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Mast Cells Produce a Unique Chondroitin Sulfate Epitope

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Keywords:	glycosaminoglycan, chondroitin sulfate, Proteoglycans, mast cells, endoglycosidase

Abstract:	The granules of mast cells contain a myriad of mediators that are stored and protected by the sulfated glycosaminoglycan (GAG) chains that decorate proteoglycans. While heparin is the GAG predominantly associated with mast cells, mast cell proteoglycans are also decorated with heparan sulfate and chondroitin sulfate (CS). This study investigated a unique CS structure produced by mast cells that was detected with the antibody clone 2B6 in the absence of chondroitinase ABC digestion. Mast cells in rodent tissue sections were characterized using toluidine blue, Leder stain and the presence of mast cell tryptase. The novel CS epitope was identified in rodent tissue sections and localized to cells that were morphologically similar to cells chemically identified as mast cells. The rodent mast cell-like line RBL-2H3 was also shown to express the novel CS epitope. This epitope co-localized with multiple CS proteoglycans in both rodent tissue and RBL-2H3 cultured cells. These findings suggest that the novel CS epitope that decorates mast cell proteoglycans may play a role in the way these chains are structured in mast cells.

Mast Cells Produce a Unique Chondroitin Sulfate Epitope

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Short Title: MAST CELLS PRODUCE A UNIQUE CS EPITOPE

Summary

The granules of mast cells contain a myriad of mediators that are stored and protected by the sulfated glycosaminoglycan (GAG) chains that decorate proteoglycans. While heparin is the GAG predominantly associated with mast cells, mast cell proteoglycans are also decorated with heparan sulfate and chondroitin sulfate (CS). This study investigated a unique CS structure produced by mast cells that was detected with the antibody clone 2B6 in the absence of chondroitinase ABC digestion. Mast cells in rodent tissue sections were characterized using toluidine blue, Leder stain and the presence of mast cell tryptase. The novel CS epitope was identified in rodent tissue sections and localized to cells that were morphologically similar to cells chemically identified as mast cells. The rodent mast cell-like line RBL-2H3 was also shown to express the novel CS epitope. This epitope co-localized with multiple CS proteoglycans in both rodent tissue and RBL-2H3 cultured cells. These findings suggest that the novel CS epitope that decorates mast cell proteoglycans may play a role in the way these chains are structured in mast cells.

Keywords: glycosaminoglycan, chondroitin sulfate, proteoglycans, mast cells, endoglycosidase

1. Introduction

Mast cells are a heterogeneous population derived from hematopoietic progenitor cells. They are widely known for their production and release of histamine in an allergic response, as well as their role in inflammation, and are predominantly found in tissues that interface the host and the external environment, such as the lungs, gastrointestinal tract and skin (Metcalfe et al., 1997). They are often characterized based on their location, as well as the proteases and cytokines they produce and store in their α-granules.

Serglycin is the most abundant proteoglycan produced by mast cells, which has shown to have a significant role in the packaging of proteases in the α-granules (Åbrink et al., 2004). A sub-set of mast cells, based on species and tissue of origin (Rönnberg et al., 2012), produce heparin that decorates the proteoglycan (PG) serglycin (Kolset and Gallagher, 1990, Metcalfe et al., 1997), while mast cell derived serglycin may also be decorated with both oversulfated chondroitin sulfate (CS) and heparin (Stevens et al., 1988, Kjellén et al., 1989). In contrast, mucosal mast cells produce serglycin decorated exclusively with CS, including CS-A, CS-E and CS-B (Stevens et al., 1986), rather than heparin (Enerbäck et al., 1985). In addition to heparin and CS, heparan sulfate (HS) also decorates PGs that are produced by mast cells (Yurt et al., 1977, Stevens et al., 1986, Seldin et al., 1985). It was previously demonstrated by Seldin *et al.* that glycosaminoglycan (GAG) chains on PGs produced by the rodent mast cell-like RBL cells were 64% CS, with the remaining 36% being either heparin or HS (Seldin et al., 1985). Mast cells also produce the CS PGs versican (Rönnberg et al., 2012) and bikunin (Ide et al., 1999), though the structure of the CS chains attached to these PGs has not been examined. RBL-2H3 cells in culture produce keratan sulfate, CS, HS and heparin from cells in both resting and activated states (Jung et al., 2013). In addition to mast cells, macrophages are also associated with sites of inflammation. Monocytes, precursors to macrophages, contain CS-A on isolation, though with *in vitro* culture transition to CS-E (Kolset et al.,

1983). Macrophages derived from monocytes have also been shown to increase their amount of CS upon lipopolysaccharide stimulation (Uhlin-Hansen et al., 1989).

The discovery of new CS structures has been enabled by a number of antibodies with epitopes against the varying CS structures. The antibody clone CS-56, detects CS-A and CS-D (Avnur and Geiger, 1984, Ito et al., 2005). The antibody clone LY-111 was developed against CS-A (Yada et al., 1992) and recognizes both CS-A and CS-D. The antibody clone MO-225 recognizes CS-D as well as CS-E (Yamagata et al., 1987). Additionally, a number of different antibodies have been developed against native CS/DS epitopes (Sorrell et al., 1990, Sorrell et al., 1993), as well as a suite of antibodies raised against chondroitinase (C'ase) ABC generated neo-epitopes (Couchman et al., 1984, Caterson et al., 1985) that have become known as anti-CS stub antibodies. The anti-CS stub antibody clones, 1B5, 2B6 and 3B3, detect the terminal unsaturated disaccharide structures following the linkage region that contain an unsulfated, 4-sulfated or 6-sulfated GalNAc, respectively. These stub neo-epitopes are generated by exhaustive digestion with the bacterial endoglycosidase chondroitinase ABC. It has also been show that the clone 3B3 detects the non-reducing terminal saturated CS disaccharide consisting of glucuronic acid (GlcA) that is adjacent to the 6-sulfated *N*-acetylgalactosamine (GalNAc) in the absence of C'ase ABC digestion (Caterson et al., 1990).

The objective of this study was to evaluate the presence of CS epitopes in tissues around material implants. In doing this, we have identified a novel CS epitope detected by the antibody clone 2B6 in the absence of endoglycosidase digestion. Furthermore, we have confirmed the presence of this novel CS epitope in cultured RBL-2H3 cells, and investigated the CSPGs that this novel CS epitope structure may be associated with.

2. Materials and Methods

2.1. Tissue sections from rat implantation model

Details of the rodent implantation model used to investigate the host response to implanted materials has previously been described (Farrugia et al., 2014). Briefly, material films (1 × 0.5 cm) were subcutaneously implanted into the lumbar region of female Spraque-Dawley rats. Animals were sacrificed at 7 days after implantation and the implant was excised and processed for histological evaluation. The *in vivo* implantation study was performed in an accredited facility by the Association for Assessment and Accreditation of Laboratory Animal Care under Institutional Animal Care and Use Committee approval. Histological results were evaluated for each test condition from multiple animals, and observations were similar between replicates. The results presented are from one material implant that are representative of the study and enable the evaluation of co-localization of markers of interest.

