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Ali Tawbeh

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Ali Tawbeh. Peroxisomal dysfunction in BV-2 microglial cells : towards a better understanding of the neurodegenerative process in X-linked adrenoleukodystrophy. Biochemistry [q-bio.BM]. Université Bourgogne Franche-Comté, 2023. English. NNT : 2023UBFCI010 . tel-04552077

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**THESE DE DOCTORAT DE L'ETABLISSEMENT  
UNIVERSITE BOURGOGNE FRANCHE-COMTE**

**Préparée à l'Université de Bourgogne**

Ecole doctorale n°554

Environnement-Santé

Doctorat de Biochimie et Biologie Moléculaire

par

**M. Tawbeh Ali**

**Dysfonctionnement peroxysomal dans les cellules microgliales BV-2 : vers  
une meilleure compréhension du processus neurodégénératif dans  
l'adrénoleucodystrophie liée à l'X**

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Peroxisomal dysfunction in BV-2 microglial cells: towards a better understanding of the neurodegenerative process in X-linked adrenoleukodystrophy



## Acknowledgement

First and foremost, I am immensely thankful to Mrs. H el ene Hirbec, Mrs. S everine Boill ee, Mr. St ephane Fourcade, and Mr. Alexandre Benani for their invaluable commitment to evaluating this work and serving as the esteemed jury of my thesis.

I extend my heartfelt appreciation to St ephane Savary, my dedicated supervisor, whose unwavering guidance, unwavering support, and exceptional mentorship have been instrumental in shaping me towards a proficient researcher. Your mentorship has been a source of inspiration throughout this journey.

Catherine Gondcaille, your persistent support and unconditional assistance have been a cornerstone of my progress. Your presence and encouragement have been a constant motivation, and I am profoundly grateful for your unwavering belief in me.

St ephane and Catherine, I am privileged to have been a part of your team. Working alongside you has been an honor and a highlight of my academic experience. Your companionship and collaboration have made these three years truly remarkable.

I am deeply appreciative to Gerard Lizard for warmly welcoming me into his lab. I extend my thanks to all members of the BioperoxIL laboratory – Anne, Doriane, Mustafa, Nathalie, Norbert, Pierre, Thomas, and Vivien.

I wish to express my sincere gratitude to Dr. Agnes Nadjar and Dr. Alain Bessis for their integral roles in forming my scientific thesis follow-up committee (CSST). Their insightful feedback has had a profound and positive impact on driving this work towards more fruitful trajectories. Many thanks to Claire Fenech, whose generous assistance in training me on a complex technique has been invaluable. Your expertise has been a guiding light in this process.

I extend my sincere gratitude to Johannes Berger and Isabelle Weinhofer for graciously welcoming me into their laboratory and providing invaluable insights. I also appreciate all laboratory members for their camaraderie and friendship.

I am grateful to our collaborators, Yannick Hamon and Francisca Di Cara, whose contributions have been instrumental in completing this work.

A special mention to our internship student Damien Loichot for his passionate involvement in a part of this research, contributing to its completion.

To my colleagues who turned into unforgettable friends - Khouloud, Imen, Soukaina, Mohamed, Juliette, Rim, and Mounia - thank you for the shared experiences and amity that have made this journey memorable.

To my friends Karim, Achour, Dayane, Hasan, and Marie. Thank you for becoming my family in Dijon.

Abeer and Caroline, your constant presence every moment of this journey, even from different areas of the world, is a testament to our true friendship. I can't imagine how it would have been without your constant company.

Lastly, to my family, I am profoundly thankful for your unconditional love, unwavering encouragement, and endless sacrifices. Your belief in me has been the foundation of my achievements.

To all those mentioned and to those who have supported me silently, my heartfelt appreciation knows no bounds. This thesis is a testament to your collective contributions.

Ali

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## List of abbreviations

|                                |  |
|--------------------------------|--|
| <b>25-HC</b>                   | 25-Hydroxycholesterol                            |
| <b>ABC</b>                     | ATP Binding Cassette                             |
| <b>ABCD</b>                    | ATP Binding Cassette Subfamily D Member          |
| <b>ACAA</b>                    | Acetyl-CoA Acyltransferase                       |
| <b>ACOT</b>                    | Acyl-CoA Thioesterase                            |
| <b>ACOX</b>                    | Acyl-CoA Oxidase                                 |
| <b>AD</b>                      | Alzheimer's Disease                              |
| <b>ALDP</b>                    | Adrenoleukodystrophy Protein                     |
| <b>ALDRP</b>                   | Adrenoleukodystrophy-Related Protein             |
| <b>ALS</b>                     | Amyotrophic Lateral Sclerosis                    |
| <b>AMACR</b>                   | 2-Methylacyl-CoA Racemase                        |
| <b>AMN</b>                     | Adrenomyeloneuropathy                            |
| <b>AMPK</b>                    | AMP-Activated Protein Kinase                     |
| <b><math>\alpha</math>-MSH</b> | Alpha-Melanocyte Stimulating Hormone             |
| <b>ApoE</b>                    | Apolipoprotein E                                 |
| <b>ATP</b>                     | Adenosine Triphosphate                           |
| <b>A<math>\beta</math></b>     | Amyloid- $\beta$                                 |
| <b>BBB</b>                     | Blood Brain Barrier                              |
| <b>BDNF</b>                    | Brain-Derived Neurotrophic Factor                |
| <b>cALD</b>                    | Childhood Cerebral Adrenoleukodystrophy          |
| <b>CC</b>                      | Co-culture                                       |
| <b>CM</b>                      | Conditioned Medium                               |
| <b>CNS</b>                     | Central Nervous System                           |
| <b>CRAT</b>                    | Carnitine O-acetyltransferase                    |
| <b>CROT</b>                    | Carnitine O-octanoyltransferase                  |
| <b>CSGA</b>                    | Centre des Sciences du Goût et de l'Alimentation |
| <b>CTSB</b>                    | Cathepsin B                                      |
| <b>DAM</b>                     | Disease Associated Microglia                     |
| <b>DAMPs</b>                   | Damage-Associated Molecular Patterns             |
| <b>DEGs</b>                    | Differentially Expressed Genes                   |
| <b>DHA</b>                     | Docosahexaenoic Acid                             |
| <b>DMSO</b>                    | Dimethyl Sulfoxide                               |
| <b>EAE</b>                     | Experimental Autoimmune Encephalomyelitis        |
| <b>ER</b>                      | Endoplasmic Reticulum                            |
| <b>FAs</b>                     | Fatty Acids                                      |
| <b>HSCT</b>                    | Hematopoietic Stem Cell Transplantation          |
| <b>IFN-<math>\gamma</math></b> | Interferon Gamma                                 |
| <b>iNOS</b>                    | Inducible Nitric Oxide Synthase                  |
| <b>LCFAs</b>                   | Long-Chain Fatty Acids                           |
| <b>LDs</b>                     | Lipid Droplets                                   |
| <b>LPS</b>                     | Lipopolysaccharide                               |
| <b>MAPK</b>                    | Mitogen-Activated Protein Kinases                |
| <b>MCFAs</b>                   | Medium-Chain Fatty Acids                         |
| <b>MCP-1</b>                   | Monocyte Chemoattractant Protein 1               |
| <b>MerTK</b>                   | MER Receptor Tyrosine Kinase                     |
| <b>MFNs</b>                    | Mitochondrial Protein Mitofusins                 |

|                                |  |
|--------------------------------|--|
| <b>MFP</b>                     | Multifunctional Protein                          |
| <b>MHC</b>                     | Major Histocompatibility Complex                 |
| <b>MRI</b>                     | Magnetic Resonance Imaging                       |
| <b>MS</b>                      | Multiple Sclerosis                               |
| <b>mTOR</b>                    | Mammalian Target of Rapamycin                    |
| <b>MUFA</b>                    | Monounsaturated Fatty Acids                      |
| <b>NBDs</b>                    | Nucleotide Binding Domains                       |
| <b>NF-<math>\kappa</math>B</b> | Nuclear Factor $\kappa$ B                        |
| <b>NOS2</b>                    | Nitric Oxide Synthase 2                          |
| <b>NRF2</b>                    | Nuclear Factor Erythroid 2-Like 2                |
| <b>P2XR<sub>s</sub></b>        | Ionotropic P2X Receptors                         |
| <b>PAMP<sub>s</sub></b>        | Pathogen-Associated Molecular Patterns           |
| <b>PD</b>                      | Parkinson's Disease                              |
| <b>PE</b>                      | Phosphatidylethanolamine                         |
| <b>Pex</b>                     | Peroxisome                                       |
| <b>PI</b>                      | Propidium Iodide                                 |
| <b>PMP69</b>                   | Peroxisomal Membrane Protein 69                  |
| <b>PMP70</b>                   | 70 kDa Peroxisomal Membrane Protein              |
| <b>POMC</b>                    | Proopiomelanocortin                              |
| <b>PPAR</b>                    | Peroxisome Proliferator-Activated Receptor       |
| <b>PRR<sub>s</sub></b>         | Pattern-Recognition Receptors                    |
| <b>RCDP</b>                    | Rhizomelic Chondrodysplasia Punctata             |
| <b>RNAseq</b>                  | RNA sequencing                                   |
| <b>RNS</b>                     | Reactive Nitrogen Species                        |
| <b>ROS</b>                     | Reactive Oxygen Species                          |
| <b>SCFAs</b>                   | Short-Chain Fatty Acids                          |
| <b>SCP<sub>x</sub></b>         | Sterol Carrier Protein X                         |
| <b>SNAP</b>                    | S-Nitro-N-acetylpenicillamine                    |
| <b>SOD</b>                     | Superoxide Dismutase                             |
| <b>TGF-<math>\beta</math></b>  | Transforming Growth Factor $\beta$               |
| <b>TLR<sub>s</sub></b>         | Toll-Like Receptors                              |
| <b>TMD<sub>s</sub></b>         | Transmembrane Domains                            |
| <b>TNF</b>                     | Tumor Necrosis Factor $\alpha$                   |
| <b>TREM2</b>                   | Triggering Receptor Expressed on Myeloid Cells 2 |
| <b>VLCFA</b>                   | Very Long Chain Fatty Acid                       |
| <b>X-ALD</b>                   | X-linked Adrenoleukodystrophy                    |
| <b>ZS</b>                      | Zellweger Syndrome                               |

# Introduction

## Chapter 1: Peroxisomes

### I. General Introduction and Function

Eukaryotic cells comprise various subcellular compartments that vary in terms of biogenesis, enzyme composition, and metabolic functions. These compartments, such as lysosomes and mitochondria, have distinct roles in the metabolism of macromolecules and aerobic metabolism, respectively. Peroxisomes are membrane-enclosed organelles present in most eukaryotic cells and serve both catabolic and anabolic functions (Wanders et al., 2023). They originate either by de novo emergence from the endoplasmic reticulum (ER), by fusion between mitochondrial and ER-derived vesicles, or by growth and division from existing organelles (J. J. Smith & Aitchison, 2013; Sugiura et al., 2017). Specific Peroxin (Pex) genes are responsible for coding proteins that are necessary for creating and sustaining the cellular population of peroxisomes.

When it comes to the characteristics of these organelles, the isolation of peroxisomes from various organisms revealed variation in the enzymatic characteristics among organisms and even between organs from the same organism. For example, only hepatic peroxisomes can catalyze the synthesis of bile acids and detoxify glyoxylate into glycine in human cells and tissues (Wanders et al., 2023). The high adaptability of these organelles is another unique characteristic. They can swiftly adjust their size, number, shape, protein makeup, location and function in response to extracellular signals (J. J. Smith & Aitchison, 2013).

Functionally, peroxisomes are involved in a large number of crucial metabolic functions. They are implicated in several important cellular processes such as the detoxification of reactive oxygen species (ROS) and reactive nitrogen species (RNS), the synthesis of ether phospholipids, bile acids and docosahexaenoic acid (DHA), and  $\alpha$ - and  $\beta$ -oxidation of fatty acids (FAs). They are solely responsible for the  $\beta$ -oxidation of very-long-chain fatty acids (VLCFAs) (FAs with 22 or more carbon atoms) and the  $\alpha$ -oxidation of branched-chain FAs (Fransen & Lismont, 2019; Wanders et al., 2023).

The correct spatiotemporal synchronization of biochemical reactions is absolutely necessary for cell function. ROS and RNS are produced naturally as a byproduct of cellular metabolism and energy production. They are unstable molecules that can react with and damage cellular components such as DNA, proteins, and lipids. When ROS and RNS levels become too high, they can cause significant damage to cells and tissues, leading to a variety of health

problems (Zhao et al., 2021). Peroxisomes constitute a signaling hub that integrates signals derived from metabolic reactions to respond to metabolic stressors. To prevent ROS/RNS damage, peroxisomes are equipped with multiple enzymatic as well as non-enzymatic antioxidant defense systems. Enzymes include the catalase which reduces H<sub>2</sub>O<sub>2</sub>, the major ROS in redox regulation of biological activities (Rhee, 1999), inducible nitric oxide synthase (iNOS) and peroxiredoxin-5, whereas non-enzymatic systems include ascorbic acid and glutathione (Fransen & Lismont, 2019; He et al., 2021; Stolz et al., 2002).

Concerning the ether phospholipids synthesis, peroxisomes contain the one and only enzyme able to generate the characteristic ether bond, and here comes another important function of these organelles. They initiate biosynthesis of ether lipids which is completed in the ER afterwards. Those lipids, among which the plasmalogens are the most common, define the structural and functional properties of the neural membrane. They are also involved in a variety of biological functions in the brain tissue, including structural roles, membrane trafficking, cell signaling and oxidative-stress homeostasis. Their levels have repeatedly been connected to some of the most abundant neurological diseases, particularly Alzheimer's disease (AD) and more recently also neurodevelopmental disorders like autism (Dorninger et al., 2020; Jové et al., 2023). Furthermore, peroxisomes are required for the production of DHA (Ferdinandusse et al., 2001; Sprecher, 2002), a FA which is of great value being a component of cell membranes and the source of eicosanoids associated with several key signaling functions (Chapkin et al., 2009), and bile acids, which play important roles in digestion and regulation of cholesterol metabolism (T. Li & Chiang, 2009).

Last but not least, the breakdown of FAs by the processes of  $\alpha$ - and  $\beta$ -oxidations are the most known functions of peroxisomes.  $\alpha$ -oxidation corresponds to chain shortening by a single carbon atom which is a process required before the total breakdown of some branched chain FAs as is pristanic and phytanic acid. This process of oxidative decarboxylation occurs solely in peroxisomes (Wanders et al., 2023). Peroxisomal  $\beta$ -oxidation is a crucial pathway for the metabolism of specific types of fatty acids and contributes to energy production and lipid homeostasis in the cell. Peroxisomes degrade the VLCFAs by the process of  $\beta$ -oxidation. This process converts VLCFAs into shorter chain fatty acids, which can then be used as energy sources by the cell. These FAs cannot be handled by the mitochondrial oxidation machinery and they need to be degraded mainly because of their toxicity when they accumulate (Wanders et al., 2023). Due to its importance in the core of this thesis, peroxisomal  $\beta$ -oxidation is detailed in the next section.



## II. Peroxisomal $\beta$ -oxidation

### II.1. Main mechanism

The chains of hydrocarbons with a carboxylic acid group (-COOH) at one end, FAs, are crucial for maintaining cellular homeostasis. They play roles in numerous biological processes, such as energy storage and supply, phospholipid synthesis, protein post-translational modifications, cell signaling, membrane permeability and fluidity, and transcription control (Calder, 2015). Human FAs are obtained from diet as well as by endogenous biosynthesis and should be oxidized in the peroxisomes or mitochondria.

FAs can be divided into four types according to the length of the carbon chain, namely short-chain fatty acids (containing 2–4 carbon atoms, SCFAs), medium-chain fatty acids (containing 6–12 carbon atoms, MCFAs), long-chain fatty acids (containing 13–18 carbon atoms, LCFAs) and VLCFAs. The  $\beta$ -oxidation process exists in mitochondria for MCFAs and LCFAs. However, VLCFAs are exclusively  $\beta$ -oxidized into the peroxisome. FA  $\beta$ -oxidation starts after they are activated and imported into the peroxisomes. The acyl-CoA synthetase in the cytosol activates the fatty acids thus forming fatty acyl-coenzyme A (fatty acyl-CoA). Afterwards, LCFA-CoAs, VLCFAs-CoAs and branched-chain fatty acyl-CoA are transported into the peroxisomes via three ATP-binding cassette (ABC) transporters, ABCD1/2/3, which use the energy of ATP hydrolysis (Tawbeh et al., 2021). Subsequently, various fatty acyl-CoAs are catabolized by a set of enzymes usually via  $\beta$ -oxidation (Nelson & Cox, 2017).

During  $\beta$ -oxidation, the FAs undergo four sequential steps of dehydrogenation, hydration, dehydrogenation again, and thiolytic cleavage. This mechanism ensures the cleavage of fatty acids at the carboxy-terminal end to produce an acetyl-CoA unit in the case of unbranched FAs and propionyl-CoA in the case of 2-methyl branched-chain FAs. The number of  $\beta$ -oxidation cycles for each lipid depends on the length of the carbon chain and generally continues until obtaining a medium-chain acyl-CoA (between 6 and 8 carbons). The acetyl-CoA and medium-chain acyl-CoA thus formed would then be complexed to carnitine via Carnitine O-acetyltransferase (CRAT) or Carnitine O-octanoyltransferase (CROT), respectively, and then exported from the peroxisome to the cytosol or mitochondria. The products formed by  $\beta$ -oxidation would also leave the peroxisome in free form after cleavage of acyl-CoA by acyl-CoA thioesterase 4 or 8 (ACOT4 or ACOT8) into free FAs and CoA (Hunt et al., 2014; Wanders et al., 2023).

## II.2. Enzymes

Peroxisomal fatty acid  $\beta$ -oxidation machinery consists of three different acyl-CoA oxidases (ACOX1, -2, and -3), two multifunctional enzymes (Multifunctional protein 1, MFP1 or LBP; and Multifunctional Protein 2, MFP2 or DBP) and two thiolases (acetyl-CoA acyltransferase 1, ACAA1; and sterol carrier protein X, SCPx).

ACOX1 is the enzyme required for the first step of peroxisomal  $\beta$ -oxidation dedicated to straight-chain fatty acids, which includes LCFAs, VLCFAs, PUFAs, and dicarboxylic acids (Baumgart et al., 1996). It primarily reacts with the CoA esters of VLCFAs catalyzing the reduction of acyl-CoA to 2-trans-enoyl-CoA and it is thus the rate-limiting enzyme in this reaction. It also plays an essential role in mediating the inflammatory response and ROS metabolism in mammals (Kong et al., 2022). ACOX2 and ACOX3 handle the dehydrogenation of branched-chain acyl-CoAs including pristanoyl-CoA, which is a product of the  $\alpha$ -oxidation of phytanic acid. The expression of ACOX2 is limited to the liver and kidney whereas ACOX3 is more widely expressed, making it safe to say that ACOX3 is probably the primary oxidase handling 2-methyl branched-chain FAs in all organs except the liver and kidneys (Ferdinandusse et al., 2018).

Concerning the two multifunctional enzymes, MFP2 is believed to be the central enzyme catalyzing the second and third steps of  $\beta$ -oxidation (the hydration and the second dehydrogenation). MFP1 role is still less clear yet it plays a crucial role in the oxidation of long-chain dicarboxylic acids (Ferdinandusse et al., 2004; Houten et al., 2012).

The two thiolases catalyze the last step of peroxisomal  $\beta$ -oxidation, the thiolytic cleavage. Although it has been argued that ACAA1 and SCP are both involved in the oxidation of VLCFAs, SCPx which is the product of the largest of the two transcripts derived from the *SCP2* gene, is shown to be the key enzyme involved in the oxidation of branched-chain FAs but not VLCFAs (Wanders et al., 2023). An additional enzyme might be required for the oxidation of some 2-methyl branched chain FAs, such as pristanic acid. This enzyme is named 2-methylacyl-CoA racemase (AMACR) and is present in both peroxisomes and mitochondria (Ferdinandusse et al., 2000; Kotti et al., 2000).

## III. Cross talk with other organelles

Recent research has provided compelling evidence highlighting the crucial role of peroxisomes in cellular metabolism, demonstrating that these organelles heavily rely on their interactions with other cellular components. Among these key interactions are those established

with the ER, mitochondria, lysosomes, and lipid droplets (LDs). These interconnections highlight the significance of organelle communication in maintaining cellular homeostasis.

### **III.1. Peroxisome-ER cross talk**

Some VLCFAs are synthesized by chain elongation of shorter-chain FAs in the ER and some require a final step in the peroxisomes. For example, DHA (C22:6 n-3), the most important n-3 PUFA which is mainly present in the brain and retina, is synthesized from C18:3 n-3 which first undergoes a number of elongation and desaturation steps in the ER to produce C24:6-CoA. It is then transported to the peroxisome and imported through an unknown mechanism. Within peroxisomes C24:6-CoA undergoes one cycle of  $\beta$ -oxidation to produce the corresponding C22:6-CoA which can then be exported out of the peroxisome for subsequent incorporation into lipids in the ER or may undergo additional sequential rounds of oxidation in peroxisomes and subsequently in the mitochondria (Wanders, 2016). ACBD5 is a peroxisomal tail-anchored protein involved in the transfer of VLCFAs from ER to peroxisomes (Costello, Castro, Hacker, et al., 2017; Hua et al., 2017; Wiese et al., 2007). The interaction between ACBD5 and VAPB, an ubiquitous ER-resident tail-anchored membrane proteins in eukaryotic cells, is believed to mediate the peroxisome-ER interaction (Kors et al., 2022). Patients with pathogenic variants of ACBD5 have been identified and they show elevated levels of plasma VLCFAs and defective oxidation of C26:0 in fibroblasts (Ferdinandusse et al., 2017). This strongly suggests that ACBD5 plays a crucial role in VLCFAs homeostasis by promoting the transfer of very long-chain acyl-CoAs from the ER to peroxisomes by bringing the two organelles together. More recently, ACBD4 was identified. It is a close homolog of ACBD5 and known to mediate the contact between peroxisomes and the ER as well (Costello, Castro, Schrader, et al., 2017). Moreover, peroxisome-ER membrane contact sites are hypothesized to play a role in peroxisomal division, though ACBD5 might not be involved in this process (C. Chen et al., 2020).

### **III.2. Peroxisome-mitochondria cross-talk**

Mitochondria and peroxisomes are two functionally close-related organelles, both playing essential roles in lipid and ROS metabolism. The importance of the interaction between peroxisomes and mitochondria comes from the fact that peroxisomal  $\beta$ -oxidation products including acetyl-CoA, propionyl-CoA, and medium-chain acyl-CoAs are shuttled

to the mitochondria either as free acids or as carnitine esters as discussed earlier. Indeed, peroxisomal  $\beta$ -oxidation only results in chain-shortened fatty acids (medium chain acyl-CoA, C6-C8), which have to be shuttled to mitochondria for full oxidation to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  (Schrader et al., 2020). Mitochondria contain both short-chain as well as medium-chain acyl-CoA synthetase activities to reactivate the free acids back into the corresponding acyl-CoAs. They are then oxidized either directly, as in acetyl-CoA, or indirectly (propionyl-CoA and medium-chain acyl-CoAs). It has not yet been determined whether the transfer of metabolites from peroxisomes to mitochondria occurs through simple diffusion to the cytosol or involves organelle interactions. There exists a biochemical evidence for direct peroxisome-mitochondrion interactions from density gradient centrifugation analyses (Islinger et al., 2006) as well as close proximity between peroxisomes and mitochondria which has been observed in ultrastructural studies many years ago (Hicks & Fahimi, 1977). In yeast, direct interaction has been documented between Pex11, a membrane-bound peroxin involved in peroxisome division and proliferation, and the mitochondrial ERMES complex, a protein complex that helps to tether the ER to the mitochondria (Esposito et al., 2019; Mattiazzi Ušaj et al., 2015). Tethering of both organelles is supposed to enhance metabolism by reducing the distance for efficient transport of metabolites from the peroxisome to the mitochondrion. Mammalian cells, however, lack ERMES so another tethering complex is supposed to perform a similar function in higher eukaryotes, including humans (Schrader et al., 2015). Another form of peroxisome-mitochondrial contact had been found after discovering that mitochondrial protein mitofusins (MFNs), the key regulators of mitochondrial fusion and fission, are in proximity to peroxisomes. After finding that MFNs are enriched at the mitochondria-peroxisome interface and regulate the contacts between these organelles, it was claimed that this new understanding of mitochondria-peroxisome interactions and the role of MFNs can provide insights into inter-organelle metabolism regulation, signaling, and the pathogenesis of mitofusin dysfunction-related diseases (Huo et al., 2022).

Considering the close interaction between peroxisomes and mitochondria, it is not surprising that mitochondrial function is impacted in peroxisomal disorders (Peeters et al., 2015). However, the precise mechanism through which dysfunctional peroxisomes lead to structural and functional abnormalities in mitochondria remains unclear. The mitochondrial defects observed in Pex5 global knockout mice, a model of peroxisomal disorders, cannot be attributed to the absence of DHA or the accumulation of phytanic acid, dicarboxylic acid,

or peroxisomal bile acid intermediates (Shinde et al., 2018). Furthermore, these mitochondrial abnormalities were only evident in hepatocytes lacking peroxisomes, suggesting that the peroxisome-mitochondria interaction may be cell type-specific, emphasizing the unique nature and significance of this interaction (Sargsyan & Thoms, 2020).

### **III.3. Peroxisome-lysosome cross-talk**

Lysosome-to-peroxisome contact sites have been identified and suggested to participate to the cellular cholesterol transport system. In fact, cholesterol is synthesized in the ER or taken up by endocytosis and released through lysosomes. It is a main component of membranes which can be stored in LDs as esters and depleted by bile acid formation. Contact between lysosomes and peroxisomes would catalyze the transfer of cholesterol from the lysosomes to peroxisomes for its incorporation into the peroxisomal membrane, its redistribution to the other organelles using further organelle contact sites, or its elimination using the peroxisomal bile acid biosynthesis pathway. Indeed, defective peroxisome biogenesis blocks cholesterol release from lysosomes (Chu et al., 2015). Consequently, plasma cholesterol levels are reduced in patients with peroxisome biogenesis disorders (Wanders & Romeijn, 1996). Cholesterol homeostasis is also found to be disturbed in fibroblasts of patients of peroxisomal disorders, specifically Zellweger disorder and X-linked adrenoleukodystrophy, where a remarkable accumulation of cholesterol was found in all cases (Chu et al., 2015; Schrader et al., 2020). Add to that, independently of cholesterol traffic, lysosome-to-peroxisome contacts would be involved in the lipid catabolism. Indeed, aggregates enriched in VLCFAs were found colocalized with lysosomes and peroxisomes in Schwann cell-specific Pex5 knockout mouse models in the peripheral nervous system resulting in secondary failure of local lysosomes and in an impaired turnover of gangliosides in myelin (Kleinecke et al., 2017).

Another form of peroxisome-lysosome crosstalk involves autophagy, or in this case, pexophagy. Autophagy is a self-eating, dynamic, cellular process involving the sequestration of cytoplasmic constituents into a double-membrane structure called the autophagosome, followed by autophagosome/lysosome fusion and degradation of the cargos in the lysosome or vacuole (J. N. S. Vargas et al., 2023). Pexophagy is a selective autophagy process that degrades damaged and/or superfluous peroxisomes in the yeast vacuole or in mammalian lysosomes. It is a major mechanism for removing damaged and/or

superfluous peroxisomes. Dysregulation of pexophagy impairs the physiological functions of peroxisomes and contributes to the progression of many human diseases (J. Li & Wang, 2021). This process is essential for the maintenance of homeostasis of peroxisomes, which is necessary in the prevention of various peroxisome-related disorders (Cho et al., 2018).

### **III.4. Peroxisome-LDs cross talk**

Lipid droplets are dynamic cellular structures involved in the storage of neutral lipids, including triacylglycerols and sterol esters. Close associations of LDs with peroxisomes and other organelles have been described (Schrader et al., 2020; Valm et al., 2017). Indeed, peroxisomes and mitochondria are involved in the lipid mobilization from LDs, a crucial process to meet cellular energy demands (Olzmann & Carvalho, 2019). LDs transfer lipids to various organelles including peroxisomes. This interaction between peroxisomes and LDs may link lipolysis, which is mediated by LDs, to peroxisomal fatty acid  $\beta$ -oxidation. Another important cross talk comes from the fact that lipids generated by peroxisomes may move into LDs. Thus, in the cases of deficiency of peroxisomal  $\beta$ -oxidation or loss of peroxisomes, enlarged LDs and alterations in their number had been observed (Dirkx et al., 2005; S. O. Zhang et al., 2010). The coordination of FA trafficking from LDs to peroxisomes is believed to be ensured by M1 Spastin, which is a membrane-bound ATPase found on LDs. M1 Spastin forms a tethering complex with the peroxisomal ABCD1 transporter to promote peroxisome-LDs contact formation which eventually facilitate LD-to-peroxisome FA trafficking (C. L. Chang et al., 2019). The transportation of fatty acids to peroxisomes through Spastin plays a crucial role in preventing the buildup of peroxidated lipids within LDs. These intriguing discoveries offer insights into the underlying mechanisms of diseases related to impaired fatty acid metabolism in both LDs and peroxisomes.

## **IV. Peroxisomal ABC transporters**

### **IV.1. Introduction**

ATP-binding cassette (ABC) transporters constitute a superfamily of membrane transporter proteins that actively translocate a wide range of molecules, from simple molecules (FAs, sugars, nucleosides, and amino acids) to complex organic compounds (lipids, oligonucleotides, polysaccharides, and proteins) (Thomas & Tampé, 2020). Active transport implies that it is dependent on the hydrolysis of Adenosine triphosphate (ATP),

which releases energy. The liberated energy can be used to import substrates into the cellular compartments or export them to the outside. ABC transporters are distributed not only in the plasma membrane of both prokaryotes and eukaryotes, but also in the membranes of the organelles of eukaryotic cells such as peroxisomes, mitochondria, lysosomes, and ER. Based on their amino acid homology and structural configuration, ABC transporters in humans are classified into seven subfamilies, A to G, comprising a total of 48 ABC transporters (Dean & Annilo, 2005). ABC transporters of subfamily D include four proteins in mammals: ABCD1 [adrenoleukodystrophy protein (ALDP)], ABCD2 [adrenoleukodystrophy-related protein (ALDRP)], ABCD3 [70 kDa peroxisomal membrane protein (PMP70)], and ABCD4 [peroxisomal membrane protein 69 (PMP69)] (Kamijo et al., 1990; Kawaguchi & Morita, 2016; Lombard-Platet et al., 1996; Mosser et al., 1993; Tawbeh et al., 2021). ABCD1, ABCD2, and ABCD3 are located in the peroxisomal membrane. ABCD4 was identified by homology search for ALDP and PMP70 related sequences in the database of expressed sequence tags, and was initially considered peroxisomal despite the absence of a membrane peroxisomal targeting signal (Shani et al., 1997). More recently, several studies have demonstrated that ABCD4 resides in the ER and lysosomes, and that its function is associated with cobalamin metabolism (Coelho et al., 2012; Kashiwayama et al., 2009; Kawaguchi & Morita, 2016).

#### **IV.2. Structure, function, and mechanism of transport**

The general structure of eukaryotic ABC transporters is a four functional unit organization, comprising two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). NBDs bind and hydrolyze ATP to trigger conformational changes in the TMDs, resulting in unidirectional transport across the membrane (Thomas & Tampé, 2020). Human peroxisomal ABC transporters have a half-transporter structure, with only one TMD and one NBD (Contreras et al., 1996). Therefore, peroxisomal ABC half-transporters need to homo- or heterodimerize in the peroxisomal membrane in order to constitute a full, active transporter (Baker et al., 2015; Geillon et al., 2014). Recently, cryo-electron microscopy structures of ABCD1 have been presented. It has been shown that each of the four inward-facing structures of ABCD1 has a vestibule that opens to the cytosol with variable sizes. They also showed that C26:0-CoA substrate binds to the TMDs and stimulates the ATPase activity of the NBDs. Moreover, ABCD1 has a unique C-terminal coiled-coil domain that negatively modulates the ATPase activity of the NBDs. The structure of ABCD1 in the

outward-facing state indicates that ATP molecules pull the two NBDs together and open the TMDs to the peroxisomal lumen for substrate release (Xiong et al., 2023).

ABCD1 and ABCD3 are mainly found as homodimers in mammalian peroxisomal membranes (Guimarães et al., 2004; Hillebrand et al., 2007; L. X. Liu et al., 1999). However, data show that ABCD1, ABCD2, and ABCD3 are able to interact as homodimers or heterodimers (Genin et al., 2011; K. D. Smith et al., 1999; Tanaka et al., 2002). The fact that nonfunctional ABCD2 has a transdominant negative effect on ABCD1 suggests that heterodimers of ABCD1 and ABCD2 are functional and can exist within cells and tissues expressing both proteins (Genin et al., 2011). Besides, chimeric proteins consisting of homo- and heterodimers of ABCD1 and ABCD2 are functionally active (Geillon et al., 2014). However, since tetrameric or more complex oligomeric structures have been evidenced, it cannot be excluded that the contacts between ABCD1 and ABCD2 result from interactions between two different homodimers (ABCD1/ABCD1 and ABCD2/ABCD2) within this larger structures (Geillon et al., 2017). Concerning ABCD3, although homodimers and heterodimers with ABCD1 and ABCD2 have been described (Geillon et al., 2017; Hillebrand et al., 2007; L. X. Liu et al., 1999; Tanaka et al., 2002; Woudenberg et al., 2010), no data is available about the functional value of the ABCD3 dimers. Actually, it remains unclear whether the oligomerization of peroxisomal ABC transporters has any influence on substrate specificity.

Functionally, since the cloning of the *ABCD1* gene in 1993 (Mosser et al., 1993), the functioning of ABCD1 has been attributed to the transport of saturated and monounsaturated VLCFAs across the peroxisomal membrane for degradation by  $\beta$ -oxidation. Due to its importance, studies concerning the structure, function, and defects of ABCD1 have never ceased. Functional complementation experiments in yeast, functional assays in mammalian cells, and studies using animal models, mainly knock-out mice, were helpful in clarifying the question of substrate specificity. The *Abcd1* knock-out mice confirmed the human biochemical phenotype, indicating that ABCD1 is indeed involved in the transport of VLCFAs (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997). The preference of ABCD1 for saturated FAs was then validated in yeast (van Roermund et al., 2008, 2011).

Cloned by homology using degenerate primers, the *ABCD2* gene was shown to code for ALDRP, the closest homolog of ALDP (Lombard-Platet et al., 1996). Both proteins display overlapping substrate specificities for saturated and monounsaturated LCFAs and



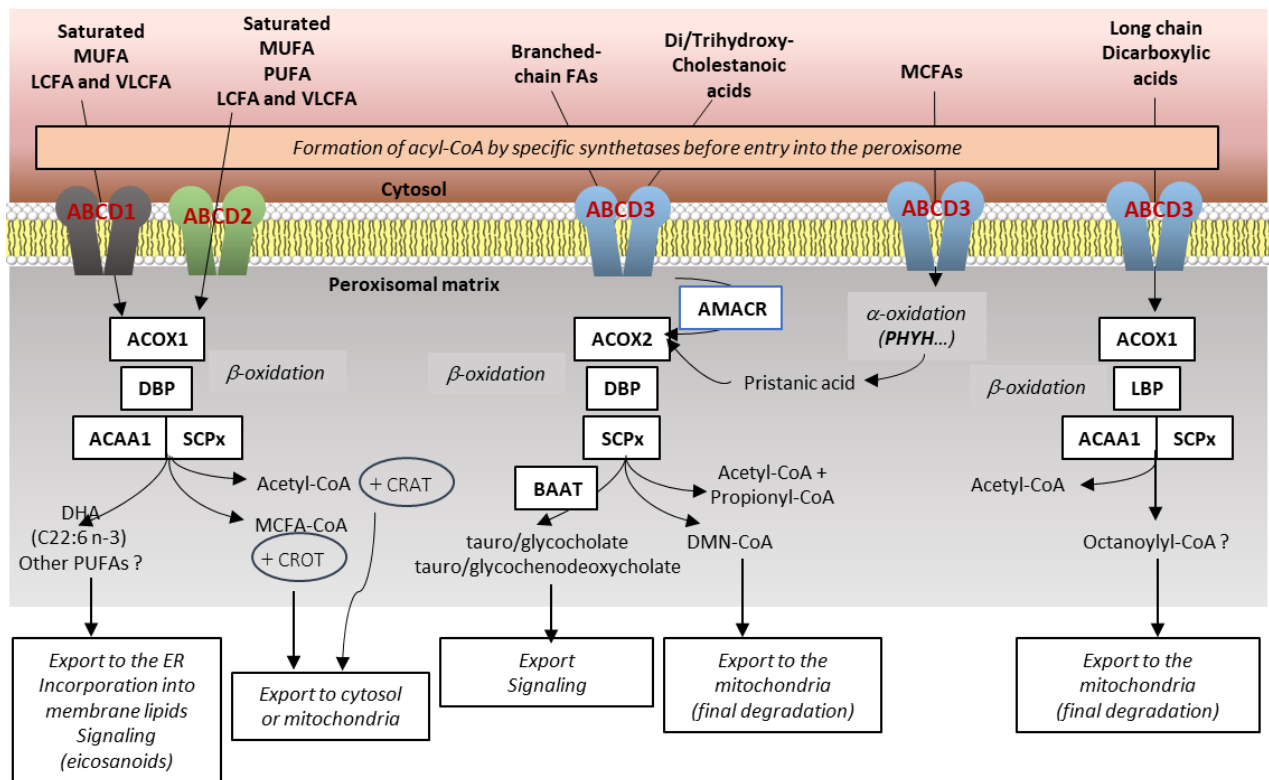
VLCFAs. In fact, exogenous expression of ABCD2 adequately restores the defect of  $\beta$ -oxidation in X-linked adrenoleukodystrophy fibroblasts in which ABCD1 is absent or nonfunctional (Kawaguchi & Imanaka, 2022). A specific role of ABCD2 in PUFA transport had been also demonstrated, especially DHA and its precursor (C24:6 n-3) (van Roermund et al., 2011). Experiments in mammalian cells confirmed such substrate preference (Geillon et al., 2014; Genin et al., 2011). Further studies using the *Abcd2* null mice demonstrated a specific role in monounsaturated FA (MUFA) transport, especially for erucic acid (C22:1 n-9) in adipose tissue (J. Liu et al., 2010, 2012) and an extended role in FA homeostasis (Fourcade et al., 2009).

PMP70, the protein coded by the *ABCD3* gene, was the first identified peroxisomal ABC transporter and is the most abundant peroxisomal membrane protein, at least in hepatocytes (Imanaka et al., 1999; Kamijo et al., 1990). ABCD3 is also involved in the transport of various lipids and shows overlapping substrate specificities with ABCD1 when overexpressed (Braiterman et al., 1998; van Roermund et al., 2014). Though, ABCD3 clearly has the broadest substrate specificity as it is involved in the transport of LCFAs and VLCFAs but also specifically in the transport of dicarboxylic acids, branched-chain fatty acids, and C27 bile acid intermediates such as di- and tri-hydroxy-cholestanoic acid (Ferdinandusse et al., 2015; van Roermund et al., 2014). Furthermore, a more recent study performed on manipulated HEK-293 cell models proved that ABCD3 is required for the transport of MCFAs across the peroxisomal membrane (Violante et al., 2019).

When it comes to the mechanism of transportation, it is already mentioned that the conversion of free FAs into CoA esters constitutes an initial activation step before peroxisomal  $\beta$ -oxidation. This reaction is catalyzed by specific acyl-CoA synthetase connected to the cytosolic side of the peroxisomal membrane (Watkins & Ellis, 2012). It was proved, using protease protection assays, that acyl-CoAs but not free FAs bind to the TMD of the transporter (Guimarães et al., 2005). It is therefore only after activation that the fatty acyl-CoAs are transported to the peroxisomal matrix through the peroxisomal ABC transporters. Fatty acyl-CoAs are captured on the cytosolic side by the TMD, enhancing the affinity of NBD for ATP. Molecules of ATP are then hydrolyzed, thus producing the energy needed to switch the conformation of TMD and eventually allowing the translocation of substrates from the cytosol into the peroxisomal matrix (Roerig et al., 2001; Tanaka et al., 2002). However, the exact mechanism of transport remains controversial. Two models are commonly considered. The first implies that esterified FAs are delivered directly to the

peroxisomal matrix, whereas in the other model, acyl-CoAs are hydrolyzed producing free FAs which are transported into the peroxisomal matrix. The free FAs are re-esterified by acyl-CoA synthetase when in the peroxisomal lumen.

Although the process of cleavage and reactivation of acyl-CoAs seems to be a waste of energy as two ATP molecules are needed for the activation reaction, such a mechanism is crucial for the specific permeabilization of the substrates of  $\beta$ -oxidation (Baker et al., 2015). Several studies have been done in an attempt to figure out the correct model for the transportation mechanism. Early studies on yeast models have demonstrated that fatty acyl-CoAs are hydrolyzed before being transported (van Roermund et al., 2012). The peroxisomal ABC transporters would release a free fatty acid that should be re-esterified inside the peroxisome before its catabolic processing. In addition, intrinsic acyl-CoA thioesterase activity has been found in COMATOSE (CTS), a homolog of human ABCD1 in *Arabidopsis thaliana*, proving again that VLCFA-CoA is hydrolyzed prior to transport (De Marcos Lousa et al., 2013). Very recently, the work of Kawaguchi et al. provided further proof of the transport mechanism (Kawaguchi et al., 2021). After expressing human His-tagged ABCD1 in methylotrophic yeast, they directly demonstrated that ABCD1 transports the FA moiety after the hydrolysis of VLCFA-CoA and that acyl-CoA synthetase is required before the  $\beta$ -oxidation of VLCFA-CoA within the peroxisomes. When it comes to the fate of the free CoA, they are released in the peroxisomal lumen, as revealed using isolated peroxisomes from *Saccharomyces cerevisiae* (Van Roermund et al., 2021). Finally, after their re-esterification, substrates are directly delivered to specific acyl-CoA oxidases to initiate the  $\beta$ -oxidation process.



**Figure 1: Peroxisomal ABC transporters and their involvement in lipid metabolism.**

Peroxisomal ABC transporters are represented as homo or heterotetramers with their preferential substrates and their involvement in metabolic routes including several enzymatic steps catalyzed by: AcylCoA oxidase 1 and 2 (ACOX1 and ACOX2), D- and L-bifunctional protein (DBP and LBP), Acetyl-CoA Acyltransferase 1 (3-ketoacylCoA thiolase, ACAA1), Sterol carrier protein 2 (SCPx), Alpha-methylacyl-CoA racemase (AMACR), Bile acid-CoA:amino acid N-acyltransferase (BAAT), Phytanoyl-CoA hydroxylase (PHYH). Adapted from (Tawbeh et al., 2021).

## V. Peroxisomal disorders and X-Linked Adrenoleukodystrophy (X-ALD)

### V.1. Peroxisomal disorders: General introduction

In 1964, Hans Zellweger and colleagues discovered the first peroxisomal disorder, initially named cerebro-hepato-renal syndrome, now known as Zellweger syndrome (ZS). This syndrome is characterized by the absence of peroxisome in the liver and kidneys of a patient having the lethal cerebrohepatorenal ZS (Goldfischer et al., 1973). It is a peroxisomal biogenesis disorder which occurs due to biallelic pathogenic variant in any of the 13 different PEX genes encoding different peroxins involved in the assembly of peroxisomes, transport of peroxisomal membrane or matrix proteins (Ebberink et al., 2011; Wanders et al., 2023; Waterham & Ebberink, 2012). After that, research on peroxisomes expanded rapidly. In the following decades, a number of other peroxisomal disorders were identified, characterized, and classified in two groups: Peroxisomal biogenesis disorders such as Rhizomelic chondrodysplasia punctata (RCDP); and single peroxisomal enzyme

deficiencies including ACOX1 deficiency as well as various disorders of peroxisomal fatty acid metabolism such as X-linked adrenoleukodystrophy (X-ALD). These defects lead to the accumulation of substrates or the loss of peroxisomal metabolic products, with dramatic consequences on several organs. It is worth noting that the central nervous system (CNS) is affected by defective peroxisomal lipid metabolism, as in defective peroxisomal  $\beta$ -oxidation. In X-ALD in particular, the accumulation of VLCFAs in the plasma and certain tissues is suspected to be the cause of the white matter abnormalities observed in some of the patients. It should be noted that X-ALD was discovered as early as 1910, however it wasn't until much later that the genetic basis of the disease and its connection to peroxisomal dysfunction were fully understood.

The defect in peroxisomal  $\alpha$ -oxidation caused by a deficiency in phytanoyl-CoA hydroxylase (PHYH) leads to the accumulation of phytanic acid and is responsible for a leukodystrophy called Refsum's disease. A defect in the synthesis of plasmalogens is responsible for RCDP in which the affected patients have skeletal abnormalities related to calcification deformities as well as intellectual deficit. On the other hand, other peroxisomal diseases of genetic origin also exist, usually less frequently. Acatlasemia, the deficiency of catalase activity, is one of these diseases in which the patients do not present abnormalities of lipid metabolism and nervous system functions.

As a result, several experimental models of peroxisomal pathologies have been developed. Baes and collaborators have largely contributed to the generation of these models and to the study of the associated neurodevelopmental anomalies by inactivating peroxisomal biogenesis genes in murine models (Baes et al., 2002). When it comes to the CNS, peroxisomes are present in all cell types and are generally smaller in size compared to other tissues. Mice with conditional peroxisomal deficiency in astrocytes, oligodendrocytes and neuronal cells develop myelination abnormalities (hypomyelination and demyelination) and axonal degeneration accompanying reactive gliosis (Hulshagen et al., 2008). Mice with a peroxisomal deficiency in oligodendrocytes alone do not show neurodevelopmental abnormalities at birth but develop demyelinating and neuroinflammatory lesions in adulthood (Kassmann et al., 2007). However, in the NEX-Pex5 model which was specifically designed to eliminate peroxisomes in neurons, no microscopic or metabolic abnormalities were observed, suggesting that the absence of functional peroxisomes in neurons does not result in axonal damage as in the case of oligodendrocytes, at least in mice. In astrocytes, the lack of peroxisomal metabolism causes

increased VLCFA levels in myelin, but this has no major impact on neurological functioning (Bottelbergs et al., 2010). Therefore, the absence of peroxisomal metabolism in neurons and astrocytes does not provoke the neurodegenerative phenotype observed after deleting peroxisomes from oligodendrocytes.

## **V.2. ACOX1 deficiency**

ACOX1 deficiency cases were first discovered in 1988 (OMIM #264470) (Poll-The et al., 1988). Currently, there are 30 identified patients in the world, at least as published in the literature. An extensive analysis of 22 patients revealed that most patients exhibited neonatal-onset hypotonia, seizures, failure to thrive, psychomotor retardation, sensorineural hearing loss, hepatomegaly, and visual loss with retinopathy (Ferdinandusse et al., 2007). Patients may exhibit some early motor development, but they typically pass away by the age of 2-3 years. This could be attributed to the neuronal and glial cell death which results from ACOX1 deficiency (Chung et al., 2020). Recently, milder and later-onset forms of ACOX1 deficiency have been documented, including two adult patients with normal early development who only developed progressive neurological symptoms in later childhood (Ferdinandusse et al., 2010).

The mouse model of ACOX1-deficiency shows an accumulation of plasma VLCFA, growth retardation and infertility and develop in adulthood a hepatic steatosis with abnormalities of peroxisomal proliferation (Fan et al., 1996). These mice also show several lipid abnormalities including a significant accumulation of polyunsaturated VLCFAs, resulting from the defect in peroxisomal  $\beta$ -oxidation (Infante et al., 2002). The unmetabolized substrates of ACOX1 enzyme function as biological ligands for peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) in liver. In ACOX1-deficient mice liver, sustained activation of PPAR $\alpha$  by ACOX1 substrates results in ER stress which in terms leads to hepatocarcinogenesis (Huang et al., 2011).

## **V.3. X-ALD: Introduction and etiology**

X-linked adrenoleukodystrophy (X-ALD; OMIM #300100) is the most common peroxisomal neurometabolic disorder (Trompier & Savary, 2013). X-ALD is characterized by a large spectrum of symptoms and has an estimated birth incidence of 1/14,700 (Turk et al., 2020) knowing that it is often underdiagnosed or misdiagnosed thus making it difficult to accurately estimate its incidence. In 1993, using positional cloning, the team of Hugo

Moser identified the *ABCD1* gene as being responsible for X-ALD (Mosser et al., 1993). Mutations in the *ABCD1* gene have been found in every X-ALD patient and are collected in the X-ALD database (<https://adrenoleukodystrophy.info/> accessed on 1 April 2023). Mutations resulting in an absent or non-functional ABCD1 protein lead to a defect in peroxisomal transport of VLCFAs thus reducing peroxisomal  $\beta$ -oxidation activity by approximately 60 to 80% (I. Singh et al., 1984). An accumulation of VLCFA is then detectable in the plasma and tissues of patients, especially in the cerebral white matter, the spinal cord and the adrenal cortex.

Logically enough, this accumulation results from the inability of the import of VLCFAs into the peroxisome, yet, increased endogenous biosynthesis had been spotted (Ofman et al., 2010). While it had been established that high levels of VLCFAs induce toxicity (Savary et al., 2012), the sequence of events leading to neurodegeneration and inflammation is still a matter of debate. Oxidative stress and cellular components, especially microglial functions, seem to play a major role in the pathogenesis of X-ALD (Bergner et al., 2019; Gong et al., 2017; I. Singh & Pujol, 2010).

This complex and fatal neurodegenerative disorder is characterized by a striking clinical variability both in the age of onset and in the symptoms with no existing genotype – phenotype correlation (Engelen et al., 2012; Wiesinger et al., 2015). The two main forms are the adult form, adrenomyeloneuropathy (AMN); and childhood cerebral ALD (cALD). AMN is a non-inflammatory slowly progressive demyelination which affects the spinal cord and peripheral nerves whereas cALD is characterized by inflammatory demyelination of the central nervous system. AMN affects virtually all male X-ALD patients reaching adulthood and about 60% of female carriers. Around 80% of all male X-ALD patients develop adrenocortical insufficiency making X-ALD the main cause of Addison's disease in which adrenal insufficiency might be its unique symptom (Dubey et al., 2005). In more than half of all male X-ALD patients, a still unknown trigger induces cALD with the breakdown of the blood brain barrier (BBB) and infiltration of mainly monocytes and T cells along with the severe rapidly progressive demyelination (Musolino et al., 2015; Powers et al., 1992; Weinhofer et al., 2018). In the majority of cases, untreated cALD results in a vegetative state or death within a few years after disease onset (Raymond et al., 2019). Since the disease is linked to chromosome X, males are most severely affected patients. Female carriers usually remain asymptomatic or present only a mild phenotype even though some severe forms have been diagnosed (Jangouk et al., 2012).

## **V.4. X-ALD pathophysiology**

### **V.4.1. VLCFA accumulation**

Cytoplasmic inclusions of lipid nature having lamellar structure were detected within the lesions of the white matter and the adrenal cortex after the first ultra-structural analysis by electron microscopy. These results as well as the presence of lipid-laden macrophages led to the conclusion that a metabolic defect was at the origin of X-ALD (Powers & Schaumburg, 1974). The accumulation of VLCFAs appears today as the main direct consequence of the absence of functional ABCD1 protein. VLCFAs accumulate in all tissues but with the greatest increase in the brain, spinal cord, adrenal cortex and the Leydig cells of the testis (Johnson et al., 1976). However, the exact mechanisms by which VLCFA accumulation contributes to the pathophysiology of X-ALD remain incompletely understood. Several hypotheses have been proposed. One hypothesis suggests that VLCFAs disrupt the structural integrity of biological membranes, leading to cellular dysfunction and death (Parisi et al., 2019). Another hypothesis involves the formation of toxic VLCFA-containing lipid species, such as sphingolipids and glycerophospholipids, which can induce oxidative stress, inflammation, and apoptosis (Kihara, 2012). In fact, accumulated VLCFAs are found in esterified form and in various complex lipids, mainly phosphatidylcholines and phosphatidylethanolamine (PE), essential components of the membranes (Igarashi et al., 1976). In the brain of ABCD1 deficient mice, saturated and monounsaturated VLCFAs were present, mainly in the sn-1 position, in numerous classes of phospholipids (75 species of phosphatidylcholine, 15 of PE, 5 of sphingomyelin, 4 of lysophosphatidylcholine, and 1 of lysophosphatidylethanolamine) (Hama et al., 2018). The accumulation of VLCFAs in the phosphatidylcholine-rich fractions of myelin is strongly likely to play a role in the initiation of the processes of demyelination and the neuroinflammatory response of cALD (Kemp & Wanders, 2010). The accumulation of VLCFAs is also accompanied by a decrease in the level of PE in the white matter of patients with cALD. These changes seem to be observed mainly in areas of inflammatory plaque and seem to be correlated with the production of ROS (Khan, Singh et al. 2008).

Moreover, several experiments have demonstrated the toxicity of VLCFAs *in vitro* (Hein et al., 2008; Zarrouk et al., 2012). These particularly hydrophobic molecules have a very strong affinity for biological membranes and a level of desorption of these membranes 10,000 times lower than that of long-chain FAs (Ho et al., 1995). They would

thus affect the structure, stability and function of these membranes, with multiple consequences in different cellular compartments. The accumulation of VLCFA induces dramatic consequences on mitochondria and ER and their functions. Mitochondrial respiration, biogenesis and significant metabolic pathways are impacted. Mitochondrial damage in X-ALD appears to be strongly related to oxidative stress abnormalities. Additionally, ER stress was demonstrated in fibroblasts from patients exposed to C26:0 (van de Beek et al., 2017).

The lack of peroxisomal  $\beta$ -oxidation is a leading cause of this accumulation. However, the seven elongases (ELOVL1-7 enzymes) located in the ER would also largely contribute to the increase in the level of VLCFA which reach up to 32 carbon atoms in certain tissues (Kemp et al., 2005). ELOVL1 is the main enzyme catalyzing the synthesis of saturated and monounsaturated 26-carbon VLCFAs. Its activity is essentially increased due to the greater availability of substrates. It would thus contribute to the physiopathology of X-ALD by increasing the intracellular levels of C26 (Ofman et al., 2010). Add to that, an increase in the activity of microsomal elongases is observed in the brain of ABCD1 deficient mice during development and the myelination phase. These activities are believed to disrupt myelin composition and play a role in triggering the pathology (Morita et al., 2015).

On the other hand, the link between VLCFA accumulation and cholesterol metabolism abnormalities had been investigated knowing that the organs most affected by a deficiency of ABCD1, brain and adrenal glands, have the highest content of cholesterol in the body. While a high-cholesterol diet normally causes a drop in cerebral VLCFA levels in wild-type mice, this same diet causes an increase in cerebral VLCFA levels in deficient mice, in particular via a drop in *Abcd2* expression. Conversely, a decrease in cholesterol intake reduces the accumulation of VLCFAs in deficient human and murine fibroblasts (Weinhofer et al., 2005). The tissue metabolic specificities highlighted in this study could be linked to the BBB. It would seem that the decrease in the level of C26:0 observed in cholesterol-depleted X-ALD fibroblasts is in fact accompanied by a higher expression of stearoyl-CoA-desaturase and an increase in the level of C26:1 and other monounsaturated fatty acids, without the  $\beta$ -oxidation being affected (Engelen et al., 2008). Phosphatidylcholine metabolism, which is altered in X-ALD, also seems to strongly impact cholesterol homeostasis in macrophages (Lagace, 2015). Furthermore, intracellular cholesterol trafficking is likely to involve lysosome-



peroxisome interactions. This traffic would be disrupted in ABCD1 deficient cells and lead to an accumulation of cholesterol in the lysosomes (Chu et al., 2015). These abnormalities in cholesterol metabolism also seem to cause its accumulation in esterified form and the formation of lipid inclusions observable by microscopy (Tangirala et al., 1994).

Another possible consequence of ABCD1 deficiency is the induction of the accumulation of ligands of several nuclear receptors and would therefore also be responsible for specific transcriptional responses. Some of these transcriptional responses are likely to contribute to the pathology and the neuro-inflammatory response, in particular by promoting the expression of 25-hydroxylase and the accumulation of 25-hydroxycholesterol (25-HC), which is responsible for the activation of the inflammasome NLRP3 and the secretion of pro-inflammatory cytokines by microglial cells (Jang et al., 2016).

Lastly, although the accumulated VLCFAs in certain classes of lipids seem to play a central role in the pathology, no correlation between the plasma level of VLCFAs and the phenotype or the severity of the pathology could be demonstrated (A. B. Moser et al., 1999). This would suggest that the plasma VLCFA levels do not reflect the tissue levels of free or esterified VLCFA. However, actors other than VLCFAs are also possibly involved in the pathology. In fact, *Abcd1* knockout mouse models present less severe phenotypes and CNS damage than in humans. They show key biochemical features of X-ALD, including VLCFA accumulation, but develop a late onset progressive neurodegenerative phenotype involving the spinal cord and sciatic nerves without brain damage (Pujol et al., 2002).

#### **V.4.2. Oxidative stress**

Oxidative stress is “a transient or long-term increase in steady-state ROS levels, disturbing cellular metabolic and signaling pathways, particularly ROS-based ones, and leading to oxidative modifications of an organism’s macromolecules that, if not counterbalanced, may culminate in cell death via necrosis or apoptosis” (Lushchak & Storey, 2021). In the three-hit hypothesis of X-ALD, oxidative stress is described as the first hit to cause axonal injury of AMN, or subsequently induce inflammatory demyelination and cell death of cALD (I. Singh & Pujol, 2010). ABCD1 deficiency and excess of VLCFA lead to dysregulation of redox homeostasis. Oxidative stress appears

as an early event in X-ALD physiopathology and causes protein damage that can be measured by markers such as lipoxidation and glycooxidation (Fourcade et al., 2008). In the affected human brain parenchyma, this oxidative stress is evidenced by an overexpression of manganese superoxide dismutase (SOD) and iNOS associated with the presence of peroxynitrite (ONOO<sup>-</sup>), malondialdehyde and 4-hydroxynonenal (Powers et al., 2005). An increase lipid peroxidation could also be observed along with an increase in SOD and catalase activities in the fibroblasts of X-ALD patients (C. R. Vargas et al., 2004). In parallel, a decrease in glutathione in reduced and oxidized form was detected in lymphocytes, confirming the redox imbalance in X-ALD (Petrillo et al., 2013). Additionally, plasma SOD activity in cALD patients is reduced compared to AMN patients. This SOD activity seems to be inversely correlated with the severity of the pathology and therefore presupposes an important role of oxidative stress in the initiation of the neuro-inflammatory response (Turk et al., 2017).

The link between VLCFA accumulation, oxidative stress and inflammation, the three presumed central players of X-ALD, is still unclear today. However, mitochondrial function seems to be one of the main victims of VLCFA accumulation and its dysfunction could be at the origin of the redox imbalance and the inflammatory response. The inner mitochondrial membrane is found to be impacted as its permeability is altered eventually inducing cell death in astrocytes and oligodendrocytes in vitro (Hein et al., 2008). Add to that, the brain's white matter and adrenal gland are metabolically active tissues with abundant mitochondria in which ROS is primarily generated (Zorov et al., 2014). Both the brain and adrenal glands are lipid-rich organs that are particularly vulnerable to oxidative stress and possess active cholesterol metabolism. The regulation of cholesterol metabolism is intricately linked to redox equilibrium since mitochondrial ROS is produced during cholesterol synthesis. This makes these organs particularly prone to oxidative stress which therefore serves as the underlying influential factor contributing to the predilection of X-ALD-affected sites (J. Yu et al., 2022).

Mitochondrial dysfunction and irregularities of oxidative phosphorylation seem to strongly contribute to axonal degeneration. These oxidative phosphorylation abnormalities appear to be common to AMN and cALD and would be involved in the induction of the pro-inflammatory transcriptional response (Fourcade et al., 2014; Schlüter et al., 2012). Additionally, oxidative stress and mitochondrial dysfunction in X-ALD could be associated with many abnormalities such as disturbance of autophagy and

its inhibitor, mammalian target of rapamycin (mTOR), as well as disruption of signaling pathways dependent on peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and its coactivator 1- $\alpha$  (PGC1 $\alpha$ ) (Fourcade et al., 2015; Launay et al., 2013, 2015). The alteration of the cellular metabolic environment associated with mitochondrial damage is also likely to modify the inflammatory response. AMP-activated protein kinase (AMPK) is known to suppress the expression of inflammatory genes and attenuate inflammatory injury (O'Neill & Hardie, 2013). A decrease in the activation of the AMPK-dependent pathway was observed in the fibroblasts and lymphocytes of cALD patients but not in AMN patients (J. Singh & Giri, 2014).

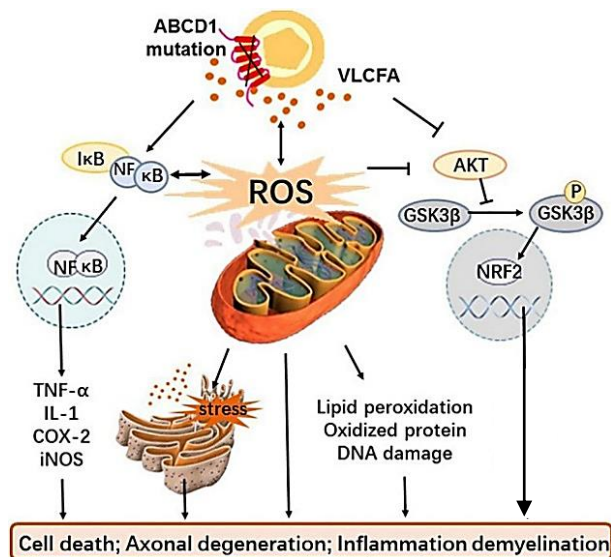
#### **V.4.3. Inflammation**

The increased secretion of pro-inflammatory cytokines including IL-1 $\beta$ , IL-2, IL-8 and Tumor Necrosis Factor  $\alpha$  (TNF) is a hallmark in X-ALD pathogenesis along with the abnormalities observed by magnetic resonance imaging (MRI). They could be measured in the plasma of patients with cALD. This inflammatory response appears to develop at an early stage as it is also observed in X-ALD patients who are still asymptomatic or suffering from AMN. It also appears to be correlated with the increase in plasma VLCFA levels (Marchetti et al., 2018). Although AMN is considered non-inflammatory, impaired glycolipid and glycerophospholipid metabolism and an atypical inflammatory profile have been demonstrated by a study of the metabolome of mononuclear blood cells from AMN patients (Ruiz et al., 2015).

Several factors suggest the involvement of a cytotoxic cellular immune response in the demyelinating damage of cALD. The hypothesis of an abnormal antigenic presentation of phosphatidylcholines resulting from the accumulation of VLCFAs has thus been proposed (Theda et al., 1992). The presence of an inflammatory infiltrate composed of cytotoxic T-lymphocytes (CD8 $^{+}$ ) suggests the involvement of lipid antigen presentation via molecules such as CD1D and potentially responsible for oligodendrocyte cytolysis. *CD1D* is expressed by microglial cells in cALD and allows the presentation of lipid antigens to T-lymphocytes (Ito et al., 2001). However, an involvement of such actors in the pathology seems insignificant given the very small differences in *CD1D* expression and the number of T-lymphocytes measured in patients (Barbier et al., 2012; Gautron et al., 2010).

When it comes to the oligodendrocytes, it is believed that their high susceptibility to VLCFAs is one of the hypotheses capable of explaining the demyelinating damage observed in cALD. Impaired peroxisomal function in oligodendrocytes is sufficient to cause demyelinating injury in mice (Kassmann et al., 2007). Besides, oligodendrocytes apoptosis has been observed in white matter lesions and appears to be correlated with demyelination in cALD patients (Feigenbaum et al., 2000). However, whether the death of oligodendrocytes is directly caused by VLCFA accumulation or indirectly via inflammatory mediators is questionable. It was observed that the increased accumulation of VLCFAs within demyelinated areas is accompanied by significant astrocyte and microglial activation (Paintlia et al., 2003), which in cALD appear to be associated with TNF secretion thus altering oligodendrocyte viability (Powers et al., 1992). This was further demonstrated when an inflammatory response through the nuclear factor  $\kappa$ B (NF- $\kappa$ B) was obtained after using RNA interference to decrease the expression of *ABCD1* and *ABCD2* in astrocytic cells in vitro (J. Singh et al., 2009).

On a different perspective, Musolino and collaborators demonstrated a major effect of ABCD1 deficiency on the cerebral microvascular endothelium. The defect resulting in the accumulation of VLCFAs leads to a decrease in the expression of endothelial tight junction proteins, in vitro. The weakening of the BBB would then be accompanied by an increased recruitment of monocyte cells. These effects seem to initiate and enhance the neuroinflammatory response in patients with X-ALD. Moreover, the absence of *ABCD2* expression in these cells would prevent any compensatory effect (Musolino et al., 2015). This same team was able to demonstrate an increase in cerebral microvascular flow and an alteration in the integrity of the BBB in the perilesional areas at early stages in patients with cALD (Lauer et al., 2017).



