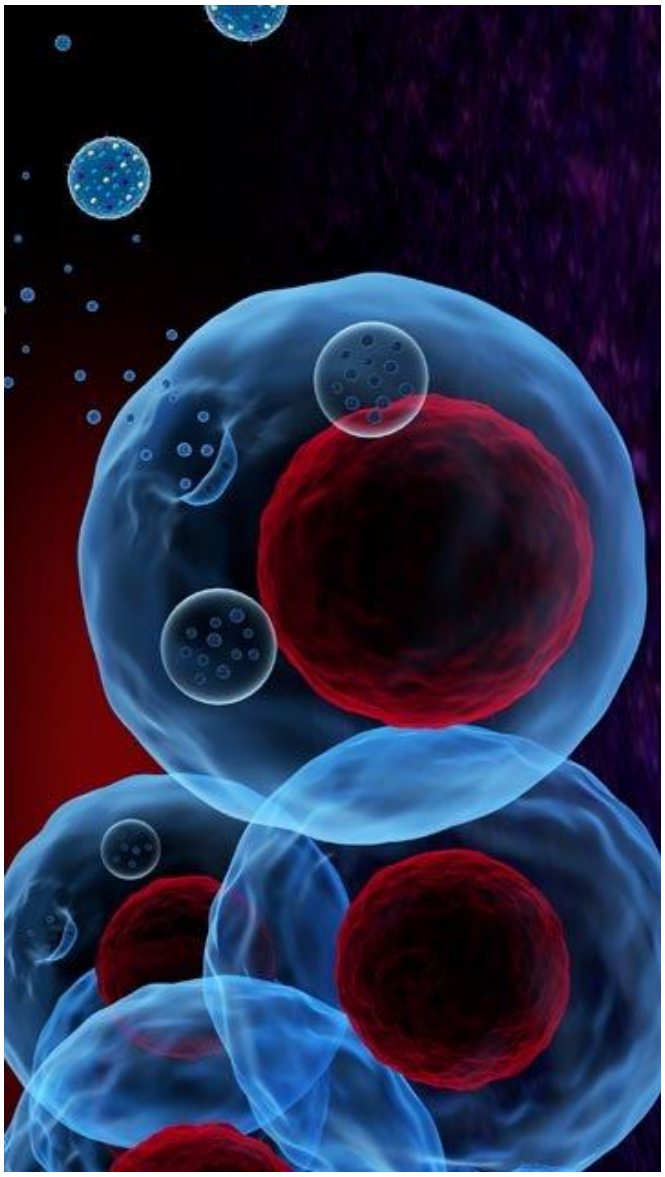

Design of engineered exosomes as a drug delivery system

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Abstract-Aims

Cells have evolved a battery of overlapping DNA repair pathways to keep their genome intact and transmit it faithfully to progeny. Nucleotide Excision Repair (NER) is a highly conserved DNA repair mechanism that cells employ to repair UV-induced DNA lesions. Defects in NER lead to enhanced cancer predisposition or to a heterogeneous group of progeroid and developmental disorders. Besides genome maintenance, multicellular organisms employ adaptive and innate immune responses to guard themselves against foreign pathogens and threats. Recent evidence points to reciprocal interactions between DNA repair mechanisms, DNA damage responses and immunity. Here, we present evidence that irreparable DNA damage in distinct cell types triggers the accumulation of double- (ds.) or single-strand (ss.) DNA moieties in the cytoplasm. In turn, ds. or ss. DNA species lead to the activation of pro-inflammatory signaling associated with a type 1 interferon response. Based on these findings, we propose a therapeutic strategy allowing us to generate engineered exosomes that will carry a nuclease that will be targeted to specific cell types. The latter strategy allows the rapid elimination of cytoplasmic DNA moieties, thereby substantially reducing chronic inflammation.

Περίληψη

Τα κύτταρα έχουν αναπτύξει έναν αριθμό αλληλεπικαλυπτόμενων μηχανισμών επισκευής του γενετικού τους υλικού για να διατηρήσουν το γονιδίωμα τους άθικτο και να το μεταβιβάσουν πιστά στους απογόνους τους. Ο μηχανισμός επιδιόρθωσης εκτομής νουκλεοτιδίων (nucleotide excision repair, NER) είναι ένας ιδιαίτερα συντηρημένος μηχανισμός επιδιόρθωσης του DNA που τα κύτταρα χρησιμοποιούν για να επιδιορθώσουν βλάβες του γενετικού υλικού που προκαλούνται από την υπεριώδη ακτινοβολία. Η μη σωστή λειτουργία του NER οδηγεί σε αυξημένη προδιάθεση για καρκίνο ή σε μια ετερογενή ομάδα προγηροειδών και αναπτυξιακών διαταραχών. Εκτός από τη συντήρηση του γονιδιώματος, οι πολυκύτταροι οργανισμοί χρησιμοποιούν έμφυτες και προσαρμοστικές ανοσολογικές αποκρίσεις για να προστατευθούν από παθογόνα και απειλές. Πρόσφατες μελέτες δείχνουν ύπαρξη αμοιβαίων αλληλεπιδράσεων μεταξύ των μηχανισμών επιδιόρθωσης DNA, αποκρίσεων στις βλάβες του DNA και ανοσολογικού συστήματος. Σε αυτή την εργασία, παρουσιάζουμε στοιχεία ότι η ανεπανόρθωτη βλάβη του DNA σε ξεχωριστούς κυτταρικούς τύπους ενεργοποιεί τη συσσώρευση δίκλωνων και μονόκλωνων μορίων DNA στο κυτταρόπλασμα. Με τη σειρά του, το κυτταροπλασματικό DNA οδηγεί στην ενεργοποίηση προ-φλεγμονώδους σηματοδότησης που σχετίζεται με την απόκριση ιντερφερονών τύπου 1. Βάσει αυτών των ευρημάτων, προτείνουμε μια θεραπευτική στρατηγική που θα μας επιτρέψει τη δημιουργία εξωσωμάτων τα οποία θα φέρουν μια νουκλεάση και θα στοχεύουν σε συγκεκριμένους τύπους κυττάρων. Αυτό θα επιτρέψει την ταχεία απομάκρυνση τμημάτων κυτταροπλασματικού DNA, μειώνοντας ουσιαστικά την επακόλουθη χρόνια φλεγμονή.

1. Introduction

1.1 DNA damage, repairing mechanisms and innate immune responses

Despite their delicate structure and fine-tuned homeostatic mechanisms, multicellular organisms, are subjected to indiscriminate damage among all their molecular structures (proteins, DNA etc.). Such damage can originate from several sources such as exposure to environmental elements (UV,x-rays), various chemical factors and byproducts of enzymatic reactions (ROS, NOS)[1].

Genomic DNA damage is the most notable among them. DNA is of indispensable value for a healthy and long lifespan of organisms. Yet its inability to be "reborn" like all other molecules makes it truly irreplaceable. It is by far the largest molecule that can accumulate damage, yet the only one that has to remain intact as it is of great importance to transmit faithfully the genetic information to progenies and preserve it in long-lived cells like neurons.

1.1.1 Nucleotide Excision Repair (NER) mechanism

A variety of repair mechanisms, damage tolerance as well as checkpoint pathways are responsible among others for the stability and preservation of the genome, in spite of its enormous length. When DNA is subjected to damage, there are different pathways activated in order to repair it and preserve its integrity during a cell's lifetime, depending on the origin and the nature of the lesion. One such pathway is Nucleotide Excision Repair (NER) which is often referred to as a "cut and patch" pathway. In mammals, it is the principle pathway activated in presence of cyclobutane pyrimidine dimers (CPD) and [6-4]pyrimidine-pyrimidine photoproducts (6-4PP) produced in human skin after prolonged exposure to UV light, as well as helix distorting chemical adducts produced by carcinogens[2]. A coordinated interplay between numerous proteins and complexes is required for its appropriate function. It can be characterized as a three step process: 1) recognition of the lesion, 2) incision of the damaged strand on both sides of the lesion 3) removal of the lesion followed by gap-filling DNA synthesis (Figure 1.1.1).

In the first step, depending on the way the lesion is detected, the pathway is subdivided into two categories, Global genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER has the ability of lesion detection throughout the genome. On the other hand, TC-NER sub-pathway can only detect lesions that are connected with genome regions that are being transcribed i.e. active genes, and disrupt the elongation machinery, as its name very well supports. In GG-NER XPC-hHR23B complex and DDB1 & DDB2 along with XPE recognize DNA damage. In TC-NER, the principal DNA damage sensor is the elongating RNAPII that is blocked at damaged DNA sites on the actively transcribed strand of genes and recruits CSB

and CSA protein. Following damage recognition by either pathway, TFIIH is recruited to the DNA damage site. XPA binds the DNA on the 5′side of the DNA insult. Together with RPA that binds the ssDNA opposite the DNA lesion, XPA stabilizes the damaged DNA for incision. RPA activates ERCC1–XPF and XPG that cleave the 5′ and 3′ side of the 24–32-nucleotide fragment respectively, containing the damaged DNA fragment. Soon after the removal of the damaged DNA fragment, the polymerase activity of the DNA replication machinery fills in the single-stranded DNA gap and the new DNA fragment is then sealed by DNA ligase I or IIIα-XRCC1. Patients with defects in GG-NER develop cancer-prone diseases like Xeroderma Pigmentosum[3]whereas defects in TC-NER promote the development of progeroid syndromes like Cockayne syndrome (CS)[4] as well as trichothiodystrophy (TTD)[5].

