Analysis of bacterial-surface-specific antibodies in body fluids using bacterial flow cytometry

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Published online 28 July 2016; doi:10.1038/nprot.2016.091

Antibacterial antibody responses that target surfaces of live bacteria or secreted toxins are likely to be relevant in controlling bacterial pathogenesis. The ability to specifically quantify bacterial-surface-binding antibodies is therefore highly attractive as a quantitative correlate of immune protection. Here, binding of antibodies from various body fluids to pure-cultured live bacteria is made visible with fluorophore-conjugated secondary antibodies and measured by flow cytometry. We indicate the necessary controls for excluding nonspecific binding and also demonstrate a cross-adsorption technique for determining the extent of cross-reactivity. This technique has numerous advantages over standard ELISA and western blotting techniques because of its independence from scaffold binding, exclusion of cross-reactive elements from lysed bacteria and ability to visualize bacterial subpopulations. In addition, less than 10^5 bacteria and less than 10 µg of antibody are required per sample. The technique requires 3–4 h of hands-on experimentation and analysis. Moreover, it can be combined with automation and mutliplexing for high-throughput applications.

INTRODUCTION

This protocol describes a technique for quantifying bacterial-surface-binding antibody titers in a variety of body fluids, using cultivated bacteria as a binding 'matrix'. We have used this extensively to study host–microbiota and host–pathogen interactions in both murine models and clinical research[1–6]. The technique builds on basic concepts developed for the analysis of pneumococcal vaccine responses[7–9] and antibody responses to *Candida albicans*[10]. The role of antibodies in host–bacterial interactions is clearly critical both in protection from pathogenic infection[11] and in influencing mutualistic interactions with the microbiota[12]. Quantification of functionally relevant bacterial-binding antibodies has mainly been carried out by ELISA/ELISPOT techniques against purified components or whole cells (e.g., refs. 13,14), or by semiquantitative techniques such as western blotting or dot blotting (e.g., refs. 2,15,16). Although all of these techniques can yield useful data, they are subject to a number of pitfalls, including high levels of cross-reactivity between highly conserved bacterial cytosolic proteins[17], variable binding to plastic or membrane scaffolds and the need to purify components to homogeneity[18]. This flow-cytometry-based technique enables quantitative analysis of host immunity to any culturable bacterial species in any available body fluid.

Most laboratory animals are raised in highly controlled environments with zero known pathogen exposure[19]. Although conventional (i.e., not germ-free) animals are colonized by a dense bacterial microbiota on all body surfaces, strong compartmentalization of the blood and mucosal immune systems renders the blood of these animals effectively naive to any bacterial exposure[20]. In these cases, parenteral vaccination or deliberate infection generates a specific immune response in the blood that can be evaluated against a near-zero background level of bacteria-specific immunity[1]. By contrast, antibodies isolated from the mucosal surfaces of conventional animals will always contain immunoglobulins induced by the presence of the microbiota[15], via either T-dependent or T-independent B-cell responses[21]. Here, the issue of high-level cross-reactivity between bacterial cytosolic components can make it extremely challenging to quantify the immune response of interest using techniques based on bulk lysed bacteria.

This technique represents a simple way to quantify exclusively antibodies binding to bacterial-surface-exposed epitopes using flow cytometry. The ability to gate on whole bacteria automatically excludes lysed bacteria and fragments. Further, the system is extremely sensitive and has a broad dynamic range; it requires only minute quantities of immunoglobulins and only very low densities of bacterial targets, thus allowing analysis of responses against fastidious species.

Overview of the procedure

In its simplest form, this technique incubates 10^5 bacterial cells from a pure culture with an antibody-containing bodily fluid (serum, plasma, intestinal/bronchial/vaginal lavage, purified antibodies from saliva, breast milk and so on). After washing, bound antibody is visualized with appropriate secondary reagents (**Fig. 1a**). The bacteria are then washed, recovered in an appropriate buffer and quantified on any standard flow cytometer with the settings adapted for bacterial cell recognition (**Fig. 1b**). Median fluorescence intensities, relative to the dilution factor or antibody concentrations, can then be analyzed to generate titers (**Fig. 1c**). As such, it is very similar to any other flow cytometry staining. However, the success of the technique depends critically on the ratio of bacterial cells to antibody, and on avoiding contaminations.

Applications of the protocol

This protocol quantifies bacterial-surface-binding antibodies. As such it is extremely useful to track the success of bacterial vaccination and to determine immunologically relevant exposure

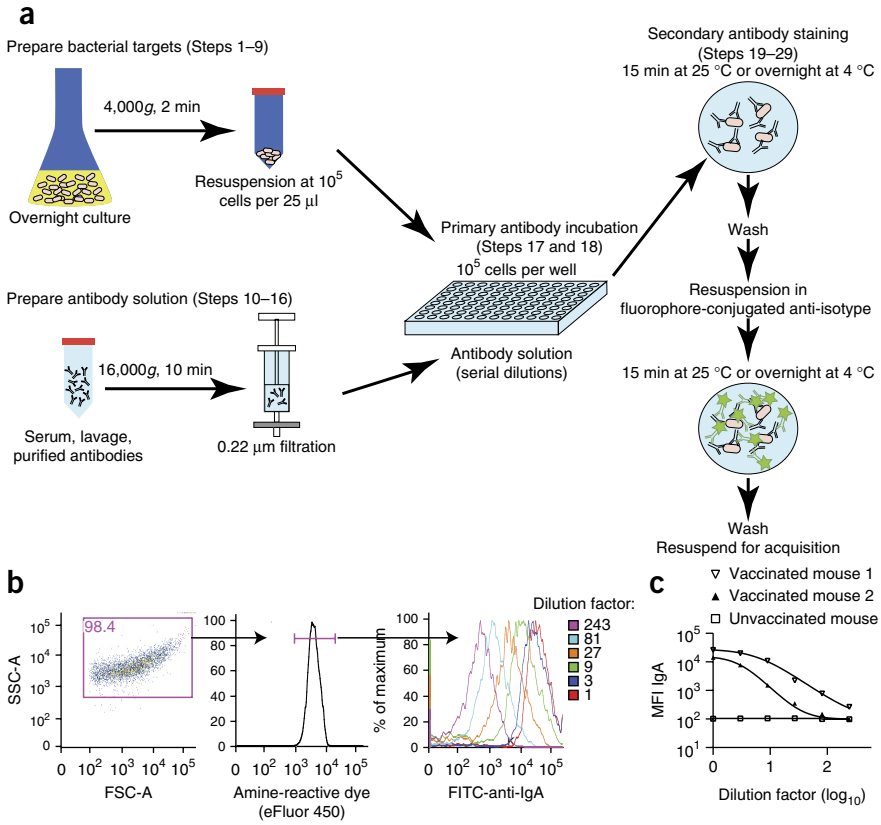

Figure 1 | Overview of the protocol. (**a**) Diagrammatic representation of how a basic bacterial flow cytometry experiment is carried out. (**b**) Intestinal lavage was collected from a mouse that had been orally vaccinated against *S. typhimurium*[22]. Typical dot plots and gating of whole *S. typhimurium* labeled with an amine-reactive dye, and overlaid histograms of the dilution series. (**c**) Typical titration curves obtained by plotting the median fluorescence intensity (MFI) of *Salmonella* staining against the sample dilution factor for intestinal lavages from two C57BL/6 mice vaccinated orally and one lavage from an unvaccinated mouse.

to defined bacterial species[1–6]. The technique may also be used to quantitatively study changes in bacterial biochemistry that alter surface antibody binding[8,22]. This protocol may also be used for a wider range of applications, such as screening of monoclonal antibodies and cell sorting[21,23,24]. Potentially, the protocol may be expanded to other cultivable microorganisms, such as fungi (e.g., *C. albicans*[10]) or eukaryotic parasites, as well as to whole purified microbiota.

Quantification of bacterial-surface-binding antibodies, when combined with cross-adsorption (i.e., removal of specific antibodies by saturating out-titration with the same bacterial strain or related strains of interest; see **Box 1**), permits definition of the species specificity of a response with much greater certainty than other techniques. It is therefore also particularly useful in quantifying antibody titers in situations in which other techniques yield high background[2,4,6]. In addition, this technique has many advantages over more traditional scaffold-based analyses, particularly for scientists working with fastidious bacterial strains or scarce antibody samples. The antigenic targets are required only at numbers such that bacteria can be reliably differentiated from electronic noise/contamination in the flow cytometer (10^5 bacteria per sample). An average assay uses between 0.1 and 100 µg of total antibody (e.g., up to 10 µl of serum for IgG1), as opposed to much larger quantities needed for western blotting applications. The quantities of antibodies required are similar to those typically used for ELISA, but the possibility of multiplexing the flow-cytometry-based analysis for multiple bacterial species further increases the efficiency. The readout is immediately quantitative and numerical analysis can calculate standard antibody titers.

Limitations of the protocol

A major requirement of this technique is the need for access to a flow cytometer and the operational knowledge to run the cytometer for bacterial analysis. Further, it is also necessary to have fluorescently labeled monoclonal or polyclonal antibodies against the immunoglobulin of interest. In addition, the technique is unsuitable for bacteria expressing high-affinity antibody-binding proteins on their surfaces (e.g., staphylococcal protein A[25,26]), as this yields nonspecific staining. We have not attempted to apply this technique to strains that produce very large amounts of capsular polysaccharide(s). However, work from the pneumococcal vaccine field suggests that, with the appropriate controls, useful data may be obtained with such strains[8,27].

An additional limitation is that bacterial culture conditions may alter the surface phenotype of your bacterial strain of interest[28–30], or the strain may display other types of phenotypic diversity such as phase variation[29]. Although it is possible to alter the culture conditions (e.g., temperature, media constituents and different growth phases) for some bacterial strains in order to influence this, it is close to impossible to accurately replicate the *in vivo* growth conditions *in vitro*. In some cases, this may lead to overestimation or underestimation of specific antibody responses. In addition, in its current form, this protocol is designed for use with culturable bacterial species. Although the fraction of microbiota species that can be successfully cultivated *in vitro* is continually expanding[31], further development of the protocol will be required to work with mixed bacterial populations (e.g., those from feces).

We would like to point out that there is considerable variation in light-scattering characteristics between bacterial species and subspecies, as well as in sedimentation efficiencies and tendency to agglutinate. Therefore, even when bacterial cell number is extremely well controlled, it is not appropriate to compare absolute binding values from a single antibody sample against two or more different species (i.e., it would be inappropriate in isolation to deduce that antibodies from donor A bind with a higher titer to bacterial species X than to bacterial species Y). However, relative binding patterns for sets of antibody donors, a donor plus appropriate controls, or cross-adsorbed samples (**Box 1**) binding to different strains can be compared. For example, donor A has a higher titer against species X than against species Y, whereas

Box 1 | Cross-adsorption for specificity testing ● TIMING 1.5 h

These antibody preparation steps should be carried out when you wish to test the specificity of a response. By first incubating the antibody-containing solution with a high number of a known bacterial species and looking for loss of signal, it is possible to distinguish nonspecific binding (which will depend only on the total immunoglobulin concentration and will be unaffected by this treatment) from specific binding (which should be completely removed by this process).

1. Cross-adsorption needs to be carried out in reasonably large volumes to permit efficient clearance and re-filtration; therefore, we suggest diluting the samples with bacterial flow cytometry buffer to a concentration giving high, but nonsaturating, binding in the bacterial flow cytometry assays (determine this in a pilot experiment).
2. Prepare (n+1) × 500 µl of the diluted sample to be cross-adsorbed, as detailed in Steps 11–14 of the PROCEDURE, where 'n' is the number of bacterial species against which cross-adsorption is planned.
3. Divide 500 µl of this solution into aliquots in 1.5-ml snap-cap tubes.
4. From stationary-phase cultures of your species of interest, centrifuge sufficient volume at 4,000–8,000*g* for 5 min at 4 °C to pellet 10^9 bacteria. Resuspend at a density of 10^9 bacteria per ml in bacterial flow cytometry buffer.
5. Add 500 µl of bacterial flow cytometry buffer to the control aliquot of your antibody solution.
6. To all other aliquots, add 500 µl of the bacterial suspensions.
7. Incubate the mixtures at 4 °C for 1 h with gentle shaking.
8. Centrifuge all tubes at 16,000*g* for 15 min at 4 °C to sediment all bacteria.
9. Recover the supernatants and filter them through a 0.22-µm filter into fresh 1.5-ml tubes to remove any remaining bacteria.
10. Proceed with staining, from Step 17 of the PROCEDURE.

▲ **CRITICAL STEP** The 'bacterial flow cytometry buffer only' cross-adsorbed sample must be included as a dilution control.

donor B has a higher titer against species Y than against species X. This concept is particularly important when using this technique to probe bacterial biochemistry that affects the nature of surface epitopes.

Finally, it should be noted that this technique was conceived specifically for the analysis of exposed surface epitopes. This does not encompass all biologically relevant epitopes (e.g., most secreted toxins cannot be analyzed). Although antibodies binding to the live bacterial surface are likely to be relevant in *in vivo* protection, the assay does not provide any direct evidence of functional protection.

Comparison with other methods

Many different assays have been developed to measure bacterial-species-specific antibodies, including plate-based assays for binding to purified surface carbohydrates, toxins or whole bacteria (e.g., refs. 13,32), or gel/membrane-based techniques such as dot-blotting and western blotting (e.g., refs. 2,15). Some of these technologies require higher amounts of antibody, which may be unobtainable from certain body fluids. In addition, even comparing between strains of the same species can yield complex results because of physical properties such as resistance to lysis and scaffold binding. Further, with other methods it can be difficult to exclude the detection of reactivity to bacterial cytosolic proteins. By specifically analyzing only intact bacteria, in the absence of any other scaffold, bacterial flow cytometry avoids many of these issues.

