Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Boolean modeling of macrophage polarization upon immune stimulation and SARS-CoV-2 infection

Master's thesis in Cell and Molecular Biology
Supervisor: Martin Kuiper
Co-supervisor: Eirini Tsirvouli
June 2022

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Abstract

Macrophages are specialized cells of the immune system that can polarize into different phenotypes depending on local conditions, classically an inflammatory macrophage (M1) and alternatively an anti-inflammatory macrophage (M2). Entities involved in eicosanoid biosynthesis play a key role in this process, including the cytosolic phospholipase A2 (cPLA2) and the eicosanoid prostaglandin E2 (PGE2). To better understand the role of these entities and the regulatory factors driving macrophage polarization, a Boolean Macrophage Polarization (MacPol) model was assembled. A previously published model of macrophage differentiation was selected and, by evaluating the literature, expanded with 38 entities and 82 interactions. The MacPol model was able to replicate the gene expression or marker activity profiles for the M1 and M2 phenotypes under polarization stimuli. Then, upon 23 different perturbations and experimental conditions, the model was able to correctly predict 15 expression profiles. The integration of eicosanoid biosynthesis pathways highlighted the role of PGE2 in the polarization of the M2 phenotype. After this, the MacPol model was converted to the Macrophage Activation in COVID-19 (MacAct-C19) model to represent the SARS-CoV-2 infection. Three specific Boolean modules of signaling pathways affected by the virus were generated from the COVID-19 Disease Map repository. The modules together with additional pathways, output process nodes, and inter-cellular interaction nodes were manually integrated into the model. Then, under viral infection conditions, the model was able to replicate the events that lead to the development of the hyperinflammatory response and acute COVID-19. Intra-cellularly, the virus induces (1) silencing of the type 1 interferon signaling pathway, (2) activation of the NLRP3 inflammasome, (3) imbalance of the renin-angiotensin pathway, and (4) constant production of inflammatory cytokines. Intercellularly, the macrophage triggers the adaptive response of the immune system by several mechanisms. In conclusion, the models provide a tool to enhance the study of eicosanoids in macrophage polarization and the macrophage response to SARS-CoV-2 infection. These can be implemented in the design of future experiments and new drugs.

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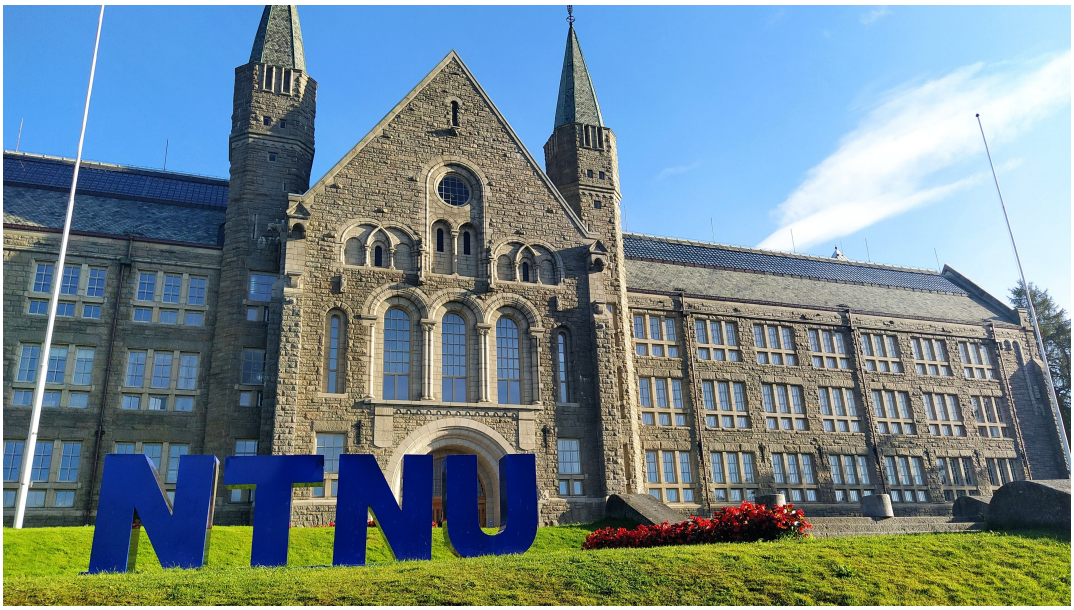
Vi



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Preface

This research is part of a project in the modeling of biological cell-type processes at the Norwegian University of Science and Technology (NTNU) Department of Biology that involves two research groups, the Kuiper group and the Johansen group. Both of these groups work towards personalized medicine and an understanding of the biological processes that drive pathological conditions but with different approaches. The Kuiper group is involved in the DrugLogics initiative to address cancer drug therapies with computational, experimental and analytical methods while the Johansen group runs wet-lab experimentation to investigate inflammatory mechanisms and their role in chronic diseases. This project is an integration of both approaches in order to understand the biological processes that govern the activation of macrophages - specialized cells of the immune system - and thus design better experiments with these cells in pathological states of inflammation. Additionally, the project was expanded in collaboration with the Disease Map Project community in an effort to generate more knowledge about the SARS-CoV-2 virus, which caused the COVID-19 pandemic. This community is oriented to establish collaborative networks with experts from different disciplines; allowing the exchange of information, practices, and tools to improve the development of disease maps.

Trondheim, June 2022

Viviam S. Bermúdez P.

Abbreviations

AA	Arachidonic acid.
ACE	Angiotensin-converting enzyme.
ACE2	Angiotensin-converting enzyme 2.
ADAM17	Disintegrin and metalloproteinase domain-containing protein 17.
AGTR1	Type-1 angiotensin II receptor.
AMs	Alveolar macrophages.
AP-1	Adaptor protein complex AP-1.
ARG-1	Arginase-1.
ATP	Adenosine triphosphate.
cAMP	Cyclic adenosine monophosphate.
CCL18	Ligand CC chemokines 18.
CCL22	Ligand CC chemokines 22.
CD206	Macrophage mannose receptor 1.
CD80	T-lymphocyte activation antigen CD80.
CI	Chronic inflammation.
COVID-19	Coronavirus disease of 2019.
CoVs	Coronavirus.
COX-2	Cyclooxygenase 2.
cPLA2	Cytosolic enzyme phospholipase A2.
DAMPs	Damage-associated molecular pattern.
EP4R	Prostaglandin E2 receptor EP4 subtype.
ERK	Extracellular signal-regulated kinase.
FBAL	Fluid of Bronchoalveolar Lavage.
FCGR	High affinity immunoglobulin gamma Fc receptor I.
GM-CSF	Granulocyte-macrophage colony-stimulating factor.
IC	Immune complexes.
IFN	Interferons.
IFN-I	Type I interferons.
IFNα	Interferon-alpha.
IFNβ	Interferon-beta.

IFNγ	Interferon-gamma.
IL-10	Interleukin-10.
IL-12	Interleukin-12.
IL-13	Interleukin-13.
IL-1Ra	Interleukin-1 receptor antagonist protein.
IL-1β	Interleukin 1-beta.
IL-4	Interleukin-4.
IL-6	Interleukin-6.
iNOS	Inducible nitric oxide synthase.
IP-10	C-X-C motif chemokine 10 or 10 kDa interferon gamma-induced protein.
JMJD3	Lysine-specific demethylase 6B.
KLF4	Krueppel-like factor 4.
LPS	Lipopolysaccharides.
M1	Classically activated macrophages type 1.
M2	Alternatively activated macrophages type 2.
MAS	Macrophage Activation Syndrome.
MIP-1α	C-C motif chemokine 3 or Macrophage inflammatory protein 1-alpha.
MyD88	Myeloid differentiation factor 88.
NF-kB	Nuclear Factor kappa B.
P2R	Purinergic P2 receptors.
PGE2	Prostaglandin E2.
PPARγ	Peroxisome proliferator-activated receptor-gamma.
RAS	Renin–angiotensin system.
RIG-I	RIG-like receptors (retinoic acid-inducible gene-I-like receptors).
S-protein	Spike-protein.
SARS	Severe Acute Respiratory Syndrome.
SARS-CoV	Severe Acute Respiratory Syndrome coronavirus 1.
SARS-CoV-2	Severe Acute Respiratory Syndrome coronavirus 2.
SOCS1	Suppressor of cytokine signaling 1.
SOCS3	Suppressor of cytokine signaling 3.
STAT3	Signal transducer and activator of transcription 3.
STAT5	Signal transducer and activator of transcription 5.
STAT6	Signal transducer and activator of transcription 6.

TAK1	Mitogen-activated protein kinase kinase kinase 7 or Transforming growth factor-beta-activated kinase 1.
TBK1	Serine/threonine-protein kinase TBK1.
TGF-β	Tumor growth factor-beta.
TLR	Toll-like receptors.
TLR4	Toll-like receptor 4.
TMPRSS2	Transmembrane protease serine 2.
TNFα	Tumor necrosis factor alpha.
TRAF6	Tumor necrosis factor receptor associated factor 6.
TRIF	TIR-domain containing adapter protein inducing interferon-β.

Chapter 1

Introduction

1.1 General aspects of Inflammation

Inflammation is the main response of the immune system to different stimuli in the body that can cause harm. At the same time, an inflammatory state is a condition underlying the development of many common diseases [2]. While inflammation is able to mitigate and minimize the damage caused by pathogens and injuries, it also contributes to tissue repair, protection, and restoration of homeostasis [3]. It is considered a vital defense mechanism and a crucial process in healing [4]. However, in cases when the resolution of inflammation and the return to homeostasis is not achieved, the ongoing immune response may be pathogenic and result in persistent inflammation [5]. This dysregulation is the causative factor of chronic diseases such as sarcoidosis, rheumatoid arthritis, psoriasis, inflammatory bowel disease, and cancer, to name a few. But it is also the process causing severe symptoms and aggravation of Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) infection. The enveloped RNA virus belonging to the *Coronaviridae* family is responsible for the Coronavirus disease of 2019 (COVID-19). The virus emerged in late 2019 in the city of Wuhan, China, and the disease was listed by the World Health Organization as a pandemic in March 2020. Both chronic diseases and the infection from SARS-CoV-2 have a significant impact on human health in a world-wide level, according to reports from the World Health Organization (WHO) [6, 7, 8]. Because of its clinical importance, it is relevant to study the inflammatory response and its pathological states to generate knowledge that can contribute to prevent and treat inflammatory diseases.

One of the key players in the activation, duration, and resolution of the immune response is the macrophage, a cell type specialized in (1) presenting antigens to other cells of the immune system, (2) phagocytosis of foreign molecules, and (3) modulation of the immune response through the production of various cytokines and growth factors [9, 3]. During the immune response, macrophages show different phenotypes and functions depending on their local environment. A first pro-inflammatory phenotype (classically activated M1-macrophages) is responsible for fighting infections and tumor growth. Then, as the stimuli in the environment change, the macrophage begins to show an anti-inflammatory phenotype

(alternatively activated M2 macrophage) that contributes to the onset of healing [3]. This transforming property is why they are commonly called remarkable plastic cells. The M1 and M2 classification of macrophages have helped to understand the different phenotypes the cell can show following different stimuli during *in vitro* experiments that simulate the inflammatory response and its resolution. Nevertheless, the reality of *in vivo* macrophages is different. Since inflammation and its resolution is a complex and dynamically changing process, the macrophage population shares characteristics of the M1 and M2 phenotype at the same time, filling a continuous spectrum of polarized states [10].

Changes in the microenvironment are responsible for the polarization of macrophages, this can involve different types of pathogen or damage molecules to the level of cytokines and cellular metabolites [3]. Even enzymes and components of the lipid metabolism have been associated with the polarization process [11]. For instance, the cytosolic phospholipase A2 (cPLA2) and the cyclooxygenase 2 (COX-2) catalyze rate-limiting steps in the conversion of arachidonic acid to the family of prostaglandins - a group of signaling lipids that play a role in the inflammatory response. They are up-regulated upon inflammatory stimuli and they are co-expressed with pro-inflammatory cytokines and markers in the M1 macrophage [12, 13]. Nevertheless, it has been suggested that the end product of these enzymes, Prostaglandin E2 (PGE2), can enhance the anti-inflammatory signaling pathways that lead to the M2 phenotype. This occurs following activation of PGE2 receptor expressed in the macrophage membrane (Prostaglandin E2 receptor EP4 subtype (EP4R)) [14, 15, 16]. The change from one phenotype to another by the products produced, or the changing molecules in the environment, is called macrophage reprogramming and many other factors of self-regulating loops participate in this process [17].

The dysregulation of the reprogramming and polarization can escalate to become harmful. For instance, the SARS-CoV-2 replicates rampantly and evades immune responses using a variety of sophisticated mechanisms [18]. Unable to eliminate the virus quickly, the inflammatory response persists. This causes an increase in pro-inflammatory cytokines released by different immune cells and their recruitment and activation at the site of infection - respiratory tract. The macrophage population increases in the Fluid of Bronchoalveolar Lavage (FBAL) [19]. Upon activation, they initiate the secretion of large amounts of cytokines and metabolites. In addition, they manage to phagocytize viral structures or infected cells. Together, this contributes to the modulation of the inflammatory response. However, failure to eliminate the virus completely generates a prolonged inflammatory response leading to hyperactivation of macrophages in the M1 phenotype [19] - generating immunopathologies such as pulmonary edema and pneumonia, among other complications [20].

Many research studies have focused on a better understanding of the mechanisms that drive that switching of phenotypes, with the overall goal of using macrophage reprogramming to treat inflammatory diseases. This requires an understanding of all the elements involved in the polarization process and how they dynamically interact with each other.

Therefore, a systems biology approach is likely to improve the understanding of its role in the immune response. In this way, the structure and dynamics of the set of components of the system are analyzed as an integrated whole to interpret the system's emerging properties [21]. In general, these analyses are done by implementing mathematical tools and computer simulation programs to build in silico models that describe and predict the behavior of the system. Such computer models can be validated by comparing their results against experimental data, and this, in turn, will indicate whether modifications should be made to optimize the model so that it reflects the real behavior of the biological system.

In the case of macrophages, several models have been constructed to predict the phenotype that results following stimulation [22, 23, 24, 1, 25, 26], mainly with the goal of understanding the underlying mechanisms that operate in the differentiation and polarization process, and to produce molecular and biomarker profiles of the differentiated states of the cell. These studies involve the assembly of gene regulatory networks and logical models of the key components and interactions involved in the differentiation of macrophages into polarized subsets (M1 and M2 phenotypes). While these models contain robust and meaningful information, they address this cellular process in specific scenarios. To give a dynamical view of macrophage activation and the role of lipid metabolism signals in this process, a logical model of macrophage polarization was assembled in this project. Taking a published model as a basis, the new model was significantly refined and expanded with relevant prior knowledge that integrates key regulatory pathways of macrophage activation and components that regulate eicosanoid biosynthesis. In addition, SARS-CoV-2 molecules that affect these pathways, as well as the virus entry pathway, were integrated to represent an extended, COVID-19 specific model of macrophage polarization. With a systems biology approach, perturbations were recreated in both the general and the COVID-19 specific model. In this way, crucial factors that drive changes at the level of genes and proteins were identified. This allowed the analysis of predicted macrophage behavior during general and perturbed immune response, as well as pathological conditions. Ultimately, these predictions can be used to design future wet-lab experiments on macrophage polarization that can point at possible drug-targets.

1.2 Objectives

The general objective of this Master's project was to develop a logical model of the signaling events in the activation of macrophages and assess the model by its ability to replicate the pattern of biomarkers expression during the mechanism of inflammation and resolution of the immune response. In this way, the model could be use to analyze the role of the eicosanoids (prostaglandin E2) in the polarization process. But also, by integrating the different viral components of the SARS-CoV-2, the objective was to identify the factors affecting macrophage functionality during infection. In this sense, this project aims to answer the following questions:

- Can a logical model coherently represent the current knowledge about macrophage polarization and be compliant with experimental result? And, thus, can it be used to predict the effects of novel perturbations and thereby help to design new experiments?
- What relevant connections and key elements can be found in the published models of macrophage cell differentiation?
- When using logical models of gene and proteins regulation, are predictive local states (activities expressed as 0 or 1) observed in the model's dynamics compatible with experimental observations of the macrophage behavior in a pro- and anti-inflammatory microenvironment?
- Can the entities related to the eicosanoid-metabolism be identified as key determinants for locking cells in a differentiated global state?
- Which pathways from macrophage polarization are constantly affected by viral SARS-CoV-2 components? and which components can be identify as key determinants for locking the cell in a inflammatory phenotype?

In order to answer these questions, the following specific objectives have been proposed:

1. Identify key regulatory components involved in macrophage activation, assemble them into a Prior Knowledge Network (PKN) and convert the PKN to a Boolean network.
2. Incorporate relevant actionable targets of the lipid metabolism in macrophages into the model, including the Cytosolic enzyme phospholipase A2 (cPLA2) and prostaglandin E2 (PGE2).
3. Perform a dynamic Boolean analysis of the stable states of the model and validate all the predicted regulations and components activity when compared with experimentally obtained expression data.
4. Identify key factors and signaling events that trigger a macrophage state change to pro-inflammation and the development of the pathological state during COVID-19.

Chapter 2

Theoretical background

2.1 The immune response

Inflammation is the adaptive response of the organism to harmful stimuli or conditions, e.g. pathogen infection or tissue damage [27]. This process, characterized already centuries ago, is described as the emergence of five signs in response to tissue injury: redness, swelling, heat, pain, and loss of sensitivity [28]. In recent years, more detailed studies allowed to elucidate the cells, mediators, and molecular mechanisms responsible for the production of these signs. The immune system carefully coordinates the response that involves the interaction of a large number of cells and specialized organs, starting with an innate response and a later adaptive response.

The innate response is immediate (minutes to hours), involves physical barriers, phagocytic and dendritic cells, natural killer cells, and plasma proteins, and has a limited and lower potency, since it recognizes general classes of pathogens with no specific distinctions [29]. While the adaptive response takes more time (weeks), it is much more potent since it involves B cells and T cells that mature to deliver a more specific response directed to the triggering agent [29]. In this case, an antigen (the substance that induces an immune response) is presented to T-cells, mainly by dendritic cells or macrophages, where it triggers the response in several ways. Once active, the T-cell differentiates into two phenotypes called T helper cells (Th1 and Th2). These cells can (1) direct an attack to the antigen-bearing cell by T-cytotoxic cells, (2) stimulate B-cells to produce antibodies that will attach to the antigen, and/or (3) induce a local inflammatory response by releasing inflammatory cytokines - small molecules involved in the activation of multiple signaling pathways. Released inflammatory cytokines, such as Interferon-γ (IFNγ), will stimulate macrophages to the production of different mediators (reactive oxygen species, lipid species, pro-inflammatory cytokines, etc.) [28]. Both types of immune response aim to eliminate the source of noxious stimuli or disturbances, followed by a resolution and repair phase.

2.2 Chronic inflammation

An abnormal response of the immune system that prolongs the release of inflammatory mediators and the activation of harmful signal-transduction pathways can lead to tissue degeneration and pathological states [30], called Chronic inflammation (CI). One of the major health problems of today with respect to morbidity and mortality worldwide stems from chronic inflammatory diseases, such as cardiovascular disease, cancer, diabetes mellitus, chronic kidney disease, non-alcoholic fatty liver disease, autoimmune diseases, degenerative and neurodegenerative disorders. Specifically, some inflammation-related diseases - stroke, chronic respiratory diseases, heart disorders, cancer, obesity, and diabetes - are among the top 10 causes of death reported by The World Health Organization and are responsible for 50% of deaths worldwide [30].

While the normal inflammatory response is a vital survival mechanism for the resolution of pathogen infections and physical injury, characterized by short-term duration and, the CI produces collateral damage over time and is characterized by long persistence and low-grade magnitude [31]. In the normal acute response, the organism detects a pathogen structure or cellular stress, sets up high-grade magnitude mechanisms, and removes the harmful stimuli in short-term to finally begin the healing process [6]. In contrast, CI can be triggered by other adverse conditions [6], such as:

- failure to eliminate a pathogen (e.g. chronic infections),
- continuous exposure to an irritant agent (e.g. industrial toxicants exposure),
- autoimmune disorder (e.g. rheumatoid arthritis and systemic lupus erythematosus),
- recurrent episodes of acute inflammation,
- biochemical inducers that cause oxidative stress and mitochondrial dysfunction.

In these cases, the activating stimulus persist causing (1) continuous migration of blood cells (monocytes) into the affected tissues, and then (2) local activation of these cells (i.e. differentiation into macrophages), and (3) interaction with resident tissue cells [28]. The outcome is a prolonged elaboration of reactive oxygen species, cytokines, procoagulants, and other small molecules that impair the tissue function such as the tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β) and interleukin 6 (IL-6) [32]. At the end, there is no resolution of the immune response and inflammation becomes chronic.

Additionally, among other causes associated to the progress of chronic inflammation diseases are gene mutations like the tumor suppressor p53 gene, the gene of hypoxia-inducible factor (HIF), and the neutrophil cytosolic factor 1 (NCF1) gene [27]. Also, other factors involving lifestyle and natural aging have been associated with the onset and progression of chronic inflammatory diseases. These include physical inactivity, poor diet and obesity, psychological stressors, emotional stress, tobacco smoking, and low sex hormones [31, 32, 6].

2.3 SARS-CoV-2 infection

Similar to chronic inflammatory diseases, infection with SARS-CoV-2 can trigger constant and uncontrolled activation of the immune system. The virus responsible for the COVID-19 belongs to the species of Severe Acute Respiratory Syndrome-related coronavirus in the subgenus of *Sarbecovirus*, (Order *Nidovirales*, suborder *Coronavirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae*) [33]. This group includes Severe Acute Respiratory Syndrome coronavirus 1 (SARS-CoV) which also affects humans and other types of Coronavirus (CoVs) that also affect animals. The SARS-CoV-2 encodes four structural proteins and sixteen nonstructural proteins (nsp) that fulfill distinct functions. The viral genome, single-stranded positive-sense RNA (+ssRNA), is packaged in the nucleocapsid (N structural protein), covered by the membrane (M) and the Spike-protein (S-protein), which interacts with cellular receptors Angiotensin-converting enzyme 2 (ACE2) of the host [33].

In most cases, infection by SARS-CoV-2 begins with entry of the virus into the respiratory tract, where it infects bronchial epithelial cells, pneumocytes and upper respiratory tract cells that express the ACE2 receptor on their cell membrane. Once inside the cell, the virus loses its envelope, releasing its viral RNA, which replicates and produces multiple copies to form new viral particles. These translocate and assemble in the cellular endoplasmic reticulum forming new viruses that are released by exocytosis to the external environment, where they infect new cells [33]. As the virus enters host cells and replicates, the innate immune response is induced where proinflammatory cells (monocytes, macrophages and neutrophils) are recruited and produce various cytokines and chemokines for the purpose of infection control. In particular, IFN are antiviral cytokines with a critical role in the containment of viral shedding [34].

Although asymptomatic cases have been reported, most patients with SARS-CoV-2 have symptoms of fever and dry cough at the onset of infection. Then, depending on the severity of the infection, the disease can be classified according to its clinical course as mild, severe, or critical [35]. In most cases the immune response is able to control the infection. However, studies suggest that the virus is capable of uncoordinating the response to the point of causing hyperinflammation syndrome and Severe Acute Respiratory Syndrome (SARS) [36]. Consequently, mild symptoms can rapidly progress to severe acute lung injury. Starting with an uncontrolled and rapid release of cytokines into the bloodstream, known as cytokine storm or hypercytokinemia [18, 36], that causes devastating effects on the cells of the pulmonary microenvironment (pneumocytes and epithelial cells) [18]. For instance, secretion of Interferon-gamma (IFNγ) and Tumor necrosis factor alpha (TNFα) leads to pneumocyte apoptosis, while other cytokines lead to increased capillary permeability and consequent recruitment of neutrophils - who contribute to the damage of cells in the microenvironment [18]. Destruction of the alveolar-capillary barrier compromises gas exchange and leads to severe respiratory pathologies such as pneumonia, pulmonary fibrosis and thrombotic events in the small pulmonary arteries [18].

2.4 Macrophages in the immune response

Macrophages are phagocytic cells, responsible for the elimination of pathogens or endogenous antigens by means of endocytosis. These cells are capable of recognizing, adhering, ingesting and degrading in acid-containing vesicles (lysosomes) the molecules or structures recognized as pathogenic or parts of a dying cell [37]. Particularly, in tissue inflammation and infection, monocytic cells are recruited from the bloodstream to the affected site, where they differentiate into macrophage cells [38]. Macrophages express numerous receptors that recognize endogenous or exogenous molecular motifs. Microorganisms or virus are recognized as exogenous (Pathogen-associated molecular patterns (PAMPs)). While self-molecules are recognized as endogenous (Damage-associated molecular pattern (DAMPs)), and can come from parts of a dying cell or danger-self molecules. The engagement of the macrophage receptors will activate signaling pathways to initiate phagocytosis and the secretion of large amounts of cytokines. Also, it will trigger the release of Arachidonic acid (AA) derived eicosanoids, which are active lipid molecules involved in multiple signaling pathways. Together, the outcome of these actions will contribute to the modulation, progress and extent of the inflammatory response [39, 40].

2.4.1 Different phenotypes and functions

Depending on the stimulus around the affected site, i.e. the cellular microenvironment, macrophages will develop different phenotypes and play specific roles in the inflammatory response. These phenotypes concern different macrophage subtypes that differ in the (1) type of receptors they express, (2) recognition of pathogens, and (3) the level and type of cytokines they release [41]. Commonly, two main phenotypes with opposite roles have been characterized in immunological studies: Classically activated macrophages type 1 (M1) and Alternatively activated macrophages type 2 (M2). However, these phenotypes represent two extremes in what is called "the hypothesis of continuous states of polarization". The hypothesis proposed that mononuclear phagocytes can combine different phenotypic characteristics depending on the amount and timing of the stimulus present in the environment [41]. In particular, the stimulus is provided by cytokines typically secreted by Th1, Th2 and T-regulatory cells but they can also be secreted by other cells of the immune system [1].

In this sense, when the macrophage cell detects fragments or molecules of bacterial origin (e.g. Lipopolysaccharides (LPS)) by membrane receptors, such as Toll-like receptors (TLR) and C-type lectin receptors, or senses pro-inflammatory stimuli and cytokines produced by Th1 cells (including Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin 1-beta (IL-1β), and IFNγ), the cell is activated by the classical pathway (phenotype M1) [1]. In this M1 state, the macrophage produces large amounts of (1) reactive nitrogen and oxygen intermediates, (2) pro-inflammatory cytokines (including TNFα, Interleukin-6 (IL-6), IL-1β, and Interleukin-12 (IL-12)) [38, 42], and (3) eicosanoids (e.g. PGE2). This state promotes a pro-inflammatory response committed to microbial clearance, tumoricidal actions, and the promotion of the Th1 response [43].

Alternatively, when the macrophage cell detects the cytokines Interleukin-4 (IL-4) and/or

Interleukin-13 (IL-13) (particularly mediated by Th2 cells), macrophage colony stimulating factor (M-CSF), Interleukin-10 (IL-10), Immune complexes (IC), as well as glucocorticoids, the cell is activated by the alternative route (M2 phenotype) [1]. In this case, the production of pro-inflammatory cytokines is limited and, instead, the anti-inflammatory cytokines IL-10, Ligand CC chemokines 18 (CCL18) and Ligand CC chemokines 22 (CCL22) are secreted [38]. This state promotes (1) an anti-inflammatory response committed to containing the pathogen, (2) tissue remodeling, (3) tumor survival and expansion, and (3) regulation of immune responses [43]. Particularly, M2-macrophages have high phagocytic activity because of the high expression of phagocytic receptors, such as Macrophage mannose receptor 1 (CD206), and dectin-1. They promote endocytosis of mannosylated ligands and other receptor-mediated endocytic processes [39]. Furthermore, different forms of the M2 phenotype (M2a, M2b, M2c) have been characterized with different functions and different activation profiles [44].

Moreover, one type of macrophage cells can also inhabit the tissue without the need to be recruited by a particular stimulus and this type is called tissue-resident macrophages. These macrophages are tissue-specialized immune sentinels with key functions in homeostasis and inflammation. In contrast with monocyte derived macrophages, this type of macrophage originates from embryonic progenitors that place them in this tissue before birth, and the population is maintain by local proliferation [45, 46]. The phenotype of these cells cannot be defined as M1 or M2 macrophages. However, it has been observed that tissue-resident macrophages exhibit mostly markers of the M2 phenotype, compared to monocyte derived macrophages [47].

In short, macrophage polarization depends on a complex network of interactions and connected stimuli. More importantly, in addition to the environmental stimuli, the molecules secreted by a particular phenotype of the macrophage end up affecting the activation of the opposite phenotype. In the immune response, a mixed macrophage population exists in the affected area involving tissue-resident macrophages and monocyte derived macrophages, that changes from M1 to M2 phenotype as the resolution of inflammation progresses [47].

2.4.2 Eicosanoid production and the role of cPLA2

As previously mentioned, macrophages are responsible for the production of cytokines and chemokines, but in addition they are also involved in the production of eicosanoids. These are biologically active 20-carbon oxidized fatty acids that derived from free AA. During the immune response, free AA is released from cellular membrane phospholipids by the group of phospholipase enzymes, which includes the Cytosolic enzyme phospholipase A2 (cPLA2) (calcium dependent and independent), and secreted phospholipase (sPLA2) (calcium dependent) [43, 39]. Furthermore, AA is metabolized by three major enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450); but it can also be oxidized by non-enzymatic pathways. In the first pathway, COX-1 and COX-2 convert AA to prostaglandins and thromboxane (together called prostanoids), while in the second pathway 5-LOX converts AA to leukotrienes. Prostanoids and leukotrienes can promote or restrain

inflammation and they have been implicated in the pathogenesis of several inflammatory diseases. The most notable diseases are asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel disease [48].

The Prostaglandin E2 (PGE2) is the main prostaglandin produced during inflammation and contributes to immune suppression. The enzyme Cyclooxygenase 2 (COX-2) mediates the biosynthesis of PGE2 from AA following its release form the plasma membrane and the action of PLA2 [49]. PGE2 can act in both autocrine and paracrine manners via a family of four membrane-spanning G-protein-coupled receptors, termed EP1, EP2, EP3, and EP4 [50]. In particular, Prostaglandin E2 receptor EP4 subtype (EP4R) has been related to the inhibition of cytokine production in macrophages mediated by an increase in intracellular Cyclic adenosine monophosphate (cAMP), representing a negative feedback loop in macrophage activation [51, 52].

The cPLA2 activity and the expression of eicosanoid enzymes, such as COX-2, is dependent on calcium concentration inside the cell. Cytosolic oscillations of this ion are well known for regulating many cellular functions. In the case of macrophages, several studies indicate that cytosolic calcium is modulated during the phagocytic process [53]. Specifically, some observations suggest that the entry of calcium ions into the cell is finely regulated by purinergic signals to ensure the efficient recruitment of phagocytes to their site of action and the subsequent elimination of invading pathogens and/or apoptotic cells [54]. The purinergic signals are mediated by the receptors for Adenosine triphosphate (ATP), called Purinergic P2 receptors (P2R), which are cation-selective. ATP can be found in the cytosolic space through the release of ATP-loaded vesicles or through the activation of large conductance channels [55], but it can also be found in the extracellular space of a pro-inflammatory environment, since cells undergoing apoptosis release ATP as a *find-me* signal that attracts phagocytes [56]. The purinergic receptors P2X4 and P2X7, once activated, mediate a regulated calcium flux inside the cell and then affect the activity of cPLA2.