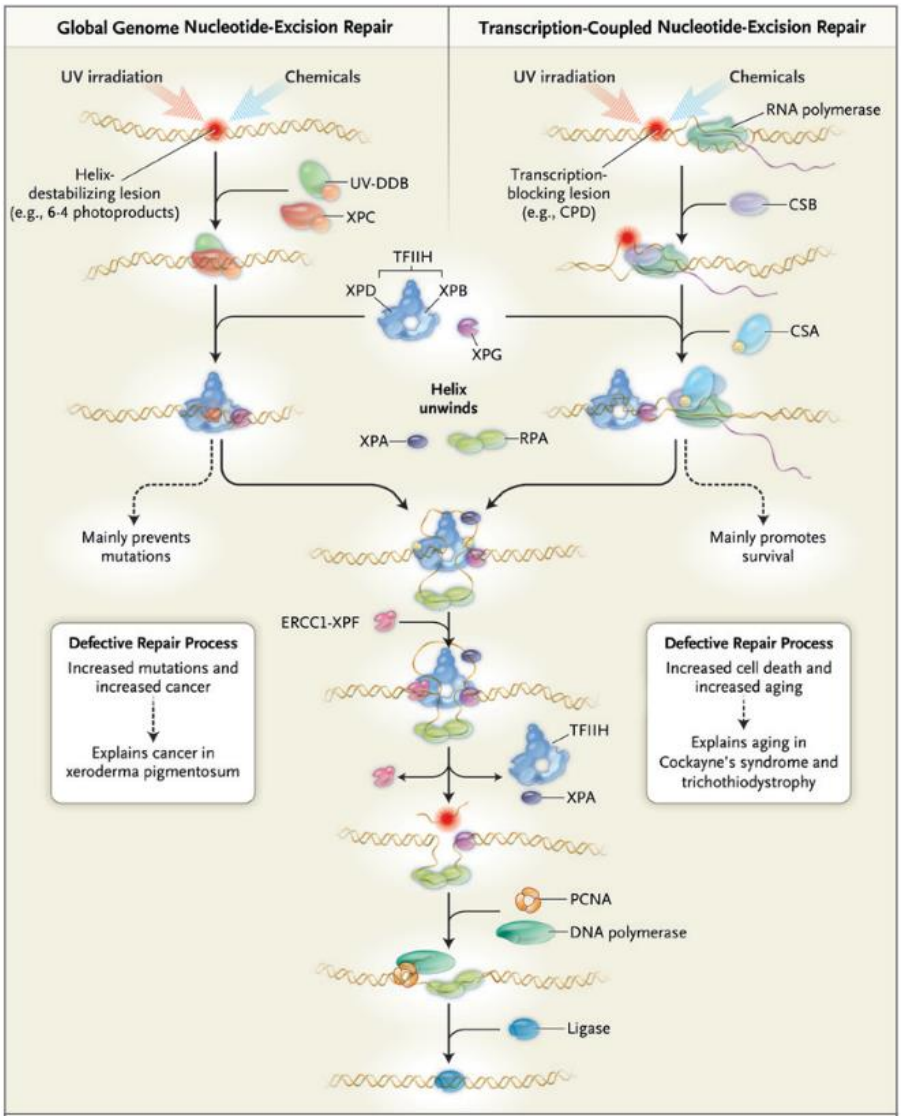

Figure 1.1.1: Nucleotide Excision Repair[6]

1.1.2 ERCC1

Repair of DNA lesions is unavoidably connected to nuclease activity. ERCC1-XPF dimer is among others (XPG, Mus81, Mre) a nuclease which participates in excision repair mechanisms like those that correct double strand breaks, single-strand breaks, inter-strand crosslinks and DNA adducts. In order to be functionally active, it forms a heterodimer complex along with XPF[7]. While ERCC1-XPF fails to recognize and cut ssDNA or dsDNA, the complex cuts DNA specifically at junctions between the two and introduces a cut in double-stranded DNA on the 5′ side of such a junction, about two nucleotides away[3]. ERCC1 provides interactions with proteins as well as with DNA, while XPF has the nuclease activity. Apart from its involvement in DNA repair pathways ERCC1-XPF complex has been involved in telomere maintenance and functions[8], in sister chromatid separation during chromosome segregation[9], as well as in fine-tuning transcription during development[10,11].

1.1.3 DNA damage and innate immunity

An effective DNA damage response is critical for maintaining genomic integrity and preventing mutations. However, the cellular response to DNA damage involves more complex signaling pathways, including innate immune responses that can promote cell survival or cell death. Moreover, gradual accumulation of DNA damage and a persistent DDR lead cells to senescence. Senescent cells are unique for their secretory phenotype; the so-called 'senescence-associated secretory phenotype'(SASP)[12]. The SASP involves the secretion of cytokines, chemokines, extracellular matrix proteins and growth factors, promotes chronic inflammation and has been associated with tissue degenerative changes. On a molecular level, several DNA damage sensors that act as innate immune transducers, as well as immune-related transcription factors (i.e NFκB), have been identified in the up-regulation of innate immune responses during DNA damage and senescence. However, the exact mechanism that activates these transcriptional regulators remain incompletely characterized.

Interestingly, patients with syndromes deriving from mutations in NER related genes [trichothiodystrophy (TTD), Cockayne syndrome] show a persistent type I interferon response comparable with those seen in the autoimmune disorder Aicardi-Goutières (AGS)[13]. Moreover, unrepaired DNA lesions in patients with mutations in ATM the central mediator of DNA damage response (Ataxia Telangiectasia syndrome) as well as in Atm−/− mice were shown to induce type I interferons (IFNs), resulting in enhanced anti-viral and anti-bacterial responses[14]. Interferon response is known to be triggered by viral-derived nucleic acid. In the case of DNA damage repair however there is growing evidence suggesting

that self-DNA byproducts deriving from these homeostatic mechanisms actually act as pathogen-associated molecular patterns (PAMPS) and fuel an interferon response.

Self-cytosolic ds-DNA has been shown in senescent cells where it is referred to as cytosolic chromatin fragments (CCFs). CCFs bud off senescent nuclei due to loss of nuclear envelope integrity in an autophagy-lysosomal dependent pathway[15]. DNA damage in the nucleus can also result in the accumulation of cytoplasmic DNA in the form of micronuclei. Micronuclei are small, DNA-containing organelles positive for lamin A/C. They are products of chromosome damage; centromere-deficient chromosome fragments deriving from nonhomologous end-joining (NHEJ) repair, centromere hypomethylation or kinetochore dysfunction can lead to the formation of micronuclei after mitosis[16].

The presence of dsDNA in the cytoplasm serves as a danger-associated molecular pattern (DAMP) to trigger immune responses. Cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS) is the sensor among others (DAI, DEAD) that detects DNA. DNA binds to cGAS in a sequence-independent manner; RNA is recognized by RIGI, whereas ssDNA cannot in principle interact with cGAS. Yet accumulation of ssDNA due to a nuclease deficiency (Trex)[17] and certain structures of ssDNA species can also trigger type I interferon in a cGAS dependent manner[18,19]. Little is known about how DNA damage leads to ssDNA accumulation in the cytosol. MUS81 is an endonuclease that may play a role in this process, but the mechanism remains unresolved[20]. A recent study also reported that extrachromosomal telomere repeat DNA (ECTR) generated by telomere elongation, which takes place in cancer through a homologous-based mechanism, can also activate IFNa/β via the cGAS–STING pathway[21].

Binding of cytoplasmic DNA to cGAS induces a conformational change of its catalytic center such that this enzyme can convert guanosine triphosphate (GTP) and ATP into the second messenger cyclic GMP-AMP (cGAMP)[22]. This cGAMP molecule serves as a ligand for the adaptor protein Stimulator of IFN Gene (STING). STING forms a transmembrane homodimer that localizes to the ER. cGAMP binding induces a conformational change in STING which triggers phosphorylation/activation of the transcription factor IRF3 via TBK1. IRF3 is able to enter the nucleus and promote transcription of inflammatory genes, and prime a type I interferon response.

There are three distinct interferon (IFN) families. The type I IFN family is a multi-gene cytokine family that encodes 13 partially homologous IFNα subtypes in humans (14 in mice), a single IFNβ and several poorly defined single gene products. The type II IFN family consists of IFNγ. The type III IFN family comprises IFNλ1, IFNλ2 and IFNλ3 which are only produced by epithelial cells. IFNα and IFNβ are the best-defined and most broadly expressed type I IFNs. Transcription of the IFN-α/β genes is controlled by proteins of the IRF family, in particular, IRF-3 and IRF-7. In most body cells as mentioned above cytosolic DNA leads to activation of IRF-3 which in turn triggers expression of only a small subset of IFN genes, in particular, IFN-β. This early IFN acts in an auto- or paracrine manner by JAK/STAT signaling to stimulate the synthesis of IRF-7, which controls transcription of many additional members

of the IFN-α gene family. Once established, secreted IFN-α/β mount-up the innate immune response by firing the production of immune related genes in an autocrine and paracrine manner. All members of the IFN-α/β family bind to a single IFN-α receptor (IFNAR resulting in the activation of the latent transcription factors STAT1 and STAT2, which then dimerize and associate with p48, IFN regulatory factor (IRF). This complex, known as IFN-stimulated gene factor 3, binds to DNA sequences (IFN-stimulated response elements) present in the promoters of hundreds of genes and promotes their transcription. The phosphoinositide 3-kinase (PI3K)–mammalian target of rapamycin (mTOR) pathway and multiple mitogen-activated protein kinase (MAPK) pathways can also be activated downstream of IFNAR in a similar manner[23,24]. The transcription of interferon stimulated genes in the microenvironment of cells with cytosolic DNA promotes an anti-viral like response in these cells as well as the activation of both the innate and adaptive immune system, more specifically myeloid cells, B cells, T cells and NK cells[24].

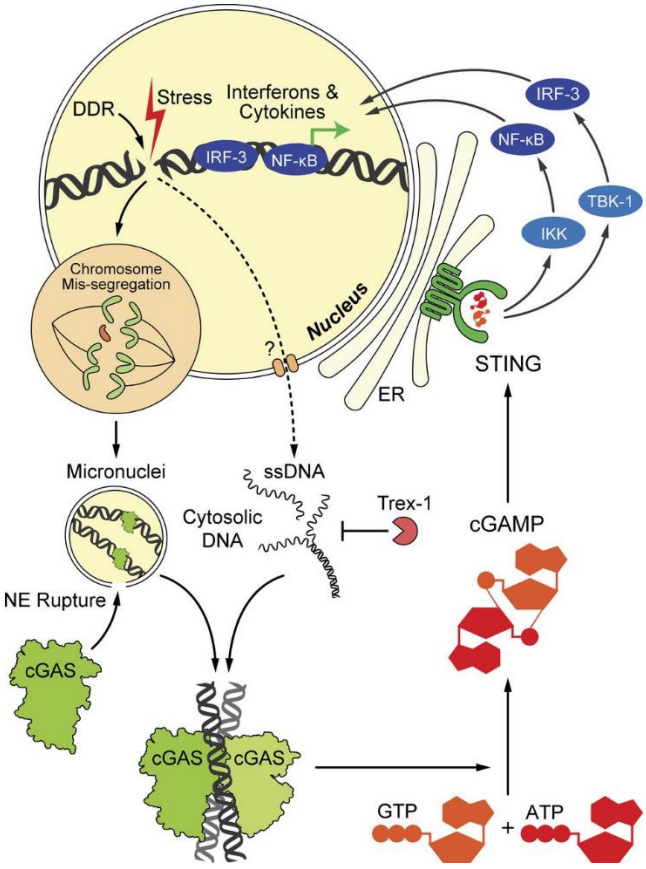

Figure 1.1.2: The cGAS–cGAMP–STING pathway[47]

1.2 Exosomes

All living cells contain endomembrane departments like ER, Golgi etc. which communicate using vesicles of the endocytic pathway. Some of these departments, endosomes, invaginate further, creating a multivesicular body (MVB) with intraluminal vesicles (ILVs). These ILVs are naturally occurring vesicles that are occasionally released in the extracellular milieu and work as mediators of intercellular communication. For decades, intercellular communication, was thought to be regulated by either cell-to-cell contact or by the release and uptake of soluble molecules acting like transmitters, as in the case of neurotransmitters. However, it is now accepted, that there is a third and crucial way of intercellular communication which is mediated by the production of extracellular vesicles[25,26,27].

