

Research Article

Omalizumab dampens type 2 inflammation in a group of long-term treated asthma patients and detaches IgE from FcεRI

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Even if omalizumab is broadly used in the treatment of severe, allergic asthma, the immunological effects in long-term treated patients have not been fully elucidated. To this aim, a cohort of 15 allergic asthmatic patients treated with omalizumab for at least three years was compared with 12 allergic asthma patients treated with standard therapy. Omalizumab treated asthmatic patients showed lower frequencies of circulating plasmacytoid DCs, and lower CD154 expression on CD4 T-helper cells than the control group. Moreover, basophils and DCs from omalizumab-treated patients had lower surface expression of IgE compared to the control group. In a longitudinal evaluation of two patients that started omalizumab treatment, we show that FcεRI free of IgE were evident on basophils just after four weeks of drug administration. Finally, in vitro experiments with basophils obtained from healthy donors confirm that omalizumab is able to detach IgE from high affinity IgE receptors. Collectively these data indicate that long-term omalizumab treatment dampens type 2 inflammation acting on different cell types that play a pivotal role in the pathogenesis of allergic asthma. Moreover, we have identified a further mechanism of action of omalizumab, such as the ability to detach IgE from its receptor.

Keywords: asthma · basophils · FcεRI · IgE · omalizumab



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Asthma is an airways, inflammatory, chronic disease, characterized by airflow limitation and bronchial hyper-responsiveness. It represents a global health problem, whose prevalence is increasing in many countries, especially among children. The

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typical symptoms, such as wheeze, shortness of breath, chest tightness, cough, and also the expiratory airflow limitation, are the consequence of the airways inflammation [1]. The quality of inflammation is different among the multiple “asthma phenotypes,” in terms of both infiltrating cells and soluble mediators, and this is the reason why some pharmacological treatments work only in selected patients [2]. Among the several phenotypes and endotypes, distinguished by distinct clinical and pathobiological expressions, the allergic/eosinophilic one is the most frequent and probably the best characterized. Current therapies for asthma patients are based on inhaled corticosteroids and β_2 -adrenergic bronchodilators, to which oral leukotriene inhibitors and inhaled anticholinergics may eventually be added [3]. These therapeutic approaches work fine in most of the cases, but in a cohort of patients, mainly the ones with severe asthma, the disease may be uncontrolled [4]. Uncontrolled asthma patients, despite optimized treatment, still experience exacerbations, poor symptom control, lung-function impairment, or their combination [5].

In the last 15 years, new targeted therapies for severe asthma have been developed, particularly acting on persistent type 2 inflammation [6, 7]. The first “biologic” approved for allergic asthma was omalizumab, a mAb (monoclonal antibody) binding free IgE, inhibiting activation of mast cells, basophils, and DCs. Omalizumab is used in patients with uncontrolled, severe, allergic asthma and is effective in reducing severe exacerbations, hospitalizations, and inhaled glucocorticoids [8]. The long-term efficacy of omalizumab in patients with persistent, uncontrolled, allergic asthma, and significant improvements in quality of life have been shown [9], but the possible effects of such a treatment on different immune cell subsets are not yet fully known.

In this study, we evaluated the immunological status in a cohort of 15 patients with allergic asthma that are currently treated with omalizumab for at least three years. Phenotypical and functional assessments on this cohort of patients were compared with the ones of a control population of allergic asthma patients treated with standard therapy (high-dose inhaled corticosteroid -ICS- plus long-acting β_2 agonist -LABA-) for at least three years. The purpose of this study was to define the effect of long-term treatment with omalizumab on both innate and adaptive immune response.

Results

OTP show low levels of circulating BDCA2+ dendritic cells

We assessed the frequencies of different cells subsets in PB of OTP and STP. No differences were observed in the two groups concerning T cells, both CD4 and CD8, B cells, NK cells, and monocytes (Fig. 1A, and data not shown). Looking at other cell subsets of innate immunity, we found, in the two cohort of patients, comparable levels of type 2 innate lymphoid cells (ILC2) (Fig. 1B), as well as of basophils (Fig. 1C). On the contrary, the frequency of

BDCA2/4+ plasmacytoid DCs (pDCs) was significantly lower in OTP than in STP, whereas the frequency of BDCA1+ myeloid DCs (mDCs) was similar in the two groups (Fig. 1D).

Reduced CD154 induction on circulating CD4⁺ T cells in OTP

Since Th2 cells, as well as ILC2, play a pivotal role in the pathogenesis of allergic diseases and bronchial asthma [7], being also able to induce IgE production [11], both cell subsets were functionally characterized in OTP and STP groups.

In both groups ILC2 were characterized by type 2 cytokines production, such as IL-13, IL-4, and IL-5, without being able to produce IFN- γ and IL-22. However, neither the frequency of cytokine producing cells nor CD154 expression differed in ILC2 between OTP and STP (Fig. 2A). Looking at the cytokines production profile of circulating T-helper lymphocytes, we found a more heterogeneous condition, in which also IFN- γ and IL-22 producing cells were detected, though no substantial differences between the two groups were found (Fig. 2B). Conversely, the expression of CD154 on T-helper cells was different in the two groups, being significantly lower in OTP respect to STP (Fig. 2B).

Low IgE and Fc ϵ RI expression on basophils and DCs in OTP

Finally, we studied the possible effects of omalizumab on cells expressing the Fc ϵ RI, DCs, and basophils. OTP virtually do not express IgE on these cells, whereas STP show very high levels (Fig. 3A and B). Then we wondered if this low expression of IgE was due to a downregulation of Fc ϵ RI. As depicted in Fig. 3C and D, only basophils showed lower expression of Fc ϵ RI in OTP compared to STP, whereas it was not the case for mDCs and pDCs. IgE expression reduction on all three cell subsets and Fc ϵ RI expression reduction on basophils, was evident in terms of both frequencies of positive cells and surface density of the molecules as assessed by MFI (Fig. 3 all panels).

Based on these findings, we speculated that Fc ϵ RI on the surface of both basophils and DCs were at least in part free of IgE in OTP. To test this hypothesis, we evaluated in vitro IgE expression on these cells before and after incubation with a high-IgE serum (1600 kU/L). All the patients assessed, showed a considerable increase in IgE expression after that incubation, in both DCs and basophils, proving that their Fc ϵ RI were, at least partially, free of IgE (Fig. 4A and B).

Subsequently, taking advantage of two patients that started omalizumab treatment during our study, we observed a substantial downregulation of IgE and Fc ϵ RI expression on basophils after the first administration of the drug (4 weeks) (Fig. 4C). At this time point, several Fc ϵ RI were free of IgE, as shown by the increase of IgE cell surface expression (MFI 711 in medium alone) when high-IgE serum was added in vitro (MFI 1545).

