RBL cells as Models for *in vitro* studies of mast cells and basophils

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Running title

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Summary

Since their establishment in 1981, RBL-2H3 cells have been widely used as a mast cell model. Their ability to be easily grown in culture in large amounts, their responsiveness to FcεRI mediated triggers and the fact that they can be genetically manipulated, have provided advantages over primary mast cells, in particular for molecular studies relying on genetic screening. Furthermore, the ability to generate clones that stably express proteins of interest, for example a human receptor, have marked the RBL cells as an attractive mast cell model for drug screening. Indeed, three RBL reporter cell lines (RS-ATL8, NFAT-DsRed, and NPY-mRFP) have been generated providing useful models for drug and allergen screening.

Similarly, RBL cells stably expressing the human MrgprX2 receptor provide a unique paradigm for analyzing ligand interactions and signaling pathways of the unique human receptor. Finally, transient co-transfections of RBL cells allow functional genomic analyses of mast cell secretion by combining library screening with simultaneous expression of a reporter for exocytosis. RBL cells thus comprise powerful tools for the study of intracellular membrane trafficking and exocytosis and the detection of allergens, vaccine safety studies and diagnosis of allergic sensitization. Their recent uses as an investigative tool are reviewed here.

[199 words]

Keywords

RBL-2H3, RS-ATL8, NPY-mRFP, NFAT-DsRed, mast cell model, *in vitro*

Animal model systems are an essential factor in disease research. An animal model that recapitulates properly the pathophysiological aspects of a human disease can facilitate the development of diagnostic means as well as the development of therapeutics, thus playing an important role in preclinical studies. However, to define the molecular basis of a disease and define the cellular pathways involved, cellular models are required to allow the study of the specific contributions of cells that are linked with the development of a disease. Such models provide invaluable tools for the identification of drug targets and for screening for genetic, environmental or pharmacological risk factors of the disease. Finally, cellular models also provide useful tools for testing the efficacy of potential drugs. In line with this premise, mast cell (MC) models have been developed for studying the molecular basis of allergy, a family of diseases, which share their onset upon the release of inflammatory mediators from activated MCs. The latter, including vasoactive amines such as histamine, proteases such as tryptase, proteoglycans such as heparin, lysosomal enzymes such as β-hexosaminidase and some cytokines, are pre-stored in cytoplasmic secretory granules (SGs) and released by exocytosis following MC activation by external triggers.[1-5] This initial event of exocytosis is followed by the *de novo* synthesis and release of a large array of biologically potent substances, including metabolites of arachidonic acid, multiple cytokines and chemokines.[6-9] Collectively, these mediators thus initiate early and late inflammatory responses. The best-characterized mode of MC activation is the immunological pathway caused by allergen/antigen (Ag)-induced crosslinking of Immunoglobulin E (IgE) antibodies that are bound to their high affinity IgE receptor, FcεRI that is expressed on the MC surface.[10-12] However, MCs can respond to many different stimuli (e.g., cytokines, bacterial products, neuropeptides, venom components, etc.) and participate in a wide variety of IgE-independent physiological and pathological processes.[13, 14]

MCs arise from committed progenitors (CD34+, c-kit positive cells) in the bone marrow, which traverses the vascular space and enter the tissues.[15-17] The cells then complete their differentiation and maturation process *in situ*. Hence, MCs are present in most tissues in the vicinity of blood vessels. However, they are especially prominent at the interface between the outside environment and the internal milieu, in tissues such as the skin and the respiratory and digestive tracts.[18]

That MCs are tissue resident cells and that even in the tissue their numbers are limited raises difficulties in obtaining large number of cells that are normally needed for studies aimed at elucidating mechanisms underlying MC processes such as MC secretion or stimulus-secretion coupling mechanisms. There are also difficulties in maintaining primary MCs in culture and almost impossible to manipulate them genetically, which is often required for identification of the machineries involved in MC function. Model systems are therefore indispensable for molecular research of MCs *in vitro*. Towards this end, a number of models have been developed. The latter are based either on *in vitro* culturing of stem cells, for example culturing of mouse bone marrow derived MCs (BMMCs),[19] or culturing of human cord blood derived MCs[20] or *in vitro* maturation of MCs from peripheral blood.[21] In addition several cell lines have been developed from human and rodent sources including the human HMC-1, LAD-2 and LUVA cell lines,[22-24] the murine MC/9 cell line[25] and the rat basophilic leukemia cell line, RBL-2H3, herein referred to as RBL.[26] The latter is the focus of this review, where we will summarize the limitations of this model vis-à-vis their value as MC model. We will also describe the newer RBL cell-based models and their important applications.

