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Mast cell regranulation requires a metabolic switch involving mTORC1 and a glucose-6-phosphate transporter

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Summary

Mast cells (MCs) are granulated cells implicated in inflammatory disorders because of their capacity to degranulate, releasing prestored proinflammatory mediators. As MCs have the unique capacity to reform granules following degranulation *in vitro*, their potential to regranulate *in vivo* is linked to their pathogenesis. It is not known what factors regulate regranulation let alone if regranulation occurs *in vivo*. We report that mice can undergo multiple bouts of MC regranulation following successive anaphylactic reactions. mTORC1, a nutrient sensor that activates protein and lipid synthesis, is necessary for regranulation. mTORC1 activity is regulated by a glucose-6-phosphate transporter, Slc37a2, which increases intracellular glucose-6-phosphate and ATP during regranulation, two upstream signals of mTOR. Additionally, Slc37a2 concentrates extracellular metabolites within endosomes which are trafficked into nascent granules. Thus, the metabolic switch associated with MC regranulation is mediated by the interactions of a cellular metabolic sensor and a transporter of extracellular metabolites into MC granules.

Graphical Abstract

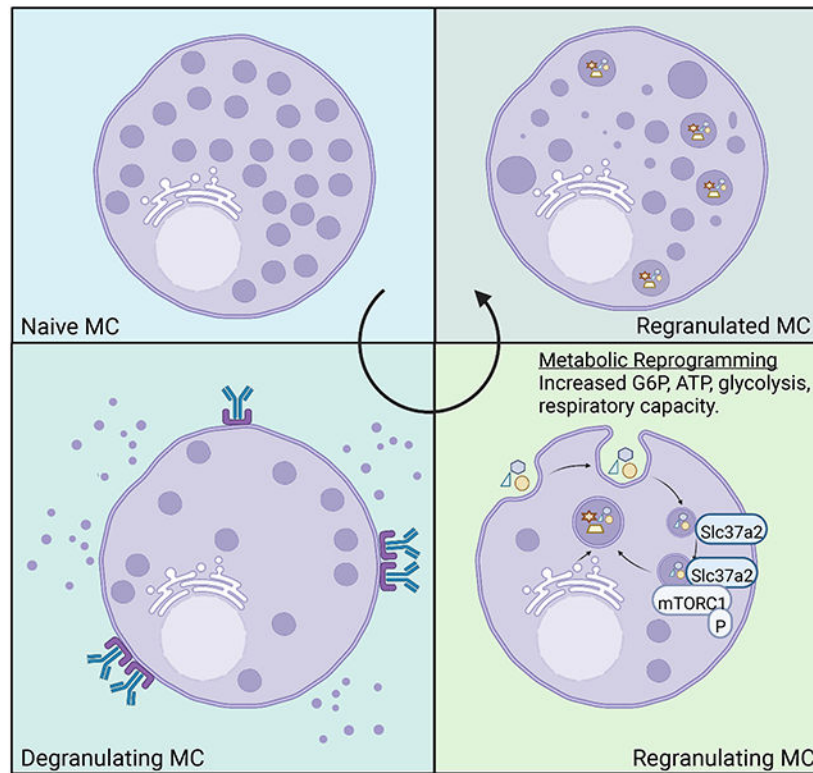
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Author Contributions

J.I. designed, performed, and analyzed most of the experiments. M.A.A. and J.S. helped perform some of the animal experiments. J.S. also performed the flow cytometry experiments. Y.M. and S.N.A. conceived the project. A.P.S.R. provided technical assistance on flow cytometry data. The manuscript was written by J.I. and S.N.A. with helpful comments from A.P.S.R. and Y.M.

Competing Interests

The authors declare no competing interests.



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Mast cells have been shown to have the capacity to reform granules following degranulation *in vitro*. Iskarpatyoti et al. demonstrate that regranulation also occurs *in vivo*. This process requires a metabolic reprogramming that is regulated by an interaction between mTORC1 and the glucose-6-phosphate transporter, Slc37a2.

Introduction

Mast cells (MCs) are long lived, highly granulated tissue resident cells of hematopoietic lineage, located at the host-environment interface (Gurish and Austen, 2012, Metcalfe et al., 1997). Although a primary physiologic role is promoting immune responses to infectious agents (Marshall, 2004, Abraham and St John, 2010, Urb and Sheppard, 2012), MCs are best known for promoting chronic inflammatory disorders such as urticaria, anaphylaxis, arthritis, and asthma (Theoharides and Kalogeromitros, 2006, Amin, 2012, Boyce, 2003, Yu et al., 2006). Many of the pathogenic contributions of MCs are linked to their capacity to release bioactive mediators that are stored within granules such as lysosomal hydrolases, amines, cytokines, chemokines, proteases, and proteoglycans for sustained periods (Wernersson and Pejler, 2014). These mediators, upon release from extracellular MC granules in a process called degranulation, promote the recruitment of various immune cells from circulation into the tissue (Abraham and St John, 2010). However, when MC release of mediators is excessive or unregulated, significant tissue damage can result.

Since MCs are long lived cells, having been described to survive *in vivo* for several months (Kiernan, 1979, Padawer, 1974), another MC trait thought to promote its pathogenesis is its unique capacity to regranulate in place following degranulation. Indeed, *in vitro* studies have reported that MC degranulation is accompanied by regranulation (Kobayasi and Asboe-Hansen, 1969, Xiang et al., 2001). This suggests that MCs have the potential to undergo multiple cycles of degranulation and regranulation *in vivo* thereby markedly enhancing their capacity to contribute to pathologies found in chronic allergic conditions such as asthma or atopic dermatitis where significant changes to tissue homeostasis are observed (Ando et al., 2013, Okayama et al., 2007). However, at this time, it is not known if multiple cycles of MC degranulation and regranulation can occur *in vivo* and if so, what factors regulate this activity.

While the functional relevance of MC degranulation is well established, the biogenesis of MC granules remains less well known. Investigations of MC granule biogenesis have been limited to mostly microscopy-based studies, and they have revealed that granule formation is initiated by uniform-sized progranules within MCs which bud off from the trans-golgi (Hammel et al., 1985, Combs, 1966). Thereafter, progranules fuse with each other forming larger, immature granules which ultimately associate with endosomes that are positive for CD63, a secretory lysosomal membrane marker (Hammel et al., 2010, Moon et al., 2014, Azouz et al., 2014). Biochemical studies of isolated MC granules have shown them to contain a dense core comprising of negatively charged serglycin proteoglycans which avidly bind to positively charged granule components such as chymase and tryptase (Rönnberg et al., 2012). Cumulatively, these ionic interactions stabilize the granule structure and keep the granule components from rapidly disaggregating immediately upon release from MCs allowing distant communications within the body (Kunder et al., 2009, St John et al., 2012).

Conceivably, mechanisms involved in the initial biogenesis of granules, such as protein and lipid synthesis, intracellular trafficking of progranules and vesicles, and packaging of granule components within MC granules are also involved in MC regranulation. Since the bulk of these activities will require significant energetic demands, we hypothesized that following degranulation, marked metabolic reprogramming within MCs would occur to accommodate regranulation. It is well known that mTOR, an evolutionarily conserved ser/thr kinase, is a critical regulator of the metabolic switch that occurs during T cell differentiation (Chi, 2012, Salmond, 2018). Specifically, mTORC1, one of two complexes formed by mTOR, is activated upon sensing sufficient glucose and amino acid levels which leads to downstream phosphorylation of 4E-BP and S6K which results in protein and lipid synthesis (Saxton and Sabatini, 2017a). Additionally, mTORC1 activation is required for increased glycolysis in T cells during differentiation (Finlay, 2012). Based on these observations, we propose that mTORC1 activation is necessary for MC regranulation. Here, we sought to validate this notion and as well as identify additional factors in MCs that contribute to MC regranulation.

Results

MCs can undergo multiple bouts of degranulation and regranulation in mice.

To date, the capacity of MCs to undergo more than one round of degranulation *in vivo* has not been studied. Here, we investigated if IgE-sensitized peritoneal MCs can undergo multiple rounds of degranulation *in vivo* following serial exposure to antigen (Ag). A mouse model of anaphylaxis was used to assess the inflammatory response induced by and correlated to the degranulation of IgE sensitized MCs (Ang et al., 2016, Choi et al., 2018). To induce MC degranulation, we passively sensitized mice with TNP-specific IgE i.p. then challenged these mice 16 hrs later with TNP-OVA Ag i.p. All the sensitized mice experienced anaphylaxis as shown by a drastic drop in core body temperature (Fig. 1a, 1st Ag challenge). We observed that temperatures began to drop in the mice within 15 minutes of Ag challenge. Temperatures continued to drop for 30 minutes before returning to baseline core body temperature (Fig. 1a).

Since MCs have demonstrated the capacity to regranulate *in vitro* (Xiang et al., 2001, Kobayasi and Asboe-Hansen, 1969), we reasoned that recently degranulated mouse peritoneal MCs should regranulate *in vivo* and participate in additional anaphylactic reactions when rechallenged with Ag. To investigate if recently degranulated MCs could regranulate *in vivo*, we collected peritoneal MCs from mice on days 1, 3, 5, and 7 following anaphylaxis and measured intracellular avidin, a probe for heparin (Tharp et al., 1985) in MCs, to assess granularity. As expected, the percentage of MCs containing high levels of avidin were significantly diminished on day 1, indicating that MCs were degranulated during anaphylaxis (Fig. 1b). MCs containing high levels of avidin increased over the 7-day time course, reaching a similar percentage of MCs as naïve mice by day 7 (Fig. 1b).

To further examine the state of granulation of these MCs, we collected peritoneal cells from mice on day 7 post Ag challenge and compared them to peritoneal cells from naïve mice via microscopy. MCs from both groups of mice were stained with anti-CD63 antibody, a probe for MC granule membranes, and Avidin-FITC. In naïve mice, Avidin-FITC⁺ and CD63⁺ MC granules occupied most of the cytoplasm of peritoneal MCs and were of relatively uniform size (Fig. 1c, top row). Although the level of granulation in MCs from Ag-challenged mice 7 days prior were comparable to naïve MCs, as shown in Fig. 1b, the granule size and shape was far more heterogeneous (Fig. 1c, middle row). As we would expect newly recruited MCs to appear similar to naïve MCs, these results confirm that 7 days after Ag-challenge, MCs are regranulated *in vivo*.

Next, we sought to examine if these regranulated MCs could function regularly by mediating anaphylaxis. A week post initial Ag challenge, we passively sensitized and challenged the same mice again. We found that mice challenged 7 days previously exhibited a comparable drop in core body temperature, reaching the lowest core body temperatures by 30 minutes (Fig. 1a, 2nd Ag challenge). 7 days after the second challenge, peritoneal MCs were examined and we found that like MCs seven days after the initial Ag challenge, MC granule size and shape were more heterogeneous than observed in naïve mice (Fig. 1c, bottom row). Also, a similar drop in body temperature was observed when these mice were sensitized and challenged a third time 7 days later (Fig. 1a, 3rd Ag challenge). Interestingly,

we observed a trend towards faster recovery time from anaphylaxis after each Ag-challenge. These observations reveal that MCs can undergo repeated bouts of degranulation without significant loss in degranulation (or regranulation) capacity.

