Thesis

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REGULATION OF MACROPHAGE INFLAMMATION BY NON-CANONICAL mRNA TRANSLATION

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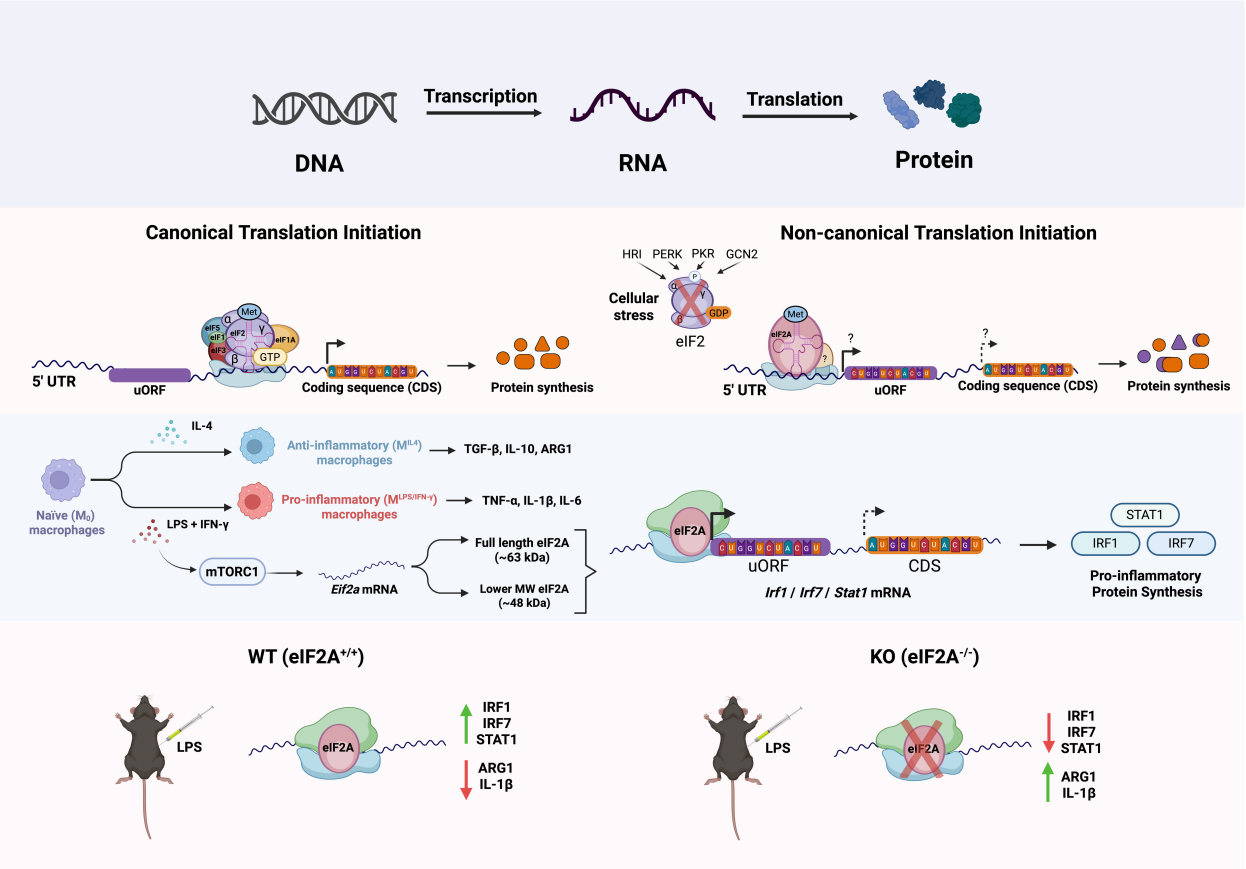
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ABSTRACT

Macrophage inflammatory responses require selective protein synthesis during polarization, yet the underlying translational mechanism is not fully elucidated. Eukaryotic initiation factor 2A (eIF2A), a non-canonical translation factor, was identified by our group to differentially regulate pro- and anti-inflammatory macrophages. We hypothesized that eIF2A plays a crucial role in macrophage polarization and functioning during the inflammatory response by exerting specific translational control. Using bone marrow-derived macrophages, we identified a full-length and a lower molecular weight eIF2A isoform predominantly in pro-inflammatory macrophages, coinciding with increased ribosomal S6 protein phosphorylation. eIF2A loss showed reduction trend in STAT1, IRF1, and IRF7 expression without altering transcripts, indicating post-transcriptional regulation, while showing an increasing trend in ARG1 and IL-1β. Upon *in vivo* LPS challenge, eIF2A was systemically induced. eIF2A knockout mice showed unaltered baseline immune cell profiles at 24 hours post-injection. These findings identify eIF2A as a selective regulator of translation during macrophage polarization.

GRAPHICAL ABSTRACT



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LIST OF ABBREVIATIONS

4E-BP	eIF4E-binding protein
AKT	V-akt murine thymoma viral oncogene homolog / PKB
ANOVA	Analysis of variance
ARE	Adenylate-uridylate-rich elements
ARG1	Arginase-1
ARS	Sodium arsenite
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
aTSS	Alternative translation start site
AUG	Adenine-uracil-guanine
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CBC	Cap-binding complex
CBP80	Cap-binding protein 80
CTIF	Cap-binding complex-dependent translation initiation factor
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's modified Eagle medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
eIF	Eukaryotic initiation factor
eIF2A$^{-/-}$	Homozygous knockout of *Eif2a* gene

ER	Endoplasmic reticulum
ETC	Electron transport chain
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN2	General Control Nonderepressible 2
GTP	Guanosine triphosphate
GWIPS-viz	Genome Wide Information on Protein Synthesis visualized
HEK293T	Human Embryonic Kidney 293 cells expressing the SV40 Large T-antigen
HeLa	Henrietta Lacks-derived cervical cancer cell line
HRI	Heme-regulated Inhibitor
Huh7	Human hepatoma 7 cells
IF-M1	Initiation factor M1
IF-MP	Initiation factor MP
IFN	Interferon
IFNAR	Interferon-α/β receptor
IFNGR	Interferon-gamma receptor
IκB	IkappaB
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IRAK	Interleukin-1 receptor-associated kinase
IRDye	Infrared fluorescent dye
IRES	Internal Ribosome Entry Site

IRF	Interferon-regulatory factor
ISR	Integrated stress response
ISRE	Interferon-stimulated response elements
JAK	Janus kinase
kDa	Kilodaltons
KO	Knockout
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAL	MyD88 adaptor-like protein
MAP	Methionine aminopeptidase
Met-tRNA$_i^{Met}$	Methionine-bound initiator transfer RNA
MIN6	Mouse insulinoma 6 cells
MMP	Matrix metalloproteinases
mORF	Main open reading frame
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ORF	Open reading frame

oxLDL	Oxidized low-density lipoprotein
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PDK-1	Phosphoinositide-dependent kinase 1
PERK	Protein Kinase R-like Endoplasmic Reticulum Kinase
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4-5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein Kinase B / AKT
PKC	Protein Kinase C
PKR	Protein Kinase RNA-activated
PRR	Pattern recognition receptor
PVDF	Polyvinylidene fluoride
Raptor	Regulatory-associated protein of mechanistic target of rapamycin
RCF	Relative centrifugal force
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mechanistic target of rapamycin
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA-IP	RNA immunoprecipitation
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature

RTK	Receptor-coupled tyrosine kinases
S	Svedberg unit
S6	Ribosomal protein S6
S6K	Ribosomal protein S6 kinase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SGK1	Serum and glucocorticoid-inducible kinase 1
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor-β
Th1	T helper 1
Th2	T helper 2
TICAM	Toll/interleukin-1 receptor domain containing adaptor molecule
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOP	Terminal oligopyrimidine
TRAF	Tumour necrosis factor receptor-associated factor
tRNA	Transfer ribonucleic acid

TSC1	Tuberous sclerosis protein 1
TSC2	Tuberous sclerosis protein 2
TYK	Tyrosine kinase
uORF	Upstream open reading frame
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WT	Wildtype

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1. Introduction

1.1 Inflammation

Inflammation is the essential reaction of the immune system in response to harmful stimuli, such as tissue injury, pathogen infection, or toxic compounds[1]. This inflammatory response is crucial, as it serves as an initial distress signal to the immune system in response to harmful agents, facilitates their elimination, and promotes the repair of local tissues, with the innate and adaptive immune systems acting as the first and second lines of defense[2,3].

The innate immune system provides a rapid, non-specific defence against harmful pathogens as the first line of defense[2]. During the encounter with the harmful pathogen, the body uses receptors that are expressed in various cells for detection and the firing of the innate inflammatory response[2]. The normal course of the innate inflammatory response involves distinct phases: onset of inflammation, onset of resolution, and return to homeostasis[3]. During the onset of inflammation, production of pro-inflammatory cytokines and chemokines increases, leukocytes such as neutrophils are recruited to the site of infection or injury, and macrophages become classically activated to function as pro-inflammatory macrophages[3]. After this response, neutrophils downregulate pro-inflammatory mediator production over time and release pro-resolving chemokines and cytokines to suppress inflammation, marking the onset of the resolution phase[3]. At the resolution phase, macrophages become alternatively activated to function as anti-inflammatory macrophages, production of anti-inflammatory cytokines and chemokines increases, and phagocytosis of apoptotic cells and neutrophils occurs in hopes of returning the tissue back to its homeostatic state[3].

This return to homeostasis is crucial, as dysfunctional pro-inflammatory and anti-inflammatory immune responses are both harmful to the body[2]. Excessive pro-inflammatory macrophage activity may promote chronic inflammation by producing reactive oxygen intermediates such as nitric oxide and pro-inflammatory mediators such as TNF-α and IL-1β, promoting tissue damage and fibrosis upon high and prolonged exposure[1,2,4]. Conversely, excessive anti-inflammatory macrophage activity may promote cancer progression and metastasis in immune-deserted "cold" tumours, in which macrophage activity facilitates angiogenesis and the cellular microenvironment is immunosuppressive due to lack of cytotoxic T cells[1,2,5]. However, this association between excessive anti-inflammatory macrophage activity and metastasis is not generalized across all tumour types, as pro-inflammatory mediators may facilitate anti-tumour immunity in immune-inflamed "hot" tumours[6,7]. Furthermore, insufficient macrophage activity is as detrimental as excessive macrophage activity, with consequences such as autoimmune diseases due to impaired clearance of apoptotic cells and autoantigen exposure, emphasizing the importance of macrophages in innate immune response[8].

The innate immune response's first encounter with pathogens initiates the adaptive immune response as the second line of defense[2]. Upon pathogen entry, antigen-presenting cells, such as macrophages, process and present proteins originating from the pathogen on major histocompatibility complex molecules found on their surface[9]. Subsequently, recognition of these antigens through lymphocyte receptors activates the adaptive immune system components T cells and B cells, leading to their differentiation into memory cells and formation of immunological memory over time[9]. This immunological memory leads to lifelong immunity against the previously encountered pathogen in most cases[2]. Thus, the adaptive immune system provides a specific, long-

lasting defence against previously encountered pathogens through the formation of immunological memory[2].

1.2 Macrophages in Inflammation

Macrophages are innate immune cells crucial for the inflammatory response, involved in both the onset of inflammation and resolution phases[10]. They promote the initiation as well as the resolution of inflammation by detecting pathogens and tissue damage, producing pro- and anti-inflammatory cytokines and chemokines, clearing out the apoptotic cells via phagocytosis, promoting tissue repair, and antigen presentation[11]. Therefore, macrophages take part in both the inflammatory response and active remodelling of their cellular environment[12].

Macrophages can be classified into two categories depending on where they originate in the body: tissue-resident macrophages and monocyte-derived macrophages[12,13]. Tissue-resident macrophages reside in most tissues and are mainly established from the fetal liver or yolk sac progenitors during embryogenesis[12]. Maintenance of tissue-resident macrophages occurs through local proliferation, as they are self-renewing and have a long lifespan, unlike monocyte-derived macrophages that require monocytic input for their maintenance and have a short lifespan[14]. Examples of tissue-resident macrophages include alveolar macrophages that phagocytose harmful pollutants and pathogens in the lungs, spleen-resident red pulp macrophages that function in iron metabolism, and large peritoneal macrophages that function in phagocytosis, microbial defence, and tissue repair in the peritoneum[14,15]. Upon sensing tissue damage or pathogens, tissue-resident macrophages release chemical signals, recruit circulating neutrophils and monocytes to the site of inflammation for microbial defence as part of their immune surveillance function and phagocytose apoptotic cells to maintain tissue homeostasis[14].

Furthermore, tissue-resident macrophages can function in apoptotic cell clearance, termed efferocytosis, by encapsulating the apoptotic cells with their membranes and degrading them[14]. Despite their crucial function of restoring tissue homeostasis and preventing detrimental inflammatory damage, tissue-resident macrophages diminish in number during the initiation of acute inflammation[14]. This reduction in tissue-resident macrophage numbers is due to excessive monocyte-derived macrophage presence at the inflammation site, causing tissue-resident macrophages' self-renewal capacity to decrease[14]. Therefore, restoring the tissue-resident macrophage population through local proliferation plays a critical role during inflammation resolution to prevent tissue damage and dynamic changes in macrophage populations are crucial during inflammation and tissue repair[14].

Unlike tissue-resident macrophages, monocyte-derived macrophages do not readily reside in tissues, have a short lifespan, and are differentiated from circulating bone marrow-derived monocytes that are recruited into tissues upon detection of pathogens or tissue damage, providing a robust pro-inflammatory response[13,14]. As part of this inflammatory response, monocyte-derived macrophages phagocytose pathogens and cells undergoing apoptosis or senescence, followed by inflammation resolution[16]. After the inflammation subsides, most of the monocyte-derived macrophages undergo efferocytosis by other macrophages, while some undergo *in situ* phenotypic transition into tissue-resident macrophages[11,14]. Therefore, macrophages are crucial immune cells in the inflammatory response, with the ability to adopt different phenotypes based on cues in their cellular microenvironment, referred to as macrophage polarization[2,11].

1.3 Macrophage Polarization

Macrophages can polarize into different phenotypes in a dynamic spectrum in response to signalling cues in their environment due to their plasticity [2,11]. Macrophage polarization towards a pro-inflammatory macrophage phenotype occurs in the early stages of pathogen or tissue damage detection for modulation of the immune response[17]. Detection of infection-causing pathogens or tissue damage by macrophages occurs through pattern recognition receptors (PRRs), most notably Toll-like receptors (TLRs)[2]. Damage-associated molecular pattern molecules (DAMPs), which are released from the host's cells upon cellular stress or microbe invasion, and pathogen-associated molecular pattern molecules (PAMPs), which are present in the foreign pathogen and absent in the host, are recognized by TLRs[17].