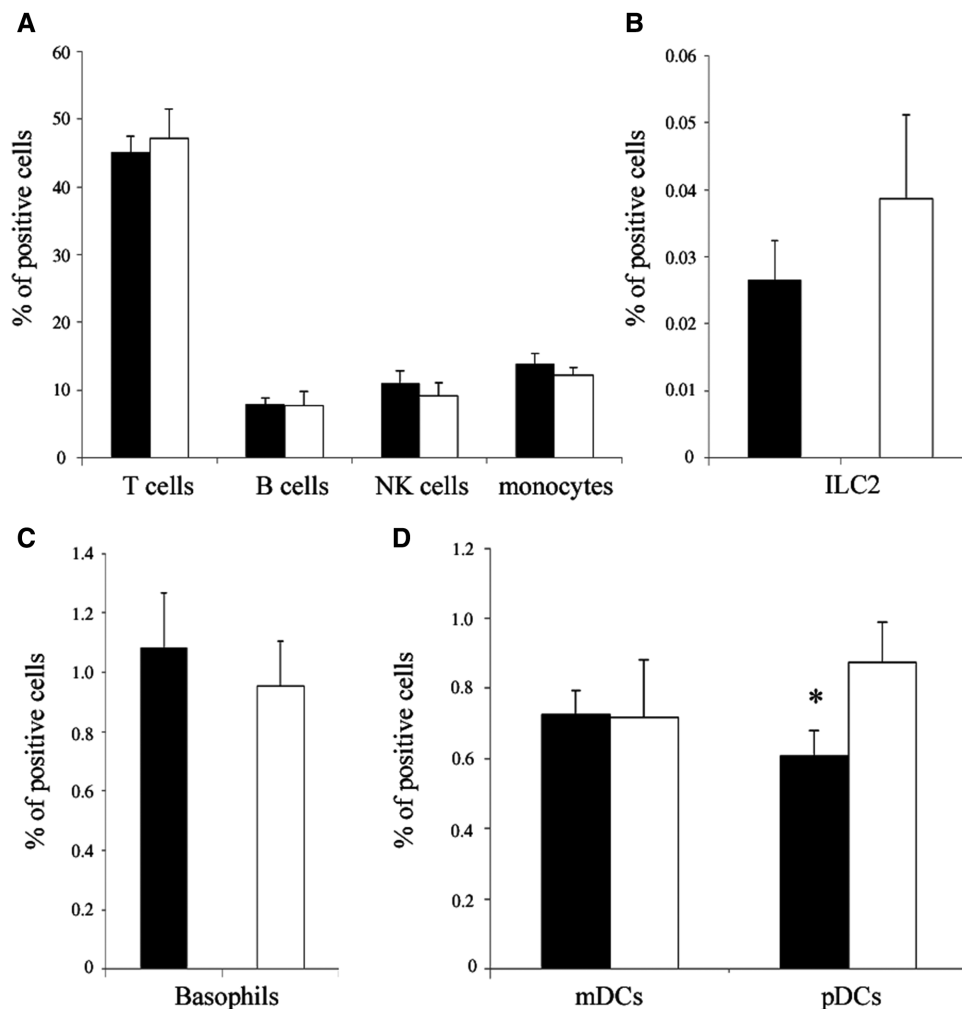


Figure 1. Evaluation of circulating immune cell subsets in asthma patients on omalizumab or standard therapy. Peripheral blood samples were obtained from patients with allergic severe bronchial asthma treated with either omalizumab or standard therapy. Frequencies of circulating T (CD3⁺), B (CD19⁺), NK (CD16⁺), cells, monocytes (CD14⁺) (A), ILC2 (lineage-CD45^{high} CD161⁺ CTH2⁺) (B), basophils (CD123+HLA-DR-) (C) on PBMCs from OTP (black columns) and STP (white columns) and (D) circulating mDCs (BDCA1⁺) and pDCs (BDCA2/4+) on CD3-CD19- from OTP (black columns) and STP (white columns) were analyzed by flow cytometry. Data are shown as mean + SE and are pooled from 15 OTP and 12 STP, respectively and are from 12–15 independent experiments with one patient sample per experiment. * $p \leq 0.05$ OTP versus STP, Student's *t* test.

Omalizumab detaches FcεRI-bound IgE from basophils

Taking account of the presence of IgE-free FcεRI on basophils surface, in both long term and early OTP, we speculated that the drug not only reduce IgE binding to its receptor but may also be able to detach IgE from FcεRI. To address this point we assessed in vitro the ability of omalizumab to decrease IgE expression on basophils obtained from healthy donors. We found a significant reduction of IgE surface expression in a dose-dependent manner on MNCs-gated basophils (Fig. 5A). The decrease of IgE surface expression started at 12 h and became more evident after 48 h (data not shown). Finally, to demonstrate that FcεRI were effectively free from IgE, we incubated PBMCs derived from 48 h in vitro culture with 1.25 mg/mL of omalizumab, with high level IgE serum and we found a significant increase of basophils' IgE surface expression (Fig. 5A and B). Then we performed the same

experiment on pure basophils population derived from PBMCs of healthy donors observing also in this case a significant reduction of IgE surface expression in presence of omalizumab (Fig. 5C). More importantly, in these culture supernatants, there was a significant increase of IgE levels compared to control culture conditions (Fig. 5D). The latter result demonstrates the ability of omalizumab to detach IgE from its receptor. Moreover, when basophils cultured 48 h with omalizumab were incubated 1 h with high-IgE serum, a significant increase of IgE surface expression was observed (Fig. 5C).

Discussion

Severe persistent asthma patients, are classically treated with high-dose inhaled corticosteroid plus long-acting β₂ agonist, or

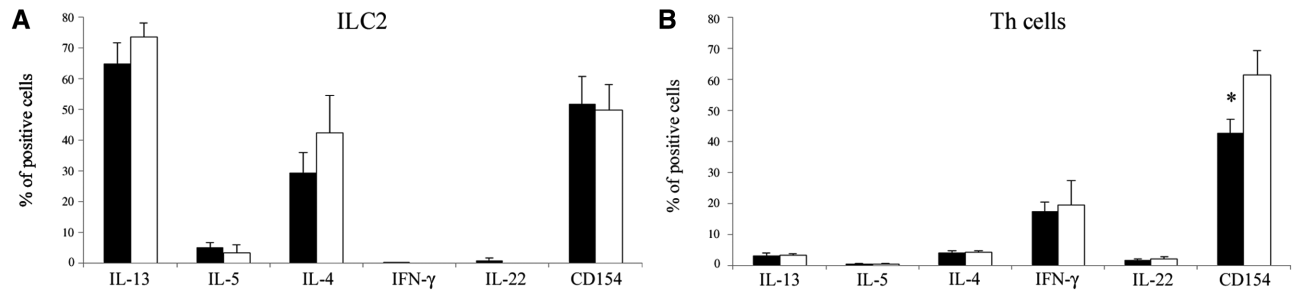


Figure 2. Intracellular cytokines production by ILC2 and CD4⁺ T cells from asthma patients on omalizumab or standard therapy. Peripheral blood samples were obtained from patients with allergic severe bronchial asthma treated with either omalizumab or standard therapy. Intracytoplasmic cytokines production and CD154 expression by ILC2 (A) and T-helper cells (B) after PMA/I stimulation of PBMCs from OTP (black columns) and STP (white columns) patients was determined by flow cytometry. Data are shown as mean + SE of the frequencies of cells producing the indicated cytokine and are pooled from 15 OTP and 12 STP, respectively and are from 12–15 independent experiments with one patient sample per experiment. * $p \leq 0.05$ OTP versus STP, Student's *t* test.

eventually also with the inhaled long-acting muscarinic agonist tiotropium. For uncontrolled asthma patients who do not respond to standard inhaled therapies, biologic agents that can interfere with the pathogenesis of the disease are now available [12]. Omalizumab, an anti-IgE mAb, was the first to be approved in allergic asthma patients with evidence of sensitization to perennial aeroallergens. The efficacy of omalizumab as add-on treatment to inhaled therapy in uncontrolled severe asthma patients is established in terms of disease control and quality of life, reduction of serious asthma exacerbations rate and of hospitalization [13, 14].

