T.C.
YEDİTEPE UNİVERSİTY
INSTITUTE OF HEALTH SCIENCES
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THE INTERACTIONS OF IGG & TRIM21/RO52 IN HUMAN MICROGLIAL CELLS.

DOCTOR OF PHILOSOPHY THESIS

NEVZAT CUMHUR DEMİRTOKA

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by the Administrative Board of Institute with decisions dated …….. and numbered……...

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Science

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Nevzat Cumhur Demirtoka

DEDICATION

I dedicate my thesis to Minat Demirtoka, the woman who despite all the difficulties, was able to leave the habit of observation and analysis while smiling as a legacy; who refused the gun given as a debt of gratitude for the life saving but asked for the opportunity of education for childrens of the village instead.

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Second key word possible is “peace” and I thank all the peacekeepers in my life.

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LIST OF ABBREVIATIONS

AID	Autoimmune disease
AIND	Autoimmune neurological diseases
ALS	Amyotrophic lateral sclerosis
ANA	Anti-nuclear antigen
APC	Antigen presentation cells
AQP4	Aquaporin 4
A-ab	Auto-antibodies
AIHA	autoimmune hemolytic anemia
BANK1	B-cell scaffold with ankyrin repeats 1
BLK	B Lymphoid tyrosine kinase
BBB	Blood brain barrier
CIA	Collagen-induced arthritis
CIDP	Chronic inflammatory demyelinating polyneuropathy
CIITA	Class II transactivator
CNS	Central Nervous System
CIDP	Chronic inflammatory demyelinating polyneuropathy
CSF	Cerebrospinal fluid
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
DHR123	Dihydrorhodamine 123
DPBS	Dulbecco’s phosphate buffered saline
EAE	Experimental allergic encephalomyelitis
EGFP	Enhanced green fluorescent protein
EBV	Epstein-Barr virus
Fas ligand	FasL
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcGR	Fc-gamma receptor
FCM	Fas ligand
FcRn	Neonatal Fc receptor
FD	Fluorescent densities

GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GBS	Guillain–Barré syndrome
GLUR	Glutamate receptors
HMC3	Human microglial clone-3 cell line
HMGB1	high-mobility group box 1
IgG	İmmunoglobulin G
IL	İnterleukin
IFN γ	İnterferon gamma
ImOL	Immune Oligodendrocytes
IRF	İnterferon regulatory factor
JEV	Japanese encephalitis virus
Jo-1	Anti-histidyl-tRNA synthetase
IBA1	Ionized calcium binding adaptor molecule 1
IC	Immune complex
IFN-γ	Interferon γ
IgG	Immunoglobulin G
IIM	Inflammatory myopathy
ITP	Immune thrombocytopenic purpura
IRF	Interferon regulatory factor
ITP	Immune thrombocytopenia
IVIG	Intravenous IgG
ITP	Immune thrombocytopenia
JEV	Japanese encephalitis virus
LE	Limbic encephalitis
LPS	Lipopolysaccharide
LPSI	LPS-incubated
MARCH-1	Membrane associated RING-CH 1
MAVS	Mitochondrial adaptor molecule
MFI	Median of Fluorescence Intensity
MHC-II	Major histocompatibility complex class II
MGluR1	Metabotropic glutamate receptor 1
MNCB	Multifocal neuropathy with conduction block
MODY	Maturity-onset diabetes of the young

MS	Multiple sclerosis
NLRs	NOD-like receptors
NMDA	N-methyl-d-aspartate
NMI	N-Myc and STAT interactor
NMO	Neuromyelitis optica
NPSLE	Neuropsychiatric Lupus
PAMPS	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PID	Primary immunodeficiency diseases
PRR	Pattern recognition receptors
PVM	Perivascular macrophages
RIP	Receptor-interacting protein
RLRS	RIG-I-like receptors
RM-ANOVA	Repeated measure analysis of variance
SAS	Subarachnoid space
SD	Standard deviation
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SPS	Stiff person syndrome
STAT	Signal transducer and activator of transcription
SS	Sjögren's syndrome
T1D	Type 1 diabet
Th1	T-helper type 1
TLR	Toll-like receptor
TNIP1	TNFAIP3-interacting protein 1
TGF-β	Transforming growth factor beta
Treg	Regulatory T cell
TRIM21/Ro52	Tripartite motif-containing protein 21
VGKC	Voltage-gated potassium channel
W	With Welch correction

ABSTRACT

The damaging mechanisms of cytosolic antigen targeting auto-antibodies and alleviating mechanism of intravenous immunoglobulin treatment (IVIG) in many neurological autoimmune diseases is not well known. TRIM21/RO52, which is itself a frequently auto-antibody targeted cytosolic antigen, a part of the innate and humoral immune system, intra-cellular FCgamma receptor (with a very high affinity) and viral defense and immune response regulator may play a role. This research investigated the time (20 and 60 min, 4, 24 and 48 hr) and the immune-response group (naive and 24 hr LPS incubated (LPSI)) based changes of the IgG and TRIM21/Ro52 levels in the IgG-incubated HMC3 microglial cells with FCM and confocal microscopy. The FCM results show: a) In both groups, the MFI of IgG changes were negligible until a sharp rise in the 48th hour. b) The MFI of TRIM21/Ro52 was significantly higher in LPSI and dropped in the first 20 min with the IgG stimulation but raised in the naive group. After 20 min the changes of the TRIM21/Ro52 levels changed parallelly in the both groups but, it was always significantly higher in LPSI (levels rose again after bottoming in the range of 60 min-4 hr) c) Cells (21% in naive group and 30% in LPSI) became TRIM21/Ro52(-) at the 48h. d) The confocal microscopic views show i) the abundance of the TRIM21Ro52 in the both groups were dropped at the 48 hour while increasing the IgG levels ii) Up to the 48th hour, IgG greens were always accompanied by a very dense TRIM21/Ro52 red, while the 48-hour images showed emerging individual and numerically more green areas. These results suggest that time and the state of the microglial cell dependent bidirectional relation between IgG and TRIM21/RO52. At the beginning, IgG looks to trigger a phasic TRIM21/Ro52 level increase (only if it is not already high), then the self destruction process of the IgG bound TRIM21/Ro52 becomes more effective. After 4 hr, some cells (probably due to the mRNA expression) show over compensation of the TRIM21/Ro52 levels while the rest depleted and IgG accumulation without TRIM21/Ro52 colocalization becomes visible. The IgG - TRIM21/Ro52 colocalization was highly probable continued with proteasomal degradation. The higher MFI of IgG in the LPSI, may be interpreted as the immune stress is a factor facilitating antibody internalization. The findings also suggest microglial cells should have an antibody absorption role in the CNS.

Keywords: IVIG, IgG, Microglia, TRIM21, Ro52, Autoimmune diseases

ÖZET

Birçok nörolojik otoimmün hastalıkta hücre içi antijeni hedef alan otoantikorların hasar verme ve intravenöz immünoglobulin tedavisinin (IVIG) hafifletme mekanizmaları iyi bilinmemektedir. Bu konuda TRIM21/RO52 (Sıklıkla oto-antikor hedefli bir hücre içi antijen, doğuştan ve humoral bağışıklık sistemlerinin bir parçası, çok yüksek afiniteli FC gamma reseptörü, viral savunma ve bağışıklık tepkisi düzenleyicisidir) rol oynayabilir. Bu araştırma, IgG ile inkübe edilen HMC3 mikroglial hücrelerinde IgG ve TRIM21/Ro52 seviyelerinin zamana (20 ve 60 dk, 4, 24 ve 48 sa) ve bağışıklık tepkisi grubuna (naif ve 24 sa LPS ile inkübe (LPSI)) bağlı değişikliklerini FCM ve konfokal mikroskopi ile inceledi. FCM sonuçları şunlarına göre: a) Her iki grupta da IgG değişikliklerinin MFI'si 48. saatte keskin bir yükselişe kadar ihmal edilebilir düzeydeydi. b) TRIM21/Ro52'nin MFI'si LPSI'de önemli ölçüde daha yüksekti ve IgG stimülasyonu ile ilk 20 dk düştü, ancak naif grupta yükseldi. 20 dk'dan sonra TRIM21/Ro52 seviyelerindeki değişiklikler her iki grupta da paralel olarak değişti ancak LPSI'de her zaman anlamlı derecede yüksekti (seviyeler 60 dk - 4 saat aralığında dibe vurduktan sonra tekrar yükseldi) c) 48'inci saatte hücreler (naif grupta %21ve LPSI'de %30) TRIM21/Ro52(-) oldu. d) Mikroskop görüntüleri şunu göstermektedir: i) her iki gruptaki TRIM21Ro52 bolluğu 48'inci saatte düşerken IgG seviyeleri arttı ii) 48'inci saate kadar IgG yeşillerine her zaman çok yoğun bir TRIM21/Ro52 kırmızısı eşlik etti 48 saatlik görüntülerde ortaya çıkan bireysel ve sayısal olarak daha fazla yeşil alan görüldü. Bu sonuçlar, IgG ile TRIM21/RO52 arasında mikroglial hücrenin durumuna ve zamana bağlı çift yönlü ilişkinin olduğunu göstermektedir. Başlangıçta IgG, fazik bir TRIM21/Ro52 seviyesi artışını tetiklemeye çalışır (ancak zaten yüksek değilse), daha sonra IgG'ye bağlı TRIM21/Ro52'nin kendi kendini yok etme süreci daha etkili hale gelir. Dört saat sonra, bazı hücreler (olası mRNA ekspresyonuna bağlı olarak) aşırı TRIM21/Ro52 telafisi diğerleri ise yoksunluğu gösterdi ve TRIM21/Ro52 eşliği (kolokalizasyon) olmadan IgG birikimi görünür hale geldi. IgG - TRIM21/Ro52 eşliğininin proteozomal bozulma ile devam etmesi kuvvetle muhtemeldir. LPSI'de IgG'nin daha yüksek MFI'si, bağışıklık stresinin antikor içselleştirmesini kolaylaştıran bir faktör olduğu şeklinde yorumlanabilir. Bulgular aynı zamanda mikroglial hücrelerin CNS'de antikor emiliminde rol oynaması gerektiğini de öne sürüyor.

Anahtar Kelimeler: IVIG, IgG, Microglia, TRIM21, Ro52, Otoimmün hastalıklar

1. Introduction

In this thesis, the relationship between immunoglobulin-G (IgG) and Tripartite motif-containing protein 21 (TRIM21/Ro52) of microglial cells (naive and with lipopolysaccharide (LPS) stimulated) was investigated. IgG is the most abundant immunoglobulin in the human body, and if it is auto-reactive, it is one of the main reasons for autoimmunity [1]. On the other hand, it is a treatment material for autoimmune diseases (AID) (by intravenous administering IgG pool from healthy people to patients (IVIG)) with positive outcomes for a significant percentage of patients [1]. TRIM21/Ro52 (as we will discuss) seems a critical role player in many steps of the mechanisms of AIDs [2]. Microglia, the CNS cells with their roles changing broadly, may harm hosts during autoimmunity [3]. Here, the following items are taken into focus around the TRIM21/Ro52. The dynamic nature of the immune system in the periphery and the CNS, meeting points of AIDs (including neuro-immunologic diseases), and the possible backgrounds of the genetic research results about AIDs.

1.1. Autoimmune disease (AID) - Epidemiological view

Since single-cell organisms, defense has been an essential requirement for survival. Although defense (immune) systems have developed and become more complex with the evolution of living species, possibly the most fundamental rule has not changed threat detection. AID is the condition in which the immune system perceives the elements (epitope) of the living being as a threat and harms them [4]. There are many defined autoimmune diseases (nearly 100 distinct AIDs) [4]. Depending on what the immune system perceives as a threat, the disease can affect almost any organ, one or more than one organ, and cause varying symptoms ranging from occasional discomfort to death [4].

Although significant developments in medicine, pharmacy, and health (especially vaccine) technologies have a tremendous positive impact on the epidemiological data of infectious diseases, the situation is different regarding AIDs [4]. There is a steady decrease in the incidence of infectious diseases (i.e., measles, mumps, tuberculosis, hepatitis-A). On the other hand, immune & allergic disorders (i.e., asthma, rhinitis, atopic dermatitis, multiple sclerosis, type 1 diabetes, Crohn's disease) have steadily risen over the past decades, especially in westernized/ developed countries [5-7]. The estimated 2019 AID incidence in the USA is between 5 to 8% (depending on the assumptions), which refers to 15 to 20 million [7]. According to the study by Lerner and friends (2015),

the rise rates of annual incidences are 7.1% for rheumatic, 6.3% for endocrinological, 6.2% for gastrointestinal, and 3.7% for neurological AIDs [6].

Epidemiological studies also point to the multifactorial nature of AIDs, i.e., race, genes, geographic conditions, socioeconomic status, childhood infections, breeding conditions, environmental factors [5,7] sex [8]. For example: a) Diabetes Mellitus type 1 (T1D) associated HLA alleles -DR3 and DR4-DQB1*0302- ratio and also the incidence of T1D is low in Japan [5] b) The prevalence of Multiple Sclerosis and T1D are higher in northern Europe and lower in eastern Europe than central Europe [5]. Similarly, there is a difference in the frequency of Multiple Sclerosis (MS), T1D, and asthma between high-income (Gross National Product per Capita >18000EU/year) and low-income (Gross National Product per Capita <14000EU/year) countries in favor of low-income countries [5]. c) Breeding in a specific pathogen-free environment is reported as an almost doubling factor of T1D incidence [5]. d) according to the statistical study in Denmark, the prevalence of almost all AIDs diseases (except Guillain Barre Syndrome and T1D), whether equal or high, in females. Especially, differences are huge for some diseases (endometriosis, Sjogren's syndrome (SS), autoimmune thyroiditis, systemic lupus erythematosus (SLE), primary biliary cirrhosis, thyrotoxicosis, and systemic sclerosis) [8]. e) Roberts & Erdei reported considerable heterogeneity for racial groups and the selected 22 AID distribution but also changed profiles by region-race combinations [7].

So many factors are underlying how the immune system's sensitivity evolves into AIDs. Probably due to the sophisticated and dynamic structure of the immune system and the fact that the immune system, in addition to the defensive task, also undertakes repair and maintenance functions opposite to the defense's destructive nature.

1.2. Immune system basics and the first ground for AIDs.

The immune system is complex, from proteins to cells, organs, and their interactions with organs [3,9]. Skin, our largest organ, is included in the immune system as a physical barrier between the body and pathogens surrounding it [3,9]. Chemical and antibacterial barriers, i.e., saliva, tears, stomach acid, mucus, are closing the gap in the organs which do not have a sufficient physical barrier, i.e., eyes, mouth, stomach, and airways [3,9]. In addition, autonomic nervous systems that trigger mechanical cleaning functions such as coughing and sneezing support these chemical barriers [3,9].

Pattern recognition receptors (PRRs), the part of the evolutionary older (innate) immune system in which the innate immune cells (i.e., dendritic cells, macrophages, monocytes,

neutrophils, and epithelial cells) express, are sensitive to molecules typical for the pathogens [10]. PRRs are also sensitive to molecules specific to the host's cell damage because cleaning the dead cells is another task of the immune system [10,11]. The PRRs sensitive to pathogens (i.e., bacterial, viral, fungal) such as lipopolysaccharide (LPS), mannose, RNA, fungal glucans, and chitin [11] are called pathogen-associated molecular patterns (PAMPs). The PRRs which sensitive to endogenous stress signals (i.e., heat, extracellular ATP, "intracellular proteins, such as high-mobility group box 1 (HMGB1), histones, S100 proteins, heat-shock proteins (HSPs), and plasma proteins, like fibrinogen, Gc-globulin, and serum amyloid A [12]") are called damage-associated molecular patterns (DAMPs).

There are many types of PRRs, membrane located (i.e., Toll-like receptors (TLRs-1,2,4,5,6 [12,13]) and C-type lectin receptors), cytoplasmic (i.e., NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), TLR-3,7,8,9 [13]) or secreted (i.e., "pentraxins C-reactive protein and pentraxin 3; the collections mannose-binding protein and surfactant proteins A and D; H-ficolin; serum amyloid A; and the complement components C3 and C5)" (sited from [14]).

Once any pathogen (i.e., bacteria, viruses, parasites) enters the body, the type of immune response they trigger depends on the molecules sensed by what type of PRRs [15]. On the other hand, as we will mention later, there are also defense mechanisms against the pathogens which can escape from PRRs.

The immune system's first response is mostly inflammation and is initiated by resident cells (i.e., macrophages, dendritic cells, epithelial cells, and mast cells) cited from [14,15]. Secretion of histamine, bradykinin, serotonin [16], leukotrienes, and prostaglandins [17] triggers pain receptors, vasodilation of the blood vessels around the infection, and attract phagocytes, especially neutrophils [11-13]. Neutrophils, the most abundant leukocyte in humans, mobilize to the inflammation zone via chemoattractants (i.e., IL-8) and have the ability to kill microbes rapidly through the granulocytes [18]). The PRR activation activates programmed cell death pathways and clears infected or damaged cells [19]. As we will mention later, replacing the dead nerve cells is impossible in contrast with the other cells in our body; therefore, the defense strategy of the central nervous system (CNS) differs. Besides the innate immune system, the humoral /adaptive immune system, which is based on antibody production, also interferes, but its' nature makes it slower but specific [20].

In summary, the immune system keeps pathogens out with the help of physical and chemical barriers, and when the pathogens can reach the body face innate immunity. The pathogens, which have a structural component within the sensing spectrum of PRRs, (for example, the gram-negative bacteria has LPS on the membrane, which is characteristic of that stimulates TLR4), face the quick response of the innate immune cells [12,13]. The molecules resulting from the stress or death of the host cells in the infected area also trigger innate immunity. Once the innate immunity is triggered, the secreted agents cause the flow to the zone, accumulation, and proliferation of the immune cells. The residuals of the attacked pathogens and dead host cells are transported by antigen-presenting cells (APCs) through the lymphatics to the lymph nodes. T and B cells with receptors recognizing the introduced antigens in the lymph nodes are formed and begin circulating [21]. However, a selection process will enable the T and B cells that may occur against the host's antigens in the lymph nodes to undergo apoptosis in the node [21]. Errors in this selection mechanism create the first ground for forming AID (Figure 1.1.)

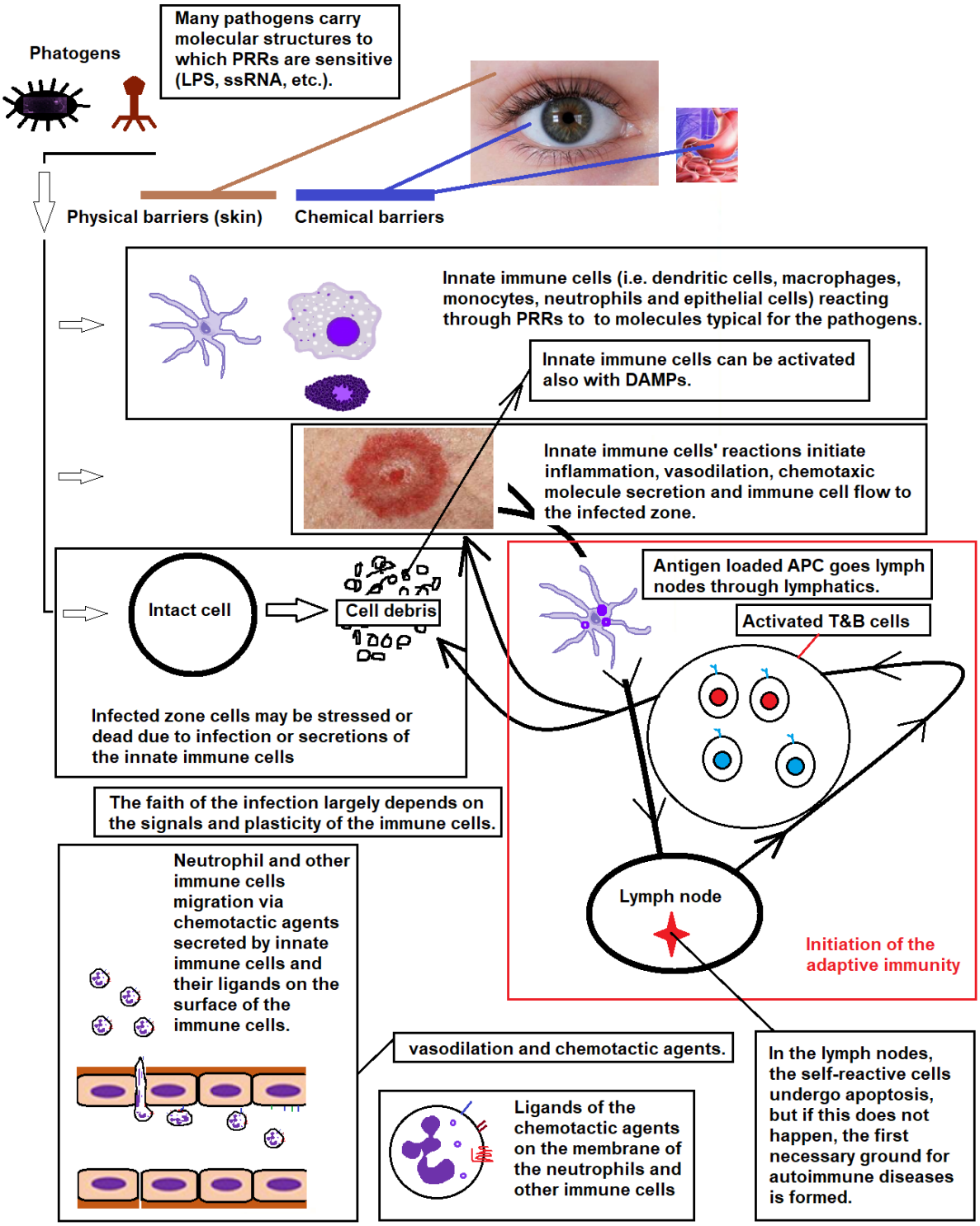


Figure 1.1 The initiation of immune responses. PRRs often initiate the innate immune response by sensing pathogen-specific molecules that can enter the body with pathogens and/or the stress/death molecules the infection creates in cells. Inflammation is mostly the first consequence of an immune response. Chemotactic agents provide immune cell flow to the region, while cytokines enhance the proliferation of immune cells and interactions. APC cells carry the antigens they collect to the lymph nodes through the

lymphatic system. In the lymph nodes T and B cells develop receptors against antigens carried to the lymph nodes and release systemic circulation, which is subject to the attraction of the chemotaxis. On the other hand, within the lymph nodes, the T or B cells that develop against host antigens undergo apoptosis. This mechanism sometimes does not work properly, and the initial ground for forming AID is formed.

