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The sources of antigenic peptides for the MHC class I pathway

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Thèse de doctorat en oncogénèse

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Titre de la thèse : Les sources de peptides antigéniques pour la voie du CMH de classe I

Résumé

Un élément clé de la capacité du système immunitaire à faire la distinction entre le soi et le non-soi est la présentation de peptides par le complexe majeur d'histocompatibilité de classe I (CMH-I). La présentation des peptides dérivés de l'intron par les molécules CMH-I pose la question de l'origine des peptides antigéniques pour la voie CMH-I. Une meilleure connaissance de l'origine des néo-antigènes conduirait à une meilleure compréhension de l'évasion immunitaire virale et cancéreuse. Pour mon doctorat, je me suis intéressée à deux sources différentes de peptides antigéniques. Premièrement, à l'autophagie en tant que mécanisme de dégradation des protéines et deuxièmement, aux produits de traduction pionniers dérivés de l'épissage des ARN pré-messagers.

Premier mécanisme

L'autophagie joue un rôle essentiel dans l'homéostasie cellulaire, en aidant notamment les cellules à se débarrasser d'agrégats protéiques nocifs qui peuvent s'accumuler et causer des troubles neurodégénératifs. La réponse immunitaire face aux cellules porteuses d'agrégats protéiques est relativement inconnue et il existe peu de connaissances du processus autophagique des peptides antigéniques dédiés à la voie de classe I du CMH. Pour évaluer la présentation des peptides antigéniques dérivés de l'autophagie par la voie CMH-I, nous avons utilisé des cellules T CD8⁺ (OT-1) reconnaissant spécifiquement l'épitope SL8 (SIINFEKL) de l'ovalbumine de poulet (OVA) présenté par les molécules murines Kb du CMH-I. Nous avons évalué deux substrats potentiels pour l'autophagie via la fusion de la séquence ovalbumin-SIINFEKL, soit à la protéine polyglutamine (PolyQ) qui a tendance à s'agréger, soit aux séquences EBNA1 codées par le virus Epstein Barr. La suppression de l'autophagie par l'inhibition d'Atg5 et Atg12 n'a pas affecté la présentation des peptides dérivés de la protéine EBNA1, alors qu'elle a réduit la présentation des peptides antigéniques dérivés d'OVA, ou OVA fusionné à la séquence PolyQ agrégée. Étonnamment, la fusion de l'ovalbumine avec la répétition glycine-alanine (GAR) d'EBNA1 (GAR-OVA) a empêché la présentation des peptides d'OVA. Ces données suggèrent une présentation de peptides antigéniques dépendants du substrat pour la

voie de classe I du MHC via l'autophagie et illustrent un nouveau mécanisme à médiation virale pour l'évasion immunitaire de la présentation d'antigènes dépendants de l'autophagie.

Deuxième *mécanisme*

Au fil du temps, l'idée selon laquelle les peptides antigéniques seraient uniquement issus de la dégradation de « vieilles » protéines a été remplacée par l'hypothèse selon laquelle les peptides antigéniques seraient également dérivés de peptides nouvellement synthétisés, via un événement de traduction alternative non-canonique qui se produit avant l'épissage de l'ARNm. Cela expliquerait la présence de peptides dérivés de l'intron sur les molécules CMH-I. En effet, nous avons observé que l'expression de la séquence SIINFEKL présente dans le second intron du gène β -globine déclenchait la prolifération des cellules T CD8+ (OT-1). Par la technique du PLA (Proximity Ligation Assay) nous avons observé une augmentation des peptides SIINFEKL dérivés d'ARNm pré-épissé après traitement avec l'inhibiteur d'épissage Isoginkgetin et un inhibiteur du protéasome MG-132. Pour caractériser ce complexe de traduction alternative, nous avons utilisé la technique de fractionnement de polysome pour identifier les ribosomes associés à l'ARNm pré épissé. L'ARNm- β -Globine pré épissé se trouve associé aux polysomes légers, contrairement à l'ARNm- β -Globine épissé qui est présent dans les fractions polysomales lourdes. Nous avons également observé par PLA, l'interaction entre la protéine ribosomale L5 (RPL5) et la séquence peptidique FEKL à partir d'ARNm pré-épissé, interaction qui est augmentée pendant le traitement avec l'inhibiteur d'élongation de traduction Cycloheximide. Ces données soutiennent la notion que les peptides antigéniques sont dérivés de la traduction des ARNm pré épissés.

Mots-clés: Présentation de l'antigène restreint du CMH de classe I, Autophagie, agrégats de protéines, EBNA1 codé par EBV, peptides dérivés d'intron présente sur la molécule MHC-I, traduction alternative non canonique, traduction d'ARNm pré-épissés, machinerie de traduction alternative.

Thesis title: The sources of antigenic peptides for the MHC class I pathway

Abstract

A key component of the immune systems' capacity to distinguish between self and non-self is the presentation of peptides on major histocompatibility complex class I (MHC-I) molecules. Non-self-peptides derived from pathogens or mutated self-proteins are recognized by cytotoxic CD8⁺ T cells leading to the destruction of the presenting cell. The presentation of intron-derived peptides on MHC-I molecules has raised the question of the origin of antigenic peptides for the MHC-I pathway. A better understanding of the origin of neo-antigens will lead to a better comprehension of viral and cancer immune evasion. For my Ph.D. study, I have focused on two different sources of antigenic peptides. Firstly, autophagy as a protein degradative mechanism, and secondly, pioneer translation products derived from pre-spliced mRNA.

First mechanism

Autophagy has an essential role in cellular homeostasis and can help rid the cells of harmful protein aggregates accumulation that can cause several diseases such as neurodegenerative disorders. Immune response towards cells carrying protein aggregates is relatively unknown and there is limited evidence for autophagy processing of antigenic peptides for the MHC class I pathway. To assess MHC-I antigen presentation of autophagy-derived antigenic peptides, we used CD8⁺ T cells (OT-1) that specifically recognize the chicken ovalbumin (OVA) SL8 epitope (SIINFEKL) presented on the murine Kb MHC-I molecules. We evaluated potential substrates for autophagy processing by the ovalbumin-SIINFEKL sequence fusion to the aggregate-prone polyglutamine (PolyQ) and the Epstein Barr Virus-encoded EBNA1 sequences. Suppressing autophagy by knocking down Atg5 and Atg12 did not affect the presentation of peptides derived from the EBNA1 protein, whereas it reduced the presentation of antigenic peptides derived from OVA, or OVA fused to the aggregate-prone PolyQ sequence. Surprisingly, fusing ovalbumin to the immune-evasive glycine-alanine repeat (GAR) of EBNA1 (GAR-OVA) prevented the presentation of peptides from OVA. These data suggest a substrate-dependent presentation of antigenic peptides for the MHC class I pathway via autophagy and illustrate a novel virus-mediated mechanism for immune evasion of autophagy-dependent antigen presentation.

Second mechanism

Over time, the assumption that antigenic peptides are solely derived from the degradation of “old” full-length proteins has been replaced with the postulation that antigenic peptides are also derived from newly synthesized peptides by a specific non-canonical alternative translation event that occurs before mRNA splicing. This would explain the presence of intron-derived peptides on MHC-I molecules. In support of this, we observed that expressing the SIINFEKL sequence in the second intron of the β -globin gene triggered OT-1 CD8⁺ T cell proliferation. Using the proximity ligation assay (PLA), we observed an increase in SIINFEKL peptides from pre-spliced mRNAs following treatment with the Isoginkgetin splicing inhibitor and an MG-132 proteasome inhibitor. To start the characterization of this alternative translation complex, we used polysome fractionation to identify ribosomes on pre-spliced mRNAs. The pre-spliced β -Globin mRNA is found on light polysomes, as opposed to the spliced β -Globin mRNA that is present in the heavy polysomal fractions. We also observed interaction between ribosomal protein L5 (RPL5) with FEKL peptide sequence from pre-spliced mRNAs, using PLA, which increased upon Cycloheximide translation elongation inhibitor. The data further supports the notion that antigenic peptides are derived from the translation of pre-spliced mRNAs.

Key-words: MHC class I restricted antigen presentation, Autophagy, protein aggregates, EBV-encoded EBNA1, intron-derived peptides on MHC-I molecule, non-canonical alternative translation, translation of pre-spliced mRNAs, alternative translation complex.

Résumé substantiel en français

La réponse immunitaire adaptative ou spécifique est composée de cellules T porteuses d'un récepteur de cellules T (T cell receptor. TCR), qui reconnaît de courts fragments des peptides antigéniques produits par la protéolyse intracellulaire. Le TCR se lie aux peptides antigéniques associés à un complexe majeur d'histocompatibilité (CMH) sur les cellules présentatrice des antigènes (CPAs).

Les cellules T sont classées en deux sous-classes fonctionnelles différentes, selon l'expression des molécules CD4 ou CD8. Les cellules CD8+ sont des cellules T cytotoxiques qui reconnaissent les peptides antigéniques associés aux molécules de classe I (CMH-I), tandis que les cellules CD4+ sont des cellules T auxiliaires qui reconnaissent les peptides antigéniques associés aux molécules de classe II (CMH-II).

La capacité du système immunitaire à faire la distinction entre le soi et le non-soi dépend de la présentation des peptides associés à la molécule CMH-I. Ces antigènes sont d'origine endogène, tels que les protéines cellulaires ou virales. Les protéines sont synthétisées dans la cellule après le processus de traduction. La traduction commence par l'initiation : la sous-unité ribosomale 40s se lie aux différents facteurs d'initiation et au PIC (complexe de pré-initiation), tandis que l'ARN messager (ARNm) est activé par d'autres facteurs d'initiation menant au recrutement du PIC. Puis, la sous-unité ribosomale 40s scanne la partie 5'UTR (région non traduite en 5') de l'ARNm jusqu'à ce qu'elle trouve le codon de départ, généralement le codon AUG. La reconnaissance du codon de départ par la sous-unité ribosomale 40s mène à la phase d'élongation. À ce stade, les facteurs d'élongation sont libérés de la sous-unité 40s du ribosome, qui attire la sous-unité ribosomale 60s et les différents facteurs d'élongation. Le ribosome 80s ainsi formé et les facteurs d'élongation synthétisent alors la chaîne polypeptidique par la polymérisation des acides aminés qui correspondent aux codons lus par les ribosomes le long de l'ARNm. Le ribosome passe de codon en codon jusqu'au codon stop. À ce stade, l'élongation s'achève et commence alors le stade de terminaison. Un facteur de libération se lie au ribosome et termine la traduction libérant le polypeptide.

Les protéines, à la fin de leur vie fonctionnelle, sont dégradées par le protéasome, générant des peptides courts, utilisés comme peptides antigéniques pour la voie CMH-

I. Les peptides antigéniques libérés par le protéasome sont transférés du cytosol au réticulum endoplasmique (RE), via un transporteur associé à la présentation de l'antigène (TAP). Une fois dans le RE, les peptides antigéniques rencontrent les molécules CMH-I avec qui ils se lient. Les complexes peptide-CMH-I quittent le RE et sont transportés vers la membrane plasmique. Ces peptides sont reconnus par les cellules T cytotoxiques CD8⁺ conduisant à la destruction de la cellule présentatrice (CP).

Aujourd'hui encore, le modèle de voie de classe I du CMH pose la question de l'origine des peptides antigéniques. De nouvelles études ont démontré la présentation des peptides dérivés d'intron par la voie CMH-I. Une meilleure connaissance de l'origine des néo-antigènes conduirait à une meilleure compréhension de l'évasion immunitaire virale et cancéreuse. Pour mon doctorat, je me suis intéressée à deux sources différentes de peptides antigéniques. Premièrement, à l'autophagie en tant que mécanisme de dégradation des protéines et deuxièmement, aux produits de traduction pionniers dérivés de l'épissage des ARN pré-messagers.

Premier mécanisme

L'autophagie est un processus de dégradation des protéines cytoplasmiques endogènes qui se produit en réponse aux différentes situations de stress, telles que la privation de nutriments. Cependant, dans des conditions basales, l'autophagie dégrade les cargos spécifiques pour se débarrasser des organelles endommagés, tels que les mitochondries ou les protéines nocives sujettes à l'agrégation.

L'autophagie commence par le recrutement de gènes liés à l'autophagie (ATG) et de différents types d'enzymes, comme les kinases, à un site spécifique près du RE nommé site d'assemblage du phagophore (PAS). L'interaction de toutes ces protéines déclenche la nucléation d'une membrane isolée qui forme une structure en forme de coupe appelée phagophore. Ensuite, ce phagophore va continuer à s'étendre, prenant avec lui une portion de cytosol, jusqu'à ce qu'il scelle la membrane. Une nouvelle vésicule à double membrane appelée autophagosome est alors formée, transportant à l'intérieur de ce repliement la cargaison autophagique. L'autophagosome se développe en libérant les ATG qui l'entourent et se déplace à travers les microtubules pour atteindre le lysosome. Enfin, l'autophagosome va fusionner avec la membrane

lysosomique pour former un autolysosome et se transformer en un seul corps autophagique de membrane, avant sa dégradation.

Dans la réponse immunitaire adaptative, l'autophagie est essentielle pour de nombreuses tâches, dont la présentation des antigènes. L'autophagie est impliquée dans la voie CMH-II et la présentation croisée, cependant, il y a peu de connaissance de la participation de l'autophagie dans la voie CMH-I. Il a été démontré que le traitement par autophagie élimine l'accumulation d'agrégats protéiques nocifs dans les cellules. Ainsi, nous nous sommes demandés si l'autophagie pouvait traiter des substrats connus pour former des agrégats et produire des peptides antigéniques pour la voie du CMH-I. Dans cette étude de doctorat, nous avons évalué deux types de substrats différents : une protéine qui a tendance à s'agréger et une protéine virale.

Les agrégats de protéines sont des complexes oligomériques formés par des composants non natifs et des interactions entre leur structure conduisent à des intermédiaires piégés, liés au repliement ou à l'assemblage des protéines. Les agrégats de protéines sont bien connus pour être associés aux maladies neurodégénératives. Par exemple, la répétition CAG qui code la polyglutamine (PolyQ) déclenche des interactions trompeuses, entraînant l'agrégation. Les maladies dues à PolyQ, comme l'Ataxie Spinocérébelleuse ou Huntington, entre autres, sont liées à différentes causes, mais toutes ont en commun que la protéine pathogène de chacune de ces maladies a plus de 40 répétitions de CAG.

L'autre substrat étudié a été la protéine virale EBNA1 (Epstein-Barr Nuclear Antigen 1), une protéine latente nécessaire à la réplication du virus Epstein-Barr et trouvée dans toutes les tumeurs positives pour ce virus. La particularité de cette protéine est son expression pendant toutes les phases de l'infection virale, mais sans déclenchement de réponse immunitaire, ainsi que sa capacité à former des agrégats. Ces caractéristiques de la protéine EBNA1 sont attribuées à la répétition d'une séquence Glycine-Alanine (GAR) capable d'inhiber sa propre traduction, diminuant ainsi la présentation antigénique sur les molécules CMH-I, ainsi qu'une tendance à une structure agrégée.

Pour évaluer la présentation des peptides antigéniques dérivés de l'autophagie par la voie CMH-I, nous avons utilisé des cellules T CD8+ (OT-1) reconnaissant spécifiquement l'épitope SL8 (SIINFEKL) de l'ovalbumine de poulet (OVA) présenté

par les molécules murines Kb du CMH-I. Nous avons évalué deux substrats potentiels pour l'autophagie via la fusion de la séquence ovalbumin-SIINFEKL, soit à la protéine polyglutamine (PolyQ), soit aux séquences EBNA1. La suppression de l'autophagie par l'inhibition d'Atg5 et Atg12 n'a pas affecté la présentation des peptides dérivés de la protéine EBNA1, alors qu'elle a réduit la présentation des peptides antigéniques dérivés d'OVA, ou OVA fusionné à la séquence PolyQ agrégée. Étonnamment, la fusion de l'ovalbumine avec la répétition glycine-alanine (GAR) d'EBNA1 (GAR-OVA) a empêché la présentation des peptides d'OVA. Ces données suggèrent une présentation de peptides antigéniques dépendants du substrat pour la voie de classe I du CMH via l'autophagie et illustrent un nouveau mécanisme à médiation virale pour l'évasion immunitaire de la présentation d'antigènes dépendants de l'autophagie.

Deuxième mécanisme

De nombreuses observations contredisent la notion selon laquelle les protéines sont dégradées par le protéasome pour produire des peptides antigéniques pour la voie CMH-I. Premièrement, le fait que les protéines virales sont moins longtemps présentes sur la molécule CMH-I comparé à la demi-vie d'une protéine et deuxièmement le petit nombre d'immunopeptides présentés sur la molécule CMH-I par rapport à l'abondance élevée du protéome. Par conséquent, un nombre croissant d'études a évalué la relation entre le protéome et l'immunopeptidome et, étonnamment, ils ont trouvé une faible corrélation entre eux.

Deux théories sont proposées pour expliquer cette mauvaise corrélation entre le protéome et l'immunopeptidome. Une théorie a proposé que la partie central 20S du protéasome peut ligaturer deux peptides via un mécanisme appelé épissage peptidique. Toutefois, cette hypothèse est très douteuse en raison de la complexité de la réaction et des études de bio-informatique ont contesté ces résultats. L'autre théorie a proposé que les substrats de peptides proviennent d'un événement spécifique de traduction d'ARNm. Plusieurs études ont révélé qu'il existerait une corrélation entre la synthèse des protéines et la génération de peptides et, plus étonnamment, des études ont montré des processus de traduction alternative qui ont potentiellement un rôle important dans la génération de peptides antigéniques. Comme notamment l'étude réalisée par Laumont et al. en 2016, qui a révélé que 10% des peptides antigéniques

de classe I des cellules B provenaient d'une séquence génomique non codante ou d'une traduction exonique hors cadre¹.

À mesure que nos connaissances progressent, les scientifiques ont classé les sources de peptides antigéniques endogènes comme des protéines à fin de vie (Retirees) ou des polypeptides rapidement dégradés, qui peuvent être sous-classés comme des produits ribosomiques défectueux (Defective ribosomal products, DRIPs) et des produits pionniers de traduction (pioneer translation products, PTPs). Les PTPs sont des peptides antigéniques dérivés de l'ARNm pré-épissé, synthétisés par le cycle pionnier de traduction dans le noyau. Des études antérieures menées au laboratoire ont utilisé comme modèle de construction la séquence codante du gène β -Globin et introduit la séquence de peptide immunitaire SL8 dans l'exon 1, juste avant un codon de terminaison prématurée, pour évaluer la présentation de l'antigène sur la molécule CMH-I. Il a été observé que la présentation des peptides antigéniques dérivés de cette construction était efficace de façon comparable à celle des peptides antigéniques dérivés de la même construction mais qui n'avait pas de codon de terminaison prématurée. L'équipe a également démontré que les PTPs sont synthétisés aux premières étapes de la maturation de l'ARNm et avant que les introns ne soient épissés. L'introduction de SL8 dans l'intron 2 de la β -Globin a induit une présentation sur la molécule CMH-I, et la translocation du pré-ARN de cette construction au cytoplasme a diminué la présentation de l'antigène.

Dans ce doctorat, nous avons tenté de fournir plus de données probantes en utilisant différentes approches pour appuyer ces nouvelles découvertes et élucider le riboprotéome responsable de la synthèse des PTPs. Nous avons observé que l'expression de la séquence SIINFEKL (SL8) introduite dans le second intron du gène β -globine déclenchait la prolifération des cellules T CD8⁺ (OT-1). Par la technique du PLA (Proximity Ligation Assay) nous avons observé une augmentation des peptides SIINFEKL dérivés d'ARNm pré-épissé après traitement avec l'inhibiteur d'épissage Isoginkgetin et un inhibiteur du protéasome MG-132. Pour caractériser ce complexe de traduction alternative, nous avons utilisé la technique de fractionnement de polysome pour identifier les ribosomes associés à l'ARNm pré-épissé. L'ARNm- β -Globine pré-épissé se trouve associé aux polysomes légers, contrairement à l'ARNm- β -Globine épissé qui est présent dans les fractions polysomales lourdes. Nous avons également observé par PLA, l'interaction entre la protéine ribosomale L5 (RPL5) et la

séquence peptidique FEKL à partir d'ARNm pré-épissé, interaction qui est augmentée pendant le traitement avec l'inhibiteur d'élongation de traduction Cycloheximide. Ces données soutiennent la notion que les peptides antigéniques sont dérivés de la traduction des ARNm pré-épissés.

Abbreviations

3-MA	3-Methoxyamphetamine
ADP	adenosine diphosphate
AMBRA1	activating molecule in Beclin 1-regulated autophagy protein 1
APCs	antigen-presenting cells
ATG	autophagy-related genes
ATP	adenosine triphosphate
Blm10	bleomycin resistance 10
CBP	cap-binding protein
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CHX	Cyclohexamide
CLIP	class II-linked li peptide
c-myc IRES	cellular Myc internal ribosome entry segment
CQ	Chloroquine
DFCP1	zinc-finger FYVE domain-containing protein 1
DMSO	Dimethylsulfoxide
DRIPs	defective ribosomal products
E.V	Empty Vector
EBNA1	Epstein-Barr virus nuclear antigen 1
EBNA1ΔGAR	EBNA1 depleted of the GAR-domain
EBV	Epstein-Barr virus
eIF	eukaryotic initiation factors
eIF1	eukaryotic initiation factors 1
eIF1A	eukaryotic initiation factors 1A
eIF2-GDP	eukaryotic initiation factors 2 Guanosine diphosphate
eIF2-GTP	eukaryotic initiation factors 2 Guanosine triphosphate
eIF3	eukaryotic initiation factors 3
eIF4A	eukaryotic initiation factors 4 A
eIF4E	eukaryotic initiation factors 4 E
eIF4F	eukaryotic initiation factors 4 F
eIF4G	eukaryotic initiation factors 4 G
eIF5	eukaryotic initiation factors 5
Epoxo	Epoxomicin
ER	Endoplasmic Reticulum
	Endoplasmic Reticulum aminopeptidase associated with antigen processing
ERAAP	
ERAD	Endoplasmic reticulum-associated degradation
FIP200	RB1-inducible coiled-coil protein 1
FISH	Fluorescence in situ hybridization
G4	G-quadruplex
GAr	glycine-alanine repeat
GAr-OVA	glycine-alanine repeat - Ovalbumin

HA	Hemagglutinin
HIV RRE-REV	The HIV-1 Rev response element
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
HPLC	High-pressure liquid chromatography
HSV-1	herpes simplex virus type 1
HTT	Huntingtin
IAV	influenza A virus
IFN- γ	Interferon Gamma
I κ B	Inhibitory Kappa B
IL-2	Interleukin-2
IRAP	insulin-regulated aminopeptidase
LAP	Leucine aminopeptidase
LC3	Protein-light Chain 3
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LIR	LC3 Interaction Motif
LMP2	low-molecular-weight protein 2
LMP7	low-molecular-weight protein7
M2	Matrix 2
m7G cap	7-methylguanosine cap
MECL-1	multicatalyticendopeptidase complex-like-1
Met-tRNA	Methionine-tRNA
MHC	Major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC-II
MIIC	the MHC-II compartment
mRNA	Messenger RNA
MS	mass spectrometry
mTOR	mammalian target of rapamycin
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMD	nonsense-mediated decay
OPTN	Optineurin
PA200	Proteasome activator 200
PABP	poly(A) binding protein
PABP2	poly-A binding protein
PAS	phagophore assembly site
PE	phosphatidylethanolamine
PI3KC3	Phosphatidylinositol 3-kinase Complex
PI3P	phosphatidylinositol-3-phosphate
PIC	43S preinitiation complex
PLA	Proximity Ligation Assay
PLC	peptide-loading complex
PolyQ	Poly-Glutamine

PolyQ-OVA	Poly-Glutamine Ovalbumin
PSA	Puromycin-sensitive aminopeptidase
PTC	premature termination codon
PTPs	pioneer translation products
RDPs	Rapidly degraded polypeptides
RNA	Ribonucleic acid
RPs	ribosomal proteins
rRNA	Ribosomal RNA
SLiPs	short-life proteins
SNAP 29	synaptosomal-associated protein 29
SNCA	synuclein alpha
SQSTM1	sequestosome 1
STX17	Syntaxin 17
TAP	Transporter associated with antigen processing
TC	ternary complex
TCR	T cell receptor
TNF- α	Tumour Necrosis Factor alpha
TOLLIP	Toll-interacting protein
TOP	Thimet oligopeptidase
TPPII	Tripeptidyl peptidase II
tRNA	Transfer RNA
ULK1	Unc-51-like kinase 1
UTR	Untranslated Region
VAMP8	vesicle-associated membrane protein 8
VPS34	vacuolar protein sorting 34
WDYF3	Autophagy linked FYVE
WIP2	WD repeat domain phosphoinositide-interacting proteins
β 2m	β 2 microglobulin

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General Introduction

Specific or adaptative immune response recognizes and responds to two different kinds of antigens. B cells synthesize and release antibodies that recognize antigens in their native state. In contrast, T cells have a T cell receptor (TCR) that recognizes short fragments of antigenic peptide chain products from intracellular proteolysis. TCR engages antigenic peptides bound with a major histocompatibility complex (MHC) molecule on the antigen-presenting cells (APCs)²⁻⁵.

T cells can either express CD4 or CD8 molecules on their surface which creates two different functional classes of T cells: cytotoxic (CD8) and helper T cells (CD4)^{4,6-8}. CD8⁺Tcells recognize antigenic peptides in association with MHC class I (MHC-I) molecules, whereas CD4⁺ T cells recognize peptides bound to MHC class II (MHC-II) molecules^{5,9}.

I. The major histocompatibility complex (MHC)

The MHC molecules are transmembrane glycoproteins encoded in the large group of genes known as MHC^{4,10-12}. In this conserved region, there are not only MHC molecules but also other products essential for MHC function. MHC genetic properties allow immune system response to be broad to possible threats. The polygenic property: the MHC region contains different MHC class I and MHC class II genes, leading to the high variability of peptide-binding specificities between individuals and the polymorphic property: each gene in the MHC has multiple alleles⁴. In many species, the MHC encodes different MHC-I and MHC-II molecules possibly generated by gene duplication⁵.

The MHC molecule is located on chromosome 6 in humans and on chromosome 17 in the mouse. In humans, the genes coding MHC are called human leukocyte antigen (HLA) and in mouse are known as H-2 genes. Human or mouse genes are organized in separate clusters of MHC class I and MHC class II genes. There are three different classes of genes either for humans or mice. The genes coding for the heavy chain of class I are HLA-A, B, and C in humans and for mice, they are named H-2K, H-2D, and H-2L. These loci in humans show a very important polymorphism for example there are 9,097 alleles for the HLA-B gene currently assigned by the WHO Nomenclature Committee for Factors of the HLA system as of September 2022. Each of them codes for different domains: $\alpha 1$, $\alpha 2$, and $\alpha 3$ for the heavy chain. The light chain or $\beta 2m$ is coded by a gene found outside the HLA or H-2 locus, specifically in chromosome 15

for humans and in chromosome 2 for mice. While genes coding for both chains of class II are HLA-DR, -DP, and -DQ in humans and H2-IA, and -IE in mice^{4,13,14}.

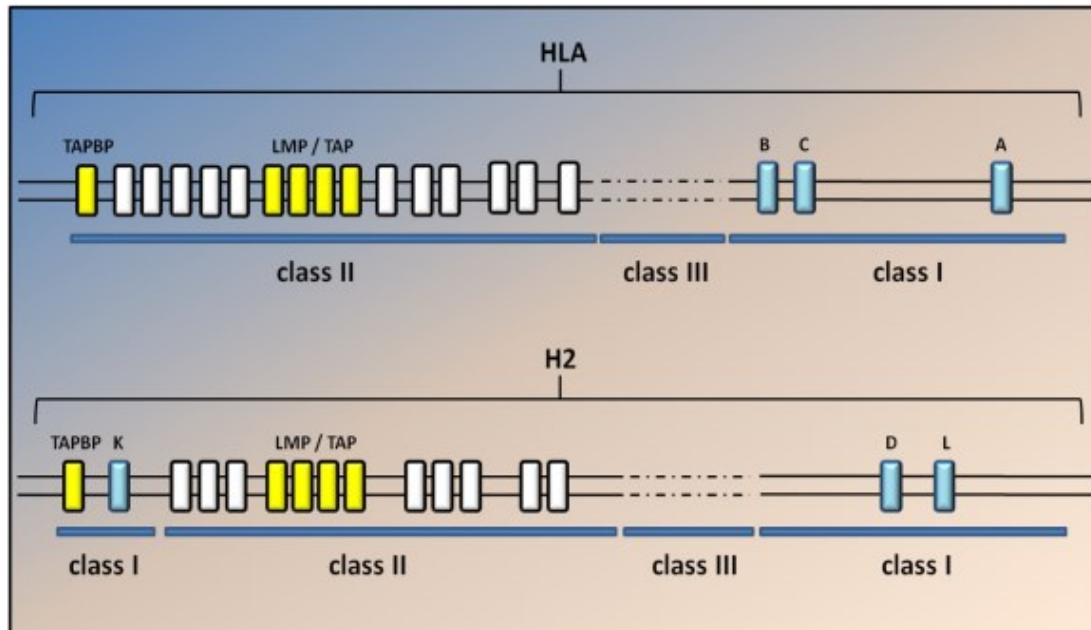


Figure 1. Schematic representation of MHC genes organization in A. Human and B. Mouse. The image depicts genes for the TAP1:TAP2 peptide transporter, the LMP genes that encode proteasome subunits, and the gene encoding the Tapasin (TAPBP) that are in the MHC class II region in both species except the TAPBP gene that is found in MHC class I in mouse^{4,13,14}(Image taken from Nardo et al 2016)¹⁵.

- The MHC molecule structure

The MHC-I and MHC-II genes are very polymorphic and have one domain with a high amino acid variation that interacts with peptides, allowing different MHC molecules to bind a broad range of peptides. The peptide binding structure is a groove formed by α -helices and β -sheets chains. For MHC-I molecules, the groove is formed by the N-terminal region of the heavy chain (α) composed of two of the three extracellular subunits $\alpha 1$ and $\alpha 2$. On the contrary for the MHC-II molecules, the groove is formed by the juxtaposition of the N-terminal region of two MHC-encoded α - and β -chains most precisely $\alpha 1$ and $\beta 1$ subunits. For both molecules, there is also a non-polymorphic domain that resembles Immunoglobulin constant region domains. For MHC-I, the $\alpha 3$ subunit of the heavy chain and the light chain $\beta 2$ microglobulin ($\beta 2m$). For MHC-II, the $\alpha 2$ and $\beta 2$ subunits. The MHC-I heavy chain and the MHC-II α and β - subunits are glycoproteins embedded in the cellular membrane having a short part exposed to the cytosol lumen^{5,16}.

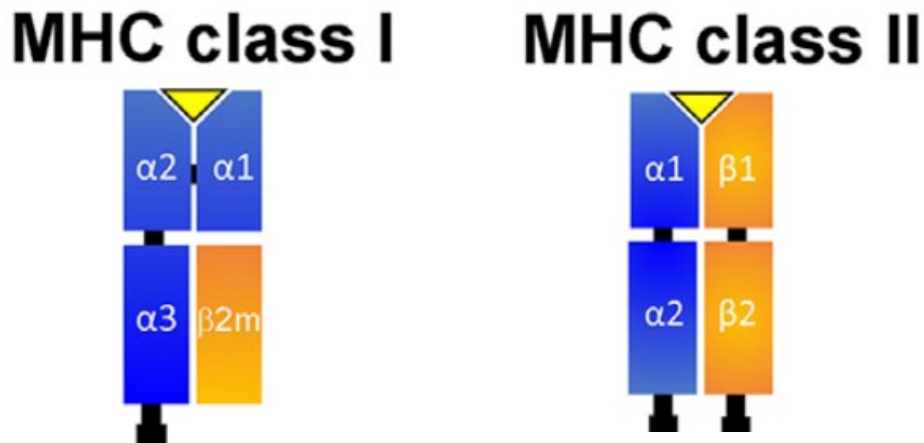


Figure 2. The MHC class molecules. The MHC molecule is composed of glycoproteins chains linked with disulfuric bonds and carboxylic extremes on the extracellular domain. Class I and II differs. MHC-I molecule has a light chain (β_{2m}) not embedded in the cell membrane, whereas the MHC-II molecule has α and β chains embedded in the cell membrane¹⁶(Image taken from Wieczorek, M. et al. et al 2017)¹⁶.

II. Processing and MHC-I presentation of endogenous antigens

The antigens presented on MHC-I can be of endogenous origin including cellular or viral antigens or from an exogenous source for example phagocytosed bacterial proteins^{2,5,9}.

- Source of antigenic peptides

Cytosolic proteins from the host or viral gene products are synthesized in the cell following the translation process. The Eukaryotic translation begins when the small subunit of the ribosome (the 40S) binds the initiator transfer RNA (tRNA) carrying Methionine (Met-tRNA) and the eukaryotic initiation factors (eIF). The stable form eIF2-GDP changes to eIF2-GTP activated form leading to the binding to Met-tRNA that forms the ternary complex (TC). The TC binds to the 40s with eIF5, eIF3, eIF1, and eIF1A to compose a larger 43S preinitiation complex (PIC). Next, eIF4F binds at the 5' 7-methylguanosine cap (m7G cap) and the poly(A) binding protein (PABP) at the 3' poly (A) tail to activate mRNAs. IF4F comprises a complex formed by eIF4E, eIF4G, and eIF4A. eIF4G has binding sites to eIF4E, PABP, and RNA that generate a closed-loop circularized. eIF4A unwinds mRNA secondary structures and facilitates the 43S PIC recruitment at the m7G cap. eIFs1 and 1A mediate the bound of the single-stranded mRNA to the 40S subunit. The 43S PIC starts the scanning along the mRNA from the m7G cap, in a 3' direction, searching for an AUG initiation codon suitable to make an mRNA codon-tRNA anticodon interaction. Once the tRNA finds the initiation

codon, eIF2 suffers a conformation change recruiting eIF5B that mediates the joining of the large subunit (the 60S). Next, the release of eIF5B and eIF1A allows the formation of an 80s ribosome attached to an mRNA with Met-tRNA bound to the AUG codon and starts the elongation phase of protein synthesis. In this stage, the different tRNAs carrying a specific amino acid bind sequentially to complementary codons in the mRNA. Each amino acid binds the growing polypeptide chain in the c-termini through a cycle of four sequential stages. The ribosome moves from codon to codon until reaches the stop codon. At this point, a releasing factor binds to the ribosome and ends the translation releasing the polypeptide¹⁷⁻¹⁹.

Proteins that are at the end of their functional lives are processed by the proteasome generating short peptides used as antigenic peptides for the MHC class I pathway.

- The proteasome

In general, the first step in endogenous antigen processing is the production of peptide fragments by the proteasome^{5,9,20}.

Classical proteasome 26S is composed of one catalytic core 20S flanked by two 19S regulatory caps. Both parts are subdivided into different protein complexes. The 20S core is a complex cylinder composed of 28 different subunits organized in four rings of seven subunits each. The two inner rings of the 20s core are composed of proteolytic subunits named $\beta 1$, $\beta 2$, and $\beta 5$, which form the catalytic chamber. On special occasions such as interferon induction, this core changes, and it is displayed by three alternative catalytic subunits called LMP2 ($\beta 1i$), MECL-1 ($\beta 2i$), and LMP7 ($\beta 5i$) that interestingly are encoded in the MHC genome region. As a result, the proteasome can be configured in two different forms the constitutive proteasome and the Immunoproteasome. The immunoproteasome configuration can produce peptides with carboxy-terminal residues that bind with high affinity to the binding groove of the MHC-I molecule^{4,21,22}.

Nevertheless, in 2007 another immune-type proteasome specifically expressed in the thymus was discovered. Murata S. et al 2007 found an unrecognized gene located adjacent to the $\beta 5$ gene encoding the $\beta 5t$ protein conserved in many mammalian species. The $\beta 5t$ is only highly expressed in cortical thymic epithelial cells, specifically located in the catalytic core along with $\beta 1i$ and $\beta 2i$, making a specific subtype of proteasome termed thymoproteasome. In response to the transcription factor Foxn1,

cortical thymic epithelial cells expressed $\beta 5t$, which exhibits a unique substrate specificity in endopeptidase proteolysis. In vivo assays performed in this study suggested that thymoproteasome conformation is crucial for optimizing the repertoire and effectiveness of the response of CD8⁺ T cells through the induction of optimal positive selection in the thymus²³.

The 19s cap, bound to both ends of the 20S core, recognizes polyubiquitinated substrates and unfolds the protein to allow the entrance of the substrate to the catalytic core of the proteasome where it is processed and short peptides that are then released into the cytosol^{4,21}.

The catalytic core 20S can also be associated with different families of regulators such as 11S complexes and PA200/Bim10. Interestingly, one regulator of the 11S family called PA28 $\alpha\beta$ (proteasome activator 28) has been implicated in the degradation of unfolded proteins and many studies have implicated this regulator in the production of MHC class I ligands. Many researchers have found that IFN- γ induces the assembling of PA28, composed of two proteins A28 α and PA28 β , to the 20S proteasome core. This special binding has been related to function as an accelerator of the antigenic peptide production^{21,24}.

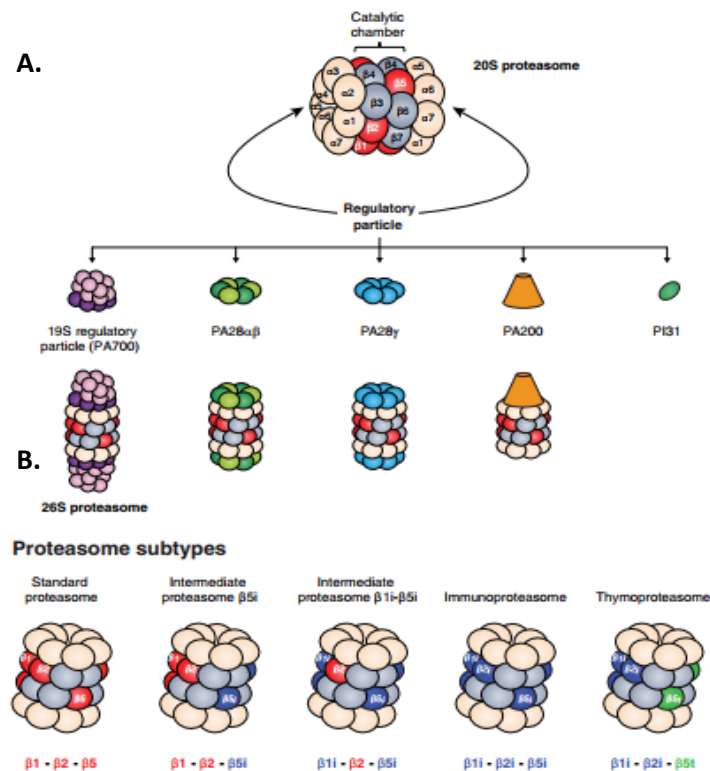


Figure 3. Structure and composition of proteasomes. A. The 20S proteasome has a cylindrical form and it is composed of four rings (α subunits and β subunits). The α subunits are located on the extremes and are the gates to the entrance of the protein to the degradation

core. The α subunits can be associated with different regulators. **B.** Proteasome subtypes, these five types of proteasomes differ in their catalytic sites. The standard proteasome includes the catalytic subunits β_1 , β_2 , and β_5 , however, in response to IFN- γ or the transcription factor Foxn1 in cortical thymic epithelial cells, the proteasome conformation changes led to two kinds of proteasomes: the Immunoproteasome and the thymoproteasome, respectively. The immunoproteasome is composed of catalytic sites β_{1i} , β_{2i} , and β_{5i} , while the thymoproteasome is composed of the catalytic site β_{5t} .^{22,24,25}(Image taken from Vigneron et al 2017)²⁶.

- Cytosolic aminopeptidases

Further studies have uncovered that many aminopeptidases tackle cytoplasmic peptides after proteasome degradation. A degradation mechanism that could explain the short half-life of a peptide of about 6 to 10 seconds and the limited peptide presentation of the MHC-I molecule compared with the high number of peptides produced from the whole proteome by the proteasome. This array of cytosolic aminopeptidases is composed of Tripeptidyl peptidase II (TPPII) and Neurolysin that cleaves large peptide fragments; Thimet oligopeptidase (TOP) that cleaves 8-15 amino acids; Leucine aminopeptidase (LAP) removes hydrophobic or aromatic amino acids; Puromycin-sensitive aminopeptidase (PSA) that cleaves small peptides and is specific for PolyQ-peptides and Bleomycin hydrolase that also targets small peptides among others²⁷.

- Peptide binding to MHC-I molecules

Peptides generated in the cytoplasm are translocated in an active ATP-dependent way into the Endoplasmic Reticulum (ER) by the protein translocator TAP (Transporter associated with antigen processing), a heterodimer composed of TAP1/TAP2 proteins encoded by genes in the MHC^{5,9,28}. TAP transport begins when ATP and/or ADP and peptides bind independently to the TAP complex in the cytoplasm. The peptide bond to the heterodimer leads to a conformational change of the TAP complex. This structural rearrangement induces the ATP hydrolysis and translocation of the peptide to the ER lumen⁵.

Within the ER, newly synthesized MHC-I molecules are retained until they bind a peptide. The folding and assembly of the MHC-I molecule depends first on the MHC-I α heavy chain associated with the light chain β_2m and then on the peptide. This process involves different proteins with chaperone functions. It starts when the synthesized MHC-I α chain enters the ER and binds to the chaperone protein calnexin, which retains the MHC-I molecule in a partially folded state. Then, the β_2m binds to

the α chain, leading to the calnexin dissociation and the binding to the peptide-loading complex (PLC)²⁹. This PLC is composed of chaperone-like calreticulin³⁰, the tapasin³⁰ that forms a wall between the MHC-I molecule and TAP, blocking the bounding of any peptide, and a third component the ERp57, a thiol oxidoreductase that has a role in breaking this complex, that allows the encounter of the peptide with the MHC-I molecule³¹. Sometimes peptides too long to bind MHC-I molecule can still be transported into the ER, when this happens, an aminopeptidase called ER aminopeptidase associated with antigen processing (ERAAP) trims the peptide amino termini in the ER lumen³². The binding of a peptide to the semi folded MHC-I molecule induces the releases from the PLC and the full folding of the MHC-I molecule. Then, the MHC-I molecule/peptide can now leave the ER and be transported to the cell surface. Importantly, not all peptides that entered the ER will bind to the MHC-I molecule. Later findings have identified a transporter ATP-dependent complex the endoplasmic reticulum-associated degradation (ERAD)³³ that can translocate back into the cytoplasm of the non-bound peptides^{9,16,34}.