In this study, we evaluated the immunological effects of omalizumab in a group of long-term treated asthma patients (three years or more). All these patients were classified as responders to the therapy as indicated by the improvement in asthma control test [15]. The immunological parameters were compared to the ones of a group of patients with severe, allergic persistent asthma treated with high-dose inhaled corticosteroid plus long-acting β_2 agonist for at least three years (standard therapy).

We analyzed both phenotypical and functional features of circulating immune cell subsets. With regard the phenotypical characterization, we showed that omalizumab treatment does not alter the proportion of the immune cell subsets in PBMCs. Indeed we didn't find any difference in the frequency of B and T cells, both CD4 and CD8, as well as of NK cells and monocytes, between OTP and STP. Looking at innate immunity subsets, particularly those playing a pivotal role in type 2 inflammation, we found no differences in frequencies of ILC2, basophils, and myeloid DCs (mDCs), whereas plasmacytoid DCs (pDCs) were lower in OTP respect to STP. Accordingly, pDCs are markedly increased during asthma exacerbations and their PB numbers positively correlate with the severity of inflammation and the risk for asthma attacks [16]. It has also been reported that the recruitment of monocytes, DCs and Th2 lymphocytes in the lung of asthmatic patients increase after allergen challenge [17] and that omalizumab treatment restored IFN- α production by pDCs in response to rhinovirus and influenza virus; this is associated with reduction of asthma exacerbation rate [18]. In this view, the low number of pDCs observed in long-term omalizumab treated patients, may be considered as a cellular biomarker of low risk of exacerbations, and of disease good

control. Noteworthy, in the two patients evaluated before and at early time points after the beginning of omalizumab treatment, we didn't find a reduction of frequency of pDCs (data not shown), suggesting that this reduction being correlated with exacerbations, becomes evident only after long term treatment.

When we moved to a functional evaluation of T cells, studying the cytokines production of ex vivo polyclonally stimulated PBMCs, we didn't find any difference between OTP and STP. These data are in agreement with previous observation that omalizumab efficacy is not dependent upon its effects on T cells [19], although we found lower expression of CD154 in PB-derived CD4 T lymphocytes in OTP compared to STP. Considering the well-known role of CD154 in Ig production [20], its low expression in OTP may support some previous reports indicating that omalizumab leads to a downregulation of IgE secretion [21, 22]. Then we evaluated ILC2, a subset of innate immune cells involved in type 2 inflammation [7, 11]. ILC2 were functionally similar in the two group of patients for both cytokines production and CD154 expression. This observation suggests that circulating ILC2 are not influenced by omalizumab treatment. However, considering that ILC2 are preferentially localized in submucosal tissues, more than in PB [7, 11], additional studies that evaluate them in the lungs need to be performed.

When we functionally assessed basophils and DCs, we found impressive differences in the two groups of patients. In particular, IgE expression on the surface of basophils and DCs, both mDCs and pDCs, was much lower in OTP than STP. To investigate if this effect was a consequence of the downregulation of Fc ϵ RI, we looked at Fc ϵ RI expression on both DCs and basophils, finding only in these latter a lower expression in OTP versus STP. In any case the downregulation of Fc ϵ RI observed on basophils can contribute, but does not explain at all, the undetectable levels of IgE on their surface. Indeed, after the in vitro incubation with high IgE serum both basophils and DCs from OTP significantly increase IgE surface expression demonstrating that free Fc ϵ RI are still present on basophils and DCs surface and that they can be reloaded.

Based on these findings, we speculate that both mechanisms, downregulation of Fc ϵ RI and prevention of IgE binding on the

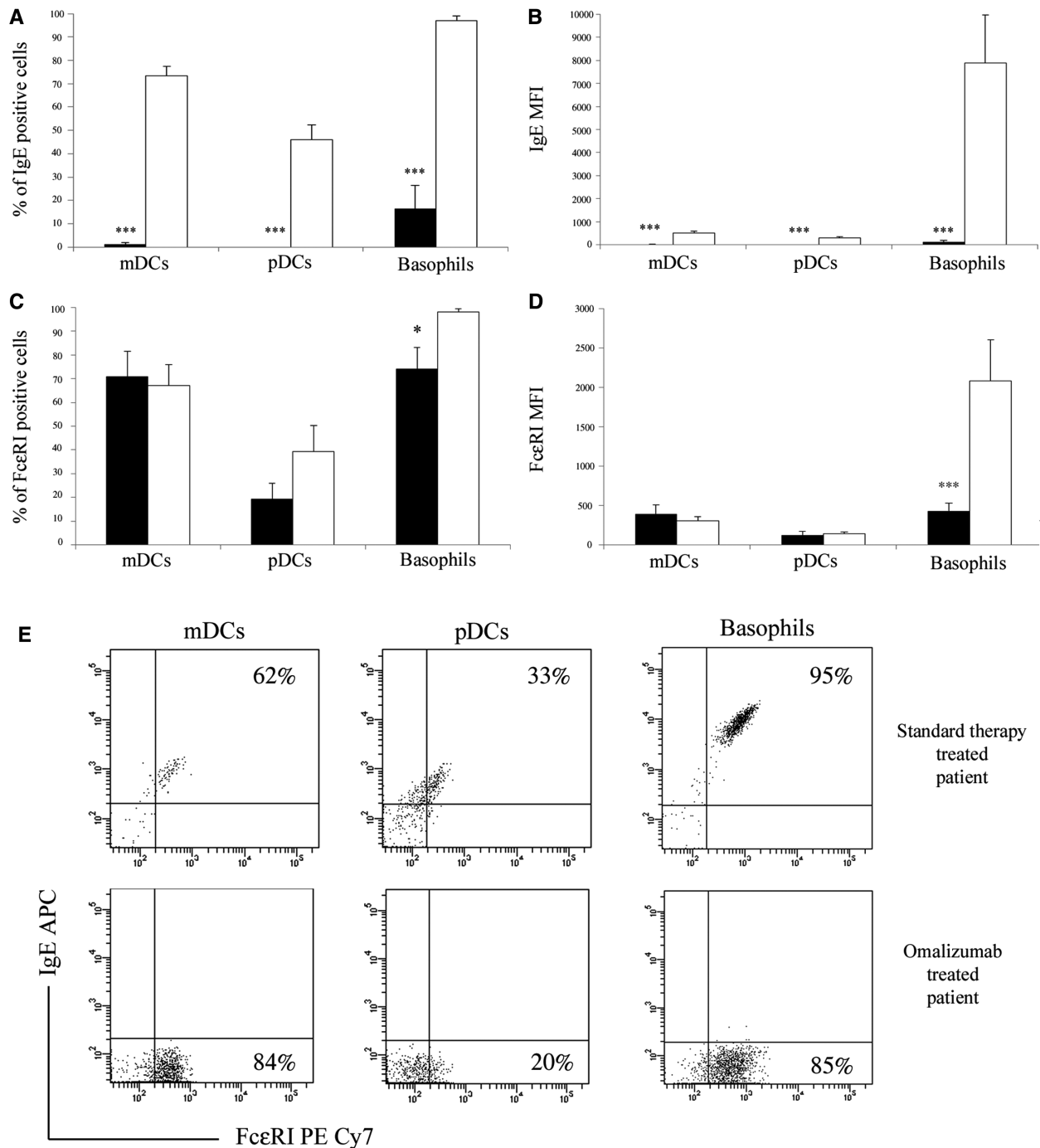


Figure 3. IgE and FcεRI expression on surface of DCs and basophils from asthma patients on omalizumab or standard therapy. Peripheral blood samples were obtained from patients with allergic severe bronchial asthma treated with either omalizumab or standard therapy. Frequencies of IgE positive cells (A) and FcεRI surface expression (C), and IgE MFI (mean fluorescence intensity) (B) and FcεRI MFI value (D) on mDCs, pDCs, and basophils were analyzed by flow cytometry. Black columns represent OTP and white columns represent STP. Two representative patients are depicted (E). Data are shown as mean + SE and are pooled from 15 OTP and 12 STP, respectively and are from 12–15 independent experiments with one patient sample per experiment. * $p \leq 0.05$, *** $p \leq 0.001$ OTP versus STP, Student's *t* test.

