

Immunotherapy in allergy and cellular tests

State of art

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The basophil activation test (BAT) is an *in vitro* assay where the activation of basophils upon exposure to various IgE-challenging molecules is measured by flow cytometry. It is a cellular test able to investigate basophil behavior during allergy and allergy immunotherapy. A panoply of critical issues and suggestive advances have rendered this assay a promising yet puzzling tool to endeavor a full comprehension of innate immunity of allergy desensitization and manage allergen or monoclonal anti-IgE therapy. In this review a brief state of art of BAT in immunotherapy is described focusing onto the analytical issue pertaining BAT performance in allergy specific therapy.

Introduction

The concept of immunotherapy has changed deeply in recent years. Immunotherapy in allergy has reached a renewed interest among clinicians due to the development of new therapeutic approaches based on standardized allergens taken into account after pivotal Phase III clinical trials.¹ Meta-analyses are powerful tool to elucidate the efficacy of immunotherapy against allergy and also provide for a glimpse on the role covered by recombinant allergens used to desensitize cellular response to allergy.²⁻⁵ Numerous comparative studies with specific immunotherapy vs placebo or pharmacological treatment have demonstrated the efficacy of this approach and its advantage in control of the disease. Allergen specific immunotherapy (AIT) induces favorable clinical, biological, and functional modifications in the course of allergic asthma. Actually, significant improvement in clinical manifestations has been demonstrated, even with levels of allergen exposure higher than those at the beginning of treatment.⁶⁻⁸ This improvement is associated with a reduced need for anti-inflammatory and bronchodilator treatment, with a decrease in healthcare expenditures.^{9,10} Specific bronchial reactivity shows a clear improvement with disappearance of delayed response and a clear increase in the threshold for immediate response to the allergen.¹¹

AIT includes a wide panoply of approaches. It consists of the administration of allergen extracts to patients with allergic disease to achieve clinical tolerance to the causative allergens. A World Health Organization (WHO) leading paper, published in the late nineties, defined immunotherapy as “the only form of treatment able to modify the natural course of allergic diseases”¹². In patients with allergic rhinitis, for example, some studies suggested that immunotherapy can modify the natural history of respiratory allergy by preventing the development of asthma in children with this disease.^{13,14} Sublingual immunotherapy (SLIT), namely a practice of administering sublingual allergens often used for respiratory allergy, is gaining an increasing diffusion worldwide as a consequence of the robust demonstration of clinical efficacy and safety provided by recent high-powered and well-designed studies.^{15,16} The recent and emerging research suggested that SLIT may have a future role in other allergic conditions such as atopic dermatitis, food, latex, and venom allergy.¹⁶ The oral route has recently overwhelmed parenteral administration as occurred in sub-cutaneous immunotherapy (SCIT), due to the supposed better efficacy, safety, and cost evaluation, albeit with some controversial opinion.^{17,18} Oral immunotherapy has been reviewed for peanut, milk, and egg allergy.¹⁹ The World Allergy Organization guidelines for the management of anaphylaxis have reviewed the current literature about the management of atopic hypersensitivity reactions and chronic allergy through AIT, recently reaching also interesting protocol suggestions and recommendations.^{20,21} However, these guidelines lack of further and detailed information about the use of diagnostic tools such as basophil activation tests (BATs). BAT is the only cellular test that is more frequently adopted in allergy investigation.^{22,23} Although different commercial kits and protocol approaches are currently available (Table 1), the increasing development of new immunotherapy strategies has compelled producers to evaluate new incoming and optimized BAT protocols (Table 2). However, the role of cellular test in AIT yet appears quite marginal or even paltry, respect to the supposed predictive potential of hallmarks such as the absence of symptomatic, skin reactive, or serological markers following an AIT protocol. The mechanism of desensitization or tolerance induction following AIT, involves directly innate immune cells,²⁴ thus suggesting that a cellular test may provide fundamental insights in the comprehension of the individual immune response to allergy therapy. BAT is being increasingly considered to test the *in vitro* efficacy of new recombinant desensitizing allergens and, moreover, it is considered also the only way to study the cell biology

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Table 1. Recent allergy immunotherapy reports and associated basophil activation tests (BATs)

Type of bat	Phenotyping strategy	Activation	Pro	Con	Cost effectiveness		References
Commercial					(A) comm/ (B) home		
FlowCAST® Bühlmann	SSC/CCR3-PE	CD63-FITC	SSC allows to well separate eosinophils from basophils	CCR3 co-expressed by CD3+ T-cells		Prep: 1 hour	Sanz et al., J Invest Allerg Clin Immunol, 2002
FlowCAST® 2 Bühlmann		CD63-FITC, CD203c-PE-DY647	Activation measured as CD63+CD203c up-regulation	CCR3 downregulated by basophil activation	A	Perf: 2 hrs	Chim and Cho, All Asthma Immunol Res, 2012
FlowCAST® Higsense Bühlmann			Performance features reported in the sheet.	Inability in evaluating CD63 or CD203c by alone and or discriminate their up-regulation in Higsense		Prep: 2.5 hrs	
					B	Perf: 1 hour	
		CD63+CD203c- PE-DY647			A ≥100-200% more expensive		
Basotest®	Anti-IgE-PE	CD63-FITC	Easy to handle	Activation based only on CD63	A = B		Rodriguez-Trabado et al., All Asthma Proc, 2008
Orpegen Pharma			Low expenditure, easy interpretation and low time-consuming	Low sensitivity respect to CD203c			
			Performance features reported in the sheet.	Dependence on CD63 response			
Basotest™	Anti-IgE-PE	CD63-FITC	Easy to handle	Activation based only on CD63	Less qualified than Orpegen		None reported
Glycotope			Low expenditure, easy interpretation and low time-consuming	Low sensitivity respect to CD203c			
				Dependence on CD63 response			
Flow cytometry protocol (1)	CD45-PC5/CRTH2/ DP2-FITC	CD203c-PE or CD63-PE	Increase in sensitivity due to CD203c	Low diffusion of CRTH2 marker (high cost)	More expensive and time consuming than a common commercial kit		Boumiza et al., Clin Mol All, 2005; Boumiza et al., Clin exp all, 2003
			Capturing marker less dependent on basophil activation status	CRTH2 co-expressed on eosinophils and Th2 lymphocytes			
				Need of a CD3 dissecting marker			

Flow2CAST. 2. Home-made; 3. Basotest® (Orpegen Pharma); OAS, Oral allergy syndrome; AIT, allergy immunotherapy; SLIT, sublingual immunotherapy.