2.2. Histological Analysis

Grafts were fixed in 10% neutral buffered formalin, paraffin embedded and sectioned (4-5 μm) as previously described (Farrugia et al., 2014). Sections were washed twice, 5 min each, with xylene to remove paraffin and the slides were immersed in a series of ethanol solutions for 3 min each (twice in 100% (v/v), once in 95% (v/v), once in 70% (v/v)) followed by several exchanges of water. Histological characterization was undertaken through the use of Leder stain, toluidine blue, and immunolocalization of PGs, glycosaminoglycans and markers of interest. Following rehydration, slides were stained with Leder stain which detects the presence of chloroacetate esterase (Naphthol AS-D chloroasetate (NADC), Sigma 91C kit)

and was carried out as per the manufacturer's protocol. Briefly, following rehydration, sections were fixed in the citrate-acetone-formaldehyde solution (30 s), washed in deionized water, and immediately placed into the NADC solution and incubated for 15 min at 37 °C. Slides were then rinsed in pre-warmed deionized water for 2 min followed by counterstaining with hematoxylin (Gill #3) for 2 min further and then rinsed with deionized water. Following rehydration, sections were stained with toluidine blue solution (0.1% w/v, pH 2.0) for 2 min, washed in RO water 3 times, dipped into 95% EtOH, followed by 100% EtOH twice, cleared in xylene and mounted. Antigen epitope retrieval was undertaken by immersing the slides, following rehydration, in 0.01 M sodium citrate (pH 6), followed by heat treatment in a decloaking chamber (Applied Medical CA, USA) at 120 °C for 4 min. The slides were then rinsed with deionized water followed by blocking of endogenous peroxidase with 3% (v/v) H_2O_2 for 5 min. Some slides were also treated with chondroitinase ABC (C'ase ABC) (0.05 U/mL**,** Seikagaku Corp. Tokyo, Japan) in 0.1M Tris-Acetate buffer (pH 8) for 3 h at 37 °C. The slides were washed with 50 mM Tris-HCl, 0.15 M NaCl, 0.05% (w/v) Tween-20, pH 7.6 (TBST) and then blocked with 1% (w/v) bovine serum albumin (BSA) in TBST for 1 h at room temperature (RT). The slides were incubated with primary antibodies diluted in 1% w/v BSA in TBST at 4 °C for 16 h. Primary antibodies and the concentrations used are detailed in Table 1. Slides were also probed with mouse IgG and IgM whole antibodies (5µg/mL, Invitrogen) as isotype controls, as well as blocking solution in place of primary antibodies prior to incubation with biotinylated secondary antibodies. Slides were then washed twice with TBST before incubating with the appropriate biotinylated secondary antibodies (1:500, GE Healthcare, Sydney, Australia) for 1 h at RT. Slides were washed twice with TBST then incubated for 30 min with streptavidin-HRP (1:250, GE Healthcare, Sydney, Australia)), rinsed four times with TBST before color development with NovaRED™ chromogen stain (Vector Laboratories, Burlingame CA, USA). The slides were then counterstained with

hemotoxylin (Gill's #3, Vector Laboratories, Burlingame CA, USA) for 6 sec and rinsed with deionized water. Slides were dehydrated through graded ethanol series and mounted.

2.3. Culture and activation of mast cell line RBL-2H3

Rat basophilic leukemia cells (RBL-2H3) were maintained in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL of streptomycin and maintained in a humidified incubator (5% CO_2 / 95% air atmosphere at 37 °C). Media was replenished every 3 – 4 days. Siriganian (SG) activation buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM Dextrose, 0.4 mM $MgCl_2$, pH 7.25) was prepared and filter sterilized and 0.1% (v/w) BSA and 1 mM $CaCl_2$ were added before use. Mast cells were chemically activated by adding a protein kinase C activator, 50 nM phorbol 12-myristate 13-acetate (PMA), and a calcium ionophore, 500 nM A23187, 0.1% (v/w) BSA, in SG buffer and equilibrated to 37 °C. Prior to activation, cells were washed twice with SG buffer (containing BSA and $CaCl_2$). Cells were activated for a period of 2 h.

2.4. Flow cytometry

RBL-2H3 cells suspended in sterile SG buffer at a concentration of 3×10^6 cells / mL were analyzed in either their resting or chemically activated state (PMA/A23187). Cells were incubated in each condition for 2 h at 37 °C, and then washed in DPBS and centrifuged at 1200 rpm for 5 min. Cells were then fixed and half were permeabilized using 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 2mM HEPES, 0.5% (w/v) Triton X-100 for 5 min on ice and blocked using 1% BSA/PBS for 20 min at RT. 500 µl of cell suspension (5×10^5 cells) was incubated with primary antibodies for 16 h at 4 °C. Primary antibodies included the 4-sulfated CS stub antibody described above (clone 2B6) as well as the mouse monoclonal anti-CS type A and C antibody (1:500, clone CS-56, Sigma Aldrich) antibody

that detects the disaccharide A-D sequence [GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)], and isotype controls, whole mouse IgG and IgM (1:500, Invitrogen). Cells were then incubated with AlexFluor-488-conjugated secondary antibodies (1:500, goat anti-mouse IgG/IgM, Invitrogen) for 30 min at room temperature and analyzed using a flow cytometer (BD Biosciences) with 10^4 events collected per sample. Data were analyzed using the commercial software, FCS 4 Express.

2.5. Immunocytochemistry

RBL-2H3 cells, at a density of 1×10^4 cells / well, were seeded into glass chamber well slides (Thermo Scientific, Rochester, NY, USA) for 2 h in either SG buffer or SG buffer with activators (PMA/A23187) in a humidified incubator (5% CO_2 / 95% air atmosphere at 37 °C). Cells were washed with PBS and fixed using 4% paraformaldehyde for 15 min at 37 °C and permeabilized as described above. After washing with 50 mM Tris-HCl, 0.15 M NaCl, pH 7.6 (TBS), non-specific binding was blocked with 5% goat serum in TBS containing 0.1% (w/v) Tween-20 (TBST) T for 1 h at RT followed by incubation with the primary antibodies at RT for 2 h. Primary antibodies used included mouse monoclonal anti-4 sulfated and anti-6 sulfated CS stub antibodies (1:500, clones 2B6 and 3B3, respectively), with cells being probed with and without C'ase ABC digestion (0.05 U/mL, 0.1 M Tris Acetate, pH 8). Slides were rinsed twice with TBST and incubated with Alexa Fluor 488 anti-mouse antibodies (1:500, Life Technologies, Sydney, Australia) for 1 h at RT before rinsing twice with TBST. Nuclei were stained with DAPI (2.5 µg/mL) for 20 min in the dark before two final washes with TBS. Slides were mounted with Prolong gold (Invitrogen) and then visualized using a confocal microscope (FluoView FV1200).

Co-localization experiments were carried out as described above with the following modifications. Following blocking of non-specific binding with 5% goat serum in TBST slides were probed with antibody clone 2B6 (1:500) and antibodies against CSPGs serglycin

(1:500, Santa Cruz, SC-292311) or perlecan (1:1000, CCN-1, raised in house against immunopurified human coronary artery endothelial cell derived perlecan (Lord et al., 2014, Whitelock et al., 1999)) at RT for 2 h. Slides were rinsed twice with TBST and incubated with Alexa Flour 488 anti-mouse IgG and Alexa Flour 594 anti-rabbit antibodies (1:500, Life Technologies, Sydney, Australia) for 1 h at RT before rinsing twice with TBST. Nuclei were stained with DAPI (2.5 μg/mL). Slides were then mounted and visualized within a confocal microscope. Co-localization was quantified using the Manders co-localization coefficient that provides a measure of co-occurrence of two channels independent of signal proportionality and is reported as the fraction of pixels with positive values in both channels.