receptors, at least in basophils, may contribute to the nearly absolute lack of IgE on the surface of these cells during omalizumab treatment. Nevertheless, it has to be emphasized that on DCs we didn't appreciate a significant decrease of FcεRI expression, and

that the more evident effect, in both basophils and DCs, was the absence of IgE on their surface. A possible explanation of the different effects of omalizumab on FcεRI on DCs and basophils could be that the same high-affinity IgE receptor presents different

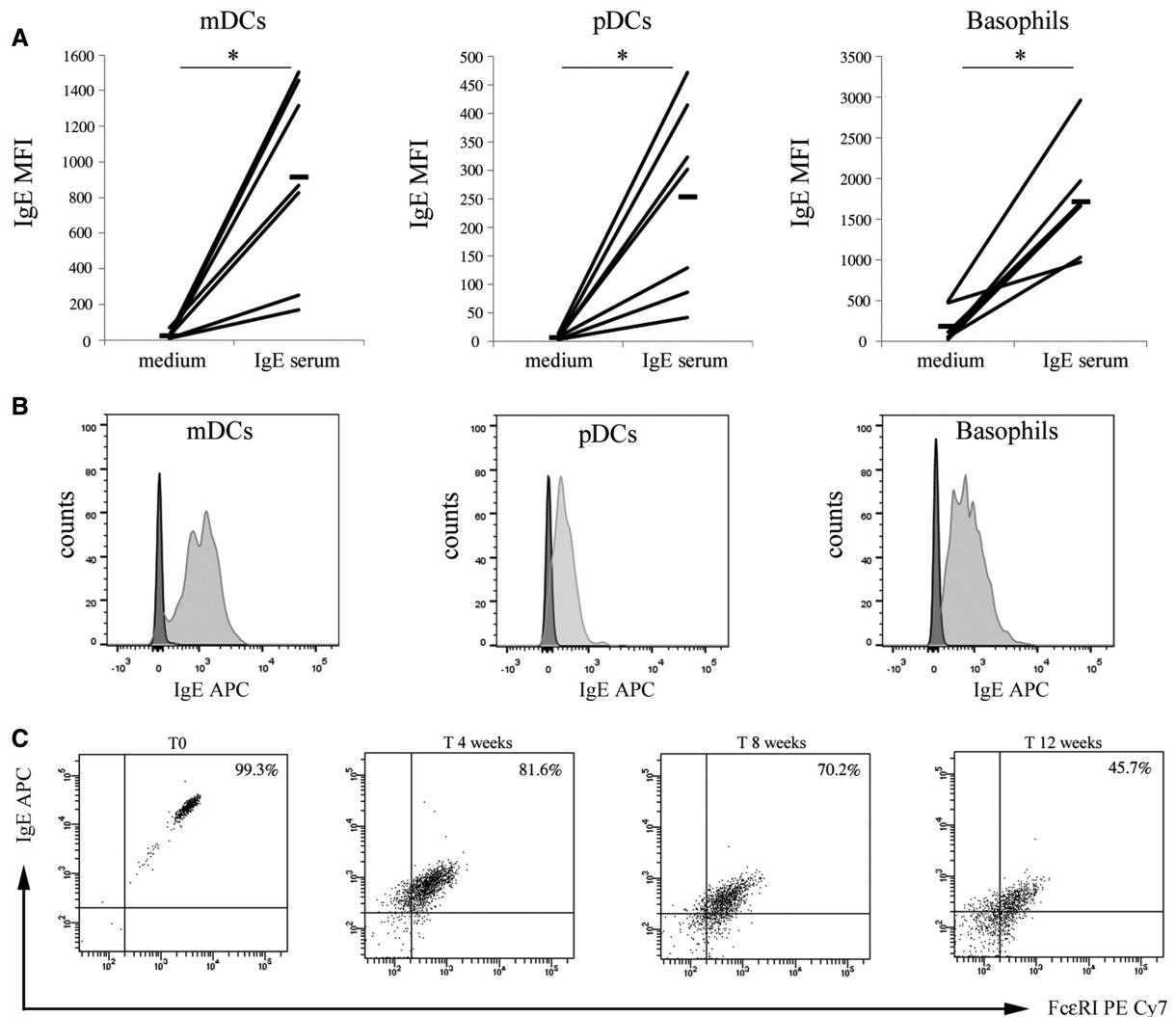


Figure 4. FC ϵ RI on basophils and DCs are free of IgE during omalizumab treatment. Peripheral blood samples were obtained from patients with allergic severe bronchial asthma treated with omalizumab to evaluate the expression of surface IgE on DCs and basophils. (A) Lines represent the MFI of IgE before and after the addition of IgE serum, in each of the seven patients. Bars represent the mean of the seven patients, from seven independent experiments. $*p \leq 0.05$, medium versus IgE serum, Student's *t* test. (B) One representative flow cytometric histogram of IgE expression before (black) and after (gray) the addition of IgE serum on the indicated cell subset. (C) Flow cytometric dot plots depicting IgE and FC ϵ RI expression on basophils, of one representative patient out of two patients evaluated before and during three months omalizumab treatment.

structure, mediates different functions, and its expression is differently regulated on the two cells subsets [23]. Moreover, FC ϵ RI expression on basophils and mast cells, but not on DCs and monocytes, is stabilized by IgE binding [24]. The capture of free IgE by omalizumab and the consequent reduced stability of FC ϵ RI due to their low binding, could reduce FC ϵ RI expression. The IgE/FC ϵ RI complex was rapidly endocytosed by DCs and monocytes to mediate the antigen presentation to T cells and the receptor expression was regulated by an active turn over [24]. This means that FC ϵ RI on basophils is important to induce signal transduction and cell activation whereas on DCs it mediates antigen internalization. Beyond the different contribution of these two mechanisms, the result is that DCs are inhibited, by omalizumab, in their capacity to capture allergens through IgE, and basophils are inhibited in their effector functions. Both these effects contribute to the

immunosuppressive activity of omalizumab, that is particularly directed toward type 2 mediated inflammation. Since the IgE downregulation on OTP basophils is very impressive, we wondered if it was only the consequence of free IgE capture or also FC ϵ RI-bound IgE detachment may occur. This latter hypothesis was supported by the evaluation of omalizumab kinetics in two patients that started the treatment during the study. After the first administration of the drug, an evident downregulation of both IgE and FC ϵ RI on basophils was observed, and also in this case a portion of FC ϵ RI were free from IgE. Obviously, being based on two subjects, our observation is purely anecdotal, but it suggests that IgE downregulation on basophils is a very early activity of omalizumab.