1.3. CNS immune system, not isolated, but restricted and integrated with the periphery

CNS was known as a hermetically sealed structure, isolated from the peripheral immune system by the blood-brain barrier (BBB), without a lymphatic system, and "Under normal physiological conditions, the CNS presents a low expression of MHC Class II molecules. Therefore, resident brain cells do not present specific antigens to lymphocytes" [22]. In 1947, Medawar showed that the existence of primed peripheral lymphocytes resulted in graft rejection in the brains of animals while naive animals (lymphocytes not primed) did not; obviously, the T cells sensitized in the periphery were passed through the BBB and reached the graft in the brain [22]. Besides, the CNS is not an entirely immune privilege zone; the regeneration disability of neurons makes CNS a sensitive zone to excessive immune responses. Accordingly, the BBB and the choroid plexus epithelial cells maintain the CNS homeostasis and keep the CNS, not as a fully sealed but restricted zone [23]. Animal studies show that systemically injected IgG in intact animals detected 0.009±0.001% of injected dose in the cortex [24]. As we will discuss later, the potential amount of IgG passes the BBB may be higher but may be subject to degradation.

CNS has many immune tools besides BBB against pathogens but also against potentially detrimental peripheral immune agents. The microglial cells and astrocytes (phagocytic cells) act as the first line of defense against the pathogens passed into the cerebrospinal fluid (CSF) [22].

Microglial cells, astrocytes, and endothelial cells also express inflammation suppressors such as transforming growth factor beta (TGF-β), galectin-9, and Fas ligand (FasL) [22]. The immunoglobulin G (IgG), which successively passes BBB, is subject to a dynamic reverse flow [25]. The capillary endothelial cells that express Fc receptor neonatal (FcRn), which mediate clearance of CSF from successive IgG passing through the BBB, are carried out through 'reverse transcytosis' from the brain to the blood [25]. As we will

discuss later, our experiment suggests a neutralization pathway of the BBB passing or CNS-borne antibodies.

CNS is an immunologically restricted zone but interacts with peripheral immune systems in health and disease conditions [26].

The following factors turn the CNS into a kind of functional virtual secondary lymphoid organ a) meningeal lymphatic vessels surrounding the brain [24], b) meningeal macrophages and other antigen presentation cells (APC) in the subarachnoid space (SAS) surveying the CSF antigens c) soluble antigens transported through the cribriform plate of the ethmoid bone -appropriately sized channels for APC passage- to nasal mucosa hosting deep cervical lymph nodes exist [26]. Besides the microglial cells [3], microvessel-associated dendritic cells (DC) [27], astrocytes, and brain perivascular macrophages (PVM) are APC populations in the CNS. PVM are, as their name suggests, brain resident myeloid (debated) cells that migrate from the yolk sac into the brain early in development [28]. PVMs contribute to BBB integrity by restricting the passage of macromolecules over ten kDa into the brain in healthy conditions in diseased status leading to BBB disruption [28]. PVMs express MHC-II more evidently in diseased status, i.e., under conditions of interferon γ (IFN-γ) treatment and CD40 treatment [28].

In addition to above mentioned olfactory & meningeal lymphatic clearance pathways, there are two known pathways: glymphatic and intramural perivascular pathways. PVMs directly access the 3rd and fourth lymphatic routes [28]. They can phagocytose large particles and may facilitate intramural perivascular drainage indirectly through the regulation of smooth muscle cell tone [28]. In disease conditions, PVMs have several effects. They increase BBB permeability, post-stroke demyelination, and granulocyte recruitment, promote VEGF expression, promote neurological dysfunction, maintain brain glucose uptake activate the secretion of chemokines for monocyte/macrophage recruitment in autoimmune diseases (AIDs) [28]. They also improve neurocognitive function by promoting Aβ clearance, facilitating leukocyte infiltration in Infectious diseases [28].

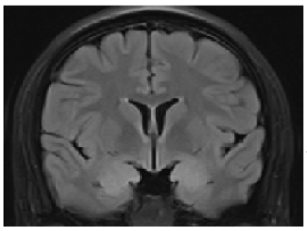
Lymphocyte infiltration to CNS also happens, and lymphocytes around veins are a marker of immune reaction in CNS parenchyma [29]. Interestingly it is reported that in experimental allergic encephalomyelitis (EAE) research, the T cells activated and specific for autoantigens are more prone to infiltrate the CNS rather than resting T cells [30]. This observation may be because naive T cells preferentially circulate through lymphoid organs [31]. The fate of lymphocytes infiltrated into CNS is varied; suppression of the

inflammation by T-Cell apoptosis is reported as a general feature of CNS (as mentioned above, microglia and astrocytes are possibly highly effective), but after the elimination of targeted antigens [30]. On the other hand, infiltrated T cells also may proliferate as organ-specific autoreactive T cells depending on the different specificities [30]. The specificities vary (i.e., mainly the HLA-DR15 haplotype is reported in Multiple Sclerosis (MS) patients as a factor of activation and "auto proliferation" of the autoreactive CD4+ T Cells by memory B Cells.[31], accumulation of DC [27], the presence of APCs[32])

The autoantibodies detection (Yahr et al., 1954) in CSF of MS patients was the first connection between autoimmune neurological diseases (AIND) and B cells in CSF[15]. Other autoantibodies connected AINDs followed (i.e., neuromyelitis optica, myelin oligodendrocyte glycoprotein antibody-associated disorder, anti-N-methyl-d-aspartate (NMDA) receptor encephalitis) [33]. Corcione et al. reported B cell differentiation in secondary lymphoid organs of CNS [34]) and Ig-secreting plasma cells in CSF as a result of favorable microenvironmental conditions in MS and other inflammatory CNS patients in parallel with their oligoclonal Ig bands [35]. CXCL12 and CXCL13 in CSF were also reported (with their ligands CXCR4 and CXCR5 (CXCR5 facilitates B cell homing) on malignant lymphocytes in "primary CNS lymphoma confined to the CNS in the absence of systemic disease" [34].

In summary, CNS should be sterile, and the immune reactions should be as much as possible non-inflammatory due to neurons' lack of regeneration capacity. For this purpose, BBB and choroid plexus try to keep pathogens and peripheral immune agents away. On the other hand, CNS is in connection with the peripheral immune system via lymphatic drains, and (as a result of this communication or independently) obviously, B and T cells not only enter but also home and activate. B and T cell's existence in the CNS happens (most probably) thanks to a highly interactive microenvironment with the CNS-specific cells (i.e., microglia, PVM), and sometimes the successful antigen clearance (with or without destruction of CNS cells) may result with apoptosis of lymphoid cells. However, it may sometimes continue with CNS autoimmunity (Figure 1.2).

A



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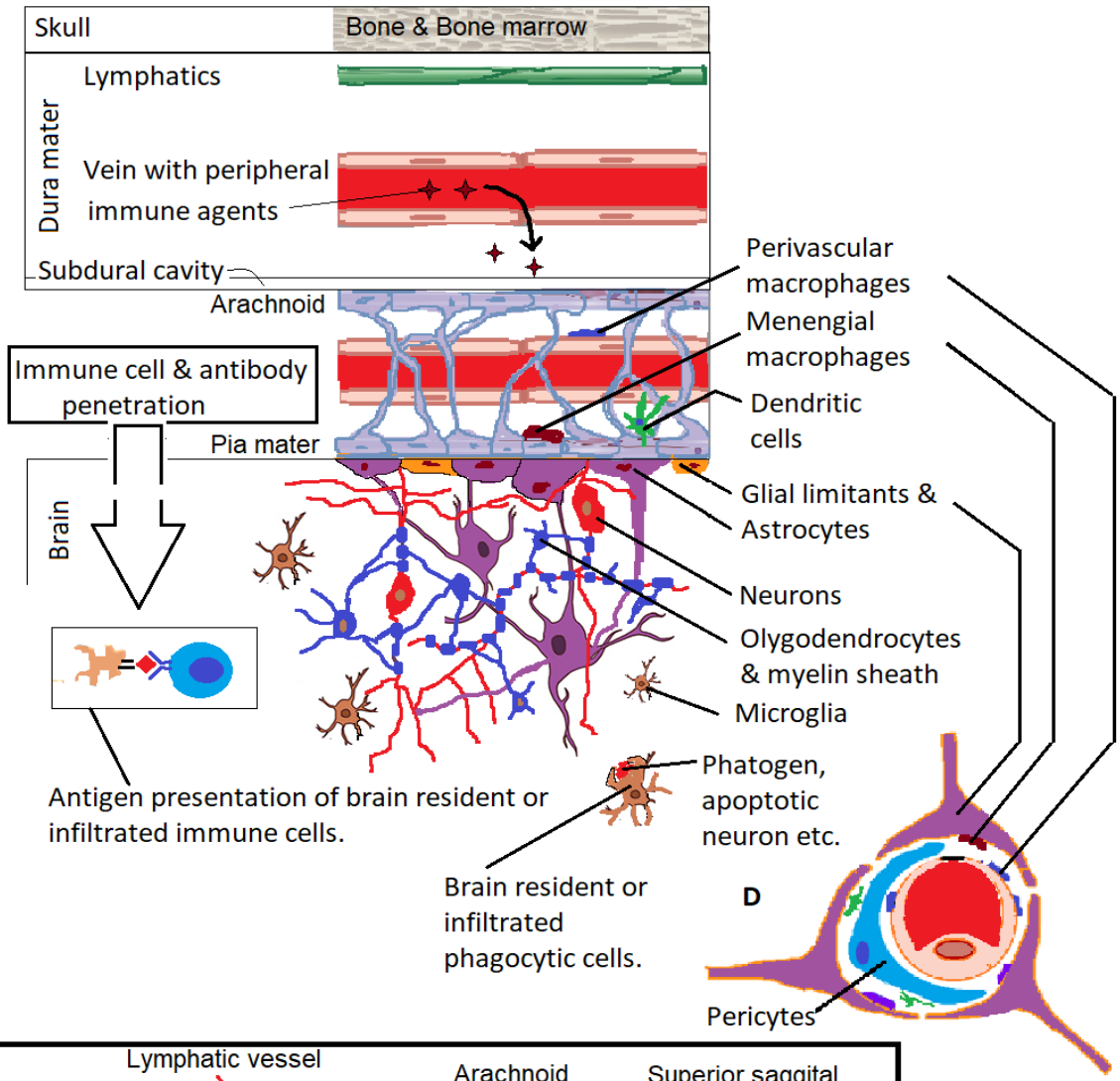
Skull
Bone & Bone marrow
Lymphatics
Dura mater
Vein with peripheral immune agents
Subdural cavity
Arachnoid
Immune cell & antibody penetration
Pia mater
Brain
Perivascular macrophages
Menengial macrophages
Dendritic cells
Glial limitants & Astrocytes
Neurons
Olygodendrocytes & myelin sheath
Microglia
Phatogen, apoptotic neuron etc.
Antigen presentation of brain resident or infiltrated immune cells.
Brain resident or infiltrated phagocytic cells.
D
Pericytes

C

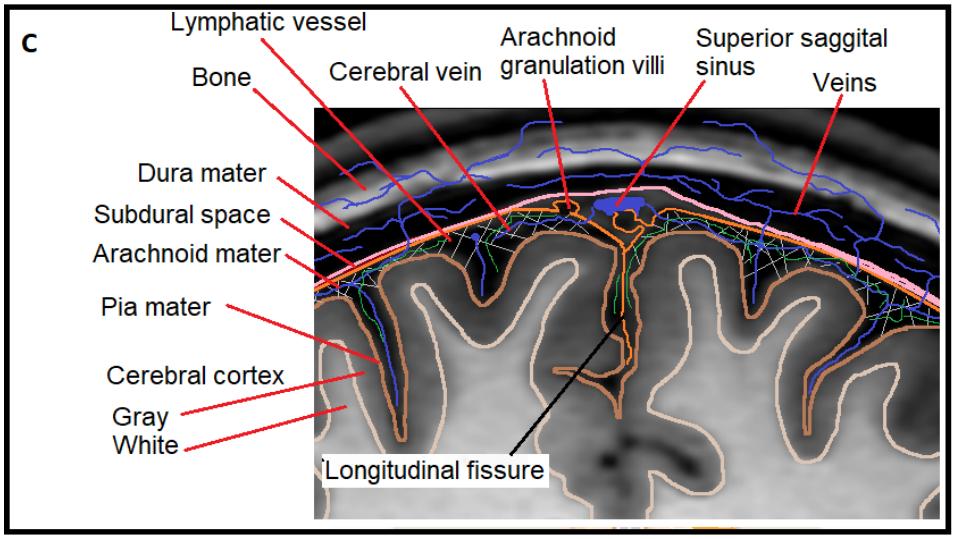
Lymphatic vessel
Bone
Cerebral vein
Arachnoid granulation villi
Superior saggital sinus
Veins
Dura mater
Subdural space
Arachnoid mater
Pia mater
Cerebral cortex
Gray
White
Longitudinal fissure

Figure 1.2. Immune System of CNS. A) Coronal view of the brain. B & C) (Schematic views of some neuro immunologic details from the skull to the cerebral cortex). In the dura mater under the skull, there are dural sinuses, veins (which may go out of the skull or go into the subarachnoid space), lymphatic vessels (i.e., draining immune cells, excess fluid and small molecules to cervical lymph nodes), arachnoid granulation villi (serving as a one-way gate from CSF to the bloodstream). There may be peripheral immune cells and other items (e.g., bacteria, viruses, antibodies) within the dura mater, depending on several factors (e.g., conformity of the tight junctions on the veins). The para ventricular macrophages and meningeal macrophages are resident macrophages serving as first-line immune cells between arachnoid and pia mater together with dendritic cells. Glia limitans and astrocytes form a second blood and immune barrier. Despite BBB, peripheral immune cells infiltrate CNS and may harm the neurons and/or glia. Oligodendrocytes form the insulation sheets (i.e., myelin) of neurons. Myelin is one of the main targets of autoimmunity-generating cells. Microglial cells are phagocytic cells and play an important role in synaptic pruning and brain architecture. They survey the brain and proliferate in case of an immune attack. The intensity difference of the microglia is shown between pathologic and nonpathologic zones. Under an immune reaction state, the morphology of the microglial cells also changes. Microglial cells are self-renewing cells; immune reactions lead them to proliferate. Microglial cells can harm neurons through phagocytosis under inflammatory conditions. Microglial cells also clean plaques through phagocytosis. Macrophages may infiltrate into CNS and create damage and inflammation, but also they may become tumor-associated macrophages. D) Schematic cross-sectional view of the capillaries of the CNS.

1.4. Glial cells

Glial cells are a group of non-neural cells with different origins and tasks in the central and peripheral nervous systems [36]. The CNS glial cells are the macro glial cells (Astrocytes -the most abundant type of glial cells-, Oligodendrocytes, Ependymal cells, Radial glial cells, Pituicytes, Tanycytes) and microglial cells [37]. The PNS glial cells are Schwann cells (provide myelination to axons) [38], satellite cells (regulate the external chemical environment of sensory neurons and sympathetic and parasympathetic ganglia) [39], and enteric glial cells [40].

1.4.1. Astrocytes

Astrocytes are essential parts of the BBB. They surround the endothelial cells of brain vasculature with pericytes and maintain tightly regulated cellular, ionic, and molecular transfer from blood to the brain [41]. Thus maintain CNS (as much as possible) pathogen and immune cells free, hormonal levels, and ionic homeostasis of CNS [41]. Astrocytic aquaporin 4 (AQP4) water channels enable the flow of CSF towards the intracranial vein by the pressure wave generated by the arterial pulse [41]. This flow, called glymphatic, transports extracellular proteins, excess fluid, and metabolic waste products like lymphatics. "Astrocytes also actively participate in synapse homeostasis through phagocytosing synapses, neuronal debris, axonal mitochondria, and pathological protein aggregates. In addition, it has also been suggested that astrocytes may regulate microglial phagocytosis by secreting molecules such as IL-33 and C3" (cited from [42]). Astrocytes are also APCs and "under inflammatory conditions, express all subunits of IL-12/IL-23" and interact with T cells. [43].

The post-mortem studies of Rostami et al. show antigen-presenting astrocytes and activated T-cells in the PD brains, highlighting the astrocytes' role in chronic inflammation [44]. The T cell modulation ability of the astrocytes puts them into focus for MS and chronic CNS inflammatory diseases in general [45]. The factors affecting astrocyte functionality reflecting neuronal immunity (i.e., the Astrocyte-microglia interactions under the existence of the AQP4 antibodies) are shown as a factor of the evolving neuromyelitis optica lesion [46]. An animal study shows that altered astrocytic characteristics of T1D patients affect neurotransmitter dynamics and result in abnormal neuronal activities [47].

One of the critical agents in the CNS, the influx and efflux that should be under strict control, is glutamate [48] because glutamate is the most abundant excitatory neurotransmitter in the vertebrate nervous system and amino acids in the human body [49]. The neurons cannot synthesize glutamate and GABA from glucose and need glutamine for glutamate or GABA production [49]. Astrocytes either synthesize glutamate from glucose from BBB or most of the glutamate (GABA as well) of the extracellular space secreted by neurons, convert them to glutamine, and release them back to synaptic cleft [50]. In summary, astrocytes are one of the critical cells of the whole brain and synaptic cleft homeostasis with immunologic roles.

1.4.2. Oligodendrocytes

Oligodendrocytes are the critical cells of the quality of the electrical signal transfer through the axons [51]. The myelin sheath they produced insulates by wrapping axons, and demyelination causes multiple sclerosis (MS), one of the prevalent neurological diseases with underlying autoimmune and/or genetic, environmental, viral (i.e., Epstein-Barr virus (EBV)) factors [52]. "Oligodendrocytes use lactate as a source of energy and as a precursor of lipids" [53], and they "oxidize lactate at a higher rate than that observed for neurons and astrocytes, and their rate of lipid synthesis from lactate was at least 6-fold higher than that found in astrocytes or neurons" [53].

Oligodendrocytes are subject to differentiation, and new studies suggest oligodendrocytes are not passive cells during myelination defects and Immune Oligodendrocytes (ImOL), differentiated by proteasomal inductions, may be linked to neuroinflammation [54]. Oligodendrocytes express MHC-II by induction with the combination of IFN-γ and dexamethasone (within the physiological range of CSF), interestingly not with IFN-γ stimulation alone [54].

In (2018), Falcão et al. reported upregulated immune cell myriad proteins in the spinal cords of EAE-induced mice [54]. These and further results suggest that oligodendrocytes as functional APCs [54]. Postmortem MS patient studies also show increased Imol phenotype [54].

The oligodendrocyte precursor cells, also known as NG2-glia, "immature cells with high proliferative capacity and widely distributed throughout the gray and white matter in the postnatal and adult brain, where they make 5–8 of the glial cells population", are crucial in demyelinating pathologies [55]. NG2-glia cells have AMPA, GABA, and glutamate receptors, and they also have other functions and interactions that need to be uncovered [55].

The glutamate receptors (GluR), which oligodendrocytes have, make them vulnerable to neurotoxicity, and GABA receptors emerge as a balancing factor similar to neurons, so the GABA receptor agonists protect oligodendrocytes against over-excitatory stimulation [56]. MHC-II expression by oligodendrocyte precursor cells is reported in vitro during co-culturing with CD45+ microglial cells [54].

If we put them together under inflammatory conditions, oligodendrocyte precursor cells may differentiate as ImOL instead of mature oligodendrocytes, and these differentiated oligodendrocytes as immune cells (with questions) may act as phagocytic APC cells and may contribute with T cells to the myelin destruction [54].

1.4.3. Ependymal cells

Ependymal cells are ciliated, cuboidal cells lining the ventricles and spinal canal in direct contact with CSF [57]. The oscillations of cilia are believed to help CSF circulation [57]. They are mitotically active cells [57]. Although they have many essential functions in development, in the adult brain, the main task of the ependymal cells is electrolyte and solute transfer between brain parenchyma and CSF [57]. As the intensive hormonal activity possible requires some specialized interactions, ependymal cells of the hypothalamus and infundibulum are specialized and called Tanycytes [58].

Studies with injured spinal cord IL-17A knockout and IL-17A neutralized with antibodies mice suggest ependymal cells positively affect axonal growth and functional recovery by secreting neurotrophic factors, which does not happen under IL-17A existence [59].

An animal study shows defective ciliary oscillation of ependymal cells after (NF-κB) signaling is reported as a probable worsening reason for the "certain types of neonatal and childhood hydrocephalus associated with hemorrhages and infections" [60].

1.4.4. Microglia

Microglia are believed to originate from "a unique, and transient RUNX1+ /cKit+ /CD45- erythromyeloid progenitor population in the embryonic yolk sac" and after the establishment of the BBB remains isolated [61]. "Microglia, unlike most other hematopoietic lineages, renew slowly at a median rate of 28% per year, and some microglia last for more than two decades" [62]. They do not need any progenitor recruitment for renewal [63]. An interesting peripherally derived macrophage infiltration into CNS (in humans, "after lethal whole-body irradiation and bone marrow transplantation") is reported in the case of microglial deficiency as compensation and then develops ramifications similar to those of microglia [64].

Microglia are multifunctional intrinsic immune cells of the CNS [65], and they are "the major cell type expressing MHC class II in the human white matter" [66]. Their existence in brain regions changes between 0.5–16.6% (microglia/total cell) [67]. Like other immune cells, microglia have PRRs that make them highly sensitive to their microenvironment [68]. They may respond to microenvironments with pro or anti-inflammatory phenotypes. However, instead of two opposite phenotypes, they can be in a phenotype in a broad spectrum in response to any chemical or proteinaceous stimuli [68]. PRRs are not the only phenotype specifiers; hormones and neurotransmitters are

also influencers [66]. Phenotyping can be region specific (i.e., "cerebellar microglia may behave differently from those in the thalamus, neocortex, and hippocampus" (animal study) [69]).

Microglia express TLRs and react to invading bacteria and viruses [70]. TLRs (except TLR3) express proinflammatory cytokines through Myd88 related pathway [70]. However, there are also Myd88 independent pathways [70]. "MyD88- independent pathway is associated with the induction of interferon (IFN)-inducible genes and maturation of dendritic cells" [70]. Age is another factor affecting TLRs expression and microglial cell phenotypes accordingly [70]. Stimuli like hypoxia, bacteria, viral agents, kainic acid, α-synuclein, and amyloid beta are reported as upregulating factors of TLRs expression in microglia [70]. Neurodegenerative diseases like AD, amyotrophic lateral sclerosis (ALS), and PD are also reported as upregulating factors of TLRs expression in microglia [70]. The contradictory reports about TLR activation and microglial cell pathological Aβ uptake [70] are probably because of the -both ends sharp- nature of inflammation. Polymorphism may always be a reason for the unbalanced reactions, and TLR2 polymorphism is reported as a factor of PD [71]. On the other hand, the report about TLR2 upregulation "in the chronically LPS-stimulated SOD1 transgenic mouse model of ALS" [m10] may be the cumulative effects of the inflammatory conditions and/or consistent TLR2 ALS relation. TLR4 activation studies are also contradictory, and the critical question is the same when the inflammation turns from beneficiary to detrimental [70].

One of the factors for microglia quiescence after inflammation (similar to other immune system cells) is IL-10, and microglia have IL-10 receptors (IL-10R) [72]. Laffer et al. reported the "loss of IL-10 promotes differentiation of microglia to an M1 phenotype" [73]. IL-10 may be expressed as Shemer et al. suggested through the "Ly49D+ NK cells and neutrophils" in the CNS or as Wu reported astrocytes and microglial cells themselves might express IL-10 in response to proinflammatory stimuli (of the leptomeninges -in the experiment of Wu) [74]. There are many similarities between microglia and macrophages. Kozicky et al. reported IL10 expressing monocytes with LPS+IgG costimulation [75].