Every cell in a living organism produces three categories of extracellular vesicles (EVs) : apoptotic bodies, microvesicles and exosomes [28]. Apoptotic bodies are the biggest in size (50-5000nm). They form during apoptosis in order to present their contents (DNA, RNA etc.) to macrophages and be engulfed by them. Microvesicles range from 50 to 1000nm, while exosomes are the smallest, with a size range between 40 and 100nm (Figure 1.2.1) [25]. Microvesicles along with exosomes aid in the intercellular communication with the difference that exosomes for from the endocytic pathway while microvesicles derive directly from plasma membrane. Their main function is to engulf proteins, RNA, lipids etc. and deliver them to recipient cells.

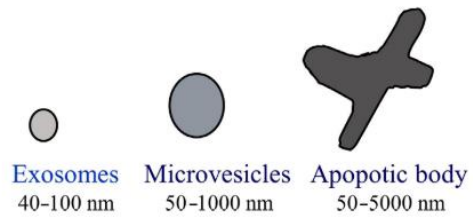

Figure 1.2.1: Extracellular vesicles

In more detail, exosomes, are lipid-bilayered vesicles whose membrane includes a variety of proteins such as tetraspanins (CD9, CD63, CD81), heatshock proteins ((Hsp70 and Hsp90) and membrane transport and fusion proteins (GTPases, Annexins, Flotillin). Tetraspanins are transmembrane proteins that are known to function as mediators of fusion, cell migration, cell-cell adhesion, and signaling events on cell basis [29]. Furthermore, they have the ability to bind with various molecules[30]. They are abundant in exosome membranes which designates them as candidate markers for exosomes. Exosomes can be found and extracted from most of the body fluids like blood, urine, saliva, amniotic fluid, bronchoalveolar lavage fluid, synovial fluid and breast milk[27].

1.2.1 Exosome biogenesis and release

Exosome biogenesis follows the pathway of Multivesicular Body (MVB) formation. It can either be ESCRT-dependent or ESCRT-independent (Figure 1.2.2). To begin with, the plasma membrane forms a bud in the cytoplasm forming an early endosome which undergoes maturation into a late endosome.

Exosomes are basically intraluminal vesicles (ILVs) that are generated by inward budding of the MVBs and are releases extracellularly by MVB fusion with the plasma membrane. There is little knowledge concerning the formation of ILVs with two possibilities existing so far. The first one utilizes the Endosomal sorting complex required for transport (ESCRT) which consists of four subcomplexes ESCRT0, -I, -II and III. It is recruited to endosomal membranes during the ILV formation with ESCRT0, -I and II leading with the recognition of ubiquitinated proteins. ESCRT-I and II along with other factors are also responsible for the beginning of the membrane invagination. Finally ESCRT-III binding to ESCRT-II leads to deubiquitylation, and abscission of the ILVs[31].

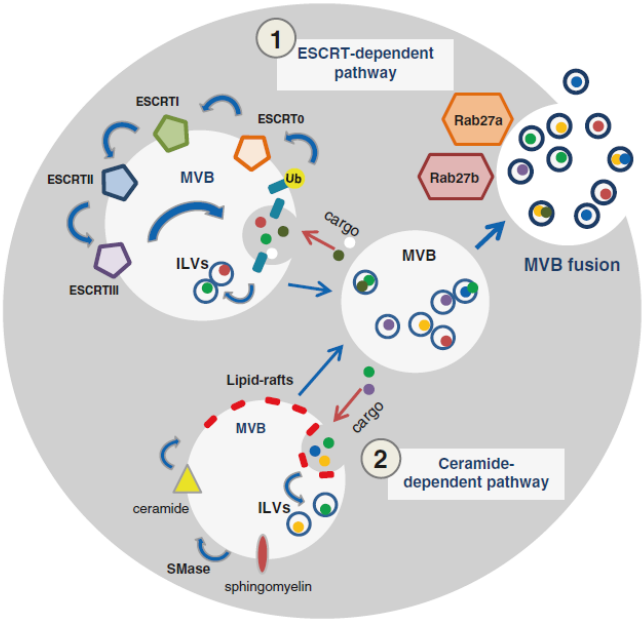

Figure 1.2.2: Exosome biogenesis

The second way of exosome formation is ESCRT-independent, as it was observed in cells lacking the complex. Instead the crucial component in this case seems to be sphingomyelinase, an enzyme that converts sphingolipids to ceramide and is present in endosome membranes[32]. This hypothesis is consistent with the large amount of ceramide in exosomes[33]. In this case exosome formation is dependent on the alteration of lipids forming the vesicles.

Following the formation of ILVs, MVBs can follow two rootes: they can either follow the degradation pathway which leads to the fusion with lysosomes or fuse with the plasma membrane and release the ILVs/exosomes to the extracellular milieu. Both these procedures are connected with Rab GTPases[34].

1.2.2 Exosome uptake

The basic idea upon which people decided to use exosomes for drug delivery is their capacity to trigger phenotypic changes in acceptor cells, which indicates that their content is released in the recipient cell.

There are a few ways by which exosomes can interact with the recipient cells as presented in Figure 1.2.3. To begin with, they can cause a cascade of reactions only by membrane proteins that interact in receptor-ligand dynamics, without delivery of their content. One such occasion is related to immune responses where EVs carrying MHC can activate T cell receptors[35]. A different scenario is the direct fusion of their membrane with the plasma

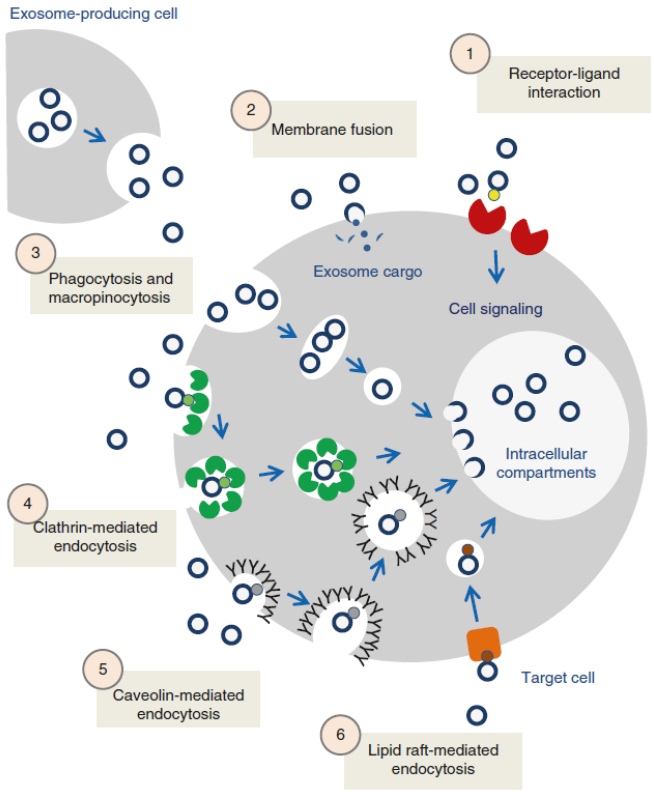

Figure 1.2.3: Exosome uptake

membrane of the recipient cell, releasing their cargo directly to the cytoplasm. If phagocytosis or micropinocytosis takes place, subsequent fusion with other endosomal structures follows, again releasing their content to the cytoplasm. However, it has been observed that in order for micropinocytosis to happen, exosomes should be isolated or very small[36]. Finally, there are other internalization processes which are either clathrin dependent or they depend on caveolin or lipid rafts and binding with specific receptors. After their internalization and/or release of their cargo, exosomes can alter the expression of genes in the target cell.

1.3 Exosomes as a drug delivery system

One of major advances of exosomes as a drug delivery system is that they possess the ability to transport their cargo intact to very distant places of the body while also protecting it from enzymatic degradation during their transit to extracellular environment[37]. Of great importance is the fact that they have low immunogenicity due to their compatibility with the host immune system, even in cases that their origin is from another organism[38]. Furthermore, once they get in contact with recipient cells they can either fuse with their plasma membrane releasing their cargo to the cytoplasm, but even if they get internalized by other pathways described before they have the potential to avoid the endosomal pathway and lysosomal degradation again by fusing with the membrane of other compartments inside the cell. What is more, they can be widely manipulated. Finally, one more unique ability is the potential to pass the blood brain barrier[39,40] which makes them a perfect candidate for neurodegenerative diseases.

In the current study, we tried to produce engineered exosomes that will be targeted to specific cell types and will carry a nuclease able to degrade the cytoplasmic DNA thus reversing the acute inflammatory phenotype.

2. Materials and Methods

2.1 Cell culture

Pancreata of C57BL/6 mice 15-20d old were extracted, minced and incubated in 37C in PBS + 2mg/ml collagenase for 15 minutes. Primary pancreatic cells (PPCs) were centrifuged and resuspended and cultured in DMEM high glucose medium supplemented with 10% FBS, 50μg/ml streptomycin, 50 U/ml penicillin (Sigma) and 2mM L glutamine (Gibco)at 37C incubator. All treatments conducted at PPCs were done 48hrs after PPCs extraction (80-90%) confluency of cells, and the medium was replaced daily.

Mitomycin C (MMC) was added at 10ug/ml final concentration for 3-4 hours along with any exosome treatment. Enzymes that were used for treatments were s1 nuclease (18001016, ThermoFisher Scientific and DNase 1 Roche (10mg/ml). Exosomes were loaded with the nucleases after incubation with 0.2% saponin in PBS for 15 minutes, RT, rotating[41].

