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ARTICLE

High-order TRAIL oligomer formation in TRAIL-coated lipid nanoparticles enhances DR5 cross-linking and increases antitumor effect against colon cancer

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Abstract

During the last years, a great effort has been invested into developing new TRAIL formulations with increased bioactivity, trying to overcome the resistance to conventional soluble TRAIL (sTRAIL) exhibited by many primary tumours. In our group, we have generated artificial lipid nanoparticles decorated with sTRAIL (LUV-TRAIL), emulating the physiological TRAIL-containing exosomes by which T-cells release TRAIL upon activation. We already demonstrated that LUV-TRAIL has greater cytotoxicity against both chemoresistant hematologic tumour cells and epithelial carcinoma cells compared to a form of sTRAIL similar to that used in clinical trials. In this study we have tested LUV-TRAIL in several human colon cancer cell lines with different sensitivity to sTRAIL. LUV-TRAIL significantly improved sTRAIL cytotoxicity in all colon cancer cell lines tested. Trying to ascertain the molecular mechanism by which LUV-TRAIL exhibited improved cytotoxicity, we demonstrated that TRAIL-coated lipid nanoparticles were able to activate DR5 more efficiently than sTRAIL, and this relied on LUV-TRAIL ability to promote DR5 clustering on the cell surface. Moreover, we show that TRAIL molecules are arranged in higher order oligomers only in LUV-TRAIL, which may explain their enhanced DR5 clustering ability. Finally, LUV-TRAIL showed significantly better antitumor activity than sTRAIL in an *in vivo* model using HCT-116 xenograft tumours in nude mice, validating its potential clinical application.

Keywords: TRAIL, colorectal cancer, lipid nanoparticles, immunotherapy, DR5 cross-linking

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1. Introduction

Colorectal cancer (CRC) is one of the most frequent solid tumours in western countries. In fact, recent estimations indicate that CRC is the third most common cancer both in males and females [1]. The survival rate throughout all stages has improved mainly due to the implementation of screening programs, leading to the detection of CRC in earlier stages, together with the development of more effective treatment options. However, CRC still accounts for about 9% of the estimated cancer-related deaths [1].

Resistance of cancer cells to apoptosis is one of the so-called hallmarks of cancer [2]. Since impairments in the intrinsic apoptotic pathway are often involved in this resistance, targeting the extrinsic apoptotic pathway is especially attractive for cancer therapy [3,4]. Among the death ligands capable of triggering apoptosis through the extrinsic pathway, CD95L showed a remarkable strong cytotoxic potential on cancer cells. However, disappointingly it also displayed a severe systemic toxicity, therefore making unrealistic its use as anti-cancer agent [5]. On the other hand, another death ligand, namely tumour necrosis factor (TNF)-related apoptosis–inducing ligand (TRAIL), was found to be able to induce apoptosis in tumour cells without affecting most normal cells [6,7]. Hence, it was soon considered as potentially useful for anti-cancer therapy [8,9]. However, despite the encouraging initial results, TRAIL-based therapies showed very limited therapeutic activity in phase II/III clinical trials carried out on a wide variety of human cancers [3,10]. In order to overcome TRAIL resistance, better sensitization strategies [11-13], as well as novel TRAIL formulations with improved bioactivity can be of great usefulness for its future clinical use [14-17].

TRAIL has four membrane-bound receptors in humans: TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 [18-24]. In addition, TRAIL

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has been described to be able to bind to the soluble receptor osteoprotegerin (OPG) [25]. Among these five receptors, only DR4 and DR5 are able to transduce the apoptotic signal upon TRAIL binding. Although they share high similarity, both receptors present certain functional differences. For example, DR5 has been described to present higher affinity for TRAIL [26]. However, apparently DR5 requires further clustering, so it can only be properly activated by membrane-bound TRAIL or by artificially cross-linked versions of the molecule [27,28]. On the contrary, DR4 can readily be activated by soluble versions of TRAIL, although cross-linked versions of TRAIL proved again to be more active than soluble TRAIL [29]. Regarding the relative contribution of both receptors to TRAIL-induced apoptosis, DR4 has been described to be the pre-eminent pro-apoptotic receptor in haematological cells, while in epithelial cells expressing both receptors DR5 appears to be the main pro-apoptotic receptor [30-34]. However, the differential contribution of both receptors is still controversial.

Our group demonstrated that, in physiological conditions, TRAIL was indeed released by activated human T cells in its transmembrane form, inserted in the membrane of lipid microvesicles called exosomes [35,36]. On this basis, we have generated artificial lipid nanoparticles containing membrane-bound TRAIL (LUV-TRAIL) resembling those natural exosomes. We already demonstrated that LUV-TRAIL was more effective inducing apoptosis than soluble TRAIL against leukemia cells that presented resistance to soluble TRAIL and chemotherapeutic drugs [37,38] and also in epithelial-derived cancer cells [39,40]. This increased cytotoxicity induced by LUV-TRAIL was due to a superior DR5 activation, which led to an enhanced DISC recruitment [40,41].

In the present work, we have tested LUV-TRAIL in several human colon tumour cell lines with different sensitivity to soluble TRAIL, finding that LUV-TRAIL was

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more efficient than soluble TRAIL inducing apoptosis in all cancer cells tested. Finally, the *in vivo* antitumor potential of LUV-TRAIL against colon carcinoma cells was tested in a model of nude mice xenografted with the human colon carcinoma cell line HCT-116. This novel TRAIL formulation showed an enhanced antitumor ability, and could be potentially useful to improve therapy against colon cancer.

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2. Materials and Methods

2.1. Generation of liposomes covered with soluble recombinant TRAIL.

Large Unilamellar Vesicles (LUV) with soluble recombinant TRAIL (hereafter sTRAIL) anchored on their surface were generated as previously described [37,42]. Briefly, after generating the lipid nanoparticles, a version of sTRAIL (rTRAIL-His$_6$, corresponding to amino acids 95–281, kindly provided by Dr. M. MacFarlane [22]) was attached to their surface by incubation at 37ºC for 30 minutes.

2.2. Cell culture.

HCT-116, HT29 and CACO-2 cell lines were obtained from ATCC.. Bax-deficient HCT-116 cells deficient (HCT-116-Bax$^{-/-}$) were kindly provided by Dr. Christoph Borner (Albert-Ludwigs-Universität Freiburg). HCT-116-Bax$^{-/-}$ cells lacking expression of Bak (thereafter HCT-116-BB) were generated in our laboratory (Fig.S.1.A). Cell lines were routinely cultured at 37 °C in DMEM medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and penicillin/streptomycin (hereafter, complete medium).

2.3. Cell viability assay.

Cell viability was quantified by the MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) assay [43] as previously described [39,40]. Data was expressed as the percentage of cell viability with respect to control cells (untreated cells for sTRAIL, and cells treated with LUVs without TRAIL for LUV-TRAIL).

2.4. Clonogenic assay.

Clonogenic survival was assessed as previously described [40]. Quantification of the clonogenic assays was performed measuring the absorbance at 550 nm after dissolving crystal-violet with DMSO. The results were expressed as the percentage of absorbance with respect to the respective control (same as indicated for cell viability assays).