Microglia have an essential role in brain structure through synaptic pruning; the agents (i.e., fractalkine, purine, and complement signaling) interfere with this process [69].

1.5 CNS and autoantibodies against surface receptor

Lack of neuronal regeneration capacity is not the only phenomenon that makes CNS immune strategies distinct from the peripheral immune system, but also excitotoxicity is another vital feature. Excessive or prolonged exposure to excitatory neurotransmitters causes unbalanced calcium homeostasis, oxidative stress, and mitochondrial perturbations, which end with neuroinflammation or apoptosis [76]. Moreover, inflamed, injured, went apoptosis neurons may lead to new consecutive excitotoxicity [76].

Long-term misregulation of neurotransmitters leads to neuroinflammation or cell death and can affect neuronal connections, synaptic architecture, and gene expressions.[77]. As a result of this fact, the autoantibodies targeting neurotransmitter receptors affect neuronal health.

One of the first autoantibodies found to cause autoimmune neuronopathy, cerebellar degeneration, and encephalitis is targeting N-methyl-D-aspartate (NMDA) receptors[78]. As a functional requirement, the neurotransmitter receptors are cell surface antigens, and it is known that autoantibodies in CSF bind them [78]. Within this group, besides NMDA receptors, we can mention Voltage-gated potassium channel (VGKC) complex antibodies [79], AMPA receptors [80], Voltage-gated calcium channels VGCC) [81], Glycine receptors [78], Metabotropic glutamate receptor 1 (mGluR1) [82]. As shown in Table 1.1, the frequent surface receptor autoantibodies may cause a wide range of neuronal or psychiatric diseases and may have associations with peripheral diseases.

Table 1.1 Disorders caused by surface receptor autoantibodies.

Targeted Receptor	Disorders	Reported Associations	Reference
AMPAR	limbic encephalitis and psychiatric symptoms	lung, breast, or thymic tumors	[80],[85]
GABAB receptors	limbic encephalitis, severe seizures or status epilepticus, cognitive impairment	lung cancer	[85,86]
Glycine receptors	encephalomyelitis, myoclonus, exaggerated startle responses,	-	[87,89]

	diplopia, ataxia, hyperekplexia, rigidity, muscle spasms		
mGluR1	cerebellar ataxia, cognitive and memory impairments	Hodgkin lymphoma	[82]
NMDAr	catatonia, agitation, seizures, problems of consciousness, movement dysfunctionality, autonomic instability	viral etiology, ovarian teratoma	[83],[84]
VGCC	Lambert–Eaton myasthenic syndrome, cerebellar degeneration	paraneoplastic with or without neurological disorders	[89]

1.6. CNS and cytosolic antigen autoantibodies

Understanding the mechanism of action of the surface receptor autoantibodies is relatively easy. They are bound to their target and create deprivation of the surface receptors. For example, the neuron (itself) endocytosis the antibody-bound NMDA receptors [90]. However, in worse examples, a cell destruction process starts. Autoantibodies do not target only surface receptors but also cytosolic antigens, and how the cytosolic antigen destruction happens and the facilitating or restricting factors of destruction are still unclear.

As an example, the autoantibodies against Anti-Glutamic acid decarboxylase (GAD) affect the Gamma-aminobutyric acid (GABA) -the primary inhibitory neurotransmitter in the nervous system- expressing neurons and cause heterogenic neurodegenerative diseases [91-93]. Autoantibodies against GABA (GABA-ab) are also one of the main reasons for type 1 diabetes (T1D) with or without neurodegeneration [91-93]. Interestingly, serum positivity of GABA-ab is possible in healthy individuals, and titrations are not correlated with disease severity [92].

As GAD is a cytosolic antigen, autoantibodies should find them in the cell, and there are in vitro [94] and in vivo [95] experiments that have already shown that. Hampe et al. incubated GAD65 expressing AF5 cell line (an immortalized rat CNS cell line) with

monoclonal GAD-ab for four hours at 37°C. Flow cytometry and confocal microscopic analysis show that dose and time-dependent antibody uptake happens [94]. Hampe did not report GAD destruction in the cells. They reported that the intrathecal human GAD-ab injected rats showed dose-dependent and epitope-specific motor and cognitive function deficiencies. However, they did not report if there were any GAD destruction clues in the rats' brain slices [94].

Gresa-Arribas et al. incubated live rat hippocampal neurons for 24 hours at 37°C with GAD-ab containing cerebellar ataxia, stiff person syndrome (SPS), limbic encephalitis (LE) with epilepsy patients CSF and they could not demonstrate the GABA-ab internalization [92]. On the other hand, within the same experiment, they used NMDR-ab positive CSF as the positive control of their methodology, and they were able to show NMDAR-ab internalization [92].

Chang et al. immunized GAD-Enhanced green fluorescent protein (EGFP) expressing mice with human GAD, and animals became GAD-ab positive. They lost some GAD-EGFP-expressing cells in the brainstem without observing behavioral abnormality [95]. However, they reported an unexpected observation. They conducted an in vitro internalization assay with the neuronal culture of GAD-EGFP mice and serum of GAD-immunized or PBS-immunized mice. Antibodies in the serum of GAD-immunized mice bound densely to GAD-EGFP expressing neuronal surfaces. In contrast, PBS-immunized mice antibodies did not. They also could not see antibodies inside the cells, although they reported GAD-EGFP dismissal [95].

Underneath these controversial reports can be the same reason for the heterogeneous characteristics and the differentially affected brain zones in GABA-ab-related diseases.

In summary, the autoantibodies against intracellular antigens do not make the same impact on all neurons (even though they may be the same type of neurons) at the same level, and there are obstacles hindering stable observations of uptaken antibodies. We should consider some major parameters to understand the fate of intracellular antigen-targeted autoantibodies. In quest of these parameters, it may be helpful to look from a broader perspective at AIDs in general.

1.7. SLE and SS: A kind of meeting point for AIDs

Statistical data highlight comorbidities of AIDs. The study of Eaton and friends reports SS and SLE showing the high odds ratio comorbidities within the list of researchers AIDs. SLE and SS do not only show high ratio comorbidity with each other.

They also show comorbidity with the same diseases pernicious anemia, dermatomyositis, scleroderma, chronic hepatitis, autoimmune hemolytic anemia, primary biliary cirrhosis, seropositive rheumatoid arthritis, dermatopolymyositis, and wegener granulomatosis (Figure 1.3) [8]. Shared serological autoantibodies (see Figure 1.4) of SS and SLE patients [96] point to the possible underlying reasons for the high comorbidity between SLE and SS exhibit. From these shared serological features (ANA, Anti-Ro52/Trim21, anti-La, anti-dsDNA, anti-Sm, anti-ribosomal P protein, antiphospholipids, anti-c2M3, rheumatoid factor, cryoglobulins, low C3, and C4 complement fractions) [96]. We focused on the protein Ro52/Trim21 not only because it is the target of common antibodies of these two distinct diseases but also (within the other reasons which will be mentioned) it is reported as the highest affinity Fc receptor in humans. Here, let us note that rheumatoid factor, actually another common autoantibody between SLE and SS, is targeting the Fc portion of Igg.

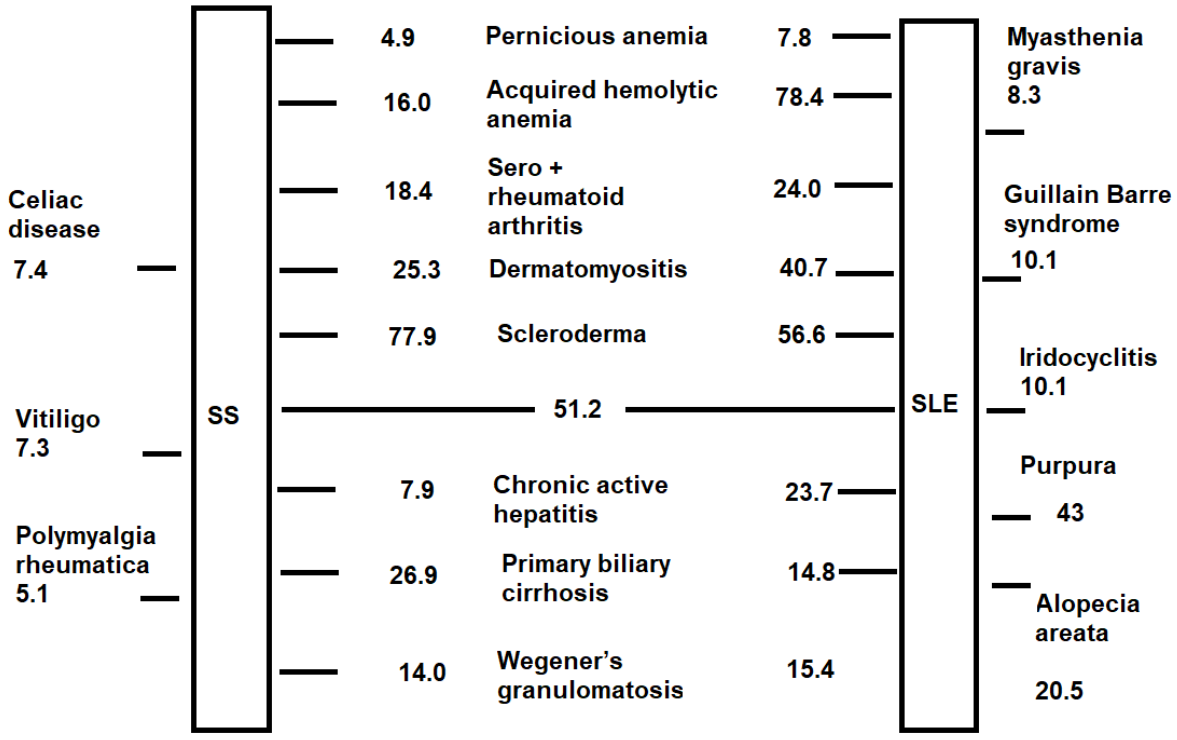

Figure 1.3 Odds ratios of comorbidities with SLE and SS. The figure is adapted from [8] ("Table 2 Comorbidity of 31 autoimmune diseases in Denmark: numbers of co-occurring cases above the diagonal, pairwise odds ratios below the diagonal").

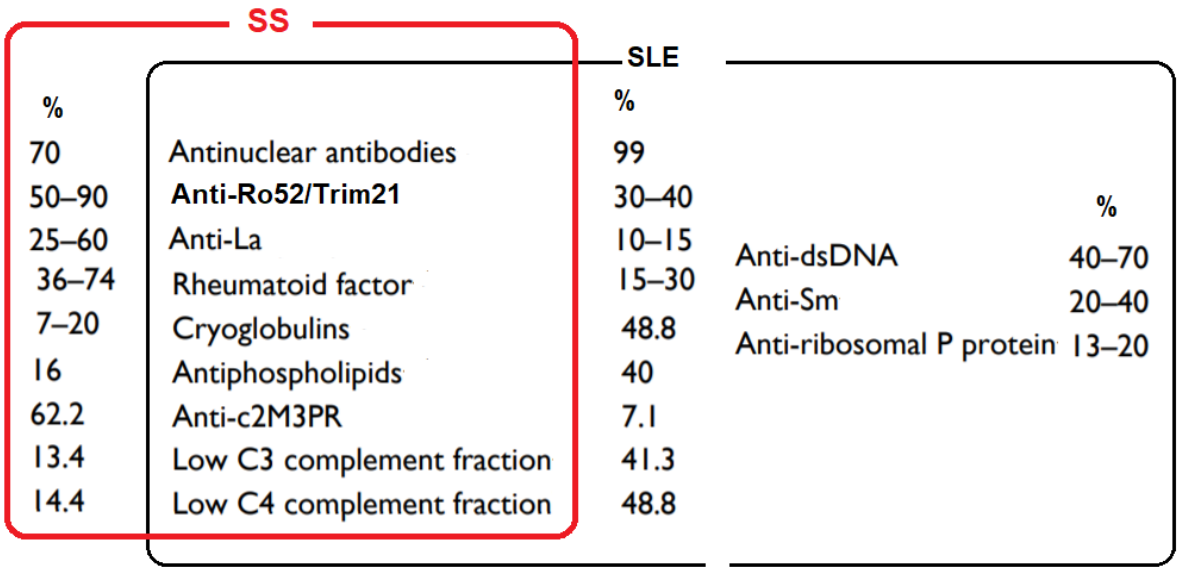

Figure 1.4 Shared serological autoantibodies of SS and SLE patients. The figure is adapted from [96] ("Table 1 Main serological features observed in pSS and SLE").

1.7.1. Susceptibility loci of SS and SLE.

The Ro52/Trim21 protein is an important intersection point between SS and SLE. However, the Ro52/Trim21 gene is not among either the over 40 susceptibility loci of SLE [97,99] or the susceptibility loci of SS [99] (see Figure 1.5). To establish a connection between reported common susceptibility genes and shared autoantibodies, we looked closely at the functions of the related proteins.

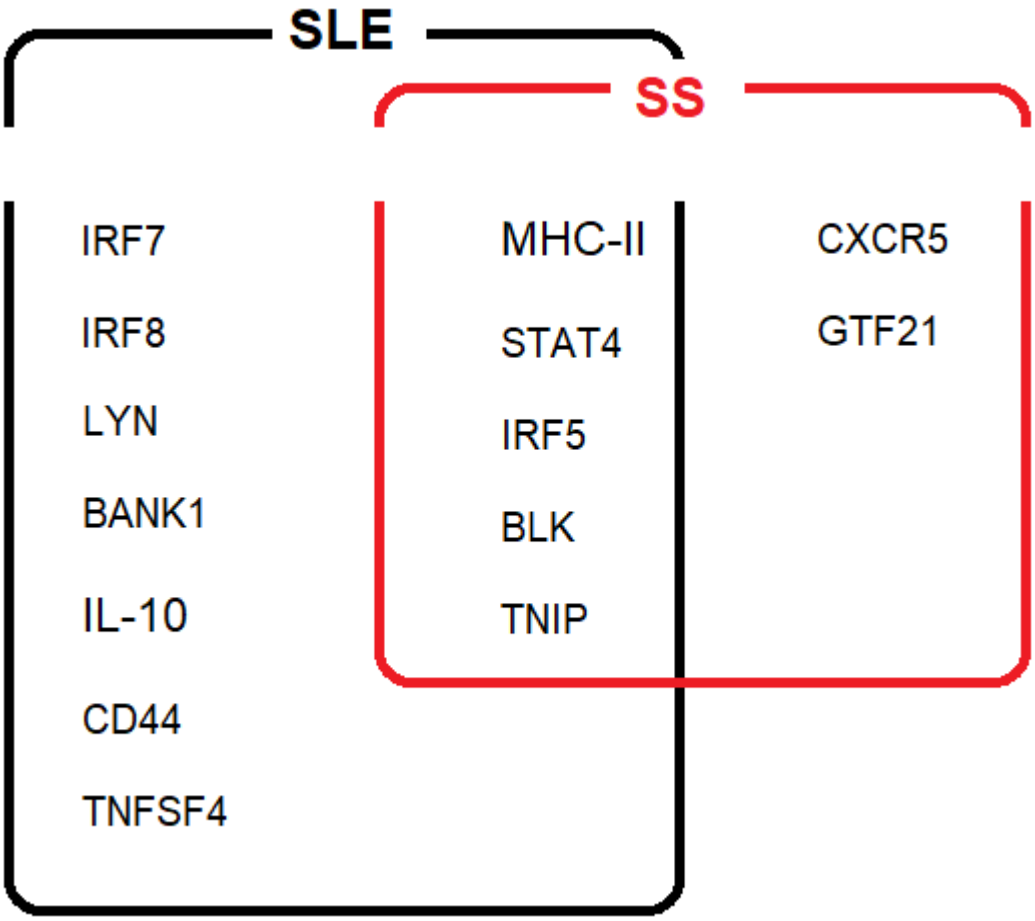

Figure 1.5 The leading susceptibility loci of SLE and SS. Major histocompatibility complex class II (MHC II), Signal Transducer and Activator of transcription (STAT) 4 (STAT4), Interferon Regulatory Factor (IRF5), B Lymphoid Tyrosine Kinase (BLK), TNFAIP3-interacting protein (TNIP) are the shared leading susceptibility loci of SLE and SS. The figure is inspired by [97-99]

1.7.1.1. Interferon regulatory factor (IRF) 5

IFR5 is "the most strongly and consistently associated SLE loci identified" (cited from 100). IRF5, like the other members of the IRF family (nine members (IRFs 1–9) in mammals) involved in signaling for virus responses as their name suggests regulating interferon production [101]. However, new studies suggest a shift from being a member to a central regulator of cellular inflammatory responses [101]. IRF5 (assisted by RelA, the subunit of NFKB [102]) mediates the expression of IL-6, IL-12, IL-23, and TNF-alpha [101]. Under inflammatory conditions, high IRF5 expression is reported in

monocytes, macrophages, B cells, and DCs [101] and adipose tissue as a novel adipose tissue marker for metabolic inflammation [103].

There are many isoforms of IRF5 (V1-11) in humans[101]. IRF5 isoforms can be grouped into three (1-A, 1-B,1-c) according to their transcriptional functions, and the group 1-B (v2, v9, v10) cause overexpression of IRF5 and linked to SLE [101].

Animal studies show that IRF5 plays a role in B-cell development and function, and "murine models of lupus lacking the IRF5 gene showed reduced ANA, glomerulonephritis, and pathogenic autoantibody production" [100]. It is reported that "IRF5 is constitutively localized to the nucleus of human SLE memory B cells and that activation of healthy donor B cells results in IRF5 nuclear localization" [100]. The role of IRF5 is in addition to cytokine expression, "differentiation, autoantibody production, and have identified a pro-effector B cell gene program that IRF5 regulates" [100]. Thus overexpression of IRF5 leads to B-cell pathology similar to SLE [100]. In contrast, the overexpression and deficiency of IRF5 are reported to cause impaired functions of B-cell [104].

The central role in inflammation of the IRF5 put the manipulation methods of IRF5 expression (i.e., siRNA, CRISPR/Cas9, adenoviral delivery) into the target of researchers, the efforts are whether in the direction of maintaining high expression (mainly against tumors) or repression against detrimental allergen reactions [102]. One of these methods is the IRF5 degradation through E3-ubiquitin ligase TRIM21/Ro52. "Following toll-like receptor 7 (TLR7) activation, TRIM21 mediates degradation of IRF5 in an isoform-specific manner, that is, it targets isoforms v1/v5, but isoforms v2/v3 are resistant" (cited from [102]). Remember that the isoforms v2, v9, and v10 are reported in correlation to IRF5 overexpression.

Another manipulation method of IRF5 expression in the focus of researchers is kinase inhibitors [101]. "Several kinases including RIP2 from the receptor-interacting protein (RIP) kinase family, and TAK1, IKKalpha, IKKbeta, IKKepsilon and TBK1 from the IKK family, are thought to phosphorylate IRF5" (cited from [101]). IKKbeta activity is shown as an actor for the activation of IRF5 (phosphorylation and nuclear translocation and an IFN response) [102]. IKKalpha also regulates IRF5 in the MyD88-IRF5 pathway but negatively [102]. As the IKKalpha and IKKbeta are the two keys of inflammatory pathways, we see the highly complex situation of immune responses of cells explain why "transcription factors are traditionally considered as 'non-druggable' targets" (cited from [102]. On the other hand, these pathways give us essential clues connecting

TRIM21/Ro52 to not only IRF5 but also to the other Susceptibility loci of SS and SLE (BLK, TNIP1, and MHC II) because they all play roles by interacting with each other.

1.7.1.2. Signal Transducer and Activator of transcription (STAT) 4

Another susceptibility loci of SS and SLE, STAT4, is a member of the STAT protein family. Stat4 responding to interleukin-12 (IL-12) sourced from stimulated macrophages, neutrophils, B cells, and dendritic cells (DC) [102] promotes T-helper type 1 (Th1) cell development, interferon IFN-γ production [103]. STAT proteins (are monomers without activation in the cytoplasm, and following their stimulation, they form homodimers [104]. These homodimers translocate to the nucleus and bind to specific promoter elements of IFN-inducible genes [104]. Polymorphism of STAT4 has an additive effect on the association of IRF5 with SS [104].

As it is a responder of IL-12, for a better understanding of the roles of IRF5 in immune response, a brief look at IL-12 should be helpful. After their discovery, Kobayashi et al. named IL12 (a hetero dimeric 70 kDa cytokine with p35 and p40 subunits) as a "natural killer cell stimulatory factor" in 1989, then other heterodimers were included in the IL-12 cytokine family which are IL-23, IL-27, IL-35, IL-39 [105]. The distinct functions of IL-12 family members and STAT4, respectively, have a central role in immune response, especially in TH1 and Th17 cells, and regulatory T cell (Treg) differentiation [105-107].

1.7.1.3. B Lymphoid Tyrosine Kinase (BLK)

From our list of susceptibility loci of SS and SLE, which we have chosen according to odd ratios, BLK is a "non-receptor tyrosine kinase of the Src family of proto-oncogenes involved in B-cell receptor signaling and B-cell development" [108]. Disease-correlated genetic variants of BLK were reported resulting in low levels of BLK and "increased B1-cell subset and lupus-related phenotypes in mice and humans" [109,110].

The importance of BLK in autoimmunity arises from its connection with the B-cell scaffold with ankyrin repeats 1 (BANK1) protein. High BANK 1 expression and regulatory B cells -with suppressing capacity of T cells proliferation- in the blood cells of kidney transplantation tolerant patients comparingly low BANK 1 expression and regulatory B cells in graft rejected patients' blood [111]. According to the suggested model by Berre et al., phosphorylated BANK1 binds to BLK and PLCg2. This complex (BANK1 & BLK / PLCg2) leads the B cell to apoptosis or tolerance. However, as in the graft-rejected patients, due to the insufficient number of this complex, this pathway (It is

not thought as the only pathway leading to B cell apoptosis) becomes less efficient [111]. Here, we speculate that due to disease-correlated genetic variants of BLK may slow down the self-degradation of B cells after antigen clearance and hinder the tolerant phase of the immune response.

BLK plays a role in pancreatic beta cells and insulin synthesis and secretion, and its deficiency also leads to the disease maturity-onset diabetes of the young (MODY). As it may affect insulin secretion, it can be expected to cause indirect metabolic secondary involvements to the diseases.

In summary, while the genetic variants of other susceptibility loci of SLE and SS encourage inflammation, pro-inflammatory T and B cell macrophage differentiation in BLK's genetic variants helps the activated B cells' prolonged life or proliferation. Thus, the autoantibody production becomes unresponsive to the suppressions.

1.7.1.4. TNFAIP3-interacting protein 1 (TNIP1)

As it has a key in cell defense, especially for the status of the immune cells, the NF-κB activation is tightly controlled by several mechanisms [112]. Ubiquitination of proteins (i.e., IkB-alpha, P100, RIP1, TRAF) is one of these controlling mechanisms, but on the other hand, deubiquitination is also among these mechanisms [112]. Remember the activation of IRF5 is sensitive to the NFKB subunits -IKKalpha and IKKbeta- which are themselves sensitive to the mechanisms mentioned above, including deubiquitination after all TNIP1, the gene of a deubiquitination protein TNFAIP3-interacting protein 1 (TNIP1) (Aliases ABIN-1, NAF1, VAN, nip40-1) are in the list of the highly associated genes with autoimmune diseases [112].

