Biochemical and Biophysical Research Communications 440 (2013) 163–167

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Syntaxin-4 is essential for IgE secretion by plasma cells

Arman Rahman [a,1], Joseph DeCourcey [a,1], Nadia Ben Larbi [a], Sinéad T. Loughran [b], Dermot Walls [b], Christine E. Loscher [a,*]

[a] Immunomodulation Group, School of Biotechnology, Dublin City University, Ireland
[b] School of Biotechnology and National Centre for Sensor Research, Dublin City University, Ireland

ARTICLE INFO

Article history:
Received 6 September 2013
Available online 18 September 2013

Keywords:
Syntaxin-4
VAMP3
Plasma cells
IgE secretion
SNARE
Multiple myeloma

ABSTRACT

The humoral immune system provides a crucial first defense against the invasion of microbial pathogens via the secretion of antigen specific immunoglobulins (Ig). The secretion of Ig is carried out by terminally differentiated B-lymphocytes called plasma cells. Despite the key role of plasma cells in the immune response, the mechanisms by which they constitutively traffic large volumes of Ig out of the cell is poorly understood. The involvement of Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in the regulation of protein trafficking from cells has been well documented. Syntaxin-4, a member of the Qa SNARE syntaxin family has been implicated in fusion events at the plasma membrane in a number of cells in the immune system. In this work we show that knock-down of syntaxin-4 in the multiple myeloma U266 human plasma cell line results in a loss of IgE secretion and accumulation of IgE within the cells. Furthermore, we show that IgE co-localises with syntaxin-4 in U266 plasma cells suggesting direct involvement in secretion at the plasma membrane. This study demonstrates that syntaxin-4 plays a critical role in the secretion of IgE from plasma cells and sheds some light on the mechanisms by which these cells constitutively traffic vesicles to the surface for secretion. An understanding of this machinery may be beneficial in identifying potential therapeutic targets in multiple myeloma and autoimmune disease where over-production of Ig leads to severe pathology in patients.

1. Introduction

Plasma cells are terminally differentiated, non-dividing final effectors of the humoral immune response. As the sole professional antibody producing cells in the body, they act as cellular factories rapidly secreting vast amounts of immunoglobulin (Ig) [1]. Plasma cells can be long lived, surviving for a number of years, and their potential to produce large amounts of Ig necessary for the clearance of pathogens means that they are tightly regulated in the immune response [2]. IgE, a variant of Ig produced in terminally differentiated plasma cells, is not stored in the cell or packaged in granules but is transported from the golgi via constitutive pathways directly to the plasma membrane where it is secreted [3]. The transportation of protein loaded vesicles from the golgi to the plasma membrane and its subsequent fusion with the plasma membrane is regulated by a conserved family of membrane associated trafficking proteins called SNAREs (*N*-ethylmaleimide-sensitive factor attachment protein receptor) [4].

SNARE proteins share a highly conserved alpha helical SNARE motif and mediate the docking and fusion of vesicular carriers critical for secretory immune functions [5]. Depending on the central functional residue amino acid in the conserved SNARE motif, the family is further divided into R-SNAREs (arginine) or Q-SNAREs (glutamine) with Q-SNAREs being further classified into Qa, Qb and Qc [6]. One SNARE motif from each group is required to form a fully functional four-helical SNARE complex with at least one SNARE present on the opposing membrane. The SNAREs are all anchored at the C-terminus allowing the SNARE helices to "zipper together" forming a stable complex that provides enough free energy to overcome opposing membrane forces and allow the membranes to fuse, resulting in the contents of the vesicle being secreted from the cell [7].

Syntaxin 4 in particular is a Qa SNARE that has been shown to play an important role in regulated secretion on the plasma membrane of neutrophils, macrophage and eosinophils (reviewed in [5]). Despite a number of studies describing the secretion pathway of IgE from plasma cells, the specific trafficking machinery involved in this process has not been fully eluci-

Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associate membrane protein; TNF, tumour necrosis factor; Ig, immunoglobulin; FBS, fetal bovine serum; PBS, phosphate buffered saline; siRNA, small interfering RNA.