Finally, we demonstrated that omalizumab detaches IgE from its receptor on basophils of healthy donors, in a dose dependent

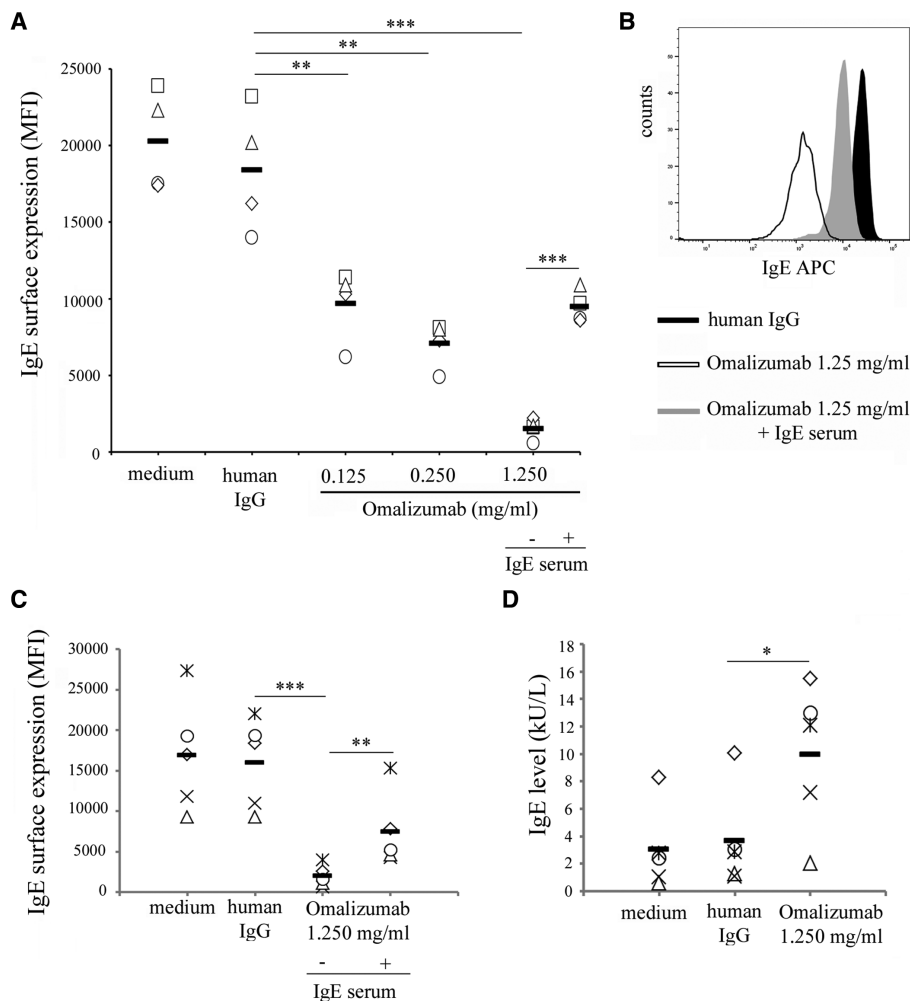


Figure 5. Omalizumab detaches IgE from FcεRI. PBMCs from healthy donors were incubated with omalizumab, washed, and subsequently incubated with high-IgE serum. (A) Each symbol represents IgE expression on basophils gated on MNCs obtained from one healthy donor, in different culture conditions as indicated. Black lines represent mean values pooled from four different experiments with one donor per experiment. $**p \leq 0.01$ $***p \leq 0.001$ as indicated by the bar, Student's *t* test. (B) One representative flow cytometry histogram of IgE expression on basophils from experiment in A is shown. (C) Basophils isolated from PBMCs of healthy donors were incubated with omalizumab, washed, and further incubated with high-IgE serum. Each symbol represents IgE expression on isolated basophils obtained from one healthy donor, in different culture conditions as indicated. (D) Each symbol represents IgE levels in culture supernatants of isolated basophils obtained from one healthy donor. In (C) and (D) each symbol represents the same healthy donor and black lines represent mean values of five different experiments with one donor per experiment. $*p \leq 0.05$, $**p \leq 0.01$ $***p \leq 0.001$ as indicated by the bar Student's *t* test

manner. The reduction of IgE expression was restored after a subsequent incubation of cells with high IgE serum, clearly demonstrating the presence of free FcεRI. Accordingly, IgE levels were increased in culture supernatants of basophils incubated in presence of omalizumab. In this view, it has been recently reported that omalizumab not only prevents IgE binding on basophils and mast cells, but may also accelerate dissociation of receptor-bound IgE from FcεRI [25, 26]. In agreement with our observations, this mechanism has been described to be dose-dependent [25, 26]. Davies et al. demonstrated that IgE are not favorable to omalizumab binding when complexed with FcεRI, however, at high omalizumab concentration the interaction between IgE and FcεRI is destabilized and a rapid IgE detachment occurs. This mechanism also takes advantage of the intrinsic flexibility of IgE that persists even when complexed with its receptors and permits omalizumab binding to the Cε3 domain [25].

The ability of omalizumab to dissociate prebound IgE from its receptor is also in accordance with the observation that, in omalizumab-treated paediatric patients, total IgE serum levels are increased at the very beginning of the treatment suggesting that an IgE detachment really occurs in this phase [27]. Our observations may also help to explain the very rapid beneficial effects of omalizumab in chronic urticaria [28]. Indeed it has been reported

that omalizumab produces a very rapid clinical response in chronic urticaria patients and that the clinical outcomes do not correlate with serum levels of free-IgE [29]. The exact mechanisms by which omalizumab detaches IgE from its receptors need to be investigated also at molecular level, even if we could speculate that it may be similar to that one described for other anti-IgE monoclonal antibodies [30–32]: their binding affinity to IgE is known to be higher than the high affinity receptor suggesting that the IgE could move from the high affinity receptor to the drug. In any case this additional property of omalizumab opens the way to additional applications of the drug. In particular, all those conditions in which a risk of anaphylaxis exists, such as mastocytosis [33, 34], allergen immunotherapy [35–37], drug desensitization, may benefit from omalizumab treatment.

In conclusion our work shows that long term treatment with omalizumab in severe allergic asthma patients, is associated with some immunological effects that involve both innate and adaptive immunity. The low expression of CD154 on T helper cells can account for the reduction of IgE production induced by omalizumab, and the low number of pDCs may explain the suppression of type 2 inflammation reducing exacerbations risk. The most evident effect of omalizumab treatment is IgE downregulation on surface of both basophils and DCs. This phenomenon is in part due to

Table 1. Patients' features

	Omalizumab-treated patients (OTP)	Standard-treated patients (STP)
Number	15	12
Sex (M/F)	7/8	6/6
Age (years)	24–79 (mean 56.3)	30–56 (mean 43.5)
FEV1 (%)	45–78 (mean 65.7)	50–80 (mean 68.4)
Serum IgE (kU/L)	124–981 (mean 447.1)	90–975 (mean 272.9)
Years of treatment	3–9 (mean 6.7)	3–9 (mean 6.3)

FEV1 values and serum IgE levels reported are referred before the beginning of the treatment.

FcεRI downregulation on these cells induced by omalizumab, but the presence of several IgE-free FcεRI on these cells, suggests that other mechanisms are involved. Among these, it is probably very important, not only the inability of FcεRI to bind omalizumab-captured IgE, but also the capacity of omalizumab to dissociate prebound IgE from its receptor.

Materials and methods

Subjects

Peripheral blood (PB) samples were obtained from 15 patients with allergic, severe bronchial asthma treated with omalizumab and from 12 allergic asthma patients treated with standard therapy, all for at least three years. Both OTP (omalizumab-treated patients) and STP (standard-treated patients) were under treatment with medium-high dosed of ICS and LABA. No differences emerged regarding smoking habit and exacerbation rate. Patients' features are listed Table 1. All OTP showed a good response to therapy as demonstrated by asthma control test value (ACT) pre- and post beginning of omalizumab treatment: 10–19 (mean 15.7) versus 19–25 (mean 21.1) $p \leq 0.001$. Two additional patients, not included in the study, were recruited before the beginning of treatment and during the next 3 months, in order to appreciate the early effects of omalizumab.

