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In vivo potential of recombinant granulysin against human melanoma

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ABSTRACT

9-kDa granulysin is a protein expressed into the granules of human cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. It has been shown to exert cytolysis on microbes and tumors. We showed previously that 9-kDa granulysin exerted cell death by apoptosis *in vitro* on hematological tumor cell lines and also on cells from B-cell chronic lymphocytic leukemia (B-CLL) patients. In addition, we have shown the anti-tumor efficiency of granulysin as a single agent in two *in vivo* models of human tumor development in athymic mice, the MDA-MB-231 mammary adenocarcinoma and the NCI-H929 multiple myeloma, without signs of overt secondary effects by itself. In this work, we have tested recombinant 9-kDa granulysin in an *in vivo* and especially aggressive model of melanoma development, xenografted UACC62 cells in athymic mice. Recombinant granulysin was administered once UACC62-derived tumors were detectable and it substantially retarded the *in vivo* development of this aggressive tumor. We could also detect apoptosis induction and increased NK cell infiltration inside granulysin-treated tumor tissues. These observations are especially interesting given the possibility of treating melanoma by intra-tumor injection.

Introduction

Granulysin is a protein that exhibits two isoforms, of 9 and 15 kDa molecular weight [1]. The 9-kDa isoform is expressed inside the granules of activated human cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and shows cytolytic activity against microbes and tumors [1]. The 15-kDa isoform is not cytotoxic and rather induces monocyte differentiation to dendritic cells [2]. It has been described that each one of these isoforms localize to different types of granules, and that their requirements for degranulation are different [3]. The main physiological function of the 9-kDa cytotoxic isoform is the killing of intracellular bacteria such as *M. Tuberculosis* in concert with perforin [4]. Granulysin is able to kill other bacteria [5], fungi such as *Cryptococcus Neoformans* [6], viruses such as *Varicella Zoster* [7], acting also in the immune response against leprosy [8]. Recently, it has been suggested that its mechanism of action against bacterial and protozoan parasites is through the generation of pores on their surface, facilitating lysis by granzyme B [9,10].

Several groups have demonstrated that recombinant 9-kDa granulysin is able to induce cell death on tumor cells [1,11,12]. Granulysin induces cell death mainly by apoptosis, although in certain cell types, a necroptotic component can be also detected [11,13]. Recombinant granulysin-induced apoptosis is initiated by its interaction with plasma membrane, altering cellular [Ca^{2+}] homeostasis, and inducing in this way a net increase in mitochondrial reactive oxygen species (ROS). These biochemical events cause finally the loss of mitochondrial membrane potential and the concomitant release of cytochrome c and apoptosis-inducing factor (AIF) from the intermembrane space of mitochondria, initiating the mitochondral apoptotic pathway [11,12,14]. Remarkably, using cytotoxic cells from granulysin transgenic mice crossed with perforin or granzyme B-deficient mice, Saini et al. demonstrated that granulysin, acting in concert with perforin activated a different apoptotic pathway, dependent on endoplasmic reticulum stress and caspase-7 activation [15].

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In any case, recombinant granulysin could be a good candidate for tumor therapy. Granulysin was able to kill *in vitro* the acute T cell leukemia Jurkat, human multiple myeloma cell lines and also leukemic cells from B-cell chronic lymphocytic leukemia (B-CLL) patients, but it was not toxic against healthy peripheral blood mononuclear cells (PBMC) [11]. In addition, granulysin transgenic mice demonstrated higher resistance to syngeneic tunor development than wild-type mice [16]. More recently, we have demonstrated the efficiency of recombinant granulysin in xenotransplantation models of human tumors in athymic mice, the mammary adenocarcinoma MDA-MB231 and the multiple myeloma NCI-H929 [17]. We showed that granulysin anti-tumoral action in these models correlated with apoptosis induction in the tumor tissue and with a prominent NK cell infiltration, indicating that granulysin-induced tumor cell death *in vivo* could be immunogenic. In the same study, we showed the lack of secondary effects of granulysin alone. In that study, human tumors were injected subcutaneolsy in mice, and treatments were performed by intratumoral injection. This type of treatment could be difficult to perform in internal tumors, in which a systemic treatment would be better indicated.

Melanoma is the most aggressive form of skin cancer. Melanoma outcome is dependent on their detection at early stages, since surgical excision of the primary tumor has over a 95% success rate at stages I/II [18]. However, melanoma lesions can remain unnoticeable for long periods and being detected only at stage IV, characterized by dissemination (metastasis) to multiple organs. Recent advances in immunotherapy of melanoma, mainly the blocking of immune checkpoint molecules such as CTLA-4 or PD-1 has given new hope to patients [19]. Although melanoma outcomes have clearly improved, with the median overall survival of patients with advanced-stage melanoma from around 9 months before 2011 to 2 years in 2017 [20], not all patients respond in the same way and many of them experiment relapses. Hence, new treatments alone or in combination with already used chemo-, radio- or immunotherpay could still improve the situation of melanoma patients.

