BRAIN-INTESTINE INTERACTION IN STROKE: DYSFUNCTION OF THE INTESTINAL BARRIER AND IMMUNE RESPONSES AS THERAPEUTIC TARGETS

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1. Project Summary

Acute brain damage induced by stroke causes multisystemic reactions altering physiological homeostasis. Therefore, far from being restricted to the brain, stroke causes disturbances of the immune system and immunodepression facilitating post-stroke infection. Infection is a frequent complication that can increase the risk of death of stroke patients. Several lines of evidence suggest that post-stroke infection is caused by commensal bacteria following translocation from the gut to the respiratory or urinary tracts. The gut and the brain are interconnected through the gut-brain axis comprising crosstalk signals derived from the gut microbiota affecting brain function and through neural control of gut function by the autonomic nervous system. This project aimed to underscore mechanisms underlying post-stroke infection focusing in the gut as a putative source of opportunistic bacteria infecting patients with severe stroke. The study of post-stroke infection has generated results collected in a manuscript that is currently under preparation, as described below. Accompanying this study, we investigated immune alterations induced by stroke involving trafficking of immune cells from the periphery to the damaged brain tissue, and the nexus of these cells with immune responses in the gut. To this end we focused on dendritic cells (DCs) for their capacity to sense danger signals generated by tissue damage and infectious agents, and capacity to migrate from peripheral organs to the stroke brain lesion. Overall these studies have contributed to three manuscripts where the support of the 'Fundació La Marató de TV3' is acknowledged (Del Fresno et al., 2018; Martínez-López et al., 2019; Gallizioli et al., 2020).

Acute brain damage causes infiltration of leukocytes; several lines of evidence support that some of these cells migrate to the brain from the gut, which is a reservoir for certain lymphocytes and DCs. DCs can sense the presence of gut bacteria through pattern recognition receptors. We reported that DCs migrate into the damaged brain tissue and exert functions different from resident microglia. Indeed, compared to microglia, infiltrating DCs are enriched in pattern recognition receptors of the C-type lectin family, such as macrophage-inducible C-type lectin (Mincle; Clec4e) and dendritic cell natural killer lectin group receptor-1 (DNGR-1; Clec9a). We identified different subsets of DCs infiltrating the brain after stroke. The signals involved in chemoattraction, and we found that the conventional cDC1 subset was beneficial in stroke (Gallizioli et al., 2020). cDC1 cells are equipped with DNGR-1, and our studies

showed that this damage sensing receptor is able to limit tissue damage, either caused by infection or under sterile conditions, by dampening neutrophil recruitment (Del Fresno et al., 2018). In contrast, in a previous study we found that the subset of cDC2 was detrimental in stroke by triggering the production of interleukin-17 (IL-17) in the brain. Independently, we identified the Mincle-FcRg-chain-Syk axis as a driver of Th17 differentiation in response to intestinal bacteria. Mincle-binding bacteria were enriched in the mouse small intestine mucosa. Moreover, Mincle played a critical role in the expression of antimicrobial molecules such as RegIIIg and the secretion of IgA. According to these findings, Mincle-deficient mice exhibited microbial translocation to the liver (Martínez-López et al., 2019). Microbial translocation from the gut could be causative of post-stroke infection. In our study (manuscript in preparation), experimental stroke caused organ colonization by commensal microbes typical of the gut rather than external pathogens, suggesting translocation of bacteria from the gut. We found stroke-induced metabolic alterations in liver and gut that can weaken the gut barrier function and detected signs of gut barrier alterations. We found that stroke induced dysbiosis in mice and stroke patients, and investigated the underlying mechanisms and the relationship between gut dysbiosis and post-stroke infection.

2. Results

The immune system is at the crossroads of the relationship between gut and brain, and has a prominent role after brain damage. Immune cells infiltrate the brain from the periphery and several lines of evidence support trafficking of lymphocytes and dendritic cells (DCs) from the gut to the brain after stroke. We studied the dynamics and functions of DCs infiltrating the brain after stroke and their participation in the regulation of inflammation and immune responses. We report that after cerebral ischemia microglia attract DCs to the inflamed brain, and astroglia produce the Flt3 ligand, supporting the development and expansion of CD11c+ cells. CD11c+ cells in the inflamed brain are a complex population derived from proliferating microglia and infiltrating DCs, including a major subset of OX40L+ conventional cDC2, and also cDC1, plasmacytoid, and monocyte-derived DCs. Despite sharing certain morphological features and markers, CD11c+ microglia and DCs display differential expression of pattern recognition receptors and chemokine receptors. Notably, infiltrating DCs are enriched in pattern recognition receptors of the C-type lectin family, such as Mincle

(Clec4e) and DNGR-1 (Clec9a), and DCs excel microglia in the capacity to present antigen through MHCI and MHCII. Of note, we found that cDC1s protect from brain injury after cerebral ischemia (Gallizioli et al., 2020).

