Stimulation of exosome release by extracellular DNA is conserved across multiple cell types

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Abbreviations: EVs: extracellular vesicles; ODNs: oligodeoxynucleotides; PS: phosphorothioate; PO: phosphodiester; TLR: Toll-like receptor; MV: microvesicles; AB, apoptotic bodies; PM, plasma membrane; MVB, multivesicular body; ESCRT, endosomal sorting complex required for transport; UC: ultracentrifugation; APCs, antigen presenting cells; MHCII: major histocompatibility class II; poly I:C: polyinosinic:polycytidylic acid; TEM: transmission electron microscopy; WB: Western blot; CHSE: Chinook salmon embryo; ASK: Atlantic salmon kidney; HEK293T: human embryonic kidney 293T; HKLs: Head kidney leukocytes; PBLs: peripheral blood leukocytes; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GO: gene ontology; SIM: Structured illumination microscopy; UbP: ubiquitinated proteins; ChQ: chloroquine; IL-1β: interleukin-1β; ERK: extracellular signal-regulated kinase

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Abstract

Exosomes are distinguished from other types of extracellular vesicles by their small and relatively uniform size (30-100 nm) and their composition which reflects their endo-lysosomal origin. Involvement of these extracellular organelles in intercellular communication and their implication in pathological conditions has fuelled intensive research on mammalian exosomes; however, currently, very little is known about exosomes in lower vertebrates. Here we show that, in primary cultures of head kidney leukocytes from Atlantic salmon (*Salmo salar*), phosphorothioate CpG oligodeoxynucleotides induce secretion of vesicles with characteristics very similar to these of mammalian exosomes. Further experiments revealed that the oligonucleotide-induced exosome secretion did not depend on the CpG motifs but it relied on the phosphorothioate modification of the internucleotide linkage. Exosome secretion was also induced by genomic bacterial and eukaryotic DNA in toll-like receptor 9-negative piscine and human cell lines demonstrating that this is a phylogenetically conserved phenomenon which does not depend on activation of immune signaling pathways. In addition to exosomes, stimulation with phosphorothioate oligonucleotides and genomic DNA induced secretion of LC3B-II, an autophagosome marker, which was associated with vesicles of diverse size and morphology, possibly derived from autophagosome-related intracellular compartments. Overall, this work reveals a previously unrecognized biological activity of phosphorothioate ODNs and genomic DNA – their capacity to induce secretion of exosomes and other types of extracellular vesicles. This finding might help shed light on the side effects of therapeutic phosphorothioate oligodeoxynucleotides and the biological activity of extracellular genomic DNA which is often upregulated in pathological conditions.

Introduction

Eukaryotic cells release different types of extracellular vesicles (EVs) implicated in unconventional protein and nucleic acids secretion, riddance of disused and harmful substances and intercellular communication [1]. The EVs are, in general, classified in three groups – apoptotic bodies (ABs), microvesicles (MVs) exfoliated from plasma membrane (PM) and exosomes [2, 3]. According to the leading model, exosomes are derived from multivesicular bodies (MVB) - late endosomal compartments which contain intraluminal vesicles. The intraluminal vesicles are precursors of exosomes and they are formed either through endosomal sorting complex required for transport (ESCRT)- or ceramide-dependent inward budding of the delimiting membrane of endosomes [4]. The MVB origin of exosomes is reflected in their nanoscale (30-100nm) size and their composition enriched in endolysosomal marker molecules [5].

The content of MVBs is usually degraded following fusion with lysosomes. Prior to merging with lysosomes, MVBs may fuse with double-membrane autophagosomes leading to generation of amphisomes which may contain vesicles with diverse size and morphology. The main function of autophagy is degradation of intracellular components; however, it has also been implicated in secretion of exosomes. While degradative autophagy has been associated with suppression of exosome secretion [6, 7], there is strong evidence that secretory autophagy promotes the secretion of these EVs [8]. Nevertheless, it is not yet clear whether the process involves direct exocytosis of amphisomes or another type of autophagosome-related compartments.

In contrast to the shedding of PM-derived microvesicles which is an inducible process triggered by extracellular stimuli [5], secretion of exosomes is, generally, regarded as a constitutive process. Nonetheless, it has been shown that signaling events which induce MV release, such as elevation of intracellular calcium, can also affect secretion of exosomes [9-11]. Exosomes perform important functions within the immune system and their secretion is triggered upon interaction between Antigen Presenting Cells (APCs) and T-cells [12]. Activation of macrophages with yeast beta glucan has also been demonstrated to induce exosome release [13] and, in a previous study, we have found that salmon head kidney leukocytes (HKLs) stimulated with phosphorothioate (PS) B-class CpG oligonucleotides (ODN 2006PS) secrete small major histocompatibility class II (MHCII)-containing EVs [14].

Since information about piscine exosomes is still scarce, the current study has been aimed to further characterize the properties of exosomes released by salmon leukocytes and to shed light on the potential involvement of pattern recognition receptors in secretion of EVs. Our results demonstrate that extracellular DNA has capacity to induce secretion of exosomes as well as larger LC3B-II-positive vesicles through TLR-independent and phylogenetically conserved mechanisms. We further speculate about the potential involvement of cell adhesion receptors and heparin binding factors in this phenomenon.

Results

ODN 2006PS induces quick, dose dependent secretion of exosomes in primary cultures of salmon HKLs.

In mammals, CpG containing ODNs activate immune cells through TLR9 [15] and we have previously shown that a salmon TLR9 homolog interacts with CpG ODNs [16]. In order to compare the capacity of different endosomal TLR ligands to modulate exosome secretion, primary salmon HKLs were stimulated with 2 µM 2006PS, 1 µg/ml of R848 (TLR7/8 ligand) and 20 µg/ml of poly(I:C) (TLR3/22 ligand) [17-19] for 24h in serum-free L-15 medium. Conditioned supernatants were precleared of cell debris and large vesicles with sequential centrifugations and subjected to ultracentrifugation (UC) as described in "Material and methods". Proteins in pellets were analyzed with WB using antibodies against exosomal marker proteins. The results shown in fig. 1A demonstrate that ODN 2006PS, and to a lesser extent R848, but not poly(I:C) upregulate the levels of alix and ubiquitinated proteins (UbP) which are common exosome markers and MHCIIβ, a marker for exosomes secreted by APCs. Note that, in addition to the predicted ~100 kDa band of the full length protein, the alix antibody recognizes an additional band of 75-80 kDa which has also been observed in human cells and exosomes [20].

Mammalian leukocytes are sensitive to stimulation with CpG ODNs and respond to concentrations <100 nM [21]. Our data demonstrates that the upregulation of exosomal markers by ODN 2006PS in UC pellets is dose dependent (fig. 1B). Salmon HKLs stimulated with 100 nM ODN 2006PS for 24 hours secreted detectable levels of exosomes which increased further with concentrations up to 2µM. A time-course experiment demonstrated that 2006PS-induced exosome secretion is a fast process as significant levels of exosomes were released as soon as 1 h post-stimulation with 2006PS (fig. 1C).

