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The role of O-GlcNAcylation in the pathophysiology of Systemic Lupus Erythematosus

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Title: The role of O-GlcNAcylation in the pathophysiology of Systemic Lupus Erythematosus

Abstract: The Hexosamine Biosynthesis Pathway (HBP) is a key metabolic pathway that links nutrient sensing to protein O-GlcNAcylation by converting glucose, glutamine, and fatty acids into UDP-N-acetylglucosamine (UDP-GlcNAc). O-GlcNAc transferase (OGT) couples this high-energy amino sugar to substrate proteins on a serine/threonine residue. The HBP has emerged as a critical pathway in immune regulation. However, its role in autoimmunity has not been fully explored. Systemic Lupus Erythematosus (SLE) is a type I interferon-mediated severe auto-immune disease that primarily affects women. Overexpression of the X-linked gene *OGT* has been proposed to contribute to SLE pathophysiology, but the precise role of O-GlcNAcylation in this pathology remains unknown. Here, we have profiled O-GlcNAcylation levels in SLE patients' blood leukocytes and show a marked upregulation of this pathway in active disease in two immune sub-populations, non-classical monocytes and plasmacytoid dendritic cells. To address causality and underlying mechanisms, we used the *Dnase1L3*$^{-/-}$ mouse model of spontaneous SLE and deleted *Ogt* specifically in myeloid cells. *Ogt*^{DMye}*Dnase1L3*$^{-/-}$ mice exhibited exacerbated disease phenotypes, particularly lupus nephritis. Macrophage O-glycome analysis unraveled several effectors of SLE, including VISTA, as possible OGT substrates. Collectively, our results point to O-GlcNAcylation as a potential regulatory mechanism induced to alleviate myeloid-driven inflammation in SLE and might have wider implications for autoimmunity and interferon-mediated diseases.

Keywords: Hexosamine Biosynthetic Pathway, O-GlcNAcylation, Systemic Lupus Erythematosus, Ogt, Immunometabolism, Innate Immunity

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Titre : Étude du rôle de l'O-GlcNAcylation dans la physiopathologie du lupus érythémateux systémique

Résumé : La voie de biosynthèse des hexosamines (HBP) est une voie métabolique clé, reliant la détection des nutriments à la glycosylation des protéines, en convertissant le glucose, la glutamine et les acides gras en UDP-N-acétylglucosamine (UDP-GlcNAc), qui, une fois couplé aux protéines par l'enzyme O-GlcNAc transférase (OGT), résulte en une modification post-traductionnelle appelée O-GlcNAcylation. L'HBP a récemment été démontrée comme impliquée dans la régulation immunitaire. Cependant, son rôle dans l'auto-immunité n'a pas été complètement exploré. Le lupus érythémateux systémique (LES) est une maladie auto-immune sévère médiée par l'interféron de type I qui affecte principalement les femmes. La surexpression du gène *OGT* (lié à l'X) a été proposée comme contributeur à la physiopathologie du LES, mais le rôle précis de l'O-GlcNAcylation dans cette pathologie reste inconnu. Dans ce travail, nous avons quantifié les niveaux d'O-GlcNAcylation dans les leucocytes sanguins des patients atteints de LES et montrons une augmentation marquée de cette modification post-traductionnelle dans le LES actif dans deux sous-populations immunitaires, les monocytes non classiques et les cellules dendritiques plasmacytoïdes. Pour étudier la causalité et les mécanismes sous-jacents à cette observation, nous avons utilisé le modèle de souris *Dnase1L3*$^{-/-}$ de LES spontané et supprimé *Ogt* spécifiquement dans les cellules myéloïdes. Les souris *Ogt*$^{\Delta Mye}$*Dnase1L3*$^{-/-}$ présentaient des phénotypes pathologiques exacerbés, en particulier la néphrite lupique. L'analyse de l'O-glycome des macrophages a révélé plusieurs effecteurs du LES, y compris VISTA, en tant que substrats possibles de l'OGT. Ensemble, nos résultats suggèrent l'O-GlcNAcylation comme un potentiel mécanisme de régulation induit pour atténuer l'inflammation des cellules myéloïdes du LES, ce qui pourrait avoir des implications plus larges pour l'auto-immunité et les maladies médiées par l'interféron de type I.

Mots clés : Voie de biosynthèse des hexosamines, O-GlcNAcylation, Lupus Erythémateux Systémique, Ogt, Immunométabolisme, Immunité innée

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2-DG: 2-Deoxy-glucose

Aco2: aconitase

AGM1: N-Acetylglucosamine-phosphate mutase

AICD: activation-induced cell death

AIM2: absent in melanoma 2

ALD-DNA: activated lymphocyte-derived DNA

ALPS: auto-immune lympho-proliferative syndrome

AMPK: AMP-activated protein kinase

ANA: Antinuclear antibodies

AP-1: Activator protein 1

APC: Antigen Presenting Cells

APRIL: a proliferation-inducing ligand

BlyS/BAFF: B Lymphocyte Stimulator/B-cell activating factor

BM: Bone Marrow

BPTES: bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CCL2: chemokine ligand 2

CD: Cluster of Differentiation

cDC: conventional dendritic cells

cGAS: cyclic guanosine monophosphate–adenosine monophosphate cGAMP synthase

CLP: common lymphoid progenitor

CLR: C-type Lectin like receptors

CMP: common myeloid progenitor

CSF1R: Colony stimulating factor 1 receptor

CX3CL1: C-X3-C motif chemokine ligand 1

DC: dendritic cells

DDIT4/REDD1: DNA-damage-inducible transcript 4/regulated in development and DNA damage response 1

DNA: Deoxyribonucleic acid

DOCK2: Dedicator of cytokinesis 2

DON: 6-diazo-5-oxo-L-norleucine

ds: double-stranded

e.g.: exempli gratia in Latin, meaning “for example”

ETC: electron transport chain

EZH2: Enhancer of zest homolog 2

F6P: fructose-6-Phospate

FADD: Fas associated death domain

FDA: Food and Drug Administration

GABA: gamma-aminobutyric acid

GC: germinal center

GFPT/GFAT: Glutamine-fructose-6-phosphate amidotransferase

GlcN-6-P: Glutamine-6-Phosphate

GlcNAc-1-P: Glucosamine-1-Phosphate

GlcNAc-6-P: Glucosamine-6-Phosphate

Gls1: Glutaminase-1

GMP: granulocyte/macrophage progenitors

GNPNAT or EMeg32: glucosamine-phosphate N-acetyltransferase

GSK-3β: Glycogen synthase kinase-3 beta

GWAS: genome-wide association studies

HBP: hexosamine biosynthetic pathway

HCQ: Hydroxychloroquine

HIV: Human Immunodeficiency Virus

HMGB1: high–mobility group box 1

HSC: hematopoietic stem cells

i.e.: id est in Latin meaning “that is”

IFN: type I interferon

IFNAR: IFNα receptor

IFNGR: IFNγ receptor

IFNΛR: IFNλ receptor

Ig: Immunoglobulin

IKK: IkappaB kinase

IL: Interleukin

ILC: innate lymphoid cells

IRF: Interferon Regulating Factor

ISRE: IFN-stimulated response element

JAK: Janus Activated Kinase

kDA: kilo-Dalton

KO: Knock-out

LN: Lupus Nephritis

Lpr: lymphoproliferation

MAMPs/DAMPs: microbial- or danger-associated molecular patterns

MAPK: Mitogen-activated protein kinases

MAVS: Mitochondrial antiviral-signaling protein

MDA5: melanoma differentiation-associated gene 5

MEP: megakaryocyte/erythrocyte progenitors

MHC: Major histocompatibility complex

MNPs: mononuclear phagocytes

moDC: monocyte-derived DC

MRL: Murphy Roths Large

mTOR: mechanistic target of rapamycin

MyD88: myeloid differentiation primary response 88

NA: nucleic acid

NET: neutrophils produce extracellular traps

NFAT: Nuclear factor of activated T-cells

NF-κB: nuclear factor-kappa B

NK: natural killer

NLR: NOD-like receptors

NLRP3: NLR family pyrin domain containing 3

NO: nitric oxide

NZB/NZWF1: New Zealand Black/New Zealand White F1

NZM: New Zealand mixed

OGA: OGlcNAcase

OGT: O-GlcNAc transferase

OXPHOS: oxidative phosphorylation

pDC: plasmacytoid DC

PI3K: Phosphoinositide 3-kinase

PRR: pattern-recognition receptors

PSGL-1: P-selectin glycoprotein ligand-1

PTM: post-translational modifications

RIG-I: retinoic acid-inducible gene I

RIPK: Receptor interacting protein kinase

RLR: RIG-I like receptors

RNA: Ribonucleic acid

RORγt: RAR-related orphan receptor gamma

ROS: reactive oxygen species

SAMHD1: sterile alpha motif and histidine/aspartic acid domain-containing protein 1

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SCID: severe combined immunodeficiency

SDH: succinate dehydrogenase

SLE: systemic lupus erythematosus

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index

SNP: single nucleotide polymorphisms

SOCS: suppressor of cytokine signaling

SP1: specificity protein 1

ss: single-stranded

STAT: signal transducer and activator of transcription

STING: stimulator of interferon genes

TBK1: TANK-binding kinase 1

TCA: tricarboxylic acid

TCM: central memory T cells

TCR: T cell receptor

TEM: effector memory T cells

TFH: T Follicular Helper

Th: T helper

TLR: Toll-Like-Receptors

TPR: tetratricopeptide repeats

T_{REG}: Regulatory T cells

TREX1: Three Prime Repair Exonuclease 1

TRIF: TIR-domain-containing adapter-inducing interferon-β

TRM: tissue resident macrophages

TULIP: Treatment of Uncontrolled Lupus via the Interferon Pathway

TYK2: Tyrosine Kinase 2

UAP1: UDP-N-acetylglucosamine pyrophosphorylase

UDP-GlcNAc: Uridine diphosphate N-acetylglucosamine

US: United States of América

UTP: Using Uridine-Triphosphate

UV: Ultraviolet

WT: wild type

XCI: X-chromosome Inactivation

XIST: X Inactive Specific Transcript

1 CHAPTER 1: INTRODUCTION, HYPOTHESIS AND RATIONALE

1.1 The Immune system: Evolution, development, and landscape

1.1.1 Evolution

The immune system has evolved to discriminate self from non-self or altered self. Its main functions are thus to recognize invading pathogens, tissue stress or neoplasia and to mount appropriate defenses to eliminate infectious agents or transformed cells and to restore homeostasis. It is composed of a first line of defense, namely innate immunity, that is fast but antigen non-specific, and a second line of defense, i.e., adaptive immunity, that is elicited later, is antigen specific and provides long-lasting immunity (1).

The innate immune system is evolutionarily ancient, existing in prokaryotes, plants, and non-vertebrate animals. Unlike adaptive immunity that utilizes “customized” defenses, innate immunity is driven by germline-encoded molecules, e.g., the restriction-modification system in prokaryotes, the resistance proteins in plants and the pattern-recognition receptors (PRR) found from plants to mammals, that sense conserved structures referred to as microbial- or danger-associated molecular patterns (MAMPs/DAMPs). Although these employ common mechanisms of recognition and defense, recent studies have highlighted their convergent rather than divergent evolution under the selective pressure of the environment (2). It is argued from studies in hydra that the diversity of the PRR repertoires and of anti-microbial peptides has evolved to deal with the commensal microbiota rather than to defend against invading pathogens (3). Evolutionary studies have also characterized the origin of phagocytes and other specialized innate lymphocytes. Phagocytes, referred to in different animals as amebocytes, hemocytes or coelomocytes, are found from nematodes to mammals, whereas innate lymphoid cells (ILC) evolved in early deuterostomes and variable lymphocyte

receptors expressed on γδ T cells, NKT cells and Mucosal Associated Invariant T cells have been identified in jawless vertebrates (4). While ancestral phagocytes evolved in early metazoan, a progressive shift towards “modern” myeloid cells as initiators and controllers of inflammatory response ago happened around 500 million years ago, that peaked with appearance of dendritic cells, specialized in the communication with the adaptive immune system (5).

Indeed, the adaptive immune system evolved more recently, around 500 million years ago, in ectodermic/cold-blooded vertebrate animals, with the apparition of lymphocytes capable of gene recombination to achieve adaptation of their receptor to a specific antigen, each antigen recognized by a small number of lymphocytes, that expand upon recognition of this antigen presented by the Major histocompatibility complex (MHC) on Antigen Presenting Cells (APC). This is thought to have happened during two evolutionary big bangs in jawless fish and cartilaginous fish as well as the extinct placoderms, which saw the dawn of most components of adaptive immunity: T and B like-cells in jawless fish, Thymus, αβ and γδ T cell receptors (TCR), immunoglobulins and adaptive cytokines, or the MHC system in cartilaginous fish and placoderms, to name a few (6).

1.1.2 Development

In mammals, the development of the immune system is initiated in the yolk sac between 7.5 and 8.5 days of embryonic life (E7.5-E8.5) with primitive hematopoiesis, followed by definitive hematopoiesis with hematopoietic stem cells (HSC) appearance, first in the fetal liver at E10.5 and then in the bone marrow, between E14.5 and E18.5 (7). Fetal monocytes and macrophages progenitors’ egress from the embryonic yolk sac and fetal liver and migrate to peripheral tissues, where they give rise to tissue resident macrophages (TRM) (such as

microglia in the brain and Kupffer cells in the liver). While this is generally the case for most TRMs, in some tissues e.g., the intestine, resident macrophages can be replaced from HSC progenitors. Mapping of TRM ontogeny was discovered by conditional labelling of Runx1, a transcription factor active in yolk sac hematopoietic cells. Such a reporter system showed that, while the Runx1 'label' faded in most macrophage compartments upon their replacement by macrophages from HSC, TRM in tissues e.g. the brain retained a Runx1 label. An HSC-independent origin of macrophages was also confirmed by parabiosis experiments, in which mice are sewn together to share the same vascular system. These experiments showed a lack of chimerism in most TRM populations, even one year after the parabiosis procedure (8). Of note, an embryonic origin of immune cells is not limited to macrophages, as it also concerns mast cells and γδ T cells, highlighting this particular ontogeny as a characteristic feature of innate immunity (7).

All other immune cells from lymphoid and myeloid lineages stem from bone marrow HSC, which first give birth to common lymphoid and myeloid progenitors (CLP and CMP, respectively). CLP leads to the development of ILC, natural killer (NK) cells, T and B lymphocytes. These cells become mature in the bone marrow except for T cells that migrate to the thymus for further maturation. CMP gives rise to megakaryocyte/erythrocyte progenitors (MEP) and granulocyte/macrophage progenitors (GMP). The erythroid lineage (red blood cells and platelets) arises from the MEP while myeloid cells encompassing granulocytes (neutrophils, eosinophils, basophils, and mast cells) and mononuclear phagocytes (MNPs) (monocytes, macrophages, dendritic cells [DC]) arise from GMP. Of note, this rather hierarchical and simplistic classification of immune cells derived from HSC progenitors has been recently challenged with the advent of single cell RNA sequencing (scRNA-seq) that revealed an unexpected continuum among HSC progenitors. Although a clear

transcriptomic signature separates megakaryocyte/erythrocyte progenitors from other hematopoietic progenitors, a co-expression of gene signatures of lymphoid and myeloid cells is possible before final commitment to a specific lineage, highlighting a more horizontal development than previously thought (9).

1.1.3 Landscape

The immune system operates through a complex interplay of cellular effectors (lymphocytes and myeloid cell populations) originating from primary (bone marrow and thymus) and secondary lymphoid organs (spleen, lymph nodes) and activated in certain contexts in tertiary lymphoid structures. Circulating immune cells infiltrate tissues guided by a web of chemokine/chemokine receptor interactions and crosstalk with tissue resident immune cells, communicating through endocrine, paracrine, and autocrine molecular signals via proteins (e.g., cytokines) and lipid mediators (10). Single cell approaches have unraveled an unprecedented diversity in immune cell phenotypes and functions, expanding our view on the immune system by refining immune cell classifications (11). These studies are revealing differences in cellular states according to ontogeny, organ-immune specificities and the metabolic environment of a tissue in health and immune-mediated diseases (12).

For instance, based on *in vitro* polarization experiments, BM-derived macrophages were classified as M1 (highly phagocytic and inflammatory) and M2 (anti-inflammatory) macrophages, however this classification does no longer stand as a spectrum of macrophage states are observed *in vivo* (13). DCs are roughly classified into conventional dendritic cells (cDC), monocyte-derived DC (moDC) and plasmacytoid DC (pDC) (14–16), but once more this is a simplification of the real complexity of the DC landscape (11). Similarly, human monocytes

have been previously divided into three subtypes, namely classical $CD14^{+}CD16^{-}$, intermediate $CD14^{+}CD16^{+}$ and non-classical $CD14^{-}CD16^{+}$ monocytes. If in humans, monocytes are divided based on the expression of the surface markers CD14 and CD16, the subdivision in mice is based on Ly6C expression (17). In mice, only classical $CD11b^{+}Ly6C^{+}$ monocytes and non-classical $CD11b^{+}Ly6C^{-}$ monocytes are described, although one team highlighted a specific subset of $Treml4^{+}$ monocytes committed to become classical, that could represent an equivalent in mice of the intermediate subtype (18). While monocytes rely on colony-stimulating factor 1 (CSF-1) to survive, to egress from the bone marrow, classical monocytes require CCL3 and non-classical monocytes S1PR5. After they egress from the bone-marrow, monocytes develop as a continuum between classical monocytes, very transient in the blood where they live around one day, towards intermediate and non-classical monocytes which are the subtype that live the longest in the blood, around one week, which is a part of the reason why they are referred to as “Patrolling monocytes”. (11,19).

Classical monocytes were thought of as the inflammatory subtype (17), while non-classical monocytes were dubbed patrolling monocytes and have been proposed as modulators or amplifiers of disease states (19). However, the monocyte landscape is more complex than initially described and monocytes can acquire context-specific states and functions. In pediatric systemic lupus erythematosus (SLE) for e.g., eight monocyte states have been identified, including two subsets enriched with a type I interferon (IFN) signature, implicating these besides pDC in the initiation and/or the response of type I IFNs in SLE (20).

Last, with the demonstration that platelets, endothelial cells, epithelial cells and fibroblasts can participate in immune responses and immune-related disorders (21–23), the ‘immune system’ becomes a much wider entity to consider.

1.2 The immune system: Functions in host defense and maintenance of homeostasis

1.2.1 Innate immunity: microbial and danger recognition, innate 'memory' and activation of adaptive immunity

The primary role of the innate immune system is to discriminate self from non-self or altered-self. Initially, it was thought of as a system that recognizes exogeneous foreign pathogens, i.e., non-self, however in 2002, Polly Matzinger proposed the danger hypothesis (24), that widened the concept of immunity to include recognition of endogenous 'danger' signals, or altered-self. While the immune system developed mechanisms to tolerate non-harmful situations, such as a fetus during pregnancy or commensal microorganisms, it reacts to danger signals through PRR induction of innate immunity.

Innate immunity harbors constitutive and inducible mechanisms. Constitutive mechanisms include physical (skin, epithelial barriers), mechanical (cilia), chemical (acidic pH of the stomach; antimicrobial enzymes in secretions), and commensal barriers (the microbiota), that limit the possibility for a pathogen to enter the host organism and expand. Inducible mechanisms arise in response to pathogen invasion or accumulation of danger signals e.g. alarmins released following tissue damage (25). Soluble and cell-bound receptors are engaged that mediate signaling events leading to immune cells recruitment, release of defense effectors such as complements and activation of phagocytes.

Soluble receptors include collectins, ficolins, pentraxins, all part of inflammatory proteins secreted by the liver and highly conserved during evolution (encompassing fibrinogen or C reactive protein, sCD14, MFG-E8/lactadherin and complement C1q). These soluble sensors bind pathogens and are also able to discern stress signals at the surface of altered cells, e.g. "eat me signals" that trigger apoptosis of stressed or dying cells (26,27).

Cell bound receptors include PRRs (Figure 1) that sense MAMPs/DAMPs but also scavenger receptors and opsonin receptors (i.e., Fc and complement receptors). MAMPs are generally structural elements common to pathogens and commensal microorganisms e.g., cell surface lipopolysaccharide (LPS) on the outer wall of Gram-negative bacteria, peptidoglycan on that of Gram-positive bacteria, flagellins or nucleic acids, to name a few. PRRs encompass Toll-Like-Receptors (TLR), NOD-like receptors (NLR), C-type Lectin like receptors (CLR) and RIG-I like receptors (RLR). Upon recognition of their cognate ligands/agonists, PRRs change conformation/topology and engage intracellular signaling pathways that converge on the activation of master transcription factors (e.g. NF-κB, AP-1 and Interferon Regulating Factors (IRFs)) or proteases (e.g. caspases within inflammasomes). The net outcome is an induction of a plethora of cytokines, chemokines, lipid mediators, and activation of cellular effectors of the innate immune systems i.e., granulocytes and MNPs that eliminate the source of danger through variable mechanisms. For instance, in response to PRR engagement, neutrophils produce extracellular traps (NET) and reactive oxygen species (ROS) (28) to capture, phagocytose and kill pathogens (29,30). Similarly, through nucleic acid (NA) sensing by PRRs, pDC produce IFNs to counter viral infections. These PRR responses protect the host, but in case of chronicity of the danger signal, or deregulation caused by genetic or epigenetic factors, they can also elicit adverse events such as inflammatory tissue destruction, cytokine storm and pathology. Auto-inflammatory diseases are a notable example of genetic diseases involving hyper-activation of innate immunity (31), and gout is caused by chronic PRR stimulation, particularly that of the NLRP3 inflammasome, by monosodium urate crystals (32). In SLE, NETs play a role in fueling the break of tolerance to self-nucleic antigens (33), and host NA sensing and type I IFN drive the pathology (15).

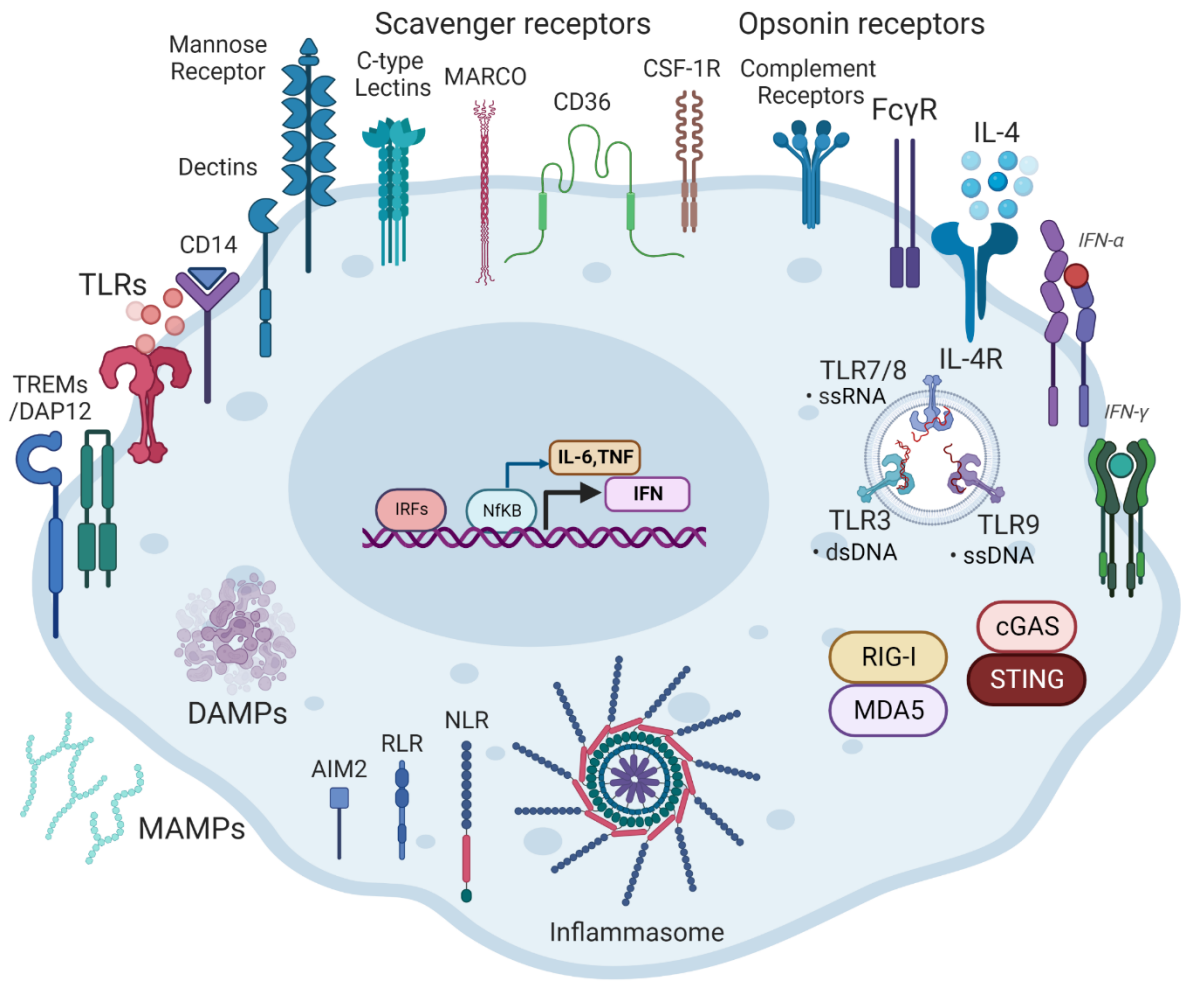

Figure 1. Pattern Recognition Receptors

Cell surface receptors (such as scavenger receptors, Toll-Like receptors (TLR) or opsonin receptors for example), and intracellular sensors (such as NOD-like receptors (NLR), RIG-I-like receptors (RLR) or endosomal TLRs) are depicted.

Besides acute responses, PRR engagement engenders two other important outcomes: a) activation of adaptive immunity, and b) induction of trained immunity (Figure 2). The first is mediated by PRRs, primarily TLRs, that ensure DC maturation, and through the induction of co-stimulatory molecules and cytokines, in naïve T cell priming and initiation of adaptive immunity (34). The second involves metabolic reprogramming and epigenetic modifications in myeloid cell progenitors resulting in an improved response upon a second encounter of the

same or another pathogen. This sort of 'innate immune memory', dubbed "trained immunity" (35) in myeloid cells, has also been reported in NK cells. For instance, mice deficient in the recombinase gene (Rag) lacking T and B cells, were able to respond to a second challenge with haptens to the same magnitude as wild-type (WT) mice (36).

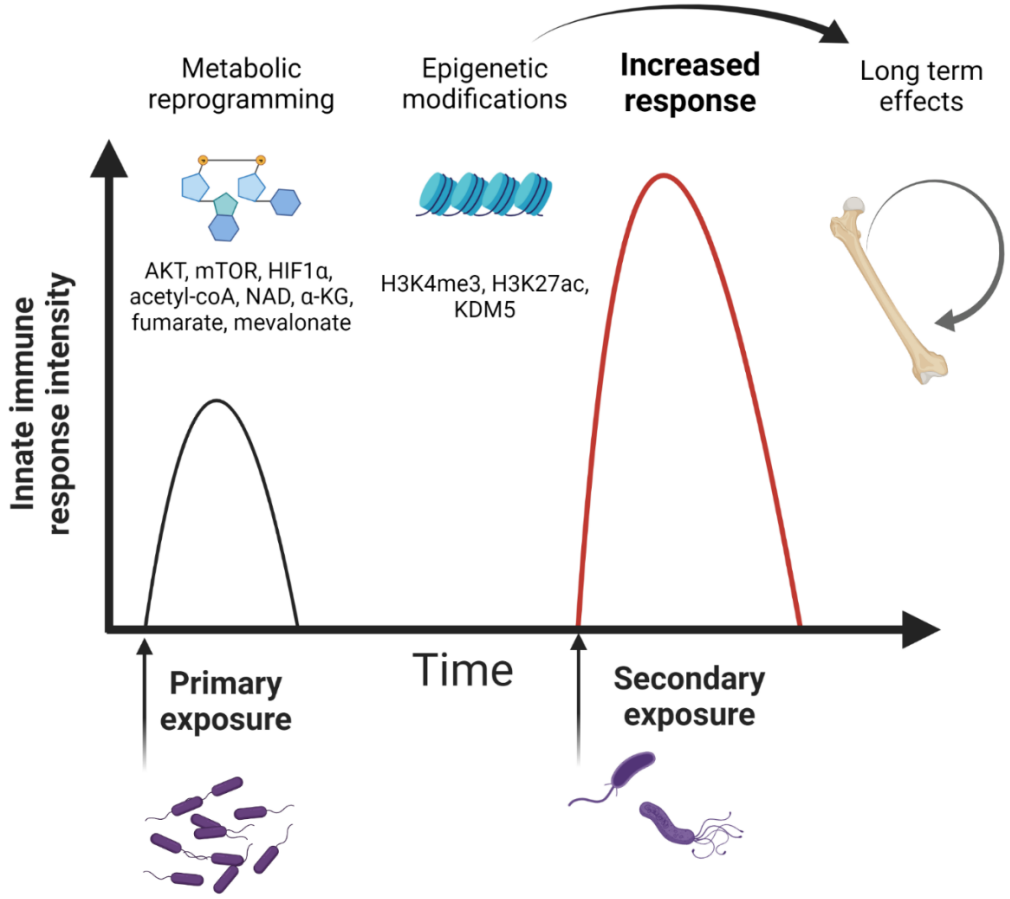

Figure 2. Trained Immunity

Primary exposition to a pathogen leads to metabolic reprogramming and epigenetic modifications of innate immune cells, resulting in an increased innate immune response upon a second exposure to the same pathogen.

1.2.2 Adaptive immunity: self-tolerance and antigen-specific long-lasting immunity

The adaptive immune system is equipped with a clever toolbox that allows lymphocytes to distinguish self from non-self/altered-self. Through somatic recombination of T cell receptor (TCR) and VDJ recombination in B cells, lymphocytes are capable to distinguish a myriad of possible antigens. This process occurs early in T and B cell development and maturation. For T cells, this occurs in the thymus, where T cell precursors rearrange their TCR and are educated to tolerate self-antigens through positive and negative selection. Thymocytes present self-antigens on MHC molecules to T cells. 95% of TCR rearrangement do not generate a reactive TCR, which results in death by neglect. T cells with a high affinity TCR for a self-antigen are killed by apoptosis through negative selection. Only T cells with low affinity for MHC-self antigens are positively selected. Bearing either a CD4 or CD8 chain, these T cells egress from the thymus to the periphery. Of note, in a narrow window between the positive selection of naïve T cells and the negative selection eliminating autoreactive clones, lies a plastic demarcation where cells with a strong affinity TCR deviate to commit to the regulatory T cells lineage (37), which participate in peripheral tolerance and the control of autoimmunity (38). Peripheral tolerance is also ensured by activation-induced cell death (AICD), induced by Fas-FADD-caspase-8 extrinsic apoptosis. The importance of Fas-mediated AICD in the control of tolerance is highlighted in the Mrl-*lpr* mice that carry a mutation in *Fas* gene and exhibit a fatal lupus-like-autoimmune disease (39). Of note, humans with FAS mutations develop an auto-immune lympho-proliferative syndrome (ALPS), also known as Canale–Smith syndrome, that differs from SLE, advocating for other factors in SLE pathogenesis than AICD defect (40).

Naïve T cells require three signals to be fully activated. Signal 1 is delivered by TCR engagement by antigen-MHC complex, signal 2 is provided by co-stimulatory molecules expressed on APC (CD80/CD86 for example) and cytokines such as interleukin (IL)-2 that

tighten the TCR signal through PI3K–AKT–mTOR, NFAT, NF-κB, and MAPK pathways (41) and prevent anergy in case of isolated signal 1. Inflammatory cytokines, , e.g. type I IFN, elicited by PRR engagement in innate immune cells, represent the third signal of activation, acting as a molecular switch towards an efficient T cell proliferation and immune response (42). Following execution of their effector functions, activated T cells are eliminated by AICD to terminate the acute response. A subset of lymphocytes is retained, namely memory T cells that provide long lasting immunity. The cytokine inflammatory milieu and the intensity of the TCR signal determine their development into central memory T cells (TCM), effector memory T cells (TEM) and tissue-resident memory T cells (TRM) subsets (43).

1.2.3 Dysregulations of the immune system

Immune system dysfunctions can be divided in two: a) immune-mediated inflammatory diseases with hyper-activated immune responses, encompassing autoinflammatory diseases, complex inflammatory diseases and allergies and or autoimmunity; and b) diseases of immunodeficiencies.

Immune-mediated inflammatory diseases form a continuum from rare monogenic autoimmune diseases like ALPS, caused exclusively by a genetic defect (e.g., *FAS* mutations), to monogenic auto-inflammatory diseases caused by self-activation of innate immunity without an adaptive response (e.g., Familial Mediterranean Fever). In between lies a whole spectrum of polygenic auto-immune or auto-inflammatory diseases with various involvement of innate and adaptative immunity (for example, Crohn's disease) (44). The complex etiology of these diseases has been uncovered by forward genetics and GWAS studies that identified inborn errors and common single nucleotide polymorphisms (SNP) associated with disease

development. In SLE, SNPs in *DNASE1L3*, involved in apoptotic bodies clearance, are associated with increased risk of disease (45,46). Similarly, variants in *TREX1*, encoding an intracellular exonuclease, have also been implicated in familial SLE cases (47). Notably, a full loss-of-function of *TREX1* leads to so called interferonopathy, a type I IFN mediated disease in humans called Aicardi-Goutieres syndrome.

In polygenic inflammatory diseases, SNPs are not sufficient to cause disease but often require interactions with environmental triggers such as viral infections, crystalline silica, UV exposure, diet, medications etc. (48). It is noteworthy to mention that certain SNPs associated with inflammatory diseases have been selected by infectious pressure during evolution, as recently demonstrated in a study on the impact of *Yersinia Pestis* on immune gene evolution (49).

Inborn errors can also lead to immunodeficiencies. For example, *RAG1* deficiency prevents efficient lymphocyte development (by preventing receptor recombination) and leads to severe combined immunodeficiency (SCID). Similarly, loss of *DOCK2*, which is required for lymphocyte migration, results in a combined immunodeficiency, less severe than SCID, but associated with impaired interferon response in hematopoietic cells (50). Inborn errors affecting type I IFN lead to high susceptibility to viral infections, are recently shown in severe SARS-CoV-2 cases (51).

1.3 Nucleic Acid sensing and type I Interferons

1.3.1 Nucleic acid sensors: TLRs and cytosolic sensors

NA sensing (Figure 3) is a major mechanism of detection of viral RNA and DNA to induce an antiviral immune response that ultimately leads to the production of type I IFN (52). NA sensing is mediated by PRR both membrane-bound and cytosolic receptors.

Membrane-bound NA sensors are TLRs: TLR3 recognizes double-stranded (ds)RNA but is able to sense double or single-stranded (ss) RNA and ssDNA, rendering it a general sensor of many viruses. TLR7 and TLR8 sense ssRNA and RNA breakdown products, whereas TLR9 detects ssDNA containing CpG motifs (53). Cytosolic NA sensors encompass several members: RIG-I binds short-dsRNA, MDA5 recognizes long dsRNA, while cGAS senses DNA, either from DNA viruses or self-DNA in autoimmune contexts, resulting in cGAMP production and the activation of STING. Last, two pyrin and hematopoietic IFN-inducible nuclear domain proteins, i.e., IFN-inducible protein 16 and AIM2 also sense DNA from viruses and/or bacteria in the cytoplasm (54–56).

1.3.2 Nucleic acid signaling pathways

Upon activation, TLR7, TLR8 and TLR9 signal through MyD88, whereas TLR3 engages TRIF. RIG-I and MDA5 both interact with MAVS (Mitochondrial antiviral-signaling protein), while cGAS activates STING through cGAMP. These pathways converge on TBK1 and IKKε that phosphorylate IRF3 and IRF7 that encode IFN genes (IFNα, β, l), while MyD88 and MAVS signaling also activate NF-kB and MAPK pathways resulting in inflammatory cytokine gene expression in addition to IRF5 phosphorylation and IFN production (52,55,57).

1.3.3 The Interferon family and its receptors

Discovered in 1957, the IFN family is a group of cytokines that "interfere" with viral replication. They can be divided into 3 subfamilies, Type I, Type II and Type III IFN, the latter discovered more recently in 2003. The IFN family is multigenic. In humans, it encompasses 21 genes, including 13 for IFNα, 3 for IFNλ and one gene each for IFNβ, g, ω, ε and κ (58,59).

Type I IFN are 18-20 kDa polypeptides. IFNα and IFNβ are ubiquitous but can be produced at massive levels by specialized cells i.e., pDC. (60). Type I IFN are secreted upon NA sensing by PRR, either in viral infection or in response to host NA in autoimmunity. Type I IFN prevent infected cells replication and participate in their destruction by inducing ribonucleases and preventing infected cell protein synthesis. Type II IFN, or IFNγ, is secreted by T cells and NK cells upon activation. It induces macrophages activation, Th1 response, cytotoxic T cell lymphocytes activation. Type III IFN, or IFN-λ, encompass IFNλ1 (IL-29), IFNλ2 (IL-28A) and IFNλ3 (IL-28B); they increase expression of genes controlling viral replication and proliferation and are mainly present at mucosal barrier surfaces (61).

Interferon receptors are heterodimers with conserved cysteine residues. All type I IFN proteins bind to a receptor termed IFNAR (IFNα receptor), Type II IFN binds to IFNGR (IFNγ receptor), and Type III IFNs bind to IFNΛR/IL-28R.

1.3.4 Interferon signaling pathways

After binding to IFN, both IFNAR and IFNGR interact with a JAK kinase (Figure 3): TYK2 for IFNAR subunit 1, JAK1 for IFNAR subunit 2 and IFNGR subunit 1 and JAK2 for IFNGR subunit 2. Following interaction with their ligands, IFN receptors dimerize and the associated JAK autophosphorylates and activates the JAK-STAT pathway. Activation of IFNAR results in

phosphorylation of STAT1 and STAT2, which associate with IRF9 to form a complex known as IFN-Stimulated Gene Factor 3 that translocates to the nucleus and promotes IFN-stimulated response elements, initiating transcriptional response. Of note, activation of IFNAR and IFNGR leads to the phosphorylation of STAT1 that dimerizes and translocates to the nucleus, where it binds GAS (IFNγ activated sites) in ISG promoters, eliciting transcriptional response (60).

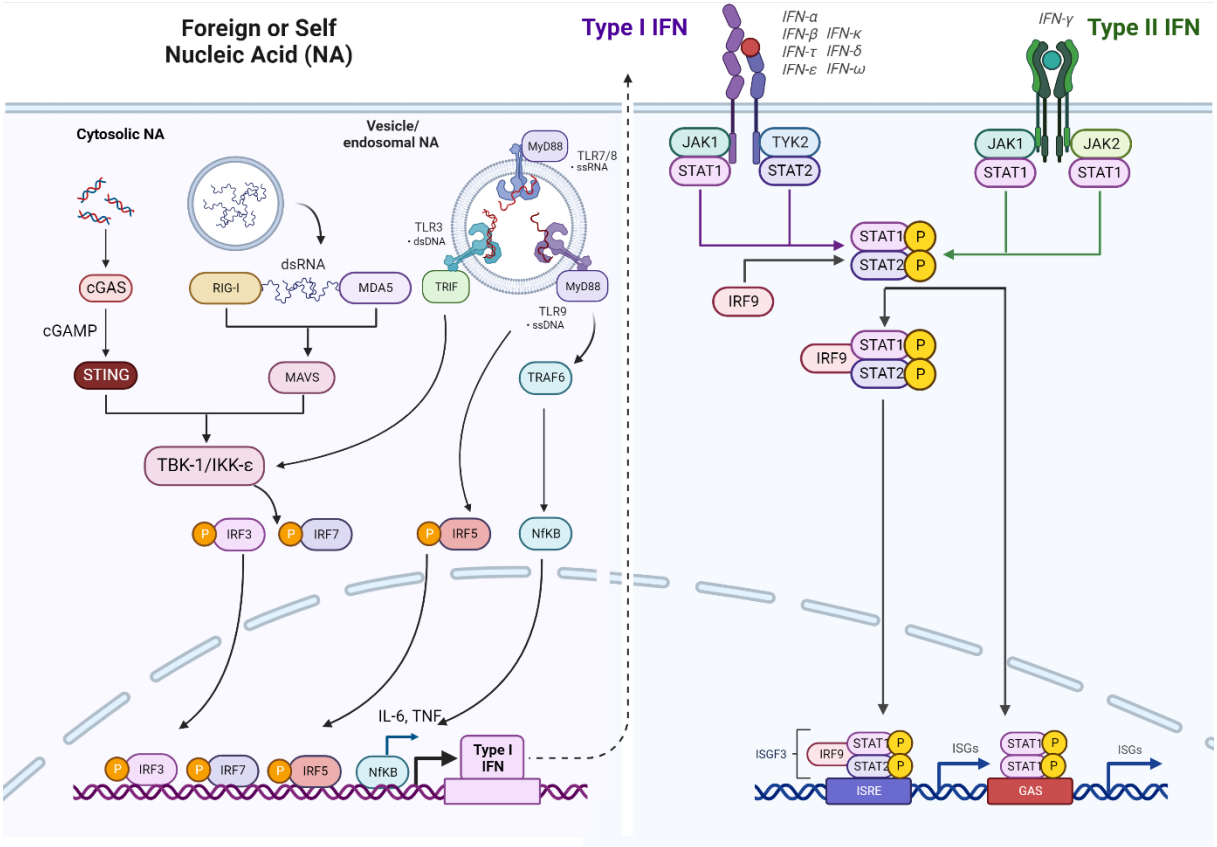

Figure 3. NA sensors and IFN signaling

Exposure to foreign or self-nucleic-acid results in activation of the cGAS-STING pathway, RIG-I-MDA5-MAVS pathway, all activating TBK-1/IKK-ε. The TBK-1/IKK-ε pathway is also induced by TLR3-TRIF activation, while TLR 7/8 and 9 activates MyD88-TRAF6. These pathways lead to Interferon Regulatory Factors (IRFs) activation and promotion of type I IFN. Type I and type II IFN activate the JAK-STAT-IRF9 pathway, leading to induction of IFN-Stimulated Genes (ISGs) through IFN-stimulated response elements (ISREs) engagement.

1.4 Immunometabolism

Cells produce energy through several bioenergetic pathways, interconnected through their intermediate or final products. The 2 main pathways are glycolysis and mitochondrial oxidative phosphorylation (OXPHOS).

Naïve immune cells require low levels of energy. However, upon activation, they upregulate their metabolism to meet their growing energy demands. In general, activated effector immune cells use aerobic glycolysis to produce building blocks required for their effector functions (e.g. cytokine production) and proliferation (62). This is particularly true for effector lymphocytes (63), inflammatory macrophages (64,65), dendritic cells (66), and granulocytes (67). In contrast, memory T cells (68), regulatory T cells (69) and alternatively activated macrophages (70–72) preferentially use OXPHOS, fueled by Acetyl-CoA produced from fatty acid oxidation (73) or driven by metabolite (e.g. glutamine) anaplerosis through the tricarboxylic acid (TCA) cycle (74) (Figure 4).

While this is a general scheme, important differences exist between T cell and macrophage immunometabolism, particularly in relation to the activity of the TCA cycle. In effector T cells, in parallel to aerobic glycolysis, the TCA is fully operational and driven by increased glutamine consumption (75). In contrast, in inflammatory macrophages, nitric oxide accumulation results in nitrosylation of two enzymes in the TCA cycle, namely aconitase (Aco2) and succinate dehydrogenase (SDH), and these events result in TCA cycle destabilization and accumulation of citrate and Succinate (76–78). Destabilization of SDH, a component of complex II of the electron transport chain (ETC), results in Reverse Electron Transport to ETC Complex I, ultimately enhancing mitochondrial ROS generation (79). Thus, inflammatory macrophages convert mitochondria from sole energy producers to inflammatory effectors.

Apart from glycolysis and the TCA cycle, several other metabolic pathways have been implicated in the regulation of immune cell phenotypes and functions. These include for e.g., the pentose phosphate pathway, arginine metabolism, glutamine metabolism, the GABA shunt, and the hexosamine biosynthetic pathway (HBP) discussed below.

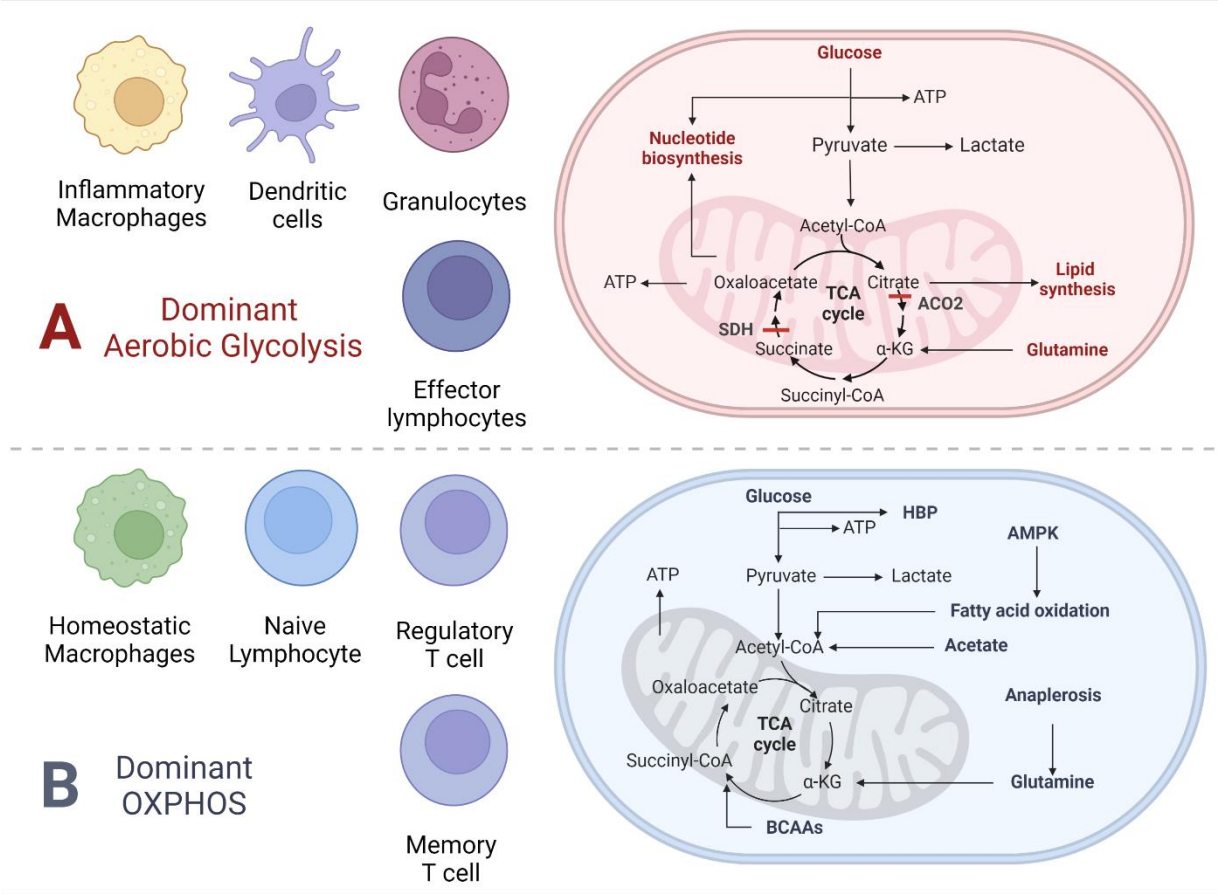

Figure 4. Immunometabolism

A. Activated leucocytes (inflammatory macrophages, DC, granulocytes, effector lymphocytes) predominantly function through aerobic glycolysis, in inflammatory macrophages in part due to destabilization of the TCA cycle on SDH and ACO2 enzymatic steps. B. Resting leucocytes (homeostatic macrophages, naïve lymphocytes, regulatory and memory T cells) function preferentially by oxidative phosphorylation.

1.5 The Hexosamine Biosynthesis Pathway and O-GlcNAcylation

1.5.1 Description of the hexosamine biosynthesis pathway (HBP)

The HBP (Figure 5) is considered as a nutrient sensor, as it integrates signals from glucose, glutamine, fatty acids and nucleotides (80). It uses 2-5% of glucose entering the cell and branches from glycolysis at the level of fructose-6-Phospate (F6P). F6P is transformed in conjunction with glutamine into Glutamine-6-Phosphate (GlcN-6-P) by the enzyme Glutamine-fructose-6-phosphate amidotransferase (GFPT/GFAT). GlcN-6-P undergoes the addition of an acetyl residue from acetyl-CoA by glucosamine-phosphate N-acetyltransferase (GNPNAT or EMeg32) to form Glucosamine-6-Phosphate (GlcNAc-6-P). GlcNAc-6-P loses 5 phosphates through the action of acetyl-CoA phosphoacetylglucosamine mutase (AGM1) to become Glucosamine-1-Phosphate (GlcNAc-1-P). Using Uridine-Tri-Phosphate (UTP), the enzyme UDP-N-acetylglucosamine pyrophosphorylase (UAP1) forms the end-product of the pathway, i.e., the high-energy metabolite Uridine diphosphate N-acetylglucosamine (or UDP-GlcNAc) (81). UDP-GlcNAc fuels different types of competing post-translational modifications (PTM): O-GlcNAcylation, versus branched O- and N-glycosylations.

GFAT is the rate limiting enzyme of the HBP. It is composed of 681 aa and weighs 77 kDA. There are two human *GFAT* genes, *GFAT1* located on chromosome 2 (82) and *GFAT2* on chromosome 5 in humans (83). While *GFAT1* is expressed ubiquitously, *GFAT2* is mainly expressed in the central nervous system. GFAT activity can be inhibited by UDP-GlcNAc (84) and Glucosamine-6P (85), but its main regulation is via phosphorylation on Serine 205 by cAMP-dependent protein kinase (86). Of note, 6-diazo-5-oxo-L-norleucine (DON) an analogue of Glutamine also modulates GFAT indirectly. Direct modulation of the HBP can be achieved using N-acetyl-D-Glucosamine (87), a nutrient that enter the HBP after the rate limiting

enzyme, overfeeding the pathway. Another possibility is the direct modulation of O-GlcNAcylation, using OGA inhibitor *in vivo* or *in vitro,* (Thiamet G (88) for example), or OGT inhibitor *in vivo* (89), or *in vitro* (OSMI-I (90) for example).

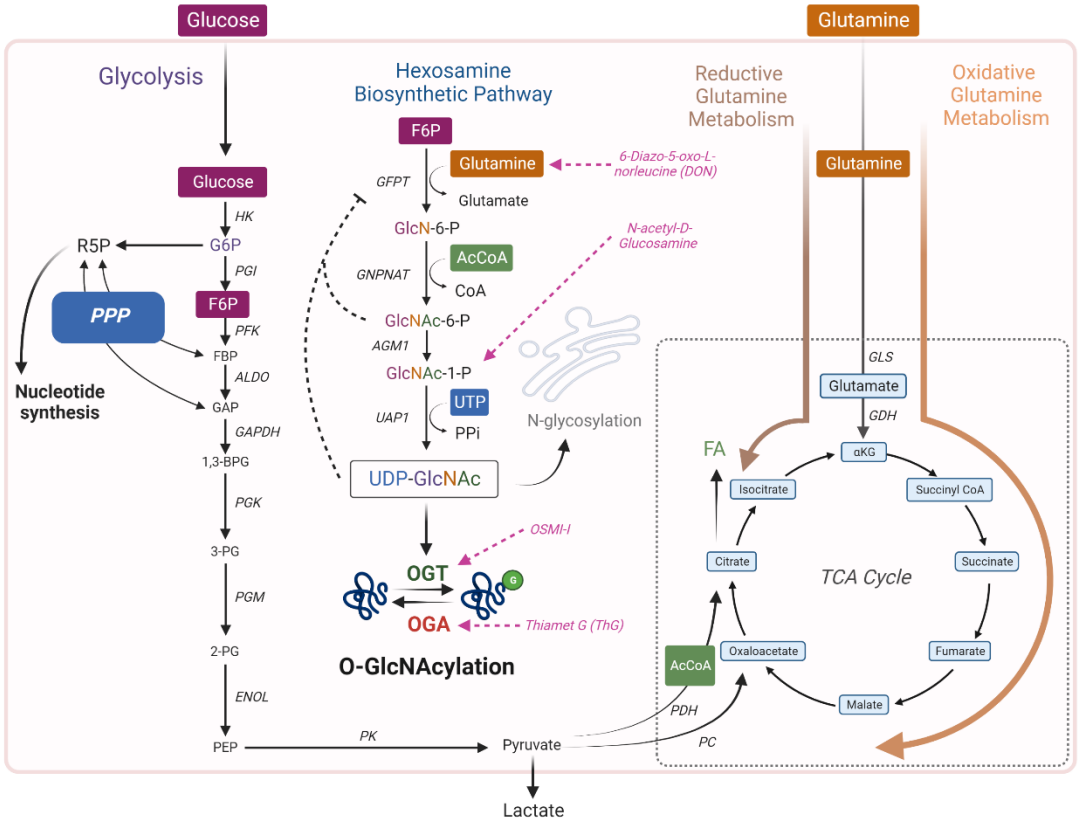

Figure 5. The HBP, O-GlcNAcylation, Glycolysis, and the TCA Cycle

The Hexosamine Biosynthesis Pathway (HBP) is depicted alongside with the two main energetic pathways in the cell (glycolysis and the TCA cycle). The HBP is fueled by F6P in purple, Glutamine in orange, Acetyl-CoA in green and UTP in blue. Potential modulators of the HBP: the glutamine analogue 6-diazo-5-oxo-l-norleucine (DON), N-acetyl-D-Glucosamine, an OGA inhibitor (Thiamet G) and the OGT inhibitor (OSMI-I) are presented in pink.