Naïve macrophages become classically activated towards pro-inflammatory macrophages upon recognition of TLR ligands such as lipopolysaccharide (LPS) and T helper 1 (Th1) cytokines such as interferon-γ (IFN-γ) by the TLRs[11,18]. Recognition of LPS by TLR4, which is a well-characterized example of an inflammation trigger, drives the translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to the nucleus and activation of interferon-regulatory factors (IRFs) through a signalling pathway involving Toll/interleukin-1 receptor domain containing adaptor molecule 1 (TICAM1) and Myeloid differentiation primary response 88 (MyD88)[2,19]. As a result, the genes involved in the inflammatory response in macrophages are transcribed, leading to the production of pro-inflammatory mediators such as tumour necrosis factor-alpha (TNF-α) and interleukins (ILs) such as IL-12 and IL-23 to elevate the immune response and eliminate the pathogen[11,18]. Other factors produced by the pro-inflammatory macrophages include IL-1β, IL-6, reactive oxygen and nitrogen intermediates, which enhance the microbicidal and tumoricidal response[13].

Following the initial pro-inflammatory response, naïve macrophages become alternatively activated towards anti-inflammatory macrophages to resolve inflammation[20]. Polarization towards anti-inflammatory macrophages occurs upon stimulation with T helper 2 (Th2) cytokines such as IL-4 and IL-13[11]. As a result, these macrophages clear the debris and produce anti-inflammatory mediators, such as IL-10, transforming growth factor-β (TGF-β), and Arginase-1 (ARG1) to promote tissue repair[13,20]. Furthermore, they produce pro-resolving mediators, such as resolvins and protectins, facilitating the return to homeostasis after the initial pro-inflammatory response[21,22]. In addition to debris clearance, anti-inflammatory macrophages produce enzymes to remodel extracellular matrix components and growth factors, such as matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF), thereby promoting angiogenesis and tissue remodelling[23,24]. During this return to homeostasis process, tissue-resident macrophages might be restored as monocyte-derived macrophages undergo cell death, or monocyte-derived macrophages might differentiate to function like tissue-resident macrophages[20].

In this thesis, LPS and IFN-γ are used to stimulate bone marrow-derived macrophages toward pro-inflammatory state, whereas IL-4 is used for stimulation toward anti-inflammatory state. These defined cues are used to push the cells in culture towards the extremes of polarization spectrum and are not assumed to represent macrophages subsets *in vivo*.

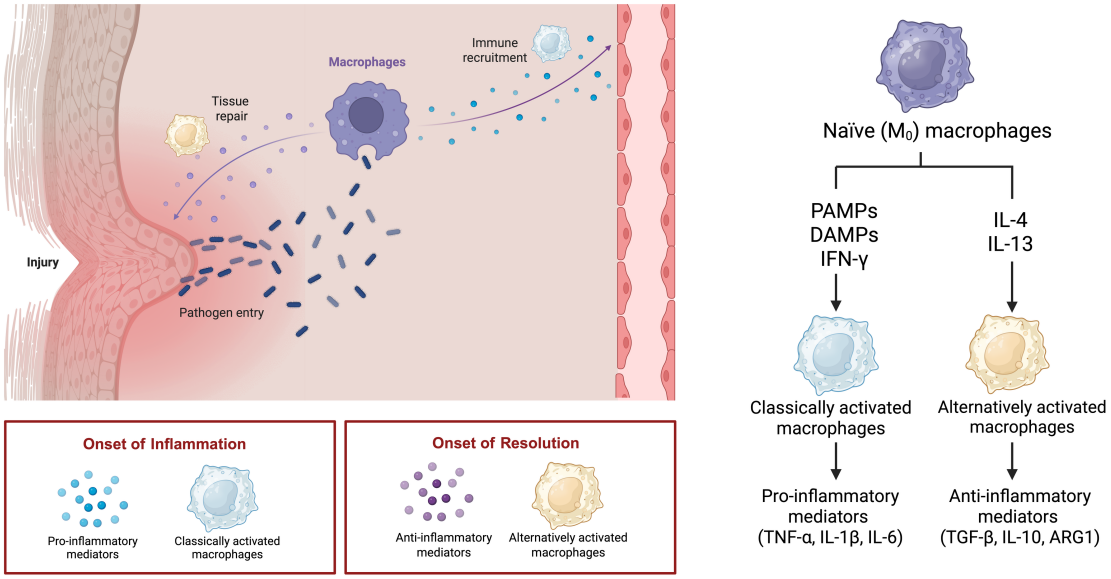


Figure 1: Macrophage polarization and its role in inflammation.

Tissue-resident and monocyte-derived macrophages, which exist in a naïve (M_0) macrophage state while unstimulated, respond to signals in the cellular microenvironment, including DAMPs, PAMPs, IL-4, and IL-13, and polarize toward functionally distinct macrophage states in a dynamic spectrum. Detecting DAMPs, PAMPs, and Th1 cytokines, such as IFN-γ, drives classical activation of macrophages, whereas IL-4 and IL-13 detection drives alternative macrophage activation. Classically activated macrophages produce pro-inflammatory mediators, such as TNF-α, IL-1β, and IL-6, and recruit more immune cells to the site of inflammation. Alternatively activated macrophages produce anti-inflammatory mediators, such as TGF-β, IL-10, and ARG1, to resolve the inflammation and repair tissues. Together, macrophage polarization plays a crucial role in coordinating both the initiation and the resolution of inflammation and maintaining homeostasis.

1.3.1 IRF/STAT Axis in Macrophage Polarization

Different TLRs can recognize different ligands depending on their subcellular localization[25]. Cell surface-expressed TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 detect extracellular pathogen components, whereas endosomal TLR3, TLR7, TLR8, and TLR9 detect nucleic acids of foreign origin[25]. For example, the Gram-positive bacteria cell wall component lipoteichoic acid (LTA) is recognized by TLR2, and the Gram-negative bacteria cell wall component LPS is recognized by TLR4[2].

Through specific ligand and receptor interactions, TLRs can initiate numerous downstream signalling pathways that play a role in macrophage polarization, such as the IRF, NF-κB, and the signal transducer and activator of transcription (STAT) transcription factor families[13]. The IRF/STAT axis is crucial in the macrophage context as it modulates polarization towards pro-inflammatory macrophages via STAT1, 2, 4, and IRF1, 5, 8, 9, whereas STAT6 and IRF3, 4 contribute to the polarization towards anti-inflammatory macrophages[26].

Type I interferons modulate the inflammatory response in a context-dependent manner, by increasing the expression of pro- or anti-inflammatory mediators, acting as facilitatory or inhibitory signals for inflammation[27]. They activate both innate and adaptive immune responses via promoting antigen presentation, mediating cytokine production, facilitating antiviral effects in infected cells, and triggering T cell and B cell responses to form immunological memory[28,29]. Type I interferon response can be triggered upon binding of LPS to TLR4, activating TICAM1-dependent and MyD88-dependent signalling pathways[30]. In the MyD88-dependent pathway, MyD88 adaptor-like protein (MAL) is recruited to MyD88 and TLR4 to form a complex at the plasma membrane[30,31]. This complex then phosphorylates interleukin-1 receptor-associated kinase (IRAK) family member IRAK4, which in turn phosphorylates IRAK1[30]. Phosphorylation of

IRAK1 activates tumour necrosis factor receptor-associated factor 6 (TRAF6), which activates the IkappaB (IκB) kinase complex, resulting in dissociation of IκB from NF-κB[30]. Subsequently, NF-κB translocates to the nucleus and promotes the transcription of early pro-inflammatory genes, such as *Il6* and *IL1b*[30,32].

In the TICAM1-dependent pathway, TICAM2 is recruited to form a complex with TICAM-1 and internalized TLR4 in endosomes[30,33]. This complex then binds to IκB kinase epsilon and TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), resulting in IRF3 phosphorylation[30]. Phosphorylated IRF3 dimerizes and translocates into the nucleus to produce IFN-β, which is a type I interferon[30,34]. IFN-β then binds to interferon-α/β receptors (IFNARs) and activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which phosphorylate STAT1 and STAT2[35]. Upon phosphorylation, the STAT1/STAT2 heterodimer binds IRF9 and translocates to the nucleus to drive transcription of interferon-stimulated genes, such as *Irf7*[35–37]. IRF7 functions in a positive amplification loop to increase the production of type I interferons IFN-α and -β, making it a crucial regulator of the type I interferon response[37–39]. However, IRF7 is constitutively expressed only in some myeloid and lymphoid cells, such as resting bone marrow-derived macrophages (BMDMs) and plasmacytoid dendritic cells[40]. Therefore, IRF7 basal expression in most cell types remains low until induction by a viral infection, unlike IRF3, which is constitutively expressed in most cell types[40].

Type II interferon IFN-γ modulates innate and adaptive immune responses, elevates TLR expression, and enhances LPS-induced activation of NF-κB[41]. The type II interferon response involves binding of IFN-γ to the interferon-gamma receptor (IFNGR)[42]. Subsequently, IFNGR binds and activates JAK1 and JAK2, which phosphorylate STAT1, leading to its homodimerization[42]. The STAT1:STAT1 homodimer translocates to the nucleus and drives

transcription of interferon-stimulated genes, such as *Irf1*[42,43]. IRF1 amplifies the transcription of interferon-stimulated genes as part of a positive amplification loop, making it a crucial regulator of type II interferon response[41]. As a result, IRF1-mediated expression of inflammatory mediators, such as inducible nitric oxide synthase (iNOS), which are crucial for the pro-inflammatory macrophage phenotype, is induced[39]. However, excessive pro-inflammatory macrophage activation is harmful and needs to be suppressed by IFN-γ-mediated feedback inhibitory loops[41]. IFN-γ mediates the inhibition of signal transduction by inducing the expression of suppressor of cytokine signalling (SOCS) proteins[41]. SOCS1 competes with STAT1 to bind to the IFNGR on the same site, leading to inhibition of the JAK/STAT signalling pathway[41]. Therefore, IFN-γ mediates signal amplification and inhibition loops to prevent cytokine storms and regulate macrophage activation[41].

1.3.2 Cellular Metabolism Reprogramming in Macrophage Polarization

In addition to the functional differences due to transcriptional output, pro- and anti-inflammatory macrophages undergo distinct metabolic reprogramming and show differences in lipid and amino acid metabolism, which affect macrophage responses[44,45].

In pro-inflammatory macrophages, production of pro-inflammatory mediators, such as reactive oxygen species, inhibits oxidative phosphorylation through damaging electron transport chain (ETC) complexes and tricarboxylic acid (TCA) cycle enzymes[46,47]. This causes a metabolic shift toward glycolysis as the main energy source, producing adenosine triphosphate (ATP) molecules and intermediates needed for several other biosynthetic pathways [44,45]. After the initial metabolic switch, maintenance of the glycolytic state involves IRF1-induced iNOS expression, where IRF1 is activated both by LPS and IFN-γ, indicating a relation between transcriptional

output and metabolic reprogramming in macrophage polarization[39,48–50]. In addition to glucose metabolism, iNOS expression plays a role in amino acid metabolism by converting arginine to nitric oxide and citrulline[51]. Citrulline is an amino acid which can be recycled into arginine, thereby producing more nitric oxide[52]. As a reactive nitrogen species, nitric oxide creates severe cellular stress, promoting inflammatory signalling and antimicrobial properties[51,52]. In terms of lipid metabolism, pro-inflammatory macrophages upregulate fatty acid synthesis to sustain pro-inflammatory properties, such as membrane remodelling required for phagocytosis[44,45,51].

Conversely, anti-inflammatory macrophages rely on oxidative phosphorylation as the main energy source, as ETC complexes are functional[51]. Amino acid metabolism of anti-inflammatory macrophages involves upregulation of ARG1, which hydrolyzes arginine into ornithine, limiting nitric oxide production[51,52]. Ornithine is further metabolized by ornithine aminotransferase, producing proline, an amino acid needed for wound healing[51]. For lipid metabolism, anti-inflammatory macrophages enhance fatty acid oxidation and produce metabolic intermediates, such as acetyl coenzyme A[51]. Subsequently, the resulting intermediates support the TCA cycle and oxidative phosphorylation, thereby producing the energy needed to sustain their pro-resolving properties[44,45,51].

Overall, macrophages play a crucial role in both pro- and anti-inflammatory responses upon detecting molecular signals, in which highly orchestrated and tightly regulated messenger ribonucleic acid (mRNA) translation is required for coordination[1].

1.4 Canonical mRNA Translation in Eukaryotes

mRNA translation process is fundamental for gene expression and vital for all living organisms, as protein production is crucial for functional and structural purposes[53]. The process of

translation involves reading and decoding of an mRNA's sequence by ribosomes along with transfer ribonucleic acids (tRNAs) into amino acid chains to synthesize a protein, which is divided into four distinct steps: initiation, elongation, termination, and ribosome recycling[53,54].

The initiation of mRNA translation in eukaryotes occurs predominantly in a cap-dependent mechanism and involves eukaryotic initiation factors (eIFs), although there are some stress response mRNAs in which translation initiation may occur in a non-canonical manner[55–57]. In canonical cap-dependent translation initiation, the heterotrimeric translation initiation factor eIF2 with α, β, and γ subunits initiates translation by binding and forming a ternary complex with the methionine-bound initiator transfer RNA (Met-tRNA$_i^{Met}$) and guanosine triphosphate (GTP)[58]. This ternary complex binds to eIF1, eIF1A, eIF3, and eIF5, and delivers the Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit to form the 43S pre-initiation complex [58,59]. Subsequently, the 43S pre-initiation complex is recruited to the 5' end of an mRNA containing a 7-methylguanosine cap by the eIF4F complex, which involves eIF4E, eIF4G, and eIF4A[58,59]. Then, the pre-initiation complex scans the mRNA strand from 5' to 3' direction for a start codon, which is commonly an adenine-uracil-guanine (AUG) codon[58]. Upon recognition, the Met-tRNA$_i^{Met}$ base pairs with this start codon via its anticodon, causing hydrolysis of the eIF2-bound GTP, and dissociation of several eIFs, including eIF2, through eIF5 and eIF5B activity[56,58]. Then, the 60S ribosomal subunit binds to the 40S ribosomal subunit via eIF1A and eIF5B activity and forms the 80S ribosome, which moves along the mRNA in the 5' to 3' direction for translation elongation[56–58]. Subsequently, GDP-bound eIF2 undergoes recycling by eIF2B, which becomes GTP-bound eIF2 and initiates more rounds of translation[57].

