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Identification of bacteria-derived HLA-bound peptides in melanoma

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Abstract

A variety of species of bacteria are known to colonize human tumours^{1–11}, proliferate within them and modulate immune function, which ultimately affects the survival of patients with cancer and their responses to treatment^{12–14}. However, it is not known whether antigens derived from intracellular bacteria are presented by the human leukocyte antigen class I and II (HLA-I and HLA-II, respectively) molecules of tumour cells, or whether such antigens elicit a tumour-infiltrating T cell immune response. Here we used 16S rRNA gene sequencing and HLA peptidomics to identify a peptide repertoire derived from intracellular bacteria that was presented on HLA-I and HLA-II molecules in melanoma tumours. Our analysis of 17 melanoma metastases (derived from 9 patients) revealed 248 and 35 unique HLA-I and HLA-II peptides, respectively, that were derived from 41 species of bacteria. We identified recurrent bacterial peptides in tumours from different patients, as well as in different tumours from the same patient. Our study reveals

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Author contributions

S.K., A.N. and Y.S. designed the study and wrote the paper. S.K. and A.N. analysed the data, and performed and supervised experiments. S.K., A.N., M.A., E.B. and P.G. performed the HLA peptidomics experiments. D.N., N.S., G.F. and R.S. analysed the bacteria composition of tumours. T.D. and S.L.-Z. performed the CLEM experiments. L.T.G., N.B., N.G.-Z., L.R., O.G. and R.E. helped with the bacteria staining techniques, imaging and imaging analysis. A.N., P.G., G.Y. and A.P. grew the cell lines for HLA peptidomics. K.V., T.D.L. and D.J.A. performed the taxonomic analysis of whole-genome sequencing data from melanoma. Y.B., W.C.S., A.W., M.L.-P. and E.S. performed whole-genome sequencing and analysis of bacteria isolates. J.R.G.-P. and S.N.P. performed bacteria isolation and sequencing. C. Barbolin, K.C., R.L., K.W., L.H. and E.R. performed computational analysis. R.R. helped with the statistical analysis. A.N. and S.T. performed the immunofluorescence experiments. M.L. helped with the TIL rapid expansion protocols. A.A. and Y.L. helped with the HLA peptidomics and mass spectrometry analyses. A.R., S.B.J., C. Bernatchez, C.H., R.A., M.P.L., M.J.B., J.S. and J.A.W. provided patient materials and information. S.L.C.K. and P.K. helped with TIL reactivity assay. G.B. performed HLA typing. All authors contributed to the final version of the paper.

Competing interests

J.A.W. is an inventor on a US patent application (PCT/US17/53.717) submitted by the University of Texas MD Anderson Cancer Center that covers methods to enhance immune checkpoint blockade responses by modulating the microbiome. J.A.W. reports compensation for speaker's bureau and honoraria from Imedex, Dava Oncology, Omniprex, Illumina, Gilead, PeerView, Physician Education Resource, MedImmune and Bristol–Myers Squibb. J.A.W. serves as a consultant/advisory board member for Roche/Genentech, Novartis, AstraZeneca, GlaxoSmithKline, Bristol–Myers Squibb, Merck, Biothera Pharmaceuticals and Microbiome DX. J.A.W. also receives research support from GlaxoSmithKline, Roche/Genentech, Bristol–Myers Squibb and Novartis.

Additional information

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Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-021-03368-8>.

that peptides derived from intracellular bacteria can be presented by tumour cells and elicit immune reactivity, and thus provides insight into a mechanism by which bacteria influence activation of the immune system and responses to therapy.

To investigate the influence of intratumoral bacteria on the immune response, we developed an experimental pipeline that comprises a 16S rRNA gene-sequencing platform⁵ coupled to HLA peptidomics and applied it to samples from melanoma tumours.

Species of bacteria identified in melanoma

Our sequencing of the 16S rRNA gene of 17 melanoma samples derived from 9 patients with melanoma (Supplementary Table 1) led to the identification of 41 distinct species of bacteria (Supplementary Table 2). Inspection of a microbial phylogenetic tree revealed high similarity in the composition of bacteria found in different metastases from the same patient (Fig. 1, Extended Data Fig. 1), but also among samples from different patients. This finding points to the existence of species of bacteria that are common to melanoma. To validate the presence of bacteria in our tumour cohort, we performed 16S fluorescence in situ hybridization staining (Extended Data Fig. 2).

We complemented this with taxonomic profiling of a whole-genome sequencing dataset of melanoma that comprised 108 paired tumour and blood samples¹⁵, focusing on DNA sequences that do not map to the human genome. Although the proportion of bacterial reads in the tumour and blood samples were the same (paired two-tailed Wilcoxon test, $P = 0.52$), the microbiota richness—measured as the number of species in a sample—was higher in tumour samples (paired two-tailed Wilcoxon test, $P = 0.0045$) (Extended Data Fig. 3a). Moreover, the bacterial composition was more conserved in tumour samples than in blood samples (Bray–Curtis distance, two-tailed Wilcoxon test, $P = 2.8 \times 10^{-8}$) (Extended Data Fig. 3b), and seven genera of bacteria exhibited greater abundance in the tumour samples (Extended Data Fig. 3c, d). Eight species of bacteria from the *Acinetobacter*, *Actinomyces*, *Corynebacterium*, *Enterobacter* and *Streptococcus* genera were also found in our cohort, supporting the microbial composition that we identified in melanoma tumours.

Presentation of bacterial peptides

We performed an HLA peptidome analysis of the HLA-I and HLA-II repertoires of the same tumours that were sequenced by 16S rRNA profiling. The raw data from each HLA peptidome were searched (using MaxQuant software) against the proteomes of the species of bacteria that we identified in the corresponding tumour, together with the human proteome. We filtered the peptides by the quality of their identification and their ability to match the HLA alleles of the patient (Extended Data Fig. 4, Supplementary Information). This analysis revealed 248 unique HLA-I-associated peptides and 35 unique HLA-II-associated peptides (Supplementary Tables 3–5). The clustering of HLA-I peptides of 8–13 amino acids in length that were identified from each patient showed a reduced amino acid complexity of the peptides (as expected for HLA-I peptides), and matched the motifs of the HLA alleles of the patient (Supplementary Fig. 1). The length distribution of the identified peptides was consistent with the expected length of HLA-I and HLA-II peptides (Extended Data Fig. 5,

Supplementary Fig. 2). The tandem mass spectra of all of the identified peptides is shown in Supplementary Fig. 3. We validated 48 of the identified peptides by comparing their tandem mass spectra to that of synthetic peptides (Supplementary Fig. 3).

In total, we obtained between 0 and 16 HLA-I and HLA-II peptides from each metastasis, and between 0 and 45 different HLA-I and HLA-II peptides from each species of bacteria (Fig. 2a). We identified 11 recurrent HLA-I-associated peptides that were derived from the bacteria *Fusobacterium nucleatum*, *Staphylococcus aureus* and *Staphylococcus capitis*. Five of the peptides appeared in different metastases from the same patient, and six appeared in different patients (Fig. 2b, Supplementary Table 7). As expected, recurrent peptides shared by patients were predicted to bind to HLA alleles shared by these patients or to contain the same HLA binding motif.

The percentage of bacterial peptides per sample that matched the *HLA-C*03:04*, *HLA-C*03:03* and *HLA-A*02:01* alleles was higher in most samples compared to the percentage of human peptides that matched the same alleles (Fig. 2c, Supplementary Fig. 4). The percentage of bacterial peptides was higher in *HLA-C*03:04* and *HLA-C*03:03* even when considering the RNA expression of the HLA molecules (Supplementary Fig. 5). When compared to the repertoire of the human-derived HLA peptidome, bacteria-derived peptides were significantly more hydrophobic (unpaired two-sample Wilcoxon test, $P = 3.71 \times 10^{-46}$) (Supplementary Figs. 5, 6, Supplementary Table 8), a property that might make these peptides preferable for antigen presentation¹⁶ and recognition by T cells¹⁷. Importantly, we found that bacterial proteomes contained a significantly higher fraction of hydrophobic amino acids compared to the human proteome (Extended Data Fig. 6) and that the *HLA-C*03:04*, *HLA-C*03:03* and *HLA-A*02:01* alleles bind to more hydrophobic peptides (Extended Data Fig. 7), which provides an explanation for the higher frequency of bacterial peptides that are presented by these alleles.

As we used bulk melanoma tumours for the HLA peptidomics assay, we were unable to determine whether the bacterial HLA peptides were derived from the melanoma cells or antigen-presenting cells. To address this, we digested two melanoma tumours to recover single cells, which we subsequently separated into two populations on the basis of their CD45 marker: immune cells (CD45⁺ cells) and nonimmune cells (CD45⁻ cells, which are primarily melanoma cells) (tumours HG38 and 422 in Supplementary Fig. 7). As previously reported, the melanoma cells in our study not only expressed HLA-I but also HLA-II molecules^{18–24} (Supplementary Fig. 8). We subjected the different populations isolated from tumour 422 to HLA peptidomics (Supplementary Table 9), which enabled us to identify both HLA-I and HLA-II peptides in the CD45⁻ population (with some overlap between CD45⁻ and CD45⁺ populations) (Fig. 3a). Although it has previously been shown that bacteria can enter antigen-presenting cells and be presented by HLA molecules^{25–28}, we wanted to assess whether the bacteria that we identified in our cohort are also presented by antigen-presenting cells. To test this, we cocultured antigen-presenting cells, the B cell line IHW01070 and THP1 cells that were differentiated into macrophages using phorbol-12-myristate-13-acetate^{29,30} with *F. nucleatum* and performed HLA peptidomics (Supplementary Fig. 8a–c, Supplementary Table 10). To ensure that the HLA peptides that we identify are indeed HLA ligands, we cocultured the HLA-I-null B cell line 721.221, with

and without overexpression of the *HLA-A*01:01* allele, with *F. nucleatum* and performed HLA peptidomics (Supplementary Fig. 8d). As expected, we observed HLA-A*01:01 bacterial peptides only in the cells that overexpressed the HLA-A*01:01 allele; none of these peptides were identified in the 721,221 cells that did not overexpress HLA-A*01:01. The HLA-II presentation did not differ between the two lines (Supplementary Table 11).

Entry of bacteria into melanoma cells

To thoroughly evaluate whether melanoma cells can present bacterial HLA peptides, we first confirmed that the species of bacteria that we identified in our cohort of patients with melanoma are capable of entering melanoma cells. To this end, we cocultured low-passage cell lines derived from the same melanoma tumours with representative intracellular bacteria (bacteria for which we identified HLA peptides), and assessed the entry of bacteria into the cells using several orthogonal methodologies. We performed a gentamicin protection assay³¹ on cocultures of 51AL and 55A3 melanoma cells with aerobically grown *Staphylococcus caprae* and anaerobically grown *Actinomyces odontolyticus*, and detected colony-forming units at both 4 and 8 h after infection (Extended Data Fig. 8a, b). As a control, we used *Lactobacillus animalis* grown under aerobic conditions and *Lactobacillus plantarum* grown under anaerobic conditions as representative species of bacteria. Both of these species are expected to possess weaker cell-invasion phenotypes and, using the gentamicin protection assay, we observed less invasion of these bacteria into 51AL and 55A3 cells compared to *S. caprae* and *A. odontolyticus* (Extended Data Fig. 8a, b, Supplementary Table 12).