Mesenchymal stem cells were collected from both bone marrow and compact bones of C57BL/6 mice as previously described[42,43]. Briefly, C57BL/6 mice two to three weeks old were killed and their hindlimbs were extracted and cleared from muscles in sterile conditions inside a laminar. Bones were stored in D-MEM medium before further processing. washed with 70% Et-OH for two minutes. The epiphyses below the end of the marrow cavity was removed and the bone marrow was flushed out using a 0.45-mm syringe needle with d-MEM medium. The bone marrow cells were centrifuged and resuspended in fresh medium DMEM supplemented with 10% FBS, 50μg/ml streptomycin, 50 U/ml penicillin (Sigma) and 2mM L glutamine (Gibco) which was replaced after 3 hours of incubation at 37 1Cwith 5% CO2 in a humidified chamber without disturbance. Subsequent to that, the medium was replaced every 8 hours for the next 72 hours. The bones were chopped to small pieces and incubated in D-MEM containing 10% (vol/vol) FBS in the presence of 1 mg ml – 1 (wt/vol) of collagenase II for 1–2 h in a shaking incubator at 37 °C with a shaking speed of 200 r.p.m. After 2-3 hours the digestion medium was aspirated and removed and the bones were washed for three times with fresh medium. Finally, they were placed in DMEM supplemented with 10% FBS, 50μg/ml streptomycin, 50 U/ml penicillin (Sigma) and 2mM L glutamine (Gibco) and cultured at 37 1Cwith 5% CO2 in a humidified chamber for 3 days.

BMDMs. Briefly, bone marrow cells were isolated from mouse femurs and tibias and cultured for 7 days in DMEM containing 10% FBS, 30% L929 conditioned media, 50 μ g/ml streptomycin, 50 U/ml penicillin (Sigma) and 2mM L glutamine (Gibco).

2.2 Exosome isolation and labeling

Mesenchymal stem cells were used for the production of exosomes. After 90% confluency of the cells media were harvested and subjected to consecutive centrifugations as described at the differential ultracentrifugation protocol[44]. Briefly culture medium was centrifuged sequentially at 300g, (10min), 2000g (10min) and 10000g (30min) to remove dead cells and cell debris. The media were collected and a final centrifugation was conducted at 100000g for 2 hours.

For EV labeling we used the PKH67 dye. EVs were incubated with PKH67 for 5mins at room temperature, using PKH67GL - PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling, Sigma-Aldrich.

For experiments including exosome treatment, the amount of exosomes used was derived from cells five times more than the recipient cells.

2.3 Immunofluorescence

For immunofluorescence assays cells were fixed in 4% formaldehyde, 1X PBS for 10mins, permeabilized with 0.5% Triton-X and blocked with 1% BSA for 45 minutes. The antibodies used are described in the following table.

Primary	**Secondary**
m-anti-ssDNA F7-26, MAB 3299, Millipore	m-Anti-IgM 488 (dylight)
γ H2AX (05-636, IF: 1:12000), Millipore	m-488 (Alexa)
m-anti HIS	

Cells were incubated with the antibodies for one hour at room temperature and DAPI was used for nuclear staining. All samples were scanned using SP8 inverted confocal microscope (Leica).

2.4 RNA extraction

RNA-extraction was performed using TRIzol reagent. Cells from 60mm cell culture well plate were extracted in 500ul of Trizol. 1/5 of the initial trizol volume of chloroform was added, mixed well and centrifuges at 12000g for 15 minutes. The upper phase was collected into a new tube and ½ of the initial volume of 2-propanol was added for the precipitation to

happen. A centrifugation followd 12000g for 15 minutes and the pellet was washed with 70% EtOH, 7500g 5minutes. The pellet was resuspended in 10-20ul of water.

2.5 DNase treatment

For DNase treatment the enzyme used was provided by Promega. Briefly, 2ug of RNA were mixed with 1ul of DNase, 1ul of 10X buffer provided by the manufacturer and H2O till 10ul. The mixture was incubated at 37C for 20-30 minutes. 1ul of stop solution (EDTA) was added and the mix was incubated at 65 for 5mins for DNase to be inactivated. Immediately after the mix was put on ice.

2.6 cDNA synthesis

cDNA synthesis was performed using minotech-RT reverse transcriptase and protocol. Briefly, a mixture of 1 μl of oligo(dT)20 (100 μM), 10 ng–2 μg total RNA, 1 μl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) and Sterile ultrapure water up to 13 μl was heated at 65C for 5 minutes. The mixture was incubated at 4C for 2 minutes and 4 μl 5X MINOTECH RT assay buffer, 1 μl 0.1 M DTT, 1 μl RNase Inhibitor (40 units/μl) and 1 μl of MINOTECH RT (~200 units/μl) were added. After being mixed gently the mixture was incubated at 42C for 60minutes and a heat inactivation step at 70C for 15 minutes followed.

2.7 qRT-PCR

Quantitative PCR (Q-PCR) was performed with a CFX Connect Real-Time PCR Detection system device according to the instructions of the manufacturer (BIO RAD). The generation of specific PCR products was confirmed by melting curve analysis. Hypoxanthine guanine phosphoribosyltransferase1 (Hprt-1) and GAPDH mRNAs were used as an external standard

The primers that were used for qRT-PCR are listed in the table 4.6.1 below.

	Forward	**Reverse**
IFNa	CTGCTGGCTGTGAGGACATA	GGCTCTCCAGACTTCTGCTC
IFNb	TGAACTCCACCAGCAGACAG	AGATCTCTGCTCGGACCACC
HPRT	CCCAACATCAACAGGACTCC	CGAAGTGTTGGATACAGGCC
GAPDH	TGTTCCTACCCCCAATGTGT	TCCTTGTCCCAAGTCACTGTC

Table 4.6.1: List of Real-Time primers

2.8 In-vivo experiments-FACS

For in vivo experiments, adult C57BL/6 mice were treated intranasally with exosomes isolated from both BMDMs as well as MSCs as described before. The peptide that was used

to paint the exosomes was a chimeric peptide based on the works of Gao et al. [45] for CPO5 and Podolnikova et al.[46] for CD11b-ligand. For peptide painting the protocol suggested from Gao et al. was followed.

Exosomes were dyed with PKH67 for their visualization. After 4-5 hours of treatment, the mice were killed and their brains extracted and stained for FACs analysis.

Briefly, brains were minced in 1xPBS/1%BSA/0.1%NaN3 and further processed with a Dounce homogenizer. Homogenized tissue was further washed in PBS-BSA buffer and passed through a 100μM wire mesh. Samples were further washed in PBS-BSA buffer. Cells were stained with fluorochrome conjugated antibodies (CD11b) for 20 min at 4C in PBS/5% FBS. Samples were acquired on a FACS Calibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

2.9 Cloning

TOPO TA (Invitrogen) subcloning method was used in order to clone all genes required before we put them into the final vectors. The multiple vectors as well as the primers that were used are listed at the tables below.

Vector	Origin	Use
PCR II TOPO	Invitrogen	Subcloning of all CDSs that will used further
pUltra #24129	Addgene	Insertion of STEAP3, STAM, Syndecan4, exosome production boost, Insertion of blasticidin (antibiotic selection for stable cell line production) First step of Stable cell line
#68421	Addgene	Template for L7Ae
#99277	Addgene	Insertion of CD63-L7Ae, CX43, SP-BBBpeptide-LAMP2, GFP Packaging, targeting and uptake of the exosomes Second step of stable cell line
#52535	Addgene	Final vector of nuclease and C/D Box insertion
#17486	Addgene	Template for blasticidin

Table 1: Table of Vectors

	Forward	Reverse
STAM	AATCTAGAATGCCTCTCTTTGCCACC	TATGGATCCCTCGAGATCATTCATTAACTTCGTATACAGTG
STEAP3	TATACCGGTATGTCGGGGGAGATGGAC	ATTGTACAACACGTGGCTTGTTTTCTC
Syndecan 4	TTAGCTAGCTGGCCGGCCATGGCGCCTGCCTGCCTGC	CGCCTGATCACATATGTCATGCGTAGAACTCATTGGTGGGG
blastisidin	GGATTAATTAAGTGTGTCAGTTAGGGTGTGGAAAGTC	GGATTAATTAATAAGATACATTGATGAGTTTGGACAAACCAC
DNase1	CTCGAGCTGAGAATTGCAGCCTTC	GATATCTCAGATTTTTCTGAGTGT
GFP	CTCGAGATGGTGAGCAAGGGCGAG	GATATCTTATCTAGATCCGGTGG
C/D-BOX 1	GATCCATGCCATCCGTGATCCGAAAGGTGAGATGGCTGCAGGTCGACCACTTCGGGTAC	CCGAAGTGGTCGACCTGCAGCCATCTCACCTTTCGGATCACGGATGGCATG
C/D-BOX2	CGTGATCCGAAAGGTGAGTACCCTGCAGGTCGACTTCGAACCGTGATCCGAAAGGTGAGATGGA	AGCTTCCATCTCACCTTTCGGATCACGGTTCGAAGTCGACCTGCAGGGTACTCACCTTTCGGATCACGGTAC
L7Ae	CATATGGGTGGTGGTGGTATGTACGTGCGCTTCGAGG	GATATCTCACTTCTGCAGGCCCTTGA
CD63	TCTAGAATGGCGGTGGAAGGAG	CATATGCATTACTTCATAGCCACT
Connexin43	GCTAGCATGGGTGACTGGAGCGCCTTG	ACGCGTCATATGAATCTCCAGGTCATCAGGCCGA
Lamp2	CTGCAGATGTGCCTCTCTCCG	GCGGCCGCCATAGTCGAGTTACCTGCATTGGACTGAACGGC
T2Aoligo fOR	TATGGGATCTGGAGAGGGCAGGGGAAGTCTACTAACATGCGGGGACGTGGAGGAAAATCCCGGCCCAGGATCTGGACTGCA	GTCCAGATCCTGGGCCGGGATTTTCCTCCACGTCCCCGCATGTTAGTAGACTTCCCCTGCCCTCTCCAGATCCCA
BBB	GGCCGCTGGATCTGGAAATCGTGGTACTGAGTGGGATGGATCTGGACAATTGGGGATATCT	CTAGAGATATCCCCAATTGTCCAGATCCATCCCACTCAGTACCACGATTTCCAGATCCAGC
LAMP2FOR	CAATTGTTGATAGTTAATTTGACAGATTCA	AGATCTGACAGACTGATAACCAGTACGAC
GFPfOR2	AGATCTGGAATGGTGAGCAAGGGCGAGG	GATATCGGATCCTTACTTGTACAGCTCGTCCATG

Table 2: Cloning oligos

3. Results

3.1 In vitro recapitulation of ERCC1 KO phenotype.

Stratigi et al., and Gkirtzimanaki et al. (unpublished data) show accumulation of ssDNA and ds DNA in pancreatic and microglia cells respectively that lack ERCC1. The generation of ssDNA and ds DNA was shown to be mainly due to DNA damage. Accumulation of cytoplasmic DNA has been shown in this case and by others to be connected with acute innate immunity responses as discussed before[21,47]. In order to further prove the previous findings, we subjected primary pancreatic cells (PPCs) to DNA damage and explored the possibility of reversing the phenotype by providing them with exosomes previously loaded with two nucleases; one with higher affinity for ssDNA (S1 nuclease) and one with affinity for both dsDNA and ssDNA (DNase1).