1.5.2 O-GlcNAcylation and OGT

Discovered by Torres and Hart in 1984 on the surface of lymphocytes (91), O-GlcNAcylation is a conserved PTM that links N-Acetyl-Glucosamine to a Serine/Threonine residue in target proteins. It has been described in all living organisms i.e., in microorganisms (92–96), plants (97) and animals (98). To date, more than 5000 O-GlcNAcylation substrates have been described, and are sensed in the O-GlcNAcome database (99) regularly updated and searchable online at https://www.oglcnac.mcw.edu. O-GlcNAcylation targets encompass nucleoporins, transcription factors, metabolic proteins, epigenetic regulators, mitochondrial proteins, kinases, enzymes and structural proteins (Figure 6), making O-GlcNAcylation a key mechanism governing a wide variety of physiological as well as pathological situations, such as memory/Alzheimer disease, cardiovascular system/cardiovascular disease, the metabolic syndrome, aging, cancer, embryonic development etc. (81). O-GlcNAcylation has been highlighted as an important regulator of innate and adaptive immunity (Figure 7 and 8), controlling immune development, differentiation and activation (100).

O-GlcNAcylation is regulated by two cellular enzymes, O-GlcNAc transferase (OGT) and OGlcNAcase (OGA) that adds and removes the O-GlcNAc, respectively (101).

OGT, also referred to as O-linked N-acetylglucosaminyltransferase or uridine diphospho-N-acetylglucosamine:polypeptide beta-N-acetylglucosaminyltransferase, was first isolated in 1992 from rat and rabbit livers (102,103), and its gene, *OGT*, described in 1997 (81,104).

The *Ogt* gene is highly conserved across species, with ~65% homology (105,106). Plants contain 2 *Ogt* genes (spindly *Ogt* and Secret agent *Ogt*) (97) while all animals, except zebrafish (107), contain only one *Ogt* gene (98).

In mammals, the *Ogt* gene is on the X chromosome, on the D region in mice and on the Xq13.1 region in humans (108), near to the *XIST* locus responsible for X-chromosome inactivation. It is composed of 22 exons and expressed in every cell type of the organism (Figure 6). Full body KO for *Ogt* is embryonically lethal because of its role in neurogenesis during development (108).

OGT exists in 3 forms (Figure 6), depending on alternative splicing, which vary by the number of tetratricopeptide repeats (TPR) at the NH_2 terminal. The rest of the protein encompasses the nuclear localization sequence and a catalytic domain separated by an linker domain of 120 aa which may play a role in protein-protein interactions (109,110).

The most abundant and ubiquitous form of OGT is the nucleo-cytoplasmic form that comprises 13 TPR and weighs 116 kDA. Mitochondrial OGT, implicated in respiratory chain protein O-GlcNAcylation, is composed of 9 TPR and weighs 103 kDA. The short form of OGT is present in the nucleus and cytoplasm and comprises only 3 TPR and weighs 78 kDa (109,111–114). Short-form OGT is regulated by O-GlcNAcylation (115) but its precise role remains unclear.

While some OGT targets interact with its catalytic domain, most substrates interact with the TPR that determine substrate specificity (116).

The activity of OGT is regulated by PTMs (115,117), including auto-O-GlcNAcylation and phosphorylation. The latter is enacted by GSK-3β or CaMKII (118–120) that enhance its activity, AMPK (121) that controls its subcellular localization, and Chk1 that enhances its stability (122). Overall, 20 sites (Ser/Thr/Tyr) in OGT can be phosphorylated (123).

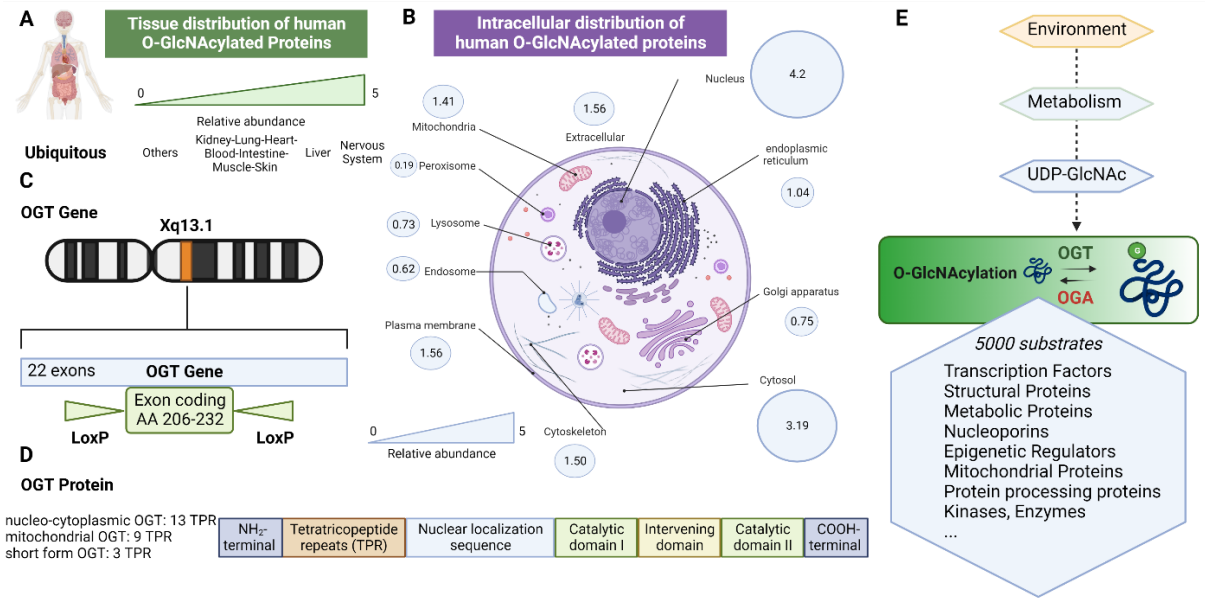

Figure 6. OGT gene, protein, O-GlcNAcylation tissue/intracellular distribution and targets

A. Tissue distribution of human O-GlcNAcylated proteins (in green), sorted by relative abundance. B. Cellular distribution of human O-GlcNAcylated proteins (in purple), sorted by relative abundance (score 0 to 5). C. OGT gene with representation of the LoxP sequence to remove it using the Cre-lox system. D. OGT proteins with numbers of TPRs depending on its isoform. E. Examples of families of proteins known to be O-GlcNAcylation targets.

1.5.3 HBP functions in immunity

1.5.3.1 Adaptive Immunity

O-GlcNAcylation has been implicated in T cell development, activation, and differentiation. Swamy and collaborators (124) have first demonstrated that thymic double negative T cells upregulate their glutamine and glucose uptake and subsequently their O-GlcNAcylation through Notch signaling. Importantly, inhibition of O-GlcNAcylation through T cell-specific *Ogt* loss resulted in reduced double positive thymocytes. T cell activation also leads to induction of O-GlcNAcylation and c-Myc, NF-κB and NFAT were demonstrated as OGT targets (125–127), leading to IL-2 production, T cell survival and IFNγ production (124). O-GlcNAcylation of FoxP3

is also required for the stability of the Regulatory T cells (T_{REG}) lineage as shown in mice (128), and loss of *Ogt* leads to a drift of the T_{REG} phenotype towards an effector phenotype. O-GlcNAcylation also mediates Th17 differentiation through O-GlcNAcylation of NF-κB and RORγt (129,130). Of note, recent work demonstrated that O-GlcNAcylation of STAT6 is a requirement for alarmin production by the epithelium upon helminth infection, leading to type II immunity promotion (131,132).

O-GlcNAcylation is also involved in B cell development, survival and activation (133). Activated B cells upregulate their glucose uptake and subsequent O-GlcNAcylation of NFAT and NF-κB, ultimately promotes B cells survival and activation. O-GlcNAcylation of Lyn on S19 is required for BCR signaling and Lyn interaction with Syk and for Germinal center B cell differentiation in lymph nodes and antibody response by plasma cells (134). Of note, O-GlcNAcylation and phosphorylation of Lsp1 is also implicated in apoptosis regulation of activated B cells, highlighting the importance of the interplay between those two PTMs (135).

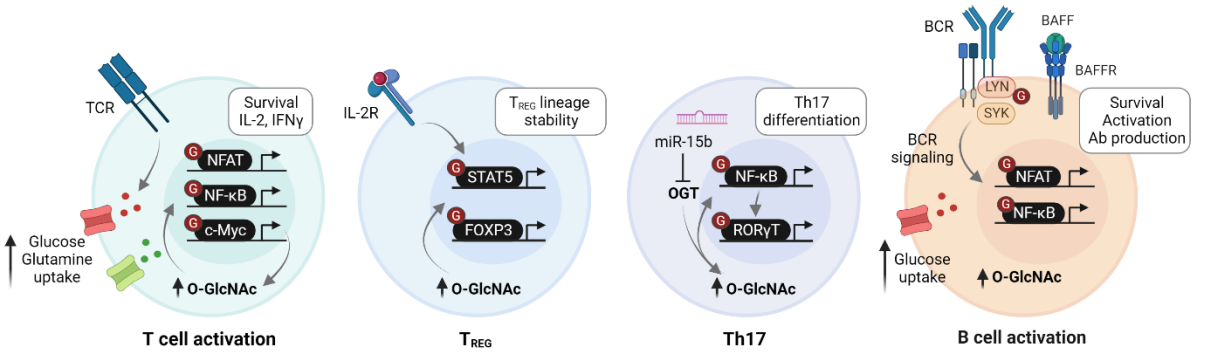

Figure 7. O-GlcNAcylation in adaptative immunity

O-GlcNAcylation is implicated in T cell activation and survival, stabilization of the T_{REG} lineage, Th17 differentiation and B cell activation, notably through O-GlcNAcylation of NFAT, NF-κB, c-Myc, FoxP3, STAT5, RORγt and Lyn (in red on the figure).

1.5.3.2 Innate Immunity

O-GlcNAcylation has also been described in innate immunity (136), albeit its impact on innate inflammation appears to be context-dependent (Figure 8).

Studies demonstrating a pro-inflammatory role of O-GlcNAcylation identified substrates in the NF-κB pathway requiring O-GlcNAcylation for full activity. For instance, the O-GlcNAcylation of RelA on T322 and T352 was associated with increased NF-κB activation (137). Another team demonstrated that RelA O-GlcNAcylation on T305 leads to efficient acetylation and subsequent transcriptional activity (138). S350 residue of c-Rel was also shown to be O-GlcNAcylated, a critical PTM for its DNA-binding activity (127). Similarly, S733 O-GlcNAcylation of IKKβ was reported to promote its activity (139). Besides the NF-κB pathway, O-GlcNAcylation of STAT3 on T717 has also been proposed to be pro-inflammatory, by preventing STAT3 phosphorylation downstream of IL-10 signaling (140), leading to induction of pro-inflammatory cytokines.

In contrast, an anti-inflammatory role for O-GlcNAcylation has also been described. For instance, the O-GlcNAcylation of RIPK3 on T467 was shown to prevent its phosphorylation and interaction with RIPK1, which inhibits necrosome formation and necroptosis-induced inflammation, while myeloid-specific loss of *Ogt* exacerbates LPS- induced endotoxemia (141). Indeed, loss of *Ogt* led to increased necroptosis, cytokine production, and decreased survival in an endotoxic shock mouse model. Necroptosis impairment through RIPK3 O-GlcNAcylation has also been shown as protective mechanism in liver fibrosis (142). These findings are consistent with the observation that enhancing O-GlcNAcylation by Glucosamine treatment alleviates sepsis in a mouse model (87). In a high-fat-diet mouse model, mTOR-mediated S6K1

phosphorylation is countered by its O-GlcNAcylation, which blunts metabolic inflammation in macrophages (143).

O-GlcNAcylation has also been proposed as a potent antiviral mechanism acting directly on viral restriction or through anti-viral immunity, which can also lead to viral infection-induced cytokine storm. N-acetyl-D-Glucosamine was shown to boost the capacity of HIV-1-infected lymphocytes to suppress viral transcription. OGT overexpression inhibited the activity of the HIV-1 LTR promoter, trough SP1 O-GlcNAcylation (144). This was also shown for Kaposi's Sarcoma-associated Herpesvirus (145,146). Two other teams have identified MAVS as an OGT target. In VSV infection of Macrophages, MAVS O- GlcNAcylation on S366 is required for its K63-linked poly-ubiquitination and downstream signaling (147). Similar findings were reported by Song and colleagues who linked MAVS O- GlcNAcylation to IRF3 activation and IFNβ production in VSV or Influenza A infection of Macrophages (148). In influenza A virus infection, OGT O-GlcNAcylates IRF5 on S430, which promotes its K63-linked poly-ubiquitination and function in viral induced cytokine storm (149). The role of O-GlcNAcylation in antiviral immunity extends beyond IRF5 and MAVS. In hepatitis B virus, O-GlcNAcylation of the interferon-induced restriction factor SAMHD1 on S93 limits viral replication by promoting SAMHD1 stability and antiviral activity (150). Independent from interferon mediated immunity, the long noncoding RNA EDAL exerts its anti-viral effects by binding to O-GlcNAcylated EZH2 and promoting its degradation (151).

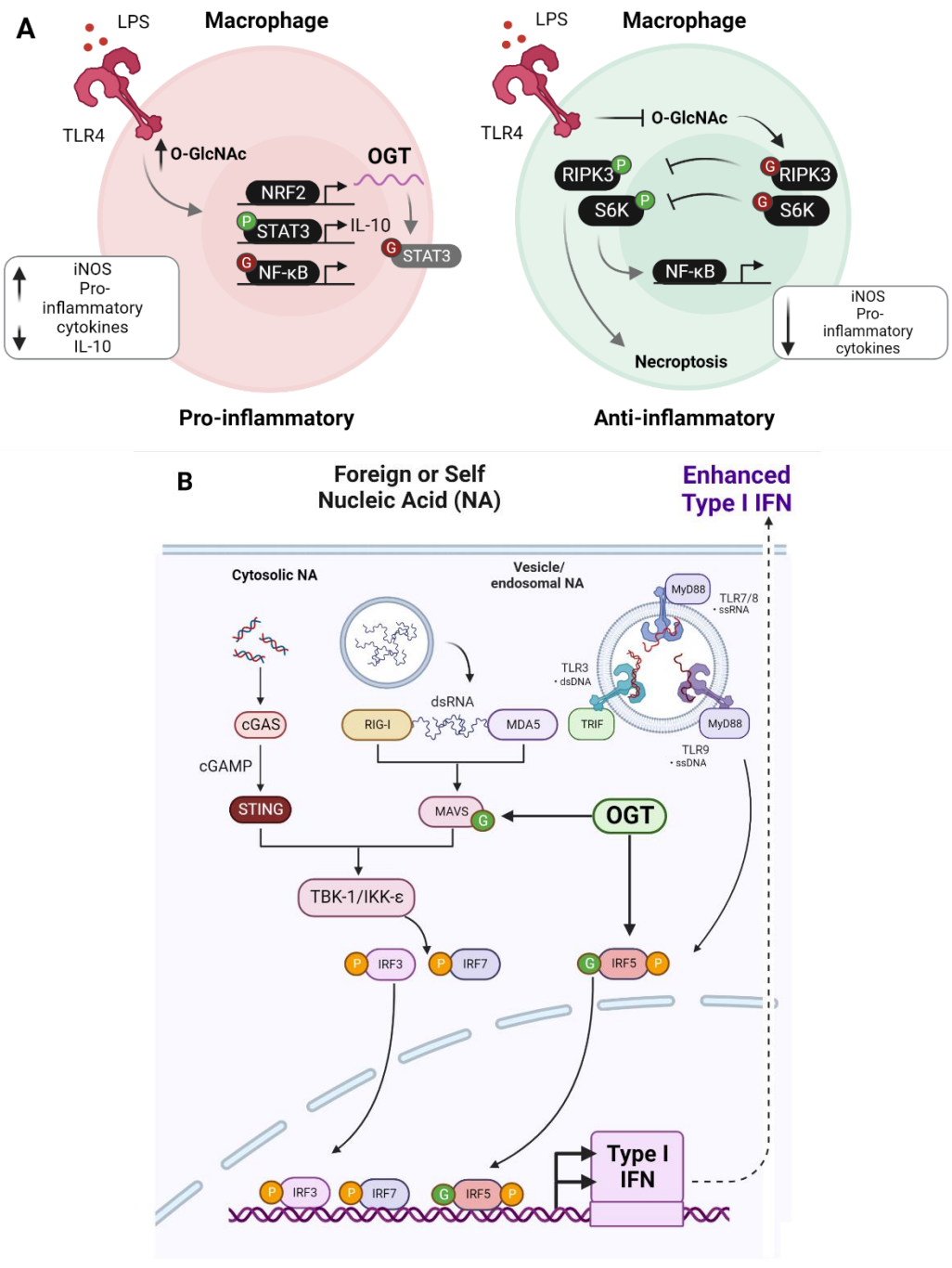

Figure 8. O-GlcNAcylation in innate immunity

A) O-GlcNAcylation can exert pro- and anti-inflammatory effects in macrophages. Left, O-GlcNAcylation promotes inflammation by preventing STAT3 phosphorylation and downstream IL-10 secretion; right, O-GlcNAcylation inhibits inflammation: on one hand, O-GlcNAcylation of RIPK3 prevents its phosphorylation and downstream necroptosis; it also blunts S6K phosphorylation and activation. B) In antiviral immunity, O-GlcNAcylation of MAVS or IRF5 is required for the promotion of type I IFN.

The notable immune effectors affected by O-GlcNAcylation are summarized below.

Table 1. Notable immune effectors that are affected by O-GlcNAcylation.

Substrate	Known Residue	Impact of O-GlcNAcylation	References
NFAT	T224, S225, S314, S333, S633, T733, S850	Activation	(152), (153), (154)
NF-κB	S65, S399, T423, T810, T859	Activation	(152), (154), (155), (156), (157), (158), (159), (160)
c-Myc	-	Activation	(125)
STAT5	T92	Activation	(156), (161), (162)
FoxP3	S33, T38, S57, S58, S59, T72, S270, S273, S285, T286	Stabilization	(128)
c-Rel	T254, T258, S350, T378, S382, S409, T425	Activation	(130), (152), (155), (157), (163), (164), (165)
Lyn	S19	Activation	(134)
STAT3	T714, T716, T717, S719, T721, S727	Activation	(152), (153), (155), (157), (158), (164), (166), (167), (168), (169), (170), (171), (172), (173), (174), (175)
RIPK3	T467	Inhibition	(141), (176), (177)
S6K1	S489	Inhibition	(143)
MAVS	T244, S246, S249, T250, T252, S253, S255, S256, S257, S284, S285, S293, T301, T307, T321, T328, S329, S330, S338, T342, S347, T365, S366, T370	Activation	(147), (148), (152), (153), (154), (157), (160), (164), (166), (169), (170), (171), (178), (179), (180), (181)
IRF5	S430	Activation	(149)
IRF7	T93	-	(170)
MyD88	S230	-	(182)
TLR3	T638	-	(153)

The known O-GlcNAcylated residue are extracted from the O-GlcNAc database (99).

1.6 Systemic Lupus Erythematosus

1.6.1 Epidemiology and Health Burden

SLE is a rare systemic autoimmune disease that affects 9 women for 1 man. Its estimated prevalence is 47.0/100,000 inhabitants in France and 48/100,000 inhabitants in Canada. Its however highly variable around the world from 13 to 7,713.5/100,000. This variation could be explained by genetic or environmental factors as well as discrepancy in study design or definition of cases (183,184).

Although rare, SLE represent a significant economic burden for society due to the overall costs of necessary care, estimated between 13494 and 55344 US dollars per patient per year (185). Economic considerations aside, SLE is primarily a serious public health problem by being one of the leading causes of death among young women (186). Patients with SLE also present higher mortality rate of all causes, in particular from cardiovascular diseases, renal diseases and infections (187). The latter is partly due to immunosuppressive treatments side effects. SLE can result in disability and impairs the quality of life of patients (188).

1.6.2 Clinical and biological manifestations

The clinical manifestations of SLE are heterogenous among patients (Figure 9), ranging from benign clinical forms to severe potentially life-threatening visceral manifestations. SLE evolves by successive flares with remission periods in-between. It is characterized by a break of tolerance to self-nuclear antigens and development of autoantibodies directed against self-nuclear antigens such as anti-Sm, SSA, SSB, RNP and anti dsDNA antibodies (189). Active SLE is characterized by elevated titers of these dsDNA antibodies that correlate with complement activation and decreased levels of C3, C4 and/or CH50. The most common manifestation is cutaneous, concerning 70-80% of patients, inaugural for 25% of patients. Other manifestations include vascular lesions e.g., livedo racemosa, an erythematous or violaceous skin discoloration. Articular manifestations also concern around 80% of patients with arthralgia and nondestructive polyarthritis. Apart from cutaneous and articular symptoms, one of the main manifestations that affects patient prognosis is lupus nephritis. It is a severe glomerular injury that leads, without immunosuppressive treatment, to terminal kidney failure and death (190). Neuro-SLE is a heterogenous appellation that regroups 19 types of

neuropsychiatric manifestations of lupus, ranging from central nervous system injuries such as aseptic meningitis, myelitis, confusion, cognitive impairment up to psychotic manifestations or peripheral nervous system involvement, like demyelinating polyradiculopathy. The activity of the disease is evaluated by the SLEDAI-2K index (191), a SLEDAI-2K ≥ 6 defining an active disease. The SLEDAI-2K score is presented in appendix 1.

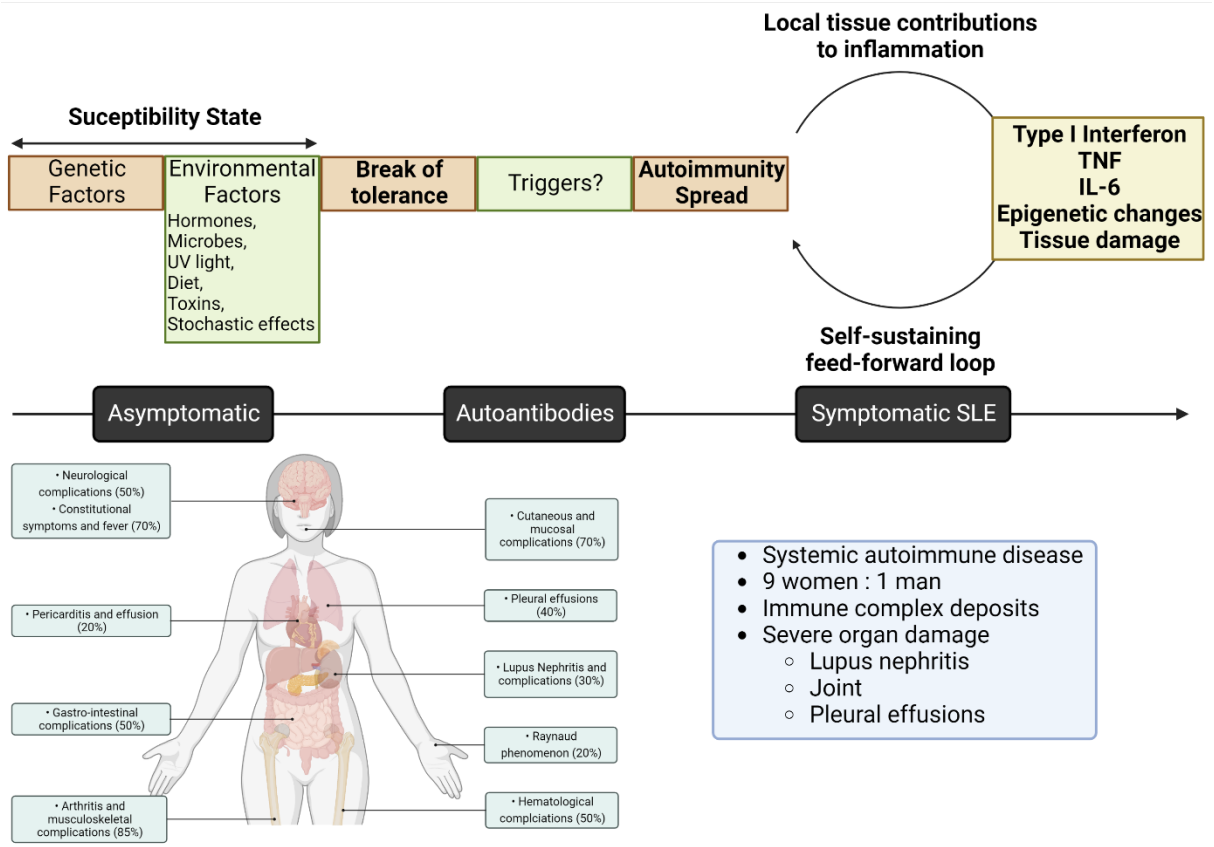

Figure 9. SLE disease course and clinical manifestations

Development of SLE, from susceptibility state to break of tolerance to self-nuclear antigens and autoimmunity spread (upper-panel). Main clinical manifestations of SLE (lower panel).

1.6.3 Standard of care

SLE management first relies on general interventions, prescribed to every patient, regardless of their symptoms or organ involvement. These encompass sun protection to prevent disease flares triggered by UV light, vaccinations to diminish risk of infections, general cardiovascular

protection with physical exercise, help to avoid or stop tobacco use, control of body weight, blood pressure or eventual dyslipidemia or associated diabetes, as SLE represents a true cardiovascular risk factor which adds up to the classical ones, described by Framingham (192). Apart from these general measures, in the absence of contraindication, all patients receive Hydroxychloroquine (HCQ), as this treatment reduces disease flare and improves survival (193). Beyond HCQ, treatment is adjusted to every patient on a treat to target basis to achieve SLE remission (defined as a SLEDAI-2K activity index at 0, prednisone discontinuation and HCQ treatment alone) or low disease activity (defined as a SLEDAI ≤ 4, HCQ treatment with prednisone ≤ 7.5 mg/day of prednisone equivalent) (194).

Glucocorticoids are used to treat disease flares and should be kept at the lowest dose possible e.g., a dose < 7.5 mg/day of prednisone equivalent to avoid side-effects. To avoid high dose prednisone durations, a steroid-free immunosuppressive regimen is used in SLE uncontrolled by HCQ alone. This associates methotrexate or azathioprine in mild to moderate SLE, calcineurin inhibitors, mycophenolate mofetil or belimumab in moderate SLE, and in severe forms of SLE, cyclophosphamide or rituximab. The SLE-associated antiphospholipid syndrome (APL) is controlled with low dose aspirin and warfarin (194,195).

1.6.4 New therapies in SLE

SLE has long been a translational graveyard. Several promising phase II disease modifying therapies failed to reach significance in phase III trials (196). Only two new targeted therapies have been approved in 50 years, namely Belimumab, an anti-Blys/BAFF monoclonal antibody (197), and more recently, Anifrolumab, an anti-type I IFN Receptor subunit-1 antibody, based on positive results from two phase III clinical trials termed “Treatment of Uncontrolled Lupus

via the Interferon Pathway" TULIP-1 (NCT02446912) and TULIP-2 (NCT02446899) (198,199). Of note, in a phase 2 trial of lupus nephritis (TULIP-LN), Anifrolumab failed to reach the primary endpoint although encouraging results were reported for the secondary endpoints (200). In addition, some concerns about increased viral infections were raised particularly with herpes zoster virus and influenza. However, a recent meta-analysis reported significantly lower incidence of severe adverse effects, with most cases of upper respiratory tract viral infections being mild or moderate and only the risk of herpes zoster was confirmed (201).

Of note, apart from global type I IFN targeting, specific targeting of pDCs, a major source of type I IFN in SLE, has been described both in humans and mice. In a phase 1 study of healthy volunteers (n=54) and a limited number of SLE patients (n=12), an anti-BDCA2 humanized monoclonal antibody against a specific receptor of pDC, showed a good safety profile and a decreased expression of IFN response genes in blood as well as reduction of skin lesions evaluated by the cutaneous lupus activity score CLASI-A (202). A monoclonal antibody against immunoglobulin-like transcript 7, a specific pDC marker, also demonstrated a reduction of pDC skin infiltration, decrease type I IFN response and improvement of clinical symptoms evaluated by the CLASI-A (203).

Calcineurin inhibitors have been success in combination with standard of care for lupus nephritis management. Voclosporin, a novel Calcineurin inhibitor with a better safety profile than the classical ones (Tacrolimus/Cyclosporine) has also been recently approved by the FDA in the US (204). Calcineurin inhibitors prevent the translocation of NFAT, which reduces the pro-inflammatory response of T cells. In podocytes, inhibition of calcineurin prevents the dephosphorylation of synaptopodin, an actin-associated protein of differentiated podocytes,

which stabilize the podocyte cytoskeletal structure, therefore reducing proteinuria. Of note, both NFAT and synaptopodin are O-GlcNAcylation targets (99).

1.6.5 Sex bias in SLE

1.6.5.1 Hypothesis 1: Hormonal influence

SLE is characterized by a strong sex-bias, affecting 9 women for one man. This has raised early on a suspicion for implication of hormones in the pathogenesis of the disease (205), an argument reinforced by SLE flares occurring in pregnancy (206) or the acceleration of the SLE phenotype or increase autoantibodies titer in lupus-prone mice treated with estrogens (207,208). Several mechanisms have been proposed including a stimulatory effect of estrogen on the cAMP pathway leading to augmented B and T lymphocyte activation and increased pro-inflammatory cytokines production (209). Betty Diamond and colleagues highlighted that estrogen upregulates CD22, SHP-1, Bcl-2, and promotes autoreactive B cells survival and activation, especially marginal zone B cells, through engagement of estrogen-receptor α (210–212). Deletion of estrogen-receptor α in B cells alleviates lupus nephritis in the NZB/NZWF1 model of lupus prone mice (213). Moreover, estrogen induces the transcription of activation-induced deaminase (AID), driving somatic hypermutation and class switch recombination (214). It also promotes type I IFN production by pDC. Indeed, estradiol treatment enhances TLR7 and TLR9 mediated IFN response in human pDC and estrogen receptor α expression in pDC promotes their cytokine secretion in mice, while its blockade during pDC development leads to decreased IFN-α production (215,216). Nonetheless, the sex bias is not limited to women of child-bearing age but extends beyond menopause, advocating for additional hormone-independent mechanisms.

1.6.5.2 Hypothesis 2: Bi-allelic expression and overexpression of X-linked genes

The X chromosome contains several immune related genes. Seminal work groups has shown that X-chromosome Inactivation (XCI) could be bypassed leading to bi-allelic expression or overexpression of immune related genes (217). An implication of X chromosome regulation in SLE is supported in humans by the increased prevalence of the disease in 47XXY Klinefelter individuals, having the same risk of developing SLE as ethnically-related women (218,219). This is also the case in the Yaa mouse model, in which mice develop SLE because of a translocation of the TLR7 locus on the Y chromosome, leading to its biallelic expression (220). Interestingly, Guéry and colleagues have highlighted that bi-allelic expression of TLR7 also occurs in pDC and B cells in Klinefelter individuals, further supporting a role of biallelic expression of X linked genes in the pathophysiology of SLE (221). This has been confirmed by Hagen *et al* in human pDC where pDC with a bi-allelic expression of TLR7 present increased IFNα and IFNβ expression (222). Guéry and colleagues also demonstrated in B cells that TLR7 bi-allelic expression is associated with increased IgG^+ B cells and plasmablast differentiation (221). Of note, Hewagama *et al.* showed that treatment of T cells with 5-azacytidine, a DNA-methyltransferase inhibitor, result in increased expression of a subset of X-linked genes, including *OGT*. Enhanced expression of OGT correlated with SLE disease activity (223), and another team demonstrated an overexpression of the Xq13 region of the X chromosome (that contains OGT) in SLE B cells (217). However, OGT is not part of the bi-allelically expressed genes identified in human B cells (224), which could advocate for a different role of OGT in SLE pathogenesis.

1.6.6 Genetic predisposition and environmental triggers

Although a partial concordance of 25% in monozygotic twins (225) has been described in SLE, genetics alone is insufficient for the development of the disease, with the exception of rare monogenic SLE. GWAS studies have identify 150 susceptibility loci in SLE (226,227). The most notable genetic risk factors are loss-of-function variants in *C2*, *C4*, *C1q*, *TREX1*, and *Dnase1L3*, the latter highlighting the impact of DNA clearance defects on the pathogenesis of SLE (45,46). For example, a recent study described a TLR7 gain of function variant, $TLR7^{Y264H}$. This missense variant identified in a pediatric human SLE case was sufficient to induce a murine SLE, highlighting the predominant role of TLR7 signaling in SLE (228). However, other genetic deficiencies have been implicated in SLE by murine models such as DEF6 and SWAP-70 (two guanine exchange factors) (229), or the kinase Lyn (230), for example.

A wide range of environmental factors have been described as potentially implicated in SLE. Among these are UV light , tobacco use, viral infections (231,232), crystalline silica exposure, diet or medications and microbiota alterations (48).

1.6.7 Immunology of SLE

1.6.7.1 Innate Immunity

1.6.7.1.1 Granulocytes

Neutrophils in SLE, especially a specific subset with low density granules, produce elevated levels of NETs in response to increased autophagy mediated by the hypoxia-response and stress-response protein DDIT4/REDD1 (233). NETosis releases DNA and DAMPs, such as HMGB1. After internalization of immune complexes through RAGE-HMGB1 in pDC, TLR9

activates, leading to elevated production of type I IFN (234–237) (Figure 10). Neutrophils phagocytotic capacity is impaired (238) and they exhibit increased ROS production (239). Low density granulocytes also promote a proinflammatory cytokine production by $CD4^+$ T cells (240). Data on eosinophils implication in SLE are surprisingly lacking in the literature, except for increased urinary eosinophils count, eosinophil cationic protein and IL-5 in lupus nephritis (241). A recent meta-analysis (242) concerning basophils in SLE highlighted that elevated IgE and activated basophils were correlated to disease severity and active lupus nephritis. Basophils increase B cell proliferation and differentiation into plasma cells and can promote Th17 differentiation. Treatment with anti-IgE monoclonal antibodies, e.g., Omalizumab, decreases type I IFN production and pDC and basophils activation (243).

1.6.7.1.2 Monocytes and macrophages

Monocytes and macrophages are enriched in SLE, in particular in Lupus Nephritis, as recently shown by single cell RNA sequencing (244). These cells could represent a therapeutic target in SLE treatment (245). Macrophages in SLE present a defect in apoptotic bodies clearance, leading to prolonged exposure of self-antigens (246,247). SLE macrophages present differences in gene expression profiles with increased M1 (STAT1, SOCS3) and decreased M2 (STAT3, STAT6 or CD163) markers (248). Endogenous macrophage depletion using clodronate worsen the ALD-DNA SLE mouse model, an effect abolished by adoptive transfer of BM derived macrophages differentiated *in vitro* into an “M2-like” or “M1-like” state, revealing a protective role of M2 macrophages in this model (249). Non-classical or patrolling monocytes, have also been implicated in SLE and other chronic inflammatory diseases (19). They patrol the kidney glomeruli to maintain its homeostasis (250) and accumulate in glomerular vessels

of SLE patients (251). Moreover, SLE patients with severe lupus nephritis present with high glomerular titers of $CD16^+$ monocytes, which could reflect organ recruitment, without indication on their exact role in SLE (252). Monocytes and macrophages can also be a secondary source of type I IFN production through TREML4-TLR7 (253) and the cGAS-STING (254) pathways, a mechanism modulated by mTOR inhibition. Non-discriminatory inhibition of myeloid cell genesis or recruitment e.g., using antagonists of CSF1R, CX3CL1 or CCL2 has proven efficient in attenuating lupus nephritis in mice (255–258). However, since some myeloid subsets are protective, it is important to characterize strategies that only deplete or inhibit deleterious myeloid subsets in human SLE for targeted therapy.

1.6.7.1.3 Plasmacytoid and conventional dendritic cells

pDC associate with disease severity and activity in SLE (259) and an elevated type I IFN signature has been confirmed by several groups in pDC (260) as well as others in myeloid cells and tubular cells (20,244,261). pDC take up immune complexes through engagement of FcγRIIa and after their endosomal internalization, these immune complexes are recognized by endosomal TLR7 and TLR9, leading to type-I IFN secretion (262). Type I IFNs produced by pDC initiate and maintain classical DC maturation, leading to their activation (259,263) and increase expression of activation molecules such as CD80, CD86 and MHCII (264). cDC present antigens to T cells, leading to their aberrant activation (265,266) and differentiation into a pro-inflammatory T helper phenotype, particularly Th17 (267). More specifically, type I IFN and DCs trigger the production of BLyS/BAFF and APRIL, which in turn promote autoreactive B cell survival (268). Moreover, Type I IFNs drive the expansion of Ig producing B cells downstream of TLR7 (269,270). Immune complexes also induce the expression of OX40L on DC, leading to

TFH differentiation, which are required for plasma cell stimulation. OX40L has also been shown to impair regulatory T cell function (271,272) (Figure 10).

1.6.7.1.4 NK cells and non-conventional lymphocytes

SLE patients present decreased peripheral NK cell counts, which correlates with disease activity. NK cells in SLE exhibit decreased cytotoxicity (273), but increased IFNγ production (274) linked to disease activity. Furthermore, they have higher expression of activating receptors and decreased expression of inhibitory molecules. However, studies on the role of NK cells in SLE are scarce (275). ILC1 and ILC3 are elevated in SLE patients and exhibit an activated phenotype e.g., increased surface expression of FAS. Their expansion correlates with a high IFN signature in patients (276). In contrast, ILC2 are decreased (277). In MRL-*lpr* lupus prone mice, kidney-resident ILC2 are decreased and inhibited by IFNγ and IL-27 produced by T cells and myeloid cells (278).

1.6.7.2 Adaptive Immunity

1.6.7.2.1 T cells

SLE is characterized by an imbalance between pro-inflammatory Th17 cells increase (Figure 10) and a decrease in tolerogenic T_{REG}. T_{REG}, characterized by their expression of CD25 and FoxP3, present impaired function and reduced numbers in SLE (279). Active SLE patient platelets interact with T_{REG} through P-selectin binding to PSGL-1 (P-selectin glycoprotein ligand-1) on T_{REG}, resulting in impaired T_{REG} function (280). Th17 cells, an effector T helper cell expressing IL-17, infiltrate tissues and mediate tissue injury alongside with

monocytes/macrophages and neutrophils by promoting their recruitment at the inflammatory site (281). IL-17 titers are elevated in patients with active LN (282), the double negative population of T cells expanded in the MRL-*lpr* model is a major source of IL-17 (283) and MRL-*lpr* deficient in *Il23r*, implicated in Th17 maintenance, present lower dsDNA and reduced LN (284). IFNγ levels are correlated to SLEDAI activity index (285) and Th1 cells, the main effector T cells producing IFNγ and expressing T-bet. IFNγ promotes B cell class switch and thus drives autoantibodies production, in part by participating in the formation of ectopic germinal center (GC) and T_{FH} accumulation in these GC (286). T_{FH} cells, $CD4^+$ T cells that differentiate in the presence of IL-6, IL-21 and ICOS, promote B cell activation, and are expanded in SLE patients' blood (287). T_{FH} cells are induced by OX40L from myeloid cells (271), promote autoantibodies production by B cells in aberrant GC (288) and accumulate in inflamed kidneys (289) (Figure 10). A recently described $CD4^+$ $CXCR5^-$ $PD\text{-}1^+$ T cell subpopulation called T_{PH} (T Peripheral Helper) are also expanded in SLE blood and are present in kidneys of patients affected with LN. T_{PH} cells produce high quantity of IL-21 and mediate SLE inflammation by promoting B cell antibody production and response in peripheral sites (290–292). Finally, circulating $CD8^+$ T cells present an impaired cytotoxic function and exhausted phenotype in the blood (293) but a cytotoxic profile is maintained in tissues, as highlighted by scRNA-seq analysis of LN (294).

1.6.7.2.2 B cells

In SLE, B cells secrete auto-antibodies directed against self-nuclear components. Several studies have investigated the reason of this break of tolerance, and two hypotheses are proposed: bone-marrow central checkpoint defects resulting in persistence of a high-affinity autoreactive repertoire of B cells (295), or expansion of autoreactive B cells in the periphery

(296). Abnormal Ig secreting B cells arise in germinal centers or are extrafollicular (297). Although complementary, extrafollicular B cells have been suggested as able to generate autoimmune plasmablast independent of the germinal center pathway, as in the Dnase1l3$^{-/-}$ model (298).

Post-GC B cells and plasma cells are increased in SLE patients (299), and the B cell repertoire is abnormally shaped towards autoimmunity with abnormal selection, increased somatic hypermutation and receptor editing (296). Apart from GC maturation, persistence of autoreactive B cells can also be induced by cytokine activation of B cells through BAFF, which promotes self-reactive B cell survival (300). A recent work has demonstrated, using the *Dnase1l3*$^{-/-}$ model, that autoreactivity can arise from extra-GC B cell differentiation into plasmablasts (298). Moreover, a specific population of CD19^{+}CD24^{high}CD38^{high} B cells, called Regulatory B cells (B_{REG}), that produce the anti-inflammatory cytokine IL-10, present an impaired function in SLE, therefore promoting inflammation (301). Finally, a CD11c^{+}Tbet^{+} B cell subset, termed ABC (age-associated B cells), or atypical memory B cell population, has recently been described in SLE (297,302). ABC produce pro-inflammatory cytokines, present impaired co-stimulatory capacity, are prone to apoptosis and exhibit increased activation of mTORC1 (303). They promote T_{FH} differentiation (304), and have been implicated in various contexts such as autoimmunity, aging or infections (302).

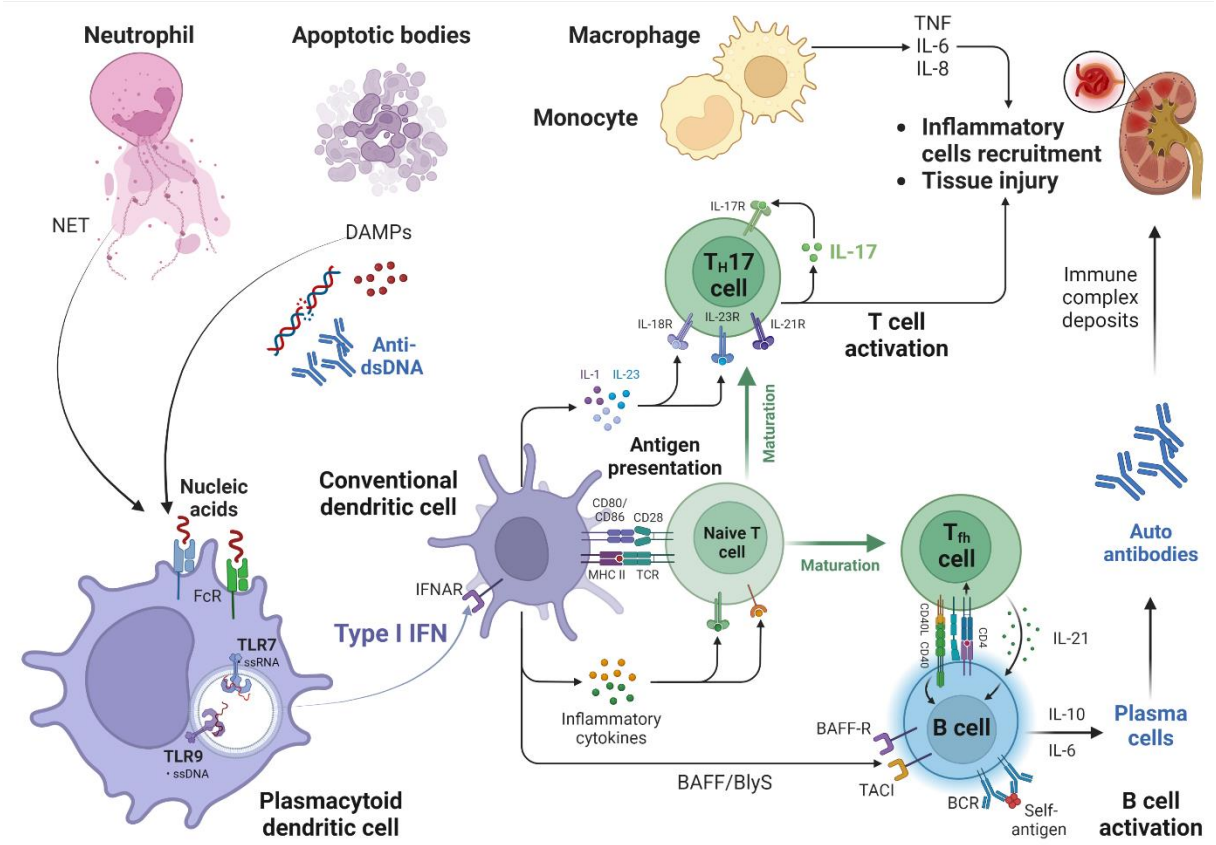

Figure 10. SLE immune physiopathology

Apoptotic cells release DAMPs, neutrophils release NETs, leading to the activation of pDC, the main cell type producing Type I IFN. Upon activation, pDC activate cDC which present the antigen to T cells. T cells can differentiate into Th17 and mediate, alongside with monocytes and macrophages, tissue injury. On the other hand, T cells cooperate with B cells, leading to the production of autoantibodies, amplifying the auto-immune loop.

1.6.8 SLE Mouse models

Several mouse models were developed to allow the investigation of the molecular pathways leading to SLE (305). These can be broadly divided into 2 groups, spontaneous (i.e., genetic) or experimental (i.e., inducible) models.

1.6.8.1 Spontaneous genetic models of SLE

One of the first model developed was the first generation of the breeding of the New Zealand Black crossed with the New Zealand White, NZB/NZWF1 mice (306). This mouse model recapitulates the secretion of autoantibodies and glomerulonephritis with mild vasculitis and affects female more than male mice but lacks other features of disease like cutaneous or articular involvement. Subsequent breeding of these NZB/NZWF1 mice gave rise to several NZM strains including the NZM2328, which develop a two stage renal disease in addition to the NZB/NZWF1 phenotypes, and the NZM2410 mouse that presents with the same phenotype as NZB/NZWF1 but without the vasculitis, and with a normal sex-ratio (307). This background was used by Laurence Morel and colleagues to generate the B6.NZM*Sle1*$^{NZM2410/Aeg}$*Sle2*$^{NZM2410/Aeg}$*Sle3*$^{NZM2410/Aeg}$ triple congenic mice also referred to as B6.NZM*Sle1/Sle2/Sle3.* This triple-congenic mouse, on a mixed NZM2410 and C57BL6/J background, expresses 3 SLE recessive susceptibility loci. *Sle1* on chromosome 1 mediates loss of tolerance to nuclear antigens through high specificity for the H2A/H2B/DNA sub-nucleosome. *Sle2* on chromosome 4 lowers the activation threshold of B cells leading to their expansion and polyclonal IgM secretion. *Sle3* on chromosome 7 mediates $CD4^+$ T cell dysregulation by impairing AICD (308). Last, the BXSB mouse or Yaa mice harbor a translocation of the *Tlr7* region of the X chromosome on the Y chromosome, in a locus called *yaa*, resulting in biallelic expression of Tlr7, increased type I IFN secretion (309–311), and an SLE like glomerulonephritis associated with dsDNA antibodies in male mice (Table 2).

1.6.8.2 Inducible models of SLE

The Pristane model of SLE is induced by the injection of a mineral oil, Pristane, in the peritoneum of BALB/c or C57BL/6 mice. This results in glomerulonephritis, arthritis and autoantibodies secondary to peritoneal irritation induced by Pristane (312). The ALD DNA model consists in immunization of BALB/c mice with subcutaneous injections of highly purified DNA from activated lymphocytes, resulting in dsDNA autoantibodies and glomerulonephritis development (313). The Resiquimod model relies on cutaneous administration of the Tlr7 ligand Resiquimod, and elicits in BALB/c mice a lupus-like phenotype with autoantibodies, splenomegaly and glomerulonephritis (314) (Table 2).

1.6.8.3 Mouse models used in this study

Because none of the mouse models recapitulates the full spectrum of human SLE, it is recommended to explore more than one model for SLE investigation. In our studies, we chose two genetic models *i.e.,* i) the MRL-*lpr* and ii) the *Dnase1l3*$^{-/-}$ mouse models.

1.6.8.3.1 MRL-*lpr* model

The MRL-*lpr* mouse is widely used. It has a complex genetic background derived from four strains (LG, B6, AKR and C3H) termed MRL-MpJ, and carries a homozygous mutation in the death receptor *Fas* (*lpr*). While MRL-MpJ mice without the *Fas* mutation develop a mild autoimmune phenotype at a late timepoint (~18 months of age) (305), MRL-*lpr* mice exhibit a lymphoproliferative syndrome, systemic autoimmunity and rapid fatal kidney failure at 18-24 weeks. Starting at 8 weeks, MRL-*lpr* mice develop numerous SLE autoantibodies (antinuclear antibodies (ANA), anti-dsDNA, anti-Sm, anti-Ro and anti-La), massive lymphadenopathy

associated with proliferation of aberrant double negative $CD3^{+}CD4^{-}CD8^{-}$ T cells, arthritis, skin rash, cerebritis and early onset immune complexes-induced glomerulonephritis (Table 2). The disease is accelerated in female mice but is present in both sexes. We initially used this model to investigate the effect of global pharmacologic modulation of O-GlcNAcylation on disease phenotypes. We chose this model for the following reasons: a) it is commercially available (JAX stock #000485); b) it leads to a strong and rapid autoimmune phenotype, c) its widely used by other groups allowing reproduction of our work by others, and d) it permits swift investigation of potential drug candidates modulating O-GlcNAcylation.

1.6.8.3.2 *Dnase1l3*$^{-/-}$ model

The *Dnase1l3*$^{-/-}$ mouse model was developed and described by Sisirak and colleagues in 2016 (315). This model recapitulates human *DNASE1L3* deficiency that is associated with early onset SLE. The *Dnase1L3* deficiency leads to accumulation of DNA in microparticles released by dying cells, leading to a break of tolerance and production of anti-DNA autoantibodies causing SLE development in mice. The increase in dsDNA autoantibodies is observed between 4 and 45 weeks, associated with increased myeloid blood count, activated T cells and decreased marginal zone B cells. At 50 weeks of age, *Dnase1l3*$^{-/-}$ mice present mild splenomegaly associated with mild kidney injury (315). A recent work highlighted that this model develop anti-dsDNA from extrafollicular plasmablasts (298). We chose to use this model to address the role of *Ogt* in specific immune compartments. We generated two triple congenic mouse strains harboring a full deletion of *Dnase1l3* together with tissue-specific deletion of *Ogt* using the Cre lox system, either in all myeloid cells (LysM-cre) or in DC (CD11c-cre).

