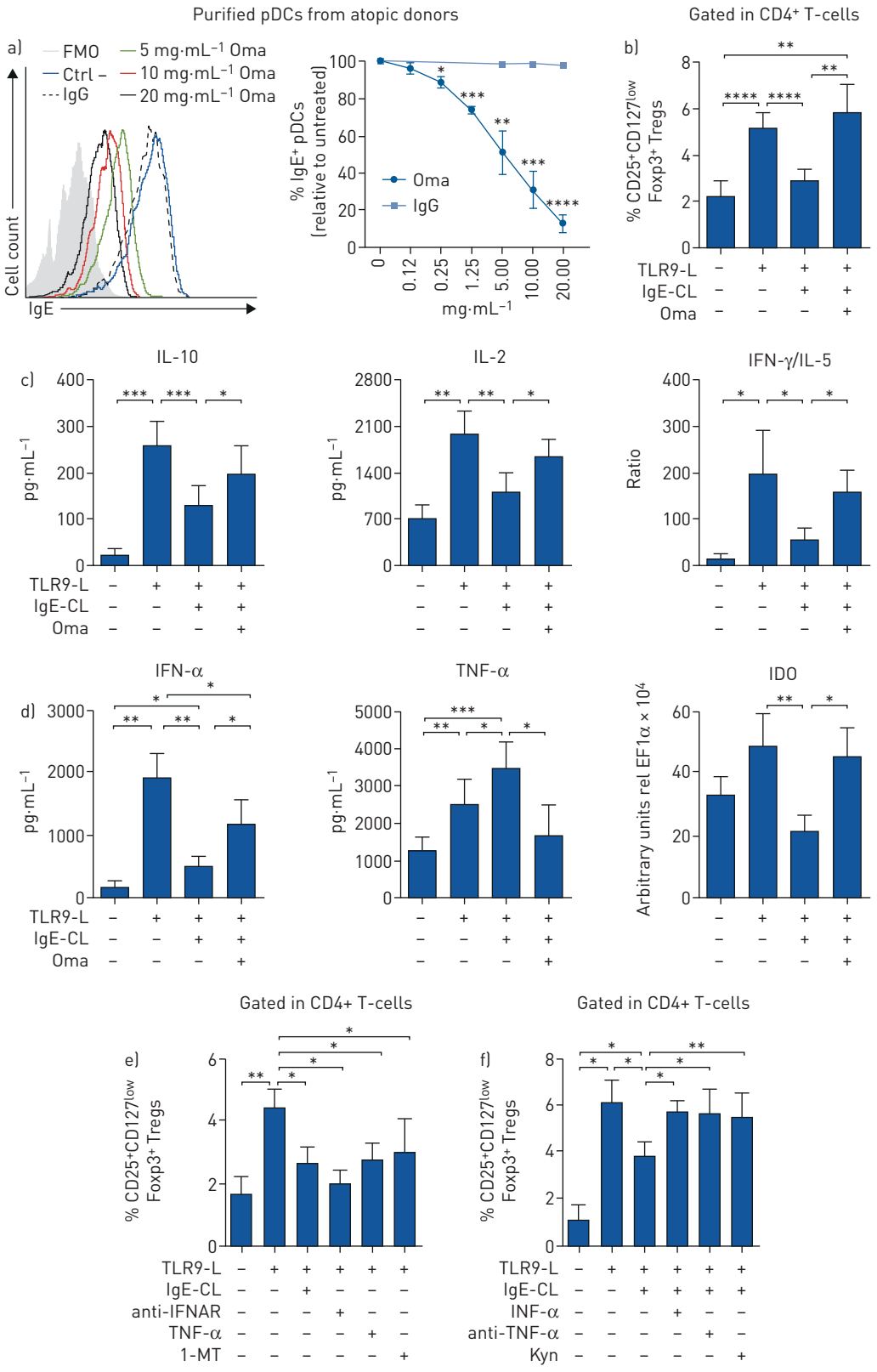

FIGURE 1 Omalizumab removes *in vitro* membrane-bound IgE from purified human plasmacytoid dendritic cells (pDCs) and restores their capacity to induce regulatory T-cells (Tregs). Purified blood pDCs from atopic donors were cultured with different concentrations of Omalizumab (Oma) or intravenous IgG for 18 h. a) Representative histogram and graph showing the levels of IgE bound to pDCs and the frequency of IgE$^+$ pDCs, respectively (n=10). FMO: fluorescence minus one. Omalizumab-treated or untreated purified pDCs