3. Results

Sections probed with antibodies that detect the epitopes 2B6 and 3B3 showed positive staining following bacterial endoglycosidase digestion with chondroitinase (C'ase) ABC (Fig. 1). Positive staining of 2B6 after C'ase ABC digestion, denoted as 2B6+ herein, was predominantly localized within the fibrous capsule surrounding the material implant (Fig. 1 A (i), indicated by the white arrow), which formed in response to the implant. 2B6+ staining was also found at lower intensity in the tissue adjacent to the fibrous capsule (Fig. 1 A (i), indicated by the grey arrow). Observation of these sections at higher magnifications revealed a population of intensely stained cells compared to the surrounding tissue (Fig. 1 A (ii) and (iii), indicated by the black arrow). These cells had a distinct morphology and were approximately 10-20 μm in diameter. Surprising, these cells were also detected with the antibody clone 2B6 without prior endoglycosidase digestion, denoted as 2B6- herein, (Fig. 1 B (ii) and (iii)) suggesting that the epitope detected by the 2B6 antibody may be a native CS epitope in addition to the 4-sulfated CS neo-epitope stub generated by C'ase ABC digestion. The fibrous capsule and surrounding tissue did not react with 2B6- (Fig. 1 B (i)), with the exception of the 2B6- cells.

Cells in proximity to blood vessels were stained positive for the antibody clone 3B3 with (3B3+, Fig. 1 C (iii)) and without (3B3-, Fig. 1 D (iii)) C'ase ABC digestion. These cells exhibited a similar morphology to the 2B6- cells (Fig. 1 A (iii) and 1 B (iii)). Antibody clone 3B3 was not reactive with the fibrous capsule or the surrounding tissue either with (Fig. 1 C) and without (Fig. 1 D) treatment with C'ase ABC. These results suggest in the absence of endoglycosidase digestion the presence of either the 6-sulfated CS neo-epitope stub generated by C'ase ABC digestion, or the presence of the non-reducing terminal end saturated CS disaccharide consisting of glucuronic acid (GlcA) that is adjacent to the 6-

sulfated *N*-acetylgalactosamine (GalNAc), or a combination of both these epitopes were present.

To control for staining with the antibody clones 2B6 and 3B3, tissue sections were probed with biotinylated secondary antibodies, mouse IgG (Fig 2 A) and mouse IgM (Fig. 2 B) where slides were incubated prior to the biotinylated secondary antibodies with blocking solution. The tissue sections revealed no significant staining when probed with either the biotinylated anti-mouse IgG or IgM secondary antibody. Cells previously presented as 2B6- and 3B3- are indicated by black arrows. These data demonstrate that non-specific binding of the biotinylated secondary antibodies did not occur. Tissue sections probed with whole mouse IgG (Fig. 2 D) and IgM (Fig 2. E) antibodies, as isotype control, demonstrated that non-specific immunoglobulin binding did not occur.

The type of inflammatory cell involved in these tissue regions was of interest. To investigate the types of inflammatory cells present tissue sections were probed for the presence of macrophages using the cell surface markers CD86 and CD206 for the detection of type 1 (Fig. 3 A) and type 2 macrophages (Fig. 3 B), respectively (Wolf et al., 2014). The fibrous capsule was positively stained for the presence of type 1 macrophages, where their presence was also observed in the surrounding tissue. The 2B6- cells were positive for CD86 on the cell surface (Fig. 3 A (iii), indicated by the black arrow). The localization of CD206 was shown to be within the fibrous capsule as well as within the adjacent tissue (Fig. 3B (i)). Similar to CD86, 2B6- cells were shown to have slight staining for CD206 (Fig. 3 B (iii), indicated by the black arrow). Controls with biotinylated anti-rabbit secondary antibody only (Fig. 2 C) were absent of positive staining, indicating that non-specific binding of biotinylated anti-rabbit antibody did not occur.

In addition to the infiltration of macrophages, mast cells also infiltrate to the implantation site of a foreign material (Farrugia et al., 2014). Sections were stained with toluidine blue (Fig. 4 A), and Leder stain (Fig. 4 B), to determine the presence of mast cells, and probed for the presence of mast cell tryptase (Fig. 4 C), a serine proteinase that is stored in mast cell α-granules and released on activation. Cells within the tissue sections showed punctate staining with toluidine blue (Fig. 4 A (ii) and (iii)). Higher magnification (Fig. 4 A (iii)) depicts violet staining of these cells, indicated by the black arrow. Leder stained cells were found within the fibrous capsule that formed around the implanted material, as well as in close proximity to blood vessels (Fig. 4 B (ii)). Cells previously shown to be stained with toluidine blue were found to be magenta following staining with Leder stain (Fig. 4 B (iii)) confirming that these cells were mast cells. Intracellular staining of mast cell tryptase was observed in cells within the fibrous capsule as well as the adjacent tissue (Fig. 4 C). Similarly to toluidine blue and Leder stain, a population of cells in proximity to blood vessels was positive for mast cells tryptase (Fig. 4 C (ii) and (iii)). These results demonstrate a population of cells with positive staining for toluidine blue, Leder stain and mast cell tryptase are characterized as mast cells. The cells chemically characterized as mast cells were observed to be of similar morphology and size (10 – 20 μm) to 2B6- and 3B3- cells. This suggests that the cells found to express the epitope detected by 2B6- may be mast cells.

Tissue sections were probed to detect the presence of the CSPGs serglycin (Fig. 5 A), perlecan (Fig. 5 B), versican (Fig. 5 C), bikunin (Fig. 5 D), and aggrecan (Fig. 5 E). Serglycin was localized within the fibrous capsule that surrounded the implanted materials, as well as in tissue adjacent to the fibrous capsule, although with decreased expression (Fig. 5 A (i)). Serglycin was shown to be present in 2B6- cells (Fig. 5 A (iii), indicated by the black arrow). Perlecan was detected in the fibrous capsule (Fig. 5 B (i)). Cells that contained the 2B6 epitope were also positive for perlecan, with intense cellular staining observed (Fig 5 B (iii),

indicated by the black arrow). Versican was found to be present in relatively low levels in the tissue adjacent to the fibrous capsule surrounding the implanted material (Fig. 5 C (i)). On further investigation versican was present with intracellular staining in 2B6- cells (Fig. 5 C (iii), indicated by the black arrow). Bikunin was expressed in the fibrous capsule as well as the adjacent tissue (Fig. 5 D (i)). Similar to serglycin, perlecan and versican, bikunin was shown to be present in 2B6- cells (Fig. 5 D (iii), indicated by the black arrow). Aggrecan is a CSPG primarily associated with cartilage. Sections probed for the presence of aggrecan showed minimal staining within tissue adjacent to the fibrous capsule (Fig. 5 E (i)), and 2B6- cells did not stain positively for aggrecan (Fig. 5 E (iii), indicated by the black arrow). These results demonstrate that a number of different CSPGs were present in the fibrous capsule in response to the implanted materials with a similar localization to 2B6- cells. This suggests that the 2B6- epitope may be associated with a number of different CSPGs.