An additional benefit of this protocol is that by using live bacteria as the adsorptive matrix ('cross-adsorption') we can demonstrate the specificity of the responses measured, as well as the nature of the epitopes recognized. This is demonstrated in **Figure 2** (see ref. 22). Here we used bacterial flow cytometry and

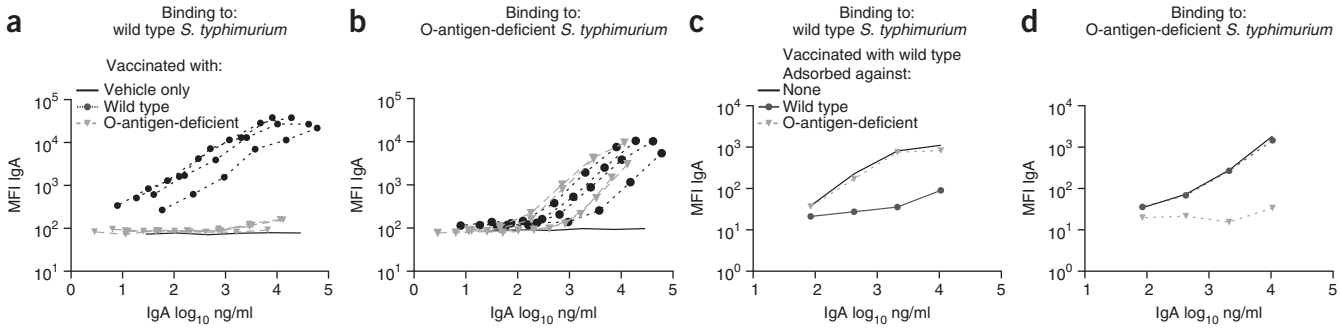

Figure 2 | Detailed analysis of surface epitopes. (**a**–**d**) C57BL/6 (wild type) mice were vaccinated once per week for 3 weeks with an oral inactivated *Salmonella* vaccine constructed from either wild type *S. typhimurium* (black circles) or an O-antigen-deficient strain (Δ*wbaP*, gray triangles) lacking the major surface carbohydrates, or with vehicle alone (PBS, no symbols). On day 21 after the first vaccination, intestinal IgA was collected by lavage and used to stain either wild type (**a**) or O-antigen-deficient *Salmonella* (**b**). n = 5 per group. Figure shows one representative experiment out of three. (**c**,**d**) A single sample generated as in **a** and **b** was cross-adsorbed against buffer only (no symbols), live wild type *Salmonella* (black circles) or O-antigen-deficient *Salmonella* (gray triangles). The cross-adsorbed samples were then used for bacterial flow cytometry staining of wild type (**c**) and O-antigen-deficient (**d**) *Salmonella*. All animal experiments were approved by the legal authorities (licenses 223/2010 and 222/2013, Kantonales Veterinäramt Zürich, Switzerland) and performed according to the applicable legal and ethical requirements. **a** and **b** adapted with permission from Moor *et al.*[22].

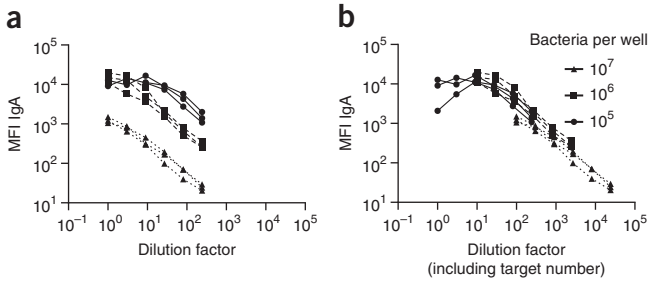

Figure 3 | Increasing the target cell number is equivalent to increasing the dilution factor. (**a**,**b**) Intestinal lavages from *S. typhimurium*-vaccinated mice (as in **Fig. 2**; see Moor *et al.*[22]) were used to stain the surface of *S. typhimurium* at a density of either 10^5, 10^6 or 10^7 cells per well, as indicated. Identical data are plotted in **a** and **b**. However, in **b**, the *x* axis dilution factor is corrected to include the increased number of bacteria per well. All animal experiments were approved by the legal authorities (licenses 223/2010 and 222/2013, Kantonales Veterinäramt Zürich) and performed according to the applicable legal and ethical requirements.

cross-adsorption to examine the intestinal IgA response induced by oral vaccination with inactivated wild type *Salmonella enterica* subsp. *enterica* serovar Typhimurium SB300 (*S. typhimurium*) and O-antigen-deficient *S. typhimurium* SB300 Δ*wbaP*[22], which lacks the major surface carbohydrate epitope. Although these strains are isogenic, apart from deletion of the galactosyltransferase *wbaP*, IgA induced by the O-antigen-deficient vaccination binds only to the surface of O-antigen-deficient bacteria (**Fig. 2a**,**b**)[22]. IgA raised by oral vaccination with the wild type vaccine binds to both strains (**Fig. 2a**,**b**). This suggested that O-antigen shields all other major epitopes on the surface of wild type *Salmonella* strains. As a confirmation, we demonstrated that cross-adsorption of the IgA induced by wild type *S. typhimurium* against wild type bacteria abrogates binding to the wild type strain, but not the O-antigen-deficient strain (**Fig. 2c**,**d**), indicating that the majority of surface-exposed non-O-antigen epitopes are indeed shielded. Vice versa, cross-adsorption of IgA induced by vaccination with wild type *S. typhimurium* against O-antigen-deficient *S. typhimurium* abrogates binding to the O-antigen-deficient strain but not to the wild type strain. We can therefore conclude that oral vaccination with an inactivated *S. typhimurium* vaccine induces IgA with specificity for both O-antigen and other surface antigens. However, on live *S. typhimurium*, the major surface epitope exposed to IgA is the O-antigen. Thus, bacterial flow cytometry can be used to interrogate the antigenic specificity of antibody responses and the nature of surface-exposed antibody epitopes in intact bacteria.

Experimental design

Bacterial number. The most important consideration when setting up a bacterial flow cytometry experiment is that bound antibodies are measured per bacterium. Therefore, in contrast to bulk assays such as western blotting or ELISA, using high numbers of bacteria per well actually dilutes rather than maximizes the specific signal. In fact, increasing the number of bacteria per sample is exactly equivalent to diluting the antibody solution (**Fig. 3**). We have therefore developed this protocol to stain only 10^5 bacteria per sample—at least 100-fold fewer than has previously been used in pneumococcal antibody assays[9]. This delivers high levels of sensitivity with very low volumes of antibody-containing body fluids.

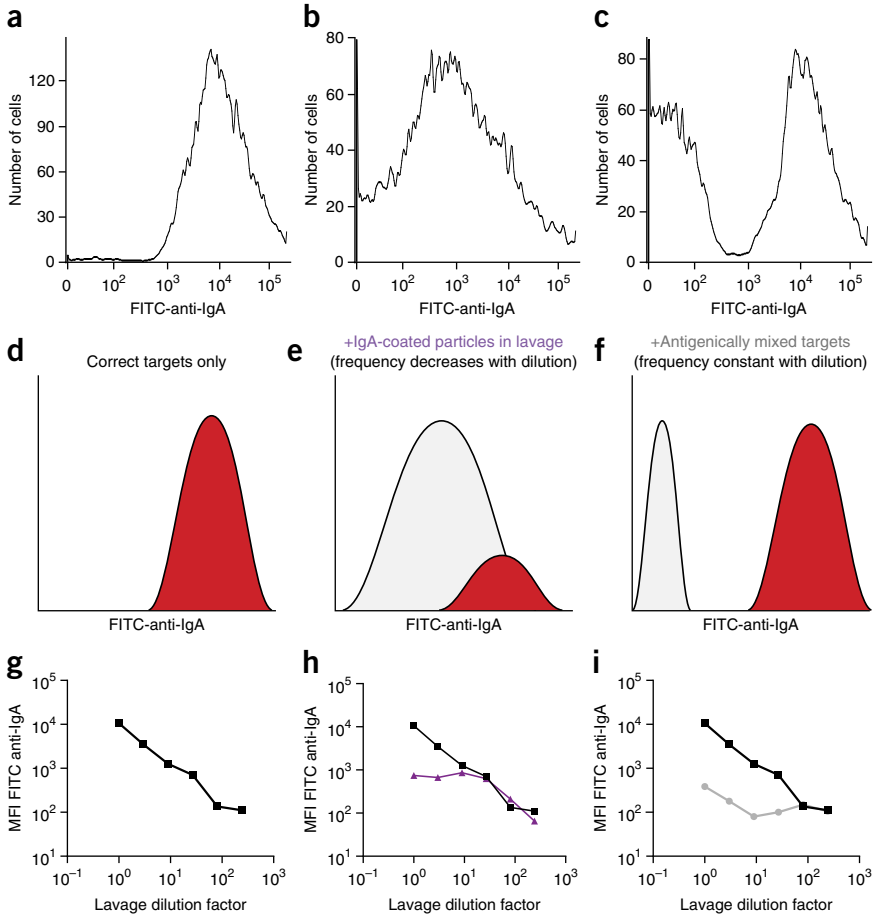

Figure 4 | The effects of different contamination sources on acquired data. (**a**–**c**) Histograms of IgA staining for a single intestinal lavage from an *S. typhimurium*-vaccinated mouse that was fully cleared by centrifugation and filtration (**a**,**c**), or was left uncleared (**b**). These antibody preparations were then used to stain a pure preparation of *S. typhimurium* (**a**,**b**) or *S. typhimurium* mixed 1:1 with *E. coli* 8178 (**c**). (**d**–**f**) Schematic representations of the effects of contaminations on the appearance of histograms at a high specific IgA concentration. Red—specific signal due to IgA bound to *S. typhimurium*, gray—nonspecific signal due to contamination. (**g**–**i**) Effect of different types of contamination on the titration curves generated by plotting median fluorescence intensity against dilution factor. Black squares—noncontaminated sample. Purple triangles—contamination with IgA-dim events present in the intestinal lavage (as in **b** and **e**). Gray circles—contamination with IgA-negative events present in the bacterial targets (as in **c** and **f**). All animal experiments were approved by the legal authorities (licenses 223/2010 and 222/2013, Kantonales Veterinäramt Zürich) and performed according to the applicable legal and ethical requirements.

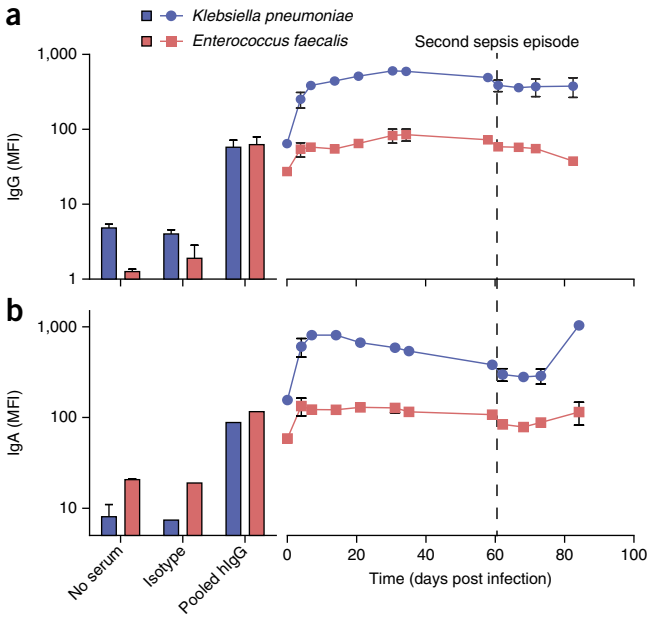

Figure 5 | Timing of serum IgG and IgA induction after confirmed *K. pneumoniae* sepsis secondary to a lung infection. (**a**,**b**) Blood samples were collected from a patient admitted to the hospital with a systemic *K. pneumoniae* infection from day 0 until day 84. A second infection with the same strain occurred during follow-up at day 62. IgG (**a**) and IgA (**b**) responses against *K. pneumoniae* (blue line, circles) and *Enterococcus faecalis* (red line, squares) were determined at a single normalized Ig concentration (10 µg/ml). Negative-control MFI levels (no primary antibodies, isotype control), as well as a standard control of pooled hIgG from healthy donors, are represented as bars. IgG and IgA median fluorescence intensity (MFI) levels are plotted for each time point. Each point or bar represents the mean of two independent experiments. Error bars represent the standard error of the mean. All human samples were obtained following acquisition of the study participants' and/or their legal guardians' written informed consent. The study protocol was reviewed and approved by the local ethics committees (Comité de Protection des Personnes, Ile de France VI).

Minimizing contamination and noise. Another major consideration is that, in the basic version of this protocol, bacteria are identified entirely based on their flow-cytometry-determined light-scattering features. This means that any bacteria or bacterial-sized particles in solutions (including dead bacteria in autoclaved solutions) or in cytometer tubing, will contaminate the final data file. This problem can be minimized by thoroughly cleaning the flow cytometer before use and by passing all buffers and media through sterile 0.22-µm filters before use (note that optically clear solutions can contain 10^6 bacteria per ml). **Figure 4** demonstrates the likely effects of such contaminations on the final conclusions drawn from the analysis. It should be noted that contaminants can be antibody positive (**Fig. 4b**,**e** and **h**) or negative (**Fig. 4c**,**f** and **i**), and can be mixed with the antibody solutions—in which case they dilute out with titrations (**Fig. 4h**)—or can be mixed with the bacterial targets or can be present in the flow cytometer (**Fig. 4i**), in which case they are present in all samples at equal concentrations. In the worst cases, excessive noise signal may render a positive signal undetectable without complex data analysis (**Fig. 4i**). In all cases, data displaying these staining patterns are unreliable (see the TROUBLESHOOTING section, Steps 31 and 32), and the experiment should be rerun with further purification steps.

An alternative solution to minimize noise is to use fluorescently labeled target bacteria, which allows specific gating. In the case of genetically tractable organisms, fluorescent protein expression is a simple and effective approach. It is highly recommended that those carrying out bacterial flow cytometry for the first time include a sample with *Escherichia coli* K-12 engineered to express an easily detectable fluorescent protein (e.g., GFP in cytometers with a 488-nm laser) in order to confidently set up the flow cytometer to detect bacteria.

For organisms that cannot easily be induced to take up plasmids and/or express fluorescent proteins, it is possible to use cell-permeable amine-binding dyes (**Fig. 1b**) or covalent surface labeling with *N*-hydroxy succinimide (NHS)-biotin compounds and fluorescent streptavidin conjugates. For multiplexing several bacterial strains, the very low off-rate of biotin–streptavidin interactions may be used with NHS-biotin labeling before mixing different target populations. A disadvantage of NHS-biotinylation of the target strain surface is that dense chemical modification of the bacterial surface may interfere with epitope recognition.

Choice of body fluid, purifications and time points for analysis. The exact choice of body fluids and time points to analyze for antibody responses depends strongly on the scientific objective. To illustrate best practices, we give four typical situations here.