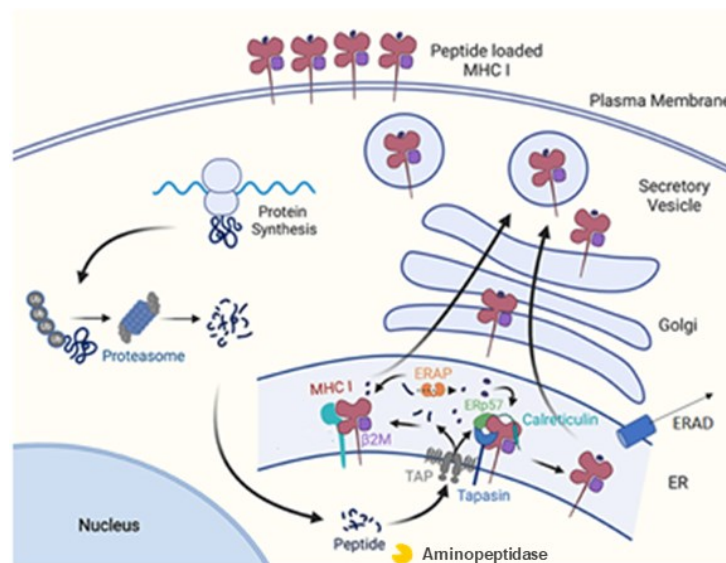


Figure 4. The MHC class I antigen presentation pathway. Intracellular proteins are processed by the proteasome and cleaved by the cytosolic aminopeptidases. The peptide products are then translocated to the ER. Within the ER the peptide is further trimmed, if necessary, by the ERAAP. Next, the peptide binds the MHC-I molecule mediated by the PLC complex composed of Tapasin, Calreticulin, and ERP57. The unbound peptides in the ER are extracted by ERAD. Following MHC-I molecule binding to the peptide, they are sent to the cell surface to activate CD8⁺ T cells⁹(Image adapted from Dhatchinamoorthy et al 2017)³⁵.

III. Processing and MHC-II presentation of exogenous and endogenous antigens

- Vacuolar pathway

Professional APCs, such as macrophages, dendritic cells, and B cells, internalized exogenous proteins and degrade them by lysosomal proteolysis. Endocytosis occurs when extracellular proteins and plasma membrane cell components are fully internalized into the cell. Internalization routes can vary depending on the cargo to three general modes of vesicular endocytic trafficking. i) Phagocytosis: the cell membrane enclosed a macromolecule or an extracellular cell and produces intracellular vesicles called phagosomes. ii) Cellular pinocytosis: cell internalize nutrients and fluids and forms vesicles smaller than phagosomes. iii) Receptor-mediated endocytosis: cells take up specific molecules via specific receptors by clathrin-mediated, caveolae-mediated, and clathrin and caveolae-independent endocytic pathways. Especially, professional APCs have several surface receptors mediating the capture of antigens in clathrin-coated domains like the B cell receptor (BCR), FC receptors, C-type lectin family receptor DEC205, and mannose and transferrin receptors. As well as they express receptors to self-proteins or microbial heat shock proteins^{5,36,37}.

Internalized cargos merge into a common endosomal network, where it is transported progressively through more acidic and proteolytically active compartments. The first vesicle formed carrying the cargo is referred to as an early endosome identified by the localization of RAB5, RAB4, RAB11, ARF1/COPI, retromer, and caveolin. Then, early endosomes become mature late endosomes characterized by the presence of RAB7-GTPase followed by late endosomes much bigger in size and acquiring more intraluminal vesicles. Late endosomes can fuse the plasma membrane to release exosomes containing protein, DNA, and RNA or move to the perinuclear region suffering several fusions among them until fusing lysosomes forming an endolysosome. In the endolysosome most of the degradation of the endocytic cargo takes place and next the endolysosome matures to a dense lysosome characterized by the location of LAMP2. Endolysosomes and lysosomes contain a pH of 4,5 and hydrolytic enzymes like cathepsins and the thiol oxidoreductase GILT that reduce the peptides to a length of 13 to 18 amino acids^{5,36,37}.

Alternatively, intracellular contents and organelles can also be transported to lysosomes through a different pathway called autophagy. This self-eating mechanism engulfs cytoplasmic components in an isolated membrane progressively sealed to form an autophagosome that is delivered to the lysosome for degradation^{36,37}.

Depending on the peptide length, short peptide products can be recycled and used for new protein synthesis, whereas large peptides can be a source for MHC-II binding^{5,9,36,37}.

Internalized exogenous antigens by phagocytosis^{38,39}, cell pinocytosis, or Receptor-mediated endocytosis are trafficked to a late endosomal compartment, also named MHC-II compartment to follow MHC class II antigen presentation⁵. As well as peptides derived from cytoplasmic and nuclear proteins are delivered by autophagy also to this MHC-II compartment⁵. It has been demonstrated that autophagy deficiency in professional APCs impairs viral and bacterial antigens in MHC class II presentation⁵ or for example, autophagy disruption in thymic epithelial cells alters the selection of thymic CD4 T cells⁴⁰.

- Peptide binding to MHC-II molecules

The MHC-II molecules composed of α - and β -chains are assembled in the ER and bound to the invariant chain (I chain), which protects the MHC-II ligand binding groove and guides the MHC-II molecules to endosomal compartments⁴¹. The resulting complex MHC-II/I chain is then transported to a late endosomal compartment called the MHC-II compartment (MIIC) by the I chain dileucine motifs via the trans-Golgi network⁴¹. Alternatively, this complex also can reach MIIC by recycling from the cell surface⁴². Inside MIIC, the I chain is digested progressively by proteolysis, resulting in a residual class II-linked I chain peptide (CLIP) located in the peptide-binding groove of the MHC-II^{43,44}. Next, CLIP is released from MHC-II molecules through the interaction of the MHC-II-CLIP with the MHC-encoded heterodimeric glycoprotein, DM, allowing the antigenic peptides to load in the MHC class II molecule^{45,46}. The DM functionality is regulated by another MHC-encoded heterodimer, DO, which inhibits DM functionality⁴⁷. After, the MHC-II molecule/antigenic peptide complex leaves the MIIC and goes to the plasma membrane, where it will present its peptide to CD4⁺ T cells⁵.

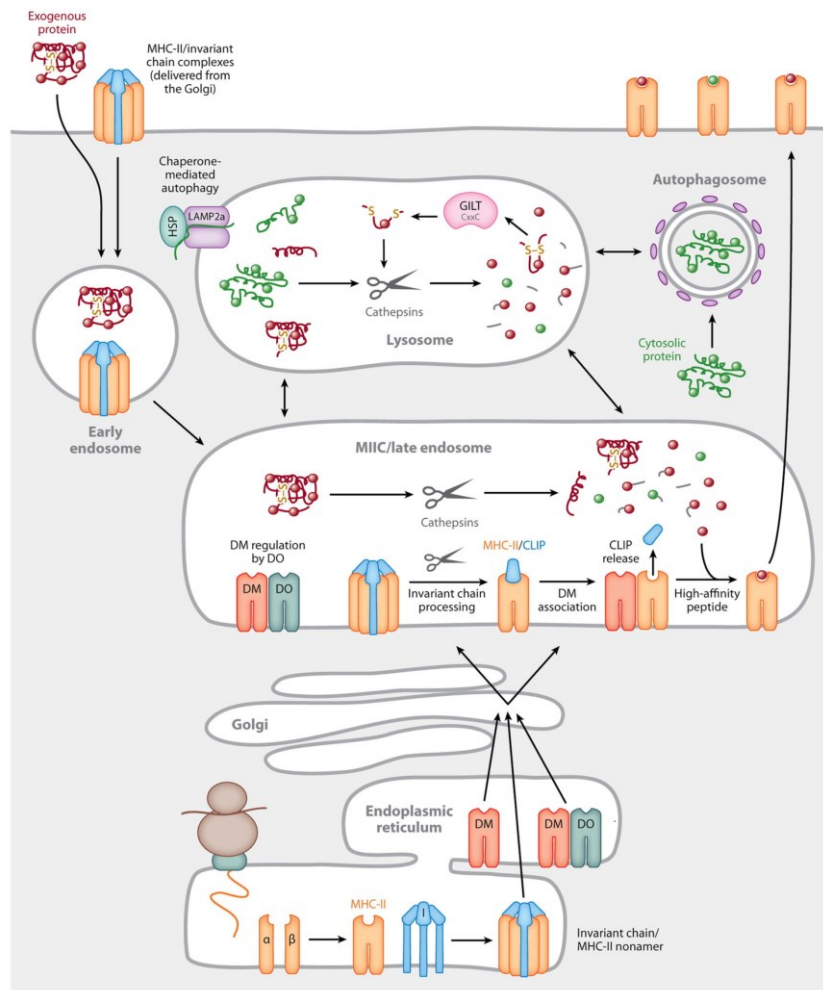


Figure 5. The MHC class II antigen presentation pathway. The MHC-II molecule assembles in the ER, where it binds to the invariant chain (I chain). The peptides and the I chain-MHC-II molecule enter the MHC-II compartment (MIIC), where the peptide binds to the MHC-II molecule. Then, the antigenic peptide bound to the MHC-II molecule is transported to the plasma membrane. Antigens transported to late endosomes or lysosomes by phagocytosis, cell pinocytosis, receptor-mediated endocytosis, or autophagy are processed by cathepsins and GILT previously to MHC-II loading^{5,8,9}. (Image taken from Blum et al 2013)⁵.

IV. Cross-presentation

In 1976, Bevan observed that mice immunization with an exogenous antigen, named H-Ag, activated CD8⁺ T cells. Indicating that professional APCs were able to present exogenous proteins internalized by phagocytosis through MHC-I molecules⁴⁸. Nowadays, this process is denominated cross-presentation.

Professional APCs can cross-present by MHC-I molecules, among them, dendritic cells are exceptional cross-presenters⁴⁹. Specific types of dendritic cells that are very efficient in mediating cross-presentation in mice are steady-state CD8 α ⁺CD11c^{high} DCs, while in humans are CD41⁺(BDCA3)⁺ DCs⁵⁰. However, other cells such as liver

sinusoidal endothelial cells, Kupffer cells, and hepatocytes have also been found to cross-present in the mouse liver for example during acute adenoviral infection⁵¹.

When exogenous antigens are engulfed by dendritic cells. The internalized antigen goes to an endocytic compartment and is processed through two different pathways.

- The cytosolic pathway

In the cytosolic pathway, material captured by DCs located in the early endosomes or phagosomes is transported into the cytoplasm by Sec61, an ER-associated degradation (ERAD) member, using the energy furnished by ATPase P97⁵².

In the cytosol, peptides are processed into antigenic peptides by proteasomes or restored their activity by Hsp90 chaperone-mediated refolding⁵³. The antigenic peptides can be further trimmed in the cytosol by accessory peptidases or they entered the ER via TAP and are trimmed by the ERAAP and loaded into the MHC-I molecule⁵⁴.

However, it is proposed that materials internalized located in early endosomes or phagosomes are not transported to the cytosol. Instead, they are trimmed by the insulin-regulated aminopeptidase (IRAP) to generate antigenic peptides and encounter MHC-I molecules in these compartments. It is known that MHC-I molecules located in these endosomes come from the ER, but more evidence suggests that MHC-I molecules are from secretory pathways or cell surface⁵⁵. Additionally, other studies elucidated that the ER soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) Sec22b mediates the vesicular relocation from the ER to the endosomes of important protein translocators necessary for the antigenic peptides entrance to the endosomes⁵⁶.

- The vacuolar pathway

Viral proteins, virus-like particles, and Escherichia coli-associated proteins for example are cross-presented through the vacuolar pathway⁵⁷⁻⁵⁹.

In this pathway, antigens remain in the phagosome to be fragmented by endosomal proteolysis from cysteine proteases, especially cathepsin S⁶⁰ (an enzyme that is induced during viral infection in response to IFN- γ ⁶¹) and IRAP. An alternative hypothesis claims that the antigens can escape the phagosome and be degraded by the proteasome, relocated to the phagosome, and be again trimmed by IRAP. The

peptides produced during these two processes encounter in both cases recycled MHC-I molecules from the plasma membrane in the phagosome, which leads to the loading and the subsequent antigen presentation⁵⁴.

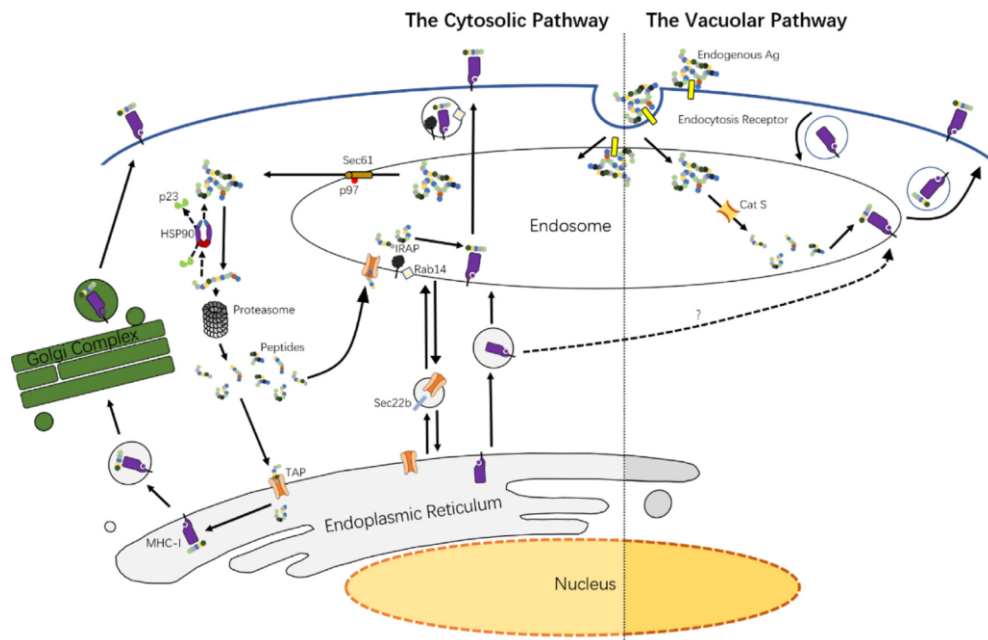


Figure 6. Cross-presentation. Exogenous antigens internalized by the dendritic cells can be processed and associated with the MHC-I molecule in the vacuolar pathway or the cytosolic pathway⁶²(Image taken from Li B. et al 2019)⁵⁰.

Until this section, I have introduced the general aspects of antigen presentation. Next, I will divide my Ph.D. dissertation into two parts to better present and discuss the two main approaches that I developed during my Ph.D. to study the origin of antigenic peptides in the MHC class I pathway.

- In the first part, I am going to focus on autophagy and its role in the generation of peptide substrates for the MHC class I pathway.
- In the second part, I am going to focus on the study of the translation machinery responsible for the production of MHC-I antigenic peptides coming from pre-spliced mRNA.

Introduction – PART 1

In the adaptive immune response, the autophagy pathway is essential for thymus selection⁴⁰, lymphocyte development⁶³, immune homeostasis, and antigen presentation^{64,65}.

Current studies propose that autophagy is tightly involved in antigen processing for MHC presentation, in which peptide products are used in the activation of T cells. However, there is not much evidence associating MHC-I presentation with the processing of antigenic peptides by autophagy⁶⁶. In this section, I'll describe first the autophagy pathway, second studies demonstrating autophagy processing of antigenic peptides presented on MHC-I molecules, and third potential autophagy substrates used for antigenic peptides production on the MHC class I pathway.

V. The Autophagy pathway

Autophagy is a key degradative process of endogenous cytoplasmic proteins that occurs in response to different forms of stress such as nutrient deprivation⁶⁷, growth factor depletion⁶⁸, infection⁶⁹, or hypoxia⁷⁰. Autophagy plays a critical role in the supply of nutrients during fasting or other types of stress. But it was later revealed that under basal conditions, autophagy has a selectivity for specific cargos to get rid of damaged organelles^{71,72}, such as harmful aggregation-prone proteins⁷³ or mitochondria⁷⁴.

There are three classes of autophagy⁷⁵:

1. Microautophagy: In this mechanism, the lysosome engulfs cytoplasmic components by inward invagination⁷⁶.
2. Chaperone-mediated autophagy: In this mechanism, substrates proteins associated with chaperones enter directly into the lysosome without the need for membrane reorganization⁷⁷.
3. Macroautophagy: In this mechanism, endogenous proteins are taken by an organelle called autophagosome and delivered to lysosomes⁷².

Macroautophagy is the major type and the most extensively studied compared to the other two types and more importantly the center of our study. Thus, we will refer to macroautophagy simply as autophagy⁷² in this manuscript.

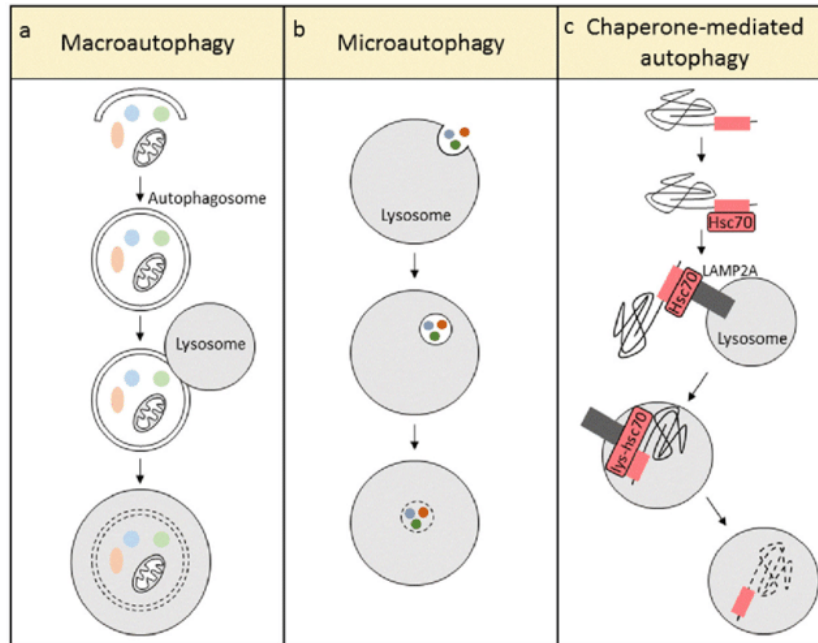


Figure 7. Different classes of autophagy. **A.** Macroautophagy: the insulation membrane takes a portion of the cytoplasm to form an autophagosome. The autophagosome fuse with the lysosome and the cargo is degraded in the lysosome. **B.** Microautophagy: cytoplasm is directly engulfed in the lysosome. **C.** Chaperone-mediated autophagy: Hsc70 recognizes KFERQ-like pentapeptide in the substrate proteins. Then, the cargo is translocated to the lysosome through Lamp-2A⁷⁵(Image taken from Li, Jin et al 2018)⁷⁵.

The autophagy mechanism

The mechanism of autophagy involves many different actors; thus, I am going to explain this process shortly, giving the main points of this pathway, and then I will provide a more detailed explanation of each stage.

Main stages of autophagy mechanism:

The activation of autophagy leads to the recruitment of autophagy-related genes (ATG) and different types of enzymes as kinases to a specific site close to the ER called the phagophore assembly site (PAS). The interaction of all these proteins triggers the nucleation of an isolated membrane that forms a cup-shaped structure called the phagophore. Then, this phagophore will continue expanding, taking with it a portion of the cytosol, until it seals the membrane. A new double-membrane vesicle is then formed termed the autophagosome carrying inside an engulfed material the autophagic cargo. The autophagosome will mature clearing the ATGs surrounding it and will move through the microtubules to reach the lysosome. Finally, the autophagosome will fuse the lysosomal membrane to form an autolysosome. Inside

this autolysosome, the autophagosome will be transformed into a single membrane autophagic body, which is followed by its degradation⁷¹.

Detailed autophagy mechanism:

- Phagophore formation

Even though other triggering signals exist, amino acid deprivation is the classical activation signal of autophagy. This deprivation will inhibit the master cell growth regulator serine/threonine kinase mTOR, but only one of the two different complexes, mTORC1. In a normal cellular state, mTORC1 is bound to Unc-51-like kinase 1 (ULK1) in a phosphorylated form, upon starvation, mTORC1-ULK sites are dephosphorylated and trigger the release of ULK1. ULK1 then is autophosphorylated, followed by the phosphorylation of ATG13, RB1-inducible coiled-coil protein 1 (FIP200), and ATG101⁷⁸. The formation of this complex triggers the nucleation of the phagophore by phosphorylating components of the classical III PI3K (PI3KC3) complex (composed of class III PI3K, vacuolar protein sorting 34 (VPS34), Beclin 1, ATG14, activating molecule in Beclin 1-regulated autophagy protein 1 (AMBRA1) and general vesicular transport factor (p115))^{79,80}. As a result, it will activate local phosphatidylinositol-3-phosphate (PI3P) production and the formation of a characteristic ER structure identified as the omegasome. PI3P then recruits WD repeat domain phosphoinositide-interacting proteins (WIPI2) and zinc-finger FYVE domain-containing protein 1 (DFCP1)⁸¹. This membrane core will be elongated with the delivery of several membranes by vesicles containing ATG9⁸². However, this source only is a part of the lipid bilayer, the rest of the membrane, still, is unknown^{71,72}.

- Phagophore expansion

WIP2 then attracts and binds ATG16L1, triggering the recruitment of Atg12~Atg5 and the lipid form of LC3. The Atg12~Atg5 and LC3 complexes are the product of two ubiquitin-like conjugation systems. The conjugation of Atg12~Atg5 occurs when Atg12 is activated by Atg7 (E1 ubiquitin-like conjugating system) and then associated with Atg5 by Atg10 (E2 ubiquitin-like conjugating system). LC3 complex then is cleaved by the Atg4 protease, resulting in an exposure of a glycine residue located in the C-terminus. When autophagy is activated, the LC3 terminus is conjugated to the polar head of phosphatidylethanolamine (PE) a component of the phospholipid bilayer. A reaction can only occur in presence of Atg7, Atg3, and Atg12-Atg5:Atg16L complex⁸¹⁻

⁸³. The LC3 associated with PE is the characteristic LC3-II marker as an autophagy activator and is located in the outer as the inner side of the autophagosome membrane, while LC3-I under normal conditions is located only in the cytosolic part⁸⁴. The conjugation system between Atg7, Atg3, and Atg12-Atg5:Atg16L complex and the lipidic LC3 promotes the phagophore expansion, mediates the cargo selective recruitment as they interact with the cargo receptors, and closure of the autophagosome membrane. The human LC3 family is composed of three members, LC3A-C and its homologs GATE-16, GABARAP1, 3, and Atg8L^{71,72}.

- Autophagosome Maturation and Lysosomal fusion

Once the autophagosome is formed it starts to mature through the release of the Atg12-Atg5:Atg16L complex and the cleavage of LC3-PE by the Atg4 protease. To fuse with the lysosomal organelles, the mature autophagosome recruits microtubule-based kinases motors in charge of lysosomal delivery and proteins implicated in the fusion with the lysosome, which includes diverse proteins from the SNARE family. For example, on the autophagosome side, Syntaxin 17 (STX17) and the synaptosomal-associated protein 29 (SNAP 29) are recruited. While on the lysosome side, it recruits some proteins such as the vesicle-associated membrane protein 8 (VAMP8) to assist in the autophagosome delivery⁸⁵. Nevertheless, the fusion mechanism between the autophagosome and the lysosome is still not fully understood^{71,72}.

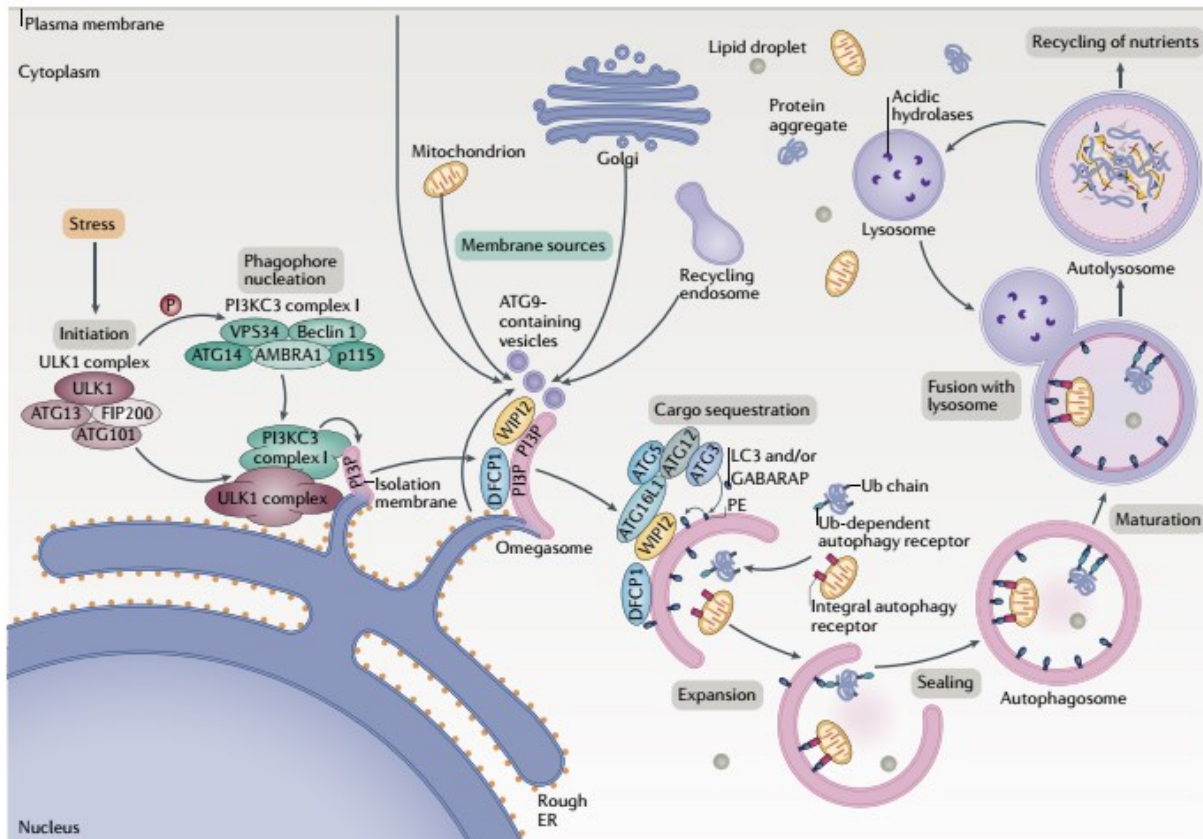


Figure 8. Autophagy mechanism. Activation of autophagy starts, for example, with starvation or protein aggregates. This stress leads the Unc-51-like kinase 1 (ULK1) complex to phosphorylate the class III PI3K (PI3KC3) complex I, triggering phagophore nucleation. The P115 component of the PI3KC3 complex I induce the production of phosphatidylinositol-3-phosphate (PI3P), elongating the ER membrane to a structure called omegasome. Next, PI3P attracts effector proteins such as WD repeat domain phosphoinositide-interacting proteins (WIPs; here WIP1) and zinc-finger FYVE domain-containing protein 1 (DFCP1). WIP1 then binds to ATG16L1 and consequently leads to the recruitment of Atg12~Atg5. Those bindings enhance the Atg3 mediated conjugation with the microtubule-associated protein light chain 3 (LC3) proteins and γ -aminobutyric acid receptor-associated proteins (GABARAPs) to the membrane-resident phosphatidylethanolamine (PE) that allowed the cargo sequestration and then its expansion. The sealing of the phagophore forms an autophagosome with a double membrane bilayer that next mature and fuses with the lysosome^{72,86} (Image taken from Dikic et al 2018)⁷¹.

V.A Intracellular quality control by selective autophagy

Under nutrient-rich conditions the contribution of autophagy was unclear but new studies have revealed that autophagy can function as a quality-control system of different cytoplasmic components and help in cell homeostasis. This quality control is made mostly through a selective autophagy mechanism, which uses ligand receptors and scaffold proteins. In the last decade, it has been a considerable number of studies

demonstrating the existence of different receptors and scaffold proteins dependent on the cargo nature⁷².

For example, the ubiquitination of substrates is a traditional “eat me signal” in the recognition and degradation by selective autophagy. There are six mammalian ubiquitin cargo receptors that are P62/SQSTM1-like receptor such as the NRB1, NDP52, OPTN, P62/SQSTM1, TAXBP1 and TOLLIP/cue5 found in autophagosomes that allow the entrance of different cargos ubiquitinated. The discovery of autophagy selectivity through ubiquitination signaling revealed that ubiquitination was not only a signal of proteasome degradation but also an autophagy signal. However, we still do not know how both systems are harmonized in the cell. The scientific community has proposed that autophagy could degrade big cargos that are not able to be destroyed by the proteasome or maybe the different length and nature of the ubiquitin chains decide the degradative destiny for such cargos^{87,88}.

In this context, I am going to outline some cargos displaying ubiquitin labeling that can be recognized by specific receptors expressed in the autophagosome membranes.

- Aggregates clearance by autophagy

Aggregation-prone proteins like Amyloid- β ⁸⁹, Huntingtin (HTT)⁹⁰, and SNCA (synuclein alpha)⁹¹ has been demonstrated to be autophagy substrates. So far, it is known that the aggrephagy mechanism is triggered only by ubiquitin. This tagging is recognized by four different receptors TOLLIP⁹², SQSTM1⁹³, NRB1⁹⁴, and OPTN⁹⁵. Moreover, later data have observed that the nucleocytoplasmic shuttling protein WDFY3 can bind the Atg5 protein, the SQSTM1 receptor, and LC3, serving as a scaffold protein⁹⁶. Interestingly, this study has also revealed that WDYF3 depletion blocked the clearance of aggregated polyQ proteins^{87,88}.

- Intracellular pathogens clearance by autophagy

Clearance of intracellular pathogens such as viruses, bacteria, and fungi via autophagy is denominated, Xenophagy. For example, some studies have observed bacterial proteins from *Salmonella typhimurium* that were ubiquitinated and recognized by the autophagosome cargo receptors SQSTM1⁹⁷ and OPTN⁹⁸. Moreover, the expression of these receptors has been associated with a growth restriction of intracellular pathogenic bacteria such as *S. Typhimurium*⁹⁹, *M. Tuberculosis*¹⁰⁰, mutant *S. Flexneri*¹⁰¹, and mutant *L. Monocytogenes*¹⁰².

Interestingly, some findings have determined that some intracellular pathogens contain LC3 Interaction Motif (LIR) to trick the host autophagy machinery. For example, the influenza A virus (IAV) Matrix 2 (M2) ion channel protein blocks autophagosome-lysosome fusion and recruits LC3 to the plasma membrane through the LIR motif present on the cytoplasmic tail of the M2 protein¹⁰³. A recent publication examined the presence of counteracting LC3 LIR sequences in databases containing more than 16000 viral sequences¹⁰⁴. The authors found several interesting candidates with a potential role similar to LIR sequences. For example, a potential LIR was identified in the HIV-1 protein Nef, which has been previously described as an inhibitor of autophagosome maturation¹⁰⁵. This LIR candidate seems to colocalize with LC3 and BECN1 to avoid autophagy degradation¹⁰⁶.

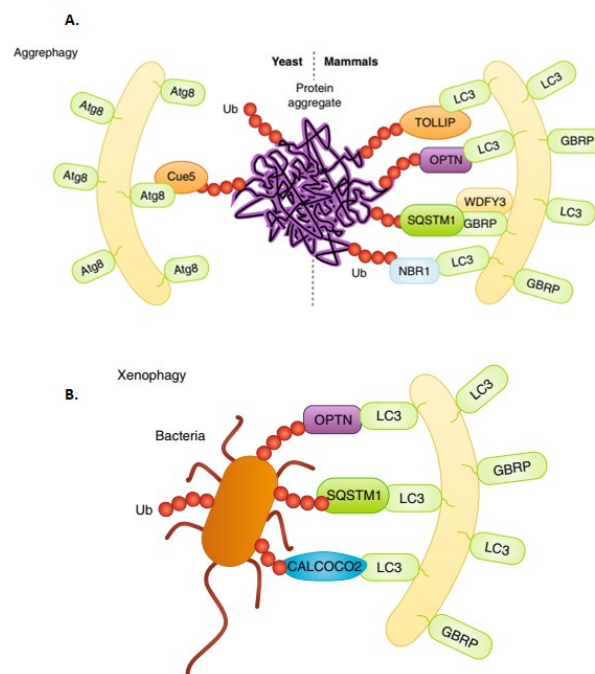


Figure 9. Aggrephagy and xenophagy. **A.** In yeast and mammals, protein aggregates are ubiquitinated and then recognized by specific receptors. In mammals, ubiquitin is recognized by TOLLIP, SQSTM1, NBR1, and OPTN. They are linked to LC3/GABARAP in the autophagosome, allowing the entrance of the protein aggregate. In yeast the receptor is Cue5. **B.** Intracellular pathogens such as bacteria are ubiquitinated and recognized by receptors embedded in the autophagosome membrane such as SQSTM1, OPTN, CALCOCO2, and NBR1⁸⁸(Image taken from Gatica et al 2018)⁸⁷.

VI. Autophagy and MHC-I presentation

The most accepted autophagy participation in the antigen presentation is through the MHC class II pathway. Traditionally, scientists have thought that only exogenous antigens engulfed by professional APCs activate CD4⁺ T cells. However, data also

suggested that intracellular sources such as viral, tumor, or other self-proteins are presented by MHC-II molecules via autophagy^{66,107,108}. The importance of autophagy in intracellular antigen processing for MHC Class II antigen presentation has been determined by loss-and- gain-of-function experiments. Loss of function assays such as ATG5 knock out decreased antiviral CD4⁺ T cell responses¹⁰⁹ and MHC class II-restricted thymic T cell selection *in vivo*⁴⁰. Similarly, the gain of function assays like the fusion of LC3B to influenza virus¹¹⁰, immunodeficiency virus¹¹¹, and tumor antigens¹¹² increased MHC class II presentation to specific CD4⁺ T cells. Furthermore, pathogens like *M. Tuberculosis* or herpes simplex virus 1 (HSV1) code autophagy proteins inhibitors to avoid MHC class II presentation, confirming the relevance of autophagy^{113,114}.

Apart from the MHC class II pathway implication, further studies also uncovered that dendritic cells, depending on the source of the antigenic peptide, can cross-present and activate CD8⁺ T cells via autophagy^{115,116}.

However, little evidence exists that points to autophagy as a processing mechanism of antigenic peptides in the MHC class I pathway. There is more available evidence showing indeed the opposite. For example, Schmid and colleagues fused the LC3 sequence to a viral epitope that induced an increase in MHC-II presentation and evaluated this same LC3-viral epitope in the MHC class I pathway. Strikingly, they did not see any increase in the MHC-I presentation as in the MHC-II presentation response¹¹⁷. Nevertheless, some evidence reveals that autophagy can contribute to MHC-I antigen presentation. English L. and colleagues have shown that macrophages, infected with herpes simplex virus type 1 (HSV-1) in an advanced stage of infection displayed autophagy processing of HSV-1 antigenic peptides for the MHC class I pathway. Furthermore, they observed that the autophagosomes activated where originated from the nuclear envelope and that these antigenic peptides were further processed by the proteasome¹¹⁸. Similarly, another study has used dendritic cells infected with the herpes simplex virus lacking the protein 34.5 (ICP34.5), which has been shown to suppress autophagy, resulting in an efficient stimulation of CD8⁺ T cells and low viral protein abundance. Interestingly the last study employs nonprofessional-APCs¹¹⁹. Tey and colleagues infected fibroblasts deficient or not in TAP expression with the adenovirus encoding the UL138 transgene and submitted them to autophagy or proteasome inhibitors. They have seen that autophagy inhibition decreased antigen

presentation of CD8⁺ T cells specific for Pul138 only in fibroblasts deficient in TAP. These studies revealed that the role of autophagy in the MHC class I pathway is still poorly understood¹²⁰.

Autophagy has not been related much to antigen processing in the MHC class I pathway, instead, it has been implicated in the localization of the MHC-I molecules on the cell surface. Dendritic cells deficient in Atg5, Atg7 or Vps34 induce an increase in MHC class I surface levels compared with wild-type dendritic cells. Thus, autophagy mediates also the endocytosis of the MHC-I molecules on the cell surface^{121,122}.

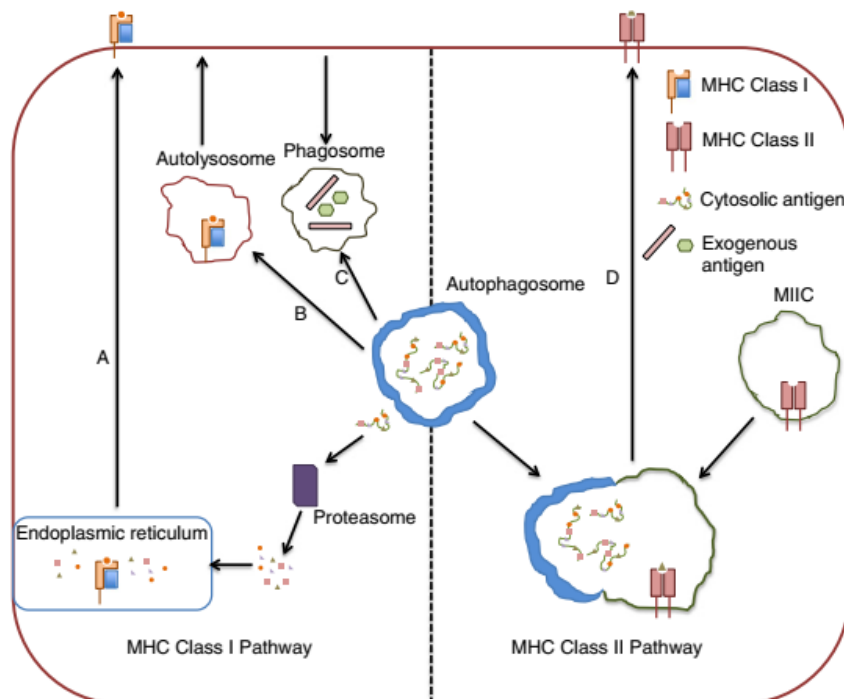


Figure 10. Autophagy and antigen presentation pathways. **A.** Following autophagosome engulfment, the antigen is released and enters the proteasome to be processed, followed by the binding to the MHC-I molecule in the ER and the display of this antigenic peptide/MHC-I molecule to CD8⁺ T cells. However, some studies claim that are proteasome-independent. **B.** MHC-I molecules may also be loaded in lysosomes before transport to the cell surface. **C.** In cross-presentation phagosomes can encounter autophagosomes carrying extracellular antigens that can then take the MHC class I pathway. **D.** For the MHC class II pathway, it is well accepted that autophagosomes fuse MHC class II compartments to display CD4⁺ T cell response from endogenous antigens^{64,123} (Image taken from Puleston et al 2014)⁶⁶.

VII. Autophagy substrates

Throughout this section, we have described the autophagy pathway and how this degradation pathway can be related to the MHC class I pathway. Various approaches have determined that autophagy can degrade long-lived proteins, aggregate-prone proteins, and proteins derived from pathogens among others. Interestingly, cells take

advantage of this mechanism to process these proteins and used the antigenic peptides to activate the immune system. Thus, recovering organism homeostasis by the clearance of infected or damaged cells.

The link between autophagy and antigenic peptides generation for the MHC class I pathway is still poorly understood. According to the available evidence, this association seems to be substrate-dependent. In this Ph.D. study, we have focused on two different kinds of substrates. An aggregate-prone protein and a viral protein.

VII.A Protein aggregation

Protein aggregates are oligomeric complexes formed by non-native components and interactions among their structure that leads to trapped intermediates related to protein folding or assembly. They tend to be insoluble and stable under physiological conditions. They can as well be observed with a structured morphology or being amorphous. Aggregation occurs when there is partial unfolding, caused by oxidative, thermal, or viral stress, or when there is an alteration in the protein's primary structure, caused by mutation, RNA modification, or translational misincorporation^{124,125}.

Initially, it was thought that protein aggregation was disorganized and random. Further analysis in bacteria has observed that defective proteins slowly started to polymerize and formed seeded foci that deposit all these defective proteins. This resulted in a protein aggregate deposit called inclusion bodies^{126,127}. However, in mammalian cells is more complicated than that, because some studies have found that cells used the microtubule machinery for the sequestration of protein aggregates within the cytoplasm. They have shown that the depolymerization of microtubules did not disperse the aggresomes conformation but instead blocked aggresome formation^{128,129}. Or in another study, Garcia-Mata has observed, using video time-lapse microscopy, the small protein aggregates delivered to the aggresomes¹²⁸. With these findings, they proposed a new theory suggesting that aggresomes or inclusion bodies are formed after the delivery of protein aggregates by the microtubule machinery¹²⁴.

- Poly-glutamine (Poly-Q) aggregates

Consecutive expansion of the trinucleotide CAG repeats encoding polyglutamine (PolyQ) is found in more than 60 human proteins¹³⁰. An study have found that PolyQ domains contribute to the maintenance of protein interactions¹³¹. The PolyQ, when is expressed in the coiled-coil region will expand to facilitate the interaction with another

coiled-coil region in another protein^{131–133}. It is believed that the continued expansion of PolyQ will trigger misleading interactions and result in pathological effects such as aggregation. PolyQ diseases such as Spinocerebellar Ataxia, Dentatorubral Pallidoluysian atrophy, Spinobulbar muscular atrophy, and Huntington's have been related to different causes, but all of them have in common that each pathogenic protein in each disease has more than 40 CAG repeats¹³⁴. Previous studies have related autophagy as a mechanism of quality control of these abnormal PolyQ proteins. For example, it has been shown that autophagy inhibition by 3-MA drug induces the accumulation of N-termini Huntingtin protein bearing different long of PolyQ sequences¹³⁵.

VII.B The Epstein-Barr virus-encoded EBNA1

The Epstein-Barr virus (EBV) belongs to the herpesvirus family. Most world's population is EBV infected, between 90 to 95%, with no apparent illness. However, sometimes EBV infection can cause diseases such as infectious mononucleosis (IM) characterized by an excessive immune response^{136–138}.

The EBV infects B lymphocytes and some epithelial cells. Infection of B cells with EBV has been associated with the development of different lymphoproliferative disorders such as Burkitt's lymphoma, Hodgkin lymphomas, and other related lymphomas in immunocompromised patients^{136–139}.

Among the proteins coded by this virus, the EBV nuclear antigen 1 (EBNA1) has been found in the majority of all tumors positive for EBV¹⁴⁰.

