SNX5 is essential for efficient macropinocytosis and antigen processing in primary macrophages

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Summary
Macropinocytosis mediates the bulk endocytosis of solute molecules, nutrients and antigens. As this endocytic pathway is considered important in functions associated with immune responses, the molecular mechanisms regulating this pathway in immune cells is of particular significance. However, the regulators of macropinocytosis in primary cells remain poorly defined. Members of the sorting nexin (SNX) family have been implicated in macropinosome biogenesis in cultured cells and here we have analyzed the role of two SNX family members, SNX1 and its binding partner SNX5, in macropinocytosis of mouse primary macrophages. We show that endogenous SNX1 and SNX5 are localised to newly-formed macropinosomes in primary mouse macrophages and, moreover, demonstrate that SNX5 plays an essential role in macropinosome biogenesis. Depletion of SNX5 in bone marrow-derived macrophages dramatically decreased both the number and size of macropinosomes. Depletion of SNX5 also resulted in dramatic reduction in uptake and processing of soluble ovalbumin in macrophages, indicating that the majority of antigen uptake and delivery to late endosomes is via macropinocytosis. By contrast, the absence of SNX1 had no effect on endogenous SNX5 localisation and macropinosome biogenesis using macrophages from SNX1 knockout mice. Therefore, SNX5 can function independently of SNX1 and is a modulator of macropinocytosis that influences the uptake and processing of soluble antigen in primary mouse macrophages.

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Key words: Macropinocytosis, Sorting nexins, Endocytosis, Antigen processing, SNX5

Introduction
Macropinocytosis is an endocytosis pathway that arises from actin-mediated membrane ruffling of the plasma membrane. Macropinosomes are derived from lamellipodia which fold back on themselves and fuse with the basal membrane creating large, irregular shaped endocytic vesicles with a diameter > 0.2 μm to 10 μm (Hewlett et al., 1994; Swanson and Watts, 1995) and which lack coat structures. Given their large size compared with other endocytic processes, macropinosomes very efficiently mediate the bulk uptake of solute molecules, nutrients and antigens as well as considerable amounts of plasma membrane. A key difference between clathrin-dependent endocytosis and macropinocytosis is that the latter requires actin cytoskeleton reorganisation whereas the former is actin independent in many circumstances. Unlike clathrin-mediated endocytosis, macropinocytosis is regulated by the activation of receptor tyrosine kinases which occur in response to growth factor stimulation such as macrophage colony stimulating factor (CSF-1), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) or tumour promoting factor such as phorbol myristate acetate (PMA) (Dharmawardhane et al., 2000; Haigler et al., 1979; Racocossin and Swanson, 1989; Swanson, 1989). Some specialised cell types such as antigen presenting cells are capable of constitutive macropinocytosis (Norbury et al., 1997; Norbury et al., 1995; Sallusto et al., 1995).

The capacity of macropinocytosis to endocytose large quantities of extracellular fluid underpins a variety of functions in development, cell motility, tumour progression and metastasis (Carpentier et al., 1991), and also innate and adaptive immune responses. Some opportunistic pathogens such as the protozoa, bacteria, viruses and prions have exploited the macropinocytotic pathway and its machineries to invade and evade the host immune system (Lim and Gleeson, 2011). In the immune system, antigen presenting cells such as the dendritic cells and macrophages utilise macropinocytosis as a major pathway for the capture of antigens from their immediate surrounding (Norbury et al., 1997; Norbury et al., 1995; Sallusto et al., 1995).

Given the physiological relevance of macropinocytosis, it is important to identify the regulatory components of this endocytic pathway. The downstream targets of phosphoinositide (PI) 3-kinase signalling, namely phosphoinositides, have been shown to be critical in the biogenesis of macropinosomes (Amyere et al., 2000; Anzinger et al., 2012; Araki et al., 1996; Clague et al., 1995; Kerr et al., 2010). Mapping of phosphoinositide derivatives during macropinocytosis has demonstrated a high level of PI(3,4,5)P3 at the site of macropinosome formation and a rapid transition from PI(3,4,5)P3 to PI(3)P as the macropinosome is closed (Yoshida et al., 2009). In addition, phosphoinositide effectors have been shown to be recruited to macropinosomes as
Results

SNX1-deficient macrophages are not affected in the biogenesis of macropinocytosis

The identification of regulators of macropinosome biogenesis in immune cells is an important prerequisite to establish the contribution of macropinocytosis to antigen processing and presentation. Given the known key roles of sorting nexins in membrane trafficking (Carlton et al., 2005), and their association with macropinosomes in different cell types (Bryan et al., 2007; Kerr et al., 2006; Lim et al., 2008; Merino-Trigo et al., 2004), we examined macropinocytosis in SNX1-deficient mouse macrophages (BMM) generated from 8–10 week old SNX1+/+ and SNX1−/− littermate mice and the biogenesis of macropinosomes assessed. BMM were incubated with FITC-conjugated 70 kDa dextran in the presence of CSF-1 for 15 min at 37°C, or on ice as control, and macropinosomes identified as FITC-70 kDa dextran-labelled structures >500 nm in diameter. No significant difference in the number of macropinosomes per cell between BMM from SNX1+/+ and SNX1−/− mice was observed in two independent experiments (Table 1). Similar results were obtained with peritoneal macrophages from SNX1+/+ and SNX1−/− mice (data not shown). Therefore, the absence of SNX1 had no apparent effect on macropinosome biogenesis.

