Published in final edited form as:
J Clin Immunol. 2012 June ; 32(3): 565–573. doi:10.1007/s10875-012-9651-x.

Modulation of mTOR Effector Phosphoproteins in Blood Basophils from Allergic Patients

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Abstract

The mammalian target of rapamycin (mTOR) pathway contributes to various immunoinflammatory processes. Yet, its potential involvement in basophil responses in allergy

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Competing interest statement The authors declare to hold no conflict of interest with the publication of the results included in this manuscript.

remains unclear. In this pilot study, we quantified two key mTOR effector phosphoproteins, the eukaryotic initiation factor 4E (peIF4E) and S6 ribosomal protein (pS6rp), in blood basophils from nut allergy patients (NA, *N*= 16) and healthy controls (HC, *N*= 13).Without stimulation in vitro, basophil peIF4E levels were higher in NA than HC subjects (*P*=0.014). Stimulation with nut (offending) but not chicken / rice (non-offending) extract increased basophil peIF4E and pS6rp levels (+32%, *P*=0.018, and +98%, *P*=0.0026, respectively) in NA but not HC subjects, concomitant with increased surface levels of CD203c and CD63, both known to reflect basophil activation. Pre-treatment with the mTOR inhibitor rapamycin decreased pS6rp and CD203c responses in nut extract-stimulated basophils in NA subjects. Thus, basophil responses to offending allergens are associated with modulation of mTOR effector phosphoproteins.

Keywords

Eosinophils; flow cytometry; food allergy; inflammation; neutrophils

Introduction

The mammalian target of rapamycin (mTOR) pathway is a canonical pathway in cell survival, growth and proliferation, due to its regulatory activity on metabolism and translation [1–3]. As such, the mTOR pathway has been studied widely in relation to normal development and cancer [4–6]. More recently, studies from various groups have established a role for the mTOR pathway in immunological and inflammatory processes. There is direct evidence for activation of mTORC1 and/or mTORC2 kinases, which control two branches of the mTOR pathway, as well as for changes in levels of downstream phosphorylated effectors, in leukocyte polarization: this includes T-cell development in memory responses to vaccination [7], macrophage effector functions in response to mycobacterial infection [8], and neutrophil re-programming in cystic fibrosis airway inflammation [9].

In allergy, the contribution of the mTOR pathway to mast cell-dependent responses is well established, with functional differences between the mTORC1 and mTORC2 branches of the pathway [5, 10]. Previous studies have also shown modulation of eosinophil-dependent allergic responses by rapamycin [11], but no direct measurement of mTOR kinases or effectors was reported. Activation of the mTOR pathway is in part dependent upon phosphatidylinositol 3-kinase (PI3K), which has been previously implicated in basophil activation [12, 13]. However, a direct role for the mTOR pathway in basophil responses in allergy is not clearly established.

Because of its increase in prevalence and severity, food allergy has become a major health concern worldwide [14]. Among food allergens, nuts are most often associated with life-threatening events [15]. Nut allergy is associated with both local and systemic reactions, such as rash, nausea/vomiting and/or anaphylaxis, during which tissue mast cells and blood basophils release histamine and other inflammatory mediators that together amplify the allergic reaction. Hence, nut allergy is a relevant context in which to look for possible functional modulation of blood basophils.

The conventional technique for phosphoprofiling, Western blot, yields data at the population level. When applied to rare, fast-responding cells such as blood basophils, Western blot suffers from two important limitations: (i) it requires large volumes of sample to yield enough material for analysis; (ii) the physical steps of cell purification necessary to sort out rare target cells are likely to alter their phosphoprotein levels. Flow cytometry-based phosphoprofiling yields data at the single-cell level that are consistent with Western blot data [16], and is particularly well-adapted to rare and fast-responding cells such as basophils. Indeed: (i) flow cytometry-based phosphoprofiling can be implemented on low volumes of sample; and (ii) rare target cells can be analyzed in their native phosphorylation state, thanks to immediate fixation [17], since analytical gating enables precise definition of target cells without a need for any physical step of cell purification.

Our group has showed that direct flow cytometry-based phosphoprofiling (without any purification) can provide unique insights into pathological signaling in leukocyte subsets from blood and other compartments [18, 19]. In the context of direct phosphoprofiling, chronically induced pathways are likely to manifest themselves through stable increases in the phosphorylation of final effectors (last elements in the pathways), since these elements are characterized by much slower on / off cycling of their phosphorylated moieties than kinases.

In the present study, we focused our attention on phosphorylated eukaryotic initiation factor 4E (peIF4E) and S6 ribosomal protein (pS6rp), two effectors downstream of the mTORC1 kinase, in blood basophils from patients with nut allergy. Results from this pilot study show that peIF4E levels are significantly altered at baseline, and that both peIF4E and pS6rp levels undergo marked increases upon stimulation with nut (offending), but not chicken / rice (non-offending), extract. Taken together, our data suggest a role for the mTOR pathway in basophil responses in allergy.