To further confirm that MCs can undergo multiple bouts of degranulation and regranulation without significant loss in functional capacity, we compared immune cell recruitment into the mouse peritoneal cavity, the site of MC activation, after each Ag challenge. Mice were euthanized 24 hours after the first, second or third Ag-challenge and peritoneal cells were collected by lavage. Inflammatory response was determined by flow cytometry (Supplemental Fig. 1). We found that after the initial Ag-challenge, the percentage of CD45⁺CD11b⁺Ly6G⁺ neutrophils, and CD45⁺Ly6G⁻Ly6C⁺ monocytes were increased compared to PBS treated controls (Fig. 1d and e). Notably, the second and third Ag-challenge, while causing anaphylaxis, had similar percentage of neutrophils in the peritoneum compared to PBS-control mice while maintaining increased monocyte recruitment. Importantly, the percentage of cKit⁺ MCs in the peritoneum remained similar after each Ag-challenge compared to PBS controls (Fig. 1f). As expected, intracellular staining with avidin showed that after each Ag-challenge there were significantly fewer Avidin⁺ MCs compared to PBS control mice, suggesting that 24 hrs after each Ag challenge, MCs were mostly degranulated (Fig. 1g). Taken together, this indicates that the second and third Ag-challenge resulted in MC degranulation capable of producing robust anaphylaxis inflammatory response.

These findings reveal that MCs have the innate capacity to regranulate *in vivo* after multiple bouts of degranulation, a distinct property that can potentially contribute to sustaining chronic MC mediated inflammatory reactions.

Kinetics of MC regranulation *in vitro*.

We sought to elucidate the molecular basis of MC regranulation. First, we investigated the kinetics of MC regranulation. For these studies, we employed cultured primary bone marrow-derived MCs (BMMCs) from mice. To ensure that our studies of MC regranulation were broadly applicable to other modes of MC degranulation, we induced MCs degranulation employing ionomycin, a Ca²⁺ ionophore that induces calcium flux, a critical signaling event preceding MC degranulation regardless of the agonist employed. We assessed degranulation and regranulation by measuring residual cellular content of β -hexosaminidase, a component of the granule that is released upon degranulation and is resynthesized within granules when MCs regranulate. As expected, following degranulation, total β -hexosaminidase in BMMCs were significantly reduced compared to untreated controls (Fig. 1h). 6 hrs post degranulation, β -hexosaminidase remained largely unchanged, however, between 24 and 48 hrs post degranulation, BMMCs had recovered β -hexosaminidase comparable to amounts seen prior to degranulation. Thus, *in vitro* MC regranulation takes between 24 and 48 hours to complete.

mTORC1 is necessary for MC regranulation.

For MCs to fully regranulate, significant protein and lipid synthesis must occur followed by vesicle trafficking, and proper packaging of various granule components into granule

structures. These processes exert increased metabolic demands on the cell, requiring substantial metabolic reprogramming to increase available energy. A potentially important cellular regulator of metabolic reprogramming is mTOR, a kinase that has been shown to act as a metabolic switch in T cell activation and innate immune cell functions (Chi, 2012, Salmond, 2018, Weichhart et al., 2015). To investigate a possible role for mTOR in MC regranulation, we induced MC degranulation with ionomycin and measured mTOR activity by Western blot. We observed that at 2 hrs post degranulation, mTOR had increased phosphorylation (Fig. 2a). Furthermore, S6K a serine/threonine kinase, and a downstream target of mTORC1, also appeared to be phosphorylated at the same time point (Fig. 2a). To assess the role of mTOR in regranulation, BMMCs were treated with torin, a selective inhibitor of mTOR activation (Liu et al., 2012), prior to degranulation with ionomycin. Torin-pretreated BMMCs exhibited no significant difference in total percent release of β -hexosaminidase upon degranulation (Fig. 2b, left). However, BMMCs maintained in torin following degranulation demonstrated a dose-dependent inhibition in their capacity to regranulate based on the reduced amounts of total β -hexosaminidase found in BMMCs 48 hrs post degranulation (Fig. 2b, right). This suggests that although mTORC1 plays no significant role in MC degranulation, its activation is necessary for MC regranulation.

To extend these findings, we investigated tamoxifen-inducible Raptor knockout (Raptor^{f/f} ER-Cre (Raptor-T-KO)) mice. Raptor (regulatory-associated protein of mTOR) is a necessary component for the formation of mTORC1. We hypothesized that we could demonstrate the role of mTORC1 by selectively inhibiting MC regranulation without inhibiting the initial granule biogenesis by treating BMMCs derived from these mice with tamoxifen only after granule maturation has been completed. Mature BMMCs from Raptor-T-KO mice were treated with 4-hydroxytamoxifen to knockout Raptor (Supplemental Fig. 2a). Raptor knockout was confirmed by Western blot (Supplemental Fig 2b). We found that upon degranulation with ionomycin, tamoxifen-treated Raptor-T-KO BMMCs contained similar amount of β -hexosaminidase compared to littermate control (Raptor^{f/f}) BMMCs and WT C57BL/6J BMMCs (Fig. 2c). Importantly, 48 hrs after degranulation, Raptor-T-KO BMMCs contained significantly reduced amount of β -hexosaminidase compared to Raptor^{f/f} and WT BMMCs confirming that mTORC1 is necessary for MC regranulation but not for degranulation (Fig. 2d).

To replicate these findings *in vivo*, Raptor-T-KO mice were treated with tamoxifen and knockout was confirmed by PCR (Supplemental Fig. 2c). We then passively sensitized Raptor-T-KO mice and littermate controls with anti-TNP IgE and Ag-challenged these mice with TNP-OVA. We found that there was no significant difference in core body temperature drop between Raptor-T-KO mice and littermate controls, suggesting that Raptor-T-KO mice maintain the capacity to undergo MC degranulation and anaphylaxis *in vivo* (Fig. 2e). 7 days after Ag challenge, peritoneal cells were collected, and granulated MCs were counted by toluidine blue staining. Compared to littermate controls, PBS-treated Raptor-T-KO mice had similar levels of granulated MCs (Fig. 2f), while Raptor-T-KO mice that were Ag-challenged had a significantly reduced number of granulated MCs. These results reveal that in Raptor-T-KO mice, the capacity of MCs to undergo regranulation is impaired. Taken together, our findings confirm that the well-known nutrient sensor mTORC1 is necessary for both *in vitro* and *in vivo* MC regranulation.

Slc37a2 is upregulated and necessary for MC regranulation.

Having identified mTORC1 as a vital regulator of MC regranulation, we sought to identify the basis of this regulation. Our strategy involved using an unbiased approach to look for highly upregulated metabolic genes following MC degranulation and seeing if they interacted with mTORC1. We examined RNA expression in BMMCs 4 hrs after ionomycin induced degranulation to selectively identify genes involved in early metabolic activity relating to regranulation while avoiding genes activated during degranulation. From our microarray analysis, we identified 44 genes that were differentially regulated in ionomycin treated BMMCs compared to naïve BMMCs, with an adjusted *p*-value less than 0.05 and at least a two-fold change (Fig. 3a). Among those genes, Slc37a2 stood out as a gene involved in glucose metabolism, a process regulated by mTOR activation (Fig. 3a). This gene is part of the Slc37 family of genes which exhibit sequence homology to the bacterial organophosphate/phosphate (Pi) antiporter (Cappello et al., 2018). Slc37a2 has been shown to have the capacity to transport glucose-6-phosphate (G6P) across membranes (Pan et al., 2011), and is involved in regulating glycolysis in macrophages (Wang et al., 2020).

We performed RT-qPCR mRNA analysis of Slc37a2 expression over a 48-hour time course following degranulation and revealed expression peaks at 6 hrs post-degranulation, displaying a >400-fold increase in mRNA. Thereafter, expression begins to subside, decreasing significantly and approaching baseline by 48 hrs (Fig. 3b).

To determine if Slc37a2 contributes to MC regranulation, we knocked down its expression in BMMCs using siRNAs. As shown in Fig. 3c, Slc37a2 mRNA in BMMCs was markedly reduced compared to control siRNA treated BMMCs. Furthermore, when we degranulated these Slc37a2-knocked down BMMCs, no significant impairment in β -hexosaminidase release was seen compared to control siRNA treated BMMCs (Fig. 3d). Notably, 48-hrs after degranulation Slc37a2-downregulated BMMCs had significantly impaired regranulation compared to controls (Fig. 3e). Furthermore, when we sought to visualize granules 48 hrs after degranulation in siRNA treated BMMCs using an antibody to the granule component serotonin as a probe, we found that in contrast to control BMMCs, very few serotonin granules were observed in Slc37a2 down regulated BMMCs (Fig. 3f, left) and there was a significant reduction in the relative integrated fluorescence intensity per cells (Fig. 3f, right). Taken together, these findings reveal that Slc37a2 is necessary for MC regranulation.

Slc37a2 concentrates extracellular medium contents acquired by fluid phase endocytosis in endosomes.

To deduce the role of Slc37a2 in MC regranulation, we first sought to visualize Slc37a2 localization in relation to their granules throughout the early stages of regranulation. For these studies, we utilized mouse peritoneal MCs which are markedly more granulated than cultured BMMCs (Malbec et al., 2007). Remarkably, in naïve MCs, Slc37a2 appeared to localize within MC granules that were encased in CD63⁺ granule membranes (Fig. 4a, top row) indicating that it was a regular MC granule component. As expected, 1 hour post degranulation, MCs appeared fully degranulated as no avidin-FITC staining residual granules were detected (Fig. 4a, middle row). Consistent with this finding, most of the CD63⁺ membranes appeared to be localized at the cell periphery (Schäfer et al., 2010).

These cells also appeared to stain poorly for Slc37a2, indicating that the cellular content of Slc37a2 was markedly reduced with the exteriorization of MC granules. However, at 6 hrs post-degranulation, which corresponded to the peak of Slc37a2 expression (Fig. 3b), newly formed Slc37a2 appeared localized to the periphery of the cell. Notably, this protein was not associated with CD63⁺ granule membranes (Fig. 4a, bottom row) suggesting that it had a function independent of the mature MC granule. In a previous study, Slc37a2 was shown to concentrate extracellular material taken up by fluid phase endocytosis into endosomes (Kim et al., 2007). To see if Slc37a2 plays a similar role in MCs, we stably over expressed Slc37a2-EGFP in an immortalized rat MC line, RBL-2H3. We incubated 10,000 MW Texas Red dextran with unstimulated Slc37a2-EGFP expressing RBL-2H3 and found that dextran was concentrated within distinct Slc37a2⁺ endosomes in MCs (Fig. 4b). To extend these findings, we knocked down expression of Slc37a2 in BMMCs using siRNAs and then incubated them with Texas Red dextran overnight. We visualized intracellular dextran and found that in Slc37a2 knocked down BMMCs, there was significantly less internalized dextran compared to control siRNA treated cells (Fig. 4c). These data reveal that following MC degranulation, recently produced Slc37a2 concentrates extracellular dextran and potentially other extracellular nutrients in endosomes.

Peripherally located Slc37a2 associates with and activates mTOR.

