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Identification of biological and pharmaceutical mast cell and basophil related targets

by

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Running head: Training school report

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Abstract

The EU COST Action BM1007 “Mast cells and Basophils – Targets for Innovative Therapies” represents a network of researchers analyzing the physiological and pathological roles of mast cells and basophils in the organism in order to come up with new treatment strategies. In this review the conclusions from a training school organized by the network in February 2015 in Uppsala, Sweden are summarized. The training school was focused on the identification of biological and pharmaceutical mast cell and basophil related targets. It is well known that these cells are important effectors in the allergic response, but in addition they have also been recognized as being relevant in innate immune responses and chronic inflammatory diseases. The topics discussed during the workshop were: i) mast cell and basophil development ii) mast cell and basophil deficient animal models iii) differences between mouse and human mast cells and basophils as well as iv) mast cells and basophils as targets for tyrosine kinase inhibitors.

Introduction

Mast cells and basophils have been studied mainly regarding their implication in IgE-mediated allergic disease. Yet, it has become clear that apart from the high affinity IgE receptor (FcεRI), these cells can respond to many different inflammatory stimuli via expression of a large variety of additional receptors making them prime effectors and regulators in inflammation and immunity. Besides highlighting mast cell involvement in

different acute and chronic inflammatory and autoimmune diseases, studies have also revealed their beneficial functions.

The COST Action BM1007 “Mast cells and Basophils – Targets for Innovative Therapies” represented a network of researchers analyzing to a large part the physiological and pathological roles of mast cells and basophils in the organism in order to come up with new treatment strategies. Within this frame the task of one working group was to identify biological and pharmaceutical mast cell and basophil related targets through the identification of relevant pathways, the means to modulate mast cell and basophil functions, the promotion of studies in the human system as well as by the validation of pharmacological approaches and preclinical models.

In February 2015 the third and last training school related to this COST action was held in Uppsala, Sweden. The school gathered 20 students together with 8 trainers from 11 countries all across Europe in a relaxed study atmosphere to discuss hot mast cell topics. These topics were: i) Mast cell and basophil development in vivo and in vitro ii) Mast cell and basophil deficient mouse strains iii) The difference between human and mouse mast cells and basophils iv) Are kinase inhibitors potential targets for mast cell and basophil driven diseases? Here we summarized the conclusion reached during this two-day training school in the form of a commentary report.

Mast cell and basophil development in vivo and in vitro

Identifying and mapping the different stages of mast cell and basophil development and differentiation is important for designing specific drugs targeting these cells. Current knowledge on the mast cell maturation and differentiation process has been reviewed recently [1], indicating an important role of transcription factors and surface molecules such as integrins and cytokine receptors. New types of regulators have been identified by a screen of microRNAs (miRNAs) expressed during differentiation of mast cells from bone marrow. Very specific miRNA patterns were found to be expressed governing the expression

of differentiation and maturation markers such as Kit and the high affinity IgE receptor (FcεRI) [2]. miRNAs play critical roles in maintaining gene expression at appropriate levels and these small molecules may thus be new potential targets to inhibit particular steps of mast cell differentiation and/or maturation.

Another important aspect of characterizing mast cell and basophil development is to establish better protocols to study these cells *in vitro*. Due to the low numbers of basophils in blood, many experimental procedures are difficult to perform. Making researchers to rely on basophil cell lines or bone marrow derived basophils differentiated *in vitro*. Likewise, mast cells reach full maturation in tissues and mature mast cells are not found in the circulation [1]. Hence, researchers generally use established cell lines or mast cells differentiated *in vitro* from progenitor cells (i.e. cord blood, bone marrow). Accordingly, better understanding of mast cell and basophil development and maturation should yield better protocols for differentiating them *in vitro*. Currently, some contradictions arise from the literature regarding the cytokines and factors critical for mast cell and basophil differentiation. For example, while differentiation of murine bone marrow derived mast cells can be achieved with IL-3 in the absence of stem cell factor (SCF), human mast cells are typically differentiated using IL-6 and SCF. Manninka et al have characterize *in vitro* culture conditions suitable for human mast cells and suggested that human mast cells arise from a committed progenitor distinct from other myeloid cells [3].

