Light-mediated discovery of surfaceome nanoscale organization and intercellular receptor interaction networks

Maik Müller, Fabienne Gräbnitz, Niculò Barandun, Yang Shen, Fabian Wendt, Sebastian N. Steiner, Yannik Severin, Stefan U. Vetterli, Milon Mondal, James R. Prudent, Raphael Hofmann, Marc van Oostrum, Roman C. Sarott, Alexey I. Nesvizhskii, Erick M. Carreira, Jeffrey W. Bode, Berend Snijder, John A. Robinson, Martin J. Loessner, Annette Oxenius & Bernd Wollscheid

The molecular nanoscale organization of the surfaceome is a fundamental regulator of cellular signaling in health and disease. Technologies for mapping the spatial relationships of cell surface receptors and their extracellular signaling synapses would unlock theranostic opportunities to target protein communities and the possibility to engineer extracellular signaling. Here, we develop an optoproteomic technology termed LUX-MS that enables the targeted elucidation of acute protein interactions on and in between living cells using light-controlled singlet oxygen generators (SOG). By using SOG-coupled antibodies, small molecule drugs, biologics and intact viral particles, we demonstrate the ability of LUX-MS to decode ligand receptor interactions across organisms and to discover surfaceome receptor nanoscale organization with direct implications for drug action. Furthermore, by coupling SOG to antigens we achieved light-controlled molecular mapping of intercellular signaling within functional immune synapses between antigen-presenting cells and CD8⁺ T cells providing insights into T cell activation with spatiotemporal specificity. LUX-MS based decoding of surfaceome signaling architectures thereby provides a molecular framework for the rational development of theranostic strategies.
Cellular function is regulated by information exchange with the outside world. The cell surface proteome (surfaceome) thereby acts as the signaling gateway to the cell by connecting external molecular cues with intracellular response pathways within dynamic surfaceome signaling domains. The spatiotemporal organization of these structures ranging in size from ~100 nm (B-cell receptor (BCR) clusters) to several microns (immune synapses) essentially determines cellular phenotypes by regulating receptor signaling, drug response, host-pathogen interactions, and intercellular communication. The tremendous therapeutic potential encoded in the surfaceome landscape catalyzes the emergence of innovative approaches that exploit receptor proximities for selective degradation of disease-causing receptors and for the targeting of cells with unmatched precision using multi-specific small molecules, antibodies, extracellular-drug conjugates (EDC) and colocalization-dependent protein switches. The ability to decipher cell-type-specific surfaceome nanoscale organizations and ligand-targeted receptor microenvironments is, therefore, a prerequisite for the understanding of fundamental cellular signaling processes and for the rational design of next-generation precision medicines.

Still today, the surfaceome landscape remains terra incognita that cannot be inferred from bulk proteomic and transcriptomic measurements. Dedicated strategies have therefore been established to profile cellular surfaceomes. For example, the extensive application of the Cell Surface Capture (CSC) technology established N-glycosylated surfaceomes for numerous cell types (collectively reported in the Cell Surface Protein Atlas, CS PA) and enabled the in silico characterization of the entire human surfaceome. While, fluorescence-based reporter assays and platforms of genetically engineered receptors established associations between receptors by high-throughput testing of binary interactions within and across surfaceomes, the Ligand-Recceptor Capture (LRC) technology enabled the identification of direct ligand interactions of N-glycosylated receptors. Most recently, localized proximity labeling by antibody-tethered enzymes (APEX, horseradish peroxidase (HRP), PUP-iT) or photocatalysts (μMap) were successfully combined with mass spectrometry for the characterization of selected surfaceome landscapes including lipid rafts, BCR neighborhoods and intact neuronal synapses in living cells and in fixed tissues. Although such antibody-based methods are useful in deciphering surface microenvironments of targeted receptors, highly versatile technologies are required to also uncover dynamic surfaceome domains that underlie the signaling function of small molecules, biomolecules, viral particles, and complex intercellular receptor interaction networks as formed within immunosynapses of interacting immune cells during T-cell activation.

Singlet oxygen generators (SOG) are well known for photocatalytic generation of short-lived singlet oxygen (SO) that promiscuously oxidize biomolecules including proteins in nanometer vicinity. The lifetime and traveling distance of photosensitized SO species thereby depend on environmental conditions and are drastically enhanced in heavy water (D₂O) based solutions. Small-molecule SOGs and genetically encodable versions thereof are extensively used throughout life sciences for chromophore-assisted light inactivation of proteins and cells, correlating light-electron microscopy and the detection of intracellular as well as extracellular protein interactions. Owing to the light-controlled activity, the tunable oxidation range, and the molecular size that is potentially compatible with any type of ligand, we recognized small molecules SOG as ideal probes for the in situ labeling of ligand-targeted surfaceome signaling domains of virtually any description.

Here, we developed a SOG-based and light-controlled proximity labeling technology termed LUX-MS enabling the cell type-specific elucidation of surfaceome nanoscale organization and ligand-targeted signaling domains on the surface of living cells of essentially any organism. The approach capitalizes on ligand-coupled small-molecule SOG for the specific and light-tunable biotinylation of ligand-proximal proteins enabling their identification using a high-throughput robot-assisted proteotyping workflow. We demonstrate the spatial specificity and broad applicability of our approach to reveal receptor microenvironments and surfaceome signaling domains of small molecules, biomolecules, and intact viruses in eukaryotic and prokaryotic systems in a discovery-driven fashion and without the need for genetic manipulation. At last, we highlight the utility of LUX-MS to elucidate intercellular surfaceome signaling domains of the highest complexity by mapping the architecture of functional immunosynapses in molecular detail.

**Results**

**Development of the LUX-MS technology.** We first tested the capability of the small-molecule SOG thiorhodamine to photo-oxidize transferrin proteins in vitro. High-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis combined with open modification peptide searching revealed the light-dependent conversion of the amino acids histidine (His), cysteine (Cys), tryptophan (Trp), tyrosine (Tyr), and methionine (Met) into previously described photo-oxidation products at varying D₂O concentrations (Supplementary Fig. 1). Next to oxidized Trp and Tyr, the two most prevalently formed modifications His+14 and His+32, previously characterized as 2-oxido (2-imidazolone) and its hydrated form, respectively, bear light-induced ketone groups that potentially offer functionalization via hydrazoamine formation using hydrazide-containing linkers. Indeed, photo-oxidation of transferrin in the presence of a biotin-hydrazone (BH) linker led to significant consumption of both His+14 and His+32 (Supplementary Fig. 1) indicating biotinylation of photo-oxidized proteins via light-activated histidines. We then tailored a liquid-handling robot-assisted processing workflow to automatically capture and proteolytically digest biotinylated proteins within streptavidin-functionalized tips for LC-MS/MS-based analysis eventually enabling the discovery-driven identification and label-free quantification of labeled proteins with excellent reproducibility and at high sample throughput (96-well format). Based on this optoproteomic workflow, we designed the LUX-MS technology (Fig. 1) where SOGs are chemically coupled to any ligand of interest and guided to selected surfaceome signaling domains on living cells to enable their light-induced labeling under physiological conditions. Proteins of the in situ labeled microenvironment are then identified in a hypothesis-free fashion by light-dependent enrichment against a non-labeled control.

**LUX-MS enables proteome-wide mapping of antibody binding targets and the surfaceome nanoscale organization.** Surfaceome interactions determine antibody specificity and signaling capacity and their elucidation is critical for the development of therapeutic antibodies such as Rituximab, a highly successful therapeutic antibody for the treatment of B-cell malignancies. Decades of research were required to eventually reveal the surface microenvironment of its primary receptor CD20 and its role in BCR-dependent cell killing. To demonstrate the utility of LUX-MS to provide insights into therapeutic antibody action, we coupled anti-CD20 antibodies to the small-molecule SOG thiorhodamine with an average degree-of-labeling (DOL) of 1.5 SOG molecules per antibody (Fig. 2a and Supplementary Fig. 2). Incubating Ab-SOG constructs with freshly isolated human peripheral blood mononuclear cells (PBMCs) resulted in antibody binding to 8% of all detected cells (Fig. 2b) corresponding to the typical fraction.
of CD20-positive cells in human PBMCs (5–10%)\(^5\). By applying light for 5 min in the presence of BH, 96% of all Ab-SOG bound but not unbound cells were surface biotinylated, demonstrating a proximity labeling reaction that is controllable by light and targetable to individual cells in a mixed cell population.