To recapitulate the DNA damage phenotype due to lack of ERCC1, we treated cells first with mitomycin C (MMC) which promotes interstrand crosslinks[48] and it has been previously shown by Stratigi et al. (unpublished data), that stimulates the accumulation of ssDNA in the cytoplasm of pancreatic cells. Our cells were in parallel stained for γH2Ax to estimate the DNA damage accumulation caused by mitomycin C (MMC)[49,50] (Figure 3.1.1). In this set up we have successful promotion of DNA damage as shown in Figure 3.1.1, which leads to accumulation of cytoplasmic ssDNA presented in Figure 3.1.2.

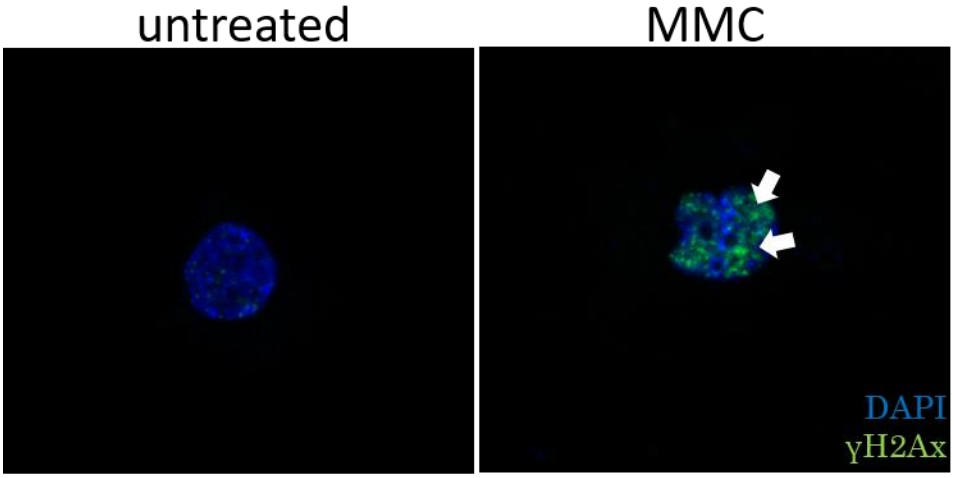

Figure 3.1.1: MMC causes DNA damage accumulation in Primary Pancreatic Cells (PPCs), DAPI (nuclei) is depicted in blue while DNA damage existence is depicted with green due to presence of phosphorylated H2Ax histone.

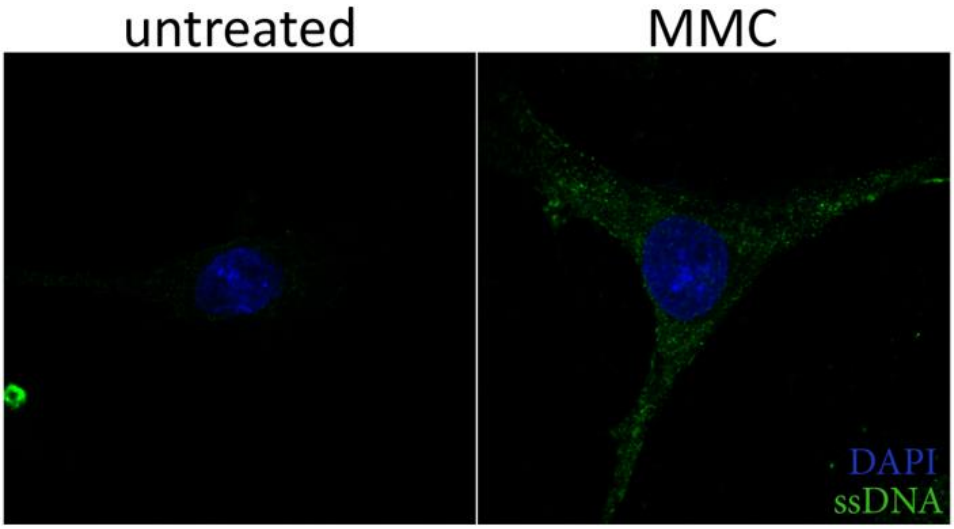

Figure 3.1.2: MMC causes cytoplasmic DNA accumulation in Primary Pancreatic Cells(PPCs) DAPI (nuclei) is depicted with blue while ssDNA is depicted with green.

3.2 Efficient uptake of exosomes by recipient cells

Since we established a set up where we have accumulation of cytoplasmic DNA due to DNA damage, we proceeded to experiments concerning exosome uptake from our pancreatic cells.

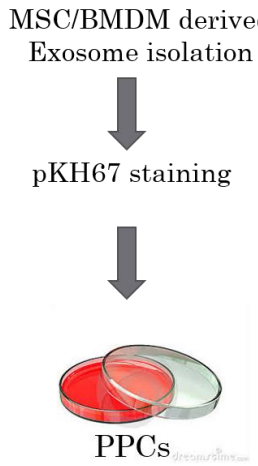

Figure 3.2.1

To begin with we wanted to test whether it was more efficient to use exosomes isolated from mesenchymal cells or bone marrow derived macrophages (BMDMs). To test this, we estimated the uptake efficiency of both exosome types from pancreatic cells. Exosomes were isolated from both Bone Marrow Derived Macrophages (BMDMs) and Mesenchymal stem cells (MSCs) and dyed with PKH67, a specific membrane marker (Figure 3.2.1). PPCs from wild type mice were treated with the dyed exosomes, fixed and stained. Confocal analysis of samples and further quantification of PKH positive cells showed that 15% of PPCs had internalised exosomes at the specific time point (Figure 3.2.2) in the case of mesenchymal cells whereas In the case of bone marrow derived exosomes the uptake efficiency was lower (<10%) (data not shown).

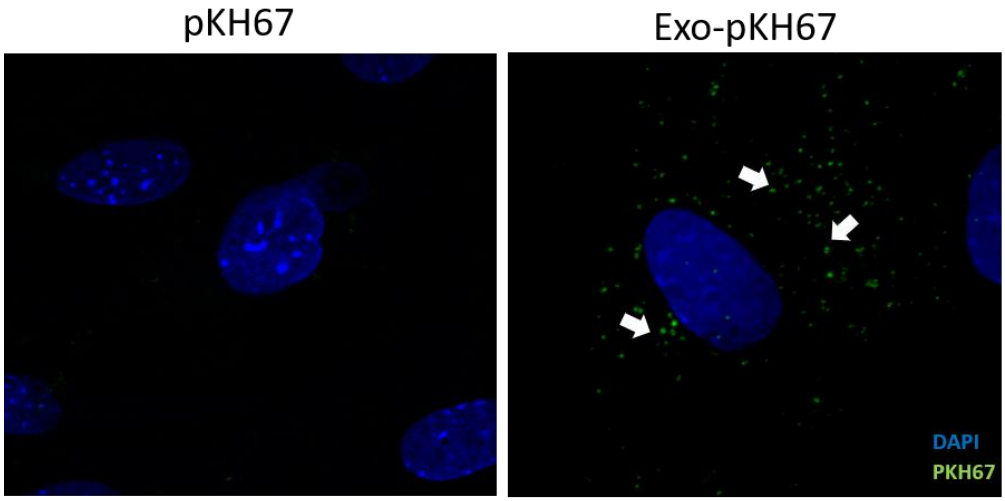

Figure 3.2.2: Successful uptake of exosomes by Primary Pancreatic Cells (PPCs). DAPI (nuclei) is depicted. MSC exosomes are depicted with PKH67 (green). Cells that were exposed to dyed exosomes (Exo-pKH67) internalized them successfully.

Haney et al.[41] showed that treatment of exosomes with the detergent saponin allows loading of exosomes with the protein or the mRNA of interest without affecting the morphology or the functionality of exosomes. To test that, we followed the same approach in order to allow entry of nucleases (DNase/S1) to the exosomes and proceeded with estimating the uptake efficiency of treated exosomes from pancreatic cells. The percentage of cells positive for PKH67 staining remained the same, indicating that saponin treatment probably doesn't compromise their functionality (Figure 3.2.3).

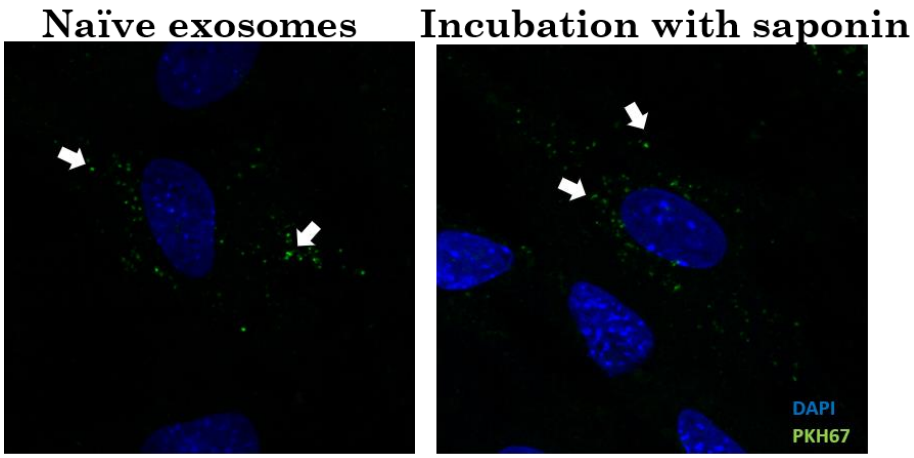

Figure 3.2.3: Saponin incubation does not affect the uptake of exosomes by recipient cells. (PPCs) DAPI (nuclei) is depicted in blue. Exosomes are depicted with PKH67 (green).

