Cryobiology 67 (2013) 163–169

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Development of a novel methodology for cryopreservation of melanoma cells applied to CSF470 therapeutic vaccine ☆

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ARTICLE INFO

Article history:
Received 12 April 2013
Accepted 28 June 2013
Available online 9 July 2013

Keywords:
Melanoma vaccine
Trehalose
Cryopreservation

ABSTRACT

CSF470 vaccine is a mixture of four lethally irradiated melanoma cell lines, administered with BCG and GM-CSF, which is currently being tested in a Phase II/III Clinical trial in stage II/III melanoma patients. To prepare vaccine doses, irradiated melanoma cell lines are frozen using dimethyl sulfoxide (Me_2SO) and stored in liquid nitrogen ($liqN_2$). Prior to inoculation, doses must be thawed, washed to remove Me_2SO and suspended for clinical administration. Avoiding the use of Me_2SO and storage in $liqN_2$ would allow future freeze-drying of CSF470 vaccine to facilitate pharmaceutical production and distribution. We worked on the development of an alternative cryopreservation methodology while keeping the vaccine's biological and immunogenic properties. We tested different freezing media containing trehalose suitable to remain as excipients in a freeze-dried product, to cryopreserve melanoma cells either before or after gamma irradiation. Melanoma cells incorporated trehalose after 5 h incubation at 37 °C by fluid-phase endocytosis, reaching an intracellular concentration that varied between 70–140 mM depending on the cell line. Optimal freezing conditions were 0.2 M trehalose and 30 mg/ml human serum albumin, at −84 °C. Vaccine doses could be frozen in trehalose at −84 °C for at least four months keeping their cellular integrity, antigen expression and apoptosis/necrosis profile after gamma-irradiation as compared to Me_2SO control. Non-irradiated melanoma cell lines also showed comparable proliferative capacity after both cryopreservation procedures. Trehalose-freezing medium allowed us to cryopreserve melanoma cells, either alive or after gamma irradiation, at −84 °C avoiding the use of Me_2SO and $liqN_2$ storage. These cryopreservation conditions could be suitable for future freeze-drying of CSF470 vaccine.

Introduction

Cells can be stored maintaining their biological functions for long periods of time after freezing at very low temperatures, typically in liquid nitrogen ($LiqN_2$). $LiqN_2$ storage is a widely used method that relies on the use of cryoprotectants to reduce cell injury during the freezing and thawing process, mainly due to intracellular ice formation [12]. Dimethylsulfoxide (Me_2SO) is a commonly used cell permeable cryoprotectant that prevents excessive dehydration during the freezing process and also inhibits intracellular ice formation. Most protocols for mammalian cell cryopreservation use a solution of Me_2SO (5–20%) combined with foetal bovine serum [19]. Other non-permeating agents like polymers and various sugars are also widely used [32].

Whole cell-based vaccines are an attractive therapeutic strategy for cancer immunotherapy, allowing the induction of anti-tumour immune responses to yet known as well as unknown antigens present in tumour cells. We have used this approach in a Phase I clinical Trial injecting melanoma patients with a mixture of gamma-irradiated melanoma cell lines plus BCG and GM-CSF in order to stimulate patienťs immune responses against the tumour [2]. A similar vaccine composed of four irradiated melanoma cell lines (CSF470) is currently being tested in a Phase II-III clinical trial in comparison with interferon alpha-2b on stage II and III melanoma

☆ *Statement of fundings:* This work has been supported by Grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto Nacional del Cáncer (INC) Ministerio de Salud, Argentina, Fundación Sales, Fundación Cáncer (FUCA), Fundación Pedro F. Mosoteguy and Fundación María Calderón de la Barca, Argentina.

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http://dx.doi.org/10.1016/j.cryobiol.2013.06.007

patients [33]. We have used the same mixture of melanoma irradiated whole cells to load autologous dendritic cells (DC) before its injection as an antitumor vaccine [26,27]. For these purposes or for their use as a source of tumour antigens for DC loading, melanoma irradiated cells (a mixture of apoptotic/necrotic cells) need to be cryopreserved in liquid nitrogen ($LiqN_2$) keeping a certain degree of cell integrity and antigen expression but not as a soluble lysate. In the case of CSF470 vaccine, we have developed a serum-free freezing medium consisting of 10% Me_2SO solution supplemented with 8% human serum albumin (HSA) in culture medium for long term $liqN_2$ storage. Although cryopreservation in $liqN_2$ is an optimal method to preserve and store CSF470 vaccine keeping post-thaw cell integrity and antigen expression, it might be impractical for pharmaceutical production. CSF470 vaccine freeze-drying would be desirable since it could allow vaccine storage at 2–8 °C or room temperature and help its distribution and patient's administration avoiding Me_2SO washing in a sterile cabinet. It would also reduce costs and regulatory issues for its distribution. Freeze-drying of eukaryotic nucleated cells is still a challenge and very few reports have achieved it successfully, with varying results mainly depending on the cell type [3,28]. For freeze-drying, melanoma cells should be frozen in the absence of Me_2SO because it is highly toxic and it could not remain in the final product.