FEV1 values were obtained by spirometric test (CareFusion, Hoechberg, Germany). Total IgE serum levels were evaluated by ImmunoCAP assay (Phadia 250, Thermo Fisher Scientific, Uppsala, Sweden). The procedures followed in the study were in accordance with the ethical standards of the Regional Committee on Human Experimentation.

Reagents

The medium was RPMI 1640 (Seromed, Germany), with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 2×10^{-5} M 2-mercaptoethanol (all from Gibco Laboratories, NY), and 10% Fetal Calf Serum (Euroclone, Italy). Fluorochrome-conjugated mAbs and isotype-matched control mAbs were from BD Biosciences (Mountain View, CA) or Miltenyi Biotec (Ber-

gisch Gladbach, Germany). Phorbol 12-myristate 13-acetate, Ionomycin, Brefeldin A, Formaldehyde and Saponin were from Sigma Aldrich Co. (St. Louis, MO). Omalizumab (Xolair®) were from Novartis (Switzerland). Human IgG (Ig VENA) were from Kedrion Biopharma (Italy).

Flow cytometric analysis

PBMCs from all patients were stained with fluorochrome-conjugated mAbs cocktails (Supporting Information Table 1) and analyzed on BD LSRII FACS (BD Biosciences).

For cytokines intracellular staining, PBMCs were stimulated with PMA/I for 6 hours (the last 4 hours with Brefeldin A), fixed in formaldehyde 2% and then stained with mAbs in 0.5% saponin.

Gating strategy for all cell subsets analyzed was reported in Supporting Information Figures 1, 2, 3, 4 and was performed following flow cytometry guidelines [10].

Loading of FcεRI with IgE

PBMCs of seven OTP were incubated 1 h at 37°C in saline buffer in presence or absence of 5% high-IgE serum (32 000 kU/L) obtained from an allergic patient and then stained for surface markers and IgE.

In vitro IgE detachment experiment

Healthy donor PBMCs were incubated 12, 24, and 48 hours at 37°C with or without omalizumab at different concentration (1.25; 0.25, and 0.125 mg/mL) in culture media. Human IgG was used as control at 1.25 mg/mL. At each time point, cells were washed and stained for basophils surface markers and IgE. After 48 hours, samples incubated with omalizumab were washed and incubated 1 hour at 37°C with high-IgE serum, then twice washed and IgE stained.

Basophils from healthy donor PBMCs were enriched using Basophils Isolation kit II (Miltenyi Biotec), following the manufacturer's instructions. Pure basophils (>90%) were incubated with high-IgE serum for 1 h at 37°C, washed twice in saline

buffer and then incubated in presence or absence of omalizumab or human IgG at 1.25 mg/mL. After 48 h at 37°C, supernatants were recovered to evaluate IgE levels and pellet cells were washed and stained for IgE surface expression; moreover, cells derived from culture condition in presence of omalizumab, after washing, were further incubated 1 h at 37°C in presence of high-IgE serum, washed twice, and IgE stained.

IgE concentration in cultured supernatants was evaluated by ImmunoCAP Total IgE Low Range (Phadia 250, Thermo Scientific, range 0–100 kU/L, sensitivity 0.005 kU/L) to allow a clear evaluation also of low IgE levels.

Statistics

Mann–Whitney or paired Student *t*-test was used for statistical analysis using Origin Software. *P* values of 0.05 or less were considered significant. **p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.001.

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- Abbreviations:** ACT: asthma control test · BDCA: blood dendritic cell antigen · BFA: brefeldin A · I: Ionomycin, · ILC2: type 2 innate lymphoid cells · MNC: mononuclear cell · PB: peripheral blood · PMA: phorbol 12-myristate 13-acetate · OTP: omalizumab-treated patients · STP: standard therapy treated patients
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Omalizumab dampens type 2 inflammation in a group of long term treated asthma patients and detaches IgE from FcεRI

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1 Supplementary information

2 Supplementary Table

Specificity	Isotype	Clone	Conjugate	Supplier
BDCA-1 (CD1c)	mouse IgG2a	AD5-8E7	FITC	Miltenyi Biotec™
BDCA-2 (CD303)	mouse IgG1κ	AC144	PE	Miltenyi Biotec™
BDCA-4 (CD304)	mouse IgG1	AD5-17F6	PE	Miltenyi Biotec™
CD3	mouse IgG1 κ	UCHT1	PB	BD Biosciences™
CD4	mouse IgG1 κ	SK3	PerCPPE-Cy™7	BD Biosciences™
CD14	mouse IgG2b κ	MφP9	APC	BD Biosciences™
CD15	mouse IgM κ	MMA	FITC	BD Biosciences™
CD16 (FcγRIII)	mouse IgG1 κ	3G8	FITC	BD Pharmingen™
CD19	mouse IgG1 κ	SJ25C1	APC-Cy™7	BD Biosciences™
CD45	mouse IgG1 κ	2D1	APC-Cy™7	BD Biosciences™
CD45	mouse IgG1 κ	2D1	APC-Cy™7PerCP	BD Biosciences™
CD123 (IL-3Rα)	mouse IgG2a κ	7G3	FITC	BD Pharmingen™
CD123 (IL-3Rα)	mouse IgG1 κ	10 F5	PE	BD Biosciences™
CD154 (CD40 ligand)	mouse IgG1 κ	TRAP1	PE	BD Pharmingen™
CD161	mouse IgG1 κ	DX12	APC	BD Pharmingen™
CD161	mouse IgG2a κ	191B8	PE-Vio ®700	Miltenyi Biotec™
CD294 (CRTH2)	rat IgG2a κ	BM16	PE	Miltenyi Biotec™
CD294 (CRTH2)	rat IgG2a κ	BM16	Brilliant Violet 510™	Biolegend®
FcεRIα	recombinant human IgG1	REA758	PE-Vio ®700	Miltenyi Biotec™
HLA-DR	mouse IgG2a κ	L243	PerCP	BD Biosciences™
IgE	mouse IgG1 κ	MB10-5C4	APC	Miltenyi Biotec™
IFN-γ	mouse IgG1κ	45-15	APC	Miltenyi Biotec™
IFN-γ	mouse IgG2b κ	25723.11	FITC	BD Biosciences™

IL-4	Rat IgG1	MP4-25D2	APC-Cy7	Biolegend®
IL-5	rat IgG2a κ	JES1-39D10	APC	Miltenyi Biotec™
IL-22	mouse IgG1κ	22URT1	eFluor® 660	eBioscience™
Lineage Cocktail 1 (lin 1): CD3 CD14 CD16 CD19 CD20 CD56	IgG1 κ IgG2b κ IgG1 κ IgG1 κ IgG1 κ IgG2b κ	SK7 MφP9 3G8 SJ25C1 L27 NCAM16.2	FITC	BD Biosciences™