2.5. Cytotoxicity assays.

CRC cells ($3x10^4$ cells) were treated with different concentrations (1 to 1,000 ng/mL) of sTRAIL or LUV-TRAIL for 24 hours. In some experiments, cell death inhibition assays were carried out pre-incubating cells for 1 hour prior to the addition of sTRAIL or LUV-TRAIL with the blocking anti-human TRAIL mAb (500 ng/mL, clone RIK2, BD Biosciences) or with the pan-caspase inhibitor z-VAD-fmk (30 μM, Bachem). Then, cell sensitivity to sTRAIL or LUV-TRAIL was analysed by annexin-V staining and in some cases, by propidium iodide staining using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

2.6. Death receptor expression.

Cell surface expression of pro-apoptotic death receptors in CRC cell lines was analysed incubating $1x10^5$ cells with anti-DR4, anti-DR5 or isotype control PE-conjugated monoclonal antibodies, (eBioscience) at 4 ºC in PBS containing 5% FCS for 30 min and analysed by flow cytometry using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

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2.7. Nuclear morphology analysis.

Cytotoxicity assays were performed seeding cells ($1x10^5$ cells) onto cover slips placed in 6-well plates with complete medium, and treated with sTRAIL and LUV-TRAIL at 500 ng/mL for 6 hours. After that, nuclear morphology was analysed by fluorescence microscopy by staining with Hoechst 33342 (Life Technologies) as previously described [39].

2.8. Western blot analysis.

For protein expression analysis, Western blot was performed as previously described [38]. The following antibodies were used to analyse the expression of the main proteins involved in the extrinsic apoptotic pathway: anti-caspase-8 (BD Biosciences), anti-caspase-3 (Cell Signaling), anti-Bid (BD Biosciences), anti-PARP-1 (BD Biosciences), anti-Bax (BD Biosciences), and anti-Bak (BD Biosciences). As loading control, expression of β-actin was analysed using a specific antibody (Sigma).

For higher-order TRAIL oligomers analysis, sTRAIL or LUV-TRAIL were incubated with the indicated amounts of the cross-linker BS3 (Thermo) for 30 minutes at room temperature. Then, Tris/HCl was added at a final concentration of 50 mM to quench the reaction, and after thorough mixing, the mix was settled for 15 minutes at room temperature. Finally, sample buffer containing 1% SDS was added to each sample. After heating for 5 minutes at 96ºC, samples were separated by SDS-PAGE in gels containing 6% acrylamide/bisacrylamide, and analysed by Western blot.

For the clustering analysis of TRAIL receptors, HCT-116 cells (10 cm Petri dishes at 80% confluence) were treated with sTRAIL or LUV-TRAIL for the indicated times. After removal of supernatants, cells were washed with cold PBS and 1 ml of lysis buffer containing 1% Triton X-100 was added to each plate. After 15 minutes of

incubation at 4ºC, cells were scraped, and centrifuged at 4ºC. Then, cells were collected and mixed with sample buffer with or without 50 mM DTT. Samples were separated by SDS-PAGE, and TRAIL receptor expression was analysed by Western blot.

2.9. In vivo antitumor activity.

Immune-deficient athymic mice, Swiss nu/nu strain, six-week old males (Charles River), were used to evaluate *in vivo* antitumor activity. All experiments in mice were carried out according to the European recommendations on animal ethics and were approved by the University of Zaragoza Animal Experimentation Ethical Committee. Mice were kept under specific standard pathogen-free conditions throughout the study.

Freshly harvested HCT-116 cells ($1x10^6$ cells/mouse) were inoculated subcutaneously into mice. Once tumours reached a volume of 0.1 cm^3, mice were randomly divided in 3 groups (4 mice/group): Control, sTRAIL and LUV-TRAIL. Thirty-six µg/ml of either sTRAIL or LUV-TRAIL, in a total volume of 500 µl, were injected intra-peritoneally daily during 5 consecutive days. Control group were injected with LUVs alone (without TRAIL). Tumours were left to grow for 17 days after last injection and then, mice were euthanized. During all the experiment, tumour volumes were daily monitored, and tumour volume was calculated using the following formula: tumour volume=$(LxW^2)/2$ (where L is longitudinal and W is transverse tumour diameter respectively). Relative tumour growth was calculated as follows: relative tumour volume = ($volume_{day\ x}$/ $volume_{day\ 0}$) x100.

In order to assess whether the tumour-derived cells exhibited a similar phenotype than the parental cells, a subcutaneous HCT-116 tumour was surgically resected and a single cell suspension was obtained by gentle physical dissociation.

Finally, death-receptor expression and sensitivity to sTRAIL and LUV-TRAIL was analysed both on tumour-derived and parental cells as above described.

2.10. Statistical analysis.

Data were analysed using Prism® software program (GraphPad Software, San Diego, CA). Student's t test was used. All values are expressed as mean ± SD and $p < 0.05$ was considered statistically significant using the Prism® software program (GraphPad Software, San Diego, CA). Student's t test was used. All values are expressed as mean ± SD and $p < 0.05$ was considered statistically significant. HPLC data was analysed using the software OriginPro 8® (OriginLab Corporation, Northampton, MA, USA).

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3. Results

3.1. LUV-TRAIL show a significant higher cytotoxic ability compared with sTRAIL in human CRC cell lines.

Firstly, we checked the surface expression of the TRAIL receptors DR4 and DR5 in all CRC cell lines studied. (Fig.S.2) finding that all cell lines expressed both pro-apoptotic TRAIL receptors. Noteworthy, all cell lines showed a higher expression of DR5 than DR4. Then, we characterized the overall sensitivity of the four CRC cell lines to both forms of TRAIL. For that, cell viability was analysed after carrying out dose-response assays using both sTRAIL and LUV-TRAIL (Fig.1.A,). CRC cell lines showed different sensitivities to TRAIL. HCT-116 resulted to be the most sensitive cell line to both sTRAIL and LUV-TRAIL, but the LC_{50} was reduced by almost 3 fold in the case of LUV-TRAIL (from around 70 ng/ml for sTRAIL to around 25 ng/ml for LUV-TRAIL). On the contrary, HCT-116-BB showed a greater resistance to sTRAIL as compared to the wild-type cells, clearly showing that these cells are type-II cells regarding TRAIL sensitivity, since the lack of Bax and Bak completely protects these cells from TRAIL-induced apoptosis. However, LUV-TRAIL managed to have a significant effect on this cell line, leading to a cell growth inhibition of almost a 40%. HT29 cells showed a low sensitivity to sTRAIL, while LUV-TRAIL reached up to 60% of reduction of relative cell viability at the highest dose used. Finally, CACO-2 proved to be highly resistant to both forms of TRAIL, showed showing a similar sensitivity pattern as HCT-116-BB, being LUV-TRAIL again able to induce a significant decrease of cell growth.

On the other hand, the long-term effect of LUV-TRAIL on the survival and proliferation of CRC cell lines was assessed by performing clonogenic assays (Fig.1.B). In all cases, treatment with LUV-TRAIL for 24 hours significantly reduced clonogenic

survival of CRC cells more efficiently than sTRAIL. These data would indicate that LUV-TRAIL not only has a stronger short term effect than sTRAIL but also that it has a long-term effect affecting to survival and proliferation of CRC cells.