Induction of serum antibodies: whole-bacterium i.v. vaccination in mice. This is an example of a robust serum antibody response induced by a very strong stimulus at a defined time point[1]. In this case, it is well documented that a peak antibody response is observed roughly 3 weeks after initial exposure. The antibodies that have been induced are present at high concentrations in blood serum. As serum is easily accessible and blood can be repeatedly sampled, one would most logically sample before vaccination and then weekly for 3–5 weeks after vaccination[1]. As only a few microliters of serum are required per assay, 50 µl of blood per time point is sufficient for a full analysis. Unless the serum samples have a very high fat content, it will be possible to use 0.22-µm-filtered serum directly for bacterial staining.

Induction of serum antibodies during documented Klebsiella pneumoniae *septicemia.* A patient presented with *K. pneumoniae* sepsis, secondary to a lung infection. We followed up the *K. pneumoniae*-specific serum IgG (**Fig. 5a**) and serum IgA (**Fig. 5b**) responses longitudinally. Serum dilutions were adjusted in order to work with single total IgG and IgA concentrations of 10 µg/ml. As a control, the IgG and IgA responses of the same individual were tested against an irrelevant strain (*Enterococcus faecalis*). These remain low and stable over the course of infection, suggesting that the *Klebsiella*-binding activity that we detected was indeed *Klebsiella* specific, or *Klebsiella* related. This longitudinal study highlights a typical 3-week kinetics in induction of specific IgG, but a much faster kinetics of IgA induction. As the IgA response is predominantly primed at mucosal surfaces, the observed response may actually have been initiated during the primary lung infection (**Fig. 5b**).

Analysis of bacterial-binding IgA in intestinal lavage of ex-germ-free mice after recolonization with a single apathogenic strain. This is an example of a relatively weak immune stimulus given at a defined time point[1]. The bacteria are given into the intestine and stimulate the mucosal immune system predominantly to induce an IgA response. Although specific IgA will be measurable in serum, the majority of the IgA is secreted across the

Box 2 | Purification of IgA from viscous or fatty samples ● TIMING 30 min–1 h

Purification of antibodies using Fc-specific gel filtration is extremely helpful for fluids with a very high viscosity or fat content—for example, fecal water or breast milk. As these tend to be mucosal-associated fluids, IgA is normally the target isotype for purification, and it should be noted that this isotype does not bind to either protein A or protein G (standard immunoglobulin purification reagents). We therefore recommend the following procedure:

1. Predilute the samples in PBS to achieve a workable viscosity, a pH >7 and a volume of between 1 and 10 ml.
2. Clear the suspensions by centrifugation at 16,000*g* for 5 min at 4 °C.
3. Filter the samples through a 0.22-µm syringe filter.

▲ **CRITICAL STEP** This is essential to avoid purification column blockage and complete loss of the sample.

4. For each sample, prepare a gravity flow column containing either peptide M/agarose or immobilized Jacalin affinity gel according to the manufacturer's instructions: typically, degas both the agarose and washing buffer, and then carefully load the agarose on top of a membrane filter, avoiding air bubbles. Wash through with 10 column volumes of wash buffer. Column size and IgA-binding protein quantities should be adjusted to the expected IgA concentration in the sample of interest. Binding capacity of peptide M: 4–6 mg/ml; binding capacity of Jacalin: 1–3 mg/ml.

▲ **CRITICAL STEP** Peptide M binds IgA1 and IgA2, whereas Jacalin binds only IgA1 (and IgD).

5. Equilibrate the columns with 10 ml of PBS.
6. Add the sample and allow it to fully enter the matrix.
7. Wash the sample with 20 column volumes of PBS.
8. Elute in 10 ml of elution buffer (0.1 M glycine, pH 2–3, for peptide M; 0.1 M α-D-galcatose for Jacalin).
9. After elution in 0.1 M glycine, pH 2–3, use 1 M Tris, pH 7.5, to neutralize the solution. As dimeric secretory IgA has a molecular mass of >300 kDa, buffer exchange can be easily performed by filtration through a 100-kDa ultrafiltration membrane (per the manufacturer's instructions) and resuspension in flow cytometery buffer.
10. These antibody solutions can be divided into aliquots and frozen at –20 °C for several months or at –80 °C for several years.

▲ **CRITICAL STEP** Re-filter the solution through a 0.22-µm syringe filter before use, in order to remove any precipitates that may have formed during the preparation process.

host mucosal membranes, and the strongest signal will therefore be measurable in an intestinal lavage[1,3]. On the basis of published literature[3,15], specific IgA is measurable from ~2 weeks post colonization, with a titer that increases over time.

Analysis of bacterial-binding IgA in human breast milk and fecal water. As these fluids are potentially viscous with a high fat content, we strongly recommend affinity-based purification of IgA before analysis to avoid high levels of nonspecific background (as detailed in **Box 2**). These purified IgA solutions can then be used to stain the bacterial species of choice using the standard protocol. In the given examples, IgA specific for a human *E. coli* isolate could be detected in fecal water. *Bifidobacterium adolescentis*-binding IgA was found in breast milk. By contrast, IgA specific for *Bacteroides dorei* was not detected in either of these donors/fluids. Nonspecific binding of the primary and secondary reagents to these bacterial strains was assessed in parallel (**Fig. 6a**,**b**).

Choice of bacterial targets and preparation methods. The species, subspecies and serovar of bacteria chosen for analysis, as well as the growth conditions used, may markedly alter the pattern of bacterial antibody binding observed. Two bacteria of the same species (for example, *S. enterica* subsp. *enterica* serovar Typhimurium and *S. enterica* subsp. *enterica* serovar Choleraesuis) share almost no exposed surface epitopes[33]. In addition, many bacterial species display considerable phenotypic variation due to growth phase, environmental influences and quorum sensing[29]. We therefore advise choosing the bacterial strains for analysis very carefully, generating frozen stocks of these strains and regularly checking for contamination by standard microbiological techniques. In addition, it may be of interest to initially screen a range of culture media and growth conditions to optimize relevant antigen expression.

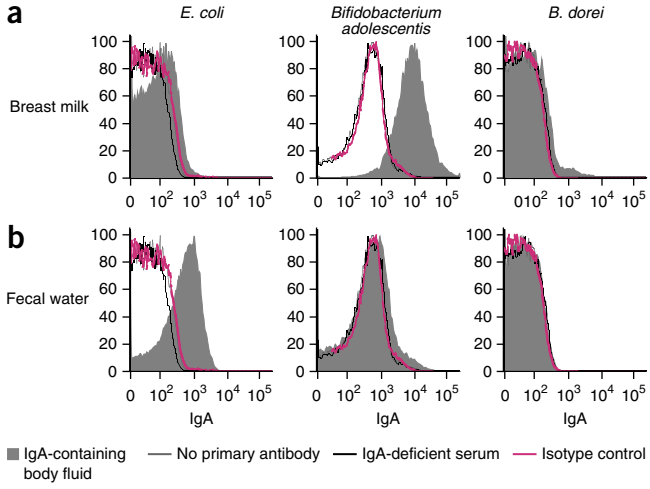

Figure 6 | Bacterial flow cytometry with purified IgA from human breast milk and fecal water. (**a**) Secretory IgA purified from breast milk (gray-filled histogram) and (**b**) secretory IgA purified from fecal water (gray-filled histogram) from two independent donors were tested for binding to *E. coli* (human isolate), *Bifidobacterium adolescentis* and *B. dorei* at a fixed, normalized, concentration (10 µg/ml). Monoclonal IgA isotype control (pink line), no primary antibody (gray line) and IgA-deficient serum (black line) are included as negative controls. Binding is revealed by anti-human IgA-FITC. All human samples were obtained following acquisition of the study participants' and/or their legal guardians' written informed consent. The study protocol was reviewed and approved by the local ethics committees (Comité de Protection des Personnes, Ile de France VI).

Box 3 | Cryo-preserving and thawing aliquots of bacterial targets culture time ● TIMING 1–3 d + 30 min

1. Cultivate bacteria and quantify density as described in Steps 1–7 of the PROCEDURE. Calculate the volume of medium that needs to be prepared and inoculated to permit production of sufficient aliquots of frozen cells for the entire experiment series (e.g., *E. coli* typically grows to a maximum density of 10^9 CFU/ml in aerobic culture in a rich medium. To generate 200 aliquots of 10^8 CFU, you will require 20 ml of overnight culture).
2. Centrifuge 10–50 ml of culture at 4 °C at 4,000–8,000*g* for 5 min. Resuspend in 10–50 ml of sterile PBS.
3. Centrifuge the culture again at 4 °C for 5 min at 4,000–8,000*g*.
4. Resuspend the pellet in cell-freezing medium to a final concentration of 5 × 10^8–10^9 CFU/ml. Make 100-µl aliquots and freeze them immediately in liquid nitrogen.

■ **PAUSE POINT** Aliquots may be stored for up to 2 years at –80 °C.

5. To perform experiments, thaw sufficient bacterial aliquots at RT for 15 min.
6. Pool all aliquots and add 1 ml of 1× PBS.

▲ **CRITICAL STEP** PBS should be at 4 °C, and it should be passed through a 0.22-µm filter before use.

7. Centrifuge the samples at 4,000–8,000*g* for 10 min at 4 °C.
8. Thaw an aliquot of 4% (wt/vol) PFA solution.
9. Resuspend the cell pellet from step 7 of this box in 4% (wt/vol) PFA ((optional) +amine-reactive dye—**Box 4**) at a density of 10^9 cells per ml. Incubate the mixture in the dark at 4 °C for 20 min.
10. Add 1 volume of 1× PBS and centrifuge (4,000*g*, 4 °C, 2 min) the mixture to gently pellet only whole bacteria.
11. Resuspend the bacteria at 5 × 10^6 cells per ml in bacterial flow cytometry buffer and use within 72 h, continuing with the Procedure from step 10.

■ **PAUSE POINT** The bacterial suspension can be stored at 4 °C for up to 72 h before flow cytometry.

For organisms with higher biosafety containment requirements, it may be necessary to fix the bacteria before staining. Although paraformaldehyde (PFA) fixation before staining very slightly reduces the specific signal for the strains we have tested, it tends to do so equally for all antibody samples and therefore does not alter the final interpretation of relative titers (**Fig. 7a**,**b**). It is also possible to fix samples at the end of staining with no alteration in signal (data not shown).

Typically, the experiment size in basic research is small and samples can be measured in a single batch. For this, freshly cultured bacteria can easily be used as targets. However, there are several situations in which it is important to have a bulk stock of bacterial targets. Typically, longitudinal clinical studies may follow patient responses to a particular strain over several years. Ideally, effects of bacterial growth conditions and genetic drift in the bacterial stocks should be excluded. In addition, some fastidious strains may require growth conditions that are unavailable at the scientists' host institution, requiring culture off-site. We have therefore established a protocol for the preparation of large batches of cryo-preserved bacterial targets (**Box 3**), and we have observed only minor uniform effects of freezing on antibody binding, which again were equal across all samples we have tested (**Fig. 7c**). This generates both a 'plug and play' system, in which bacteria are always available for experiments, and much better reproducibility of experiments over time.

Controls. The value of this technique rests on the use of good controls for nonspecific binding and contamination. Bacterial species that interact with mammalian hosts have experienced selective pressure from adaptive immunity, and some have the ability to nonspecifically bind antibodies[25,26,34]. In addition, it is important to include sufficient controls to distinguish

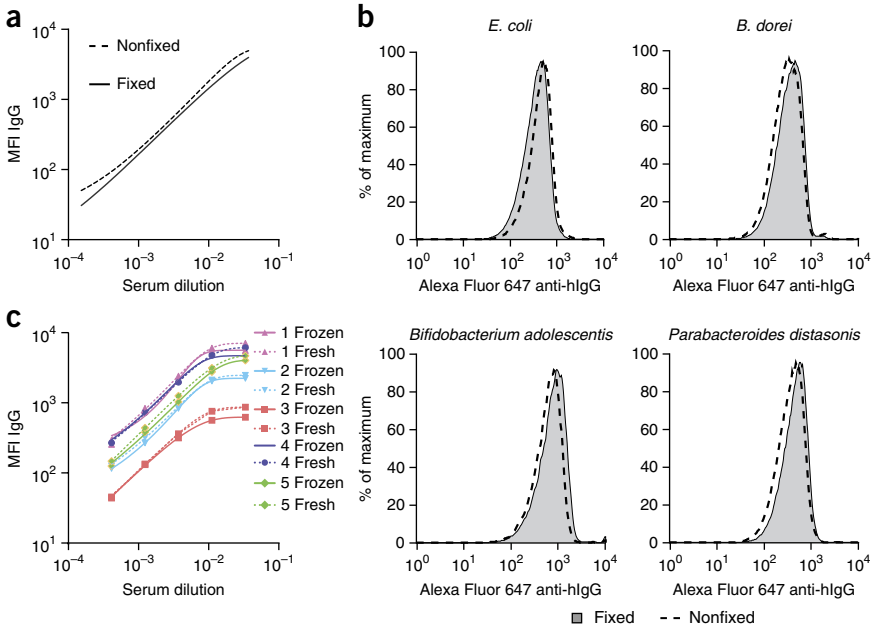

Figure 7 | Effects of bacterial cryo-preservation and fixation on IgG binding. (**a**) Analysis of healthy donor serum IgG responses against paraformaldehyde-fixed and live (nonfixed) freshly grown *K. pneumoniae*. (**b**) A similar analysis of *E. coli*, *B. dorei*, *Bifidobacterium. adolescentis* and *Parabacteroides distasonis* stained with 10 µg/ml pooled healthy donor serum IgG. (**c**) Analysis of IgG responses from five healthy donors against either fresh (dotted lines) or frozen (solid lines) *K. pneumoniae*. All human samples were obtained following acquisition of the study participants' and/or their legal guardians' written informed consent. The study protocol was reviewed and approved by the local ethics committees (Comité de Protection des Personnes, Ile de France VI).