**Figure 2: The role and mechanism of oxidative stress and inflammation in X-ALD.**

Mutations in *ABCD1* gene obstruct the transport of VLCFA for  $\beta$ -oxidation, resulting in VLCFA buildup. Malfunctioning peroxisomes and accumulated VLCFA not only trigger mitochondrial production of excessive ROS and oxidative stress but also directly disrupt the GSK3 $\beta$ -NRF2 antioxidant pathway, causing redox imbalance and activating the NF- $\kappa$ B pathway thus inducing inflammation. Moreover, excessive ROS can impact antioxidant systems and inflammatory pathways, inducing ER stress, and generating peroxidation damage to macromolecules. These changes ultimately culminate in cell death, axonal degeneration, and inflammatory demyelination. Adapted from (Yu et al., 2022).

### V.5. X-ALD treatment

In 1984, a first trial of therapy by allogeneic hematopoietic stem cell transplantation (HSCT) was performed on a 13-year-old boy with cALD, but the benefits of this therapy were outweighed by the risks associated with the transplant, and the trial proved unsuccessful (H. W. Moser et al., 1984). Six years later, the same intervention in a patient at an early stage of the disease showed much more encouraging results as the white matter lesions observed by MRI have disappeared 18 months after the transplant (Aubourg et al., 1990). As myeloablative chemotherapy preceded an allogeneic HSCT, the beneficial effects are probably attributable to a partial replacement of microglia and perivascular macrophages: up to 25% of the cells in these populations would then come from the donor, restoring normal metabolic function in these cells (Asheuer et al., 2004; Priller et al., 2001). Ideal candidates for such intervention are individuals without any neurologic deficits, who receive HLA matched sibling or related donor HSCT. However, this intervention has a high-morbidity and long-term sequelae related to immunosuppression and graft versus host disease. It is important to note that disease progression continues for some 6–9 months following HSCT (Miller et al., 2011; Peters et al., 2004; Raymond et al., 2019) and that

adrenal dysfunction is not corrected following HSCT transplant for cerebral disease (Burtman & Regelman, 2016). In order to limit the main complications and the high mortality (35%) associated with allogeneic transplants, gene therapy trials on autologous stem cells have been carried out. This therapy involves transfer of DNA encoding *ABCD1* into autologous stem cells (CD34 positive) by lentiviral vector (modified HIV-1) ex vivo before the cells are transplanted. Two years after transplantation, 9 to 14% of leukocytes express the *ABCD1* gene. Gene therapy presents neurological benefits comparable to those of allogeneic transplantation and therefore appears to be a major advance (Cartier et al., 2009). It is suggested that gene therapy would be the safest therapeutic option, with lentiviral vectors presenting a reduced risk of mutagenesis (F. Eichler et al., 2017). However, although preventing the neuroinflammatory damage associated with cALD, these therapies do not seem to prevent the development of myelopathy in adulthood (van Geel et al., 2015).

Several attempts for the development of nutritional therapy for X-ALD have also emerged. A part of the VLCFAs has an exogenous origin, however, a dietary restriction in VLCFAs does not seem sufficient. The endogenous synthesis of VLCFAs therefore seems to contribute in a major way to the total mass of VLCFAs. In 1986, Augusto Odone, with the help of Professor Moser's laboratory, developed a therapy for his son Lorenzo who suffered from cALD. The developed Lorenzo's oil constitutes of a mixture of triglycerides formed of oleic acid (C18:1) and erucic acid (C22:1) with a ratio of 4:1. This mixture helps to normalize plasma levels of C24:0 and C26:0 (A. B. Moser et al., 1987). This effect is at least partly attributable to the inhibitory effect of oleic and erucic acid on the elongase ELOVL1 (Sassa et al., 2014). However, although the treatment appears to reduce the risk of abnormalities appearing on MRI (H. W. Moser et al., 2005), it does not seem to affect the progression of neurological damage (Aubourg et al., 1993). Thus, the effectiveness of this treatment in X-ALD is still debated.

The existence of a functional redundancy between the *ABCD1* and *ABCD2* transporters generates interesting prospects for pharmacological intervention. In humans, the low level of expression of *ABCD2* in several cell types such as macrophages or endothelial cells supports the strong potential of therapeutic possibility. 4-phenylbutyrate is one of the first molecules demonstrating an ability to induce the expression of *ABCD2* and *ABCD3*, to improve the activity of peroxisomal  $\beta$ -oxidation and to decrease tissue levels of VLCFA in vivo (Kemp et al., 1998). However, the rapid degradation of this molecule in

vivo makes its use limited in the context of X-ALD. Valproic acid, in addition to possessing antioxidant properties, appears to have a similar mechanism of action (Fourcade et al., 2010). However, its use in humans causes considerable side effects, including significant liver toxicity, thus limiting its potential therapeutic benefits in X-ALD (T. K. H. Chang & Abbott, 2008).

Due to the dysregulation of cholesterol metabolism observed in ABCD1 deficient mice, lipid-lowering molecules, blocking the cholesterol synthesis pathway, have been tested in vitro and in vivo, including lovastatin (Khan et al., 2008; I. Singh et al., 1998; Yamada et al., 2000). Cholesterol depletion was shown to increase the expression of *ABCD2* and reduce VLCFAs accumulation in vitro (Weinhofer et al., 2002). However, a clinical trial carried out in 14 patients concluded that lovastatin has a non-specific effect on VLCFAs and that there is no therapeutic benefit (Engelen et al., 2010). Add to that, treatment with simvastatin, another cholesterol-lowering substance, was found to further exacerbates VLCFA accumulation in the CNS of ABCD1 deficient mice (Cartier et al., 2000).

In addition to the *ABCD2* expression induction pathway, several molecules have been tested in vitro or in vivo aiming at improving mitochondrial function and limiting the inflammatory response in X-ALD. They include AMPK activators such as metformin (J. Singh et al., 2016), PPAR $\gamma$  activators such as pioglitazone (Morato et al., 2013), "nuclear factor erythroid 2-like 2" activators (NRF2) such as dimethylfumarate (Ranea-Robles et al., 2018) or antioxidant molecules, such as cyclophilin D (López-Erauskin et al., 2011), which have cytoprotective properties in vitro and in vivo . However, few of these molecules have been able to demonstrate their effectiveness in clinical trials. Immuno-modulatory or immuno-suppressive therapies, including intravenous injection of immunoglobulins or interferon- $\beta$ , have also been tested without demonstrating conclusive effects (Cappa et al., 1994; Korenke et al., 1997). Lately, an exciting new treatment approach has been studied. Preclinical treatment with a selective agonist of cannabinoid receptor 2 (CB2r) halted axonal degeneration and associated locomotor deficits, along with normalization of microgliosis (Parameswaran et al., 2022).

The failure of these therapies, despite the inflammatory nature of demyelinating lesions in cALD, highlights important pathophysiological differences between X-ALD and other demyelinating diseases such as multiple sclerosis. This also sheds light on the importance of the follow-up of X-ALD patients. Indeed, newborn screening for X-ALD was added to the Recommended Uniform Screening Panel in February 2016. The implementation of this

screening continues to expand in the United States and is being considered in other countries (A. B. Moser et al., 2022). However, affected males may experience adrenal disease at any stage of life, and more than a third of boys develop cALD. In order to prevent significant morbidity and mortality, and to be able to provide best evidence-based care for children and their families, especially with a focus on preventing the devastating advanced forms of cALD, the establishment of reliable imaging criteria and biomarkers is essential (Turk et al., 2020). In other words, we are in need of reliable biomarkers to monitor X-ALD disease progression as well as the efficacy of the provided treatments.



## **Chapter 2: Microglia**

### **I. General introduction and origin**

Microglial cells are the innate immune cells of the CNS representing between 5 and 12% of CNS cells depending on the region. They play an essential role in maintaining CNS homeostasis through performing routine surveillance and continuously monitoring their surroundings with motile protrusions. They have the ability to detect any abnormalities or lesions within the brain parenchyma and resolve any disturbance (Nimmerjahn et al., 2005; Sevenich, 2018).

The term "microglia" emerged during the first decade of the twentieth century. Nicolás Achúcarro was among the pioneering scientists who identified brain cells possessing phagocytic capabilities, which are now recognized as microglia. Subsequently, Achúcarro's student, Pío del Río-Hortega, refined the techniques and successfully examined the intricate morphological details of microglia. In 1924, Río-Hortega's findings gained global recognition, establishing microglia as a distinct cell type (Tremblay et al., 2015).

A hematopoietic origin of microglia has long been debated and it is only recently that their origin has been highlighted. Microglia derive from myeloid progenitors called primitive macrophages originating from the yolk sac before the 9<sup>th</sup> embryonic day in mice and 4.5 weeks in the human fetus (Ginhoux et al., 2010). In mammals, these primitive macrophages migrate through the circulatory system, as soon as it is established, to colonize the cerebral parenchyma. Once reached, this cell population first proliferates in the form of amoeboid cells before colonizing the entire cerebral parenchyma and adopting a branched morphology, a process that occurs from the second trimester in humans (Monier et al., 2006).

Microglia differentiate prior to the establishment of hematopoiesis and the formation of the BBB, i.e., before 13.5 embryonic days in mice. The conservation of primitive macrophages, from which microglia originate, suggests their crucial role during embryonic development in vertebrates. Therefore, microglia have a distinct origin compared to other mononuclear phagocytic cells. The presence of monocytic cells capable of differentiating into cells resembling microglia within the brain parenchyma has contributed to the ambiguity surrounding the origin of microglia for a long time (Ginhoux et al., 2013).

Microglia possess the remarkable ability to maintain their population through local self-renewal. In humans, their renewal occurs at a slow rate (28% per year), with certain cells persisting for several decades, indicating that the entire microglial cell population is expected to be renewed throughout a person's lifetime (Réu et al., 2017). This longevity and capacity for

self-renewal are notable features when considering the significant phenotypic plasticity exhibited by these cells.

Under certain pathological conditions, blood or bone marrow precursors have the ability to generate myeloid cells colonizing the cerebral parenchyma and adopting a microglial cell phenotype, similar to infiltrating macrophages colonizing tissue in response to the secretion of chemoattractant molecules. Infiltrating microglial cells have been demonstrated in models of hematopoietic stem cell transplantation, after irradiation and alteration of the BBB, or in models of chronic neurodegenerative diseases or even in mice deficient for the PU.1 gene, where embryonic endogenous microglia are absent (Beers et al., 2006; Ginhoux et al., 2013). Recent studies suggest that other CNS macrophage populations including perivascular macrophages, meningeal macrophages and choroid plexus macrophages originate from primitive macrophages and form stable populations comparable to microglial cells. These cells are likely to contribute to the maintenance of CNS homeostasis and to provide functions similar to those of microglia in normal and pathological conditions (Goldmann et al., 2016).

## **II. Role during normal physiological conditions**

### **II.1. Sensing and homeostasis**

Under resting conditions in the adult brain, microglia represent highly ramified motile processes that constantly extend and retract to survey the local environment (Bernier, York, Kamyabi, et al., 2020). They are in constant and dynamic motion, surveying the brain parenchyma through their extremely motile thin processes and protrusions. Microglia express several genes encoding various cell receptors, which allow them to sense minute changes in the tissue microenvironment (Afridi et al., 2020; Nimmerjahn et al., 2005). This ensures a rapid reaction to any loss of homeostatic equilibrium. Microglia then have the ability to adopt an amoeboid morphology and a so-called “activated” phenotype. This process is accompanied by a rapid rearrangement of the actin cytoskeleton, an increased expression of membrane receptors and an increase in its phagocytic or cytotoxic activity. They can therefore present varied activation profiles, likely to promote a pro-inflammatory or anti-inflammatory response, allowing tissue regeneration and the survival of neuronal and glial cells. This plasticity has a potential role in clearing the brain parenchyma of degraded metabolic products and tissue components (Nimmerjahn et al., 2005).

Microglia have a very low activation threshold and have the ability to detect a very wide range of signals. They differ from other myeloid cells by the presence of a large

number of neurotransmitter receptors which are useful for their interaction with other CNS cells (Neumann et al., 2009). Ionotropic and metabotropic purinoceptors are also expressed on microglial membranes and are activated by ATP. ATP appears to be the essential mediator of the rapid response of microglia as they express the purinergic metabotropic P2Y receptors. Ionotropic receptors P2X4 and P2X7 are also expressed by microglia. P2X7 is up-regulated under pathological conditions, contributing to cytokine release and microglial activation. A local increase in the level of extracellular ATP seems sufficient to induce a microglial response (Caruso et al., 2023; Davalos et al., 2005).

The limited regenerative capacity of nervous tissue could explain this specialization of the immune cells within the CNS to prevent the damage of an inflammatory nature in this tissue. Tissue damage of a necrotic nature is likely to induce the release of mediators of inflammatory reaction. In the mature CNS, microglial cells possess a unique molecular signature dependent on the "Transforming Growth Factor  $\beta$ " (TGF- $\beta$ ), distinguishing them from other infiltrating myeloid cells. This molecular signature regulates the expression of the metabotropic purinergic receptor P2RY12 which is responsible for the homeostatic function of these cells (Butovsky et al., 2014).

Lastly, it would be safe to say that there are no resting microglia. Their sensing, housekeeping, and protecting functions keep them constantly engaged.

## **II.2. Phagocytosis**

### **II.2.1. General mechanism**

Phagocytosis is the cellular process by which a large extracellular component ( $>0.5 \mu\text{m}$ ), another cell or parts of cells are engulfed and digested. It comprises three main steps: recognition, engulfment, and digestion. During recognition (and often engulfment as well), "eat me" signals are recognized by phagocytic receptors. These signals comprise phosphatidylserine or calreticulin, as well as opsonins such as growth arrest-specific 6 (Gas6), milk fat globule epidermal growth factor 8 (MFG-E8), or complement factors present on the surface of target cell. Microglia express several families of receptors that enable phagocytosis or endocytosis of apoptotic cells, protein aggregates, and lipoprotein particles. These receptors include scavenger receptors, such as CD36, SR1, and MARCO (Areschoug & Gordon, 2009); LDL receptor family members, such as LDLR, ApoER2, and VLDL (Rebeck et al., 2006); and three receptor tyrosine kinases, Tyro3, Axl, and MER receptor tyrosine kinase (MerTK) (Lemke, 2013). MerTK and Axl are expressed

in surveying and activated microglia, respectively (Fourgeaud et al., 2016). Microglia also capture and endocytose immune complexes and complement-opsonized protein complexes through Fc receptors and complement receptors. Other phagocytic receptors include complement receptor 3 (CR3) and triggering receptor expressed on myeloid cells 2 (TREM2) (Vilalta & Brown, 2018).

TREM2 appears as one of the main phagocytosis receptors as it has the ability to recognize a large number of ligands and affect the microglial response. These signals would modulate the metabolic response and promote the survival, proliferation and phagocytosis activity of microglial cells, in particular via the activation of the adapter protein DAP12 and the Wnt/ $\beta$ -Catenin and mTor signaling pathways (Ulland et al., 2017; Zheng et al., 2017). Signaling via this receptor would also have an anti-inflammatory effect by facilitating the phagocytosis of myelin debris and by increasing the lysosomal activity of phagocytic cells. The decrease or loss of expression of TREM2 is associated with defective tissue repair and increased expression of pro-inflammatory cytokines (Takahashi et al., 2007). Finally, Apolipoprotein E (ApoE) acts as an opsonin by binding to exposed phosphatidylserine and acts as a ligand of TREM2 (Atagi et al., 2015).

After the engulfment step, the process progresses to the next stage, which is the maturation and degradation of the internalized material. This involves the fusion of the phagosome, the vesicle containing the engulfed material, with a lysosome. Lysosomes play a crucial role in phagocytosis by microglia. They are specialized organelles within the cell that contain various hydrolytic enzymes, including proteases, nucleases, lipases, and carbohydrases. These enzymes are responsible for breaking down and degrading the internalized material. During the process, the phagosome fuses with a lysosome, resulting in the formation of a phagolysosome. This fusion brings the hydrolytic enzymes of the lysosome into contact with the engulfed material, allowing for its degradation. The enzymes within the lysosome break down proteins, nucleic acids, lipids, and carbohydrates into smaller molecules that can be recycled by the cell or expelled (Kono et al., 2021). The lysosomal enzymes function optimally in an acidic environment, and upon fusion, the pH of the phagolysosome decreases due to the action of proton pumps on the lysosomal membrane, the V-ATPases. This acidic pH facilitates the activity of the hydrolytic enzymes, enhancing their effectiveness in breaking down the internalized material (Marshansky & Futai, 2008).

### **II.2.2. Phagocytosis of myelin debris**

Microglia play a vital role in myelin phagocytosis by engulfing debris from damaged myelin, thereby preventing the release of harmful intracellular substances (Fukumoto et al., 2019). Under homeostatic conditions, microglia selectively phagocytose myelin sheaths to maintain myelination balance. However, excessive phagocytosis of myelin sheaths can accelerate demyelination, leading to detrimental consequences (Hughes & Appel, 2020). In many neurodegenerative conditions, microglia, actively participating in myelin repair, accumulate cholesterol and lipid-rich debris along with pro-inflammatory characteristics, including increased TREM2 expression. Single-cell RNA sequencing suggests that these microglia upregulate genes coding for APOE and LPL, as well as other phagocytic signature genes associated with lipid clearance and metabolism and extracellular matrix reshaping (Zareba & Peri, 2021). In multiple sclerosis, aiming at reinforcing remyelination by oligodendrocytes, microglia can synthesize sterols using the internalized cholesterol-rich myelin debris (Berghoff et al., 2021). Thus, a timely activation of the pro-inflammatory program in microglia appears to be associated with demyelinating conditions as it is required for myelin clearance, oligodendrogenesis, and remyelination (F. Yu et al., 2022).

### **II.2.3. Efferocytosis**

The clearance of apoptotic cells, termed ‘efferocytosis’, is a crucial mechanism that ensures tissue homeostasis in normal physiological conditions and aids in tissue repair, inflammation resolution and restoring homeostasis after diseases (Arandjelovic & Ravichandran, 2015; Doran et al., 2020; Ge et al., 2022). It has three main outcomes: cessation of inflammatory reactions, facilitation of self-tolerance, and activation of proresolving pathways. Phagocytes identify and locate cellular apoptotic bodies, then internalize them through reorganization of the plasma membrane (Boada-Romero et al., 2020). Actin remodeling triggers invagination, causing localized protrusion of the plasma membrane and formation of phagosomes, which would be followed by the fusion with lysosomes as discussed earlier (Boada-Romero et al., 2020; Doran et al., 2020). The components of the lysosomes facilitate the digestion of apoptotic cells to maintain equilibrium (Viaud et al., 2018).

However, when efferocytosis is hindered and its homeostatic functions are compromised, inflammation is increased thus leading to various diseases. In several

chronic inflammatory disorders, due to the impairment of efferocytosis, deceased cells accumulate. These dead cells can undergo secondary necrosis, triggering autoimmune reactions, tissue necrosis, and abnormal inflammation (Kawano & Nagata, 2018; Szondy et al., 2014). Aberrant efferocytosis appears to be involved in several inflammatory and autoimmune disorders, including rheumatoid arthritis, diabetes, multiple sclerosis, and autoimmune lymphoproliferative syndrome (Ge et al., 2022). Aberrant efferocytosis could be also related to neurological disorders such as epilepsy. Interestingly, mouse models and human biopsy samples have shown impaired microglial efferocytosis during epilepsy caused by hyperactivity of the neuronal network (Abiega et al., 2016).

Due to its important implication in inflammation, efferocytosis's impact on anti-inflammatory and tolerogenic pathways has been thoroughly examined in previous studies (Blander, 2017; Doran et al., 2020; Elliott et al., 2017; Ge et al., 2022).

#### **II.2.4. Synaptic pruning**

The process of phagocytic elimination of non-functional synapses by microglia during development is named synaptic pruning. This process was first discovered in 1968 as displacement of synaptic terminals by microglial cells but without entirely elucidating its functional significance (Blinzinger & Kreutzberg, 1968). It is now known that it contributes to neuronal network refinement and learning between birth and late adolescence in humans (Neniskyte & Gross, 2017). The selective elimination of inappropriate synaptic connections depends on several signals such as the complements or the chemokine fractalkine CX3CL1. The complement cascade initiated in the concerned neurons makes it possible to target unsolicited synapses. The latter can then be phagocytosed by microglia expressing the receptor for the C3 component of complement (Stevens et al., 2007). In mice deficient for the fractalkine receptor CX3CR1, which is expressed only by microglia within the CNS, an increase in the number of dendritic spines and immature synapses is observed, characteristic of an immature neural network and consistent with insufficient synaptic pruning (Paolicelli et al., 2011).

### III. Role during inflammatory conditions

#### III.1. Sensing, activation, and secretions

Being the cells mediating the innate immune response in the CNS, microglia play a crucial role in detecting and responding to inflammatory conditions. Inflammatory reactions in the CNS can be triggered by various factors, such as injury, infection, or neurodegenerative diseases. Microglial sensing and recognition come as a first step in responding to CNS disturbances. This is ensured by a set of receptors expressed by microglial cells as the pattern-recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) or tissue damage-associated molecular patterns (DAMPs). PAMPs comprise molecular structures unique to microbes such as lipopolysaccharides (LPS), a microbial cell wall component (Medzhitov, 2007). DAMPs are internal molecules typically confined within cells unless they are released due to injury. In physiological states, these DAMPs remain concealed from the detection of innate immune cells. Nevertheless, during instances of cellular stress or damage, DAMPs can be liberated into the extracellular environment from injured cells, subsequently triggering the activation of innate immune cells (G. Y. Chen & Nuñez, 2010).

Microglial PRRs include Toll-like receptors (TLRs), such as TLR4 and TLR1/2, and their coreceptors, such as CD14; NOD-like receptors (NLRs), such as the NLRP3 inflammasome (Heneka et al., 2015; Saijo et al., 2013); receptors for nucleic acids (J. Wu & Chen, 2014); and C-type lectin receptors (CLRs), such as CLEC7A (Sancho & Reis e Sousa, 2012). TLR4 has the ability to recognize LPS of bacterial origin and to induce an inflammatory response via NF- $\kappa$ B signaling pathway (Ransohoff & Perry, 2009). Add to that, the reciprocal interaction between the microglial chemokine receptor and the neuronal ligand CX3CL1 allows precise and effective communication between neurons and microglial cells and thus plays a key role in coordinating many aspects of brain function. The altered functionality of the CX3CL1/CX3CR1 pathway in several different pathological states may promote the activation of microglia and stimulate the release of inflammatory factors (Pawelec et al., 2020).

After the recognition step, microglia have the ability to activate and implement an adapted polarized phenotype. Microglia activation is often categorized as either classical (M1) or alternative (M2), following the paradigm used for macrophages. M1 activation is a proinflammatory and neurotoxic state typically induced by simultaneous triggering of TLR and interferon gamma (IFN- $\gamma$ ) signaling pathways thus inducing the production of

proinflammatory cytokines and chemokines, such as TNF, IL-6, IL-1 $\beta$ , IL-12, and monocyte chemoattractant protein 1 (MCP-1, also named CCL2). M1 microglia also express the NADPH oxidase, which generates superoxide and ROS, as well as iNOS, which converts arginine into NO. iNOS is largely overexpressed in response to LPS, and its product, NO, has a bactericidal effect and regulates apoptosis (Lee et al., 2001). ROS synthesis is accompanied by mitochondrial, metabolic and morphological rearrangements involving the “Mitogen-activated protein kinases” (MAPK) signaling pathway (Kato et al., 2017). M1 microglia also express high amounts of Fc receptors as well as Major Histocompatibility Complex (MHC) class II which are recognized by CD4<sup>+</sup> helper T cells. Ultimately, M1 microglia induce inflammation and neurotoxicity (Colonna & Butovsky, 2017).

M2 activation describes the anti-inflammatory and healing activities of microglia. It can be induced by IL-4, IL-13, IL-10, ligation of Fc receptors by immunocomplexes, and detection of apoptotic cells, as well as activation of the transcription factors PPAR $\gamma$ , liver X receptor (LXR), and retinoic acid receptor (RXR) by fatty acids, oxysterols, and 9-cis-retinoic acid, respectively (Saijo et al., 2013). M2 activation promotes the release of anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$  and the secretion of neurotrophic factors brain-derived neurotrophic factor (BDNF), neurotrophins (NT) 4/5, and glial cell–derived neurotrophic factor (GDNF). Neurotrophic factors engage a family of receptor tyrosine kinases known as Trk receptors, which regulate synaptic strength and plasticity. M2 microglia also release the pro-survival factor progranulin (Colonna & Butovsky, 2017).

While the M1 and M2 categories have proven useful in understanding microglia behavior in vitro, it is increasingly acknowledged that this M1/M2 framework is insufficient when describing microglia and macrophage activation in vivo. Microglia rarely exhibit a significant inclination towards either the M1 or M2 phenotype, as evidenced by transcriptome studies that demonstrate diverse and context-dependent microglia activation. Under normal CNS functioning, microglia maintain a "resting" or homeostatic transcriptome profile reflecting their surveillance activity (Butovsky et al., 2014; Gosselin et al., 2014; Hickman et al., 2013; Ransohoff, 2016; Wes et al., 2016; Y. Zhang et al., 2014). Conversely, in neurodegenerative models, microglia express both neurotoxic and neuroprotective factors, as well as genes involved in oxidative phosphorylation, lysosome, ribosome, and spliceosome functions associated with responses to misfolded proteins, stress, neuronal death, or injury (Chiu et al., 2013; Wes et al., 2016). Ongoing investigations



are expected to identify numerous microglia phenotypes linked to aging, various neuropathological conditions, and different stages of diseases. In the time being, an assembly of a group of multidisciplinary microglia experts discussed the current understanding of microglial states. They agreed that the dualistic classification of good or bad microglia is inconsistent with the wide repertoire of microglial states and functions in development, plasticity, aging, and diseases that were elucidated in recent years. They therefore provided a conceptual framework and recommendations on the use of microglial nomenclature for researchers, reviewers, and editors (Paolicelli et al., 2022).

Last but not least, the varying levels of microglial activation, which progress from a mild reactive phenotype to more severe neurotoxic phenotypes, are suggested to be controlled by metabolites. The microglial inflammatory outputs are ensured by fine-tuning mitochondrial activity and metabolic reprogramming itself. These findings reveal that microglial immunometabolism is a key factor in regulating neuronal communication, with possible wide-range effects on brain function. The process of glycolytic metabolic reprogramming can potentially redirect metabolites to generate crucial molecular intermediates, supporting not just cell proliferation but also the production of cytokines and inflammatory proteins. As metabolic reprogramming plays a vital role in facilitating neuroinflammatory processes in microglia, it raises the question of whether immunometabolism has a direct impact on CNS disorders (Bernier, York, & MacVicar, 2020).

### **III.2. Microglia in neurodegenerative and demyelinating diseases**

Neurodegeneration refers to chronic and progressive loss of neurons in the brain and spinal cord. In the context of neurodegenerative diseases, microglia exhibit distinct behavior and responses contributing to the pathogenesis and progression of these conditions. The dysregulation of any of the microglial functions results in an imbalance that initiates or propagates neurodegeneration. There is ample evidence documenting the activation of microglia in these disorders, leading to the expression of a wide range of immune mediators such as IL-1 $\beta$ , TNF, ROS, and NO (Banati et al., 1993; Gehrmann et al., 1995; Hopkins & Rothwell, 1995). The most common neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, and amyotrophic lateral sclerosis (ALS), are all characterized by microglia-mediated neuroinflammation, and increase oxidative stress (Q. Wu & Zou, 2022). Under normal circumstances, these immune

mediators can function as scavengers, aiding in the elimination of necrotic neurons and managing their regular turnover. However, in neurodegenerative diseases, persistently activated microglia may worsen the condition by excessively releasing these cytotoxic factors. For instance, in AD, rather than efficiently clearing Amyloid- $\beta$  (A $\beta$ ) deposits, microglia can be triggered to secrete proinflammatory cytokines, causing further damage to neurons (Fuhrmann et al., 2010; Subhramanyam et al., 2019). The A $\beta$ -plaque-associated microglia, also known as disease-associated microglia (DAM) present a pro-inflammatory signature which was revealed through transcriptomic analysis. *Axl*, *ApoE*, *Ctsb*, *Grn*, *Gpnmb*, *Lgals3*, *Spp1*, and *Trem2* are among the upregulated genes in DAM and are now known to be part of a common neurodegenerative signature (Butovsky & Weiner, 2018; Krasemann et al., 2017). DAM have been observed in other conditions including ageing, ALS and frontotemporal dementia (FTD) as well as in an inducible CK-p25 mouse model of severe neurodegeneration, and it appears that the DAM expression phenotype is nonspecifically triggered by altered brain homeostasis (Keren-Shaul et al., 2017). It is important to note that there are likely to be multiple microglial phenotypes associated with disease and that DAM microglia as defined may have either beneficial or detrimental function (Butovsky & Weiner, 2018). It should be also noted that DAM signature cannot be exactly shared in all neurodegenerative diseases and their models, as microglial activation status really depends on a great variety of factors.

Dysregulated and excessive activation of microglia are closely associated with the development of multiple sclerosis (MS), which is a chronic inflammatory autoimmune disease characterized by demyelination of neurons in the CNS. In human MS, microglia lose the expression of the P2RY12 homeostatic marker and adopt a pro-inflammatory phenotype (Zrzavy et al., 2017). Experimental autoimmune encephalomyelitis (EAE) serves as the most prevalent model for MS and the involvement of microglia in this disease. In EAE mouse model, microglia have a transcriptional phenotype that is similar to that observed in ALS and AD models (Krasemann et al., 2017). In *CD11b-HSVTK* transgenic mice, effectively depleting microglia significantly reduced the severity of the disease and demyelination in EAE (Heppner et al., 2005). Additionally, remarkable attenuation of clinical symptoms in EAE was observed upon the inhibition of microglia/macrophage activation by macrophage inhibitory factor (TKP) (Heppner et al., 2005). These studies indicate that the activation of microglia/macrophage potentially contributes to the development of EAE (Tian et al., 2023). Several other studies have demonstrated that

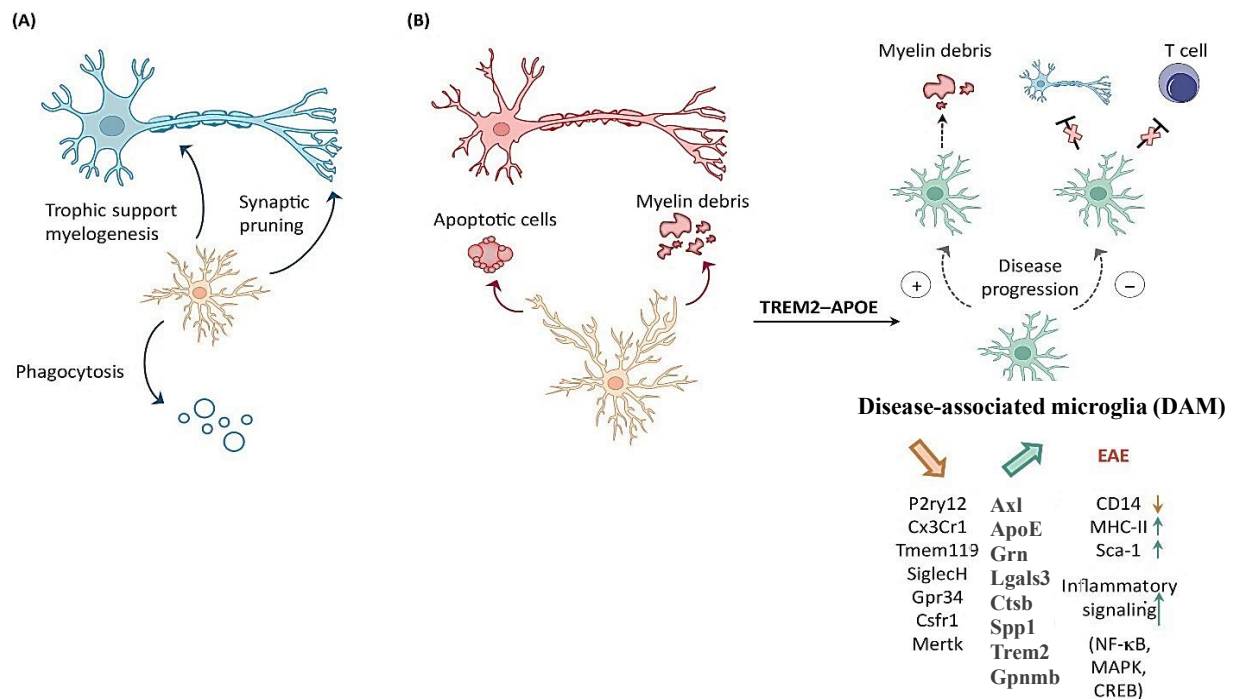
microglial secretions such as IFN- $\gamma$ , TNF, ROS, IL-1 $\beta$ , IL-6, IL-18, IL-12 and IL-23 and also chemokines such as CCL2, CCL3, CCL4, CCL5, CCL7, CCL12 and CCL22 contribute to detrimental effects in the pathogenesis of MS (Mado et al., 2023; Merson et al., 2010; Tsouki & Williams, 2021; Voet et al., 2019).

Another common characteristic of neuroinflammation-mediated neurodegeneration is the disruption of the BBB, which is observed in patients with neurodegenerative diseases. The tight junction protein complexes, responsible for maintaining the integrity of the BBB, possess a dynamic nature and can undergo reorganization in response to neurological diseases. Consequently, these alterations in tight junction protein complexes lead to an increased permeability of the BBB, allowing for the passage of circulating substances. Remarkably, the activation of microglia in CNS pathological conditions is closely associated with dysfunction of the BBB (Ronaldson & Davis, 2020). Normally, vessel-associated microglia play a vital role in preserving the integrity of the BBB by expressing the tight-junction protein Claudin-5 and establishing physical connections with endothelial cells. Through simultaneous *in vivo* imaging, it has been observed that systemic inflammation triggers the migration of microglia residing in the brain towards the cerebral vasculature. In situations of prolonged inflammation, these microglia engage in the

phagocytosis of astrocytic end-feet, leading to impaired BBB function (Haruwaka et al., 2019).

### III.3. Microglia in adrenoleukodystrophy

Previous research has predominantly focused on the toxicity of VLCFA in oligodendrocytes due to their high lipid content (Engelen et al., 2014). However, the crucial role of microglia in cerebral and spinal demyelination is becoming increasingly evident. Recent studies in mouse models of adrenoleukodystrophy have revealed that microglial activation precedes synaptic loss, and ABCD1-deficient spinal cord microglia are primed for neuronal phagocytosis and show elevated TREM2 expression (Gong et al., 2017). Analysis of brain tissue samples from adrenoleukodystrophy patients by Bergner et al. corroborates these findings, showing minimal changes in oligodendrocytes and neuronal morphology in prelesional areas, while microglia display signs of activation such as reduced



**Figure 3: Homeostatic and DAM microglia**

(A) Microglia's role in maintaining homeostasis involves continuous environmental surveillance for potential threats, facilitation of synapse formation and myelogenesis, secretion of neurotrophic factors, and phagocytosis of CNS debris. (B) The transition to a DAM phenotype occurs in response to various triggers during EAE, including apoptotic neurons and myelin debris (red arrows). Microglia undergo a loss of their normal molecular signature (orange) and acquire an activated signature (green), which relies on TREM2-APOE signaling. This DAM signature is shared among several neurodegenerative disorders, such as AD, ALS, PD, and is also observed during aging. The DAM-related genes depicted in the figure are commonly upregulated in EAE and other neurodegenerative conditions. However, microglia in EAE exhibit a distinct pro-inflammatory profile, displaying unique signaling activities and gene expression patterns compared to other neurodegenerative diseases. The impact of this DAM signature on disease progression (dashed arrows) remains a subject of debate, with its effects being either beneficial (+) or detrimental (-). These DAM cells can lose their ability to regulate T cells and contribute to neuronal loss, yet they also play a crucial role in the initial response to neuronal injury by clearing myelin and neuronal debris. Adapted from (Voet et al., 2019)

TMEM119 expression and a transition to an amoeboid phenotype (Bergner et al., 2019). Moreover, a zone lacking microglia within perilesional white matter was found in human X-ALD autopsies. Surrounding this zone, clusters of activated and apoptotic microglia within subcortical white matter were observed. These studies, along with previous ones, indicate a depletion of microglia in prelesional areas compared to healthy white matter and demyelinated regions, suggesting that activated microglia may undergo apoptosis just before demyelination occurs (F. S. Eichler et al., 2008).

The treatment of adrenoleukodystrophy further emphasizes the crucial role of immune cells in the pathophysiology of neurodegeneration resulting from *ABCD1* mutations. Early administration of bone marrow transplantation has demonstrated the ability to slow down or halt the progression of adrenoleukodystrophy (Shapiro et al., 2000). Recently, there have been further refinements in therapy, with promising results seen in clinical trials using autologous transplants of CD34+ cells carrying a functional copy of *ABCD1*. These transplants have shown similar outcomes to conventional bone marrow transplantation, but notably without the occurrence of graft-versus-host disease or other complications associated with transplantation (Cartier et al., 2009; F. Eichler et al., 2017). This advancement holds clinical and scientific significance as it suggests that the disease-modifying effects of CD34+ transplants may be attributed to the influence of properly functioning macrophages, which can migrate into the brain and potentially occupy the microglial niche, rather than the immunosuppressive medications required after conventional HSCT (Sirkis et al., 2021).

#### **III.4. In vitro cell models**

In vitro cell models of microglia play a crucial role in studying the functions and behaviors of these specialized cells within the CNS. In recent years, significant advancements have been made in developing in vitro models of microglia that mimic their characteristics and functions in vivo. These models involve culturing primary microglial cells obtained from animal brains or human postmortem tissues, as well as utilizing immortalized microglial cell lines. Several cell lines have been established in order to avoid the constraints associated with primary culture, the most common being the murine BV-2 and N9 lines, with evident predominance for BV-2 cells (4716 results for Pubmed search “microglia AND BV2 OR BV-2” versus 427 results for “microglia AND N9” as of June 26,

2023). The availability, reproducibility and immortalization of the cell lines represent a huge advantage in research.

The BV-2 cell line was established in the laboratory of Dr. Barbara L. Hempstead at Weill Cornell Medical College in New York, USA. The exact details of the creation and characterization of BV-2 cells were published by Blasi et al. in the *Journal of Neuroscience Research*. These cells were derived from a culture of primary mouse microglial cells and were immortalized by infecting them with a v-raf/v-myc oncogene-carrying retrovirus (Blasi et al., 1990). These cells were extensively characterized to confirm their microglial origin and to assess their properties. This included evaluating their morphology, surface marker expression, phagocytic activity, response to stimuli, and functional properties. These characterizations helped establish the identity and usefulness of BV-2 as a microglial cell line which is believed to retain the phenotypic and immunological properties such as phagocytosis, cytokine secretion and response to pro-inflammatory stimuli such as exposure to LPS. Although not recapitulating all the characteristics of microglia in vivo, they do represent a valid practical substitute (Henn et al., 2009). Additionally, the cells showed a normal regulation of NO production, a functional response to IFN- $\gamma$ , and important characteristics for appropriate interaction with T cells and neurons. BV-2 cells were also able to stimulate other glial cells. They can trigger the translocation of NF- $\kappa$ B, and a subsequent production of IL-6 in astrocytes. Thus, BV-2 cells appear to be a valid substitute for primary microglia in many experimental settings, including complex cell-cell interaction studies. But again, this cell line is not fully representative of primary cells especially upon LPS treatment with a lower reactivity than what is observed with primary cells (Das et al., 2016).

On the other hand, both primary microglia as well as cell lines appear to exhibit a highly dysregulated homeostatic molecular signature in vitro. This is attributed to the fact that microglia in vitro do not undergo the influence of the mature brain microenvironment in the presence of a functional BBB, and the culture conditions could promote a pro-inflammatory phenotype of these cells (Butovsky et al., 2014). Recent advances have helped in the development of human microglial cells derived from induced pluripotent cells and which appear to be a promising alternative for the study of microglia (Abud et al., 2017).

Aiming at investigating the effects of peroxisomal dysfunctions in microglia and the underlying pathogenesis of X-ALD, ABCD1 and/or ABCD2 deficient microglial cell lines have been obtained in our lab using CRISPR/Cas9 gene editing in the BV-2 cells. The

ABCD1/ABCD2 deficient cells were generated to avoid masking effects due to functional redundancy. They show classical X-ALD biochemical hallmarks such as increased levels of saturated and monounsaturated VLCFAs as well as increased levels of some LCFAs and PUFAs (Raas, Gondcaille, et al., 2019). ACOX1 deficiency microglia have been obtained as well. They mimic the condition of ACOX1 deficiency through deactivating the enzyme responsible for the first step of peroxisomal  $\beta$ -oxidation (Raas, Saih, et al., 2019).

## Objectives

The pathogenesis of X-ALD, the most common peroxisomal disease, continues to present numerous unresolved aspects, challenges, and uncertainties. Despite notable advancements, such as the implementation of neonatal screening in several countries, treatment options remain strictly limited. The emergence of gene therapy offers hope for individuals with cALD, but its potential is accompanied by significant scientific, regulatory, and financial challenges. One major hurdle hindering our understanding of the disease's pathological mechanisms and the development of effective therapies is the lack of relevant experimental models. The existing mouse models deficient in ABCD1 and/or ABCD2, while valuable, fall short in recapitulating the neuroinflammatory demyelinating damage characteristic of cALD. Hence, it becomes imperative to explore alternative paths. Our research aims to address a significant gap in the current understanding of X-ALD pathogenesis. Although extensive investigations have been conducted on the in vitro molecular aspects of X-ALD, the role of microglia has been notably overlooked. This aspect forms the central focus of our study, bringing a novel perspective to the field. In this context, our laboratory has successfully generated knockout BV-2 cells using CRISPR/Cas9 genome editing. The knockout cells are *Abcd1*<sup>-/-</sup>, *Abcd2*<sup>-/-</sup>, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> (Raas, Gondcaille, et al., 2019; Raas, Saih, et al., 2019). These cells provide a promising and valid alternative for molecular investigations into the impact of peroxisomal dysfunctions in microglia. By utilizing these cells, we aim to shed light on the intricate interplay between peroxisomal  $\beta$ -oxidation abnormalities and microglial function. This research would give an insight about the relations between peroxisomal-specific microglial functions, holding the potential to uncover the molecular mechanism underlying X-ALD pathology and opening new avenues for therapeutic intervention. We aim to unravel the crucial role played by peroxisomes in the functions of the specialized CNS immune cells and the maintenance of an optimal immune response and homeostasis. By comprehensively characterizing the pathology-associated markers in these cells and how they are leading to neurodegeneration, it would become possible to establish a foundation for identifying potential biomarkers and therapeutic molecules capable of restoring normal cellular function. Ultimately, our findings have the potential to contribute significantly to the advancement of targeted therapeutic strategies after understanding the molecular pathogenesis of X-ALD in vitro.



Thus, the precise objectives of my thesis were as follows:

- Objective 1: Characterization of the BV-2 cells knocked out of *Abcd1*, *Abcd2* and *Acox1*.
- Objective 2: Assessment of the impact of the secretions of knockout cell on neuronal properties and oligodendrocytes' viability.

The first objective will be achieved through a comprehensive analysis of the transcriptomic profile, protein expression and secretion patterns as well as their phagocytic activity and their ability to present antigen to T lymphocytes and induce their immune response. This would give an insight into their inflammatory profile and their response to stress and inflammatory stimulation. By investigating these aspects, we aim to gain a better understanding of the molecular alterations resulting from the absence of peroxisomal proteins in BV-2 cells. The high plasticity of microglial cells, which can easily be demonstrated during LPS stimulation, is strongly dependent on their metabolism. This study therefore aims to assess the effects of a defect in peroxisomal metabolism on this plasticity.

The second objective focuses on investigating the effects of secretions from the knockout cells on cells of the CNS. Specifically, we aim to evaluate the influence of these secretions on neuronal viability, morphology, activity and the viability of oligodendrocytes. By elucidating the impact of the knockout cell secretions on neurons, we hope to identify potential molecular targets that could serve as therapeutic interventions to impede the progression of the peroxisomal disorder X-ALD. This research will thus pave the way for developing targeted strategies aimed at halting the progression of X-ALD.

This project therefore is multifaceted and is an enormous undertaking that has spanned several years and involved multiple collaborators. The project commenced with the creation of knockout cells, which were successfully generated by Quentin Raas, the previous PhD student of the team. Once the cells were obtained, RNA sequencing was conducted by Céline Keime from the GenomEast platform (IGBMC, University of Strasbourg) thanks to funding from the French “Fondation Maladies Rares”, therefore opening up a world of possibilities for analysis. The next step was to start characterizing these cells, initially through treating and sorting the transcriptomic data according to gene functional families, a task mostly performed by Stéphane Savary and Quentin Raas. When I joined the lab, my initial focus was on understanding the various aspects of the project and familiarizing myself with the accumulated data. Subsequently, I actively contributed to the characterization of the cells through confirming gene expressions

at the protein level using techniques such as western blot, immunofluorescence, and ELISA. Even though it was shortlisted, we had an extensive list of genes we were interested in. Throughout this process, I received assistance from Mounia Tahri Joutey, a PhD student working on *Acox1* knockout cells and lysosome genes activity, as well as my co-supervisor, Catherine Gondcaille, who provided unwavering support.

Having achieved my initial objective of establishing cell characterization, I embarked on investigating the impact of microglial secretions on neurons. This required me to develop co-culture and conditioned medium systems and adapt them for the used cell lines. After numerous trials, I successfully designed the experimental protocol. Meanwhile, Catherine Gondcaille focused on oxidative stress-related proteins, building upon the work previously conducted by Fatima-Ezzahra Saih, a PhD student who focused on studying the oxidative stress markers of *Acox1* knockout cells.

With the co-culture and conditioned medium system in place, I delved into studying the effects on neurons. For that purpose, I reached out to Alexandre Benani at “Centre des Sciences du Goût et de l’Alimentation (CSGA)” who provided immense support. I gained access to the CSGA which was extremely helpful in realizing several experiments on neurons. I was also trained by Claire Fenech at CSGA thus providing me with valuable theoretical and technical insights and into calcium imaging in neurons.

A collaborative effort was crucial for the functional analysis of microglial cells. Dr. Yannick Hamon in Marseille contributed to the antigen presentation aspect, while I traveled to Vienna to receive training from Johannes Berger, Isabelle Weinhofer, and their team at the Medical University of Vienna for the phagocytosis analysis. During my time at their esteemed laboratory, I conducted various tasks, including isolating myelin sheath debris from mice brain, assessing the phagocytosis activity of BV-2 cells incubated with labeled myelin sheath, and studying the impact of myelin-activated BV-2 cell secretions on neurons in a conditioned medium system.

In summary, this research project encompasses various branches and requires a significant level of collaboration. I actively engaged in a substantial portion of the experimental work, while at other points, my role involved gathering and analyzing the data, thereby contributing to the comprehensive narrative of the project.

## Results

### **Transcriptomic analysis and characterization of BV-2 cells with peroxisomal defects**

The results of this thesis are presented in the form of published articles (“article 1” and “article 2”), and additional unpublished results which will ultimately be included in a third paper (“article 3”).

The first article is entitled “Peroxisomal defects in microglial cells induce a disease-associated microglial signature” and published in *Frontiers in Molecular Neuroscience*. It presents the results of the characterization of the cells at the basal level, i.e., without any treatment. The aim of this article was to reveal the different modified pathways in microglia upon the knockout of the peroxisomal genes and shed light on the importance of peroxisomal functions in microglial and CNS homeostasis. The mentioned publication, attached below, presents the characterization of BV-2 cell models with knockouts of *Abcd1*, *Abcd2*, *Acox1*, and a double knockout of *Abcd1* and *Abcd2*. These knockout BV-2 cells were created in 2018, and subsequently, a comprehensive transcriptomic analysis was conducted. To further elucidate the effects of the peroxisomal gene knockout, it involved sorting the differentially expressed genes (DEGs) into specific families using databases such as Gene Ontology and KEGG pathway. This approach allowed us to gain insight into the pathways influenced by the peroxisomal genes knockout and subsequently verify the pathways of interest through investigating the DEGs at the protein level. Indeed, the characterization of these knockout cells is important for several reasons. First, it helps in uncovering the microglial phenotype of X-ALD, thus providing an in-vitro insight into the disease mechanisms. Then, the comparative analysis of the knockout cell models with their WT counterparts helps to shed the light on the involvement of peroxisomal molecular pathways with various cellular functions.

In our initial publication, we directed our attention towards four gene groups: lipid metabolism, cholesterol regulation, lysosomes, and autophagy. These terms came out as the most enriched pathways identified through gene enrichment analysis conducted on the mutant BV-2 cells, utilizing gene ontology biological processes and KEGG pathway databases. By focusing on these gene families, we aimed to gain insights into the fundamental functions of microglia. We also aimed at investigating DAM signature as the microglia of neurodegenerative diseases show the existence of a DAM transcriptomic signature (Butovsky & Weiner, 2018).

Identifying this signature in the mutant cells has an important significance showing that peroxisomal mutations induce neurovegetative phenotype in microglia. Additionally, any differential expression observed in lysosomal genes can indicate alterations in phagocytosis, a pivotal microglial function which is also closely associated with lipid metabolism, cholesterol and consequently membrane fluidity. However, it is crucial to conduct further functional analyses to confirm such hypotheses. In our subsequent analyses, we delve deeper into the functional implications.



## OPEN ACCESS

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## SPECIALTY SECTION

This article was submitted to  
Brain Disease Mechanisms,  
a section of the journal  
Frontiers in Molecular Neuroscience

RECEIVED 21 February 2023

ACCEPTED 27 March 2023

PUBLISHED 17 April 2023

## CITATION

Raas Q, Tawbeh A, Tahri-Joutey M,  
Gondcaille C, Keime C, Kaiser R, Trompier D,  
Nasser B, Leoni V, Bellanger E, Boussand M,  
Hamon Y, Benani A, Di Cara F, Truntzer C,  
Cherkaoui-Malki M, Andreoletti P and  
Savary S (2023) Peroxisomal defects in  
microglial cells induce a disease-associated  
microglial signature.  
*Front. Mol. Neurosci.* 16:1170313.  
doi: 10.3389/fnmol.2023.1170313

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Cherkaoui-Malki, Andreoletti and  
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# Peroxisomal defects in microglial cells induce a disease-associated microglial signature

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Microglial cells ensure essential roles in brain homeostasis. In pathological condition, microglia adopt a common signature, called disease-associated microglial (DAM) signature, characterized by the loss of homeostatic genes and the induction of disease-associated genes. In X-linked adrenoleukodystrophy (X-ALD), the most common peroxisomal disease, microglial defect has been shown to precede myelin degradation and may actively contribute to the neurodegenerative process. We previously established BV-2 microglial cell models bearing mutations in peroxisomal genes that recapitulate some of the hallmarks of the peroxisomal  $\beta$ -oxidation defects such as very long-chain fatty acid (VLCFA) accumulation. In these cell lines, we used RNA-sequencing and identified large-scale reprogramming for genes involved in lipid metabolism, immune response, cell signaling, lysosome and autophagy, as well as a DAM-like signature. We highlighted cholesterol accumulation in plasma membranes and observed autophagy patterns in the cell mutants. We confirmed the upregulation or downregulation at the protein level for a few selected genes that mostly corroborated our observations and clearly demonstrated increased expression and secretion of DAM proteins in the BV-2 mutant cells. In conclusion, the peroxisomal defects in microglial cells not only impact on VLCFA metabolism but also force microglial cells to adopt a pathological phenotype likely representing a key contributor to the pathogenesis of peroxisomal disorders.

## KEYWORDS

peroxisome, adrenoleukodystrophy (X-ALD), microglia, lysosome, lipid metabolism, autophagy

## Introduction

Microglial cells are small stellate cells that play an essential role in brain homeostasis (Wright-Jin and Gutmann, 2019). They are involved in synaptogenesis, neurotransmission and neurogenesis, which gives them the ability to control the activity of neuronal circuits. Moreover, they regulate blood brain barrier permeability and can sense metabolic circulating signals from the periphery supporting their role in neuroendocrine functions. As the main immune cells in the brain, they can perform phagocytosis, antigen presentation, and secrete chemoattractant substances and inflammatory mediators in response to injury and infection.

Dysfunction of microglia and/or modification of the microglial homeostatic state clearly contributes to the pathogenesis of some brain diseases (Sirkis et al., 2021; Paolicelli et al., 2022), including peroxisomal leukodystrophies such as X-linked adrenoleukodystrophy (X-ALD, MIM 300100). X-ALD, the most frequent peroxisomal disorder, is associated with mutations in the *ABCD1* gene and is characterized by an impaired peroxisomal  $\beta$ -oxidation pathway and very long-chain fatty acid (VLCFA) accumulation in plasma and tissues of patients (Mosser et al., 1993; Trompier and Savary, 2013; Kemp et al., 2016). Functional replacement of microglia by monocyte/macrophage cells using allogeneic hematopoietic stem cell transplantation and cell-based gene therapy has proven to be therapeutically effective for X-ALD patients (Cartier et al., 2009; Eichler et al., 2017; Weinhofer et al., 2018). Metabolic defects in microglia are indeed suggested to be key contributors to the pathology of X-ALD. Moreover, it has also been proposed that a primary microglial dysfunction might directly initiate neurodegenerative processes since microglial alteration precedes myelin breakdown in X-ALD (Gong et al., 2017; Bergner et al., 2019).

Using the CRISPR/Cas9 gene editing technology, we recently established novel cell models to study the impact of peroxisomal defects in BV-2 murine microglial cells (Raas et al., 2019a,b). The BV-2 cells were chosen because they maintain transcriptomic signature resembling that of primary cells and many microglial features such as phagocytosis and the ability to respond to inflammatory stimulation (Henn et al., 2009; Das et al., 2015). Despite thousands of articles using BV-2 cells as a model, the cell line is not fully representative of primary cells especially upon LPS treatment with a lower reactivity than what is observed with primary cells (Das et al., 2016). Moreover, primary microglia present a spatial and temporal heterogeneity particularly under pathological status (Masuda et al., 2019). While fully aware of the limitations of the model, we decided to use the BV-2 cell line and targeted the *Abcd1* and *Abcd2* genes, which encode for partially redundant peroxisomal ABC transporters of CoA esters of VLCFAs (Mosser et al., 1993; Lombard-Platet et al., 1996; Genin et al., 2011; Tawbeh et al., 2021), and the Acyl-CoA oxidase 1 (*Acox1*) gene, which controls the first step of

peroxisomal  $\beta$ -oxidation and whose defect is associated with a rare leukodystrophy (MIM 264470; Fournier et al., 1994; Nohammer et al., 2000; Ferdinandusse et al., 2007; Vamecq et al., 2018). Peroxisomal ABC transporters show overlap in their substrate specificity, with ABCD1 being more specialized in the transport of saturated and monounsaturated VLCFAs while ABCD2 would show an extended specificity to polyunsaturated fatty acids (PUFAs; Mosser et al., 1993; Lombard-Platet et al., 1996; Genin et al., 2011; Tawbeh et al., 2021). Both transporters provide the substrates of ACOX1 for further degradation. Since BV-2 cells express both *Abcd1* and *Abcd2* genes, VLCFA accumulation was only observed in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> microglial cells, as expected (Raas et al., 2019a,b). Lipid droplets and lipid inclusions likely due to cholesteryl esters of VLCFA, some of the hallmarks of X-ALD, were also observed, especially in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> genotype (Raas et al., 2019a,b).

To further clarify how peroxisomal defects impact microglia, we used next generation sequencing (NGS)-based approach to obtain an accurate and unbiased vision of the differentially expressed genes (DEGs) in microglial cells with specific peroxisomal gene mutations. RNA-sequencing (RNA-seq) indicated the induction of specific transcriptomic programs in the mutant genotypes. Nevertheless, intersectional analysis revealed a common transcriptomic program in the four genetic mutants. Gene ontology analysis permitted to highlight deregulated gene clusters involved in immune system process, signaling pathways, lipid metabolism, lysosome, and autophagy. We also demonstrated the induction of a disease-associated microglial (DAM) signature in microglia cells bearing one or several peroxisomal gene mutations. A DAM signature is a transcriptomic response shared by most common neurodegenerative diseases (Keren-Shaul et al., 2017; Butovsky and Weiner, 2018; Dubbelaar et al., 2018). We confirmed these transcriptional modifications at the protein level for several dysregulated genes and explored some functional consequences on plasma membrane (cholesterol accumulation), lysosomal alterations and autophagy. Moreover, we observed an increased secretion of five selected proteins belonging to the DAM signature, which prompted us to launch a pilot study in X-ALD adult patients.

## Materials and methods

### Resource availability

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE200022.<sup>1</sup>

The data supporting the findings and the excel files containing the gene lists used to create the figures are available from the corresponding author (SS) upon request.

### Cell culture

Mouse microglial BV-2 cell line was purchased from Banca-Biologica e Cell Factory (catalog no. ATL03001). Single or double

Abbreviations: AA, Arachidonic acid; ABC, ATP-binding cassette; ACOX1, Acyl-CoA oxidase 1; AMN, Adrenomyeloneuropathy; cALD, Cerebral adrenoleukodystrophy; CRISPR, Clustered regularly interspaced short palindromic repeat; DAM signature, Disease-associated microglial signature; DEGs, Differentially expressed genes; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; FA, Fatty acids; FBS, Fetal bovine serum; log<sub>2</sub> FC, log<sub>2</sub> fold change; GC-MS, Gas chromatography mass spectrometry; L/VLCFA, Long/very long-chain fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; RNA-seq, RNA sequencing; WT, Wild type; X-ALD, X-linked adrenoleukodystrophy.

<sup>1</sup> <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE200022>

mutant BV-2 cells, deficient for the peroxisomal proteins *Abcd1*, *Abcd2* or *Acox1* (*Abcd1*<sup>-/-</sup>, *Abcd2*<sup>-/-</sup>, *Abcd1*<sup>-/-</sup>/*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup>), were obtained by CRISPR/Cas9 editing (Raas et al., 2019a,b). Sanger sequencing confirmed the absence of CRISPR/Cas9-induced mutations in the two genomic sites with the highest predicted likelihood of off-target binding strongly, suggesting the absence of off-target events. WT and mutant BV-2 cells were grown in DMEM supplemented with 10% heat-inactivated FBS (Corning), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. For RNA-seq experiments, as BV-2 WT and mutant cells were included as controls in a study involving treatments (study which will be the subject of another article), a treatment with 0.01% ethanol and 0.5 mM α-cyclodextrin corresponding to the vehicle was applied.

## Blood and plasma samples

Frozen plasma samples from male X-ALD patients were obtained from the X-ALD Biobank of Dijon (agreement for research usage CRB.FC-MAD 12236.4, Centre de Ressources Biologiques (CRB) Ferdinand Cabanne, Dijon, France). There is no restriction on the use of these human samples to identify putative biomarkers. Control plasma samples were obtained after centrifugation (1,300 g for 10 min) from fresh blood samples obtained from male healthy donors (agreement for the transfer of blood products for non-therapeutic use between Etablissement français du sang Bourgogne Franche-Comté, France and University of Bourgogne DECO-21-0051). Analyses were carried out in samples from 3 patients with arrested cALD (ages 36, 16, and 30 years, with onset ages of 21, 10, and 14 years, respectively for patients cALD1, cALD2 and cALD3), 3 AMN patients (ages 43, 42, and 44 years, with onset ages of 33, 25, and 36 years, respectively for AMN1, AMN2, and AMN3), and 10 healthy donors aged 40 to 45 years. Last examination of cALD patients revealed a severe dementia (cALD1), a mildly abnormal gait and moderate dementia (cALD2) and a near-normal situation (cALD3). Their C26:0 plasma level at the date of sampling was 2.40 µmol/L (cALD1), 3.39 µmol/L (cALD2), 5.96 µmol/L (cALD3; normal value < 1.45 µmol/L). Patients cALD1 and 2 received Lorenzo's oil.

## RNA-sequencing

Total RNA was extracted from 3 independent batches of BV-2 cells for each genotype (WT, *Abcd1*<sup>-/-</sup>, *Abcd2*<sup>-/-</sup>, *Abcd1*<sup>-/-</sup>/*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup>) using RNeasy kit (Qiagen). RNA sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA LT Sample Preparation Kit and sequencing was performed on Illumina HiSeq4000 sequencer as single-end 1×50 base reads. Reads were preprocessed using cutadapt (Martin, 2011) version 1.10 in order to remove adapter and low-quality sequences (Phred quality score below 20) and reads shorter than 40 bases were discarded for further analysis. Reads mapping to rRNA sequences were also discarded [this mapping was performed using bowtie (Langmead and Salzberg, 2012) version 2.2.8]. Reads were then mapped onto the mm10 assembly of *Mus musculus* genome using STAR (Dobin et al., 2013) version 2.5.3a. Gene expression was quantified using htseq-count (Anders et al., 2015) version 0.6.1p1 and annotations from Ensembl version 91.

Differential gene expression analyses were performed using R 3.3.2 and DESeq2 version 1.16.1 Bioconductor library (Love et al., 2014). *p* values were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

## Differential gene expression analysis and bioinformatic

Venn diagrams were obtained using Venny 2.1.<sup>2</sup> MA-Plot and Volcano plot were obtained using the VolcanoR web app.<sup>3</sup> Gene ontology analysis and gene set enrichment analysis were performed using the DAVID resources<sup>4</sup> (Huang et al., 2007; Huang da et al., 2009) for association with KEGG pathways and the Enrichr platform<sup>5</sup> (Kuleshov et al., 2016) for association with Wikipathways 2019 Mouse and Elsevier pathway collection. Significance level of 0.05 was used when not correcting for multiple testing. A cutoff of 0.05 for adjusted *p*-value was chosen for multiple testing correction using the method from Benjamini and Hochberg (BH). DEGs were selected from genes presenting an adjusted *p*-value lower than 0.05 and an absolute log<sub>2</sub> FC higher than 0.58. Expression-based heatmaps and hierarchical clustering were performed with Heatmapper<sup>6</sup> (Babicki et al., 2016) using average linking and Pearson distances.

## Lipid analysis

The levels of cholesterol precursors were measured by gas chromatography–mass spectrometry (GC–MS) from cellular homogenates, prepared from pellets of 10<sup>7</sup> cells suspended in water (100 µL) and mixed with structural homologous internal standards as previously described (Raas et al., 2019a). Peak integration was performed manually. Metabolites were recognized by retention time and fragmentation patterns, and quantified from total-ion count against internal standards using standard curves for the measured sterols (Leoni et al., 2017).

## Western blotting

Cell lysate proteins (30 µg) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were first blocked in 5% fat-free milk in PBST (Phosphate buffer saline (PBS), 0.1% Tween 20) and then probed with primary antibodies in 1% fat-free milk in PBST. The following antibodies were used with the indicated dilution: anti-APOE (1:2,000, Abcam ab183597), anti-ATG13 (1:1,000, Abcam ab201467), anti-ATP6V1B2 (1 µg/mL, Abcam ab73404), anti-ATP6V0D2 (1:1,000, Sigma-Merck SAB2103221), anti-CTSB (1:500, Sigma-Merck IM27L), anti-CTSK (1:1,000, Santa Cruz sc-48353), anti-CD36 (1:1,000, R&D Systems AF2519), anti-DHCR24 (1:500, Cell Signaling 2033), anti-GPNMB (1:5,000, Abcam ab188222),

<sup>2</sup> <https://bioinfogp.cnb.csic.es/tools/venny/index.html>

<sup>3</sup> <https://huygens.science.uva.nl/VolcanoR/>

<sup>4</sup> <https://david.ncifcrf.gov/tools.jsp>

<sup>5</sup> <https://maayanlab.cloud/Enrichr/>

<sup>6</sup> <http://www.heatmapr.ca/>

anti-GRN (1:1,000, Abcam ab187070), anti-LAMP-2 (1:250, Santa Cruz sc-20,004), anti-LAMTOR4 (1:500, Cell Signaling 12,284), anti-LC3 (1:1,000, Sigma-Merck L8918), LGALS3 (1:1,000, Abcam ab190167), anti-mTOR (1:1,000, Cell Signaling 2,983), anti-p-mTOR(Ser2448; 1:1,000, Cell Signaling 5,536), anti-P62 (1:1,000, Abcam ab56416), anti-SPP1 (1:3,000, R&D Systems AF808), Ulk1 (1:1,000, Cell Signaling 8054) and p-Ulk1(Ser757; 1:1,000, Cell Signaling 14,202). Membranes were washed in PBST and incubated with the appropriate HRP-conjugated secondary antibodies (1:5,000) in 1% fat-free milk in PBST. After 5 washes, immunoreactivity was revealed by incubating membranes with the HRP SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) and the signal was detected by the Chemidoc XRS system (Bio-Rad). Densitometric analysis was performed using the Image Lab software (Bio-Rad) on membranes obtained from 3 biological replicates for each genotype. Proteins amount loading control and normalization was achieved by probing the membranes with  $\alpha$ -tubulin (1:4,000, Sigma-Merck T5168) or  $\beta$ -actin (1:10,000, Sigma-Merck A5441) antibodies.