The density of mammalian exosomes reported in different studies ranges between 1.08 and 1.20 g/ml [22-24]. In the current study, the density of the vesicles present in the 2006PS-induced UC pellets was analyzed using Optiprep density gradient ultracentrifugations. The results from the SDS PAGE/silver staining and WB shown in fig. 2A demonstrate that most of the vesicles accumulate in fractions with density >1.09 and <1.20 g/ml. A small portion of the proteins remained in the top fraction with density <1.03 g/ml indicating these were probably soluble proteins transiently bound to the EVs. Notably, all of the exosome markers were detectable in fractions with

density >1.09 and <1.20 g/ml with a peak at 1.12 g/ml which confirms that the UC pellets are enriched in exosomes. This was further supported by TEM analysis showing that the UC pellets from conditioned medium of 2006PS-stimulated HKLs contain vesicles whose size and morphology are typical for exosomes (fig. 2B). Using TEM we also observed what appears to be a process of exocytosis of MVBs and release of exosome-like vesicles by HKLs stimulated with ODN 2006PS for 2 h (fig. 2C).

HKL-derived exosomes are enriched in homologs of mammalian exosome marker proteins.

UC pellets from serum-free supernatants of non-stimulated HKLs and cells stimulated with 2µM 2006PS for 24h were analyzed with mass spectrometry. The total numbers of proteins identified through at least two peptides in three replicate experiments with cells from different individuals in NS and 2006PS-stimulated samples were 166 and 355 respectively. Of these, 132 proteins were identified in both NS and 2006PS samples. The numbers of unique proteins identified only in NS or 2006PS samples were 34 and 223, respectively (fig. 3A).

To investigate the presence of exosome marker proteins we used the information available in ExoCarta (www.exocarta.org) - an exosome database which provides information about the contents of exosomes isolated from different organisms. Figure 3B shows a list with some of the 50 most frequently identified proteins in exosome preparations whose homologs were identified in at least one of the three replicate samples in NS and 2006PS-induced EVs. The identified proteins include homologs of classical mammalian exosome markers such as CD9, CD63, CD81 and PDCD6IP (alix).

In order to perform a gene ontology enrichment analysis we mapped the list with the gene IDs of the identified proteins in 2006PS exosome samples against the human gene database using a web-based tool provided by the Gene Ontology Consortium (http://www.geneontology.org/). The results from PANTHER Overrepresentation Test showing 15 most significantly overrepresented GO Cellular component categories in 2006PS-induced salmon EV samples based on the P-value are shown in fig. 3C. The most significantly enriched category was the “extracellular exosome” with a P-value of 5.09E-20.

ODN 2006PS affects the release of exosomes but not cell debris and AB.

In vitro, primary leukocytes, especially lymphocytes, may undergo quick apoptosis due to lack of essential growth factors and negative selection mechanisms. This may lead to release of significant amount of apoptotic bodies and cell debris which are biochemically distinct compared to exosomes and may contaminate exosome preparations [25]. To investigate the influence of ODN2006PS on secretion of different types of EVs, conditioned serum-free medium from HKLs incubated for 24h was subjected to differential centrifugation (fig. 4). Protein analysis of the contents of the pellets with Western blot and micro BCA demonstrated that both stimulated and non-stimulated cells released considerable amounts of particles pelleted through sequential centrifugations at 1 500 g and 10 000 g (fig. 4A). The particles pelleted at 1 500 g are, typically, enriched in cell debris and apoptotic bodies (AB). In addition to MHCIIβ and flotillin-1 these pellets also contained detectable levels of calnexin and high amounts of prohibitin-1 which indicates these samples contained material derived from endoplasmic reticulum and mitochondria. Neither the total protein amount nor the levels of the proteins analyzed with WB in 1 500 g pellets were significantly influenced by the 2006PS treatment. PM-derived microvesicles (MV) are usually isolated through centrifugation at 10 000 g [26]. In the current study, the 10 000 g pellets from supernatants of non-stimulated cells contained high levels of MHCIIβ, flotillin-1, actin and calnexin and stimulation with 2006PS slightly upregulated the protein levels in these samples. Of the proteins analyzed with WB, the 2006PS stimulation upregulated the levels of UbP and the lipidated, membrane-bound form of LC3B – LC3B-II with an apparent molecular weight of ~14 kDa. In contrast to pellets isolated through 1 500 and 10 000 g centrifugation, the stimulation with 2006PS upregulated significantly the amount of proteins in the UC pellets (114 000 g). Most importantly, alix, MHCIIβ, flotillin-1, UbP and LC3B-II were clearly upregulated in the UC pellets whereas actin, calnexin and prohibitin-1 were not - indicating that the pellets were enriched in exosomes and not cell debris.

Analysis of the RNA contents of the pellets from the differential centrifugation demonstrated that the 2006PS stimulation upregulated significantly the RNA levels only in the exosome samples (fig. 4B). In the 1 500 g pellets, the size of the RNA molecules was generally low - <1000 nucleotides and the 18S and 28S RNA bands were very faint indicating that these RNA samples were highly degraded. The RNA in 2006PS-induced exosomes was also depleted of 28S rRNA, yet, the average size of the RNA molecules was larger in exosomes as compared to that present in cell debris/AB (1 500 g). In addition, the banding pattern shown in fig. 4B was very consistent between

RNA samples isolated from different exosome preparations indicating this was not merely degraded RNA but, rather, a relatively specific pool of RNA molecules - a feature typical for exosomes [27].

Genomic DNA and PS-modified but not phosphodiester ODNs induce exosome secretion in HKL cultures through a chloroquine-insensitive mechanism.

To investigate whether the capacity of ODN 2006PS to induce exosome release depends on its nucleotide sequence, HKLs were also incubated with 2 µM ODN 2216PO/PS (class A CpG ODN), and ODN 2137PS (inverted CpG control for 2006PS). The results presented in figure 5A show that the effect was not dependent on the presence of CpG motifs since ODN 2137PS induced release of comparable amounts of exosomes. In ODN 2216PO/PS only 8 of 19 bonds are PS-modified while the rest are phosphodiester (PO) and, compared to ODNs with fully PS-modified backbone, it was a relatively weak inducer of exosome secretion. This prompted us to investigate the influence of the PS nucleotide linkage on the capacity of ODNs to induce exosomes. As shown in fig. 5B, in contrast to PS-modified ODN 2137, no significant exosome upregulation was detected when HKLs were incubated with an ODN with the same sequence but with phosphodiester sugar-phosphate backbone.

We further tested the capacity of genomic salmon DNA (*S . sal.* DNA) to induce exosome secretion. Due to the variable size and the considerably higher MW of the sonicated genomic DNA molecules, ranging between 100 and 1500 bp, we stimulated the cells with 15 µg/ml of *S. sal.* DNA which equals the mass concentration of 2 µM 2006PS. The results demonstrated that, like PS ODNs, treatment of HKLs with *S. sal.* DNA also induced exosome secretion (fig. 5C). Proteomic analysis demonstrated that the composition of exosomes induced by *S. sal*. DNA was similar to that of PS ODN-induced exosomes (fig. 5D) and they contained the major markers for mammalian exosomes (fig. 5E).

