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Published
2006

Journal Title
Infection and Immunity

DOI
https://doi.org/10.1128/IAI.74.1.625-631.2006

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Title:

Microbial Pattern Recognition Receptors Mediate M-Cell Uptake of Bacteria

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Abstract:

The receptors involved in the sampling of particulate microbial antigens by the gut are largely unknown. Here we demonstrate for the first time in an in vitro M-cell model and in situ in isolated murine intestinal segments that the pattern recognition receptors, TLR-4, PAF-R and α5β1 integrin are all involved in mediating bacterial uptake. The pattern of TLR-4 and α5β1 integrin expression differed on M-cells and enterocytes. Inhibition of TLR-4 and PAF-R, but not TLR-2, reduced bacterial uptake by both cell types whereas inhibition of the apically expressed α5β1 integrin was very significant for the M-cell translocation of bacteria. Hence, the involvement of each receptor was dependent on functionality, upregulation and localisation suggesting that pathogen-associated molecular pattern interactions with pattern recognition receptors are key factors in M-cell recognition of intestinal antigens for mucosal immune priming.

Text:

Uptake of particulate microbial antigen by epithelial cells of the gastrointestinal tract resulting in stimulation of the mucosal immune system has long been thought of as a specific receptor driven event. These receptors are considered to be specific to the microfold (M)-cells that are found within the follicle-associated epithelium (FAE) that overlie the mucosal immune inductive tissue of the Peyer’s patches of the intestine (1-3). Researchers have demonstrated that interactions between pathogenic bacteria and the gut epithelia rely on several pathways, such as receptor-mediated uptake as demonstrated by: Yersinia enterocolytica invasin-β1 integrin interactions (4); type III secretion systems such
as the translocated intimin receptor, Tir, -dependent disruption of the intestinal epithelia by enteropathogenic *Escherichia coli* (5); and, the paracellular invasion strategy used by *Campylobacter enteritis* (6). However, the species-specific variability of potential M-cell receptors (7) and a failure to find receptors that are specific to human M-cells that are not present elsewhere on the gut lining has thwarted research efforts to target mucosal antigen uptake for the induction of mucosal immune responses.

Prokaryotes contain molecular motifs that are unique to microorganisms. These motifs, known as common microbial pathogen-associated molecular patterns (PAMPs), are recognised by the innate immune system through pattern recognition receptors (PRRs) either, through direct receptor-bacterial ligand or endogenous adaptor-bacterial molecule interactions (8). For example, toll-like receptor (TLR)-4, in association with CD14 and MD-2, recognises lipopolysaccharide (LPS) expressed by Gram negative bacteria (9); TLR-2 recognises lipotechoic acids expressed by Gram positive bacteria (10); platelet-activating factor receptor (PAF-R) binds to the phosphorylcholine moiety of the surface lipooligosaccharide bacterial molecules (11); and, α5β1 integrin binds, through endogenous fibronectin, to fibronectin-binding proteins expressed by many bacteria (12). To date, with the exception of α5β1 integrin, the expression of PRRs by follicular associated epithelial cells has not been described and neither has their involvement in gut microbial particulate antigen sampling been demonstrated. We approached this challenge from two directions and have now identified specific receptors for particulate microbial antigen uptake and functional differences that allow M-cells rather than enterocytes to recognise and deliver
antigen to the underlying gut-associated lymphoid tissues (GALT). Our development of a validated in vitro M-cell model (13) has allowed us to describe the relative levels of four specific PRRs on M-cells, demonstrate their involvement in antigen sampling and substantiate these results in situ in a mouse intestinal segment model.

Results and Discussion
To study the bacterial-M-cell interactions in vitro, we modified the M-cell model originally described by Kerneis (14). This model consisted of a bicameral co-culture of human enterocyte-like Caco-2 cells and freshly isolated murine Peyer’s patch cells, separated by a semi-permeable membrane (13). These in vitro M-cells were shown to display many characteristics exhibited by M-cells in vivo, such as reduced apical expression of digestive enzymes and enhanced energy dependent transcytosis of particulates.