Localization of SNX1 and SNX5 on macropinosomes in primary mouse macrophages

Given the finding that SNX1 deficiency has no effect on macropinocytosis in macrophages, we therefore investigated the localization of endogenous SNX1 and its binding partner, SNX5, to ascertain the relationship of these sorting nexins with newly formed macropinosomes in these primary cells. To label primary immune cells would provide a significant advance in the field as it would allow the opportunity to investigate the precise roles of macropinocytosis by antigen presenting cells in vitro and in vivo. Here we have investigated the role of endogenous SNX1 and SNX5 in macropinocytosis and antigen processing in primary macrophages. Our findings demonstrate that SNX5, but not SNX1, regulates macropinocytosis, antigen uptake and ultimately levels of antigen processing in primary macrophages.

Table 1. Absence of SNX1 does not affect macropinosome biogenesis in bone marrow-derived macrophages. BMM were incubated with FITC-conjugated 70 kDa dextran in the presence of CSF-1 for 15 min at 37°C and the macropinosomes enumerated as described in Materials and Methods.

|                          | Mean number of macropinosomes per cell ± SD |
|--------------------------|--------------------------------------------|
| Experiment 1             |                                            |
| SNX1+/+                  | 12.76 ± 5.97                               |
| SNX1−/−                  | 11.48 ± 3.83                               |
| Experiment 2             |                                            |
| SNX1+/+                  | 12.64 ± 4.51                               |
| SNX1−/−                  | 14.32 ± 6.26                               |
macropinosomes, primary peritoneal macrophages were incubated with FITC-conjugated 70 kDa dextran at 37°C for 15 min. After 15 min, 70 kDa dextran labelled predominantly “donut-like” large structures and also to a lesser extent smaller endosomal structures (Fig. 1A,B). The donut appearance arises from the association of the fixable dextran with biomolecules associated with the membranes of the macropinosomes and the loss of non-fixed, luminal, dextran during permeabilization steps. The majority of the 70 kDa dextran-labelled “donut-like” structures were >500 nm in diameter (Fig. 1A,B), a size compatible with classical macropinosomes. Moreover, the 70 kDa dextran-labeled “donut-like” structures observed in paraformaldehyde-fixed peritoneal macrophages were reduced in number by >95% in cells treated with the selective macropinosome inhibitor, 5-(N-ethyl-N-isopropyl) amiloride (Koivusalo et al., 2010; West et al., 1989), confirming their identity (data not shown).

Primary peritoneal macrophages were incubated with FITC-conjugated 70 kDa dextran at 37°C for 15 min and then stained for endogenous SNX1 and SNX5 using antibodies we generated to these mouse SNXs (Materials and Methods; supplementary material Fig. S3). Newly-formed macropinosomes >500 nm in diameter stained strongly for both endogenous SNX1 and SNX5 (Fig. 1A,B). The early endosome marker, EEA1, which has shown to be also a marker of newly-formed macropinosomes in cultured cells (Kerr et al., 2006; Merino-Trigo et al., 2004), was detected on the FITC-dextran labelled macropinosomes in peritoneal macrophages. Both SNX1 and SNX5 juxtaposed with EEA1 on boundary of the macropinosomes (Fig. 1A,B).

We then assessed whether both SNX1 and SNX5 were co-localised on the same macropinosomes or whether they may label distinct subpopulations. As co-staining with the rabbit anti-SNX1 and anti-SNX5 antibodies could not be performed, peritoneal macrophages were transduced with recombinant adenovirus encoding green fluorescent protein (GFP)-SNX5 and stained for EEA1 and SNX1. For this analysis, macropinosomes were identified based on their size (>500 nm diameter), circular structure and the staining pattern for EEA1 which resembled a halo. Despite a high background due to cytosolic GFP-SNX5, the analysis showed that GFP-SNX5 and SNX1 were localised on the same macropinosome structures juxtaposing with EEA1 (Fig. 1C). Collectively, these data indicate that both SNX1 and SNX5 are recruited to the same population of newly formed macropinosomes in primary mouse macrophages.

SNX1 and SNX5 have been reported to physically interact, based on yeast two-hybrid analyses and biochemical assays of transfected cells overexpressing sorting nexins (Kerr et al., 2006; Liu et al., 2006; Wassmer et al., 2009). Using the reagents we had generated, we investigated whether endogenous SNX1 and SNX5 in mouse primary macrophages can exist as a heterodimer. Peritoneal macrophages were isolated from BALB/c mice, lysed and endogenous SNX5 immunoprecipitated using anti-SNX5 antibodies. The immunoprecipitated complex was then analysed by immunoblotting for endogenous SNX1. The 70 kDa endogenous SNX1 co-immunoprecipitated with endogenous SNX5 (Fig. 1D). SNX1 was not detected in the control immunoprecipitation (Fig. 1D) demonstrating that the interaction was specific. A SNX1/SNX5 interaction was also detected by immunoprecipitation from bone-marrow derived macrophages (BMM) and NIH3T3 cells (data not shown). These data confirm that endogenous SNX1 and SNX5 can interact in vivo in primary cells and is consistent with the presence of a heterodimer.

We have previously demonstrated that elevated levels of SNX5 results in enhanced macropinocytotic activity (Lim et al., 2008). Given the finding that SNX1 deficiency does not perturb macropinocytosis, and that SNX1 and SNX5 physically interact in primary macrophages, we assessed the level and localization of SNX5 in SNX1-deficient macrophages. Immunoblotting revealed that endogenous SNX5 was present at a similar level in BMM

![Fig. 1. SNX1 and SNX5 are localized on newly-formed macropinosomes.](image_url)
from SNX1\(^{+/+}\) and SNX1\(^{-/-}\) mice, and furthermore, SNX5 was localized to both small and large (>500 nm diameter) EEA1-positive structures in SNX1-deficient BMM (Fig. 2). These results indicate that the absence of SNX1 does not affect the localization of SNX5 to endosomal and macropinosomal membranes in macrophages. Therefore, the removal of SNX1, or both SNX1 and SNX5, may be required to have an impact on macropinocytic activity.