Pretreatment of HKLs with chloroquine (ChQ,) which interferes with endosomal acidification, did not significantly affect the capacity of 2006PS to induce exosome secretion (fig. 5F). This indicates that, unlike upregulation of immune genes by ODN 2006PS (fig. 5G), the PS ODN-induced exosome upregulation does not require endosomal acidification

To compare the immunostimulatory capacity of different types of DNA, HKLs were stimulated with 15 µg/ml of ODNs 2137PS, 2216PO/PS, 2006PS and genomic *E. coli* and *S. sal.* DNA for 48 h. The expression of IL-1β and Mx proteins was analyzed in cell lysates with WB (fig. 5H). The results demonstrated that 2006PS and *E. coli* DNA induced considerable expression of both IL-1β and Mx while cells stimulated with ODNs 2137PS and 2216PO/PS did not upregulate significantly IL-1β but expressed high levels of Mx. In contrast, *S. sal.* DNA did not induce detectable levels of neither IL-1β nor Mx.

ODN 2006PS induces exosome release in different types of piscine cell cultures

Next, we compared exosome secretion in primary cultures of Atlantic salmon leukocytes derived from different tissues. Equal numbers of leukocytes isolated from HK, blood (PBLs) and spleen were stimulated with 2 µM 2006PS and exosomes were isolated through UC. The UC pellets were analyzed by WB – exosomes secreted by 20 x 10^6 cells stimulated for 24 hours were loaded in each lane. The results presented in fig. 6A demonstrate that, unlike HKLs, the stimulation did not induce significant exosome secretion in PBL and splenocyte cultures. The levels of MHCIIβ and UbP were comparable in lysates of HKLs, PBLs and splenocytes and, therefore differences in the expression of these proteins within cells could not account for the differences of their levels in exosome preparations. In contrast to PBL and splenocyte cultures, incubation of ASK and CHSE-214 cells with 2 µM 2006PS consistently and significantly upregulated exosome release (fig. 6B).

Flow cytometry analysis demonstrated that, compared to splenocytes and PBLs, which contained mostly lymphoid cells, the HKL cultures contained higher percentage of myeloid cells including granulocytes and mononuclear phagocytes (fig. 6C). In addition, compared to PBLs, the HKLs cultures contained considerably higher percentage of adherent cells (fig. 6D).

PS-modified ODNs, genomic DNA and cell adhesion induce EV secretion in cultures of HEK293T cells

In the following set of experiments we investigated the capacity of PS-modified ODNs, as well as genomic eukaryotic and prokaryotic DNA from *S. salar* and *E. coli*, respectively, to induce EV secretion in human HEK293T cells. The cells were seeded in cell culture flasks at 100% confluence (10 x 10^6 cells/sample) in MEM

supplemented with 5% exosome-depleted FBS and allowed to adhere overnight followed by a 1 hour incubation with 2 μM 2006PS and 15 μg/ml of *S. sal.* and *E. coli* DNA. All of the treatments induced secretion of exosomes (fig. 7A) indicating the process is controlled through phylogenetically conserved mechanisms within vertebrates. The density of the PS-ODN-induced HEK293T vesicles present in the UC pellets was investigated using an Optiprep density gradient ultracentrifugation. The results from WB analysis of 11 fractions collected from the gradient demonstrate that the exosome markers accumulate mostly in fractions with density >1.07 and <1.13 g/ml (fig. 7B).

It has been shown that, in HT1080 cultures, cell adhesion concurs with secretion of exosomes [28]. In the current study, we observed that HEK293T cells adhere relatively quickly (within 1 hour) in multilayers when seeded at high density (>1 x 10^6 cells/cm^2). Under these conditions, addition of *S. sal.* DNA obviously interfered with the adhesion of the cells to the well bottom and most of the cells formed suspended cell clumps whereas those seeded in absence of DNA remained firmly attached to the well bottom even after shaking of the cell culture plate (fig. 8A). Next, we collected conditioned medium 5 and 45 min after the cells had been seeded with or without 15 μg/ml of *S. sal.* DNA. Exosomes were isolated through UC and analyzed with WB. Five minutes after seeding, the cells were still in suspension and there was no detectable exosome secretion in control samples whereas *S.sal.* DNA had already induced detectable levels of alix, flotillin-1 and UbP in UC pellets (fig. 8B). After 45 min, most of the cells had attached and there were detectable exosome levels in conditioned medium of both non-stimulated and *S.sal.* DNA-treated cells. Figure 8C shows results from an experiment in which conditioned media of non-stimulated cells were harvested after 60 min when the cells had adhered (adh.). The cells were further incubated in presence of fresh growth media for another 60 min (post-adh.). The WB analysis demonstrated that, following adhesion, HEK293T cells secreted significantly lower amounts of exosomes. In this group of experiments we also demonstrated that incubation of HEK293T cells on ice, which blocked cell adhesion to the cell culture well, also inhibited exosome secretion (Fig. 8D). The genomic DNA preparations used in the current study had 260/280 nm absorbance ratios >2 indicating they were relatively pure and experiments in which *S. sal.* DNA had been pretreated with 50U of DNase I showed that the intact DNA, rather than presence of impurities in the DNA preparation upregulated the secretion of exosomes (fig. 8E). This latter experiment serves as another example

demonstrating that adhesion *per se* (in the absence of external stimuli) causes exosome secretion as high levels of alix were detected in UC pellets derived from non-stimulated cells after 60 min of incubation.

Alix and LC3B colocalize in PS-ODN-induced EVs from HEK293T cells.

Like in HKL cultures, treatment of HEK293T cells with ODN 2006PS induced secretion of LC3B-II whose level, unlike that of alix, was significantly higher in the 10 000 g pellets as compared to exosome pellets (fig. 9A) indicating it associated predominantly with vesicles larger than exosomes.

To investigate whether alix and LC3B colocalize within EVs, 2137PS-induced exosomes from HEK293T cells were spotted on glass coverslips, stained with alix and LC3B antibodies and subjected to Structured Illumination Microscopy (SIM) imaging. The images in fig. 9B show that while most of the observed vesicles appeared to be single-positive either for alix or LC3B, there were some vesicles which were positive for both proteins. Further, we investigated the localization of alix and LC3B in EV preparations from 2137PS-stimulated HEK293T cells through immunogold labeling and TEM. The supernatants had been precleared of large cell debris with 1500 g centrifugation and the remaining EVs were pelleted through UC without prior centrifugation at 10 000 g and 0.2 µm filtration to isolate exosomes and larger EVs together. The pellets were subjected to high pressure freezing in capillary tubes and processed for immunogold labeling with LC3B and alix antibodies. The images in fig. 9C demonstrate that alix and LC3B do colocalize in vesicles with diverse size and morphology. Some of the vesicles appeared to be composed of double or multi-membrane layers indicating they might be derived from intracellular compartments such as autophagosomes, amphisomes and multilamellar bodies.

PS-modified ODN, genomic DNA and heparin induce secretion of exosomes by Jurkat cells.

Jurkat cells are a human leukemic T lymphocyte cell line [29] which grows in suspension and has lymphoblast morphology. In the current study, like in HEK293T cultures, stimulation of Jurkat cells with 2 µM PS ODNs and 15 µg/ml of *S.sal.* DNA induced secretion of exosomes (fig. 10A) and the induction was inhibited by pretreatment of the genomic DNA with DNase I (fig. 10B).

