Figure S1

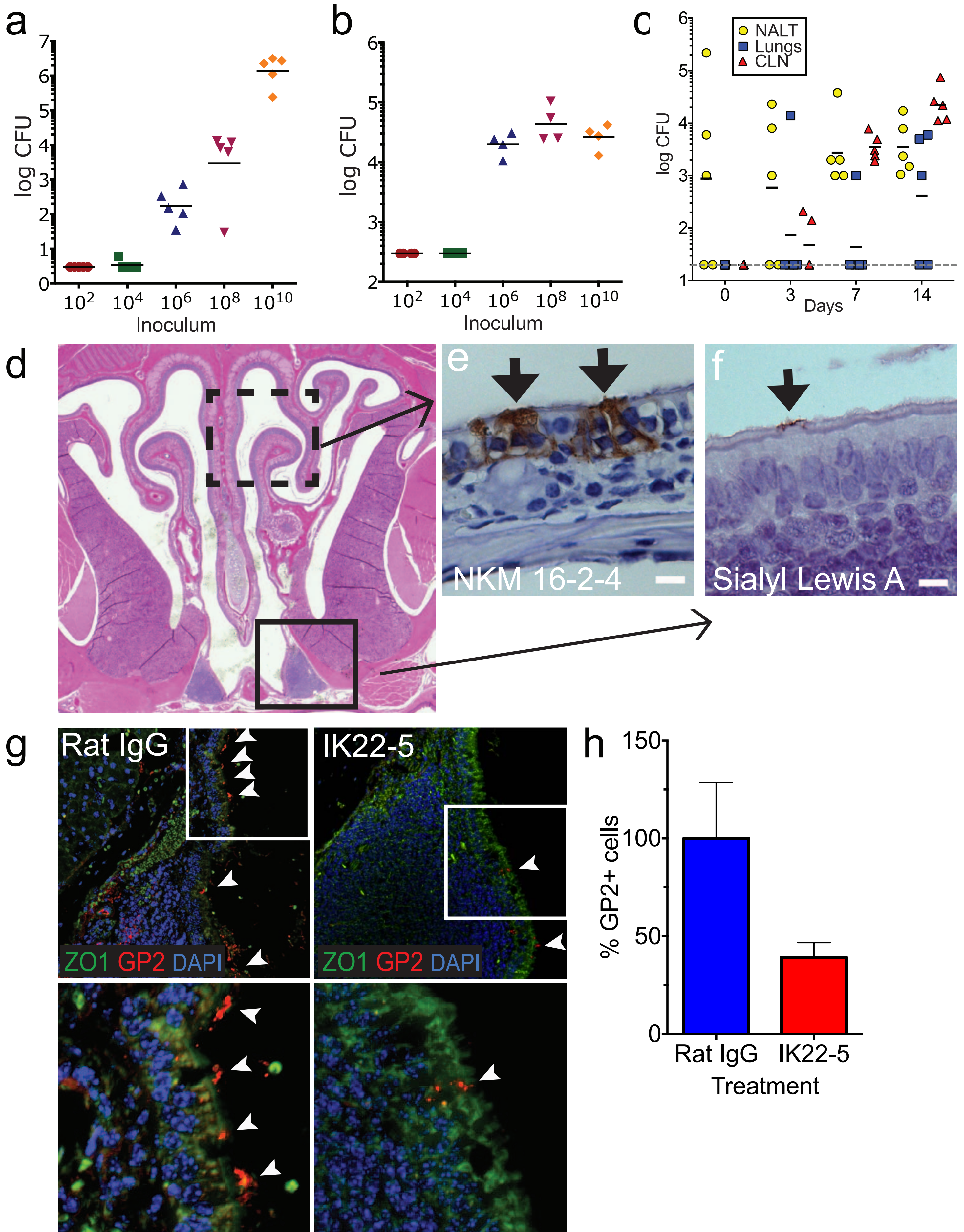


Figure S2

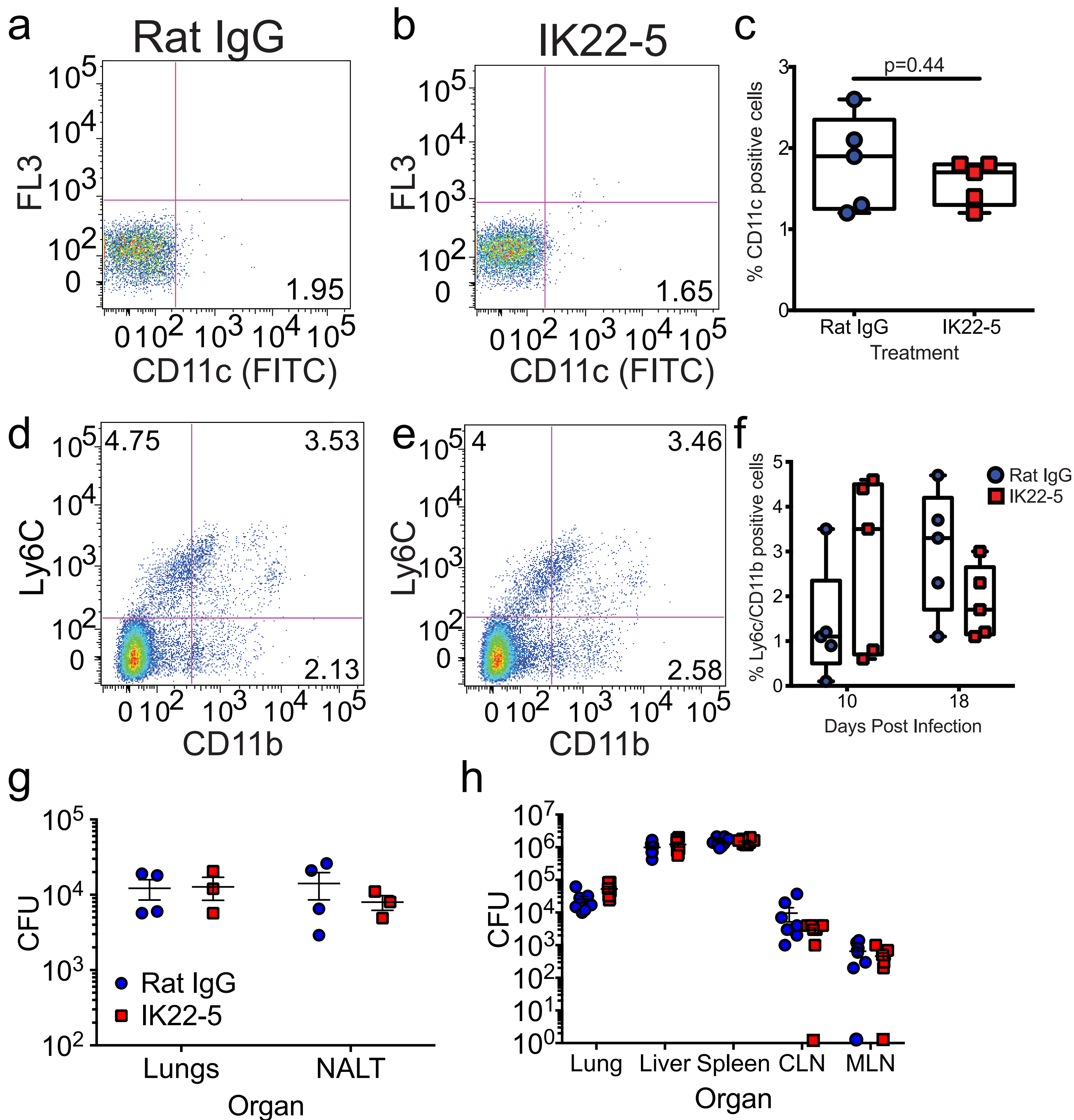