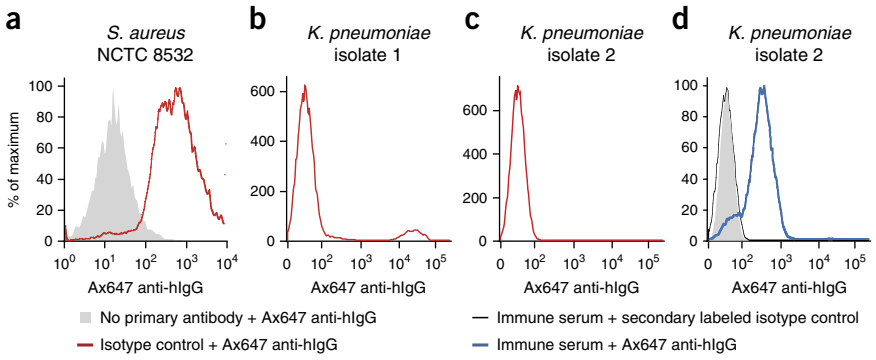

Figure 8 | Nonspecific antibody binding. (**a**–**d**) *S. aureus* strain NCTC 8532 (**a**) and two primary isolates of *K. pneumoniae* (**b**,**c**) were stained without primary anti-sera ('no primary antibody', gray shading) or were incubated with a humanized anti-TNF IgG monoclonal antibody ('isotype control', red). Binding was detected with Alexa Fluor 647-conjugated polyclonal goat-anti-human IgG (Ax647 anti-hIgG). (**d**) For *K. pneumoniae* isolate 2, an additional positive control (serum from a patient with documented *K. pneumoniae* sepsis ('immune serum', blue) and negative control (serum from an IgA-deficient patient, black line) are shown. All human samples were obtained following acquisition of the study participants' and/or their legal guardians' written informed consent. The study protocol was reviewed and approved by the local ethics committees (Comité de Protection des Personnes, Ile de France VI).

'natural antibody' binding, background priming levels and antibody complexes from real signals.

Testing for antibody Fc binding should be carried out before starting any experiments on the bacterial strain of interest. Monoclonal antibodies with irrelevant specificities (isotype controls) are the ideal controls. These are widely available in the mouse, rat and rabbit systems. In the human setting, purified Fc fragments or clinical humanized antibodies specific for cytokines can be used. **Figure 8a**,**b** shows examples in which either the whole population (*Staphylococcus aureus*) or a small subpopulation (*K. pneumoniae* Isolate 1) displays strong binding to human IgG Fc. If this test is positive, it may be worth screening related isolates or genetic mutants to find one lacking Fc-binding activity (**Fig. 8c**). It is important to use secondary staining reagents that are identical to those planned for the main experiments when investigating nonspecific binding, in order to exclude nonspecific binding of the secondary antibody (**Fig. 8d**).

'Natural' broadly cross-reactive antibody responses and background priming can be identified using appropriate negative controls. In the murine system (and other experimental animal models), it is usually possible to obtain negative control samples from animals that have never been exposed to the bacterium of interest (**Fig. 9**; Slack *et al.*[1]). If substantial antibody binding is observed here, even if it is lower than that in actively immunized animals, it may be worth obtaining appropriate antibody preparations from germ-free animals to exclude the role of microbiota-driven responses (**Fig. 9a**,**b**)[21].

Technical controls for adequate clearance of antibody solutions and buffers, as well as biological controls for pre-existing specific antibodies, are essential to demonstrating the reliability of data generated using this technique. Large antibody complexes can sometimes form in antibody solutions, and, if the sample were insufficiently cleared before the bacterial staining, these may be hard to distinguish from bacteria (**Fig. 4b**). The best control for sufficient sample preparation is to examine the presence of a positive antibody-binding signal against a bacterial strain to which the host is highly unlikely to have a functional antibody response. In mice, this can be any strain excluded under the definition of hygienic housing in the local facility (e.g., *S. enterica* serovar Typhimurium; **Fig. 9a**,**b**)[1]. In humans, we have previously used a plant symbiont, *Bradyrhizobium japonicum*, as a negative control[4]. Ideally, such bacteria should be stained in parallel with the test species so that exactly the same antibody preparation is used.

In clinical scenarios, appropriate controls are harder to obtain. Microbial exposure is universal and highly variable, making biological negative controls almost impossible for most experimental purposes. The best controls for Fc- or light-chain binding available are therefore isotype controls—typically monoclonal antibodies of known irrelevant specificity such as the humanized antibodies used clinically for cytokine blockade (**Figs. 8** and **10a**,**b**). Correspondingly, for most species, there are no active human immunizations to generate positive controls. However, positive controls can be obtained in cases of recent and documented infections with the bacterium of interest or a strain of identical

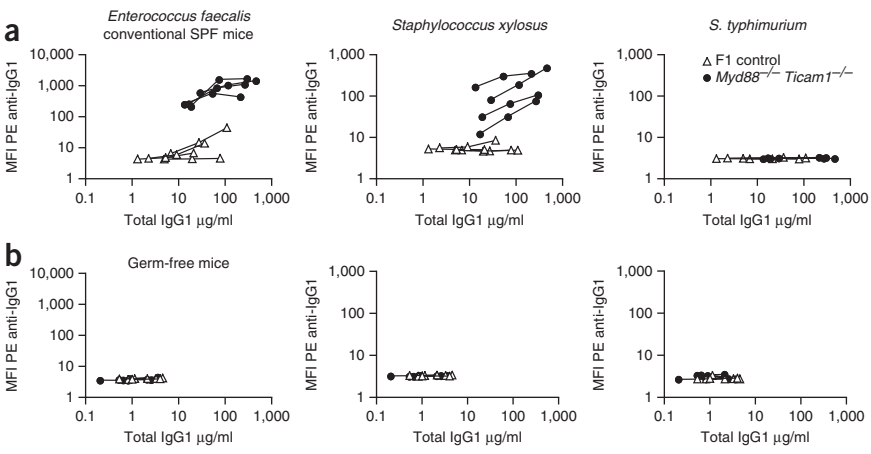

Figure 9 | Use of irrelevant bacterial species and germ-free mice as negative controls in the murine system. (**a**) Sera from conventionally housed *MyD88*$^{-/-}$*Ticam1*$^{-/-}$ mice (black circles) and F1 control mice (open triangles) were used to stain two autologous aerobically culturable microbiota species, *Enterococcus faecalis* and *Staphylococcus xylosus*, as well as the irrelevant bacterial strain *S. typhimurium* (to which the mice had never been exposed). (**b**) Sera from germ-free *MyD88*$^{-/-}$*Ticam1*$^{-/-}$ mice and F1 control mice were used to stain the same bacterial strains. IgG1 staining was visualized using phycoerythrin (PE)-conjugated rat anti-mouse IgG1 monoclonal, and the median fluorescence intensity (MFI) was calculated for each dilution. $n = 4$ per group. From Slack, E. *et al.*[1]. Adapted with permission from the American Association for the Advancement of Science.

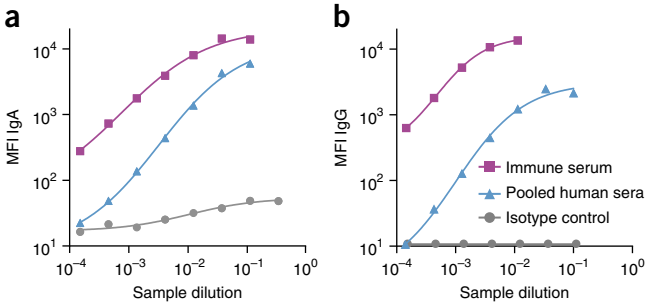

Figure 10 | Controls in the human system. (**a**,**b**) Serum from a patient with *K. penumoniae* sepsis 'immune serum' (purple squares), 'pooled human sera' (blue triangles) and humanized monoclonal IgA or IgG Isotype controls (gray circles) were used to stain *K. pneumoniae* (isolate 2, non-Fc-binding) followed by detection with human (**a**) IgA- and (**b**) IgG-specific reagents. All human samples were obtained following acquisition of the study participants' and/or their legal guardians' written informed consent. The study protocol was reviewed and approved by the local ethics committees (Comité de Protection des Personnes, Ile de France VI).

serotype (**Figs. 5** and **10a**,**b**). Although for practical reasons this will not always be possible, introduction of such positive controls greatly improves data interpretation. A further advantage of such positive control samples is that they can be used as internal standards to facilitate data comparisons in longitudinal measurements.

Pooled polyclonal human IgG (either replacement therapy Ig (IVIg) or human reference serum for research purposes) and IgA (human reference serum) may also be used as standard controls. These are also useful tools that allow a global assessment of the average healthy population status (**Fig. 10a**,**b**). Cross-adsorption, as shown in **Figure 2**, may also be applied in the human system to generate appropriate negative controls.

A final essential set of controls is required to determine appropriate flow cytometer settings and compensation for fluorescence spillover. Ideally, these controls should be generated using the same bacterial species as the main analysis to account for background fluorescence. For each fluorophore, you should generate a pair of samples: one stained with positive control antibodies and one stained with negative control antibodies, as detailed in the PROCEDURE (Steps 1–28). These samples are then stained with a single fluorophore-conjugated secondary antibody to generate single-stained controls. For highly complex stainings, cocktails of secondary antibodies can be generated for 'fluorescence-minus-one' controls. In the case that no positive control is available, a last resort is to use cytometer setup beads to determine cytometer settings, but, given the very different size and scatter characteristics of beads and bacteria, this is only really suitable for fluorophores with little or no spectral overlap. A further control for longitudinal experiments in which data must be compared over several different days of analysis is the use of fixed gating templates and calibration beads. When the first samples are run, the calibration beads should be run on the fully set up cytometer and the file should be saved. A 'gate' can then be drawn on each bead population in the fluorescence channels of interest, and this gating template and statistics can be saved. As the bead fluorescences remain constant over time, exactly replicating the distribution of beads in the saved gates can generate fully comparable cytometer settings[35].

Choice of FACS machine. Samples can theoretically be acquired on any flow cytometer with a laser/filter compatible with the chosen fluorophores. The procedures suggested here have been used successfully to analyze samples on a FACSCalibur (Becton Dickinson, (BD)), a LSRII (BD), FACS CANTO II (BD) and a FACSArray (BD). It is beyond the scope of this protocol to provide instructions for all possible flow cytometers, and considerable differences exist between older and newer machines. Rather, we suggest contacting the manufacturer or local experts to determine an appropriate setup protocol for bacterial detection on your specific flow cytometer, as well as for advice on how best to generate comparable data on different days.

How detailed an analysis is planned? Full titration curves versus single antibody concentrations. ELISA-based techniques to measure antibody titers typically measure either the total signal for a given antibody concentration or generate a full titration curve by applying serially diluted sample to an antigen-coated plate. Exactly the same types of quantitative data can be generated by bacterial flow cytometry. The requirement for either type of analysis depends on the aim of the experiment.

When serially diluted antibody-containing solutions are used to stain the bacterial strain of interest, the resulting fluorescence intensities can be plotted against the dilution factor and will form a four-parameter logistic curve (**Fig. 11a**,**b** and **Supplementary Data**). This generates a very data-rich data set that can be further interpreted. Typically, the horizontal displacement of curves is used to determine the relative titer. In cases in which a monoclonal antibody of known specificity for the strain of interest is analyzed in parallel, this permits the calculation of a titer as the concentration of surface-binding antibodies present in the sample (**Fig. 11c** and **Supplementary Data**). It should be noted that avidity and epitope availability will also affect the shift when comparing a monoclonal with a polyclonal response, so this is not an entirely accurate description of the read-out. It is also possible to calculate relative titers in the absence of a monoclonal control (**Fig. 11d–f** and **Supplementary Data**). Note that although the units and values differ from the absolute values calculated in **Figure 5c**, the proportional difference between the 'test' and 'control' values is identical.

For very large sample sets, analysis of full titration curves increases the workload multiplicatively for each titration step and generates complex statistical issues relating to curve fitting and multiple testing. In these cases, it may be more reasonable to carry out pilot dilution-series stainings on positive control samples to determine a total antibody concentration giving binding levels around the inflection point of the logistic curve (pseudo-linear range; **Fig. 11g**). All samples can then be diluted to exactly this total antibody concentration and a single well can be analyzed for each donor (**Fig. 11h**,**i**). The disadvantages of this approach are that although it will consider a reasonably narrow window of high-titer responses, it may miss quantitative differences between samples showing saturating binding at these concentrations and it will score all low titers as 'zero'. Nevertheless, this can generate data almost equivalent to those obtained by analysis of full titration curves, with considerably lower cost, fewer lab hours and fewer computer hours.

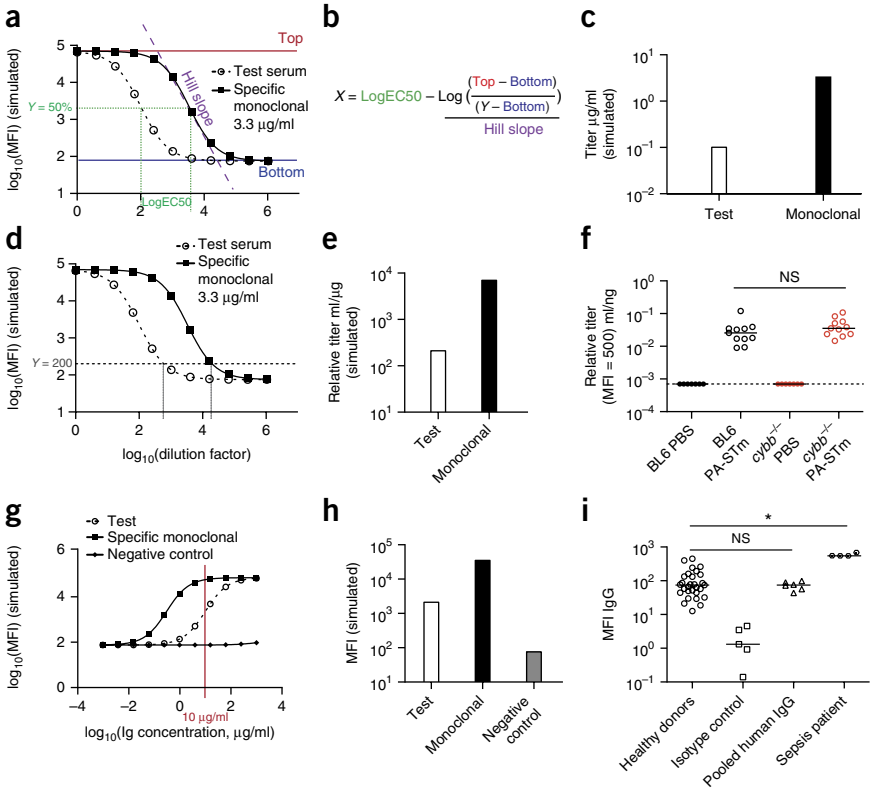

Figure 11 | Data analysis methods. (**a**) Two *in silico*-generated 'perfect' data sets for a monoclonal antibody of known specificity and a test sample fitted using the four-parameter logistic equation to demonstrate the meaning of all parameters generated by the nonlinear regression. (**b**) The four-parameter logistic equation rearranged to give *X* in *Y*. (**c**) Simulated absolute titers calculated from the curves in **a**. (**d**) *In silico*-generated curves, as in **a**, showing the setting of a *y* axis limit for the calculation of relative titers. (**e**) Relative titers calculated from the curve shown in **d**. (**f**) Relative titers calculated using the method shown in **d** and **e** for IgA in the intestinal lavages of orally vaccinated (PA-STm) or mock-vaccinated (PBS) wildtype (B6) and gp91Phox-deficient mice (*cybb*$^{-/-}$) against *S. typhimurium*. Statistics: two-way ANOVA. $n = 8$ or more per group. (**g**) Use of single immunoglobulin concentration measurements to simplify data acquisition and analysis. From full titration curves (simulated as in **a**,**d**), a value can be identified that gives a good window for detection of positive and negative signals (10 µg/ml in this case). (**h**) Plot of the MFI values for the total concentration obtained in **g**. (**i**) Binding of 10 µg/ml IgG of the indicated sources to *K. pneumoniae*. Lines indicate medians. Kruskal–Wallis test with Dunn's post-test. **P* value < 0.05. All human samples were obtained following acquisition of the study participants' and/or their legal guardians' written informed consent. The study protocol was reviewed and approved by the local ethics committees (Comité de Protection des Personnes, Ile de France VI). NS, not significant. **f** adapted with permission from Moor *et al.*[22].