* Corresponding author. Address: Immunomodulation Group, School of Biotechnology, Dublin City University, Dublin 9, Ireland.
E-mail address: christine.loscher@dcu.ie (C.E. Loscher).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.09.058

dated [8]. In this study we propose a role for syntaxin-4 in the transport of IgE from plasma cells. We demonstrate that siRNA induced knock-down of syntaxin-4 reduces IgE secretion and results in the accumulation of IgE within the cell due to interference with vital SNARE mediated transport machinery. Secretion of large volumes of antibodies from terminally differentiated plasma cells targeted against auto-antigens and immunogens can lead to severe autoimmune disease in patients. Furthermore, development of multiple myeloma can result in toxic levels of Ig accumulating in the blood stream [1,9]. The identification of SNARE trafficking proteins regulating secretion in plasma cells will lead to a better understanding of the trafficking pathway involved in the secretion of immunoglobulin from plasma cells and potential therapeutic targets in autoimmune disease and multiple myeloma.

2. Materials and methods

2.1. Cell culture

The human multiple myeloma cell line U266B1 (IgEλ) was cultured in RPMI-1640 medium supplemented with 15% FBS (Gibco, Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell culture was maintained at 37 °C in humidified air with 5% CO_2.

2.2. Antibodies

Polyclonal anti-VAMP3 was purchased from Synaptic System, anti-IgE was purchased from Bethyl laboratories and goat polyclonal-anti-syntaxin-4 was purchased from Santa Cruz. Species specific secondary antibodies, Alexa Fluor 546® and Alexa Fluor 488® were purchased from Molecular Probes, Invitrogen. HRP labelled anti rabbit IgG secondary anti body was purchased from Sigma Aldrich. A FITC conjugated polyclonal goat anti-human IgE antibody was used for intracellular IgE staining and purchased from Alpha Diagnostic Intl.

2.3. Small interfering RNA (siRNA) treatment

Knock-down of syntaxin-4 and VAMP3 was performed using two specific siRNA duplex sets (Silencer® Select Pre-designed siRNA) from Applied Biosystems targeted to syntaxin-4 (s13595, s13596), and VAMP3 (s17856, s17857). Silencer® Select Negative Control #1 siRNA and Cy3 labelled GAPDH positive control siRNA (Applied biosystem) served as negative and positive control. TransIT-siQUEST® transfection reagent was used according to the manufacturer's instructions. For knock-down of the SNARE genes, cells were transfected with a final concentration of 50 nM of each siRNA duplex set. The cells were incubated for 48 h in culture medium before centrifuging the cells and placing in fresh media for 24 h. Supernatants were collected to assess IgE concentration and the cells were collected for real time RT-PCR and Western blot analysis to confirm the knock-down of SNARE proteins.

2.4. ELISA for IgE and IL-6

Culture supernatants from the transfection experiment were analysed in duplicate with the appropriate controls and standards. IgE and IL-6 ELISA were used according to manufacturer's instruction (Mabtech) and analysed on the Versa Max ELISA plate reader with accompanying software.

2.5. Flow cytometry

For the detection of intracellular IgE, post-transfected cells were stained with anti-human IgE-FITC (Alpha Diagnostics) using the Saint PhD protein delivery system (Synvolux Theraputic) according to the manufacturer's instruction. Briefly, the siRNA transfected cells were collected, washed in PBS and transferred into a 24 well plate. IgE-FITC was complexed with 20 μl protein transfection solution and the final volume was adjusted to 250 μl of culture medium before adding to the cells. After 24 h incubation, cells were washed in PBS supplemented with Tween20 prior to analysis by flow cytometry. In order to exclude the possibility that unspecific staining could occur during intracellular staining due to the transfection method, control cells were intracellularly stained with anti mouse CD4-FITC antibody (ebioscience) which showed no non-specific staining (data not shown). Non transfected cells were used as a positive control. Ten thousand events were acquired for each sample on a FACS Aria I (Becton Dickinson). Data analysis was performed using FlowJo software (Treestar).