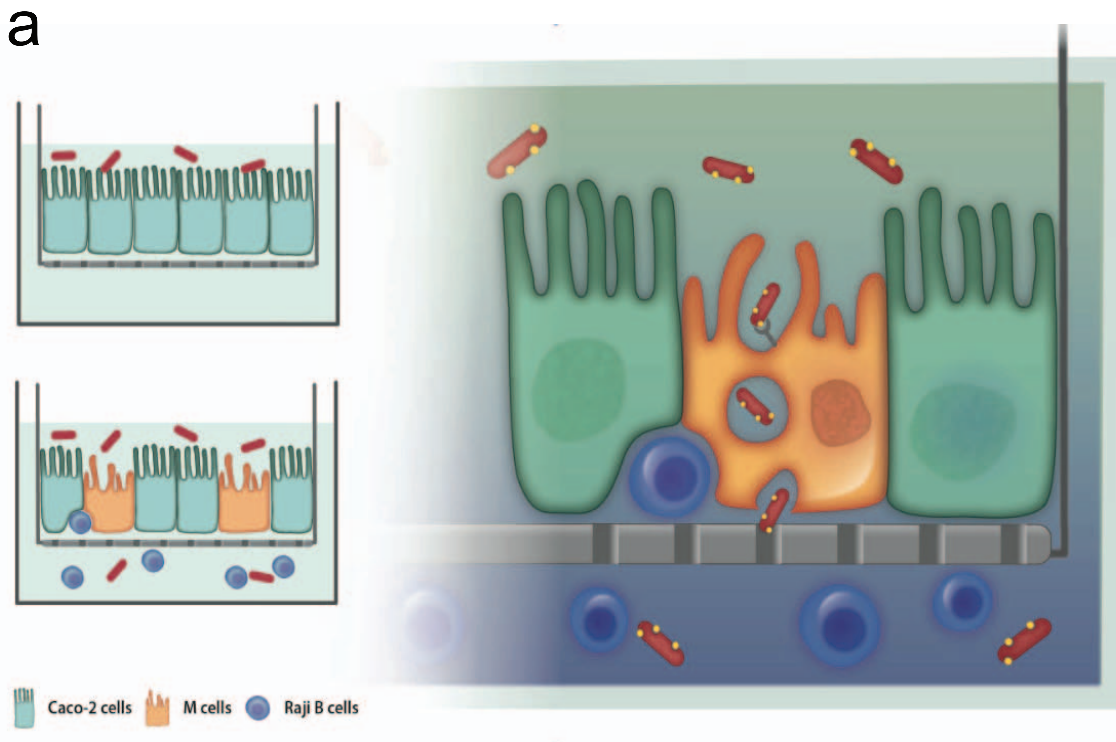
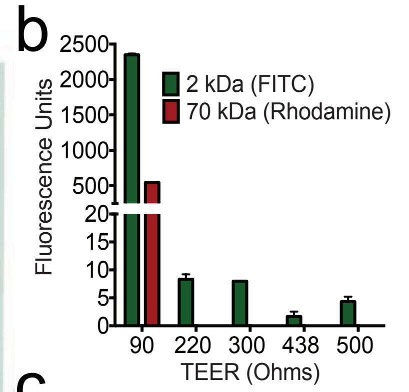