We then examined LUX-labeling dynamics using Ab-SOG on a human B-lymphoma cell line and observed light-dependent cell surface biotinylation to be fine-tunable by the duration of illumination (Fig. 2c and Supplementary Fig. 2). The extent of labeling was further tunable by modulating buffer conditions, i.e., increasing D\(_2\)O concentration and pH in line with enhanced photo-oxidation (Supplementary Fig. 4) and histidine conversion\(^5\), respectively. We then performed a CD20-targeted LUX-MS experiment by treating cultured human B-lymphoma cells with Ab-SOG prior illumination for 0–5 min. In total, 1674 proteins were quantified by LUX-MS with at least two unique proteotypic peptides per protein (Fig. 2d and Supplementary Fig. 2). Most proteins were equally abundant in the non-illuminated conditions, however, 63 proteins showed striking enrichment that correlated with the duration of illumination and that culminated in a fivefold abundance increase after 5 min of light. Of these, 84% were bona fide surface proteins while only 1% of all non-enriched proteins were surface associated (Fig. 2e). We thereby specifically retrieved 43 of the 215 CSPA-reported surfaceome members for this cell line including non-glycosylated surface proteins that typically evade CSC- or LRC-based surfaceome interrogations (Supplementary Fig. 3). LUX-MS, therefore, enables unbiased spatial proteotyping with sub-surfaceome resolution.

Fig. 1 The LUX-MS strategy for the light-controlled discovery of surfaceome nanoscale organization and ligand-targeted signaling domains. Small-molecule singlet oxygen generators (SOG) are chemically coupled to antibodies or ligands of any description (purple) and activated by visible light to establish spatiotemporally controlled oxidation of targeted surfaceome landscapes on living cells (red). Light-induced conversions of amino acids such as histidine into 2-oxo-histidine (gray) enable the stable biotinylation of SOG-proximal proteins under physiological conditions using biotin-functionalized hydrazide linkers (biocytin-hydrazide, green). In situ labeled proteins are automatically captured in streptavidin-functionalized tips and processed using a liquid-handling robotic platform ensuring fast and reproducible mass spectrometry-based quantification across a large number of samples. Relative protein quantification between labeled (light) and unlabeled (i.e., no light) samples ultimately enables discovery-driven and proteome-wide identification of cell surface proximity networks that constitute acute antibody- and ligand-targeted surfaceome signaling domains on living cells.

replicates, we used statistical testing to identify bona fide SOG-proximal candidates (enrichment fold change >1.5 and \(p\) value < 0.05, Fig. 2e and Supplementary Data 1). Among the top hits, we found immunoglobulin G2 (IgG2) isoforms of the Ab-SOG, the primary binding target CD20, human leukocyte antigen class II, tetraspanin CD37, CD298, and CD71 that are physically or functionally associated with CD20 on the surface of B-lymphoma cells\(^12,54-61\). Furthermore, LUX-MS revealed the proximity of CD20 to the IgM BCR complex (CD79A, CD79B, IgM heavy, and light chain) and BCR-associated CD19, CD38, and ITGA4 in line with a model where rituximab binding to CD20 is able to eradicate continuously activated B-lymphoma cells by altering survival-promoting BCR signaling\(^5\). On resting human B cells that express lower levels of CD20, we found CD20 in proximity to the BCR in presence of CD40 and partial absence of IgM indicating its segregation to quiescent IgD BCR nanoclusters as demonstrated previously\(^51\) (Supplementary Fig. 4). Performing LUX-MS in D\(_2\)O recapitulated these results and, in addition, provided CD20-proximity candidates that were not identified in H\(_2\)O alone, thereby increasing the number of CD20-proximity candidates from 118 to 172 (Fig. 2d and Supplementary Fig. 4). In contrast, CD20-proximity labeling using HRP-coupled antibodies yielded 220 candidates with a partially overlapping set, including the target receptor and interactors. Furthermore, using SOG-coupled antibodies against CD38, CD54, CD166, and CD220 revealed distinct receptor-specific surfaceome neighborhoods that covered a wide range of protein functionalities (Fig. 2f). LUX-MS, therefore, enables the discovery of cell-type-specific surfaceome microenvironments of selected receptors on living cells with fine-tunable...
spatial precision and as such provides a means for the systematic mapping of the global surfaceome nanoscale organization of a cell on a proteome-wide scale.

LUX-MS decodes surfaceome signaling domains of small-molecule drugs and biomolecules. Next to therapeutic antibodies, identifying receptors and surfaceome microenvironments targeted by small molecules is crucial for the development of small-molecule drugs and next-generation precision therapies such as drug-conjugated bispecific antibody (DBA)\(^1\) and EDC technologies\(^2\). EDC exploit cell surface proximities between disease-relevant antibody targets and small-molecule payload receptors for efficient cell ablation. However, the identity of small-molecule receptors and their
PCA analysis of CD38, CD54, CD166, and CD220 receptor microenvironments identifies Basigin (CD147), which is a functional target of CG1-loaded on the surface of HL60 cells (Fig. 3c and Supplementary Data 2) including transferrin and the cation-independent mannose-6-phosphate receptor or insulin-like growth factor 1 receptor (IGF1R) homo-dimers, and the transferrin receptor (TFRC), respectively. Hormone insulin and the plasma glycoprotein transferrin that selectively inhibits the active ion pumping subunit (ATP1B1) of the ubiquitously expressed human Na⁺/K⁺-ATPase complex (NKA) (Fig. 3a) [12]. A single-cell chemosensitivity screen using cultured human promyelocytic leukemia (HL60) cells showed potent cell ablation by both CG1 (IC₅₀ = 3.5 nM) and CG1-IOG (IC₅₀ = 93 nM) after 48 h, whereas the coupling of CG1 to HRP with an average DOL of 2.5 CG1 molecules per enzyme essentially abolished its drug activity (Fig. 3b and Supplementary Fig. 6). Flow cytometric analysis of HL60 cells LUX-labeled with increasing CG1-SOG concentrations showed light-induced and concentration-dependent cell-surface biotinylation (Supplementary Fig. 6). In a subsequent LUX-MS experiment, 48 CG1 proximity candidates were identified on the surface of HL60 cells (Fig. 3c and Supplementary Data 2) including the beta3 subunit of the NKA (ATP1B3), indicating that LUX-MS enables the discovery-driven identification of small-molecule receptors. Furthermore, we found the cell-surface receptor Basigin (CD147), which is a functional target of CG1-loaded EDCs on the cell type used for this experiment [12]. Therefore, the data suggest that small-molecule-targeted receptors and associated surfaceome signaling domains can be revealed by LUX-MS eventually enabling systematic target discovery for DBA or EDC development.

We next asked whether LUX-MS can be applied to uncover surfaceome interactions of more complex ligands, i.e., secreted biomolecules that are key elements of intercellular communication. We, therefore, coupled thiorhodamine SOG to the peptide hormone insulin and the plasma glycoprotein transferrin that regulate cellular homeostasis through interactions with insulin receptor or insulin-like growth factor 1 receptor (IGF1R) homo- or heterodimers, and the transferrin receptor (TFRC), respectively (Fig. 3d). LUX-MS was performed on human B-lymphoma cells that were treated with either conjugate and illuminated for 5 min. Results demonstrated specific enrichment of insulin or transferrin in conjunction with their cognate receptors INSR and IGF1R or TFRC, respectively (Fig. 3e and Supplementary Data 3). Transferrin-guided LUX-MS further revealed CD22, a B-cell specific lectin shown to weakly bind to sialylated N-glycoproteins including transferrin [65], and the cation-independent mannose-6-phosphate receptor M6PR, a sorting enzyme involved in the clathrin-mediated endocytosis and recycling of transferrin-bound receptors that was shown to co-localize with TFRC prior to internalization into sorting endosomes [65]. Thus, in addition to small molecules, LUX-MS can also be used to unravel surfaceome signaling domains of biomolecules providing molecular insights into long-range intercellular communication.