Methods

Human subjects

The Stanford A dministrative Panel of Human Subjects in Medical Research approved the study. All 13 healthy control (HC) and 16 nut allergic (NA) subjects (or parents, for minors) included in the study signed informed consent forms before they underwent study procedures. A few samples were limited in volume and did not yield sufficient starting material to perform the basophil stimulation assay and control experiments (rapamycin pre-treatment and time-course). Samples from at least 12 HC and 15 NA subjects were used in each instance. Clinical nut allergy (to peanut, N= 13; to cashew, N=2; to walnut, N= 1; see Table I) was diagnosed by clinical history of food allergy reaction, and nut-specific IgE ≥15 kU/L (ImmunoCAP, Phadia, Uppsala, Sweden) or positive skin prick test to nut extract. Severity was graded based on published scores of anaphylaxis symptoms, as noted elsewhere [20]. Total blood IgE levels were also measured in allergic subjects by the Stanford Hospital Clinical Laboratories using the ImmunoCAP assay.

Sample collection and processing

Blood was collected by venipuncture in ethylene diamine tetraacetic acid (EDTA) Vacutainer tubes (BD Biosciences, San Jose, CA, USA) and immediately placed on melting ice. Within 30 min, blood was centrifuged at 400 G for 10 min at 4°C to separate the erythrocyte / leukocyte pellet (kept on melting ice) from platelet-rich plasma. Platelet-rich plasma was further spun at 3000 G for 10 min at 4°C to remove platelets and yield platelet-free plasma. The erythrocyte / leukocyte pellet was reconstituted to its original volume with platelet-free plasma, yielding platelet-free, reconstituted blood. We used platelet-free reconstituted blood to assess blood basophil function, to avoid platelet-triggered aggregation and clotting in the course of the 10-minute, 37°C incubation with saline or extracts (see below). We showed previously that basophils from allergic patients respond to allergen stimulation equally well in the absence or presence of plasma, emphasizing the importance of surface (pre-bound) Ig molecules and other receptors involved in this activation process [21].

Incubation with saline or extracts

We assessed blood basophil function under 3 conditions: (i) incubation with phosphate-buffered saline (PBS, referred to as “without stimulation”); (ii) incubation with offending nut (peanut, cashew, or walnut) extract; and (iii) incubation with non-offending (chicken / rice) extract. Extracts were from clinical-grade preparations used for skin testing (Greer, Lenoir, NC, USA).We used extracts, rather than purified allergens, because we aimed to mimic the conditions of in vivo stimulation as occurring during skin testing, which assesses effector cell reactivity to mixed preparations, rather than to specific molecular determinants. For each of the three incubations, 600 μl of platelet-free, reconstituted blood (processed as above), was mixed with either 9 μ;l of PBS or relevant extracts for 10 min at 37°C. The 10-minute time point appeared to be optimal based on our previous data quantifying surface CD203c and CD63 in nut extract-stimulated basophils from NA patients [21], as well as based on time-course studies analyzing phosphoproteins, as presented in Supplementary Figure 2. The incubation was stopped by adding ice-cold PBS-EDTA (EDTA [2.5 mM final] is a calcium chelator that blocks cell activation) and cells were pelleted by centrifugation (490 G, 5 min, 4°C) for surface and intracellular staining (see below). To demonstrate the specificity of mTOR signaling in nut extract-stimulated basophils, blood was pre-treated in five experiments (5 NA and 5 HC subjects in total; one NA and one HC subject per experiment) with the mTOR inhibitor rapamycin [22] (10 nM, 30min, 37°C), prior to incubation with PBS or nut extract.

Cell staining

Each incubation condition (without stimulation or stimulation with nut extract or chicken / rice extract) was characterized with surface and intracellular staining combinations. Surface staining for CD203c and CD63 was performed prior to fixation, as detailed elsewhere (see [21], with some modifications, as detailed in the Methods section of the Online Repository). Intracellular phosphoprotein staining was performed after surface staining, fixation and

Electronic supplementary material The online version of this article (doi:10.1007/s10875-012-9651-x) contains supplementary material, which is available to authorized users.

methanol-based permeabilization (see [17], with some modifications, as detailed in the Methods section of the Online Repository).

Flow cytometry data acquisition and analysis

We used a LSRII digital flow cytometer (BD Biosciences) equipped with 4 lasers (535, 488, 633, 405 nm), 2 light scatter detectors (yielding forward and side scatter data) and 18 fluorescent detectors. Several steps were taken to standardize flow cytometric data acquisition (see Methods section in the Online Repository). Compensation was done using single-stained beads or cells upon analysis in the FlowJo software (Treestar, Ashland, OR, USA), as previously described [23]. The gating strategy to identify basophils and measure surface markers and intracellular phosphoproteins is illustrated in Fig. 1. Analytical gates for basophils included at least 400 cells. Eosinophils and neutrophils were also amenable to gating and downstream measurements with this staining protocol, and served as control subsets for the basophils from the same sample (see Supplementary Figure 4 for the eosinophil/neutrophil gating strategy). Analytical gates for eosinophils and neutrophils included at least 1000 cells.