Table 2. Main spontaneous and inducible SLE Mouse models

Model	Kidney	Cut.	Art.	Other	Anti-dsDNA antibodies	Other auto antibodies	Sex Ratio
NZB/NZWF1	+	-	-	Vasculitis	+	-	F>M
NZM2328	2 stages renal disease	-	-	Vasculitis	+	-	F>M
NZM2410	+	-	-	-	+	-	F=M
B6.NZM*Sle1 /Sle2/Sle3*	+	-	-	Splenomegaly	+	-	F=M
BXSB *Yaa*	+	-	-	-	+	-	M
MRL-*lpr*	+	+	+	Cerebritis	+	+	F=M
***Dnase1l3*$^{-/-}$**	+	-	-	Splenomegaly	+	+	F=M
Pristane	+	-	+	-	+	+	-
Resiquimod	+	-	-	Splenomegaly	+	-	-
ALD DNA	+	-	-	-	+	-	-

1.6.9 Targeting metabolism in SLE

Since the first observations of increased mTOR activation and enhanced glycolysis in SLE T cells, immunometabolism studies have unraveled several metabolic alterations in SLE immune cells that contribute to pathogenesis (316) and investigators have seeked to modulate SLE phenotype through metabolic intervention. Indeed, treatment of the B6.NZM*Sle1/Sle2/Sle3* with the glycolysis inhibitor 2-DG that blocks the function of hexokinase, impairs the autoimmune T_{FH} and B cells but not the humoral response towards viral infection, highlighting the metabolic differences between autoreactive and non-autoreactive T and B cells (317). Moreover, targeting glutamine metabolism with 6-diazo-5-oxo-l-norleucine (DON) impairs the T cell-dependent production of autoantibodies in the same model (318). The role of glutamine metabolism in SLE is supported by the inhibition of Glutaminase-1, impacting glutamine entry in the mitochondria, that alleviates the MRL-*lpr* model (319). The modulation of cellular metabolism is therefore a valid approach to modulate SLE phenotypes in mouse models, but could also be envisioned as a new avenue to explore for the treatment of patients with SLE. In the clinic, classic anti-inflammatory drugs, such as salicylate or methotrexate, act by targeting metabolic effectors, including AMPK and dihydrofolate reductase, respectively

(320,321). As mentioned above, Calcineurin inhibitors are also used in SLE. In addition, Sirolimus, an inhibitor of mTOR is efficient in dampening SLE disease activity (322). Similarly, metformin, a mild inhibitor of the complex I of the ETC and a modulator of the AMPK-mTOR pathway, is a biguanide antihyperglycemic treatment used as a first line of treatment in Type 2 diabetes that also reduces SLE flares (323).

1.7 Hypothesis and Rationale

O-GlcNAcylation is an essential homeostatic nutrient-sensing pathway. We hypothesize that it is elicited in inflammatory contexts as a protective regulatory mechanism. Indeed, N-acetyl-D-Glucosamine, a nutrient that boosts O-GlcNAcylation, protects from chronic inflammatory pathologies. We explore this hypothesis in two models initiated by NA sensing and driven by type I IFN and inflammatory cytokines: SLE and viral infection. In SLE, OGT is overexpressed in T cells and the Xq13 region containing *OGT* is bi-allelically expressed in SLE B cells (217,223). In viral infection, O-GlcNAcylation controls MAVS signaling and acts as a rheostat of virus-induced cytokine production.

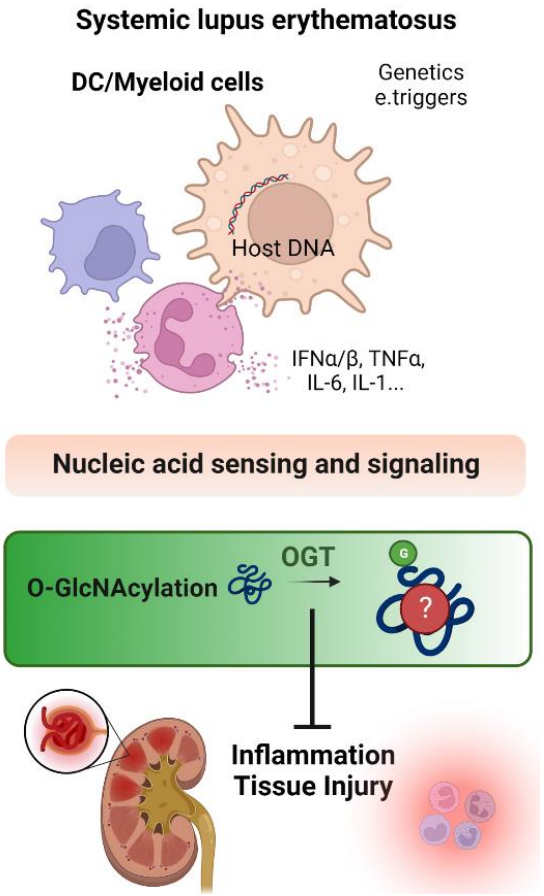

Figure 11. Proposed hypothesis for O-GlcNAcylation in SLE

O-GlcNAcylation in myeloid cells attenuates the severity of Systemic Lupus Erythematosus

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ABSTRACT

The Hexosamine Biosynthesis Pathway (HBP) is a key metabolic pathway that links nutrient sensing to protein O-GlcNAcylation by converting glucose, glutamine, and fatty acids into UDP-N-acetylglucosamine (UDP-GlcNAc). O-GlcNAc transferase (OGT) couples this high-energy amino sugar to substrate proteins on a serine/threonine residue. The HBP has emerged as a critical pathway in immune regulation. However, its role in autoimmunity has not been fully explored. Systemic Lupus Erythematosus (SLE) is a type I interferon-mediated severe auto-immune disease that primarily affects women. Overexpression of the X-linked gene *OGT* has been proposed to contribute to SLE pathophysiology, but the precise role of O-GlcNAcylation in this pathology remains unknown. Here, we have profiled O-GlcNAcylation levels in SLE patients' blood leukocytes and show a marked upregulation of this pathway in active disease in two immune sub-populations, non-classical monocytes and plasmacytoid dendritic cells. To address causality and underlying mechanisms, we used the *Dnase1L3*$^{-/-}$ mouse model of spontaneous SLE and deleted *Ogt* specifically in myeloid cells. *Ogt*^{DMye}*Dnase1L3*$^{-/-}$ mice exhibited exacerbated disease phenotypes, particularly lupus nephritis. Macrophage O-glycome analysis unraveled several effectors of SLE, including VISTA, as possible OGT substrates. Collectively, our results point to O-GlcNAcylation as a potential regulatory mechanism induced to alleviate myeloid-driven inflammation in SLE and might have wider implications for autoimmunity and interferon-mediated diseases.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease induced by loss of tolerance to nuclear antigens. It is characterized by the production of autoantibodies and immune complex deposition ultimately leading to organ damage[1]. Chronic inflammatory diseases (CIDs) like SLE are a significant health and economic burden in developed countries[2,3], and despite current treatments, the morbidity and mortality of SLE remain high, and the vital or organ prognosis of patients may be affected by disease flare-ups[4]. It is therefore crucial to further understand the pathophysiology of the disease towards the development of new therapeutic approaches.

SLE pathophysiology is complex and not fully understood. It involves innate and adaptive immunity[1]. One hallmark of the disease is the production of type I interferon (IFN) downstream of nucleic acid accumulation and sensing e.g. by TLR7 and TLR9[5]. The main producers of type I IFN are plasmacytoid dendritic cells (pDC), but other cell-types such as monocytes can also be an important source[6–8]. The modulation of cellular metabolism has been demonstrated as a valid approach to alleviate SLE phenotypes in mouse models[9–11], and classic anti-inflammatory drugs, such as salicylate or methotrexate, act by targeting metabolic effectors[12,13]. Immunometabolism studies have revealed that distinct cellular metabolism pathways differentially modulate immunological phenotypes[14,15]. For instance, inflammatory macrophages, DCs and effector T cells shift their metabolism to aerobic glycolysis upon activation, a feature initially observed in cancer by Otto Warburg and dubbed the Warburg effect[16]. By increasing the availability of glycolysis intermediates, precursors of various anabolic pathways, tumor cells and inflammatory leukocytes meet their energetic and biosynthetic needs associated with proliferation and effector functions.

In parallel to aerobic glycolysis, the hexosamine biosynthesis pathway (HBP), has emerged as an important regulator of immunity[17]. Besides consuming 2-5% of cellular glucose, HBP also

integrates signals from glutamine, fatty acid (acetyl-CoA) and nucleotide (UTP) metabolism. Through the production of the high-energy metabolite uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), HBP links nutrient sensing into a tractable cellular modification of substrate proteins, namely O-GlcNAcylation. This dynamic post-translational modification of serine/threonine (S/T) residues in target proteins often overlaps with phosphorylation. O-GlcNAcylation is regulated by two unique cellular enzymes, O-GlcNAc transferase (OGT) that is encoded by a gene on the X-chromosome, which catalyzes the addition of UDP-GlcNAc to substrates and O-GlcNAcase (OGA) that hydrolyzes it[18,19].

The *OGT* gene is located on the X chromosome in a region (Xq13) adjacent to the X chromosome inactivation (XCI) center harboring the XIST locus[20] that encodes the long non-coding RNA XIST responsible for chromosome X inactivation. SLE mainly affects women[21] and several immune-related genes in Xq13 region, e.g., *CD40L*, *CXCR3* and *OGT* are overexpressed in T cells from women affected with SLE treated with the DNA methyltransferase inhibitor 5-azacytidine[22]. The HBP is significantly upregulated by enhanced glucose and glutamine uptake, a feature of activated inflammatory leukocytes[23]. O-GlcNAcylation regulates pathways associated with the Warburg effect in inflammation, through stimulatory modification of phosphofructokinase 1 (PFK1)[24], HIF-1α[25], c-Myc[26,27] and glucose-6-phosphate dehydrogenase (G6PD)[28] for example. O-GlcNAcylation is upregulated in activated T cells, where it is required for self-renewal, peripheral clonal expansion and IL-2 production[23,29], but it is also needed for regulatory T (T_{reg}) cell lineage stability[30] and Th17 differentiation[31,32]. It is similarly required for B cell homeostasis and antibody production[33]. The role of O-GlcNAcylation in innate immunity is context-dependent. For instance, O-GlcNAcylation of STAT3 prevents its phosphorylation downstream of IL-10 signaling leading to enhanced production of inflammatory cytokines[34]. In contrast, O-GlcNAcylation prevents necroptosis, acute inflammation and endotoxic shock by inhibiting the phosphorylation of

RIPK3[35]. Similarly, it prevents metabolic inflammation downstream of mTOR signaling by dampening S6K1 phosphorylation[36]. In viral infections, O-GlcNAcylation exerts dual effects. On one hand, it promotes anti-viral innate immunity by activating Mitochondrial Antiviral Signaling (MAVS) protein[37–39], ultimately leading to IRF3 translocation to the nucleus and enhanced immune response. On the other hand, it promotes IRF5 nuclear translocation and influenza-induced cytokine storm[40], through the O-GlcNAcylation of Serine 430. Interestingly, IRF5 is a risk factor for SLE[41–43], is mainly expressed in innate immune cells[44,45], and its blockade or knock-out alleviates SLE in mouse models[46–50].

Because of its dual role in innate immunity and type I IFN-mediated diseases, alongside with biallelic expression of *OGT* in women, we hypothesized that O-GlcNAcylation is implicated in SLE innate immune responses, either as a promoter or a regulatory phenomenon, and might represent a promising target for immunometabolic intervention.

In this work, we describe the total level of O-GlcNAcylation in immune cells of patients with in SLE compared to healthy donors (HD). We identify a selective upregulation of the pathway in non-classical monocytes and pDC. To address the role of O-GlcNAcylation in myeloid cells in SLE, we generated an *Ogt* myeloid-specific KO mouse that we bred to *Dnase1l3*$^{-/-}$ mice, a monogenic model of spontaneous SLE[51]. Our results reveal a protective role of myeloid O-GlcNAcylation in this model. Through the characterization of the macrophage O-Glycome in SLE, we identify several SLE effectors as OGT substrates, highlighting their potential as therapeutic entrypoints.

MATERIALS AND METHODS

Patients

Blood samples from patients with SLE were obtained through a transversal study called METABOLUPS (clinicaltrials.gov number: NCT04440566). The research was approved by the ethical committee CPP Sud-Est IV, with the help of the French Blood Center for HD samples and the Center of Reference for Rare Systemic Autoimmune Diseases East-Southwest (RESO, Bordeaux University Hospitals). PBMC were isolated from whole blood using Ficoll technique from 33 HD and 53 SLE patients (25 inactive SLE and 28 active SLE). Activity of the disease was defined by the SLEDAI-2K index[52] $\geq$ 6. Demographic characteristics, clinical and biological characteristics of SLE patients and HD are described in Table S1.

Flow cytometry analyses of human peripheral blood leukocytes

Flow cytometry analysis was performed on monocytes, DC, NK cells, T and B lymphocytes using 6 antibody panels. The details of panels used, and antibody references are listed in Table S2a. The gating strategy is described in Supplemental Figure S1. To quantify total O-GlcNAcylation, intracellular staining using the anti-O-GlcNAc RL2 antibody (eBiosciences Cat# 51-9793-42) was performed, after intranuclear permeabilization (Miltenyi Biotec Inc. Cat# 130-122-981). Representative FACS histograms and scatter plots of O-GlcNAcylation levels in human and mouse myeloid cells is depicted in Supplemental Figure S3. Flow cytometry data were acquired using a BD LSRFortessa™ 16 colors Cell Analyzer with BD FACSDiva Software Version 8.0.1 or 9.0.1 and analyzed using FlowJo™ Software for Windows 10 (Version 10.6.2. Ashland, OR: Becton, Dickinson, and Company; 2022). All statistical analysis were performed using GraphPad Prism for Windows (Version 8.3.0, GraphPad Software, La Jolla, California, USA; 2022). The data were described as geometric

means, standard deviations, or percentages. Data were compared using unpaired-t-test or Mann-Whitney test or Kruskal Wallis test, depending on the sample size, group numbers and the possibility of a non-parametric distribution of the data. Correlations were established by the Spearman coefficient. Longitudinal data were analyzed using a mixed model analysis. A *p*-value < 0.05 was considered significant.

Mouse models

Mice were housed and bred in an appropriate mouse facility, kept under pathogen-free conditions (Animal facility A2, University of Bordeaux, France, approval B33-063-916). Experiments were performed in an accredited laboratory for the manipulation of genetically modified organisms (declaration 3153). The animal breeding and experimental plan was approved by an Ethical Review Committee at the University of Bordeaux (DAP 23458-V1-2020012317578212). Myeloid-specific *Ogt* KO mice ($Ogt^{\Delta Mye}$) were generated by breeding $Ogt^{F/F}$ mice (JAX stock #004860) with the LysM-cre myeloid deleter strain (JAX stock #004781). $Ogt^{\Delta Mye}$ were then bred to $Dnase1l3^{-/-}$ mice that develop spontaneous lupus[51] to obtain $Ogt^{\Delta Mye}Dnase1l3^{-/-}$ lupus-prone mice lacking Ogt in the myeloid compartment. Representative genotyping images (Figure S4a), western blot (Figure S4b) and flow-cytometry analysis (Figure S4c) of these $Ogt^{\Delta Mye}$ and $Ogt^{\Delta Mye}Dnase1l3^{-/-}$ mice are presented. MRL-*lpr* female mice (JAX stock #000485) aged 8 weeks were treated with an OGA inhibitor, Thiamet G (Abcam, Cat# ab146193) at 20 mg/kg, three times a week by intraperitoneal injections until 16 weeks of age.

SLE phenotypic characterization

Ogt$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mouse and littermate controls were monitored from birth till 11 months of age and regularly sampled for blood to quantify serum dsDNA antibodies by homemade ELISA, and urines to measure proteinuria by biochemical dosage. At the 11-month endpoint, blood, spleen, kidneys, and peritoneal macrophages (PEM) were collected. After mechanical dissociation of the spleen and red blood cell lysis in blood and spleen samples, FACS analysis of blood leukocytes and splenocytes was performed (the detailed panels used and antibody references are listed in Table S2b, the gating strategy in Supplemental Figure S2a). The kidneys were fixed in 10% formamide, included in paraffin and stained using Hematoxylin & Eosin (HE) or Masson's Trichrome staining. A composite score assessing glomerular enlargement (0-1), interstitial infiltration (0-1), mesangial proliferation (0-4), glomerular thrombosis (0-2), fibrosis (0-2) was used to assess kidney damage, ranging from 0 (normal kidney) to 10 (kidney destruction). Blinded histological analysis was performed by a trained pathologist. For MRL-*lpr* mice, lymph nodes volumes were quantified as length x width²)/2 as a readout of lymphoproliferation burden. O-GlcNAcylation was quantified in lymph nodes immune cells by flow cytometry (Panel and antibodies used listed in table S2b and gating strategy in figure S2b). Serum dsDNA antibodies and kidney histology were measured as described above for the *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mouse model.

Isolation of peritoneal macrophages

Peritoneal macrophages (PEM) were retrieved by peritoneal lavage and adhesion on plastic. Peritoneal cells were plated in 12 wells plates at 10^6 cells/ml in RPMI medium containing 10%FBS, 1%Glutamine and 1%Penicillin-Streptomycin for 1h30 at 37°C, 5% CO_2. After 3 washes, macrophages were retrieved by scrapping, pooled, and lysed in RIPA lysis buffer.

Whole cell extracts (between 3423 and 3601 µg/ml) were precipitated on Wheat Germ Agglutinin (WGA) beads. WGA-bound proteins were then processed for mass-spectrometry analysis.

Label-free quantitative proteomics

Beads were boiled in Laemmli buffer. The supernatants were loaded on a 10% acrylamide SDS-PAGE gel and proteins were visualized by Colloidal Blue staining. Migration was stopped when samples had just entered the resolving gel and the unresolved region of the gel was cut into only one segment. The steps of sample preparation and protein digestion by the trypsin were performed as previously described[53]. NanoLC-MS/MS analysis were performed using an Ultimate 3000 RSLC Nano-UPHLC system (Thermo Scientific, USA) coupled to a nanospray Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, California, USA). The LC and MS parameters used as previously described[54]. Protein identification and Label-Free Quantification (LFQ) were done in Proteome Discoverer 2.5. MS Amanda 2.0, Sequest HT and Mascot 2.5 algorithms were used for protein identification in batch mode by searching against a UniProt Mus musculus database (55338 entries, release January 2022). Two missed enzyme cleavages were allowed for the trypsin. Mass tolerances in MS and MS/MS were set to 10 ppm and 0.02 Da. Oxidation (M) and acetylation (K) were searched as dynamic modifications and carbamidomethylation (C) as static modification. Peptide validation was performed using Percolator algorithm[55] and only "high confidence" peptides were retained corresponding to a 1% false discovery rate at peptide level. Minora feature detector node (LFQ) was used along with the feature mapper and precursor ions quantifier. The quantification parameters were selected as follows: (1) Unique peptides (2) Precursor abundance based on intensity (3) Normalization mode: none (4) Protein abundance calculation: summed abundances (5) Protein ratio calculation: pairwise ratio based (6)

Imputation mode: Low abundance resampling and (7) Hypothesis test: t-test (background based). Quantitative data were considered for master proteins, quantified by a minimum of 2 unique peptides and a fold change above 2.

RESULTS

O-GlcNAcylation is increased in non-classical monocytes of patients with active SLE and correlates with disease activity

We enrolled 33 HD, 25 patients with inactive SLE and 27 patients with active SLE. Using multi-parametric FACS analysis, we first quantified the frequencies and activation phenotypes of peripheral blood leukocytes. Among the monocytes, we analyzed the classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$), and non-classical ($CD14^{-}CD16^{+}$) subsets and observed an expansion of the $CD16^{+}$ monocyte subsets (intermediate and non-classical) accompanied by decreased frequencies of classical monocytes in the blood of patients with active SLE compared to HD (Figure 1a). Serum complement proteins are quantified to gauge disease activity in SLE, and active disease exhibits reduced total levels (CH50), as complements are consumed. Interestingly, the frequency of non-classical monocytes, but not that of other monocytes, positively correlated with CH50 (Figure 1b), suggesting that this subset may play a regulatory role in SLE.

FACS quantification of O-GlcNAcylated proteins by intracellular staining with the anti-O-GlcNAc antibody RL2 revealed that among monocytes, only non-classical monocytes exhibited disease-associated increase in O-GlcNAcylation, as evident in active SLE compared to HD or inactive SLE (Figure 1c). Non-classical monocytes O-GlcNAcylation levels correlated with SLE disease activity index (SLEDAI-2K) (Figure 1d). Moreover, O-GlcNAcylation levels in classical and intermediate monocytes increased to those of non-classical monocytes in patients with inactive SLE that received cyclophosphamide or Mycophenolate Mofetil (Figure 1e). Collectively, these results suggest that O-GlcNAcylation may have a regulatory role in SLE and could be modulated by immunosuppressive drug-based interventions.

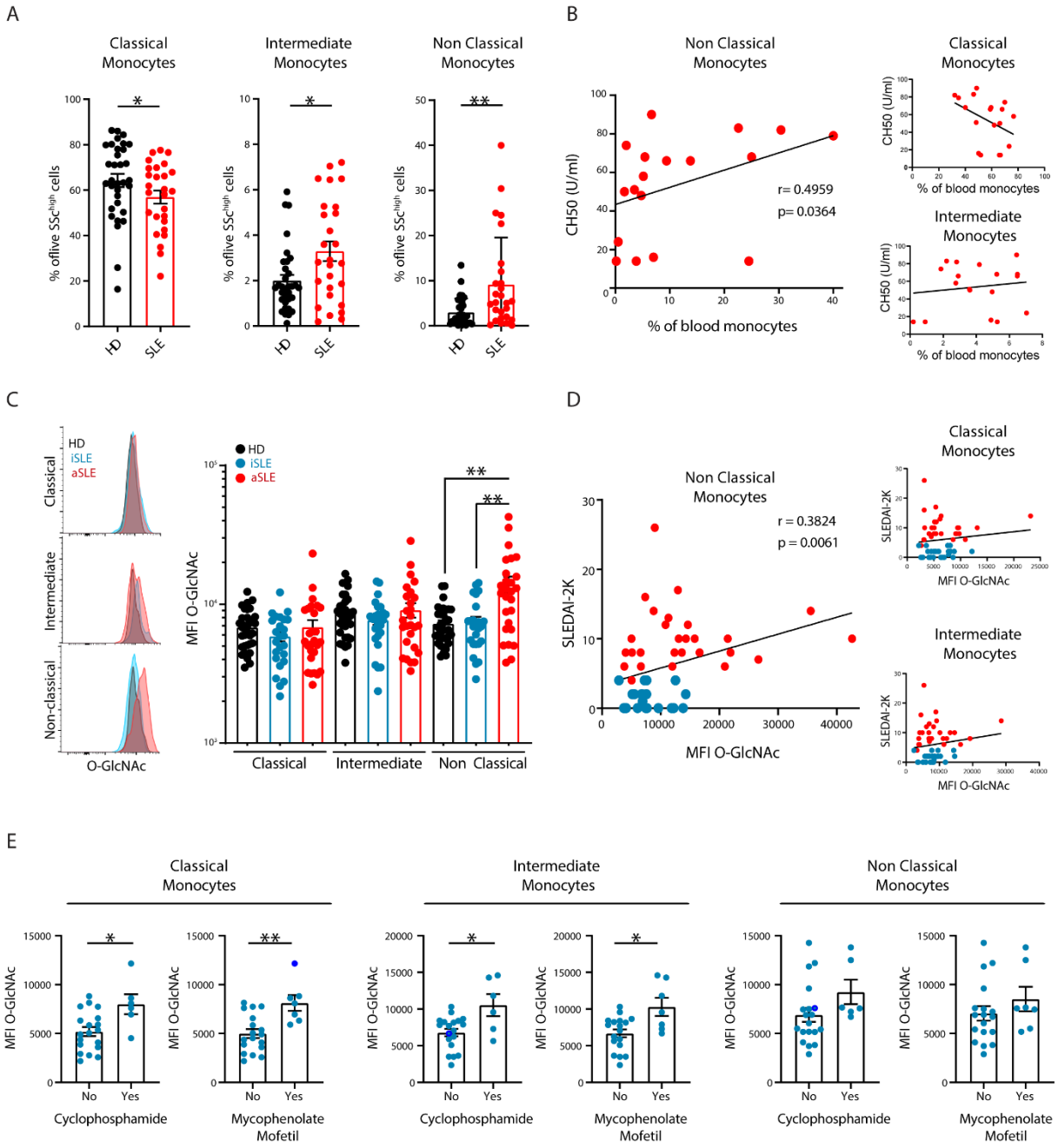

Figure 1. O-GlcNAcylation is increased in non-classical monocytes of patients with active SLE and correlates with disease activity

A. Frequencies of classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and non-classical ($CD14^-CD16^+$) monocyte subsets in HD and patients with active SLE (SLEDAI-2K ≥ 6). Mann-Whitney test, * = p<0,05; ** = p<0,01. **B.** Spearman's rank correlation between CH50 and the frequencies of classical, intermediate, and non-classical monocyte subsets. **C.**

Representative FACS histograms and pooled quantification of intracellular O-GlcNAc MFI in monocyte subsets from HD or patients with inactive SLE (iSLE) or active SLE (aSLE). Kruskal-Wallis test, ** = p<0,01. **D.** Spearman's rank correlation between SLEDAI-2K activity index and O-GlcNAc MFI in classical, intermediate, and non-classical monocyte subsets. **E.** O-GlcNAc MFI in classical, intermediate, and non-classical monocyte subsets of patients with inactive SLE (SLEDAI-2K < 6), who received immunosuppressive treatment with Cyclophosphamide or Mycophenolate Mofetil. Mann-Whitney test, * = p<0,05; ** = p<0,01.

O-GlcNAcylation in pDC correlates with SLE disease activity and pDC activation

Our analysis of the DC compartment revealed that only pDCs, but not conventional DCs (cDC) or monocyte-derived DCs (moDC), exhibited a 'hyper-activated' phenotype associated with active disease, as assessed by enhanced surface expression of the activation marker CD86 (Figure 2a).

As for non-classical monocytes, O-GlcNAcylation levels were markedly higher in pDCs from patients with active SLE compared to inactive disease (Figure 2b). pDC CD86 and O-GlcNAcylation levels positively correlated with disease activity (SLEDAI-2K) (Figure 2c, d). Consistently, there was a positive correlation between pDC activation, i.e., CD86 surface expression, and O-GlcNAcylation levels (Figure 2e). These correlations were not observed in moDC or cDC (Figure 2f). Of note, patients with inactive SLE who received immunosuppressive treatment by Mycophenolate Mofetil displayed enhanced O-GlcNAcylation in the moDC subset[56] (Figure 2g), in line with the observations in classical and intermediate monocytes (Figure 1e).

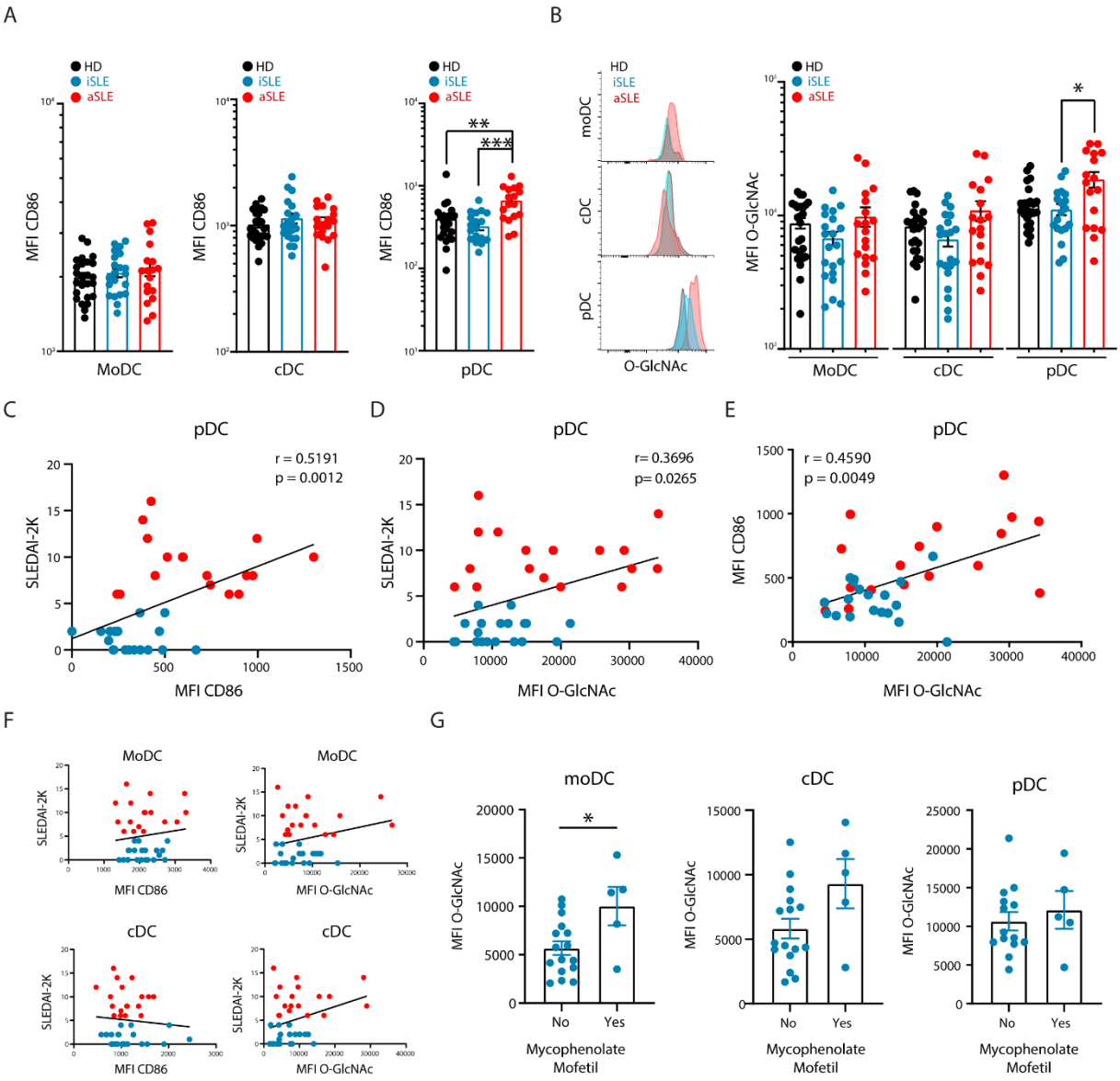

Figure 2. O-GlcNAcylation in pDC correlates with SLE disease activity and pDC activation

A. The MFI of CD86 on monocyte-derived DCs (moDC), conventional DCs (cDC) and plasmacytoid DCs (pDCs) from HD, patients with inactive SLE (iSLE) or patients with active SLE (aSLE, SLEDAI-2K ≥ 6). Kruskal-Wallis test, ** = p<0,01; ***= p<0,001. **B.** Representative FACS histograms and pooled quantification of intracellular O-GlcNAc MFI in DC subsets from HD, patients with inactive SLE (iSLE) or patients with active SLE (aSLE). Kruskal-Wallis test, * = p<0,05. **C.** Spearman's rank correlation between SLEDAI-2K activity

index and the MFI of CD86 in pDC. **D.** Spearman's rank correlation between SLEDAI-2K activity index and the MFI of O-GlcNAc in pDC. **E.** Spearman's rank correlation between the MFI of CD86 and that of O-GlcNAc in pDC. **F.** As in C and D but for moDCs and cDCs. **G.** The MFI of O-GlcNAc in moDC, cDC, pDC subsets from patients with inactive SLE (SLEDAI-2K < 6) who received immunosuppressive treatment with Mycophenolate Mofetil. Mann-Whitney test, * = p<0,05.

Increasing O-GlcNAcylation by pharmacological blockade of OGA alleviates the SLE phenotype in MRL-*lpr* lupus prone mice

To explore the impact of targeted modulation of O-GlcNAcylation on SLE phenotypes, we first used a pharmacological approach in the MRL-*lpr* lupus-prone mouse model. To augment global O-GlcNAcylation, we treated female mice with an OGA inhibitor (Thiamet G at 20 mg/kg) three times a week for 8 weeks. We confirmed the efficacy of Thiamet G by quantifying O-GlcNAcylation levels in lymphocytes and monocytes isolated from lymph nodes of MRL-*lpr* mice. Treated mice displayed increased O-GlcNAcylation in immune cells from the lymph nodes compared to untreated mice (Figure 3a). OGA inhibition markedly decreased the lymphoproliferative phenotype of MRL-*lpr* mice as revealed by smaller lymph nodes volume (figure 3b, c) and dampened serum dsDNA antibodies titers (figure 3d). Furthermore, Thiamet G-treated MRL-*lpr* mice had reduced kidney glomerular damage compared to untreated littermate controls and improved kidney architecture (figure 3e). These data highlight O-GlcNAcylation as a targetable metabolic pathway *in vivo*, potentially eligible for drug intervention in SLE.

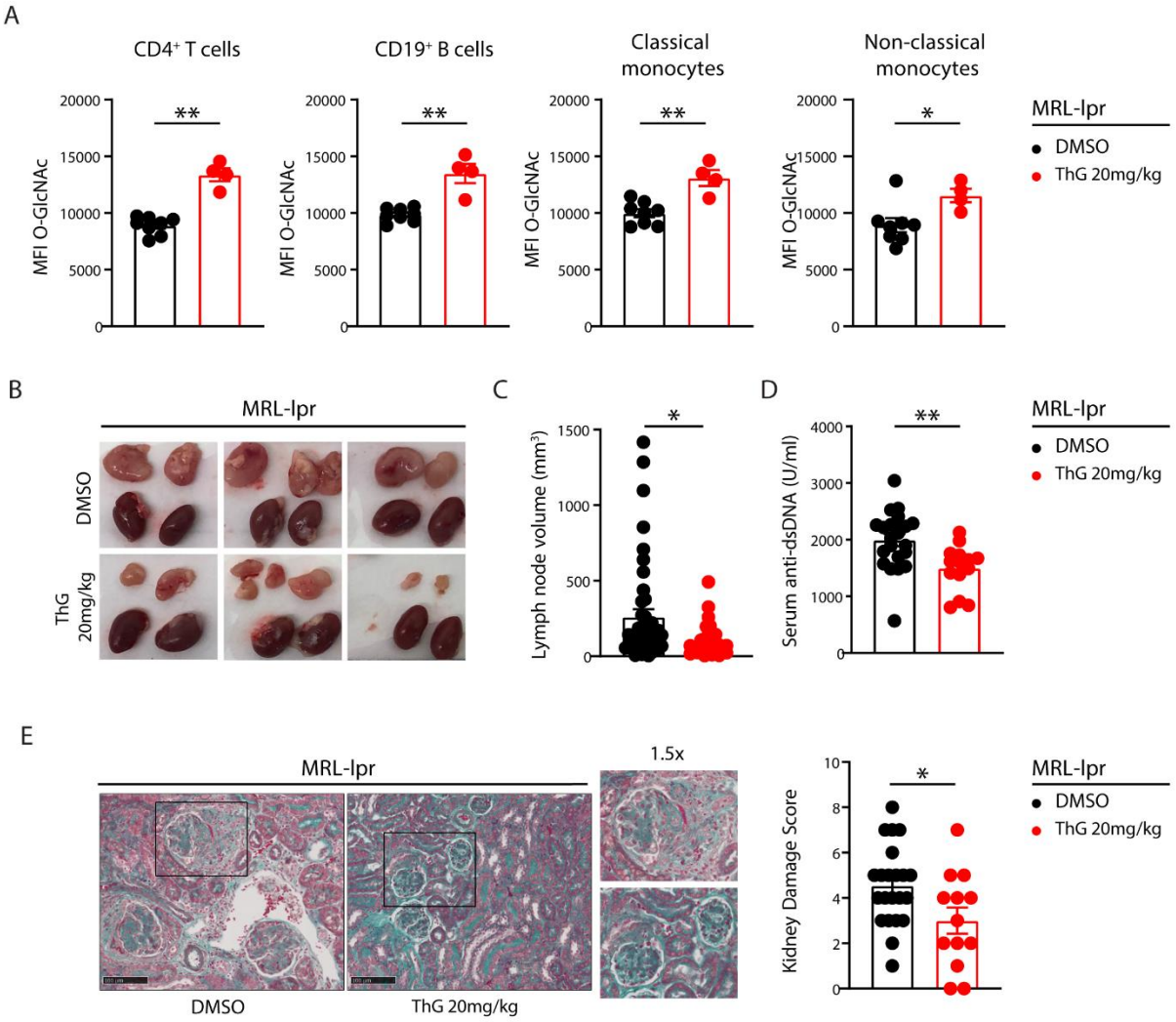

Figure 3. Increasing O-GlcNAcylation by pharmacological blockade of OGA alleviates the SLE phenotype in MRL-*lpr* mice

A. The MFI of O-GlcNAc in $CD4^+$ T cells, $CD19^+$ B cells, classical ($Lin^-CD11b^+Ly6C^{hi}$) and non-classical ($Lin^-CD11b^+Ly6C^{lo}$) monocytes in lymph nodes of MRL-*lpr* mice treated intraperitoneally with the OGA inhibitor Thiamet G (ThG) at 20mg/kg or with 5% DMSO solution as a control. The treatment regimen was three times a week from 8 to 16 weeks of age. Mann-Whitney test, * = p<0,05; ** = p<0,01. **B.** Representative images of lymph nodes and kidneys from ThG (20mg/kg) or control (5% DMSO) treated MRL-*lpr* mice. **C.** Lymph nodes volume quantification from ThG (20mg/kg) or control (5% DMSO) treated MRL-*lpr* mice. Mann-Whitney test, * = p<0,05. **D.** Anti dsDNA antibodies ELISA quantification from ThG

(20mg/kg) or control (5% DMSO) treated MRL-*lpr* mice. Mann-Whitney test, ** = p<0,01. **E.** Representative images of kidney sections colored by Masson's trichrome, and kidney damage quantified by a subjective composite score ranging from 0 (normal kidney) to 10 (kidney destruction), from ThG (20mg/kg) or control (5% DMSO) treated MRL-*lpr* mice. Mann-Whitney test, * = p<0,05.

Myeloid-specific deletion of *Ogt* exacerbates the SLE phenotype in *Dnase1l3*$^{-/-}$ mice

Based on the observed dynamic regulation of O-GlcNAcylation in the myeloid compartment in patients with SLE, we next wished to investigate the contribution of myeloid O-GlcNAcylation to disease progression and severity. We thus generated a triple-congenic mouse model in which *Ogt* is deleted in myeloid cells using the LysM-cre recombinase deleter strain, which we crossed to *Dnase1l3*$^{-/-}$ mice. A simplified breeding strategy is depicted in Figure 4a, and representative genotyping results are shown in Supplemental Figure 4a. As published by others[33], the *Ogt* deletion was more penetrant in male mice, thus we focused the investigation on male *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice. *Dnase1l3*$^{-/-}$ mice on the C57BL/6 background produce anti-dsDNA as early as 4 weeks of age[51]. As the disease progresses, they exhibit increased myeloid blood count, activated T cells, and decreased marginal zone B cells[51]. Later, at around 50 weeks of age, they develop mild kidney injury[51]. Accordingly, we monitored serum anti-dsDNA antibodies and urine proteinuria regularly from 3 to 11 months of age. At endpoint, we evaluated immune cell phenotypes in the blood and spleen and performed kidney histology to quantify the extent of nephritis. Levels of serum anti-dsDNA antibodies (Figure 4b) were significantly higher in *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice compared to *Dnase1l3*$^{-/-}$ littermate controls. A pattern of flares was observed in both genotypes. As of month 8, *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice exhibited enhanced protein/creatinine ratio compared to *Dnase1l3*$^{-/-}$ mice (Figure 4c). At endpoint, *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice had a more severe lupus nephritis (LN) (Figure 4d), with

more mesangial proliferation, interstitial fibrosis, and inflammatory infiltrate (Figure 4e). Assessment of immune cell phenotypes revealed that *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice had enhanced activation of myeloid and lymphoid cells in the spleen than *Dnase1l3*$^{-/-}$ control mice. This was revealed by FACS analysis of neutrophils, classical- and non-classical monocytes, evaluated by MHCII surface expression, and effector memory CD4^{+} T cells, evaluated by ICOS surface expression (Figure 4f). Last, *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice displayed higher circulating levels of pDC (Figure 4g).

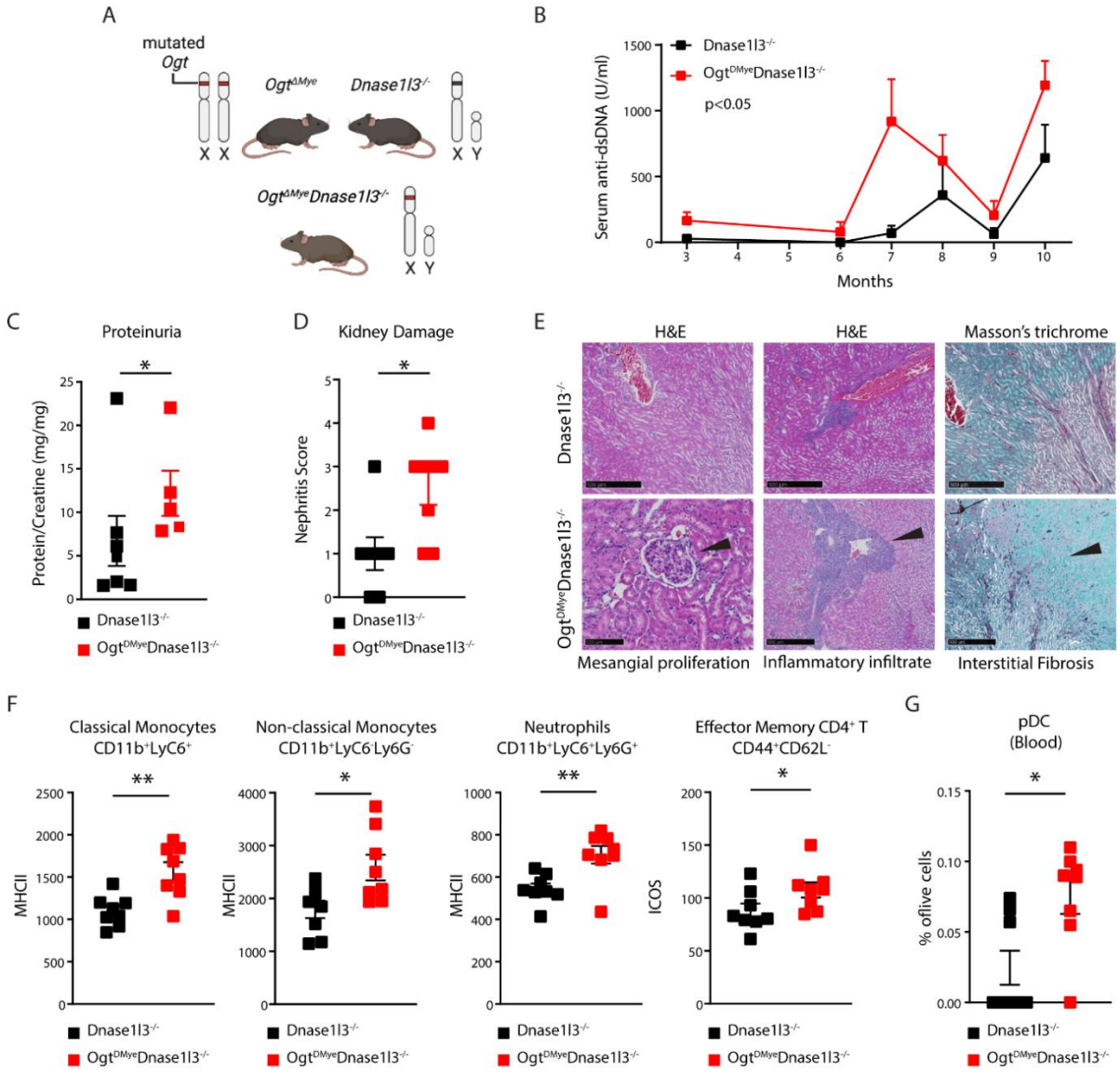

Figure 4. Myeloid-specific deletion of *Ogt* exacerbates the SLE phenotype in *Dnase1l3*$^{-/-}$ mice

A. Simplified representation of the breeding strategy to generate *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice. **B.** Longitudinal ELISA quantification of anti-dsDNA antibodies in serum from *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice and *Dnase1l3*$^{-/-}$ littermate control mice. Mixed-model analysis. **C.** Proteinuria evaluated by protein/creatinine ratio in the urine of *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ and *Dnase1l3*$^{-/-}$ mice at 8 months of age. Mann-Whitney test, * = p<0,05. **D.** Kidney damage quantification at 11 months by a subjective composite score ranging from 0 (normal kidney) to 10 (kidney destruction), from *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ and *Dnase1l3*$^{-/-}$ mice. Mann-Whitney test, * = p<0,05. **E.** Representative images of mesangial proliferation, interstitial fibrosis, and inflammatory infiltrate on kidney sections colored by Hematoxylin & Eosin (H&E) and Masson's trichrome from 11-month-old *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ and *Dnase1l3*$^{-/-}$ mice. **F.** The MFI of MHCII on classical and non-classical monocytes and neutrophils, and the MFI of ICOS on effector memory CD4^{+} T cells from *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ and *Dnase1l3*$^{-/-}$ mice at 11 months of age. Mann-Whitney test, * = p<0,05; ** = p<0,01. **G.** pDC blood frequencies in *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ and *Dnase1l3*$^{-/-}$ mice at 11 months of age. Mann-Whitney test, * = p<0,05.

The macrophage O-Glycome in SLE

To decipher the molecular mechanism underlying the exacerbated lupus phenotype elicited by myeloid-specific loss of *Ogt*, we isolated peritoneal macrophages from *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ and *Dnase1l3*$^{-/-}$ littermate controls and analyzed their differentially glycosylated proteins by mass spectrometry. This analysis retrieved a total of 8243 peptides. After exclusion of contaminants and adjustment of the *p*-value by the Benjamini-Hochberg method to account for false-discovery rate, we identified a total of 50 proteins glycosylated in *Dnase1l3*$^{-/-}$ mouse

macrophages but absent from *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ macrophages. Interrogation of the literature and the O-GlcNAc database[57] revealed, that among the 50 hits in our analysis, 12 proteins are implicated in SLE and 6 of which reported as O-GlcNAcylated (Table 1). The latter encompassed Protein Phosphatase 2A (PP2A, subunits α and β), Protein disulfide isomerase (Creld2), Cytosolic endo-beta-N-acetylglucosaminidase (Engase), Apolipoprotein E (Apoe), Ribose-phosphate pyrophosphokinase 2 (Prps2) and V-type Ig domain-containing suppressor of T-cell activation or PD-1H (VISTA). Interestingly, inhibitory signaling of VISTA has recently been shown to prevent systemic and cutaneous lupus in mice[58], partly through myeloid cells. Other hits in our analysis, implicated in SLE but not previously described as O-GlcNAcylation targets, included macrophage receptor with collagenous structure (MARCO), complement factor H (Cfh), fMet-Leu-Phe receptor (Fpr1), ABC-type glutathione-S-conjugate transporter (Abcc3), Hemopexin (Hpx) and P-selectin (Selp). We also identified Extracellular matrix protein 1 (Ecm1), implicated in inflammatory bowel diseases, Feline leukemia virus subgroup C receptor-related protein 1 (Flvcr1) implicated in ferroptosis, and Zinc transporter 1 (Slc30A1).

Protein	Gene	Protein known to be implicated in SLE or immune mediated diseases	Protein Known to be O-GlcNAcylated	Site identified	Abundance Ratio (WT/KO)	p value
Serine/threonine-protein phosphatase 2A catalytic subunit alpha	*Ppp2ca*	SLE	yes	no	31.25	0.0000001318
VISTA (V-type immunoglobulin domain-containing suppressor of T-cell activation)	*Vsir*	SLE	yes	T37	15.15	0.0000306265
Protein disulfide isomerase CreId2	*CreId2*	SLE	yes	no	9.71	0.0006171303
Cytosolic endo-beta-N-acetylglucosaminidase	*Engase*	TREX1 associated SLE	yes	S397	4.29	0.0000015581
Serine/threonine-protein phosphatase 2A catalytic subunit beta	*Ppp2cb*	SLE	yes	no	4.22	0.0436206078
Apolipoprotein E	*Apoe*	SLE	yes	no	4.12	0.0000499582
Ribose-phosphate pyrophosphokinase 2	*Prps2*	SLE	yes	no	3.64	0.0392756524
Macrophage receptor MARCO	*Marco*	SLE	no	no	11.90	0.0000019317
Complement factor H	*Cfh*	SLE	no	no	7.46	0.0000001318
fMet-Leu-Phe receptor	*Fpr1*	SLE	no	no	5.59	0.0000488992
ABC-type glutathione-S-conjugate transporter	*Abcc3*	SLE	no	no	4.81	0.0045278960
Hemopexin	*Hpx*	SLE	no	no	4.41	0.0244134232
P-selectin	*Selp*	SLE	no	no	3.18	0.0000467526
Feline leukemia virus subgroup C receptor-related protein 1	*Flvcr1*	Indirect: implicated in Ferroptosis	yes	no	13.16	0.0002412373
Tyrosine-protein kinase Fgr	*Fgr*	Pulmonary fibrosis	yes	S181, S183	6.67	0.0000001752
Fibronectin	*Fn1*	Implicated but non specific	yes	T432, T1008	5.85	0.0000015471
Keratin, type II cytoskeletal 72	*Krt72*	Implicated but non specific	yes	no	4.78	0.0000122685
Junction plakoglobin	*Jup*	Implicated but non specific	yes	T14, T21	4.61	0.0012270480
Complement C3	*C3*	Implicated but non specific	yes	no	4.46	0.0000015471
Plasma membrane calcium-transporting ATPase 1	*Atp2b1*	ARDS	yes	no	2.78	0.0216788640
EMILIN-1	*Emilin1*	Psoriasis	yes	S795	2.07	0.0049718040
Autophagy-related protein 9	*Atg9a*	IBD	yes	T518	2.04	0.0177827583
Extracellular matrix protein 1	*Ecm1*	IBD	no	no	17.54	0.0000623605
Chitinase-like protein 3	*Chil3*	IBD	no	no	9.26	0.0000000001
Histocompatibility 2, Q region locus 1	*H2-Q1*	Implicated but non specific	no	no	5.59	0.0125877142
Immunoglobulin heavy constant gamma 2C	*Ighg2c*	Implicated but non specific	no	no	4.81	0.0003117337
B-lymphocyte antigen CD19	*Cd19*	Implicated but non specific	no	no	4.05	0.0289091642
Immunoglobulin kappa variable 14-126	*Igkv14-126*	Implicated but non specific	no	no	3.91	0.0001513427
Monocyte differentiation antigen CD14	*Cd14*	Implicated but non specific	no	no	3.79	0.0000569589
Arylsulfatase A	*Arsa*	Neuroinflammation	no	no	3.53	0.0374458473
Tryptase beta-2	*Tpsb2*	Indirect: implicated in Ferroptosis	no	no	3.27	0.0001443637
Cationic amino acid transporter 2	*Slc7a2*	IBD - Pulmonary fibrosis	no	no	2.61	0.0002539155
Integrin beta-7	*Itgb7*	Implicated but non specific	no	no	2.33	0.0005496729
Sulfate transporter	*Slc26a2*	Athma	no	no	2.20	0.0139813607
Leukocyte immunoglobulin-like receptor subfamily B member 3	*Lilrb3*	IBD - Pulmonary fibrosis	no	no	2.17	0.0244134232
Vam6/Vps39-like protein	*Vps39*	Vitiligo	no	no	2.12	0.0158518395
Zinc transporter 1	*Slc30a1*	no	yes	no	15.38	0.0001127364
Myosin-11	*Myh11*	no	yes	S1347	7.19	0.0045278960
Cleavage and polyadenylation specificity factor subunit 6	*Cpsf6*	no	yes	S426	6.58	0.0046845258
ATP-binding cassette sub-family C member 3	*Abcc3*	no	yes	no	4.81	0.0045278960
tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6	*Trmt6*	no	yes	No	4.02	0.0046513143
Plastin-3	*Pls3*	no	yes	S122	3.94	0.0316095050
Omega-amidase NIT2	*Nit2*	no	yes	no	3.82	0.0190627066
Ras-related protein R-Ras	*Rras*	no	yes	No	3.26	0.0177827583
Threonyl-tRNA synthetase	*Tars2*	no	yes	no	2.13	0.0237571148
Threonine--tRNA ligase, mitochondrial	*Tars2*	no	yes	no	2.13	0.0237571148
Chitinase-like protein 4	*Chil4*	no	no	no	5.92	0.0032485675
Aldo-keto reductase family 1 member C13	*Akr1c13*	no	no	no	3.39	0.0454237260
Aspartoacylase	*Aspa*	no	no	no	3.08	0.0454237260
Fibronectin type-III domain-containing protein 3A	*Fndc3a*	no	no	no	3.07	0.0214214291

Table 1. The macrophage O-Glycome in SLE

Mass-spectrometry analysis of glycosylated proteins in peritoneal macrophages identifies 50 proteins glycosylated in *Dnase1l3*$^{-/-}$ but not *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ macrophages. Identified proteins and their corresponding genes are ordered in 3 categories: 1) implicated in SLE, 2) implicated in CIDs, and 3) no reported role in SLE or CIDs. Each category is ordered according to their O-GlcNAcylation status (known or not) according to the O-GlcNAc database(99). The specific O-GlcNAcylation site is depicted according to the O-GlcNAc database. Abundance ratio (WT: *Dnase1l3*$^{-/-}$ / KO: *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$) and *p*-value adjusted by the Benjamini-Hochberg method are presented.

