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GUT EPITHELIAL REGENERATION AND THE MICROBIOME: DECRYPTING SIGNALS IN THE CRYPT

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SUMMARY

The intestinal crypt represents a strategic niche where signals are integrated to regulate epithelial regeneration, due to the presence of stem cells and their associated lineages such as Paneth cells and proliferative cells of the transit-amplifying compartment.

We have selected the intestinal crypt as a model to study microbiota-host cross-talks. Our starting hypothesis was that among the complex assemblage of luminal and mucosal commensal species that compose the microbiota, a limited set may enter into a mutualistic interaction with the cells in the intestinal crypt reflecting a long co-evolution in which the epithelial regenerative apparatus may benefits from microbiota-mediated protection.

We are currently developing a cellular microbiology of the intestinal crypt, particularly in the caecum and the colon where we have identified a "crypt specific core microbiota" (CSCM) that is composed of a restricted set of strictly aerobic, non-fermentative microorganisms whose possible function of crypt "gatekeeper" is currently under study. In this context, we have provided the first evidence that a bacterial product, the peptidoglycan fragment muramyl-dipeptide (MDP), exerts a strong and direct NOD2-dependant cytoprotective effect on intestinal stem cells upon induction of a cytotoxic stress.

THE GUT MICROBIOTA: THE FORGOTTEN ORGAN

The microbiota is a complex ecosystem populated by a diverse community of microorganisms, mainly dominated by bacteria, but also comprised of archaea, fungi, protozoa, and viruses, that inhabit a specific area of the. The most studied is the intestinal microbiota, particularly abundant in the colon, in which Bacteroidetes and Firmicutes represent the dominant phyla.

The microbiota has been defined as a "forgotten organ" (*O'Hara* and *Shanahan*, 2006), playing a central role in health and disease. The microbiota provides protective functions by forming a natural defence barrier against pathogens, by occupying niches and receptors, and by producing colicins. It has metabolic functions such as the fermentation of non-digestible dietary residues, the synthesis of vitamins, and the detoxification of dietary carcinogens (*Sansonetti*, 2008). The microbiota contributes to the regulation of the intestinal barrier, modulating physical properties, such as the composition and the thickness of the mucus layer or the tight junctions between the epithelial cells (*Jakobsson* et al., 2015; *Ulluwishewa* et al., 2011). Moreover,

the gut microbiota interacts with the immune system, stimulating its development and maturation (*Chow* et al., 2010).

The microbiota, as well as bacterial products and bacterial metabolites, provide continuous stimuli to the entire epithelial layer, possibly indirectly affecting stem cells that could sense signals from neighbouring cells responding to bacterial agonists. These signals could influence the survival of stem cells and therefore control both proliferation and regeneration of the whole epithelium. The dialogue between the microbiota and the intestinal cells (immune and epithelial) is mainly due to the expression of innate immune receptors (pattern recognition receptors - PRRs) that recognize conserved bacterial motifs defined as "microbe-associated molecular patterns" (MAMPs) (*Kawai* and *Akira*, 2011).

Principal members of the PRRs are the Toll-like receptors (TLRs) and the nucleotide oligomerization domain receptors (NODs). TLRs are transmembrane proteins located at the cellular plasma membrane or at the endosomal membranes. Instead, the NODs are cytosolic proteins.

Upon MAMPs recognition, PRRs initiate a signalling cascade that usually trigger to an inflammatory response, through the activation of the transcriptional factor NF-κβ responsible of the production of pro-inflammatory cytokines such as IL-1β, IL-6, or IFN-γ. Indeed, in the gut, PRRs seem to be crucial for bacterial-host communications and for maintaining intestinal homeostasis (*Abreu* et al., 2005).