Depletion of SNX5 in primary macrophages

To determine the influence of SNX5 in macropinocytosis in macrophages, we examined the effect of silencing SNX5 using the BLOCK-IT\(^{TM}\) Pol II miR RNAi expression system. Out of five RNAi targets examined by immunofluorescence, SNX5 miRNA-1 was the optimum RNAi; however, SNX5 miRNA-1 reduced the level of SNX5 by only 40% as determined by immunoblotting (Fig. 3A). To further improve the efficiency of SNX5 depletion, a recombinant adenovirus construct with 4 copies of miRNA-1 in one primary transcript was generated, designated miRNA-1.4. Transduction of NIH3T3 cells with the recombinant SNX5 miRNA-1.4 adenovirus showed 100% transduction efficiency (data not shown) and the level of SNX5 in NIH3T3 cells was reduced by \(\approx 92\%\) compared to control miRNA (Fig. 3B,C).

To determine if SNX5 miRNA-1.4 was able to silence SNX5 in peritoneal macrophages, BMM were transduced with the recombinant adenovirus and cells analysed 96 hours after transduction. Unfortunately, GFP\(^{+}\) transduced BMM had a similar level of SNX5 compared to the untransduced, wild-type (GFP-negative) cells (data not shown). Likewise peritoneal macrophages showed no reduction in SNX5 after transduction with SNX5 miRNA-1.4 adenovirus (data not shown). On the other hand, expression of an unrelated miRNA (GCC185 miRNA) in peritoneal macrophages showed effective silencing of GCC185 (not shown), demonstrating that the miRNA vector was functional in BMM.

SNX5 expression has been reported to be lower in hematopoietic stem cells than committed progenitor cells (Eckfeldt et al., 2005). As the silencing of SNX5 may be more efficient in hematopoietic stem cells we therefore introduced the SNX5 miRNA into hematopoietic progenitor bone marrow stem cells, prior to differentiation into macrophages. Hematopoietic progenitor bone marrow stem cells from BALB/c mice were

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**Fig. 2.** Absence of SNX1 does not affect the level and localization of SNX5.

(A) BMM from SNX1\(^{+/+}\), SNX1\(^{+/2}\) and SNX1\(^{-/-}\) mice were lysed in reducing sample buffer and proteins resolved in a 10% NuPAGE\(^{\oplus}\) gel. Proteins were then transferred onto a PVDF membrane and probed with affinity-purified rabbit anti-SNX1 or anti-SNX5 antibodies followed by HRP-conjugated anti-rabbit IgG. Anti-SNX5 antibodies were stripped from the membrane to allow subsequent incubation with mouse monoclonal anti-\(\alpha\)-tubulin antibodies followed by HRP-conjugated anti-mouse IgG. Bound antibodies were detected using chemiluminescence. (B) BMM from SNX1\(^{-/-}\) mice were fixed in 4% paraformaldehyde, permeabilised, and stained with rabbit anti-SNX5 antibodies followed by Alexa 558-conjugated anti-rabbit IgG and anti-EA1 antibodies followed by Alexa 647-conjugated anti-human IgG. Magnification of boxed region is shown in the bottom panel of B. Bars = 5 \(\mu\)m.

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**Fig. 3.** SNX5 knockdown in NIH3T3 and primary macrophages.

(A) NIH3T3 cells were transfected with control miRNA or miRNA-1, GFP\(^{+}\) cells sorted 96 hours later and then lysed in reducing sample buffer.

(B) NIH3T3 cells were transduced with control miRNA or miRNA-1.4 recombinant adenovirus, and 96 hours later lysed in reducing sample buffer.

(A,B) Proteins were resolved in a 10% NuPAGE\(^{\oplus}\) gel, transferred onto a PVDF membrane and probed with rabbit anti-SNX5 antibodies followed by HRP-conjugated anti-rabbit IgG. Rabbit anti-SNX5 antibodies were stripped from the membrane to allow subsequent incubation with mouse monoclonal anti-\(\alpha\)-tubulin antibodies followed by HRP-conjugated anti-mouse IgG. Bound antibodies were detected using chemiluminescence.

(C) NIH3T3 were fixed in 4% paraformaldehyde and permeabilised 72 hours after transduction and stained with rabbit anti-SNX5 antibodies followed by Alexa 647-conjugated anti-rabbit IgG. Asterisks (*) indicate transduced macrophages with reduced SNX5 expression. Bars = 10 \(\mu\)m.
transduced with SNX5 miRNA recombinant adenovirus and the cells cultured in the presence of CSF-1 for 72 hours to generate bone marrow-derived macrophages. The majority (65%) of bone marrow-derived macrophages were GFP-positive indicating that miRNA-1.4 was successfully introduced into the cells. The cells were positive for the macrophage marker, F4/80+, and had the decisive characteristics of bone marrow-derived macrophages (data not shown). Therefore, the expression of the miRNA construct did not influence macrophage differentiation. Two distinct populations were observed when GFP-positive BMM were stained for SNX5. Approximately 50% of the GFP-positive cells showed a dramatic reduction in SNX5 staining (Fig. 3D) while the remaining 50% of the GFP-positive cells showed normal levels of SNX5 staining. This result was reproducible in more than 5 separate experiments. Quantitative analysis of the SNX5 staining intensity indicated that the SNX5-silenced population of GFP+ BMM had SNX5 levels reduced by 89% compared with control BMM. Although the reason why only 50% of the GFP+ cells are effectively silenced in SNX5 is not clear, this strategy provided primary macrophages depleted in SNX5.