Expression intensity and location of PRRs differs on M-cells compared to enterocytes
The expression and function of three PRRs, TLR-2, TLR-4 and PAF-R, were examined in M-cell co-cultures and Caco-2 monocultures by immunofluorescence microscopy and quantitative image analysis (Fig 1). TLR-4 expression was significantly upregulated in the in vitro M-cell co-cultures compared to the Caco-2 cell monocultures. In contrast, TLR-2 and PAF-R were equally expressed in both culture systems. These in vitro findings were substantiated in situ by immunofluorescence photomicrography of paraffin-embedded sections of murine villi and FAE (Fig 2). In vivo and in vitro observations showed that TLR-4 expression was restricted to the apical surfaces of M-cells. In contrast, PAF-R was abundantly expressed throughout the murine intestine and TLR-2 was poorly expressed in
situ and it was not possible to obtain quality photomicrographs. Visual examination of the sections indicated that there was no apparent difference in the expression of TLR-2 by M-cells and enterocytes.

While this is the first study to identify the distribution of TLR-2, TLR-4 and PAF-R, in an M-cell model in vitro and by M-cells and FAE overlying the Peyer’s patches, in situ, earlier studies have examined the expression of these receptors in the intestinal villi (15, 16). These earlier studies demonstrated low level expression of TLR-2 and TLR-4 in the villi of the jejunum and these patterns of receptor expression were considered to reflect hyporesponsiveness of the mucosal immune system in response to the high levels of immunostimulatory bacterial products, such as LPS, within the lumen of the gut (16). In contrast, the Peyer’s patches are immune inductive sites and one may expect that the expression of receptors involved in the generation of immune responses could be upregulated. We have demonstrated that the expression of the LPS receptor, TLR-4, is upregulated in vitro in M-cell co-cultures and on the apical surfaces of murine M-cells and hence may play an important role in the induction of mucosal immune responses to Gram negative bacteria.

Expression of the lipotechoic acid receptor TLR-2 was low in both co-cultures and monocultures and barely detectable in murine villi and Peyer’s patches. While this pattern of expression suggests a bias in the gut for providing mechanisms for inducing immune responses against Gram negative bacteria, receptors, such as PAF-R, can bind both Gram negative and Gram positive bacteria (11, 17, 18). Low levels of TLR-2 expression by
intestinal epithelial cells, including on Caco-2 cells, has also previously been reported and these cells were found to be broadly unresponsive to a range of Gram-positive ligands (19).

**M-cells preferentially transport whole killed cell bacteria**

Oral immunisation as a vaccination route is not a new concept and has significant potential application, however, this approach for widespread use in vaccine delivery has been limited by the challenges in targeting such vaccines to the GALT, and more specifically to M-cell antigen sampling receptors. It is well established that oral immunisation of humans and animals with whole killed cell non-typeable *Haemophilus influenzae* (NTHi) induces protection against acute exacerbations of chronic obstructive pulmonary disease (COPD) in humans (20) and enhances bacterial clearance in animals challenged with live NTHi in the middle ear and lung (21). The intestinal antigen sampling mechanisms responsible for the induction of these immune processes have not yet been elucidated. Therefore, we hypothesised that the intestinal sampling of NTHi, and hence induction of protective mucosal immune responses, was initially mediated by PRRs on the surface of M-cells.

To provide evidence in support of this hypothesis, we incubated formaldehyde-killed chloromethylfluorescein diacetate (CMFDA) labelled NTHi in the lower chambers of M-cell co-cultures and Caco-2 monocultures and used flow cytometry analysis to measure the number of bacteria moving from the lower to the upper chambers, representing transcytosis from the apical to the basolateral surfaces of the cells. In this model, NTHi was preferentially transported by the M-cell co-cultures (Fig 3A).
Blocking of PRRs specifically inhibits whole killed cell bacterial transport

Blocking of specific receptors with either receptor specific antibody or a mimetic antagonist, demonstrated that blocking of the PRRs TLR-4 and PAF-R similarly reduced the capacity of both M-cell co-cultures and Caco-2 monocultures to translocate NTHi (Fig 3B and 3C). In contrast, blocking α5β1 integrin did not affect translocation of NTHi by the Caco-2 monocultures, but did significantly inhibit the translocation by M-cell co-cultures. Inhibition of TLR-2 did not affect the translocation of NTHi in either culture system, an expected outcome for a Gram negative microorganism.