Table 1. Recent allergy immunotherapy reports and associated basophil activation tests (BATs) (continued)

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Commercial					(A) comm/ (B) home	
Flow cytometry protocol (2)	SSC/CD203c-PE	CD63-FITC	CD203c is a basophil specific marker	CD203c shift basophil gate following activation = loss in CD63 expressing cells	Cost likewise a test equipped 2-colors FC	Mikkelsen et al., Clin Mol All, 2010
Flow cytometry protocols (3)	CD45/CD123/	CD63 or/and CD203c	Better discrimination of basophil capture	Elevated cost	Feasibility and handling to address	Chirumbolo et al., 2008
-4	HLA-DR		Electronic purification (>95%)	Time consuming		Ford et al. JACI, 2013
	CD45/CD123/		CD123 does not change the gate with activation			
	HLA-DR/CD41a					

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of peripheral blood basophils with an easily handling procedure.²³ This would mean that BAT further provides to be a cellular test able to dissect basophil response mechanisms to allergy onset and downregulation, a feature that should reappraise BAT as an unavoidable diagnostic tool, even in AIT. Basophil response to allergens is a complex mechanism on which polychromatic flow cytometry may shed a light and enhance the potential of BAT itself as a diagnostic tool, also during allergy therapy and AIT follow up. This review tries to address BAT application and feasibility in the context of AIT.

Role of Basophils in Immune System Following Immunotherapy: Introduction

During immunotherapy, a cellular assay such as BAT might give important insights about AIT efficacy and maintenance dose protocol. Basophils are easily to obtain by a simple venous peripheral blood withdrawal and flow cytometry proved to be a reliable approach to evaluate in vitro response of these cells to many allergens, including food, inhalants, or chemical compounds such as latex.^{22,25} Notwithstanding, very few reports exist dealing with the role of BATs in AIT, while most of them regards immunotherapy of *Hymenoptera* venom allergy.²⁶⁻³⁰ The effect of *A. mellifera* venom on basophils has a nonlinear behavior, namely the *Hymenoptera* venom allergens used in immunotherapy (VIT) showed a biphasic or hormetic pattern on basophil IgE-mediated response,³¹ then making difficult to use BAT as a simple allergy-non allergy probing test. Low doses of honey bee venom (HoBV) can diffuse into the systemic circulation and induce an activated state, while high doses elicit a primed state, but they induce IgE-FcεRIβ desensitization, as allergen/IgE cross-linking increases, a mechanism that allows

many researchers to suggest that the higher the priming is, the greater the desensitization.³¹ This occurred in all stung subjects, both sensitized asymptomatic and hymenoptera allergic subjects, raising issues about VIT maintenance time and doses.^{31,32} VIT effectiveness is strictly dependent on allergy desensitization mechanisms, and an understanding of this represents a major goal of immunotherapy.³³⁻³⁵ This would mean that performing a BAT by simply investigating membrane upregulation of CD63 and CD203c, must take into account the complexity of HoBV interaction with cell response to VIT and the BAT gating protocol employed in the test, in order to better optimize its performance.²³ Such a consideration raised from AIT with hymenoptera venom, may fit to many other immunotherapy protocols with defined allergens, such as dust mites.³⁶

The usefulness of a cellular test likewise BAT has been demonstrated for immune vaccination besides allergy diagnosis.³⁷⁻³⁹ Notwithstanding, basophil response to allergens used in immunotherapy does not seem to fulfil all the expectations raised from a commonly accepted model of the basophil behavior toward an IgE-mediated mechanism.^{31,40} For example, recent evidence showed that treatment with omalizumab results in a markedly increased sensitivity of basophils to an IgE-mediated stimulation in terms of the number of IgE molecules required to produce a given response.⁴¹ Treatment with omalizumab in patients suffering from cat or peanut allergy enhanced the intrinsic sensitivity of circulating basophils as evaluated by histamine and LTC₄ release.⁴¹ The mechanism underlying this increase in IgE-mediated response is yet unclear. During the formation of omalizumab-IgE heterodimers in the serum,⁴² the monoclonal IgG₁-anti-Cε3(IgE) complex, can be recognized by FcγRs in immune and dendritic cells and removed from systemic circulation.⁴³ This evidence suggests that omalizumab may interact with basophil FcγRs and regulate cell response to

