Molecular and cellular bases of autoinflammatory diseases-Focus on the neutrophilic dermatoses
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Neutrophilic dermatoses (NDs) are a group of inflammatory disorders mainly characterized by local or widespread cutaneous lesions of neutrophil infiltrates associated with fever and elevated blood neutrophils without evidence of infection *(Wallach and Vignon-Pennamen, 2006)*. NDs appear in isolation or in the context of an underlying inflammatory condition. Due to the similarities in the clinical phenotype and cytokine profile, NDs are now considered as part of the **systemic auto inflammatory diseases (SAIDs)**, a spectrum of rare, clinically and genetically heterogeneous disorders characterized by recurrent flares of systemic inflammation and cutaneous manifestations *(Marzano et al., 2014, 2019)*. **The aetiology and pathophysiology of NDs remain poorly understood today.**

Neutrophils (also known as polymorphonuclear leukocytes, PMNs) are the most abundant circulating white blood cells and key players of innate immunity. They are characterized by a multi-lobulated nucleus and a granule-packed cytoplasm that mediates the primary defence against infections. Upon detection of a Pathogen-Associated Molecular Pattern(s) (PAMP) or Damage-Associated Molecular Pattern(s) (DAMP), neutrophils emigrate from the circulation into the tissues (extravasation) through a cascade of events which include adhesion to activated endothelium, chemotaxis to the site of infection, phagocytosis and clearance of the pathogen. The last steps occur through a combination of proteases, formation of reactive oxygen species (ROS), and anti-microbicide peptides stored in intracellular neutrophil granules. Most importantly, activated neutrophils also release extracellular traps (NETs), web-like structures of decondensated chromatin, histones and antimicrobial peptides that can capture and kill microbes and prevent collateral damage by localizing proteases and degrading cytokines/chemokines *(Papayannopoulos, 2018)*. **Although NETs are important modulators of inflammation, the mechanisms leading to their release remain unclear**. Recently it was shown that inflammasomes, multiprotein complexes which regulate caspase 1-dependent secretion of interleukin (IL)-1b, can also induce NETosis through the activation of another lytic form of death known as pyroptosis. Interestingly, inflammasomes are formed around a cytoplasmic family of innate immune receptors which when mutated lead to SAIDs. Although the majority of NDs are mainly idiopathic, the neutrophil-rich cutaneous inflammation is also a cardinal feature of several SAIDs. This raises the question of the implication of genes causal for monogenic SAIDs in NDs.

The aim of this project is to study the molecular and cellular bases of NDs using complementary genetic and cell biology approaches: The approaches of this project are complementary between the two groups (INSERM UMRS 933 and Fiocruz).

On the genetic level: Assess the involvement of known SAID mutations in NDs and identify new causal genes

Molecular and analyses will be performed through the following two approaches.

a. Candidate-gene approach: A targeted sequencing approach will be performed by high-depth next-generation sequencing (NGS) on a NextSeq500 or MiSeq (Illumina) available in the lab *(Louvrier C, JACI 2020)*, using a custom targeted capture (SeqCap EZ Choice system; Roche) of the exons and the flanking intronic sequences of (i) the ~40 known SAID causing genes and (ii) ~100 additional candidate genes for inflammatory diseases (SAID and ND). This high-depth NGS approach allowed identification of germline and somatic mosaic mutations in the studied genes. Indeed, somatic mutations can be a possible disease mechanism, as the majority of ND patients are sporadic cases.

b. Whole exome sequencing: WES will be performed in the patients with no mutation identified after targeted sequencing of the genes present on our panel. This will be done using the Nextseq500 (Illumina) available in the lab.

On the cell biology level: Assess the pathogenicity of known SAID mutations in NDs. Explore the role of neutrophils in NDs and SAIDs by (i) NET formation and (ii) by the interplay between neutrophil activation and inflammasome(s)

Cellular analyses:

a. Functional validation of variations in known SAID genes as well as in newly identified sequence variants in ND samples: The lab has cellular models based on THP1 cells and on human monocytes from patients or healthy controls in order to study the causality of the identified variations *(Assrawi J Invest Dermatol 2020).*

b. Neutrophil isolation and activation from ND and SAID patients and healthy controls. Isolated neutrophils will be activated with specific stimuli to induce NET formation and inflammasome activation. Inflammasome inhibitors and will be used in order to test how inflammasome participates in neutrophil activation. Protease release and cell death assays (necrosis, MLKL dependent necroptosis and gasdermin dependent pyroptosis) will also be used.

c. Cytokine secretion will be measured in the above tested conditions

d. The impact of drugs (used as primary therapy in ND and SAID) in neutrophil and inflammasome activation will be tested