1. The creation of the RBL-2H3 cell line

RBL cells were generated by injecting rats with the chemical carcinogen β-chlorethylamine.[27] Tumors generated by the injected rats were subsequently adapted to culture.[28] Binding assays revealed that the cells, termed RBL-1, bound specifically IgE.[28, 29] Nevertheless, RBL-1 failed

to release histamine in response to either an IgE/antigen trigger or chemical stimulation by a Ca^{2+} ionophore.[30] However, subsequent cloning of the cells gave rise to the isolation of a responsive subline, that degranulated in response to an IgE trigger, hence the RBL-2H3 subline.[26] Since their establishment in 1981, RBL cells underwent two phases. Shortly after their formation, RBL cells were considered a reliable model, which was extensively used to study the IgE receptor. However, their credibility and suitability was later questioned.[31, 32] In this review, we will revisit the question of reliability of the RBL cells as an adequate model for MC research.

2. The role of RBL cells in IgE receptor research

The availability of the RBL cells that could be grown in culture in large quantities provided a unique opportunity to study the properties of the FcεRI. The binding characteristics of IgE, i.e. dependence on temperature, sensitivity to metabolic inhibitors[33] as well as quantitative aspects of the binding reaction[34] could be analyzed by the use of this cell line. Moreover, the receptor could be solubilized,[35, 36] an achievement that has eventually paved the road for the cloning of the receptor,[37-40] receptor crystallization[41] and generation of transgenic mice.[42] RBL cells also provided an excellent paradigm to decipher the signaling cascades of the FcεRI.[43] Signaling pathways elicited by the receptor were delineated and adaptors involved in the stimulus secretion coupling mechanisms in MCs were identified.[12, 44-46] Finally, RBL cells were used to analyze the fate of the receptor,[47-49] eventually leading to the discovery of the role played by ubiquitination and E3 ligases in receptor regulation.[50-52]

3. RBL cells: MCs or basophils?

Whether the phenotype of RBL cells is closer to basophils or to MCs is an old dispute.[31, 32, 53] Here, we will briefly attempt to show that the debate is not only unresolved, but perhaps unresolvable — but: does it really need to be resolved?

From a morphological point of view, RBL cells grow firmly attached to the culture vessels, forming monolayers and displaying an elongated morphology which does not fully resemble MCs but even less so that of basophils, and is much more reminiscent of fibroblast morphology. [54] In contrast, transmission electron microscopy of RBL-2H3 cells [55, 56] suggests a heterogeneous distribution of granule sizes and densities which is closer to what is seen in peripheral blood basophils than the dense and more homogeneously sized granules of MCs.[57] At the molecular level, there are clear discrepancies from (human) basophils, such as the lack of detectable expression of N-formyl peptide (FPR) or complement (C5aR) receptors.[58] In fact, several studies have introduced fMLP receptors into RBL-2H3 by transfection.[58, 59]

Additional differences between RBL and basophils have been described regarding the effects of Toll-like receptor (TLR) agonists, e.g. by the TLR-4 ligand Lipopolysaccharide (LPS). While both peripheral blood basophils[60] and RBL cells[61] appear to express TLR-4, RBL cells are not activated by LPS, possibly because they lack the essential co-receptor CD14.[61] However, LPS also does not directly activate peripheral blood basophils,[62] despite the apparent presence of CD14,[63] while it appears to synergize with IgE-dependent stimuli.[62] We have not found any information about the presence of TLR-2 on RBL cells. Therefore, it appears that several receptors linked to RBL activation are differentially expressed between RBL cells and basophils.

But what about the highly studied markers CD63 and CD203c on human basophils? Are these shared with RBL cells? Human basophil activation via the high affinity IgE receptor can be monitored by measuring changes in the surface expression of activation markers, such as CD63 and CD203c. CD63 is a glycoprotein member of the transmembrane 4 superfamily

(TM4SF) and is also known as lysosome associated membrane protein-3 (LAMP-3). Its expression is not unique to human basophils, but due to its strong (up to 100-fold) upregulation during anaphylactic degranulation,[64] it is eminently useful for measurement of basophil activation. CD203c is a type II transmembrane glycoprotein belonging to the family of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP3) enzymes, and is a specific marker for basophils in peripheral blood,[65] CD63 and/or CD203c, usually in combination with other surface antigens needed to unambiguously identify basophils in peripheral blood samples by flow cytometry, form the basis of most commercial or experimental basophil activation tests.[66] Typically, resting basophils have very low levels of surface CD63, while CD203c levels are intermediate. CD63 is located in the granular membrane and inserted into the cytoplasmic membrane during degranulation, an event that involves fusion of granular and plasma membrane.[67, 68] In contrast, CD203c appears to be recruited from other pre-existing intracellular pools, leading to its rapid (3-5-fold) upregulation on the cell surface post-activation.[69] Can these two surface activation markers help resolve the MC vs basophil dispute? Unfortunately, there is little knowledge regarding the expression of CD203c on the RBL surface, perhaps due to the lack of suitable specific antibodies. We and others have found CD63 levels on RBL cells to be elevated also on resting cells; Kitani *et al.*[70] describe 1.1 x 10^5 CD63 molecules per cell: a striking difference to the barely measurable levels of CD63 expression on resting human basophils.