To assess if the observed decrease of cell viability implied an apoptotic process, several features of apoptosis were analysed. First of all, nuclear morphology after TRAIL treatment was studied (Fig.2.A). HCT-116 cells showed typical morphological features of apoptotic cell death such as blebbing and cell shrinkage (yellow arrows). Remarkably, apoptotic nuclear changes were more numerous and apparent when cells were treated with LUV-TRAIL, and importantly these nuclear changes correlated with cell death analysed in parallel using PI staining. On the other hand, phosphatidyl-serine translocation was studied by annexin-V staining upon treatment with both forms of TRAIL (sTRAIL and LUV-TRAIL, Fig.2.B). In HCT-116, HT29 and CACO-2 cells, LUV-TRAIL exhibited a greater ability to induce apoptosis than sTRAIL. Altogether, these results perfectly correlated with the cell viability assays showed in Fig.1.A in all cases except for HCT-116 BB cells, where no apoptosis was observed neither with sTRAIL nor with LUV-TRAIL.

To assess that LUV-TRAIL-induced apoptosis was due to a TRAIL-specific effect, pre-incubation with the TRAIL-blocking antibody RIK2 was performed before treatment with both TRAIL formulations. In all cases, apoptosis induced by either sTRAIL or LUV-TRAIL was entirely abrogated (Fig.2.B) corroborating that apoptosis induced by LUV-TRAIL exclusively relied on the presence of TRAIL on the liposome surface. In this line, it is important to point out that LUVs alone (without TRAIL on their surface) did not show any pro-apoptotic effect in any CRC cell line (see controls in Fig.2) Therefore, it could be concluded that the cytotoxic effect of LUV-TRAIL was

exclusively due to the action of TRAIL, discarding any non-specific effect that could be attributed to the liposomal particles.

3.2. LUV-TRAIL are able to induce a stronger activation of the extrinsic apoptotic pathway than sTRAIL in CRC cell lines.

To further characterize that cell death induced by LUV-TRAIL was an apoptotic process, inhibition assays were carried out using the pan-caspase inhibitor z-VAD-fmk (Fig.S3). z-VAD-fmk was able to fully inhibit cell death both in HCT-116 and HT29 cells when treated with forms of TRAIL indicating that LUV-TRAIL-induced cell death is a caspase-dependent process.

Next, activation of the extrinsic apoptotic pathway was assessed by Western blot (Fig.3. and Fig.S.4.A and B). For that, CRC cell lines were treated with sTRAIL or LUV-TRAIL for the indicated times (up to 6 hours). As it is shown, LUV-TRAIL induced a faster and stronger cleavage of the initiator caspase-8 and the effector caspase-3 in HCT-116 and HT29 cells. Furthermore, their specific substrates, Bid and PARP1 respectively, were also clearly cleaved, indicating that both caspases were indeed functionally activated. In parallel, aliquots taken from every time-point were analysed to measure the apoptotic levels by annexin-V staining (Fig.3., lower panels), finding that the differences observed in the Western blot were actually reflected in terms of apoptosis-induction ability, perfectly correlating. On the other hand, the resistant cell line HCT-116 BB showed a clear activation of the initiator caspase-8 and subsequently cleavage of its substrate Bid, to the same extent as their parental cell line HCT-116 (Fig.S4.A). In contrast, caspase-3 cleavage was virtually absent. Noteworthy, while parental HCT-116 cells showed high levels of a caspase-3 cleaved fragment of about 30 kDa upon LUV-TRAIL treatment, in HCT-116 BB only the fragment of 19 kDa was

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detected. Finally, no apoptosis could be detected (Fig.S.4.C), correlating with the results showed in Fig.2. However, surprisingly PARP1 cleavage was quite clear, starting after only 1 hour of treatment with LUV-TRAIL. To better characterize this apparent discrepancy, caspase-3 activation was quantified by using a colorimetric assay. As shown in Fig. S4.E, the activation pattern of caspase-3 in wild-type HCT-116 was in agreement with the Western blot in Fig. 3. For HCT-116 BB, caspase-3 activity was clearly diminished in comparison to the wild-type cells. However, a marginal activity could be detected, which could account for the PARP1 cleavage observed, but would not be able to fully undergo apoptosis. Altogether, this is the typical pattern expected from a type-II cell line, in which the activation of the mitochondrial apoptotic branch is mandatory for the execution of the apoptotic program. Finally, the highly resistant cell line CACO-2 showed certain activation of caspase-8 upon LUV-TRAIL treatment, but to a much lesser extent than the other cell lines, (Fig.S4.B). Accordingly, some activation of caspase-3 and cleavage of PARP-1 could be detected, which correlated with the observed slight induction of apoptosis (Fig.S4.D).

3.3. LUV-TRAIL enhanced pro-apoptotic potential relies on the formation of supra-molecular TRAIL oligomers.

Up to this point it was clear that LUV-TRAIL exhibited a higher pro-apoptotic potential than the soluble version of TRAIL. Our hypothesis was that the membrane-bound TRAIL present in the LUV-TRAIL formulation would form supra-molecular TRAIL oligomers that, in turn, would activate the main pro-apoptotic TRAIL receptor DR5 more efficiently than sTRAIL. To confirm that assumption, we carried out a cross-linking analysis in order to see the formation of supra-molecular TRAIL oligomers on the liposomal surface. The artificial cross-linker BS3 was added to sTRAIL or LUV-

TRAIL at increasing concentrations to stabilize the different TRAIL-oligomer populations, and samples were resolved by SDS-PAGE and Western Blot analysis (Fig.4.A). BS3 cross-linking of LUV-TRAIL allowed the detection of supra-trimeric populations of high molecular order. However, these higher-order oligomers could not be detected in sTRAIL. In order to study in more detail these high-order oligomers seen in LUV-TRAIL, the cross-linker BS3 was added to sTRAIL or LUV-TRAIL to stabilize the different TRAIL populations, and samples were analysed by HPLC (Fig.4.B and C). In LUV-TRAIL, a population of up to 840 kDa was detected, corresponding to approximately 32 TRAIL monomers (around 11 trimers, Fig.4.C), while in sTRAIL oligomers were hardly detected without incubation with BS3 (Fig.S.5) and it only reached approximately 23 TRAIL monomers (around 8 trimers) after BS3 addition (Fig.4.B).

On the other hand, the actual ability of sTRAIL and LUV-TRAIL to cluster TRAIL receptors on the cell surface was assessed on HCT-116 cells by allowing the cells to be stimulated with either sTRAIL or LUV-TRAIL for up to one hour, and then performing non-reducing SDS-PAGE followed by immunoblotting. As shown in Fig.4.D, only treatment with LUV-TRAIL allowed the clear formation of DR5 oligomers on the target cells. This oligomerization could be detected already from the first time point studied (10 minutes), and increased along time. Noteworthy, no DR4 oligomeric populations could be detected in any case.

Finally, to further characterize the relative importance of both TRAIL receptors in this scenario, we generated HCT-116 derived sub-lines stably down-regulating the expression of DR4 or DR5 (Fig.5.A and Fig.S.6). For that, the sub-lines were treated with different concentrations of sTRAIL and LUV-TRAIL to check their relative sensitivity. As shown in Fig.5.B, silencing of DR4 induced a remarkable reduction of

sensitivity to both TRAIL formulations. This would be clearly indicating that DR4 is the main pro-apoptotic receptor in these cells. However, importantly, significant differences between sTRAIL and LUV-TRAIL could still be detected at the highest concentrations (300 and 1000 ng/ml).

On the other hand, knockdown of DR5 did not have an important effect on the overall sensitivity of the cells neither to sTRAIL nor LUV-TRAIL, but the differences between sTRAIL and LUV-TRAIL disappeared (Fig.5.B, right panel). These data strongly suggested that the main difference between sTRAIL and LUV-TRAIL is their relative ability to activate DR5. Altogether, these results clearly indicated that the presence of higher order TRAIL oligomers on the liposomal surface was directly linked to the clustering of DR5 on the cell surface, ultimately leading to a higher cytotoxic effect.