Depletion of SNX5 influences macropinocytosis
To investigate the physiological relevance of SNX5 in macropinocytosis, progenitor bone marrow stem cells from BALB/c mice were transduced with either miRNA-1.4 or control miRNA recombinant adenovirus, then differentiated into BMM by culturing in the presence of CSF-1. BMM, stimulated with 50 ng/ml CSF-1, were incubated with Texas Red (TxR)-conjugated 70 kDa dextran at 37°C for 15 min to label newly-formed macropinosomes, identified as TxR-dextran-positive structures >500 nm in diameter. Cells were fixed and stained with anti-SNX5 antibodies to identify cells with reduced SNX5. BMM depleted of SNX5 had significantly reduced uptake of TxR-conjugated 70 kDa dextran (Fig. 4B) compared to BMM expressing control miRNA (Fig. 4A). To quantify the impact of SNX5 depletion on macropinocytosis, the mean number of TxR-dextran labelled macropinosomes per cell was determined for BMM expressing control miRNA and miRNA-1.4 with low levels of SNX5. BMM depleted of SNX5 showed a 60% reduction (P < 0.001) in number of macropinosomes compared to cells expressing control miRNA (Fig. 4C). This result was reproducible (Table 2). The data were also analysed according to the number of macropinosomes per cell (Fig. 4D; Table 2). For cells depleted of SNX5, the majority of BMM (60%) had <6 macropinosomes per cell and none had >16 macropinosomes per cell (Table 2). In comparison, all cells expressing control miRNA had 6 or more macropinosomes per cell and > 40% of cells had 16 or more macropinosomes per cell (Table 2). Therefore, there was a dramatic reduction in the formation of macropinosomes in SNX5-depleted cells. A duplicate experiment showed reproducible distribution pattern in the number of macropinosomes formed per cell (Table 2).

In addition to the reduced number of macropinosomes, depletion of SNX5 resulted in the formation of smaller macropinosomes. The size of macropinosomes in either untransduced BMM (data not shown) or BMM expressing control miRNA can be up to ~4 μm in diameter (Fig. 4A). In BMM depleted of SNX5, the majority of macropinosomes were considerably smaller, ~1–2 μm in diameter (Fig. 4B). Collectively, the data show a reduced level of SNX5 affects not only macropinocytic activity but also the size of macropinosomes formed.

To eliminate the possibility of off-target effects of SNX5 miRNA-1.4, a second independent miRNA target sequence was identified, miRNA-2, and a multiple target copy of this target sequence, miRNA-2.4 reduced the level of endogenous SNX5 in NIH3T3 cells by 83% (supplementary material Fig. S1). BMM expressing miRNA-2.4 and depleted of SNX5 showed 50–60% decrease (P < 0.001) in macropinocytosis compared to cells expressing control miRNA (Table 2). Silencing using miRNA-2.4 resulted in a similar decrease in number of macropinosomes per cell as for miRNA-1.4 (Table 2).

Depletion of SNX5 in SNX1+/− BMM
Although SNX1-deficiency does not influence macropinosome biogenesis in BMM, given the association of SNX1 and SNX5,
depletion of both SNX1 and SNX5 may have a greater impact on macropinocytosis than SNX5 depletion alone. To investigate this possibility, SNX5 was silenced in SNX1<sup>2/2</sup>BMDM using SNX5 miRNA-1.4. Progenitor bone marrow stem cells from 8–10 week old SNX1<sup>2/2</sup> mice were either left untransduced or transduced with miRNA-1.4 or the control miRNA using recombinant adenoviruses. Transduced cells were then differentiated into BMM and then incubated with TxR-conjugated 70 kDa dextran in the presence of 50 ng/ml CSF-1 stimulation to label newly-formed macropinosomes. SNX1<sup>2/2</sup>BMM depleted of SNX5 showed a 50% reduction in macropinocytosis compared to untransduced cells or cells expressing control miRNA (supplementary material Fig. S2). The level of macropinocytosis between untransduced and cells expressing control miRNA were similar, indicating that macropinocytosis was unaffected by adenovirus transduction (supplementary material Fig. S2). In untransduced SNX1<sup>2/2</sup>BMM and SNX1<sup>−/−</sup>BMM expressing control miRNA, the majority of the cells (> 80%) had between 6–15 macropinosomes per cell. In contrast, very few (4%) of SNX1<sup>−/−</sup>BMDM depleted of SNX5 had >10 macropinosomes per cell (supplementary material Fig. S2). Therefore the absence of both SNX1 and SNX5 did not have a greater impact on the reduction of macropinocytosis than the depletion of SNX5 alone. Collectively, the data show that SNX5, and not SNX1, is the main modulator of macropinocytosis in BMM.

### Influence of SNX1 and SNX5 on antigen (ovalbumin) uptake and processing by macrophages

To investigate the relevance of macropinocytosis on the uptake of soluble antigen, BMM were incubated with a mixture of FITC-70 kDa dextran and Alexa555-ovalbumin for 30 min at 37°C, or on ice as control. Alexa555-ovalbumin was detected in dextran-positive macropinosomes (Fig. 5A). As expected, neither dextran nor ovalbumin was internalised when the cells were incubated on ice (Fig. 5A). After 30 min internalisation, some of the ovalbumin-positive punctate structures stained positive for LAMP-1 (Fig. 5B), indicating the delivery of ovalbumin to the late endosome/lysosome.

Processing of soluble antigen in acidic endosomal compartments is required for antigen presentation. To further explore the role of macropinocytosis in ovalbumin uptake and processing, DQ-ovalbumin was used as it has intrinsic fluorescent properties after exposure to a processing compartment. DQ-ovalbumin is a self-quenched conjugate of ovalbumin that exhibits fluorescence upon proteolytic degradation and can be used to study antigen uptake and processing in the late endosome/lysosome (Daro et al., 2000; Santambrogio et al., 1999). A 3 min pulse of BMM with DQ-ovalbumin followed by a 30 min chase resulted in the gradual increase in fluorescence at 615 nm, suggesting that ovalbumin was processed in the late endosome/lysosome.

