A human intestinal M-cell-like model for investigating particle, antigen and microorganism translocation

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The specialized microfold cells (M cells) in the follicle-associated epithelium (FAE) of intestinal Peyer's patches serve as antigen-sampling cells of the intestinal innate immune system. Unlike 'classical' enterocytes, they are able to translocate diverse particulates without digesting them. They act as pathways for microorganism invasion and mediate food tolerance by transcellular transport of intestinal microbiota and antigens. Their ability to transcytose intact particles can be used to develop oral drug delivery and oral immunization strategies. This protocol describes a reproducible and versatile human M-cell-like *in vitro* model. This model can be exploited to evaluate M-cell transport of microparticles and nanoparticles for protein, drug or vaccine delivery and to study bacterial adherence and translocation across M cells. The inverted *in vitro* M-cell model consists of three main steps. First, Caco-2 cells are seeded at the apical side of the inserts. Second, the inserts are inverted and B lymphocytes are seeded at the basolateral side of the inserts. Third, the conversion to M cells is assessed. Although various M-cell culture systems exist, this model provides several advantages over the rest: (i) it is based on coculture with well-established differentiated human cell lines; (ii) it is reproducible under the conditions described herein; (iii) it can be easily mastered; and (iv) it does not require the isolation of primary cells or the use of animals. The protocol requires skills in cell culture and microscopy analysis. The model is obtained after 3 weeks, and transport experiments across the differentiated model can be carried out over periods of up to 10 h.

INTRODUCTION

In vitro models of the intestinal barrier have been commonly used to study the permeability of orally administered drugs and the transport of newly developed formulations (e.g., nanoparticles). Permeability studies of drugs have been performed mainly on enterocyte-like cell monolayers, typically obtained by growing Caco-2 cells on permeable supports[1]. However, the intestinal barrier contains another cell type, M cells, which are part of the mucosal immune system and are predominantly located in the FAE of Peyer's patches. They are specialized epithelial cells with the ability to transport particulate matter, including antigens, bacteria and viruses, from the lumen (apical side of cells) to the underlying lymphocyte pocket (transcytosis). M cells therefore have an important role in food tolerance and mucosal immune response induction, but they are also exploited by many pathogens as a route of intestinal invasion. Because of their high transcytotic capacities and their ability to transport a broad range of materials, including therapeutic microparticles and nanoparticles, these cells have potential in the oral delivery of encapsulated therapeutic peptides and vaccines[2,3].

M cells are highly variable in proportion and phenotype between different species[4]. The limited availability of human tissues for research purposes and the lack of success in maintaining differentiated primary human intestinal M cells in culture are two of the reasons why an *in vitro* model of human M cells containing 'M-like' cells is essential to understanding human M-cell physiology and function. A major challenge regarding M-like cell models is to obtain reproducible levels of M-like-cell function on every filter, but a key advantage is the use of human cell lines and not primary cell cultures.

To obtain a model for human M cells, enterocyte-like Caco-2 cells were cocultured with Raji cells (a human B-lymphocyte cell line)[5,6]. Although it is well accepted that Raji cells are responsible for Caco-2 cell conversion to M-like cells, the biochemical factors that mediate this conversion are currently unknown. It seems that inflammatory mediators could be involved, but further studies are required to clearly identify the responsible molecules[7,8]. The two cell types are expanded separately. Caco-2 cells are initially seeded on Matrigel (a basement membrane preparation rich in extracellular matrix proteins)-coated cell culture filter inserts (apical side), where they are differentiated for ~14 d. From that point, the inserts are either maintained in their normal orientation[6,9] or inverted[10] (as performed in this protocol) to mimic the physiological condition in which B lymphocytes come into direct contact with enterocytes via migration through the filter pores. Raji cells are then placed in the basolateral compartment and cocultured with Caco-2 cells for 5 d. The conversion of Caco-2 cells to M-like cells is then assessed by a set of electrophysiological, histological, biochemical and functional readouts—e.g., decreased transepithelial electrical resistance (TEER), increased nanoparticle translocation, loss of microvilli, decreased apical alkaline phosphatase activity and increased bacterial adherence and translocation.

Development of the M-like cell *in vitro* model and comparison with other methods

The first M-cell *in vitro* model to be reported was developed by Kerneis *et al.*[5] This model consisted of a 'mixed' coculture of (human) Caco-2 cells, seeded on the basolateral side of porous Transwell inserts, with mouse B lymphocytes isolated from Peyer's patches added to the apical compartment. The authors observed that B lymphocytes presenting B-cell markers triggered a higher transcytotic activity of particles. To obtain a model based strictly on

human cells and that is easier to set up, Gullberg *et al.*[6] replaced the mouse primary B lymphocytes with a human cell line (Raji cells), thus enabling a fully human model. A human M-like cell model could enable the identification of surface markers, allow for the study of the mechanisms involved in microorganism translocation and contribute to the development of drug and vaccine delivery systems that target M cells[11–13]. For this purpose, Caco-2 cells were seeded on the upper side of the Transwell insert in a normal configuration, and Raji cells were grown on the lower side. This model had the advantage of keeping the two cell types separated to enable gene expression studies in a search for M-like cell surface targets for ligands that could be used in oral vaccine studies[14]. Moreover, this method allowed the study of the impact of B-cell-released type 1 cytokines and other growth factors, including interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), on nanoparticle transport[8]. This 'inverted' protocol addresses two main limitations encountered when working with the 'non-inverted' Gullberg model: (i) inconsistencies between different experiments in the functionality of the M-cell model regarding nanoparticle transport and (ii) lack of intimate contact between Caco-2 cells and Raji cells as observed *in vivo*[10].

The inversion of the inserts has resulted in increased model reproducibility and allowed for the creation of an environment closer to the *in vivo* situation by placing the Raji cells in direct contact, via filter pores, with the Caco-2 cells growing on the opposite side of the membrane.

Since the development of the model described by Gullberg *et al.*[6], several groups have been working on further modifications of the initial human model. Recently, a triculture model has been proposed by two groups, combining Caco-2 cells and mucus-secreting HT29-MTX cells with Raji cells[15,16]. In addition, a murine model and a bovine/murine mixed M-cell model have been reported[17,18]. A recent paper describes a comparable triple cell culture method using Caco-2 cells, HT29 mucus-secreting cells and Raji cells. We note that when comparing our protocol with the triple-culture method, the data showing uptake and translocation of nanoparticles are very similar, with Raji cell presence being the most important factor in driving uptake[19]. Ragnarsson *et al.*[20] also reported that Caco-2 cell incubation with *Yersinia pseudotuberculosis* resulted in increased transport of nanoparticles similar to that observed with coculture M-cell models, perhaps also via an acute inflammatory trigger mechanism.

Application of the protocol

Our model is especially useful for studying (i) the transport of drug delivery particulates, (ii) the interaction of bacteria and viruses with the intestinal epithelium and (iii) M-cell physiology. The M-cell-like model is particularly adapted to the study of oral delivery of bioactive molecules in therapeutic microparticulate and nanoparticulate systems. Researchers can examine the role of M cells in oral drug or protein/peptide delivery[21], in oral vaccination[22] or in the development of drug-laden (nano)particles targeting M-cell-expressed receptors[23,24]. The interaction of bacteria and viruses (including *Salmonella* or HIV) with the intestinal epithelium can also be evaluated, especially to study their mechanisms of entry and to develop novel drugs to prevent this[25].

Box 1 | Evaluation of Caco-2 cell monolayer conversion to a functional M-cell-like cell monolayer ● TIMING 2–4 h

Here we provide guidelines for evaluating Caco-2 conversion to a functional M-cell-like cell monolayer. These procedures take ~2–4 h, depending on the method or the combination of methods selected.