Statistics

We used the JMP8 software (SAS Institute, Cary, NC, USA) for all statistical analyses presented here. For between- and within-group comparisons, we used the nonparametric Wilcoxon rank sum and signed-rank tests, respectively. Differences were considered significant with $P<0.05$ for single intracellular assays (pS6rp levels in allergen stimulation experiments with rapamycin pre-treatment) and single surface assays (CD203c levels in allergen stimulation experiments with rapamycin pre-treatment), and $P<0.025$ (=0.05 divided by 2) for dual intracellular assays (pS6rp and peIF4E levels in allergen stimulation experiments without rapamycin pre-treatment) and dual surface assays (CD203c and CD63 levels in allergen stimulation experiments without rapamycin pre-treatment), to correct for multiple assays.

Results

peIF4E levels in basophils from nut allergic patients are increased without stimulation in vitro

Live blood basophils (minimum of 400 per stain, per subject) were gated as the Live/Dead^{lo} / CD3^{-} / CD16^{-} / CD20^{-} / CD56^{-} / CD66b^{-} / HLA-DR^{-} / CD123^{+} subset (Fig. 1a), in which phosphoproteins of interest were quantified (Fig. 1b) using flow cytometry. All phosphoproteins tested were distributed unimodally and their levels were measured using median fluorescence intensity (MFI) values on the whole basophil population. Since food allergy is a chronic disease, we first investigated whether blood basophils from nut allergic patients (NA) exhibited changes in intracellular phosphoprotein levels without stimulation (incubation in saline), as compared to healthy controls (HC). We found minimally increased baseline levels of peIF4E ($P=0.014$, Fig. 2a) in basophils from NA subjects, while values for the other mTORC1 target, pS6rp, did not differ significantly from those of HC subjects (Fig. 2b).

peIF4E and pS6rp levels in basophils from nut allergic patients are not affected by stimulation with chicken / rice (non-offending) extract

We showed previously that basophils from NA subjects stimulated with nut extract, but not a non-offending extract, undergo significant increases in surface levels of CD63 and CD203c [21]. Here, we used these surface markers as experimental positive controls, to which we compared changes in phosphoproteins after stimulation with nut (offending) *vs.* chicken / rice (non-offending) extracts. First, we checked whether our stimulation protocol induced non-specific changes in phosphoprotein levels in basophils. After stimulation with a non-offending extract, basophils from either NA or HC subjects did not exhibit significant differences in surface levels of CD203c or CD63, as assessed by within-group (chicken / rice extract stimulation vs. without stimulation for each subject) and between-group (chicken / rice extract stimulation in NA vs. HC groups) statistics (Supplementary Figure 1). Consistently, non-offending extract stimulation did not induce significant changes in levels of peIF4E or pS6rp (Fig. 2a and b, respectively).

peIF4E and pS6rp levels in basophils from nut allergic patients are markedly increased upon stimulation with nut (offending) extract

In contrast with the non-offending extract, nut extract stimulation induced highly significant increases in intracellular levels of peIF4E (+32%, P=0.017) and pS6rp (+98%, P=0.0006) in blood basophils from NA, compared to HC, subjects (Fig. 2b). As an experimental control, we investigated whether total eIF4E and S6rp levels were changed in blood basophils from NA subjects (#13 and #16, Table I) upon nut extract stimulation (see Supplementary Material, for details). As shown in Supplementary Figure 1, total elF4E and S6rp levels in blood basophils from NA patients are also increased upon stimulation with nut allergen. However, the increase in total proteins is not as substantial as the increase in their respective phosphorylated forms. Consistent with increased peIF4E and pS6rp levels, nut extract stimulation also induced marked increases in surface CD203c and CD63 levels (as described in [21], and illustrated for this study in Supplementary Figure 2). Nut extract stimulation for 30 rather than 10 min did not enhance peIF4E and pS6rp levels further (Supplementary Figure 3).

Rapamycin inhibits nut extract-induced increase pS6rp levels in blood basophils from NA subjects

As additional evidence for the activation of the mTOR pathway in the context of basophil stimulation with nut extract, we found that pre-treatment with the mTOR inhibitor rapamycin (10 nM, for 30 min) inhibited the nut extract-induced increase in pS6rp levels in basophils from NA subjects (P=0.01, Fig. 3). Pre-treatment with rapamycin (10 nM, for 30 min) also inhibited the nut extract-induced increase in CD203c levels in basophils from NA subjects (P=0.031, Supplementary Figure 4), which confirms that the mTOR pathway is involved in the basophil activation process. However, we did not observe a significant increase in the phosphorylation of Akt (the main, but not sole, intermediate kinase upstream of the mTOR pathway) in basophils from NA subjects stimulated with nut extract, as measured at the 10-minute timepoint (data not shown).