### Table 2. Reduced levels of SNX5 affect macropinosome biogenesis in bone marrow-derived macrophages.

| Target sequence 1: miRNA-1.4 | Distribution of number of macropinosomes per cell (%) | Mean number of macropinosomes per cell ± SD |
|-----------------------------|------------------------------------------------------|-------------------------------------------|
| Experiment 1 Control miRNA  | 14.28 ± 4.60                                         | 0–5 6–10 11–15 16–20 > 20                 |
| Experiment 2 miRNA-1.4      | 5.64 ± 3.83                                          | 60 28 12 0 0                              |
| miRNA-1.4                   | 15.92 ± 6.40                                         | 0 16 36 20 28                            |
| miRNA-1.4                   | 5.99 ± 4.87                                          | 48 32 12 8 0                            |

| Experiment 2 Control miRNA  | 14.12 ± 6.16                                         | 0–5 6–10 11–15 16–20 > 20                 |
| Experiment 2 miRNA-2.4      | 5.73 ± 3.44                                          | 4 24 28 32 12                            |
| miRNA-2.4                   | 13.96 ± 6.46                                         | 52 40 4 4 0                            |
| miRNA-2.4                   | 7.04 ± 4.02                                          | 48 36 12 4 0                            |

**Fig. 5. Ovalbumin is internalized into LAMP-1 positive macropinosomes.** BMM were rendered quiescent by culturing overnight in complete RPMI. The next day (A) BMM were incubated with 500 μg/ml of FITC-conjugated 70 kDa dextran and 50 μg/ml of Alexa 555-conjugated ovalbumin for 30 min at 37°C or on ice in the presence of 50 ng/ml CSF-1 and then fixed in 4% paraformaldehyde. In (B) BMM were incubated with 50 μg/ml of Alexa 555-conjugated ovalbumin for 30 min at 37°C and then fixed in methanol and stained with rat anti-LAMP-1 antibodies followed by Alexa 488-conjugated anti-rat IgG. FITC-dextran, Alexa 555-conjugated ovalbumin and LAMP-1 are shown in grey. Bars = 5 μm.
indicative of the processing of the DQ-ovalbumin by proteases in MHCII loading compartments (Fig. 6A). To determine the role of SNX5 on ovalbumin processing, progenitor bone marrow stem cells from BALB/c mice were transduced with either miRNA-1.4 or control miRNA recombinant adenovirus and then differentiated into bone marrow-derived macrophages as previously. BMM were then pulsed with DQ-ovalbumin for 3 min and chased in the presence of 50 ng/ml CSF-1 at 37°C for 30 min, the time required for ovalbumin to reach LAMP-1 positive compartments. As a control, cells were continuously incubated in DQ-ovalbumin on ice. To identify cells with reduced SNX5 expression, fixed cells were stained with anti-SNX5 antibodies. BMM depleted of SNX5 have significantly reduced DQ-ovalbumin intensity as determined by immunofluorescence analysis (Fig. 6C) compared to BMM expressing control miRNA (Fig. 6B). Quantitative analyses showed a 60% reduction ($P < 0.001$) in DQ-ovalbumin fluorescent intensity in SNX5-depleted BMM compared with control (Fig. 7A). The level of reduction in DQ-ovalbumin intensity is similar to the reduction in macropinosome biogenesis after SNX5 depletion (Fig. 4C), indicating that SNX5 is regulating antigen uptake and processing in primary macrophages. In contrast, no reduction in DQ-ovalbumin fluorescent intensity was observed in SNX1−/− deficient BMM (Fig. 7B), indicating that SNX1 is not required for antigen uptake and processing. Therefore, these data show that SNX5 is a major regulator of antigen uptake and processing by primary macrophages.

**Discussion**

Primary macrophages undergo a very high rate of constitutive macropinocytosis and represent an excellent cell type to identify the regulators of this pathway in specialised cells of the immune system (Tsang et al., 2000). Microarray data have indicated that...

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**Fig. 6.** SNX5 is required for DQ-ovalbumin uptake and processing.

(A) Time course of DQ-ovalbumin processing by BMM. BMM were pulsed with DQ-ovalbumin for 3 min, washed and chased for time periods indicated in the presence of CSF-1. Bar = 10 μm. (B,C) Control miRNA or miRNA-1.4 was introduced into progenitor bone marrow cells from BALB/c mice by recombinant adenovirus. Cells were then differentiated into bone marrow-derived macrophages (BMM) by culturing in the presence of CSF-1. After 72 hours, BMM were rendered quiescent by culturing overnight in complete RPMI. The following day, BMM were pulsed with 50 μg/ml of DQ-ovalbumin for 3 min and chased in the presence of CSF-1 for 30 min at 37°C. A control was performed as a continuous incubation with DQ-ovalbumin in the presence of CSF-1 for 30 min on ice. BMM were then fixed in 4% paraformaldehyde, permeabilised, and stained with rabbit anti-SNX5 antibodies followed by Alexa 647-conjugated anti-rabbit IgG. Bars = 5 μm.