2.6. SDS–PAGE immunoblotting

U266 cells (post transfection and control) were washed three times in PBS and lysed in buffer A (10 nM Tris, pH 7.4, containing 1 nM EDTA, 0.5% Triton X-100 and Complete protease inhibitors (Roche Applied Science)), by passing through a series of successively smaller needles and centrifuged for 10 min at 17,000*g*. Supernatants were assayed for protein content (Pierce® BCA Protein Assay Kit). Protein (10 μg) was subjected to 12% SDS–PAGE gel separation and transferred to PVDF membrane using an iblot system (Invitrogen). Blocking and probing were performed using the SNAP-ID system (Millipore, Bedford, MA) followed by visualization using BM chemiluminescence substrate (Roche Diagnostic Systems).

2.7. PCR

RNA was extracted from cells using the Nucleospin® RNA isolation kit (Machery Nage) and cDNA was prepared using High capacity cDNA reverse transcription kit (Applied Biosystem, CA). Real time quantitative PCR (qPCR) was carried out using TaqMan® Gene Expression assay kits (Applied Biosystems) for syntaxin-4 and VAMP-3. SYBR Green master mix (Roche diagnostics) was used on a 7500 Real time PCR system (Applied Biosystems). GAPDH was used as internal control, and samples were compared using ΔCT expression relative to control cells.

2.8. Confocal microscopy

Cytospin preparations were prepared by centrifugation of 100 μl of human B cell suspension (1×10^6 cells/ml) on polysine-coated slides at 1000 rpm for 3 min. The slides were air dried and fixed in cold acetone at −20 °C for 15 min. Slides were washed and background staining was blocked for 1 h at room temperature with 100 mM PBS–Glycine, 1.2% cold fish gelatin. The slides were then incubated with antibodies diluted in the blocking buffer. The next day, slides were washed and incubated with Alexa Fluor® species specific secondary antibodies (Molecular Probes, Invitrogen). Slides were washed and mounted with anti-fade medium (Dako). Slide preparations were observed using a Zeiss Axio Observer Z1 equipped with a Zeiss 710 and ConfoCor 3 laser scanning confocal head (Carl Zeiss, Germany). Co-localisation signals were analysed using ZEN 2008 software.

3. Results

3.1. Knock-down of syntaxin-4 reduces IgE secretion from U266 plasma cells

Syntaxin-4 has previously been associated with regulation of protein secretion from immune cells at the plasma membrane [5]. In order to investigate the role of syntaxin-4 in the secretion of IgE from terminally differentiated U266 plasma cells, cells were transfected with siRNA targeted to syntaxin-4 and VAMP3. Flow cytometry analysis of Cy3 labeled GAPDH siRNA indicated $\geqslant$65% transfection efficiency of siRNA with cell viability $\geqslant$80% 48 h post-transfection (data not shown). Relative quantification of syntaxin-4 and VAMP3 mRNA after siRNA induced knock-down of these genes showed a significant reduction using two siRNA targets for each SNARE (Fig. 1A). Following transfection, syntaxin-4 and VAMP3 knock-down cells were cultured in fresh media for 24 h. Western blot analysis of syntaxin-4 and VAMP3 siRNA induced knock-down cell lysates confirmed significant reduction in the protein level of these SNAREs when compared to the controls cells (Fig. 1B). A significant reduction in the concentration of IgE was observed in the syntaxin-4 knock-down cell supernatants compared to the controls ($p < 0.001$) (Fig. 2A). No decrease was observed in the VAMP3 knock-down cells indicating that syntaxin-4 and not VAMP3 is directly involved in regulating the secretion of IgE from human plasma cells. In order to confirm no off target effects on syntaxin-4 following VAMP3 siRNA transfection, syntaxin-4 and VAMP3 knockdown cells were lysed and probed for syntaxin-4 protein expression. Immunoblotting with an anti syntaxin-4 antibody revealed almost no syntaxin-4 in the knock-down protein isolates as expected. VAMP3 knock-down and control cell lysates resulted in strong bands when probed for syntaxin-4 indicating that VAMP3 siRNA interference had no effect on syntaxin-4 expression in these cells (Fig. 2B). The significant reduction observed in IgE secretion appears to be due to an absence of syntaxin-4, suggesting a critical role for syntaxin-4 in the exocytosis of IgE from U266 plasma cells.