surface IgE-FcεRI complexes. Although basophil response to further allergens during anti-IgE immunotherapy has not been yet investigated, the accepted model suggests that clearance of allergens by immunocomplexes (ICs)-mediated competition or trap mechanism, elicits also a downregulation mechanism of FcεRI on basophil membrane, then reducing basophil IgE-mediated response and allergy symptoms.^{41,46} The use of humanized anti-IgE antibodies, such as omalizumab,^{44,45} may raise some concern about the role of mast cell and basophil Fc-gamma receptors in resolving allergy by an anti-IgE immunotherapy approach.⁴⁶ Mast cells, as like as basophils, compete for FcRβ in the allergy IgE-mediated mechanism.⁴⁷ The subunit FcRβ is shared both by FcεRI and FcγRIII, and functions as an amplifier of allergy response, able to increase Syk phosphorylation and activity.⁴⁸ FcγRIII are downregulated by high density surface FcεRI-IgE and in atopic subjects, it is presumable therefore that a decrease in FcεRI may enhance basophil FcγRIII function.⁴⁹ Although basophils may express CD16b (FcγRIII) besides to CD32 (FcγRII), no BAT has been planned or performed to evaluate this issue to date. At the same time, this evidence appears quite paradoxical respect to previous reports, showing a basophil desensitization following omalizumab immunotherapy.⁵⁰ Certainly, immunotherapy may affect basophils directly and their expression of surface markers, such as CD63, CD193, CD203c, CD69, CD164, usually upregulated during cellular activation, may be significantly modified during treatment. Despite to the many difficulties in interpreting BAT,²³ this consideration prompted researchers to evaluate BAT as a promising tool during immunotherapy follow up. In a mastocytosis model, BAT showed a sensitivity of 87% and specificity of 100% in diagnosing wasp venom allergy and an ability in tracing immunotherapy against hymenoptera culprit.⁵¹ Previous reports suggested the routine use of BAT to facilitate prescription of immunotherapy.⁵²⁻⁵⁴ Basophils might exert a quite different task in progressing the hypersensitivity reaction than mast cells and eosinophils, as probably they play a modulating and regulating action in allergy rather than ruling an effector mechanism.⁵⁵ Both the innate and acquired immune networks play an outstanding role in AIT but may hinder a full comprehension of which role basophils exert in the field. Actually, from an immune network perspective, AIT involves an early suppression of innate effector cells of allergic inflammation, including mast cells, eosinophils, and basophils, besides to affect the regulation of pro-allergic T helper 2 type (Th2) and IgE+ B cell responses in the tissue and in peripheral blood.⁵⁶ The role of basophil during AIT is yet far to be fully understood when one considers this complex pathway, fundamentally because of the many puzzling and unsolved issues still existing about the biology of basophils within the immune network.⁵⁷ Frequently, the allergen-tolerant state is associated with local and systemic induction of distinct populations of allergen-specific T regulatory cells, including IL-10 expressing Tregs (Tr1 cells), TGF-β+ Tregs, and FoxP3+ memory Tregs. B lymphocytes are switched in favor of producing IgG (particularly IgG₄) antibodies and associated blocking activity for IgE-dependent events, including basophil activation and IgE-facilitated allergen binding to B cells. Furthermore, an induction of IL-10+ B regulatory cells and alterations in dendritic

cell subsets have also recently been described.⁵⁸ These events are followed by the induction of T regulatory cells, suppression of allergen-specific T cell proliferation and immune deviation from Th2 in favor of Th1 responses. Alternative mechanisms of tolerance include apoptosis/deletion of antigen-specific memory Th2 cells and/or a failure of co-stimulation leading to T cell anergy. The role of T-reg in allergy and immunotherapy has been elucidated quite recently. When the allergen is taken up by regional APCs, such as dendritic cells, as occurs during AIT, it leads to the induction of T-reg.⁵⁸ CD4+ naïve T cells differentiate into distinct T cell subsets such as Th1, Th2, Th9, Th17, and Th22 type memory and effector cells, depending on the cytokines, other molecules and cells present in the micro-environment.⁵⁹ The involvement of acquired immunity and regulatory T-cells in allergy and in immunotherapy with allergens or anti-IgE mAbs, has been reviewed elsewhere,^{56,60-63} while a thorough role of basophils appears yet unclear. Immune tolerance induced by AIT should involve these mechanisms and dampen basophil response to allergen by a T-cell mediated mechanism.⁵⁸ However, very few reports have addressed the active participation of basophil in allergy tolerance following AIT.^{56,64,65}

Basophil Activation Test in Immunotherapy: Allergen/FcεRI Regulation as a Possible Pitfall

Although encouraging evidence has been reported, basophil interaction with innate and acquired immunity appears highly complex to endeavor a comprehensive functional model able to highlight BAT performance during AIT. The first concern is about the allergen dose/FcεRI membrane expression ratio and its regulation on sensitized and/or activated basophils. A sub-threshold desensitization of basophils following the induction of allergy tolerance may lead to loss of Syk and cell surface FcεRI.^{66,67} Both FcεRI membrane recycling and the downstream receptor associated tyrosine kinase signaling, depend on sub-stimulatory or long lasting allergen doses. Kinetics of antigen binding on FcεRI/IgE complex has been reviewed in the past⁶⁸⁻⁷⁴ and may give an interpretation to the complex responsive pattern expressed by basophil during AIT. Past reports showed that the complexity of FcεRI/IgE-allergen interaction, where the allergen may be replaced by an anti-IgE antibody able to cross link the high affinity receptor, should imply that early activation events may be not altered by the process of desensitization and could be maintained at an increased level of activity indefinitely with the maintenance of IgE crosslinks.⁷⁵ Therefore, AIT not necessarily exerts a *tout court* desensitization effect on basophil but a more complex receptor-mediated signaling probably occurs. Single basophils responded in a graded manner,^{76,77} leading to a differential CD63 intracellular endowment and membrane expression mechanism, a fact that may affect the reliability of BATs.⁷⁸ Furthermore, a percentage of basophils show anergy or a non responding phenotype, probably due to mechanisms related to calcium influx,⁷⁹ where basophils showed marked oscillations whose magnitudes were partially dependent on the strength of stimulus,⁸⁰ or intracellular receptor-associated signaling.⁸¹ The

