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TITLE: **Understanding Microbial Sensing in Inflammatory Bowel Disease Using Click Chemistry**

PRINCIPAL INVESTIGATOR: Dennis L. Kasper, Ulrich von Andrian

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14. ABSTRACT

The surface molecules of bacteria contribute significantly to the host's response to both pathogens and commensals. However, the technology available to track these molecules in host cells and tissues remains primitive. To address this limitation, we have developed an expanded metabolic labeling approach that chemically tags lipopolysaccharide, capsular polysaccharide, and peptidoglycan simultaneously in live anaerobic commensal bacteria. This technology enabled us to track the entry of differentially labeled surface molecules from live, luminal bacteria into specific host intestinal immune cells and their subsequent degradation in host phagocytes. Notably, this approach also enabled live imaging of the tagged bacterial surface molecules from endogenous commensals in the intestine of the living murine host.

15. SUBJECT TERMS

Bioorthogonal click-chemistry, bacterial cell wall, bacterial outer membrane, peptidoglycan, lipopolysaccharide, endotoxin, capsular polysaccharide, inflammatory bowel disease, microbiome, microbiota, carbohydrate chemistry, fluorescent microscopy, 2-photon microscopy, confocal microscopy

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

In this proposal, we will apply and expand our bioorthogonal click-chemistry strategy to simultaneously label an unprecedented three components of the bacterial cell wall: PSA, lipopolysaccharide (LPS), and peptidoglycan (PGN). With this approach, we will be able to track a TLR2 ligand (PSA), a TLR4 ligand (LPS), and a ligand for NLR signaling (PGN) in living bacteria and their host and to follow these molecules into specific host immune cells in order to define the pathways by which protective or inflammatory responses develop. This methodology will also allow us to visualize the spatial and temporal changes in bacterial product acquisition within diseased tissue. Beyond adding to the basic knowledge of host-commensal communication, an understanding of microbial sensing in the gut will allow us to design new treatments for IBD patients. Our long-term objective is to use fluorescently labeled commensal bacteria as a less invasive tool for the study of IBD in patients. This approach leverages the Kasper lab's expertise in commensal microbiology and carbohydrate chemistry with the von Andrian lab's skills in fluorescence microscopy and immunology to develop a new interdisciplinary platform for imaging and tracing bacterial products in the gut.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Bioorthogonal click-chemistry, cell wall, outer membrane, peptidoglycan, lipopolysaccharide, endotoxin, capsular polysaccharide, IBD, microbiome, microbiota, carbohydrate chemistry, fluorescent, 2-photon, confocal microscopy

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

There were three specific aims in our proposal.

Aim 1. Incorporate non-natural sugars and amino acids into lipopolysaccharide, capsular polysaccharide, and peptidoglycan for live bacterial-cell labeling.

Aim 2. Use click labeling to elucidate the acquisition and distribution of commensal material *in vitro* and in host intestinal tissue.

Aim 3. Use live intravital microscopy to monitor changes in bacterial-component acquisition during chemical and pathogen-induced colitis.

We have made significant progress on all three specific aims as summarized below and the published manuscript is attached in the appendix. The Kasper lab has done the chemistry, microbiology and worked with the von Andrian lab on the immunology and microscopy. All work was done between the two labs at Harvard Medical School.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We have completed all the goals of this project and we have published a major original research article in *Nature Microbiology (2017; vol. 2, 17099)* entitled, "Illuminating vital surface molecules of symbionts in health and disease." We used a combination of metabolic labeling and bioorthogonal click chemistry (that is reactions performed in living organisms) to specifically tag up to 3 prominent surface immunomodulatory macromolecules – peptidoglycan, lipopolysaccharide and capsular polysaccharide – either simultaneously or individually in live anaerobic commensal bacteria. Importantly, the peptidoglycan labeling enables, for the first time, the specific labeling of live, endogenous anaerobic bacteria within the mammalian host. This approach has allowed us to image and track the path of labeled surface molecules from live, luminal bacteria into specific intestinal immune cells in the living murine host during health and disease. The chemical labeling of three specific macromolecules within a live organism offers the potential for in depth visualization of host-pathogen interactions. These specific studies are presented in the manuscript as Appendix A.

We will continue to advance the biologic insights gained using the technological advances reported in our manuscript. We have already met the great majority of the technological goals in all three specific aims. We will now apply these advances to understand disease mechanisms and interactions between immune cells and microbes in inflammatory bowel disease and in colon cancer. Our basic approach is outlined as found in the manuscript (Figure 2). In Figure 2 we have shown that in inflammatory bowel disease peptidoglycan can be followed into specific immune cells by HADA labeling. This provides a powerful tool for understanding which bacterial molecules impact of specific subsets of immune cells during inflammation.

In addition to the manuscript, we have also generated some important preliminary data that will allowed us to focus on IBD in the small intestine and colon and may may result in innovative strategies to selectively target inflammatory responses in the intestinal microvasculature without impacting immune responses elsewhere. The technology we developed from this grant will allow us to further understand immune cell recruitment in the guts and the effect of bacterial components in this process.

Our work builds on the methods we have developed in this grant for metabolic labeling of bacteria (Specific Aim 1) and tracking of bacterial components in the gut (Specific Aims 2 and 3). In our published study (attached to the appendix), we used a combination of metabolic labeling and bioorthogonal click chemistry (reactions performed in living organisms) to specifically tag up to 3 relevant surface immunomodulatory macromolecules – peptidoglycan, lipopolysaccharide and capsular polysaccharide – either simultaneously or individually - in live, anaerobic commensal bacteria. Importantly, this strategy enables the specific labeling of live, endogenous anaerobic bacteria within a mammalian host and allows us to image and track the path of labeled surface molecules from live, luminal bacteria into specific intestinal immune cells in the living murine host during health and disease. The chemical labeling of up to three specific macromolecules within a live organism also offers the potential for in depth visualization of host-pathogen interactions. Our experiments done in the last year of the grant revealed that the frequency for the venular segment of the vasculature (the $DARC^{+}$ venular endothelial cell) was higher in germ free mice compared to specific pathogen free mice in

small intestine, colon, caecum and Peyer's patches, but not in MLN (**Figure 1)**. The DARC$^+$ V-EC are part of the microvasculature that line the segments that allows cell to extravasate from blood into tissues. Therefore, a difference in the frequency of these V-EC could potentially impact the kinetics or magnitude of inflammatory cell recruitment, which might in turn influence immuno-pathologies.. It should be cautioned that even though there appear to be more V-EC in germ free mice, these animals have actually *fewer* immune cells in their intestines. DARC is a marker that allows for identification of differentiated V-EC, but it does not report the functional activity of V-EC.

In the future, we will ask which intestinal cell type(s) must sense commensal bacteria to affect venular differentiation. Imaging of metabolically labeled bacteria in conjunction with flow cytometry will allow us to identify candidate subsets of hematopoietic and stromal cells that interact with and/or engulf intestinal bacteria. Although we consider it unlikely that the microbiota is directly interacting with vascular EC at steady state, we will also carefully explore this possibility.