Elucidation of surfaceome signaling interactions across domains of life. Protein proximity labeling and detection by LUX-MS primarily rely on light-induced modifications of histidine, whose cellular biosynthesis is evolutionary well conserved [64]. Thus, LUX-MS may uncover ligand-targeted surfaceome signaling domains in non-eukaryotic systems such as bacteria where LRC-like approaches are not applicable, aiding in the further development of anti-infective agents such as Thanatin to fight the growing threat of drug-resistant bacteria. Thanatin is a naturally occurring peptide that was recently shown to inhibit the outer membrane biogenesis complex Lpt in Escherichia coli (E. coli) by binding to LptA [65]. To demonstrate the utility of LUX-MS to identify such important surfaceome interactions in bacteria, we coupled Thanatin to the SOG methylene blue (Fig. 4a) and added it to E. coli cells with or without competition by an excess of unconjugated Thanatin. All cells were subsequently illuminated for 5 min with red light at 656 nm and subjected to LUX-MS for identification of proximity labeled proteins. Numerous outer membrane and periplasmic proteins were significantly enriched (Fig. 4b and Supplementary Data 4), indicating membrane-localized labeling by SOG-coupled Thanatin. LUX-MS thereby revealed the functional binding partners of Thanatin LptA and LptD as top enriched hits and suggested proximity to outer membrane proteins including BamA and BamC (Fig. 4b). Indeed, the Bam complex was shown to catalyze the folding and insertion of outer membrane proteins including LptD forming supramolecular surfaceome clusters on living bacteria [66]. LUX-MS therefore enables deconvolution of ligand-targeted receptors and associated surfaceome microenvironments across domains of life.

Unraveling the surfaceome interaction network of intact viral particles using LUX-MS. In addition to drug-resistant bacteria, pathogenic viruses pose a growing threat to global human health. Extracellular host-pathogen interactions are thereby key for cellular tropism and infection and, as such, are primary research targets for the classification and diagnosis of infectious diseases and vaccine discovery. To demonstrate the utility of LUX-MS to reveal viral surfaceome interactions, we coupled thiorhodamine SOG to bacteriophages that recognize the ubiquitous cell wall component teichoic acid of Gram-positive Listeria monocytogenes (Lm), a highly infectious food-borne bacteria that causes listeriosis [67] (Fig. 5a). Lm was incubated with SOG-coupled phages at a multiplicity of infection (MOI) of 1 or 10 and LUX-labeled by illumination for 0–15 min. Flow cytometry analysis revealed the light-dependent cell-surface biotinylation of individual bacteria that reached saturation after 15 min of illumination at an MOI of 10 (Fig. 5b). Subsequent LUX-MS analysis revealed light-induced, significant enrichment of 146 proteins including 19 phage and 127 Lm proteins (Fig. 5c and Supplementary Data 5). We found components of bacteriophage capsids (Cps, Gp5, Gp9),...
sheath (Gp3), baseplate (Gp20), and tail (Gp17, Gp18, Gp19, Gp23, Tmp, Tsh) and enzymes involved in DNA binding (Gp37), replication (Gp45), recombination (Gp44), transcription (Gp7), and immunoprotective functions (Gp32) indicating thorough LUX-labeling of host cell-attached bacteriophages. For \( Lm \) proteins, gene ontology analysis revealed 81 extracellular and only 8 intracellular proteins (Fig. 5d) including major virulence factors such as cell wall-associated internalins A and B that mediate host cell invasion, membrane-localized ActA that governs cell-to-cell spreading, and ATP-binding cassette (ABC) transporters that mediate multi-drug resistance (Fig. 5e). Using HA-tagging and immunofluorescence microscopy, we further validated the \( Lm \)
surface expression of a putative cell wall-anchored protein and an ABC transporter that were strongly enriched by LUX-MS (Fig. 5f). Altogether, our results demonstrate the capability of LUX-MS to unravel host surfaceome interactions of intact viruses and its use to map the cell surface of pathogenic bacteria eventually facilitating the design of anti-infective strategies.

Elucidating intercellular surfaceome signaling domains in functional immunosynapses. Contact-dependent intercellular communication is an important regulator of immune system function and relies on highly complex intercellular surfaceome signaling architectures - in the case of T lymphocyte activation by antigen-presenting cells (APCs)—the immunological synapse. The contextual interplay of immunosynaptic receptor interactions between APCs and CD8+ T cells leads to T-cell activation, proliferation, and differentiation enabling these cells to exert effector functions against infected, tumorogenic, or foreign cells. Thus, the ability to resolve functional immunosynapses at the molecular level with spatiotemporal specificity would enable discoveries in the field of immunology and immuno-oncology. We thus coupled thiorhodamine SOG to the lymphocytic choriomeningitis virus (LCMV)-derived peptide gp33-41 (gp33, sequence: KAVYN-FATM) (Fig. 6a), an immunodominant epitope of the LCMV glycoprotein (GP) that is presented by MHC class I H-2Db and H-2Kb and that is recognized by T-cell receptor (TCR) transgenic CD8+ T cells of P14 mice. In a two-cell system, we showed that SOG-coupled gp33 is still presented by mouse dendritic cells (DCs) and is able to activate freshly isolated P14 CD8+ T cells with comparable efficacy than its unconjugated counterpart (Supplementary Fig. 7). Applying light led to cell surface biotinylation of both cell types, validating the photocatalytic function of SOG-coupled gp33 and our ability to efficiently perform trans-cellular surfaceome labeling during immunosynaptic cell-to-cell contacts (Supplementary Fig. 8). We then implemented stable isotope labeling by amino acids in cell culture (SILAC) for isotopic barcoding of interacting DCs and T cells (TC) and performed LUX-MS in data-independent acquisition (DIA) mode after 30 min of co-culture to analyze mature immunological synapses (Fig. 6b). Overall, we identified 4811 protein groups that could clearly be assigned to TC (e.g., TCR, CD3, and CD8), DC (e.g., MHC class I H-2Db and H-2Kb), or both based on observed SILAC-ratios (Fig. 6c). We observed light-dependent enrichment of numerous cell surface proteins including components of the primary signaling axis such as MHC class I on DC and the gp33-specific TCR as well as CD8 on TC (Fig. 6d-e, Supplementary Data 6 and 7). Furthermore, we found co-stimulatory signaling components essential for early T-cell priming such as CD86 of the CD86-CD28 axis and the entire CD2-CD48 axis with reported cellular directionality. Our results further indicate immunosynaptic localization of CD5 and CD6 on TC. Indeed CD5 was shown to interact with TCR, CD8, and CD2 in the immune synapse to fine-tune TCR-mediated signaling.

![Fig. 4 Hypothesis-free identification of surfaceome signaling interactions in prokaryotic systems. a Schematic of coupling the peptidomimetic antibiotic Thanatin to the singlet oxygen generator (SOG) thiorhodamine. b Volcano plot showing relative abundance changes of LUX-MS quantified proteins from Thanatin-SOG treated Escherichia coli illuminated for 5 min with and without Thanatin competition, tested using a two-sided Student’s t test. Blue dots represent significantly enriched proteins and red dots represent the direct binding targets of Thanatin previously found by photo-crosslinking experiments. The main targets and selected surfaceome interactors are highlighted. c Schematic representation outlining the molecular mechanism of action of Thanatin in E. coli cells with spatial context. Source data are provided as a Source Data file and interactive volcano plots (Supplementary Data 4).](https://doi.org/10.1038/s41467-021-27280-x)
**Fig. 5** Mapping virus-targeted surfaceomes on living hosts using bacteriophage-guided LUX-MS. 

**a** Schematic of coupling the *Listeria monocytogenes* (*Lm*)-specific bacteriophage to the singlet oxygen generator (SOG) thiorhodamine. **b** Histogram plot showing light-dependent cell surface biotinylation of *Lm* cells after LUX-labeling with bacteriophage-SOG (>30,000 cells per condition). **c** Volcano plot showing relative abundance changes of LUX-MS quantified proteins from bacteriophage-SOG treated *Lm* with and without illumination for 15 min, tested using a two-sided Student’s *t*-test. Blue and green dots represent significantly enriched *Lm*- or phage-derived proteins, respectively, whereas gray dots indicate non-enriched proteins. Candidates selected for microscopic characterization are highlighted. **d** GO-term enrichment analysis of significantly enriched *Lm* proteins. **e** Schematic representation of the spatial coverage of LUX-MS based on subcellular annotations of significantly enriched proteins. Candidates selected for microscopy are marked with arrows. **f** Immunofluorescence of the indicated *Lm* strains stained with an anti-HA monoclonal antibody conjugated with Alexa Fluor 555 (Red). Images are representative of three experiments. Source data are provided as a Source Data file and interactive volcano plots (Supplementary Data S5).
while the structurally related CD6 physically associates with CD5 and its ligand CD166 (also known as ALCAM) that was also captured on DC. LUX-MS further revealed immunosynaptic adhesion complexes including LFA-1 (TC), ICAM-1 (DC), and VLA-4 (both) that enhance intercellular proximity and T-cell activation and identified highly membrane-embedded tetraspanins CD9, CD81, CD82, and CD151 on DC that organize immunosynaptic signaling domains on APCs. In contrast, the ubiquitous protein tyrosine phosphatase receptor type C (PTPRC, CD45) was not identified on TC in line with its immunosynaptic exclusion to permit TCR phosphorylation and signaling (Fig. 6e). Taken together, our results demonstrate the utility of LUX-MS to comprehensively resolve the intercellular surfaceome signaling architecture of functional immune synapses with cell-type specificity and spatiotemporal control by light, providing a molecular framework for drug discovery in immunology and immuno-oncology.

