Protein Tyrosine Phosphatase Inhibition Mimics FcεRI-induced Anergy and Reveals the Immunomodulatory Role of Hydrogen Peroxide as a Signaling Molecule in Human Basophils

Caitlin Woo, Steven Yoo, Winnie Chan, Hugh A. Sampson, Wayne G. Shreffler *

New York, New York

Key words: human basophils, desensitization, basophil anergy, syk kinase, Erk, lyn kinase, protein tyrosine phosphatase, hydrogen peroxide, intracellular phosphoflow, CD63, CD203c

1 This study was supported by AI-079544 and CoFAR
* Reprint Requests: The Immunology Institute and the Jaffe Food Allergy Institute of Mount Sinai School of Medicine - box 1198, One Gustave L Levy Place, New York, NY 10029
Email address: wayne.shreffler@mssm.edu (Wayne G. Shreffler).
URL: http://www.iisinai.org/shreffler (Wayne G. Shreffler).

Preprint submitted to Elsevier Science 1 August 2017
Abstract

- anergy induction for CD63 upregulation has the same characteristics reported for the suppression of histamine secretion
- CD203c is upregulated independently of CD63 and could be useful as a marker for desensitization
- Basophil anergy is pathway, but not antigen specific
- hydrogen peroxide is produced by oxidative burst in basophils and it can modulate the response to IgE-mediated activation
- hydrogen peroxide augments the phosphorylation of Syk, Btk, Erk by IgE cross-linking
- degranulation, however, is suppressed (as is 203 upregulation) – thought to be via PTPs (orthovanadate also has this effect).
- A distinct pathway for Erk phosphorylation is activated, and those cells are anergized for IgE, but not fMLP stim
- while peroxide effect is thought to be secondary to PTPs, neither lyn, nor syk are activated

Word Count = 1701
Introduction

Successful immunotherapy (IT) of IgE-mediated allergic disease is defined clinically as a long-lasting diminution of immediate hypersensitivity, i.e., greater tolerance, that is induced by repeated antigen exposure over a relatively long time period (e.g. weeks-to-months). In contrast, the clinical term ‘desensitization’ is generally used to describe a transient hyporesponsiveness that is rapidly induced over hours by repeated administration of increasing, but – ideally for the patient – sub-threshold antigen exposure. The mechanisms of this desensitization or anergy-induction are thought to be distinct from IT as evidenced by its contrasting transience and antigen non-specificity.

In settings of IgE-sensitization and ongoing antigen exposure anergy, hypersensitivity reactions may also be suppressed by effector cell anergy. For example, patients with clinical tolerance despite readily detectable specific IgE to food allergens have been observed to experience new onset hypersensitivity after a period of avoidance. We recently reported evidence of non-specific suppression of basophils in milk-allergic children with partial tolerance when they begin to regularly ingest milk protein.

Given new and accumulating evidence for the immunomodulatory role of effector cells such as basophils, it is important to better evaluate the nuance of their responses to antigen in both models of disease as well as human subjects. Suppression of degranulation by chronic allergen exposure, may or may not be associated with the suppression of other effector responses, such as leukotriene synthesis or cytokine production.

Mast cell or basophil anergy, as defined by the suppression of histamine release
after FcεRI cross-linking, can be readily induced \textit{in vitro}, but it is unknown whether the mechanisms of this effector cell down-regulation are induced during clinical desensitization. In this study, we tracked surface and intra-cellular events occurring in basophils at the single-cell level during stimulation via FcεRI in order to evaluate the role of hydrogen peroxide as a second messenger and better characterize the phenotype of anergized basophils in a manner that could be applied to future human studies.
Methods

Reagents.

Basophil isolation.

Measurement of oxidative burst.

In vitro basophil activation and desensitization.

Measurement of surface and intracellular basophil activation.

Data analysis.
Results

The kinetics and distribution of IgE-induced LAMPs and CD203c up-regulation are distinct.

We have compared the expression of CD107 (LAMP-1), CD63 and CD203c over time following anti-IgE stimulation (Figure 1). The distribution of both LAMPs is clearly bi-modal and tightly correlated, consistent with an all-or-nothing classical degranulation response. The peak percent positive response is at 15 minutes and by 120 minutes, the majority of the cells no longer express high levels of LAMPs on the plasma membrane. In contrast, the distribution of CD203c remains more unimodal during activation for most donors, is near maximal by 5 minutes, and remains elevated at 120 minutes.

The expression of CD63 and CD107 is highly correlated, though CD107 appears to be down-regulated slightly ahead of CD63, resulting in the emergence of CD63+ CD107dim cells by 45 minutes. CD63+ cells are also CD203c^bright and consistently appear as a subset of cells that have up-regulated CD203c (Figure 1).