In response to immune stimuli, the PLA2 enzymes play key roles in the correct function of macrophages, mainly the phagocytic mechanism that is accompanied by the rapid generation of AA-derived eicosanoids that promote acute inflammatory responses [57]. Specifically, sPLA2 has been implicated in the regulation of phagocytosis by human macrophages treated with IL-4 (alternatively activated macrophages), possibly involving the generation of ethanolamine lysophospholipid (LPE) at the plasma membrane [39].

2.5 Macrophage response during SARS-CoV-2 infection

In severe cases of patients infected with SARS-CoV-2, the number of macrophages with an inflammatory phenotype predominates within the immune cell population present at the site of infection. Liao et al (2020) [58] studied the microenvironment of bronchioles and alveoli of patients with SARS-CoV-2 admitted to Shenzhen Third People's Hospital, Shenzhen from January to February 2020. They were not only able to characterize the macrophage population, but could also determine that the proportion of macrophages was higher than myeloid and plasmacytoid dendritic cells in patients with critical disease, compare to mild

disease or control. Additionally, they were able to determine that macrophages in patients with severe SARS-CoV-2 expressed high levels of the proinflammatory cytokines IL-1β, IL-6, TNFα and several chemokines (CCL2, CCL3, CCL4 and CCL7) - capable of binding to CCR1 and CCR2 membrane receptors on monocytes. Thus, the study suggested that proinflammatory macrophages predominate in the cellular microenvironment of patients with severe SARS-CoV-2 and contribute to immunopathology by releasing increased amounts of proinflammatory cytokines and recruiting monocytic cells and neutrophils to the site of infection [58].

During the progression of COVID-19, the macrophages can be infected by SARS-CoV-2 through interaction with the membrane receptor of Angiotensin-converting enzyme 2 (ACE2) and contribute to the pathology [59]. Although most infected cells are those of the respiratory tract (small airway epithelium, nasal epithelium and masticatory mucosa), macrophages can be infected by the virus in general. Virus entry is determined by the expression of the receptor ACE2 and extracellular proteases specific for S-protein. Some of these are Transmembrane protease serine 2 (TMPRSS2), the paired basic amino acid cleavage enzyme furin, proprotein convertase subtilisin/kexin 3 (PCSK3), the neuropilin-1 receptor (NRP1), and dipeptidyl peptidase 4 (DPP4) [60, 61]. Thus, the proportion of infected macrophages may vary depending on the level of receptor and protease expression in different macrophage phenotypes.

2.5.1 Angiotensin-Converting Enzyme 2 (ACE2) imbalance

On the other hand, the ACE2-Protein S interaction not only results in cell infection but has implications for macrophage cell signaling and the hyperinflammatory response. Since virus entry involves the internalization of ACE2, the Renin–angiotensin system (RAS) (of which ACE2 participates) is affected [62]. The system involves two axes: the ACE2/Angiotensin (Ang)-(1-7)/Mas receptor, and the Angiotensin-converting enzyme (ACE)/ Angiotensin II/ Type-1 angiotensin II receptor (AGTR1) axis. ACE2 favors the expression of Ang-(1-7) and decreases the bioavailability of Ang II. When ACE2 does not participate in the system, Ang II production is favored, which contributes to the proinflammatory response in several ways. Ang II acts on the AGTR1 receptor. It can (1) modulate the cytokine milieu, (2) direct monocyte migration and inflammatory response, (3) induce inflammatory cytokine production in macrophages via Nuclear Factor kappa B (NF-kB) signaling, (4) increase oxidative stress, and (5) participate in autocrine/paracrine mechanisms between macrophages and other neighboring cells [62].

2.5.2 Evasion of antiviral response

It has been suggested that the virus is able to evade the antiviral response by several mechanisms, one of which is to avoid detection by the Alveolar macrophages (AMs). These immune cells are tissue-resident and constitute the first line of defense. They are responsible for the first wave of pro-inflammatory cytokines released, including the production of Interferons (IFN). These are activated when they detect viral RNA through their Toll-like receptors 3 or

7, or the RIG-like receptors (retinoic acid-inducible gene-I-like receptors) (RIG-I) [63]. In turn, IFN act as second messengers by stimulating neighboring cells and modulating their immune response. However, infection of SARS-CoV-2 compromises IFN production. The study by Dalskov et al. (Denmark, 2020) [34] shows that infection of SARS-CoV-2 in AMs does not result in any measurable induction of IFN. Although the virus is able to enter the cell (viral protein N expression), replication is not productive (absence of viral RNA in the cytoplasm), and viral proteins are capable of altering the expression of IFN genes [64, 65]. Yet, it appears that the expression of proinflammatory cytokines (TNFα, IL-8, C-X-C motif chemokine 10 or 10 kDa interferon gamma-induced protein (IP-10), C-C motif chemokine 3 or Macrophage inflammatory protein 1-alpha (MIP-1α), IL-1β) is unaffected [64]. Hence, the evidence indicates that there is a failure of viral detection at the macrophage level. Therefore, the proper activation of the early immune response is compromised. Suppressing the production of IFN also suppresses the cellular response to these antiviral cytokines. This affects the critical barrier to stopping viral spread and the development of severe disease [34].

2.5.3 Increased inflammasome activity

Effective replication of the SARS-CoV-2 induces cell death in infected epithelial cells [36]. In the inflammatory response, this event occurs programmatically via the pyroptosis or necrosis pathway. Pyroptosis is caused by a variety of external stimuli that lead to the activation and formation of NLRP3 (NOD-like receptor protein 3) inflammasome. This is mediated by the converting enzyme of IL-1β (also called caspase-1). The result of this process is the formation of gasdermin D (GSDMD) pores in the cell membrane. This allows the release of cellular contents (including Damage-associated molecular pattern (DAMPs) and inflammatory cytokines) into the environment [66]. Hence, an exacerbated infection leads to the presence of a proinflammatory environment at the affected site. In the case of severe SARS-CoV-2, it is evidenced by cytokine storm and the Macrophage Activation Syndrome (MAS) [19].

Moreover, the study by Ferreira et al. (Brazil, 2021) [67] found that SARS-CoV-2 is capable of engaging the inflammasome and inducing pyroptosis in primary human monocytes. By treating human monocytes with pharmacological inhibitors of inflammasome proteins, the researchers observed that cell death caused by SARS-CoV-2 was abolished. Also, they determined that the virus induced (1) caspase-1 activation, subsequent (2) production of SARS-CoV-2 and (3) activation of GSDMD - events that were abolished by using the corresponding inhibitors. Taken together, the results suggest that SARS-CoV-2 activates pyroptosis in monocytes and possibly this event contributes to cytokine storm and SARS-CoV-2.

2.6 Benefits of a Systems Biology approach

All the crucial processes described above are based on interactions among several chemical constituents and biological components of the entire system -the organism-. Systems biology provides a way to characterize and evaluate the interactions between the components and to explain emergent properties of biological processes. Following such an approach, the

first step is to represent the system of study as a network of interacting factors that together govern the system's emerging properties. This approach is based on the "general systems theory" proposed by Ludwig von Bertalanffy in 1968 and it advocates the understanding of a the system as a whole to gain insight into the functionality of its subcomponents; or in other words, studying how the system is structured and connected instead of studying the separated components to elucidate the specific functional properties of the system. Systems biology attempts to model physiological processes with computational tools in order to predict functional outcomes in response to perturbations (i.e., environmental factors). In this context, a cell is understood as a network-based system composed of nodes (the compounds) and links (chemical transformations) between them [68]. To study the structure (how the components interact) and dynamics (how the system behaves over time), an approach of four steps is described by [69]:

1. Define all the components of the system and combine them in a prior knowledge network. This refers to the identification of the system structure and requires the use of technologies that monitor the biological process in bulk (high-throughput technology).

2. Systematically perturb and monitor components of the system. This is done to analyze and understand the system's behavior or system-level characteristics.

3. Reconcile the experimentally observed responses with those predicted by the model. This is done in order to validate the resulting model and adjust it to the biological reality.

4. Design and perform new perturbation experiments to distinguish between multiple or competing model hypotheses.

In addition, two ways may lead the model building and the approach of the study: one based on static network representation (gene regulatory networks), omics data analysis and extensive perturbations of the nodes; and the other based on mathematical models (with Boolean or differential equations) to dynamically analyses, explain and predict the system's behavior.

2.7 Regulatory networks and Boolean models

In the systems biology framework, the identification of the system structure is the first step to understand it. This is done by constructing mathematical abstractions, the so-called gene regulatory networks (GRN): graphs that represents genes, proteins, mRNA, and/or metabolites as nodes, and molecular interactions as edges connecting these nodes [70]. The result of this may be a network (N, E) with the molecules as nodes N and the relationships as edges $E = E^{+}(activation) \bigcup E^{-}(inhibition)$. Levels of gene expression or activity are represented by a binary state: expressed/active (value of 1), or not expressed/inactive (value of 0) [71]. The interactions in this set determine the regulation of gene expression levels, which

in turn rules the biological processes, such as, maintaining homeostasis, cell growth, cell movement, among others. In this way, the graphical approach provides a conceptual view of biological processes and also a way of studying the functions of the system.

The further step in a systems biology approach is to study the system behavior and its response to perturbations, i.e. the dynamics of the system. This is achieved by transforming the static network representation into a dynamic representation, where each of the nodes would have an associated variable (x_i) that represents its state or abundance and the value of this variable will be controlled by the interaction and states of other nodes in the network [72]. Two different approaches can be used to describe this: continuous or discrete modeling. The use of one or the other depends on how the state variables are defined (discrete or continuous) and also the amount and quality of information gathered about the system's interactions. Continuous models require sufficient mechanistic details and kinetic parameters of each interaction, which makes them suitable for well-characterized systems [72], while discrete models can be made with qualitative data and very few or no kinetic parameters, which makes them suitable for modeling larger regulatory networks when only basic regulatory functions of system components (activation and inhibition) are known. In this case, the organization of the regulatory network is more relevant than the kinetic details of individual interactions [72], in part because the change of state variables can be predicted by the regulatory interactions in the network.

Boolean networks fall under the category of discrete dynamic models that operate with binary values. In these networks, the associated variable *xi* of each node represents an "on" or "off" state (0 or 1), meaning that the entity (gene, protein, transcription factor, etc.) is or is not currently active, expressing, or above a threshold - at which the specific value may or may not be given [72]. The dynamic behavior of the system can represent a continuous time stream over a certain period (e.g., an experiment) [73]. The abstract representation of biochemical changes taking place in the cell is represented by a series of states of the system (N-nodes and N-dimensional vectors of 0s and 1s) [73]. In case of synchronous updating, at every time-point, the global state of the system is described as a vector $(x_1, ..., x_N)$ and, since variables will change with time, the system will traverse through a sequence of global states called a trajectory. The simulation can start from a fixed point - where the states of the nodes are defined in a specific global state (e.g. all nodes at 0) -, or just follow random activation. Then, the system will evolve from one state to another depending on the activation of regulatory relationships established in the network. The transition between states is determined by the regulatory relationships that govern the node state, which is defined by Boolean regulatory functions - Boolean rules - that are written in terms of the three logical operators AND, OR, and NOT. After a certain series of updates of the system following these logical rules, the network dynamics will either reach one of a number of possible states and stay there (the stable state) or will keep cycling forever across the same set of states (the limit cycle) [74, 75]. These stable states are called "attractors". Together, the state of the nodes in the system can indicate the functional outcome of a biological process or a specific cell behavior. Commonly, these attractors can be associated with specific phenotypes in the study system [76].

Boolean dynamic models can be developed using knowledge bases and sets of causal interaction statements, and they can be configured to a specific cell type or process. Several resources can be used to validate this, such as gene expression, proteomic measurements, time series, knock-out data, among others, where the information is used to establish the binary states in the known conditions and/or inferred the relationships between entities. The prior knowledge can be available from a knowledge base (e.g. STRING, and SIGnaling Network Open Resource 2.0 (SIGNOR 2.0)), as a regulatory network graph, or as a Boolean model already. In this case, more data can be integrated depending on the goals of new research investigations, i.e. combining biological processes, constructing a PKN for representing a specific scenario, improving previous models with new data, among others. Starting from a published PKN or Boolean model, an expansion can be accomplished by integrating new information from prior knowledge and updating the Boolean regulatory functions of the nodes. Then, after the new information is integrated, the model behavior is analyzed and the results are compared with established experimental results with the aim to refine it and make it accurate with the system's real behavior. At this point, the model can be used to make predictions about the effect of perturbations in the system, that is, to simulate and study the dynamical behavior and resulting stable state upon a new set of conditions. Different conditions can be studied as perturbations of the nodes in the system, such as gene knock-outs, constitutive expression/activity of a node, usage of inhibitors, etc. In this case, the nodes may adopt new state values of "on" or "off", when running the simulations. Perturbations analysis can lead to the identification of key components in the system that govern system behavior and experimental perturbation data can be used to test the model behavior by comparing the simulation results with the *in vivo* systems behavior.

The development of PKN and Boolean network models is of great importance in today's biology and biomedical research as it is a valuable resource in understanding the cellular processes of biological systems at a macro level. The resulting network or module would serve as a working model for researchers to form novel hypotheses and assist in experimental design [77]. The discovery of key molecules in the regulation of the system, the importance of the relationships that are established, and the predictions that can be made about the behavior of the system under certain conditions are some key points that are generated with the help of these network models. This new knowledge is then applied, developed, and compared with previous knowledge, which helps to recognize future challenges in the field of biology, medicine, and the design of new drugs.

Chapter 3

Materials and Methods

This project was divided in two parts. The first corresponds to the building of the Macrophage Polarization (MacPol) model that represents polarization conditions common in the immune response. It also includes the validation of its in silico predictions with the macrophage experimental literature. This first part of the project was done individually, while the second was done in collaboration with Marco Fariñas. The second part corresponds to the integration of the Coronavirus disease of 2019 (COVID-19) modules into the MacPol model. The new Macrophage Activation in COVID-19 model aims to represent the pathological state of macrophages during the viral infection of SARS-CoV-2. Additionally, inter-cellular interactions were added to generate the Macrophage Activation and Cellular Communication in COVID-19 (MacAct-CC) model. This part includes the verification of both models predictions on the pathological state with the literature available at the time of the study.

Additionally, the project mainly focuses on the bioinformatic analysis of experimental data, either found in the public domain or produced in the Johansen group laboratory. Thus, the production of new experimental data was not carried out in this project, only the bioinformatics protocols are extensively described. While the laboratory protocols are only mentioned or briefly described.

3.1 PART I: Macrophage polarization under immune stimulation

3.1.1 Analysis of current models and expansion of a selected model

Current macrophage models

To evaluate the state of the art in macrophage models, a review of the available models and studies about the differentiation, polarization, and activation of the macrophage was performed. Three published models [25, 24, 1] were selected. Then, in order to determine the dynamic functionality of these models, a validation of their behavior was performed using the GINsim (Gene Interaction Network simulation) tool [78]. The validation consisted of recreating the models (encoding them in GINsim) and carrying out a stable state analysis to

verify that the results and predictions described by the authors of the model could be replicated. The models that were considered in this project to use as a base for the expansion are published in the following articles:

- *Deriving a Boolean dynamics to reveal macrophage activation with in vitro temporal cytokine expression profiles* [25]. This article describes a Boolean network model of three macrophage activation patterns representing M1, M2a, and M2c phenotype. It is based on cytokine expression profiles from published results in the literature and it combines methods of clustering, data discretization, Boolean function determination, and machine learning to quantify the activation patterns. The proposed method can predict macrophage activation patterns using extracellular cytokine measurements.

- *Gene Regulatory Network Modeling of Macrophage Differentiation Corroborates the Continuum Hypothesis of Polarization States* [1]. This article describes a Boolean network model of the gene regulation driving macrophage polarization to the M1, M2a, M2b, and M2c phenotypes. This model is based on data available in the literature and represents the shift among the different phenotypes.

- *Logical modeling of lymphoid and myeloid cell specification and transdifferentiation* [24]. This article contains a Boolean network model of hematopoietic cell specification including progenitor, B-cells, and macrophages. It is based on available data and represents the gene expression patterns and signaling cascades in the transition from the progenitor to a B-cell state or macrophage state.

The validation process allowed to verify that Palma's model of macrophage differentiation [1] corresponded to the dynamics reported by the authors. In addition, its description and functional characteristics were considered adequate to be used as a parent model for this project. Some of the characteristics of this model that were taken into account are:

- It represents the macrophage polarization of the 4 canonical phenotypes in one network of 30 nodes and 50 interactions.

- It evidences the continuum hypothesis of macrophage polarization by pointing out the possibility of finding hybrid macrophage phenotypes.

- It describes the plasticity of macrophages in silico and provides a map to follow the phenotypes with the switch from one phenotype to another, annotated with the gene expression patterns.

The characteristics of this model were considered adequate as they can provide a good basis for further expansion of the MacPol model. The model covers signature signaling pathways in the macrophage polarization, integrates the polarization of the canonical phenotypes in a single network, and it also highlights the interaction and switches between the different phenotypes. The other analyzed models were considered not suitable as they focus

on specific conditions, e.g., relevant cytokine signature profiles and activation patterns [25], or a specific process, e.g., macrophage differentiation from common progenitors [24].

In order to work with an updated model of macrophages based on recent literature, a validation of the interactions presented in the Palma model was carried out. In the previous step, the dynamics of the model was verified. While, in this next step, the interactions reported in the model are verified to correspond to the literature from which they were taken by the authors. To do so, the literature reported in the article was manually reviewed. The sense of the interaction and the logical rules established by the authors were checked on the source publications. This analysis allowed to verify that (1) the interactions have a solid scientific basis, (2) they come from a specific study for macrophage cells, and (3) the information is recent and not in conflict with new findings. Those interactions that could not be fully validated were further investigated in the following steps (Figure 4.1).

Expansion of key regulatory components

In order to integrate into the MacPol model relevant components in the activation and differentiation of macrophage phenotypes, as well as actionable targets of the lipid metabolism, an expansion of regulatory nodes and key markers of phenotypes was carried out. The expansion process included an extensive search on selected databases and compilation of the literature to integrate new entities and connections (interactions). Mainly studies where a relationship between entities was demonstrated on an experimental basis were taken into account. Thus, the role of the entities and the interactions between them were inferred from the experimental data or as established by the authors in the text.

To correctly establish the interactions and the role of the components, the diverse literature was carefully reviewed. Some studies indicate direct interactions or regulations, as protein-protein or transcription factor-gene interactions. While others only suggest an indirect regulatory relationship (e.g., gene knockouts). In the case of indirect relationships, the experimental evidence was carefully analyzed and interpreted to properly define the interactions in the network. For example, a change in the activity level of a protein after the cell has been subjected to a stimulus suggests that the protein is responding to that stimulus. Also, if a gene is over-expressed after experimentally inhibiting the activity of another gene or enzyme, it means that there is negative regulation by this inhibited entity towards the over-expressed gene. Other inferences in the literature are more complex, for example when an entity promotes a process that results in the activation of another entity; or when there are studies that suggest the interaction between two molecules but there are no experiments that evidence such a relationship. Additionally, there were cases where the conclusions of different studies contradict each other. To determine which of the experimental observations best validates the interaction, an additional examination was carried out. This considered the methods, the cell lines, and the conditions under which the experiments were conducted.

To keep track of the expansion process, two data tables were constructed with manual annotations. The first one listed the entities and their most relevant information: the short

and complete name of the entity according to Uniprot, its entry in the UniProt database [79], its function and/or relevance in the immune response, and whether it was associated with a particular macrophage phenotype or lipid metabolism. The second table contained the interactions between the entities, both those belonging to the Palma model and the new ones found. The interactions were annotated in the table by identifying the source and target entity, the publication from which it was extracted, a text fragment from the publication that evidenced the relationship, whether it was specific to macrophage cell lines, and whether it belonged to a signaling pathway associated with a phenotype or lipid metabolism. By keeping a record of what had been investigated, a complete and rigorous extraction of the literature was achieved.

Although the intention was to add many more molecules of interest in the polarization of the macrophage into the network, care was taken to minimize the number of interactions and possible nodes that were integrated. This was to preserve the simplicity of the network. Mainly because the analysis of large and complex regulatory networks is challenging at the statistical, mathematical, computational, and theoretical levels [80]. Most importantly, an attempt was made to capture the complex biological processes of the macrophage in a single, coherent regulatory network. However, biological models face some challenges in describing the behavior of the system in real life. For example, factors such as the abundance of the molecules, the probability of effective interaction between them, the change in the timing of the interactions in response to a stimulus, and internal processes of the cell, can be complicated to model and therefore are not fully captured in the MacPol model [80]. Also, a large amount of data is currently generated, which constantly challenges the usefulness of these models to capture all the factors affecting the process being modeled.

The databases used to gather more information on molecular signaling pathways and biological processes related to macrophages are described below. During this step, emphasis was given to reported biomarkers for M1, M2a, and M2c, and the cPLA2 related lipid metabolism. The mining for information was also focused on macrophage cell lines from human and mouse. However, some interactions were only validated in other cell lines such as monocytes, smooth muscle cells, astrocytes or epithelial cells. In all cases, this type of provenance for the interactions was recorded.

- STRING, a database of known and predicted protein-protein interactions [81]. Using queries with multiple proteins, a list of the polarized macrophage markers related to a unique or to multiple macrophage phenotypes was submitted. Searches were for *Homo sapiens*, and the minimum required interaction score was set to high confidence (0.700). The evidence suggesting the functional link was checked in the PubMed abstracts and the association in curated databases. Among the cell line data, the links related to macrophages were prioritized.

- The SIGnaling Network Open Resource 2.0 (SIGNOR 2.0), a public repository that stores signaling information as binary causal relationships between biological entities [82]. The query was done with one or more entities, selecting *Homo sapiens* and prioritizing the macrophage cell-line. Then, the relationships found were analyzed by the sentence

published in the database and additional information presented in the corresponding publication.

- Myogenesis and Muscle Regeneration Portal (Myo-REG), a public repository of functional relationships (cellular and molecular) between and within cells involved in muscle regeneration. The intracellular pathways in macrophages were checked and more molecular interactions between signaling molecules were retrieved from the tables presented in the portal.

- The BioGateway resource, as a Cytoscape App (version 3.7.1) that accesses the Semantic Systems Biology database [83]. Queries were done by importing some entities into Cytoscape -an open source software platform for visualizing molecular interaction networks and biological pathways [84]- and visualizing the resulting graphs of the proteins and gene interactions after building the query in the App. The interactions of interest were checked further through the links provided by the App.

3.1.2 Representation in a static and dynamic regulatory network

In order to visualize the polarization process as a static system where the components are interconnected, a previous knowledge network was assembled. Taking the Palma model as a base and the information gathered in the previous steps, the regulatory entities and their interactions were represented as a network in GINsim. Each node in the expanded network can represent a protein, a transcription factor, a small molecule, or a gene. All of these entities are associated in some way with the regulatory process of macrophage polarization. GINsim allows to manually create annotations to each node. Thus, all entities are annotated with a link to their UniProt entry, a link to their entry in another database, or to a scientific publication where a description of the entity can be accessed. In addition, the GINsim options also allow saving annotations in text form. Thus, the long and/or short name, as well as the biological function and/or biological effect of the entity that can be found in the provided link was saved. For this part, mainly the entities data table from the previous step was used.

To establish the interactions in GINsim, the interactions data table from the previous step was used. The interactions between the entities are represented by pointed (activating) or blunted (inhibiting) arrows connecting the nodes. The arrow goes from the source node to the target node. Interactions were inferred from the literature and each of them was documented with links to the publication(s) and the exact statement(s) from which they were inferred.

Logical functions

In order to observe the polarization process dynamically and thus study the properties of the system, the next step is to transform the static regulation network into a Boolean dynamic model - the Macrophage Polarization (MacPol) model. Specifically, a discrete dynamical model operating with binary values. For this purpose, the Boolean rules of each node in

the static network were defined. That is, the regulatory relationships exerted on each node by other entities in the network were described in terms of Boolean operators NOT (!), AND (&), and OR (|). The combination of the operators determine the entity status (i.e., its activity). They are equivalent to the regulations and biochemical changes that take place in the cell. In this way, the binary state of each node depends on the interactions that govern it and the state of its regulators.

At first, the Boolean rules were established as defined in Palma's parent model. However, after a careful assessment of the relevant literature, some of these rules were updated with more precise experimental evidence. The same assessment was performed to define the conditions enabling the activation of each new entity integrated in the extension. Eventually, the logical rules were refined multiple times as new entities and interactions were included in the MacPol model, until the model was able to correctly replicate the entities' states observed experimentally. The final Boolean rules for each node are shown as a table in Appendix D.

In all cases, the Boolean rules were inferred from literature. When an entity was governed by two regulators (e.g., in a biosynthesis reaction) it was represented with the AND operator. When the activation of one entity was negatively regulated by another (e.g., the inhibition of an enzyme) it was represented with the NOT operator. When two entities regulated another one in a independently manner, with no synergy, or one could be replaced by the other, the OR operator was used. In cases where the entities were governed by many regulators, the literature was carefully examined to generate the appropriate combination that would express the observed experimental behavior.

3.1.3 Comparative analysis of the initial model and the expanded MacPol model

To evaluate the work done during the extension process, the constructed MacPol model was subjected to a comparative graphical analysis against the initial Palma model. This was done by running the "Compare graphs" tool in GINsim. The Palma's model file was used as master graph and the MacPol model as the second graph. GINsim runs a network integration of both graphs and searches for new and matching interactions, Boolean rules and node names between the two files. The result is an integrated network with nodes and interactions from both files. The differences between the networks are shown through the layout colors. The resulting GINsim network was exported into Cytoscape for better visualization and interpretation of the graph. The layout was set to "Circular" for each set of node's type (common nodes, new nodes, and nodes with changed Boolean rules). Finally the key network changes are gathered in the Table 4.1. And, the result of the graph comparison is shown in the Figure 4.3.

3.1.4 Polarization stable states and validation of the MacPol model behavior

Analysis of polarization stimulatory conditions

Once the network can display a dynamic Boolean behavior, the next step is to analyze its performance as a model of macrophage polarization. To evaluate this, initial conditions representing macrophage polarization stimuli were established and stable states were determined using the "compute stable state" tool in GINsim. In this way, the simulation starts from a fixed point with all nodes at zero, except for the corresponding stimulus for an M1, M2a or M2c phenotype (set at 1). In addition, these inputs were combined with the PGE2 stimulus, to assess the effect of lipid metabolism on the polarization. In total, six fixed inputs were set and the resulting stable states are presented as a heatmap (Figure 4.4).

The final stable states indicate the entity activity after stimulation. These results were compared to the macrophage literature indicating marker activity under each stimulus. If the results were consistent with the literature, i.e., the activity of the nodes corresponded to that reported in the available studies, the module was left as it was. On the other hand, if the activity of the nodes did not correspond to those reported in the studies, the MacPol model was re-examined. The source of the inconsistency was sought, the logical rules were corrected and/or the interactions were changed. Modifications were made until the model was able to display an approximation of the functional behavior in the cell.

The heatmap was made in R-Studio using the superheat package [85]. This package produces visualizations of complex and/or large datasets. They can be easily modified and more extensions of the analysis of the data can be added, like scatterplot, clusters, boxplots, and correlation information as barplots, among others, for further interpretation of the dataset.

Perturbation analysis and refinement of the MacPol model

To get a better impression of the model´s behavior and to validate the in silico outcomes with the literature, a determination of stable states using perturbed polarization conditions was performed. The aim was to reproduce known results from experimental observations in the Boolean model. In this case, a perturbation can represent the use of inhibitors, gene knockouts, constitutive gene expression, and/or pretreatment with molecules that affect the output. They are defined in the Boolean model as the change of a node state: setting the value of the node to "0", in knockout or inhibitors cases, or to "1", in constitutive expression or pretreatment cases. The fixed point was established with the polarization stimuli used in the perturbation study and the perturbed node with the corresponding state (1 or 0). Then, the simulation was run with the "compute stable state" tool in GINsim using the established input or fixed point.

Finally, the stable state reached by the MacPol model was compared with the markers activity reported in the perturbation study. If inconsistencies were found in the activity of the nodes, the literature was reviewed again and appropriate modifications were made in order to obtain, as far as possible, more accurate results consistent with the selected studies. Such

modifications could be (1) changing the logical rules, (2) adding or simplifying interactions, (3) adding more regulatory nodes to the model, among others. The simulations were then rerun with the new corrections and ultimately the comparison results are shown through a heatmap (Figure 4.5). The matrix was built with the same package used in the previous step.

For this analysis, 23 sets of conditions of systems perturbations were selected from experimental studies. These studies analyze the disruption of genes or molecules, or the addition of external chemicals to macrophage cells. Including all of the above-mentioned perturbations. They were chosen by the entities being analyzed. That is, whether the entities in the objective of the study were represented in the MacPol model. In this way, the conditions could be recreated by the model and subjected to analysis. Most of them use macrophage cell lines in mice and only two of them use human cell lines. In addition, most of the experiments were performed by recreating or examining specific macrophage polarization scenarios. Information about the perturbations can be found in the Appendix E, Table E. It is important to mention that the studies do not come from a single research group, with the same line of study, nor do they follow the same experimental protocols, which has to be taken into account when analyzing the results.

3.1.5 Gene expression data from new experiments run at NTNU

The gene expression data of the polarized M1 macrophages was obtained from the experiments carried out in the laboratory of the Johansen group. These consist of analyzing the expression profiles of THP-1 cells (CLO:0009348), a human acute monocytic leukemia cell line, differentiated into macrophages and their different phenotypes (M1, classically activated). The THP-1 cell line of leukemic monocytes is commonly used in different areas of research and represents a suitable model for the *in-vitro* study of monocyte-macrophage differentiation and macrophage polarization.

THP-1 cells were cultured in RPMI1680 media supplemented with 10% fetal bovine serum), L-Glutamine, Gentamycin and 0.05mM 2-β-mercaptoethanol and maintained under 5% CO2 at 37^{o}C. From the monocyte state, they were differentiated into macrophage cells (M0) when treated with phorbol-12-myristate-13-acetate (PMA) for 4, 24 and 28 hours. A concentration of 10nM PMA was used for treatment and a non-PAM vehicle control (DMSO) was maintained to compare results. After treatment, cells were taken for RNA isolation using Qiagen's mini RNA kit [86]. The RNA was reverse transcribed into cDNA using the Quantitect - Qiagen reverse transcription kit. The transcribed cDNA was used for real-time PCR (LC480 Sybr green 1 Master mix - Roche, Mannheim, Germany). The primers in Table 3.1.5 were used to determine the expression of the selected surface markers together with the reference gene (surface markers - CD14, CD36; reference genes - GAPDH, RSP18, ACTB). The markers CD14 and CD36 are preferentially expressed in macrophages compared to monocytes [38]. Finally, in order to polarize cells to the M1 phenotype, macrophages previously differentiated by PMA were treated with a fresh medium rich in pro-inflammatory stimuli (IFNγ (10ng/ml) + LPS ((10pg/ml)), TNFα ((10ng/ml)) and/or PGE2 ((100µg/ml))) for about 16 hours before the RNA was extracted. Then, the level of expression of the following genes was determined:

TNFα, CCL2, COX2, SOCS3, and compared to the expression levels of the reference genes.