Several studies have shown that bacteria or bacterial motifs are necessary for proper host development not only of the gut but also, for instance, of the immune system. The recognition of commensal microflora by TLRs expressed by epithelial cells is required for intestinal homeostasis (*Rakoff-Nahoum* et al., 2004), while the interaction between fragments of peptidoglycan and NOD1 receptors are necessary and sufficient to induce the genesis of isolated lymphoid follicles (*Bouskra* et al., 2008). One can reasonably hypothesize that stem cells, located in the intestinal crypts, could also express PRRs to recognize bacteria, therefore directly responding themselves to the microbiota. It represents a rare situation in which a differentiating and proliferative epithelium is directly exposed to bacteria, both permanent symbionts and occasional pathogens. One can thus hypothesize that co-evolution of mammals with their gut microbiota has led to a balance, protecting the crypt against microbial insults while maintaining a capacity to sense and integrate microbial signals to convert them into signals boosting epithelial regeneration. We have selected the intestinal crypt as a model to study microbiota-host cross-talks. On one side exploring the possibility that a particular microbiota (i.e., crypt specific core microbiota [CSCM]) is selected to survive in the crypt environment particularly because of its adaptation to the niche environment. Such a CSCM may play a homeostatic role by acting as a gatekeeper, preventing the proliferation of more aggressive symbiotic microorganisms (i.e., pathobionts) (*Chow* and *Mazmanian*, 2010) and pathogens, and by providing optimal signalling to the crypt and its environment.

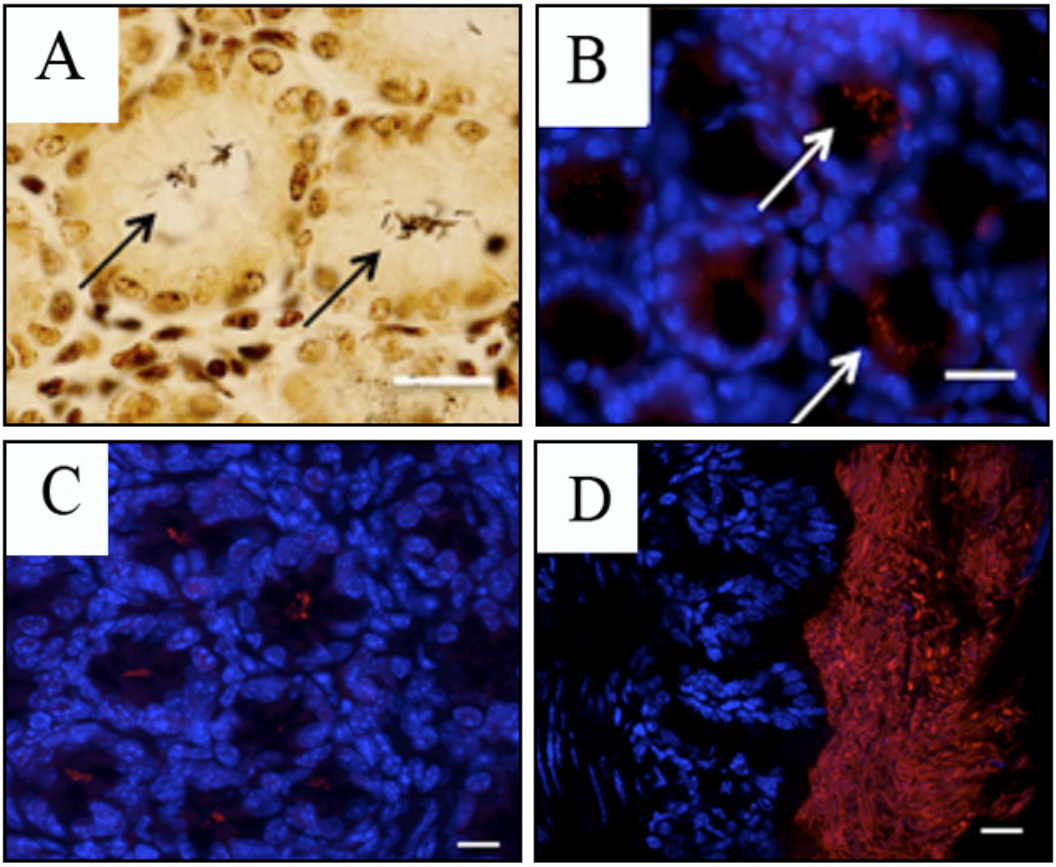

Figure 1: Bacteria reside in the murine proximal colonic crypts (modified from *Pédron* et al., 2012).
A: Warthin-Starry staining, and B: FISH with the universal 16S rRNA gene-targeted probe are shown. Black and white arrows indicate the presence of bacteria. Using specific probes, *Acinetobacter* (C) and the Firmicutes (D) are localized mainly in the crypts and lumen of the colon, respectively.