1. When coculturing Caco-2 cells with Raji cells, it is necessary to ensure that the conversion of the Caco-2 monolayer to an M-cell-like monolayer has actually occurred. The easiest way to do that routinely is to compare the transport rate of commercial 200-nm fluorescent nanoparticles (FluoSpheres, see REAGENTS) by monocultures and cocultures. The converted Caco-2 monolayers are incubated with nanoparticles 5 d after addition of Raji cells, and the number of nanoparticles that cross the cell monolayer in the coculture from the apical to the basolateral side can be quantified by FACS (**Box 2**). The number of nanoparticles transported by cocultures should be higher than the number of particles transported by monocultures. In our experience, we typically detect translocation of ~80,000 nanoparticles across cocultures versus ~100 for monocultures over 120 min, an ~100-fold difference[36]. This control must be performed for each experiment.

2. Cocultures have lower TEER values than Caco-2 cell monolayers (100–150 $\Omega \cdot cm^2$ and >200–250 $\Omega \cdot cm^2$, respectively (values obtained in our laboratory when using a measurement chamber)).

3. Alkaline phosphatase (AP) activity can be estimated by incubation of the inserts with the AP substrate *p*-nitrophenyl phosphate (500 µl per insert) and then measuring optical density at 405 nm. Significant AP downregulation is observed in cocultures as compared with monocultures ($P < 0.05$).

4. Another way of evaluating the degree of conversion of Caco-2 cells to M-cell-like cells is to measure the apparent permeability (P_{app}) of the monolayers using [^{14}C]-mannitol, a paracellular marker. We observed that the P_{app} of [^{14}C]-mannitol is approximately twofold higher in normally oriented cocultures than in inverted cocultures, and in general the increased flux is reciprocal to the reduced TEER values. These P_{app} values are in the range of 10^{-6} cm/s in Caco-2 monolayers. However, these values are approximately fivefold higher in inverted cocultures[10,37].

5. Other techniques can be used to validate the conversion of Caco-2 cells to M-cell-like cells but are more difficult to routinely implement and should be used only when substantial changes are made to the culture conditions—for example, when a new batch of serum or new Caco-2 cells are used. SEM and TEM can be used to visualize morphological changes that are characteristic of M cells (e.g., lack of microvilli at the apical surface, presence of desmosomes) (**Fig. 2**). SEM analysis of the monolayer apical surface can be used to quantify the percentage of cells in cocultures that have lost the brush border.

Box 2 | Determination of nanoparticle transport in the apical-to-basal direction by monocultures and cocultures ● TIMING 30 min–2 h without the FACS analysis (add 1 h for FACS analysis); start 1 h before the experiment

1. Nanoparticle transport by cocultures is temperature-dependent, so all solutions and buffers that are going to be used during the transport experiment should be prewarmed to 37 °C (30 min minimum).
2. Prepare the donor solution at a concentration of 4.5×10^9 nanoparticles per ml in HBSS and incubate at 37 °C. When using FluoSpheres, this concentration is obtained when dispersing 5 µl of nanoparticles in 5 ml of HBSS.
3. Prepare receiver plates. Prepare 12-well plates containing 1.2 ml of HBSS per well for each insert for a total number of wells equal to the number of sampling points +1, as described in Step 7 of the PROCEDURE. Incubate the 12-well plates at 37 °C for 30 min.
4. Label the FACS tubes for nanoparticle quantification.
5. Wash the cell monolayers twice with HBSS (0.5 ml and 1.5 ml for the apical and basolateral sides, respectively) in 12-well plates and incubate them at 37 °C for 30 min.
6. Measure TEER values as in Steps 22 and 23 of the PROCEDURE.
7. Remove the HBSS from the apical side and place the inserts in the prewarmed 12-well plate prepared in step 3 of this box.
8. Add 400 µl of the donor solution to each insert apically. Be careful not to spill any of the donor solution onto the basolateral compartment (filled with 1 ml of HBSS). Keep a volume of the donor solution (500 µl) to measure the exact number of nanoparticles in the donor solution by FACS.
9. Incubate the plate at 37 °C and 10% CO_2 in a humidified chamber.
10. Transport can be evaluated as one end point or as a kinetics (nanoparticle transport can be evaluated every 30 min, for instance, or only after 2 h). For each time point, move each insert from one HBSS-containing well to a new well containing 1 ml of HBSS. Be careful not to transfer one solution to the other if a kinetics is performed. Note that the duration of the experiment can be related to the subsequent transport experiment.
11. Add 500 µl of the basolateral compartment to 2.5 µl of red beads (Sphero beads, Gentaur) (used as internal standard for FACS) and 5 µl of FBS in FACS tubes for FACS analysis. FBS is added to avoid the adherence of the nanoparticles to the tube walls.
12. To visualize the nanoparticles in the monolayer by confocal microscopy (**Box 3**), transfer the inserts to a separated 12-well plate, wash the cells in cold HBSS (0.5-ml for the apical side and 1-ml for the basolateral side) and fix them for 15–20 min at 4 °C in 4% (vol/vol), PFA if you want to mount them onto microscopy slides.

■ **PAUSE POINT** Inserts fixed in 4% (vol/vol) PFA can be kept in the refrigerator at 4 °C until they are mounted on microscopy slides. We recommend not keeping them longer than 1 week in the refrigerator.

! **CAUTION** Use PFA in a chemical hood and avoid inhalation, as this product is toxic.

13. In the remaining receiver plates, add 10 µl of FBS to each well to avoid nanoparticle adsorption on plastic (in FACS tubes). Transfer HBSS from the basolateral side of each well in the FACS tubes and add 5 µl of red beads (Sphero beads, Gentaur) per ml of cell suspension as an internal standard for FACS analysis.

Advantages and limitations of the protocol

The main advantages of this model are that it (i) is based on coculture using well-established differentiated human cell lines, (ii) is reproducible under the conditions described in this protocol, (iii) can be easily mastered and (iv) does not require the isolation of primary cells and thus contributes to support of the three Rs (replacement, reduction and refinement)[26]. M-cell properties and characteristics vary between species, so it is critical to work with human cells. In this regard, *in vitro* models are easier to set up and to use when the objective is to compare uptake and translocation of different types of particles or their mechanisms of endocytosis, or when studying bacterial interactions with M cells. Validation of these observations can be then performed on human intestinal explants, but the model is less suitable for screening and mechanistical studies.

Inversion of the inserts prevents growth of Caco-2 cells on the basolateral side of the filter, thus averting formation of a bilayer, and it allows direct contact between the Raji cells and the Caco-2 cells, ensuring a successful conversion of Caco-2 cells to M-like cells. However, this model has some limitations, including a higher proportion of M-like cells (15–30%)[7] as compared with the proportion of M cells in Peyer's patches *in vivo* (~10% in rodents and ~5% in humans).

A recent culture system for M cells came from 'enteroid' cultures treated with the cytokine receptor activator of NF-κB (RANKL)[27]. RANKL and the transcription factor Spi-B are required for epithelial stem cell differentiation into M cells. This culture system consists of a 3D culture technique using a growth-factor-containing scaffold established from small intestinal crypts from mice[28]. This model has been used to investigate the signaling pathways involved in M-cell differentiation.

Overview of the procedure

The present protocol consists of four main parts: (i) Caco-2 cell seeding onto permeable supports (Steps 1–9), (ii) inversion of the inserts (Steps 10–14), (iii) Raji cell seeding in the basolateral compartment (Step 15) and (iv) transport studies across M cells (Steps 16–28). Part ii is illustrated by **Supplementary Video 1**. **Box 1** describes controls for evaluating the functionality of the cocultured M-like-cell model. **Box 2** describes the steps for evaluating the transport of commercial nanoparticles across M-cell monolayers. **Box 3** provides the protocol for visualization of fluorescent particles within cell monolayers by confocal microscopy. **Box 4** gives examples of the evaluation of bacterial transport across M cells.

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Box 3 | Visualization of nanoparticles in the cell monolayers by confocal microscopy ● TIMING 30 min–2 h, not including confocal analysis, which takes between 1 and 4 h, depending on the number of samples

▲ **CRITICAL** Nanoparticle localization can be visualized by confocal microscopy only if the nanoparticles are fluorescently labeled.

1. Remove fixed inserts from the refrigerator (Step 27 of the PROCEDURE and **Box 3**) and wash them twice in HBSS (0.5 ml for the apical side and 1 ml for the basolateral side) at RT.
2. To stain the cell actin cytoskeleton with phalloidin labeled with a fluorescent dye (rhodamine or Alexa 568-phalloidin if the nanoparticles are 'green', for instance), incubate each insert with 250 µl of fluorescent phalloidin (4 U/ml) in HBSS + 0.2% (vol/vol) Triton X-100 for 10 min in the dark.