DISCUSSION

Our work points to O-GlcNAcylation as a regulatory mechanism that alleviates myeloid-driven inflammation in SLE.

A regulatory role of O-GlcNAcylation in inflammatory diseases has been previously reported. This has been demonstrated experimentally using N-acetyl-D-Glucosamine, a commercially available nutrient, which drives O-GlcNAcylation by bypassing the rate-limiting step of HBP or using OGA inhibitors. For instance, these approaches were shown to alleviate experimental sepsis inflammation[59–61], and protect from adjuvant-induced arthritis [62].

Analysis of O-GlcNAcylation profiles in peripheral blood leukocytes of patients with SLE revealed disease-associated upregulation of this pathway in non-classical monocytes and pDCs specifically. Non-classical monocytes have been implicated in SLE and other CIDs[63]. They patrol the kidney glomeruli to maintain its homeostasis[64] and accumulate in glomerular vessels of SLE patients[65]. SLE patients with severe LN present high glomeruli titers of $CD16^{+}$ monocytes[66], and their blood frequency is inversely correlated to proteinuria in LN patients[67], suggesting a regulatory role. While global targeting of myeloid cells e.g. e.g., with CSF-1R or CX3CL1 blockade, has proven effective in LN in mice[68–70], subset-specific depletion approaches have not been reported. Previous work has described distinct transcriptional profiles in monocyte subsets. For instance, topical administration of the TLR7 agonist imiquimod to mice elicits an IFNα signature in classical monocytes but not in $Ly6^{lo}$ non-classical monocytes[71]. Furthermore, in an experimental mouse model of SLE, adoptive transfer of IL-4 differentiated BM-derived macrophages (BMDM) (“M2” polarization state) alleviates the SLE phenotype, while IFNγ differentiated BMDMs (M1) do not[72]. Together, these findings suggest that modulation of myeloid cells towards an anti-inflammatory and/or wound-healing profile might represent a potential therapeutic strategy in lupus.

A regulatory role of O-GlcNAcylation in non-classical monocytes and pDCs is aligned with previous findings identifying a protective role of this pathway in septic shock and the metabolic syndrome, specifically by inhibiting the inflammatory functions of RIPK3[35] and S6K1[36], respectively. As for DCs, there is scarce data regarding the role of O-GlcNAcylation in the functions of DC subsets. A recent report showed that OGA blockade drives DC differentiation from hematopoietic stem cells progenitors[73], while another described that inhibition of OGT by OSMI-I[74] altered moDC differentiation *in vitro*[75].

Besides non-classical monocytes and pDCs, our analysis also revealed disease-associated induction of O-GlcNAcylation in naïve but not in memory B cells (Figure S5c). Wu *et al*. have previously demonstrated that O-GlcNAcylation of the tyrosine kinase Lyn was required for B cell receptor (BCR) signaling and antibody production[33], suggesting that the observed O-GlcNAc signal in naïve B cells might also confer a regulatory mechanism. Of note, DC-specific deletion of *Lyn* results in spontaneous autoimmunity[76], but whether O-GlcNAcylation of Lyn in DCs controls its function in autoimmunity remains unclear.

Our study also unraveled heightened O-GlcNAcylation in NK cells of patients with active SLE (Figure S5d). Of note, the frequency of cytotoxic and terminally differentiated NK cells is increased in patients with active SLE[77,78], and O-GlcNAcylation has been shown as required for NK cell-mediated cytotoxicity[79], but the role of this modification in NK function in SLE remains an open question.

Last, we did not observe differences in O-GlcNAcylation in $CD4^+$ or $CD8^+$ T cells between HD and patients with SLE (Figure S5a, b). This is at odds with the reported increase in *OGT* expression in T cells from women with SLE, observed only in response to T cell treatment with the DNA methyltransferase inhibitor 5-azacytidine[22]. This could be because we did not modulate X methylation, which might be required to observe differential O-GlcNAcylation in

T cells. Of note, O-GlcNAcylation is a protective mechanism in regulatory T cells in autoimmune hepatitis[80].

Among the potential macrophage O-GlcNAcylation targets in SLE, our proteomic analysis identified VISTA. VISTA is upregulated in cutaneous lesions of patients with SLE and *Vista*$^{-/-}$ BALB/c mice spontaneously develop a lupus-like disease and exhibit enhanced frequencies of $CD4^+$ T cells and myeloid cells systemically and in cutaneous lesions[58]. Furthermore, VISTA agonist treatment alleviates the lupus-like phenotype of MRL-*lpr* mice[58]. VISTA agonist blunts innate immunity including type I IFN-mediated inflammation[81], and VISTA expressing macrophages promote kidney repair after ischemic injury[82]. Together, these findings unravel VISTA as a protective mechanism in SLE, in part through its action in inhibiting myeloid cell function in innate inflammation and type I IFN. Moreover, VISTA can bind PSGL-1 at acidic pH [83] and PSGL-1 interaction with P-selectin impairs regulatory T cells in SLE[84], posing PSGL-1 as a potential target of VISTA checkpoint function in SLE. How O-GlcNAcylation controls the activity of VISTA in SLE remains to be addressed.

In this work, we show that OGA inhibition leads to improvement of the SLE phenotype in lupus prone MRL-*lpr* mice. Previous studies have demonstrated that modulation of glucose[9] or glutamine[10] metabolism by 2-deoxyglucose (2-DG) and bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), respectively, alleviates SLE in mouse models. The protective effect of these treatments could be mediated by O-GlcNAcylation, as the HBP is driven by glucose and glutamine availability. Treatment of MRL-*lpr* mice in our lab with 6-diazo-5-oxo-L-norleucine (DON), an analogue of Glutamine, able to modulate GFAT the rate limiting enzyme of the HBP, was also able to alleviate the lupus phenotype (data not shown), as demonstrated by the team of Laurence Morel in their triple-congenic lupus model[9,85].

Repositioning drugs is an effective approach to accelerate treatment of autoimmune diseases[86]. Based on our results and the reported protective effects of glucosamine in murine models of sepsis[59,60] along with prospective studies in humans showing lower all causes mortality[87–92] associated with long term glucosamine intake, we propose to envision repositioning of glucosamine or use of OGA blockade as an adjuvant therapy in SLE in combination with the standard of care.

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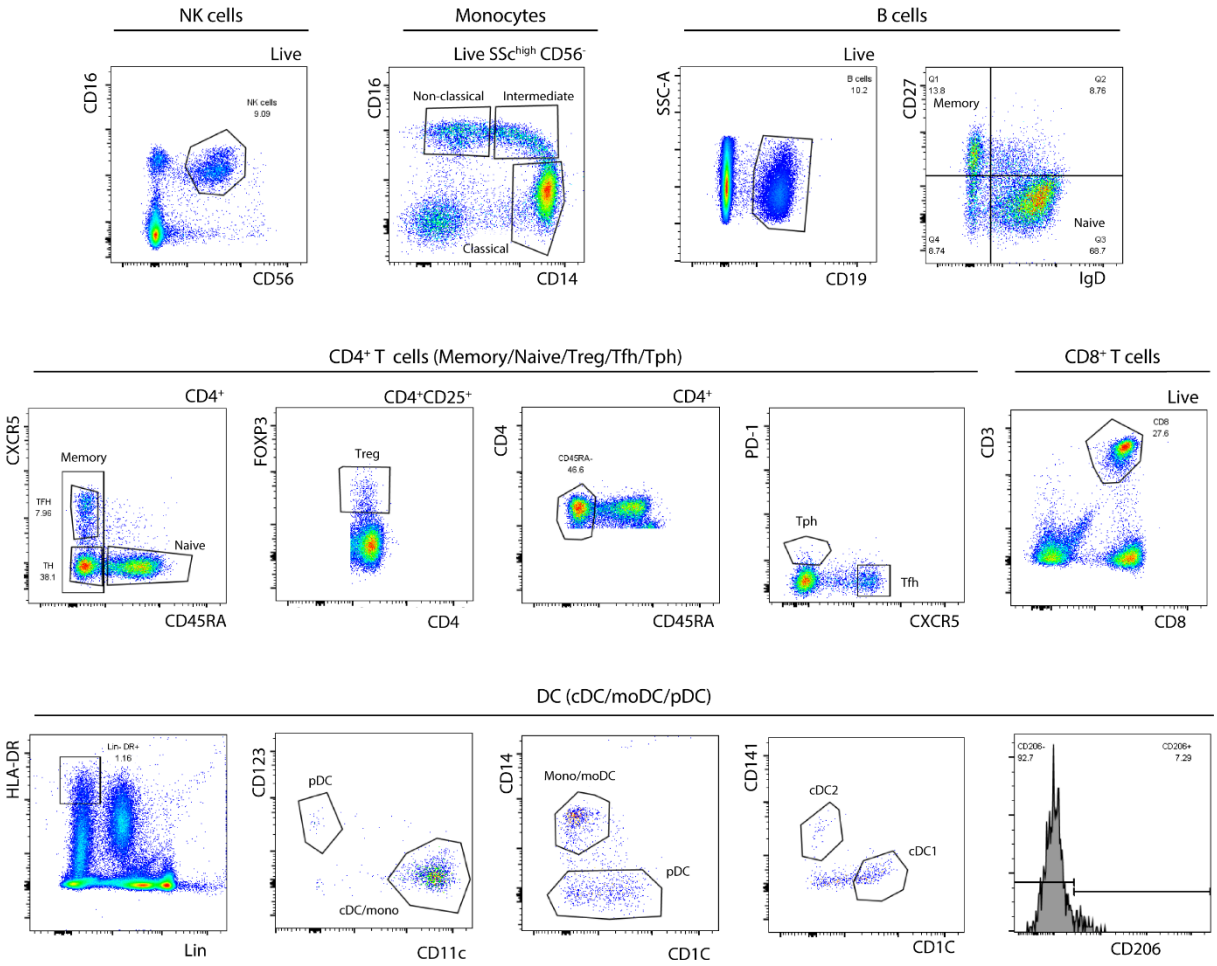
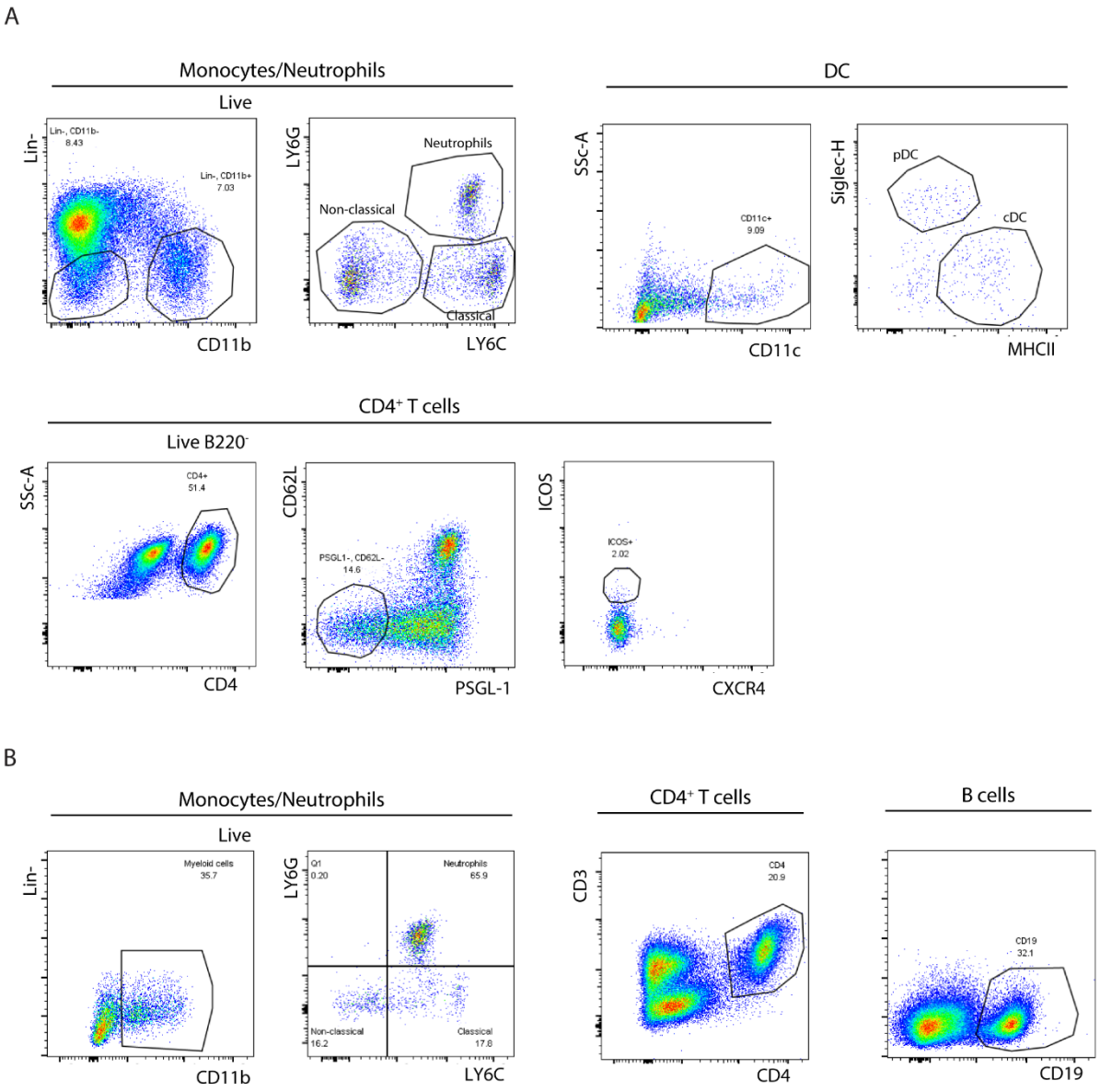
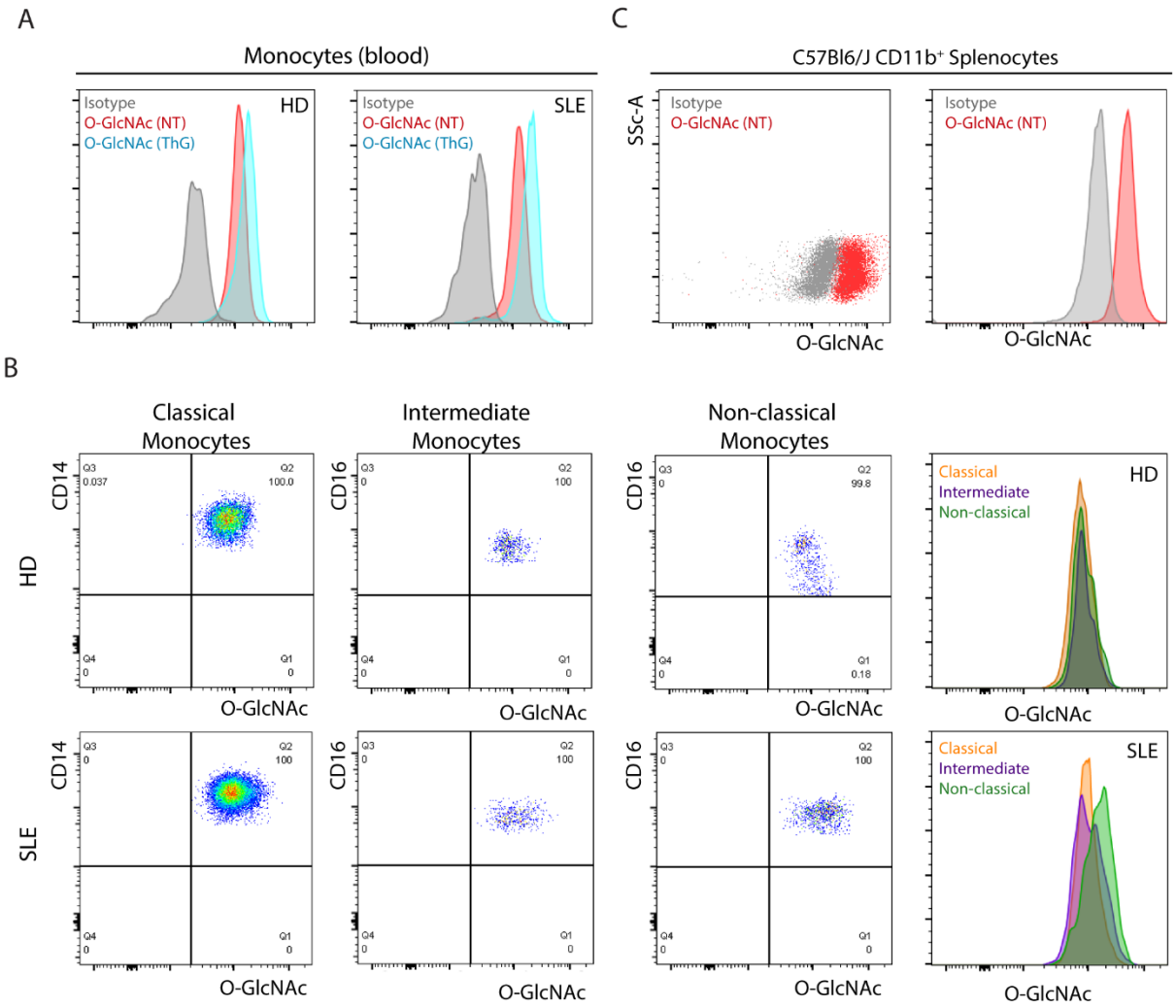
Supplemental figures and tables



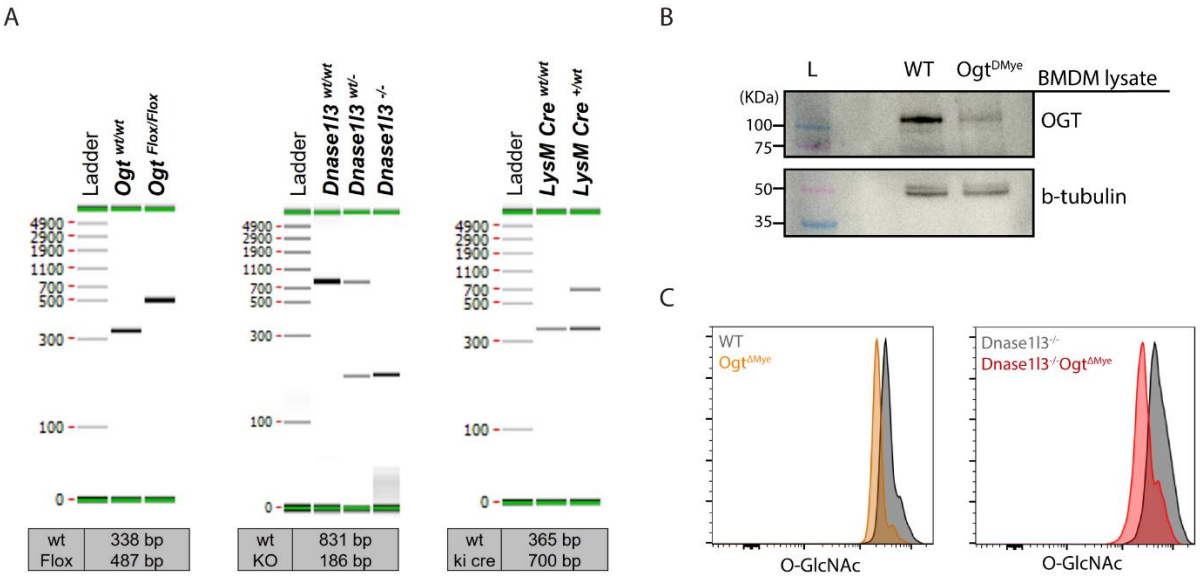
Supplemental Figure S1. Human flow cytometry gating strategy (NK cells, monocytes, B cells, $CD4^+$ T cells, $CD8^+$ T cells and DC subsets).



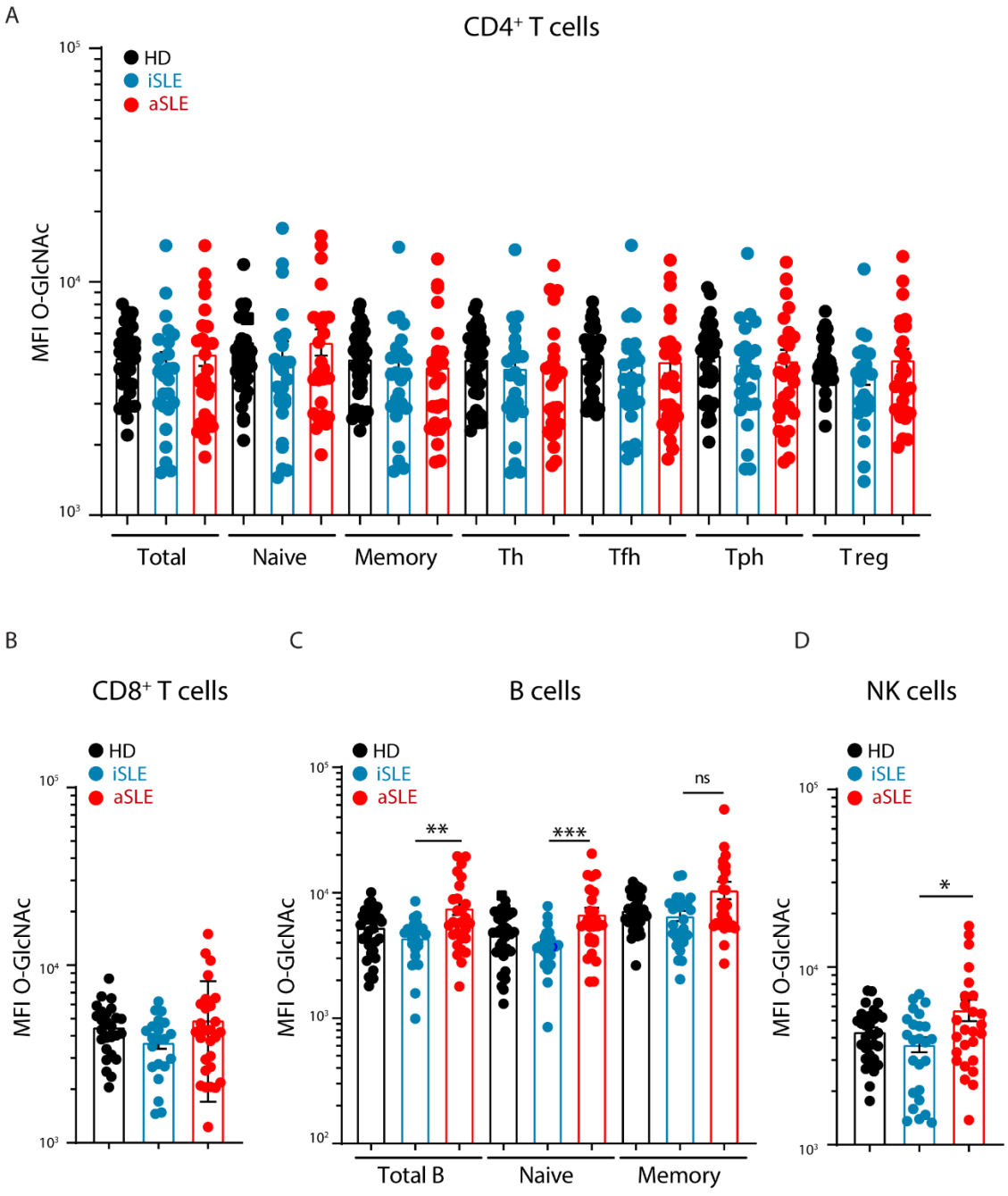
Supplemental Figure S2. Mouse flow cytometry gating strategy**. A.** *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ experiments (monocytes, neutrophils, DC, CD4^{+} T cells). **B.** MRL-lpr experiments (monocytes, neutrophils, CD4^{+} T cells, B cells).



Supplemental Figure S3. A. Representative histogram of the MFI of O-GlcNAc and control isotype in blood monocytes, untreated or treated with the OGA inhibitor ThG (10µM) for 2 hours. **B.** Representative dot-plots and histogram representation of the staining and MFI of O-GlcNAc in classical, intermediate, and non-classical monocytes subsets from HD and patients with SLE. **C.** The MFI of O-GlcNAc and control isotype in C57Bl6/J splenocytes.



Supplemental Figure S4. A. Representative genotyping of *Ogt*, *Dnase1l3* and *LysM-cre* from WT, $Ogt^{\Delta Mye}$, and $Ogt^{\Delta Mye}Dnase1l3^{-/-}$ mice. **B.** Representative western-blot analysis of OGT and β-Tubulin in Bone Marrow Derived Macrophages (BMDM) from WT or $Ogt^{\Delta Mye}$ mice. **C.** Representative MFI of O-GlcNAc from WT, $Ogt^{\Delta Mye}$ and $Ogt^{\Delta Mye}Dnase1l3^{-/-}$ mice.



Supplemental Figure S5. Pooled quantification of the intracellular MFI of O-GlcNAc in peripheral blood lymphocyte subsets from HD, patients with inactive SLE (iSLE) or patients with active SLE (aSLE) **A.** $CD4^+$ T cells subsets, Kruskal-Wallis test. **B.** $CD8^+$ T cells, Kruskal-Wallis test. **C.** B cells subsets, Kruskal-Wallis test, ** = p<0,01; *** = p<0,001. **D.** NK cells subsets, Kruskal-Wallis test, * = p<0,05.

Supplemental table 1. Demographic characteristics of HD and SLE patients included in the METABOLUPS study (NCT04440566), and SLE patients clinical, biological manifestations, activity of the disease and treatment received.

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Supplemental table 2. Flow cytometry panels used and antibodies references. **A.** Human experiments. **B.** Mouse experiments.

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3 CHAPTER 3: THE ROLE OF O-GLCNACYLATION IN DENDRITIC CELLS IN THE CONTEXT OF INFLUENZA INFECTION OR SLE

3.1 Generation of *Ogt*$^{\Delta DC}$ and *Ogt*$^{\Delta DC}$/*Dnase1l3*$^{-/-}$ mice

Following our observation of dynamic changes in O-GlcNAcylation levels in the pDC compartment of SLE patients compared to healthy donors, and because of scarce data on the role of O-GlcNAcylation in DCs in general (324,325), we decided to further investigate this pathway in DCs. We first generated DC-specific *Ogt* mutant (*Ogt*$^{\Delta DC}$) mice. We bred Ogt$^{F/F}$ mice (JAX stock #004860) with WT C57B6/J mice expressing a CD11c-cre (JAX stock #008068) (326) to specifically delete *Ogt* in DCs. *Ogt* was successfully deleted in DCs in both male and female mice, as demonstrated by flow cytometry of splenic DC and western analyses on FACS-sorted splenic DCs after negative magnetic selection (Miltenyi Biotec Inc. 130-100-875) from *Ogt*$^{\Delta DC}$ male and female mice, respectively (Figure 12a, b). We tested the response of these mice in an acute model of influenza infection at McGill University. In parallel, we crossed *Ogt*$^{\Delta DC}$ with *Dnase1l3*$^{-/-}$ mice to explore the role of DC O-GlcNAcylation on SLE. A simplified breeding strategy and representative genotyping images are depicted (Figure 12c, d). We generated two cohorts of seven to eleven mice/genotype/cohort, which are currently between four and five months of age for further study.

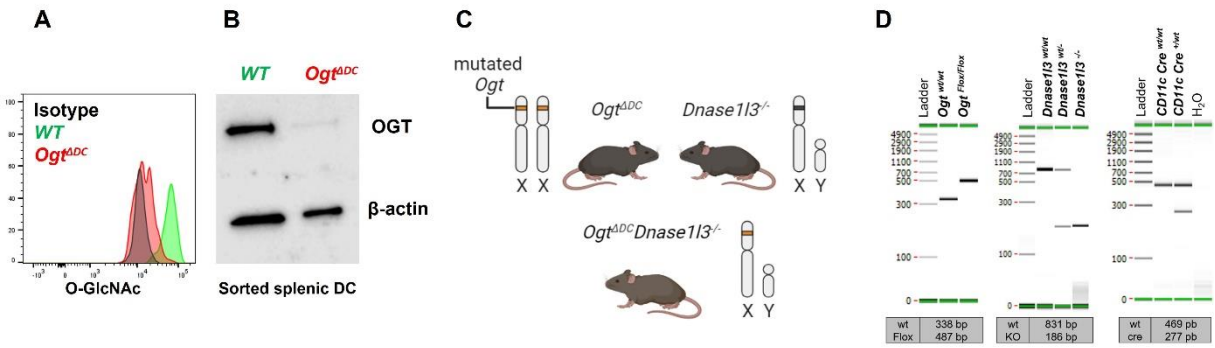

Figure 12. Generation of Ogt$^{\Delta DC}$ and Ogt$^{\Delta DC}$Dnase1l3$^{-/-}$ mice

A. Representative MFI of O-GlcNAc and control isotype in splenic DC from WT and *Ogt*$^{\Delta DC}$ male mice. **B.** Representative western-blot analysis of OGT and β-actin in FACS-sorted splenic DC from WT and *Ogt*$^{\Delta DC}$ female mice (3 spleen per genotype). **C.** Simplified representation of the breeding strategy to generate *Ogt*$^{\Delta DC}$*Dnase1l3*$^{-/-}$ mice. **D.** Representative genotyping of *Ogt*, *Dnase1l3* and *CD11c-cre* from WT, *Ogt*$^{\Delta DC}$, and *Ogt*$^{\Delta DC}$*Dnase1l3*$^{-/-}$ mice.

3.2 Investigation of O-GlcNAcylation in influenza infection

As previously mentioned, the HBP is a central regulator of type I IFN production and antiviral immunity through the O-GlcNAcylation of two substrates in particular: 1) MAVS protein (147,148,181) and IRF5 (149). We hypothesized that O-GlcNAcylation mediates antiviral immunity by impacting IFN-producing innate immune cells, particularly DCs. To test this, we infected *Ogt*$^{\Delta DC}$ mice with 250 pfu/20g of H1N1 PR/8 or H3N2 HK/68 MA20 influenza virus, monitored their clinical phenotypes at 3 days post infection (dpi). In parallel, we investigated the effect of pharmacological modulation of the pathway by treating WT mice with 220mg/kg of N-Acetyl-D-glucosamine (Sigma-A8625), a commercially available nutrient that bypasses the rate limiting step of the HBP leading to increased O-GlcNAcylation. We observed that increased O-GlcNAcylation with glucosamine treatment alleviated weight loss at 3 dpi after H3N2 infection (250pfu/20g) in WT male mice, with a similar trend in female mice infected with H1N1. This mechanism appears independent of viral control, as viral loads were not different between conditions at this time point as evaluated by plaque-assays. Consistent with a protective role of O-GlcNAcylation, *Ogt*$^{\Delta DC}$ mice were more susceptible to influenza-induced weight loss at 3 dpi in male mice, independently of viral loads, suggesting that O-GlcNAcylation in DCs could protect the host from influenza infection by enhancing host disease tolerance.

The same trend was observed with H1N1 in females albeit to a lesser extent. These influenza experiments were performed once with small mouse n numbers and need to be repeated for confirmation. Nonetheless, it appears that O-GlcNAcylation in DCs provides a protective mechanism in the host inflammatory response to influenza infection.

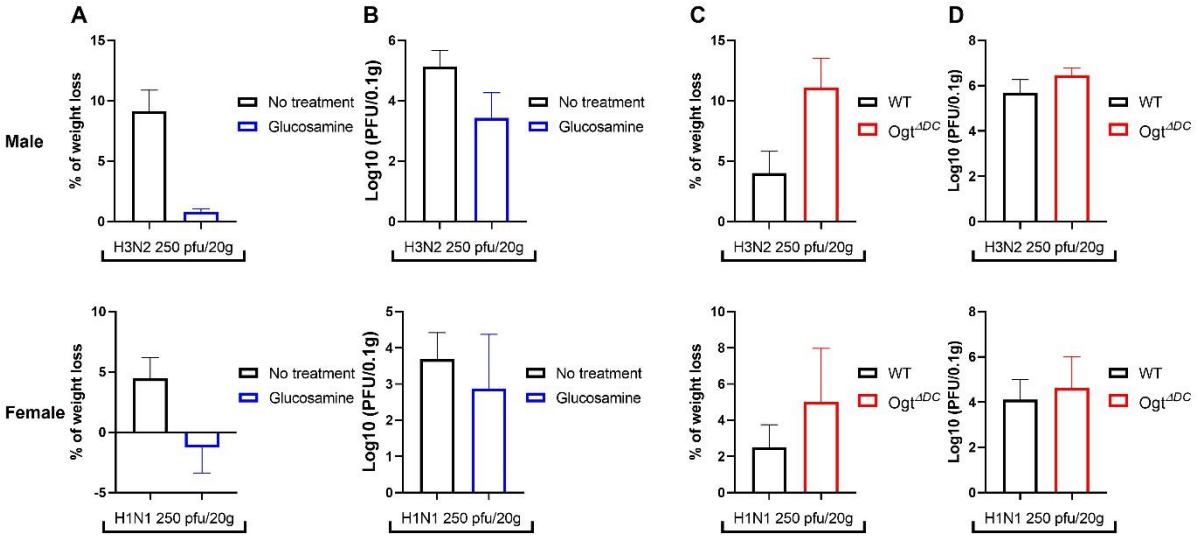

Figure 13. O-GlcNAcylation of DC could protect against influenza infection by enhancing host disease tolerance (preliminary results).

A. Weight loss and **B.** Lung viral loads (evaluated by plaque-assay on MDCK cells) at 3 dpi, in male and female WT mice treated intraperitoneally with 220mg/kg of N-acetyl-D-glucosamine or water and infected with 250 pfu/20g of H1N1 PR/8 or H3N2 HK/68 MA20 influenza virus. The treatment regimen was one per day from 2 days before to 2 days after infection. **C.** Weight loss and **D.** Lung viral loads (evaluated by plaque-assay on MDCK cells) at 3 dpi, in male and female WT and *Ogt*$^{\Delta DC}$ mice infected with 250 pfu/20g of H1N1 PR/8 or H3N2 HK/68 MA20 influenza virus.

4.1 Monocyte and macrophage O-GlcNAcylation in SLE and other CIDs

Our work reveals that O-GlcNAcylation alleviates myeloid-driven inflammation in SLE, especially through the non-classical monocyte subset (327). Non-classical monocytes have been implicated in SLE and other CIDs (19). They patrol the kidney glomeruli to maintain its homeostasis (250) and accumulate in glomerular vessels of SLE patients (251). SLE patients with severe LN present high glomeruli titers of $CD16^{+}$ monocytes (252) and their blood frequency is inversely correlated to proteinuria in patients with LN (328). While global targeting of myeloid cells e.g. e.g., with CSF-1R or CX3CL1 blockade, has proven effective in LN in mice (255–257), subset-specific depletion approaches have not been reported. One team highlighted that administration of topical Tlr7 agonist (Imiquimod) in mice increased pro-inflammatory cytokine *IL-6* and chemoattractant *Ccl5* and *Cxcl13* transcription in kidney $Ly6^{lo}$ monocytes but only found an IFNα signature in the classical subset (329). Adoptive transfer of "M2-like" IL-4 differentiated BM-derived macrophages in the ALD-DNA SLE mouse model depleted of endogenous macrophages using clodronate, alleviated the SLE phenotype, while "M1-like" IFNγ differentiated BM-derived macrophages did not, highlighting a potent proinflammatory role for M1-like macrophages and a possible protective role for M2-like macrophages (249). A regulatory role of O-GlcNAcylation in non-classical monocytes is aligned with previous findings identifying a protective role of this pathway in septic shock and the metabolic syndrome, specifically by inhibiting the inflammatory functions of Ripk3 (141) or S6k1 (143), respectively. Together, these findings suggest that modulation of myeloid cells towards an anti-inflammatory and wound-healing profile might represent a potential therapeutic strategy in lupus.

Of note, increasing O-GlcNAcylation by OGA blockade reduces synovial fibroblast inflammatory cytokines *in vitro* but promotes IL1β signaling through TAK1 O-GlcNAcylation *in vivo* in a rat adjuvant-induced arthritis (AIA) model (330). This discrepancy could rely on different mechanisms of O-GlcNAcylation in genetically induced SLE models versus acute inducible models of arthritis.

To functionally assess the role of O-GlcNAcylation in myeloid cell response to TLR stimulation, we tried to isolate human $CD16^{+}$ monocytes from HD and SLE patients blood samples. We also attempted to isolate sufficient numbers of pDC from HD cytapheresis ring/buffy coats, by negative magnetic selection. We wished to stimulate them with TLR agonists (e.g. LPS for monocyte treatment, or Resiquimod for pDC treatment) and assess their surface activation markers, cytokine production and transcriptional status following OGT or OGA inhibition *ex vivo*. Unfortunately, we only managed to retrieve few cells, insufficient for *ex vivo* experiments. This led us to consider alternative approaches to investigate rare cells. For instance, we will modulate O-GlcNAcylation pharmacologically in whole blood to investigate effects on TLR responses.

One of the limitations of our human study is the relatively low number of patients, to be able to use O-GlcNAc profiles to stratify patients according to clinical involvement: LN, cutaneous-articular, seritis, neurologic, etc., or response to treatment.

Last, by working in a center of reference laboratory investigating other autoimmune diseases, I had the opportunity to evaluate O-GlcNAcylation levels in 7 systemic sclerosis patients. These patients did not present higher O-GlcNAcylation in non-classical monocytes and pDC compared to HD, as we observed in SLE. These preliminary data need to be confirmed but suggest context-specific induction of O-GlcNAcylation in SLE.

4.2 O-GlcNAcylation, a pro- or anti-inflammatory mechanism?

We argue that O-GlcNAcylation is a rheostat that controls the amplitude of the inflammatory response. In contexts of acute infection, O-GlcNAcylation might boost the inflammatory response to rid the organism of the infectious agent. For instance, in viral infections, O-GlcNAcylation is required for anti-viral immunity by activating MAVS signaling and IRF5 activity (147–149). However, uncontrolled responses could result in adverse events. This is the case of IRF5-induced cytokine storm in influenza virus infection (149). Otherwise, O-GlcNAcylation limits overt inflammation to protect the organism from inflammatory tissue damage. Several pieces of evidence support this hypothesis. a) the HBP is linked to alternative activation of macrophages (331); b) O-GlcNAcylation inhibits necroptotic cell death by inhibiting RIPK3 phosphorylation (141); c) it also blunts metabolic inflammation by limiting S6K phosphorylation downstream of mTOR (143); d) N-acetyl glucosamine administration is therapeutic in different inflammatory pathologies models (135,141,240); e) O-GlcNAcylation is reduced in IBD patients' tissues (332); and f) it is a protective mechanism in autoimmune hepatitis by driving Treg activity (333). In SLE, in the Dnase1l3$^{-/-}$ model deficient for Ogt in myeloid cells, as well as the MRL-*lpr* model, O-GlcNAcylation is a protective mechanism.

4.3 O-GlcNAcylation in dendritic cells

As previously stated, O-GlcNAcylation is increased in pDC of active SLE and correlates to their activation, a feature that we confirmed both in humans and in the *Dnase1l3*$^{-/-}$ mouse model (data not shown). Moreover, we also confirmed that the pDC subset displayed more O-GlcNAcylation than cDC or moDC subsets in HD as well as SLE. This richer O-Glycome in pDC advocates for a wider role of O-GlcNAcylation in this cell type, not limited to SLE. There is scarce data regarding O-GlcNAcylation implication in DC. A recent work highlighted that OGA

blockade, leading to increased O-GlcNAcylation, drives DC differentiation of hematopoietic stem cells progenitors, through STAT3 and STAT5 activation (325). Another team described that pharmacological inhibition of OGT by OSMI-I (90) leads to decrease switch from monocytes to moDC (334) *in vitro,* by decreasing phosphorylation of AKT and mTOR (324), but the precise role of O-GlcNAcylation in DC subsets remains to be elucidated.

Isolation of pDC for *ex vivo* studies is challenging, as they represent the rarest PBMC subtype. BMDC differentiation using GMCSF or FLT3L (335,336) allows for greater number of cells to investigate DC responses upon stimulation. However, these *in vitro* cultures require one week to obtain differentiated DCs and a subsequent step of magnetic negative selection is useful to allow a good DC purity, which distances the subsequent evaluations (cytokines, RNA) from the original cells found in a living organism. To stay closest to physiological and pathological contexts, we chose to focus our DC investigation on *in vivo* approaches.

We were particularly interested in deleting *Ogt* specifically in the pDC subset, given the observation in humans. Although depletion of pDCs, using a BDCA2 promoter-inducible diphtheria toxin approach exists (JAX stock #014176) (337), a pDC-specific cre deleter strain is to our knowledge not available. Therefore, we chose to create an $Ogt^{\Delta DC}$ mouse model, lacking *Ogt* expression in all DC subsets. At first, we bred $Ogt^{F/F}$ female mice with male bearing the CD11c-cre transgene. However, offsprings did not exhibit efficient deletion of *Ogt*. We attributed this to an insufficient cre expression and decided to move the cre on female mice, as it could result in better expression (338). Fortunately, this resulted in efficient *Ogt* deletion in both male and female mice (Figure 12a, b). We encountered the same inconsistency in cre expression in our myeloid breeding where *Ogt* deletion was more efficient in male mice, a phenomenon that could possibly be explained by a mosaicism in females (338). Of note, the

Ogt$^{\Delta Mye}$ phenotype seems to be more penetrant in younger animals, maybe because of increased recombination of the floxed allele (339).

Finally, while the original publication using the CD11c-cre deleter strain did not observe an effect on B cells (326), we cannot rule out at this stage of the study a role of O-GlcNAcylation in ABC, an important B cell population in SLE that is CD11c^{+}. In the event, we observe altered ds-DNA autoantibodies secretion in our *Ogt*$^{\Delta DC}$ mice, we will explore this population more closely.

4.4 VISTA, a potential target of O-GlcNAcylation in myeloid cells

Our proteomic analysis identified 6 O-GlcNAcylation targets implicated in SLE. Among them, VISTA appears as an interesting candidate mediating the regulatory effect of O-GlcNAcylation. Indeed, VISTA is upregulated in cutaneous lesions of patients with SLE and *Vista*$^{-/-}$ BALB/c mice spontaneously develop lupus-like disease (340), with increased numbers of CD11b^{+} myeloid cells, pDCs, CD4^{+} T cells, NK cells, T_{FH}, decreased regulatory T cells, increased circulating IFNα after TLR7/8 agonist injection. *Vista*$^{-/-}$ animals cutaneous lesions exhibit more CD4^{+} T cells, pDCs, myeloid cells, especially neutrophils, and VISTA agonist treatment alleviated the MRL-*lpr* phenotype. VISTA agonist is also able to widely blunt innate inflammation including the type I IFN pathway (341), and VISTA expressing macrophages promote kidney repair after ischemic injury (342). Together, this highlight VISTA as an inhibitory mechanism in SLE, in part through its action on innate inflammation and type I IFN. Moreover, VISTA can bind PSGL-1 at acidic pH (343). As mentioned above, P-selectin impairs regulatory T cells in SLE by binding to PSGL-1 on their surface (280). However, whether VISTA promotes Treg activity in an O-GlcNAcylation-dependent manner remains an open question.

4.5 Metabolism modulation and drug repositioning in SLE

Glucose (317) or glutamine (319) modulation (by 2-DG and BPTES, respectively) alleviates SLE in mouse models. These treatments could influence the HBP indirectly. In this work, increased O-GlcNAcylation by OGA inhibition led to improvement of the SLE phenotype in lupus prone mice. Of note, treatment of MRL-*lpr* mice in our lab with 6-diazo-5-oxo-L-norleucine (DON) an analogue of Glutamine, able to modulate GFAT the rate limiting enzyme of the HBP, was also able to alleviate the lupus phenotype (data not shown), as demonstrated by the team of Laurence Morel in their triple-congenic lupus model (317,318). Repositioning drugs is an effective approach to accelerate treatment approvals for autoimmune diseases (344). N-acetyl-D-Glucosamine, a substrate of the final step of the HBP after its rate limiting step, alleviates sepsis inflammation in mice (87,345,346). In humans, long term glucosamine intake has been associated with lower all-cause mortality, as reported in a prospective cohort of 495,077 men and women (347), a finding aligned with other studies (348–351). Of note, selection bias in those studies prevents strong conclusions on the impact of glucosamine use on mortality (352) but point to the potential of repositioning glucosamine as an adjuvant therapy to the SLE standard of care.

4.6 Future directions.

Concerning the SLE project, we wish to further characterize the phenotype of *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice, by performing immunohistochemistry analyses on the kidneys and performing additional analyses of urine samples.

Next, we wish to study the role of VISTA O-GlcNAcylation in SLE. First, we will validate VISTA as an OGT substrate by performing immunoprecipitation (IP) and western blot analyses.

We will IP Vista from *Dnase1l3*$^{-/-}$ and *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ peritoneal macrophages using anti-VISTA antibodies on Protein G agarose beads, and probe for an O-GlcNAc signal on western blot using anti-RL2 antibodies. Second, we will identify the S/T residue in VISTA that is O-GlcNAcylated, using site-directed mutagenesis. Third, we will explore VISTA binding to PSGL1 and the impact of O-GlcNAcylation on this binding. For this, we will express WT VISTA or a (S/T→A) mutant form deficient in O-GlcNAcylation in a model cell-line *in vitro* and perform binding assays using recombinant PSGL-1. In parallel, we will co-IP VISTA and PSGL-1 from a myeloid/Treg co-culture in the presence of OGT or OGA inhibitors to assess whether O-GlcNAcylation modulation alters their binding affinity. Fourth, we will synthesize VISTA peptides harboring the O-GlcNAc residue and test their therapeutic potential in SLE mouse models.

As we did with *Ogt*$^{\Delta Mye}$*Dnase1l3*$^{-/-}$ mice, we also wish to characterize the phenotype of *Ogt*$^{\Delta DC}$*Dnase1l3*$^{-/-}$, to validate the functional impact of *Ogt* loss in DCs on the SLE phenotype.

Concerning the influenza project, we wish to replicate our early data and investigate in depth the functional consequences of Ogt loss in DCs in influenza infection, by analyzing DC activation by flow cytometry, their O-glycome and transcriptome (by bulk RNAseq) upon influenza challenge of WT and *Ogt*$^{\Delta DC}$ mice. Of note, this model could be used to assess other viral infections or to study the role of Ogt in DC in other disease contexts, such as cancer.

From a translational point of view, to further investigate O-GlcNAcylation in SLE, we wish to design a follow up clinical study, in which we sample patients at enrolment and before treatment and sample them again at different time points after treatment and at remission to investigate O-GlcNAcylation changes longitudinally. However, this would require a longer period of inclusion as naïve patients at diagnosis are rare, especially to obtain homogeneous

groups of patients to stratify by clinical involvement. Other measures could be also included in this follow-up study, for e.g., O-GlcNAcylation evaluation at a specific time of the day, a precise nutritional assessment, and exclusion of confounding treatments e.g. with mTORi or Metformin. Finally, a pilot study to evaluate repositioning glucosamine as an adjuvant therapy to standard of care could be rapidly envisioned as glucosamine has a safe tolerance profile and is already approved by the European Medicines Agency, Health Canada, and the FDA.

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6 APPENDIX

6.1 Appendix 1: SLE Disease Activity Index 2000 (SLEDAI-2K)

Point	Symptom	Definition
8	**Seizure**	Recent onset seizure. Exclude metabolic, infectious, or drug causes.
8	**Psychosis**	Altered ability to function in normal activity due to severe disturbance in the perception of reality (include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, and bizarre, disorganized, or catatonic behavior); exclude uremia and drug causes
8	**Organic brain syndrome**	Altered mental function with impaired orientation, memory, or other intellectual function (with rapid onset and fluctuating clinical features), inability to sustain attention to environment, and ≥2 of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, and increased or decreased psychomotor activity; exclude metabolic, infectious, or drug causes
8	**Visual disturbance**	Retinal changes of SLE (include cytoid bodies, retinal hemorrhages, serous exudates or hemorrhages in choroid, and optic neuritis); exclude hypertensive, infectious, or drug causes
8	**Cranial Nerve Involvement**	New onset sensory or motor neuropathy involving cranial nerves
8	**Lupus headache**	Severe, persistent headache (may be migrainous but must be nonresponsive to narcotic analgesia)
8	**Stroke**	New onset stroke. Exclude arteriosclerosis

8	**Vasculitis**	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages or biopsy, and angiogram proof of vasculitis
4	**Arthritis**	≥2 joints with pain and signs of inflammation (i.e., tenderness, swelling, or effusion)
4	**Myositis**	Proximal muscle aching/weakness associated with elevated CPK/aldolase, EMG changes, or a biopsy showing myositis
4	**Urinary casts**	Heme-granular or Red Blood Cells (RBC) urinary casts
4	**Hematuria**	>5 RBC/high-power field; exclude stone, infection, or other cause
4	**Proteinuria**	Recent onset or increase >0.5 g/24 hours
4	**Pyuria**	>5 White Blood Cells (WBC)/high-power field; exclude infection
2	**New onset Rash**	Inflammatory-type rash
2	**Alopecia**	New onset or relapse
2	**Oral or nasal mucosal ulcers**	New onset or relapse
2	**Pleuritis**	Pleuritic chest pain with pleural rub/effusion or pleural thickening
2	**Pericarditis**	Pericardial pain with ≥1 of the following: rub, effusion, or EKG/echocardiogram confirmation
2	**Low complement**	CH50, C3, or C4 decreased below lower limit of normal for lab
2	**High DNA binding**	Increased above normal range for lab
1	**Fever**	Temp > 38°C (100.4 °F). Exclude infectious causes
1	**Thrombocytopenia**	Platelets <100 x 10^9/L
1	**Leucopenia**	WBC <3 x 10^9/L Exclude drug causes

6.2 Appendix 2: other works

During these 3 years of PhD, I worked on 4 reviews alongside with investigators from my two laboratories in Bordeaux and McGill, three as first author or co-first author and one as third author. They are presented in this appendix.

Immunometabolism of Macrophages in Bacterial Infections. Galli G, Saleh M. Front Cell Infect Microbiol. 2021 Jan 29;10:607650. doi: 10.3389/fcimb.2020.607650. eCollection 2020.

Soluble CD95L in cancers and chronic inflammatory disorders, a new therapeutic target? Kurma K, Boizard-Moracchini A, Galli G, Jean M, Vacher P, Blanco P, Legembre P. Biochim Biophys Acta Rev Cancer. 2021 Dec;1876(2):188596. doi: 10.1016/j.bbcan.2021.188596. Epub 2021 Jul 26.

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Identifying Markers of Emerging SARS-CoV-2 Variants in Patients With Secondary Immunodeficiency. Markarian NM*, Galli G*, Patel D, Hemmings M, Nagpal P, Berghuis AM, Abrahamyan L, Vidal SM. Front Microbiol. 2022 Jul 1;13:933983. doi: 10.3389/fmicb.2022.933983. eCollection 2022. * Co-first author

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REVIEW
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Immunometabolism of Macrophages in Bacterial Infections

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Macrophages are important effectors of tissue homeostasis, inflammation and host defense. They are equipped with an arsenal of pattern recognition receptors (PRRs) necessary to sense microbial- or danger-associated molecular patterns (MAMPs/DAMPs) and elicit rapid energetically costly innate immunity responses to protect the organism. The interaction between cellular metabolism and macrophage innate immunity is however not limited to answering the cell's energy demands. Mounting evidence now indicate that in response to bacterial sensing, macrophages undergo metabolic adaptations that contribute to the induction of innate immunity signaling and/or macrophage polarization. In particular, intermediates of the glycolysis pathway, the Tricarboxylic Acid (TCA) cycle, mitochondrial respiration, amino acid and lipid metabolism directly interact with and modulate macrophage effectors at the epigenetic, transcriptional and post-translational levels. Interestingly, some intracellular bacterial pathogens usurp macrophage metabolic pathways to attenuate anti-bacterial defenses. In this review, we highlight recent evidence describing such host-bacterial immunometabolic interactions.