## Cytometry

BV2 WT and mutant cells were recovered by trypsinization and resuspended in DMEM-Ham F-12 (1:1) supplemented with 1% Nutridoma-SP (Roche 11011375,001; lipid free synthetic serum complement) at a concentration of  $10^6$  cells/mL. Filipin III (Merck F4767) binding kinetics (256 s) were performed on a LSR II UV flow cytometer (Becton Dickinson) equipped with a 355 nm UV laser line. Emitted fluorescence was filtered by a 450 nm $\pm$ 25 nm dichroic mirror. Filipin III (final concentration of 1  $\mu$ g/mL) was added to the cells after a 60 s-recording of the natural fluorescence baseline. Cells

## Electron microscopy

Transmission electron microscopy was used to visualize WT and mutant BV-2 cells cultured for 24 h. Cell sample preparation and diaminobenzidine staining to visualize peroxisomes were performed as previously described (Raas et al., 2019a).

## ELISA

BV2 cells were cultured in 6-well plates for 48 h. Cell culture supernatants were subjected to enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits to detect cathepsin b (CTSB; Abcam ab119585), osteoactivin (GPNMB; Invitrogen EM59RB), progranulin (GRN; Invitrogen EMGRN), galectin-3 (LGALS3; Invitrogen EMLGALS3) and osteopontin (SPP1; Invitrogen EMSPP1) according to manufacturers' instructions. Samples were diluted to meet the concentration range of the standard curve. At least three biological replicates for each sample were tested in triplicates and each experiment was repeated at least two times. Osteopontin was quantified in plasma samples (control, AMN, cALD) using Human osteopontin ELISA kit (Invitrogen BMS2066) according to manufacturers' instructions. Measurements were performed from 1:50

dilution of plasma samples in triplicates and the experiment was repeated twice. Absorbance measurements were performed on TECAN M200 Infinite Pro microplate reader.

## Statistical analyses

The different statistical tests which were applied for densitometric analyses of western blotting experiments and ELISA quantifications are indicated in the figure legends.

## Results

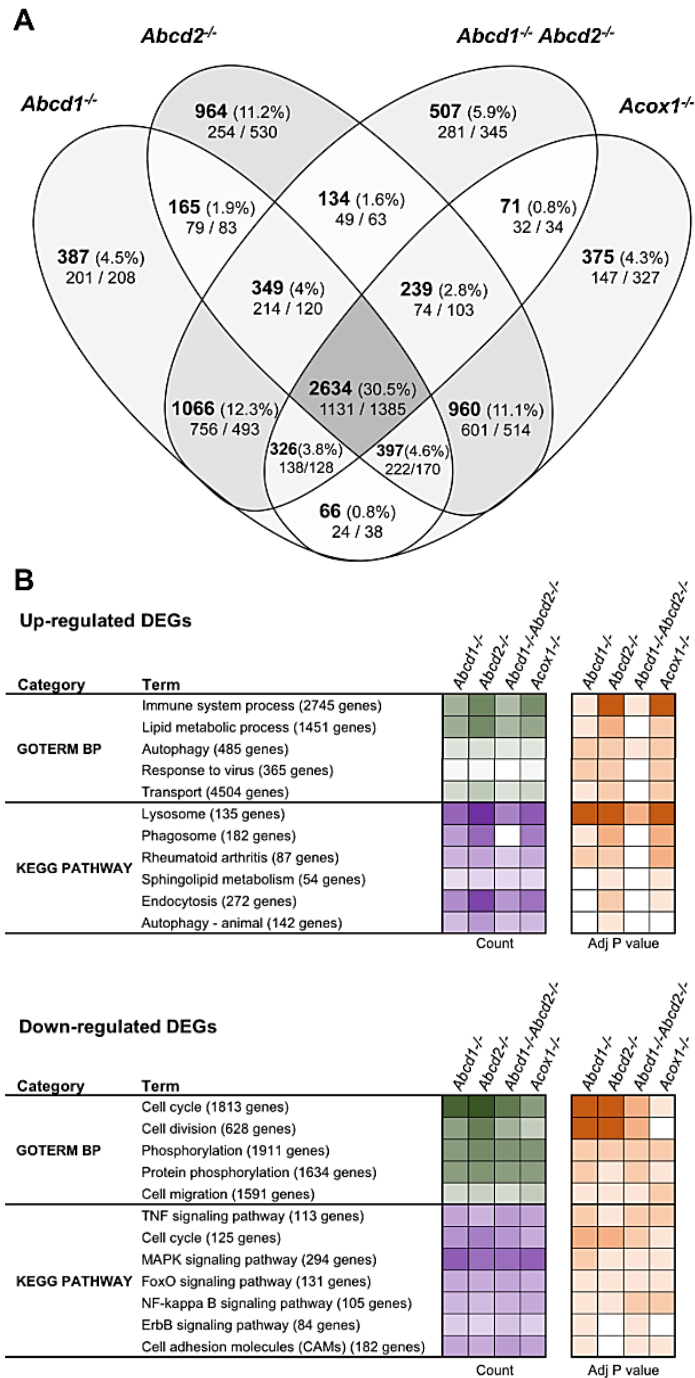
### Genotype comparison

Using a cut off adjusted *p*-value lower than 0.05 and an absolute log<sub>2</sub> fold change (log<sub>2</sub> FC) higher than 0.58 (1.5-fold induction or repression), we found more than 5,000 genes significantly and differentially expressed for each genotype as compared to the WT cells (Figure 1A). Of these DEGs, a total of 2,634 genes representing about one-third of the total DEGs were shared by each mutant genotype. In the 200 most expressed genes in WT cells, 27 were found differentially expressed in the 4 mutant cell lines. Of note, most of these genes were found associated either with lysosomes (induction of *Ctsb*, *Ctsk*, *Ctsc*, *Lamp1*, *Atp6v1b2*, *Atp6v1c1*, *Grn*, *Psap*) or with the DAM signature (induction of *Tyrobp* (DAP12), *ApoE*, *Gpnmb*, *Spp1*, *Grn*, *Lgals3* and repression of *Isyna1*). Analysis performed exclusively on the upregulated genes revealed 1,131 genes shared by all mutant cells. Counterpart analysis on the repressed genes revealed 1,385 DEGs.

Single genotypes analysis revealed unique gene expression profile for each genotype. The comparison of genotypes two by two by two *Acox1*<sup>-/-</sup> cells with 4,230 intersecting DEGs comprising 960 genes DEGs exclusive to these two genotypes (Figure 1A). When we compared the transcriptional profiles of *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells, we observed 4,105 intersecting DEGs including 1,066 DEGs exclusive to these two genotypes. When we look at the transcriptional profiles of mutant cells presenting VLCFA accumulation and supposed to have the most important cellular dysfunction, i.e., *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> (Raas et al., 2019a,b), they showed 3,270 DEGs including 3,025 genes differentially expressed in the same direction (1,375 upregulated and 1,650 downregulated) and 245 genes with a mirror regulation. Only 71 genes were found differentially expressed exclusively in these two genotypes (Figure 1A).

To characterize the enriched biological processes and pathways in our screen, the DEGs were subjected to a functional enrichment analysis using the DAVID online toolkit. A selection of the most enriched terms with Benjamini adjusted *p*-values (false discovery rate) below 0.05 is presented in Figure 1B. From the gene set enrichment analysis restricted to upregulated genes in mutant cells compared to WT cells, "Immune system process" (GO:0002376) was the most significant enriched GO terms in the four deficient genotypes and was followed by "Lipid metabolic process" (GO:0006629) and "Autophagy" (GO:0006914). When analyzing downregulated DEGs, "cell cycle" (GO:0007049), "Cell division" (GO:0051301), and "Phosphorylation" (GO:0016310) were found most enriched. Enrichment analysis





**FIGURE 1**  
 Differentially expressed genes (DEGs) between mutant and WT cells. **(A)** Comparative analysis of the sets of DEGs using Venn diagram ( $n=3$  for each genotype). The significant genes (number and percentage) were selected using a cut off adjusted  $p$ -value (DESeq2 Wald test with Benjamini and Hochberg  $p$ -value adjustment) lower than 0.05 and an absolute  $\log_2$  FC higher than 0.58 (higher than 1.5-fold change in both directions) and are indicated in each group [ $Abcd1^{-/-}$  (5,390 DEGs: 2,765 up, 2,625 down),  $Abcd2^{-/-}$  (5,842 DEGs: 2,894 up, 2,948 down),  $Abcd1^{-/-}Abcd2^{-/-}$  (5,326 DEGs: 2,675 up, 2,651 down), and  $Acox1^{-/-}$  (5,068 DEGs, 2,369 up, 2,699 down)]. Numbers below correspond to the genes found in each group when the analysis was performed exclusively on upregulated genes (left) or downregulated genes (right). **(B)** Gene set enrichment analysis in mutant BV-2 cells presenting a selection of the main gene ontology biological process and KEGG pathway terms found in the 4 mutant genotypes. Functional annotation was obtained using DAVID online tools from upregulated and downregulated differentially expressed genes, respectively, in each mutant genotype. Gene count (green for GO Biological process and magenta for KEGG pathway) and Benjamini-corrected  $p$ -values are mentioned in color-coded form and available in a source data file. The number of genes contained in each term is indicated.

restricted either to upregulated or downregulated genes uncovered significant involvement in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Figure 1B). From the analysis of the upregulated gene sets, “Lysosome” (mmu04142), “Phagosome” (mmu04145), and “Autophagy - animal” (mmu04140) ranked first. Interestingly, “Phagosome” was not found in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells illustrating genotype specificities. On the other hand, the analysis of the downregulated DEGs demonstrated a significant enrichment in many signaling pathways related to inflammation control (Figure 1B).

We extended our analysis to Wikipathways 2019 Mouse and Elsevier Pathway collection in order to perform statistical analyses that highlight the biological processes significantly altered as a consequence of gene expression changes. From the 1,131 upregulated genes found in the intersection of the 4 mutant genotypes, “TYROBP causal network” (WP3625; Zhang B. et al., 2013) was the first and most significantly enriched pathway (Adjusted *p*-value = 0.0101). From the repressed DEGs and gene set enrichment analysis using the Elsevier Pathway collection, “Lipoxins and Resolvins in Inflammation Resolution” (Adjusted *p*-value = 0.0253) was the most significant hit.

## Lipid related genes

Peroxisomal defects *in cellulo* and *in vivo* are known to affect lipid metabolism in an extensive manner (Lodhi and Semenkovich, 2014). With their ability to interact with diverse organelles, peroxisomes affect both anabolic and catabolic pathways. It leads to the accumulation or defects of various lipid metabolites, which serve as metabolic precursors, signaling molecules impacting membrane and cellular functions, and regulators of transcription factors including nuclear receptors. In the BV-2 microglial cell models, saturated VLCFA accumulation was observed in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells and *Acox1<sup>-/-</sup>* mutants along with increase in lipid droplets (Raas et al., 2019a,b). Depending on the genotypes, we noticed some modifications of the levels of monounsaturated and polyunsaturated long-chain fatty acids. In addition, we observed cholesterol accumulation in the *Abcd1<sup>-/-</sup>* and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells as well as modifications of oxysterols levels. Whorled lipid inclusions, which likely result from the accumulation of cholesteryl esters of VLCFA, were observed in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells. The high rank of the GO term “Lipid metabolic process” in our functional enrichment analysis is in agreement with the observed modifications impacting lipids and reflects a wide reprogramming of lipid metabolism.

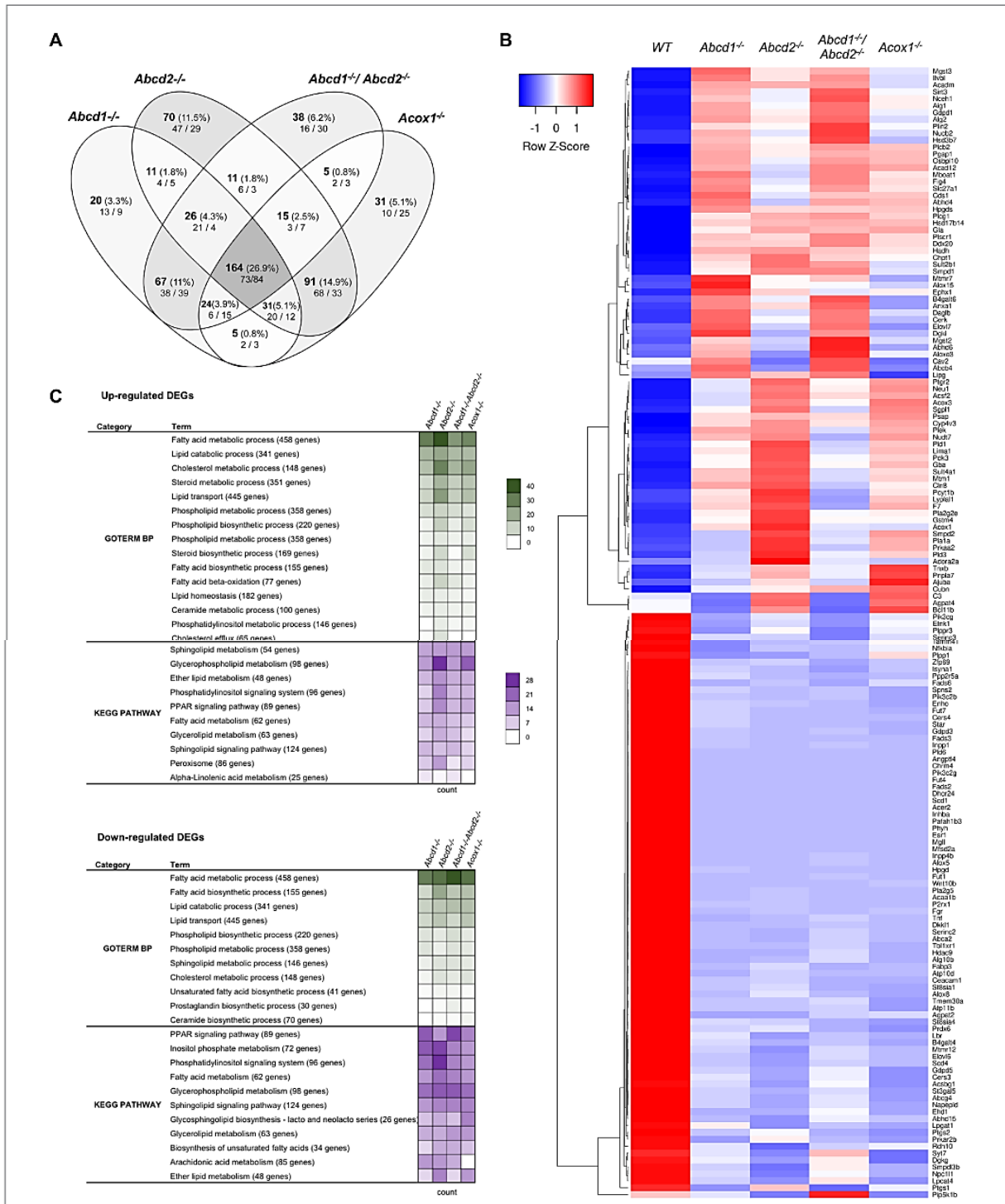
From the 1,190 genes associated with lipid metabolism and expressed in BV-2 cells, a total of 609 genes were found differentially expressed with the chosen cut off (348 for *Abcd1<sup>-/-</sup>*, 419 for *Abcd2<sup>-/-</sup>*, 350 for *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*, and 366 for *Acox1<sup>-/-</sup>* cells; Figure 2A). Like in the global analysis, Venn diagram demonstrated that the most important group of DEGs (164 genes) belong to the intersection of the 4 mutant genotypes (Figure 2A). The heatmap analysis of these genes indicates a greater overlapping between *Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* cells with 91 DEGs in common, and between *Abcd1<sup>-/-</sup>* and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells with 67 shared DEGs (Figure 2B). MA Plot showed that hits concern both low- and high-expressed genes (e.g., *ApoE*) while Volcano plot illustrated an equilibrated repartition of DEGs with the most significantly and differentially expressed genes being downregulated (Supplementary Figure 1). A majority of the genes belonging to these pathways were indeed moderately induced

or repressed but several genes such as *Fads2*, *Scd1*, *Acer2*, *Dhcr24* were found drastically downregulated in every mutant genotype. GO and KEGG pathway enrichment demonstrated an overall modification of the expression of genes associated with lipid metabolism widely impacting fatty acids catabolism and biosynthesis, membrane lipid metabolism, lipid transport, cholesterol metabolism, ether-lipid metabolism, and lipid-related signaling pathways (Figure 2C).

## Fatty acid metabolism

We found many genes belonging to both fatty acid biosynthesis and oxidation pathways (Figures 2B,C; Supplementary Figure 1). Both peroxisomal and mitochondrial catabolic pathways were impacted. Among the peroxisomal  $\beta$ -oxidation genes, the most striking observation was a strong repression of *Acaa1b* (3-ketoacyl-CoA thiolase B). Genes controlling mitochondrial  $\beta$ -oxidation *Cpt2* (carnitine palmitoyltransferase 2), *Acads* and *Acadm* (short and medium fatty acyl-CoA dehydrogenase encoding genes respectively), *Hadh* (hydroxyacyl-CoA dehydrogenase, which plays an essential role in the mitochondrial  $\beta$ -oxidation of short chain FA) were also among the hits (Figure 2B; Supplementary Figure 1). Since VLCFA accumulation is not exclusively related to the  $\beta$ -oxidation defect and may also be associated with an increase in *de novo* synthesis, we looked at the expression of the *Elovl* genes encoding FA elongases (Jump, 2009; Kihara, 2012). *Elovl6* (elongation of saturated and monounsaturated fatty acids C16:0-CoA or shorter) was found repressed in all mutant cells while *Elovl4* (elongation of extremely long saturated FAs and PUFAs > C26) and *Elovl7* (elongation of C16–C20-CoA) were induced except in *Acox1<sup>-/-</sup>* cells. *Elovl7* was among the most significantly upregulated DEGs in both *Abcd1<sup>-/-</sup>* and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells (Figure 2B; Supplementary Figure 1). *Elovl1*, which encodes for the main enzyme responsible for C26:0 synthesis (Ofman et al., 2010), was found very weakly but significantly induced in all mutant genotypes. Moreover, among the four 3-hydroxyacyl-CoA dehydratase encoding genes involved in VLCFA synthesis (Ikeda et al., 2008), *Hacd4* was found upregulated and *Hacd3* weakly repressed in every mutant genotype (Supplementary Figure 1). Concerning the acyl-CoA synthetase encoding genes which participate both in biosynthesis and oxidation pathways, *Acsbg1* was found repressed, especially in *Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* cells (Figure 2B; Supplementary Figure 1). On the contrary, the genes encoding acyl-CoA synthetase genes for medium-chain (*Acsf2* and *Acsf3*), long-chain (*Acsf1*), and very long-chain (*Slc27a1*), were found upregulated (Figure 2B; Supplementary Figure 1).

Unsaturated fatty acid metabolism was in the enriched pathways of mutant cells in accordance with the observed variations in the levels of these fatty acids (Raas et al., 2019a,b). Four stearoyl-CoA desaturases encoded by *Scd* genes catalyze the rate-limiting step in the production of MUFAs from C16:0 and C18:0. *Scd2*, the most expressed gene (10-fold more than *Scd1*), remained unchanged regardless of the genotype. *Scd1* expression was considerably reduced in every mutant genotype (Figure 2B; Supplementary Figure 1). *Scd3* and *Scd4*, although poorly expressed in BV-2 cells, were also repressed in every mutant genotype (Figure 2B; Supplementary Figure 1). The high expression level of *Scd2* and the substrate specificity shared with *Scd1* may explain why total MUFA levels have remained almost unchanged. Concerning PUFAs, the levels of AA and DHA remained unchanged in the *Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* cells but were increased in *Abcd1<sup>-/-</sup>* and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells (Raas et al., 2019a,b). *Fads2* encodes for a



**FIGURE 2** Reprogramming of lipid metabolism in mutant cells. **(A)** Comparative analysis of the sets of DEGs using Venn diagram ( $n=3$  for each genotype). From the 1,190 genes associated with the GO term "Lipid metabolic process" and expressed in BV-2 cells, the significant genes (number and percentage) were selected using a cut off adjusted  $p$ -value (DESeq2 Wald test with Benjamini and Hochberg  $p$ -value adjustment) lower than 0.05 and an absolute  $\log_2$  FC higher than 0.58 (higher than 1.5-fold change in both directions) and are indicated in each group [*Abcd1*<sup>-/-</sup> (348 DEGs: 177 up, 171 down), *Abcd2*<sup>-/-</sup> (419 DEGs: 242 up, 177 down), *Abcd1*<sup>-/-</sup> *Abcd2*<sup>-/-</sup> (350 DEGs: 165 up, 185 down), and *Acox1*<sup>-/-</sup> (366 DEGs: 184 up, 182 down)]. Numbers below correspond to the genes found in each group when the analysis was performed exclusively on upregulated genes (left) or downregulated genes (right). **(B)** Expression-based heatmap showing hierarchical clustering (left) using average linking and Pearson distances based on the 164 DEGs of the intersection between the 4 mutant genotypes (right). Each point corresponds to the Row Z-score collected from the means of the reads normalized

(Continued)

## FIGURE 2 (Continued)

and divided by median of transcripts length in kb from 3 independent cell samples for each genotype. (C) Biological processes and gene ontology pathways related to lipid metabolism corresponding to over and under-expressed genes in mutant BV-2 cells. Functional annotation was obtained using DAVID online tools from upregulated and downregulated DEGs in each mutant genotype. A selection of the most represented terms is presented with their number of associated genes mentioned in color-coded form (green for GO Biological process and magenta for KEGG pathway) and available in a source data file. The number of genes contained in each term is indicated.

$\Delta 6$ -desaturase which is the rate-limiting enzyme of the long-chain PUFA biosynthesis such as arachidonic acid (AA, C20:4, n-6) and eicosapentaenoic acid (EPA, 20:5, n-3) and which controls the last step of desaturation for docosahexaenoic acid (DHA, 22:6, n-3) and docosapentaenoic acid (DPA, 22:5, n-6) synthesis. Paradoxically, *Fads2* expression was hugely decreased in the 4 mutant cells (Figure 2B; Supplementary Figure 1). *Fads3* and *Fads6* genes, which are weakly expressed, were also found repressed in every mutant genotype while *Fads1* (the gene encoding a  $\Delta 5$ -desaturase forming AA and EPA) remained unchanged (Figure 2B; Supplementary Figure 1). Mobilization of fatty acids or other lipid derivatives from membrane lipids or triacylglycerols represents a very abundant source of signaling molecules which play a major role in the control of inflammation, especially in the brain (Joensuu et al., 2020). A large number of genes encoding lipases and phospholipases was observed among the hits, most of them being upregulated. A few ones such as *Mgll*, an important regulator of neuroinflammation in the brain, were strongly repressed (Figure 2B; Supplementary Figure 1). *Mgll* encodes for the monoglyceride lipase which controls hydrolysis of the endocannabinoid 2-arachidonoylglycerol in the brain generating AA and glycerol (Grabner et al., 2016). Interestingly, the entire pathway leading to produce endocannabinoid arachidonoyl ethanolamine (anandamide), which plays an important role in microglia and brain homeostasis (Tanaka et al., 2020), was found particularly deregulated (induction of *Abhd6*, *Camk2a*, *Daglb*, *Faah*, and repression of *Mgll*, *Dagla*, *Alox8*, *Napepld*; Figure 2B; Supplementary Figure 1). Since inhibition of FAAH was shown to be neuroprotective (Tanaka et al., 2020), the observed alterations may contribute to neurodegenerative process in peroxisomal disorders.

Many other phospholipase encoding genes were found among the DEGs indicating that the production of several signaling lipids such as diacylglycerol, inositol-trisphosphate, N-acylethanolamine, or phosphatidic acid is likely affected. Among the *Pla2* genes which control the release of unsaturated fatty acids such as AA, a precursor of inflammatory mediators, we observed a repression of *Pla2g4c* and *Pla2g5*, as well as an induction of *Pla2g2e* in every mutant genotype (Figure 2B; Supplementary Figure 1). *Pla2g2d* and *Pla2g7* were induced in *Abcd1<sup>-/-</sup>*, *Abcd2<sup>-/-</sup>*, and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells (Supplementary Figure 1). Interestingly, genes involved in AA or DHA metabolism were found deregulated (induction of *Aloxe3* and *Alox15*, repression of *Alox5* and *Alox8*; Figure 2B; Supplementary Figure 1). Among the genes associated with leukotriene metabolism, *Ltc4s*, *Mgts2* and *Mgts3* were induced while *Lta4h* remained unchanged. Genes involved in the synthesis of the prostaglandins were also among the hits. Finally, *Tbxa1s*, which is responsible for thromboxane synthesis was repressed in *Abcd1<sup>-/-</sup>* and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells (Supplementary Figure 1). In summary, our transcriptional analysis suggests a large modification of the metabolic pathways controlling the levels of pro-inflammatory eicosanoids (leukotrienes and prostaglandins) and anti-inflammatory docosanoids (resolvins) in the mutant microglial cells.

## Lipid droplets

Lipid droplets play a major role in fatty acid homeostasis and participate actively to the inter-organelle exchanges associated with lipids (Renne and Hariri, 2021). From the preliminary observations in the BV-2 mutant cells showing lipid droplets and lipid inclusions (Raas et al., 2019a,b), modification of the transcriptome associated with lipid droplets was expected. About 40 genes related to lipid droplets were indeed found differentially expressed, a majority being upregulated. Lipid droplets are now considered to be highly dynamic organelles consisting of a core of triglycerides and cholesteryl-esters surrounded by a monolayer of phospholipids and proteins such as perilipins. Among the main actors regulating the generation and the maturation of lipid droplets, we noticed the induction of the perilipin encoding genes, *Plin2* and *Plin3* (Figure 2B; Supplementary Figure 1). The *Rsad2* gene, which encodes for Viperin was the most induced gene of this group. Viperin is localized in the lipid droplets and participates in the antiviral response and in the regulation of lipid synthesis (Hinson and Cresswell, 2009). Concerning the lipid composition of the lipid droplets, the increased labeling of neutral lipids observed by Oil Red O staining in mutant cells (Raas et al., 2019a,b) is likely due to cholesteryl-esters and not triglycerides. The *Soat1* and *Soat2* genes encoding sterol-O-acyltransferase-2 that produces cholesteryl esters from cholesterol and LCFA or VLCFA were indeed upregulated. Interestingly, *Bscl2*, which encodes for Seipin, was found significantly repressed but only in *Abcd1<sup>-/-</sup>* and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells and could be associated to the ultrastructure differences concerning lipid droplets observed in the mutant cells (Supplementary Figure 1). Seipin was indeed shown to facilitate contacts between lipid droplets and endoplasmic reticulum and its deficiency was shown to result in lipid droplet size heterogeneity (Salo et al., 2019).

## Glycerophospholipids and sphingolipids

Pathway analysis of DEGs revealed a strong representation of genes associated with membrane lipids (glycerophospholipids, plasmalogens and sphingolipids). This was particularly true for upregulated DEGs. *De novo* synthesis of phospholipids from glycerol-3-phosphate to form phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol and their remodeling consisting of various exchanges of fatty acids are essential for membrane functions (Yamashita et al., 2014). In general, saturated fatty acids are present at the sn-1 position while unsaturated occupy the sn-2 position. Besides their role associated with membrane properties, phospholipids also act as reservoirs for bioactive lipids (eicosanoids, docosanoids, lysophospholipids, anandamides) thanks to the activity of various phospholipases, transacylases or acyltransferases. Many genes of the glycerophospholipid metabolism were found differentially upregulated in the 4 mutant genotypes (Figure 2B; Supplementary Figure 1). We observed a repression of *Agpat2* and *Plpp1* and upregulation of *Cds1* in the mutant cells, genes associated with the formation of

phosphatidic acid (*Agpat2*) and then its conversion to diacylglycerol to yield phosphatidylcholine and phosphatidylethanolamine (*Plpp1*) or cytidine diphosphate diacylglycerol to yield phosphatidylinositol and cardiolipin (*Cds1*). Genes encoding phospholipases D which control conversion of phosphatidylcholine or phosphatidylethanolamine into phosphatidic acid, were induced. Genes encoding diacylglycerol kinases phosphorylating diacylglycerol to phosphatidic acid, were differentially expressed (induction of *Dgki* and repression of *Dgka* and *Dgkg*). The phosphatidylcholine pathway appears to be induced since we observed upregulation of *Pld1*, *Pcyt1b*, *Chpt1*, and *Lpcat2* (Figure 2B; Supplementary Figure 1). Remodeling of glycerophospholipids and lysophospholipids is also apparently widely affected and differs between the genotypes. For instance, we observed the induction of and *Cept1* in *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells. Conversely *Agpat4* was repressed in *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells. Many other acyltransferase encoding genes and phospholipid phosphatase encoding genes figured in the DEGs (Supplementary Figure 1).

Sphingolipid metabolism and sphingolipid catabolic processes came out on a high rank in the sorting of gene ontology (Figure 2C). Sphingolipid metabolism has progressively received attention in neurodegenerative diseases and is now considered as a key component of neuroinflammation regulation (Assi et al., 2013; Lee et al., 2020). We observed that a majority of genes related to sphingolipid metabolism were significantly upregulated in the mutant cells (Figure 2B; Supplementary Figure 1). A few genes were downregulated and among them, *Acer2*, which controls the hydrolysis of long- and very long-chain ceramides and dihydroceramides to yield sphingosine, and *Cers4*, the ceramide synthase 4 encoding gene (Figure 2B; Supplementary Figure 1). It is noteworthy that the most expressed ceramide synthase encoding genes in BV-2 cells (*Cers2*, *Cers5*, *Cers6*) were not differentially expressed. Some of these results (induction of *Smpd1*, *Cerk*, *Sphk1*, and repression of *Acer2*, *Cers3*, *Cers4*) are in agreement with the transcriptomic data obtained from patient fibroblasts with cerebral or adrenomyeloneuropathy forms of X-ALD showing that the anabolic pathway toward sphingolipids and glycosphingolipids is downregulated (Lee et al., 2019). Concerning glycosphingolipid biosynthesis, many genes belonging to this pathway were repressed and *Fut4*, which is involved in the synthesis of lactosphingolipids, was among the most downregulated genes (Figure 2B; Supplementary Figure 1).

## Cholesterol

Chronic neurodegenerative disorders are increasingly thought to be associated with dysregulation of cholesterol homeostasis in the brain. Enrichment analysis revealed many genes participating to cholesterol metabolism, transport and homeostasis. *Trem2*, which was found induced in all mutant genotypes, was recently described as a key transcriptional regulator of cholesterol transport and metabolism (Nugent et al., 2020). The expression level of genes encoding the main transcriptional regulators (*Srebf1* and *Srebf2*) was found unchanged. However, a significant but moderate upregulation (Log2FC varying between 0.42 and 0.62 depending on genotypes) of *Insig2* was observed in all mutants (*Insig1* remaining stable) while *Scap* was repressed in *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells. In the presence of elevated levels of cholesterol, *Insig2* is known to bind SCAP and prevent the proteolytic processing of SREBPs, thereby inhibiting cholesterol synthesis. *Nr1h3*, which encodes for the oxysterol nuclear receptor

LXR $\alpha$ , was also induced. *Hdac9*, which was shown to repress cholesterol efflux (Cao et al., 2014) was strongly downregulated, especially in *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells. *Nfkbia*, which is associated with positive regulation of cholesterol efflux was also repressed. This observation is consistent with the discrepancy found between mutant cells showing that cholesterol accumulate selectively in the *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells (Raas et al., 2019a,b).

Although many genes of the cholesterol and oxysterol biosynthetic pathways remained quite stable, genes encoding key enzymes of the initial steps of cholesterol synthesis (from acetyl-CoA to lanosterol) were weakly but significantly induced especially in *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells (Figures 2B, 3A,C; Supplementary Figure 1). The involvement of peroxisomes in these initial steps of cholesterol biosynthesis remains controversial with conflicting reports about peroxisomal location of several enzymes such as HMGCR of MVK for instance (Kovacs et al., 2002; Hogenboom et al., 2004a,b; Figure 3C). In the Bloch and Kandutsch-Russell pathways which drives conversion of lanosterol to cholesterol through several steps of reduction, *Lbr* and *Dhcr24* were found repressed in all mutants while *Tm7sf2* was repressed only in the *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells (Figures 3B,C; Supplementary Figure 1). *Nsdhl* and *Ebp* were weakly induced in the *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells (Figure 3B; Supplementary Figure 1). It is noteworthy that the Kandutsch-Russell pathway (lathosterol pathway) is used for more than 70% of cholesterol biosynthesis in the brain. The expression of *Dhcr24*, which encodes for the 24-dehydrocholesterol reductase (also called seladin-1) catalyzing the final conversion of desmosterol into cholesterol in the Bloch pathway but also the exchange between the Bloch and Kandutsch-Russell pathways, was found hugely decreased in every mutant genotype (Figures 2B, 3B; Supplementary Figure 1). Western blotting experiments confirmed the loss of expression of *Dhcr24* in mutant BV-2 cells (Figure 3D). Although there was a moderated upregulation of genes of the squalene synthesis pathway, the analysis of the cholesterol precursors revealed an approximately 2-fold decrease in lanosterol content and of the lanosterol/cholesterol ratio in the mutant cells (Figure 3E). The level of lathosterol and the lathosterol/cholesterol ratio were also reduced in the mutant cells suggesting that the Kandutsch-Russell pathway is inhibited. The defect in *DHCR24* is associated with a rare autosomal recessive developmental disorder called desmosterolosis characterized by desmosterol accumulation (Waterham et al., 2001). Surprisingly, the desmosterol level remained quite stable except in the *Acox1*<sup>-/-</sup> cells (37% increase; Figure 3E). The desmosterol/cholesterol ratio was weakly increased in the *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells and weakly decreased in the *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells in which cholesterol accumulates (Raas et al., 2019a).

Once synthesized, cholesterol can be esterified, oxidized, used for steroidogenesis or transported. As cited above, the sterol-O-acyltransferase encoding genes *Soat1* and *Soat2* were indeed found upregulated. The expression of *Agt*, which positively regulate cholesterol esterification, was also induced (Figure 2B; Supplementary Figure 1). On the other hand, *Nceh1*, which catalyzes the hydrolysis of intracellular cholesteryl-esters, was found induced (Figure 2B; Supplementary Figure 1). Several important genes involved in cholesterol trafficking and lipoprotein metabolism such as *ApoE* and *ApoA2*, were also found upregulated. Interestingly, APOE is the main cholesterol carrier in the brain and was shown to mediate a switch toward a neurodegenerative

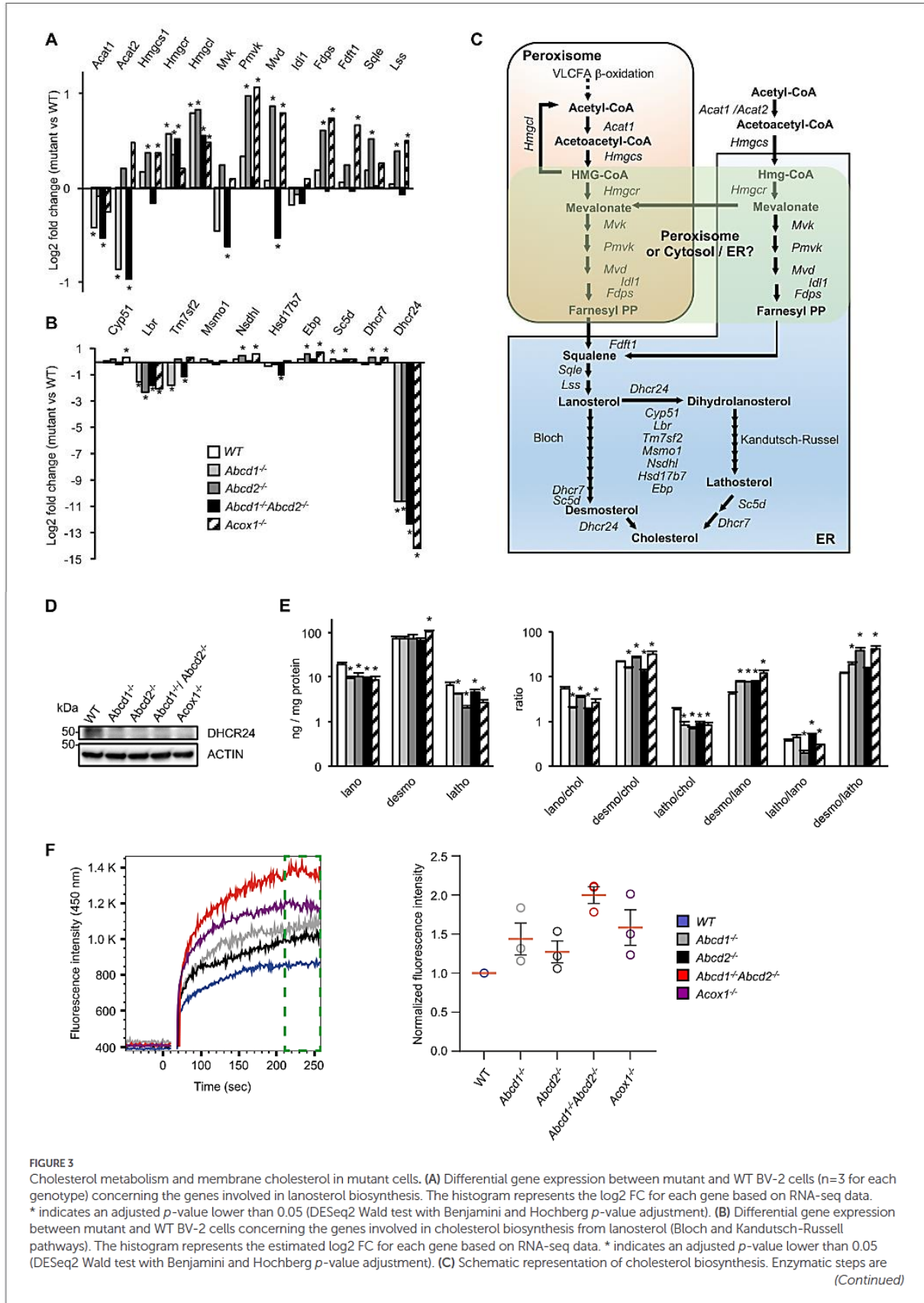


FIGURE 3

Cholesterol metabolism and membrane cholesterol in mutant cells. (A) Differential gene expression between mutant and WT BV-2 cells (n=3 for each genotype) concerning the genes involved in lanosterol biosynthesis. The histogram represents the log<sub>2</sub> FC for each gene based on RNA-seq data.

\* indicates an adjusted *p*-value lower than 0.05 (DESeq2 Wald test with Benjamini and Hochberg *p*-value adjustment). (B) Differential gene expression between mutant and WT BV-2 cells concerning the genes involved in cholesterol biosynthesis from lanosterol (Bloch and Kandutsch-Russell pathways). The histogram represents the estimated log<sub>2</sub> FC for each gene based on RNA-seq data. \* indicates an adjusted *p*-value lower than 0.05 (DESeq2 Wald test with Benjamini and Hochberg *p*-value adjustment).

(Continued)

## FIGURE 3 (Continued)

represented by arrows and the associated genes are indicated. The peroxisomal location of the initial steps of cholesterol biosynthesis remains controversial especially the steps concerning *Hmgcr*, *Mvk*, *Pmvk*, and *Mvd* (green windows). (D) Western blotting analysis of DHCR4 from cell lysates of WT and mutant BV-2 cells. For densitometric analysis, see Supplementary Figure 3. Source data are available online for this figure. (E) Cholesterol precursors levels in WT and mutant BV-2 cells and their ratio. Gas chromatography mass spectrometry (GC-MS) analysis was performed from cellular homogenates (3 independent experiments). Histograms represent the mean levels of cholesterol precursors (ng/mg protein) with their respective standard deviation and their ratios. Statistical significance at  $p < 0.05^*$  determined by Mann-Whitney test analysis is indicated. (F) Analysis of cholesterol accumulation in plasma membrane of WT and mutant BV-2 cells. Left panel, Filipin III binding kinetics in living cells recorded by flow cytometry during 256s. Right panel, median fluorescence intensities calculated from the last minute filipin III binding kinetics (green gate on the left panel) were normalized as a fold of the BV2 WT fluorescence ( $n=3$  independent experiments).

phenotype in human microglia in association with TREM2 (Krasemann et al., 2017). *Lrp1*, which encodes low density lipoprotein receptor-related protein 1, was found induced (Figure 2B; Supplementary Figure 1). Of note, among the triglyceride lipase encoding genes which contribute to lipoprotein degradation (Loving and Bruce, 2020), *Lipg* was induced except in the *Acox1*<sup>-/-</sup> cells, *Lipn* was induced except in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells, and *Lipa* (lysosomal lipase) was weakly induced in every mutant genotype (Figure 2B; Supplementary Figure 1). Among the ABC transporters associated with cholesterol efflux (Wang et al., 2008), many inductions were observed, suggesting increased efflux of cholesterol but with genotype specificities (Figure 2B; Supplementary Figure 1). Of note, *Npc1* and *Npc2* genes, which are involved in the transport of LDL-derived cholesterol from lysosomes to the cytoplasm (Saha et al., 2020), were induced in *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells while *Npc11*, although weakly expressed, was repressed in every mutant genotype (Figure 2B; Supplementary Figure 1). The loss of these genes triggers endolysosomal accumulation of unesterified cholesterol and lipids and is associated to Niemann-Pick disease (Vance, 2012). In contrast, the expression of *Syt7*, which is involved in the contact between peroxisomes and lysosomes and in the cholesterol transport from lysosomes to peroxisomes (Chu et al., 2015), was decreased in every mutant genotype. *Star*, which transports cholesterol from the outer to the inner mitochondrial membrane and regulates the rate limiting step in steroidogenesis, was repressed in all mutant cells (Figure 2B; Supplementary Figure 1).

To document the consequences of the alterations concerning genes related to cholesterol metabolism and transport, we evaluated the free cholesterol content of the plasma membrane by Filipin III binding kinetics in BV-2 living cells by flow cytometry. We evidenced that Filipin III accumulates more in the plasma membrane of the mutant cell lines compared to BV2 WT cells and in a more pronounced manner in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells (Figure 3F). Excess of free cholesterol in the plasma membrane might originate from either a modification of the equilibrium between ordered versus disordered phases affecting lipid membrane organization or modified exchanges between the cellular compartments or enzymatic conversions (Litvinov et al., 2018).

### Oxysterols

Oxysterol levels were found modified in mutant cells (Raas et al., 2019a) and either oxidative stress or gene regulation may explain this observation. Among the genes involved in oxysterol metabolism (Mutemberezi et al., 2016), only a few genes were deregulated. *Ephx1*, which encodes for cholesterol epoxide hydrolase and forms dihydroxycholesterol, *Hsd3b7*, which converts 7 $\alpha$ -hydroxycholesterol

to 7 $\alpha$ -hydroxy-4-cholesten-3-one, and *Sult2b1*, involved in the formation of sulfoconjugates of 25- and 24-hydroxysterols, were found induced in every mutant genotype (Figure 2B; Supplementary Figure 1). *Sult4a1*, whose function is unknown was also found induced. *Cyp11a1* which encodes for the enzyme catalyzing the synthesis of 22R-hydroxycholesterol was induced in every mutant genotype except *Acox1*<sup>-/-</sup> cells (Supplementary Figure 1). CYP46A1 converts cholesterol to 24S-hydroxycholesterol, a transport derivative of cholesterol from the brain to the systemic circulation, allowing cholesterol excess elimination in human brain (Petrov and Pikuleva, 2019). Although its expression level seems to be very low in BV-2 cells, *Cyp46A1* was weakly induced in mutant cell lines. The expression level of *Cyp27a1* remained unchanged while that of other main cytochrome P450 genes (*Cyp7a1*, *Cyp7b1*, *Cyp3a4*, *Cyp3a5*, *Cyp39a1*) and other genes (*Ch25h*, *Sult2a1*) involved in oxysterol metabolism were undetectable.

### A “disease-associated microglial signature”-like including upregulation of lysosomal and autophagy-related genes

Lysosome, together with endosome and autophagy, emerged as main terms of gene ontology enrichment analysis (Figure 1B). Moreover, the “Tyrobp (DAP12) causal network” figured in first position of the Wikipathways upregulated in the BV-2 mutant cells. *Trem2* and *Tyrobp* are considered as hub genes for microglial functions and the TREM2-APOE pathway has been shown essential to drive the transcriptional phenotype associated with dysfunctional microglia in neurodegenerative diseases (Jay et al., 2017; Krasemann et al., 2017; Gratuze et al., 2018; Ulland and Colonna, 2018; Deczkowska et al., 2020). *Trem2* encodes a membrane receptor for diverse ligands (LPS, phospholipids, glycolipids, lipoproteins and apolipoproteins) playing an essential physiological role in addition to be central in pathological conditions being responsible to the DAM signature (Butovsky and Weiner, 2018). Indeed, TREM2 recruits DAP12/TYROBP to mediate a complex signaling cascade controlling phagocytosis, cell survival, cell differentiation and inflammation (Konishi and Kiyama, 2018). The three hub genes (*ApoE*, *Tyrobp*, *Trem2*) were found significantly induced in the mutant cells (except for *ApoE* in *Acox1*<sup>-/-</sup> cells). Of note, the expression of *CD36* which encodes for a FA translocase playing an important role in microglial activation (Stuart et al., 2007) and phagocytosis of myelin debris (Grajchen et al., 2020), was found repressed in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells and induced in *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells. Microglial dysfunction and lysosomal activity are tightly connected (Gabandé-Rodríguez et al., 2019). In a first step, we therefore focused on lysosome-associated genes and then explored

the DEGs in mutant cells with reference to the transcriptomic DAM signature found in microglia in case of neurodegenerative disease.

### Lysosome and autophagy

The KEGG term “Lysosome” was the first term to come out from upregulated DEGs genes and from global analysis. From the 513 genes associated with the term “Lysosome” (list combined from KEGG and Gene Ontology search) and expressed in BV-2 cells, a total of 316 were found differentially expressed with a majority of upregulated genes (158 in *Abcd1*<sup>-/-</sup>, 192 in *Abcd2*<sup>-/-</sup>, 130 in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, and 161 in *Acox1*<sup>-/-</sup>; Figure 4A; Supplementary Figure 2). Eighty-five genes were found upregulated and 28 downregulated in the 4 mutant genotypes, showing two clustering patterns indicating a higher proximity between *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and between *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> respectively (Figure 4B). Genes involved in metabolism and hydrolysis of various substrates, transport, motility, autophagy, and acidification were found in the hits. Among these genes, *Lamtor4*, *Map2*, *Pla2g5*, *Cpq*, and *Ctsb* emerged as the most repressed genes with log<sub>2</sub> FC ratio exceeding -8 (Supplementary Figure 2). Of the most expressed DEGs, *ApoE*, cathepsin encoding genes (*Ctsa*, *Ctsb*, *Ctsk*, *Ctsl*, *Ctsz*), *Lamp1*, *Grn*, and vacuolar ATPase encoding genes (*Atp6v1b2*, *Atp6v1c1*), were all upregulated in mutant cells (Supplementary Figure 2). Noteworthy, *Lamp2*, although less expressed than the genes above, was also upregulated (Supplementary Figure 2). The expression of *Tfeb*, which encodes for a transcription factor controlling lysosomal biogenesis (Napolitano and Ballabio, 2016) was not modified suggesting that biogenesis of lysosomes was likely not modified by peroxisomal defect. In contrast, lysosomal metabolic, degradative, and signaling functions appear to be widely affected and mainly activated. In addition, motility and positioning of lysosomes should be affected regarding the virtual knock out of *Map2*, which encodes for a microtubule-associated protein 2 (Pu et al., 2016), and the induction of *Hook1* (Hook microtubule tethering protein 1) and *Plekhm2* (SKIP, kinesin-interacting protein). Cathepsins form an important group of proteases in the endosomal-lysosomal compartment and 14 cathepsin genes were found expressed in BV-2 cells. *Ctsk* and *Ctsb*, which code for two lysosomal proteins also known to be secreted and play an important role in the brain, particularly in microglia (Dauth et al., 2011; Nakanishi, 2020a), figured in the most expressed cathepsin genes (Supplementary Figure 2). With the exception of the poorly expressed *Ctsw* gene, cathepsin genes were found mostly upregulated in the mutant cells (Figures 4B,C). We also observed upregulation of all V-ATPase encoding genes expressed in BV-2 cells along with the *Atp6ap1* and *Atp6ap2* genes which encode accessory subunits involved in V-ATPase regulation (Jansen and Martens, 2012; Figures 4B,D). Western blotting analysis of several lysosomal proteins was performed to confirm the transcriptomic data. Upregulation of both cathepsins B and K and two other lysosomal proteins, progranulin (GRN) and galectin-3 (LGALS3, a cytosolic protein recruited to damaged lysosomes), was observed in the mutant cells (Figure 5A; Supplementary Figure 3). Upregulation of ATP6V0D2 was observed in the mutant cells except in the *Abcd2*<sup>-/-</sup> genotype. However, we failed to show increased expression of ATP6V1B2 and LAMP2, except in *Abcd1*<sup>-/-</sup> cells for the latter (Figure 5A; Supplementary Figure 3).

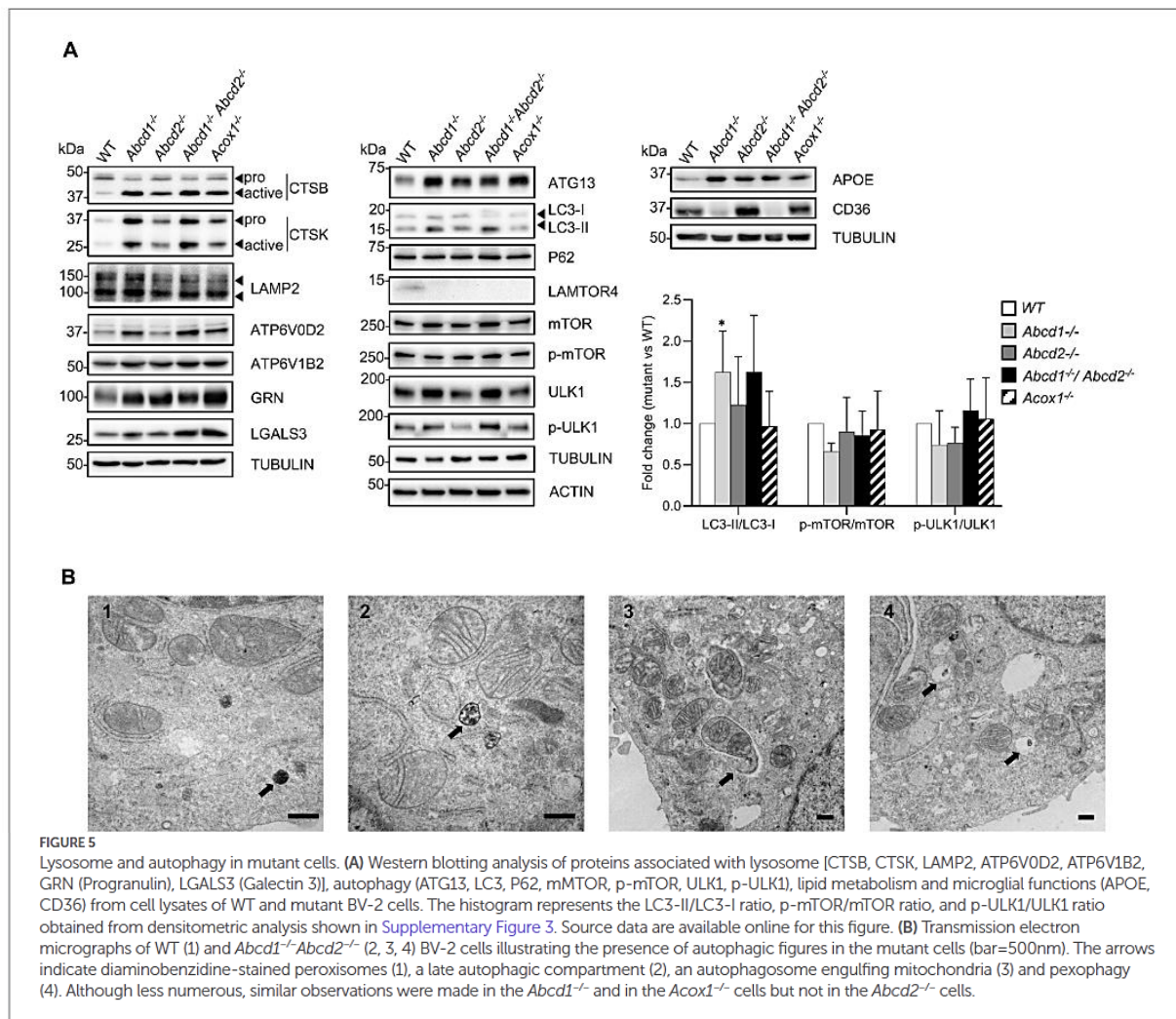
Autophagy is tightly coupled to lysosomal activity and plays an important role in microglia regarding neuroinflammation and brain homeostasis (Julg et al., 2021). Moreover, peroxisomal β-oxidation has

recently been linked to the lysosomal localization and activation of mTORC1 complex (He et al., 2020). Besides, the heterodimeric Rag GTPases, the Lamtor/Ragulator complex that tethers the Rags to the lysosome, and the V-ATPase form a signaling system coupled to the rapamycin complex I (mTORC1) activity (Bar-Peled et al., 2012; Colacurcio and Nixon, 2016; Shen et al., 2016). *Rragc* and *Rragd* were u-regulated in mutant cells while *Rraga* expression remained unchanged. With the exception of the strong repression of *Lamtor4*, we observed a weak but significant induction of *Lamtor1*, *Lamtor3*, and *Lamtor5*. *Lamtor2* demonstrated a significant induction but below the 0.58 cut off. We observed a majority of autophagy-related (*Atg*) genes non differentially expressed but *Atg10* and *Atg13* were induced while *Atg9a* was repressed. Of note, *Atg7* expression was also found upregulated in *Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells. Moreover, *Sqstm1* (P62), *Rubcnl*, *Map1lc3a* (which exhibit two forms, LC3-I and LC3-II, the latter being induced in response to autophagic stimulus), and *Map1lc3b* genes were weakly upregulated in the mutant cells. To confirm the impact of peroxisomal mutations on autophagy, we carried out western blotting analysis of various autophagy markers and explored the phosphorylation of mTOR and ULK1, which represents the main mechanism of autophagy induction. mTORC1 directly downregulates autophagy by phosphorylating ULK1 on Ser757. LAMTOR4 repression and ATG13 induction were confirmed in each mutant genotype (Figure 5A; Supplementary Figure 3). We observed a weak induction of LC3-II, mTOR, ULK1 and p-ULK1 especially in the *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells while the expression of P62, LC3-I, and p-mTOR remained almost unchanged in the mutant cell lines (Figure 5A; Supplementary Figure 3). In addition to the autophagic and lysosomal markers, we analyzed the expression at the protein level of important genes associated with microglial functions related to lipids, i.e., *ApoE* and *CD36*. Western blotting results were in accordance with the transcriptomic data (Figure 5A; Supplementary Figure 3). The increased LC3-II/LC3-I ratio in the *Abcd1*<sup>-/-</sup> and in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells suggest increased autophagy in these genotypes even though the p-mTOR/mTOR ratio and p-ULK1/ULK1 ratio remained almost unchanged (Figure 5A). Ultrastructure analysis by electron microscopy using diaminobenzidine staining to identify peroxisomes indeed revealed an increased number of autophagic figures in the mutant cell lines, especially in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells (Figure 5B).

### DAM-like signature

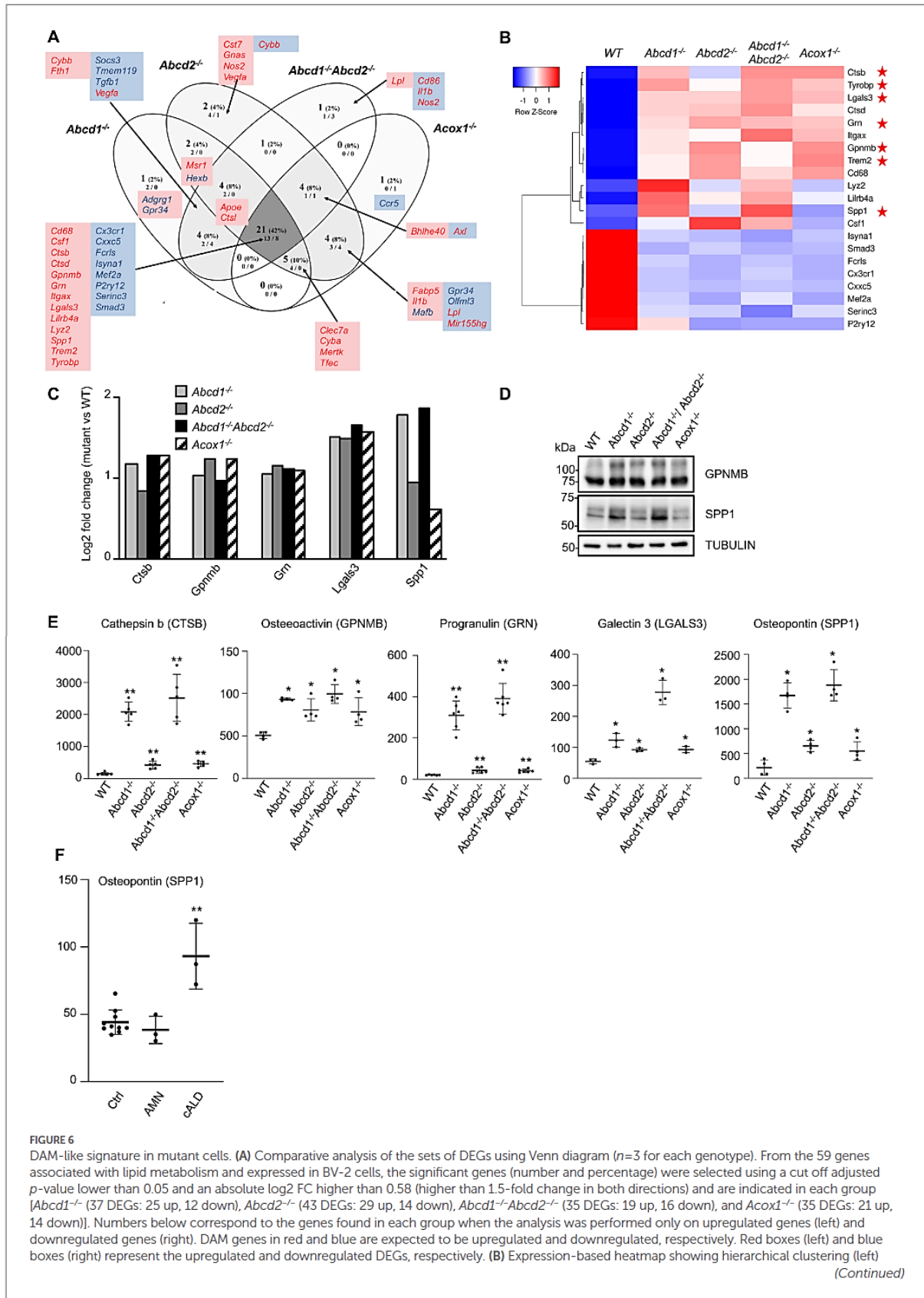
A list of 59 genes expressed in BV-2 microglial cells was established from the review of Butovsky and Weiner and other transcriptomic analyses on microglia, which established a common signature associated with neurodegenerative pathologies (Keren-Shaul et al., 2017; Krasemann et al., 2017; Butovsky and Weiner, 2018; Deczkowska et al., 2018; Bonham et al., 2019). The DEGs belonging to this list include 27 repressed genes and 32 induced genes and define a DAM-like signature distinct from that of microglial activation or polarization. In the BV-2 mutant cells, the variations of expression of the majority of these genes were significant and their orientation was in agreement with those of the DAM signature. We found a total of 50 genes differentially expressed with 8 downregulated “homeostatic genes” (*Cx3cr1*, *Cx3c5*, *Fcrls*, *Isyn1*, *Mef2a*, *P2ry12*, *Serinc3*, *Smad3*) and 13 upregulated genes (*Cd68*, *Csf1*, *Ctsb*, *Ctsd*, *Gpnmb*, *Grn*, *Ilgax*, *Lgals3*, *Lilrb4a*, *Lyz2*, *Spp1*, *Trem2*, *Tyrobp*) shared by the 4 mutant genotypes (Figures 6A,B; Supplementary Figure 4). If the intersection





Progranulin (*Grn*), Galectin-3 (*Lgals3*), Osteopontin (*Spp1*; [Figure 6C](#)). Cathepsin B has an important function in microglia and inflammatory control with its participation to the secretion of IL1 $\beta$  ([Nakanishi, 2020b](#)). Elevated levels of cathepsin B have been detected in biological fluids of patients with brain diseases ([Hook et al., 2020](#)). Osteactivin, encoded by *Gpnmb*, is a transmembrane glycoprotein that would function as a negative regulator of inflammatory processes and that can be secreted as a soluble fragment upon lysosomal stress ([van der Lienden et al., 2019](#)). Osteactivin is thought to carry anti-inflammatory and reparative functions and has been demonstrated to be neuroprotective ([Budge et al., 2018](#)). Progranulin, encoded by *Grn*, is a secreted protein that not only contributes to neuroinflammation but also actively participates in the regulation of lysosomal functions and autophagy ([Chitramuthu et al., 2017](#); [Paushter et al., 2018](#); [Elia et al., 2019](#); [Mendsaikhani et al., 2019](#)). Regarding the lysosomal trafficking of progranulin, it depends on prosaposin and sortilin; it impacts the production of granulin peptides and interferes with the secretory pathway ([Du et al., 2022](#)). Interestingly, *Sort1* was found highly repressed ( $\log_2$  FC = -5) while *Psap* was upregulated ( $\log_2$  FC = 1) in the 4 mutant cell lines. Galectin-3, which is also secreted by

microglia, has demonstrated pro-differentiating effects on oligodendrocyte progenitor cells and pro-inflammatory properties acting as a TLR4 ligand in an autocrine regulation ([Burguillos et al., 2015](#); [Thomas and Pasquini, 2018](#)). It is worth noting that both *Lgals3* and *Tr4* genes were upregulated in the four mutant genotypes. Galectin-3 was also recently described as an endogenous ligand of TREM2 and would therefore participate to the TREM2/DAP12 signaling cascade in an autocrine manner ([Boza-Serrano et al., 2019](#)). Osteopontin, encoded by *Spp1*, is a pro-inflammatory secreted protein which has been shown to inhibit autophagosome-lysosome fusion ([Tang et al., 2020](#)) and is thought to play a role in pathogenesis of neurodegenerative diseases or in neuroprotection by regulating the activation and function of microglia ([Yu et al., 2017](#)). We confirmed an increased expression of *Ctsb*, *Gpnmb*, *Grn*, *Lgals3*, and *Spp1* by western blotting ([Figures 5A, 6D](#); [Supplementary Figure 4](#)). We performed ELISA experiments on the culture medium of each mutant cell line to assess the secretion levels of these 5 proteins. Significant increase of secretion of each protein was found in the mutant cells as compared with the WT cells ([Figure 6E](#)). The highest relative increase compared with the WT cells was found in the



**FIGURE 6 (Continued)**  
 using average linking and Pearson distances based on the 21 DEGs of the intersection between the 4 mutant genotypes (right). Each point corresponds to the Row Z-score collected from the means of the reads normalized and divided by median of transcripts length in kb from 3 independent cell samples for each genotype. Red stars point the five DAM genes further studied and the *Trem2* and *Tyrobp* genes known as major hubs of the microglial activation. (C) Differential gene expression between mutant and WT BV-2 cells concerning the Cathepsin b (*Ctsb*), Osteoactivin (*Gpnmb*), Progranulin (*Grn*), Galectin-3 (*Lgals3*), and Osteopontin (*Spp1*) genes that belong the DAM signature. The histogram represents the log2 FC [with an adjusted *p*-value lower than 0.05 (DESeq2 Wald test with Benjamini and Hochberg *p*-value adjustment)] for each gene based on RNA-seq data. (D) Western blotting analysis of DAM proteins [Osteopontin (SPP1) and Osteoactivin (GPNMB)] from cell lysates of WT and mutant BV-2 cells. Western blotting showing Cathepsin b (CTSB), Progranulin (GRN), and Galectin-3 (LGALS3) expression has been shown in Figure 5A. For densitometric analysis, see Supplementary Figure 3. Source data are available online for this figure. (E) Increased secretion of DAM markers in BV-2 mutant cells. Dot plots representation of the concentration (ng/mL) of the DAM markers obtained by ELISA from the supernatant of mutant and WT BV-2 cells. Statistical significant differences are indicated: \**p*<0.05, \*\**p*<0.01 (Mann–Whitney test from 3 to 6 experiments). (F) Increased plasma level of osteopontin in cALD. Dot plots representation of the plasma level (ng/mL) of osteopontin (SPP1) obtained by ELISA from healthy controls (10), AMN patients (3), and cALD patients (3). Statistical significant differences are indicated: \*\**p*<0.01 (Mann–Whitney test).

*Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells (CTSB: 2518 ng/mL, 16.2-fold; GPNMB: 99.5 ng/mL, 1.96-fold; GRN: 391 ng/mL, 19-fold; LGALS3: 278 ng/mL, 5.08-fold; SPP1: 1,881 ng/mL, 8.75-fold).

Osteopontin is an inflammatory protein that has recently been described as a biomarker of Alzheimer's disease (Chai et al., 2021) and multiple sclerosis (Agah et al., 2018). In X-ALD, microglial defect is thought to precede the neurological involvements (Eichler et al., 2008; Bergner et al., 2019). Since osteopontin appears to be oversecreted from microglial cells in case of peroxisomal defect, osteopontin could therefore represent an interesting biomarker for X-ALD. To address this point, we conducted a pilot study on the plasma level of osteopontin using samples from 3 adult patients diagnosed with a cerebral form of X-ALD (cALD) and 3 adult patients with adrenomyeloneuropathy. The results were compared with control values taken from 10 healthy controls. While there was no significant difference in AMN patients, a 2.11-fold increased concentration was observed in cALD patients (Figure 6F).

## Discussion

The transcriptomic analysis of the mutant cell lines revealed 1,000 of DEGs. As expected, genes related to lipid metabolism belong to the DEGs but "immune system process" ranked first in the significant enriched GO terms, which support a role of peroxisomal metabolism far beyond its main known metabolic functions. Several recent studies have documented a functional role of peroxisomes on immune functions and cell signaling (Di Cara et al., 2019; Di Cara, 2020; Di Cara et al., 2023) and the present study reinforces this statement. Although many signaling pathways, cell division and cell cycle features, phagocytosis, and immune response of microglial BV-2 mutant cells appear to be largely affected, we focused on lipid metabolism, lysosome, autophagy, and microglial plasticity in link with neurodegenerative processes.

Although all 4 mutant genotypes result in a defect in peroxisomal  $\beta$ -oxidation, different phenotypes were expected because of the predicted severity of the mutations, in descending order: *Acox1*<sup>-/-</sup>, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, *Abcd1*<sup>-/-</sup>, and finally *Abcd2*<sup>-/-</sup>. Indeed, there is currently no disease associated with a mutation in the *ABCD2* gene. Moreover, since both *Abcd1* and *Abcd2* genes are expressed in BV-2 cells and these genes have partial functional redundancy, a single KO should have resulted in an attenuated phenotype compared to the double KO. Finally, since ACOX1 is the rate-limiting enzyme in

peroxisomal  $\beta$ -oxidation, the most severe phenotype was expected for this KO. Autophagic alterations and increased secretion of DAM proteins was indeed more important in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> genotype. However, it was quite puzzling to observe in some cases that the dysregulations are less marked in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells than in the single mutants. These indirect effects of peroxisomal defects are probably coming along with compensatory mechanisms, which differ depending on the genotypes. More pronounced feedback loop may exist in the case of the double knock out. VLCFA accumulation, as a marker of peroxisomal defect, was seen only in the *Acox1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells and cannot be the sole cause of the phenotype differences (Raas et al., 2019a,b). Surprisingly, several clusters of DEGs were found associated either with *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> or with *Acox1*<sup>-/-</sup> and *Abcd2*<sup>-/-</sup> cells and sometimes in a counterintuitive manner. Lipidomic differences have already been described between ACOX1 and ABCD1 human fibroblasts which reflects the heterogeneity of the peroxisomal disorders ACOX1 deficiency and X-ALD (Herzog et al., 2018). Accumulation of phospholipids with VLCFAs was observed in the majority of phospholipid classes in ACOX1-deficient cells but was limited to phosphatidylcholine and phosphatidylethanolamine classes in ABCD1-deficient fibroblasts. The observed proximity between *Acox1*<sup>-/-</sup> and *Abcd2*<sup>-/-</sup> BV-2 genotypes observed with several genes question about a functional link between these two genes. Is it associated with a specific substrate of ABCD2, which is metabolized by ACOX1? One possible explanation could be linked to the accumulation of a specific lipid (probably a PUFA not transported by ABCD1) leading to the sustained activation of transcription factors such as PPARs. PUFAs and some of their derivatives have been demonstrated to bind PPARs and regulate microglial functions (Ajmone-Cat et al., 2012; Leyrolle et al., 2019). Interestingly, *Ppar $\beta/\delta$*  appears to be the most expressed isotype in the BV-2 cells and its expression was found repressed in both *Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells, but not in the other mutants, whereas *Ppar $\gamma$*  was found upregulated in both *Acox1*<sup>-/-</sup> and *Abcd2*<sup>-/-</sup> cells.

In spite of this observation, the most numerous group of DEGs was found in the intersection group concerning the four mutant genotypes. It was both true for the global analysis and for the specific analyses related to lipid metabolism and other pathways. If LCFA and VLCFA accumulation is considered as the hallmark of peroxisomal diseases, from our results, we can conclude that peroxisomal defects have induced an in-depth reprogramming of genes involved in lipid metabolism. Fatty acid oxidation and elongation, fatty acid release and

signaling, PUFA metabolism and production of bioactive PUFA derivatives, metabolism of membrane lipids, cholesterol metabolism and trafficking, have all been found impacted in the mutant cells. From these data, we could not conclude whether the mutations were leading to a clear upregulation or downregulation of these lipid metabolic pathways since most of the pathways were represented in both upregulated and downregulated DEGs. An extended lipidomic study is needed to explore in detail the impact of the mutations on the various classes of lipids and complete the preliminary analysis on total FA and cholesterol precursors of the mutant cell lines (Raas et al., 2019a,b). Further studies are also needed to understand the origin of cholesterol accumulation, especially in the context of DHCR24 loss, and document to which extent the observed modifications in FA content, in membrane cholesterol and in cholesterol precursors, alter the biophysical properties of membranes, as well as their signaling and inflammatory associated functions.

Nevertheless, such metabolic reprogramming is known to impact microglial functions related to inflammatory signaling, oxidative stress, phagocytosis, and brain homeostasis (Nadjar, 2018; Fitzner et al., 2020; Loving and Bruce, 2020; Chausse et al., 2021; Navia-Pelaez et al., 2021). Peroxisomal  $\beta$ -oxidation defect in microglia using the *Cx3cr1-Mfp2<sup>-/-</sup>* mice (targeted gene *Hsd17b4*) led to an inflammatory state (Beckers et al., 2019). In *Drosophila* plasmatocytes, fly equivalent of mammalian macrophages, defective peroxisomal  $\beta$ -oxidation was also shown to modify glycerophospholipids with consequences on cytoskeleton and signaling related to inflammation (Nath et al., 2022). Therefore, from our observations and the microglial literature, the defect in peroxisomal ABC transporters and ACOX1 in BV-2 cells likely contribute to a shift toward a pro-inflammatory state and a modification of phagocytosis ability independently of the supposed severity of the metabolic defects associated with the different mutant genotypes. In agreement with this hypothesis, in all the mutant microglial cells, we found upregulation of the *Trem2*, *Tyrobp* and *ApoE* genes, which constitute essential hub genes for microglial functions (Loving and Bruce, 2020). Further experiments of phagocytosis using myelin debris or apoptotic brain cells for instance, and an in-depth analysis of the inflammatory response of the mutant cells are needed to confirm this hypothesis.

A striking observation of the analysis of DEGs in our study was the virtual knock out of genes associated with lipid metabolism such as *Scd1*, *Fads2*, *Acer2*, or *Dhcr24*. Both *Scd1* and *Dhcr24* genes impact the polarization of microglial cells and are clearly associated with microglial functions such as phagocytosis and inflammatory control (Bogie et al., 2020; Zu et al., 2020). Reduced expression of *DHCR24* was observed in Alzheimer's disease (Iivonen et al., 2002; Zerenturk et al., 2013). The functional loss of *Scd1* was shown to trigger complex metabolic changes (Flowers and Ntambi, 2008) and has also been associated to inflammation and cellular stress (Liu et al., 2011). Interestingly, decreased SCD1 activity has been shown to increase the accumulation of saturated VLCFA (Raas et al., 2021). Regarding the  $\Delta 6$ -desaturase gene (*Fads2*), its disruption not only impacts PUFA content but also triggers triglyceride and cholesterol accumulation (Hayashi et al., 2021). Membrane lipid modifications and the activation of various signaling pathways related to LXR or PPAR for example, are also likely (Bogie et al., 2013; Zerenturk et al., 2013) but remain to be clarified in our mutant cell lines. Regarding the shutdown of *Acer2*, which is involved in the hydrolysis of long chain and very-long chain ceramides and dihydroceramides to generate

sphingosine, important signaling consequences affecting microglia are expected (Li et al., 2018). Of note, impairment of alkaline ceramidase 3, which is specific for unsaturated long chain ceramides causes early-onset progressive leukoencephalopathy (Pant et al., 2020).

Alterations of lipid metabolism, lysosomal function, phagocytosis and autophagy, appear to form a cluster of microglial abnormalities found in common neurodegenerative diseases. Autophagy is connected to lipid metabolism and its alteration has been shown to impact the role of microglia in neuroinflammatory control (Julg et al., 2021; Xu et al., 2021). Here, we presented several evidences of increased autophagy in the mutant cell lines, mainly in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells, with an increased expression of key autophagic genes, an increased LC3-II/LC3-I ratio together with the observation of autophagic figures by electron microscopy. The full repression of *Lamtor4* also in agreement with an induction of the autophagic process (Schweitzer et al., 2015). Since the phosphorylation of mTOR and ULK1 was not clearly altered, our results suggest an mTOR-independent induction of autophagy. This mechanism remains to be characterized but could be related to the increase lysosomal activity (Zhang X. D. et al., 2013) and/or to the inositol signaling pathway since many related phospholipase genes were strongly deregulated (Sarkar, 2013). The autophagy induction observed mainly in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* genotype seems to be in contradiction with the impaired autophagic flux observed in X-ALD fibroblasts and presented as a neuroprotective mechanism (Launay et al., 2015) but fits with the increased autophagy observed in case of *Acox1* defect (He et al., 2020). Concerning lysosomes, beyond the fact that lysosomal DEGs were sorted first in GO analysis, that cathepsin and V-ATPase genes were almost all upregulated, we confirmed at the protein level the upregulation of several lysosomal markers (ATP6V0D2, CTSS, CTSK, GRN, LGALS3) and their increased secretions (CTSS, GRN, LGALS3). Of note, the activation of various lysosomal enzymatic activities by VLCFA observed in skin fibroblasts, was suggested to participate in the pathogenesis of X-ALD (Dhaunsi et al., 2005). Moreover, the increased expression and secretion of cathepsins, especially CTSS, could represent a key component of the pathogenesis of X-ALD as a major driver of neuroinflammation (Ni et al., 2015; Lowry and Klegeris, 2018; Nakanishi, 2020b). Alteration of the lysosomal acidification has been associated with aging and adult-onset neurodegenerative diseases (Colacurcio and Nixon, 2016). In contrast, overexpression of V-ATPases has been observed in tumors and likely participates in the activation of lysosomal hydrolases, autophagic flux, and various signaling pathways (Pamarthy et al., 2018). Altogether, our transcriptomic study has clearly demonstrated that peroxisomal defects induce autophagic and lysosomal alterations like in common neurodegenerative diseases. Does it mean that peroxisomal functions are affected in most of the neurodegenerative diseases or does it mean that different triggering defects converge toward a common microglial pathologic state? It is too early to conclude even though several peroxisomal markers have been described in Alzheimer's disease or multiple sclerosis (Zarrouk et al., 2020). It is however clear that lysosomes and peroxisomes are tightly connected and that peroxisomal defects impact lysosomal lipid transport and intracellular clearance, and therefore microglial functions (Chu et al., 2015; Schrader et al., 2020).

Beyond the metabolic reprogramming, we identified a transcriptomic signature resembling that of the DAM signature

identified in common neurodegenerative diseases (Keren-Shaul et al., 2017; Butovsky and Weiner, 2018). Despite a few exceptions, most of the “homeostatic genes” of the DAM signature were found repressed in the mutant cells while the “neurodegenerative genes” were found upregulated. Therefore, our findings identify peroxisomal defect as a new triggering event to lose the signature of homeostatic microglia and shift toward the DAM phenotype along with aging and the most common neurodegenerative diseases. A puzzling question concerns *Abcd1* null mice which do not develop brain demyelination contrary to human. To our knowledge, primary microglia was not investigated in these mice with respect to the DAM signature but was shown to overexpress *Trem2* (Gong et al., 2017). Such discrepancy between human and mice could be due to *Abcd2* since human microglia do not express *Abcd2* (Olah et al., 2020) but *Abcd1/Abcd2* double knockout mice do not show demyelination in spite of an earlier and more severe phenotype. Species differences may involve not only microglia but also other cell types and possibly the blood–brain barrier. In any cases, correcting the peroxisomal defect in microglia and restoring homeostatic state represents a promising strategy in peroxisomal leukodystrophies.

In the context of X-ALD diagnosis and follow up, with the expansion of neonatal screening and improved diagnosis, a complementary tool in addition to brain MRI follow-up is needed. Since VLCFA levels are not correlated with neurodegenerative involvements and since brain MRI is not easy to manage, there is an urgent need for prognostic biomarkers to evaluate disease progression and improve the follow-up of patients (Honey et al., 2021). Recent studies identified and evaluated neurofilament light chain as a potential biomarker for cALD (Weinhofer et al., 2021; Wang et al., 2022). In this study, we identified 5 proteins of the DAM signature which are over secreted by the mutant cells that could virtually represent relevant biomarkers of disease progression. Cathepsin B, progranulin, galectin-3, osteactivin and osteopontin are secreted proteins that have been found in biological fluid of patients suffering from neurodegenerative disorders (Kramer et al., 2016; Agah et al., 2018; Moloney et al., 2018; Batzu et al., 2019; Siew et al., 2019; Chai et al., 2021). Here, we confirmed in this pilot study that the plasma level of osteopontin is significantly higher in patients with cALD than in AMN or healthy controls. In spite of this interesting result, a clinical study conducted on a larger cohort and including longitudinal follow-up of young boys is necessary to investigate the use of osteopontin as a biomarker of the cerebral form of X-ALD and to explore whether the 4 other proteins may also be useful. During the preparation of the manuscript, the level of osteactivin (GPNMB) in cerebrospinal fluid was found correlated with MRI disease severity score in cALD patients (Taghizadeh et al., 2022), which reinforces the relevance of our results.

In conclusion, our study demonstrates that BV-2 microglial cells with a peroxisomal defect lose their homeostatic signature and adopt a DAM-like signature accompanied with a large reprogramming in lipid metabolism, signaling, lysosomal and autophagic genes. It would be interesting to confirm such observations in primary microglial cells from peroxisomal knock out mice or human induced pluripotent stem cells into microglia-like cells for instance (Pandya et al., 2017) but functional consequences including secretion of inflammatory cytokines and immune response, phagocytosis, are likely. While these BV-2 murine microglial cell models have limitations relative to primary microglial cells and their relevance to humans, they have

offered the opportunity to explore in detail and robustly the consequences of a peroxisomal defect in a key cell type of the peroxisomal leukodystrophies and to discover potential biomarkers for which further clinical studies may confirm value.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/GSE200022>.

## Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## Author contributions

QR, AT, MT-J, and CG were in charge of cell culture, mRNA, and protein purification. QR, AT, MT-J, and CG performed experiments related to protein expression. VL performed lipid analysis. RK and CK performed RNA-seq and the related bioinformatics and statistical analyses. QR, AT, PA, and SS performed heatmap, gene ontology analyses, V-Plot, and MA-Plot analyses. EB, MB, and YH, performed Filipin labeling and cytometry analyses. QR, AT, MT-J, CG, AB, PA, YH, CT, and MC-M analyzed the data. CT, CG, and SS performed statistical analyses. QR, AT, MT-J, CG, PA, and SS mounted the figures. SS conceived the project, performed analyses, and wrote the manuscript with QR, AT, MT-J, CG, YH, FC, MC-M, PA, DT, AB, and BN. All authors contributed to the article and approved the submitted version.

## Funding

We warmly acknowledge the Fondation Maladies Rares which supported our transcriptomic analysis project (GenOmics: High throughput sequencing and rare diseases, call 2017-20170615). Sequencing was performed by the GenomEast platform, a member of the “France Génomique” consortium (ANR-10-INBS-0009). We would like to acknowledge the SATT Sayens of Dijon, the CHU of Dijon (project CRBSEP-EMATSEP grant), the Canadian New Frontiers Research Funds Exploration (NFRF-E 19-00007), and the regional council of Bourgogne Franche-Comté (Project PERSIL 2019) for their support. We are also grateful to networking support by the COST Action CA 16,112 NutRedOx (Personalized Nutrition in aging society: redox control of major age-related diseases), supported by COST (European Cooperation in Science and Technology). We thank the Centre d’Immunologie de Marseille-Luminy (CIML) flow cytometry facility. This work was supported by institutional grants from INSERM, CNRS and Aix-Marseille University to the CIML and

program grant from the French National Research Agency (ANR-17-CE15-0032). The project leading to this publication has received funding from Excellence Initiative of Aix-Marseille University—A\*MIDEX, a French “Investissements d’Avenir” program. The laboratory BioPeroXIL was funded by the Ministère de l’Éducation Nationale et de l’Enseignement Supérieur et de la Recherche (France) and by the University of Bourgogne. MT-J was funded by CNRST (PhD excellence grant number: 17UHP2019, Morocco) and by the Action Intégrée of the Comité Mixte Inter-universitaire Franco-Marocain (n° TBK 19/92 n° Campus France: 41501R) from the PHC Toubkal program, Ministère des Affaires Étrangères.

## Acknowledgments

We are grateful to N. Bancod for her helpful contribution in figure conception, as well as C. Le Manach, and G. Gomes-Martins for their technical support. We also acknowledge Isabelle Hamer and Michel Jadot (URPhyM, University of Namur, Belgium) for the training period concerning MT-J and the preliminary work on lysosomes.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2023.1170313/full#supplementary-material>

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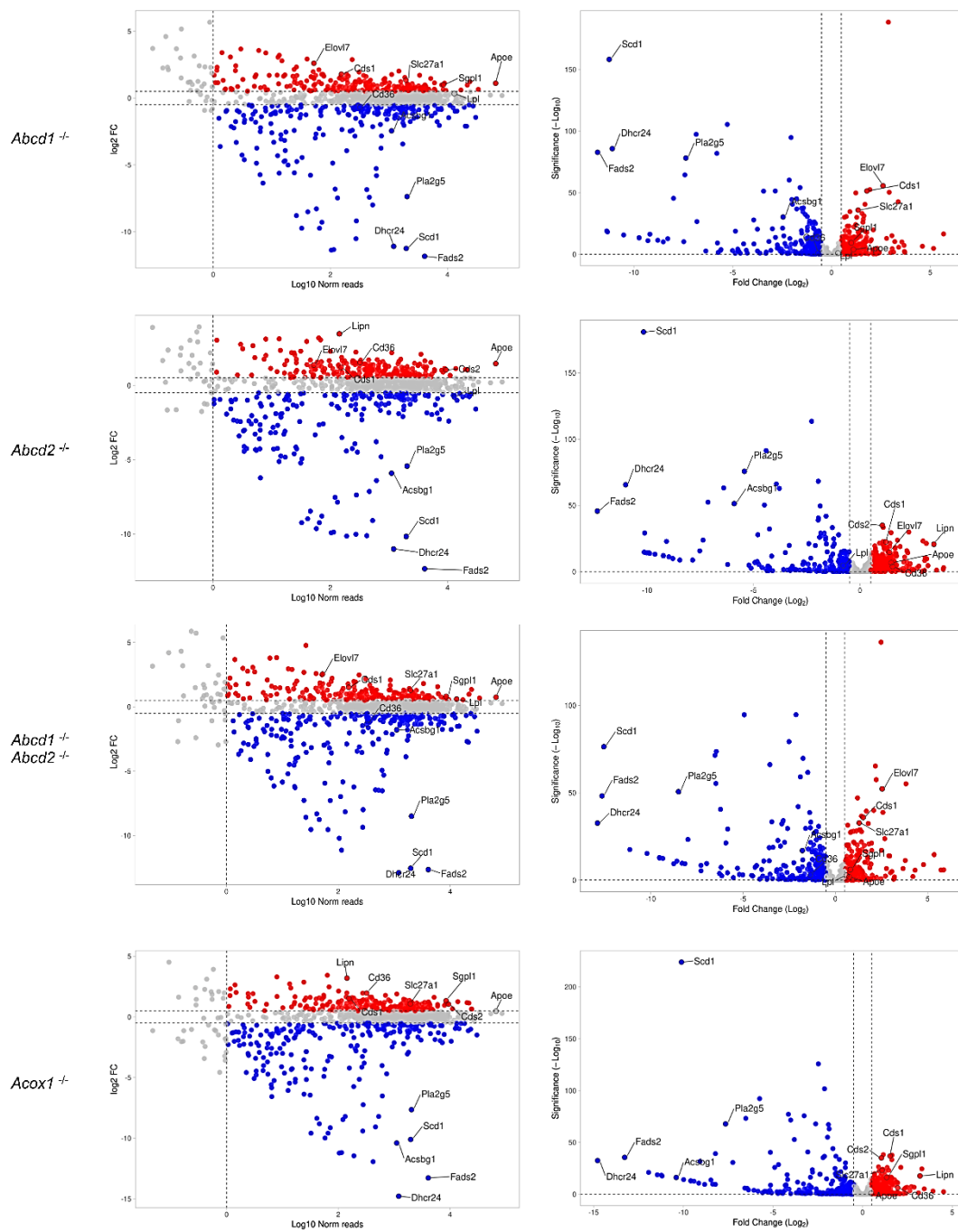
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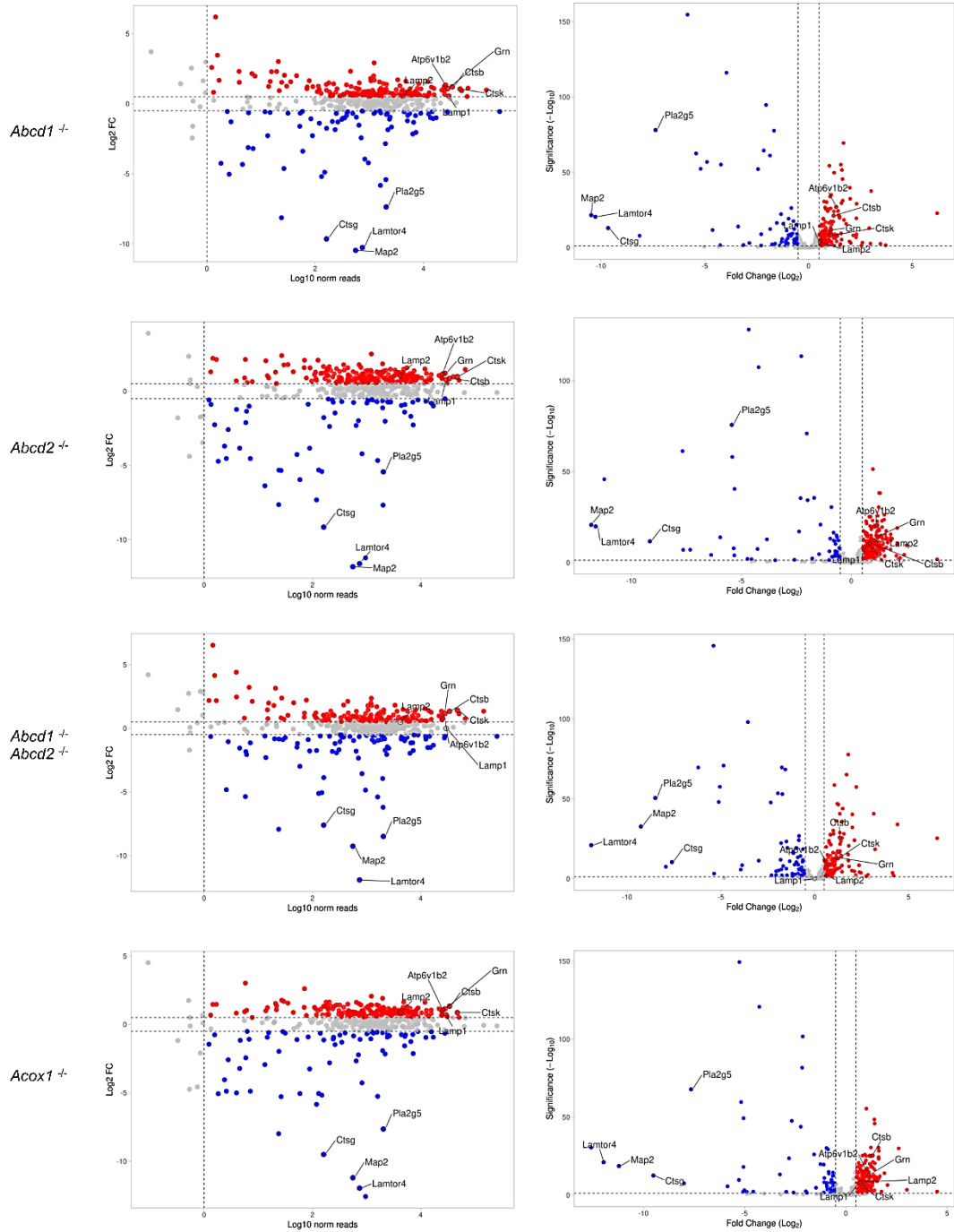


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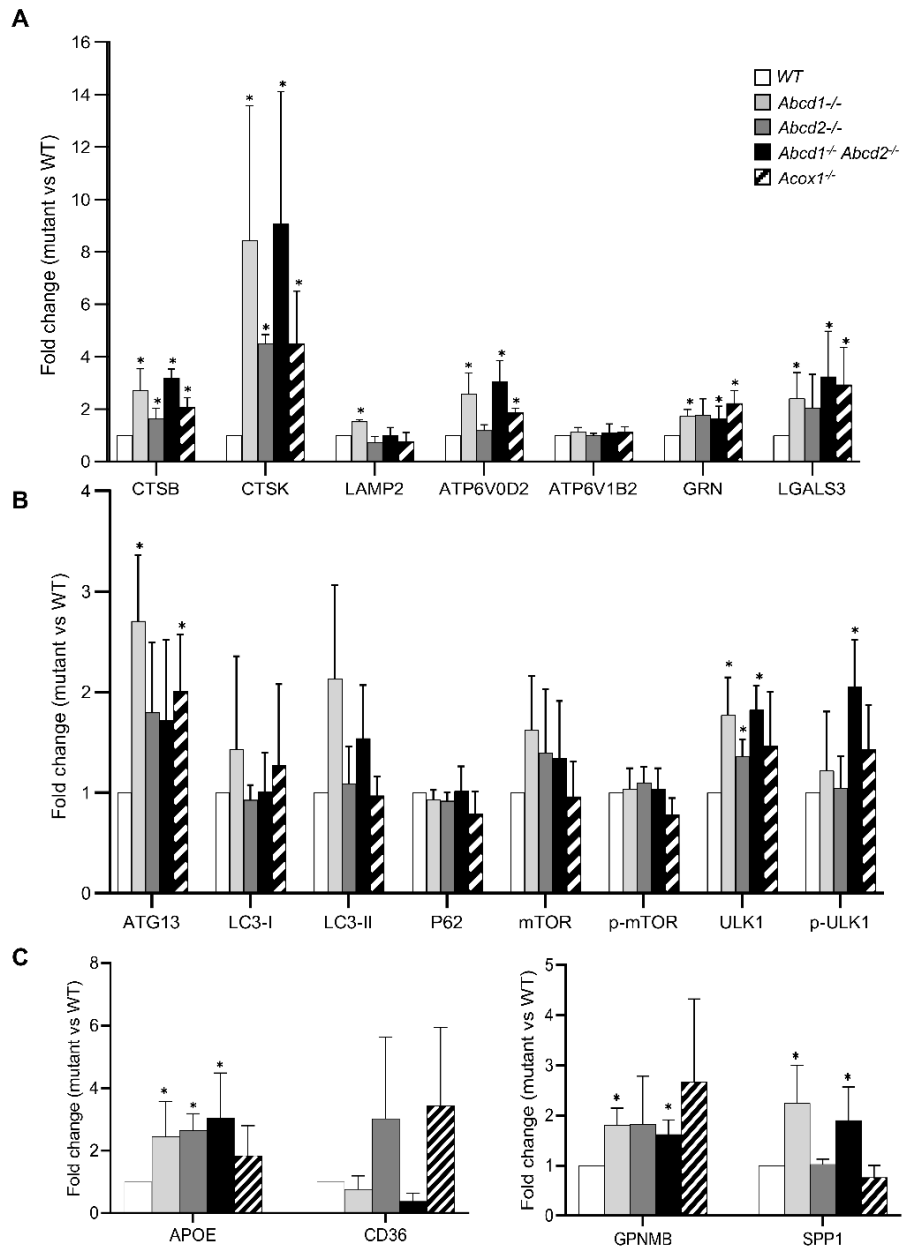
## Supplementary figures



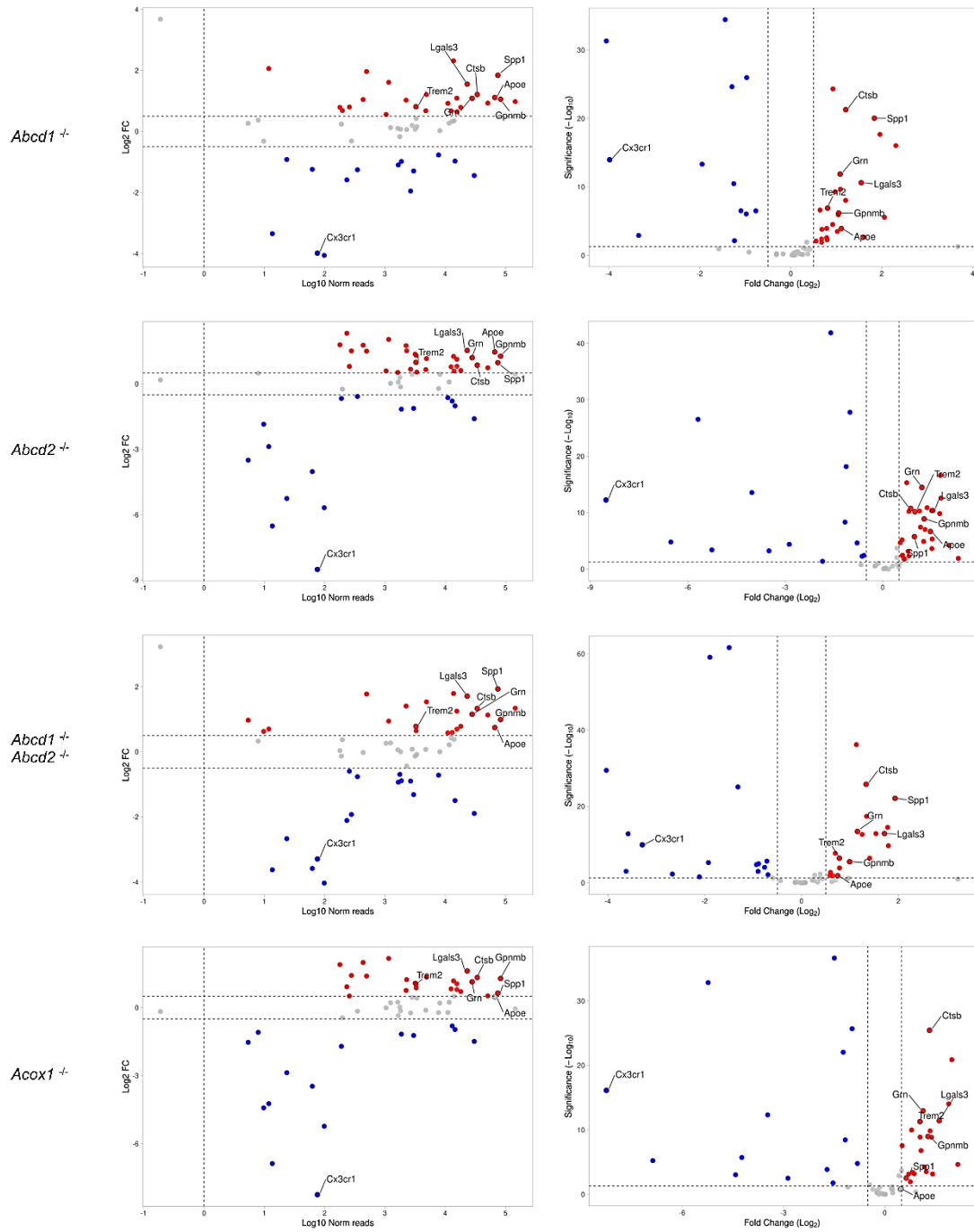
**Supplementary Figure 1.** MA plot and Volcano plot of DEGs associated with lipid metabolism, related to **Figure 2**.



**Supplementary Figure 2.** MA plot and Volcano plot of DEGs associated with lysosome and autophagy, related to **Figure 4**.



**Supplementary Figure 3.** Densitometric analysis of western blotting experiments, related to **Figure 5A**.



**Supplementary Figure 4.** MA plot and Volcano plot of DEGs associated with the DAM signature Figure 6A, 6B, 6C.

## **Key Findings of Article 1 and Future Directions**

This paper investigates the impact of peroxisomal defects in BV-2 microglial cells. Our findings reveal that these mutations not only affect VLCFA metabolism but also prompt microglial cells to adopt a pathological phenotype, which likely contributes significantly to the pathogenesis of peroxisomal disorders. RNA-sequencing analysis uncovers extensive reprogramming of genes involved in lipid metabolism, immune response, cell signaling, lysosome, autophagy pathways, and other indicatives of a DAM-like signature. Indeed, these cells exhibit a DAM signature, characterized by the downregulation of homeostatic genes and the upregulation of genes associated with neurodegenerative diseases. The observed changes in gene expression were further confirmed at the protein level for selected genes, which consistently supported our observations.

The huge modification of the expression of genes involved in lipid metabolism can impact several aspects of cellular functions through modifying fatty acid metabolism, membrane biophysical properties, and membrane signaling evidenced by deregulation of several phospholipases. Such modifications of lipid genes expression also suggest alterations in the control of inflammation by lipid molecules such as the docosanoids and eicosanoids which are known to have anti-inflammatory and pro-inflammatory roles respectively.

The differential gene expression in the mutant cells also revealed a huge impact on lysosomal functions and autophagy, making it possible to question the convergence of the consequences of mutations of both peroxisomal and lysosomal genes toward lysosome defects. In fact, lysosomal activity seems to be fundamental in the function of microglia (Dou et al., 2012; Kreher et al., 2021), and similar to peroxisomal diseases, lysosomal diseases are known to cause demyelination and neurodegeneration (Gabandé-Rodríguez et al., 2019). Indeed, dysfunctional microglia seems to exist in common lysosomal disorders, such as Niemann-Pick disease (Cognoux et al., 2018). Would it therefore be possible to say that lysosomal dysfunctions in microglia lead to demyelinating disorders? Such links between lysosomal dysfunctions in microglia and neurodegeneration were discussed in a recent review (Quick et al., 2023). Additionally, the modifications in the expression of genes controlling autophagy question whether the deregulation of this essential cellular process has a protective effect or further amplifies the phenotype observed in the mutant cells.

Such metabolic reprogramming is known to affect microglial functions related to inflammatory signaling, oxidative stress, phagocytosis, and brain homeostasis (Chausse et al., 2021; Loving & Bruce, 2020; Nadjar, 2018; Navia-Pelaez et al., 2021).

The upregulated expression of DAM genes, which was confirmed through increased protein levels and secretions, is a breakthrough in this field. The peroxisomal defects in microglial are shown, for the first time, to trigger DAM signature. The increased secretion of DAM proteins could be used to identify novel biomarkers for X-ALD.

## **Transcriptomic analysis and functional characterization of LPS-stimulated BV-2 microglial cells with a peroxisomal defect**

Based on these findings, our next article focuses on investigating the modification of the immune response in mutant BV-2 cells. This article is entitled “Immune response of BV-2 microglial cells is impacted by peroxisomal beta-oxidation” and is published in *Frontiers in Molecular Neuroscience* journal.

As we aim to study the immune response in the mutant versus the WT cells, we treated the BV-2 cells with LPS, which is widely used as a potent activator of the immune response triggering a robust inflammatory reaction. It provides a standardized and controlled method to induce inflammation and study microglial activation. We particularly examined cytokine secretion through multiplex analysis, and antigen presentation, with the collaboration of the laboratory of Dr. Yannick Hamon in Marseille. Additionally, we observed an excess of free cholesterol in the mutant cells' plasma membrane, impacting lipid membrane organization, as well as differential expression of lysosomal genes, indicating altered lysosomal functions. These modifications could potentially lead to significant changes in phagocytosis mechanisms. To shed light on these processes, we collaborated with the Department of Pathobiology of the Nervous System at the Center for Brain Research, Medical University of Vienna, working closely with Dr. Johannes Berger and Dr. Isabelle Weinhofer.

This paper is attached below.



**Article 2: Immune response of BV-2 microglial cells is impacted by peroxisomal beta-oxidation** DOI: <https://doi.org/10.3389/fnmol.2023.1299314>

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**Footnotes**

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**Keywords:** Adrenoleukodystrophy, Immune response, Inflammation, Microglia, Peroxisome

**Abstract**

Microglia are crucial for brain homeostasis and dysfunction of these cells is a key driver in most neurodegenerative diseases, including peroxisomal leukodystrophies. In X-linked adrenoleukodystrophy (X-ALD), a neuroinflammatory disorder, very long-chain fatty acid (VLCFA) accumulation due to impaired degradation within peroxisomes result in microglial

defects, but the underlying mechanisms remain unclear. Using CRISPR/Cas9 gene editing of key genes in peroxisomal VLCFA breakdown (*Abcd1*, *Abcd2* and *Acox1*), we recently established easy accessible microglial BV-2 cell models to study the impact of dysfunctional peroxisomal  $\beta$ -oxidation and revealed a disease-associated microglial signature in these cell lines. Transcriptomic analysis suggested consequences on immune response. To clarify how impaired lipid degradation impacts the immune function of microglia, we here used RNA-sequencing and functional assays related to immune response to compare wild-type and mutant BV-2 cell lines under basal conditions and upon pro-inflammatory lipopolysaccharide (LPS) activation. A majority of genes encoding proinflammatory cytokines, as well as genes involved in phagocytosis, antigen presentation and co-stimulation of T lymphocytes were found differentially overexpressed. The transcriptomic alterations were reflected by altered phagocytic capacity, inflammasome activation, increased release of inflammatory cytokines including TNF and upregulated response of T lymphocytes primed by mutant BV-2 cells presenting peptides. Together, the present study shows that peroxisomal  $\beta$ -oxidation defects resulting in lipid alterations including VLCFA accumulation directly reprogram main cellular functions of microglia. The elucidation of this link between lipid metabolism and immune response of microglia will help to better understand the pathogenesis of peroxisomal leukodystrophies.

## INTRODUCTION

Recent neuroscience research has highlighted the crucial role of microglia, the resident immune cells of the central nervous system, in regulating neural activity, synaptic plasticity, and neuroinflammation (Wright-Jin and Gutmann, 2019). Microglia play important roles in brain development and function, and alteration of its homeostatic state has been linked to various neurological disorders (Sirkis et al., 2021; Paolicelli et al., 2022). Microglia respond to injury and infection by releasing inflammatory mediators, phagocytosing myelin debris and dying cells, while promoting myelin repair and regeneration. Despite extensive research into their immune functions and signaling pathways, little attention has been paid to microglia's organelle-related functions.

We recently engineered novel easily accessible murine BV-2 models to study the impact of peroxisomal defects in microglial cells (Raas et al., 2019a; Raas et al., 2019b). The final aim was to gain insight into the pathogenesis of peroxisomal leukodystrophies such as X-linked adrenoleukodystrophy (X-ALD, MIM 300100) and acyl-CoA oxidase 1 (ACOX1) deficiency (MIM 264470). X-ALD, the most frequent peroxisomal disease, is a complex neurodegenerative disorder with two major clinical forms: the childhood cerebral form (cALD) showing rapid *progressive inflammatory* cerebral demyelination and the adult form called adrenomyeloneuropathy (AMN) showing a slowly progressive and non-inflammatory spinal cord disease. Independent of the variability of clinical symptoms, X-ALD is associated with mutations in the *ABCD1* gene, which encodes a transporter of very long-chain fatty acids (VLCFAs) localized at the peroxisomal membrane (Mosser et al., 1993). The disease is characterized by an impaired peroxisomal  $\beta$ -oxidation and VLCFA accumulation (Trompier and Savary, 2013; Kemp et al., 2016). In agreement with the functional redundancy between *Abcd1* and *Abcd2*, its closest homologue (Mosser et al., 1993; Lombard-Platet et al., 1996; Genin et al., 2011; Tawbeh et al., 2021), VLCFA accumulation was observed exclusively in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* double knockout mutant BV-2 cells, and not in the single knockouts of either *Abcd1* or *Abcd2*. The ACOX1 enzyme controls the first step of peroxisomal  $\beta$ -oxidation and its defect is associated with a very rare and severe leukodystrophy resulting also in the accumulation of VLCFA in plasma and tissues (Fournier et al., 1994; Nohammer et al., 2000; Ferdinandusse et al., 2007; Vamecq et al., 2018). Accordingly, *Acox1<sup>-/-</sup>* BV-2 cells also demonstrate VLCFA accumulation, although less marked than in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells.

Despite some limitations including a lower reactivity than primary cells upon LPS treatment (Das et al., 2016), the BV-2 cell line displays many microglial features such as phagocytosis and the ability to respond to inflammatory stimulation through the secretion of nitric oxide (NO) and pro-inflammatory cytokines (Henn et al., 2009; Das et al., 2015). Thus, these cells serve as a useful tool and continue to be the most used cell line for studying microglial behavior and inflammatory response *in vitro*. Using the BV-2 cell models, we could demonstrate that a defect in peroxisomal fatty acid  $\beta$ -oxidation results in reprogramming of gene expression linked to lipid metabolism, lysosome, autophagy and in the emergence of a disease-associated microglial (DAM) signature (Raas et al., 2023). Interestingly, some of the main hub genes of microglia (*Apoe*, *Tyrobp*, *Trem2*) were found to be significantly up-regulated in the knockout genotypes. TREM2, with its downstream adaptor TYROBP/DAP12, constitutes a receptor for diverse ligands, including LPS, that plays a key role in microglial differentiation, phagocytosis, and inflammatory responses (Jay et al., 2017; Krasemann et al., 2017; Gratuze et al., 2018; Ulland and Colonna, 2018; Haure-Mirande et al., 2022). Moreover, the gene ontology term “Innate immune response” was the most enriched term among the differentially expressed genes (DEGs) found in this study.

Recent research has highlighted the physiological role of peroxisomes and their importance in the regulation of immune and inflammatory responses (Di Cara et al., 2019; Di Cara, 2020; Di Cara et al., 2023; Wanders et al., 2023). However, there is currently a lack of studies investigating the relationship between peroxisomes and microglia. Peroxisome proliferator-activated receptor delta (PPAR $\delta$ )-deficient microglia were demonstrated to overexpress genes associated with phagocytosis and inflammation (Doroshenko et al., 2021). MFP2 deficiency, leading also to a defect of peroxisomal  $\beta$ -oxidation, was shown to provoke a microgliosis and a chronic inflammatory profile (Beckers et al., 2018; Beckers et al., 2019). In X-ALD, defective microglia have been supposed to prime and amplify the neurodegenerative process (Gong et al., 2017; Bergner et al., 2019). Blood-brain barrier rupture allowing the infiltration, recruitment and activation of peripheral immune cells such as macrophages and T lymphocytes would contribute to neuroinflammation and demyelination (Griffin et al., 1985; Ito et al., 2001; Zierfuss et al., 2022). Allogeneic hematopoietic stem cell transplantation and more recently, cell-based gene therapy have been shown to halt disease progression (Cartier et al., 2009; Eichler et al., 2017). The exact mechanism by which this therapeutic strategy proves to be efficient in brain is unclear but the functional replacement of microglia by monocyte/macrophage cells derived from hematopoietic stem cells is suspected (Weinhofer et al., 2018). X-ALD macrophages were described as pro-inflammatory skewed and unable to complete anti-inflammatory response and peroxisomal  $\beta$ -oxidation of VLCFAs appears central in the resolution of inflammation (Weinhofer et al., 2018; Zierfuss et al., 2022). Immune system alterations including macrophage and T cell activation as well as cytokine production may explain some of the variability observed in clinical picture. In addition to gene mutations, environmental factors, including infections and other inflammatory stimuli, may also influence disease manifestation (Wiesinger et al., 2015). The clinical variability observed in X-ALD, even in identical twins (Korenke et al., 1996), indeed suggests that other factors beyond genetic mutations contribute to the pathogenesis.

To investigate in more details the functional consequences of the peroxisomal defects in BV-2 microglial cells related to immune response, we performed RNA-sequencing in WT and mutant cell lines and compared the response to a 24-hour treatment with LPS. We investigated the inflammatory response of the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> BV-2 microglial cells by studying the expression and the secretion of key inflammatory cytokines and chemokines. We explored the consequences of the peroxisomal  $\beta$ -oxidation defects on the phagocytic capacity of microglial cells. Finally, using calcium imaging, we studied the effect of peroxisomal dysfunction on microglial antigen presentation, a crucial role in priming T lymphocyte response.

## **MATERIALS AND METHODS**

### **Resource Availability**

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE200022 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE200022>) for the genome comparison and GSE237635 for the LPS effect. The data supporting the findings and the excel files containing the gene lists used to create the figures are available from the corresponding author (SS) upon request.

### **Cell culture**

Mouse microglial BV-2 cell line was purchased from Banca-Biologica e Cell Factory (catalog no. ATL03001). Single or double mutant BV-2 cells, deficient for the peroxisomal

proteins ACOX1 (*Acox1*<sup>-/-</sup>) or ABCD1 and ABCD2 (*Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>) were obtained by CRISPR/Cas9 editing (Raas et al., 2019a; Raas et al., 2019b). Sanger sequencing confirmed the absence of CRISPR/Cas9-induced mutations in the two genomic sites with the highest predicted likelihood of off-target binding strongly, suggesting the absence of off-target events. WT and mutant BV-2 cells were grown in DMEM supplemented with 10% heat-inactivated FBS (Corning), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. WT and mutant BV-2 cells were seeded in 6-well plates at a density of 2×10<sup>5</sup> cells/well. The day after, cells were treated for 24 h with 1 µg/mL LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich). A vehicle containing 0.01% ethanol and 0.5 mM α-cyclodextrin was applied in each condition.

T lymphocytes were obtained from transgenic mice and maintained in DMEM F12 medium (Lonza). Animal experiments respected French and European directives. Animals were housed in cages with water and food ad libitum in the CIML animal house facility, Marseille. OT-1 mice specific for H-2Kb/ovalbumin (SIINFEKL) were kept on a *Rag-1*<sup>-/-</sup> C57BL/6 background. Splens and lymph nodes were recovered from heterozygous *Rag1*<sup>+/-</sup> OT-1 <sup>+/-</sup> transgenic mice originating from crossing of OT-1 mice with C57/Bl6 mice (Charles River). Organ dilacerations were done onto nylon membrane in DMEM F12 medium (Lonza), splenic erythrocytes were removed in Geyes lysis solution. CD8<sup>+</sup> T cells were isolated by depleting the CD8 negative cells according to manufacturer instructions (DynaMouse CD8 Negative Isolation Kit, Invitrogen).

### **RNA-sequencing, Differential gene expression analysis and bioinformatic**

Total RNA was extracted from 3 independent batches of BV-2 cells for each genotype (WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup>) using RNeasy kit (Qiagen). RNA-seq libraries preparation, sequencing and analysis were performed as previously described (Raas et al., 2023). Differential gene expression analyses were performed using R 3.3.2 and DESeq2 version 1.16.1 Bioconductor library (Love et al., 2014). To analyze the effect of the treatment, we define a model with one factor (the corresponding condition). To test if the treatment effect differs between two genotypes, we define a model with two factors (genotype and treatment) and their interaction. Wald test *p*-values were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). DEGs were selected using the following thresholds: adjusted *p*-value lower than 0.05 and absolute log<sub>2</sub> Fold Change (FC) higher than 1. Venn diagrams were obtained using Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>). Gene ontology analysis and gene set enrichment analysis were performed using ShinyGO online tools (<http://bioinformatics.sdstate.edu/go/>) (Ge et al., 2020). Expression-based heatmaps and hierarchical clustering were performed with Heatmapper (<http://www.heatmapper.ca/>) (Babicki et al., 2016) using average linking and Pearson distances.

### **Analysis of protein expression by western blotting**

Cell lysate proteins (30 µg) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were first blocked in 5% fat-free milk in PBST (Phosphate buffer saline (PBS), 0.1% Tween 20) and then probed with primary antibodies in 1% fat-free milk in PBST. The following antibodies were used with the indicated dilution: anti-CASP1 (1:1,000, Adipogen AG-20B-0042), anti-CD36 (1:1,000, R&D Systems AF2519), anti-FCGR2B (1:1,000, Cell Signaling 96397), anti-IL1B (1:1,000, GeneTex GTX 74034), anti-MRC1 (1:1,000, Abcam ab64693), anti-NLRP3 (1:3,000, Adipogene AG-20B-0014), anti-TLR2 (1:500, R&D Systems AF1530), anti-TLR4 (1:1,000, Santa Cruz sc-293072). Membranes were washed in PBST and incubated with the appropriate HRP-conjugated secondary antibodies

(1:5,000) in 1% fat-free milk in PBST. After 5 washes, immunoreactivity was revealed by incubating membranes with the HRP SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) and the signal was detected by the Chemidoc XRS system (Bio-Rad). Proteins amount loading control and normalization was achieved by probing the membranes with  $\alpha$ -tubulin (TUBA1A) (1:4,000, Sigma-Merck T5168) or  $\beta$ -actin (ACTB) (1:10,000, Sigma-Merck A5441) antibodies.

### **Analysis of protein expression by cytometry**

BV2 cells staining was performed in 1X PBX with 1% BSA after blocking of the Fc receptors with 2.4G2 (BD Bioscience). Expression of H2-K1 (H2Kb, MHC I), H2-AB1 (Iab, MHCII), CD80, CD86, CD54 (ICAM) was assessed using the following antibodies, respectively: anti-Ia/Ie AF647, clone M5/114.15.2 (BD Bioscience), anti-H2Kb AF647, clone AF6-88.5 (Biolegend), anti-CD80-PE, clone 13-10A1 (eBioscience), anti-CD86-PE, clone g11 (eBioscience), anti-CD54-APC, clone YN1/1.7.4 (Biolegend). Surface marker analysis was performed using an LSR II flow cytometer (Becton Dickinson) using the FACSDiva software. Data analysis was performed with FlowJo (Tree star).

### **Multiplex analysis of cytokines**

The WT and mutant BV-2 cells were seeded in two 6-well plates in 3 mL DMEM supplemented with 10% FBS and 1% penicillin-streptomycin antibiotics. Twenty-four hours later, the cells of one plate were treated with 1  $\mu$ g/mL LPS whereas the other was kept at basal conditions. After 48 h in culture, the supernatants of the BV-2 cells were collected and centrifuged at 300 g for 7 min then stocked at -80°C until used. At the day of the experiment, the cell culture supernatants were subjected to multiplex analysis using Milliplex Map kit #MCYTOMAG-70K according to manufacturers' instructions. Samples were tested under 2 different conditions (undiluted or diluted 50-fold) in order to meet the concentration range of the standard curve for all the cytokines being tested. For each of the dilution conditions, three biological replicates for each sample were tested. The immunoassay was performed using Bio-Plex 200 (Bio-Rad) equipped with Bio-Plex Manager for acquisition and data analysis.

### **Myelin isolation and labelling**

Myelin was isolated from brain tissue of 6-8 week-old male wild type C57BL/J6 mice by ultracentrifugation according to the protocols of Norton and Poduslo (1973) and Larocca and Norton (2007). The isolated myelin was recovered in PBS and tested for putative LPS contamination using an endotoxin detection kit (Lonza). The isolated myelin was fluorescently labelled by incubating 1 mg myelin in PBS with 10  $\mu$ l pHrodo<sup>®</sup>-Green succinimidyl ester in DMSO (1 mg/mL, Life Technologies) for 45 min at room temperature in dark. Excess dye was removed by a short centrifugation and the labelled myelin was resuspended in PBS (PH 7.4) and stored in aliquots at -80°C.

### **Phagocytosis analysis of pHrodo<sup>™</sup> Green-labelled myelin and *E. coli* BioParticles**

The phagocytic ability of BV-2 WT and KO cells was assessed using pHrodo<sup>™</sup> Green-labelled myelin and *E. coli* BioParticles<sup>™</sup> Conjugate (ThermoFisher, P35366). Cells were seeded in 96-well plates, allowed to settle before being treated or not with LPS 1  $\mu$ g/mL for 24 h. Cells were then incubated with 10  $\mu$ g/mL labelled myelin or 25  $\mu$ g/mL with fluorescent bioparticles. Afterwards, the plates were transferred to IncuCyte SX5 live-cell analysis system (Sartorius), kept in cell culture conditions (37°C and 5% CO<sub>2</sub>) and the fluorescent activity was recorded every 30 min for 16 h. Five replicates were included for each condition and three

repetitions for each experiment were carried out. Data were analyzed and extracted using the InCuCyte software.

### **Phagocytosis analysis of Fluoresbrite® Yellow Green (YG) carboxylate beads**

BV-2 cells (100,000 cells/well) were seeded in 12-well plates overnight for 24 hours. Fluoresbrite® Yellow Green (YG) carboxylate microspheres (1  $\mu\text{m}$ ) (Polysciences) were washed and added to BV-2 cells at a cell/beads ratio of 1: 200 at 37°C for 1 hour. Phagocytosis was stopped by washing the cells with ice-cold PBS. Phagocytosis was immediately quantified on a LSRII flow cytometer (BD Biosciences), with addition of Trypan blue 0.04% in PBS 1x to quench extracellular fluorescence from attached but not internalized YG beads. For negative controls, equivalent cell samples were pre-treated with the phagocytosis inhibitor cytochalasin-D (37°C, 4  $\mu\text{M}$ , Biotrend) then kept on ice and maintained in 4  $\mu\text{M}$  cytochalasin-D during cell exposure to YG beads. Data are expressed as the percentage of YG beads-positive cells (i.e., cells that have ingested at least 2 beads) and as phagocytic index (PI) calculated using the following formula:  $\text{PI} = (\% \text{ YG beads-positive cells}) \times (\text{Mean Fluorescence Intensity of YG beads-positive cells})$ . Data were analyzed using FlowJo X 10.0.7 software.

### **Barcoding T cell calcium response**

Loading protocol:  $10^6$  primary OT1 CD8+ T cells per well were seeded in 96 plates in 100  $\mu\text{L}$  of DMEM F12 (Gibco), Nutridoma-SP 1% (Roche diagnostic). Cells were loaded with BD PBX diluted in 1X dye loading solution (according to manufacturer instructions) at 1/1666 for primary T cells i.e.  $\sim 0.6 \mu\text{M}$ . 100  $\mu\text{L}$  of this solution were dispatched to each well before incubation at 37°C during 30 min in the dark. Cells were then washed twice in Hank's balanced salt solution (HBSS) Hepes 0.01M and resuspended in the same medium. Five wells were pooled ( $5 \cdot 10^6$  cells) and seeded onto BV2 cells ON treated with LPS (LPS from E.coli 055.B5, Sigma, 1 $\mu\text{g}/\text{mL}$ ) then loaded for 1h with various concentration of the OVA SIINFELKL peptide (Eurogentec) and analyzed by live imaging microscopy.

Acquisition procedure: Movies were realized on a Zeiss LSM 780 confocal microscope. Pictures were taken with a C-Apochromat 40X/1.4 oil immersion objective, using an argon laser at 488 nm dichroic and a 505 nm long pass filter at 37°C using a hot plate. During acquisition, T cells were added to the medium containing BV-2 as antigen presenting cells. Time lapse movies are composed of 600 images taken every 6 seconds.

Analytical parameters: The cell tracking and the calcium signal analysis was achieved by the MAAACS software as described in (Salles et al., 2013). All parameters obtained in calcium experiment analysis (fluorescence amplitude, percentage of responding cells) were adapted for naïve CD8+ T cells by taking into consideration a 1.7 threshold level upon optimizing the product of their probability of detection times their probability of false alarm ( $\text{PD} \times (1-\text{PFA})$ ) as mentioned in (Salles et al., 2013). Analysis of calcium signals are represented in dot plot only for cells that respond specifically (above threshold). Each point corresponds to the value of a parameter defined above.

Statistical analysis: All statistical analyses were performed using GraphPad Prism 9.4.

### **Statistical analyses**

The different statistical tests which were applied are indicated in the figure legends.

## RESULTS

### Global analysis of the LPS response

One notable finding from the transcriptomic analysis of the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> mutant BV-2 cell lines was that the term "Innate immune response" consistently ranked as the top hit across all mutant genotypes (Raas et al., 2023). Genes encoding cytokines and chemokines, defense receptors such as Toll-like receptors (TLRs), proteins involved in immune signaling, response to viruses or bacteria, and inflammasome figured among the DEGs. We can cite *Ighm*, *C1rb*, *Nlrp1*, *Grp*, *Grp2*, *Il23*, *Cd14*, and *Nlrp3* among the most down-regulated genes, and *Rsad2*, *Clec4n*, *Nlrc3*, *Ifit3*, and *Tlr1* among the most up-regulated DEGs (Raas et al., 2023). These changes in gene expression may suggest that the ability of microglial cells to respond to immune stimuli has been altered, rather than a commitment to a specific inflammatory state although genes encoding pro-inflammatory cytokines (*Il6*, *Tnf*) and the main actor of inflammasome (*Nlrp3*) were mostly repressed. Paradoxically, genes encoding pro-inflammatory chemokines (*Ccl3* and *Ccl4*) were induced in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and the gene encoding interleukin 1 beta (*Il1b*) was induced in *Acox1*<sup>-/-</sup>. We thus compared the transcriptional response of WT and mutant BV-2 cells treated for 24 h with LPS (1 µg/mL). We found 891 genes significantly and differentially expressed in the WT cells while 1723 and 2096 DEGs were revealed in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> respectively, indicating a stronger response to pro-inflammatory stimulation in peroxisomal mutants than in control cells (Figure 1A). It is noteworthy that when conducting statistical analysis to compare the LPS response across different genotypes, it was found that a higher percentage of repressed DEGs, specifically 9.5% in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and 14.7% in *Acox1*<sup>-/-</sup>, as well as a higher percentage of up-regulated DEGs, specifically 20.1% in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and 25.4% in *Acox1*<sup>-/-</sup>, exhibited a significant modulation of expression (Figure 1A).

### LPS response and inflammatory cytokines

Enrichment analysis of the up-regulated DEGs revealed quite similar results between genotypes for the first hits in link with immune response and pro-inflammatory response (innate immune response, response to cytokine, defense response to other organisms for GO terms; TNF signaling pathway, NF-κB signaling pathway, cytokine-cytokine receptor interaction, NOD-like receptor signaling pathway for KEGG pathways). Of note, "NF-kappa B signaling" came out in higher position in the KEGG enrichment for mutant genotypes likely illustrating the amplified response of mutant cell lines (Figure 1B). Among the down-regulated DEGs, enrichment results were however divergent between genotypes. Venn diagrams illustrate a substantial proportion of genes responding in a similar way to LPS stimulation but also in a genotype-dependent manner. Intersection analysis illustrates the huge amount of specific DEGs found in one mutant genotype. About one third of these DEGs were exclusive to one single mutant genotype (Figure 1C). Statistical analysis of the LPS response showed that 18% (81/439) and 28% (128/463) of DEGs were significantly more up-regulated in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> than in WT cells, respectively. In the down-regulated genes, 4% (7/172) and 8% (23/285) of the down-regulated DEGs were found to be significantly more repressed, respectively in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> genotypes. Altogether, these data indicate a stronger response both in number of DEGs and in fold change in mutant cells impaired for peroxisomal β-oxidation.

As expected from previous studies in WT BV-2 cells (Lund et al., 2006; Henn et al., 2009; Das et al., 2015; Das et al., 2016), LPS treatment resulted in the up-regulation of pro-inflammatory genes encoding cytokines (*Tnf*, *Il1b*, *Il6*, ...) chemokines (*Ccl3*, *Ccl4*, *Ccl5*, *Cxcl2*, *Cxcl3*, ...), transcription factors (*Nfkb1* and *Nfkb2*), and various markers of microglial activation

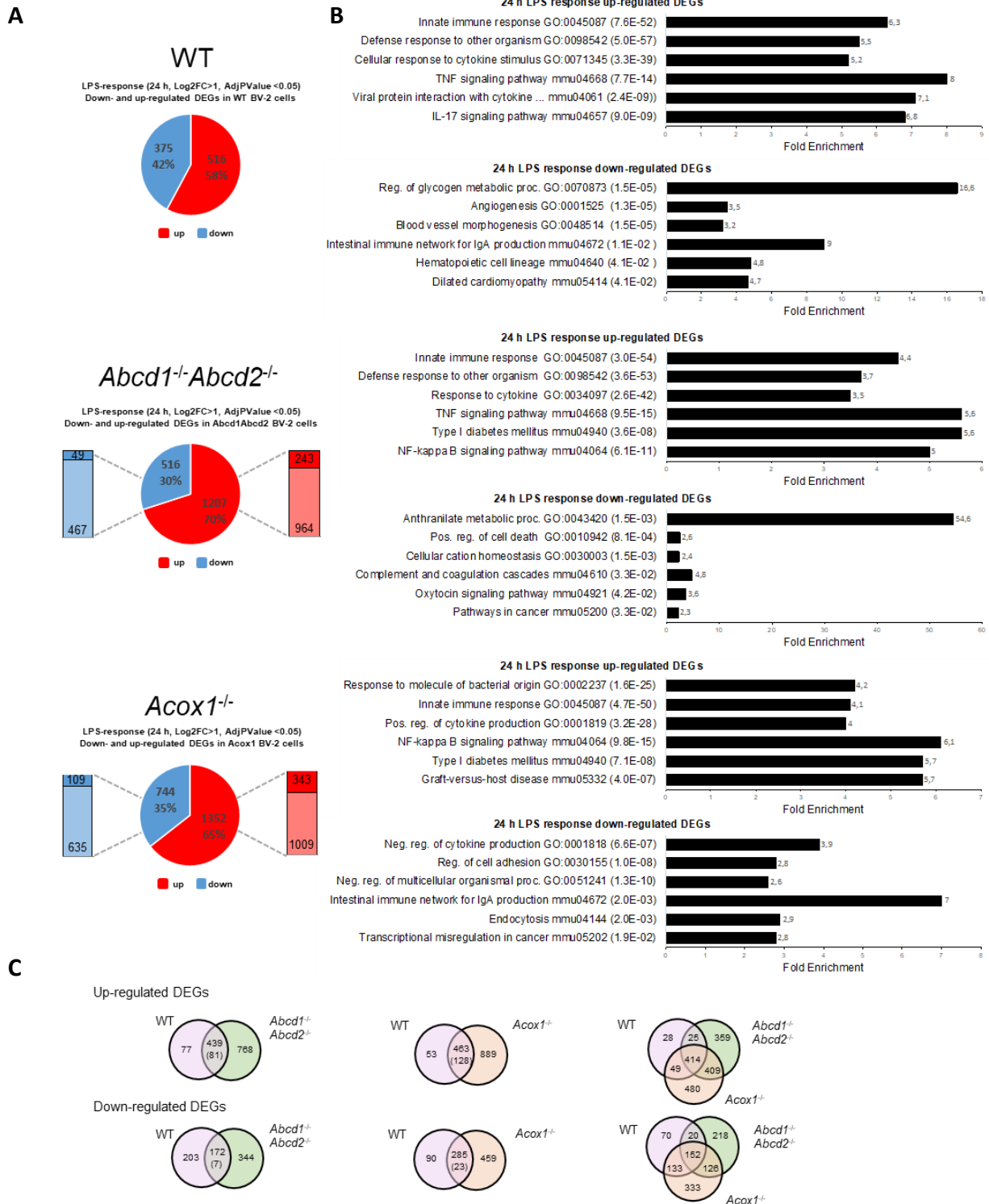


such as *Nos2* along with the down-regulation of *Arg1*. All of these genes figured in the intersection list of the 3 genotypes illustrating similar LPS response in regard to these genes between WT and mutant genotypes (Figure 1C).