The novel CS epitope recognized by the antibody clone 2B6 in rodent tissues was explored further in the rat derived mast cell-like line RBL-2H3. Expression of CS epitopes by RBL-2H3 cells detected with the antibody clones 2B6 and CS-56 was investigated via flow cytometry (Fig. 6). Expression of the 2B6- epitope showed a similar fluorescence intensity when the cells were either in their resting or activate state, irrespective of permeabilization prior to antibody probing. Whereas, the fluorescence intensity of the CS-56 antibody reactivity increased upon permeabilization of the cell membrane when cells were either resting or in their activated state. These results demonstrate that the 2B6- and CS-56 epitopes were present in the RBL-2H3 cells in different cellular location. Cells were also incubated with whole mouse IgG and IgM, and blocking solution in place of a primary antibody, followed by incubation with AlexFluor488 conjugated secondary antibodies as controls for non-specific immunoglobulin and secondary antibody binding. Background fluorescence levels were detected for each of these controls and compared to cells probed with either

antibody clones 2B6 or CS-56.These data demonstrated that immunoglobulin binding, either IgG or IgM, nor non-specific binding of the fluorophore conjugated secondary antibody did not occur. These data confirmed the specificity of both antibody clones 2B6 and CS-56 in the RBL-2H3 cells.

Immunocytochemistry was carried out on RBL-2H3 cells in their resting state and following chemical activation to further investigate the presence and localization of the epitope detected by the antibody clone 2B6. Epitopes detected by the antibody clone 2B6 with (Fig. 7 A (i)) and without (Fig. 7 A (ii)) endoglycosidase digestion, were shown to be present when cells were in their resting state. No significant staining was detected for cells probed with the fluorophore conjugated secondary antibody only (Fig. 7 A (iii)). Following chemical activation (Fig. 7 B) the presence of the 2B6 epitope was found to be more punctate, particularly when cells were endoglycosidase digested prior to antibody probing (Fig. 7 B (i)). Positive staining for the 2B6- epitope was also observed when cells were in their activated state (Fig. 7 B (ii)). Cells probed with the fluorophore conjugated secondary antibody only showed minimal background fluorescence with the absence of the punctate staining (Fig. 7 B (iii)). RBL-2H3 cells did not contain either the 3B3+ (Fig. 7 C (i)) or 3B3- (Fig. 7 C (ii)) epitopes. Additionally, cells probed with the fluorophore conjugated secondary antibody only were also absent of staining (Fig. 7 C (iii)). As observed within the rodent tissue, and supporting the flow cytometry data, the 2B6- epitope was present in rat derived mast cells and was shown not to be dependent on their activation state. Furthermore, the punctate staining suggested a granular localization of the both the 2B6+ and 2B6- epitopes. These data suggest that RBL-23 cells produce a native CS epitope detected by the antibody clone 2B6 in the absence of C'ase ABC, in addition to the 4-sulfated CS stub generated by endoglycosidase digestion.

To further investigate the CSPGs that may be associated with the novel CS epitope, RBL-2H3 cells were stained for the presence of 2B6-, and either serglycin or perlecan (Fig. 7). Positive staining for the 2B6- was observed (Fig. 7 A (i) and (ii)). These cells also showed reactivity for both perlecan (Fig. 7 B (i)) and serglycin (Fig. 7 B (ii)). Cells probed with anti-mouse Alexa Fluor 488 (Fig. 7 A (iii)) or anti-rabbit Alexa Fluor 594 (Fig. 7 B (iii) where the blocking solution was used in place of a primary antibody showed minimal staining, which indicated that non-specific binding of the secondary antibodies did not occur. Co-localization of the 2B6- epitope with perlecan (Fig. 7 C (i) and D (i)), or serglycin (Fig. 7 C (ii) and D (ii)) was observed as indicated by the presence of yellow. Both perlecan and serglycin were detected in regions of positive staining that co-localize with the 2B6- epitope, as well as regions where the 2B6- epitope did not co-localize with either CSPG (Fig. 7 D (i) and (ii)). Quantification of co-localization was carried out using the Manders co-localization coefficient (Fig. 7 E). An average coefficient of 0.59 was shown for co-localization of the 2B6- epitope with perlecan, and 0.65 for perlecan with 2B6-. The Manders co-localization coefficient of 0.55 was shown for 2B6- with serglycin, and 0.54 for serglycin with 2B6-. Thus demonstrating that the 2B6- epitope did not solely co-localize with either of the CSPGs and either perlecan or serglycin did not solely co-localize with the 2B6- epitope. These results demonstrate the 2B6- does not associate with a specific CSPG and suggests these CSPGs may not be decorated exclusively with CS that contains the 2B6- epitope.

4. Discussion

The present study investigated the presence of a novel CS epitope detected using the antibody clone 2B6 in the absence of C'ase ABC digestion, in mammalian tissue and derived cells. The mammalian tissue was obtained from a rodent plantation model where the inflammatory response to implanted materials including Surgicel and chitosan was evaluated (Farrugia et al., 2014). In this study mast cells infiltrated into the wound site in response to all of the implanted materials and the expression of the 2B6- epitope was not dependent on material implant. The implantation of a foreign material will invoke an inflammatory response involving a range of cells including neutrophils, monocytes, macrophages and mast cells. Macrophages, and their precursor monocytes, are inflammatory cell types that are also known to contain serglycin decorated with CS-E (Uhlin-Hansen et al., 1989). Unlike mast cells where serglycin is stored in its granules and released upon activation, serglycin is continuously secreted from macrophages and is involved in the regulation of tumor necrosis factor α (Zernichow et al., 2006). Serglycin has been shown to be produced by various inflammatory cells where it is involved with the storage and secretion of various proteases and cytokines (Grujic et al., 2005, Niemann et al., 2007).

The role that mast cells play in the immune system is largely due to the constituents of their secretory granules that includes various enzymes, cytokines, and proteases (Wernersson and Pejler, 2014). Heparanase is contained in the secretory granules with roles in the cleavage of heparin and HS, where the ability of heparanase to intracellularly cleave heparin in mast cells (Wang et al., 2011) has been demonstrated and shown that it influences the storage and release of secretory granular components. Cleavage of macromolecular heparin by heparanase is crucial for accelerating the release of heparin and chymase from the secretory granules to the extracellular matrices (Higashi et al., 2014). Heparanase, however, does not

cleave CS chains, and therefore would not be responsible for the results presented with regards to the CS structure detected by 2B6.

The 2B6 antibody detects the unsaturated terminal disaccharide stub structure containing 4-sulfated GalNAc that is revealed after bacterial C'ase ABC digestion (Couchman et al., 1984, Caterson et al., 1985). Evidence of the 2B6- epitope has previously been reported in human osteoarthritic cartilage (Asari et al., 1996) with the epitope localized within the chondrocytes, however, the possible function of this epitope was not further explored. The data presented in this study demonstrated that cells in rodent tissue that were morphologically similar to and chemically characterized as mast cells produce a CS structure detected by the 2B6 antibody without endoglycosidase digestion. The bacterial endoglycosidase, chondroitinase (C'ase) ABC, belongs to the family of lysases where endolytic cleavage of CS chains occurs at β(1 → 4) galactosaminic bonds between GalNAc and GlcA. This results in an unsaturated terminal disaccharide structure following the linkage region. The suite of antibodies 2B6, 3B3 and 1B5 (Couchman et al., 1984, Caterson et al., 1985) enables the detection of the terminal unsaturated disaccharide structures that remain following C'ase ABC digestion, and whether these structures are sulfated, 4S or 6S, or unsulfated, respectively. The results presented demonstrated that mammalian cells produce an epitope recognized by the antibody clone 2B6 in the absence of endoglycosidase digestion. This suggests that the 2B6- epitope may be generated *in situ* or the structure that was detected may occur naturally. To the best of our knowledge there is no known mammalian CS digesting enzyme that reveals the epitope of antibody clone 2B6.