We further verified the presence of bacteria within melanoma cells by performing immunofluorescence staining of 51AL and 55A3 GFP-expressing cells that were cocultured with *S. caprae*, using anti-lipoteichoic acid (Extended Data Fig. 9). Alternatively, we used copper-free click chemistry to label anaerobically grown *F. nucleatum* and *A. odontolyticus* with DIBO–Alexa Fluor 488³², and cocultured these bacteria with 51AL and 55A3 cells stained with anti-HLA-I (Fig. 3b, Extended Data Fig. 10). The images presented in Extended Data Figs. 9, 10 are derived from the centre of z-stack images of the cells cocultured with the bacteria (Supplementary Video 1–8). We also constructed 3D representations of the z-stack images (Supplementary Videos 10–18). We detected all three species of bacteria in both of the melanoma cell lines. We performed correlative light and electron microscopy (CLEM) analysis of cells cocultured with *F. nucleatum*, *A. odontolyticus* and *S. caprae* and stained with anti-lipoteichoic acid, which confirmed that the bacteria indeed entered the melanoma cells (Fig. 3c, Extended Data Fig. 11).

Bacterial presentation is specific

We assessed whether we could use the bacteria that were identified in tumours to identify the bacteria-derived HLA-bound peptides of the corresponding patient in cocultures of 51AL cells with *F. nucleatum*, and 55A3 cells with *S. caprae*. Subjecting these cocultures to HLA peptidomics revealed 105 HLA-I and 130 HLA-II peptides in cells cocultured with bacteria (Supplementary Table 13). Knockout of the β -microglobulin (*B2M*) or class II major histocompatibility complex transactivator (*CIITA*) genes in the cells using genome editing reduced the expression of HLA-I and HLA-II, respectively (Supplementary Fig.

8e), and led to a decrease in the number of HLA-I- and HLA-II-associated peptides after coculture with bacteria compared to a scrambled non-targeting single-guide RNA control (Fig. 3d). Furthermore, coculturing the cells with *L. plantarum* or *L. animalis*—which are less cell-invasive than *F. nucleatum* and *S. caprae* (Extended Data Fig. 8a, b)—yielded fewer HLA-I- and HLA-II-bound peptides (Fig. 3d). To control for nonspecific contamination in the HLA immune purification process, we performed HLA peptidomics on bacterial pellets using the same amount as was used for the cocultures, and observed none or few peptides—none of which were identified in the coculture samples (Supplementary Table 14). Both the HLA-knockout and less-invasive-bacteria controls suggest that the bacterial peptides that we identified are HLA ligands, as their presence was strongly reduced in both control experiments.

Tumour-isolated bacterial presentation

To further validate the results of our cultured cell studies, we isolated bacteria from melanoma tumours and checked whether the specific isolated strains can invade melanoma cells. Our gentamicin protection assay and immunofluorescence staining of the isolated bacteria from tumours (*S. capitis* from tumour 58A and *Staphylococcus succinus* from tumour 261MS) revealed that both of these bacteria invade melanoma cells (Extended Data Figs. 8c, d, 9, Supplementary Videos 1–18). We cocultured *S. capitis* from tumour 58A with a melanoma-derived cell line produced from this tumour and performed HLA peptidomics on the cells, which led to the identification of 13 HLA-I and 11 HLA-II bacterial peptides (Supplementary Table 15).

For our HLA peptidomics analyses, we used published proteomes (from UniProt³³) of the species of bacteria that we identified using 16S rRNA sequencing. We performed whole-genome sequencing of the bacteria isolated from the tumours and of the commercial bacteria used for the cocultures. We then constructed a proteome database from the whole-genome sequencing data and analysed the HLA peptidomics data in a similar manner to that from the UniProt database. The bacterial peptides identified in each of the experiments are shown in the peptide tables provided in Supplementary Tables 9–11, 13–15. The overlap in the identified peptides between the two analyses was high in most experiments, which suggests that the use of published proteomes—even if they are not of the specific strain that was identified in the tumour—can give a sufficiently close peptide identification (Supplementary Table 16). This emphasizes that—although there is a clear advantage in using a database derived from the specific strain of bacteria isolated from the tumour to increase specificity—using a combination of 16S rRNA sequencing and the UniProt database avoids the need to isolate and sequence the bacteria from the tumour (which may be very difficult for many bacteria), thus increasing the number of bacterial HLA-bound peptides that it is possible to identify. A schematic overview of bacteria sequencing stages that we applied before the HLA-I and HLA-II peptidomics workflow is presented in Supplementary Fig. 9.

Recurrently presented bacterial peptides

We were able to identify two HLA-I peptides in the HLA peptidome of the cocultured cell lines that were also identified in the corresponding tumour samples: GLDLGTLTY,

which was identified in metastasis 27 of patient 51, and GVDLGTLTY, which was identified in metastasis 51BR of the same patient—these peptides were noted also in 51AL cells that were cocultured with *F. nucleatum*. We identified the peptide ETTLVVTEY both in tumour 27 and in 721.221 cells that were cocultured with *F. nucleatum*. We found two additional peptides, one in the 51AL cell line (peptide IASDVSAIL) and one in macrophages and 721.221 cells (peptide NSIKIIGDKTDLY) cocultured with *F. nucleatum*. This demonstrates that some bacterial peptides can be presented by both melanoma cells and antigen-presenting cells, similar to what we observed in tumour 422 (in which we identified two peptides (EELSRQNL and LSNAKSLEL) that were present in both the CD45⁺ and CD45⁻ cell fractions) (Supplementary Table 9).

In addition, we identified 39 bacterial protein groups from which we received multiple bacterial peptides (2–15 different peptides per gene) (Supplementary Table 17). The bacterial protein with the corresponding highest number of different peptides was pyruvate-ferredoxin (flavodoxin) oxidoreductase from *F. nucleatum* (Fig. 3e). Twelve of these peptides were HLA-I peptides, and three were HLA-II peptides. Most of the peptides that we identified were from the 51AL cell line cocultured with *F. nucleatum*, although we also identified some in macrophages and the B cell line 721.221 overexpressing HLA-A*01:01. One peptide was found in both the 51AL melanoma cells and the 721.221 B cells, and two different pairs of peptides had an overlapping sequence. Other genes showed a higher number of overlapping peptides, which created presentation ‘hot spots’. Among these genes was ATP synthase subunit- δ from *S. caprae* (from which 10 different HLA-II peptides were derived, protein group 9) (Supplementary Table 17).

Bacterial peptides are immunogenic

As recent studies have reported that tumour microbiota can affect responses to therapy by modulating the immune system^{12–14} and as we identified antigens derived from bacteria on tumour HLA-I and HLA-II molecules, we hypothesized that intratumoural bacteria may not only shape the immune tumour microenvironment but also affect T cell immune reactivity. To evaluate this hypothesis, we analysed the reactivity of tumour-infiltrating lymphocytes (TILs) isolated from the analysed tumours towards the HLA-I-bound bacterial antigens that we identified. Specifically, we assessed the reactivity of bacterial peptides identified in the tumour that were derived from bacteria (such as *F. nucleatum* and *S. aureus*) that are known to negatively affect the host and immune system response by mechanisms other than peptide presentation^{34–39}, as well as the recurrently presented bacterial peptides (Supplementary Table 7), owing to their biological relevance and potential prevalence in the population. We pulsed synthetic peptides onto Epstein–Barr-virus-transformed B cells that expressed the tumour-matched HLA alleles. We then cocultured the loaded B cells with the autologous TILs, and analysed TIL reactivity by using flow cytometry to detect IFN γ -secreting TILs (Extended Data Fig. 12). We detected an increase in IFN γ -secreting TILs of twofold or more for eight different bacterial peptides, compared to control B cells that were not loaded with these peptides (Fig. 4, Extended Data Fig. 12, Supplementary Fig. 10). These included seven immunogenic peptides that were derived from patient 51 and one peptide that was derived from patient 55. Of the reactive antigens, one peptide (VLTDTYLTL) was identified in two different metastases of patient 51, two peptides

(ITELNSPVL and SLTDKISII in patients 51 and 92) were identified in two different patients, one peptide was also identified in the matching in vitro coculture of cells from the same patient (GVDLGTLTY in patient 51), and one peptide was identified from two potential species of bacteria that were identified in the same metastasis (ALSDMSLAL was derived from *Sphingomonas dokdonensis* or *Sphingomonas melonis*; both of these species were observed in metastasis 55B3). We confirmed these results by flow cytometry analysis of the CD69 T cell reactivity marker (Fig. 4, Supplementary Fig. 11).

Discussion

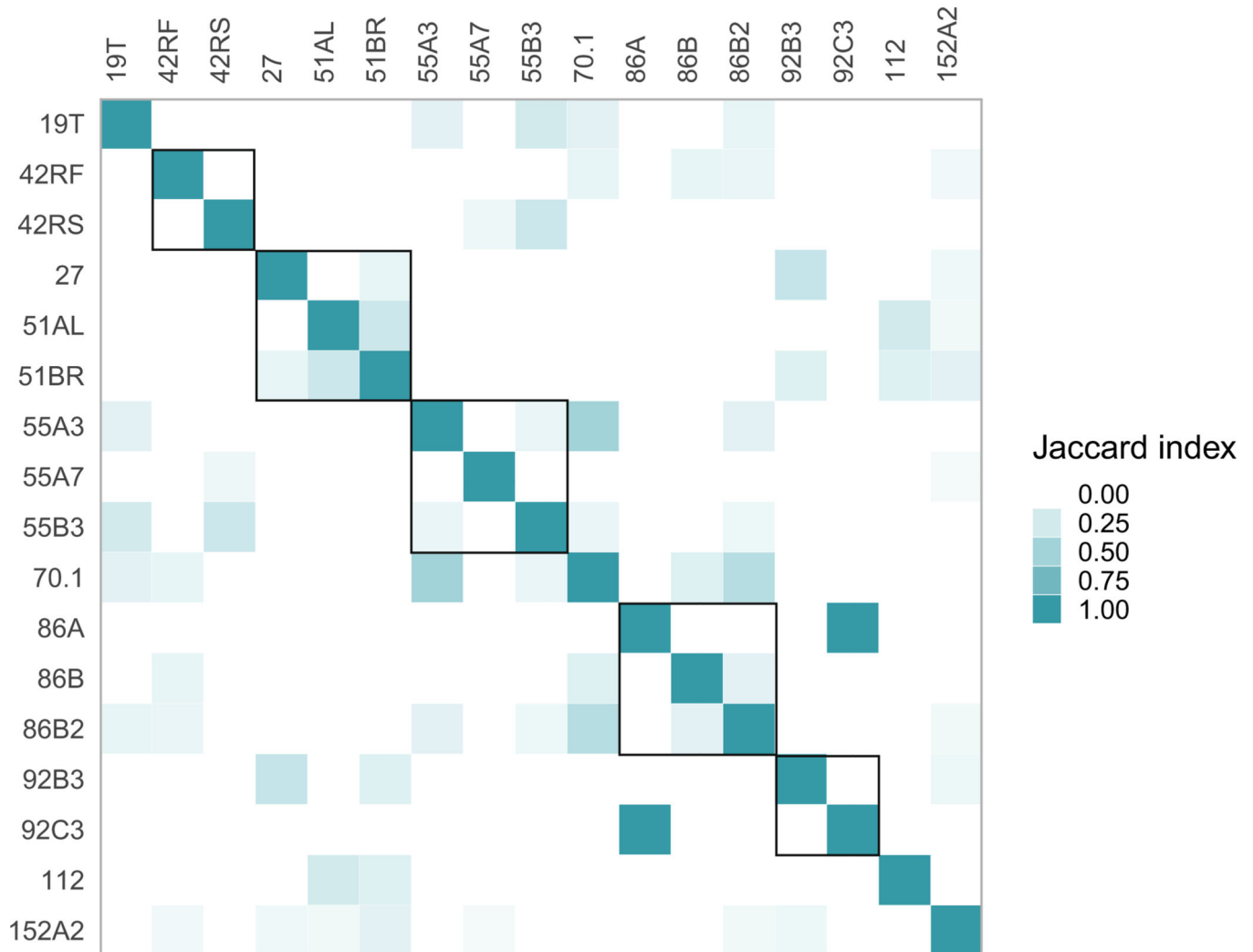
In summary, here we demonstrate the presence of HLA peptidomic signatures derived from bacteria in tumours from patients with melanoma, and characterize the species of bacteria involved. We demonstrate that bacterial HLA-I and HLA-II peptides can be presented by both antigen-presenting cells (as has previously been shown^{25–28}) and by melanoma cells, by applying HLA peptidomics to a tumour sample that was separated into CD45⁻ and CD45⁺ populations as well as to antigen-presenting cells and melanoma cell lines that were cocultured with bacteria. Among the peptides that we identified were ones shared by different metastases from the same patient or by metastases from different patients. Some of these recurrent antigens bind to HLA alleles that are highly prevalent in the melanoma cohort of The Cancer Genome Atlas (Supplementary Fig. 12). Moreover, a few of the identified peptides that elicited an immune response from the autologous TILs of the patient are recurrent peptides. As the bacterial antigens are non-self, they could serve as targets for immunotherapy. The selection of the species of bacteria for immunotherapy should be carefully considered, and should favour species of bacteria that are known to negatively affect the response of the host and immune systems (rather than more ‘protective’ bacteria^{40–42}). When focusing on the presented bacterial proteins, we observed genes that have overlapping peptides, which create presentation hot spots that possibly indicate the existence of protein domains and may further assist investigations into selecting the targets for immunotherapy.