As a first step toward preserving melanoma cells by freeze-drying, we describe a new methodology for cryopreservation of CSF470 vaccine using trehalose and HSA instead of Me_2SO. We show here that trehalose can be loaded into melanoma cells in a temperature-dependent manner, presumably by fluid-phase endocytosis. Trehalose loaded melanoma cells frozen at −84 °C in trehalose-containing medium showed a similar performance after freeze–thawing in terms of melanoma cell integrity and growth before gamma irradiation as compared to standard Me_2SO cryopreservation. To prepare CSF470 vaccine doses, trehalose loaded melanoma cells were gamma-irradiated and further cryopreserved in trehalose at −84 °C, keeping several biological characteristics that define CSF470 vaccine for at least 4 months.

Materials and methods

Cell lines

Four melanoma cell lines (MEL-XY1, MEL-XY2, MEL-XY3 and MEL-XX4) were established from tumour biopsies from metastatic melanoma patients at the Centro de Investigaciones Oncológicas, FUCA (Buenos Aires, Argentina). Cells were cultured as previously described [26].

Cell counts and integrity

Cell counts (total cells) and cell integrity were assessed by Trypan Blue exclusion (Life Technologies) using a haemocytometer (Neubauer) before and after freezing under each experimental condition. For CSF470 vaccine stability determinations, cell integrity was assessed immediately after quick thawing at 37 °C in a water bath, at 1, 2, 3 and 5 h post thawing. To assess long-term storage, cells were frozen at −84 °C for 7, 15, 30, 60, 90 and 120 days, subsequently thawed and cell integrity was determined as above.

LYCH loading and detection

The fluorescent dye Lucifer Yellow carbohydrazine (LYCH) (Sigma Aldrich) was used to monitor solute uptake by fluid phase endocytosis (FPE) in melanoma cell lines. Cells were grown until 70% confluence. Cells were incubated with 0.5 mg/ml LYCH for different time points at 37 °C in a CO_2 incubator. Following incubation, cells were washed with phosphate buffered saline (PBS) and harvested using a 0.2 g/l EDTA solution. After one PBS wash, cell counts and integrity were assessed as described above. For each cell line, a control culture was performed incubating the cells at 4 °C to inhibit FPE. LYCH uptake was analysed by FACS (FACSCalibur, BD Biosciences), acquiring a minimum of 20,000 cells/experimental point using CELLQuest software. Results are shown as MFI∗% (mean fluorescence intensity *x* percentage of positive cells). LYCH uptake inside melanoma cells was also visualized under a BX40 epifluorescence microscope (Olympus), equipped with a DP72 camera and DP-2 BSW software (Olympus).

Trehalose loading

Melanoma cells growing in monolayers were loaded with 200 mM D(+) Trehalose dihydrate (TRE) (Sigma Aldrich) added to the culture medium and incubated at 37 °C for 5 h. Control cells (CRL) were not loaded with TRE. After being washed twice with PBS, cells were harvested with EDTA solution and collected by centrifugation (1300 rpm). Total sugars were extracted in 80% (v/v) methanol at 80 °C for 2 h, dried under N_2 and quantified by the Anthrone-Sulfuric assay, by a micro method in 96 well strip-plates in quadruplicate [18]. A standard curve was set for each determination using purified TRE (sensibility range 0.05–0.4 g/L). Intracellular TRE concentration (mM) was calculated using the formula:

$$\text{Intracellular TRE}\frac{(\text{Sugars in TRE}-\text{CRL})}{\text{Number of cells}}\times\frac{1000\times1000\ (\text{ml})}{378.3\times\text{Free}-\text{CV}}$$

Cellular volume (CV) was calculated using the formula of the sphere $4/3\ \pi\ r^3$ after measuring the mean cell ratio (r) for each cell line (n = 16 cells/cell line) by analysis of microscope pictures using a phase contrast microscope (BX40, Olympus) and DP-2 BSW software. Free-CV was considered as 50% of CV. MW trehalose = 378.3 g/mol.