**Fig. 7.** Quantitation of DQ-ovalbumin processing in SNX5− and SNX1−/− deficient macrophages. (A) BMM expressing control and miRNA-1.4 and (B) BMM from SNX1+/+ and SNX1−/− mice were cultured in the presence of CSF-1 and were pulsed with 50 μg/ml of DQ-ovalbumin for 3 min and chased in the presence of CSF-1 for 30 min at 37°C. The intensity of DQ-ovalbumin per pixel for 25 cells was analysed using Metamorph software. Each data-point represents one BMM cell. The mean is shown for each data-set. Data were analysed by unpaired two tail t-test. *** $P < 0.001$. NS = not significant.
monocytes and immature dendritic cells express high levels of SNX1 and SNX5 transcripts (Su et al., 2004) coinciding with their high macroinocytic activities. Our previous studies using cell lines showed that SNX5 is a molecular component of the macroinocytosis pathway (Lim et al., 2008) and that macroinocytic activity is increased when SNX5 levels are raised (Lim et al., 2008). Here we have extended these earlier findings and shown that SNX5 is a key regulator of macroinocytosis in primary macrophages. Depletion of SNX5 resulted in a dramatic reduction in macroinocosome biogenesis and, moreover, resulted in a substantial reduction in antigen uptake and processing by primary macrophages, highlighting the importance of this sorting nexin and the macroinocytosis pathway in antigen presentation. The identification of SNX5 as a key regulator of the macroinocytic pathway of antigen presenting cells is an important finding as it provides a potential strategy to perturb macroinocytosis selectively in vivo and to analyze the biological relevance of this endocytic pathway in a range of primary specialized cells.

Macropinosomes were identified in this study using 70 kDa dextran as a fluid-phase marker. 70 kDa dextran is expected to label predominantly macropinosomes due to size constraints of entry into endocytic structures. The majority of 70 kDa dextran labelled intracellular structures by macrophages were >500 nm in diameter and the formation of these dextran labelled structures was inhibited in the presence of the macroinocosome selective inhibitor, amiloride, confirming their identity. Another advantage of using the large sized 70 kDa dextran is the low level of content mixing between “older” and “newer” macroinosomes (Berthiaume et al., 1995), thereby restricting the labelling to newly-formed macropinosomes.

By generating specific antibodies to mouse sorting nexins, we have shown that endogenous SNX1 and SNX5, along with EEA1, are juxtaposed on the same newly formed macropinosome in primary macrophages. In addition endogenous SNX1 and SNX5 were shown to interact by co-immunoprecipitation, a finding consistent with previous studies that demonstrated an interaction between overexpressed exogenous SNX1 and SNX5 (Liu et al., 2006). Importantly, the studies performed here have revealed an interaction between these sorting nexins under physiological conditions in primary cells. It is likely that SNX1 and SNX5 are recruited to newly emerging macropinosomes through interaction between their PX domains and PI(3)P and/or PI(3,4)P₂. High levels of sorting nexins have been shown to tubulate membranes in vitro and drive tubulation in vivo (Carlton et al., 2004). Notably tubules emanating from maturing macroinosomes have been shown to label predominantly macroinosomes in HEK293 cells (Kerr et al., 2006), and we have also detected both SNX1 and SNX5 on tubular cytoplasmic structures in macrophages (unpublished data), findings consistent with the behaviour of these sorting nexins in cultured cells. Thus the behaviour of the endogenous SNXs in primary macrophages parallels their reported activity in overexpressing cultured cells.

Using a miRNA based silencing system, we have shown that depletion of SNX5 resulted in decreased macropinosome biogenesis in bone marrow-derived macrophages. This was verified using 2 different miRNA target sequences for SNX5, reducing the possibility of off-target effects. SNX5 was depleted at the progenitor stem cell stage and SNX5 depleted stem cells then differentiated into macrophages. It is possible that a deficiency of SNX5 could result in perturbation of macropinosomes as well as general endosomal transport pathways, given the potential role of SNX5 also in retromer function (Wassmer et al., 2007). However, the differentiation of phenotypically normal macrophages from SNX5-depleted stem cells would argue that general endosomal transport pathways, including those dependent on retromer, remained intact. Furthermore, deficiency of SNX5 did not affect size of macrophages or the level of surface marker F4/80, and in addition SNX5 deficiency had no effect on transferrin receptor endocytosis in mouse NIH 3T3 cells (unpublished data). Taken together, these data indicate a selective role for SNX5 associated with macropinosome biogenesis.

In contrast to the findings for SNX5, the absence of SNX1 did not affect macroinocytosis biogenesis or antigen uptake. As SNX5 was localised to macroinosomes in the absence of SNX1, membrane recruitment and function of SNX5 was independent of SNX1. We have previously shown that SNX5 can be recruited to the plasma membrane of EGF stimulated cells in the absence of SNX1 (Merino-Trigo et al., 2004) and the findings here concur that SNX5 has a role independent of SNX1 in macroinocytosome biogenesis. We have also previously demonstrated that SNX5 does not form a homodimer (Kerr et al., 2006), and given the dimerization properties of BAR domain proteins, is therefore likely to exist as heterodimers with SNX1 and also possibly other sorting nexins such as SNX2 which has 63% amino acid sequence identity to SNX1 (Rojas et al., 2007). Regardless, our data clearly show the SNX1-independent role of SNX5 in membrane recruitment and macroinocytosome biogenesis.

How might SNX5 regulate macroinocytosis in primary macrophages? The presence of the BAR domain can regulate membrane curvature (Carlton et al., 2004; Peter et al., 2004) and the BAR domain of SNX5 may be a key factor in orchestrating macroinocosome formation. Following phosphoinositide dependent recruitment, SNX5 complexes may distort the plasma membrane of macrophages to subsequently form a macropinosome. The finding that depletion of SNX5 not only reduced the number, but also the size, of macroinosomes in BMM supports a role for SNX5 in defining the curvature of budding process. Also, given the potential role of SNX5 in tubulation and maturation of fully formed macroinosomes (Kerr et al., 2006), SNX5 may have multiple roles in macroinocytosis in macrophages, firstly in their biogenesis at the plasma membrane and secondly by contributing to the tubulation and maturation process of fully formed macroinosomes.