The secretion of cytokines and chemokines is considered as one of the main immunomodulatory functions of microglial cells directly affecting neurons. In basal conditions, we previously noticed that the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* mutants present a different expression pattern at the mRNA level for several cytokine encoding genes (Raas et al., 2019b; Raas et al., 2023). Regarding the apparent amplified LPS response of mutant genotypes at the mRNA level, it was therefore important to pay attention to this group of genes. RNA-seq analysis confirmed that cytokine and chemokine encoding genes figure among the DEGs both in the absence and presence of LPS. In basal condition, *Ccl3* and *Ccl4* genes were specifically up-regulated in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells and *Il1b* was found up-regulated in the *Acox1<sup>-/-</sup>* cells. *Tnf* and *Il6* genes were found to be down-regulated in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* cells (Figure 2A). The LPS treatment mainly resulted in the up regulation of pro-inflammatory genes, mutant genotypes displaying a more important number of induced genes (Figure 2B). The Venn diagram illustrating the LPS response indicates that the largest group of genes is the intersection of the 3 genotypes, which includes *Ccl3*, *Ccl4*, *Il1b*, *Il6* and *Tnf* (Figure 2C and D). The *Ccl2* gene was up-regulated in the WT and *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* genotypes only. The up regulation of these genes was found significantly increased in the mutants as compared to the WT cells. Of note, both the inflammasome *Nlrp3* gene and the non-canonical inflammasome *Casp4* gene (Casson et al., 2015) were overexpressed upon LPS treatment, their induction being significantly higher in the mutants than in the WT BV-2 cells.

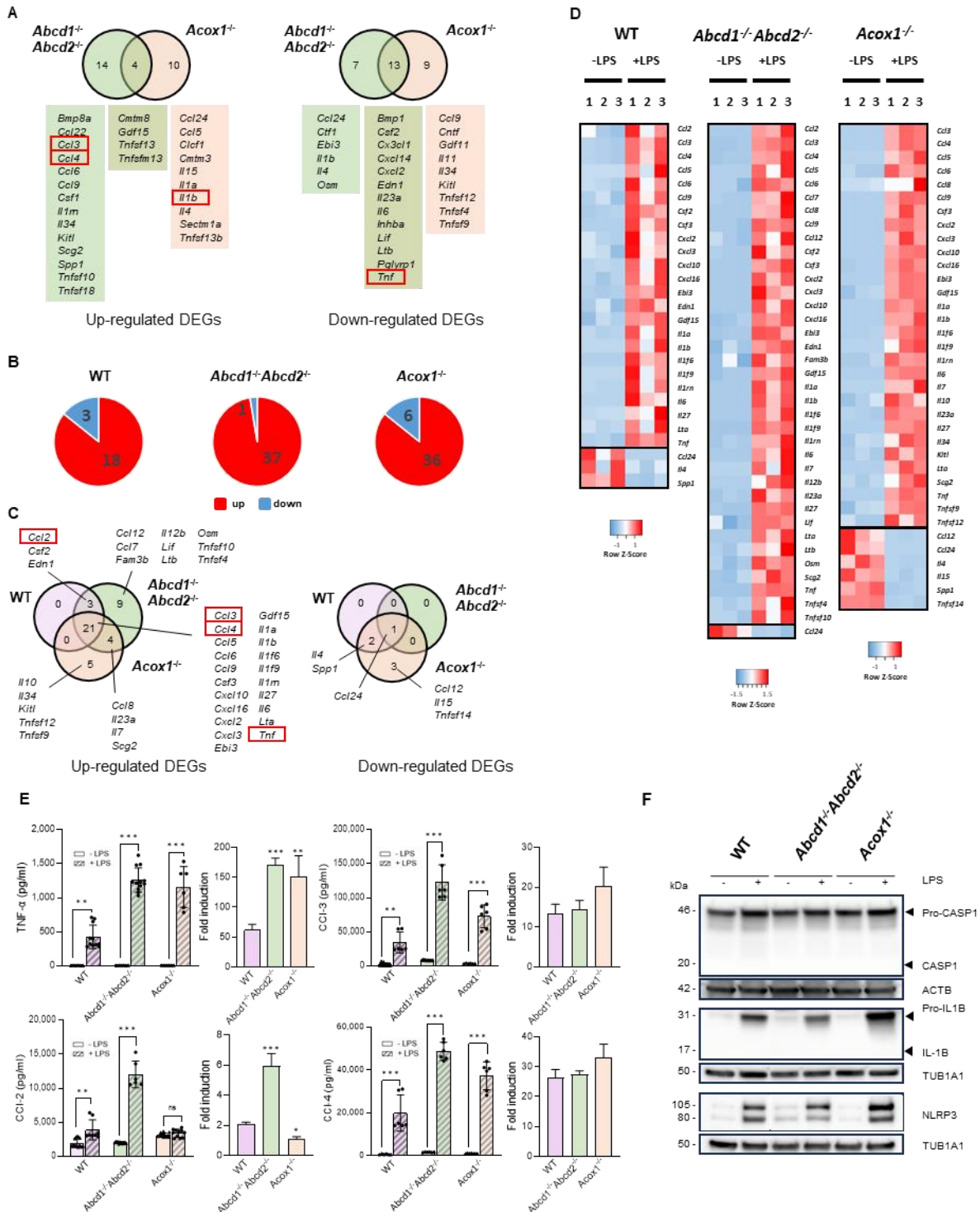
To confirm these results and check whether secretion of these pro-inflammatory chemokines and cytokines is increased, we performed Bio-Plex analysis to measure the secretion of CCL2 (MCP-1), CCL3 (MIP-1 $\alpha$ ), CCL4 (MIP-1b), IL1B, and *TNF*. LPS treatment resulted in a significant increased secretion of CCL3, CCL4, and TNF whatever the genotype; the CCL2 level being also significantly increased except in the *Acox1<sup>-/-</sup>* cells (Figure 2E). The highest concentrations of the four analyzed cytokines were found in the supernatant of the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells. However, we failed to detect secretion of IL1B. Interestingly, if we analyze the ratio between the levels of the cytokines in a single genotype, the LPS-associated increase in TNF secretion was significantly higher in the mutant genotypes (Figure 2E). The LPS-associated increase in CCL2 secretion was also significantly higher in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* genotype (Figure 2E). These significant LPS-dependent over secretion were not detected for CCL3 and CCL4 (Figure 2E).

As no IL1B secretion was detected, we wondered whether there was a maturation problem and also investigated the expression of the NLRP3/CASP1 inflammasome complex, which controls the release of IL1B and IL18 (Li and Jiang, 2023). Western blotting analysis confirmed the increase in pro-IL1B expression upon LPS treatment in each genotype but the absence of maturation of the protein (Figure 2F). We can notice that the pro-IL1B signal is higher in the *Acox1<sup>-/-</sup>* cell lines than in the control (Figure 2F). This maturation depends on the activation of caspase 1 (*Casp1*) by ASC (*Pycard*). Expression of pro-CASP1 was high and remained stable upon LPS treatment but CASP1 activation was not detected (Figure 2F), which is likely explained by a very low level of *Pycard* expression in BV-2 cells rendering impossible maturation and secretion of IL1B. However, we measured the expression of NLRP3 and confirmed its increased levels in each genotype upon LPS treatment, the signal intensity being higher in the *Acox1<sup>-/-</sup>* cells (Figure 2F).



**Figure 1:** Transcriptomic analysis of the LPS response of WT and mutant (*Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>*) BV-2 cells ( $n=3$  for each genotype). **(A)** Pie chart displaying the number of up- or down-regulated DEGs after a 24 h LPS treatment. The significant genes (number and percentage) were selected using a cut off adjusted  $p$ -value (DESeq2 Wald test with Benjamini and Hochberg  $p$ -value adjustment) lower than 0.05 and an absolute  $\log_2$  FC higher than 1. The proportion of DEGs significantly ( $p<0.05$ ) more repressed (dark blue) or induced (dark red) is indicated (statistical significance of the genotype effect on the LPS induction was measured by using Wald test). **(B)** Gene set enrichment analysis in each genotype presenting a selection of the 3 main Gene Ontology biological process and KEGG pathway (up and down) terms selected by FDR (in brackets) and sorted by Fold Enrichment (abscissa axis). Functional annotation was obtained using ShinyGO online tools (<http://bioinformatics.sdstate.edu/go/>) (Ge et al., 2020). **(C)** Comparative analysis of the sets of DEGs using Venn

diagram illustrating the large increase of up-regulated (up) and down-regulated (down) DEGs in mutant genotypes. The number in brackets represents the number of DEGs belonging to the intersection significantly more deregulated ( $p < 0.05$ ) than in the WT genotype (Wald test).



**Figure 2:** Impact of peroxisomal defect on the expression of inflammatory cytokine-related genes in WT and mutant (*Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup>) BV-2 cells. **(A)** Venn diagrams illustrating the up- and down-regulated DEGs ( $\text{Log}_2\text{FC} > 1$ ,  $\text{AdjP Value} < 0.05$ ) encoding for cytokines and chemokines in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> non-stimulated BV-2 cells. Genes whose expression level was measured at the protein level are boxed. **(B)** Pie chart displaying the number of up- or down-regulated inflammatory cytokine-related DEGs after a 24 h LPS treatment ( $\text{Log}_2\text{FC} > 1$ ,  $\text{AdjP Value} < 0.05$ ) in WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> BV-2 cells. **(C)** Venn diagrams

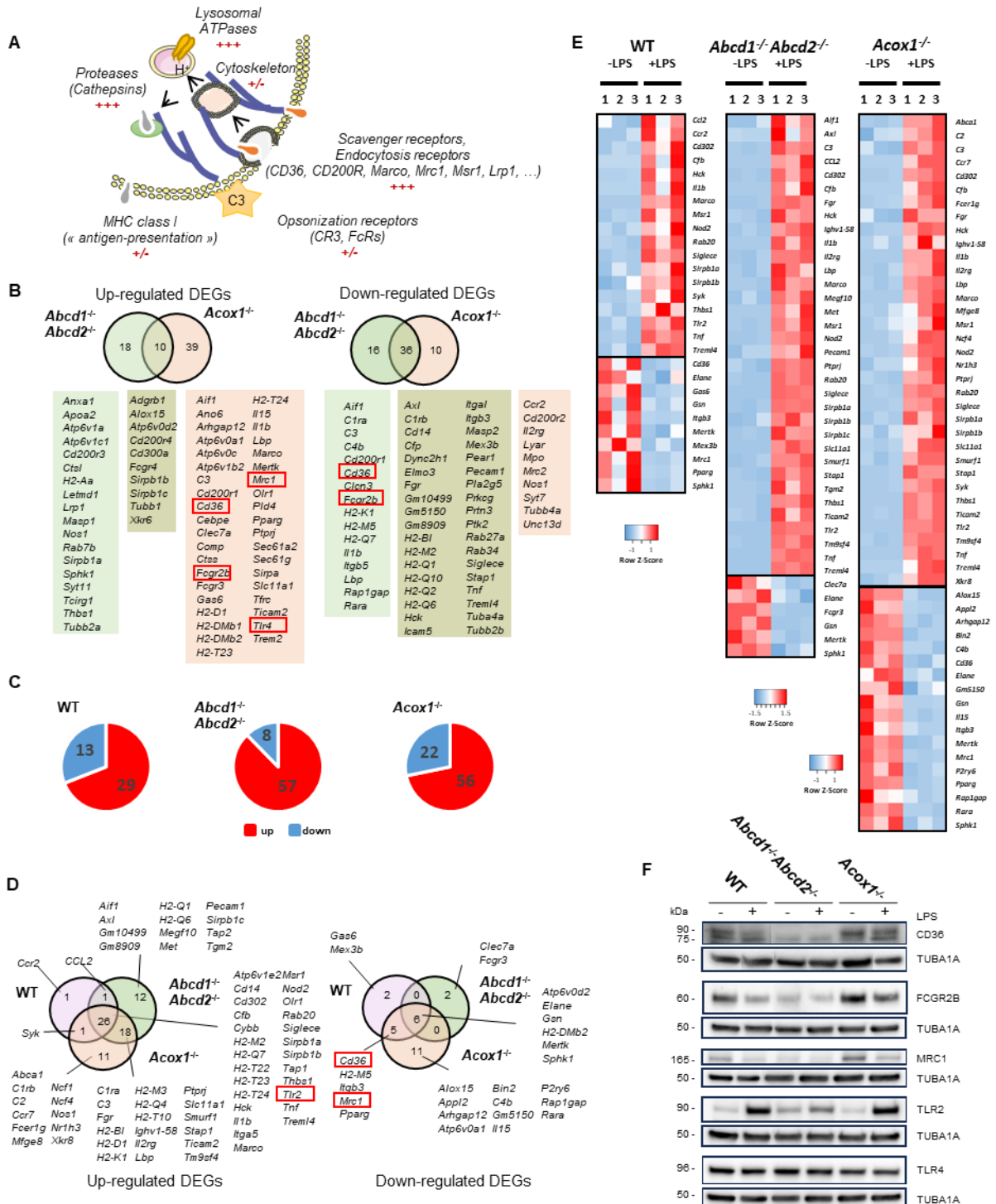
representing the LPS-response in each genotype. (D) Heat map representing RNA-Seq gene expression of DEGs at 24 h after LPS stimulation in WT, *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*, and *Acox1<sup>-/-</sup>* BV-2 cells (3 independent batches of BV-2 cells for each genotype). (E) Multiplex analysis of the concentration of secreted cytokines and chemokines (CCL2 (MCP-1), CCL3 (MIP-1 $\alpha$ ), CCL4 (MIP-1b), *TNF*). Dot plots histograms present the single values and mean (+/-SD) of 6 to 12 measurements in the absence of LPS (empty bars) or after a 24 h-treatment with LPS (hatched bars). Statistical differences relative to the untreated genotype are indicated (two-way ANOVA, \* p<0.01, \*\* p<0.01, \*\*\* p<0.001). Relative fold induction is presented on the right histogram for each cytokine and statistical differences relative to the fold induction observed in WT cells are indicated (one-way ANOVA, \* p<0.01, \*\* p<0.01, \*\*\* p<0.001). (F) Representative image of western blotting analysis (3 independent experiments) of the expression levels of Caspase 1 (CASP1 and Pro-CASP1), Interleukin 1b (IL1b and ProIL1b), and NLRP3 in WT, *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*, and *Acox1<sup>-/-</sup>* BV-2 cells untreated (-) or treated with LPS for 24 h (+). Expected molecular weights are indicated. Source data are available online for this figure.

## Phagocytosis

To further define how impaired peroxisomal  $\beta$ -oxidation modifies other important functions of microglial cells, we first focused on the DEGs belonging to the terms “phagosome”, “Fc $\gamma$  Receptor” and “engulfment”. Phagocytosis is a complex phenomenon allowing uptake and removal of various targets (apoptotic cells and cell debris, bacteria) (Fu et al., 2014; Cockram et al., 2021). It involves many proteins of the plasma membrane serving as receptors (specific receptors for “find me” and “eat me” signals, Fc receptors and scavenger receptors) (Figure 3A). Cytoskeleton proteins facilitate the engulfment and internalization of particles into a phagosome, while proteins in the endo-lysosomal compartment aid in the removal of degradation products, recycling, and antigen presentation (Figure 3A). In the brain, this process, mainly linked to microglia activity, participates in brain homeostasis, neural development and plasticity, synaptic connectivity, immune response, control of inflammation, and myelin repair (Galloway et al., 2019; Li et al., 2022). Several genes known to have an important role in microglial phagocytosis, especially at the cell surface, were found in the up-regulated DEGs in the *Acox1<sup>-/-</sup>* genotype as compared to the WT cells (*C3*, *Cd200r1*, *Cd36*, *Fcgr2b*, *Fcgr3*, *Lbp*, *Marco*, *Mrc1*, *Tlr4*, *Trem2*) (Figure 3B). In the enrichment analysis of the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells, we did not find the term “phagosome”. Although *Fcgr3*, *Tlr4*, and *Trem2* figured in the up-regulated DEGS, their fold changes were below the threshold ( $0.78 < \log_2 \text{FC} < 1$ ) and the other phagocytosis-associated genes were either not dysregulated or found in the down-regulated DEGs (Figure 3B). LPS treatment resulted in a more profound change in expression of phagocytic genes in mutant cells than in WT cells (Figure 3C and D). Some DEGs belonging to the intersection of the 3 genotypes were induced such as *Msr1*, *Marco* and *Tlr2* while other being repressed like *Cd36*, *Mertk*, or *Mrc1* (Figure 3D and E). Of note, the expression levels of *Trem2* and *Tyrobp* (DAP12), which together promote microglial proliferation and phagocytosis, were not modified upon LPS treatment. Altogether, the transcriptomic data suggested different behaviors of the cells depending on the genotype, the conditions of activation, and the nature of the substrates to bind and engulf (Cockram et al., 2021).

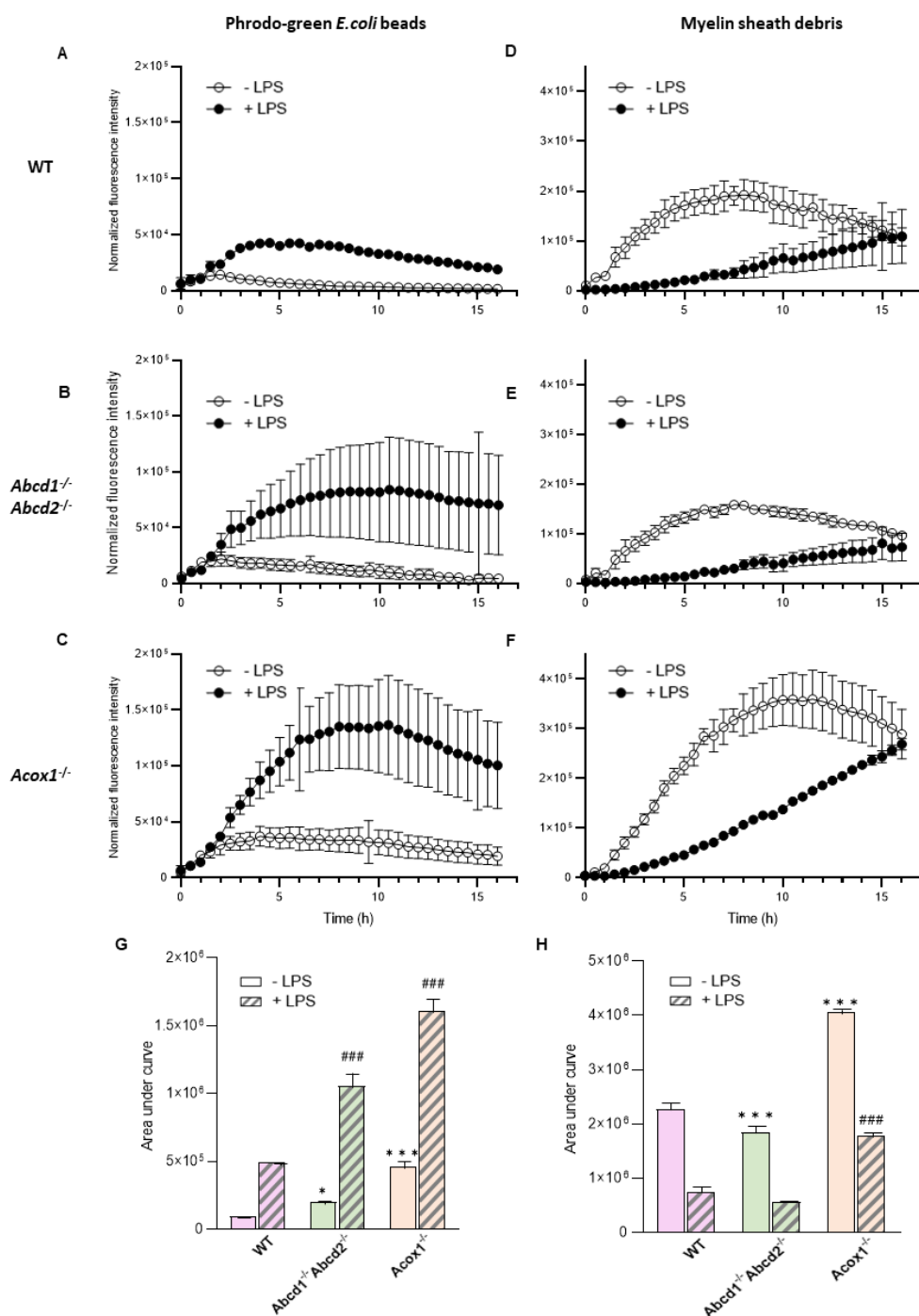
To confirm these observations, we analyzed the expression of some of the phagocytosis markers cited above by western blotting (Figure 3F). The lower expression of FCGR2B (Fc Gamma Receptor IIb), MRC1 (CD206) and CD36 in the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells and their higher expression in the *Acox1<sup>-/-</sup>* cells were confirmed (Figure 3F). Upon LPS treatment, we observed a weak reduction of the signal intensity for each receptor in accordance with the transcriptomic results (Figure 3F). Concerning TLR2 and TLR4 expression, we did not observe major differences between genotypes in basal conditions. However, upon LPS treatment, we confirmed the up regulation of TLR2 with no modification of the TLR4 expression. Of note, TLRs are not directly considered as phagocytic receptors but they directly modulate phagocytosis (Fu et al., 2014).

We then investigated the phagocytic capacity of WT and mutant BV-2 cells. First, we used pHrodo<sup>®</sup>-Green *E. coli* BioParticle conjugate, which are pH-sensitive dye conjugates that only produce bright green fluorescence when taken up into the acidic phagosome compartment. Cells were pre-incubated with LPS (1 µg/ml) for 24 h before pHrodo<sup>®</sup>-Green bioparticles with beads (25 µg/mL) were added. Incucyte live imaging was used to monitor the engulfment (live-cell images were acquired every 30 minutes for 16 hours and fluorescence intensity was measured and normalized). Internalization was detectable 1 h after incubation with beads. The fluorescence increased to reach a peak (2 h for the WT and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells and 4 h for the *Acox1*<sup>-/-</sup> cells), and then decreased in a time-dependent manner, possibly because of the release of the fluorochrome from the internalized particles. (Figure 4A). The mutant cells demonstrated a stronger phagocytic ability than WT cells, with *Acox1*<sup>-/-</sup> cells showing the most prominent activity. In the presence of LPS, phagocytosis of fluorescent bioparticles was increased for each genotype but the peak was delayed (5 h for the WT cells and 10 h for the mutants). We next evaluated phagocytosis of YG carboxylate beads by flow cytometry one hour after incubation with the beads (Supplementary Figure 1). While *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells demonstrated almost no phagocytosis of these beads, we were able to confirm the increased phagocytic capacity for the *Acox1*<sup>-/-</sup> cells. Again, the 24 h LPS pre-treatment resulted in an increased phagocytosis capacity for each genotype. Since a main function of microglial cells is the phagocytosis of myelin debris under conditions of demyelination, we next compared the phagocytic capacity of WT and mutant BV-2 cells using pHrodo-Green labeled murine myelin as substrate. Accordingly, BV-2 microglial cells were incubated with pHrodo<sup>®</sup>-Green-labeled myelin and the uptake was followed by Incucyte live-imaging for up to 16 h (Figure 4B). Internalization was detectable 4 h after incubation, increased to reach a peak (8 h in for the WT and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells and 12 h for the *Acox1*<sup>-/-</sup> cells), and then decreased along with the incubation time. While the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells showed a lower phagocytic ability than WT cells, the phagocytic rate was significantly increased in *Acox1*<sup>-/-</sup> cells. Of note, the LPS treatment resulted in a decreased phagocytic capacity for each genotype, thus reflecting the downregulation of phagocytic receptors observed in Western blot analysis (Figure 3F). At 16 h, continuous increase of fluorescence was observed for LPS-treated cells. In summary, peroxisomal mutations impact phagocytosis both for myelin debris and fluorescent beads with different composition. In agreement with the transcriptomic data, the *Acox1*<sup>-/-</sup> cells showed increased phagocytic properties while *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells are less efficient than WT cells.



**Figure 3:** Impact of peroxisomal defect on the expression of phagocytosis-related genes in WT and mutant (*Abcd1*<sup>-/-</sup>/*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup>) BV-2 cells. (A) Schematic representation of the main actors of phagocytosis. (B) Venn diagrams illustrating the up- and down-regulated encoding for phagocytosis-related DEGs in *Abcd1*<sup>-/-</sup>/*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> non stimulated BV-2 cells. Genes whose expression level was measured at the protein level are boxed. (C) Pie chart displaying the number of up- or down-regulated phagocytosis-related DEGs after a 24 h LPS treatment (Log2FC > 1, AdjP Value < 0.05) in *WT*, *Abcd1*<sup>-/-</sup>/*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> BV-2 cells. (D) Venn diagrams representing the LPS-response in each genotype (3 independent batches of BV-2 cells for each genotype). (E) Heat map representing RNA-Seq gene expression of DEGs at 24 h after LPS stimulation in *WT*, *Abcd1*<sup>-/-</sup>/*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup> BV-2 cells. (F) Representative image of western blotting analysis (3 independent experiments) of the expression levels of CD36, FCGR2B, MRC1, TLR2 and TLR4 in *WT*, *Abcd1*<sup>-/-</sup>/*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup> BV-2 cells

untreated (-) or treated with LPS for 24 h (+). Expected molecular weights are indicated. Source data are available online for this figure.



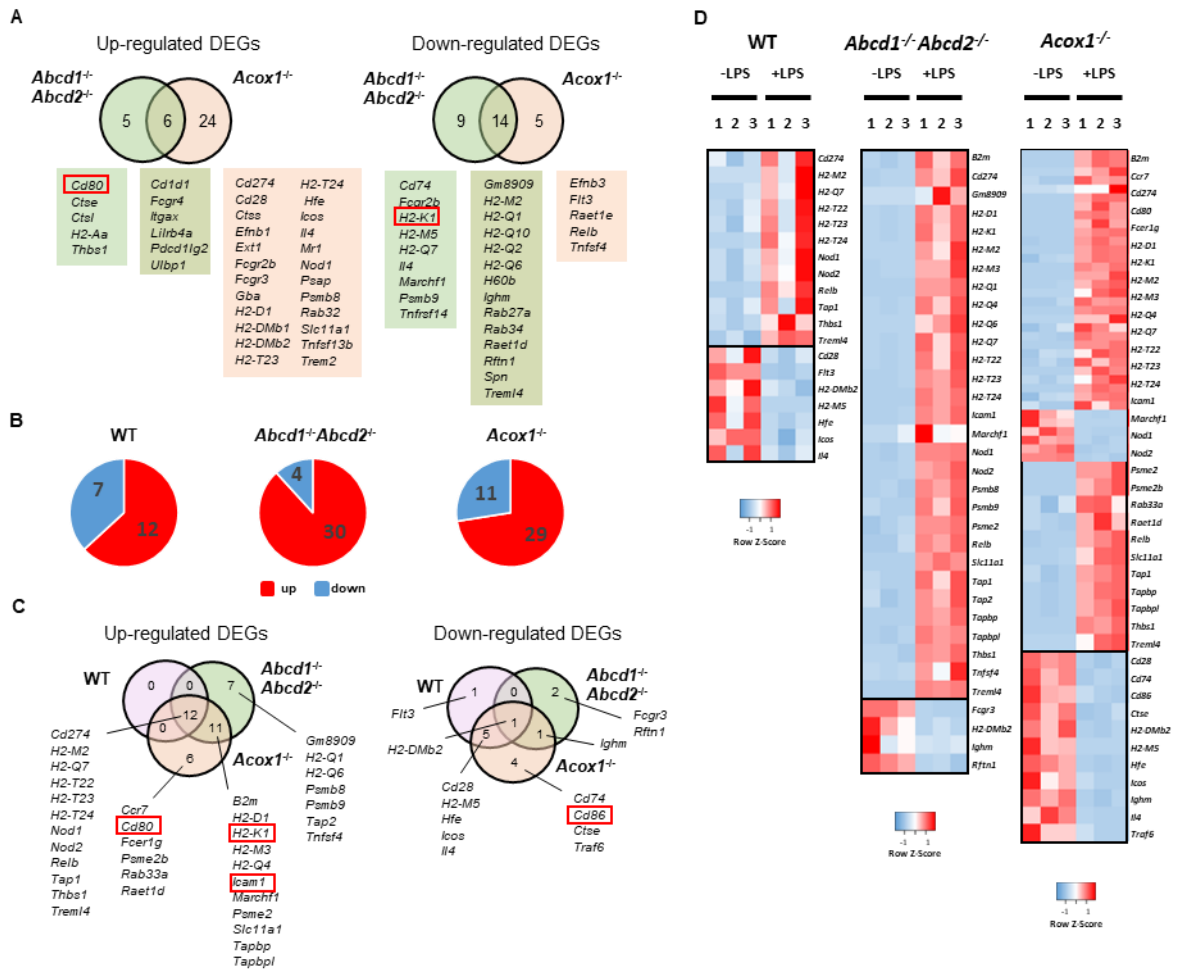
**Figure 4:** Phagocytosis of Phrodo-green *E. coli* beads and Phrodo-green labeled myelin sheath debris by BV-2 WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup> cells. BV-2 cells were incubated with 25  $\mu\text{g}/\text{mL}$  Phrodo-green *E. coli* beads (A, B, C) or 10  $\mu\text{g}/\text{mL}$  pHrodo<sup>®</sup>-green-labeled myelin (D, E, F) in the presence or absence of LPS (LPS 1  $\mu\text{g}/\text{mL}$  for 24h). Incucyte was used to monitor the phagocytosis. The time course of pHrodo signal quantification for 16 hours is represented for WT (A, D); *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> (B, E); and *Acox1*<sup>-/-</sup> (C, F) with bioparticles and myelin respectively. The curves show the mean  $\pm$  S.E.M. from n=3 independent experiments for the beads (left) and n=4 for the myelin (right). Quantification of pHrodo signal over 16 hours represented as area under the curve for phagocytosis of beads (G) and myelin (H). The statistical differences were calculated using 2-way ANOVA followed by correction for false discovery rate and represented as ‘\*’ to compare the phagocytosis of LPS KO to WT cells and as ‘#’ to compare the LPS-treated KO versus LPS-treated WT cells (\* $p < 0.05$ , \*\*\* $p < 0.001$ , ### $p < 0.001$ ).

## Antigen presentation to T lymphocytes

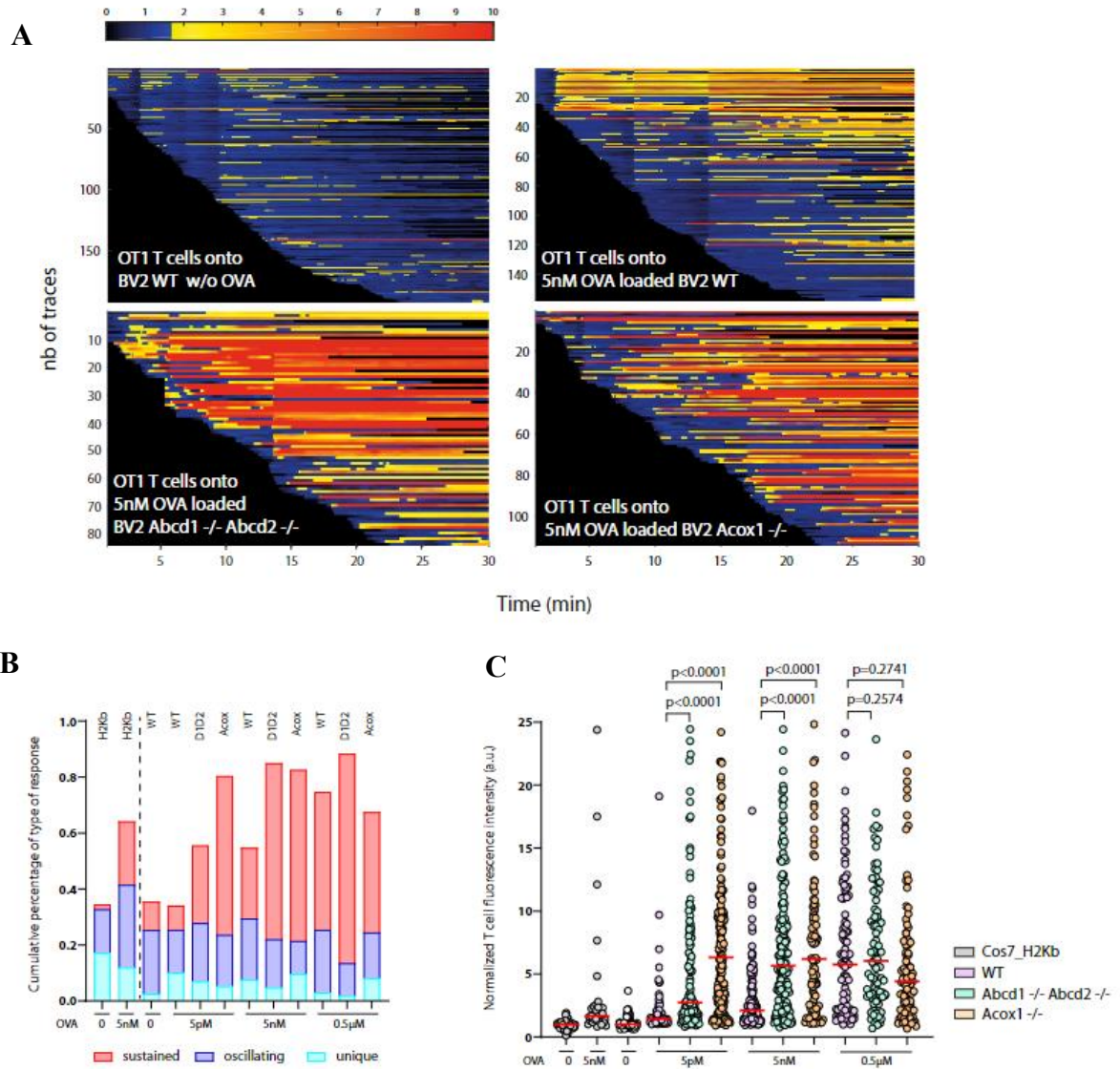
Microglia plays a crucial role in the immune response as the primary antigen presenting cell (APC) in the brain parenchyma, particularly during neurodegeneration. They express both major histocompatibility complex (MHC) class I and class II molecules, enabling them to activate infiltrating CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes, respectively (Schetters et al., 2017). Our transcriptomic analysis revealed a significant number of DEGs among the genes involved in antigen processing and presentation, particularly in the *Acox1*<sup>-/-</sup> genotype (Figure 5A). Many of these genes were H2 genes, which encode major histocompatibility complex (MHC) proteins (Figure 5A). The response to LPS was more pronounced in the mutant cell lines compared to the WT cells (Figure 5B and C). As expected, LPS treatment primarily induced the expression of genes involved in antigen processing such as *Tap1* and antigen presentation (H2 genes). Interestingly, MHC class I genes *H2-D1* and *H2-K1* were only induced in the mutant cells along with *B2m* suggesting a skewing towards CD8<sup>+</sup> T lymphocyte activation (Figure 5C and D). Besides, genes involved in adhesion (*Icam1*, *Cd54*) and co-stimulation (*Cd80*) were also up-regulated only in the mutant genotypes (Figure 5C and D). Of note, the expression of *Gpnmb* and *Lilrb4* genes, which encodes for reliable markers of microglial activation, considered as anti-inflammatory and immunosuppressive modulators of adaptive immunity (Kretzschmar et al., 2021; Saade et al., 2021) has been found induced in BV-2 mutant cells (Raas et al., 2023). Moreover, we observed modifications of the expression of several genes involved in the suppression of microglia and APC-mediated T cell activation (induction of *Trem2*, *Tyrobp* (DAP12), *CD200r3*, *CD200r4*, repression of *Cx3cr1* and *Itgam* (CR3), while *Cd200r1* was repressed in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells and induced in *Acox1*<sup>-/-</sup> cells). Altogether, these data suggest that peroxisomal defects alter immune response and modify antigen presentation to T lymphocytes.

To address this hypothesis, we first analyzed by flow cytometry the expression of some cell surface proteins involved in antigen presentation H2-K1(H2-Kb), H2-AB1 (I-Ab), co-stimulation (CD80, CD86), and adhesion (ICAM1 (CD54)). In basal conditions, the protein results did not perfectly match RNA-seq data but upon LPS, the results globally confirmed a higher expression of H2-K1, CD80, and ICAM1 in the mutant genotypes (Figure 5E). To functionally link these expression levels to T cell activation, we performed calcium response imaging in ex-vivo naïve CD8<sup>+</sup> OT1 T cells by live imaging. Calcium fluctuations were further analyzed using our home-made algorithm MAAACS (methods for automated and accurate analysis of *cell* signals) (Salles et al., 2013; Sadoun et al., 2021) of the calcium flux in T lymphocytes upon stimulation by OVA-loaded LPS-treated BV2 cells acting as antigen presenting cells (Figure 6). We mainly analyzed the calcium fluctuations, the intensity, and the duration of the response over the threshold. This enables us to classify responses as unique, oscillating or sustained according to the duration of the calcium increase over the threshold and the frequency of calcium peaks. Throughout a 1-hour timelapse, 35% to 90% T cells displayed a calcium response over the threshold but mostly dependent (in term of intensity) when T cells were seeded onto antigen loaded BV2 cells. We evidenced that the mutant BV2 cells, compared to WT BV2, were inducing in T cells a strong calcium response, dominantly sustained even for low doses of antigen (in the range of few pM). This may originate from the enhanced surface expression of adhesion and co-stimulatory molecule that drive T cell response upon a sustained calcium response by the co-engaging of the TCR, CD28 and LFA-1, consistent with our previous observations (Xia et al., 2018). Indeed, when CD8<sup>+</sup> OT1 cells were seeded onto COS7 APC expressing only MHC1 molecules both the frequency of activated T cells, the strength and the shape of the response (sustained vs oscillating) were far less intense in T cells than in contact with mutant BV2 cells (Figure 6).





**Figure 5:** Impact of peroxisomal defect on the expression of genes related to antigen presentation and T lymphocyte co-stimulation in WT and mutant (*Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup>) BV-2 cells. **(A)** Venn diagrams illustrating the up- and down-regulated DEGs (Log<sub>2</sub>FC > 1, AdjP Value < 0.05) encoding genes related to antigen presentation and T lymphocyte co-stimulation in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> non-stimulated BV-2 cells. Genes whose expression level was measured at the protein level are boxed. **(B)** Pie chart displaying the number of up- or down-regulated DEGs after a 24 h LPS treatment (Log<sub>2</sub>FC > 1, AdjP Value < 0.05) in WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> BV-2 cells. **(C)** Venn diagrams representing the LPS-response in each genotype. **(D)** Heat map representing RNA-Seq gene expression of DEGs at 24 h after LPS stimulation in WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup> BV-2 cells (3 independent batches of BV-2 cells for each genotype).



**Figure 6:** T cell calcium fluctuations as a consequence of BV2 antigen presentation. OT1 CD8<sup>+</sup> T cells were loaded with PBX dye before being seeded onto WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup> BV-2 cells previously treated over night with LPS and pulsed with various concentrations of OVA SIINFEKL peptide. (A) Barcoding representing the T cell response, (B) Type of response (C) Normalized fluorescence intensity.

## DISCUSSION

In this study, we further characterized mutant microglial BV-2 cells with peroxisomal defects by focusing on the immune response and investigated their response to LPS stimulation. Our aim was to better understand the impact of peroxisomal  $\beta$ -oxidation on microglial functions and to help clarify the pathophysiology of X-ALD and other peroxisomal leukodystrophies. Microglia play an essential role in mediating the immune response in the brain through their ability to detect danger, participate in the elimination of damaged or dead cells, trigger, amplify and resolve inflammation, present antigens and induce the lymphocyte response, and control the blood-brain barrier. Inflammation is not always harmful, but rather an adaptive response to damage that can sometimes become unmanageable (Sochocka et al., 2017). Neuroinflammation occurs when microglia become overactive and release inflammatory cytokines and reactive

oxygen species, leading to infiltration of peripheral immune cells and the destruction of healthy neurons and synapses. The abnormal activation of microglia can lead to devastating outcomes, including neurodegenerative diseases. Therefore, understanding the mechanisms of microglial activation and how to regulate their activity is crucial for developing therapies for neurodegenerative diseases. Although highly dynamic and complex, microglia has been commonly classified into two states, namely resting and activated, with the activated state encompassing two polarization states: M1, characterized by pro-inflammatory properties, and M2, known for its anti-inflammatory characteristics (Guo et al., 2022). Recently, a consortium of scientists has recommended to consider microglia and its complexity with a new nomenclature referring to homeostatic for microglia in basal condition and reactive with its entire specificities (Paolicelli et al., 2022). The induction of *Trem2* and the DAM signature (down-regulation of *Cx3cr1*, *P2ry12* and *Tmem119*, up-regulation of *ApoE*, *Clec7a*, *Ctsb*, *Lyz2*, *Spp1*, and *Trem2*), which was observed in the mutant BV-2 cells, clearly indicate that the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells have lost their homeostatic state (Raas et al., 2023). Concerning inflammation, in basal conditions, despite the up-regulation of *Trem2* and of the presence of a DAM signature in mutant BV-2 cells, pro-inflammatory genes were mostly repressed (Raas et al., 2023). A clear polarization towards an anti-inflammatory state was however absent.

LPS-stimulated BV-2 microglial cells are often used as in vitro models of neuroinflammation and accordingly, BV-2 cells adopt a classical pro-inflammatory state (induction of *Il1b*, *Il6*, *Tnf*). In our study, LPS treatment induced an inflammatory response of both WT and mutant BV-2 cells illustrated by the induction of NLRP3 and the increased secretion of several pro-inflammatory cytokines and chemokines. It is important to note that our results recapitulate most of the results already published on WT BV-2 cells but punctuate discrepancies exist and probably result from the use of different LPS types along with different concentrations (from 10 ng/mL to 1 µg/mL) and lengths of treatment (from 4 h to 24 h) (Henn et al., 2009; Dai et al., 2015; Das et al., 2015; Das et al., 2016; Bussi et al., 2017). Of note, we observed that the overall LPS response was largely increased in the mutant genotypes both in the number of DEGs and in their range of variation. TLR4, which mediates inflammatory responses by recognizing lipopolysaccharide (LPS) and TLR2 (Lehnardt et al., 2002; Hwang et al., 2016), could be directly involved in this amplified response since their expression is increased in the mutant cell lines, in basal condition for *Tlr4*, and upon LPS stimulation for *Tlr2*. Of note, other TLR encoding genes figured among the DEGs both in basal conditions (induction of *Tlr1*, *Tlr3*, and *Tlr13* in the mutant cells) and after LPS treatment (induction of *Tlr3* and *Tlr12* and repression of *Tlr5* and *Tlr8*) suggesting that peroxisome defect impacts not only neuroinflammation but also adaptive immunity and antiviral response (Kumar, 2019; Dabi et al., 2023).

Confirmation of the induction at the protein level was obtained from selected proteins (CCL2, CCL3, CCL4, and TNF). The increased LPS-dependent secretion of TNF, which is considered as a major cytokine in neurodegenerative processes (Montgomery and Bowers, 2012), was significantly higher in mutant cells than in WT cells consistently with the LPS response of peripheral blood mononuclear cells of X-ALD patients (Lannuzel et al., 1998; Di Biase et al., 2001). The *Tnf* gene was investigated as a possible modifying gene related to the different clinical phenotypes of X-ALD but the conclusions rule out this hypothesis. (McGuinness et al., 1995). More recent analyses failed to demonstrate correlation between plasma levels of TNF and the severity of the disease (Marchetti et al., 2018). Regarding IL1B, we failed to detect secretion of this cytokine both in mutant and WT BV-2 cells, which is in contradiction with previous studies for the WT cells (Dai et al., 2015; Bussi et al., 2017). Our results are consistent with the lack of maturation of CASP1 and IL1B, likely attributed to the significantly low expression level of *Asc* in our cell lines. Nevertheless, we observed an

enhanced expression of *Iib* mRNA and pro-IL1B protein in all genotypes following LPS treatment. This finding aligns with the enrichment of *NF-κB* signaling in the up-regulated DEGs. Notably, in basal condition, we did not observe a significant upregulation in mRNA of *Iib* when compared to WT contrary to what was seen in *Abcd1<sup>-/-</sup>* mice (Gong et al., 2017). As expected from previous studies (Lu et al., 2013), the CCL2, CCL3, and CCL4 chemokines showed increased secretion levels upon LPS treatment. Noteworthy, the highest concentrations were obtained with the mutant supernatants. These chemokines are small cytokines that play a key role in the regulation of immune cell migration and actively participate in neurodegenerative processes (Wojcieszak et al., 2022). CCL2 (MCP-1) activates CCR2 receptor and is involved in the recruitment of peripheral immune cells to sites of inflammation. CCL3 (MIP-1α) binds CCR5 and has a similar function as CCL2 but has been also reported to mediate immune cells cytokine secretion and promote aggregation and migration of various cells. CCL4 (MIP-1β), another ligand of CCR5, is implicated in the attraction of natural killer cells, monocytes and some other inflammatory cells. *Tnf*, *Ccl2*, *Ccl4* mRNA have been shown to be increased in the inflammatory areas of brains affected by cALD (Paintlia et al., 2003). Cerebrospinal fluid revealed higher levels in cALD samples compared to controls for CCL2 and CCL3 and serum CCL2 significantly correlated with disease severity as determined by MRI (Lund et al., 2012). Altogether, our results suggest that peroxisomal defect in BV-2 microglial cells is responsible for an inflammatory over response when stimulated by LPS and that the excessive release of pro-inflammatory cytokines and chemokines by microglia would be a major cause of neurotoxicity. Despite the involvement of other cell types (mainly astrocytes or infiltrating immune cells) in the release of these cytokines within the brain, we can therefore speculate that neuroinflammation may arise due to an excessive reaction of microglia. In X-ALD patients, such hyper response would be triggered by internal or external stimuli and amplified by the progressive accumulation of VLCFA. Such hypothesis is in agreement with a recent study in human macrophages showing that ABCD1 defect and VLCFA accumulation increase their proinflammatory response (Zierfuss et al., 2022).

In this study, we further explored phagocytosis, another important function of microglia. In physiologic situation, phagocytosis is critical by maintaining brain homeostasis, participating in the synaptic plasticity and the continuous removal of debris and dying cells (Galloway et al., 2019; Hammel et al., 2022). Clearance of dying cells and debris allows the recruitment of oligodendrocyte progenitor cells and remyelination (Lloyd and Miron, 2019). Moreover, microglial phagocytosis of myelin was shown to be tightly linked to a decreased inflammatory response (Liu et al., 2006). Conversely, in disease state, phagocytosis alteration largely contributes to neuroinflammation and demyelination and thus, constitutes a therapeutic target in neurodegenerative disorders. Microglial autophagy-associated phagocytosis is responsible for myelin degradation and is essential for recovery from neuroinflammation (Berglund et al., 2020).

Here, we showed that peroxisomal defect in BV-2 cells impact phagocytosis of myelin and *E. coli* bioparticles in different ways depending on the nature of the substrates. Carboxylate beads were almost not engulfed by *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells while *Acox1<sup>-/-</sup>* cells displayed a 2-fold increased ability as compared to WT. *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells engulfed other beads (pHrodo<sup>®</sup>-Green *E. coli* BioParticle conjugate) slightly more efficiently than WT cells, but were less potent than *Acox1<sup>-/-</sup>* cells. Whatever the genotype and the type of beads, LPS treatment resulted in an increased phagocytosis ability. Regarding phagocytosis of labeled myelin debris, while *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells were slightly less potent than WT cells, *Acox1<sup>-/-</sup>* cells again displayed much more efficiency. Unlike artificial beads, the LPS treatment resulted in a decreased phagocytic ability of myelin debris independent of the genotype. Surprisingly, knockouts of peroxisomal ABC transporters and ACOX1 enzyme gave different results, which could be

explained by the differential expression of several key genes involved in phagocytosis. Among them, *Cd36* was found to be associated with the uptake of myelin debris (Grajchen et al., 2020). Other receptors including complement-receptor-3 (CR3) (van der Laan et al., 1996; Reichert and Rotshenker, 2003) (Zorina et al., 2018), low-density lipoprotein receptor-related protein 1 (LRP1) (Gaultier et al., 2009), Fc-receptors (Kuhlmann et al., 2002), MerTK (Healy et al., 2017; Shen et al., 2021), macrophage scavenger receptor 1 (MSR1, CD204) (Kong et al., 2020), scavenger receptors (SR-AI/II and collectin placenta 1 (CL-P1)) (Reichert and Rotshenker, 2003), and TREM2 (Cignarella et al., 2020). Microglia expressing high level of mannose receptor MRC1 (CD206) are abundant in active lesions and this receptor would also promote remyelination (Lloyd and Miron, 2019). Most of the genes encoding these receptors were found up-regulated only in the *Acox1*<sup>-/-</sup> genotype. In addition, *Cd36* and *Fcgr2b* were down-regulated in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells. Regarding the different results obtained with LPS, we can point out the down-regulation of *Mertk* in each genotype as well as *Cd36* and *Mrc1* in WT and *Acox1*<sup>-/-</sup>. Upon LPS treatment, the decreased expression of *Cd36*, which was shown to promote myelin phagocytosis (Grajchen et al., 2020) and of *MertK*, known as a positive regulator of myelin phagocytosis (Healy et al., 2017), are in agreement with the reduced phagocytosis of myelin observed in BV-2 cells. Regarding fluorescent beads, LPS treatment resulted in increased phagocytosis suggesting that the beads we used do not involve the same receptors involved in myelin phagocytosis. TLR2 and MARCO, whose expression was increased by LPS, could be one of the surface proteins involved in the increased phagocytosis of coated beads. The use of blocking antibodies in another phagocytosis assay with BV-2 cells and phycoerythrin-conjugated polystyrene microspheres demonstrated their involvement (Rangaraju et al., 2018).

In the context of X-ALD, changes in the process of phagocytosis in the brain could serve as a crucial event triggering the development of demyelination and neurodegeneration and impacting myelin repair (Gong et al., 2017; Bergner et al., 2019; Bergner et al., 2021). Phagocytosis indeed permit to eliminate damaged myelin, to resolve inflammatory signals and to scavenge the lipotoxic effects of VLCFA in the other brain cell. However, we can speculate on the negative outcomes associated with both an increase and a decrease in the ability to undergo phagocytosis. An increased phagocytosis of myelin debris containing VLCFA could form foamy microglia, which, once surpassing a certain threshold, could become fully dysfunctional, particularly in terms of their role in regulating inflammation. Conversely, a reduced phagocytosis capability would hinder the elimination of accumulated VLCFA and abnormal myelin, thus promoting cell death. In our study, contrarily to *Acox1*<sup>-/-</sup> genotype, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells displayed a decreased ability to myelin phagocytosis. *Abcd1*<sup>-/-</sup> mouse primary microglia, in both unstimulated and LPS-challenged conditions, demonstrated modification of the expression level of phagocytosis-related genes including *Trem2* but not *C3* were upregulated (Gong et al., 2017). Phagocytosis of labeled neurons exposing phosphatidylserine were weakly increased in the absence or presence of LPS (Gong et al., 2017).

The defective interplay between microglia and T lymphocytes is now recognized as essential in various neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD) (Schettters et al., 2017; Dressman and Elyaman, 2022; Xu et al., 2023). Microglia and T lymphocytes are indeed tightly linked in terms of activation and function. Many microglial secreted cytokines and chemokines are known to recruit and control T lymphocyte activity. Microglia also play a major role in the priming of T lymphocyte response through their ability to present antigens (Aloisi et al., 1999). Several alleles HLA genes, which code for components of major histocompatibility complex (MHC) class I or class II expressed by microglia, are now considered as risk factors in these diseases (Xu et al., 2023). Here, we demonstrated that a defect in peroxisomal  $\beta$ -oxidation in BV-2 cells modify the expression of

numerous genes involved in this cellular crosstalk between microglia and T lymphocytes. Upon LPS activation, expression of key markers of antigen presentation and co-stimulation were also modified. More important, we showed for the first time that peroxisomal mutations in microglia, acting as antigen-presenting cells (APCs), have a significant impact on the T lymphocyte response. This effect likely stems from alterations in the expression of adhesion and co-stimulatory surface molecules, especially upon LPS activation. In X-ALD, accumulation of T lymphocytes have been found in brain lesions (Griffin et al., 1985) and likely result from the microglial-dependent rupture of blood-brain barrier and the secreted chemoattractants. Together with infiltrating macrophages, T lymphocytes form a substantial portion of inflammatory cells following the demyelination's forefront. This observation implies that infiltration and inflammatory activation are subsequent events that follow demyelination (Eichler and Van Haren, 2007). Over-reactive microglia would permit to initiate and amplify such situation. Our results reinforce the importance of microglia interplay with T lymphocytes, possibly participating in a loop of amplification leading to chronic inflammation and neurodegeneration. In the context of X-ALD, targeting microglia and their capacity to prime T lymphocytes makes sense in a therapeutic point of view as it was proven to be efficient in a mouse model of Alzheimer's disease (Chen et al., 2023). However, the lack of predominant T-cell Vbeta receptor usage in CALD patients effectively ruled out the hypothesis suggesting a link between T-cell repertoire and clinical variability in X-ALD (Picard et al., 2005).

While the alterations in the expression levels of various surface proteins related to antigen presentation, T cell co-stimulation, and phagocytosis, provide a plausible explanation for the observed outcomes, we cannot ignore that lipid changes and biophysical characteristics of the plasma membrane play a role. Both phagocytosis (Nadjar, 2018; Rubio et al., 2018) and T cell activation can indeed be impacted by such modifications (Chouaki Benmansour et al., 2018). We have previously demonstrated a vast reprogramming of lipid metabolism and changes in the fatty acid composition in the mutant cell lines, together with the accumulation of cholesterol, especially in the plasma membrane (Raas et al., 2019a; Raas et al., 2019b; Raas et al., 2023). Additional research is required to analyze the biophysical properties of the plasma membrane in BV-2 microglial cells and investigate how these putative peroxisomal-dependent alterations on membrane reorganization may affect phagocytosis and cell-to-cell interactions.

Taken together, our study reveals that impaired peroxisomal  $\beta$ -oxidation transform murine BV-2 microglial cells into over-reactive microglia in which the secretion of inflammatory cytokines, the phagocytosis ability, and the antigen presentation to T lymphocytes are drastically modified, especially upon stimulation. This finding further highlights the pivotal role of immunometabolism in the context of X-linked adrenoleukodystrophy (X-ALD) and peroxisomal leukodystrophies, shedding light on the pathophysiological mechanisms underlying these disorders. These insights might hold the potential for development of novel therapeutic approaches targeting microglia to prevent or slow down the neuroinflammatory process in peroxisomal disorders.

## **Acknowledgements**

We warmly acknowledge the Fondation Maladies Rares which supported our transcriptomic analysis project (GenOmics: High throughput sequencing and rare diseases, call 2017-20170615). Sequencing was performed by the GenomEast platform, a member of the 'France Génomique' consortium (ANR-10-INBS-0009).

We would like to acknowledge the Canadian New Frontiers Research Funds Exploration NFRF-E 19-00007), and the regional council of Bourgogne Franche-Comté (Project PERSIL 2019) for their support. We are also grateful to networking support by the COST Action CA

16,112 NutRedOx (Personalized Nutrition in aging society: redox control of major age-related diseases), supported by COST (European Cooperation in Science and Technology). We thank the Centre d'Immunologie de Marseille-Luminy (CIML) flow cytometry and microscopy facility. This work was supported by institutional grants from INSERM, CNRS and Aix-Marseille University to the CIML and program grant from the French National Research Agency (ANR-17-CE15-0032). The project leading to this publication has received funding from Excellence Initiative of Aix-Marseille University - A\*MIDEX, a French "Investissements d'Avenir" programme.

The laboratory BioPeroXIL was funded by the Ministère de l'Éducation Nationale et de l'Enseignement Supérieur et de la Recherche (France) and by the University of Bourgogne. M.T-J was funded by CNRST (PhD excellence grant number: 17UHP2019, Morocco) and by the Action Intégrée of the Comité Mixte Inter-universitaire Franco-Marocain (n° TBK 19/92 n° Campus France: 41501RJ) from the PHC Toubkal program, Ministère des Affaires Étrangères. IW was supported by the Austrian Science Fund KLI 837-B and by the European Leukodystrophy Association (ELA) Germany.

### **Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### **Author contributions**

AT, QR, MT-J, and CG were in charge of cell culture, mRNA and protein purification. AT, QR, MT-J, CG performed western blotting experiments. AT and SM performed bioplex analysis of cytokine secretion. RK and CK performed RNA-seq and the related bioinformatics and statistical analyses. AT, QR, PA, and SS performed heatmap, gene ontology analyses. EB, MD, YH, performed analyses of expression by cytometry and MAAACS. AT, AVG, IW, T, DT performed phagocytosis assays. AT, QR, MT-J, CG, AT, PA, YH, CT, MCM analyzed the data. AT, CG, and SS performed statistical analyses. AT, QR, CG, PA, YH, and SS mounted the figures. SS conceived the project, performed analyses and wrote the manuscript with AT, QR, MT-J, CK, BN, YH, FDC, AB, MCM, PA, and CG.

### **Funding**

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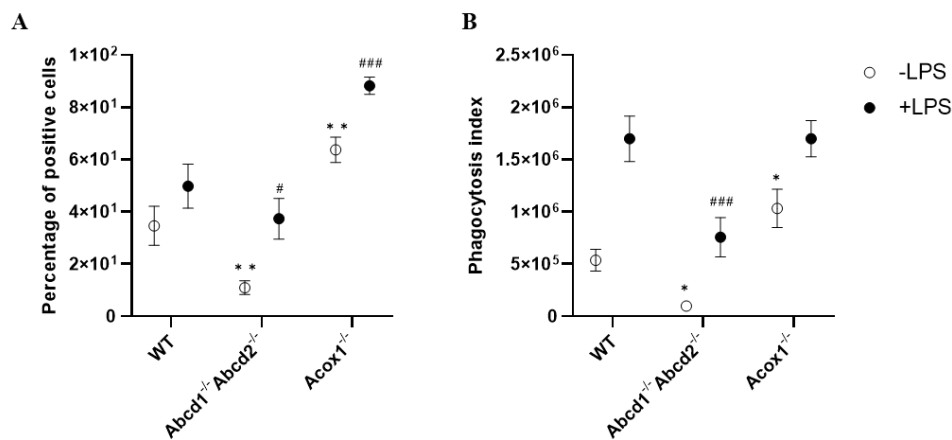
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## SUPPLEMENTARY FIGURE



**Supplementary Figure 1:** Phagocytosis of YG beads by BV-2 cells. Following treatment (+) or not (-) with LPS 1  $\mu\text{g}/\text{mL}$  for 24 hours, the ability of BV-2 cells (WT, *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>*) to phagocytose fluorescent YG carboxylate beads were performed at 37°C as indicated. **(A)** Percentage of YG-beads positive cells were determined by flow cytometry and shown as mean  $\pm$  SEM, with n=4 separate experiments for wild type cells and n=5 separate experiments for KO cells. **(B)** Phagocytic index were calculated ((% YG beads-positive cells) x (mean fluorescence intensity of YG beads-positive cells)) and shown as mean  $\pm$  SD, with n=4 separate experiments for wild type cells and n=5 separate experiments for KO cells. Statistical significance was calculated using 2-way ANOVA followed by correction for false discovery rate and represented as ‘\*’ to compare the phagocytosis of -LPS KO to WT cells and as ‘#’ to compare the LPS-treated KO versus LPS-treated WT cells (\* $P < 0.05$ , \*\* $P < 0.01$ , # $P < 0.05$ , ### $P < 0.001$ ). Negative controls for which phagocytosis was blocked by cytochalasin D treatment and by keeping cells on ice were performed with no positive cells obtained (data not shown here).

## Key Findings of Article 2 and Future Directions

In this study, we chose to focus our analysis on the double knockouts *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and the *Acox1*<sup>-/-</sup>, rather than considering the single knockouts of *Abcd1* and *Abcd2*. This decision was based on the substantial amount of data generated when comparing the different genotypes. Including all the results would have made the findings less clear and the discussion more difficult to understand. In fact, we faced this challenge in our previous paper, where we had to eliminate certain results as requested by the reviewers. Additionally, by comparing the absence of VLCFA importation into the peroxisomes (in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells) with the defects in peroxisomal  $\beta$ -oxidation while VLCFA are still being imported (in *Acox1*<sup>-/-</sup> cells), we can establish a solid baseline for the consequences of peroxisomal defects in microglia.

In this paper, we presented more results of the characterization of the mutant cells, with a specific focus on the immune response induced by LPS treatment. We demonstrated increased expression and secretion of pro-inflammatory cytokines in the mutant cells, specifically TNF which was found to be hugely induced in the LPS-treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells' secretions, suggesting a pro-inflammatory skewing and exaggerated inflammatory response in these cells. We also showed modified phagocytic activity in the mutant cells, as expected from the transcriptomic data. Interestingly, the *Acox1*<sup>-/-</sup> cells showed increased phagocytic activity whereas a repressed activity was revealed in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells. This could be explained by the increased expression of phagocytosis receptors on the membranes of *Acox1*<sup>-/-</sup> cells and their decrease on the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells. We also showed modified antigen presentation activity and amplified CD8<sup>+</sup> T-lymphocytes activation by the LPS-treated mutant cells compared to the WT cells. Such results emphasize on the importance of microglia interplay with T lymphocytes, possibly participating in a loop of amplification leading to chronic inflammation and neurodegeneration, and proves the implication of microglial peroxisomal functions on T lymphocytes activation.

Furthermore, another important aspect of our cell characterization involves studying oxidative stress. However, including the results of oxidative stress markers and secretion in this paper would make it more complex and significantly longer. Therefore, we have decided to prepare a third paper that will encompass these findings.

## **Impaired peroxisomal beta-oxidation in microglial BV-2 cells: oxidative stress and impact on neurons and oligodendrocytes**

Our objective here turns toward exploring the neurodegenerative features including redox homeostasis. Oxidative stress is a hallmark of neurodegeneration. Along with the elevated and dysregulated ROS production from DAM, it is hypothesized to contribute to neuronal death (Simpson & Oliver, 2020). We investigated the expression levels of oxidative stress markers and the impact of the secretions of the mutant cells on neurons and oligodendrocytes, which we explored using conditioned medium and co-culture systems, thus providing an insight on neurodegeneration induced by peroxisomal defects in microglia. These data will provide the core of a third article in which I will be positioned as a first author. While the final format is not yet ready, I will present the results subsequently, utilizing the figures that will ultimately shape our next publication.

### **Article 3 (in preparation):**

Tentative title: Impaired peroxisomal beta-oxidation in microglia triggers oxidative stress and impacts neurons and oligodendrocytes

Before presenting the obtained results, I shall detail below the materials and methods which are optimized and used for the purpose of meeting our hypotheses. The great majority of the materials and methods are already described in their corresponding articles. However, as this paper is still unpublished, its materials and methods are listed below. This section also includes the details of the optimization of specific protocols.

## **Materials and Methods**

### **I. Cell culture**

BV-2 cell line (WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup>) were used and cultured as described previously in (Raas, Gondcaille, et al., 2019; Raas, Saih, et al., 2019) (Raas et al., 2023). The murine mHypoA-POMC/GFP-1 cell line, which is an immortalized adult-derived POMC-expressing/a-MSH-secreting cell model (Nazarians-Armavil et al., 2014), was purchased from Cerdarlane Labs (reference CLU-500). The murine 158N cell line, an immortalized oligodendrocytes cell line was created and provided by Said Ghandour CNRS-ER 2072, Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France (Feutz et al., 2001). All these

cell lines were cultured in the same medium, DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotics (penicillin-streptomycin) and incubated at 37°C, 5% CO<sub>2</sub>.

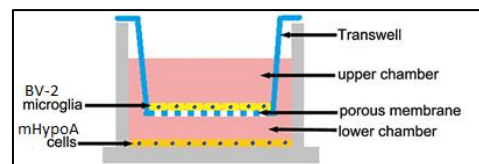
## II. Co-culture and conditioned medium

To examine whether peroxisomal dysfunctions in microglia induce the secretions of pro-inflammatory molecules which could consequently induce neurotoxicity, we implemented co-culture (CC) and conditioned medium (CM) experiments. Several protocols have been tested as described below.

### II.1. Co-culture using transwell inserts (CC):

Transwell cell culture inserts are generally used to co-culture two different cell lines in separate chambers of the same well, separated by a porous membrane allowing the communication between the cells only through secreted substances. The upper part (the Transwell itself) is removable allowing the microscopic observation of the cells of the lower chamber (the well of the plate), however it is not possible to observe the cells seeded in the upper chamber. Transwells are available with different membrane porosities (0.4, 1, 3, 5, 8... μm) depending on the purpose of the experiments (secretion, cell migration...). The polyester membrane is considered ideal for cell culture experiments. The Transwells used for our research were purchased from Sarstedt Company. They are TC-inserts of 0.4μm pores (REF: 83.3930.040 for 6-well-plates and 83.3832.040 for 24-well-plates).