Since both mTORC1 and Slc37a2 are essential for regranulation, we wondered if these MC components interact with each other. In view of our finding that Slc37a2 concentrates extracellular nutrients within endosomes, it is conceivable that this activity may result in mTORC1 activation, analogous to how late endosomes signal to mTORC1 (Flinn et al., 2010). Support for this comes from our finding that at 6 hrs post degranulation, a subset of mTOR colocalized with nutrient bearing Slc37a2⁺ vesicles at the periphery of the MC (Fig. 5a). To see if Slc37a2 contributes to mTOR activation, we compared phosphorylation of mTOR following degranulation in control siRNA treated BMMCs and Slc37a2 knocked down BMMCs. While control siRNA treated BMMCs displayed increased mTOR phosphorylation by 2 hrs post degranulation, Slc37a2 knocked down BMMCs exhibited limited mTOR phosphorylation (Fig. 5b), suggesting mTOR phosphorylation requires Slc37a2.

To further support the notion that co-localization of Slc37a2 with mTOR in the cell periphery is critical to mTOR phosphorylation and subsequent MC regranulation, we blocked the trafficking of newly synthesized Slc37a2 to the cell periphery. When BMMCs were treated post degranulation with nocodazole, a microtubule depolymerizer (Vasquez et al., 1997), recently synthesized Slc37a2 accumulated in the perinuclear region of the cell (Supplemental Fig. 3a) whereas in vehicle control treated BMMCs Slc37a2 localized as expected to the cell periphery. In contrast to control treated BMMCs, mTOR in nocodazole treated BMMCs failed to get phosphorylated (Fig 5c) and these cells regranulated poorly (Fig. 5d), indicating that localization of Slc37a2 at the periphery of MCs during regranulation may be critical for mTOR phosphorylation and subsequent regranulation.

Previous studies have shown that G6P signals glucose levels to mTORC1 in cardiomyocytes (Karlstaedt et al., 2020, Roberts et al., 2014). In addition, Slc37a2 has

the capacity to transport G6P(Pan et al., 2011). Therefore, we wondered whether Slc37a2 increased intracellular G6P during regranulation to regulate mTOR activation in MCs. To test this, we measured intracellular G6P concentrations in Slc37a2 siRNA treated BMMCs before degranulation and at 2 hrs and 6 hrs post degranulation and compared them to control siRNA treated BMMCs. We found no significant difference in intracellular G6P between Slc37a2 siRNA treated BMMCs compared to control siRNA treated cells prior to degranulation. 2 hrs post activation by ionomycin, Slc37a2 siRNA treated cells trended towards lower G6P concentrations ($p=0.07$) compared to control siRNA treated cells. Finally, at 6 hrs post activation, there was a significantly lower concentration of G6P in Slc37a2 siRNA treated cells compared to controls (Fig. 5e). These results suggest that Slc37a2 is necessary to increase intracellular G6P during regranulation.

Slc37a2 directs metabolic reprogramming linked to MC regranulation.

The capacity of Slc37a2 to concentrate nutrients from the extracellular medium into endosomes associated with mTOR, leading to mTOR phosphorylation, suggests that Slc37a2 may play a critical role in regulating MC energy levels to accommodate MC regranulation. Indeed, previous studies have shown that Slc37a2 regulates glycolysis in macrophages(Wang et al., 2020) and the related protein G6PT (Slc37a4) has been shown to be necessary in maintaining G6P, lactate, and ATP amounts in neutrophils(Jun et al., 2014). Since Slc37a2 has the capacity to transport G6P across membranes(Pan et al., 2011), we tested whether blocking G6P metabolism using 2-deoxyglucose (2DG), a glucose analog that cannot be fully metabolized could inhibit regranulation. We found that treating BMMCs with 2DG 1 hr after degranulation significantly reduced MCs capacity to regranulate (Fig. 6a) suggesting that glycolysis is indeed necessary for regranulation.

We next sought to investigate the role Slc37a2 has in glycolysis and oxidative phosphorylation during regranulation. We treated BMMCs with Slc37a2 siRNAs or control siRNAs and performed a mitochondrial stress test prior to degranulation or 6 hrs after MC degranulation, corresponding to the peak expression of Slc37a2 in WT BMMCs then we used Seahorse XF to measure extracellular acidification and oxygen consumption rates (Fig. 6b). From these experiments we found that basal glycolysis is increased 6 hrs post degranulation in control siRNA treated BMMCs, but a corresponding increase in Slc37a2 siRNA treated cells was not observed (Fig. 6c). Additionally, ATP production is increased 6 hrs post degranulation in control siRNA treated cells, but not in Slc37a2 siRNA treated cells (Fig. 6d). Notably, maximum respiration is significantly increased in control siRNA treated BMMCs 6 hrs post degranulation, but not in Slc37a2 siRNA treated BMMCs (Fig. 6e).

To demonstrate the importance of Slc37a2 localization on peripheral endosomes to metabolic reprogramming during regranulation, a mitochondrial stress test was performed 6 hrs post degranulation on nocodazole treated BMMCs (Supplemental Fig. 3b). We found that both basal respiration and glycolysis were inhibited when Slc37a2 localization was blocked (Fig 6f-g). In addition, ATP production and maximum respiration were significantly reduced (Fig. 6h-i). These results suggest that the specific localization of Slc37a2 to peripheral endosomes during regranulation is critical for the metabolic reprogramming that occurs during regranulation. Furthermore, the presence of Slc37a2 in the cell periphery is

necessary for increases in glycolysis, ATP production, and maximum respiration associated with the metabolic shift linked to MC regranulation.

Slc37a2 conveys components of the extracellular medium into MC granules.

Slc37a2 is found within mature MC granules of naïve primary MCs (Fig 4a), suggesting that Slc37a2 on the periphery ultimately traffics to and become incorporated in nascent MC granules. To investigate if Slc37a2 associates with MC granule contents, we transduced the RBL-2H3 MC line expressing serglycin mCherry to mark and visualize the core of MC granules with plasmids expressing either Slc37a2-EGFP or an EGFP empty vector control. Both sets of transduced serglycin mCherry RBL-2H3 cells were probed for CD63 and visualized by microscopy. As expected, serglycin was localized within CD63-marked vesicles in EGFP empty vector control transduced RBL-2H3 cells (Fig. 7a). Interestingly in Slc37a2 EGFP expressing RBL-2H3 cells, serglycin appeared encased in Slc37a2⁺ vesicles with CD63⁺ membranes seemingly serving to encase granules comprised of serglycin and Slc37a2⁺ vesicles. Presumably, Slc37a2⁺ vesicles appeared to have a stronger affinity for MC granules than CD63⁺-vesicles that typically encase MC granules (Fig. 7a). Thus, peripherally located Slc37a2⁺ vesicles may be destined to become components of MC granules.

If Slc37a2 vesicles are trafficked into MC granules, then cargo acquired from the extracellular medium that are stored and concentrated in these vesicles may also be incorporated into MC granules. To investigate this possibility, we degranulated peritoneal MCs and exposed them to Texas Red dextran, a marker of nondescript extracellular medium components. We found that at 48 hrs, Slc37a2 was associated with nascent Avidin⁺ granules. Furthermore, these granules were also associated with dextran (Fig. 7b). This suggests that extracellular components are trafficked in Slc37a2⁺ endosomes into newly formed granules. To further confirm this, we incubated BMDCs with Texas Red dextran for 24 hrs and then degranulated these BMDCs using ionomycin. We found that a significantly increased amount of dextran was released along with granules compared to unactivated BMDC controls (Fig. 7c).

Thus, taken together, Slc37a2 appears to have an additional property of concentrating cargo acquired from the extracellular medium into vesicles that are directly trafficked into MC granules, suggesting that Slc37a2 has the capacity to regulate the composition of MC granules.

Discussion

MCs are best known for their high content of electron-dense secretory granules that occupy most of the cytoplasm of the cell (Wernersson and Pejler, 2014). The capacity of MCs to simultaneously exocytose most of their granules upon activation is one of the primary reasons that MCs are regarded as the early triggers of a wide range of inflammatory disorders such as asthma, arthritis, and anaphylaxis. Although MCs were shown to regranulate *in vitro* over 50 years ago (Kobayasi and Asboe-Hansen, 1969), their capacity to regranulate *in vivo* has remained unclear. Importantly, we found that MCs *in vivo* are capable of regranulating after each successive IgE mediated activation, and that each

activation is capable of inducing inflammatory responses comparable to the initial activation of naïve MCs. This shows that the regranulation capacity of MCs *in vivo* is comprehensive and restores function. It is noteworthy that *in vivo*, MC regranulation required 7 days to attain granulation levels seen in naïve MCs, which is significantly longer than what it takes for BMDCs to regranulate *in vitro*. It is possible this delay *in vivo* is due to the presence of other inflammatory cells. However, the innate capacity of MCs to undergo at least two rounds of regranulation and degranulation *in vivo* after an initial degranulation response would suggest that in addition to initiating inflammatory responses upon activation, MCs can contribute to sustaining these inflammatory activities for as long as the activating agent is present.

While MCs maintained their capacity to undergo multiple cycles of degranulation and regranulation *in vivo*, changes to the inflammatory response were observed in the second and third MC activation. Our studies were specifically designed to reveal IgE mediated MC degranulation, and to exclude the contribution of other immune cells, such as lymphocytes capable of memory responses. Rather than actively immunizing test mice with OVA Ag, we passively immunized mice with OVA-specific IgE antibodies so that, for the most part, only MCs were sensitized and capable of reacting to each challenge with Ag. Furthermore, Ag challenges of the mice were undertaken 7 days apart, by which time the inflammation induced by the previous challenge had subsided and MCs, which had previously degranulated had regranulated to similar levels of granule content as naïve MCs (Fig. 1b). Thus, the immune responses evoked after each challenge were primarily a result of MCs with little or no contribution from innate or adaptive immune cells. Notably, an influx of monocytes was observed after each Ag challenge, however, neutrophil recruitment was reduced in the second and third Ag challenges. This matches the trend towards an increased rate of recovery from anaphylaxis after each subsequent Ag challenge and suggests a possible adaptation of MCs to repeat Ag challenge. Interestingly, a similar observation was made after repeat FcεRI triggering in human cord blood MCs *in vitro* resulted in modulated MC function (Suurmond et al., 2016). It is also notable that 7 days after Ag challenge, regranulated MCs appeared slightly diminished in size (Fig. 1c), which may suggest a correlation between MC size and inflammatory response during anaphylaxis. This may be consistent with previous findings that suggest MCs continuously form granules overtime, increasing their granule contents for six months or more (Hammel et al., 2010). This would suggest that MCs may increase in size over time when not degranulated.

This capacity of MCs to undergo multiple cycles of degranulation and regranulation is unique. While much is known regarding how MCs degranulate and release their granules, comparatively, little is known regarding the molecular events associated with initial MC granule formation, and no information is available on granule reformation. Conceivably, for MCs to fully regenerate the repertoire of granules populating their cytosol, significant protein and lipid synthesis in concert with intracellular vesicle trafficking and packaging of various granule components into the granule structure would need to occur. These activities require significant energy and so MCs would need to undergo significant metabolic reprogramming. Our studies have implicated the protein kinase mTOR, the well-known mediator of cellular metabolism, growth, and proliferation as the cellular switch initiating MC regranulation associated activities (Saxton and Sabatini, 2017b). This conclusion is

based on the findings that (i) mTOR and S6K a serine/threonine kinase, a signaling substrate downstream of mTOR, become phosphorylated following MC degranulation, (ii) impeding mTOR activation with a chemical inhibitor, torin, prevents MCs from regranulation *in vitro* without impeding their capacity to degranulate and (iii) genetically knocking out Raptor, a necessary gene for mTORC1 expression, blocks the capacity of MCs to regranulate *in vivo* without affecting their capacity to degranulate. Additionally, mTOR activation during MC regranulation was closely linked to increased glycolysis, ATP production and maximum respiration in MCs (Fig. 6). That mTOR is important for MC regranulation following MC degranulation is consistent with previous reports showing that activation of mTOR in MCs inhibited IgE mediated MC degranulation and cytokine release as well as their capacity to proliferate (Blatt et al., 2012, Rakhmanova et al., 2018). Presumably, activating mTOR triggers a metabolic switch in steady state MCs to accommodate regranulation which interferes with both degranulation and MC proliferation activities.