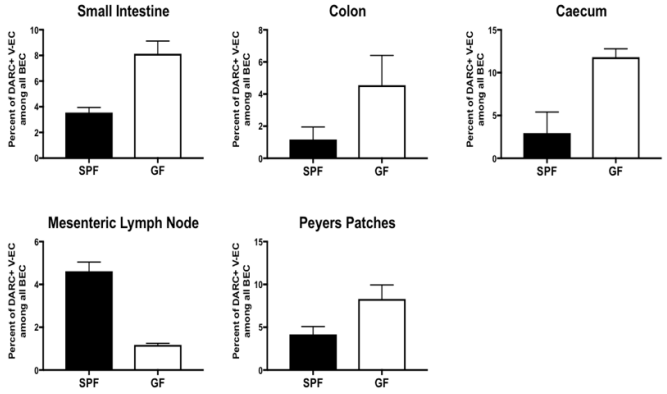

Figure 1. Venular endothelial cells from SPF and germ free mice. Quantitation of the frequency of DARC$^+$ V-ECs among total BECs in a panel of fresh murine gut tissues based on the gating strategy from figure 4. Bars depict mean +/- SD of V-EC frequencies in small intestine, colon, caecum, mesenteric lymph node and Peyer's patches from SPF and germ free mice.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

There have been four postdoctoral fellows and one technician working on this project and the work has afforded significant training in solving the chemical, immunologic, and microbiologic aims of this grant. Three fellows from the Kasper lab do the chemistry, microbiology and work with the fourth fellow from the von Andrian lab on the immunology and microscopy. The technician has been responsible for the synthesis of chemical reagents for fluorescent labeling. Teaching has come from several sources including direction and advice from PI's and senior lab members, conferences, and regular lab meetings.

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science,

Dissemination of knowledge to the community is in the form of a major research article to be found in Appendix A.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The techniques we have developed provide important tools that will enable research at the interface of host microbe interactions during disease. An understanding of these interactions provides key insights into potential avenues for therapeutic intervention.

These chemical techniques are also a potential method for delivery of antibiotics directly to the target in the bacterial cell. We are exploring the possibility of commercial impact of this approach.

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page*

numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Published

Hudak JE, Alvarez D, Skelly A, **von Adrian UH**, **Kasper DL**. Illuminating vital surface molecules of symbionts in health and disease. Nature Microbiology 2017; 2:Article number: 17099. PMCID: PMC5546223.

Acknowledgement of federal support: Yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing To Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing To Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing To Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing To Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing To Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: **Dennis Kasper**
Project Role: Principal Investigator
No change

Name: **Ulrich von Andrian**
Project Role: Principal Investigator
No change

Name: **Naama Geva Zatorsky**
Project Role: Research Fellow
Nearest person month worked: 11
Contribution to Project: *Dr. Geva-Zatorsky is a postdoctoral fellow working on this project and worked on the microbiologic component of these studies.*

Name: **Xinyang Song**
Project Role: Research Fellow
Nearest person month worked: 6
Contribution to Project: *Dr. Song is a postdoctoral fellow working on this project and is directly involved with the immunologic component of this study.*

Name: **Meng Wu**
Project Role: Research Fellow
Nearest person month worked: 6
Contribution to Project: *Dr. Wu is a postdoctoral fellow working on creating bacterial mutants to specifically explore mechanisms of organism immune interaction.*

Name: **Kailyn Stefan**
Project Role: Graduate Student
No Change

Name: **Jason Daugherty**
Project Role: Technician
Nearest person month worked: 3
Contribution to Project: *Mr. Daugherty is a technician responsible for synthesis of chemical reagents required for bio-orthogonal chemistry.*

Name: **Bella Printseva**
Project Role: Research Administrator
No Change

Name: **Munir Mosaheb**
Project Role: Research Fellow
Nearest person month worked: 5
Contribution to Project: *Dr. Mosaheb is a postdoctoral fellow working on this project. He is responsible for conducting intravital two photon microscopic studies.*

Name: **Pavel Hanc**
Project Role: Research Fellow

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing To Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing To Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Nature Microbiology (2017; vol. 2, 17099).

nature microbiology

LETTERS

PUBLISHED: 26 JUNE 2017 | VOLUME: 2 | ARTICLE NUMBER: 17099

Illuminating vital surface molecules of symbionts in health and disease

Jason E. Hudak[1], David Alvarez[1], Ashwin Skelly[1], Ulrich H. von Andrian[1,2] and Dennis L. Kasper[1]*

The immunomodulatory surface molecules of commensal and pathogenic bacteria are critical to microorganisms' survival and the host's response[1,2]. Recent studies have highlighted the unique and important responses elicited by commensal-derived surface macromolecules[3–5]. However, the technology available to track these molecules in host cells and tissues remains primitive. We report, here, an interdisciplinary approach that uses metabolic labelling combined with bioorthogonal click chemistry (that is, reactions performed in living organisms)[6] to specifically tag up to three prominent surface immunomodulatory macromolecules—peptidoglycan, lipopolysaccharide and capsular polysaccharide—either simultaneously or individually in live anaerobic commensal bacteria. Importantly, the peptidoglycan labelling enables, for the first time, the specific labelling of live endogenous, anaerobic bacteria within the mammalian host. This approach has allowed us to image and track the path of labelled surface molecules from live, luminal bacteria into specific intestinal immune cells in the living murine host during health and disease. The chemical labelling of three specific macromolecules within a live organism offers the potential for in-depth visualization of host–pathogen interactions.

Current methods to study commensal–host interactions *in situ*, such as fluorescence *in situ* hybridization, remain limited in scope. Despite significant advances in updating this technology[7,8], it still suffers from many drawbacks[9]. Chemical-based probes have been used to image and track bacterial components that are otherwise recalcitrant to conventional genetic tagging methods[10,11]. Furthermore, the predominantly anaerobic environment of the intestinal lumen presents an additional hurdle to genetically encoded tags such as green fluorescent protein (GFP), which require oxygen to mature. In this vein, we previously reported a method to tag and trace the capsular polysaccharides (CPSs) of various live commensal bacteria in cells and animal hosts[12]. This approach uses the metabolic incorporation of a non-natural sugar, *N*-azidoacetylgalactosamine (GalNAz)[13], into bacterial CPS to tag and track the bacterium and its CPS. However, fewer than 50% of anaerobic microorganisms tested could be reliably labelled with this approach. Therefore, we sought to expand and improve this method to (1) label a larger subset of commensals that did not incorporate GalNAz and (2) extend the tagged targets to other immunomodulatory surface molecules, especially more common bacterial molecules.

For our first target, the peptidoglycan (PGN) component of bacteria seemed an obvious choice as it is a highly conserved structural feature of most bacterial phyla. PGN is sensed by the innate nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) in mammalian cells, and the strong link of mutations in these receptors to inflammatory bowel disease (IBD) highlights their importance in maintaining healthy commensal–host interactions[14,15]. The promiscuity of PGN biosynthesis was recently exploited in developing a method to install non-natural fluorescent D-amino acids into bacterial PGN (Fig. 1a)[16,17]. Because cells use only a defined set of L-amino acids for protein synthesis, only the provided D-amino acids can specifically label the PGN. We wanted to determine whether this approach could be used to label and track anaerobic commensal bacteria both *in vitro* and *in vivo*.

Incubation of anaerobic cultures with the fluorescent D-amino acid hydroxycoumarin amino-D-alanine (HADA) resulted in the successful labelling of a wide range of anaerobic and facultative commensal bacteria, including *Bacteroides fragilis*, *Bacteroides vulgatus*, *Parabacteroides merdae*, *Clostridium clostridioforme*, *Clostridium ramosum*, *Enterococcus faecalis*, *Escherichia coli* and *Bifidobacterium adolescentis* (Fig. 1a,b and Supplementary Fig. 1a). As a control, we incubated the bacteria with the L-enantiomer of the fluorescent amino acid referred to as HALA, which does not incorporate into PGN or other macromolecules. The minimal background we observed supported the specific labelling of the PGN layer by D-amino acid incorporation. This labelling is both time- and concentration-dependent, as previously reported (Supplementary Fig. 1b)[16], although overnight incubation with HADA was optimal, as previously reported for labelling with GalNAz[12].