Despite its strengths, our study has several shortcomings. Specifically, the resolution needed for the identification of specific species of bacteria is limited, owing to the high similarity of the 16S sequences between some species of bacteria. We also acknowledge the limitations of identifying bacterial peptides using HLA peptidomics, as it relies on peptide intensity: this may sometimes be below the detection level, especially when the amount of sample material is limited. This also explains the higher number of identified bacterial peptides in cell lines, as the amount of cells used was higher in these experiments. Additional studies that assess intratumoral bacteria-derived peptides—in combination with clinical and therapeutic information on large-scale cohorts—are likely to shed further light on the clinical role of bacterial peptides and to provide a higher-resolution view of the fundamental trends outlined in this Article.

Our comprehensive analysis of the intratumour microbiota in melanoma and the HLA peptides derived from these bacteria demonstrates that the bacteria that colonize melanoma tumours can enter melanoma cells, and that their peptides can be presented by the HLA-I and HLA-II molecules of melanoma tumours. As both classes of HLA molecule have

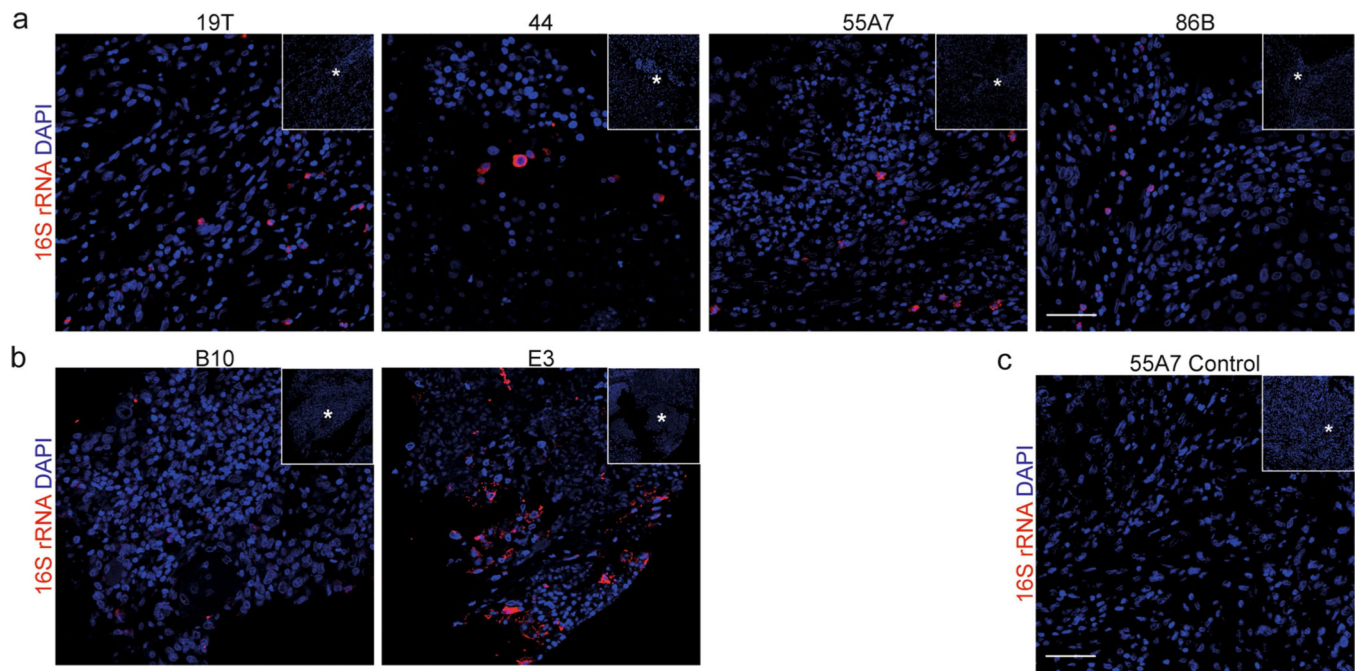
previously been shown to have a central role in CD8⁺ and CD4⁺ T cell immunity^{43–49}, they are expected ultimately to modulate immune function. Finally, as the gut and tumour microbiota can affect the survival of patients with cancer and their responses to therapy^{8,14,40–42,50}, our findings are of particular relevance, as they suggest that a mechanism that involves antigen presentation may underlie these effects.

Extended Data



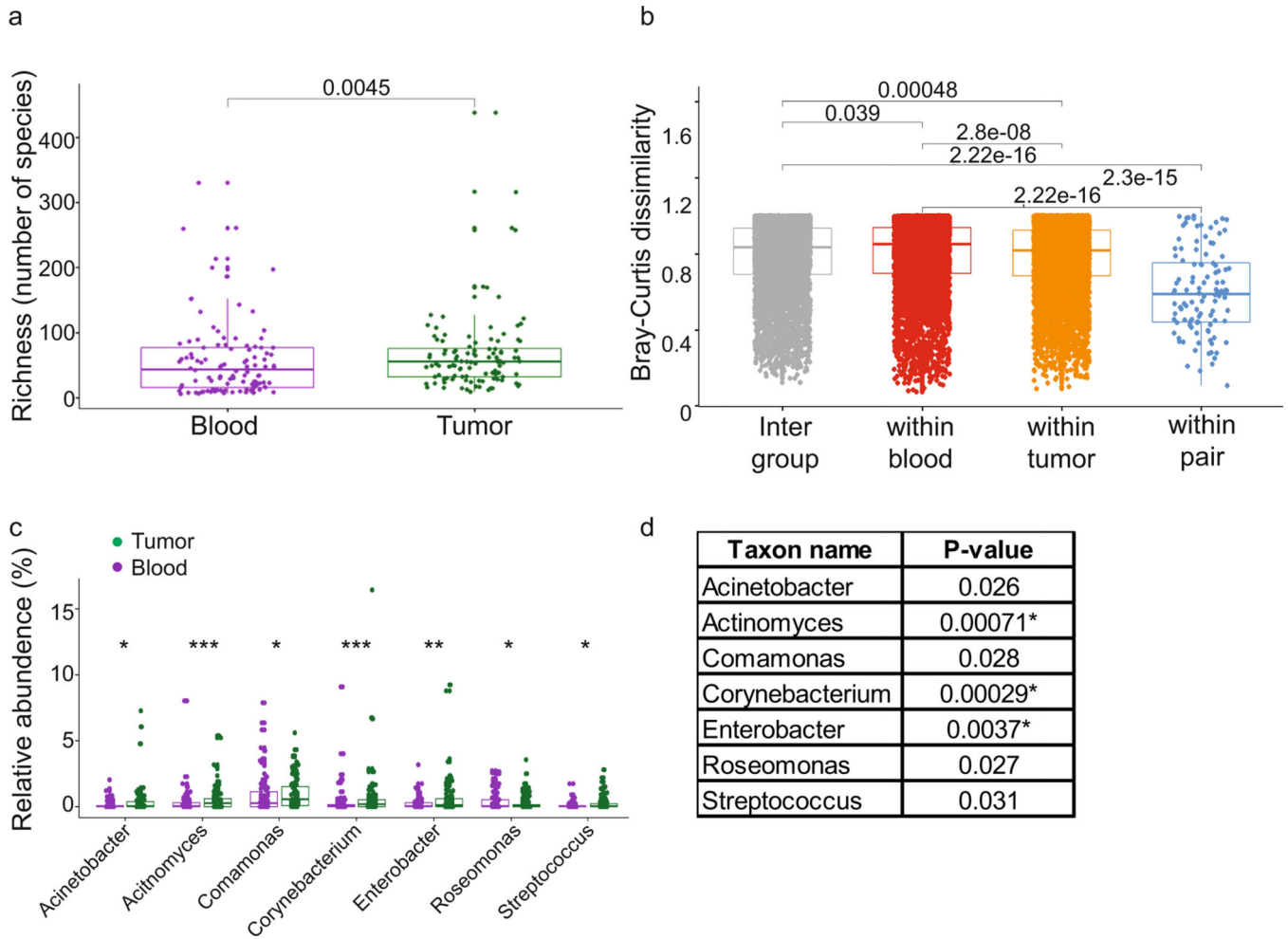
Extended Data Fig. 1 |. Similarity of bacterial composition between metastases.

A Jaccard index was calculated to determine the similarity between the bacterial composition of the different metastases on the species level. Colour code indicates the Jaccard index. The highest similarity was observed between metastases from the same patient, but metastases of different patients also showed similarity. Black boxes indicate tumour samples taken from the same patient.