Melanoma is a type of tumor that could be suitable for treatment by intratumoral injection in the clinic and that has not been previoulsy tested as a target for granulysin therapy. In the present work, we have tested recombinant granulysin in a xenotransplant model of the aggressive human melanoma UACC62 in athymic mice, obtaining promising results.

Material and methods

Cell culture

Jurkat and NCI-H929 cells were cultured in RPMI 1640 medium supplemented with 5% FCS and penicillin/streptomycin (Pan Biotech, Aidenbach, Germany) and GlutaMAX (Invitrogen, Barcelona), at 37ºC and 5% CO_2. SKMEL103, UACC62 and A549 cell lines were cultured in DMEM medium (PAN Biotech GmbH) supplemented with 10% fetal bovine serum (FBS, Sigma). Cell lines were tested for mycoplasma contamination by PCR. SKMEL103 and UACC62 melanoma cell lines were kindly provided by Dr. María Soledad Soengas, CNIO, Madrid, Spain

Recombinant granulysin expression and purification

Recombinant granulysin was produced in *E. coli* and purified as described in [11,17], using an on-column renaturation approach following the protocol described in [21]. Protein purity was checked by SDS-PAGE in 12% polyacrylamide gels and Coomassie blue staining and also by immunoblot with specific rabbit polyclonal anti-9 kDa granulysin antibody, gift of Drs. Alan Krensky and Carol Clayberger, Northwestern University, Chicago, USA. The content of LPS was determined before and after removal of endotoxin using the ToxinEraser Endotoxin Removal Kit from Genscript, as indicated in [17]. In the stock solutions, the concentrations were 22 EU/ml before removal and 11 EU/ml after removal. The volume injected in mice was of 50 µl, so the total endotoxin injected was around 0,5 EUs.

In vitro granulysin cytotoxicity assays

For *in vitro* assays, cells were incubated with granulysin or with the same amount of PBS in control wells at 37ºC and then cell death was analyzed by PS exposure and loss of membrane integrity. These parameters were analyzed at the same time by flow cytometry using Annexin-V-FITC (BD Biosciences, Madrid) and 7AAD (Inmunostep, Salamanca, Spain), respectively, and results analyzed using a FACSCalibur (BD, Madrid).

Determination of calreticulin exposure after granulyin treatment

Cells in suspension were directly treated with a 30 µM dose of GRNLY for approximately 24 h, while adherent cells were treated approximately 12–24 h later to allow time for the cells to adhere to the surface of the well. Then a centrifugation was carried out to eliminate the supernatant and proceed to the labeling. The cells were labeled with an anti-calreticulin mAb (Invitrogen, Madrid) and with 7-AAD to be able to select and analyze only 7-AAD negative cells to ensure that only living cells that expose calreticulin on their surface are analyzed. An isotype control was also carried out. Specifically, 50 ng of 7-AAD per well and 5 µg per 10^6 cells of the anti-calreticulin or of the isotype control mAb were added in PBS buffer with 5% FBS and incubated for 30 min at 4°C in darkness before analyzing the results by flow cytometry. To perform the histogram, 10,000 cell events with negative 7-AAD labeling were analyzed.

In vivo experiments in athymic mice

In vivo experiments were performed using six-week-old athymic males of the Swiss nu/nu strain (Charles River, Barcelona). Animal experimentation was performed according to the European recommendations on animal ethics and the University of Zaragoza Animal Experimentation Ethical Commission previously approved the housing and experimental protocols, with permission number PI17/16.

1×10^6 or $2{,}5 \times 10^5$ UACC62 melanoma cells were injected s.c. in 10 nude mice in each experiment. When tumor volume had arrived to 0.1 cm^3 (15 days or less), mice were divided in two groups, the control group and the group treated with granulysin, being $n = 5$ for each experimental group. In the granulysin-treated group, 44 µg of granulysin in 50 µl of phosphate buffered saline (PBS) were injected every 2 days for 10 times in each mouse. In the control group, mice were injected in the same way with 50 µl of PBS alone. Tumor growth was analyzed every 2 days by measuring tumor size with a precision caliper. To calculate the tumor volume, the following formula was applied:

$$V = LxA^2/2$$

where A is the width and L the length of the tumor. At day 22 after the initiation of the treatments, mice were sacrificed following ethical rules and tumors were excised. Tumor tissues were stored in 10% buffered formalin until examination in histological studies.

Histological studies on tumor sections

Hematoxylin-eosin histological staining

Tissue was fixed in 4% formaldehyde and embedded in paraffin. 5-µm sections were cut from the tissue blocks and deparaffinized by immersion in xylene twice for 5 min each. After that, tissue sections were rehydrated by immersion in decreasing concentrations of ethanol. Then, immersing in GILL II Hematoxylin for 3 min stained nuclei. Afterwards, cell cytoplasm was stained by immersing in 0.5% of eosin, which contains 0.2% of glacial acetic acid, for 3 min. Finally, sections were

dehydrated by immersion in solutions with ascending alcohol percentages. All sections were cleaned by xylene, dried and mounted on glass coverslips with DPX.