Ribosomes are crucial ribonucleoprotein complexes functioning in protein synthesis[60]. They have 3 different sites for tRNA binding: aminoacyl, peptidyl, and exit sites[61]. The aminoacyl

site is where the new tRNAs that are charged with amino acids, the aminoacyl-tRNA, enter the ribosome[62]. When a three-letter codon in the mRNA strand enters the aminoacyl site of the ribosome, the aminoacyl-tRNA is recruited to the aminoacyl site for base pairing via its matching anticodon[53,62]. Then, the tRNA that was bound to the aminoacyl site moves to the peptidyl site, allowing the next set of codons in the mRNA to enter the aminoacyl site and the recruitment of a new aminoacyl-tRNA that matches the codon[53]. This continues the elongation process, as the adjacent amino acids on the tRNAs form a peptide bond, the tRNA on the peptidyl site releases its amino acid and moves to the exit site to leave the ribosome, and the tRNA on the aminoacyl site moves to the peptidyl site with its growing chain of polypeptides, leaving the aminoacyl site empty for the next round of tRNA binding[53,62]. Translation elongation continues until a UAG, UGA, or UAA stop codon enters the aminoacyl site, which causes the translation to terminate as there are no tRNAs with a matching anticodon for stop codons[63]. As a result, release factors recognize the stop codon and bind to the aminoacyl site, leading to the hydrolysis of the newly synthesized polypeptide chain from the tRNA and the recycling of the ribosome for more rounds of translation[53,62]. Recycling of the ribosomes involves the separation of 40S and 60S ribosomal subunits and the dissociation of the mRNA strand and the deacylated tRNA from the 40S ribosomal subunit[64]. Inefficient recycling may lead to consequences such as ribosome accumulation at the stop codon and translation reinitiation downstream of the stop codon, which could impair the efficiency of translation[64]. Therefore, tight regulation of the mRNA translation process holds a crucial role in the maintenance of efficient protein synthesis in accordance with cellular needs[59].

1.5 Mammalian Translational Control

Translational control is crucial for organisms to regulate cellular processes, such as proliferation and growth, as well as immune cell functioning[1,56]. The ability of macrophages to produce pro- and anti-inflammatory mediators in response to signals in their cellular microenvironment does not occur merely by transcriptional control[65]. Translational control is required to orchestrate how much and when a protein is produced[66]. For example, upon sensing DAMPs and PAMPS, naïve macrophages must polarize toward the pro-inflammatory phenotype and produce pro-inflammatory mediators at a precise amount and time to prevent cytokine storm and detrimental tissue damage[67]. Subsequently, polarization toward the anti-inflammatory phenotype and anti-inflammatory mediator production must occur at a precise amount and time to resolve inflammation and prevent chronic inflammatory diseases[68].

Furthermore, specific mRNAs that are involved in the immune response are translationally repressed during the resting state and selectively translated through becoming derepressed upon sensing harmful stimuli[65]. For example, the expression of TNF-α is under translational control via the adenylate-uridylate-rich elements (AREs) found in the 3'UTR of *Tnf* mRNA[1]. Under normal conditions, translation of TNF-α is repressed due to occupation of AREs by the RNA-binding proteins[1]. When the immune response is activated, the affinity of AREs for RNA-binding proteins decreases, which allows translation to proceed in a transcript-independent manner, since TNF-α protein levels are affected by translational repression rather than the abundance of the transcript[1]. Therefore, translational control offers precise and selective protein output required for immune cell activation, which is crucial for the immune response and cell survival[1,2,11,65].

In mammals, the mechanistic target of rapamycin (mTOR) is a major regulator of translation in response to signals from growth factors, availability of nutrients, cellular stress, and

energy sufficiency[56,59]. During translation initiation, mTOR facilitates the formation of the eIF4F complex on the 5' end of the mRNA strand, which recruits the 43S pre-initiation complex to the mRNA for the subsequent cap-dependent translation process[59]. Therefore, translation control occurs mostly at the initiation step, where the rate at which ribosomes are added to the mRNA is regulated by a complex process[56].

mTOR is involved in the formation of two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)[56]. Upon binding of an extracellular signalling molecule to a receptor-coupled tyrosine kinase (RTK), phosphoinositide 3-kinase (PI3K) becomes activated, leading to phosphorylation of phosphatidylinositol-4-5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-triphosphate (PIP_3)[56]. Subsequently, phosphoinositide-dependent kinase 1 (PDK-1) is recruited to the plasma membrane along with protein kinase B (PKB), also known as AKT[56]. AKT requires phosphorylation of its Threonine 308 and Serine 473 residues by PDK-1 and mTORC2, respectively, which activates and phosphorylates tuberous sclerosis proteins 1 and 2 (TSC1 and TSC2)[56]. This phosphorylation dissociates TSC1 from TSC2 and prevents the inhibition of mTOR, causing GTP-bound Ras homolog enriched in brain (Rheb) to be hydrolyzed to the GDP-bound form, thereby activating mTORC1[56]. The mTORC1 complex involves the regulatory-associated protein of mTOR (Raptor), regulates proliferation and growth of cells, whereas the mTORC2 complex involves the rapamycin-insensitive companion of mTOR (Rictor) and regulates cell metabolism, survival, and cytoskeletal structure[59,69]. The mTORC1 complex functions by promoting the translation of ribosomal proteins and transcription of ribosomal RNAs (rRNAs) for ribosome biogenesis[56]. Upon activation through the lysosomal membrane, mTORC1 phosphorylates the eIF4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K), both of which are recruited by the Raptor protein, regulating mRNA translation, cell proliferation, and

lipid metabolism[56,69,70]. Phosphorylation of S6K leads to its activation, which phosphorylates and thereby activates the ribosomal protein S6, a component of the 40S ribosome, to induce mRNA translation[56]. Conversely, phosphorylation of 4E-BP leads to its inactivation, which allows the eIF4E-eIF4G interaction, as eIF4E is no longer bound and sequestered by the 4E-BPs, leading to the 43S pre-initiation complex recruitment to the 5' end cap of mRNA for canonical translation initiation[56]. The mTORC2 complex phosphorylates and activates several protein kinases at the plasma membrane, such as protein kinase C (PKC) and serum and glucocorticoid-inducible kinase 1 (SGK1), to regulate cytoskeletal structure and cell survival[70,71]. Furthermore, mTORC2's phosphorylation of AKT at Serine 473, which functions in the mTORC1 pathway, leads to AKT's activation and enhances mTORC1 activity[56,70,71].

Beyond its role in cellular proliferation and survival, mTOR signalling integrates various molecular signals in the cellular environment during the immune response[69,72]. In immune cells, mTORC1 activity has been shown to regulate their functioning and differentiation via Raptor[69]. For example, BMDMs that are deficient in Raptor protein have been reported to show decreased mTORC1-S6K signalling and have impairment in differentiating into osteoclasts[69]. In macrophage polarization, the role of mTORC1 and mTORC2 in promoting macrophage phenotypes is suggested to be highly context-dependent[73]. mTORC2 has been primarily proposed to promote macrophage polarization toward an anti-inflammatory phenotype to maintain homeostasis[73]. Conversely, multiple reports have shown conflicting results on mTORC1's role in promoting macrophage phenotypes[73]. In most cases, active mTORC1 has been shown to promote the anti-inflammatory macrophage phenotype[73,74]. However, chronically active mTORC1 has been proposed to promote the pro-inflammatory macrophage phenotype during inflammation upon LPS treatment in a nutrient-rich environment[73,74]. Together, this suggests that although mTORC1 is a

major regulator responsible for translational control under favourable conditions, alternative mechanisms of translational control under cellular stress may exist[1,16,56].

1.6 mRNA Translation Under Stress

In various stress conditions, such as hypoxia or the presence of viral infection, the integrated stress response (ISR) suppresses global mRNA translation by using four ISR kinases to sense the cellular stress-causing factors[56]. These four ISR kinases include GCN2, which responds to amino acid deficiencies; PKR, which responds to infections by viruses; PERK, which responds to endoplasmic reticulum (ER) stress and the unfolded protein response; and HRI, which responds to heme availability[56,75]. Upon sensing cellular stress by these four ISR kinases, the translation initiation factor eIF2α gets phosphorylated at serine 51, causing the availability of eIF2 to form the 43S pre-initiation complex to become limited, and thereby suppressing global protein synthesis[56]. However, selective translation of some mRNAs still occurs under ISR conditions to overcome the cellular stress, even though eIF2 is inactivated[56].

During cellular stress, mRNA translation favours the stress response and cell survival[55]. Non-canonical translation initiation may take place via cap-dependent or cap-independent mechanisms[55]. In cap-independent translation initiation, the scanning process does not occur and the recruitment of the 40S ribosomal subunit to the 5’ UTR of mRNA occurs directly at or upstream of the start codon using an internal ribosome entry site (IRES)[55]. In the absence of IRES elements, translation can also selectively initiate in a cap-independent manner but still utilize the 5’ mRNA scanning process, using a cap-independent translational enhancer (CITE) element[55]. Furthermore, the 5’UTR of mRNAs that contain N6-methyladenosine can be translated through the direct binding of eIF3 and the 40S ribosomal subunit in a cap-independent manner[55].

In cap-dependent non-canonical translation initiation, recruitment of initiator tRNA to the 40S ribosomal subunit can be performed by other carriers, such as eIF2D, MCTS1-DENR, and eIF2A, to selectively translate specific mRNAs, independent of GTP[16,58,76,77]. Furthermore, the eIF2D protein and MCTS1-DENR heterodimer function in ribosome recycling by separating the deacylated tRNA and mRNA from the unrecycled 40S ribosomal subunits and in translation reinitiation by delivering Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit when the start codon is positioned in its peptidyl site[64,77,78]. Translation reinitiation downstream of some uORFs, such as in the ATF4 5' untranslated region (UTR) upon cellular stress, occurs by using non-canonical translation factors[64,78]. Under normal conditions, the 5' UTR of the *Atf4* mRNA with upstream open reading frames (uORFs) causes main open reading frame (mORF) translation to be suppressed due to ribosome dissociation prior to mORF translation[56,76]. However, under cellular stress, selective translation of *Atf4* mRNA may occur by eIF2D and MCTS1-DENR recycling the ribosome, which enables mORF translation, although the exact mechanism remains unclear[76–78]. This suggests that eIF2D and MCTS1-DENR's role in translation might be primarily in ribosome recycling and translation reinitiation steps, whereas eIF2A's precise role in eukaryotic translation remains unclear[58,79].

eIF2A, formerly known as IF-M1, is a monomeric non-canonical translation factor with a molecular weight of 65 kDa[58]. In the 1970s, eIF2A was proposed to deliver Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit to initiate translation, although it is less efficient compared to trimeric eIF2, formerly known as IF-MP, based on methionyl-puromycin formation[80]. However, further research showed that eIF2A delivers Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit under cellular stress, in which ISR causes eIF2α to be phosphorylated at serine 51 and suppresses global mRNA translation[16,56,58]. Upon delivery by eIF2A, the Met-tRNA$_i^{Met}$ can pair with a non-AUG start codon

in the upstream ORF, without binding to GTP, leading to selective translation of specific mRNAs[58]. However, multiple reports have challenged eIF2A's role in translation initiation[58,81–83]. Ribosome profiling data in studies involving yeast, HEK293T, and HeLa cell lines found little to no evidence that eIF2A plays a role in translation under cellular stress[81–83]. Separately, one study involving mammalian *in vitro* translation extracts reported that eIF2A plays a role in translation inhibition by binding and sequestering the 40S ribosomal subunit directly, and cells could lower phosphorylated eIF2α levels by sensing elevated levels of eIF2A[58]. However, a conclusive role for eIF2A protein in eukaryotic translation, particularly in macrophages where translational control is crucial for immune response, has yet to be established.

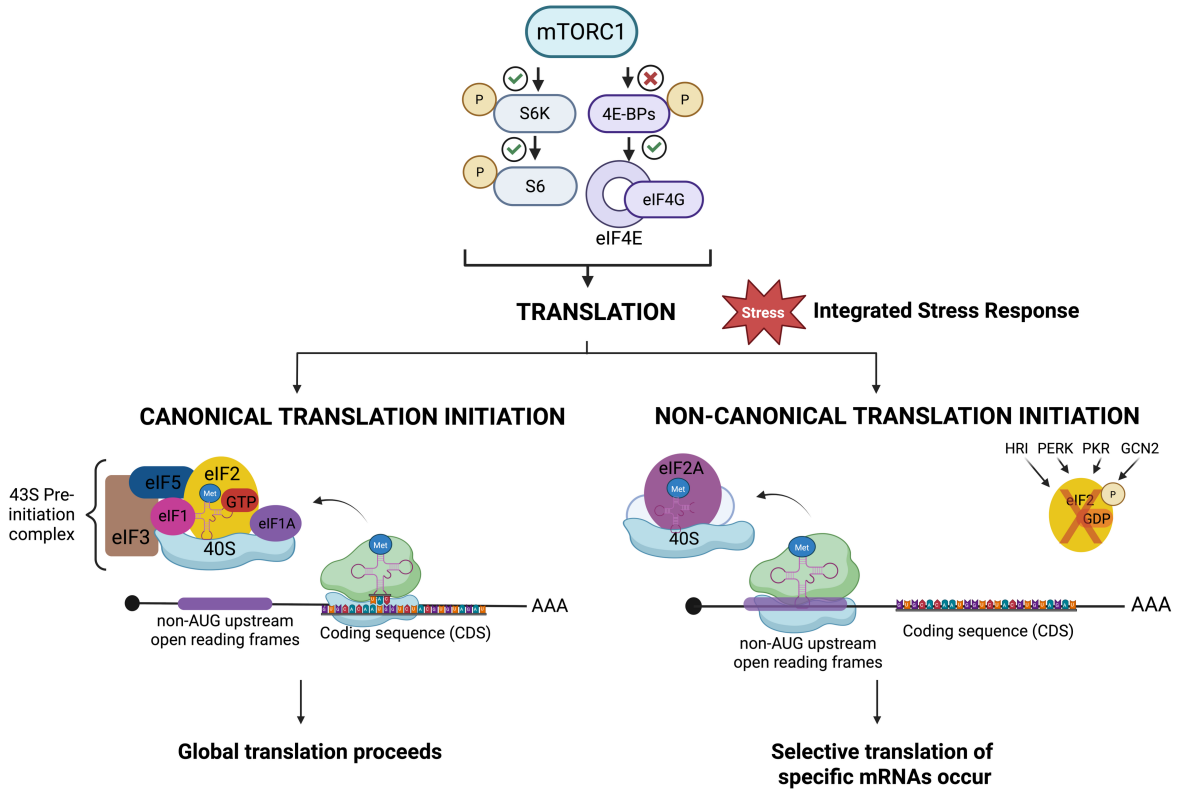


Figure 2: Canonical and non-canonical translation initiation pathways downstream of mTORC1.

mTORC1 signalling phosphorylates S6K and 4E-BP, leading to phosphorylation and activation of S6 and interaction of eIF4E with eIF4G, thereby promoting cap-dependent translation. Under normal conditions, canonical translation initiation involves eIF2-mediated delivery of initiator tRNA to the 40S ribosomal subunit via the 43S pre-initiation complex, allowing global translation to proceed. Under cellular stress, eIF2 is phosphorylated by stress-sensing kinases, as part of the integrated stress response, thereby repressing its function. Under these conditions, eIF2A has been proposed to recruit initiator tRNA to the 40S ribosomal subunit and initiate translation at upstream open reading frames at non-AUG start codons. This enables selective translation of specific mRNAs under stress, despite suppression of global translation.

