Vincristine-induced bystander effect in human lymphocytes

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Abstract

Bystander effect is a known radiobiological effect, widely described using ionizing radiations and which, more recently, has also been related to chemical mutagens. In this study, we aimed to assess whether or not a bystander response can be induced in cultured human peripheral lymphocytes by vincristine, a chemotherapeutic mutagen acting as spindle poison, and by mitomycin-C, an alkylating agent already known to induce this response in human lymphoblastoid cells. Designing a modified *ad hoc* protocol for the cytokinesis blocked micronucleus (MN) assay, we detected the presence of a dose dependent bystander response in untreated cultures receiving the conditioned medium (CM) from mitomycin-C (MMC) or vincristine (VCR) treated cultures. In the case of MMC, MN frequencies, expressed as micronucleated binucleates, were: 13.5±1.41 at 6 μM, 22±2.12 at 12 μM or 28.25±5.13 at 15 μM vs. a control value of 4.75±1.59. MN levels for VCR, expressed as micronucleated mononucleates were: 2.75±0.88 at 0.0 μM, 27.25±2.30 at 0.4 μM,

46.25±1.94 at 0.8 μM, 98.25±7.25 at 1.6 μM. To verify that no mutagen residual was transferred to recipient cultures together with the CM, we evaluated MN levels in cultures receiving the medium immediately after three washings following the chemical treatment (unconditioned medium). We further confirmed these results using a cell-mixing approach where untreated lymphocytes were co-cultured with donor cells treated with an effect inducing dose of MMC or VCR. A distinct production pattern of both reactive oxygen species and soluble mediator proteins by treated cells may account for the differences observed in the manifestation of the bystander effect induced by VCR. In fact, we observed an increased level of ROS, IL-32 and TGF-β in the CM from VCR treated cultures, not present in MMC treated cultures.

Keywords: bystander effect; micronuclei; spindle poisons; human lymphocytes; ROS; cytokines

1. Introduction

Over the last twenty years many authors have shown that unexposed cells in irradiated cultures can experience significant biochemical and phenotypic changes that are often similar to those observed in targeted cells; this event is known as bystander effect [1-5]. According to the current model, several factors such as reactive oxygen or nitrogen species (ROS or RNS) and cytokines are produced by targeted cells in response to the radiation-induced damage [6-7]. These signals can reach neighbouring cells via gap-junctions or via the extracellular medium, and are able to induce a number of different effects in the unexposed cells such as sister chromatid exchanges [8,9], micronuclei [10], apoptosis [11] or changes in patterns of gene or miRNA expression [12,13]. Several studies have also demonstrated the existence of the bystander effect *in vivo*, highlighting the relevance of this response in therapies based on the irradiation of neoplastic tissues [14-18], thus providing important hints for the optimization of radiotherapy. As cancer therapies are often based on the use of chemotherapeutic drugs, it would also be important to understand if these molecules can also induce a bystander effect. In this regard, melanoma cultures from mice treated with N-

chloroethyl-N-nitrosourea induced growth inhibition, cytoskeletal and metabolic alterations in bystander cells [19]. In addition, mitomycin-C and phleomycin were able to increase micronuclei frequency in bystander cells and to provoke the activation of MAPK pathways [20,21]. Other studies have shown that the treatment with actinomycin-D conditioned medium can induce the activation of intrinsic apoptotic pathway [22]. More recently, the exposure of several cell types to different concentrations of bleomycin and neocarzinostatin, produced the bystander effect in cells not receiving the mutagens, increasing the rate of micronuclei [23]. Bearing this background in mind, we aimed to verify whether or not the bystander effect was induced in cultures of peripheral blood lymphocytes by using, as a reference or test chemical, the bi-functional alkylating agent mitomycin-C (MMC), already known to induce the bystander response, or the spindle poison vincristine (VCR), respectively. VCR prevents the polymerisation and depolymerisation of microtubules, hence inhibiting their dynamic instability, which is essential for the formation of the mitotic spindle [24]. After analysing the induction kinetics of the effect in the reference mutagen, 1) we attempted to detect the presence of a bystander response, expressed as micronuclei (MN) formation, transferring medium from treated to untreated cultures (principal experiments), 2) we confirmed the results using a cell-mixing approach, 3) we analysed the type of MN induced in bystander cultures, and 4) we assessed the involvement of ROS and soluble mediator proteins (IL-32 and TGF-β), which may be involved in the manifestation of the observed effect. To do this, we performed the entire study under standardized experimental conditions, obtained by designing a modified *ad hoc* protocol for the cytokinesis blocked micronucleus assay (CBMN), a well-characterized assay to detect genotoxic and cytotoxic damage [25,26].