Table 3.1: Forward and reverse primers of surface and reference markers for gene expression analysis by qPCR

NCBI ID	Oligo Name	Sequence
929	FH1_CD14	5'- GATTACATAAACTGTCAGAGGC -3'
929	RH1_CD14	5'- TCCATGGTCGATAAGTCTTC -3'
948	FH1_CD36	5'- AGCTTTCCAATGATTAGACG -3'
948	RH1_CD36	5'- GTTTCTACAAGCTCTGGTTC -3'
2597	FH1_GADPH	5'-ACAGTTGCCATGTAGACC -3'
2597	RH1_GADPH	5'- TTTTTGGTTGAGCACAGG -3'
6347	FH1_CCL2	5'- AGACTAACCCAGAAACATCC-3'
6347	RH1_CCL2	5'- ATTGATTGCATCTGGCTG-3'
5742	FH1_PTGS1	5'- GTTCTGGGAGTTTGTCAATG-3'
5742	RH1_PTGS1	5'- GAGTGTAATAGCTCACGTTG-3'
5743	FH1_PTGS2	5'- AAGCAGGCTAATACTGATAGG-3'
5743	RH1_PTGS2	5'- TGTTGAAAAGTAGTTCTGGG-3'
9021	FH1_SOCS3	5'- CCTATTACATCTACTCCGGG-3'
9021	RH1_SOCS3	5'- ACTTTCTCATAGGAGTCCAG-3'

The experimental results of gene expression were used to compare with the stable state results from the MacPol model. Only the experimental condition of TNFα in combination with PGE2 had representative results that could be used in this project. Therefore, this condition was set in the stable state analysis. Since TNFα is not described as a stimulus in the network, PGE2 was set as the stimulus instead. In the network, TNFα is an internal node that can activate the NF-kB pathway, hence the value of TNFα was fixed to 1 - representing a constant expression of the node - to meet the experimental condition. The comparison of the laboratory gene expression measures with the stable state results is shown in Figure 4.5, using the same logic described in the previous section for this matrix.

3.2 PART II: Macrophage activation under SARS-CoV-2 infection

3.2.1 Creation of SARS-CoV-2 modules

Selection of COVID-19 Disease Map process diagrams

To represent the pathological conditions of Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) infection in macrophages, it is first necessary to evaluate the pathways used by the virus to enter the cell and to affect the functionality of the cell. For this purpose, the COVID-19 Disease Map repository of SARS-CoV-2 virus-host interaction was used [87]. This open-access resource gathers diagrammatic representations of mechanisms used by the virus to affect host cells. The information is carefully curated by multiple experts and shared with Systems Biology Mark-up Language (SBML) (http://www.sbml.org). Therefore,

the diagrams can be easily access using different resources. In this case, the MINERVA ((Molecular Interaction NEtwoRk VisuAlization) Platform [88] was used to assess the diagrams and select the ones possibly affecting macrophages. The platform allows the visualization of the molecular interaction networks that are storage using the Systems Biology Graphical Notation (SBGN)-compliant format [88]. Three diagrams where selected from the repository:

1. Renin-angiotensin pathway or the ACE/ACE2 axes.
2. Interferon I pathway.
3. NLRP3 inflammasome activation.

Conversion of process diagrams to executable modules

Process diagrams are static, more detailed, complex representations of biological process [89]. For example, nodes can be grouped together to represent protein complexes. Also, nodes can be represented more than once in the diagram to establish the different forms of the same entity (e.g. expressing on the nuclear, cytosolic, or extracellular space). Furthermore, diagrams represent biochemical reactions in multiple steps. Often, these details are not shown in Boolean models, or they are represented in a different way, for example, through one step interactions or through the logical rules describing complex regulations.

Therefore, to integrate the static process diagrams in the new Macrophage Activation in COVID-19 (MacAct-C19) model, the diagrams were transformed into dynamically executable modules that could be easily integrated in GINsim. In this step, diagrams must be reduced, simplified, and adapted to describe regulations correctly through logical rules. To this end, the CaSQ bio.tool was used [90], as it was developed to convert process diagrams into Boolean models. The software infers the logical rules base on the information present in the diagram, e.i., topology and semantics of the interactions and biochemical reactions. First, the selected process diagrams were downloaded from the COVID-19 Disease Map GitHub repository. Then, they were entered and converted in the CaSQ sofware. This results in a Systems Biology Marked Up Language-qualitative (SBML-qual) type of file that can be opened in GINsim.

However, information regarding annotations and references present in the diagrams is lost when the output file is opened in GINsim. This is a minor complication regarding formats and interoperability between the software tools [90]. GINsim displays the node name in the form of the species ID instead of the short entity name. This makes the interpretation of the network more complicated. Consequently, the network had to be manually adjusted by taking the information directly from the process diagram. The logical rules were also evaluated. In the case of inconsistencies, the information in the process diagram was reviewed and the logical rules were modified.

Refinement to macrophage-specific modules

The previous step resulted in GINsim readable networks that gather all the necessary information for interpretation. However, as mentioned previously, process diagrams are made

with a considerable amount of details that are reduced when building Boolean networks. The CaSQ tool was able to reduce many steps described in the diagram. Nevertheless, some particularities such as "double entities" and protein complexes were still present. Double entities refer to nodes representing the same entity but in different cell spaces.

For this reason, to reduce the computational and human effort of processing and interpreting the modules, the networks obtained in CaSQ were manually reduced. For comparison, Table 4.2 was constructed with the graphical characteristics before and after reduction and refinement. Nodes that were not of interest for macrophage polarization (e.g., extracellular particles) or that did not fit the model objective (i.e., representation of different cellular spaces) were suppressed.

Additionally, the diagrams from the COVID-19 Disease Map repository are built and curated using data that are not specific to a particular cell type. That is, they are general for virus target cells, including macrophages but not exclusively. Therefore, to properly integrate these modules into the MacAct-C19 model, the module must be macrophage-specific - meaning that nodes and interactions must be validated or curated using macrophage literature. To this end, information from the process diagrams was carefully reviewed. In the case of nodes, the literature was rigorously examined to validate that the node was part of active macrophage regulatory pathways or that the entity was expressed in macrophages. In the case of interactions, an effort was made to validate them with macrophage literature. However, in the case of direct interactions with viral proteins, the assessment was more flexible, as at the time of the study, the amount of macrophage-specific literature was still limited in SARS-CoV-2 studies.

3.2.2 Adaptations to the MacPol model

Additional pathways and changes

In order to establish a complete picture of virus-triggered changes in macrophages in the MacAct-C19 model, additional pathways and modifications to the MacPol model were made. Based on the literature on viral infections and SARS-CoV-2, the pathway of Toll-like receptor 4 (TLR4) was modified, and the pathway of TLR2 was added. In this step, the protocols used for the MacPol model expansion were followed (see sections 3.1.1 and 3.1.2 in Materials and Methods). Thus, essential nodes and interactions of the TLR2 pathway were added in a small module and logical rules were defined based on the literature (Appendix G).

Additionally, the MacPol model used to develop the MacAct-C19 model was a new version of the model presented in Part I of this work. This new version was performed by Marco Fariñas as part of his master's project for NTNU [91]. The manuscript submitted for the university details all the changes made, the reasons for these changes and their analysis. Briefly, among the changes presented in this version are: the integration of the TNFα and TGF-β signaling pathways, the refinement of NF-kB signaling, and the extension of eicosanoid regulation. However, the nodes representing eicosanoid regulation were omitted in the version used in the following steps. This due to two main reasons: (1) to limit the total size of the network, and (2) to maintain the confidentiality of the work done for the Johansen lab, since

the MacAct-C19 model developed in this project was shared with the COVID-19 Disease Map community.

Another modification to the MacPol model was the High affinity immunoglobulin gamma Fc receptor I (FCGR) signaling pathway. This was omitted because the pathway required further refinement regarding the SARS-CoV-2-specific immune response and research in this regard was sparse and not specific enough at the time of this work. Thus, in order to meet the project timelines, it was decided to suggest this refinement in future steps of the project.

Biological process output nodes

By integrating the modules described above into the main MacPol model the total number of nodes would increase. This implies considerable efforts when interpreting the final results, meaning: assessing the correctness of the dynamics and stable states that the MacAct-C19 model can reach under certain conditions. Therefore, in order to facilitate and speed up the verification of the functionality of the final MacAct-C19 model, nodes describing biological conditions or processes were created. These nodes describe the processes of: (1) phagocytosis, (2) inflammation response, (3) antiviral response, (4) anti-inflammation response, and (5) viral replication. They represent the end point of the simulation and can be associated with macrophage phenotypes. As end points, the nodes don't have any interactions departing from them (output nodes). Instead, the different markers of the MacPol model would be connected to the output nodes depending on the process with which they are associated. In this sense, instead of evaluating the activity of individual markers, a verification of the dynamics and functionality of the regulatory pathways can be made by assessing the activity of the output node.

The markers of the MacPol model had to be correctly associated with the biological process nodes. For this purpose, an analysis of the characteristics of each of the final markers was carried out. The analysis consisted of manually classifying the markers by: (1) the macrophage phenotype with which they are associated, (2) the type of entity (chemokine, cell surface receptor, cytokine, or enzyme), and (3) the function it performs within the immune response. A table was constructed with the results of this analysis (Table F, Appendix F), which also included the link to the scientific article from which the information was extracted. Finally, depending on the function and phenotype of the macrophage to which the entities were associated, a biological process output node was attributed to them.

To complement the assessment of the function and association of the nodes to the biological processes, an analysis of Gene Ontology (GO) terms associated with the initial model markers was performed. GO-terms are functional group classifications of a gene (the protein or non-coding RNA derived from the gene) [92]. The terms are associated to a gene according to the related function it has. For the classification and grouping it uses the scientific annotations developed by the Gene Ontology Consortium (available at http://geneontology.org). In this project, the analysis of GO-terms was performed in two ways. The first consisted of an enrichment analysis using the ClueGO Cytoscape plug-in [93]. For this, the entities classified in the previous table (Table F, Appendix F) were first separated into two lists, one with

the entities associated with the inflammation phenotype and the other associated with the anti-inflammation phenotype. The list of markers was inserted into the "Load Marker List" option of ClueGO. The parameters were then adjusted to include the "BiologicalProcess" and "ImmuneSystemProcess" ontologies. The network specificity was adjusted above the "medium" and the "GO Term Fusion" option was used to obtain more accurate results, i.e., close to the real function of the entity. The rest of the parameters were kept as shown in the original configuration of the tool. The result of the functional analysis can be seen as a pie chart and as a table, with the most relevant GO-terms associated to the list of markers. The GO-terms resulting from the two lists were included in the annotations of the inflammation and anti-inflammation output nodes, respectively.

The new entities contained in the modules created in the previous steps also had to be correctly associated to the other output nodes (phagocytosis, antiviral response, and viral replication). To this end, the entities were first associated to the biological process (output nodes) according to the process diagrams taken from the COVID-19 Disease Map repository. Then a second GO-term analysis was carried out with these entities to verify that they were associated with the selected process (output node). The analysis was a manual search of the terms using the QuickGO search engine [94]. The tool uses the manual annotations available from the Gene Ontology Annotation (GOA) resource (http://www.ebi.ac.uk/GOA). For the search, the UniProtKB ID of each entity was individually inserted into the QuickGO browser. The taxon "*Homo sapiens*" was selected and only the qualifiers "involve in", "enables", and "contributes to" were included in the query. Among the list of GO-terms associated with the entity, it was verified that some of them (or several) were related to the established biological process output node.

3.2.3 Integration of modules to the MacPol model

Verification of the module's dynamic functionality

First, to ensure that the modules had adequate dynamic behavior and were performing correctly, a steady state analysis was carried individually. Adequate behavior refers to the model being able to reach steady states consistent with what is described in the literature. This indicates that the logical rules are correctly defined and are able to represent as good as possible the regulations occurring in vivo. For this step, the same stable state verification protocol described in the previous steps of this project was followed (see sections 3.1.4 in Materials and Methods). Fixed points where established according to each module input (e.g., viral entry). Then, the stable states were obtained with the "compute stable state" option in GINsim. Upon finding inconsistencies in the in silico results with the literature, a review of the module was carried and modifications were made if needed. These corrections were made until the stable states were able to reflect as closely as possible the functional behavior of the module according to the available studies.

Manual integration

Finally, once the modules and adaptations of the MacPol model were completed, validated, and performing in accordance with the literature (as close as possible), the next step was the integration into the MacAct-C19 model. For this, the modules were manually created inside the MacPol model file in GINsim. Nodes that were new to the MacPol model (e.g., viral proteins) were created as new nodes and the information from the annotations was copied to the main model file. For nodes and interactions that were already included in the main macrophage model, they were kept in the same form and only new annotations were added. For the logical rules, a careful assessment was performed. Initially, the previously defined rules were respected. If the new regulation affected the rules already described, the literature was evaluated. Depending on the information found, the rules were modified and the new regulations were added.

3.2.4 Pathological stable states and validation of MacAct-C19 model behavior

Definition of the pathological conditions as fixed points

To analyze the effect of SARS-CoV-2 infection on the modules and the complete MacAct-C19 model, it is first necessary to define a generalized fixed point that represents the pathological condition. During the development of the SARS-CoV-2 infection, two conditions are observed in the macrophage microenvironment: (1) the general state of inflammation, characterized by the presence of cytokines and other inflammatory molecules in the surroundings, and (2) the viral infection, characterized by the presence of intra- and extracellular viral particles. Additionally, in the general immune response an anti-inflammatory state is reached for the resolution of inflammation. According to these conditions, 3 fixed points were described with the respective inputs for each scenario (Table 3.2). The inflammation fixed point was defined with all nodes at zero (stimulus absent) minus cytokines and inflammatory molecules (IFNγ, IL-1β, oxidized phospholipids, IFNα, IFNβ, TNFα) defined with binary value 1 (stimulus present). Then, the viral infection fixed point was defined with all nodes at zero minus the viral RNA (double and single stranded RNA, and different Open Reading Frames (ORFs)) and the viral proteins (including structural and non-structural proteins (Nsps)) with a value of 1. In particular, the ACE/ACEII module is activated by the Angiotensin I input, and because during viral infection this module is affected, this input was defined with a value of 1 for the viral infection fixed point. Finally, the anti-inflammatory fixed point was defined with all nodes at zero except for the anti-inflmmatory cytokines IL-4, IL-13, IL-10, TGF-β, and the pleiotropic cytokine IL-6.

Analysis of individual modules upon different pathological conditions

Before evaluating the effect of SARS-CoV-2 infection on the complete MacAct-C19 model, an analysis of the dynamic functionality of each module integrated in the model was first

Table 3.2: **Fixed points describing pathological condition.** Fixed points (stimuli) are set as a starting point of the MacAct-C19 model simulation. They describe the conditions observed during the development of SARS-CoV-2: inflammation, anti-inflammation, infection, and their combination. The active nodes are shown for each stimulus. If the nodes are not listed, it means that they are inactive for that fixed point.

Fixed point	Active nodes
Inflammation	IFNg, IL1b, OxPLs, IFNa, IFNb, TNF.
Infection	VdsRNA, VssRNA, Nsp1, Nsp3, Nsp13, Nsp15, S, E, N, Orf6, Orf7a, Orf8, Orf9, AngI.
Inflammation + Infection	IFNg, IL1b, OxPLs, IFNa, IFNb, TNF, VdsRNA, VssRNA, Nsp1, Nsp3, Nsp13, Nsp15, S, E, N, Orf6, Orf7a, Orf8, Orf9, AngI.
Anti-inflammation	IL6, TGFb, IL4, IL13, IL10.
Anti-inflammation + Infection	IL6, TGFb, IL4, IL13, IL10, VdsRNA, VssRNA, Nsp1, Nsp3, Nsp15, S, E, N, Orf6, Orf7a, Orf8, Orf9, AngI.
Combination of all three	All nodes in inflammation, anti-inflammation, and infection.

performed under different conditions of stimulation. A similar verification of dynamic functionality of the modules was described in section 3.2.3, where a steady state analysis was performed using the inputs for each specific module. In this section the verification protocol is based on the same principle, however, the difference is that (1) the steady state analysis was performed on the complete model, (2) several fixed points were defined to evaluate the particular response of each module, and (3) the analysis was not performed in GINsim but in the Jupyter Notebook server (available at https://jupyter.org/) using the bioLQM (Logical Qualitative Modelling) tool [95].

The Jupyter Notebook is a computational environment web application that integrates several programming languages and allows the use of different software and tools from the scientific community. In this case, for the stable state determination the bioLQM tool was used, which is included among the software developed by the logical modeling consortium CoLoMoTo (Consortium for Logical Models and Tools) (http://www.colomoto.org/). Within the command line of the Jupyter Notebook, all bioLQM options can be accessed. First, the GINsim model (.zginml) was loaded in SBML qual format with the option "biolqm.load". Then, the particular stimulation conditions for each module were defined with the option "biolqm.perturbation" and the stable states were determined starting from these conditions with the option "biolqm.fixpoints". The results of each determination were stored as numerical data tables using the open source Pandas library [96] with the "pd.DataFrame" option. Pandas is a Python package that allows the storage of lower dimensional data as "objects" for easy mathematical manipulation. To visualize the steady state results, a table was constructed by concatenating all the "objects" with the pandas option "pd.concat". Then, for a better interpretation of the data matrix, a heatmap was constructed using the Python data visualization library Seaborn [97]. In the final version of the heatmap (Figure H.1 in Appendix H), data from certain nodes were omitted ("pandas.DataFrame.drop") and the nodes were put together in subplots ("plt.subplots") according to their relationship with each module

or their overall role in the MacAct-C19 model. In total, the data matrix was divided into 7 horizontal sections and a two-color code was used to differentiate the activity of the nodes (active/inactive). The final Notebook is included as a supplementary material (Appendix K).

Analysis of complete MacAct-C19 model upon different pathological conditions

The last step was to evaluate the functionality of the MacAct-C19 model when the pathological conditions of SARS-CoV-2 infection and inflammation were established. Once the small modules were analyzed individually, the model was ready to be analyzed as a whole. For this purpose, a stable state analysis was performed starting from the pathological conditions established earlier (inflammation, infection, anti-inflammation, and their combinations). Similar to the previous step, estimation was carried out in Jupyter Notebook using the bioLQM tool with the same options as described above. Similarly, the Pandas and Seaborn packages were used to organize the results into a stable state table and heatmap with the data. These are styled in the same way as the previous results.

3.2.5 Representation of inter-cellular communication

To complete the analysis of the effect of SARS-CoV-2 on macrophage functionality, we proceeded to include the inter-cellular interactions that this cell has with the rest of the immune system in a new model - the Macrophage Activation and Cellular Communication in COVID-19 (MacAct-CC) model. The objective was to generate a multi-cellular perspective of the model and to evaluate the innate and adaptive response in a global manner. For this purpose, the same strategy was followed as for the output nodes of biological processes. First, the most relevant intracellular interactions and processes in which the macrophage participates were selected after a critical analysis of the literature. Then, nodes were created that function as or represent a bridge connecting macrophage markers with new cellular communication nodes in a new small module (Figure I.1, Appendix I). Additionally, a small module representing the general phagocytosis process was built (Figure J.1, Appendix J), since this process is relevant for the inter-cellular communication. Both modules were integrated into the MacAct-C19 model and, finally, to evaluate the intracellular response of the macrophage under the pathological conditions, a stable state analysis was performed. Jupyter Notebook and the bioLQM tool were again used to run the analysis and organize the results. The procedures were follow in the same way as described in Section 3.2.4 (Materials and methods).

Chapter 4

Results and discussion

In the first part of this project, a workflow following a systems biology approach was used to build a logical model that represents the known interactions between key regulatory components, mainly of the immune response, that lead to macrophage polarization. In the initial steps, a review of the existing logical models and literature associated with macrophage activation and polarization was conducted to generate a list of genes and proteins crucial to this process. Entities were selected from models and signaling pathways for their relevance in the process and supplemented with new findings in the literature. An additional focus was to include a set of macrophage biomarkers that will be measured in future experiments run by the Johansen group at NTNU. Of the available models, the Palma *et al.* (2018) model of macrophage differentiation [1] was used as a base for expansion since it covers the differentiation process into the canonical phenotypes while providing the possibility to find hybrids, which displays the plasticity of macrophages. Next, with the selected key components and interactions, a network was assembled and Boolean logical rules were defined for each interaction. The new Macrophage Polarization (MacPol) model was represented and analyzed using the GINsim tool [78]. During the expansion of the model, the logical rules of the model were optimized until the MacPol model was able to reach one or more stable states that correspond to the observed behavior of macrophage polarization, as described in the literature and various experimental settings. Stable states should represent configurations of gene expression patterns of network components in which the system tends to evolve from established initial conditions [1]. Finally, the MacPol model's predictions were validated by benchmarking against datasets obtained experimentally in the Johansen group's laboratory and datasets from the literature.

In the second part of this project, the MacPol model was modified and adapted to represent the pathological state of Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) infection. First, the COVID-19 Disease Map repository of SARS-CoV-2 virus-host interaction [87] was used to evaluate and select the pathways employed by the virus to enter the cell and to affect its functionality. From the repository information and literature available at the time of this project, small macrophage-specific modules were created using GINsim software. Their functionality was verified with a stable state analysis, and modifications were made until the modules were able to represent the behavior described in the

literature. At this point, the modules were manually integrated into the MacPol model in GINsim and additional adaptations were made. This resulted in the new Macrophage Activation in COVID-19 (MacAct-C19) model, that included modifications to existing pathways and the creation of "biological process" output nodes. Additionally, from the interactions of the macrophage with other cells of the immune response, a cellular communication module was assembled. The integration of this module resulted in the new Macrophage Activation and Cellular Communication in COVID-19 (MacAct-CC) model. Next, both model's ability to represent macrophage dynamic behavior during SARS-CoV-2 infection was assessed by means of a stable state analysis performed with the bioLQM tool in a Jupyter Notebook. For this purpose, initial stimulus representing pathological conditions of inflammation, viral infection, anti-inflammation, and their combination were defined. In the end, both models predictions were in agreement with the scenarios reported in the literature for moderate and severe COVID-19 cases.

4.1 PART I: Macrophage polarization under immune stimulation

4.1.1 Analysis of current models

For the evaluation of the state of the art in macrophage models, three published models were considered, which were recreated in GINsim to verify their dynamic functionality by means of a stable state analysis. In this analysis the results obtained corresponded reasonably well with the results reported by the authors of the models. Some discrepancies were found concerning the stable states of some models reported in the literature but these could be attributed to the potential differences of the algorithms used to obtain the stable states. For instance, GINsim is able to find all the stable states that a model can reach, however, some of them do not occur in vivo and authors may decide to only show the results that can be biological possible or the ones of interest, i.e. simplified state results. In other cases, the authors will only show the experimental stable states of the molecules of interest in their study even if their model comprises more entities, limiting the extent to which model output can be compared. Comparing the number of stable states reached by the analysis to published results is therefore somewhat problematic. In summary, this limits the validation of the models since, although similar results could be obtained, the information generated does not support whether the model has been replicated in its entirety as described by the authors.

As stated previously in the Materials and Methods section, the Palma *et al.* (2018) [1] model was selected as a parent model for the following work of this project. During the validation analysis, it was possible to verify that the dynamics of the model recreated in GINsim corresponded to that reported by the authors. Although in the recreation the model presented fewer stable state results than reported in the scientific publication, overall, the stable state results were consistent with the results stated by the authors. The inconsistency

may be due to the way the simulations were conducted in this analysis or the interpretation of stable states from the authors. Additionally, the overall coverage of polarized macrophage states that the model supports, were in agreement. More details and characteristics that were taken into account for the selection of this model were explained in Materials and Methods (section 3.1.1. Current macrophage models). The model represents the macrophage polarization into four different phenotypes (M1, M2a, M2b, and M2c). It contains four types of nodes, depending on their cellular location and function: input nodes (extracellular stimuli), receptors, internal regulators, and main gene and protein products of each distinct type of macrophage (Figure 4.1). The interactions and logical functions of this model were derived from the available literature. The dynamics of the network were analyzed by the authors and the model simulations found five sets of steady states fitting the macrophage phenotypes:

1. M0: no nodes active;
2. M1: Interleukin-12 (IL-12) and at least one among Signal transducer and activator of transcription 1 and 5 (STAT1 and STAT5), or Nuclear Factor kappa B (NF-kB) are active;
3. M2a: all of Peroxisome proliferator-activated receptor-gamma (PPARγ), Signal transducer and activator of transcription 6 (STAT6), Lysine-specific demethylase 6B (JMJD3) and Interleukin-10 (IL-10) are active;
4. M2b: Extracellular signal-regulated kinase (ERK) and Interleukin-10 (IL-10) are active; and
5. M2c: Signal transducer and activator of transcription 3 (STAT3) and IL-10 are active.

4.1.2 The expanded Macrophage Polarization (MacPol) model

Starting with Palma's model, the expansion with key regulatory components allowed the generation of a Macrophage Polarization (MacPol) model with a higher level of detail in regulatory pathways, macrophage phenotype markers and actionable components of lipid metabolism. In the first instance, the exhaustive manual verification of the interactions in Palma's model resulted in some inconclusive references or interactions that were not validated in detail in the literature used by the authors. Therefore, the expansion step started from these interactions (Figure 4.1). Then, to integrate new entities into the model, the extensive manual compilation of relevant information from the literature and databases resulted in a list of selected components of macrophage polarization (Appendix A, Table A). Each component was annotated with its complete name and entry from the UniProtKB database [79]. Some entities were not found in UniProtKB since they do not represent unique proteins, these include: ions (e.g. calcium ion), and protein or molecule complexes (e.g. Immune complexes (IC) or the Adaptor protein complex AP-1 (AP-1)). Similarly, the compilation process resulted in a manually curated list of interactions (Table B, Appendix B), which includes: (1) the entities involved (source and target node), (2) the sign of the interaction (positive, i.e., activator, or negative, i.e., inhibitor), and (3) the literature supporting

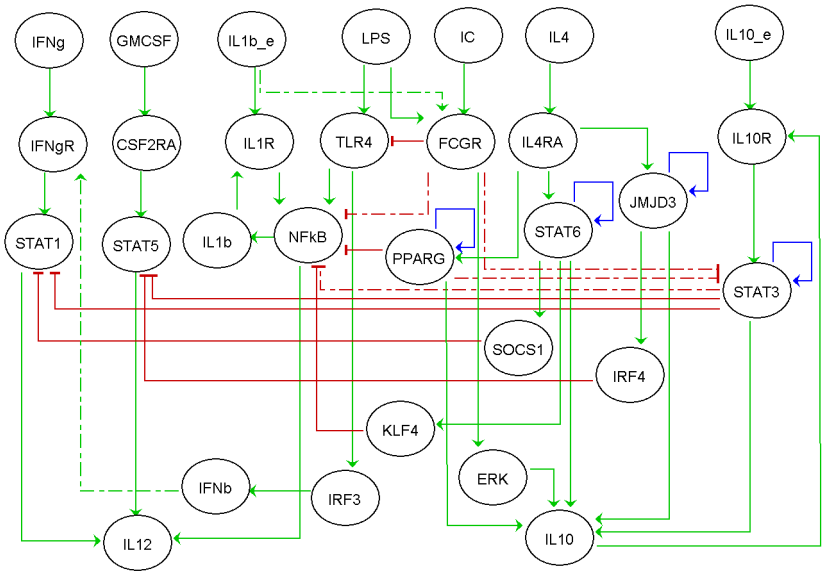

Figure 4.1: **Reconstruction of the Palma *et al.* (2018) macrophage differentiation model [1] in GINsim.** Nodes can represent either proteins, small molecules, transcription factors or genes. Edges can represent either protein-protein regulatory interactions or transcriptional regulations. Blue edges represent self-loops, green edges represent positive (i.e. activating) interactions, red edges represent negative (i.e. inhibiting) interactions, and dashed edges represent interactions that could not be validated with recent literature.

such relationship between the entities, including a fragment of the publication text describing the relationship and the PubMed reference ID (PMID), or link where the publication can be accessed.

Following the prior-knowledge driven expansion, a logical model describing the signaling events that govern macrophage activation was constructed (Figure 4.2). The network comprises a total of 68 nodes, with 132 interactions among them. The main stimuli, receptors, and signaling pathways of macrophage polarization are represented. The positive interactions are represented by green pointed arrows and the negative interactions by red blunted arrows. The information that confirms the validity of each one, in addition to the link of the reference used, was added in the GINsim file as an annotation, together with more information about the nodes. This information can be found in Appendix B.

To have a visual distinction of the nodes in the network and to be able to intuitively arrange the signature signaling pathways of each macrophage phenotype, the nodes were arranged by color and position in the graph 4.2. This was done to highlight and differentiate stimuli, receptors, and internal nodes or macrophage markers, and to follow their order in the signaling cascade - from the external stimulus to the secreted products or expressed genes.

Stimuli, receptors and signalling cascades

Ten input nodes are shown at the top of the network. They represent external molecules that, when bound to the corresponding receptor in the macrophage membrane, can elicit

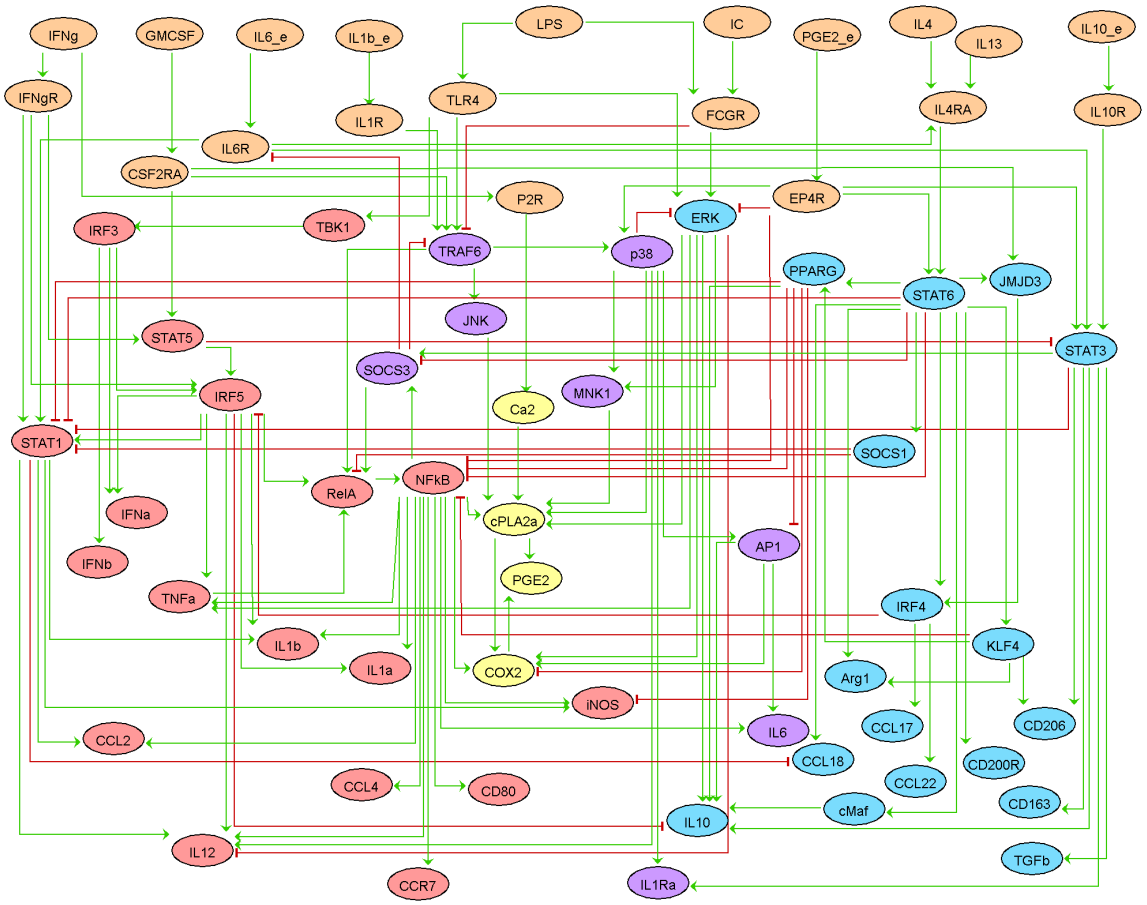

Figure 4.2: **Macrophage Polarization (MacPol) model.** Nodes represent proteins, transcription factors, genes, or ions. The relationships between them are represented as green arrows connecting the nodes, if they cause an activating effect, or red blunted arrows, if they cause inhibitory effects on a target node. Top nodes (organge): external stimuli and receptors. Middle nodes: intracellular signaling molecules and transcription factors. Bottom nodes: molecules secreted or expressed (cytokines, chemokines, and membrane markers). Red nodes: entities related to the M1 phenotype. Blue nodes: entities related to the M2 phenotype. Purple nodes: entities related to both phenotypes. Yellow nodes: entities related to the lipid metabolism mediated by Cytosolic enzyme phospholipase A2 (cPLA2).

the activation of different signaling pathways inside the cell, i.e. the cellular stimuli. For the polarization of the Classically activated macrophages type 1 (M1), Lipopolysaccharides (LPS) and Interferon-gamma (IFNγ) are the main stimuli, since the phenotype is activated by microbial products or pro-inflammatory cytokines. However, this phenotype can also be activated by other inflammatory signals in the environment, such as Granulocyte-macrophage colony-stimulating factor (GM-CSF) [98], or Tumor necrosis factor alpha (TNFα) [99]. In contrast, for the polarization of the M2 phenotype (alternatively activated macrophage) the anti-inflammatory cytokines Interleukin-4 (IL-4), IL-13, and IL-10 are the classic main stimuli. In addition, the Alternatively activated macrophages type 2 (M2) can be activated by other signals, such as Interleukin-6 (IL-6) [99], or IC trapped by antibodies [100]. In the case of Interleukin 1-beta (IL-1β), this stimulus is associated with both phenotypes. It has been shown that IL-1β promotes the activation of the pro-inflammatory phenotype by directly augmenting TNF signaling in macrophages [101]. Yet, IL-1β in combination with LPS can stimulate the activation of the M2b phenotype [98].

