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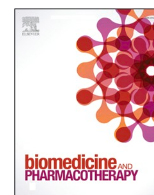

Lymphocyte infiltration and antitumoral effect promoted by cytotoxic inflammatory proteins formulated as self-assembling, protein-only nanoparticles

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ABSTRACT

Two human proteins involved in the inflammatory cell death, namely Gasdermin D (GSDMD) and the Mixed Lineage Kinase Domain-Like (MLKL) protein have been engineered to accommodate an efficient ligand of the tumoral cell marker CXCR4, and a set of additional peptide agents that allow their spontaneous self-assembling. Upon production in bacterial cells and further purification, both proteins organized as stable nanoparticles of 46 and 54 nm respectively, that show, in this form, a moderate but dose-dependent cytotoxicity in cell culture. *In vivo*, and when administered in mouse models of colorectal cancer through repeated doses, the nanoscale forms of tumor-targeted GSDMD and, at a lesser extent, of MLKL promoted $CD8^+$ and $CD20^+$ lymphocyte infiltration in the tumor and an important reduction of tumor size, in absence of systemic toxicity. The potential of these novel pharmacological agents as anticancer drugs is discussed in the context of synergistic approaches to more effective cancer treatments.

1. Introduction

Despite many innovative therapeutic initiatives, cancer therapies are still largely supported by low molecular weight cytotoxic agents [1–3] that, upon primary tumor resection, are administered systemically to prevent or delay tumor relapse and metastasis. Being untargeted, the systemic toxicity and severe side effects linked to chemotherapies [3] push to develop new drug delivery approaches, that being highly selective for target cells, could enhance the local cytotoxic effect and reduce undesired off-target toxicities. Selectivity in drug delivery for cancer was initially based on plain nanoscale carriers [4–6], designed to exploit the enhanced retention and permeability (EPR) effect through the so-called passive targeting [7–10]. In a step further, several categories of materials have been engineered in form of nanoparticles to be loaded with a payload drug, and functionalized with tumor-homing peptides for active and very precise receptor-mediated targeting through the interaction with cell-surface tumor markers [11–13]. Such strategies, even though being conceptually promising, have not been generically successful [14], as only around 1% of the systemically administered drug dose reaches the tumor [15]. In part, such failure is attributed to the protein corona, which dramatically reduces the interactivity of the targeting ligands displayed on the surface of carrier nanoparticles [16–18]. On the other hand, immunotoxins and antibody-drug conjugates, even reaching the clinics, also fail to produce generically good therapeutic outcomes because of a low uptake in cancer tissues [19] and systemic toxicity due to high accumulation and

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metabolism in the liver. In this context, some of such approved drugs have been discontinued [20].

Among the materials tested for cancer nanomedicines, oligomeric protein complexes at the nanoscale result from either spontaneous or ion-assisted self-assembling [21–26]. These materials benefit from the robust, scalable and repetitive procedures for recombinant protein production that have been developed during the last 40 years and that sustain the protein drug industry [27,28]. The multiple and geometrically regular presentation of the homing peptides or other functional protein domains, in a virus-like fashion [29,30], favors cell receptor binding and internalization what in turn allows a high local concentration of the payload. Also, the interactivity of these protein-based particles with tumor cells is not shadowed by any protein corona. The formation of protein coronas around protein nanomaterials has not been observed [31], since protein-protein contacts are exquisitely specific [32,33]. Finally, proteins allow, by recruitment of multiple biologically active domains [34], the construction of nanoparticles that are intrinsically multi-functional (*e.g.* a tumor homing peptide, a cytotoxic domain and a self-assembling tag in the same polypeptide), thus achieving the rising nanomedical concept of self-assembled, self--delivered, cell-targeted nanoscale drugs [35] that do not require any driving, non-drug material as vehicle [36].

Under this concept, we have recently generated tumor-targeted nanoparticles based on plant [37] or microbial toxins [38], pro-apoptotic factors [39] or venoms [40]. Being intrinsically cytotoxic, these materials were aimed to tumor reduction and/or metastatic control. When administering tumor-targeted microbial toxins (namely a modified *Pseudomonas aeruginosa* exotoxin and the diphtheria toxin) to different animal models of human cancers, pyroptotic routes were unexpectedly involved [41,42], and an important and clinically promising tumor remission was observed [41,42]. Being both pyroptosis and necroptosis inflammatory forms of cell death [43], promoting those routes in cancer cells has been recently proposed as a novel and interesting therapeutic approach [44–46]. Then, proteins that are directly involved in inflammatory cell death cascades might be of great interest as anticancer drugs. In this context, and by exploiting the versatility in the design and engineering of multidomain proteins, we have designed, produced and functionally characterized two type of protein nanoparticles targeted to the tumoral marker CXCR4. They are based on Gasdermin D (GSDMD) and on the Mixed Lineage Kinase Domain-Like (MLKL) proteins, which are essential agents in the pyroptotic and necroptotic cascades, respectively [47,48]. The results presented here demonstrate an antitumor effect and a clinical potential of these inflammatory proteins formulated as self-assembling, tumor-targeted multimeric nanoparticles.

2. Materials and methods

2.1. Protein design, production and purification

The synthetic genes encoding the modular proteins T22-GSDMDmut-S19-H6 and T22-MLKLmut-S19-H6 were designed in house and provided by GeneArt (Thermo Fisher), as subcloned in a pET22b plasmid (Novagen). T22-GSDMDmut-S19-H6 contains a mutation (L290D) in the GSDMD scaffold that prevents the C-terminal auto-inhibition of the N-terminal effector domain [49]. T22-MLKLmut-S19-H6 carries phosphomimetic mutations (T357E and S358D) in the MLKL scaffold to bypass RIPK3 activation [50]. Both constructions also contain the S19 peptide, which stimulates the intracellular delivery [51]. The N-terminal peptide T22, is a polyphemusin II derivative [52] initially adapted as a blocker of the human immunodeficiency virus (HIV) infection [53], as it specifically binds the cytokine receptor CXCR4 [54]. Incorporated to protein nanoparticles and imaging agents, T22 promotes their highly selective binding and penetration *in vitro* into $CXCR4^+$ cultured cells [55] and *in vivo*, in $CXCR4^+$ tumoral cells, with a highly precise tumor biodistribution [56–59].