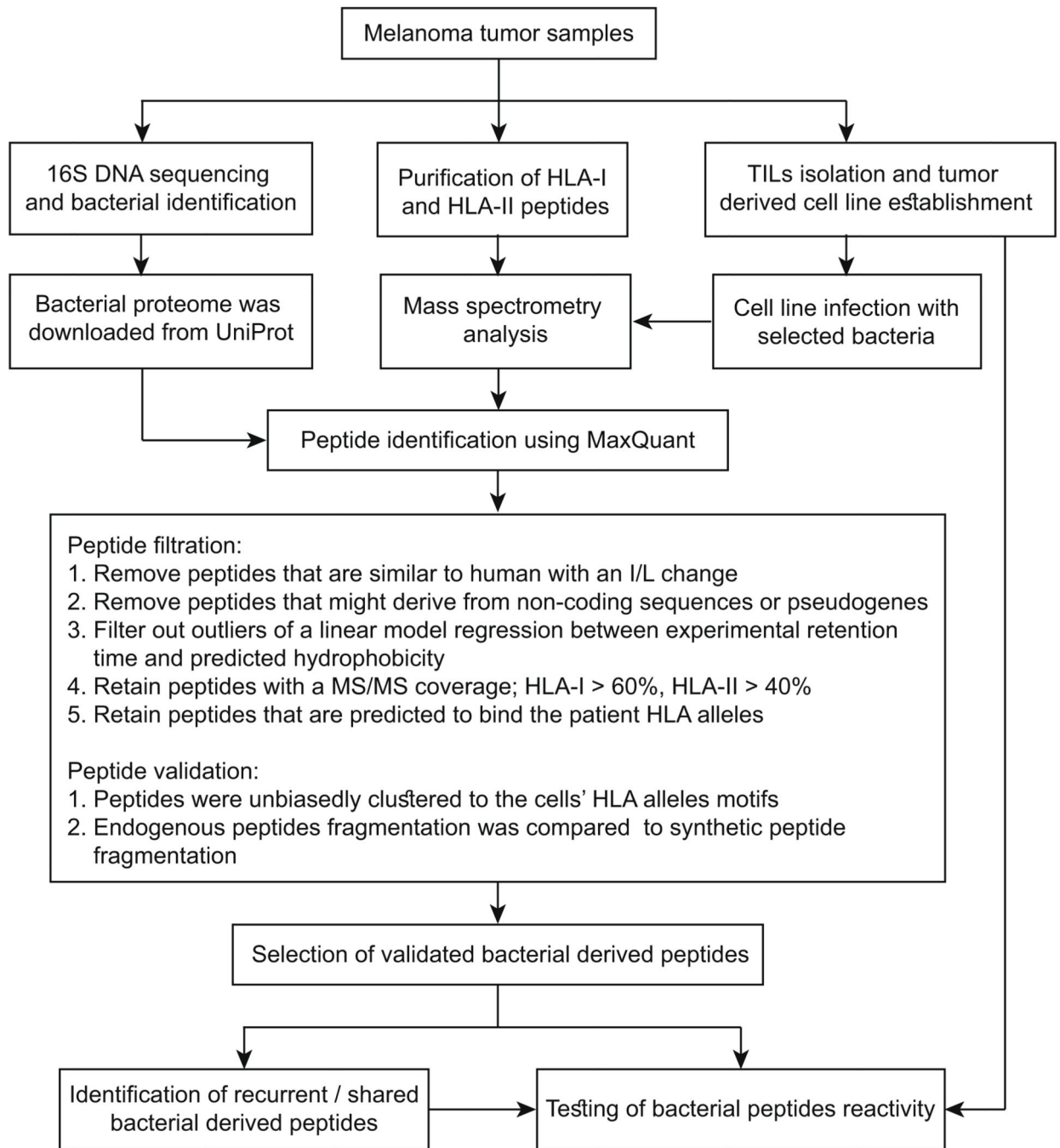
Extended Data Fig. 2 | Visualization of bacterial 16S rRNA in tissue sections from melanoma tumours.

a, 16S rRNA fluorescence in situ hybridization (FISH) staining of tissue sections from melanoma tumours using pan-bacteria EUB338 probe (red) and DAPI (blue). **b**, 16S rRNA FISH staining of tissue microarray sections of melanoma tumours (red) and DAPI (blue). Slice name indicates the position in the tissue microarray. Images are presented at 20 \times magnification. Scale bars, 100 μ m. **c**, Representative control using 16S FISH nonspecific control probe. Asterisks mark the region that was selected for higher magnification. Figures are representative of at least three independent experiments.

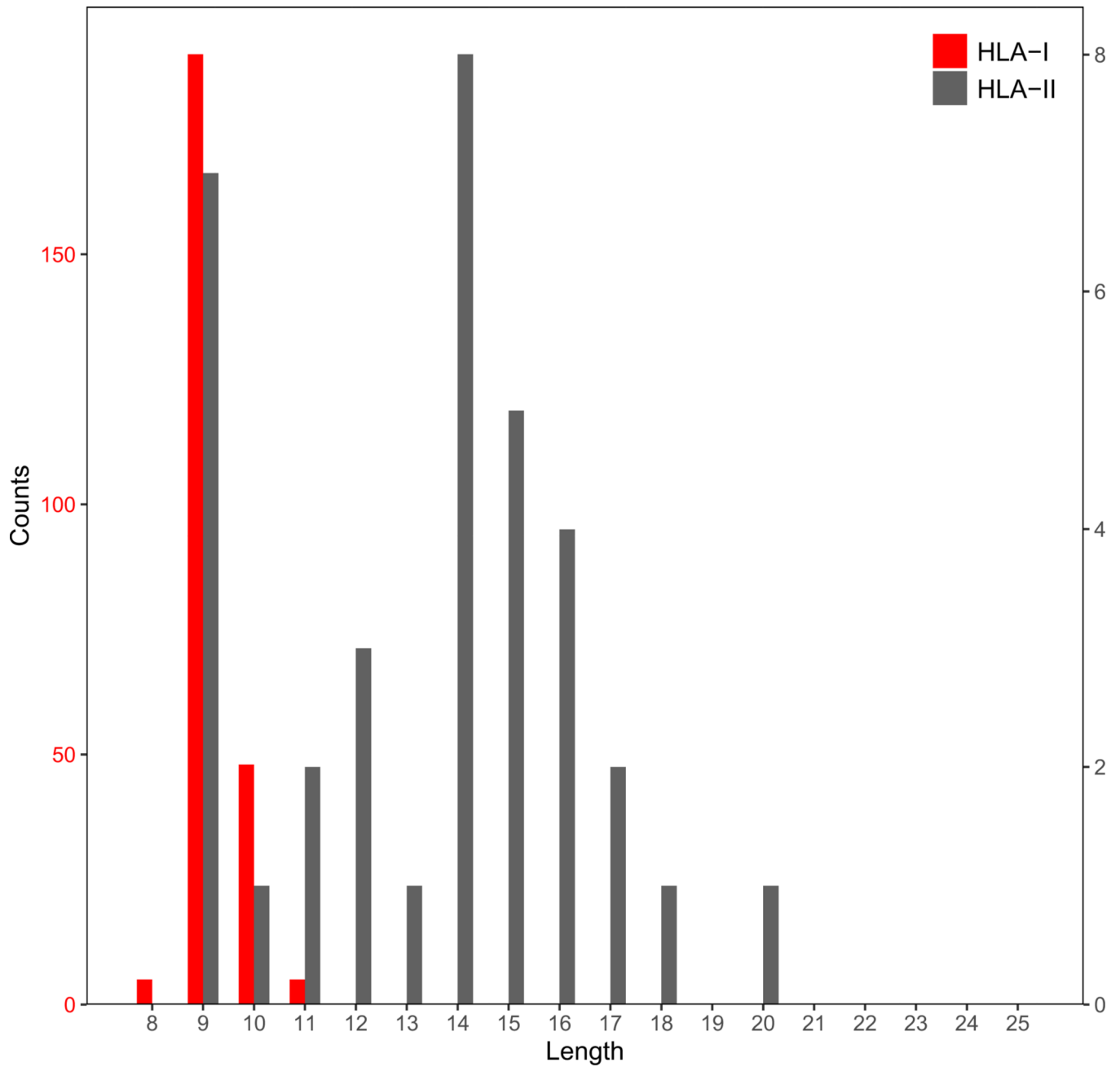


Extended Data Fig. 3 | Taxonomic analysis of 108 whole-genome-sequenced melanoma samples identify a bacterial composition similar to that found in our tumour cohort.

a, Alpha diversity, measured as the number of observed species in a tumour (green) or blood (purple) sample. *P* values from paired two-tailed Wilcoxon test between tumour and blood taxonomic diversity. **b**, Microbiome similarity within and between groups. Bray–Curtis dissimilarity measured between each pair of samples, then stratified into four groups. *P* values from two-tailed Wilcoxon test. **c**, Comparison of the relative abundance between tumour samples and associated blood samples. *P* values from paired two-tailed Wilcoxon test between tumour and blood taxonomic abundance. ****P* < 0.001, ***P* < 0.01, **P* < 0.05. **d**, List of groups of bacteria that are more abundant in the tumour samples plotted in **c**. *P* values from paired two-tailed Wilcoxon test between tumour and blood taxonomic abundance. *P* values with asterisks survived multiple hypothesis correction (false-discovery rate of 5%). In the box plots, the centre lines represent the medians, the boxes represent the range between the 25th and 75th percentile, and the whiskers represent the range between the smallest and largest data point.

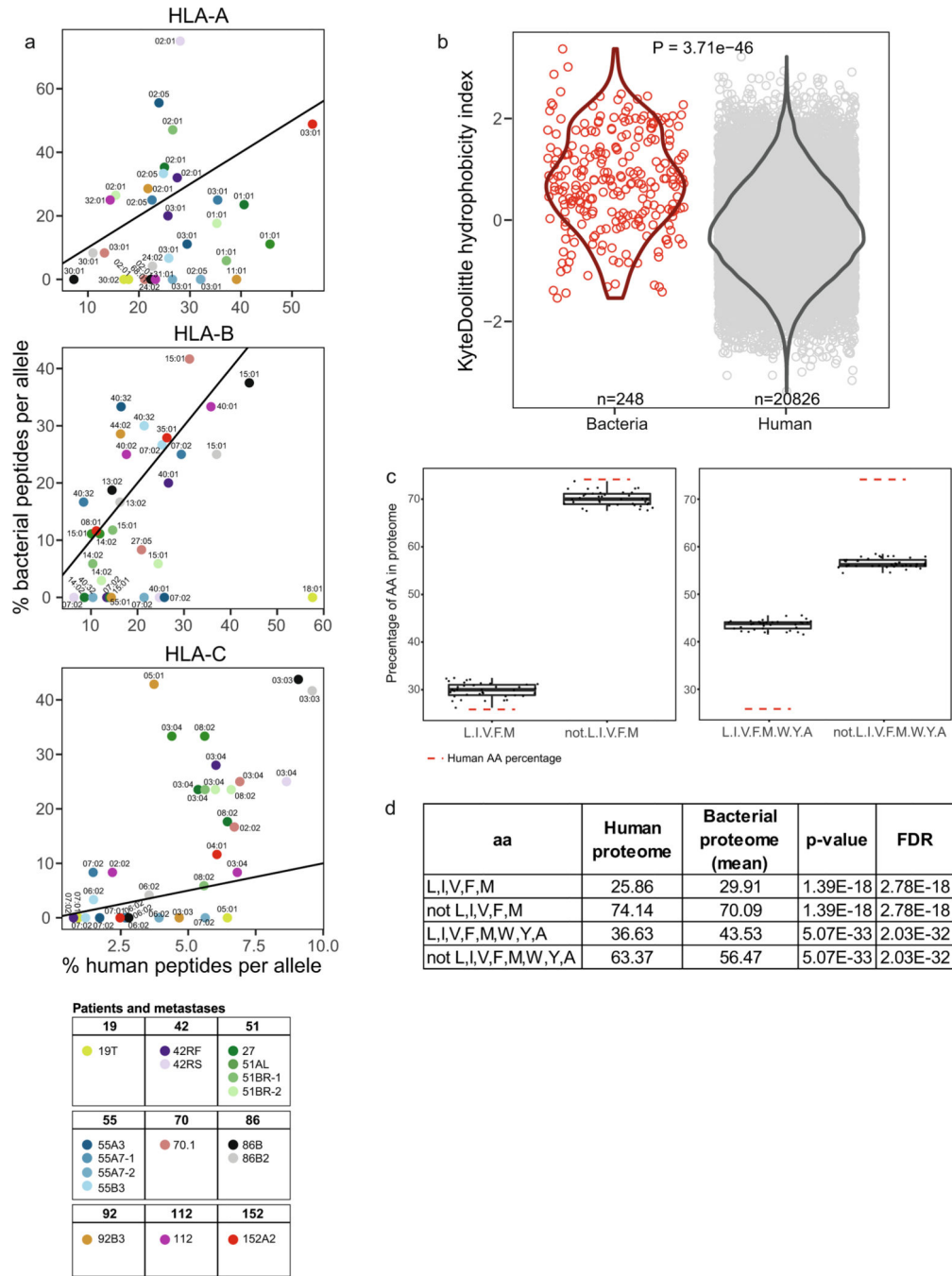


Extended Data Fig. 4 | Pipeline for the identification of bacteria -derived HLA peptides. Tumour samples were 16S-sequenced to identify their bacterial composition and analysed using HLA peptidomics. Searching the data according to the bacteria resulted in the identification of bacteria-derived peptides. Peptides were filtered according to their identification quality and ability to bind to the HLA alleles of the patient. Selected bacterial peptides were then tested for reactivity. Identification of bacterial peptide presentation was validated by a HLA peptidomics analysis of cell lines cocultured with bacteria.