EBNA1 escapes immune surveillance

Even though EBNA1 protein is always expressed, it manages to escape immune surveillance. This is due to the low antigen presentation of EBNA1-derived peptides on MHC-I molecules. Several studies have related the domain glycine-alanine repeat (GAR) of EBNA1 with the absence of CD8⁺ T cells response. For example, one study has observed that individual CD8⁺ T cells clones from EBV seropositive donors had antigen-specific cytotoxicity in cells expressing EBNA1 lacking GAR but not in cells expressing endogenously the full-length^{140,141}. In another study, Levitskaya and colleagues observed that the fusion of the GAR domain to another EBV protein (EBNA4) also inhibited antigen presentation to CD8⁺ T cells^{140,142}.

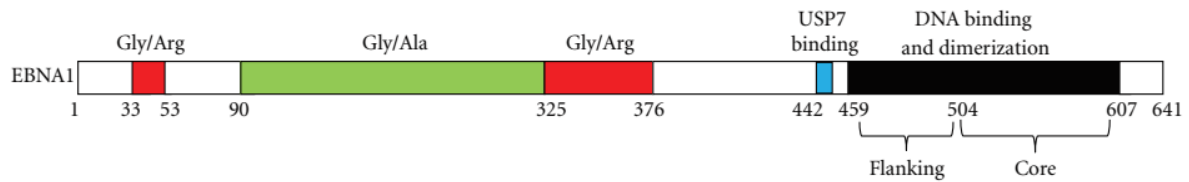


Figure 11. EBNA1 protein sequence arrangement. Amino acids numbers are shown below¹⁴³ (Image taken from Frappier et al 2012)¹⁴⁰.

Two years after the same group tried to explain how the GAR inhibits EBNA1 antigen presentation on the MHC class I pathway. They evaluated antigenic peptide production by proteasome degradation through ATP addition of cells either expressing EBNA4, GAR fused to EBNA4 and EBNA1. Upon ATP addition protein levels decrease only in EBNA4 but not in EBNA1 or GAR fused to EBNA4. Claiming with this result that GAR inhibits proteasome function¹⁴⁴. However, other studies demonstrated that the GAR on its own does not explain the low EBNA1 turnover in cells¹⁴⁵.

These observations did not explain GAR association with the MHC-I antigen presentation. Years later, research developed in our lab found that the GAR mediates suppression of antigenic peptides for the MHC class I pathway by inhibiting EBNA1 mRNA translation *in cis*¹⁴⁶.

The GAR domain of EBNA1

- Translation inhibition

Research carried out in the lab has shown that GAR peptide length and the position of GAR fusion to ovalbumin controlled synthesis and antigenic peptides production¹⁴⁷. Kinetic pulse-chase experiments and analysis of the ribosomal profile of GAR-ovalbumin expressing cells revealed that GAR targets the initiation step of translation¹⁴⁷. This result was then confirmed when they introduced a sequence known to allow alternative cap-independent mechanisms of mRNA translation, the *c-myc* IRES, in the 5' UTR of the GAR-ovalbumin. They observed that GAR-ovalbumin fused to *c-myc* IRES increased protein levels and antigen presentation, suggesting that the GAR inhibition mechanism targets only some translation initiation mechanisms¹⁴⁸.

Proteins binding RNA structures affect gene expression. Among the secondary structures, G-quadruplex (G4) are non-canonical nucleic acid structures formed by the stacking of several G-quartets. These structures are planar arrangements of four guanines connected by Hoogsten hydrogen bonds. When these RNA G4 structures

are found in the 5' UTR of the mRNA has been associated with translation suppression^{149–151}.

GAr domain in EBNA1 encodes a guanine-rich sequence that forms G4 structures. Research studying G4 structures formed by GAr has demonstrated that destabilizing the RNA G4, through mutation insertion or using oligos in vitro, prevented inhibition of antigen presentation and increased mRNA translation^{152,153}.

Further studies uncovered that the multifunctional protein Nucleolin interacts with the GAr-encoding G4 RNA structures and that this interaction mediates the repression of translation and antigen presentation. Nucleolin overexpression enhanced GAr mediated inhibition of EBNA1 protein expression and treatment with a G4 ligand competitor of Nucleolin, PhenDC3, prevented Nucleolin binding and reversed the GAr inhibition of translation and antigen presentation¹⁵⁴.

- GAr prone to form aggregates

It is reported that EBNA1 exists as an aggregate. Luka and colleagues observed protein aggregation of EBNA1 during the purification of this protein from human lymphoid cell lines¹⁵⁵. Another study states that EBNA1 has a similar coding sequence to the silk protein. This study proposed that EBNA1 protein might have some β -sheets structure as the silk protein, but also aggregation structure due to the irregular periodicity of the GAr copolymer domain¹⁵⁶.

- EBNA1 is presented in MHC-II molecule via autophagy

Previously the available evidence about EBNA1 antigen presentation suggested that only extracellular peptides could be presented to CD4⁺ T cells. However, Munz C. demonstrated that the nuclear EBNA1 derive peptides can also be presented in the MHC-II molecule and activated CD4⁺ T cells via autophagy. They observed that upon lysosomal inhibition, EBNA1 protein was accumulated in autophagosomes and more interestingly the knock down of atg12 decreases the MHC-II antigen presentation of EBNA1 derived peptides¹⁵⁷.

- EBNA1 nuclear location prevents autophagy processing to generate MHC class II restricted antigenic peptides

One more study assessed autophagy function in EBNA1-derived antigenic peptides for the MHC class II pathway. Leung C. et al 2009 used stable transfected lymphoblastoid cell lines expressing three epitopes in the EBNA1 sequence specific for CD4⁺ T cell clones. They observed that the MHC-II antigen presentation of one of the three epitopes was affected by autophagy inhibition induced by the 3-MA drug. Although, the relocation of the EBNA1 protein to the cytosol through mutation of the nuclear localization sequence made two out of three peptides affected by autophagy inhibition through 3-MA and atg7 siRNA. In parallel, they tested CD8⁺ T cell response to EBNA1 inserting the Human Papillomavirus peptide in the EBNA1 sequence. They observed no influence of autophagy inhibition in MHC class I antigen presentation, even with the relocation of this protein in the cytosol¹⁵⁸.

VIII. Aim of Ph.D. study – PART 1

Initially, autophagy was thought to be an unspecific degradative process, however, over time scientists have uncovered the selectivity of this pathway. Among the different intracellular constituents, aggregates are specifically degraded by autophagy. Currently, a growing body of studies is interested to know the different functionalities of autophagy in the steady-state of cells, and antigen presentation is one of them.

There is little evidence of autophagy processing in the MHC class I pathway and is unknown the role of the immune system to target and destroy aggregate-carrying cells.

The first part of my Ph.D. aimed to evaluate MHC class I antigenic peptides production from autophagy processing of different types of substrates.

- The EBNA1 viral protein is characterized by the GAR domain conferring an aggregate-prone protein conformation and to be autophagy processed, via Atg5/12, to produce antigenic peptides for the MHC class II pathway.
- Ovalbumin protein fused to aggregate-prone glutamine repetitions (OVA-PolyQ).

IX. Material and Methods – PART 1

Plasmids

The pCDNA3-EBNA1, pCDNA3-EBNA1ΔGAR, pCDNA3-Ovalbumin (OVA), pCDNA3-GAr-OVA, pCDNA3-*c-myc* GAr-OVA, GFP and GAr GFP constructs were obtained as described previously^{148,159} respectively.

c-myc EBNA1 and *c-myc* EBNA1ΔGAR were generated by amplification of full-length human *c-myc* by polymerase chain reaction (PCR), using a 5' sense primer containing a HindIII site 5' AATA**AAGCTT**CCACTGCTTACTGGCTTATCG 3' and a 3' antisense primer 5' TAA**AAGCTT**CGGCCGTTACTAGTGGATCC 3' containing another HindIII site. The fragment was cloned into the 5'UTR digested pCDNA3-EBNA1 and EBNA1ΔGAR constructs.

The OVA Poly 125 glutamine (Q) construct was made by digestion of OVA construct with EcoRI and XbaI enzyme and introducing 125 glutamine repetition sequence contained in a vector already mentioned previously¹⁴⁵.

Cell culture and Transfection

H1299 cells (Human non-small cell lung carcinoma) were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 1% Penicillin- Streptomycin and mouse cell atlas (MCA-205) cells were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate and 1% Penicillin-Streptomycin. For antigen presentation, western blot, and qRT-PCR experiments, cells were cultured in 6 wells plates (8×10^4 cells/well) at 37°C with 5% CO₂. The day after seeding and Atg5/12 siRNA induction, transfections were performed using 3 µl of Gene Juice reagent according to the manufacturer's protocol (Merck Bioscience). Cells were co-transfected with 0.5 µg of murine MHC class I molecule Kb and 1 µg of EBNA1, EBNA1ΔGAR, GAr-OVA, and PolyQ-OVA cDNA carrying the SIINFEKL (SL8) epitope coding sequences in its open reading frame (ORF). In all antigen presentation assays, 1 µg of an OVA cDNA was used as positive control and the same quantity for the empty vector as a negative control.

siRNA against Atg5/12

The day after seeding, cells were transfected with Human siRNAs or Murine siRNAs at 20 pM using Jet Prime reagent (Polyplus) following the manufacturer's instructions.

Material and Methods – PART 1

The knock down of these proteins was evaluated by Real-time PCR (qRT-PCR) and Western Blot at the end of 72 hours of incubation and 120 hours.

Human siRNAs used were: two siRNAs against Atg12 (SI02655289 and SI04335513, Qiagen) and three siRNAs against Atg5 (SI02655310, SI02633946, and SI00069251, Qiagen).

Murine siRNAs used were: two siRNAs against Atg12 (SI00900319|S0 and SI00900333|S0, Qiagen) and three siRNAs against Atg5 (SI02696806|S0, SI02720186|S0, and SI02745435|S0, Qiagen).

Autophagy drug modulator treatment

The day after seeding, cells were treated with Chloroquine at [30µM] for 36 hours. The autophagy inhibition was evaluated by Western Blot assessing LC3-II accumulation.

Proteasome drug modulator treatment

The day after seeding, cells were treated with Epoxomicin at [20µM] for 6 hours. The proteasome inhibition was evaluated by Western Blot assessing P21 accumulation.

Antigen Presentation assay: OT1 CD8⁺ T cells proliferation

To determine the levels of antigen presentation, we used CD8⁺ T cells that express specific receptors to the OVA epitope, SIINFEKL, recognized by H-2 Kb. These CD8⁺ T cells were purified from OT1 transgenic mice expressing a transgenic TCR specific for SIINFEKL-Kb. Spleen and lymph nodes from OT1 transgenic mice were passed through a 70 µm cell strainer and red blood cells were lysed with ACK buffer treatment for 5 minutes. After several washes with PBS-FBS 5%, CD8⁺ T cells were negatively selected using a CD8⁺ T cell isolation kit (MACS Miltenyi Biotec) according to the manufacturer's instructions. Afterward, the CD8⁺ T cells were stained with CellTrace™ Violet at 5µM for 10 minutes (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

Two days after transfection, H1299 cells used as presenting cells were briefly washed with PBS, trypsinized, resuspended in splenocytes medium (RPMI-1640), supplemented with 10% (FBS), 4mM L-glutamine, 1% Penicillin-Streptomycin, 0.05 mM 2-Mercaptoethanol, and 5 mM HEPES) and seeded in 48 wells plates (1.25×10^5) cells per well. Except for the antigen presentation upon proteasome inhibition, H1299

cells were fixated with 0.1% v/v glutaraldehyde during 20 s and 0.2 M of glycine was used to block fixation. Then, cells were washed three times with PBS and seeded also in 48 wells plates (1.25×10^5) in Splenocytes medium. Then, 5×10^5 CellTrace™ labelled T-cells were added per well and the co-cultures were incubated at 37°C with 5% CO₂. The levels of antigen presentation were deduced from the percentage of T-cell proliferation verified by flow cytometry.

Flow Cytometry analysis: OT1 CD8⁺ T cells proliferation

After 3 days, cells were harvested, stained with anti-mouse CD45.2-PE-Cy7 (BD Pharmingen), fixable viability dye eFluor® 506 (eBioscience, USA), and analyzed on a CANTO II flow cytometer (BD Biosciences, USA). Cells were gated for live CD45.2+ cells (4×10^5 events collected) and data were analyzed using FlowJo software version 8 (Tree Star). The percentage of live CD8⁺ T cells in each generation was calculated using the FlowJo proliferation platform and this value was considered for statistical analysis.

Antigen Presentation assay: Direct measurement in the presenting cells

H1299 cells co-expressing murine MHC I Kb and the constructs mentioned above were submitted to Chloroquine treatment. Then, cells were harvested and stained with APC anti-mouse H-2 Kb bound to SIINFEKL Antibody (Biolegend) and Fixable viability 506 (eBioscience, USA). These cells were analyzed on a CANTO II flow cytometer (BD Biosciences, USA) and were gated for live cells. Data were analyzed using FlowJo software version 8 (Tree Star).

Direct measurement of MHC I Kb and HLA-ABC

MCA-205 and H1299 cells were submitted to murine or human Atg5/12 siRNA transfection. Then, cells were harvested and stained. MCA-205 cells with anti-mouse H-2 Kb Antibody (Biolegend) and FITC anti-mouse IgG2a Antibody (Biolegend); H1299 cells with HLA-ABC FITC antibody (Invitrogen). Both cell types were also stained with Fixable viability 780 (eBioscience, USA). These cells were analyzed on a CANTO II flow cytometer (BD Biosciences, USA) and were gated for live cells. Data were analyzed using FlowJo software version 8 (Tree Star).

RNA extraction, RT-PCR and qRT-PCR

At 72 hours post siRNA Atg5/12 transfection, H1299 cells were washed with PBS, and RNA was purified using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. cDNA synthesis was carried out using M-MLV reverse transcriptase and oligo(dT) primers (Invitrogen). For qRT-PCR, the StepOne (Applied BioSystems) real-time PCR system was used, and the reactions were performed with the Perfecta SYBR green Fast mix ROX (Quanta) using specific primer pairs for human Atg5 (Forward: 5' GCTGCAGATGGACAGTTGCA 3' and Reverse: 3' TGTTCACTCAGCCACTGCAG 5'), human Atg12 (Forward: 5' ATGACTAGCCGGGAACACCA 3' and Reverse: 3' CACGCCTGAGACTTGCAGTA 5'), murine Atg5 (Forward: 5' TGTGCTTCGAGATGTGTGGTT 3' and Reverse: 3' GGTCCCCTTTGCACACTTACA 5') and murine Atg12 (Forward: 5' GCCATCTCACCAGCCCAATA 3' and Reverse: 3' CATGCCTGGGATTTGCAGT 5').

LC3-GFP induction

To confirm the blockage of autophagosome formation by Atg5 and Atg12 siRNA, we performed epifluorescence microscopy. For this, we seeded 1.5×10^4 H1299 cells in a 24 well plate over a sterile 22x22mm coverslip. Then, cells were transfected with 20 pM of siRNA against Atg5/12 and 0.1 μ g of an LC3-GFP construct at 24 and 48 hours after seeding, respectively. After 72 hours of culture, cells were treated with a starvation buffer described elsewhere¹⁶⁰ (140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 20 mM Hepes, pH 7.4) for 2 hours and a complete RPMI-1640 medium was used for the negative control cells. Images were taken at 63x using the Axio Imager D2 microscope. All images were analyzed in Fiji software and the number of green dots was calculated as previously described¹⁵¹.

Western Blot

Cells were trypsinised and the obtained pellets were resuspended with 50 μ l of lysis buffer (20 mM HEPES KOH, 50mM β -Glycerol phosphate, 1mM EDTA, 1mM EGTA, 0.5mM Na₃VO₄, 100 mM KCL, 10% Glycerol and 1% Triton x-100, protease inhibitor cocktail Roche). Total lysates were obtained after the mechanic hitting and freezing at -80°C for at least 2 h. After, samples were centrifuged at 13 000 RCF for 10 min at 4°C, and supernatants were collected. Samples were quantified using Bradford Reagent (BioRad) and 50 μ g of protein were separated on 4-12% SDS-PAGE gels (Thermo Fisher Scientific) and transferred to nitrocellulose blotting membranes (Pall

Material and Methods – PART 1

Corporation). After saturation of membranes with TBS- 0.5% Tween containing 5% non-fat milk, membranes were overnight incubated with anti-EBNA1 (16216-1-AP Abnova), anti-Atg12 (R&D systems), anti-chicken egg albumin (C6534 Sigma), anti-LC3B (L75443 Sigma), anti-GFP (11814460001 Roche), anti-P21 (cell signaling) and anti-actin (AC-15 Sigma) antibodies. After washing with TBS-Tween, bound antibodies were detected using a rabbit anti-mouse (Dako) or a mouse anti-rabbit (Dako) secondary antibody conjugated to horseradish peroxidase (1:1000; 1 h at room temperature). Immunocomplexes were then revealed with ECL (Thermo Scientific) and imaged using a MyECL Imager (Thermo Scientific).

Immunofluorescence

H1299 cells were seeded as described for LC3-GFP induction experiments and transfected with 0.8 μ g of EBNA1, EBNA1 Δ GAr, GAr-OVA, EBNA1 *c-myc*, EBNA1 Δ GAr *c-myc*, GAr-OVA *c-myc*, OVA, PolyQ-OVA or empty vector. Samples were fixed with 4% paraformaldehyde and permeabilized with 0.4% Triton x-100 0.05% CHAPS PBS. Afterward, cells were blocked with 3% Bovine serum albumin (BSA) Saponin 0.1% PBS for 1 hour and then incubated with mouse anti-EBNA1 (16216-1-AP Abnova) or rabbit anti-egg albumin (C6534 Sigma) for 1 hour at room temperature. After two washes with PBS, samples were incubated with an anti-mouse Alexa 488 or anti-rabbit Alexa 647 antibodies for 1 hour at room temperature. Next, samples were washed with PBS, stained with DAPI, and mounted with a fluorescence mounting media (Dako). Samples were examined in an LSM 800 confocal laser microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and images were treated using the Fiji software.

Statistics

Data were analyzed by two-tailed unpaired Student's t-test or One sample T-test using GraphPad Prism 6 for Windows (GraphPad Software). Data shown are mean \pm sd. of minimum three independent experiments. *P < 0.0332; **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0,1234 ns, not significant.

X. Results - PART 1

Transfection efficiency

The research developed during this Ph.D. is based on transient lipid transfection of different cDNA constructs or siRNA. I have tested the transfection efficiency of the Gene Juice reagent into H1299 cells. Two different GFP cDNA constructs were transfected at [1 μ g] and 48 hours later harvested cells were quantified by flow cytometry. We observed that percentage of GFP expressing cells in both transfections was between 31% to 48% (**Fig. 12**). Furthermore, no cell toxicity was observed (data not shown) why this approach was chosen.

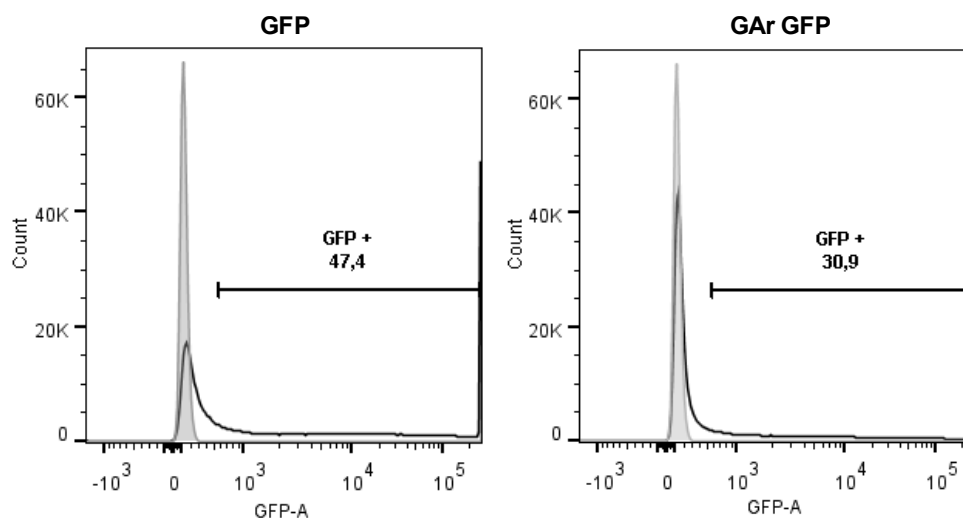


Figure 12. Quantification of transfection efficiency is around 31 to 50%. H1299 cells were transfected with 1 μ g of GFP or GAr GFP cDNA constructs. After 48 hours cells were harvested and quantified by Flow Cytometry. Gray histogram shows cells expressing empty vectors (not fluorescent cells) and open histograms of the corresponding cDNA constructs.

Knocking down Atg5 and Atg12 blocks autophagy in H1299 cells

To evaluate the role of autophagy in antigen presentation to the MHC class I pathway, we knocked down the expression of Atg5 and Atg12 using specific siRNAs. These proteins are crucial for the conjugation system that allows the formation of autophagosomes and their downregulation is reported to block the autophagy pathway^{109,157,161}. The efficiency of siRNA treatments was confirmed by the downregulation of Atg5/12 mRNA (**Fig. 13A**) and protein levels (**Fig. 13B, upper lane**). siRNA treatments resulted in a decreased ratio of LC3 II-I (**Fig. 13B, middle lane**) and suppressed autophagy flux following serum starvation (**Fig. 13C, upper part**). Of note, LC3-GFP protein levels did not change under serum starvation (**Fig. 13C, bottom part**). Together, these data show that the siRNA against Atg5/12 interferes with the autophagy pathway in H1299 cells.

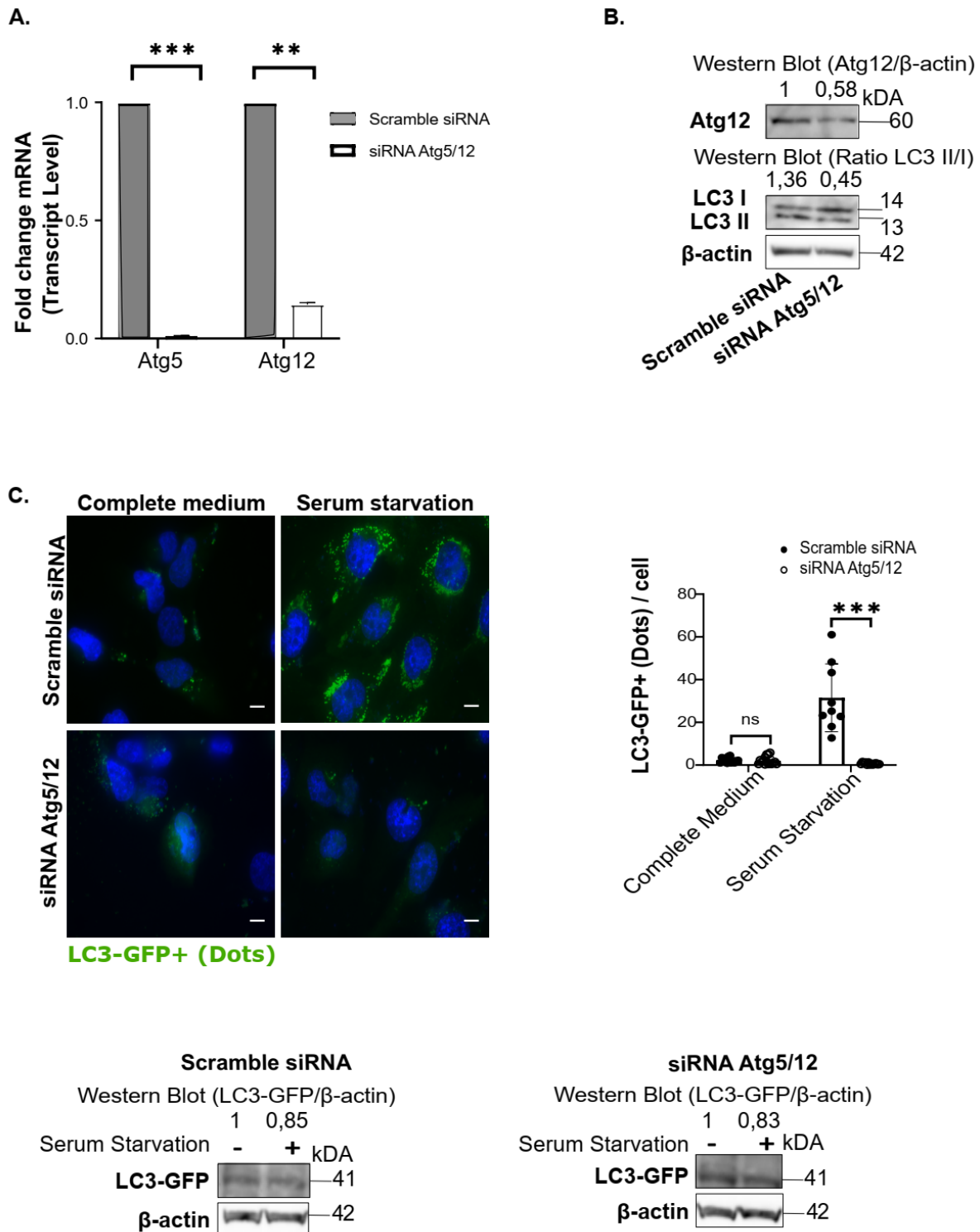


Figure 13. Autophagy Inhibition. **A.** The Atg5/12 mRNA levels were confirmed using RT-qPCR seventy-two hours following transfection of [20 pM] human siRNA against Atg5/12 or scramble siRNA **B.** Western Blots depicts the expression of Atg12, LC3 I, and LC3 II. Values above the bands show the densitometry analysis normalized against β -actin and the fold change compared with the scramble siRNA. Autophagy suppression was estimated by the ratio between LC3 II and LC3 I **C.** H1299 cells were transfected with a plasmid encoding LC3-GFP 24 hours after treatment with siRNAs as in A. 48 hours later, cells were treated without serum for two hours, and then fixed. One representative image is shown from the three independent

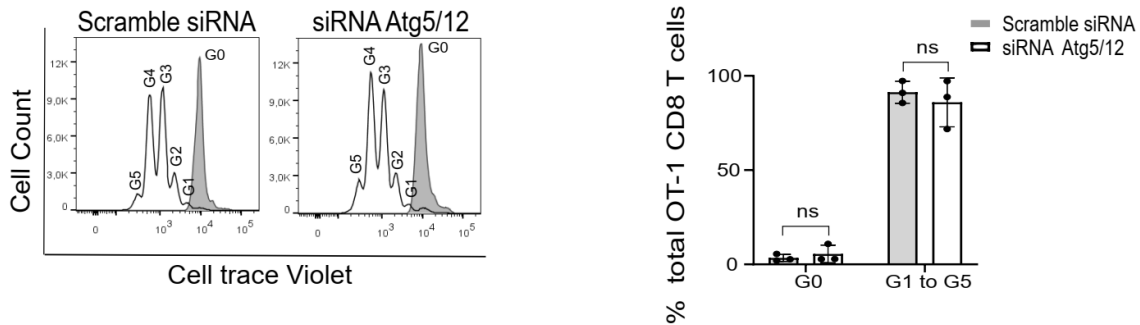
experiments performed. LC3-GFP fluorescence was observed as green dots, indicating autophagosome formation. The number of GFP dots was quantified (top right graph). LC3-GFP expression was determined by Western Blot (bottom panels). Values above the bands show the densitometry analysis of bands normalized with β -actin and the fold change comparing the complete medium with the serum starvation treatment. Significant values were calculated using Multiple paired groups T-test. ***P < 0.0002; **P < 0.0021; 0,1234 ns, not significant. White scale bars denote 10 μ m.

Atg5 and Atg12 knock down does not affect MHC class I cell surface expression

Previous studies have found that dendritic cells impaired autophagy via siRNA knock down of Atg5 and Atg7 proteins increased MHC class I cell surface localization. Loi and colleagues uncovered that these two proteins mediate the endocytosis of the MHC I molecule from the cell surface and its later degradation through autophagy¹²².

To further pursue our antigen presentation research, first, we needed to evaluate the influence of Atg5/12 knock down on MHC class I cell surface localization. Our research in the MHC class I pathway has been based on the use of the SIINFEKL (SL8) immune epitope from chicken ovalbumin. This peptide is specifically recognized by CD8⁺ T-cells derived from transgenic OT-1 mice in the context of the murine (Kb) MHC class I molecule. We have transfected murine MHC-I (Kb) into human H1299 cells. Transfected cells were subjected to autophagy inhibition through Atg5/12 siRNA treatment. Later, these presenting cells were co-cultured with OT1 CD8⁺ T cells and free SL8 peptide [1 μ g/ml]. The relative level of antigen presentation was estimated by OT-1 CD8⁺ T-cells proliferation using flow cytometry in response to the free SL8 bound to MHC-I (Kb) on the surface of the APCs. We observed no effect in OT-1 CD8⁺ T cells proliferation upon Atg5/12 knock down compared to the scrambled control cells, showing that Atg5/12 down-regulation does not affect the MHC class I pathway per se (**Figure 14A**). Moreover, we analyzed the changes in the membrane localization of endogenous MHC-I (Kb) molecules in murine MCA-205 cells or MHC-I (HLA-ABC) molecules in human H1299 cells and we observed no difference in MHC class I cell surface localization (**Figure 14B, left panels**). Of note, murine Atg5/12 knock down showed the same marked downregulation as the Human Atg5/12 siRNA (**Figure 14B, right graphs**).

A.



B.

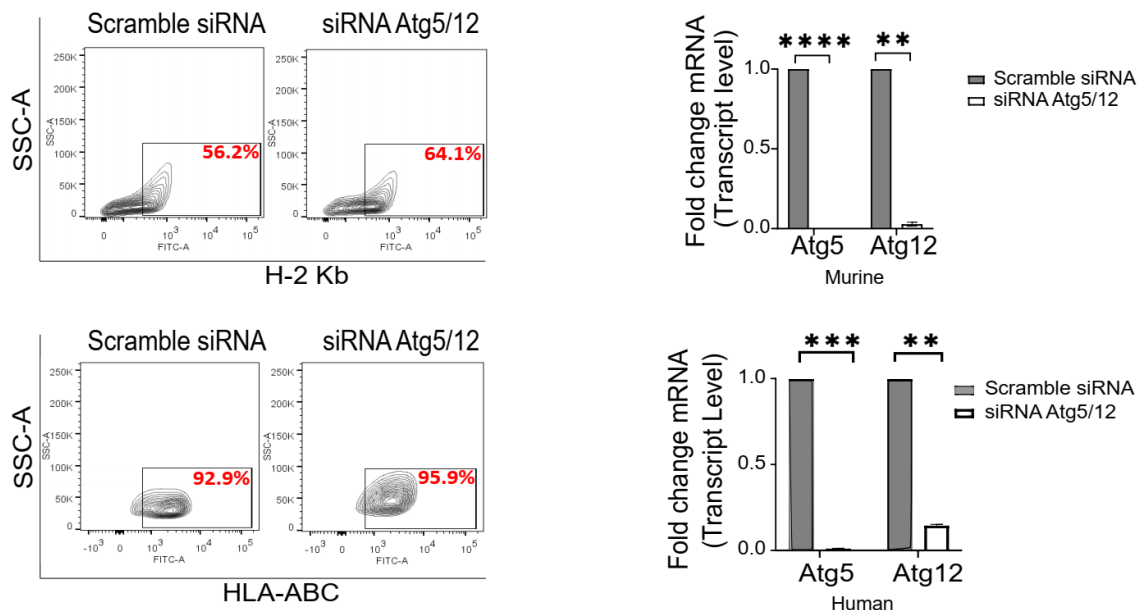


Figure 14. Presentation of exogenous SL8 peptide and membrane location of MHC Class I molecule is not affected by Atg5/12 knock down. **A.** H1299 cells co-expressing murine MHC-I (Kb) were transfected with human siRNA Atg5/12 [20 pM] or scramble siRNA for 72 hours. Next, three days after these presenting cells were co-incubated with OT-1 CD8⁺ T cells labeled with Cell-trace violet and free SL8 peptide [1ug/ml]. The levels of OT-1 CD8⁺ T cells proliferation were analyzed by Flow Cytometry. Open peaks in the histogram represent the proliferating populations and grey peaks denote unstimulated populations (Empty Vector transfected cells) (Left panel). The percentage of total OT1 CD8⁺ T cells was calculated using the number of cells in each generation generated by the modeling of the Proliferation tool in Flow Jo software. The graph shows the sum of the percentage of cells from generation 1 to 5 compared with the percentage of non-divided cells (generation 0) from 3 independent experiments. **B.** MCA-205 and H1299 cells were transfected with murine/human siRNA Atg5/12 [20 pM] or scramble siRNA respectively for 72 hours. HLA-ABC FITC or H-2 Kb FITC antibodies were used to measure MHC class I molecules in live cells by Flow Cytometry. Murine or human Atg5/12 siRNA knock down was confirmed by qRT-PCR. Significant values were calculated using Multiple paired groups T-test. **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0,1234 ns, not significant.

Preventing autophagy reduces MHC class I antigen presentation independently of protein aggregate formation

Autophagy can degrade harmful cytosolic proteins, including aggregates^{71,87,88}. We tested the capability of this pathway to process protein substrates for the MHC-I pathway. We used a chicken OVA construct whose secretion is blocked by the deletion of the first 50 amino acids¹⁶². This construct enabled us to study the antigen presentation via the MHC-I pathway using OT-1 CD8⁺ T cells. We also fused a poly-glutamine repetition (PolyQ), known to cause aggregates and to be processed by autophagy^{163–165}, to OVA (**Fig. 15A**). Immunohistochemistry assays using anti-OVA antibodies showed that PolyQ-OVA forms approximately 10 aggregates per cell (white arrowheads) while OVA was uniformly stained throughout the cells and no visible aggregate was detected (**Fig. 15B**). Expression of the reporter constructs was not significantly affected by siRNA against ATG5/12 (**Fig. 15C and annexes suppl. Fig. 1**). We did not detect any accumulation of PolyQ-OVA upon ATG5/12 knock down, presumably because the PolyQ-OVA is not only present in the aggregate conformation (**Fig. 15B**) due to the limited time (24 hours) of expression. To test the role of autophagy in the processing of antigenic peptide substrates for the MHC class I pathway, we co-expressed the indicated SL8-carrying constructs together with the (Kb) MHC cDNA in human H1299 cells. Transfected cells were subject to autophagy inhibition through Atg5/12 siRNA treatment and antigen presentation was evaluated by co-culture with OT1 CD8⁺ T-cells. The relative level of antigen presentation was estimated by OT1 CD8⁺ T-cells proliferation using flow cytometry. For every assay, we confirmed suppression of autophagy by, in parallel estimating the LC3 I/II ratio (Fig. 13B and data not shown). We observed that under Atg5/12 knock down, OVA and PolyQ-OVA showed a higher percentage of cells in the non-proliferating OT1 CD8⁺ T cell population (G0) and a corresponding decrease in the proliferating population (G1 to G5), indicating a reduction of antigen presentation (**Fig.15D**). The percentage of OT1 CD8⁺ T cells in each generation is shown in (**Annexes suppl. Fig. 2**). Despite being uniformly expressed and showing no apparent formation of aggregates, it was surprising to see that knocking down Atg5/12 affected the presentation of antigenic peptides from OVA as much as from PolyQ-OVA.

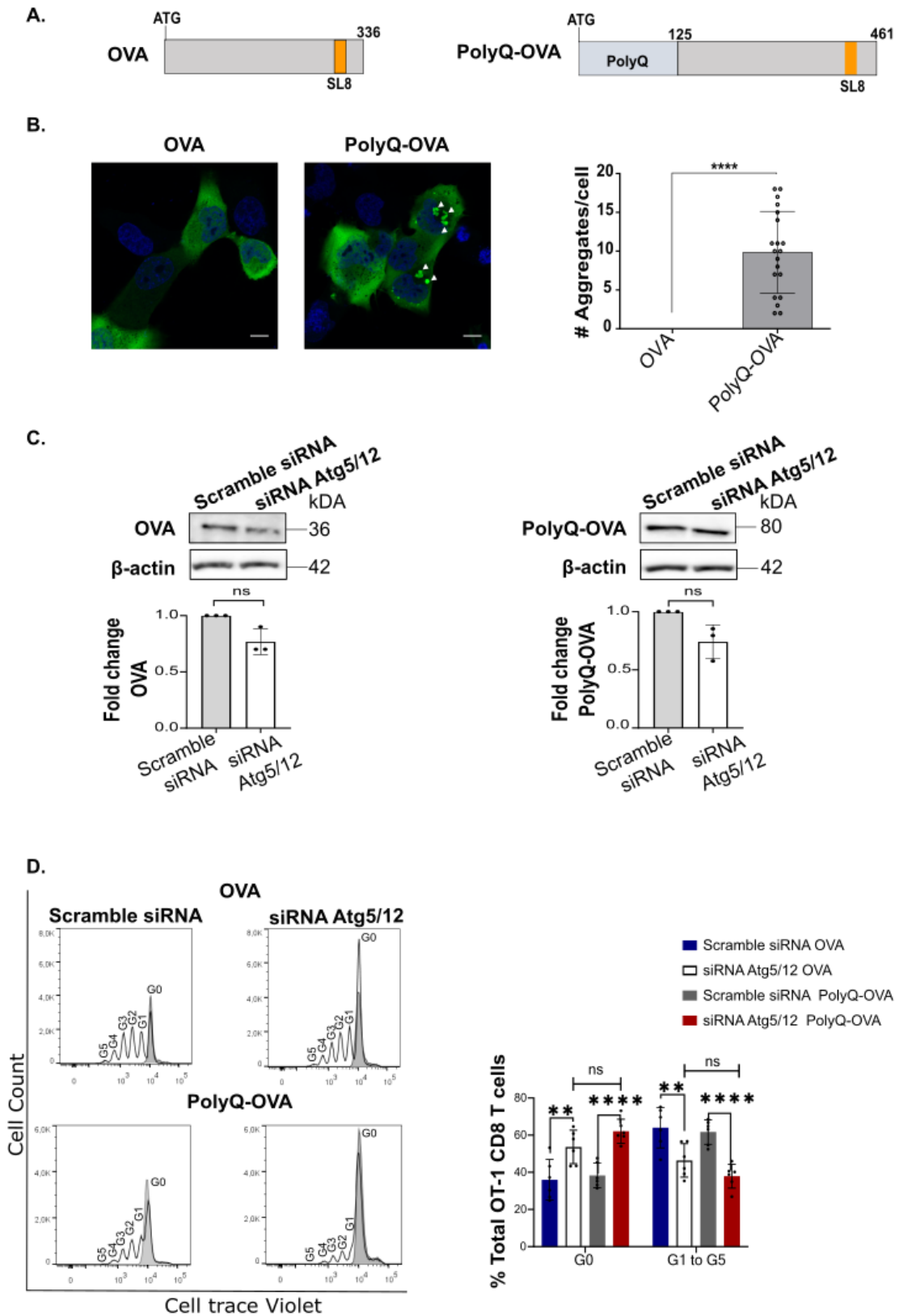


Figure 15. Autophagy affects antigen presentation of Ovalbumin and Ovalbumin fused to the polyglutamine peptide **A.** Cartoon illustrating chicken ovalbumin (OVA) sequence with the location of the immune peptide SL8 and the glutamine repeat (PolyQ) **B.** Representative

immunofluorescence image of OVA and PolyQ-OVA. White arrow heads indicate an aggregation pattern. The graph shows the average number of aggregates observed **C**. Western Blot represents the effect of 72 hours of Atg5/12 human siRNA transfection on OVA and PolyQ-OVA expression. The graphs below show the densitometry analysis, normalized against β -actin and expressed in fold change compared with the scramble siRNA **D**. H1299 were transfected with human siRNA Atg5/12 [20 pM] or scrambled siRNA. 24 hours later they were transfected with murine MHC-I (Kb) and indicated constructs. After 48 hours they were incubated with OT-1 CD8⁺ T cells labeled with cell-trace violet for 3 days. OT-1 CD8⁺ T cell proliferation was analyzed by Flow Cytometry. A higher rate of proliferation indicates more antigen presentation. Open peaks in the histogram represent the proliferating populations and grey peaks denote unstimulated populations (Empty Vector transfected cells) (left panel). The graph shows the sum of the percentage of cells from generation 1 to 5 compared with the percentage of non-dividing cells (generation 0) from 6 independent experiments (right graph). Significant values were calculated using Multiple paired groups T-test. *P < 0.0332; **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0,1234 ns, not significant. White scale bars denote 10 μ m.

Proteasome affects the presentation of Ovalbumin derive antigenic peptides

The proteasome is known to be one of the main processing mechanisms that produce antigenic peptides in the MHC class I canonical pathway¹⁶⁶. As the proteasome can be another possible degradation machinery besides autophagy, we evaluated antigen presentation of OVA-derived peptides under proteasome function inhibition. H1299 cells co-expressing MHC-I (Kb) and OVA were treated with or without Epoxomicin (Epoxo.) for 6 hours. Next, harvested cells were fixed and co-culture with OT-1 CD8⁺ T cells. The levels of OT-1 CD8⁺ T cell proliferation were analyzed by Flow Cytometry. We demonstrated that OT-1 CD8⁺ T cells proliferation was significantly decreased in response to OVA antigenic peptide presentation upon Epoxo. treatment compared with DMSO (**Figure 16A**). In parallel, we observed an increase in the OVA protein levels during proteasome inhibition by western blot (**Figure 16B**). We also confirmed proteasome inhibition, as the specific proteasome target P21 cell cycle protein¹⁶⁷ was accumulated with this drug treatment (**Figure 16B**). This is in line with previous studies stating that OVA-derived antigenic peptides are degraded by the proteasome^{20,168}. The autophagy pathway, as well as the proteasome, could both tackle OVA protein because OVA is very susceptible to polyubiquitination and this tag signal is recognized by both mechanisms^{87,169}.