Freezing media

Standard freezing conditions (Me_2SO medium) for melanoma cell lines and CSF470 vaccine were: Freezing medium- 10% Me_2SO (Merck) plus 8% human serum albumin (HSA) (Universidad Nacional de Córdoba Hemoderivados, Argentina) in Dulbecco's Modified Eagle Medium (Life Technologies) (Me_2SO medium). Cells were suspended in ice-cold freezing media and immediately placed in at −84 °C ultrafreezer protected with a cotton wrap for 24–48 h. Afterwards, cells were stored under $LiqN_2$. 17.6×10^6 - cells were frozen in 3 ml/cryotubes.

Freezing conditions were set after testing different concentrations of TRE plus HSA in PBS. After 5 h TRE loading, cells were harvested with EDTA solution and suspended in ice-cold freezing medium: 200 mM TRE plus 30 mg/ml HSA in PBS (TRE medium). 17.6×10^6 cells were frozen in 1 ml/glass vial. Cells were suspended in ice-cold TRE freezing medium in glass vials and immediately placed in a −84 °C ultrafreezer. Cooling rate was calculated inserting a digital temperature probe (Sp Scientific) inside the vials during freezing from 0 °C to −40 °C inside the −84 °C ultrafreezer.

Cell growth

After thawing, cell growth curves were performed in quadruplicate by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [8]. 3000 cells/well were plated for MEL-XY1, MEL-XY2 and MEL-XY3 cell lines, and 5000 cells/well for MEL-XX4 cell line. At the different time points relative cell growth was calculated as:(sample Abs for each time-Abs_0)/Abs_0; Abs_0 = sample Absorbance at time 0.

Preparation of CSF470 vaccine doses

To prepare CSF470 standard doses, a batch of 200–250 $\times 10^6$ - cells for each cell line was frozen in Me_2SO medium under liquid nitrogen, thawed, pooled, gamma-irradiated (70 Gy, Siemens Lineal Accelerator, Instituto Alexander Fleming, Buenos Aires, Argentina) and frozen again as vaccine doses (17.6×10^6 cells/3 ml) in Me_2SO medium. Alternatively, we prepared CSF470 vaccine first loading each of the four melanoma cell lines with 200 mM TRE in the culture medium for 5 h prior to freezing in TRE medium and stored for 1–2 weeks at −84 °C. Cells were thawed, pooled, gamma irradiated and frozen again in TRE medium as vaccine doses (17.6×10^6 cells/1 ml). A clonogenic assay was performed in sextuplicate for each vaccine batch to ensure the complete abrogation of cell growth in CSF470 doses [2].

Apoptosis–Necrosis detection after gamma irradiation

Apoptosis and necrosis were assessed by Annexin-V and Propidium iodide (PI) staining (BD Biosciences) and FACS analysis in samples of the mixture of the four gamma-irradiated (70 Gy, Siemens Accelerator, Instituto Alexander Fleming) melanoma cell lines, either frozen in Me2SO or TRE-medium as indicated. As a control for this experiment non-irradiated cells were also stained. A minimum of 20,000 cells were analysed in each case.

Indirect immunofluorescence assays

mAbs were used to assess membrane expression of HLA-A0201 (clone BB7.2) and gangliosides GD2 (clone 14.62a) and GD3 (clone MB3.6) by indirect immunofluorescence and FACS (all mAbs were from BD Biosciences). Intracellular MART-1 (clone 2A9, [1]) and gp100 (clone HMB45, Dako) expression was assessed by incubating mAbs after cell fixation and permeabilisation with 0.1% saponin. Isotype-matched controls and mAbs were revealed with RPE goat anti-mouse F(ab')2 (Dako). FACS analysis was performed acquiring at least 20,000 events in each case.

Statistics

Comparisons between TRE and Me_2SO freezing media were analysed using Student's *t*-test to determine the *P*-values. $P < 0.05$ was considered significant.

Results

Fluid phase endocytosis in CSF470 melanoma cell lines

In order to protect cells from intracellular ice formation and injury during the freezing step, sugars like TRE should be present both inside and outside melanoma cells, possibly allowing the stabilization of intracellular membranes and proteins as it was demonstrated for other cell types [31]. TRE is not incorporated into eukaryotic cells by simple diffusion. In some cell types, TRE loading requires an active process of fluid phase endocytosis (FPE). This can be achieved by incubation at 37 °C in the presence of TRE before freezing. We tested whether the four melanoma cell lines included in the CSF470 vaccine were able to perform FPE using the fluorescent dye Lucifer yellow (LYCH). LYCH is an anionic dye and does not cross the cell membrane by passive diffusion and is a well-studied probe for FPE. [22]. As can be observed in Fig. 1A, all four cell lines presented LYCH uptake at 37 °C, reaching a plateau by 5 h incubation. This was completely inhibited at 4 °C, evidencing FPE capacity in the melanoma cell lines. Fig. 1B shows LYCH-containing vesicles observed in the cytoplasm of every cell after 5 h incubation.