In association with mTOR, we found that Slc37a2 was a critical regulator of MC regranulation. Slc37a2, a transmembrane protein implicated previously in G6P transport in macrophages (Wang et al., 2020), was the most upregulated gene involved in glucose metabolism that we encountered (Fig. 3a) increasing 400-fold over baseline by 6 hrs after MC degranulation (Fig. 3b). Furthermore, newly produced Slc37a2 was found to specifically localize at the MC periphery and on vesicles that had recently endocytosed nutrients found in the extracellular medium (Fig. 4). MC uptake of labelled extracellular dextran is analogous to findings in Slc37a2 over-expressing COS cells where dextran from the extracellular medium was found to be harbored within Slc37a2+ endosomes (Kim et al., 2007). Conceivably, peripherally located Slc37a2 contributes to the uptake of nutrients from the extracellular medium. This is supported by our studies showing that nocodazole treatment of BMDCs, which prevents Slc37a2 trafficking to endosomes, reduced basal respiration, basal glycolysis, ATP production, and maximal respiration 6 hrs post degranulation, similar to what is observed when Slc37a2 is knocked down by siRNAs (Fig. 6 c-i).

So how are these extracellular nutrient-encapsulating Slc37a2+ endocytic compartments in MCs connected to mTOR mediated metabolic switch and MC regranulation? One possibility is that cargo encased in Slc37a2+ endosomes signals mTOR in a manner analogous to how amino acids, glucose, and ATP borne in lysosomes activates the cellular nutrient sensor mTOR found associating with the lysosome surface and triggering a metabolic switch (Saxton and Sabatini, 2017b). Support for this possibility comes from the finding that nutrient-bearing Slc37a2+ endosomes found in the cell periphery appear to associate with mTOR. Furthermore, knockdown of Slc37a2 results in reduced G6P and ATP within MCs during regranulation, two critical signals for mTORC1 activation. Finally, knocking down of Slc37a2 or preventing the trafficking of Slc37a2 to the cell periphery where it colocalizes with mTOR was found to prevent MC regranulation, emphasizing the critical nature of the Slc37a2-mTOR interactions to this activity.

Another intriguing finding was that during MC regranulation, peripherally located Slc37a2+ endosomes travel to nascent MC granules or progranules which are located in the “progranule zone” to become MC granule components. While we are not able to ascertain

its function within the granule, one possibility is that Slc37a2 contributes to concentrating granule components making it more compact and particulate, a phenomenon sometimes known as condensation(Hammel et al., 2010). This role for SLC37a2 was recently observed when intracellular Slc37a2⁺ vesicles within microglia were found to exhibit the capacity to concentrate vesicular contents and reduce vesicle volume to a fraction of its original size. Loss of Slc37a2 blocked this vesicular shrinkage, resulting in its expansion and bloating of the cell(Villani et al., 2019).

What drives SLC37a2⁺ endosomes to traffic into nascent MC granules during regranulation is unclear. When SLC37a2 was over expressed in RBL-2H3 cells, SLC37a2 rapidly gathered around MC granules and seemed to displace the CD63⁺ membranes from around granule cores (Fig. 7a). Apparently, SLC37a2 has a stronger affinity for the serglycin-rich and negatively charged granule core than CD63⁺ membranes. It is noteworthy that when SLC37a2⁺ endosomes fuse with nascent MC granules they do so bearing cargo endocytosed from the extracellular medium. Indeed, dextran acquired from the extracellular medium by SLC37a2⁺ vesicles were translocated to nascent MC granules within 48 hrs of degranulation. Incorporation of dextran in granules was confirmed by the observation that when MCs were activated, dextran was released along with granules. This finding is consistent with previous claims that histamine and TNF found in the extracellular medium could be incorporated into MC granules and released when MCs degranulate(Olszewski et al., 2007, Ohtsu et al., 2002). These studies also reveal that Slc37a2 is potentially a key regulator of MC granule composition. Previous studies have reported that mature MC granules are formed when progranules leave the “progranule zone” near the golgi and endoplasmic reticulum and fuse with other progranules, immature granules, endosomes, or lysosomes. Our studies reveal that regranulation also involves the active participation of vesicles recruited from the plasma membrane bearing cargo from the extracellular medium and reveal the critical role of Slc37a2 as a regulator of MC granule content.

Finally, now that we have demonstrated that MCs have the capacity to regranulate *in vivo*, future studies can be designed to assess the specific contribution of the regranulation property to the pathogenesis of MCs in various inflammatory disorders. If this property is deemed to be significant, selectively targeting it may be an efficient way to temper the excessive activities of MCs without impairing its beneficial role in promoting innate and adaptive immune responses to infectious agents and in maintaining homeostasis.

Limitations of the Study

While the Raptor-T-KO mice demonstrated the significance of mTORC1 in MC regranulation, the current study does not completely rule out the effect a global knockout of Raptor may have on MC regranulation. Additionally, our study was limited to mice and cell lines from mice and rats, therefore the role Slc37a2 has in human MCs has yet to be demonstrated. Finally, in our microarray study, Slc37a2 isoform 2 was identified, however, the role additional isoforms may have in MC regranulation is unclear.

Star Methods

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Soman N. Abraham (soman.abraham@duke.edu).

Materials availability—The cell lines and plasmids generated in this work can be requested from the lead contact with a completed MTA. This study did not generate new unique reagents.

Data and code availability

- Microarray data have been deposited at GEO and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—Six- to eight-week-old female C57BL/6J and Raptor^{f/f} mice were purchased from The Jackson Laboratory and housed in Duke University animal facilities. Raptor^{f/f}-ER Cre mice were generously donated by Dr. Xiao Ping Zhong at Duke University. Six- to twelve-week-old male and female C57BL/6J and Raptor^{f/f} ER-Cre mice were used for our experiments. Cre negative littermates were used as controls. Raptor ER-Cre mice and littermate controls were treated with 2 mg tamoxifen (Tocris) in 100 μ L corn oil via gavage on 5 consecutive days. All animal experiments were performed according to approved protocols by the Duke University Institute Animal Care and Use Committee.

Cell lines—BMMCs were derived from femur bone marrow of WT C57BL/6J, Raptor^{f/f}-ER Cre, and Raptor^{f/f} Cre-negative littermate controls. Cells were cultured in RPMI-1640 cell media containing 2 mM L-glutamine (ThermoFisher), 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 1 mM nonessential amino acids (ThermoFisher), 25 mM HEPES (ThermoFisher), 1 mM sodium pyruvate (ThermoFisher), 1X Antibiotic-Antimycotic (ThermoFisher), 5 ng/mL recombinant murine IL-3 (R&D Systems, and 5% stem cell factor-containing supernatant from CHO-KL cells for 4-6 weeks until maturation. CHO-KL cells were a gift from M. Arock (Laboratoire de Biologie et Pharmacologie Appliquée, Paris, France). RBL-2H3 cells (ATCC) and transduced RBL-2H3 expressing SDLC37a2-EGFP were cultured in MEM media containing Earl salt's, 2 mM L-glutamine (ThermoFisher), 15% heat-inactivated FBS, and 1 mM sodium pyruvate. Amphopack-293 cells (BD Biosciences) were cultured in DMEM cell media containing 10% heat-inactivated FBS, 2 mM L-glutamine, and 1X Antibiotic-Antimycotic. BMMCs and RBL-2H3 cells were authenticated by flow cytometry employing mast cell markers and transduced RBL-2H3 cells were authenticated by PCR.

METHOD DETAILS

Anaphylaxis Experiments—To induce anaphylaxis, mice were sensitized i.p. with 10 µg anti-TNP IgE (BD Biosciences) in 100 µL sterile PBS. Mice were Ag-challenged 16 hrs later i.p. with 50 µg TNP-OVA (Biosearch Technologies) in 100 µL sterile PBS. Control mice were challenged with sterile PBS. Core body temperatures were monitored using a rectal thermometer immediately prior to Ag-challenge and then every 15 minutes for up to 3 hrs. Repeat Ag-challenges were separated by 7 days and mice were resensitized with 10 µg anti-TNP IgE and Ag-challenged 16 hrs later with 50 µg TNP-OVA.

Flow cytometry analysis.—Mice were Ag-challenged one, two, or three times, as described above in Anaphylaxis Experiments. 24 hrs after the final Ag-challenge, peritoneal cells were collected after lavage with 5 mL cold 10 mM EDTA in PBS. Samples were washed with PBS containing 5 mM EDTA and 3% FBS (FACS Buffer) and then blocked with 1% anti-mouse CD16/CD32 (BD biosciences), 5% normal rat serum and 5% normal mouse serum in FACS buffer for 10 minutes at RT. Then, surface staining was performed using 7-AAD (Biolegend) and fluorochrome-tagged antibodies to CD45 and CD117 (both from Biolegend), and CD11b, Ly6G, SiglecF, Ly6C, and F4/80 (all from BD Biosciences). Intracellular staining for Avidin (BD Biolegend) was performed using the BD Cytotfix/Cytoperm Fixation/Permeabilization Kit and following manufacturer's instructions. Data was collected using BD Fortessa X-20 (BD Biosciences) and analyzed with FlowJo software (TreeStar). The gating strategy used is described in Supplemental Fig. 1.

Immunofluorescence staining and microscopy—For visualization of BMMCs and peritoneal cells, cells were cytospun onto charged glass slides. For RBL-2H3 cells, cells were seeded onto sterilized glass coverslips and allowed to adhere overnight. Cells were then fixed using 4% paraformaldehyde for 20 minutes at room temperature (RT). Cells were washed in PBS three times and permeabilized in 0.1% saponin (Sigma) and blocked in 1% BSA (USBiological Life Sciences) in PBS for 20 minutes at RT. Cells were then incubated with 1% anti-CD63 (MBL), 0.5% anti-Slc37a2 (GeneTex), 1% anti-serotonin (Dako), or 1% anti-mTOR (EMD Millipore) overnight at 4°C. The following day, cells were washed in 0.1% saponin and 1% BSA in PBS three times and incubated with 0.25% fluorescent secondary antibodies (Jackson ImmunoResearch) or 0.2% avidin conjugated with FITC (BD Biosciences) for 1 hr at RT. Cells were then washed twice with 0.1% saponin and 1% BSA in PBS and twice with PBS. Slides were mounted with ProLong Diamond (Invitrogen). Images were acquired using the Leica Thunder Imager and analyzed by ImageJ.