Given the success of *in vitro* PGN labelling, we examined whether the labelled bacteria could be imaged and traced within the natural niche of the host intestine. *B. adolescentis* and *E. faecalis* were labelled as described and administered to mice via oral gavage and direct intestinal injection, respectively. The bacteria retained the PGN label and were successfully imaged in tissue sections of the small intestine and colon (Fig. 1c and Supplementary Fig. 2a). *E. faecalis* in particular was found close to the tissue in the proximal colon and in the vicinity of $CD11c^+$ antigen-presenting cells (APCs, Fig. 1c). We also synthesized fluorescein-D-lysine (FDL)[16] and labelled *C. clostridioforme* in culture. Imaging of this organism in the murine colon (Supplementary Fig. 2b,c) demonstrated the capacity for multicolour labelling.

Because exogenous D-amino acids are not used by mammalian cells[10], we reasoned that feeding conventionally raised mice the fluorescent D-amino acid might allow selective labelling of endogenous bacteria. Confocal imaging of tissue histology slices from HADA-gavaged specific pathogen-free (SPF) mice showed robust labelling of the commensal bacteria already present in the lumen of the small intestine and the colon 2 and 4 h, respectively, after gavage (Fig. 1d). By contrast, the HALA-gavaged controls showed little background. This approach overcomes a significant methodological obstacle by providing the ability to specifically label the endogenous microbiota within a living host.

Further examination of the luminal contents demonstrated that the bacteria in the small intestine were quickly labelled (within 45 min) after HADA administration, but this signal was washed out within 3 h (Fig. 1e). This result is in accord with previous reports that small-intestinal niches are less populated and rapidly

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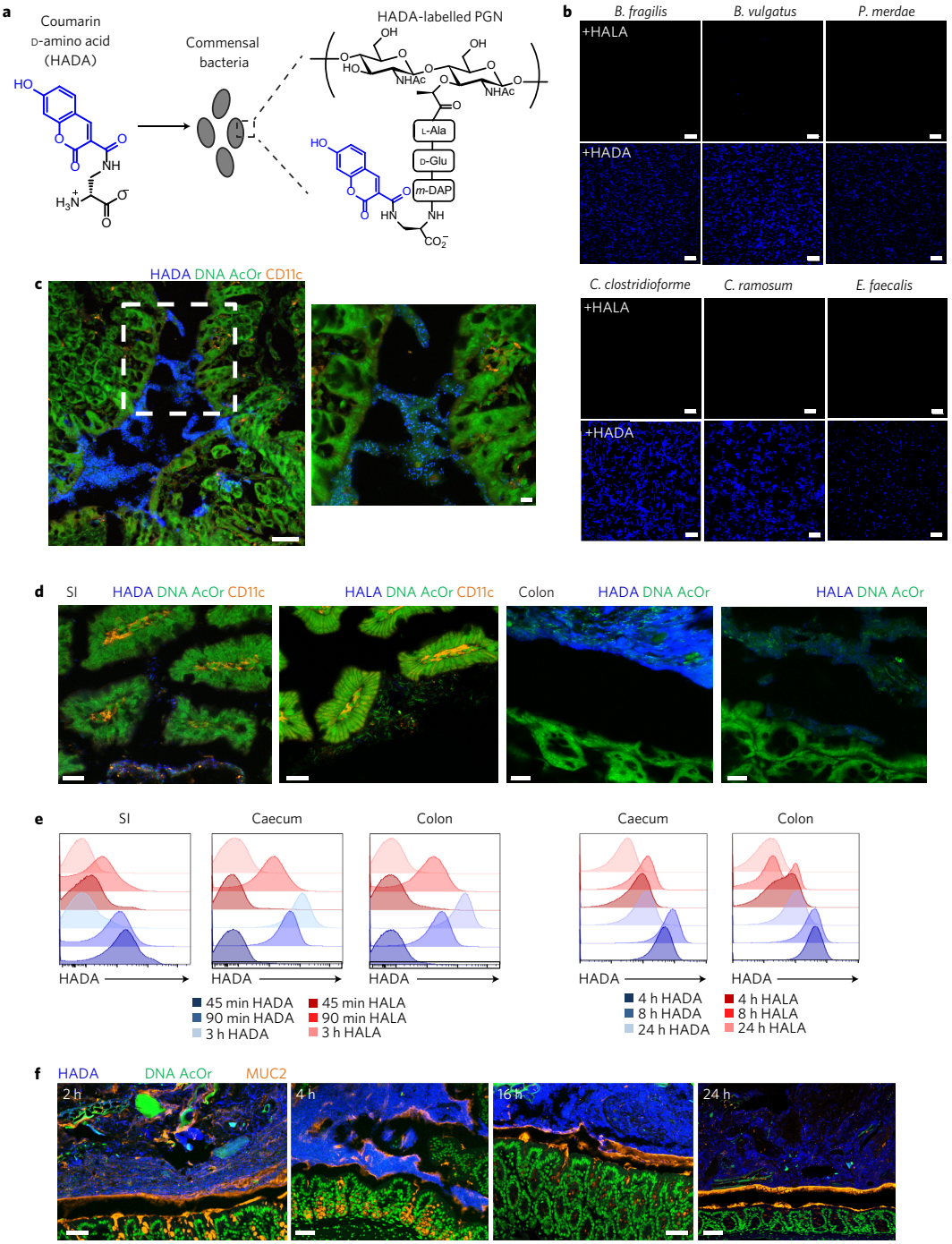

Figure 1 | Fluorescent D-amino acid labels PGN in commensal bacteria. a, Schematic of metabolic labelling of the PGN of commensal bacteria with fluorescent D-amino acid derivatives. Addition of the fluorescent coumarin moiety is highlighted in blue. **b**, Images of various commensal bacteria grown overnight in medium with a fluorescent amino acid: HADA or (as a negative control) HALA. Scale bars, 10 μm. **c**, Confocal image and expanded region (right) of frozen, fixed tissue slice of mouse colon 1 h after injection of HADA-labelled *E. faecalis*. AcOr, acridine orange. Scale bars, 50 μm (left) and 10 μm (right). **d**, Confocal images of Carnoy's-fixed, paraffin-embedded tissue slices from mice 2 h (small intestine (SI), left) or 4 h (colon, right) after oral gavage with HADA or (as a negative control) HALA. Scale bars, 20 μm. **e**, Flow cytometry histogram plots of luminal contents from mice given HADA or HALA orally demonstrate decay of fluorescent signal over time. **f**, Confocal images of Carnoy's-fixed, paraffin-embedded tissue slices from mice given HADA orally. The mice were killed and tissue was fixed at the specified times after oral gavage. The images show significant retention of fluorescent signal over 24 h. MUC2, mucin 2. Scale bars, 50 μm. Data in **c**–**f** are representative of at least three independent experiments.