To date, the importance of macroinocytosis in antigen presentation in vivo has only been investigated using broad-based drug strategies such as amiloride, Sanglifehrin A or rapamycin, agents (Hackbarth et al., 2007; Hackstein et al., 2002; von Delwig et al., 2006). However, in vivo application of these drugs is not specific. Strategies are required to selectively manipultate macroinocytosis in the whole animal system. This study identifying the role of SNX5 as a regulatory component of macroinocytosis in antigen presenting cells could therefore provide a more specific approach to determine the effect of inhibiting macroinocytosis on the immune system in vivo.

**Materials and Methods**

**Antibodies and reagents**

CSF-1 was purchased from Gibco (USA). Human auto-antibodies to EEA1 (Mu et al., 1995) have been described. Rat anti-mouse CD16/CD32 (Mouse BD Fe Block™) and rat anti-mouse LAMP-1 was purchased from BD Bioscience (USA). Horse-radish peroxidase (HRP)-conjugated swine anti-rabbit IgG and
rabbit anti-mouse IgG were from DAKO Corporation (Denmark). Mouse monoclonal anti-bovine α-tubulin, goat anti-human IgA Alexa FluorTM 488, goat anti-rabbit IgG Alexa FluorTM 568, goat anti-rabbit IgG Alexa FluorTM 647 and goat anti-rat IgG Alexa FluorTM 488, lissamine-fluorescein Trans-conjugated 70 kDa dextran, fluorescein isothiocyanate (FITC)-conjugated 70 kDa dextran, Alexa Fluor® 555-conjugated ovalbumin and DQ-ovalbumin were purchased from Molecular Probes (USA).

Recombinant DNA constructs
cDNA was obtained from mouse testis mRNA of BALB/c mice by reverse transcription PCR. The full length cDNA of SNX1 was amplified from mouse testis cDNA and the resulting PCR product was cloned into EcoRI/Xhol sites of pEGFP-C1 (Clontech, USA) to generate pEGFP-C1-SNX1 construct. To generate pEGFP-C1-SNX5, the full length cDNA of SNX5 was amplified from mouse testis cDNA and the resulting PCR product was cloned into KpnI/BamHI sites of pEGFP-C1.

cDNAs or mRNA were cloned into pDC315 (Microbix Biosystems, Canada) for generating recombinant adenovirus (rADV). To generate pDC315-GFP-SNX5, full length cDNA encoding SNX5 was inserted at the C-terminal GFP was amplified from pEGFP-C1-SNX5 and cloned into BamHI site of pDC315. For pDC315-GFP-SNX5, the full length cDNA of SNX5 was amplified from mouse testis mRNA according to manufacturer’s instructions. Bound antibodies were separated and identified using a BDSan 100 SDS-PAGE gel and transferred to a nitrocellulose membrane (Pall Corporation, USA). The membrane was incubated with the primary antibody (1:1000 dilution) and then with the corresponding secondary antibody (1:5000 dilution) and developed with an ECL Plus detection system (Amersham, USA).

Molecular Probes (USA).

Generation of antibodies specific for mouse SNX1 and SNX5

A region corresponding to the N-terminal 108 residue of mouse SNX1 (SNX11–108) was injected subcutaneously into New Zealand white rabbits. Two additional boost injections were given 27 days and 48 days after the initial immunisation. Terminal bleed was carried out 90 days after the initial immunisation. All immunisation procedures were performed by Millipore (USA). Antibodies were affinity purified from the terminal bleed serum using a column of SNX11–108 protein conjugated to CNBr-activated Sepharose 4B beads (GE Healthcare, UK) according to manufacturer’s instructions. Bound antibodies were subjected to alternate high and low salt washes prior to low pH elution. Affinity-purified antibodies reacted by immunoblotting with a 88 kDa GFP-full length SNX1 fusion protein from transfected NIH3T3 cells, in addition to the predicted endogenous ~70 kDa SNX1 protein (supplementary material Fig. S3A). The antibodies co-localised with the punctate staining pattern of GFP-SNX1 and detected punctate structures in untransfected NIH3T3 cells, which correspond to the expected location of endogenous SNX1 (supplementary material Fig. S3C), confirming that the antibodies are specific for SNX1.

Anti-SNX5 antibodies

A synthetic peptide corresponding to the N-terminal 35 residues of mouse SNX5 (SNX51–35), with an additional cysteine residue at the C-terminus was synthesised (Research Transfer Facility, Bio21 Institute). Sequence of peptide is: MAAVPELLEDEQRESLRKVSV5DVLNPDSQIDC. SNX51–35 peptide was directly injected subcutaneously into New Zealand white rabbit. Three additional boost injections were given 21, 42 and 63 days after the initial immunisation. A terminal bleed was carried out 99 days after the initial immunisation. Immunisation was performed by Invitrogen (USA). Antibodies were affinity purified from the terminal bleed serum using a column of SNX51–35 peptide conjugated to SulfoLink Coupling Gel (Pierce, USA) according to manufacturer’s instructions. Affinity-purified antibodies recognised the expected size of the endogenous SNX5 (~50 kDa) and also a ~76 kDa band which corresponded to the size of GFP-SNX5 (supplementary material Fig. S3B). The affinity-purified antibodies stained punctate structures in untransfected NIH3T3 cells and co-localised with punctate staining pattern of GFP-SNX5 of transfected NIH3T3 demonstrating specificity for SNX5 (supplementary material Fig. S3D).

Cell culture and transfection

L-cells were maintained in RPMI (Invitrogen, USA) supplemented with 10% (v/v) foetal calf serum (JRH, Australia), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. NIH3T3 and 293 cells were maintained as monolayers in DMEM (Gibco Laboratories, USA) supplemented with 10% (v/v) foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. All cells were cultured in a humidified 37°C incubator with 10% CO2. Transient transfections of cells with vector-based constructs were performed using Fugene 6 transfection reagent (Roche, USA) according to the manufacturer’s instructions. Cells were incubated for 48–96 hours, as indicated, to allow expression of construct.