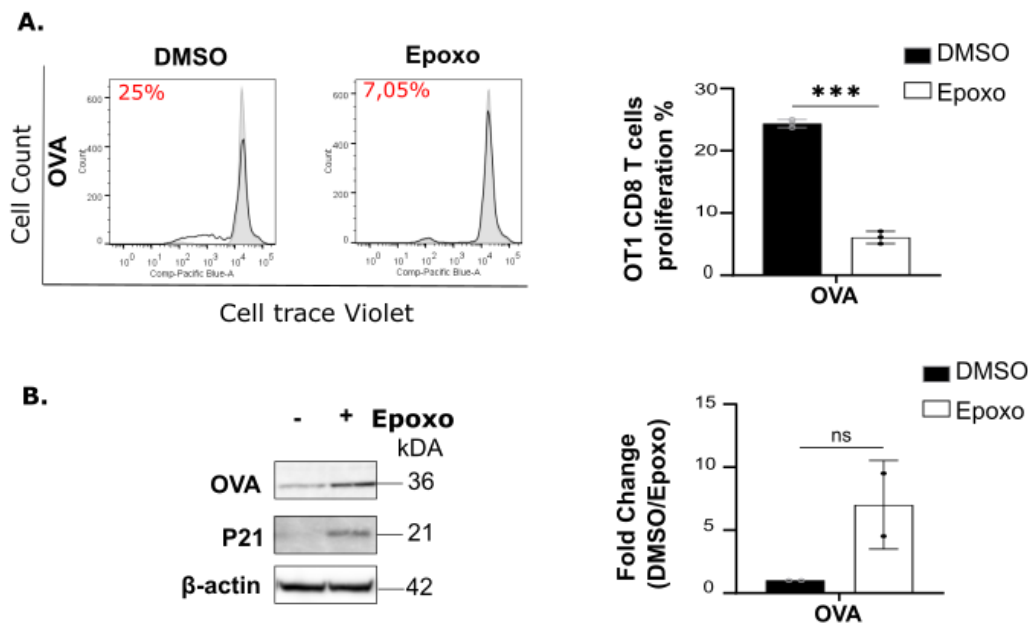


Figure 16. Proteasome also affects the presentation of Ovalbumin derived antigenic peptides. **A.** H1299 cells co-expressing MHC-I (Kb) and OVA were treated with Epoxomycin (Epoxo.) [20 μ M] or without (DMSO) for 6 hours. Then, these cells were fixated and co-culture with OT-1 CD8+ T cells labeled with Cell-trace violet for 3 days. The levels of OT-1 CD8+ T cells proliferation percentage were analyzed by Flow Cytometry. A higher rate of proliferation indicates more antigen presentation. Open peaks in the histogram represent the proliferating populations and grey peaks denote unstimulated populations (Empty Vector transfected cells) (left panel). The graph shows three independent experiments **B.** Western Blot showing OVA expression upon [20 μ M] Epoxo. treatment for 6 hours. P21 protein accumulation confirmed proteasome inhibition during Epoxo treatment. The graph shows the densitometry analysis, normalized against β -actin and expressed in fold change compared with DMSO. Significant values were calculated using Multiple paired groups T-test. ***P < 0.0002; 0,1234 ns, not significant.

MHC class I-restricted presentation of peptides derived from EBNA1 is not affected by the suppression of autophagy

The Epstein-Barr virus-encoded EBNA1 protein has been reported to be an aggregate-prone protein due to its long repeat of non-polar gly-ala residues (GAR) ^{155,156}. Since EBNA1-derived antigenic peptides are processed for the MHC class II pathway via autophagy ^{157 71,87,88}, we wanted to know if EBNA1-derived peptides can also be presented for the MHC class I pathway through autophagy. We inserted the antigenic SL8 peptide into the EBNA1 open reading frame (ORF), or in an EBNA1 depleted of the GAR-domain (EBNA1 Δ GAr). We also used a construct carrying the GAR-domain fused to OVA cDNA (GAR-OVA) (**Fig. 17A**). To test if EBNA1 shows the same

aggregation pattern observed for PolyQ-OVA, we performed immunohistochemistry assays. However, we observed no obvious aggregates of EBNA1, EBNA1ΔGAR, or GAR-OVA and no differences in subcellular localization with, or without, the GAR (**Fig.17B**). The GAR mediates suppression of antigenic peptides for the MHC class I pathway by inhibiting *EBNA1* mRNA translation *in cis*¹⁴⁶. In agreement with this, we observed a low percentage of CD8⁺ T cell proliferation in response to SL8 derived from EBNA1 and GAR-OVA, as compared to EBNAΔGAR (**Fig. 17C**) and OVA (**Fig.15D**). Importantly, we observed no significant difference between percentages of OT-1 CD8⁺ T cells in the undivided (G0) or the proliferating populations (G1 to G5), for any of the tested conditions following Atg5/12 siRNA treatment (**Fig. 17C and annexes suppl. Fig. 2B**). We also showed that Atg5/12 knock down had no significant effect on EBNA1, EBNA1ΔGAR, and GAR-OVA expression (**Fig. 17D and annexes suppl. Fig. 1**). These results support the idea that the autophagy pathway does not provide EBNA1-derived antigenic peptides for the class I pathway and that the fusion of the GAR prevents OVA from being presented via autophagy. Hence, EBNA1 can be processed by autophagy and presented to the class II pathway but not the class I pathway.

Drug inhibition of autophagy does not affect antigen presentation of EBNA1, EBNA1ΔGAR, or GAR-OVA in the MHC class I pathway

To confirm the absence of effect in the autophagy inhibition by Atg5/12 siRNA on EBNA1 derive antigenic peptides presentation, we treated H1299 cells co-expressing MHC I (Kb) and EBNA1, EBNA1ΔGAR, GAR-OVA with Chloroquine (CQ) for 36 hours. We evaluated antigen presentation directly in the presenting cells with the labeling of SL8 H-2 Kb. Cells having SL8 H-2 Kb on the cell surface were measured by Flow Cytometry using the APC anti-mouse H-2Kb bound to the SL8 antibody. We showed that autophagy inhibition with CQ treatment had no effect in presenting antigenic peptides derived from EBNA1, EBNA1ΔGAR, or GAR-OVA (**Fig. 18A**). Next, western blots showed that Chloroquine decreased EBNA1 protein levels and blocked autophagy efficiently, as LC3 II protein levels were accumulated compared with the control condition water (**Fig. 18B**). These observations support the data obtained using siRNA against Atg5/12 and that the GAR sequence prevents OVA from being presented to the class I pathway via autophagy.

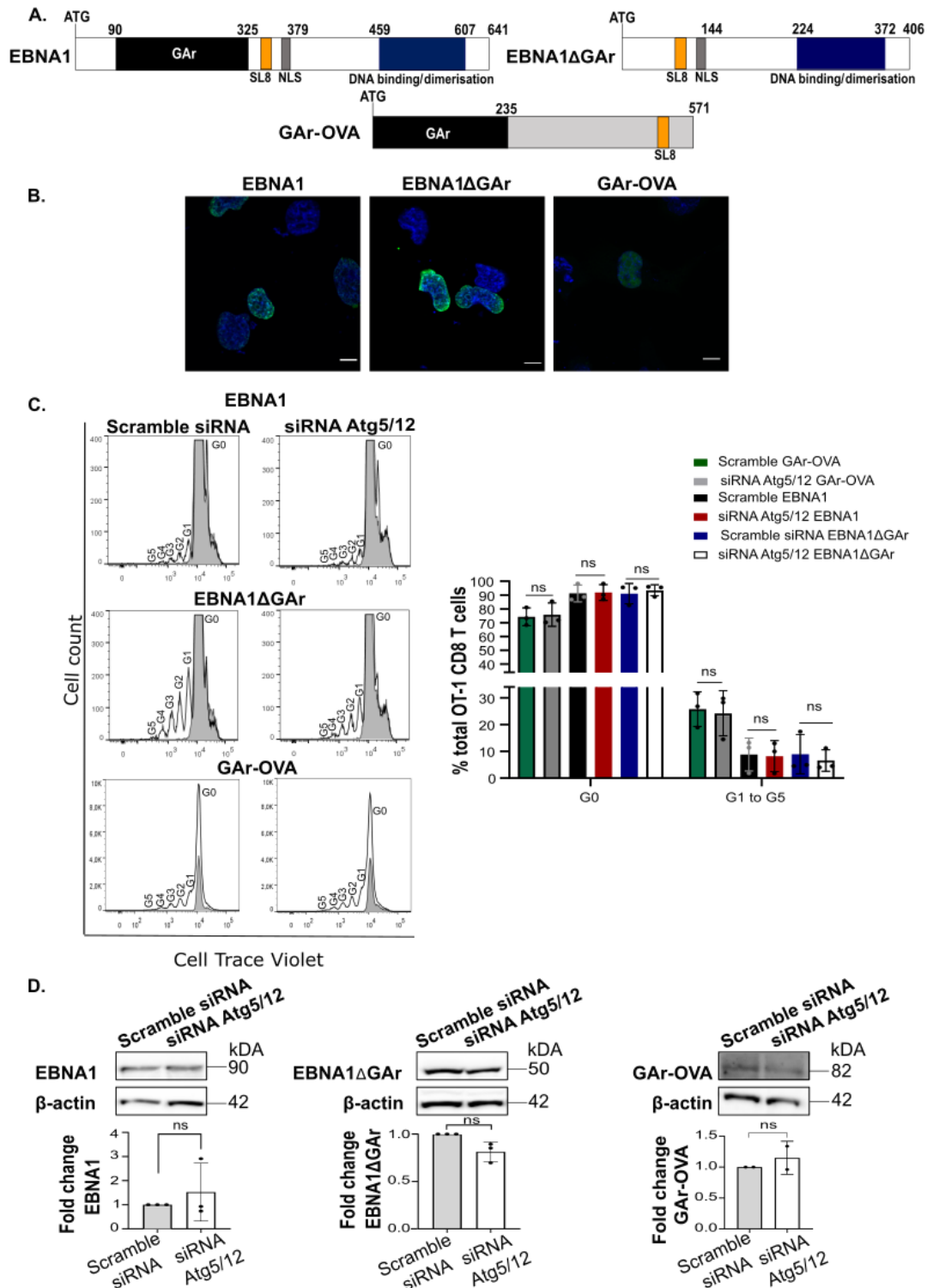


Figure 17. Fusion of the EBNA1-derived gly-ala repeat (GAR) sequence suppresses Atg5/12-dependent antigen presentation. **A.** Cartoon illustrating different EBNA1 constructs with, or without, the GAR (EBNA1ΔGAR) and GAR fused to Ovalbumin. The location of the nuclear localization signal (NLS), the DNA binding/dimerization sequence in EBNA1, and the SL8 epitope are indicated. **B.** Representative immunofluorescence image of EBNA1, EBNA1ΔGAR, and GAR-OVA. **C.** H1299 cells co-expressing murine MHC-I (Kb) and the indicated constructs were transfected with human siRNA Atg5/12 [20 pM] or scramble siRNA for 72 hours like in figure 17D. The graph shows the percentage of cells from generations 1 to 5 compared with

the percentage of non-divided cells (generation 0) from 3 independent experiments (right graph) **D**. Western Blots show one out of three representative experiments on the effect of autophagy inhibition on EBNA1, EBNA1ΔGAR, and GAR-OVA protein expression levels. The graphs show densitometry analysis normalized against β -actin and expressed in fold change compared with the scramble siRNA. Significant values were calculated using Multiple paired groups T-test. Not significant ns: 0,1234. White scale bars denote 10 μ m.

The level of protein expression does not determine MHC class I restricted antigen presentation via the autophagy pathway.

The above results (Fig.15 and Fig.17) were surprising considering that OVA alone, or OVA fused to the PolyQ, presents antigenic peptides in an Atg5/12-dependent fashion, while this antigen presentation pathway is prevented by the fusion of the GAR. We next set out to test if the effect of the GAR on antigen presentation via autophagy is associated with its effect on suppressing mRNA translation *in cis*. For this, we fused the *c-myc* 5'UTR to the 5' of the GAR-OVA (**Fig. 19A**). The presence of the *c-myc* sequence overcomes the translation inhibitory capacity of the GAR and restores protein synthesis without altering the coding sequence¹⁴⁸. Initially, we confirmed by western blot and immunofluorescence that the insertions of the *c-myc* sequence resulted in the expected increase in expression of GAR-OVA (**Fig. 19B**). When we compared antigen presentation we observed the expected significant increase in presentation from the *c-myc*-carrying GAR-OVA construct, as compared to GAR-OVA alone (**Fig. 19C**).

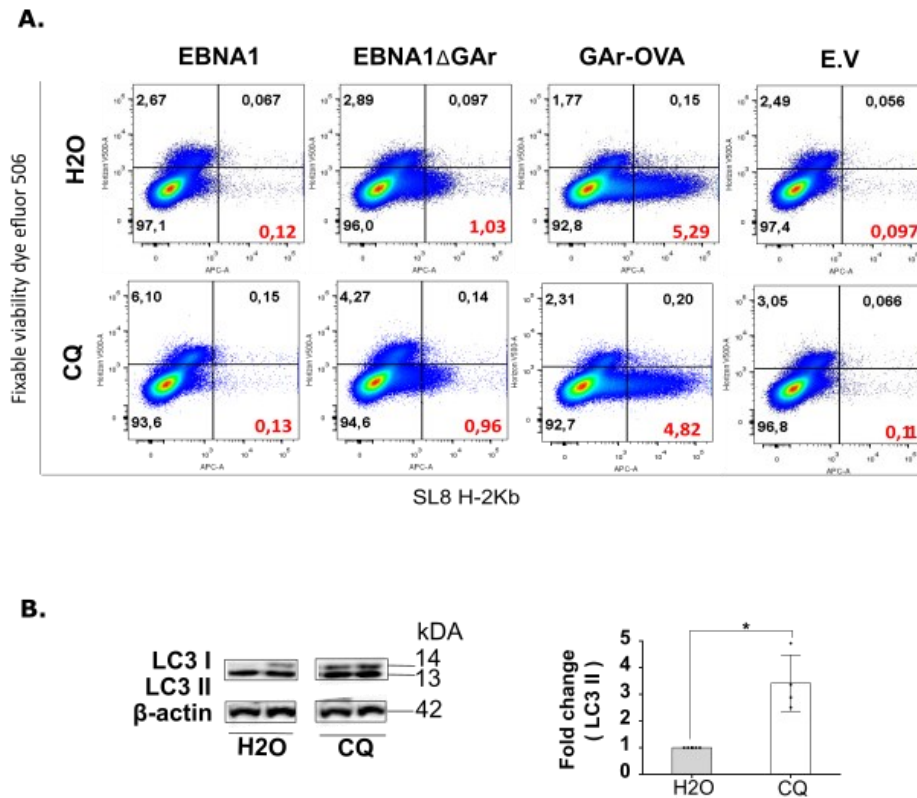


Figure 18. Chloroquine treatment does not affect EBNA1, EBNA1ΔGAR, or GAR-OVA antigen presentation **A.** H1299 cells co-expressing murine MHC-I (Kb) and EBNA1, EBNA1ΔGAR, GAR-OVA or E.V were treated with Chloroquine [30 μM] during 36 hours. Then, cells were harvested and labeled with SL8 H-2 Kb and fixable viability dye. Cells having SL8 H-2 Kb on the membrane were measured by Flow Cytometry. **B.** Western Blots show the effect of 36 hours of Chloroquine treatment at [30μM] on EBNA1 and EBNA1ΔGAR protein expression. Autophagy inhibition was confirmed by the accumulation of LC3 II. The graphs (Right side) show the densitometry analysis, normalized against β-actin and expressed in fold change compared with water. Significant values were calculated using Multiple paired groups T-test. *P < 0.0032; 0,1234 ns, no significant.

Once we verified that *c-myc* insertion increased GAR-OVA protein synthesis and GAR-dependent antigen presentation, we assessed if an increase in protein levels has an effect on the presentation of substrates to the class I pathway via autophagy. Additionally, to the fusion of *c-myc* 5' UTR to the 5' of the GAR-OVA, we also fused the *c-myc* to the 5' of the EBNA1 and EBNA1ΔGAR (**Fig. 20A**). Western blots showed that the insertion of the *c-myc* sequence resulted in the expected increase in expression of EBNA1 and GAR-OVA but not EBNAΔGAR (**Fig. 20B**). Immunofluorescence showed that *c-myc* insertion did not change the subcellular localization of EBNA1, EBNA1ΔGAR, and GAR-OVA proteins (**Fig. 20C**), and western blots showed that Atg5/12 knock down did not affect the expression of either construct (**Fig. 20D and**

annexes suppl. Fig.1). Next, we compared antigen presentation between the different constructs and we observed no significant difference in antigen presentation between *c-myc* carrying constructs following Atg5/12 knock down (Fig. 20E and annexes suppl. Fig.2). Taking together these results suggest that the levels of EBNA1 or GAR-Ova protein expression do not affect the autophagy-dependent presentation of antigenic peptides for the MHC class I pathway.

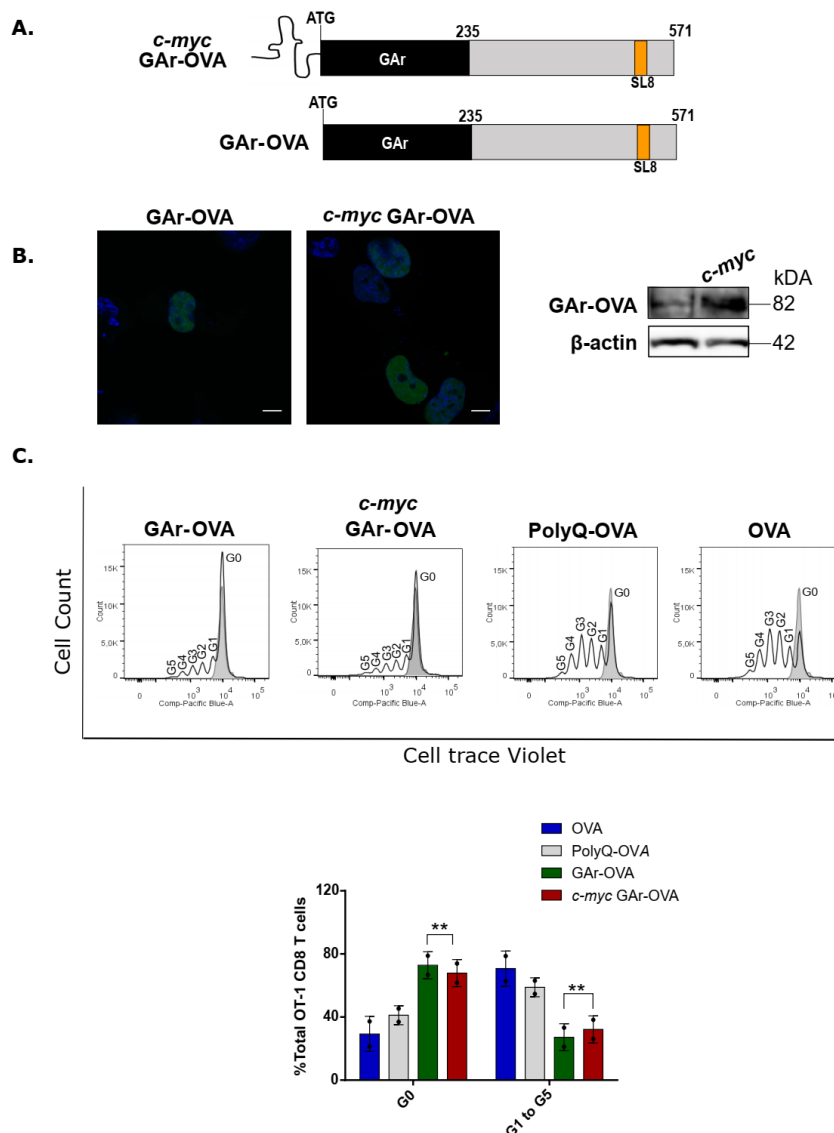


Figure 19. *c-myc* sequence increases protein expression of GAR-OVA and antigen presentation of GAR-OVA derive antigenic peptides. **A.** Cartoon illustrating the location of *c-myc* 5' UTR RNA sequence inserted in the 5'UTR of GAR-OVA and the unmodified GAR-OVA sequence. **B.** The *c-myc* fused to the 5' UTR of the GAR-OVA construct overcomes GAR-mediated mRNA translation suppression. Immunofluorescence and Western blot show the differences in protein expression levels. **C.** H1299 cells co-expressing murine MHC-I (Kb) and GAR-OVA, *c-myc* GAR-OVA, PolyQ-OVA, or OVA were co-cultured with OT1-CD8+ T cells for three days. OT1 CD8+ T cells proliferation was measured by Flow Cytometry. The graph shows the percentage of cells from generations 1 to 5 compared with the percentage of non-

divided cells (generation 0) from 2 independent experiments (bottom graphs). Significant values were calculated using Multiple paired groups T-test. *P < 0.0332; **P < 0.0021; 0,1234 ns, no significant.

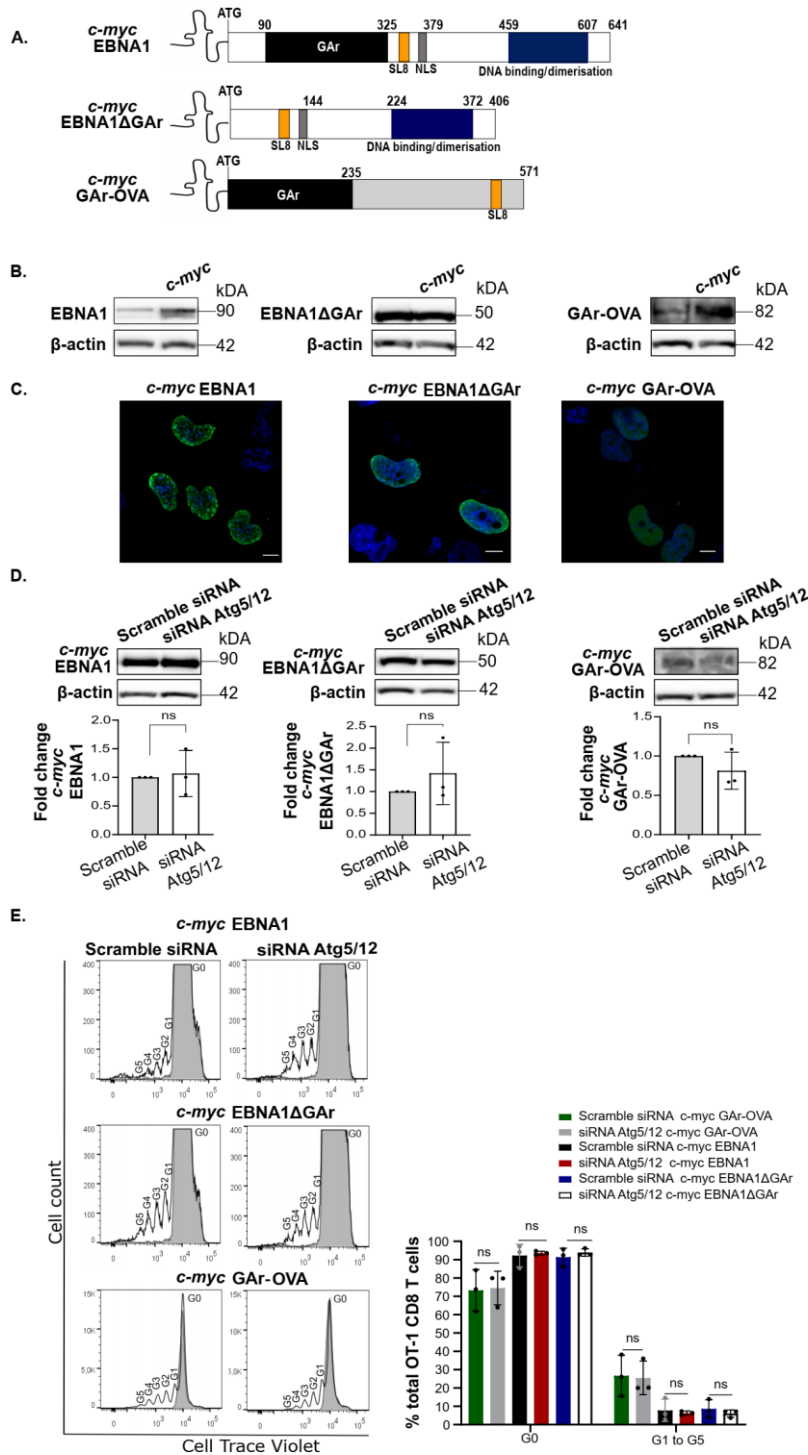


Figure 20. Protein levels do not change autophagy-dependent antigen presentation. **A.** Cartoon illustrating the location of *c-myc* 5' UTR RNA sequence inserted in the 5'UTR of EBNA1, EBNA1ΔGAR, and GAR-OVA **B.** The *c-myc* fused to the 5' UTR of EBNA1, EBNA1ΔGAR, and GAR-OVA constructs overcome GAR-mediated mRNA translation suppression. Western blots show the differences in protein expression

levels **C.** Representative immunofluorescence of EBNA1, EBNA1 Δ GAr, and GAr-OVA constructs carrying the *c-myc*. **D.** Western Blot showing the effect of autophagy inhibition on protein levels of the indicated constructs. The graphs show densitometry analysis, normalized against β -actin for all targeted proteins and expressed in fold change compared with the scramble siRNA. **E.** H1299 cells co-expressing murine MHC-I (Kb) and the indicated constructs following human siRNA Atg5/12 [20 pM] or scramble siRNA treatment for 72 hours. The antigen presentation was estimated as described in figures 5 and 7. The graph shows the percentage of cells from generations 1 to 5 compared with the percentage of non-divided cells (generation 0) from 3 independent experiments (right graph). Significant values were calculated using Multiple paired groups T-test. Not significant ns: 0,1234. White scale bars denote 10 μ m.

XI. Discussion – PART 1

Antigenic peptide substrates processed by autophagy for the MHC class I pathway

Alternative sources of peptides for the MHC class I pathway have been proposed but if, and to what extent, peptides derived from the processing of substrates via the autophagy pathway can be presented to the class I pathway is unknown. We have tested different types of substrates that were potentially good candidates to be presented in the MHC class I pathway via autophagy which gave us a broader idea of the relationship between autophagy and the different kind of peptides.

The SIINFEKL peptide derived from chicken ovalbumin (OVA) has been used as a gold standard method to study antigenic peptides for the direct or cross-presentation MHC class I pathways. Initial studies with OVA used as an antigenic peptide have been focused on proteasome degradation mechanism and little is known about autophagy influence in ovalbumin derived peptide presentation via MHC class I pathway^{170,171}. Strikingly, during this Ph.D. study, we observed that OVA-derived peptides were significantly reduced upon autophagy inhibition by Atg5/12 siRNA, without showing any visible aggregates of OVA protein and confirming that it was not related to an effect of siRNA methodology per se in the MHC class I functionality. Importantly, indirect measurement of the free SL8 in the murine H2-Kb transfected molecule in H1299 cells co-culture with OT1-CD8⁺ T cells did not show any difference during Atg5/12 knock down. Secondly, when we measured endogenous MHC class I (Kb) of murine MCA-205 cells or HLA-ABC molecules in human H1299 cells during Atg5/12 siRNA there was no difference in MHC class I cell surface location under Atg5/12 siRNA compared with the scramble. Furthermore, the presentation of SL8 fused to EBNA1 was not affected by autophagy knock down. Hence, these observations show that Atg5/12 knock down, does not affect the MHC class I pathway per se.

In line with our results, Liu and colleagues implicated OVA as a substrate for autophagy and showed that mice immunized with OVA caused an allergic reaction and induced activation of autophagy accompanied by a relative increase of LC3 II compared to LC3 I in eosinophils cells from lung tissues¹⁷². Our study shows an autophagy-dependent presentation of OVA for the direct class I pathway, but other studies have associated autophagy with cross-presentation. For example, it was found that the high accumulation of OVA in the phagosome, triggered the cross-presentation of the OVA derive peptides in a TAP-independent manner, partially due to the processing by the cysteine protease cathepsin S in the lumen of the phagophore⁶⁰. Another example is

the study done by Tabachnick and colleagues that demonstrated that polyQ fused to OVA was shown to be presented to the MHC class I pathway following injection into mice¹⁶³.

EBNA1 is a viral protein expressed in all Epstein-Barr virus (EBV)-infected cells¹⁴⁰, which bears a repetition disorder called GAR known to cause aggregates^{155,156}. EBV needs to ensure that EBNA1-expressing cells are not detected and destroyed by the immune system. It has been previously proved that the GAR sequence has a translation-mediated control in cis on EBNA1 protein expression¹⁴⁶. Essentially, EBV uses this mechanism to minimize EBNA1 synthesis to evade the MHC class I pathway and CD8⁺ T cell recognition but keeps sufficient EBNA1 expression to maintain the virus alive¹⁷³. However, although autophagy has been shown to contribute to the processing of EBNA1 for the MHC class II pathway¹⁵⁷, our data suggest that this mechanism is not involved in the production of EBNA1-derived substrates for the MHC class I pathway. This raises the possibility that EBNA1 has evolved a mechanism to evade autophagy-mediated class I- but not class II-restricted antigen presentation. An idea that is supported by the fact that GAR fused to OVA prevents OVA from being presented via autophagy as we have evidenced in this study. This suggests that evasion of autophagy-mediated MHC class I-restricted antigen presentation is another mechanism employed by viruses to remain undetected by the immune system.

By inserting *c-myc* 5' UTR upstream of GAR-carrying constructs we could override its translation inhibitory capacity and show that protein expression levels have little effect on GAR-mediated evasion of antigen presentation via autophagy. This points towards a more selective mechanism for how peptide substrates are presented to the class I pathway by autophagy and have interesting implications for understanding not only the cell biological aspects of how proteins are processed by autophagy, but also in terms of disease. As shown by animal studies, which have suggested that the inflammasome plays a role in Alzheimer's disease, indicating that the immune response can play a role in the etiology of neurological disease associated with protein aggregates^{174,175}. It is an interesting possibility that selective autophagy-dependent processing of cellular disease-associated substrates for the MHC I and II pathways could exist. Further studies using more substrates and deeper analysis of autophagy pathways shall confirm, or not, this possibility. Nevertheless, published new data have revealed protein aggregates clearance by autophagy in polyglutamine disorders such as

Huntington disease¹³⁴. Qin and colleagues showed that autophagy inhibition reduced cell viability and increased Huntingtin protein aggregation¹³⁵.

OVA protein did not show any visual sign of aggregate formation but showed an Atg5/12-dependent antigen presentation. Fusion of the PolyQ sequence to OVA resulted in the formation of aggregates but this had a little significant effect on Atg5/12-dependent antigen presentation. Moreover, we were not able to detect aggregates caused by GAR in GAR-OVA or EBNA1 expressing cells, although it is known that the disordered gly-ala domain affects protein folding and unfolding¹⁴⁵. Nevertheless, our antigen presentation assays indicate that GAR-OVA or EBNA1 are not substrates for autophagy in the MHC class I pathway.

The constructs that we used carry the PolyQ and the GAR sequences in the N-terminus of the OVA reporter construct and even though the GAR is located inside the EBNA1 protein, it is likely that GAR and PolyQ location can affect how substrates are presented to the class I pathway via autophagy. In some studies in available databases, it was noted that PolyQ appears often at the c-terminus of coiled-coil regions. Schaefer et al 2012 demonstrated that PolyQ serves to increase the length of the coiled-coil to extend to a neighboring coiled-coil region and facilitate its interaction with another protein, causing abnormal interactions and protein aggregation¹³². In our study, PolyQ was fused to the N-termini and did indeed cause aggregates but it cannot be ruled out that the location of the PolyQ within the protein can have an effect on how the substrate is processed by autophagy.

Proteasome as alternative processing machinery

Generally, the proteasome is the principal degradation organelle in the MHC class I pathway. For this reason, we wanted to determine if the degradation of the different antigenic substrates was processed exclusively by autophagy or not.

We observed a significant autophagy dependence of OVA-derived antigenic peptides, but also on the proteasome. Although we are aware that antigen presentation upon proteasome inhibition showed very low levels of OT1 CD8⁺ T cell proliferation in response to OVA derived antigenic peptides as it is usually observed, because of our fixation strategy. Interestingly, we have observed protein accumulation only during proteasome and not during autophagy inhibition. This result suggests that the majority of OVA full-length protein is degraded by the proteasome and not by the autophagy

pathway. However, OVA-derived antigenic peptide presentation was affected by both pathways. This observation is explained by the fact that OVA protein is highly ubiquitinated¹⁶⁹. Some work carried out several years ago has found that reducing the ubiquitin-ligation by the inducible inactivation of the E1 enzyme in ts20 cells greatly decreased OVA antigen presentation¹⁶⁹. Ubiquitination mechanism that serves as targeting signal for degradation either by proteasome or autophagy^{168,176}.

The challenge of measuring murine H2-Kb directly with antibodies

Our model of antigen presentation was based on human H1299 cells transfected with H2-Kb to be recognized by OT1-CD8+ T cells. To demonstrate that H2-Kb cell surface localization was not affected by the Atg5/12 siRNA, we measured OT-1 CD8+ T cell proliferation by adding the synthetic SL8 peptide to the co-culture. Given that this experiment was an indirect measurement, we tried to estimate the H2-Kb cell surface by Flow Cytometry. However, we observed that H2-Kb transfected into H1299 cells did not give any positive signal (**Annexes supplementary figure 3**). We may hypothesize that the double chain structure of the H2-Kb transfected molecule might have one chain human and the other murine, explaining why the murine anti-H2-Kb does not bind.

As a result, we instead measured endogenous MHC class I membrane localization in the human H1299 and murine MCA-205 cells using anti-HLA ABC and anti-H2-Kb, following Atg5/12 siRNA treatment. We confirmed that cell surface localization of the MHC-I molecule was not affected by the Atg5/12 down-regulation in either species. Of note, MCA-205 cells had a lower H2-Kb positive population (60%) than H1299 cell's HLA-ABC positive population (80%) because the murine antibody only recognizes one class of H-2 the K.

XII. Concluding Remarks – PART 1

Concluding Remarks – PART 1

After the discovery of autophagy-related proteins, many studies have found the importance of autophagy in human health. However, there is still plenty of discoveries to be made in the autophagy field. For example, it has been proposed that certain types of autophagy can take place without Atg 5/7 intervention in mice¹⁷⁷.

My Ph.D. study aimed to evaluate autophagy (via Atg5/12) as an antigenic peptide processing mechanism for the MHC class I pathway.

This work has proved that autophagy via Atg5/12 can interfere with the availability of antigenic peptide sources for the MHC class I pathway. Demonstrated by the downregulation of OVA-derived peptides in antigen presentation upon Atg5/12 siRNA.

This study suggests that protein aggregation is not a key feature to provide antigenic peptides for the MHC class I pathway via Atg5/12-dependent autophagy. But the fact that we observed substrate-specific processing suggests that other mechanisms select which substrates shall be presented or not, for the class I pathway. It is interesting to point out that EBNA1 is presented to the class II pathway via autophagy

We have shown that autophagy is not involved in the production of EBNA1-derived substrates for the MHC class I pathway and that the GAR sequence prevented OVA from being presented via autophagy. This led us to suggest that EBNA1 has evolved a mechanism to evade autophagy-mediated class I, but not class II, antigen presentation.

Taken together, this study shows a substrate-specific presentation of peptides via autophagy that is selective for the MHC class I pathway. It has interesting implications for viral immune evasion and for inflammatory reactions associated with disease in which cellular proteins are processed by autophagy.

Introduction – PART 2

XIII. The classical conception of peptides derived from the degradation of the full-length protein comes into question

The median protein half-life is approximately 46 hours until it reaches obsolescence and is degraded by the proteasome. But this did not fit with observations on the presentation of viral antigenic peptides, which took much less time. Therefore, the scientific community started to challenge the theory that antigenic peptides used for the MHC class I presentation come from the degradation of full-length proteins by the proteasome. Furthermore, if we think carefully, the abundance of the proteome is much higher than the immunopeptidome (repertoire of peptides presented by MHC-I molecules). Each cell expresses approximately 10^4 to 10^5 MHC-I molecules on its cell surface. Thus, if all proteome peptides have access to the class I pathway, the sensitivity of detecting viral or tumor neoantigens would be much lower^{166,178,179}.

These and other observations prompted our group to test to which extent antigenic peptides originate from full-length protein degradation via the proteasome pathway. The method used was based on the NF- κ B pathway. Activation of NF- κ B starts with an extracellular stimulation, such as the TNF- α receptor, that results in I κ B phosphorylation that triggers ubiquitination and degradation via the proteasome. This causes the release of NF- κ B and its transfer to the nucleus. Introducing a mutation on I κ B prevented phosphorylation and, therefore proteasomal degradation. When an antigenic peptide OVA257-264 (SIINFEKL, SL8) was fused to the wild type I κ B or the mutated I κ B, it was shown that both constructs synthesized a similar amount of SL8 peptide. When cells were treated with TNF- α , they observed that the wild-type I κ B was rapidly degraded and, as expected, there was no effect on the mutant I κ B. Importantly, despite the rapid degradation of the wild type I κ B, there was no effect on antigen presentation. This shows, that the degradation of full-length protein via the proteasome is not a source for the production of antigenic peptides¹⁸⁰. Interestingly, using a different approach, they found that transfected capped ovalbumin mRNAs produced full-length proteins up to eight hours after translation, while antigen peptides from the same mRNA were only produced for 2 hours¹⁸⁰. Other studies by other groups also supported this statement, such as Yewdell et al., who engineered a recombinant vaccinia virus that produced different cytosolic peptides with high affinity to mouse class I H-2K^b and GFP tagged. They showed that those antigenic peptides derived

from the vaccinia virus competed with each other for the MHC class I binding but not with peptides derived from full-length protein¹⁸¹.

In this context, a growing number of studies have evaluated the relationship between the proteome and the immunopeptidome. Many studies have observed that antigenic peptides were found at a high rate, while the protein source remained low^{182,183}. Interestingly, one study compared the MHC-I peptidome with the proteome of the same culture cells, using pulse-chase experiments based on capillary chromatography and Tandem mass spectrometry (MS). They have uncovered that the immunopeptidome and the proteome only have approximately 6% of correlation¹⁸⁴.

There are potentially two theories proposed to explain this poor correlation between the proteome and the immunopeptidome.

1) Peptide splicing by the proteasome as a source of MHC class I antigenic peptides

The first theory suggests that the poor correlation is due to the ligation of two peptides by the 20S core of the proteasome in a mechanism called peptide splicing²⁶. In 2004, Hanada et al. observed that CD8⁺ T cells were specific for a renal carcinoma peptide composed of two fragments distant in the original FGF-5 protein¹⁸⁵. Later, this finding was supported by another study which found also CD8⁺T cells activation to non-contiguous fragments of the melanoma differentiation antigen gp100¹⁸⁶. They have demonstrated that the production of this peptide involves the excision of four amino acids and splicing of the fragments¹⁸⁶. Furthermore, the authors reproduced the experiment in vitro with isolated proteasomes and observed that splicing occurred by transpeptidation involving an acyl-enzyme intermediate of the proteasome¹⁸⁶. However, this theory is questioned for two reasons. First, biochemistry assays argue that transpeptidation happens in an aqueous solution and is a reaction very hard to obtain because the water will compete with the peptide substrate. Thus, peptide splicing is very inefficient and unlikely to occur. Second, new studies questioned the bioinformatics employed in these studies¹⁸⁷.

2) Alternative mRNA translation as a source of MHC class I antigenic peptides

The second theory proposed that peptide substrates originate from a specific mRNA translation event. In a major advance in 2013, Croft et al. showed a correlation between

protein synthesis and peptide generation. Demonstrating that antigenic peptides are newly synthesized polypeptides. They used MS selection-reaction monitoring and evaluated eight different peptides. For the early vaccinia virus protein, peptides were detected on the first time point evaluated at 30 minutes. Strikingly, for almost all peptides studied the levels remained the same or declined when the synthesis of different genes was downregulated¹⁸⁸. Moreover, previous studies have revealed that specific translation mechanisms synthesizing antigenic peptides differ from the canonical translation. There are alternative translation processes that potentially have an important role in the generation of antigenic peptides. For example, some studies have observed an alternative translation mechanism that synthesizes polypeptides with a very short half-life^{178,179}. These observations could explain why there is a production of viral antigenic peptides before the expression of the viral protein in the host cell^{178,179}. In a 2016 study, Laumont and colleagues searched MHC class I antigenic peptides on the six-frame translation of the B-cells transcriptome. To determine antigenic peptides derived from non-canonical reading frames, they performed high-throughput MS. The authors found that approximately 10% of MHC class I antigenic peptides originate from noncoding genomic sequences or exonic out-of-frame translation¹. More recently, new techniques such as ribosome profiling have moved forward our knowledge about proteome. Ribosome profiling has revealed that ribosomes can be detected in many regions of the transcriptome previously thought to be noncoding including 5' UTRs and long noncoding RNAs. This opened the door to new studies that have found antigenic peptides synthesized by a cryptic translation pathway^{178,179}.

XIV. Types of antigenic peptide sources in the MHC class I pathway

Over the years scientists have classified the sources of endogenous antigenic peptides using the time of degradation of the precursors as criteria. Therefore, they have classified the sources of antigenic peptides into 1. Retirees (stable proteins degraded by the proteasome with a general turn-over of approximately two days). 2. Rapidly degraded polypeptides (RDPs) which are much faster degraded than retirees and are composed of three subcategories: defective ribosomal products (DRIPs), very short-life proteins (SLiPs), and pioneer translation products (PTPs)^{178,189}.

- DRIPs

Initially, DRIPs were categorized as unstable or immature ribosomal products rapidly degraded after synthesis. They were thought only to be misfolded proteins or prematurely terminated proteins. Then, an increasing number of studies have revealed more exceptions where the peptide translation event has occurred. Therefore, the DRIPs conception has evolved, and now there is more evidence showing that antigenic peptides can derive from alternative mRNAs, ribosomal frameshifting, downstream initiation on bona fide mRNAs, tRNA-amino acid misacylation, or transcription errors¹⁸³.

- SLiPS

SLiPs are usually subunits of multiprotein complexes that require binding to a partner to achieve a stable conformation. If they do not find the appropriate partner in a short period they are quickly degraded^{183,190}. For example, ubiquitinated short-lived proteins¹⁹¹.

- PTPs

PTPs are antigenic peptides products derived from pre-spliced mRNA synthesized through pioneer round of translation in the nucleus^{180,189,192}.

