

Chapter 15

Basophil Stimulation and Signaling Pathways

Edward F. Knol and Bernhard F. Gibbs

Abstract

Despite growing use of flow cytometry to analyze the functional characteristics of primary basophils the intracellular signaling cascades that control their ability to elaborate various inflammatory mediators and cytokines remain comparatively obscure. Additionally, some studies require the analysis of pro-allergic and inflammatory mediators, such as histamine, LTC₄, and various basophil-derived cytokines (e.g., IL-4 and IL-13). Elucidation of intracellular signaling proteins by Western blotting, cytosolic free calcium concentration by spectrofluorophotometry, and detection of mediator releases, as well as analysis of gene expressions by RT-PCR, generally require relatively large numbers of purified basophils. In selected assays, flow cytometry can enable the analysis of relatively low cell numbers and purity for the expression of intracellular signaling proteins or measurement of cytosolic free calcium concentrations by basophil-specific gating strategies. Unfortunately, many aspects of signal transduction relevant to human basophils cannot be readily extrapolated from the use of basophil or mast cell lines. This chapter therefore focuses on how to employ primary human basophils for studying mediator releases and signaling characteristics.

Key words Basophils, Signaling, IgE receptors, Western blotting, Flow cytometry, Inhibitors

1 Introduction

Human basophilic granulocytes are inflammatory cells that appear to be involved in the pathogenesis of a variety of allergic diseases (reviewed in [1]). Upon activation by various stimuli they release several biologically active mediators, such as histamine and leukotriene C₄, as well as cytokines such as IL-4 (*see* Chapter 10). Due to the limited availability of human basophils of high purity, precise knowledge of the signal transduction pathways leading to release reactions is still sparse. Activation of basophils through FcεRI signaling is mostly studied via anti-IgE or allergen-induced activation. Other receptor-mediated stimuli for human basophils include the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP) and the complement fragment C5a. Calcium ionophores A23187 or ionomycin as well as phorbol-myristate acetate (PMA) activate

Edward F. Knol and Bernhard F. Gibbs have contributed equally to this Chapter.

Bernhard F. Gibbs and Franco H. Falcone (eds.), *Basophils and Mast Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1192, DOI 10.1007/978-1-4939-1173-8_15, © Springer Science+Business Media New York 2014

basophils independent of cell membrane receptors. Priming of basophils can be achieved by interleukin-3 or platelet-activating factor which markedly potentiate mediator release caused by various stimuli.

It is clear that the receptor-mediated stimulation of basophils results in a strong donor-dependent activation determined, for instance, by histamine release. For FcεRI-induced degranulation the strength of activation of Syk has been hypothesized to be causal [2]. For many other stimuli this is not clear yet and we need better insights into basophil signal transduction cascades that control degranulation or the release of cytokines and other mediators.

2 Materials

2.1 Chemicals and Kits

1. Recombinant human IL-3: Prepare aliquots of a stock solution containing 10 µg/mL of IL-3 in Ca²⁺-free HEPES-buffered Tyrode's solution to avoid repeated freeze-thaw cycles. Use at 0.1–1 ng/mL final concentration as a priming factor to enhance IgE-dependent basophil stimulation.
2. Anti-human IgE: Polyclonal IgG antibodies are most often employed. These need to be titrated in a pilot experiment to determine optimum basophil mediator release and concentrated aliquots prepared accordingly and frozen at –20 °C for subsequent experiments. Note that some basophil mediators, e.g., IL-4, are released at five- to tenfold lower concentrations than those required for histamine release or CD63 expression. It is therefore advisable to use concentrations of anti-IgE (or other IgE-dependent stimuli) that are slightly suboptimal with respect to histamine release or CD63 expressions.
3. IgE-independent stimuli: 1 mM stock solutions in DMSO to avoid freeze-thaw issues (which are particularly of concern regarding calcium ionophores) and perform pilot basophil stimulation experiments to determine optimum (or suboptimum) basophil stimulation. For A23187 this is usually between 1 and 10 µM (note that there is a sharp bell-shaped response for this stimulus): for fMLP 100–1,000 nM, for C5a 1–10 nM, and for PMA 10–100 ng/mL. For C5a and IL-3, use either buffer or 0.9 % NaCl supplemented with 1 mg/mL BSA to prepare stock solutions.
4. ELISA kits for histamine, LTC₄, and pro-allergic cytokines (e.g., IL-4): Note that for detection of cytokine release the most sensitive ELISA kits are usually required (i.e., minimum detection at ca. 1–3 pg/mL). Histamine releases also are mostly determined spectrofluorometrically based on the method described by Shore et al. [3].
5. Laemmli-lysis buffer for SDS-PAGE/Western blotting: Prepare lysis buffer consisting of 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 5 mM DTT, 1 % Nonidet P-40,