Extended Data Fig. 5 |. Length distribution of bacteria-derived peptides.

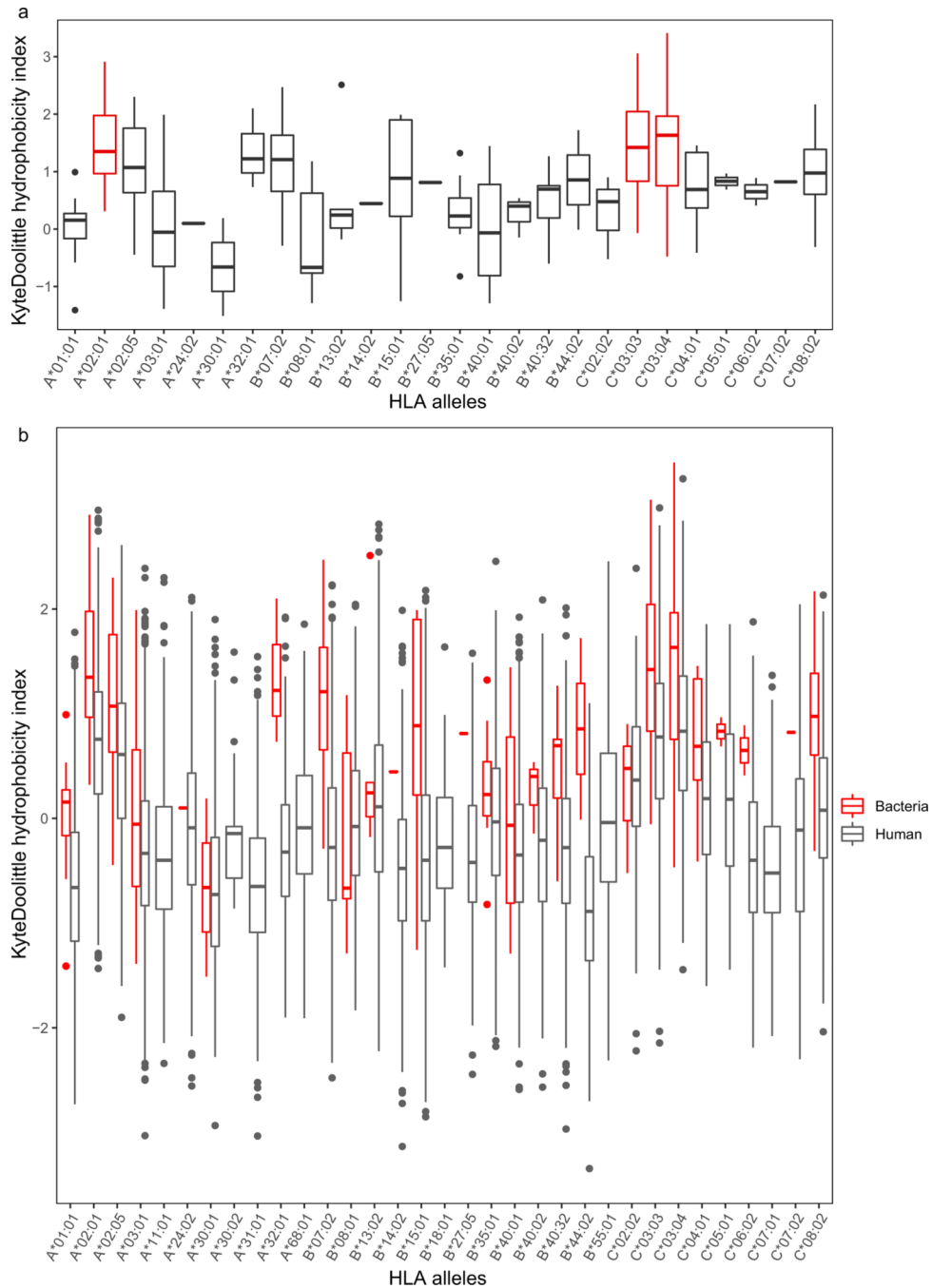
The length distribution of bacteria-derived peptides is similar to the expected length of HLA-I and HLA-II peptides.



Extended Data Fig. 6 | Bacterial proteomes contain a higher amount of hydrophobic amino acids compared to the human proteome.

a, For each metastasis, the percentages of bacterial and human peptides that match each *HLA-A*, *HLA-B* and *HLA-C* allele of the patient is indicated. The allele with the best per cent rank binding prediction by NetMHCpan was assigned to each peptide. **b**, Kyte-Doolittle hydrophobicity index was calculated for bacterial and human peptides. The hydrophobicity of HLA-I bacterial peptides is higher than that of human-derived peptides (indicated *P* value is from an unpaired two-sample Wilcoxon test). **c**, The percentage of hydrophobic

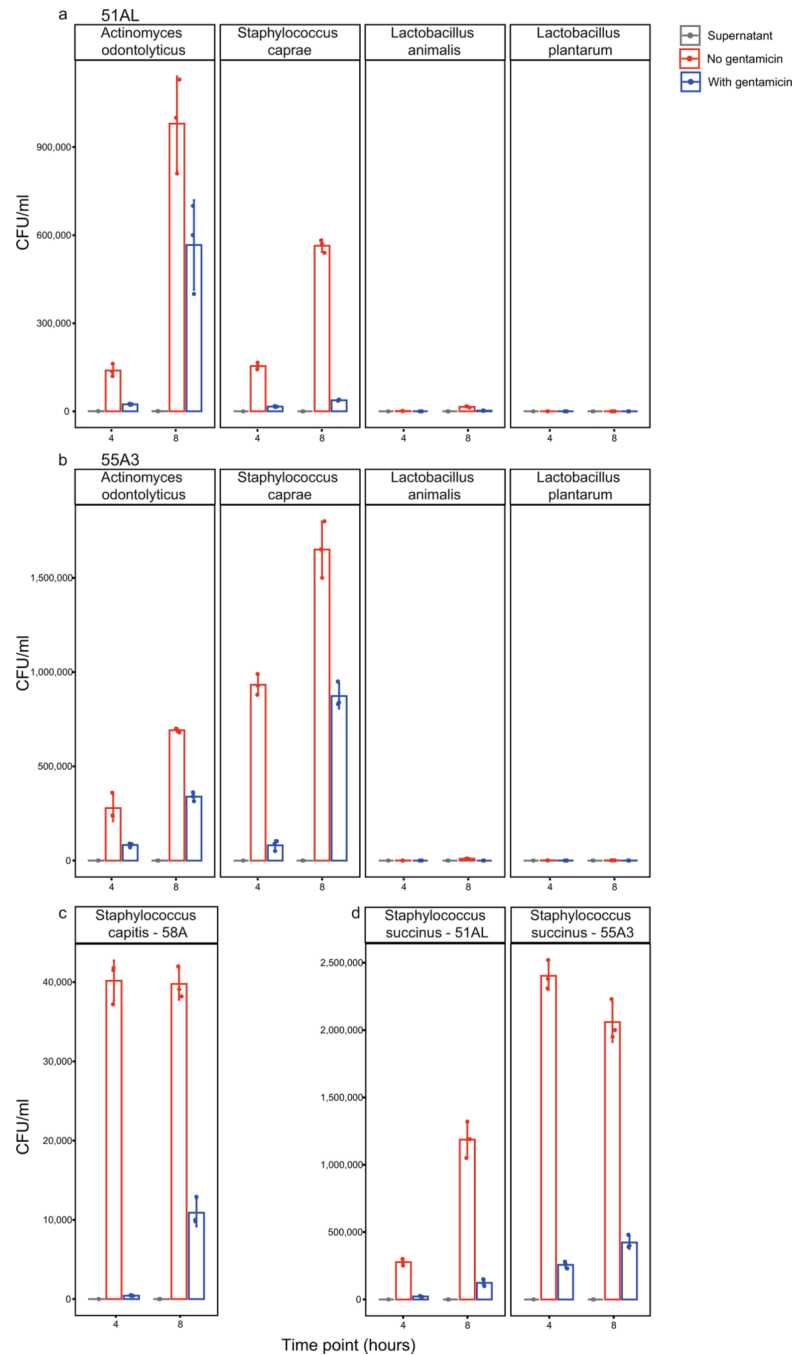
and nonhydrophobic amino acids was calculated for bacterial proteomes and the human proteome. Two groupings were used for selecting hydrophobic amino acids: L, I, V, F and M, or L, I, V, F, M, W, Y and A. The percentage of hydrophobic and nonhydrophobic amino acids from bacterial proteomes is plotted in the box plot. In the box plots, the centre lines represent the medians, the boxes represent the range between the 25th and 75th percentile, and the whiskers represent the range between the smallest and largest data point. The percentages representing the human proteome are marked by a red dashed line. **d**, Two-sided Student's t-test comparing the percentage of hydrophobic and non-hydrophobic amino acids between bacterial proteomes and the human proteome. The *P* values and false-discovery rates are indicated in the table.



Extended Data Fig. 7 | Hydrophobicity of bacterial and human peptides per allele.