The receptors are located below the stimulus nodes in Figure 4.2. Some of the external molecules can activate more than one receptor, such as the LPS molecules. These components are found in the outer membrane of gram-negative bacteria. Their presence can be recognized by Toll-like receptor 4 (TLR4) in the macrophage membrane but they can also interact with the High affinity immunoglobulin gamma Fc receptor I (FCGR) to enhance its activity [102, 103]. On the other hand, different external molecules can activate the same receptor, such as IL-4 and IL-13. These cytokines can trigger M2 macrophage polarization, among other immune responses, and both molecules signal through different subunits of the same receptor [104]. In the case of IFNγ stimulus, this molecule can activate its corresponding receptor but can also affect the activation of ATP-receptors (purinergic receptors P2) by increasing the gene expression of it, leading to an up-regulation of purinergic signaling [105].

Below the receptors, the signaling cascades activated by each of them are represented with the main molecules of the pathway. These receptors can trigger several signaling pathways involved in multiple cell functions. However, only those directly related to the immune response, both inflammatory and anti-inflammatory, are represented in the network. A complete list of the signaling pathways triggered by each of the receptors is shown in Appendix C. Additionally, only some of the early adaptors and intracellular signaling molecules are represented in the network. This was done in order to keep the number of nodes in the network to a preferred minimum (see Materials and Methods). And, since at this level many of the receptors share adaptor proteins in their signaling cascades, only those that are characteristically used by each receptor are represented. For instance, in the case of Tumor necrosis factor receptor associated factor 6 (TRAF6), an adaptor protein that mediates a wide array of protein-protein interactions via its TRAF domain and a RING finger domain, the decision was to include it in the network since many regulatory processes in the macrophage are directly related to this molecule [106]. That is, many other signaling cascades in the cell, both at the level of receptors and internal molecules, can act through the activation or inhibition of TRAF6. For example, it has been suggested that the Suppressor of cytokine signaling 3

(SOCS3) causes its effect to inhibit the inflammatory response (activated by IL-1β and/or LPS) by inactivating TRAF6 and thus stopping the release of pro-inflammatory cytokines produced in this signaling pathway [107, 72, 108, 109, 110].

Most of the intracellular signaling molecules that are represented in the MacPol model are associated with TLR4 activation. This receptor plays a critical role in the recognition of infectious agents and it is one of the most studied pathogen recognition receptors (PRRs) in macrophages [111]. There was a dedicated effort to integrate some of the key molecules associated with this receptor, because they are involved in multiple signaling cascades that lead to the activation of pro-inflammatory molecules. After forming a complex with LPS -the stimulus-, it can activate the adaptor of Myeloid differentiation factor 88 (MyD88) and/or the adaptor TIR-domain containing adapter protein inducing interferon-β (TRIF) signaling pathway [112]. Both signaling pathways are represented in the network by the activation of TRAF6 (for the MyD88-pathway), and Serine/threonine-protein kinase TBK1 (TBK1) (for the TRIF-pathway). Downstream of the cascade, TBK1 activation results in the translocation of Interferon Regulatory Factor 3 (IRF3) into the nucleus, and Type I interferons (IFN-I) production; whereas TRAF6 activation results in Nuclear Factor kappa B (NF-kB) translocation and expression of various pro-inflammatory genes but also c-Jun N-terminal kinases (JNK), Mitogen-activated protein kinase (p38), and AP-1 transcription factor (TF) family translocation and expression of different genes [112, 113].

Although many details about the exact regulations involved in macrophage polarization remain unknown, the classic signaling pathways associated with each phenotype are represented in the MacPol model. Three signaling pathways are related to M1 polarization, three are related to M2 polarization, and two are related to the polarization of both phenotypes. They involve: (1) interferon regulatory factors (IRF), (2) signal transducers and activators of transcription (STAT), and (3) suppressor of cytokine signaling (SOCS) proteins. Briefly, the IRF5/STAT1 pathway activated upon IFNγ stimulus, and the TRIF and MyD88 pathways activated upon LPS stimulus regulate the transcription of M1-related genes [114]. Alternatively, STAT6, STAT3, and Extracellular signal-regulated kinase (ERK) activation and translocation upon IL-4/IL-13, IL-10, and IC stimulus, respectively, trigger the M2 polarization in its different variations (M2a, M2c, and M2b, respectively) [114]. Finally, ERK and p38 activation following IC and LPS stimulus, respectively, can lead to the transcription of genes related to both M1 and M2 phenotype.

Markers of macrophage differentiation

The signaling pathways represented in the MacPol model trigger the expression of a large number of pro-inflammatory and anti-inflammatory genes. A selected set of these markers were included in the network, both those classically used to differentiate macrophage phenotypes and markers currently used in macrophage polarization assessments. The integration of new markers is important, as it increases the usefulness of the MacPol model. The model is more complete and provides the latest information on the regulation of these markers. Thus, the MacPol model can be implemented in new experiments and clinical studies

that evaluate these new markers.

There was an effort to integrate the markers of the M2 phenotype. In previous years the view of this phenotype as a single state of activation has expanded. With the observation of different patterns in the gene expression profile a subdivision of the generalized M2 phenotype was made. The recent subsets are defined depending on the stimulus that generates its differentiation and the resulting gene expression profile.

A total of 11 of the nodes included in the MacPol model correspond to markers of the M1 phenotype, 10 nodes correspond to markers of the M2 phenotype, while three of the included nodes have been associated with both phenotypes. These markers include expressed genes and translated proteins, as well as cytokines and chemokines. As mentioned before, some entities have been reported in the expression profile of both M1 and M2 phenotypes. Additionally, some of the genes represented in the network have basal expression levels in the resting macrophage. Yet, upon a stimulus, the cell will begin to express more of one polarization-biased subset of genes than the other. In the network, only the nodes SOCS3, IL-6, and the Interleukin-1 receptor antagonist protein (IL-1Ra) are represented as common to both M1 and M2 phenotypes because they are considered markers for both phenotypes. Nevertheless, many of the others may be equally present in the expression profile of polarized subsets. This is the case with IL-12 and IL-10 interleukins, which are expressed in M1 and M2 macrophages but are represented in the MacPol model as unique markers for each phenotype since some studies show that they are expressed in different proportions. M2 macrophages express more IL-10 than IL-12, while M1 macrophages express more IL-12 than IL-10 [115].

To begin with the common markers in the network, the role of SOCS3 in macrophages is many times controversial. Some studies point out that SOCS3 signaling is required for the anti-inflammatory effects of macrophages since it is involved in repressing the M1 pro-inflammatory phenotype [116]. Studies indicate that SOCS3 inhibits LPS and IL-1 signaling at the level of NF-kB activation and mitogen-activated protein kinase (MAPK or MAP kinase) by inhibiting ubiquitination of TRAF6, thus preventing activation of Mitogen-activated protein kinase kinase kinase 7 or Transforming growth factor-beta-activated kinase 1 (TAK1)) and thereby blocking further signaling of pro-inflammatory cytokines during cell immune response [107, 108, 109]. Moreover, SOCS3 inhibits STAT1 and regulates IFNγ signaling, in response to LPS stimulation by binding to phosphorylated tyrosine sites of the JAK2 receptor domain to control macrophage anti-bactericidal effects [117]. On the other hand, it has also been observed that, in a pro-inflammatory environment, macrophages show strong up-regulation of SOCS3 and this directs the efficient production of pro-M1 cytokines [118, 119]. Studies of SOCS3-deficient macrophages confirm that SOCS3 positively regulates TLR4 signaling and M1 activation by inhibition of Interleukin 6 receptor (IL-6R)-mediated STAT3 activation, as well as Tumor growth factor-beta (TGF-β) mediated SMAD3 activation, which is critical for the negative regulation of TLR-induced TNFα and IL-6 production [118, 119, 120, 121].

Next, IL-1Ra is part of an auto-regulatory loop that attenuates the inflammatory reaction. IL-1Ra binds to the IL-1 receptor with high affinity, thereby competing with IL-1α and

IL-1β and preventing the activation of the receptor and its signaling of pro-inflammatory cytokines [122, 123]. IL-1Ra is regulated by the activation of p38 in the TLR signaling following LPS stimulus [124, 125], and also by the activation of STAT3 following IL-10 stimulus [126]. Since STAT3 can make the IL-1Ra promoter accessible to readily available nuclear NF-kB in LPS-treated phagocytes, it is said that STAT3 cooperates with other transcription factors to synergistically enhance IL-1Ra transcription.

Finally, IL-6 is a pleiotropic cytokine, since it is associated with the perpetuation and enhancement of several diseases in inflammatory situations, and is also necessary for the resolution of inflammation and adequate wound healing to occur in other situations [127]. As a secreted pro-inflammatory cytokine, IL-6 expression is regulated by NF-kB, p38, and ERK upon inflammatory stimuli [128, 129]. However, it can also be induced by STAT3 activation and then co-express with M2 macrophages-related cytokines (IL-10 and TGF-β) [130]. As a stimulus, studies suggest that IL-6 reinforces the IL-4 + IL-13 M2 polarization of macrophages [127]. Co-treatment of alternatively activated macrophages with IL-6 results in a spontaneous release of IL-10, suppressed LPS-induced nitric oxide production and inhibited cytokine production by activated CD40+ T cells; but in the presence of IFNγ, IL-6 promotes the production of IL-1β and TNFα suggesting that this cytokine can enhance the phenotype to which a macrophage has committed [127].

The lipid metabolism mediated by cPLA2

Seven entities of the macrophage cPLA2-related lipid signaling are represented in the network, including the key enzymes in the metabolism of arachidonic acid (AA) and eicosanoid production. The signaling cascade begins with the activation of the Purinergic P2 receptor (P2R) by IFNγ stimulus. Macrophages express a wide variety of adenosine triphosphate (ATP) receptors, both ionotropic P2X and metabotropic P2Y receptors [131]. Although these receptors detect extracellular nucleotides and nucleosides, the stimulus that triggers its activation in the MacPol model is IFNγ since it represents the characteristic cytokine of an inflammatory environment which is rich in extracellular nucleotides, such as ATP, that derive from dying, necrotic, and apoptotic cells [132, 54]. Subsequently, the activation of P2 receptors can elicit Ca^2+ mobilization inside the cell from the intracellular storage (P2Y receptors) or extracellular microenvironment (P2X receptors) [131]. The increase in calcium concentration positively cooperates with the activity of cPLA2, since this enzyme requires the presence of this ion as a cofactor [133]. At the same time, the expression of cPLA2 and the Cyclooxygenase 2 (COX-2) are up-regulated upon LPS stimulus by Nuclear Factor kappa B (NF-kB) and intracellular signaling molecules of the TLR4 MyD88-pathway. Finally, the expression of both cPLA2 and COX-2 are equally important in regulating Prostaglandin E2 (PGE2) biosynthesis in macrophages exposed to LPS [134].

Furthermore, PGE2 has a dual role in macrophage polarization. Some studies prove that PGE2 enhances inflammasome activation and promotes the expression of M1-related markers, such as IL-1β and IL-12, by the activation of its receptor Prostaglandin E2 receptor EP4 subtype (EP4R) in the membrane of macrophages [12] or through undefined mitochondrial-

membrane-potential-regulated circuits [135]. Other studies point out that activated EP4R following PGE2 stimulus plays a key role in anti-inflammation and immune suppression functions by suppressing the expression of pro-inflammatory cytokines activated in the NF-kB signaling pathway [136, 137], as well as increasing the levels of IL-10 and IL-6 by the activation of p38 MAP kinase activity [14], and the up-regulation of STAT3 and STAT6 activity [15, 16]. At this point is important to note that the literature reports conclusions that are based on experiments performed in different conditions, which may explain some of the differences of the role of PGE2. However, the literature pointing at the enhancement of the M2 phenotype was more conclusive and taken as reference in the incorporation of PGE2 signals in the MacPol model.

4.1.3 Comparative analysis of the Palma model and the MacPol model

Graphical comparisons were made between the Palma model and the new MacPol model to highlight the work done during the expansion process. As expected, the MacPol model features a larger number of nodes and interactions, as the goal of the project was to add more regulatory details and macrophage biomarkers in the network. Knowledge mining in databases and macrophage literature led to the integration of 38 nodes and 82 interactions in the Palma model (Table 4.1). Three new stimulus nodes were added: IL-6, IL-13, and PGE2. Additionally, 20 new product or output nodes, including secreted molecules and expressed genes from the different macrophage phenotypes were added. Similarly, the regulatory wiring of the MacPol model was improved: the number of inhibitory interactions doubled after the expansion while positive interactions increased to 109 after the expansion. This is also due to the attempt to connect the biomarkers with their transcription factors in the network. Many of the secreted molecules or expressed genes require several transcription factors to be produced by the cell, and that is why the number of positive interactions is higher in the network, both in Palma's model and in the MacPol model.

Table 4.1: **Graph properties of Palma's model and the new MacPol model after the expansion.** The total number of nodes and interactions (edges) is shown, and they are also broken down by category, i.e., input and output nodes, activation (positive) and inhibition (negative) interactions.

Property	***Palma et al. (2018) model***	***MacPol model***
Number of nodes	30	68
Number of links	50	132
Number of promotion links	39	109
Number of inhibition links	11	23
Number of input nodes	7	10
Number of output nodes	2	22

In order to visualize the results of the expansion and the differences with the previous model, Figure 4.3 was built. The nodes that were added during the model expansion can be seen as green circles, while the nodes that were preserved from Palma's model are colored in

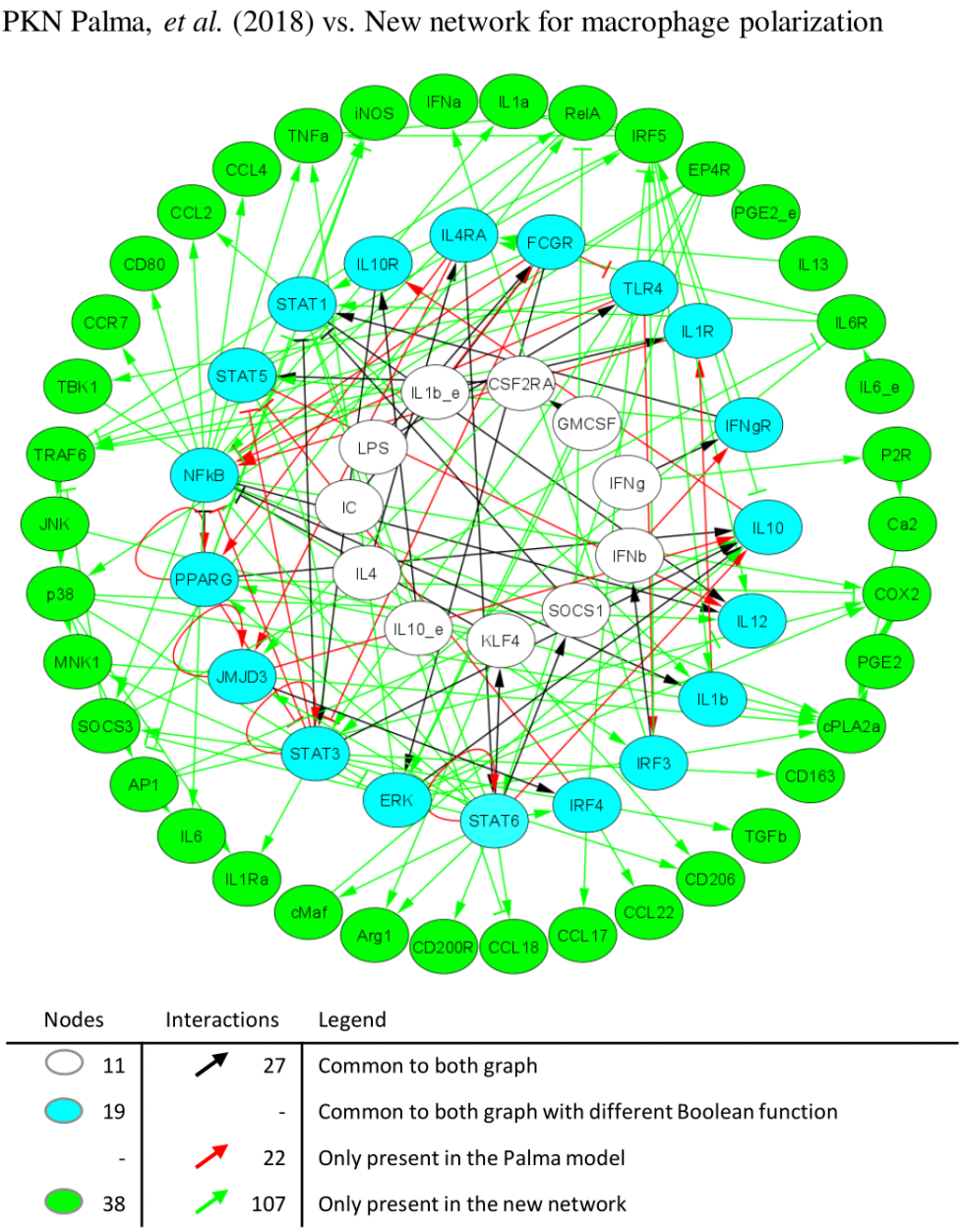

Figure 4.3: **Comparative graph of the nodes and interactions of Palma model [1] and the MacPol model.** In the center are the nodes that are common to both models (white and blue circles) and the outer circle shows the nodes that were added in the extension of the MacPol model (green circles). The interactions of the Palma model that have been maintained after the expansion are shown in black and those that have not been included are shown in red. Newly added interactions are shown in green. Finally, a table with the total number of nodes and interactions of each category is shown as a legend.

white and blue. Furthermore, some nodes, colored blue, were preserved but their Boolean functions were updated as more regulatory interactions were added to the MacPol model. For the same reason, several of Palma's model interactions were not kept after the expansion, either because they could not be validated or because new forms of regulation on the entities were found in the literature. The omitted interactions are presented in red.

Most of the nodes preserved with the same Boolean functions correspond to stimuli (a total of 7 nodes) but also include two intracellular signaling molecules (Suppressor of cytokine signaling 1 (SOCS1), and Krueppel-like factor 4 (KLF4)), one receptor (Granulocyte-macrophage colony-stimulating factor receptor subunit alpha (CSF2Ra)), and one secreted cytokine (IFNβ)). This is due to the fact that the stimuli are presented in the network as input nodes and do not have upstream regulators, so they remain the same in both Palma's model and the MacPol model. In the case of the other unchanged nodes, no differences were found after the literature analysis, as all their relevant upstream regulators were already present in the model. However, some interactions were added downstream from these nodes. For example, SOCS1 is still activated by STAT6 but it inhibits the Transcription factor p65 (RelA) in the new MacPol model, as well as STAT1, like in Palma's model.

On the other hand, most of the nodes preserved but with different regulations are intracellular signaling molecules (a total of 10 nodes), but they include also six receptors and three interleukins. There are several reasons for these updated regulations but the main ones are listed below.

1. The MacPol model has three new stimuli that trigger some of the intracellular signaling molecules that were in Palma's model. This means that now these signaling molecules have more regulators. Such is the case with STAT3, which is activated upon IL-10, IL-6, and PGE2 stimulus in the MacPol model whereas it was only activated upon IL-10 stimulus in Palma's model.

2. Many regulatory molecules associated with the receptor in the signaling cascade were added in the MacPol model. This means that now the regulatory cascade has more intermediate nodes compared with the Palma model, and what was once represented as a direct interaction between two nodes now has more regulatory entities in between. For example, IRF3 was directly regulated by TLR4 in the Palma model but now this interaction is mediated by TBK1 -representing the TRIF-pathway of TLR4.

3. The Boolean functions of some entities have been changed due to new evidence in the literature. For instance, IL-12 could be activated by either STAT1, STAT5, or NF-kB in Palma's model but in the MacPol model it is activated only when STAT1, NF-kB, IRF5, and p38 are active and ERK is not. The literature that validates the new interactions is shown in Appendix B, and the Boolean functions can be found in Appendix D.

4. Some interactions from Palma's model could not be validated and were not added to the new MacPol model. An example of this is the activation of FCGR by IL-1β.

4.1.4 Stable state analysis and validation of MacPol model behavior

Analysis of polarization stimulatory conditions

To analyze the performance of the MacPol model as a representation of macrophage polarization, a stable state analysis was carried out using polarization stimulatory conditions. The stable states reached by the MacPol model after the simulation in GINsim represent the dynamic behavior as a consequence of the Boolean rules. The binary states of the nodes are interpreted as states of activity that the entities possibly present in a real polarization situation, i.e. in experimental results. Six fixed inputs were set to represent the polarization stimuli and each input resulted in a stable state, plotted through a heatmap in Figure 4.4. The inputs were defined according to the literature on the activation of the different macrophage phenotypes, i.e. IFNγ and LPS for M1, IL-4 for M2a, and IL-10 for M2c. The M2b phenotype was not analyzed in the project since there was not a lot of literature regarding the gene expression profiles and new experiments run in the Johansen laboratory considered only the mentioned phenotypes. Additionally, the effect of PGE2 stimulus in the stable states was tested by including PGE2 in the aforementioned fixed inputs. These results comprise the MacPol model's predictions for macrophage polarization under the canonical conditions of differentiation.

Each stimulus causes the evolution of the system to a new stable state (attractor). The system's resulting state is represented by a column in the heatmap, representing activity states of all nodes in the system. The new stable state allows generating an expected expression profile that can be associated with the experimentally observed macrophage phenotype. The stable states of the receptors were omitted, since they will be active (value of 1) by default after the interaction with the specific stimulus (established before in the fixed input). Additionally, the nodes were grouped in the heatmap by the signaling pathways they belong and the phenotype that they are related to, i.e. if they are commonly found in the expression profile of a specific phenotype (M1, M2a and M2c).

The system reached one stable state after each stimulus and this corresponds to the expression profile reported for each macrophage phenotype in the literature. When the inflammatory stimulus is provided to a resting system, the markers of the M1 phenotype become activated (value of 1). Alternatively, when the anti-inflammatory stimulus is provided, the markers of the M2 phenotype get activated, both for the M2a and the M2c sub-types. It is important to note that the heatmap also includes the stable state of adaptor molecules, and intracellular signaling molecules of the pathways activated, such as the nodes triggered by TLR4 activation (TRIF and MyD88 pathway), even though these entities are not markers of the polarization of macrophages to a particular phenotype.

To begin with, the M1 polarization is reached after IFNγ and LPS stimulation. IFNγ triggers the STAT1 and STAT5 signaling pathways, and LPS triggers the TLR4 signaling pathways. These pathways lead to the transcription of pro-inflammatory target genes, such as IL-12, IL-1β, IL-6, TNFα, Inducible nitric oxide synthase (iNOS), C-C motif chemokine 2 (CCL2), T-lymphocyte activation antigen CD80 (CD80), and COX-2, among other markers represented in the MacPol model. In this condition, the IL-1Ra gets activated following TLR4 activation

of p38, since this molecule functions as a negative feedback loop to attenuate the inflammatory response. Similarly, SOCS3 is induced following TLR4 activation of NF-kB, which may function as a negative feedback loop since it inhibits the TLR4 signaling at the level of TRAF6.

Next, the M2a polarization is reached after IL-4 stimulation. With the activation of the STAT6 signaling pathway, PPARg and JMJD3 get activated, and thus, the three entities are responsible for the transcription of anti-inflammatory genes, such as Arginase-1 (ARG-1), IL-10, C-C motif chemokine 17 and 22 (CCL17 and CCL22), Cell surface glycoprotein CD200 receptor 1 (CD200R), and Macrophage mannose receptor 1 (CD206).

Finally, the M2c polarization is reached after IL-10 stimulation. The key STAT3 signaling pathway gets activated and leads to the transcription of anti-inflammatory target genes, such as IL-10 and CD206 (common with the M2a phenotype), SOCS3 and IL-1Ra (common with the M1 phenotype), the Scavenger receptor cysteine-rich type 1 protein M130 (CD163), and the TGF-β - all of them crucial for this phenotype.

In addition to the canonical stimulation, the PGE2 stimulus resulted in an enhancement of the M2 phenotype in all the observed stable states. As expected, in combination with either IL-4 or IL-10 the system assumes an M2 phenotype. Since the STAT3 and STAT6 pathways get activated following PGE2 stimulation, all markers related to the M2 phenotype, both M2a and M2c, get activated. On the other hand, PGE2 in combination with the inflammatory stimulus (IFNγ + LPS) produces a stable state characteristic of the M2-profile. Yet, some entities related to the inflammatory signaling pathways have an active state in the final result. This indicates that the pro-inflammatory signaling pathways get activated upon the stimulus but the inhibitory pathways triggered by PGE2 are crucial for deactivating the expression of pro-inflammatory markers. It is possible that the cell passes through an M1-like state before it reaches the functional M2 configuration. Overall, the PGE2 stimulus reinforces the polarization of the macrophage towards the anti-inflammatory phenotype regardless of the combination with other stimuli.

It is important to mention that the work carried out in this project gave rise to new experiments in the Johansen laboratory, including the polarization of M2 macrophages and the stimulation with PGE2. The knowledge generated by the regulatory network was able guide the choice of markers to be measured and to understand and interpret the gene and protein expression measured in the experiments. Moreover, the expansion work on the MacPol model was continued in the Master's project of Marco Fariñas, who integrated new components of eicosanoid metabolism and some extra signaling cascades that were suggested at the end of this study [91]. The aim of continuing the project was to further refine the MacPol model and to have a more detailed representation of the regulations that occur during polarization. This allows for more accurate comparisons of the in silico behavior with the values that can be obtained in laboratory experiments.

Perturbation analysis and refinement of the MacPol model

In order to test whether the MacPol model was able to represent the observed experimental behavior of the macrophages in the literature, a perturbation analysis was performed by test-

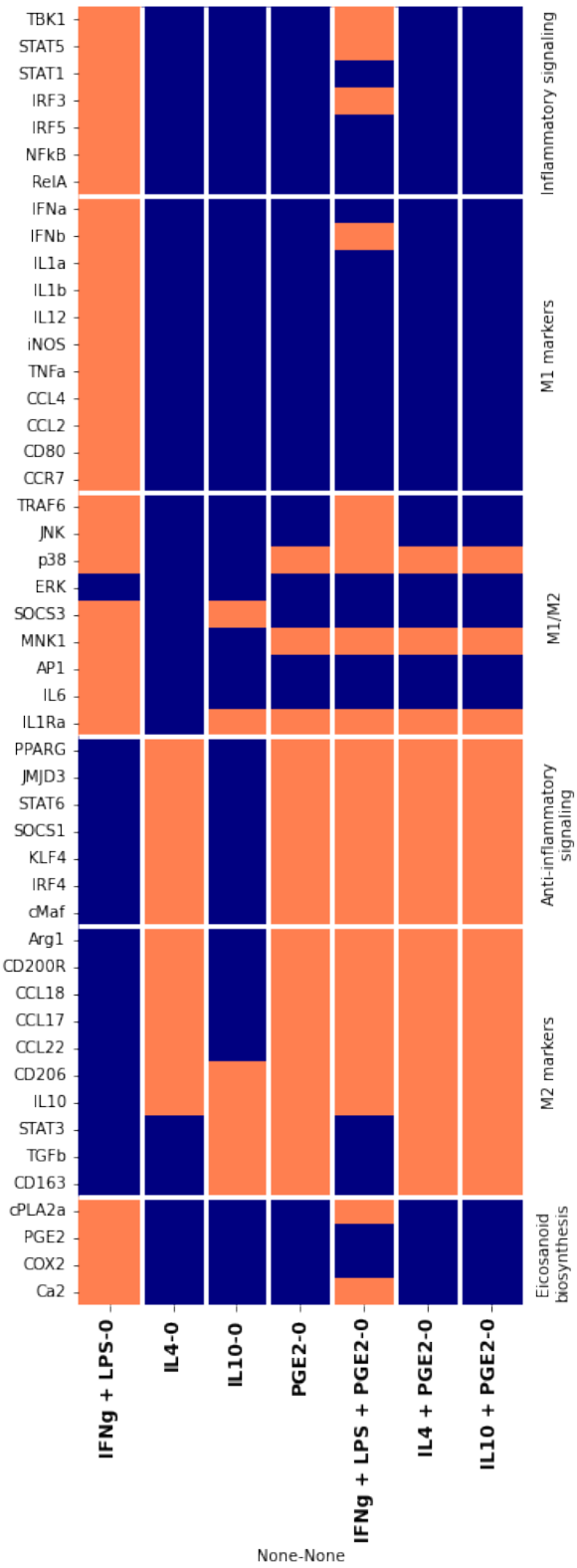

Figure 4.4: **Heatmap of the stable states reached by the MacPol model using experimental macrophage polarization conditions.** The system reaches one stable state after setting the respective fixed input. Each column therefore represents one complete stable state with the stimulus indicated above the column. The entities that present an active or high expression state (value of 1) are shown in coral while those with an inactive state (value of 0) are shown in navy. The entities are organized by their role in the network and it's association with the different phenotypes, i.e. if they are commonly found in the expression profile of a specific phenotype. The high activity or expression of marker nodes is decisive to define the macrophage phenotype.

ing the model's stable state predictions under different experimental conditions found in the macrophage literature. Fixed inputs were used to define experimental conditions, including the use of inhibitors, gene knockouts, constitutive gene expression, and/or pre-treatments. The perturbations were recreated in the analysis by setting the value of the nodes to "0", in knockout or inhibitors cases, or to "1", in constitutive expression or pretreatment cases. In the first stage, the results of the analysis were compared to the results reported in the respective perturbation study. If the in silico results were different from those in the studies, changes to the MacPol model were made to make the network behavior approximate the experimental behavior better. These steps comprise the refinement of the MacPol model. After each improvement of the model, the stable states following polarization stimuli were evaluated once again to determine if the corrections affected more entities. Once all the perturbation conditions were evaluated and the improvements in the MacPol model were added, the optimized model was used to perform all the previous stable state analyses again -including polarization stimuli and node perturbation conditions.