3.4. LUV-TRAIL shows a significantly higher *in vivo* antitumor activity than soluble TRAIL in a human tumour CRC xenograft model.

Before performing *in vivo* experiments, cytotoxicity assays in 3-D cell cultures were carried out. For this purpose, we used a novel technology based on microfluidic devices which resembles more accurately the *in vivo* conditions in which cancer cells grow [44-46]. Confirming the *in vitro* results, LUV-TRAIL showed a higher cytotoxic effect than sTRAIL against HCT-116 cells also in 3-D cell cultures (Fig.6.A and B).

Finally, antitumor efficacy of both sTRAIL and LUV-TRAIL was tested using xenografted subcutaneous HCT-116 tumours. In any case, before performing the experiment, we wanted to assess whether during the tumour engraftment process the cells could undergo a selection process that might affect their phenotype regarding TRAIL sensitivity when compared to parental cells. Therefore, HCT-116 cells were first

analysed regarding their phenotype before and after forming tumours in nude mice. After obtaining a single-cell suspension from a tumour, cells were analysed for TRAIL-DR expression and overall sensitivity to sTRAIL and LUV-TRAIL in comparison to the parental cells (Fig.S7.A and B). As it shown, tumour-derived HCT-116 cells showed a similar surface expression pattern of both pro-apoptotic receptors DR4 and DR5 as parental cells. Concerning the relative sensitivity to sTRAIL and LUV-TRAIL, the tumour derived cells surprisingly showed similar sensitivity to both TRAIL formulations. Despite this change in relative sensitivity, we decided to carry out an *in vivo* experiment using a HCT-116 xenograft model (Fig.6.C and D). Mice were treated intraperitoneally with daily subcutaneous injections of 36 µg of either sTRAIL or LUV-TRAIL for 5 consecutive days, and tumour growth was monitored. Of note, to reduce the volume needed to reach 36 µg of TRAIL, the stock solutions of sTRAIL and LUV-TRAIL were prepared at 72 µg/ml, which represented six times the usual concentration of LUV-TRAIL (routinely prepared at a TRAIL concentration of 12 µg/ml). As this was an exceptional condition, to discard any unexpected effect due to the high concentration of TRAIL, an *in vitro* cytotoxic assay using LUV-TRAIL (6x) in comparison with normal LUV-TRAIL was performed on HCT-116 cells (Fig.S7.C). As it is shown, both LUV-TRAIL formulations showed a similar cytotoxic potential. Therefore, it could be assumed that the antitumor activity of LUV-TRAIL (6x) against HCT-116 tumours was equivalent to that of LUV-TRAIL. As shown in Fig.6.C and D, LUV-TRAIL clearly showed a better antitumor effect than sTRAIL against HCT-116–derived tumours. The difference between both forms of TRAIL was clear, being significant already at day 13 (Fig.6.C). Surprisingly, sTRAIL did not reduce nor delay tumour growth respecting to the Control group (Fig.6.D). This would be most likely due to the poor stability and

pharmacokinetic profile of sTRAIL [47,48], which would inactivate the molecule by the time it reached the tumour site.

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4. Discussion

Despite the initial optimism that TRAIL incited as a possible new and highly selective antitumor agent, the clinical effectiveness of TRAIL, both in monotherapy and in combined regimes, has been disappointing [3,4,10]. It is currently accepted that in order to overcome the poor responsiveness of some cancers to recombinant TRAIL formulations currently available [17] it appears as absolutely indispensable to improve its pro-apoptotic potential by the development of novel TRAIL formulations [15].

In this line, our group previously demonstrated that human lymphocytes secrete TRAIL preferably associated with lipid vesicles called exosomes [35,36] Aiming to mimic this exosome-bound TRAIL, we generated the novel TRAIL formulation LUV-TRAIL, based on anchoring this death ligand on Large Unilamellar Vesicles (LUV) resembling these natural exosomes. LUV-TRAIL has been proved to be much more potent than soluble TRAIL both in haematological malignancies [37,38,41] and solid tumours [39,40]. Remarkably, the soluble version of TRAIL used in all those studies is virtually identical to the version used in clinical trials (Dulanermin®), which confers more significance to LUV-TRAIL improved efficiency.

Pursuing to further analyse the effectiveness of LUV-TRAIL in solid tumours, we laid our focus on CRC cancer. We tested our liposomal formulation on a panel of four different CRC cell lines presenting a heterogeneous sensitivity to sTRAIL induced apoptosis. Although in some particular cases LUV-TRAIL showed similar pro-apoptotic ability than sTRAIL, in general LUV-TRAIL was shown to be more active than sTRAIL. Noteworthy, LUV-TRAIL was capable of overcoming the resistance to sTRAIL of some CRC cell lines highly resistant to sTRAIL, and induced a more robust activation of the caspase cascade than the soluble ligand. Moreover, LUV-TRAIL not only showed a greater cytotoxic potential *in vitro* in the short term, but it also showed a

significant advantage over sTRAIL at reducing the clonogenic survival in all cell lines tested.

Among the two TRAIL pro-apoptotic receptors, DR5 has been described as the main DR responsible for TRAIL-induced apoptosis in some epithelial-derived cancer cells such as breast and ovarian carcinoma [32,34]. Regarding CCR cells, both DRs have been implicated in TRAIL-induced apoptosis [52,53]. On the other hand, TRAIL exhibits a much stronger cytotoxicity when it is artificially cross-linked, and this increased bioactivity directly relies on its ability to cluster its pro-apoptotic receptors in supra-molecular structures larger than merely trimers [27,54]. Indeed, it has been previously demonstrated that the enhanced clustering of TRAIL receptors is related with an improved DISC recruitment [49-51]. In this line, it has been described that DR5 requires further cross-linking for a correct activation [27,28]. In fact, co-treatment of soluble TRAIL with an agonistic anti-DR5 antibody allowed an enhanced DR5 activation mediated by the artificial cross-linking of this receptor facilitated by the agonistic antibody [55,56]. We previously demonstrated that the enhanced cytotoxicity showed by LUV-TRAIL in human leukemic cells relied on their higher efficiency for promoting DR5 clustering leading to a higher DISC recruitment [41]. In the present study, we wanted to corroborate this feature also in CRC cells. First, we were able to demonstrate in cell-free assays by Western blot and liquid chromatography the existence of high molecular weight TRAIL oligomers only on LUV-TRAIL. Moreover, clustering analysis in CRC cells clearly demonstrated that LUV-TRAIL induced a more efficient clustering of DR5 than sTRAIL. Altogether, these results strongly suggest that the more effective apoptotic signal triggered by LUV-TRAIL relies on its more pronounced ability to induce a better clustering of the pro-apoptotic receptors on the target cell surface. To fully corroborate that the pro-apoptotic potential of LUV-TRAIL

is due to their more efficient activation of DR5, HCT-116 cells stably down-regulating expression of either DR4 or DR5 were generated using shRNA. As expected, the HCT-116 -derived shDR4 or shDR5 cells presented different sensitivities to both TRAIL formulations. Thus, in the shDR4 clones, the overall cell death levels were remarkably reduced, but a significant difference between sTRAIL and LUV-TRAIL could still be observed. On the other hand, down-regulation of DR5 did not produce in general any important effect on the cytotoxic potential of either sTRAIL or LUV-TRAIL. However, importantly, in shDR5 cells the significant difference between sTRAIL and LUV-TRAIL disappeared, indicating that DR5 activation was the main differential feature determining the improved outcome of LUV-TRAIL. Considering our results altogether, although in HCT-116 cells DR4 appeared to be the main pro-apoptotic TRAIL receptor, apoptosis induction could indeed be further favoured by the concomitant activation of DR5 provided by LUV-TRAIL. This activation was possible by the clustering of DR5, only achieved upon LUV-TRAIL stimulation.