Animal studies suggest that the loss of function of TNIP1, "enhanced production and activations of myeloid and B cells," plays a role in developing SLE and lupus nephritis [113].

1.7.1.5. Major histocompatibility complex class II (MHCII)

MHCII molecules (cell-surface glycoproteins) are essential for initiating adaptive immunity via the presentation of pathogen-derived peptides to the antigen receptor of CD4+ T cells [114]. MHCII shows extensive polymorphism and is associated with many autoimmune diseases [114]. By induction, APCs constitutively and the other cells express MHCII molecules [114]. MHCII expression is tightly controlled by the class II transactivator (CIITA) and posttranslational regulations. As a result of this tight control

after the presentation, both the expression and degradation of MHCII (depending on the cytokine regulation) may stop so the long-lived MHCII-pathogen-related peptide complexes can reach the lymph nodes [114]. Degradation of MHCII at the end of the activation of the APCs starts via ubiquitination-triggered internalization [114]. The ubiquitination happens via membrane-associated RING-CH 1 (MARCH-1), which is why under inflammatory conditions, MARCH-1 is subject to downregulation in conventional DCs [114]. "MARCH-1 also mediates cytokine regulation of MHCII turnover in monocytes and B cells" (cited from [114]). It is reported that the MHCII turnover rate in non-obese diabetic (NOD) mice is high, and there is a debate about fast MHCII turnover rate results in impaired T-cell tolerance and may be related to autoimmunity [114].

The endothelial upregulation of MHCII molecules is reported in several autoimmune diseases, including MS [115]. Activating endothelial cells means expressions of immune cells guiding proteins and less tight vascular systems [115]. Endothelial cells do not express costimulatory B7, but this is not the case for brain microvascular, which may be a factor of T cell transmigration [115]. GAD65 (the protein which can cause several types of neurological diseases) presentation to the specific T cell through the endothelial cells is reported (but not in the brain) [36]. Endothelial cells stimulated by IFN-γ can present the autoantigen GAD65 from the islets of Langerhans to GAD-65-specific T cells [36]. Furthermore, effective antigen presentation enhanced the migration of GAD65-specific T cells across endothelial cell monolayers and migrated T cells retained full proliferative capacity [115].

Molecular mimicry may also be a reason for the association of the MHCII with Auto immunities [115].

1.8 TRIM21/Ro52

The susceptible loci of SS and SLE we have been over (IRF5, STAT4, BLK, TNIP1, MHCII) are parts of the typical immune response pathways and orchestration of different types of immune cells. Now let us look at where TRIM21/Ro52 is in the immune response and what the interactions are.

1.8.1 The protein turns the cells into an active part of the innate and humoral immune systems.

In 2007 Leo et al. described the C-terminal PRYSPRY domain of TRIM21/Ro52 as very fast and with a very high-affinity binding capacity to the FC fragment of IgG (salt-

dependent but - in contrast to the pH-dependent binding of the neonatal Fc receptor (FcRn)- pH-independent) [116,117]. In 2010 Mallery et al. infected several types of cells (HeLa, TE671, and HT1080) with AdV5-GFP and reported rapid viral cleaning by TRIM21/Ro52 with the help of antibodies against the virus. After all," humoral immunity is not limited to extracellular protection but can neutralize a virus even after it has entered a cell" [118].

It has also been shown that TRIM21/Ro52 is an important part of innate immunity. "The mitochondrial adaptor molecule MAVS plays a critical role in the innate immune response to viral infection," and TRIM21/Ro52 "interacts with MAVS to positively regulate innate immunity" and "under viral infection, TRIM21 is upregulated through the IFN/JAK/STAT signaling pathway" [119]. let's put it on the record, “some E3 ligases, like AIP4, RNF5, RNF125, Smurf1, Smurf2, TRIM25, and MARCH5, negatively regulate the activity of MAVS by catalyzing K48-linked polyubiquitin for its degradation” and TRIM3, TRIM44 and TRIM21/Ro52 enhancing MAVS activity [120]. McEwan et al. reported the neutralization capacity of TRIM21/Ro52 of antibody-labeled tau assemblies in a tau "seeding" assay in humans [121]. As an FcγR, TRIM21/Ro52 should have the potential neutralization capacity of any cytosolic antigen bound to antibodies. However, the matter is that the antibody-antigen complex should be inside the cell. In 2017 Clift et al. developed an acute and rapid degradation of endogenous proteins named -Trim-Away-. Basically, they inserted (in several experiments) into different types of cells nine different endogenous antibodies with several methods (microinjection, electroporation, lipofectamine) and have consistent results giving the following confident conclusions ((see Table 1.2) [2,122].

Researchers obviously concentrated on and also succeeded in developing a new protein depletion method. If TRIM21/Ro52 plays a role in a scientific protein depletion model, isn't it possible it has a role in depleting auto-antibody-targeted cytosolic proteins? Suppose degradation of autoantibody and its cytosolic target happens within minutes of proteins. In that case, this may also explain why some researchers could not detect the autoantibodies inside the cells, and some were able to. If the number of antibodies flowing into the cell is higher than the TRIM21/Ro52, the antibodies are highly probable detectable. However, if the situation is vice versa, they should already be degraded through protease. Here, suppose naive antibodies flow into a cell. In that case, another question may arise, as the target antigen is absent in the cytosol -this must be the case in many cells during an IVIG therapy- do they still subject to degradation through protease?

Table 1.2 Some of the details of the Trimaway* applications reported in [123].

* Trimaway is a rapid cytosolic protein depletion method with antibodies.

Cell line	**antibody**	**Antibody insertion method**	**The Outcome in addition to protein degradation**	**Time**
NIH 3T3 cells overexpressing mCherryTRIM21 (a fibroblast cell line)	anti-GFP antibody - Free GFP	microinjection	MG132 (protease inhibitor) suppressed.	80 min.
isolated mouse oocytes (Primary Cells)	anti-GFP antibody - Membrane anchored GFP	microinjection	Control IgG had no effect on GFP protein levels.	60 min
isolated mouse oocytes (Primary Cells)	anti-Eg5 antibody	microinjection	Eg5-mEGFP expression transformed the monopolar spindles back to a bipolar state	50 min.
NIH 3T3 cells expressing disease-causing variants of huntingtin protein together with mCherry-TRIM21	antibody (3B5H10) which specifically binds to the disease-causing variant of huntingtin	microinjection	normal huntingtin protein was preserved	80 min
oocytes that expressed both the normal and the disease-causing variant of huntingtin simultaneously	antibody (3B5H10) which specifically binds to the disease-causing variant of huntingtin	microinjection	normal huntingtin protein was preserved	2.5 hour
NIH 3T3 and HEK293T (bulk)	anti-pericentrin antibodies	electroporation	Altered Cdk5rap2 localization as a result of insufficient pericentrin	3 hr

overexpressing mCherry-TRIM21 HEK293T cells	anti-mTOR antibody	electroporation	Degradation was not complete, perhaps because a subset of mTOR resides within intracellular compartments inaccessible to antibody	
HEK293T cells overexpressing mCherryTRIM21	anti-IkBa antibody	electroporation	degradation of IkBa and the substantial induction of NF-kB activity	-

1.8.2. TRIM21/Ro52 part of immune regulation

TRIM21/Ro52 expression is reported in many tissues (brain, thymus, lung, heart, intestine, liver, spleen, kidney, testis, bone marrow), including immune (B, T, NK, myeloid and dendritic) cells [124]. TRIM21/Ro52-KO embryonic fibroblasts are reported with upregulated proinflammatory cytokines (IL-1, IL-6, TNF-alpha, and CXCL10), higher TLR-mediated NF-B activation, impaired ubiquitylation of IRF3 and IRF8 but without detected abnormalities in the composition or functions of immune cells [124]. TRIM21/Ro52 regulates IRFs through ubiquitination [124], which is important for the necessary signal termination [124]. On the other hand, some viruses affect the TRIM21/Ro52 and consequently suppress an immune response. For example, the influenza A virus causes upregulation of the N-Myc and STAT interactor (NMI), which rise the ubiquitination of IRF7 by TRIM21/Ro52 while the Sendai virus and FoxO1 virus causes a rise of IRF3 degradation [124]. Unlike the viruses using TRIM21/Ro52 for immune response, "peripheral blood mononuclear cells (PBMC) isolated from SS patients displayed elevated TRIM21 transcript expression, correlating with increased IRF1 and IRF2 mRNA" [124]. Interestingly in SS patients, besides the upregulated TRIM21/Ro52, upregulated IRF3 and IRF5 levels are reported as clues of more complex interactions and immune response homeostasis [124].

Dysregulated, activated autoantibodies secreting B cells are another characteristic of SLE and SS patients [122,123]. Animal studies with TRIM21/Ro52 deficient (C57BL/6) mice

show a) increased NF-κB dependent proinflammatory cytokine production b) increased IL-23-dependent Th17 differentiation, and type I IFN [2]. Another animal study with "MRL/lpr lupus-prone mice in which Trim21 gene is deleted" shows symptoms of SLE pathology, high (auto and not auto) antibody production, high urine protein levels, aberrant B-cell differentiation, increased expression of transcription factors, IRF5 and BLIMP-1 [2]. Tan and Xia reported similar results, aggravation of epithelial keratitis in C57BL/6J mice after herpes simplex virus (the leading cause of infectious blindness in the developed world) through the elevated TRIM21/Ro52 suppressed IRF3 activation [125]. Another study using human microglial cell line (CHME3 cells) by knocking down TRIM21/Ro52 draws attention to rising TRIM21/Ro52 levels in response to Japanese encephalitis virus (JEV), which suppresses immune response (IFN-Beta) in control groups while significantly higher IFN-Beta levels in TRIM21/Ro52 KO group because of the missing TRIM21/Ro52 ubiquitylation of IRF3[126].

The murine macrophage experiment of Kong et al. displayed IRF8 ubiquitylation by TRIM21/Ro52 and enhancement of IL-12p40 expression [127]. Let's remember IL-12 triggers the phosphorylation, and nuclear translocation of STAT4 initiates a cascade of events in B cells leading to Th1-like differentiation [128]. One suggestion to explain this situation is that disrupted ubiquitylation of IRF 5 promotes IRF4 activation (although it is known such a relation, it needs to be clarified for the question of how) and leads to B cell activation and antibody secretion.

In summary, TRIM21/Ro52 has multiple contradictory roles in intracellular immune response, and the reflections of these responses are not limited to the cell. However, the differentiations of surrounding immune cells are also part of these reflections.

1.9. Intravenous immunoglobulin (IVIG)

IVIG is an intravenous application of a mix of serum IgG (other immunoglobulin isotypes can also be found) of ~3,000–60,000 healthy blood donors [1]. The first application in the 1950s was "by Bruton in pediatric patients with primary immunodeficiency diseases (PID)" [110]; even though the application started with immunodeficient patients (doses were approximately 0.5g per kg body weight), in contrast to PID, the usage for the suppression of autoimmune diseases, i.e. ("at much higher doses, which range from 1–3g per kg body weight") come into practice [1].

"Besides the licensed indications of IVIG therapy for immune thrombocytopenia (ITP), Guillain–Barré syndrome, Kawasaki's disease, and chronic inflammatory

demyelinating polyneuropathy (CIDP), it is used off-label for a large number of chronic inflammatory diseases, such as multiple sclerosis, systemic vasculitis, rheumatoid arthritis, systemic lupus erythematosus (SLE), autoimmune hemolytic anemia (AIHA), autoimmune skin-blistering diseases, graft-versus-host disease, and sepsis" (sited from [1]), multifocal neuropathy with conduction block, and acute myasthenia gravis [129], GAD-ab related neuropathies [130,132].

The immunomodulatory functions of IVIG lay underneath these controversial applications, from enhancing to suppressing the immune system [133]. On the other hand, the results of the IVIG therapies vary, i.e., "not all patients with SPS seem to respond to IVIG, and a partial response is frequently reported in patients with anti-GAD" [134].

1.9.1. Mechanism of action of IVIG

IgG fragmentation via pepsin at a pH of 4.0 Figure 1.6 is a widely used method to understand the Mechanism (s) of action of IVIG [135,136], and many reports are suggesting that both fragments have roles in immune regulation. The Fab part plays a role in autoantibody neutralization by anti-idiotypic IgG (Figure 1.7) in case there are enough doses of the specific anti-idiotypic IgG in a lot of IVIG [136]. The autoantibody neutralization effect of anti-idiotypic antibodies is reported for autoantibodies against self-antigens, i.e. (dsDNA, acetylcholine receptor, desmoglein 1+3, factor VIII, GM1 ganglioside, thyroglobulin) [138]. The second Fab part that plays a role is self-antigen neutralization [138]. The proteins becoming harmful for patients in specific conditions, e.g., BAFF, APRIL, GM-CSF, IFN-α, IL-1α, Fas, SIGLEC-(8 and 9), adhesion molecules, T-cell receptor, FcγRs, Amyloid beta [138]. The third way is scavenging complement components C3a, C3b, and C5a through anti-idiotypic binding [138].

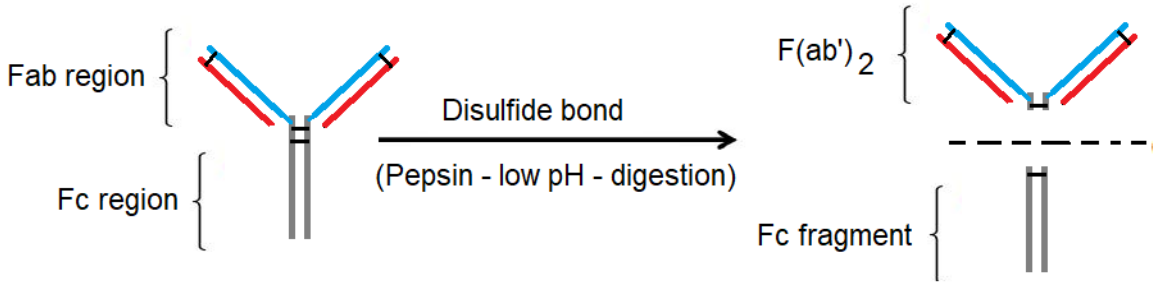

Figure 1.6. IgG fragmentation via pepsin at a pH of 4.0. a widely used method to understand the mechanism(s) of action of IVIG, and many reports suggest that both fragments have roles in immune regulation.

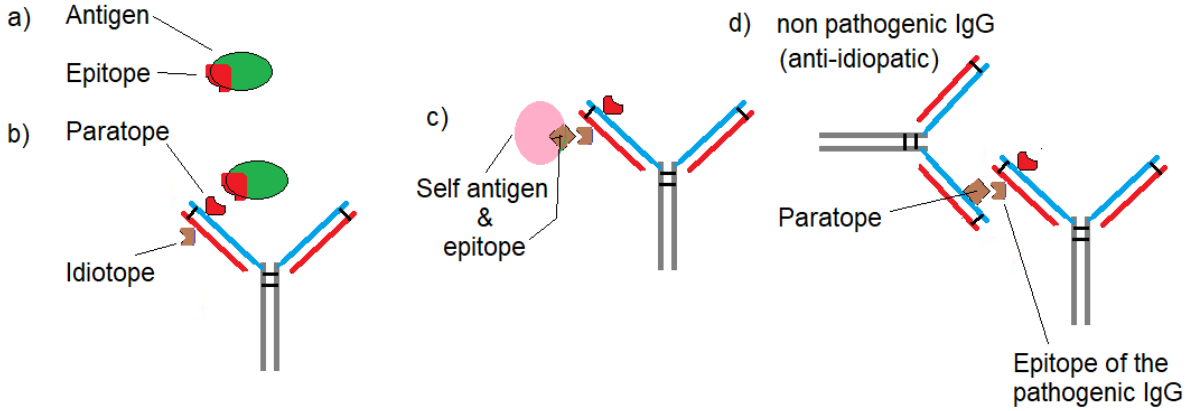

Figure 1.7. Neutralization of pathogenic self IgG via IVIG. We call the parts of antigens recognized by antibody epitopes (a). Antibodies have paratopes that match the epitopes of the antigens, but they may be found in other matching points which match any other antigens called idiotopes (b). These idiotopes may match self-antigens (c), and these idiotopes may be the epitopes of the IgG of IVIG(d).

The role of the IVIG through the Fc part of the IgG possibly happens via Fc receptors. Within the Fc receptors, the neonatal Fc receptors (FcRn) are preventing the catabolism of IgG and prolonging the half-life of immunoglobulins (Figure 1.8.), so the saturation of the FcRn by IVIG shortens the half-life of the patient's IgGs which eventually diminishes the pathogenic autoantibody levels [138]. Supporting this hypothesis, FcRn-deficient mice were reportedly resistant to autoantibody-induced symptoms [138]. Blocked FcγRs of the phagocytic cells (Figure 1.9.) is another suggested reason for breaking the presenting of the self-antigen vicious cycle by the Fc part of the IgG [138].

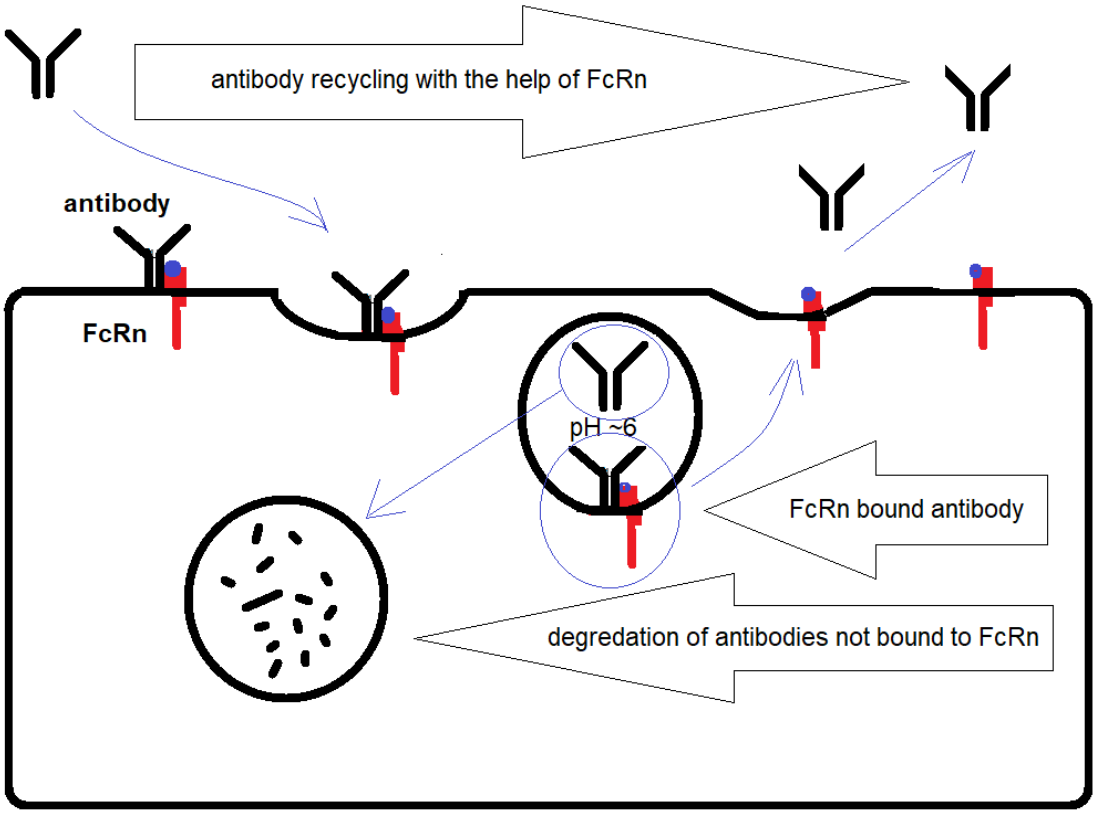

Figure 1.8. IVIG and the role of the Fc receptors neonatal (FcRn). While the IgGs bound to FcRn are subject to recirculation, the IgGs bound to FcγRs are subject to lysosomal degradation (escape from the lysosome may end with proteasomal degradation, not shown in the figure)

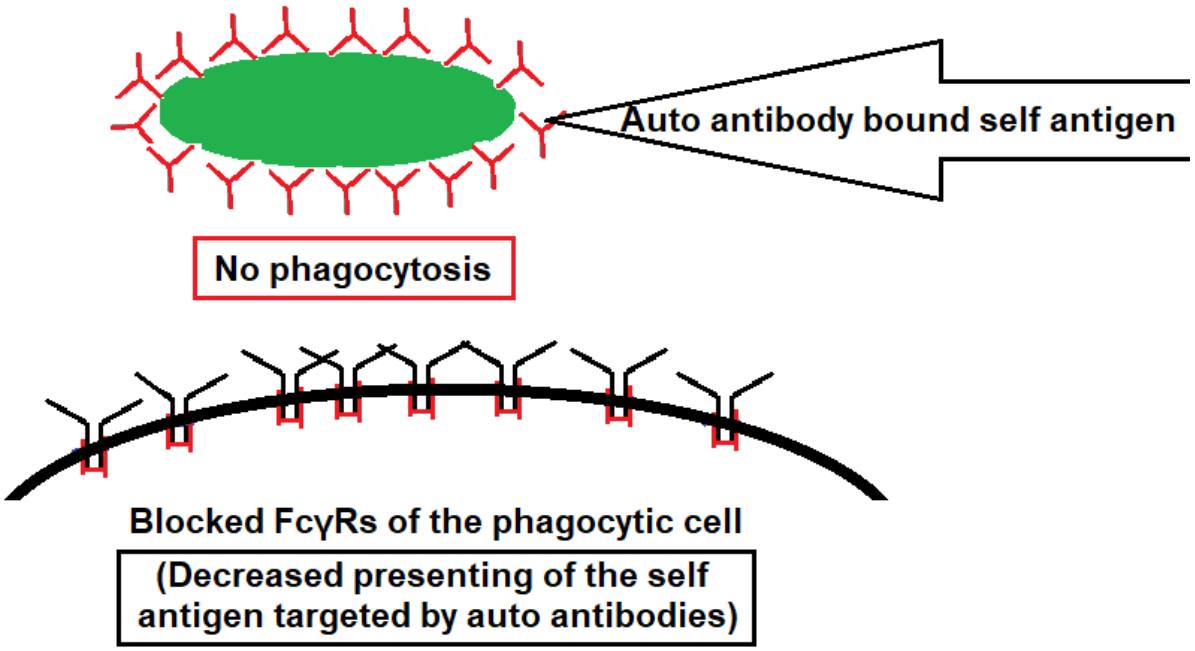

Figure 1.9. Saturation of FcγRs of the phagocytic cells by IVIG-sourced IgG. One of the mechanisms of action of the IVIG is the FcγR blockage by the non-harmful IgGs, which compete with self-destructive autoantibodies.

As the Fc fragment of IVIG binds to the FcγRs, it is expected that FcγR blockade and studies demonstrate the FcγR blockage and respective outcomes, i.e., an increase in platelet numbers in immune thrombocytopenic purpura patients [139].