Since mast cells reach final maturation in tissues, it is plausible that other tissue-resident cells supply additional maturation signals that may be responsible for the existing considerable heterogeneity. Consistent with this notion mast cells in various types of tissues present different phenotypes, such as connective tissue or mucosal mast cells. Hsieh et al. [4] have shown that airway epithelial cells can provide necessary factors for expression of tryptase/chymase in cord blood differentiated mast cells. They also demonstrated that culture conditions affect their capacity to release leukotrienes. This finding is important since most studies tend to characterize human mast cells only by their protease content.

Importantly, when attempting to study mast cells *in vitro*, one should consider the type of mast cell that is relevant for the physiological aspect of the study and adjust culture conditions, accordingly. Thus, to model human lung mast cells, special culture conditions may be required such as co-culture with airway epithelial cells along with IL-3 and IL-4 [5]. Cell differentiation is tightly regulated by transcription factors. Mast cell and basophil maturation and differentiation requires GATA and STAT transcription factors families [6, 7]. However, new transcription factors continue to be discovered. In mice, interferon regulatory factor-8 (IRF8), a transcription factor essential for the development of several myeloid lineages, also regulates basophil and mast cell development [8]. It is expressed in granulocyte progenitors but not in basophils, mast cells, and basophil/mast cell-restricted progenitors. However, in IRF8 knock-outs, mast cell and basophil development is inhibited supporting a role in the generation of mast cell and basophil progenitors, likely involving its function as a transactivator of GATA2 [8]. The complexity of the gene expression regulation during cell differentiation highlights the need for searching additional candidate transcription factors.

Current drugs used for mast cell and basophil-induced pathologies are non-specific or target only a single mediator like anti-histamines calling for more selective targets and knowledge about factors determining mast cell and basophil development. Improved *in vitro* systems to model the *in vivo* mast cell and basophil phenotypes are also crucial for the research community.

Mast Cell & Basophil Deficient Mouse Strains

The role of mast cells was originally studied in mouse models carrying natural mutations in Kit, i.e. WBB6F-Kit W/W-v and C57BL6-Kit W-sh/W-sh, which resulted in 99% depletion. However, due to altered KIT expression in other cell types, abnormalities of haematopoiesis and in the immune system have been described [9]. Basophil *in vivo* research for long time was impossible lacking naturally occurring deficient mouse strains. First attempts were made using basophil depleting monoclonal antibodies: anti-FcεRIα (clone Mar-1) or anti-CD200R3

(clone Ba103) an IgE-independent activating receptor. Despite basophil depletion (>90%), reports of mast cell activation leading to anaphylaxis [10], depletion of dendritic cells [11] via FcεRI and activation of myeloid cells and natural killer cells through CD200R [11, 12], complicated the interpretation and needed further validation.

This triggered new mast cell and basophil depletion strategies, e.g Cre recombinase models (usually under the control of mast cell or basophil-associated proteases). Cre mediates constitutive depletion either by toxicity (Cpa3cre/Cre Master; Mcpt8-Cre) [10, 13] or by crossing with Rosa-DTα (R-DTA) mice expressing the diphtheria toxin (DT) α subunit downstream a loxP-flanked stop cassette from the Rosa locus (Mcpt5-Cre-R-DTA, Basoph8) [14-16]. Conditional depletion is achieved after injection of DT into DT Receptor-knock in mice, either by Cre-inducible DTR (iDTR), in which DTR expression is blocked by an upstream LoxP-flanked stop sequence (Mcpt5-Cre-iDTR) [15, 16]; or by a mast cell/basophil protein specific DTR transgenic mice (Mas-TRECK, Bas-TRECK, Mcpt8 DTR) [17, 18]. A recent model, the red mast cell and basophil model (RMB) [19] allows tracking and depletion of both granulocytes selectively due to the insertion of the bright red td-Tomato (tdT) fluorescent protein and the human DTR in the Ms4a2 gene encoding the FcεRI β chain. Mast cells and basophils are traditionally thought of in the context of TH2 type responses, however, recent studies indicated roles beyond allergic and anti-helminth responses [20, 21]. Furthermore, significant differences in mast cell phenotypes derived from different mouse strains, different environment and different investigators have been identified influencing disease susceptibility [9]. Hence, emphasis should be made to carefully report experimental conduct, as well as detailed genetic backgrounds of mouse strains, animal husbandry, and origin of mice.