Discussion

The nanoscale organization and extracellular interaction network of the cellular surfaceome is a key mediator in antibody signaling, drug action, hormone function, viral infection, and intercellular communication. In this study, we developed and applied a
SOG-based and highly versatile proximity detection technology termed LUX-MS that enables to specifically resolve surfaceome signaling domains in all these scenarios with spatiotemporal control by light. In contrast to larger enzymes (APEX2: 28 kDa; miniTurbo: 27 kDa)\textsuperscript{75}, the use of small molecular SOG (<1 kDa) as labeling probes offers the following advantages: (i) Broad ligand-compatibility enabling target deconvolution and surfaceome interaction profiling of ligands of basically any description ranging from small molecules, antibodies and viral particles to whole cells. (ii) Covalent SOG-coupling with ligand-compatible chemistry preserving structure-function relationships and circumventing the need for genetic manipulation. (iii) Light-controlled generation of SO species with modifiable reactivity allowing for fine-tunable in situ labeling with tight spatiotemporal control. (iv) Protein labeling via conserved amino acid residues enables proteome-wide surfaceome interrogations across domains of life. Recently, photocatalysts producing short-lived carbene with nanosecond half-life were utilized for antibody-guided mapping of protein microdomains that could not be resolved using enzyme-based strategies (intermediates with half-lives of 0.1 ms to 60 s)\textsuperscript{28}. Proximity labeling by LUX-MS is mediated by photosensitized SO species with a lifetime of 3.5 μs\textsuperscript{36} that is tunable by modulating buffer conditions indicating spatial resolution in between enzyme- and carbene-based approaches. Indeed, although CD20 microenvironment mapping using LUX-MS provided a defined list of proximity candidates that was extendable by the use of D\textsubscript{2}O, an HRP-based approach provided a substantially longer list of complementary CD20 candidates. Furthermore, the direct coupling of SOG to signaling competent ligands confines labeling to signaling relevant surfaceome domains and circumvents the use of antibodies that provide structural flexibility. Thus, LUX-MS complements currently available technologies and provides an analytical stepping stone for system-wide elucidation of the cellular surfaceome landscape.

Using antibodies to guide proximity labeling, we demonstrated the ability of LUX-MS to specifically reveal distinct surfaceome proximities on living cells that form a molecular framework for the reconstruction of the surfaceome nanoscale organization. Given the broad availability of high-quality antibodies for established surface targets and the high-throughput capability of the LUX-MS workflow, profound surfaceome interaction mapping is feasible providing the cell surface level information that is currently lacking in ongoing systems biology efforts to map and understand the structure and function of the human interactome\textsuperscript{26–29}.

By applying LUX-MS to small molecules, we mapped the surfaceome signaling domains of the small-molecule drug cardiac glycoside CG1. In contrast to LRC methodologies, LUX-MS not only enabled proteome-wide target deconvolution of small molecules but also provided cell-type-specific surfaceome proximity information that could be leveraged for targeted drug delivery, e.g., using EDC\textsuperscript{72}. While this approach is expected to be transferrable to other drug modalities, the accumulating knowledge of drug-targeted cellular surfaceomes eventually allows for the identification of disease-associated receptor proximities enabling the rational design of next-generation precision medicines that go well beyond the targeting of individual receptors.

Using LUX-MS, we decoded viral surfaceome interactions in bacterial hosts that are not detectable using state-of-the-art technologies such as CSC, LRC, and HATRIC owing to the structural complexity of the cell envelope and a general lack of protein glycosylation. Using bacteriophages that recognize a ubiquitous cell wall component of Listeria monocytogenes, we mapped the bacterial surfaceome (>80 proteins) with substantial coverage and specificity that superseded common profiling strategies such as trypsin shaving (11 proteins identified), surface-restricted biotinylation (27 proteins identified), and cell fractionation (21 proteins identified)\textsuperscript{80}. Depending on the binding specificity of the employed viral particle, LUX-MS enables the spatiotemporal elucidation of viral attachment sites, target receptors, and global surfaceomes of live prokaryotic and eukaryotic cells in complex biological systems. This is of particular interest considering the broad application of advanced phage display assays\textsuperscript{81} and related screening platforms\textsuperscript{82,83} in basic and translational research.

Finally, we employed LUX-MS for the spatiotemporal analysis of intercellular receptor interaction networks within immunological synapses. Previous attempts were limited to molecular analysis in model systems that use soluble, plate-bound, or membrane-associated factors to mimic immunosynaptic T-cell activation\textsuperscript{69} or to fluorescence-based reporting of transient intercellular immune cell interactions\textsuperscript{28,84}. Here, we performed a gp33 immunogen-guided LUX-MS in an isotopically barcoded two-cell system of millions of synchronized and functional immunosynapses and thereby obtained a holistic view of the mature immunosynaptic surfaceome signaling architecture, fostering the formulation of original biological hypotheses. For example, we found several lysosomal proteins (LAMP-1, LAMP-2, CD68, and cathepsins B, D, and Z) within the immunosynaptic cleft on naive CD8\textsuperscript{+} T cells but not antigen-presenting DCs. This indicates local fusion of lysosomal granules in T cells that is typically observed in cytotoxic T cells during degranulation-mediated target cell killing\textsuperscript{85}. However, while being a feature of antigen-experienced cells, degranulation and thus cytolytic activity was shown to be absent in naive CD8\textsuperscript{+} T cells. Indeed, we did not identify any cytolytic effector molecules such as perforin or granzymes, indicating the secretion of non-lytic granules in naive CD8\textsuperscript{+} T cells within 30 min of initial contact with APCs (Fig. 6f). Albeit, the cellular machinery for immunosynapse-directed transport of vesicles is in place within 6 min of initial cell contact, the exact secretion mechanism and functional role of such non-lytic granule secretion in antigen-dependent T-cell priming remains to be further explored.
In conclusion, we provide a high-throughput, optoproteomic technology termed LUX-MS to discover protein networks within specific surfaceome signaling domains on living cells and we demonstrate its utility and versatility in a broad range of biomedically important scenarios including antibody signaling, drug action, hormone function, viral infection and intercellular communication. Given the biological and therapeutic importance of the surfaceome signaling landscape, we envision LUX-MS to impact basic and applied research areas by unraveling the spatiotemporal interconnectivity of the cell surface signaling gateway in health and disease.

Methods

Reagents. All chemicals were purchased from Merck unless stated otherwise. SOGs in form of NHS-functionalized thiorhodamine (cat: AD-Thio12-41) and azide-functionalized methylene blue (cat: AD-MB2-31) were purchased from ATTO-TEC GmbH (Siegen, Germany). Biocytin-hydrazide was purchased from Pitsch Nucleic Acids AG (Stein am Rhein, Switzerland). The following antibodies were used for LUX-MS experiments: mouse IgG1 Isotype control antibody (Invitrogen, cat: 10400 G), human anti CD20 mouse IgG2 monoclonal antibody clone 2H7 (Invitrogen, cat: 14-0209-82), anti-human CD38 chimeric monoclonal antibody kindly provided by Centrode LLC, anti-human CD54 mouse IgG1 monoclonal antibody clone HCD54 (BioLegend, cat: 322704), anti-human CD166 mouse IgG1 monoclonal antibody clone J56 (BioLegend, cat: 343960), anti-human CD220 mouse IgG2 monoclonal antibody clone B6.220 (BioLegend, cat: 352062).