The production of both constructs was preliminary assayed at 16 ºC (overnight), 20 ºC (overnight) and 37ºC (3 h). As in all the tested conditions the only detected product occurred in the insoluble cell fraction, the proteins were finally produced at 37ºC for three hours to maximize protein yield and recovery from aggregates. For harvesting, bacterial cultures were centrifuged at 5000 g for 15 min and the pellets washed with PBS and stored at − 80ºC until use. Cell disruption was performed by sonication (40% amplitude, 8 min, 1 s ON, 4 s OFF) in a 20% sucrose, 20 mM Tris-HCl buffer. After the centrifugation (15,000 g, 45 min), the supernatants were discarded and pellets washed with PBS (15,000 g, 15 min). Then, the pellets were weighted and 40 mM Tris-HCl, 0.2% N-Lauroyl Sarcosine were added to 50 mL/g. These mixtures were left overnight in agitation. The next day, samples were centrifuged (15,000 g, 45 min), pellets discarded and supernatants kept for further use.

Protein purification was done by immobilized metal affinity chromatography (IMAC). Before such step, the solubilized product was dialyzed against 20 mM Tris-HCl, 500 mM NaCl, pH 8.0, in two rounds of two hours and one additional overnight round. The soluble protein was recovered and charged in a HisTrap HP 5 mL column (GE Healthcare). Elution from the columns was achieved by a linear gradient of elution buffer (20 mM Tris, 500 mM NaCl, 500 mM imidazole) and the fractions that contained the target proteins were dialyzed against 100 mM NaCl, 20 mM Tris-HCl, pH 8. The engineered proteins were detected by Western Blot using an anti-His monoclonal antibody (GenScript) and their concentration was determined by Bradford (BioRad).

2.2. Dynamic light scattering

The size distribution of T22-GSDMDmutS19-H6 and T22-MLKLmutS19-H6 particles was determined by dynamic light scattering (DLS) at 633 nm (Zetasizer Pro, Malvern Instruments). 50 μL of each protein (ranging from 1 mg/mL to 2 mg/mL) were measured in triplicate at 25ºC. Size data were later processed and analyzed in the ZS Explorer software (Malvern Instruments).

2.3. Protein stability

T22-GSDMDmutS19-H6 and T22-MLKLmutS19-H6 were incubated at a concentration of 1 mg/mL in human serum (Sigma) for 24 h at 37ºC without agitation. Samples were extracted at 0, 1, 5 and 24 h. The proteolytic stability was determined by Western Blot using a monoclonal antiHis antibody (GenScript). Protein quantification was assessed with the ImageLab software (Bio-Rad) comparing the proteins at different times with the samples at time 0 (100%).

2.4. Cell culture and in vitro cell viability

HeLa cells (ATCC, CCL-2) were maintained in MEM alpha medium (Gibco) with 10% Fetal Bovine Serum (Gibco) in a humidified atmosphere at 37ºC and 5% CO_2. To assess cell viability, HeLa cells were cultured on 96-well plates (3500 cells/well) for 24 h. The next day, the proteins to be tested were diluted with MEM alpha medium until the desired final concentration. Then, 10 μL of the protein dilution were added to the correspondent well. The toxicity of the protein buffer was also assessed as a control. Protein incubations were performed in triplicate. Cell viability was determined using CellTiter-Glo Luminiscent Cell Viability Assay (Promega), in the presence of 1 μg/mL LPS as described [60]. Plates were read in a Victor 3 luminescent plate reader (Perkin Elmer) at 48 and 72 h. The final data were expressed as the mean of the viability percentage (± standard deviation) referred to the control wells in which only MEM alpha was added.

2.5. Animal procedures and histology

Seven-week-old BALB/cByJ females were purchased from Charles River (France). Upon arrival, mice were kept in quarantine for 1 week

and maintained in specific pathogen-free conditions with sterile food and water *ad libitum*. All animal procedures were approved by the Hospital de la Santa Creu i Sant Pau Animal Ethics Committee and Catalonia authorities (Authorization number 9721). CT26 cells (ATCC, CRL-2638), which are colon carcinoma mouse cells, were transduced with mouse CXCR4 receptor (pLV-EF1A-Puro-mCXCR4, VectorBuilder). Mice were subcutaneously injected with 2.5 × 105 CT26-CXCR4 cells into the right flank in 200 μL RPMI 1640 medium (Gibco, Life technologies). On day 9 post cell inoculation, those animals with palpable tumors were randomized into 3 experimental groups (n = 5–6 per group) and the treatment started. Such mice were treated intravenously three times per week either with 8 g/Kg of T22-GSDMD-H6 or T22-MLKL-H6 or the equivalent volume of Tris-HCl for control animals. Mice's well-being, tumor volume (V= length x width^2 x 0.5) and mouse body weight were recorded through the experiment. Twenty-four hours after the fifth dose, animals were euthanized and tumors were collected, fixed in 4% formaldehyde and embedded in paraffin. For histological analysis, 4 µm sections were stained with hematoxylin and eosin and the necrotic area and tumor-infiltrated lymphocytes (TILs) were quantified by a pathologist. Representative images were acquired using an Olympus DP73 digital camera. For immunostaining, 4 µm paraffin embedded sections were dewaxing and submitted to an antigen retrieval procedure using a high pH buffer (PTLink, Agilent Technologies). CD3, CD8, CD20 (ready to use antibodies, Agilent Technologies) and CXCR4 (1:200, Abcam) staining were performed in a DAKO Autostainer Link48 (Agilent Technologies) following manufacturer's directions. Stained slides were scanned and quantified using Panoramic Scan II and DensitoQuant image analysis center from 3DHISTECH Ltd. Representative images were acquired using Slide Viewer software.

2.6. Cell cytometry

CXCR4 membrane expression in CT26-CXCR4 was confirmed by MACSQuant® Analyzer 10 flow cytometer (Miltenyi Biotech) using PE rat anti-mouse CXCR4 antibody. The isotype PE rat IgG2b antibody was used as a negative control (Biolegend).

3. Results

Modified versions of human GSDMD and MLKL have been engineered as multidomain proteins (Fig. 1), intended as building blocks for self-assembling as nanoscale oligomers. The occurrence of C-terminal polyhistidine tails (here an hexahistidine, H6) [21,61] combined with the cationic peptide T22 (a specific ligand of CXCR4 [62] and an efficient tumor homing peptide [58]), are expected to promote the formation of regular oligomers assisted by cations from the media [63–65]. This category of H6-driven nanoparticles is structurally robust and stable during circulation in blood [31], what make them excellent drugs or drug vehicles for nanomedical purposes [57,59]. Both modular proteins were well produced in recombinant *Escherichia coli* and recovered upon purification as discrete bands with the predicted migration and molecular mass (Fig. 1). Also, both protein constructs organized, as expected, as monodisperse nanoparticle populations, sizing around 46 nm (T22-GSDMDmut-S19-H6) and 54 nm (T22-MLKLmut-S19-H6) respectively (Fig. 1). When exposing these oligomeric materials to denaturing plus chelating conditions they disassembled into the forming monomers (Fig. 1).