Table 2. Basophil activation tests (BATs): potential, handling, cost-effectiveness, and reliability

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	CD45/CD123/		CD123 does not change the gate with activation			
	HLA-DR/CD41a					

mechanism of basophil desensitization should involve a panoply of different factors, which in turn may hamper a full elucidation of the issue. Probably, several forms of downregulation appear to occur during IgE-mediated stimulation of human basophils and, actually, previous studies reported downregulation in 2 regions of signaling; involving the molecular microenvironment of Syk kinase and PIP3, with a slight involvement of SHIP, which undergoes modest non-specific desensitization.⁸² These results demonstrated a further process that accompanies nonspecific desensitization besides Syk and FcεRI, a persistent mechanism, which may be related to the loss of signaling components such as Syk kinase. These kind of considerations proved to be very important to esplicate the effect of anti-IgE immunotherapy, more than AIT. However, one should expand the debate about the role of basophil response to AIT when a BAT is performed.

Immunotherapy with Allergens and BAT in Sub-Cutaneous (SCIT) and Sub-Lingual (SLIT) Immunotherapy

Immunotherapy with allergens represents a promising way to dampen allergy response against molecules eliciting an hypersensitivity mechanism, although some controversy still exists regarding the potential role of specific immunotherapy (SIT) as a therapeutic intervention for patients with atopic dermatitis, eczema, and asthma.⁸³⁻⁸⁶ A 3-y sub-cutaneous immunotherapy treatment (SCIT), combined with inhaled corticosteroids, resulted as an effective immunotherapy for children with allergic asthma, showing also a reduction of the required corticosteroids dose.⁷ SCIT or sub-lingual immunotherapy (SLIT) approaches were successfully reported also for *D. pteronyssinus* or house dust mite allergy,⁸⁷⁻⁸⁹ besides to rhinitis⁹⁰ and SLIT has been attempted with success also for rhinoconjunctivitis, using ragweed AIT.^{91,92} BAT used in sub-cutaneous immunotherapy against wasp venom allergy, showed an increase in sensitivity during the up-dosing phase of SCIT, an evidence reported by others also for humanized anti-IgE allergy therapy.^{93,94} This transient increase has not yet

fully elucidated. Basophils increased their sensitivity after 3 wk of specific immunotherapy, probably due to a mechanism reflecting competing amounts of immunoglobulin on basophil membrane (IgE) and free immunoglobulin in circulation⁹³ and coincides with the observed early decrease in histamine release and increase in IgG4 and IgA immunoglobulins.^{93,95} This may reduce BAT performance as it could not predict hymenoptera allergy using an allergen challenge at the range 0.1–1.0 μg,⁹³ despite the use of reliable activation markers such as CD63 and CD203c.⁹⁶ More encouraging results were recently obtained by using BAT in SLIT for latex allergy,⁹⁷ where the cellular test proved to be useful in evaluating SCIT efficacy in this form of hypersensitivity.⁹⁸ BAT has been also reported to be a reliable tool to monitor immunotherapy in patients with mastocytosis.^{51,53,93} Bidad and colleagues showed that basophils were less sensitive to wasp venom allergens after reaching the maintenance dose, a result apparently in agreement with previous reports.^{53,93} Encouraging results from early open-label studies have recently sparked great interest both in oral and sublingual immunotherapy, and thus several randomized controlled trials have recently been conducted to establish the safety and efficacy of these treatment strategies.¹⁹ Although some contrasting opinions have been raised about SLIT or oral immunotherapy (OIT) in children,⁹⁹⁻¹⁰² the methodology is gaining increasingly interest. Particularly due to its safety and tolerability,¹⁰³ though issues have been raised about the opportunity to use either sublingual (SLIT) or sub-cutaneous (SCIT) immunotherapy.¹⁰⁴

AIT is also included in the panel against allergic rhinitis,¹⁰⁵ with grass pollen allergens,¹⁰⁶ allergy to *Dermatophagoides pteronyssinus*,¹⁰⁷ asthma,^{108,109} atopic dermatitis.^{110,111} However, immunotherapy with allergens might be a still puzzling research field because of the complex cross reactivity occurring between different allergens, e.g., food or inhalatory allergens,^{112,113} which further affects the reliability of BAT in AIT.¹¹⁴ Some interesting papers on AIT, such as egg or cow milk allergy, did not include or take into consideration BAT in diagnosis and therapy follow up,¹¹⁵⁻¹¹⁸ probably because clinical markers are still preferred than in vitro assays. However, BAT might still give fundamental

insights on the cellular involvement in allergy development and desensitization^{23,119} and this fact may give further chances to optimize BAT for the cellular investigation of AIT.