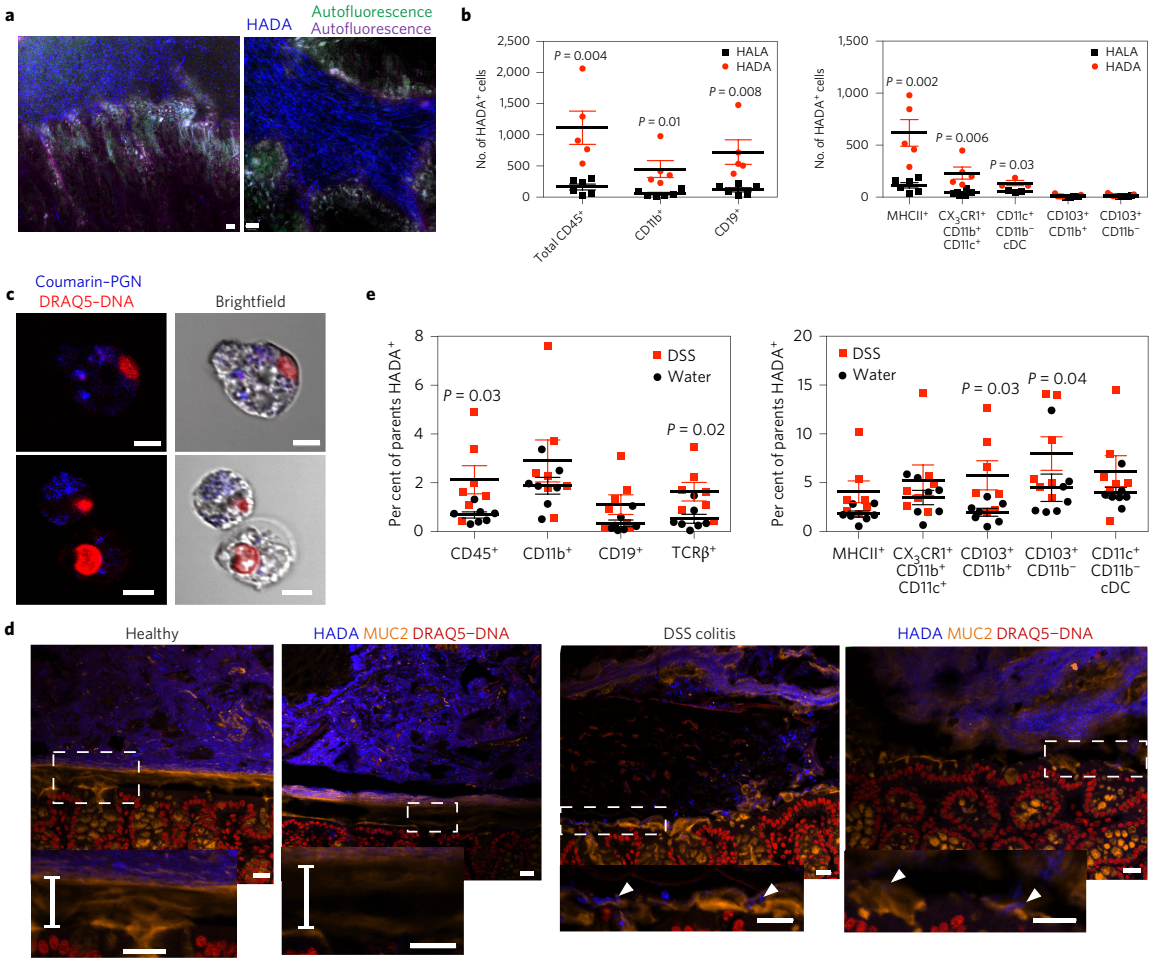

Figure 2 | Use of HADA-labelled PGN to track live commensals in the host. a, Representative live images from intravital two-photon microscopy of the murine colon 16 h after administration of HADA. Mice were anaesthetized and intestinal loops were surgically extracted and mounted for imaging. Purple and green represent tissue autofluorescence (Supplementary Videos 1 and 2). Scale bars, 10 μm. **b**, Numbers of colonic lymphocytes bearing a HADA$^+$ signal 4 h after oral gavage of HADA. Data are presented as mean ± s.e.m. values for biological replicates of $n = 5$ mice (P values calculated by paired Student's t-test). **c**, Representative confocal images of live CD11c$^+$ (top) or CD11c$^+$CX$_3$CR1$^+$ (bottom) mononuclear phagocytes (sorted for a HADA$^+$ signal) bearing HADA-labelled bacteria/PGN. Scale bars, 5 μm. DRAQ5, red DNA dye. **d**, Representative images of Carnoy's-fixed, paraffin-embedded tissue slices obtained 4 h after oral gavage of HADA from mice given normal drinking water (left) or drinking water containing 3% DSS (right) for the preceding 4 days. The damaged mucosal layer and breach with endogenous bacteria are evident in the DSS-induced colitic colons. Bars show that mucus thickness is ~50 μm in healthy mice and arrowheads point at bacteria at the epithelial layer in DSS-treated mouse colons. Scale bars, 20 μm. **e**, Percentages of the parent subpopulation of colonic lymphocytes bearing a HADA$^+$ signal 4 h after oral gavage with HADA in healthy mice and mice with DSS-induced colitis. Data are presented as mean ± s.e.m. values for biological replicates of $n = 7$ mice (P values calculated by paired Student's t-test). Data in **a**–**e** are representative of at least three independent experiments.

flush luminal content[18]. Correspondingly, the caecum and colon showed maximal labelling of endogenous bacteria within 3–8 h (Fig. 1e). The high signal from HALA samples at earlier time points suggests that several hours are required for the excess label to flush out naturally. Surprisingly, commensal bacteria remained significantly labelled up to 24 h after initial HADA administration by oral gavage (Fig. 1e,f and Supplementary Figs 3a,b and 4). This result suggests that, despite clearance of the PGN label within 6–8 h in rich media *in vitro* (Supplementary Fig. 3c), the label *in vivo* is retained much longer, perhaps because of slow growth rates in the normal gut. Repetition in germ-free (GF) mice showed minimal background signal, again suggesting that the observed fluorescence staining is dependent on PGN from endogenous bacteria (Supplementary Fig. 5a,b).

Because our approach labels living bacteria, we also used multiphoton intravital microscopy to view the dynamics of the host–microbiota interaction in real time[19]. Our results showed robust labelling of the commensal microorganisms in the small and large intestines (Fig. 2a and Supplementary Fig. 6). Videos of these dynamics highlight the ability to view endogenous bacteria moving within the lumen and adhering to the mucosal surface of the epithelial barrier (Supplementary Videos 1 and 2).

If the PGN of HADA-labelled bacteria could be imaged and traced in the lumen, then this approach would have the potential to track PGN from live commensals into specific intestinal cells. Several studies have investigated how free luminal protein or pathogens gain access to gut-associated lymphoid tissue (GALT); however, much less is known about bacterial structural-component