peIF4E and pS6rp levels in blood eosinophils and neutrophils from nut allergic patients are normal without stimulation and marginally changed by stimulation with nut extract

We gated live eosinophils and neutrophils in the same samples as basophils, as shown in Supplementary Figure 5, and measured peIF4E and pS6rp levels in them.Without stimulation, eosinophils (Supplementary Figure 6) and neutrophils (Supplementary Figure 7) showed similar peIF4E and pS6rp levels in NA and HC subjects. In eosinophils, no difference in peIF4E levels was observed (Supplementary Figure 6a) while pS6rp levels were minimally increased in NA compared to HC subjects under both chicken / rice and nut extract stimulation (P=.014 and .015, respectively), but no significant changes were measured in within-group analyses (Supplementary Figure 6b). In neutrophils, stimulation with either chicken / rice or nut extract did not induce any significant changes in peIF4E and pS6rp levels in NA or HC subjects (Supplementary Figure 7a and b).

Discussion

The mTOR pathway plays a key role in various immune response and inflammatory processes [5, 7–10]. In this pilot study, we show that peIF4E levels are increased in blood basophils from nut allergic subjects under conditions of no stimulation in vitro, and that peIF4E levels, along with pS6rp levels, undergo a marked upregulation upon stimulation by nut extract. Hence, basophil stimulation is associated with modulation of mTOR effector phosphoproteins.

In patients with allergy, chronic exposure of blood basophils to priming / activating conditions may lead to an extension of their survival at baseline [24]. Supporting this notion is our observation that peIF4E levels are increased in basophils from NA compared to HC subjects, as assessed under conditions of no stimulation in vitro. Indeed, peIF4E is a major effector phosphoprotein in the pro-survival mTORC1 branch of the mTOR pathway. It is important to note, however, that the other mTORC1 effector phosphoprotein, pS6rp, was not altered without stimulation, suggesting only partial activation of the pathway at baseline.

Next, we investigated levels of mTOR effector phosphoproteins in blood basophils upon stimulation with clinical extracts used to screen allergic patients. Our objective was to mimic in vivo stimulation protocols and test blood basophil responses in “real-life” conditions, rather than to isolate specific molecular determinants potentially responsible for such responses. Indeed, clinical extracts contain multiple molecular determinants that may activate basophils, not only via immunoglobulin cascades (mediated by IgE and other isotypes of immunoglobulins), but also via lectin- or damage-associated molecular pattern receptor-dependent cascades [25–28]. Previously, we showed that nut extract stimulation induces a marked increase in surface CD203c and CD63 levels on basophils from NA subjects [21]. Here, we confirmed that nut extract stimulation also resulted in increases of both peIF4E and pS6rp levels in basophils, suggesting that the mTORC1 branch of the mTOR pathway is fully active in this context.

The two antibodies used here are thought to be specific to peIF4E and pS6rp, respectively, and we provide evidence that rapamycin leads to decreased pS6rp levels, which further establishes the identity of this pathway. However, the possibility remains that the antibodies

used may also recognize other basophil-associated phosphoproteins. Confirmatory experiments using Western blot are necessary to address this question. Such experiments are not without limitations themselves since large volumes of blood followed by sorting would be required to yield enough basophils for analysis, which bears strong potential for artifacts.

Interestingly, the increase in intracellular peIF4E and pS6rp levels induced by nut extract stimulation in basophils from NA subjects occurred in the absence of plasma factors. This results is consistent with our previous observation of increased surface CD203c and CD63 levels occurring upon nut extract stimulation in basophils from NA subjects in the absence of plasma factors [21]. Taken together, these results suggest that surface and intracellular activation processes in nut extract-stimulated blood basophils do not critically depend on soluble plasma factors, but rather on existing surface (prebound) Ig molecules, lectin- and damage-associated molecular pattern receptors in basophils.

Prior attempts at profiling basophil phosphoproteins by flow cytometry have used saponin-based permeabilization [34, 35]. However, the detection of some phosphoproteins by specific antibodies is either suboptimal or unachievable unless cells have been treated with methanol (which permeabilizes membranes and linearizes epitopes), as opposed to saponin (which only permeabilizes membranes) [36]. Unfortunately, approaches employing methanol are problematic, since some surface markers used for basophil gating are destroyed by methanol treatment [17]. We illustrate here a method that employs methanol-resistant surface markers for basophil gating from blood and methanol-based phosphoprotein profiling. In principle, our approach is applicable to any clinical condition thought to be associated with basophil activation.