Trehalose can be loaded in melanoma cells by FPE

Since LYCH and TRE are of similar size (molecular mass 457 and 378.3 Da, respectively) and uptake of the two molecules from the extracellular medium is likely to be similar as previously described [22], we investigated whether the four melanoma cell lines that compose the CSF470 vaccine could uptake TRE from the culture medium. We incubated MEL-XY1, MEL-XY2, MEL-XY3 and MEL-XX4 melanoma cell lines in the presence of 200 mM TRE at 37 °C for 5 h in the culture medium and quantified TRE uptake after a methanolic extraction of total sugars as described under Methods. As observed in Fig. 2, all four cell lines could be loaded with TRE achieving between 70.6 and 143.2 mM intracellular TRE, depending on the cell line. When the same experiments were performed incubating cells at 4 °C, TRE incorporation was substantially lower for MEL-XY1, MEL-XY3 and MEL-XX4. However, for MEL-XY2 TRE uptake decrease at 4 °C was less pronounced. Altogether, these results suggest that TRE loading in melanoma cells is an active temperature-dependent process.

Melanoma cells can be cryopreserved using trehalose and human serum albumin

Using only one of the melanoma cell lines (MEL-XY3), we compared its cryopreservation using a freezing medium with TRE at −84 °C and the standard Me_2SO freezing procedure in $liqN_2$. Early attempts to freeze cells in <200 mM TRE containing freezing medium or without TRE loading or HSA addition only yielded less than 5% cell integrity after thawing (data not shown). Loading with 200 mM TRE for 5 h at 37 °C and addition of HSA to the freezing medium allowed us to cryopreserve MEL-XY3 cells similarly to the Me_2SO standard condition. As observed in Table 1, after TRE loading, the addition of 30 mg/ml HSA to 200 mM TRE solution in PBS and freezing at −84 °C resulted in 72.6% ± 6.2 cell integrity as compared to 74.6% ± 8.4 in Me_2SO, respectively. Under the same TRE conditions, we also cryopreserved the other three melanoma cell lines MEL-XY1, MEL.XY2 and MEL-XX4 successfully (cell integrity: 62.9 ± 13.5; 75.9 ± 7.3 and 60.6 ± 7.3, respectively in TRE vs. 54.3 ± 7.4; 76.3 ± 17.7; and 66.2 ± 7.1, respectively, frozen in Me_2SO and $liqN_2$; $n \geqslant 3$ independent experiments, $p > 0.05$). Cooling rate measured with a digital probe during cooling in the ultrafreezer was relatively rapid, −1.5 °C/ min from 0 to −15 °C and −7.6 °C/min from −15 to −40 °C. After thawing, melanoma cells cryopreserved in TRE or Me_2SO were cultured for several days showing no morphologic differences as assessed by phase contrast microscopy (not shown). Also, cell growth curves were not significantly different for MEL-XY1, MEL-XY3 and MEL-XX4 (Fig. 3). However, MEL-XY2 cells grew significantly slower after freeze–thawing in TRE medium as compared to Me_2SO.

CSF470 vaccine can be cryopreserved in trehalose keeping its biological properties

Next, we prepared TRE-cryopreserved CSF470 vaccine doses, first loading each of the four melanoma cell lines with 200 mM TRE for 5 h at 37 °C in serum-free culture medium, prior to freezing in TRE medium, storage at −84 °C, thawing, pooling and gamma irradiation. The mixture of the four gamma-irradiated cell lines (CSF470 doses) was frozen again in TRE freezing medium as described under Methods. CSF470 vaccine doses could be successfully cryopreserved in TRE at −84 °C for at least four months as compared to Me_2SO frozen in $liqN_2$. Thawed vaccine doses showed similar cell integrity and morphology (Fig. 4A), apoptosis/necrosis

