Identification and characterization of an M cell marker in nasopharynx- and oropharynx-associated lymphoid tissue of sheep

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ABSTRACT

M cells play a pivotal role in the induction of immune responses within the mucosa-associated lymphoid tissues. M cells exist principally in the follicle-associated epithelium (FAE) of the isolated solitary lymphoid follicles as well as in the lymphoid follicles of nasopharynx-associated lymphoid tissue and gut associated lymphoid tissue (GALT). Through lymphatic cannulation it is possible to investigate local immune responses induced following nasal Ag delivery in sheep. Hence, identifying sheep M cell markers would allow the targeting of M cells to offset the problem of trans-epithelial Ag delivery associated with inducing mucosal immunity. Sheep cDNA from the tonsils of the oropharynx and nasopharynx was PCR amplified using Glycoprotein-2 (GP$_2$)-specific primers and expressed as a poly-His-tagged recombinant sheep GP$_2$ (56 kDa) in HEK293 cells. The recombinant GP$_2$ protein was purified using Ni-NTA affinity chromatography and polyclonal serum against the protein was raised in rats. The antiserum recognized the recombinant sheep GP$_2$ and purified rat IgG against GP$_2$ stained M cells in sections of sheep tonsils from nasopharynx and oropharynx. M cells were found to be present in epithelium of the palatine tonsils (oropharynx), pharyngeal tonsils as well as tubal tonsils (nasopharynx). They were also present in the FAE of the scattered lymphoid follicles over the base of the nasopharynx. Thus, GP$_2$ has been identified to be an important M cell marker of nasopharynx and oropharynx-associated lymphoid tissues in sheep.

1. Introduction

The mucosal immune system plays a crucial role through immune-surveillance against potential pathogens at the mucosal surfaces of the gastro-intestinal, respiratory and uro-genital tracts. The luminal area of these surfaces is covered with epithelium and is exposed to commensals as well as pathogens. Microfold cells (M cells) are specialized epithelial cells found in the follicle-associated epithelium (FAE) of Peyer's patches of gut associated lymphoid tissue (GALT), appendix (Sansonetti and Phalipon, 1999; Wang et al., 2011) and solitary lymphoid follicles found in lymphoid tissues of nasal associated lymphoid tissue (NALT) (Hameleers et al., 1989) and bronchial associated lymphoid tissue (BALT; Pankow and Von Wichert, 1988 Gebert et al., 1999). They play a pivotal role in induction of mucosal immune responses by facilitating adhesion and transport of luminal Ags to the APCs seated in their basolateral pockets (Debard et al., 1999; Claeys and De Belder, 2003). M cells differ morphologically from adjacent epithelial and goblets cells in having a thinner glycocalyx and insignificant microvilli (cilia in case of NALT and airway M cells). M cells *per se* do not act as APCs since very little proteolytic processing of Ag occurs within these cells and they do not present Ags in the context of MHC molecules. Therefore, they principally serve as sampling/surveillance cells, which by virtue of transcytosis deliver molecules to APC. In recent studies, M cell deficient mice could not raise satisfactory mucosal immune response against *Salmonella typhimurium* and *Yersinia enterocolitica* (Kanaya et al., 2012; Kishikawa et al., 2017) and display a delay in maturation of germinal centres of Peyer's patches and consequently a delay in the endogenous IgA production across epithelia is observed (Rios et al., 2016). Thus, M cells are a lynchpin in the development of mucosal immune response.

M cells are specialized cells but have highly divergent developmental pathways e.g. Peyer's patches M cells develop from cryptic stem cell populations (Giannasca et al., 1994; Lelouard et al., 2001) and are