The new deficient mouse models should allow to validate previous work performed with the KIT-dependent mast cell deficiencies. Previous work in Kit^{W/W-v} and Kit^{W-sh/W-sh} mice showed that mast cells can contribute to orchestrating neutrophil recruitment in various inflammatory responses. IgE-dependent local and systemic anaphylaxis reactions in both

KIT-dependent and independent mast cell-deficient mice, (KitW/W-v mice, KitW-sh/W-sh mice, and mast cell-depleted-Mcpt5-Cre;iDTR mice [22-25] have consistently demonstrated a critical role of mast cells. However, conflicting results regarding their role in contact hypersensitivity (CHS) responses have been generated. Researchers demonstrated that CHS responses to dinitrofluorobenzene (DNFB) were enhanced in KitW/W-v and KitW-sh/W-sh mice while mast cells were critical for CHS responses to DNFB and fluorescein isothiocyanate when using Mcpt5 Cre-iDTR mice or Mcpt5 Cre- R DTA mice. Additionally, immediate ear swelling response was abolished in Mcpt5 Cre-iDTR mice but remained intact in the Kit-mutant strains. Further studies are required to clarify the mechanisms that might explain these contradictory results. Similar validation and/or controversies applied also to other pathologies. Despite these discrepancies, previous studies on KIT-dependent and recent work on KIT-independent mast cell deficiencies should be seen as complementary.

Concerning basophils, generation of (inducible) basophil-deficient mice allowed profound advances in the understanding of their immune regulatory and effector roles. Thus basophil involvement in acquired tick feeding resistance demonstrated in the 1980's in guinea pigs [26] has been validated just recently, with the help of the Mcpt8-DTR mice[17, 18]. However, conflicting results regarding the role of basophils in antigen presentation or TH2 response to papain have arisen in the literature when antibody-mediated and genetically-mediated basophil depletions have been compared [10, 27-30]. Despite these controversies, basophil-deficient mice represent new tools that allow deciphering the role of basophils in health and disease ranging as well as their regulatory role in humoral immune responses and autoimmune diseases [31].

Taken together, while no “cleaner models” for the study of mast cells and basophil functions have become available, the previous studies using KIT-dependent MC deficiencies and antibody-mediated basophil depletion should still be considered as valid, despite the observed differences. One may want to keep in mind that the true mechanisms involving mast cells and basophils in pathologies may reside precisely in these differences.

Differences between human and mouse mast cells and basophils

Mouse mast cell and basophil models have been the mainstay of *in vivo* and *in vitro* investigations to study the causes and to identify cures for mast cell and basophil driven diseases in humans [32, 33]. Their use has provided valuable insight and triggered various hypotheses about mast cell and basophil driven diseases and remain the prime tools for the identification of biological and pharmaceutical mast cell and basophil related targets [32, 34]. However, questions remain as to whether these models have been very useful in the identification of these targets for applications in humans [23, 35, 36]. Concise studies that examine the predictability of these models in human disease is still scanty. For example, recent large scale studies have shown that the small differences between the mouse and human genome can give rise to significant differences in their immune system and inflammatory response [36, 37]. It is therefore not surprising that several drugs for mast cell and basophil related disease like asthma that were reported to be effective in preclinical models have failed in clinical trials [38, 39]. This warrants careful consideration in experimental design and the extrapolation of data to humans considering known differences between mouse and human mast cell and basophils. In this respect some of the differences between mouse and human mast cells and basophils are summarized in table 1 and 2. These differences suggest that to enhance the chances of identifying clinically useful mast cell and basophil related targets new models that precisely mimic human disease are needed. This will not only accelerate the identification of relevant targets, but will also result in efficient use of the already scarce resources available for research.