As illustrated in figure 4, we seeded the BV-2 WT and KO cells in the upper chamber (the Transwell) whereas the mHypoA neurons were seeded in the lower chamber (bottom of 6-well or 24-well plates).



**Figure 4: Co-culture system**

To implement this system, several parameters have to be set up. First, several seeding densities for each cell line were tested in order to find the density which allows appropriate confluency after the incubation period.

mHypoA cells: The confluency of the cells was checked 24, 48, 72 and 96 h after the seeding for each condition. The seeding density which leads to 80 – 90% confluency after each incubation period was selected.

BV-2 cells: The set-up was more complicated as the confluency was not detectable by microscopic observation of the Transwell. Additionally, the multiplication of WT cells is



faster than *Acox1*<sup>-/-</sup> cells, which in turns is faster than *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells. This is also demonstrated by our transcriptomic data which revealed that gene families related to cell cycle and cell division are downregulated in KO cells. Therefore, for each BV-2 cell line, the cell density was evaluated by assaying the proteins contained in the lysate obtained from the totality of the cells cultured on the Transwell membrane. Several seeding numbers were tested in the transwells of different sizes (growth area of 4.5 cm<sup>2</sup> for the 6-well-plates inserts and 0.3 cm<sup>2</sup> for the 24-well-plates inserts). The seeding conditions were selected to provide the same protein quantity after the desired incubation time for each of the BV-2 genotypes.

Next, the volume of culture medium to be used in the wells and in the Transwell inserts was determined. Actually, in the presence of the Transwell, the medium is distributed between the well and its Transwell. The standard volume which is conventionally used in the wells of 6-well-plates is 3 ml and in those of 24-well-plates is 1 ml. Due to the presence of the Transwells with the plate wells, the volume of medium should be reduced.

Finally, the duration of the incubation and the time point at which the BV-2 and mHypoA should be co-cultured was determined. According to the bibliography, protocols greatly differ from one lab to another depending on the final objective of the experiment. Several protocols, adapted from those published in the bibliography, were therefore tested and presented in table 1 (Hsiao et al., 2016, 2017; Z. Liu et al., 2019; Qie et al., 2020; Wang et al., 2017). After comparing the results of the applied protocols, and due to some limitations of the use of Transwells, the protocol of choice was the 5<sup>th</sup>, in which the BV-2 and the mHypoA are seeded at the same time and directly co-cultured. The effect on neurons is checked 48 h after the incubation time.

Hence, the seeding densities and the volumes of medium used for 48 h of coculture were as follows:

mHypoA cells: 70,000 cells seeded 6-well-plates in 1.7 ml of medium or 20,000 cells in 24-well plates in 500 µl of medium.

BV-2 cells:

- Seeded in Transwells of 6-well plates: 40,000 WT cells; 150,000 *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells; 60,000 *Acox1*<sup>-/-</sup> cells in 800 µl of medium.
- Seeded in 24-well plates: 5000 WT cells; 7000 *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells; 5500 *Acox1*<sup>-/-</sup> cells in 300 µl of medium.

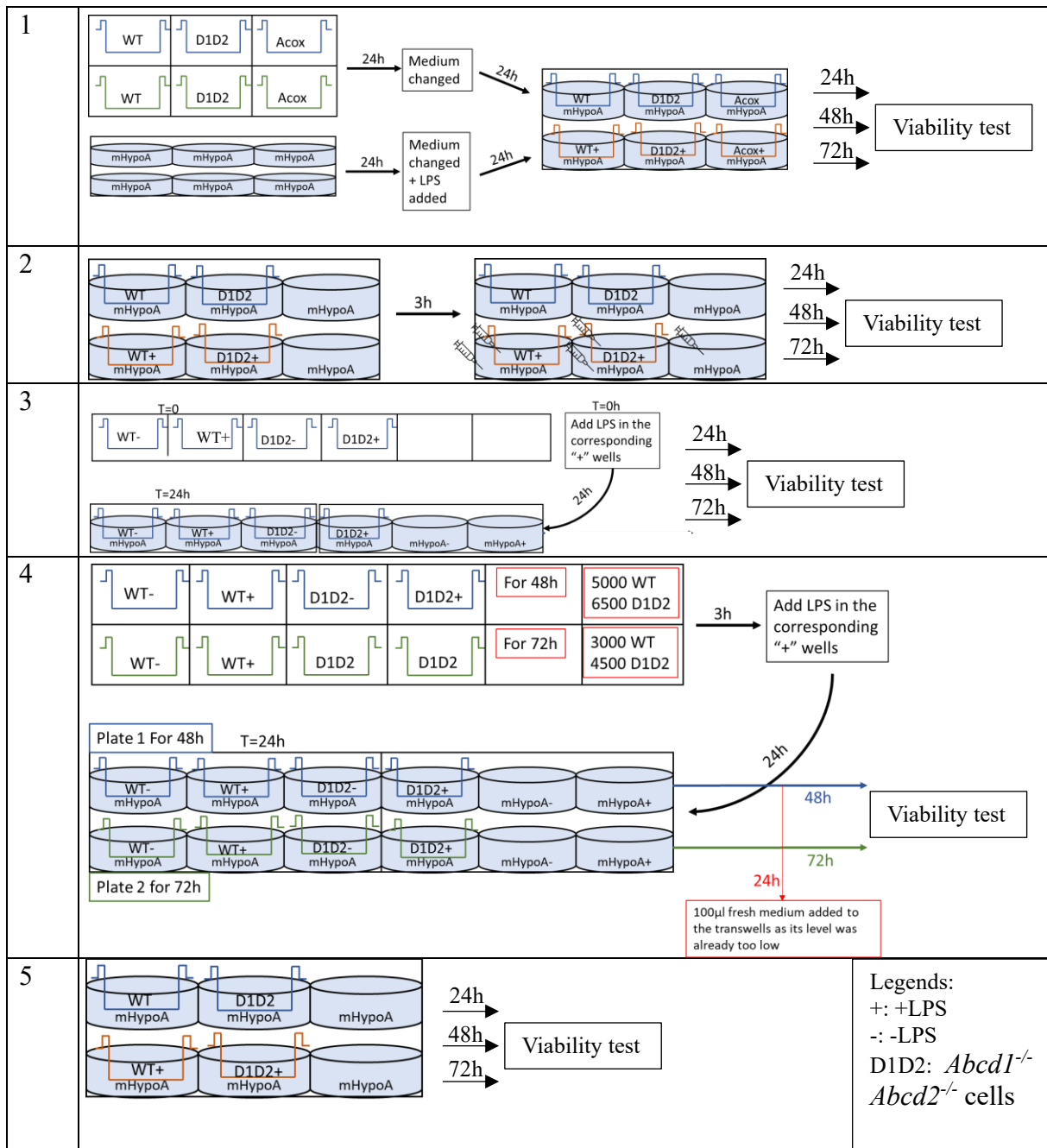


Table 1

## II.2. Conditioned medium (CM)

As shown in figure 5, conditioned medium experiments consist of growing cells, in our case neurons, in the medium collected from the culture of another cell type, in this case BV-2 cells.

Here again, several parameters were tested before measuring the impact of BV-2 secretions on neurons.

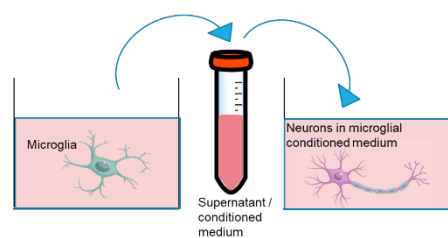


Figure 5: Conditioned medium

First, seeding conditions of BV-2 and neurons were determined after testing several seeding densities followed by confluency observation, cell counting and protein quantification. BV-2 cells were seeded as follows: 75,000 WT cells; 350,000 *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells; 150,000 *Acox1<sup>-/-</sup>* cells in 6-well-plates in 3 ml culture medium for 48 h. mHypoA seeding numbers were determined based on the final confluency as described earlier.

Then, the percentage of BV-2 CM in fresh medium in mHypoA culture and the time of incubation were regulated. Several protocols exist in the bibliography in which 30% to 100% of CM is used in the neuronal culture (Puzi & Vidyadaran, 2020). After testing several concentrations, it appeared that incubating the neurons in 100% BV-2 KO CM shows a clear impact on neuronal viability with yet no impact when neurons were incubated with the CM of BV-2 WT cells. Therefore, this concentration was fixed for all the experiments.

The time of incubation is also a matter of question. Incubation times of 6 h, 12 h, 16 h, 24 h, 48 h and 72 h have been tested. No changes were found for any duration less than 24 h. A mild decline in neuronal viability was observed at 24 h whereas an important change was observed at 48h. When the neurons were kept for 72 h in the BV-2 CM, we observe neuronal death at all conditions probably due to the depletion of the nutrients in the medium. Indeed, in this case, the medium was used for BV-2 culture for 48 h and next for the culture of neurons for 72 h making a total of 120 h of culture. To confirm this assumption, neurons were cultured for 48 h, then their supernatant was collected (will be referred as HypoCM for simplicity) and used to culture freshly seeded neurons. No impact was observed after 24 h and 48 h of incubation whereas decreased neuronal viability was observed after 72 h of incubation with HypoCM. HypoCM has no secretions which could impact the mHypoA cells. This shows that the culture medium would be depleted after 96h in culture, and this would be applied for any other CM.

Finally, experimental protocol was the following: BV-2 WT or KO cells were seeded in 6-well-plates according to previously determined seeding densities and kept cultured for 48 h. Their supernatants were then collected and centrifuged at 300 g for 7 min to remove all floating cells and debris. The medium was then stored at -80°C or freshly used to seed neurons, either in 6-well plates (70,000 neurons in 2 ml of BV-2 CM) or in 24-well-plates (20,000 neurons in 1 ml of BV-2 CM) or in 96-well-plates (5,000 neurons in 200 µl of BV-2 CM). The neurons were cultured for 48 h before analysis. It should be noted that we performed some tests which showed no difference when fresh or frozen media were used.

### III. Viability assay and cell death characterization

Cell viability was determined by MTT assay. The principle of the test is based on the use of the water-soluble MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which is reduced to formazan by mitochondrial succinate dehydrogenase in living cells. The quantity of formazan produced is proportional to the metabolic activity of the cells. This insoluble, violet-colored formazan crystal must be solubilized in Dimethyl sulfoxide (DMSO) or isopropanol to enable spectrophotometric determination at 570 nm. For this test, mHypoA or 158N cells were cultured in 24-well and 96-well plates at a density of 20,000 and 5000 cells/well respectively. Conditioned medium and co-culture experiments were performed as mentioned earlier. At the end of the culture, the supernatants were replaced with DMEM containing 1 mg/ml MTT (Sigma Aldrich, reference M2128-1G) and the cells were incubated at 37°C and 5% CO<sub>2</sub> for 3 h. The medium was then removed and DMSO was added (500 µl for 24-well plates or 100 µl for 96-well plate) to dissolve the formazan crystals. Absorbance of the assay solution was measured at 570 nm using a microplate reader (TECAN M200 Infinite Pro). MTT assay was performed in triplicates and repeated for at least 3 independent times (biological replicates) for reproductivity.

To characterize the cell death induced by coculture or conditioned medium, i.e. to determine the percentage of apoptotic or necrotic cells in each condition, Annexin V-FITC and propidium iodide (PI) staining was performed using the “Annexin V-FITC/PI Apoptosis Detection Kit” (Cohesion Biosciences, reference CAK2001). For this purpose, after CC or CM experiments, cells were trypsinized then washed with cold DPBS, resuspended in binding buffer at a density of  $3 \times 10^6$  cells / ml. A 100 µl suspension was then stained with 5 µl Annexin V-FITC, incubated for 5 min at room temperature in the dark then stained with /10 µL PI. After 5 min of incubation, 200 µl DPBS was added, and the resulting suspension was homogenized gently and subsequently analyzed using a LSRII Becton Dickinson Flow cytometer. For each sample, 10,000 events were captured and data analysis was performed using FlowJo V10 software.

### IV. Sholl analysis

Sholl analysis is a quantitative method used to characterize the morphologic parameters of neurons. For this purpose, neurons are stained and imaged. Neurons were seeded at 20,000 cells/ml on circular 10-mm diameter glass coverslips in 24-well plates in the CM or CC conditions for 48 h. Fluorescent immunocytochemistry was performed on neurons following

fixation with PFA 4% for 10 min, permeabilization with Triton x100 0.1% for 10 min and blocking with 5% mouse serum for 20 min, with PBS wash between each step. Neurons were stained with a global neuritic marker, mouse anti-MAP2ab antibody (Arigo Biolaboratories, reference ARG52328), diluted 1:200 in PBS containing 0.05% saponin and 2% mouse serum, incubated for 40 min then washed with PBS containing 0.05% saponin. Afterwards, the samples were incubated with the secondary antibody, AlexaFluor 546 goat anti-chicken (Invitrogen, reference A11040), diluted 1:1000 in PBS, 0.05% saponin, 2% mouse serum. After the last wash, coverslips were mounted in a fluorescent preservative medium.

The stained cells were observed at  $\times 20$  magnification using a Zeiss Axio Imager M2 epifluorescence microscope, equipped with an AxioCamMRm CCD camera and Axiovision software v4.8.2 (Carl Zeiss). Photos were taken  $\times 20$  mosaic option with the groups being blinded. Four different images were taken in 4-5 different zones. Positions are chosen randomly but with preference to those showing individualized neurons. From each image, 2 to 4 neurons were analyzed in order to have a total of 10-12 neurons studied at each condition. Two biological replicates were conducted obtaining a total of 20-22 isolated neurons. Neurite outgrowth was assessed using Fiji software (Schindelin et al., 2012) and the following parameters were examined: the number of neuritic branches, neuronal length and Sholl profile (Sholl, 1953). Neuronal length was defined as the total length a neuron measure between the farthest extremities. To generate the Sholl profile, the number of neuritic branches was plotted against the distance from the cell soma. Graphs were plotted using GraphPad prism version 9.4.1.

## **V. Calcium imaging**

Calcium imaging is a microscopy technique allowing the observation of calcium status of isolated cells using a probe (Fura-2 in our study) which chelates calcium ions, making it fluorescent. It represents a functional readout of neuronal activity. In our experiments, 70,000 mHypoA neurons were cultured on sterile coverslips in the CM of BV-2 WT and KO cells for 48 h in 6-well plates. Cells were loaded with 5  $\mu$ M Fura 2-AM (Invitrogen, reference F1221) for 30 minutes at 37°C and 5% CO<sub>2</sub> in HEPES-buffered saline solution. The Fura2-AM solution was removed, and cells were washed then mounted and observed using an inverted microscope (Olympus IX70). Fura-2 fluorescence images were acquired every 0.5 or 1 s by alternating excitation at 340 and 380 nm and emissions 420 and 600 nm, and were collected using a cooled, charge-coupled device camera with TiLL Photonics Live Acquisition software v2.4.0.18.

Values for the 340/380 nm fluorescence ratio, representative of  $[Ca^{2+}]_i$ , were obtained after correction for background fluorescence values. For baseline, intracellular  $Ca^{2+}$  levels were recorded for at least 60 seconds. Cells were then stimulated with ATP solution (100  $\mu$ M) using the integrated automated injection system and recorded for another 90 seconds. Afterwards, cells were reinjected with the HEPES-buffered saline solution and recorded for at least 90 seconds. Data were exported using Live Acquisition software version 2.4.0.18 by Till Photonics FEI. The percentage of fluorescence for each studied cell was then calculated considering the fluorescence for the first 60 sec (before ATP stimulation) as a baseline. The amplitude of the response and the response time to ATP stimulation were subsequently calculated and compared among the different culture conditions.

## **VI. Secretion of $\alpha$ -MSH**

The mHypoA cells were seeded in 6-well plates at a density of 70,000 cells per well using BV-2 WT or KO CM as their cell culture medium. After 48 h in culture, the supernatant was removed, cells were washed once with DPBS and 1.5 mL serum-free DMEM was added to the cells which were cultured for additional 4 h. To induce the secretion of alpha-melanocyte stimulating hormone ( $\alpha$ -MSH), the medium was replaced with serum-free DMEM containing 60mM KCl (1.5 mL per well) and the cells were cultured for additional 20 min. The media were then collected as 500  $\mu$ l aliquots and transferred to SpeedVac concentrator until the total evaporation of the supernatants (around 8 h). Afterwards, the samples were kept at  $-80^{\circ}C$  until the day of analysis. Samples were collected in triplicate for each experimental repeat. This protocol was published by (Nazarians-Armavil et al., 2014), and we obtained the detailed one after contacting their laboratory.  $\alpha$ -MSH secretion was determined using the  $\alpha$ -MSH (Human, Rat, Mouse) enzyme immune assay (EIA) kit (Phoenix Pharmaceuticals, Inc., reference EK-043-01). For this assay, the dried samples were rehydrated in 50  $\mu$ l assay buffer and the totality of the sample was loaded onto the assay plate according to the manufacturers' instructions. Absorbance measurements were performed on TECAN M200 Infinite Pro microplate reader.  $\alpha$ -MSH secretion values were normalized to 1/3 of the total number of cells per well, as 500  $\mu$ l were aliquoted out of the 1500, which is the volume of each well. Results were expressed as  $\alpha$ -MSH (pg) per 100,000 cells.

## VII. Treatment with purified TNF

To evaluate the toxic effect of TNF on the viability of neurons and oligodendrocytes, cells were treated with TNF at 0.447 ng/mL (corresponding to the concentration measured in the CM of LPS-treated BV-2 WT cells) or at 1.26 ng/mL (corresponding to the concentration measured in the CM of LPS-treated mutant BV-2 cells). The cells (mHypoA or 158N) were seeded in 24-well at a density of 20,000 cells per well in 1 mL of fresh medium and treated for 48 h with the mentioned concentrations of recombinant mouse TNF (R&D Systems, reference 410-MT). TNF was previously resuspended in PBS at a concentration of 100 µg/mL. MTT test was performed 48 h after the treatment, which corresponds to the duration of incubation of the neurons and oligodendrocytes in the CM of the BV-2 cells.

## VIII. Treatment with NO donor (SNAP)

To evaluate the toxic effect of NO on the viability of neurons and oligodendrocytes, cells were treated in the presence of a NO donor, the S-Nitro-N-acetylpenicillamine (SNAP) and the viability was evaluated. The concentration of NO measured in the CM of LPS-treated WT, *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* is null, 15 µM and 2.5 µM respectively. Several tests were carried out to determine which concentration of NO donor (SNAP) should be used to obtain a concentration of 15 µM and 2.5 µM of NO in the medium. Then, the mHypoA neurons and the 158N oligodendrocytes were seeded in 24-well plates at a density of 20,000 cells per well in 1 ml of fresh medium and treated for 48 h in the presence of 50 µM SNAP, which is the dose necessary to produce 15 µM of NO in the culture medium, or in presence of 6 µM SNAP, dose necessary to produce 2.5 µM of NO in the culture medium. The viability was then estimated using MTT assay.

## IX. Treatment with purified CTSB

To evaluate the toxic effect of Cathepsin B (CTSB) on the viability of neurons and oligodendrocytes, cells were treated for 48 h with cathepsin B at concentrations of 155 ng/ml or 460 ng/ml or 2518 ng/ml (concentrations measured in the CM of BV-2 WT, *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* respectively) for 48 hours. The cells (mHypoA or 158N) were seeded in 24-well plates at a density of 20,000 cells per well in 1 mL of fresh medium and treated with the mentioned concentrations of CTSB (Acro Biosystems, reference CTB-M52H9). MTT test was performed 48 h after the treatment, which is similar to the case of incubating the neurons and oligodendrocytes in the CM of the BV-2 cells.

## **X. Incubating neurons with the CM of myelin-exposed BV-2 cells**

To test the effect of the secretions of myelin-exposed BV-2 cells on the viability of neurons, BV-2 cells were seeded in 96 well plates for 24 h, then treated with 10 µg/mL myelin sheath debris prepared as described in the second paper. After the treatment, the supernatants were recovered, centrifuged at 1000 g for 10 min (to remove myelin debris from the medium) and stocked at -80° C until used.

Next, 5000 mHypoA cells were seeded in 96-well plates in 200 µl of the collected CM and cultured for 48h before MTT test was performed.

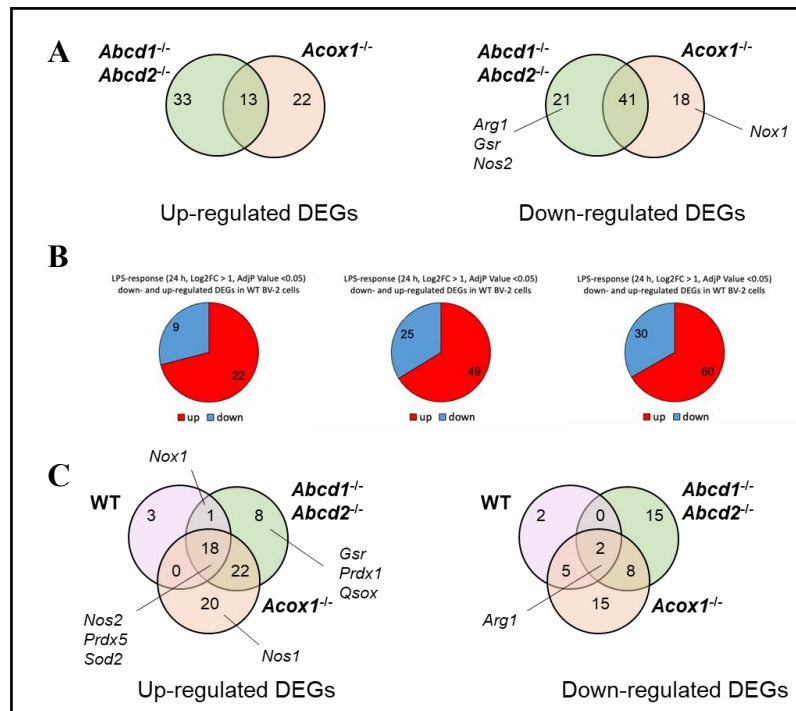


## I. Oxidative stress markers in BV-2 mutant microglial cells

### I.1. Oxidative stress: Transcriptomic data

The investigation of the oxidative stress markers in the WT and the mutant BV-2 cells was among the first areas of interest after the creation of the cells. This part was basically done by former students and other members of the team, however due to its importance in the story of my thesis, I mounted the figures and performed the necessary statistical analysis and thus presenting some important data below. The technical protocols of the experiments presented are described in the “Annex” section of this manuscript.

Genes related to oxidative stress are found to be differentially expressed in the mutant cells compared to the WT cells at basal conditions as well as upon LPS treatment as shown in figure 6. For example, nitric oxide synthase 2 (NOS2), which produces NO and is inducible by LPS and certain cytokines, is found to be upregulated in the WT as well the mutant cells when treated with LPS. However, it was found to be upregulated in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells at basal conditions. Furthermore, GSR gene, which encodes for the enzyme glutathione reductase, was found to be downregulated in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells at the basal conditions, but upregulated after LPS treatment only in these cells. The mentioned genes belong to a large list of oxidative stress related DEGs in the mutant cells, pointing out to an impaired redox regulation in these cells. To complement the transcriptomic data, the protein level of several modified genes is currently being analyzed by western blotting.



**Figure 6: Impact of peroxisomal defect on the expression of oxidative stress-related genes in WT and mutant (*Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>*) BV-2 cells.**

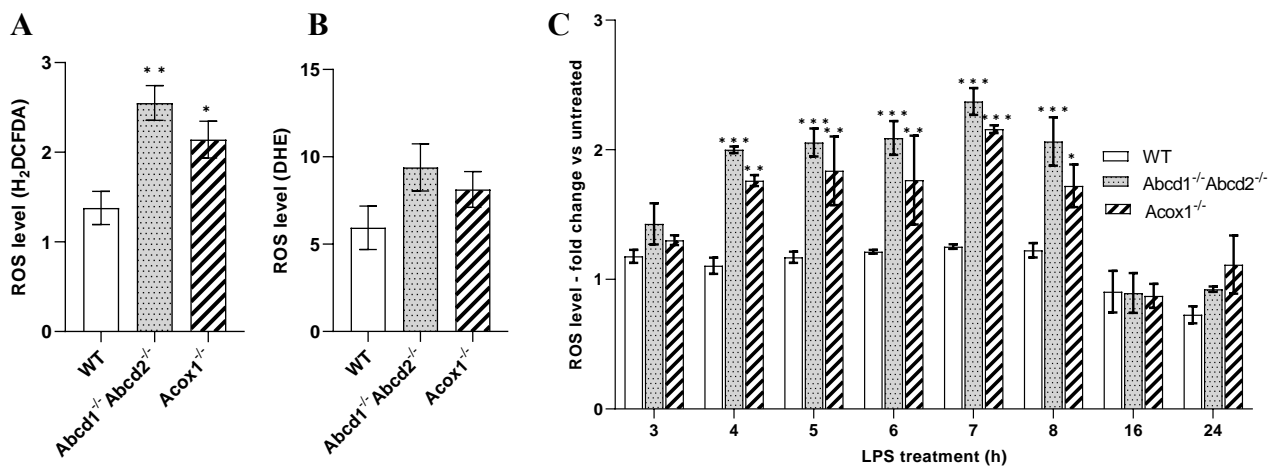
(A) Venn diagrams illustrating the up- and down-regulated DEGs (Log2FC > 1, AdjP Value < 0.05) encoding for oxidative stress markers in *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* non stimulated BV-2 cells. (B) Pie chart displaying the number of up- or down-regulated inflammatory cytokine-related DEGs after a 24 h LPS treatment (Log2FC > 1, AdjP Value < 0.05) in WT, *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* BV-2 cells. (C) Venn diagrams representing the LPS-response in each genotype.

## I.2. Increased ROS production in activated mutant BV-2 cells

ROS are kept in a state of dynamic balance through a combination of processes that generate ROS and cellular mechanisms that defend against them. Oxidative stress refers to the harm that arises when ROS overwhelms the body's antioxidant defense systems. This occurrence is a result of disrupted internal stability, either due to excessive ROS production or a decrease in defense mechanisms (Salim, 2017). Microglia, acting as phagocytes, have the ability to trigger an "oxidative/respiratory burst" as a response to damaging molecules and substances associated with pathogens, such as aggregated proteins and cellular debris (Claude et al., 2013; Herzog et al., 2019).

To complete the transcriptomic analysis of oxidative stress related DEGs, we studied the ROS production in the BV-2 cells with and without LPS treatment (figure 7). Using two ROS indicators, CM-H<sub>2</sub>DCFDA (large ROS specificity) and DHE (specific for superoxide), we showed significant changes in ROS production among *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* cells compared to WT cells at basal level, *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells having the most oxidative status.

The relative comparison of the ROS production between WT and KO cells using CM-H<sub>2</sub>DCFDA shows increases that are greater than those observed using the DHE probe. It would mean that KO cells produced not only superoxide but also other ROS species. However, the specificity of these probes being a matter of debate, the interpretation must be careful. Moreover, when treated with LPS, a higher induction in ROS production was observed in mutant cells compared to the WT cells. Such levels contribute to oxidative stress, which is hypothesized to cause neuronal death as there is a strong correlation between immune activation and oxidative damage in neurodegenerative disease (Simpson & Oliver, 2020; Tönnies & Trushina, 2017). A pattern of secretion with a peak of ROS production 7 h after LPS treatment was observed in both the WT and the mutant cells (figure 7C). This would imply that a correction of the redox balance begins to take place 7 h after the exposure to LPS. After 16 h of LPS treatment, ROS levels were almost the same between WT and mutant cells, suggesting that the antioxidant defenses of mutant cells are highly effective. Nevertheless, 24 h after LPS treatment, ROS levels were again higher in KO than in WT cells, as is the case under basal conditions. Altogether, these findings confirm the imbalance of the redox status in the peroxisomal mutant cells, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> presenting the most pro-oxidant status.



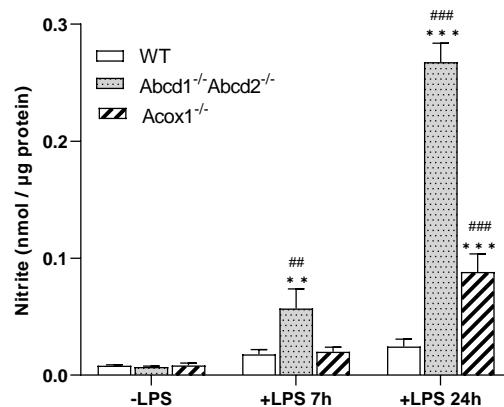
**Figure 7: Intracellular ROS level in WT and KO BV-2 cells.**

After 24h in culture, the cells were incubated with CM-H<sub>2</sub>DCFDA (A) or DHE (B). ROS fluorescence was standardized to Hoechst fluorescence. For each experiment, the triplicate fluorescence mean of KO BV-2 cells was compared to the triplicate fluorescence mean of WT BV-2 cells. Values are the means of four independent experiments. (C) Cells were treated with LPS (1 μg/ml) for 3 to 24 h. Following treatment and PBS washing, cells were incubated with CM-H<sub>2</sub>DCFDA (5 μM) for 70 min. For each experiment, the triplicate fluorescence means of LPS-treated BV-2 cells were compared to the triplicate fluorescence mean of untreated BV-2 cells. Values are the means of three independent experiments. Statistical significance was calculated by 1-way ANOVA at basal conditions (A, B) and 2-way ANOVA for LPS treatment (C). (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001) used for comparison of KO versus WT BV-2 cells.

### I.3. Increased NO production in mutant BV-2 cells

NO, considered an RNS, is a signaling molecule responsible for a large number of physiological functions, and can be harmful when produced in excessive concentrations. Its implication in many neurodegenerative diseases has been suggested.

As transcriptomic data showed that NOS2 gene was upregulated in the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells at basal conditions, NO production was analyzed in the WT and mutant cells, at basal level and upon activation with LPS. As shown in figure 8, a significant increase in nitrite production was observed in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> 7 h after LPS treatment, whereas a slight and insignificant increase was observed in WT and *Acox1*<sup>-/-</sup> cells. After 24 h of LPS treatment, a significant increase of NO production was observed in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> cells, around three times higher in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> than in *Acox1*<sup>-/-</sup> cells. These results show an increased NO production in the mutant BV-2 cells, especially the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, upon LPS treatment compared to WT cells. Such increase in NO levels is believed to exacerbate neuronal damage, a hypothesis we tried to investigate later.



**Figure 8: NO production by LPS-treated WT and KO BV-2 cells.**

Cells were treated with LPS (1 µg/ml) for 7 or 24 h. The level of nitrite, the final product of NO oxidation, was determined in the medium by Griess reaction and reported to protein quantity per well. Values are the means of four independent experiments. Statistical significance was calculated by 2-way ANOVA (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ) for comparison of LPS-treated cells versus untreated cells and (# $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ ) for comparison of KO cells versus WT cells within each condition.

## II. Impact of BV-2 secretions on neurons and oligodendrocytes

After detecting pro-inflammatory skewing in the mutant cells along with the increased secretion of DAM proteins and production of oxidative stress markers, we wondered how this alteration in microglial plasticity would impact the different aspects of neurons and oligodendrocytes. Therefore, using the designed co-culture and conditioned medium experiments, we investigated the viability of both neurons (mHypoA) and oligodendrocytes (158N) in the presence of WT and mutant cells' secretions. We further analyzed neuronal morphology and function under these conditions. The results below show the data obtained from conditioned medium experiments. The co-culture system showed similar results in the viability test in neurons. However, due to time limitations, we only used the CM system for the morphological and functional analysis of neurons.

### II.1. Viability of neurons and oligodendrocytes

#### II.1.1 Effects of CM of LPS-treated BV-2 cells

Using a classical cell viability test, MTT, we estimated the percentage of viable cells at each condition. No difference was observed when the neurons and oligodendrocytes were incubated with the CM of WT cells to those incubated in fresh medium, so the cells incubated in the CM of WT BV-2 cells were considered as control. When mHypoA neurons were incubated with the CM of LPS-treated WT cells, the viability decreased to 78% compared to the control. When exposed to the CM of *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells, the viability was also decreased to 79%. Interestingly, CM of LPS-treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells resulted in a significant decrease in neuronal viability, reducing it to 47%. Similarly, incubation with the CM of *Acox1*<sup>-/-</sup> cells led to a viability of 77% in neurons, which further dropped to 36% upon LPS activation (figure 9A).

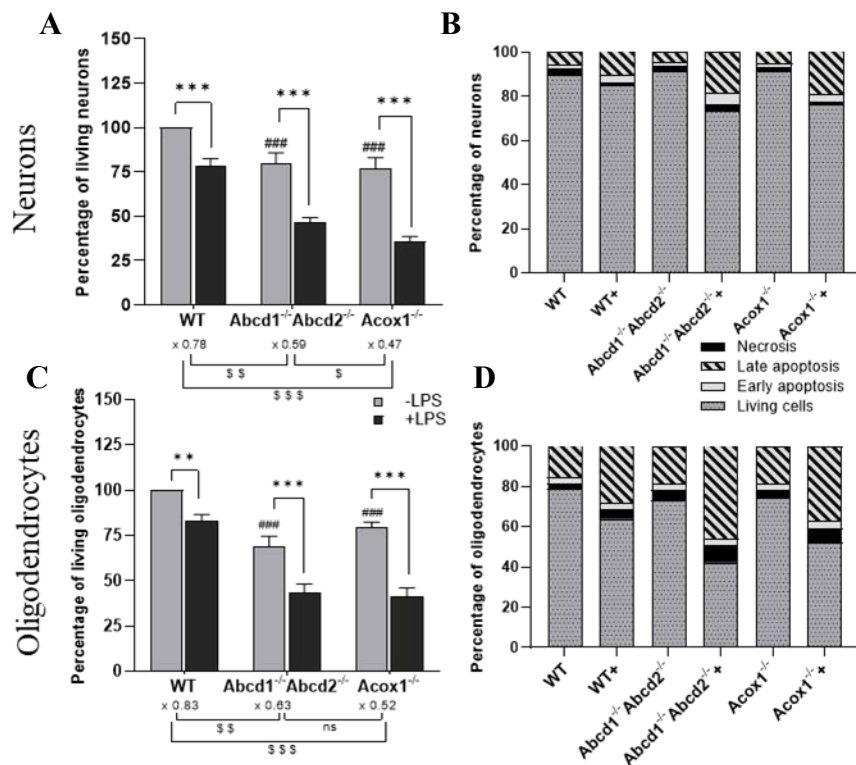
In the case of 158N oligodendrocytes, incubation with the CM of LPS-treated WT BV-2 cells led to a decrease in viability to 83% compared to the control. When exposed to the CM of *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells, the viability of oligodendrocytes decreased to 69%. CM of LPS-treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells further exacerbated this effect, reducing viability to 44%. Likewise, the CM of *Acox1*<sup>-/-</sup> cells resulted in an 80% viability of oligodendrocytes, which declined to 42% following LPS activation (figure 9C).

Therefore, culturing the neurons and oligodendrocytes in the CM of the mutant BV-2 cells leads to a significant reduction in cell viability, particularly when the cells were subjected to LPS activation. This finding strongly indicates that the CM derived from

mutant BV-2 cells possesses cytotoxic properties towards neurons and oligodendrocytes. To determine whether the observed decrease in cell viability corresponds to apoptotic cell death, we employed annexin-V-FITC/propidium iodide staining in combination with flow cytometric analysis.

Analysis of the flow cytometry data revealed that the cytotoxic effects induced by KO cells' CM are predominantly associated with apoptotic cell death (Figure 9B, D). In the case of neurons treated with LPS-activated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM, the percentage of viable cells was 73.1%, while apoptotic and necrotic populations accounted for 23.7% and 3.1%, respectively. Similarly, when oligodendrocytes were exposed to LPS-treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM, the viable cell population decreased to 42.2%, with apoptotic and necrotic populations comprising 48.9% and 8.9% respectively.

These compelling results confirm that the secretions of KO BV-2 cells exert cytotoxic effects on neurons and oligodendrocytes primarily through apoptotic cell death. The observed increase in apoptotic populations suggests that specific factors released by the KO BV-2 cells, which are absent in the WT cells' secretions, contribute to the apoptotic signaling pathways. Indeed, Kim et al. characterized the secretome, i.e. the CM, from BV-2 microglia that were co-stimulated with LPS and IFN $\gamma$  and demonstrated that such CM was neurotoxic when placed on primary cortical mouse neurons (Kim et al., 2007). Our results when using CM of LPS-treated WT BV-2 cells confirmed such neurotoxicity, but interestingly, it was more pronounced when the CM of the mutant cells was applied to the neurons, signifying that microglia with peroxisomal defects promote a further toxic environment.



**Figure 9: mHypoA and 158N cells' viability when incubated in the CM of KO BV-2 cells +/- LPS**

After being cultured in the CM of WT and KO BV-2 cells +/-LPS for 48 h, (A, C) represent the percentage of viable neurons and oligodendrocytes was determined by MTT test. The comparison of the viability of cells incubated with the CM of the mutant cells to those incubated in the CM of WT cells without LPS is shown as “#” (# $p$  < 0.05; ### $p$  < 0.001). “\*” shows that statistical comparison of the effect of LPS treatment within each genotype (\*\* $p$  < 0.01, \*\*\* $p$  < 0.001) and “\$” compares the impact of LPS treatment in the BV-2 WT and KO on the viability of the neurons and oligodendrocytes (\$ $p$  < 0.05; \$\$ $p$  < 0.01; \$\$\$ $p$  < 0.001; i.e the secretions of LPS treated WT cells decreased the viability of neurons by a factor of 0.78 compared to the secretions of WT -LPS whereas the secretions of LPS treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells decreased the viability of neurons by a factor of 0.59 compared to the secretions of *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> -LPS and the difference of LPS effect between WT and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> is significant with  $p$  < 0.01). The mode of cell death was identified by staining with Annexin-V-fluorescein-isothiocyanate (FITC) and propidium iodide using an apoptosis detection kit followed by FACS. (B, D) show the distribution of cells in living state, late apoptotic, early apoptotic and necrotic. The statistical differences were calculated using 2-way ANOVA followed by correction for false discovery rate for  $n=3$  independent repetitions for each experiment.

### II.1.2 Effects of TNF, NO donor, and CTSB

Proteomic studies have detected 187 and 155 proteins in supernatants of murine BV-2 and primary microglia, respectively, and demonstrated a significant difference in secretomes between unstimulated and immune-stimulated cells. A much larger number of proteins (4938) has been detected in supernatants of IFN- $\gamma$  or LPS-stimulated BV-2 cells (Woo et al., 2017). A component of this microglial secretome includes cytotoxic molecules capable of causing damage and death to CNS cells, thus contributing to the

pathogenesis of neurodegenerative disorders. Microglia neurotoxins include cytokines such as TNF, enzymes such as proteases, amino acids as glutamate, and small reactive molecules as hydroxyl radicals (Brown & Vilalta, 2015; Takeuchi, 2010).

Upon observing the demise of neurons and oligodendrocytes in the CM of the activated mutant cells, we became intrigued by the secretions which led to the death of these cells. To delve into this matter, we subjected mHypoA and 158N cells to TNF, NO, and CTSB, all of which were excessively present in the mutants' secretions, and are believed to have cytotoxic effect on CNS cells (Lindhout et al., 2021). The concentrations of TNF, NO and CTSB were previously measured in the secretome of the WT and KO BV-2 cells, thus we treated the cells with the same concentrations found in the WT versus that of the KO secretome. With these treatments, we aim at confirming the toxicity of these secreted molecules on the neurons and oligodendrocytes and to verify whether the higher doses present in the KO CM increase the mortality of these cells. Interestingly, we observed a substantial toxicity induced by TNF, with a dose-dependent effect on mHypoA. Specifically, at concentrations of 0.447 ng/ml and 1.26 ng/ml, cell viability plummeted by 35% and 51% respectively, in comparison to the control medium. While the impact on 158N cells was somewhat lesser, there was still a noticeable toxicity, with a decrease in viability of approximately 10% for both tested doses, still significantly lower than the control group (figure 10A).

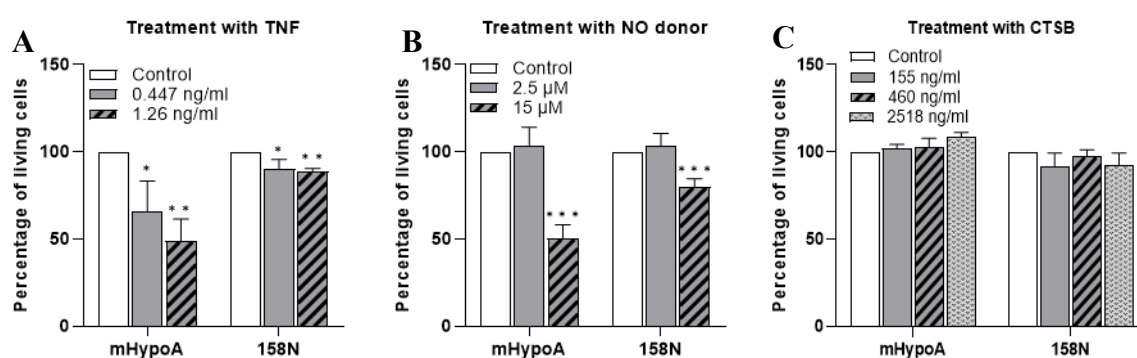
Neurons are particularly sensitive to oxidative damage. As more NO are produced by LPS-induced mutant cells, particularly *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*, and no production of NO was observed in the secretome of WT cells. We aimed at confirming that such concentration can impact neuronal and oligodendrocytes viability. We treated the neurons and oligodendrocytes with a concentration of NO donor (SNAP) that allows to obtain a concentration of 15  $\mu$ M and 2.5  $\mu$ M corresponding to that measured in the secretome of *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* respectively. With the treatment with NO donor, we witnessed a significant decrease in viability, with neurons experiencing a 53% reduction and oligodendrocytes experiencing a 22% reduction, at a concentration equivalent to that found in the LPS-treated *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* secretions (15  $\mu$ M). However, no toxicity was detected at a dosage of 2.5  $\mu$ M (figure 10B).

Cathepsin B (CTSB) belongs to a family of lysosomal cysteine proteases and plays an important role in intracellular proteolysis (Cavallo-Medved et al., 2018). It is also one of the DAM signature proteins which are found to be upregulated in neurodegenerative



diseases (Butovsky & Weiner, 2018). It was found that overloading microglial lysosomes through myelin debris accumulation induces lysosomal damage and CTSB extracellular release by lysosomal exocytosis, and that the inhibition of CTSB prevents neuronal death (Gabandé-Rodríguez et al., 2019). We aimed at testing whether the concentrations of CTSB secreted by the WT and the KO cells induce cytotoxicity in the neurons and oligodendrocytes in vitro. Our findings revealed no significant change in cell viability across all tested concentrations (figure 10C). This indicates that CTSB alone, even at high levels, does not induce cytotoxicity in the mHypoA and 158N cells. While previous research has demonstrated the involvement of microglial CTSB in driving neurodegenerative diseases primarily through neuroinflammation induction (Nakanishi, 2020; Pišlar et al., 2021), our results suggest that it might be one of several microglial secretions which together trigger signaling cascade that aggravate neurodegeneration, at least in the case of X-ALD.

These results underscore the vulnerability of neurons and oligodendrocytes to cytotoxicity, attributed to the inflammatory environment and oxidative stress induced by the mutant BV-2 cells. These findings could potentially explain the observed apoptotic death in mHypoA and 158N cells incubated in the mutant cells' secretions. It is important to note, however, that these results represent our initial exploration into understanding the cytotoxic factors secreted by microglia with peroxisomal defects, which contribute to neurodegeneration. Further analysis is imperative to fully understand the complexity of this phenomenon and its underlying mechanisms.



**Figure 10: Treatment of mHypoA and 158N with TNF, NO donor and CTSB.**

The percentage of living cells was determined using MTT test after 48 h treatment with purified TNF (A), NO donor (B) and purified CTSB (C) at chosen concentrations corresponding to those previously measured in the secretome of the BV-2 WT and KO cells as described in the material and methods. Data are shown as mean  $\pm$ SD from 3 to 4 independent experiments. Data were analyzed within each cell type using 1-way ANOVA. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\*  $P$ <0.001 for the comparison with the control.

### II.1.3 Effects of CM from myelin-exposed BV-2 cells

Microglia respond to neural injury and neurodegeneration by undergoing pronounced morphological and cell biological transformations to clear myelin debris and promote repair (Santos & Fields, 2020). Such exposure to myelin debris has been shown to suppress or reduce microglial inflammatory activities in vitro in demyelinating disorders such as MS (Grajchen et al., 2020; Y. Liu et al., 2006). Thus, we would hypothesize that exposing the KO BV-2 cells to myelin debris would promote an anti-inflammatory response and consequently decrease the neuronal death observed when incubating neurons with the CM of the mutant cells.

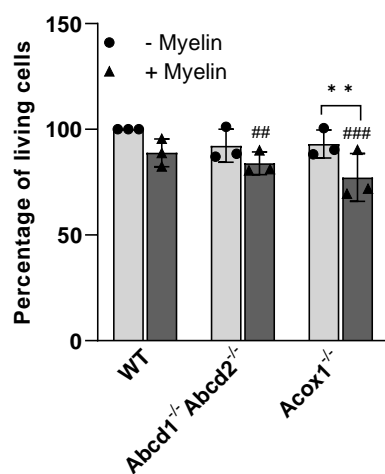
We therefore treated BV-2 cells for 24 h with myelin debris then collected their supernatants which are consequently used to culture mHypoA for 48 h. The results of the viability test indicated that the secretions from the myelin-exposed mutant cells still had a detrimental impact on neuronal viability although the negative effects were not as pronounced as those observed with LPS treatment. There was a significant decrease in the number of neurons when they were incubated with the CM obtained from myelin-treated *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* cells compared to control (neurons incubated with WT CM). A significant decrease in neuronal viability was also observed with the CM of myelin-exposed *Acox1<sup>-/-</sup>* cells compared to the those in the CM of *Acox1<sup>-/-</sup>* cells (Figure 11).

These results are intriguing and highlight the complex nature of microglial responses in the presence of myelin debris. While microglia are generally involved in promoting repair and reducing inflammation, our results suggest that under the absence of proper peroxisomal functioning, microglia may not respond as anticipated. Instead, the secretions from these mutant cells may still exert harmful effects on neurons. A possible explanation would be that the KO cells are already overwhelmed with the accumulated VLCFA, phagocytosing more lipids would further stress these cells thus rendering them more pro-inflammatory. A more pronounced decrease in neuronal viability was noticed in the presence of CM of myelin-exposed *Acox1<sup>-/-</sup>* compared to *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*, this would be linked to what we showed earlier as these cells phagocytose more myelin than the *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*.

Our results also demonstrated a slight but insignificant decrease in neuronal viability when incubated with the CM of myelin-exposed WT cells. We thus question whether it is BV-2 specific effect or if it is the used protocol. We would be certain about

the significance of the variation if we analyze the components of the secretome of the myelin-exposed cells, a step which was unfortunately not done due to the restrained time.

These results add to the growing body of knowledge on the intricate interplay between microglia, myelin debris, and neuronal health but with additional focus on peroxisome-specific microglial functions. Further research is warranted to elucidate the underlying mechanisms and identify potential therapeutic targets that could modulate microglial responses in autoimmune disorders and neurodegenerative diseases associated with myelin damage. Understanding these mechanisms is crucial for developing interventions that can harness the reparative potential of microglia while minimizing their detrimental effects, ultimately promoting neuroprotection and repair in the context of neural injury and neurodegeneration.



**Figure 11: Cell viability of mHypoA cultured in myelin-exposed CM of BV-2 cells.**

After exposing the BV-2 WT and KO cells for myelin sheath debris for 24 h, their CM was used to incubate the neurons. MTT test was used to investigate the cell viability at each condition. Data are shown as mean  $\pm$ SD from 3 independent experiments and analyzed using 2-way ANOVA followed by correction for false discovery rate. “\*” is used to compare the myelin effect within the same cell type (\*\* $P < 0.01$ ) whereas “#” is used to compare each condition with the control, i.e., neurons with unexposed WT secretions (## $P < 0.01$ ; ### $P < 0.001$ ).

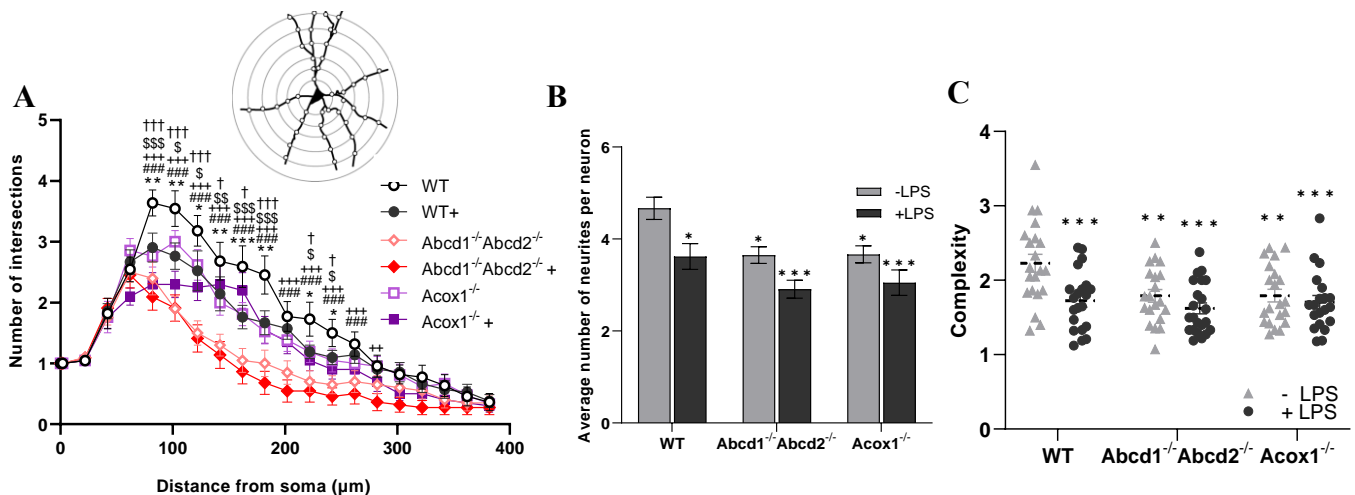
## II.2. Morphology of neurons

The release of inflammatory molecules by activated microglia can lead to synaptic dysfunction, impair neuronal signaling pathways, and promote neuronal death. These processes can ultimately result in a reduction in neuronal complexity, which refers to the structural and functional intricacy of neuronal connections and processes (Muzio et al., 2021). To assess whether the morphological parameters of neurons were also affected by the CM derived from our KO BV-2 cells, we performed Sholl analysis, a classical morphological test, on neurons subjected to different CM conditions. Two-way ANOVA analysis of the number of neuritic branches in each neuron revealed a significant effect of LPS treatment, but a more pronounced impact was observed with the KO CM. The average number of neurites per neuron decreased from 4.6 in the presence of CM from WT cells to

3.6 with LPS-treated WT CM. Notably, the number of neurites decreased from 3.6 with *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* CM to 2.9 with LPS-treated *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* CM. In the case of neurons incubated with *Acox1<sup>-/-</sup>* CM, the number of neurites decreased from 3.6 to 3 upon LPS treatment (Figure 12B).

The Sholl analysis, depicted in Figure 12A, demonstrated a significant difference among the different conditions. Further analysis was performed to assess neuronal complexity under each condition. Neuronal complexity was calculated by dividing the sum of interactions, which represents the sum of circles-neurites crossovers, by the number of circles intersecting a cell. Notably, neuronal complexity was significantly decreased when neurons were incubated with KO CM compared to WT CM. However, upon LPS treatment, no significant difference was observed when comparing the CM of LPS-treated WT or KO (Figure 12C).

These findings firstly indicate that microglia play a crucial role in regulating neuronal growth and connectivity. They also show that the CM derived from KO BV-2 cells exerts a detrimental impact on the morphological parameters of neurons. The reduction in the number of neurites and the overall decrease in neuronal complexity suggest impaired neurite outgrowth and ramifications. These alterations in neuronal morphology may have implications for neuronal connectivity and proper functioning, and are a characteristic of neurodegenerative diseases (Kovacs, 2019). The underlying cause of the observed alterations in neuronal morphology could be attributed to the reduced trophic support, as the absence of proper peroxisomal functioning alters a wide range of microglial functions and may lead to reduced secretion of these neurotrophic factors (such as Neurotrophin-3, Brain-derived Neurotrophic Factor, or even TGF- $\beta$ ) thereby impacting neuritic outgrowth. The excessive inflammation induced by LPS treatment and demonstrated by increased pro-inflammatory cytokines' secretion as well as the increased production of NO and ROS can be detrimental to neuronal health and morphology, as is the case of neurodegenerative diseases. Further investigations are warranted to elucidate the underlying mechanisms through which the KO CM modulates neuronal morphology. Such investigation would start by repeating the experiment on different neuronal cell type to eliminate the possibility of mHypoA-specific effects. This would be followed by investigating the cytoskeletal rearrangements in the neurons including the assembly of cytoskeletal proteins forming neuritic extensions and the expression of related genes.



**Figure 12: The impact of KO BV-2 cells secretions +/- LPS on the neuronal morphology.**

(A) shows a schematic illustration of the Sholl analysis method for an isolated neuron and a graph showing the Sholl profile of mHypoA cells incubated in the secretions of BV-2 cells for 48 h. Neurons seeded in WT secretions were considered as control. \* $P < 0.05$ , \*\* $P < 0.01$  \*\*\* $P < 0.001$  (neurons in WT +LPS CM), #### $P < 0.001$  (in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM), ++ $P < 0.01$ , +++ $P < 0.001$  (in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> +LPS CM), \$ $P < 0.05$ , \$\$ $P < 0.01$  \$\$\$ $P < 0.001$  (in *Acox1*<sup>-/-</sup> CM), † $P < 0.05$ , ††† $P < 0.001$  (in *Acox1*<sup>-/-</sup> +LPS CM) compared to neurons incubated in WT CM. (B) shows the average number of neurites per neuron as determined through Sholl profile, and (C) represents the complexity of neurons at each condition. Complexity is calculated by dividing the sum of interactions (between neurites and concentric circles) over the number of circles intersecting a cell. \* $P < 0.05$ , \*\* $P < 0.01$  \*\*\* $P < 0.001$  compared to WT -LPS. Data expressed as mean ± SEM (n = 18-20 cells from 2 independent experiments). The statistical differences were calculated using 2-way ANOVA followed by correction for false discovery rate.

## II.3. Functions of neurons

### II.3.1. Calcium trafficking

Calcium ions ( $\text{Ca}^{2+}$ ) serve as a ubiquitous secondary messenger, playing a crucial role in regulating various essential cellular processes across all eukaryotic cells.

This reliance on  $\text{Ca}^{2+}$  signaling is perhaps more pronounced in neurons compared to other eukaryotic cells. In neurons, the role of  $\text{Ca}^{2+}$  in signaling has distinctive and significant features. Specifically,  $\text{Ca}^{2+}$  signals regulate synaptic transmission, which involves the release of neurotransmitters and a direct correlation between plasma membrane depolarization and intracellular  $\text{Ca}^{2+}$  increase. These processes are crucial for neuronal function and their proper functioning is vital to neuronal survival. To connect the  $\text{Ca}^{2+}$  signal to their biochemical machinery, neurons have evolved complex and intricate  $\text{Ca}^{2+}$  signaling pathways. The influx of  $\text{Ca}^{2+}$  into neurons is facilitated through voltage-dependent ion channels and plasma membrane receptors (Brini et al., 2014).

Given the critical significance of  $\text{Ca}^{2+}$  signaling in neuronal operation, it is not surprising that its abnormalities would be associated with neurological defects. The

malfunction of  $\text{Ca}^{2+}$  regulation is progressively being established as a causal factor in various crucial neuronal pathologies, especially those marked by neurodegeneration (Cali et al., 2012; Camandola & Mattson, 2011; Duchen, 2012; Foskett, 2010).

Based on what is mentioned above, and to verify whether the general functioning of the neurons is impacted by the presence of the secretions of KO BV-2 cells, we were interested in studying the calcium flux in neurons after the incubation in CM. We used ATP as a stimulant to induce the calcium influx into the neurons.

We performed  $\text{Ca}^{2+}$  imaging experiments following the 48-hour incubation period, using ATP (100  $\mu\text{M}$ ) as a stimulus. Out of the analyzed parameters, we found that “the maximum attained fluorescence intensity” and “the response time” were among the most dysregulated ones.

Our results revealed significant modifications in these parameters in neurons exposed to different CM conditions. Specifically, the amplitude of fluorescence, which reflects the concentration of  $\text{Ca}^{2+}$  entering the cell upon ATP stimulation, was significantly increased in neurons incubated with *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM compared to neurons incubated with WT CM. However, when cultured with LPS-treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM, the amplitude of fluorescence was significantly reduced compared to non-treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM. No significant changes were observed with the other conditions (Figure 13B).

In neurons incubated with WT CM, the amplitude of fluorescence reached its maximum 60 seconds after ATP stimulation (corresponding to 120 sec of recording time), and this remained relatively unchanged in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM (61 seconds). However, it slightly decreased to 56 seconds with LPS-treated WT CM and significantly decreased to 53 seconds with non-treated *Acox1*<sup>-/-</sup> CM. These findings indicate that neurons incubated with *Acox1*<sup>-/-</sup> CM exhibited increased sensitivity to ATP stimulation or a faster response, leading to a faster calcium influx compared to control conditions.

Interestingly, in the presence of LPS-treated *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> CM, the neurons exhibited a delayed response to ATP stimulation, taking 77 seconds to reach the maximum fluorescence intensity. This response time was significantly higher than in all other conditions. Additionally, a slight increase to 64 seconds was observed with LPS-treated *Acox1*<sup>-/-</sup> CM. Therefore, in the presence of LPS-treated KO CM, the response of the neurons to ATP stimulation was late compared to the control conditions (Figure 13C).

These findings suggest that the calcium signaling dynamics in neurons are altered when exposed to KO CM, particularly in response to ATP stimulation. The observed changes in amplitude and response time indicate disrupted calcium homeostasis and altered neuronal responsiveness in the presence of KO CM.

The ionotropic P2X receptors (P2XRs) respond to extracellular ATP inducing membrane depolarization and  $\text{Ca}^{2+}$  influx (Pankratov et al., 2009). Recent evidence suggests that ATP released from astrocytes can activate these receptors which have a relatively high calcium permeability allowing calcium entry at both hyperpolarized and depolarized membrane potentials (Egan & Khakh, 2004; Pougnet et al., 2014). Verifying differential expression or activation of these receptors as a result of incubation with the CM of mutant cells could be a key in understanding the observed differences.

To sum up, our data showed a difference between conditions, precisely a smaller yet insignificant peak of calcium flux when the neurons were incubated with the CM of *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* and *Acox1<sup>-/-</sup>* suggesting modified functions of neurons which need to be further confirmed through several biological repetitions. A delay in the response to ATP stimulation was also observed in neurons in activated *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* secretions thus further indicating irregularity in the neuronal functioning at this condition.

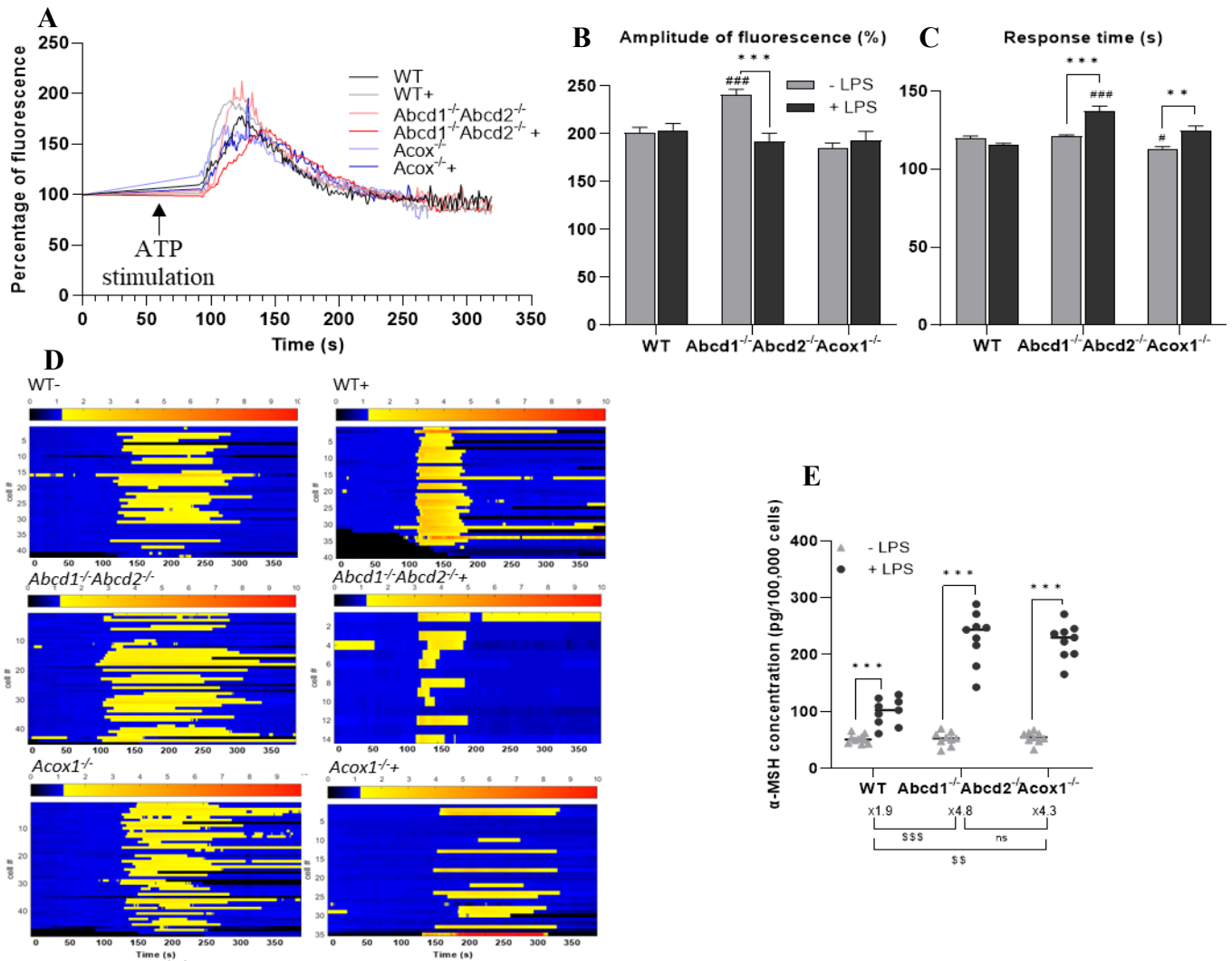
### **II.3.2. Secretion of $\alpha$ -MSH**

Our study aimed to investigate how peroxisomal impairment in microglia can contribute to neuronal defects through elucidating the influence of microglial secretions on the functional aspects of the neurons. mHypoA cell line is considered as a specific type of hypothalamic proopiomelanocortin (POMC) neurons known to produce various peptides, including  $\alpha$ -MSH (Belsham et al., 2004; Nazarians-Armavil et al., 2014). Given that  $\alpha$ -MSH secretion represents a distinct function of POMC neurons, we sought to investigate whether the CM derived from mutant BV-2 cells impacts  $\alpha$ -MSH secretion in these neurons. In other words, even if it is not related to the context of X-ALD, we were interested in checking whether microglial secretions impact the specific functioning of these neurons. The results of our investigation revealed a significant impact of microglial secretions on the production and release of  $\alpha$ -MSH from mHypoA neurons. Treatment of microglia with LPS led to a pronounced induction of  $\alpha$ -MSH secretion by mHypoA cells. Specifically, incubation of mHypoA neurons with the CM of WT BV-2 cells resulted in  $\alpha$ -MSH production of 52 pg/100,000 cells. However, when exposed to the CM from LPS-

activated WT BV-2 cells, the  $\alpha$ -MSH production increased to 99 pg /100,000 cells. Similarly, incubation with the CM of *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells yielded  $\alpha$ -MSH production of 52 pg/100,000 cells, but drastically increased to 230 pg/100,000 cells when the CM was derived from LPS-activated *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* cells. Likewise, the CM from *Acox1<sup>-/-</sup>* cells resulted in  $\alpha$ -MSH production of 54 pg/100,000 cells, which increased to 223 pg/100,000 cells upon LPS activation. Notably, the induction of  $\alpha$ -MSH secretion was more pronounced when mHypoA neurons were incubated with secretions from LPS-activated BV-2 KO cells (Figure 13E).