Jurkat cells maintain constitutively high levels of Extracellular Signal–Regulated Kinase (ERK) activity. It has been reported that heparin may inhibit ERK activity [30, 31] and that inhibition of ERK activity, in cells in which this kinase is constitutively active, upregulates exosome secretion [32]. Therefore, we further investigated the influence of heparin and *S. sal.* DNA on ERK phosphorylation and the influence of an inhibitor of the ERK pathway (PD184352) on the secretion of exosomes by Jurkat cells. The cells, grown in MEM supplemented with 5% exosome-depleted FBS, were pretreated with 10 μM PD184352 or vehicle (DMSO) for 20 min and stimulated with 15 μg/ml of *S. sal.* DNA and heparin for 1h. Cell lysates and exosomes isolated through UC were analyzed with WB (fig. 10C) showing that, under these conditions, neither DNA nor heparin had a negative effect on ERK phosphorylation which was effectively downregulated by the inhibitor. Within the timespan of this experiment, PD184352 slightly upregulated exosome secretion by non-stimulated cells while, on the other hand, the inhibitor suppressed moderately the heparin-induced exosome secretion (fig. 10D).

Discussion

The data presented here reveal a novel aspect of the biological activity of extracellular DNA – its capacity to induce secretion of EVs.

The current study has been intended to follow up on a previous research in which we discovered that, in cultures of salmon head kidney leukocytes, stimulation with immunostimulatory CpG ODN 2006PS activate unconventional secretory mechanisms including release of exosome-like vesicles [14].

The major findings and conclusions based on the presented data are as follows:

i. In cultures of primary salmon head kidney leukocytes, ODN 2006PS induces dose-dependent secretion of exosomes with fast kinetics (< 1 h). This suggests that the process involves quick exocytosis of intracellular compartments which contain preformed exosome precursors.
ii. In addition to exosomes, incubation of cells with PS-modified ODNs induces secretion of LC3B-II-positive vesicles with diverse size and morphology suggesting secretory autophagy might be involved in their biogenesis.
iii. Non-immunostimulatory genomic salmon DNA also has capacity to induce EVs release in cultures of HKLs and non-immune cell types suggesting the process does not depend on activity of immune sensors for DNA, such as TLR9.
iv. DNA-induced exosome secretion is observed both in piscine and human cell cultures demonstrating that this phenomenon is controlled through phylogenetically conserved mechanisms.

Based on their morphology, density, accumulation of RNA, depletion of intact ribosomal RNA and enrichment in exosome markers, including alix, syntenin, TSG101, CD63, CD9 and CD81, it could be inferred that the PS2006-induced exosomes are derived from classical MVBs. In the current study, we did observe examples of what appears to be MVB exocytosis in 2006PS-stimulated HKLs. However, in addition to MVBs, it has been reported that exosome-like vesicles may originate from other cellular compartments, including plasma membrane of Jurkat T cells [33] and budding of cytoplasmic vesicular aggregates in human platelets [34]. Therefore, we

cannot exclude the possibility that intracellular compartments other than MVBs might also contribute to the inducible EV secretion we observed in the current study.

The differential centrifugation approach demonstrated that the 2006PS stimulation has an effect mostly on the exosome secretion and it did not significantly affect the release of cell debris/AB. This concurs with previously published data showing that 2006PS does not affect cell viability [14]. However, the 2006PS stimulation did affect the release of larger vesicles/vesicle aggregates pelleted through 10 000 g centrifugation. Interestingly, in contrast to alix, which is a marker for ESCRT-dependent exosomes [35], the levels of LC3B-II were relatively higher in the 10 000 g pellets suggesting it associates mostly with larger vesicles and, to a lesser extent, with exosomes. Similar to what has been reported for exosomes derived from prostate cancer cells [8], in EVs from salmon leukocytes and HEK293T cells we identified only the lipidated, membrane-associated form - LC3B-II (~14 kDa MW). This is a major autophagosome marker protein and SIM and immunogold/TEM analyses demonstrated that LC3B-II colocalized with alix in both exosome-like as well as larger vesicles some of which appeared to have multilayer membrane structure. It could be speculated that these vesicles might be derived from amphisomes or autolysosomes; however, in proteomic analysis of EVs from salmon leukocytes and HEK293T cells, we could not identify other major autophagy markers which had previously been detected in exosomes from PC-3 cells [8]. Therefore, it is possible that the LC3B-positive vesicles we identified in the current study might be generated through alternative LC3B-dependent mechanisms rather than classical autophagy.

Activation of leukocytes through innate immune, pattern recognition receptors may influence secretion of EVs. For example, it has been demonstrated that activation of macrophages by yeast beta glucan, a ligand for dectin-1, induces secretion of exosomes [13]. ODN 2006PS is a ligand for the endosomal receptor TLR9 [15] and therefore, we wanted to find out whether immune activation through endosomal TLR-signaling might be implicated in regulation of exosome release. Albeit much weaker, stimulation with R848, which is a ligand for TLR7/8 [18] also induced exosome secretion supporting the potential involvement of endosomal TLRs in secretion of exosomes. Nevertheless, several lines of evidence presented here indicate that TLR-independent pathways might be essential for the PS ODN-induced exosome upregulation. Firstly, it has been demonstrated that PS-modified ODNs without CpG motifs act as TLR9 inhibitors and that the TLR9 can be activated by the sugar-

phosphate backbone of phosphodiester ODNs, independently of their CpG contents [36]. In contrast, in the current study, exosome upregulation depended on the PS modification of the ODNs but not on the CpG motifs and it was also observed following incubation of leukocytes with non-immunostimulatory genomic salmon DNA. In addition, cell lines from salmon (ASK and CHSE) as well as human HEK293T cells which do not express detectable levels of TLR9 [16, 37] and are not activated by CpG motifs also upregulated exosome secretion following incubation with PS ODNs and genomic DNA. Moreover, inhibition of endosomal acidification and CpG-induced immune gene upregulation with chloroquine did not interfere with the 2006PS-induced exosome secretion. Binding of CpG ODNs to salmon TLR9 is pH-dependent [16] and it has been demonstrated that, in mammals, endosomal acidification is essential for interaction of endosomal TLRs, including TLR9, with their ligands [38].

The fact that ODN 2006PS consistently induce EV release by primary salmon HKLs and non-immune, piscine and human cell lines indicates that this response is inherent to diverse cell types. Remarkably, no significant exosome upregulation by 2006PS could be observed in cultures of primary salmon PBLs splenocytes. The ASK, CHSE-214 and HEK293T cell cultures are composed of adherent cells with relatively homogeneous epithelial morphology. In contrast, primary cell cultures contain diverse types of leukocytes whose percentage may vary considerably in immune organs of different individuals. Nevertheless, the salmon HKL cultures contain consistently high percentage of adherent myeloid cells, including granulocytes and mononuclear phagocytes [39]. In contrast, the splenocyte and PBL cultures are composed predominantly of non-adherent lymphoid cells and a much lower percentage of adherent cells.