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short-lived, while the airway M cells are longer-lived and are derived from basal cells (Rawlins and Hogan, 2008). Consequently, there is currently no universal M cell marker as M cells differ greatly in their carbohydrates, glycoproteins and enzyme expression profiles, (including the type of cellular filaments) between species and tissues (Casteleyn et al., 2013). For example, clusterin was identified as a specific M cell marker in the human palatine tonsils, adenoids and Peyer's patches (Verbrugghe et al., 2008). In horses, cytoplasmic vimentin filaments as well as galactose epitopes on the apical surface were found to be specific for equine tonsillar M cells (Kumar et al., 2001). In mice the presence of specific sugar molecule on the M cells surface (i.e. $\alpha(1,2)$fucose) lead to the targeting of M cells using *Ulex europeaus agglutinin-I* (UEA-1) (Foster et al., 1998) and also by the NKM 16-2-4 mAb raised against UEA-1^{+} WGA^{-} cells (Nochi et al., 2007). Other targeting strategies include the use of *Anguila anguila* agglutinin and *Aleuria aurantia* lectin (Clark et al., 1994). Transcriptomic profiling meta-analysis studies were performed by Kobayashi et al., (2012) and identified the genes for GP$_2$, TNF-α expressed-induced protein 2 (Tnfaip2) and Ccl9 to be highly expressed in mature M cells of mouse.

Glycoprotein-2 (GP$_2$) was originally identified as a glycosylphosphatidylinositol (GPI)-anchored protein, which is specifically expressed in zymogen granules of pancreatic acinar cells (Hoops and Rindler, 1991; Fukuoka et al., 1991) but later shown to be highly expressed by M cells of mice and in humans Peyer's patches (Terahara et al., 2008; Hase et al., 2009). GP$_2$ is a specific receptor for the binding FimH protein, which is a major component of the type 1 pilus on the outer membrane of a subset of gram-negative enterobacilli such as *E. coli* and *Salmonella enterica* (Hase et al., 2009; Yu and Lowe, 2009).

In domestic animals, there is very limited information on M cell markers (Casteleyn et al., 2013). The six tonsils present in sheep are: the lingual, palatine, paraepiglottic, pharyngeal, and tubal tonsils and the tonsil of the soft palate (Casteleyn et al., 2011). In addition, their arrangement is quite similar to that of humans, making sheep an important model animal for nasal immunization (Scheerlinck et al., 2008).

In addition, lymphatic cannulation in sheep allows for the collection of lymphocytes from lymph nodes with known draining areas including those draining from nasal mucosal sites (Yen et al., 2006). Hence, sheep is an excellent animal model for mucosal immunization studies. However, the lack of M cell markers in sheep precludes the targeting of M cells to enhance mucosal immunization, as suggested by Kim and Jang, (2014), in this species. This study therefore aims to identify and characterize some of the M cell markers in the nasal associated lymphoid tissue of sheep.

2. Material and methods

2.1. Cloning, expression and purification of GP$_2$

Sheep heads were collected from a local slaughterhouse and sagittally sectioned at the atlanto–occipital joint. The nasal septum was then removed, exposing both halves of the nasopharyngeal cavity. The palatine tonsil, pharyngeal tonsil, lingual tonsil and dispersed lymphoid follicles along the distal aspect of the base of nasopharynx were collected in PBS. Samples were also preserved in OCT medium and stored at $-80\,°C$ for cryo-sectioning.

RNA was isolated from each of the collected lymphoid tissue and cDNA was prepared. Coding sequence of 1.5 kb length of GP$_2$ was PCR amplified using primers (GP$_2$F GCCCTGGCCTCCTACATTCT and GP$_2$R, TCCTGCAGTGTTGGGTGTTCC). The cycling conditions were: 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1.5 min, with a final extension of 72 °C for 10 min. GP$_2$ was cloned in pGEM®-T Easy Vector using TA cloning strategy and sequenced using the BigDye™ Terminator v3.1 Cycle sequencing protocol.

The PCR product was amplified using the primers containing an N-terminal His-tag as well restriction sites XbaI and KpnI for directional cloning into pcDNA3.1 (GP$_2$F$_1$: CGGGGTACCATGCATCATCACCATCA