The potential cytotoxicity of these nanoparticles was tested over cultured HeLa (CXCR4$^+$, [66]) cells. A moderate but significant reduction of the cell viability was observed at 72 h in both cases, ranging between 15% and 20% depending on the protein concentration (Fig. 2). Since these data were in the range of previous reports about the effect of plain unformulated pyroptotic proteins in cell culture [67], we thus confirmed the retention of the biological activity of both products formulated as nanoscale oligomeric forms. The observed deleterious effect over cultured cells was dose-dependent (Fig. 2), a fact especially apparent after 72 h of exposure when the cytotoxicity of the nanoparticles was more obvious.

The systemic context of the immune- and inflammatory-based mechanisms of pyroptosis or necroptosis prompted us to expect an *in vivo* magnified impact of these drugs. Such a biological effect should be more evident than the mild cell killing observed in cell culture, an event restricted to a mere local context. Before moving to *in vivo* experiments, the stability of T22-GSDMDmut-S19-H6 and T22-MLKLmut-S19-H6 was determined in human serum during incubation at 37 °C. As observed, a slight decrease in the full-length forms of the protein was observed throughout the incubation time (Fig. 3A), indicating a half-life of around 5 h (Fig. 3B). Although this value was not very high, most of the intravenously injected dose of the T22-driven nanoparticle GFP versions accumulate in tumor around 5 h [55]. A half-life around this value was then considered as promising and sufficient to observe a biological effect of the proteins.

To assess such expected effect of both nanoparticles *in vivo*, an immunocompetent mouse model of aggressive, CXCR4$^+$ colorectal cancer was developed, through the implantation of CXCR4$^+$ CD26 cells (Fig. 3C) in immunocompetent animals. The resulting tumors were, as expected, CXCR4$^+$ (Fig. 3D). The animals were treated with repeated intravenous doses of each protein once the tumors were palpable (9 days post cell implantation). The selected tentative dosage was 8 µg of each nanoparticle per g of animal weight, administered intravenously 3 times per week, with a total of 5 doses.

As observed (Fig. 4A), the weight of the treated animals remained unmodified in comparison with the buffer-treated group, indicative of absence of systemic toxicity associated to the administration of both protein nanoparticles. Notably, the treatment with T22-GSDMDmut-S19-H6 significantly reduced tumor growth since its implantation compared with the buffer-treated group (Fig. 4B, adjusted P value = 0.008, Tukey post-hoc test following an ANOVA test), while T22-MLKLmut-S19-H6 also showed a similar therapeutic trend (adjusted P value = 0.095). This antitumoral effect was evident along the experimental time and very pronounced at 13, 15 and 19 days post-implantation (Fig. 4B). Unfortunately, the scattering of data within the control group rendered a few comparative points in which the differences resulted in the border of significance, while the global healing effect was evident along the treatment time (Fig. 4B). In the last sampling analysis (20 days post implantation), the tumor size was similar in all the groups, suggesting a loss of antitumoral activity while the drug was still administered (Fig. 4B). Interestingly, the histological analysis of the tumors at day 20 (Fig. 5A, B) indicated infiltration of TILs in tumor tissue at a statistic significant level, for the animals treated with T22-GSDMDmut-S19-H6 nanoparticles and also for those treated with T22-MLKLmut-S19-H6 nanoparticles (Fig. 5C). Moreover, at day 20, there was also a trend towards an increase in necrotic tumor area in nanoparticle-treated mice, compared to control mice, again in the border of statistical significance (Fig. 5C).

To more precisely assess lymphocyte infiltration in tumor, CD3, CD8 and CD20 lymphocyte subpopulations, relevant to the emerging immunotherapies of cancer [68–70], were analyzed in tumors of treated animals, through immunohistochemistry. As observed (Fig. 6 A, B), tumors of animals treated with T22-GSDMDmut-S19-H6 or T22-MLKLmut-S19-H6, exhibit an increased number of infiltrated B-lymphocytes (CD20) and cytotoxic T-Lymphocyte (CD8) when compared with buffered treated mice. CD3, a pan T lymphocyte marker did not show differences, suggesting a specific anti-tumor immune cell recruitment.

4. Discussion

Pyroptosis and necroptosis are related types of programmed cell death [71] characterized by the activation of inflammatory caspases, including caspase-1, caspase-4, caspase-5, and caspase-11 [72], which lead to the release of pro-inflammatory cytokines and

