Studying the impact of novel narrow-spectrum β-lactam antibiotics on the release of peptidoglycan fragments by the microbiome and the host response to these fragments.

β-lactam antibiotics are arguably the most used antibiotics in human medicine. Unfortunately, their extensive use has also led to a systematic attack against our beneficial commensal flora and a gradual loss of diversity which is linked to the rise of non-communicable diseases such as diabetes, chronic inflammatory diseases including Crohn's or neurodegenerative diseases such as Parkinson. There is a growing appreciation for the need to develop new antibiotics that overcome existing resistance mechanisms while preserving at best as possible the microbiome diversity. β-lactam treatment often leads to bacterial lysis and the release of peptidoglycan (PG) fragments and their associated biological activity on the host (1). The detection of these fragments by the host is mediated by pattern recognition receptors (PRRs), such as NOD2. Binding of PG fragments to NOD2 triggers innate immunity inflammatory response. Interestingly, mutations in the *card15* gene, encoding NOD2, are associated with increased risk of the chronic diseases cited above (2-4). β-lactam antibiotics target penicillin-binding proteins which are central for the assembly of the essential PG layer of the bacterial cell wall. The release of PG fragments is mediated by autolysins, enzymes that cleave bonds in mature PG. The role of autolysins has been mainly investigated by determining the cleavage specificity of purified enzymes using as substrate the purified PG macromolecule or fragments thereof. In contrast, little is known on the autolysins that actually participate in β-lactam-induced bacterial cell lysis and the mechanisms underlying the dysregulation of their activity triggered by drug exposure.

Our two labs (I Boneca, Institut Pasteur, and G Sezonov, Sorbonne-Université) are currently collaborating in a project funded by PPR-AMR (Programme de recherche prioritaire-Antibiorésistance), which is coordinated by Partner 1 (NASPEC project, https://anr.fr/ProjetIA-20-PAMR-0007). The goal of this project is to develop new antibiotics that function as dual drugs by combining two β-lactams in prodrugs that will be selectively activated by the WHO-defined critical priority pathogens (5). β-lactam resistance in these pathogens is achieved by the production of β-lactamases [carbapenemases and Extended Spectrum β-lactamases (ESBL)], enzymes that cleave the antibiotics. In our approach, selectivity will be achieved as hydrolysis of the first "sacrificial" β-lactam by carbapenemases and ESBLs will be required to release the second β-lactam "war head" in an active form. By selectively targeting the resistant strains and sparing the commensal flora, these drugs reduce drastically the triggering of endogenous autolysins to the resistant strains and the proinflammatory potential of the lysed PG layer induced by classical beta-lactam exposure. The originality of this project lies on a synergy between Partner 1 (Sorbonne Université), who will focus on the molecular aspects of PG fragments release and Partner 2 (Institut Pasteur), who will evaluate the activities of antibiotics and prodrugs on the microbiota. The novelty of this project is to establish the connection between antibiotics, PG turnover and inflammation. These fundamental aspects will be strengthened by the evaluation of the prodrugs using organ-on-chips and animal models.

Task 1 (Partner 1 - SU). Contribution of autolysins to the β-lactam-induced release of PG fragments in Gram-positive and Gram-negative bacteria.

Members of the intestinal microbiota, in particular the enterococci, have been shown to release PG fragments that modulate the host immune system (6,7). This phenomenon has not been explored in the context of antibiotic exposure. Our hypothesis is that β-lactam exposure enhances the production of PG fragments by autolysins leading to an increase in the host inflammatory response. The goal of this task is to identify the set of autolysins that promote the release of PG fragments in the presence of β-lactams using both a Gram-negative (*Escherichia coli*) and a Gram-positive (*Enterococcus faecalis*) model. Partner 1 has generated a large collection of mutants lacking specific combinations of autolysins in both species. PG fragments will be quantified (see Task 2) in these mutants and in wild-type strains exposed to representatives of the main classes of β-lactams. The pathway underlying PG degradation by autolysin will

be inferred from both the structure of the released fragments and the known specificity of the enzymes. The regulation of the production of the autolysins and of the activators that control their activity will be investigated by classical approaches (RNA seq, Western blot, and zymogram).

Task 2 (Partners 1 and 2 – SU and IP). Evaluation of currently-used β-lactams and pro-drugs to induce release of peptidoglycan fragments by commensal and MDR bacteria. Susceptible and isogenic resistant strains will be grown *in vitro* in chemically defined media and exposed to selected β-lactams and pro-drugs (concentration range from 0.4 to 4 times the MIC). Sixteen hours post-exposure, supernatant of culture will be collected, filtered at 0.2 µm, desalted on SPE cartridges and subjected to LC-MS/MS for the identification of released PG fragments (8). Identification will be done semi-automatically on a Dyonex Ultimate 3000 - QExactive Focus (Thermo) with an in-house library of PG fragments. Quantification will be done using standard curves of synthetic PG fragment. Efficiency of recovery and standard curves will be done by spiking the chemical defined media with known concentrations of synthetic PG fragments. Supernatants will also be assessed for their ability to stimulate the host PG receptors, NOD1 and NOD2, in standard luciferase (or alkaline phosphatase) NF-kB activation reporter assays in HEK293 cells (9).

Task 3 (Partner 2 - IP). Evaluation of β-lactams and pro-drugs to induce release of PG fragments in organoids and a gut organ-on-chip (OOC) system. Most of the PG is absorbed and translocated in the small intestine, in particular, the ileum (8). First, we will use isolated ileal crypt Lgr5+ stem cells to generate and propagate ileal organoids (10). Organoids are maintained in matrix gel and are oriented with the lumen inside the organoid, requiring microinjection to study the impact of bacteria and their products. This is time consuming and prevents high through put studies. To overcome this limitation, we will invert them by the method described by Amieva and colleagues (11). Supernatants and/or bacteria of interest treated or not with β-lactams and pro-dugs will be incubated with organoids and the activation of NF-kB will be analyzed at different time points (usually between 30 minutes and 6 hours) by immunofluorescence microscopy on an Opera Phenix confocal microscope. In parallel, organoids will be isolated and bacteria washed away by differential centrifugation. Purified organoids will be dissociated and their content collected for analysis by LC-MSMS for the presence of translocated PG fragments. Dissociated cells will be further lysed and their intracellular content also analyzed by LC-MSMS for PG fragments.

Task 4 (Partner 2 -IP). Evaluation of β-lactams and pro-drugs to induce release of PG fragments *in vivo*. Mice colonized with the oligoMM12 community or conventional mice, with or without enterobacteria producing KPC-2, NDM1, OXA48, or CTX-M-15, will be treated with β-lactams or prodrugs. The lumen content of the small intestine will be collected, centrifuged, and filtered to remove bacteria and solid debris. The filtrate will be analyzed by LC-MS/MS for the identification and quantification of released PG fragments. In parallel, the gut tissue will be collected, dissociated, filtered and analyzed by LC-MS/MS for the identification and quantification of PG fragments taken up by the intestine. Both lumen and intestinal fractions will be tested in the luciferase NF-kB activation reporter as Task 2.

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