Immunotherapy with Allergens and BAT: Oral Immunotherapy (OIT) and Food Allergy

Oral immunotherapy for food allergy was attempted for very few allergens, such as peanut^{120,121} or eggs,¹²² still remaining controversial opinions, therefore standing on a pivotal experimental stage.^{19,123-128} Systematic reviews have tried to focus onto the issue and shed a light about current approaches in diagnosing and managing food allergy.¹²⁹ In peanut allergy, for example, many efforts have been recently made,^{120,123,130} albeit doubts remain about reliability and clinical effectiveness of peanut allergy oral immunotherapy (OIT).¹²⁷ Concern about peanut allergy rises up to some years ago^{131,132} but many AIT approaches are quite recent.^{115,133} Well designed, prospective clinical trials are urgently needed to determine whether OIT is a safe, effective form of therapy for food allergy.^{120,121,134,135} In food allergy, immunotherapy is usually performed by oral or sublingual administration of allergens: several research studies have assessed that oral and sublingual immunotherapy for food allergy is generally well-tolerated and safe in highly controlled clinical settings.¹³⁶⁻¹⁴⁰ Reports from the NIAID-sponsored Expert Panel provided concise clinical recommendations and additional guidance on points of current controversy in patient management about food allergy and immunotherapy, yet identifying gaps in the current scientific knowledge to be addressed through future research in the field.^{141,142} Taken these reports altogether, a possible conclusion is that acquisition of oral tolerance to the diverse array of ingested food antigens and intestinal microbiota is an active immunologic process that is successfully established in the majority of individuals. In subjects who develop food allergy, there is a failure or loss of oral tolerance acquisition to a limited number of food allergens. OIT may offer a promising approach to induce specific oral tolerance to selected food allergens, though yet restricted to a small group of molecules, and can represent a potential strategy for long-term curative treatment of food allergy,¹²⁵ though many controversial problems about protocol and allergen dose yet exist, even for other AIT protocols.¹⁴³⁻¹⁵⁰ The application of BAT in oral immunotherapy should consider also the role of secretory IgAs and their receptor on granulocytes.¹⁵¹⁻¹⁵³ Past reports have shown that secretory IgAs induce degranulation in IL-3 primed basophils.^{154,155} The role of secretory mucosal IgA (sIgAs) is fundamental in OIT and SLIT, both for food and inhalant allergens¹⁰⁷ and the investigation of FcαRI (CD89) expression on basophil membrane¹⁵⁶ during a BAT performance would provide important insights about SLIT or OIT efficacy.

Immunotherapy with Monoclonal Anti-IgEs and BAT

Immunotherapy with monoclonal anti-IgEs (moAbs) represents a novelty in the field of allergy therapeutics.¹⁵⁷⁻¹⁵⁹

The recent introduction of monoclonal anti-IgEs, such as omalizumab, allowed physicians to successfully face allergic asthma in patients with refractory asthma or severe asthma that is difficult control with conventional treatment, in which AIT is not indicated.¹⁶⁰⁻¹⁶² Xolair® omalizumab has been investigated in various other conditions including perennial and seasonal allergic rhinitis, idiopathic anaphylaxis, mastocytosis, peanut allergy, latex allergy, atopic dermatitis, chronic urticaria, eosinophilic gastroenteritis, and nasal polyposis, with increasing success (see ref.¹⁶³ for a review). In this context, the analytical assessment of allergy desensitization subsequent to immunotherapy may promote BAT as an eligible tool to highlight allergy in individuals undergoing treatment.^{40,164}

Particularly for omalizumab physicians and researchers have suggested this anti-IgE moAb as a reliable and promising tool against allergy, though with some raised criticism.¹⁶⁵⁻¹⁶⁹ Omalizumab is an IgG₁ recombinant humanised monoclonal antibody which specifically binds to the Cε3 domain of immunoglobulin E, targeting the site of high-affinity IgE receptor binding.¹⁷⁰ The anti-IgE therapy has been also considered in association with specific AIT in seasonal allergic rhinitis or asthma, providing encouraging results particularly for omalizumab,^{171,172} an approach aimed at addressing atopic asthma in childhood, which still represents a big concern in allergy.¹⁷³ However, questions remain about the feasibility of omalizumab-related therapy, mainly because of difficulties in patient eligibility.¹⁶⁹ Committees, such as the NIH and Clinical Excellence (NICE) Appraisal Committee and the resulting NICE guidance for the Evidence Review Group (ERG), were planned and made up by Governmental foundations to assess the use of omalizumab in AIT.¹⁷⁴ This recombinant anti-IgE moAb has been investigated in several other allergic conditions, and has become a promising immunotherapeutic for several forms of allergy.^{163,175} The use of BAT in omalizumab therapy does not seem to account on a significant amount of practitioners, except for very few reports.^{176,177} Xolair®-Omalizumab is probably the most known anti-IgE moAb used in allergy therapy; however, many further attempts have been recently made to optimize immunotherapy with monoclonal antibodies.¹⁷⁸⁻¹⁸⁰ Monitoring omalizumab or other moAbs effect on innate immune cells involved in allergy, particularly basophils, may represent a cumbersome issue for health practitioners, due to the many reasons described below. First, the use of an anti-IgE-fluorochrome labeled to gate basophils, such as using Orpegen Basotest®, may give bias because of the dramatic effect caused by anti-IgE monoclonal antibodies on membrane FcεR1s.⁴¹ Khan and colleagues used a BAT where cells were captured following the expression of IgE (anti-IgE-FITC) and CD123-PECy5 and non-expression of HLA-DR-APC, besides the absence of CD3, CD8, CD14, CD19. While they excluded subjects undergoing omalizumab treatment, their BAT was affected by a significant proportion of false-positive results, due to the anti-IgE phenotyping approach.¹⁸¹ The possible cross-reaction with surface FcγRs on basophils by the IgG₁-omalizumab may affect the upregulation of those markers related to the degranulation process, such as CD63. CD63 is actually highly sensitive to the many aspects

regarding the complex pattern of response to allergens and anti-IgEs exhibited by basophils,^{22,23,36,40,93} Granulocytes FcγRs, when activated, induce Th1 differentiation in allergy.¹⁸² Furthermore, past reports showed that the expression of IgG₄ is IL-4 related, likewise IgE.¹⁸³ These facts assess that the role of FcγRs in basophil response to omalizumab might play an utmost role also in CD63 expression and basophil releasability and influence the interpretation of the assay. The increasing diffusion of Xolair®-omalizumab in treating allergy^{175,184,185} raises important issues about the opportunity to reappraise and review BAT protocols and application in subjects undergoing omalizumab treatment.