Another molecule expressed on the surface of RBL cells, is c-kit, the proto-oncogene tyrosine kinase acting as receptor for stem cell factor (scf).[71] In contrast, basophils are c-kit negative. Vice versa, the receptor for IL-3 (CD123), which is highly expressed on basophils,[72] has not been described on RBL-2H3. Considering the central roles of IL-3 and scf for growth and development of basophils and MCs, respectively, this dichotomy clearly appears to put RBL cells in closer proximity to MCs rather than basophils. However, bearing in mind the

highly aberrant karyotype of RBL-2H3 cells, whether or not they can be considered a suitable model for MCs, depends on the context of what is studied.

4. RBL cells as reliable model for studies on exocytosis

Numerous studies indicate similar mechanisms of exocytosis of BMMCs and RBL cells thus marking the RBL cells as an appropriate model for studies on MC exocytosis. In support of this notion are the reports that demonstrated the involvement of same SNARE proteins in mediating the fusion events that lead to degranulation in either cell type. Immunohistochemistry studies have further supported these studies by demonstrating similar cellular locations of the SNARE proteins in primary MCs and RBL cells, whereby SNAP23 and syntaxin 4 localize to the plasma membrane and syntaxin 3 and VAMP-2, −3, −7 and −8 primarily localize to the SGs (reviewed in[73]). Furthermore, similar protein interactions between SNARE proteins were noted in the primary MCs and the RBL cells, demonstrating protein complexes consisting of SNAP-23, Syntaxin 4 and VAMP-2, VAMP-8, VAMP-7 and VAMP-3 (reviewed in[73]). Functional assays further confirmed this notion by comparing MCs derived from knockout mice and knockdown RBL cells. For example, BMMCs derived from VAMP8-deficient mice exhibit reduced degranulation[74] and similarly, VAMP8 was identified as major SNARE in the release of β hexosaminidase from RBL cells.[75]

Not only the fusion machineries used by BMMCs and RBL are similar, but this is also true for the accessory proteins that regulate SNARE function, SG motility or SG size. The latter include the Sec/Munc (SM) protein Munc18-2 that regulates SNARE function and SG motility,[76] the Rab GTPases, Rab 12[77] and Rab27[78] that respectively regulate retro and anterograde transport of the SG and Rab5 that regulates SG fusion and size.[79]

MC exocytosis is associated with remarkable cytoskeletal changes. To gain insight into the factors that govern these cytoskeletal changes, a pharmacological screen of Rho family

inhibitors was recently conducted testing the impact of inhibitors of members of the Rho family of GTPases on MC secretion.[80] The results of this study conducted on both FcεRI-activated BMMCs and RBL cells revealed similar drug sensitivities, therefore implicating similar actin signaling linked with BMMC or RBL stimulated exocytosis.[80] These results are consistent with previous studies of direct measurements of actin rearrangements that occur during exocytosis of BMMC or RBL that documented similar actin remodeling.[81, 82]

5. The new generation of RBL cell based models

5.1. Humanized RBL cells as model system for screening allergens/anti allergic drugs

5.1.a. RBL-SX-38; RBL-48; RBL30/25: the human FcεRIα expressing RBL cells:

The expression of the high affinity IgE receptor FcεRI on RBL cells suggested that this cell line could be used for detection of allergen-specific IgE and allergens. However, while the human IgE receptor is able to bind both rodent and human IgE,[83] the rodent receptor does not bind human IgE.[84] Thus, for use with human samples, the RBL cells first had to be transfected with the human FcεRI receptor.[85] The suitability of RBL cells for screening purposes became clear in the early 90s when RBL cells were transfected with the α chain of the human FcεRI and shown to maintain functionality. Stable transfection of RBL cells with the human FcεRI α chain yielded a cell surface expressed receptor that mediated antigen-induced signaling and mediator release.[85-88] Such humanized RBL cells were then used to test the capacity of purified peanut allergens to induce degranulation[89-91] or food allergens.[92] Since then, the RBL cells have also been transfected to express equine[93] and canine[94] high affinity IgE receptor α chains, extending their use to other mammalian species.