1 mM PMSF, 100 µg/mL aprotinin, 20 µg/mL leupeptin, and 10 mM benzamidine and then mix with equal volumes of 2× concentrated Laemmli sample buffer (obtained from commercial sources). Freeze aliquots at -20 °C. Ensure complete dissolution of contents prior to use.

6. SDS-PAGE reagents: Tris base pH 8.8, Tris-HCl pH 6.8, 30 % acrylamide-Bis solution, glycerol, glycine, SDS, TEMED, APS. Blotting and transfer buffers.
7. Western blotting reagents: Tris-buffered saline and Tween-20 (TBST) solution (containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1 % Tween 20), skimmed milk powder, antibodies to signaling proteins, secondary antibodies (e.g., labeled with either HRP or dyes if using LiCor imaging systems).
8. Calcium indicators: The free Ca^{2+} indicators indo-1 and fura-2 are acetoxymethyl ester labelled. Basophils are incubated with approximately 1 µM in a Ca^{2+} -containing buffer. Within basophils the acetoxymethyl ester, which can diffuse freely through the membrane, is cleaved by cytosolic esterases, resulting in a charged, cell-impermeable compound form of indo-1 and fura-2.

2.2 Buffers for Cell Culture and Stains

1. *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-buffered Tyrode's solution (pH 7.4): NaCl 137.0 mM (8.0 g/L), glucose 5.6 mM (1.0 g/L), KCL 2.7 mM (201 mg/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.0 mM (147 mg/L), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.0 mM (203 mg/L), HEPES 10.0 mM (2.9 g/L), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.4 mM (62.5 mg/L). The pH is adjusted to 7.4 with NaOH (1 M). This buffer will keep for 1 week at 4 °C and may be used for brief periods (<1 h) of basophil stimulation.
2. HEPES-buffered Tyrode's solution (pH 7.4) without MgCl_2 and CaCl_2 (Ca^{2+} -free buffer): Used for washing basophils before experimentation and for terminating reactions at the end of incubation periods (though this is not usually done following long-term incubation periods, especially where ELISAs for LTC_4 and cytokine releases are required and dilution of samples must be avoided).
3. RPMI 1640 with L-glutamine, 1 % penicillin-streptomycin, and 1 mg/mL BSA: Used for experiments requiring long incubation periods (>1 h).
4. Alcian blue staining of basophils (adapted from [4]): Dissolve cetylpyridinium chloride (76 mg), lanthanum chloride (700 mg), NaCl (900 mg), Tween 20 (21 µL), and alcian blue (143 mg) in 100 mL distilled H_2O and stir for at least 2 h at room temperature. Filter using a 1 µm filter or, alternatively, centrifuge at $1,000 \times g$ for 15 min and carefully collect the supernatant. The solution is stable for at least 1 year at 4 °C.

To 10 μL of cell suspension add 65 μL of NaCl solution (1 mg/mL containing 0.1 % EDTA) followed by 65 μL of alcian blue solution (described above). Finally, add 5 μL of 1 M HCl, gently resuspend, and determine the total number and purity of basophils using a hemocytometer.

2.3 Basophils

1. Enrichment and purification of primary human basophils are described in detail in Chapter 2.
2. Ensure sufficient basophil purity and number using alcian blue stain and a hemocytometer (*see* Chapter 2, Subheading 2.2, item 4), or flow cytometer with basophil-discriminating antibodies (*see* Chapter 7). For determining mediator release, at least 100,000 basophils/vial are usually necessary. For RT-PCR and Western blotting, use at least 200,000–300,000 basophils/vial.