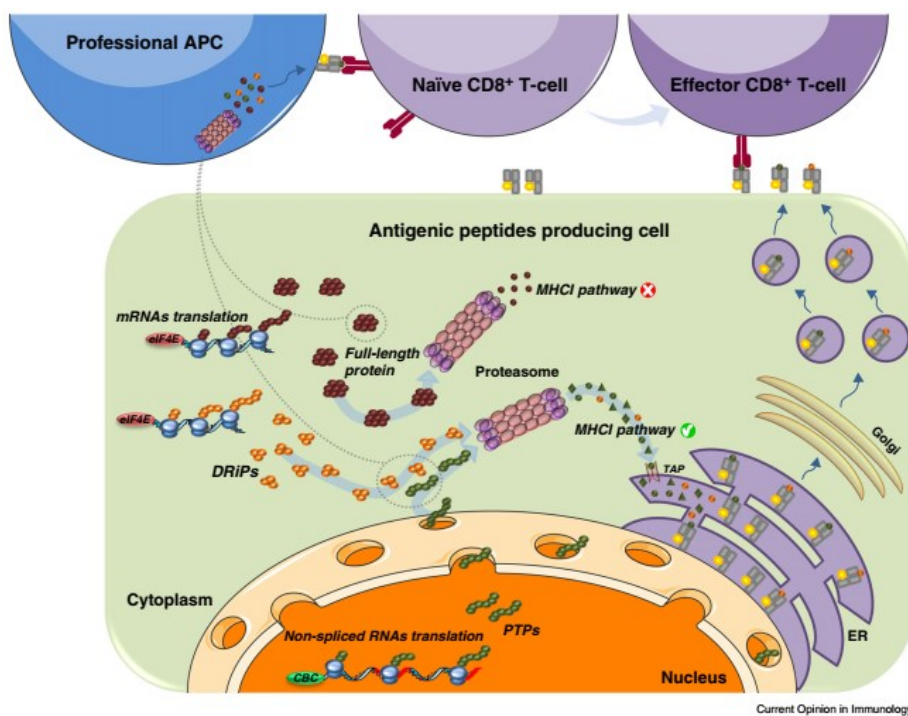


Figure 1. Major Sources of antigenic peptides for the MHC class I pathway. Full-length proteins or retirees degraded by the proteasome were thought to be the source of antigenic peptides for the MHC class I pathway. However, new reports have discovered that defective

ribosomal proteins (DRiPs) and pioneer translation products (PTPs) are the major sources. Once PTPs or DRiPs are synthesized in the nucleus or the cytoplasm. They reach the proteasome to be further processed. The peptide products are then translocated to the ER. Within the ER the peptide binds to the MHC class I and is transported to the cell membrane. A previous study by Duvallet et al 2016 has shown that PTPs from tumoral cells are transported to antigen-presenting cells (APC) through exosomes, serving as sources for cross-presentation^{183,193,194}(Image taken from Apcher et al 2016)¹⁸⁹.

XV. Alternative mRNA translation as a source of MHC class I antigenic peptides

XV.A Antigenic peptide synthesis by alternative translation initiation

Shastri N. was one of the first to present proofs of cryptic translation products used for MHC class I antigenic peptides. Using antigen-presenting cells transfected with cDNA constructs coding OVA257-264 (SIINFEKL, SL8) with variations in the translation initiation codons, he found that besides ATG, other codons such as ATT, ACG, CTG, GCG, TGG, and GAT still were able to activate CD8⁺ T cells in ovalbumin transfected cells. However, he and his team realized that the SIINFEKL peptide generated from alternative codons in the cell was much lower than the concentration of ATG SIINFEKL peptide¹⁹⁵. They later confirmed these previous findings using a different immune epitope detection system called JAL8/K^b. In this publication, they went further, showing that these cryptic antigenic peptides were not a subproduct of replication or transcription errors. Interestingly, they observed that these antigenic peptides could use the CUG coding for leucine as a start codon using High-pressure liquid chromatography (HPLC) analysis¹⁹⁶.

Later, Shastri's team tried to understand the mechanism involved in the translation initiation using CUG as a start codon. In 2004, They tested if their alternative start codon CUG required the standard eukaryotic translation initiation factor 2 (eIF2 α). To answer this question, they transfected HeLa cells with the YL8 vector containing an immunogenic peptide bearing CTG or ATG initiation sequences. After, cells were treated with sodium arsenite to induce the phosphorylation of eIF2 α and prevent canonical translation. Surprisingly, when they evaluated the antigen presentation of those cells, they realized that CD8⁺ T cells still were activated. They proposed that eIF2 α might not be required for the CUG translation initiation¹⁹⁷. Later in 2012, Shastri et al. published new information showing that CUG translation indeed was mediated by leucine-tRNA. In this publication, they transfected cos 7 cells with YL8 and blocked

the binding of the met-t-RNA using a synthetic inhibitor. They observed that under these conditions, CD8⁺ T cells were still activated¹⁹⁸.

Moreover, the work of Shastri not only showed antigenic peptide synthesis from alternative codon initiation. He also revealed that the 3' untranslated region of mRNA could produce antigenic peptides and shape the TCR repertoire in mice¹⁹⁹.

Interestingly, the production of MHC class I antigenic peptides via alternative translation is also reported in viruses. A recent study used SIINFEKL antigenic peptide fused to influenza M1-M2 sequences and evaluated the MHC-I antigen presentation by infected cells upon splicing inhibition. They observed a complete downregulation of mRNA and protein synthesis. Interestingly, K^b-SIINFEKL complexes were still highly expressed. This result suggested an alternative non-canonical initiation in the reading frame. Importantly, in the same study, they inserted synonymous changes in CUG codons in the M1-M2 sequences upstream of SIINFEKL and treated cells with splicing inhibitors. They found a significant reduction of MHC-I antigen presentation compared to the non-mutated M1-M2 sequence fused to SIINFEKL¹⁷⁸.

XV.B Antigenic peptides derived from intron sequences

Antigenic peptides have also been identified to originate from intron sequences. A study carried out in 1995 observed that CD8⁺ T cells recognized a human melanoma antigen that is also expressed in normal cells. They performed a PCR comparing the antigenic peptide sequence in normal and melanoma cells. They identified a point mutation that replaced serine with isoleucine at position 5 of the antigenic peptide in both cells. Strikingly, the coding sequence of this antigenic peptide came from a point mutation located in an intronic sequence²⁰⁰.

XV.C Antigenic peptides derived from the pioneer round of translation

To control the quality of mRNA production and destroy abnormal mRNA, the cell uses a post-transcriptional mechanism called nonsense-mediated decay (NMD) during ribosomal scanning. Generally, eukaryotic mRNAs have an average of over 7 to 8 splicing-generated exon-exon junctions. NMD is triggered when a premature termination codon (PTC) is found in 50 to 55 nucleotides upstream of an exon-exon junctions²⁰¹.

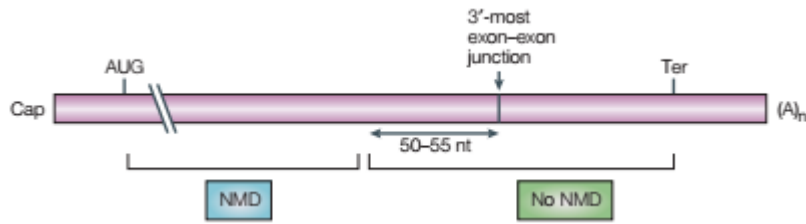


Figure 2. Nonsense-mediated decay mechanism. NMD is triggered when a stop codon is located more than 50-55 nucleotides upstream of an exon-exon junction of the mRNA (blue site)²⁰¹ (Image taken from Maquat et al 2004)²⁰².

When mRNA is transcribed, the first round of translation occurs, called the “pioneer round of translation”. This translation is triggered when pre-mRNA is bound to cap-binding protein (CBP) heterodimer CBP80-CB20 at the 5' cap. Then after splicing, the poly-A binding protein (PABP2) is associated with the 3' poly-A tail and the exon-exon junction complex remains attached to the mRNA. In turn, the exon-exon junction complex recruits the NMD factors, forming the pioneer translation initiation complex. Next, one or more ribosomes translate mRNA during the pioneer round. However, it is unclear if the pioneer round of translation happens in the nucleus or cytoplasm. Since, the CBP80-CB20 complex binds to the cap soon after transcription and during splicing, which is a nuclear process. The pioneer translation mechanism ends with the exchange of the pioneer translation initiation complex with the eIF4E at the 5' cap and PABP1 at the 3' poly (A) tail leading to the canonical translation or it ends with the finding of PTC leading to NMD²⁰¹.

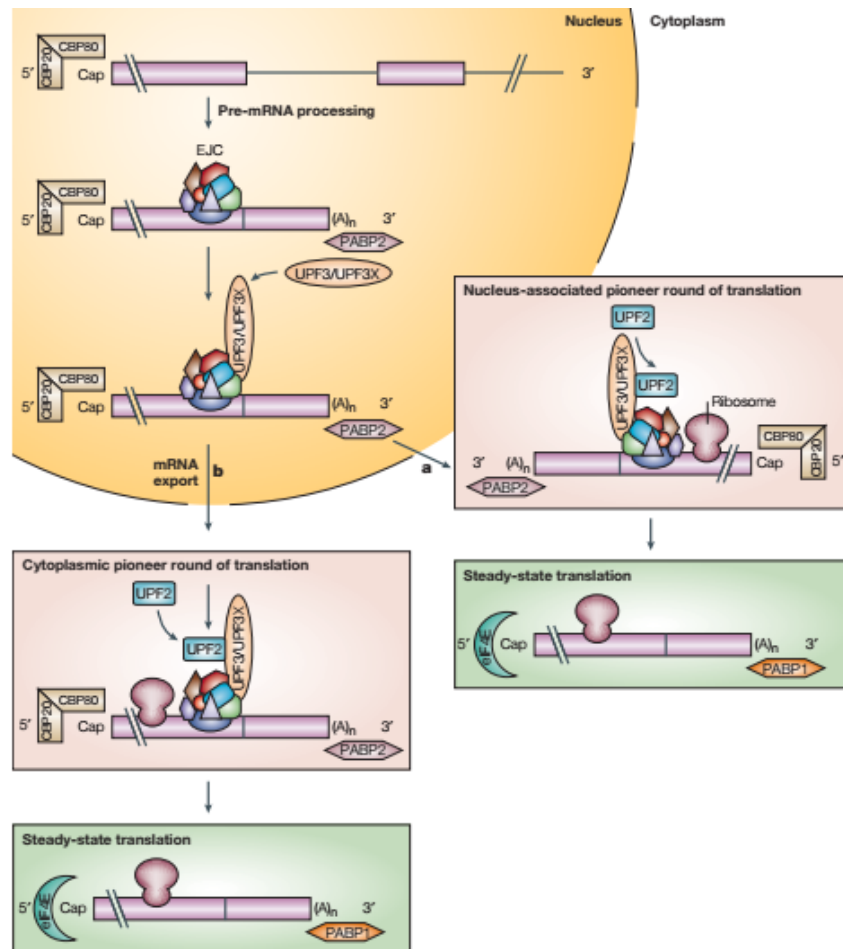


Figure 3. Pre-mRNA splicing, pioneer round of translation, and steady-state translation.

Pre-mRNA binds to the CBP80 and CBP20 at the 5' cap; Once spliced the 3' poly(A) binds PABP2 and the exon junction complex (EJC) remains upstream of the exon-exon junction. The EJC attracts the UPF3X, which in turn recruits UPF2. This complex is subjected to a pioneer round of translation either in the nucleus or in the cytoplasm. Then, depending on the mRNA, steady-state translation or NMD begins²⁰¹. (Image taken from Maquat et al 2004)²⁰².

To evaluate the implication of the pioneer round of translation in the generation of antigenic peptides, Fahraeus's team has engineered a construct in which the OVA257-264 (SIINFEKL, SL8) peptide sequence was inserted in the β -globin gene. The SL8 sequence was located in exon 1 of the β -globin gene, just before a PTC located 53 nucleotides upstream of the exon junction complex in exon 2. Evaluating RNA levels, they observed that β -globin RNA was degraded efficiently, indicating the NMD induction. Interestingly, the SL8 immune epitope presentation was very efficient, almost like the presentation levels of the same construct that lacked PTC. In this same experiment, they also used the β -globin construct bearing the PTC at 53 nucleotides upstream of the exon junction complex in exon 2, but having the SL8 after the PTC. They observed no SL8 antigen peptide presentation. Together these results

demonstrated that the production of antigenic peptides can be associated with the pioneer round of translation that precedes the NMD process. Moreover, to understand better how these antigenic peptides translation occurs, they assessed the role of some initiation factors. Using inhibitors targeting eiF4E association to the mRNA cap structure and also to the eiF4G that is necessary for the binding of eiF4E and the CBP heterodimer that initiate ribosomal quality control scanning. They showed that eiF4E inhibition did not affect antigen presentation, while eiF4G inhibition decreased significantly. This result suggested that antigenic peptide synthesis is governed by a different translation mechanism. They named these antigenic peptides as pioneer translation products (PTPs)¹⁸⁰.

Fahraeus's team has continued the research using these peptides, demonstrating that PTPs are synthesized in the early steps of the mRNA maturation and before introns are spliced out. The innovative idea of introducing an immune epitope sequence in the introns, allowed them to probe the possibility of immune peptides derived from pre-spliced mRNAs. Interestingly, they demonstrated that PTPs are synthesized in the nucleus. In this work, they employed the ability of puromycin to be incorporated in the nascent peptide sequence translated by the ribosome. Next, they transfected cells with a β -globin construct carrying an HA tag in the intron sequence. Using antibodies against puromycin and HA they observed the colocalization in the nucleus¹⁹⁴.

XV.D Immunoribosomes

Ribosomes are complex and highly conserved machinery. It consists of four ribosomal RNA (rRNA) species and 80 ribosomal proteins (RPs)²⁰³. The eukaryotic 80S ribosome consists of a small 40S subunit and a large 60S subunit. The 40S subunit is composed of the 18S rRNA and 33 different RPs designated with the letter S for the small subunit followed by their number. In contrast, the 60S subunit consists of 25S, 5,8S, and 5S rRNA together with 47 RPs designated by the letter L for the large subunit followed by the number²⁰⁴. The ribosomal subunits are assembled in the nucleolus, where the new modified and coded rRNA binds to the RPs. Those have been translocated from the cytoplasm to the nucleus after their synthesis. Once they are assembled, the two ribosomal subunits are then translocated to the cytoplasm, where they associate and carry out protein synthesis forming polysomes²⁰⁴. Structural studies on ribosomes claimed that rRNA is responsible for the ribosomal structure, the location of the tRNA over the mRNA, and their catalytic activity. Thus, rRNA is located in the core of the

ribosome, and RPs are located on the surface²⁰⁵. The classical view of RPs claims that their functionality is to stabilize the RNA inside the ribosome²⁰⁴. However, it is intriguing that the translation machinery is composed of RPs with high heterogeneity. This poses intriguing questions if there is a functional reason for the RPs diversity. A new hypothesis suggests that the mRNA 40s subunits binding and scanning is influenced by RPs composition. Interestingly, other studies suggest that its importance is linked to the differences in RPs over different tissues or during embryonic development. Some others have identified differences in RP composition and the number of ribosomes bound to each mRNA. According to some studies, RPs seem to be non-essential in mammalian¹⁷⁸.

More recent evidence²⁰⁶ suggests that RPs can regulate MHC class I peptide generation. Wei and colleagues knocked down each of the 80 RPs and identified RPs that regulates MHC class I antigenic peptide generation, but did not alter source protein expression. The knock down was carried out in cells infected with a recombinant influenza A virus expressing SIINFEKL fused to the neuraminidase or in steady-state cells. They screened all RPs and selected the ones with the highest effects on MHC class I expression or viral/peptide MHC-I complex generation, without affecting the overall translation. They found five possible candidates. Then, they evaluated the effect on the transcriptome by microarray and selected only 3 RPs with a higher resemblance to the scramble control cells. They discovered that in infected cells, depleting RPL6 decreases ubiquitin-dependent viral peptide presentation, while the RPL28 depletion increases both ubiquitin-dependent and -independent peptide presentation. In uninfected cells, depletion of RPS28 increased peptide supply. Even though it is the first evidence revealing the potential RPs specialization related to antigen peptides production, it still unknown if the heterogeneity of the RPs is associated with the different variants in the alternative translation mechanism responsible for antigen peptide production²⁰⁶.

XV.D Nuclear Translation

The first studies of nuclear translation were reported in 1954. Allfrey and colleagues observed that nuclei isolated from the thymus (primary lymphoid organs from the immune system) incorporated the radiolabeled C¹⁴-alanine into nuclear proteins. It was also shown that ribonuclease treatment did not affect the uptake of C¹⁴-alanine on isolated thymus nuclei²⁰⁷.

A cutting-edge paper of 2001, using the lysyl-tRNA tagged with biotin has found that biotin immunolabeling was observed mostly in the cytoplasm but also in the nucleus. Surprisingly, electron microscopy evaluated Immune gold labeling of biotin-lysine-tRNA and Br-RNA. They observed both signals associated inside the nucleus. Moreover, RPL7 Immune gold labeling was also found together with biotin inside the nucleus. These results suggested that coupled transcription and translation can occur within the nucleus^{208,209}.

Two studies have tried to determine whether antigenic peptides can be synthesized in the nucleus by nuclear translation. Both studies have employed similar strategies to block the mRNA export from the nucleus. The first study used the RNA polymerase inhibitor called DRB on cells infected with the influenza A neuraminidase fused to SIINFEKL. They observed that indeed the neuraminidase mRNA was accumulated in the nucleus after DRB treatment. Inhibiting neuraminidase protein translation, but surprisingly K^b-SIINFEKL presentation was detectable²¹⁰. Likewise, another work in 2013 used the human immunodeficient virus (HIV) RRE-REV machinery, which is used by HIV to promote the nuclear export of transcripts and efficiently translate viral particles. Generally, in infected cells host ribosomes synthesize REV proteins. These proteins would enter the nucleus and bind the RRE segment in the newly translated HIV pre-mRNA. Then, the mRNA-REV complexes interact with nuclear transporters and trigger the export of the unspliced HIV RNA. This system was used¹⁹² to address the hypothesis that RRE-REV machinery would induce an export of unspliced RNA, inhibiting the nuclear translation of antigenic peptides. Indeed, when they measured the antigen presentation in cells expressing the SL8 peptide overexpressing REV protein, they observed a progressive decrease in antigen presentation¹⁹².

Furthermore, a new improved methodology has been used to visualize nuclear translation sites. Brogna and colleagues detected ribosomal subunits at the transcription sites of *Drosophila salivary* chromosomes, using chromosomal immunostaining and in situ hybridization²¹¹. Another study used the ribopuromylation method (RPM), which is based on the visualization of the puromycin entrance in the immobilized ribosome induced by the antibiotic treatment. They observed RPM signals in the nucleoplasm and nucleolus²¹². Then, with another approach, Al-Jubran and colleagues fused halves of fluorescent proteins to RPs. When RPs were closed, a strong fluorescence appeared. They observed that S18 and L18 RPs signal was

located in the cytoplasm, but also clearly in the nucleolus of cultured cells and fly tissues²¹³.

Also, imaging evidence was obtained by Apcher et al¹⁹² who visualized HA-tagged signals in the nucleus from cells transfected with β -Globin bearing HA-tag sequence in the intron 1. They further confirmed this observation with the visualization of the interaction between puromycin or RPS6 with HA through proximity ligation assay (PLA) in the nucleus.

Even though nuclear translation remains controversial, over the years new studies have agglomerated novel evidence demonstrating that nuclear translation and synthesis of antigenic peptides for the MHC class I pathway. Although, it remains uncovered the translation factors and cellular machinery engaged in nuclear translation.

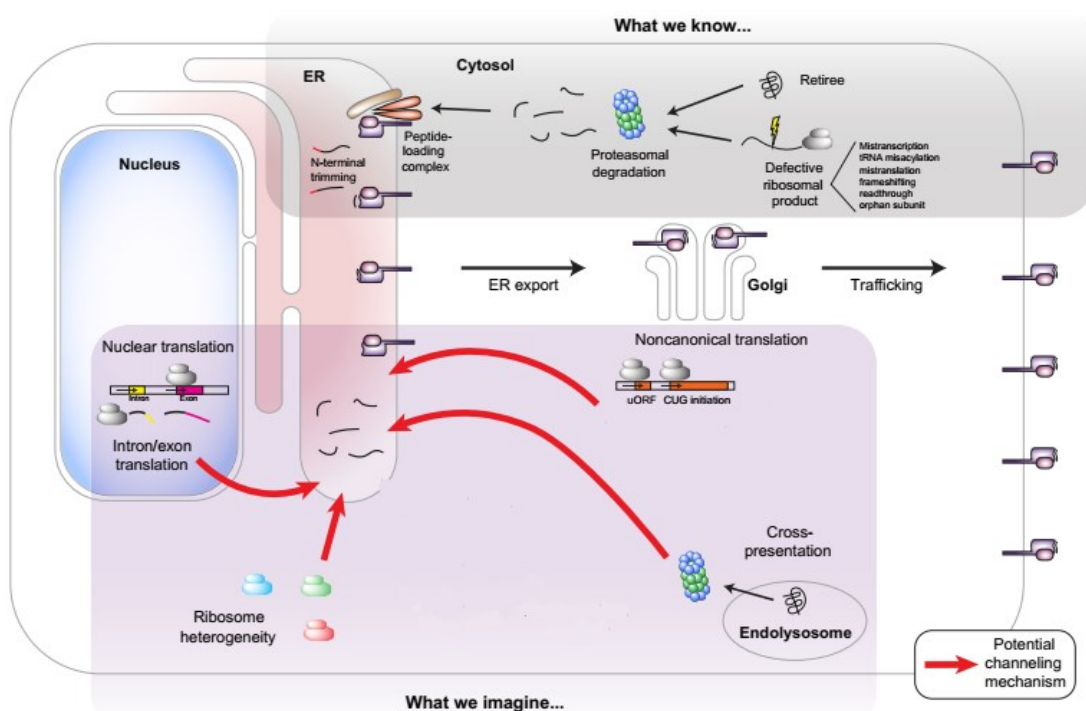


Figure 4. The classical vs new perspectives for the MHC class I pathway. A growing body of studies has demonstrated different approaches and theories that explain the selectivity and rapid MHC class I antigen presentation. Antigenic peptides from pre-spliced mRNA (PTPs), DRIPs, and non-canonical translation, could be associated with specialized ribosomes responsible for these newly synthesized peptides used for MHC-I molecule^{178,183,189}(Image adapted from Yewdell et al 2019)¹⁷⁹.

XIV. Aim of my Ph.D. study – PART 2

Previous studies have demonstrated that some MHC class I antigenic peptides can be originated from pre-spliced mRNA, these peptides are called PTPs. In this Ph.D., we attempted to provide more evidence using different approaches to support these novel findings and elucidate the riboproteome responsible for PTPs synthesis with a first preliminary approach.

XV. Material and methods – PART 2

Plasmids

The human/murine pCDNA3- β -Globin SL8-Exon1 and human/murine pCDNA3- β -Globin SL8-Intron1 constructs^{180,192}, and the GFP was obtained as described previously²¹⁴. The murine pCDNA3- β -Globin SL8 Intron2 was a gift from Dr. Rodrigo Martins.

The SL8-GFP was prepared as follows. The SL8 insert was made by annealing the Forward: oligo 5' GGATCCATGTTTCAGGGTGAGTCTGATGGGCACCTCCAGTATAA TCAACTTTGAAAACTGTGGGTTTCCTTCCCCTGGCTATTCTGCGAATTC 3' containing BamHI and EcoRI sites and the Reverse: oligo 5' GAATTCGCAGAATAG CCAGGGGAAGGAAACCCACAGTTTTTCAAAGTTGATTATACTGGAGGTGCCCAT CAGACTCACCTGAACATGGATCC 3'. containing BamHI and EcoRI. The fragment was cloned into the 5'UTR digested pCDNA3-GFP construct.

Cell culture and Transfection

H1299 cells (Human non-small cell lung carcinoma) were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 1% Penicillin- Streptomycin. HEK 293 cells (Human embryonic kidney) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 1% Penicillin- Streptomycin. For antigen presentation experiments, cells were cultured in 6 wells plates (8×10^4 cells/well) at 37°C with 5% CO₂. The day after seeding, transfections were performed using 3 μ l of Gene Juice reagent according to the manufacturer's protocol (Merck Bioscience). Cells were co-transfected with 0.5 μ g of murine MHC class I molecule Kb and 1 μ g of β -Globin SL8-Exon1, β -Globin SL8-Intron1, and β -Globin SL8 Intron 2 cDNA. In all antigen presentation assays, 1 μ g of an OVA cDNA was used as positive control and the same quantity for the empty vector (E.V) as a negative control. For microscopy experiments, cells were cultured in 24 wells plates (3×10^4 cells/well) at 37°C with 5% CO₂. The day after seeding, transfections were performed as mentioned before. Cells were transfected with 0,5 μ g of murine β -Globin, SL8-GFP, GFP, and E.V constructs. For polysome fractionation, HEK 293 cells were cultured in a 10 cm dish (1×10^6 cells/well) at 37°C with 5% CO₂. The day after seeding, transfections were performed as mentioned before. Cells were transfected with 2 μ g of the murine β -Globin construct.

Antigen Presentation assay: OT1 CD8⁺ T cells proliferation

To determine the levels of antigen presentation, we used CD8⁺ T cells that express specific receptors to the OVA epitope, SIINFEKL, recognized by H-2 Kb. These CD8⁺ T cells were purified from OT1 transgenic mice expressing a transgenic TCR specific for SIINFEKL-Kb. Spleen and lymph nodes from OT1 transgenic mice were passed through a 70 µm cell strainer and red blood cells were lysed with ACK buffer treatment for 5 minutes. After several washes with PBS-FBS 5%, CD8⁺ T cells were negatively selected using a CD8⁺ T cell isolation kit (MACS Miltenyi Biotec) according to the manufacturer's instructions.

Two days after transfection, H1299 cells used as presenting cells were briefly washed with PBS, trypsinized, resuspended in splenocytes medium (RPMI-1640), supplemented with 10% (FBS), 4mM L-glutamine, 1% Penicillin-Streptomycin, 0.05 mM 2-Mercaptoethanol, and 5 mM HEPES) and seeded in 48 wells plates (1.25x10⁵) cells per well with OT1-CD8⁺ T cells (5x10⁵). Antigen presentation was measured through the IL-2 release by activated CD8⁺ T-cell using ELISA.

Quantification of IL-2 release by ELISA

The day after co-culture, the release of IL-2 in the supernatant was quantified by ELISA using the Mouse IL-2 ELISA MAXTM Standard kit (Biolegend). This ELISA procedure is based on the sandwich method, which uses an anti-IL2 capture antibody and a detection antibody. Immunocomplexes were revealed by a colorimetric enzyme-substrate reaction using Avidin-HRP and TMB substrate. Finally, absorbance levels were quantified in a plate spectrophotometer (Fluo StarOptima) at 450 nm.

Proximal Ligation Assay (PLA)

H1299 cells were grown over sterile 22x22mm coverslips and transfected with murine β-Globin, SL8-GFP, GFP, and E.V constructs. For PTPs observation, cells were treated with [30 µM] Isoginkgetin (Merck) for 22 hours or with [25 µM] MG-132 (Merck) for 3 hours. For RPL5-PTPs interaction asses, cells were treated with Cycloheximide 100 µg/ml for 10 minutes. Then, the cells were fixed in 4% paraformaldehyde for 20 min before being permeabilized in PBS and 3% BSA containing 0,1% saponin. Primary antibodies- Rabbit FRVSLMGTSS**SIIN** with Goat **FEKLWVSFPWLFC** (Eurogentec) and Rabbit RPL5 (Abcam) with **FEKLWVSFPWLFC** (Eurogentec) were incubated in the

same buffer overnight. After the cells were washed, PLA probes were added, followed by hybridization, ligation, and amplification according to the manufacturer's protocol (Duolink, Thermo Fisher). Then, we performed immunofluorescence using mouse anti- β Globin and Anti-mouse Alexa488. Coverslips were mounted on slides using slow fade diamond antifade mounting medium (Thermofisher) with Hoescht. Slides were analyzed by confocal microscopy. PLA dots were quantified in H1299 cells with or without β -Globin immunofluorescence signal by a custom-made automated script in FIJI.

Polysome Fractionation

Five–fifty percent wt/vol linear sucrose gradients were freshly cast on SW41 ultracentrifuge tubes (Beckmann) using the Gradient master (BioComp instruments) following the manufacturer's instructions. H1299 or HEK 293 cells were transfected and treated on the same day with Isoginkgetin at [30 μ M] or [10 μ M] respectively. Twenty-two hours post-treatment, cells (with 80% confluency) were treated with cycloheximide 100 μ g/ml for 5 min at 37 °C and then washed twice with 1 \times PBS (Dulbecco modified PBS, GIBCO) containing cycloheximide 100 μ g/ml. Cells were then scraped, lysed with polysome lysis buffer (100mM KCL, 50mM HEPES KOH, 5mM MgCl₂, 0.1% NP-40, 1 mM DTT, cycloheximide 100 μ g/ml, pH 7.4) and spin at 2348xg for 10 minutes at 4°C. Lysates were then loaded on a sucrose gradient and centrifuged at 222228 \times g for 2 h at 4 °C in an SW41 rotor. Samples were fractionated using a Foxy R1 fraction collector (Teledyne ISCO) at 0.5 min intervals ¹⁵⁹.

RNA extraction for RT-qPCR analysis

RNA purifications from fractions were performed using ethanol precipitation combined with RNeasy Mini Kit (Qiagen). RT and qRT-PCR were performed as described above using primers described in (**Annexes table 1**). The relative distribution of target mRNA was calculated using fraction 1 as reference according to Panda et al.²¹⁵.

Protein extraction for Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis

Protein purifications from fractions were performed using Chloroform-Methanol precipitation. Precipitated proteins were resuspended in LDS sample buffer 4x (Life Technologies) and sample reducing agent 10x (Life Technologies), heated for 10 min

Material and Methods – PART 2

at 95°C, and loaded in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were subjected to electrophoresis (10% gel) using MOPS SDS running buffer 20x (Invitrogen). The gels were subsequently stained with Coomassie brilliant blue R-250 staining solution (BioRad) for 2 hours. The gels were destained in 40% (v/v) ethanol, 10% (v/v) glacial acetic acid, 50% (v/v) water until the background became clear.

In-gel digestion for LC-MS/MS analysis (Collaboration done with Dr. Lenka Hernychova from Masaryk Memorial Cancer Institute)

Gel bands were excised out of the gel, washed with deionized water, cut into small pieces and decolorized with a freshly prepared 200 mM solution of ammonium hydrogen carbonate (NH₄HCO₃, pH 7.8) in 40% (v/v) acetonitrile for 20 min at 30 °C and equilibrated in 50 mM NH₄HCO₃ (pH 7.8) in 5% (v/v) acetonitrile for 30 min at 30 °C. The supernatant was removed and the gels were dehydrated with acetonitrile. The supernatant was removed and the samples were reduced by the addition of 10 mM DTT for 1 h at 60 °C, followed by alkylation with 55 mM iodoacetamide in the dark for 45 min at room temperature. The supernatant was removed and the gel pieces were washed three times with equilibration buffer and dehydrated with acetonitrile. Trypsin digestion was carried out at 37 °C overnight using sequencing-grade trypsin (Promega). Digested peptides were extracted using acetonitrile, vacuum dried and desalted using C-18 micro spin columns (Harvard Apparatus) according to the manufacturer's guidelines.

LC-MS/MS analysis (Collaboration done with Dr. Lenka Hernychova from Masaryk Memorial Cancer Institute)

Before mass spectrometry analysis, the evaporated peptide samples were dissolved in 2% acetonitrile with 0.05% aqueous TFA. LC-MS/MS analysis was carried out using an Orbitrap Fusion™ mass spectrometer (Thermo Fisher Scientific) with a New Objective digital PicoView 565 nanospray source (Scientific Instrument Services) coupled to a Dionex™ UltiMate™ 3000 RSLC Nanoliquid chromatograph.

The peptides were loaded into an Acclaim PepMap™ 100 nano trap column (nanoViper™ C18, 0.3 × 5 mm, 5 μm particle size, 100 Å pore size; Thermo Fisher Scientific); loading solvent 2% (v/v) acetonitrile with 0.05% (v/v) aqueous TFA at flow rate 5 μl/min. The trap column was directly connected to an Acclaim PepMap™ RSLC

Material and Methods – PART 2

C18 analytical column (nanoViper™ 75 µm × 25 cm, 2 µm particle size, 100 Å pore size, Thermo Fisher Scientific) kept at 50 °C. From 4-min of initial organic phase concentration with 2% B, the tryptic peptides were eluted with 36-min linear gradient from 2-25% B and 5-min gradient from 25-60% B, followed by a 7-min wash step with 98% B, and 7-min of equilibration with 2% B. Mobile phase A was composed of LC-MS grade water and 0.1% formic acid (FA) while B was acetonitrile 80% with 0.1% aqueous FA (v/v). Flow rate was 300 nl/min.

The Orbitrap mass analyser was operated in positive ion mode, with the static positive ion spray voltage set to 2.75 kV, and ion transfer tube temperature to 275 °C. The master scan was acquired at resolving power settings of 120,000 (FWHM @ m/z 200), precursor mass range 350-1400 m/z. The MS/MS spectra of multiply charged ions were collected in data-dependent mode with the cycle time 3 s. Dynamic exclusion was set to 30 s. The peptides were fragmented using collision-induced dissociation (CID) with the normalized collision energy setting at 35 %. The peptide fragments generated via CID were detected in an ion trap (rapid scan rate). MS and MS/MS data were recorded in profile and centroid data type, respectively.

Data processing (Collaboration done with Dr. Lenka Hernychova from Masaryk Memorial Cancer Institute)

Data analysis was performed with the software Proteome Discoverer™ version 2.5 (Thermo Fisher Scientific). The database search was performed with Sequest HT search engine with the following search settings: database SwissProt (TaxID=9606, validated fasta, updated 04/19/2021, # sequences 42,153; taxonomy: Homo sapiens) and cRap protein (<ftp://ftp.thegpm.org/fasta/cRAP>) databases. The search engine settings employed 10 ppm precursor mass tolerance and 0.6 Da fragment mass tolerance, enzyme Trypsin (full), considering up to two missed cleavages and the following dynamic modifications: methionine oxidation (+15.995 Da); protein N-terminal acetylation (+42.011 Da), and static modification: cysteine carbamidomethylation (+57.021 Da). The results of the search were further submitted to generate the final report considering 1% FDR on both PSM and peptide group levels using Percolator.

Bioinformatics analysis

Material and Methods – PART 2

Identified peptides were selected in each sample according to a high false discovery rate (FDR) and identified peptides to be more than two unique peptides that mapped the identified protein in all individual sets of samples.

Venn diagrams indicating the constantly identified proteins between replicates and shared identified proteins between the 80s & light polysome fractions were created using the online bioinformatics tool <https://bioinformatics.psb.ugent.be/webtools/Venn/>.

Gene ontology analysis was carried out using the online DAVID bioinformatics resource tool.

Statistics

Data were analyzed by two-tailed unpaired Student's t-test or One sample T-test using GraphPad Prism 6 for Windows (GraphPad Software). Data shown are mean \pm sd. of minimum three independent experiments. *P < 0.0332; **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0,1234 ns, not significant.

XVI. Results - PART 2

Antigenic peptides for MHC class I are produced from pre-spliced mRNA

Previous studies in the lab have shown that antigenic peptides for the MHC class I pathway are synthesized mainly during the initial ribosomal scanning of pre-spliced mRNAs^{180,216}. To study these unique antigenic peptides Apcher S. et al. employed the sequence from the β -globin gene and introduced the highly immunogenic SIINFEKL (SL8) peptide in either intron or exons.

In this Ph.D. study, we have used these same constructs of β -Globin containing chimeras half-murine and half-human (carrying the SL8 peptide in the exon 1 or intron 1) used by S. Apcher and an alternative construct composed of the murine β -Globin gene with the sequence of the SL8 peptide incorporated in the intron 2 (**Fig. 5A**).

Using these constructs, we evaluated the antigen presentation of transfected H1299 cells. Two days after transfection, presenting cells were co-cultured with OT-1 CD8⁺ T cells and antigen presentation was determined using IL-2 quantification. As previously published data, we observed that the constructs with the SL8 sequences introduced in the intronic regions exhibited a significant IL-2 production by CD8⁺ T cells compared with the empty vector that produced non. Of note, IL-2 levels were lower compared with SL8 sequences introduced in the exon of the β -globin gene or OVA. (**Fig. 5B**). These results support the idea that antigenic peptides for the MHC class I pathway can come from pre-spliced mRNA and potentially from pioneer translation products (PTPs).

PTPs peptides identification by PLA assay

Previous experiments done in the lab performed intron-derived peptides imaging using β -Globin sequence bearing the HA tag epitope in the intron 1¹⁹². They observed a specific signal in the nucleus of cells expressing this construct using anti-HA antibodies¹⁹². Nevertheless, this approach only showed us how PTPs could be visualized, assuming that their behavior would be similar to the HA-tagged intron. To visualize PTPs directly, we used polyclonal antibodies against the SIINFEKL peptide and flanking sequences from the murine β -Globin intron 2. Hence, immune peptides are rare and rapidly degraded we used proximity ligation assay (PLA). An assay that is based on the strong fluorescent signal emitted from a fluorescent oligos chain reaction between two antibodies in close proximity.

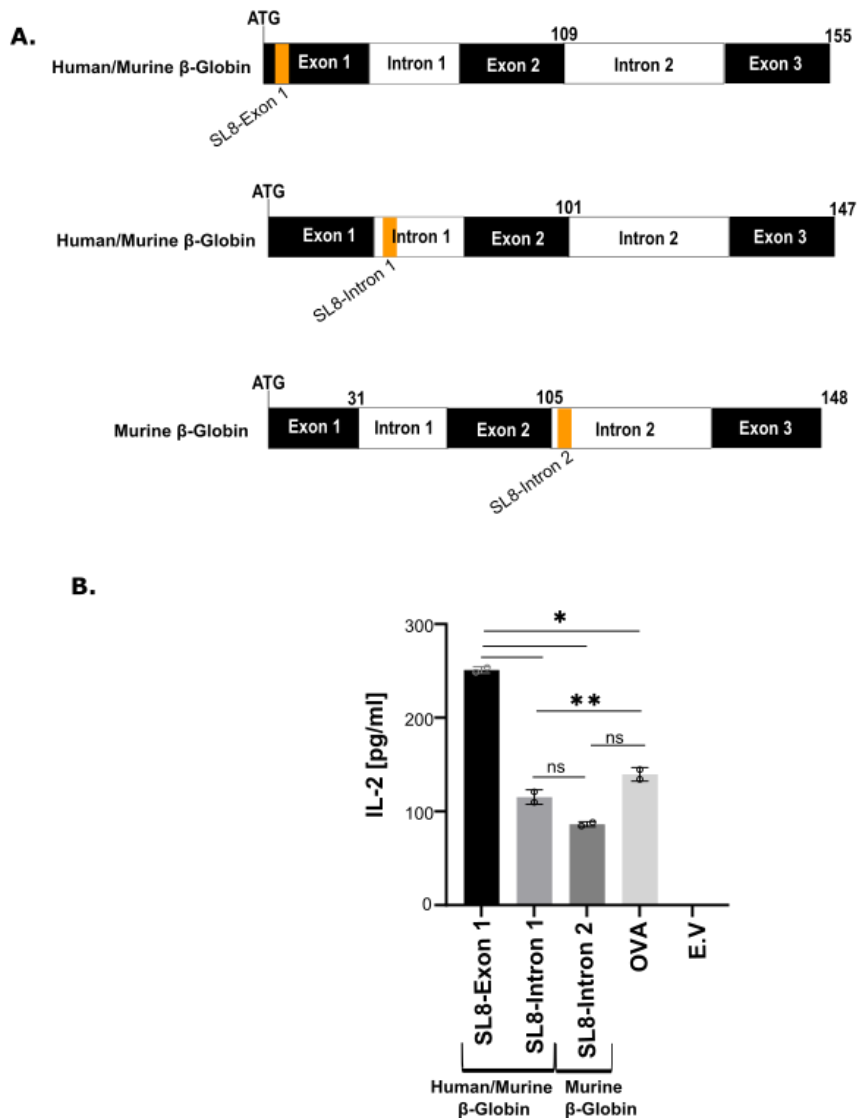


Figure 5. Antigenic peptides for MHC class I are produced from non-spliced mRNA. **A.** Cartoons illustrate Human/Murine or fully Murine β -Globin constructs. The exonic and intronic regions are depicted in A. SIINFEKL (SL8) peptide was introduced in Exon 1 or Intron 1 of Human/Murine β -Globin gene sequence or intron 2 of Murine β -Globin gene sequence. **B.** H1299 cells co-expressing MHC-I (Kb) and the SL8-containing constructs were co-cultured for 3 days with OT-1 CD8⁺ T cells. Culture media was collected and IL-2 concentration was measured with an ELISA Kit. Significant values were calculated using the multiple paired groups T-test. *P < 0.0332; **P < 0.0021; 0,1234 ns, not significant.

For the PLA experiments, we employed an antibody targeting FRVSLMGTTSSIIIN and another antibody against the FEKLWVSFPWLFC sequence from the β -globin gene construct (**Fig. 6A**). Transfected H1299 cells with the construct mentioned before were treated with the splicing inhibitor, Isoginkgetin, for 22 hours. Then cells were fixed and labelled with anti- β Globin and submitted to PLA assay. As expected, all the

transfected cells were positive for β -globin (in green). Equally, we could observe that the Isoginkgetin treatment partially impaired the synthesis of β -globin. Therefore, the cells treated with DMSO showed a higher green fluorescent signal corresponding to β -globin proteins when compared to Isoginkgetin-treated cells. (**Fig. 6B**). Importantly, PLA signals (white dots) were increased under Isoginkgetin treatment when compared with DMSO (**Fig. 6B**). Graphs represent the quantification of PLA dots comparing samples with or without treatment in transfected (green positive) or non-transfected cells (green negative) (**Fig. 6B**). The Quantification showed that Isoginkgetin treatment significantly increased PLA dots compared to DMSO treatment, whereas non-transfected cells did not show any difference (**Fig. 6B**). In the same line when we treated β -globin expressing cell with the proteasome inhibitor MG-132 at 25 μ M for 3 hours. We observed a significant increase of PLA dots upon MG-132 compared to DMSO in transfected cells, while non-transfected cells did not show difference (**Fig. 6C**). In parallel, other control constructs were used to show the specificity of the PLA assay (**Fig. 6D**). The use of the antibodies specific to the SL8 intronic sequence in PLA assay allows us to identify PTPs peptides in the nuclear and cytoplasmic compartments. Through the drug treatment, we confirmed the pre-mRNA origin and the use of this peptide for the MHC-I pathway.

SL8 peptides derived from pre-spliced β -Globin mRNA interact with the ribosomal protein RPL5

To further demonstrate the translation of antigenic peptides from pre-spliced mRNA, PTPs, we assessed the interaction of SL8 peptide from pre-spliced β -Globin mRNA with a ribosomal protein RPL5, using the PLA assay (**Fig. 7A**). We used H1299 cells expressing SL8 peptide sequence in the intron 2 of β -Globin gene and treated them with Cycloheximide for 10 min to freeze translation and be able to see RPL5-SL8 interaction. We observed a significant increase of PLA dots upon Cycloheximide treatment compared to DMSO or cells transfected only with an empty vector (**Fig. 7A, B**). In addition, mostly localization of the PLA signal was found in the nucleus (**Fig. 7A**). This data support other previous findings from the team, where they have found RPS6 interaction with HA peptide derived from an intron sequence. The interaction between RPL5 and SL8 peptides during Cycloheximide treatment suggests that this interaction potentially happens during translation.