In situ studies using isolated murine intestinal loops concurred with the in vitro data for TLR-4 and PAF-R. Unlike the Caco-2 cell monocultures, blocking of α5β1 integrin did reduce translocation of NTHi across the intestinal epithelia, however, a greater reduction was observed in the Peyer’s patches confirming the importance of α5β1 integrin in M-cell sampling and transport to the Peyer’s patches. Inhibition of these PAMP–PRR interactions demonstrates their importance in the uptake of bacteria from the intestinal lumen, in particular the significance of α5β1 integrin in the translocation of antigen through the M-cells to the GALT.

PRR location a major factor in whole killed cell bacterial transport

The level of PRR expression was not the sole indicator of receptor functionality for the uptake of NTHi by M-cells. Our previous study demonstrated that α5β1 integrin relocates from basolateral and lateral surfaces to the apical surface (13) with differentiation of Caco-
2 cells into M-cells which is a classical differential characteristic between M-cells and enterocytes (22). While the pattern of expression of TLR-4 (Fig 1F) and α5β1 integrin (13) differed on M-cells and enterocytes in vitro, it was only the increase in apical expression of α5β1 integrin and not TLR-4 (Fig 3) that was shown to enhance the translocation of NTHi across M-cells. This suggests that upregulation of receptor expression alone does not necessarily reflect enhanced functionality and that it is both the expression and location of this receptor on the apical surface that is critical for M-cells to transcytose NTHi. Such differential functionality may reflect downstream intracellular signalling initiated by the different receptors on the M-cells.

In conclusion, our data suggest that there are multiple PAMP-PRR interactions that ensure adequate redundancy in M-cell antigen sampling so that immune priming can occur in response to specific pathogens. In support of our hypothesis that the protective responses following oral immunisation with killed NTHi were initially mediated by PRRs, the PRR α5β1 integrin was the most significant receptor associated with translocation of NTHi through M-cells. In developing prophylactics and therapeutics, where M-cell recognition is important for mucosal immune priming, the ability to target and optimize PAMP-PRR interaction is important.
Materials & Methods

Cell Culture

The growth of the Caco-2 and the culture of M-cells was performed as described by Tyrer et al (2002). Briefly, Caco-2 cells were cultured in 75 cm² tissue culture flasks in Caco-2 Medium (Dulbecco’s Modified Eagles Medium (DMEM) with GlutaMax I, 4,500 g/l glucose and pyridoxal (Invitrogen, Mount Waverley, Victoria Australia) supplemented with 10 % v/v foetal calf serum (CSL, Parkville, Victoria, Australia), 1 % v/v non-essential amino acids (Invitrogen) and, 1 % v/v antibiotic and antimycotic (Invitrogen)) in a humidified atmosphere at 37 °C in 5 % CO₂, until they had reached confluence. After trypsinisation of the Caco-2 cells, the cells were seeded onto the uppermost surfaces of inverted 6.5 mm, 3.0 μm pore, polycarbonate Transwells (Corning Costar, Lindfield, NSW, Australia) and incubated overnight at 37 °C to allow the cells to attach. The Transwells were reinserted into their 24-well dishes and cultured in Caco-2 Medium for 21-28 days to allow the cells to fully differentiate. Prior to co-culture, the transepithelial electrical resistances of the cells were checked by use of an EVOM (World Precision Instruments, Coherent Scientific Pty, Ltd, Hilton, South Australia) and cultures with readings greater than 150 ohms.cm² were considered suitable for co-culture.

M-cell cocultures were produced by introducing 0.1 ml of 10⁶ cells/ml freshly isolated murine Peyer’s patch cells in Caco-2 medium. The cultures were then incubated at 37 °C in 5 % CO₂ for two days, prior to experimentation.
Identification and Quantification of PRRs In Vitro