A CRYPT-SPECIFIC CORE MICROBIOTA RESIDES IN THE MOUSE COLON

Our starting hypothesis was that among the complex assemblage of luminal and mucosal commensal species that compose the microbiota, a limited set may enter into a mutualistic interaction that may reflect a long co-evolution that established a situation in which the epithelial regenerative apparatus may benefit from microbiota-mediated protection.

At stake is the homeostasis of a particularly sensitive zone where adult stem cells are directly exposed to the gut flora.

Preliminary evidence collected from Whartin-Starry (silver/nitrate) staining of various segments of the intestinal tract of various mouse lines reproducibly showed the presence of a small cluster of bacteria located at the crypt bottom in the caecum and proximal colon. These bacteria were not seen in general in the duodeno-jejunum and in the distal colon.

These data were confirmed by fluorescent *in situ* hybridization (FISH) using universal 16S rRNA gene-targeted probe, indicating that these bacteria were alive and metabolically active (Figures 1A and 1B).

We next developed a dedicated pipeline to molecularly identify the relevant bacteria. We combined laser capture microdissection (LCM), DNA amplification with primers flanking the V5-V6 hypervariable regions of 16S rRNA encoding sequences, and 454 sequencing. We clustered the sequences into species-level operational taxonomic units (OTUs) of 97% sequence similarity by the furthest-neighbour method, using the Mothur software program. The analysis was carried-on on mice with different backgrounds and obtained from several providers. We compared samples obtained from luminal and crypt regions and fourteen bacterial phyla were detected, but most sequences could be assigned to five phyla: Firmicutes (73%), Beta- and Gamma-proteobacteria (16%), Actinobacteria (3.5%), and Bacteroidetes (1.7%).

We have compared samples obtained from luminal versus crypt content and we have found that whereas members of the Bacteroidetes were rather poorly represented within both crypt and luminal samples, the Firmicutes represented the majority of luminal sequences (95.5%). The Proteobacteria represented the most abundant sequences found in crypts (47.6%, versus 2.7% for the lumen). Interestingly, the major bacterial family identified was the Moraxellaceae (23.7%), with 23% of *Acinetobacter* spp. sequences in crypts versus 1.6% in the lumen. OTUs from *Acinetobacter* spp. were shared among all crypts, representing a possible common bacterial phylogroup with possible quantitative variations according to the mouse line studied. However, in all cases, levels of *Acinetobacter* spp. in crypts were significantly higher than those observed in luminal samples, in other words a strictly aerobic, non-fermentative genus belonging to the gamma-proteobacterial family.

Thus, using FISH with probes specific for bacterial families and/or genera, the presence of *Acinetobacter* spp. was unequivocally confirmed in crypt samples from different murine strains (more than 10% of the crypts were colonized by *Acinetobacter*, as visualized by FISH) whereas members of the Firmicutes were localized in the lumen (Figures 1C and 1D).

In order to confirm the tropism of *Acinetobacter*, germfree mice were colonized using a conventional microbiota originating from littermates. After 26 days of colonization, bacteria were observed by silver staining in colonic crypts, and the presence of *Acinetobacter* spp. was clearly demonstrated by FISH.

PROTECTIVE EFFECT OF COMMENSALS ON INTESTINAL STEM CELLS

In this context, it was clear that our hypothesis also needed to be validated by demonstrating the existence of a true cross-talk between the microbiota, possibly more specifically the CSCM and the crypt. For this we took advantage from the recently described culture system for intestinal crypts developed by Hans Clever's group: the "miniguts" or "organoids" (*Sato* et al., 2009).