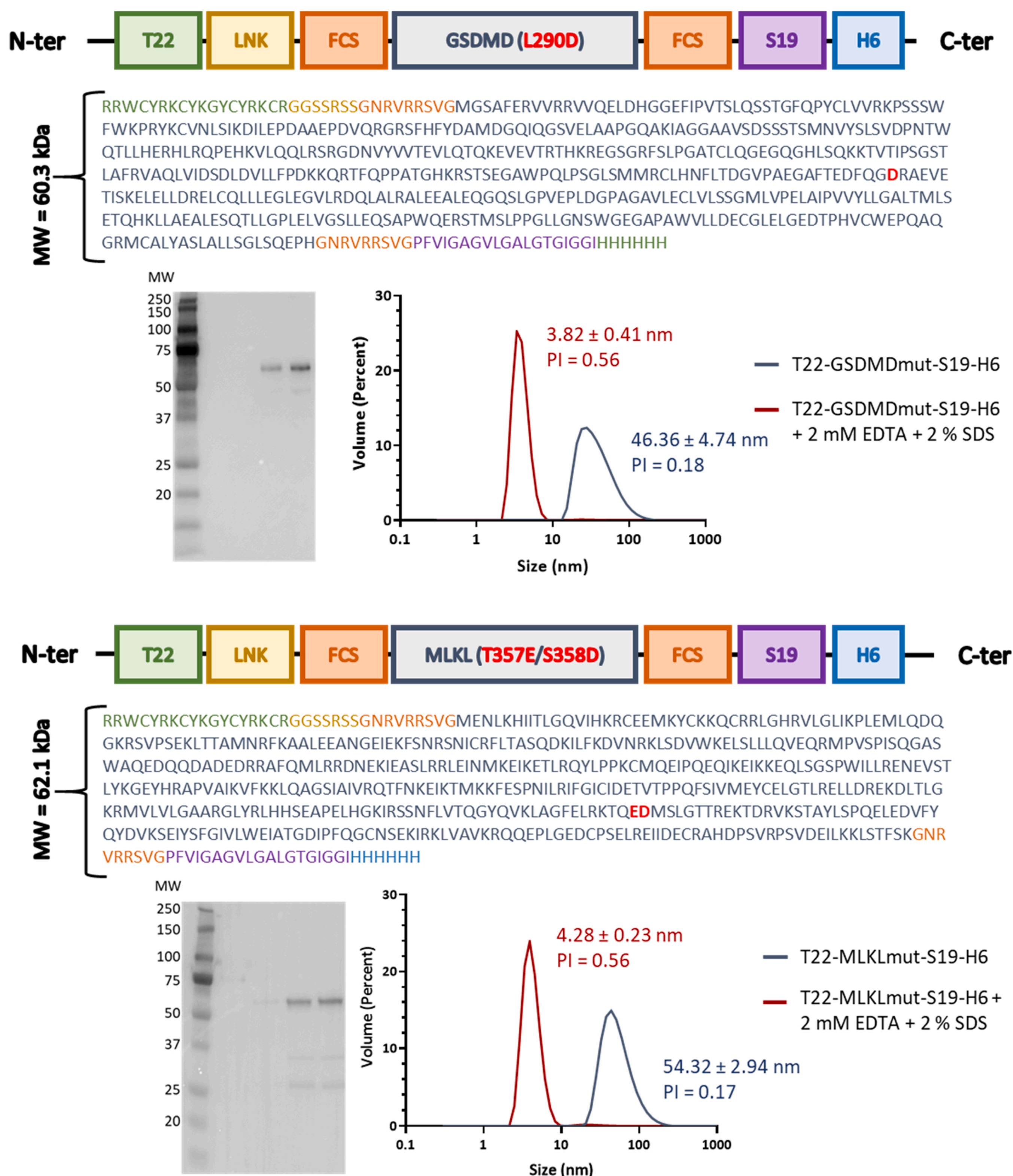

Fig. 1. Modular organization of building blocks and characterization of the resulting nanoparticles. Modular design and amino acid sequence of T22-GSDMDmut-S19-H6 (up) and T22-MLKLmut-S19-H6 (bottom). The CXCR4-ligand T22 (green), a peptide spacer (dark yellow) and two furin-cleavable sites (orange) are included in the construct, that also harbors the endosomal escape peptide S19 (purple) and the end terminal H6 tag (blue). In red, there are indicated the point mutations over the original sequences known to enhance the biological activity of either GSDMD [49] or MLKL [50]. Linked to each construction map, the western blot detection of purified protein is shown, in which protein bands were visualized using an anti-His antibody. The size of the soluble protein nanoparticles and their monomeric forms (upon disassembling with 2 mM EDTA + 2% SDS), were determined by DLS. PI stands for polydispersity index.

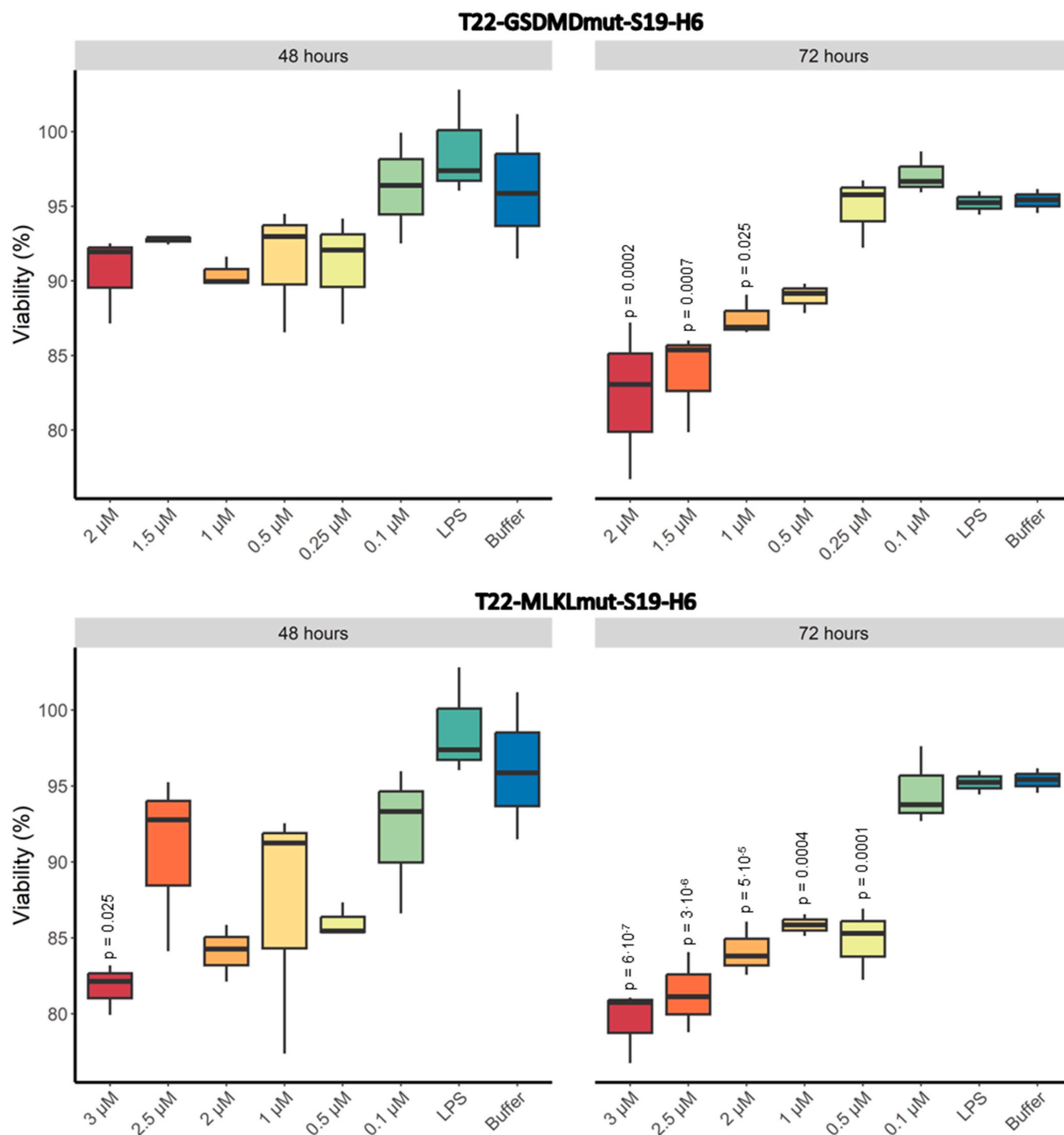

Fig. 2. Cytotoxicity of protein nanoparticles on cultured cells. HeLa cells were incubated with increasing concentrations of GSDMD- (up) or MLKL-based (down) nanoparticles together with 1 μg/mL LPS for 48 or 72 h. PBS and LPS 1 μg/mL were used as controls. Significance was assessed with an ANOVA test and the adjusted p-value (p in the plot) was calculated using a Tukey post-hoc test comparing the different protein concentrations with the PBS control.

damage-associated molecular patterns (DAMPs) [73,74]. They are key players in the regulation of both inflammation and immune responses, and pyroptotic and necrotic events have been associated with several pathological conditions, including cancer [73,75–77]. Both pyroptosis and necroptosis are lytic cell death types that upon induced in cancer cells release cytokines and DAMPs that trigger inflammation. These events attract circulating lymphocytes to cancer tissues. The resulting activation of the antitumoral immunogenic cell death is then a potential new approach for cancer treatment [78–81]. This is of special interest regarding 'cold tumors', which are highly relevant in human clinics due to their poor recognition by the immune system and their limited responsiveness to conventional treatments [82].