IVIG modulates the components of the complement system, i.e., amplifying the C3 convertase level (in patients with the complement-dependent autoimmune disease "with an immediate and long-lasting attenuating effect") [140]. For example, It is known under ischemic conditions a) increasing C5a levels increase CD11b and ICAM1 expression b) increasing iC3b upregulates the CD11a expression[141]. It is reported, "IVIG treatments suppressed ischemia-induced increases in the levels of ICAM-1, CD11a, and CD11b, markers of endothelial cell adhesion, lymphocyte infiltration, and microglial activation, respectively" [141]. The study of Basta and friends with a mouse model of asthma confirms C3a and C5a IVIG relation and indicates one of the interactions happens through a constant region of F(ab)′2-IVIG [142].

In summary, the IVIG treatments' mechanisms of action depend on many factors. Furthermore, the treatment alleviates or not the symptoms of AID patients under the dynamic nature of the immune system. As we will discuss later, the results of this research

suggest IgG neutralization through TRIM21/Ro52 as a factor of efficacy of the IVIG treatment.

1.10. HMC3 Cell line

The human microglial clone-3 cell line (HMC3) (ATCC®CRL-3304, (established in 1995 through SV40-dependent immortalization of human embryonic microglial cells) is one of the preferable microglia in-vitro study cell lines with satisfactory properties of cytokine and chemokine production, phagocytosis, free oxygen, and nitrogen species production, migratory capacity and secretion of matrix metalloproteinases, cell morphology, and antigenic profile[143]. HMC3 was used in around seventy microglial cell research listed in 'Pubmed' by August 2022. As the different names are given to the same cell line by other suppliers (CHME3 and CHME5), the number of scientific studies using HMC3 is around a hundred and forty. The production of cytokines, chemokines, and other inflammatory mediators by HMC3 lines slightly differs from the original microglial cells [143]. However, this expected result of the immortalization seems negligible when considering the opportunity to work with a human-derived microglia cell.

1.11. The aim of the thesis

The thesis aimed to investigate whether IgG has a direct impact on cytosolic TRIM21/Ro52 in a human microglial cell line (HMC3) and if the answer is yes, what the acute and relatively long term (within the times of 20 min, 60 min, 4 hours, 24 hours and 48 hours) impacts are under naive and immune stress conditions. First, the expression of the characteristic microglial membrane proteins; IBA1, CD11b, and CD45 is analyzed with flow cytometry. Then the immune response of the HMC3 cell to the LPS is investigated with the DHR123 method by flow cytometry to determine optimum LPS concentrations. Finally, the IgG and the TRIM21/Ro52 levels of the HMC3 cells (naive and 24 hours with LPS (500ng/ml) preincubated) incubated with 100ug/mL IgG for (0min, 20min, 60min, four h, 24h, 48h) are evaluated with flow cytometry and confocal microscopy.

The thesis hypothesized that IgG and the TRIM21/Ro52 levels in the cells would change in a time and immune-state-dependent manner. In the beginning, (at least a group of) IgG subject to internalization will be cathed by TRIM21/Ro52 proteins and degraded together. As a result of this phenomenon, the flow cytometric measurements and confocal microscopy imaging will suggest only a negligible amount of IgG inside the cells and accompanying reduction of the TRIM21/Ro52 levels. However, the cells will adapt

themselves against the level of TRIM21/Ro52 reduction (supposed to happen around 4 hours). The IgG and TRIM21/Ro52 existence in the cells will vary according to the balance between the IgG influx and the ability of the cells' TRIM21/Ro52 expression. The immune state (activation with LPS) is expected as an IgG influx-raising factor.

Together, in the beginning, the internalized IgG will be degraded together with TRIM21/Ro52, and the IgG's existence in the cell will be detectable only under TRIM21/Ro52 depletion and within the period between the internalization and degradation. Unfortunately, the concepts (FcγR bound IgG and IgG in lysosome) are not taken into consideration in the experiment. However, the existence of any major effect of these concepts could be understandable from the results.

2. Materials and methods

The steps of our study (details are below) were a) evaluation of protein expressions (IBA1, CD11b, and CD45) of HMC3 cells with flow cytometry, b) Determining optimum LPS dosage for generating immune response c) Time and immune state-dependent (naive and LPS primed HMC3 cells) evaluation of TRIM21/RO52 and IgG level's change (by flow cytometry and confocal microscopy) during IgG incubation. The flow chart of the experiment is given in Figure 2.1.

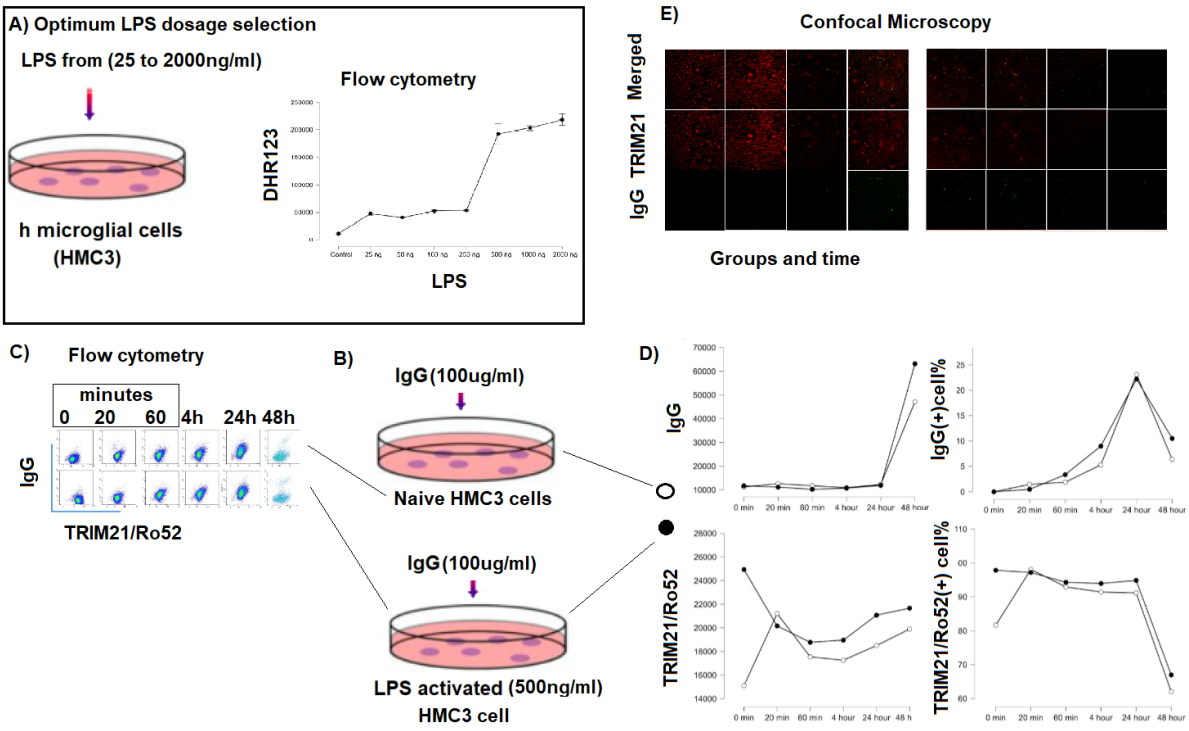

Figure 2.1. The flow chart of the experiment. a) First evaluation of the expression of the proteins (IBA1, CD45, and CD11b) was done by flow cytometry (not shown in the figure). Then the HMC3 cells were stimulated with LPS ranging from 25ng/ml to 2000ng/ml for selecting the optimum dose, evaluations were done by staining cells with DHR123 evaluating with flow cytometry. b) The selected optimum dose for LPS was 500ng/ml. The naive cells and with 500ng/ml LPS pre-stimulated (24h) cells were then incubated with 100ug/ml IgG for (0min, 20min, 60 min, 4h,24h,48h) c) The cells were evaluated with flow cytometry. d) The total IgG and TRIM21/Ro52 fluorescences measured and also changes the percentages of the IgG(+)cells and TRIM21/Ro52(+) cells were evaluated. Further mathematical analysis is done to understand the average TRIM21/Ro52 levels of the (+) cells and the per-minute change of the total

TRIM21/Ro52 levels of the colony. e) The IgG and TRIM21/Ro52 is evaluated with confocal microscopy.

2.1. Cell culture, reagents.

We obtained the human microglial clone 3 cell line, HMC3 (established in 1995, through SV40-dependent immortalization of human embryonic microglial cells, ATCC®CRL-3304) from Yeditepe University, Engineering Faculty, Genetics And Bioengineering Department as 5th passage/ frozen on 14th of June 2021, courtesy of Dr. Burcu Kasapoğlu. All experiments were conducted in triplicate (n = 3).

The reagents, used during the experiments are, EMEM (with Earle's salts, L-Glutamine, and sodium bicarbonate, liquid, sterile-filtered: Sigma Aldrich, #M4655), Fetal Bovine Serum (FBS- Sterile-filtered, suitable for cell culture: Sigma-Aldrich, #F7524.), Antibiotic mixture (50 mg/mL streptomycin in 0.9% NaCl (as sulfate) and 50,000 U/mL penicillin in 0.9% NaCl: Sigma-Aldrich, #11074440001). IgG from human serum was: Sigma Aldrich, #I2511.

For culturing HMC3 cells, EMEM supplemented with 10% FBS and 1%Penicillin-streptomycin solution was used (complete medium).

Lipopolysaccharide (LPS): Santa Cruz Biotechnology, #sc-3535. LPS was dissolved in sterile molecular grade water to obtain a 5 mg/mL stock solution. Further dilutions were obtained by diluting stock solution in a complete culture medium.

Dulbecco's Phosphate Buffered Saline (DPBS), Thermo Fisher Scientific, #14190250. DPBS was used for washing cells prior to detachment to remove residual complete culture media as well as washing cells after centrifugation for flow cytometric analyses.

Trypsin -EDTA solution. 0.25% (Sterile-filtered, suitable for cell culture), Sigma-Aldrich #T4049, used for detaching cells.

Dihydrorhodamine 123 (DHR123): Santa Cruz Biotechnology, #sc-203027. DHR123 stock solution was prepared by dissolving in DMSO at 10 mM concentration. Aliquots (10 uL) were stored at -20°C and were not subject to repeated freeze-thaw cycles. The working solution at 10 μM was obtained by dilution with DPBS.

2.2. Antibodies.

The antibodies were Recombinant PE Anti-Iba1 antibody (clone EPR6136, PE-conjugated: Abcam, #ab209942), Rabbit anti-human TRIM21 antibody (NOVUS

Biologicals, #NBP1-87122), Goat Anti-Rabbit IgG H&L antibody (Alexa Fluor® 647 conjugated: Abcam, #ab150079), Anti-human IgG antibody (FITC conjugated: Abcam, #ab6854), Anti CD45 antibody (clone J.33, Krome Orange conjugated: Beckman Coulter, #A96416), Anti CD11b antibody (clone Bear1, APC conjugated: Beckman Coulter, #A87782)

2.3 Equipment

The following list of equipment was used. Incubator (Galaxy 170 S, New Brunswick, 272007456), Light microscope (Olympus, CKX41), Laminar cabin (ESCO, LA2-4A1), JuLI Br Imaging System (NanoEnTek, JULI FL), Centrifuge (HITACHI, CTGEL), DxFLEX flow cytometry system coupled with blue (488 nm), red (635 nm) and violet (405 nm) lasers, Leica inverted confocal microscope.

2.4. Cell treatment

HMC3 cell line was cultured in EMEM supplemented with 10 % FBS, and antibiotics (100 IU/mL of penicillin and 100 µg/mL of streptomycin) in a cell culture incubator at 37°C, under a humid environment containing 5% CO2. Cells were seeded at the density of 2×10^4 cells/cm2 in 75 cm^2 flasks. The medium was replaced twice a week, and cells were passaged once they reached 90-95 % confluency. All studies were conducted with the cells in passage 7.

2.5. Determination of the Ideal Lipopolysaccharide Dose for Inducing Microglial Cell Activation

For determining the ideal concentration of LPS, cells were plated at 5×10^5 cells per 60 mm culture dishes, let to recover overnight, and stimulated with varying doses of LPS diluted in complete culture medium (25, 50, 100, 250, 500, 1000 ng/mL) the day after. Untreated cells were used as control. After 24 hours of LPS incubation, cells were detached with Trypsin-EDTA, washed twice with DPBS, and suspended in 500 µL DPBS, followed by labeling with DHR123 at the final concentration of 10 µM. Cells were incubated for 40 minutes under dark conditions at room temperature, and tubes were immediately acquired with DxFLEX flow cytometry system as triplicates. 2×10^4 cells at medium speed (30µl/minute) was read for each tube. Analyses were conducted with CytExpert for DxFLEX software.

2.6. Evaluation of the interaction between TRIM21/Ro52 and IgG Protein Levels in a Time-Dependent Manner

Upon internalization of the IgG, the interaction between TRIM21 and the internalized protein was analyzed with flow cytometry by labeling the cells with anti-human IgG antibody (FITC conjugated) and Rabbit anti-human TRIM21 antibody, followed by labeling with Goat Anti-Rabbit IgG H&L antibody (Alexa Fluor® 647 conjugated). Before the experiments, ideal antibody concentrations were determined by evaluating different concentrations 1/(500,1000,2000,500) and the results were presented in Appendix H.

Cells were seeded into 60 mm tissue culture dishes as 5×10^5 cells per dish and incubated overnight at 37°C to allow adherence, followed by either treatment with IgG (100µg/mL) or they were first primed with LPS (500 ng/mL) and then treated with IgG (100µg/mL).

Cells were then collected by detaching with Trypsin-EDTA on 20 min, 60 min, 4 hours, 24 hours, and 48 hours. Cell suspensions were washed twice with DPBS and fixed with 1% paraformaldehyde solution (pH: 7.4). Fixative was discarded and cells were permeabilized with DPBS containing 0.1% Triton X-100 (Sigma Aldrich Cat. No: T9284) and 1% Blocker™ BSA (Thermo Fisher Scientific, Cat. No: 37525). Fixation and permeabilization steps were carried out at 4°C in the dark for 30 minutes.

Cells were suspended in 500 µL DPBS containing 1% Blocker™ BSA and labeled with Rabbit anti-human TRIM21/Ro52 antibody (Novus Biologicals, #NBP1-87122) and anti-human IgG antibody-FITC conjugated (Abcam #ab6854) by incubating one hour incubation at 4°C under dark. By centrifuging at 400g for 5 minutes, primary antibody solutions were discarded, and cells were labeled with secondary antibody, Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647) (ab150079 -final concentration: 0.1mg/mL) for TRIM21/Ro52 evaluation by incubating cells at room temperature for 30 minutes under dark conditions. Following the removal of the antibody solutions by centrifugation, the cells were suspended in 500 µL of DPBS containing 1% Blocker™ BSA. Cells were immediately acquired with DxFLEX flow cytometry system as triplicates. 2×10^4 cells at medium speed (30µl/minute) was read for each tube. Analyses were conducted with CytExpert for DxFLEX software.

2.7. Characterization of the microglia

For determining IBA1, CD45, and CD11b protein levels, cells were seeded into 60mm tissue culture dishes as 5×10^5 cells per dish and incubated overnight to allow adherence. To stimulate cells, the culture medium was replaced with a fresh complete medium containing 500 ng/mL LPS and cells were further incubated for 24 hours. Cells were detached with trypsin-EDTA, washed twice with DPBS and suspended in 500 µL DPBS containing 1% Blocker™ BS. Cell surface proteins C45 and CD11b were labeled by incubating cell suspension with respective antibodies at room temperature under dark conditions for 15 minutes. Cells were then fixed with 1% paraformaldehyde solution (pH: 7.4) and permeabilized with DPBS containing 0.1% Triton X-100 (Sigma Aldrich Cat. No: T9284) and 1% Blocker™ BSA (Thermo Fisher Scientific, Cat. No: 37525). Fixation and permeabilization steps were carried out at 4°C in the dark for 30 minutes. Fixed and permeabilized cells were suspended in 500 µL DPBS containing 1% Blocker™ BSA and labeled with Anti-Iba1 antibody (Abcam, #ab209942) by incubating at 4°C under dark for 30 minutes. The primary antibody solution was discarded, and cells were labeled with secondary antibody PE conjugated donkey anti-rabbit antibody (Abcam Cat. No: ab98441, final concentration: 0.1mg/mL). Following removal of the antibody solutions by centrifugation, the cells were suspended in 500 µL of DPBS containing 1% Blocker™ BSA. Cells were immediately acquired with DxFLEX flow cytometry system as triplicates. 2×10^4 cells at medium speed (30µl/minute) was read for each tube. Analyses were conducted with CytExpert for DxFLEX software.

2.8. Fluorescence imaging by confocal microscopy.

For fluorescence imaging, cells were seeded on 12-well cell culture plates and treated as above mentioned. At the end of incubation, cells were fixed with 2% glutaraldehyde solution by incubating for 15 min at 4˚C. For quenching residual aldehydes, fixed cells were incubated with glycine solution (0.1M) at RT. Cells were then permeabilized with 0.25% Triton X-100 in PBS (one hour-RT), followed by blocking with TBS containing 1% Blocker™ BSA (one hour-RT) Cells were then washed three times with PBS (5 min each), and labelled with antibodies Rabbit anti-human TRIM21/Ro52 antibody at 1: 500 dilution (Novus Biologicals, #NBP1-87122) and anti-human IgG antibody-FITC conjugated at 1: 500 dilution (Abcam #ab6854) overnight, at 4°C under dark. Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647) (ab150079 -final concentration: 0.1mg/mL) was used as the secondary antibody for TRIM21/Ro52.

Antibody solutions were prepared in TBS containing 3% Blocker™ BSA. Cells were visualized with Leica inverted confocal microscope.

2.9. Statistical analysis.

Statistical analysis was done in JASP 0.16.00 software and data are represented as mean ± standard deviation. Time dependent experiments were analyzed using repeated measure analysis of variance (RM-ANOVA) or using Paired Sample t-test whether the data meet the assumptions or not. Post Hoc tests were done by using Bonferroni. Comparisons between groups were conducted with independent samples t-test was used with or without Welch correction (the Welch corrections was noted with W). Mann Whitney U test was denoted 'MWU'. P values lower than 0.05 were considered statistically significant (*p<0.05, **p<0.005, ***p<0.001).

3. RESULTS

3.1. Determination of optimum LPS concentration

The measured MFI of DHR123, which shows mitochondrial stress of the HCM3 cells (after incubation with 0, 25, 50, 100, 250, 500, 1000, and 2000 ng/ml LPS at 37°C and 5% CO2 in EMEM supplemented with 10 % FBS, and antibiotics) are shown in Figure 3.1. The one-way ANOVA analysis (as the homogeneity of variances assumption failed) with Welch correction (shows significant changes in the MFI of DHR123 ($F(5.0,5.219)=9305$; $P<.001$), the Post Hoc analysis (Appendix A) suggested a stable (not significantly changing) plateau between (500-2000)ng/ml. The dose of 500ng/ml is selected for further experiments.

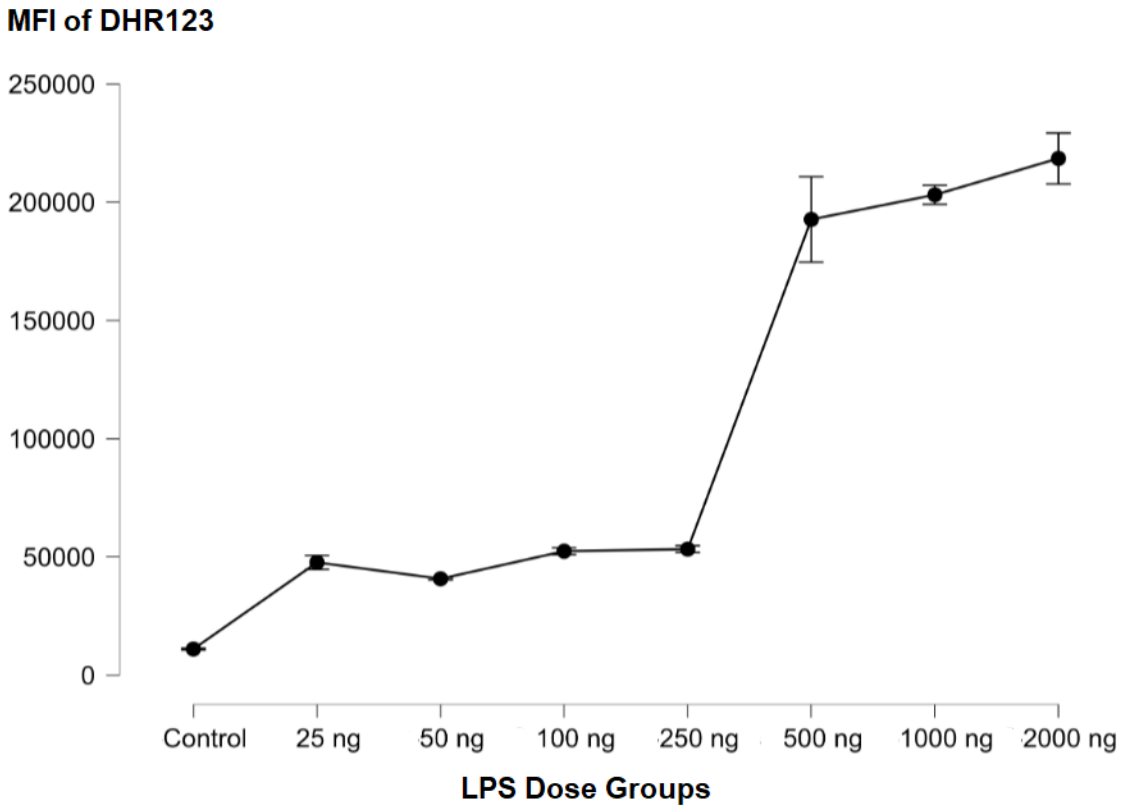

Figure 3.1. The MFIs of DHR123 change by LPS dose. The MFIs of DHR123 after incubation of HMC3 cells with (0, 25, 50, 100, 250, 500, 1000, and 2000 ng/ml LPS at 37°C and 5% CO2 in EMEM supplemented with 10 % FBS and antibiotics). The dose of 500ng/ml is selected.

3.2. Characterization of Microglial Cells

The measured MFI Levels of IBA1, CD45, and CD11b Proteins of HMC3 cells (control and stimulated with 500 ng/ml LPS at 37°C and ~5% CO2 in EMEM supplemented with 10 % FBS and antibiotics) are shown in Figure 3.2 (Statistics are presented in Appendix B). After LPS stimulation, the IBA1(+) cell ratio changed sharply from 2.88% to 98.6, and the MFI of the IBA1 raised threefold with p<.001. The expression of the IBA1, CD45, and CD11b proteins is observed.