Hyaluronidase -4, a member of the hyaluronidase family, has shown to behave as a chondroitin hydrolyze in *C. elegans* (Kaneiwa et al., 2008). Most hyaluronidases degrade both hyaluronidase and CS, including hyaluronidase 1 and testicular hyaluronidase, particularly CS-A (Honda et al., 2012) where HYAL-4 cleaves glactosmainidic bonds of CS,

specifically GalNAc(4S)-GlcUA and GalNAc(6S)-GlcUA, with optimal pH of 4.5 – 5 (Kaneiwa et al., 2010). Thus, it is hypothesized that the 2B6- epitope detected may be the result of hyaluronidase cleavage of CS. The CSPG that the novel CS epitope may be associated with was investigated where serglycin, perlecan, versican and bikunin were shown to localize in cells of similar morphology as those that contain the novel CS epitope. Furthermore, co-localization of 2B6- with both perlecan and serglycin in RBL-2H3 cells demonstrated that the 2B6- epitope was present on both of these CSPGs. This demonstrates that the unique CS structure is not specific to a certain CSPG, and supports the hypothesis that the novel epitope is generated by a mammalian chondroitinase, or synthesized by the cells.

Serglycin is the prominent CS PG that is produced by mast cells. Though, the results presented demonstrating the presence of serglycin within the rodent tissue was relatively faint. It is noted that the primary antibody used to probe for the presence of serglycin was raised against an amino acid sequence of full length human serglycin (aa 1 – 158) and not a rodent sequence which could explain this result. Murine mast cell tryptase 5 binds to heparin containing PGs (Matsumoto et al., 1995) demonstrating importance of GAGs in the protection and packing of contents of mast cell granules. Furthermore, serglycin is important for the assembly of secretory granules, transport of secretory compounds into the granules, as well as maturation of the granules (Braga et al., 2007). The function of GAGs that are present in the mast cell granules include roles in the storage (Åbrink et al., 2004) and retention (Henningsson et al., 2006) of proteases through complex formation (Serafin et al., 1986). Heparin is important in the storage of mast cell proteases (Humphries et al., 1999), where *N*-deacetylase/*N*-sulphotransferase-2 knockout mice that are unable to express heparin and show defects in their granules including reduced storage of proteases and histamine (Forsberg et al., 1999). Proteases and cytokines are packaged, in an inactive state, in mast cell granules at

pH 5.5 through strong ionic interactions with GAGs (De Young et al., 1987). Heparanase is involved in the cleavage of heparin/HS from the PGs in mast cell granules and the release of bound proteases and cytokines into the surrounding tissue. It is hypothesized that this process also occurs for CS in the mast cell granules and that cleavage occurs by a member of the hyaluronidase family, thus enabling release of CS bound proteases and cytokines. Further investigation is underway to test this hypothesis.

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Author Contribution

BF, JW, BC and ML contributed to the experimental design, data interpretation, and preparation of the manuscript. RO'G contributed to data interpretation and preparation of the manuscript. BF carried out the experiments. All authors have read and approved the final manuscript.

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Figure Legends

Figure 1: Characterization and localization of chondroitin sulfate structures. Using immunohistological methods sections probed for the presence of the epitopes detected by antibody clones 2B6 (A and B) and 3B3 (C and D). Sections were probed with the antibody clones with (A and C) and without (B and D) prior endoglycosidase digestion with chondroitinase ABC. Scale bars represent 3 mm (i), 60 µm (ii), and 20 µm (iii).

Figure 2: Immunohistochemical controls included probing the tissue sections without primary antibodies with biotinylated mouse IgG (A), mouse IgM (B) and rabbit (C) secondary antibodies. Isotype controls were carried out with sections probed with mouse IgG (D) and IgM (E). Scale bars represent 200µm.

Figure 3: Localization of macrophages. Using immunohistological methods sections were probed to investigate the presence of macrophages, type 1 (A) and type-2 (B). Scale bars represent 3 mm (i), 60 µm (ii), and 20 µm (iii).

Figure 4: Characterization and localization of mast cells. Mast cells were chemically characterized via staining with toluidine blue (A), Leder stain (B) and immunohistological methods to probe for the presence of mast cell tryptase (C). Scale bars represent 3 mm (i), 60 µm (ii), and 20 µm (iii).

Figure 5: Localization of CS proteoglycans. Using immunohistological methods sections were probed for the presence of serglycin (A), perlecan (B), versican (C), bikunin (D) and aggrecan (E). Scale bars represent 3 mm (i), 60 μm (ii), and 20 μm (iii).

Figure 6: Flow cytometry probing for the presence of the CS epitopes detected by the antibody clones CS56 and 2B6, in the absence of chondroitinase ABC digestion, in RBL-2H3 cells in their resting state (A) and following chemical activation (B). Prior to incubation with primary antibodies cells were not permeabilized (A (i) and (B (i)) or permeabilized (A (ii) and (B (ii)).

Figure 7: Immunolocalization of epitopes detected using the antibody clones 2B6 and 3B3 produced by mast cells (RBL-2H3). Cells were probed for the presence of 2B6 with (A (i) and B (i)) and without (A (ii) and B (ii)) endoglycosidase digestion with chondroitinase ABC. Localization of 2B6 was investigated when cells were in their resting (A) or chemically activated states (B). Cells were probed for the presence of 3B3 with (C (i)) and without (C (ii)) chondroitinase ABC digestion. Localization was investigated when cells were in their resting state. Negative controls were not incubated the presence of the primary antibody prior to incubation with Alexa Fluor 488 conjugated anti-mouse secondary antibody (A (iii), B (iii) and C (iii)). Scale bars represent 10 μm (A and B) and 50 μm (C).

Figure 8: Co-localization CS proteoglycans with 2B6, in the absence of chondroitinase ABC digestion. Cells were probed for the presence of 2B6 (A (i) and (ii)) and either perlecan (B (i)) or serglycin (B (ii)). Cells were then probed with anti-mouse-Alexa Fluor 488 and anti-

rabbit-Alexa Fluor 594 secondary antibodies to detect the presence of 2B6 and the CS proteoglycans respectively. Merged channels demonstrate co-localization of 2B6 and perlecan (C (i) and D (i), as well as 2B6 and serglycin (C (ii) and (D (ii)). Co-localization of 2B6 and serglycin or perlecan was quantified using the Manders co-localization coefficient (E). Scale bars represent 50 μm (A, B and C) and 10 μm (D).

Table Legends:

Table 1: Details of the primary antibodies utilized for immunohistological staining.