5.1.b. MrgprX2-expressing RBL cells as model for basic secretagogue-induced signaling and secretion

Alongside their well characterized Ag/IgE stimulated and FcεRI-mediated response, MCs can also be activated independently of IgE by numerous ligands, including ligands of Toll like receptors[95] and adenosine.[96] Intriguingly, a subset of MCs, connective tissue type MCs in rodents or MCs that contain both tryptase and chymase in their SGs (MC^{TC}) in humans, selectively respond to a family of cationic stimuli and polybasic compounds. The latter collectively known as the basic secretagogues of MCs include neuropeptides such as substance P, toxins such as the wasp toxin mastoparan and numerous positively charged chemicals such as the synthetic compound 48/80 (c48/80) and many drugs.[13] Basic secretagogues are enigmatic because they share no sequence homology, apart from their common positive charge, and yet they seem to share a common mechanism of action reflected in the requirement for a high, micromolar range concentration to elicit their responses, their relatively rapid secretory response and sensitivity to pertussis toxin (PTX).[97] Studies by others and us addressing the mode of action of basic secretagogues in rat peritoneal MCs, a model for connective tissue type MCs in rodents, have suggested that these secretagogues activate MCs in a receptor-independent manner, by directly activating Gi proteins. We have then identified Gi3 as a crucial mediator of basic secretagogue-induced MC degranulation.[98] Interestingly, basic secretagogues stimulated secretion and basic secretagogues stimulated signaling, reflected in the activation of protein tyrosine kinases including the syk kinase and phosphorylation of the ERK1/2 MAP kinases[99, 100] displayed distinct dose response relationships suggesting the existence of two cellular targets of basic secretagogues. In consistence with such notion, recently, the Kulka Lab has shown that MCs' differential response to basic secretagogues is dependent upon the expression of the Mrgpr receptor.[14] This extremely important observation

thus provided new mechanistic insights into the mode of action of basic secretagogues and their differential activity.

MAS-related G protein coupled receptors (Mrgprs) or sensory neuron-specific receptors (SNSRs) comprise a multigene family of G protein coupled receptors (GPCRs) that have expanded in a species-dependent manner through evolution (reviewed in [101]). Interestingly, most amino acid alterations occurred in the extracellular domains of Mrgprs, implying that Mrgpr evolution was adaptive affecting their capacity to recognize diverse ligands according to each species need (reviewed in [102]). Humans (and other primates) express four MrgprX genes: MrgprX1-X4, whereas rodents have one gene each of MrgprA, MrgprC, and MrgprD and 10 genes of MrgprB (reviewed in [102]). Though the receptors are still considered "orphan", many ligands, including amino acids and peptides, have been shown to bind to this receptor class (reviewed in [103]). Mrgprs are predominantly expressed in dorsal root ganglia (DRG) and are therefore considered sensory neuron-specific receptors (SNSRs), whose function is linked with nociception and pain.[104] However, apart from DRG, the second major site of Mrgprs expression is in MCs. Human MCs, including cord blood-derived MCs (CBMCs), skin MCs and the LAD2 MC line, but not lung MCs, express considerable levels of MRGPRX2 and small amounts of MRGPRX1 (reviewed in [102, 103]). Strikingly, human MCs contain more MRGPRX2 than FcεRI,[105] thus indicating the importance of this receptor in MC function. Several lines of complementary evidence implicate Mrgprs as the ‘basic secretagogue receptors’ in MCs. First, screening of peptide and chemical libraries using a reporter gene assay demonstrated that a number of known basic secretagogues of MCs could increase reporter gene expression in HEK293 cells transfected to express MRGPRX2;[106] second, endogenous expression of Mrgprs in MCs correlates with their responsiveness to basic secretagogues;[106] third, studies by the Ali Lab have demonstrated that RBL cells, that do not normally respond to basic secretagogues, acquired such responsiveness upon stable transfection with MRGPRX2;[107] finally, Kulka and

Dong demonstrated that basic secretagogue-induced responses are inhibited in an Mrgprb2-receptor deficient mouse model, thus identifying Mrgprb2 as the basic secretagogue receptor in mouse MCs.[108] Collectively, these findings strongly implicate Mrgprs as mediators of neurogenic inflammation, that is initiated by MC activation by neuropeptides such as substance P (SP), vasoactive intestinal peptide (VIP) and calcitonin gene related peptide (CGRP) that were shown to activate MCs independently of the canonical receptors by a mechanism shared with other basic secretagogues (reviewed in [109, 110]). Furthermore, in view of the fact that many FDA approved drugs act as basic secretagogues capable of activating MCs, MRGPRX2 is likely to mediate pseudoallergic reactions caused by such drugs (reviewed in [109, 110]). However, the species variability of these receptors and the potential variations in their ligand recognition raise important concerns regarding the applicability of experiments in animal models for predicting the pseudoallergenic potential of drugs in humans [14] (reviewed in [109]). In this regard, the model developed by the Ali group of RBL cells that are stably transfected with the human MRGPRX2, offers excellent opportunities to explore the role of the human receptor in MCs, while overcoming the drawbacks of using human MCs that express endogenously the receptor. Indeed, unlike CBMCs whose source is limiting or the LAD2 human MC line, whose doubling time is one week, RBL cells grow fast and can be easily grown in 96- or 348-well plates that are suitable for high throughput screening. Moreover, the selective responsiveness of MRGPRX2 expressing, but not native RBL cells to basic secretagogues allows discrimination of drug toxicity, reflected as toxic release of mediators from native RBL cells, from genuine MRGPRX2 mediated release, as well as discrimination between responses mediated by canonical receptors of a subset of basic secretagogues, for example, responses to SP that are mediated by the canonical Neurokinin 1 receptor (NK-1R) receptor that is endogenously expressed in RBL cells and MRGPRX2-mediated responses. Indeed, by using this system of stably transfected RBL cells, the Ali group was able to demonstrate that PMX-53, an