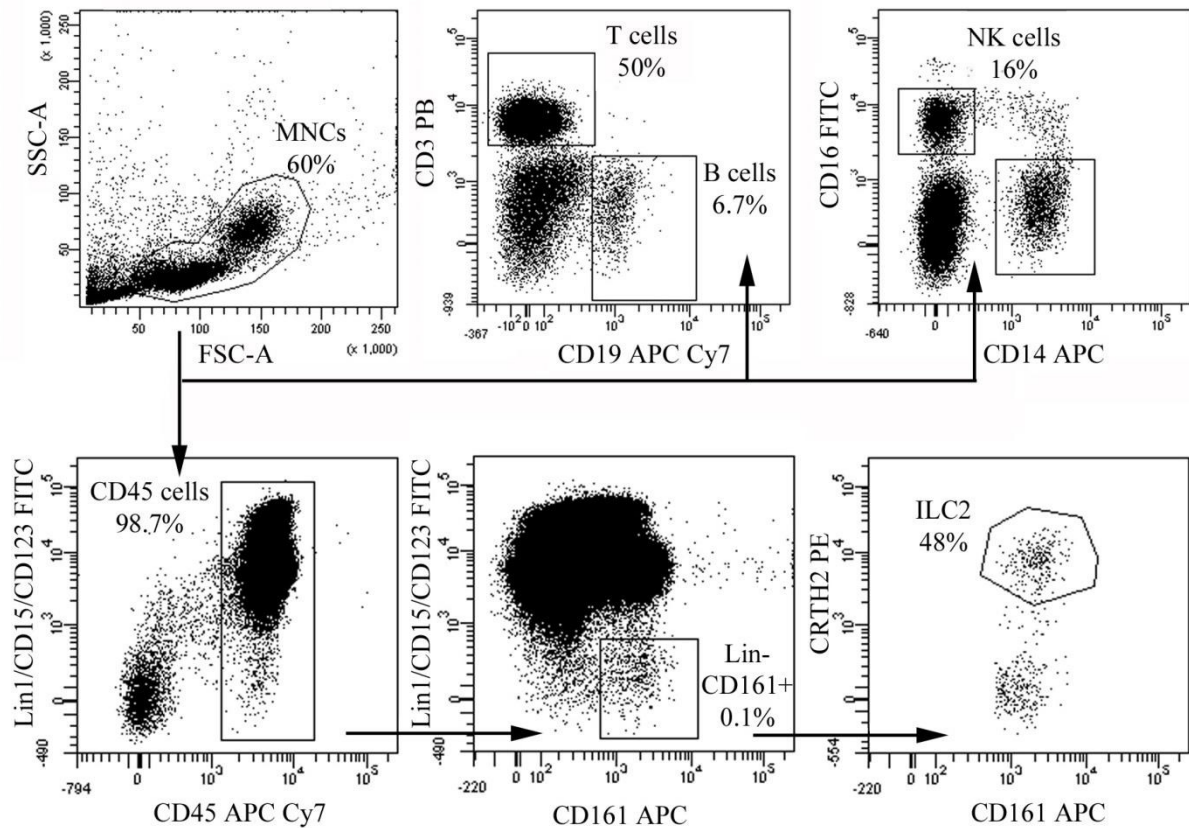
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4 **Table S1: mAbs used for flow cytometry staining.**

5 Fluorochrome conjugated-mAbs used for both surface and intracellular flow cytometry
6 staining.

7

8 **Supplementary Figures**

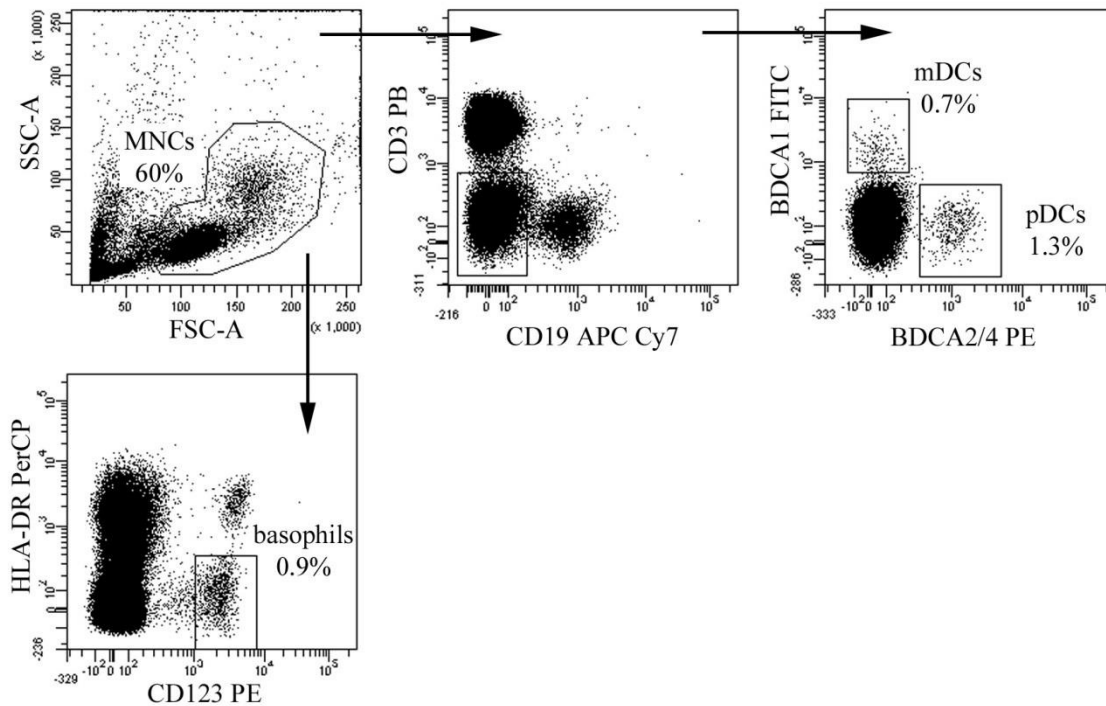


9

10 **Figure S1: Flow cytometry gating strategy used to determine T cells, B cells, NK**
11 **cells, monocytes and ILC2 frequency in PBMCs obtained from OTP and STP.**

12 MNCs were gated on a forward scatter (FSC) vs side scatter (SSC) plot. In the first tube (
13 upper panels) CD3 vs CD19 plot was used to identify T and B cell population while CD14
14 vs CD16 plot was used to select monocytes and NK cells. ILC2 were evaluated on a
15 separate tube (lower panels). Within the CD45 positive population, staining with Lin1, anti-
16 CD15 and anti-CD123 in FITC channel allowed the exclusion of T, B, NK, monocytes,
17 platelets and basophils. ILC2 were defined within the Lin- CD161+ population by CRTH2
18 expression.