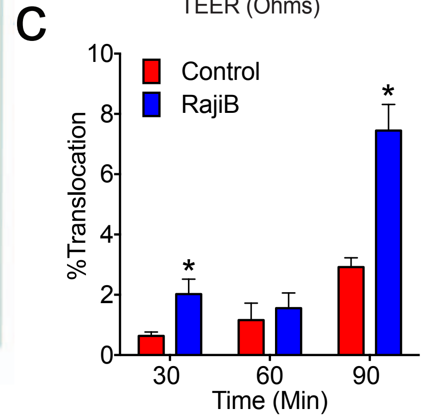
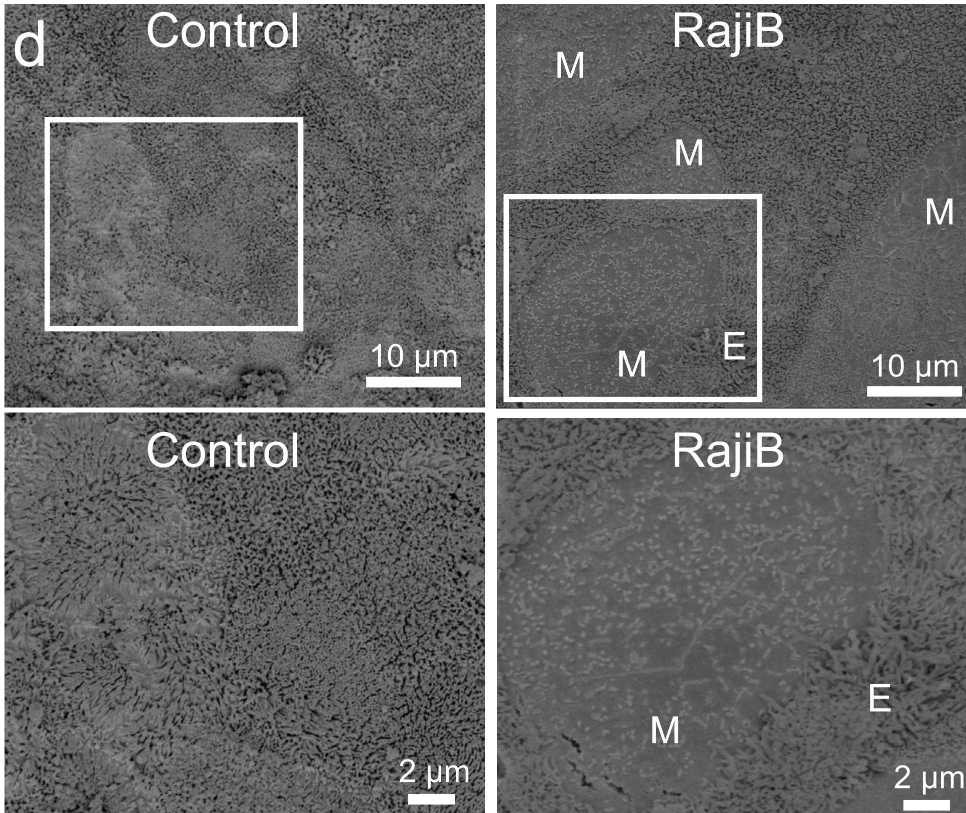