In this regard, the relative apoptotic contribution of DR4 or DR5 in HCT-116 has been indeed addressed in the literature by using different approaches. Those reports showed somehow contradictory results: whilst in some of them DR4 appears to be the pre-eminent pro-apoptotic receptor, in others it is DR5 [52,53,57-59]. These differences might as well be explained by the use of different agonistic molecules in those studies. Some of those studies were conducted using different monoclonal agonistic antibodies against DR4 or DR5, however, the use of monoclonal antibodies as TRAIL-receptor agonists is a delicate matter. Although originally seen as a more specific and reliable way to activate DR4 or DR5, it was later shown that in certain cases those antibodies were not able to induce apoptosis on their own and required further crosslinking. An additional layer of complexity in this regard relies on the actual localization of the

receptors within specific membrane micro-domains on the cell surface, which could be directly involved in the requirement for further oligomerization of DR5 between different cell lines [60-63]. In any case, it appears that using improved TRAIL-derived formulations instead of antibodies might be advantageous given that TRAIL bear the potential to simultaneously activate both DR4 and DR5.

Although LUV-TRAIL has been proved to be more active than sTRAIL in all experimental settings tested so far, there are still some caveats regarding its possible use as a therapeutic agent. Before testing the *in vivo* antitumor potential of LUV-TRAIL, we first tested its performance in a more complex scenario based on culturing the cells in a three-dimensional matrix embedded in a central chamber in small polymer chips [64,65]. In this model, sTRAIL or LUV-TRAIL were perfused through lateral channels, and the agents should be able to diffuse into the matrix and reach the cells inside the chamber, somehow similarly to physiological extravasation processes. In accordance with its much bigger size, previous results showed that LUV-TRAIL exhibited a much slower diffusion rate through the matrix than sTRAIL (data not shown), which could actually represent a serious handicap regarding its pro-apoptotic efficiency. However, despite its low diffusion rate, LUV-TRAIL not only retained its cytotoxicity, but it showed again a much higher pro-apoptotic ability than sTRAIL.

Finally, we tested the *in vivo* potential of sTRAIL and LUV-TRAIL using xenografted HCT-116 tumours. Remarkably, LUV-TRAIL showed a significant tumour size reduction effect, as compared both to sTRAIL and Control groups. Moreover, strikingly no antitumor effect was observed for sTRAIL, which, taking into consideration the high sensitivity of HCT-116 to sTRAIL *in vitro*, clearly suggests that the systemic administration seriously affected the stability of the molecule. Although no specific pharmacokinetic profiles were measured, these results indirectly suggested that

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the immobilization of TRAIL on the liposomal surface greatly improved the stability of TRAIL, retaining its cytotoxic potential *in vivo.* In this regard, LUV-TRAIL formulation would also be taking advantage of the well-known *enhancing permeability and retention* (EPR) effect. According to the EPR effect, thanks to their size, nanoparticles in the range of 100 nm of diameter tend to spontaneously localize in tumour sites by extravasation through the leaky defective blood capillary irrigating the tumour. This EPR effect has been described to be an inherent property of nanoparticles independently of the composition of the particles [66].

Although the use of liposomes for the treatment of diseases of different kind has been extensively described, liposome platforms have been always used as vehicle to encapsulate drugs inside their lumen, improving their stability and pharmacodynamic properties [67-69]. However, the original idea of using of liposomes as a platform to attach TRAIL on their surface, increasing its bioactivity, was firstly developed and described by our group. The present study validates this TRAIL formulation not only as a potential tool for the treatment of haematological malignancies as previously reported, but also as a novel antitumor therapeutic strategy for colorectal cancer.

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Conflict of Interest

The authors have not conflict of interest. Alberto Anel and Luis Martinez-Lostao have filed a patent application (W02011020933) for the use of liposome-bound Apo2L/TRAIL.

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Figure 1.

(A) Analysis of cell viability of CRC cell lines after treatment with sTRAIL or LUV-TRAIL. Cells were treated with sTRAIL or LUV-TRAIL, and left overnight. The following day, cell growth was measured by the MTT assay method. Graphs show the mean ± SD of several independent experiments. The number of experiments is indicated in every case. **(B) Effect of sTRAIL and LUV-TRAIL on clonogenic survival of CRC cell lines.** Cells were treated with the indicated concentrations of sTRAIL or LUV-TRAIL for 24 hours. The following day, medium was carefully replaced, and cells were left to grow for 10 more days. Finally, cells were stained with crystal-violet. Quantification was performed by dissolving the crystal violet with pure DMSO, and measuring the absorbance at 600 nm. Effect of sTRAIL and LUV-TRAIL on the cells is presented as percentage of colony formation respecting to control. Bar-plots represent the mean ± SD of at least three independent experiments. $^{**}p<0.005$, $^{***}p<0.001$).

Figure 2.

(A) Nuclear staining of HCT-116. Cells were treated with sTRAIL or LUV-TRAIL at indicated doses (100 and 1000 ng/ml) alone or after pre-incubation with the TRAIL-blocking antibody RIK for 1 hour, and then subjected to nuclear staining by using Hoechst 33342. Yellow arrows indicate apoptotic cells. Cell death was measured in parallel by flow cytometry after PI staining. **(B) Apoptosis induction of sTRAIL and LUV-TRAIL on CRC cell lines.** Cells were treated or with sTRAIL or LUV-TRAIL at indicated concentrations, and left overnight. The following day, apoptosis induction was measured by annexin-V staining. In all CRC cells, pre-incubation with the TRAIL-blocking antibody RIK for 1 hour, was carried out. Graphs show the mean ± SD of at least three independent experiments. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$.

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Figure 3.

Analysis of time-course caspase activation in HCT-116 and HT29 cells. Cells were treated with sTRAIL or LUV-TRAIL (1000 ng/ml) at the indicated times. Finally, cells were lysed, and lysates were subjected to SDS-PAGE and to Western blot analysis (upper panels). Aliquots from every time point were collected, and apoptosis levels were measured by annexin-V staining (lower panels).

Figure 4.

(A) Oligomerization status of TRAIL molecules in sTRAIL and LUV-TRAIL. Same amount of BS3 cross-linked sTRAIL and LUV-TRAIL was loaded in an acrylamide gel. Next, samples were reduced and separated by SDS-PAGE. After transferring to a PVDF membrane, different TRAIL populations were assessed by Western blot. **(B and C) Size-exclusion chromatography of sTRAIL and LUV-TRAIL oligomer populations.** Samples of sTRAIL and LUV-TRAIL cross-linked with 1 mM BS3 were loaded in a HPLC column, and molecular masses were determined. Separation was performed using a Superdex 200 10/300 GL column. Oligomer populations identified by HPLC in sTRAIL and LUV-TRAIL samples were indicated below each size-exclusion chromatography plot. **(D) Clustering of DR4 and DR5 upon activation with sTRAIL or LUV-TRAIL.** HCT-116 cells (2-3 x 10^7 cells for each condition) were treated with 1000 ng/ml of either soluble TRAIL (sTRAIL) or LUV-TRAIL at indicated times (0, 10, 30, and 60 min) and then performing both reducing and non-reducing SDS-PAGE followed by immunoblotting. Western blot analysis of the DISC components (DR4 and DR5) using specific antibodies was performed.