! **CAUTION** Triton X-100 is used to permeabilize the cell membranes, but it is also known to remove the signals of certain dyes from the carbocyanine family (e.g., DiD)[38]. In these cases, Triton X-100 should not be used, and the incubation time should be prolonged from 10 min to 2 h.

3. Wash the inserts gently with PBS (0.5 ml for the apical side and 1 ml for the basolateral side) and cut the filter out of the insert.
4. Place the filter on a microscopy slide, cell side up, and add a coverslip on top using a hard-setting mounting medium containing DAPI, such as VectaShield HardSet Antifade Mounting Medium (REAGENTS), to stain the cell nuclei blue.
5. Let the mounting medium polymerize overnight protected from light.

■ **PAUSE POINT** Mounted inserts can be kept at –20 °C for better preservation until confocal analysis.

6. Analyze using a confocal microscope to acquire *x–y*, *x–z* and *y–z* views of the cell monolayer to localize nanoparticles at the cell apical side, in the cells or at the cell basolateral side (**Fig. 3b**).

Experimental design

The further analytical procedures of this protocol depend on whether the transport of particles or interactions with selected bacteria/viruses with the epithelium are investigated.

Regarding the detection of fluorescent (nano)particles in the basolateral compartment, analysis can be conducted based on the nanoparticle's flux *per se* by measuring the absolute number of particles, the fluorescence associated with particles or the concentration of the encapsulated molecule. In particular, investigation of the human M-cell role in particle and antigen translocation across the intestinal barrier can be studied quantitatively by FACS or by using a fluorescence plate reader; it can be studied qualitatively by use of confocal microscopy.

Quantification by FACS analysis. The number of fluorescent nanoparticles transported to the basolateral compartment can be quantified by flow cytometry. The nanoparticle donor solution (diluted) is used to calculate the percentage of transported nanoparticles or the apparent permeability (P_{app}). It is important to note that this analysis can be done only when nanoparticle fluorescence intensity is high enough.

Quantification of nanoparticle fluorescence. The quantification of the fluorescence present in the basolateral compartment can also be done by fluorimetry. Dilutions of the nanoparticle donor solution can be used to obtain a calibration curve. This technique is easier and less time-consuming than flow cytometry. It is important to note that if the fluorescent marker is not covalently linked to the nanoparticle (i.e., when the fluorescent marker is encapsulated within the nanoparticle), the fluorescence signal measured in the basolateral compartment might correspond, at least partially, to free dye that has leaked from the nanoparticle. The rate and/or extent of release from the nanoparticle for each dye and nanoparticle, as well as nanoparticle stability, should be assessed in the same transport medium before conducting *in vitro* transport studies. The background fluorescence of the basolateral compartment (inserts cultured with medium only) must always be subtracted from the measurement. If the signal-to-noise ratio is too low, this technique is not appropriate.

Quantification of payload transport. If the nanoparticles are not fluorescently labeled, it is still possible to estimate nanoparticle transport across the cocultures by directly measuring the fluxed payload. The quantification of the cargo can be carried out using different techniques depending on its nature. For instance, (i) HPLC or liquid chromatography (LC)–mass spectrometry (MS)/MS can be used to quantify small molecules (e.g., atropine (passive transport) or digoxin (P-gp substrate)[1]) or peptides (e.g., helodermin[21]); (ii) the cargo can also be radiolabeled and quantification can be done by liquid scintillation counting, ^{14}C being the label of choice[29]; (iii) peptides and antigens can be quantified by ELISA; and (iv) antibodies can be quantified by fast protein liquid chromatography. The transport of peptides and proteins can be quantified in the basolateral compartment using specific kits and/or assays[30]. Specific ELISA kits are used in most cases. Quantification of bacterial translocation across M cells is summarized in **Box 4**. We assume that, in most cases, nanoparticles that can cross M cells and reach the basolateral side are intact. Hence, to quantify the encapsulated cargo, nanoparticles must first be disrupted. One way to disrupt these nanoparticles is by diluting the basolateral solution with an equal volume of the mobile phase used in the analytical method (e.g., HPLC or LC–MS/MS).

Visualization by confocal microscopy. Cell monolayers can be visualized using fluorescent dyes (REAGENTS). Fluorescence-labeled nanoparticles (e.g., with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate (DiD)) or bacteria (e.g., with GFP) can be localized within the cell monolayers using this technique. When quantifying the transport of fluorescent nanoparticles, the fluorescent dyes incorporated into the nanoparticles should be carefully selected, as some dyes diffuse from the nanoparticles and stain the surrounding tissues/cells[31], causing artifacts. This will preclude quantification.

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Box 4 | Study of the interaction of M-like cells with bacteria ● TIMING 4 h

Peyer's patch M cells have specific receptors for uptake and transport of bacteria (e.g., *S. typhimurium*, *V. parahaemolyticus*) and viruses (reovirus, poliovirus). M-cell-like cocultures can therefore also be used to study the translocation of bacteria and viruses. The procedures below can be used to study *S. typhimurium* adherence and internalization and to study translocation of *V. parahaemolyticus*.

***Salmonella typhimurium* adherence and internalization**

1. Using Mueller–Hinton (MH)-2 agar plates at 37 °C, a fluorescently labeled colony is used to inoculate 50 ml of MH-2 broth and grown overnight at 100 r.p.m. at 37 °C on an orbital shaker incubator. GFP-transfected *Salmonella* are used[39].
2. Inoculate 100 µl of an overnight culture into 25 ml of MH-2 broth and grow the culture for 180 min at 37 °C to obtain mid-log-phase bacteria for the M-like cell association assay (i.e., measuring both adherence and internalization). Centrifuge 1 ml of mid-log-phase bacteria at 15,000*g* for 2 min at room temperature. Wash the pellet twice with 2 ml of HBSS and resuspend it in 1 ml of HBSS.
3. Infect filter-grown monolayers and cocultures at day 21 (Step 25 of the PROCEDURE; 5 d in coculture) apically for 60 min by diluting the bacteria in an appropriate volume of HBSS to yield OD_{600} values (optical density of a sample measured at a wavelength of 600 nm) equivalent to the required multiplicity of infection (MOI) of 100 (e.g., 100 bacteria per epithelial cell). OD_{600} values are measured using a plate reader.
4. Visualize GFP-labeled *Salmonella* on inserts associated with M-like cells using the FITC filter of a fluorescence microscope. The assay does not discriminate between adhered and internalized bacteria, but this can be done effectively using an anti-*Salmonella* primary antibody followed by a TRITC-conjugated secondary antibody, neither of which can access intracellular locations and which therefore provide a measure of adhered bacteria.
5. Express the results as mean bacterial count per high-powered microscopic field (0.4 mm^2). The bacteria preferentially adhere to and invade cocultures with a high proportion of M-like cells. Sample data of coculture-associated bacteria versus monocultures are shown in **Figure 4**.

***Vibrio parahaemolyticus* translocation**

1. Grow *V. parahaemolyticus* at 37 °C in Luria–Bertani medium (LBM) and add1.5% (wt/vol) agar where appropriate.
2. Grow the bacteria to mid-log phase in LBM at 37 °C with agitation. Wash the bacteria with 2 ml of PBS, and measure OD_{600} values to determine bacterial numbers.
3. Fresh DMEM minus PEST is added 2 h before infection. In all experiments, infect the cells at an MOI = 5. Add bacteria to the apical chamber of the inserts, and incubate the monolayers at 37 °C for 1 h with agitation. After incubation, all basolateral medium is removed and replaced with prewarmed DMEM minus PEST.
4. Incubate the monolayers at 37 °C for another hour with agitation, and again collect the basolateral medium.
5. The basolateral medium is then serially diluted and plated on LBM.
6. Measure the TEER values at the 0-, 1- and 2-h time points. Incubate all LBM plates at 37 °C overnight.
7. Determine the number of translocated bacteria by plating serial dilutions from the basolateral compartments on MH-2 agar plates and calculating the number of CFU per ml. Express the results as percentage of bacteria translocated across cocultured monolayers relative to control monolayers[25].