Following purification of murine intestinal crypts and their embedding in

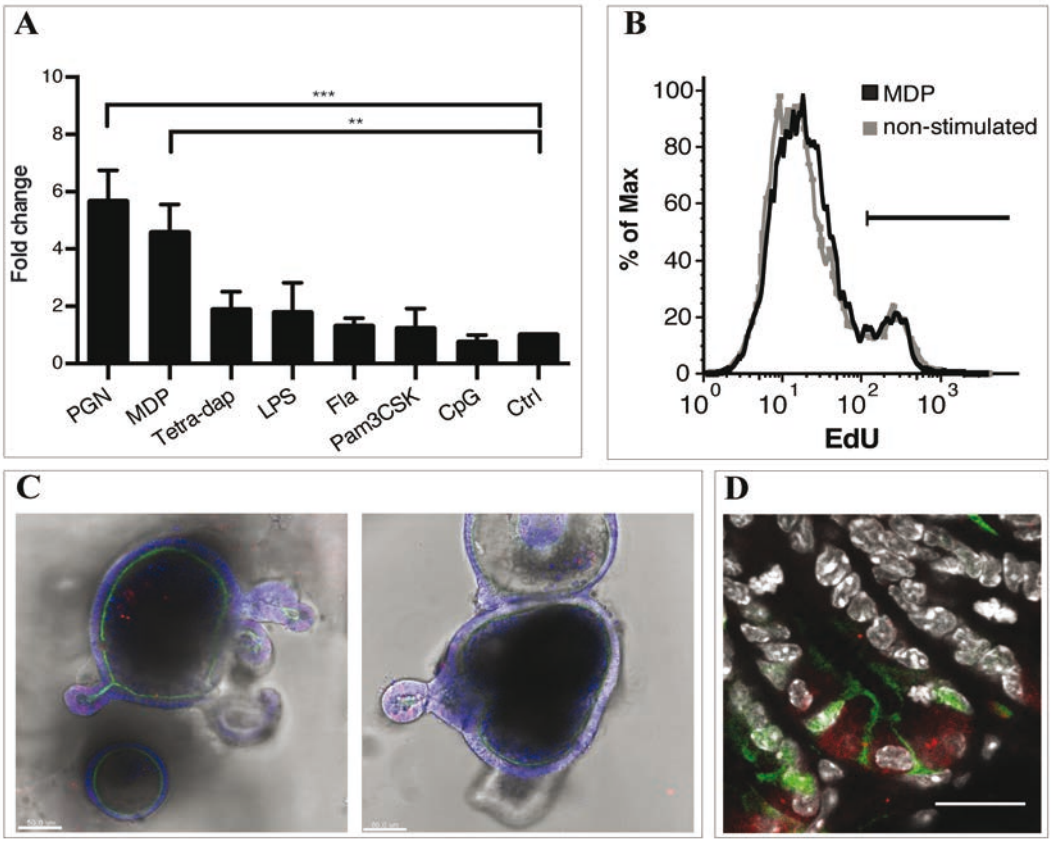

Figure 2: Protective Effect of MDP on intestinal stem cells (modified from *Nigro* et al., 2014).
A: Crypts were stimulated with soluble sonicated peptidoglycan (PGN), muramyl-dipeptide (MDP), muramyl-tetrapeptide (Tetra-dap), *Escherichia coli* lipopolysaccharide (LPS), flagellin (Fla), synthetic lipoprotein (Pam3CSK), or unmethylated CpG dinucleotides (CpG). The fold change in the number of organoids over non-stimulated organoids (Ctrl) was calculated after 4 days of culture.
B: Cell proliferation was analysed by cytometry, monitoring EdU incorporation after 2 h. Representative profiles of MDP-treated (black) and non-treated organoids (grey) are shown.
C: Organoids stimulated (left) or not (right) with MDP were stained with anti-Ki67 (red). Nuclei are in blue and phalloidin in green.
D: The capacity of stem cells to express Nod2 was tested on small intestine sections from Nod2 KO mice. Cells expressing Nod2 are in green, and Paneth cells stained with lysozyme are in red; nuclei are in white.

Matrigel in the presence of essential growth factors such as R-Spondin, Noggin and Wnt ligands, organoids can be grown and maintained *in vitro* showing the progressive appearance of crypt-like structures composed of stem cells, as well as a transit-amplifying compartment followed by cell cycle arrest and differentiation into the various epithelial lineages: Paneth cells in close apposition to stem cells, bona fide epithelial cells, goblet cells and entero-endocrine cells (*Sato* et al., 2009). The organoids will form a three-dimensional structure, recapitulating the crypt-villus architecture, with an internal lumen.

When crypts were exposed, before embedding, to bacterial MAMPs (microbe-associated molecular patterns),

such as peptidoglycan (PGN) muramyl-dipeptide (MDP), muramyl-tri and tetrapeptide, lipopolysaccharide (LPS), flagellin (Fla), unmethylated CpG dinucleotides (CpG), and lipoproteins (Pam3CSK), only crypts exposed to PGN and MDP yielded 4-5 fold more organoids compared to not stimulated organoids (Figure 2A).