β-hexosaminidase assay—For BMMC degranulation assays, BMMCs were plated at 1×10^5 cells/well in a 96 well V-bottom plate. Cells were washed in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose, 1% BSA, 20 mM HEPES, pH 7.4) and then incubated with 1 µg/mL ionomycin (Sigma) in Tyrode's buffer for 1 hr at 37 °C. BMMCs in Tyrode's buffer alone were used as a negative control. After incubation, supernatants were collected and cell pellet was lysed in 0.1% Triton-100X (Spectrum Chemical) in Tyrode's buffer. Collected supernatant and lysed pellet were separately incubated with 3.4 mg/mL p-nitrophenyl-N-acetyl-β-D-glucosaminide (NAG, Sigma) in citrate buffer (pH 4.5) for 1 hr at 37°C. Reactions were stopped at 1 hr

with 0.1 M carbonate buffer (pH 10). Colorimetric measurements were performed at 405 nm using a Synergy H1 microplate reader (BioTek). Percent degranulation was determined as percent β -hexosaminidase activity in supernatant compared to total β -hexosaminidase activity in supernatant and pellet.

To measure regranulation, 1 hr post-activation with ionomycin, BMMCs were resuspended in BMMC media and incubated at 37°C for 48 hrs, unless otherwise noted. Total β -hexosaminidase activity was measured by plating 1×10^5 cells/well in a 96 well V-bottom plate. BMMCs were washed in Tyrode's buffer then lysed in 0.1% Triton-100X (Spectrum Chemical) in Tyrode's buffer. Total β -hexosaminidase activity in lysed pellet was determined as described above.

For regranulation experiments using 1 mM 2-deoxyglucose (Sigma), 33 μ M nocodazole (Sigma), or 20 nM torin (Tocris), BMMCs were treated 1 hr post degranulation with each respective inhibitor in BMMC medium. Total β -hexosaminidase activity was measured 48 hrs post degranulation.

Western Blot—Cells were lysed at indicated time points post degranulation using RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails 2 and 3 (Sigma). Lysates were incubated at 4°C for 30 minutes and supernatants were collected after centrifugation at 20,000xG at 4°C for 15 minutes. Total protein concentrations were determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit according to manufacturer's instructions (ThermoFisher). Equal concentrations of cell lysates were prepared in Laemmli buffer (BioRad) containing a 5% β -mercaptoethanol (Sigma) and denatured at 95°C for 5 minutes. Proteins were separated on a 4% to 20% mini-PROTEAN TGX precast gel (Biorad) and transferred onto a nitrocellulose membrane by wet transfer. Membranes were blocked in 5% BSA or 5% milk dissolved in TBS (Biorad) containing 0.1% Tween 20 (Sigma) for 45 minutes and then incubated at 4°C overnight with primary antibodies in blocking buffer. Membranes were washed five times in TBST then incubated with secondary antibody conjugated to horseradish peroxidase (HRP) for 1 hr at RT. Membranes were washed five times in TBST then proteins were detected using enhanced chemiluminescent HRP substrate (ThermoFisher). Antibody dilutions were 1:1000 for anti-mTOR, anti-p-mTOR S2448, anti-S6K, and anti-p-S6K S371 (Cell Signaling), and 1:5000 for anti- β -actin (Sigma).

Toluidine Blue Assay—7 days after mice were Ag-challenged, peritoneal cells were collected after lavage with 5 mL cold 10 mM EDTA in PBS. 100 μ Ls of cells were cytospun onto glass slides and fixed with Carnoy's fixative (60% ethanol, 30 % chloroform, and 10% glacial acetic acid) for 20 minutes at RT and stained with 0.1% toluidine blue dissolved in PBS containing 0.5N HCl for 20 min. Granulated MCs were visualized and counted under a brightfield microscope.

Microarray—RNA was collected from untreated BMMCs or BMMCs 4 hrs after treatment with 1 μ g/mL ionomycin using the RNeasy Mini Kit. Total RNA was assessed for quality with Agilent 2100 Bioanalyzer G2939A (Agilent Technologies) and Nanodrop 8000 spectrophotometer (Thermo Scientific/Nanodrop). Hybridization targets were prepared

with the Affymetrix Whole Transcriptome Plus Kit (Affymetrix) and Affymetrix GeneChip Whole Transcriptome Terminal Labeling Kit (included with Affy Whole Transcriptome Plus) from total RNA, hybridized to GeneChip[®] Mouse Transcriptome Array 1.0 in Affymetrix GeneChip[®] hybridization oven 645, washed in Affymetrix GeneChip[®] Fluidics Station 450 and scanned with Affymetrix GeneChip[®] Scanner 7G according to standard Affymetrix GeneChip[®] Hybridization, Wash, and Stain protocols. (Affymetrix, Santa Clara, CA).

Affymetrix Gene Chip microarray data was processed using the *oligo* (Carvalho and Irizarry, 2010) package in the Bioconductor suite (Gentleman et al., 2004) from the R statistical programming environment. Log-scale Robust Multiarray Analysis (RMA) was used to normalize the data and eliminate systematic differences across the arrays. Differential expression between the naïve and the wild-type samples was calculated with an empirical Bayes moderated test statistic using the *limma* package (Ritchie et al., 2015). The False Discovery Rate (FDR) was used to control for multiple hypothesis testing. Gene set enrichment analysis (Mootha et al., 2003) (GSEA) was performed using the *fgsea* (Korotkevich et al., 2021) package to identify gene ontology terms and pathways associated with the differentially expressed genes.

RT-qPCR—Total RNA was extracted from BMMCs at indicated time points post ionomycin treatment using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA was then synthesized using the iScript cDNA synthesis kit (BioRad) and following manufacturer's instruction. Finally, RT-qPCR was performed using iQ SYBR Green Supermix (BioRad) and run on in triplicate on a StepOnePlus Real-Time PCR System (Applied Biosciences) and analyzed by StepOne Software v2.3. mRNA amounts were normalized using β -actin and calculated using the 2^{-CT} method. The following primer pairs purchased from IDT were used: Slc37a2, 5'-GCTGCTCCCATGATGTTCCCT-3' and 5'-TTGGCGTTACCCTTCAGGCT-3', β -ACTIN, 5'-GATTACTGCTCTGGCTCCTAGC-3' and 5'-GACTCATCGTACTCCTGCTTGC-3'.

siRNA Treatment—Slc37a2 was knocked down in BMMCs using siGENOME SMARTpool siRNAs (Dharmacon). Transfection of siRNAs into BMMCs was performed using the Amaxa Nucleofector Kit T (Lonza). BMMCs were pelleted and resuspended at a concentration of 2×10^6 cells/100 μ L Nucleofector T solution. 300 nM Slc37a2 siRNAs or Silencer[™] negative control siRNA (Ambion) was added to the solution. BMMCs were nucleofected using the Amaxa Nucleofector Program U-023. Transfected BMMCs were then resuspended in cell media for 48 hrs at 37°C prior to assaying.

Dextran Uptake Assay—RBL-2H3 cells stably expressing Slc37a2-EGFP or EGFP vector control seeded onto coverslips, or BMMCs treated with Slc37a2 siRNAs or negative control siRNAs were incubated with 0.5 mg/mL Dextran Texas Red 10,000 MW in cell media for 16 hrs at 37°C. Cells were washed twice with cell media and then incubated an additional 2 hrs in cell media at 37°C. For RBL cells, cells were fixed with 4% paraformaldehyde, washed three times in PBS, and mounted onto a glass slide using Prolong Diamond. For BMMCs, cells were cytospun onto charged glass slides, fixed with 4% paraformaldehyde, washed three times in PBS and then a coverslip was mounted using

ProLong Diamond. Images were acquired using the Leica Thunder Imager and analyzed by ImageJ.

Glucose-6-Phosphate Assay—BMMCs, treated with Slc37a2 siRNA or control siRNA, were activated using 1 $\mu\text{g}/\text{mL}$ ionomycin in Tyrode's buffer. 1 hr after ionomycin treatment, BMMCs were washed three times and resuspended in BMMC medium. G6P concentrations were determined at 2 hrs and 6 hrs post activation and compared to unactivated controls using a G6P kit (Sigma) and following manufacturer's instructions.

Seahorse XF Assays—For Slc37a2 siRNA knockdown experiments, Slc37a2 siRNA or control siRNA treated BMMCs were sensitized overnight with 1 $\mu\text{g}/\text{mL}$ anti-TNP IgE. Cells were washed three times and resuspended in Tyrode's buffer containing 10 ng/mL TNP-OVA or PBS. After 1 hr, cells were washed three times and resuspended in BMMC media. For nocodazole experiments, BMMCs were treated with 1 $\mu\text{g}/\text{mL}$ ionomycin or PBS in Tyrode's buffer. 1 hr after ionomycin treatment, BMMCs were washed three times and resuspended in BMMC medium containing 33 μM nocodazole. For BMMC adherence, XF96 cell culture microplates were precoated with Cell-Tak following manufacturer's instruction. At 6 hrs post TNP-OVA or ionomycin treatment, BMMCs were seeded onto Cell-Tak treated XF96 cell culture microplates at a density of 1×10^5 cells/well. Basal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using the Seahorse XF96e Analyzer followed by sequential addition of 1 μM oligomycin (Sigma), 0.5 μM carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP, Sigma), and 0.75 μM rotenone (Sigma) with 1.5 μM antimycin A (Sigma). Basal respiration, basal glycolysis, ATP production, and maximal respiration were evaluated based on the manufacturer's instructions.

RBL-2H3 expressing Serglycin-mCherry and Slc37a2-EGFP—RBL-2H3 cells stably expressing serglycin-mCherry were previously generated in our lab (Ang et al., 2016). To generate Slc37a2-EGFP expressing RBLs, Slc37a2 was amplified by PCR from BMMC cDNA and inserted into a pEGFP-C1 plasmid (Takara Bio). Slc37a2 pEGFP-C1 plasmid or plasmid vector control were transfected into Amphopack-293 cells to produce viral particles that were used to infect RBL-2H3 cells or RBL-2H3 cells stably expressing serglycin-mCherry. Slc37a2-EGFP stably expressing cells were selected using neomycin.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism v.9.0.0 (GraphPad Software). For comparison between two groups, results were analyzed using a two-tailed unpaired *t* test. For comparison between multiple groups, results were analyzed using a two-tailed ANOVA with Tukey's post test. A *p*-value less than 0.05 was considered statistically significant. Error bars are represented as SD unless otherwise noted in the figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mast cells can regranulate after each successive anaphylactic reaction in mice.
- The interaction of a G6P transporter, Slc37a2, and mTORC1 contributes to regranulation.
- Slc37a2, is needed for the metabolic switch regulating regranulation
- Slc37a2 also conveys extracellular medium components into nascent mast cell granules.