Perturbations in the MacPol model affect the network functionality and, consequently, the resulting marker profile and phenotype of the macrophage. Implementing the different experimental conditions from the literature affect the model dynamics, and the expression or activity of the nodes will respond accordingly. The comparison of the stable state results with the expression results reported in the literature is shown through a heatmap with marker's activity levels reached in the different conditions (Figure 4.5). The expression values reported in the literature are shown with numbers (1 = high expression/activity; 0 = low expression/activity); if the obtained stable state of a model component has the same value as the one reported in the literature, the corresponding cell in the heatmap is colored green, if not, the cell is colored red. This means that green cells represent results that match and the red cells denote apparent model failures.

Furthermore, perturbed nodes were grouped by their role in the network, that is, whether they were related to lipid metabolism, to the signaling pathways that lead to the polarization of the M1, the M2, or both phenotypes (M1/M2). Similarly, markers were organized by the phenotype they are related to, that is, if they are commonly found in the expression profile of a specific phenotype. The organization of the perturbed nodes and markers allows to easily see which of the measured entities are affected upon the perturbation. For instance, perturbations of nodes related to the M1 phenotype will affect the signaling pathways and the expression of pro-inflammatory markers, so the organization in the heatmap allows to locate these markers and see the value of expression measured experimentally. Also, it is important to note that the perturbations have been carried out with either pro- or anti-inflammatory stimuli and this has to be examined carefully to understand why some markers get activated or not.

In general, 15 of the stable states predictions were consistent with the experimental results described in the literature but 8 of them presented some inconsistencies. In the generated matrix it seems that there is some accumulation of errors at some columns and row levels. At the column level, this suggests that there may be problems in the definition of the node regulations - the logical rules - of the MacPol model. While at the row level, it suggests

that there may be problems with the conditions recreated and the effect of the disturbed entities on the network topology. However, this must be carefully considered because when analyzing the errors as a whole and individually, there are in fact quite a number of possible reasons for these differences:

- Overall, the MacPol model aims to represent a general macrophage polarization without taking into account specific details of the polarization protocol. Thus, discrepancies can be attributed to the experimental variety and diversity that exists between studies, given that each recreated experimental condition corresponds to a particular study. These studies were carried out in different years, by different groups of investigators, and with different objectives. This means that not all the 23 sets of results considered in the analysis were produced under the same conditions, using the same cell lines, and following the same protocols. Thus, some of the discrepancies can be attributed to this.

- Another reason that can be broadly considered for the discrepancies found are the limitations posed by the use of mouse models for the study and analysis of human cell behavior and pathologies. Although the MacPol model attempts to reflect the behavior of human macrophages - by including biomarkers and validated interactions in studies with human macrophages - some of the interactions in the network come from studies in mice. This is due to the fact that the mouse model is widely used in biomedical research because of its advantages of easy manipulation at the genome level, ease of breeding and maintaining the species, economic and protocol reasons, among others [138]. However, multiple differences in the immune system of the mouse relative to the human system have been observed, indicating that the regulation and expression of markers may differ at the species level [139, 140, 141]. Most of the studies considered use the mouse model and this may explain some errors in the comparative analysis of stable states of a human oriented model and experimental observations.

- Additionally, interpreting the experimental gene expression levels of the studies is done after discretization of the data. The studies measured the expression level as a continuous value. Yet, to compare the results to the stable states reached by the MacPol model, these continuous datasets were translated to binary datasets. Therefore, some discrepancies may arise from this.

- In a more specific observation of the discrepancies, it should be noted that genes have basal expression levels in the cells. Therefore, it is possible to find significant levels of expression experimentally, even if the entity is not fully active as such in a biological pathway. Yet, the MacPol model does not simulate this basal expression and only represents the complete activation of the node. This means that the corresponding stimulus is needed for the node to become active. As an example, the cytokines IL-10 and IL-12 are constantly produced by macrophages. Some studies have found significant levels of expression of these cytokines even though the experimental conditions do not stimulate their expression. This also happens when the studies use pro-inflammatory

stimuli with an M1-related perturbation and consequently find significant levels of expression of M2-related markers. This scenario will not be reflected in the stable state results because the M2-related nodes need the corresponding stimuli to get activated in the network. This is the case for the ARG-1 and CD206 markers related to the M2 phenotype. Some studies show high values of expression after pro-inflammatory stimulation. However, the MacPol model will never reflect these results since, as the network is described, these markers need the M2 stimuli to be activated (IL-4 stimulus). Overall, the threshold used in experimental studies must be examined very carefully to make an accurate comparison, because this type of experimental results could reflect only a partial activation or the constant basal level of expression of the nodes.

- Another particular reason for the discrepancies found are those experimental scenarios that are complex to recreate in silico and thus result in differences with the data and predictions generated by the MacPol model. That is the case of some studies that use an already polarized macrophage and then induce a perturbation (e.g. use an inhibitor) with another set of conditions. This is more complicated to simulate in the MacPol model and that is why, in a direct way, the results do not meet the literature. This is the case for the COX-2 inhibitor used in an M1 polarized macrophage population stimulated with the IL-4 anti-inflammatory cytokine. The study points out that M1-related genes are restored with COX-2 inhibition [142] but the simulation was set with only IL-4 stimulus and this condition is not sufficient to activate the M1-related entities in the model.

Nevertheless, the analysis performed in this step allowed to refine the MacPol model to meet many of the expected results described in the literature. Some of the Boolean regulations and rules were changed in the process. In this way, the behavior of the model was optimized to represent as close as possible the behavior of macrophages under different experimental conditions, in as much as the time frame available for this project allowed.

Figure 4.5: **Heatmap of the stable states using different experimental conditions of perturbed nodes reported in the literature.** The conditions include perturbations of the nodes (use of inhibitor (INB), knockouts (KO), constitutive expression (E1), or pretreatment (PT)), and the stimulus for macrophage activation. The cells inside the heatmap represent the stable state activities obtained in the analysis. The numbers indicate the value reported in the literature; 1 for high, and 0 for low expression/activity. The colors indicate stable states discrepancies - green for matching results (Agreement), red for not matching (Disagreement), and black when the level was not measured in the study. On the left, nodes perturbed are grouped by their role in the network, that is, if they belong to the lipid metabolism or if they are related to the pathways that lead to the polarization of M1, M2, or both phenotypes. At the top, markers are grouped by the phenotype they are related to - M1, M2a, M2c, or both M1 and M2. To the right of the figure, a legend lists the above mentioned specifications.

4.2 PART II: Macrophage activation under SARS-CoV-2 infection

4.2.1 SARS-CoV-2 modules and additional pathways

The first step in adapting the MacPol model to represent the pathological conditions of Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) infection was the selection, conversion, and refinement of process diagrams from the COVID-19 Disease Map repository. This step resulted in three small modules representing signaling pathways relevant to macrophage polarization and utilized or affected by SARS-CoV-2. The modules are dynamically executable networks in GINsim, provide information about the entities and interactions present in the network, and were almost entirely validated or curated using macrophage-specific literature.

Table 4.2: **Graphical properties of the modules before and after refinement.** The total number of nodes and interactions (edges) of the modules for the file generated from CaSQ throughout and the module after reduction and refinement are shown. The last column shows the percentage reduction of the module and is calculated with the average reduction of nodes and edges.

Module	File	Nodes	Edges	Reduction (%)
Type I Interferon	CasQ	102	157	
	Final	54	88	45.5
ACE/ACE2 axes	CasQ	58	78	
	Final	27	40	51.1
NLRP3 inflammasome	CasQ	35	30	
	Final	19	24	32.9

The files generated in CasQ represent the conversion of process diagrams to Boolean models. Then, the final file represents the module after manual reduction and refinement with macrophage-specific literature. Table 4.2 shows that the modules have been reduced by a considerable percentage. The final ACE-ACE2 module presents the highest percentage of reduction (51.1%), followed by the Type I interferon module (45.5%), and finally the NLRP3 inflammasome module (32.9%). The reduction represents the effort that was made to minimize the steps described in the process diagrams while keeping the flow of information consistent. The final size of the modules makes them suitable for integration into the overall MacPol model, as they can be processed and interpreted in a more direct way and with less computational effort. The modules and modifications made to the new Macrophage Activation in COVID-19 (MacAct-C19) model are explained next.

Renin-angiotensin pathway or the ACE/ACE2 axes module

Figure 4.6 shows the final module for the Renin-angiotensin pathway or the ACE/ACE2 axes (Angiotensin-converting enzyme (ACE) - Angiotensin-converting enzyme 2 (ACE2)). It con-

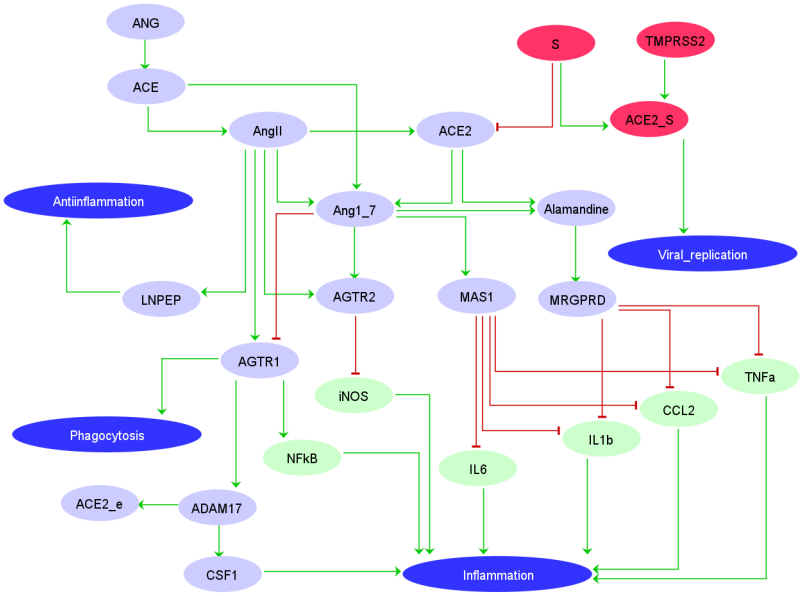

Figure 4.6: **ACE/ACE2 axes module.** Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows and negative interactions with red blunted arrows. Red nodes: viral entities and complexes. Lila nodes: cellular entities from the process diagram. Green nodes: cellular entities already present in the MacPol model. Blue nodes: biological process.

tains a total of 27 nodes and 40 interactions, mostly positive regulation. Only three nodes are related to ACE2: viral Spike-protein, the ACE2 and S-protein complex, and Transmembrane protease serine 2 (TMPRSS2), which facilitates viral entry. It also has four nodes that represent biological processes: viral replication, inflammation, anti-inflammation, and phagocytosis. The rest of the nodes are either part of the Renin–angiotensin system (RAS) or are entities regulated by it (i.e., cytokines and/or receptors).

This module represents the direct infection of macrophages mediated by ACE2 - S-protein interaction (viral replication node), such as the imbalance entailed by this interaction in the Renin–angiotensin system (RAS). This system includes several important entities in the regulation of the inflammatory and anti-inflammatory response. To begin with, Angiotensin I is metabolized by the enzyme Angiotensin-converting enzyme (ACE) to generate Angiotensin II, which acts through the Type-1 angiotensin II receptor (AGTR1) receptor and contributes to inflammatory response by inducing the production of inflammatory cytokines via NF-kB signaling. On the other hand, Angiotensin I itself can be degraded by ACE2 to give rise to Angiotensin - 1-7 acting through the MAS receptor, which promotes the anti-inflammatory response in macrophages by reducing the expression of proinflammatory cytokines (IL6, IL-1β, TNFα, CCL2). Thus, ACE2 maintains the balance between the two axes by favoring the expression of Ang-(1-7) and decreasing the bioavailability of Ang II. In the case that ACE2 does not participate in the system (when forming a complex with S-protein) Ang II accumulates. Therefore, the imbalance contributes to inflammatory damage and leads to the development of Macrophage Activation Syndrome (MAS) [62].

In addition to the imbalance of ACE2 by viral entry, this receptor can be released from

the cell membrane by the effect of Disintegrin and metalloproteinase domain-containing protein 17 (ADAM17). In this case the regulation can be seen as a positive feedback loop. First, ADAM17 is activated by the effect of AGTR1, which is upregulated by Ang II [62]. Then, ADAM17 cleaves the membrane-anchored ACE2, affecting its availability in the Renin – angiotensin system (RAS) [143]. Hence, ACE2 imbalance causes the accumulation of Ang II and starts the loop again. Additionally, ADAM17 is an important regulator of other membrane receptors associated with the inflammatory response (e.g. TNFα and IL-6 receptors, not included in the module) [62]. Therefore, this protein not only affects the Renin–angiotensin system (RAS), but also affects the overactivation of the inflammatory response.

Interferon I pathway module

Figure 4.7 shows the final module for the Type I interferons (IFN-I) pathway (IFNα and IFNβ). Since this module was developed by Marco Fariñas, the details of its construction and the signaling of the different cascades are found in his MSc manuscript for NTNU [91]. Here only an introduction to the module and its functional purpose is given.

The network contains 54 nodes and 88 interactions. It is branched into two main cascades: (1) the Toll-like receptors (TLRs) 7 and 3 (TLR7 and TLR3, respectively) cascade activated by pathogen-associated molecular patterns (PAMPs) that give rise to the inflammatory response, and (2) the interferon-α/β receptor (IFNAR) cascade that, together with the viral replication signal, give rise to the antiviral response. Thus, the biological processes in the network are: viral replication, antiviral response, inflammation, and pathogen-associated molecular patterns (PAMPs) signaling.

The two branches of this module represent on the one side the production of IFN-I, and on the other side the effect exerted by these interferons on the receptors present on adjacent cells. The main purpose of the module is to show the effect of viral entities on the signaling of these processes in macrophages. Thus, it is observed that viral proteins inhibit the antiviral response and the production of IFN-I, both at the level of receptors (e.g. RIG-like receptors (retinoic acid-inducible gene-I-like receptors) (RIG-I)) and at the level of signaling molecules (e.g. nuclear factor I$\varkappa$Bα). As such, the virus is able to evade the innate immune system response in macrophages [65, 64, 34]. This is of great relevance as both the production of IFN-I and the cellular response to these antiviral cytokines constitute a critical barrier to stopping viral dissemination and the development of severe disease [34].

NLRP3 inflammasome activation module

Figure 4.8 shows the final module for the NLRP3 inflammasome activation pathway. This module was processed in direct collaboration with the authors of the original diagram (Julia Somers and Ebru Kocakaya, Disease Maps consortium), and later refined by Marco Fariñas, so the details are in his MSc thesis for NTNU [91]. This section gives only a brief presentation of the module. The network contains 19 nodes and 24 interactions, with only two of them inhibitory. Four of the nodes represent viral entities and two nodes represent biological processes (inflammation and pyroptosis activation).

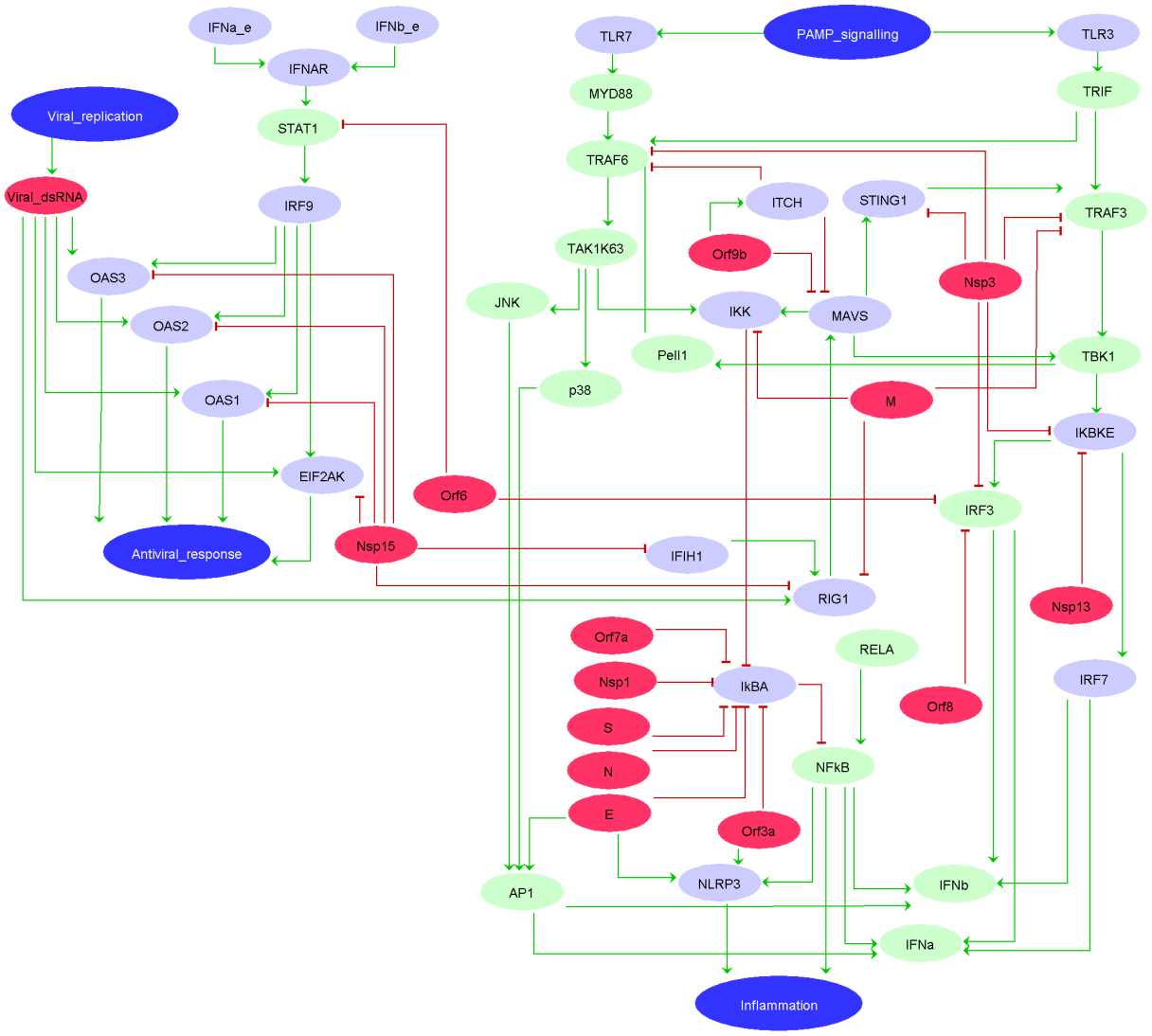

Figure 4.7: **Type I Interferon module.** Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows and negative interactions with red blunted arrows. Red nodes: viral entities and complexes. Lila nodes: cellular entities from the process diagram in the COVID-19 Disease Map repository. Green nodes: cellular entities already present in the MacPol model. Blue nodes: biological process.

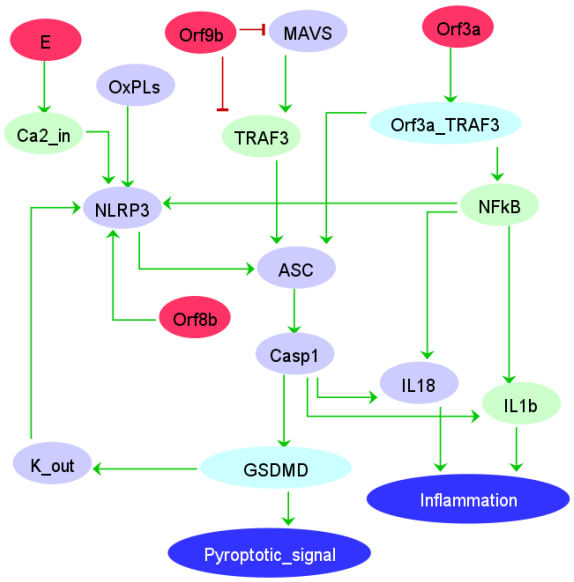

Figure 4.8: **NLRP3 inflammasome activation module.** Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows and negatives are shown with red blunted arrows. Red nodes: viral entities and complexes. Lila nodes: cellular entities from the process diagram. Green nodes: cellular entities already present in the MacPol model. Blue nodes: biological process.

This module represents in a few steps the activation of the inflammasome, the consequent pyroptosis response, and how the virus influences both processes. The network includes (1) the activation of NLRP3 (NOD-Like Receptor Protein 3) receptors to assemble the inflammasome, (2) the consequent formation of gasdermin D (GSDMD) pores in the cell membrane leading to pyroptosis, and (3) the activation and secretion of IL-1β leading to the inflammatory response. In the case of effective replication of SARS-CoV-2 in the cell, the inflammasome is induced by viral proteins E, 3a, and 8b to induce pyroptosis [144]. Thus, upon release of cellular contents, newly formed viruses capable of infecting new cells are released. Damage-associated molecular patterns (DAMPs) and inflammatory cytokines are also released in this process [66], which contributes to cytokine storm and MAS.

Additional changes

One of the additional changes made to the MacPol model was the integration of the TLR2 receptor signaling pathway and the modification of the TLR4 receptor signaling pathway. In the case of TLR2, an 12-node module with 13 positive interactions and a single negative interaction was constructed (Figure G.1, Appendix G). As the pathway of TLR4 was mainly modified by Marco Fariñas, the changes are explained in detail in his MSc thesis for NTNU [91]. This section presents the comparison of the original TLR4 pathway designed for the MacPol model and the modified pathway for the MacAct-C19 model (Figure 4.9). In addition, TLR2 signaling was incorporated in the figure for an integrated visualization of both changes in the new MacAct-C19 model.

The TLR2 pathway is a small module that represents the activation of this membrane re-

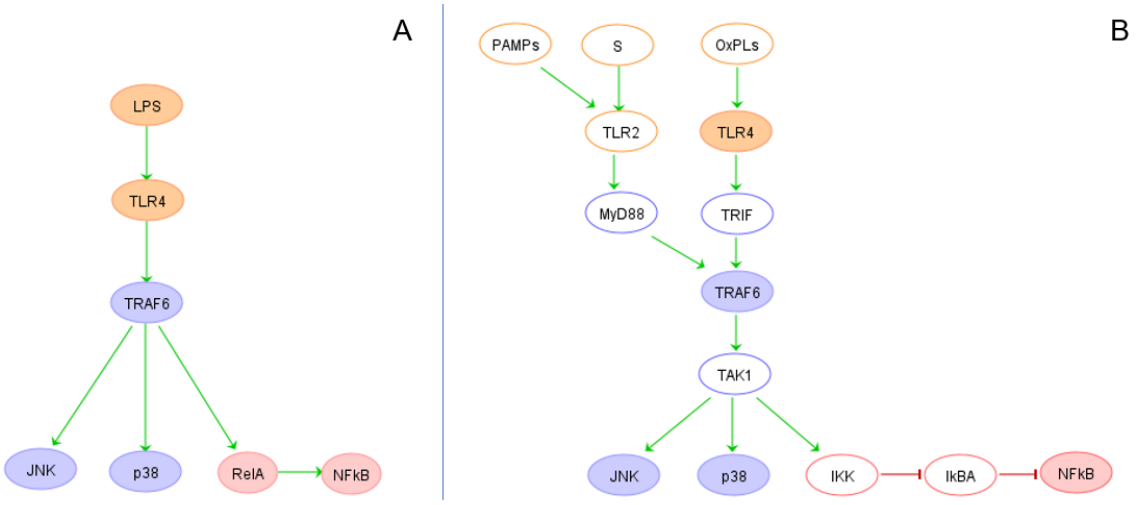

Figure 4.9: **TLR2 and TLR4 signaling pathways.** A: Representation of part of the TLR4 signaling pathway in the MacPol model. B. Representation of part of the TLR2 and TLR4 signaling pathways in the MacAct-C19 model. Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows and negative are shown with red blunted arrows. White nodes with colored borders: new integrated entities. Nodes with colored fill: entities present in the MacPol model. Lila nodes: cellular entities that lead to M1 or M2 polarization. Yellow nodes: stimuli and receptors. Red nodes: entities that lead to M1 polarization.

ceptor by viral particles (S-protein and PAMPs) and it's common ligand LPS (which is not included in the MacAct-19 model). The interaction with these stimuli results in the activation of the transcription factor NF-kB by the route of adaptor protein Myeloid differentiation factor 88 (MyD88) and the consequent release of inflammatory cytokines. This pathway is of great importance for the MacAct-C19 model since it has been reported that TLR2 is sensitive to SARS-CoV-2 viral particles, even when the infection is not effective at the intracellular level [145]. Moreover, the consequent activation of the MyD88 pathway triggers the release of cytokines and chemokines, which in the case of SARS-CoV-2 contributes to cytokine storm and MAS.

On the other hand, regarding the TLR4 pathway, the main changes are at the stimulus level and in the regulation of the transcription factor NF-kB. In the MacAct-C19 model, TLR4 is activated upon detection of oxidized phospholipids (OxPLs) in the extracellular space instead of lipoproteins (LPS) from bacteria. According to the literature, TLR4 is a receptor that is mainly activated by LPS in macrophages [146]. However, in the case of SARS-CoV-2 infection, TLR4 expressed on alveolar cells (including macrophages) can be activated by DAMPs, among them OxLPs and High mobility group box 1 protein (HMGB1) - both released by lysed cells [147]. Activation of the receptor and the transcription factor NF-kB ultimately leads to the release of proinflammatory cytokines, so that the continued state of activation of this pathway contributes to hyperinflammation and the development of severe COVID-19 disease [147].

4.2.2 The Macrophage Activation in COVID-19 (MacAct-C19) model

Figure 4.10 shows the pathological model of macrophage activation (Macrophage Activation in COVID-19 (MacAct-C19) model) that integrates different viral components of SARS-CoV-2 and different signaling pathways affected by the infection. The network represents the final result of the process of integration, refinement and manual modification of both the modules described above and the MacPol model. It contains a total of 131 nodes and 271 interactions, distributed as shown in Table 4.3. Most of the nodes come from the MacPol model representation (66 nodes), which keeps the polarization stimuli found in a pro- and anti-inflammatory microenvironment (with some modifications, e.g., TLR4 stimulus). Also, most of the signaling pathways derived from these stimuli have been conserved. However, cytokines, secreted molecules, and/or expressed receptors are now connected to nodes representing biological processes (5 nodes).

Table 4.3: **Graphical properties of the MacAct-C19 model.** Source or type of nodes inside the network are shown with the color in which they are presented, total number of nodes, and interactions coming from these nodes (edges). The last row shows the total number of nodes and interactions in the network (not a sum of the columns).

Source or type	Nodes	Edges
Type 1 IFN module	22	76
ACE/ACE2 axes module	13	32
NLRP3 module	10	26
Virus	15	32
Base model	66	199
Biological process	5	
TOTAL	**131**	**271**

As for the nodes coming from the previously assembled modules, it can be observed that the number of entities in the final model is lower than the original number for each module (see Table 4.2). This is because sometimes the modules shared entities (e.g., viral proteins), or presented entities that were already represented in the MacPol model, so they were not included twice. In addition, some entities were omitted from the modules to maintain the simplicity of the final model and to ease the evaluation. This is the case with TMPRSS2, an additional input in the ACE/ACE2 axes module that modulates the viral entry to the cell [143]. In the final MacAct-C19 it is assumed that TMPRSS2 is performing its proteolytic action on the ACE2-S-protein complex, avoiding the inclusion of further inputs in the model and simulations.

In terms of visualization, an attempt was made to retain the distribution and order presented in the MacPol model (stimuli at the top, signaling pathways in the middle, and secreted or expressed molecules at the bottom). However, most of the integrated nodes were grouped according to the module from which they originate. On the other hand, the viral entities (15 nodes) are distributed throughout the MacAct-C19 model and are not grouped with any module, so they present a different color (cyan with pink border). Yet, in the case of

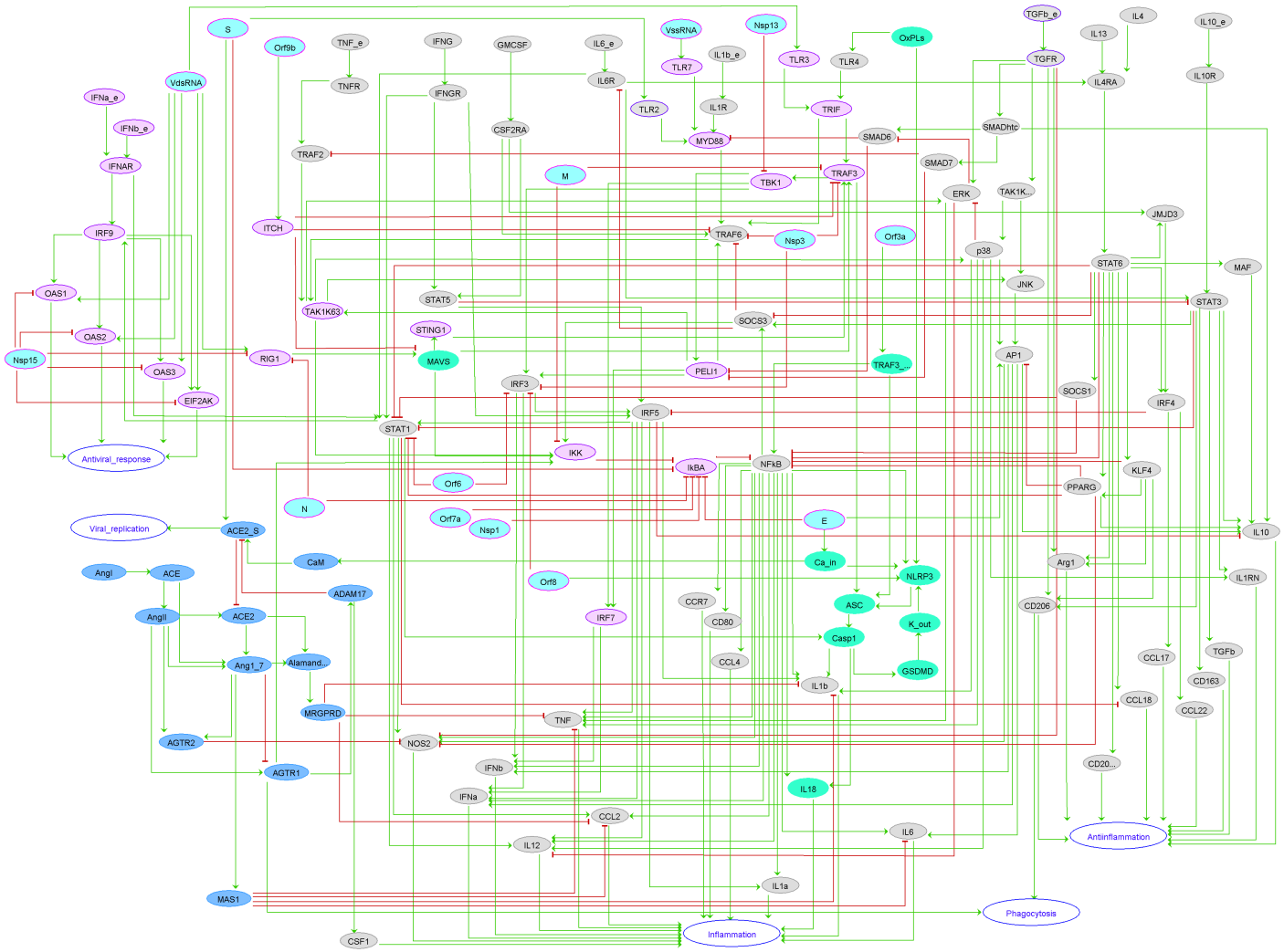

Figure 4.10: **Macrophage Activation in COVID-19 (MacAct-C19) model.** The network represents different pathways that lead to macrophage polarization and how they are affected by SARS-CoV-2 infection. Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows and negative are shown with red blunted arrows. White nodes with blue border: biological process. Cyan nodes with pink border: viral entities. Gray nodes: cellular nodes. Gray nodes with blue border: new receptors (TLR2 and TGF-β receptor). Blue nodes: ACE/ACE2 axes module. Green nodes: NLRP3 inflammasome module. Purple nodes: Type I Interferon module.

those viral entities that form a complex with a cellular entity (e.g. ACE2-S-protein complex), these are identified as cellular entities and are represented with the colors of the module to which they belong.