SNX11–108 mice

SNX11–108 mice were purchased from Mutant Mouse Regional Resource Centres (USA) and have been previously described (Schwarz et al., 2002). Litters of weaned pups were genotyped as described (Schwarz et al., 2002) with annealing temperature for the PCR reactions set at 59.3°C.

RNA interference

For knockdown of SNX5 expression in cells, a miRNA system was employed. The following primer pairs were designed using the Invitrogen BLOCK-iT™ RNAi Designer (https://rnanodesigner.invitrogen.com/express), annealed and cloned into pcDNA™ 6.2GW/EmGFp-miRNA expression vector which contains a GFP expression cassette.

miRNA-1: 5’-TGGTGTGTTAGCAGGAATCTCGGATGTTTGCGCAG-3’ and 5’-CTGGTTTACACCGTACGTACGAGCTAGTC-3’, miRNA-2: 5’-TGGTGTGACGTCTAAACTGACCTATGGCAG-3’ and 5’-CTGGTGCAGTCTGAAATG-3’. Each batch of recombinant virus was analysed for the presence of GFP by fluorescence microscopy (3×105 cells/well per replicate). Viral transduction was performed using Fugene 6 according to manufacturer’s instructions. A single viral colony (rADV1.2) (contains 2 tandem copies of miRNA-1) was picked. Virus harvested from rADV1.2 was then digested using BamHI and Xhol restriction enzymes to obtain miRNA-1 insert. The resulting 291 bp fragment was cloned into the BglII/Xhol sites of a pcDNA™ 6.2GW/EmGFp-miRNA-1 from a separate restriction enzyme digest generating pcDNA™ 6.2GW/EmGFp-miRNA-1.2 (contains 4 tandem copies of miRNA-1). The cloning strategy was used to obtain miRNA-2.4.

Isolation of primary peritoneal and bone marrow-derived macrophages

Eight to ten week old BALB/c mice were killed by CO2 asphyxiation in accordance with animal ethics guidelines. For isolation of primary peritoneal macrophages, 9 ml of ice-cold serum-free RPMI (Invitrogen, USA) was injected into the peritoneal cavity. Macrophages were liberated by massage and the peritoneal cells collected back into the syringe. Cells were pelleted at 394 g for 5 min and resuspended in 5 ml of 0.9% (w/v) NH4Cl for 5 min at 37°C to lyse contaminating red blood cells. Cells 0.1% (v/v) BSA/PBS (5 ml) was then added and cells were collected by centrifugation. Cells were resuspended and cultured overnight in RPMI supplemented with 10% (v/v) foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidified 37°C incubator with 10% CO2. Cells were then lysed and washed twice in serum-free RPMI to remove any non-adherent cells.

For isolation of bone marrow-derived macrophages, cells were obtained from the femurs and tibias of mice. Red blood cells were then lysed and cells pelleted by centrifugation. Cells were resuspended in complete bone marrow medium (RPMI supplemented with 15% (v/v) FCS, 20% (v/v) L-cell conditioned medium, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) seeded onto a tissue culture dish and grown in a humidified 37°C incubator with 10% CO2 overnight. The medium containing the progenitor bone marrow stem cells were obtained the next day and differentiated into bone marrow-derived macrophages by culturing in the presence of CSF-1 (from the L-cell conditioned medium) for 5–7 days in a humidified 37°C incubator with 10% CO2.

Recombinant adenovirus production and transduction

Recombinant adenovirus (rADV) shuttle plasmid containing the gene of interest and rADV genomic plasmid, pHBlGloA1.3Cre (Microbix, Canada) containing a modified adenovirus type-5 genome, were co-transfected into 293 cells using Fugene 6 according to manufacturer’s instructions. A single viral colony (rADV LM-GFP-Cre) was isolated and amplified in 293 cells. Virus harvested from the 293 cells was titrated in NIH3T3 cells by scoring for the expression of the fluorescent GFP tag. Each batch of recombinant virus was analysed for the expression of the construct by immunoblotting and immunofluorescence analysis. Aliquots of virus were stored at −80°C.

For transduction of NIH3T3 cells, cells were incubated with rADV in the presence of serum-free DMEM at a multiplicity of infection (MOI) of 10–20 at 37°C, 10% CO2 for 2 hours with shaking every 15 min. The medium containing rADV was then replaced with complete DMEM and cells were grown in a humidified 10% CO2 atmosphere at 37°C as indicated.

For transduction of primary peritoneal macrophages, cells were incubated with rADV at a MOI of 1 in the presence of complete RPMI in a humidified 10% CO2 atmosphere at 37°C overnight.

For transduction of progenitor bone marrow cells, cells were incubated with rADV at a MOI of 0.5–2 in the presence of complete bone marrow medium in a humidified 10% CO2 atmosphere at 37°C for 24 hours. Cells were then pelleted at

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(Invitrogen, USA) and then transferred onto Immobilon-P PVDF membranes (100 mM Tris-HCl, 4% (w/v) SDS, 0.2% (w/v) Bromophenol Blue, 20% (v/v) glycerol, 1% (w/v) SDS, PBS, pH 2) as necessary. Membranes were then washed and blocked, as previously, and incubated with different primary antibodies.

Statistical analysis

The unpaired two-tailed Student t test was used to determine statistical significance. A p-value of <0.05 (*) was considered as significant, p-value of <0.01(**) was highly significant and P<0.001 (***) was very highly significant. The absence of a p value indicates the differences were not significant.

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Competing Interests

The authors have no competing interests to declare.

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