One of the most promising strategies for inducing inflammatory cell death in cancer would be the targeting to tumor cells of pore-forming inflammatory proteins, specifically gasdermin D (GSDMD) [83]. Gasdermins are a family of pore-forming proteins that are involved in various cellular processes, including cell death. In particular, GSDMD has been shown to be a key mediator of pyroptosis, as its activation leads to the formation of membrane pores and subsequent release of inflammatory signals [84]. On the other hand, MLKL induces necroptosis in

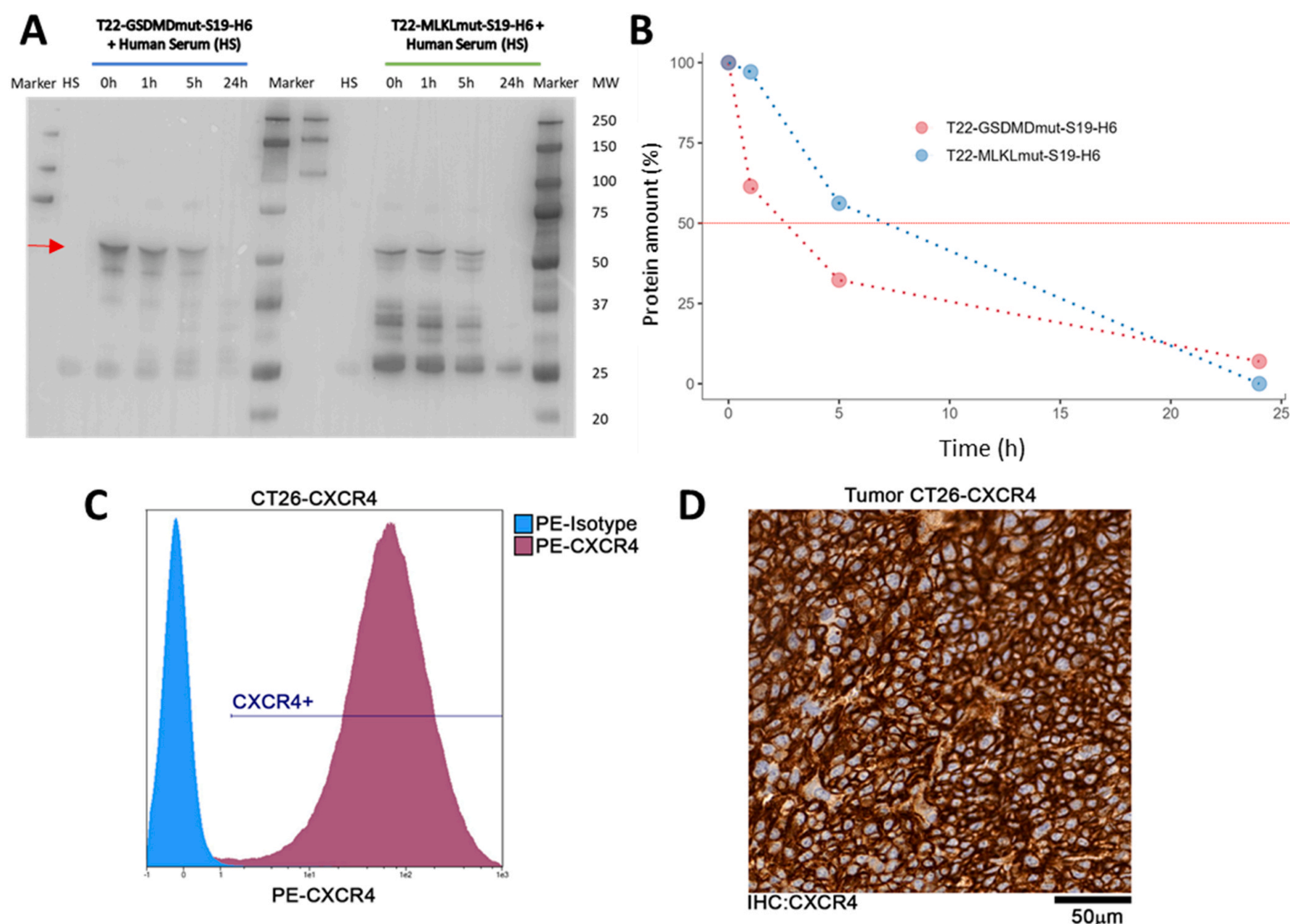

Fig. 3. Stability of GSDMDmutS19-H6 and T22-MLKLmutS19-H6 in human serum. A. Western blot analysis of both proteins incubated in human serum at 37ºC. The full length forms (red arrow) were quantified for further analysis. **B**. Declining of full length protein forms throughout incubation time. The horizontal line indicates 50% of the initial protein amount. C. Histogram of CXCR4-membrane expression detected by flow cytometry in transduced CT26 cells. Cells incubated with the PE-isotype antibody were considered as negative. D. CXCR4 expression in subcutaneous CT26-CXCR4 tumor grown in BALB/c mice. Scale bar: 50 µm.

several cancer cell lines, and its expression has been found to be downregulated in various types of cancer, suggesting that it may function as a tumor suppressor [85]. MLKL expression and cancer progression have been shown as inversely correlated [86–88]. This whole set of observations places both GSDMD and MLKL as potential protein-based anticancer drugs candidates.