Finally, the MacAct-C19 model developed here is intended to illustrate the full picture of the internal changes that the macrophage undergoes when it encounters SARS-CoV-2 infection. The effects of those internal events triggered by the virus are reflected in the activated biological processes (inflammation, anti-inflammation, phagocytosis, antiviral response, and viral replication). Thus, rather than representing macrophage polarization, the model aims to show the pathological state to which the cell evolves after SARS-CoV-2 infection, both due to the internal viral infection and the external inflammatory microenvironment.

4.2.3 Pathological stable states and validation of MacAct-C19 model behavior

In order to evaluate the dynamic behavior and performance of the MacAct-C19 model, the complete model was subjected to a stable state analysis using the different conditions that take place during SARS-CoV-2 infection. The results are shown in Figure 4.11 as a heat map, similar to those presented in previous sections. Three conditions of stimulation (fixed points) and the combination between them were used to simulate the different pathological scenarios that the macrophage may face. In each of these conditions the MacAct-C19 model reached a different stable state (rows within the matrix). In the model, the polarization markers and the new output entities are connected to the biological process nodes (grouped in the final column), which facilitates the global interpretation of the stable states reached. However, the individual activity or state of the nodes (grouped by their relation to a signaling pathway or their relation to a macrophage phenotype) enables the understanding of the evolution towards these biological processes. This is why it is important to analyze the results as a whole.

Under the condition of inflammation, the system evolves to the corresponding biological process. Inflammatory stimuli activate signaling pathways associated with the inflammatory response in the macrophage, leading to the expression of M1 phenotype markers. All M1 markers are activated, except Colony Stimulating Factor 1 which requires ADAM17 activation (belonging to the ACE/ACE2 module). Also, the NLRP3 pathway, associated with the inflammatory response, is activated almost entirely. On the other hand, it is observed that most of the entities shared in the M1 and M2 phenotype (M1/M2 column) are active, and only JMJD3 of the anti-inflammatory signal is active. However, this is not sufficient to activate the anti-inflammatory response. First, among the active M1/M2 entities there are some that are shared only in the signaling pathway. Thus, as these entities are not released cytokines or expressed receptors, they do not cause effects on the activation of the final biological processes. The same happens with JMJD3, which is only a part of the signaling pathway but not a marker of a phenotype. Then, among the activated M1/M2 entities are also the cytokine IL-6 and the receptor IL-1Ra, which have a pleiotropic nature. These markers can

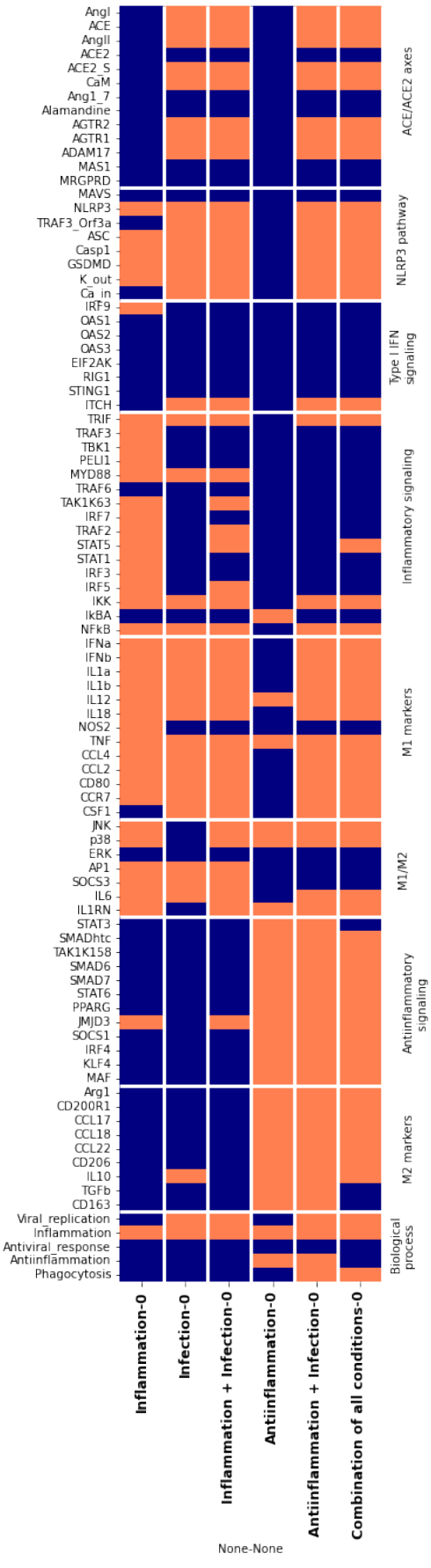

Figure 4.11: **Heatmap of the stable states reached under pathological conditions of stimulation in the MacAct-C19 model.** The system reaches one stable state after setting the respective fixed input. Therefore, each row represents a complete stable state with the stimulus indicated to the left of it. At the bottom, the names of the network entities are shown and within the matrix their state of activity or expression: active/high expression (value of 1) in coral; inactive/low expression (value of 0) in navy. At the top, entities are grouped by their relationship to a signaling pathway or their relationship to a macrophage phenotype (M1 and M2 markers). The M1/M2 column represents signaling pathways and pleiotropic markers. Finally, the last column groups the biological processes that result in the stable state, these are decisive to define the overall response to the pathological condition that is induced.

promote the anti-inflammatory response but individually are not sufficient to establish such a response in the macrophage. In this way, it can be concluded that the MacAct-C19 model is able to replicate the expected expression profile when the macrophage is in a purely inflammatory condition.

Under the condition of viral infection, the system evolves to an inflammatory response and viral replication. In this condition all viral entities in the fixed input are active, but not the cytokines that may be present in an inflammatory microenvironment. This is why, in principle, the signaling pathways associated with the inflammatory response should not be activated in the final stable state. However, almost all markers of the M1 phenotype are active. This is because viral entities can affect signaling pathways in the cell. For example, the viral TRAF3 - Orf3a complex is able to (1) mediate the activation of the transcription factor NF-kB (which is responsible for inducing almost all M1 markers), and (2) mediate the activation of the NLRP3 inflammasome. Similarly, viral protein E is able to induce the AP-1 complex (pleiotropic in nature) and activate other anti-inflammatory cytokines such as IL-6 and IL-10. However, as mentioned above, individually these would not be able to activate the anti-inflammatory response. Instead, more active anti-inflammatory markers are needed at the same time to induce this response. Another pathway affected is the ACE/ACE2 axes pathway, in which the formation of the viral S-protein - ACE2 complex promotes infection and causes the system to become unbalanced towards Ang II production and this contributes to the inflammatory response. The effect of SARS-CoV-2 is also reflected in the type 1 interferon signaling pathway, where most entities are blocked or inhibited by the virus. Still, IFNα and IFNβ production appears to be activated by other pathways (NF-kB and AP-1).This suggests that the virus is able to block interferon production by its main upstream regulators [148]. But the signal may be activated to a lesser extent by alternate pathways [149] and possibly interferon production is not sufficient to be effective, as seen in several studies [34, 65, 64]. Taken together, the stable state that results after a SARS-CoV-2 infection is as expected: the production of inflammatory cytokines leading to a cytokine storm and the silencing of the Type 1 interferon signaling pathway that reduces the secretion of this important second messengers. This is consistent with the cases of severe COVID-19, where complications arise from the disproportionate release of inflammatory cytokines coupled with inadequate induction of interferon signaling [150, 148].

When faced with the third condition: inflammation and infection, the system evolves to inflammatory response and viral replication. In this case the stimulus is the combination of the two previous fixed inputs: inflammatory cytokines and viral entities active in the microenvironment. As in the previous results it is observed that the viral entities affect several signaling pathways in the cell: the ACE/ACE2 axes pathway, the NLRP3 inflammasome, the type 1 interferon signaling pathway, and the inflammatory signaling pathways. In the first three cases, the reason for the results is explained in the previous paragraph. What is interesting in this stable state is the effect that the viral entities have on the inflammatory signaling pathways. Despite having the stimuli that activate them to begin with, the pathways are silenced at several points because of the effect of the viral entities. Even so, the M1 markers are activated almost entirely, promoting the inflammatory response and amplification of

the cytokine storm. As in previous findings, these are consistent with clinical evidence for hyperinflammation syndrome and Severe Acute Respiratory Syndrome (SARS) in the most severe cases of COVID-19 [36].

On the other hand, when evaluating the anti-inflammatory response, the system evolves to the corresponding biological process and also to the inflammatory response. Initially, the anti-inflammatory stimuli activate the corresponding signaling pathways and consequently all M2 markers in the MacAct-C19 model are activated. Thereby verifying that the model behaves appropriately to the provided stimulus. However, the inflammatory response is triggered as a consequence of the activation of Mitogen-activated protein kinase 11 (p38), an essential component in the shared M1/M2 pathways. This results in the expression of pro-inflammatory cytokines (IL-12 and TNFα), which can promote the inflammatory response. However, the amount of cytokines would only result in a mild inflammatory response. Thus, the anti-inflammatory signal would have more power in the final response and the MacAct-C19 model results are as expected in the given condition.

Now, when the MacAct-C19 model is subjected to the combined condition of viral infection and anti-inflammation, the system evolves into several biological processes: viral replication, inflammation, anti-inflammation, and phagocytosis. First, anti-inflammatory stimuli are able to induce the corresponding signaling pathways and thus fully activate M2 markers. In this condition, the process of phagocytosis is also induced, promoted by the expression of the specific membrane receptors CD206 and AGTR1. The former is induced by the signaling of the anti-inflammatory pathways [151] and the latter by the imbalance of the ACE/ACE2 axes pathway caused by the entry of the virus into the cell [152]. At the same time, viral entities are able to activate the inflammatory response and also initiate viral replication, as explained in the previous results. Through the ACE/ACE2 axes pathway, the NLRP3 inflammasome pathway, and the type 1 interferon signaling pathway, the virus is able to activate M1 markers. Furthermore, in this case there is a cross-talk between M1 and M2 signaling. Several entities of the anti-inflammatory pathways are able to silence their counterpart for effective resolution of inflammation. Compared to the single stimulatory condition of infection, it is observed that in this case several entities of the inflammatory signaling pathway are inactive. This reflects the cross-talk between the pathways. Yet, the model still results in an inflammatory cytokine profile. This suggests that, the anti-inflammatory microenvironment is not sufficient to induce an effective shift toward the anti-inflammatory response. Therefore, the result is as expected in the presence of viral infection and is consistent with the literature. SARS-CoV-2 induces mechanisms to evade the normal immune response (from inflammation to resolution) and deregulate cytokine production [153].

The final stable state results from the combination of all the above stimuli (inflammation, anti-inflammation, and viral infection). In this case the system evolves towards the biological processes of inflammation, viral replication and phagocytosis. In general terms this result can be understood by the analysis carried out in the previous paragraphs. Here, it is interesting that the anti-inflammation process is not activated when most of the M2 markers are active. This is because the Scavenger receptor cysteine-rich type 1 protein M130 (CD163) is key to defining the M2 phenotype and in this condition is inactive. CD163 activ-

ity is affected by the cross-talk that exists between the M1 and M2 pathways, where STAT5 activation (induced by GM-CSF and/or IFNγ stimulus) generates an inhibition on STAT3, responsible for inducing CD163. Even so, given the large number of active M2 markers, it could be concluded that the anti-inflammatory response is induced in this condition. However, as explained above, SARS-CoV-2 generates a dysregulation of the immune response that promotes viral replication and inflammatory cytokine production. So under this combination of stimuli, the MacAct-C19 model results in a mixed cytokine profile that could be associated with the transitional stage between inflammation and its resolution [28]. In this mixed scenario, the amount and timing of the stimulus present in the environment is critical to define the final phenotype of the macrophage and the biological process that is induced [41]. These two factors are not taken into account in the Boolean model. Even so, it can be interpreted that when adding the viral infection stimulus the result will lean toward a hyperinflammatory response. This result is consistent with multiple COVID-19 studies highlighted in the above analysis.

4.2.4 Cellular communication and Macrophage Activation under SARS-CoV-2 infection

The last step in this study was the evaluation of the functionality of the macrophage in connection with the rest of the immune system, especially the adaptive response. Assessment of the literature led to the selection of key processes that are summarized in Figure 4.12 and explained next. To begin with, several scenarios involving the macrophage can occur during SARS-CoV-2 viral infection. Epithelial cells in the lungs are the first to come into contact with the virus. Infection leads to pyroptosis which, by releasing multiple inflammatory molecules (PAMPs), activates neighboring cells [154]. Thus, activated alveolar macrophages generate inflammatory cytokines and chemokines in order to recruit more cells (monocytes, macrophages and T cells) to the site of infection and promote inflammation. Once here, the recruited macrophages can perform different functions. By recognizing apoptotic cells and other mediators of cellular stress, the process of phagocytosis is activated [155]. Also, during phagocytosis of infected cells, the macrophage can take up viral particles contained in the lysosomes. In this case, the macrophage engages antigen presentation and sets off the adaptive response. The activated cell expresses specific membrane receptors that allow it to migrate by a chemokine stream from the lungs to the lymph nodes. Here, the viral antigen is presented by the antigen presenting cell (macrophage) on major histocompatibility complex class I (MHCI) to naive CD8+ T lymphocytes [156, 157]. Back at the site of infection, macrophages can also be directly infected by the virus. This, in addition to activating the production of inflammatory cytokines, can lead to the fragmentation of viral particles in the cytosol. These fragments are processed by the cell and expressed in the major histocompatibility complex class II (MHCII) [158]. The activated cell migrates to the lymph nodes and presents the antigen to CD4+ T lymphocytes. Both activated CD4+ and CD8+ cells become specialized cells of the immune system with the goal of shutting down the viral infection [156]. CD8+ cells turn into cytotoxic T lymphocytes. Whereas, in the presence of IL12, CD4+

cells become helper T lymphocytes (Th1 cells). Meanwhile, macrophages at the site of infection express specific membrane receptors (Chemokine ligand 9 (CXCL9), and Chemokine ligand 4 (CCL4)) to direct the migration of activated lymphocytes from the lymph nodes to the site of infection. At the same time, the released cytokines TNFα and IL-8 participate in the chemokine gradient that enables the recruitment and subsequent activation of neutrophil cells [159, 160]. Taken together, under optimal conditions of the immune response, all these steps and processes allow the rapid clearance of the virus and stop the spread of damage in the lungs [154].

The processes described above were gathered together in a small module shown in Appendix I, that was integrated next into the MacAct-C19 model. Figure 4.13 shows the resulting Macrophage Activation and Cellular Communication in COVID-19 (MacAct-CC) model, with the most relevant inter-cellular interactions. This new version contains a new viral node called "SARS-CoV-2" that activates all the viral entities that were previously defined in the MacAct-C19 model. The purpose of this change was to facilitate the definition of fixed inputs of viral infection and the analysis of stable states. With respect to the other cells of the immune system, seven new inter-cellular communication nodes have been included. With respect to apoptotic cells that may be in the microenvironment, a new module was developed to represent the process of phagocytosis (see Appendix J). Also, four new internal nodes were created to connect with the cell communication nodes. First, the molecules MHCI and MHCII, crucial for antigen presentation and T cell stimulation. Then, the cytokine IL-8 that stimulates neutrophil cells, and Chemokine (C-X-C motif) ligand 9 (CXCL9) that recruits cytotoxic T cells (CTLs). Other entities mentioned in the previous explanation were already present in the MacAct-C19 model and were only connected to the new cellular communication nodes in the new MacAct-CC model.

Once the inter-cellular communication connections were established, the dynamic behavior and performance of the MacAct-CC model was evaluated through a stable state analysis. The results are shown in Figure 4.14 as a heat map, similar to those presented in previous sections. A new stimulation condition was used to evaluate the phagocytosis process. In this one, all other stimuli are at zero and only the apoptotic cell input is activated. In addition, the three fixed points defined for the previous analyses (infection, inflammation and anti-inflammation) were used. In this case, specific combinations were selected: the inflammation stimulus and viral infection (to represent the inflammatory scenario), the anti-inflammation stimulus and apoptotic cells (to represent the resolution scenario), and the combination of these two (to represent the transition between both). For each of these conditions the MacAct-CC model reached one different stable state (rows within the matrix). The analysis of the results follows the same logic used in the previous results: the global interpretation of the stable state and the activity or individual state of the nodes to explain the predictions of the model.

Under the condition of inflammation and infection, the system evolves to the biological process of viral replication and inflammation. This profile is consistent with that obtained in the stable state evaluation of the MacAct-C19 model. Both biological processes result in the production of inflammatory cytokines and several membrane receptors associated

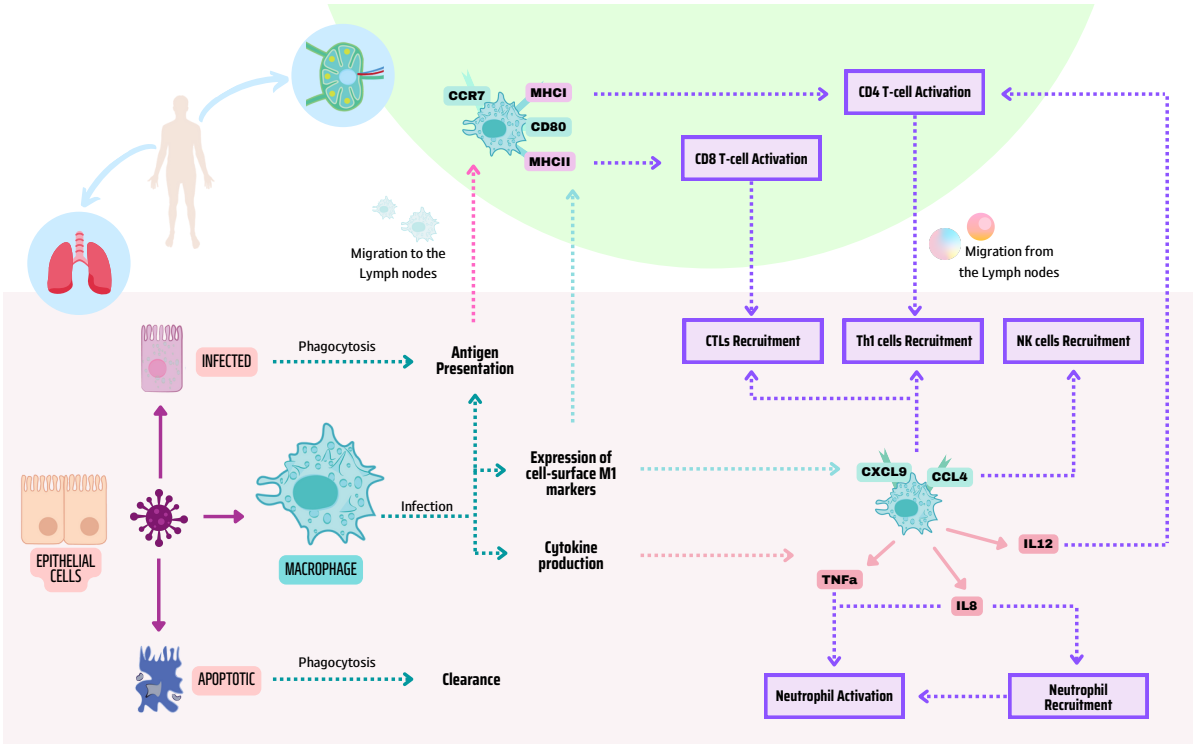

Figure 4.12: **Macrophage role in adaptive immune response during SARS-CoV-2 viral infection**. Following virus entry into the pulmonary epithelium, three scenarios involving the macrophage are depicted: (1) phagocytosis of apoptotic cells, (2) phagocytosis of infected cells, and (3) direct infection of the macrophage. The series of processes that are activated in each scenario are explained in the text. With respect to inter-cellular communication, the seven new communication nodes that have been integrated into the MacAct-CC model are shown in purple boxes. CTLs: cytotoxic T lymphocytes. Th1: helper T lymphocytes. NK: natural killer. MHCI: major histocompatibility complex class I. MHCII: major histocompatibility complex class II.

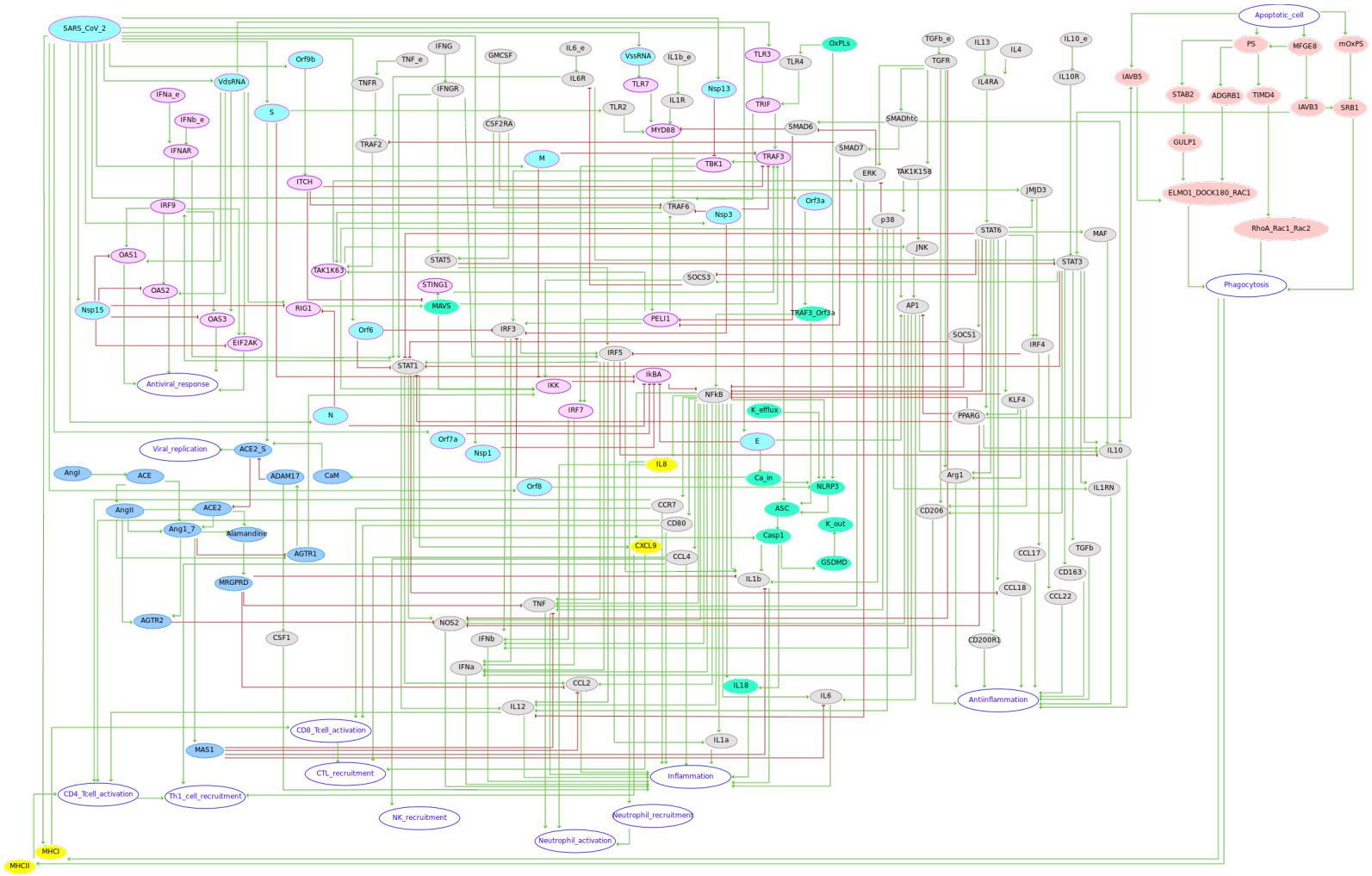

Figure 4.13: **Macrophage Activation and Cellular Communication in COVID-19 (MacAct-CC) model.** The interior of the network is the same as represented in the MacAct-C19 shown previously. The nodes represent entities in the network and the edges represent interactions. Positive interactions are shown with green arrows and negative interactions are shown with blunt red arrows. White nodes with blue border: biological process and inter-cellular communication. Cyan nodes with pink border: viral entities. Blue nodes: ACE/ACE2 axis module. Green nodes: NLRP3 inflammasome module. Purple nodes: type I interferon module. Yellow nodes: new internal nodes that function as bridges to the inter-cellular communication nodes. Light red nodes: phagocytosis module.

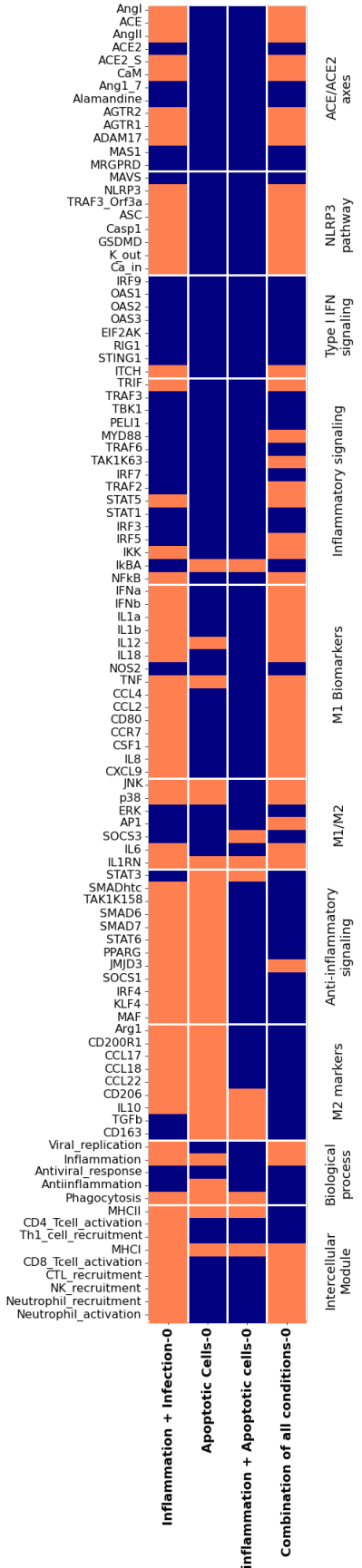

Figure 4.14: **Heatmap of the stable states reached under pathological conditions of stimulation of the MacAct-CC model.** The system reaches one stable state after setting the respective fixed input. Therefore, each row represents a complete stable state with the stimulus indicated to the left of it. At the bottom the names of the network entities are shown and within the matrix their state of activity or expression is indicated: active/high expression (value of 1) in coral; inactive/low expression (value of 0) in navy. At the top, entities are grouped by their relationship to a signaling pathway or their relationship to a macrophage phenotype (M1 and M2 markers). The M1/M2 column represents signaling pathways and pleiotropic markers. Finally, the biological processes column represent the cellular response of the macrophage to the stimulus. Whereas the inter-cellular module column represents the process activated by the macrophage in regards to inter-cellular communication.

with the inflammatory response. Among them, the release of TNFα and IL-8 has been reported at high levels in patients with COVID-19 [161]. These cytokines lead to neutrophil recruitment and activation. In addition, upon virus entry, the cell can fragment viral particles for presentation to CD8+ T lymphocytes via the MHCI complex [157]. Together with the expression of membrane receptors CCR7 and CD80 (co-stimulatory signal), this allows the activation of CD8+ T-cells in the lymph node. At the same time, expression of CXCL9 and CCL4 ligands by macrophages at the site of infection induces recruitment of differentiated lymphocytes (Natural killer (NK) cells and Cytotoxic T lymphocytes (CTLs)) to the site of infection. These chemokines have been reported at high levels in patients with COVID-19 and are thought to be crucial in the development of the cytokine storm [162]. Taken together, the MacAct-CC model predicts that in this condition the macrophage is able to activate the adaptive response. This is in agreement with reports from acute COVID-19 where high levels of cytotoxic cell markers (IFNγ, granzyme B, perforin, among others) and high production of neutrophil extracellular traps (NETs) have been detected [156]. Both cytotoxic lymphocyte and neutrophil responses are specific to eliminate infected cells and stop virus propagation. This is why the participation of the macrophage in the activation of these cells is crucial to stop the infection.

The next condition evaluated was the presence of apoptotic cells in the microenvironment without any other stimuli. This condition does not represent any scenario of the immune response, since in real conditions there would be a combination of several stimuli. However, the isolated stimulus allows us to evaluate the behavior of the module with respect to the rest of the network. In this case, the corresponding biological process - phagocytosis - is activated and with it the activation of the MHCI and MHCII complexes. However, the presentation of antigens in the lymph node mediated by these complexes and the activation of specialized immune cells is not enabled. These processes are dependent on the presence of inflammatory cytokines in the environment and specific membrane receptors on the macrophage [157]. Due to the absence of the inflammatory stimuli in the environment, the markers are not activated and the processes are not carried out. This is an indicator that the connections made with respect to inter-cellular communication are appropriate. On the other hand, the phagocytosis module induces the activation of STAT3, triggering the activation of markers of the M2 phenotype [163] (see Appendix J for details). This is consistent with activation of the resolution of inflammation, which includes phagocytosis-mediated clearance of apoptotic cells and other DAMPs in the microenvironment and the reprogramming to the anti-inflammatory phenotype [163]. Taken together, the module appears to work with the other pathways in the MacAct-CC model and the results are as expected: activation of phagocytosis and induction of an early anti-inflammatory macrophage.

Upon the combination of the anti-inflammatory stimulus and apoptotic cells, the MacAct-CC model evolves to the corresponding biological processes of anti-inflammation and phagocytosis, but also to the inflammatory response. In addition, the MHCI and MHCII complexes of the inter-cellular communication module are activated. As in the previous stable state, activation of the adaptive response does not take place in this case. This is due to the lack of expression of specific co-stimulatory receptors to mediate inter-cellular communication

[157]. Furthermore, it has been reported that antigen presentation does not take place when macrophages are polarized to the M2 phenotype [157]. Furthermore, activation of the inflammatory response in the stable state is due to the activation of some M1 phenotype markers (IL-12, TNFα, and IL-1Ra). However, as explained in similar results, this does not reflect the full activation of inflammation and the M1 phenotype. Rather, in contrast to the amount of activated M2 markers (due to the anti-inflammatory stimulus), it is possible to say that the activated response and phenotype leans much more to the anti-inflammatory profile. Thus, under the established stimuli, it can be concluded that the behavior of the MacAct-CC model is as expected and indicates that there is a progress towards the second phase of the normal inflammatory response (resolution and clearance).