2.4 Equipment and Other Materials

1. Water purification system.
2. Microscope and hemocytometer.
3. CO₂ incubator or water bath set to 37 °C.
4. Centrifuges (400–1,000 $\times g$).
5. Heating block for 1.5 mL tubes.
6. ELISA plate reader.
7. LiCor Odyssey or comparable imaging system or darkroom for ECL detection using photographic film.
8. Flow cytometer.
9. Spectrofluorophotometer.
10. 50 and 10 mL test tubes (polypropylene) and 1.5 mL Eppendorf tubes.
11. Pipette tips.

3 Methods

The following methods describe the basic procedure for analyzing basophil responses as well as variations depending on whether the release of various mediator types is being assessed or signal transduction studies conducted. It is vital that cell handling and preparation of purified basophils are kept as short as possible.

3.1 Basophil Stimulation Assay: General Procedure

1. Wash basophils and resuspend in either Tyrode's buffer (short incubations) or RPMI 1640 medium (long incubations to determine cytokine release), at room temperature, and aliquot (400 μL) into test tubes at a density of ca. 50,000–150,000 basophils/mL. Include tubes for positive control (e.g., anti-IgE and/or IgE-independent stimulus) and negative control (buffer with cells alone).

2. Place all tubes in a water bath or culture cabinets set at 37 °C.
3. Add 50 μ L of prewarmed buffer, priming factors (e.g., IL-3), or signal transduction inhibitors to the tubes and preincubate for 15 min. Avoid cooling.
4. Stimulate all tubes except negative control with 50 μ L anti-IgE (or IgE-independent stimulus) for the required times (the total volume for each vial should now be 500 μ L).

3.2 Histamine Release Analysis

1. Prepare and treat basophils as described above (Subheading 3.1; *see Note 1*) and stimulate for 30 min (unless release kinetics are being addressed, *see Note 2*).
2. Quench reactions by adding 1 mL of ice-cold Ca^{2+} -free HEPES-buffered Tyrode's solution into each tube and centrifuge immediately ($850\times g$, 2 min, 4 °C).
3. Decant supernatants into fresh tubes and add 1.45 mL Ca^{2+} -free HEPES-buffered Tyrode's solution to the pellet tubes. For automated histamine analysis deplete proteins, including enzymes, in the supernatant by adding 2 % w/v perchloric acid. Lyse the pellet tubes by adding 50 μ L perchloric acid (70 %) for automated analysis of histamine release according to the method described by Shore et al. [3]. Alternatively, freeze-thaw cell pellet samples (without perchloric acid) three times.
4. Briefly vortex pellet tubes and centrifuge ($850\times g$, 10 min, 4 °C) to remove debris.
5. The tubes can be stored at -20 °C until ready for assay.
6. Assay for histamine (either using commercial kits or fluorometric analysis).
7. Histamine release is calculated as a percentage of the total content (present in the cell pellet and corresponding supernatant). Results are typically expressed as either relative percent releases (the % release into the supernatant for each condition/vial) or corrected percent release (the net % release after subtracting spontaneous releases obtained from the negative controls). Spontaneous release should be below 10 %, preferably below 5 %, to check for any nonspecific activation (e.g., caused by cell-handling issues).

3.3 Eicosanoid and Cytokine Release Analysis

1. Experimental conditions are the same as described in Subheading 3.1 except for the variable incubation periods and termination of reactions.
2. Unless the basophil density is very high (e.g., 200,000 basophils in 500 μ L total reaction volume), it is not recommended to stop reactions using ice-cold Ca^{2+} -free HEPES-buffered Tyrode's solution since the mediators of interest may be diluted too much to detect. Instead, briefly place all tubes in ice water and then centrifuge ($850\times g$, 2 min, 4 °C).

3. Decant supernatants into fresh vials and freeze immediately (-20°C or lower) until ready for assay.
4. Eicosanoid (e.g., LTC_4) release is usually complete after 30-min stimulation and is measured only in the supernatants.
5. Optimum cytokine production from basophils varies from 4 h, for the detection of IL-4 release, to over 16 h for IL-13 releases. At the end of each stimulation period harvest the supernatants as described above and freeze immediately for storage prior to assaying cytokine content (*see* **Note 3**).