antagonist of CD88, the receptor of C5a, displays dual activities. Hence, while PMX-53 inhibits CD88, it induced degranulation of MRGPRX2-expressing RBL cells that do not express CD88.[102] Similarly, the human β defensins, hBD2, hBD3, and CST, small cationic antimicrobial peptides that play an important role in host defense against pathogens, induced substantial degranulation of MRGPRX2 expressing RBL cells demonstrating the capacity of these antimicrobial peptides to bind to and activate the human MRGPRX2.[111] Therefore, collectively, these results mark MRGPRX2 expressing RBL cells as a useful model for future screening and evaluation of allergic potential of polycationic molecules.

5.2. Reporter expressing RBL cells

5.2.1 RS-ATL8: The luciferase expressing humanized RBL cell line

A further improvement of humanized RBL cells consisted in introducing a reporter gene (firefly luciferase), allowing easy and highly sensitive measurement of cellular activation via the IgE receptor. The first humanized reporter cell line was created by Nakamura and colleagues;[112] the system is a NFAT-firefly luciferase reporter, which, rather than reporting degranulation, is linked with the branch of IgE-dependent signal transduction resulting in *de novo* cytokine synthesis. Nuclear Factor of Activated T-cells (NFAT) proteins belong to a five-membered family of transcription factors, whose subcellular localization is ultimately determined by intracellular calcium concentrations and the existence of a Nuclear Localization Signal (NLS) located in the N-terminal third of the molecule.[113] In a resting state, this NLS is masked by multiple phosphorylation as the result of serine/threonine kinases, such as casein kinase and glycogen synthase kinase 3,[114] which phosphorylate serine residues in serine-rich regions (SRRs) and three SPXX-repeat motifs (SPs) found in NFAT. Stimulation of the RBL cells via the high affinity IgE receptor results in sustained calcium influx, leading to activation of Calcineurin, a phosphatase that dephosphorylates NFAT, leading to unmasking of the NLS

and subsequent nuclear translocation.[115] In the nucleus, NFAT binds to specific promoter sites and initiates gene transcription, and in the case of the RS-ATL8 cells, transcription of the luciferase reporter gene. Thus with these reporter cells, activation can be measured, peaking 3-4 hours after activation, by lysing the cells and measuring luciferase activity with appropriate substrates and a luminescence detector.

The RS-ATL8 is a very sensitive reporter system, able to detect an allergen concentration of 15 fg/ml, using a threshold for positivity defined as the double of the background (without serum).[112] The high sensitivity also allows to get around issues of serum toxicity, partially due to activated complement; such issues are avoided when diluting the sera to be tested 1:100.[112] Such dilution would result in likely failure to detect activation if measuring beta-hexosaminidase, the traditional way of measuring RBL cell degranulation, using a fluorimetric assay first described by Leaback and Walker in 1961.[116] When using RS-ATL8 cells, it is not necessary to use additional chemicals such as deuterated water[117] or N-ethylcarboxamidoadenosine (NECA),[118] sometimes used for enhancement of degranulation.

This sensitive RS-ATL8 assay has since been used for detection of food or other allergens and is amenable to high throughput format.[119,120] It has also been suggested for assessment of allergenicity of anti-schistosome vaccine candidates[121] and for other purposes, as described recently by us.[122]

5.2.2. Humanized NFAT-DsRed expressing RBL cells

While the RS-ATL8 is exquisitely sensitive, it has two main drawbacks. Firstly, its routine use is relatively expensive due to the need for luciferase substrates. This factor can be reduced by transferring the assay from e.g. 96-well to 384-well plates, without major loss of sensitivity.[119] More importantly, the RS-ATL8 is not suitable for assessment of activation on allergen arrays, as luciferase detection requires cell lysis, thus disconnects the fluorescent signal from the

location of the allergen spot causing activation on an array slide. To overcome this limitation, we developed a fluorescent reporter system, which similarly to the RS-ATL8, reports IgE-dependent NFAT nuclear translocation by inducing DsRed protein expression (NFAT-DsRed RBL).[123, 124]