3.3 Efficient delivery of the protein of interest to the recipient cells

Due to lack of a specific marker for nuclease s1/DNase 1, we then proceeded to exosome loading with a HIS-tagged protein in order to be able to visualize the delivery efficiency of their cargo to PPCs. To exclude the possibility of passive delivery of proteins to recipient cells, we treated them with a HIS-tagged protein alone.

PPCs treated with exosomes carrying the His-tagged protein were positive for that protein (Figure3.3.1, Exos, HIS-tagged protein). On the other hand, PPCs treated with the protein alone (Figure3.3.1, HIS-tagged protein) haven't internalized it. As a result, we established that His-tagged protein is efficiently translocated to the cytoplasm of recipient cells via exosomes.

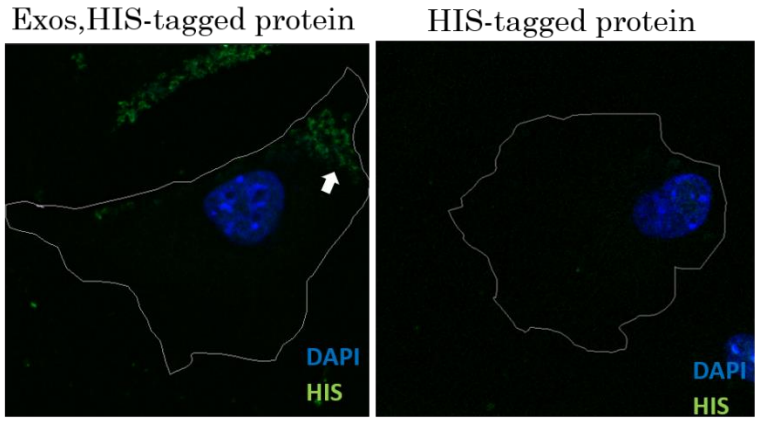

Figure 3.3.1

Exosomes have the capacity to deliver the protein of interest (Exos, HIS-tagged protein). When cells are treated with the protein alone, it cannot be internalized. (PPCs), DAPI (nuclei) is depicted in blue. His-tagged protein is depicted with green.

3.4 Reduction of ssDNA and Type I Interferon Response upon treatment with exosomes carrying S1nuclease/DNase1.

After establishing the experimental set-up we wished to answer whether providing DNA damaged cells with exosomes carrying nuclease S1/DNase1 would lead to a reduction of ssDNA from the cytoplasm and dampening of the innate immune responses. Again, we isolated exosomes from mesenchymal stem cells, loaded them with the nucleases using saponin and put them in PPCs along with MMC. We utilized immunofluorescence assay (IFA) for cytoplasmic DNA detection and q-RT PCR for detection of IFNa/b mRNA levels (Figure 3.4.1).

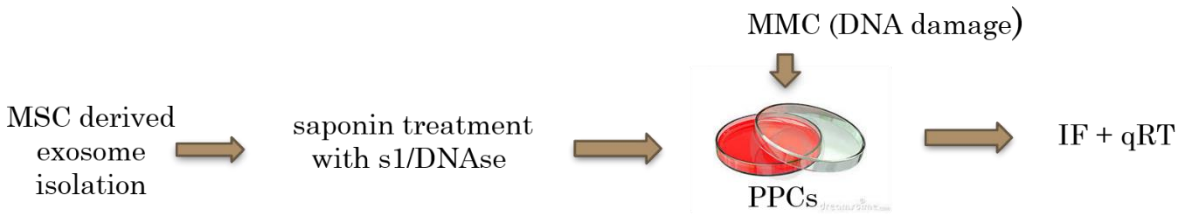

Figure 3.4.1: Experimental set up

For the IFA we used an antibody that binds specifically to the ssDNA in the cytoplasm and it is presented in green in Figure 3.4.2. Untreated PPCs show low levels of cytoplasmic ssDNA, which amplifies a lot when cells are treated with MMC, as expected and shown before. When MMC treated cells receive naïve exosomes, cytoplasmic ssDNA persists. On the contrary, in the case of PPCs treated with both MMC and exosomes that carry a nuclease, either s1 nuclease or DNase 1 we observed a clear reduction in ssDNA in the cytoplasm. These data suggest a possible phenotype rescue of damaged cells following treatment with exosomes carrying a nuclease.

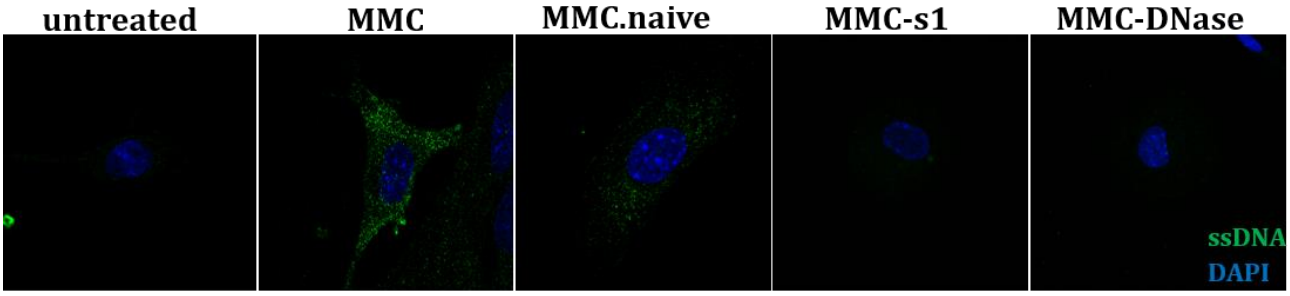

Figure 3.4.2: (PPCs) DAPI (nuclei) is depicted in blue while ssDNA is depicted in green. Untreated cells do not present any signs of cytoplasmic DNA. However, it is increased in the case of MMC treatment, sustained with naïve exosomes and degraded again while treating cells with exosomes carrying either s1 nuclease or DNase1.

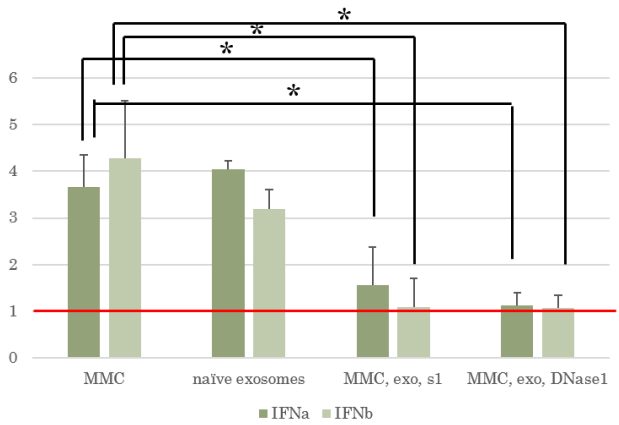

Figure 3.4.3: mRNA levels of IFNa and IFNb. Both interferon mRNA levels follow the same pattern; mRNA is upregulated with MMC, upregulation is sustained when we add naïve exosomes and downregulated upon addition of exosomes that carry either s1 nuclease or DNase1. The basal level of IFN expression is indicated by the red line.

Cytoplasmic ssDNA is known to trigger a cascade of innate immune responses with the more prominent one being type one interferon response. IFNa and IFNb are known to be the final products of the type one interferon cascade as discussed above. For that reason, we measured the mRNA levels of both genes in cells treated with MMC alone and cells treated with MMC and exosomes carrying s1 nuclease or DNase1. In the diagram above we can see that both IFN levels increase upon MMC and are restored almost to their basal levels only when exosomes carrying either one of the nucleases are added (Figure 3.4.3).

3.5 Preferential uptake of MSC produced exosomes by brain cells in vivo

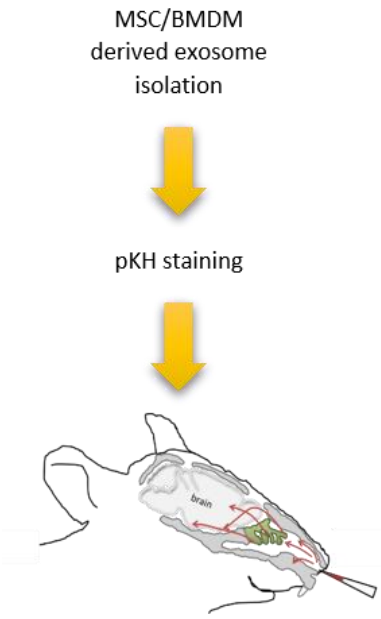

Figure 3.5.1

In parallel with the cellular phenotype triggered by genomic DNA accumulation, we were interested in the physiological effect this could have in an organism. Gkirzimanaki et al. (unpublished data) has revealed a mechanism where absence of ERCC1 in tissue resident macrophages and more specifically in microglia leads to accumulation of cytoplasmic dsDNA and persistent immune response resulting in neurodegeneration and slow paralysis of the back legs of the mice. Microglia represent the majority of immune cells of the brain. The initial origin of most microglial cells is from the yolk sac, where microglial cells arise and populate the neuroepithelium during early embryogenesis[51]. The resident macrophages/microglia that derive from the yolk sac, present a typical macrophage morphology and high expression of CD11b as well as F4/80[52]. However, it is less clear whether in later stages of embryogenesis as well as in postnatal stages there is assistance from other sources like bone marrow myeloid progenitors. What is clear though, is that as soon as the brain undergoes any kind of damage and/or gets injured, microglia become activated and highly responsive. This process includes change in their shape and their size as well as increase of the amount of cytokines that they produce and secrete. There is also increase of their proliferation rate and the cell surface antigens they present[53].

In the case of Gkirzimanaki et al. removal of cytoplasmic dsDNA could result in alleviation of the phenotype, further proving the specificity of the mechanism as well as suggesting a novel therapeutic approach. Our goal was to deliver molecules with nuclease activity specifically to the brain, targeting primarily the microglia and observe overtime the possible change of the outcome.