Keywords: innate immunity, bacteria, inflammation, immunometabolism, infection

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PREFACE

Derived from hematopoietic precursors, macrophages are central innate immune cells that function in host defense and maintenance of tissue homeostasis. First described by Ilya (Elie) Mechnikov in 1882, macrophages are essentially found in every tissue, seeding the tissue during embryonic development and acquiring specialized organ-specific identities and functions through transcriptional and epigenetic programs governed by factors released by the organ's stroma (Lavin et al., 2015). In case of perturbations to tissue homeostasis, bone marrow-derived monocytes are recruited from the blood to the affected site where they differentiate into macrophages. Both tissue-resident and monocyte-derived macrophages activate innate and adaptive immunity (Lavin et al., 2015). They act as scavengers that engulf and destroy microbes, particulate matters or altered host cells, while alerting the immune system through the secretion of cytokines, chemokines and lipid mediators. In addition, macrophages contribute to wound healing and tissue repair processes. In contrast, dysregulated activation of macrophages leads to inflammatory tissue damage and inflammatory diseases, cancer promotion, granulomas and chronic infections, atherosclerosis and the metabolic syndrome (Murray and Wynn, 2011). Macrophages express scavenger receptors and immunoglobulin receptors, which promote phagocytosis (Kumar, 2020), antibody-dependent cell phagocytosis (ADCP) and antibody-dependent cell cytotoxicity (ADCC). In addition, they are equipped with germ-line encoded pattern recognition receptors (PRRs) that sense

microbial- or danger-associated molecular patterns (MAMPs/DAMPs). Following PRR stimulation, signal transduction cascades converge on the activation of master transcription factors, proteases, and effectors of phagocytosis, allowing a quick innate immune response (Kumar, 2020) (**Figure 1A**). PRR engagement also ensures durable responses through metabolic and epigenetic establishment of an "innate memory", termed trained immunity. This term coined in 2011 (Netea et al., 2011) refers to the ability of innate immune cells, such as monocytes and macrophages to develop a heightened secondary response, that is rather unspecific, as it occurs following rechallenge by the same or other pathogens. Trained immunity has been demonstrated primarily in studies exploring the response to β-glucan of *Candida albicans* or *Mycobacterium tuberculosis* (*Mtb*) bacillus Calmette-Guérin (BCG). Unlike the classical immunological memory of the acquired immune system, which involves gene recombination events, trained immunity is established by metabolic and epigenetic reprogramming of transcriptional pathways in myeloid progenitors (Netea et al., 2020) (**Figure 1B**).

According to their function in inflammation and host defense versus wound healing and tissue repair, macrophages have been broadly designated as classically activated macrophages or alternatively activated macrophages (also referred to as M1 and M2, respectively). However, such an M1/M2 classification is an *in vitro* paradigm that does not reflect the heterogeneity of macrophages observed *in vivo*, as recently revealed by single cell approaches (Guilliams et al., 2018; Bonnardel and Guilliams, 2018). Previous studies have relied on *in vitro* polarization of murine bone marrow-derived macrophages (BMDM) with bacterial lipopolysaccharide (LPS) + interferon (IFN)γ to obtain the M1 phenotype or interleukin (IL)-4 and/or -13 for M2 polarization. These two macrophage states can be distinguished based on the expression of inducible nitric oxide (NO·) synthase (iNOS) and arginase in M1 and M2 cells, respectively (MacMicking et al., 1997). These markers highlight a primary metabolic difference in the metabolism of arginine into the "killer" molecule NO· in M1 cells or the "repair" metabolite ornithine in M2 cells. Macrophage metabolic adaptations are however not limited to the arginine pathway. As we discuss below, a number of studies have reported a "break" in the TCA cycle and a shift to aerobic glycolysis in M1 macrophages versus a preference for oxidative phosphorylation (OXPHOS) with enhanced glutamine and fatty acid utilization in M2 macrophages. Metabolic intermediates and effectors (e.g. NO·, reactive oxygen species [ROS], TCA derivatives, itaconate, prostaglandins, tryptophan metabolism etc.) were shown to regulate macrophage phenotypes and functions by acting as direct bactericidial agents or through the modulation of innate immunity signaling pathways, leading to the production of cytokines, antimicrobial peptides or tissue repair factors (O'Neill et al., 2016). Here, we focus on recent examples of immunometabolic adaptations following bacterial challenge and of bacterial strategies that target metabolic effectors to modulate host defense mechanisms. The discovery of such immunometabolic interactions provide novel therapeutic entry points to treat immunological disorders and infectious diseases.

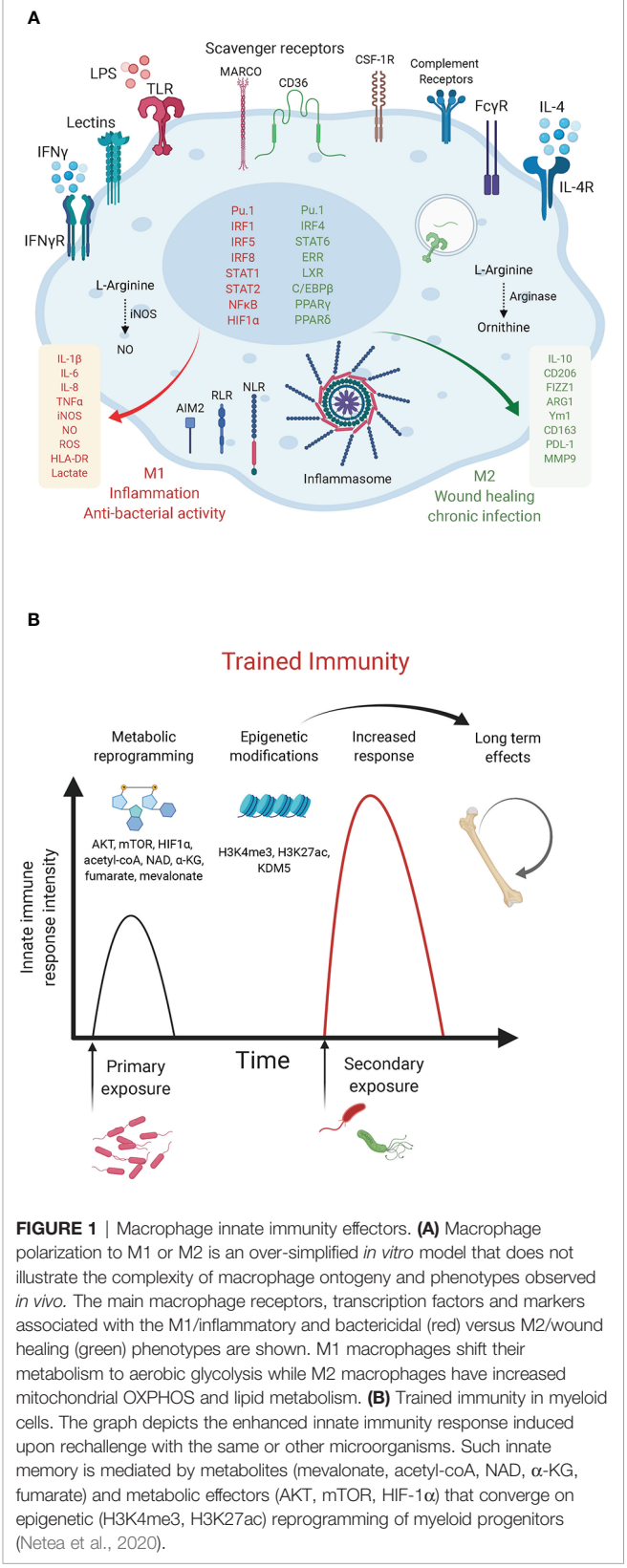

FIGURE 1 | Macrophage innate immunity effectors. **(A)** Macrophage polarization to M1 or M2 is an over-simplified *in vitro* model that does not illustrate the complexity of macrophage ontogeny and phenotypes observed *in vivo*. The main macrophage receptors, transcription factors and markers associated with the M1/inflammatory and bactericidal (red) versus M2/wound healing (green) phenotypes are shown. M1 macrophages shift their metabolism to aerobic glycolysis while M2 macrophages have increased mitochondrial OXPHOS and lipid metabolism. **(B)** Trained immunity in myeloid cells. The graph depicts the enhanced innate immunity response induced upon rechallenge with the same or other microorganisms. Such innate memory is mediated by metabolites (mevalonate, acetyl-coA, NAD, α-KG, fumarate) and metabolic effectors (AKT, mTOR, HIF-1α) that converge on epigenetic (H3K4me3, H3K27ac) reprogramming of myeloid progenitors (Netea et al., 2020).

MACROPHAGE METABOLIC REWIRING AND ASSOCIATED FUNCTIONAL OUTCOMES

Prior to delving into macrophage metabolic rewiring, we briefly provide a snapshot of the key cellular bioenergetic pathways described to impact macrophage functions upon bacterial challenge, with a focus on glycolysis and the mitochondrial TCA cycle (Box 1) (O'Neill et al., 2016). Early studies examining macrophage immunometabolism have explored the impact of Toll-like receptors (TLR) engagement, in particular that of TLR4, on metabolic rewiring. Follow-up studies examined live bacterial infections and interrogated the functional outcome of metabolic adaptations on macrophage function. Whether the early metabolic changes impact macrophage polarization or are a consequence of inflammatory signaling is currently debated. Nonetheless, there is ample evidence that metabolic mediators and effectors control both innate immunity and trained immunity in a feedforward manner.

Nitric Oxide· Kick-Starts the Metabolic Rewiring in Lipopolysaccharide-Activated Macrophages

Macrophages stimulated with LPS upregulate the expression of iNOS and metabolize arginine to produce high levels of NO· (MacMicking et al., 1997). Besides its anti-microbial effects, NO· has been shown, almost 30 years ago, to inhibit the ETC (Granger and Lehninger, 1982; Stadler et al., 1991; Cleeter et al., 1994). More recently, NO· was demonstrated as the main driver of metabolic rewiring in LPS-activated macrophages, as demonstrated using murine BMDM (Palmieri et al., 2020). Previous studies have described two metabolic "breaks" in the TCA cycle in such inflammatory macrophages leading to the accumulation of citrate and succinate. Citrate accumulation was attributed to changes in isocitrate dehydrogenase (IDH1) expression (Jha et al., 2015) and activity (Bailey et al., 2019; De Souza et al., 2019). However, the recent report by Palmieri et al. demonstrated that the break is rather mediated by NO·-dependent inactivation of aconitase 2 (ACO2) (Palmieri et al., 2020) (**Figure 2A**). NO· also inhibited pyruvate dehydrogenase (PDH), potentially through Cysteine nitrosylation of the PDH-E3 subunit (dihydrolipoyl dehydrogenase, DLD), which blunts the entry of pyruvate in the TCA cycle. Cessation of glucose flux thus increases glutamine uptake and its anaplerotic utilization. Concomitantly, NO· impairs SDH function (Jha et al., 2015), although this has also been attributed to itaconate-mediated inhibition (Cordes et al., 2016; Lampropoulou et al., 2016). In either case, SDH inhibition leads to succinate accumulation (**Figure 2A**).

Reactive Oxygen Species and NAD^+ as Determinants of the Macrophage Inflammatory and Bactericidal Response

In 2011, West et al. reported that stimulation of TLR1, TLR2, and TLR4 on murine BMDM augmented mitochondrial ROS. They demonstrated juxtaposition between phagosome and mitochondria and an interaction between the TLR adaptor TRAF6 and ECSIT (Evolutionarily Conserved Signaling Intermediate In Toll Pathway), a protein involved in CI assembly in the inner mitochondrial membrane. This TLR-induced interaction leads to TRAF6-dependent ubiquitination of ECSIT which alters its localization to the outer mitochondrial membrane resulting in CI disassembly (West et al., 2011) (**Figure 2A**). Depletion of either ECSIT or TRAF6 impaired the ability of murine BMDM to clear intracellular bacteria, such as *Salmonella typhimurium*, supporting a role of CI-derived mROS in antibacterial defense (West et al., 2011). Follow up studies showed that engagement of different TLRs on human monocytes by lysates from different bacteria including *Escherichia coli*, *Mtb* and *Staphylococcus aureus* resulted in a universal increase in aerobic glycolysis, but changes in OXPHOS and lipid metabolism were restricted to some but not all TLRs (Lachmandas et al., 2016). This suggests that bacterial infection might induce context-specific metabolic adaptations in macrophages, different from what has been reported with the LPS challenge model.

In parallel to enhanced aerobic glycolysis, Tannahill et al. were first to show that LPS-activated murine BMDM accumulate succinate (Tannahill et al., 2013). Succinate enhances the activity of SDH (CII), which overloads coQ with electrons, forcing the electrons to flow backwards to CI in a process referred to as reverse electron transport (RET). RET was proposed to trigger CI-dependent generation of mitochondrial ROS (mROS), which has been linked to the induction of pro-inflammatory gene expression (Mills et al., 2016) (**Figure 2A**). In line with these findings, Garaude et al. showed that murine BMDM challenged with live bacteria remodel their ETC, decreasing CI assembly and switching to CII preferential utilization. This switch was dependent on sensing bacterial viability by TLRs and the NLRP3 inflammasome and was mediated by phagosomal NADPH oxidase and the ROS-dependent tyrosine kinase FGR phosphorylating and activating SDH (CII) (Garaude et al., 2016). Reciprocally, inflammasome activation was driven by CII, as its inhibition diminished the production of IL-1β (while increasing IL-10) (**Figure 2A**) (Garaude et al., 2016). Importantly, inhibition of CII rendered mice more susceptible to infection with *S. typhimurium* or *E. coli* (Garaude et al., 2016). More recently, the role of RET and CI as the source of mROS has been challenged (Cameron et al., 2019). CIII was shown to produce mROS in LPS-stimulated murine BMDM. Inhibition of CIII which is hypothesized to drive RET, reduced rather than increased inflammatory cytokine production. Consistently, deletion of the CI subunit NDUFS4 leads to systemic inflammation in *Ndufs4*$^{-/-}$ mice (Jin et al., 2014).

One of the effects of mROS accumulation in LPS-activated murine BMDM is the depletion of the cellular NAD^+ pool, leading to inhibition of NAD^+ dependent mitochondrial respiration. On one hand, NAD^+ is consumed by poly(ADP-ribose) polymerase activation in response to mROS-induced DNA damage (Cameron et al., 2019). On the other hand, the *de novo* synthesis of NAD^+, derived from tryptophan metabolism *via* the kynurenine pathway, is inhibited in

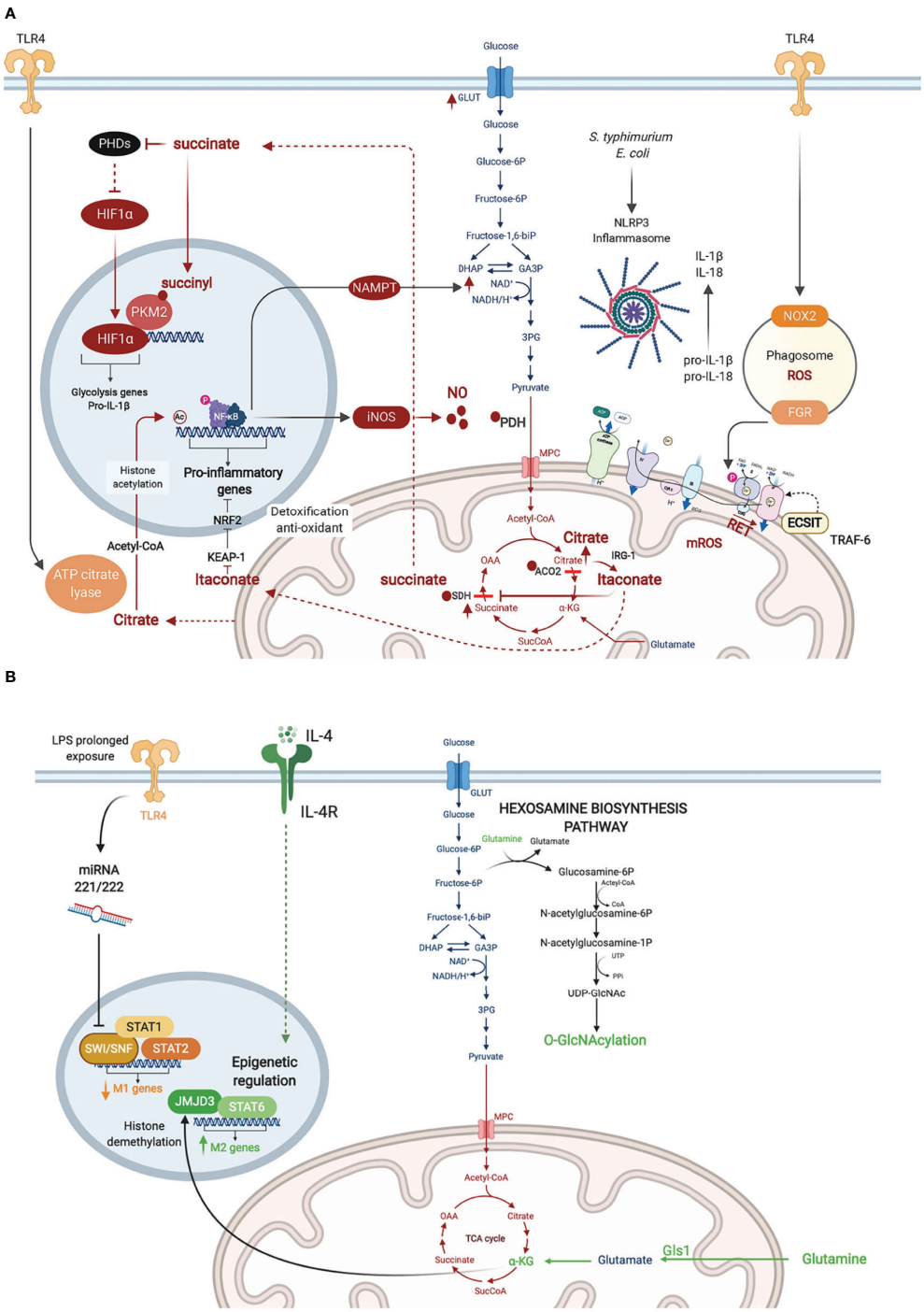

FIGURE 2 | Metabolic adaptations in activated macrophages. **(A)** Inflammatory macrophages are characterized by NO· -mediated inhibition of glucose flux in the Tricarboxylic Acid (TCA) cycle. NO· inhibits pyruvate dehydrogenase (PDH), aconitase 2 (ACO2), and SDH presumably through cysteine nitrosylation. This results in citrate accumulation and its conversion to itaconate which also blocks SDH. Citrate conversion to acetyl coA by ATP citrate lyase in the cytosol leads to histone acetylation and activation of inflammatory gene loci. SDH inhibition results in succinate accumulation, which inhibits PHDs, stabilizing HIF-1α and enhancing its transcriptional induction of glycolytic and inflammatory genes (e.g. pro-IL-1b). Succinylation of PKM2 leads to its inhibition and translocation to the nucleus where it promotes HIF-1α activity. RET mediated by succinate accumulation leads to ROS production. **(B)** Metabolic adaptations in M2 macrophages are represented in green. Macrophage tolerance in response to prolonged LPS exposure are in yellow.

response to TLR4 engagement as revealed by Isotype tracer studies (Minhas et al., 2019). To replenish NAD^+ levels and sustain GAPDH activity in glycolysis, TLR4 quickly induces the NAD^+ salvage pathway by upregulating the expression of nicotinamide phosphoribosyltransferase (NAMPT) (Cameron et al., 2019). Together, these studies demonstrate that PRR-stimulated murine BMDM convert their mitochondria from ATP- to ROS-producing factories, which depletes NAD^+ levels and inhibits mitochondrial respiration while promoting glycolysis. Of note, NAD^+ also controls the Sirtuins, a family of NAD^+ dependent type III deacetylases that modulate inflammation by deacetylating various substrates, including transcription factors involved in macrophage activation such as NF-κB and AP-1 (Galli et al., 2011). A competition among the SIRT family members can fine-tune the macrophage inflammatory response, as recently shown for SIRT5 using murine peritoneal macrophages (Qin et al., 2017).

Itaconate, an Anti-Inflammatory and Bactericidal Derivative of the Tricarboxylic Acid Cycle

As a consequence of the break in the TCA cycle at the level of ACO2, inflammatory macrophages convert citrate to itaconate through the mitochondrial enzyme immune-responsive gene 1 (IRG1)-mediated decarboxylation of cis-aconitate, as shown using murine peritoneal macrophages (Michelucci et al., 2013). Itaconate blocks SDH, resulting in impaired succinate oxidation, diminished oxygen consumption (Németh et al., 2016) and reduction in the levels of inflammatory cytokines (IL-1β, IL-18, IL-6, IL-12), NO and HIF-1α. Mechanistically, itaconate inhibits inflammation *via* the alkylation and inactivation of KEAP1 (Kelch-like ECH-associated protein 1) (Mills et al., 2018), which is a repressor of the transcription factor Nrf2 (Bellezza et al., 2018), allowing Nrf2 to exert its anti-oxidant and anti-inflammatory effects. The anti-inflammatory property of itaconate was also observed *in vivo* for e.g. in murine models of LPS-induced lethality (Liao et al., 2019), ischemia-reperfusion injury (Lampropoulou et al., 2016), and *Mtb* infection (Nair et al., 2018). In the latter, *Irg1*$^{-/-}$ mice were shown to be more susceptible to *Mtb* infection than wild-type animals, due to a more severe immunopathology. Besides its anti-inflammatory activity, itaconate is a potent anti-bacterial, as it inhibits the key enzyme of the bacterial glyoxylate cycle isocitrate lyase, and has been shown to restrict the growth and virulence of *Mtb* (Michelucci et al., 2013), *S. typhimurium* (Michelucci et al., 2013) and *Legionella pneumophila* (Naujoks et al., 2016).

HIF-1α and Aerobic Glycolysis Govern the Macrophage Inflammatory and Anti-Bacterial Response

HIF-1α is a master transcription factor best known for its role in cellular adaptation to hypoxia. In bacterial infection, HIF-1α levels are upregulated at the transcriptional level by NF-κB, as shown in murine BMDM infected with group A *Streptococcus* or *Pseudomonas aeruginosa* or in LPS challenge of the murine macrophage cell-line (RAW264.7) (Rius et al., 2008). The stability of HIF-1α is also regulated at the post-translational level. The latter is induced by succinate accumulation in inflammatory macrophages. On one hand, succinate inhibits prolyl hydroxylases (PHD) (Tannahill et al., 2013), a family of α-KG-dependent dioxygenases (α-KGDD) involved in HIF-1α degradation at steady state (Bruick and McKnight, 2001). PHDs are also indirectly inhibited by mROS leading to HIF-1α stabilization (Mills et al., 2016). On the other hand, succinylation of the glycolysis rate limiting enzyme pyruvate kinase M2 (PKM2) converts it from an active homotetramer into an inactive monomer/dimer that binds to and activates HIF-1α (Palsson-McDermott et al., 2015). Among the key inflammatory genes with a HIF-1α response element is the gene encoding pro-IL-1β. Thus HIF-1α couples the metabolic shift to aerobic glycolysis in murine BMDM to the induction of IL-1β-mediated inflammatory response (**Figure 2A**). In chronic infection with some intracellular bacteria, HIF-1α stabilization leads to reduced citrate levels, and such a nutritional depletion prevents bacterial replication but without impacting bacterial survival leading to bacterial persistence (Hayek et al., 2019). This was demonstrated for *Coxiella burnetii*, the causative agent of Q fever, using human monocyte-derived macrophages (hMDM) and in murine BMDM and for *L. Pneumophila*, the causative agent of Legionnaires' pneumonia, in a murine BMDM infection model.

The Role of Glutaminolysis, α-KG, and the Hexosamine Biosynthetic Pathway (HBP) in M2 Macrophage Polarization

Using metabolomics and transcriptomics approaches on murine BMDM cultured under LPS+IFNγ elicited M1 or IL-4-induced M2 polarizing conditions, Jha et al. identified different metabolic pathways important for each macrophage state. In particular, an enrichment of effectors and metabolites of the glutaminolysis and the HBP was observed in M2 macrophages. Concordantly, inhibition of N-glycosylation or glutamine deprivation reduced M2 polarization (Jha et al., 2015). The same was seen with inhibition of the glutaminase Gls1, which was demonstrated to promote M2 polarization through elevation of α-KG production and epigenetic upregulation of M2-associated genes by Jumonji domain-containing protein D3 (JMJD3) (Liu et al., 2017), a histone demethylase belonging to the α-KGDD superfamily (Loenarz and Schofield, 2008). Glucosamine treatment, which engages the HBP, suppressed LPS-induced proinflammatory gene expression in BMDM and improved clinical outcomes in the cecal ligation and puncture (CLP) mouse model of sepsis (Hwang et al., 2019) (**Figure 2B**). Conversely, myeloid-specific knockout of murine *Ogt*, which encodes the HBP effector enzyme O-GlcNAc transferase (OGT) led to heightened susceptibility to LPS-induced septic shock, mediated by exacerbated macrophage inflammation (Li et al., 2019a; Li et al., 2019b).

Metabolic Epigenetic Control of Inflammation and Trained Immunity

As mentioned earlier, α-KG exerts important epigenetic regulation of murine BMDM polarized to the M2 phenotype

through the histone demethylase activity of JMJD3 (Liu et al., 2017). Another layer of control of macrophage inflammatory response is mediated by acetyl-CoA production and histone acetylation. Using metabolic tracing of glucose and glutamine and metabolic assays, Lauterbach et al. recently demonstrated that TLR4 stimulation of murine BMDM activates ATP-citrate lyase, which converts citrate pumped out of the mitochondria to acetyl-CoA in the cytosol. Acetyl-coA is then used for histone acetylation and activation of several inflammatory gene loci, including the *Il-12* locus, linking cellular metabolism to epigenetic activation of innate immunity (Lauterbach et al., 2019) (**Figure 2B**). A new post-translational modification (PTM) of histone lysine residues, derived from lactate and referred to as lactylation, has recently been added to the metabo-epigenetic armamentarium of macrophage regulation (Zhang et al., 2019). Interestingly, this PTM appears to occur later in the course of murine BMDM polarization than histone acetylation, upregulating genes involved in wound healing (e.g. Arg1) presumably to restore homeostasis (Zhang et al., 2019).

The immune tolerance state observed in macrophages following prolonged exposure to LPS, which provides a model to study immune paralysis, as observed in sepsis (Kumar, 2018), is similarly controlled by chromatin remodeling. Seeley et al. showed that sustained LPS promoted murine BMDM tolerance by inhibiting STAT1/2-dependent upregulation of inflammatory genes. Prolonged LPS treatment induced two microRNAs, miR-221 and miR-222, that inhibited the chromatin remodeling complex SWI/SNF by targeting its core component brahma-related gene 1 (Brg1). Interestingly, expression of miR-221/-222 correlated with increased organ damage in sepsis patients, and may potentially serve as a biomarker of sepsis-related immune paralysis (Seeley et al., 2018). Beyond the regulation of innate immunity, the metabolic-epigenetic crosstalk exerts a key role in the establishment of trained immunity. The two main epigenetic marks linked to trained immunity are histone methylation (H3K4me3) in promoters and histone acetylation (H3K27ac) in distal enhancers of poised innate immunity genes within specific loci in the genome. In human monocytes stimulated with β-glucan (to induce trained immunity), Arts et al. showed that cholesterol synthesis, aerobic glycolysis and glutamine anaplerotic use in the TCA cycle, lead to fumarate accumulation, which induces epigenetic rewiring of macrophages by inhibiting the histone demethylase lysine demethylase 5 (KDM5) (**Figure 1B**). Inhibition of glutaminolysis and cholesterol synthesis in mice reduced trained immunity induction *in vivo* (Arts et al., 2016). β-glucan inhibits IRG1 thus limiting itaconate inhibition of SDH; as a consequence succinate is converted to fumarate in β-glucan-trained human MDM (Dominguez-Andres et al., 2019).

BACTERIA REWIRE MACROPHAGE METABOLISM AS A STRATEGY TO GROW AND EVADE INNATE IMMUNITY

Some intracellular bacterial pathogens evolved multiple and overlapping mechanisms to survive within the threatening environment of a macrophage (**Figure 3**). These include exploiting macrophage metabolic resources to survive and rewiring macrophage metabolism to attenuate bacterial sensing and innate anti-bacterial defenses. Furthermore, some bacteria adapt to macrophage innate immunity responses to tolerate antibiotics.

Exploiting the Macrophage Glycolytic Pathway as a Nutrient Source

Among the intracellular bacteria shown to rewire macrophage metabolism toward aerobic glycolysis are *L. pneumophila*, *Brucella abortus*, *Mtb* and *Listeria monocytogenes*. While *L. pneumophila* depends on serine metabolism in its exponential growth phase, it switches to glycerol and glucose use in the post-exponential phase (Hauslein et al., 2017; Oliva et al., 2018). *L. pneumophila* establishes a permissive niche by impairing macrophage OXPHOS in a type IV secretion system (T4SS)-dependent manner (Escoll et al., 2017). Using human MDM and the murine RAW264.7 macrophage cell-line, Escoll et al. showed that *L. pneumophila* triggered mitochondrial fission through the secreted effector MitF, a Ran GTPase activator that interacts with host DNM1L, a GTPase involved in mitochondrial fission. In parallel, *L. pneumophila* enhances glycolysis in a T4SS-independent manner, although the mechanism controlling this pathway has not been determined. Using macrophages derived from the human monocytic cell-line THP-1, Czyż et al. showed that *B. abortus* increased macrophage aerobic glycolysis to promote its survival, which depended on lactate as its sole carbon source. This was demonstrated using a *Brucella* strain deficient in lactate dehydrogenase or by pharmacological inhibition of host glycolysis, both resulting in impaired *Brucella* growth (Czyz et al., 2017). *Mtb* was reported to use lactate instead of pyruvate as a carbon source. Using WT and KO mutant strains, Billig et al. showed that this ability to use lactate relied on oxydation by the L-lactate dehydrogenase LldD2. ^{13}C tracing experiments proved that lactate was used in the bacterial TCA cycle and for gluconeogenesis *via* phosphoenolpyruvate carboxykinase. This pathway was key for *Mtb* intracellular survival in human macrophages (Billig et al., 2017). The cytosolic bacteria *Listeria monocytogenes* poorly metabolizes lactate and pyruvate, but relies on glycerol and glucose-6-phosphate for energetic and anabolic needs, respectively (Grubmuller et al., 2014). Through its toxin Listeriolysin O (LLO), *L. monocytogenes* induces transient mitochondrial fragmentation (Stavru et al., 2011), and takes advantage of the increased glycolysis elicited in inflammatory macrophages to proliferate (Gillmaier et al., 2012).

Dampening Macrophage Glycolysis to Promote Chronic Infection

B. abortus chronic intracellular infection preferentially occurs in alternatively activated or M2 macrophages, relying on PPARγ that contributes to increased glucose availability for the bacteria (Xavier et al., 2013). Similarly, *S. typhimurium* hijacks glucose from the host cell requiring the transcription factor PPARδ to sustain chronic infection as shown using murine BMDM (Eisele et al., 2013). Unlike the findings by Billig et al. described above (Billig et al., 2017), Cumming et al. demonstrated that enhanced aerobic glycolysis was only observed with *M. bovis* BCG or dead *Mtb*. In contrast, live virulent *Mtb* directed human macrophage metabolism to exogenous

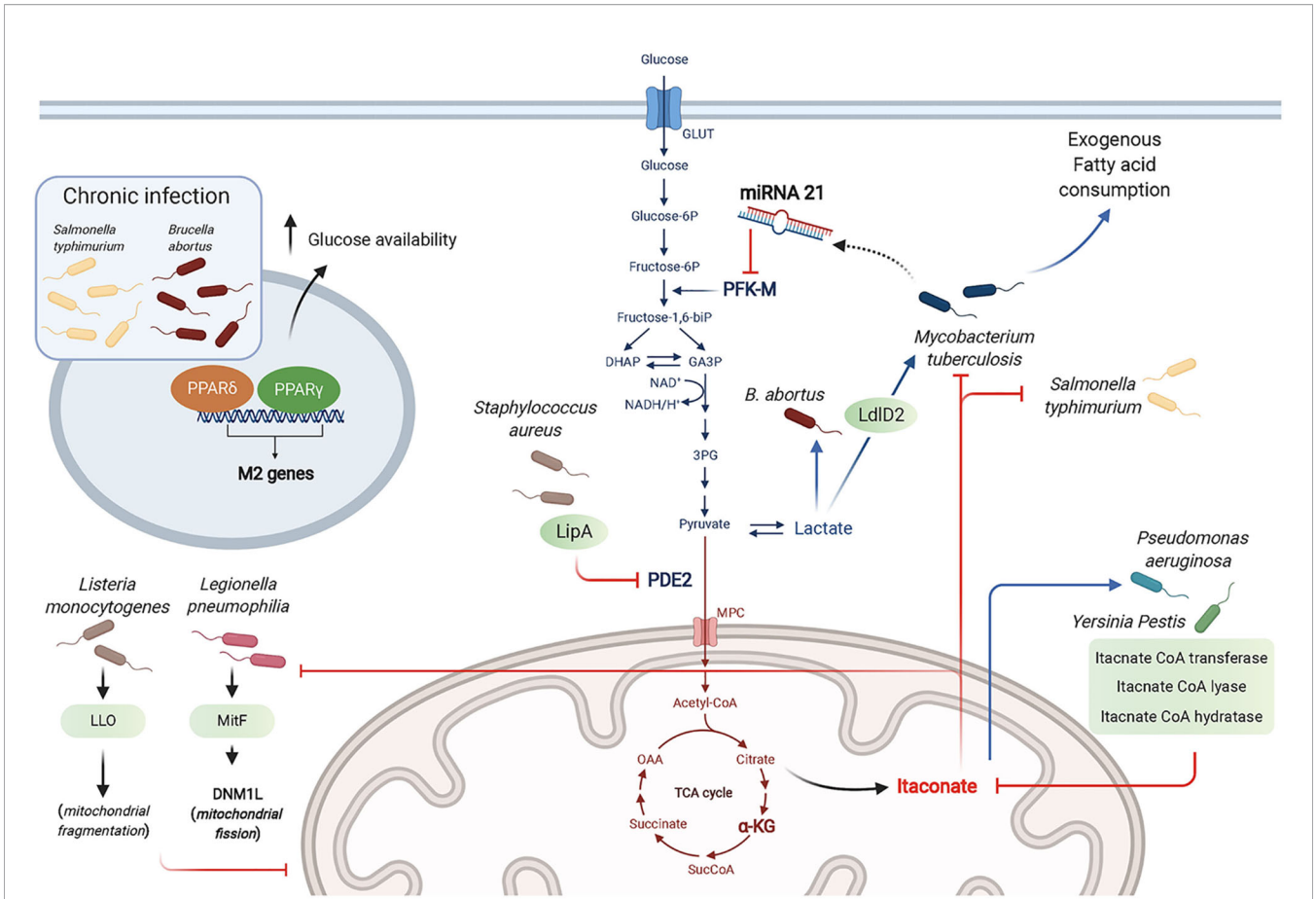

FIGURE 3 | Bacterial strategies targeting macrophage metabolism toward a successful infection. Bacteria manipulate metabolic pathways to enhance the necessary nutrient resources required for their survival, including glucose and lactate as carbon sources. The antibacterial effects of itaconate is countered by some bacteria through expression of itaconate degradative enzymes. Bacteria can "hide" from innate sensors by altering host metabolic enzymes (e.g. PDE2 conversion into lipoyl-E2-PDH that blocks TLR1/2 stimulation by bacterial lipopeptides). Chronic infection by intracellular bacteria is promoted through the actions of PPARδ and PPARγ that promote a wound healing phenotype and enhance glucose availability for the bacteria.

fatty acid consumption instead of glucose while globally reducing both glycolysis and the TCA cycle. *Mtb* thus reprograms macrophage metabolism into a "quiescent state" to facilitate its intracellular survival (Cumming et al., 2018). Among the mechanisms by which *Mtb* dampens macrophage glycolysis is through the induction of microRNA-21 (miR-21) that targets the glycolysis limiting enzyme phosphofructokinase-M (PFK-M). Using WT or miR-21 deficient mice and *in vitro* assays with human and mouse macrophages, Hackett et al. demonstrated that this anti-inflammatory miR-21 dampened glycolysis and ultimately decreased IL-1β production, promoting bacterial growth. Interestingly, IFNγ secreted in response to *Mtb* infection counters miR-21 induction, restoring the macrophage anti-bacterial response (Hackett et al., 2020).

Countering the Anti-Bacterial Effect of Itaconate

Several bacteria, including *Yersinia Pestis* and *P. aeruginosa* degrade itaconate as a common survival strategy, by expressing three enzymes, namely itaconate coenzyme A (CoA) transferase, itaconyl-CoA hydratase, and (S)-citramalyl-CoA lyase (Sasikaran et al., 2014). Furthermore, *P. aeruginosa* exploits itaconate as a carbon source allowing it to produce biofilm as in cystic fibrosis patients lungs. Riquelme et al. recently reported that itaconate-adapted *P. aeruginosa* accumulate mutations in the LPS-assembly protein IptD, and upregulate extracellular polysaccharides, which in turn promotes itaconate production by macrophages in a feedforward mechanism (Riquelme et al., 2020).

Targeting Metabolic Effectors to "Hide" From Macrophage Pattern Recognition Receptors and to Tolerate Antibiotics

Using murine BMDM, Grayczyk et al. determined that *S. aureus* is able to secrete a lipoic acid synthetase, LipA, that modifies pyruvate dehydrogenase E2 subunit (PDE2) by adding a lipid moiety, lipoic acid. This yields the secreted metabolic protein lipoyl-E2-PDH that blocks TLR1/2 stimulation by bacterial lipopeptides. Altogether, these data suggest a key role for LipA in bacterial escape from innate immunity (Grayczyk et al., 2017).

S. aureus also benefits from host-derived ROS to tolerate antibiotics. Rowe et al. showed that ROS attenuated the metabolism of *S. aureus* by attacking iron-sulfur (Fe-S) clusters-containing proteins including bacterial TCA cycle enzymes, namely SDH and acotinase. This metabolic state confers *S. aureus* resistance to killing by multiple antibiotics, highlighting a situation where innate immunity is exploited by the bacteria for a successful infection (Rowe et al., 2020).

CONCLUSION AND FUTURE PERSPECTIVES

The last decade has witnessed an impressive growth in the understanding of the intricate immunometabolic network governing macrophage activation in bacterial infections. Furthermore, several studies have now described strategies used by intracellular bacterial pathogens to survive in macrophages, evade innate immunity and establish a chronic infection. These advances provide exciting perspectives for developing new therapies targeting macrophage metabolic effectors to treat infectious and inflammatory diseases. Notable example of currently approved anti-inflammatory drugs that target metabolic effectors include methotrexate, rapamycin and metformin, that respectively inhibit dihydrofolate reductase, mammalian target of rapamycin (mTOR), and CI. Additional metabolic modulators include dimethyl fumarate (DMF) that inhibits KEAP1, NF-κB and the inflammasome, among others, and TEPP-46 that promotes PKM2 tetramerization [reviewed in (Palsson-McDermott and O'Neill, 2020)]. However, to expand this armamentarium, the focus must be steered away from tissue culture models of macrophage infection and *in vitro* macrophage polarization studies to fully grasp organ-specific immunometabolic mechanisms of macrophages of different lineages involved in fighting or containing bacterial infections. Exploring the efficacy of new immunometabolic modulatory drugs might provide urgently needed therapeutic options for emerging infectious diseases or those resistant to approved therapies.

AUTHOR CONTRIBUTIONS

GG and MS both wrote the text and illustrated the figures. All authors contributed to the article and approved the submitted version.

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Review

Soluble CD95L in cancers and chronic inflammatory disorders, a new therapeutic target?

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A B S T R A C T

Although CD95L (also known as FasL) is still predominantly considered as a death ligand that induces apoptosis in infected and transformed cells, substantial evidence indicate that it can also trigger non-apoptotic signaling pathways whose pathophysiological roles remain to be fully elucidated. The transmembrane ligand CD95L belongs to the tumor necrosis factor (TNF) superfamily. After cleavage by metalloprotease, its soluble form (s-CD95L) fails to trigger the apoptotic program but instead induces signaling pathways promoting the aggressiveness of certain inflammatory disorders such as autoimmune diseases and cancers. We propose to evaluate the various pathologies in which the metalloprotease-cleaved CD95L is accumulated and analyze whether this soluble ligand may play a significant role in the pathology progression. Based on the TNFα-targeting therapeutics, we envision that targeting the soluble form of CD95L may represent a very attractive therapeutic option in the pathologies depicted herein.

1. Introduction

CD95 (Fas or APO-1) is undergoing a paradigm change, which could lead to a therapeutic revolution. In addition to its apoptotic function, the death receptor (DR) CD95 can also favor tumor growth and metastasis through the induction of non-apoptotic signaling pathways [1,2]. In this regard, although the interaction between transmembrane CD95L and CD95 leads to an expected and well-known apoptotic response, which is involved in the elimination of infected and transformed cells [3], the metalloprotease-cleaved CD95L induces multiple non-apoptotic signaling pathways, resulting in chronic inflammation [2,4–6] and tumor development [2,7]. Although CD95L-expressing immune cells including lymphocytes and natural killer (NK) cells infiltrate tumor tissues to kill CD95-expressing cancer cells [3], the loss of CD95 in cancer cells is rarely observed supporting that CD95 might exert oncogenic functions. In this regard, the maintain of CD95 expression by ovarian and liver cancers in mice promotes tumor growth through the constitutive induction of the c-Jun N-terminal Kinase (JNK) signaling pathway [1]. In addition, CD95 participates in the metastatic dissemination of cancer cells [7–10], and contributes to maintain the population of cancer stem cells (CSCs) [11–14]. The CSCs present tumorigenic properties allowing them to replenish the heterogeneous tumor population after an initially successful therapy [15] and thereby, their elimination is crucial to achieving remission. These seminal studies highlight that by inhibiting the CD95-mediated non-apoptotic functions, new generation of drugs could revolutionize the therapy efficiency in these pathologies. Despite the fact that a list of autoimmune, infectious, and cancer diseases with elevated s-CD95L levels was published over fifteen years ago [16], the role of this cytokine in the pathogenesis of these diseases has yet to be elucidated. This review intends to describe the pathologies in which s-CD95L have been found up-regulated and could therefore represent a potential therapeutic target. Associated with this analysis, we will emphasize some of the incorrect conclusions about the CD95/CD95L pair, mainly drawn due to technical issues.

2. Structure, aggregation level and biological functions of CD95 and CD95L

CD95 is a type I transmembrane glycoprotein encompassing 335 amino acids in its pre-mature form (Fig. 1), and the removal of its peptide signal engenders a mature protein containing 319 amino acids (Fig. 1) [17]. The ligand of CD95, namely CD95L (FasL), is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) family. CD95L exhibits a long intracellular domain, a transmembrane

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domain, a stalk region, and a C-terminal TNF homology domain (THD) (Fig. 2). The long intracellular region of CD95L harbors different motifs, including a tandem casein kinase I phosphorylation site and a proline-rich domain, which serves as a binding site for SH3 (Src homology 3) domain-containing proteins [18]. Although neglected, the interaction between CD95 and CD95L also induces a "reverse signaling" in the CD95L-bearing cells increasing in the proliferation rate in CD8+ lymphocytes and inducing cell death in CD4+ T cells [19,20]. The bi-directional signaling induced within CD95L- and CD95-expressing cells renders it difficult to draw definitive conclusions from the phenotype of CD95L or CD95 knockout (KO) mice. In other words, the lupus-like phenotype developed by these mice [21] is the consequence of the combined failures to trigger not only the apoptotic and non-apoptotic signaling pathways by CD95, but also the reverse signaling by CD95L rendering difficult to decipher the role of each signal in the pathological phenotype of these mice.

The transmembrane CD95L designated m-CD95L for membrane-CD95L, can be processed within its stalk region (amino acids 102 to 136) by metalloproteases (MMPs) [22]. The soluble extracellular CD95L (s-CD95L) released into the extracellular medium corresponds to a homotrimer due to the presence of a trimerization domain in its C-terminal region [23]. Initial studies on s-CD95L suggested that this ligand was neutral in terms of cell signaling and could compete with its membrane-bound counterpart to prevent the implementation of the apoptotic response [24,25]. Thus, a homotrimeric CD95L fails to induce apoptosis, while a recombinant hexameric counterpart does [26], indicating that the increase in CD95L stoichiometry exerts a pivotal role in the biological function of the ligand. After cleavage in the cornea, s-CD95L seems to interact with fibronectin within the extracellular matrix to increase in its stoichiometry and retrieve its cytotoxic activity [27]. Interestingly, plasmin can also cleave CD95L between arginine at position 144 and lysine 145 [28], but the soluble ligand seems to preserve its pro-apoptotic activity involved in the elimination of endothelial cells and the inhibition of angiogenesis [28]. In the serum, another form of CD95L exists as a transmembrane ligand present at the surface of exosomes [29]. Many cells can secrete these small extracellular vesicles (20-200 nm), transferring lipids, proteins, and RNAs between cells [30]. These CD95L+ exosomes can be secreted by Epstein-Barr virus (EBV)-infected B-cells [31] or Vδ2 T-cells [32], contributing to kill follicular T cells and dampen the humoral response or to eliminate EBV-associated tumors, respectively. Overall, CD95L is found accumulated in the serum of patients suffering from various diseases and the biological function (*i.e.,* driver or bystander) of this ligand in such pathologies remains to be elucidated.

It is important to keep in mind that CD95 is not an enzyme and thereby, to trigger cell signaling, it requires the recruitment of proteases, ubiquitin ligases, kinases or phosphatases through protein-protein interactions (PPIs). In the absence of stimulation, the amino terminal region of CD95, namely pre-ligand assembly domain (PLAD) [33], and the proline motif within its transmembrane domain [34] are responsible for its pre-association as homodimers or homotrimers. The multi-aggregation of CD95 mandatory for the induction of the apoptotic program has been described as a two-step mechanism involving first, a certain degree of CD95 aggregation engendered by its interaction with CD95L and second, the induction of a caspase-8-dependent intracellular signal, promoting the accumulation of unstimulated CD95 into lipid rafts [35] and the induction of the apoptotic signal [36]. The initial CD95-mediated caspase-8 activation stimulates ceramide production, promoting receptor aggregation and cell death induction [37].

CD95 and CD95L undergo numerous post-translational modifications (PTMs), regulating the downstream signaling pathways. These consist of S-palmitoylation of CD95 at C199 (C194 in mouse CD95) [38,39], triggering the receptor distribution into lipid rafts and sensitizing re-activated T-cells to cell death [40]. Similarly, S-nitrosylation on both C199 and C304 enhances the distribution of CD95 into lipid rafts and tumor cell death [41]. S-glutathionylation of CD95 at cysteine 294 in mouse is another PTM promoting the receptor aggregation and apoptosis induction [42]. We observed that co-activation of CD95 with both the transmembrane protein CD28 and the glycosylphosphatidylinositol (GPI)-linked factor CD59 induces the CD95 distribution into lipid rafts but these plasma membrane re-localizations affect the induction of cell death contrarily in activated T-cells [43]. This suggests that different types of lipid rafts may exist and affect the initial steps of the death receptor signaling differently.

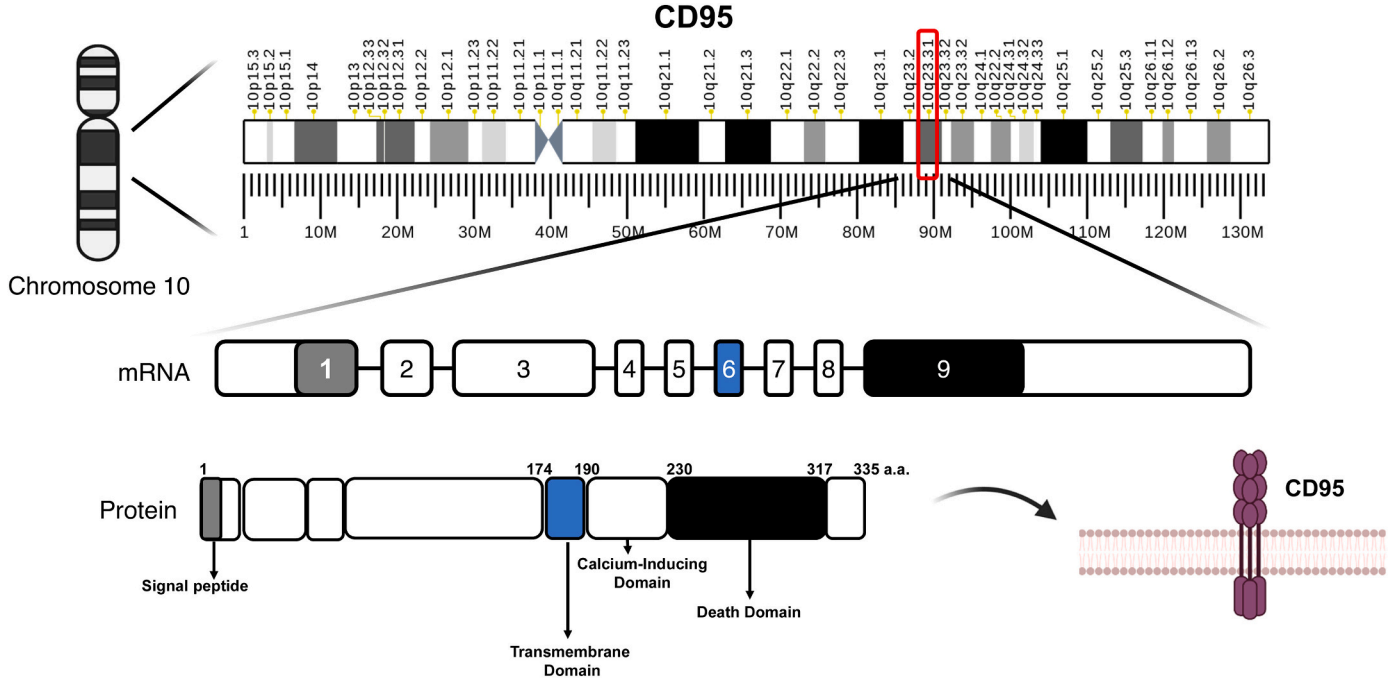

Fig. 1. CD95 from gene to protein.
Schematic diagram illustrating gene localization of CD95 (Fas) on human chromosome 10 and its mRNA and protein structures. Figure created with BioRender.com.

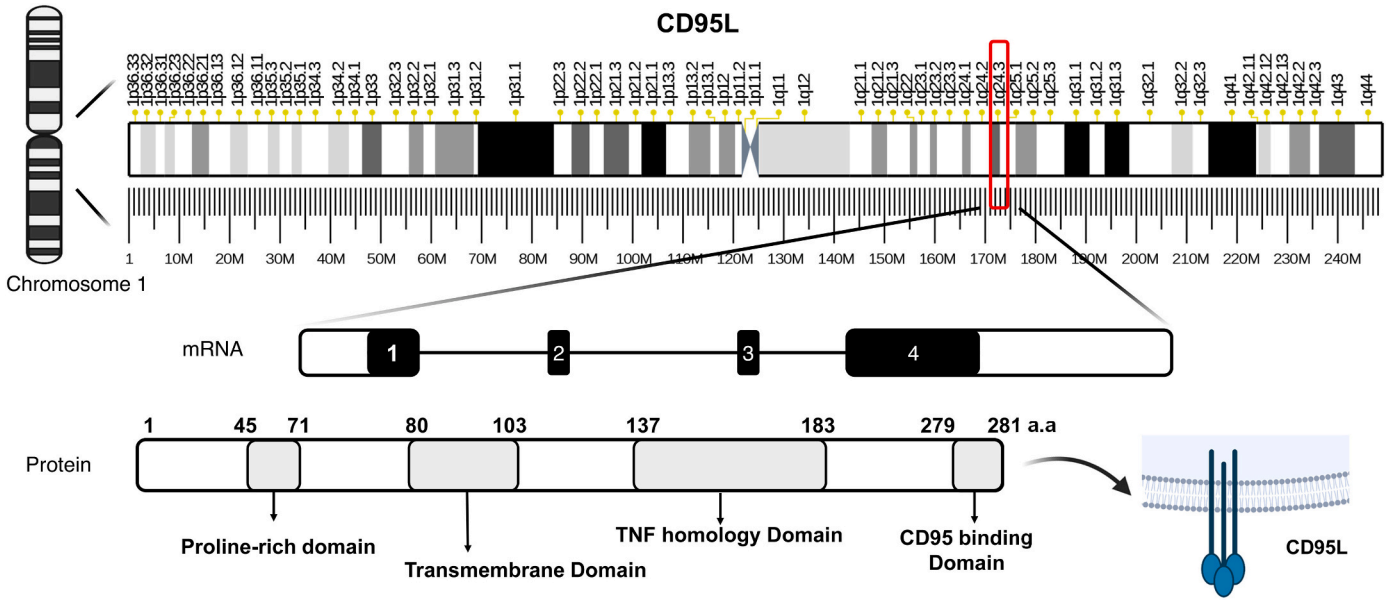

Fig. 2. CD95L from gene to protein.
Schematic diagram illustrating gene localisation of CD95L (FasL) on human chromosome 1, and its mRNA and protein structures. Tumor Necrosis Factor; TNF. Figure created with BioRender.com.