The Basophil Activation Test (BAT) During Immunotherapy: Technical Issues

Aside from some technical issue or detail and the recognized performance of CD63, BAT protocols has not yet fully optimized to date to fit as a reliable tool in evaluating immunotherapy follow up and recovery, despite to some promising discussion about.⁴⁰ Current BATs are usually interpreted as a simple test-bench of IgE-mediated response, without considering important effects on CD63 membrane expression and releasability from IgAs or IgGs.¹⁸⁶ Furthermore, recent research advances have suggested the existence of at least 2 subpopulations of basophils, IL-3-elicited and TSLP-elicited basophils, rendering quite tangled the meaning and diagnostic potential of a BAT in immunotherapy, especially for CD203c.⁵⁷

Table 1 summarizes most recent BAT performing in AIT. The response of basophils to any defined allergen is evaluated by the membrane upregulation of proteins, usually related to basophil releasability, i.e., histamine and LTC₄ release, such as CD63 and CD203c.^{22,40} Most of recent BATs were performed in *Hymenoptera* venom AIT.^{51,52} During *Hymenoptera* venom immunotherapy (VIT) a rise in CD63 was previously reported.¹⁸⁷ The increase in CD63 membrane expression during VIT indicates a partial basophil degranulation with release of stored protein mediators, including IL-4, a cytokine that may cause in turn a transient upregulation of different surface antigens in an autocrine manner.⁵⁵ Thereafter, cytokines released by T cells, which as a result of VIT have changed from a Th2 type to a more Th1 type, downregulate the activation of the basophilic granulocytes. In a phase I clinical trial on peanut AIT, BAT, assessed at baseline and at weeks 16 and 20 following AIT with *E. coli* encapsulated peanut recombinant allergens (Ara h 1, Ara h 2, Ara h 3), reported a single increase of CD63% from baseline at the 0.01 µg/mL concentration ($P = 0.05$), while no other differences between the reactive and non reactive subjects, were shown.¹⁸⁸ In those subjects with peanut allergy, about 50% included individuals who were unable to complete the AIT protocol, leading to the conclusion that the vaccine, through a rectal route of administration, led to frequent and sometimes severe allergic reactions, in spite of the approved scientific rationale and reliable preclinical studies behind its development and the very low positive results in BAT.¹⁸⁸ MacGowan and Saini recently investigated the role of BAT in immunotherapy, reaching

the conclusion that BAT is a useful in vitro tool for the diagnosis of allergy.⁴⁰ The main goal of a BAT during immunotherapy or allergy vaccination should be restricted to its ability in testing allergy desensitization following an AIT maintenance dose protocol or alternatively to provide instructions about the opportunity to interrupt or change the allergen dose. The many reports dealing with the issue, though some encouraging evidence, do not seem to shed a light on BAT reliability in performing an AIT follow up, however. Some concern raises from BAT protocol in itself. Following CD63 membrane up-load should be reviewed and revised taking into account the fact that this marker is highly influenced by basophil ability to respond to any allergen or anti-IgE challenge^{78,189-191}; BAT CD63 sensitivity ranges from 17% to 94%, with a specificity of 79–100%,⁴⁰ while CD203c resulted in a higher performance¹⁹²; this would suggest that CD203c should be a more reliable marker to ascertain allergy desensitization but it is highly influenced by IL-3 activity and is not always directly related to degranulation.⁵⁵ Figure 1 suggests a possible approach of BAT application during an AIT protocol. Before carrying out a BAT for immunotherapy follow up, some “analytical barriers” (indicated with capital letters) may generate bias or weakness in the analytical performance of the test. Furthermore, performers should select whether purchase or build up the assay with a non commercial protocol. This condition may enrich BAT endowment and potential, both in phenotyping and evaluation of basophil activation, but the addition of many markers might hamper the readability of the test in diagnosing allergy.²² A second phase of the BAT application in a planned AIT protocol should involve an accurate investigation of basophil sensitivity to the allergen, associate to its IgE responsivity, in order to better elucidate the responsive status of basophilic cells (section D in Fig. 1). Building up a new flow cytometry protocol by considering a previously reported method and adding new activation markers besides CD63, may enhance BAT reliability and diagnostic performance but the resulting BAT may be characterized by a more expensive or time-consuming pattern, respect to a simple purchasable kit. Basophil activation tests, which are routinely employed in allergy, allow cell electronic capture by targeting surface IgE in a flow cytometry protocol. During an anti-IgE immunotherapy, detect basophils by targeting high affinity FcεRI-IgE receptor complex, may result in a time-consuming procedure and low affinity FcεRII bearing cells might affect significantly basophil yield in the gating strategy¹⁷⁶; this may be the case to gate basophils using other phenotyping markers. Table 2 summarizes the main hallmarks of BAT performance useful to take a decision for AIT. Most commercial kits permit to make a number of about 100 tests, obviously including controls, both negative and positive, thus yielding an average number of 30 triplicate samples in the same analytical run. This is approximatively the half amount of tests that can be performed by a *home made* BAT with a defined panel of fluorochrome-conjugated monoclonals, if optimizing the staining protocol.^{189,193} Phenotyping markers, likewise molecules tracing the activation mechanism of basophil, are restricted to very few molecules in available commercial kits. Each marker has its own analytical hallmark, which may affect the performance of