These intriguing findings shed light on the regulatory role of microglia in modulating the functions of POMC neurons, particularly in the context of inflammation and peroxisomal dysfunction. As far as we know, there has been no link between peroxisomal diseases and  $\alpha$ -MSH secretion, yet we investigated the secretion of this neuropeptide as a part of studying the functional communication between microglia and neurons. Further investigation into the underlying pathways involved in the induction of  $\alpha$ -MSH secretion will provide valuable insights into novel roles of microglia in regulating POMC neuronal functions under normal and pathological conditions.





**Figure 13: General and specific functioning of mHypoA cells examined by calcium imaging and  $\alpha$ -MSH secretion respectively.**

The time course of fluorescence signal in neurons with the ATP stimulation at 60 sec. Representative curves (with no error bars) are presented (A). The average amplitude of neurons at each condition (B) and the response time which corresponds to the time at which the amplitude of fluorescence is reached (C) and the intensity of activation of each neuron over time (D) “#” represents the significance of comparison with WT -LPS. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  for the comparison of LPS effect within a genotype. (E) shows  $\alpha$ -MSH secretion by mHypoA cells after 48 h incubation in BV-2 WT, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>, and *Acox1*<sup>-/-</sup> CM treated or not with LPS. Data were normalized over the number of cells and expressed as pg / 100,000 cells. \$\$ represents statistical significance for the LPS effect between the three conditions with  $P < 0.01$ ; \$\$\$ for  $P < 0.001$ . “\*” is again used for the comparison of LPS effect within a genotype. The statistical differences were calculated using 2-way ANOVA followed by correction for false discovery rate from three biological repetitions accounting for  $n = 37$  to  $n = 129$  neurons for the calcium imaging. For  $\alpha$ -MSH secretion, the values represent 3 technical and 3 biological replicates.

## **Key Findings of Article 3 and Future Directions**

In this study, the investigation into the effects of peroxisomal defects in the mutant BV-2 cells has provided an insight into the cellular dynamics of oxidative stress and its implications for neuronal and oligodendrocyte health. The findings highlight a significant elevation in oxidative stress markers within the mutant BV-2 cells, particularly following exposure to LPS, suggesting a compromised capacity of the mutant BV2 cells to manage oxidative insults effectively. This susceptibility could be linked to impaired ROS detoxification mechanisms or altered antioxidant defense pathways. Moreover, the conditioned medium from mutant BV-2 cells was observed to induce apoptotic death in both neurons and oligodendrocytes, underscoring the deleterious impact of these activated mutant cells. Interestingly, the susceptibility of neurons and oligodendrocytes to cell death was evident upon treatment with TNF and NO, further linking inflammation and oxidative stress induced by the mutant BV-2 cells to cellular demise.

The study's implications extend beyond cell death induction, as the secretions of mutant BV-2 cells were shown to influence neuronal morphology and activity, notably affecting calcium signaling. This emphasizes the broad impact of these mutant BV-2 cells on neuronal health, underscoring the potential relevance of peroxisomal functions in maintaining proper neuronal function.

Additionally, the incubation of mHypoA cells in the secretions of myelin-exposed mutant BV-2 cells was found to compromise neuronal viability. These intriguing results warrant further investigation into the specific factors involved in this detrimental interaction.

These findings collectively raise intriguing questions about the broader implications of peroxisomal dysfunction in neurodegenerative processes and highlight potential therapeutic avenues for mitigating oxidative stress-induced neuronal damage.

# General discussion

## Transcriptomic analysis

The RNA sequencing (RNAseq) offers tremendous opportunities for various types of analysis, but constraints like limited time and funds force us to make choices and prioritize our investigations. In our study, we focused on confirming the overexpression of specific genes such as those associated with lipid metabolism, phagocytosis, lysosomes, autophagy, DAM signature, antigen presentation, inflammation and oxidative stress. However, due to the depth and complexity of the involved pathways, we were unable to conduct a thorough analysis of all the altered pathways resulting from peroxisomal gene knockout in the microglia, primarily due to time limitations. For instance, investigating the expression of cannabinoid receptors would have added a new horizon especially after the recent treatment approach which was proposed by Parameswaran and collaborators (Parameswaran et al., 2022). Some other examples of possible investigations include analyzing cell cycle and cell division pathways as this would have given an explanation about the decreased multiplication observed in the knockout cells, or NF- $\kappa$ B and MAPK pathways for deeper exploration of the induced inflammatory profile and the control of different cellular processes.

The RNAseq experiments were conducted under basal conditions and after subjecting the cells to 24-hour LPS treatment. Additionally, it was performed following a 24-hour treatment with 25-HC. In fact, it has been shown that the synthesis of 25-HC can be upregulated in some neurological disorders including X-ALD (Odnoshivkina et al., 2022), and that 25-HC mediates the neuroinflammation of X-ALD via activation of the NLRP3 inflammasome (Jang et al., 2016). Therefore, analyzing these data sets could have provided us with further insights and revealed additional altered pathways, but unfortunately, I was unable to do so within my given time frame.

Interestingly, we observed that ROS reached their peak levels 7 hours after LPS treatment and then decreased at the 24-hour mark. To gain more valuable data, conducting RNAseq after a 6-hour LPS treatment could have been beneficial. However, we must be realistic and acknowledge that even if we had performed this additional experiment, it would have been challenging to analyze the data thoroughly given our existing limitations.

To sum up, while RNAseq presents numerous opportunities for research, the constraints of time and funds necessitate careful selection and prioritization of investigations. Despite the limitations, our study provided valuable insights into the expression of certain genes involved

in several microglial functions. Although we were unable to perform in-depth analysis of all the pathways affected by peroxisomal gene knockout in microglia or explore the RNAseq data from 25-HC treatment, the study still yielded significant findings that contribute to our understanding of the underlying processes.

## **Defects associated with the knockout of the peroxisomal genes**

Several alterations in different pathways and cellular processes have been discovered in the BV-2 cells after the knockout of the peroxisomal genes. However, the four mutant cell lines did not show the same phenotype, and sometimes showed unexpected differences. The observed differences can be attributed to several factors, primarily arising from the distinct genetic alterations.

As expected, a more severe phenotype was observed in most cases in the double knockout (*Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>) cells. To explain the difference between the double knockout and the single knockout (*Abcd1*<sup>-/-</sup>, *Abcd2*<sup>-/-</sup>) cells, the substrate specificity and the functional redundancy of the transporter should be taken into consideration. The knockout of *Abcd2* prevents the possible compensation of VLCFA transportation thus increasing the severity of the phenotype in the double knockouts. However unexpectedly, more pronounced modifications were sometimes observed in the single knockout cells. We could hypothesize that there might be certain metabolic compensatory pathways that are not activated in the single knockouts due to a mild phenotype but are activated in the double KO due to a more severe disturbance and consequently masking the entire phenotype.

Even though ABCD1 and ABCD2 have overlapping substrate specificities, some FAs are still transported by one transporter and not the other. Thus, if a specific FA is not metabolized correctly in a single knockout, it would directly or indirectly trigger modifications of gene expression and cellular functions, providing a possible explanation about the observed phenotype difference among the knockout cells. Another factor which should be taken into consideration is the role of the peroxisomal ABC transporters besides the importation of VLCFAs, such as the interaction of ABCD1 with spastin which was found to mediate the crosstalk between peroxisomes and LDs (C. L. Chang et al., 2019). It is currently unknown whether ABCD2 has the same tethering properties or other unidentified functions. To gain deeper insights, it is essential to closely characterize the single knockout cells and compare them with the *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells. This comparison would unravel the direct and indirect roles of each transporter on microglial functions and shed light on the consequences of accumulated

fatty acids on general and specific cell functions. A more specific characterization of lipid metabolites or biosynthetic pathway intermediates affected by the ABCD1 and ABCD2 defects in these cells should thus be considered.

The knockout of peroxisomal ABC transporters (*Abcd1* and *Abcd2*) leads to impaired fatty acid import into the peroxisomes, resulting in disrupted  $\beta$ -oxidation. Conversely, the knockout of the *Acox1* gene affects the enzyme responsible for the initial step of peroxisomal  $\beta$ -oxidation. This could partially explain the differences with the *Acox1*<sup>-/-</sup> cells. Here it should also be noted that what is imported might be mainly catalyzed by ACOX1, but there are other ACOX enzymes and probably other possible compensatory pathways activated in the peroxisome. As the peroxisomal metabolism is tightly connected to lipid metabolism, its activity and products depend on the activity of elongases, desaturases, esterases, phospholipases and other enzymes located in the ER, the mitochondria, or the cellular membranes. Thus, we would also expect the activation of compensatory pathways in different cellular compartments.

It is well known that different genetic modifications have varying impacts on cellular metabolism, lipid homeostasis, and downstream signaling pathways. However, the number of DEGs among the knockout cells was unexpectedly high, demonstrating a huge modification in key microglial functions. Trying to explain this, we might attribute it to the huge availability of the ligands of PPARs or LXR, and the activation of other transcription factors related to fatty acids, sterols and steroids, or oxidative stress which would suppress gene expression. The increased levels of some signaling lipids would cause changes in various signaling pathways. Additionally, varying lipid levels might cause modifications in the biophysical properties of the membranes as well as their signaling properties. Some cellular consequences can be linked to the accumulated VLCFA and cholesterol. Such accumulation could block lysosomes, induce ER stress, oxidative stress and consequently the activation of NF- $\kappa$ B signaling pathway. However, this cannot explain all observed differences, simply due to the fact that VLCFA accumulation was observed in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Acox1*<sup>-/-</sup> whereas cholesterol accumulation was especially prominent in *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup> cells. Thus, understanding the alterations in lipid metabolism caused by these genetic modifications will provide valuable information about the underlying mechanisms and potential therapeutic targets for the associated disorders. A detailed lipidomic analysis, with a special focus on cholesteryl esters and membrane lipids would help in uncovering such alterations. Additionally, the investigation of the derivatives of some lipids, such as prostaglandins and leukotrienes which are derived from arachidonic acid (Lakshmanan, 2021), would provide an insight into the cytokine-

independent inflammatory mechanisms in the mutant cells. Anti-inflammatory mediators such as maresins and neuroprotectins are also worth investigating.

### **Signature of the knockout cells**

The decrease in the expression of P2RY12 and CX3CR1 receptors in all deficient microglial cells serves as a reliable indicator of the loss of the homeostatic state associated with the TGF- $\beta$  signaling pathway, as demonstrated by Butovsky et al. (Butovsky et al., 2014). This indication is further supported by the increased secretion of pro-inflammatory cytokines and the production of oxidative stress markers.

*Abcd1*<sup>-/-</sup> and *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells showed a similar expression pattern of DAM signature genes even though they do not show similar accumulation of VLCFAs (Raas, Gondcaille, et al., 2019). The confirmation of the upregulated expression at the protein level provided strong evidence that these cells exhibit the phenotype commonly associated with neurodegenerative diseases. This finding further validates these cells as an appropriate model for studying neurodegeneration associated with X-ALD. Such findings also allow us to question the possible involvement of peroxisomal dysfunctions in other neurodegenerative diseases such as in AD and MS, as reviewed in (Zarrouk et al., 2020), or even in other neurodegenerative diseases. We showed that peroxisomal defects led to DAM signature, but it has been also shown that lysosomal disorders, mitochondrial dysfunction, and accumulation of A $\beta$  all lead to DAM as well (Cognoux et al., 2019; Y. Li et al., 2022). This allows us to wonder about the role of specific lipids, cholesterol, or the crosstalk between organelles in the development of DAM.

A pilot study on possible biomarkers performed using these microglial cells has shown promising results, highlighting their potential in basic research. However, to consider these biomarkers for clinical application in X-ALD follow-up and treatment, a large-scale study confirming their reliability and accuracy, followed by a longitudinal study on young cALD boys as well as in treated patients is necessary. Yet, before moving forward with clinical studies, we believe that it is crucial to address several scientific concerns and conduct further basic research. This would pave the way for future breakthroughs in X-ALD diagnosis, follow-up, and treatment.

### **The impact on neurons and oligodendrocytes**

Although the use of the mHypoA neuronal cell line may not be ideal for studying neurodegeneration, the observed changes in viability, apoptosis, morphology, and calcium

signaling indicate that the KO microglial secretions modify normal neuronal structure and activity. These neurons have limited dendritic extensions and they grow and differentiate without the need for astrocytes in the culture, this questions the practicability of the Sholl analysis. Yet, this morphological analysis had been applied as a classical morphological test on neuronal cell lines and published in several peer reviewed articles.

The decreased viability of neurons and oligodendrocytes suggests the presence of cytotoxic factors secreted by the mutant microglial cells. The direct impact of TNF and NO on both cell types yielded similar patterns of decreased viability, highlighting their potential role in the observed cytotoxicity. However, this finding adds little to the existing literature, necessitating further analysis of the conditioned medium's contents to identify the factors responsible for altering neuron and oligodendrocyte viability as well as neuronal morphology and activity. Several approaches can be considered for such analysis, including a proteomic analysis of the secretome of the WT and KO BV-2 cells, which would complement our transcriptomic data, allowing the identification of all the possible cytotoxic factors secreted by the KO cells. This would be followed by targeting specific secreted substances, using blocking antibodies for example, and verifying whether the cytotoxic impact is reduced. In one of our experiments, we used purified TNF and demonstrated its cytotoxicity at the concentration found in the KO cells' CM. Blocking its action using anti-TNF in the CM of KO cells, and probably blocking other cytotoxic factors such as NO in parallel, would confirm our speculations and help in identify potential molecular mechanisms leading to cytotoxicity.

In this study, CC system was utilized aiming at investigating the potential crosstalk between neurons and microglia which might supplement the results obtained with CM. Although the optimization of both systems was time-consuming, the study mainly focused on CM, and a detailed comparative analysis between the two systems was not pursued due to time limitations. Mixed culture system, which would allow to study the influence of cell contact and the possible involvement of CX3CL1/CX3CR1 axis, was also considered (Pawelec et al., 2020). It was however not executed due to the restricted time.

## **Peroxisomal defects in microglia, VLCFA accumulation, inflammation, oxidative stress and neurodegeneration**

The pathophysiological mechanisms of X-ALD remain unclear, but some in vitro/in vivo research showed that VLCFA could induce oxidative stress and inflammation, leading to cellular damage (J. Yu et al., 2022). Some evidences have supported the involvement of

microglia in the pathogenesis of X-ALD. The zones of activated microglia in the demyelinating regions in the brain, corresponding to areas where myelin contains higher levels of VLCFAs, along with the elevated levels of pro-inflammatory chemokines in the cerebrospinal fluid of cALD patients allowed to suggest that microglial dysfunction and apoptosis might constitute an early pathogenic change in cerebral X-ALD, contributing to neuroinflammation and the alteration of the neurovascular unit. It also allowed to suggest that the abnormal microglial functions could impair the ability to provide neuroprotective factors to deficient oligodendrocytes (Cartier et al., 2014; F. S. Eichler et al., 2008).

Here, we demonstrated that the microglial cell model of X-ALD, accumulating VLCFA, shows secretions of proinflammatory cytokines and production of oxidative stress markers which we proved to be cytotoxic on neurons and oligodendrocytes. This sheds light not only on the involvement of microglia in the pathogenesis of this neurodegenerative disease, but also on its role in the initiation of the pathogenesis of X-ALD.

## **Limitations**

The limitations of the current study are multifaceted and should be considered when interpreting the results. Firstly, one major limitation is the use of a microglial cell line. Microglia, being specialized cells in the CNS, have a unique and delicate environment. It has been demonstrated by Das et al. that BV-2 microglial cell line differs from primary cell lines in several aspects (Das et al., 2016). These differences could potentially affect the behavior and responses of microglia in vitro, limiting the generalizability of the findings. Yet, the fact that BV-2 show diminished response to LPS compared to primary cells allows us to expect even more important effects in primary cells such as increased pro-inflammatory cytokines secretions with LPS treatment. Besides, the in-vitro culture of microglia is by itself a limitation, as both primary microglia and cell lines exhibit dysregulated homeostatic molecular signatures in vitro. This dysregulation is thought to occur due to the absence of influence from the mature brain microenvironment and the functional BBB. The culture conditions may also contribute to promoting a pro-inflammatory phenotype in microglial cells. This limitation highlights the need for caution when extrapolating in vitro findings to the in vivo scenario. Although, it is worth noting though that in order to avoid any bias, and to obtain reproducible results, we completely controlled the cell culture conditions (seeding densities, passage number, duration of culturing, etc.). Some researchers might also doubt the use of the fetal bovine serum in the culture medium



as it might introduce bias, but its use was absolutely necessary as serum deprivation promotes immediate activation of the BV-2 cells and modifies their adhesion properties.

Similarly, the use of a specific neuronal cell line, particularly a hypothalamic one (POMC), might pose doubts. However, in the cerebral form of X-ALD, there's no single type of impacted neurons, but rather a pathology impacting mainly the corpus callosum regardless of the neuronal type. Indeed, confirmation of the results using primary neurons would strengthen the study's conclusions. Additionally, it would be more logical if the neurons being studied exhibited the accumulation of VLCFA, a characteristic feature of the condition under investigation, or had peroxisomal genes knocked out or knocked down. But we can still do investigations using normal neuron as it was shown in the literature that neurons are not directly impacted by their own peroxisomal defects but rather by the peroxisomal defects in microglia, infiltrating macrophages, astrocytes, and oligodendrocytes (Bottelbergs et al., 2010). Furthermore, the reliance on a single oligodendrocyte cell line (158N) introduces limitations. Using additional cell lines or primary oligodendrocytes would provide more robustness to the results.

In terms of experimental setup, the use of conditioned medium or co-culture systems, while informative, should be supplemented with a more complex culture system. Incorporating multiple cell types such as neurons, microglia, oligodendrocytes, and astrocytes would better mimic the cellular interactions and communication that occur in the brain. For instance, a brain organoid, which allows direct contact between these cell types, or a cerebral organoids through differentiating iPSCs from ALD patients to microglia cells could offer deeper insights into the pathogenesis underlying X-ALD. In fact, the latter model had been developed but not characterized (Lindborg et al., 2016; Muffat et al., 2016). Such models would better represent the complexity of the disease and provide a stronger basis for understanding its mechanisms.

Last but not least, despite setbacks and constraints, this research reveals a significant and unexpected link between peroxisomal functions and microglial processes, ultimately impacting the central nervous system's homeostasis. By shedding light on peroxisome-specific microglial functions, this study contributes to a better understanding of the complex interactions within the CNS and opens new avenues for future research in microglial biology and related neurological conditions.

## Conclusion and perspectives

In the course of this investigation, we demonstrated the pivotal role that peroxisomes play in facilitating optimal microglial functionality and settled a link between the pathogenesis of X-ALD and the disruption of microglial homeostasis. We confirmed the vast impact of peroxisomal dysfunction on lysosomes, autophagy, lipid metabolism, DAM signature, immune response, cytokine secretion, phagocytosis, T cell response and oxidative stress in the BV-2 cells. We established a causal relationship between mutant microglia and their capacity to elicit cytotoxic responses in neurons and oligodendrocytes. A deleterious impact which appears to be mediated by the release of pro-inflammatory cytokines, including but not limited to TNF, accompanied by a surge in oxidative stress. A particularly intriguing aspect of our study lies in the heightened reactivity of the mutant microglia upon exposure to LPS stimulation. This hyper-responsiveness allows us to imagine an involvement of environmental factors in modulating the disease's phenotypic manifestations, thereby offering a plausible explanation for the observable variabilities even among identical twins. Unraveling the precise nature of these environmental triggers could unveil novel avenues for both risk assessment and preventive strategies, empowering us to anticipate, mitigate, or even intercept the disease progression.

Looking forward, the insights gained from this research pave the way for prospective clinical interventions aiming at ameliorating the impact of X-ALD. While translating our findings into practical treatments requires rigorous *in vivo* investigations, the potential is tantalizing. Targeting microglial secretions or signaling pathways holds promise as a means to modulate the disease trajectory. Whether through the application of antioxidants to counteract oxidative stress, specialized anti-inflammatory agents designed to selectively influence microglial secretions, or interventions designed to intercept harmful signaling cascades, our work offers a blueprint for a novel therapeutic approach.

Finally, our investigation illuminates the intricate connections between peroxisomes, microglial function, and the pathogenesis of X-ALD. By deciphering the molecular basis of this complex relationship, we not only deepen our comprehension of a specific disorder but also enrich our understanding of neurodegenerative diseases. While anticipating the therapeutic potential our findings hold, we must commit to unraveling the remaining mysteries.

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## Annex

### Material and Methods - ROS and RNS analysis

#### I. Intracellular ROS production

BV-2 cells oxidative stress was assessed using two ROS indicator: a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein (CM-H<sub>2</sub>DCFDA, ThermoFisher, reference C6827) and dihydroethidium (DHE, ThermoFisher, reference D11347). CM-H<sub>2</sub>DCFDA and DHE are molecules which can enter by passive diffusion in the cells where they are oxidized by intracellular ROS what produce fluorescent DCF and ethidium, respectively. CM-H<sub>2</sub>DCFDA is a general oxidative stress indicator. Its chloromethyl group offers a better intracellular retention than H<sub>2</sub>DCFDA and is thiols reactive. DHE is more specifically oxidized by superoxide.

BV-2 cells were seeded in 96-well black/clear bottom plates as follows: 20,000 WT or *Acox1*<sup>-/-</sup> cells; 25,000 *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells per well in 100 µl phenol red-free DMEM/10% FBS medium. They were cultured for 24 h and next treated with LPS (1 µg/ml) for 3 to 24 h as indicated (or not treated in the case of the basal ROS production analysis). Following the incubation (48 h total culture time), the culture medium was removed and cells were washed one time with pre-warmed PBS then incubated with either CM-H<sub>2</sub>DCFDA (5 µM in PBS) for 70 min or DHE (10 µM in PBS) for 30 min at 37°C and 5% CO<sub>2</sub>. Using plate reader (TECAN infinite M200Pro), the probes were excited at 485 nm or 518 nm and the fluorescence emitted was collected at 535 nm or 605 nm to detect DCF and ethidium, respectively. For each experiment, values are calculated as the fluorescence means of a triplicates.

For the ROS basal production comparison, as KO and WT BV-2 cells have different growing kinetic, ROS production was standardized to cells quantity which was evaluated using Hoechst 33342. For this purpose, in identical but distinct wells, cells were treated and dyed in the same maner with Hoecht 33342 (3.5 µM in PBS) for 10 min. The Hoechst fluorescence was measured using an excitation wavelenght of 350 nm and an emission wavelenght of 461 nm. The ROS fluorescence was reported to Hoechst fluorescence.

For the kinetics of ROS production under LPS treatment, the fluorescence of LPS-treated BV-2 cells was compared to the fluorescence of untreated BV-2 cells.

Four and three independent experiments were performed for basal and LPS-induced ROS production, respectively.

## II. NO production

The production of nitric oxide by BV-2 cells was quantified using the colorimetric Griess assay. Briefly, NO being unstable, the final product of NO oxidation, the nitrite ion  $\text{NO}_2^-$  as well as nitrates react with the N-(1-Naphthyl)-ethylene-diamine contained in the Griess reagent to form a pink diazo product which absorbs at 546 nm. The ion concentration is proportional to the intensity of the pink coloration.

For this analysis, mutant and WT BV-2 cells were seeded in 6-well plates at a density of 250,000 *WT*, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> or *Acox1*<sup>-/-</sup> cells per well in 2,5 ml complete medium. They were cultured for 24 h and next treated with LPS (1  $\mu\text{g/ml}$ ) for 7 or 24 h. At the end of the incubation, supernatants were collected, centrifuged at 300 g for 5 min, to eliminate cells and debris, and freezeed at  $-20^\circ\text{C}$  until used for NO assay. In parallele, cells were lyzed for protein content quantification.

Griess assay was performed as follows: 50  $\mu\text{L}$  culture medium was mixed with an equal volume of Griess reagent (0.1% N-1-naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid; Sigma-Aldrich) and incubated for 15 min at room temperature in the dark. Absorbance was measured at 540 nm using a plate reader (TECAN Sunrise). Nitrite concentration was calculated from a standard curve obtained with sodium nitrite diluted in culture medium at a concentration ranging from 0 to 100  $\mu\text{M}$ .

Cells protein content was quantified as follows: cells were washed with PBS and homogenized in 200  $\mu\text{L}$  solubilization buffer (100 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100) containing 1% PMSF and protease inhibitor mixtures (Roche Applied Science). After incubating 30 min at  $4^\circ\text{C}$ , the lysate was centrifuged at 10,000 g for 10 min at  $4^\circ\text{C}$  to eliminate nuclei. The lysate was collected and the protein amount was quantified using BiCinchoninic Acid (BCA) kit (Pierce, reference 23227) according to the manufacturer's instructions.

Nitrite quantity was reported to protein amount per well for each condition.

Four independent experiments were performed.

# Résumé en français du manuscrit de la thèse

## Introduction

### Chapitre 1 : Peroxysome et adrénoleucodystrophie

Les cellules eucaryotes comprennent divers compartiments subcellulaires qui varient en termes de biogenèse, de composition enzymatique et de fonctions métaboliques. Ces compartiments, tels que les lysosomes et les mitochondries, ont des rôles distincts dans la dégradation des macromolécules et le métabolisme aérobie, respectivement. Les peroxysomes sont des organites présents dans la plupart des cellules eucaryotes remplissant à la fois des fonctions cataboliques et anaboliques (Wanders et al., 2023).

Ils sont impliqués dans un grand nombre de fonctions métaboliques cruciales. Ils interviennent dans plusieurs processus cellulaires importants, tels que la détoxification des espèces réactives de l'oxygène (ROS) et des espèces réactives de l'azote (RNS), la synthèse de plasmalogènes (étherphospholipides), des acides biliaires et de l'acide docosahexaénoïque (DHA). Ils participent également à la dégradation des acides gras (AG) et divers composés lipidiques par  $\alpha$ - et  $\beta$ -oxydation. Ils sont les seuls organites responsables de la  $\beta$ -oxydation des acides gras à très longue chaîne (AGTLC) (AG comportant 22 atomes de carbone ou plus) et de l' $\alpha$ -oxydation des AG à chaîne ramifiée (Fransen & Lismont, 2019; Wanders et al., 2023). En effet, ces AG ne peuvent pas être traités par la machinerie d'oxydation mitochondriale.

La dégradation des AG par les processus d' $\alpha$ - et de  $\beta$ -oxydation est la fonction la plus connue des peroxysomes. L' $\alpha$ -oxydation correspond au raccourcissement de la chaîne d'un seul atome de carbone, un processus nécessaire avant la dégradation totale de certains AG à chaîne ramifiée. Cette réaction d'oxydation-décarboxylation se produit exclusivement dans les peroxysomes (Wanders et al., 2023). La  $\beta$ -oxydation peroxysomale est une voie cruciale pour le métabolisme de certains acides gras et contribue à l'homéostasie lipidique de la cellule et indirectement à la production d'énergie en fournissant à la mitochondrie de l'octaboyl-CoA. Les peroxysomes dégradent les AGTLC par le processus de  $\beta$ -oxydation. Ce processus, qui se produit chez la plupart, voire tous les organismes, convertit les AGTLC en acides gras à chaîne plus courte, pouvant ensuite être utilisés comme sources d'énergie par la cellule. Ces AG doivent être dégradés principalement en raison de leur toxicité lorsqu'ils s'accumulent (Wanders et al., 2023).

Les AGTLC sont exclusivement  $\beta$ -oxydés dans le peroxysome. La  $\beta$ -oxydation des acides gras commence après leur activation et leur importation dans le peroxysome. L'acyl-CoA synthétase dans le cytosol active les acides gras, formant ainsi des thioesters d'acyl-CoA. Ensuite, les acyl-CoA à longue ou très longue chaîne sont transportés dans les peroxysomes via deux transporteurs ABC (« ATP binding cassette »), ABCD1 et ABCD2, tandis que les acyl-CoA des acides gras à chaîne ramifiée ou des acides dicarboxyliques entrent dans le peroxysome grâce à ABCD3 (Tawbeh et al., 2021). Une fois dans le peroxysome, les acyl-CoA sont catabolisés par un ensemble d'enzymes, généralement par  $\beta$ -oxydation (Nelson & Cox, 2017). La machinerie de  $\beta$ -oxydation peroxysomale des acides gras est composée de trois différentes acyl-CoA oxydases (ACOX1, -2 et -3), de deux enzymes multifonctionnelles (protéine multifonctionnelle 1, MFP1 ou LBP ; et protéine multifonctionnelle 2, MFP2 ou DBP) et de deux thiolases (acétyl-CoA acyltransférase 1, ACAA1 ; et la protéine transporteuse de stérol X, SCPx). ACOX1 est l'enzyme nécessaire à la première étape de la  $\beta$ -oxydation. Elle réagit principalement avec les esters de CoA des AGTLC, catalysant la réduction de l'acyl-CoA en 2-trans-énol-CoA, et est ainsi l'enzyme limitante de cette réaction. Elle joue également un rôle essentiel dans la médiation de la réponse inflammatoire et du métabolisme des ROS chez les mammifères (Kong et al., 2022).

Les transporteurs à cassette de liaison à l'ATP (ABC) constituent une superfamille de protéines de transport membranaire qui transloque activement un large éventail de molécules, des molécules simples (AG, sucres, nucléosides et acides aminés) aux composés organiques complexes (lipides, oligonucléotides, polysaccharides et protéines) (Thomas & Tampé, 2020). Les transporteurs ABC de la sous-famille D comprennent quatre protéines chez les mammifères : ABCD1 [protéine d'adrenoleukodystrophie (ALDP)], ABCD2 [protéine apparentée à l'adrenoleukodystrophie (ALDRP)], ABCD3 [protéine membranaire peroxysomale de 70 kDa (PMP70)] et ABCD4 [protéine membranaire peroxysomale 69 (PMP69) mais qui n'est en fait pas localisée dans le peroxysome] (Kamijo et al., 1990; Kawaguchi & Morita, 2016; Lombard-Platet et al., 1996; Mosser et al., 1993; Tawbeh et al., 2021).

L'adrénoleucodystrophie liée à l'X (X-ALD ; OMIM #300100) est la maladie peroxysomale la plus fréquente avec une incidence estimée à la naissance de 1/14 700 (Turk et al., 2020), sachant qu'elle est souvent sous-diagnostiquée ou mal diagnostiquée, rendant ainsi difficile une estimation précise de son incidence. En 1993, en utilisant le clonage positionnel, l'équipe de Hugo Moser a identifié le gène *ABCD1* comme étant responsable de l'X-ALD (Mosser et al., 1993). Des mutations dans le gène *ABCD1* ont été trouvées chez chaque patient

atteint d'X-ALD et sont répertoriées dans la base de données X-ALD (<https://adrenoleukodystrophy.info/> consulté le 1er avril 2023). Les mutations à l'origine d'une protéine ABCD1 absente ou non fonctionnelle entraînent un défaut dans le transport peroxysomal des AGTLC, réduisant ainsi l'activité de  $\beta$ -oxydation peroxysomale d'environ 60 à 80 % (I. Singh et al., 1984).

Ce trouble neurodégénératif complexe et fatal est caractérisé par une variabilité clinique tant dans l'âge de début que dans les symptômes, sans corrélation génotype-phénotype (Engelen et al., 2012; Wiesinger et al., 2015). Les deux principales formes sont la forme adulte, l'adrénomyeloneuropathie (AMN) et la leucodystrophie cérébrale de l'enfance (cALD). L'AMN est une démyélinisation progressive non inflammatoire qui affecte la moelle épinière et les nerfs périphériques, tandis que la cALD se caractérise par une démyélinisation inflammatoire du système nerveux central. L'AMN affecte pratiquement tous les patients masculins atteignant l'âge adulte et environ 60% des porteuses féminines. Environ 80% de tous les patients X-ALD masculins développent une insuffisance corticosurrénalienne, faisant de l'X-ALD la principale cause de la maladie d'Addison dont l'insuffisance surrénalienne pourrait être le seul symptôme (Dubey et al., 2005). Chez plus de la moitié des patients masculins, un événement déclenchant encore inconnu induit la cALD avec la rupture de la barrière hémato-encéphalique (BHE) et l'infiltration principalement de monocytes et de lymphocytes T, ainsi qu'une démyélinisation sévère et rapide (Musolino et al., 2015; Powers et al., 1992; Weinhofer et al., 2018). Dans la majorité des cas, une cALD non traitée conduit à un état végétatif ou à la mort dans les quelques années suivant le début de la maladie (Raymond et al., 2019). Étant donné que la maladie est liée au chromosome X, les hommes sont les patients les plus gravement atteints. Les porteuses féminines restent généralement asymptomatiques ou présentent seulement un phénotype léger, même si certaines formes graves ont été diagnostiquées (Jangouk et al., 2012).



## Chapitre 2 : La Microglie

La microglie correspond aux cellules immunitaires innées du système nerveux central (SNC), représentant entre 5 et 12 % des cellules du SNC selon la région. Elles jouent un rôle essentiel dans le maintien de l'homéostasie du SNC en effectuant une surveillance continue de leur environnement grâce à des protrusions motiles ainsi qu'à leur capacité de phagocytose et de sécrétion de divers facteurs trophiques et chimiotactiques ou de molécules pro- ou anti-inflammatoires. Elles participent à la plasticité synaptique et au maintien de l'intégrité de la barrière hématoencéphalique. Elles ont la capacité de détecter toute anomalie ou lésion dans le parenchyme cérébral et de résoudre toute perturbation (Nimmerjahn et al., 2005; Sevenich, 2018).

En tant que cellules médiatrices de la réponse immunitaire innée dans le SNC, les cellules microgliales jouent un rôle crucial dans la détection et la réponse aux conditions inflammatoires. Les réactions inflammatoires dans le SNC peuvent être déclenchées par divers facteurs, tels que des lésions, des infections ou des maladies neurodégénératives. La détection et la reconnaissance par les microglies constituent la première étape de la réponse aux perturbations du SNC (Medzhitov, 2007).

La neurodégénérescence fait référence à la perte chronique et progressive de neurones dans le cerveau et la moelle épinière. Dans le contexte des maladies neurodégénératives, la microglie présente un comportement et des réponses distinctes, contribuant à la pathogenèse et à la progression de ces affections. En effet, la dysrégulation d'une quelconque fonction microgliale entraîne un déséquilibre qui initie ou propage la neurodégénérescence. Il existe de nombreuses preuves documentant l'activation des cellules microglies dans les troubles neurodégénératifs, conduisant à l'expression d'un large éventail de médiateurs immunitaires tels que l'IL-1 $\beta$ , le TNF $\alpha$ , les ROS et le NO (Banati et al., 1993; Gehrman et al., 1995; Hopkins & Rothwell, 1995). Les maladies neurodégénératives les plus courantes, telles que la maladie d'Alzheimer (AD), la maladie de Parkinson (PD), la maladie de Huntington et la sclérose latérale amyotrophique (SLA), sont toutes caractérisées par la neuroinflammation médiée par la microglie et une augmentation du stress oxydatif (Q. Wu & Zou, 2022).

La microglie semble jouer un rôle clé dans la pathogenèse de l'adrénoleucodystrophie liée à l'X (X-ALD). Des études récentes menées sur des modèles murins de l'adrénoleucodystrophie ont révélé que l'activation des cellules microgliales précède la perte synaptique, et que la microglie déficiente en ABCD1 de la moelle épinière est dans un état de pré-activation pour la phagocytose neuronale avec une expression élevée de TREM2 (Gong et al., 2017).

Le traitement de l'adrénoleucodystrophie met en évidence le rôle crucial des cellules immunitaires dans la physiopathologie de la neurodégénérescence résultant des mutations *ABCD1*. Une transplantation de moelle osseuse allogénique ou de cellules souches hématopoïétiques génétiquement corrigées a démontré la capacité de ralentir ou d'arrêter la progression de l'adrénoleucodystrophie. On suppose que les précurseurs hématopoïétiques exprimant *ABCD1* forment une population de monocytes/macrophages qui, une fois dans le cerveau, pourront remplacer fonctionnellement la microglie déficiente (Asheuer et al., 2004; Priller et al., 2001).

Les modèles cellulaires *in vitro* de microglie jouent un rôle crucial dans l'étude des fonctions et des comportements de ces cellules spécialisées au sein du SNC. Ces dernières années, des avancées significatives ont été réalisées dans le développement de modèles *in vitro* de microglie qui imitent leurs caractéristiques et leurs fonctions *in vivo*. Ces modèles impliquent la culture de cellules microgliales primaires obtenues à partir de cerveaux d'animaux ou de tissus post-mortem humains, ainsi que l'utilisation de lignées cellulaires de microglies immortalisées. Plusieurs lignées cellulaires ont été établies afin d'éviter les contraintes liées à la culture primaire, les plus courantes étant les lignées murines BV-2 et N9, avec une prédominance évidente pour les cellules BV-2 (4716 résultats pour la recherche PubMed "microglia AND BV2 OR BV-2" contre 427 résultats pour "microglia AND N9" au 26 juin 2023). La disponibilité, la reproductibilité et l'immortalisation des lignées cellulaires représentent un énorme avantage dans la recherche.

Dans le but d'étudier les effets des dysfonctionnements peroxisomaux dans la microglie et la pathogenèse sous-jacente de l'adrénoleucodystrophie liée à l'*X* (*X*-ALD), des lignées cellulaires de microglies déficientes en *ABCD1* et/ou *ABCD2* ont été obtenues dans notre laboratoire en utilisant l'édition génique CRISPR/Cas9 dans les cellules BV-2. Les cellules double déficientes en *ABCD1/ABCD2* ont été générées pour éviter les effets masquants dus à une redondance fonctionnelle. Elles présentent les caractéristiques biochimiques classiques de l'*X*-ALD, telles qu'une augmentation des niveaux d'AGTLC saturés et monoinsaturés, ainsi qu'une augmentation des niveaux de certains AG à chaîne longue et d'AG polyinsaturés (Raas, Gondcaille, et al., 2019).

## Objectifs

La pathogenèse de l'adrénoleucodystrophie liée à l'X (X-ALD), la maladie peroxysomale la plus courante, continue de présenter de nombreux aspects, défis et incertitudes non résolus. Malgré des avancées notables, telles que la mise en place de dépistages néonataux dans plusieurs pays, les options de traitement restent strictement limitées. L'émergence de la thérapie génique offre de l'espoir pour les individus atteints de la forme cérébrale de l'X-ALD, mais son potentiel est accompagné de défis scientifiques, réglementaires et financiers importants. Un obstacle majeur entravant notre compréhension des mécanismes pathologiques de la maladie et le développement de thérapies efficaces est le manque de modèles expérimentaux pertinents. Les modèles murins existants déficients en ABCD1 et/ou ABCD2, bien que précieux, ne parviennent pas à reproduire pleinement les dommages neuroinflammatoires démyélinisants caractéristiques de la forme cérébrale de l'X-ALD. Il devient donc impératif d'explorer des voies alternatives. Notre recherche vise à combler un important écart dans la compréhension actuelle de la pathogenèse de l'X-ALD. Bien que des investigations approfondies aient été menées sur les aspects moléculaires *in vitro* de l'X-ALD, le rôle de la microglie a été notablement négligé. Cet aspect constitue le centre d'intérêt principal de notre étude, apportant une nouvelle perspective au domaine. Dans ce contexte, notre laboratoire a réussi à générer des cellules BV-2 knockout en utilisant l'édition du génome CRISPR/Cas9. Les cellules knockout sont *Abcd1*<sup>-/-</sup>, *Abcd2*<sup>-/-</sup>, *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> et *Acox1*<sup>-/-</sup> (Raas, Gondcaille, et al., 2019; Raas, Saih, et al., 2019). Ces cellules offrent une alternative prometteuse et valide pour des investigations moléculaires sur l'impact des dysfonctionnements peroxysomaux dans la microglie. En étudiant et en utilisant ces cellules, nous avons comme objectif de comprendre l'incidence du défaut peroxysomal sur les fonctions microgliales et ainsi de mieux comprendre le lien complexe entre les anomalies de la  $\beta$ -oxydation peroxysomale et la mise en place des processus neurodégénératifs. Cette recherche doit nous permettre d'élucider des mécanisme moléculaire et cellulaires sous-jacents à la pathologie de l'X-ALD et ainsi, ouvrir de nouvelles perspectives pour des interventions thérapeutiques. Nous visons à élucider le rôle crucial des peroxysomes dans les fonctions des cellules immunitaires spécialisées du SNC et le maintien d'une réponse immunitaire optimale et de l'homéostasie cérébrale. En caractérisant de manière approfondie les marqueurs associés à la pathologie dans ces cellules et en comprenant comment ils conduisent à la neurodégénérescence, il serait possible d'établir une base pour identifier des biomarqueurs potentiels et des molécules thérapeutiques capables de restaurer la fonction cellulaire normale.

Les objectifs spécifiques de ma thèse se sont articulés autour des points suivants. Premièrement, l'Objectif 1 a visé la caractérisation des cellules BV-2 déficientes en ABCD1, ABCD2 et ACOX1, en se concentrant sur plusieurs aspects. Tout d'abord, une comparaison des génotypes a été entreprise, accompagnée d'une exploration de la réponse au LPS. Cette approche a permis d'entamer une analyse transcriptomique approfondie, éclairant les conséquences des KO et identifiant les axes d'étude prioritaires. Des enrichissements ont émergé, mettant en avant des groupes de gènes différentiellement exprimés dans les divers génotypes et conditions. Une validation partielle des données d'expression a été réalisée au niveau protéique, tandis qu'une exploration fonctionnelle a été entreprise. Cela incluait des altérations dans la composition en cholestérol de la membrane plasmique, une surexpression de protéines lysosomales, ainsi qu'une exploration préliminaire des mécanismes de l'autophagie. De plus, la sécrétion de protéines de la signature DAM, l'expression et la sécrétion de cytokines et de chimiokines pro-inflammatoires, ainsi que l'analyse de la fonction phagocytaire et de la capacité à induire une réponse lymphocytaire T ont été examinées. Le statut redox en réponse à une stimulation au LPS a également fait l'objet d'une investigation approfondie.

En ce qui concerne l'Objectif 2, une évaluation des effets des sécrétions des cellules knockout sur les propriétés neuronales a été entreprise. Cela a inclus une analyse des effets cytotoxiques sur les neurones et les oligodendrocytes, ainsi qu'une évaluation des répercussions sur la morphologie et la fonction neuronale.

## Matériels et méthodes

S'agissant d'une thèse basée sur des articles, cette section est donc incluse dans les documents qui forment le cœur de cette thèse. L'une des principales méthodologies qui n'est pas mentionnée dans les articles figurant dans la thèse (et qui sera au cœur d'un prochain article) a été incluse et détaillée dans la thèse. Il s'agit des expériences de coculture et d'utilisation de milieux conditionnés (MC). La section MC est donc présentée ci-dessous.

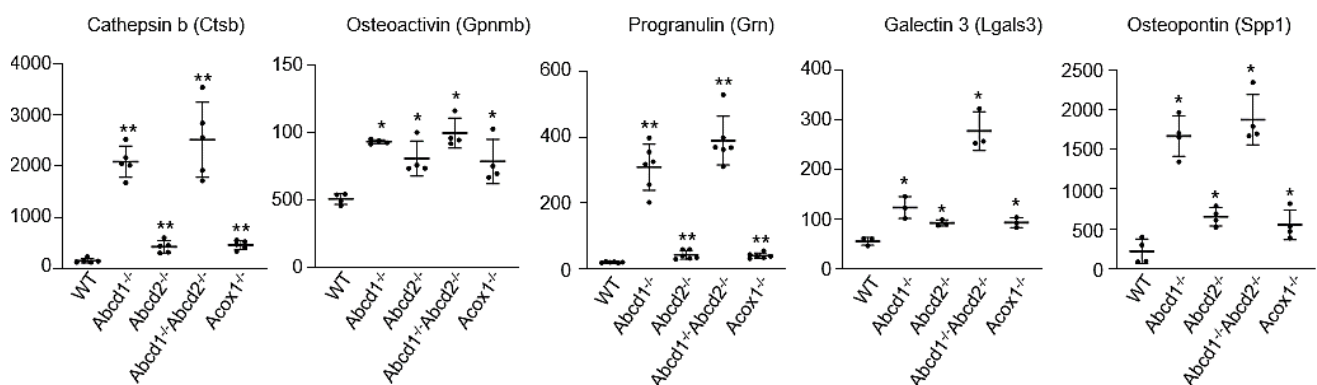
## Résultats

Les résultats de cette thèse sont présentés principalement sous forme d'articles. Les résultats les plus importants sont décrits ci-dessous.

Dans le premier article, les cellules ont été caractérisées au niveau basal, c'est-à-dire sans aucun traitement. L'objectif de cet article était de révéler les différentes voies modifiées dans la microglie lors de l'inactivation des gènes peroxysomaux et de mettre en lumière l'importance des fonctions peroxysomales dans l'homéostasie de la microglie et du SNC. La publication mentionnée présente la caractérisation de modèles de cellules BV-2 avec des knockouts d'*ABCD1*, *ABCD2*, *ACO1*, et un double knockout d'*ABCD1* et *ABCD2*. Ces cellules BV-2 knock-out ont été soumises à une analyse transcriptomique par séquençage de l'ARN. Pour mieux comprendre les effets de l'inactivation des gènes peroxysomaux, il a fallu classer les gènes différentiellement exprimés (DEGs, « Differentially expressed genes ») dans des familles spécifiques en utilisant des bases de données telles que Gene Ontology et KEGG pathway. L'analyse comparative des modèles de cellules knock-out avec les cellules sauvages permet ainsi de mettre en lumière l'implication des voies moléculaires peroxysomales dans diverses fonctions cellulaires. Il reste à confirmer les données d'expression au niveau protéique et de vérifier si les variations d'expression identifiées se traduisent par des modifications fonctionnelles. La caractérisation des cellules knock-out permet ainsi de découvrir sans a priori le phénotype microgliale dans le cas d'une déficience peroxysomale en lien avec l'X-ALD, donnant ainsi potentiellement un aperçu in vitro des mécanismes pathologiques de la maladie. Dans notre première publication publiée en mars 2023 (Raas, Tawbeh et al. 2023), nous avons concentré notre attention sur quatre groupes de gènes associés avec le métabolisme des lipides (acides gras et leurs dérivés, lipides membranaires, cholestérol ...), les lysosomes et l'autophagie en laissant de côté les gènes associés à la réponse immunitaire. Ces termes sont apparus comme les voies les plus enrichies identifiées par l'analyse d'enrichissement génique menée sur les cellules BV-2 mutantes, en utilisant les processus biologiques de l'ontologie des

gènes et les bases de données des voies KEGG. Nous avons également identifié une signature ressemblant à la signature microgliale pathologique (signature DAM, « disease-associated microglia ») retrouvée dans les maladies neurodégénératives les plus fréquentes (Butovsky & Weiner, 2018). L'identification de cette signature dans les cellules mutantes a une signification importante puisqu'elle montre pour la première fois que les mutations peroxysomales induisent dans la microglie un phénotype pathologique semblable à celui retrouvé dans des maladies neurodégénératives d'origine non peroxysomales. J'ai confirmé sur certains gènes analysés que les variations d'expression en ARN se retrouvaient bien au niveau protéique et pu ainsi valider en partie les conclusions de l'analyse transcriptomique.

Parmi les résultats importants présentés dans ce document figure la sécrétion des protéines DAM. Le graphique ci-dessous montre l'augmentation de la sécrétion de ces protéines par les cellules *Abcd1*<sup>-/-</sup> et *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>. De tels résultats constituent une percée dans ce domaine. Il est démontré, pour la première fois, que les défauts peroxysomaux dans les microglies déclenchent la signature des DAM. La sécrétion accrue de protéines DAM pourrait être utilisée pour identifier de nouveaux biomarqueurs pour l'X-ALD.



**Figure 1 : Signature de type DAM dans les cellules mutantes.**

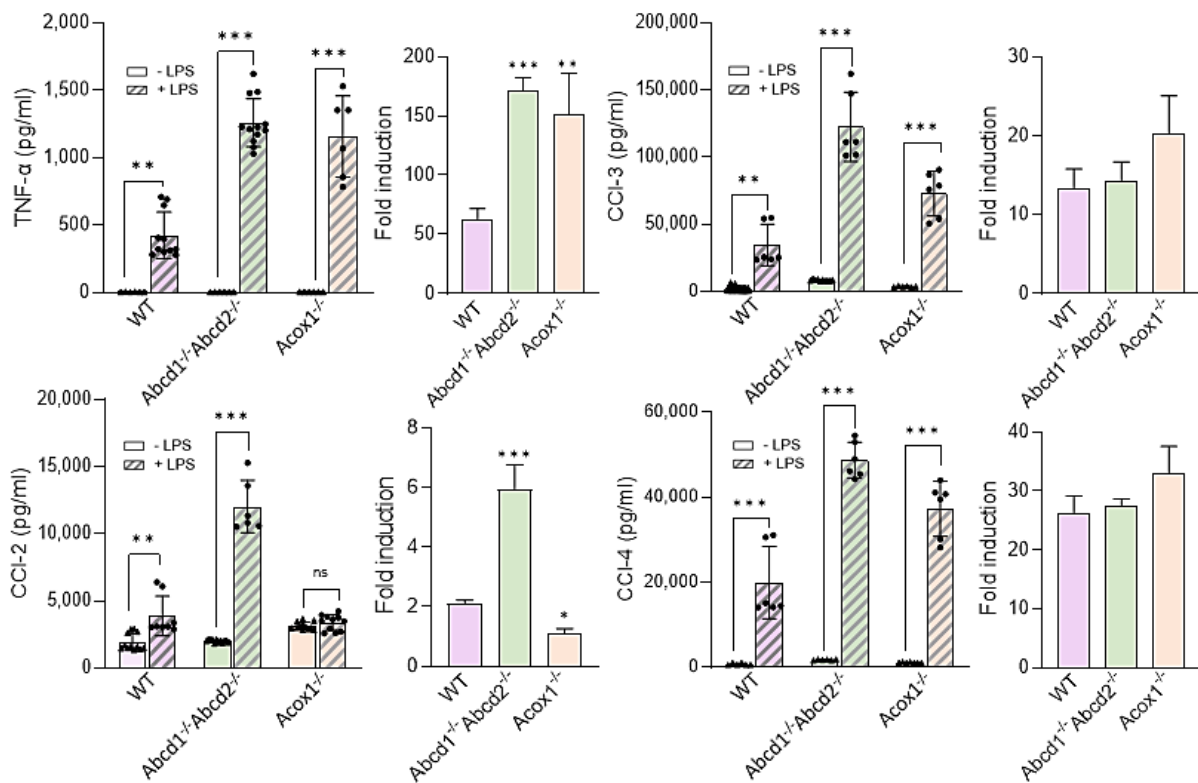
Augmentation de la sécrétion des marqueurs DAM dans les cellules mutantes BV-2. Graphique en pointillés représentant la concentration (ng/mL) des marqueurs DAM obtenus par ELISA à partir du surnageant des cellules BV-2 mutantes et WT. Les différences significatives sur le plan statistique sont indiquées : \* $p \leq 0,05$ , \*\* $p \leq 0,01$  (test de Mann-Whitney de 3 à 6 expériences).

La confirmation de l'altération de l'expression de protéines lysosomales, de protéines en lien avec l'autophagie, de protéines en lien avec la synthèse du cholestérol (ceci étant corrélé avec une augmentation du cholestérol libre dans les membranes des cellules mutantes) laisse penser que les mutations peroxysomales influencent la cellule au niveau métabolique mais aussi au niveau de ses fonctions, telles que la phagocytose. La phagocytose est une fonction microgliale centrale qui est également étroitement associée au métabolisme des lipides, au

cholestérol et, par conséquent, à la fluidité membranaire. Il était important de mener des analyses fonctionnelles plus poussées pour confirmer ces hypothèses et d'explorer la capacité des cellules microgliales à effectuer une réponse immunitaire (terme principal de l'analyse transcriptomique laissée de côté dans le premier article).

Les résultats inclus dans le second article portent sur la modification de la réponse immunitaire dans les cellules BV-2 mutantes. Le LPS est largement utilisé comme un puissant activateur de la réponse immunitaire qui déclenche une forte réaction inflammatoire. Il constitue une méthode standardisée et contrôlée pour induire une inflammation et étudier l'activation microgliale. L'analyse transcriptomique des cellules BV-2 mutantes traitées au LPS a montré d'une part, une augmentation du nombre de DEGs par rapport aux cellules sauvages et d'autre part, une augmentation des niveaux de variation induits pour une proportion importante de gènes répondeurs, parmi lesquels des gènes codant des cytokines pro-inflammatoires et des gènes associés à la présentation antigénique. Dans le but de documenter et confirmer ces résultats au niveau protéique, j'ai examiné l'expression par western blotting et la sécrétion de plusieurs cytokines en analyse multiplexe en cytométrie. En collaboration avec le laboratoire de Yannick Hamon (CIML, Marseille), nous avons étudié l'expression de protéines de surface impliqués dans l'adhésion, la co-stimulation lymphocytaire et la présentation antigénique par cytométrie. Enfin, j'ai pu explorer l'impact supposé du défaut peroxysomal sur la phagocytose. Pour faire la lumière sur ces processus, nous avons collaboré avec le « Department of Pathobiology of the Nervous System » du « Center for Brain Research » du « Medical University of Vienna ».

Les résultats de la sécrétion de cytokines présentés ci-dessous montrent une sécrétion induite de la cytokine pro-inflammatoire TNF dans les cellules mutantes après traitement au LPS et de CCL2 dans les cellules doublement knock-out traitées au LPS.

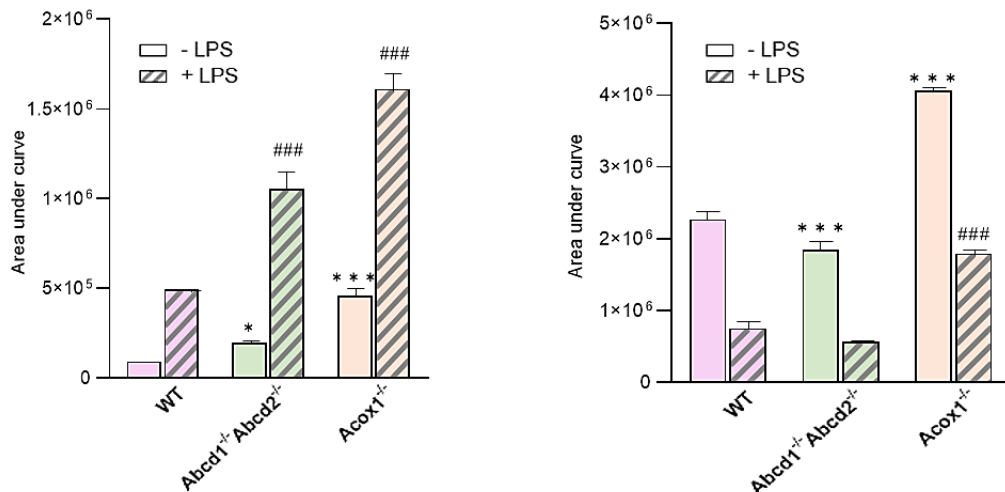


**Figure 2 : Impact de l'anomalie peroxysomale sur l'expression des gènes liés aux cytokines inflammatoires dans les cellules BV-2 WT et mutantes (*Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* et *Acox1<sup>-/-</sup>*).**

Analyse multiplex de la concentration des cytokines et chimiokines sécrétées (CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1b), TNF). Les histogrammes en pointillés présentent les valeurs individuelles et la moyenne (+/- SD) de 6 à 12 mesures en l'absence de LPS (barres vides) ou après un traitement de 24 h avec du LPS (barres hachurées). Les différences statistiques par rapport au génotype non traité sont indiquées (ANOVA à 2 facteurs, \* p<0,01, \*\* p<0,01, \*\*\* p<0,001). L'induction relative est présentée sur l'histogramme de droite pour chaque cytokine et les différences statistiques par rapport à l'induction observée dans les cellules WT sont indiquées (1-way ANOVA, \* p<0,01, \*\* p<0,01, \*\*\* p<0,001).

Un autre résultat important de cet article est la différence d'activité phagocytaire entre les cellules WT et les cellules mutantes. La figure ci-dessous montre l'aire sous la courbe de la courbe temporelle représentant la capacité phagocytaire de ces cellules. Elle montre ainsi une phagocytose accrue des billes dans les cellules knock-out, en particulier après un traitement au LPS. En revanche, la phagocytose des débris de gaine de myéline a diminué dans les cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* par rapport aux cellules WT, mais a été induite dans les cellules *Acox1<sup>-/-</sup>* dans des conditions basales. Le traitement au LPS entraîne une diminution de l'activité phagocytaire dans toutes les cellules, qu'elles soient WT ou mutantes.





**Figure 3 : Quantification du signal pHrodo sur 16 heures représenté par l'aire sous la courbe pour la phagocytose des billes (gauche) et de la myéline (droite). Les différences statistiques ont été calculées à l'aide d'une ANOVA à 2 facteurs suivie d'une correction pour le taux de fausse découverte et représentées par "\*" pour comparer la phagocytose des cellules KO LPS à celle des cellules WT et par "#" pour comparer les cellules KO traitées au LPS par rapport aux cellules WT traitées au LPS (\*p < 0,05, \*\*\* p < 0,001, ### p < 0,001).**

Dans cette étude, nous avons choisi de concentrer notre analyse sur les doubles knockouts *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* et *Acox1<sup>-/-</sup>*, plutôt que de considérer les knockouts simples d'*ABCD1* et *ABCD2*. Cette décision a été prise en raison de la quantité importante de données générées lors de la comparaison des différents génotypes. L'inclusion de tous les résultats aurait rendu les conclusions moins claires et la discussion plus difficile à comprendre. En fait, nous avons été confrontés à ce défi dans notre article précédent, où nous avons dû éliminer certains résultats à la demande des réviseurs. En outre, en comparant l'absence d'importation d'AGTLC dans les peroxysomes (dans les cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*) avec les défauts de la  $\beta$ -oxydation peroxysomale alors que les AGTLC sont toujours importés (dans les cellules *Acox1<sup>-/-</sup>*), nous pouvons établir une base solide pour les conséquences des défauts peroxysomaux dans la microglie.

Dans cet article, nous avons présenté d'autres résultats de la caractérisation des cellules mutantes, en mettant l'accent sur la réponse immunitaire induite par le traitement au LPS. Nous avons démontré une augmentation de l'expression et de la sécrétion de cytokines pro-inflammatoires dans les cellules mutantes, en particulier le TNF qui s'est avéré être fortement induit dans les sécrétions des cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* traitées au LPS, ce qui suggère un biais pro-inflammatoire et une réponse inflammatoire exagérée dans ces cellules. Nous avons également montré une modification de l'activité phagocytaire dans les cellules mutantes,

comme prévu par les données transcriptomiques. Il est intéressant de noter que les cellules *Acox1*<sup>-/-</sup> ont montré une activité phagocytaire accrue alors qu'une activité réprimée a été révélée dans les cellules *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>. Cela pourrait s'expliquer par l'expression accrue des récepteurs de phagocytose sur les membranes des cellules *Acox1*<sup>-/-</sup> et leur diminution sur les cellules *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>. Nous avons également montré une modification de l'activité de présentation de l'antigène et une activation amplifiée des lymphocytes T CD8<sup>+</sup> par les cellules mutantes traitées au LPS par rapport aux cellules WT. Ces résultats soulignent l'importance de l'interaction entre la microglie et les lymphocytes T, participant éventuellement à une boucle d'amplification conduisant à l'inflammation chronique et à la neurodégénérescence, et prouvent l'implication des fonctions peroxysomales microgliales sur l'activation des lymphocytes T.

De plus, un autre aspect important de notre caractérisation cellulaire concerne l'étude du stress oxydatif et la confirmation des variations d'expression relatives induites par le LPS identifiées dans l'analyse transcriptomique. Nous avons montré une propension des cellules mutantes à répondre de manière exagérée à la stimulation au LPS que ce soit dans la production de ROS ou de NO. Cependant, inclure les résultats des marqueurs et de la sécrétion du stress oxydatif dans cet article le rendrait plus complexe et nettement plus long. C'est pourquoi nous avons décidé de préparer un troisième document qui englobera ces résultats. L'article suivant inclura également l'impact des sécrétions des cellules mutantes sur les neurones et les oligodendrocytes, que j'ai exploré en utilisant le milieu conditionné et la co-culture, donnant ainsi un aperçu de la neurodégénérescence induite par les défauts peroxysomaux dans la microglie.

### **Impact des sécrétions BV-2 sur les neurones et les oligodendrocytes**

Après avoir détecté une tendance pro-inflammatoire dans les cellules mutantes ainsi qu'une production accrue de marqueurs du stress oxydatif, nous nous sommes demandé comment cette altération de la plasticité microgliale pouvait avoir un impact sur les différents aspects des neurones et des oligodendrocytes. Par conséquent, en utilisant les expériences de co-culture et de milieu conditionné conçues, nous avons étudié la viabilité des neurones (mHypoA) et des oligodendrocytes (158N) en présence des sécrétions des cellules WT et mutantes. Nous avons également analysé la morphologie et la fonction des neurones dans ces conditions. Les résultats ci-dessous montrent les données obtenues à partir des expériences sur le milieu conditionné (MC).

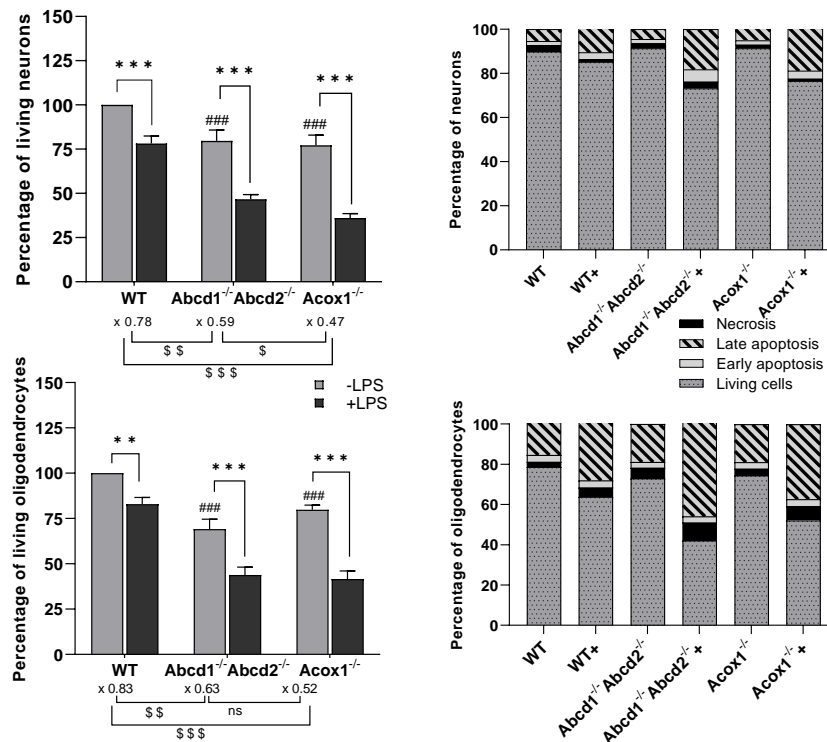
En utilisant un test classique de viabilité cellulaire, le MTT, nous avons estimé le pourcentage de cellules viables dans chaque condition. Aucune différence n'a été observée entre les neurones et les oligodendrocytes incubés avec le MC de cellules WT et ceux incubés dans du milieu frais, de sorte que les cellules incubées dans le MC de cellules BV-2 WT ont été considérées comme le contrôle. Lorsque les neurones mHypoA ont été incubés avec le MC de cellules WT traitées au LPS, la viabilité a diminué de 78 % par rapport au contrôle. Lors de l'exposition au MC de cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*, la viabilité a également été réduite à 79%. Il est intéressant de noter que la MC des cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* traitées au LPS a entraîné une diminution significative de la viabilité neuronale, la réduisant à 47 %. De même, l'incubation avec le MC de cellules *Acox1<sup>-/-</sup>* a conduit à une viabilité de 77% dans les neurones, qui a encore chuté à 36% lors de l'activation du LPS (figure 4A).

Dans le cas des oligodendrocytes 158N, l'incubation avec le MC de cellules BV-2 WT traitées au LPS a conduit à une diminution de la viabilité à 83% par rapport au contrôle. Lors de l'exposition au MC de cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>*, la viabilité des oligodendrocytes a diminué jusqu'à 69%. La MC de cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* traitées au LPS a encore exacerbé cet effet, réduisant la viabilité à 44%. De même, la MC des cellules *Acox1<sup>-/-</sup>* a permis d'obtenir une viabilité des oligodendrocytes de 80 %, qui a chuté à 42 % après l'activation par le LPS (figure 4C).