We previously showed concomitant increases in peIF4E and pS6rp in activated airway neutrophils from cystic fibrosis patients [9]. Additionally, we observed that stimulation of mouse mast cells triggers activity of the p70-S6 kinase, which is a key activator in the mTOR pathway [29], and later studies confirmed a role for both mTORC1 and mTORC2 in mast cell activation [10, 30]. In addition, the mTOR pathway contributes to chemoattraction and mediator release in mouse mast cells [10]. The observed inhibition of nut extract-induced surface CD203c increase by rapamycin further suggests that the mTOR pathway plays a mechanistic role in the basophil activation process, which warrant additional experimental and clinical studies (e.g., in allergic patients treated with rapamycin for other indications, such as certain forms of cancer). Taken together, the evidence provided here that peIF4E and pS6rp are modulated in basophils in nut allergy is consistent with previous data and suggests a functional role for the mTOR pathway in this pathological context.

Phosphatidylinositol 3-kinase, a classical upstream kinase in the mTOR pathway, has been previously implicated in basophil activation [31–34]. The kinase Akt is the main (but not exclusive) intermediate between phosphatidylinositol 3-kinase and mTORC1 and mTORC2 kinases. In this study, we did not observe any significant increase in phosphorylated Akt in nut extract-stimulated basophils from NA patients at the 10-minute timepoint. One possible explanation for this result is that Akt may undergo an increase in phosphorylation occurring quickly and transiently (a process typical of intermediate kinases), with a return to baseline levels by 10 minutes. Alternatively, mTOR pathway activation in nut extract-stimulated

basophils from NA patients may not depend on Akt. Indeed, the mTOR pathway can be triggered in many different ways (including small metabolite transport) that do not require concomitant activation of the phosphatidylinositol 3-kinase / Akt axis [1, 2]. Further studies detailing the early time-course of nut extract-induced stimulation of basophils from NA patients, using multiple modulators of small metabolite transport and phosphatidylinositol 3-kinase / Akt are needed to explore this question.

Irrespective of the upstream cascade of events, our findings that basophils from patients with nut allergy show substantial activation of mTOR effector phosphoproteins upon nut extract stimulation, while basophils from healthy controls do not, are in accord with current understanding of the role of effector phosphoproteins in activated effector cells. Circulating basophils, although rare, represent a much more accessible subset of effector cells for studies of human allergy than mast cells, and additional studies using the direct phosphoprofiling approach outlined here are warranted. Previously, we showed that anti-immunoglobulin E therapy in NA patients results in diminished levels of surface CD203c in blood basophils [21]. Thus, it will be interesting to assess whether anti-immunoglobulin E, other conventional allergy treatments, or possibly treatments that target mTOR [22], can result in decreased levels of the peIF4E and pS6rp effector phosphoproteins in basophils, in possible correlation with clinical improvement.

Our data showing concomitant increases in phosphorylated and total levels of eIF4E and S6rp proteins in nut extract-stimulated blood basophils is consistent with previous reports showing that phosphorylation as well increased translation of these two effector proteins are indicative of mTOR pathway activation [37, 38]. Thus, it will be of interest for future studies to explore the short- and long-term impact on basophil survival and effector mechanisms of mTOR signaling (considering phosphorylated levels, primarily, but also total levels of effector proteins), both in vitro and in vivo.

Acknowledgments

We thank our subjects for their participation; C. Crumpton and J. Van Dyke at the Stanford FACS Facility for technical support; Drs. K. Atkuri, E. Ghosn, A. Kumar, K. Mukai, P. Sadate-Ngatchou, and M Tsai, as well as C. McDonald-Hyman for critical advice; Dr. V. Saper for recruiting two subjects; and M. Miglianico and E. Hoyte for technical assistance.

Funding Stanford School of Medicine's Dean Fellowship (YG), Stanford Morgridge and Gallo Fellowship (YG) and the Stanford Institute of Immunity, Transplantation and Infectious Diseases Seed Grant (KCN), Orsak Family Fund (KCN), the Skippy Frank Foundation (RT and YG), the Stanford School of Medicine SPARK/SPECTRUM program (YG and KN), and United States Public Health Service grants AI23990, AI070813 and CA72074 (SJG).

Abbreviations

EDTA	ethylene diamine tetraacetic acid
eIF4E	eukaryotic Initiation Factor 4E
HC	healthy control
MFI	median fluorescence intensity
mTOR	mammalian target of rapamycin

NA	nut allergy
PBS	phosphate buffered saline
p	phosphorylated
S6rp	S6 ribosomal protein