CD203c up-regulation is not dependent on extracellular calcium.

IgE-induced histamine release from basophils is dependent on extracellular calcium. We compared the sensitivity of CD63 and CD203c up-regulation to extracellular calcium depletion using EDTA. Consistent with the hypothesis that CD63 up-regulation corresponds to degranulation, anti-IgE-induced CD63 up-regulation was suppressed by calcium depletion, CD203c up-regulation, how-
ever, was not (Figure 2).

**FcεRI cross-linking under calcium free conditions induces a pathway-specific anergic state in basophils that can be measured by flow cytometry.**

In vitro induction of anergy in basophils (and mast cells) has been studied for many years as a method for interrogating the FcεRI signaling pathway and its regulation and for its potential relevance to in vivo desensitization. FcεRI cross-linking on basophils in the absence of extracellular calcium, renders them unable to degranulate upon subsequent stimulation through the same pathway, as measured by histamine release.[5] We hypothesized that the CD63 response would be similarly suppressed in anergized cells. Normal donor basophils were isolated (>80% purity) and stimulated first in the absence of extracellular calcium with anti-human IgE, washed and then stimulated again in physiological concentrations of calcium. As expected, in those cells that were first stimulated in the absence of calcium, there was a dose-dependent suppression of the CD63 response following the secondary stimulus (Figure 3).

Cells anergized by stimulation via the IgE receptor have been reported to be suppressed in pathway specific, but non-antigen specific manner.[6] To determine whether the same was true of the CD63 response in our experiments, we also sensitized normal donor basophils with monoclonal IgE specific for the hapten, 4-hydroxy-3-nitrophenylacetyl (NP). In this way, the small fraction of unoccupied FcεRI receptors of the cell become occupied with NP-specific IgE, while the majority are occupied with polyclonal donor IgE. When those cells are stimulated with NP-BSA after mock desensitization with unconjugated BSA, a small fraction are induced to upregulate CD63. This sub-optimal
stimulation, however, is sufficient to induce anergy: the median percent CD63 response in nine experiments was reduced from approximately 20 to 5% (Figure 4).

*Basophils produce hydrogen peroxide during FεRI cross-linking.*

Nolan and others have shown that hydrogen peroxide is generated during B cell receptor (BCR) cross-linking and acts as a second messenger to enhance tyrosine kinase-dependent signaling by specifically and transiently inhibiting protein tyrosine phosphatases (PTPs).[7] FεRI signaling, like BCR, involves activation of Src family kinases (SFKs) and recruitment of Syk to phosphorylated ITAMs present in the cytoplasmic tails of receptor complex membrane proteins. It has been previously reported from cultures of mast cell lines and basophil-enriched leukocyte preparations that activation by IgE cross-linking produces reactive oxygen species (ROS).[8] We hypothesized that PTP inhibition may be important for basophil anergy.

Using the fluorochrome dihydrorhodamine (DHR), we demonstrated that reactive oxygen species are induced by FεRI cross-linking (Figure 5). Increased DHR was detectable at 30 minutes post stimulation and was strongly inhibited by the superoxide dismutase inhibitor, diethyldithiocarbamic acid (DETC) and partially inhibited by N-acetylcystein (NAC).

In order to test the hypothesis that H$_2$O$_2$ would enhance signaling, we investigated the effect of exogenous H$_2$O$_2$ on the levels of phosphorylated Syk and Erk 1/2. Like Nolan, we found that the addition of H$_2$O$_2$ at the time of receptor cross-linking enhanced downstream phosphorylation of Syk and Erk (Figure 5).
H$_2$O$_2$ by itself also modestly induced detectable pErk. Reth has shown that H$_2$O$_2$ is sufficient by itself to mimic some effects of BCR activation. 

H$_2$O$_2$ was unable to induce degranulation by itself at any concentration tested up to 10 mM (not shown).

PTP inhibition is sufficient to induce anergy.

Because H$_2$O$_2$ enhanced phosphorylation of Syk and Erk, we next hypothesized that H$_2$O$_2$ would also enhance degranulation. The effect of H$_2$O$_2$ on degranulation, however, proved to be dependent on the timing of its administration relative to receptor cross-linking. Stimulation with H$_2$O$_2$ at time points -5, 0, +1 minutes relative to anti-IgE was inhibitory, while stimulation at +5, and +15 enhanced degranulation (Figure 7). The specificity of this effect to PTPs was suggested by the absence of any suppressive effect on fMLP signaling (not shown). Treatment of basophils with the irreversible PTP inhibitor, orthovanadate, also had the same suppressive effect (not shown). Cell viability was also unaffected by concentrations up to 10 mM (not shown).