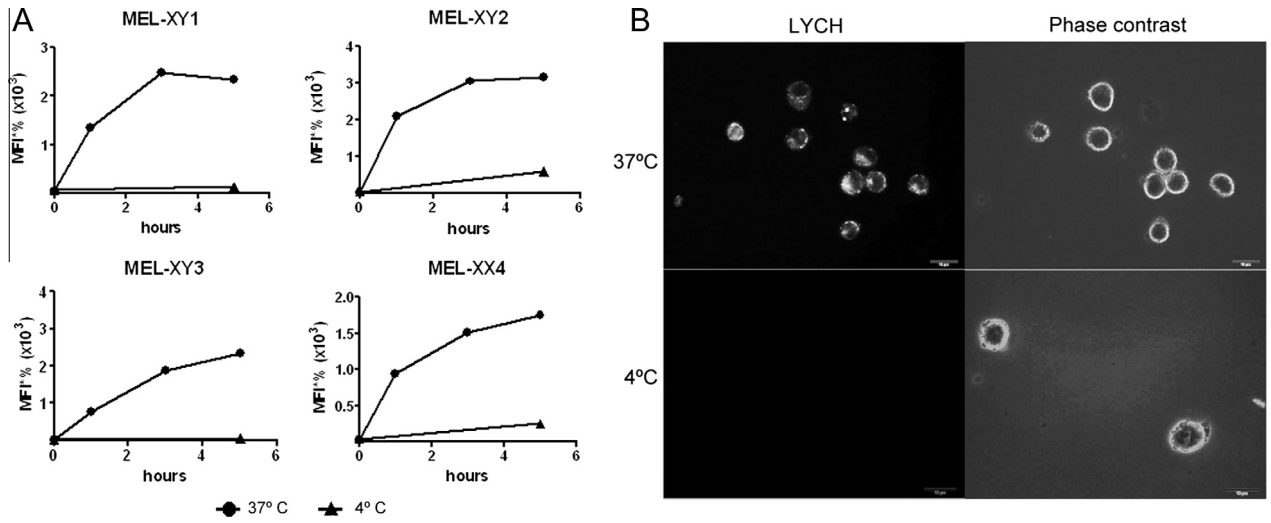

Fig. 1. Melanoma cell endocytosis evidenced by LYCH uptake. A-LYCH endocytosis was tested in the four melanoma cell lines that comprise CSF470 vaccine at different incubation times at 37 °C. Control cultures were incubated at 4 °C for endocytosis inhibition. LYCH uptake was measured by FACS as described under Methods. MFI∗% = mean fluorescence intensity *x* percentage of positive cells. B- MEL-XY3 cells showing LYCH intracellular pattern after 5 h FPE at 37 °C (upper pictures) and negative control at 4 °C. Original magnification = 400×. Scale bar = 10 μm.

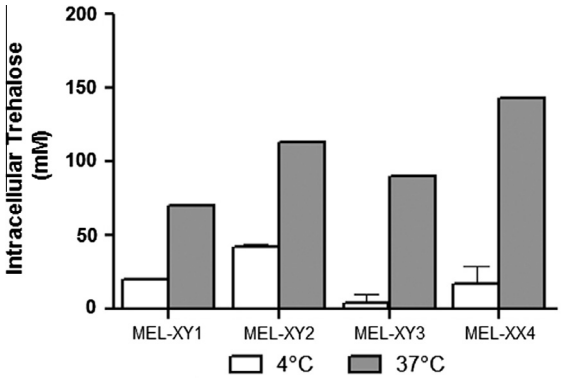

Fig. 2. Trehalose loading in melanoma cell lines. TRE intracellular concentration was determined for each melanoma cell line after 5 h incubation in the TRE-containing culture medium as described under Methods. Cells were either incubated at 37 °C or 4 °C. TRE quantification was performed in quadruplicate. Results are shown as mean ± SD of two independent experiments.

pattern (Fig. 4B) and expression of HLA-A0201 (present in two of the four cell lines), GD2 and GD3 gangliosides [25], melanoma antigen differentiation Ags gp100 [14] and MART-1 [5,13] (Fig. 4C), that are characteristics of CSF470 vaccine. Furthermore, CSF470 thawed doses, cryopreserved either in TRE at −84 °C or Me_2SO in $liqN_2$, suspended in Dulbecco's Modified Eagle Medium (Life technologies) and kept in ice, were equally stable for at least 3 h, losing less that 10% cell integrity (not shown).

Discussion

The biological response to cryopreservation ranges from innocuousness to irreversible damage depending on the cell type [20]. Concerning cell cryopreservation three major variables are critical: cooling rate, type and concentration of protective additive, and warming rate [21]. In this work, we focused on the use of a freezing medium that would allow us to further freeze-dry melanoma cells and the cellular vaccine CSF470. Despite the satisfactory results that we have got additional efforts could be made to optimise cooling rate for our biological system.