MATERIALS

REAGENTS

- Caco-2 cells (C2BBe1 clone; ATCC, cat. no. CRL-2102) ! **CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma. ▲ **CRITICAL** Caco-2 cell passage number is critical depending on the type of experiments that are going to be conducted[32]. We thus recommend using early passages. We use Caco-2 cells between passages *x*+12 and *x*+30.
- Raji cells (ATCC, cat. no. CCL-86) ! **CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma. ▲ **CRITICAL** Raji cell passage number is critical when developing the *in vitro* M-cell model. Use only Raji cells from early passages. We use Raji cells between passages *x*+1 and *x*+10.
- *Salmonella enterica* serovar Typhimurium SL1344, GFP-labeled and unlabeled, was donated by M. Jepson, School of Medical Sciences, University of Bristol
- *Vibrio parahaemolyticus*, RIMD2210633, O3:K6 serotype sequence was defined in 2000 by Osaka University, and was described in the study by Makino *et al.*[33]
- DMEM (with 4.5 g/l D-glucose and L-glutamine and without pyruvate) (Thermo Fisher Scientific, cat. no. 41965-039). ▲ **CRITICAL** DMEM contains a sodium bicarbonate buffer system (3.7 g/l), and thus it requires a 5–10% CO_2 environment to maintain physiological pH.
- Roswell Park Memorial Institute (RPMI) medium with L-glutamine (Thermo Fisher Scientific, cat. no. 21875-034). ▲ **CRITICAL** RPMI contains a sodium bicarbonate buffer system (2.0 g/l), and thus it requires a 5–10% CO_2 environment to maintain physiological pH.
- HyClone FBS (Thermo Scientific, cat. no. SV30160.03). ▲ **CRITICAL** The FBS batch can substantially affect cell behavior. Ideally, different batches should be tested and the FBS batch that ensures the fastest Caco-2 cell growth should be selected.
- Dulbecco's PBS (Thermo Fisher Scientific, cat. no. 14190-094)
- Mueller Hinton (MH)-2 agar plates (Sigma-Aldrich, cat. no. 70191)
- Luria–Bertani medium (Sigma-Aldrich, cat. no. L3022)
- Penicillin (10,000 U/ml)–streptomycin (10,000 µg/ml) (PEST; Thermo Fisher Scientific, cat. no. 15140-122)
- Nonessential amino acids (NEAA; 100×; Thermo Fisher Scientific, cat. no. 11140-035)
- L-glutamine (200 mM; Thermo Fisher Scientific, cat. no. 25030-054)
- Hank's Balanced Salt Solution (HBSS), phenol red free (Thermo Fisher Scientific, cat. no. 14025-050)
- Trypsin-EDTA (0.05% (vol/vol); Thermo Fisher Scientific, cat. no. 25300-054)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- HEPES (Sigma-Aldrich, cat. no. H3375)

- 2-(N-Morpholino)ethanesulfonic acid hydrate (MES) (Sigma-Aldrich, cat. no. M8250)
- DMSO (Sigma-Aldrich, cat. no. D2650)
- Trypan blue solution (Sigma-Aldrich, cat. no. T8154)
- Matrigel basement membrane matrix, phenol red free (BD Bioscience, cat. no. 356237)
- Dyes for cell monolayer staining: Alexa Fluor488–phalloidin or rhodamine–phalloidin to stain cell cytoskeleton (Molecular Probes, cat. nos. A12379 and R415, respectively) and DAPI to stain cell nuclei (Molecular Probes, cat. no. D21490). ▲ **CRITICAL** Different dyes are used for the localization of the nanoparticles within the cell monolayers. The dyes selected for the visualization of the cell monolayers should be chosen based on the emission wavelength of the dye used for the nanoparticle preparation.
- Mounting medium (e.g., VectaShield HardSet Antifade Mounting Medium with DAPI (Labconsult, cat. no. VEC.H-1500))
- Solvents for analytical methods (e.g., acetonitrile for HPLC)

EQUIPMENT

- Two cell incubators (37 °C), one set to 5% CO_2 (for RPMI medium for Raji cell culture) and another set to 10% CO_2 (for DMEM medium for Caco-2 cell culture), with a water-saturated atmosphere
- Orbital shaker incubator (e.g., Heidolph Unimax 1010; VWR, cat. no. 63400-138)
- Epithelial volt/ohm meter for transepithelial electrical resistance (TEER) measurement (EVOM, World Precision Instruments) or similar device connected to a chopstick electrode or a measurement chamber. ▲ **CRITICAL** The volt/ohm meter should be charged before measuring the TEER, as the connection of the volt/ohm meter to an electric socket can alter TEER values. Equilibrate the volt/ohm meter before taking measurements.
- Laminar flow hood
- A heating plate
- Confocal microscope (e.g., Zeiss, model no. LSM 150)
- Flow cytometer (e.g., BD FACSVerse)
- Cell culture flasks, 75 cm^2 (Corning Costar, cat. no. 430641U)
- Cell culture flasks, 162 cm^2 (Corning Costar, cat. no. 3151)
- Inserts: the most commonly used inserts are Transwell 12-well filters, 12-mm diameter, 3-µm pore size, 1.12-cm^2 growth area, polycarbonate membrane (Corning Costar, cat. no. 3402). ▲ **CRITICAL** The pore size is a limiting factor that can affect the successful conversion of M cells. However, other culture scales, e.g., 6-well plates or 24-well plates, could potentially be applicable to this protocol. It should be possible to scale the model up or down, as long as the proper ratio of Caco-2/Raji cells is retained.
- 12-Well cell culture plates (Corning Costar, cat. no. 3512)
- FluoSpheres carboxylated 0.2-µm yellow-green nanoparticles (Molecular Probes, cat. no. F8811)
- Sphero fluorescent Nile red 2.27-µm particles (Gentaur, cat. no. FH-2056-2)
- Silicon tubes presenting an internal diameter of 14 mm and an external diameter of 20 mm (Labo-Moderne, cat. no. AX38824)
- Large glass Petri dishes (diameter: 185 mm, height: 40 mm). ▲ **CRITICAL** Sterilize large Petri dishes using a laboratory oven set to 180 °C for 1 h, to avoid water condensation inside the Petri dishes observed when autoclaved.
- Pipettes (volumes 1–10 µl, 10–100 µl and 100–1,000 µl)
- Flow cytometry tubes, 5-ml polystyrene 12 × 75-mm round-bottom tubes (Corning Falcon, cat. no. 352054)
- Falcon 15-ml and 50-ml tubes
- Microscope slides, Superfrost Plus, 25 × 75 × 1-mm (Thermo Scientific, cat. no. J1800AMNZ)
- Microscope cover glasses, 24 × 50 mm
- Neubauer hemocytometer (VWR, cat. no. 15170-263)
- Analytical equipment: fluorescence plate reader (e.g., SpectraMaxM3 (Molecular Devices)), scintillation counter (e.g., Packard, model no. Radiomatic FSA 150TR), HPLC or LC–MS/MS (e.g., Agilent 1100 Series HPLC system)

REAGENT SETUP

Caco-2 culture medium Prepare different media types, depending on whether the medium will be used to maintain the cells in flasks (flask medium: 500 ml of DMEM + 5 ml of NEAA + 5 ml of L-glutamine + 50 ml of FBS), to seed the cells in inserts (insert medium: 500 ml of DMEM + 5 ml of NEAA + 5 ml of L-glutamine + 50 ml of FBS + 5 ml of PEST) or to freeze the cells (Caco-2 freezing medium: flask medium (95% (vol/vol)) and DMSO (5% (vol/vol))). These media can be stored in the refrigerator at 4 °C for up to 1 month. ▲ **CRITICAL** We add antibiotics to the insert medium in order to avoid contamination during the culture in the Petri dishes. Inverting the inserts requires more manipulation than changing the flask medium, and, in addition, Petri dishes have no filtered ventilation. Therefore, the contact with the environment is more extensive, and the risk of contamination is higher in these steps. Provided that cell growth is not affected by addition of antibiotics, insert medium can also be used throughout the experiment. The insert medium can be stored in the refrigerator at 4 °C for up to 1 month.