3.4 Measurement of Cytosolic Free Calcium Concentrations by Spectrofluorophotometer or Flow Cytometer

1. Prepare and treat basophils as described above (Subheadings 2.1, item 8, and 3.1).
2. For the spectrofluorophotometer and indo-1-loaded basophils excitation and emission wavelengths should be taken at 340 nm and 390 nm, respectively.
3. Before each measurement the cells are diluted in medium with 1 mM CaCl_2 to a density of approximately 1×10^6 cells/mL.
4. Calibration of the signal is obtained by permeabilization of the cells with 5 μM digitonin to obtain maximum fluorescence and then quenching with 0.5 mM MnCl_2 to obtain minimal fluorescence (*see* Fig. 1).

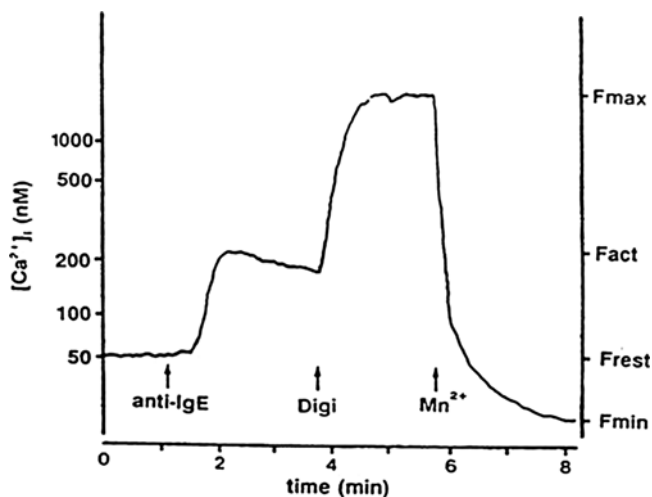


Fig. 1 Increase in $[\text{Ca}^{2+}]_i$ in a purified basophil suspension after anti-IgE stimulation. Basophils were loaded with indo-1 and fluorescence measurements were performed using a spectrofluorophotometer, as described in Subheading 3.4. In this recording the calibration of indo-1 fluorescence as a function of $[\text{Ca}^{2+}]_i$ was also carried out. All trapped indo-1 was saturated by permeabilization of the cells with 5 μM digitonin (Digi), resulting in a maximal fluorescence (F_{max}). Subsequently, the indo-1 signal was quenched by addition of 0.5 mM MnCl_2 (Mn^{2+}) resulting in minimal fluorescence (F_{min}). Fluorescence of the unstimulated population is marked as F_{rest} and the anti-IgE-stimulated population as F_{act} [5]

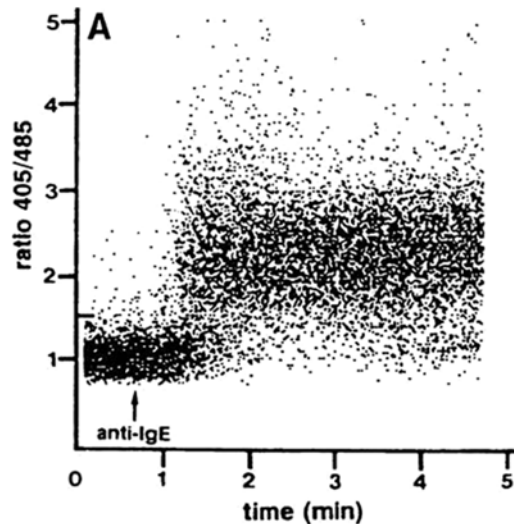


Fig. 2 Increase in $[Ca^{2+}]_i$ determined by flow cytometer in a purified basophil suspension after anti-IgE stimulation. Basophils were loaded with indo-1 and fluorescence measurements were performed in a flow cytometer, as described in Subheading 3.4. In contrast to the data shown in Fig. 1, the $[Ca^{2+}]_i$ in individual basophils is depicted [6]

5. Cytoplasmic free calcium concentration is calculated by the equation $[Ca^{2+}] = K_d \times ((F - F_{min}) / (F_{max} - F))$, using 250 nM as the K_d for the Ca^{2+} /indo-1 complex.
6. For flow cytometry it is not possible to determine the precise $[Ca^{2+}]$ using the calibration strategy described above; only the relative ratio of $[Ca^{2+}]$ can be obtained (*see* Fig. 2).
7. Flow cytometry emission and excitation wavelengths are determined somewhat by the filters available, but should be within the range described above.
8. For flow cytometry, a sampling chamber with low magnetic stirring and heating at 37 °C is required.
9. Addition of EGTA (1.5 mM) or buffering Ca^{2+} concentrations in incubation buffers to about 100 nM selectively will prevent Ca^{2+} influx and will only show Ca^{2+} increase due to release from intracellular stores (*see* Fig. 3).