Figure S3









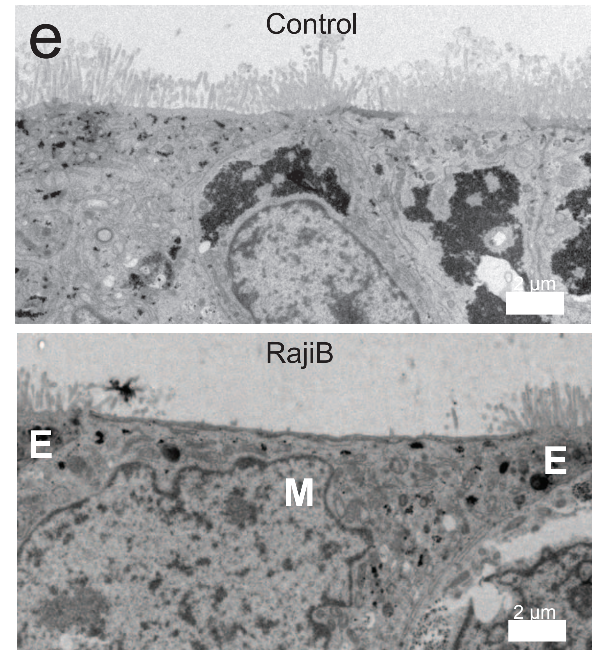
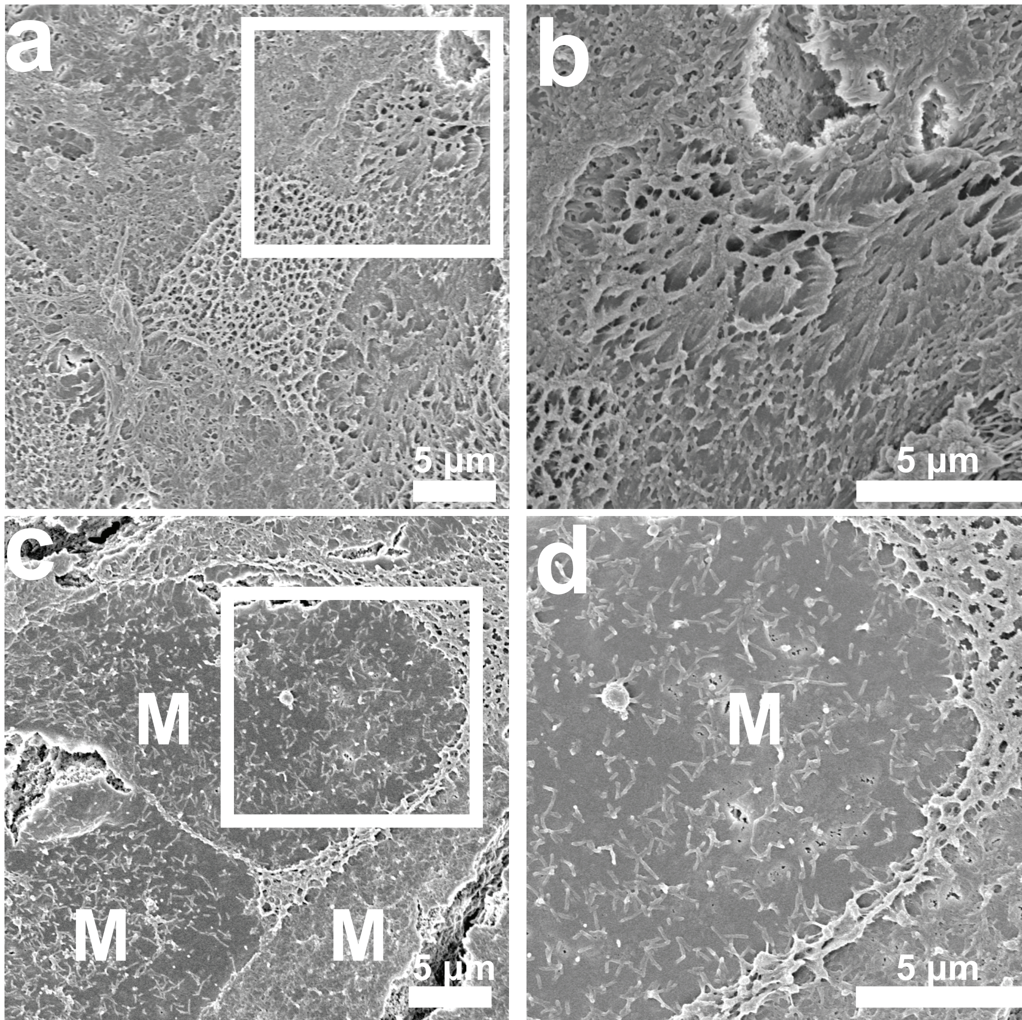
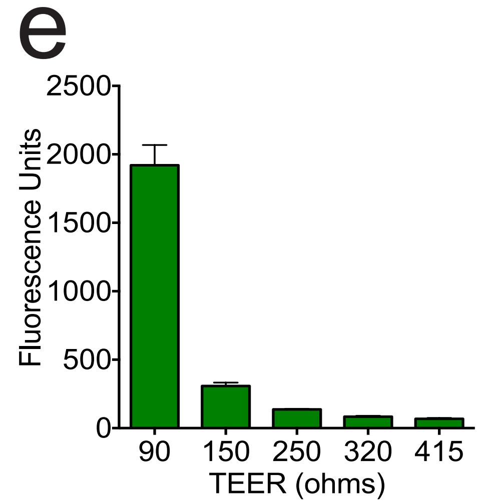
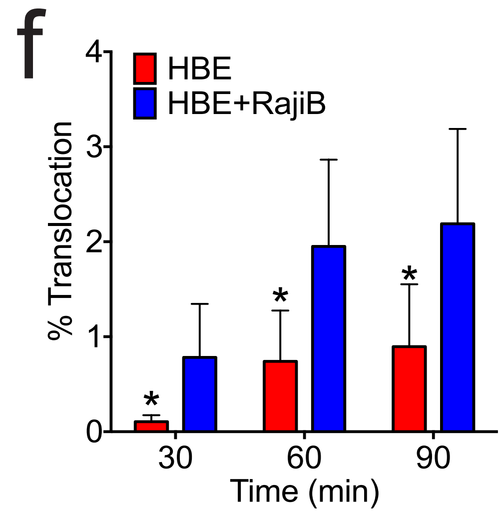
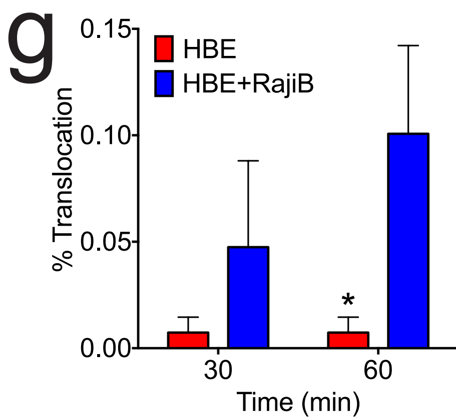