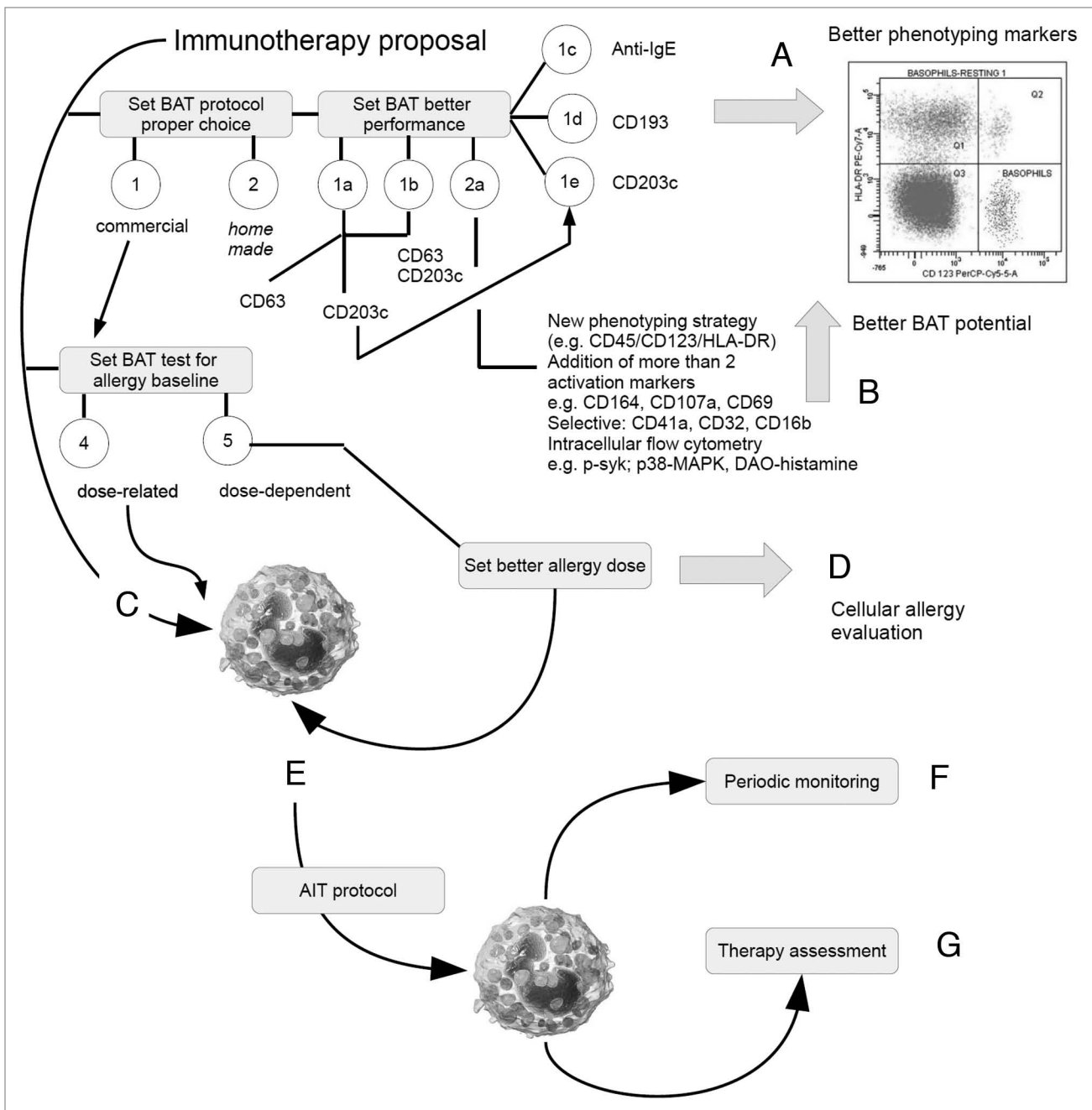


Figure 1. Cartoon showing a possible panel of decision strategy for the optimization of BAT use during AIT. Each step, indicated by a capital letter, represent an “analytical barrier,” which the operator must address in order to reach the best quality performance of the assay. Starting from a dual choice (commercial or *home made*) a BAT must address “analytical barriers” represented by the proper choice of gatig markers (**A**) and activation/intracellular markers (**B**). Usually, due to limited needs regarding cost/effectiveness and feasibility/handling, practitioners select commercial one-step, dose-related 2-colors FC protocols (**C**). Once chosen the methodology, further barriers are represented by the allergen sensitization protocol (**D**), desensitization process (**E**), and follow up during or post maintenance (**F**). *Home made* protocols with a dose-dependent method of allergen response calibration (**D**) allow a better interpretation of the cellular response during AIT.

the assay,^{190,194} therefore a BAT made up within one's own laboratory might provide the researcher with more diagnostic advantages. Building up a CD45/CD123 gating protocol and investigating the expression of CD63 and CD203c, Subbarayal et al., showed that BAT was sensitive to IgG₄ blocking immunoglobulins produced by birch pollen allergens as well as

other cross reactive ones, such as Mal d 1 (hazelnut) and Cor a 1 (apple) for food allergy.¹⁹⁵ The protocol by which practitioners succeed in capturing basophils through the electronic gate in flow cytometry, plays a fundamental role in ensuring the reliability of a BAT, therefore. However, in this field, handling preventing a time-consuming approach, associated to a rapid

interpretation of the result, prompts operators to select a commercial kit rather than building up a new BAT protocol. The best suggestion, if the flow cytometry method includes more than 2 markers, is to privilege the best phenotyping protocol rather than adding more than one activation markers. At the same time, basophils are hardly ever investigated about their responsiveness status, which may influence markers such as CD63²³ or CD203c.¹⁹⁶ Intracellular flow cytometry should address many disturbing issues, which may hamper a clear interpretation of BAT in AIT and then provide a more reliable diagnostic tool.¹⁹⁷ However, once selected the best BAT to perform for AIT, a further barrier is represented by the sensitivity to the allergen provided in the immunotherapy protocol. Depending on allergen cross-reactivity or competition with other allergy eliciting molecules, on surface IgE density and LAMP-3 (or CD63) granule-associated intracellular endowment, the response of basophils to different doses of allergens used for immunotherapy, may be significantly complex and should ask for a careful interpretation. Moreover, well conducted randomized controlled clinical trials in AIT, may use very simple and poorly predictive BATs, so possibly underestimating the role of a cellular test in the study.¹³⁰ The recommended use of BAT in vaccination may give possible suggestions²³ but criticisms about its routine use and meaning are yet far to be fully prevented. However, most of the reported studies show BATs as simple confirmatory assay of allergen-induced desensitization,^{198,199} while cellular test should indicate fundamental parameters in innate immunity involved in allergy desensitization, even during the maintenance protocol. Furthermore, BAT is frequently used to assess in vitro allergen immunotherapeutics, often as a probe to detect AIT working.^{199,200} These circumstances might deject researchers to use BAT as a finest diagnostic tool to probe basophilic cell behavior during allergy desensitization with immunotherapy. Even the role of membrane markers, the expression of which is elicited by an anti-IgE mediated response, merits further attention.