In conclusion, when designing a bacterial flow cytometry experiment, the following key steps should be taken: determine the optimal time points, body fluids to collect and whether antibody purification will be required; identify the bacterial species of interest and obtain pure frozen bacterial stocks; establish robust culture protocols for this species and whether fresh or frozen targets are most appropriate; establish whether fixation is necessary for practical or biosafety reasons; before any experiment is started, be sure to exclude Fc binding by the bacterial strain of interest by the use of isotype controls; establish the relevant positive and negative control antibody solutions and bacterial strains; and decide whether full titrations or single concentrations will be analyzed. If single concentrations are to be used, establish the optimal concentration in pilot experiments.

MATERIALS

REAGENTS

- PBS, pH 7.4, no calcium or magnesium (standard lab reagent or 10× PBS, Eurobio, cat. no. CS3PBS01-01)
- BSA factor V (GE Healthcare, cat. no. K41-001)
- Sodium azide (Sigma-Aldrich, cat. no. 71289) **! CAUTION** This is a hazardous chemical. Avoid contact with skin, eyes and airways.
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. no. 158127)
  ! CAUTION PFA is toxic and flammable. When you are weighing the crystalline solid or preparing solutions, work should be performed in a fume hood.
- Human blood, breast milk, feces or other fluid of interest, collected in the context of an approved clinical trial or from an approved biobank.
  ! CAUTION For human studies, informed consent must be obtained from all study subjects. **! CAUTION** For studies using human or animal samples, all experiments must be conducted according to the relevant guidelines and official permissions/ethical approval must be obtained
  ! CAUTION Such samples are potential infection hazards. Handle them at an appropriate biosafety level.
- Murine blood, intestinal lavage, feces or other fluid of interest collected in the course of ethically approved animal experiments **! CAUTION** For studies using human or animal samples, all experiments must be conducted according to the relevant guidelines and official permissions/ethical approval must be obtained **! CAUTION** Such samples are potential infection hazards. Handle them at an appropriate biosafety level.
- Appropriate liquid bacterial culture medium: e.g., lysogeny broth medium (e.g., liquid medium, MP Biomedicals, cat. no. 3001-031), brain–heart infusion broth (Oxoid, cat. no. CM1135) **! CAUTION** Many liquid media contain components that are potentially harmful to human health. Follow the manufacturer's instructions for safe handling.
- Appropriate solid bacterial culture media: e.g., Columbia agar + 5% sheep blood (Biomerieux, cat. no. 43049), LB agar (Thermo Fischer Scientific, Life Technologies, cat. no. 22700-025) **! CAUTION** Many solid media contain components that are potentially harmful to human health. Follow the manufacturer's instructions for safe handling.
- Frozen stocks of the bacteria of interest (e.g., *Klebsiella pneumoniae* (American Type Culture Collection (ATCC), cat. no. BAA-1705))
  ! CAUTION Such stocks are potential infection hazards. Handle them according to the appropriate biosafety regulations for the species in question.
- Appropriate liquid disinfectant (e.g., Clidox, Pharmacal Research Labs, cat. nos. 95120F and 96120F)
- Taq PCR kit (e.g., Promega GoTaq Green, Promega, cat. no. M7122)
- Agarose LE (e.g., Promega, cat. no. V3125)
- 1× Tris–EDTA (TE) buffer (e.g., Promega, cat. no. V6232)

- Gel extraction kit (e.g., Wizard SV Gel and PCR Clean-Up System, Promega, cat. no. A9281)
- ACD tubes (BD Vacutainer, cat. no. 364606)
- Lymphocyte separation medium (Eurobio, cat. no. CMSMSL0101)
- 1.2-ml serum-activating gel tubes (Sarstedt, cat. no. 41.1395.005)
- 0.5 M EDTA (Applichem, cat. no. A1104) ! CAUTION EDTA is an irritant. Wear suitable personal protective equipment while handling this compound.
- Soybean trypsin inhibitor (Sigma-Aldrich, cat. no. T9128)
- EZ-Link NHS-PEG4-Biotin (Thermo Fischer Scientific, Life Technologies, cat. no. 21330)
- Glycine (AppliChem, cat. no. A1067)
- Cell proliferation dye eFluor 450 (eBiosciences, cat. no. 65-0842-85)
- Fixable viability dye eFluor780 (eBiosciences, cat. no. 65-0863-14)
- LIVE/DEAD Fixable Green Dead Cell Stain Kit (Life Technologies, cat. no. L-23101) ▲ CRITICAL We have noticed that the Aqua version (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Life Technologies, cat. no. L-34957) is inefficient for bacterial staining.
- CellTrace 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE; Life technologies, cat. no. C34554) ▲ CRITICAL CFSE is efficient only for a subset of bacterial species. As dye loading is heavily dependent on bacterial biochemistry, optimal dye-type, concentration and loading conditions should be determined for each bacterial species tested.
- IgA-binding peptides: peptide M/agarose (Invivogen, cat. no. gel-pdm-2), immobilized Jacalin (Thermo Fischer Scientific, cat. no. 20395) ▲ CRITICAL M-peptide binds IgA1 and IgA2, whereas Jacalin binds only IgA1 (and IgD).
- Human reference serum (Bethyl, cat. no. RS10-101)
- Pooled human IgG (Hizentra, CSL Behring Laboratories)
- Infliximab (monoclonal chimeric IgG1 anti-human TNF-α, Janssen Biotech)
- Chimeric IgA anti-hRSV protein F (B cell Design, cat. no. A1Trsv11)
- Glycerol (Sigma-Aldrich, cat. no. G7757)
- Fluorescent calibration beads (e.g., Rainbow calibration particles eight peaks, 3.0–3.4 µm, Becton Dickinson, cat. no. 559123)
- Flow-count fluorosphere beads (Beckman Coulter, cat. no. A91346)
- ELISA kits to determine total concentrations of your antibody isotypes of interest (e.g., Human IgG ELISA Quantitation Set, Bethyl, cat. no. E80-104)
- Standard Taq PCR mix
- Neutralization buffer: 1 M Tris HCl, pH 7.5 (e.g., Life Technologies, cat. no. 15567-027)

16S rDNA primers

- FD1: 5′-AGAGTTTGATCCTGGCTCAG-3′ (Microsynth AG)
- FD2: 5′-AGAGTTTGATCATGGCTCAG-3′ (Microsynth AG)
- RC1: 5′-ACGGGCGGTGWGTRCAA-3′ (Microsynth AG)

Elution buffers

- Peptide M: 100 mM glycine, pH 2–3 (AppliChem, cat. no. A1067)
- Jacalin: 0.1 M α-D-galcatose (Sigma-Aldrich, cat. no. G0625)

EQUIPMENT

- Polypropylene 96-well V-bottom plates (e.g., VWR, cat. no. 732-2620)
- Library tubes, 1.2 ml polypropylene, 5.8 × 47.4 mm (Milian SA, cat. no. 077210)
- Sterile serological pipettes (e.g., Sarstedt, cat. no. 86.1254.001)
- Syringes
- 0.22-µm Bottle-top filters (rapid Filtermax, 0.22-µm PES, TPP, cat. no. 99505)
- 0.22-µm Syringe filters (>1 ml, polyethersulfone 0.22 µm, TPP, cat. no. 99722)
- 0.22-µm Syringe filters (<1 ml, Millex-GV, 0.22-µm polyvinylidene difluoride, 4 mm, Merck Millipore, cat. no. SLGVR04NL)
- 1.5-ml Snap-cap tubes, autoclaved at 121 °C for 12 min (e.g., Sarstedt, cat. no. 72.695.500)
- Sterile pipette tips
- Petri dishes (e.g., Sarstedt, cat. no. 82.1472)
- Sterile inoculation loops (e.g., Nunc, Sigma-Aldrich, cat. no. I7648-1PAK)
- Parafilm (Sigma-Aldrich, cat. no. P7793-1EA)
- 0.2-µm Centrifugation columns (VWR, cat. no. 82031-356)
- Disposable gravity-flow columns (e.g., Pierce, Life Technologies, cat. no. 29925)
- Amicon ultracentrifugal filters, 100 kDa cutoff (Merck Millipore, cat. no. UCF910096)
- Glass bacterial culture flasks/tubes with rubber stoppers and aluminum seals (e.g., Chemglass Life Sciences, cat. no. CLS-4209-01)
- Any flow cytometer with appropriate laser/detector combinations for the fluorophores used. For example, LSRII (Becton Dickinson) 488-, 405- and 633-nm lasers and with nine detectors. Associated computer running acquisition software—for example, FACS Diva (BD) ! CAUTION Class I laser product.
- Robotic sample loaders for the flow cytometer (not essential)
- Bench-top centrifuge capable of centrifuging 96-well plates (e.g., Eppendorf 5810 R, A-4-81 rotor)
- Refrigerated minifuge for 1.5- to 2-ml tubes (e.g., Eppendorf 5417 R with rotor FA-45-30-11)
- Multichannel pipettes (e.g., Gilson Pipetman L multichannel 12× 20–200 µl, cat. no. FA10012)
- Laminar flow cabinet certified up to biosafety level II (e.g., Scanlaf Safe 1200, Vitaris, cat. no. 61200-LAB)
- Fume hood
- For anaerobic culture of targets: anaerobic tent (e.g., type B vinyl anaerobic chamber) supplied with gas mixture (e.g., 10% H_2, 5% CO_2 and 85% N_2) for anaerobic culture
- For anaerobic culture of targets only: anaerobic culture jar (e.g., Oxoid, cat. no. HP0011)
- Stationary incubator (e.g., Heraeus B12, Thermo Fischer Scientific)
- Shaking incubator (e.g., Kuhner shaker ISF-1-W, Adolf Kühner AG)
- Micropipettes
- Aspirator pump (e.g., Vacusafe, Vitaris)
- Waterbath or thermomixer programmable to 37–56 °C
- Orbital shaker
- Spectrophotometer capable of measuring OD_{600nm}. (e.g., Agilent Technologies, Agilent 8543)
- PCR machine (e.g., T100 thermal cycler, Bio-Rad)
- Agarose gel equipment and power pack (e.g., 1704467 and PowerPac Universal Power Supply, Bio-Rad)
- Analysis computers running FlowJo (Treestar), Excel (Microsoft) and Prism (GraphPad). It would be possible to run all analysis in the 'R' statistical programming language, and open-source flow cytometry analysis solutions (Flow (https://galen.dulci.duhs.duke.edu/flow/) or Bioconductor (http://bioconductor.org/) and thus to have an open-source software solution. However, we have not currently implemented this solution.

REAGENT SETUP

PBS Dilute 10× PBS to 1× with dH_2O.

Bacterial flow cytometry buffer The bacterial flow cytometry buffer is PBS with 2% (wt/vol) BSA and 0.02% (wt/vol) sodium azide. Filter-sterilize the buffer and store it at room temperature (RT, 20–25 °C) for up to 1 year. ! CAUTION Sodium azide is harmful. Avoid contact with skin, eyes or airways. Wear suitable personal protective equipment when you are handling the solid or solutions containing this chemical.

4% (wt/vol) PFA in PBS Dissolve PFA powder in PBS with heating at high pH. Adjust the pH to 7 using hydrochloric acid. Pass the solution through a sterile 0.22-µm filter. Divide the solution into aliquots, and store them at −20 °C for up to 3 months. ! CAUTION PFA is toxic and flammable. When you are weighing the crystalline solid or preparing solutions, work should be performed in a fume hood.

Collection of antibody-containing body fluids: serum/plasma Collect serum/plasma using any standard protocol. Serum and plasma should be separated away from blood cells rapidly after collection and stored at −80 °C until use (indefinite storage at −80 °C). ! CAUTION For human studies, informed consent must be obtained from all study subjects. ! CAUTION For studies using human or animal samples, all experiments must be conducted according to the relevant guidelines, and official permissions/ethical approval must be obtained. ! CAUTION Such samples are potential infection hazards. Handle them at an appropriate biosafety level.

Collection of antibody-containing body fluids: mouse intestinal lavages Collect the samples as described[15]. Make intestinal lavage buffer <1 h before use: it contains 1× PBS, 5 mM EDTA and 1 mg/ml soybean trypsin inhibitor. Dissect the entire intestine from the stomach to the sigmoid colon. Inject 5 ml of intestinal lavage buffer into the duodenal lumen over a Petri dish. Collect the fluid into a Petri dish by cutting open the cecum. Transfer the fluid to a 50-ml falcon tube and clear it by centrifugation at 16,000*g* at 4 °C for 30 min. The supernatant can be divided into aliquots and stored at −80 °C for several years. ! CAUTION For human studies, informed consent must be

TABLE 1 | Suitable secondary reagents.