CCATGCCCTGGCCTCCTACATTCT and GP2R1: TGCTCTAGATCCTGCAGTGTTGGGTGTTCC). The purified insert was digested using XbaI (NEB, Massachusetts, USA) and KpnI (NEB) in a 20 μL reaction. Similarly, the pcDNA3.1 was digested using the same enzymes and dephosphorylated using calf intestinal phosphatase enzyme. Insert and Vector in a 3:1 ratio, were ligated in a 20 μL reaction using T4DNA ligase (NEB). The ligated product was transformed into the DH5alpha competent cells produced by using modified protocol of Chung et al., (1989). The GP$_2$ containing plasmid was isolated and transfected into HEK293 T cells using the standard protocol at 70–80% confluence. HEK293 T cells were lysed and the soluble poly-his-tagged GP$_2$ was purified using the Ni-NTA affinity chromatography. The eluate was dialysed against PBS and the recombinant protein was concentrated using the Amicon ultra-4 centrifuge filter device (Millipore, County Cork, Ireland) with a membrane cut-off of 10 kDa. The concentration of purified protein was determined by modified Lowry's protein assay kit according to the manufacturer's protocol (Biorad, California, USA, DC protein assay kit).

### 2.2. Production, testing and purification of hyperimmune sera in rats

Two rats were injected with 10 μg of purified GP$_2$ protein adjuvanted with Titermax Gold (Sigma Aldrich, St Louis, USA) and boosted twice with 5 μg of protein in the same adjuvant at days 21 and 42. Animals were terminally bled at day 60 and the IgG fraction was purified from hyperimmune sera. Normal rat serum was used as a control.

For the ELISA, polystyrene microwell plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 μL/well of 5 μg/mL of GP2 in 0.05 M Carbonate-Bicarbonate coating buffer, pH 9.6 coating buffer. After overnight incubation at 4 °C, the coated wells were washed four times and left to block with 5% skim milk powder in PBS with 0.05% Tween-20 (blocking buffer) for 1 h. The rat sera were added to the wells in dilutions (1/20, 1/50, 1/100, 1/250 in blocking buffer) and were incubated for 1 h. Subsequently, the wells were washed three times and goat anti-rat horseradish peroxidase (HRP) secondary Abs (Southern Biotech, Birmingham, USA) was added at 1/3000 dilution in blocking buffer, for 1 h at room temperature. The wells were washed three times with PBST and the presence of HRP was detected using Single-Component TMB Peroxidase EIA Substrate (Biorad) for 15 min after which the reaction was stopped with 100 μL/well 1 M H$_2$SO$_4$. The OD was measured at 450 nm.

2.3. Cryo-sectioning and histological examination of ovine nasopharynx- and oropharynx-associated lymphoid tissue stained with anti-GP$_2$

Cryo-sectioning of 6 μm sections from nasopharynx-associated lymphoid tissue (Tonsils, lymphoid epithelium) was performed using a Leica CM3050 cryostat. Sections were fixed with cold ethanol and endogenous peroxidase activity was inhibited using 3% H$_2$O$_2$ for 10 min. Slides were washed three times with PBS and blocking was done with 1% BSA in PBS for 30 min. Anti-GP$_2$ IgG (1/100) were used to cover sections for primary Ab staining for 2 h. Normal rat IgG was used as a negative control. Slides were rinsed with PBS and goat anti-rat HRP secondary Abs (Southern Biotech, Birmingham, USA) at 1/3000 dilution were used for 1 h. Slides were rinsed three times with PBS and labeling was demonstrated using substrate solution (2.6 mM Di-amino benzidine in citrate buffer and 0.1% of hydrogen peroxide solution). Slides were counterstained with Hematoxylin for 5 min before rinsing, dehydrating, clearing, and covering.

Frozen cryo-sections (6 μm) were fixed with 4% paraformaldehyde for 10 min at room temperature and washed three times with ice cold PBS. The sections were blocked with 3% BSA in PBST for 1 h. Anti-GP$_2$ purified IgG were used as primary Abs (1/100) and were incubated with section for 2 h at room temperature. Slides were washed three times with PBS and incubated for 1 h with goat anti-rat IgG H&L Alexa