Raji culture medium Prepare different media types, depending on whether the medium will be used to maintain the cells in 75-cm^2 cell culture flasks (Raji cell medium: 500 ml of RPMI + 5 ml of NEAA + 5 ml of L-glutamine + 50 ml of FBS + 5 ml of PEST) or to freeze the cells (Raji freezing medium: Raji cell medium (95% (vol/vol)) and DMSO (5% (vol/vol))). Caco-2 cell insert medium will be used for seeding Raji cells on Transwell inserts (Step 15). These media can be stored in the refrigerator at 4 °C for up to 1 month.

Luria–Bertani medium Luria–Bertani medium is 10.0 g/l tryptone, 5.0 g/l yeast extract and 10.0 g/l sodium chloride supplemented with 3% (wt/vol) NaCl.

Transport buffer Typically, HBSS buffered at pH 7.4 with HEPES (25 mM final concentration) is used as the transport buffer on both sides of the monolayer. However, the type of transport buffer will depend on the molecule/nanoparticle to be tested (e.g., some molecules are pH-sensitive). ▲ **CRITICAL** Do not use FBS in the HBSS buffer when evaluating nanoparticle transport across cell monolayers, as FBS can influence nanoparticle transport across cells by forming a corona around the particle[34]. Before any nanoparticle transport study, the dispersion and stability of the nanocarrier in the transport medium should be assessed. This medium should be made up fresh before each experiment.

PROCEDURE

Cultivation of Caco-2 and Caco-2/Raji cell monolayers on permeable supports ● TIMING start 30–35 d ahead of the planned experiment

1| *Defrosting of Caco-2 cells*. Defrost one vial of Caco-2 cells (3 × 10^6 cells) at room temperature (RT; 20–25 °C). Resuspend the cells in 10–15 ml of flask medium and seed them in 75-cm^2 cell culture flasks. Change the medium every other day for 1 week or until the cells reach 90% confluence. Passage them to expand in 165-cm^2 cell culture flasks. Place the cells in an incubator at 10% CO_2 and 37 °C.

? TROUBLESHOOTING

2| *Defrosting of Raji cells*. Defrost one vial of Raji cells (3 × 10^6 cells) at RT. Resuspend the cells in 40 ml of Raji cell medium and seed them in 75-cm^2 cell culture flasks placed vertically in the incubator (5% CO_2). Raji cells grow in suspension, so just let them settle at the bottom of the flask. Change the medium two times a week by aspirating 30 ml of

the medium and adding 30 ml of fresh medium without disturbing the cells. Passage the cells once every 10 d by resuspending the cells, retrieving 10 ml of cell suspension and adding 30 ml of fresh medium. Place the cells in an incubator set to 5% CO_2 and 37 °C.
▲ **CRITICAL STEP** Passage the cells at least once between thawing and seeding them in the inserts. Be careful not to aspirate Raji cells while changing the medium.

3| Place the desired number of inserts into 12-well cell culture plates. Coat the inserts with Matrigel (10 µl/ml of medium—200 µl of medium per insert to cover the whole insert surface), and allow it to adsorb at the insert surface for 1 h under a laminar flow hood at RT.
▲ **CRITICAL STEP** Coating the inserts with Matrigel is critical to ensuring better adhesion of the cells to their surface before the inversion process at days 3–5 (Step 12). It is also critical to inhibit migration of Caco-2 cells from one side of the Transwell to the other through filter pores[35].

4| After 1 h, rinse the inserts three times with PBS (0.5 ml) to remove traces of nonadsorbed Matrigel. After washing, either directly add Caco-2 cells at the apical side of the inserts (Steps 5–9) or add medium, but do not allow the inserts to dry.
▲ **CRITICAL STEP** For this model, use Matrigel-coated Transwell inserts (or equivalent) with a 12-mm diameter. The pore size should be 3 µm to allow the Raji cells to cross the insert membrane to an extent and to partially intercalate the Caco-2 cell monolayer. Transport experiments are always carried out in parallel in both Caco-2 cell (monocultures) and Caco-2/Raji cell (cocultures) monolayers in order to compare transport under the same conditions and with the same number of test and control inserts. Do not let the inserts dry, and do not open the plates outside the laminar flow hood, as this is a risk for contamination.
? TROUBLESHOOTING
■ **PAUSE POINT** Inserts can be coated with Matrigel and stored for up to 2 d in the refrigerator at 4 °C. It is important to add medium to the apical and the basolateral chambers to prevent their drying.

5| Trypsinize Caco-2 cells from 90% confluent Caco-2 cultures in 165-cm^2 flasks (90% confluency corresponds to ~15×10^6 cells per flask): remove the medium, rinse the cells gently with PBS (30 ml per 165 cm^2 flask), remove the PBS and add trypsin-EDTA (5 ml per 165-cm^2 flask). Ensure that the whole flask is covered with trypsin. Incubate the flask at 37 °C under a 10% CO_2/90% air atmosphere for ~15 min. Once the cells are detached, immediately add a volume of flask medium that is at least twice the volume of the trypsin to stop trypsinization. For example, per 165-cm^2 flask, we normally use 5 ml of trypsin and 25 ml of medium to inactivate it.

6| Transfer the cell suspension to a Falcon tube (evaluate the volume with the pipette) and pipette gently in order to disperse cell aggregates. Take an aliquot and count the cells using a Neubauer hemocytometer.
▲ **CRITICAL STEP** Use a Neubauer chamber to ensure accurate cell counting and Trypan blue solution to distinguish between dead and living cells. The percentage of dead cells should not exceed 5%. Caco-2 cells aggregate, so carry out repeated mixing using the pipette to avoid clumping.

7| Take the desired number of Caco-2 cells to be placed on the inserts (5×10^5 cells per insert × number of inserts +1 (the +1 being used to ensure that you have enough inserts in case any might be damaged) and centrifuge them at 4 °C for 5 min at 250*g*.

8| Remove the supernatant and resuspend the cells in medium in order to obtain 10^6 cells per ml.
? TROUBLESHOOTING

9| Remove PBS or the medium from the inserts (Step 4) and seed 5×10^5 Caco-2 cells in 0.5 ml of insert medium per insert. Place the 12-well plates containing the inserts back into the incubator.
▲ **CRITICAL STEP** Avoid touching the inserts with the pipette in order to prevent compromising the integrity of the insert.

10| Replace the medium supporting the Caco-2 cells every second day. The maximum volume used for 12-well plates is 0.5 ml for the apical side and 1.5 ml for the basolateral side.