The most important question to answer is how to overcome these differences in order to avoid errors when we interpret our results and extrapolate them from mouse or other models to human. One important step is to validate results in the most suitable human model for a specific physiological or pathophysiological context. Furthermore protocols to obtain, culture and characterize primary mast cells and basophils should be improved in order to approach them as close as possible to this context. Yet, it should be clear that, *in vitro* and *ex vivo* approaches have still important limitations and do not necessarily reflect the complex

relationships in the human body. Several groups have proposed “humanized mouse models” to study the behavior of human cells into a whole body [40]. In 2004, Kambe and collaborators were able to populate mouse tissues with human mast cells with the same distribution pattern to human body [41] by using an immunodeficient mouse model (NOD/SCID)/gamma(c)(null) (NOG model). This model allows studying key molecules involved in cell differentiation, cell migration or cell function in a whole body context.

In summary, accumulated evidences show that a large part of animal validated drugs does not make it into clinics. While this may be in part due to incoherence in animal studies themselves (see chapter above) important differences exist also when comparing animal models and humans. For this reason, the identification of the differences between mouse (or any other animal model) and humans will help us to know our limitations and will enable us to extrapolate animal data to humans better.

Mast cells and Basophils as targets for tyrosine kinase inhibitors

The non-redundant roles of mast cells and basophils in allergic disorders and systemic mastocytosis are well known. However, mast cells and basophils are increasingly recognized as important contributors to non-allergic inflammatory and autoimmune disorders. Several of the mast cell and basophil signalling pathways are regulated by tyrosine kinases (TKs). There are about 90 human TKs which regulate cell signalling and protein function by transferring a phosphate group from ATP to the hydroxyl group of a target protein tyrosine [42]. About 90% of mastocytosis patients present the D816V mutation within the cytoplasmic tail of the receptor tyrosine kinase Kit. It results in constitutional Kit activation leading to the pathologic mast cell proliferation and mediator release [43, 44]. TK signalling can be suppressed by tyrosine kinase inhibitors (TKI) preventing phosphorylation of target proteins. As TKs are involved in mastocytosis, inhibitors have been considered as a feasible way for treatment. Furthermore, specific mast cells eradication may also be a means to treat mast cell driven diseases.

Several TKIs that target mast cells and basophils are approved by the FDA for clinical use or are in the clinical trial phase. Imatinib was the first TKI used in mastocytosis patients, however, patients carrying the D816V mutation are resistant, as well as for Nilotinib and Ponatinib, which all target KIT. Subsequent trials focused on other compounds with a more broad inhibitory spectrum. Thus, Dasatinib is inhibiting the SRC family protein TKs, whereas Midostaurin also targets protein kinase C (PKC) [45]. Dasatinib and Midostaurin, in addition to act on clonal mast cells and basophil diseases, have also been shown to be potent inhibitors of mast cell and basophil degranulation *in vitro* by targeting both the KIT and FcεRI receptor signalling pathways [46, 47]. Nilotinib inhibits passive cutaneous anaphylaxis in mice and Imatinib has shown effectiveness in ameliorating diarrhea in a murine food allergy model [48] [49]. Currently, a trial study is testing the effect of Imatinib on treatment of resistant asthma (ClinicalTrials.gov identifier: NCT01097694). So far, all these studies have focused on the short-term effects of TKIs on KIT and IgE-mediated signalling. Results from long term Imatinib use in chronic myeloid leukaemia reveal that mast cell depletion can be achieved with less severe side effects compared to short-term treatment, indicating that mast cell depletion can be another possible TKI therapeutic stratagem [50].