Tables

TABLE 1:

Primary Antibody epitope	Species	Isotype	Clone / Catalogue No.	Supplier	Dilution	Endoglycosidase Digestion*
Mast Cell Tryptase	Mouse	IgG	AA1	Abcam	2µg/mL	-
Chondroitin-4 sulfate stub	Mouse	IgG	2B6		1:500	Yes
Chondroitin-6 sulfate stub	Mouse	IgM	3B3		1:500	Yes
Serglycin	Rabbit	Polyclonal	sc-292311	Santa Cruz	1:100	-
Perlecan	Rabbit	Polyclonal	CCN-1	**	1:1000	-
Versican	Mouse	IgG	5D5	***	1:250	Yes
Bikunin	Rabbit	Polyclonal	Ab43073	Abcam	1:400	-
Aggrecan	Mouse	IgG_1	969D4D11	Invitrogen	0.2µg/mL	Yes
CD86	Rabbit	IgG	EP1158Y	Abcam	1:200	-
CD206	Rabbit	Polyclonal	ab64693	Abcam	1:500	-

* Digestion with chondroitinase ABC, 0.05U/mL, pH 8.0, 0.1M Tris-Acetate buffer
** Raised in house against immunopurified human coronary artery endothelial cell derived perlecan (Lord et al., 2014, Whitelock et al., 1999)
*** A gift from Dr Firoz Rahemtulla, University of Alabama, Birmingham, AL, USA

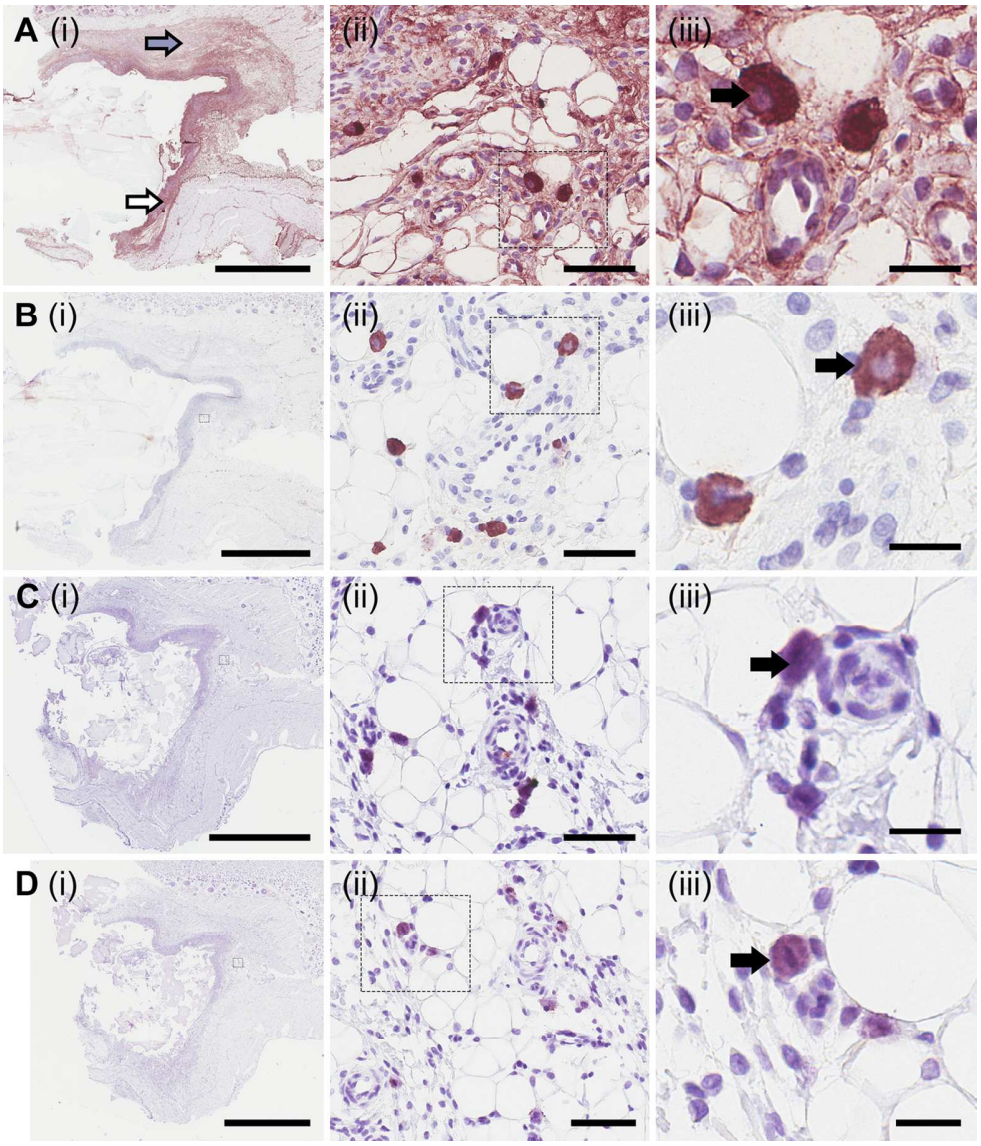

Figure 1
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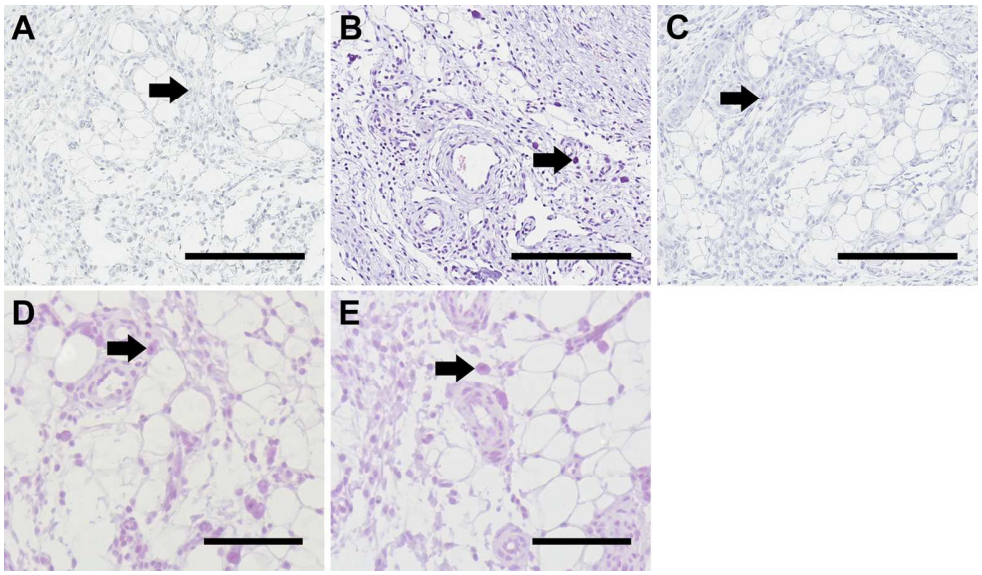