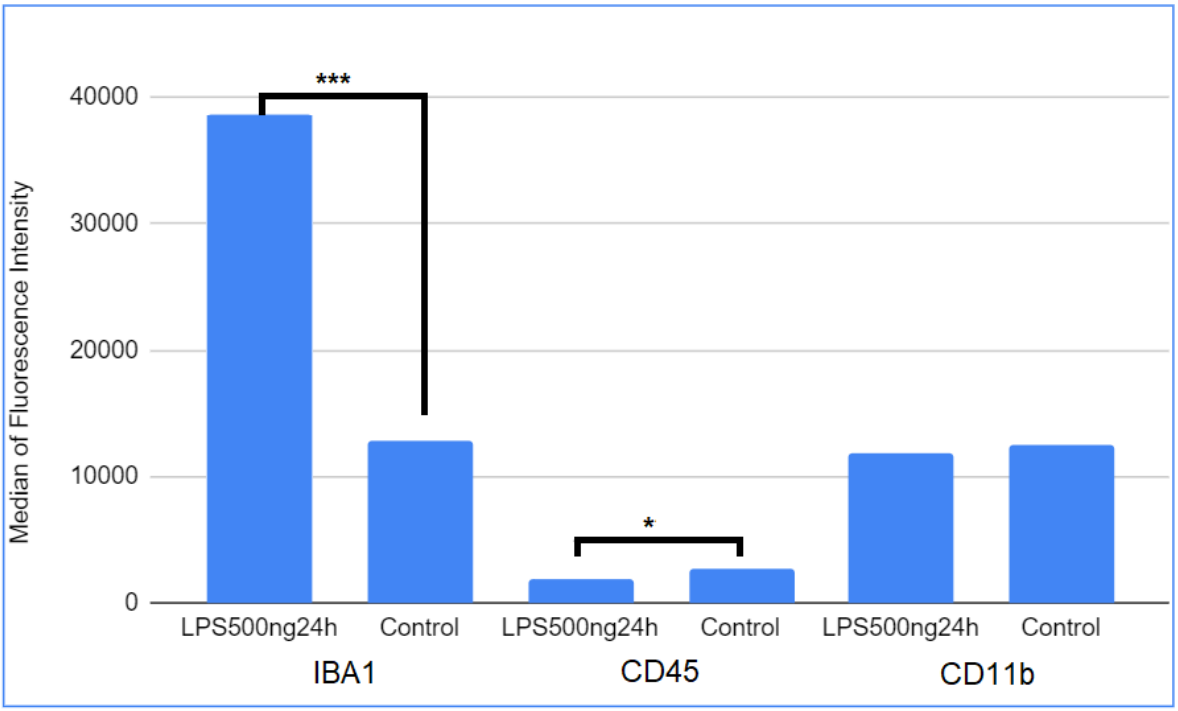

Figure 3.2 The MFI of the IBA1, CD45, CD11b of the Control and LPS-stimulated HMC3 cells. The MFI of the IBA1, CD45, and CD11b of the control and with 500ng LPS for 24-hour stimulated HMC3 cells show that cells expressing IBA1, CD45, and CD11b proteins.

3.3. Comparisons of the IgG and IgG+LPS groups

The flow cytometric results of the two main groups IgG and LPS+IgG (incubated with IgG, preincubated with 500ng/ml LPS before incubation with IgG, respectively) and their time-based subgroups (0min, 20min, 60min, four h, 24h, 48h) are listed in Appendix D. As it is shown in Figure 3.3 the scatter plots of MFI levels of IgG (bound to FITCH) and TRIM21/Ro52 (bound to Alexa Fluor 647) proteins were initially in a locally concentrated form turned into a scattered shape, especially within the 24-48 h.

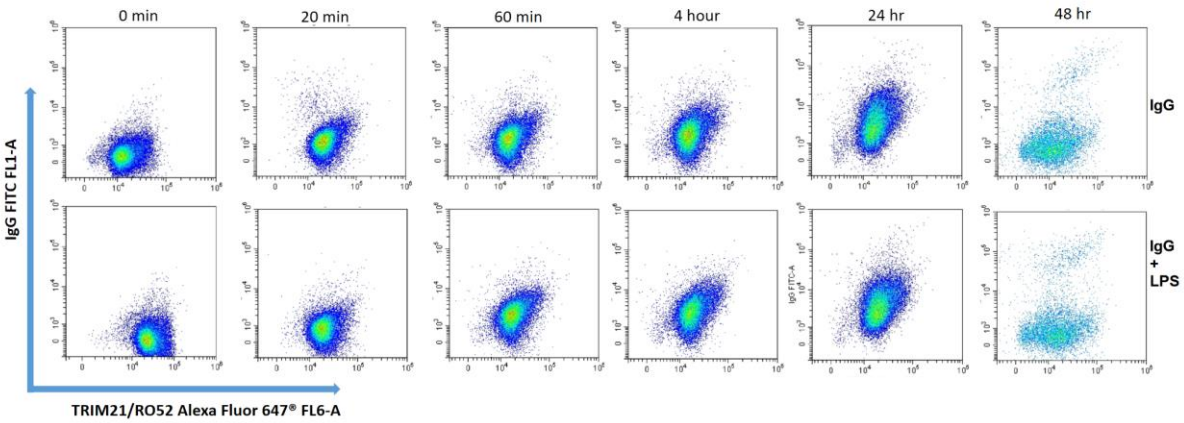

Figure 3.3. Representative dot plots (IgG versus TRIM21/Ro52) of HMC3 cells. The naive and the stimulated by LPS pretreatment HMC3 cells were incubated with IgG for (0min, 20min, 60min, four h, 24h, and 48h) and MFI levels of IgG (bound to FITC) and TRIM21/Ro52 (secondary antibody: to Alexa Fluor 647) proteins are measured with flow cytometry. At minute 0, the MFI of TRIM21/Ro52 was significantly lower in the IgG group, while at the end of the 20th minute, the gap between the two groups in terms of TRIM21/Ro52 level was closed without an increase in the MFI of the IgG. In the 20th minute and 24-hour range, especially in the four h 24h range, we see that the gathering area on the graph is growing, mostly in the direction of MFI of the IgG increase. In the 24h 48h range, as a result of some cells in which TRIM21/Ro52 obviously decreased while in others, IgG levels increased, thus the dot plot which was initially locally concentrated form turned into a scattered shape.

3.3.1. Flow cytometry results for IgG and TRIM21/Ro52 measurements on cells.

The MFI changes of the IgG were negligible within the time until 48 hours when a sharp rise was observed (Figure 3.4.a.). The repeated measure ANOVA analysis revealed that both the groups (LPS pre-stimulation) and the time are statistically significantly effective factors (Appendix C).

The MFI levels of TRIM21/Ro52 are presented in Figure 3.4.b, the 24-hour LPS stimulation increased the MFI levels of TRIM21/Ro52 1.65 fold. Interestingly, the IgG also resulted in a similar effect on the naive HMC3 cells within the first 20 min as the MFI values of TRIM21/Ro52 raised 1.40 fold though the effect of IgG on the LPS+IgG group was opposite (TRIM21/Ro52 levels were already high in this group). The MFI levels of TRIM21/Ro52 decreased by 23,6% in the LPS+IgG group. Statistical analysis is shown in Appendix D.

The percentage of the IgG(+) cells (for both groups) made a pick at the 24h and dropped back to the levels around the four h (0min<20min<60min<4hour<48hour<24hour, p<0.001) (Figure 3.4.C.)

The percent of TRIM21/Ro52(+) cells for all groups is shown in Figure 3.4.C. In IgG 20 min groups (Parallel to the rise of the MFI-TRIM21/Ro52), the TRIM21/Ro52(+) cell ratio increased, but in both groups, between 24 hours and 48 hours, this ratio decreased sharply. Statistical analysis between groups is in Appendix E.

The average fluorescent densities (FD) (calculated by dividing the MFI values by the percentage of the positive cells to estimate the average of the positive cells) for IgG and TRIM21/Ro52 also rose between (24-48) hours period in both groups (Figure 3.4 e and f). The statistical analysis is given in Appendix F.

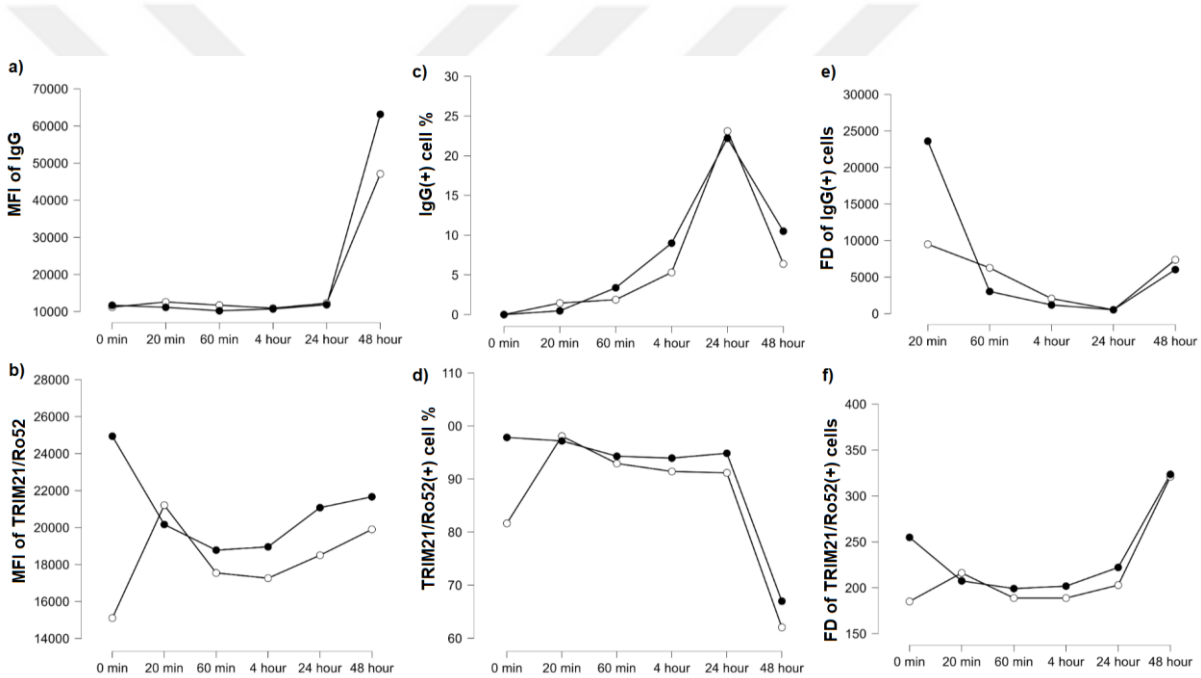

Figure 3.4 IgG and TRIM21 protein levels of the HMC3 cells upon IgG incubation. The naive HMC3 cells (group IgG, the white circles) and LPS+IgG group (pre-incubated with LPS for 24 hours, the black circles) were incubated with IgG (x) for 20 min, 60 min, 4 hours, 24 hours, 48hour). The measured MFI changes of the IgG were negligible in both groups within the time until the 48 hours when a sharp rise was shown (a), The MFI levels of TRIM21/Ro52 after 24-hour LPS stimulation was 1.65 fold higher, but interestingly, the IgG was also made a similar effect on the naive HMC3 cells, increasing MFI of TRIM21/Ro52 1.40 fold within the 20 min in the IgG group. On the other hand, the effect of IgG on the LPS+IgG group was the opposite as it led to a decrease of 23,6% in terms of TRIM21/Ro52 (b). The percentage of the IgG(+) cells (for both groups) reached the

peak point at the 24-hour and dropped back at the 48-hour (0min<20min<60min<4hour<48hour<24hour) (c). In parallel to the percentage of the IgG(+) cells, the percentage of TRIM21/Ro52(+) cells decreased between 24h and 48 hours. In contrast to the low percentages of the IgG(+) cells, the percentage of the TRIM21/Ro52(+) cells was high. In the IgG group, TRIM21/Ro52(+) cells were lower than in the LPS+IgG group. The percentage of the TRIM21/Ro52(+) cells were increasing in the IgG group while decreasing in the LPS+IgG group between 0 and 20 minutes (d). The average fluorescent densities (FD) of IgG(+) cells decreased until 24h and then increased (d). The FD values of TRIM21/Ro52 were also increased between 24 and 48 hours, and in the first 20 minutes, despite the decrease in the LPS+IgG group, an increase was observed in the IgG group(e).

The IgG (positive & negative) and TRIM21/Ro52 (positive & negative) levels of HMC3 cells' distribution for the IgG and LPS+IgG groups and their IgG incubation time-based subgroups are shown in Figure 3.5. The abundance of the IgG(-)/TRIM21(+) zone decreases continuously in the LPS+IgG group; the same trend was the case for the IgG group except in the first 20 minutes. IgG(+)/TRIM21(21) zone made a pick in both groups. The IgG(-)/ TRIM21(-) cells increased between 24 and 48 hours sharply in both groups. The IgG group showed a decrease between the start and 20min. The IgG(+)/TRIM21(-) zone shows an increase, especially between 24 and 48 hours. The t-test results comparing groups for each time segment are in Appendix G.

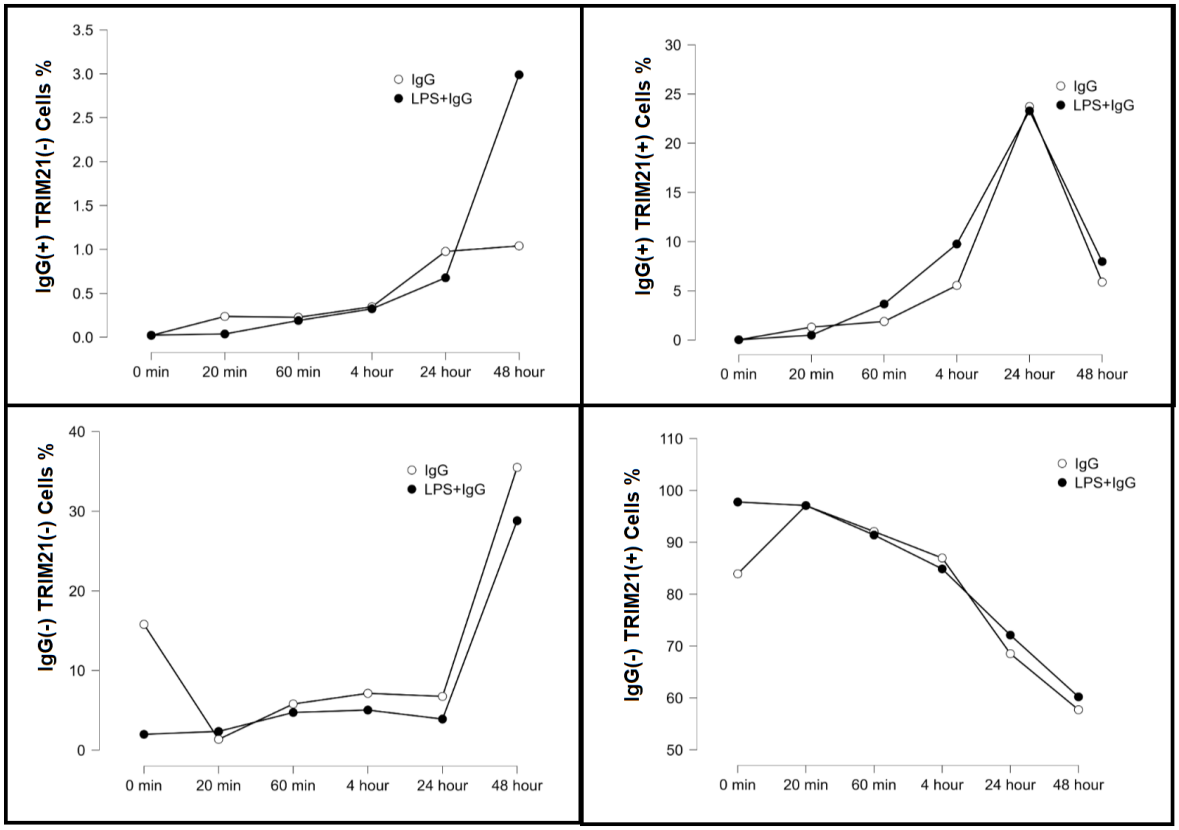

Figure 3.5 The distribution of the cells into zones. The time-based cell percentages in the zones (i.e., IgG positive and negative, TRIM21/Ro52 positive and negative) for IgG and LPS+IgG groups.

The average TRIM21/Ro52 MFI changes per minute between time segments were contradictory in the first 20 min but, after 60 min, very similar for each group, suggesting homeostasis that aims to preserve the cell populations' TRIM21/Ro52 inventory (Figure 3.6.)

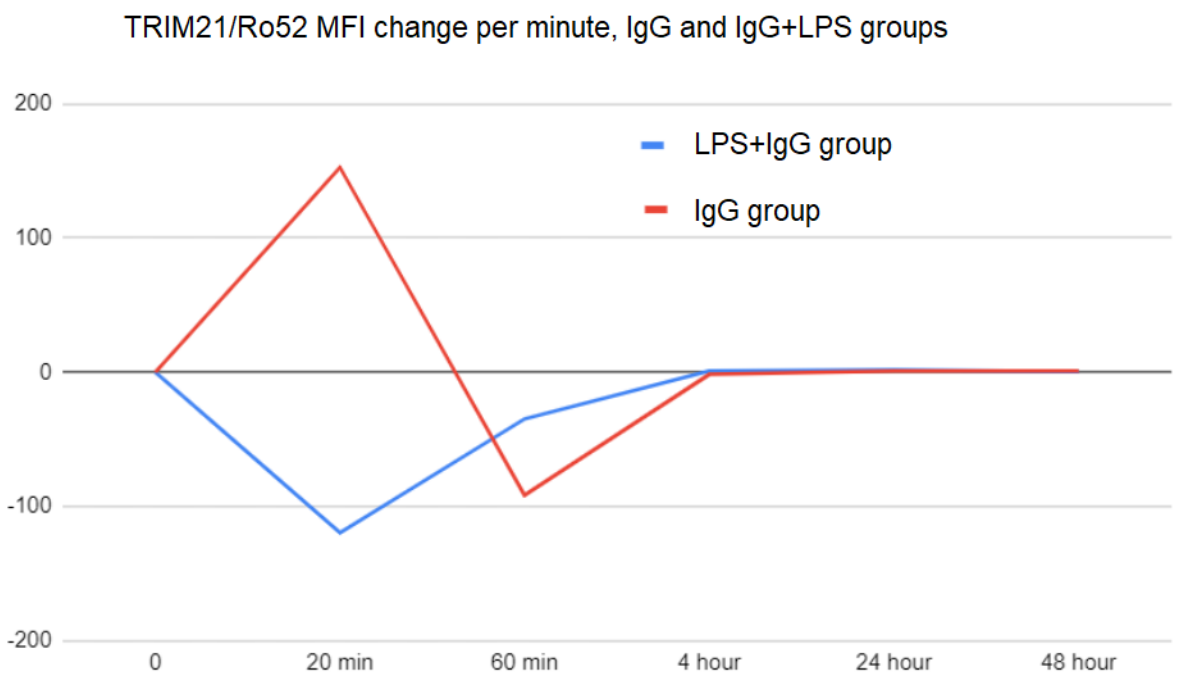

Figure 3.6 The (calculated average) TRIM21/Ro52 MFI changes per minute between time segments. In the first 20 minutes, the reactions of the groups were contradictory. Between 20-60 minutes, both groups seem to respond oppositely, and after 60 minutes, they both reach equilibrium.

The transition ratios of the cells from the IgG(-) & TRIM21/Ro52(+) zone (the highest ratio in both groups) to the other zones (via gaining IgG or losing TIM21/Ro52) between 0 min and 48 hours are presented in Figure 3.7. The highest transition ratio was observed to the IgG(-) and TRIM21/Ro52(-) zone, followed by the IgG(+) and TRIM21/Ro52(+) zone and finally, IgG(+) and TRIM21/Ro52(-) zone, respectively.

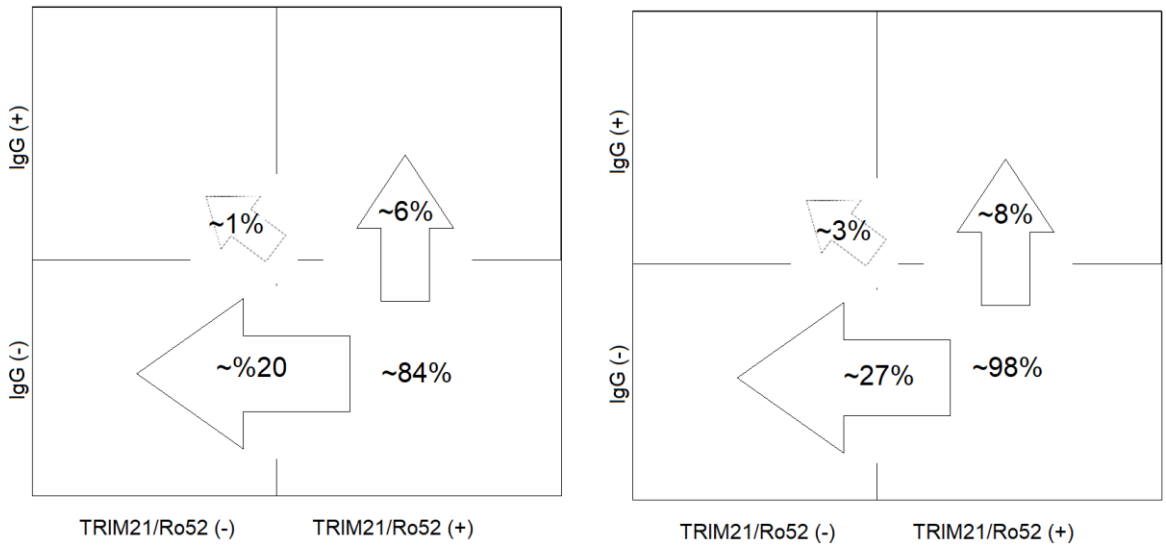

Figure 3.7. The (interzonal) transition ratios of the cells from IgG(-) and TRIM21/Ro52(+) between 0 min and 48 hours. Initially, the TRIM21/Ro52(+) cell ratio of the IgG group was 14% less than the LPS+IgG group. In both groups, the transition from IgG(-) and TRIM21/Ro52(+) zone to IgG(-) and TRIM21/Ro52(-) zones were the most prominent, the second zone was the IgG(+) and TRIM21/Ro52(+) zone and the last zone was IgG(+) and TRIM21/Ro52(-).

3.3.2. Confocal microscopy analyses of the cells.

The confocal micrographs of the cells for up to 48 hours (Figures 3.8, 3.9, and 3.10) are compatible with the flow cytometry analyses' results. In the beginning, green dots (IgG) were rare and accompanied by shiny red dots TRIM21/Ro52. The dense red dots degraded with time, and green dots emerged, but not as much as in the beginning.