cDC1s express DNGR-1, encoded by the gene Clec9a, which senses tissue damage and favors cross-presentation of dead-cell material to CD8+ T cells. We found that DNGR-1 mediates a reduction of damaging inflammatory responses induced by sterile and infectious tissue injury in mice. DNGR-1 deficiency leads to exacerbated inflammatory tissue damage in different organs and damaging conditions. This effect is B and T cell independent, and is attributable to increased neutrophilia in DNGR-1-deficient mice. Mechanistically, DNGR-1 engagement activates SHP-1 and inhibits MIP-2 (encoded by Cxcl2) production by cDC1s. This effect limits chemoattraction and recruitment of neutrophils to the lesion site and promotes disease tolerance. Thus, DNGR-1-mediated sensing of injury by cDC1s serves as a rheostat for the control of tissue damage, innate immunity, and immunopathology (del Fresno et al., 2018).

In contrast, we showed in a previous study that cDC2 played detrimental effects in brain ischemia by promoting the generation of interleukin-17 (IL-17) in the brain (Gelderblom et al., 2018,doi: 10.1161/STROKEAHA.117.019101). In independent studies we found that production of IL-17 in the gut in response to the gut microbiota ensures maintenance of intestinal barrier function and prevents bacterial translocation. These results highlight the critical balance between the detrimental effect of excessive inflammatory/immune responses for the brain lesion and the important role of these responses in the gut to prevent bacterial translocation and reduce the risk of infection. Accordingly, while anti-inflammatory interventions may be protective for the stroke brain lesion, they may increase the risk of stroke patients developing life-threatening post-stroke infections. In the gut, we found that the mechanism underlying the commensal-dependent production of IL-17 and other cytokines by CD4+ T cells involved a regulatory Syk-kinase-coupled signaling pathway in DCs. The Syk-coupled C-type lectin receptor Mincle detected mucosal-resident commensals in the Peyer's patches (PPs), triggered IL-6 and IL-23p19 expression, and thereby regulated function of intestinal Th17- and IL-17-secreting innate lymphocytes. Mice deficient in Mincle or with selective depletion of Syk in CD11c+ cells had impaired production of intestinal RegIIIg and IgA and increased systemic translocation of gut microbiota. Consequently, Mincle deficiency led to liver inflammation and deregulated lipid metabolism. Thus,

sensing of commensal microbiota by Mincle and Syk signaling in CD11c+ cells reinforces intestinal immune barrier and promotes host-microbiota mutualism, preventing systemic inflammation (Martínez-López et al., 2019).The DC-mediated inflammatory reaction necessary to combat bacteria translocation through the gut epithelium, could in turn be locally detrimental in the brain by increasing inflammation and exacerbating the stroke lesion. While Mincle deficiency is expected to increase the risk of post-stroke infection and was an initial target in our studies, a report showed that Mincle deficiency was protective in brain ischemia possibly due to reduced production of IL-17 in the brain as well as the expression of Mincle in brain cells other than DCs (Arumugam et al., 2017; doi: 10.1177/0271678X16661201).

Opportunistic bacteria of the commensal microbiota are likely involved in post-stroke infection. Moreover, several lines of evidence suggest that post-stroke infection follows intestinal dysbiosis and bacterial translocation from the gut to the lungs or other organs. Post-stroke infection is associated to immunodepression as illustrated by lymphopenia amongst other manifestations. This study aimed to identify mechanisms underlying post-stroke infection and its relation with changes in gut microbiota by studying immune, molecular, physiological, metabolic and microbiological alterations induced by stroke, and combining animal studies with studies in stroke patients. Transient middle cerebral artery occlusion in mice facilitated bacterial overgrowth in various organs, including lungs, liver, spleen, and mesenteric lymph nodes (mLN). To investigate whether gut bacteria could translocate from the gut to different organs, we generated mice with chemiluminescent Escherichia coli in the gut that could be visualized with bioluminescence imaging. We investigated possible alterations in gut barrier permeability by studying the permeability to fluorescent tracers given by gavage, the properties of the intestinal epithelial layer in *ex vivo* isolated preparations and the levels of IgA in the intestinal wash of ischemic versus sham-operated or control mice. We studied inflammatory reactions in the gut by investigating the lymphocyte populations in different parts of the gut, including the PP, intraepithelial lymphocytes, lamina propria and the mLN, as well as the mRNA expression of inflammatory molecules in the small intestine. Given that the inflammatory status of the gut is regulated by metabolic products generated by the microbiota as well as by the host, we studied main metabolic pathways in ischemic mice versus sham-operated and control mice. The metabolic alterations that we found pointed to stroke-induced changes in the composition of the gut microbiota. To assess the bacterial presence in