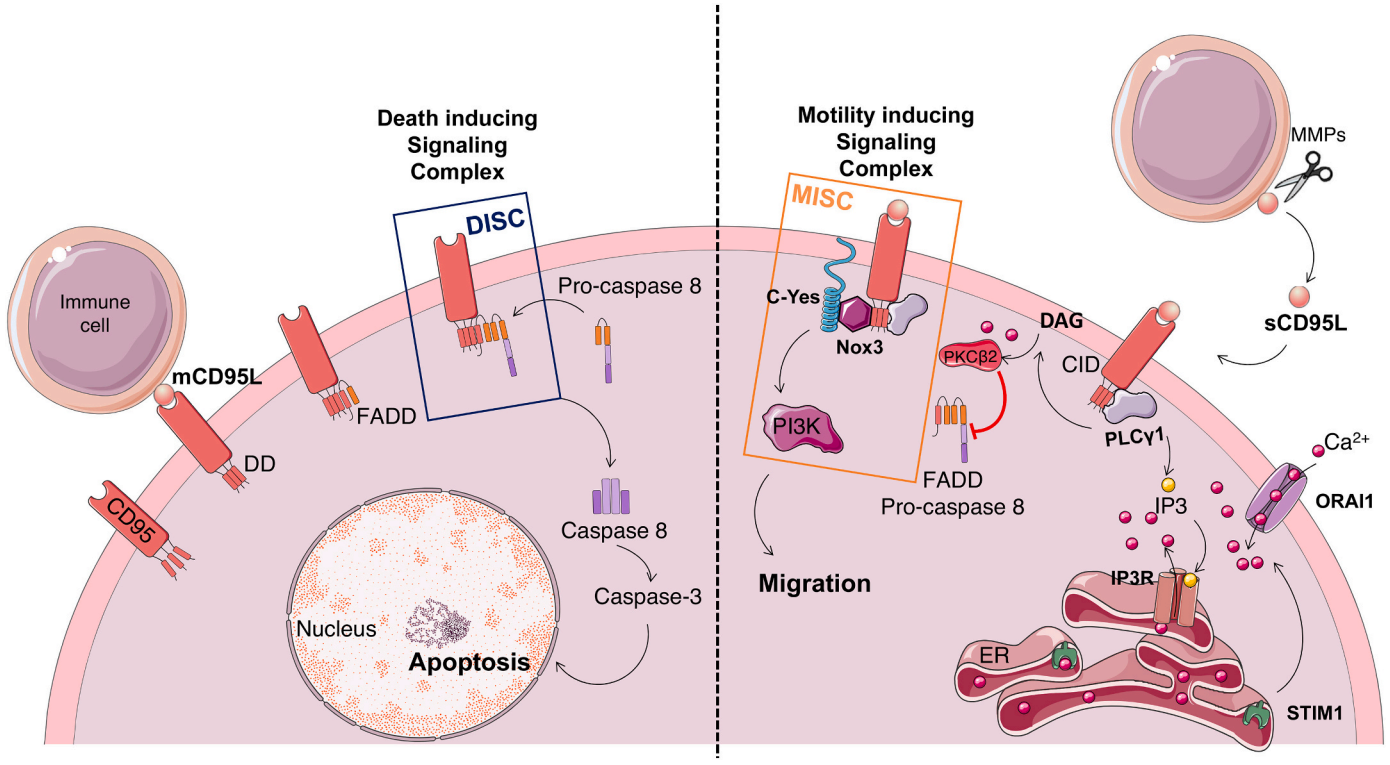

Fig. 3. CD95-mediated signaling pathways.
Left panel: Binding of m-CD95L to CD95 leads to the recruitment of the adaptor protein FADD (Fas-associated protein with death domain) through the death domain (DD) of CD95. This leads to the formation of the death inducing signaling complex (DISC) resulting in the induction of apoptosis. *Right panel:* CD95L can be cleaved by MMP (Metalloprotease) to release a soluble ligand (s-CD95L). s-CD95L binds CD95, which in turn recruits PLCγ1 (Phospholipase C gamma 1) *via* its calcium-inducing domain (CID). Plasma membrane recruitment of PLCγ1 triggers the generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its receptors (IP3Rs) at the endoplasmic reticulum (ER) to release Ca^{2+} (calcium) from the ER, leading to the redistribution of calcium sensor STIM1 to the plasma membrane where it activates an ORAI1 channel-driven entry of extracellular calcium. This accumulation of calcium combined with the production of DAG activates PKCβ2, which inhibits FADD recruitment and DISC formation. This s-CD95L-mediated signal promotes the formation of a Motility inducing signaling complex (MISC) containing among others, NADPH oxidase (Nox)3 and c-yes in triple negative breast cancer cells (TNBC).

In patients suffering from acute respiratory distress syndrome (ARDS), s-CD95L undergoes oxidation at methionine 224 and 225, causing its aggregation and promoting its cytotoxic function [44]. Oxidation of methionine at position 121 in CD95L prevents its cleavage by MMP7 and thereby, s-CD95L in ARDS patients preserves its stalk region [44]. Nagata and colleagues previously demonstrated that a recombinant and soluble CD95L encompassing amino acid residues 101 to 279, containing the stalk region, can still induce apoptosis [45]. A recent study confirmed that a soluble CD95L containing the stalk region triggers cell death in the human T-cell line Jurkat and in human small airway epithelial cells [46]. In accordance with these findings, conservation of the CD95L stalk region has been shown to promote the efficiency of the CD95/CD95L interaction and the level of s-CD95L aggregation [47], contributing to the release of an apoptotic cytokine. To render the understanding of the biological role of this domain more complex, the amino acid residues 105 to 130 of CD95L can interact with the hepatocyte growth factor (HGF) receptor (also known as c-Met), promoting cell migration of cancer cells in an autocrine fashion [48]. Overall, these findings suggest that both the THD and the juxtamembrane stalk region of CD95L contribute to the ligand multimerization, rendering it difficult to predict the biological function of serum CD95L prior to its sequencing.

3. A new complex formed upon s-CD95L/CD95 interaction

CD95 harbors three main intracellular domains, a juxtamembrane calcium inducing domain (CID), a death domain (DD), and a C-terminal region (Fig. 1). While the CID and the C-terminal region are involved in implementing non-apoptotic signals, the DD plays a crucial role in the induction of the apoptotic response. The binding of m-CD95L to CD95 induces a homotypic interaction between the DDs of CD95 and Fas-Associated protein with death domain (FADD) (Fig. 3). Then, FADD recruits caspases -8 and -10 to trigger the caspase cascade and induce cell death (Fig. 3). The CD95/FADD/Caspase complex has been designated DISC for death-inducing signaling complex [49].

Although the homotrimeric s-CD95L fails to form the DISC, this ligand is not neutral and promotes the formation of a different complex that we designated MISC for motility-inducing signaling complex (Fig. 3) [6,7]. The main feature of the MISC is the recruitment of the phospholipase C (PLC)-γ1 *via* the interaction of its SH3 domain to the CD95 CID (Fig. 1) [5,50]. It is noteworthy that unlike DD, CID is a disordered structure involved in weak and transient PPIs [4], rendering it difficult to identify its partners. Recruitment of PLCγ1 at the plasma membrane catalyzes the cleavage of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) acting as second messengers to activate an intracellular calcium (Ca^{2+}) response (Fig. 3). The CD95-driven IP3 production induces the release of the endoplasmic reticulum (ER)-stored Ca^{2+} through the stimulation of the IP3 receptors (Fig. 3). The increased intracellular Ca^{2+} concentration is further enhanced by the re-localization of stromal interaction molecule 1 (STIM1) from the ER to the plasma membrane and the opening of the plasma membrane-localized calcium channel ORAI1, creating high and localized calcium spikes, which limit CD95/FADD interaction by activating protein kinase C (PKC)-β2 (Fig. 3) [51]. Of note, triple-negative breast cancer (TNBC) cells exposed to s-CD95L also undergo a Ca^{2+} flux between the endoplasmic reticulum (ER) and the mitochondria, modulating the metabolism of the organelle (*i.e.,* ATP and reactive oxygen species (ROS) productions) and promoting cell migration [52,53].

In addition, MISC contains nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (*e.g.*, Nox3) responsible for ROS production, which activates Src kinases such as c-yes, promoting cell migration in TNBC cells [7]. ROS stimulates Src kinases through the oxidation of cysteine SH groups [54]. The involvement of Src kinases in the CD95-mediated non-apoptotic response has been observed not only in cancer cells [5,8,9] but also in Th17 cells [6] and neutrophils [55]. Nonetheless, the role of Nox and src kinase families in the CD95 signaling pathways remains ambivalent because these enzymes can also participate in the induction of the apoptotic signal in hepatocytes [56], pushing for further investigations to better apprehend the role of ROS and Src kinases in the CD95 signal. Interestingly, src kinases can also recruit tyrosine kinase receptors (TKRs) such as epidermal growth factor receptor (EGFR), thereby connecting CD95 with growth-stimulating effectors [7,9,10,57,58]. Src participates in the phosphoinositide 3-Kinase (PI3K) signaling pathway by the phosphorylation of caspase-8, a molecular process that might favor the switch between DISC and MISC [59–61]. Indeed, phosphorylation of tyrosine at position 380 in caspase-8 abrogates the protease activity and generates a binding site for the SH2 domain of the PI3K subunit p85 and the subsequent activation of the PI3K/Akt signaling pathway [59–61]. This CD95-mediated PI3K/Akt signaling pathway is essential in cancer cell migration [7,10]. Taken together, these studies demonstrate that CD95-mediated, and in particular s-CD95L-mediated non-apoptotic signaling can induce cell migration.

4. s-CD95L and cancer

The s-CD95L expression has been found to be increased in several cancers and chronic inflammatory disorders since its discovery [24,25], with biological functions ranging from pro-apoptotic to pro-inflammatory ligand (Fig. 4). Although most studies on CD95L have focused on its apoptotic function and thus its putative role in the elimination of tumor-infiltrating immune cells by cancer cells ("tumor counterattack" theory [62]) or its failure to induce the immune contraction in autoimmune disorders, more recent evidence emphasize that the non-apoptotic and pro-inflammatory functions of s-CD95L could also affect the progression of these pathologies. To identify the cancers in which novel drugs targeting CD95/CD95L pair could represent attractive therapeutic options, we describe the pathologies in which the concentration of s-CD95L has been investigated.

4.1. Hematological malignancies

s-CD95L has been initially detected up-regulated in sera from patients affected with natural killer-large granular lymphocyte (NK-LGL) leukemia, T-LGL leukemia, and NK-lymphoma [63]. In contrast, no trace of this ligand has been found in adult T-cell leukemia (ATL), acute myelogenous leukemia (AML), acute promyelocytic leukemia (APL) or acute lymphocytic leukemia (ALL) [63]. Contradicting this initial study, a prospective analysis on childhood lymphoproliferative malignancies, including 18 ALLs and 7 non-Hodgkin lymphomas (NHLs) patients revealed a significant increase in s-CD95L concentration [64]. Moreover, patients with elevated s-CD95L levels exhibited a detrimental effect on relapse-free survival (RFS) and overall survival (OS) [64]. This discrepancy could be explained by the cancer stage and also by the comparison of patients at diagnosis or in the course of the treatment. Indeed, a follow-up of NK lymphoma patients treated with CHOP protocol (a chemotherapeutic regimen including cyclophosphamide, hydroxydaunorubicin, oncovin and prednisolone) revealed that this treatment not only reduced the number of lymphoma cells but also decreased in serum CD95L concentration [63].

In multiple myeloma (MM), the concentration of s-CD95L was higher than that in control subjects and increased with the disease progression [65]. Although the CD95L-expressing cells have yet to be identified in this cancer, the quantity of this ligand in MM patients serum was correlated with that of interleukin-6 (IL-6), a pro-inflammatory cytokine associated with MM oncogenesis, and s-CD95L seemed to favor bone marrow infiltration *via* an unknown mechanism that needs to be elucidated [65].

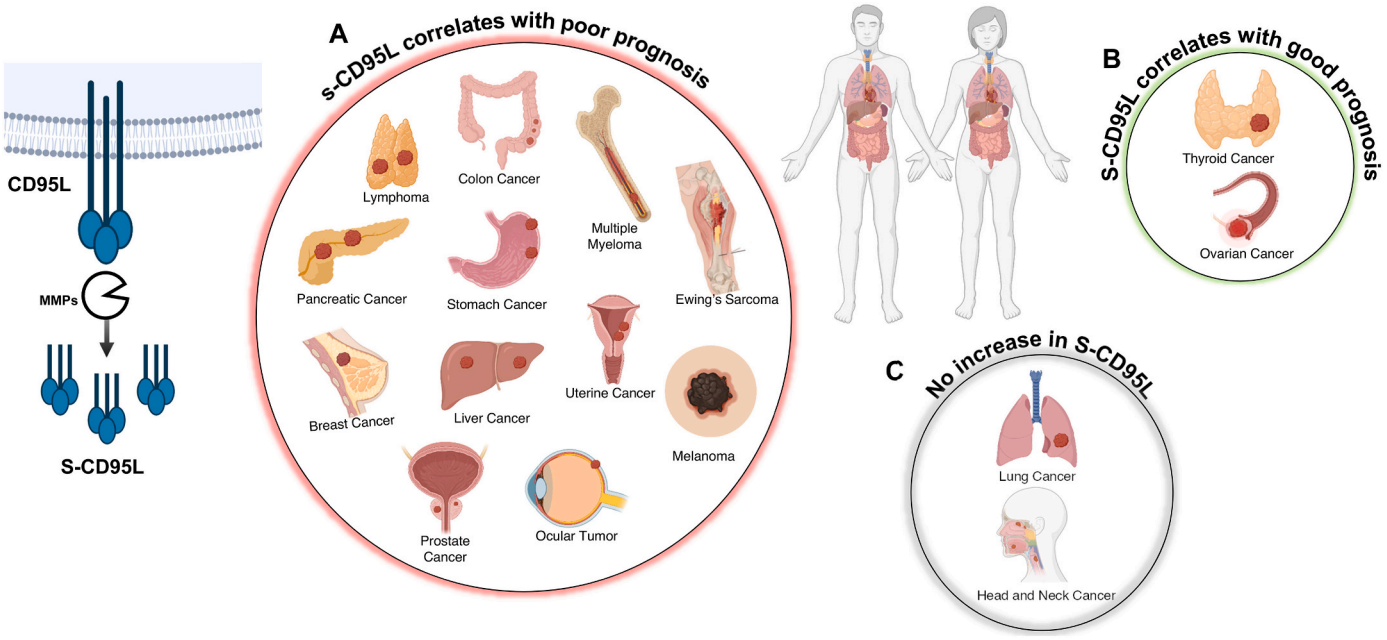

Fig. 4. Deregulation of s-CD95L expression in cancers.
A. The concentration of s-CD95L correlates with poor prognosis in lymphoma, multiple-myeloma, melanoma, colon cancer, pancreatic cancer, stomach cancer, Ewing's cancer, breast cancer, liver cancer, uterine cancer, prostate cancer and ocular tumors.
B. s-CD95L concentration correlates with poor prognosis in thyroid cancer and ovarian cancer.
C. No variation of s-CD95L was detected in patients affected by lung cancer and head and neck cancer as compared to healthy subjects. Figure created with BioRender.com.

4.2. Solid tumors

4.2.1. Gastro-intestinal cancers

The concentration of s-CD95L has been found increased in colon cancer patients' sera as compared to that in healthy donors [66]. Furthermore, supernatants of cultured colon cancer cells containing s-CD95L induced apoptosis of CD95-sensitive cells, while supernatants without s-CD95L did not [66]. Interestingly, serum CD95L levels dropped after tumor resection suggesting that the tumor or its environment was the main source of s-CD95L, but further investigations are needed to identify these CD95L-expressing cells. In contrast to this study, additional experiments on colon cancer cell lines observed CD95L transcription and translation but did not detect the presence of CD95L at the plasma membrane [67], and colon cancer cells did not kill CD95-sensitive immune cells [67].

To explain this discrepancy, it is noteworthy that most of the initial analyses involving tumor cells as the primary source of CD95L were carried out using anti-CD95L antibodies, which turned out to be unspecific in immunohistochemistry (IHC) and flow cytometry experiments rendering uncertain the conclusion of these studies [68]. In agreement with these data, a study evaluating the selectivity of twelve anti-CD95L antibodies confirmed that the polyclonal antibodies N20 and C20 from Santa Cruz and the monoclonal clone 33 from Transduction Laboratory displayed unspecific staining using flow cytometry and IHC methods [69]. On the other hand, anti-CD95L monoclonal antibodies (mAbs) designated G247-4 and NOK-1 showed strong selectivity for human CD95L [62,70].

Pancreatic carcinoma patients expressed higher levels of s-CD95L as compared to healthy subjects, and this concentration increased with the pathology aggressiveness [71]. Unfortunately, the CD95L staining performed by IHC in this latter study also relied on unspecific mAbs, rendering difficult to apprehend the source of CD95L in this tumor.

Hepatocellular carcinoma (HCC) also exhibited some plasma membrane CD95L, but as aforementioned, the IHC was performed with unspecific polyclonal anti-CD95L antibodies (*i.e.,* C20 from Santa Cruz) [69]. Interestingly, the authors observed that these tumors expressed a splicing variant of CD95 rendering it soluble, allowing them not only to block the CD95L/CD95 interaction but also to reduce the quantity of transmembrane CD95 in these tumor cells [72], a cellular process associated with inhibition of oncogenesis [1]. Infection with Hepatitis C virus (HCV) can result in cirrhosis and HCC, and an interesting gradient of s-CD95L concentration has been reported between healthy subjects, HCV-infected patients, and HCV-infected patients with HCC, suggesting that s-CD95L accumulation could be a non-invasive marker of the disease progression to cancer [73].

In stomach carcinoma, although the concentrations of serum CD95L were significantly different as compared to those in healthy donors [74], this cytokine was accumulated later in the disease progression. Interestingly, the quantity of s-CD95L was correlated with the metastatic dissemination of the pathology, and patients with high levels exhibited a worse prognosis than those with low levels, signifying that this cytokine could exert a pro-metastatic activity in this pathology [74].

4.2.2. Central nervous system cancers

Although s-CD95L was increased in the cyst fluids of astrocytomas, it only contributed to a minor part (11.4%) of the cell death signal observed in the CD95-sensitive Jurkat T-cell line exposed to these fluids [75], suggesting that this ligand could exert other biological functions in this pathology. In an ocular tumor model, the metalloprotease-cleaved CD95L promoted the metastatic dissemination of cancer cells by recruiting neutrophils suggesting that s-CD95L could act as an inflammatory and pro-metastatic cytokine [76].

4.3. Sarcoma

In Ewing's sarcoma, membrane-bound CD95L was detected and associated with the metastatic dissemination of these tumors [77]. This ligand was cleaved by unidentified metalloproteases, releasing a soluble ligand that accumulated in this pathology. Even though the anti-CD95L G247-2 mAb was used in this study, the authors only used it for western

blotting and not for IHC analyses rendering it difficult to conclude from their IHC results showing CD95L staining in cancer cells. It is also worth noting that the authors had to concentrate supernatants by 15 to achieve 24% of cell death in the CD95-sensitive Jurkat T-cells. Since protein concentration promotes unnatural CD95L aggregation, favoring its cytotoxic activity [78], the apoptotic role of s-CD95L in this pathology remains doubtful. On the other hand, as recently reported, this metalloprotease-cleaved CD95L could exert a pro-migration effect on these tumors [7–9].

4.4. Breast cancers

Breast cancer cells express less CD95 than normal surrounding tissues and, despite the fact that immune cells, endothelial cells, and cancer cells seem to express CD95L in tumor tissues, these cells remained sensitive to the CD95-mediated apoptotic signal, implying that the reduction of CD95 expression was associated with a different function than resistance to cell death [79]. Moreover, the anti-CD95L antibody used in IHC images was not detailed rendering it difficult to determine the staining specificity. Because the mix of breast cancer cells with CD95-sensitive cells triggered a trivial apoptotic response, we could conclude from this study that either CD95L was not expressed by these cancer cells or CD95L exerted a non-apoptotic function at the surface of these cells [79].

Breast cancer is a heterogeneous disease with a molecular classification allowing to distinguish between the luminal A and B expressing hormonal receptors including estrogen and/or progesterone receptors (PR), basal/TNBC, and human epidermal growth factor receptor 2 (HER2)-overexpressing tumors [80]. Basal/TNBC patients show the poorest clinical outcomes, and no targeted therapies exist compared to other molecular subtypes. We observed that serum CD95L concentration was increased among TNBC women compared to that in healthy subjects, and this was associated with an increased risk of relapse. This ligand was a potent chemoattractant for TNBC cell lines [7], suggesting that s-CD95L could promote metastatic dissemination in these women. Moreover, we established that CD95L was ectopically expressed by neovessels in the tumors [7]. These findings suggest that inhibition of the CD95-mediated signal and more accurately, the inhibition of the non-apoptotic signaling pathways might represent a novel and attractive therapeutic option in this pathology in which it exists an urgent therapeutic need.

4.5. Gynecologic cancers

4.5.1. Ovary

In an initial study on ovarian cancers, despite the use of a non-selective anti-CD95L mAb for IHC experiments [81], the authors observed that a highly glycosylated CD95L was expressed in the endo-lysosomal organelles of the ovarian cancers cells. These CD95L-containing microvesicles were secreted and detected in the ascites of ovarian cancer patients. Hence, CD95L-expressing exosomes triggered apoptosis in CD95-sensitive T-cells, suggesting that it could have an immunosuppressive effect in this pathology [81]. Besides that, we recently established that the serum concentration of s-CD95L is increased in women with advanced high-grade serous ovarian cancer (HGSOC) as compared to healthy women [82]. In contrast to TNBC [7], higher levels of this ligand were associated with a good prognosis and with increased tumor-infiltrating T- ($CD8^+$ and $CD4^+$) and B-lymphocytes [82]. Interestingly, similar to TNBCs, endothelial cells covering newly formed blood vessels inside the tumor expressed transmembrane CD95L [82].

4.5.2. Uterus

s-CD95L was also increased in the serum of women affected by uterine tumors compared to healthy controls [83]. The concentration of s-CD95L increased with the clinical stages of the disease, suggesting that in this pathology, s-CD95L could exert an oncogenic effect [83] and therapeutic regimens targeting this cytokine might be of interest to treat these women.

4.6. Prostate cancers

Liu et al. demonstrated that prostate cancer cell lines (*i.e.*, LNCaP, DU145, and PC3 cells) expressed CD95L and secreted a cleaved ligand, but NOK-1 or G247-4 clone was not used in this study, rendering these findings questionable [84]. Nonetheless, the authors observed that elevated concentrations of serum MMP-7, a metalloprotease involved in the CD95L cleavage [85], were associated with poor prognosis in prostate cancer patients [86].

4.7. Melanoma

Although soluble CD95L was not significantly up-regulated in melanoma patients, the only two stage IV patients with distant metastases in this cohort showed an increased amount of s-CD95L as compared to healthy subjects [87], suggesting again that s-CD95L did not play a pivotal role in the primary tumor growth but could exert a pro-metastatic function. The putative pro-metastatic role of s-CD95L has been confirmed by another study [88]. Of note, some melanoma cells have been reported to degranulate CD95L-expressing exosomes that could exert a cytotoxic activity [89].

4.8. Head and Neck cancers

s-CD95L concentration has been found increased in patients with laryngeal squamous cell carcinoma as compared to healthy donors, and the surgical treatment applied to these patients engendered a drop of the soluble cytokine concentration, indicating that s-CD95L stemmed from the primary tumor. However, the CD95L-expressing cells in the tumor tissue remains to be identified [90].

While oral squamous cell carcinoma showed no increase in s-CD95L when compared to healthy subjects [91], another study performed with head and neck cancers exhibited a significant reduction in s-CD95L compared to healthy subjects, indicating a different regulation of this ligand in these pathologies [92].

4.9. Thyroid cancer

Similar to HGSOC [82], high levels of s-CD95L in thyroid cancer patients were associated with disease-free survival [93]. Plasminogen activator inhibitor-1 (PAI-1) inhibits plasmin activity [94]. As aforementioned, DeClerck *et al.* observed that PAI-1 expression by endothelial cells protected angiogenesis from a CD95L-mediated apoptotic program in neuroblastoma tumors [28]. By blocking the plasmin activity, PAI-1 inhibited the release of a soluble and cytotoxic CD95L from the surface of endothelial cells [28]. Unexpectedly, both PAI-1 and s-CD95L were associated with progression-free survival in thyroid cancers, suggesting that the combination of these markers as a prognostic factor cannot be generalized.

4.10. Lung cancers

No variation of serum levels of s-CD95L in malignant (lung cancer patients) or tuberculous pleurisy patients were detected compared to healthy donors, regardless the fact that the values depicted in the manuscript were in the μg/mL range, far higher than the concentrations in the other cancers which were in ng/mL [95]. Interestingly, serum MMP7 concentration was also associated with poor survival in patients with non-small-cell lung cancer treated with cisplatin-based chemotherapy [96].

Overall, these studies indicate that although the concentration of s-CD95L seems to be up- (*i.e.,* colon, pancreas, liver, stomach, breast with

the triple-negative breast cancer, uterus, prostate, melanoma, larynx, and sarcoma) or down- (*i.e.*, ovary, thyroid) regulated in cancers, its role in the pathology progression necessitates further investigations. In addition, the presence of the MMP-cleaved CD95L raises the question of the identification of the CD95L-expressing cells and the metalloprotease (s) involved in the shedding process using validated tools. Finally, the high concentrations of s-CD95L in certain cancers suggest that drugs targeting the CD95/CD95L pair could represent potential therapeutic options for these patients.

5. s-CD95L and chronic inflammatory disorders

5.1. Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease targeting the central nervous system. In MS, the immune system damages myelin engendering deterioration of the nerves. Of note, CD95 stimulation can induce the production of inflammatory cytokines, which are involved in the activation and recruitment of innate immune cells such as neutrophils and macrophages. To do so, CD95 engagement relies on different mechanisms including the induction of a caspase-dependent apoptotic program in monocytes and macrophages [97,98], although this receptor can also promote an inflammatory program in a caspase-independent manner in macrophages [98]. Combined with TNF, CD95L can trigger the caspase-1 activation and the production of the pro-inflammatory IL1β *via* a caspase-8-dependent mechanism in antigen-presenting cells such as macrophages or dendritic cells [99]. This CD95L-mediated IL1β production is responsible for the pathological progression in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS). In this study, CD95 deficient (*lpr* mice) and non-functional CD95L-expressing mice (*gld* mice) resisted to a myelin oligodendrocyte glycoprotein (MOG) model of EAE [100]. Because the concentration of s-CD95L is not up-regulated in the sera of MS patients, we may consider that the transmembrane CD95L contributes to this pathology [101].

5.2. Lupus and Sjögren's syndrome

Lupus and Sjögren's syndrome (SS) are systemic autoimmune disorders. While in Sjögren's syndrome, the immune system targets mainly salivary and tear glands engendering dryness in the eyes and mouth, these diseases can affect other organs including skin, kidneys, articulations, lungs, and brain. In Lupus and SS, accumulating evidence points to s-CD95L as a pivotal effector of the disease aggressiveness [6,102], with a recent study confirming that s-CD95L levels were higher in lupus patients [103]. We previously observed that s-CD95L concentrations were associated with the disease progression in lupus patients, and that this ligand promoted the Th17 trafficking in inflamed organs [4,5]. We generated a cell-permeant peptide based on the CID sequence that prevented the CD95/PLCγ1 interaction and thereby, selectively inhibited the non-apoptotic signaling pathway [5]. Injection of this peptide alleviated clinical symptoms in lupus-prone mice. Finally, to improve the pharmacokinetic properties of this drug, we switched the peptide structure to that of a protease-resistant peptidomimetic (*i.e.,* an AApeptide designated DB550) and confirmed the therapeutic value of this novel generation of CD95-targeting drugs [4]. Interestingly, CD95 stimulation could also promote Th17-driven inflammatory pathologies *via* its role in Th17 cell differentiation [104]. Indeed, a comprehensive analysis of the regulatory network controlling the differentiation of mouse Th17 cells revealed CD95 as a key factor [105], capable of sequestering signal transducer and activator of transcription (STAT1), thus promoting STAT3 transcriptomic activity [104]. Beyond s-CD95L, membrane-bound CD95L may also play a role in lupus inflammation, as CD95L expressing Th1 cells ($Tbet^{+}$) were involved in the severity of cutaneous lupus erythematosus (CLE) in a Toll like receptor 9 (TLR9)-KO murine model [106]. These CD95L+ Th1 T-cells induced the synthesis of inflammatory chemokines such as IL-1, IL-6, CXCL2 or CXCL1 known to mediate skin lesions by interacting with CD95+ cells, which could be keratinocytes or skin-infiltrating myeloid CD11b+ cells [106]. Interestingly, knock-in mice in which the wild type CD95L was replaced by its soluble counterpart developed more aggressive lupus as compared to that observed in CD95L-KO mice and died to kidney damage and histiocytic sarcoma cancer suggesting that the presence of s-CD95L could aggravate the auto-immune pathology and promote oncogenesis [2]. Nonetheless, we cannot rule out that this aggressiveness was due to the lack of "reverse signaling", this question must be addressed in the future.

Initial studies in SS patients showed a significant increase in s-CD95L as compared to healthy donors, and more interestingly, the concentration of this ligand dropped after steroid therapy (prednisolone or methylprednisolone), indicating that s-CD95L dosage in humans must be performed using samples from patients at the diagnosis [107].

5.3. Ocular pathologies

Nabel *et al.* highlighted that grafts of CD95L-expressing colon carcinoma cells were eliminated faster than those of wild type tumors because CD95L promoted the recruitment of neutrophils and the induction of a robust inflammatory program [108]. Because of the presence of TGF-β, this difference was lost when tumors were grafted into the anterior chamber of the eye [108]. Because Marshak-Rothstein and colleagues have recently described the role of CD95L in ocular pathologies [109], we will not go into further details.

5.4. Rheumatoid arthritis (RA)

Rheumatoid arthritis is a systemic disease and the most frequent form of chronic arthritis. In addition to affect articulations, RA also exhibits a variety of extra-articular manifestations. In Rheumatoid arthritis patients, s-CD95L was found to be elevated in the synovial fluid (SF) and was associated with the pathology's aggressiveness, despite serum levels of s-CD95L being comparable to those dosed in healthy subjects. Another study using sera from RA patients, as well as insulin-dependent diabetes mellitus, systemic lupus erythematosus, gastric cancer, and leukemia patients observed a significant increase in s-CD95L in these pathologies [110], rendering it difficult to conclude whether s-CD95L was only up-regulated in SF or in both SF and sera of RA patients. Moreover, s-CD95L concentration in SF was found increased in RA patients as compared to that in osteoarthritis patients and correlated to the knee joint score, a disease progression marker [111]. Of note, in this pathology, MMP1, MMP2, MMP-8 and MMP9 failed to cleave membrane-bound CD95L, whereas MMP3 and MMP7 did. Additionally, the observed correlation between s-CD95L and MMP3 concentrations suggested that this metalloprotease could be responsible for the accumulation of the soluble form of CD95L in RA patients. However, as the authors failed to quantify MMP7 in SF of RA patients, we cannot rule out the possibility that MMP7 was also involved in the cleavage of CD95L in these pathology [111]. Overall, it remains to address how s-CD95L could contribute to the pathology progression in RA patients.

5.5. Familial Mediterranean fever (FMF)

FMF is a monogenic inflammatory disease caused by mutations in the MEditerranean FeVer (MEFV) gene encoding pyrin [112,113]. These mutations cause recurrent episodes of fever and serositis. Although the genetic aspect of this pathology is well described, the pathogenesis of inflammatory flares remains poorly understood. Ceri *et al.* reported no significant increase in serum s-CD95L levels in FMF patients *versus* controls, and s-CD95L concentrations did not vary between attack and attack-free periods [114], suggesting that s-CD95L do not exert any effect in this pathology.

5.6. Psoriasis

Psoriasis is a chronic skin disease that progresses in a cyclic fashion, with flare up and remission. This chronic inflammatory disease is characterized by immune cell infiltration and psoriatic keratinocytes resistant to apoptosis. Although, the concentration of s-CD95L in the sera of psoriasis patients did not vary compared to that in healthy subjects, it increased in patients undergoing Goeckerman therapy [115], a daily dermal application of crude coal tar containing polycyclic aromatic hydrocarbons (PAH), combined with exposure to ultraviolet radiation (UVR) or a topical treatment of dithranol [116]. Unfortunately, the role of s-CD95L in treatment efficiency was not investigated in these studies.

5.7. Atherosclerosis

Atherosclerosis is a systemic inflammatory disease mediated by several factors and often associated with hypertension. Okura *et al.* wondered whether s-CD95L levels could be elevated in atherosclerosis patients, in which transmembrane CD95L was detected on endothelial cells and MMPs were found to be over-expressed [117]. Interestingly, the endothelial dysfunction in atherosclerosis, measured by the intima–media thickness in the carotid artery, was correlated with the s-CD95L concentration [117]. In agreement with this pioneering study, a positive correlation between s-CD95L and atherosclerotic lesions was confirmed in a second analysis [118], suggesting that the release of membrane-bound CD95L by metalloproteases could represent a robust and non-invasive biological marker for severe coronary artery diseases. Nonetheless, no molecular or cellular mechanism has been proposed to rationalize this correlation.

5.8. Toxic epidermal necrolysis (TEN) and Stevens-Johnson syndrome (SJS)

TEN and SJS are severe blistering diseases associated with keratinocyte apoptosis in the epidermis, which results in dermo-epidermal separation and bullae. s-CD95L was found up-regulated in TEN and SJS patient's sera compared to healthy subjects [119]. In this pathology, s-CD95L could exert a cytotoxic activity on keratinocytes *via* a molecular mechanism that remains to be elucidated [119,120]. If s-CD95L kills keratinocytes through induction of apoptosis, we hypothesize that s-CD95L aggregation may be increased in these pathologies by an oxidative process such as the one described in ARDS patients [121].

5.9. Hashimoto's thyroiditis (HT) and graves disease

Graves' disease and chronic thyroiditis (also designated Hashimoto's thyroiditis) are autoimmune diseases targeting the thyroid gland. While the Graves' disease is caused by the stimulation of the thyroid-stimulating hormone (TSH, or thyrotropin) receptor promoting the hyperplasia of the thyroid gland, patients affected by Hashimoto's thyroiditis undergo a reduction of the TSH hormone activity leading to the organ atrophy. Some connections exist between these two pathologies since certain patients suffering from Hashimoto's disease have been diagnosed following the treatment of Graves' disease episode [122].

Carla Giordano and colleagues reported that thyrocytes from HT glands expressed CD95 while healthy thyrocytes did not [123]. This group also observed the expression of CD95L at the mRNA and protein levels in normal and HT thyrocytes, and suggested that because normal thyrocytes did not express CD95, the thyroid damage only occurred in the HT patients. However, using similar methods and antibodies, another group failed to confirm these findings [68]. Questioning the conclusion from the former study, a technical note evaluating the selectivity of commercial anti-CD95L antibodies revealed that the two anti-CD95L antibodies used in HT studies (*i.e.,* the monoclonal clone 33 from Transduction Laboratories and the rabbit polyclonal IgG C-20, from Santa Cruz Biotechnology) were unspecific [69].

Although it remains to address whether thyroid glands express the transmembrane CD95L, s-CD95L has been found increased in sera of patients affected by Graves' hyperthyroidism as compared to those of Graves' disease in remission and s-CD95L concentration was correlated with the concentration of a disease marker, namely the anti-TSH receptor antibodies (TRAb) [124].

Overall, these studies on chronic inflammatory disorders suggest that s-CD95L could exert a potent pro-inflammatory activity that could contribute to the aggressivity of these pathologies. Accordingly, we hypothesize that small drugs or antibodies inhibiting selectively the pro-inflammatory signal induced by s-CD95L could alleviate clinical symptoms in the diseases exhibiting elevated serum concentrations of s-CD95L.

6. s-CD95L and infections

Initial studies on s-CD95L showed that it is up-regulated in patients infected with *Plasmodium falciparum* malaria [125], tuberculosis [126], and human immunodeficiency virus (HIV) [127]. We recently confirmed that s-CD95L levels were elevated in HIV patients [4]. Interestingly, increased s-CD95L concentration was associated with lymphopenia in malaria patients, which the authors hypothesized, was caused by the CD95L apoptotic function. Soluble CD95L was reported accumulated in sera of hepatitis B virus patients [128], while a Korean cohort of patients exhibited an opposite trend [129], rendering it difficult to conclude for the presence of this ligand in this infection.

7. s-CD95L and transplantation

Acute graft-versus-host disease (aG*v*HD) is a major complication associated with allogeneic bone marrow transplantation (BMT). During the preparative stage and the period of myelosuppression, serum s-CD95L concentrations drop and then increase with hematopoietic reconstitution after BMT. Moreover, sera of patients with aG*v*HD exhibit higher CD95L concentrations than those without aG*v*HD [130]. Unexpectedly, administration of s-CD95L to BALB/c mice receiving C57BL/6 strain transplant (graft skin) extended the survival time of grafted mice. According to this study, s-CD95L induced graft tolerance in mouse models by decreasing IL-2 and IFN-γ expressions while increasing IL-4 expression [131]. These findings render it difficult to apprehend the role of s-CD95L in aG*v*HD and would require further investigations.

It has also been reported that activated T cells were more susceptible to CD95-mediated apoptosis than naive counterparts because CD95 redistribution into lipid rafts in activated T cells rendered these cells more prone to re-stimulation [132]. Accordingly, *ex vivo* incubation of T cells with agonistic anti-CD95 mAb (Jo2 clone) or a recombinant and cytotoxic CD95L selectively deleted alloreactive T-cells, while sparing the graft *versus* tumor (G*v*T) T cell response [133]. The G*v*HD inhibition initially attributed to the elimination of alloreactive T cells could be, in fact, caused by the expression of CD95L by memory T-cells. Indeed, the presence of these CD95L-expressing memory T cells during adoptive cell transfer (ACT), to treat patients with advanced cancer, accelerated the conversion of the naive T cells into differentiated memory effector cells through a CD95-mediated PI3K signaling [134]. This accelerated conversion of naive T cells reduced their anti-tumor capacity during ACT and was designated "precocious differentiation", a biological process that could synchronize the immune response and terminate it.

8. s-CD95L and aging

Few studies have analyzed the concentration of s-CD95L in relation to age. Werner's syndrome is a typical progeroid syndrome manifesting a variety of age-related symptoms, which have been shown to be associated with chronic inflammation. Interestingly, the concentration of s-

CD95L in healthy subjects is strongly correlated with natural aging and this ligand is found to be increased in Werner's syndrome patients [135]. Although the pathophysiologic role of serum CD95L in aging is not elucidated, its pro-inflammatory functions in neutrophils [55,108] and Th17 cells [4,5,105] or its role in "precocious differentiation" could contribute to aging.

9. Natural inhibitors of CD95/CD95L interaction

DcR3 is a TNFR member, lacking transmembrane and intracellular sequences. DcR3 interacts with CD95L [136], and genomic amplification of this soluble receptor has been reported in approximately half of the lung and colon cancers [136], as well as its overexpression has been demonstrated in 44% of gastrointestinal cancers [137]. Although DcR3 mRNA was found to be highly expressed in pancreatic cancer cells, the expression level of this soluble protein was not associated with clinical markers rendering it difficult to confirm a putative role of this factor in the pathology progression [138].

Several forms of soluble CD95 (sCD95) are derived from alternative splicing phenomena [139], and soluble CD95 was found increased in sera of silicosis patients and systemic lupus erythematosus patients [140]. These spliced variants of CD95 conserve their amino-terminal oligomerization domain, termed PLAD [33], allowing them to interact with wild-type CD95 [141] and thereby dominantly interfere with the downstream signaling pathway by generating non-functional homodimers or trimers. Some of these soluble forms of CD95 can still interact with CD95L and prevent its interaction with wild-type CD95, acting as a decoy receptor [142]. The major CD95 spliced variant is devoid of its exon 6 (*i.e.*, encoding for TM domain) (Fig. 1) and corresponds to a soluble receptor that can interact with transmembrane CD95L and by doing so, triggers a CD95L-induced "reverse signaling" leading to cell death [143] and rendering complex the evaluation of its pathophysiologic role. Of note, the heterozygous expression of an exon 6-spliced CD95 gene should also engender a reduction of the CD95 level at the plasma membrane (the remaining wild type allele). It is noteworthy that the loss of plasma membrane CD95 leads to the inhibition of tumor growth in different tumor cells [1]. Similarly, the presence of CD95 in lung adenocarcinomas can exert protection against tyrosine kinase inhibitors by evoking a yet uncharacterized NFkB signal that contributes to tumor relapse [144]. We recently observed that the genetic loss of CD95 in TNBC cells reduced tumor growth by releasing an immune checkpoint on NK cells [145]. Accordingly, CD95 loss in TNBC cells triggered a CD95L-independent inflammatory transcriptomic signature modulating the immune landscape in these breast cancers [145].

The reduction or the loss of the transmembrane CD95 is also observed in ALPS patients type 0 (ALPS-FAS in the revised classification) [146] and in Lpr mice [147,148] leading to lupus-like disorder.

10. Concluding remarks

Three decades after the cloning of CD95 [17], although the CD95-induced apoptosis is crucial for the elimination of infected and transformed cells or the immune contraction [3], the role of its non-apoptotic functions has been overlooked, and we are only beginning to understand the molecular mechanisms underlying the induction of these responses.

The dichotomous role of CD95 reflects the development of drugs with either antagonist or agonist properties. APO010 is a CD95 agonist that mimics hexameric CD95L whose administration prolonged the survival of glioma-bearing mice [149]. The short peptide residue YLGA designated Met12, disrupts CD95 homotrimerization [150], inhibiting the induction of both the apoptotic and non-apoptotic responses. In a rat model of age-related macular degeneration, intravenous injection of Met12 significantly reduced retinal degeneration [151]. Strategies for targeting the CD95L/CD95 system are similar to those used for the TNF system, with Asunercept (APG101), a CD95 decoy receptor, which is currently tested in phase I clinical trial of myelodysplastic syndrome (MDS), and in a phase II trial of glioma showing a beneficial progression-free survival for patients treated with a combination of radiotherapy and APG101 as compared to radiotherapy alone [152]. Based on the pivotal role of the CD95-mediated apoptotic signal in the anti-tumor and anti-infectious responses, as well as in the immune homeostasis, we can envision that chronic administration of Asunercept in patients might lead to unwanted effects such as chronic infection, autoimmune symptoms or oncogenesis which might be circumvented with the generation of novel drugs specifically inhibiting the non-apoptotic signaling pathways of CD95. As such, we recently demonstrated that targeting CD95 non-apoptotic signaling is pharmacologically achievable and therapeutically beneficial in a pre-clinical model of lupus [4]. If these chemical derivatives might be useful in other s-CD95L- and Th17-driven pathologies remains to be evaluated. In addition, aforementioned cancer and inflammatory disorders, in which elevated concentrations of s-CD95L are detected push us to compare the therapeutic value of large spectrum inhibitors, including neutralizing antibodies or decoy receptors targeting CD95/CD95L pair with that of more selective inhibitors such as DB550 [4] abrogating the CD95-mediated non-apoptotic signal. Further unraveling the PPIs required for the apoptotic and non-apoptotic cellular functions of CD95 at the level of the CD95-associated complex and defining the precise forms of s-CD95L that accumulate in various pathologic conditions, will also likely prove therapeutically meaningful in the context of both autoimmune diseases and cancers.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

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Authors' contributions

K.K., A.B-M, G.G, M.J., P.V., P.B. and P.L. combined data and wrote the manuscript.

Declaration of Competing Interest

M.J., P.V., P.B. and P.L. have filled patents covering drugs targeting CD95/CD95L in inflammatory disorders (WO2014118317, WO2015189236, WO2015158810, WO2015104284, WO2017149012, WO2018130679).

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Review

Fas/CD95 Signaling Pathway in Damage-Associated Molecular Pattern (DAMP)-Sensing Receptors

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Abstract: Study of the initial steps of the CD95-mediated signaling pathways is a field of intense research and a long list of actors has been described in the literature. Nonetheless, the dynamism of protein-protein interactions (PPIs) occurring in the presence or absence of its natural ligand, CD95L, and the cellular distribution where these PPIs take place render it difficult to predict what will be the cellular outcome associated with the receptor engagement. Accordingly, CD95 stimulation can trigger apoptosis, necroptosis, pyroptosis, or pro-inflammatory signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and phosphatidylinositol-3-kinase (PI3K). Recent data suggest that CD95 can also activate pattern recognition receptors (PRRs) known to sense damage-associated molecular patterns (DAMPs) such as DNA debris and dead cells. This activation might contribute to the pro-inflammatory role of CD95 and favor cancer development or severity of chronic inflammatory and auto-immune disorders. Herein, we discuss some of the molecular links that might connect the CD95 signaling to DAMP sensors.

Keywords: apoptosis; auto-immunity; CD95; inflammasome; necroptosis; pyroptosis; sting

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1. Introduction

Although CD95 (Fas) has long been viewed as the death receptor prototype, only involved in the induction of the apoptotic signaling pathway, more recent and accumulating evidence point out that this transmembrane receptor contributes to chronic inflammatory disorders and cancer by inducing non-apoptotic signaling pathways [1,2]. Herein, we first discuss the pathologies associated with genetic mutations in the CD95 signaling pathway and in damage-associated molecular patterns (DAMP) sensors. Second, we expose the different links existing between CD95 or its downstream apoptotic machinery including the adaptor protein Fas-Associated protein with Death Domain (FADD) and the initiator protease caspase-8 in the activation/regulation of the DAMP-sensing complexes. The main goal of this review is to question whether CD95 might act as an alarmin for Pattern Recognition Receptors (PRRs).

2. CD95 and DAMP-Sensor-Mediated Pathologies

2.1. Genetic Mutations in CD95 and DAMP Sensors and Chronic Inflammatory Diseases

2.1.1. CD95-Associated Genetic Disorders

In humans, CD95 mutation leads to the development of a disease called auto-immune lymphoproliferative syndrome or ALPS, also known as Canale–Smith syndrome [3]. ALPS involves the development of early onset polyclonal lymphoproliferation (splenomegaly,

adenopathy) associated with expansion of a population of aberrant double-negative $B220^{+}CD3^{+}TCR\alpha\beta^{+}CD4^{-}CD8^{-}$ T cells. To qualify as ALPS, the evolution of the disease must be superior to 6 months and with exclusion of a secondary etiology for the lymphoproliferation (hematological malignancies or solid cancers, for example) [4,5]. This cardinal presentation is associated with mutation of CD95, CD95L, or caspase 10, a defect in lymphocyte proliferation, autoimmune cytopenia (mainly autoimmune hemolytic anemia and immune thrombocytopenic purpura), augmentation of Immunoglobulin A and G titers, associated with elevated dosage of B12 vitamin, augmentation of IL-10 or IL-18 and an increase in soluble-CD95L in patient serum. Of note, ALPS present an increased risk of hematological malignancies, which can complicate its diagnosis [6]. ALPS are divided into five subtypes, showing mutations in CD95, CD95L, or caspase-10 (see Table 1). Although most of the ALPS patients exhibit CD95 mutations, when Oliveira et al. revised the ALPS classification in 2010, lymphoproliferative disorders showing no mutations in CD95, CD95L, or caspase 10 were also classified as ALPS disorders (i.e., caspase 8, NRAS/KRAS, or SH2D1a mutations). In addition, Dianzani Autoimmune Lymphoproliferative Disease (DALD) was also reported as an ALPS-related disorder even though still no genetic defect was identified [4,5].

Table 1. Auto-immune lymphoproliferative syndrome (ALPS) classification.

ALPS Type	Mutation Type
ALPS-FAS	CD95 germline homo- or heterozygous mutation
ALPS-sFAS	CD95 somatic mutation
ALPS-FASLG	CD95L germline mutation
ALPS-CASP10	Caspase 10 germline mutations
ALPS-U	ALPS phenotype with no known-ALPS mutation

Similarly, CD95 (Lpr and Lpr^{cg} mice) or CD95L (Gld) mutations in mice lead to a similar phenotype with aberrant T cell proliferation and lymphoproliferation, but also arthritis and early death from acute glomerulonephritis. These mice exhibit the development of autoantibodies, such as those found in Systemic Lupus Erythematosus (SLE) patients, and have led to their use as a model for SLE physio-pathological studies. However, this autoantibody development in CD95-mutated mice represents a striking difference with the human pathology in which autoantibodies do not represent a common clinical feature of human ALPS [7].

2.1.2. DAMP-Sensor-Associated Genetic Disorders

DAMP are non-infectious triggers of the immune system released by dying or damaged cells [8] and detected by various membrane-bound or intracellular sensors, notably through a cytosolic complex called inflammasome [9]. These DAMP sensors have been implicated in several inflammatory diseases, on a spectrum ranging from autoimmune polygenic phenotypes such as in systemic lupus erythematosus, rheumatoid arthritis or Crohn's disease, to monogenic autoinflammatory disorders [10].

In these monogenic autoinflammatory syndromes, enhanced activation of the inflammasome pathway due to genetic mutations (i.e., nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain 1/NLRP1, NLRP3, NLR family CARD domain-containing protein 4/NLRC4 or Pyrin), leads to common clinical phenotypes involving fever, skin rash, or dermatologic lesions and systemic symptoms (such as arthralgia or arthritis for example), associated with elevated inflammatory markers in the blood of affected patients [11]. For instance, NLRP1 mutations lead to NLRP1-associated Auto-Inflammation with Arthritis and Dyskeratosis (NAIAD) [12] and NLRC4 gain-of-function mutations triggers Syndrome of enterocolitis and Autoinflammation associated with mutation in NLRC4 (SCAN4) [13] or recurrent Macrophage Activation Syndrome (MAS) [14], (see Table 2).

Table 2. DAMP-sensor-associated genetic disorders.

DAMP-Sensor Mutation	Mutation Type	Autoinflammatory Disorder
NLRP1	Loss of function	NAIAD
NLRP3	Gain of function	CAPS = NOMID, FCAS, MWS
NLRC4	Gain of function	SCAN4, MAS
Pyrin	Gain of function of MEFV or loss of function of MVK	FMF, HIDS, PAAND
PSTPIP1	Loss of function	PAPA

CAPS: Cryopyrin-Associated Periodic Syndrome; DAMP: Damage-Associated Molecular Patterns; FCAS: Familial Cold Autoinflammatory Syndrome; FMF: Familial Mediterranean Fever; HIDS: Hyper-IgD Syndrome; MAS: Macrophage Activation Syndrome; MVK: Mevalonate Kinase; MWS: Muckle-Wells Syndrome; NLRP: Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain; NLRC4: NLR Family CARD Domain Containing 4; NOMID: Neonatal-Onset Multisystem Inflammatory Disease; PAAND: Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis; PAPA, Pyogenic sterile Arthritis, Pyoderma gangrenosum, and Acne; PSTPIP1: Proline Serine Threonine Phosphatase-Interacting Protein 1; SCAN4: Syndrome of enterocolitis and Autoinflammation associated with mutation in NLRC4; TNFR1: TNF-Receptor-1; TRAPS: TNF-Receptor-Associated Periodic Syndrome.

Diseases induced by mutation in NLPR3 correspond to a group named Cryopyrin-Associated Periodic Syndrome (CAPS). CAPS represents a spectrum of related clinical phenotypes due to sporadic or autosomal dominant mutations in NLPR3 and previously described as three distinct diseases: NOMID (Neonatal-Onset Multisystem Inflammatory Disease, also known as Chronic Infantile Neurological Cutaneous Articular syndrome or CINCA), FCAS (Familial Cold Autoinflammatory Syndrome) and MWS (Muckle-Wells Syndrome) [15].