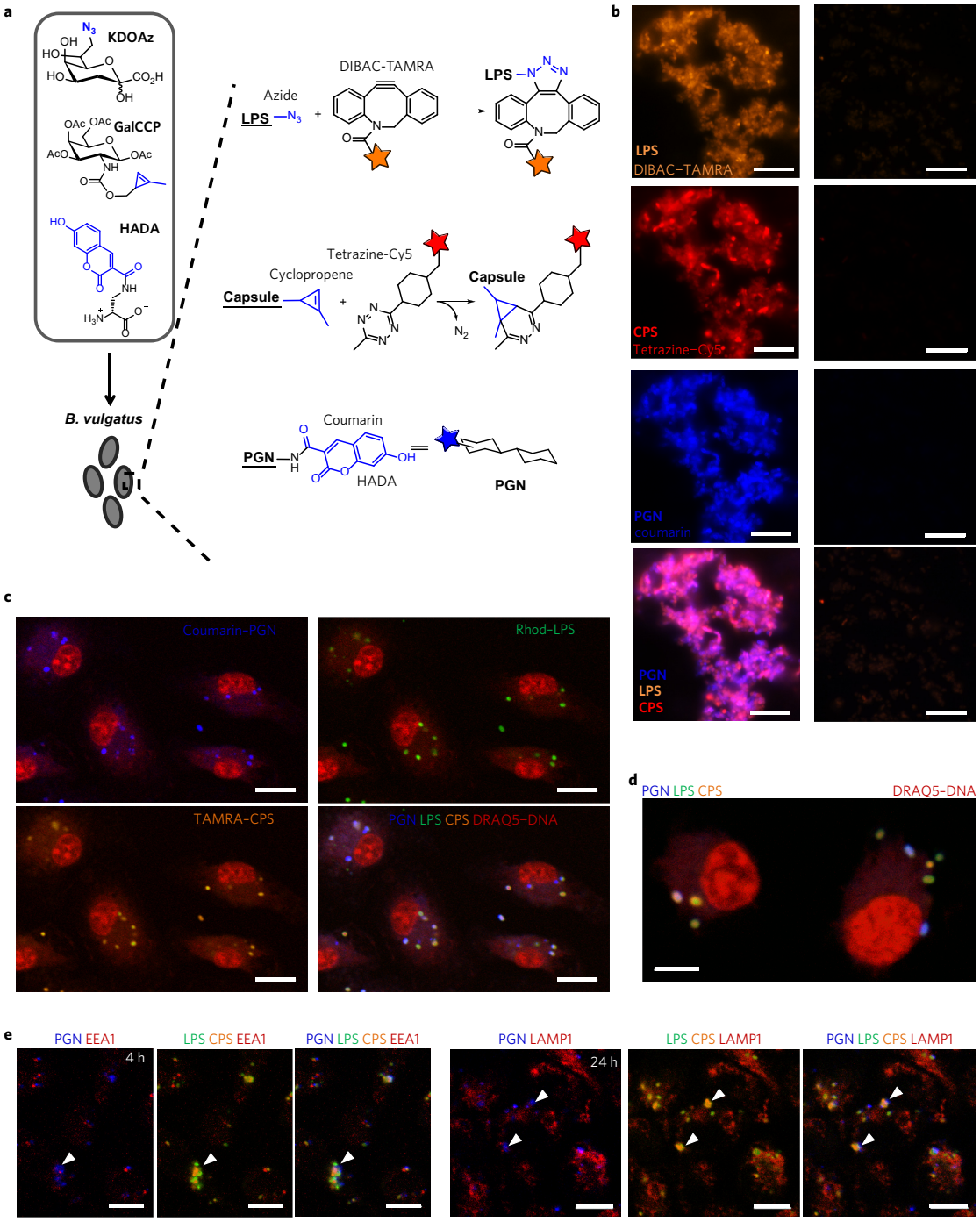

Figure 3 | Simultaneous labelling of three cell surface molecules in commensal bacteria. a, Schematic of the metabolic labelling approach using the non-natural metabolites KDOAz (LPS), GalCCP (CPS) and HADA (PGN) to simultaneously label three cell surface macromolecules in the commensal bacterium *B. vulgatus*. The reactive or fluorescent moieties are highlighted in blue. DIBAC-TAMRA, dibenzo-aza-cyclooctyne-carboxytetramethylrhodamine; Cy5, cyanine-5. **b**, Images of *B. vulgatus* grown overnight in medium with KDOAz, GalCCP and HADA (left) or, as a negative control, with GalNAc and HALA (right) and reacted with DIBAC-TAMRA and tetrazine-Cy5. Scale bars, 10 μm. **c**, Confocal images of fixed bone-marrow-derived macrophages 1 h after inoculation of three-component-labelled *B. vulgatus*. Rhod, rhodamine green; DRAQ5, red DNA dye. Scale bars, 5 μm. **d**, Zoomed confocal image of bone-marrow-derived macrophages bearing three-component-labelled *B. vulgatus*. Scale bar, 5 μm. **e**, Representative images of J775A.1 murine macrophages 4 h and 24 h after inoculation of three-component-labelled *B. vulgatus*. Arrows highlight where PGN is seen separate from colocalized CPS/LPS. Scale bars, 10 μm. Data in **b**–**e** are representative of at least three independent experiments with $n = 3$ technical replicates.

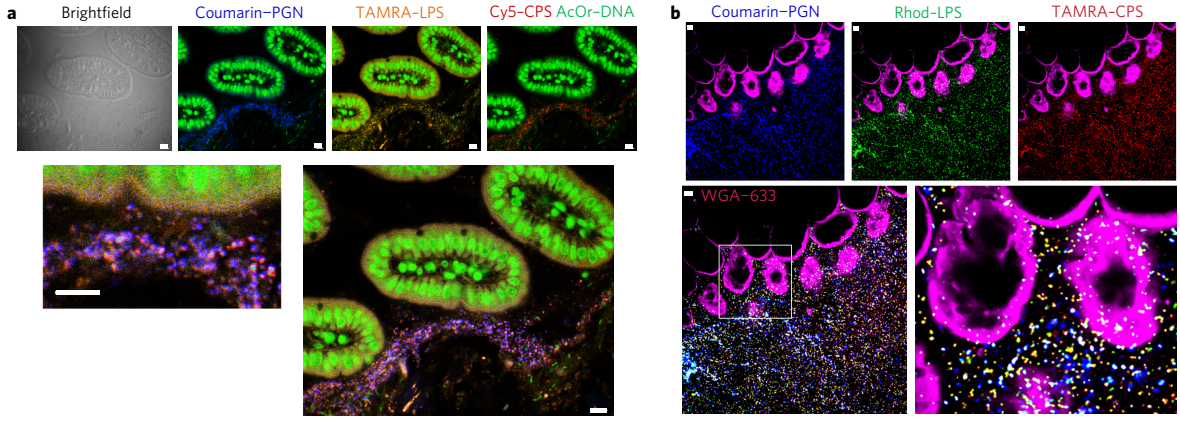

Figure 4 | Simultaneous labelling of three cell surface molecules in commensal bacteria. a, Confocal image of Carnoy's-fixed, paraffin-embedded tissue slices obtained from mice 1 h after intestinal injection of labelled *B. vulgatus*. Zoomed image (lower left) is shown to highlight the interaction with the host epithelial layer. Scale bars, 10 μm. **b**, Representative live images from intravital two-photon microscopy of the murine small intestine after direct inoculation with three-component-labelled *B. vulgatus*. Purple represents lectin staining of the epithelium with WGA-633 (Supplementary Videos 3 and 4). Scale bars, 10 μm. Data are representative of at least three independent experiments with $n = 3$ mice per experiment.

transport, especially in commensals[20,21]. Flow cytometry analysis of colonic lamina propria cells from mice receiving HADA or HALA revealed a significant portion of $CD45^+$ lymphocytes containing HADA fluorescence (Fig. 2b and Supplementary Fig. 7). Among these $HADA^+$ lymphocytes, $CD11b^+$ phagocytes and $CD19^+$ B cells predominated. This large number of $HADA^+CD19^+$ B cells was a surprise. Although dendritic cells (DCs) are known to stimulate B cells in the GALT[22], few studies have cited such high levels of direct commensal association with resident B cells.

Aside from $CD19^+$ B cells, the APCs in the $MHCII^+HADA^+$ population of the colon consisted mainly of $CX_3CR1^+CD11c^+CD11b^+$ macrophages and conventional $CD11c^+CD11b^-$ DCs. CX_3CR1^+ macrophages are known to directly sample luminal bacteria and to play key roles in phagocytosis and antigen presentation in the lamina propria[21,23]. $CD103^+$ cells are the main DCs to migrate to the mesenteric lymph nodes[21]. However, we did not see a significant population of $HADA^+CD103^+$ DCs in the colonic lamina propria (Fig. 2b). This result may be due to the small numbers of this cell type in the lamina propria or may highlight the limited ability of these cells to directly acquire luminal bacteria. To confirm that $HADA^+$ cells did indeed contain PGN or PGN-labelled bacteria, we sorted and imaged the $CD11c^+CX_3CR1^+$ and $CD11c^+CX_3CR1^-$ cells containing HADA fluorescence (Fig. 2c and Supplementary Fig. 8).