Par conséquent, la culture des neurones et des oligodendrocytes dans la MC des cellules BV-2 mutantes entraîne une réduction significative de la viabilité cellulaire, en particulier lorsque les cellules sont soumises à l'activation du LPS. Ce résultat indique fortement que la MC dérivée des cellules BV-2 mutantes possède des propriétés cytotoxiques pour les neurones et les oligodendrocytes. Pour déterminer si la diminution observée de la viabilité cellulaire correspond à une mort cellulaire apoptotique, nous avons utilisé la coloration à l'annexine-V-FITC/iodure de propidium en combinaison avec une analyse par cytométrie en flux.

L'analyse des données de cytométrie en flux a révélé que les effets cytotoxiques induits par la MC des cellules KO sont principalement associés à une mort cellulaire apoptotique. Dans le cas des neurones traités avec les MC *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* activées par le LPS, le pourcentage de cellules viables était de 73,1 %, tandis que les populations apoptotiques et nécrotiques représentaient respectivement 23,7 % et 3,1 %. De même, lorsque les oligodendrocytes ont été exposés à des MC *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* traités au LPS, la population de cellules viables est tombée à 42,2 %, les populations apoptotiques et nécrotiques représentant 48,9 % et 8,9 %, respectivement (figure 4B, D).

Ces résultats convaincants confirment que les sécrétions des cellules BV-2 KO exercent des effets cytotoxiques sur les neurones et les oligodendrocytes principalement par le biais de la mort cellulaire apoptotique. L'augmentation observée des populations apoptotiques suggère que des facteurs spécifiques libérés par les cellules BV-2 KO, qui sont absents des sécrétions des cellules WT, contribuent aux voies de signalisation apoptotiques. En effet, Kim et al. ont caractérisé le sécrétome, c'est-à-dire le MC, des microglies BV-2 co-stimulées avec du LPS et de l'IFN $\gamma$  et ont démontré que ce MC était neurotoxique lorsqu'il était placé sur des neurones primaires corticaux de souris (Kim et al., 2007). Nos résultats ont confirmé cette neurotoxicité, mais il est intéressant de noter qu'elle était plus prononcée lorsque la MC des cellules mutantes était appliquée sur les neurones, ce qui signifie que les microglies présentant des défauts peroxysomaux favorisent un environnement toxique.



**Figure 4 : Les sécrétions des cellules BV-2 KO +/- LPS endommagent les neurones mHypoA et les oligodendrocytes 158N principalement par apoptose.**

Après culture dans la MC de cellules BV-2 WT et KO +/-LPS pendant 48h, (A, C) le pourcentage de neurones et d'oligodendrocytes viables déterminé par le test MTT. La signification de la variation de la viabilité des cellules incubées dans la MC des cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* ou *Acox1<sup>-/-</sup>* par rapport à celles incubées dans la MC des cellules WT est indiquée par "#" (#p < 0.05 ; ###p<0.001). "\$" montre l'impact de la MC des BV-2 WT et KO traités au LPS sur la viabilité des neurones et des oligodendrocytes (\$p < 0,05 ; \$\$p < 0,01 ; \$\$\$p < 0,001 ; c'est-à-dire que les sécrétions des cellules WT traitées au LPS ont diminué la viabilité des neurones d'un facteur de 0.78 par rapport aux sécrétions de WT -LPS alors que les sécrétions des cellules *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* traitées au LPS ont diminué la viabilité des neurones d'un facteur de 0,59 par rapport aux sécrétions d'*Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* -LPS et la différence d'effet du LPS entre WT et *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* est significative avec p < 0,01). Le mode de mort cellulaire a été identifié par coloration à l'annexine-V-fluorescéine-isothiocyanate (FITC) et à l'iodure de propidium à l'aide d'un kit de détection de l'apoptose suivi d'un FACS. (B, D) Distribution des cellules vivantes, apoptotiques tardives, apoptotiques précoces et nécrotiques. Les différences statistiques ont été calculées à l'aide d'une ANOVA à 2 facteurs suivie d'une correction pour le taux de fausse découverte pour n=3 répétitions indépendantes pour chaque expérience.

Pour évaluer si les paramètres morphologiques des neurones étaient également affectés par la MC dérivée de nos cellules BV-2 KO, nous avons effectué une analyse de Sholl, un test morphologique classique, sur les neurones soumis à différentes conditions de MC.

L'analyse de Sholl a montré une différence significative entre les différentes conditions. Une analyse plus poussée a été réalisée pour évaluer la complexité neuronale dans chaque condition. Notamment, la complexité neuronale était significativement réduite lorsque les neurones étaient incubés avec KO MC par rapport à WT MC.

Ces résultats indiquent tout d'abord que la microglie joue un rôle crucial dans la régulation de la croissance et de la connectivité neuronales. Ils montrent également que la MC dérivée des cellules BV-2 KO exerce un impact négatif sur les paramètres morphologiques des neurones.

Les ions calcium ( $Ca^{2+}$ ) sont des messagers secondaires omniprésents, qui jouent un rôle crucial dans la régulation de divers processus cellulaires essentiels dans toutes les cellules eucaryotes. Cette dépendance à l'égard de la signalisation  $Ca^{2+}$  est peut-être plus prononcée dans les neurones que dans les autres cellules eucaryotes.

Afin de vérifier si le fonctionnement général des neurones est affecté par la présence des sécrétions des cellules BV-2 WT et KO, nous avons voulu étudier le flux de calcium dans les neurones après incubation dans le MC. Nous avons utilisé l'ATP comme stimulant pour induire l'influx de calcium dans les neurones.

Nos résultats ont révélé des modifications significatives de ces paramètres dans les neurones exposés à différentes conditions de MC. En particulier, l'amplitude de la fluorescence, qui reflète la concentration de  $Ca^{2+}$  entrant dans la cellule lors de la stimulation par l'ATP, était

significativement plus élevée dans les neurones incubés avec le MC *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* que dans les neurones incubés avec le MC WT. Cependant, lorsque les neurones sont cultivés avec la MC *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* traitée au LPS, l'amplitude de la fluorescence est significativement réduite par rapport à la MC *Abcd1<sup>-/-</sup>Abcd2<sup>-/-</sup>* non traitée. Aucun changement significatif n'a été observé dans les autres conditions.

## Discussion

Le RNAseq offre d'énormes possibilités pour divers types d'analyses, mais des contraintes telles que le temps et les fonds limités nous obligent à faire des choix et à établir des priorités dans nos recherches. Dans notre étude, nous avons décidé de nous concentrer sur la confirmation de la surexpression de gènes spécifiques associés au métabolisme lipidique, à la phagocytose, aux lysosomes, à l'autophagie, à la signature DAM, à la présentation antigénique, à l'inflammation et au stress oxydatif. Cependant, en raison de la profondeur et de la complexité des voies impliquées, nous n'avons pas été en mesure d'effectuer une analyse approfondie de toutes les voies altérées résultant du knock-out du gène peroxysomal dans la microglie, principalement en raison du manque de temps.

Plusieurs altérations dans différentes voies et processus cellulaires ont été découvertes dans les cellules BV-2 après l'inactivation des gènes peroxysomaux, mais elles ne sont pas toujours partagées par les quatre inactivations. Les différences observées entre les cellules knock-out peuvent être attribuées à plusieurs facteurs, principalement aux altérations génétiques distinctes induites par le knock-out de gènes spécifiques impliqués dans les fonctions peroxysomales. L'inactivation des gènes des transporteurs peroxysomaux (*ABCD1* et *ABCD2*) entraîne une altération de l'importation des acides gras dans les peroxysomes, ce qui perturbe la  $\beta$ -oxydation. Inversement, l'invalidation du gène *ACOX1* affecte l'enzyme responsable de l'étape initiale de la  $\beta$ -oxydation. Ces différentes modifications génétiques ont des impacts variés sur le métabolisme cellulaire, l'homéostasie lipidique et les voies de signalisation en aval.

Les mécanismes physiopathologiques de la X-ALD restent obscurs, mais certaines recherches *in vitro/in vivo* ont montré que les AGTLC pouvaient induire un stress oxydatif et une inflammation, entraînant des dommages cellulaires (J. Yu et al., 2022). Certaines données ont confirmé l'implication de la microglie dans la pathogenèse de l'X-ALD. Les zones de microglie activée dans les régions démyélinisantes du cerveau, correspondant aux zones où la myéline contient des niveaux plus élevés d'AGTLC, ainsi que les niveaux élevés de chimiokines pro-inflammatoires dans le LCR des patients cALD ont permis de suggérer que le dysfonctionnement microgliale et l'apoptose pourraient constituer un changement pathogénique précoce dans l'X-ALD cérébrale, contribuant à la neuroinflammation et à l'altération de l'unité neuro-vasculaire. Cela a également permis de suggérer que les fonctions microgliales anormales pourraient nuire à la capacité de fournir des facteurs neuroprotecteurs aux oligodendrocytes déficients (Cartier et al., 2014; F. S. Eichler et al., 2008)

Ici, nous avons démontré que le modèle de cellules microgliales BV-2 de l'X-ALD (*Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup>), accumulant les AGTLC, présente un phénotype de microglie pathologique (DAM), ainsi qu'une altération de ses fonctions de cellule phagocytaire et de cellule présentatrice d'antigènes. En réponse au LPS, les cellules *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> augmentent leur sécrétion de cytokines pro-inflammatoires et la production de marqueurs de stress oxydatif dont nous avons prouvé qu'ils étaient cytotoxiques pour les neurones et les oligodendrocytes. Ceci est en accord avec l'hypothèse de l'implication de la microglie dans la pathogénèse de l'X-ALD, mais aussi de son rôle dans l'initiation de la neurodégénérescence.

Enfin, malgré les revers et les contraintes, cette recherche révèle un lien significatif et inattendu entre les fonctions peroxysomales et les processus microgliaux, ce qui a un impact sur l'homéostasie du système nerveux central. En mettant en lumière les fonctions microgliales spécifiques aux peroxysomes, cette étude contribue à une meilleure compréhension des interactions complexes au sein du SNC et ouvre de nouvelles voies pour la recherche future sur la biologie microgliale et les maladies neurologiques associées.



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Review

# Peroxisomal ABC Transporters: An Update

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**Abstract:** ATP-binding cassette (ABC) transporters constitute one of the largest superfamilies of conserved proteins from bacteria to mammals. In humans, three members of this family are expressed in the peroxisomal membrane and belong to the subfamily D: ABCD1 (ALDP), ABCD2 (ALDRP), and ABCD3 (PMP70). These half-transporters must dimerize to form a functional transporter, but they are thought to exist primarily as tetramers. They possess overlapping but specific substrate specificity, allowing the transport of various lipids into the peroxisomal matrix. The defects of ABCD1 and ABCD3 are responsible for two genetic disorders called X-linked adrenoleukodystrophy and congenital bile acid synthesis defect 5, respectively. In addition to their role in peroxisome metabolism, it has recently been proposed that peroxisomal ABC transporters participate in cell signaling and cell control, particularly in cancer. This review presents an overview of the knowledge on the structure, function, and mechanisms involving these proteins and their link to pathologies. We summarize the different *in vitro* and *in vivo* models existing across the species to study peroxisomal ABC transporters and the consequences of their defects. Finally, an overview of the known and possible interactome involving these proteins, which reveal putative and unexpected new functions, is shown and discussed.

**Keywords:** ABC transporters; peroxisome; adrenoleukodystrophy; fatty acids



**Citation:** Tawbeh, A.; Gondcaille, C.; Trompier, D.; Savary, S. Peroxisomal ABC Transporters: An Update. *Int. J. Mol. Sci.* **2021**, *22*, 6093. <https://doi.org/10.3390/ijms22116093>

Academic Editor: Thomas Falguières

Received: 28 April 2021

Accepted: 3 June 2021

Published: 5 June 2021

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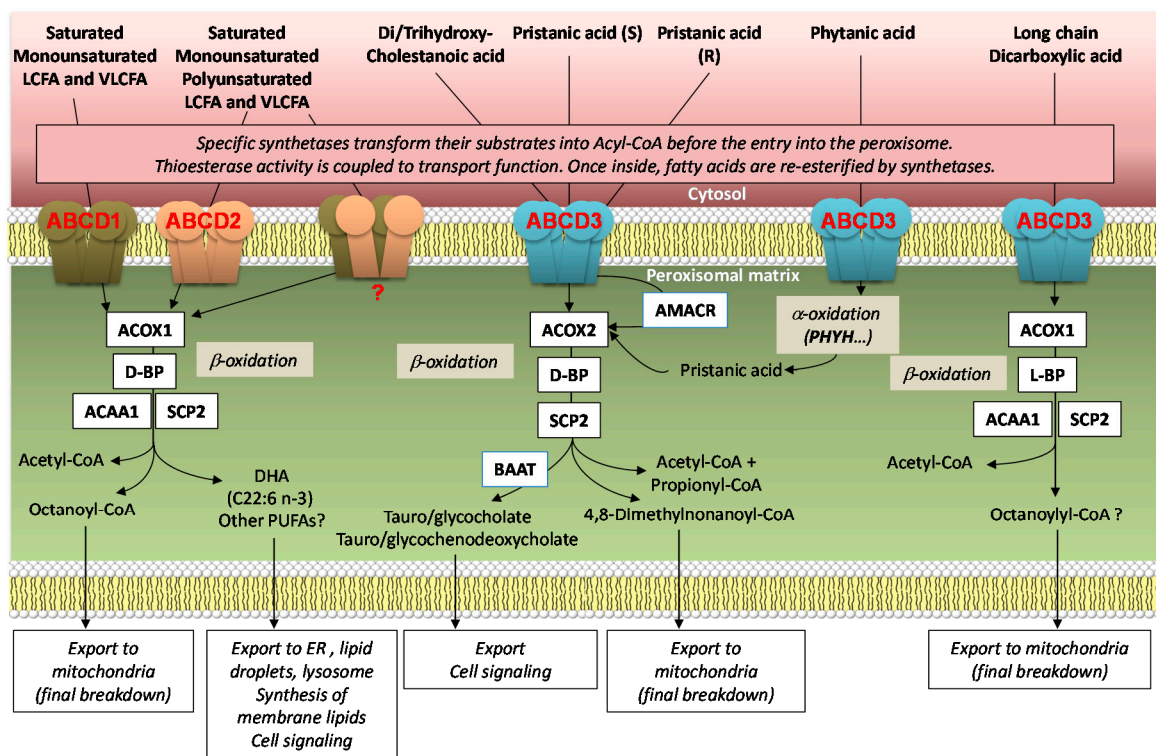


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## 1. Introduction

ATP-binding cassette (ABC) transporters constitute a superfamily of membrane transporter proteins that actively translocate a wide range of molecules, from simple molecules (fatty acids (FAs), sugars, nucleosides, and amino acids) to complex organic compounds (lipids, oligonucleotides, polysaccharides, and proteins) [1]. Transport of substrates is dependent on the hydrolysis of ATP, which releases energy that can be used to accumulate substances in the cellular compartments or export them to the outside. ABC transporters are distributed not only in the plasma membrane of both prokaryotes and eukaryotes, but also in the membranes of the organelles of eukaryotic cells such as peroxisomes, mitochondria, lysosomes, and endoplasmic reticulum (ER). Based on their amino acid homology and structural configuration, ABC transporters in humans are classified into seven subfamilies, A to G, comprising a total of 48 ABC transporters, many of which are implicated in diseases [2]. ABC transporters of subfamily D include four proteins in mammals: ABCD1 [adrenoleukodystrophy protein (ALDP)], ABCD2 [adrenoleukodystrophy-related protein (ALDRP)], ABCD3 [70 kDa peroxisomal membrane protein (PMP70)], and ABCD4 [peroxisomal membrane protein 69 (PMP69)] [3]. ABCD1, ABCD2, and ABCD3 are located in the peroxisomal membrane. ABCD4 was identified by homology search for ALDP and PMP70 related sequences in the database of expressed sequence tags, and was initially considered peroxisomal despite the absence of a membrane peroxisomal targeting signal [4]. More recently, several studies have demonstrated that ABCD4 resides in the endoplasmic reticulum and lysosomes, and that its function is associated with cobalamin metabolism [3,5,6].

The three human peroxisomal ABC transporters play an important role in the transport of various lipid substrates into the peroxisome for their shortening by  $\beta$ -oxidation (Figure 1).  $\beta$ -oxidation of FAs is a conserved process of peroxisomes by which acyl groups are degraded two carbons at a time after being activated to form the corresponding CoA derivative by a specific acyl-CoA synthetase located at the peroxisomal membrane [7]. The  $\beta$ -oxidation process exists in mitochondria for medium- and long-chain fatty acids (MCFAs and LCFAs) and is necessary to terminate degradation of octanoyl-CoA coming from peroxisomes. However, very long-chain fatty acids (VLCFAs, number of carbon atoms >22) are exclusively  $\beta$ -oxidized into the peroxisome, and this organelle is therefore essential, especially in the brain [8]. Moreover, polyunsaturated fatty acid (PUFA) synthesis may require a peroxisomal cycle of  $\beta$ -oxidation, as in the case of docosahexaenoic acid (DHA, C22:6 n-3) synthesis from its precursor (C24:6 n-3) [9]. It is important to note that DHA is not only of great value by itself as a component of cell membranes, but is also the source of eicosanoids associated with several key signaling functions [10].



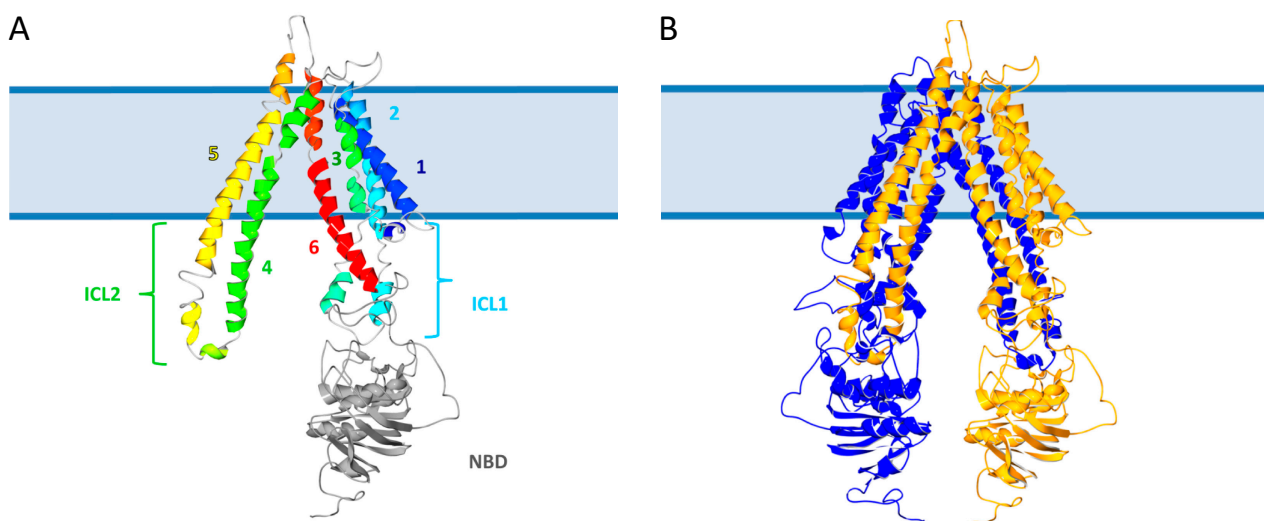
**Figure 1.** Peroxisomal ABC transporters and their involvement in lipid metabolism. Peroxisomal ABC transporters are represented as homo or heterotetramers with their preferential substrates and their involvement in metabolic routes, including several enzymatic steps, catalyzed by acyl-CoA oxidase 1 and 2 (ACOX1 and ACOX2), D- and L-bifunctional protein (D-BP and L-BP), acetyl-CoA Acyltransferase 1 (3-ketoacyl-CoA thiolase, ACAA1), sterol carrier protein 2 (SCPX thiolase, SCP2), alpha-methylacyl-CoA racemase (AMACR), bile acid-CoA:amino acid N-acyltransferase (BAAT), and phytanoyl-CoA hydroxylase (PHYH).

Thus, peroxisomal  $\beta$ -oxidation may not be considered a simple catabolic process of fatty acids. The role of peroxisomal ABC transporters is therefore not restricted to the catabolic function of peroxisomes, but is fully associated with their various metabolic functions including synthesis and degradation of lipids, cell signaling, inflammation control, and redox homeostasis [11–15].

## 2. Structure, Function, and Mechanism of Transport

### 2.1. Structure

The general structure of eukaryotic ABC transporters is a four functional unit organization, comprising two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). NBDs bind and hydrolyze ATP to trigger conformational changes in the TMDs, resulting in unidirectional transport across the membrane [1]. Human peroxisomal ABC transporters have a half-transporter structure, with only one TMD and one NBD [16]. In 2017, we proposed a structural model of human ABCD1 based on the crystal structure of the mitochondrial ABC transporter ABCB10, which shows not only the putative structure of ABCD1 in a membrane context but also the complex intricacy of  $\alpha$ -helices that constitute the whole transmembrane domain (Figure 2) [17]. Therefore, peroxisomal ABC half-transporters need to homo- or heterodimerize in the peroxisomal membrane in order to constitute a full, active transporter [18,19].



**Figure 2.** Structural model of human ABCD1 (reprinted from [17]). (A) Ribbon representation of the ABCD1 monomer. TMD helices are numbered from 1 to 6 and rainbow colored from dark blue to red. NBD is in light grey, and intracellular loops (ICL) 1 and 2 are indicated. (B) Ribbon representation of the ABCD1 homodimer with the two subunits respectively colored in dark blue and yellow.

Data shows that ABCD1, ABCD2, and ABCD3 are able to interact as homodimers or heterodimers [20–22], although both ABCD1 and ABCD3 are mainly found as homodimers in mammalian peroxisomal membranes [23–25]. Moreover, ABCD1 and ABCD2 homodimers are functional [26,27]. However, the fact that nonfunctional ABCD2 has a transdominant negative effect on ABCD1 [20] suggests that heterodimers of ABCD1 and ABCD2 are also functional and can exist within cells and tissues expressing both proteins. Besides, chimeric proteins consisting of homo- and heterodimers of ABCD1 and ABCD2 are functionally active [19]. Concerning ABCD3, although homodimers and heterodimers with ABCD1 and ABCD2 have been described [22,23,25,28,29], no data is available about the functional value of the ABCD3 dimers. Surprisingly, ABCD1 and ABCD3 were found in different detergent-resistant microdomains [29], implying that these proteins have a different environment in the peroxisomal lipid bilayer, questioning the biological relevance of the ABCD1 and ABCD3 heterodimers. Additionally, native PAGE experiments concerning complex oligomerization confirm that ABCD1 and ABCD2 exist predominantly as homo-tetramers, although both homo- and hetero-tetramers are present [28]. Therefore, we cannot rule out the possibility that hetero-interaction between ABCD1 and ABCD2 occurs in hetero-tetramers composed of two distinct homodimers rather than in complexes composed of two heterodimers. Finally, it remains unclear whether the oligomerization of peroxisomal ABC transporters has any influence on substrate specificity.

## 2.2. Substrate Specificity

Since the cloning of the *ABCD1* gene in 1993 and its association with X-ALD [30], ABCD1 function has been attributed to the transport of saturated and monounsaturated VLCFAs across the peroxisomal membrane for further degradation by  $\beta$ -oxidation. Accumulation of saturated and monounsaturated VLCFAs indeed occurs in the plasma and tissues of X-ALD patients, and is used for diagnosis [31,32]. Due to its importance, studies concerning the structure, function, and defects of ABCD1 have never ceased. Functional complementation experiments in yeast, functional assays in mammalian cells, especially cells coming from X-linked adrenoleukodystrophy (X-ALD) patients, and studies using animal models, mainly knock-out mice, were helpful in clarifying the question of substrate specificity. The *Abcd1* knock-out mice confirmed the human biochemical phenotype, indicating that ABCD1 is indeed involved in the transport of VLCFAs [33–35]. Transfection of X-ALD skin fibroblasts with ABCD1 cDNA corrected the  $\beta$ -oxidation defect and restored normal levels of VLCFAs [36,37]. The preference of ABCD1 for saturated FAs was also confirmed in yeast [26,27].

Cloned by homology using degenerate primers, the *ABCD2* gene was shown to code for ALDRP, the closest homolog of ALDP [38]. Both proteins display overlapping substrate specificities for saturated and monounsaturated LCFAs and VLCFAs. It explains the correction of  $\beta$ -oxidation defect in X-ALD fibroblasts in case of *ABCD2* overexpression after transfection [39]. Using transgenic expression of *Abcd2* in the *Abcd1* knock-out mouse, Pujol et al. demonstrated that VLCFA accumulation and disease phenotype could be corrected in vivo [40]. This set the basis for a new therapeutic strategy for X-ALD patients aiming at inducing *ABCD2* expression with pharmacological, hormonal, or nutritional management [41,42]. Pharmacological induction of *ABCD2* was indeed shown to compensate for ABCD1 defect in vitro and in rare cases, in vivo, opening the way for clinical trials [43–58].

Functional complementation in yeast model and X-ALD fibroblasts confirmed the functional redundancy for saturated VLCFAs, but also demonstrated the specific role of ABCD2 in PUFA transport, especially DHA and its precursor (C24:6 n-3) [26]. Experiments in mammalian cells confirmed such substrate preference [19,20]. Further studies using the *Abcd2* null mice demonstrated a specific role in MUFA transport, especially for erucic acid (C22:1 n-9) in adipose tissue [59,60] and an extended role in FA homeostasis [61].

PMP70, the protein coded by the *ABCD3* gene, was the first identified peroxisomal ABC transporter and is the most abundant peroxisomal membrane protein, at least in hepatocytes [62,63]. Wrongly associated with peroxisome biogenesis [64], ABCD3 is also involved in the transport of various lipids and shows overlapping substrate specificities with ABCD1 when overexpressed [37,65]. Though, ABCD3 clearly has the broadest substrate specificity as it is involved in the transport of LCFAs and VLCFAs but also specifically in the transport of dicarboxylic acids, branched-chain fatty acids, and C27 bile acid intermediates such as di- and tri-hydroxy-cholestanoic acid [65–67]. The *Abcd3* knock-out mice indeed revealed a marked accumulation of bile acid intermediates, and ABCD3 was recently associated with a congenital bile acid defect (CBAS5, see below) [67]. Furthermore, a more recent study performed on manipulated HEK-293 cell models proved that ABCD3 is required for the transport of MCFAs across the peroxisomal membrane [68].

## 2.3. Mechanism

Conversion of free FAs into CoA esters constitutes an initial activation step before peroxisomal  $\beta$ -oxidation. This reaction is catalyzed by specific acyl-CoA synthetase connected to the cytosolic side of the peroxisomal membrane [69]. It was proved, using protease protection assays, that acyl-CoAs but not free FAs bind to the TMD of the transporter [70]. It is therefore only after activation that the fatty acyl-CoAs are transported to the peroxisomal matrix through peroxisomal ABC transporters. Fatty acyl-CoA are captured on the cytosolic side by the TMD, enhancing the affinity of NBD for ATP. ATP molecules are then hydrolyzed, thus producing the energy needed to switch the conformation of TMD and eventually allowing the translocation of substrates from the cytosol into the peroxisomal



matrix [22,71]. However, the exact mechanism of transport remains controversial. Two models are commonly considered. The first implies that esterified FAs are delivered directly to the peroxisomal matrix, whereas in the other model, free FAs are transported into the peroxisomal matrix after the hydrolysis of acyl-CoAs, which are re-esterified by acyl-CoA synthetase when in the peroxisomal lumen.

Although the process of cleavage and reactivation of acyl-CoAs seems to be a waste of energy as two ATP molecules are needed for the activation reaction, such a mechanism is crucial for the specific permeabilization of the substrates of  $\beta$ -oxidation [18]. Several studies have been done in an attempt to figure out the correct model for the transportation mechanism. Early studies on yeast models have demonstrated that fatty acyl-CoAs are hydrolyzed before being transported. This hydrolysis occurs when acyl-CoAs interacts with the heterodimer Pxa1p-Pxa2p at the cytosolic side of the peroxisomal membrane [72]. The peroxisomal ABC transporters would release a free fatty acid that should be re-esterified inside the peroxisome before its catabolic processing. In addition, intrinsic acyl-CoA thioesterase activity has been found in COMATOSE (CTS), a homolog of human ABCD1 in *Arabidopsis thaliana*, proving again that VLCFA-CoA is hydrolyzed prior to transport [73]. Very recently, the work of Kawaguchi et al. provided further proof of the transport mechanism [74]. After expressing human His-tagged ABCD1 in methylotrophic yeast, they directly demonstrated that ABCD1 transports the FA moiety after the hydrolysis of VLCFA-CoA and that acyl-CoA synthetase is required before the  $\beta$ -oxidation of VLCFA-CoA within the peroxisomes. When it comes to the fate of the free CoA, they are released in the peroxisomal lumen, as revealed using isolated peroxisomes from *Saccharomyces cerevisiae* [75].

Finally, after their re-esterification, substrates are directly delivered to specific acyl-CoA oxidases to initiate the  $\beta$ -oxidation process. Peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) catalyzes the first and rate-limiting step of the  $\beta$ -oxidation pathway dedicated to straight-chain fatty acids, which includes LCFAs, VLCFAs, PUFAs, and dicarboxylic acids [76]. Other acyl-CoA oxidases also exist, ACOX2 and ACOX3. ACOX2 is specific to bile acid intermediates [76] whereas the oxidation of branched-chain FAs depends on both ACOX2 and ACOX3 enzymes [77,78]. Of note, mitochondria catalyze the  $\beta$ -oxidation of the majority of short, medium, and long chain FAs but not that of VLCFAs [79]. In yeast and plants, this process of FA  $\beta$ -oxidation occurs exclusively in peroxisomes, whereas in higher eukaryotes, the catabolism of VLCFAs is initiated solely in the peroxisomes [7,80].

### 3. Human Diseases

#### 3.1. X-Linked Adrenoleukodystrophy

X-linked adrenoleukodystrophy (X-ALD, OMIM # 300100) is the most frequent peroxisomal disorder but is still classified as a rare disease, with an estimated incidence of 1:17,000 [81]. Recent therapeutic successes [82,83], and the feasibility and reliability of a diagnosis method based on VLCFA quantification from blood spot [84,85], have prompted some countries to establish systematic screening of newborns. This complex and fatal neurodegenerative disorder is characterized by a huge clinical variability both in the age of onset and in the symptoms [31]. The two main forms are the childhood cerebral ALD (ccALD), characterized by inflammatory demyelination of the central nervous system and the adult form, called adrenomyeloneuropathy (AMN), consisting of a non-inflammatory, slowly progressive demyelination affecting the spinal cord and peripheral nerves. X-ALD is also the main cause of Addison's disease and adrenal insufficiency may remain the unique symptom of the disease. Since the disease is linked to chromosome X, boys and men are the most severely affected patients. Female carriers usually remain quasi asymptomatic or present only a mild phenotype, but severe forms have also been described [86].

In 1993, using positional cloning, the team of Hugo Moser identified the ABCD1 gene as being responsible for X-ALD [30]. Mutations in the ABCD1 gene have been found in every X-ALD patient and are collected in the X-ALD database (<https://adrenoleukodystrophy.info/> accessed on 1 June 2021). In spite of almost 900 non-recurrent mutations, no genotype-

phenotype correlation has been described. It is important to note that the majority of missense mutations affect protein stability and result in the absence of the protein. ABCD1 defect results in the accumulation of VLCFAs, mainly C26:0 and C26:1, which accumulate as free FAs or in esterified forms in membrane lipids and cholesteryl esters. This accumulation results from the impossibility of their entry into the peroxisome for their degradation by  $\beta$ -oxidation, but also from an increased endogenous biosynthesis [87]. While the toxicity of VLCFAs has been recognized [88], the sequence of events leading to neurodegeneration and inflammation is still debated. Oxidative stress and cellular components, especially microglial functions, seem to play a major role in the pathogenesis of X-ALD [89–91].

Therapeutic strategies depend on the clinical symptoms of patients. A majority of X-ALD patients present adrenal insufficiency and require a careful patient follow-up, but hormone-replacement therapy successfully manages the adrenal defect and prevents a potentially fatal Addisonian crisis. Hematopoietic stem cell transplantation (HSCT) has proven efficiency to halt neurological involvement in X-ALD. Since 1990 and the first success of this therapy [92], allogeneic graft has been indicated for boys with ccALD at an early stage of the disease when a compatible donor exists. In 2009, autologous HSCT was demonstrated to be successful to halt cerebral demyelination in two boys with no compatible donors who received their own genetically corrected stem cells [82]. Lentiviral correction of bone marrow derived stem cells and autologous transplantation proved effective in 15 patients in 2017 [83]. These promising results suggest that such a therapeutic strategy may be as effective as allogeneic HSCT. In addition, efforts to find pharmacological strategies targeting oxidative stress, inflammation, or compensatory mechanisms (antioxidant cocktail [93], leriglitazone [94], sobetirome [50,54,55]) are still present. It remains to be evaluated whether such treatments would be useful per se or in combination with HSCT strategies, at least to delay the onset of neurological concerns and permit a lengthening of the time window to allow transplantation.

### 3.2. Congenital Bile Acid Synthesis Defect Type 5

Although mutations were found in the ABCD3 gene of a Zellweger patient [64], further evidence showed that ABCD3 has no link with peroxisomal biogenesis and is definitively not associated with Zellweger Syndrome [95]. ABCD3, which presents partial functional redundancy with ABCD1, has been shown to transport branched-chain FAs, dicarboxylic acids, and bile acid precursors. A few years ago, the accumulation of peroxisomal C27-bile acid intermediates DHCA and THCA, as well as VLCFAs, was described in a young Turkish girl whose parents were consanguineous [67]. The patient presented hepatosplenomegaly and a severe progressive liver disease and she died of complications after liver transplantation. Patient fibroblasts showed reduced numbers of enlarged peroxisomes, as well as reduced  $\beta$ -oxidation of pristanic acid, compared to controls. Immunofluorescence confirmed the absence of ABCD3 in the peroxisomal membrane. A homozygous truncating mutation was identified in the ABCD3 gene of the patient, and the disease was named congenital bile acid synthesis defect (CBAS) type 5 (OMIM # 616278). It should be noted that CBAS type 1, 2, 3, 4, and 6 are associated with mutations in HSD3B7, AKR1D1, CYP7B1, AMACR, and ACOX2 respectively. These genes control key reactions in bile acid synthesis and all the CBAS forms present an autosomal recessive inheritance.

### 3.3. Peroxisomal ABC Transporters and Cancer

Beyond its recognized role in metabolism and redox homeostasis, the peroxisome is now increasingly regarded as a signaling platform and a key organelle in cellular metabolic reprogramming with major consequences on the immune response, cell cycle, and cell differentiation [12,96]. Elegantly presented in the state of the art of Hlaváč and Souček, several studies have revealed a significant association between the level of expression of peroxisomal ABC transporters and various cancers [97]. This suggests a role of these ABC transporters in cell cycle control, cell differentiation, and tumorigenesis. Downregulation of peroxisomal ABC transporters has been observed in several cases: *ABCD1* in

melanoma [98] and renal cell carcinoma [99], *ABCD2* in breast cancer [100], and *ABCD3* in ovarian cancer [101] and colorectal cancer [102]. Moreover, a lower prognostic value has been associated with low expression of *ABCD1* in ovarian cancer [103] and low expression of *ABCD3* in colorectal cancer [102]. On the contrary, *ABCD1* and *ABCD3* were found upregulated in breast carcinoma [104], and a positive correlation was observed between *ABCD3* expression and glioma tumor grades [105]. Recently, VLCFA accumulation was associated with colorectal cancer [106]. Increased endogenous elongation appears to be primarily responsible for this observation, but peroxisomal ABC transporters are also likely involved, and the ability to regulate their expression could potentially represent a therapeutic interest in such cancers. Altogether, further studies are required to understand the link between the transport function and metabolic role of peroxisomal ABC transporters and the control of cell cycle with regard to the complexity of tumor heterogeneity.

#### 4. Cell, Plant, and Animal Models

Phylogenetic analysis of peroxisomal ABC transporters in eukaryotes shows strong conservation, highlighting their fundamental and specific role in the cellular functions. Interestingly, their substrate specificity seems to become more restrictive with the complexification of the biological systems. Although the number of peroxisomal ABC transporters and their specific functions vary between species, each model of study is of scientific interest and has contributed significantly to the knowledge of peroxisomal ABC transporters. Here are described the main eukaryote models.

##### 4.1. Yeast

In addition to its convenience for genomic modification, *Saccharomyces cerevisiae* is a particularly interesting model for studying the peroxisomal metabolism of lipids, since it can use FAs as its only carbon source and  $\beta$ -oxidation of FAs of all length takes place only in peroxisome. The yeast model expresses only two peroxisomal ABC transporters, called Pxa1p and Pxa2p, which function as a strict heterodimer to import fatty acyl-CoAs into the peroxisomal matrix [107–109]. Functional assays and functional complementation experiments of *pxa1/pxa2*Δ yeast mutants with mammalian peroxisomal ABC transporters were particularly important in studying their transport mechanism and substrate specificity [26,65,75].

##### 4.2. Plant

In *Arabidopsis thaliana*, CTS, the human *ABCD1* ortholog, is an integral peroxisomal membrane protein composed of two fused half-size transporters. CTS is involved in the import of FAs and phytohormone precursors into the peroxisome where they are  $\beta$ -oxidized [110,111]. The products of this oxidation are involved in the transition from dormancy to germination, root growth, seedling establishment, and fertility [112]. Expression of human *ABCD1* in *A. thaliana* CTS mutant cannot restore the germination and establishment, whereas human *ABCD2* only restores the germination phenotype [113]. These results are related to the physiological differences between plants and mammals, and highlight the differences in substrate specificity between *ABCD1* and *ABCD2*. The plant model was also very important as it showed for the first time the existence of CTS in high molecular weight complexes and allowed the study of the transport mechanism, especially the role of its thioesterase activity [73].

##### 4.3. Nematode

*Caenorhabditis elegans* is a well-known worm model in neurobiology studies, but the interest of this model in the field of X-ALD has been shown only very recently. *PMP-4* is one of the five putative peroxisomal ABC transporters identified in *C. elegans* and is the ortholog of human *ABCD1* and *ABCD2*. It is mainly expressed in gut and hypodermis, the main fat storage tissues in the *C. elegans*. Moreover, hypodermal cells have similarities with vertebrate glial cells and participate in neuronal migration [114]. *PMP-4* deficient worms

have a normal growth and maturation but show several hallmarks of X-ALD (global VLCFA accumulation, redox imbalance, axonal damage, motility alteration) [115]. Interestingly, the number and the size of lipid droplets (LDs) are increased and can be normalized using a mitochondrial targeted antioxidant. *C. elegans* is therefore a valuable model to study the involvement of FA accumulation and oxidative stress in the pathogenesis of X-ALD but has some limitations since its nervous system is not myelinated.

#### 4.4. Insect

An X-ALD fly model has been generated in *Drosophila melanogaster* using RNA interfering of *dABCD*, the ortholog of *ABCD1*. These flies survive to adulthood but exhibit a specific brain neurodegenerative phenotype with retinal defects including holes and loss of pigment cells associated with death of neurons and glia [116]. Interestingly, cellular targeted disruption of *dABCD* in neurons, but not in glia, triggers the retinal defects. The phenotype is indistinguishable from the one observed in *bgm* (bubblegum) and *dbb* (double-bubble) deficient flies [117]. Both *bgm* and *dbb* genes code for long/very-long-chain acyl-CoA synthetases. The shared neurodegenerative features in *dABCD* and *bgm/dbb* deficient flies show that the lipid metabolic pathway is a key component of the X-ALD-like neurodegenerative disease in *Drosophila*. More specifically, experiments achieved with *bgm* and *dbb* deficient flies indicate that the loss of metabolites is the cause of neurodegenerative disease rather than accumulation of substrates (V/LCFAs), as was commonly thought.

#### 4.5. Fish

Zebrafish (*Danio rerio*) has recently been proved to be a useful model for studying the pathogenesis of X-ALD. Indeed, *Abcd1* (the zebrafish ortholog of *ABCD1*), is expressed during development in spinal cord and in the central nervous system especially in the oligodendrocytes and motor neuron precursors, but also in the interrenal gland (functional equivalent of the adrenal cortex) [118]. Zebrafish *Abcd1* mutant models show key biochemical and nervous system alteration features of X-ALD (increased level of C26:0, accumulation of cholesterol, hypomyelinated spinal cord, modified development of interrenal gland and brain, early alteration of motor behavior, decreased survival, and modified oligodendrocytes pattern associated with apoptosis). Interestingly, the motor alteration and the oligodendrocytes pattern can be corrected by human *ABCD1* expression. Moreover, a recent drug screening study showed that chloroquine can improve motor activity in zebrafish *Abcd1* mutant and reduce saturated VLCFA levels [119].

#### 4.6. Rat and Mouse

Various cellular models have been created in rodent species to study the function of peroxisomal ABC transporters and the consequences of their defect. Considering that the liver is a platform for peroxisomal lipid metabolism in mammals, the hepatic H4IIEC3 cell line was used to create a specific cell model allowing the inducible expression of a normal or mutated rat *Abcd2* protein fused to green fluorescent protein [120]. It allowed to precise the substrate specificity of *Abcd2* as well as its dimeric status, and even, to demonstrate for the first time its supradimeric structure. [19,20,28]. To better understand the role of peroxisomal ABC transporters in the glial cells, models of ALD astrocytes have been developed. Astrocytes are known to regulate the inflammatory response. In neurodegenerative diseases, reactive astrocytes secrete inflammatory cytokines, which allow the permeability of the blood-brain barrier (BBB) to peripheral infiltrating immune cells. When *Abcd1* and/or *Abcd2* genes are silenced in mouse primary astrocytes, X-ALD biochemical hallmarks are present (decreased C24:0  $\beta$ -oxidation, increased C26:0 level), but so are redox imbalance and pro-inflammatory features (increased cytokines expression and nitric oxide production) [121]. These characteristics are inverted by treatment with Lorenzo oil and increased by a long-term VLCFA treatment showing the link between VLCFA accumulation and the pro-inflammatory response of these glial cells [122]. These first results obtained in primary astrocytes led to the development of an immortalized

astrocyte cell line [123]. This model should be very useful for studying the mechanisms of astrocyte activation and was used to screen therapeutic compounds such as SAHA, an HDAC inhibitor that normalizes ROS production as well as iNOS and TNF expression [53].

Microglia is also considered a major player in the X-ALD pathogenesis, especially in the inflammatory process. To proceed further, *Abcd1* and/or *Abcd2* deficient microglia cell lines have been obtained using CRISPR/Cas9 gene editing in the mouse BV-2 cell line [124]. The *Abcd1*<sup>-/-</sup>*Abcd2*<sup>-/-</sup> cells, generated to avoid masking effects due to functional redundancy, show classical X-ALD biochemical hallmarks (increased levels of saturated and monounsaturated VLCFAs) but also increased levels of some LCFAs and PUFAs. Like in brain macrophages from X-ALD patients [125], whorled lipid inclusions, probably corresponding to cholesterol esters of VLCFAs, were observed, making these cells particularly interesting for modelling the human disease. Further studies using these cell lines, alone or in co-culture with glial and/or neuronal cells, should bring new insights for understanding the impact of *Abcd1/Abcd2* deficiencies in the microglial function, and could be used for the screening of pharmaceutical compounds useful to halt chronic inflammation in the brains of cALD patients.

In order to study the function of peroxisomal ABC transporters and the pathogenesis of X-ALD in integrated mammalian models, *Abcd1*-, *Abcd2*-, and *Abcd3*-deficient mouse models have been generated [33–35,40,67,126]. The *Abcd1* knock-out mice show key biochemical features of X-ALD but develop a late onset progressive neurodegenerative phenotype involving the spinal cord and sciatic nerves without brain damage [127]. In the spinal cord, inflammation is observed in old mice and includes microglia and astrocyte activation [40]. However, microglia activation seems to occur early, probably from eight months of age [91]. VLCFA excess would induce an early oxidative stress leading to mitochondria structural and functional damages as well as an ER stress concomitant with autophagy disruption [128–132]. Although no cerebral phenotype is observed, *Abcd1* knock-out mice can be considered a physiological model of AMN or female myelopathy and can be useful for screening pharmaceutical compounds. Several molecules have thus been tested and have demonstrated their efficacy, including antioxidant compounds that have been proven to reverse oxidative stress in vitro and reduce locomotor impairment [133–135]. These hopeful results led to a prospective phase II pilot study that was carried out for 13 AMN patients treated with a cocktail of antioxidant molecules [93]. The study showed that biomarkers of oxidative damage and inflammation were normalized and that patients' locomotion was improved, paving the way for a hopeful Phase III study.

Even if the mouse model is attractive because of its phylogenetic proximity to humans, it doesn't reproduce the human brain phenotype of X-ALD. One possible explanation could be related to species and cell-type differences in the expression levels of *ABCD1–3* and functional redundancy issues. Sustaining this hypothesis, a transcriptomic analysis showed that *ABCD2* is not expressed in human microglia and *ABCD3* is 1.6-fold more expressed than *ABCD1* [136], whereas in mouse BV-2 microglial cells, *Abcd2* is 2.5-fold more expressed than *Abcd1* and *Abcd3* is 1.6-fold more expressed than *Abcd1* [124]. In addition, the biochemical and neurological defects observed in the *Abcd1* knock-out mice can be corrected by ubiquitous transgenic expression of *Abcd2* [40]. On the contrary, *Abcd1/Abcd2* double knock-out mice have an earlier and more severe neurological phenotype associated with inflammatory T lymphocyte infiltration in the spinal cord [40]. The *Abcd2* knock-out mice also develop progressive motor disabilities specifically involving sensitive peripheral neurons and spinal cord dorsal and ventral columns and share subcellular abnormalities with the *Abcd1* knock-out mice (axonal degeneration, C26:0 accumulation, oxidative stress, organelle abnormalities concerning mitochondria, lysosome, endoplasmic reticulum, and Golgi apparatus). This model also revealed the key role of *Abcd2* in adrenals [137] and in adipose tissue and lipid physiology [59–61].

In contrast to the *Abcd1* and *Abcd2* knock-out models, the *Abcd3* knock-out mice do not develop peripheral or central neurodegeneration (like *ABCD3* deficiency in humans),

but exhibit hepatomegaly associated with abnormalities in peroxisomal FA metabolism, which seems to represent a suitable model for CBAS5 [67].

#### 4.7. Human

X-ALD patient skin fibroblasts have, for several years, constituted one of the rare in vitro models of the disease. In 1980, Moser et al. demonstrated for the first time that the accumulation of VLCFAs observed in the brain and adrenals of patients is also present in primary fibroblasts, thus validating this model for X-ALD studies, at least at the biochemical level [138]. Since then, this cellular model has become a platform for a broad variety of analyses concerning lipid metabolism, X-ALD diagnosis, functional characterization of peroxisomal ABC transporters, cellular consequences of *ABCD1* deficiency, and screening of therapeutic compounds. Great scientific advances have emerged from this handy model, but its skin origin is a limitation in pathogenesis studies. Indeed, the gene regulation and function in skin fibroblasts are very far from those of neural, glial and microglial cells.

The involvement of peripheral blood mononuclear cells (PBMCs) in the inflammation feature of X-ALD was early suspected, since PBMCs from X-ALD patients produce higher levels of inflammatory cytokines than control ones [139,140]. Used in gene therapy, the CD34+ PBMCs (lymphoid and myeloid progenitors) transduced with normal *ABCD1* can efficiently correct the clinical phenotype of the X-ALD patients [82]. Moreover, AMN monocytes have a pro-inflammatory expression pattern and, after differentiation into macrophages, are not able to switch to an anti-inflammatory regenerative state [141]. *Abcd2*, whose expression level is extremely low in these cells, could be a therapeutic target [142]. Therefore, human monocytes can be used to study the inflammatory process and identify compounds capable of inducing *ABCD2* expression, correcting VLCFA level,  $\beta$ -oxidation, and inflammatory features [44,58].

The development of the iPSC (induced pluripotent stem cell) technology offers the opportunity to study disease-involved cells with a chosen mutation and a phenotype matching physiology. Several iPSC models have successfully been obtained from skin fibroblasts of cALD and AMN patients [143–147]. Gene expression profiling shows that X-ALD iPSCs have differentially expressed genes compared to control iPSCs, among which some are positively correlated to the severity of the disease (cALD versus AMN) [148]. When iPSCs are differentiated into oligodendrocytes or astrocytes, the VLCFA level is increased and is higher in cALD differentiated cells than in AMN cells, whereas no VLCFA accumulation is observed in neurons [144]. iPSC-derived astrocytes show pro-inflammatory features that also correlate with the severity of the phenotype. The differentiation of microglia from iPSC also seems to be a promising model, as differentiated microglia show the main phenotype of primary fetal and adult human microglia including phagocytic and inflammatory capacity [146]. In addition, cALD iPSCs differentiated in brain microvascular endothelial cells show impaired BBB function as well as lipid metabolism modifications and interferon activation [149], and could lead to the study of an important factor of brain pathogenesis in X-ALD. Altogether, these works show that iPSC-derived brain cells should allow the study of the pathogenesis of X-ALD in detail, permit the identification of biomarkers, and screen new therapeutic molecules. Co-culture experiments are expected to provide new insight into intercellular communication in the brain.

In conclusion, for forty years, enormous progress has been made in the knowledge of peroxisomal ABC transporters thanks to the development and the use of cell and animal models. If no model exactly mimics the human X-ALD, there is no doubt that the new technological developments will offer opportunities to progress in the study of the role of peroxisomal ABC transporters in the neuronal, glial, and microglial intercellular communications.

## 5. Protein Interactions and Unexpected Roles

Physical interaction between peroxisomal ABC transporters and other proteins have been reported in several studies. Most binding partners are involved in lipid metabolism.

Here, we propose to review these binding partners for which strong interaction experiments have been obtained, or for which further investigations are needed to be reliable.

Peroxisomal membrane insertion, substrate binding, transport mechanism, and the potential novel functions of peroxisomal ABC transporters require protein interactions. Since their first identification, many efforts have been developed to understand how peroxisomal ABC transporters are targeted to the peroxisomal membrane. PEX19p, a cytosolic peroxin, was identified as an interactor of ABCD1, ABCD2, and ABCD3 by using the yeast two-hybrid system and in vitro GST pull-down assays [150]. In addition to being involved (in association with PEX3) in the correct peroxisomal targeting of peroxisomal membrane proteins (PMPs), PEX19p may also function as a protein chaperone to prevent aggregation of newly synthesized PMPs [151]. It's worth noting that PEX19p is the only ABCD2 binding partner that has been reported in the literature, probably due to the fact that ABCD2 is much less closely studied than ABCD1 and ABCD3 since it is not responsible for a genetic disease when mutated. To identify potential ABCD2 binding partners, we used the inducible H4IIEC3 cell model, which expresses ABCD2-EGFP depending on the presence of doxycycline [28]. We performed quantitative ABCD2 co-immunoprecipitation assays coupled with tandem mass spectrometry. Differential analysis between cell samples was done to limit detection of false-positive interactions. The list of potential binding partners of ABCD2 is given in Table 1 and includes 13 non-redundant proteins exclusively detected in the positive samples [28]. Only one subunit of the oligosaccharyl transferase (OST) complex that catalyzes the N-glycosylation of newly translated proteins in the endoplasmic reticulum was identified as a potential ABCD2 binding partner: the dolichyl-diphosphooligosaccharide protein glycosyltransferase subunit 2 (RPN2) (Table 1). On the other hand, RPN2 has also been identified by proteomic analyses in a subclass of peroxisome expressing ABCD2 [152]. These data are still quite surprising since peroxisomal ABC transporters, such as most PMPs, are known to be synthesized on free polysomes and to further insert directly from the cytosol into the peroxisomal membrane. It is worth noting that an indirect peroxisomal targeting pathway exists via the ER since several PMPs are found glycosylated [153]. The potential interaction of ABCD2 with the OST complex involved in N-glycosylation is inconsistent with the absence of routing through the ER with respect to peroxisomal ABC transporters. Nevertheless, proteomic data leading to identification is not robust since the protein probability for RPN2 is low (0.7224) (Table 1).

**Table 1.** List of proteins identified in co-immunoprecipitated ABCD2-EGFP complex by liquid chromatography coupled with tandem mass spectrometry (modified from [28]).

| Protein Accession | Protein Name | Protein Probability   | Fold Change <sup>a</sup> |       |
|-------------------|--------------|---|--------------------------|-------|
| Q9QY44            | ABCD2        | ATP-binding cassette sub-family D member 2                              | 1                        | 12.42 |
| P97612            | FAAH1        | Fatty-acid amide hydrolase 1  | 1                        | 5.02  |
| P11507            | AT2A2        | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2                     | 1                        | 4.71  |
| P07340            | AT1B1        | Sodium/potassium-transporting ATPase subunit beta                       | 1                        | 2.48  |
| P55159            | PON1         | Serum paraoxonase/arylesterase 1  | 1                        | 2.32  |
| D3ZHR2            | ABCD1        | ATP-binding cassette sub-family D member 1                              | 1                        | <2    |
| P16970            | ABCD3        | ATP-binding cassette sub-family D member 3                              | 1                        | <2    |
| Q7TS56            | CBR4         | Carbonyl reductase family member 4                                      | 1                        | <2    |
| P11505            | AT2B1        | Plasma membrane calcium-transporting ATPase 1                           | 1                        | <2    |
| P16086            | SPTN1        | Spectrin alpha chain, non-erythrocytic 1                                | 1                        | <2    |
| Q63151            | ACSL3        | Long-chain acyl-CoA synthetase 3  | 0.9997                   | <2    |
| O88813            | ACSL5        | Long-chain acyl-CoA synthetase 5  | 0.9994                   | <2    |
| P14408            | FUMH         | Fumarate hydratase, mitochondrial                                       | 0.8013                   | <2    |
| P25235            | RPN2         | Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 2 | 0.7224                   | <2    |

<sup>a</sup> Statistical significance was obtained for proteins identified with a fold-change >2.

Besides the question of routing and peroxisomal targeting, the main putative ABCD2 partners revealed in this study were associated with lipid metabolism. Unsurprisingly, several binding partners identified have a role in FA activation, which is required on both sides of the peroxisomal membrane. At the cytoplasmic side of the peroxisomal membrane, a complex FA synthesis-transport machinery was evidenced by using a multi-approach method, combining GST pulldown experiments, mass spectrometry (LC/MS), co-immunoprecipitation assays, and bioluminescence resonance energy transfer (BRET) measurements [154]. This machinery consists of the binary interaction of ABCD1/3 with proteins carrying functions associated with FA activation/transport (ACSVL4) and FA synthesis (ACLY, ATP citrate lyase; FASN, FA synthase). On the inner surface of the peroxisomal membrane, studies using a yeast two hybrid system and surface plasmon resonance techniques indicate that the very long-chain acyl-CoA synthetase 1 (ACSVL1) interacts with ABCD1 [155]. In *Saccharomyces cerevisiae*, peroxisomal ABC transporters (Pxa1p and Pxa2p) functionally interact with the acyl-CoA synthetase Faa2p on the inner surface of the peroxisomal membrane for subsequent re-esterification of the VLCFAs [72]. In this model, whether or not a physical interaction with acyl-CoA synthetases exists remains to be investigated. In *Arabidopsis thaliana*, peroxisomal long-chain acyl-CoA synthetases (lacs6 and lacs7) physically and functionally interact with CTS, as assessed by co-immunoprecipitation experiments [73].

In our study aiming at identifying ABCD2 binding partners, the fatty-acid amide hydrolase 1 (FAAH1) exhibited the highest fold change (Table 1, FC = 5.02). This endoplasmic reticulum enzyme is the main enzyme involved in anandamide hydrolysis and plays an important role in endocannabinoid metabolism degrading the FA amides to the corresponding fatty acids, with a PUFA preference over MUFAs and saturated fatty acids [156,157]. Interestingly, FAAH1 catalyzes the conversion of the ethanolamine amide form of DHA (N-docosahexaenoyl ethanolamine) to DHA [158]. The interaction of FAAH1 with ABCD2 could be consistent with the role of FAAH1 as a supplier of ABCD2 substrates (DHA and other PUFAs) for further degradation in the peroxisome by  $\beta$ -oxidation.

Concerning ether lipid biosynthesis, the peroxisomal enzyme alkyl-dihydroxyacetone phosphate synthase (AGPS) is suggested to interact with ABCD1, as assessed by an integrative global proteomic profiling approach based on chromatographic separation [159]. Ether lipid biosynthesis starts in the peroxisome with the transfer of the acyl group of fatty acyl-CoAs to dihydroxyacetonephosphate (DHAP), generating an acyl-DHAP. The second peroxisomal step is catalyzed by AGPS, which exchanges the acyl chain for an alkyl group, yielding an alkyl-DHAP. After a final peroxisomal step, the ether lipid biosynthesis is completed in the ER. This global proteomic analysis showed that AGPS failed to interact with ABCD2, just as our co-immunoprecipitation coupled to proteomic analysis [28].

$\beta$ -oxidation of MCFAs to LCFAs mainly takes place in the mitochondria, whereas VLCFAs are first metabolized down to octanoyl-CoA in the peroxisome for further degradation in the mitochondria. Surprisingly, proteomic data supported by co-immunoprecipitation experiments evidenced a physical interaction between a long-chain acyl-CoA synthetase 1 (ACSL1) localized in the ER and ABCD3 [159]. This could be in agreement with the role of ABCD3 in the  $\beta$ -oxidation of lauric and palmitic acids [68]. In addition, ACSL1 has been shown to interact with ACBD5 [160], a peroxisomal membrane protein suggested to function as a membrane-bound receptor for VLCFA-CoA in the cytosol to bring them to ABCD1 [161]. Whether ACSL1 transfers other unidentified lipid species to ACBD5, ABCD1, or ABCD3 for peroxisomal degradation needs further investigation.

Other potential binding partners of ABCD2 identified are involved in mitochondrial FA metabolism, such as the carbonyl reductase family member 4 (CBR4), the long-chain acyl-CoA synthetase 3 (ACSL3), and the long-chain acyl-CoA synthetase 5 (ACSL5) (Table 1). These enzymes were identified with less confidence (fold change <2). Although linked to lipid metabolism, CBR4 is a matrix mitochondrial enzyme. ACSL3 and ACSL5 do not activate VLCFAs, nor does ACSL1, which nevertheless has been found to interact with ABCD3 [160] as discussed above.



Peroxisomes contain enzymes involved in the  $\alpha$ -oxidation of phytanic acid. Large-scale mapping of protein–protein interactions by mass spectrometry identified a single interaction between peroxisomal proteins i.e., the peroxisome matrix phytanoyl-CoA 2-hydroxylase (PHYH) and ABCD3 [162]. This interaction makes sense since, after activation of phytanic acid, phytanoyl-CoA is imported into the peroxisome by ABCD3 and enters in the peroxisomal  $\alpha$ -oxidation pathway of which PHYH is the first enzyme (Figure 1).

The recent demonstration of ABCD1 interaction with M1 spastin, a membrane-bound AAA ATPase found on LDs, suggests the involvement of ABCD1 in inter-organelle FA trafficking [163]. Actually, ABCD1 forms a tethering complex with M1 spastin as assessed by co-immunoprecipitation experiments to connect LDs to peroxisomes. Furthermore, by recruiting IST1 and CHMP1B to LDs, M1 spastin facilitates LD-to-peroxisome FA trafficking. Whether M1 spastin-ABCD1 interaction directly promotes fatty acids channeling into peroxisomes remains unclear. It is worth noting that among proteins detected in our ABCD2 interactome study, the spectrin alpha chain, non-erythrocytic 1 (SPTN1) was identified as a potential ABCD2 binding partner (Table 1). As a cytoskeletal protein, SPTN1 is known to be involved in stabilization of the plasma membrane and to organize intracellular organelles [164]. These data corroborate the existence of peroxisome interconnection with LDs and the cytoskeleton [165,166].

Related to calcium signaling, the sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (AT2A2) was identified with a high fold change (FC = 4.71), which ensures a specific interaction with ABCD2 (Table 1). Besides, its homolog (ATPase1) has been identified as well by proteomic analyses in a subclass of peroxisome expressing ABCD2 [152]. AT2A2 transfers Ca<sup>2+</sup> from the cytosol to the ER and is then involved in calcium signaling. Coincidentally, disturbed calcium signaling was suggested to be associated with the pathogenesis of X-ALD [122]. Involved in maintaining intracellular calcium homeostasis, the plasma membrane calcium-transporting ATPase 1 (AT2B1) was identified, though with less confidence (fold change <2). Actually, its physical interaction with ABCD2 remains questionable since it is expressed at the plasma membrane. Nevertheless, several high throughput studies using robust affinity purification-mass spectrometry methodologies to elucidate protein interaction networks have revealed the interaction of ABCD1 with AT2B2 [167] and ABCD3 with AT2B2 and AT2A2 [168,169]. Hence, clusters of arguments indicate that peroxisomal ABC transporters could be linked to calcium signaling, but deciphering molecular interaction networks would be required to confirm this hypothesis.

Identification in the putative ABCD2 partners of the serum paraoxonase/arylesterase 1 (PON1), an antioxidant enzyme synthesized and secreted by the liver in the serum [170] where it is closely associated with high density lipoprotein (HDL), could be at first glance intriguing (Table 1). Nevertheless, in the liver, PON1 is primarily localized in microsomal fraction where the enzyme is associated with vesicles derived from the ER [171]. The potential intracellular interaction with ABCD2 remains to be elucidated. Noteworthy, PON1 activity and polymorphisms have been associated with neurodegenerative diseases [172], of which X-ALD is not evoked.

The binding partners of peroxisomal ABC transporters discussed in this review are mainly linked to lipid metabolism (PUFA metabolism,  $\alpha$ -oxidation pathway, and ether lipid biosynthesis) and are consequently found in the cytosol, in the peroxisomal membrane, or in the peroxisomal matrix. However, binding partners were identified in other cell compartments. Since peroxisomal lipid metabolism requires cooperation and interaction with mitochondria, ER and LDs, peroxisomal ABC transporters, through their interactome, could therefore actively participate in this intracellular metabolic network. Peroxisome-organelle interactions have physiological relevance [166,173], and peroxisomes are increasingly considered important intracellular signaling platforms that modulate physiological processes such as inflammation, innate immunity and cell fate decision [12,174,175]. Peroxisomal ABC transporters would play an essential part in this emerging role of peroxisomes in signaling pathways such as calcium signaling as highlighted in this review.

## 6. Conclusions

Transcriptomic, proteomic, and lipidomic studies, which have multiplied in the last few years, have confirmed and/or revealed the involvement of peroxisomal metabolism in various biological processes essential for cellular adaptation, brain homeostasis, or even immune response and inflammation. Peroxisomal ABC transporters constitute a pathway for the entry of various lipid substrates into the peroxisome mainly for their degradation but also for the synthesis of bioactive lipids impacting membranes and signaling pathways. It is therefore quite logical that the role of peroxisomal ABC transporters is now extended to unexpected biological processes. Since their cloning in the 90s, the lack of good antibodies, the rarity of relevant cell models, the fragility of the peroxisomal membrane, and other difficulties have constituted a real handicap towards performing functional assays and *in vitro* transport reconstitutions, and progressing in the understanding of the role of peroxisomal ABC transporters. The emergence of new cell models and the rise of model organisms, as well as cell reprogramming and CRISPR gene editing technologies, suggest that major new discoveries will be made soon that reveal their role in physiological and pathological situations.

**Author Contributions:** A.T., C.G., D.T., and S.S. contributed to the bibliography and writing equally. S.S. coordinated and finalized the elaboration of the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** The authors of this review received no external funding for this bibliographical work.

**Acknowledgments:** The authors acknowledge the French Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation, the University of Bourgogne, as well as the NFRF-Exploration stream (NFRF-E-2019-00007) (Canada) for funding the current scientific projects of the Bio-PeroxiL laboratory.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

|       |   |
|-------|---|
| ABC   | ATP-binding cassette                    |
| ACOX1 | Acyl-coenzyme A oxidase 1               |
| AMN   | Adrenomyeloneuropathy                   |
| BBB   | Blood-brain barrier                     |
| cALD  | Cerebral adrenoleukodystrophy           |
| CBAS  | Congenital bile acid synthesis defect   |
| CNS   | Central nervous system                  |
| CoA   | Coenzyme A                              |
| CTS   | Comatose                                |
| DHA   | Docosahexaenoic acid, C22:6 n-3         |
| ER    | Endoplasmic reticulum                   |
| FA    | Fatty acid                              |
| FC    | Fold change                             |
| HSCT  | Hematopoietic stem cell transplantation |
| iPSC  | Induced pluripotent stem cell           |
| LCFA  | Long-chain fatty acid                   |
| LD    | Lipid droplet                           |
| MCFA  | Medium-chain fatty acid                 |
| MUFA  | Monounsaturated fatty acid              |
| NBD   | Nucleotide binding domain               |
| PBMC  | Peripheral blood mononuclear cell       |
| PMP   | Peroxisomal membrane protein            |
| PUFA  | Polyunsaturated fatty acid              |
| TMD   | Transmembrane domain                    |
| VLCFA | Very long-chain fatty acid              |
| X-ALD | X-linked adrenoleukodystrophy           |

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