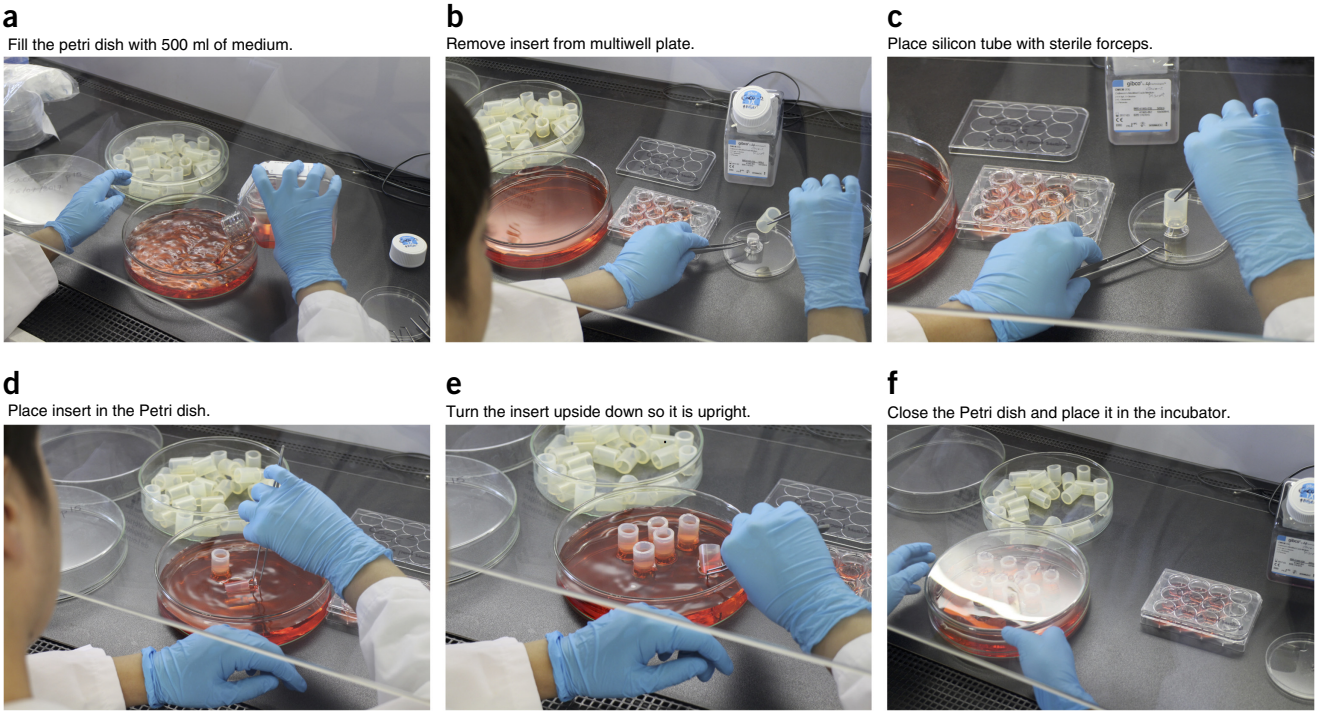

Figure 1 | Graphical description of Steps 11–14, representing the insert inversion.

11| On days 3–5, remove the inserts from the 12-well plates. Fill one Petri dish per cell culture (one for monoculture and one for coculture) with one bottle (500 ml) of insert medium (**Fig. 1a**).
▲ **CRITICAL STEP** As transport experiments will be carried out in parallel using monocultures and cocultures, two glass Petri dishes are needed—one for Caco-2 monolayers on inserts and another for Caco-2/Raji cell cocultures on inserts.
▲ **CRITICAL STEP** This model was originally used without the inversion of the insert step (e.g., Gullberg *et al.*[6]). Reasons as to why the 'inverted model' was developed are discussed in the INTRODUCTION.
? TROUBLESHOOTING

12| Invert the inserts and wrap a silicon tube (EQUIPMENT) around the insert's basolateral side using forceps so that the tube exceeds the height of the insert by 1 cm (**Fig. 1b**,**c**, **Supplementary Video 1**).
▲ **CRITICAL STEP** Before use, sterilize the silicon tubes and the forceps in an autoclave at 120 °C for 20 min. Silicon is a biocompatible, flexible material. It is inert, can be sterilized by autoclaving and can be reused.

13| Immerse the inverted insert with the attached silicon tube in a large Petri dish filled with 500 ml of insert medium (**Fig. 1d**).
? TROUBLESHOOTING

14| Place the inserts upside down (with the apical side facing down and the basolateral side with the silicon tube protruding facing up; **Fig. 1e**) to create a new basolateral chamber on the underside of each insert with a volume of ~500 µl. When all the inserts have been inverted, close the Petri dishes and place them in the 10% CO_2 incubator (**Fig. 1f**). Each Petri dish has an insert capacity equivalent to two 12-well plates (24 inserts in total).
▲ **CRITICAL STEP** The level of the medium in the Petri dish is very important. This level must be high enough to fill the apical side of an inverted insert, but not so high that the inserts float.

15| On days 14–16, remove the basolateral medium from the inserts by decanting and seed 2.5 × 10^5 Raji cells per insert.
▲ **CRITICAL STEP** Raji cells should be thawed at least 15 d before the experiments for them to grow sufficiently before their addition to the Caco-2 cells. Once Raji cells have been added to the basolateral compartment, do not remove the medium from the basolateral side of the inserts of cocultures (e.g., the compartment created by the silicone tube) until the day of the experiment. Treat monocultures similarly.

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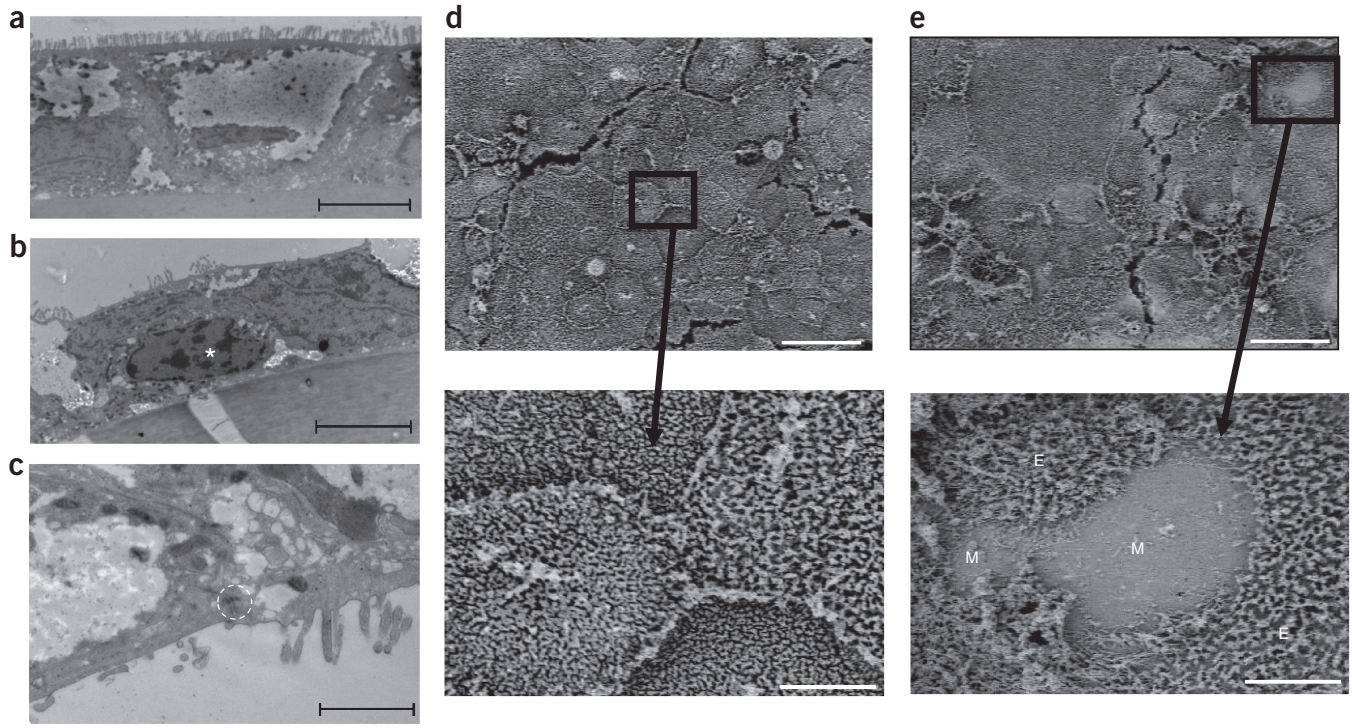

Figure 2 | Images of monocultures and cocultures obtained by TEM and SEM microscopy. (**a**–**c**) TEM images. Caco-2 cell monolayers present a columnar shape and a brush border (**a**). M cells were identified by their lack of microvilli at their apical surface (**b**) and the presence of desmosomes (**c**, dashed circle). A Raji lymphocyte can be identified within the enterocyte monolayer (**b**, asterisk). (**d**,**e**) SEM images. M cells were identified by their lack of, or fewer, microvilli at their apical surface (**e**). Monocultures were used as a control (**d**). **d**,**e** (bottom) are higher-magnification versions of boxed elements in **d**,**e** (top). Scale bars, 5,000 nm (**a**,**b**); 1,000 nm (**c**); 20 µm (**d**,**e** (top)); 5 µm (**d**,**e** (bottom)). E, enterocyte; M, M cell. Image adapted with permission from ref. 10, Elsevier.