3.2. Knock-down of syntaxin-4 is specific to IgE secretion and does not affect IL-6

SNARE proteins are commonly involved in more then one pathway in a cell and proteins may also share vesicles for transport to

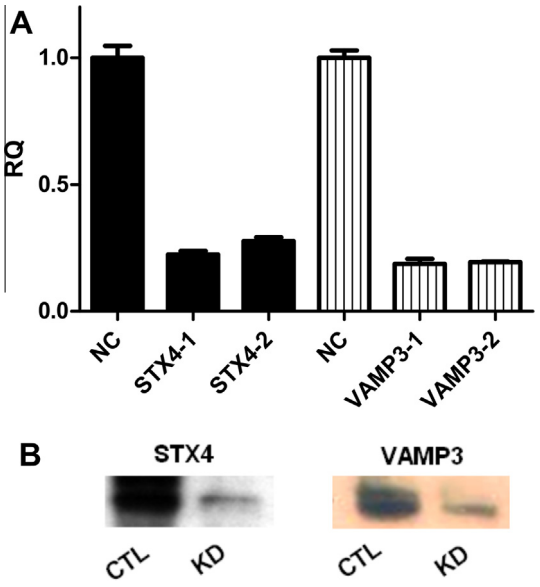

Fig. 1. Knock-down of SNAREs at mRNA and protein level in U266 multiple myeloma plasma cells. (A) qRT-PCR shows relative quantification of syntaxin 4 and VAMP-3 mRNA after siRNA induced knock-down in plasma cells. Significant reduction in mRNA is seen for all three SNARE genes in U266 cells. (B) Western blot analysis of protein extracted from siRNA induced knock-down cells confirms significant reduction of expression of syntaxin-4 and VAMP3 proteins in plasma cells.