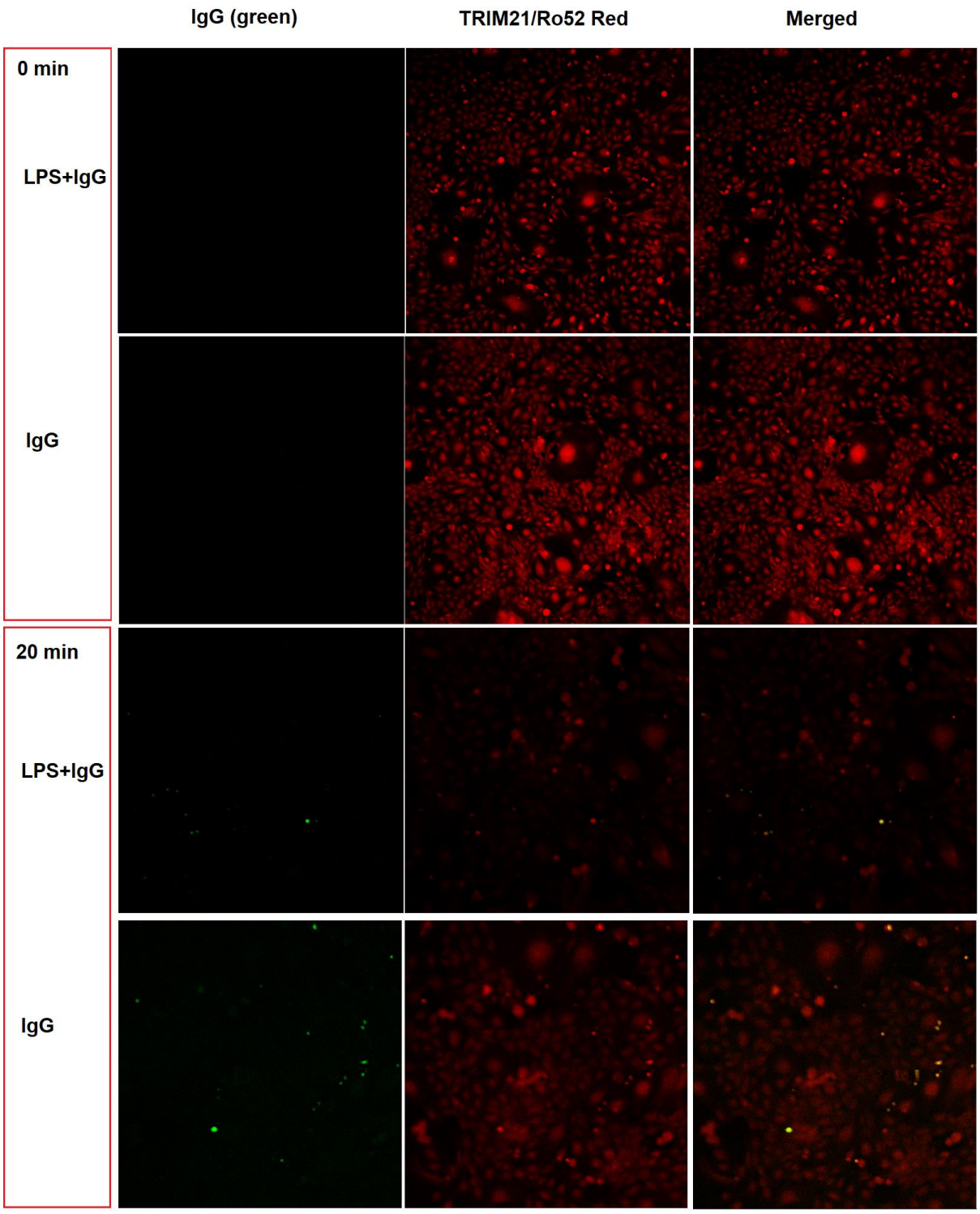

Figure 3.8. The confocal micrographs of the cells on 0 and 20 minutes. The micrographs of both the HMC3 cells group, the IgG group (incubated with IgG), and the LPS+IgG group (pre-incubated with LPS before IgG incubation) are presented. Green colors indicate IgG, and red color indicates TRIM21/Ro52. The abundance of TRIM21/Ro52 in both groups was detected at the beginning, and in the 20 min, The LPS+IgG group

obviously lost some TRIM21/Ro52 protein while green dots emerged (with accompanying bright red signal) in both groups.

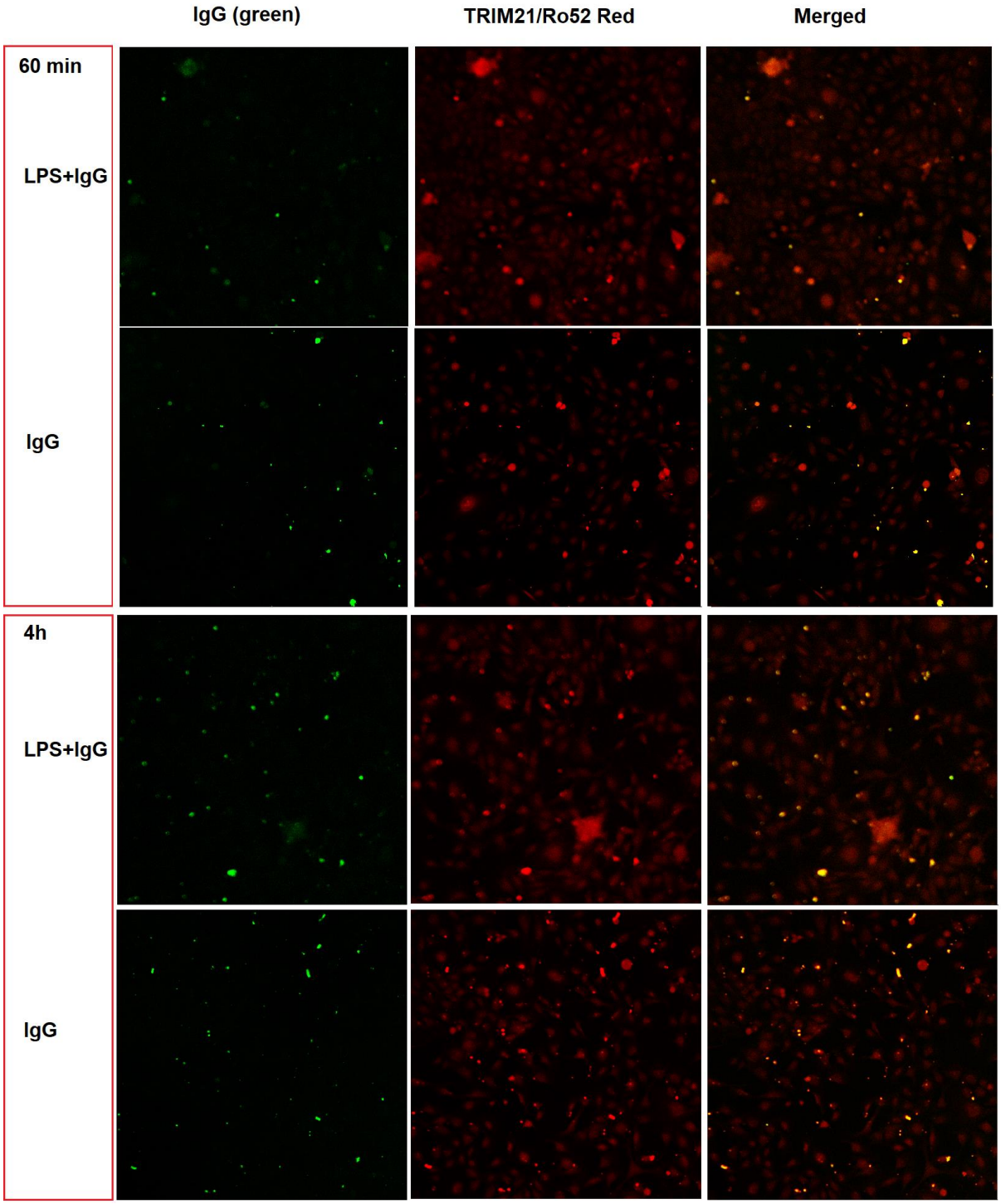

Figure 3.9. The confocal micrographs of the cells at 60 minutes and 4 hours. The amount of tiny green dots with accompanying bright redness increased to some degree. The red signal in both groups vanished compared with the 0 min.

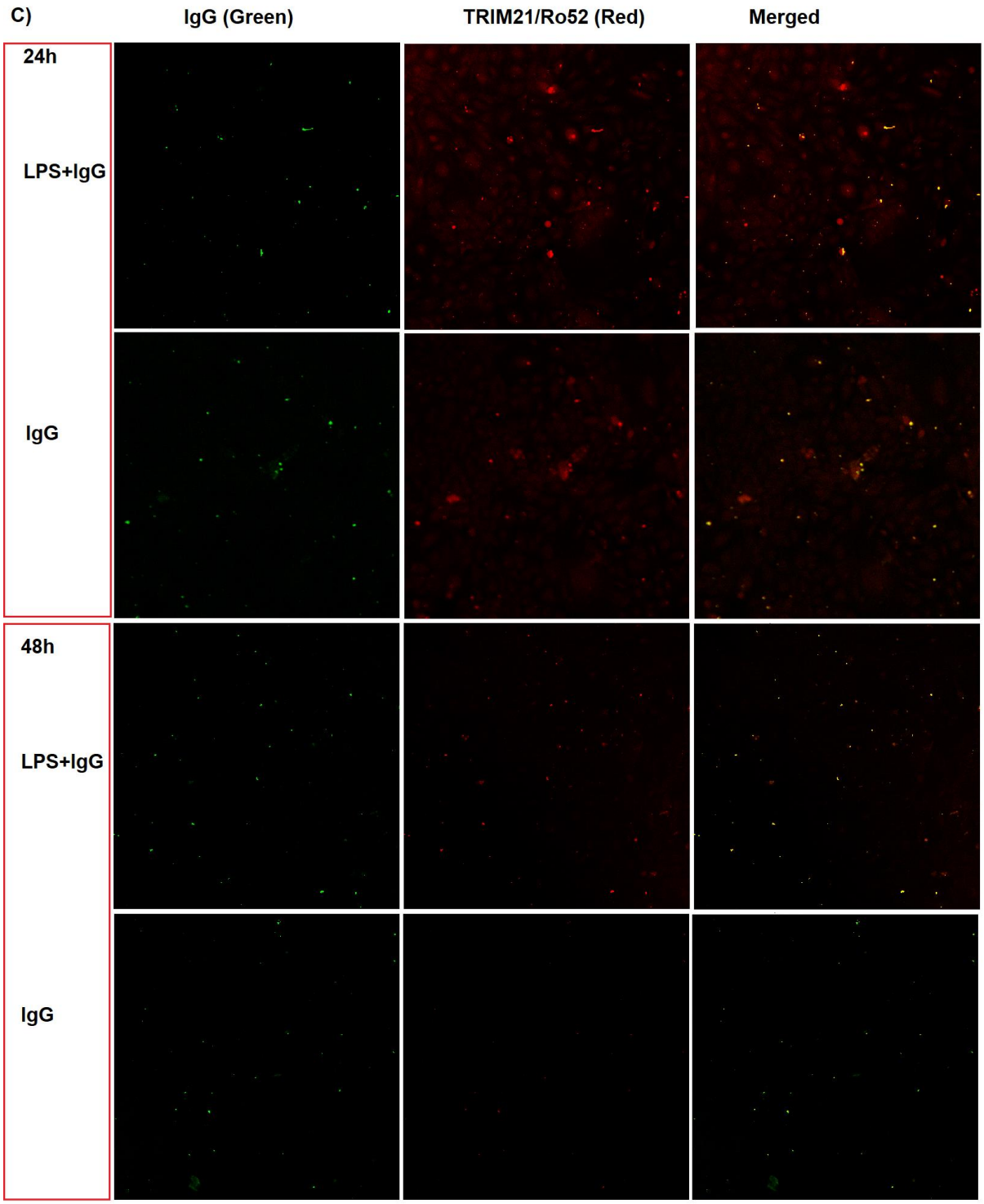

Figure 3.10. The confocal micrographs of the cells at 24 hours and 48 hours. In the images of the 24-hour IgG incubation, the overlapping shiny red and green dots were still prominent, but at the 48th hour, in addition to a decrease of the red and green signals, the presence of green dots was not accompanied by bright red signals.

3.4. Summary of the Results

Together, The DHR123 measurements indicate that HMC3 cells respond to LPS stimulation starting from 25ng/ml and with the dose of 500ng/ml, the highest difference in terms of mitochondrial activation was observed (Figure 3.1). HMC3 cells express IBA1, CD45, and CD11b, and LPS stimulation has a great effect on the IBA1 expression (Figure 3.2). The MFI levels of the internalized IgG show a plato around almost zero levels in the first 24h but at the 48th hour shows a sharp increase and a TRIM21/Ro52 level decrease accompanied in the same range. LPS preincubation upregulates TRIM21/Ro52. IgG incubation and TRIM21/Ro52 levels are time and the state of the cell (whether the TRIM21/Ro52 levels are already upregulated or not, incubation time is more or less than four h -probably depends on mRNA-). At the end of the incubation periods, while some cells lost their TRIM21/Ro52 proteins, others become overly expressive, and interestingly the change of the MFI of TRIM21/Ro52 protein per minute for the HMC3 cell population is zero after the 4th hour. The micrographs obtained by the confocal microscopy are in line with our flow cytometric analysis. The small numbers of bright IgG fluorescence are always accompanied by bright TRIM21/Ro52 fluorescence except in the micrographs taken on the 48th hour: at the 48th hour, the sole IgG signal emerged.

4. DISCUSSION

This study aims to examine the effect of inflammation triggered by LPS administration in microglial cells and the interaction between TRIM21/RO52 and IgG in a time-dependent manner. It was shown in many cells (but not microglia) TRIM21/RO52 and IgG+antigen complexes are subjected to degradation. Herein, for the first time, immune state-dependent interactions of the TRIM21/RO52 and non-pathogenic IgG on microglial cells.

Intravenous IgG administration in autoimmune neurological diseases to alleviate flare-ups in autoimmune diseases since the 1980s, though the exact mechanism is unknown, and the success rates vary between cases[75]. The main target of this research was to investigate the interaction between IgG and TRIM21/Ro52 protein in human microglial cells and how their interaction changes under immune stress over time. For this purpose, LPS is chosen as the immune stressor because there is vast information about the pathways it affects, and it is widely used as a TLR4 ligand found on Gram-negative bacteria cell walls [75]. LPS stimulation is commonly used to promote inflammation, and in the literature, it has been applied in a very wide dose range in similar studies.

To determine the ideal dose of the stimulant, we incubated the HMC3 cells with LPS ranging from 25 to 2000 ng/mL concentration for 24 hours and determined intracellular ROS accumulation with DHR123 assay via flow cytometry. In line with the literature, HMC3 cells showed an immune response starting from the lowest dose (25 ng/mL), indicating ROS accumulation within the cells. The ROS level was almost horizontal in the range of 25-250 ng/mL and then plateaued four times higher in the range of 500-2000 ng/ml. Thus, 500ng/mL was selected for pre-incubating HMC3 cells to promote a pro-inflammatory state in further evaluations. The activation of HMC3 cells was also confirmed with the increase in IBA1 protein expression levels upon LPS incubation at the dose mentioned above for 24 hours. Moreover, TRIM21/Ro52 levels increased almost two-fold after 24-hour LPS incubation, suggesting activation.

Next, untreated and LPS-primed (500 ng/mL) cells were treated with (100 ug/mL) IgG, and the interaction between IgG and TRIM21/Ro52 was evaluated with flow cytometry in a time-dependent manner. In the first 20 minutes of the IgG treatment, the change of the TRIM21/Ro52 levels of the naive and 24-hour LPS preincubated HMC3 cells was in opposite directions: the MFI values of TRIM21/Ro52 protein were significantly decreased in the LPS+IgG group while it was significantly increased in the untreated group. In the first 20 minutes, the MFI of the IgGs was almost zero in both groups,

indicating that internalization of IgG requires longer than this incubation duration, or internalized IgG was subject to quick degradation. This finding is also confirmed with confocal microscopy results where a significant amount of TRIM21/Ro52 and a negligible amount of IgG in both groups were observed. The results from the group that was LPS-primed prior to IgG treatment results suggested that within 20 minutes, TRIM21/Ro52 started to decrease and disappeared with the internalized IgG through proteasomal degradation.

On the other hand, in the untreated group, it is evident that the IgG contacts the cell surface, then being internalized and eventually degraded IgG led to TRIM21/Ro52 synthesis. These findings suggest that self-regulatory characteristics of TRIM21/Ro52 might inhibit these pathways in the LPS+IgG group due to already high TRIM21/Ro52 levels, though further studies are needed to clarify this phenomenon.

In contrast to the first 20 minutes, between the 20th and 60th minutes, both groups' TRIM21/Ro52 MFI values decreased. The decrease in the IgG group was higher than in the LPS+IgG group. These results suggest that the cells may have a limited phasic TRIM21/Ro52 synthesis potential. After the disposal of this potential, IgG influx ends with decreased TRIM21/Ro52 levels; moreover, IgG influx may be higher in the LPS pre-stimulated group (possibly due to the already increased expression of FCγRs).

Between one and four hours, no significant change in IgG MFI values was observed; similarly, TRIM21/Ro52 MFI levels also remained unaltered. This finding may suggest that the TRIM21/Ro52 degradation and production have reached an equilibrium state due to decreased IgG influx. However, the 24-hour IgG incubation data might rule out this possibility as the IgG MFI increased in groups with IgG-positive cells. In 48 hours, IgG MFI values showed a sharp increase along with the TRIM21/Ro52 MFI values, though the latter was not as prominent as the previous one. These results contradict the hypothesis that internalized IgGs are subjected to proteasomal degradation. However, it should be considered that the cells did not react homogeneously: TRIM21/Ro52 levels were increased in some cells, while the others lacked TRIM21/Ro52. Thus, while the TRIM21/Ro52 MFI values did not change much between the 20th minute and 48 hours, the cells were divided into two groups in terms of TRIM21/Ro52 protein expression. TRIM21/Ro52 negative cells make up more than thirty percent of cells appearing at 48 hours and explain the sharp rise in IgG MFI values in this measurement period, while a significant decrease in IgG positivity along with TRIM21/Ro52 positive cells between 24-48 hours indicates the co-extinction of IgG and TRIM21/Ro52.

When considering cells that were not primed with LPS, 15% of the total cell population was found to be TRIM21/Ro52 negative, and these cells rapidly synthesized TRIM21/Ro52 in the first 20 minutes. As mentioned previously, this phase was already completed in the LPS+IgG group. The TRIM21/Ro52 positive cell percentage was ~98%, which was ~84% in the naive group. At the 48th hour, only ~1% (naïve group) and ~3% (LPS-primed groups) of the IgG-TRIM21/Ro52+ cells were switched to the IgG+TRIM21/Ro52- state. Though the mechanism behind the low proportion IgG+ TRIM21/Ro52- cells need to be elucidated by additional studies. In parallel with the TRIM21/Ro52 protein levels in the cell, IgG supplementation may be decreased.

Other mechanisms that may be involved in breaking down the IgG taken into the cell or allowing it to be excreted outside the cell, including neonatal FCγRs or exosomes, can be considered. The cells that were initially IgG-TRIM21/Ro52+ and became IgG+TRIM21/Ro52+ region was ~6% in the naïve group and ~8% in the LPS primed group. Here, three possibilities can be considered: 1. The IgGs may be protected from proteasomal degradation due to neonatal FcRs, 2. The IgGs may still be in the endosomes or 3. Proteasomal degradation speed is slow. However, the large proportion of IgG-TRIM21/Ro52- cells suggests that IgG and TRIM21/Ro52 co-extinction is the main mechanism.

When the fluorescence densities of IgG+TRIM21/Ro52+ cells were calculated for each timepoint (FD=MFI/ positive cell %), it was observed that the average fluorescence intensity of positive cells (IgG and TRIM21/Ro52 separately) increased in 24-48 hours. IgGs likely accumulate in the cells where TRIM21/Ro52 proteins are degraded, while expression is further increased in the cells where TRIM21/Ro52 synthesis continues. Here, the TRIM21/Ro52 MFI change per minute calculation gives a graph that does not change after the 4th hour. This stable graph may suggest that the cells first achieve a collective balance by influencing each other as if they distribute certain tasks between different cells with either high or low TRIM21/Ro52 expression.

In the LPS-primed group, TRIM21/Ro52 MFI values were always statistically higher than the naïve group except on the 20th minute, which we have already discussed. On the other hand, IgG MFI did not significantly differ between groups until the 48th hour. At the 48th hour where a sharp increase in IgG MFI was observed in both groups, and LPS-priming led to statistically higher IgG levels even at this point in comparison with the naïve group. These findings suggest that long-term TLR4 stimulation may alter the cell surface

molecules, leading to higher IgG internalization capacity; this situation may be detected after TRIM21/Ro52 degradation in some of the cells.

In summary, IgGs are taken into HMC3 microglia cells and form complexes that eventually lead to the degradation of the IgG- TRIM21/Ro52 complexes. There may be a possible phasic TRIM21/Ro52 synthesis mechanism triggered by IgG binding to the cell surface or internalization of IgG, yet, if the TRIM21/Ro52 levels are high, this mechanism is unlikely to work. After four hours, the TRIM21/Ro52 levels start to rise again, which may be attributed to the newly synthesized mRNAs; after 24 hours, almost one-third of the cells become TRIM21/Ro52 negative which may be the result of high IgG influx and/or decreased TRIM21/Ro52 expression. LPS priming increases TRIM21/Ro52 levels which seems to prevent cells from responding to a phasic TRIM21/Ro52 increase upon IgG incubation. In addition, LPS pretreatment probably accelerates the flow of IgG into the cells, but this can only be monitored by intracellular IgG buildup due to TRIM21/Ro52 burnout at 48 hours.

Although it is very well known the IgG antigen complex is the natural target of all phagocytic cells, to our knowledge, IgG (alone, without bound antigen) internalization by microglia and the fate of the IgGs inside a cell are not studied. Our results reveal a very solid interaction between IgG and TRIM21/Ro52 proteins in microglia, and time along with cells' immune status, determines this relationship. The co-extinction of the IgG and TRIM21/Ro52 may be a part of the answer to the questions arising about the heterogeneity of neuroimmunological disorders. Regardless of their source (CNS or periphery), IgGs' homogenous distribution throughout the CNS is not likely. Instead, the density might decrease as the distance from the source increases due to their degradation via microglia (Figure 4.1). Most probably, this IgG-clearance is not limited to microglial cells; other cells in the CNS cells may play the same role, but this hypothesis should be further confirmed. A similar question is also valid for autoimmune neurological diseases arising from the presence of auto-reactive autoantibodies; do the cells around the immune reactive zone express TRIM21/Ro52? Do self-antigens are also degraded via IgG and TRIM21/Ro52? If so, is it possible to find a way to destroy autoreactive IgGs before they find their target? Similar to our experiment, some cells may have naturally low levels of TRIM21/Ro52 that may lead to longer IgG half-life inside the cell, and this may promote the destruction of self-antigens due to the absence of IgG-dampening mechanisms of the cells. Finally, what happens in the periphery? As the macrophages behave similarly to microglia, does the IVIG administration dampen macrophages? If so, targeting specific

techniques may be required for the sake of the efficiency of the IVIG treatments to suppress auto-immunity in CNS. In conclusion, many questions arise about this mechanism, and some of them can be answered by simply changing the cell type (i.e., macrophages - to understand the situation in the periphery, endothelial cells, astrocytes, etc., to understand the situation in the BBB, and neurons and other types of glial cells for understanding the situation in the CNS). Strategies to destroy auto-antibodies before they reach their target may be an option, e.g., may methods similar to dialysis be employed to clear pathogenic autoantigens? As time is one of the main players, live imaging studies involving transduced cell lines with fluorescent protein transfections would be helpful, especially by covering other proteins such as FcRs, FcR-neonatal, IRFs, cytokines, and so many others. The hypothesis we have suggested could also be investigated in vivo, and the effects of several drugs (i.e., antiepileptics, antidepressants, antipsychotics) on antibody neutralization may be taken into account for the patients suffering from autoimmunity.