CD63 membrane expression has been reported to be associated with degranulation and releasability (histamine and LTC₄) in basophils but not in mast cells.^{193,201,202} Notwithstanding, CD63 expression may be dissociated from releasability and induced by other non-IgE mediated stimuli, such as PAMPs²⁰³; this should suggest that the use and interpretation of CD63 must be reviewed in light of the many issues raised from recent reports about innate immunity and allergy.¹¹⁹ The nonlinear behavior of the main molecules used in BATs has suggested the introduction of further activation markers^{194,204,205} but none of them was highlighted as molecules actually able to improve BAT in immunotherapy. CD164 was reported as a reliable marker to investigate pollen allergy diagnosis²⁰⁶ but its evidence in immunotherapy is yet scanty. The 4-color flow cytometry protocol CD123/HLA-DR with CD63 and CD203c^{189,190} was used in peanut-allergic children with encouraging results.²⁰⁷ BAT protocol was built within the research laboratory and allowed to assess the hypothesis that effector cell anergy could contribute to clinical desensitization.²⁰⁷ Other protocols likewise, most introducing also CD41a, were used to diagnose and treat milk allergy^{208,209} and showed a good linearity between basophil desensitization and heat-denatured

milk tolerant children. Therefore, questions remain. As BAT still remains the only cellular test that is widely used to assess allergy diagnosis and AIT follow up, further efforts have to be made to ameliorate its performing and meaning.

Though this technique has not yet been standardized, IgE, CCR3 (CD193), CRTH2 (CD294), and CD123 are commonly used to identify basophils in the peripheral blood as positive markers, often by alone or in association with negative markers such as HLA-DR, whereas CD63 and CD203c are frequently used as markers of IgE receptor activation, although many other molecules have been envisaged to probe basophil activation.

Some concluding remarks, to be kept in mind before approaching to a BAT protocol, are suggested as follows:

- 1) BATs based on a 2-colors flow cytometry (FC) are easy to handle, less expensive, and short time-consuming. They detect basophil response by targeting only CD63, which has a good relationship with cell releasability but is affected by the many issues raised in this article;
- 2) FC 2-colors BATs based on anti-IgE basophil capture may create bias in omalizumab-mediated allergy desensitization;
- 3) FC 2-colors BATs based on CD193 (CCR3), the eotaxin receptor as a phenotyping marker, have to solve possible bias related to CCR3 dependency on activation and CCR3 expression on CD3+ cells;
- 4) BATs using CD203c as a phenotyping marker should address the fact that CD203c expression is significantly related to IL-3 and moves with allergen or anti-IgE challenge on FcεRI;
- 5) Commercial FC 2–3-colors BATs are apparently cheaper but may prevent a full and thorough use of BAT as a cellular test;
- 6) Better phenotyping markers should possess most of these hallmarks: (1) highest specificity, they should be expressed only on target cells; (2) bright, highly expressed molecules; (3) not affected by activation;
- 7) Activation followed by CD63 should be optimized by adding other markers, even including intracellular FC.
- 8) A good BAT protocol should be set by considering a (bright + negative) highly specific/selective couple of phenotyping markers and 2 activation FC molecular probes (membrane markers and/or intracellular molecules).

Conclusions

Despite the encouraging role attributed to BAT, yet the behavior of basophils in allergy desensitization through allergen vaccination or immunotherapy with anti-IgEs is yet far to be elucidated. So, when performing a BAT following AIT, which is the meaning of a CD63 or CD203c membrane expression in a laboratory perspective, so to shed a light on AIT follow up and dose maintenance? The current status of our knowledge does not permit yet to find a conclusive remark and an outstanding or reliable answer to this question. The basophil activation test is considered an in vitro assay in which the activation of basophils upon exposure to various stimuli triggering allergy is measured by flow cytometry. In this sense, the basophil activation test has

been validated in many IgE-mediated conditions, including drug allergy, food allergy, venom hypersensitivity, and pollen allergy. Furthermore, in recent years, the application of this test has been expanded to include quinolone and NSAID drug allergy, differentiating sensitization from true allergy, determining the allergenic potential of CCD determinants, and monitoring the success of immunotherapy. State-of-art of BATs in immunotherapy can be described as a progressing context where physicians and allergologists more frequently adopt this strategy to confirm or deny an occurred allergy desensitization. The use of BAT should give insights about how AIT is progressing, rather than focusing onto the desensitization process by an all-or-none

mechanism, captured by analyzing the amount of molecules on cell membrane through flow cytometry. The basophil activation test continues to be a useful in vitro tool for the study of allergic disease but needs further insights in order to better fit its feasibility to AIT diagnosis and follow up. Cellular tests possess the fundamental potential to focus onto the innate immunity causing allergy desensitization: in this sense, BATs represent irreplaceable tools to investigate allergy mechanism during allergen or anti-IgE immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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