1.7 eIF2A in Macrophage Polarization

Given that eIF2A's role in the macrophage polarization context has not been established in the literature, unpublished preliminary data from our group started to address eIF2A's role in macrophage polarization and provided the rationale for this study. Translation efficiency data used for pathway enrichment analysis showed that eIF2 signalling is differentially regulated in pro-inflammatory ($M^{LPS/IFN\text{-}\gamma}$) and anti-inflammatory (M^{IL4}) macrophage states (**Figure 3A**). This macrophage polarization state-dependent regulation of eIF2 signalling prompted a closer look into the factors involved in eIF2-dependent translational machinery, where our group identified eIF2A as a candidate factor involved in these distinct translational programs. Further analysis of eIF2A in translational kinetics among $M^{LPS/IFN\text{-}\gamma}$ and M^{IL4} macrophages by puromycin labelling showed that M_0, $M^{LPS/IFN\text{-}\gamma}$, and M^{IL4} BMDMs exhibit translational kinetics peaking at different time points (**Figure 3B**). $M^{LPS/IFN\text{-}\gamma}$ macrophages showed peak protein synthesis until 24 hours post-polarization, whereas M^{IL4} macrophages sustained protein synthesis until 48 hours post-polarization. These distinct translational kinetics and differential pathway enrichment data observed in $M^{LPS/IFN\text{-}\gamma}$ and M^{IL4} macrophages suggested the presence of a translational regulator involved in the distinct translational programming during macrophage polarization.

We then examined eIF2A's expression and the mTORC1 pathway component ribosomal protein S6, as total protein and phosphorylated forms, through Western blotting to observe whether there are distinct expression patterns for these proteins among different macrophage polarization states. eIF2A protein was detected to have two distinct bands, a full-length (~ 63-65 kDa) and a lower molecular weight (~48 kDa) isoform (**Figure 3C**). The lower molecular weight eIF2A isoform was present across all macrophage phenotypes, whereas the full-length eIF2A isoform was observed prominently in pro-inflammatory ($M^{LPS/IFN\text{-}\gamma}$) macrophages. Expression of ribosomal

protein S6 remained comparable among all macrophage phenotypes, whereas $M^{LPS/IFN\text{-}\gamma}$ macrophages showed increased phosphorylated S6 (p-S6) protein expression, suggesting activation of mTORC1 signalling during polarization toward the pro-inflammatory macrophage state.

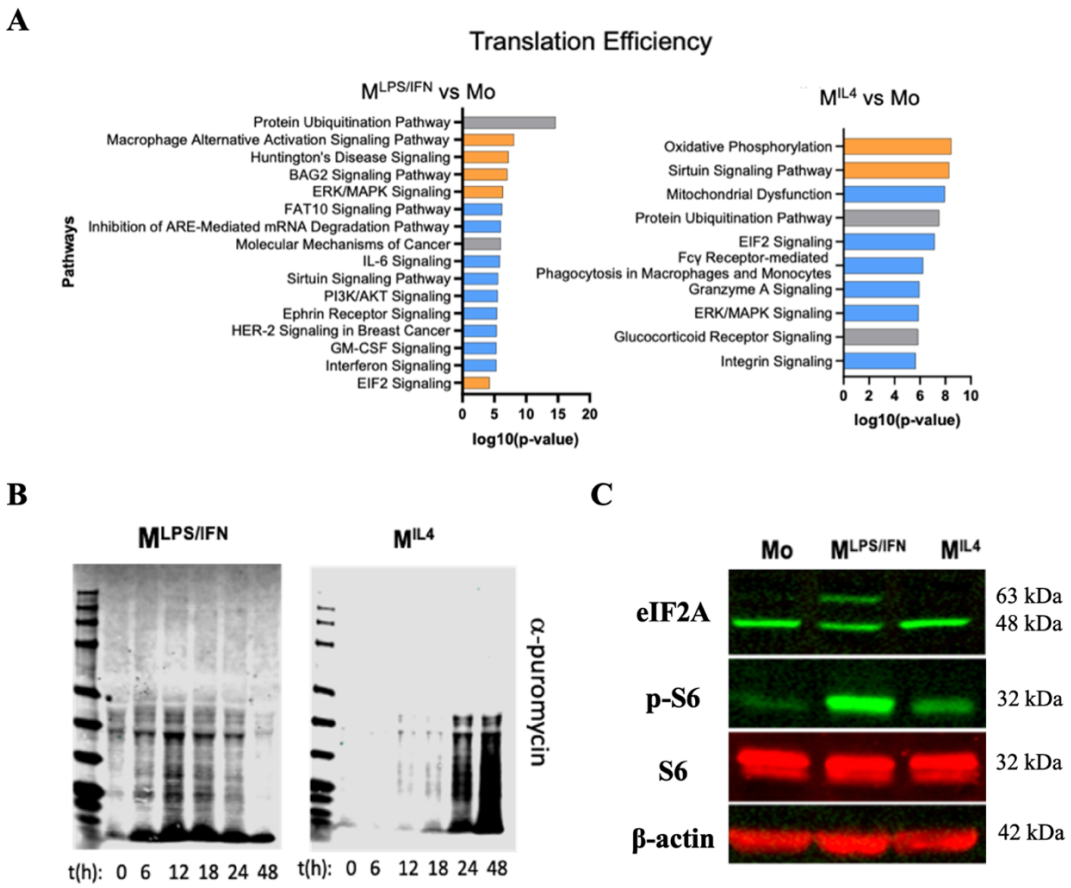


Figure 3: Pro- and anti-inflammatory BMDMs have distinct translational programs.

(A) Pathway enrichment analysis of translation efficiency (Ribo-seq/RNA-seq) in $M^{LPS/IFN-\gamma}$, and M^{IL4} BMDMs compared to M_0. Orange bars show activated pathways and blue bars show inhibited pathways. **(B)** Western blot depicting the puromycin-labelled $M^{LPS/IFN-\gamma}$ and M^{IL4} BMDMs at 0-, 6-, 12-, 18-, 24-, and 48-hour time points post-polarization. **(C)** Western blot depicting the expression of eIF2A protein in BMDMs from WT M_0, $M^{LPS/IFN-\gamma}$, and M^{IL4} macrophages, along with mTOR pathway targets phosphorylated S6 (p-S6) and S6 proteins. β-actin was used as the loading control. Data were obtained by Dr. Huy-Dung Hoang and Dr. My-Anh Nguyen (unpublished).

1.8 Rationale

Preliminary data generated by Dr. Huy-Dung Hoang and Dr. My-Anh Nguyen (unpublished) show that eIF2 signalling is differentially regulated in pro-inflammatory and anti-inflammatory macrophages. The eIF2 signalling pathway was predicted to be upregulated in $M^{LPS/IFN\text{-}\gamma}$ macrophages, whereas downregulated in M^{IL4} macrophages in comparison to the M_0 macrophages (**Figure 3A**). Puromycin labelling showed distinct translational kinetics between the $M^{LPS/IFN\text{-}\gamma}$ and M^{IL4} macrophages (**Figure 3B**), indicating the presence of macrophage polarization state-dependent translational programming. Together, these findings raised the possibility of a translational regulator involved in this distinct translational programming of macrophages. The candidate factor eIF2A showed distinct protein expression patterns across polarized BMDMs, with the discovery of two distinct eIF2A bands in $M^{LPS/IFN\text{-}\gamma}$ macrophages (**Figure 3C**). Furthermore, $M^{LPS/IFN\text{-}\gamma}$ macrophages showed increased phosphorylated ribosomal protein S6 levels, suggesting an mTORC1-eIF2A axis in translation regulation. Thus, the non-canonical translation factor eIF2A might be involved in the polarization of macrophages toward inflammatory or reparative states.

1.9 Hypothesis and Objectives

We hypothesize that eIF2A plays a crucial role in the polarization and functioning of macrophages during the inflammatory response by exerting specific translational control.

Objectives

Aim 1: Explore the role of eIF2A in macrophage inflammatory responses *in vitro.*

Aim 2: Explore the role of eIF2A in macrophage polarization *in vivo*.

Aim 3: Explore the protein and RNA binding partners of eIF2A.

2. Materials and Methods

2.1 Isolation of Bone Marrow-Derived Macrophages

Bone marrow contents were isolated from C57BL/6 wildtype (WT) and *Eif2a* homozygous knockout mice (*eIF2A*$^{-/-}$) by flushing the femur and tibia with BMDM media using a syringe. The BMDM media for cell culture was prepared using 20% v/v L929 fibroblast-conditioned media with 10% v/v heat-inactivated fetal bovine serum (HI-FBS) (Gibco, 12483-020) and 1% v/v Penicillin-Streptomycin (Gibco, 15140-122) in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 11965-092). The isolates from the bone marrow were cultured for 6 days in an incubator with 5% CO_2 and 37°C conditions, with the supplementation of BMDM media 3 days post-isolation for optimal differentiation into macrophages.

2.2 Cell Treatments

BMDMs were harvested 6 days post-isolation. The naïve macrophages were treated with 100 ng/ml of lipopolysaccharide (LPS) (Sigma, L4391) and 100 ng/ml of interferon-γ (IFN-γ) (PeproTech, 315-05-100UG) to obtain pro-inflammatory $M^{LPS/IFN-\gamma}$ macrophages and 10 ng/ml of interleukin-4 (IL-4) (PeproTech, 214-14-20UG) to obtain anti-inflammatory M^{IL-4} macrophages. Cells were incubated for 24 hours at 37°C and 5% CO_2 for optimal macrophage polarization.

2.3 Protein Isolation and Quantification

The cells were washed 2 times with ice-cold 1X PBS after aspiration of the media and lysed using an ice-cold RIPA lysis buffer containing 2 mM $MgCl_2$ (Fisher, BP214), 0.5% w/v SDS (Fisher, BP1311), 10 mM Tris-HCl (pH 8.0) (Fisher, BP153), 90 mM NaCl (Fisher, BP358), 1 mM EGTA (Fisher, O2783) along with 20% PhosSTOP™ Phosphatase Inhibitor cocktail (Roche,

04906837001), and 25% cOmplete™ EDTA-free Protease Inhibitor cocktail (Roche, 04693132001). After lysis, the plates containing polarized macrophages were scraped using a cell scraper, spun in a cold centrifuge at 4°C for 20 minutes, and the resulting supernatants were collected. 10 µL of Bio-Rad Pierce™ Pre-diluted Protein Assay Bovine Serum Albumin (BSA) Standards (ThermoFisher, 22660) were loaded into a 96-well plate (Evergreen, 290-8115-901F) in duplicate. 8 µL of 1X PBS and 2 µL of the cell lysate for analysis (1:5 dilution) were loaded into other wells in duplicate. 150 µL of Bio-Rad Pierce™ 660 nm Protein Assay Reagent (ThermoFisher, 22660) was added into each well without forming bubbles. The plate was incubated for 5 minutes at room temperature and read at 660 nm on Agilent BioTek Synergy H1 Hybrid multi-mode microplate reader (Fisher Scientific) at 660 nm absorbance.

2.4 Western Blotting

Samples were prepared by adding 1X PBS and 5X Sample Buffer (0.25M Tris (pH 6.8), 5% w/v SDS, 50mM EDTA, 25% v/v glycerol (Fisher, BP229), 0.016% w/v bromophenol blue (Bio-Rad, 161-0404), 11.1% v/v ß-mercaptoethanol) into cell lysates, boiled at 95°C for 5 minutes, and centrifuged at 20817 RCF for 1 minute in Eppendorf 5417C Centrifuge (Sigma-Aldrich). Between 30-60 µg of protein was loaded into each well of a 7.5-12% Mini-PROTEAN TGX™ Precast Protein Gels (Bio-Rad) or 10-12% TGX™ Stain-free acrylamide gels (Bio-Rad). Gels were run at 100 V for 1.5 hours and transferred to 0.45 µm polyvinylidene fluoride (PVDF) membranes (Millipore, IPVH00010) at 80 V for 2 hours in an ice box at 4°C using PowerPac™ Electrophoresis Power Supply (Bio-Rad). Stain-free acrylamide gels were activated on ChemiDoc MP Imaging System (Bio-Rad) using ultraviolet light for imaging the total protein loaded per well.

Membranes were blocked with EveryBlot Blocking Buffer (Bio-Rad, 12010020) and incubated at 4°C overnight with primary antibodies. Primary antibodies that were used are: Rabbit anti-eIF2A (Abcam, ab169528), Mouse anti-eIF2A (Invitrogen, MA5-31724), Rabbit anti-IRF1 (Cell Signalling, 8478), Rabbit anti-IRF7 (Invitrogen, MA5-41165), Mouse anti-STAT1 (Abcam, ab281999), Rabbit anti-p65 (Abcam, ab16502), Rabbit anti-GAPDH (Millipore, ABS16), Mouse anti-beta actin (Sigma, A5441), Mouse anti-HSP90 (Santa Cruz Biotechnology, sc-13119), Rabbit anti-Arginase 1 (Cell Signalling, 93668S), and Rabbit anti-IL-1beta (Abcam, ab9722). Membranes were washed 3 times with 1X TBS-Tween for 5 minutes and incubated in Goat anti-rabbit IRDye800CW (LI-COR Biosciences, 926-32211), Goat anti-mouse IRDye680RD (LI-COR Biosciences, 926-68070), Goat anti-mouse HRP (Invitrogen, A16072), and Goat anti-rabbit HRP (Jackson ImmunoResearch, 111-035-144) secondary antibodies at a 1:5000 dilution in EveryBlot Blocking Buffer (Bio-Rad, 12010020). After secondary antibody incubation, membranes were washed 3 times in 1X TBS-Tween for 5 minutes and imaged on a ChemiDoc MP Imaging System (Bio-Rad) using the fluorescent IRDye settings or the chemiluminescence setting with Clarity ECL substrates (Bio-Rad, 1705061). Quantifications of bands were performed on Image Lab software (Bio-Rad) and normalized to housekeeping proteins or Stain-free gel total proteins.