Interestingly, integrin/syndecan-mediated cell adhesion has been associated with secretion of exosomes as the latter directly precedes the assembly of focal adhesions and promotes cell attachment and migration [28]. As polyanions, DNA molecules have potential to interact electrostatically with cationic proteins and they share interacting partners with heparin and heparan sulfate proteoglycans such as syndecans [40]. The latter are phylogenetically conserved receptors which are essential for growth factor signaling and cell adhesion [41]. In addition, syndecans are involved in exosome biogenesis and secretion through a process which implicates their heparan sulfate chains [42]. Here we also present evidence that, upon adhesion HEK293T cells secrete exosomes and that treatment of the cells with genomic DNA interferes with the cell adhesion to plastic cell cultureware. It

may be argued that tyipsinizing and washing of the cells prior to seeding may have contributed to release of cellular debris, including exosome-like particles. However, in our study, this possibility can be dismissed since no significant levels of EVs were detected in conditioned supernatants of HEK293T cells seeded on ice which also did not adhere.

In addition to enhanced DNase resistance [43], the PS modification enhances significantly the affinity of ODNs for heparin binding proteins [44]. Therefore, our observation that EVs upregulation by ODNs requires a PS-modified backbone further supports the possibility that the induction of EVs by DNA might depend on its heparin/heparan sulfate-mimicking properties. Our observation that both genomic DNA and heparin induce exosome secretion in cultures of Jurkat cells further supports this hypothesis. Jurkat cells are a leukemia T-cell line which grows in suspension; however, these cells do express cell adhesion receptors, including integrins and syndecans whose interaction with extracellular matrix molecules can be inhibited by PS ODNs [44]. It should also be considered that, Jurkat cells maintain high basal ERK activity. Treatment of cells with heparin is known to inhibit the ERK activity [30, 31], which in turn may lead to upregulation of exosome secretion as it has been described previously in cells in which ERK activity is constitutively high [32]. Our results do not support this scenario since neither DNA nor heparin had any detectable negative effect on basal ERK phosphorylation in Jurkat cells. In addition, incubation of the cells with an inhibitor of the ERK signaling pathway only weakly upregulated EV secretion and, on the opposite, it had a slight negative effect on the heparin-induced exosome secretion.

In vivo, DNA is discharged from cells mostly through leakage from dead cells and ejection of extracellular chromatin traps [45]. In addition, damaged genomic DNA may translocate to the cytoplasm, whence it can be incorporated in exosomes [46]. Normally, circulating exDNA has a relatively short half-life [47] and its concentrations in plasma samples of healthy human individuals are relatively low, ranging between undetectable levels up to 100 ng/ml [47]. However, under pathological conditions, such as physical trauma, autoimmune disorders and metastatic cancer, circulating exDNA amounts may increase reaching up to several micrograms per ml of plasma [48, 49]. Very likely, these values are much higher in tissues where exDNA is released and where it may reach concentrations comparable to that used in the current study (15 μg/ml). The biological activity of exDNA is still poorly understood and the finding that it has a capacity to regulate EV secretion might help shed light

on pathological processes such as autoimmune disorders and cancer metastasis in which both exDNA and exosomes are implicated [50-52].

Conclusions: In summary, we have demonstrated that, in cultures of piscine and human cells, PS ODNs induce secretion of exosomes as well as larger, LC3B-positive vesicles, likely derived from different intracellular compartments. Exosome release is also induced by genomic eukaryotic and prokaryotic DNA as well as heparin. The process does not correlate with activation of DNA-sensing immune mechanisms but it rather relies on phylogenetically conserved mechanisms which may be implicated in cell adhesion and may involve heparin-binding factors.

Materials and methods

Reagents

The sequences of the ODNs purchased from Thermo Scientific are as follows: 2006PS: T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T; 2216PO/PS: G*G*G*GGACGATCGTCG*G*G*G*G*G; 2395PS: T*C*G*T*C*G*T*T*T*TC*G*G*C*G*C*G*C*G*C*C*G, where phosphorothioate modifications are marked with asterisks. The sequence of ODN 2137 is the non-CpG control for 2006PS in which the CpG dinucleotides are inverted. The synthetic dsRNA, polyinosinic:polycytidylic acid (poly I:C) was purchased from Invivogen (tlrl-pic). Salmon testes DNA was purchased from Pharmacia (cat. #: 27-4564-01). The wild type E. coli strain 33W1485 (F+) was obtained from the Bulgarian National Collection for Microorganisms and Cell Cultures, and chromosomal DNA was isolated as described elsewhere [53]. Salmon and bacterial DNA was sheared by sonication to fragments ranging in size from 100 bp to 1500 bp. The spectral 260/280 ratios for both DNA preparations were ~2. Optiprep (D1556) and the MEK inhibitor PD184352 (PZ0181) were obtained from Sigma Aldrich. The salmon MHCIIβ and CD18 antibodies were produced in rabbits using synthetic peptides (DGREVKSDVTSTEEL for MHCIIβ and DFNREVDKQRISGNLDSPE for CD18). The monoclonal mouse antibody specific for salmon interleukin-1β (IL-1β) was produced using a synthetic peptide with the following sequence: GFRNWFISTDMQQDNTKPVD. Antibodies against alix (ab117600), flotillin-1 (ab41927) and calnexin (ab137336) were obtained from Abcam. Antibodies against actin (A2066) and LC3B (L7543) were from Sigma Aldrich. Monoclonal antibody recognizing mono- and polyubiquitinated conjugates (FK2) was purchased from Enzo Life Sciences (BML-PW8810). Anti prohibitin-1 (sc-28259) and secondary anti-rabbit (sc- sc-2004) and anti-mouse IgG (sc-2005) HRP-conjugated antibodies were obtained from Santa Cruz Biotechnology. Secondary anti-mouse Alexa Fluor 488 (A-1101) and anti-rabbit Alexa Fluor 555 (A-21430) were purchased from Thermo Fisher Scientific. Proteins in samples were quantified using reagents provided with the Thermo Scientific Pierce Micro BCA Protein Assay Kit (cat. #: 23235) and following manufacturer's instructions. Salmon testes DNA was purchased from Pharmacia (cat. #: 27-4564-01). The wild type E. coli strain 33W1485 (F+) was obtained from the Bulgarian National Collection for Microorganisms and Cell Cultures, and chromosomal DNA was isolated as described elsewhere [53].

Cell cultures and EV isolation

Non-vaccinated Atlantic salmon, Salmo salar L., strain Aquagen standard (Aquagen, Kyrksæterøra, Norway), 500–1000 g, was obtained from Tromsø Aquaculture Research Station (Tromsø, Norway). The fish were kept at 6–12°C in tanks supplied with running filtered sea water and were fed on commercial, dry food. The animal treatment was performed in accordance with relevant guidelines and regulations given by the Norwegian Animal Research Authority. Head kidney (HK) and spleen leukocytes were isolated as described by Jørgensen et al. [54]. Briefly, HK and spleen tissues were passed through 100-mm pore size cell strainers (Falcon) in L-15 medium containing penicillin (10 U/ml), streptomycin (10 mg/ml), 2% fetal bovine serum (FBS) (Gibco – cat.# A25904DG) and heparin (20 U/ml). Blood samples were diluted 1:4 in medium with the same composition. The HKL and splenocyte suspensions were placed on a 25/54% discontinuous Percoll gradient and centrifuged at 400 g for 40 min at 4°C. The diluted blood was layered on 54% Percoll cushion and centrifuged at 400 g for 20 min at 4°C. The cells at the interface were collected and washed twice in L-15 medium. The density of the leukocyte suspensions was adjusted to 14 x 10^6 cells/ml and the HKLs were further incubated in serum-free L-15 or medium supplemented with 5% exosome-depleted FBS as indicated in the description of the different experiments.