Kyte–Doolittle hydrophobicity index was calculated for bacterial and human peptides and plotted in a box plot for each HLA allele. In the box plots, the centre lines represent the medians, the boxes represent the range between the 25th and 75th percentile, and the whiskers represent the range between the smallest and largest data point. **a**, The hydrophobicity of the bacterial peptides that bind to the HLA-C*03:04, HLA-C*03:03 and—to a lesser extent—HLA-A*02:01 was higher compared to the hydrophobicity of other alleles (marked in red). Additional alleles also show this trend, but they were derived from

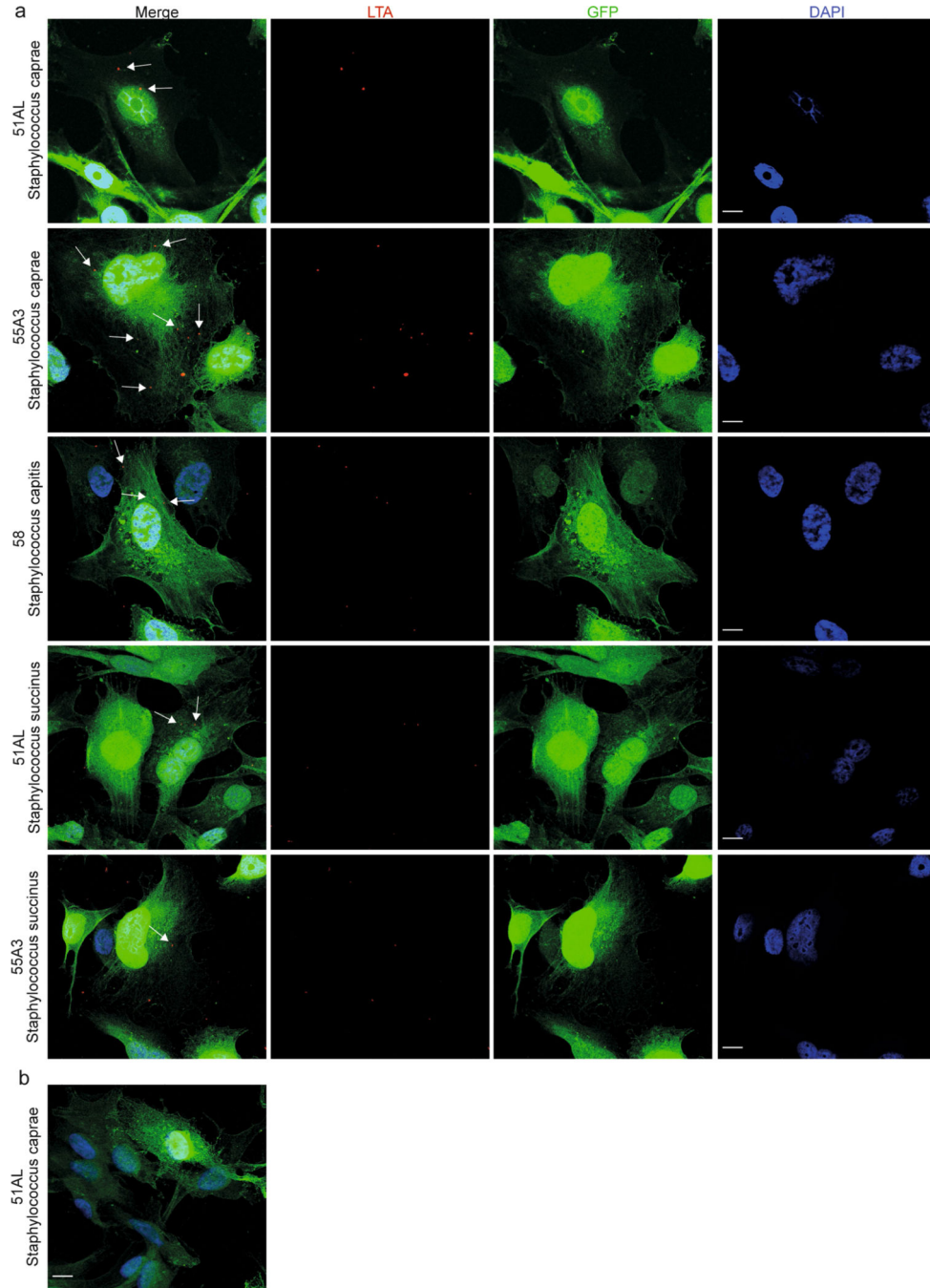
a lower number of tumour samples and therefore are not indicated. **b**, Bacterial peptides are marked in red and human peptides are marked in grey.



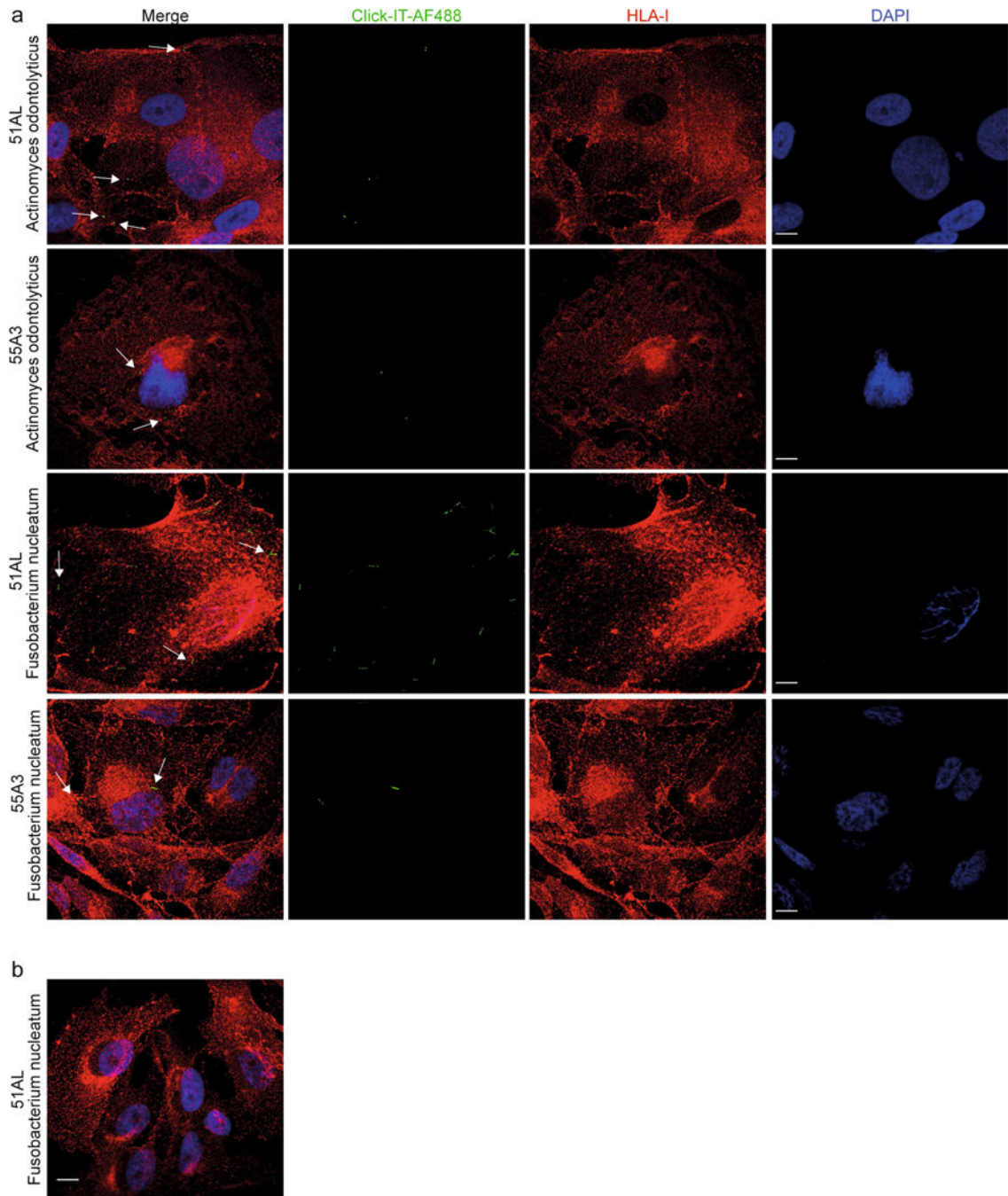
Extended Data Fig. 8 |. Gentamicin assay demonstrating the entry of bacteria into melanoma cells.

a, b, Colony-forming units (CFU) of *A. odontolyticus* and *S. caprae* after coculture with 51AL and 55A3 melanoma cells. Less-invasive bacteria (*L. animalis* and *L. plantarum*) were used as a control, and show lower CFU. **c, d**, CFU of *S. capitis* (**c**) (isolated from tumour 58) after coculture with 58A melanoma cells, or *S. succinus* (**d**) (isolated from tumour Me1261)

after coculture with 51AL or 55A3 cells. Cells were cultured with the indicated bacteria for 4 and 8 h. ‘Supernatant’ refers to the CFU of medium taken from samples incubated with gentamicin after coculture with the bacteria (grey). ‘No gentamicin’ refers to samples not treated with gentamicin after the coculture (red). ‘With gentamicin’ refers to samples treated with gentamicin for 1 h after the coculture (blue). Bars represent the average of s.e. between biological replicates ($n = 3$). P values from Student’s t -test between the supernatant sample and the without gentamicin or with gentamicin samples; P values between *S. caprae* or *A. odontolyticus* with gentamicin to *L. animalis* or *L. plantarum* with gentamicin control samples are from a one-way analysis of variance followed by Tukey’s test, and are presented in Supplementary Table 4.



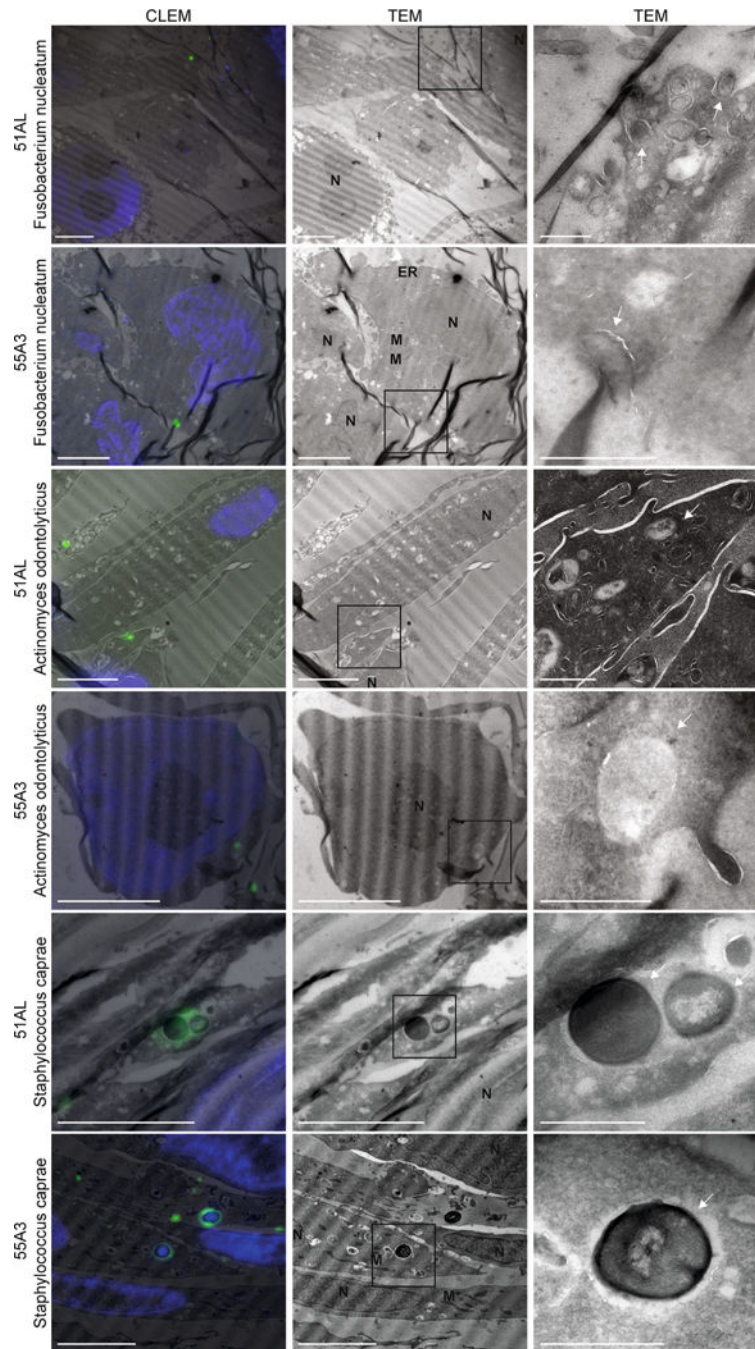
Extended Data Fig. 9 | Immunofluorescence staining of melanoma cells cocultured with aerobically grown bacteria, demonstrating the ability of the bacteria to enter the cells.
a, Melanoma cells expressing GFP (green) were cocultured with the aerobically grown bacteria *S. caprae*, *S. capitis* or *S. succinus* stained with antibacterial antibody lipoteichoic acid (LTA) (red); cell nuclei were stained with DAPI (blue). White arrows indicate the location of bacteria that entered the melanoma cells. **b**, A representative image of 51AL cells expressing GFP (green) cocultured with *S. caprae* and stained without a primary LTA antibody (red), to exclude nonspecific staining. Images are presented at 63× magnification. Scale bars, 10 μm. Figures are representative of at least three independent experiments.