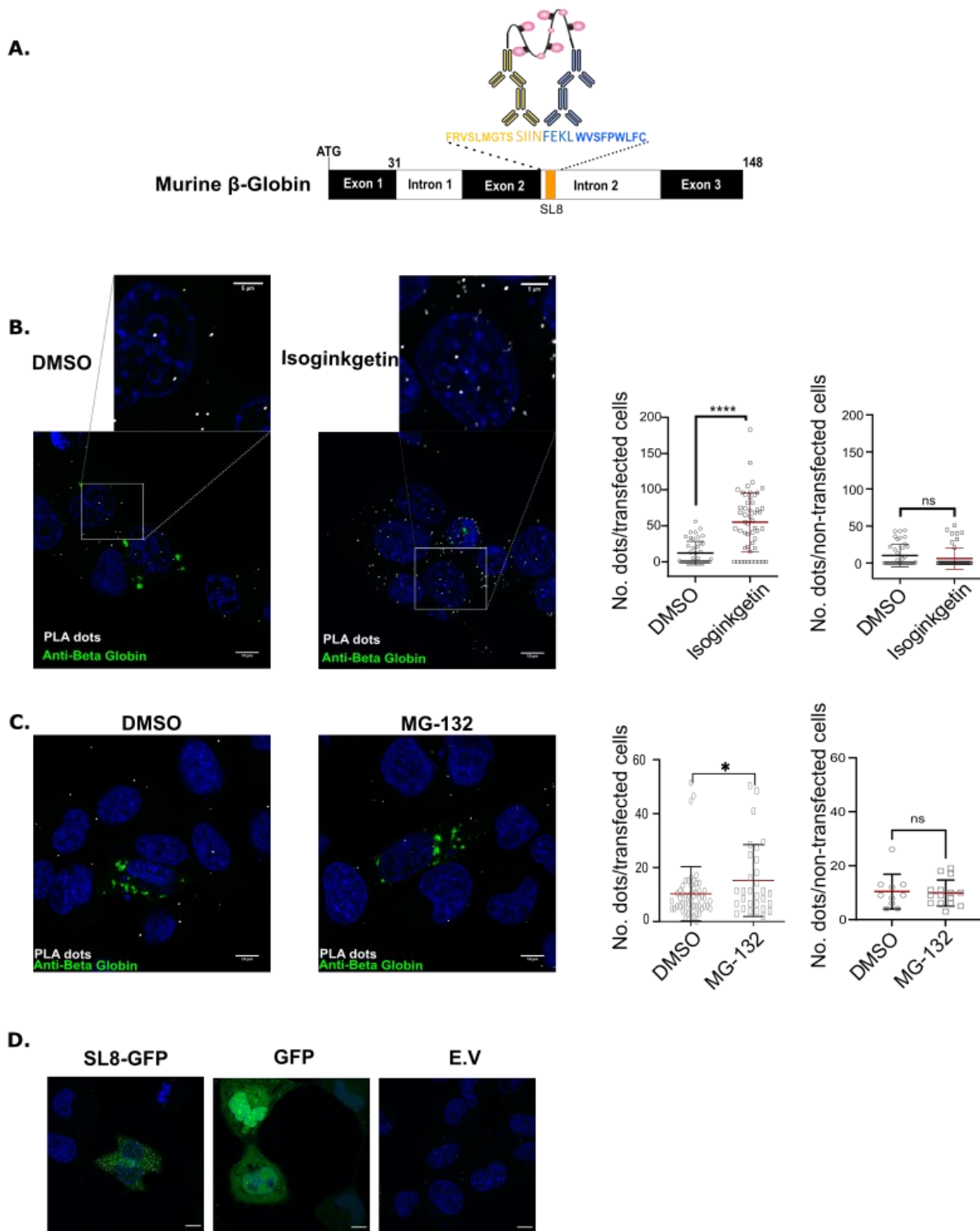


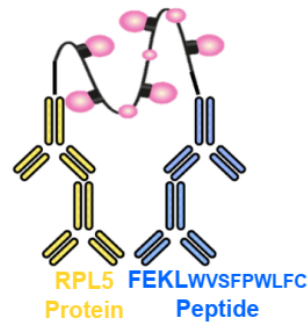
Figure 6. Identification and location of PTPs from murine β -globin gene construct. **A.** Cartoon illustrating the location of SIINFEKL (SL8) in the intron 2 of murine β -globin gene construct. PLA allows the identification of two primary antibodies in close proximity. We used primary antibodies targeting the SIIN and the FEKL sequence from the β -globin gene construct containing the SL8 sequence insert. The primary antibodies will be in turn recognized, by secondary PLA-labelled antibodies emitting then a bright specific signal. **B.** PTPs observation by PLA assay in H1299 cells expressing murine β -globin gene constructs, which were treated with the spliced inhibitor, Isoginkgetin, at 30 μ M for 22 hours, and **C.** proteasome inhibitor, MG-

132 at 25uM for 3 hours. Images show PLA dots in white and β -Globin immunofluorescence signal in green. A selected area is zoomed in for better appreciation. Graphs (Bottom side) represent the quantification of PLA dots in transfected or non-transfected cells. Significant values were calculated using the multiple paired groups T-test. ****P < 0.0001; *P < 0.0332; 0,1234 ns, not significant. **C.** PLA assay performed in H1299 cells expressing GFP control constructs containing the SL8 peptide sequence or GFP alone or, empty vector (E.V). The white scale bars are equivalent to 10. Of note, this result was done in collaboration with Ewa Sroka Ph.D. student at Gdansk University.

The pre-spliced mRNA SL8 from the intron sequence is found in the light polysomes

To study the translation machinery in charge of the PTPs synthesis and better understand how this translation happens. We used the polysome fractionation approach. This technique allowed us to determine the localization of mRNAs in the different ribosomal conformations. H1299 cells were transfected with β -Globin carrying the SL8 peptide sequence in the intron 2. The day after, cells were treated or not with Isoginkgetin for 22 hours and then with Cycloheximide for 10 min. Next cells were lysed and fractionated through a sucrose gradient. Polysome fractionation showed a standard RNA polysome profile, displaying initially free RNA, the 40s and 60s subunits, the 80s monosomes, light polysomes, and then the heavy polysomes. (**Fig. 8A**). In total, we obtained 23 different fractions that were pooled by pairs from fractions 8 to 23 (**Fig. 8A**). Next, RNA was purified from each fraction pooled and RT-qPCR analysis was performed using different pairs of primers. To quantify the β -Globin pre-spliced mRNA levels in the samples, we used a pair of primers binding to the exon 2-SL8 sequences, the exon 2-Neo sequences, the Neo sequences, and the SL8 sequences.

A.



B.

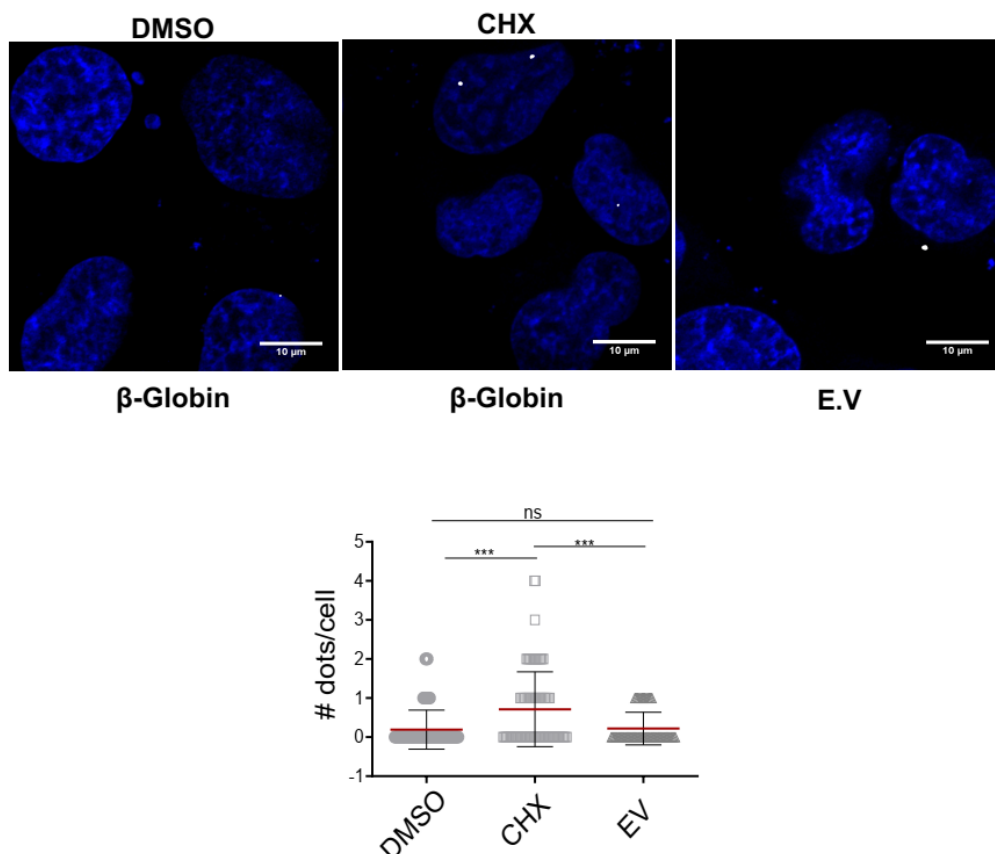


Figure 7. *RPL5* interacts with PTPs from murine β -globin gene construct. **A.** Cartoon illustrating the primary antibodies used in PLA targeting the RPL5 (Ribosome protein from the large subunit 5) and the FEKL sequence from the β -globin gene construct containing the SL8 sequence insert. **B.** PLA dots signal in H1299 cells expressing murine β -globin gene constructs, which were treated with the translation elongation inhibitor, Cycloheximide (CHX), at 100 μ g/ml for 10 minutes. Empty vector (E.V) was used as a negative control. Graphs (Bottom side) represent the quantification of PLA dots of β -globin transfected cells with or without CHX treatment and E.V transfected cells. Significant values were calculated using the multiple paired groups T-test. *** $P < 0.0002$; 0,1234 ns, not significant. Of note, this result was done in collaboration with Ewa Sroka Ph.D. student at Gdansk University.

For spliced mRNA quantification, we used primers binding to exon 1-exon 2, exon 2, and actin gene sequence. The primers localization in the construct's sequences is shown in (**Fig. 8B**). We demonstrated that the pairs of primers used amplified correctly the pre-spliced or spliced targeted sequences of the β -Globin, showing its correct synthesis (**Fig. 8C**). Analysis of the relative mRNA levels in the different fractions showed that pre-spliced β -Globin mRNA was mainly found in the monosomes and light polysomes fractions (early fractions) (**Fig. 8D**), whereas spliced β -Globin mRNA was mainly found in the heavy polysome fractions (last fractions) (**Fig. 8E**). Importantly, we observed that upon splicing inhibition (Isoginkgetin treatment) pre-spliced β -Globin mRNA levels carrying the SL8 sequence were increased in the light polysome fractions (fractions 3 and 4) compared with DMSO (**Fig8F**). On the other hand, splicing inhibition decreased spliced β -Globin mRNA levels in the heavy polysome fractions compared with DMSO (**Fig. 8F**). Taking together, these results showed that the immune peptide sequence in the pre-mRNA is found in the light polysomes. This suggests that this immunogenic peptide could be synthesized by a specialized ribosome that differs from the machinery used in the canonical translation.

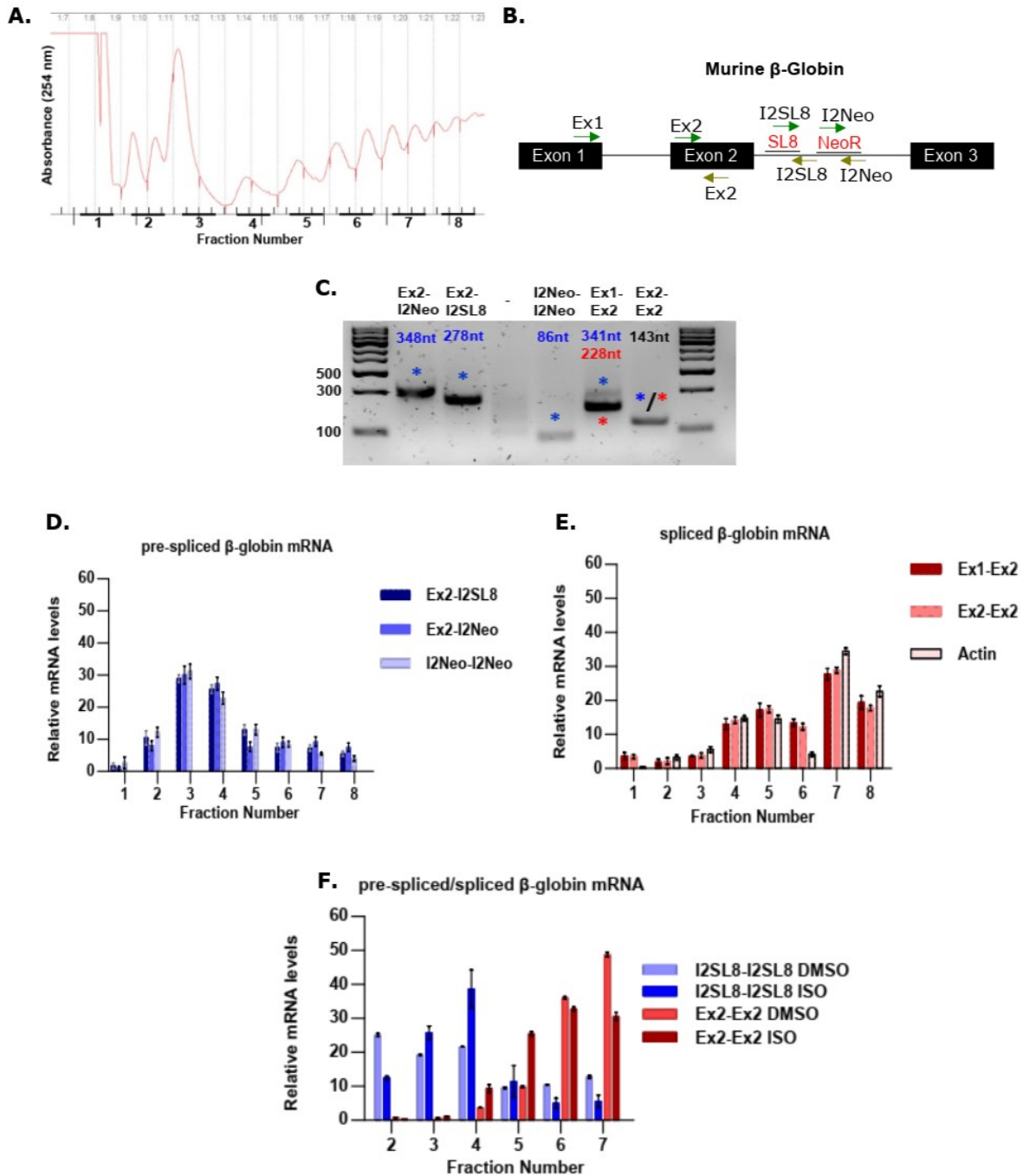


Figure 8. The pre-spliced RNA SL8 from the intron sequence is found in the light polysomes. **A.** Cells expressing murine β -Globin bearing the SL8 in the intron 2 were lysed and fractionated through sucrose gradients. Global RNA polysome profile generated by density gradient fractionation is shown. **B.** Primers used targeting pre-spliced mRNA and spliced mRNA of β -Globin sequences are indicated. **C.** Fractions 3 to 8 were pooled and RT-qPCR was performed using indicated primers. RT-qPCR products obtained were visualized in agarose gel. Pre-spliced mRNA product size is illustrated in blue, while spliced-mRNA product size is in red. Ex2f and Ex2r products are visualized in both colors because those primers do not distinguish between pre-spliced-mRNA and spliced mRNA **D and E.** RT-qPCR analysis targeting pre-spliced mRNA and spliced mRNA sequences using different primers (B.) in eight collected gradient fractions. The relative distribution of target RNAs is shown as % and was calculated

using fraction 1 as reference. **F.** RT-qPCR analysis of polysome gradient fraction from cells treated with [10 μ M] Isoginkgetin or DMSO for 22 hours. Of note, this result was done in collaboration with Ewa Sroka Ph.D. student at Gdansk University.

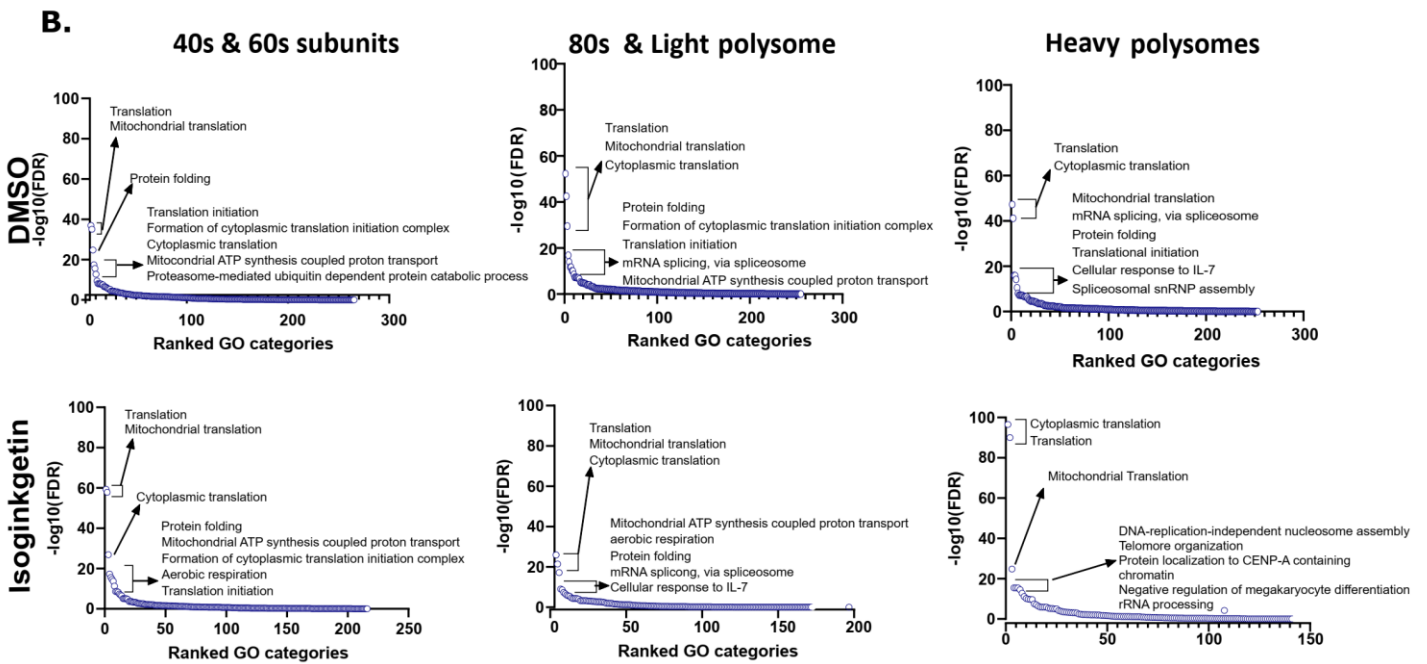
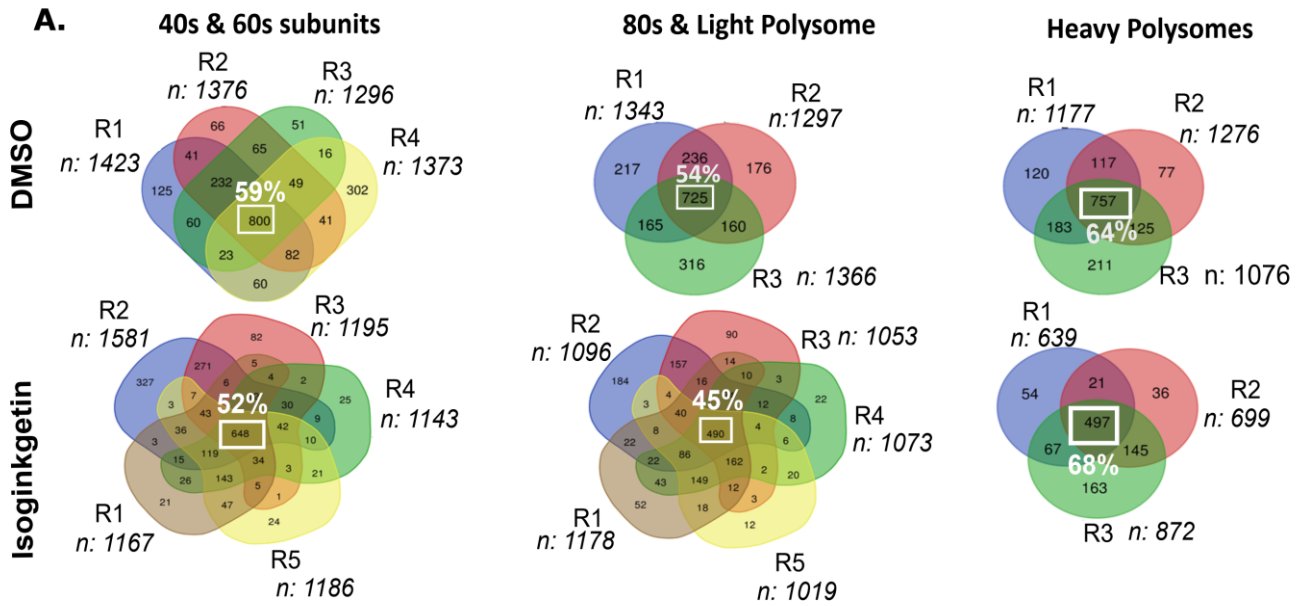
LC-MS/MS analysis revealed no unique ribosomal proteins upon splicing inhibition in the 40s & 60s and 80s & light polysome fractions.

Next, we thought to continue exploiting polysome fractionation assay to identify these specific RPs found in the 80s & light polysomal fraction upon Isoginkgetin treatment compared with other fractions using Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

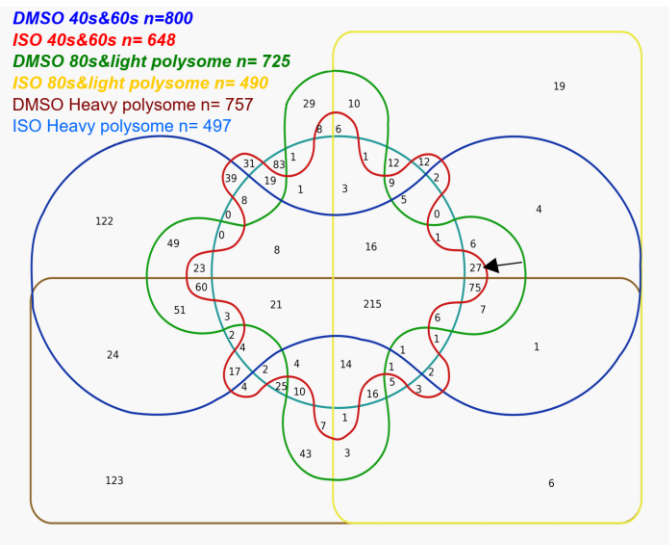
We used HEK cells coding for the SL8 in the intron 2 of the β -Globin gene and treated them with or without Isoginkgetin. Then, we performed polysome fractionation and selected three representative fractions to be analyzed by LC-MS/MS: the 40s & 60s subunits fraction, the 80s & light polysome fraction, and the heavy polysomes fraction (**Supplementary Figure 4, Annexes**). Proteins from the different fractions were extracted using the Chloroform-methanol precipitation protocol. Then, proteins precipitated were resuspended in loading buffer and reducing agent, loaded in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 10%, and subjected to electrophoresis for only 10 min. Then, gels were stained with Coomassie brilliant blue R-250 and visible bands were excised out of the gel. Gel digestion was performed followed by trypsin digestion. LC-MS/MS analysis was carried out using an Orbitrap fusion™ mass spectrometer. The data processing was performed with the software Proteome Discoverer™. Identified peptides were selected in each sample according to a high false discovery rate (FDR) and identified peptides to be more than two unique peptides that mapped the identified protein. Samples showed 68% to 45% common hits between replicates (three to five replicates per fraction) that were used in the following analysis (**Fig. 9A**).

To better understand the changes induced by the Isoginkgetin treatment, we used DAVID to perform a gene ontology-based functional characterization of the proteins identified. This analysis showed a considerable group of proteins obtained were associated with translation function and other's minor part to mitochondrial respiration or ATP synthesis, proteasome or cellular response to IL-7 among others (**Fig. 9B**). Critically, we identified proteins associated with function of mRNA splicing, via spliceosome only in 80s & light polysome fraction, which correlates with the increase

observed of the pre-spliced β -Globin mRNA levels upon splicing inhibition (Fig.8F). According to the Gene ontology analysis, the response to the splicing inhibitor, Isoginkgetin compared to DMSO, induced an increase of proteins identified associated with translation, cytoplasmic translation, and mitochondrial translation function only in 40s & 60s and heavy polysome fraction (**Fig. 9B**). Strikingly, opposite to the others fractions, the 80s & light polysome fraction showed a lower number of translational associated proteins (**Fig. 9B**). To identify the RPs responsible for the synthesis of antigenic peptides from pre-spliced mRNA, we hypothesise that specific RPs would be only identified in 40s & 60s and 80s & light polysome fractions. Upon splicing inhibition induction, we believe these specific RPs would increase. For this reason, we looked for common hit proteins between the 40s & 60s, and 80s & light polysome fractions treated or not with splicing inhibitor, Isoginkgetin, using Venn diagrams (**Fig. 9C**). We found 27 common proteins between those four fractions, however, none of them were RPs (**Fig. 9C**). We observed that RPs found in the subunits or the light polysome fractions were also identified in the heavy polysomes fractions (Data not shown). With this approach, we hypothesized that RPs used for the synthesis of antigenic peptides from pre-spliced mRNA might be identified with the protein level quantification of RPs upon splicing inhibition between the different fractions.



C.

**Proteins identified**

26S proteasome non-ATPase regulatory subunit 11
 39S ribosomal protein L21, mitochondrial, L21mt
 Chloride intracellular channel protein 4
 Coatomer subunit epsilon
 Coatomer subunit zeta-1
 Cyclin-dependent kinase 1, CDK1
 DnaJ homolog subfamily C member 8 (Splicing protein spf31)
 Dynactin subunit 2
 eIF5-mimic protein 1 (Basic leucine zipper and W2 domain-containing protein 2)
 Eukaryotic translation initiation factor 5, eIF-5
 Haloacid dehalogenase-like hydrolase domain-containing 5 (Cat eye syndrome critical region protein 5)
 Histidine--tRNA ligase, cytoplasmic, EC 6.1.1.21 (Histidyl-tRNA synthetase, HisRS)
 Mitochondrial import receptor subunit TOM34, hTom34 (Translocase of outer membrane 34 kDa subunit)
 NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial
 N-alpha-acetyltransferase 50, hNaa50p
 Nucleosome assembly protein 1-like 1
 Phosphatidylinositol transfer protein beta isoform, PI-TP-beta
 Polymerase delta-interacting protein 2
 Programmed cell death protein 4 (Neoplastic transformation inhibitor protein)
 Prostaglandin E synthase 3, EC
 Proteasome subunit beta type-7
 Small glutamine-rich tetratricopeptide repeat-containing protein alpha
 Transcription elongation factor A protein 1
 Transforming protein RhoA, EC 3.6.5.2
 Tubulin-folding cofactor B
 Tubulin-specific chaperone A
 Uridine-cytidine kinase 2

Figure 9. LC-MS/MS analysis revealed no unique ribosomal proteins upon splicing inhibition in the 40s & 60s and 80s & light polysome fractions. **A.** Venn diagrams depicting shared proteins identified in each of the different samples replicates by the LC-MS/MS analysis from three different polysome fractions. Identified proteins having a high false discovery rate (FDR) and more than two unique peptides mapping the identified protein, ranged between 639 to 1581 of the individual data sets. The shared percentage between replicates of each polysome fraction was calculated **B.** Gene ontology (GO) analysis of the three different polysome fractions from cells treated with DMSO or Isoginkgetin highlights multiple functional groups enriched in the polysome fractionation. **C.** Venn diagram illustrating the identified proteins shared only between the 40s & 60s and 80s & light polysome fractions with and without Isoginkgetin treatment (bold italics). Out of the total identified proteins, 27 are shared between these four groups, indicated with a black arrow. A table illustrating the 27 identified proteins shared only between the 40s & 60s and 80s & light polysome fractions with and without Isoginkgetin treatment is shown.

XVII. Discussion and Perspectives-PART 2

Discussion and Perspectives – PART 2

A large number of studies suggest alternative sources of antigenic peptides for the MHC-I pathway, including translation of pre-spliced mRNA^{216,217}. During my studies, we have obtained some results that demonstrate, with two different approaches, the existence of these PTPs and show preliminary results that can provide some clues to identifying the riboproteome in charge of the PTPs translation.

Initially, we have shown that the immune SL8 peptide derived from the intron 2 of the murine β -Globin was presented to OT-1 CD8⁺ T cells. We obtained a similar response as Apcher and colleagues regarding the antigen presentation levels²¹⁶.

Importantly, for the first time, we have been able to directly image the SL8 immune peptide synthesized from an intron sequence through PLA assay. We observed an intron-derived peptide increased following treatment with splicing or proteasome inhibitors. Labeling cells with an anti- β -Globin antibody was used to demonstrate that the anti-SL8 antibodies used were specific by identifying the cells that were transfected. However, the SL8 intron-derived immune peptide is translated by an alternative translation mechanism, while the β -Globin full length protein is translated by the canonical machinery, meaning that the SL8 intron-derived immune peptide might be detected on cells not labeled with anti- β -Globin. Therefore, an assay combining Fluorescence in situ hybridization (FISH) assay against β -Globin RNA and PLA assay targeting the SL8 immune peptide derived from the intron would be necessary to confirm our findings. In addition, SL8 PLA positive labeling was detected in the nucleus as in the cytoplasm. Opposite to the previously reported study by the group, where they observed the HA peptide from the intron sequence located in the nucleus²¹⁶. The localization difference could be explained by the fact that Apcher and colleagues used elongation inhibitors, visualizing the nascent HA peptide from the ribosome, whereas, with the splicing or proteasome inhibitor, we did not control translation.

Furthermore, Apcher et al. 2013 found that the HA tag coded from an intron sequence interacted with the RPS6, within the nucleus²¹⁶. A very interesting RP because RPS6 interacts with the chromatin in primary hepatocytes and is phosphorylated within the nucleus in response to hormones²¹⁸. Interestingly, we also showed interaction between a RP RPL5 with the SL8 immune epitope expressed in the pre-spliced β -Globin mRNA. RPL5 protein is known to transport the 5s RNA to the nucleolus for assembly into the

Discussion and Perspectives – PART 2

60s subunit. These data suggest that the specific translation of antigenic peptides from pre-spliced mRNA might involve RPs from the small and large subunit of the ribosome.

To elucidate the complete riboproteome involved in the synthesis of antigenic peptides from pre-spliced mRNA, we used polysome fractionation that allowed us to visualize the ribosome assembling process. In this preliminary experiment, we have shown that the splicing inhibition induces the increase of the pre-spliced mRNA in the light polysome fraction, suggesting that specialized riboproteome synthesize specific antigenic peptides.

Following this result, we hypothesized that unique RPs responsible for the synthesis of pre-spliced β -Globin mRNA would be only found in the 80s & light polysome and 40s & 60s subunits fractions, but not in heavy polysome fraction. We believed that splicing inhibitor would enhance pre-spliced β -Globin mRNA synthesis and therefore identify these specific RPs. However, we did not see any specific ribosomal proteins corresponding only to the 40s & 60s subunits and 80s & light polysome fractions in all conditions. All RPs identified in these fractions were also found in the heavy polysome fraction. It seems likely that RPs composing ribosomes synthesizing antigenic peptides from pre-spliced mRNA might be identified by evaluating the quantity of the RPs upon splicing inhibition between the different fractions. Future work could evaluate protein levels of RPs from the different fractions, upon splicing inhibition, by Western Blot or RT-qPCR targeting all RPs, or by more precise SILAC-based quantitative mass spectrometry.

Even though the LC-MS/MS analysis did not show specific RPs in the 40s & 60s subunits and 80s & light polysome fractions, we did see a difference in mitochondrial translation. Gene ontology analysis revealed that identified proteins associated with mitochondrial translation increased upon splicing inhibition in the 40s & 60s subunits and heavy polysome fractions, but not in 80s & light polysome fraction. Looking for the mitochondrial translation proteins, we observed that mitoribosomal proteins appear upon splicing inhibition in the 40s & 60s subunit fraction, while in the 80s & light polysome fraction several disappears. The heavy polysome fraction, contrary to the mentioned fractions, showed mitoribosomal proteins that appear or disappear upon splicing inhibition. We are currently in the process to validate these data by mRNA

Discussion and Perspectives – PART 2

sequencing and RT-qPCR towards mitoribosomal RNA from the polysome fraction samples.

These findings are interesting because mitoribosomes are very different in composition, function, and structure from their bacteria ancestors but also from the cytoplasmic ribosomes²¹⁹. Mitoribosomes specialize only in the mitochondrial membrane protein synthesis necessary for the mitochondrial respiratory chain²¹⁹. These specialized mitoribosomes are an example of the possibility to find ribosomes with a functional specialization such as antigenic peptides synthesis from pre-spliced mRNA.

Interestingly, alteration of mitoribosomal protein expression is associated with the development of cancer²¹⁹ and mitochondrial fission has also been related to the decrease of MHC-I surface expression²²⁰. These findings may reveal the importance of mitochondria in cancer immune evasion.

XVIII. Concluding Remarks – PART 2

So far, a significant amount of research has shown evidence for intron and nuclear translation. However, translation of introns and nuclear translation are two controversial topics that have so far been neglected in the molecular field. The scientific community argues that the nucleus has a low abundance of many translation factors and nascent ribosomes found in the nucleus are likely inactive^{221,222}.

Nevertheless, our study provides additional support for intron translation theory. i) We have shown that MHC class I antigenic peptides are derived from intron sequences. ii) we have visualized intron-derived peptides, iii) We have shown an interaction between the ribosomal protein RPL5 and SL8 peptides, and iv) identified pre-spliced mRNA antigenic peptide sequence in the light polysome.

These data open the possibility that there are RPs in charge of the antigenic peptide translation. In the team, we believed that the highly conserved features and the RPs heterogeneity in ribosomes support the idea that there is specialized ribosomal machinery synthesizing antigenic peptides for the MHC class I pathway.

XIX. General discussion

Understanding how different intracellular antigenic peptide sources are governed to activate an effective immune response through CD8⁺ and CD4⁺ T cells will be useful for the design of novel immunotherapy and performant approaches.

We have observed that ovalbumin protein alone or fused to glutamine repetitions are substrates for autophagy processing to generate MHC class I restricted antigenic peptides, opposite to EBNA1 viral protein. Interestingly, the fusion of the GAR sequence to ovalbumin blocked the processing of this protein via autophagy and induced OT1 CD8⁺ T cell proliferation. This observation suggests that the GAR sequence could be key in a mechanism used by the EBV to avoid MHC class I antigen presentation via autophagy a potentially common strategy used by other viruses carrying similar domains. For example, the Kaposi's sarcoma-associated herpesvirus synthesizes a protein named latency-associated-nuclear antigen 1 (LANA1) carrying a central repeat-rich in glutamine, glutamate, and aspartate. In 2011 Kwun H. reported that LANA1 transfected cells treated with Chloroquine or Rapamycin did not change LANA1 MHC class I antigen presentation²²³.

We proposed that the presence of repetition sequences like glutamine or glycine-alanine triggering aggregates protein conformation (no evidence GAR causes aggregates in this study, but other studies have reported this^{155,156}) does not condition the protein to be processed by autophagy to generate antigenic peptides. On the contrary, the glycine-alanine repetition (GAR) seems to be an evasion mechanism of the MHC class I pathway via autophagy, not related to the GAR effect on translation inhibition in cis of EBNA1 mRNA. We could imagine that the GAR sequence impacts protein structure conformation or activates an autophagy regulator to avoid autophagy processing in the MHC class I pathway.

Assessing autophagy's role in the MHC-class I antigenic peptides derived from protein aggregates derived from glutamine repetitions and the EBNA1 viral protein. We have found unexpectedly that our control ovalbumin protein was a substrate of autophagy. An observation that could be supported by the reports made by other studies, for example i) mice immunization with ovalbumin caused an allergic reaction and induced an activation of autophagy in eosinophils cells from lung tissues¹⁷², ii) accumulation of ovalbumin in the phagosomes triggered the cross-presentation of the OVA derived peptides in a TAP-independent manner⁶⁰, iii) PolyQ fused to OVA was shown to be

presented to the MHC class I pathway following injection into mice¹⁶³ and iv) ovalbumin synthesized endogenously access to an endosomal/lysosomal compartment via an intracellular route and be processed and presented in association with class II-MHC molecules²²⁴.

Others researches made in viral antigen presentation has revealed that viruses inhibit protein synthesis to avoid CD8⁺ T cell activation, indicating a relation between the translation mechanism with MHC class I antigen presentation^{146,148,149}. Supporting the idea that antigenic peptide substrates originate from a specific mRNA translation event.

During this Ph.D. study and the evidences obtained in the lab^{180,192}. We have demonstrated that antigenic peptides can be derived from pre-spliced mRNA in antigen presentation assays. Additionally, in this study the sl8 peptide coded from pre-spliced mRNA was observed directly using PLA assay, proving its synthesis.

To understand how this SL8 is synthesized we based our research on polysome fractionation. We identified an increase of pre-spliced β -globin mRNA carrying the SL8 in the intron sequence in the 80s and light polysome fraction upon splicing inhibition. This result point to the idea that SL8 synthesis from pre-spliced mRNA could be performed by ribosomes composed of specific ribosomal proteins localized in the 40s, 60s, 80s, and light polysome fractions. We performed LC/MS analysis from proteins precipitated from three representative fractions 40s&60s subunits, 80s&light polysome, and Heavy polysomes fraction of cells expressing the sl8 in the intron 2 of β -globin. We observed that ribosomal protein composition from either of these fractions did not differ from ribosomal proteins from ribosomes found in heavy polysomes fraction, where happens the classical translation mechanism.

We believed that quantification of the ribosomal proteins from these fractions using quantitative LC/MS will reveal the ribosomal proteins that increase when the splicing inhibitor Isoginkgetin is used. Then, ribosomal proteins found can be targeted with siRNA and observe the effect of knockdown of these proteins in the MHC class I antigen presentation assay. However, we cannot exclude the possibility that the translation mechanism responsible for this antigenic peptide synthesis could be different from the classical ribosomal mechanism.

More and more studies revealed a very complex cellular defense mechanism to generate antigenic peptides and through antigen presentation activate T cell response.

Interestingly, viruses have evolved to avoid these mechanisms, thus studying viral immune response can elucidate new mechanisms.

Autophagy processing to produce antigenic peptides for the MHC class I pathway depends on the kind of substrates, although it is not subjected to aggregate protein conformation. Understanding this mechanism can prompt the development of new therapeutics for diseases with inflammatory components, including infections, autoimmunity, cancer, metabolic disorders, neurodegeneration, and cardiovascular and liver diseases.

Research by the group has elucidated that the presence of intron-derived peptides on class I molecules of tumor cells generates immune tolerance¹⁹⁴. Understanding antigenic peptide synthesis could be the base for new therapeutics for modulating immunosurveillance by targeting for example the ribosomes.