The cell lines were propagated as follows: HEK293T cells were cultured in minimum essential medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO2. Chinook salmon embryo CHSE-214 cells originate from Chinook salmon (*Oncorhynchus tschawytscha*) embryo and ASK cells were isolated from Atlantic salmon kidney. These cells were cultured at 19 °C in L-15 medium with supplemented with 10% and 8% FBS for ASK and CHSE cells, respectively.

EVs were isolated through a centrifugation protocol [26]. Briefly, conditioned supernatants were centrifuged sequentially at 500 g (10 min), 1500 g (15 min), 10 000 g (40 min) to isolate cells, AB/cell debris, and MVs respectively. The supernatants were then filtered through 0.2 µm filters (VWR) and ultracentrifuged at 114 000 g for 2 h using a SW50.1 rotor and an Optima L-80 XP ultracentrifuge (Beckman Coulter). The pellets were resuspended in 5 ml PBS and centrifuged again at 114 000 g. After the washing, the pellets were resuspended in PBS and analyzed immediately or stored at -70°C until further use.

Optiprep gradient ultracentrifugation

To obtain discontinuous density gradients, Optiprep (Sigma-Aldrich) solution was diluted with PBS to obtain 40% (1.216 g/ml), 30% (1.163 g/ml), 20% (1.111 g/ml), 10% (1.059 g/ml) and 5% (1.03 g/ml) iodixanol solutions. Aliquots of 1 ml with increasing density were loaded sequentially in 5 ml ultracentrifuge tubes. Exosomes resuspended in 100 µl PBS were layered atop the 5% solution and centrifuged at 114 000 g for 3 hours. Ten 0.5 ml fractions along with ~100 µl of the pellet were collected from top. The samples were concentrated with 10 kDa cutoff Amicon® Ultra 0.5mL Filters (Merck), lysed in LDS loading buffer (Invitrogen) and subjected to SDS/PAGE – silver staining using a SilverQuest™ Silver Staining Kit (Invitrogen™) and Western blotting.

Western Blotting (WB) and densitometry analysis

EVs and cell pellets were lysed in LDS sample buffer (Invitrogen) supplemented with 50mM dithiothreitol (DTT) and denatured at 70°C for 10 min. The samples derived from equal numbers of cells within each experiment were run on NuPAGE Novex Bis-Tris 4–12% gels (Invitrogen) in either MES or MOPS running buffer. The proteins were transferred to PVDF membranes, blocked (Tris-buffered saline, 5% BSA, 0.1% Tween-20) for 1 h, and incubated overnight with primary Abs (1:1000 dilution) followed by 1 h of incubation with the secondary HRP-conjugated antibodies (1:10 000 dilution). The blots were developed with either SuperSignal West Pico or Femto Chemiluminesccent Substrates (Pierce).

The densitometry was performed on selected exosome markers using the ImageJ software: (http://rsb.info.nih.gov/ij/index.html). Statistical analyses were performed using the GraphPad Prism 6 software. The value of $p < 0.05$ was considered to be significant.

RNA isolation and analysis

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). EV pellets were lysed in 0.5 ml of QIAzol. The homogenate was mixed with 100 µl of chloroform for 15 s and incubated for 2–3 min at room temperature. The samples were centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred to another tube and mixed with 300 µl 70% EtOH. After this RNA isolation was performed following the

manufacturer's instructions. On-column DNase digestion was performed using the RNase-Free DNase set (Qiagen, Hilden, Germany).

The composition of the RNA samples was analyzed through capillary electrophoresis using RNA 6000 Pico Kit and an Agilent 2100 Bioanalyzer® instrument (Agilent Technologies). One microliter of RNA dissolved in water was analyzed according to the manufacturer's protocol.

The cDNA synthesis and SYBR® Green PCR analyses were performed using primers and protocols as previously described [39]

Transmission Electron Microscopy (TEM)

Drops of PBS containing resuspended exosomes were transferred to carbon-coated formvar films on copper-grids for 3–5 min, washed in distilled water and treated with 1.8% methylcellulose/0.3% uranyl acetate for 5 min before drying.

To visualize HKLs and vesicles pelleted through 10 000 g centrifugation, pellets were fixed with 0.5% glutaraldehyde, 4% formaldehyde, 0.05% malachite green, 0.1 M PHEM buffer using a microwave protocol and stained as previously described [55]. Ultrathin cryosections cut on a Leica EMUC6 Ultramicrotome were mounted on Formvar-coated EM- grids.

Supernatants from HEK239T cells were cleared of large cell debris through centrifugation at 1 500 g for 15 min and ultracentrifuged (114 000 g) for 1 h to isolate exosomes and larger EVs together. Pellets were resuspended in PBS, subjected to high pressure freezing in capillary tubes [56] and processed for immunocytochemical labeling as previously described [37, 57]. The samples were examined with Jeol JEM1010 (Tokyo, Japan) and micrographs were taken with a Morada camera system (Olympus).

Proteomic analysis

Protein samples were run on NuPAGE Novex Bis-Tris 4–12% gels for ~5 min. The gels were stained with Coomassie G-250 (Invitrogen) and the whole lanes (~1 cm long) containing proteins were cut and subjected to in

gel reduction, alkylation, and tryptic digestion using 6 ng/µl trypsin (V511A, Promega, Wisconsin, USA) [58]. OMIX C18 tips (Varian, Inc., Palo Alto, CA, USA) was used for sample cleanup and concentration. Peptide mixtures containing 0.1% formic acid were loaded onto a Thermo Fisher Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2µm, 100 Å, 50 cm x 50µm). Peptides were fractionated using a 2-100% acetonitrile gradient in 0.1 % formic acid over 50 min at a flow rate of 200 nl/min. The separated peptides were analyzed using a Thermo Scientific Q-Exactive mass spectrometer. Data was collected in data dependent mode using a Top10 method. The raw data were processed using the Proteome Discoverer 2.1 software. The fragmentation spectra were searched against a NCBInr *Salmo salar* database downloaded 01/2017 using an in-house Mascot server (Matrix Sciences, UK). Peptide mass tolerances used in the search were 10 ppm, and fragment mass tolerance was 0.02 Da. Peptide ions were filtered using a false discovery rate set to 5 % for protein identifications.

To perform Gene Ontology (GO) enrichment analysis, the accession numbers of the proteins identified through at least two different peptides in three samples of 2006PS-induced exosomes were mapped to 221 Gene IDs using www.uniprot.org. The salmon gene IDs were subjected to PANTHER Overrepresentation Test using the web based tool provided by The Gene Ontology Consortium (http://www.geneontology.org). The used GO Ontology database (Homo sapiens) was released on 2017-12-27; the test type was Fisher's Exact with FDR multiple test correction.