Culture media from the 2 day-old M cell cocultures and Caco-2 monocultures were discarded and the cells were washed 3 times with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen). The cells were then fixed in 10% neutral buffered formalin for 10 minutes, and then washed again. Non-specific binding sites were blocked by incubation of the samples in 2% w/v bovine serum albumin (Sigma, Sydney, NSW, Australia) in DPBS for 1 hour at 37 °C. The samples were probed with 1μg/ml each of rabbit anti-TLR-2 (Zymed, Bio-Scientific Pty, Ltd, Gymea, NSW, Australia), anti-TLR-4 (Zymed) or anti-PAF-R (Cayman Chemical, Sapphire Bioscience Pty Ltd, Crows Nest, NSW, Australia) for 1 hour at 37 °C, then washed three times in DPBS. Primary antibodies were probed with donkey anti-rabbit Alexa 488 conjugate (1:200; Molecular Probes, Mount Waverley, Victoria Australia) for 1 hour at 37 °C, before washing and counterstaining with 10 μg/ml 4',6-diamidino-2-phenyindole (Sigma) for 15 minutes at room temperature. The Transwell membranes were then excised with a scalpel blade and mounted onto poly-L-lysine coated microscope slides. Glass coverslips were mounted with ProLong AntiFade Kit (Molecular Probes) according to the manufacturer’s instructions.

The slides were imaged by fluorescence microscopy and a MicroMax cooled CCD camera (Princeton Instruments, Trenton, New Jersey, USA). The total area of staining of TLR-2, TLR-4 and PAF-R for each slide was analysed by MetaMorph software (Universal Imaging Corporation Ltd, Marlow, Buckinghamshire, UK). Three areas of five Transwells for each receptor and culture system were analysed. A student’s t-tests was used to test for statistical
significance of differences (SPSS software). P values less than 0.05 were considered to be significant.

**Identification of PRRs in Murine Intestine**

Eight week-old male specific pathogen free Balb/c mice were killed by cervical dislocation and their intestines were excised. Rings of tissue approximately 5 mm in length from a section of the jejunum proximal to the stomach containing Peyer’s patches and villus epithelium were removed and fixed overnight in 10% v/v neutral buffered formalin. Tissue samples were then processed for paraffin embedding with a Hypercenter XL tissue processor and 5 μm sections were cut from the embedded material with a microtome (model HM325, Microm GmbH, Waldorf, Germany), and mounted onto poly-L-lysine coated microscope slides. The samples were stored at 4 °C until further processing. The samples were then dewaxed and rehydrated according to standard procedures before immunofluorescence staining.

To unmask antigens that were cross-linked by the fixation procedure and incapable of binding to primary antibodies, the sections were subjected to antigen retrieval. A 10x stock of pH 7.8 citrate buffer (containing 3.2 g/l sodium citrate, 5.0 g/l sodium EDTA, 2.5 g/l Tris base) was diluted 1/10 and 500 μl Tween-20 added. This solution was dispensed into 30 ml microscope slide staining chambers and immersed into a water bath at 95 °C to equilibrate. Once the citrate buffer solution had reached 95 °C, the slides were inserted into
the chambers and immersed into the water bath for 20 minutes. The slides were then removed and rinsed three times in deionised water.

Non-specific binding sites in the tissue sections were blocked in blocking buffer (2 % w/v BSA in D-PBS) for 1 hour at 37 °C, and then primary antibodies directed against TLR-2, TLR-4 and PAF-R were added at a concentration of 1 μg/ml in blocking buffer and the samples incubated for 1 hour at 37 °C. After washing 3 times in D-PBS, secondary conjugate antibodies (1 in 200 dilution) and 1 μg/ml TRITC *Ulex europeaus* I lectin (UEA-1; to identify murine M-cells; Sigma), each in blocking buffer, were added for 1 hour at 37 °C. Control sections were stained with only conjugate to control for non-specific staining by the secondary antibodies. After washing the samples, the sections were counterstained with 1 μg/ml DAPI for 15 minutes, washed in deionised water, and then coverslips were mounted with ProLong mounting medium. The media was allowed to set overnight before the slides were sealed with clear nail varnish. The slides were photographed as described above for the *in vitro* samples.

**Receptor Antagonism of In Vitro Bacterial Translocation**

NTHi was grown overnight on brain heart infusion agar at 37 °C in 5 % CO₂, then harvested. The bacteria were labelled with 10 μM chloromethylfluorescein diacetate (CMFDA; Molecular Probes) in D-PBS overnight at 37 °C, then washed in D-PBS. The bacteria were killed by suspension in 1 % v/v formaldehyde for 2 hours at 37 °C, and then
washed three times. The killed, labelled bacteria were stored at 4 °C, protected from light, until required (within 1 week).