Sensing of PGN by the host immune system plays a role in the development and progression of IBD[14]. We used the D-amino acid metabolic labelling approach in a mouse model of colitis to track and image the PGN of commensals during disease-associated inflammation. As seen in Fig. 2d, the mucosal layer within the colon was greatly diminished and this change allowed direct interaction of the labelled endogenous bacteria with the colonic epithelium, a finding previously described in IBD[24,25]. This interaction correlated with an increase in $HADA^+CD45^+$ lymphocytes in the lamina propria (Fig. 2e). Of these lymphocytes, T-cell receptor β (TCRβ)-positive T cells and $CD103^+$ DCs were significantly associated with bacteria/bacterial PGN in the colitic versus healthy host, a finding that further supports a key role for these cells in microbial influences on colitis[25].

The utility of the PGN D-amino acid labelling approach impelled us to seek yet more surface molecules that could be tagged to trace commensals *in vivo*. Recently, an azide-containing 2-keto-3-deoxy-D-mannooctanoic acid (KDO) derivative, 8-azido-8-deoxy-KDO (KDOAz), was shown to incorporate into the lipopolysaccharide (LPS) of *E. coli* (Supplementary Fig. 9a)[26]. Once incorporated, the azido group can be labelled by reaction with a fluorescent-stained alkyne to form a stable, covalent triazole[6]. We anticipated that this approach would be broadly applicable because the LPS of many other Gram-negative bacteria consists of an acylated lipid A with a KDO linker in its core oligosaccharide[27]. As confirmed by microscopy, flow cytometry and gel electrophoresis, KDOAz incorporated into LPS and labelled a wide variety of Gram-negative intestinal resident bacteria, including *E. coli*, *Klebsiella*, *B. vulgatus*, *Bacteroides uniformis*, *Bacteroides eggerthii* and *Fusobacterium necrophorum* (Supplementary Figs 9b,c and 10).

At this point we had successfully shown labelling of CPS, PGN and LPS on a variety of commensal bacteria. Could we then label all three of these immunomodulatory surface components simultaneously on live commensals and thereby overcome a significant previous obstacle—the simultaneous metabolic labelling of multiple distinct molecules in one organism? GalNAz or KDOAz labelling relies on the same azide chemical handle for fluorophore attachment, so we devised an orthogonal labelling approach for CPS, using a GalNAc derivative that contains a cyclopropene, *N*-cyclopropenyl galactosaminyl carbamate (GalCCP) (Fig. 3a)[28]. Like azides, cyclopropenes are small and biologically inert functional groups, but they react selectively with tetrazines and at the same time as the azide–alkyne reaction[29]. Thus, LPS and CPS could conceivably be labelled simultaneously with different fluorophores.

We tested our three-component labelling scheme on the commensal *B. vulgatus* because it performed well with the individual components. By flow cytometry and microscopy, we documented distinct labelling of all three components, with minimal background in controls incubated with HALA and GalNAc (Fig. 3b and Supplementary Fig. 11a). The distinct labelling of the LPS and CPS components was confirmed by SDS–PAGE, by which the high-molecular-weight (M_w) CPS for tetrazine–GalCCP can be distinguished from the lower-M_w LPS ladder for cyclooctyne–KDOAz (Supplementary Fig. 11b).

A main appeal of this approach is its ability to track how individual commensal components are broken down and sensed by immune cells. To test this utility, we incubated three-component-labelled *B. vulgatus* with the murine macrophage cell line J755A.1 or bone-marrow-derived macrophages. At early time points, all

three components could be seen distinctly in association with phagocytosed bacteria in the macrophages (Fig. 3c,d and Supplementary Fig. 12a,b). Within 4–24 h, the components were often seen to separate into distinct areas: LPS and CPS were located in large encapsulated endosomes, while the HADA-PGN signal was frequently found in punctate areas around these lysosomal LAMP-1$^+$ compartments (Fig. 3e, indicated by arrowheads). In cells treated with bafilomycin, which halts endosome acidification, these large LPS$^+$CPS$^+$ compartments could not be found, a result implying that this phenomenon is a product of cell-mediated bacterial degradation (Supplementary Fig. 12c).

The three-component-labelled bacteria were also introduced and imaged within the intestinal lumen of the murine host. This experiment was performed (1) on fixed tissue sections from mice given labelled *B. vulgatus* by direct intestinal injection and (2) with two-photon intravital microscopy on surgically exposed intestinal loops in live mice given labelled *B. vulgatus* by direct application (Fig. 4a,b and Supplementary Fig. 13a,b). Live videos (Supplementary Videos 3 and 4) highlight the ability to track each component as the bacteria traverse the lumen and interact with host epithelial cells.

The metabolic labelling and click chemistry approach we present here allows, for the first time, simultaneous labelling of three bacterial components: CPS, LPS and PGN. Because most of the mammalian microbiota resides in the intestine, deciphering how and when these bacterial molecules are obtained by the host is especially important in understanding the progression of intestinal diseases, including IBD and colorectal cancer[30]. The ability to track these inflammatory (and anti-inflammatory) inducers will enable the study of where they accumulate, which cell types they encounter in GALT, and, ultimately, whether differences in their localization correlate with disease.

Methods

Mice. Male and female 6- to 8-week-old C57BL/6 mice were purchased from Jackson Laboratory. Mice were housed in SPF conditions with food and water *ad libitum*. GF C57BL/6 mice were bred and maintained in sterile vinyl isolators in the animal facility at Harvard Medical School and were provided with sterile food (LabDiets 5K67), water and bedding. All experiments were conducted in accordance with the guidelines of the US National Institutes of Health and approved by the Harvard Medical Area Standing Committee on Animals.

Bacteria and growth media. The following bacterial species were used: *Bacteroides fragilis* (NCTC9343), *Bacteroides eggerthii* (DSM 20697), *Bacteroides uniformis* (ATCC8452), *Bacteroides vulgatus* (ATCC8482), *Parabacteroides merdae* (CL03T12C32), *Fusobacterium necrophorum* (AO43), *Clostridium clostridioforme* (2_1_49FAA), *Clostridium ramosum* (AO31), *Escherichia coli* (K12), *Bifidobacterium adolescentis* (L2-32), *Klebsiella* spp. (4_1_44FAA) and *Enterococcus faecalis* (TX0104). All bacteria were grown in a basal peptone-yeast broth containing (per litre) 5 g of yeast extract, 20 g of protease peptone, 5 g of NaCl, 5 mg of hemin, 0.5 mg of vitamin K1 and 5 g of K_2HPO_4. Hemin, vitamin K1 and K_2HPO_4 were added through a filter after the basal medium had been autoclaved. All non-natural metabolites for metabolic labelling were synthesized in house (for details see Supplementary Note 1).