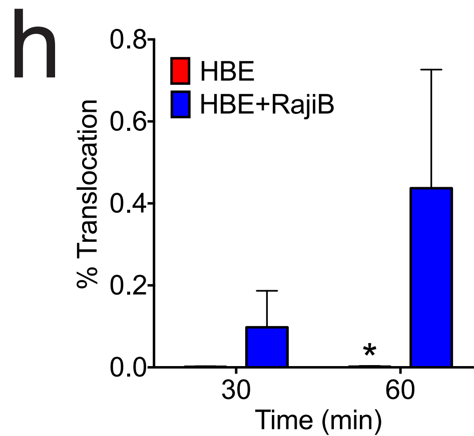
Figure S4











Supplemental Figure Legends

Fig. S1 (related to Fig. 1 and Fig. 4) Establishment of an intranasal Mtb infection model and identification of NALT M-cells. a, b, NALT was inoculated with increasing doses of Mtb in 10 μl and CFU measured at day 0 in the NALT (**a**) or at day 30 in the draining cervical LN (**b**). **c,** NALT was inoculated with 10^8 CFU, and NALT (yellow circles), lungs (blue squares) and cervical LN (red triangles) plated for CFU at various time points after infection (n=5 mice/time point, line indicated geometric mean). The dashed line represents the lower limit of detection. Note the absence of Mtb from lungs at day 0, 3 and 7. **d,** Coronal section through mouse cranium showing diffuse (dashed square) and organized (solid square) NALT (H&E). **e, f**, Immunohistochemistry for two markers of M-cells, NKM 16-2-4 (**e**) and Sialyl Lewis A (**f**). Arrowheads identify NKM and SLA positive cells. **g, h** BALB/c mice were treated with control or IK22-5 antibody for 8 days prior to analysis. NALT was collected from uninfected mice and GP2 staining (red) of frozen sections was performed. ZO1 staining (green) is used to identify cell boundaries. The boxed area in the top panel is enlarged in the bottom panel. Arrowheads identify GP2 positive M-cells. **h**, M-cells were quantified in the NALT of control and IK22-5 treated mice. The number of M-cells in control mice was averaged and used to normalize percent positive cells for individual mice (n=3 mice/group).