Since TKIs have a broad target profile and are not mast cell specific, they are associated with many adverse events depending on the particular TKI compound. These include periorbital oedema, nausea, cytopenia, folliculitis and pancreatitis, thereby limiting the usefulness in mild diseases [51]. However, recent studies have shown the possibility of combining TKIs for synergistic effect. The big advantage is that patients can be treated by using lower drug concentrations, with possible fewer side effects but, fascinatingly, with much higher anti-neoplastic or even anti-allergic effect. As an example, the combination of Ponatinib and Midostaurin requires 75% less Midostaurin and 66% less Ponatinib in comparison to the individual use of the drugs to halve the proliferation speed (IC₅₀) of HMC1.2 (KIT^{D816V}) cell-lines [52]. Moreover, combinations with non-KIT based therapies are currently being developed. For instance,

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synergistic apoptotic effect is observed after calcineurin phosphatase and KIT inhibition in KIT-mutant mast cell lines [53]. Thus, TKIs can be used in multiple synergistic ways to still get a desired outcome in patients with the least systemic problems due to reduced side effects.

In conclusion, TKIs are potentially attractive compounds thus far reserved for severe treatment and resistant diseases. New developments with increasing specificity or the use of synergistic dosing schedules are promising to broaden the therapeutic spectrum i.e. include less severe diseases. The benefit would be the low degree of side effects, which can be seen in the TKIs used in the clinic today.

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Table 1. Difference between human and murine mast cells

Feature	Murine mast cells	Human mast cells	Reference
General phenotype	MMC, CTMC Based on granule proteoglycan Relatively well correlated with tissue distribution	MC _T , MC _{TC} , MC _C Based on granule protease	[54]
Proteoglycan	MMC: Chondroitin sulfate di-A, B E CTMC: Heparin, Chondroitin sulfate-E	Heparin, Chondroitin sulfate-A, E	[54]
Protease	Several tryptases and chymases with different specificities (e.g., mouse MMCs predominantly express MMCP-1 and -2, whereas mouse CTMCs predominantly express MMCP-4, -5, -6, -7, and carboxypeptidase)	MC _T : Tryptase MC _{TC} : Tryptase, chymase MC _C : chymase Three tryptases (α , β , and γ) and one chymase	[54]
Functional IL-3 receptor	+	(+) Human intestinal MCs (-) Human lung, skin, uterus, kidney, or tonsils MCs and HMC-1 (+/-) CBMCs [#] (-) PBDMCs	[55] [56, 57] [58, 59]
Fc ϵ RI	+ Obligatory tetrameric $\alpha\beta\gamma_2$ structure ; IL-4, IL-10 and TGF β 1 inhibit Fc ϵ RI expression ; Binds murine IgE	+ Fc ϵ RI β chain is dispensable for Fc ϵ RI cell-surface expression; IL-4 enhances Fc ϵ RI α chain expression; Binds human and murine IgE	[54] [60]
Fc γ RI	Fc γ RIIb1, Fc γ RIIb2, Fc γ RIII: constitutively expressed in mouse BMMCs, RBL-2H3, and increased expression in mouse mature PMCs	Fc γ RI: IFN γ increases Fc γ RI expression in PBMCs Fc γ RII: PBMCs constitutively express Fc γ RIIa. Human skin MCs constitutively express Fc γ RIIa but not Fc γ RIIb, whereas CBMCs constitutively express Fc γ RIIb but neither Fc γ RIIa nor Fc γ RIIc	[54]
MHC	MHC-I: constitutively expressed in mouse BMMCs MHC-II: IFN γ and LPS enhance	MHC-1: constitutively expressed in human	[54]