A
B
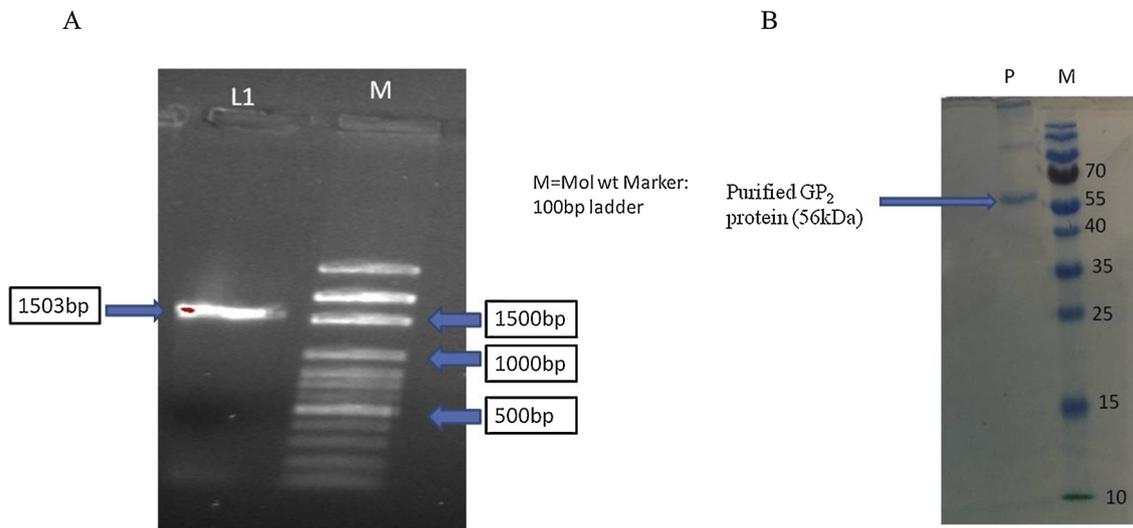
Fig. 1. Cloning and expression of sheep GP_2. (A) PCR amplification of the 1503 bp fragment of GP2 and (B) SDS-PAGE of purified GP_2 protein.

Fluor 594 secondary Abs (1/2000) (Abcam, Cambridge, UK). Slides were washed with PBS and a quenching step using 0.3% Sudan black was performed to quench the autoflourscence of lymphocytes. Sudan Black (0.3% w/v) in ethanol (70% v/v) was stirred in the dark for 2 h and was applied to slide for 10 min after the secondary Ab application. Slide was rinsed quickly with PBS and counterstained with DAPI for 5 min. Slide was washed with PBS and mounted.

3. Results and discussion

3.1. GP_2 cloning and expression

Since GP_2 was an uncharacterized gene in sheep, primers were designed based on genomic sequences and a nearly complete CDS sequence (1503 bp) was amplified from sheep tonsils (Fig. 1a). GP_2 was detected in all of the collected tonsils as well the lymphoid epithelium and the fragment was cloned into pcDNA3.1. Colony PCR, restriction enzyme digestion and sequencing were independently performed to confirm the in-frame cloning of the insert into the vector.

Following expression in HEK293 T cells, a 56 kDa protein was purified using Ni-NTA affinity chromatography (Fig. 1b), which resulted in a yield of purified protein of 50 μg/mL in phosphate buffer.

3.2. GP_2 expression in normal ovine tonsils and NALT tissue

Hyperimmune sera raised against GP_2 in rats could detect GP_2 in ELISA in a dose-dependent manner (Fig. 2), confirming the reactivity of Abs to GP_2 at dilutions ranging from 1/20 to 1/250. The IgG fractions

purified from sera of GP_2 immunized rats yielded 1.1 mg/mL, while normal rat serum yielded 0.33 mg/mL.

Histologically, epithelial cells of the tonsils could be distinguished from the underlaying lamina propria, which has a follicular distribution of lymphocytes (Fig. 3a). The lymphoid cells in the sub-mucosal compartment were very densely packed in comparison to epithelial cells in the epithelium above it. Tubular invaginations of the epithelium in the form of crypts divide the tonsils into various planes and segments. Between the crypts lymphoid tissue in the form of nodules could be identified.

Immuno-histochemistry demonstrated the presence of M cells in the epithelium of tonsils as well as in the cryptic invaginations (Fig. 3b) while the negative control using normal rat serum was negative (Fig. 3b). However, there was no staining in the regions of the lymphocyte-rich sub-mucosa with either of the sera. Stratified squamous epithelium of the anterior oral cavity did not show any staining with anti-GP_2 Abs.