To show if the growth rate is affected by the presence of the MDP, all the organoids were imaged and their area measured. No difference was observed in the maximum size of organoids compared to controls, suggesting that stimulation with MAMPs did not affect the growth rate. To confirm this observation, treated and control organoids were tested after 4 days of culture for both EdU incorporation and Ki67 staining (Figures 2B and 2C). We did not observe any variation in the cell proliferation rates between organoids stimulated with MDP and controls, either globally or specifically in the transit-amplifying compartment. Therefore, epithelial proliferation was not affected by MDP stimulation. Moreover, we repeated the *in vitro* experiment of stimulation by using crypts from NOD1KO and NOD2KO mice and we observed the same increase in the yield only in the NOD1KO organoids, indicating that the observed phenotype is linked to NOD2 expression. Further experiments indicated that Lgr5+/CD24middle+ crypt cells corresponding to the stem cells expressed high levels of NOD2 transcripts, the intracellular cytosolic sensor involved in MDP recognition (Figure 2D). Using co-culture of single intestinal stem cells and Paneth cells from wildtype (wt) and/or NOD2KO mice, we demonstrated that this effect was due to the expression of NOD2 in stem cells and not in Paneth cells. To better evaluate the cytoprotective effect of MDP on the stem cells, we first performed *in vivo* experiments on mice in which the microflora was depleted by antibiotic treatment. We showed that mice gavaged with MDP were protected from the effects of doxorubicin, a DNA-intercalating agent that induces high levels of oxidative stress. To test the existence of a NOD2-dependent pathway of stem cell cytoprotection in the presence of microbiota-produced MDP, we carried out similar experiments in conventional wt and NOD2KO mice. We observed that wt mice, not NOD2KO mice, were able to regenerate the gut upon treatment with doxorubicin. Moreover, the wt mice presented higher numbers of crypt survival compared to NOD2KO mice, indicating a protective effect of NOD2. We also showed that crypts extracted from doxorubicin-treated mice were much more responsive to MDP regarding the yields of organoids, thereby indicating that the MDP-NOD2 pathway is rather protective than following cytotoxic aggression in homeostatic conditions. This altogether provides strong support to the concept of a protective cross-talk between the microbiota and the regenerative apparatus of the crypt with particular targeting to the stem cells.

CONCLUSION

Our approach has attempted to set the basis for a cellular microbiology of the mutualistic symbiosis established between elements of the intestinal microbiota and the gut mucosal tissues. We have shown that the caecal and colonic crypts harbour resident microbiota in the mouse and this bacterial population

is unexpectedly dominated by aerobic genera.

Interestingly, this microbiota resembles the restricted microbiota found in the midgut of invertebrates. Thus, the presence of our so-called "crypt-specific core microbiota" in the mouse colon potentially reflects a co-evolutionary process under selective conditions that can now be addressed. We suggest that CSCM could play both a protective and a homeostatic role within the colon.

Our central hypothesis is that the CSCM may act as a crypt "gatekeeper" with multiple complementary functions selected by the co-evolutionary process:

(i) Protection against the intrusion of pathogens or pathobionts that may disturb the fragile homeostasis required to preserve the balance of epithelial regeneration in physiological conditions or following a cytotoxic aggression, and "buffering" of inflammation that may be transiently caused by the accidental passage of a pro-inflammatory pathobiont.

(ii) Biodegradation of the xenobiotic molecules that may gain access to the crypt and induce strong genotoxic damage, particularly on stem cells.

It must be noticed that the CSCM isolates are typical environmental microorganisms with strong and diverse biodegradative activities, as assessed by the annotation of their genomic sequences (*Saffarian* et al., 2015).

Therefore, in the presence of stress, such as doxorubicin, the stem cells are more prone to respond to the MDP released by the microflora that enhances their protection from injury. This work highlighted a new role for NOD2 in intestinal homeostasis. In a steady-state condition, the bacteria perhaps do not give any specific advantage to stem cells, as indicated by the fact that NOD2KO mice are viable and do not present any particular difference compare to wt mice. However, upon injury, the presence of the microbiota and particularly the released MDP, has a protective effect on stem cells, making them more reactive to MDP itself and more resistant to death.

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