The lower chambers (apical surface) of the M-cell co-cultures and Caco-2 monocultures were incubated in the presence of the specific receptor antagonists in Caco-2 medium for 1 hour at 37 °C, in 5 % CO₂, as indicated in Table S1.

Table S1: Concentrations of Specific Receptor Antagonists used to Inhibit Bacterial Translocation.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Anti-TLR-2 receptor antibody</td>
<td>0.5 µg/ml</td>
</tr>
<tr>
<td>Anti-TLR-4 receptor antibody</td>
<td>0.5 µg/ml</td>
</tr>
<tr>
<td>PAF-16, antagonist</td>
<td>10 µM</td>
</tr>
<tr>
<td>Anti-α5β1 integrin antibody</td>
<td>0.5 µg/ml</td>
</tr>
<tr>
<td>Control</td>
<td>No antagonist</td>
</tr>
</tbody>
</table>

The inhibitor solutions were replaced with 600 µl of 5 x 10⁸ cfu/ml of CMFDA labelled killed NTHi in respective inhibitor solution and the cultures incubated in a shaking incubator (Bioline, Edwards Instrument Company) at 37 ºC for 2 hours. The numbers of bacteria translocated from the lower chamber (apical surface) to the upper chamber (basolateral surface) was analysed by counting labelled NTHi in the upper chamber media.
by a Coulter EPICS-XL/MCL flow cytometer. The Mann-Whitney test was used to
determine statistical significance, with p values less than 0.05 considered significant.

**Receptor Antagonism of Ex Vivo Bacterial Translocation**

Male specific pathogen free Balb/c mice were killed by cervical dislocation and their
intestines were excised above the caecum. 4 cm lengths of jejunum, just below the stomach
and containing at least one Peyer’s patch, were removed. One end of each isolated intestinal
segment was closed by tying with suture thread. The intestinal segments were filled by
injection with labelled NTHi (3 x 10^{10} cfu/ml) in HEPES buffered Kreb’s Ringer
bicarbonate solution (20 mM HEPES, 107 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 1 mM
MgSO₄, 7 mM NaHCO₃, 0.1 % w/v BSA and 10 mM glucose) with antagonist
(concentrations indicated in Table S1). Control segments were filled with only bacteria and
buffer. The open end was tied with suture and the segments incubated in the aerated
HEPES buffered Kreb’s Ringer bicarbonate solution for two hours at 37 °C. One segment
was used for each combination of antagonist and bacteria

Following incubation, the intestinal segments were removed and the Peyer’s patches cut
out. The Peyer’s patch segments were fixed, processed, paraffin embedded and mounted on
poly-L-lysine coated slides. The samples were washed in deionised water prior to
immunofluorescence staining. The tissue sections were permeabilised in 0.05 % Tween-20
for 15 minutes at room temperature to allow access to intracellular antigens. The slides
were then blocked in 2 % v/v BSA in D-PBS to block non-specific binding by antibodies.
The CMFDA dye, conjugated to bacterial proteins, was probed with 1 μg/ml anti-
fluorescein antibody in 2 % BSA in D-PBS followed by 1 µg/ml Alexa 488 conjugate. M-cells were detected with 1 µg/ml TRITC UEA-1. The slides were counterstained with 1 µg/ml DAPI and coverslips mounted with the ProLong Antifade Kit and allowed to set overnight before sealing with nail varnish.

The slides were observed with a Nikon TE300 inverted epifluorescence microscope using a 100x PlanFluor objective. The degree of uptake of bacteria by Peyer’s patches and villus segments in untreated and treated intestines was measured by scoring each section between 0 and 5 - described in Table S2. These data are presented as mean score ± standard error of the mean. Statistical significance between samples was tested by use of the 1-sided multivariate General Linear Model with Dunnet’s post hoc analysis with SPSS software. P values less than 0.05 were considered statistically significant.

Table S2: Scoring system for analysis of uptake of whole-cell killed bacteria into murine Peyer’s patches.