Finally, the last stable state results from the combination of all the previous stimuli (inflammation, anti-inflammation, apoptotic cells and viral infection). Here the system evolves into a combination of several biological processes (inflammation, viral replication, and phagocytosis) and the activation of all the inter-cellular communication nodes. With the virus replication and phagocytosis process, the cell is able to process or take up viral antigens to be presented in the MHCI and MHCII complexes, respectively. Then, the inflammatory stimulus maintains a continuous flow of inflammatory cytokines into the environment and induces the expression of specific membrane receptors on the macrophage. This enables inter-cellular communication to activate and recruit the other specialized cells of the adaptive response. In particular, with the activation of the MHCII complex and the production of IL-12, the specific CD4+ T-cell response is activated. This is consistent with studies of SARS-CoV-2 where activation of these cells has been detected by measurement with T cell-specific antigens [164]. During viral infection, activation of CD4+ T cells is of great importance for infection control as they can differentiate into specialized cells (such as the Th1 lymphocytes included in the MacAct-CC model) [164]. The differentiated cells can carry out antiviral activities, promote tissue repair, and enhance protective immunity by their relationship with other cells of the adaptive response. Taken together, the MacAct-CC model appears to match the inter-cellular communication processes that take place during viral infection.

Furthermore, the combination of stimuli results in a profile of markers that can be associated with both M1 and M2 phenotypes. However, as explained above, in the face of a mixed stimulus the polarization of the macrophage will depend on the amount and timing of the stimulus. The prediction of this stable state should not be interpreted such that the macrophage will express all of these markers. Instead, it could be interpreted that, under these stimuli, the macrophage population could differentiate into several separate phenotypes and carry out various biological processes. Thus, the diversity of phenotypes would be able to contribute to the inflammatory response in multiple ways. Inflammatory stimuli and viral infection would polarize the population toward the M1 phenotype and engage in the adaptive response. Whereas anti-inflammatory stimuli and apoptotic cells would polarize the population towards the M2 phenotype and trigger tissue resolution and clearance.

In conclusion, the results indicate that the MacAct-CC model is able to correctly reflect macrophage behavior with respect to selected multi-cellular interactions. Under a multi-cellular perspective, the model is able to reflect interactions with other specialized cells ca-

pable of initiating the adaptive response. Furthermore, the activated intra-cellular processes go hand in hand with the inter-cellular communication that can be established in each scenario evaluated. Globally, the MacAct-CC model is thus able to predict the role that the macrophage may play during SARS-CoV-2 infection.

4.3 Concluding remarks

4.3.1 PART I: Macrophage polarization under immune stimulation

- Overall, the MacPol model has a greater level of detail in the signaling pathways because of the extensive effort to add more regulatory entities, 'actionable targets', and biomarkers routinely monitored in experimentation. As a result of the manual validation, the MacPol model provides a much better basis to support experimentation of the Johansen group than the initial Palma model. The integration of additional biomarkers and the refinement of Boolean functions with updated literature is key to following and understanding the effects of regulations in the cells. By comparing the stable states of the MacPol model with, for example, gene expression data, it is possible to correlate the result of in silico stimuli with the experimental result (phenotype or gene expression profile). Taken together, the MacPol model is (1) more complete, (2) constitutes a next step in representing the biological reality of macrophages, and (3) can help to understand the behavior of the cells both in general polarization or specific conditions.

- The steady state behavior of the MacPol model under polarization stimuli is consistent with the behavior described in the experimental observations of macrophage studies. The model replicated the expected profiles of markers after the stimulation with either pro- or anti-inflammatory molecules. In particular, combining either of the stimulus with PGE2 drives the MacPol model to a global anti-inflammatory state, with the expression of both M2a and M2c markers. Specifically, the stimulation with IFNγ and LPS together with PGE2 suggests that the cell passes through an M1 state before it reaches the M2 configuration. This highlights the importance of PGE2 and lipid metabolism in the polarization of macrophages. Additionally, it highlights the importance of interconnected pathways inside the cell which can inhibit the expression of genes and proteins even though their corresponding signaling pathways get activated.

- The inconsistencies in the MacPol model predictions when replicating perturbed experimental conditions highlight two facts: (1) the complex behavior of macrophages, and (2) the complex task of manually discretizing continuous experimental observations to binary data sets (used to compare model results). To begin with, genes in a cell are always expressed at the basal level. Depending on the strength and timing of the stimulus present in the environment, the activity and expression levels of the entities in the cell may change. Thus, macrophages may exhibit different phenotypic characteristics of polarized subtypes depending on these two factors. However, these variables cannot be represented in the MacPol model with the current simulation tools.

Only a profile of all-or-nothing can be obtained. In this sense, the continuous expression of the entities in the in vivo macrophage are not represented in the MacPol model. It is for this reason that the integration of these variables in future model simulations should lead to a better interpretation of the system. Furthermore, the inconsistencies found may also lead to the design of new experiments to clarify these observations.

- In particular, the MacPol model developed in this study will be implemented by the Johansen research group at NTNU in support of their research on macrophages. Initially, the network itself and the generated predictions will be used to determine the markers of interest in the overall polarization of the M1, M2a and M2c phenotype. Next, the model will contribute to the interpretation of macrophage behavior under special conditions, as well as the use of inhibitors. To refine the study, more lipid metabolism entities can be integrated into the MacPol model, and popular or new drug candidates can be included to predict synergies. In this way, the above analyses can be used to propose hypotheses and solutions in the investigation of various inflammatory diseases in which macrophages play a role.

4.3.2 PART II: Macrophage activation under SARS-CoV-2 infection

- The integration of specific SARS-CoV-2 modules and the modification of some features of the MacPol model presented in the first part has allowed the generation of a specific pathological model. The new MacAct-C19 model allows to evaluate the macrophage cell fate in terms of phenotype polarization, but also with respect to its role in the immune response during viral infection. Thus, the effects of SARS-CoV-2 on the macrophage can be seen at the macro level as well as at the micro level.

- At the intra-cellular level in the MacAct-C19, it could be identified that viral entities constantly affect inflammatory signaling pathways. Even in the absence of inflammatory stimuli, the virus is able to activate most of the M1 phenotype markers due to its effect on the transcription factor Nuclear Factor kappa B (NF-kB). Activation of NF-kB results from the formation of the TRAF3 and Orf3a complex which, in turn, induces the formation of the NLRP3 inflammasome. The effect on these two pathways results in a constant inflammatory state in the macrophage. Thus, the cytokines released by this phenotype contribute to the cytokine storm and the Macrophage Activation Syndrome observed during acute COVID-19. Another pathway affected by the virus is Type 1 Interferon signaling. Although the MacAct-C19 model predicts the production of these molecules by alternative pathways (NF-kB and Adaptor protein complex AP-1), the main pathway is inhibited by the virus. These results are consistent with the low interferon production observed during acute COVID-19. Finally, the virus uses the Angiotensin-converting enzyme 2 receptor as a means of cell entry and infection. This not only promotes virus propagation, but also generates an imbalance of Renin–angiotensin system that potentiates the inflammatory response.

- At the inter-cellular level in the MacAct-CC, the model is able to represent the communication processes between the innate and adaptive responses of the immune system. The stable states in which this model results highlight the ability of the macrophage to generate diverse responses depending on the stimuli involved. Each of the profiles can be associated with a specific scenario during COVID-19 development. The definition of stimuli and the predictions of the MacAct-CC model can be used to understand the changes that occur during the immune response and the role of the macrophage in this response.

- Finally, the steps performed in this project can be taken as a guide for the expansion of logical models and the conversion of these into pathological models. The work done shows the diversity of systems biology resources (databases, modeling and data analysis tools) and the great use that can be made of them. In principle, it is advisable to have a base model. But also, having a repository of information about the specific disease is crucial to include the particular responses of the cell to the pathology. The combination of both elements in this work allowed the generation of a logical model of a specific pathology on a specific cell. However, the steps can be used to evaluate other pathologies and other cells of interest.

Chapter 5

Conclusions and Recommendations for Further Work

5.1 Summary

The work presented here has been divided into two parts due to its evolution over time. When the objectives of the first part were completed, the Coronavirus disease of 2019 (COVID-19) pandemic arose and this motivated the extension of the analysis on the effects of Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) on macrophages. In both parts a logic model has been refined and expanded in order to generate a tool that can be used and integrated in macrophage research and experimental workflows. In the first part, a Macrophage Polarization (MacPol) model has been developed to support experimental work on lipid metabolism in the Johansen laboratory at NTNU. This model integrates key entities of lipid metabolism and signaling pathways important for macrophage polarization. In the second part, a Macrophage Activation in COVID-19 (MacAct-C19) model has been developed to interpret macrophage behavior and dynamics in response to SARS-CoV-2 infection. This model will serve as a tool in COVID-19 research and will be included in the COVID-19 Disease Map repository of SARS-CoV-2 virus-host interaction.

In the first part of the project, a logical model of the signaling events that regulate the activation of macrophages was refined and expanded from a previously published model - the model of Palma, *et al.* (2018). The Macrophage Polarization (MacPol) model aims to represent the most recent findings in macrophage polarization studies, and also integrate additional biomarkers and key entities involved in the lipid metabolism. The work resulted in the addition of a total of 38 nodes and 82 regulatory interaction to the initial model. The new MacPol model comprises a total of 68 nodes and 132 manually validated interactions from the literature. The stable states reached by the model were analyzed and compared with available experimental observations. Under polarization conditions using pro- and anti-inflammatory stimuli, the stable states reached were correctly related to the profiles of expression or level of marker activity described in the literature for phenotype M1, M2a and M2c. That is, the stable states reached by the MacPol model are compatible with previous experimental observations. This suggests that the model is capable of making predictions

consistent with the experimental behavior of macrophages. Finally, 23 different experimental conditions from macrophage studies were recreated in the model and a stable state analysis was performed. Observed stable states were compared with the experimental observations of the different studies. The conditions included the use of inhibitors, gene knockouts, gene constitutive expression, and pre-treatment with molecules that affect the polarization of macrophages. Of the stable state results, 15 were consistent with the experimental observations from the studies, while eight had some inconsistencies. Mainly, differences were related to the fact that the MacPol model only represents the complete activation of the nodes (all or nothing) upon the corresponding stimulus. Therefore, it is not well equipped to replicate constant or basal levels of expression of the nodes - which is sometimes seen in the experimental observations. Other discrepancies can be attributed to details of the experimental designs, including used thresholds, cell lines, and polarization conditions. Nevertheless, the analysis and results are promising and provide a solid basis for further refining the MacPol model. In this sense, the MacPol model provides a tool for the identification of crucial entities and regulations in macrophage polarization, such as the role of PGE2 in the polarization of the global M2 phenotype.

In the second part of the project, a logical model of the signaling events that may be affected by the viral components of SARS-CoV-2 in the macrophage was assembled. The intention was to represent the pathological states that may develop during infection and to identify the key factors that lead to the critical state of inflammation in severe cases of COVID-19. The MacPol model produced in the first part of this work was taken as a basis. This model was expanded and modified using curated information from the Disease Map repository and relevant literature. The resulting Macrophage Activation in COVID-19 (MacAct-C19) model had a total of 131 nodes and 271 interactions, including three modules specific to SARS-CoV-2 infection and five biological process output nodes. Additionally, seven nodes representing cellular communication were added to expand the analysis in a multi-cellular approach. This resulted in the Macrophage Activation and Cellular Communication in COVID-19 (MacAct-CC) model, with a module of phagocytosis and interactions of cellular communication. Then, to determine the dynamics of both models, they were tested under three conditions of stimulation (and their combination) common in the immune response and viral infection: inflammation, viral infection, anti-inflammation, and apoptotic cell stimulus (only in the MacAct-CC model). The stable states were determined and the resulting expression profile predictions were compared with previous experimental and clinical evidence. Given the established conditions both models, MacAct-19 and MacAct-CC, evolve to display active states of the expected biological processes and the adaptive immune response - trigger by inter-cellular communication. The predicted cytokine and biomarker profiles were correctly explained by the literature. In both models the viral entities induce inflammatory cytokine production, silence interferon production and promote viral replication. This result is consistent with clinical evidence from severe COVID-19 cases. Thus, the overall performance of both models is optimal under the established pathological conditions and may be used to validate and generate new advances in drug therapy design and basic research of viral infection processes. New conditions and signaling pathways can be added, as

well as drugs that can generate a change in the final pathological state of the macrophage.

The work done in this study will lead to a more precise interpretation of the mechanisms and regulations that act together in macrophage polarization and behavior. The experimental observations can be explained when the elements and interactions of the system are considered as a complete dynamic entity. Especially in the case of macrophages, where their activity and function are constantly dependent on the conditions of the microenvironment. With the tool generated in this work, complex processes and conditions, such as macrophage reprogramming and pathological environments, can be represented. The expected results can then (1) be used to provide answers to experimental observations, (2) provide help in the design of new experiments, and, especially, (3) could indicate new potential drug targets and drug effects.

5.2 Recommendations for Further Work

The function and phenotype of macrophages depend on the conditions of the microenvironment, and macrophages themselves contributes to that. Mainly, the cytokines produced and released by the cell can affect that same macrophage population. Entities that contribute to this behavior were represented in the MacPol model both as a stimulus and as a secreted product, e.g. IL-6 and PGE2. However, some of them were only represented as secreted products, and the integration of their signaling function as a stimulus did not take place due to the limitations of the available project time. This is the case for the cytokines TGF-β and TNFα, represented only as secreted products. The TGF-β signaling plays a role in the polarization of the M2 phenotype by controlling the expression of genes characteristic of the anti-inflammatory response [165]; while the TNFα signaling plays a role in the polarization of the M1 phenotype by orchestrating the inflammatory cytokine cascade in many inflammatory diseases. Given their importance in macrophage polarization and the development of pathological conditions, their integration as stimuli (input nodes) in the MacPol model is recommended.

Considering further the consequences of a continuously changing microenvironment, the definition of pure M1 and M2 phenotypes does not exist under real conditions. Instead, the cell activation varies along a spectrum of continuous stimuli in a process called macrophage reprogramming [17]. Starting from a pro-inflammatory early stage that activates M1 macrophages to an anti-inflammatory later stage that activates M2 macrophages, the conditions in the environment are constantly changing. Factors like the type, time of exposure, and amount of cytokines present act simultaneously under in vivo conditions - which affects the expression of markers. Consequently, the macrophage population is a mix of many hybrid states. This process is important for the adequate functioning of macrophages in a away that the tissue needs it and even the functioning of the immune response [17]. Their dysregulation is what gives rise to the development of pathologies. Therefore, from a therapeutic perspective, the integration of the reprogramming process in the MacPol model is recommended.

The study of the reprogramming process can be carried out with model simulations and

stable state analysis. Specifically, instead of starting from a resting state, the simulations should be performed from a polarized state and then new stimuli should be defined. The study must also include the evaluation of the ratios of polarized macrophages in different conditions, since the macrophage population is a mix of many hybrid states. Finally, the variable time and amount of cytokines should be included. To conduct these analyses, a review of available methodologies must be performed in order to select the most appropriate ones. Initially, the use of the MaBoSS software - which applies the kinetic Monte-Carlo algorithm (or Gillespie algorithm) to Boolean networks - [166] is recommended because estimation of time evolution probabilities, simulations of mutations, drug treatments, and sensitivity analyses can be performed with this tool.

On the other hand, SARS-CoV-2 remains a topic of great interest in scientific and medical research. By the time the construction of the MacAct-C19 model was stopped, many more studies have been published regarding the integrated modules. More information is now available that can be used to refine the interactions and regulations established in the network. For example, SARS-CoV-2 uses specific membrane proteins to efficiently enter the cell, such as Transmembrane protease serine 2 (TMPRSS2), furin-like enzymes, and the neuropilin-1 receptor (NRP1) [167, 61]. However, it is recommended to investigate the cell specificity of new entities before integrating them into the model. As a starting point, it is suggested to use the information contained in the Disease Map repository of SARS-CoV-2 virus-host interaction [87], since this has been validated by experts in the scientific community.

It would also be relevant to include in the MacAct-C19 model drugs that are currently being used or are being suggested to treat SARS-CoV-2 infection. In this way, the MacAct-C19 model could be used to evaluate whether there are synergies between different drugs and/or between natural stimuli of the inflammatory response. This approach was continued by Marco Fariñas for his MSc thesis project [91]. However, to date, more drugs and combinations have been suggested that could prevent the development of acute COVID-19. For example, inhibitors of molecules that allow virus entry into the cell (ACE2), or inhibitors of entities that induce the constant production of inflammatory cytokines (AGTR1) [153] have been described.

Additionally, one of the signaling pathways that was not extensively modeled is that of the Fcγ receptors. Although it was briefly included in the MacPol model, with the High affinity immunoglobulin gamma Fc receptor I (FCGR) node, due to the complexity and diversity presented by this group of receptors and the time frame set for the completion of this project, it was not included in the MacAct-C19 model. However, recent studies in SARS-CoV-2 suggest that this group of receptors (expressed in the macrophage) seems to have a great influence in the activation of the NLRP3 inflammasome and the development of the cytokine storm [168]. It is therefore recommended to further refine these receptors, their respective stimulus, and signaling pathway. Initially, it is suggested to build a small module with all this information. Then, it can be included in the MacAct-C19 model and the respective stable state analyses can be carried out.

Finally, although several ways of inter-cellular communication between the macrophage

and other cells of the immune system were considered for the MacAct-CC model, many more remain to be included. However, due to the size of the model constructed in this project, it may be advantageous to continue the expansion of inter-cellular communication in a separate model. In this way fewer details of the macrophage internal regulatory network could be considered and more interactions with the innate and adaptive response can be included. Furthermore, it is recommended to focus the expansion on the effect that viral entities have on inter-cellular communication. The MacAct-CC model constructed in this project only represents an optimal scenario of cellular communication, but recent studies suggest that the virus may limit these interactions. For example, antigen presentation at the major histocompatibility complex class I (MHCI) is inhibited by several SARS-CoV-2 mechanisms [169]. Thus, the response of the adaptive system may be limited by the the virus preventing the activation of CD8+ T cells. Also, the virus may block the response of the CD8+ T cells by inducing their apoptosis or causing their exhaustion [156, 164]. Finally, a model that includes these features could be relevant in a multi-cellular modeling framework. Thus, the research could be continued in collaboration with researchers such as Laurence Calzone and Vincent Noël from the Institut Curie - who use the PhysiBoSS tool that integrates spatial-temporal dimensions and the dynamical interactions between different cells [170].

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Appendix A

List of nodes

Table A.1: **Annotations of the nodes in the expanded MacPol model.** The formal name of the node, the node type, and entry in the UniProt database are shown. Some nodes have several entries in the database, while others do not have any (NA) because of the type of node, e.g. protein complex or ion, respectively.

Node	Name	Type	Entry
AP1	AP-1 complex	Protein complex	NA
Arg1	Arginase-1	Enzyme	P05089
Ca2	Calcium (2+)	Ion	NA
CCL17	C-C motif chemokine 17	Chemokine	Q92583
CCL18	C-C motif chemokine 18	Chemokine	P55774
CCL2 / MCP-1	C-C motif chemokine 2	Chemokine	P13500
CCL22	C-C motif chemokine 22	Chemokine	O00626
CCL4 / MIP-1b	C-C motif chemokine 4	Chemokine	P13236
CCR7	C-C chemokine receptor type 7	Protein	P32248
CD163	Scavenger receptor cysteine-rich type 1 protein M130	Protein	Q86VB7
CD200R	Cell surface glycoprotein CD200 receptor 1	Protein	Q8TD46
CD206 / MRC1	Macrophage mannose receptor 1	Protein	P22897
CD80	T-lymphocyte activation antigen CD80	Protein	P33681
cMaf	Transcription factor Maf	Transcription factor	NA
COX2 / MTCO2	Cytochrome c oxidase subunit 2	Protein	P00403

Table A.1 – *Continued from previous page*

Node	Name	Type	Entry
cPLA2	Cytosolic phospholipase A2	Enzyme	P47712
CSF2Ra	Granulocyte-macrophage colony-stimulating factor receptor subunit alpha	Protein	P15509
EP4R	Prostaglandin E2 receptor EP4 subtype	Protein	P35408
ERK	Extracellular signal-regulated kinase (ERK) / Mitogen-activated protein kinase (MAPK)	Enzyme	P27361; P28482
FcgR	High affinity immunoglobulin gamma. Fc receptor for IgG (FcγRs)	Protein family	NA
GMCSF_e	Granulocyte-macrophage colony-stimulating factor external stimulus	Cytokine	P04141
IC_e	Immune complexes external stimulus	Complex	NA
IFNa	Interferon alpha-2	Protein	P01563
IFNb	Interferon beta	Protein	P01574
IFNg_e	Interferon gamma external stimulus	Protein	P01579
IFNgR	Interferon gamma receptor	Protein complex	P15260; P38484
IL10_e	Interleukin-10 external stimulus	Cytokine	P22301
IL10_out	Interleukin-10 output	Cytokine	P22301
IL10R	Interleukin-10 receptor	Protein complex	Q13651; Q08334
IL12_out	Interleukin-12	Cytokine	P29459; P29460
IL13	Interleukin-13	Cytokine	P35225
IL1a	Interleukin-1 alpha	Cytokine	P01583
IL1b	Interleukine 1 beta	Cytokine	P01584
IL1b_e	Interleukine 1 beta external stimulus	Cytokine	P01584
IL1R	Interleukin-1 receptor type 1	Protein	P14778
IL1Ra	Interleukin-1 receptor antagonist protein	Protein	P18510
IL4	Interleukin-4	Cytokine	P05112
IL4RA	Interleukin-4 receptor subunit alpha	Protein	P24394
IL6	Interleukin-6	Cytokine	P05231
IL6R	Interleukin-6 receptor	Protein complex	P08887; P40189

Table A.1 – *Continued from previous page*

Node	Name	Type	Entry
iNOS / NOS2	Nitric oxide synthase, inducible	Enzyme	P35228
IRF3	Interferon regulatory factor 3	Transcription factor	Q14653
IRF4	Interferon regulatory factor 4	Transcription factor	Q15306
IRF5	Interferon regulatory factor 5	Transcription factor	Q13568
JMJD3	Lysine-specific demethylase 6B	Enzyme	O15054
JNK / MAPK8	Mitogen-activated protein kinase 8	Enzyme	P45983
KLF4	Krueppel-like factor 4	Transcription factor	O43474
LPS_e	Lipopolysaccharide external stimulus	External stimulus	NA
MNK1 / MKNK1	MAP kinase-interacting serine/threonine-protein kinase	Enzyme	Q9BUB5
NFkB	Nuclear factor NF-kappa-B	Transcription factor complex	P19838; Q00653
P2R	Purinergic P2 receptors (P2RX4 and P2RX7)	Protein	Q99571; Q99572
p38	Mitogen-activated protein kinase 11	Transcription factor	Q15759
PGE2 / PTGES2	Protaglandin E2	Enzyme	Q9H7Z7
PGE2_e	Prostaglandin E2 external stimulus	Prostanoid	NA
PPARg	Peroxisome proliferator-activated receptor gamma	Protein	P37231
RelA	Transcription factor p65	Transcription factor	Q04206
SOCS1	Suppressor of cytokine signaling 1	Protein	O15524
SOCS3	Suppressor of cytokine signaling 3	Protein	O14543
STAT1	Signal transducer and activator of transcription 1	Transcription factor	P42224
STAT3	Signal transducer and activator of transcription 3	Transcription factor	P40763
STAT5	Signal transducer and activator of transcription 5	Transcription factor complex	P42229; P51692
STAT6	Signal transducer and activator of transcription 6	Transcription factor	P42226
TBK1	Serine/threonine-protein kinase TBK1	Transcription factor	Q9UHD2
TGFb	Transforming growth factor beta-1 proprotein	Protein	P01137

Table A.1 – *Continued from previous page*

Node	Name	Type	Entry
TLR4	Toll-like receptor 4	Protein complex	O00206
TNFa	Tumor necrosis factor alpha	Protein	P01375
TRAF6	TNF receptor-associated factor 6	Protein	Q9Y4K3

Appendix B

List of interactions

Table B.1: **Annotations of the interactions in the expanded network.** The source node, interaction type, and target node are shown with the PubMed ID reference (PMID) that was used to validate the interaction. PMIDs are marked with an asterisk (*) when the literature does not refer to macrophages, rather the interaction has been established in other cell lines. Additionally, the "Network" column shows if the interaction belongs to the selected Palma model (PKN) or if it was added into the MacPol model (New).

Source	Type	Target	PMID	Network
IFNg_e	Positive	IFNgR	23898330	PKN
IL10_e	Positive	IL10R	10347215	PKN
IL4_e	Positive	IL4Ra	18852293	PKN
STAT6	Positive	SOCS1	17093501	PKN
LPS_e	Positive	TLR4	9851930	PKN
STAT6	Positive	KLF4	22378047	PKN
STAT5	Negative	STAT3	*23716595	PKN
STAT3	Negative	STAT1	*25921060	PKN
STAT1	Positive	IL12_out	19029990	PKN
SOCS1	Negative	STAT1	16628196 28901399	PKN
PPARg	Positive	IL10_out	*17681149	PKN
PPARg	Negative	NFkB	18276926	PKN
NFkB	Positive	IL1b	29158945 20975042	PKN
KLF4	Negative	NFkB	22378047 21670502	PKN
IFNgR	Positive	STAT1	23898330	PKN
IL1b_e	Positive	IL1R	20086235	PKN
IC_e	Positive	FcgR	18064051	PKN

Table B.1 – *Continued from previous page*

Source	Type	Target	PMID	Network
IL4Ra	Positive	STAT6	23124025	PKN
IL10R	Positive	STAT3	12626585	PKN
NFkB	Positive	IL12_out	7565674	PKN
GM-CSF_e	Positive	CSF2Ra	11867689	PKN
JMJD3	Positive	IRF4	27525438	PKN
CSF2Ra	Positive	JMJD3	27525438	PKN
FcgR	Negative	TRAF6	31057544 19734236 28439271	PKN
LPS_e	Positive	FcgR	*18064051	PKN
IRF3	Positive	IFNb	31024544	PKN
FcgR	Positive	ERK	20670655	PKN
ERK	Positive	IL10_out	20670655	PKN
CSF2Ra	Positive	STAT5	7716810 25506346	PKN
cPLA2	Positive	COX2	12841340	New
EP4R	Positive	STAT3	16818766	New
ERK	Positive	COX2	12234923	New
p38	Negative	ERK	10586030	New
NFkB	Positive	CD80	*12860928	New
IL6R	Positive	STAT1	21752694	New
NFkB	Positive	CCL2	9916692	New
TRAF6	Positive	p38	15385464 25700345	New
CSF2Ra	Positive	TRAF6	15385464	New
TRAF6	Positive	JNK	25700345 9395283	New
TNFa	Positive	RelA	9916692	New
TLR4	Positive	ERK	17507094	New
TBK1	Positive	IRF3	17275323	New
TLR4	Positive	TRAF6	17275323	New
NFkB	Positive	CCR7	*22158872	New
STAT1	Positive	IL1b	17032168	New
PGE2_e	Positive	EP4R	23523686	New
SOCS1	Negative	RelA	14690596	New
ERK	Positive	MNK1	18032482	New
p38	Positive	MNK1	18032482	New
STAT5	Positive	IRF5	25506346	New
SOCS3	Negative	IL6R	12626585 21752694	New

Table B.1 – *Continued from previous page*

Source	Type	Target	PMID	Network
IFNgR	Positive	IRF5	21240265	New
EP4R	Positive	p38	10762076	New
STAT6	Negative	NFkB	9786931	New
STAT6	Negative	STAT1	9786931	New
STAT1	Positive	CCL2	28391993	New
SOCS3	Negative	TRAF6	16543409	New
SOCS3	Positive	RelA	24088176	New
NFkB	Positive	CCL4	29669317	New
p38	Positive	AP1	24771982	New
NFkB	Positive	COX2	17114486 9296354	New
NFkB	Positive	iNOS	11134171	New
MNK1	Positive	cPLA2	10978317	New
TLR4	Positive	TBK1	17275323	New
IFNgR	Positive	STAT5	32142888	New
AP1	Positive	IL6_Out	20086235	New
IRF5	Positive	IL12_out	21240265	New
IRF5	Negative	IL10	25159141 *20237317	New
IRF3	Positive	IRF5	29361124	New
IRF5	Positive	STAT1	26366410 21240265	New
IRF5	Positive	RelA	*20237317 25159141	New
IRF5	Positive	IFNa	*11303025	New
NFkB	Positive	TNFa	20237317 25159141 25506346	New
AP1	Positive	COX2	17114486	New
NFkB	Positive	CCL2	20086235 9916692	New
IRF4	Negative	IRF5	22378047 20580461	New
STAT3	Positive	SOCS3	12626585	New
NFkB	Positive	IL1a	25159141	New
NFkB	Positive	SOCS3	19643666	New
IRF3	Positive	IFNa	31024544	New
NFkB	Positive	IL6	25159141	New
IRF5	Positive	IL1b	*20237317	New
STAT1	Negative	CCL18	10671296	New
p38	Positive	IL1Ra	11877429	New

Table B.1 – *Continued from previous page*

Source	Type	Target	PMID	Network
RelA	Positive	NFkB	9450761 20086235	New
TRAF6	Positive	RelA	16543409	New
ERK	Negative	IL12_out	16394015 10586030	New
STAT6	Positive	PPARg	28804688	New
KLF4	Positive	PPARg	21670502	New
STAT6	Negative	SOCS3	28391993	New
cMaf	Positive	IL10_out	15749884	New
PPARg	Negative	AP1	18276926	New
PPARg	Negative	STAT1	18276926	New
STAT6	Positive	CD200R	27742835	New
KLF4	Positive	Arg1	21670502	New
IRF4	Positive	CCL22	27007158	New
IL6R	Positive	IL4Ra	24681566	New
STAT6	Positive	JMJD3	19567879	New
STAT6	Positive	CCL18	27788604	New
PPARg	Negative	iNOS	11595817	New
STAT6	Positive	cMaf	15749884	New
IL13_e	Positive	IL4Ra	12704343	New
STAT3	Positive	CD206	*29152078	New
STAT3	Positive	IL10_out	*29152078	New
STAT3	Positive	TGFb	*29152078	New
IL6R	Positive	STAT3	12626585	New
IRF4	Positive	CCL17	27525438	New
KLF4	Positive	CD206	21670502	New
STAT3	Positive	CD163	*29152078	New
STAT6	Positive	Arg1	21670502	New
STAT3	Positive	IL1Ra	20032313	New
STAT6	Positive	IRF4	29152078	New
IL6_e	Positive	IL6R	18923185	New
IL1R	Positive	TRAF6	16543409	New
IRF5	Positive	IL1a	25159141	New
COX2	Positive	PGE2	12841340	New
p38	Positive	IL12_out	10202148	New
Ca2	Positive	cPLA2	9525925	New
cPLA2	Positive	PGE2	12841340	New
PPARg	Negative	COX2	12740443 20508742	New
EP4R	Positive	STAT6	29374192	New
ERK	Positive	TNFa	9921718	New

Table B.1 – *Continued from previous page*

Source	Type	Target	PMID	Network
EP4R	Negative	ERK	23523686	New
EP4R	Negative	NFkB	23523686	New
AP1	Positive	IL10	22428854	New
STAT1	Positive	iNOS	11310846	New
P2X4/7R	Positive	Ca2	30943393	New
ERK	Positive	cPLA2	*16176925	New
JNK	Positive	cPLA2	19625654	New
NFkB	Positive	cPLA2	*21520062	New
p38	Positive	cPLA2	*16176925	New
IFNgR	Positive	P2X4/7R	30943393	New

Appendix C

List of Signaling pathways trigger by the receptors

Table C.1: **Signaling molecules and pathways below the receptor level in macrophages.** Some of the receptors represented in the MacPol model are shown; each receptor with its adaptive molecules and then the first and second molecules of each signaling cascade that is activated by that receptor. The function or outcome that triggers the signaling cascade in the cell is also shown. Finally, the column "MPM" indicates if that pathway is represented in the MacPol model. Only the signaling pathways related to the immune response are included in the network representation. The "*" in the signaling cascade of IL-1R indicates that this signaling cascade is not represented in the network but the activation of AP-1 by IL-1R can be achieved through TRAF6 activation, another pathway activated by the same receptor.