	MHC-II expression in mouse BMMCs and spleen-derived MCs. IL-3 inhibits MHC-II expression in mouse BMMCs. IFN γ and IL-4 enhance MHC-II expression in mouse PCMCs. IFN γ enhances MHC-II expression in rat MCs isolated from pleural cavity.	skin, lung, liver and uterus MCs, and HMC-1 MHC-II: IFN γ and TNF enhance MCH-II expression in HMC-1	
CD14	+	-	[61]
Functional TLRs expression [#]	+++	+/-	[62]
Sex Hormone Receptor	ER α : constitutively expressed in BMMCs PR: constitutively expressed in BMMCs	ER α : constitutively expressed in human MCs in nasal polyps, abdominal aortic vessels (fertile women), HMC-1, and LAD2 PR: constitutively expressed in human MCs in nasal polyps and HMC1	[54]
EP2 receptor	(+) PCMCs (-) BMMCs	(+++) PBDMCs, LAD2	[63]
CCR3	(-) BMMCs	(+) Immature and mature CBMCs, human gut, lung, skin MCs	[64] [65]
IL-4 production	(+) Spontaneously or after stimulation by Fc ϵ RI aggregation	(-) Under normal conditions (+) Under allergic conditions	[61] [66]
IL-5 production	+? *	+++	[61]
TNF production	+++	+	[61]
NOS	Mouse MCs: iNOS, eNOS Rat MCs: nNOS, iNOS, eNOS	nNOS, iNOS, eNOS	[54]

[#]See references for details. *Murine mast cells express IL-5 but IL-5 protein synthesis and release from murine mast cells has not been quantified. BMMC, bone marrow-derived mast cells; CBMC, human cord blood-derived mast cell; CTMC, connective tissue-type mast cell; eNOS, endothelial nitric oxide synthase; ER α , estrogen receptor α ; IFN γ , interferon- γ ; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; MMC, mucosal type mast cell; MMCP, mouse mast cell protease; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; PBMC, human peripheral blood-derived mast cell; PCMC, peritoneal cell-derived mast cell; PMC, peritoneal mast cell; PR, progesterone receptor; RBL-2H3, rat basophilic leukemia 2H3 cell line; TLR, toll-like receptor; TNF, tumor-necrosis factor

Table 2. Difference between human and mouse basophils

Feature		Murine basophils	Human basophils	References
Act as antigen presenting cells (APC)		Yes	?	[67]
Phenotypical markers	FcεRI	+	+	[67]
	CD11b	+/-	+ (on IL-3 activation)	[68, 69]
	CD15	?	+	[70]
	CD16a (FcγRIIIA)	+	-	[67, 70, 71]
	CD16b (FcγRIIIB)	-	+	[67, 71]
	CD41	+ (activation marker ↑)	?	[72]
	CD63	?	+ (activation marker ↑)	[73, 74]
	CD117	+/-	+/-	[67, 75]
	CD123(IL-3R)	++	+++	[68, 70]
	CD191 (CCR1)	?	+	[76]
	CD192(CCR2)	++	+	[67, 68]
	CD193(CCR3)	-	+	[67, 76, 77]
	CD195 (CCR5)	-	+	[68, 76]
	CD200R	+ (activation marker ↑)	+	[74, 78]
	CD200R3	+	?	[12]
	CD203c	-	+(activation marker ↑)	[70, 74]
	CD300a	?	+	[79]
	protease activated receptor	+	-	[67, 80]
Cytokine production	IL3	?	+	[67, 81]
	IL-5	-	+	[67, 82]
	TSLP	+	?	[28, 67]

MCP-2, monocyte chemotactic protein 2; MIP-1 α , macrophage inflammatory protein-1 α ; TPA, 12-o-tetradecanoyl-phorbol-13-acetate; TSLP, thymic stromal lymphopoietin; RANKL, Receptor activator for nuclear factor kappaB (RANK) ligand.