Fluorescence study of apoptotic nuclei and immunohistochemistry for detection of activated caspase-3 and NK1.1 staining

Tissue sections 5-μm thick were deparaffinated, rehydrated and stained by immersing in GILL II Hematoxylin followed by eosin staining. For the study of apoptotic nuclei, tissue sections were stained with DAPI Fluoromont-G (EMS, Madrid) for 10 min and detected in a fluorescence microscope (E600/E400, Nikon) equipped with a digital photography machine (DXM1200F, Nikon).

The expression of activated caspase-3 was investigated by immunohistochemistry using a rabbit polyclonal anti-human caspase-3 antibody (Cell Signaling, Barcelona), which recognizes the active, cleaved caspase-3 form. After blocking with 5% horse serum diluted in PBS for 1hr at room temperature, sections were incubated at 4 °C in humid chambers with the anti-caspase-3 antibody at 1/150 dilution for 1hr followed by ready to use secondary anti-rabbit antibody (Vector Laboratories, Peterborough, UK) for 30 min. As a chromogenic substrate, 3,3′-Diaminobenzidine (DAB; Agilent, Madrid) was used, followed by hematoxylin counterstaining. Appropriate negative control stainings were also performed. For the study of NK cell infiltration in tumor tissue, sections were stained with a rabbit polyclonal antibody anti-human NKp46 from Biorbyt (Cambridge, UK), and revealed in the same way as indicated for caspase-3 activation. Isotype control staining were also performed, showing no substantial signal.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 program (GrandPath Software Inc). Statistical significance was evaluated by using Student t-test for non-paired variants, considering a $p < 0.05$ value as significant.

Results

In vitro cytotoxic activity of recombinant granulysin on human melanoma cell lines

In previous studies, we have demonstrated the high sensitivity of the Jurkat T cell leukemia to recombinant granulysin cytotoxicity [11]. We tested the *in vitro* toxicity of recombinant granulysin on two human melanoma cell lines, SK-MEL103 and UACC62, and comparatively also on Jurkat cells. SKMEL103 cells are p53 and BRAF wt, but exhibit the Q16R mutation in N-Ras and PTEN deletion; UACC62 cells are p53 and N-Ras wt, but exhibit the V600E mutation in BRAF, together with p16 and PTEN deletion, making it a specially aggressive tumor [22].

As shown in Fig. 1, while Jurkat cells were very sensitive to 50 μM granulysin after 24 h of incubation, SK-MEL103 cells showed low sensitivity, and UACC62 cells showed a significant level of sensitivity, although not as high as Jurkat cells. In principle, mutations in UACC62 cells would make it a more aggressive tumor than SKMEL103, but, at the same time, it resulted more sensitive to granulysin. Granulysin has been shown to be inactive against normal T lymphocytes, and the higher or lower sensitivity of several multiple myeloma cell lines to granulysin correlated rather with the ratio between pro-apoptotic and anti-apoptotic long members of the Bcl-2 family [11].

In vivo granulysin treatment experiments on UACC62-induced tumors

Due to its higher *in vitro* sensitivity to recombinant granulysin, and given that it easily generates tumors in immune-deficient mice [22], we chose the UACC62 cell line to perform xenograft experiments in athymic mice, essentially as described in our previous work on mammary adenocarcinoma and multiple myeloma [17].