M cells were clearly distinguishable from adjacent epithelial and goblet cells through immunofluorescence staining based on anti-GP_2 and alexa flour 594 secondary staining (Fig. 4a). Immuno-staining with control normal rat serum Abs was found to be negative (Fig. 4b). M cells were also found in epithelium of the palatine tonsils, pharyngeal tonsils as well as tubal tonsils and in the FAE of the scattered lymphoid nodules over the base of the nasopharynx (data not shown).

Taken together these data show that the anti-sera against GP_2 could distinguish M cells from epithelial and goblet cells, which are present in much larger numbers in nasal surfaces. Immuno-histochemistry and immuno-flourscence using purified anti-GP_2 Ig, clearly indicated the presence of M cells which are present intermittently in lower numbers, among the epithelial cell population, suggesting that GP_2 could be a marker for M cells in sheep NALT tissues. We also found that GP_2 is present in the tonsil tissues of nasopharynx (pharyngeal and tubal tonsils), oropharynx (palatine tonsils) and FAE associated with the NALT tissue in sheep.

4. Conclusion

Recombinant sheep GP_2 protein was produced in HEK expression system. Anti-GP_2 IgGs were successfully used to detect recombinant GP_2 protein by ELISA and dot blot. GP_2 has been identified to be ubiquitous M cell marker of NALT tissues (FAE of NALT as well as in all the tonsils) in sheep.
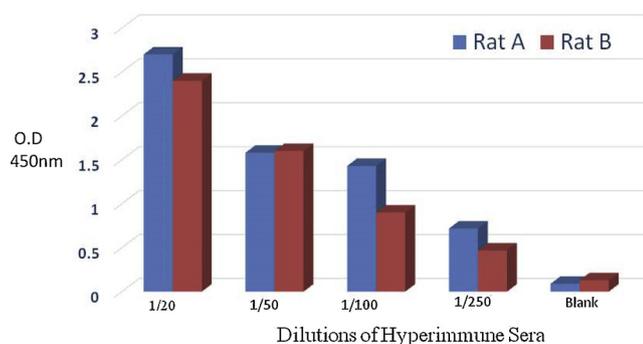
Fig. 2. Recognition of purified GP_2 by hyperimmune sera from immunized rats in ELISA. The ELISA O.D. is shown in function of the dilution of the sera.
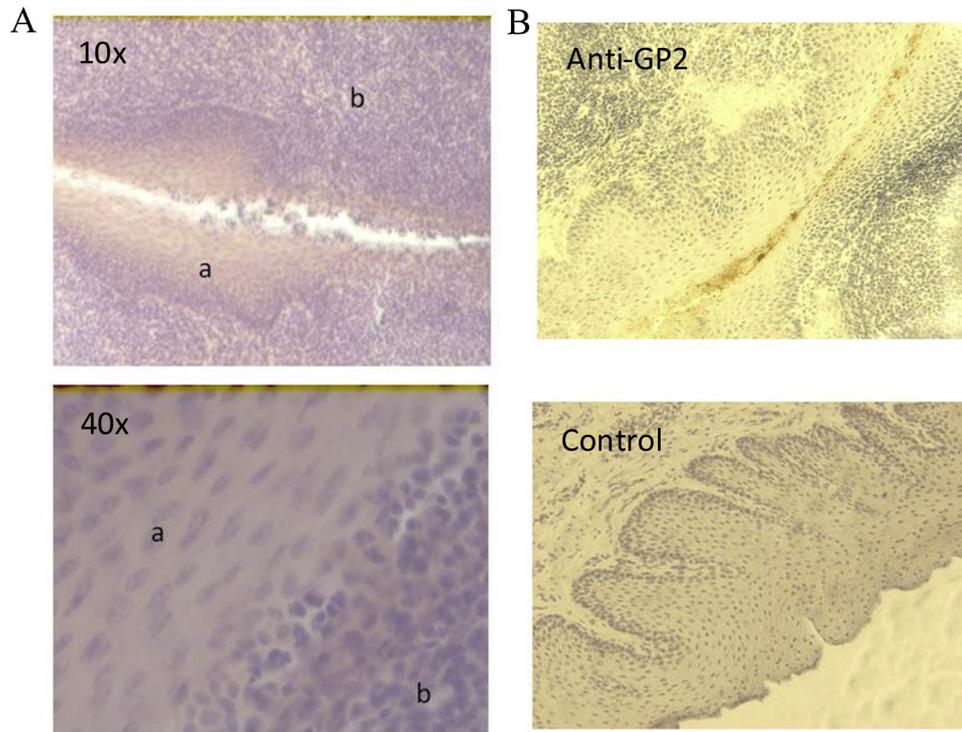
Fig. 3. (A) Histological staining of palatine tonsils showing epithelium (a) and lymphocytic follicles (b) and (B) immuno-histochemistry of palatine tonsils (20x) demonstrating staining with anti-GP$_2$ antiserum (top panel) and control normal rat serum (bottom panel).
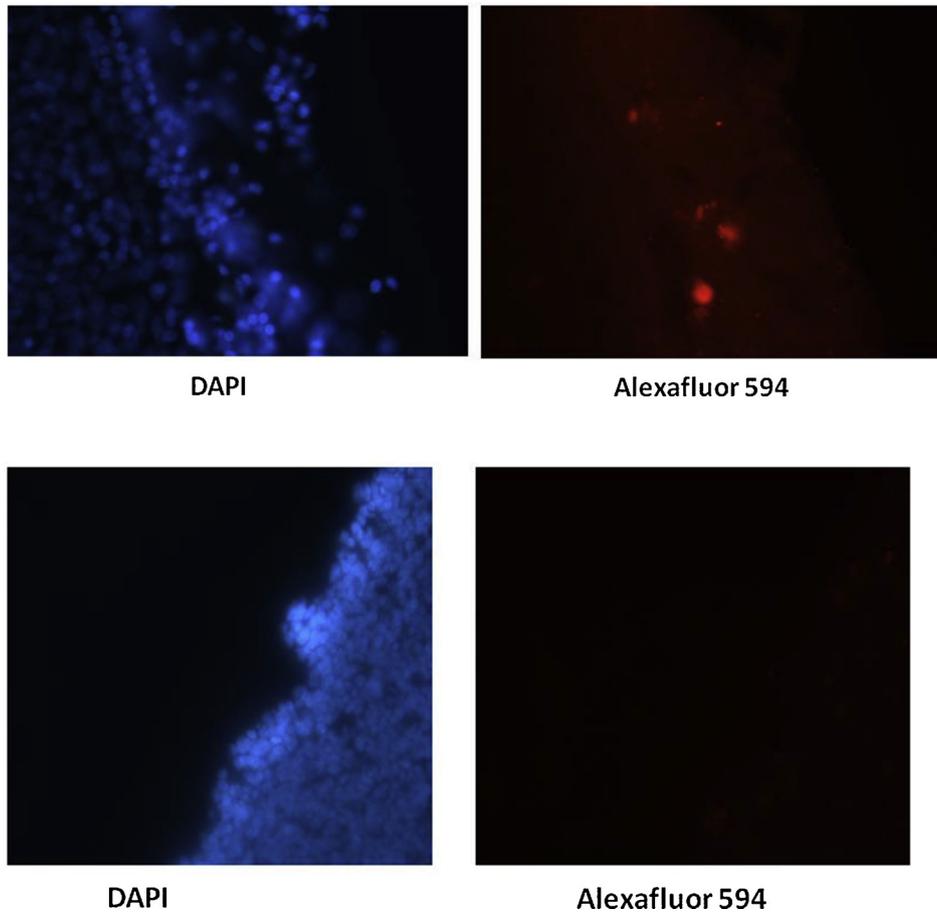
Fig. 4. Immuno-fluorescence of palatine tonsils stained with DAPI to demonstrate the presence of tissue (left panels). Same sections stained with secondary Ab labeled with Alexafluor 594 (right panel) in the presence of anti-GP$_2$ antiserum (top panel) and normal rat serum (bottom panel).

Acknowledgments

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