Fig. S2 IK22-5 treatment does not affect NALT dendritic cell numbers, recruitment of inflammatory monocytes during aerosol infection or bacterial spread via intravenous infection (related to Fig. 1 and Fig. 4). For all experiments, mice were treated with control or IK22-5 antibody for 8 days prior to analysis or infection. **a-c**, NALT was collected from uninfected mice and CD11c+ dendritic cells counted. Shown are scatter plots for representative control (**a**) or IK22-5 (**b**) treated mice. **c**, Quantification of percent CD11c+ cells (5 mice/group). **d-f** Mice were aerosol infected with ~200 CFU Mtb, lungs collected, and $Ly6c^+/CD11b^+$ cells counted at 10 and 18 days after infection. Shown are scatter plots for representative control (**d**) or IK22-5 (**e**) treated mice at the 10 day time point. **f**, Quantification of percent $Ly6c^+/CD11b^+$ cells (5 mice/group) per time point. **g,h** Mice underwent retro-orbital (intravenous) infection with 10^7 CFU Mtb, and bacteria enumerated in various organs on day 0 (**e**) or day 7 (**f**).

Fig. S3 Establishment of a Caco-2/RajiB coculture model (related to Fig. 2 and Fig. 3). a, Schematic of M-cell model and Mtb translocation. **b,** Two fluorescent dextrans of different sizes were monitored for translocation across the transwell at various TEER. **c**, Fluorescent polystyrene Dragon Green beads (1.9 μm) were added to the apical surface and percent translocation determined by flow cytometry. **d,** Control or RajiB-cocultured transwells were fixed and imaged by scanning (**d**) and transmission (**e**) electron microscopy. Boxes (**d, top**) are magnified in lower panels. Control wells have a cobblestone appearance of epithelial (E) cells with prominent microvilli while RajiB wells have many flat cells lacking microvilli characteristic of M-cells (M). Scale bars are 10 μm (d, top), 2 μm (d, bottom and panel E).

Fig. S4 Characterization of the HBE M-cell model (related to Fig. 2). a-d, Control (**a,b**) or RajiB-cocultured (**c,d**) transwells containing HBE cells on the apical surface were fixed and imaged by scanning electron microscopy. Boxes (**a, c**) are magnified in adjacent panels. Control wells have a mucoid surface appearance with prominent extensions while RajiB-cocultured wells have many flat cells lacking microvilli characteristic of M-cells (M). **e**, Translocation of a 2kD dextran dye was measured at various TEER values. TEER > 300 prevents dye translocation consistent with extensive tight junction formation. **f**, Fluorescent polystyrene Dragon Green beads (1.9 μm) were added to the apical surface and percent translocation determined by flow cytometry. **g,h** *Streptococcus pneumonia* (**g**) or *Pseudomonas aeruginosa* were added to the apical surface and percent translocation determined by quantifying CFU. * $p<0.05$ comparing control versus Raji-B cocultured HBE cells.

Supplemental Experimental Procedures

Antibodies

For depletion of M-cells in vivo, we used the rat monoclonal antibody IK22-5 as described (Knoop et al., 2009). Rat anti-M-cell antibody, also known as NKM16-2-4 antibody (D279-3), or rat anti-GP2 antibody (D278-3) were obtained from MBL and mouse anti-SLA antibody (clone KM231) was obtained from Kamiya Biomedical Company. Rabbit anti-ZO-1 was obtained from Invitrogen (#61-7300). All secondary antibodies were from Jackson Immunochemicals and were pre-adsorbed against mouse antigens. Flow cytometry antibodies were purchased from BD Biosciences.

Cell culture

The human colorectal adenocarcinoma cell line Caco-2 (HTB-37) and human Burkitt lymphoma cell line Raji-B (CCL-86) were from ATCC (Manassas, VA, USA). 16HBE14o- cells (Cozens et al., 1994) were provided by Dieter Gruenert (University of California, San Francisco). Caco-2 or HBE cells were grown in DMEM with 20% FBS (Hyclone), 1% sodium pyruvate (Hyclone), 1% non essential amino acids (Hyclone), glutamine and 1 mM HEPES. Raji B cells were grown in suspension in RPMI with 10% FBS and glutamine. All cells were maintained at 37°C and 5% CO_2 in a humidified incubator and tested routinely for mycoplasma contamination.

Dye Translocation

The 2 kD FITC dextran and 70kD rhodamine dextran dyes were obtained from Life Technologies. Fluorescent dextran was added to the apical compartment of the transwell to achieve a concentration of 2 mg/ml and 4 hours later samples from the basolateral transwell were taken for fluorescence measurement.