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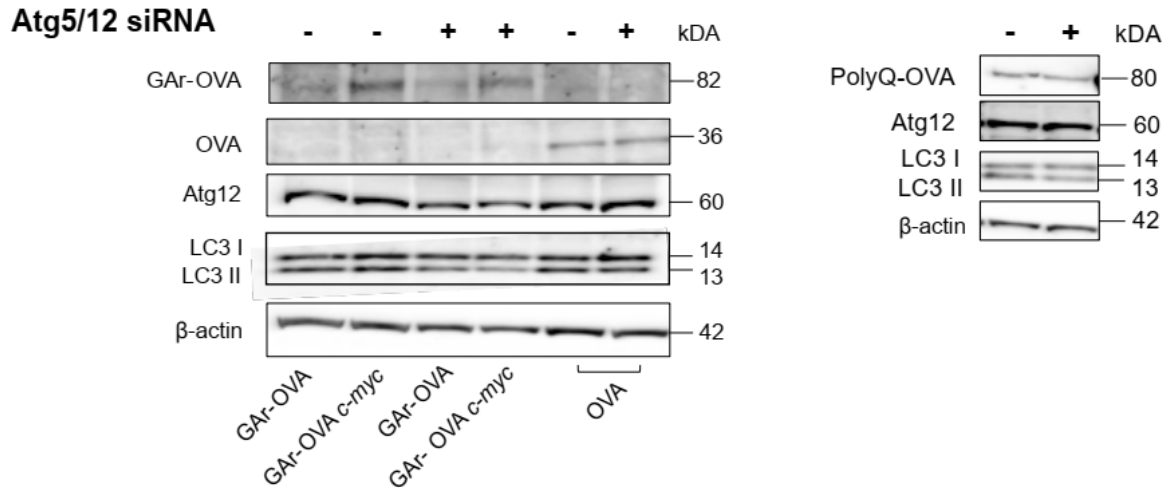
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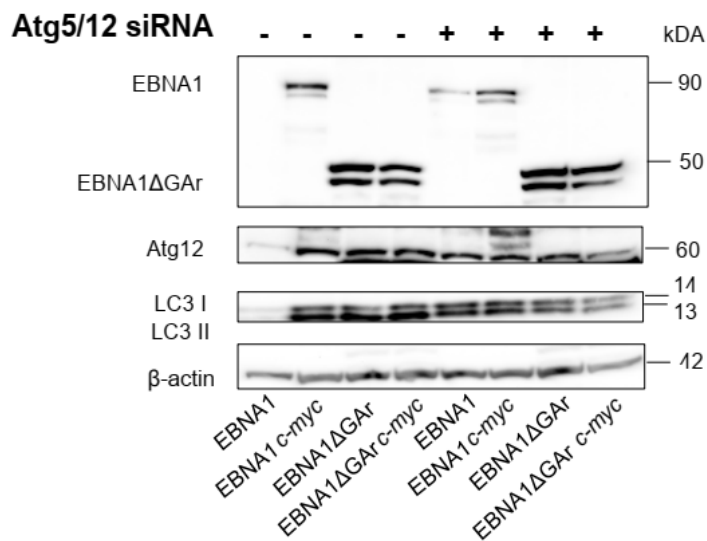
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XXI. Annexes

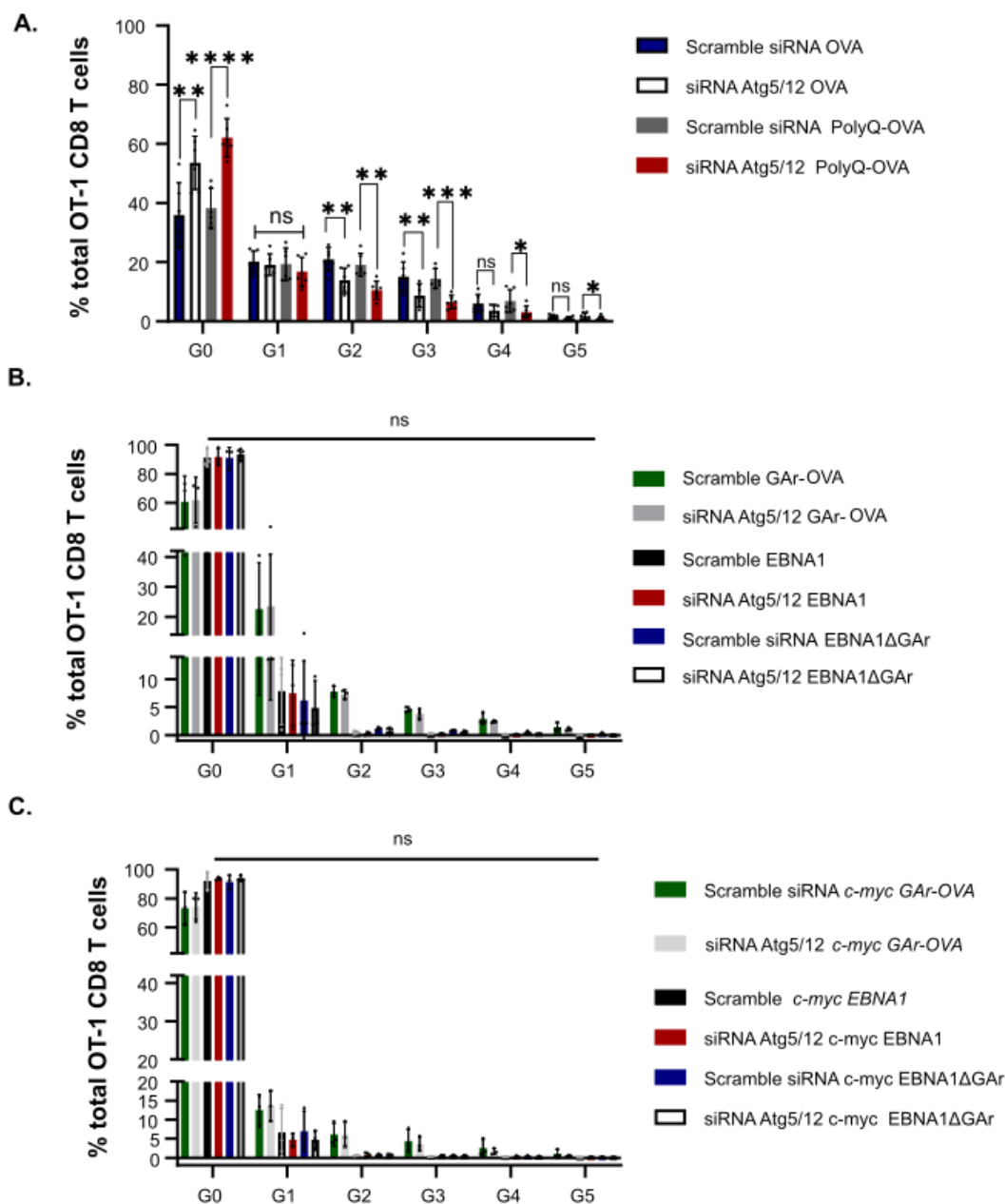
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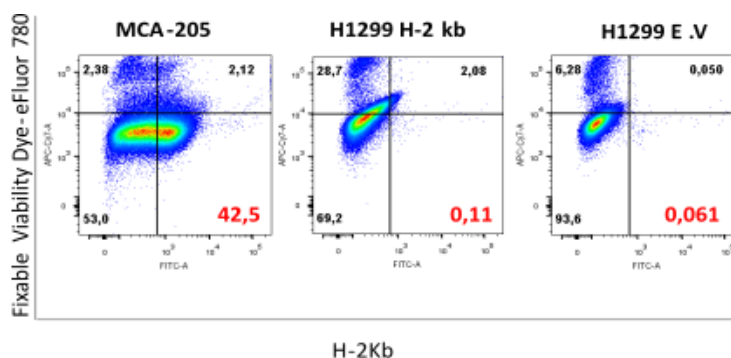
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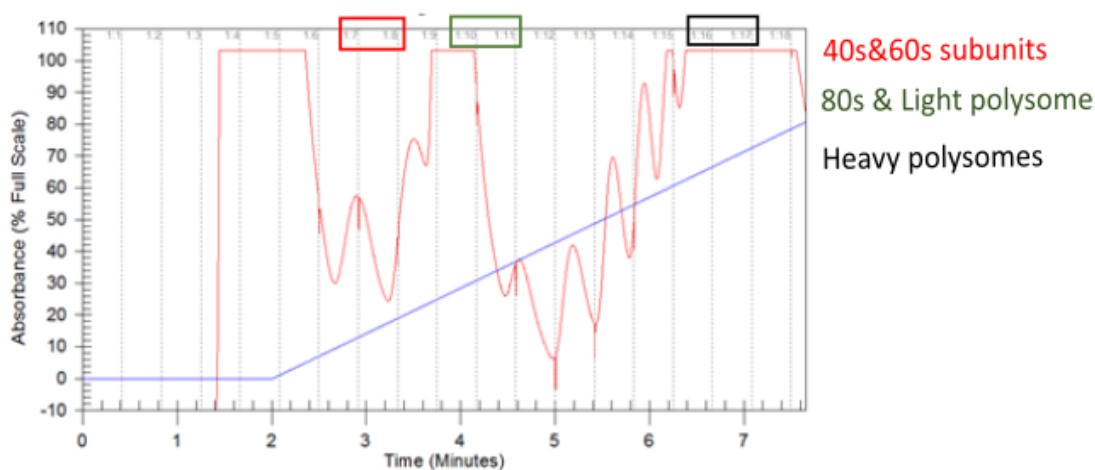
Supplementary Figure 1. Autophagy inhibition downregulates Atg5/12 protein levels and decreased LC3 II-I ratio of the presenting cells after co-culture. Western Blot showing the effect of autophagy inhibition in protein levels of **A.**GAr-OVA, OVA and PolyQ-OVA with, or without the *c-myc* in the 5' UTR **B.** EBNA1 and EBNA1ΔGAr with, or without, the *c-myc* in the 5' UTR. One representative experiment out of three is shown.



Supplementary Figure 2. Evaluation of autophagy dependence in antigen presentation assays. Co-culture of H1299 presenting cells with different endogenous antigenic substrates and OT-1 CD8⁺ T cells labeled with Cell-trace violet. The levels of OT-1 CD8⁺ T cells proliferation were analyzed by flow Cytometry. Percentage of total OT1 CD8⁺ T cells was calculated using the number of cells in each generation generated by the modeling of the Proliferation tool in Flow Jo software. The graphs show the percentage of cells from each generation compared with percentage of non-divided cells (generation 0). **A.** Presentation of antigenic derive peptides from OVA and PolyQ-OVA **B.** Presentation of antigenic derive peptides from GAR-OVA, EBNA1 and EBNA1ΔGAR **C.** Presentation of antigenic derive peptides from same constructs as B., but fused with the *c-myc* in the 5' UTR. Graphs represent 6 or 3 independent experiments. Significant values were calculated using Multiple paired T test grouped. *P < 0.0332; **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0,1234 ns, no significant.



Supplementary Figure 3. Antigen presentation measured from presenting cells. Directly H-2Kb was measured by cytometry in murine MCA cells, human H1299 cells expressing H-2kb and human H1299 cells expressing E.V.



Supplementary Figure 4. Representative polysome profiling from HEK cells. Square shapes show the fractions pooled representing the 40s & 60s subunits, 80s & light polysome, and heavy polysomes fractions selected to be submitted to LC-MS/MS analysis.

Table 1. Primers used in β -Globin polysome fractionation (Results Part2, Figure 7)

Primers qPCR	Sequence (5'-3')
I2Neo Forward	CTCGAGCTCGCGAAAGCTAGC
I2Neo Reverse	CCTCGAGATCTAGATATCGATGAATTG
I2SL8 Forward	GGGCACCTCCAGTATAATCAACT
I2SL8 Reverse	CTTCGCGAGCTCGAGTTTC
Ex1 Forward	GGTGAACGCCGATGAAGTTG
Ex2 Reverse	GCCATGGGCCTTCACTTTGG
Ex2 Forward	GCTGGTTGTCTACCCTTGGA
Actin Forward	TCACCCACACTGTGCCCATCTACGA
Actin Reverse	TGAGGTAGTCAGTCAGGTCCCG



Substrate-specific presentation of MHC class I-restricted antigens via autophagy pathway

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ABSTRACT

The accumulation of protein aggregates is toxic and linked to different diseases such as neurodegenerative disorders, but the role of the immune system to target and destroy aggregate-carrying cells is still relatively unknown. Here we show a substrate-specific presentation of antigenic peptides to the direct MHC class I pathway via autophagy. We observed no difference in presentation of peptides derived from the viral EBNA1 protein following suppression of autophagy by knocking down Atg5 and Atg12. However, the same knock down treatment suppressed the presentation from ovalbumin. Fusing the aggregate-prone poly-glutamine (PolyQ) to the ovalbumin had no effect on antigen presentation via autophagy. Interestingly, fusing the EBNA1-derived gly-ala repeat (GAR) sequence to ovalbumin rendered the presentation Atg5/12 independent. We also demonstrate that the relative levels of protein expression did not affect autophagy-mediated antigen presentation. These data suggest a substrate-dependent presentation of antigenic peptides for the MHC class I pathway via autophagy and indicate that the GAR of the EBNA1 illustrates a novel virus-mediated mechanism for immune evasion of autophagy-dependent antigen presentation.

1. Introduction

The cellular CD8⁺ T cell immune response is based on the recognition of antigenic peptides presented on the surface of host cells on the major histocompatibility complex (MHC) class I molecules. The presentation of antigenic peptides via the direct MHC class I pathway involves the degradation of the substrate by the proteasome, transport into the endoplasmic reticulum and further processing by peptidases and loading onto the MHC I molecules [1,2]. On the other hand, exogenous antigens endocytosed by professional presenting cells, such as dendritic cells or macrophages, are translocated to endosomal compartments and presented to the MHC I pathway via the so-called cross presentation pathway [1]. It was thought early on that peptides for the MHC I & II pathways were derived from processing of full length proteins but

studies have since discovered a more complex origin of MHC-I antigenic peptides, including peptides derived from the 3' untranslated sequences (UTRs) of mRNAs [3] and from introns [4–6], supporting a model in which non-canonical translation can provide antigenic peptide substrates.

The latent Epstein-Barr (EBV) and Kaposi's sarcoma viruses both target mRNA translation to evade the MHC-I pathway [7,8]. The EBV-encoded EBNA1 uses a glycine-alanine repeat (GAR) consisting of small non-polar amino acids that is prone to cause aggregates [9,10]. The GAR suppresses translation of any mRNA to which it is fused and this has been shown to minimize the presentation of antigenic peptides for the direct MHC class I pathway [7]. It consists of a stretch of up to 250 single glycine residues separated by one, two or three alanines. Inserting a single serine in every eight residue renders the GAR non-functional

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[11]. EBNA1 has been reported to be presented to the class II pathway via autophagy [12] but whether, or not, peptides for the class I pathway can be generated from processing of full length proteins via autophagy remains an open question. A cross-presentation study reported MHC I molecules on endolysosomal compartments [13] and it was suggested that endogenous human cytomegalovirus latency-associated protein (pUL138) can be presented to CD8⁺ T cells through autophagy [14].

The poly glutamine (PolyQ) is well known to cause aggregates to which it is fused and is implicated in various neurodegenerative diseases such as Huntington disease (HD), dentatorubral pallidolusian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA) and six spinocerebellar ataxias (SCA) [15].

Autophagy is a key degradative process of endogenous cytoplasmic proteins [16,17]. It was first defined as non-specific degradation process, but it was later revealed that autophagy has selectivity for specific cargos including, but not exclusively, aggregated proteins tagged with ubiquitin chains that are recognized by autophagy receptors bound to the autophagosomes membrane protein LC3 (Light chain 3) [18–20]. There are different autophagy such as microautophagy, chaperon mediated autophagy and macroautophagy [21]. In this study, we focused on the macroautophagy pathway that has been implicated in presenting EBNA1 to the class II pathway [12]. It involves the recruitment of ATG proteins, such as Atg5 and 12, to specific phagophore assembly sites (PAS) that elongates and traps a portion of the cytosol until it is sealed in the double membrane autophagosome vesicle. After trapping the engulfed cytosolic cargo, autophagosomes fuse to the lysosome to clear the cargo and the autophagic body [20]. It was recently proposed that the trafficking route of autophagosomes carrying cytoplasmic molecules fuse with endosomes carrying MHC class II molecules and thereby facilitate presentation of endogenous antigens on MHC II molecules [22].

In this study we have used the EBNA1 protein that is known to be processed by the autophagy pathway as well as protein aggregates caused by the poly-glutamine (PolyQ) repeat to address if autophagy is a source of peptide substrates for the MHC class I pathway.

2. Material and methods

2.1. Plasmids

The pCDNA3-EBNA1, pCDNA3-EBNA1ΔGAR, pCDNA3-Ovalbumin (OVA), pCDNA3-GAr-OVA and pCDNA3-*c-myc* GAr-OVA constructs were obtained as described previously [23].

c-myc EBNA1 and *c-myc* EBNA1ΔGAR were generated by amplification of full-length human *c-myc* by polymerase chain reaction (PCR), using a 5' sense primer containing a *Hind*III site 5' AATAAGCTTC-CACTGCTTACTGGCTTATCG 3' and a 3' antisense primer 5' TAAAAGCTTCGGCCGTTACTAGTGGATCC 3' containing another *Hind*III site. The fragment was cloned into the 5'UTR digested pCDNA3-EBNA1 and EBNA1ΔGAR constructs.

The OVA Poly 125 glutamine (Q) construct was made by digestion of OVA construct with *Eco*RI and *Xba*I enzyme and introducing 125 glutamine repetition sequence contained in a vector already mentioned previously [24].

2.2. Cell culture and transfection

H1299 cells (Human non-small cell lung carcinoma) were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% Penicillin- Streptomycin and mouse cell atlas (MCA-205) were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate and 1% Penicillin-Streptomycin. For antigen presentation experiments, cells were cultured in 6 wells plates (8x10⁴ cells/well) at 37 °C with 5% CO₂. The day after seeding and Atg5/12 siRNA induction, transfections were performed using 3 μl of Gene Juice reagent

according to the manufacture's protocol (Merck Bioscience). Cells were co-transfected with 0.5 μg of murine MHC class I molecule Kb and 1 μg of EBNA1, EBNA1ΔGAR, GAr-OVA and PolyQ-OVA cDNA carrying the SIINFEKL (SL8) epitope coding sequences in its open reading frame (ORF). In all antigen presentation assays, 1 μg of an OVA cDNA was used as positive control and the same quantity for the empty vector as negative control.

2.3. siRNA against Atg5/12

The day after seeding, cells were transfected with Human siRNAs or Murine siRNAs at 20 pM using Jet Prime reagent (Polyplus) following the manufacturer's instructions. The knock down of these proteins was evaluated by Real time PCR (qRT-PCR) and Western Blot at the end of 72 h incubation and 120 h.

Human siRNAs used were: two siRNAs against Atg12 (SI02655289 and SI04335513, Qiagen) and three siRNAs against Atg5 (SI02655310, SI02633946 and SI00069251, Qiagen).

Murine siRNAs used were: two siRNAs against Atg12 (SI00900319|S0 and SI00900333|S0, Qiagen) and three siRNAs against Atg5 (SI02696806|S0, SI02720186|S0 and SI02745435|S0, Qiagen).

2.4. Chloroquine treatment

The day after seeding, cells were treated with Chloroquine at [30 μM] during 36 h. The autophagy inhibition was evaluated by Western Blot assessing LC3-II accumulation.

2.5. Antigen presentation assay: OT1 CD8⁺ T cells proliferation

To determine the levels of antigen presentation, we used CD8⁺ T cells that express specific receptors to the OVA epitope, SIINFEKL, recognized by H-2 Kb. These CD8⁺ T cells were purified from OT1 transgenic mice expressing a transgenic TCR specific for SIINFEKL-Kb. Spleen and lymph nodes from OT1 transgenic mice were passed through a 70 μm cell strainer and red blood cells were lysed with ACK buffer treatment during 5 min. After several washes with PBS-FBS 5%, CD8⁺ T cells were negatively selected using a CD8⁺ T cell isolation kit (MACS Miltenyi Biotec) according to manufacturer's instructions. Afterwards, the CD8⁺ T cells were stained with CellTrace™ Violet at 5 μM during 10 min (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

Two days after transfection, H1299 cells used as presenting cells were briefly washed with PBS, trypsinized, resuspended in splenocytes medium (RPMI-1640), supplemented with 10% (FBS), 4 mM L-glutamine, 1% Penicillin-Streptomycin, 0.05 mM 2-Mercaptoethanol and 5 mM HEPES) and seeded in 48 wells plates (1.25x10⁵) cells per well. Then, 5x10⁵ CellTrace™ labelled T-cells were added per well and the co-cultures were incubated at 37 °C with 5% CO₂. The levels of antigen presentation were deduced from the percentage of T-cell proliferation verified by flow cytometry.

2.6. Flow cytometry analysis: OT1 CD8⁺ T cells proliferation

After 3 days, cells were harvested, stained with anti-mouse CD45.2-PE-Cy7 (BD Pharmingen), fixable viability dye eFluor® 506 (eBioscience, USA) and analyzed on a CANTO II flow cytometer (BD Biosciences, USA). Cells were gated for live CD45.2 + cells (4x10⁵ events collected) and data was analyzed using FlowJo software version 8 (Tree Star). The percentage of live CD8⁺ T cells in each generation was calculated using FlowJo proliferation platform and this value was considered for statistical analysis.

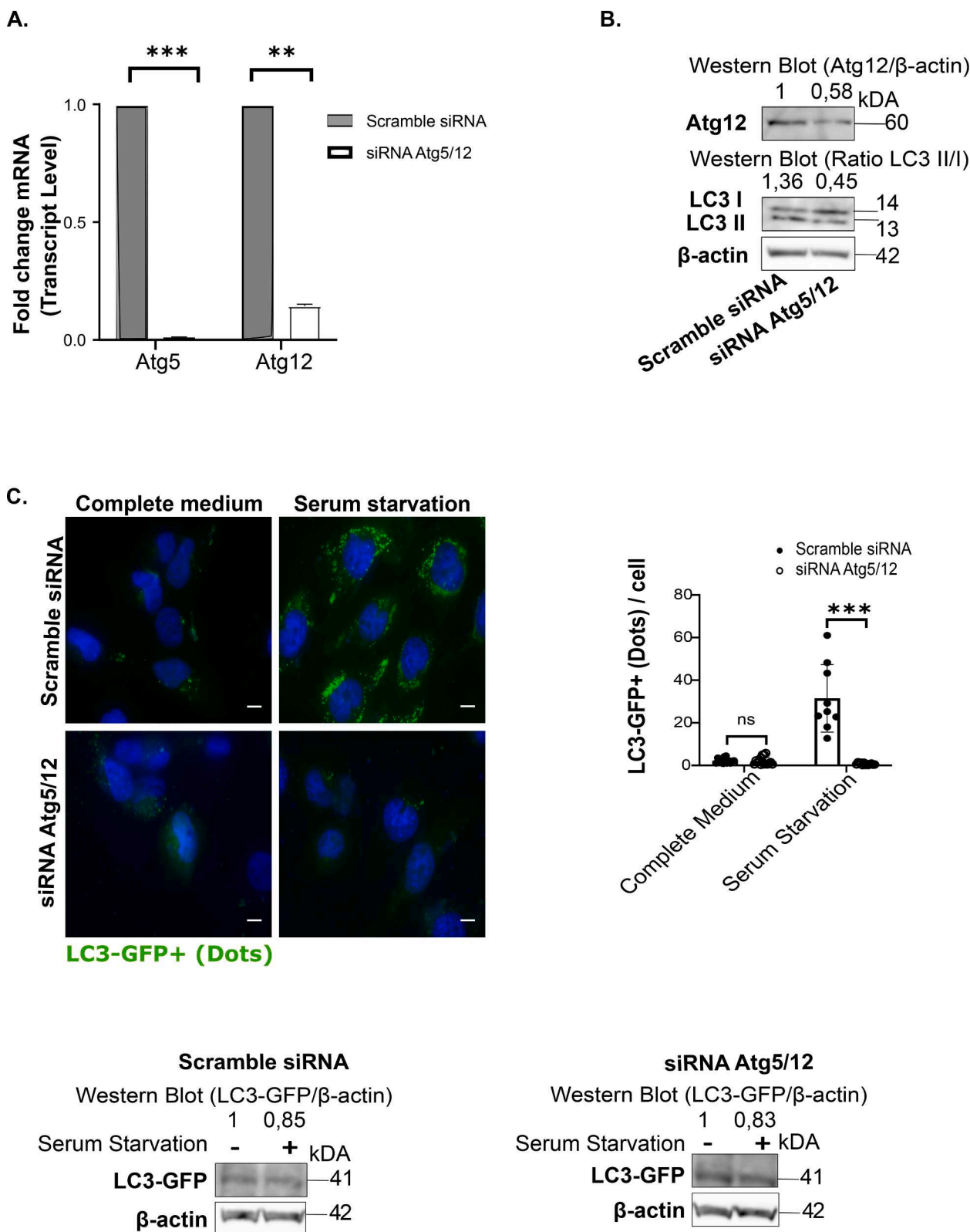
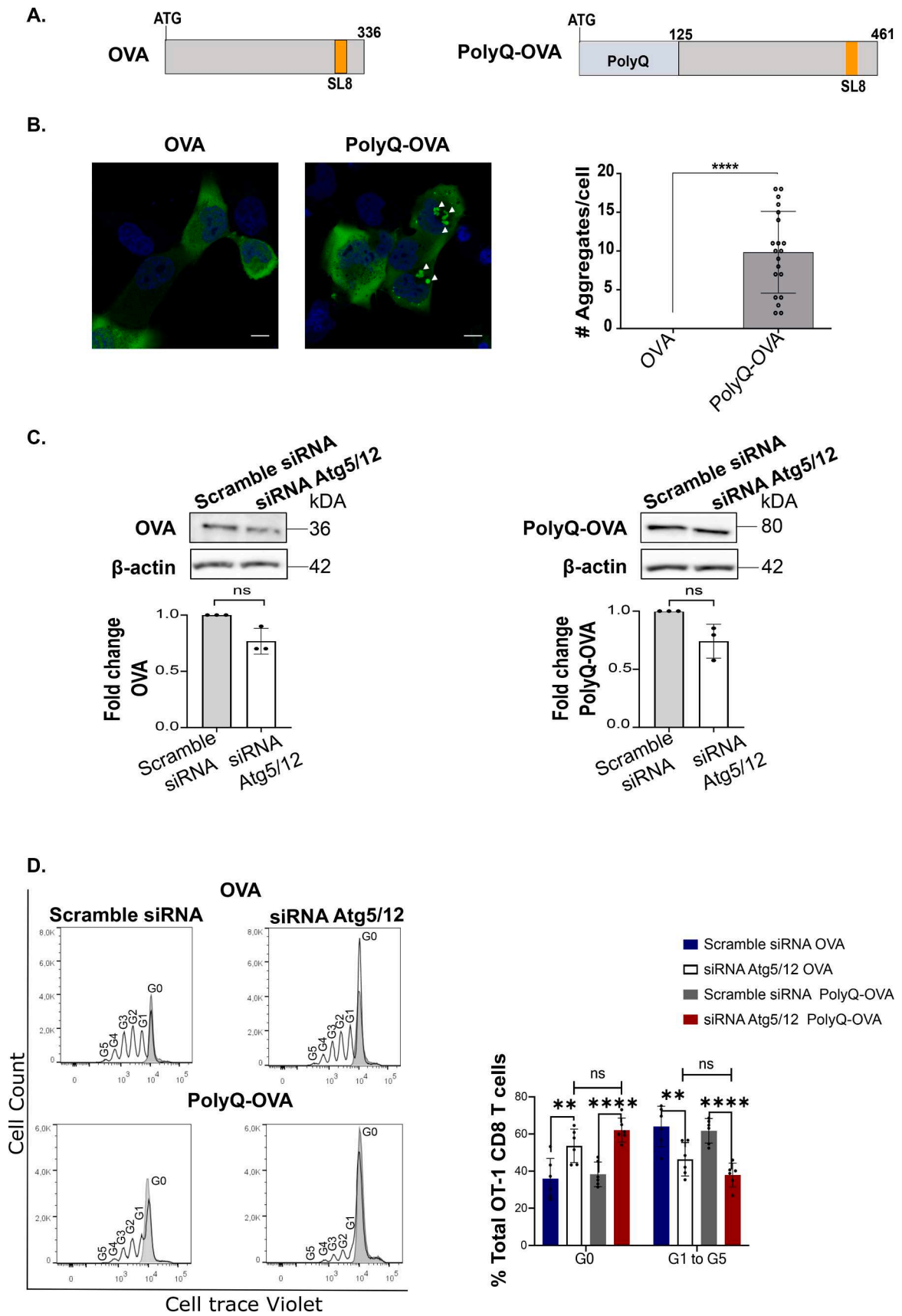


Fig. 1. Autophagy Inhibition. A. The *Atg5/12* mRNA levels were confirmed using RT-qPCR seventy-two hours following transfection of [20 pM] human siRNA against *Atg5/12* or scramble siRNA B. Western Blots show the expression of *Atg12*, *LC3 I* and *LC3 II*. Values above the bands show the densitometry analysis normalized against β -actin and the fold change compared with the scramble siRNA. Autophagy suppression was estimated by the ratio between *LC3 II* and *LC3 I* C. H1299 cells were transfected with a plasmid encoding *LC3-GFP* 24 h after treatment with siRNAs as in A and B. 48 h later, cells were treated without serum during two hours and then fixed. One of 10 fields is shown from one of three similar experiments. *LC3-GFP* fluorescence was observed as green dots, indicating autophagosomes formation. Number of GFP dots was calculated (top right graph). *LC3-GFP* expression was determined by Western Blot (bottom panels). Values above the bands show the densitometry analysis of bands normalized with β -actin and the fold change comparing the complete medium with the serum starvation treatment. Significant values were calculated using Multiple paired T test grouped. *** $P < 0.0002$; ** $P < 0.0021$; 0,1234 ns, not significant. White scale bars denote 10 μ m.



(caption on next page)

Fig. 2. Autophagy affects antigen presentation of Ovalbumin and Ovalbumin fused to the poly glutamine peptide **A.** Cartoon illustrating chicken ovalbumin (OVA) sequence with the location of the immune peptide SL8 and the glutamine repeat (PolyQ) **B.** Representative immunofluorescence image of OVA and PolyQ-OVA. White arrows heads indicate aggregation pattern. The graph shows the average number of aggregates observed **C.** Western Blot showing the effect of 72 h Atg5/12 human siRNA transfection on OVA and PolyQ-OVA expression. The graphs below show the densitometry analysis, normalized against β -actin and expressed in fold change compared with the scramble siRNA **D.** H1299 were transfected with human siRNA Atg5/12 [20 pM] or scrambled siRNA. 24 h later they were transfected with murine MHC-I (kb) and indicated constructs. After 48 h they were incubated with OT-1 CD8⁺ T cells labeled with cell-trace violet for another 72 h. OT-1 CD8⁺ T cell proliferation was analyzed by flow cytometry. Higher rate of proliferation indicates more antigen presentation. Open peaks in the histogram represent the proliferating populations and grey peaks denote unstimulated population (Empty Vector transfected cells) (left graph). The graph shows the sum of percentage of cells from generation 1 to 5 compared with percentage of non-dividing cells (generation 0) from 6 independent experiments (right graph). Significant values were calculated using Multiple paired T test grouped. *P < 0.0332; **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0.1234 ns, not significant. White scale bars denote 10 μ m.

2.7. Antigen presentation assay: direct measurement in the presenting cells

H1299 cells co-expressing murine MHC I Kb and the constructs mentioned above were submitted to Chloroquine treatment. Then, cells were harvested and stained with APC anti-mouse H-2 Kb bound to SIINFEKL Antibody (Biolegend) and Fixable viability 506 (eBioscience, USA). These cells were analyzed on a CANTO II flow cytometer (BD Biosciences, USA) and were gated for live cells. Data was analyzed using FlowJo software version 8 (Tree Star).

2.8. Direct measurement of MHC I Kb and HLA-ABC

MCA-205 and H1299 cells were submitted to murine or human Atg5/12 siRNA transfection. Then, cells were harvested and stained. MCA-205 cells with anti-mouse H-2Kb Antibody (Biolegend) and FITC anti-mouse IgG2a Antibody (Biolegend); H1299 cells with HLA-ABC FITC antibody (Invitrogen). Both cell types were also stained with Fixable viability 780 (eBioscience, USA). These cells were analyzed on a CANTO II flow cytometer (BD Biosciences, USA) and were gated for live cells. Data was analyzed using FlowJo software version 8 (Tree Star).

2.9. RNA extraction, RT-PCR and qRT-PCR

At 72 h post siRNA Atg5/12 transfection, H1299 cells were washed with PBS and RNA was purified using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. cDNA synthesis was carried out using M-MLV reverse transcriptase and oligo(dT) primers (Invitrogen). For qRT-PCR, the StepOne (Applied BioSystems) real-time PCR system was used, and the reactions were performed with the Perfecta SYBR green Fast mix ROX (Quanta) using specific primer pairs for human Atg5 (Forward: 5' GCTGCAGATGGACAGTTGCA 3' and Reverse: 3' TGTTCACTCAGCCACTGCAG 5'), human Atg12 (Forward: 5' ATGAC-TAGCCGGGAACACCA 3' and Reverse: 3' CAGCCTGAGACTTGAGTA 5'), murine Atg5 (Forward: 5'TGTGCTTCGAGATGTGTGGTT 3' and Reverse: 3' GGTCCCCTTGCACACTTACA 5') and murine Atg12 (Forward: 5'GCCATCTCACCAGCCCAATA 3' and Reverse: 3'CATGC CTGGGATTTGCAGT 5').

2.10. LC3-GFP induction

To confirm the blockage of autophagosomes formation by Atg5 and Atg12 siRNA, we performed epifluorescence microscopy. For this, we seeded 1.5×10^4 H1299 cells in a 24 well plate over a sterile 22x22mm cover slip. Then, cells were transfected with 20 pM of siRNA against Atg5/12 and 0.1 μ g of a LC3-GFP construct at 24 and 48 h after seeding, respectively. After 72 h of culture, cells were treated with a starvation buffer described elsewhere [25] (140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 20 mM Hepes, pH 7.4) during 2 h and complete RPMI-1640 medium was used for the negative control cells. Images were taken at 63x using the Axio Imager D2 microscope. All images were analyzed in Fiji software and the number of green dots was calculated as previously described [26].

2.11. Western Blot

Cells were trypsinised and the obtained pellets were resuspended with 50 μ l of lysis buffer (20 mM HEPES KOH, 50 mM β -Glycerol phosphate, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na₃VO₄, 100 mM KCl, 10% Glycerol and 1% Triton x-100, protease inhibitor cocktail Roche). Total lysates were obtained after mechanic hitting and freezing at -80 °C for at least 2 h. After, samples were centrifuged at 13 000 RCF during 10 min at 4 °C and supernatants were collected. Samples were quantified using Bradford Reagent (BioRad) and 50 μ g of protein were separated on 4–12% SDS-PAGE gels (Thermo Fisher Scientific) and transferred to nitrocellulose blotting membranes (Pall Corporation). After saturation of membranes with TBS- 0.5% Tween containing 5% non-fat milk, membranes were overnight incubated with anti-EBNA1 (16216–1-AP Abnova), anti-Atg12 (R&D systems), anti-chicken egg albumin (C6534 Sigma), anti-LC3B (L75443 Sigma), anti-GFP (11814460001 Roche) and anti-actin (AC-15 Sigma) antibodies. After washing with TBS-Tween, bound antibodies were detected using a rabbit anti-mouse (Dako) or a mouse anti-rabbit (Dako) secondary antibody conjugated to horseradish peroxidase (1:1000; 1 h at room temperature). Immunocomplexes were then revealed with ECL (Thermo scientific) and imaged using a MyECL Imager (Thermo scientific).

2.12. Immunofluorescence

H1299 cells were seeded as described for LC3-GFP induction experiments and transfected with 0.8 μ g of EBNA1, EBNA1 Δ GA_r, GAR-OVA, EBNA1 *c-myc*, EBNA1 Δ GA_r *c-myc*, GAR-OVA *c-myc*, OVA, PolyQ-OVA or empty vector. Samples were fixed with 4% paraformaldehyde and permeabilized with 0.4% Triton x-100 0.05% CHAPS PBS. Afterwards, cells were blocked with 3% Bovine serum albumin (BSA) Saponin 0.1% PBS during 1 h and then incubated with mouse anti-EBNA1 (16216–1-AP Abnova) or rabbit anti-egg albumin (C6534 Sigma) during 1 h at room temperature. After two washes with PBS, samples were incubated with an anti-mouse Alexa 488 or anti-rabbit Alexa 647 antibodies during 1 h at room temperature. Next, samples were washed with PBS, stained with DAPI and mounted with a fluorescence mounting media (Dako). Samples were examined in a LSM 800 confocal laser microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and images were treated using the Fiji software.

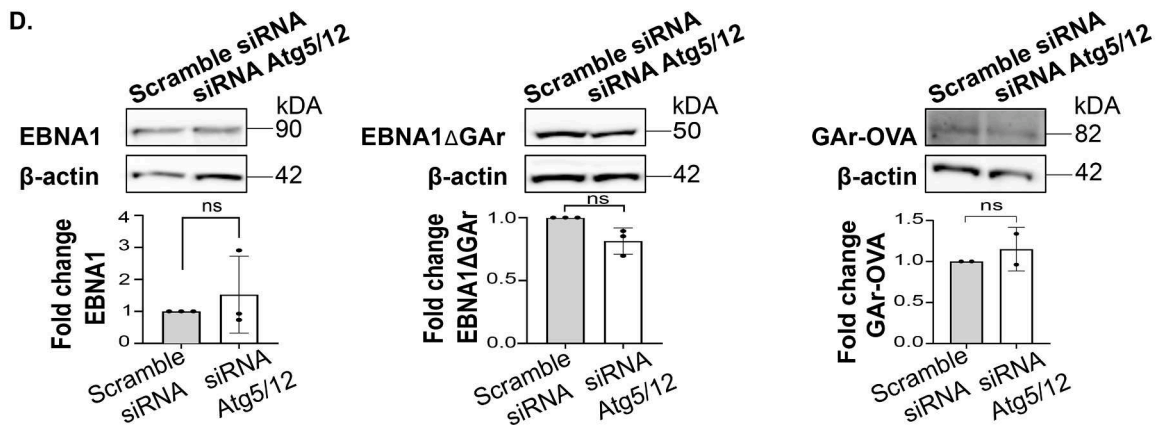
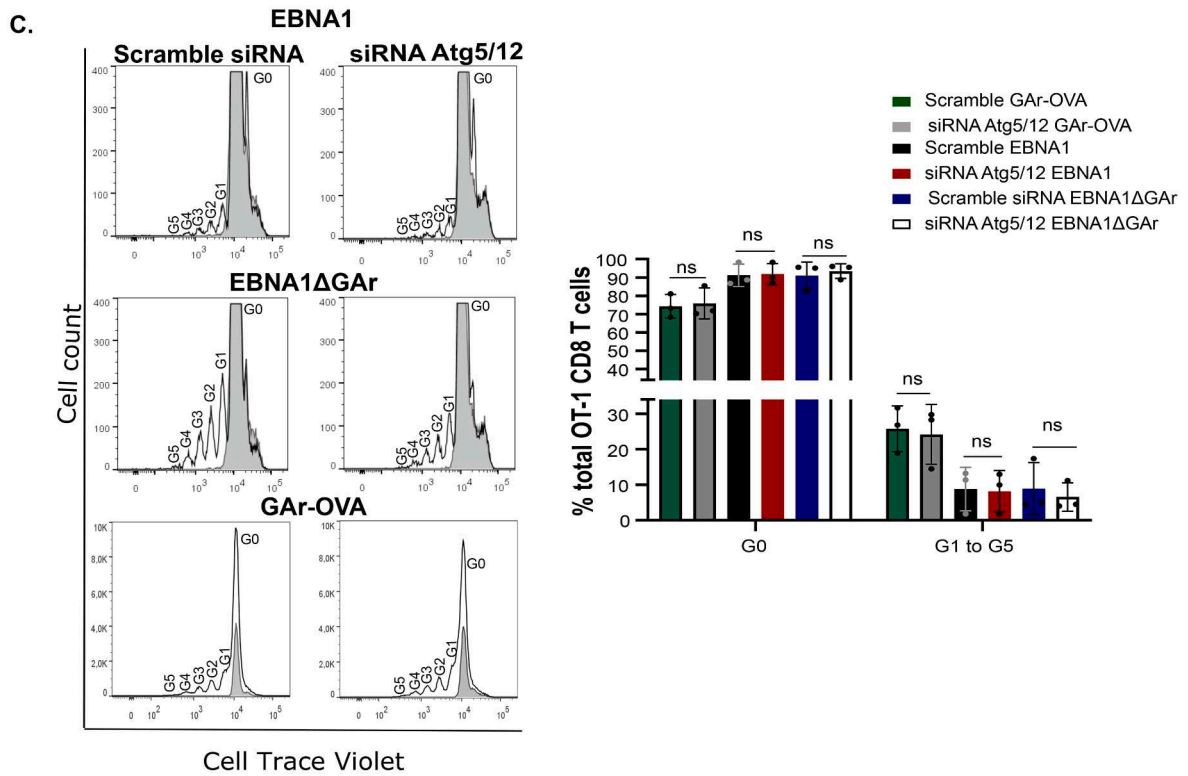
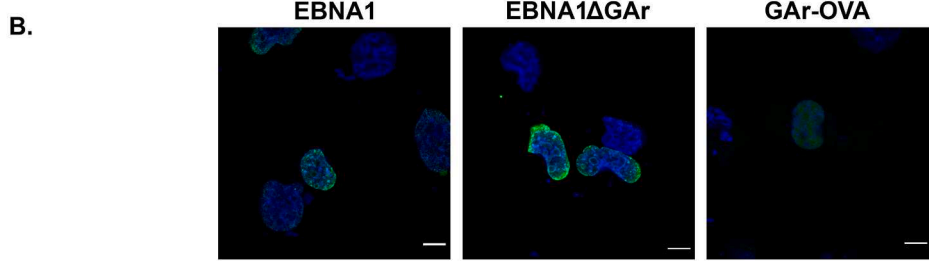
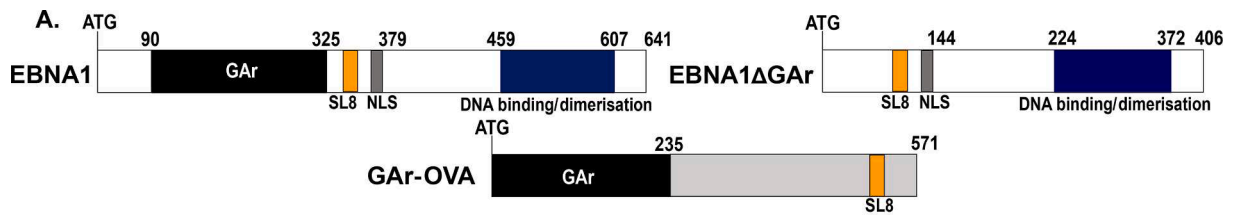
2.13. Statistics

Data were analyzed by two-tailed unpaired Student's *t*-test or One sample *T*-test using GraphPad Prism 6 for Windows (GraphPad Software). Data shown are mean \pm sd. of minimum three independent experiments. *P < 0.0332; **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0.1234 ns, not significant.

3. Results

3.1. Knocking down Atg5 & Atg12 blocks autophagy in H1299 cells.

In order to evaluate the role of autophagy in antigen presentation to the MHC class I pathway we knocked down the expression of Atg5 and



(caption on next page)

Fig. 3. Fusion of the EBNA1-derived gly-ala repeat (GAR) sequence suppresses Atg5/12-dependent antigen presentation. **A.** Cartoon illustrating different EBNA1 constructs with, or without, the GAR (EBNA1ΔGAR) and GAR fused to Ovalbumin. The location of the nuclear localization signal (NLS), the DNA binding/dimerization sequence in EBNA1 and the SL8 epitope are indicated. **B.** Representative immunofluorescence image of EBNA1, EBNA1ΔGAR and GAR-OVA. **C.** H1299 cells co-expressing murine MHC-I (Kb) and the indicated constructs were transfected with human siRNA Atg5/12 [20 pM] or scramble siRNA during 72 h like in Fig. 2D. The graph shows the percentage of cells from generation 1 to 5 compared with percentage of non-divided cells (generation 0) from 3 independent experiments (right graph) **D.** Western Blots show one out of three representative experiments on the effect of autophagy inhibition on EBNA1, EBNA1ΔGAR and GAR-OVA protein expression levels. The graphs show densitometry analysis normalized against β-actin and expressed in fold change compared with the scramble siRNA. Significant values were calculated using Multiple paired T test grouped. Not significant ns: 0.1234. White scale bars denote 10 μm.

Atg12 using specific siRNAs. These proteins are crucial in the conjugation system that allows the formation of autophagosomes and their downregulation is reported to block the macroautophagy pathway (from here on simply referred to as autophagy) [12,27,28]. The efficiency of siRNA treatments was confirmed by the downregulation of Atg5/12 at both mRNA (Fig. 1A) and protein levels (Fig. 1B, upper lane). siRNA treatments resulted in a decrease of LC3 II-I ratio (Fig. 1B, middle lane) and suppressed autophagy flux following serum deprivation (Fig. 1C, upper part) Of note, LC3-GFP protein levels did not change under serum starvation (Fig. 1C, bottom part). Together, these data show that the siRNA against Atg5/12 interfere with the autophagy pathway in H1299 cells.