Figure 5.

(A) DR expression of HCT-116 clones stably down-regulating DRs. After obtaining two different HCT-116 clones stably down-regulating expression of DR4 or DR5 were generated by shRNA technology DR4 and DR5 surface expression were analysed by flow cytometry. (**B) Cytotoxic assays of sTRAIL and LUV-TRAIL on HCT-116 clones stably down-regulating DRs.** HCT-116 clones stably down-regulating expression of DR4 or DR5 were treated with increasing concentrations of sTRAIL or LUV-TRAIL, and cell growth was measured by MTT. In every graph, results from HCT-116 wild-type cells are depicted in light grey to easily compare the results. Graphs show the mean ± SD of at least three independent experiments. $^{*}p<0.05$, $^{***}p<0.001$.

Figure 6.

(A) Polystyrene-based microdevices used in 3-D cytotoxic assays. For cytotoxic assay in 3D cell cultures, Polystyrene-based microdevices were used. For 3-D cell culture, $4x10^6$ HCT-116 cells/ml were incubated in a collagen hydrogel within the central microchamber. After 24 hours, treatment with TRAIL (sTRAIL and LUV-TRAIL) was perfused through the lateral microchannels for 24 h. Cells were stained using a solution containing CA (green, alive cells) and PI (red, dead cells) and photographed along all the microdevice height (300 μm) using a Nikon Eclipse Ti microscope. Confocal images of one of three independent experiments are shown. Original magnification 20x. Scale bar is 400 μm. **(B)** Cell viability was quantified by manual counting of live (green)/dead (red) cells. Graphics show the average of the cell viability of treated cells expressed as percentage. The results are expressed as the mean ± SD of at least three experiments. **(C) *In vivo* antitumor activity of sTRAIL and**

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LUV-TRAIL. Nude mice bearing HCT-116 tumours received five consecutive intra-peritoneal injections of LUVs-alone, sTRAIL (36 μg/injection) or LUV-TRAIL (36 μg/injection) on the arrow-pointed days. Relative tumour growth is depicted, calculated as the percentage of increase in tumour volume relative to the volume at day 1 (n=4 tumours/group; mean ± SD). **(D).** Tumour volumes on the different experimental groups at day 22 expressed as the mean ± SD of animals of each group. *$p<0.05$, ** $p<0.01$; *** $p<0.001$.

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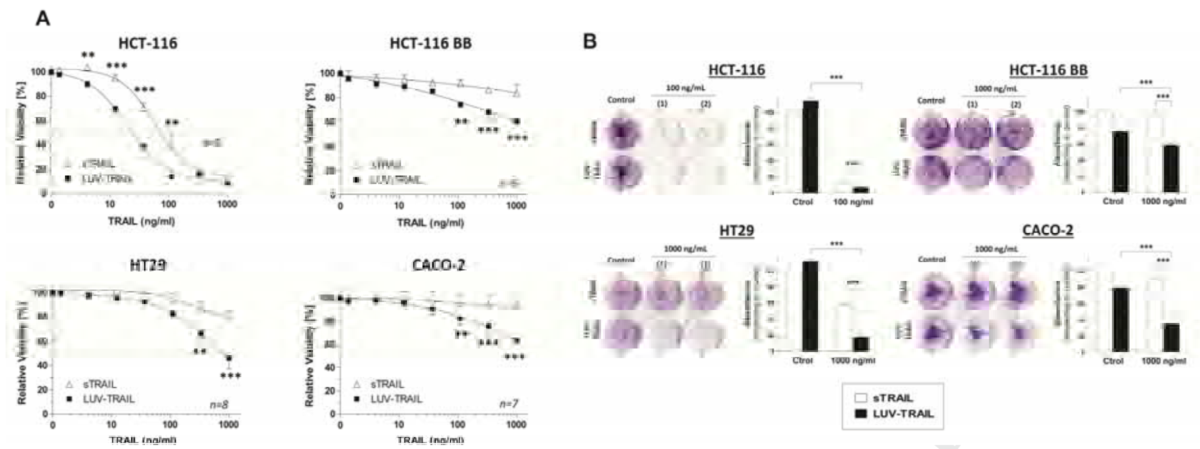
Figure 1



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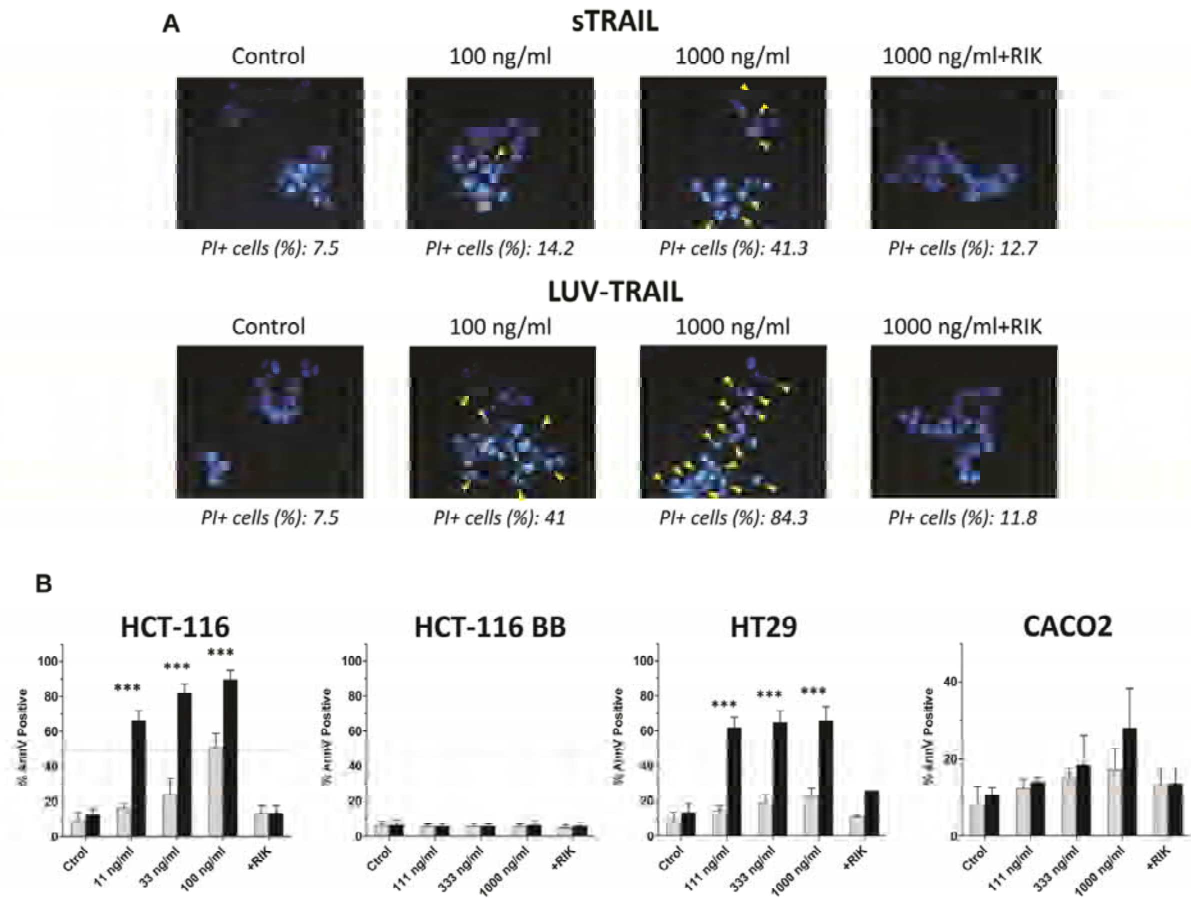