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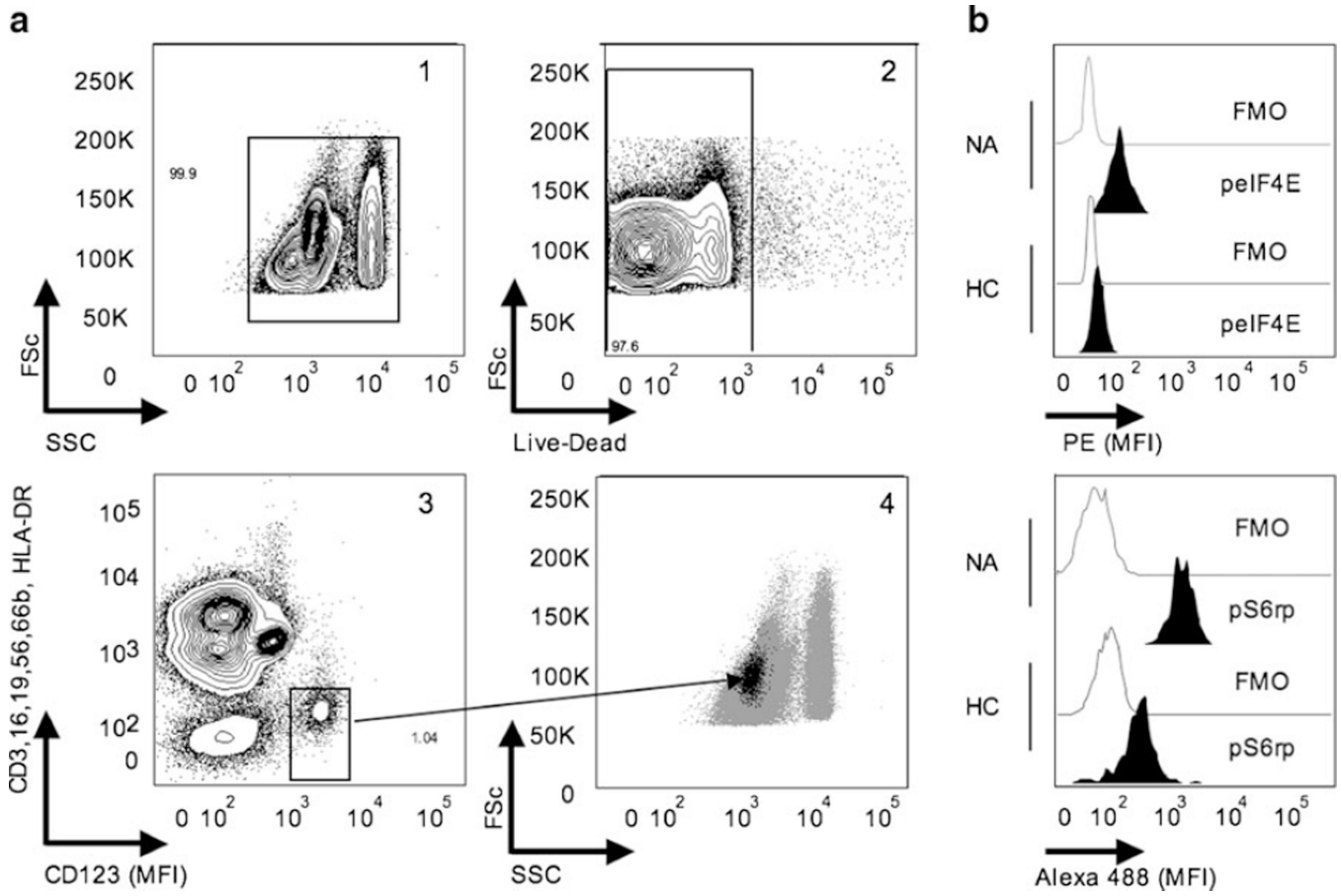

Fig. 1.

Blood basophil phosphoprotein profiling. a: Basophils were selected as the Live/Dead^{lo} / CD3^{-}/ CD16^{-}/ CD20^{-}/ CD56^{-}/ CD66b^{-}/ HLA-DR^{-}/ CD123^{+} subset. b: pelF4E and pS6rp were then quantified in gated basophils. FMO: intracellular phosphoprotein fluorescence control (see Methods section of Online Repository for details). Shown here are representative subjects (one NA and one HC), whose basophils were studied after nut extract stimulation