were stimulated with 10 μg·mL^{-1} rabbit anti-human IgE (IgE-FcεR1-crosslinker, IgE-CL) or isotype control in the presence of 2 μM CpG class B TLR9-ligand ODN 2006 (TLR9-L). After 18 h, the pDCs were washed and cocultured with purified allogeneic naïve CD4$^+$ T-cells (1:5 pDC:T-cell ratio, as previously described [9]) for 5 days. b) Graph showing the percentage of induced CD25$^+$CD127^{low}Foxp3$^+$ Tregs gated over CD4$^+$ T-cells under the different assayed conditions (n=20) and c) concentration of interleukin (IL)-10 and IL-2 and the interferon (IFN)-γ/IL-5 cytokine ratio in the coculture supernatants (n=12). d) After TLR9-L stimulation, pDCs were washed and the concentration of IFN-α (n=6) and tumour necrosis factor (TNF)-α (n=9) in culture supernatants and mRNA expression of indoleamine-2,3 dioxygenase (IDO) under the different assayed conditions (n=9) were measured. Data are presented as arbitrary units relative to elongation factor 1α (EF1α). Then, the pDCs were cocultured (1:5 pDC:T-cell ratio) in the presence of e) 5 μg·mL^{-1} anti-IFN-α/β receptor blocking antibody (anti-IFNAR), 10 ng·mL^{-1} TNF-α and 250 μM 1-Methyl-D-Tryptophan (1-MT) (n=14), or f) 5 ng·mL^{-1} IFN-α, 5 μg·mL^{-1} anti-TNF-α blocking antibody or 0.5 μM L-kynurenine (Kyn) (n=5) for 5 days and the induced Tregs analysed. Values are given as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 in a mixed-effects model with pairwise two-stage Benjamini–Yekutieli's *post-hoc* comparisons.

of Omalizumab needed to detach FcεR1-bound IgE in our study is probably due to the extremely high local concentration of FcεR1-IgE complexes at the single cell level. Omalizumab at the range of clinical concentrations did not detach FcεR1-bound IgE. In real life clinical treatment, Omalizumab traps free IgE, thus gradually reducing FcεR1-bound IgE and FcεR1 expression on pDCs [13]. The Omalizumab concentrations used in our *in vitro* experimental setting will not be achieved in treated patients. Herein, our goal is just to mimic *in vitro* the Omalizumab clinical effect to support the relevance of our main novel fundamental finding: IgE-CL on pDCs breaks Treg induction, which could be restored by Omalizumab.

To gain insights into the molecular mechanisms underlying these effects, we analysed changes in expression levels of molecules involved in T-cell polarisation and tolerogenicity on pDCs. TLR9-L-activated pDCs from atopic donors produced higher levels of IFN-α and tumour necrosis factor (TNF)-α than unstimulated pDCs (figure 1d). Similarly, the mRNA levels of indoleamine-2,3 dioxygenase (IDO), an enzyme involved in tryptophan catabolism, were also increased in TLR9-L-activated pDCs. IgE-CL in TLR9-L-activated pDCs impaired the production of IFN-α and IDO expression, whereas it increased the production of TNF-α (figure 1d). Omalizumab partially or completely restored the levels of all these molecules, suggesting they might represent potential candidates involved in the capacity of IgE-CL to impair the generation of Foxp3$^+$ Tregs by TLR9-L-activated pDCs.

The IFN-α/TNF-α axis and IDO expression have been previously associated with the capacity of pDCs to polarise Tregs [8]. To verify whether the downregulation of IFN-α and IDO, or TNF-α upregulation could be involved in the impaired ability of pDCs to generate Tregs after IgE-CL, we performed functional experiments. Blocking the IFN-α receptor (anti-IFN-α/β receptor blocking antibody (anti-IFNAR), Millipore), inhibiting IDO activity with 1-Methyl D-tryptophan (1-MT, Sigma-Aldrich) and exogenous TNF-α (PreproTech) impaired the capacity of TLR9-L-activated pDCs to induce Tregs (figure 1e). Supporting these data, the capacity of TLR9-L-activated pDCs under IgE-CL to generate Tregs was significantly restored after adding exogenous IFN-α (Biolegend) or kynurenine (a metabolite of downstream tryptophan catabolism that might bypass IDO downregulation; Sigma-Aldrich), or after blocking TNF-α with an anti-TNF-α antibody (Biolegend) (figure 1f). Collectively, our data show that IgE-CL in TLR9-L-activated pDCs reduces the production of IFN-α and IDO expression whereas it increases TNF-α production leading to the impairment of pDCs' capacity to polarise Tregs, which is completely restored by Omalizumab.

In conclusion, we show for the first time that IgE-FcεR1 cross-linking on human pDCs from atopic donors is associated with an impaired capacity of pDCs to polarise Tregs *in vitro*. We provide a molecular mechanism that might well help to explain how Omalizumab treatment increases Tregs frequency in asthmatic children [12]. The induction and maintenance of functional Tregs is essential for healthy immune responses to allergens [8]; therefore, our findings might have important clinical implications also for other allergic conditions [1]. The molecular mechanism described herein might also pave the way for the identification of potential novel biomarkers to assess Omalizumab clinical efficacy and to identify responder patients. Thus, future prospective clinical studies evaluating the capacity of pDCs to induce Tregs and its association with asthma control in Omalizumab treated patients are warranted. In the long run, these studies might well also help to elucidate and monitor whether Omalizumab could display potential long-term disease-modifying capacity for some specific patients.

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