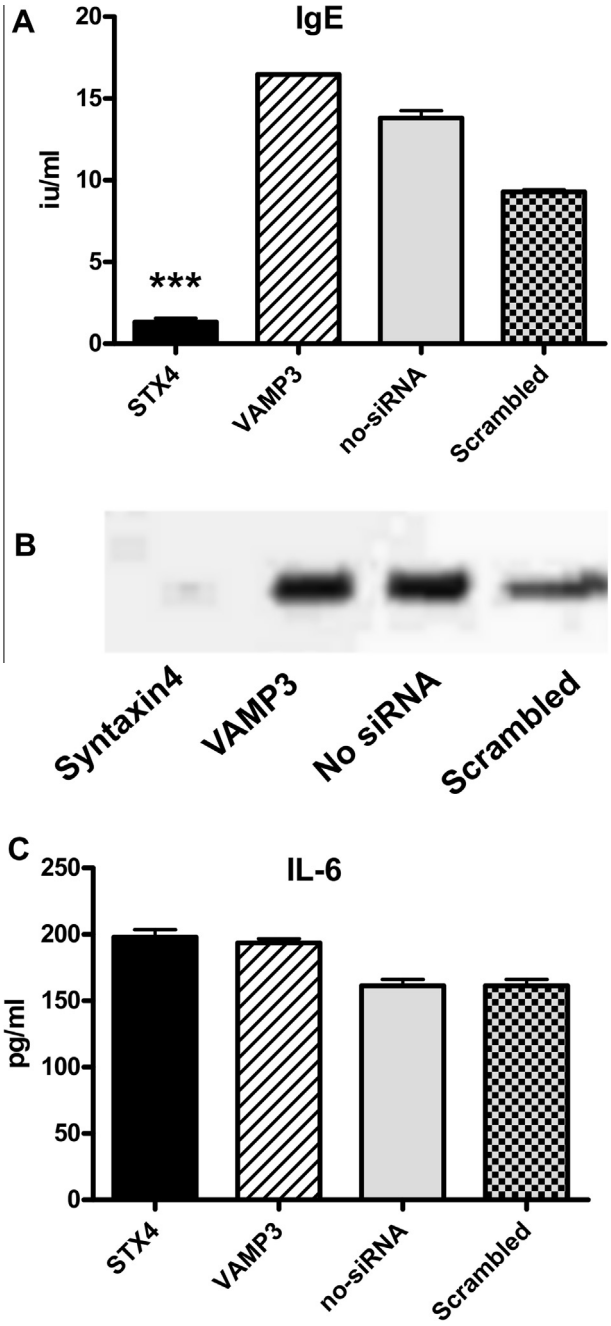

Fig. 2. Knock-down of syntaxin-4 inhibited IgE but not IL-6 secretion from U266 plasma cells (A) IgE concentrations in supernatants collected from the SNARE siRNA transfected cells shows marked reduction in IgE secretion in syntaxin-4 knockdown cells in comparison to VAMP3 and controls cells. (B) Syntaxin-4 and VAMP3 protein extract from knock-down cells show no off target effect of VAMP3 knock-down on syntaxin-4 protein expression. (C) IL-6 concentration was assessed in supernatants from knock-down cells. No changes were observed in IL-6 in the supernatants from syntaxin-4 and VAMP3 siRNA transfected cell supernatants compared to controls. Results are ±SD of three experiments. One-way ANOVA was used to determine if differences between groups were statistically significant ($^{***}p < 0.001$).