In order to recapitulate the observed in vitro results in an in vivo experiment, we administrated naïve exosomes in wt adult mice (Figure 3.5.1). In order to test if there is preference regarding internalization by the desired cells (microglia) we used both BMDM and MSC derived exosomes. To mark exosomes, PKH staining was performed, as in previous experiments, and exosomes were given to mice via intranasally administration, as this route of administration is considered quite efficient for brain treatment purposes. 4-5 hours post administration, mice were sacrificed and brains were utilized for flow cytometry analysis. Microglia were discriminated using CD11b cell surface marker, while cells with internalized exosomes were positive for PKH staining. Despite the fact that not many microglia had internalized the exosomes, with a corresponding percentage of 2.20% in MSC and 0.9% in BMDMs of the total microglia (CD11b positive), it is quite clear that exosomes do get internalized by brain cells in vivo (Figure 3.5.2). Furthermore, we should take into consideration that microglia represent only a small percentage of the total population of brain cells, 10-15% of total brain cells[54]. Based on the higher percentage of uptake from MSC derived exosomes we decided to carry the following experiments using these cells as exosome producing cells. However, it is to be noted that recent studies suggest that the recipient cells rather than the origin of the exosomes are important as to whether exosomes will be engulfed.

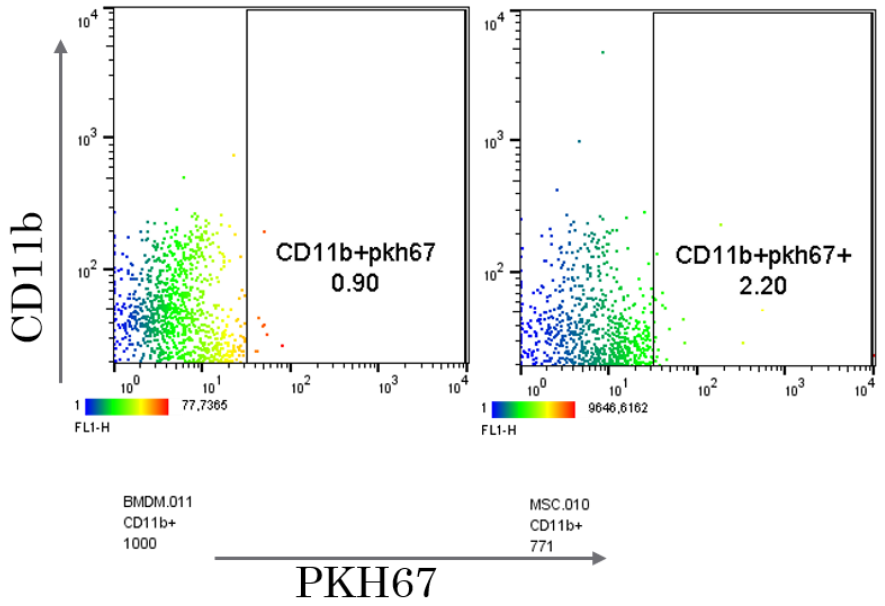

Figure 3.5.2

BMDM and MSC derived exosomes uptake by microglia. All cells depicted are CD11b positive. MSC derived exosomes are internalized more efficiently (2.20 % of microglia) compared to BMDM derived exosomes (0.90% of microglia)

3.6 Higher concentration of exosomes does not lead to more efficient uptake from microglia in the cerebellum

Further on, we wanted to investigate which concentration of exosomes was suitable for providing sufficient uptake by the cerebellum. Cerebellum is a brain area of great interest since preliminary data from our lab suggest that in mice lacking ERCC1 protein in microglia there is impairment in the movement of back legs, a function mainly controlled by the cerebellum. Therefore, for this set of experiments cerebella were extracted and prepared separately from the remaining brain tissue.

As presented in Figure 3.6.1 when we use triple the concentration of exosomes, compared to what we used in previous experiments we detected a higher population of exosomes reaching microglia in brain (from 5.67% to 20.6 %). However, there is not much difference in the amount of exosomes that reach the cerebellum (from 4.32% to 4.79%). One possible

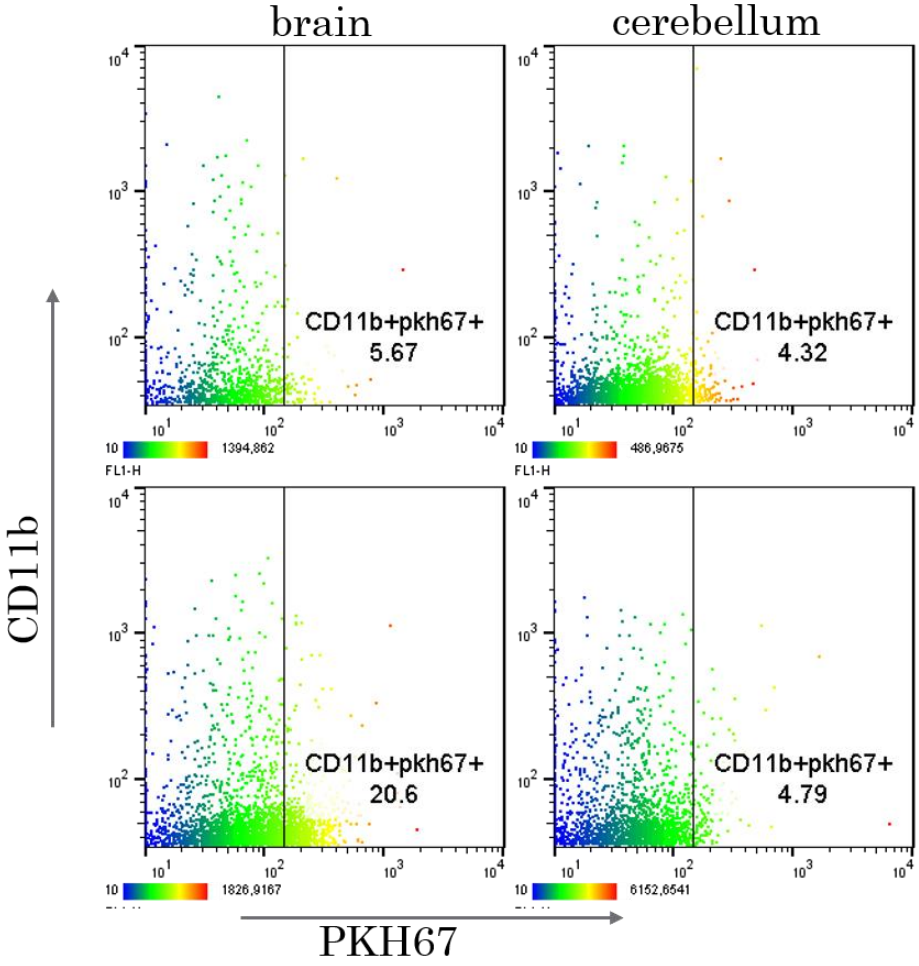

Figure 3.6.1

reason for the poor CD11b positive stained cells in the cerebellum area, might also be its low density of microglia[52].

To that extend, we tried to make our exosomes more specific to microglia cells in order to increase their uptake from cerebellum microglia.

3.7 Exosome specific peptide efficiently delivers exosomes in cerebellum

We decided to decorate our exosomes with a Cd11b ligand[46] aiming to target preferentially microglia cells. For that reason we fused a peptide specific for the extracellular domain of CD63, a known tetraspanin and marker of exosomes (Gao et al[45]) .with the CD11b ligand (Figure 3.7.1), and incubated our exosomes with it.

CP05 **CD11b-ligand**
CRHSQMTVTSRL - RKLRSLWRR

Figure 3.7.1: microglia specific peptide

This set of experiments was carried out as before (Figure 3.5.1), with the difference that this time we performed an additional step of exosome painting with the peptide followed by PKH staining. Stained exosomes were intranasally administered in wt adult mice that were sacrificed after a period of 4-5 hours. Brain tissues were isolated; the cerebellum was again separated from the rest of the tissue and cells were marked with CD11b staining.

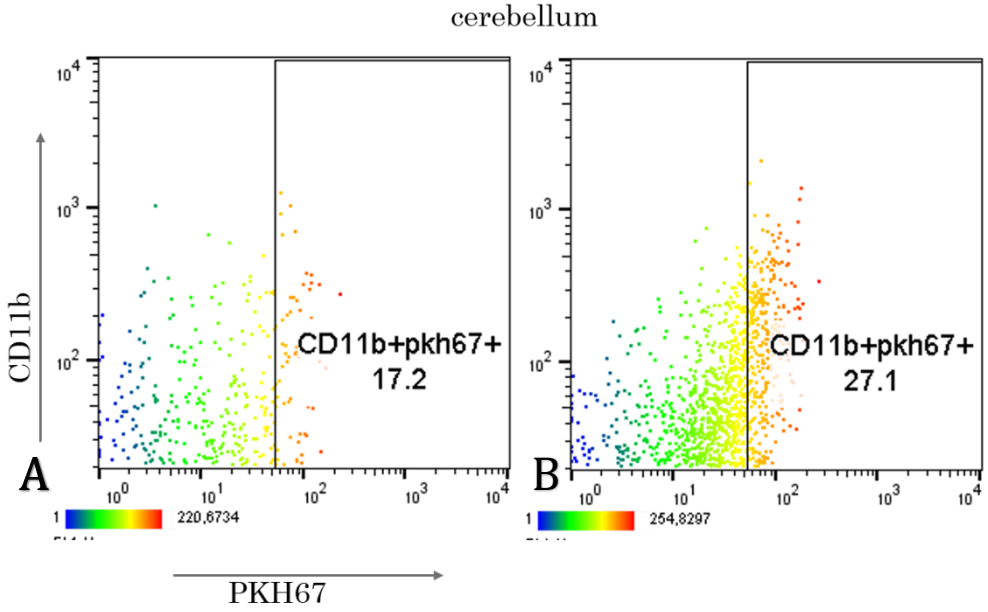

Figure 3.7.2: A) microglial cells, 17.2% of microglia are PKH positive, thus they have internalised exosomes. B) after microglia-specific peptide addition, 27.1% of microglia have internalised exosomes

Our data suggest that upon treatment with the peptide exosomes are internalized more efficiently by CD11positive microglial cells in the cerebellum where the percentage of double positive cells for CD11b and PKH staining is upregulated from 17.2% to 27.1% (Figure3.7.2). However, we should mention that cerebellum cell populations seem to differ which could justify for the significant difference observed, thus the experiment should be repeated with more accuracy.