2.5 Immunoprecipitation

Immunoprecipitation (IP) buffer was prepared using 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% v/v NP-40 (BioVision), 1% v/v Triton X-100 (Sigma-Aldrich), and UltraPure™ Distilled Water (Invitrogen).

Pierce™ Protein A/G Plus Agarose (Fisher Scientific) bead slurry was washed 3 times with ice-cold IP buffer, spun down for 2 minutes at 4.0 RPM between each wash, and resuspended in

IP buffer. For the binding of antibodies to beads, 5 µg of Rabbit anti-eIF2A (Abcam, ab169528) and Rabbit IgG (Abcam, ab172730) antibodies were bound to 100 µl of bead slurry per group and spun overnight on a rotator at 4°C.

Cell lysates were obtained from polarized macrophages, and 25 µl from each protein sample were stored at -20°C in 2X Sample buffer to serve as inputs for Western blot analysis. The rest of the protein samples were divided per group, and clean bead slurries were added for pre-clearance, followed by a spin in the rotator for 1 hour at 4°C. Samples were centrifuged at 4.0 RPM for 2 minutes at 4°C, and 25 µl of the pre-cleared supernatants were collected and stored at -20°C in 2X Sample buffer for Western blot analysis. The rest of the protein samples were saved for further use.

The antibody-bound beads that were in the rotator overnight were centrifuged at 4.0 RPM for 5 minutes, and the supernatants were collected as antibody flowthrough and stored at -20°C for Western blotting in 2X Sample buffer. The rest of the antibody-bound beads were washed 3 times with ice-cold IP buffer, spun down for 2 minutes at 4.0 RPM between each wash, and resuspended in ice-cold 1X PBS. 30 µl of each antibody-bound bead slurry was put into respective groups of protein samples and incubated in the rotator for 4 hours at 4°C. After rotation, samples were centrifuged at 4.0 RPM for 3 minutes at 4°C, and the supernatants were collected and stored at -20°C in 2X Sample buffer as post-IP flowthroughs for Western blotting. The beads were washed 3 times with ice-cold IP buffer and 1 time with ice-cold 1X PBS, spun down for 2 minutes at 4.0 RPM between each wash. Supernatants were discarded, 2X Sample Buffer was added to each sample, and the samples were boiled for 5 minutes to perform Western blot analysis. For detection, membranes were incubated with a 1:1000 dilution of mouse anti-eIF2A monoclonal antibody (Invitrogen, MA5-31724) overnight at 4°C, followed by secondary antibody incubation.

2.6 *In vivo* Mouse Studies

C57BL/6 wildtype (WT) and *eIF2A*$^{-/-}$ mice were used for the experiments. *eIF2A*$^{-/-}$ mice were obtained from Dr. Anton A. Komar at Cleveland State University. Mice were housed and bred at the University of Ottawa Heart Institute.

2.6.1 Mouse Injections

WT and *eIF2A*$^{-/-}$ mice (n=3/group) were injected intraperitoneally with 0.6 - 2 µg lipopolysaccharide per gram of mouse weight to mimic bacterial infection *in vivo* and saline as a control. After 24 hours post-injection, mice were sacrificed, and blood, bone marrow, and spleen samples were collected and kept on ice for further processing to use in flow cytometry.

2.6.2 Flow Cytometry

1 million cells from each cell suspension were blocked using Fc block (BD, 553142) for 5 minutes and stained with B220-BV786 (BD, 563894), Ly6C-BV605 (BD, 563011), CD8a-BV510 (BD, 563068), CD11b-BV421 (BD, 562605), CD4-PerCP-Cy5.5 (BD, 561115), Ly6G-FITC (BD, 551460), CD115-PE (BD, 565249), CD45-APC-Cy7 (BD, 561037), CD3e-APC (BD, 553066), F4/80-PE-Cy7 (BioLegend, 123114) for 30 minutes. After staining, Fixable Viability Stain 700 (BD, 564997) was used to stain the cells for 30 minutes. Cells were washed with FACS buffer containing 2% FBS (Gibco, 12483-020) and 1mM EDTA (1:500) in 1X PBS and resuspended in BD Cytofix Fixation Buffer (BD, 554655) for 15 minutes, protected from light. Cells were washed 2 times with FACS buffer and stored at 4°C to use within 24 hours after staining, protected from light. Data were processed and acquired on FACSAriaTM III Cell Sorter (BD) and analyzed using FlowJo software.

2.7 Statistical Analyses

Comparisons were performed using two-way ANOVA followed by Tukey's or Šídák's multiple comparison tests using GraphPad Prism 10 software. For experiments with $n < 3$, individual data points are shown, and statistical tests were not performed. Data are presented as mean ± SD and the statistical significances are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

3. Results

3.1 eIF2A expression is modulated in different macrophage polarization states.

To characterize the expression of eIF2A protein in different macrophage polarization states, BMDMs from WT and *eIF2A*$^{-/-}$ mice were left untreated (M_0) or polarized into pro-inflammatory $M^{LPS/IFN\text{-}\gamma}$ and anti-inflammatory M^{IL4} macrophages, followed by Western blotting on the lysates. eIF2A was detected as a single band with a molecular weight of ~48 kDa in M_0 and M^{IL4} macrophages. However, in $M^{LPS/IFN\text{-}\gamma}$ macrophages, a higher molecular weight (~63-65 kDa) band was detected along with the lower molecular weight band, which is consistent with the full-length eIF2A protein published in the literature (**Figure 4A**). eIF2A bands were only observed in WT BMDMs, confirming the validity of *eIF2A*$^{-/-}$ mice and the specificity of the eIF2A antibody (**Figure 4A**). Quantification of relative expression of eIF2A isoforms showed that the higher molecular weight isoform is prominently present in the pro-inflammatory macrophages, while the lower molecular weight isoform is expressed across all macrophage polarization states (**Figure 4B**). These results demonstrate that eIF2A expression could be modulated depending on the polarization state of the macrophages, distinguishing the pro-inflammatory macrophage state from the naïve and anti-inflammatory macrophages.

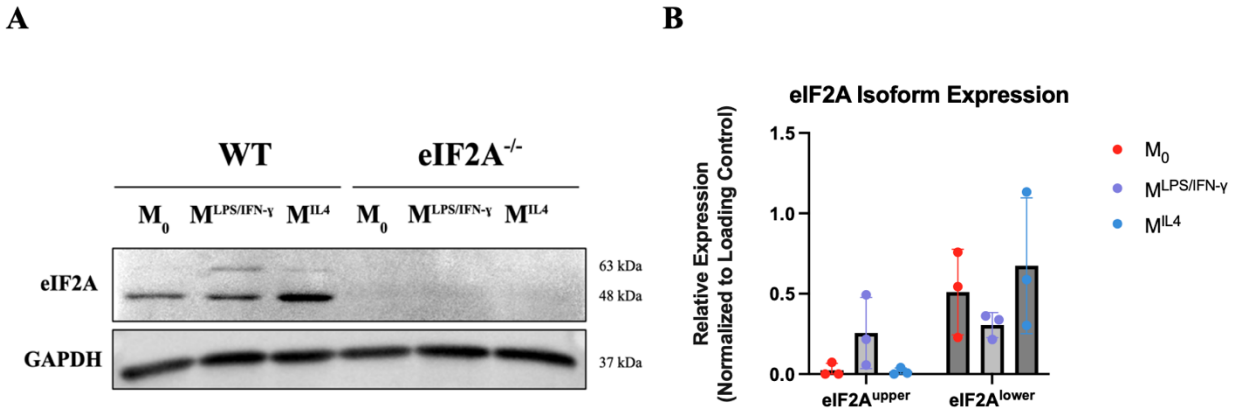


Figure 4: eIF2A is detected as two distinct bands in pro-inflammatory macrophages.

(**A)** Western blot depicting the eIF2A expression in polarized BMDMs derived from WT and *eIF2A*$^{-/-}$ mice. GAPDH was used as the loading control. (**B)** Relative expression of the higher molecular weight (eIF2A^{upper}) and lower molecular weight (eIF2A^{lower}) bands in wildtype M_0, $M^{LPS/IFN-\gamma}$, and M^{IL4} macrophages, normalized to GAPDH and β-actin as loading controls. Data were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test (n = 3).

3.2 eIF2A may be associated with post-transcriptional regulation of inflammatory transcription factors IRF1, IRF7, and STAT1.

Based on the appearance of two distinct eIF2A bands in pro-inflammatory macrophages, we asked whether eIF2A is involved in the pro-inflammatory programming of macrophages at the mRNA level. Pathway enrichment analysis of $M^{LPS/IFN-\gamma}$ BMDMs compared to M_0 BMDMs showed enrichment in interferon signalling. However, differential expression analysis of specific transcription factors involved in interferon pathways has yet to be performed. Therefore, we directly examined mRNA counts of *Irf1*, *Irf7*, and *Stat1*, which are involved in interferon pathways, through RNA sequencing across M_0, $M^{LPS/IFN-\gamma}$, and M^{IL4} BMDMs from WT and *eIF2A*$^{-/-}$ mice (**Figure 5A**). *Irf1*, *Irf7*, and *Stat1* transcripts were induced in pro-inflammatory $M^{LPS/IFN-\gamma}$ macrophages in both the presence and absence of eIF2A at similar levels, indicating that loss of eIF2A may not impair the induction of *Irf1*, *Irf7*, and *Stat1* at the transcriptional level.

To establish whether eIF2A interacts with these transcripts directly, RNA immunoprecipitation (RNA-IP) of endogenous eIF2A was performed in M_0, $M^{LPS/IFN-\gamma}$, and M^{IL4} BMDMs and normalized to *eIF2A*$^{-/-}$ BMDMs. Given that there is no eIF2A protein present in the *eIF2A*$^{-/-}$ BMDMs to be immunoprecipitated, fold enrichment was calculated as the ratio of WT to knockout (KO), as any signal from the KO samples shows non-specific binding due to sticky RNA and antibody rather than endogenous eIF2A pulldown. The RNA-IP result showed ~8-fold enrichment of 18S rRNA in M_0 and $M^{LPS/IFN-\gamma}$ BMDMs, which confirms ribosome association. Furthermore, pro-inflammatory macrophages showed ~22-fold enrichment of *Irf1* mRNA and ~9-fold enrichment of *Irf7* mRNA (**Figure 5B**). Together, these results indicate that eIF2A may be selectively interacting with *Irf1* and *Irf7* mRNAs in the pro-inflammatory macrophage state, suggesting potential regulation of these transcripts by eIF2A.

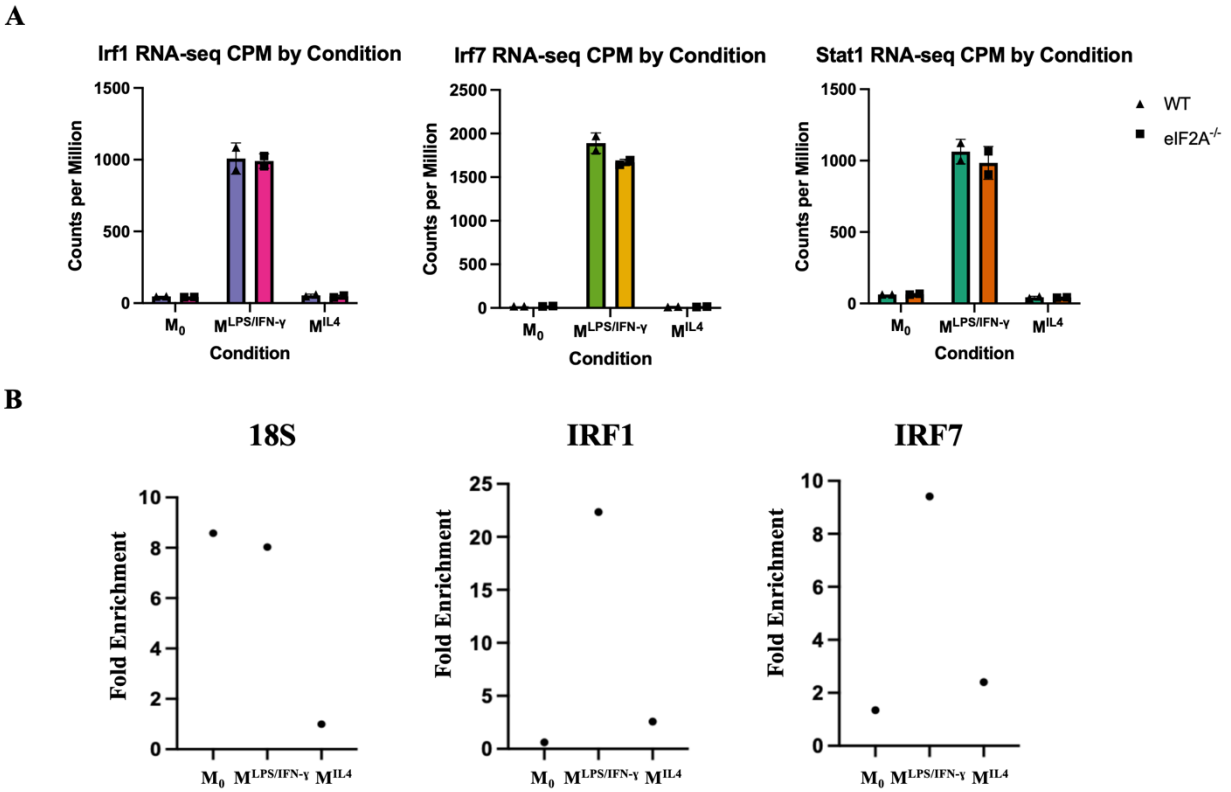


Figure 5: eIF2A may be associated with post-transcriptional regulation of inflammatory transcription factors IRF1 and IRF7 through interacting with *Irf1* and *Irf7* mRNAs.

(A) RNA sequencing data of *Irf1*, *Irf7*, and *Stat1* mRNA counts per million by conditions of polarized BMDMs derived from WT and *eIF2A*$^{-/-}$ mice (n = 2). **(B)** Fold enrichment of *Irf1* and *Irf7* mRNA and 18S rRNA in polarized BMDMs from WT mice, normalized to background from *eIF2A*$^{-/-}$ mice. Data were obtained by Dr. Huy-Dung Hoang and Dr. My-Anh Nguyen (unpublished).

3.3 Loss of eIF2A impairs IRF1, IRF7, and STAT1 protein expressions *in vitro*.

Based on findings indicating that mRNA levels of *Irf1*, *Irf7*, and *Stat1* are not affected by eIF2A's presence, we performed Western blotting on $M^{LPS/IFN\text{-}\gamma}$ BMDMs from WT and *eIF2A*$^{-/-}$ mice. In contrast with the previous result indicating comparable mRNA levels of *Irf1*, *Irf7*, and *Stat1* in both WT and KO conditions, expression of IRF1, IRF7, and STAT1 proteins showed a decreasing trend upon loss of eIF2A **(Figure 6).** Considering that the transcript levels remain comparable and protein expression showed a reduction in the absence of eIF2A, this suggests that eIF2A could be regulating this subset of interferon-pathway transcripts post-transcriptionally.