Structured illumination microscopy (SIM)

For visualization of exosomes through fluorescence microscopy we used a protocol based on a previously published procedure [59]. Exosomes from HEK293T cells resuspended in PBS were spotted onto #1.5 coverslips and allowed to adhere for 20 min in a humidified chamber. Coverslips were then incubated for 20 min with 200 µl 4% formaldehyde, washed three times with 200 µl PBS, permeabilized with 0.1% Triton X-100 for 10 min and blocked for 30 min with 5 % BSA. Coverslips were incubated for 30 min with mouse anti-alix and rabbit anti-LC3B antibodies (1:400 dilution), washed three times with PBS, incubated for 30 min with secondary anti-mouse/Alexa Fluor 488 and anti-rabbit/Alexa Fluor 555 antibodies (1:1000 dilution). To obtain controls for background fluorescence, exosomes were stained only with secondary antibodies. The coverslips were mounted with glycerol

containing 1% DABCO. The samples were imaged on a OMX V4 Blaze structured illumination microscope (GE Healthcare) equipped with a 60X, 1.42 NA oil immersion objective (Olympus) and three sCMOS cameras. Sequential excitation was provided by 568, 488, and 405 nm laser lines; with emission filters of 609/37, 528/48, and 436/31, respectively. A 2 µm z-stack was imaged by collecting 15 images per z-plane with a 0.125 µm step size to enable 3D SIM reconstruction. Image reconstruction was performed using the manufacturer-provided softWoRx program.

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Supporting Information

Table S1: Complete proteomic dataset for fig. 3

Table S2: Complete proteomic dataset for fig. 5D&E

Figure legends

Figure 1. ODN 2006PS induces quick, dose dependent secretion of exosomal marker proteins in primary cultures of salmon HKLs. A, influence of endosomal TLR ligands on exosome secretion by HKLs (NS – non-stimulated control). The cells were stimulated as indicated in serum-free medium for 24h. Conditioned supernatants were precleared of cell debris and large vesicles as described in "Materials and methods" and exosomes were pelleted through UC. The pellets were analyzed with WB using the indicated antibodies. Samples derived from equal number of cells were loaded in each lane. B, dose-dependent induction of exosome secretion by ODN 2006PS. HKLs were stimulated with the indicated concentrations of ODN 2006PS for 24 hours and samples were collected and analyzed as in panel A. C, time-course of 2006PS-induced exosome secretion. HKLs were stimulated with 2 µM 2006PS for the indicated duration and samples were collected and analyzed as in panel A. Within each panel, the histograms below the blot images show the relative densitometry values for the MHCIIβ and alix bands; the error bars show the standard error (SE), n=3. The data was analyzed with one-way ANOVA and all of the stimulations were compared to the non-stimulated controls using Dunnett's post hoc test; *p<0.05. , **p<0.005, ***p<0.0005.

Figure 2. The density and the morphology of exosomes released by ODN 2006PS-treated salmon HKLs is similar to that of mammalian exosomes. A, UC pellets isolated from supernatants of 2006PS-stimulated HKLs were ultracentrifuged on discontinuous Optiprep gradients. Eleven fractions were collected from the top of the gradient and their protein content was analyzed using SDS PAGE/silver staining and WB using the indicated exosome marker antibodies. B, TEM analysis demonstrates that UC pellets from supernatants of HKLs stimulated with 2006PS contain vesicles with typical exosome size and morphology. C, TEM analysis showing MVB-like organelles near PM (arrowhead) and an apparent exocytosis of exosome like vesicles (arrows) in HKLs stimulated with 2006PS for 2 h.

Figure 3. Proteomic analysis of HKL-derived exosomes demonstrating enrichment in homologs of mammalian exosome marker proteins. UC pellets from serum-free SNs of non-stimulated (NS) HKLs and cells stimulated with 2 µM 2006PS for 24 h were analysed using a Q-Exactive mass spectrometer. A, Venn diagram showing the total number of unique and common proteins identified in NS and 2006PS-stimulated samples from three replicate

experiments. B, a table showing the identified salmon homologs of some of the 50 most frequently identified proteins in exosomes according to Exocarta (www. http://exocarta.org/exosome_markers_new). Identification of homologs in each of the three replicate samples is indicated with a "+". C, Gene ontology enrichment analysis demonstrating that 2006PS-induced salmon exosome samples are enriched in homologs of human exosome marker proteins. The accession numbers of the proteins identified in the three 2006PS samples were mapped to 221 Gene IDs using www.uniprot.org. The salmon gene IDs were subjected to a PANTHER Overrepresentation Test by using the human gene database. The histogram shows fifteen of the most significantly overrepresented GO cellular component categories based on the P-value. Raw proteomic data is presented in Table S1.

Figure 4. Differential centrifugation analysis – 2006PS affects the release of exosomes but not cell debris/AB. Conditioned medium from control HKLs and cells stimulated with 2µM 2006PS for 24h in serum-free medium was subjected to sequential centrifugations at 500g (cells), 1500g (debris/AB), 10 000 g (MV) and 114 000 g (Exo). A, protein composition of the pellets was analyzed with WB and micro BCA (histogram). B, RNA derived from the pellets was analyzed through capillary electrophoresis using a Bioanalyzer instrument. The size of the RNA and the position of the 18S and 28S rRNA bands are shown to the left of the gel-like image. The RNA concentrations are shown in the histogram. Proteins and RNA released from equal number of cells were loaded in each well of the SDS-PAGE gels and the RNA 6000 Pico Kit chips, respectively. The error bars in both histograms show the SE; n=3. Data was analyzed using the Student's t test; *p<0.05.

Figure 5. Genomic DNA and PS-modified but not phosphodiester ODNs induce exosome secretion in HKL cultures through a chloroquine-insensitive mechanism. A, HKLs were stimulated with 2 µM ODN 2137PS (an inverted CpG control of 2006PS), 2216PO/PS (with 8 PS and 11 PO bonds) and 2006PS. Exosomes were isolated through UC and analyzed with WB. B, HKLs were incubated with 2 µM ODN 2137 with phosphorothioate (PS) or phosphodiester (PO) nucleotide linkage and samples were analyzed as in panel A. C, HKLs were incubated with 15 µg/ml of salmon (*S. sal.*) genomic DNA (corresponding to the mass concentration of 2 µM 2006PS) and 2 µM 2137PS and samples were analyzed as in panel A. D, Exosome preparations from non-stimulated cells (NS) and HKLs incubated for 1 h with 15 µg/ml of ODN 2006PS and *S. sal.* DNA were analyzed with mass spectrometry. The Venn diagram shows the number of unique and common proteins identified in the exosome preparations. E, a table showing the

identified homologs of some of the most abundant exosome markers according to ExoCarta (www.exocarta.org). The presence of proteins identified through at least two peptides is indicated with (+). Raw proteomic data is presented in table S2. F, HKLs were treated with 2006PS and 10 µg/ml of chloroquine (ChQ), as indicated, and samples were isolated and processed as in panel A. G, QPCR analysis showing the relative expression of immune genes in HKLs treated with 2006PS and 10 µg/ml of ChQ as indicated. H, activation of HKLs by different types of DNA. The cells were incubated with 15 µg/ml of the indicted types of DNA for 48h and whole cell lysates were subjected to WB using antibodies against IL-1β and Mx. The Ponceau staining was used as a loading control. The results in panel H were confirmed in experiments with cells from at least two individuals. In panels C and H intervening lanes on the same gel were removed as designated by the splice mark. The histograms shown on the right of the WB images in panels A-C and F show the relative densitometry values of the alix bands. The error bars in the histograms show the SE; n=3. Statistical analyses was performed with one-way ANOVA followed by Tukey's multiple comparisons test; $p<0.05$.