3.2. Preventing autophagy reduces MHC class I antigen presentation independently of protein aggregate formation.

Autophagy can degrade harmful cytosolic protein, including aggregates, [18–20] and we tested the capacity of this pathway to process protein substrates for the MHC-I pathway. We used a chicken OVA construct whose secretion was blocked by the deletion of the first 50 amino acids [29]. This construct enabled us to study the antigen presentation via the MHC-I pathway using CD8⁺ T cells from OT-1 mice that specifically recognize the OVA-derived SL8 antigenic peptide in the context of the murine Kb MHC class I molecule. We also used a poly-glutamine repetition (PolyQ), well known to cause aggregates and to be processed by autophagy [30–33] that we fused to OVA (Fig. 2A). We used a GFP construct to estimate transfection efficiency of approximately 30% to 50% of cells (Suppl. Fig. 1). Immunohistochemistry assays using anti-OVA antibodies showed that PolyQ-OVA forms approximately 10 aggregates per cell (white arrow heads) while OVA was uniformly stained throughout the cells and no visible aggregate detected (Fig. 2B). Expression of the reporter constructs were not significantly affected by siRNA against ATG5/12 (Fig. 2C and suppl. Fig. 2). We did not detect an accumulation of PolyQ-OVA upon ATG5/12 knock down, presumably due to the fact that the PolyQ-OVA is not present only in the aggregate conformation (Fig. 2B) due to the limited time (24 h) of expression. To test the role of autophagy on the processing of antigenic peptide substrates for the MHC class I pathway, we co-expressed the indicated SL8-carrying constructs together with the Kb MHC cDNA in human H1299 cells. Transfected cells were subject to autophagy inhibition through Atg5/12 siRNA treatment and antigen presentation was evaluated by co-culture with OT1 CD8⁺ T-cells. The relative level of antigen presentation was estimated by OT1 CD8⁺ T-cells proliferation using flow cytometry. For every assay we confirmed suppression of autophagy by in parallel estimating the LC3 I/II ratio (Fig. 1B and data not shown). We observed that under Atg5/12 knock down, OVA and PolyQ-OVA showed a higher percentage of cells in the non-proliferating OT1 CD8⁺ T cell population (G0) and a corresponding decrease in the proliferating population (G1 to G5), indicating a reduction of antigen presentation (Fig. 2D). The percentage of OT1 CD8⁺ T cells in each generation is shown in (Suppl. Fig. 3A). Despite being uniformly expressed and showing no apparent formation of aggregates, it was surprising to see that knocking down Atg5/12 affected the presentation of antigenic peptides from OVA as much as from PolyQ-OVA.

3.3. MHC class I-restricted presentation of peptides derived from EBNA1 is not affected by suppressing autophagy.

The Epstein-Barr virus-encoded EBNA1 has been reported as an aggregate prone protein and this feature has been attributed to the long repeat of non-polar gly-ala residues (GAR) [9,10]. Since EBNA1-derived antigenic peptides are processed for the MHC class II pathway via autophagy [12] and autophagy is associated to the clearance of proteins, including aggregates [18–20], we wanted to know if EBNA1-derived peptides can also be presented for the MHC class I pathway through autophagy. We inserted the antigenic SL8 peptide into the EBNA1 open reading frame (ORF), or in an EBNA1 depleted of the GAR-domain (EBNA1ΔGAR). We also used a construct carrying the GAR-domain fused to OVA cDNA (GAR-OVA) (Fig. 3A). To test if EBNA1 shows the same aggregation pattern observed for PolyQ-OVA, we performed immunohistochemistry assays. However, we observed no obvious aggregates of EBNA1, EBNA1ΔGAR or GAR-OVA and no differences in subcellular localisation with, or without, the GAR (Fig. 3B). The GAR mediates suppression of antigenic peptides for the MHC class I pathway by inhibiting EBNA1 mRNA translation *in cis* [7]. In agreement with this, we observed a low percentage of CD8⁺ T cell proliferation in response to SL8 derived from EBNA1 and GAR-OVA, as compared to EBNAΔGAR (Fig. 3C) and OVA (Fig. 2D). Importantly, we observed no significant difference between percentages of OT-1 CD8⁺ T cells in the undivided (G0) or in the proliferating populations (G1 to G5), for any of the tested conditions following Atg5/12 siRNA treatment (Fig. 3C and suppl. Fig. 3B). We also showed that Atg5/12 knock down had no significant effect on EBNA1, EBNA1ΔGAR and GAR-OVA expression (Fig. 3D and suppl. Fig. 2). In addition, we observed no effect on antigen presentation of EBNA1, EBNA1ΔGAR or GAR-OVA following treatment with the autophagy inhibitor drug Chloroquine (Suppl. Fig. 4). These results support the idea that the autophagy pathway does not provide EBNA1-derived antigenic peptides for the class I pathway and that the fusion of the GAR prevents OVA from being presented via autophagy.

3.4. The level of protein expression does not determine MHC class I restricted antigen presentation via the autophagy pathway.

The above results were surprising considering that OVA alone, or OVA fused to the PolyQ, present antigenic peptides in an Atg5/12-dependent fashion, while this antigen presentation pathway is prevented by the fusion of the GAR. We next set out to test if the effect of the GAR on antigen presentation is associated with its effect on suppressing mRNA translation *in cis*. For this we fused the *c-myc* 5'UTR to the 5' of the EBNA1, EBNAΔGAR and GAR-OVA (Fig. 4A). The presence of the *c-myc* sequence overcomes the translation inhibitory capacity of the GAR and restores protein synthesis without altering the coding sequence [23]. Western blots and Immunofluorescence showed that the insertion of the *c-myc* sequence resulted in the expected increase in expression of EBNA1 and GAR-OVA but not EBNAΔGAR (Fig. 4B and suppl. Fig. 5A), and did not affect the subcellular localization (Fig. 4C). Atg5/12 knock down did not affect the expression of either construct (Fig. 4D and suppl. Fig. 2). When we compared antigen presentation we observed the expected increase in presentation from the *c-myc*-carrying GAR-OVA construct, as compared to GAR-OVA alone (Suppl. Fig. 5B). Importantly, there was no significant difference in antigen presentation between *c-myc* carrying constructs following Atg5/12 knock down.

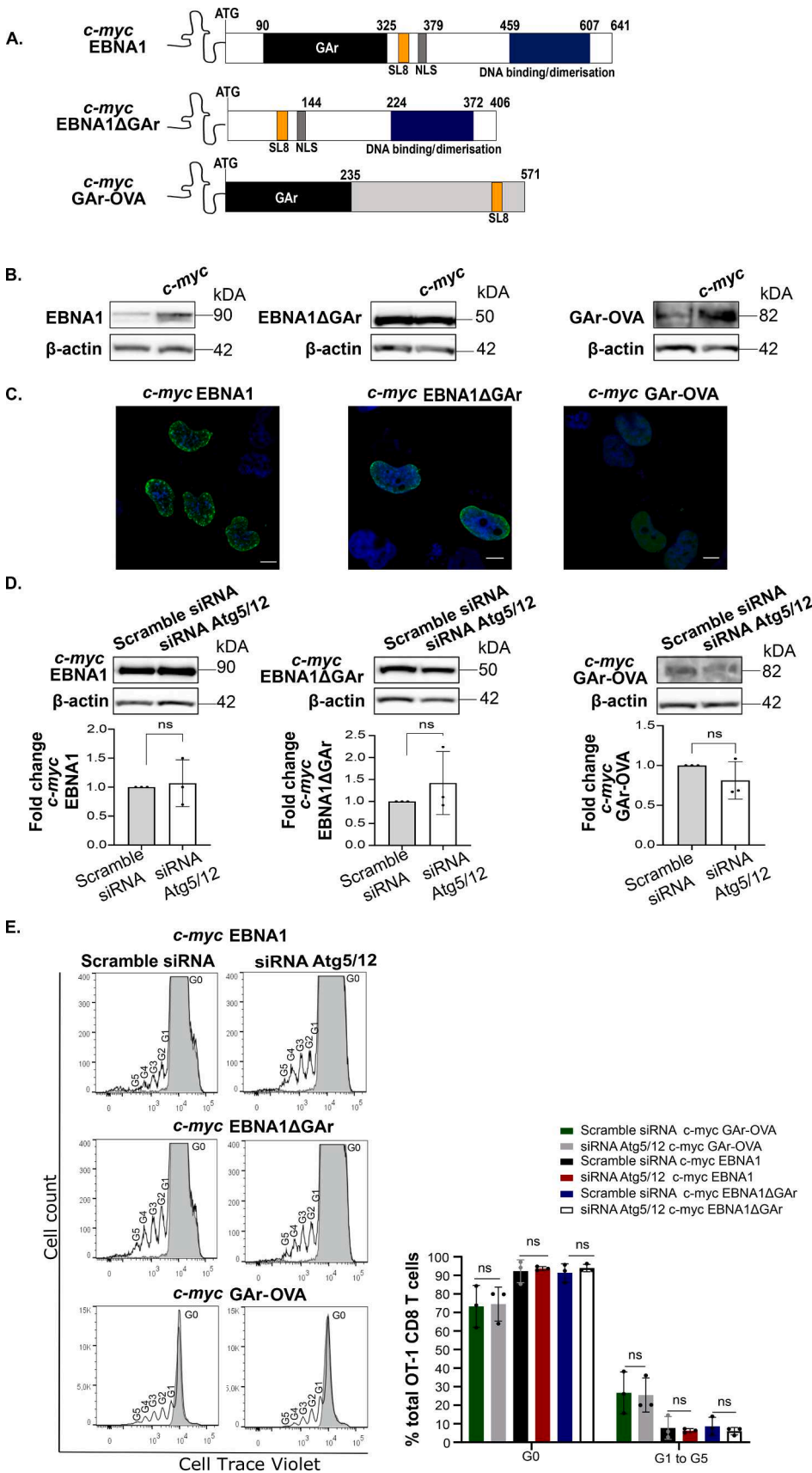


Fig. 4. Protein levels do not change autophagy-dependent antigen presentation. A. Cartoon illustrating the location of *c-myc* 5' UTR RNA sequence inserted in the 5'UTR of EBNA1, EBNA1ΔGAR and GAR-OVA. B. The *c-myc* fused to the 5' UTR of EBNA1, EBNA1ΔGAR and GAR-OVA constructs overcomes GAR-mediated mRNA translation suppression. Western blots show the differences in protein expression levels. C. Representative immunofluorescence of EBNA1, EBNA1ΔGAR and GAR-OVA constructs carrying the *c-myc*. D. Western Blot showing the effect of autophagy inhibition on protein levels of the indicated constructs. The graphs show densitometry analysis, normalized against β-actin for all targeted proteins and expressed in fold change compared with the scramble siRNA. E. H1299 cells co-expressing murine MHC-I (Kb) and the indicated constructs following human siRNA Atg5/12 [20 pM] or scramble siRNA treatment during 72 h. The antigen presentation was estimated as described in Figs. 2 and 3. The graph shows the percentage of cells from generation 1 to 5 compared with percentage of non-divided cells (generation 0) from 3 independent experiments (right graph). Significant values were calculated using Multiple paired T test grouped. Not significant ns: 0.1234. White scale bars denote 10 μm.

(Fig. 4E and suppl. Fig. 3C). These results show that the levels of protein expression do not affect autophagy-dependent presentation of antigenic peptides derived from EBNA1 or from GAR-Ova for the MHC class I pathway.

4. Discussion

Alternative sources of peptides for the MHC class I pathway have been proposed but if, and to what extent, peptides derived from the processing of peptide substrates via the autophagy pathway can be presented to the class I pathway is poorly investigated. The PolyQ sequence is linked to several neurodegenerative diseases, including Huntington's disease, and is well known to cause aggregates of proteins to which it is fused [15,34]. The GAR is a disordered domain derived from the EBNA1, a viral protein expressed in all Epstein-Barr virus (EBV)-infected cells [35], and known to cause aggregates [9,10]. EBV needs to ensure that EBNA1-expressing cells are not detected and destroyed by the immune system and it has previously been shown that EBNA1 uses a mechanism based on minimizing EBNA1 synthesis to evade MHC class I pathway and CD8⁺ T cell recognition. At the same time, EBNA1's low turnover rate ensures that a sufficient amount of EBNA1 is expressed to support the virus [36]. The inhibition of synthesis and stability are both mediated by the GAR sequence [7]. However, although autophagy has been shown to contribute to the processing of EBNA1 for the MHC class II pathway [12], our data suggest that this mechanism is not involved in the production of EBNA1-derived substrates for the MHC class I pathway. This raises the possibility that EBNA1 has evolved a mechanism to specifically evade autophagy-mediated class I- but not class II-restricted antigen presentation. In line with the notion of an active EBNA1-mediated mechanism to evade class I-restricted antigen presentation, we observed that when the GAR is fused to OVA it prevents OVA from being presented via autophagy. This suggests that evasion of autophagy-mediated MHC class I-restricted antigen presentation is another mechanism employed by viruses to remain undetected by the immune system.

Although the fusion of the PolyQ sequence to the OVA led to the formation of aggregates, it did not alter Atg5/12-dependent change in MHC class I-restricted antigen presentation, suggesting that aggregates alone is not the key to antigen presentation via autophagy. This is supported by the observation that OVA alone, nor EBNA1, results in any obvious aggregate formation, at least which could be detected by the methods used here. Nevertheless, it is interesting that the disordered glyala domain that is known to affect protein folding and unfolding, [24] prevents presentation to the class I pathway via synthesis and autophagy suppression. If this reflects a more general mechanism to evade the class I pathway, or if it is restricted to the GAR, remains to be seen. The reporter constructs we used carries the PolyQ and the GAR sequences in the N-termini of the OVA reporter constructs and even though the GAR is located inside the EBNA1 protein, it is possible that the location of the GAR and the PolyQ can affect how substrates are presented to the class I pathway via autophagy.

By inserting *c-myc* 5' UTR upstream of GAR-carrying constructs we could override its translation inhibitory capacity and show that protein expression levels have little effect on GAR-mediated evasion of antigen presentation via autophagy. This points towards a more selective mechanism for how peptide substrates are presented to the class I pathway by autophagy and has interesting implications for understanding not only the cell biological aspects of how proteins are processed by autophagy, but also in terms of disease etiology. Animal studies have suggested that the inflammasome plays a role in Alzheimer disease, indicating that the immune response can play a role in the etiology of neurological disease associated with protein aggregates [37,38]. It is an interesting possibility that there could be a selective autophagy-dependent processing of cellular disease-associated substrates for the MHC I and II pathways. Further studies using more substrates and deeper analysis of autophagy pathways shall confirm, or not,

this possibility. The implication of autophagy in the clearance of intracellular protein aggregates associated with polylglutamine disorders such as Huntington disease (HD) is known [15] and Qin and colleagues showed that autophagy inhibition reduced cell viability and increased Huntingtin protein aggregation [34].

It is unlikely that the knock down of Atg5/12 affects the MHC class I pathway *per se* as the effect we observed are substrate-specific and secondly, that the addition of synthetic SL8 peptide to the Kb class I molecules did not show any difference during Atg5/12 knock down (Supplementary Fig. 6A) and neither in the membrane location of endogenous MHC-I Kb molecules in murine MCA-205 cells or HLA-ABC molecules in human H1299 cells (Supplementary Fig. 6B).

In line with our results, Liu and colleagues implicated OVA as being a substrate for autophagy and showed that mice immunized with OVA caused an allergy reaction and induced activation of autophagy accompanied by a relative increase of LC3 II compared to LC3 I in eosinophils cells from lung tissues [39]. Our study shows autophagy-dependent presentation of OVA for the direct class I pathway but other studies have associated autophagy with cross-presentation via uptake of substrates by dendritic cells. For example, polyQ fused to OVA was shown to be presented to the MHC class I pathway following injection into mice [30].

Taken together, this study shows a substrate-specific presentation of peptides via autophagy that is selective for the MHC class I pathway. It has interesting implications for viral immune evasion and for inflammatory reactions associated with disease in which cellular proteins are processed by autophagy.

Acknowledgements

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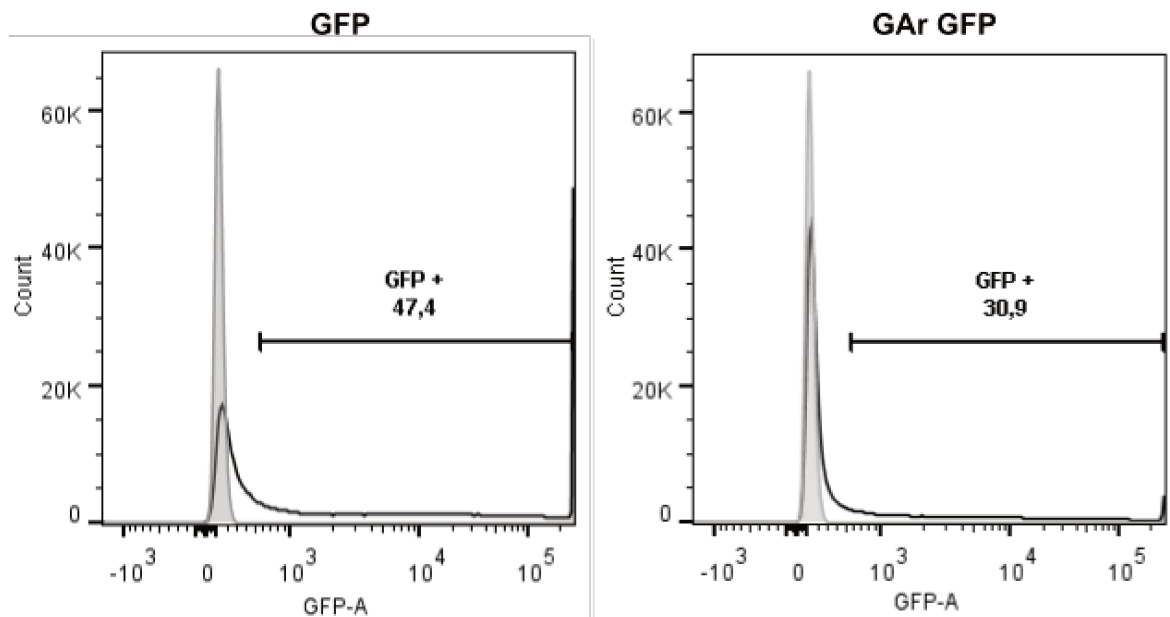
Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2022.104484>.

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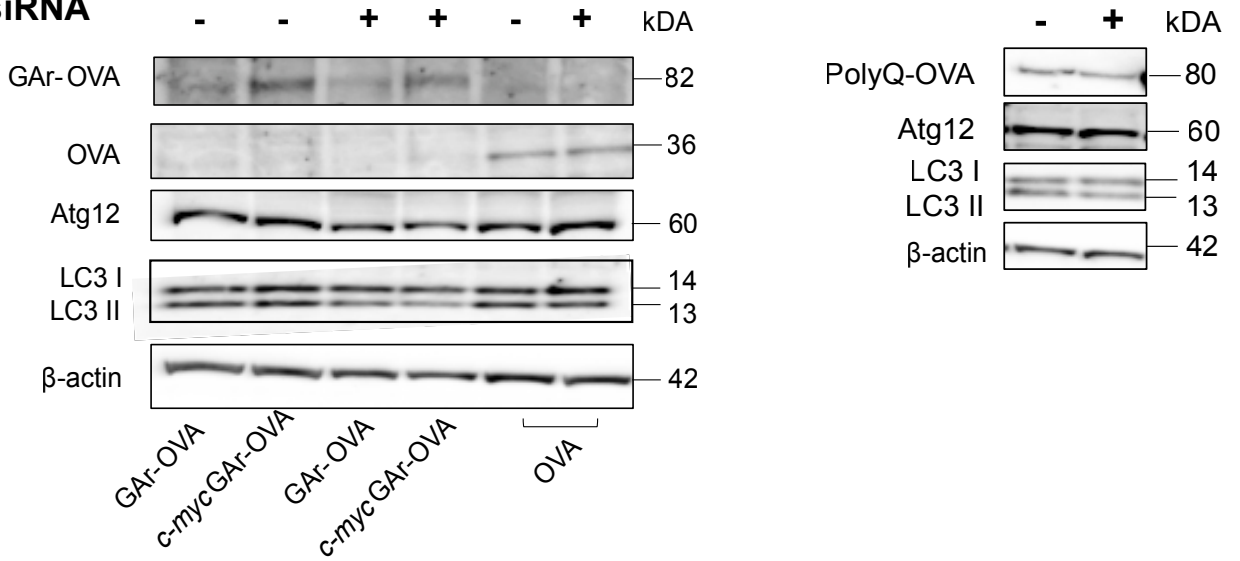
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Supplementary Figure 1. Quantification of transfection efficiency it is around 31 to 50%. H1299 cells were transfected with 1 μ g of GFP or GAr GFP cDNA constructs. After 48 hours cells were harvested and quantified by flow Cytometry. Gray histogram shows cells expressing empty vector (not fluorescent cells) and open histograms the corresponding cDNA constructs.

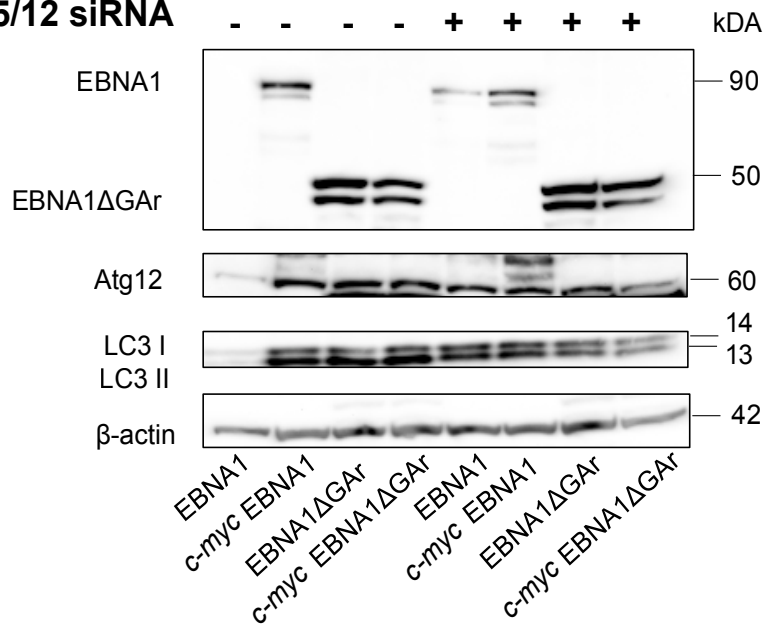
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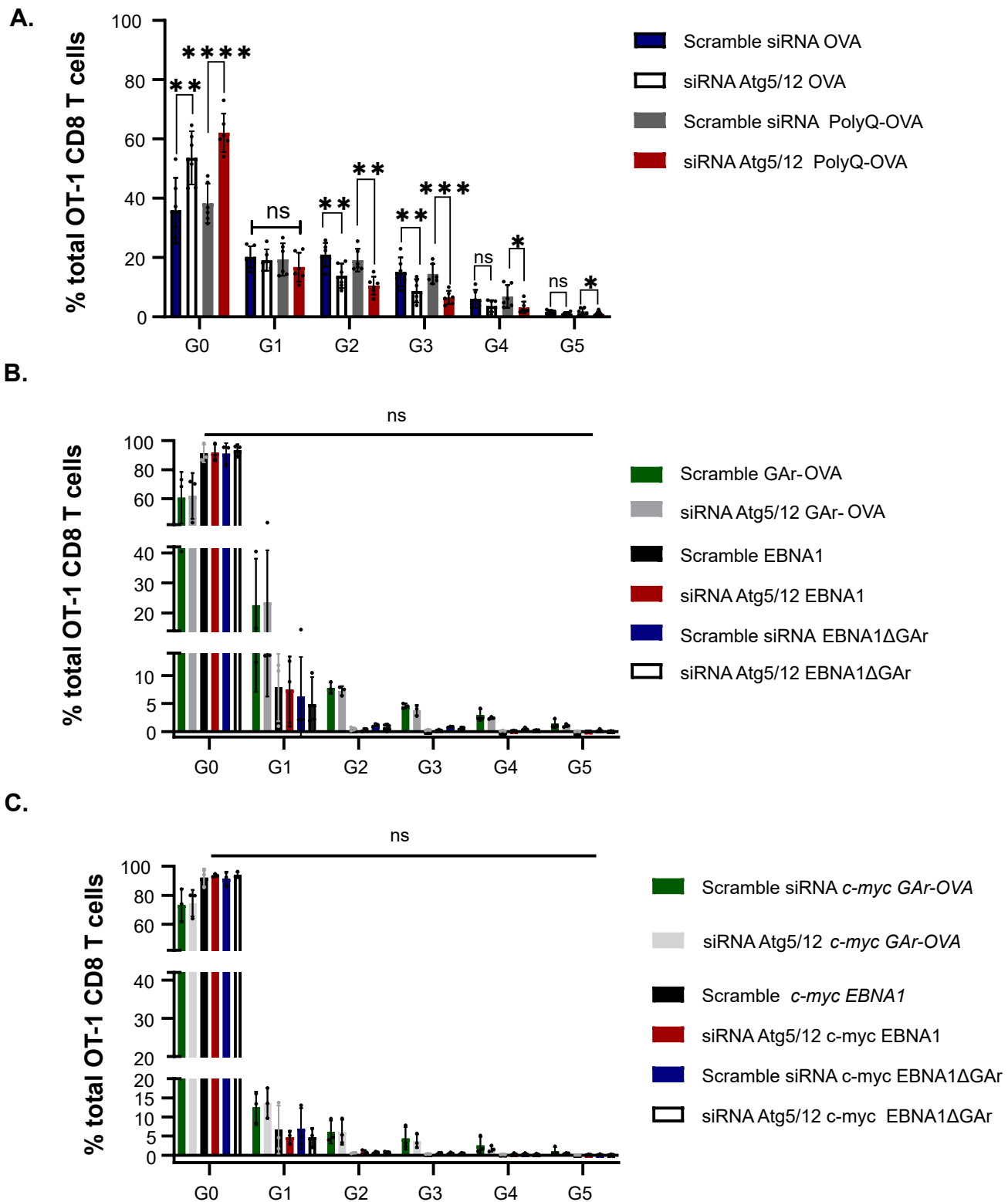


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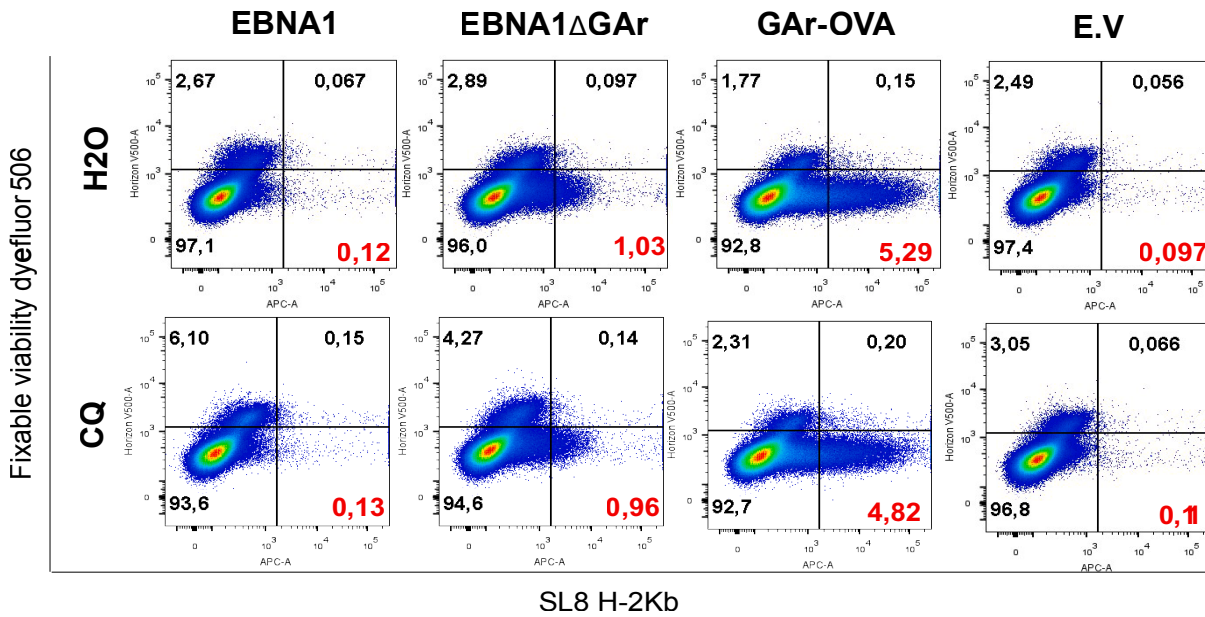


Supplementary Figure 2. Autophagy inhibition downregulates Atg5/12 protein levels and decreased LC3 II-I ratio of the presenting cells after co-culture. Western Blot showing the effect of autophagy inhibition in protein levels of **A.** GAR-OVA, OVA and PolyQ-OVA with, or without the *c-myc* in the 5' UTR **B.** EBNA1 and EBNA1 Δ GAr with, or without, the *c-myc* in the 5' UTR. One representative experiment out of three is shown.

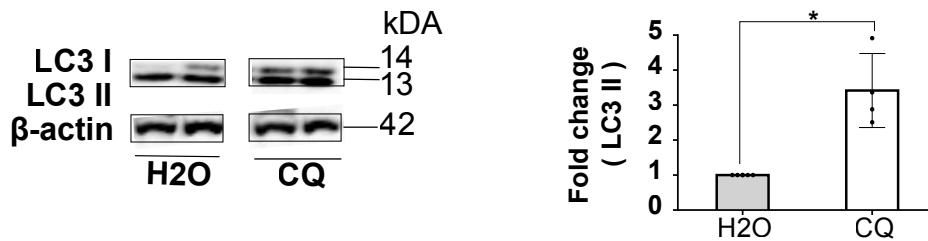


Supplementary Figure 3. Evaluation of autophagy dependence in antigen presentation assays. Co-culture of H1299 presenting cells with different endogenous antigenic substrates and OT-1 CD8⁺ T cells labeled with Cell-trace violet. The levels of OT-1 CD8⁺ T cells proliferation were analyzed by flow Cytometry. Percentage of total OT1 CD8⁺ T cells was calculated using the number of cells in each generation generated by the modeling of the Proliferation tool in Flow Jo software. The graphs show the percentage of cells from each generation compared with percentage of non-divided cells (generation 0). **A.** Presentation of antigenic derive peptides from OVA and PolyQ-OVA **B.** Presentation of antigenic derive peptides from GAR-OVA, EBNA1 and EBNA1ΔGAR **C.** Presentation of antigenic derive peptides from same constructs as B., but fused with the *c-myc* in the 5' UTR. Graphs represent 6 or 3 independent experiments. Significant values were calculated using Multiple paired T test grouped. *P < 0.0332; **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0,1234 ns, no significant.

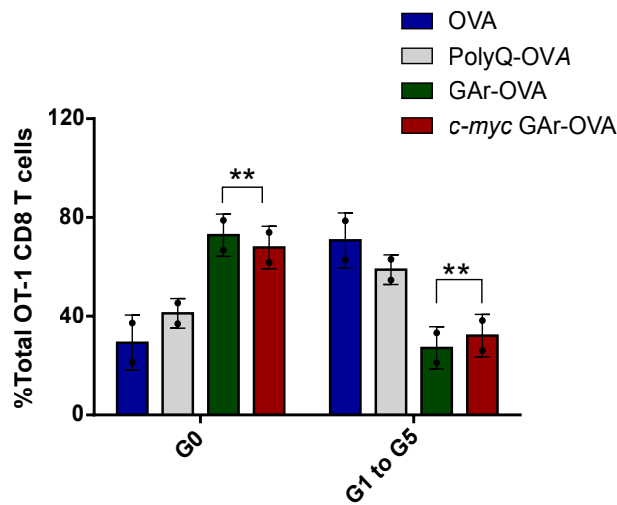
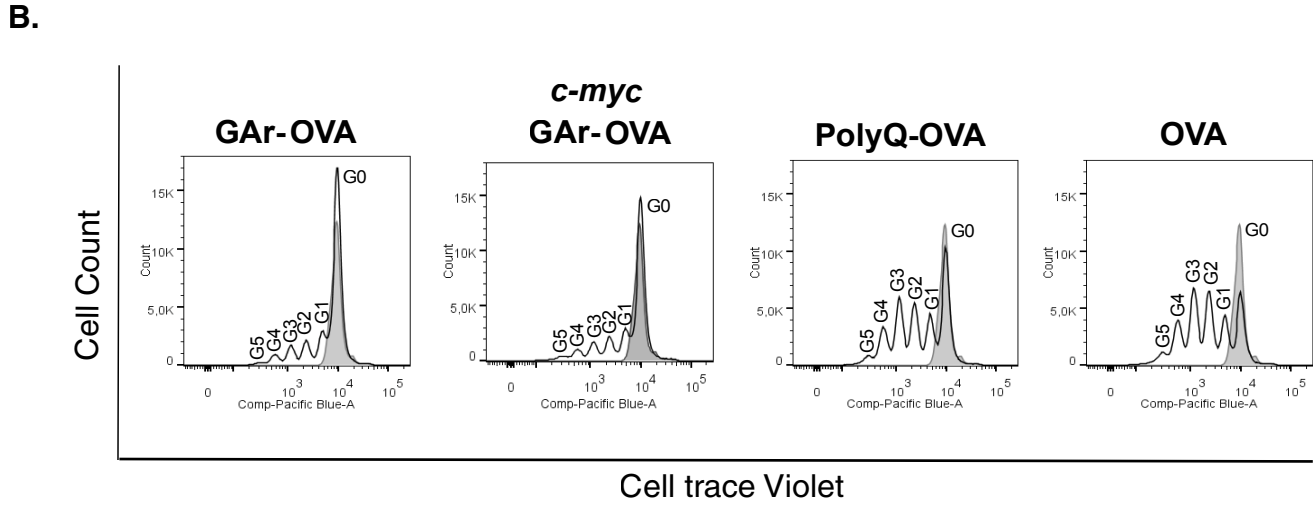
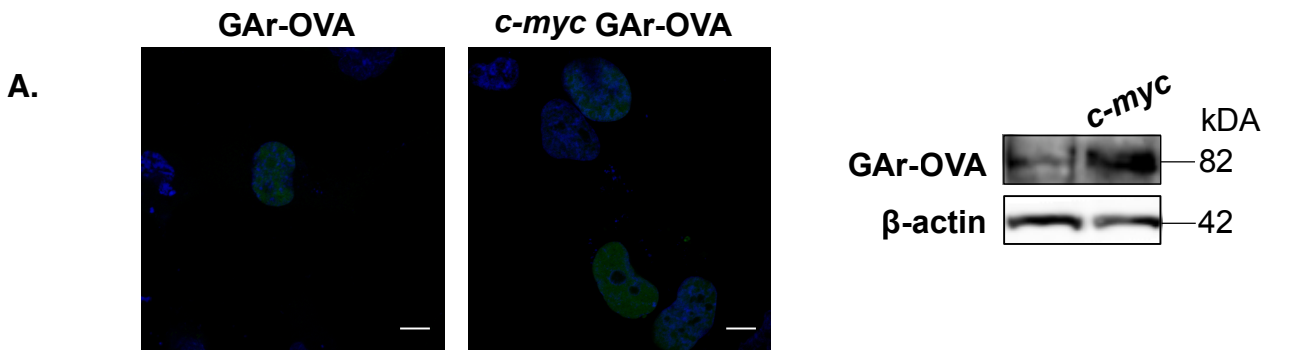
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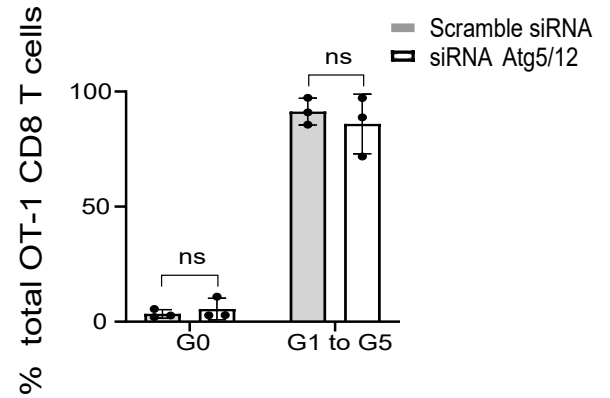
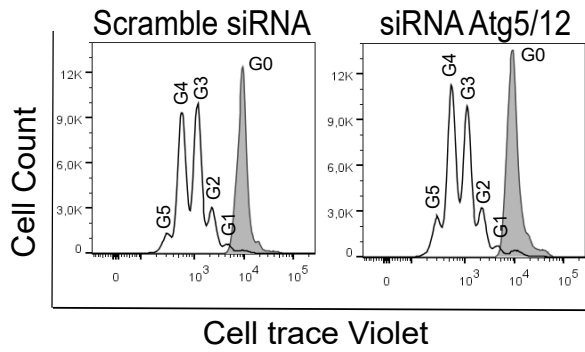
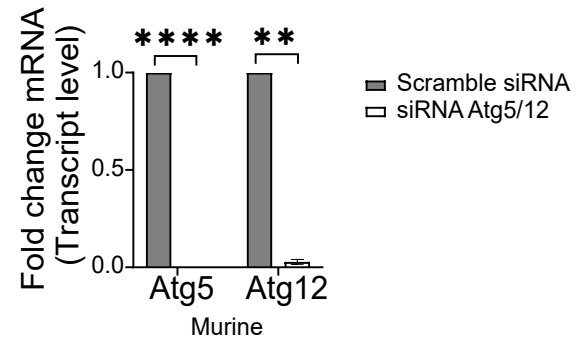
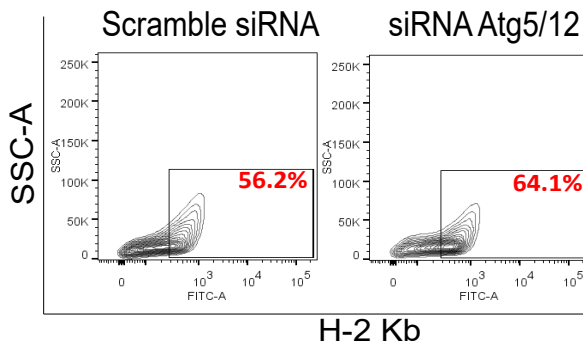
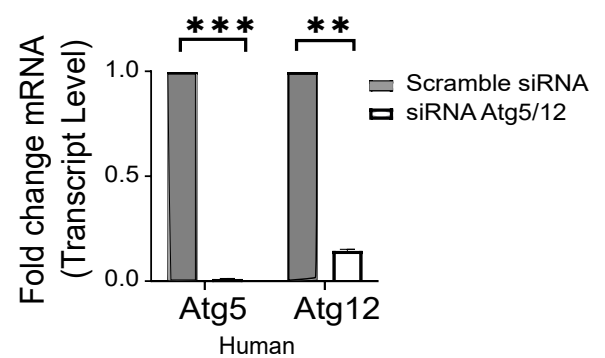
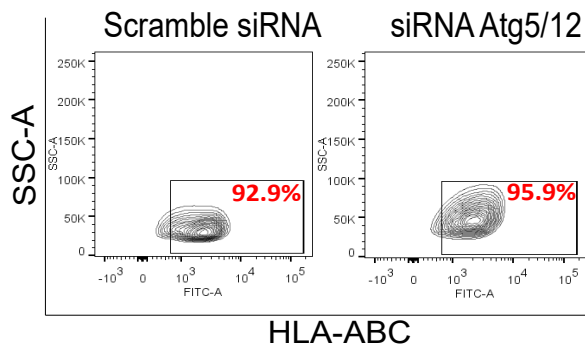
B.



Supplementary Figure 4 Autophagy inhibition with Chloroquine (CQ) does not affect antigen presentation of EBNA1 or GAR-OVA derive peptides in MHC-I pathway. **A.** H1299 cells co-expressing murine MHC-I (Kb), EBNA1, EBNA1ΔGAR, GAR-OVA and E.V were treated with Chloroquine [30 μM] during 36 hours. Then, cells were harvested and labeled with SL8 H-2Kb APC and fixable viability dye 506. Cells having SL8 H-2Kb on the membrane were measured by flow cytometry. **B.** Western Blots shows autophagy inhibition by accumulation of LC3 II, after 36 hours of [30μM] Chloroquine treatment. The graphs below show the densitometry analysis, normalized against β-actin and expressed in fold change compared with water. Significant values were calculated using Multiple paired T test grouped. *P < 0.0332.



Supplementary Figure 5 *c-myc* sequence increase protein expression of GAr-OVA and antigen presentation of GAr-OVA derive antigenic peptides. **A.** Immunofluorescence and Western Blot comparing GAr-OVA with their fusion to *c-myc* sequence **B.** H1299 cells co-expressing murine MHC-I (Kb) and the indicated constructs were co-cultured with OT1-CD8 T cells during three days. OT1 CD8 T cells proliferation was measured by flow cytometry. The graph shows the percentage of cells from generation 1 to 5 compared with percentage of non-divided cells (generation 0) from 2 independent experiments (bottom graphs). Significant values were calculated using Multiple paired T test grouped. *P < 0.0332; **P < 0.0021; 0,1234 ns, no significant.

A.**B.**

Supplemental Figure 6. Presentation of exogenous SL8 peptide and membrane location of MHC Class I molecule is not affected by Atg5/12 knock down. **A.** H1299 cells co-expressing murine MHC-I (Kb) were transfected with human siRNA Atg5/12 [20 pM] or scramble siRNA during 72 hours. Next three days, these presenting cells were co-incubated with OT-1 CD8+ T cells labeled with Cell-trace violet and free SL8 peptide [1ug/ml]. The levels of OT-1 CD8+ T cells proliferation were analyzed by Flow Cytometry. Open peaks in the histogram represent the proliferating populations and grey peaks denote unstimulated population (Empty Vector transfected cells). Percentage of total OT1 CD8+ T cells was calculated using the number of cells in each generation generated by the modeling of the Proliferation tool in Flow Jo software. The graph show the sum of percentage of cells from generation 1 to 5 compared with percentage of non-divided cells (generation 0) from 3 independent experiments. **B.** MCA-205 and H1299 cells were transfected with murine/human siRNA Atg5/12 [20 pM] or scramble siRNA respectively during 72 hours. HLA-ABC FITC or H-2 Kb FITC antibody were used to measure MHC class I molecule in alive cells by Flow Cytometry. Murine or human Atg5/12 siRNA knock down was confirmed by qRT-PCR. Significant values were calculated using Multiple paired T test grouped. **P < 0.0021; ***P < 0.0002; ****P < 0.0001; 0,1234 ns, not significant.