Tissue Bilayer Model

To establish the tissue bilayer, Caco-2 or HBE cells were grown on the upper compartment of a 3.0 µm polyester transwell (Corning #3462) and Raji B cells were grown in the lower compartment. In experiments comparing control versus Raji-B treated cells, control wells had media alone in the lower compartment. The transwells were maintained at 37°C and 5% CO_2 in a humidified incubator for 2 weeks until the transepithelial resistance measured using a EVOM2 Volt-Ohm Meter (World Precision Instruments) reached >350Ω.

In vitro Mtb infections

For in vitro infections, Mtb cultures were washed twice with PBS, resuspended in PBS and centrifuged to remove clumps. The supernatant was sonicated three times to generate a single cell suspension. Bacteria were then resuspended in DMEM plus 20% fetal bovine serum. For translocation assays, the upper wells of the tissue bilayer were infected with Mtb at an MOI of 5:1 and the translocated bacteria were collected from the lower wells over a 60 minute time period. The samples were plated on 7H11 agar plates and grown in a 37°C incubator for 3 weeks.

Antibody mediated M-cell depletion

For the antibody-mediated M-cell depletion, wild-type BALB/c mice were injected intraperitoneally with 250 µg of either rat IgG or monoclonal rat IK22-5 antibody every other day for a total of four injections over an 8 day period as previously reported (Knoop et al., 2009). For immunofluorescence or immunohistochemistry, the NALTs and Peyer's patches were collected on the ninth day. For intranasal and aerosol experiments, mice were infected with Mtb on the ninth day.

Flow cytometry

To stain mouse NALT DCs, 5 mice per group were injected with rat IgG or IK22-5 per our standard protocol. The NALT was removed and the lymphoid pockets disaggregated in PBS. Red blood cells were lysed and the cells were passed through a cell strainer. After cell counting, cells were stained with anti-CD11c antibody, counted on FACSCalibur flow cytometer and analyzed using FlowJo software. To stain Ly6C+/CD11b+ inflammatory monocytes, mice were injected with rat IgG or IK22-5 per our standard protocol. Mice were then infected via aerosol with 200 CFU of Mtb and sacrificed at 10 and 18 days after infection (5 mice/group per time point). Lungs were collected, minced with scissors and digested in a buffer consisting of RPMI 1640, HEPES (10mM), 5% FCS (heat inactivated), 30 µg/ml Dnase I (Roche), 1 mg/ml Collagenase II (Worthington) at 37°C for 45 minutes as described (Wolf et al., 2007). Cells were

then passed through a cell strainer, washed and then resuspended in PBS with Bovine Serum Albumin (10 mg/ml) and EDTA (5mM). Cells were stained with anti-Ly6c and anti-CD11b. After staining, cells were fixed with paraformaldehyde 1% in PBS overnight and then counted.

Immunohistochemistry and Immunofluorescence microscopy

To stain mouse NALT using NKM16-2-4 and anti-SLA antibodies, mice were killed by cervical dislocation, their heads removed and then immersed in 4% paraformaldehyde (PFA, pH 7.4) for 24 hours. The heads were then decalcified with 5% EDTA for 2 weeks at 4 °C. After decalcification, heads were embedded in paraffin, sectioned to approximately 5 um thickness and mounted on glass slides. Paraffin embedded specimens were deparaffinized in xylene, subjected to heat mediated antigen-retrieval in 10 mM sodium citrate (pH 6.0) and blocked in 10% donkey sera. For the anti-Sialyl Lewis A antibody we also used a mouse Ig blocking reagent (Vector Labs, MKB-2213). To stain mouse NALT using the anti-GP2 antibody, mice were killed by cervical dislocation, their heads removed and then immersed in 4% paraformaldehyde (PFA, pH 7.4) for 24 hours. The heads were then decalcified with 5% EDTA for 2 weeks at 4 °C. The decalcified tissues were placed in 30 % sucrose solution overnight at 4 °C, embedded in OCT compound, and rapidly frozen in liquid nitrogen. Frozen sections, approximately 10-μm thick, were mounted on gelatin-coated glass slides. After pretreatment with 0.3 % Triton X-100 in phosphate buffered saline for 30 min and preincubation with 10 % normal donkey serum, the sections were incubated with a rat anti-GP2 monoclonal antibody (1:200, MBL) For immunofluorescence and immunohistochemistry in mouse tissue, M-cells were identified using rat anti-M cell (NKM16-2-4 antibody, 1:100, MBL) or mouse anti-Sialyl Lewis A (1:100, Kamiya Biomedical) and rabbit anti-ZO-1 (1:100 Invitrogen, #61-7300) for tight junctions. For detection of primary antibodies against NKM or SLA we used HRP-conjugated secondary antibodies (1:500, Jackson Immunochemicals) followed by amplification with tyramide (1:50, Perkin Elmer) for immunoflorescence or 3,3-diaminobenzidine (DAB) for immunohistochemistry (Vector Labs, SK-4100) followed by hematoxylin counterstain. For detection of ZO-1 we used Cy3 labeled donkey anti-rabbit antibody and Hoechst stain to visualize nuclei. Controls for each immunohistochemical and immunofluorescence experiment were carried out by replacing the primary antibodies with the same amount of isotype control antibody. Sections were mounted using Prolong Gold and images were acquired using a Zeiss AxioImager Z2 microscope equipped with a Photometrics CoolSnap HQ2 camera. For cryosections stained with anti-GP2 antibody, images were acquired using a Zeiss PLAN APOCHROMAT 20X/0.8 NA air objective. The same acquisition times were used for each channel for all samples. *Z*-stacks were deconvolved with AutoDeBlur (Bitplane) and then analyzed using Imaris version 8.2 (Bitplane).