Extended Data Fig. 10 | Immunofluorescence staining of melanoma cells cocultured with anaerobically grown bacteria, demonstrating the ability of the bacteria to enter the cells.

a, Melanoma cells stained an anti-HLA antibody (red) were cocultured with anaerobically grown bacteria *F. nucleatum* or *A. odontolyticus*. These bacteria were labelled with click chemistry (green). Cell nuclei were stained with DAPI (blue). White arrows indicate the location of bacteria that entered the melanoma cells. **b**, A representative image of 51AL cells stained with the anti-HLA antibody (red) cocultured with *F. nucleatum* that were not grown with D-GalNAz and labelled with Alexa Fluor F488 (green), to exclude nonspecific staining.

Images are presented at 63× magnification. Scale bars, 10 μm. Figures are representative of at least three independent experiments.



Extended Data Fig. 11 | CLEM images showing entry of bacteria into melanoma cells. *Fusobacterium nucleatum* was grown with D-GalNAz and then labelled with DIBO-Alexa Fluor 488. *Actinomyces odontolyticus* and *S. caprae* were incubated with an anti-LTA antibody, and then with an anti-mouse secondary antibody labelled with Alexa Fluor 488. The 51AL and 55A3 cell lines were coincubated with the bacteria for 8 h. Ultra-thin

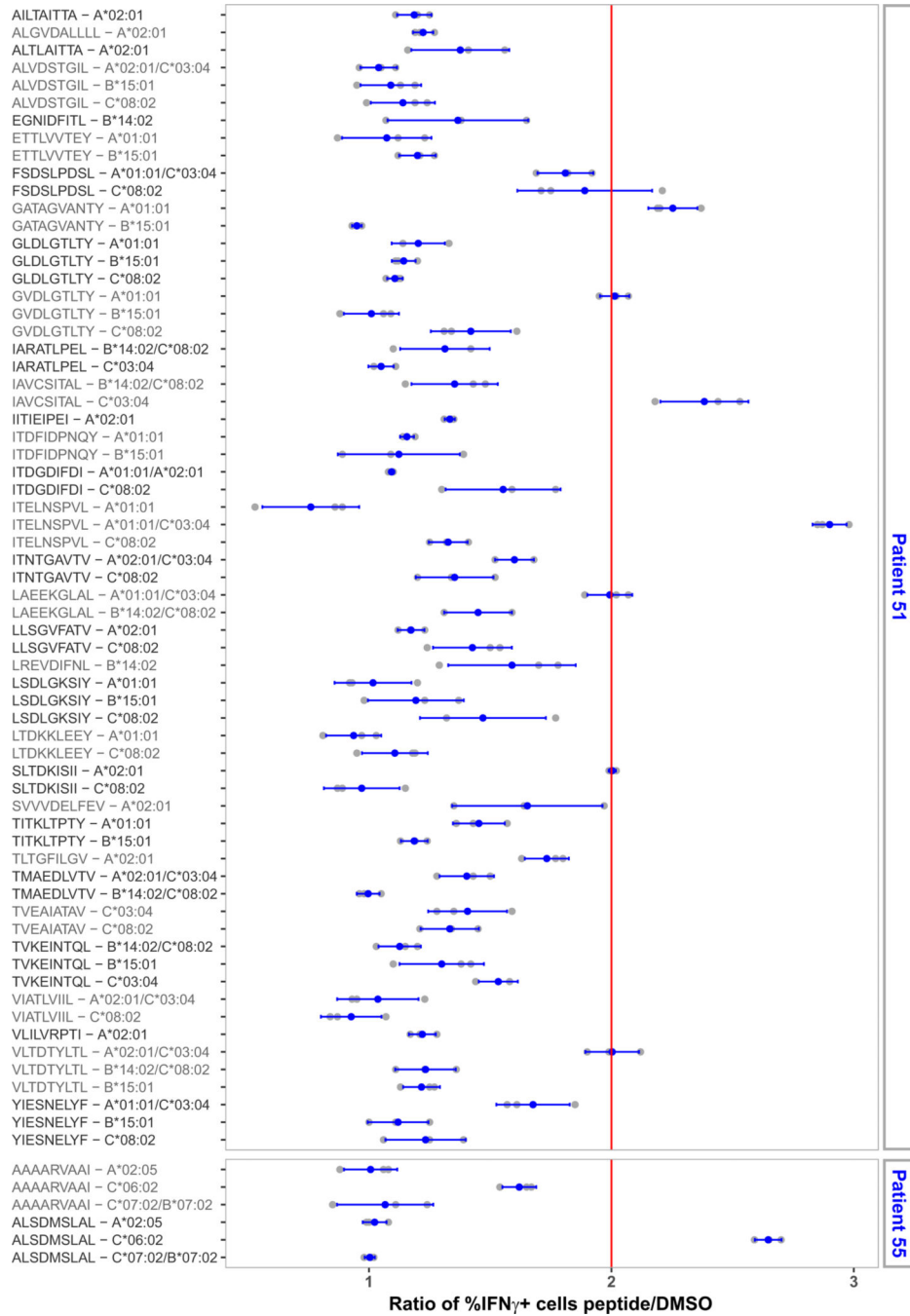
sections were analysed by fluorescence microscopy to identify the bacteria (green and blue labelling are for bacteria and nucleus, respectively), followed by transmission electron microscopy (TEM) of the same cells for high-resolution morphology. Left and middle panels show CLEM and TEM images, respectively. Scale bars, 5 μm . The right panel shows high-magnification TEM image of the area in the black box in the corresponding middle panel. Scale bars, 1 μm . Bacteria that entered the melanoma cell are indicated with a white arrow. N, nucleus; M, mitochondrion; ER, endoplasmic reticulum. Figures are representative of at least three independent experiments.

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Extended Data Fig. 12 | TIL reactivity towards bacteria-derived antigens.

Flow cytometry analysis of IFN γ -secreting TILs after coculture of 51AL and 55A3 TILs with B cells loaded with the indicated bacterial peptide. Each peptide was loaded on a different B cell that exhibited the HLA alleles to which the peptide was predicted to bind. TILs were stained with anti-IFN γ and anti-CD45 bifunctional antibody, which binds secreted IFN γ . The value indicates the ratio of IFN γ -secreting cells with the peptides to those with the DMSO control. Grey dots indicate the results of $n = 3$ biological replicates, and blue dots represent the average of replicates. Bars represent s.e. between replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

All raw mass spectrometry files, as well as human and bacteria proteomes and the MaxQuant version used for analysing the data, have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository, with the dataset identifier PXD022150. Raw sequence data of the whole-genome sequencing of isolated bacteria have been deposited in the NCBI Sequence Read Archive (SRA), under BioProject accession number PRJNA669827. Source data are provided with this paper.

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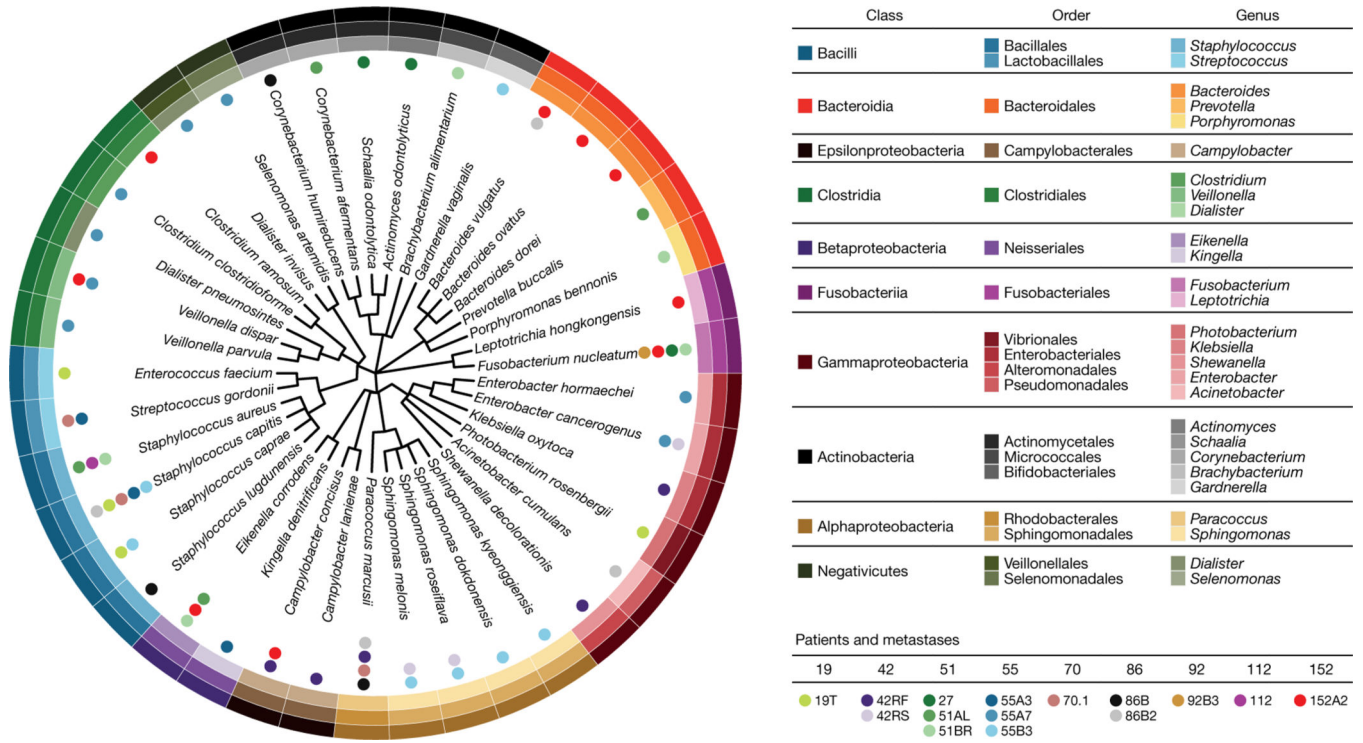


Fig. 1 | Identification of intratumoral bacteria in melanoma. Schematic phylogenetic tree of the bacterial composition of 17 melanoma metastases that originated from 9 patients. The analysis is based on rRNA 16S gene sequencing. The different colours and shades in the circles indicate the different classifications of bacteria at the genus (inner circle), order (middle circle) and class (outer circle) level. Each patient is colour-coded (as in the index), and different metastases from the same patient are depicted in different shades of the same colour.