Cell culture. All chemicals for cell culture were purchased from ThermoFisher Scientific unless stated otherwise. All other chemicals were purchased from Sigma-Aldrich unless stated otherwise. Cell lines were purchased from ATCC and grown at 37 °C and 5% ambient CO2. Patient-derived B-lymphoma cell line SUHL6 (ATCC: CRL-2599) and human Burkitt lymphoma B cell-line Ramos (ATCC, CRL-1596, kindly provided by Michael Reth) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 1.5 mM GlutaMAX, 1% penicillin–streptomycin, and 10% fetal bovine serum. Patient-derived promyelo- cytic leukemia cell line HL60 (ATCC CCL-240) was grown in RPMI with 1.5 mM GlutaMAX (Gibco), 1% penicillin–streptomycin (Gibco), and 10% human serum (Chemicon). Mouse dendritic cell line MutuDC1949 was kindly provided by Hana Acha-Orbea (Department of Clinical Science, University of Lausanne, Switzerland) and was cultured and isotopically labeled in Iscove’s Modified Dulbecco’s Medium for SILAC (Thermo, cat: 88367) supplemented with glucose (final: 4.5 g/l), l-Arg1-13C6-15N2 (final: 42 μg/ml), l-Lys1-13C6-15N2 (final: 73 μg/ml), 1.5 mM GlutaMAX, 10 mM HEPES, 50 μM l-mercaptoethanol, 1% penicillin–streptomycin, non-essential amino acids, and 10% diazylated fetal bovine serum.

Mice. Female or male mice of six to eight weeks of age were used for animal experiments described in this study. CD45.1 P14 mice with T-cell receptors on CD8+ T cells specific for the glycoprotein GP33-41 epitope of LCMV71 crossed with Nurr77-GFP mice (P14 Nurr77-GFP) were bred at the ETH Phenomics Center at a light–dark cycle of 12 h, the ambient temperature of 22 °C ± 2 °C and humidity of 55% ± 10%. All animals were bred and held under specific-pathogen-free conditions prior to use. All animal experiments were performed in accordance with institutional policies and Swiss federal regulations, following guidelines and being approved by the veterinary office of the Canton of Zürich (animal experimental permissions: 115/2017).

Light sources. Photo-oxidation reactions were controlled using Precision LED spotlights operated via a BioLED Light Control Module (BLS-PLD4-US) in continuous wave mode (Mightex Systems, Pleasanton, USA). For LUX-MS applications with thiorhodamine SOG, spotlights BLS-PLS-050-030-05 were used for illumination at 590 nm with a light intensity of 4.6 mW/cm2. For LUX-MS applications with methylene blue as SOG, spotlights BLS-PLS-065-030-07-S were used for illumination at 650 nm with a light intensity of 14.9 mW/cm2.

Light-controlled production of SO. The SOG thiorhodamine was mixed with 1 mM Singlet Oxygen Sensor Green (SOG, Invitrogen, cat: S36002) in phosphate-buffered saline at increasing D2O/H2O ratios and illuminated at a working distance of 30 cm with 590 nm Precision LED spotlights for 0–15 min. The fluorescence of photo-oxidized SOG was monitored using a Synergy HT Multi-Mode Plate Reader (BioTek) at excitation 485 ± 20 nm and emission 528 ± 20 nm. Quantified intensities were blanked and normalized to non-illuminated control using a customized R-script.

Discovery and tuning of light-induced protein labeling sites. Human holo- transferrin (Sigma, cat: T4132) was coupled with thiorhodamine SOG at a molar ligand-to-SOG ratio of 1:1 and purified using 7-KDa ZebaSpin Columns (Thermo, cat: 89882) according to manufacturer’s recommendations. SOG-coupled transferrin in D2O-based phosphate-buffered saline (PBS) containing 0 or 5 mM D2O was photo-oxidized with 590-nm hydrazide at either 15 or 10 min of left in the dark. The buffer was complemented with 50 mM 2-(aminomethyl)imidazole dihydrochloride catalyst for 50 min to catalyze hydrazone formation. Treated proteins were digested overnight at 37 °C using sequencing-grade trypsin (Promega, cat: V511C) at an enzyme-to-protein ratio of 1:100. For 18-purified peptides from LUX-MS, the discovery protein data were subjected to an open modification search using MSStagger (v.20190628) and Crystal-C in the FragPipe pipeline (v.9.4) using standard settings and a SwissProt-reviewed human protein database (downloaded June 2014) containing common contaminants. Observed modifications were implemented in a subsequent closed search of all acquired samples using Comet (v.2015.01) within the Trans Proteomic Pipeline v.4.7 (SPC/ISB Seattle) as variable modification (carboxylation of His, Cys, and Trp; single oxidation of Met, Trp, and Tyr; double oxidation of Met, Trp, and His; triple oxidation of Met, Cys, and Trp) to identify fully tryptic peptides with a maximum of two missed cleavage sites and a maximum of five variable modifications. The precursor and fragment mass tolerance was thereby set to 20 ppm and 0.02 Da, respectively. Peptides were quantified by integration of chromatographic traces using Progenesis Q I v.4.0 (Nonlinear Dynamics) and filtered to a false discovery rate of <1%. Transferrin peptides were assigned to dummy proteins representing unmodified or single modification types. Within the R computing environment, mixed-effects models for fold changes (expressed in log2) were calculated using a linear mixed-effect model and tested for statistical significance using a two-sided t test with the appropriate degree of freedom in the R package MStas (v.3.8.6)90. Modification types with an abundance fold change >1.5 upon illumination and an adjusted p value < 0.05 were considered products of light-induced photo-oxidation. Analyses were performed in R using a custom-designed R-script.

Collection and purification of human PBMCs. Buffy coats were obtained from healthy donors provided by the Blood Transfusion Service Zurich. To isolate PBMCs, human buffy coat samples were diluted 1:1 in PBS (Gibco) and mono-nuclear cells were isolated with a Histopaque-1077 density gradient (Sigma-Aldrich) according to the manufacturer’s instructions. PBMCs at the interface were collected, washed once in PBS, and resuspended in media until further processing.

Generation of antibody-SOG and HRP conjugates. For antibody-SOG conjugates, 10 μg antibody per sample was purified using 7-KDa ZebaSpin Desalting Columns (Thermo Scientific, cat: 89882), following manufacturer’s guidelines, and eluted in 10 mM sodium bicarbonate in PBS, pH 8.3. Antibody was coupled with...
thiorhodamine SOG to a molar antibody to SOG ratio of 1:5, purified using 7-kDa ZebaSpin Columns, and immediately used for LUX-MS experiments.

For antibody-HRP conjugates, 10 μg anti-CD20 antibody (Invitrogen, clone 2H7) per sample was purified using 7-kDa ZebaSpin Desalting Columns (Thermo Scientific, cat: 898882), following manufacturer’s guidelines, and eluted in 0.2 M carbonate-bicarbonate buffer, pH 9.4. Purified anti-CD20 antibody was conjugated to pre-activated amine-reactive HRP (Thermo Scientific™ EZ-Link™ Plus Activated Peroxidase, Thermo Scientific, cat: 31489) for 1.5 h in 0.2 M carbonate-bicarbonate buffer, pH 9.4 with a coupling ratio of antibody to HRP of 1:10. The reaction mixture was cleaned with 7-kDa ZebaSpin Desalting Columns, eluted in PBS, pH 7.2, and further processed according to the manufacturer’s manual. Anti-CD20-HRP conjugates were immediately used for the HRP-based proximity labeling experiment.

For CG1-HRP conjugates, pre-activated amine-reactive HRP (Thermo Scientific™ EZ-Link™ Plus Activated Peroxidase, Thermo Scientific, cat: 31489) was coupled to amine-containing cardiac glycoside CG1 (kindly provided by Centrose LLC, Madison, Wisconsin) for 1.5 h in 0.2 M carbonate-bicarbonate buffer, pH 9.4 with a coupling ratio of HRP to CG1 of 1:10. CG1-HRP conjugate was re-buffered into PBS, pH 7.2 using 7-kDa ZebaSpin Desalting Columns and further processed as recommended by the manufacturer’s guidelines. HRP-CG1 conjugates were purified and separated from free CG1 using both 7-kDa ZebaSpin Desalting Columns (twice) and 10 kDa molecular weight cut-off filters (Merek, cat: UFC651024) and subsequently used for single-cell chemosensitivity screening.

**Degree of labeling (DOL) calculation.** Average DOL of antibodies labeled with thiorhodamine SOG probes was determined by measuring absorbance as previously described. Specifically, the absorbance of conjugates (Conjugate), antibody (Ab), and free thiorhodamine (SOG) at both 280 nm and 582 nm were measured using a NanoDrop spectrophotometer (Thermo Scientific). Next, the next two following ratios were calculated: Ratio\_Ab = AS582/\_Ab / AS280/\_Ab and Ratio\_SOG = AS582/\_SOG / AS280/\_SOG. Then, the absorbance of the antibody-SOG conjugate was measured yielding A280\_Conjugate and AS582\_Conjugate. The actual concentration of antibody and SOG contained in the conjugate solution were determined as follows: [Ab] = (\( \text{A280} - \text{Ratio}\_\text{Ab} \times \text{AS280}\) / \( \text{Ratio}\_\text{Ab} \times \text{AS280}\)), [SOG] = (\( \text{A582} - \text{Ratio}\_\text{SOG} \times \text{AS280}\) / \( \text{Ratio}\_\text{SOG} \times \text{AS280}\)). The following extinction coefficients (ε) were thereby used: εA582 = 210,000 M⁻¹ cm⁻¹, εA582 = 110,000 M⁻¹ cm⁻¹. Finally, the DOL was determined: DOL = [SOG]/[Ab]. Per conjugate, an average DOL of six replicate measurements was calculated in the R computing environment.