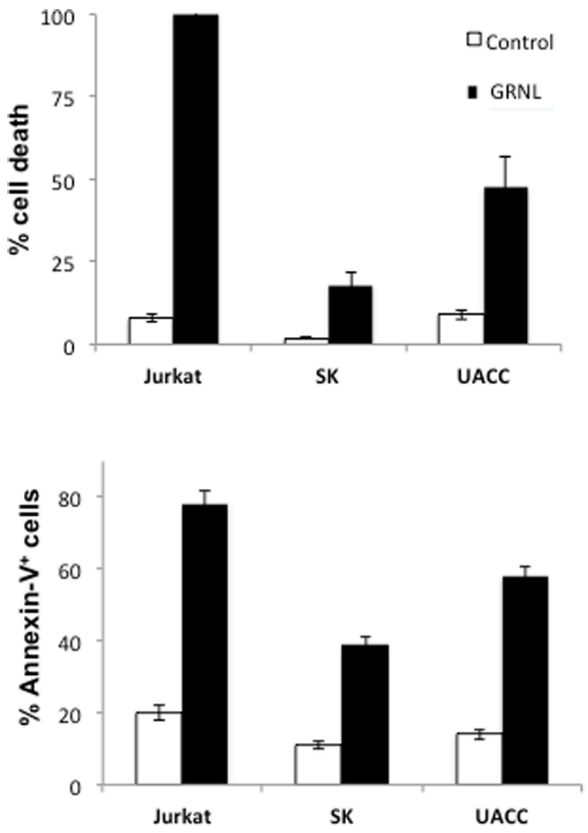

Fig. 1. *In vitro* granulysin-induced death of Jurkat, SK-MEL103 and UACC62 cells. Jurkat, SK-MEL103 (SK) or UACC62 cells (UACC) were incubated (black bars, GRNL) or not (white bars, Control) with 50 μM recombinant granulysin during 24 h. A, cell death was estimated using Trypan blue staining. B, apoptotic cell death was determined by detection of phosphatidylserine exposure by staining with Annexin-V-FITC. Results are the mean ± SD of 3 different experiments.

1×10^6 UACC62 cells were injected subcutaneously (s.c.) in groups of athymic mice and treatments started when tumor volume arrived to 100 mm^3. Tumor growth was extremely aggressive, growing to a size of 100 mm^3 in 2 weeks or less. Mice were then divided into two groups, the group treated with granulysin and the control group. In the granulysin group, 44 μg of granulysin suspended in 50 μl PBS was injected intratumorally every 2 days for a total of 10 injections. In the control group, 50 μl PBS was injected following the same temporal schedule. After the 10 injections, mice were sacrificed and tumors resected. As shown in Fig 2A, the external, apparent mean tumor volume was not greatly affected by treatment with recombinant granulysin in this aggressive tumor model. However, when tumors were resected, and the inflammatory surroundings eliminated, the mean size of granulysin-treated melanomas were significantly reduced with respect to the control group (Fig 2B). The mean tumor size was reduced from 5036 mm^3 in the control group to 2830 mm^3 in the treated group, meaning a 44% reduction, that arrived to be statistically significant ($P = 0.05$ in the Student's t-test).

Given the aggressiveness of the tumor, we performed a second set of experiments, injecting s.c. $2{,}5 \times 10^5$ tumor cells instead of 1×10^6, while following a similar protocol as above. As shown in Fig 3A, in these experimental conditions, the external volume of the tumor was reduced by granulysin treatment throughout the experiment, with a reduction at the time of sacrifice from a mean of 7325 mm^3 in the control group to 4282 mm^3 in the treated group, a 42% decrease. However, due to the high SD in the control group, differences were not statistically significant. When the size of the tumors was measured after resection, the reduction was greater, from a mean size of 4247 mm^3 in the control

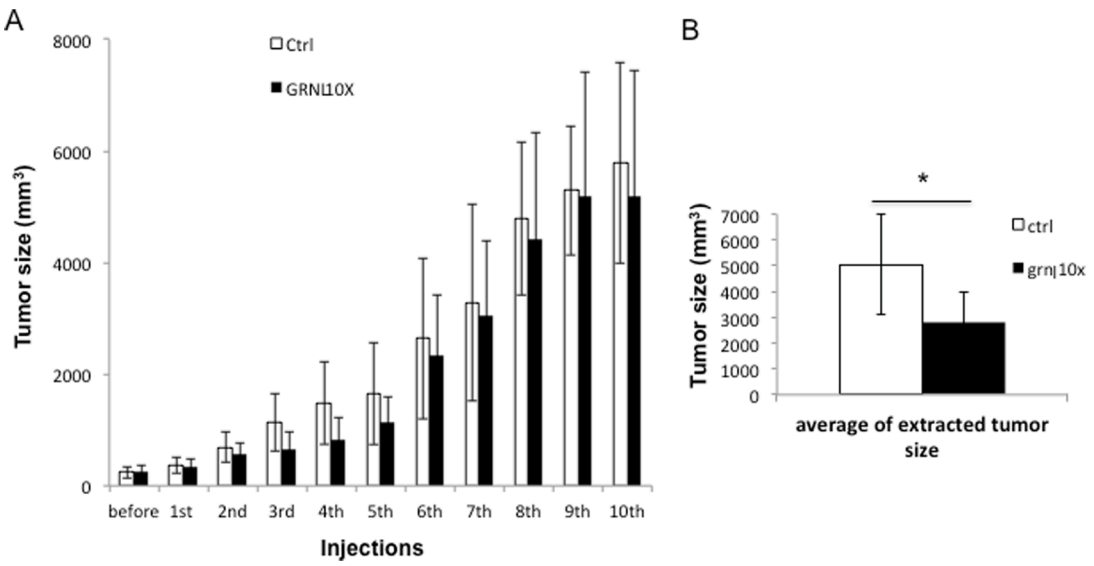

Fig. 2. Granulysin treatment of nude mice bearing UACC62 human melanoma cancer xenografts. 1×10^6 UACC62 melanoma cells were injected s.c. in 10 nude mice. When tumor volume had arrived to 0.1 cm^3 mice were divided in two groups, Ctrl (white bars) and GRNL-treated (black bars), $n = 5$. Mice in the GRNL group received intra-tumor injections of GRNL and mice in the Ctrl group of PBS every 2 days for 10 times and then mice were sacrificed. A, data are the mean ± SD of the tumor volume in each group of the study. B, data show the tumor volumes after resection. *, $P = 0.05$.