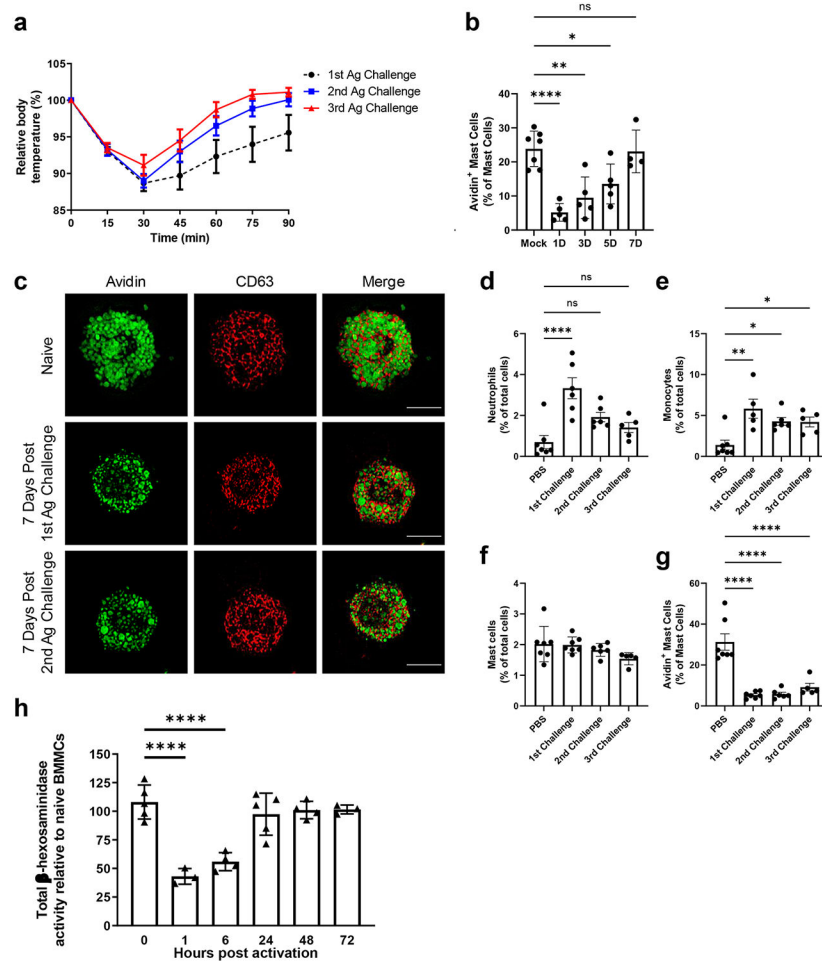


Figure 1. Regranulated MCs are capable of causing repeat anaphylaxis *in vivo*.

(a) Core body temperatures following three Ag challenges, each separated by 7 days ($n = 7$ mice). (b) Flow cytometry analysis of CD45⁺cKit⁺avidin⁺ MCs in peritoneal lavages at time points indicated post initial Ag challenge ($n = 4$ mice). (c) Immunofluorescence images of naïve peritoneal MCs or 7 days post Ag challenge a first or second time. Cells are immunostained with avidin (green) and CD63 (red). Images are representative of at least 2 independent experiments. Scale bars represent 10 μ m. (d-g) Flow cytometry analysis of peritoneal lavages 24 hrs after the first, second, or third Ag challenge or from PBS treated control mice. Quantification of these results is shown for (d) CD45⁺CD11b⁺Ly6G⁺ neutrophils (e) CD45⁺CD11b⁺Ly6C⁺ monocytes, (f) CD45⁺cKit⁺ MCs, and (g) CD45⁺cKit⁺Avidin^{Hi} intracellular MC staining ($n = 6$ mice). See also Supplemental Fig 1. (h) Total β -hexosaminidase in BMDCs at time points indicated post activation using ionomycin. Error bars represent SEM from $n = 3$ independent experiments. Data were analyzed by a 1-way ANOVA with a Tukey's post-test (b and d-g), or a 1-way ANOVA with a Dunnett's post-test comparing each group with untreated BMDC controls (h). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

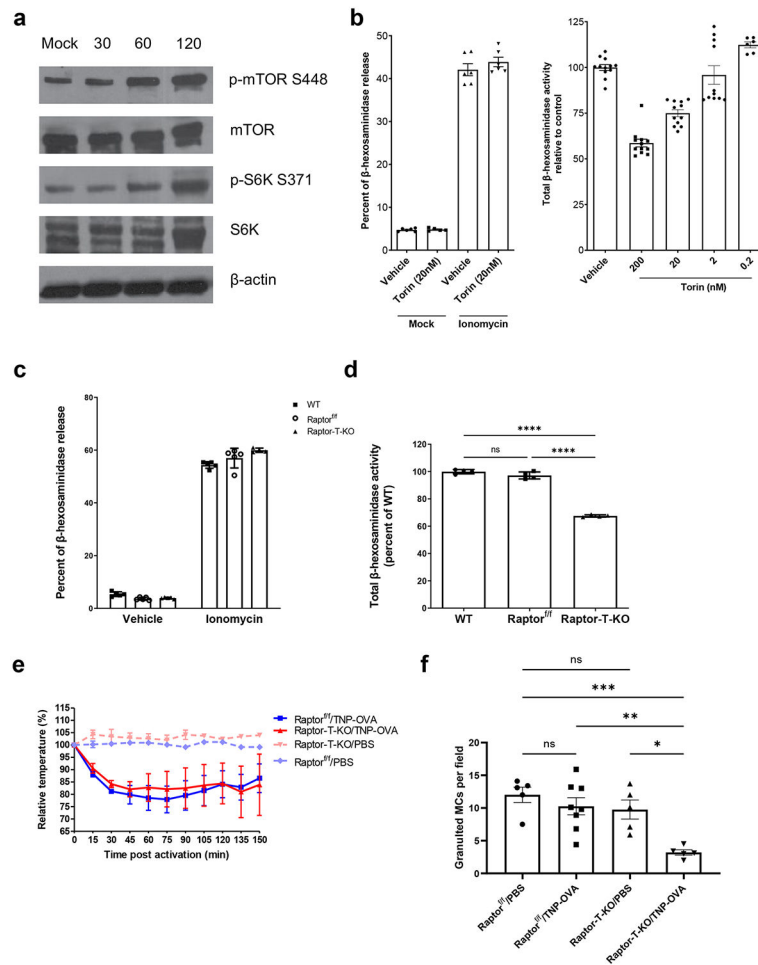


Figure 2. mTORC1 is necessary for MC regranulation *in vitro* and *in vivo*.

(a) Western blots of p-mTOR S2448, mTOR, p-S6K S371, S6K, and β -actin at time points indicated post activation by ionomycin. Two independent experiments were performed with similar results. (b, left) Percent β -hexosaminidase release of BMMCs pretreated with torin or DMSO (vehicle control) and then activated by ionomycin in Tyrode's buffer or treated with Tyrode's buffer alone (Mock) for 1 hr. β -hexosaminidase release was measured and expressed as a percent of total. (b, right) Total β -hexosaminidase at 48 hrs in BMMCs treated with indicated concentrations of torin or DMSO (vehicle) 1 hr post activation by ionomycin in Tyrode's buffer. Results are displayed as a percent of vehicle control ($n = 6$ replicates). Three independent experiments were performed with similar results. (c) Percent β -hexosaminidase release of BMMCs from C57BL/6J mice (WT), $Raptor^{f/f}$, or $Raptor-T-KO$ after activation by ionomycin in Tyrode's buffer or Tyrode's buffer alone (vehicle control) for 1 hr. β -hexosaminidase release was measured and expressed as a percent of total ($n = 5$ replicates). Data are representative of three independent experiments. (d) Total β -hexosaminidase in BMMC from C57BL/6J (WT), $Raptor^{f/f}$, or $Raptor-T-KO$ 48 hrs post activation by ionomycin in Tyrode's buffer. Results are normalized to WT BMMCs ($n = 5$ replicates). Data are representative of three independent experiments. See also Supplemental Fig. 2a. (e) Core body temperatures of $Raptor^{f/f}$ or $Raptor-T-KO$ mice

following antigen challenge. Results are expressed as relative body temperature compared to initial temperatures ($n = 5$ mice). (f) Total number of toluidine blue stained cells (granulated MCs) in peritoneal lavages of C57BL/6J (WT), Raptor^{f/f}, and Raptor-T-KO mice 7 days post Ag challenge. Granulated MCs were counted in ten fields of focus per mouse. See also Supplemental Fig. 2b and c. Error bars represent SEM from $n = 5$ mice. Data were analyzed by a Student's t test (b) or a 1-way ANOVA with a Tukey's post-test (c-f). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

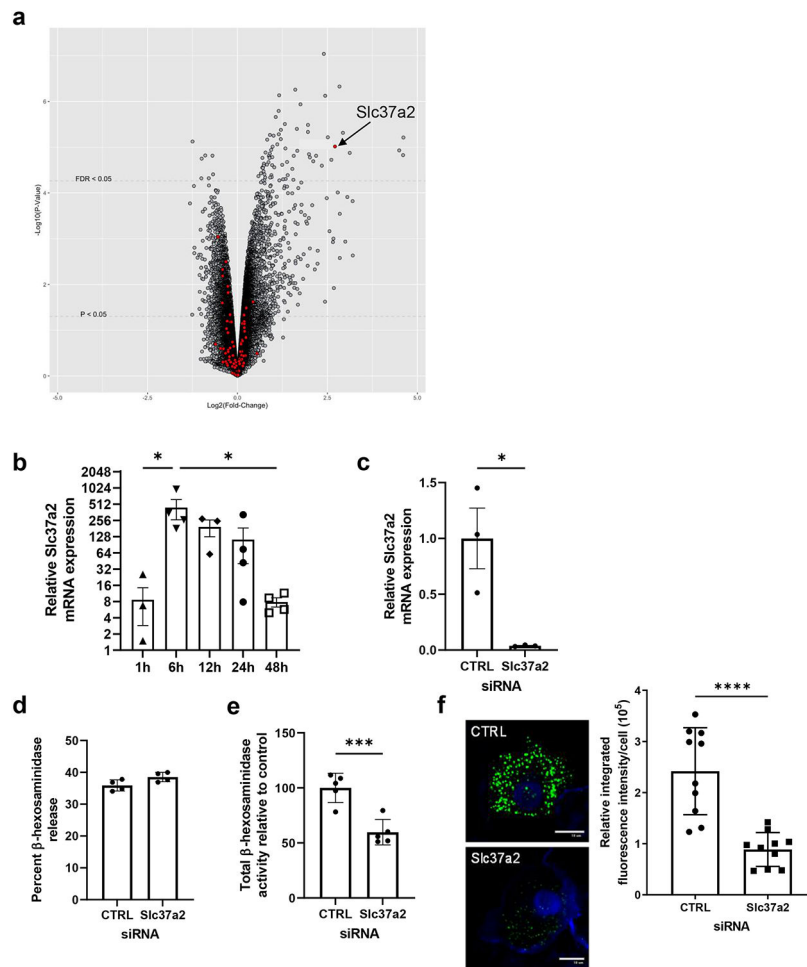


Figure 3. Slc37a2 is a necessary gene in MC regranulation.

(a) Volcano plot of microarray analysis of total RNA from three independent samples of naïve BMMCs and BMMCs 4 hrs after activation by ionomycin. Genes with a GO Term of Glucose Metabolism Pathway are highlighted in red. (b) RT-qPCR analysis of Slc37a2 mRNA in BMMCs at timepoints indicated post activation with ionomycin. Results are normalized to untreated BMMCs. Error bars represent SEM from $n = 3$ independent experiments. (c) RT-qPCR analysis of Slc37a2 mRNA in Slc37a2 siRNA treated BMMCs compared to control siRNA (CTRL) treated BMMCs. Results were normalized to control siRNA treated BMMCs. Error bars represent SEM from $n = 3$ independent experiments. (d) Percent β -hexosaminidase release of siRNA treated BMMCs compared to control siRNA treated BMMCs after activation with ionomycin for 1 hr. Results were expressed as a percent of total β -hexosaminidase ($n = 4$ replicates). Data are representative of three independent experiments. (e) Total β -hexosaminidase activity relative to control in Slc37a2 siRNA treated BMMCs compared to control siRNA treated BMMCs 48 hrs post activation with ionomycin. Results were normalized to control siRNA treated BMMCs ($n > 4$ replicates). Data are representative of three independent experiments. (f) Immunofluorescence images of Slc37a2 siRNA treated BMMCs compared to control siRNA treated BMMCs 48 hrs post activation with ionomycin. Cells are immunostained with serotonin (green) and phalloidin (blue). Scale bars: 10 μm .