feces and cecum, we first investigated changes in well characterized bacterial groups by studying the gene encoding 16S ribosomal RNA by quantitative RT-PCR. These groups included *Lactobacillus, Akkermansia, Bacteroidetes, Cluster IV, Cluster XIVa, Bifidobacteria*, and *Enterobacteria*. Although the levels of some of these bacteria changed slightly from experiment to experiment, we found robust changes induced by ischemia that we consistently detected in five independent experiments. We validated the findings in mice obtained from different commercial suppliers to ensure that this effect was not dependent on specific breading conditions of the mice. The results were confirmed through metataxonomics by sequencing the 16S rRNA gene in the feces obtained before and after ischemia, or before and after sham-operation. In parallel to studies of ischemia-induced changes in gut microbiota composition in mice, we carried out a study in humans where we performed a time-course study and tested whether different gut microbiota populations could be related to stroke outcome. We extracted DNA from feces samples of stroke patients and sequenced the V3-V4 region of 16S rRNA gene using the Illumina MiSeq platform. The time course study in stroke patients showed results in agreement with the experimental studies. Moreover, analysis of independent clinical and microbiological variables using Lasso regression revealed a relationship of microbiota composition and stroke outcome at 3 months as assessed with the modified Rankin score. Therefore, the results in stroke patients support that stroke induces a disequilibrium in gut microbiota composition associated with stroke outcome.

We designed experimental strategies to prevent the microbiota alterations induced by stroke exploring the role of the sympathetic nervous system as well as the hypothalamic-pituitary-adrenal (HPA) axis amongst others. One of the treatments prevented certain changes in microbiota composition and made it possible to investigate the relationship between dysregulation of the microbiota composition and post-stroke infection in mice.

3. Relevance and future implications

Infections in stroke patients are frequent life-threatening complications. Understanding the biology underlying post-stroke infection will help to design adequate strategies for treatment and prevention. Novel treatments are necessary given that prophylaxis with

antibiotics is ineffective in stroke patients, probably because opportunistic commensal bacteria are often carriers of antibiotic resistances. Our study identified common bacteria involved in post-stroke infection and gut dysbiosis in mice and stroke patients, and their relation to metabolic and immune alterations. In addition, we identified molecular and immune mechanisms in the gut that are crucial to prevent gut bacterial translocation under steady-state. Moreover, our studies illustrate the opposite role of certain pattern-recognition receptors in dendritic cell subsets due to generation of inflammatory reactions that are critical for preventing gut bacterial translocation while they can trigger excessive local inflammation detrimental for the ischemic brain tissue. We hope these findings will pave the way for the development of novel strategies to prevent post-stroke infection while avoiding excessive brain inflammation.

4. Generated Bibliography

Del Fresno C, Saz-Leal P, Enamorado M, Wculek SK, Martínez-Cano S, Blanco-Menéndez N, Schulz O, Gallizioli M, Miró-Mur F, Cano E, Planas A, Sancho D. DNGR-1 in dendritic cells limits tissue damage by dampening neutrophil recruitment. Science. 2018 Oct 19;362(6412):351-356. doi: 10.1126/science.aan8423. PMID: 30337411.

Gallizioli M, Miró-Mur F, Otxoa-de-Amezaga A, Cugota R, Salas-Perdomo A, Justicia C, Brait VH, Ruiz-Jaén F, Arbaizar-Rovirosa M, Pedragosa J, Bonfill-Teixidor E, Gelderblom M, Magnus T, Cano E, Del Fresno C, Sancho D, Planas AM. Dendritic Cells and Microglia Have Non-redundant Functions in the Inflamed Brain with Protective Effects of Type 1 cDCs. Cell Rep. 2020 Oct 20;33(3):108291. doi: 10.1016/j.celrep.2020.108291.

Martínez-López M, Iborra S, Conde-Garrosa R, Mastrangelo A, Danne C, Mann ER, Reid DM, Gaboriau-Routhiau V, Chaparro M, Lorenzo MP, Minnerup L, Saz-Leal P, Slack E, Kemp B, Gisbert JP, Dzionek A, Robinson MJ, Rupérez FJ, Cerf-Bensussan N, Brown GD, Bernardo D, LeibundGut-Landmann S, Sancho D. Microbiota Sensing by Mincle-Syk Axis in Dendritic Cells Regulates Interleukin-17 and -22 Production and Promotes Intestinal Barrier Integrity. Immunity. 2019 Feb 19;50(2):446-461.e9. doi: 10.1016/j.immuni.2018.12.020.

In preparation

Díaz-Marugán L, Gallizioli M, Márquez-Kisinousky L, Miró-Mur F, Arboleya S, Mastrangelo AL., Casals C, Ramos S, Trasserra-Call, Ruiz-Jaén F, Gueimonde M, Torres JL, Jiménez M, Gonzalez de los Reyes-Gavilán C, Sancho D, Urra X, Planas AM. Relationship between gut dysbiosis and stroke-associated infection.