Figure 2
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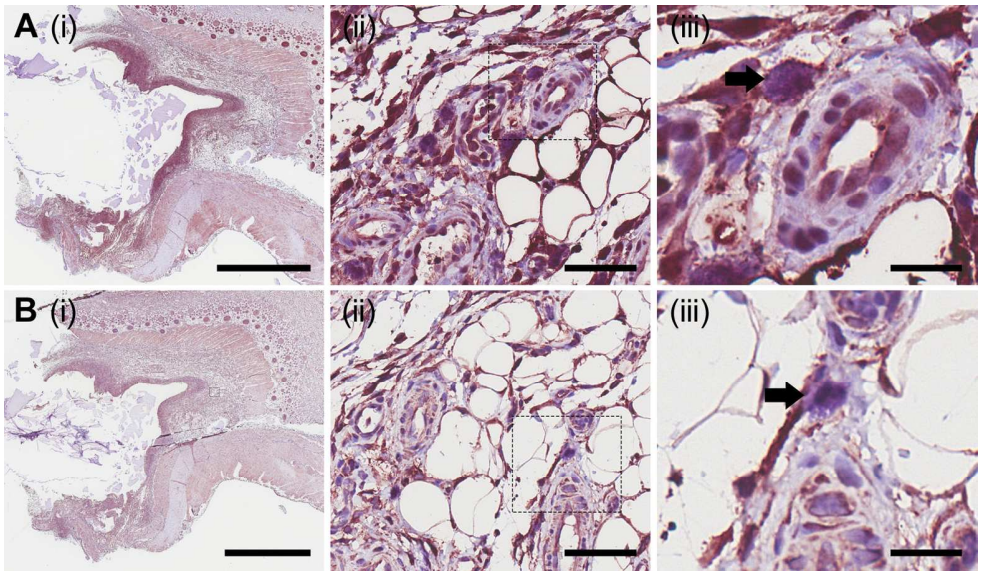

Figure 3
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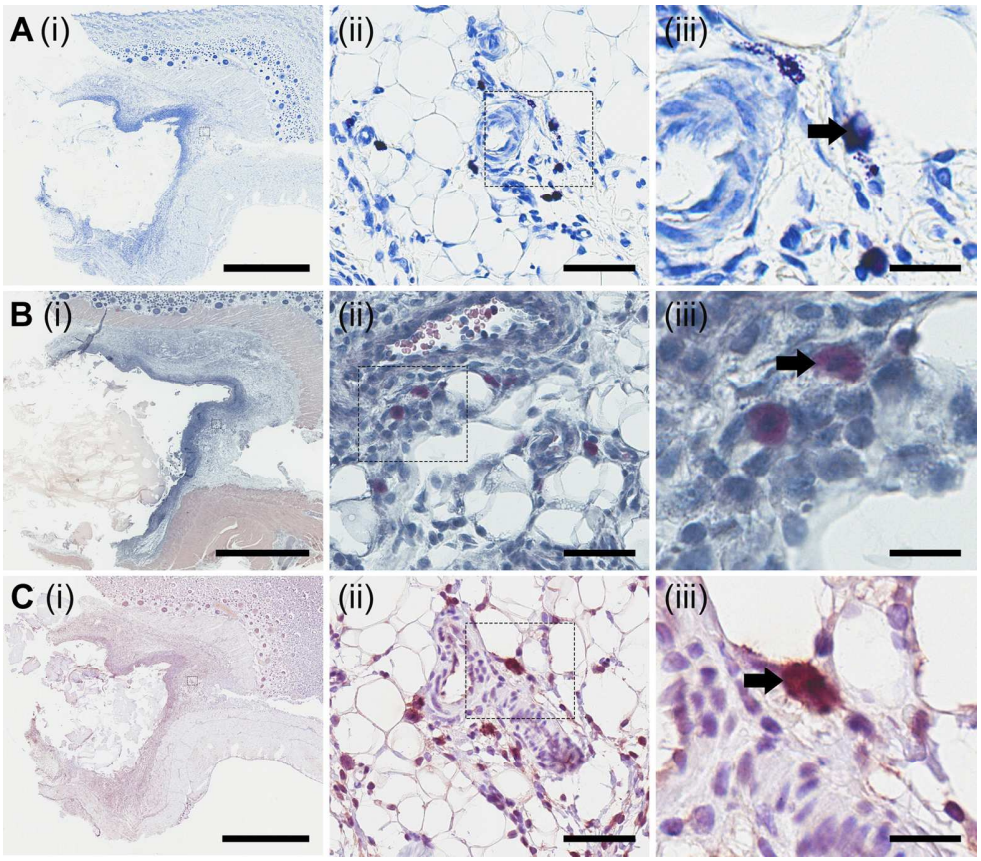

Figure 4
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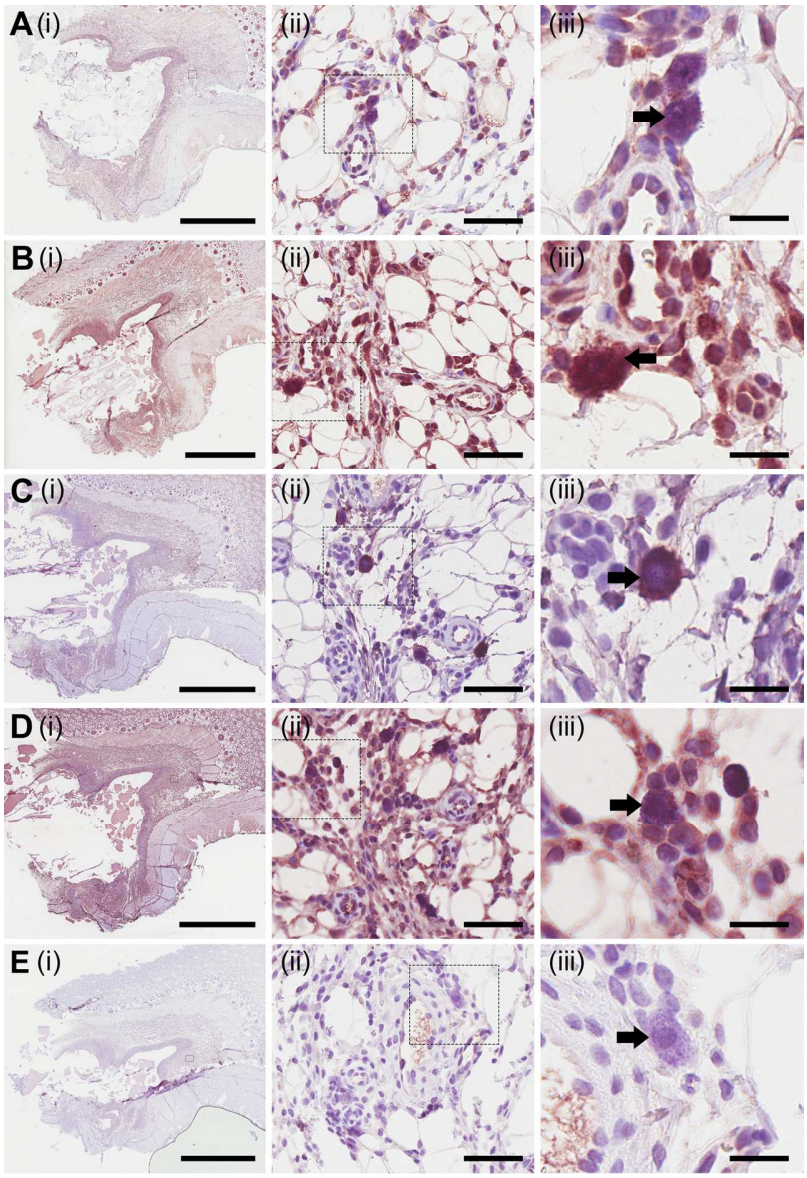