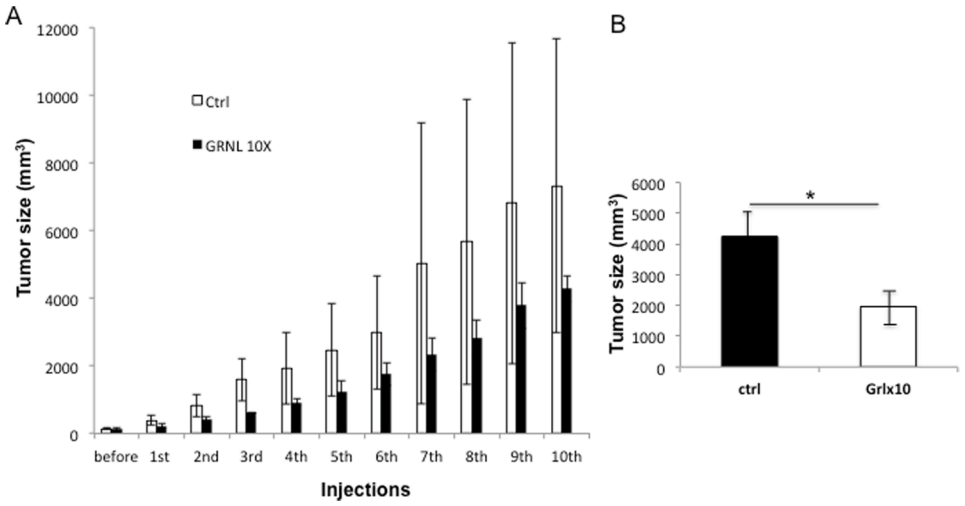

Fig. 3. Granulysin treatment of nude mice bearing UACC62 human melanoma cancer xenografts. 2.5×10^5 UACC62 melanoma cells were injected s.c. in 10 nude mice. When tumor volume had arrived to 0.1 cm^3 mice were divided in two groups, Ctrl (white bars) and GRNL-treated (black bars), $n = 5$ for each experimental group. Mice in the GRNL group received intra-tumor injections of GRNL at 44 μg in 50 μl of PBS every 2 days for 10 times and then mice were sacrificed. Mice in the Ctrl group received injections of 50 μl PBS with the same time schedule. A, data are the mean ± SD of the tumor volume in each group of the study. B, data show the tumor volumes after resection in the two experimental groups described. *, $P < 0.05$.

group to 1964 mm^3 in the treated group, a 54% reduction, which was statistically significant (Fig 3B). These data indicate that intra-tumor administration of recombinant granulysin retards tumor growth in the aggressive UACC62 melanoma xenograft.

H&E and DAPI staining, caspase-3 activation and NK cell infiltration on resected UACC62-derived tumors

H&E staining was performed on paraffin-embedded tumor samples. Regarding tumors obtained from the experiment shown in Fig. 2, tumors in the control group showed a compact tissue organization, forming nested structures, sometimes separated by connective tissue walls (Fig 4A, upper left panel), and sometimes extremely well irrigated (Fig 4A, upper middle panel). Hemorrhagic areas could be also observed in these tumors, accompanied by the presence of defined regions containing cells with heavily stained, picnotic nuclei (Fig 4A, upper right panel). In the case of tumors treated with granulysin, although it could be observed regions with compact structure, formed by healthy, well irrigated cells (*e.g.*, Fig 4A lower left panel), the most frequent tissue aspect, especially in the internal part of the tumor, was a less compact structure, with frequent gaps, and with a predominance of cells with heavily stained, picnotic nuclei (Fig 4A, lower middle and right panels). In H&E stainings made on tumors obtained in the experiment shown in Fig. 3, we can observe again in the controls the indicated nested and well irrigated structures (Fig 4B, upper panels), while the aspect of the tumors treated with granulysin were characterized by the presence of gaps and of areas devoid of nucleated cells, probably made of collagen or other extracellular matrix proteins. The presence of picnotic nuclei was also clearly detected in these treated tumors (Fig 4B, lower panels).