Diseases associated with Pyrin mutations encompass the Familial Mediterranean Fever (FMF), which is due to a gain-of-function mutation in the MEFV gene encoding Pyrin [16]. S242R and E244K mutations in pyrin sequence disrupt its interaction with the regulatory 14-3-3 protein and leads to the inflammasome activation and the constitutive IL1β and IL-18 secretion in a monogenic pathology called Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND) [17]. Additionally, the related PAPA syndrome (for Pyogenic sterile Arthritis, Pyoderma gangrenosum, and Acne) implicates mutations (A230T or E250Q) in the phosphatase PSTPIP1 that better interact with Pyrin promoting IL-1β and IL-18 secretion [18].

All these DAMP-sensor mutations and their associated diseases are summarized in Table 2. Interestingly, the high systemic inflammatory symptoms/systemic clinical manifestations observed with these pathologies seem to be shared with those observed in mice exhibiting an uncleavable caspase-8 and deficient for the necroptotic factors mixed-lineage kinase domain-like protein (MLKL) or receptor-interacting protein kinase 3 (RIPK3) [19]. Indeed, these mice die to a systemic and exacerbated inflammatory response that can be abrogated by the elimination of one CD95L allele [19], suggesting that a molecular link might exist between CD95 stimulation and the inflammasome activation in certain pathophysiological contexts.

3. CD95/Fas

CD95 is a member of the tumor necrosis family receptors (TNF-Rs) and it is the prototype receptor to study the apoptotic signaling pathway. TNF-Rs are devoid of any enzymatic activity and necessitate protein-protein interactions (PPIs) to recruit enzymes such as proteases, kinases, and ubiquitin ligases to implement dynamic and complex signaling pathways.

At the plasma membrane, CD95 auto-aggregates as homotrimer independently of its natural ligand, CD95L (also known as FasL or CD178) [20–23]. This trimeric structure is mandatory to implement cell death and rapidly forms larger signaling platforms in the presence of its natural ligand [20]. CD95 engagement induces the recruitment of the

adaptor protein FADD, which in turn aggregates pro-caspase-8 in a complex-designated death-inducing signaling complex (DISC) [24]. Beyond DISC formation and induction of the apoptotic signal, FADD and caspase-8 contribute to different complexes involved in the induction of necroptosis or pyroptosis (discussed below).

The switch between apoptosis and necroptosis has been discussed previously [25]. Briefly, ubiquitination of RIPK1 is a pivotal post-translational modification for the induction of the TNF-R1-mediated NF-κB activation [26,27] and its deubiquitination leads to the induction of cell death. The deubiquitinated RIPK1 is released from the death receptor and recruits TRADD, Fas-associated death domain (FADD), pro-caspase-8, and the long isoform of FLICE-like inhibitory protein ($FLIP_L$) to trigger apoptosis [28]. In this complex, the caspase-8-mediated RIPK1 cleavage extinguishes the kinase activity. Degradation of c-IAP1 and c-IAP2 prevents the K63 ubiquitination of RIPK1 [29] and promotes the formation of another cellular complex in which FADD, pro-caspase-8, and $FLIP_L$ associate to trigger apoptosis.

When caspase-8 is inactivated in these two complexes, the necrosome is formed. RIPK1 associates with RIPK3 to form this complex, in which RIPK3 phosphorylates MLKL to promote its plasma membrane distribution and the induction of necrosis [30–32]. Although CD95 engagement displays a broad range of cellular outcomes [33], whether such complexes occur in a CD95-dependent manner remains to be elucidated.

Interestingly, the elimination of FADD or caspase-8 in mice does not lead to hyperplasia but instead is responsible for embryonic lethality, and these mice can survive post weaning when a double-ko mouse is realized with one of the necroptosis-mediating genes, RIPK3 [34,35] or MLKL [36] pointing out the tight control of the necroptotic machinery by the apoptotic one. Interestingly, caspase-$8^{-/-}$/RIPK$3^{-/-}$ or caspase-$8^{-/-}$/MLKL$^{-/-}$ double-ko mice develop hyperinflammation and lymphadenopathy, a phenotype resembling that observed in CD95-deficiency Lpr mice strongly suggesting that these factors control an additional mechanism in which CD95 could be involved too.

3.1. Classical Apoptotic Program

In the presence of CD95L, CD95 death domain (DD) recruits the adaptor protein FADD through homotypic interaction [37,38]. Then, the death effector domain (DED) of FADD binds the caspase-8 DED1 and the complete activation of the protease will require two molecular steps, (i) the oligomerization of caspase-8 via DED2/DED1 interaction [39,40], and (ii) its autocleavage to release a cytosolic active caspase. Mice lacking caspase-8 [34,35] or its activity (caspase-8 mutant C362S) [41,42] die during gestation because of the loss of the apoptotic control over the necroptotic process. Interestingly, caspase-8 mutants that fail to auto-aggregate or to undergo auto-cleavage do not trigger a strong CD95-mediated apoptotic signal but still exert a control over necroptosis through a residual caspase-8 activity [19]. Therefore, mice expressing a caspase-8 D387A (DA, uncleavable) or caspase-8 FGLG (mutations in the DED1 leading to no auto-aggregation) can block necroptosis during embryogenesis and are viable [19].

In agreement with the abrogation of the CD95-mediated apoptotic signal in these mice, caspase-8 DA or caspase-8 FGLG mice are resistant to fulminant hepatitis induced by injection of the agonistic anti-CD95 antibody Jo2 [19]. Nonetheless, elimination of RIPK3 and MLKL in these mice unravels an inflammatory burst when they are injected with the anti-CD95 agonistic mAb Jo2 [19]. Therefore, CD95 engagement triggers a pro-inflammatory program when caspase-8 cleavage and necroptosis are abrogated. Another TNF-R member designated TRAIL-R activates a complex called FADDosome, containing FADD and caspase-8 that induces the expression of pro-inflammatory genes independently of the caspase activity [43,44].

From a molecular standpoint, immune cells exposed to CD95L form a FADDosome in which FADD recruits caspase-8. A decreased caspase-8 activity, probably through a c-FLIP-dependent mechanism, impinges on the necroptotic signal but still allows the induction of a RIPK1-dependent inflammatory signal [19]. Interestingly, the kinase activity of RIPK1 was

not required to secrete the inflammatory cytokines in this context. This original signaling pathway contributes to an inflammatory response via a process independent of caspases-1 and -11 activities [19].

Of note, caspase-8 cleavage site between its large and small catalytic subunits correspond to the amino acid residues L384/E385/V386/D387 and elimination of E385 (ΔE385) within this sequence generates an uncleavable caspase-8 [45]. Like caspase-8 DA/Mlkl$^{-/-}$ mice [19], the hematopoiesis in Casp8ΔE385/Ripk3$^{-/-}$ or Mlkl$^{-/-}$ mice is skewed toward myeloid development [45]. The inflammatory response in Casp8ΔE385/Ripk3$^{-/-}$ mice also relies on the scaffold property of RIPK1 [45] confirming that caspase-8 and RIPK1 form, in apoptotic altered and necrotic deficient immune cells, a scaffold involved in hematopoiesis and in the induction of an inflammatory response (Figure 1, green complex). In the presence of TNF, Casp8ΔE385 fails to trigger apoptosis [45], and enhances necroptosis by preventing the cleavage of RIPK1 questioning whether the interplay between apoptosis, necroptosis and inflammation is similarly regulated between TNF and CD95L receptors. Overall, these observations suggest the existence of a molecular link between CD95, the FADDosome (FADD/RIPK1/Casp8) and a pro-inflammatory signal.

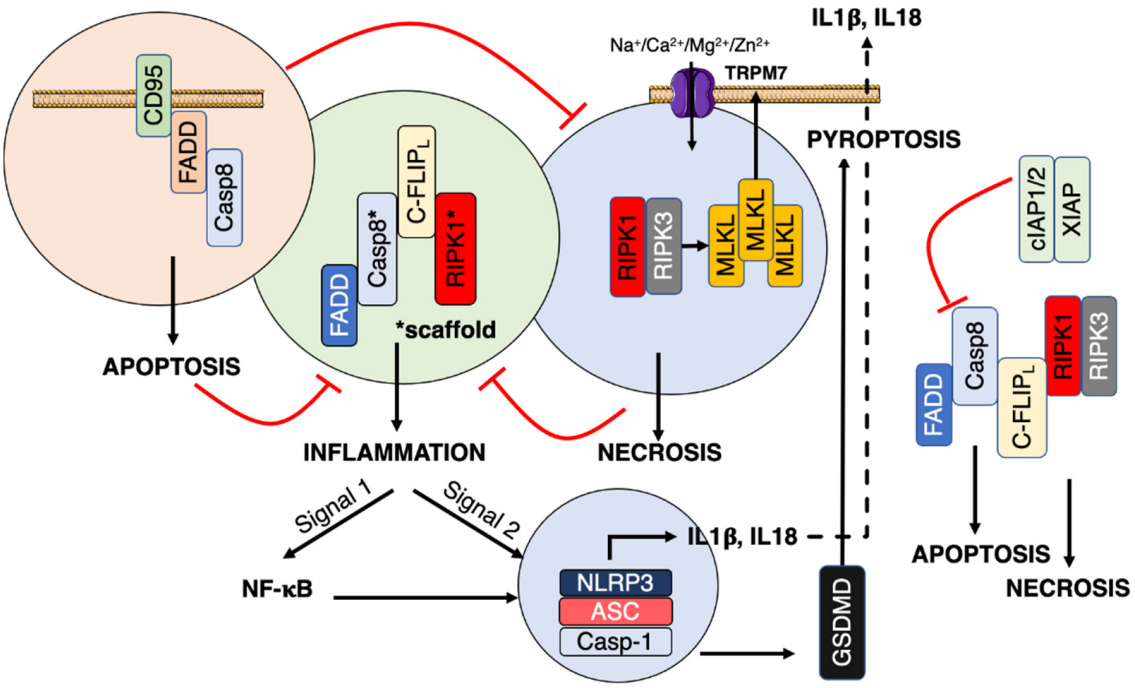

Figure 1. CD95 stimulation and potential links with inflammasome activation. At least three molecular complexes could occur upon CD95 engagement to trigger either apoptosis (**pink circle**), inflammation (**green circle**), or necrosis (**blue circle**). Apoptotic and necroptotic complexes control each other and the inflammatory complex (**red lines**). Necroptosis and pyroptosis signaling pathways lead to the break of the plasma membrane. IAPs also control an additional complex and these members are known to participate in the TNF-R-mediated signaling pathway. The NLRP3 inflammasome, which is activated by signal 1 + 2 is depicted. (GsdmD = Gasdermin D).

3.2. Necroptosis

On a morphological basis, cell death occurs by apoptosis, necrosis, or autophagy [46]. Necroptosis is a well-regulated mechanism inhibited by Necrostatin-1 (Nec-1), which abrogates the kinase activity of RIPK1 [47]. The caspase-8 counteracts necroptosis by cleaving different necrotic factors such as RIPK1, RIPK3 and mixed-lineage kinase domain-like protein (MLKL) [48]. Upon different stimuli including TNF, CD95L, or IAP (inhibitor of apoptosis proteins) antagonists, the inhibition of caspase 8 (i.e., using chemical inhibitors or viral proteins) leads to the association of RIPK1 and RIPK3, their autophosphorylation,

and transphosphorylate forming microfilament-like complexes [49] called necrosomes [50]. Although RIPK3 directly phosphorylates RIPK1, the opposite is not true [51] and the RIPK1 kinase activity seems instead responsible for stabilizing the necrosome [51]. The RIP homotypic interaction motifs (RHIMs) of RIPK1 and RIPK3 contribute to the necrosome formation [49]. RIPK3 is mandatory for necroptosis by phosphorylating MLKL and triggering its homotrimerization [31]. RIPK3 phosphorylates MLKL at the T357 and S358 [52] to induce its translocation to the plasma membrane and the disruption of cell membrane integrity [30,31]. At the plasma membrane, MLKL aggregation contributes to the activation of calcium channel TRPM7, which in turn mediates Na^+, Ca^{2+}, Mg^{2+}, and Zn^{2+} influxes [32] (Figure 1). The MLKL translocation into the inner leaflet of the plasma membrane breaks the cell integrity.

Of note, DAMPs released by necroptotic cells stimulate immune cells through ligation of pattern recognition receptors (PRRs) to secrete pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18, which subsequently activate host immune defenses against various pathogens. The maturation of IL-1β and IL-18 is mediated by cytosolic NOD-like receptors (NLRs) and HIN domain-containing family member AIM2, which associate with other proteins to form a large multimeric complex designated the inflammasome [53,54]. The most extensively studied inflammasome consists of NLRP3, the apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 [54]. In the NLRP3 inflammasome, NLRP3 recruits ASC, which aggregates pro-caspase-1, triggering its auto-cleavage and the release in the cytosol of an activated protease. Activated caspase-1 cleaves and matures the pro-inflammatory cytokines IL-1β and IL-18 [55]. The inflammasome necessitates two signals, designated signals 1 and 2, to trigger the release of mature IL-1β and IL-18 [56]. Signal 1 leads to the NF-κB activation and the transcriptional upregulation of the inflammasome machinery and pro-IL-1β and pro-IL-18. Signal 2 is mandatory to promote the assembly of the inflammasome machinery in the cytosol and the subsequent caspase-1 activation releasing mature IL-1β and IL-18. Signal 2 is provided by a broad range of stimuli (i.e., DAMPs) including ATP and ions such as Cl^- and K^+ effluxes [57] or Ca^{2+} influx [58]. Many links exist between necrosome and inflammasome since MLKL and RIPK3 can also contribute to the NLRP3 inflammasome activation by inducing signal 1, which is NFκB activation [59]. Although CD95 has been associated with necroptosis [60], a robust link between CD95 engagement and necrosome still remains to be established.

3.3. Non-Apoptotic Signals

The five members of the NF-κB family, consisting of RelA (p65), RelB, c-Rel, NFκB1 (p105), and NFκB2 (p100), share a conserved Rel homology domain (RHD) responsible for DNA binding. While p65, RelB, and c-Rel encompass a transactivation domain in their C-terminal regions, NFκB1 (p105) and NFκB2 (p100), which are degraded into p50 and p52, respectively, are devoid of transactivation domains [61]. Therefore, nuclear accumulation of p50/p50 homodimers might exert a transcriptional repression over the NF-κB response [61–66]. Stimulation of CD95 in cancer cells resistant to the apoptotic pathway triggers cell migration by inducing NF-κB signals [67–69]. CD95 can also induce a PI3K response that promotes cell migration [70–77] and endothelial transmigration of inflammatory neutrophils or Th17 T cells [78–80].

We recently established that the CD95 expression in triple-negative breast cancer (TNBC) cells is responsible for the partial degradation of p105 into p50 via the ubiquitin ligase KPC2/KPC1 [81]. The C-terminal region of CD95 directly binds KPC2, which in turn recruits KPC1 [81], and the loss of CD95 in TNBC cells impinges on this KPC2/KPC1-mediated p105 ubiquitination, leading to a decrease in p50. The p50 drop favors the formation of active p50/p65 heterodimers at the expense of p50/p50 homodimers and the induction of the pro-inflammatory NFκB response in CD95-deficient TNBC cells [81]. Interestingly, the induction of this pro-inflammatory signal seems to stimulate a natural killer (NK)-mediated anti-tumor response in TNBCs [82]. Overall, these findings suggest

that CD95 expression in TNBCs behave as an immune checkpoint preventing the NK-mediated anti-tumor response.

Associated with c-FLIPL, the caspase-8 activity can induce the NF-κB signaling pathway in a complex consisting of caspase-8, c-FLIP$_L$, RIPK1, and FADD to promote the differentiation of monocytes to macrophages in the presence of macrophage-colony-stimulating factor (M-CSF) [83]. The caspase-8-dependent cleavage of RIPK1 prevents the sustained NF-κB activation in this differentiation process. Although the death receptors do not seem to be involved in the monocyte differentiation [83], further investigation could address whether such a complex could account for the pro-inflammatory signal observed with non-cleavable caspase-8 [19]. In a similar manner, association of caspase-8 and c-FLIP$_L$ favors CD8+ T-cell proliferation by recruiting RIPK1 and activating NF-κB [79]. From a molecular standpoint, c-FLIP$_L$ is processed by caspase-8 into a p43 form that recruits RIPK1 to activate NF-κB [84]. CD95 could be involved in this process since pioneering studies highlighted that CD95 activation in T cells enhanced the CD3-mediated activation [85,86].

The activation of PI3K by CD95 engagement was described in the late 1990s [87], but its biological roles remained difficult to apprehend. Although initial reports described that CD95-mediated PI3K activation was crucial for ceramide synthesis and cell death, and this signal was induced by caspase, Ras [88], and p56Lck activities [89], contradicting findings highlighted that in fact, the CD95-mediated PI3K activation exerts an inhibitory function on caspase cleavage and apoptosis [90]. Although the CD95-mediated PI3K activation seems to rely on different members of the src kinase superfamily, how CD95 recruits these kinases remains difficult to apprehend. Epidermal growth factor receptor (EGFR) can phosphorylate caspase-8 at tyrosine 380 (Y380), in a src-dependent fashion, to inhibit the induction of the CD95-mediated apoptotic signal [91]. Although the protease activity is prevented, Y380 phosphorylation promotes cell migration by recruiting the p85α-regulatory subunit of phosphatidylinositol 3-kinase through its SH2 domain [92]. CD95 can associate with the receptor tyrosine kinases (RTK) EGF-R in cancer cells [74,93], the integrin LFA-1 in neutrophils [78] or PDGFR-β in colon cancer cells [75] to activate src kinases and promote cell migration. Therefore, CD95 can trigger different complex signaling pathways by recruiting transmembrane RTKs [33], and how this recruitment occurs remains to be elucidated.

4. Inflammation

Inflammation after infection or injury relies on the detection of pathogen-associated and damage-associated molecular patterns (PAMPs/DAMPs). Examples of sterile DAMPs includes cholesterol crystals (atherosclerosis), β-amyloid (Alzheimer's), islet amyloid polypeptide, ceramide, saturated fatty acids (type II diabetes), asbestos, silica dioxide (pulmonary fibrotic disorders), and monosodium urate (gout) [8,94]. Pattern recognition receptors (PRRs) translate the cell stress into proinflammatory signals.

The cytosolic PAMPs/DAMPs activate inflammasome, which in turn induces caspase-1 activity leading to the maturation of interleukin-1β (IL-1β) and IL-18 [95,96]. Inflammasome can also induce pyroptosis by cleaving gasdermin D (GsdmD) [97,98]. GsdmD forms plasma membrane pores responsible for the induction of cell death and the release of mature IL-1β and IL-18 [99,100] (Figure 1). The signal 2 activating NLRP3 is diverse and includes mitochondrial reactive oxygen species (ROS), potassium efflux, and/or chloride influxes [101] (Figure 2), pointing out that NLRP3 is a sensor for a broad spectrum of cellular disturbances.

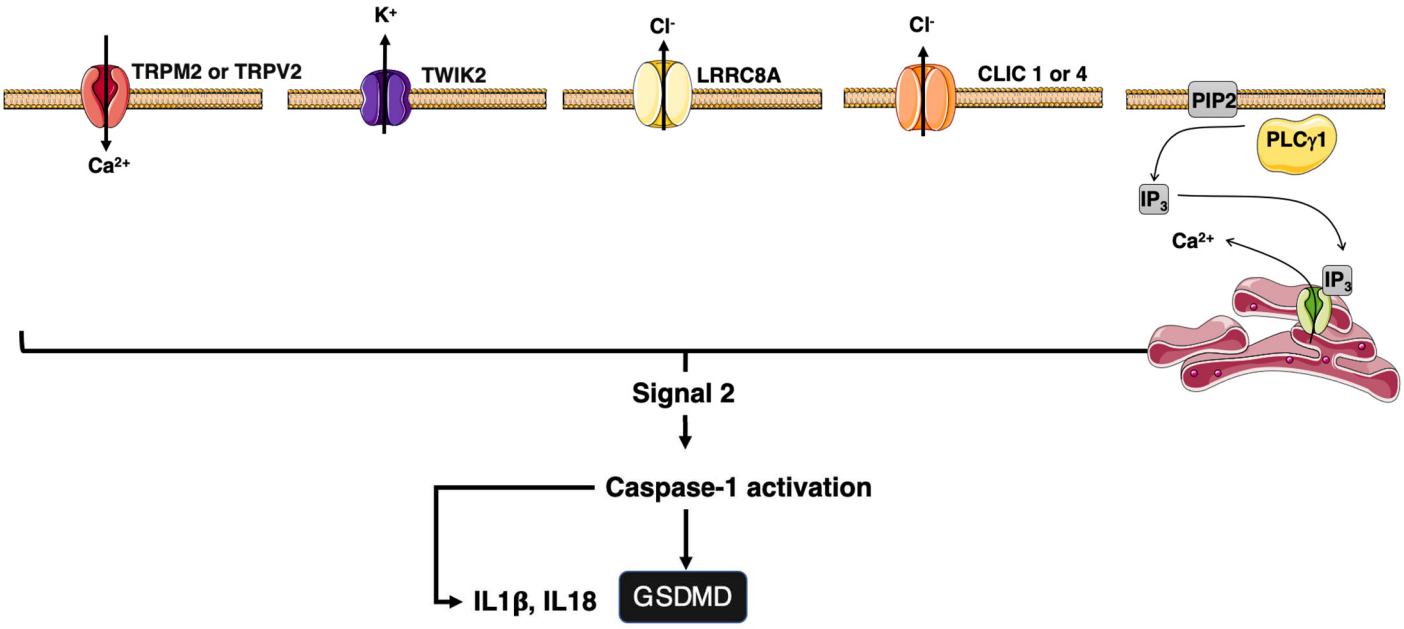

Figure 2. Representation of ion channels involved in the inflammasome activation. Different plasma membrane or endoplasmic reticulum ion channels have been involved in ion fluxes (**black arrows**) responsible for the signal 2 activating the inflammasome.

4.1. Inflammasome

Inflammasome activation requires NF-κB induction (priming signal or signal 1) for the upregulation, among others, of NLRP3 and pro–IL-1β; next, a danger signal (signal 2) activates inflammasome. FADD and caspase-8 form the FADDosome to activate NF-κB [102]. The caspase-8 activity does not contribute directly to this signal, but it serves as a scaffold [103] (Figure 1). On the other hand, the cytoplasmic dsRNA sensor requires caspase-8 activity to relieve the inhibition of RIPK3 on the NLRP3 inflammasome assembly [103]. Although this study highlights the pivotal role of caspase-8 and FADD in the activation of inflammasome, whether the death receptors contribute to this process remains to be elucidated. Interestingly, activation of the NLRP3-mediated inflammasome in macrophages induces FADD secretion [104]. It is tempting to envision that this secretion corresponds to a negative feedback loop to dampen the FADD-dependent inflammatory response by reducing the intracellular quantity of the adaptor protein.

GsdmD is cleaved at D275 in humans (D276 in mice) by caspase 1 in the canonical pathway and in the noncanonical pathway by caspases 4/5 in humans and caspase-11 in mice (orthologue). Interestingly, caspase-8 can also cleave GsdmD at the same site, providing an alternative method of activation [105,106]. Caspase-8 can also cleave IL-1β [107].

It is noteworthy that pioneer studies with injection of CD95L-expressing tumor cells into wild-type mice provokes a massive neutrophil infiltration [108] through an IL-1α/β-dependent but caspase-1-independent mechanism [109]. The caspase-1-independent release of IL-1β by macrophages exposed to CD95L has been confirmed [109,110]. More recently, it has been reported that CD95 engagement activates caspase-8 in macrophages and dendritic cells, leading to the maturation of IL-1β and IL-18 [111].

Antagonists of apoptosis proteins (IAPs) promote CD95-mediated cell death signals (i.e., apoptosis and necroptosis) [112]. IAP inhibitors also trigger IL-1β maturation via the NLRP3 inflammasome and caspase-1, as well as via a caspase-8–dependent pathway [107]. Elimination of IAPs engenders the IL-1β maturation through a RIPK3-dependent mechanism [107]. Therefore, the molecular targets of IAP antagonists including IAP1, 2, and XIAPs proteins control a RIPK3- and caspase-8-dependent inflammasome [107]. In agreement with the tight interplay between necroptosis, apoptosis, and inflammation, cIAP1, cIAP2, and XIAP also inhibit the RIPoptosome, a complex consisting of FADD/RIPK1/RIPK3/Caspase-

8/FLIP$_L$ and involved in apoptosis and necroptosis [113,114]. Whether CD95 can contribute to these signals remains to be defined. Rendering more complex the manner how CD95 engagement could secrete IL-1β, it has been reported that CD95 induces IL-1β maturation via a FADD- and caspase-8-dependent but NLRP3-, caspase-1-, caspase-11- and ASC-independent process in Bone-Marrow-Derived Macrophages (BMDMs) [111].

A catalytically inactive caspase-8 in which cysteine 362 has been replaced by an alanine (C362A) fails to trigger apoptosis and thereby to inhibit necroptosis, but can still induce ASC specks and the caspase-1-dependent cleavage of GsdmD [42]. Accordingly, while Casp8$^{-/-}$Mlkl$^{-/-}$ mice are viable, C362A$^{+/+}$/Mlkl$^{-/-}$ mice die during the perinatal period, confirming that the caspase-8 scaffold contributes to a toxic signal independently of its apoptotic role or its anti-necroptosis function. Caspase-1, caspase-11, and their adaptor ASC contribute to this signal [42]. From a molecular standpoint, the inactive caspase-8 adopts a different conformation as compared to its active counterpart, enabling its prodomain to engage ASC and promote a caspase-1 and caspase-11-mediated inflammatory signal [42]. The lethal phenotype of C362A$^{+/+}$/Mlkl$^{-/-}$ mice is not prevented by elimination of Nlrp3 [42], raising the question of the Nod-like receptor (NLR) responsible for inflammasome activation in these animals.

As aforementioned, although the CD95-mediated apoptotic signal in immune cells expressing an oligomerization-deficient or a non-cleavable caspase-8 mutant is impaired, the protease can still counteract the necroptotic signal [19]. Nonetheless, elimination of RIPK3 or MLKL in these mice unleashes an inflammatory response when CD95 is stimulated, suggesting that the necrosome and the caspase-8 activity control a CD95-dependent inflammatory response. This signal occurs through the FADDosome (FADD/RIPK1/caspase-8) formation [19]. In epithelial cells (i.e., intestinal barrier), the elimination of FADD in Casp8DA/Mlkl$^{-/-}$ animals causes an inflammatory burst resulting in mouse death within 2 weeks of life [19]. Therefore, FADD promotes inflammation in immune cells and inhibits it in epithelial cells [19]. The inflammasome inhibited by FADD in epithelial cells probably encompasses NLRP6 [19].

4.2. PAMP Sensors and CD95 Crosstalk

Extensive cross-talks exist between cell death pathways, and the simultaneous induction of pyroptotic, apoptotic and necroptotic molecules led to the concept of PANoptosis. AIM2 inflammasome senses double-stranded DNA (dsDNA) [115,116]. Unlike classical AIM2 inducer (i.e., poly(dA:dT)), HSV1 and F. novicida infections induce a large complex containing AIM2, pyrin, ZBP1, ASC, caspase-1 and -8, RIPK1, RIPK3, and FADD that induces PANoptosis. The authors called this complex the AIM2 PANoptosis [117]. AIM2 activation in macrophages induces the over-expression of CD95L in an IL-1β-dependent mechanism. This process contributes to the elimination of T-cells after severe tissue injury, and thereby increases susceptibility for life-threatening infections [118]. These findings indicate that although a link between CD95 stimulation and inflammasome remains to be identified, the activation of inflammasome can, on the other hand, modulate the immune landscape by triggering the CD95-mediated apoptotic signal.

cGAS and Stimulator of Interferon Genes (STING) have been identified as the sensors of cytosolic dsDNA [119]. Upon binding to dsDNA cGAS (a cyclic GMP/AMP synthase) converts GTP and ATP to the cyclic nucleotide called cGAMP [120,121]. STING binds cGAMP undergoing a conformational change, thereby facilitating the phosphorylation of the transcription factor IRF3, and finally leading to the expression of Type I IFN genes [120,121]. Both CD95 and INF signatures have been involved in the progression of lupus, but whether a link exists between these two pathways remains to be elucidated. Although the elimination of the INFγR in MRL/Lpr mice (CD95 deficient mice) protected them from glomerulonephritis, it remains inefficient to prevent lymphadenopathy and accumulation of double-negative T-cell (B220+CD3+CD4-CD8-) [122]. Interestingly skin lesions were higher in the INFγR$^{-/-}$ MRL/Lpr mice as compared to their MRL/Lpr counterparts, suggesting a protective role of this signal in the skin inflammation [122]. cGAS or

STING elimination in different models of lupus, in which CD95/Fas has been involved, also suggests that these factors could protect from the pathology progression. These factors alleviated the clinical symptoms by preventing the endosomal TLR signaling [123]. These data indicate that while TLR7 and TLR9 and its downstream transcription factor IFN regulatory factor 5 (IRF5) contribute to the SLE pathogenesis [124–126], this could occur in a cGAS/STING-independent pathway.

4.3. Ion Regulation of Inflammation

4.3.1. Ion Channels and Inflammasomes

Intracellular ion homeostasis is sensed by inflammasome signaling. Non-isosmotic conditions implement NLRP3- and NLRC4-mediated caspase-1 activation in LPS-primed macrophages [127]. Potassium (K^+) [128] and chloride (Cl^-) [129] effluxes and calcium (Ca^{2+}) [58] influx represent different but probably interconnected mechanisms contributing to the osmotic regulation and inflammasome activation. The K^+ channel TWIK2 (two-pore domain weak inwardly rectifying K^+ channel 2) [130] is responsible for K^+ efflux (Figure 2), which promotes the association of NEK7 with NLRP3, activating the NLRP3-inflammasome [57]. Leucine-rich repeat-containing protein 8A (LRRC8A) is a channel allowing the passage across the plasma membrane of chloride (Cl^-) ions [131]. The volume-regulated anion channels (VRAC), formed by Leucine-rich repeat-containing protein 8 (LRRC8) heteromers, also contribute to the NLRP3 inflammasome activation in hypotonic medium, while it does not participate in DAMP-induced inflammasome [132]. Cl^- efflux seems to be essential for the formation of ASC specks, while K^+ efflux promotes the cleavage and maturation of IL-1β [57]. Interestingly, although the DAMP-mediated NLRP3 activation does not rely on VRAC, chloride efflux still modulates this signal suggesting that other chloride channels are involved in the inflammasome activation [132]. The chloride intracellular channel (CLIC) protein family consists of six evolutionary conserved proteins (CLIC1–CLIC6) implicated in various cellular processes [133]. CLIC1, CLIC4, and CLIC5 are expressed in macrophages. CLIC 1 and 4 promote the NLRP3 inflammasome and the subsequent pyroptotic response in macrophages [134,135] (Figure 2). Interestingly, K^+ efflux alters the mitochondrion functions and promotes ROS production, which in turn favors the translocation of CLICs from ER to the plasma membrane for the induction of Cl^- efflux [135].

Phospholipase C hydrolyzes phosphatidyl inositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-trisphosphate (IP3), a second messenger, which binds to inositol-1,4,5-trisphosphate receptors (IP3-R) at the endoplasmic reticulum (ER). IP3 binding to the IP3 receptors releases calcium from ER stores, leading to a transient peak of free calcium in the cytosol [2,33], involved in the assembly of inflammasome [58,136] (Figure 2). Calcium channels TRPM2 [137] and TRPV2 [129] contribute to the sustained Ca^{2+} response necessary for NLRP3 inflammasome activation. Interestingly, TRPM2 also contributes to the inflammatory response induced by TNFα in macrophages [138] and the activation of these channels in the CD95-mediated signaling pathway remains to be evaluated.

4.3.2. Ion Channels and CD95

Of note, cell volume modulation also affects the CD95-mediated signaling pathway, suggesting a possible interplay between death receptors and VRACs. CD95 has been reported to activate different ion channels including K^+, Cl^-, and Ca^{2+}. CD95 engagement invokes the Cl^- efflux [139] in a ceramide- and p56Lck-dependent mechanism [140,141]. Nonetheless, the chloride channel involved in the CD95-mediated signaling pathway and its role in the induced signal remain to be identified.

Interestingly, lymphocytes exposed to CD95L undergo an inhibition of K^+ channel Kv1.3 through the production of ceramide and activation of Src-like tyrosine kinases [142,143] suggesting that Cl^- and K^+ channels are regulated in an opposite fashion by CD95, and thereby, whether an effect exists on the inflammasome remains difficult to apprehend.

CD95 engagement stimulates the calcium response. The PLCγ1-mediated IP3 production and the subsequent activation of IP3-Rs leads to the release of ER-stored Ca^{2+} (Figure 2). This reduction in Ca^{2+} in ER lumen is sensed by ER transmembrane proteins STIM1 and STIM2 that traffic to the plasma membrane to activate Orai channels, allowing a sustained Ca^{2+} influx from the extracellular space [144–147], a molecular mechanism designated store-operated calcium entry (SOCE). CD95 can directly interact with PLCγ1 [79,80] to trigger IP3-R activation and SOCE-dependent Ca^{2+} response [148] suggesting that at least the signal 2 exists when this death receptor is stimulated. Because CD95 can also trigger NF-κB [67,69], we could postulate that CD95 might stimulate signal 1 and the inflammasome.

5. Conclusions and Open Questions

In invertebrates, TNF receptors share with inflammasome the biologic property to fight against pathogens. Indeed, ancestors of the TNF/TNFR superfamily present in phylum Mollusca (e.g., oysters, mussels, and clams) contribute to the elimination of Gram-negative bacteria [149]. In the lancelet fish, Amphioxus, lipopolysaccharide challenge promotes the increase in TNF and TNFR expression suggesting that this family might contribute to the elimination of pathogens [150]. Elegant in vivo data highlight that a molecular link exists between CD95 engagement and activation of the inflammasome, but this occurs in a cellular context in which the caspase activity is dampened and the necroptotic signal is inhibited [19]. Characterization of the molecular mechanism leading to inflammasome activation upon CD95 engagement, and the patho-physiological roles of this signal have still to be elucidated. We can envision that some apoptotic and necroptotic resistant tumor cells or certain infected cells, in which both cell death signals are under control, could undergo this signal.

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REVIEW
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Identifying Markers of Emerging SARS-CoV-2 Variants in Patients With Secondary Immunodeficiency

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Since the end of 2019, the world has been challenged by the coronavirus disease 2019 (COVID-19) pandemic. With COVID-19 cases rising globally, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to evolve, resulting in the emergence of variants of interest (VOI) and of concern (VOC). Of the hundreds of millions infected, immunodeficient patients are one of the vulnerable cohorts that are most susceptible to this virus. These individuals include those with preexisting health conditions and/or those undergoing immunosuppressive treatment (secondary immunodeficiency). In these cases, several researchers have reported chronic infections in the presence of anti-COVID-19 treatments that may potentially lead to the evolution of the virus within the host. Such variations occurred in a variety of viral proteins, including key structural ones involved in pathogenesis such as spike proteins. Tracking and comparing such mutations with those arisen in the general population may provide information about functional sites within the SARS-CoV-2 genome. In this study, we reviewed the current literature regarding the specific features of SARS-CoV-2 evolution in immunocompromised patients and identified recurrent *de novo* amino acid changes in virus isolates of these patients that can potentially play an important role in SARS-CoV-2 pathogenesis and evolution.

Keywords: SARS-CoV-2, viral evolution, secondary immunodeficiency, mutations, spike protein, COVID-19

INTRODUCTION

In late 2019, a new viral outbreak in Wuhan city, China (World Health Organization [WHO], 2020a), rapidly identified as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), resulted in the coronavirus disease 2019 (COVID-19) pandemic (World Health Organization [WHO], 2020b), which still continues with the rise of novel variants of concern (VOCs) and of interest (VOIs).

Increased age is perhaps the strongest risk factor for severe COVID-19 (Bonanad et al., 2020); obesity, male gender, and various comorbidities such as hypertension, cardiovascular disease, and

diabetes also contribute to an increased odds ratio of severe disease (Hu and Wang, 2021). However, among infected individuals, patients with secondary immunodeficiency, due to preexisting health conditions, and those undergoing immunosuppressive treatment are particularly susceptible to SARS-CoV-2 (Gao et al., 2020a; Liu and Hill, 2020; Hoffmann et al., 2021; Jones et al., 2021). Many research groups have reported chronic infections and the accumulation of viral protein-coding mutations in such individuals in the presence of anti-COVID-19 treatments, with potential relevance at both biological and epidemiological levels. We hypothesized that two main kinds of mutations could be observed in such immunodeficient setting, namely, (1) variations selected by antiviral treatment and (2) variations reflecting the adaptation of the virus to the human host, particularly in the context of an environment with reduced immune responses, allowing niches of selective pressure.

To gain insights into the mutational signatures of secondary immunodeficiency in SARS-CoV-2 genetic profiles, we have queried the literature to review SARS-CoV-2 genome data from 44 patients with secondary immunodeficiency who underwent treatment against COVID-19. We retrieved 148 full genomes from 21 patients and partial genomes for 24 patients. By analyzing the viral genomes detected in these patients in comparison with circulating variants, we identified numerous new protein-coding mutations and inspected their predicted structural or functional impact at the protein level.

SARS-CoV-2

SARS-CoV-2 is a betacoronavirus that shares 96% of its genomic identity with the RaTG13 bat coronavirus and is hypothesized to be of zoonotic origin (Zhou et al., 2020; Banerjee et al., 2021). It is a positive sense ribonucleic acid virus (RNA), with a genome spanning around 30 kilobases in length (Wu et al., 2020; V'kovski et al., 2021). Notably, two-thirds of its genome is composed of overlapping open reading frames (ORF) 1a and 1b, which together encode for an RNA-dependent RNA polymerase (RdRp) and other non-structural proteins important for viral replication and transcription (**Figure 1A**; Wang Q. et al., 2020; Wang Y. et al., 2020; Yan et al., 2021). The remainder of the viral genome is composed of ORFs 2–10 encoding for structural and accessory proteins (**Figure 1A**; Davidson et al., 2020; Jiang et al., 2020; Michel et al., 2020; Mohammad et al., 2020; Pancer et al., 2020).

Of the structural proteins, the spike is a large accessible homotrimeric protein of great importance in viral tropism and viral entry, making it a great target in therapeutic development (Conceicao et al., 2020). With a molecular weight of around 180 kDa, the spike protein is composed of 2 major subunits per monomer: the S1 (residues 14–685) and S2 (residues 686–1273) (**Figure 1B**; Huang et al., 2020; Martí et al., 2021). The former is the most variable part of the spike among coronaviruses and contains the amino (N)-terminal domain (NTD) and the receptor-binding domain (RBD) (**Figure 1B**; Huang et al., 2020; Martí et al., 2021). As for the S2, its domains, which are essential for viral fusion with the host cell membrane, are more conserved in structure and sequence (**Figure 1B**; Huang et al., 2020; Martí et al., 2021).

The main target of the spike is the angiotensin-converting enzyme (ACE2) (Li et al., 2020). The broad expression of ACE2 explains in part SARS-CoV-2 pathogenesis in a multitude of organs from respiratory, circulatory, urogenital, gastrointestinal, and nervous systems (Lopes-Pacheco et al., 2021).

Following cell entry, the replication of SARS-CoV-2 takes place in the cytoplasm with the help of the host ribosomal machinery, translating the ORF 1a and 1b genes into two large replicase polyproteins, namely, pp1a and pp1ab (V'kovski et al., 2021). Together, both pp1a and pp1ab polyproteins undergo proteolytic cleavages *via* the viral-encoded proteinases papain-like protease (PL-pro, Nsp3) and 3C-like protease (3CL-pro, Nsp5) to generate 16 mature non-structural proteins, i.e., Nsp1 to Nsp16 (Astuti and Ysrafil, 2020). Proteolysis is an essential step for viral replication, which is why antivirals targeting proteases are of interest (Dampalla et al., 2021; Roe et al., 2021). Later, the RNA-dependent RNA polymerase (RdRp and Nsp12), helicase (Nsp13), and Nsp7 to Nsp9 form the replication/transcription complex (RTC), allowing the synthesis of viral RNA in double-membrane vesicles (DMV) at the periphery of the endoplasmic reticulum (Brant et al., 2021).

MUTATIONS IN EMERGING VARIANTS

Like most RNA viruses, SARS-CoV-2 continues to mutate as it spreads, resulting in different variants, where the Pango numeric system assigns lineages with a number or letter such as B.1 (Oude Munnink et al., 2021). Among the variants circulating as of May 08 2022, five are known VOCs defined by the WHO based on their epidemiology and their association with disease severity or potential to escape available treatments or vaccines (**Figure 2**; Harvey et al., 2021; World Health Organization [WHO], 2021). Martin et al. (2021) reported that there has been a shift in the mutational landscape of some VOCs with the N501Y spike amino acid substitution (alpha, beta, and gamma) where there have been mutations arising independently and repeatedly in different viral lineages at 29 genome sites from 15 March 2021 to 1 June 2021. Such converging evolution in these sites could likely occur in variants of the same and different lineages (Martin et al., 2021). Variations in spike proteins that define VOCs are highlighted in **Figure 2**.

As a nidovirus, SARS-CoV-2 encodes a unique proofreading enzyme 3′ to 5′ exonuclease (ExoN) involved in excising faulty nucleotides inserted by RNA polymerases, thus ensuring replication fidelity (Shannon et al., 2020; Gribble et al., 2021). Despite this proofreading mechanism, SARS-CoV-2 has shown a capacity to accumulate a wide range and high number of mutations (Chen et al., 2020). A study of samples from the first wave and second wave of COVID-19 in Japan noted a mutation rate of $1.16–1.87 \times 10^{-3}$ base substitutions/site/year (Ko et al., 2021). This is relatively low compared with the human immunodeficiency virus (HIV) subtype B, which can have a nucleotide substitution rate ranging from 5.25×10^{-3} to

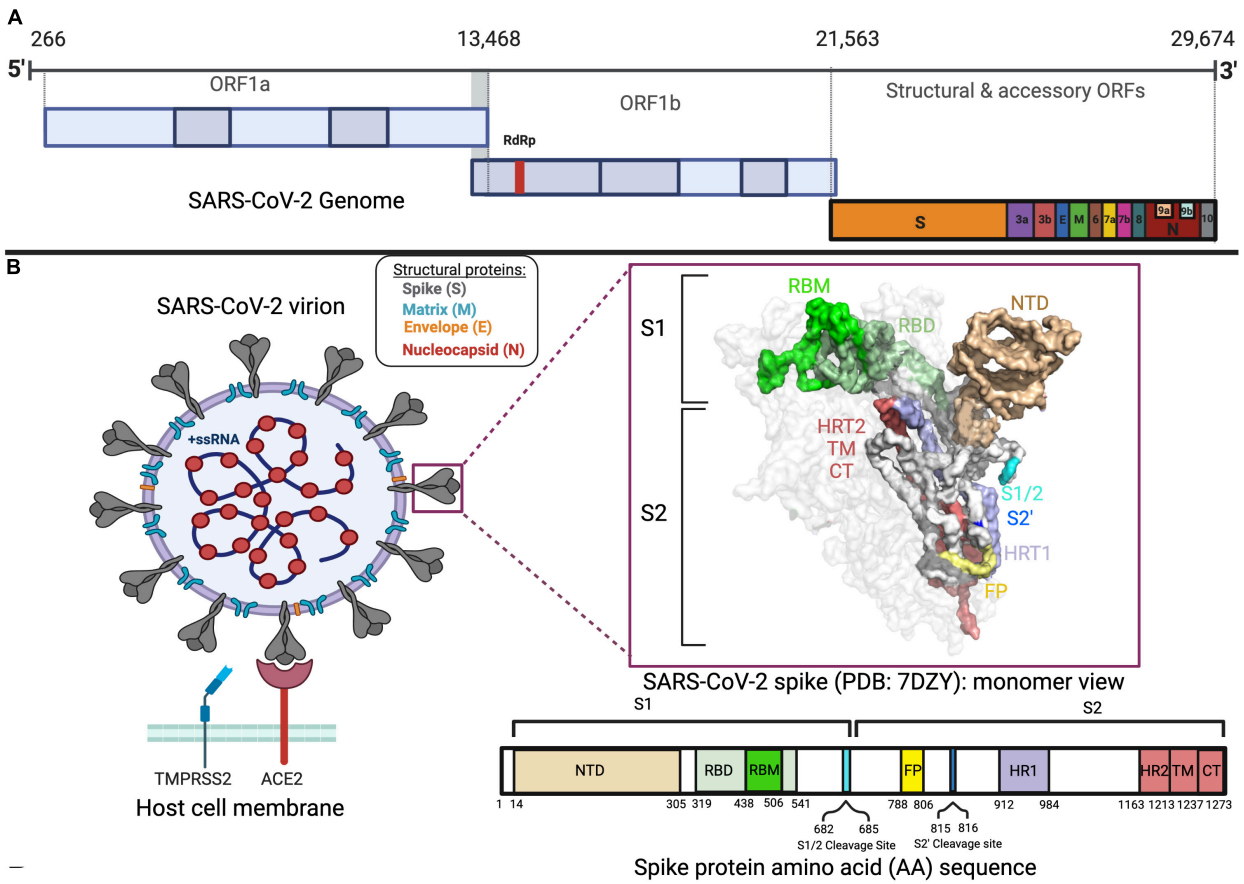

FIGURE 1 | SARS-CoV-2 genome, spike, and virion. **(A)** The genomic sequence of SARS-CoV-2 with different open reading frames (ORFs) is displayed in different colors. **(B)** Representation of the SARS-CoV-2 virion structure, spike protein and amino acid sequence of spike protein, and its domains in different colors. The binding of SARS-CoV-2 virion to the ACE2 receptor adjacent to the TMPRSS2 protein is also shown.

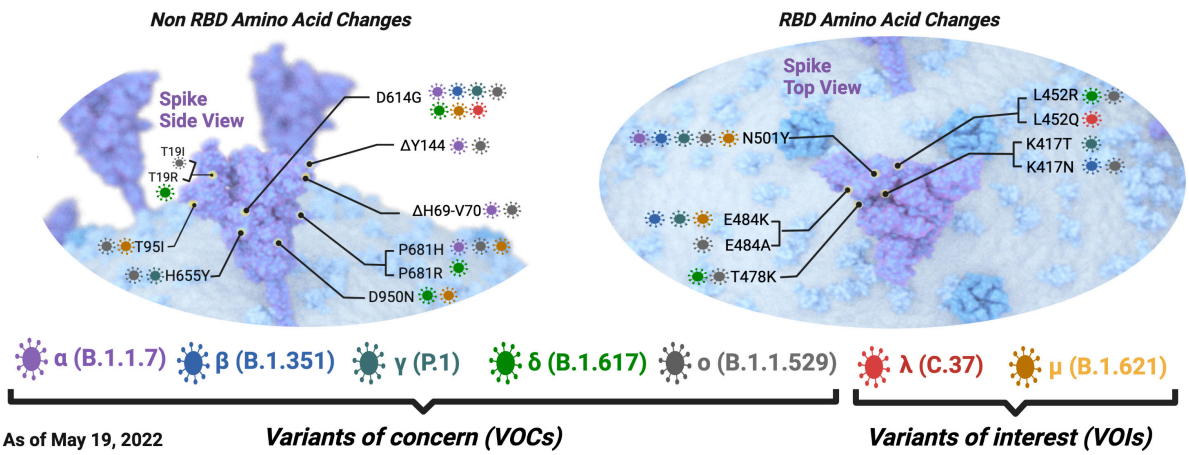

FIGURE 2 | Defining spike amino acid changes in SARS-CoV-2 variant of concerns (VOCs) and interest (VOIs). The variants of concern are depicted in purple, blue, dark green, pale green, and pale gray for the alpha(α), beta (β), gamma (γ), delta (δ), and omicron (ο) variants, respectively. The variants of interest are depicted in red and dark yellow for the lambda (λ) and mu (μ) variants, respectively. The envelope is shown in blue, and the spike protein is shown in purple. Common amino acid changes in different variants are also depicted. Non-RBD amino acid changes are shown on the left, and RBD amino acid changes are shown on the right. Defining amino acid changes are those appearing at the phylogenetic root of a variant (Hodcroft, 2021). Adapted from "The SARS-CoV-2 Variants of Concern," by BioRender.com (2021).

1.60×10^{-2} substitutions/site/year in gag and env-gp120 genes (Dapp et al., 2017).

However, there are more ways of generating genetic diversity; viral recombination, which is the generation of new progeny from two distinct strains of virus co-infecting a cell, is a way to generate viral genetic variation (Simon-Loriere and Holmes, 2011). In the case of SARS-CoV-2, Pollet et al. (2021) performed a recombination analysis of a variety of coronaviral sequences including 100,000 SARS-CoV-2 sequences. Through this analysis, they showed eight SARS-CoV-2 recombination events, two of them in the spike gene (Pollet et al., 2021). Earlier in 2021, a SARS-CoV-2 co-infection event of a single patient was reported with two strains with distinct lineages, which raises concern for the recombination of SARS-CoV-2 evolution (Francisco et al., 2021).

Furthermore, the genetic variability of viruses is shaped through the selection pressure of their host cell or environment. The host has multiple immune defense mechanisms at cellular, tissue, and systemic levels that can interfere with viral replication and spread. Letko et al. (2018) showed an example of MERS-CoV causing observable cytopathic effect due to the accumulation of amino acid variations in the spike protein after eight viral passages in BHK cells expressing the bat DPP4 receptor. Antiviral treatments that target specific viral proteins are another selective pressure that can result in the development of treatment-resistant mutants. For instance, the emergence of two mutations in the RdRp of murine hepatitis virus (MHV) conferred a 5.6-fold increased resistance to remdesivir (based on EC_{50} values) (Agostini Maria et al., 2018). The study of virus sequences that emerge in chronically infected patients could reveal regions of the virus genome that will be important as we prepare for and predict future variants.

SECONDARY IMMUNODEFICIENCIES

The most common cause of immunodeficiency is acquired immunodeficiency, meaning impaired immune response secondary to a condition or its treatment. This review will focus on the four main types we have found to be associated with COVID-19, namely, cancer, organ transplantation, HIV infection/AIDS, and autoimmune diseases.

Indeed, it has been documented that immunosuppression leads to poorer prognosis in hospitalized patients (Ponsford et al., 2021), especially in cancer and organ-transplanted patients (Elkrief et al., 2020; Bhogal et al., 2021; Coll et al., 2021), as well as in HIV-infected patients (Suwanwongse and Shabarek, 2020; Kanwugu and Adadi, 2021). In cancer, this impaired immune response can result from the medical condition itself, for example, impaired humoral response in a chronic lymphocytic leukemia or bone marrow infiltration by an acute leukemia preventing the development of normal leukocytes. But immunosuppression can also be induced by the malignancy treatment: hypogammaglobulinemia induced by B cell depletion after rituximab use or by alkylating agent that impairs DNA from replicating cells (including cancer cells and leukocytes). The same kind of treatment-induced immune impairment happens in organ transplantation contexts, with the immunosuppressive regimen used for the prevention of graft rejection.

In HIV-affected patients, with incomplete or without antiretroviral treatment, HIV infection leads to low CD4 + T cells count, and the decrease in these cells gives rise to opportunistic diseases (Chinen and Shearer, 2010).

Autoimmune diseases are a heterogenous group of diseases characterized by loss of tolerance to self-antigens, leading to the development of autoantibodies and activation of the immune system, resulting in immune complex deposits, organ failure, and ultimately death in the most severe cases (Kaul et al., 2016; Denton and Khanna, 2017). Treatment options involve mainly the use of non-specific immunosuppressive agents such as high-dose corticosteroids or cyclophosphamide (alkylating agent), as well as targeted therapies such as rituximab (anti-CD20) and anti-TNFα (infliximab and adalimumab). The increased risk of infectious disease upon immunosuppressive therapies is well documented (Lode and Schmidt-Ioanas, 2005; Barber and Clarke, 2020; Mitratza et al., 2021), but the impact of autoimmune diseases and their therapies on COVID-19 disease course remains debated (Kastritis et al., 2020; Murtas et al., 2020; Pablos et al., 2020; Zen et al., 2020; Zhong et al., 2020; Liu et al., 2021b).