Like FcεRI cross-linking in calcium free conditions, peroxide treatment alone induced pathway-specific anergy. Hydrogen peroxide is a short-lived mediator with a half-life measured in seconds. We hypothesized that H$_2$O$_2$-induced anergy was secondary to a phosphorylation event occurring as a result of transient PTP inhibition.
H$_2$O$_2$-induced anergy is dependent on a Src-family kinase, but not dependent on Syk activation.

MacGlashan et al. has previously reported that the pathway-specific anergic state induced by Fc$\varepsilon$RI stimulation is a membrane proximal event that is not blocked by specific pharmacological inhibition of PI3K or Syk,[5] though it is blocked by SFK inhibitors, such as PP1 and PP2.[10] We found a dose-dependent inhibition of degranulation as measured by upregulation of CD63 using inhibitors of SFKs (PP2), Erk (PD98059) and Syk (picetannol) PI3K (wortmannin); PP2 and wortmannin also blocked CD203c upregulation (not shown). Neither wortmannin nor picetanol were able to block H$_2$O$_2$-induced anergy (not shown), however, PP2 treatment did prevent anergy induction as predicted from MacGlashan’s findings (Figure 8). There are nine mammalian SFKs that have been described. Two members, Lyn and Fyn, have been repeatedly implicated in basophil activation. We specifically looked for evidence that PTP inhibition by H$_2$O$_2$ induced phosphorylation of either of these molecules. Phosphorylation was detectable with control anti-IgE stimulation, but not with peroxide treatment (not shown). Other SFKs have been shown to play a role in Fc$\varepsilon$RI signaling, including Fgr in the mast cell-like line, RBL-2H3, and Hck in mast cells.[11,12] It may be that activation of one or another of these SFKs leads to the anergic state and identification of that event may represent a specific marker of anergy that could be used for ex vivo translational studies.
Discussion

Lichtenstein and MacGlashan demonstrated in vitro anergy and have carefully discriminated antigen-specific and non-specific forms and at least some of the mechanisms that underly them. Antigen-specific anergy has been associated with weaker signaling, involving fewer IgE receptor complexes and transient phosphorylation of syk.[13]
References


List of Figures

1. Kinetics and distribution of LAMP (CD107 and CD63) versus CD203c during basophil activation with anti-IgE. 15
2. Effect of extracellular calcium on the upregulation of activation markers. Solid open histo-grams with calcium, filled histograms without. Broken line histogram unstimulated. 16
3. Basophils are anergized following FceRI cross-linking. First stim indicated on x-axis. In comparison to PBS treated control, stim with anti-IgE rendered cells hyporesponsive to a second round of stimulation (indicated by color, see legend). 17
4. Anergy is not antigen-specific. The hapten, NP, induces anergy in basophils that have been sensitized with anti-NP. 18
5. Anti-IgE stimulation of basophils induces reactive oxygen species. 19
6. H$_2$O$_2$ enhances anti-IgE induced phosphorylation of Erk and Syk. Solid lines indicate median; broken lines represent 25-75th percentile; n=9. 20
7. PTP inhibition at early time points relative to IgE receptor cross-linking blocks degranulation. 21
8. The SFK inhibitor, PP2, blocks H$_2$O$_2$-induced anergy. 22
Fig. 1. Kinetics and distribution of LAMP (CD107 and CD63) versus CD203c during basophil activation with anti-IgE.
Fig. 2. Effect of extracellular calcium on the upregulation of activation markers. Solid open histo-grams with calcium, filled histograms without. Broken line histogram unstimulated.
Fig. 3. Basophils are anergized following FcεRI cross-linking. First stim indicated on x-axis. In comparison to PBS treated control, stim with anti-IgE rendered cells hyporesponsive to a second round of stimulation (indicated by color, see legend).
Fig. 4. Anergy is not antigen-specific. The hapten, NP, induces anergy in basophils that have been sensitized with anti-NP.
Fig. 5. Anti-IgE stimulation of basophils induces reactive oxygen species.
Fig. 6. H$_2$O$_2$ enhances anti-IgE induced phosphorylation of Erk and Syk. Solid lines indicate median; broken lines represent 25-75th percentile; n=9.
Fig. 7. PTP inhibition at early time points relative to IgE receptor cross-linking blocks degranulation.
Fig. 8. The SFK inhibitor, PP2, blocks H$_2$O$_2$-induced anergy.