**Antibody-guided receptor microenvironment mapping.** For SOG-based LUX-MS, 20 × 10⁶ cells were incubated with 10 μg SOG-antibody (final 5 μg/ml) for 30 min at 4°C in the dark to minimize background light-induced oxidation. Cells were washed with ice-cold PBS, resuspended in chilled photo-oxidation buffer (5 mM biocytin-hydrazide in D2O-based PBS, pH 7.5 if not stated otherwise), and incubated overnight for 24 h at 37°C and 5% CO₂. Cell number and viability were determined by the use of a Countess II Cell Counter (Thermo Fisher). Cells were subsequently treated with a drug screen library of three single compounds (CG1, CG1-SOG, and CG1-HRP) in four concentrations each with ten technical replicates each. Cells were then analyzed using an Accuri C6 Flow Cytometer and FlowJo (v.10.07). The FSC/SSC pattern of the unlabeled condition was thereby used to gate for live cells (Supplementary Fig. 12). For LUX-MS analysis, cells were extensively washed with PBS, snap-frozen as cell pellets in liquid nitrogen, and stored at −80°C until further processing.

**Identification of small-molecule drug-targeted surfaceome structures.** Fifty million cells were incubated with 8 μg CG1-SOG (A in μM for LUX-MS experiment) for 30 min at 4°C in the dark to minimize background light-induced oxidation. Cells were washed with ice-cold PBS, resuspended in chilled photo-oxidation buffer, and illuminated at a working distance of 20 cm with 590-nm Precision LED spotlights or left in the dark. For the anti-CD20 time course LUX-MS, one and three biologically independent samples were analyzed for each illumination time point and the non-illuminated control, respectively. To determine the DOL, 20 × 10⁶ Ramos cells were incubated with 1 mM biotin-tyramide (Pitsch Nucleic Acids AG). The HRP-based proximity labeling condition. For the control samples, PBS-based Mock buffer (without \( \text{H}_2\text{O}_2 \)) was used. After 1 min, the reaction was stopped with a quenching buffer containing 10 mM \( \text{Na}_2\text{SO}_4 \), 10 mM sodium ascorbate, and 1:10,000 catalase (Sigma-Aldrich, C30) in ice-cold PBS, immediately followed by three washes with quenching buffer to remove excess HRP. Cells were then lysed in liquid nitrogen and stored at −80°C until further processing. In total, three biologically independent replicates were used per condition.

**Synthesis of SOG-coupled carbohydrate glycoside CG1.** The primary amine-containing carbohydrate glycoside CG1 was kindly provided by Centrose LLC (Madison, Wisconsin). A solution of CG1 (25 μg, 0.0081 mmol) in N,N-dimethylformamide (1 mL) was added to Et₃N (0.011 mL, 0.08 mmol). The reaction mixture was stirred at RT for 1 h and product formation was confirmed by LC-MS. The final product CG1-SOG 3 was purified by preparative RP-HPLC on a Waters Eclipse XDB (X18, 250 × 21.2 mm, 7 μm) column with a gradient of 5–50% MeCN in 0.1% TFA in water (A) and 0.1% TFA in MeCN (B) at a flow rate of 1 cm³/min. Further purification was performed using preparative RP-HPLC on a Waters Eclipse XDB (C18, 250 × 21.2 mm, 5 μm) column with a gradient of 5–50% MeCN in 0.1% TFA in water (A) and 0.1% TFA in MeCN (B) at a flow rate of 1 cm³/min, each column volumes.

**Single-cell chemosensitivity screening.** A single-cell suspension of HL60 cells was seeded (2000 cells/well with 50 μl/well) in CellCarrier 384 Ultra, clear-bottom, tissue culture-treated plates (PerkinElmer) containing antineoplastic agents (see below) and incubated overnight for 24 h at 37°C and 5% CO₂. Cell number and viability were determined by the use of a Countess II Cell Counter (Thermo Fisher). Cells were subsequently treated with a drug screen library of three single compounds (CG1, CG1-SOG, and CG1-HRP) in four concentrations each with ten technical replicates each. Cells were then analyzed using an Accuri C6 Flow Cytometer and FlowJo (v.10.07). Single cells detection and nuclear segmentation were performed by maximum correlation thresholding on the DAPI channel. To extract cell contours, measurement calculation was calibrated by a circular expansion of 10 pixels around the nucleus. To measure the local background intensity around every single cell, a second larger expansion of 30 pixels was performed. Standard CellProfiler raw fluorescent intensities were extracted, log₁₀ transformed, and normalized towards the local cellular background.

**Biomolecule-guided discovery of intercellular surfaceome signaling domains.** The human peptide hormone insulin and glycoprotein holo-transferrin were coupled with thiorhodamine SOG at a molar ligand:SOG ratio of 1:1 and 1:13, respectively according to the manufacturer’s recommendations. The SOG-coupled ligand constructs were kept at 4°C and immediately used. Twenty million cells were incubated with 1 μM Insulin-SOG or 75 nM Transferrin-SOG for 10 min at 4°C in the dark to minimize background light-induced oxidation. Cells were washed with ice-cold PBS, resuspended in chilled photo-oxidation buffer and illuminated at a working distance of 20 cm with 590-nm Precision LED spotlights for 5 min or left in the dark (three biologically independent samples each). Cells were then pelleted by centrifugation and resuspended in chilled labeling buffer for 50 min at 4°C in the dark. For flow cytometric analysis of cell surface biotinylation, cells were extensively washed with PBS and stained with NeutrAvidin Protein DyLight 488 (Invitrogen, cat: 228322) 1:200 for 20 min prior analysis using an Accuri C6 Flow Cytometer (BD Biosciences) and FlowJo (v.10.07). The FSC/SSC pattern of the unlabeled condition was thereby used to gate for live cells (Supplementary Fig. 12). For LUX-MS analysis, cells were extensively washed with PBS, snap-frozen as cell pellets in liquid nitrogen, and stored at −80°C until further processing.
blue as SOG (0.5 mg, 0.009 mol/mL) in 100 µL DMSO and CuSO4 (0.1 M, 30 µL, 0.003 mmol) was added. After 15 min the reaction mixture was directly injected into a prep HPLC C18 column with a gradient of 10-40% ACN/H2O (0.1% TFA). The purity of the Thanatin-SOG compound was confirmed by UPLC analysis and the identity was verified by HR-ESI with a calculated mass of 652.1353 (M + 6H)+ and a measured m/z value of 652.1360 (M + 6H)+ (Supplementary Fig. 10).

**Proteome-wide and light-activated target deconvolution in prokaryotic systems.** Castaniol and colleagues (J. Bacteriol. 259(22)4478) were illuminated and analyzed with NeutrAvidin Protein DyLight 488 1:50 for 20 min prior analysis using an automated antibody detection system. Promoters were cloned into the pPL2 integration vectors with an HA tag using PCR cloning. The wall anchor domain protein (WAP) and A0A0E1R383 (ABC transporter), and their native promoters were used. Surface proteins were detected using a high-throughput flow cytometric assay. The phage-SOG complex was precipitated by adding 1/5 volume of 20% PEG/2.5 M NaCl and incubated for 2 h at 4 °C. The supernatant was removed and unreacted SOG molecules, and the 3-dye pellet was removed in 1 ml of distilled water subjected to PFU determination. The phage assay, 990 µl overnight cultures of L. monocytogenes cells were mixed with 10 µL of serially diluted phage stock solution (in SM buffer). The culture was then mixed and vortexed with 5 ml of melted soft agar (50 °C) and poured onto the solid agar plates. Solid agar plates were incubated at 30 °C for a day and phage titer was determined based on observed plaque numbers.