19

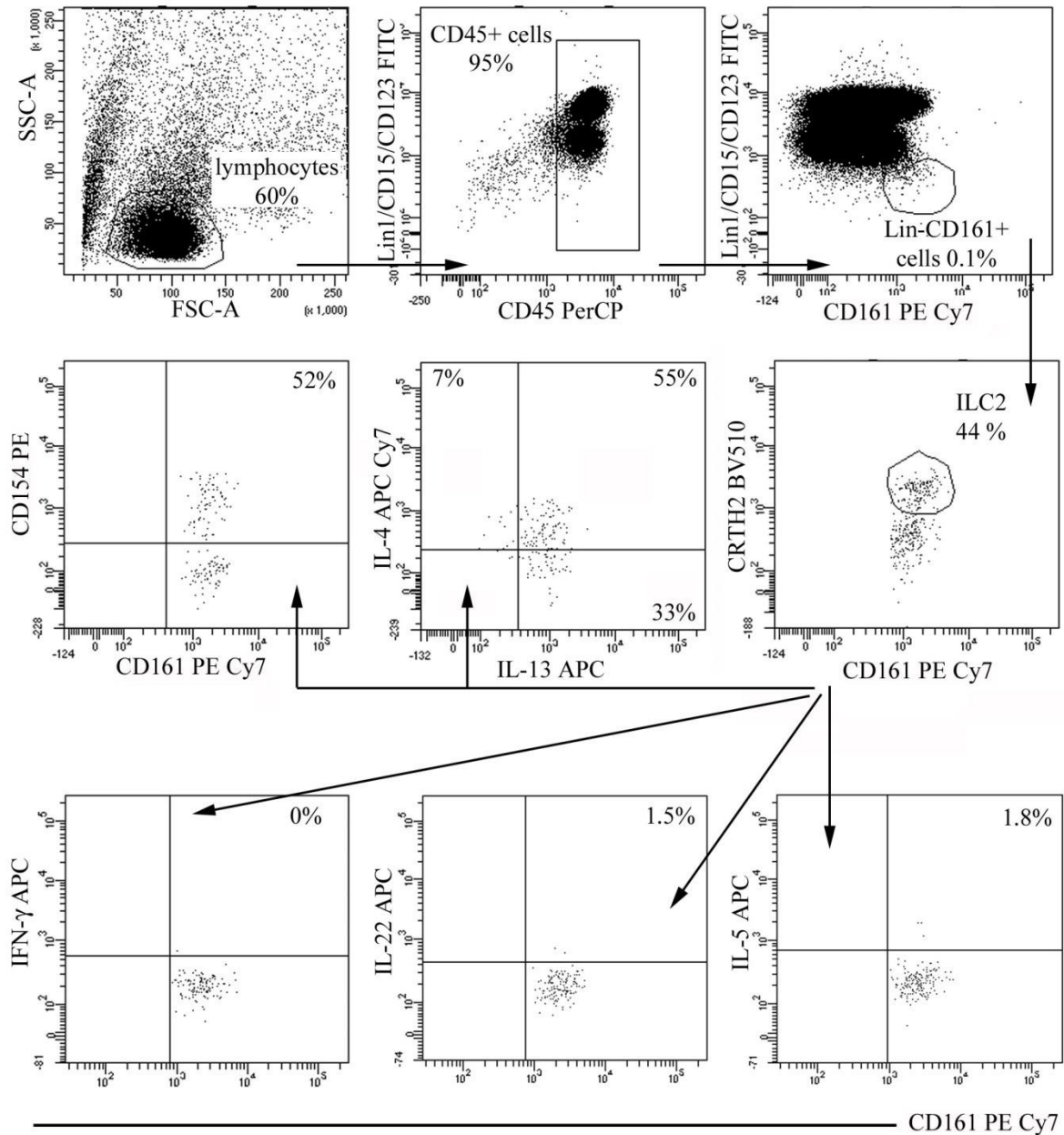


Fig

20

21 **Figure S2: Flow cytometry gating strategy used to identify mDCs, pDCs and**
 22 **basophils in PBMCs obtained from OTP and STP.**

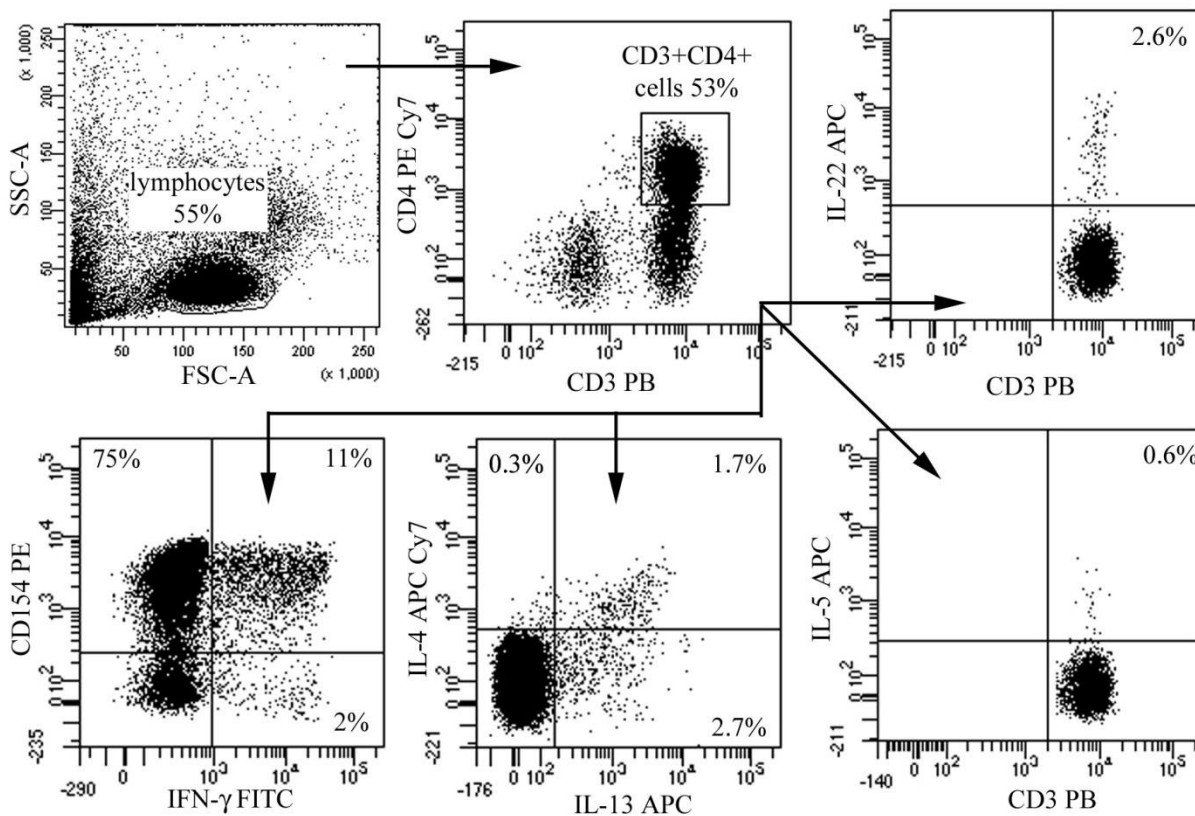
23 MNCs were gated on a forward scatter (FSC) vs side scatter (SSC) plot. In the first tube
 24 (upper panles) CD3 vs CD19 plot was used to exclude T and B cells. mDCs and pDCs
 25 populations were identified by BDCA1 and BDCA2/4 expression respectively. On a
 26 separate tube (lower panel), basophils were gated on the HLADR-CD123+ cell population.



27

28 **Figure S3: Flow cytometry gating strategy used to determine ILC2 cytokines**
 29 **production and CD154 expression in response to polyclonal stimulation in PBMCs**
 30 **obtained from OTP and STP.**

31 Within the lymphocytes population, ILC2 were gated following the same strategy used for
 32 the membrane staining (Figure S1). IL-4, IL-13 production and CD154 expression were
 33 evaluated in the same tube (middle panels). IFN-γ, IL-22 and IL-5 production was
 34 assessed in three separate tubes (lower panels).



35

36 **Figure S4: Flow cytometry gating strategy used to determine CD4⁺ T cells cytokines**
 37 **production and CD154 expression in response to polyclonal stimulation in PBMCs**
 38 **obtained from OTP and STP.**

39 CD3⁺CD4⁺ T cells were gated within the lymphocytes population. IL-22 and IL-5
 40 production were evaluated in two separate tubes (upper- right and lower- right panels). IL-
 41 4, IL-13, IFN- γ production and CD154 expression were evaluated in the same tube (lower
 42 panels).