Our interest is focused on the use of irradiated melanoma cells as adjuvant therapeutic antitumor vaccines for high risk, stages II-III melanoma patients. Gamma-irradiated apoptotic/necrotic melanoma cells can serve as a source of Ags either interacting *in vivo* with antigen presenting cells (APC), mainly DC and/or macrophages [1] at the vaccinal site or instead, used for *ex vivo* loading of tumour Ags on purified DCs that are afterwards injected into the patient [27]. These strategies rely on further migration of APC to the lymph nodes to initiate T cell proliferation. Although gamma-irradiated cells are not able to proliferate, cell integrity, tumour Ag expression as wells as signals induced during the apoptotic/necrotic death process are required for DC phagocytosis and maturation, to generate an adequate immune response [11,26]. Our mixture of melanoma irradiated cells, CSF470 vaccine, must be cryopreserved in $liqN_2$ before its clinical use with Me_2SO as a cryoprotectant. Alternatively, the same cells could be freeze-dried, allowing a cost-effective storage and avoiding the need of Me_2SO removal before injection into patients or their co-culture with clinical grade DC. As a first step towards CSF470 vaccine lyophilisation, we tested different freezing media alternative to Me_2SO.

Table 1
MEL-XY3 cells freeze-thawing testing in TRE medium containing HSA.

Pre-incubation	200 mM TRE 5 h				Me_2SO Control
Freezing medium	PBS + 200 mM TRE				
HSA concentration	10 mg/ml	20 mg/ml	30 mg/ml	40 mg/ml	
% Recovered[a] cells	54.8 ± 14.6	85.2 ± 8.8	88.7 ± 12.8	75.4 ± 6.5	92.8 ± 11.1
% cell integrity[b]	66.1 ± 4.4	66.6 ± 3.4	72.6 ± 6.1	66.7 ± 22.1	74.6 ± 8.4

[a] The % recovered cells was calculated as the total n° cells thawed/total n° cells frozen ×100.
[b] The % cell integrity was calculated as the n° non-stained cells/n° total thawed cells ×100 as determined by Trypan blue exclusion. Results are shown as mean ± SD; $n = 3$ experiments.

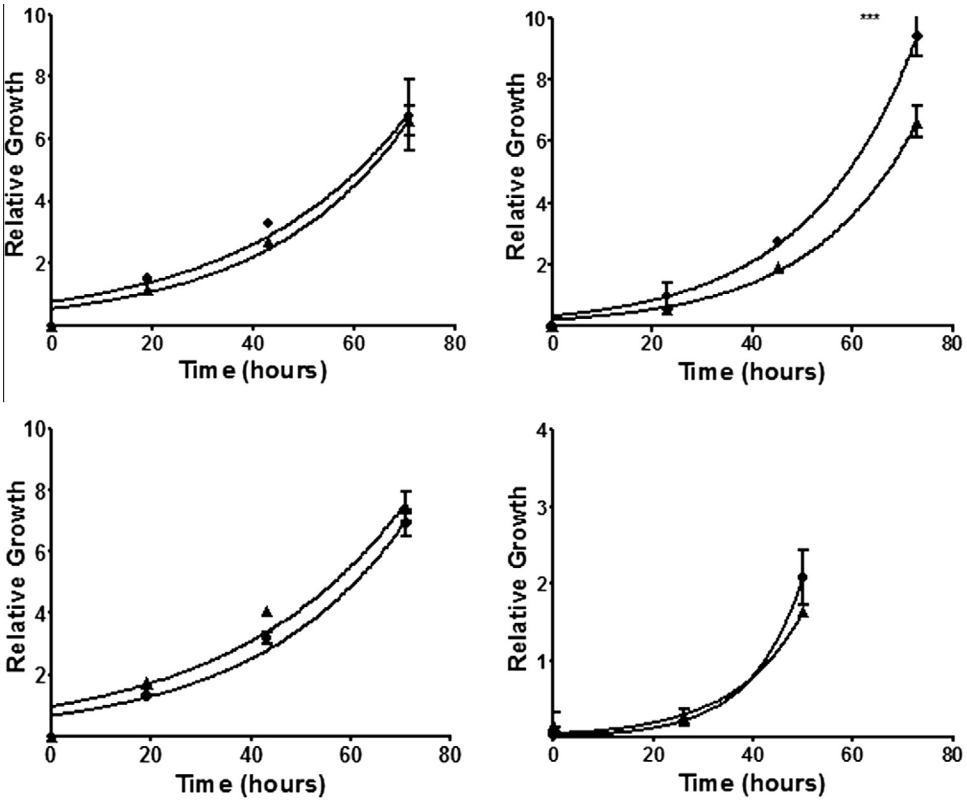

Fig. 3. Melanoma cell integrity after cryopreservation with trehalose. Cells were thawed 7 days after freezing in TRE or Me_2SO and plated to evaluate cell growth as described under Methods. A representative experiment for each cell line is shown, and each point represents mean ± SD.