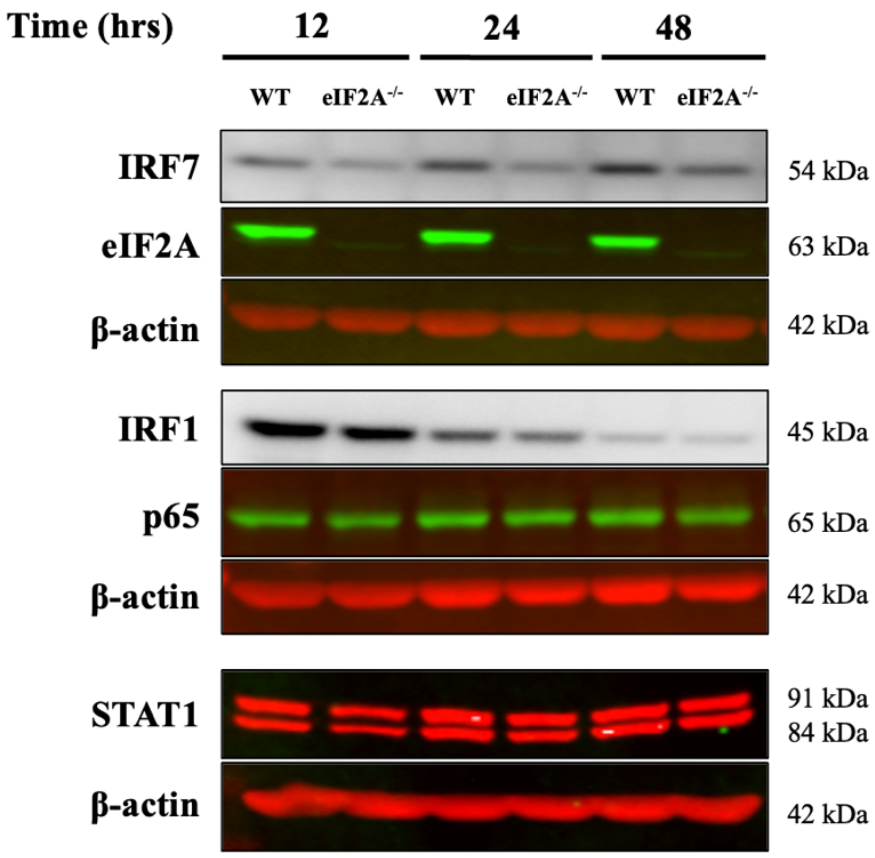


Figure 6: Expression of STAT1, IRF1, and IRF7 proteins shows a decreasing trend *in vitro* in $M^{LPS/IFN-\gamma}$ BMDMs in the absence of eIF2A.

Western blot depicting IRF7, eIF2A, IRF1, p65, and STAT1 protein levels in $M^{LPS/IFN-\gamma}$ BMDMs from WT and *eIF2A*$^{-/-}$ mice at 12-, 24-, and 48-hours post-polarization. β-actin was used as the loading control. Data were obtained by Dr. Huy-Dung Hoang and Dr. My-Anh Nguyen (unpublished).

3.4 Loss of eIF2A impairs IRF1, IRF7, and STAT1 protein expressions *in vivo*.

To examine the mechanism underlying eIF2A's regulation of these transcripts, publicly available ribosome profiling data were visualized using the Genome Wide Information on Protein Synthesis visualized (GWIPS-viz) tool. Ribosome profiling data available for *Stat1*, *Irf1*, and *Irf7* showed the presence of ribosome footprint densities upstream of the cognate ATG start codon found in the coding sequence, suggesting that *Stat1*, *Irf1*, and *Irf7* have alternative translation start sites at their 5' UTRs **(Figure 7A)**. This finding raised the possibility that eIF2A modulates the translation of these transcripts by using the alternative translation start sites in their uORFs.

Furthermore, to establish whether these protein expression changes *in vitro* also occur physiologically, we injected WT and *eIF2A*$^{-/-}$ mice (male, 4 months old) intraperitoneally with LPS (2 μg/g body weight) and saline and collected spleen for analysis. Western blotting was performed for the proteins STAT1, eIF2A, IRF1, and IRF7 **(Figure 7B).** Band intensities for these proteins were quantified and normalized to total protein from the stain-free gel. Expression of eIF2A increased upon *in vivo* LPS administration, confirming that eIF2A induction occurs systemically in response to inflammatory challenge **(Figure 7C)**. In contrast, a decreasing trend in STAT1, IRF1, and IRF7 proteins were detected upon LPS administration in the absence of eIF2A **(Fig. 7B and 7C).** This decrease in protein expression was consistent with the *in vitro* data, confirming that the findings on the subset of interferon-pathway transcription factors are not limited to cultured BMDMs.

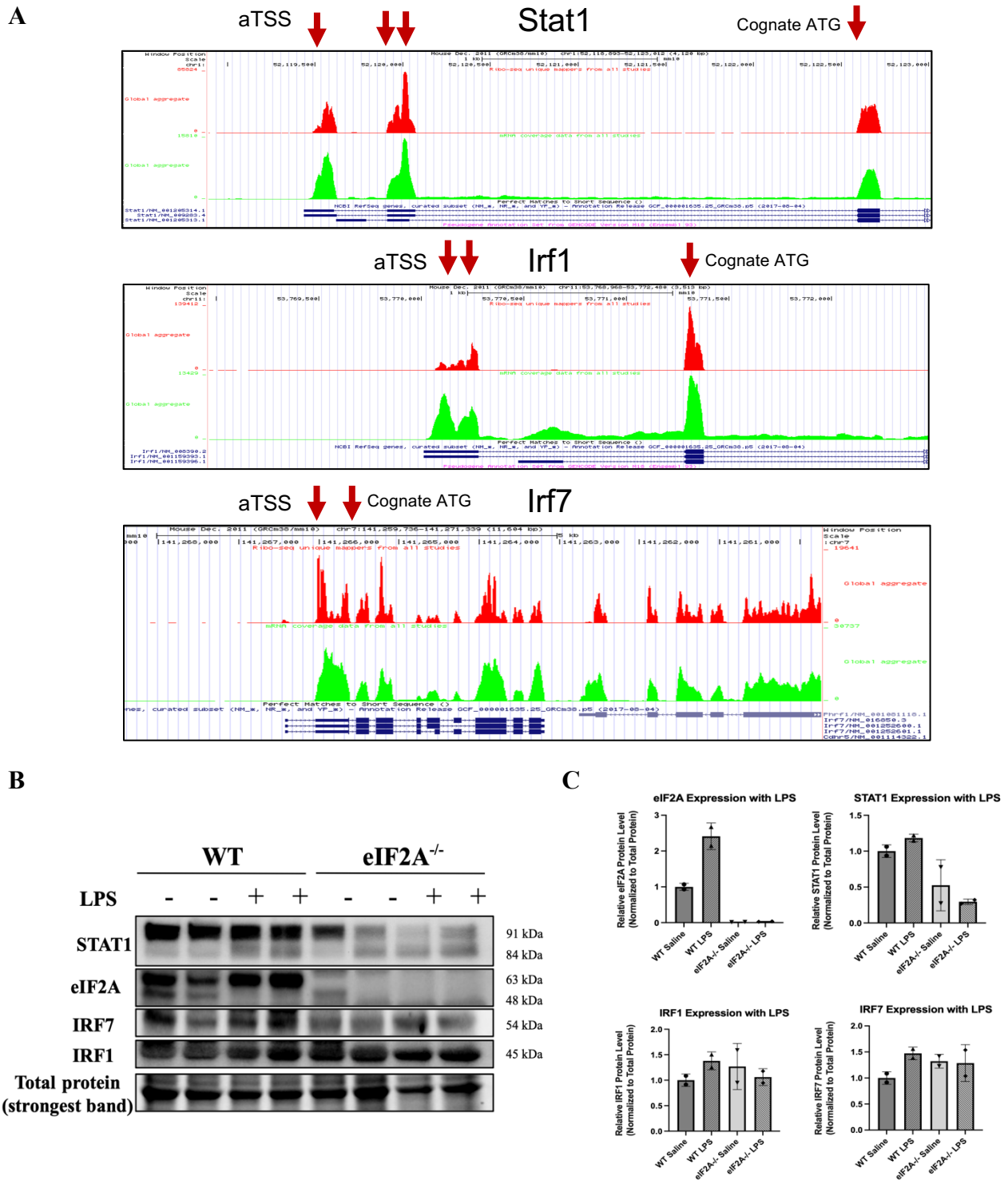


Figure 7: Loss of eIF2A shows a trend toward reduced STAT1, IRF1, and IRF7 protein expression upon *in vivo* LPS administration in the spleen.

(**A)** Ribosome profiling-identified alternative translation start sites (aTSSs) within the 5′ untranslated regions of the *Stat1, Irf1,* and *Irf7* genes visualized using GWIPS-viz. (**B**) Western blot depicting eIF2A, STAT1, IRF1, and IRF7 protein levels in spleens from WT and *eIF2A*$^{-/-}$ mice (male, 4 months old) following intraperitoneal injection of LPS (2 µg/g body weight) or saline control. The stain-free total protein signal was used as the loading control. (**C**) Quantification of eIF2A, STAT1, IRF1, and IRF7 protein levels normalized to total protein (strongest band is shown) (n = 2).

3.5 Loss of eIF2A shows a potential trend toward increased ARG1 and IL-1β protein expression in macrophages

Given the established role of eIF2A in translation initiation at uORFs and the possible findings of eIF2A-dependent post-transcriptional regulation of STAT1, IRF1, and IRF7, we asked whether eIF2A is involved in the translation initiation of key markers during macrophage polarization. IL-1β, a key marker of pro-inflammatory macrophages, and ARG1, a key marker for anti-inflammatory macrophages, were investigated for potential alternative translation start sites. Ribosome profiling data publicly available on the GWIPS-viz tool revealed the presence of alternative translation start sites based on ribosome footprint densities upstream of the cognate start codon, seen in both *Arg1* and *IL1b* 5'UTRs (**Figure 8A**). This suggests that the translation of *Arg1* and *IL1b* transcripts might start at uORFs via non-canonical translation initiation.

Then, Western blotting was performed on M_0, $M^{LPS/IFN\text{-}\gamma}$, and M^{IL4} BMDMs from WT and *eIF2A*$^{-/-}$ mice to determine if the presence of eIF2A impacts ARG1 and IL-1β protein expression during non-canonical translation initiation (**Figure 8B**). Quantification of band intensities normalized to GAPDH as a loading control showed that loss of eIF2A elevated ARG1 and IL-1β protein expression in comparison with their respective WT expressions, M^{IL4} for ARG1 and $M^{LPS/IFN\text{-}\gamma}$ for IL-1β (**Figure 8C**). This raises the possibility that eIF2A might be modulating translation initiation of these specific transcripts and the absence of eIF2A allows an increase in the production of ARG1 and IL-1β proteins.

To understand the basis for these changes in protein expression, RNA-sequencing of *Arg1* and *IL1b* was performed across all polarized BMDM states in WT and *eIF2A*$^{-/-}$ mice. In contrast to the previous findings of comparable *Stat1*, *Irf1*, and *Irf7* transcript levels, *Arg1* and *IL1b* transcript levels showed an increasing trend in *eIF2A*$^{-/-}$ BMDMs compared to the WT (**Figure 9**),

indicating that eIF2A might modulate these factors at the transcription level. Together, opposite findings from the macrophage markers and a subset of interferon-pathway transcription factors raise the possibility that eIF2A is not an activator or a repressor, but rather a multi-purpose modulator of selective transcripts whose underlying selection mechanism has yet to be determined.

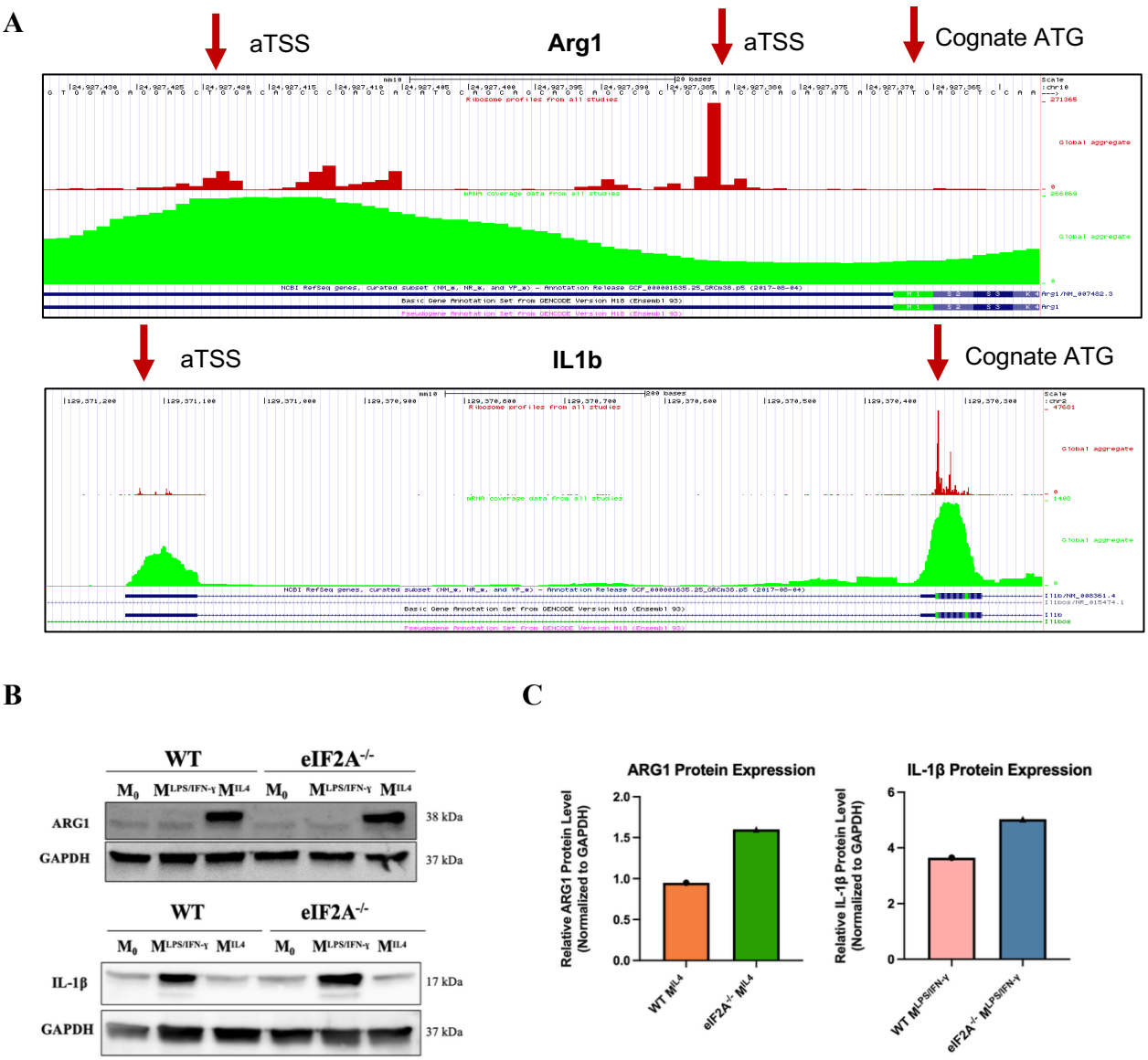


Figure 8: Loss of eIF2A shows a potential trend toward increased IL-1β and ARG1 protein expression in polarized BMDMs.