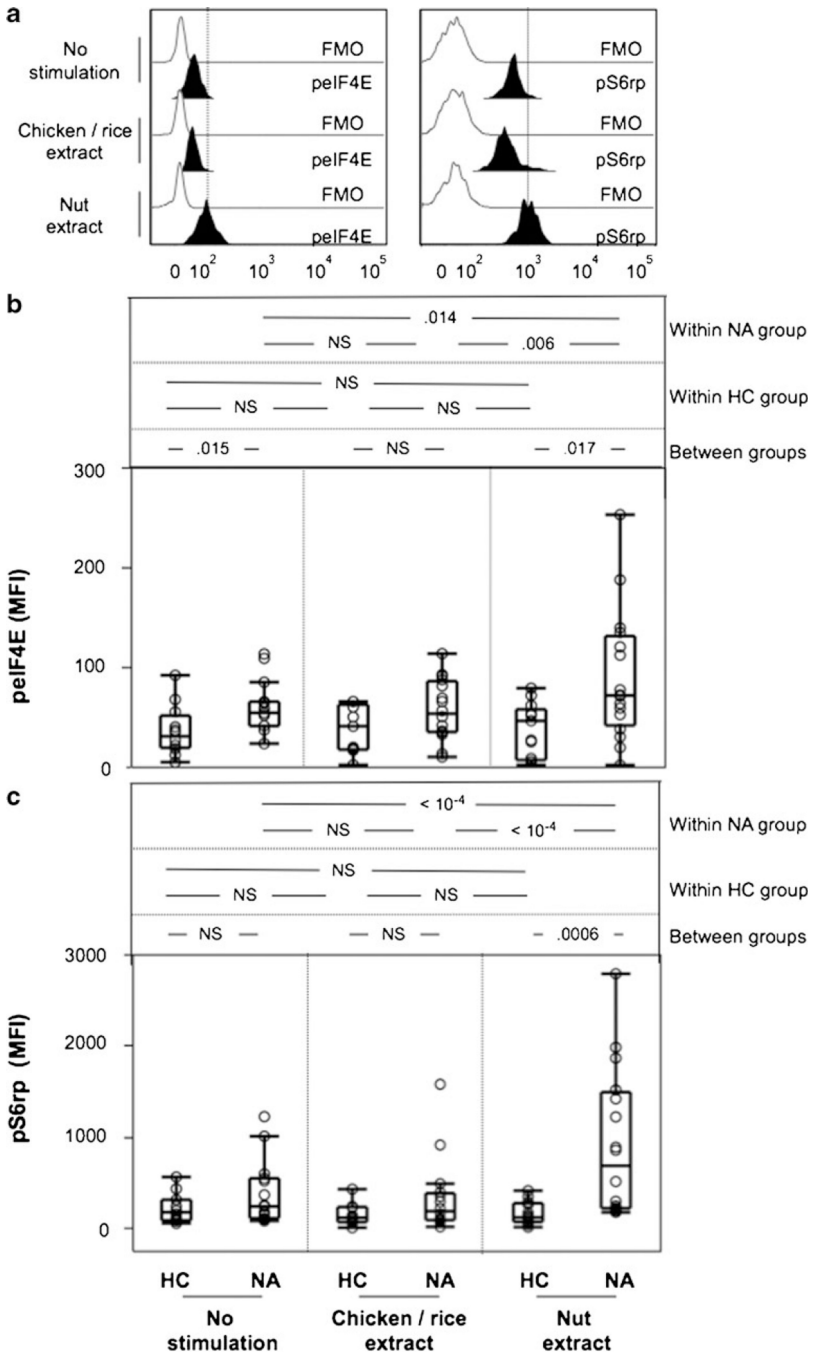

Fig. 2.

Phosphoprotein profiling in blood basophils without stimulation and after 10-minute stimulation with nut or chicken / rice extracts. **a**: pelF4E (left panel) and pS6rp (right panel) were quantified in gated basophils. FMO: intracellular phosphoprotein fluorescence control (see Methods section of Online Repository for details). Shown here is a representative NA subject, whose basophils were studied under no stimulation, and after chicken/rice and nut extract stimulations. **b**: Shown are box plots (delimited by 25th and 75th percentiles, with median line inside and outside bars marking 10th and 90th percentiles) for pelF4E and

pS6rp levels (top to bottom) in blood basophils from healthy control (HC, *N*= 12) and nut allergy (NA, *N*= 15) subjects. Each point represents an individual subject. The significance of differences between HC and NA cohorts was evaluated using the Wilcoxon rank sum test (between-group statistics). Within-group (paired) statistics for this dataset were calculated using the paired test. NS for not significant