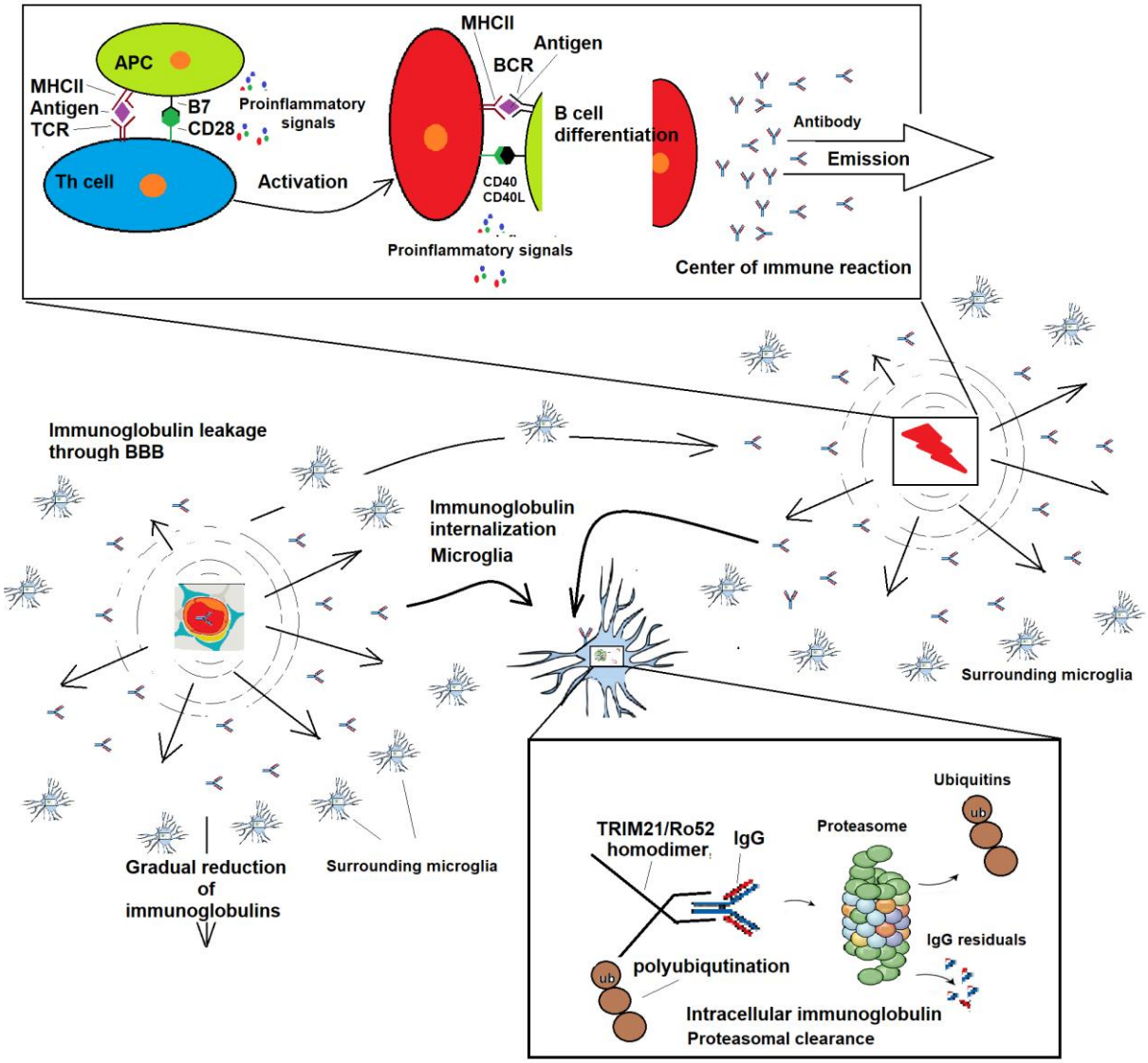

Figure 4.1 Hypothetical immunoglobulin suppression in CNS. Either successive antibodies cross the BBB or antibodies secreted by T, B, and APC cell complexes under pro-inflammatory conditions should be neutralized by microglia (and also possibly by astrocytes and even by neurons) through internalization, and TRIM21/Ro52 mediated proteasomal degradation. In the case of autoimmunity, the damage on the cells (i.e., neurons, myeloids) far from BBB leakage location or far from antibody-producing immune cell complex may be gradually less because of gradually descending antibody titrations. APC antigen-presenting cells, BBB blood-brain barrier, BCR B cell receptors, CD40 Cluster of differentiation 40, CD40L Cluster of differentiation 40 ligand, IgG immunoglobulin G, MHC II, major histocompatibility complex 2 molecules; TCR, T cell receptors

As mentioned before, many items about this topic need to be answered. What is the mechanism of IgG internalization? What is the proteasomal, lysosomal degradation ratio? Is there IgG recycling that we should have been aware of? Did the other trim family members play a role? How were the IRF and interleukin family members' expressions? How could the neuron glial cell interactions change? Do the neurons also degrade IgGs? Is IgG degradation via TRIM21/RO52 a general mechanism through the body, and if so, does it differ individually? Why did in a group of cells' TRIM21/RO52 exhausted contrary to the other groups' TRIM21/RO52 raised? What immunological role differences are there between TRIM21/RO52 exhausted and rich microglial cells? What would change in an in vivo study? How the drugs like antiepileptics, antidepressants, or cortisol which most of the patients get during IVIG therapies.

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APPENDIX

Appendix A. Table of the One Way ANOVA Post Hoc (Games-Howell) Analysis Results of the MFI -DHR123 of the LPS Dose Groups of the HMC3 Cells.

Groups	Mean Difference	SE	t	df	ptukey	
Control - 25 ng	-36634	692	-53	2.1	< .001	***
Control - 50 ng	-29718	132	-226	3.7	< .001	***
Control - 100 ng	-41372	339	-122	2.2	< .001	***
Control - 250 ng	-42257	348	-121	2.2	< .001	***
Control - 500 ng	-181686	4191	-43	2.0	< .001	***
Control - 1000 ng	-192094	932	-206	2.0	< .001	***
Control - 2000 ng	-207464	2499	-83	2.0	< .001	***
25 ng - 50 ng	6916	695	10	2.1	0.038	*
25 ng - 100 ng	-4738	762	-6	2.9	0.055	
25 ng - 250 ng	-5623	766	-7	2.9	0.034	*
25 ng - 500 ng	-145052	4246	-34	2.1	0.002	**
25 ng - 1000 ng	-155460	1155	-135	3.7	< .001	***
25 ng - 2000 ng	-170830	2591	-66	2.3	< .001	***
50 ng - 100 ng	-11654	347	-34	2.4	0.001	**
50 ng - 250 ng	-12539	355	-35	2.4	< .001	***
50 ng - 500 ng	-151968	4191	-36	2.0	0.002	**
50 ng - 1000 ng	-162375	934	-174	2.1	< .001	***
50 ng - 2000 ng	-177746	2500	-71	2.0	< .001	***
100 ng - 250 ng	-885	473	-2	4.0	0.621	
100 ng - 500 ng	-140314	4203	-33	2.0	0.003	**
100 ng - 1000 ng	-150722	985	-153	2.5	< .001	***
100 ng - 2000 ng	-166092	2520	-66	2.1	< .001	***

250 ng - 500 ng	-139429	4204	-33	2.0	0.003	**
250 ng - 1000 ng	-149837	988	-152	2.5	< .001	***
250 ng - 2000 ng	-165207	2521	-66	2.1	< .001	***
500 ng - 1000 ng	-10407	4292	-2	2.2	0.476	
500 ng - 2000 ng	-25778	4878	-5	3.3	0.068	
1000 ng - 2000 ng	-15370	2665	-6	2.5	0.083	

Appendix B. MFI of the IBA1, CD45, and CD11b of the Control and LPS-stimulated HMC3 cells.

Protein	Group	N	Mean	SD	SE	Test	Statistic	df	p		Effect Size
IBA1	LPS500 ng24h	3	38598	1354	781	Student t	32.623	4	< .001	***	26.636
	Control	3	12786	210	121						
CD45	LPS500 ng24h	3	1955	55	32	Student t	-3.523	4	0.024	*	-2.876
	Control	3	2695	359	207						
CD11b	LPS500 ng24h	3	11917	6	3	MWU	0	-	0.1		
	Control	3	12505.2	551	318						

Appendix C. Table of the Repeated Measures ANOVA analysis of the MFI of IgG between groups and time groups.

Repeated Measures ANOVA						
Within Subjects Effects						
Cases	Sum of Squares	df	Mean Square	F	p	
Time	9.55E+09	5	1.91E+09	1058.635	< .001	***
Time ⁎ group	3.51E+08	5	7.02E+07	38.922	< .001	***
Residuals	3.61E+07	20	1.80E+06			
Post Hoc Comparisons - Time						
		Mean Difference	SE	t	pbonf	
0 min	20 min	-428	775	-0.552	1	
	60 min	470	775	0.607	1	
	4 hour	633	775	0.816	1	
	24 hour	-617	775	-0.796	1	
	48 hour	-43665	775	-56.319	< .001	***
20 min	60 min	899	775	1.159	1	
	4 hour	1061	775	1.368	1	
	24 hour	-189	775	-0.244	1	
	48 hour	-43237	775	-55.767	< .001	***
60 min	4 hour	162	775	0.209	1	
	24 hour	-1088	775	-1.403	1	

	48 hour	-44135	775	-56.926	< .001	***
4 hour	24 hour	-1250	775	-1.612	1	
	48 hour	-44297	775	-57.135	< .001	***
24 hour	48 hour	-43048	775	-55.523	< .001	***
Post Hoc Comparisons - group						
		Mean Difference	SE	t	pbonf	
IgG	(LPS+IgG)	-2162.828	389.203	-5.557	0.005	**
Post Hoc Comparisons - group ⁎ Time		Mean Difference	SE	t	pbonf	
IgG, 0 min	(LPS+IgG, 0 min)	-506.1	1073	-0.471	1	
	IgG, 20 min	-1405.5	1096	-1.282	1	
	(LPS+IgG, 20 min)	43.333	1073	0.04	1	
	IgG, 60 min	-533.467	1096	-0.487	1	
	(LPS+IgG, 60 min)	968.3	1073	0.902	1	
	IgG, 4 hour	275.333	1096	0.251	1	
	(LPS+IgG, 4 hour)	483.733	1073	0.45	1	
	IgG, 24 hour	-1074.4	1096	-0.98	1	
	(LPS+IgG, 24 hour)	-666.467	1073	-0.621	1	
	IgG, 48 hour	-35899.033	1096	-32.741	< .001	***

	(LPS+IgG, 48 hour)	-51936.833	1073	-48.362	< .001	***
(LPS+IgG, 0 min)	IgG, 20 min	-899.4	1073	-0.837	1	
	(LPS+IgG, 20 min)	549.433	1096	0.501	1	
	IgG, 60 min	-27.367	1073	-0.025	1	
	(LPS+IgG, 60 min)	1474.4	1096	1.345	1	
	IgG, 4 hour	781.433	1073	0.728	1	
	(LPS+IgG, 4 hour)	989.833	1096	0.903	1	
	IgG, 24 hour	-568.3	1073	-0.529	1	
	(LPS+IgG, 24 hour)	-160.367	1096	-0.146	1	
	IgG, 48 hour	-35392.933	1073	-32.957	< .001	***
	(LPS+IgG, 48 hour)	-51430.733	1096	-46.906	< .001	***
IgG, 20 min	(LPS+IgG, 20 min)	1448.833	1073	1.349	1	
	IgG, 60 min	872.033	1096	0.795	1	
	(LPS+IgG, 60 min)	2373.8	1073	2.21	1	
	IgG, 4 hour	1680.833	1096	1.533	1	
	(LPS+IgG, 4 hour)	1889.233	1073	1.759	1	
	IgG, 24 hour	331.1	1096	0.302	1	
	(LPS+IgG, 24 hour)	739.033	1073	0.688	1	
	IgG, 48 hour	-34493.533	1096	-31.459	< .001	***

	(LPS+IgG, 48 hour)	-50531.333	1073	-47.053	< .001	***
(LPS+IgG, 20 min)	IgG, 60 min	-576.8	1073	-0.537	1	
	(LPS+IgG, 60 min)	924.967	1096	0.844	1	
	IgG, 4 hour	232	1073	0.216	1	
	(LPS+IgG, 4 hour)	440.4	1096	0.402	1	
	IgG, 24 hour	-1117.733	1073	-1.041	1	
	(LPS+IgG, 24 hour)	-709.8	1096	-0.647	1	
	IgG, 48 hour	-35942.367	1073	-33.468	< .001	***
	(LPS+IgG, 48 hour)	-51980.167	1096	-47.408	< .001	***
IgG, 60 min	(LPS+IgG, 60 min)	1501.767	1073	1.398	1	
	IgG, 4 hour	808.8	1096	0.738	1	
	(LPS+IgG, 4 hour)	1017.2	1073	0.947	1	
	IgG, 24 hour	-540.933	1096	-0.493	1	
	(LPS+IgG, 24 hour)	-133	1073	-0.124	1	
	IgG, 48 hour	-35365.567	1096	-32.255	< .001	***
	(LPS+IgG, 48 hour)	-51403.367	1073	-47.865	< .001	***
(LPS+IgG, 60 min)	IgG, 4 hour	-692.967	1073	-0.645	1	
	(LPS+IgG, 4 hour)	-484.567	1096	-0.442	1	
	IgG, 24 hour	-2042.7	1073	-1.902	1	
	(LPS+IgG, 24 hour)	-1634.767	1096	-1.491	1	

	IgG, 48 hour	-36867.333	1073	-34.329	< .001	***
	(LPS+IgG, 48 hour)	-52905.133	1096	-48.251	< .001	***
IgG, 4 hour	(LPS+IgG, 4 hour)	208.4	1073	0.194	1	
	IgG, 24 hour	-1349.733	1096	-1.231	1	
	(LPS+IgG, 24 hour)	-941.8	1073	-0.877	1	
	IgG, 48 hour	-36174.367	1096	-32.992	< .001	***
	(LPS+IgG, 48 hour)	-52212.167	1073	-48.618	< .001	***
(LPS+IgG, 4 hour)	IgG, 24 hour	-1558.133	1073	-1.451	1	
	(LPS+IgG, 24 hour)	-1150.2	1096	-1.049	1	
	IgG, 48 hour	-36382.767	1073	-33.878	< .001	***
	(LPS+IgG, 48 hour)	-52420.567	1096	-47.809	< .001	***
IgG, 24 hour	(LPS+IgG, 24 hour)	407.933	1073	0.38	1	
	IgG, 48 hour	-34824.633	1096	-31.761	< .001	***
	(LPS+IgG, 48 hour)	-50862.433	1073	-47.361	< .001	***
(LPS+IgG, 24 hour)	IgG, 48 hour	-35232.567	1073	-32.807	< .001	***
	(LPS+IgG, 48 hour)	-51270.367	1096	-46.76	< .001	***
IgG, 48 hour	(LPS+IgG, 48 hour)	-16037.8	1073	-14.934	< .001	***

Appendix D. Table of the MFI of TRIM21/Ro52 of LPS and LPS+IgG groups in time

The MFI of TRIM21/Ro52 of LPS and LPS+IgG groups in time								
Group	**Time**	**Mean**	**SD**	**t**	**df**	**p**		**Cohen's d**
IgG	0 min	15108.77	130.02	-25.85	4.00	< .001	***	-21.11
LPS+IgG		24940.47	645.72	-25.85				
IgG	20 min	21207.97	1215.91	1.43	4.00	0.23		1.17
LPS+IgG		20165.83	328.86	1.43				
IgG	60 min	17546.87	280.10	-6.47	4.00	0.00	**	-5.28
LPS+IgG		18779.10	174.60	-6.47				
IgG	4 hour	17260.67	163.63	-14.15	4.00	< .001	***	-11.56
LPS+IgG		18959.30	128.23	-14.15				
IgG	24 hour	18501.70	191.45	-12.28	4.00	< .001	***	-10.02
LPS+IgG		21076.10	308.64	-12.28				
IgG	48 hour	19899.43	618.77	-4.42	4.00	0.01	*	-3.61
LPS+IgG		21664.80	309.99	-4.42				

Appendix E. The TRIM21/Ro52(+) cell percentages of LPS and LPS+IgG groups in time

The TRIM21/Ro52(+) cell percentages of LPS and LPS+IgG groups in time								
Group	**Time**	**Mean**	**SD**	**t**	**df**	**p**		**Cohen's d**
IgG	0 min	81.64	2.08	-13.49	2.01	0.005	*	-11.02

LPS+IgG		97.84	0.11	**(w)**				
IgG	20 min	98.09	0.54	2.59	4.00	0.061		2.11
LPS+IgG		97.19	0.27					
IgG	60 min	92.93	0.72	-2.80	4.00	0.049	*	-2.29
LPS+IgG		94.30	0.45					
IgG	4 hour	91.43	0.55	-6.44	4.00	0.003	**	-5.26
LPS+IgG		93.95	0.39					
IgG	24 hour	91.18	0.45	-7.42	4.00	0.002	**	-6.06
LPS+IgG		94.86	0.73					
IgG	48 hour	62.05	2.72	-3.12	2.04	0.087		-2.55
LPS+IgG		66.98	0.26	(w)				

Appendix F. The TRIM21/Ro52 FD of LPS and LPS+IgG groups in time

Group	**Time**	**Mean**	**SD**	**t**	**df**	**p**		**Cohen's d**
IgG	0 min	185.11	3.39	-16.76	4.00	< .001	***	-13.68
LPS+IgG		254.90	6.37					
IgG	20 min	216.17	11.19	1.30	4.00	0.263		1.06
LPS+IgG		207.49	2.81					
IgG	60 min	188.80	1.56	-9.70	4.00	< .001	***	-7.92
LPS+IgG		199.15	0.99					
IgG	4 hour	188.78	0.75	-16.38	4.00	< .001	***	-13.37
LPS+IgG		201.80	1.16					
IgG	24 hour	202.92	1.62	-14.06	4.00	< .001	***	-11.48
LPS+IgG		222.18	1.74					

IgG	48 hour	320.84	4.26	-0.63	4.00	0.561		-0.52
LPS+IgG		323.47	5.77					

Appendix G. Table of the Independent Sample t-Test Comparisons of LPS and LPS+IgG groups in time.

Independent Sample t-Test Comparisons of LPS and LPS+IgG groups in time										
		Group	**Mean**	**SD**	**t**	**df**	**p**		**Cohen's d**	
IgG(-) Trim21(-)%										
	0 min	IgG	15.80	1.92	12.43	2.01	0.01	**	10.15	W
		LPS+IgG	2.00	0.11						
	20 min	IgG	1.37	0.57	-2.83	4.00	0.05	*	-2.31	
		LPS+IgG	2.36	0.21						
	60 min	IgG	5.80	0.66	2.47	4.00	0.07		2.01	
		LPS+IgG	4.74	0.35						
	4 hour	IgG	7.14	0.52	5.68	4.00	0.01	**	4.64	
		LPS+IgG	5.04	0.37						
	24 hour	IgG	6.76	0.30	6.61	4.00	0.00	**	5.40	
		LPS+IgG	3.91	0.68						
	48 hour	IgG	35.51	2.47	4.64	2.09	0.04	*	3.79	W
		LPS+IgG	28.81	0.36						
IgG(+) Trim21(-)%										

	0 min	IgG	0.00	0.00					-0.26	NA
		LPS+IgG	0.00	0.00						
	20 min	IgG	0.24	0.13	2.61	4.00	0.06		2.13	
		LPS+IgG	0.04	0.02						
	60 min	IgG	0.23	0.02	1.89	4.00	0.13		1.54	
		LPS+IgG	0.19	0.03						
	4 hour	IgG	0.35	0.04	0.80	4.00	0.47		0.65	
		LPS+IgG	0.32	0.03						
	24 hour	IgG	0.98	0.06	4.41	4.00	0.01	*	3.60	
		LPS+IgG	0.68	0.10						
	48 hour	IgG	1.04	0.18	-16.87	4.00	< .001	***	-13.77	
		LPS+IgG	2.99	0.08						
IgG(-) Trim21(+)%										
	0 min	IgG	83.93	1.90	-12.64	2.01	0.01	**	-10.32	W
		LPS+IgG	97.78	0.08						
	20 min	IgG	97.09	0.32	-0.11	4.00	0.92		-0.09	
		LPS+IgG	97.11	0.18						
	60 min	IgG	92.09	0.76	1.39	4.00	0.24		1.13	

		LPS+IgG	91.42	0.36						
	4 hour	IgG	86.98	0.45	6.83	4.00	0.00	**	5.58	
		LPS+IgG	84.88	0.28						
	24 hour	IgG	68.54	0.68	-7.28	4.00	0.00	**	-5.94	
		LPS+IgG	72.12	0.52						
	48 hour	IgG	39.77	29.86	-1.19	2.00	0.36		-0.97	W
		LPS+IgG	60.22	0.35						
Igg(+) Trim21(+)%										
	0 min	IgG	0.00	0.00						
		LPS+IgG								
	20 min	IgG	1.31	0.46	3.09	2.01	0.09		2.52	W
		LPS+IgG	0.49	0.03						
	60 min	IgG	1.88	0.12	-19.98	4.00	< .001	***	-16.31	
		LPS+IgG	3.66	0.10						
	4 hour	IgG	5.54	0.12	-41.95	4.00	< .001	***	-34.25	
		LPS+IgG	9.76	0.13						
	24 hour	IgG	23.72	0.62	1.11	4.00	0.33		0.91	
		LPS+IgG	23.29	0.27						

	48 hour	IgG	5.89	0.04	-5.73	4.00	0.01	**	-4.68	
		LPS+IgG								

Appendix H. IgG and TRIM21/Ro52 Antibody titration results for flow cytometric analyses

Antibody and dilution	(A) Median IgG, IgG(+)	(B) Median IgG, IgG(-)	(C) rSD IgG (IgG(-)	SI
1/5000 igg	15205	966.8	545.8	13.04
1/2000 igg	26150.1	1844.6	1055.4	11.51
1/1000 igg	16463.1	3489.9	1407.1	4.61
1/500 igg	13814.8	4468.4	1476.1	3.17
1/5000 TRIM21/Ro52	56829.8	10254.5	2224.5	10.47
1/2000 TRIM21/Ro52	58607.2	10349.5	2447	9.86
1/1000 TRIM21/Ro52	65058.7	10539.9	24644.4	1.11
1/500 TRIM21/Ro52	70340.5	7424.1	3757.3	8.37