Figure 6. Induction of exosome release by PS-modified ODNs in different types of piscine cell cultures. A, ODN 2006PS induces exosome secretion in primary cultures of salmon HKLs but not peripheral blood leukocytes (PBLs) and splenocytes. Samples derived from ~20 x 10^6 cells incubated for 24 h were loaded in each lane. The samples were run on same gel and intervening lanes were removed as designated by the splice mark. Cell lysates were also subjected to WB with MHCIIβ and UbP antibodies showing expression of comparable levels of these proteins in the different types of leukocyte cultures. B, Induction of exosome release by 2006PS in fish cell lines - ASK (*Salmo salar*) and CHSE-214 (*Oncorhynchus tshawytscha*). The cells were grown to confluence in cell culture flasks in complete growth media, washed with PBS and stimulated with 2 µM 2006PS. Exosomes were prepared through UC and analyzed with WB. Exosomes, isolated from 4 x 10^6 (ASK) and 10 x 10^6 (CHSE) cells were loaded in each lane. The histograms shown to the right of the WB images in panels A and B show the relative densitometry values of the alix bands; error bars – SE; n=3. C, flow cytometry analysis of salmon splenocytes, PBLs and HKLs. Cells from the indicated gates within the HKL dot plots were stained with MayGrünwald–Giemsa and representative images are shown as insets. D, 10 x 10^6 PBLs and HKLs were seeded in 24-well cell culture plates and allowed to adhere

overnight. The cells were washed three times and adherent cells were counted. The histogram shows the average number of adherent cells counted in four random microscope view fields of cells from two individuals; error bars - SE. Statistical analyses was performed with Student's t test; **p<0.005, ***p<0.0005, nd – not detected.

Figure 7. PS-modified ODNs and genomic DNA from salmon and *E. coli* induce exosome secretion in human HEK293T cell cultures. A, the cells were seeded in cell culture flasks in MEM supplemented with 5% exosome-depleted FBS and allowed to adhere overnight followed by a 1 h incubation with 15 µg/ml of the indicated types of DNA. Exosomes isolated through UC were analyzed with WB. The histograms shown to the right of the WB images show the relative densitometry values of the alix bands. The error bars show the SE; n=3. Statistical analysis was performed with Student's t test; **p<0.005, ***p<0.0005. B, Optiprep density gradient ultracentrifugation of HEK293T exosomes. Exosomes from HEK293T cells stimulated with PS ODNS were centrifuged on discontinuous Optiprep density gradient for 3 hours accumulating mostly in fractions with density 1.08 -1.11 g/ml.

Figure 8. Genomic salmon DNA interferes with adhesion of HEK293T cells to plastic cell cultureware; adhesion is implicated in exosome secretion by HEK293T cells. A, seeding of HEK293T cells in presence of salmon DNA interferes with cell adhesion to cell culture well bottom. The picture was taken 1 h after the cells were seeded in a 6-well plate in culture medium with or without 15 µg/ml of *S. sal.* DNA. The control cells remained firmly attached to the bottom of the wells whereas a significant part of the cells seeded in presence of DNA formed suspended cell clumps. B, HEK293T cells were seeded in medium supplemented with 5% exosome-depleted FBS in presence or absence of 15 µg/ml of salmon DNA. Media was harvested at the indicated time points and exosomes were isolated through UC and analyzed with WB. C, HEK293T cells were seeded as in panel B. Conditioned media was harvested after 60 min when the cells had adhered (adh.). The cells were further incubated in presence of fresh growth media for another 60 min (post-adh.). Samples were processed and analyzed as in panel B. D, HEK293T cells were seeded and incubated on ice or at 37°C for 1 h in cell culture medium with or without *S. sal.* DNA and samples were harvested and analyzed as in panel B. E, HEK293T cells were seeded as in panel B in presence of untreated *S. sal.* DNA or DNA pretreated with 50U of recombinant DNase I. Control cells were untreated or incubated with the same amount of DNase I. Samples were collected after 1 hour of incubation and analyzed as in

panel B. The histogram shows the relative densitometry values of the alix bands. The error bars show the SE; n=3. Data was analyzed with two-way ANOVA and Tukey's multiple comparisons test; $p<0.05$.

Figure 9. PS-modified ODNs induce secretion of LC3B-II-postive EVs with diverse size and morphology. A, HEK293Tcells were stimulated for 1 h with 2137PS and supernatants were subjected to differential centrifugation. Pellets collected with sequential 10 000g and 114 000g centrifugations were analyzed with WB. Exposures for two durations are shown for the LC3B blot to visualize both the lipidated and the non lipidated forms in the whole cell lysate (WCL) and the presence of LC3B-II in exosomes. The histogram shows the relative densitometry values of alix and LC3B-II bands. The error bars show the SE; n=3. Data was analyzed separately for each protein with two-way ANOVA and Tukey's multiple comparisons test; $p<0.05$. B, SIM image showing colocalization of alix and LC3B in 2137PS-induced exosomes. Samples from cells stimulated with ODN 2137PS were spotted on glass coverslips and stained with antibodies as indicated. The arrows indicate the position of double-positive vesicles in the projected image; scale bars – 500 nm. C, Supernatants from HEK293T cells incubated for 1 h with ODN 2137PS were pre-cleared of cell debris (1500 g, 15 min) and ultracentrifuged to isolate exosomes and larger EVs together. Pellets were resuspended in PBS, subjected to high pressure freezing in capillary tubes and processed for immunogold labeling with LC3B and alix antibodies; LC3B: 10 nm, arrows; alix: 5nm – arrowheads. Regions with apparent multi-membrane structure are indicated with asterisks.

Figure 10. PS-modified ODNs, genomic DNA and heparin induce exosome secretion by Jurkat cells. A, Jurkat cells were treated with 15 µg/ml of *S. sal*. DNA or 2 µM ODN 2395PS for 1 h. Exosomes were isolated through UC and analyzed with WB. B, Jurkat cells were stimulated with untreated *S. sal.* DNA or DNA preincubated with 50U of recombinant DNase I, as indicated. The samples were run on same gel and intervening lanes were removed as designated by the splice mark. C, Jurkat cells were pretreated with the ERK pathway inhibitor PD184352 or 0.1% DMSO for 20 min and stimulated for 1h with 15 µg/ml of *S. sal.* DNA and heparin prior to exosome isolation. Lysates from the cells were collected at the time of supernatant harvesting and were analyzed with WB using an antibody against phosphorylated ERK. Ponceau staining was used to confirm equal cell lysate loading. D, the histograms show the relative densitometry values for alix and flotillin-1 WB bands detected in

control and heparin-induced exosomes from cultures incubated with or without the PD184352 inhibitor. The error bars show the SE; n=3. The data was analyzed with two-way ANOVA and Tuckey's multiple comparison test; $p<0.05$.