Receptor (adaptors)	**Signaling**		**Function Outcome**	**MPM**
	1st molecules	2nd molecules		
IL6R alpha	JAKs	STAT3/1/5	Inflammation response	Yes
	PI3K - Akt	NFkB / mTOR	Cell survival / proliferation	No
	p38	JNK/MAPK	Cell proliferation	No
	MEK	ERKs	Transcription of target genes	No
IL1R (MyD88, IRAK1/2/4)	MAP3K1	ERK1/2 p38 / AP1	Transcription of target genes Pro-inflammatory response	No*
	PI3K - Akt	NFkB / mTOR	Protein synthesis / Proliferation	No
	TRAF6	JNK/p38 NFkB	Pro-inflammatory response	Yes
IL4R alpha (JAK1/3)	STAT5/6	-	Inflammation response	Yes
	PI3K	AKT	Anti-apoptotic	No
	MEK1/2	ERK1/2	Mitogenic	No
IL10R (Tyk2/JAK1)	STAT3/1/5		Inflammation response	Yes
	PI3K	mTORC1	Cell survival / proliferation	No
TLR4 (MyD88, IRAKs, TRAF6)	TAK1	NFkB	Pro-inflammatory response	Yes
	PI3K - Akt	STAT / IRF	Anti-inflammatory response / Type I or III IFN	Yes
	MAP3K1	MEK1/2 - ERK1/2	Pro-inflammatory response	No
	p38 / JNK	AP1	Pro-inflammatory response	Yes
IFNg R1/R2 (JAK 1/2)	PI3K	STAT1	Inflammation response	Yes
	PI3K - Akt	NFkB / mTOR	Survival signal Expression of Antigen Processing-presentation signals	No
	MEK1/2	ERK1/2	Transcription of target genes	No
FCGR	PI3K - Akt	FoxO / mTOR	Cell survival / Protein synthesis	No
	JAK	STAT	Cell proliferation Differentiation	No
	Ras	p38	Stress response	No
	MEK1/2	ERK1/2	Cell proliferation	Yes
IL13R	PI3K - Akt	NFkB	Pro-inflammatory response	No
	JAK1/2	STATs	Inflammation response	Yes
CSF2aR	JAKs	STAT5	Inflammation response	Yes

Appendix D

Boolean functions

Table D.1: **Boolean functions of the nodes in the MacPol model.** The logical rules for each nodes are defined using the Boolean operators NOT (!), AND (&), and OR (|).

Target node	Boolean function
AP1	p38 & ! PPARG
Arg1	STAT6 & KLF4
CCL17	IRF4
CCL18	! STAT1 & STAT6
CCL2	! STAT1 & NFkB | STAT1
CCL22	IRF4
CCL4	NFkB
CCR7	NFkB
CD163	STAT3
CD200R	STAT6
CD206	! KLF4 & STAT3 | KLF4
CD80	NFkB
COX2	NFkB & ! AP1 & ! ERK & ! PPARG & cPLA2a | NFkB & ! AP1 & ERK & ! PPARG | NFkB & AP1 & ! PPARG
CSF2RA	GMCSF
Ca2	P2R
EP4R	PGE2_e
ERK	! TLR4 & FCGR & ! EP4R & ! p38 | TLR4 & ! EP4R & ! p38
FCGR	LPS & IC
GMCSF	GMCSF
IC	IC
IFNa	IRF5 & IRF3
IFNb	IRF3
IFNg	IFNg
IFNgR	IFNg

Table D.1 – *Continued from previous page*

Target node	Boolean function
IL10	! IRF5 & ! AP1 & ! ERK & ! PPARG & ! cMaf & STAT3 | ! IRF5 & ! AP1 & ! ERK & ! PPARG & cMaf | ! IRF5 & ! AP1 & ! ERK & PPARG | ! IRF5 & ! AP1 & ERK | ! IRF5 & AP1
IL10R	IL10_e
IL10_e	IL10_e
IL12	STAT1 & IRF5 & NFkB & p38 & ! ERK
IL13	IL13
IL1R	IL1b_e
IL1Ra	! p38 & STAT3 | p38
IL1a	IRF5 & NFkB
IL1b	! STAT1 & IRF5 & NFkB | STAT1 & NFkB
IL1b_e	IL1b_e
IL4	IL4
IL4RA	! IL6R & ! IL4 & IL13 | ! IL6R & IL4 | IL6R
IL6	NFkB & AP1
IL6R	IL6_e & ! SOCS3
IL6_e	IL6_e
IRF3	TBK1
IRF4	JMJD3 & STAT6
IRF5	! IFNgR & ! STAT5 & IRF3 & ! IRF4 | ! IFNgR & STAT5 & !IRF4 | IFNgR & ! IRF4
JMJD3	! CSF2RA & STAT6 | CSF2RA
JNK	TRAF6
KLF4	STAT6
LPS	LPS
MNK1	! p38 & ERK | p38
NFkB	! EP4R & RelA & ! PPARG & ! STAT6 & ! KLF4
P2R	IFNg
PGE2	cPLA2a & COX2
PGE2_e	PGE2_e
PPARG	STAT6 & KLF4
RelA	! IRF5 & ! TNFa & TRAF6 & SOCS3 & ! SOCS1 | ! IRF5 & TNFa & TRAF6 & ! SOCS1 | IRF5 & TRAF6 & ! SOCS1
SOCS1	STAT6
SOCS3	! NFkB & ! STAT6 & STAT3 | NFkB & ! STAT6
STAT1	! IFNgR & ! IL6R & IRF5 & ! PPARG & ! STAT6 & ! SOCS1 & ! STAT3 | ! IFNgR & IL6R & ! PPARG & ! STAT6 & ! SOCS1 & ! STAT3 | IFNgR & ! PPARG & ! STAT6 & ! SOCS1 & ! STAT3
STAT3	! IL6R & ! IL10R & EP4R & ! STAT5 | ! IL6R & IL10R & ! STAT5 | IL6R & ! STAT5
STAT5	! IFNgR & CSF2RA | IFNgR
STAT6	! IL4RA & EP4R | IL4RA
TBK1	TLR4

Table D.1 – *Continued from previous page*

Target node	**Boolean function**
TGFb	STAT3
TLR4	LPS
TNFa	! IRF5 & ERK | IRF5 & ! NFkB & ERK | IRF5 & NFkB
TRAF6	! CSF2RA & ! IL1R & TLR4 & ! FCGR | ! CSF2RA & ! IL1R & TLR4 & FCGR & ! SOCS3 | ! CSF2RA & IL1R & ! FCGR | ! CSF2RA & IL1R & FCGR & ! SOCS3 | CSF2RA & !FCGR | CSF2RA & FCGR & ! SOCS3
cMaf	STAT6
cPLA2a	! NFkB & ! JNK & ! p38 & ! MNK1 & ERK & Ca2 | ! NFkB & ! JNK & ! p38 & MNK1 & Ca2 | ! NFkB & ! JNK & p38 & Ca2 | ! NFkB & JNK & Ca2 | NFkB & Ca2
iNOS	STAT1 & NFkB & ! PPARG
p38	! EP4R & TRAF6 | EP4R

Appendix E

List of mutation experiments

Table E.1: **Experimental conditions of perturbed nodes found in the literature.** The perturbed node, type of mutation, stimulus, and cell line details used in the studies are shown. The PubMed-ID or doi of the study is shown in the "Reference" column. Mutations include knockout (KO), use of inhibitor (INB), constitutive expression (E1), pretreatment (PT).

Condition	Stimuli	Cell line		Reference
COX2 (INB)	IL4/13	Mice	Mouse polyps. Tumor-associated macrophages (TAMs)	21730361
COX2 (INB)	IL10	Mice	Mouse polyps. Tumor-associated macrophages (TAMs)	21730361
cPLA2 (CE)	LPS	Mice	Mouse resident peritoneal macrophages	23950842
ERK (INB)	PAM/LPS	Mice	Mice bone marrow-derived macrophages	26445168
ERK (INB)	LPS	Mice	Murine bone marrow-derived macrophages	25776754
ERK (INB)	LPS	Mice	Primary Human Macrophages	15792794
IRF4 (KO)	LPS	Mice	Mice macrophage line RAW264.7	16243976
IRF5 (CE)	LPS	Human	Differentiated human monocytes	21240265
IRF5 (CE)	LPS	Human	Human embryonic kidney (HEK)-293 cells	10.25560/9045
IRF5 (KO)	IFNg + LPS	Mice	Mice bone marrow-derived macrophages	29762983
IRF5 (KO)	Virus	Mice	Mice cell line	17360658
IRF5 (KO)	IFNg	Mice	Mice macrophages residing in myocardial infarcts (MI)	24361318

Table E.1 – *Continued from previous page*

Condition	Stimuli	Cell line		Reference
JMJD3 (KO)	LPS	Mice	Mice bone marrow-derived macrophages	20729857
KLF4 (KO)	IL4	Mice	Mice macrophage line RAW264.7	21670502
KLF4 (KO)	LPS	Mice	Mice macrophage line RAW264.7	21670502
NFkB (INB)	PGN	Mice	Mice macrophage line RAW264.7	15007072
NFkB (INB)	IFNg + LPS	Mice	Mice macrophage line RAW264.7	26993378
NFkB (INB)	LPS	Mice	Mice macrophage line RAW264.7	18057724
NFkB (INB)	LPS	Mice	Murine J774 Macrophage-Like Cells	11278990
p38 (INB)	LPS + PGE2	Mice	Mice bone marrow-derived macrophages	20637838
p38 (INB)	IL6 + LPS	Mice	Mouse myeloid leukemia M1 cell line	12112010
PGE2 (PT)	LPS	Human	Human THP-1 cells	30429830
PGE2 (PT)	IL4	Mice	Mice bone marrow-derived macrophages	30566880
PPARg (KO)	IL4	Mice	Mice bone marrow-derived macrophages	17515919
SOCS1 (KO)	LPS	Mice	Mice macrophage line RAW264.7	12433365
SOCS3 (KO)	LPS	Mice	Mice bone marrow-derived macrophages	22925925
SOCS3 (KO)	IFNg + LPS	Mice	Mice bone marrow-derived macrophages	18424750
STAT1 (KO)	IFNg + LPS	Mice	Mice bone marrow-derived macrophages	14977926
STAT3 (KO)	IL10 + LPS	Mice	Mice bone marrow-derived macrophages	12193690
STAT3 (KO)	IL10	Mice	Mice macrophage line RAW264.7	26260587
STAT3 (KO)	LPS	Mice	Mice macrophage line RAW264.7	26260587
TRAF6 (INB)	LPS	Mice	Mice macrophage line RAW264.7	22925919
TRAF6 (KO)	LPS	Mice	Mice bone marrow-derived macrophages	16306937
TRAF6 (KO)	LPS	Mice	Mice macrophage line RAW264.7	17507094

Appendix F

Nodes associated to Biological Process

Table F.1: **Macrophage phenotype markers associated to a Biological process node.** Each marker is shown with its respective macrophage phenotype, the entity short name, the entity type, and the entry where the function can be found (PMID or doi link). Next, the biological process (BP) designated to the marker, e.i., the immune response that is triggered by the entity.

Phenotype	Node	Type	PMID / doi link	BP
M1	TNFa	Cytokine	10891884	Inflammation
M1	CCL2	Chemokine	31921102 10.3389/fimmu.2019.02759	Inflammation
M1	CCL4	Chemokine	10.1007/978-1-4614-6438-9_10-1	Inflammation
M1	CCR7	Chemokine receptor	25359998 10.1189/jlb.1A0314-170R	Inflammation
M1	CD80	Cell surface receptor	31093509	Inflammation
M1	IFNa	Cytokine	19161415	T1 IFN response
M1	IFNb	Cytokine	19161415	Inflammation
M1	NOS2	Enzyme	25451639	Inflammation
M1	IL1b	Cytokine	22019906	Inflammation
M1	IL1a	Cytokine	27434011	Inflammation
M1	IL12A	Cytokine	26918147	Inflammation
M1	CSF1	Cytokine	16337366	Inflammation
M1	IL18	Cytokine	11203186	Inflammation
M1&M2	IL1RN	Cytokine	10085034	Antiinflammation
M1&M2	IL6	Cytokine	25339958 32834892	Inflammation
M2	Arg1	Enzyme	30613266	Antiinflammation
M2	CCL17	Chemokine	30910796	Antiinflammation
M2	CCL18	Chemokine	22117697 10.21203/rs.3.rs-97834/v1	Antiinflammation
M2	CCL22	Chemokine	30910796	Antiinflammation
M2	CD163	Cell surface receptor	19777868	Antiinflammation
M2	CD200R1	Cell surface protein	12072366	Antiinflammation
M2	CD206	CLR	24672807	Phagocytosis
M2	TGFb1	Cytokine	19481975 33178221	Antiinflammation
M2	IL10	Interleukin	25004819	Antiinflammation

Appendix G

TLR2 pathway Module

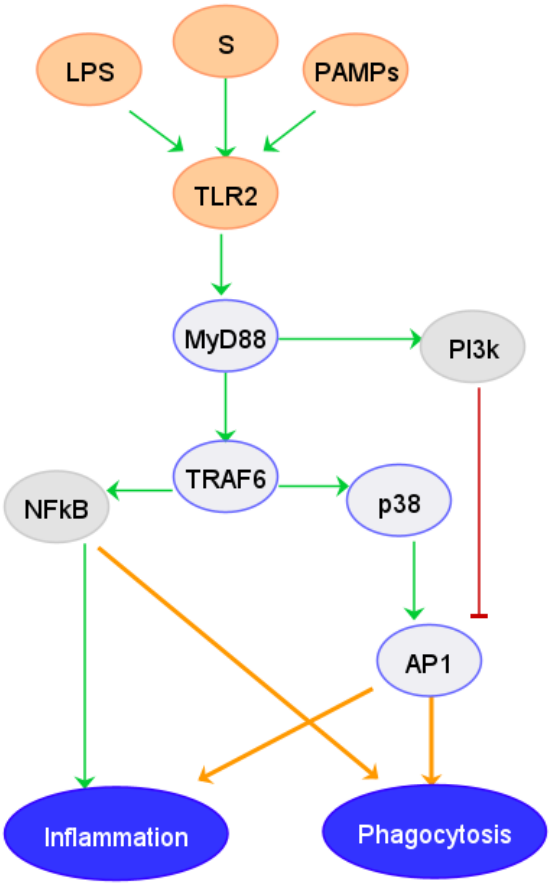

Figure G.1: **TLR2 pathway Module.** Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows. Negative interactions are shown with red arrows. Interactions that are only triggered by LPS stimulus are shown in orange. Purple node: biological process. Orange nodes: stimuli and receptor. Gray nodes: cellular entities. Gray node with purple border: entities related to the M1 and M2 signaling pathways.

Appendix H

Stable States of the individual SARS-CoV-2 modules

In order to evaluate the dynamic behavior and performance of each module integrated in the MacAct-C19 model, the complete model was subjected to a stable state analysis using the different conditions that stimulate each particular module. First, the specific stimuli that activate each module were evaluated individually. Then, in some cases, a knockout mutation was defined and evaluated in combination with the module-specific stimulus. This was done with the purpose of eliminating interference that could be caused by entities from other signaling pathways in the evaluated module. Finally, the module-specific stimulus was evaluated in combination with the viral infection stimulus. The results are shown in Figure H.1 as a heatmap.

For each of the conditions evaluated, the MacAct-C19 model reached one or two different stable states in each case (columns within the matrix). If the system reaches two stable states, the first one is indicated by a 0 after the defined stimulating condition, and the second one by a 1 at the same place. The stimulating conditions are indicated at the bottom of each column within the matrix. At the left, the names of the network entities are shown and within the matrix their state of activity or expression: active/high expression (value of 1) in coral; inactive/low expression (value of 0) in navy. At the right, entities are grouped by their relationship to a signaling pathway or their relationship to a macrophage phenotype (M1 and M2 markers). The M1/M2 row represents signaling pathways and pleiotropic markers. Finally, the last row groups the biological processes that result in the stable state, these are decisive to define the overall response to the pathological condition that is induced.

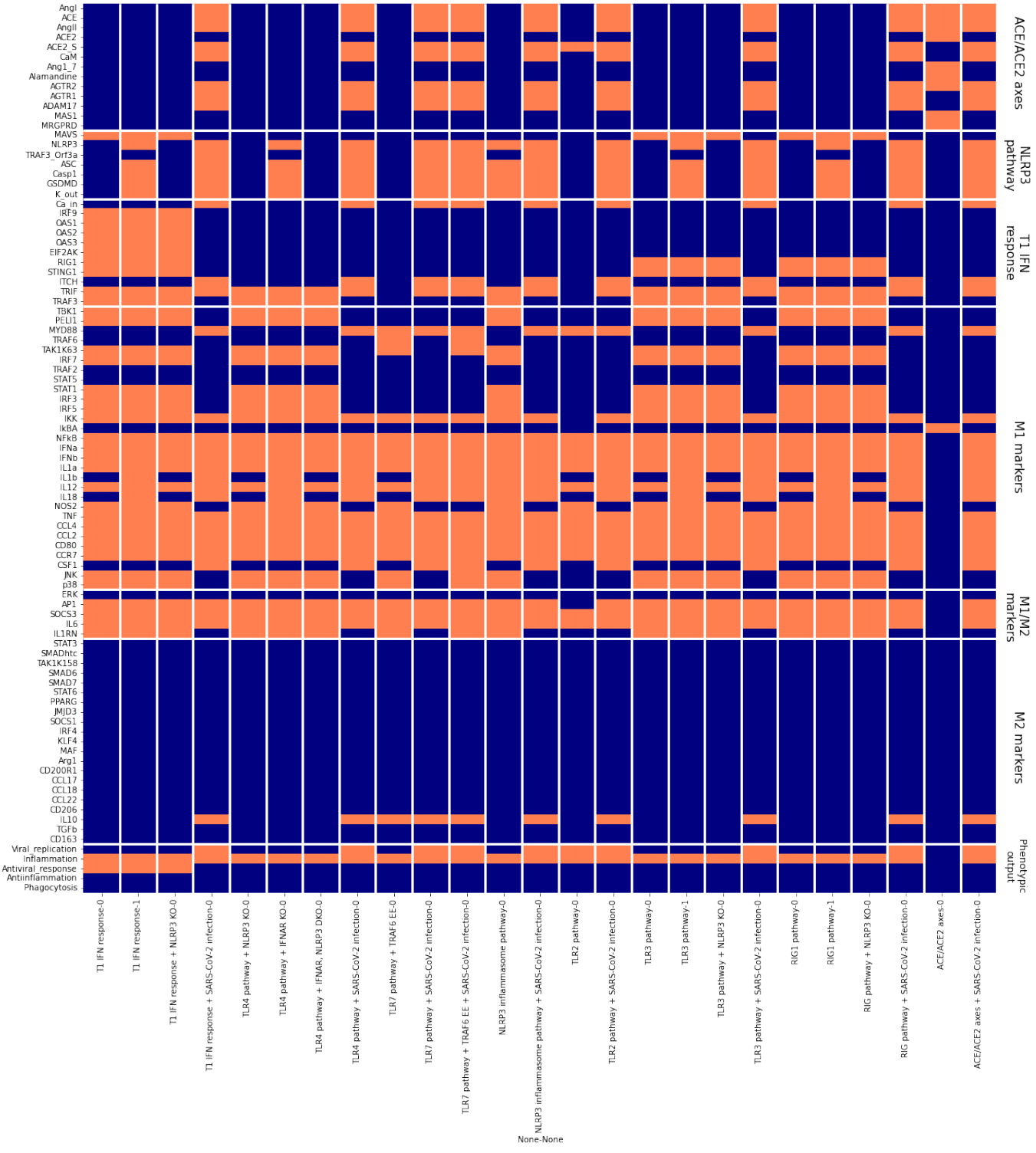

Figure H.1: **Heatmap of the stable states of the SARS-CoV-2 modules.** The system reaches one or two stable state after setting the corresponding stimulus as fixed input. Each column represents a complete stable state with the stimulus indicated at the bottom of the matrix.

Appendix I

Cellular Communication Module

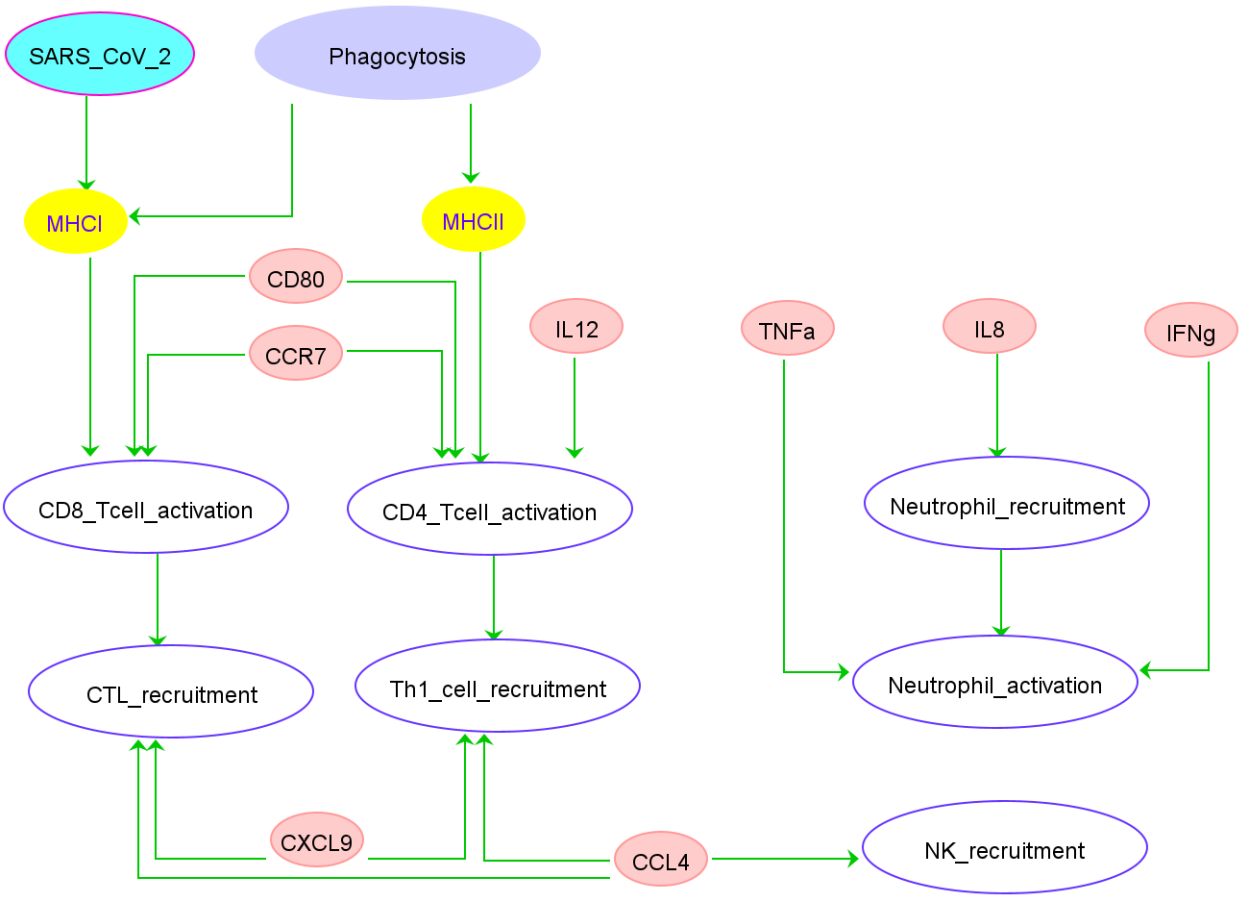

Figure I.1: **Inter-cellular communication Module.** Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows. Purple node: biological process. White node with purple border: cellular communication process. Yellow nodes: major histocompatibility complex. Blue node with pink border: viral node. Light red nodes: cellular entities.

Appendix J

Phagocytosis Module

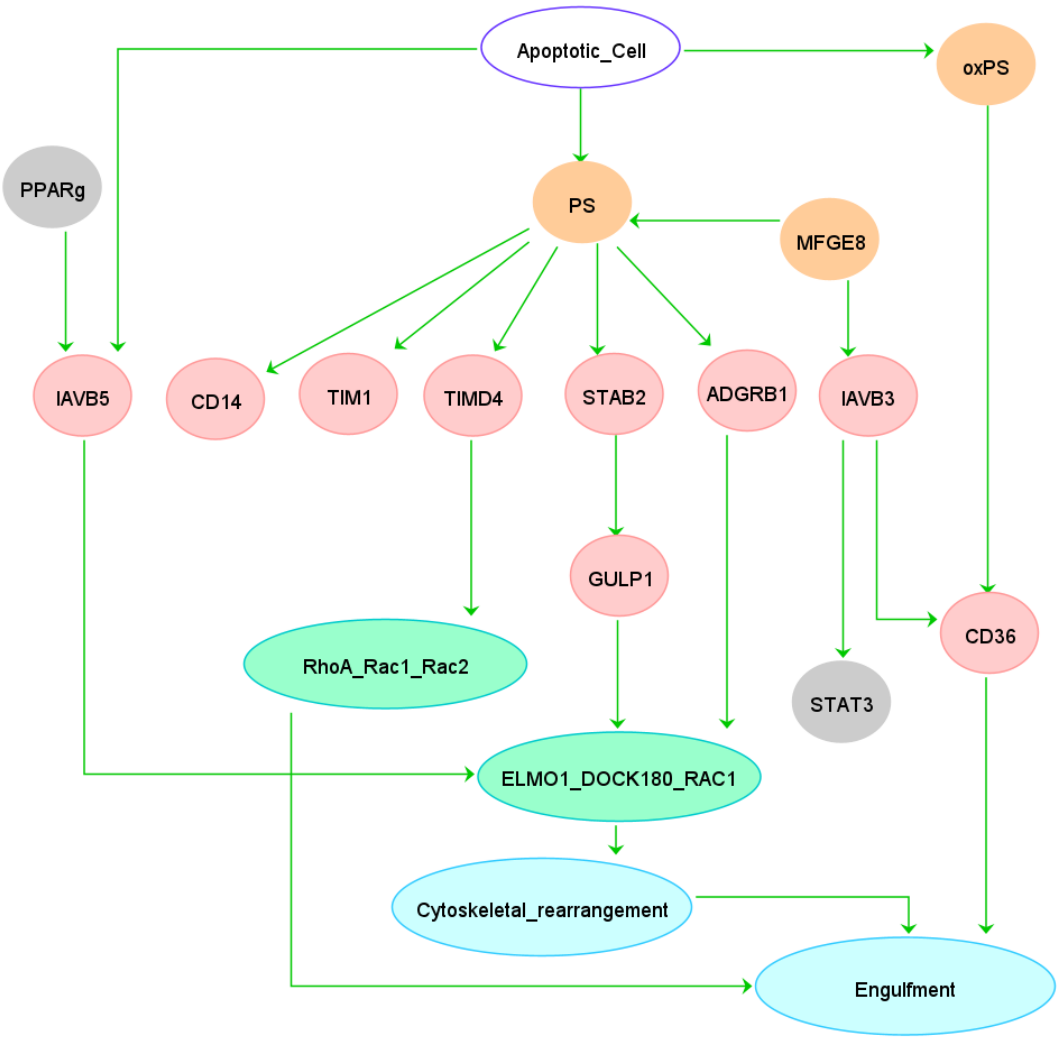

Figure J.1: **Phagocytosis Module.** Nodes represent entities in the network and edges represent interactions. Positive interactions are shown with green arrows. White node with purple border: apoptotic cell stimulus. Orange nodes: apoptotic signal proteins ("find-me" and "eat-me"). Light red nodes: receptors and adaptor proteins. Green nodes: signaling pathways. Gray nodes: cellular entities already present in the MacPol model. Light blue nodes: biological process.

The phagocytosis module contains a total of 19 nodes and 22 positive interactions. This module is not SARS-CoV-2 specific, but represents the generalized process of phagocytosis activated by an apoptotic cell (which may have been previously infected by the virus). To begin with, cells initiating the phagocytosis process expose specific proteins on their surface that act as "find-me" and "eat-me" signals to activate phagocytic cells [171]. The module depicted phosphatidylserine (PS) and oxidized PS (oxPS) ligands presented on the cell membrane of apoptotic cells [172]. Milk fat globule epidermal growth factor (EGF) factor 8 (MFG-E8) protein, which acts as an enhancer of the apoptotic process by recognizing the PS ligand, was also included [163].

The module includes 8 receptors and an adaptor protein (macrophage specific) responsible for the recognition of the above mentioned signals. From here on, the activated signaling pathways are not characterized for all receptors. This is the case for the receptors Cluster of differentiation 14 (CD14) and T cell immunoglobulin mucin 1 (TIM1), that do not continue to the phagocytosis process in the module. Since the information was not complete, these receptors were not included in the MacAct-CC model. However, for the other receptors we represented (1) the RhoA/Rac1/Rac2, or (2) the ELMO1/DOCK180/Rac1 pathway. Both pathways induce morphological changes in the cell that allows the formation of phagosomes to then engulf apoptotic cells [173, 174]. The components of the two pathways were grouped into singular nodes representing the complete signaling pathway. This was done to allow the simplicity of the module and to facilitate subsequent integration into the MacAct-CC model. For the same reason, the "Cytoskeletal rearrangement" and "Engulfment" nodes were grouped in the MacAct-CC model as a single biological process node ("Phagocytosis").

In addition, two nodes previously represented in the MacPol model were included. The first, Peroxisome proliferator-activated receptor-gamma (PPARγ), functions as an enhancer of phagocytosis by positively regulating the expression of integrin $\alpha V\beta 5$ - one of the membrane receptors capable of mediating interaction with apoptotic cells and inducing the process of phagocytosis via the ELMO1/DOCK180/Rac1 pathway [175]. The second, Signal transducer and activator of transcription 3 (STAT3), is induced when MFG-E8 facilitates the recognition of apoptotic cells by integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ [163]. Both PPARγ and STAT3 induce the expression of M2 phenotype markers in the macrophage - which is associated with the inflammation-resolving response. Thus, the two transcription factors enable the connection between the MacAct-CC model and the phagocytosis module.

Appendix K

Supplementary material

Organization of the supplementary material:

1. Figures. The folder is organized in four subfolders containing the different heatmaps, models, modules, and other figures included in this document. All files are in PNG format.

2. GINsim Files. The folder is organized in four subfolders containing the final models, the pathological modules used for the MacAct-C19 model (including the processed CaSQ files), and the cellular communication modules used for the MacAct-CC model. All files are in the GINsim format and can be open in the same tool or through a Jupyter Notebook.

3. Other Analysis Files. The folder contains extra tables and files used in this project. This includes information about the analysis of the different models, other visualization files, and extra information about the nodes and interactions.

4. Notebook. The folder includes the Jupyter Notebook (ipynb and HTML formats) made for this project. In the Notebook, different figures presented in this document are displayed. They are included with the aim to provided a better visualization of them. Also, the Notebook includes the code used to generate the stable state analyses (using bioLQM) and the heatmaps of this project.

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Boolean modeling of macrophage polarization upon immune stimulation and SARS-CoV-2 infection

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