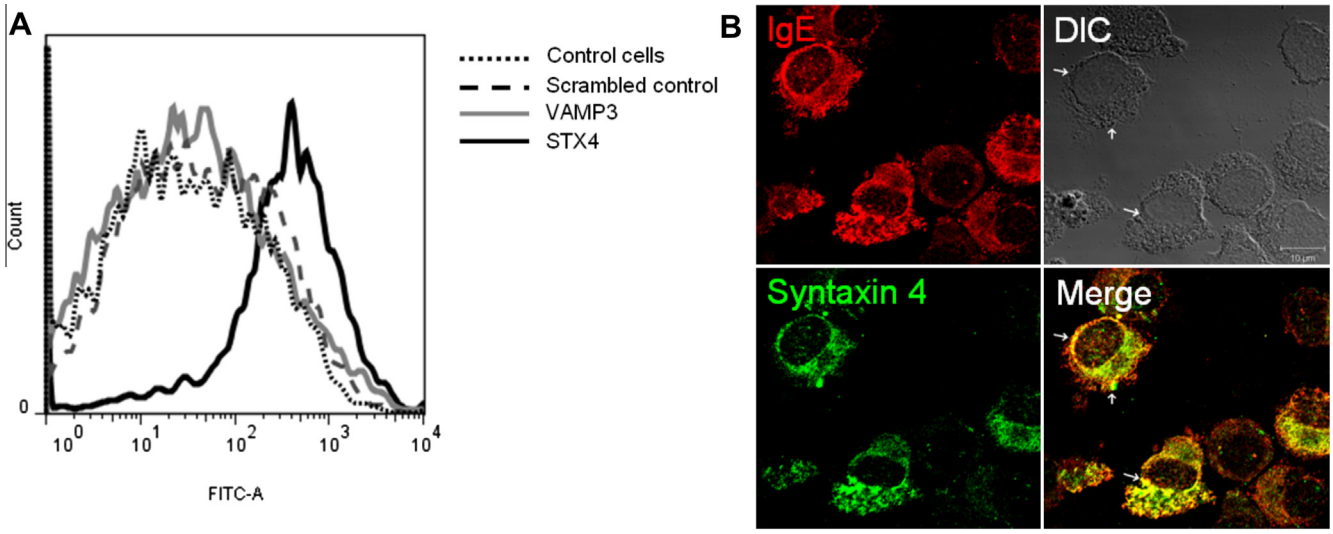

Fig. 3. Knock-down of syntaxin-4 resulted in accumulation of IgE due to its direct involvement in exocytosis as demonstrated by co-localisation of IgE and syntaxin-4 in U266 plasma cells. (A) Intracellular IgE staining following siRNA transfection and measured by flow cytometry. Syntaxin-4 knock-down resulted in increased IgE accumulation inside the cells as demonstrated by in increase in fluorescence intensity compared to VAMP3 and control cells. VAMP3 knock-down showed similar accumulation of IgE as scrambled siRNA and no siRNA controls. (B) Confocal microscopy was used to show co-localisation of syntaxin-4 with IgE in U266 plasma cells suggesting direct involvement in the delivery of IgE from the cell.

the plasma membrane [10]. In order to show that syntaxin-4 regulation was specific to IgE secretion we measured the concentration of IL-6 in the supernatants of syntaxin-4 knock-down cells. Levels of IL-6 were unaffected in syntaxin-4 and VAMP3 knock-down U266 plasma cells compared to control cells (Fig. 2C). These data indicate that syntaxin-4 is specific to IgE secretion and is not involved in the secretion of IL-6 from plasma cells.

3.3. IgE accumulates inside plasma cells as a result of syntaxin-4 knock-down

We have shown that the knock-down of syntaxin-4 resulted in low concentrations of IgE being detected in the supernatants of plasma cells after 24 h. Syntaxin-4 has been localised at the surface and implicated in the secretion of protein from different immune cells such as macrophage [11] and adipocytes [12]. Furthermore, the secretion of IgE is constitutive and is transported from the golgi directly to the plasma membrane for delivery out of the cell [3]. Therefore it is reasonable to assume that if syntaxin-4 is essential in the exocytosis of IgE at the plasma membrane, there should be an accumulation of IgE in plasma cells that are deficient in syntaxin-4. In order to investigate the fate of IgE once syntaxin-4 is diminished, intracellular IgE was stained in knock-down cells and analysed using flow cytometry. As shown in Fig. 3A, higher levels of IgE were measured in syntaxin-4 knock-down cells. In contrast, VAMP3 knock-down cells showed minimum accumulation of IgE inside the cells implying syntaxin-4, not VAMP3, is essential for the secretion of IgE from plasma cells (Fig. 3A). Non-specific binding was not observed when using an off-target antibody (data not shown). Minimal IgE staining was observed in the scrambled siRNA and in the cells transfected with transfection solution only (no siRNA control) (Fig. 3A).

3.4. Syntaxin-4 co-localises with IgE in plasma cells

The intracellular accumulation of IgE in plasma cells following the knock-down of syntaxin-4 suggests that this SNARE protein is directly involved in the secretion of IgE from plasma cells. Therefore the sub cellular localization of syntaxin-4 and IgE in a U266 plasma cell line was examined using indirect immunofluorescence detection with affinity purified polyclonal antibodies (Fig. 3B). Double labelling with antibody directed against syntaxin-4 (green) and IgE (red) in fixed U266 plasma cells was analysed by confocal microscopy. Sytnatxin-4 and IgE showed good co-localisation indicating that syntaxin-4 is directly involved with the secretion of IgE from plasma cells (Fig. 3B, indicated by white arrows).