16| On days 19–21 after Caco-2 cell seeding (4–6 d after coculture), inverted monocultures and cocultures are ready for transport studies.

▲ **CRITICAL STEP** Before using the monocultures and cocultures, it is important to carry out a quality control check of the cultures—e.g., to check for functionality of the cocultures, absence of Caco-2 cells at the basolateral side of the membrane and TEER readings (**Box 1**). **Figure 2** shows the typical morphology observed in monocultures (enterocyte-like cells) and cocultures (M-cell-like cells) by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Perform phalloidin staining, a simple routine method to observe the absence of microvilli, on the M-cell-transformed cells. This could also be used to localize cell borders, as explained in **Box 3**.

17| Remove the inserts containing silicon tube wrapping one by one from the Petri dishes.

18| Remove the silicon tube from each of the inserts, and place the inserts in 12-well plates for transport studies.

19| Wash the inserts twice with prewarmed HBSS (0.5 ml for the apical side and 1 ml for the basolateral side).

Particle and microorganism transport assays ● TIMING up to 2 h, in the case of particle, drug or microorganism transport excluding further analysis

20| Prepare donor solutions containing the particles or microorganisms in suspension (see Reagent Setup and **Box 2** for control nanoparticle settings).

▲ **CRITICAL STEP** All solutions should be preheated to 37 °C before proceeding.

21| Incubate the inserts for at least 30 min in the incubator.

22| Prepare 12-well plates in duplicate (containing exactly 1.2 ml of transport buffer in the basolateral compartment) and allow them to equilibrate in the incubator for at least 30 min. One plate will be used for TEER measurement and the other one for transport experiments.

? TROUBLESHOOTING

23| Measure the TEER values of both Caco-2 and Caco-2/Raji cell monolayers using an Endohm epithelial volt/ohm meter connected to a chopstick electrode or to a measurement chamber according to the manufacturer's instructions. Do not forget to equilibrate the inserts at 37 °C before measuring the TEER, as this value is highly influenced by the temperature (**Box 1**).
▲ **CRITICAL STEP** The TEER measurement at the beginning and end of particle translocation studies is highly variable, depending on temperature[1]. Perform the measurement at 37 °C after letting the cells equilibrate in prewarmed HBSS at 37 °C.
▲ **CRITICAL STEP** When using chopstick electrodes, the background resistance is normally higher as compared with that observed when using a measurement chamber. In this case, the background should be corrected by subtracting the values obtained with unseeded Transwell inserts. Using this particular Caco-2 cell clone, only monocultures presenting TEER values >200 $\Omega \cdot cm^2$ and cocultures presenting TEER values >100 $\Omega \cdot cm^2$ can be used; discard inserts with lower values.

24| After measuring the TEER values, transfer the inserts to the plates designated for transport experiments and remove the medium from the inserts by decanting.
▲ **CRITICAL STEP** Remove the medium by decanting, not by aspiration, as this can compromise monolayer integrity. Place the edge of the filter support on the edge of the 12-well plate and pour the contents onto the basolateral compartment. The residual volume is thus reduced.

25| Add the particle or microorganism donor solution to the apical compartment on which either monocultures or cocultures are grown (0.4 ml per insert). *In vivo*, M cells transport particles from the lumen (apical side of cells) to the underlying lymphocyte pocket. Thus, *in vitro* transport experiments should be carried out in the apical-to-basolateral direction.
▲ **CRITICAL STEP** Be careful not to spill or accidently add drops of the donor solution to the basolateral side. Generally, we retain at least three inserts each of monocultures and cocultures as controls for quality assurance. These controls are incubated with commercial polystyrene nanoparticles (e.g., FluoSpheres carboxylated 0.2-µm yellow-green nanoparticles) (**Box 2**). The objective is to assess the functionality and reproducibility of the coculture model (INTRODUCTION) by evaluating the ability of the cocultures to transport at least twofold more nanoparticles as compared with monocultures (**Box 2**). When performing bacterial adherence translocation assays, the apical buffer will depend on the bacteria type and strain (**Box 4**).

26| Typically, particle transport is carried out for 2 h, and a single measurement is recorded at this time point (**Box 2**). It is also possible, however, to quantify the number of particles transported at intermediate time points. In this case, half of the 1.2-ml volume of the basolateral compartment is sampled and replaced by the same volume of prewarmed HBSS. The inserts are then further incubated until the next time point. For bacterial translocation protocols, see **Box 4**.

27| At the end of the incubation period, remove the donor solution from the apical side by decanting. At this point, follow option A to measure TEER values or option B to image the inserts.

(A) TEER measurement

(i) Wash the inserts three times with prewarmed HBSS (0.5 ml for the apical side and 1 ml for the basolateral side).
(ii) Repeat the TEER measurements as described in Steps 22 and 23.

(B) Imaging the inserts

(i) Transfer the inserts to a new 12-well plate containing cold HBSS to stop further particle transport (0.5 ml for the apical side and 1 ml for the basolateral side).
(ii) Transfer the inserts to a new 12-well plate containing cold 4% (vol/vol) paraformaldehyde (PFA) to fix the cells (0.5 ml for the apical side and 1 ml for the basolateral side).
■ **PAUSE POINT** Fixed cells can be stored at 4 °C (**Box 3**). We store them at 4 °C for up to 1 week before mounting the inserts for confocal analysis.
(iii) Perform staining and confocal analysis.
▲ **CRITICAL STEP** Use inserts and the corresponding basolateral solutions for further analysis only if their TEER values are not significantly different ($P > 0.05$) from the initial values obtained before the transport experiment.

28| Sample the basolateral solution into a FACS tube for analysis of fluorescent nanoparticle translocation or use a fluorescence plate reader. It is also possible to evaluate drug transport by HPLC or LC–MS/MS. In the case of microorganisms, the number of translocated bacteria is determined by plating serial dilutions from the basolateral compartments on agar plates and calculating the number of colony-forming units per milliliter (CFU per ml) (**Box 4**).
▲ **CRITICAL STEP** 1% (vol/vol) serum (final concentration) can be added to FACS tubes to prevent particle adsorption at their surface.

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? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Cells do not grow properly	The FBS used was not appropriate	Change FBS batch
		Passage number was too high	Use cells from earlier passages
4	Cells do not detach	Cells have been allowed to grow too confluent	Trypsinize the cells earlier, at lower confluency
8	Cells are not homogeneously distributed on the insert	The Matrigel was not well distributed or absorbed	Ensure homogeneous dilution of Matrigel in the medium Allow the Matrigel to incubate on the insert for longer
		Batch of Matrigel used was of poor quality or too old	Change to a new batch of Matrigel
		The cells have aggregated	Increase homogenization of the cell suspension
11	Medium is leaking from the basolateral compartment	The silicon tube width used was not appropriate	Change to a different size of silicon tube
13	Monolayers are not homogeneous	Air bubbles formed in the apical/basolateral compartment	Remove any bubbles present above or below the inserts when inverting them
		Not enough culture medium was present in the Petri dish	Add more medium to the Petri dish
22	Low or no TEER values	Vigorous pipetting The inserts were broken	Pipette carefully before seeding the cells Avoid touching the inserts while changing the medium or during manipulation
	The TEER values of cocultures are not lower than the values for monocultures	Poor conversion of Caco-2 cells to M-like cells (passage number for Raji or Caco-2 cells was too high or previous problems in the table occurred)	Use new Raji or Caco-2 cells of a lower passage number
	The TEER values are different between cocultures	The monolayers/filters were damaged	Discard the inserts presenting different TEER values
	The commercial nanoparticle transport is not significantly ($P > 0.05$) higher in cocultures as compared with monocultures	Poor conversion of Caco-2 cells to M-like cells (passage number for Raji or Caco-2 cells was too high or previous problems in the table occurred)	Use new Raji or Caco-2 cells of a lower passage number

● TIMING

Steps 1–19, cultivation of Caco-2/Raji cell monolayers on permeable supports: 30–35 d
Steps 20–28, particle transport assays: up to 2 h
Box 1, evaluation of Caco-2 cell monolayer conversion to a functional M-cell-like cell monolayer: 2–4 h
Box 2, determination of commercial nanoparticle transport in the apical-to-basal direction by monocultures and cocultures: 30 min–2 h without the FACS analysis (add 1 h for FACS analysis); start 1 h before the experiment
Box 3, visualization of nanoparticles in the cell monolayers by confocal microscopy: 30 min–2 h, not including confocal analysis (add between 1 and 4 h for confocal analysis, depending on the number of samples)
Box 4, study of the interaction of M-like cells with bacteria: 4 h

ANTICIPATED RESULTS

The images of monocultures and cocultures typically obtained by TEM and SEM microscopy to study cell morphology are shown in **Figure 2**. M cells can be identified by a lack of microvilli at the apical side (**Fig. 2b**) and the presence of desmosomes (**Fig. 2c**, circle).