5.2.3. Humanized NPY-mRFP expressing RBL cells

The NFAT reporter cell lines RS-ATL8 and NFAT-DsRed RBL sensitively report IgE/FcεRI-mediated cell activation. However, these reporters protein do not directly mirror the degranulation event. Thus, the aforementioned reporter lines are not suitable for studies addressing degranulation. A new reporter cell line had to be created for such experiments, using the subcellular compartment targeting properties of Neuropeptide Y (NPY). NPY is a 36 amino acid peptide, discovered in 1982 by Tatemoto *et al.*.[125] which localizes to electron-dense secretory granules. Lang *et al.* first showed that expression of NPY fused with a fluorescent protein could be used to label granules in a neuronal cell line, PC12, using a NPY-GFP fusion.[126] This system has also been used for *in vivo* imaging in mice by Ramamoorthy *et al.*. [127] In the Lang *et al.* study, a specific variant of GFP had to be used, as earlier variants of GFP were not stable in secretory granules, due to the acidic environment and dense protein matrix. Degranulation of PC12 cells could be conveniently measured by quantifying fluorescence released from the cells. Functional NPY-Venus and NPY-mRFP fusion proteins in neurons were demonstrated by Yizhar and Ashery.[128]

We have shown that NPY-mRFP can also be used to label granules in non-neuronal RBL cells for exocytosis measurements.[129] It may seem curious that NPY can be localized in granules of a non-neuronal cell line such as RBL-2H3, in particular considering the high quantity of proteases present in the granules. In 1983, Greenberg and Burnstock discovered that, in addition to phagocytosis of MCs granules by phagocytes, rat MCs could exchange

granules between other cell types via another mechanism, which they termed "transgranulation", although in their study, transgranulation was only observed between MCs and fibroblasts or vascular endothelial cells. [130] Transgranulation between MCs and neurons was first described by Wilhelm *et al.* in 2005, who investigated mast cell-rich brain tissue from doves.[131] The transgranulation mechanism may be a possible means of communication between MCs and neurons, and may give a clue as to how MC granules, or in this case, RBL granules, may be similar enough to neuron granules to display similar cellular NPY localization. In addition, neurons may also secrete NPY in response to increases in cytosolic Ca^{2+},[126] similar to MC and basophil degranulation in response to Ca^{2+} influx, which demonstrates more similarities between MC, basophil and neuron behavior. Similarly to the NFAT luciferase or fluorescent protein reporters, we hypothesized that RBL cells stably transfected with NPY-mRFP and HsFcεRIα could result in a convenient way to detect allergic sensitization in human patient sera. Stable transfectants could be sensitized with the sera and exposed to allergen. Presence of clinically relevant allergen-specific IgE would cause degranulation in response to allergen stimulation, and release of NPY-mRFP, measurable by quantifying fluorescence in the supernatant (or, if needed, the decrease of red fluorescence in the cells after removing the supernatant and a few washes) (Figure 1). The advantages over the NFAT-luciferase and NFAT-DsRed RBL reporters consists in the lack of a need for expensive luciferase substrates and a shorter assay time (0.75-1 hour vs. 18-24 hours), respectively (Figure 1). Although this assay is a soluble mediator assay, and unlike the aforementioned NFAT-DsRed cells, it would not be suitable for use in allergen microarrays. However, this assay would be faster than the traditionally used β-hexosaminidase assay, as there would be no requirement for a substrate-developing step, and would be a direct reporter of degranulation, unlike the cytokine induction reporters.

<Insert Figure 1 here>

Finally, it is suggested here that when using RBL reporter cell lines for screening of anti-allergic compounds, there may be some clear benefit of using a combination of NFAT reporters (e.g. luciferase or fluorescent protein) with degranulation reporters (e.g. NPY-mRFP RBL) during screening. Assuming lack of toxicity, hits obtained with a candidate molecule in both cell lines, would point to inhibition of early events in IgE-dependent signal transduction, such as inhibition of syk or lyn kinases. In contrast, inhibition of one reporter, but not the other, would point to an inhibition of later steps in the signal transduction cascade (e.g. to events involved in membrane fusion or cytokine gene induction).