Target	Clone name/ polyclonal	Supplier	Catalog number	Conjugate	Tertiary	Working conc.
Mouse IgA	C10-3	BD	559354	FITC	NA	10 µg/ml
Mouse IgA	RMA-1	Biolegend	407004	Biotin	Pacific Blue–streptavidin Invitrogen, S-11222, 2 µg/ml	10 µg/ml
Mouse IgG2a/c	RMG2a-62	Biolegend	407106	FITC (others not tested)	NA	5 µg/ml
Mouse IgG2b	RMG2b-1	Biolegend	406706	FITC (others not tested)	NA	5 µg/ml
Mouse IgG3	RMG3-1	Biolegend	406803	Biotin	Pacific Blue–streptavidin Invitrogen, S-11222, 2 µg/ml	5 µg/ml
Mouse IgM	RMM-1	Biolegend	406509	Allophycocyanin (APC-Cy7 also tested)	NA	4 µg/ml
Human IgM (Fcµ)	Donkey polyclonal	Jackson Immuno-research	709-116-073	Phycoerythrin (PE)	NA	7 µg/ml
Human IgG (Fcγ)	Goat polyclonal	Jackson Immuno-research	109-605-098	Alexa Fluor 647	NA	7 µg/ml
Human IgA (Fcα)	Goat polyclonal	Jackson Immuno-research	109-095-011	FITC	NA	7.5 µg/ml

These reagents can be combined in cases in which fluorophores are compatible, permitting analysis of multiple antibody isotypes in parallel. There are many suppliers and many possible secondary reagents that could encompass almost any host species. The optimal concentration for reagents that are not listed here should be determined by testing a range of concentrations on a known positive control sample. NA, not applicable.

obtained from all study subjects. ! **CAUTION** For studies using human or animal samples, all experiments must be conducted according to the relevant guidelines, and official permissions/ethical approval must be obtained. ! **CAUTION** Such samples are potential infection hazards. Handle them at an appropriate biosafety level.

Collection of antibody-containing body fluids: human fecal water Homogenize 0.2 g of feces in 1 ml of PBS. Centrifuge the suspension at 21,000*g* for 15 min at 4 °C. Carefully collect the supernatant by micropipetting. Add a second 1 ml of PBS, homogenize the mixture and centrifuge it again. Pool this supernatant with that collected initially, and store it at −80 °C until required. ! **CAUTION** For human studies, informed consent must be obtained from all study subjects. ! **CAUTION** For studies using human or animal samples, all experiments must be conducted according to the relevant guidelines and official permissions/ethical approval must be obtained. ! **CAUTION** Such samples are potential infection hazards. Handle them at an appropriate biosafety level.

Preparation of secondary staining reagents Make up all secondary staining solutions <1 h before use in bacterial flow cytometry buffer, as indicated in **Table 1**. ▲ **CRITICAL** Filter all staining solutions through a 0.22-µm syringe filter immediately before use to remove antibody complexes.

Preparation of bacterial agar plates Make up and autoclave the solid bacterial medium according to the manufacturer's instructions. In a clean environment, pour 25 ml of agar per Petri dish. Close the lids on the plates and allow them to set at RT. Follow the manufacturer's guidelines on agar plate storage. For anaerobic bacterial culture, it is recommended to pre-reduce the plates by incubation in an anoxic environment for 3–7 d before inoculation.

Preparation of liquid bacterial culture media Make up, sterilize and store the liquid bacterial culture media according to the manufacturers' instructions. Follow the manufacturer's guidelines on media storage.

▲ **CRITICAL** Most of the rich liquid culture media contain poorly defined ingredients such as yeast extract and may contain dead bacterial particles. To avoid these as a source of incorrect target material, all liquid media should be first autoclaved and then filtered with a sterile 0.22-µm filter to clear these particles. If correct aseptic technique is then used, all bacterial-sized particles present in the final culture will be your species of interest. For anaerobic bacterial culture, it is recommended that the medium be reduced before use by incubating it in an anoxic environment for at least 7 d before inoculation.

Cell-freezing buffer The cell-freezing buffer is 1× PBS with 20% (vol/vol) glycerol. Filter the buffer through a sterile 0.22-µm filter into a sterile sealable vessel. As along as good aseptic technique is followed, this solution may be stored at 4 °C for up to 6 months.

EQUIPMENT SETUP

For users of FACSCalibur (BD), LSRII (BD), FACS CANTO II (BD) and FACSArray (BD), bacteria are easily visualized by changing the standard cytometer settings. First, both forward-scatter and side-scatter parameters should be acquired on logarithmic scales. Second, standard flow cytometer settings 'threshold' the acquired events to only record data from particles of mammalian cell size or higher (typically electronically discarding all values with a low forward-scatter value). Bacterial cells have low values for both side scatter and forward scatter. We therefore recommend to threshold the acquisition on both forward-scatter and side-scatter at a very low, but nonzero, value (e.g., FSc = 200, SSc = 200 on LSRII and FACS CANTO II). The thresholds should be combined with an 'AND' logic gate. ▲ **CRITICAL** Ensure that the flow cytometer is clean by acquiring a tube containing 0.22-µm-filtered 1× PBS or water before commencing acquisition. If >100 events are acquired in 1 min, reclean the machine and repeat.

PROCEDURE

Preparation of bacterial targets ● TIMING 2–7 d

1| Work in sterile conditions (e.g., laminar flow cabinet). Streak out the bacterial target strain from a frozen stock onto solid media using a sterile inoculation loop (media as suggested in **Table 2**, or appropriate to your strain of interest).

! CAUTION This is a potential biohazard. Wear personal protective equipment and follow local guidelines for biosafety level 1 or 2.

▲ **CRITICAL STEP** If the strain is an anaerobe, carry out this procedure in an anaerobic tent using pre-reduced agar plates and inoculation loops.

2| Incubate the inverted plates at an appropriate temperature and in an appropriate atmosphere (e.g., sealed in anaerobic culture jars for anaerobes or unsealed for aerobes in a 37 °C incubator) until single colonies are visible: typically 16–48 h for aerobes and 48 h or longer for anaerobic strains.

3| Prepare an appropriate sterilized bacterial culture vessel containing 5 ml of 0.22-μm-filtered bacterial culture medium (**Table 2**) using good aseptic technique.

▲ **CRITICAL STEP** For anaerobes, pre-reduce this medium for 1 week by incubating it unsealed at RT in an anaerobic tent.

4| Inoculate a single colony from Step 2 into the 5 ml of culture medium from Step 3 using good aseptic technique under an appropriate atmosphere.

5| Incubate the culture at 37 °C (or the appropriate temperature) under an appropriate atmosphere until stationary phase growth is reached.

! CAUTION These are potential infectious hazards. Always wear appropriate personal protective equipment. Handle bacterial strains according to their designated biocontainment risk level.

▲ **CRITICAL STEP** Growth phase, medium and oxygen availability may affect the expression of relevant surface antigens. Carry out pilot experiments to determine the optimal growth conditions for the detection of antibody binding.

▲ **CRITICAL STEP** For further details on anaerobic culture, see Speers *et al.*[36].

? TROUBLESHOOTING

TABLE 2 | Typical bacterial growth conditions of species that have been tested in bacterial flow cytometry.

Strain	Growth medium	Incubation
Facultative/obligate aerobes		
E. coli K-12, *E. coli* Nissle 1913, human and mouse primary *E. coli* isolates, *S. enterica* species, *K. pneumoniae*, *Enterococcus faecalis*, *Streptococcus epidermidis*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Lactococcus lactis*, altered schaedler flora, *Lactobaclli* strains	Liquid: lysogeny broth medium or brain–heart infusion medium	Tubes and flasks of liquid media should be incubated at 37 °C for 18 h in a standard shaking incubator. Plates should be incubated at 37 °C for 18 h in a standard incubator
Citrobacter rodentium, *Yersinia enterocolitica*, *Burkholderia cepacia* complex	Solid: LB agar plates or Columbia agar + 5% sheep blood	
Strict anaerobes		
Clostridium perfringens, altered Schaedler flora *Clostridia* species, *Bifidobacterium adolescentis*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Mucispirillum schaedleri*	Liquid: brain–heart infusion medium Solid: Columbia agar + 5% sheep blood	Both plates and liquid cultures should be stationary while incubated at 37 °C for 18–48 h in an anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2)
Anaerobic or microaerophillic		
B. dorei, *B. fragilis*, *B. thetaiotamicron*, *B. vulgatus*, *Parabacteroides distasonis*	Liquid: brain–heart infusion medium	Liquid media at are incubated 37 °C with gentle shaking for 18–36 h in anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2)
	Solid: Columbia agar + 5% sheep blood	Plates are incubated for 48 h at 37 °C in anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2)

Box 4 | Use of an amine-reactive dye or NHS-biotin labeling for target identification and multiplexing ● TIMING amine-reactive dyes, 20 min; surface biotinylation, 2 h

These procedures can be used to chemico-fluorescently label bacterial targets, either to positively gate these away from debris in the flow cytometer or to be able to mix multiple bacterial species to multiplex the analysis.

Amine-reactive dyes

1. Either prepare fresh live bacterial targets as in Steps 1–6 of the PROCEDURE or thaw frozen bacterial targets as described in **Box 3**.
2. Centrifuge at 4,000–8,000*g* for 2 min at 4 °C.
3. Thaw a fresh aliquot of 4% (wt/vol) PFA (1 ml per 10^9 CFU) and add an amine-binding dye to the recommended concentration (e.g., e-Fluor 450 cell proliferation dye, 20 µM), and pass the solution through a 0.22-µm filter.
4. Resuspend the bacterial pellet in the dye solution and incubate it for 20 min at RT in the dark with shaking to ensure even dye labeling.

? TROUBLESHOOTING

5. Centrifuge the mixture at 4,000–8,000*g* for 2 min at 4 °C. Resuspend the sample in 1 ml of bacterial flow cytometry buffer and proceed to assess and adjust the bacterial density to 5 × 10^6 per ml, as described in Steps 7–9 of the PROCEDURE.

Surface NHS-biotin labeling

1. Prepare fresh, live bacterial targets as described in Steps 1–6 of the PROCEDURE. Measure the OD_{600nm} value and adjust the density to 5 × 10^8 bacteria per milliliter with bacterial flow cytometry buffer. Determine the volume of this suspension required for the experiment (0.25 µl per well to be analyzed).
2. Make up an equal volume of EZ-Link NHS-PEG4-Biotin per the manufacturer's instructions, to 2 mM in 1× PBS.
3. Mix the NHS-biotin solution 1:1 with the bacterial suspension.
4. Incubate the mixture at RT for 1 h, with shaking (500 r.p.m., orbital shaker).
5. Centrifuge the mixture at 4,000–8,000*g* for 5 min at 4 °C, and discard the supernatant.
6. Resuspend the pellet in 1 ml of 1× PBS 100 mM glycine to quench staining.
7. Centrifuge the mixture at 4,000–8,000*g* for 5 min at 4 °C, and discard the supernatant.
8. Resuspend the pellet in 1 ml of bacterial flow cytometry buffer. Repeat the washing steps of this box (Steps 7 and 8) with bacterial flow cytometry buffer twice.
9. Resuspend in 1 ml of fluorophore–streptavidin conjugate solution in bacterial flow cytometry buffer (e.g., Pacific Blue–streptavidin, 2 µg/ml). Incubate the mixture for 30 min at RT in the dark.
10. Wash three times by centrifugation at 4,000–8,000*g* for 5 min at 4 °C, and resuspend in 1 ml of bacterial flow cytometry buffer to remove all unbound streptavidin.
11. Bacterial targets labeled with different colors of streptavidin conjugates may then be pooled and the final concentration adjusted to 5 × 10^6 bacteria for each species per milliliter of bacterial flow cytometry buffer.

▲ **CRITICAL STEP** Check that surface labeling is homogeneous by running a small aliquot (25 µl of the 5 × 10^6 CFU/ml final suspension, diluted to 300 µl with bacterial flow cytometry buffer) through the flow cytometer. A single fluorescent peak should be visible in the relevant fluorescence channel. If multiple peaks are observed, the targets should not be analyzed further.

? TROUBLESHOOTING

6| Centrifuge 1 ml of culture at 4,000*g* for 2 min at 4 °C to gently pellet only whole bacteria (bacterial fragments will bind antibodies but they will later be lost from the analysis, so they may reduce the specific signal). Optionally, it is possible to label the bacteria with an amine-reactive dye or NHS-biotin at this stage (**Box 4**).

It is advisable to freeze an aliquot of this culture in cell-freezing medium for later confirmation of culture purity (**Box 5**).

▲ **CRITICAL STEP** Anaerobic and aerobic strains may be handled identically from this step onward.

▲ **CRITICAL STEP** Bacterial species vary enormously in size, shape and resistance to physical force. Although centrifugation speeds given in this protocol have worked for all species listed in **Table 2**, you should experimentally determine optimal speeds to give full sedimentation without excessive toxicity for all other species.

? TROUBLESHOOTING

7| Resuspend the bacteria in 1 ml of bacterial flow cytometry buffer and determine the bacterial density. For *E. coli* and *Salmonella* cultures, a 1-ml pathlength OD_{600nm} measurement of 1.0 roughly corresponds to 5 × 10^8 bacterial CFU/ml. For other species, it will be necessary to test the relationship between CFU and OD_{600nm} values by measurement and plating. Alternatively, bacteria may be quantified by flow cytometry counting—i.e., mixing a known density of counting beads with the bacterial sample and determining the ratio of beads to bacteria in the cytometer. Optionally, it is possible to prepare large batches of frozen bacteria in aliquots for longitudinal experiments at this stage (**Box 3**).

? TROUBLESHOOTING

Box 5 | Confirmation of bacterial sample identity and purity ● TIMING 2 h plus 1–3 d culture time

A bacterial sample frozen at –80 °C in cell-freezing buffer may be stored for several months before analysis if necessary. We recommend that you carry out one or more of the following controls to confirm that the correct species was grown and purity was maintained:
1. Resuspend the pellet at a density of 10^8 bacteria per ml in 1× PBS that has been freshly filtered through a sterile 0.22-µm filter. Place 20 µl of this suspension onto a glass slide and cover it with a coverslip. Image the bacteria using 100× phase-contrast light microscopy. Confirm that a single bacterial morphology is present.
2. Dilute the bacteria to 5,000 bacteria per ml in bacterial flow cytometry buffer and plate 50 µl on rich agar (e.g., Columbia agar + 5% sheep blood). Confirm that the expected number of bacteria grow and that a single colony morphology is present.
▲ CRITICAL STEP This is recommended only for easily culturable aerobes because of oxygen exposure during the preparation steps.
3. Pellet 1 ml of overnight culture by centrifugation at 8,000*g* at 4 °C for 2 min.
4. Resuspend the pellet in 200 µl of sterile water and heat it to 99 °C for 15 min to release chromosomal DNA.
5. Vortex the suspension and then add 1 µl as a template to a 16S PCR (standard Taq PCR mix containing 10 nmols each of primers FD1, FD2 and RC1). Prepare a second reaction with no template added and, if possible, a third reaction, which you inoculate with a single colony from the plates generated in step 2 of this box.
6. Run PCR for 35 cycles of 94 °C for 5 min, 94 °C for 1 min, 43° for 1 min, 72° for 2 min, plus a final 5-min elongation step.
7. Load the entire PCR product onto a 1% (wt/vol) agarose gel. Run it at 100 mV constant voltage for 45 min.
8. Cut out bands and send them for Sanger DNA sequencing with specific primer RC1.
9. Examine the sequencing results and align the 16S sequences using NCBI Blast. Mixed sequences are a strong indication of contamination.
10. If available, analyze the bacteria using a clinical mass spectrometer. For more details, see Schulthess *et al.*[37].