(**A**) Ribosome profiling-identified alternative translation start sites in the 5′ UTR of *Arg1* and *IL1b* genes visualized in GWIPS-viz. (**B**) Western blot depicting ARG1 and IL-1β protein expression in polarized BMDMs from WT and *eIF2A*$^{-/-}$ C57BL/6 mice. GAPDH was used as the loading control. (**C**) Quantification of relative ARG1 expression in M^{IL4} BMDMs and IL-1β expression in $M^{LPS/IFN\text{-}\gamma}$ BMDMs from WT and *eIF2A*$^{-/-}$ mice, normalized to GAPDH (n=1).

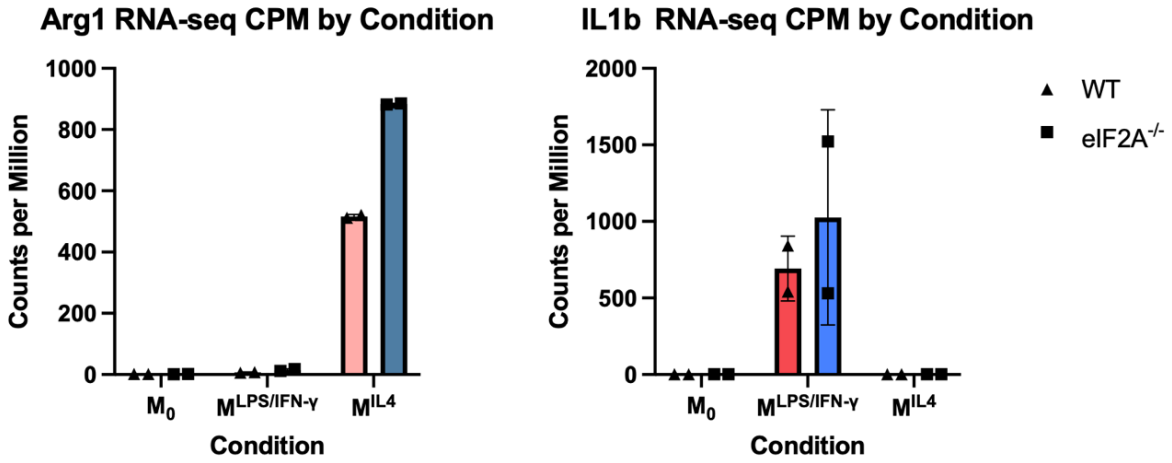


Figure 9: eIF2A may be regulating the expression of *Arg1* and *Il1b* at the transcriptional level.

RNA sequencing data of *Arg1* and *IL1b* mRNA counts per million by conditions of polarized BMDMs derived from WT and *eIF2A*$^{-/-}$ mice (n = 2). Data were obtained by Dr. Huy-Dung Hoang and Dr. My-Anh Nguyen (unpublished).

3.6 *In vivo* LPS administration increases eIF2A and affects immune cell profiles.

To understand the physiological impacts of eIF2A's presence and its effects on immune cells, mice were intraperitoneally injected with 0.6 μg/g body weight of LPS or saline and sacrificed 24 hours post-injection. Western blot of spleen lysates showed that WT mice had increased eIF2A protein expression in the spleen upon receiving LPS injection compared to the saline controls at 24 hours post-injection (**Figure 10**). There were no eIF2A bands detected in the *eIF2A*$^{-/-}$ mice upon saline and LPS administration, confirming the validity of the KO animal model. Due to baseline eIF2A expression in the spleen, Western blotting on spleen lysates indicated the presence of eIF2A as two distinct bands even in the saline-injected WT group 24 hours post-injection, whereas bone marrow lysates showed two eIF2A bands upon LPS administration due to low baseline eIF2A levels at the time point (**Figure 11**).

To examine whether the loss of eIF2A has physiological consequences, we performed flow cytometry on blood, spleen, and bone marrow lysates collected 24 hours post-injection. Baseline immune cell profiles were comparable between genotypes in both sexes, therefore, flow cytometry results of the male cohort (n=3) and female cohort (n=3) were pooled (**Figure 12**). Upon LPS administration, the percentage of live T and B cells in blood showed a decrease in both genotypes. A similar pattern was observed in the spleen, where the percentage of live B cells increased while the percentage of live T cells decreased in both genotypes. Furthermore, the percentage of live neutrophils in bone marrow showed a decrease in both genotypes. Overall, this suggests that the changes in immune cell profiles are due to an acute inflammatory response to LPS and not due to the presence of eIF2A.

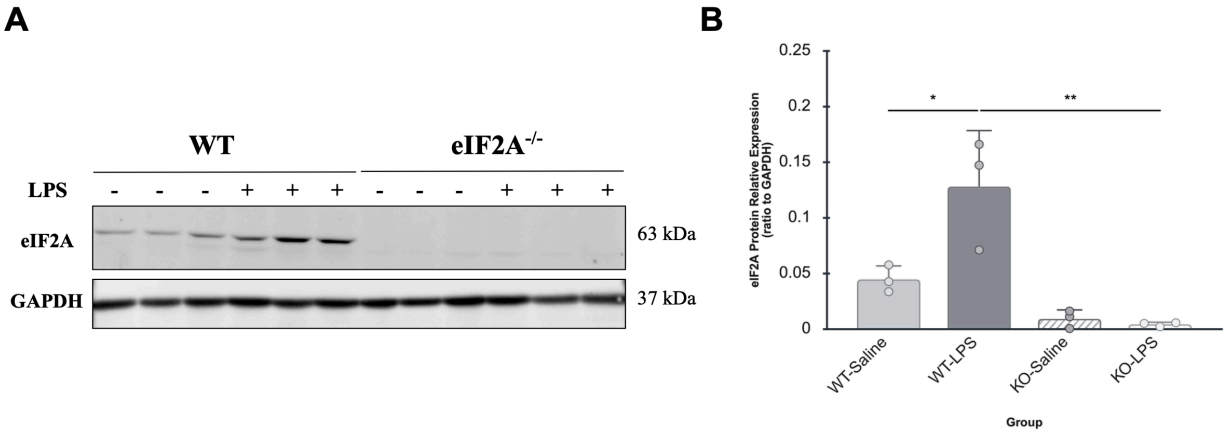


Figure 10: *In vivo* LPS administration increases eIF2A protein expression in the spleen.

(**A**) Western blot depicting eIF2A expression in the spleen of WT and *eIF2A*$^{-/-}$ mice following intraperitoneal injection of LPS (0.6 µg/g body weight) or saline. GAPDH was used as the loading control. (**B**) Relative expression of eIF2A to GAPDH. Data represent mean ± SD and were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test (n = 3). *$p < 0.05$, **$p < 0.01$.

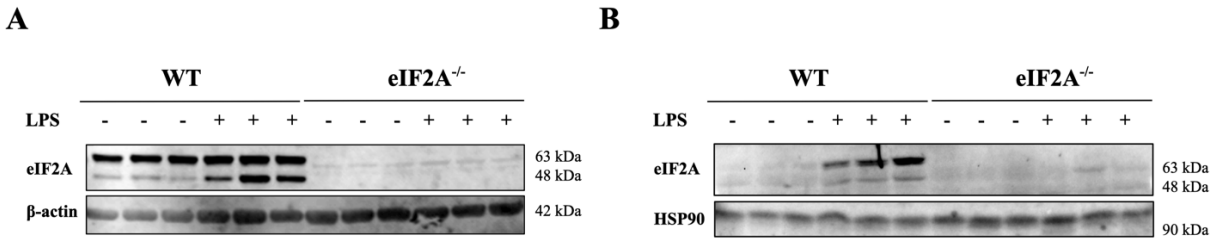


Figure 11: *In vivo* LPS administration increases eIF2A protein expression in the spleen and bone marrow.

Western blot depicting eIF2A expression in the spleen (**A**) and bone marrow (**B**) of WT and *eIF2A*$^{-/-}$ mice following intraperitoneal injection of LPS (0.6 μg/g body weight) or saline. β-actin and HSP90 were used as the loading controls.

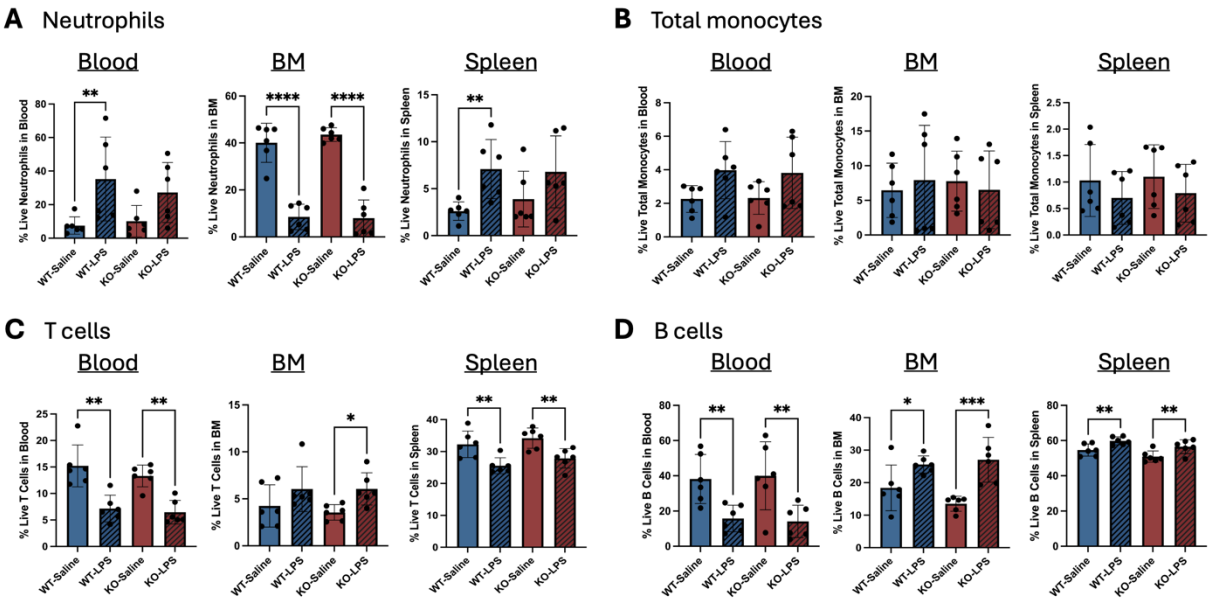


Figure 12: Immune cell profiles of LPS-injected WT and *eIF2A*$^{-/-}$ mice compared to saline controls.

Flow cytometry was performed on blood, bone marrow, and spleen samples collected 24 hours from WT and *eIF2A*$^{-/-}$ mice following intraperitoneal injection of LPS (0.6 µg/g body weight) or saline. Percentages of live neutrophils **(A)**, total monocytes **(B)**, T cells **(C)**, and B cells **(D)** are shown. Data were pooled from male mice (n=3, 9 months old) and female mice (n=3, 7 months old), for a total of n=6. Data represent mean ± SD and were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4. Discussion

Inflammatory response is vital for organisms to eradicate the infection, repair the damage and maintain a homeostatic state for survival. In both the innate and adaptive immune systems, macrophages play a crucial role by undergoing translational reprogramming that affects their phenotype and functioning. In eukaryotes, canonical mRNA translation occurs via eIF2 and is controlled by the mTOR signalling pathway. However, cells such as macrophages may use non-canonical translation to translate mRNAs required for stress response and survival under cellular stress, in which eIF2 is inactivated by the ISR. This work identifies eIF2A, with two distinct isoform expressions, as a factor involved in non-canonical mRNA translation under stress conditions. Loss of eIF2A was found to impair the expression of IRF1, IRF7, and STAT1 transcription factors, which are involved in the interferon pathway and are crucial for mediating inflammatory responses, both *in vitro* and *in vivo*. Furthermore, loss of eIF2A was found to impact immune cell profiles in blood, bone marrow, and spleen of mice after LPS stimulation. However, the exact mechanism by which eIF2A functions in eukaryotic translation is debatable and continues to be an active area of research.

4.1 The Role of eIF2A in Translation

eIF2A was proposed to function in delivering the Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit during stress, in which canonical translation initiation factor eIF2 is phosphorylated and functionally repressed by ISR kinases[16]. However, Roiuk *et al*., 2025 published that human eIF2A does not have a significant effect on translation initiation under stress conditions in HeLa and HEK293T cells based on reporter assays and ribosome profiling data[82]. This finding is consistent with earlier work published by Ichihara *et al*., 2021, reporting that HEK293T cells showed only a

slight translation initiation dependence on eIF2A upon eIF2 inhibition based on ribosome footprinting data[81]. Furthermore, these data are consistent with Gaikwad *et al*., 2024's work, which indicates that there is little evidence that eIF2A is involved in translation initiation *in vivo* in yeast when eIF2 is repressed by amino acid deprivation based on ribosome profiling data[83]. Together, these findings suggest that eIF2A does not play a major role in translation in yeast and mammalian cell lines. However, it could be argued that the triggers used to induce the ISR in these studies were not sufficient to suppress canonical translation. For example, the concentrations of sodium arsenite (ARS) that were used in the HeLa and HEK293T cells to induce oxidative stress or amino acid deprivation in yeast might not be sufficient to suppress canonical translation fully, thereby being insufficient to show eIF2A-dependent translation events during stress.

As an alternative to the translation initiator role, Grove *et al*., 2023 proposed that eIF2A plays a regulatory role in translation by showing that elevated levels of eIF2A inhibit translation *in vitro* through directly binding and sequestering the 40S ribosomal subunit[58]. If elevated eIF2A levels cause translation to be inhibited, this might explain the non-cytosolic localization of eIF2A published in multiple reports, as a way of preventing eIF2A from interacting with the translational machinery and inhibiting translation[58]. If this finding is generalizable to macrophages, it raises a possibility that the reduced IRF1, IRF7, and STAT1 protein expressions observed in our work upon eIF2A's absence might not be explained by the loss of non-canonical translation initiation.