Metabolic labelling and click chemistry of bacteria. Bacteria were inoculated from a plate culture and grown overnight at 37 °C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) in an anaerobic chamber in basal peptone-yeast broth with HADA or HALA (0.8 mM), KDOAz (5 mM) and/or GalCCP (250 μM). Bacteria were pelleted at 5,000*g* and washed twice in PBS and once in PBS supplemented with 1% BSA. For the subsequent click reactions with KDOAz and GalCCP, the final pellet was resuspended in one-tenth the original culture volume (for example, 100 μl for a 1 ml culture) of 1% BSA in PBS with 5 μM DIBAC-Rhod, DIBAC-Cy5 or DIBAC-TAMRA (Click Chemistry Tools) for reaction with azide or 5 μM tetrazine-TAMRA/tetrazine-Cy5 (Click Chemistry Tools) for reaction with GalCCP. The suspension was incubated with agitation for 1 h at 37 °C, after which the bacteria were pelleted and washed twice with 1% BSA in PBS and once in PBS. For three-component labelling, the bacteria were first reacted with 5 μM DIBAC for 1 h at 37 °C, washed once with PBS and then reacted with 5 μM tetrazine for 1 h at 37 °C. After the final wash, cells were resuspended in PBS and were administered to mice or fixed in 1% formalin and mounted for microscopy or run on flow cytometry. To monitor label decay, bacteria were resuspended in basal broth and aliquots were removed and fixed in 1% formalin at specified time points.

Tissue culture studies and immunofluorescent microscopy. *B. vulgatus* was grown overnight in basal peptone-yeast broth with HADA (0.8 mM), KDOAz (5 mM) and GalCCP (250 μM) to an optical density at 600 nm (OD_{600}) of ~0.6. As a control, bacteria were grown in HALA (0.8 mM) and GalNAc (250 μM). The bacteria were labelled with tetrazine-Cy5 and DIBAC-TAMRA or tetrazine-TAMRA and DIBAC-Rhod as described in the previous section. C57BL/6 primary-bone marrow-derived macrophages or J775A.1 murine macrophages were isolated from mouse bone marrow or received directly from the ATCC, respectively, without further authentication or mycoplasma testing. A total of 5×10^5 macrophage cells were seeded onto 18 mm coverslips in 12-well plates and grown to confluency under humid conditions in an atmosphere of 5% CO_2 at 37 °C in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% nonessential amino acids, 1% penicillin–streptomycin, 0.15% sodium bicarbonate and 2 mM L-glutamine. Cells were washed with PBS and each well was filled with RPMI medium containing no penicillin–streptomycin. A 10 μl volume of labelled bacterial cells (multiplicity of infection of ~10) was added to each well and spun at 300*g* for 5 min. For some studies, 0.1 μM bafilomycin (Enzo Life Sciences) was added from a DMSO stock to inhibit lysosome maturation. After incubation for 30 min in 5% CO_2 at 37 °C, the medium was removed and coverslips were washed with sterile RPMI containing no penicillin–streptomycin and left in RPMI. Coverslips were removed from the plates at 1, 4 and 24 h, washed with PBS, and fixed for 20 min in 4% paraformaldehyde in PBS. Cells were blocked and permeabilized by incubation with 10% FBS, 1% BSA and 0.1% Triton X-100 in PBS for 1 h, washed twice with Tris-buffered saline with 0.1% Tween (TBST), and incubated with antibodies to LAMP-1 (sc-19992, clone 1D4B, lot H1612, Santa Cruz Biotechnology) or EEA-1 (sc-6415, clone N-19, lot B1813, Santa Cruz Biotechnology) (1:200 in 2% BSA/PBS) for 16 h (protected from light) at 4 °C. Nuclear staining was performed with acridine orange (Biotium) or DRAQ5 (BioLegend) (1 μg ml^{-1}) for 10 min. Coverslips were washed three times with TBST, mounted on glass slides with Mowiol medium, and viewed on an Olympus Fluoview BX50WI inverted confocal microscope. All images were analysed with Fluoview Viewer Software and prepared in Adobe Photoshop.

Tracking of labelled bacteria in the murine host. For tracking of endogenous bacteria, C57BL/6 mice received 250 μl of 3 mM HADA/HALA in PBS by oral gavage. At specified time points, mice were euthanized with CO_2 and the intestinal tissue and mesenteric lymph nodes were removed. In most experiments, the selected time point was 2 h after gavage for the small intestine and 4 h after gavage for the colon. For tracking of exogenously labelled bacteria, organisms were grown in basal medium containing metabolic labels as described under 'Bacteria and growth media'. Approximately 10^8–10^9 organisms in 100 μl of PBS were directly injected into the lumen of the ileum or proximal colon of live SPF C57BL/6 mice anaesthetized under isofluorane. At 1 h after injection, mice were euthanized with CO_2 and the intestine and mesenteric lymph nodes were removed. In the dextran sodium sulfate (DSS)-induced colitis model, mice received 3% DSS in the drinking water as previously described[31] and were monitored daily. Colonic tissue was collected from mice on day 4 after administration of DSS (at the onset of intestinal inflammation)[31] and lymphocytes were isolated.

For fluorescence imaging, intestinal tissue was fixed overnight in Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) and submitted to the Harvard Cancer Center Research Pathology Core for paraffin embedding and sectioning. Paraffin was removed by two incubations in xylene substitute (ThermoFisher) for 10 min at 37 °C followed by rehydration with sequential incubation in 100, 95, 70 and 50% ethanol and then in distilled water. Alternatively, tissue was fixed in 4% formalin/DMEM for 4 h at 4 °C, embedded in OCT compound (Tissue-Tek), frozen into tissue moulds and later sectioned at a thickness of ~12 μm with a cryostat. The tissue sections were blocked and permeabilized by incubation with 10% FBA, 1% BSA and 0.1% Triton X-100 in PBS for 1 h, washed twice with TBST, and incubated with antibodies to CD11c (clone N418, Santa Cruz Biotechnology) or MUC2 (clone H-300, lot F2314, Santa Cruz Biotechnology; 1:200 in 2% BSA/PBS) for 16 h (protected from light) at 4 °C. Secondary antibody to rabbit (AF568-goat IgG α-rabbit, Life Technologies; 1:1,000 in 2% BSA/PBS) was applied for 1 h at room temperature for MUC2 staining. Nuclear staining was performed with acridine orange (Biotium) or DRAQ5 (BioLegend) (1 μg ml^{-1}) for 10 min. Coverslips were washed three times with TBST and mounted on glass slides with Mowiol medium. Confocal images were acquired with an Olympus Fluoview BX50WI inverted confocal microscope. All images were analysed with Fluoview Viewer Software and prepared in Adobe Photoshop.

For analysis of single-cell isolates, intestinal tissues were cleaned and dissociated as previously described[32]. Intestinal luminal contents were flushed in 5 ml of PBS and fat was removed from tissue. For analysis of luminal bacteria, the flushed contents were allowed to settle and then filtered through nylon mesh, and material was pelleted and fixed overnight in 1% formalin/DMEM at 4 °C. The cleaned tissue was treated with RPMI containing 1 mM DTT, 2.5 mM EDTA and 1.5% FBS at 37 °C for 15 min to remove epithelial cells, minced and dissociated in collagenase solution (collagenase II (Gibco), 1.5 mg ml^{-1}; dispase (Gibco), 0.5 mg ml^{-1}; DNAse I (Worthington), 0.05 mg ml^{-1} and 1% FBS in RPMI) with constant stirring at 37 °C for 45 min. Single-cell suspensions were then filtered and washed in 5% FBS RPMI. Cell suspensions were filtered through nylon mesh and resuspended in

DMEM without phenol (Gibco/Life Technologies). This process was followed by staining on ice for 20 min (1:200 dilution) with fluorescently labelled antibodies to CD45 (30-F11), CD4 (GK1.5), CD11b (M1/70), CD11c (N418), CD19 (6D5), I-A/I-E (M5/114.15.2), CX_3CR1 (SAO11F11), CD103 (2E7) or TCRβ (H57-597), all from Biolegend. The fixable viability dye eFluor-780 (eBioscience) was used to gate out dead cells. The stained samples were immediately run or fixed in 1% formalin/DMEM overnight and run on flow cytometry (MacsQuant, Miltenyi or LSR-II, BD Biosciences) and analysed with FlowJo software. Cell suspensions were run on a FACSAria and sorted for $HADA^+$I-A/I-E^+CD11c^+ and for CX_3CR1^+ and CX_3CR1^-.