In previous studies, we were successful in promoting the self-assembling of cytotoxic proteins (namely venoms, toxins or pro-apoptotic factors) as both scaffolding and functional building blocks of tumor-targeted protein-only nanoparticles [89]. This fact prompted us to engineer GSDMD and MLKL (Fig. 1) for this purpose through minor amino acid sequence modifications. The assembling of the materials as nanoparticles was successful (Fig. 1) and the cytotoxicity *in vitro* was observed at the expected levels (Fig. 2). The particles proved to be sufficiently stable in human serum (Fig. 3) to be moved towards *in vivo* testing for systemic administration in repeated doses. MLKL showed a mild tendency towards an antitumoral effect once administered in an animal model of colorectal cancer (Fig. 4B), while GSDMD-based nanoparticles promoted a clear reduction of tumor growth several days upon starting the administration regimen (Fig. 4B). The fact that systemic toxicities were not observed (Fig. 4 A) demonstrated the precise T22-mediated selective delivery of the nanoparticles into CXCR4^{+} tumor cells, as previously seen in related T22-empowered oligomeric vehicles used for the delivery of highly cytotoxic small molecular weight drugs [90–92]. On the other hand, both proteins induced a potent lymphocyte infiltration in tumors (Fig. 5), what can be seen as turning 'cold' tumors into 'hot' tumors [82,93,94]. As far as we know, this is the first report in which such activity is described for a pore-forming inflammatory protein. In agreement with this concept, tumors from treated mice exhibited an increased number of infiltrated B-lymphocytes (CD20) and also of cytotoxic T-lymphocytes (CD8). Interestingly, a general T-lymphocyte increase was not detected (no changes in of CD3^{+} cell infiltration), suggesting an antitumoral specific recruitment within the tumor when compared with control mice (Fig. 6 A,B). Cytotoxic lymphocytes are observed as antitumoral lymphocytes, as they participate in antigen recognition and correlate with better prognosis in several cancers, including colorectal cancer [95,96]. In this tumor type, CD20 infiltration has been particularly associated with improved survival. The proteins tested here efficiently induce the activation of the antitumor adaptive immune system mediated by B and T lymphocytes.

Regarding the antitumoral effect, that was marked the first two weeks upon stating the treatment, the functional nanoparticle T22-GSDMDmut-S19-H6 promoted an important reduction of tumor growth followed by a relapse (Fig. 4B). This particular outcome would need further analyses at the cell and tissue level to discriminate between appearing of resistance or other issues. However, even if transient, the cancer tissue destruction and the consequent lymphocyte recruitment (Figs. 5 and 6) initiated by the pyroptotic window might be of great clinical value when used in combination with synergistic treatments. In the clinics, it has been generically observed a correlation between the

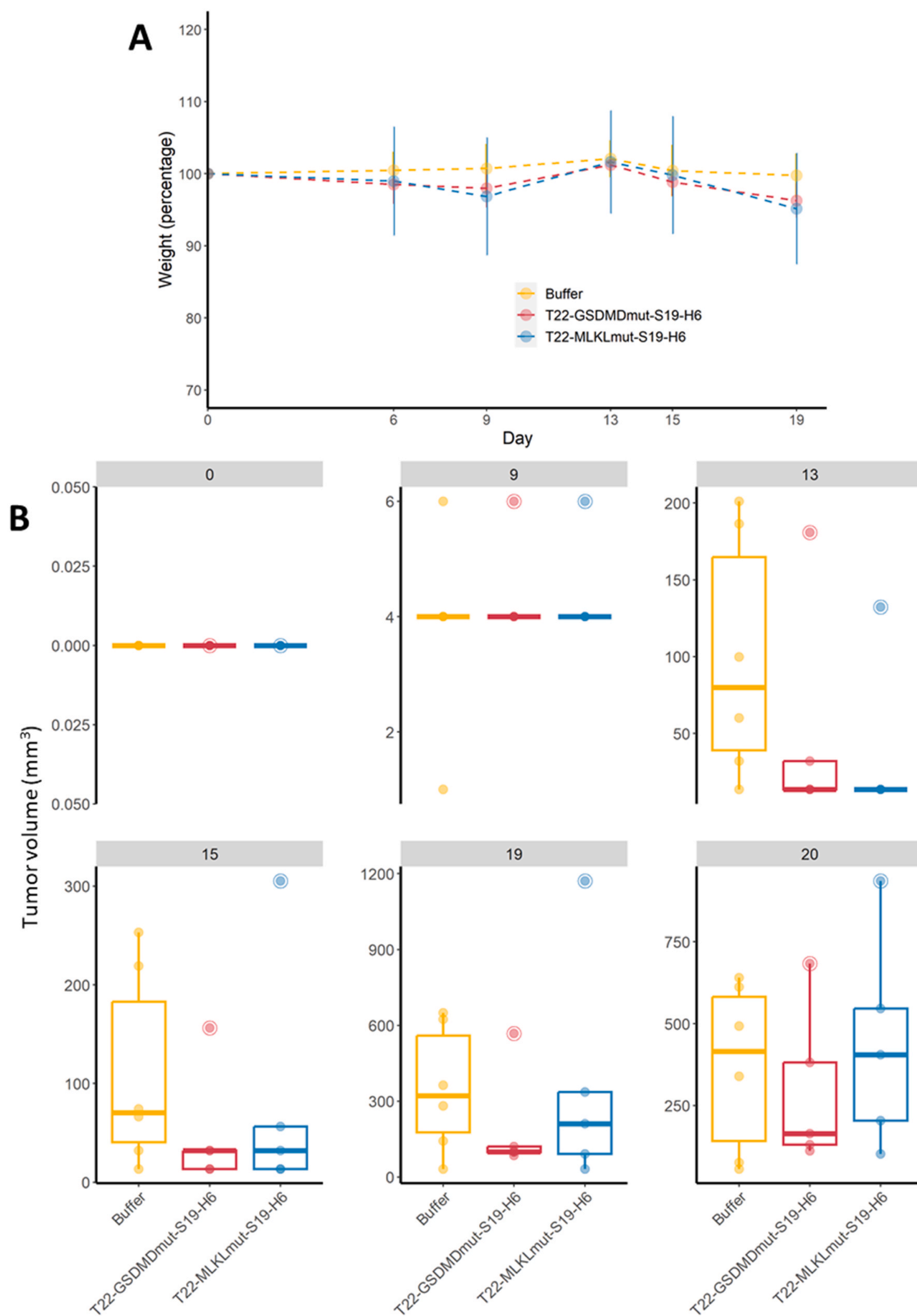

Fig. 4. Impact of protein nanoparticles on tumor size in a colorectal cancer mouse model. A murine colorectal carcinoma cell line was implanted in several mice (n = 16); the animals were divided into three groups depending on the treatment: a saline buffer (n = 6), T22-GSDMDmutS19-H6 (n = 5) and T22-MLKLmutS19-H6 (n = 5). A. Mouse body eight evolution for every group. Data are the mean for each time point and the bars represent the standard deviation. B. Tumor growth was analyzed at day 9, 13, 15, 19 and 20 post-implantation. Two mice (one for each nanoparticle type) had a disproportionately higher tumor size at each measure and were not considered for statistical analysis. These mice are highlighted in the figure with a double circle. Statistical significance was assessed with an ANOVA test and a Tukey post-hoc test.