Electron microscopy

Transwells prepared as above were either fixed directly with a 1:1 ratio of 2.5% glutaraldehyde and 4% EM grade PFA, or were infected with Mtb at an MOI of 25:1 by adding Mtb containing medium to the apical surface of the transwell. After 30 min infection at 37°C, 5% CO_2, the cells were fixed as above for 30 minutes at room temperature. For scanning electron microscopy, transwell membranes were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C. After three rinses in 0.1 M sodium cacodylate buffer, they were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 45 minutes. Cells were rinsed with water and dehydrated with increasing concentration of ethanol. The membranes were then removed and critical point dried. They were air dried under the hood, mounted on SEM stubs and sputter coated with gold in a Cressington 108 auto sputter coater. Images were acquired on a Field-Emission Scanning Electron Microscope (Zeiss Sigma).

For transmission electron microscopy, transwell membranes were fixed with 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer. After five rinses in 0.1 M sodium cacodylate buffer, they were post-fixed in 1% osmium tetroxide and 0.8 % K3[Fe(CN6)] in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The membranes were rinsed with water and en bloc stained with 2% aqueous uranyl acetate overnight. After five rinses with water, specimens were dehydrated with increasing concentration of ethanol, infiltrated with Embed-812 resin and polymerized in a 60oC oven overnight. Blocks were sectioned with a diamond knife (Diatome) on a Leica UC7 ultramicrotome (Leica Microsystems) and collected onto copper grids, post stained with 2% Uranyl acetate in water and lead citrate. Images were acquired on a Tecnai G2 spirit transmission electron microscope (FEI) equipped with a LaB6 source.

Intranasal Infection

For intranasal infections, liquid Mtb cultures were washed twice with PBS, resuspended in PBS and centrifuged to remove clumps. The supernatant was sonicated three times to generate a single cell suspension. Bacteria were resuspended in PBS at a concentration of 1 x 10^8 bacteria per 10 µl. Each mouse was infected intranasally with 10 µl of the inoculum. NALT from 5 mice from each group was collected and plated on 7H11 plates, 30 minute after the infection to determine the inoculum. At 7 days after infection, cervical lymph nodes, lung, liver, and spleen were collected, homogenized and lyates plated on 7H11 plates that were then incubated in a 37°C incubator for 3 weeks to determine CFU.

Intravenous Infection

For intravenous infection, bacteria were prepared as above. Antibody depletion was performed as above with rat IgG or IK22-5. Mice (n=12 per group) were infected via the retroorbial venous plexus with 10^7 CFU in 100 µl using a tuberculin syringe. Thirty minutes after infection, lungs and NALT (n=4 per group) were collected, homogenized and plated for CFU to enumerate initial inocula. At day 7, lungs, liver, spleen, cervical and mediastinal lymph nodes were collected (n=8 per group), homogenized and plated for CFU.

Aerosol Infection

For aerosol infections, liquid Mtb cultures were washed twice with PBS, resuspended in PBS and centrifuged to remove clumps. The supernatant was sonicated three times to generate a single cell suspension. Bacteria were resuspended in PBS at an OD of 0.1 and mice were infected using a GlasCol aerosol chamber so that each mouse received a bacterial load of ~200 bacteria. At day zero, both lungs from 5 mice of each treatment group were plated to detemine the inoculum. Mice were then either sacrificed at day 18 for organ CFU determination or followed over time for survival as previously described (Zacharia et al., 2013).

Bibliography

Zacharia, V.M., Manzanillo, P.S., Nair, V.R., Marciano, D.K., Kinch, L.N., Grishin, N.V., Cox, J.S., and Shiloh, M.U. (2013). cor, a novel carbon monoxide resistance gene, is essential for Mycobacterium tuberculosis pathogenesis. MBio *4*, e00721-00713.