Recently, Oh *et al*., 2026 showed that eIF2A interacts with the nuclear cap-binding complex components cap-binding protein 80 (CBP80) and cap-binding complex-dependent translation initiation factor (CTIF) according to their immunoprecipitation results in HEK293T cells upon ARS treatment[76]. Therefore, suggesting a new role for eIF2A in functioning in cap-binding complex-dependent translation when eIF2α is phosphorylated through strong ISR

induction[76]. Furthermore, their work suggested that eIF2A's role in cap-binding complex-dependent translation is the reason for the translation of selective mRNAs when cap-dependent canonical translation is suppressed[84]. Further research is crucial for the possibility of eIF2A's involvement in the pioneer round of translation rather than cap-dependent translation initiation, as it might explain the occurrence of protein synthesis despite the inhibitory effects of eIF2A published in the literature.

4.2 Cellular Localization of eIF2A

There are several studies in the literature about where eIF2A is localized, with some suggesting that eIF2A localizes to the nucleus, ER, centrosome, and mitochondria under normal conditions, which is not typical for a translation initiation factor[58]. Roiuk *et al*., 2025 published that human eIF2A localizes predominantly to the cytosol, with some fractions still present in the nucleus, whereas Kim *et al.*, 2011 published that eIF2A localizes predominantly to the nucleus and partially to the cytoplasm under normal conditions and shuttles out of the nucleus as a cellular stress response in Huh7 cells[82,85]. Other non-cytosolic localizations of eIF2A were reported, such as eIF2A in MIN6 cells colocalizes with the ER under normal conditions by Panzhinskiy *et al.*, 2021, and human eIF2A has a mitochondrial localization pattern in human osteosarcoma (U2OS) and epidermoid carcinoma (A-431) cell lines by the Human Protein Atlas, but only one of the antibodies tested showed this result and control mitochondrial co-staining was lacking in the latter[58,86]. Recently, Jungfleisch *et al.,* 2025 have shown that eIF2A is involved in cell migration in cancer by colocalizing with the centrosome and promoting the direction of migration through appropriate centrosome positioning in metastatic human melanocytes[49]. The cellular localization of eIF2A remains undetermined in our cell line of interest, due to the limitations of vendor-

validated anti-eIF2A antibodies. Therefore, non-cytosolic localization of eIF2A in multiple cell lines might mean that eIF2A has alternative functions other than translation initiation, which occurs in the cytosol, and future work is needed to determine the exact mechanism in which eIF2A functions[58].

4.3 The Two Isoforms of eIF2A

Western blot data implicated that naïve (M_0), pro-inflammatory (M_1, $M^{LPS/IFN\text{-}\gamma}$), and anti-inflammatory macrophages (M_2, M^{IL4}) express the low molecular weight isoform of eIF2A (~48 kDa), which we believe is truncated or cleaved. Strikingly, only the $M^{LPS/IFN\text{-}\gamma}$ macrophages were found to express the full-length eIF2A isoform (~63-65 kDa) in addition to the shorter isoform, upon inflammatory activation. Mass spectrometry (MS) data showed this size difference between the two isoforms is not explained by post-translational modifications, raising the possibility that the size difference between the isoforms (~15 kDa) could be due to truncation. Furthermore, MS data showed possible N-terminal methionine loss on a peptide, suggesting a co-translational processing event taking place.

Although the exact mechanism underlying the presence of the shorter eIF2A isoform remains undetermined, multiple papers in the literature have shown cleavage of factors involved in eukaryotic translation initiation. In 1998, Marissen and Lloyd reported that apoptosis inducers cause eIF4G to be cleaved in HeLa cells, potentially through caspase 3 activity, leading to translation inhibition[87]. Similarly, Marissen *et* al., 2000 showed that eIF2α is cleaved at the C-terminus by several caspases, notably caspase-3, 6, 8, and 10 in apoptotic cells, leading to alteration of translation in HeLa cells[88]. Furthermore, Vosler *et al*., 2011 showed that calpain, a calcium-activated protease, cleaves the eIF4F complex component eIF4G1 in HeLa cells, impairing cap-

dependent translation[58,89]. Another eIF4F complex component, eIF4E, was shown to be degraded by proteasomes through ubiquitination at Lysine 159 in HEK293T cells[90]. Therefore, these findings suggest that some factors in translation initiation machinery are targets for proteolytic cleavage and the shorter 48 kDa isoform of eIF2A observed in polarized macrophages may be a proteolytic cleavage product of the full-length eIF2A. In the case of the full-length isoform being predominantly present in $M^{LPS/IFN\text{-}\gamma}$ macrophages, this could be explained by inflammatory signalling partially evading this cleavage event, resulting in expression of both the full-length and shorter eIF2A isoforms. It is important to note that this is only a hypothesis and needs further investigation by treating polarized macrophages with inhibitors for caspases, calpains, and proteasomes to determine if this leads to any changes in eIF2A band observations in Western blot analysis.

Alternatively, the presence of two distinct eIF2A isoforms may be explained by alternative translation initiation leading to protein truncation or an alternative splicing event of the eIF2A transcript. In the case of alternative translation initiation, a ribosome may be initiating translation at a site with an AUG or non-AUG start codon downstream of the cognate start codon, leading to production of an N-terminally truncated eIF2A isoform. In the case of alternative splicing, the eIF2A transcript may undergo splicing of exons, leading to production of a shorter eIF2A protein. However, further investigation is required to detect the mechanism underlying the presence of two eIF2A isoforms.

Our preliminary MS data, in collaboration with Dr. Sasha Singh at Harvard Medical School, shows that the difference between the two isoforms of eIF2A is not a post-translational modification according to the peptide abundance mapping, but instead identified a peptide with a methionine loss at the N-terminus (Sequence: MAPSTPLLTVR) and another peptide lacking the

first methionine (Sequence: APSTPLLTVR), suggesting the presence of methionine excision modification in eIF2A at its N-terminus with high confidence level. This removal of the first N-terminal methionine could be a result of co-translational methionine aminopeptidase (MAP) activity, which removes the first methionine in most proteins in the cytosol in which translation initiation occurs[58,84]. Based on our sequence coverage data, which includes the upper eIF2A band from M^{LPS} and $M^{LPS/IFN\text{-}\gamma}$ macrophages and the lower eIF2A band from M_0, $M^{LPS/IFN\text{-}\gamma}$, M^{IL4}, and M^{LPS} macrophages, peptides that correspond to the C-terminus were not detected in the lower band. This suggests the disproof of the alternative translation initiation explanation for the short eIF2A isoform and favours proteolytic cleavage or a splicing event at the C-terminus, although the results are not conclusive with a high confidence level.

4.4 eIF2A in Macrophage Inflammatory Responses *In Vitro* and *In Vivo*

This work showed that eIF2A is present as two distinct isoforms prominently in pro-inflammatory macrophages and immune organs such as spleen and bone marrow, which are crucial for immune response. Consistent with the appearance of two eIF2A isoforms, pro-inflammatory macrophages also showed higher phosphorylated S6 protein compared to naïve and anti-inflammatory BMDMs, suggesting that the emergence of both isoforms might be related to a mTORC1-eIF2A axis, although further research is required.

The occurrence of two distinct isoforms of eIF2A has not been published in the literature, raising the possibility that the findings in previous literature could be relevant for the full-length or lower molecular weight isoform, which has not been exclusively specified. Another possibility is that eIF2A may not function in translation initiation at upstream ORF non-AUG start codons

but instead function in the pioneer round of translation, interacting with the nuclear cap binding complex, although further research is needed.

The regulatory role of eIF2A was examined on different markers crucial for macrophage polarization and transcription factors involved in interferon pathways, which indicates possible eIF2A-dependent modulation of STAT1, IRF1, and IRF7 expression at a post-transcriptional level and *Arg1* and *IL1b* at the transcriptional level based on RNA-IP, RNA sequencing, ribosome profiling, and Western blotting data. Publicly available ribosome profiling data indicated the presence of alternative translation start sites at the 5' UTR of these transcripts upstream of the cognate start codon, raising the possibility that eIF2A could be controlling their translation by affecting start codon selection or translation reinitiation. Although the decreasing trend in STAT1, IRF1, and IRF7 and the increasing trend in ARG1 and IL-1β upon loss of eIF2A serves as a paradox for eIF2A's regulatory role, it could be explained by eIF2A not being a uniform inducer or repressor, but rather a selective modulator upon the crosstalk of signals in the cellular microenvironment, such as the effects of unfolded protein response.

This work showed that intraperitoneal LPS administration induces eIF2A systemically 24 hours post-administration and the loss of eIF2A shows a decreasing trend in the protein expression of STAT1, IRF1, and IRF7 *in vivo.* These findings conflict with current literature indicating a minimal role of eIF2A in translation in yeast, HeLa cells, and HEK293T cells. One possibility could be that eIF2A's function may be cell type-dependent, as evidenced by localization differences, context-dependent, as in the inflammatory stimulus used for pro-inflammatory macrophages and not the stress factors used in other cells in the literature, or time-dependent. Considering the lack of eIF2A KO mouse usage in these reports and the usage of eIF2A overexpression systems, which potentially excluded the 5'UTR region of eIF2A, current literature

might not be capturing the physiological effects of eIF2A to a full extent. The decreased lifespan and metabolic syndromes in lipid metabolism in the eIF2A KO mice, which were published by Anderson *et al*., 2021, were not observed in *eIF2A*$^{-/-}$ mice in comparison with WT mice[91].

Flow cytometry results of blood, bone marrow, and spleen from both male and female WT and *eIF2A*$^{-/-}$ C57BL/6 mice demonstrated that loss of eIF2A does not affect baseline immune cell composition significantly. The changes observed in populations require further investigation to determine whether these effects are eIF2A-dependent, time-dependent, or dosage-dependent, as they were not statistically significant at 24 hours post-injection with the LPS dosage administered.

4.6 eIF2A's Protein and RNA Binding Partners

Preliminary immunoprecipitation experiments of eIF2A in BMDMs polarized with LPS and IFN-γ ($M^{LPS/IFN\text{-}\gamma}$) showed the capture of only the shorter 48 kDa isoform of eIF2A with rabbit anti-eIF2A antibody and rabbit IgG isotype control and not the full-length eIF2A isoform, potentially due to limitations of commercially available eIF2A antibodies for immunoprecipitation. We attempted to detect CBP80 protein after eIF2A pulldown for a potential physical interaction between proteins, but CBP80 was not detected in the input. This could be a limitation due to the antibody, as the antibody was not tested in any cell lines other than human cells, which presents a significant limitation to test eIF2A's interaction partners in CBC-dependent translation. Therefore, further investigation is needed with IP-validated eIF2A antibodies that could target both the full-length and lower molecular weight eIF2A isoforms to perform immunoprecipitation experiments in mouse BMDMs reliably.

4.7 Significance and Limitations

The findings of this work contribute to the understanding of eIF2A's role in macrophage inflammatory responses and protein synthesis, which is crucial for the immune system. By demonstrating that eIF2A exists as two isoforms in pro-inflammatory macrophages and in the immune organs of LPS-challenged mice such as bone marrow and spleen, this work identifies eIF2A as a factor involved in translational control during macrophage inflammatory activation, both *in vitro* and *in vivo*. eIF2A was shown to selectively impact protein output of STAT1, IRF1, and IRF7 *in vitro*, which also holds physiological relevance *in vivo* with the use of eIF2A KO mice receiving LPS challenge, which is lacking in the literature. This contributes to a broader understanding that the activation of immune response is not only regulated at the level of transcription, but also post-transcriptionally through translational machinery.

Despite these findings, there were limitations in this study which should be taken into consideration while interpreting the data. The presence of two eIF2A bands in pro-inflammatory macrophages, spleen, and bone marrow was not consistently reproduced, suggesting that there could be a trigger that leads to the emergence of full-length and lower molecular weight isoforms of eIF2A, which remains undetermined. Experiments with $n < 3$ are shown as individual data points, as they do not support statistical comparisons. For *in vivo* Western blots, only male cohort at 4 months of age were used, whereas 9-months-old male and 7-months-old female cohorts were used for flow cytometry experiments. Future experiments with sex- and age-matched cohorts are needed to test these findings. Furthermore, the anti-eIF2A antibodies that were used were not vendor-validated for some experimental techniques, such as flow cytometry and immunoprecipitation, serving as a limitation for downstream analyses.

5. Conclusions

In conclusion, this work demonstrates that eIF2A exists as two isoforms prominently in pro-inflammatory macrophages and immune organs such as spleen and bone marrow crucial for the immune response. This emergence of two eIF2A isoforms coincides with an increase in the phosphorylated ribosomal S6 protein expression in pro-inflammatory macrophages, suggesting the presence of a potential mTORC1-related eIF2A axis in translational control. Building on preliminary findings, this work confirmed induction of eIF2A both *in vitro* following classical activation of macrophages and *in vivo* using intraperitoneal LPS administration. Loss of eIF2A showed a decreasing trend in protein expression levels of STAT1, IRF1, and IRF7, although the mRNA levels remained comparable, suggesting a potential post-transcriptional regulation of this subset of interferon pathway transcription factors by eIF2A. Paradoxically, loss of eIF2A showed an increasing trend in ARG1 and IL-1β protein in both protein output and mRNA levels, suggesting a transcriptional control mechanism. Together, this diversity of potential eIF2A targets suggests that eIF2A does not act merely as a uniform translational inducer but incorporate transcriptional and post-transcriptional mechanisms to selectively translate key markers crucial for macrophage inflammatory responses. These effects are not limited to cultured BMDMs, as evidenced by protein output changes in these proteins after *in vivo* LPS challenge, used to systemically induce eIF2A. Administration of LPS mimicked bacterial infection, prompting the upregulation of eIF2A levels in the spleen and bone marrow 24 hours post-injection. Flow cytometry results showed that the changes in immune cell profiles were consistent in the absence and presence of eIF2A, displaying that the changes are due to an acute inflammatory response to LPS.

This work contributes to a broader understanding of macrophage inflammatory response and identifies eIF2A as a selective regulator of translation in macrophage inflammatory responses with both *in vitro* and *in vivo* relevance. However, the exact mechanism by which eIF2A functions, the protein and RNA interaction partners of eIF2A, and the molecular identity and functional differences between the two isoforms of eIF2A remain not fully elucidated and need future investigation. Considering the link between dysfunctional inflammation resolution and vascular diseases, this work may have relevance to understanding eIF2A-dependent selective translational control during inflammatory diseases such as atherosclerosis, although future investigation is required in this area of research.

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