3.5 Determination of Activation Pathways by Flow Cytometry

1. Prepare and treat basophils as described above (Subheading 3.1). Depending on the system unprocessed blood, peripheral blood mononuclear cell preparation, or purified (partly or fully) basophils can be activated (*see* Note 4).
2. Stop reactions by adding ice-cold buffer with EDTA.
3. Antibodies to characterize basophils, such as CD123⁺/CD203C⁺/HLA-DR⁻, or CCR3 are added together with CD63 (in order to estimate activated basophils) for about 30 min at 4 °C.

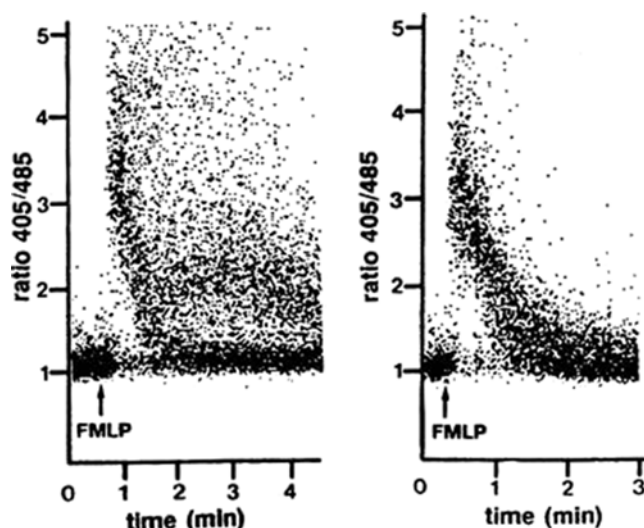


Fig. 3 Changes in $[Ca^{2+}]_i$ determined by flow cytometer in a purified basophil suspension following the addition of fMLP. Experiments were performed as described above. In the *right* panel, cells were stimulated without Ca^{2+} , but with 1.5 mM EGTA to deplete extracellular Ca^{2+} . The *right* panel depicts the increase in $[Ca^{2+}]_i$ from intracellular stores only [5]

4. If unprocessed blood was used, residual erythrocytes should be lysed.
5. To stain for intracellular proteins cells should be fixed and permeabilized by resuspending them in 300 μ L of 0.3 % saponin and 3.7 % formaldehyde in PBS for 30 min at 4 $^{\circ}$ C.
6. Staining of phosphorylated (activation) proteins, such as phospho-p38 mitogen-activated protein kinase (p38 MAPK), is performed on the permeabilized cells at relatively early time points (e.g., 1, 3, 5, 10 min) following stimulation (*see Note 5*).
7. Control cells that are stored in ice-cold buffer are used to determine baseline phosphorylation.
8. Flow cytometric analysis should be performed on either labeled basophils or purified basophils.

3.6 Signal Transduction Experiments and Preparation of Samples for SDS-PAGE and Western Blotting

1. Prepare and treat basophils as described above (Subheading 3.1) and stimulate for the required times (*see Note 6*).
2. Stop cell incubations either by briefly placing tubes on ice water (if concentrated supernatants are required for mediator release assays) or by adding 1 mL of ice-cold Ca^{2+} -free HEPES-buffered Tyrode's solution.
3. Centrifuge immediately (850–1,000 $\times g$; 2 min).

4. Remove supernatants and lyse cell pellets on ice by vigorous mixing in 15–30 μ L of Laemmli-lysis buffer (*see Note 7*).
5. Immediately heat all tubes to over 95 °C for 3 min and either store at –70 °C for later analysis or proceed to SDS-PAGE.
6. Separate proteins by SDS-PAGE and transfer to nitrocellulose membranes. Include a molecular weight (MW) marker, e.g., pre-stained MW rainbow markers for each SDS-PAGE run.
7. Block membranes for 2 h in 5 % skimmed milk in TBST buffer.
8. Briefly wash twice with TBST and incubate membranes overnight at 4 °C with specific antibodies to either phosphorylated, total signaling proteins or beta-actin on a swirling platform.
9. Wash membranes 4–5 times in TBST for a total of 15 min.
10. Incubate with secondary antibodies (e.g., anti-mouse or anti-rabbit Ig horse radish peroxidase or LiCor-labeled antibodies for Odyssey imaging) for 1 h at room temperature.
11. Remove unbound secondary antibodies by washing membranes 4–5 times in TBST for a total of 15 min.
12. Visualize proteins either by chemiluminescence according to the manufacturer's instructions (e.g., ECL plus) or using a LiCor imaging system.
13. After detection, if additional signaling proteins require analysis, strip membranes for 15–25 min using commercial stripping reagents, wash in TBST (4 $\times$ 5 min), re-block, wash briefly and then re probe depending on the manufacturer's instructions.