**Bacteriophage-guided exploration of virus-targeted host surfaceomes.** Listeria monocytogenes cells (strain WSLC 1042) were treated with 40 µg/mL gentamicin for 1 h at RT before incubation with SOG-coupled Listeria A500 phages at an MOI of 10 for 30 min at 4 °C in the dark. For each sample, bacteria corresponding to 3.5 × 10^6 colony-forming units (CFU) were washed with ice-cold PBS, resuspended in chilled photo-oxidation buffer, and illuminated at a working distance of 20 cm with 590-nm Precision LED spotlights for 15 min or left in the dark. The pH was then adjusted to 4.2 and the samples were centrifuged and resuspended in chilled labeling buffer for 50 min at 4 °C in the dark. Cells were extensively washed with PBS, snap-frozen as cell pellets in liquid nitrogen, and stored at −80 °C until further processing.

**Generation and characterization of SOG-coupled bacteriophages.** Gram-positive L. monocytogenes specific bacteriophages were coupled with thiorehodamine SOG by mixing 1.2 mL of Listeria A500 phage solution (a titer of 10^13 plaque-forming units (PFU)/ml) with 60 µl of 0.2 M NaHCO3 and 30 µl of 0.15 mM NHS-functionalized thiorehodamine. The mixture was incubated at RT in the dark for 1 h. The phage-SOG complex was precipitated by adding 1/5 volume of 20% PEG/2.5 M NaCl and incubated for 2 h at 4 °C. The supernatant was discarded to remove unreacted SOG molecules, and the 3-dye pellet was resuspended in 1 ml of chilled labeling buffer for 50 min at 4 °C in the dark. For the PFU assay, 990 µl overnight cultures of L. monocytogenes cells were mixed with 10 µl of serially diluted phage stock solution (in SM buffer). The culture was then mixed and vortexed with 5 ml of melted soft 1/2 BHI agar (50 °C) and poured onto the solid agar plates. Solid agar plates were incubated at 30 °C for a day and phage titer was determined based on observed plaque numbers.

**Flow cytomtery analysis of murine immune cells.** Cells were stained for flow cytometry in PBS for 20 min at RT. The following antibodies were purchased from BioLegend (San Diego, United States) and used for flow cytometry: BV510 (cat: 100752, clone: 53-6.7, dilution 1:200), CD45.1-PacificBlue (cat: 110722, clone: A20, dilution 1:200), CD44-APC (cat: 559250, clone: IM7, dilution 1:100), CD11c-PerCP (cat: 117326, clone: N418, dilution 1:200), CD69-PeCy7 (cat: 104512, clone: H1:6F3, dilution 1:100), CD25-FITC (cat: 555072, clone: 3C7, dilution 1:100). The viability of cells was determined by using fixable near-IR dead cell staining (LifeTechnologies, Carlsbad, United States). Biotinylation was assessed by streptavidin-PE (BioLegend) staining for 10 min at RT. T-cell activation was determined by the expression of GFP expression under the control of the Nur77 promoter. Data was acquired on a Canto^TM flow cytometer (BD Bioscience, Allschwil, Switzerland) and analyzed using FlowJo software (Treestar, Ashland, OR, USA) (see Supplementary Fig. 12 for gating strategy).
8 min with 98% buffer B. Samples were acquired in DDA mode with a fixed cycle time of 3 s (universal method). Electrospray voltage and capillary temperature were thereby set to 2.2 kV and 320 °C, respectively. A high-resolution survey mass spectrum (from 395 to 1500 m/z) acquired in the Orbitrap with a resolution of 120,000 at m/z 200 (automatic gain control target value 5 × 105 and maximum injection time 100 ms) was followed by MS/MS spectra with a resolution of 30,000 of most-abundant peptide ions with a minimum intensity of 2 × 106 that were selected for subsequent higher-energy collision-induced dissociation fragmentation with a collision energy of 28% and an isolation window of 1.4 Da. Fragmented precursors were dynamically excluded for 30 s. For antibody-guided LUX-MS samples acquired in DIA mode, peptides were separated by reversed-phase chromatography on an HPLC column (75-μm inner diameter, New Objective) that was packed in-house with a 30-cm stationary phase (ReproSil-Pur C18-AQ, 1.9 μm) and connected to a nano-flow HPLC with an autosampler (EASY-nLC 1200, Thermo Scientific). The HPLC was coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source (Thermo Scientific). Peptides were loaded onto the column with 100% buffer B (99.9% H2O, 0.1% FA) and eluted at a constant flow rate of 300 nl/min with a 70-min linear gradient from 6–28% buffer B (99.9% ACN, 0.1% FA) followed by a 4-min transition from 28 to 50% buffer B. After the gradient, the column was washed with 10 min with 98% buffer B, 4 min with 10% buffer B, and 8 min with 98% buffer B. Electrospray voltage was set to 2.2 kV and capillary temperature to 320°C. A survey scan acquired in the orbitrap with 120,000 resolution, 50 ms max injection time, and an AGC target of 3 × 106 was followed by 24 precursor windows with fragmentation at a collision energy of 28% and MS/MS spectra acquisition in the orbitrap with 30,000 resolution. The injection time was set to auto, AGC target 1 × 106, and mass range to 300–1700 m/z. For the spectral library, the samples were additionally measured on the Q-Exactive Plus platform in DDA mode as described above for gp33-guided LUX-MS samples acquired in DIA mode, peptides were separated by reversed-phase chromatography on an HPLC column (75-μm inner diameter, New Objective) that was packed in-house with a 30-cm stationary phase (ReproSil-Pur C18-AQ, 1.9 μm) and connected to a nano-flow HPLC with an autosampler (EASY-nLC 1200, Thermo Scientific). The HPLC was coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source (Thermo Scientific). Peptides were loaded onto the column with 100% buffer A (99.9% H2O, 0.1% FA) and eluted at a constant flow rate of 250 nl/min with a 70-min linear gradient from 6 to 28% buffer B (99.9% ACN, 0.1% FA) followed by a 4-min transition from 28 to 50% buffer B. After the gradient, the column was washed for 10 min with 98% buffer B, 4 min with 10% buffer B, and 8 min with 98% buffer B. Electrospray voltage was set to 2.2 kV and capillary temperature to 320°C. A survey scan acquired in the orbitrap with 120,000 resolution, 50 ms max injection time, and an AGC target of 3 × 106 was followed by 24 precursor windows with fragmentation at a collision energy of 28% and MS/MS spectra acquisition in the orbitrap with 30,000 resolution. The injection time was set to auto, AGC target 1 × 106, and mass range to 300–2000 m/z. For the spectral library, the replicate of the illuminated and non-illuminated conditions were pooled and analyzed on the same instrumental setup in DDA mode using a universal method with equal gradient and collision energy of 27% and 30%.

Data analysis. Raw files acquired in DDA mode were searched against corresponding SwissProt-reviewed protein databases containing common contaminants using Comet (v.2015.01) within the Trans Proteomic Pipeline v.4.7 (SPC/BSB Seattle). Peptides were required to be fully tryptic with a maximum of two missed cleavage sites, carbamy- lation, and methionine oxidation in all search options. Peptides were filtered for high confidence using the following criteria: ions with charge state unassigned, 1 or >6 were excluded from fragmentation. For bacteriophage-guided LUX-MS samples acquired in DDA mode, peptides were separated by reversed-phase chromatography on an HPLC column (75-μm inner diameter, New Objective) that was packed in-house with a 30-cm stationary phase (ReproSil-Pur C18-AQ, 1.9 μm) and connected to a nano-flow HPLC with an autosampler (EASY-nLC 1200, Thermo Scientific). The HPLC was coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source (Thermo Scientific). Peptides were loaded onto the column with 100% buffer A (99.9% H2O, 0.1% FA) and eluted at a constant flow rate of 300 nl/min with a 70-min linear gradient from 6–28% buffer B (99.9% ACN, 0.1% FA) followed by a 4-min transition from 28 to 50% buffer B. After the gradient, the column was washed with 10 min with 98% buffer B, 4 min with 10% buffer B, and 8 min with 98% buffer B. Electrospray voltage was set to 2.2 kV and capillary temperature to 320°C. A survey scan acquired in the orbitrap with 120,000 resolution, 50 ms max injection time, and an AGC target of 3 × 106 was followed by 24 precursor windows with fragmentation at a collision energy of 28% and MS/MS spectra acquisition in the orbitrap with 30,000 resolution. The injection time was set to auto, AGC target 1 × 106, and mass range to 300–2000 m/z. For the spectral library, the replicate of the illuminated and non-illuminated conditions were pooled and analyzed on the same instrumental setup in DDA mode using a universal method with equal gradient and collision energy of 27% and 30%.
References

1. Bausch-Fluck, D., Milani, E. S. & Wollscheid, B. Surfaceome nanoscale organization and extracellular interaction networks. Curr. Opin. Chem. Biol. 48, 26–33 (2019).