<table>
<thead>
<tr>
<th>Score</th>
<th>Degree of Uptake (number of bacteria seen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Greater than 50 bacteria detected</td>
</tr>
<tr>
<td>4</td>
<td>16-50 bacteria detected</td>
</tr>
<tr>
<td>3</td>
<td>6-15 bacteria detected</td>
</tr>
<tr>
<td>2</td>
<td>3-5 bacteria detected</td>
</tr>
<tr>
<td>1</td>
<td>1 or 2 bacteria detected</td>
</tr>
<tr>
<td>0</td>
<td>No bacteria detected</td>
</tr>
</tbody>
</table>
Acknowledgements:

The authors would like to thank Professor Robert Clancy and Dr Paul Pavli for critical reading of the manuscript. This work was funded by an ARC SPIRT grant and PT was supported by Provalis plc, UK.

The authors have no conflicting financial interests.
References:


Figure Legends:

Figure 1. Expression of PRRs by *in vitro* M-cell co-cultures and Caco-2 monocultures as measured by immunofluorescence microscopy and image analysis with MetaMorph software.

A-C shows expression of TLR-2. TLR-2 was probed with anti-TLR-2 antibody and antibody conjugated Alexa 488 (green). (A) Expression of TLR-2 in M-cell co-cultures, (B) expression of TLR-2 in Caco-2 monocultures, and (C) image analysis of expression of TLR-2. Image analysis demonstrates that there was no difference in expression between the culture systems (*p* = 0.217). D-F shows expression of TLR-4. TLR-4 was probed with anti-TLR-4 antibody and antibody conjugated Alexa 488 (green). (D) Expression of TLR-4 by M-cell co-cultures, (E) expression of TLR-4 by Caco-2 monocultures, and (F) image analysis of expression of TLR-4. Image analysis demonstrates that TLR-4 was significantly upregulated in M-cells (*p* = 0.043). G-L shows expression of PAF-R. PAF-R was detected with anti-PAF-R antibody and antibody conjugated Alexa 488 conjugate (green). (G) Expression of PAF-R by M-cell co-cultures, (H) expression of PAF-R by Caco-2 monocultures, and (I) image analysis of PAF-R expression. Image analysis demonstrated that PAF-R was expressed equally by both cell culture systems (*p* = 0.432). In each photomicrograph, nuclei are counterstained blue with DAPI. Size bar indicates 100 μm.
Figure 2. Expression of PRRs by murine M-cells and enterocytes as shown by immunofluorescence microscopy.

M-cells were identified by the M-cell specific *Ulex europeaus* (I) TRITC conjugate (red). TLR-4 and PAF-R were probed with anti-TLR-4 and anti-PAF-R, and stained with antibody conjugated Alexa 488 (green). (A) TLR-4 (green) was expressed on the apical surfaces of murine M-cells (red) and intracellularly in adjacent follicle-associated enterocytes. Size bar indicates 5 μm. (B) PAF-R (green) was expressed ubiquitously throughout the murine intestine including M-cells (red). The section is counterstained blue with DAPI. Size bar indicates 50 μm.

Figure 3. NTHi translocation by M-cell co-cultures and Caco-2 monocultures.

Translocation of fluorescently labelled NTHi from the lower chamber (apical surface) to the upper chamber (basal surface) was measured by flow cytometry analysis of the culture media. (A) M-cell co-cultures translocated more NTHi than Caco-2 monocultures (p=0.009). (B) In the M-cell co-cultures, specific blocking of TLR-4 (p = 0.046), PAF-R (p = 0.004) and α5β1 integrin (p = <0.001) inhibited transcytosis of killed NTHi. (C) In the Caco-2 monocultures, blocking of TLR-4 (p = 0.017) and PAF-R (p = <0.001) inhibited translocation of NTHi by Caco-2 cells. ★ denotes significantly different to control.
Figure 4. Blocking PRRs with specific receptor antagonists inhibits translocation of NTHi \textit{in situ} in mouse intestinal segments.

Specific blocking of (A) TLR-4 (p = 0.004), PAF-R (p = 0.013) and $\alpha 5\beta 1$ integrin (p < 0.001) inhibited uptake of NTHi into Peyer's patches, and (B) TLR-2 (p = 0.003), TLR-4 (0.001), PAF-R (p = <0.001) and $\alpha 5\beta 1$ (p = <0.001) integrin inhibited uptake of NTHi into the intestinal villus. $\star$ denotes significantly different to control.
Figure 1.

| M-Cell Cocultures | Caco-2 Monocultures | Image Analysis |
Figure 2.
Figure 3.
Figure 4.

A

B