4 Notes

1. If histamine release is being determined alone, i.e., without Western blotting or cytokine/LTC₄ releases, basophil preparations of lower purity may be used, especially for pilot experiments to gauge the optimum concentrations of stimuli required for subsequent experiments. In this case, even Ficoll-density enrichment may suffice for determining basophil histamine releases since, other than platelets in some donors, most of the blood histamine content is basophil derived.
2. Optimum kinetics for IgE-dependent histamine releases is usually around 30-min stimulation. However, this may vary if highly suboptimal or supraoptimal stimulus concentrations are used. IgE-independent stimuli, such as fMLP, display faster release kinetics. This is not usually a problem if a 30-min incubation period is employed (e.g., when fMLP or A23187 is being used as a positive control compared to anti-IgE or allergens) since the released histamine will not usually be broken down under the conditions described in this chapter.

When basophils are stimulated with PMA at least 60-min stimulation is required for maximal histamine release.

3. Optimum cytokine production from basophils varies from 4 h, for the detection of IL-4 release, to over 16 h for IL-13 release. However, since some preformed cytokines may be released very rapidly (within the first 30 min) it is recommended to initially use several stimulation periods, e.g., 30 min, 4 h, 8 h, 16 h, and more in order to gauge the kinetics of both preformed and de novo-generated cytokines as well as for assessing gene expressions by RT-PCR.
4. Analysis of basophil activation by flow cytometry enables to use basophil preparation of low purity, or even whole blood. In this case selective basophil markers (CD203c⁺, CD123⁺/HLA-DR⁻, CCR3⁺/low side scatter) should be used.
5. Many signaling proteins are optimally phosphorylated within the first 15 min after basophil stimulation (e.g., Syk, p38 MAPK, ERK1 and 2). It is strongly advised to determine the optimal activity of the signal of interest by performing a kinetics experiment using basophils stimulated, for example, for 1, 5, 15, and 30 min. Other signaling proteins, e.g., HIF-1 α , are maximally expressed at far greater periods (hours). Note that the concentrations of the stimulus and the degree of priming substantially affect signal transduction kinetics.
6. IgE-independent stimuli can be differentiated in receptor-dependent (e.g., fMLP, C5a) and receptor-independent (e.g., A23187) stimuli. The bacterial peptide fMLP serves as important positive control for basophil degranulation (especially in determining the so-called non-responders to IgE-dependent triggers; if the IgE-independent controls fail to elicit a response this may indicate cell-handling issues). C5a can be an alternative for fMLP for degranulation, but requires preceding stimulation with IL-3 for release of LTC₄. Calcium ionophores (e.g., A23187) or activators of protein kinase C (PKC), such as PMA, can be informative if receptor-mediated stimuli have no effect to check for the status of basophil function.
7. Care must be taken to immediately disrupt cell pellets with Laemmli-lysis buffer. On some occasions cell pellets are not clearly visible but cells are deposited on the sides of 1.5 mL tubes on fixed-angle rotors. Ensure, therefore, that Laemmli-lysis buffer comes into contact with this material. If pellet debris does not immediately disrupt (i.e., becomes turbid and difficult to resuspend) proceed to the next step of heating the vials and vortex after 30 s of the heating/boiling step. If protein concentrations need to be determined prior to SDS-PAGE and Western blotting disrupt the cells in lysis buffer alone on ice for 30 min before assessing protein concentrations

(Bradford Assay). The remaining lysed cells are then treated with Laemmli for SDS-PAGE and Western blotting. These procedures can also be carried out at a later date by freezing the lysed cells at -80°C .

Acknowledgements

This work was supported by the COST Action BM1007 Mast Cells and Basophils—Targets for Innovative Therapies.

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