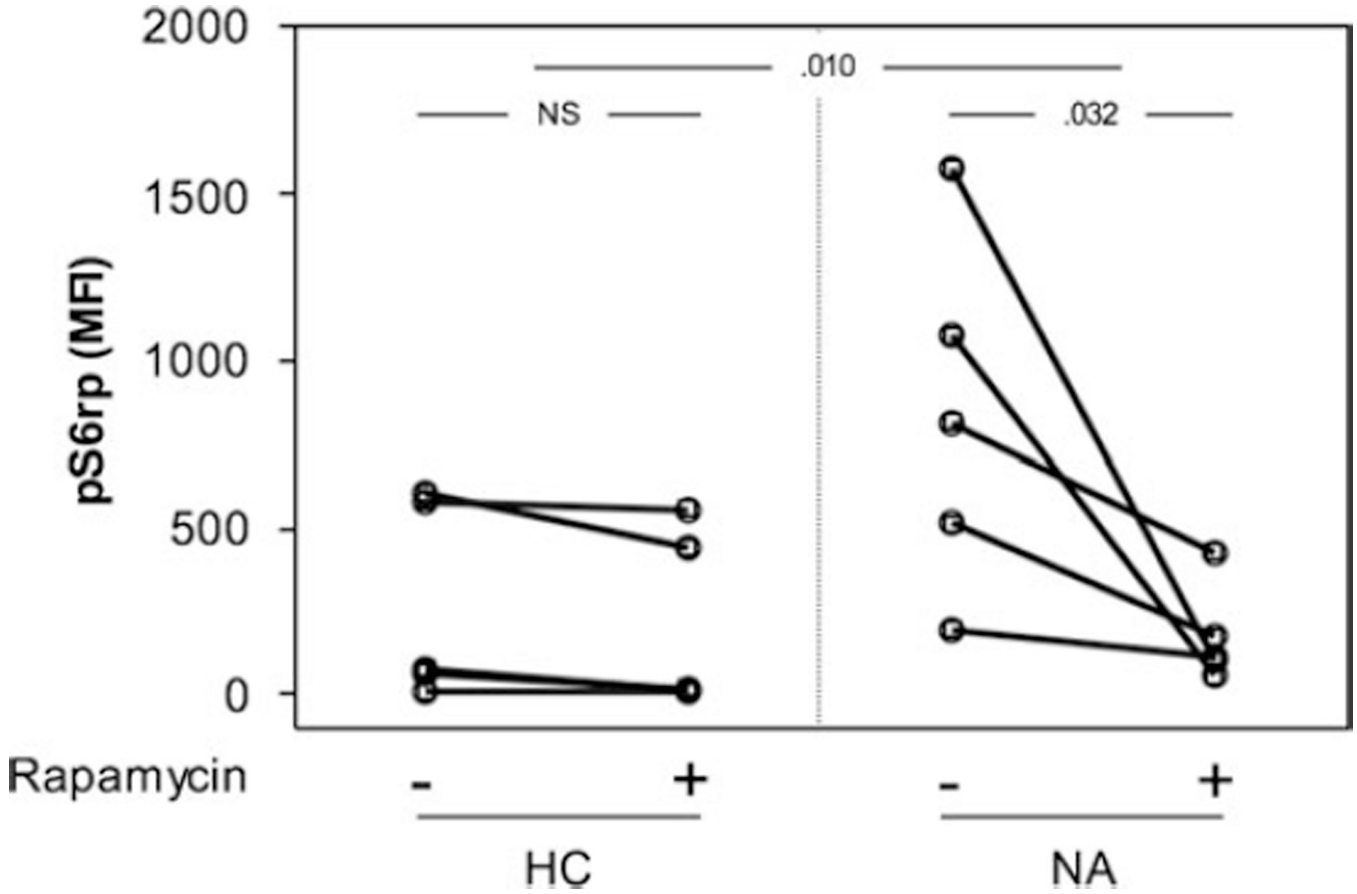

Fig. 3.

Effect of rapamycin pre-treatment on blood basophil pS6rp levels following nut extract stimulation. Shown are levels of pS6rp in blood basophils measured upon nut extract stimulation in HC (*N*=5, left panel) and NA (*N*=5, right panel) subjects, with or without rapamycin pre-treatment. The effect of rapamycin pre-treatment within each cohort was calculated with the Wilcoxon signed-rank test. The difference in the effect of rapamycin pre-treatment between cohorts was calculated with the Wilcoxon rank-sum test

Table I

Characteristics of nut allergy subjects included in the study

Subject	Age (yrs)	Gender	Nut allergen	Nut IgE[a]	Total IgE[a]	Reaction to peanut	Co-morbid allergy
1	10	F	Peanut	12.4	180	Drooling, vomiting	Large local reaction to yellow jacket venom
2	19	F	Peanut	4.5	800	Angioedema, urticaria	None
3	5	F	Peanut	100	527	Angioedema, urticaria, wheezing	Allergic rhinitis, asthma,
4	7	M	Peanut	100	1495	Angioedema, urticaria	Asthma, atopic dermatitis, rhinitis
5	7	F	Peanut	100	279	Abdominal pain, vomiting	Asthma
6	11	M	Peanut	819	2403	Angioedema, urticaria	Allergic rhinitis, asthma
7	6	M	Peanut	100	421	Angioedema, urticaria	Allergic rhinoconjunctivitis, asthma
8	6	M	Peanut	100	2813	Abdominal pain	None
9	4	M	Cashew	13.3	152	Lethargy, vomiting,	None
10	2	M	Peanut	9.33	36	Eye swelling, vomiting	Eczema
11	6	M	Peanut	>100	529	Vomiting	Allergic rhinitis, atopic dermatitis
12	5	M	Peanut	50	721	Abdominal pain, vomiting	Allergic rhinitis, asthma
13	18	M	Walnut	57	431	Angioedema	Atopic dermatitis, rhinitis
14	15	F	Peanut	34.5	596	Cough, throat swelling	Allergic rhinitis
15	9	M	Peanut	>100	–	Cough, emesis, flushing, hives, rhinorrhea	Allergic rhinitis, asthma
16	24	M	Cashew	33.3	471	–	Allergic rhinitis, asthma

[a] Nut and total IgE levels are indicated in kU/L