Intravital two-photon microscopy of the intestine. Intravital two-photon microscopy of mouse intestine was performed as previously described[12,33]. Mice were anaesthetized with ketamine–xylazine–acepromazine and positioned on a customized heated stage. A ~2 cm segment of intestine was exteriorized through the peritoneum, immobilized with tissue-adhesive glue and kept hydrated with a mixture of PBS and lubricant gel. Because of the thickness of the intestinal muscularis, the lumen interior was exposed by micro-incision along the intestinal wall followed by careful removal of large faecal matter and mounting on the customized stage. In some preparations, labelled bacteria were injected directly into the intestinal loop; otherwise, SPF mice were given 3 mM HADA in 250 µl by oral gavage at least 4 h before imaging. For delineation of the intestinal epithelial layer, the exposed luminal section was incubated in wheat germ agglutinin-AF633 (Life Technologies/ThermoFisher; 0.5 mg ml^{-1} in PBS) for 10 min to counterstain before imaging. Two-photon imaging was performed on an Ultima Two-Photon Microscope (Prairie Technologies/Bruker) equipped with a Tsunami Ti:sapphire laser with a 10 W MilleniaXs pump laser (Spectra-Physics) and a ×20/0.95 NA water-immersion objective (Olympus). The two-photon excitation wavelength was set to 815 nm for optimal fluorescence excitation of the HADA coumarin fluorophore while maintaining excitation of additional rhodamine green and TAMRA fluorophores for three-colour labelling. Fluorescence emission was detected with 665/65 nm, 590/50 nm, 525/50 nm and 450/50 nm bandpass filters for four-colour imaging. Raw image sequences were first processed with Volocity software (v.6.0, PerkinElmer). Autocontrasted images were processed with fine noise-reduction filters and each image channel was assigned a pseudo-colour according to emitted light wavelengths (bp665/65 nm, magenta; bp590/50 nm, red; bp525/50 nm, green; bp455/70 nm, blue). For time-lapse videos of the small intestine, processed image sequences were corrected for motion artefacts with ImageJ/Fiji (v.2.0.0). Images were motion-corrected by recursive registration of stacks/StackReg[34] with Affine transformation, followed by Elastic Stack Alignment[35] with Rigid transformation.

Statistical analyses. All statistical analyses were performed in Prism (GraphPad Software) and were based on normally distributed data sets with equal variance (Bartlett's test). Gender- and age-matched mice were randomly assigned to groups for *in vivo* experiments. Sample size was chosen for adequate numbers for statistical analyses. Investigators were not blinded during the experiments or outcome assessments. Where applicable, data points are presented as mean ± s.e.m. values unless otherwise stated. Data were inferred as statistically significant if P values were <0.05. The significance of differences between two groups was determined by two-tailed paired Student's t-test.

Data availability. The data that support the findings of this study are available from the corresponding author upon request, including, but not limited to, raw data, original images and further technical details.

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Author contributions

J.E.H. designed the experiments, analysed the data and wrote the manuscript with help from D.A. and A.S. D.A. provided expertise in two-photon intravital microscopy. D.L.K. supervised the study, edited the manuscript and provided helpful comments, with assistance from U.H.v.A.

Additional information

Supplementary information is available for this paper.

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Competing interests

The authors declare no competing financial interests.

Understanding Microbial Sensing in Inflammatory Bowel Disease Using Click Chemistry

Insert ERMS/Log Number and Task Title Here

Award Number: W81XWH-15-1-0368 / W81XWH-15-1-0367

PI: Dennis L. Kasper / Ulrich von Andrian **Org:** Harvard Medical School **Award Amount:**2,519,970

Aims

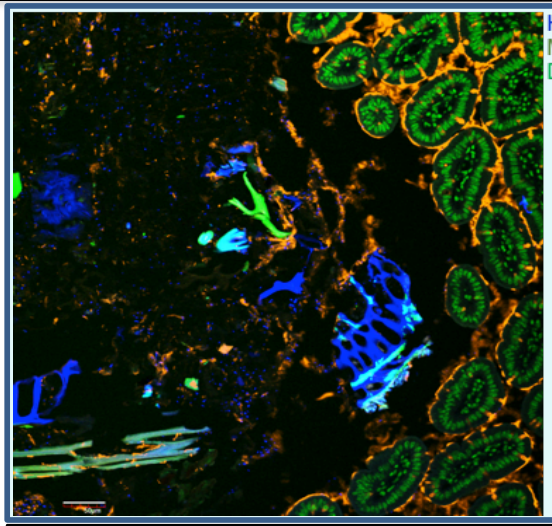
1. Incorporate non-natural sugars and amino acids into lipopolysaccharide, capsular polysaccharide, and peptidoglycan for live bacterial cell labeling.

2. Use click labeling to elucidate the acquisition and distribution of commensal material *in vitro* and in host intestinal tissue.

3. Use live intravital microscopy to monitor changes in bacterial component acquisition during chemical and pathogen-induced colitis.

Approach

Using bioorthogonal "click" chemistry strategy we have simultaneously labeleld three components of the bacterial cell wall: PSA (TLR2 ligand), LPS (TLR4 ligand), and PGN (ligand for NLR signaling). This has allowed us to track these components in living bacteria and their host and to follow these molecules into specific host immune cells in order to define the pathways by which protective or inflammatory responses develop. This methodology also allows us to visualize the spatial and temporal changes in bacterial product acquisition within diseased tissue.



Confocal image of HADA-labeled *B. adolescentis* in Carnoy's-fixed murine small intestine 1 h after direct luminal injection. Scale bar, 50 µm.

Accomplishment: We have made significant progress on all three aims. 1.) We have successfully labeled PSA, LPS and PGN in live bacteria. 2.) We developed a novel way to specifically labeled endogenous gut bacteria using D-amino acid labeling and 3.) and done preliminary experiment looking at labeled bacterial interactions using intravital microscopy to view the dynamic of host-microbiota interaction in real-time.

Timeline and Cost

Activities	CY 15	16	17	
Aim 1				
Aim 2				
Aim 3				
Budget ($K)	**$839,990**	**$839,990**	**$839,990**	

Goals/Milestones:

CY15 Goal – Aim 1: Bioorthogonal click chemistry labeling of bacterial components in live bacteria.
☑Aim 1 has been completed.

CY16 Goals – Aim 2: Elucidate the acquisition and distribution of commensals materials *in vitro* and in host intestinal tissues.
we have successfully labeled endogenous bacteria by feeding mice with fluorescently labeled D amino acids (HADA).
We have done confocal microscopy experiments demonstrating the presence of labeled bacteria in the intestinal lumen upon gavage of HADA lab ☐ X Aim 2 has been completed.

CY17 Goal – Aim 3: Use of intravital microscopy to assess the bacterial components acquisition by immune cells.

☐X Aim 3 is completed, we have performed experiments using multiphoton intravital microscopy and were able to observe robust labeling of bacteria in the small and large intestine upon gavage with HADA labeled bacteria.

4/15/19