3.8 Cloning results

Parallel to the previous experiments we wanted to create a more efficient way of exosome production in order to make them more efficient for "drug delivery" purposes. For this purpose, we needed to intervene in the following steps a) Exosome biogenesis (boost), b) Packaging of specific/desired mRNAs in the exosomes (in our case DNase1), d) Targeting and e) delivery of mRNA into the cytosol of target cells.

We followed the work of Kojima et al[55] and decided to clone all the genes needed for the above mentioned process, aiming to provide a stable cell line in the end, that will have the capacity to produce a high number of exosomes that will be targeted to a specific cell type. The only vector that will need to be inserted through transient transfection will be the one with the desired mRNA which will be able to change depending on the occasion.

3.8.1 Cloning strategy

The goal is to clone 3 different vectors. Vector 1 and Vector 2 as indicated below are lentiviral vectors that will be used in order to produce a stable cell line that will 1) produce a large number of exosomes, 2) provide a packaging method for DNase mRNA and 3) target the exosomes to the brain. Vector 3 is the only one that will be transfected each time and will contain the DNase mRNA.

In more detail:

Vector 1: boosting of exosome biogenesis (Figure 3.8.1)

The genes that will be cloned are **STEAP3, syndecan 4 and STAM**. P2A and T2A peptides[56] ensure concomitant expression of all three proteins.

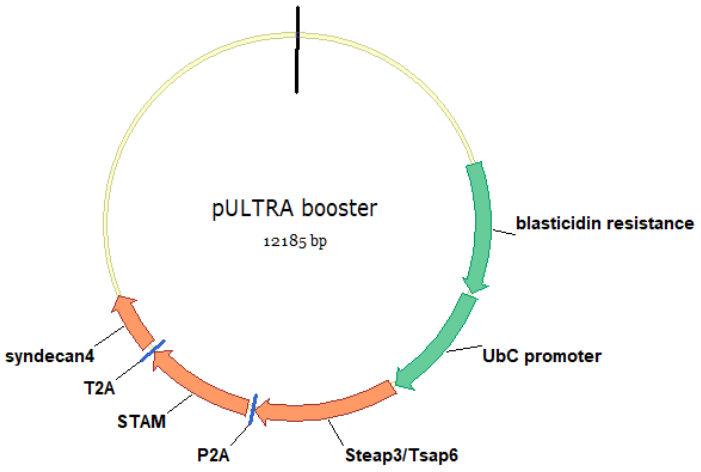

Figure 3.8.1: Vector 1, exosome biogenesis boost

STEAP3 also known as TSAP6 plays a role in the exosome biogenesis and it has been shown that this procedure is compromised upon its deletion [57].

STAM is part of the ESCRT 0 complex which along with Vps27/HRS initiate the MVB(multivesicular body) biogenesis pathway through binding to ubiquitinated proteins attached on the membrane[31].

Syndecan 4 interacts with many signaling and adhesion molecules including Alix through syntenin. Alix in turn drives the syntenin-syndecan complex to the ESCRT(II/III) machinery playing a role in the generation of MVBs and the exosome budding procedure [5859].

To sum up all the genes that will be cloned play a role in the biogenesis of exosomes through the ESCRT mechanism and were chosen according to Kojima et al [55]. The genes will be expressed under a ubiquitin C promoter and a blasticidin resistance will ensure the final step selection (creation of stable cell line).

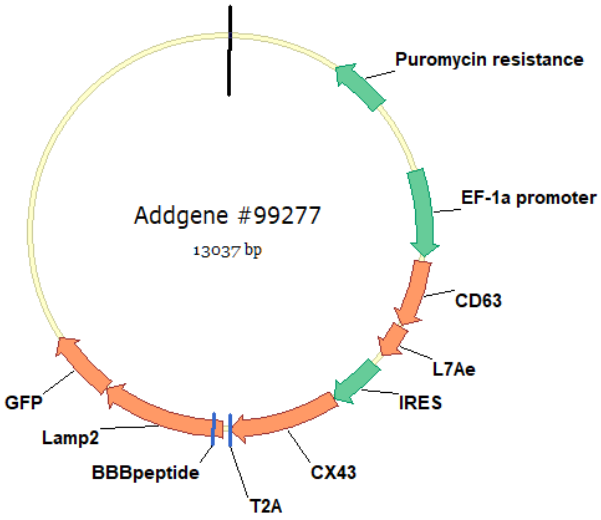

Figure 3.8.2: Vector 2, packaging, targeting, uptake

The enhanced exosome biogenesis will be tested using common exosome markers (Alix, CD9, CD81).

Vector 2: packaging and targeted delivery (Figure 3.8.2)

The final vector2 construct will contain a **CD63- L7Ae fusion, connexin 43, Lamp2SP-targeting peptide-Lamp2-GFP** in this order, under EF-1A promoter. T2a peptide and an IRES sequence this time will ensure simultaneous expression of all three sequences.

L7Ae is used to establish the packaging of the desired mRNA as it binds the C/D box RNA structure[60] which will be conjugated with the DNase mRNA(vector 3). L7Ae will be fused in the C-terminal of CD63 in order to be delivered in the exosomes.

Connexin 43 (CX43) is a gap junction protein that will be used as an exosome delivery helper as it was indicated by [55] because of its potential to form channels that can deliver exosomes to the recipient cell more efficiently[61].

Lamp2 will be used as a carrier for the targeting peptide as in (Alvarez-Erviti et al., 2011)[62] and [63]. Shortly a targeting peptide will be added after the signal peptide sequence of Lamp2. This will facilitate rapid uptake of exosomes from the brain minimising peripheral losses. Finally Lamp 2 will be tagged with GFP for easier visualization. Genome integration will be achieved via lenti-viral packaging (puromycin selection.)

Vector 3: DNase mRNA packaging (Figure 3.8.3)

The final construct will contain DNase1, a secreted endonuclease which acts on both ssDNA and dsDNA as well as chromatin. Dnase I is already used as a drug (dornase alfa) in cystic fibrosis [64].

DNase I will be fused to a C/D box (3' UTR) and will be tagged with an HA and a FLAG tag N-terminally. DNase I will be cloned without its native signal peptide to prevent its secretion from the targeted cells.

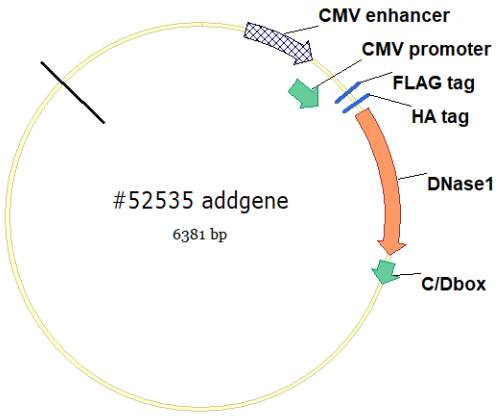

Figure 3.8.3: Vector3

The stable cell line that will be used is NIH-3T3 which is a fibroblast cell line easily transfected and handled in order to produce a high number of exosomes.

Vector 3 has already been produced along with initial steps of the other two vectors.

4. Discussion

Genome is subjected to a wide variety of damages throughout an organism's life. In order to maintain, preserve and faithfully transmit the information, organisms have a employed a wide variety of DNA repair mechanisms. Among them, NER is a very complex and delicate pathway, using more than 20 protein complexes to finely tune and extrude damages caused to DNA from UV. It has an effect both throughout the genome as well as in regions that are being transcribed. NER shares proteins and enzyme complexes with other repair pathways. One such example is ERCC1-XPF complex which acts as a nuclease and participates in many damage repair mechanisms. Lack of this nuclease from primary pancreatic cells as well as from microglia of mice lacking ERCC1 in all resident macrophages leads to accumulation of DNA damage, subsequent accumulation of ssDNA/dsDNA or both in the cytoplasm and innate immune response that includes type one interferon response through cGAS-STING pathway as shown by Stratigi et al. and Gkirtzimanaki et al. (unpublished data). In the case of microglia cells cytoplasmic DNA accumulation was additionally shown to be connected with animal paralysis.

In an attempt to reverse such a phenotype, we decided to utilize engineered exosomes that will provide the targeted cell population with a nuclease capable of degrading cytoplasmic DNA, thus leading to phenotypic rescue. This is extremely appealing especially in the case of microglial cells due to the exosomes' unique ability of passing the blood brain barrier (BBB).

To begin with we managed to establish that exosomes that were extracted from MSCs are able to be internalized by recipient cells both in vitro (PPCs) and in vivo (microglia). Furthermore, we show evidence, like others before us, that exosomes can carry and preserve proteins and deliver them efficiently inside acceptor cells. We managed to show the significant decrease of cytoplasmic DNA in the case of PPCs that have received nucleases enclosed in exosomes. Moreover, in the same cells the innate immune response pathway was significantly dampened. Furthermore, our in vivo studies provided information about the capacity of brain cells and especially microglia to internalize exosomes. Finally, we generated a peptide following the work of Gao et al. that decorates exosomes with a microglia specific ligand thus enhancing significantly the uptake of exosomes from the targeted cell population.

However, all in vivo experiments need to be repeated at the experimental mice lacking ERCC1 form resident macrophages with exosomes loaded with a nuclease in order to observe any phenotypic change macroscopically (mouse movement impairment) as well as at the cellular level (presence of cytoplasmic DNA in microglia cells and mRNA levels of type I interferons).

Although more work needs to be done, the data presented in this study suggest that exosomes can be considered a promising tool for drug delivery purposes in neurodegenerative /neuroinflammatory disorders.

Alongside these, we started a procedure of generating a stable cell line, that will be used as a tool for production of exosomes with specific qualities/characteristics. In more detail, the stable cell line will be able to produce an increased amount of exosomes following the work of Kojima et al.[55], which will be also preferentially targeted to microglia and will have the capacity of encapsulating the mRNA of interest, in our case that of a nuclease (S1 nuclease/DNAse1).

The conundrum here is which host cell line should be used. One would assume that an oligodendrocyte cell line would be appropriate due to its normal exchange of exosomes with microglia. However, upon microglia activation like in our case there are studies suggesting that there is no exosome preference from oligodendrocytes[65]. NIH cells could be a first suggestion, due to their easy manipulation and accessibility. Many studies suggest that cell-line derived exosomes do not cause any kind of immune response[38].

Furthermore, more studies need to be made in order to make exosomes more targeted against specific cell types. One way to do that would be finding other specific markers of cells undergoing damage and cytoplasmic DNA accumulation and use them to specifically direct exosomes against them.

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