2. Jacobson, K., Liu, P. & Lagerholm, B. C. The lateral organization and mobility of plasma membrane components. Cell 177, 806–819 (2019).

3. Mattila, P. K., Batista, F. D. & Tetreau, B. Dynamics of the actin cytoskeleton mediates receptor cross talk: an emerging concept in tuning receptor signaling. J. Cell Biol. 212, 267–280 (2016).

4. Casalotto, J. B. & McClatchey, A. I. Spatial regulation of receptor tyrosine kinases in development and cancer. Nat. Rev. Cancer 12, 387–400 (2012).

5. Garcia-Fernandez, E. et al. Membrane microdomain disassembly inhibits immune checkpoint protein PD-L1.

6. Viswanathan, K., Verweij, M. C., John, N., Malouli, D. & Früh, K. Quantitative mass spectrometry data generated in this study have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository96 under the accession code PXD020481 (LUX-MS data set).

7. SwissProt-reviewed human protein databases were downloaded from https://www.uniprot.org/. Source data are provided with this paper.

Received: 4 November 2020; Accepted: 9 November 2021; Published online: 02 December 2021

Using CytoScape (v.3.7.1)24. Protein topology information was retrieved and visualized using the PROTER interactive webtool25. Protein structures were visualized using PyMOL. Molecular Graphics System (v.2.0.4, Schrodinger, LLC.). Mass spectrometry data were visualized using ggplot2 (v.3.3.2). Interactive volcano plots were created using an in-house developed R-script using the plotly package (https://plotly-r.com). The 3D-modeled pipette tip of Fig. I was created by Thomas Spletstoeßer (www.scistyle.com). All other schematic figures were created using the BioRender webtool (https://biorender.com/).
52. Kleiverland, C. R. Peripheral Blood Mononuclear Cells. In The Impact of Food Bioactives on Health: in vitro and ex vivo models (eds. Verhoeckx, K. et al.) 161–167 (Springer International Publishing, 2015).

53. Matheson, I. B. C. & Lee, J. Chemical reaction rates of amino acids with singlet oxygen. Photochem. Photobiol. 29, 879–881 (1979).

54. Oostindie, S. C. et al. CD20 and CD37 antibodies synergize to activate complement by Fc-mediated clustering. Haematologica 104, 1841–1852 (2019).

55. Gentner, E. et al. Association of CXCR4 with IgM and IgD BCR isotypes: role in B cell malignancies. Blood 132, 1852–1852 (2018).

56. Klašener, K., Maity, P. C., Hobeika, E., Yang, J. & Reth, M. B cell activation involves nanoscale receptor reorganizations and inside-out signaling by Syk. Elife 3, e02069 (2014).

57. Matilla, P. K. et al. The actin and transferrin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling. Immunity 38, 461–474 (2013).

58. Malavasi, F. et al. CD38 and chronic lymphocytic leukemia: a decade later. Blood 118, 3470–3478 (2011).

59. Polyak, M. J., Li, H., Shariat, N. & Deans, J. P. CD20 homo-oligomers physically associate with the B cell antigen receptor. Dissociation upon receptor engagement and recruitment of phosphophosferates and calmodulin-binding proteins. J. Biol. Chem. 283, 18545–18552 (2008).

60. Petric, R. J. & Deans, J. P. Colocalization of the B cell receptor and CD20 followed by activation-dependent dissociation in distinct lipid rafts. J. Immunol. 168, 8866–8891 (2002).

61. Szöllősi, J., Horejsí, V., Bene, L., Angelisová, P. & Damjanovich, S. Supramolecular complexes of MHC class I, MHC class II, and CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. J. Immunol. 157, 2939–2946 (1996).

62. Powell, L. D. & Varki, A. The oligosaccharide binding specificity of the B cell antigen receptor. Dissociation upon receptor engagement and recruitment of phosphophosferates and calmodulin-binding proteins. J. Biol. Chem. 283, 18545–18552 (2008).

63. desk, S., Stassen, H. A. & Wall, J. M. Toward understanding B cell receptor signaling. J. Immunol. 177, 329–336 (2001).

64. Esbelin, J. et al. Comparison of three methods for cell surface proteome extraction of Listeria monocytogenes biofilms. OMICS 22, 779–787 (2018).

65. Wu, C.-H., Liu, I.-J., Liu, R.-M. & Wu, H.-C. Advancement and applications of peptide phage display technology in biomedical science. J. Biomed. Sci. 23, 8 (2016).

66. Pollock, S. B. et al. Highly multiplexed and quantitative cell-surface protein profiling using genetically barcoded antibodies. Proc. Natl. Acad. Sci. USA 115, 2836–2841 (2018).

67. Dunne, M. et al. Reprogramming bacteriophage host range through structure-guided design of chimeric receptor binding proteins. Cell Rep. 29, 1336–1350.e4 (2019).

68. Pasqual, G. et al. Monitoring T cell-dendritic cell interactions in vivo by intercellular enzymatic labelling. Nature 553, 496–500 (2018).

69. Ritter, A. T. et al. Actin depletion initiates events leading to granule secretion at the immunological synapse. Immunity 42, 864–876 (2015).

70. Fuertes Marraco, S. A. et al. Novel murine dendritic cell lines: a powerful auxiliary tool for dendritic cell research. Front. Immunol. 3, 331 (2012).

71. Moran, A. E. et al. T cell receptor signal strength in Treg and INKt cell development demonstrated by a novel fluorescent reporter mouse. J. Exp. Med. 208, 1279–1289 (2011).

72. Kong, A. T., Lepreost, F. V., Avtonomov, D. M., Mellacheruvu, D. & Nesvizhskii, A. I. MSfragger: ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. Nat. Methods 14, 513–520 (2017).

73. Chang, H.-Y. et al. Crystal-C: a computational tool for refinement of open search results. J. Proteome Res. 19, 2511–2515 (2020).

74. Esbelin, J. et al. MSstats: an R package for statistical analysis of quantitative mass spectrometry-based proteomic experiments. Bioinformatics 30, 2524–2526 (2014).

75. Carpenter, A. E. et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 7, R100 (2006).

76. Suender, B. et al. Image-based ex-vivo drug screening for patients with aggressive haematological malignancies: interim results from a single-arm, open-label, pilot study. Lancet Haematol. 4, e695–e606 (2017).

77. Gibson, D. G. et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–349 (2009).

78. Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504 (2003).

79. Oinas, U., Ahrens, C. H., Müller, S. & Wollcheid, B. Protrin: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics 30, 884–886 (2014).

80. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. 47, D442–D450 (2019).

Acknowledgements
We acknowledge Julia Boshard, Heidi Funke, and Dr. Patrick Pedrioli for constructive discussions and specific comments on the manuscript. We specifically acknowledge Dr. Damaris Bausch-Fluck and Dr. Andreas Frei for their strategic input and critical discussion with the conceptual stages of the project. We are grateful to the members of the B.W. research group for suggestions and support at all stages of the project. We thank J.R. Wyatt for scientific text editing and T. Spletstoesser for graphical support. We thank Stephan Schneider for the technical assistance on phag endocytosis and coupling with SOGs. We further acknowledge Dr. Sebastian Müller, Dr. Patrick Pedrioli, and Dr. Silvana Albert for assistance in analyzing the SILAC-DHA LUX-M5 data set and David Vogel for contributing to antibody-guided LUX-M5 experiments. The following agencies contributed and are acknowledged for their generous support: ETH grant ETH-30 17-1 and grant ETH-25 15-2 and Swiss National Science Foundation (grant 31003A_160259 for B.W. and NIH grants R01-GM-094231 and U24-CA210967 for A.L.N.

Author contributions
M.M. performed all experiments except those noted below. F.G. and N.B. performed isolation and FACS analysis of murine immune cells. Y.S. produced SOG-coupled bacitracin, and various beta-lactam antibiotics. M.M., F.G., N.B., Y.S., J.R.P., E.M.C., J.W.B., B.S., J.A.R., M.J.L., A.O., and B.W. contributed and are acknowledged for their generous support: ETH (grant ETH-30 17-1 and grant ETH-25 15-2) and Swiss National Science Foundation (grant 31003A_160259 for B.W. and NIH grants R01-GM-094231 and U24-CA210967 for A.L.N.

Competing interests
James R. Prudent is an employee/CEO of Centrose LLC, Madison, Wisconsin, USA, a private company. All other authors declare no competing interests.