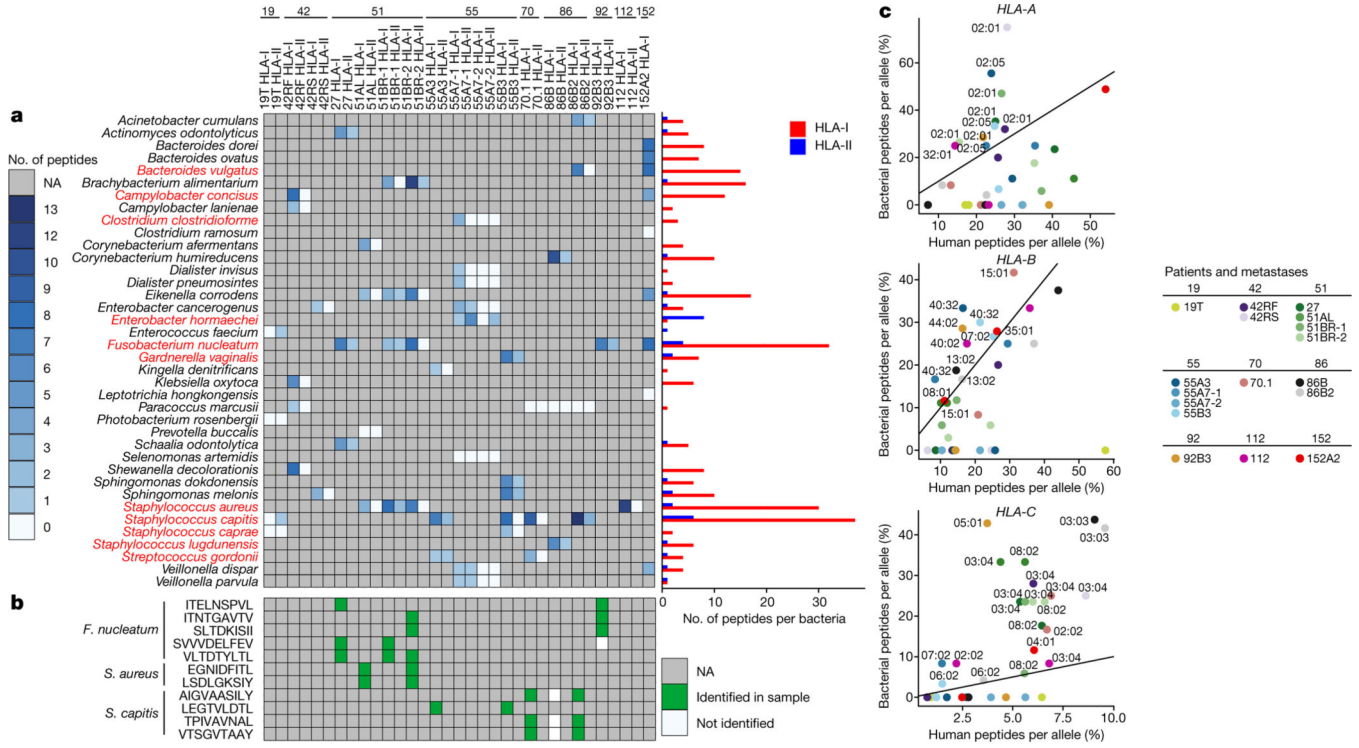


Fig. 2 | Characteristics of bacterial peptides.

a, The number of bacterial peptides presented on HLA-I and HLA-II in each patient sample (patient number indicated at top) is indicated in a blue colour scale (left). White indicates that no peptides were identified in the sample, and grey indicates that the bacterium was not identified in this metastasis (NA, not applicable). The total number of bacterial HLA-I and HLA-II peptides from each bacterium is noted in the bar plot on the right. Species names marked in red are known to be intracellular bacteria (Supplementary Table 6). **b**, Bacterial peptides that were identified in a few metastases from the same patient or in different patients are indicated. Peptides identified in the sample are marked green, and white denotes peptides that were not identified in the sample (although the metastasis has the required HLA allele for this peptide presentation and the species of bacteria). Grey indicates samples that lack the HLA allele and bacteria to produce the peptide. **c**, For each metastasis, the percentages of bacterial and human peptides that match each *HLA-A* (left), *HLA-B* (middle) or *HLA-C* (right) allele of the patient is indicated. The allele with the best per cent rank binding prediction (by NetMHCpan) was assigned to each peptide; the full allele list is indicated in Extended Data Fig. 6.

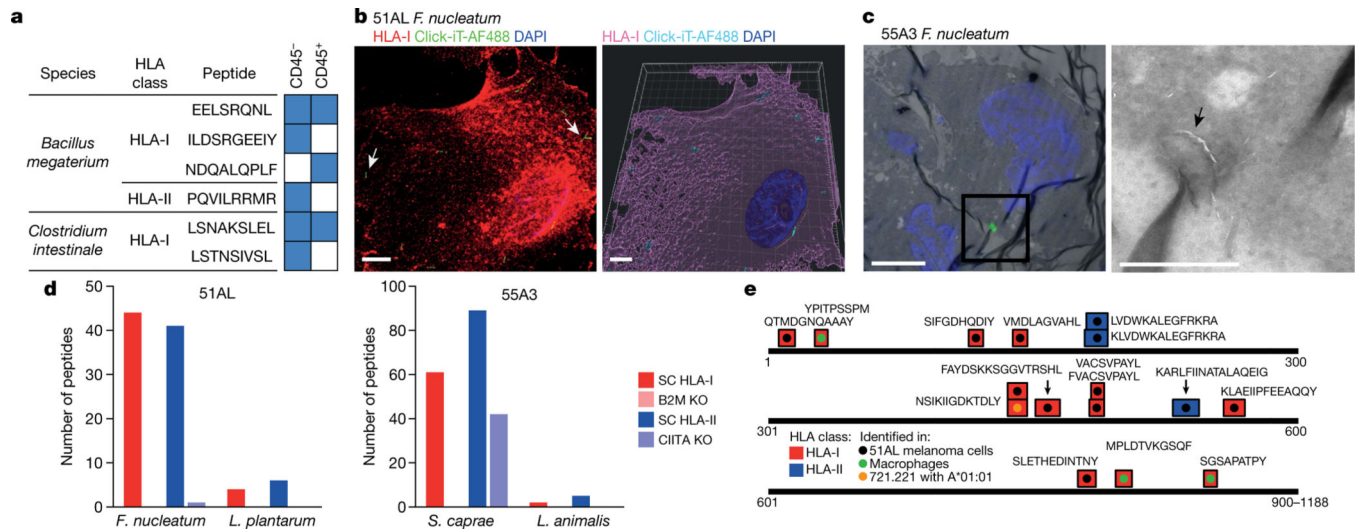


Fig. 3 | Evidence of bacteria entry and presentation by melanoma cells.

a, Tumour 422 was digested and CD45⁺ and CD45⁻ populations were subjected to HLA peptidomics. The table lists the peptides identified in each sample. **b**, Immunofluorescence staining detecting bacteria in 51AL cells stained with an anti-HLA antibody (red) that were cocultured with *F. nucleatum* (green). Cell nuclei were stained with DAPI (blue). Left, representative merged image from the *z*-stack centre, presented at 63× magnification. Right, *z*-stack 3D image (bacteria, turquoise; melanoma cells, magenta; nucleus, blue). Scale bars, 10 μm. **c**, We co-incubated 55A3 cells with *F. nucleatum* (green). Cell nuclei were stained with DAPI (blue). Ultramicrotome sections were analysed by CLEM. Left, the area of intracellular bacteria is marked by a black box. Scale bar, 5 μm. Right, zoomed-in view of the bacteria. Scale bar, 1 μm. **d**, Number of bacterial peptides identified by HLA peptidomics in the 51AL and 55A3 cell lines that were cocultured with *F. nucleatum* and *S. caprae*, respectively. *B2M* and *CIITA* were knocked out (KO) to reduce the levels of HLA-I and HLA-II, respectively. Compared with cells infected with scrambled control single-guide RNA (SC), the cells with *B2M* or *CIITA* knocked out show a lower number of identified bacterial peptides. Less-invasive species of bacteria (*L. animalis* and *L. plantarum*) were used as a control to show that the identified peptides are HLA ligands that resulted from intracellular bacteria. **e**, Different peptides were derived from the pyruvate-ferredoxin (flavodoxin) oxidoreductase protein from *F. nucleatum*. The protein sequence is described until the 900th amino acid rather than the end of the protein (1,188 amino acids in total), as no peptides were identified after this region of the protein.

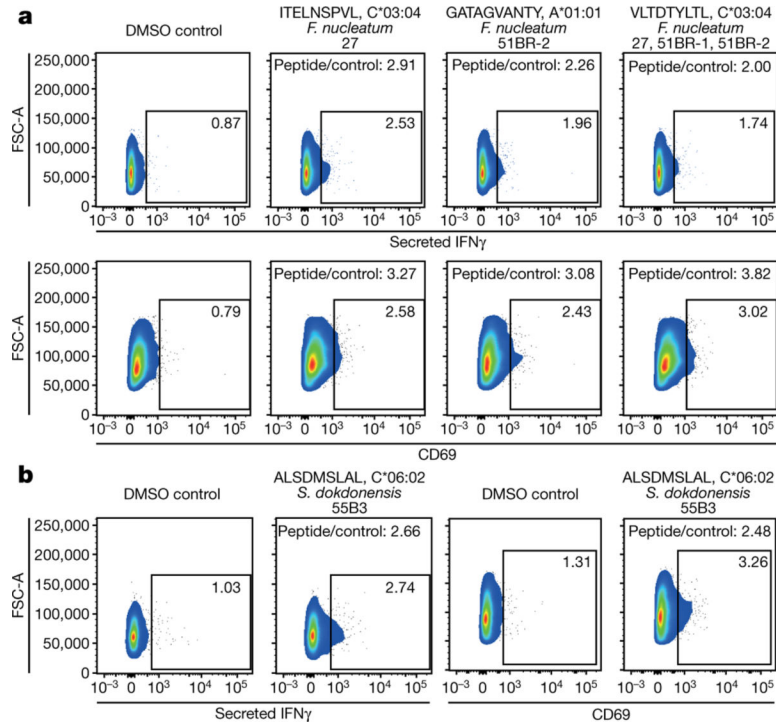


Fig. 4 |. TIL reactivity towards bacteria-derived antigens.

IFN γ -secreting 51AL and 55A3 TILs were detected after 6 h of coculture with B cells loaded with a bacterial peptide or dimethyl sulfoxide (DMSO) control, using flow cytometry. TILs were also tested for the presence of the CD69 reactivity marker. The image presents 4 representative immunogenic peptides (3 out of 7 immunogenic peptides of patient 51 and 1 out of 1 peptides of patient 55) that showed at least a 2-fold change between the peptide and DMSO control. The percentage of positive IFN γ -secreting or CD69-expressing TILs is an average of three independent experiments (Extended Data Fig. 12, Supplementary Figs. 10, 11). **a**, Patient 51. **b**, Patient 55.