Feasibility

Our research unit (UMR_S933) has a long-standing experience in genetics and functional biology and in establishing the causality of new genes in diseases. The autoinflammatory group focuses on the pathophysiology of innate immune factors involved in SAIDs and our research is tightly linked to our molecular diagnostics lab (UF de génétique moléculaire from the Département de Génétique – APHP/Sorbonne Université). This means that, on the one hand, we screen on a routine-basis genes that are involved in SAIDs, and, on the other hand, we have developed molecular and cellular approaches to identify new causative genes in those conditions and to assess the functional consequences of the identified sequence variants. Samples patients are available in the lab through our reseau Immunaid H2020 (https://www.immunaid.eu/) and our involvement in the national rare disease center of SAIDs. The diagnostic part of the lab is a Reference laboratory for the molecular diagnostics of SAIDs (expertise recognized by the Organisation de la Direction Générale de l'Offre de Soins - DGOS from the Ministry of Health). Our contribution to the molecular bases of autoinflammation is the following: demonstration of the diagnostic value of the analysis of *MEFV,* the first SAID gene identified; this data validated the first objective criterion for the diagnosis of FMF *(Cazeneuve C et al Am J Hum Genet 1999)*; demonstration of the prognostic value of *MEFV* molecular analysis, by establishing the association of a particular genotype (M694V homozygous), at risk for the development of renal amyloidosis *(Cazeneuve C et al Am J Hum Genet 1999)*; identification of the first modifier gene for the FMF phenotype (*SAA1*), of which a particular genotype (alpha/alpha) is associated with an increased risk of developing inflammatory amyloidosis (OR=6.9) *(Cazeneuve C et al Am J Hum Genet 1999)*; identification of two SAID genes (*NLRP12* and *TNFRSF11A*) *(Jeru I PNAS 2008 & Arthritis & Rheumatology 2014)* demonstration of the existence of a FMF-like condition unrelated to *MEFV (Cazeneuve C Arthritis Rheum. 2003)* ; identification of 14-3-3 proteins as Pyrin partners *(Jeru Arthritis & Rheumatology 2005)*, with key implications for the understanding of Pyrin function and the pathophysiology of FMF and other *MEFV*-related auto-inflammatory disorders *(Masters Sci Transl Med 2016);* identification of the phenotypic and molecular characteristics of germline vs. somatic mosaic *NLRP3* mutations in autoinflammation *(Louvrier J Allergy Clin Immunol 2019);* role of mosaic *NLRP3* mutations in late onset urticarial *(Assrawi J Inv Derm 2020)* and establishment of the pathogenicity of the NLRP3 p.A441V mutation in SAIDs (*ACR Open Rheumatol. 2019*). More recently we have discovered a novel disease condition characterized by persistent urticarial lesions and clinically bundled hypercytokinemia caused by the loss of RNF213 function. This discovery highlights the crucial role of RNF213 in innate immunity *(Louvrier et al, J Allergy Clin Immunol. 2022)*. We have demonstrated the involvement of a new gene, *LYN*, which encodes a non-receptor kinase essential for regulating innate and adaptive immune responses, in a severe early-onset SAID *(Louvrier et al., Arthritis Rheumatol. 2022)*. As somatic mosaic variations pose a new challenge in the diagnosis of genetic diseases, particularly in SAIDs high-depth sequencing techniques and the creation of tailored pipelines in our lab showed that some *NLRP3* mutations found only in a mosaic state as otherwise would be incompatible with life *(Louvrier et al. J Allergy Clin Immunol. 2020). TNFRSF1A* mosaicism may be an overlooked cause of SAID, emphasizing the importance of using a high-depth next-generation sequencing (NGS) approach to prevent misdiagnosis *(Assrawi et al. Rheumatology Oxford 2022)*.

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Since 2017, our research group has initiated the structuring of a diagnostic network for Inborn Errors of Immunity in the city of Rio de Janeiro, with 7 specialized allergy and immunology services called RECIP (Carioca Network for Primary Immunodeficiency), in partnership with public specialized allergy and immunology care units (Ferreira et al. DOI: 10.1186/s12863-023-01148-z, Junior et al. DOI: 10.1186/s12887-022-03245-x). We have performed about 440 whole exomes sequencing (WES) with 30% efficiency in identifying genetic variants that explain the clinical phenotype. In specific inconclusive cases where a candidate variant is present, our team investigates the association of genotype with the clinical phenotype presented by the patient through molecular strategies and biological functional assays (Reis et al. DOI: 10.3390/genes12101476, Zin et al. DOI: 10.3390/ijms241511876, Zin et al. DOI: 10.3390/genes12071069). Currently, we are expanding this network to operate nationally and have created RENOMIEII (Brazilian Genomic Network for Inborn Errors of Immunity), involving 10 Brazilian states and 17 specialized allergy and immunology centers in partnership with the Brazilian Society of Clinical Immunology. In this new project, we will use the strategy of whole-genome sequencing (WGS) followed by other omics strategies for the reclassification of variants of uncertain significance. We hope this initiative can bring the possibility to Brazilian patients access the benefits of genomic tests in our universal health system (SUS), paving the way to advanced therapies and precision medicine (Lira et al. DOI: 10.1038/s41467-022-28762-2).

Benefit: This collaborative PhD project between INSERM UMRS 933 and Fiocruz -specialized labs in the genetics of rare and inflammatory/tropical disorders respectively will foster an international cooperation which can lead to faster diagnosis of rare diseases through complementary discovery of causative genes and access to diverse patient populations and infrastructure. The project can lead to significant advancements in understanding and treating rare diseases in both countries. By pooling resources and expertise from different European and Brazilian populations the project can promote knowledge exchange, innovation, and help in the development of new therapies benefiting a broader range of patients worldwide. This partnership will strengthen the scientific network between Sorbonne and Fiocruz, and address globally the challenges associated with rare diseases effectively.