To analyze nuclear apoptotic features *in vivo* we stained tumor tissue sections with DAPI. Control tumor samples show the typical nuclear staining of living cells, while in treated tumors, it could be easily detected the higher intensity of labeling, due to chromatin condensation, together with the very frequent observation of fragmented nuclei, especially clear in the high magnification pictures, and indicated with arrows (Fig 5A). In addition, there were also observed cells with peripheral nuclear condensed chromatin (indicated with circles in Fig 5A), typical of apoptosis-inducing factor (AIF)-mediated cell death, that our

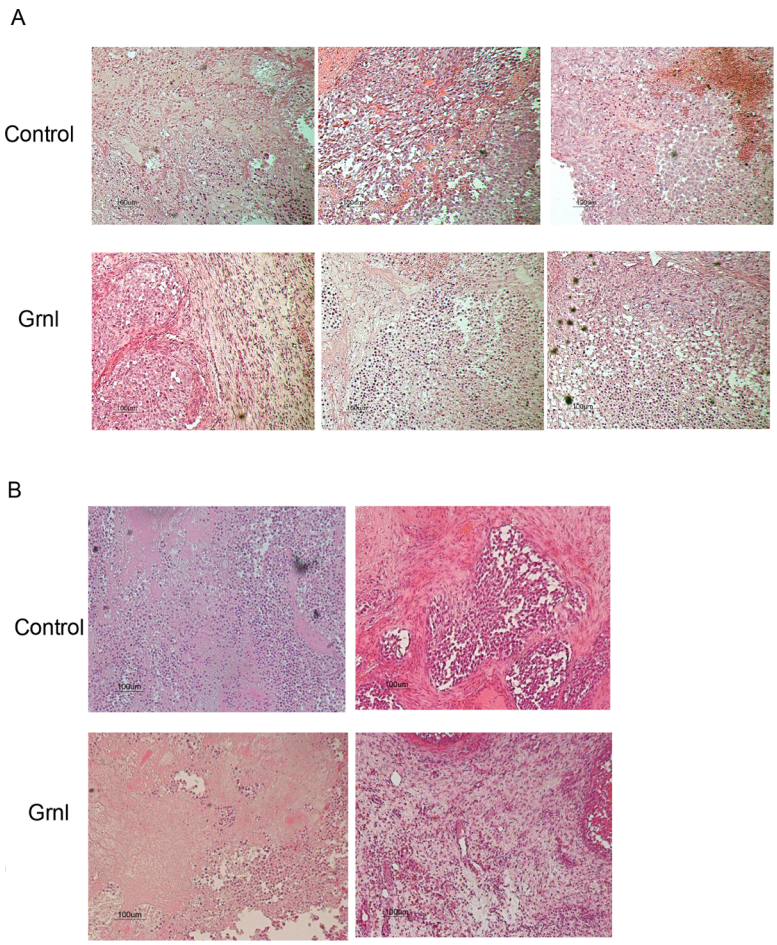

Fig. 4. H&E staining in histological sections of UACC62-derived tumors. A, representative images of resected UACC62-derived tumors from the control group or from the granulysin-treated group (Grnl) of the experiment depicted in Fig 2 are shown. B, representative images of resected UACC62-derived tumors from the control group or from the granulysin-treated group (Grnl) of the experiment depicted in Figs 3 are shown. Tissue sections were stained with hematoxylin and eosin, as indicated in Materials and Methods, and photographed at a 100X magnification in all images.

group demonstrated previously was implicated in granulysin-induced cell death [14].

To confirm apoptosis induction *in vivo*, we performed anti-caspase 3 immunostaining on tumor samples. For that, we used an antibody that detects the active, cleaved form of caspase-3. As shown in Fig. 5B, peroxidase staining was clearly observed in tumor samples obtained from treated tumor samples, both at low and high magnification, while it was absent in tumor samples from control mice.

In our previous study [17], we also showed that granulysin treatment induced the infiltration of NK cells in the tumor tissue. Athymic mice used in these studies are deficient in T cells, but other cells characteristic of the innate immune response, such as neutrophils, macrophages or NK cells are normally expressed. Therefore, we studied NK cell infiltration in the melanoma model using immunostaining with an anti-NKp46 antibody.

We could not detect intratumoral staining of NK cells in tumors from control, untreated mice (Fig 6, upper panels). In the case of tumors from granulysin-treated mice, although NK infiltration was not massive and there were tumor zones devoid of peroxidase labeling, we could detect in almost all samples analyzed some cells clearly stained with peroxidase, indicating some NK cell infiltration due to the intratumoral granulysin treatment (Fig 6, lower panels). In the image obtained at a higher magnification (Fig 6, lower right panel), a group of NK cells is close to a blood vessel, indicating NK cell extravasation in the proximity of the tumor mass.

Determination of calreticulin exposure upon granulysin treatment in tumor cell lines

The observed NK cell infiltration in the tumor mass could indicate that granulysin is inducing an immunogenic type of cell death. To further study this possibility, we perfomed *in vitro* assays to determine if granulysin treatment was able to induce calreticulin (CRT) exposure on the surface of tumor cells, one of the most important markers of this type of cell death. As shown in Fig. 7, granulysin increased CRT exposure in two different types of cells. In the NCI-H929 multiple myeloma, which grows in suspension, the increase was of 40%, while in the lung carcinoma A549, which grow as adherent cells, similar to the UACC62 melanoma, the increase was of 27%.