STUDY POPULATION

Our literature review found 44 patients with prolonged SARS-CoV-2 infection affected with secondary immunodeficiency, summarized in **Table 1**. A more detailed version of **Table 1** is attached in **Supplementary Table 1**. These patients were described in papers found using the two search engines: PubMed and Google Scholar with key phrases "SARS-CoV-2 chronic infection," "SARS-CoV-2 evolution immunocompromised," and "SARS-CoV-2 evolution immunodeficiency" queried until May 08 2022.

Among this population, 27 patients were affected with cancer, one with cholangiocarcinoma (P27) and 26 with hematopoietic malignancies. These encompass chronic lymphocytic leukemias (P1–6), acute leukemias (P7–12), lymphomas (P13–25), and multiple myeloma (P26). All these patients presented with a humoral deficiency, either due to the initial pathology or received treatments that combine anti-CD20 monoclonal antibodies that deplete B cells. This wide spectrum of antibody and chemotherapy regimens, including, for example, bendamustine or cyclophosphamide, has a broad effect on innate and adaptive immune responses. Four patients (P28–P31) were solid organ recipients with drug-induced immunosuppression designed to prevent graft rejection using a wide spectrum of immunosuppressors, such as mycophenolate mofetil, tacrolimus, cyclosporine, azathioprine, and steroids. Five patients (P32–P36) were HIV-infected individuals with $CD4^+$ T cells impairment due to the viral infection. Two patients (P37 and P38) were affected with autoimmunity: one with antiphospholipid syndrome and one with ANCA-associated vasculitis. Finally, six patients (P39–P44) were immunocompromised by other associated comorbidities such as diabetes, chronic heart, or

TABLE 1 | Secondary immunodeficient patient population.

Patient data				Timeline and outcome		Anti-Spike mAb			Antivirals		Ig and plasma			References
N°	Age	Sex	Medical conditions	End-point (days)	Outcome (cause of death if not COVID)	BAM	ETE	CAS – IMD	REM	L-R	IV Ig	CP	HP	Study
P1	71	F	CLL	105	R						x	x		Avanzato et al., 2020
P2	75	M	CLL	197	R				x			x		Monrad et al., 2021
P3	late 60s	M	CLL	91	R	x								Jensen et al., 2021
P4	72	M	CLL	61	R	x			x			x		Truffot et al., 2021
P5	76	F	CLL	72	R				x			x		Martinot et al., 2021
P6	68	M	CLL	43	R	x			x		x	x		Bronstein et al., 2021
P7	23	M	ALL	410	R			x	x			x		Bailly et al., 2021
P8	3	F	ALL	91	R									Truong et al., 2021
P9	21	M	ALL	45	R				x			x		Truong et al., 2021
P10	2	M	ALL	51	R				x					Truong et al., 2021
P11	21	F	ALL	98	D				x					Leung et al., 2022
P12	55	F	AML	42	R	x			x					Lohr et al., 2021
P13	Early 60s	M	FL	103	R	x						x		Jensen et al., 2021
P14	52	M	FL	194	D				x	x			x	Pérez-Lago et al., 2021
P15	47	M	FL	120	R				x	x	x		x	Pérez-Lago et al., 2021
P16	63	F	FL	69	D				x	x	x		x	Pérez-Lago et al., 2021
P17	52	F	FL	100	R						x			Lynch et al., 2021
P18	Unkn	F	FL	165	R									Mancon et al., 2022
P19	61	F	DLBCL	58	R				x					Borges et al., 2021
P20	48	F	DLBCL	335	R				x		x	x		Nussenblatt et al., 2022
P21	70	F	NHL	292	R			x	x		x			Gandhi et al., 2022
P22	70	M	MBCL	102	D				x			x		Kemp et al., 2021
P23	60	M	MCL	39	R				x			x		Baang et al., 2021
P24	33	M	HL	45	R but still PCR +	x								Bronstein et al., 2021
P25	63	F	CTCL	40	R but still PCR +	x	x							Guigon et al., 2021
P26	73	M	Multiple myeloma	74	D				x			x		Hensley et al., 2021
P27	73	M	Cholangio-carcinoma	21	D	x	x							Focosi et al., 2021b
P28	Early 50s	M	Kidney transplant	64	R							x		Chen L. et al., 2021
P29	Late 60s	M	Heart transplant	40	R	x								Jensen et al., 2021
P30	Mid 60s	F	Kidney transplant	26	R	x						x		Jensen et al., 2021
P31	58	M	Kidney transplant	189	R				x					Weigang et al., 2021
P32	Early 40s	F	AIDS (HIV-Toxo)	32	R	x			x			x		Jensen et al., 2021
P33	66	M	AIDS (HIV-LEMP)	Unkn.	Unkn – probable D (LEMP)									Tarhini et al., 2021
P34	28	M	AIDS (HIV-P. jiroveci-M. avium)	103	R									Álvarez et al., 2022
P35	Late 30s	F	HIV	216	R but still PCR +									Cele et al., 2022
P36	61	F	HIV	93	R									Hoffman et al., 2021
P37	45	M	APL syndrome	154	D			x	x		x			Choi et al., 2020
P38	Early 70s	M	AAV	20	D	x						x		Jensen et al., 2021
P39	87	M	PAOD, Diabetes, HBP, CHD, CKD	27	R	x								Peiffer-Smadja et al., 2021
P40	35	M	Diabetes, HBP, CKD, RVD, JIA	38	R	x								Peiffer-Smadja et al., 2021
P41	61	M	Stroke, PAOD, Diabetes, HBP, CHD, CKD	18	R	x								Peiffer-Smadja et al., 2021
P42	97	M	Dementia, HBP and Diabetes	37	D (decubitus complications)	x								Peiffer-Smadja et al., 2021
P43	64	M	Stroke, Diabetes, HBP, CHD (heart transplant)	48	R	x								Peiffer-Smadja et al., 2021
P44	66	M	Stroke, Diabetes, HBP, RA, CKD (kidney transplant)	45–50	D	x								Peiffer-Smadja et al., 2021

x, Treatment used; mAb, monoclonal antibody; N°, patient number; F, female; M, male; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; FL, follicular lymphoma; DLBCL, diffuse large B cell lymphoma; MBCL, marginal B cell lymphoma; MCL, mantle cell lymphoma; NHL, non-HL; HL, Hodgkin's lymphoma; CTCL, cutaneous T cell lymphoma; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; LEMP, progressive multifocal leukoencephalopathy; Toxo, toxoplasmosis; P. jiroveci, Pneumocystis jiroveci; M. avium, Mycobacterium avium; APL, antiphospholipid; AAV, ANCA (anti-neutrophil cytoplasmic antibodies) associated vasculitis; PAOD, peripheral arterial occlusive disease; HBP, high blood pressure; CHD, coronary heart disease; CKD, chronic kidney disease; RVD, restrictive ventilatory disorder; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis; Unkn, unknown; R, recovery; D, death; diag, diagnosis; BAM, bamlanivimab; ETE, etesevimab; CAS-IMD, casirivimab – imdevimab; REM, remdesivir; Lopi-Rito, lopinavir-ritonavir; IV Ig, intravenous immunoglobulins; CP, convalescent plasma; HP, hyperimmune plasma. "Endpoint (days)" refers to the time frame between the earliest positive sample of the patient that confirms the diagnosis and the most recent sample available before recovery, death, or discharge.

kidney disease. Of note, two of these patients (P40 and P44) were affected by autoimmune diseases (juvenile idiopathic arthritis and rheumatoid arthritis, respectively). (No information on an eventual immunosuppressive regimen was recorded for those two patients).

Among the 44 patients, 38 received treatment directly targeting the virus (associated or not to treatment for the cytokine storm or host-based therapies for immunomodulation). Notably, twenty-one (21) patients received anti-spike monoclonal antibodies (mAb) that target the receptor-binding domain (RBD) of the S protein (Kumar S. et al., 2021). Three patients (P7, P21, and P37) received an association of 2 mAb, casirivimab–imdevimab (Regeneron/Roche REGEN-COV/Ronapreve®), and 18 patients received bamlanivimab (16 in monotherapy, two in association with etesevimab, both from Lilly).

Of note, 21 patients received direct antiviral treatments; 21 patients were associated with remdesivir (an adenosine analog that directly inhibits the viral RNA polymerase, Gilead), and 3 patients were associated with lopinavir-ritonavir (HIV-1 protease inhibitors that bind to the catalytic site of the protease and impair virion production). Sixteen patients received convalescent plasma (CP), three patients in association with polyvalent immunoglobulins. Eight patients received polyvalent immunoglobulins intravenously [three patients with CP as mentioned, two patients with hyperimmune plasma (HP), and three patients in monotherapies]. Three patients received hyperimmune plasma (HP), two with IV immunoglobulins and one in monotherapy.

All these therapeutic data are summarized in **Table 1**, and previous immunosuppressive regimen, cytokine storm treatment, and host-based therapies are described in **Supplementary Table 1**.

Furthermore, viral genomes of patients were analyzed for predicted novel amino acid changes found in the different proteins. We defined novel amino acid changes as those detected as different from the earliest viral sequence obtained from the patient at admission. The underlying variations discussed are based on recurrence and/or structural significance, if reported in other studies. To have a common nomenclature for the corresponding amino acid changes, the ORF1ab polyprotein amino acid changes provided in some papers were converted to the corresponding Nsps. First, the amino acid sequence of the Nsp of interest was aligned with the sequence of polyprotein 1ab using BLAST. Second, the corresponding position of the amino acid change was determined and manually annotated. When only nucleotide sequences were available, the nucleotide changes in the different codons of different genes were manually determined. When our compiled studies showed amino acid changes with respect to the ORF1b gene only, we added nine amino acids to the position of the change to account for the ribosomal slippage in which the first nine amino acids are read in ORF1a and the following amino acids in the ORF1b (Bhatt et al., 2021).

In addition, in all patients examined (**Table 1**), the number of amino acid changes occurring in each SARS-CoV-2 protein reported by studies that performed full-length genome sequencing of SARS-CoV-2 was aggregated and divided by the number of amino acids per respective protein to generate a variation frequency and allow us to better visualize which proteins are the most changed (**Figure 3**). The full list of these variations is shown in **Supplementary Table 2**. To compare with the total number of SARS-CoV-2 sequenced viruses, the percentage of each amino acid change was calculated from the GISAID database as of May 18 2022 (using 10,900,892 as the total number of deposited sequences, and shown in **Supplementary Table 3**). The subsequent sections focus on the proteins with the most substitutions (in red-dark yellow heat map color on **Figure 3**) or substitutions with functional significance.

From these data, it could be deduced that most variations occur in the spike protein, more particularly in the receptor-binding motif (RBM) and the NTD. Of note, the E protein has the second highest frequency, followed by Nsp12, Nsp1, Nsp8, ORF7a, and M. Variations in these proteins and others that are either recurrent and/or with functional significance are discussed in the following paragraphs.

SPIKE PROTEIN VARIATIONS

Spike proteins had the highest number of amino acid changes, mainly in the RBM and in the NTD. Interestingly, the emergence of the E484K amino acid substitution (AAS) was observed in 43% of the patients studied (P3, P4, P12, P13, P28, P29, P32, P35, P37, P38, P39, P40, P41, P42, and P43). Secondary immunodeficient patients treated with bamlanivimab showcased a potential example of antiviral-induced selective pressure. Indeed, in April 2021, the emergency authorization use license of bamlanivimab monotherapy was revoked in the United States due to concerns about inefficiencies, as most circulating variants, especially the dominant delta variant, were resistant to neutralization (Gottlieb et al., 2021). Soon after, in January 2022, combined therapy of bamlanivimab and etesivimab, as well as casirivimab and imdevimab, was discouraged in the United States due to less neutralization activity against the now dominant omicron variant (Cavazzoni, 2022). All variations detected in SARS-CoV-2 spike proteins in the 44 patients are shown in **Figure 4**.

VARIATIONS IN THE RECEPTOR-BINDING MOTIF

The spike RBM is a 69 amino acid motif (aa 438–506) involved in binding to the host cell receptor. From the selected patients, a total of 19 amino acid changes were found, with the most frequent change being the E484K substitution which was noted in 15 out of 44 patients (P3, P4, P12, P13, P28, P29, P32, P35, P37, P38, P39, P40, P41, P42, and P43). From these patients, 13 out of these 15 (29.5% of total patients) had received monoclonal antibody therapy, and of those, 12 (27% of total patients) were treated with bamlanivimab (**Table 1**). Furthermore, other variations at the same amino acid position (484) have also been reported. This includes the E484G, E484A, and E484Q substitutions that occurred in 1/44 (P31 – 2.3% of total patients), 2/44 (P37 and P40 – 4.5% of total patients), and 5/44 (P4, P6, P10, P36, and P38 – 11.4% of total patients) patients,

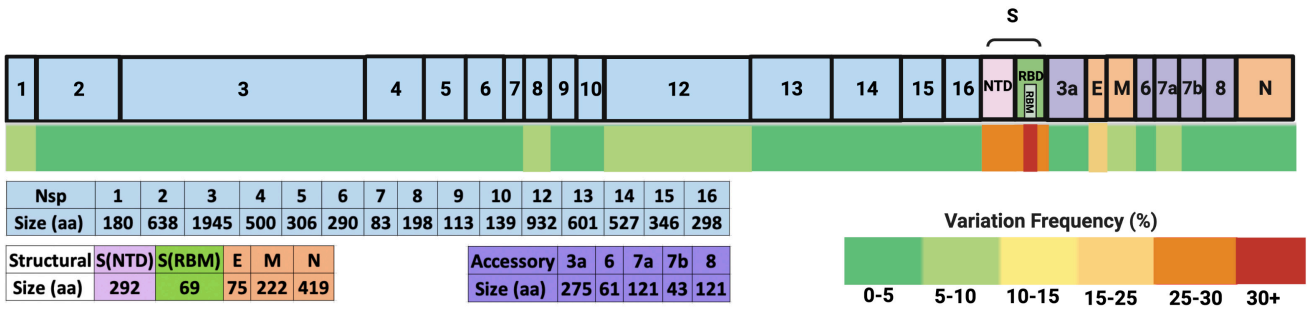

FIGURE 3 | Frequency of amino acid variation in SARS-CoV-2 proteins from the analyzed secondary immunodeficient patients. The SARS-CoV-2 proteins are depicted on top, where the light blue boxes represent non-structural proteins (Nsps) generated from polyprotein 1ab (pp1ab). The NTD and RBM spike domains are shown as pink and green boxes, and other structural proteins are depicted in orange boxes (E, envelope; M, membrane; N, nucleocapsid). The accessory proteins are shown in purple. The total number of amino acids of each protein is depicted below. The heat map scale is shown on the bottom right. The changes shown were isolated from a total of 148 full-length SARS-CoV-2 genomic sequences from 21 patients (shown in S2).

respectively. For E484Q, 3 of 5 patients (6.8% of total patients) with this substitution received bamlanivimab, and both patients (4.5% of total patients) with E484A had received casirivimab and bamlanivimab, respectively, but P31 did not receive any monoclonal therapy. In recent studies, Jangra et al. (2021) showed that recombinant SARS-CoV-2 virus harboring the E484K AAS reduced *in vitro* antibody neutralization of human convalescent and post-vaccination sera relative to control virus without this variation. This result was also confirmed by Collier et al. (2021), showing loss of neutralizing activity by vaccine-elicited antibodies and monoclonal antibodies. *In silico* results by Wang et al. (2021) predicted that this AAS could result in favorable electrostatic interactions and tighter binding with the ACE2 receptor. In combination with another change not found in these patients (L452R), it has also been shown that a pseudotyped virus with the E484Q substitution resulted in a reduced neutralization of immune sera from vaccinated (against RBD) non-human primates, convalescent COVID-19 patients, as well as double-dose vaccinated individuals (also against RBD) (Li G. et al., 2021). This result was also confirmed by Ferreira et al. (2021) who also reported a decreased neutralization of pseudotyped virus with both E484Q and L452R alone or in combination using sera of vaccinated individuals. The AAS E484K is observed in beta, gamma, omicron, and mu variants, and E484A is found in the omicron variant (Hodcroft, 2021). Additionally, the 484 residue is in the proximity of suspected bamlanivimab- and casirivimab-binding sites (**Figure 5**), suggesting that these antibody therapies may have exerted selection pressure.

Another common variant observed in these secondary immunodeficient patients was the Q493R substitution, present in 6/44 (13.6% of total patients) patients (P4, P12, P25, P27, P28, and P44). All of them, except for P28, received bamlanivimab treatment. In combination with bamlanivimab, P25 and P27 also received etesivimab treatment, which binds the RBM in close proximity to this residue (Q493) (**Figure 5**). At the same position (493), the Q493K substitution was also reported in P28 as well as P37, where the latter had received casirivimab/imdevimab in combination (Choi et al., 2020). In terms of their abundance in the total number of SARS-CoV-2 sequences in GISAID, Q493R and Q493K have a frequency of 30.9 and 0.0079% respectively, suggesting an increased representation of the variation in secondary immunodeficient patients (Elbe and Buckland-Merrett, 2017). Clark et al. (2021) showed that SARS-CoV-2 spike pseudotyped viruses harboring the Q493K substitutions significantly decreased the neutralization of the REGN10933 (Casirivimab) and C1A-VH3-53 antibodies. This decreased neutralization was also noted in the case of Q493R pseudotyped virus (Clark et al., 2021). Similarly, a study by Starr Tyler et al. (2021) also investigated the potential of antibody escape mutations and demonstrated the escape of Q493K from the REGN10987 (imdevimab). Another nearby AAS is S494P, which has been observed in 4 patients (P11, P12, P37, and P39 – 9.1% of total patients), and is in 0.15% of all SARS-CoV-2 sequences in GISAID. This change has also led to the neutralization reduction in antibodies from convalescent and post-vaccinated sera, especially in combination with E484K and N501Y substitutions (Alenquer et al., 2021). These data suggest that Q493K and Q493R AAS could contribute to SARS-CoV-2 resistance to anti-spike monoclonal antibodies in immunodeficient patients.

Moreover, the N501Y AAS was also identified to emerge *de novo* in five patients (P4, P7, P22, P35, and P37), three of whom were treated with monoclonals (P4: bamlanivimab; P7 and P37: casirivimab/imdevimab). This AAS seems to be near the casirivimab-binding site to RBM (**Figure 5**). Furthermore, N501Y has also been determined to be present in alpha, beta, gamma, omicron, and mu variants. Having this AAS increases the binding affinity of spike proteins to human ACE2 as shown by Tian et al. (2021) and Liu et al. (2021a). Moreover, it has been shown that this substitution decreases neutralization by both H00S022 and 10F9 neutralizing monoclonal antibodies and increases the infectivity of pseudotyped virus by 5-fold compared with the 614G variant in HEK293T cells expressing mouse ACE2 (Li Q. et al., 2021). Interestingly, Niu et al. (2021) also noted that pseudotyped virus with the N501Y change effectively infected mouse-ACE2 expressing 293T cells and detected a successful infection of wild-type BALB/c with a SARS-CoV-2 strain bearing the substitution. A recent study by Liu et al. (2021a) reported

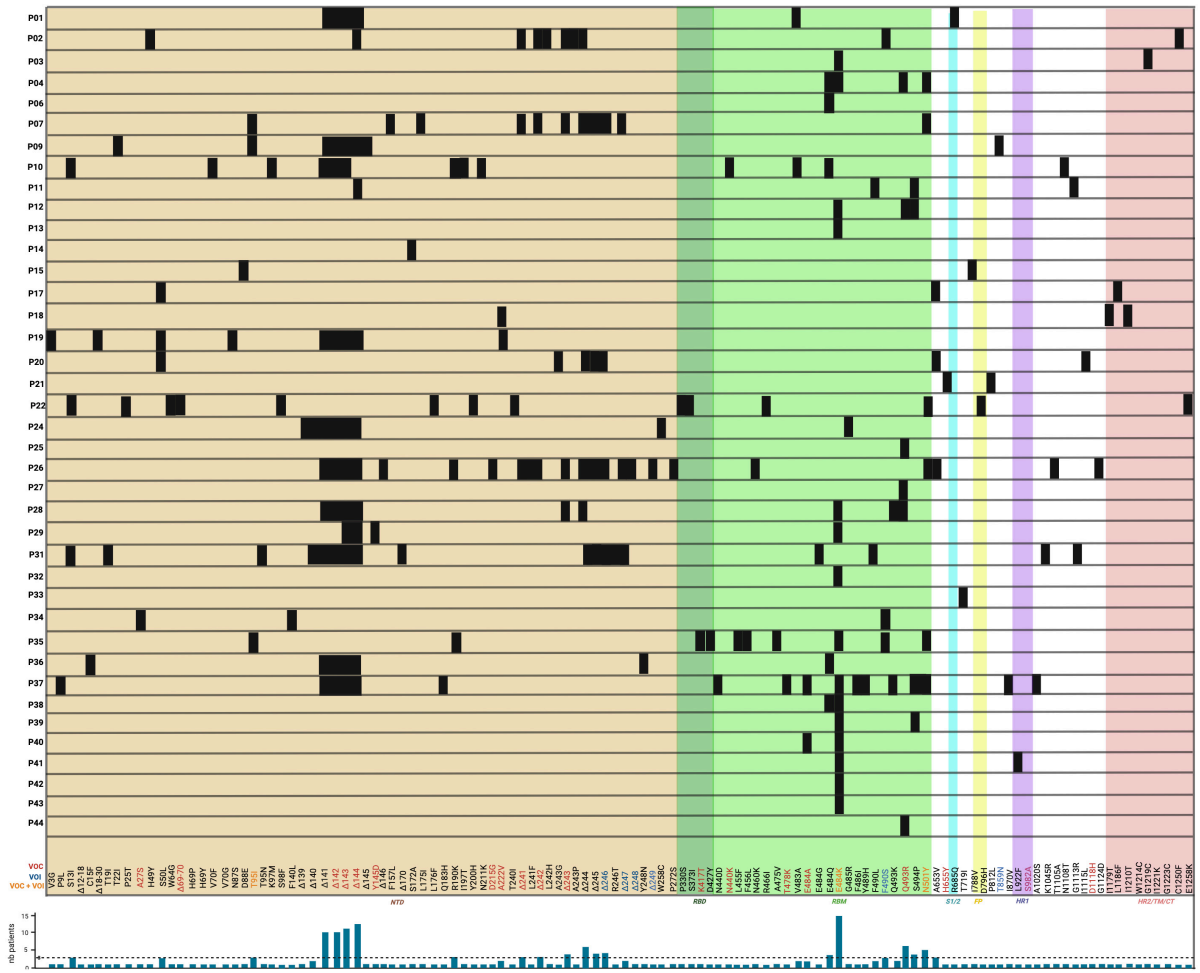

FIGURE 4 | Location of novel SARS-CoV-2 amino acid changes in the spike protein emerging in some immunocompromised patients during chronic infection. The variations are shown as black boxes and represent either amino acid substitutions or deletion with their corresponding identity at the bottom. The different colored areas around the boxes are representative of the spike protein domains corresponding to those shown in **Figure 1B**: the brown area corresponds to the NTD; the dark green corresponds to the RBD; the pale green corresponds to the RBM of the RBD; the cyan corresponds to the S1/2; the yellow corresponds to the FP; the purple corresponds to the HR1, and the pale red corresponds to either HR2, TM, or CT domains. The amino acid change commonly found only in variants of concern (VOC) are in red font; those only in variants of interest (VOI) are in blue and those found in both VOC and VOI are in orange. Patients P5, P8, P16, P23, P30 did not present any novel amino acid changes in the spike protein and thus are not shown. A histogram depicts the occurrence of variations in the number of patients (shown on *y*-axis as "nb patients") with the threshold of selection of three patients depicted by a dashed line.

that *in vivo*, this substitution enhanced viral fitness in intranasally infected hamsters and intra-cage transmissions. This suggests that N501Y may play a role in a potential viral spillover to mice (Huang et al., 2021).

Besides the aforementioned, other RBM changes were also found in the reviewed population, including the N440K (P10), T478K (P37), and F490S (P2, P34, and P35), which are found in the omicron, delta, and lambda variants, respectively. The N440K variant has been reported to evade the REGN10987 antibody (Starr Tyler et al., 2021), while F490S has been shown to allow resistance to vaccine-elicited sera (Kimura et al., 2021). Furthermore, among millions of GISAID sequences, the frequency of F490S, N440K, and T478K AASs is 0.17, 25.9, and 70.9%, respectively. Other AASs have also been identified in casirivimab/imdevimab receiving patients. In **Figure 5**, we showed the molecular structures and positions of the monoclonal antibodies and the AASs identified in the RBM of the spike. These findings suggest that either therapy (vaccine or antibody therapy) or convergent evolution explains their emergence.

(N)-TERMINAL DOMAIN AND OTHER SPIKE VARIATIONS

Besides the RBM, spike amino acid changes were identified in other domains, especially in the 292 amino acid NTDs (aa 14–305). Among the NTD variations reported, the most frequent ones occurred in the range of residues between amino acid positions 139 and 146 and this occurred in 13 out of 44 patients (29.5% of the total patients, P1, P2, P9, P10, P11, P19, P24,

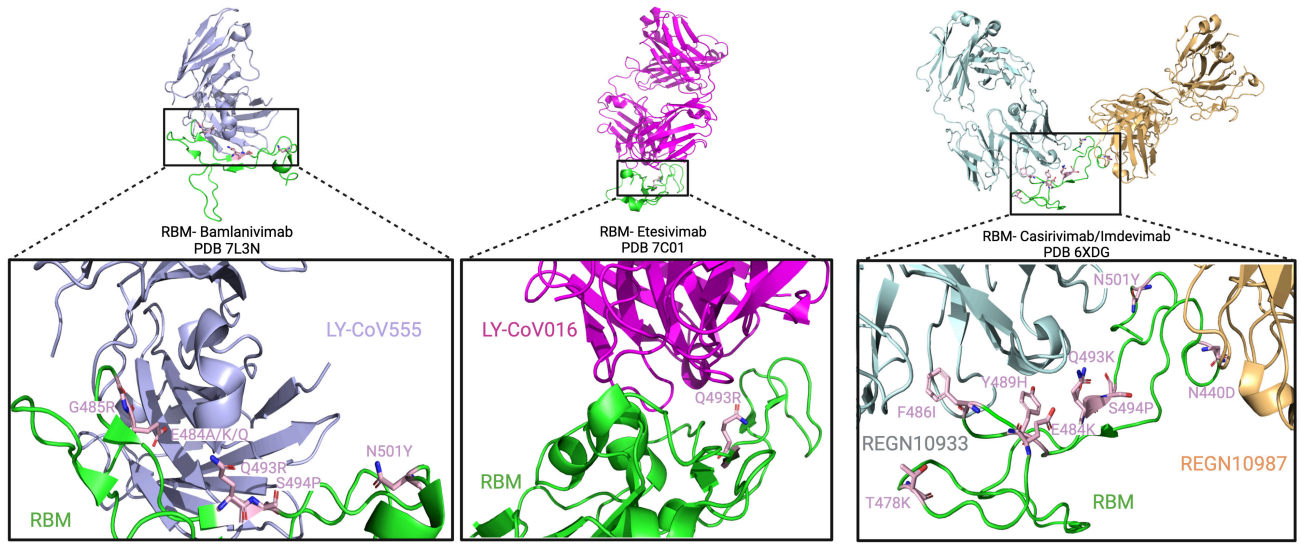

FIGURE 5 | Spike RBM amino acid substitutions in patients treated with monoclonal antibodies. The interaction between the RBM and bamlanivimab, etesivimab, and casirivimab/imdevimab (light blue, pink, gray/yellow) is depicted on top from right to left, respectively. Below, a close-up view of the RBM/antibody interaction is shown with substitutions shown in light pink.

P26, P28, P29, P31, P36, and P37). The second most common variant was a deletion occurring in a range of residues between amino acid positions 241 and 249 in 6 out of 44 patients (13.6% of total patients, P2, P7, P20, P26, P28, P31). In terms of substitutions, S50L (P17, P19, and P20), T95I (P7, P9, and P35), and R190K (P10, P26, and P35) were identified in 3 patients (6.8%). Moreover, other deletions were also reported at position 69–70 (Δ69–70) for P22, and both patients P19 and P37 had deletions from aa 18 to 30 (Δ18–30) and 12 to 18 (Δ12–18), respectively. Several of these amino acid changes were also present in VOCs and/or VOIs: a spike deletion at position 141 is present in the alpha variant, and the ones spanning from position 142–144 (Δ142–144) are in the omicron variant. Furthermore, the beta variant contains deletions from residue 241 to 243 (Δ241–243), whereas the lambda variant has a deletion from position 246 to 249 (Δ246–249). Both alpha and omicron variants contain the Δ69–70 deletion, and the lambda variant contains the T19I substitution (Hodcroft, 2021). Functionally, Mccarthy et al. (2021) showed that the combination of deletions (Δ69–70 and Δ 144/145), (Δ141–144, Δ144/145, and Δ146), and Δ243–244 all abolished binding to the 4A8 neutralizing antibody, indicating these regions in the NTD to be possible immunodominant epitopes for neutralization. Such an effect was also tested by Graham et al. (2021) where a significant reduction of neutralization by NTD targeting antibodies was also noted in pseudoviruses with the Δ141 spike deletion in combination with the D614G substitution. Furthermore, the researchers studying the chronic infection of P19 showed that double deletion Δ69–70, and another substitution reported in the spike fusion peptide D796H decreased the sensitivity to convalescent plasma *in vitro* (Kemp et al., 2021). It was also revealed that the Δ69–70 deletion had higher infectivity than a wild-type SARS-CoV-2 and that D796H was the main contributor to escaping neutralization while showing reduced infectivity (Kemp et al., 2021). The mutations seen in the NTD are functional mutations that overlap domains and have been observed in both VOCs and VOIs, and thus are worthy of significant focused interest for surveillance of future variants with altered biology. Besides the NTD and RBD, other amino acid changes occur in the spike such as the recurrent S13I in three patients (7.5% of total patients, P10, P22, and P31), the T859N (P9), and D1118H (P31) substitutions (2.5% of total patients). The T859N is also found in the lambda variant, and the D1118H is found in the alpha variant. The significance of these changes has not yet been determined. In sum, these results encompass the most recurrent amino acid changes observed in the spike protein of secondary immunodeficient patients described in the literature.

VARIATIONS IN NON-SPIKE PROTEINS

Envelope

The SARS-CoV-2 envelope (E) is a 75 amino acid hydrophobic transmembrane protein that is crucial for infecting host cells (Boson et al., 2021). It is composed of three domains including the N-terminal domain (NTD; aa 1–8), transmembrane domain (TM; aa 9–38), and the C-terminal domain (CTD; aa 39–75) (Mandala et al., 2020). In other coronaviruses, it is thought that the TM acts as an ion channel and that the CTD interacts with other proteins like cellular adapters (Schoeman and Fielding, 2019). From our analysis of patients, all five AASs in the E protein were located in the TM and the CTD. From those, 9 out of 44 reviewed patients (20.4% of total patients) presented the T30I AAS (P10, P11, P15, P17, P18, P19, P20, P22, and P37) which is found in the transmembrane domain of this protein.

A search of the 10,900,892 SARS-CoV-2 sequences recorded by GISAID, as of May 18 2022, indicated that this very rare variation is only found in 1,156 sequences (0.011%) (Elbe and Buckland-Merrett, 2017). Using FoldX, one study predicted that this change could be a stabilizing substitution (Rahman et al., 2021). To gain more insight on its structural effects, we modeled the T30I AAS into previously determined NMR structures of the SARS-CoV-1 (PDB: 5X29) and SARS-CoV-2 (PDB: 7K3G) envelope protein (**Figure 6**). Despite sequence similarity, there are notable differences between the structures, among them the positions of residue 30. In the 5X29 structure, Thr30 is in an interhelical position, whereas this residue is in a lipid-facing position in the 7K3G structure. It is unclear if these variations result from differing experimental techniques or simply plasticity of the protein complex. Due to the ambiguous position of Thr30, we additionally generated models using DeepMind AlphaFold 2. However, these models also suffered from inconsistent Thr30 positions, and thus, the precise position of this residue is uncertain. Nonetheless, in both the interhelical and lipid-facing positions, the T30I AAS increases the hydrophobicity of the transmembrane domain. The substitution from threonine, a hydrophilic amino acid, to isoleucine, a hydrophobic amino acid, would likely have a stabilizing effect, as the surrounding residues and lipid environment are also hydrophobic. Although the function of this precise change in SARS-CoV-2 is unknown, Nieto-Torres et al. (2014) have investigated the ion channel activity of the E protein in *in vitro* and *in vivo* pathogenesis of SARS-CoV; interestingly, they observed a lesser disease severity in mice infected with viruses lacking ion-channel (IC) activity, as opposed to those infected with viruses lacking IC activity with the T30I AAS, suggesting an impact on the presentation of the SARS-CoV-2 pathogenesis. The E protein can be sensed by TLR2-dependent host cell signaling to produce proinflammatory cytokines (Tasakis et al., 2021), suggesting that variations may have multiple effects on ion conductivity, pathogenesis, and inflammation.

Besides T30I, five other AASs in the E protein were reported in the studied population, including the N48D and S50I (P10), T9I (P18), L19I (18), and L21F (P23) but as of yet, none has been reported to have an impact on the TM and CTD domains. Furthermore, as of May 18 2022, the only variation detected in the TM of E is the T9I substitution, with a variation frequency of 33.5% in 10,900,892 GISAID sequences, present in VOCs (Hodcroft, 2021). From our analysis, it could be speculated that the T30I AAS could perhaps be selected in immunodeficient settings, but more research on SARS-CoV-2 E AASs is needed to clarify whether this is the case.

Membrane Protein

The coronaviral membrane (M) is a 222 amino acid protein known to play a role in virion assembly and morphogenesis, among other processes (Liu et al., 2007; Jörrißen et al., 2021). In studied patients, the most common AAS identified was the H125Y, in five patients (11.4% – P10, P17, P20, P22, and P33). In addition, other AASs such as the A2S AAS were identified in 2 patients (4.5% – P1, P37). Yet, no functional impact has been reported from the literature, and the variation frequency of both H125Y and A2S in the total GISAID sequences is 0.11 and 0.24%, respectively. However, some defining AASs in the studied immunodeficient patients have been noted in VOCs, particularly in the delta (A82T) and omicron variants (D3G, Q19E, and 63T) (Hodcroft, 2021).

Nsp1

Nsp1 coronavirus proteins are known to shut down host protein translation to inhibit the expression of key genes involved in viral control (Nakagawa and Makino, 2021). As an 180 amino acid protein, it has been predicted to be made of an NTD (aa 1–128), a linker (aa 129–148), and a CTD domain (aa 149–180) (Schubert et al., 2020). The CTD has been shown to inhibit cellular gene expression by binding to the 40S ribosomal entry channel, whereas the NTD allows SARS-CoV-2 mRNA to escape this inhibition by binding to its leader sequence and stabilizing its interaction with the ribosome (Mendez et al., 2021).

In the analyzed secondary immunodeficient patients, several AASs and deletions were detected in the Nsp1 protein in patient viruses whose genomes were fully sequenced (P1, P2, P9, P10, P16, P17, P19, P21, P22, P28, and P31). From those, there were recurrent changes in the NTD including the amino acid deletion at position 85, which occurred in 2 (4.5%) patients (P1 and P31), and the AASs R124C (P10 and P22) and I114T (P2 and P19), which were also detected in two patients, respectively. The M85 deletion, R124C, and I114T substitutions are, respectively, present in 1.78, 0.14, and 0.05% of total GISAID sequences.

Structurally, the Nsp1 deletion at amino acid 85 has been previously shown to lead to a lower type I interferon response in infected Calu-3 cells, in contrast to wild-type Nsp1 (Lin et al., 2021). In the case of the AAS R124C, Mou et al. (2021) previously predicted *in silico* that this SNP has a destabilizing effect on the Nsp1 protein structure. Later, an *in vitro* study by Mendez et al. (2021) found that the R124A amino acid change at the same position along with the K125A AAS promotes host RNA decay, reduces host mRNA translation levels by destabilizing the binding to the 40S ribosomal subunit, and reduces the repression of SARS-CoV-2 leader containing transcripts. Moreover, Kim et al. (2021) also showed that the R124A/K125A changes did not have any effect on the levels of caspase-1 proteins *in vitro*, in contrast to the wild-type Nsp1, which significantly reduced caspase-1 levels and blocked its cleavage.

In summary, we identified recurrent Nsp1 variations in the NTD domain that could be involved in interfering with the host defenses. It would be of interest to investigate if there is a selection of Nsp1 NTD variations in immunodeficient individuals.

Nsp3

Nsp3 is the largest multi-domain coronaviral protein with a total of 1,945 amino acids (Lei et al., 2018; Gruca et al., 2021). It is involved in the proteolytic cleavage of polyproteins pp1a and pp1ab and the removal of K18-linked polyubiquitin and interferon-stimulated gene 15 (ISG15) from cellular proteins (Lei et al., 2018; Armstrong et al., 2021). From its many domains, its protease activity is conferred by the papain-like protease domain (aa 813–1076) (Armstrong et al., 2021). In this domain, two recurrent AASs were identified in the studied patients including T820I (4.5% of total patients, P11 and P23) and P822L (4.5%, P15 and P23). These occur with a frequency of 0.04 and 3.8% in the

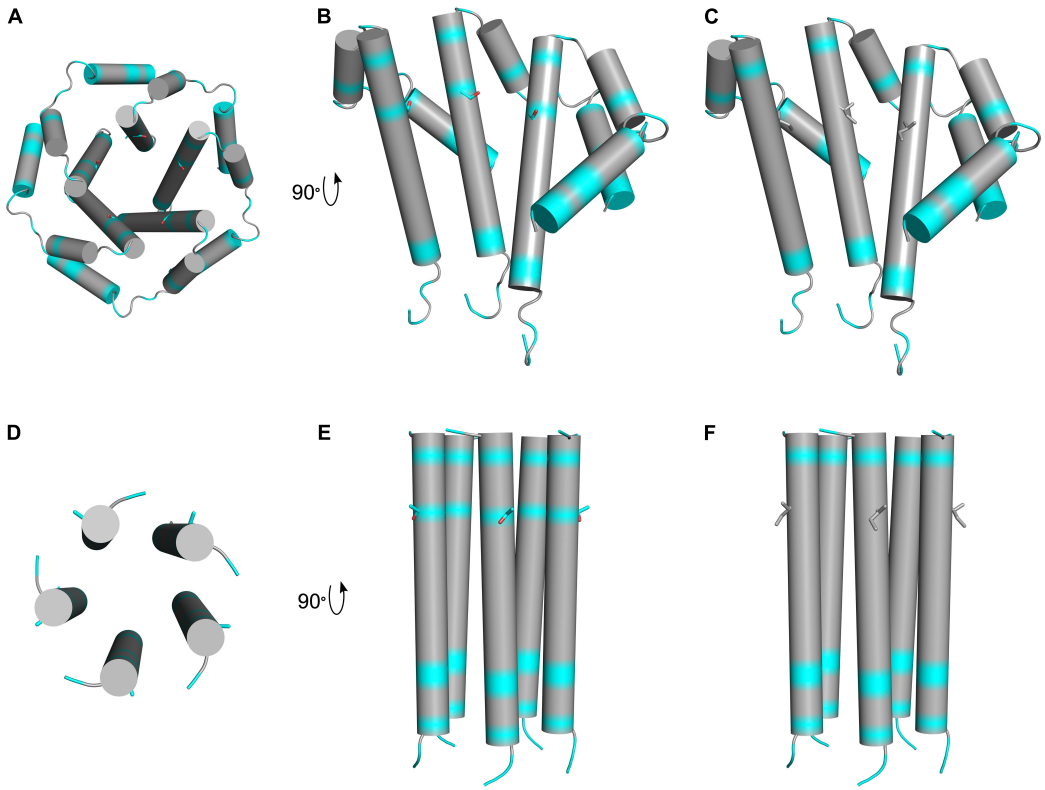

FIGURE 6 | Schematic presentation of hydrophobic regions of T30I mutant E protein. The top row depicts LMPG micelle solution NMR structures of truncated SARS-CoV-1 E protein residues 8–65 in homopentameric channel complex (PDB: 5 × 29). Hydrophobic residues are colored in gray, and hydrophilic residues are colored in cyan. Oxygen atoms are colored in red. **(A)** Schematic view of the pore formed by the wild-type protein complex showing threonine 30 side chains. **(B)** Three subunits of the wild-type complex showing threonine 30 side chains. **(C)** Three subunits of the T30I mutant complex showing isoleucine 30 side chains. The bottom row depicts solid-state NMR structures of SARS-CoV-2 E protein transmembrane domain in homopentameric channel complex (PDB: 7K3G). Hydrophobic residues are colored in gray, and hydrophilic residues are colored in cyan. Oxygen atoms are colored in red. **(D)** View of the pore formed by the wild-type protein complex showing threonine 30 side chains. **(E)** Wild-type complex showing threonine 30 side chains. **(F)** T30I mutant complex showing isoleucine 30 side chains.

total GISAID sequences, and their impact on the role of Nsp3 is not yet studied. Other changes were also identified (S2), with the P1228L AAS (P7) being present in 35.6% of total GISAID sequences. This falls in the α-helical loop (aa 1,177–1,333), which is not yet well characterized. Therefore, it would be of great interest to investigate the role of the identified variations in Nsp3 and their possible impact on its catalytic activity.

Nsp6

Nsp6 is a transmembrane protein that is not very well characterized in SARS-CoV-2 infection (Kumar A. et al., 2021). Sun et al. (2022) showed that this protein can target the ATPase proton pump component involved in lysosomal acidification, ATP6AP1, to trigger NLRP3-dependent pyroptosis in lung epithelial cells. In the reviewed patients, the L37F protein-coding change was noted to emerge *de novo* in three different cases (11.4% of total patients, P10, P17, P20, P21, P31), and this AAS is part of around 2.0% of GISAID sequences (Lynch et al., 2021; Truong et al., 2021; Weigang et al., 2021). Recently, Benvenuto et al. (2020) predicted that such an amino acid change led to a lower stability of the Nsp6 protein structure and suggested a role of Nsp6 in binding with the ER. This AAS was also studied by Wang R. et al. (2020) who analyzed around 76,000 sequences in GISAID up to 19 October 2020, and correlated it with lower death ratios and transmission rates. Through bioinformatic analysis, it was also shown to be destabilizing and less functional compared with the wild-type (Wang R. et al., 2020). Furthermore, this same AAS has also been observed to weaken the interaction of Nsp6 with ATP6AP1, thus reducing lysosome acidification and pyroptosis induction (Sun et al., 2022). These observations stress the importance of the L37F AAS in both immunodeficient and immunocompetent individuals, given the relatively high variation rate in the reviewed patients and in GISAID, which might suggest an unappreciated fitness benefit conferred by this variation. The study of patients with chronic SARS-CoV-2 infection would be another source of data for predicting the transmissibility and lethality of SARS-CoV-2, especially in the context of immunodeficiency.

RdRp/Nsp12

The RNA-dependent RNA polymerase (RdRp) involved in SARS-CoV-2 genome replication and transcription of genes is

composed of a catalytic subunit known as Nsp12 as well as two accessory subunits, Nsp8 and Nsp7 (Gao et al., 2020b). The Nsp12 domain resembles a right hand, comprising the fingers subdomain, which interact with the template strand RNA and direct it into the active site, and the palm domain, which forms the catalytic active center. The RdRp is the target for antiviral drugs such as remdesivir (RDV and GS-5734), which can incorporate itself in the nascent viral RNA chains, causing premature transcriptional termination (Warren et al., 2016). In the study population, many RdRp variations were identified in patients who were treated with remdesivir (nine patients: 20.4%, P2, P5, P9, P14, P16, P21, P22, P23, and P31). From these, a few AASs were identified in the RdRp palm domain including V792I (5%, P16 and P22), E796D (P9), C799R (P14), and E802D, respectively. In the whole GISAID database, these occur at a frequency of less than 0.0025%. Interestingly, an *in vitro* study by Szemiel et al. (2021) showed that a palm domain substitution in a conserved residue (E802D) of the RdRp decreases the sensitivity to remdesivir and viral fitness in a competition assay; this same amino acid change (E802D) was found in P21, decreased binding to remdesivir, and has a fitness cost (Gandhi et al., 2022). As this substitution was found to be close to residues involved in binding with nascent RNA (aa 813–815), it was suggested that the RdRp could have structural changes that could allow elongation of template RNA, even when remdesivir is incorporated (Szemiel et al., 2021). Indeed, this highlights those substitutions in the RdRp finger, and palm domain should be studied more carefully to determine if these play a role in conferring resistance to antivirals against the RdRp.

Nsp13

The helicase protein (Nsp13) plays a role in unwinding duplex RNA and DNA in a 5′ to 3′ direction (Vazquez et al., 2021). In the patients we studied, the D374E substitution identified in P22 is an AAS that occurs in one of the residues identified in the NTP hydrolysis active site, and its functional effect is yet to be determined (Jia et al., 2019). Although this AAS is very rare (9/10,900,892 GISAID sequences), since this Nsp13 is one of the proteins involved in the RTC formation, it would be of interest to investigate such changes and their role in viral replication.

ORF3a

The SARS-CoV-2 ORF3a is an integral membrane protein that has been shown to play a role in inducing apoptosis (Ren et al., 2020) in infected cells, in promoting lysosomal exocytosis (Chen D. et al., 2021), and in blocking the formation of autolysosomes (Miao et al., 2021; Qu et al., 2021). It has also been shown to inhibit STAT1 phosphorylation *in vitro* (Xia et al., 2020). In the described patients, nine variations were identified including the S171L (P9, 0.69% GISAID frequency). Chen D. et al. (2021) showed that the SARS-CoV-2 ORF3a S171E AAS abolished the production of *trans*-soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex proteins involved in fusing the lysosome to plasma membranes; additionally, the amino acid substitution abolished the ability of ORF3a to increase the Ca^{2+} levels in the cytoplasm. Furthermore, the S171L substitution was predicted *in silico* to increase protein instability with a turn structure replaced by a coiled coil (Azad and Khan, 2021), suggesting that it could be a functional mutation selected in patients.

ORF7a

The ORF7a accessory protein is believed to play a role in modulating host immune responses (Redondo et al., 2021). In the case of the 44 patients described here, the AASs S81P occurred twice in P14 and P22 (4.5%), and the A105V, which occurred in P9, has been characterized before. In a 62-patient Romanian cohort, Lobiuc et al. (2021) showed that 27.5% were infected with the A105V AAS, and they experienced a twofold higher death rate than others without A105V. The researchers then did a bioinformatic analysis of this change and predicted an increased stability by allosteric effects (Lobiuc et al., 2021).

In summary, we have identified many recurrent SARS-CoV-2 amino acid changes to emerge *de novo* in immunodeficient patients in a variety of proteins that have previously been identified to have a structural effect. This could be the result of different host selection pressures as some proteins (E, Nsp1, M, and ORF7a) had a relatively higher frequency compared with others (**Figure 3**). More investigation and a bigger study population are needed to make a definitive conclusion.

DISCUSSION

Prolonged infections resulting from weakened impaired immune responses allow the virus to persist, providing opportunities for increased viral replications and accumulations of mutations, some of which may be novel (Avanzato et al., 2020; Monrad et al., 2021). Thus, as the COVID-19 pandemic continues, it is crucial to track mutations arising in circulating and novel strains that can potentially become VOCs and VOIs to help predict their role in transmission and pathogenesis.

Case studies of chronically infected individuals with immunodeficiency could help gain insights into how the virus evolves in such settings. In this review, we highlighted 44 patients with secondary immunodeficiencies that were chronically infected with SARS-CoV-2 and received a variety of treatments, some of which may exert a selective pressure on the virus (De Vlaminck et al., 2013) (e.g., antiviral drugs targeting a specific protein site). Early studies showed that treatments with monoclonal antibodies should be used with great caution as they have been demonstrated to exert selective pressures on viruses. Focosi et al. (2021a) reviewed case series and reports and observed frequent emergence of single-nucleotide changes in the RBD regions of the spike gene when under the pressure of monoclonal antibodies; additionally, they noted that the mutational pressure from convalescent plasma was different in nature with deletions being more present, presumably due to the polyclonal nature of the antibodies. Alternatively, polyclonal antibodies recognizing different spike epitopes or combination therapy could be used to reduce selection pressure and treatment resistance. Efforts are being made to design broadly neutralizing SARS-CoV-2 and pan-coronavirus antibodies, some relying on the principle of targeting conserved regions that have a high

fitness cost if altered (Cameroni et al., 2021; Martinez et al., 2021; Saunders et al., 2021; Shrestha et al., 2021; Tan et al., 2021). Such consideration and strategies when designing new therapies are needed to deliver therapeutics with longevity for the use of the current pandemic and future ones that will inevitably arise.

A careful approach is required in administering future antivirals targeting a specific site of SARS-CoV-2 proteins, especially as novel therapies such as molnupiravir, which targets the RdRp (Fischer et al., 2021), and paxlovid, which targets the SARS-CoV-2 main protease (Nsp5) by reacting reversibly with a cysteine residue at its active site (Pavan et al., 2021), are slated for approval. If these antivirals do not have a high genetic barrier for mutational escape, lengthy efforts and enormous resource commitments could be wasted on therapies that the world has already begun to hail as an end to the pandemic.

Besides the spike protein, it was interesting to find a recurrent AAS T30I in the E protein transmembrane motif in 9 out of 44 different patients (20.4% of total patients) who presented the T30I AAS (P10, P11, P15, P17, P18, P19, P20, P22, and P37). Like the latter, other variations were recurrent in the reviewed patients with a frequency greater than those found in the GISAID database. This was the case of the M protein A2S (4.5% of total patients, 0.24% of GISAID) and H125Y (11.4% of total patients, 0.11% of GISAID) AASs, as well as the Nsp1 R124C (4.5% of total patients, 0.14% of GISAID) and I114T (4.5% of total patients, 0.05% of GISAID), among others. One explanation for this difference could be that these mutations are specific to an immunodeficient environment, where certain immune selective pressures could be different, e.g., weakened. On the contrary, other AASs present in circulating VOCs were noted to emerge in the reviewed patients such as the Nsp3 P1228L (35.6% of GISAID), which could reflect the adaptation of the virus to the human host; however, the functional effect of such variations remains to be elucidated in further studies.

Although of interest, this literature review of SARS-CoV-2 variations in immunocompromised patients has limitations. Previous immunosuppressive regimens were not always known in detail, and this could lead to incomplete evaluation of the extent of immunodeficiency in the studied population. Another confounding factor is the variation in the standard of care for SARS-CoV-2 infection throughout the pandemic. Indeed, variation in treatment regimens over time makes the rise of mutations difficult to interpret, especially considering the variable time from treatment to the moment of infection. Moreover, virus sequencing is often from samples originating from the nasopharynx, which is part of the upper respiratory tract. These samples are not necessarily representative of the virus composition in the lower respiratory tract. Furthermore, the virus replicating in the lower respiratory tract may experience different selection pressures than the upper respiratory tract. In addition, the GISAID frequencies obtained are derived from uploaded consensus sequences in contrast to our patients' sequencing data that have variable consensus agreement; therefore, the AASs we note may be more noisy and non-selective compared with the GISAID AASs. GISAID frequencies can also include immunocompromised individuals although we expect these to be in the minority. Of note, in the 44 patients that we compiled, all viruses were not sequenced at the beginning of SARS-CoV-2 infection or sequenced at the same timepoint during the disease, once again leading to comparison discrepancies. In addition, some of the papers focus on spike proteins only. Finally, although we chose to focus on secondary immunodeficiencies due to the larger number of patients with analyzed viral genomes, mutations in primary immunodeficient patients (Bucciol et al., 2021; Ciuffreda et al., 2021; Cabañero-Navalon et al., 2022) are slowly being characterized that could add to the topic of viral evolution in the context of immunodeficiencies at large.

CONCLUSION

In this review, several variations were found in the spike or Nsp12 proteins, which are important therapeutic targets. We also identified several recurrent variations in E, Nsp1, M, and ORF7a proteins that may play an important role in SARS-CoV-2 pathogenesis. Determining whether these variations emerged through selection in the immunodeficient patients or resulted from the adaptation of SARS-CoV-2 to the human host will require further study. However, the breadth and impact of mutations characterized in patients with secondary immunodeficiency highlight the relevance of monitoring the evolution of SARS-CoV-2 in immunocompromised individuals, not only to identify potentially adaptive novel mutations but also to mitigate the risk of introducing variants that may pose increased health threats to communities.

AUTHOR CONTRIBUTIONS

SV and LA: conceptualization and supervision. NM, GG, DP, MH, PN, and AB: writing—original draft preparation. NM, GG, DP, MH, PN, AB, LA, and SV: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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