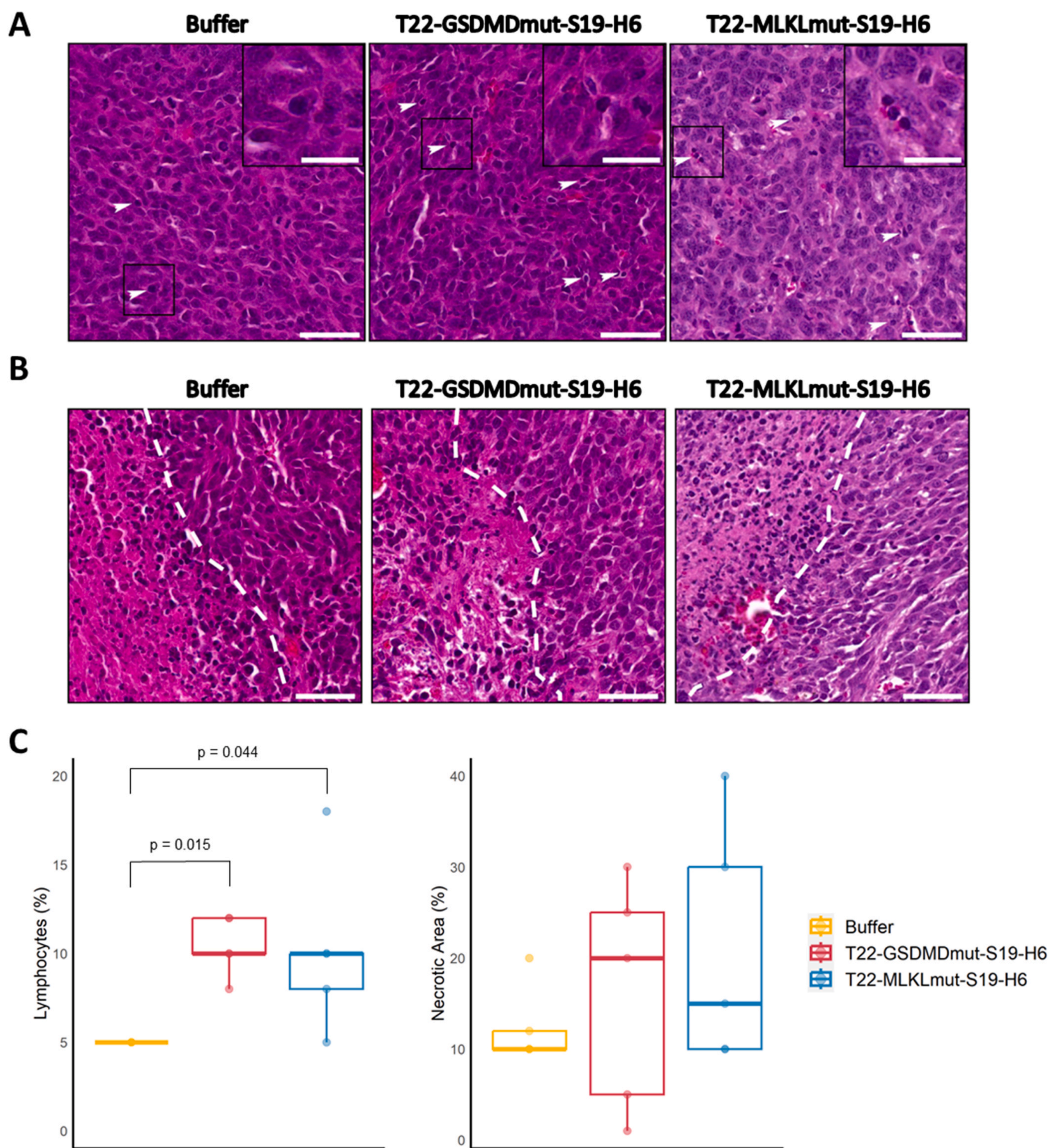

Fig. 5. Lymphocyte infiltration and necrotic tumor area in mice treated with protein nanoparticles. A. Tumor-infiltrating Lymphocytes (TIL) in hematoxylin and eosin (H&E) stained sections. Arrows point to TILs. Scale bar represents 50 µm in the general view and 20 µm in the zoomed view. **B.** Necrotic areas in H&E-stained tumor sections. The necrotic tissue is at the left and the viable tumor is at the right. Scale bar represents 50 µm. **C.** Percentage of TIL (left) and necrotic area (right) in each condition. Statistical significance was assessed with a Kruskal-Wallis test and a Dunn post-hoc test (the p value was adjusted using the Benjamini-Hochberg procedure).

intensity of TILs infiltration and a low tumor volume, limited metastatic progression and good prognosis [97,98]. However, some authors have reported, such as in the case of meningioma, that the infiltration of TILs is linked with a tumor size reduction for large tumors but in contrast, it is associated to a slight increase in the tumor size in small tumors [99]. Also, in Non-Small-Cell Lung cancer, an inverse correlation between TILs infiltration and tumor recurrence was only observed in large (>5 cm in diameter) tumors but not in smaller ones [100]. In fact, a diversity of controversial outcomes from the crosstalk between inflammatory processes and tumor development have been also repeatedly stressed [101,102], also depending on the sub-populations of involved lymphocytes [103]. Among these observations, tumors might develop