Figure 5
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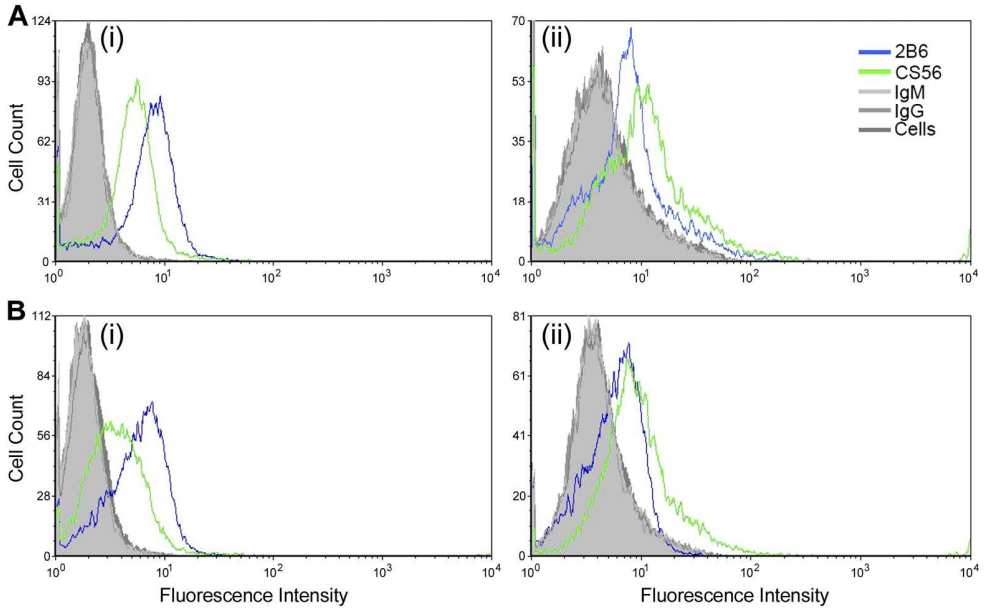

Figure 6
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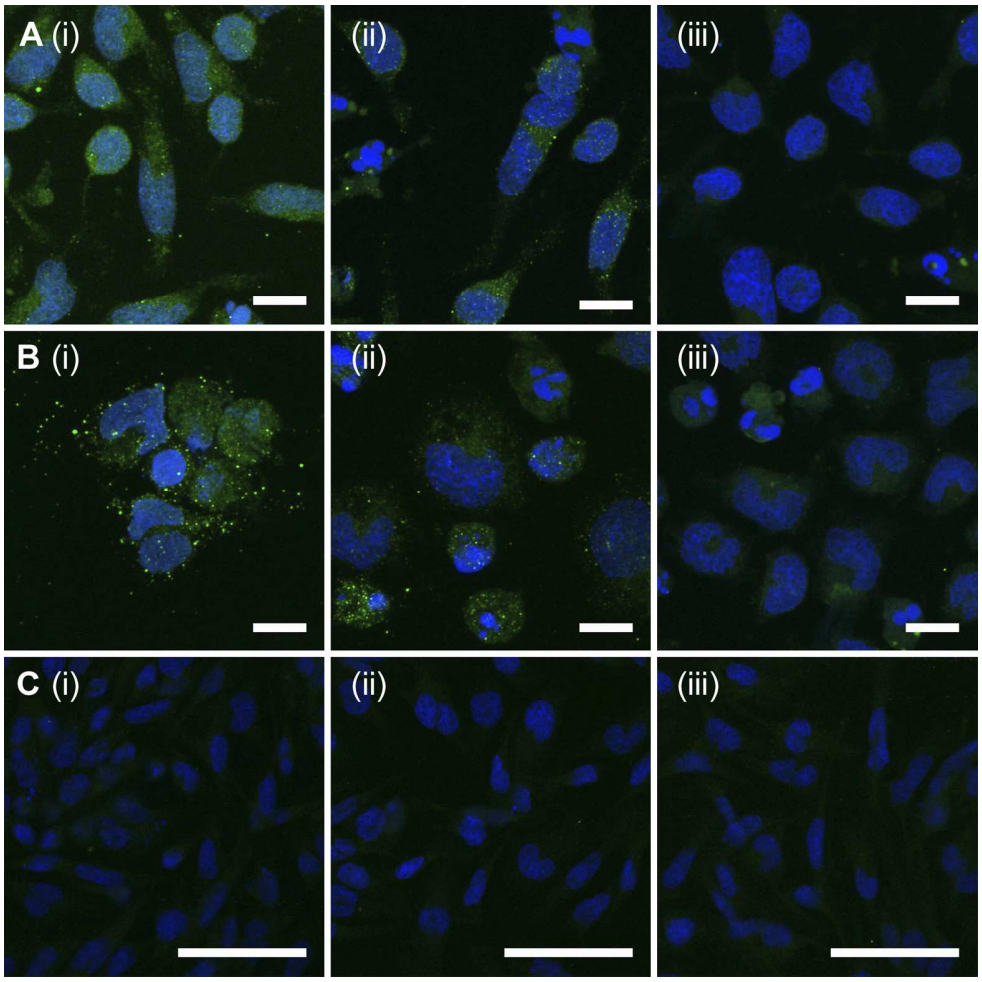

Figure 7
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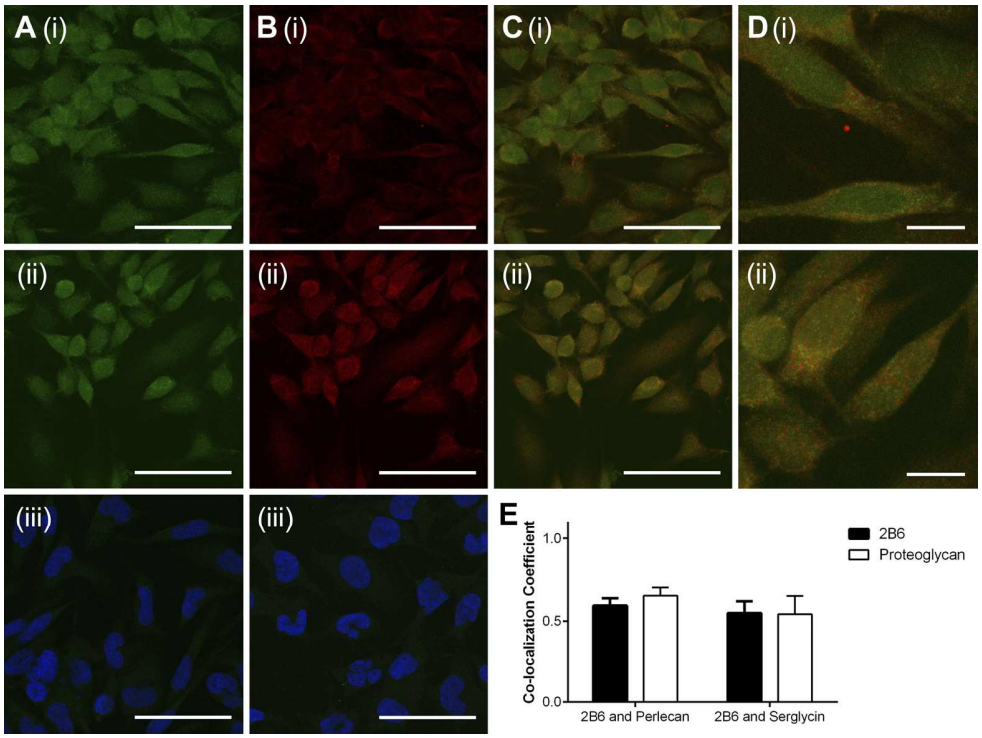

Figure 8
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