Serotonin was quantified using ImageJ; ($n = 10$ cells). Data were analyzed by a 1-way ANOVA with a Tukey's post-test (**b**), or a Student's t test (**c-f**). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

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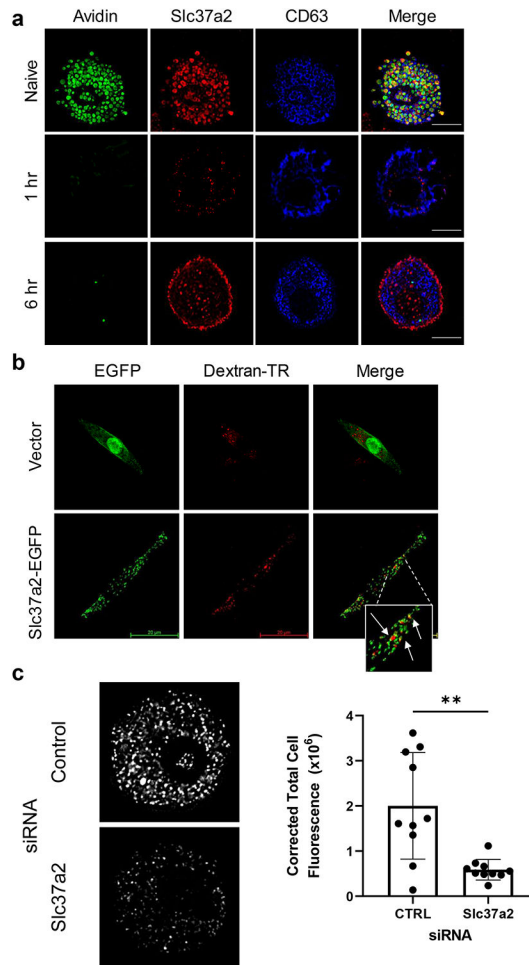


Figure 4. Slc37a2 localizes to endosomes during MC regranulation where it concentrates dextran.

(a) Immunofluorescence images of peritoneal MCs at time points indicated post activation with ionomycin. Cells are immunostained with avidin (green), slc37a2 (red), and CD63 (blue) Scale bars: 10 μ m. (b) Immunofluorescence images of Slc37a2-EGFP or EGFP vector expressing (green) RBLs incubated with Texas Red dextran (red) overnight. Arrows indicate Texas Red Dextran found within Slc37a2+ vesicles. All Images are representative of at least 2 independent experiments. (c) Immunofluorescence images of Slc37a2 siRNA or control siRNA treated BMMCs incubated overnight with Texas Red dextran (grey). Texas Red Dextran was quantified using ImageJ; ($n = 10$ images). Data were analyzed using a Student's t test. ** $P < 0.01$.

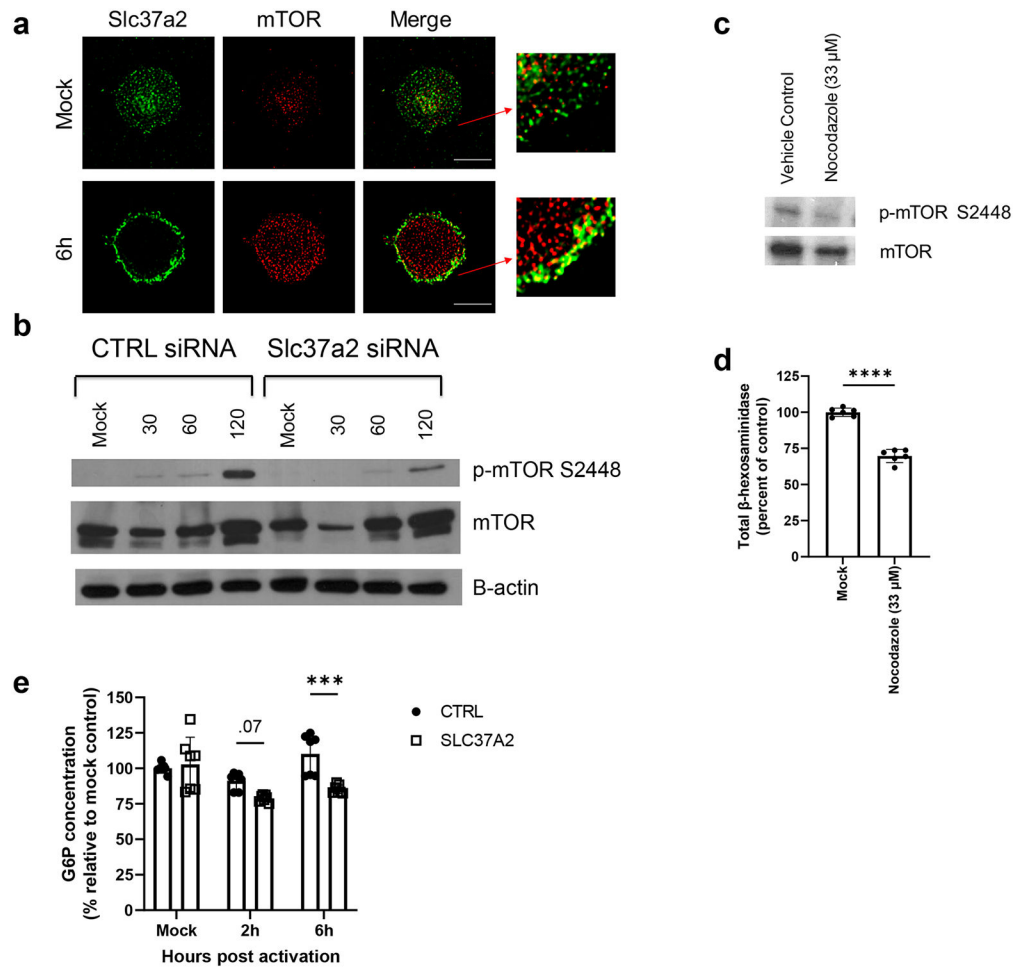


Figure 5. Trafficking of Slc37a2 to endosomes is necessary for mTOR activation.

(a) Immunofluorescence images of BMMCs 6 hrs post activation with ionomycin in Tyrode's buffer or Tyrode's buffer alone (mock). BMMCs were immunostained with Slc37a2 (green) and mTOR (red). (b) Western blots of p-mTOR S2448, mTOR, and β-actin of Slc37a2 siRNA treated BMMCs or control siRNA treated BMMCs at time points indicated. Two independent experiments were performed with similar results. (c) Western blots of p-mTOR S2448 and mTOR of BMMCs treated for 2 hrs with nocodazole or vehicle control post activation with ionomycin. (d) Total β-hexosaminidase at 48 hrs in BMMCs treatment with nocodazole or vehicle control post activation with ionomycin. Results were normalized to vehicle control ($n = 5$ replicates). Data are representative of 3 independent experiments. See also Supplemental Fig. 3a. (e) G6P concentrations in Slc37a2 siRNA treated BMMCs or control siRNA treated BMMCs at time points indicated post activation with ionomycin. Results were normalized to untreated control siRNA BMMCs. Data were analyzed using a Student's t test (d) or a 2-way ANOVA with a Bonferroni post-test. (e) *** $P < 0.001$, **** $P < 0.0001$.

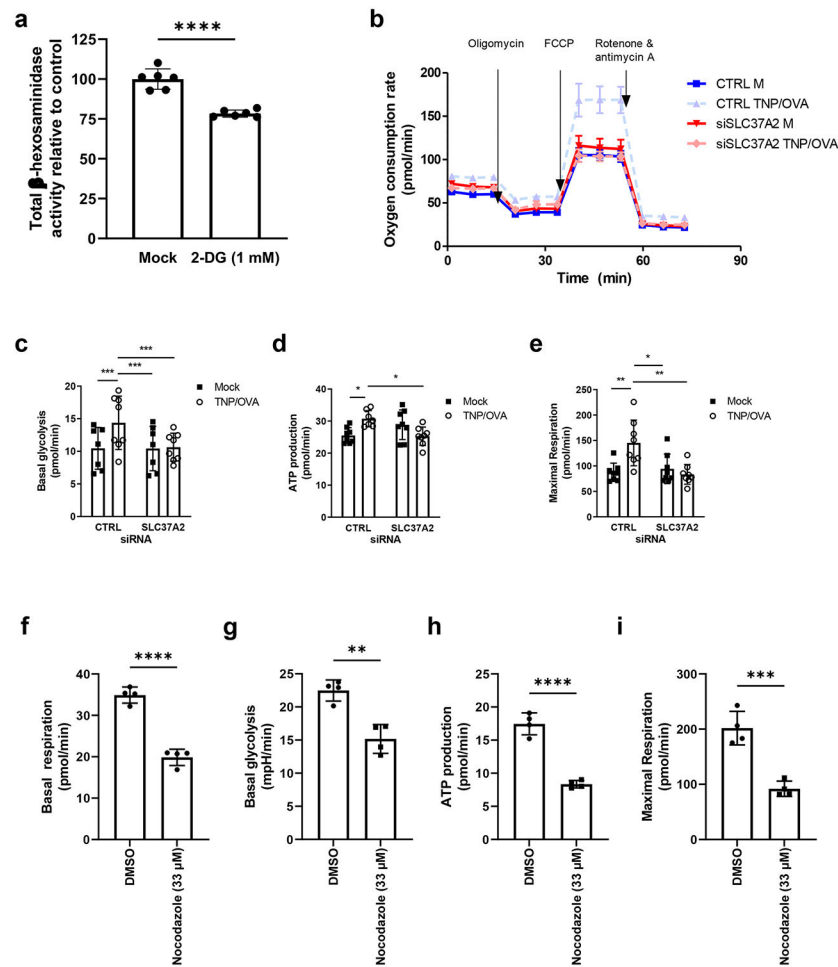


Figure 6. Slc37a2 trafficking to endosomes is necessary for metabolic reprogramming that occurs during MC regranulation.