8| (Optional) To reduce operator risk, it is possible to PFA-fix bacteria at this stage, although it should be considered that chemical modification of surface epitopes by PFA may alter antibody binding. To do so, centrifuge the culture at 4,000*g* for 2 min at 4 °C and resuspend it in 1 ml of freshly thawed 4% (wt/vol) PFA. Incubate the culture for 20 min at RT. Centrifuge it at 4,000*g* for 2 min at 4 °C and resuspend it in 1 ml of bacterial flow cytometry buffer.

9| Adjust the bacterial concentration to 5 × 10^6 per ml with bacterial flow cytometry buffer.
■ PAUSE POINT The bacterial suspension can be stored at 4 °C for up to 72 h before flow cytometry[37].

Preparation of antibody-containing bodily fluids ● TIMING 1 h

10| Thaw antibody-containing fluids rapidly at 37 °C in an incubator or a water bath.

11| Dilute the solution to twice the desired starting concentration in bacterial flow cytometry buffer (**Table 3**). The total volume required depends on the number of assays being performed. We recommend 50 µl multiplied by the number of bacterial strains to be analyzed. Be sure to include sufficient volume for positive and negative control samples that are required for the cytometer setup.
? TROUBLESHOOTING

12| Inactivate complement and other heat-labile antimicrobials by heating the antibody solution to 56 °C for 30 min in a water bath or a heating block.

13| Centrifuge the solution at 16,000*g* for 5 min at 4 °C to pellet all bacterial-sized particles and antibody complexes.

14| Carefully remove the supernatant and pass it through a 0.22-µm syringe filter (if >500 µl total volume) or a 0.22-µm spin filter column (if <500 µl total volume).
▲ CRITICAL STEP Fluids containing a high fat concentration or with a high viscosity give high background in this assay even after 0.22-µm filtration. These fluids (e.g., breast milk and fecal water) must be purified (as described in **Box 2**) before continuing.
▲ CRITICAL STEP For specificity testing by cross-adsorption, see **Box 1**.
? TROUBLESHOOTING

15| Make serial dilutions in bacterial flow cytometry buffer, as suggested in **Table 3**.
▲ CRITICAL STEP The exact scale and number of titration steps ideal for your experiment will vary depending on the experiment type, and the values given in **Table 3** should be considered as suggestions only.

TABLE 3 | Recommended titration schemes.

Sample type	Expected response strength	Suggested starting concentration	Dilution step size	Number of steps
Mouse serum	Strong IgG response (e.g., after i.v. vaccination)	≤100 µg/ml IgG	Fourfold	6–8
Mouse intestinal lavage	Strong IgA response	≤5 µg/ml IgA	Threefold	6–8
Mouse serum	Weak/endogenous response	≤1 mg/ml IgG	Threefold	6–8
Human serum (sepsis)	Strong response	≤100 µg/ml IgG ≤100 µg/ml IgA	Threefold	6–8
Human serum (unknown)	Unknown	<200 µg/ml IgG <50 µg/ml IgA	Threefold	6–8
Human fecal water	Weak response	50 µg/ml IgA	Threefold	6–8
Human breast milk	Weak response	100 µg/ml IgA	Threefold	6–8
Human serum/purified antibodies	Strong response where a single concentration should be analyzed across multiple donors	10 µg/ml IgA or IgG	NA	NA

NA, not applicable.

16| Transfer 25 µl of the titrated antibodies to V-bottom 96-well plates.
▲ **CRITICAL STEP** The exact volume of antibody solution will vary with the details of the experiment. 25 µl is our suggested minimum volume.

Primary antibody incubation ● TIMING 45 min to overnight
17| The minimum number of bacteria that can be reasonably detected in a single sample is 10^5. We therefore suggest as a minimum to mix 25 µl of bacterial suspension at a density of 5×10^6 bacteria per ml (from Step 9) with 25 µl of antibody-containing solution (from Step 16) prepared according to **Table 3** in a 96-well V-bottom plate.
▲ **CRITICAL STEP** If higher numbers of bacterial targets are used, the volume of antibody-containing solution must be correspondingly increased.

18| Incubate the samples at RT for 15 min or at 4 °C for 1 h to overnight. (These incubation times give equivalent results and can be adapted for convenience.)

Primary antibody washing ● TIMING 30 min
19| Add 200 µl of sterile bacterial flow cytometry buffer to each well and centrifuge the plates at 4,000*g* for 10 min at 4 °C in a bench-top centrifuge (e.g., Eppendorf centrifuge 5810 R, A-4-81 rotor).
! CAUTION If potentially infectious material is present, seal the plates with Parafilm before centrifugation to prevent aerosol formation.
▲ **CRITICAL STEP** You should not see any cell pellets with 10^5 bacteria per well.

20| Remove the supernatant by decanting.
! CAUTION Decant potentially infectious samples in a biosafety flow cabinet into a large vessel of disinfectant, not directly into the sink.

21| Repeat Steps 19 and 20 (without actively resuspending the pellets in the bacterial flow cytometry buffer) to remove all residual antibody solution.

Secondary incubation ● TIMING 30 min to overnight
22| Add 50 µl of secondary staining reagent, which is made up as in **Table 1**, to each well.

23| Actively resuspend the bacteria by pipetting.
▲ **CRITICAL STEP** Vortexing or shaking is not recommended for this, as resuspension is poor using these methods and there is a high risk of contamination between wells.

24| Wrap the plates in Parafilm and incubate them in the dark at RT for 15 min or at 4 °C for 1 h to overnight (with equivalent results).

Secondary antibody washing ● TIMING 30 min

25| Repeat Steps 19–21.

Tertiary reagent staining (e.g., streptavadin–fluorophore conjugates) ● TIMING 30 min to overnight

▲ **CRITICAL** The steps in this section are optional; if you are not performing them, skip to Step 29.

26| Make up the tertiary reagent solution (typically 2 µg/ml fluorophore–streptavadin in bacterial flow cytometry buffer) and add 50 µl per well. Actively resuspend the bacteria by pipetting. Incubate the mixture at RT for 30 min or at 4 °C overnight (these incubations yield identical results and can be adapted to the available time schedule).

27| Repeat Steps 19–21.

28| (Optional) If the bacteria are not already fixed, and samples must conform to biosafety level 1 for cytometry analysis, resuspend the pellet in 200 µl of 2% (wt/vol) PFA in 1× PBS, and pass through a 0.22-µm filter. Incubate the samples for 20 min at RT in the dark, and then repeat Steps 19 and 20.

Resuspension for acquisition ● TIMING 10 min per plate

29| Resuspend the samples in a minimum of 300 µl of bacterial flow cytometry buffer or 2% (wt/vol) PFA (depending on local flow cytometry recommendations) for acquisition. For acquisition on a FACSArray or another cytometer with plate-loading capacity, the stained bacteria plus buffer can be left in the 96-well plates and directly loaded into the machine. For flow cytometers without plate-loading capabilities, we recommend transferring the samples to 1.2-ml library tubes, which can be arrayed with an arrangement identical to that of the 96-well plates, thus avoiding extensive tube labeling.

Cytometer setup and acquisition ● TIMING 20 s per sample

▲ **CRITICAL** The exact setup procedure will depend on the model of flow cytometer used. The steps below are suggested for LSRII (BD) and FACS CANTO II (BD) machines. For other cytometers, we recommend contacting the local support team to establish an optimal setup for bacteria detection.

30| Using the cytometer setup described in the 'Equipment Setup' section, first acquire positive control single-stained samples to set photomultiplier tube (PMT) voltages and compensation matrices. It is recommended that <2,000 events per second be acquired, even on cytometers that are capable of detecting mammalian cells at five times this rate. Optionally, for longitudinal analyses, it may be beneficial to work with a fixed gating template and calibration beads. In this case, acquire calibration beads and adjust the cytometer settings so that the beads fall into the predetermined gates[35].

? TROUBLESHOOTING

31| Acquire all samples. For robust statistics, it is recommended that a minimum of 10,000 positively identified target events be acquired per sample. Plate-loaders should be set up to acquire this sample size, according to the manufacturer's instructions.

? TROUBLESHOOTING

Data analysis: example workflow using FlowJo, Excel and Graphpad Prism ● TIMING 30 min per plate

▲ **CRITICAL** This could be implemented entirely in open-source frameworks by anyone who is familiar with the following tools: Flow (https://galen.dulci.duhs.duke.edu/flow/) or Bioconductor (http://bioconductor.org/), and the R project (https://www.r-project.org).

32| Import raw data from the flow cytometer (ideally, .fcs3 files) into FlowJo (this analysis is functional in all FlowJo versions).

Draw a gate on the main bacterial population based on Fsc/Ssc. Optionally, carry out software compensation based on single-stained positive control samples, if this was not performed during acquisition[38].

▲ **CRITICAL STEP** Generate a layout displaying all fluorescence parameters for each sample to screen for errors in data acquisition, multiple peaks and evidence of contamination. Omitting this step may lead to misinterpretation of the data.

? TROUBLESHOOTING

33| Calculate median fluorescence intensity (MFI) for each sample and each fluorophore/isotype combination present.

▲ **CRITICAL STEP** The arithmetic mean should not be used because of the log-normal distribution of fluorescence data, as well as the presence of potentially more complex distributions (multiple peaks and so on). For purely log-normal distributions (i.e., clean log-symmetrical single peak data), the geometric mean will be identical to the median. However, as all parametric statistics are more sensitive to low numbers of outliers with very high or even negative values, we recommend using the median as a robust single value readout of fluorescence intensity.

34| Export the MFI values to Excel. If a single antibody concentration was used, this is the end result and can be statistically evaluated between groups.
▲ **CRITICAL STEP** Where multiple isotypes are quantified in parallel, statistical significance levels must be corrected for multiple testing—for example, using Bonferroni corrections or one-way ANOVA of normalized values to analyze the data.

35| If titrations were generated, rearrange the data to generate a table in which the first column contains the dilution factor and each adjacent column contains the MFI titration values for a single sample. Generate the $\log_{10}$ values of the entire table.

36| Import these data into Graphpad Prism as an *xy* dot-plot. Inspect this plot for smooth logistic dilution curves.
? TROUBLESHOOTING

37| Use the nonlinear regression function with the least-squares method to fit a four-parameter logistic equation.

$$Y = \text{Bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{(\log\text{EC50} - X) \times \text{Hill slope}}}$$

▲ **CRITICAL STEP** At least five points, plus a baseline, need to be present to fit with reasonable accuracy. Constrain the bottom of the curve to a single value for all samples (typically the MFI of the negative control). It may be necessary to limit the top asymptote if titrations do not reach saturation to avoid nonsensical results. Optionally, the MFI values may also be plotted against the total antibody concentration for each sample dilution, rather than the dilution factor. This is particularly important when no further analysis is planned, as differences between curves, as revealed by dilution values, may be due simply to changes in total antibody concentrations.
? TROUBLESHOOTING

38| Export the results of the curve fitting back to Excel. For each curve, you should have values for 'top', 'bottom', 'logEC50' and 'Hill slope'. The 'logEC50' is the $\log_{10}$ of the dilution factor at the curve inflexion point.

39| Rearrange the four-parameter logistic equation to give *X* in *Y*

$$X = \text{LogEC50} - \left(\frac{\text{Log}\left(\frac{\text{top} - Y}{Y - \text{bottom}} \right)}{\text{Hill slope}} \right)$$

and use this with your above-exported parameters ('top', 'bottom', 'logEC50' and 'Hill slope') to calculate the dilution factor (*X*) giving a defined 'above-background' MFI (*Y*) for all data sets (**Fig. 5** and **Supplementary Data**). This is your titer in terms of the dilution factor. As long as '*Y*' falls within the pseudolinear range of the fitted curves, the actual value you choose to set for *Y* should not markedly affect the relative data interpretation.