5.2.4 RBL cells transiently transfected with NPY-mRFP as model for functional genomics analyses of MC secretion

In the current post-genomic era functional genomics analyses are extensively applied to decipher signaling networks and protein interaction maps of cellular processes. In particular, high-throughput analyses employing RNAi or gain of function screens have opened new avenues for the systematic analyses of pathways. Systematic knock-down of proteins allows the identification of essential proteins versus those that can be deleted without hampering the integrity of the network, thus contributing to the robustness of the system. Computational biology then allows recapitulation of data into predictive models, which can form the basis for future experiments and analyses. Moreover, by subsequent rescue experiments the network hierarchy can be defined and crucial points for networks cross-talk identified. In MCs, functional genomics analyses have been limited due to their low (10-30%) transfection efficiency. For example, genetic manipulations are unlikely to leave an impact on readouts of average secretion measured by conventional methodologies if only a small fraction of the

releasing cells is transfected. Therefore, the major approaches to investigate MC activation involved studies on MCs derived from gene specific knock-out mice,[132] or stably transfected MCs. However, these approaches do not allow high-throughput analyses of cellular processes. To overcome this problem, we have recently introduced a model system that is based on the transient co-transfection of RBL cells with a gene of interest (i.e. WT, mutant or targeting shRNA) and NPY-mRFP, as a reporter for exocytosis.[5, 129, 133] We have shown that NPY-mRFP is targeted to the SGs where it colocalizes with serotonin, the endogenous mediator, and is released alongside the endogenous mediators serotonin and β-hexosaminidase.[129] Because the fluorescence of mRFP is on the whole pH insensitive, the reporter allows visualization of the acidic SGs in both fixed and living cells. Optimizing the transfection conditions to yield 100% co-transfection of the two genes, the reporter and the gene of interest thus allows to measure release exclusively from the transfected, hence, genetically manipulated cells, irrespectively of the actual efficiency of transfection (see Figure 2).

<Insert Figure 2 here>

Since RBL cells can be grown in 96- or 384-well plates, and the release of NPY-mRFP can be measured by the use of a fluorescence plate reader, this model allows functional genomics analyses of MC secretion. For example, we have used this model to screen the family of Rab GTPases on their impact on exocytosis triggered by either the IgE receptor or the chemical combination of Ca^{2+} ionophore and the phorbol ester TPA.[129] These studies could be further extended using the same model system to screen the kinome or any other gene family for their functional impact on secretion. Furthermore, a phenotypic screen can complement the functional screen, by taking advantage of the fact that the NPY-mRFP labeled SGs are easily

detected, allowing characterization of their morphology, quantification of their size and number as well as their spatiotemporal positioning in resting and activated cells.[5, 133] Indeed, taking advantage of these possibilities, we were able to define a role of the small GTPase Rab5 in SG fusion by observing an increase in SG size linked with the reduction of SGs numbers in RBL cells that were co-transfected to express NPY-mRFP and a constitutively active mutant of Rab5.[79] Conversely, co-transfection of NPY-mRFP with shRNAs directed against Rab5A/B/C the Rab5 isoforms that are endogenously expressed in RBL cells resulted in an increased number of smaller SGs.[79] This model also allowed us to demonstrate a role of Rab12 in regulating retrograde transport of the SGs. Specifically, we have shown that co-transfection of NPY-mRFP with a constitutively active mutant of Rab12, resulted in the perinuclear clustering of the SGs by a mechanism involving the motor protein dynein and its interacting protein RILP. Therefore, this model could be used in the future to screen for proteins that affect SG exocytosis as well as screen for proteins that impact SG morphology or transport.

Concluding statements

Overall, these examples show how RBL cells can and have been used for a multitude of purposes, ranging from powerful tools for the study of basic cellular mechanisms of intracellular membrane trafficking and exocytosis, to detection of allergens, vaccine safety studies and diagnosis of allergic sensitization. However, to which extent RBL cells can be used as model for studies on mechanisms underlying MC growth and survival or MC metabolics, needs to be determined experimentally in each individual case and would require verification before extrapolation of results.

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Figure Legends

Figure 1: Schematic representation of the three currently available RBL reporter cell lines RS-ATL8, NFAT-DsRed, and NPY-mRFP. In all three, RBL cells are sensitized overnight with serum or other IgE-containing solutions (sensitization step). The next day, cells can be challenged with a matching allergen, an anti-IgE antibody, or other stimuli leading to cell activation, such as Concanavalin A. Allergen will crosslink the IgE bound to the FcεRIα receptor chain and induce a kinase cascade involving the intracellular-tyrosine activating motifs (ITAM) on the FcεRIβ and/or γ-chains (not shown). This cascade will ultimately open Calcium release-activated channels (CRAC) in the membrane, leading to a sustained influx of Ca^{2+} ions into the cytosol. The elevation of intracellular Ca^{2+} activates calmodulin, which in turn activates the phosphatase calcineurin. Calcineurin will dephosphorylate cytosolic Nuclear Factor of Activated T-cells (NFAT), unmasking a nuclear localization signal (NLS). The NLS will lead to NFAT translocation from the cytosol into the nucleus, where it will bind to regulatory elements. In RS-ATL8 and NFAT-DsRed cells, NFAT nuclear translocation will result in transcription of luciferase and DsRed fluorescent protein, respectively. In NPY-mRFP, activation will result in the release of preformed, red fluorescence, which can be measured in the supernatant 45-60 minutes after activation, while luciferase and DsRed measurements require 3-4 hours or 18-24 hours of incubation, respectively.