After quantification of the encapsulated compound of interest in the basolateral compartment (e.g., by HPLC or LC–MS/MS), the apparent permeability coefficient (P_{app}, in cm/s) can be calculated according to the following equation:

$$P_{app} = dQ/dt \times 1/AC_0$$

where dQ/dt is the transport rate (in µg/s), A is the surface area of the membrane filter (in cm^2) and C_0 is the initial drug concentration on the apical side (in µg/ml).

After the quantification of transported fluorescent nanoparticles, the data can be expressed as a percentage of, or P_{app} of, fluorescence or nanoparticles, in the event that it can be assessed that the fluorescence detected belongs to the nanoparticle and not to an artifact (e.g., nanoparticles are covalently linked to a dye) and the integrity of the nanoparticles is preserved. **Figure 3a** shows the influence of temperature on the transport of commercial FluoSpheres carboxylated 0.2-µm yellow-green nanoparticles (**Box 1**) across cocultures and monocultures. Fluorescent nanoparticles can also be visualized within the monolayers by confocal microscopy and can be localized by taking *y–z*, *x–y* and *x–z* sections of the monolayers (**Fig. 3b**)[36]. In **Figure 3b**, lipidic nanoparticles labeled with coumarin-6 (green) can be observed within Caco-2 cell monolayers (nuclei stained with DAPI (blue) and cell membranes stained with rhodamine–phalloidine (red)). The *y–z* and *x–z* views can be used to localize particles within the cell monolayers (top to bottom orientation), and the *x–y* view shows a section of the *z*-stack at a selected *z*-level.

Bacterial association with epithelia can be measured using fluorescence microscopy. In the example in **Figure 4a**, fluorescently labeled *S. typhimurium* was used to assess the functionality of the model. After the washing steps, the fluorescent bacteria could be visualized and quantified. The number of translocated bacteria across M cells can be determined by calculating the number of CFU per milliliter. Results can be expressed as a percentage of bacteria translocated across cocultured monolayers relative to control monolayers, or as CFU. **Figure 4b** reveals an increased number of *V. parahaemolyticus* CFU traversing cocultures as compared with monocultures.

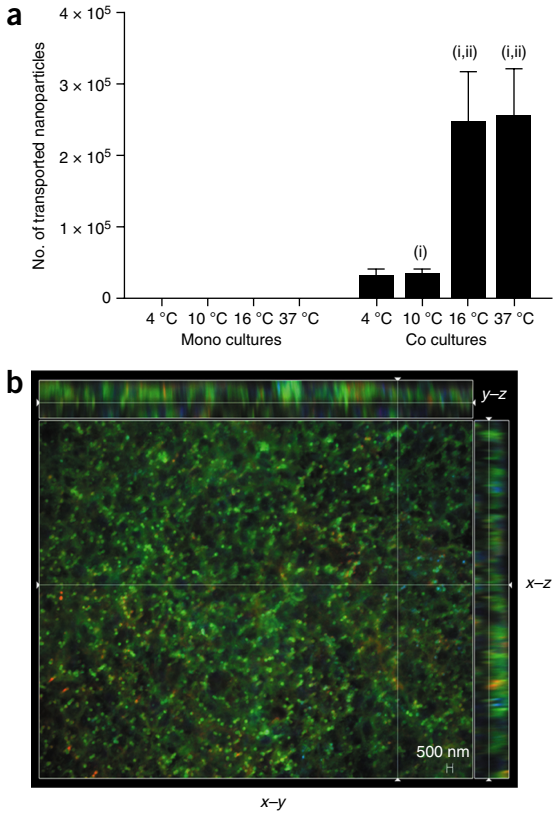

Figure 3 | Quantification and visualization of transported nanoparticles. (**a**) Influence of temperature on the transport of FluoSpheres carboxylated 0.2-µm yellow-green nanoparticles (4.5×10^9 nanoparticles per ml) suspended in HBSS. Results were expressed as the number of transported nanoparticles, ascertained by flow cytometry. Mann–Whitney nonparametric test was carried out for (i) $^*P < 0.05$ versus monocultures and (ii) $^*P < 0.05$ versus cocultures when incubated at 4 and 10 °C ($n = 4$ and 8 for monocultures and cocultures, respectively; n = number of inserts). The results are expressed as mean ± s.d. (**b**) *y–z*, *x–y* and *x–z* sections of saquinavir-loaded lipid-based nanoparticles labeled with coumarin-6 within Caco-2 cell monolayers. Nanoparticles (green) can be localized within the cell monolayers. Cell membranes are stained in red with rhodamine–phalloidine and cell nuclei are stained in blue with DAPI. Scale bar, 500 nm. Images adapted with permission from refs. 10,36, Elsevier.

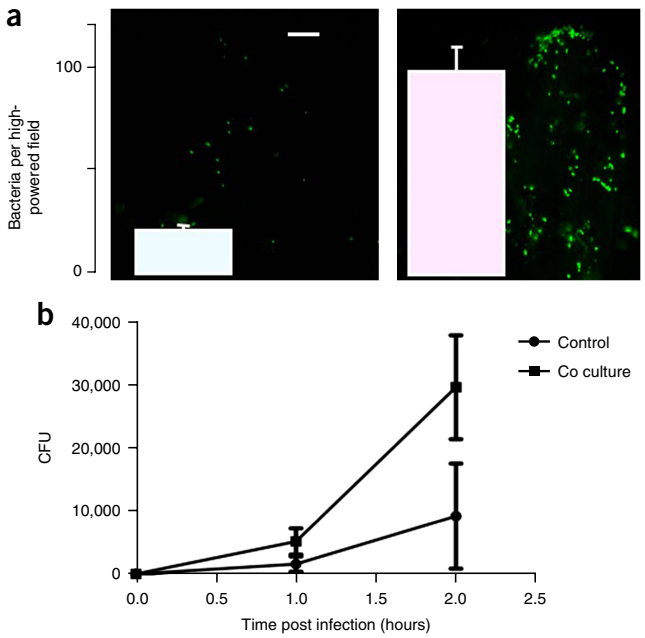

Figure 4 | Adherence to and translocation of different bacterial species across M-like-cell cocultures. (**a**) Adherence of fluorescently labeled *S. typhimurium* to monocultures and cocultures can be quantified. Significantly greater levels of interaction occur over 60 min when cocultures (right) as opposed to monocultures (left) are exposed to the bacteria ($n = 3$; $N = 4$; $P < 0.05$). Scale bar, 10 µm. The results are expressed as mean ± s.e.m. (**b**) Passage of *V. parahaemolyticus* across the coculture model versus Caco-2 cell monolayers (control). Cells were infected with wild-type bacteria at a multiplicity of infection (MOI) = 5. Basolateral medium was collected 1 and 2 h post infection and the number of colony-forming units (CFU) was determined; unpaired Student's *t*-tests were carried out with $^*P < 0.05$ ($n = 3$; $N = 3$). **a** adapted with permission from ref. 3, Elsevier. **b** adapted with permission from ref. 25, Federation of European Microbiological Societies.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS A.B. and A.d.R. wrote the draft protocol, excluding the discussion of data for the transport of bacteria. D.J.B. wrote the discussions of experimental data related to the transport of bacteria and viruses across M cells, including Box 4, and edited the English language. All authors contributed to the composition, correction and editing of the protocol and have jointly supervised the work.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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