40| To generate relative titers, divide the total antibody concentration (as determined by commercial sandwich ELISA following the manufacturer's instructions) by the titer in terms of dilution factor. This reveals the total antibody concentration required to give the chosen level of bacterial coating for each sample/donor. As this value is necessarily low with a high titer response—and this is often confusing to readers—the inverse of this value is usually plotted—i.e., a value in nanograms per milliliter.
? TROUBLESHOOTING

41| As an alternative, titers giving an approximate idea of the concentration of specific antibodies per milliliter can be calculated by reference to binding of a defined monoclonal antibody (**Fig. 5** and **Supplementary Data**). It should be noted that this is not quite an accurate description, as the shift in binding curve depends not only on the number but also the affinity/avidity of antibodies; however, it can be a more intuitive readout where such standards are available. Here, the value in nanograms per milliliter is calculated as follows:

$$\text{Titer} = (\text{concentration of monoclonal}) \times \frac{\text{dilution factor for MFI = N:sample}}{\text{dilution factor for MFI = N:monoclonal}}$$

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	No bacterial growth	Stock too old/dead	Obtain fresh bacterial frozen stock
	Unexpectedly rapid bacterial growth	Inappropriate growth conditions	Check recommended growth conditions
		Stock or media contaminated	Check culture identity (**Box 5**)
			Re-sterilize glassware. Re-prepare and sterilize culture media
			Obtain fresh bacterial frozen stocks
6	Poor sedimentation after centrifugation at 4000*g*, 2 min	Small/buoyant bacteria	Increase centrifugation speed and time
	Sticky pellet after centrifugation	Fragile bacteria may lyse if pelleted with excessive force, releasing DNA	Decrease centrifugation speed
7	Highly variable OD_{600} readings after overnight culture	Culture conditions are not constant	Check media and gas compositions. Check that incubator temperatures are constant
		OD_{600} readings are affected by bacterial aggregation	Decrease centrifugation speeds to avoid bacterial lysis. Try disrupting aggregates by physical force, or consider counting by flow cytometry
11	Extensive precipitates and very high viscosity in antibody-containing body fluids	Cell debris or lipids are present in the fluid	If this is hard to clear by centrifugation and filtration, consider antibody purification before analysis (**Box 2**)
	Insufficient antibody to follow recommended concentrations in **Table 3**	NA	Use undiluted samples with minimum numbers of bacterial targets to maximize the chance of observing a specific signal
14	Frequent blockage of 0.22-µm syringe filters during antibody-containing fluid preparation	Large quantities of cell debris or precipitated biomolecules in solutions	Increase the length of the 16,000*g* centrifugation step before filtration
			Use larger-volume 0.22-µm filters
			Consider antibody purification (**Box 2**)
30	High event numbers in the cytometer when running 0.22-µm-filtered water or PBS	Contamination in cytometer tubing or sheath fluid	Thoroughly clean the cytometer with bleach- and detergent-containing solutions. Pass the in-house-produced sheath fluid through a 0.22-µm filter
			If this does not solve the issue, the flow cell may be damaged. Ideally, this should be replaced. However, an alternative solution is to chemico-fluorescently label the bacterial targets (**Box 4**) to permit specific gating
30 and 31	Zero events in the flow cytometer	Incorrect cytometer settings	Ensure that threshold values are not set too high, resulting in exclusion of all bacteria-sized events
		Cytometer blocked or misfunctioning	Check for normal detection of setup beads. Clean the machine to remove blockages. If this fails, contact the manufacturer's support engineers
30 and 31	No bacteria observable in the cytometer	Insufficient cells	Check the relationship between the OD_{600nm} and CFU values
		Centrifugation failure	Include one well with a high CFU number to check that centrifugation has pelleted cells
		Incorrect cytometer settings	Obtain a fluorescently labeled bacterial sample (e.g., brightly GFP-positive *E. coli*, or chemically stained cells) at reasonably high density (e.g., 5×10^7 per ml). Alter the cytometer settings until the population is visible

(continued)

TABLE 4 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
30 and 31	Very low fluorescence signal in positive control sample	Incorrect bacterial strain or growth conditions	Streak out the bacterial culture and pick a single colony. Confirm the species by biochemistry and genetics (**Box 5**)
		Too many CFUs per well	Recheck the relationship between the OD_{600nm} and CFU/ml values. Correct these such that only 10^5 bacteria are loaded per well
30 and 31	High background in negative control	Fc- or nonspecific binding	Check the binding of isotype controls. Test closely related strains to identify one without Fc binding activity
		Antibody complex contamination	Ensure that all antibody solutions are filtered through 0.22-µm filters before adding them to the bacterial targets
30–32	Multiple peaks	Phenotypic variation or stochastic gene expression in targets	Alter the culture conditions or try to work with phase-locked variants, if genetically accessible
		Inefficient detection by cytometer (typically second peaks appear on the axes)	Decrease the acquisition rate
		Contamination	See **Box 5**. Obtain fresh bacterial stocks, culture media and sterile culture vessels
36	'Bell-shaped' dilution curves when MFI is plotted against dilution factor	Toxicity and/or massive agglutination at very high antibody concentrations	Exclude all values with high total antibody concentrations to permit a curve fit. As efficient agglutination is what antibodies 'do' at high concentrations, this is unavoidable. Consider repeating the measurements with lower starting antibody concentrations
37	Poor r^2 value for curve fitting, or unable to fit four-parameter logistic equation	No antibody binding detected Sample contamination Bell-shaped curve	As you cannot fit a four-parameter logistic curve to a flat horizontal line, the titer for samples displaying no binding must be set to the detection limit (typically, the inverse undiluted antibody concentration). For samples showing some positive binding, this may be indicative of some kind of interference in the samples. Examine scatter plots in FlowJo for evidence of multiple peaks. See TROUBLESHOOTING for multiple peaks (Steps 30–31) or see TROUBLESHOOTING for bell-shaped dilution curves (Step 37)
40	No mathematical solution for relative titer calculations	Top of the curve does not reach the chosen *y*-axis value	Check that your chosen *y*-axis value is reasonable (i.e., falls in the pseudolinear part of the curve for the positive control samples). If this is OK, then samples that do not reach this value have a titer at the detection limit
Box 4	Multiple peaks after chemical labeling	Insufficient mixing after dye addition	Repeat chemical labeling with gentle vortexing

NA, not applicable.

● TIMING

Steps 1–9, preparation of bacterial targets: 2–7 d, depending on bacterial growth characteristics
Steps 10–16, preparation of antibody-containing bodily fluids: 1 h
Steps 17 and 18, primary antibody incubation: 45 min to overnight
Steps 19–21, primary antibody washing: 30 min
Steps 22–24, secondary incubation: 30 min to overnight
Step 25, secondary antibody washing: 30 min
Steps 26–28, (optional) tertiary reagent staining: 30 min to overnight

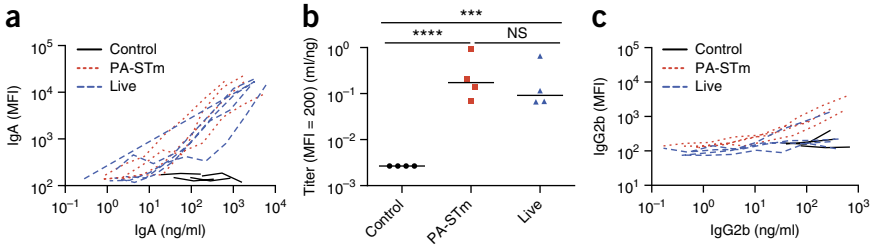

Figure 12 | Vaccination with oral peracetic-acid-inactivated *S. typhimurium* and infection with live-attenuated *S. typhimurium* induce a similar magnitude of intestinal IgA response. (**a**–**c**) C57BL/6 specific opportunistic pathogen–free (SOPF) mice were either pretreated with 1.0 g/kg streptomycin and 1 d later infected orally with 5×10^7 CFU of the oral vaccination *S. typhimurium* strain M556 (SB300 Δ*sseD*; 'live') or were gavaged once a week with 10^{10} particles of peracetic-acid-killed *S. typhimurium* ('PA-STm') over 3 weeks. (**a**) Intestinal lavage IgA titer curves and (**b**) intestinal lavage IgA titers, as calculated in **Figure 11d,e** (Kruskal–Wallis test on log-normalized values, *P* value < 0.0001. Pairwise comparisons calculated by Dunn's post-tests). Lines indicate medians. (**c**) Serum IgG2b titer curves at day 21 after the first vaccination/infection, as determined by bacterial flow cytometry. $n = 4$ per group. All animal experiments were approved by the legal authorities (licenses 223/2010 and 222/2013, Kantonales Veterinäramt Zürich, Switzerland) and performed according to the applicable legal and ethical requirements. Image adapted with permission from Moor *et al.*[22].

Step 29, resuspension for acquisition: 10 min per plate
Steps 30 and 31, Cytometer setup and acquisition: 20 s per sample
Steps 32–41, data analysis: 30 min per plate
Box 1, cross-adsorption for specificity testing: 1.5 h
Box 2, purification of IgA from viscous or fatty samples: 30 min–1 h
Box 3, cryo-preserving and thawing aliquots of bacterial targets: culture time (1–3 d) + 30 min
Box 4, use of an amine-reactive dye or NHS-biotin labeling for target identification and multiplexing: 20 min (amine-reactive dyes), 2 h (surface biotinylation)
Box 5, confirmation of bacterial sample identity and purity: 2 h plus culture time (1–3 d)

ANTICIPATED RESULTS

If this protocol is carefully followed, clean data on species-specific antibodies can be generated in

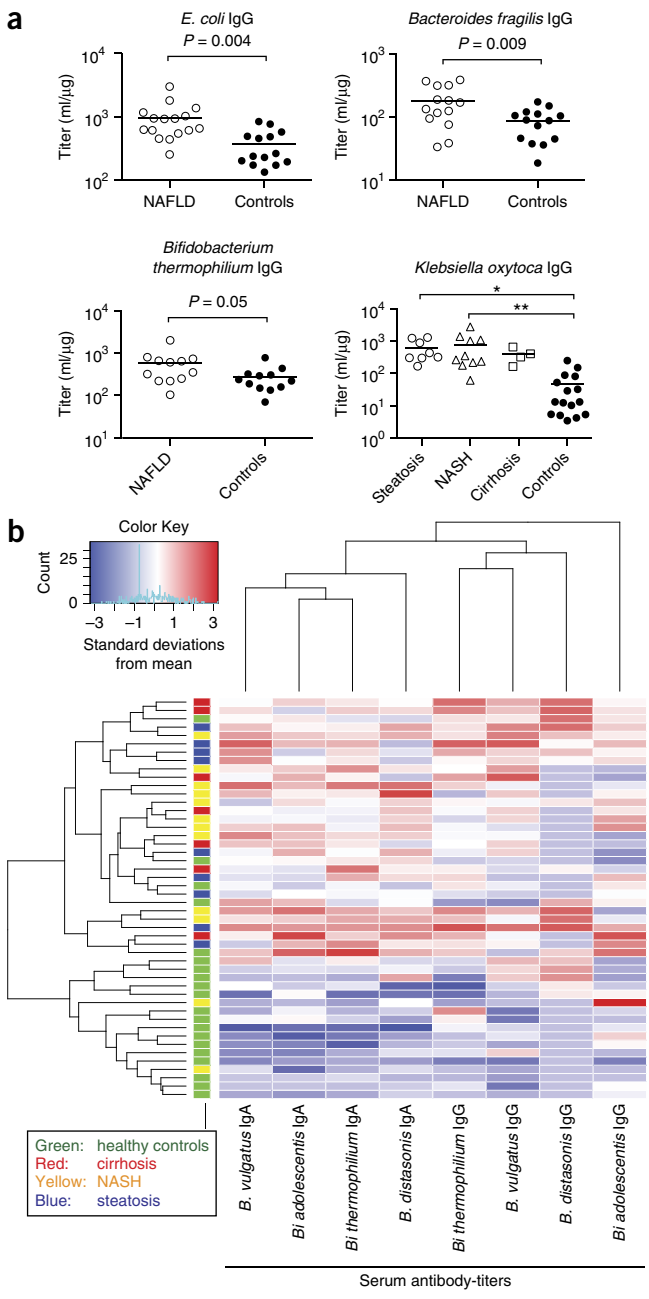

Figure 13 | Bacterial flow cytometry reveals increased microbiota-specific serum antibodies in metabolic liver disease. (**a**) Serum IgG titers against the indicated bacteria in nonalcoholic fatty liver disease (NAFLD) patients (open symbols) compared with age- and sex-matched healthy controls (filled dots). Pure cultures of the indicated bacteria were stained with dose titrations of serum from patients or controls. Serum antibody coating of bacteria was visualized using monoclonal DyLight 647-conjugated anti-human IgG and quantified per bacterium by flow cytometry. Resulting MFI was plotted against total IgG added to the assay, as determined by ELISA and IgG titers calculated by fitting four-parameter logistic curves to each donor and determining the concentration of IgG required to obtain a median fluorescence intensity binding of 80. The inverse of this IgG concentration is shown, for ease of interpretation. Each point represents an individual subject and lines show means. Unpaired *t*-test or one-way ANOVA and Tukey's post-test were used to compare the groups; $*P \leq 0.05$, $**P \leq 0.01$. (**b**) Cluster-analysis from Basel NAFLD patients with different stages of liver disease (steatosis = blue, nonalcoholic steatohepatitis (NASH) = yellow and cirrhosis = red) and age- and sex-matched healthy controls (green). Heatmaps were generated using a euclidean distance function with complete linkage clustering in the statistical package R using the package 'gplots version 2.8.0', function 'heatmap.2'. Red indicates increased and blue indicates decreased titers as compared with the mean of the entire population. From Balmer, M.L. *et al.* The liver may act as a firewall mediating mutualism between the host and its gut commensal microbiota. *Sci. Transl. Med.* **6**, 237–266 (2014). Adapted with permission from the American Association for the Advancement of Science.

a wide range of situations. The technique has been successfully used in a range of publications both in murine model systems and in the human system[1–6].

In the first published example, taken from Moor *et al.*[22], C57BL/6 specific pathogen–free (SPF) mice either received three oral doses of 10^{10} peracetic-acid-inactivated *S. typhimurium* over 3 weeks or were orally infected with a live-attenuated *S. typhimurium* vaccination strain. 3 weeks after the initiation of vaccination, the intestinal IgA response was quantified from intestinal lavages (**Fig. 12a**,**b**), and serum IgG2b (**Fig. 12c**); IgG1 and IgM were quantified from serum (data not shown). This demonstrates the effective induction of a mucosal IgA response by the inactivated and live oral vaccines, with minimal induction of serum IgG at these time points. The data analysis leading from the raw data in **Figure 12a** to the analyzed data in **Figure 12b** is described in detail in the **Supplementary Data**.

In the second published example, taken from Balmer *et al.*[6], bacterial flow cytometry was used to quantify serum antibody responses against a panel of anaerobic intestinal microbiota species in patients with varying stages of metabolic liver disease and healthy donors. Relative titers were calculated for each sample/species combination (**Fig. 13a**), and the final large data set was analyzed by hierarchical clustering (**Fig. 13b**). In this example, we demonstrated elevated microbiota-directed serum IgG responses in patients with metabolic liver diseases, independent of disease stage. This suggested that increased immune exposure to the intestinal microbiota is an early event in the progression of metabolic diseases.

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

ACKNOWLEDGMENTS We acknowledge J. Doré and C. Juste (Institut National de la Recherche Agronomique (INRA), UMR1319 Micalis, Jouy en Josas, France) for access to well-characterized bacterial strains, and C. Parizot and D. Gouas for technical help during initial experimental setup. The authors also acknowledge the following funders: M.L. was supported by the Institut national de la santé et de la recherche médicale (Inserm), Agence Nationale de la Recherche (MetAntibody, ANR-14-CE14-0013) and Fondation pour l'Aide a la Recherche sur la Sclerose En Plaques (ARSEP). E.S. was supported by an SNF Ambizione fellowship (PZ00P3_136742) and an ETH research grant (ETH-33 12-2).

AUTHOR CONTRIBUTIONS K.M., J.F., A.T. and D.S. generated the data shown in **Figures 1–11**. M.L.B. generated the data shown in **Figure 13**. A.J.M. provided support during the development of the method and reviewed the manuscript. G.G. provided support for adaption of the method to clinical scenarios and reviewed the manuscript. M.L. further developed the method for application to human research, generated data shown in **Figures 1–11** and wrote the manuscript. E.S. established the methodology and analysis protocols, generated data shown in **Figures 1–12** and wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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