We have chosen TRE as an alternative cryoprotectant because it is a non-toxic, non-reducing disaccharide of glucose that is produced by anhydrobiotic organisms to prevent cell damage from desiccation [7]. Importantly, TRE could be present as an excipient in the reconstituted injectable vaccine (Food and Drug Administration and European Medicines Agency approved excipient). TRE has been previously used to cryopreserve several human cell types like hematopoietic stem cells [23], platelets [10] and endothelial cells [4,15], due to their ability to stabilise and preserve cells and cellular structures during freezing [6,29]. TRE has also been used as a cryoprotectant for freeze-drying ofbiological products, since it has the ability to form stable glasses during cooling, preventing lethal ice crystals formation and minimizing cell damage due to high electrolyte concentration during cooling [6,9,17,24].

It has been shown that TRE must be present both inside and outside cells to preserve eukaryotic cells like human platelets [31]. Although mammalian cells do not have receptors for TRE incorporation, certain cell types can uptake soluble solutes from the extracellular *milieu* utilizing their own endocytosis machinery [22]. Fluid phase endocytosis (FPE) would thus allow TRE internalisation from the culture medium in a temperature-dependent fashion [30]. After using LYCH as a FPE probe, Oliver et al. [22] have shown that human mesenchymal stem cells can be loaded with TRE from the extracellular milieu. Similarly, we observed that melanoma cell lines incorporated LYCH presumably by FPE reaching a plateau at 5 h of incubation, an active process that was inhibited at 4 °C. This mechanism could also account for TRE uptake in the individual melanoma cell lines comprising CSF470 vaccine. We added TRE to melanoma cell lines in the final 5 h of their culture, in the presence of HSA instead of FBS. We confirmed that TRE can be successfully loaded into melanoma cells at 37 °C, when added to the serum-free culture medium at 200 mM. TRE loading was also dependent on incubation temperature, since uptake at 4 °C was about 60–90% lower than uptake at 37 °C, showing some differences in TRE incorporation among the four melanoma cell lines tested. After 5 h TRE loading, we tested freezing media and found that for each live cell line as well as for the four irradiated cell lines mixture (CSF-470), the best cryopreservation condition was freezing cells in 200 mM TRE plus 30 mg/ml HSA at −84 °C (TRE freezing medium).

Live TRE-loaded cells can be frozen at −84 °C in TRE medium without significant membrane integrity loss, exhibiting a slightly different performance in terms of freezing-thawing survival. A recent report has shown that continuous exposure to TRE induced malignant melanoma cell growth inhibition, both *in vitro* and *in vivo* [16]. We only cultured melanoma cells in the presence of 200 mM TRE for 5 h to achieve loading. In fact, we showed here that after thawing, three out of four TRE cryopreserved melanoma cells grew equally well as compared to Me_2SO cryopreserved controls, while only MEL-XY2 cells grew significantly slower. Thus, TRE cryopreservation can be used instead of Me_2SO standard freezing procedure to store the four melanoma cell lines tested at −84 °C.

CSF470 vaccine was prepared with the four TRE-loaded melanoma cell lines rendered apoptotic/necrotic after gamma irradiation, and cryopreserved in TRE freezing medium at −84 °C, keeping its biological properties such as cell integrity, antigenic expression and apoptosis/necrosis pattern. CSF470 vaccine could be preserved for at least four months stored at −84 °C. As far as we know this is the first report showing that human melanoma cells either alive or gamma-irradiated can be cryopreserved in the absence of Me_2SO at −84 °C. The same procedure could be extended to other cell types for clinical use, i.e. peripheral blood mononuclear cells, tumour infiltrating lymphocytes, *ex vivo* expanded dendritic cells and stem cells, provided they can be loaded with TRE by FPE.