4. Discussion

We report for the first time that syntaxin-4 plays a vital role in exocytosis of IgE from plasma cells. Knock-down of syntaxin-4, but not VAMP3 dramatically reduced IgE secretion from U266 plasma cells causing it to accumulate in the cell.

Stow et al. recently reported that siRNA induced knock-down of syntaxin-4 reduced TNF-α secretion significantly in macrophage. Syntaxin-4 has also been described as a regulator of TNF-α trafficking and over expression of syntaxin-4 led to an increase in TNF-α delivery, while knock-down of syntaxin-4 inhibited TNF-α secretion from these cells [11]. Further work from this group also demonstrated that knocking down syntaxin-4 ablated the surface TNF-α but did not affect golgi TNF-α in macrophage cell [13]. Our data demonstrates that syntaxin-4 co-localises with IgE in plasma cells and syntaxin-4 knock-down, but not VAMP3, resulted in a dramatic loss of IgE secretion. Furthermore, synthesised IgE could not be exocytosed from the cell and consequently accumulated in syntaxin-4 knock-down cells indicating a similar role for syntaxin-4 in regulating IgE to that reported for TNF-α regulation in macrophage.

It has been previously reported that SNAP23 is essential for IgE secretion in U266 myeloma cells and it is possible that sytnaxin-4 forms a complex with SNAP23 at the cell surface to facilitate the secretion of IgE [8]. Indeed SNAP-23 and syntaxin-4 have been previously implicated in secretion of protein from macrophage [11] and neutrophils [14].

IL-6 is reported to function as a growth factor for multiple myeloma plasma cells and to maintain long-term plasma cell survival [15,16]. IL-6 is secreted by multiple myeloma U226 plasma cells and the secretion of IL-6 form these cells acts in an autocrine fash-

ion maintaining the cells in culture [15]. In order to investigate the specificity of syntaxin-4 regulation in IgE secretion from U266 plasma cells, IL-6 production was assessed in syntaxin-4 knockdown cells. Levels of IL-6 were unaffected by knocking down VAMP3 or syntaxin-4 in these cells suggesting that syntaxin-4 is critical in IgE secretion and independent of IL-6 secretion in these cells. In activated macrophage cells, IL-6 and TNF-α have been shown to use different secretory mechanisms [17]. Although the TNF-α secretion pathway described in macrophage is through recycling endosomes to the cell surface and therefore intrinsically linked to phagocytosis, differential pathways of cytokine secretion using different SNARE proteins has also been described in T-cells, another non-phagocytic immune cell [10]. It is entirely possible that this is also the case in U266 multiple myeloma plasma cells, in which IgE and IL-6 use two distinct secretory pathways and different sets of SNARE proteins to facilitate delivery of protein from the cell.

The conventional belief that the sole function of plasma cells is to secrete antibody has been severely challenged in the recent years. Previous studies have shown that both intestinal plasma cells and the multiple myeloma cell lines, including U266, produce antimicrobial peptides [18]. Fritz et al. has also recently demonstrated that intestinal IgA^+ plasma cells produce TNF-α and inducible nitric oxide synthase (iNOS) that are essential in maintaining gut homeostasis [19]. The intra-cellular trafficking of these molecules in plasma cells is poorly understood and multiple pathways may exist that are regulated by different SNARE complexes.

Here we have identified a SNARE that is involved in constitutive release of IgE from a U266 multiple myeloma plasma cell line. There is an opportunity now to explore the specific SNAREs and their complex partners involved in the secretion of immunoglobulin and potential immunomodulating molecules from plasma cells. This would lead to a better understanding of the intracellular trafficking of proteins and the development of potential therapeutics targeting the essential trafficking mechanisms in these cells.

Acknowledgments

The authors acknowledge funding provide under the Programme for Research in Third Level Institutions (PRTLI) Cycle 4. The PRTLI is co-funded through the European Regional Development Fund (ERDF), part of the European Union Structural Funds Programme 2007–2013.

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