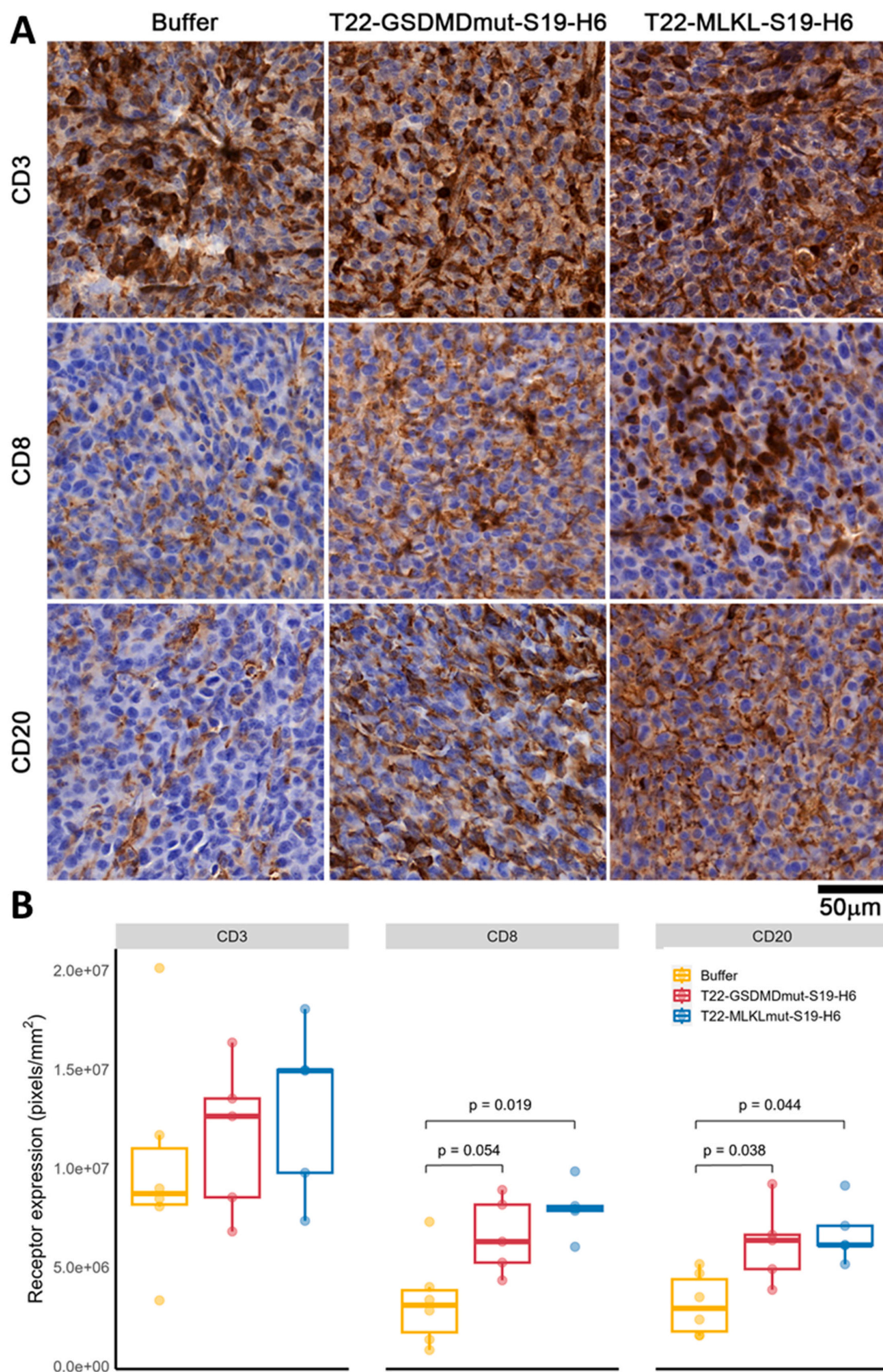

Fig. 6. Characterization of tumor infiltrating lymphocytes. A. Representative images of immunohistochemical staining against CD3, CD8 and CD20 proteins in tumor sections. Scale bar: 50 µm. B. Quantification of positive staining per mm^2 of alive tumor tissue. Data are shown as mean±SEM. Statistical significance was assessed with a Kruskal-Wallis test and a Dunn post-hoc test (P value was adjusted using the Benjamini-Hochberg Procedure).

tolerance to the immune response through upregulation of PD-L1, an immune checkpoint [104]. In the trends of using combined drug formulations in immunotherapy [105], inhibitors of PD-L1 have been developed for such type of synergistic approaches [106].

The presented data demonstrate that the pyroptotic inflammatory cell death induced by T22-GSDMDmut-S19-H6 nanoparticles (Figs. 2, 4) was able to release DAMPs and cytokines capable of recruiting TILs (Fig. 5 C, 6) which, according to the expected outcome [67,107], had an impact on the tumor volume size (Fig. 5). Importantly, the cellular events triggered by GSDMD and more moderately by MLKL promote a lytic cell death that could engage the antitumor activity of TILs and, therefore, inducing immunogenic cell death. The observed cell death type is in stark contrast to the classical induction of apoptosis by chemotherapy, which is not immunogenic [108]. In fact, and because of such inflammatory character, even a low level of pyroptosis in tumors appears to be sufficient for recruiting the actors of the immune system relevant to potent anticancer activities [67]. Thus, this novel approach based on tumor-targeted pro-inflammatory proteins might be especially suited for the treatment of tumors resistant to chemotherapy by an artificially induced upregulation of anti-apoptotic proteins [109,110]. In the particular case of colorectal cancer, it is believed that immunotherapy resistance may be related to insufficient tumor antigen presentation, tumor antigen presentation damage, T cell exclusion and immunosuppressive signaling in the tumor microenvironment [111].

In the context of the multifaceted nature of human cancer and the increasingly recognized need of synergistic treatments to reach efficient therapeutic effects [112–115], the induction of a potent pyroptotic event by the treatment with functional protein-only drugs might create new opportunities for a potent multimodal approach to cancer treatment. In this context, the use of oligomeric, virus-like nanoparticles based on self-assembling, self-delivering concepts [116] might not only provide precision targeting but also a high concentration of the active drug at the local site of action.

5. Conclusions

The inflammatory proteins GSDMD and the MLKL have been genetically engineered for their assembly as regular and stable nanoparticles of around 50 nm that are targeted to the tumoral marker CXCR4. Both drug formulations induce a moderate target cell destruction *in vitro* but an important antitumoral effect, in absence of side toxicity, when intravenously administered in an animal model of colorectal cancer. The therapeutic impact involves arrest of tumor growth, necrosis and specific infiltration of TILs in tumor tissue at a statistically significant level. Importantly, both tested proteins induce the activation of the antitumor adaptive immune system mediated by B and T lymphocytes. The observed effects point out the exploration and further development of tumor-targeted pro-inflammatory proteins as suited novel drugs for the primary or synergistic treatment of cancer through the exploitation of immunogenic cell death routes.

CRediT authorship contribution statement

Carlos Martínez-Torró: Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Lorena Alba-Castellón:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. Luis Miguel Carrasco-Díaz: Methodology, Formal analysis, Investigation. **Naroa Serna:** Methodology, Formal analysis, Investigation. **Laura Imedio:** Methodology, Investigation. **Alberto Gallardo:** Methodology, Formal analysis, Investigation. **Isolda Casanova:** Methodology, Formal analysis, Investigation. **Ugutz Unzueta:** Conceptualization, Supervision, Resources, Writing – review & editing. **Esther Vázquez:** Conceptualization, Project administration, Supervision, Resources, Writing – review & editing. **Ramón Mangues:** Conceptualization, Project administration, Supervision, Resources, Writing – review & editing. **Antonio Villaverde:** Project administration, Supervision, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

In this study, we have developed a new prototype of nano-organized antitumoral drugs based on two different inflammatory proteins, namely Gasdermin D, involved in the pyroptotic cell death pathway and MLKL, involved in the necrotic cell death pathway. The effectivity of these new drugs has been demonstrated both *in vitro* and particularly *in vivo*, in an animal model of human colorectal cancer. In this model, by the administration of nanoparticles based on the above-mentioned proteins, we have observed lymphocyte infiltration in tumor, necrosis and tumor volume reduction upon systemic administration, in absence of detectable side effects. The novelty of the approach, the easy and scalable biofabrication of the drugs in cell factories and their potential application in nanomedicine makes the study of interest for innovative treatments for cancer.

Data Availability

Data is available on the following link: https://doi.org/10.34810/data735.

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