(a) Total β -hexosaminidase at 48 hrs in BMBCs treatment with 2-DG or PBS (mock) post activation with ionomycin. Results were normalized to mock treated cells ($n = 6$ replicates). Data are representative of 2 independent experiments. (b-e) Mitochondrial stress test using the Seahorse XF on Slc37a2 siRNA treated BMBCs or control siRNA treated BMBCs 6 hrs post activation using TNP-specific IgE and TNP/OVA or mock. (b) Representative Seahorse plot from three independent experiments, (c) Basal glycolysis, (d) ATP production and (e) maximal respiration were quantified ($n = 8$ replicates). (f-i) Mitochondrial stress test using the Seahorse XF on BMBCs treated with nocodazole post activation with ionomycin. (f) Basal respiration, (g) basal glycolysis, (h) ATP production, and (i) maximal respiration were quantified ($n = 4$ replicates). Data are representative from two independent experiments. See also Supplemental Fig. 3b. Data were analyzed by a 1-way ANOVA with a Tukey's post-test (c-e), or a Student's t test (f-i). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

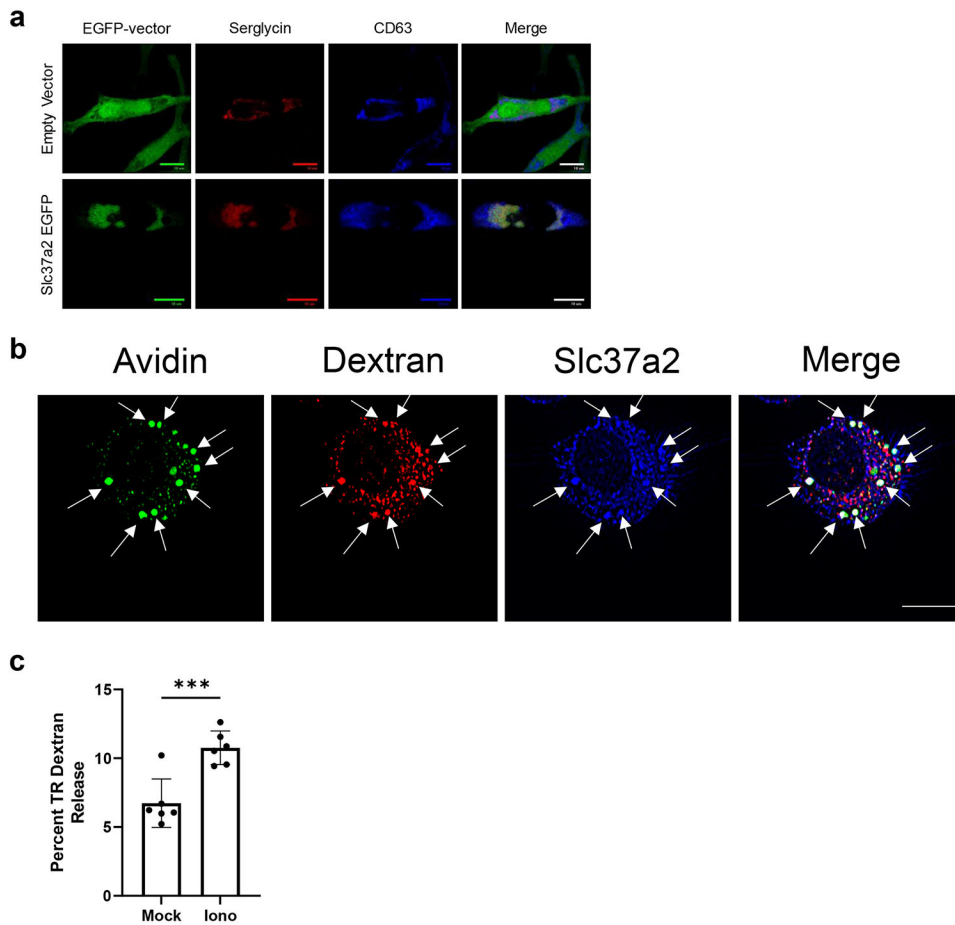


Figure 7. Slc37a2 traffics to nascent granules, carrying dextran into newly formed granules. (a) Immunofluorescence images of RBLs stably expressing serglycin mCherry (red) transduced with lentivirus expressing EGFP or Slc37a2-EGFP (green). Cells were immunostained for CD63 (blue). (b) Immunofluorescence images of BMMCs incubated for 48 hrs with Texas Red Dextran (red) 1 hr post activation with ionomycin. BMMCs were immunostained with avidin (green) and Slc37a2 (blue). Arrows indicate colocalization of avidin, dextran, and Slc37a2. (c) Quantification of Texas Red Dextran released from BMMCs incubated with Texas Red dextran in cell medium overnight and then activated with ionomycin in tyrode's buffer or in Tyrode's buffer alone (mock) for 1 hr. Percent released dextran was determined by quantifying fluorescence signal in supernatant and comparing it to total fluorescence in supernatant and lysed pellet ($n = 6$ replicates). Results are representative of two independent experiments. Data were analyzed using a Student's t test. *** $P < 0.001$.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-TNP IgE	BD Biosciences	Cat#557079
Rat anti-CD63	MBL	Cat#D263-3
Rabbit anti-Slc37a2	GeneTex	Cat#GTX85198
Mouse anti-serotonin	Dako	Cat#M0758
Mouse anti-mTOR	EMD Millipore	Cat#05-1592
Rabbit anti-mTOR	Cell Signaling	Cat#2983S
Rabbit anti-p-mTOR S2448	Cell Signaling	Cat#5536S
Rabbit anti-S6K	Cell Signaling	Cat#92902S
Rabbit anti-p-S6K S371	Cell Signaling	Cat#9208S
Mouse anti- β -actin	Sigma	Cat#A2228
Anti-mouse CD16/CD32	BD Biosciences	Cat#553142
7-AAD	Biolegend	Cat#424404
Pacific Blue anti-mouse CD45	Biolegend	Cat#103126
PE/Cyanine7 anti-mouse CD117	Biolegend	Cat#105813
BV510 Rat Anti-CD11b	BD Biosciences	Cat#562950
Brilliant Violet 650 anti-mouse Ly-6G	BD Biosciences	Cat#127641
BD Optibuild BUV395 Rat Anti-Mouse SiglecF	BD Biosciences	Cat#740280
Brilliant Violet 785 anti-mouse Ly-6C	Biolegend	Cat#128041
APC anti-mouse F4/80	BD Biosciences	Cat#123116
Avidin-FITC	BD Biosciences	Cat#554057
Chemicals, peptides, and recombinant proteins		
Tamoxifen	Tocris	Cat#6342
TNP-Ovalbumin	Biosearch Technologies	Cat#T-5051-10
Recombinant Mouse IL-3	Biolegend	Cat#575508
Neomycin	Sigma	Cat#N1142
Saponin	Sigma	Cat#8047-15-2
Ionomycin	Sigma	Cat#I0634
Triton-100X	Spectrum Chemical	Cat#9036-19-5
p-nitrophenyl-N-acetyl- β -D-glucosaminide	Sigma	Cat#487052
2-deoxyglucose	Sigma	Cat#D3179
Nocodazole	Sigma	Cat#M1404
Torin 1	Tocris	Cat#4247
BD Cytotfix/Cytoperm Fixation and Permeabilization Kit	BD Biosciences	Cat#554722
Prolonged Diamond	Invitrogen	Cat#P36970
Paraformaldehyde	Electron Microscopy Sciences	Cat#15710
Protease Inhibitor Cocktail	Sigma	Cat#P8340

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Phosphatase Inhibitor Cocktail 2	Sigma	Cat#P5726
Phosphatase Inhibitor Cocktail 3	Sigma	Cat#P0044
Pierce™ Coomassie (Bradford) Protein Assay Kit	ThermoFisher	Cat#23200
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	ThermoFisher	Cat#34578
Toluidine blue	Millipore Sigma	Cat#TX0875-2
Nucleofector T Kit	Lonza	Cat#VVCA-1002
Dextran Texas Red 10,000 MW	Thermo Fisher	Cat#D1828
Cell-Tak	Millipore Sigma	Cat#CLS354240
Oligomycin	Sigma	Cat#75351
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine	Sigma	Cat#C2920
Rotenone	Sigma	Cat#R8875
Antimycin A	Sigma	Cat#A8674
Critical commercial assays		
RNeasy Mini Kit	Qiagen	Cat#74104
Affymetrix Whole Transcriptome Plus Kit	ThermoFisher	Cat#902280
Affymetrix GeneChip Whole Transcriptome Terminal Labeling Kit	ThermoFisher	Cat#900671
GeneChip Mouse Transcriptome Array 1.0	AffyMatrix	Cat#901171
iScript cDNA synthesis kit	BioRad	Cat#1708890
iQ SYBR Green Supermix	BioRad	Cat#1708880
Glucose-6-phosphate Assay Kit	Sigma	Cat#MAK014-1KT
Deposited Data		
Microarray Data	This Paper	GEO Accession # GSE207235
Experimental models: Cell lines		
RBL-2H3 cells	ATCC	Cat#CRL-2256
CHO-KL cells	Dr. Michel Arock(Bibi S et al., 2016)	N/A
Amphopack-293 cells	BD Biosciences	Cat#C3201-1
Serglycin-mCherry RBL-2H3	Soman Abraham Lab(Ang WXG et al., 2016)	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6J	Jackson Laboratory	Cat#000664
Mouse: Raptor ^{fl/fl} ; B6.Cg-Rp107 ^{tm1.1Dmsa/J}	Jackson Laboratory	Cat#013188
Mouse: Raptor ^{fl/fl} ER-Cre	Dr. Xiao Ping Zhong Lab(Shin J et al., 2014)	N/A
Oligonucleotides		
Slc37a2 Forward qPCR Primer, 5'-GCTGCTCCCATGATGTTCCCT-3'	This Paper	N/A
Slc37a2 Reverse qPCR Primer, 5'-TTGGCGTTACCCTTCAGGCT-3'	This Paper	N/A
B-actin Forward qPCR Primer, 5'-GATTACTGCTCTGGCTCCTAGC-3'	This Paper	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
B-actin Reverse qPCR Primer, 5'-GACTCATCGTACTCTGCTTGC-3'.	This Paper	N/A
Mouse Slc37a2 siGENOME SMARTpool siRNAs	Dharmacon	Cat#M-060760-01-0005
Silencer™ negative control siRNA	Ambion	Cat#AM4611
Recombinant DNA		
pEGFP-C1 plasmid	Clontech	Cat#6058-1
Software and algorithms		
FlowJo v10	FlowJo, LLC	https://www.flowjo.com/solutions/flowjo/downloads
ImageJ	NIH	https://imagej.nih.gov/ij/
R statistical Program	R Core	https://www.r-project.org/
Bioconductor	https://bioconductor.org	https://bioconductor.org/install
<i>oligo</i> (R Package)	Carvalho BS, et al., 2010	https://www.bioconductor.org/packages/release/bioc/html/oligo.html
<i>limma</i> (R Package)	Ritchie ME et al., 2015	https://bioconductor.org/packages/release/bioc/html/limma.html
<i>fgsea</i>	Korotkevich G et al., 2021	https://bioconductor.org/packages/release/bioc/html/fgsea.html
StepOne Software v2.3	Applied Biosciences	https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/StepOne-and-StepOnePlus-Real-Time-PCR-System.html
Prism v.9.0.0	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
BD Fortessa X-20	BD Biosciences	N/A
Thunder Imager	Leica	N/A
Synergy H1 microplate reader	BioTec	N/A
Agilent 2100 Bioanalyzer G2939A	Agilent Technologies	N/A
Nanodrop 8000 spectrophotometer	Thermo Scientific	N/A
Nucleofector 2b Device	Lonza	Cat#AAB-1001
Seahorse XF96e Analyzer	Agilent	N/A
Affymetrix GeneChip hybridization oven 645	ThermoFisher	Cat#00-0331
Affymetrix GeneChip Fluidics Station 450	ThermoFisher	Cat#00-0079
StepOnePlus Real-Time PCR System	Applied Bioscience	N/A