Figure 2: Schematic representation of the use of RBL cells for functional genomics analyses of exocytosis. In this method, RBL cells are transiently co-transfected with the SG reporter NPY-mRFP and a second gene of interest or corresponding control plasmid. Normally, cell electroporation in the presence of the reporter and the manipulating gene at a 1:1 ratio will result in their co-expression in the majority of cells. Occasionally, increasing the amount of the manipulating gene is required to ascertain that all NPY-mRFP expressing cells, do also co-express the manipulating gene. Therefore, regardless to the percentage of transfected cells (i.e. efficiency of transfection), measuring the amount of NPY-mRFP in cell supernatants exclusively monitors release from the genetically manipulated cells, unlike measurements of endogenous mediators that are also released by non-transfected cells. Following their electroporation, cells are immediately seeded in 96/384-well plates and sensitized with IgE, if required. Twenty-four hours later, cells are activated by either the corresponding antigen (allergen), anti IgE or any other stimulus for the desired times. Supernatants containing released NPY-mRFP are then transferred to 96/384-well plates and fluorescence of released and residual cellular mRFP are measured using a fluorescence plate reader, allowing the quantification of release (i.e. percentage of NPY-mRFP released).

Figure 1

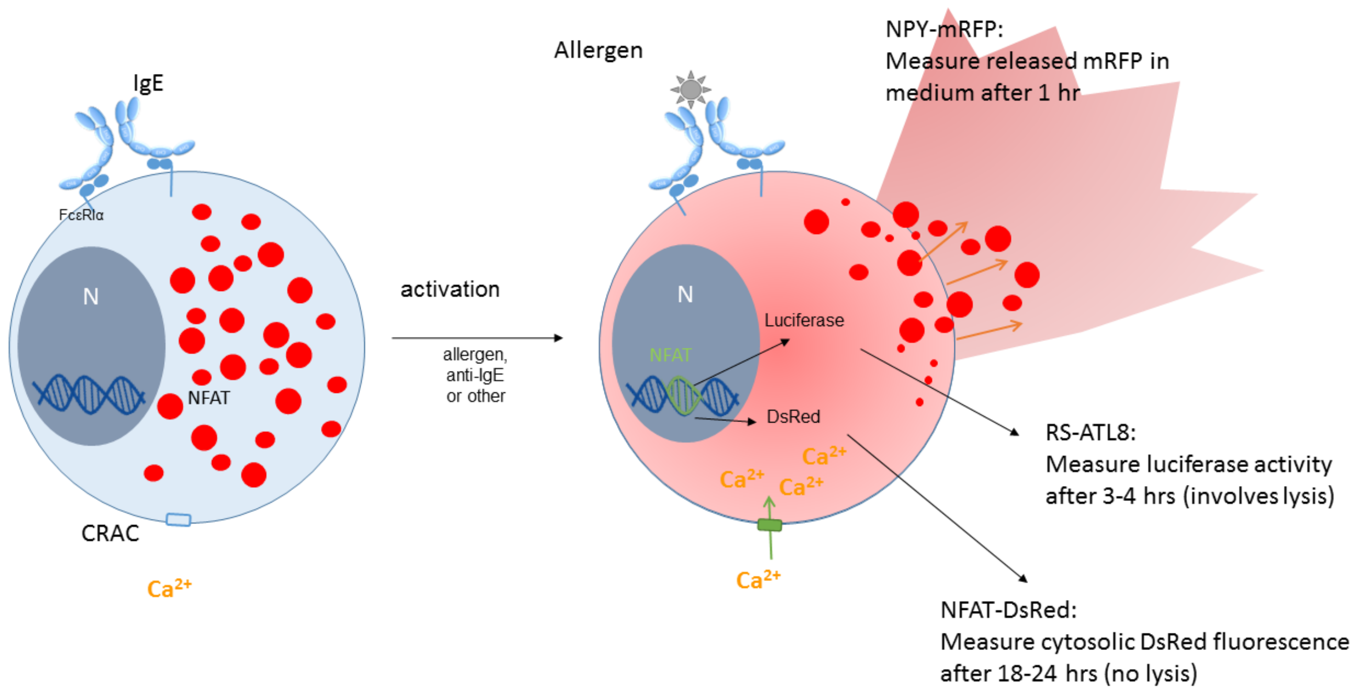

Figure 2