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Figure 3

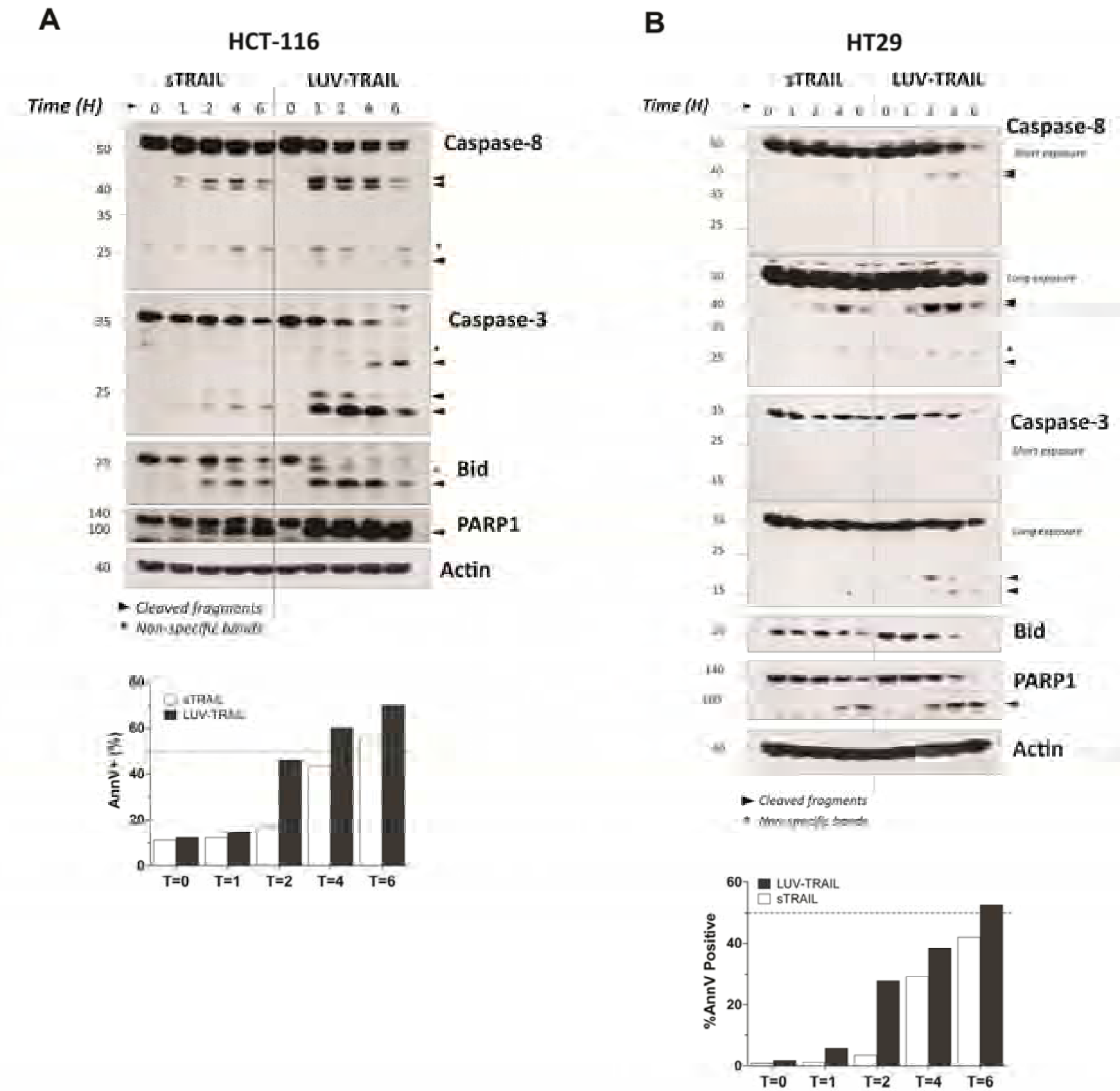

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Figure 4

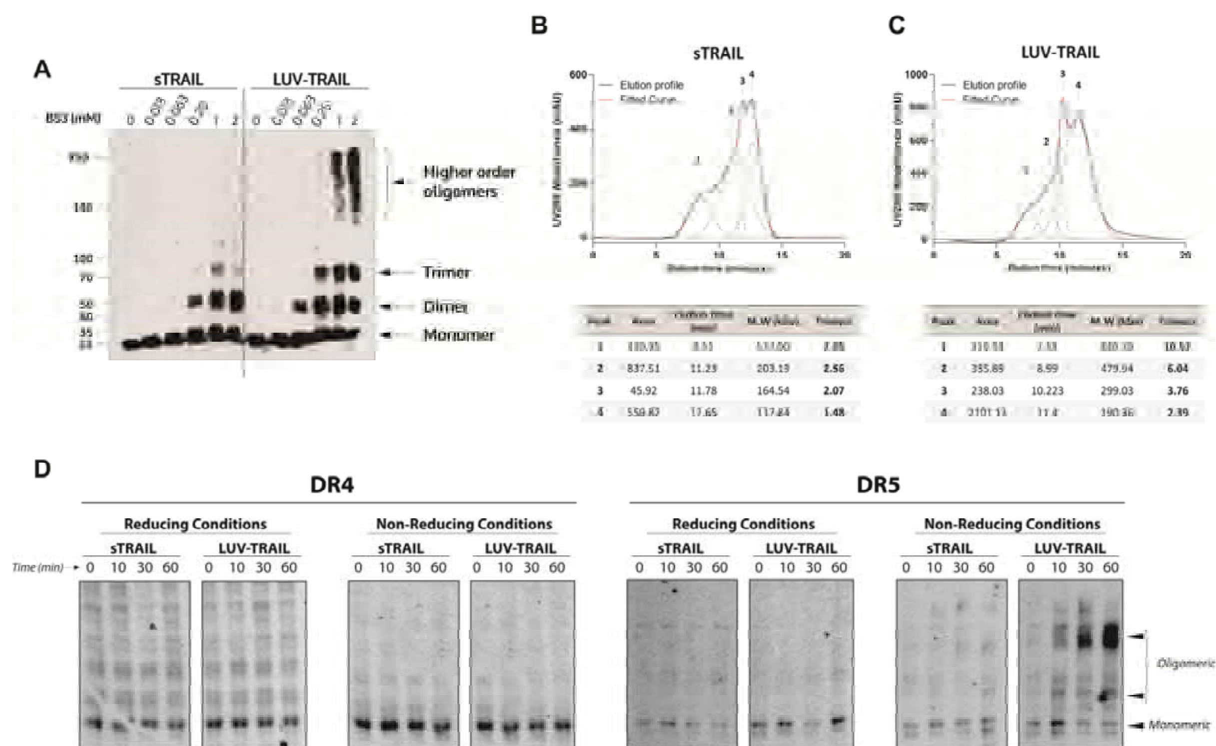

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Figure 5

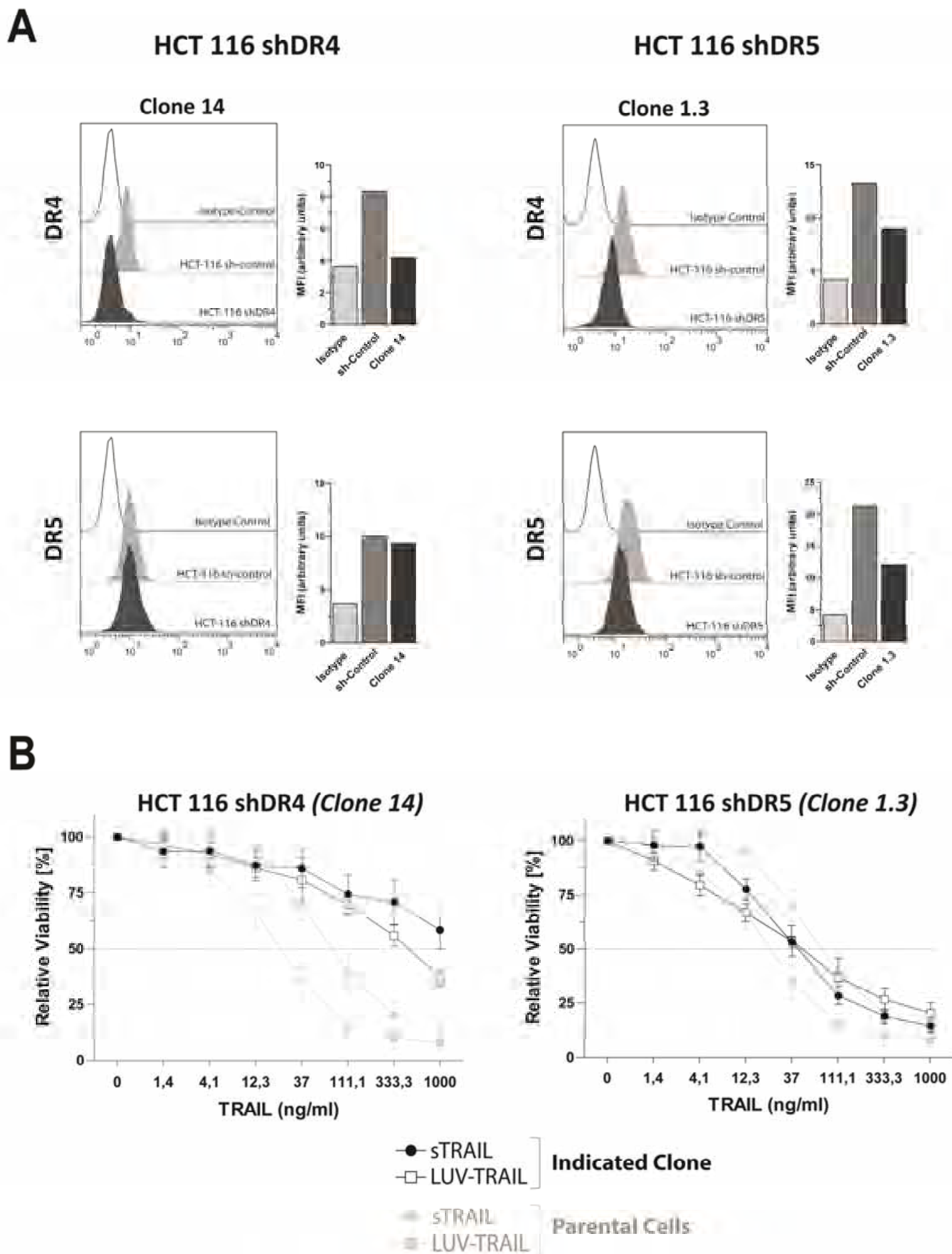
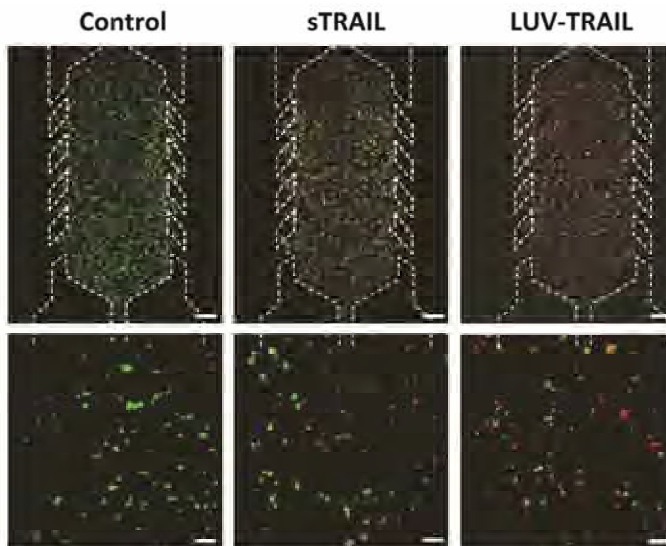
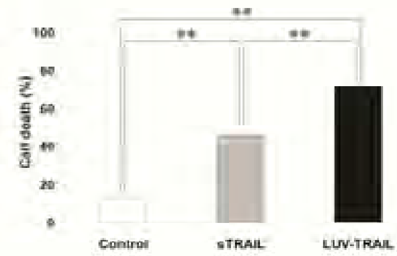
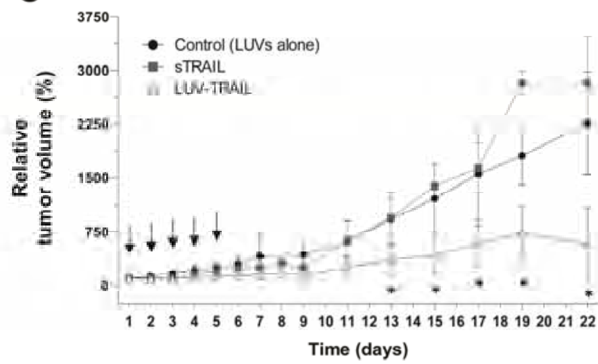
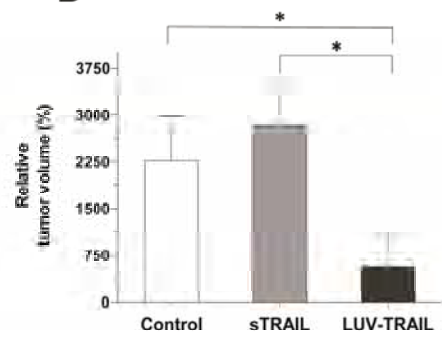


Figure 6









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HIGHLIGHTS

High-order TRAIL oligomer formation in TRAIL-coated lipid nanoparticles enhances DR5 cross-linking and increases anti-tumor effect against colon cancer

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Highlights:

1. Liposomes with TRAIL show greater cytotoxicity than soluble TRAIL in colon cancer cells.

2. Liposomes with TRAIL overcome resistance to soluble TRAIL in colon cancer cells.

3. TRAIL anchored to liposome surface form high-order oligomers which in turn, induce a more potent clusterization of DR5.

4. Liposomes with TRAIL show greater in vivo anti-tumor activity than soluble TRAIL.

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