Discussion

In the initial characterization paper of granulysin, it was demonstrated that recombinant 9-kDa granulysin was able to kill tumor cells in the absence of perforin [1]. Afterwards, it was demonstrated that granulysin expression correlated with good prognosis in different types of cancer patients [23-26]. Although the antitumor role of endogenous 9-kDa granulysin has not been clearly demonstrated, the recombinant molecule could be tested as an anti-tumor agent. In previous *in vitro* studies from our group, it was demonstrated its activity against the human acute lymphoblastic leukemia (ALL) Jurkat [14,27], against several human multiple myeloma cell lines [11] and, most importantly, against primary leukemic cells from B-cell chronic lymphocytic leukemia (B-CLL) patients [11]. Subsequently, we also showed the *in vivo* potential of recombinant granulysin in xenotransplant models of human breast adenocarcinoma MDA-MB-231 and human multiple myeloma NCI-H929 [17]. In this study, we showed that intratumoral injection of recombinant granulysin induced apoptosis in the *in vivo* treated tumors, and also the infiltration of NK cells.

A recent concept in cancer treatment is that of immunogenic tumor

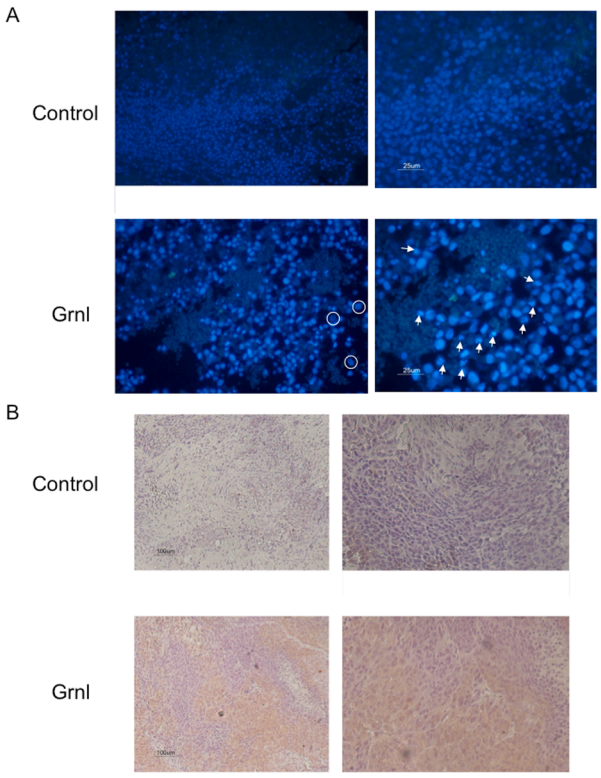

Fig. 5. Nuclear staining and immunostaining for caspase-3 in histological sections of UACC62-derived tumors. A, B, representative images of resected UACC62-derived tumors from the control group or from the granulysin-treated group (Grnl) of the experiment depicted in Figs 3 are shown. A, nuclei were stained using the DAPI nuclear dye and photographed in a fluorescence microscope at 200X (left panels) or 400X magnification (right panels). B, tissue sections were immunostained with anti-caspase-3 antibody and photographed at 100X (left panels) or 200X magnification (right panels).

cell death. Some cytotoxic drugs normally used in chemotherapy, such as anthracyclines, are able to kill cancer cells in such a way that they promote a subsequent antitumor immune response [28,29]. We have confirmed in the melanoma model described in this work that granulysin generate some type of immunogenic tumor cell death *in vivo*, resulting in intratumoral NK cell recruitment. This effect could be due in part to the presence of small amounts of endotoxin in the granulysin preparation, although we have used an endotoxin-removal kit. However, in our previous study [17], we demonstrated that the *in vivo* antitumoral effect of recombinant granulysin was not dependent on the higher or lower amount of endotoxin in the preparations. In addition, we have demonstrated in several tumor cell models, that granulysin is able to induce calreticulin exposure on their plasma membrane, one of the hallmarks of immunogenic cell death [29].

Results presented in this work suggest that intra-tumor administration of granulysin could be used as a novel anti-melanoma treatment. Of course, more pre-clinical and translational research would be needed to ascertain this. The results obtained in the melanoma model are especially interesting for two reasons. First, the intratumoral injection, although difficult to perform in internal tumors, could be easily adapted to melanoma treatment. Second, given the *in vitro* and *in vivo* immunogenic potential of granulysin-induced cell death, observed here and in our previous study [17] associated with its capacity to recruit NK cells, granulysin can be used to transform non-immunogenic tumors in immunogenic [30], and then combine with immunotherapy regimens, such as immune checkpoint inhibitor blockers, which are achieving impressive results in melanoma patients [31]. It should be noted than in the athymic mice model, only NK cells are immune anti-tumor effectors, but that in immunocompetent settings, this immunogenicity would probably extend to specific anti-tumoral cytotoxic T lymphocytes.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Author contribution

SAW produced and purified recombinant granulysin and performed most of the animal experimental work. He prepared Figs 1, 2 and 3.