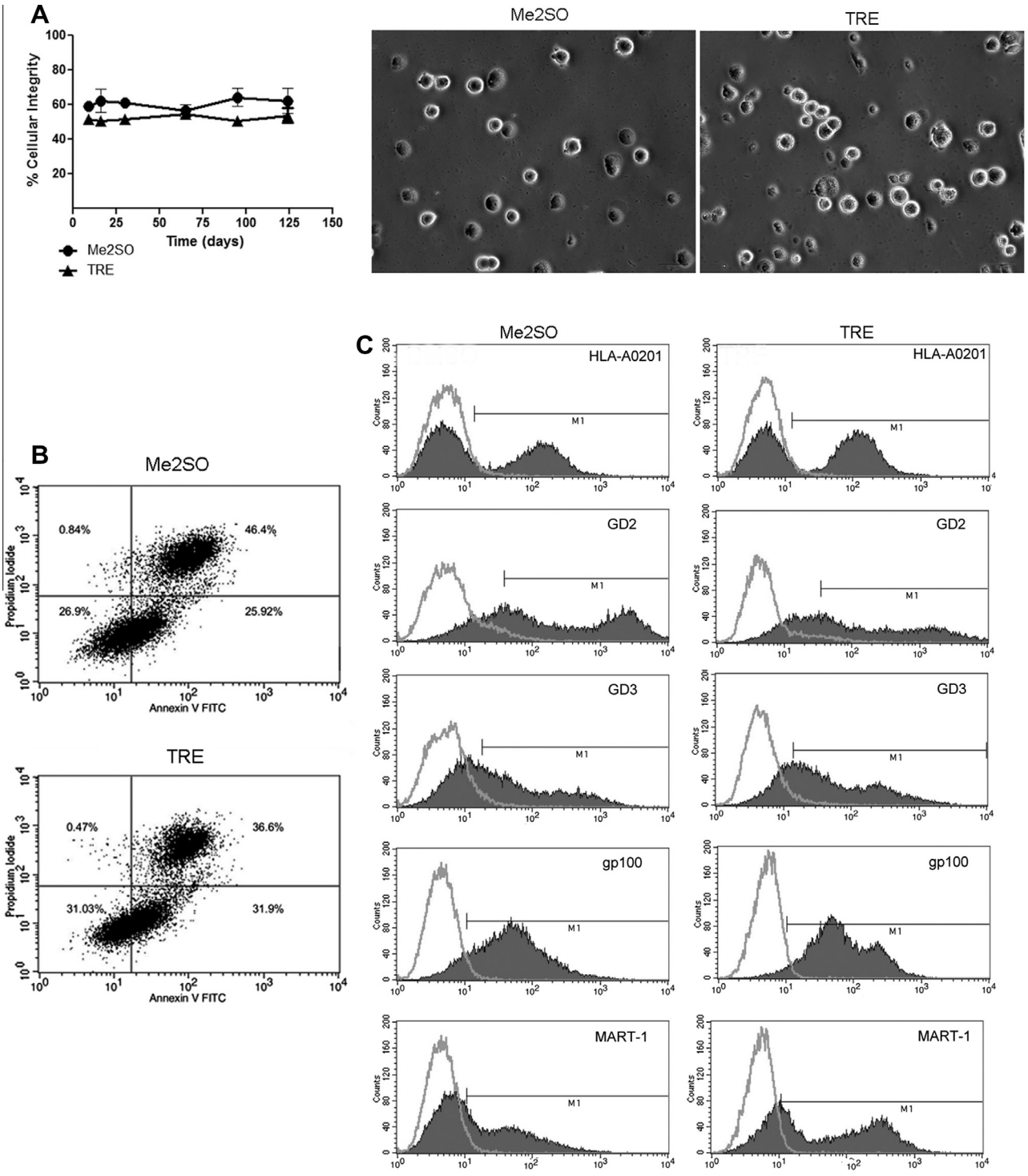

Fig. 4. CSF470 vaccine can be cryopreserved in trehalose instead of Me_2SO keeping its biological properties. CSF470 vaccine doses were cryopreserved in Me_2SO or TRE, thawed at different time points and percentage cellular integrity was assessed by Trypan blue exclusion ($n = 2$ experiments); representative pictures are shown for each case, scale bar = 20 μm (A); the pattern of apoptosis/necrosis was evaluated by Annexin V/PI staining (one representative experiment out of three is shown) (B) and expression of HLA-A0201, GD2 and GD3 gangliosides, and gp100 and MART-1 melanoma Ags were analysed by FACS, as described under methods (one representative experiment out of three is shown) (C).

Also, preservation of melanoma cells in TRE freezing medium would allow their lyophilisation. In particular, freeze drying of nucleated eukaryotic cells other than spermatozoids, is still hard to achieve but it is a field of intensive research, particularly to preserve cells for clinical purposes [3]. Combining TRE cryopreservation with freeze-drying would allow the production of CSF470 vaccine at a lower cost enabling a more convenient distribution and storage. TRE freezing medium that we have developed is suitable for such purpose and these experiments are now underway. CSF470 vaccine frozen in TRE medium could also be stored and distributed avoiding the use of $liqN_2$ and allow direct injection to patients after thawing without the need of Me_2SO removal. This sole fact represents an advantage over standard cryopreservation of CSF470 vaccine in $liqN_2$.

Author's Information

JM, FM and MMB are members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). JM is the Principal Investigator of the CASVAC0401 Clinical Study. IJT and JMA are fellows (PhD students) from CONICET. MA is a fellow from Fundación Sales. The author(s) declare that they have no competing interests.

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