PGO analyzed and photographed H&E samples, caspase-3 immunohistochemistry and DAPI nuclear staining. She prepared Figs 4 and 5.

RIP and RS performed the experiments shown in Fig. 7 and prepared the Figure. RIP produced and purified recombinant granulysin used in these experiments.

BC coordinated the animal experimentation studies and was aware of permissions and correct procedures.

LML interpreted the results in the histology sections, especially those related with H&E staining, caspase-3 immunohistochemistry and DAPI nuclear staining and wrote some sections of the manuscript.

AA coordinated the study, obtained financial support for the

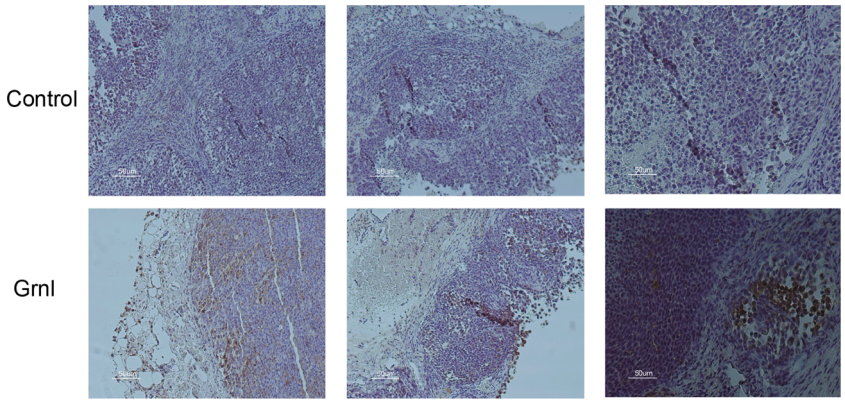

Fig. 6. Immunostaining for NKp46 in histological sections of UACC62-derived tumors. Representative images of resected UACC62-derived tumors from the control group or from the granulysin-treated group (Grnl) of the experiment depicted in Figs 3 are shown. Tissue sections were immunostained with anti-NKp46 antibody to detect NK infiltration and photographed at 100X (left and middle panels) or 200X magnification (right panels).

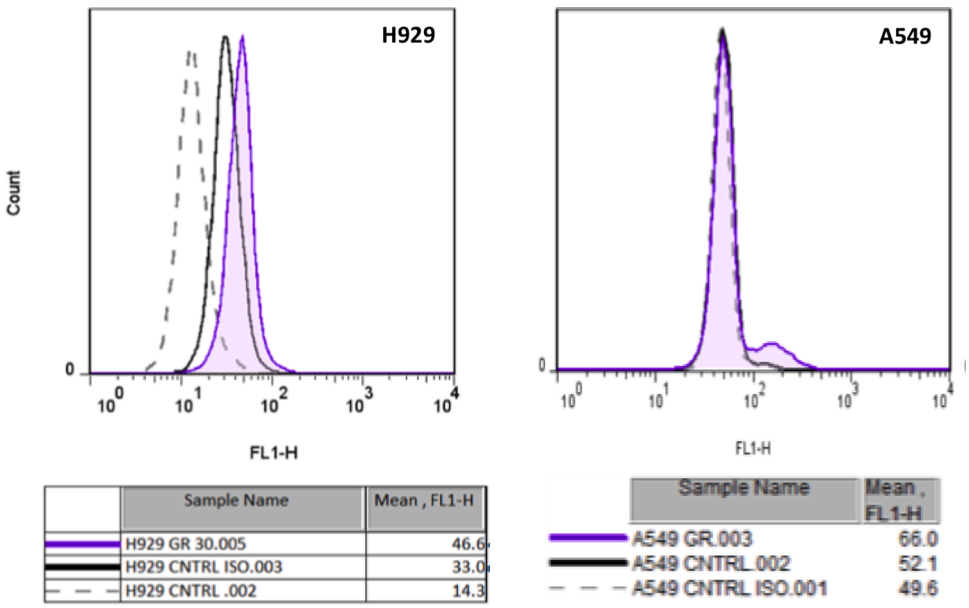

Fig 7. Induction of calreticulin exposure upon granulysin treatment. Calreticulin exposure was analyzed by flow cytometry on the surface of H929 and A549 cells after treatment with 30 μM granulysin for 24 h. Solid black histograms, labeling of control cells, non-treated with granulysin, with an anti-calreticulin mAb conjugated with FITC; cyan histograms, labeling of cells treated with granulysin with the anti-calreticulin mAb; discontinuous histograms, labeling of cells with an isotype control mAb conjugated with FITC.

development of the work, wrote the manuscript and analyzed and photographed NK cell immunohistochemistry samples, preparing Fig. 6.

Acknowledgements

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