2. Materials and methods

2.1 Cell cultures, treatment and harvesting

Heparinised whole blood samples were obtained by venipuncture from four healthy 23- to 26-year-old donors (3 males and 1 female) previously proved to give a comparable response to mutagen treatment. The study was performed according to the Pisa University Ethical Committee.

Two experiments, consisting of a series of two independent cultures per experimental point, were performed for each mutagen treatment. One male and the female were used in all the cell-mixing experiments, the other two males were used in all the conditioned medium experiments. Each culture tube was set up with 300 μl of whole blood and 4.7 ml of RPMI-1640 medium (Life Technologies, Milan, Italy) supplemented with 20% foetal bovine serum (Life Technologies, Milan, Italy), 1% antibiotic/antimitotic (Life Technologies, Milan, Italy) and 1.5% phytohaemagglutinin (Life Technologies, Milan, Italy) and incubated at 37°C for a total time of 72 h. Independently of the specific experimental approach applied, cultures were treated after 24 h from the start of culturing with different doses of mitomycin-C (6, 12 and 15 μM, final concentration) (MMC; Sigma-Aldrich, Milan, Italy) and vincristine (0.2, 0.4, 0.8 and 1.6 μM, final concentration) (VCR; Sigma-Aldrich, Milan, Italy) for 1 and 2 h, respectively. All mutagens were dissolved in sterile H_2O and control cultures not treated with mutagens were also set up. To block cell cytodieresis, cytochalasin B (Cyt B; Sigma-Aldrich, Milan, Italy, 6 μg/ml final concentration) was added at 44 h, and all cultures were harvested at 72 h (end of cell culturing). Lymphocytes were harvested by 4-min centrifugation at 2400 rpm, and the remaining cell pellet was treated with 5 ml of 0.075 mM KC1 for a few min to lyse erythrocytes, prefixed in methanol/acetic acid (3:5), re-centrifuged, fixed in 100% methanol for at least 30 min, washed twice in methanol/acetic acid (9:1), and dropped onto clean glass slides. Harvesting procedure was performed at room temperature.

2. 2. Conditioned medium transfer

As shown in Fig. 1, for each mutagen we set up two groups of cultures, *donor cultures* (DC) and *recipient cultures* (RC). DC were cultures in which cells come in direct contact with the chosen mutagen. To remove the mutagen at the end of the treatment, DC underwent three rounds of washes, each consisting in a 4-min centrifugation, removal of the supernatant and re-suspension of

the cell pellet in 10 ml of fresh medium. Then, according to the standard procedure described elsewhere [27], each DC tube was passed through a 0.20 μm sterile filter (Sarstedt, Verona, Italy) to ensure that no cell factors/debris remained in the media. The filtered medium harvested from these cultures (called *conditioned medium*, CM) was transferred to the corresponding RC at different established times ($T_{transf} > 0$ h) measured after the end of the three washes. RC were then analysed for the induction of a bystander response. To avoid the possibility that residuals of chemicals might be responsible for the effect observed in RC, we also set up a parallel series of cultures called respectively *washing donor cultures* (WDC) and *washing recipient cultures* (WRC) that reproduced the same experimental procedures as DC and RC with the difference that WRC received the medium from WDC immediately after the washes ($T_{transf} = 0$ h) in order to prevent bystander signalling molecules being released in the medium.

2.3 Temporal kinetic and bystander effect induction analysis by conditioned medium transfer

For the temporal kinetic analysis, we treated only DC with the reference mutagen MMC. At the end of treatment, DC were washed three times and CM was transferred to RC at four different times: $T_{transf} = 1$ h, 1.5 h, 2 h and 2.5 h. For the bystander induction analysis, DC and WDC treatments were performed with VCR and the reference mutagen MMC at the previous mentioned doses, plus negative control. After the treatment, DC and WDC were washed and the medium was transferred to the corresponding cultures (to RC at time $T_{transf} = 1.5$ h and to WRC at time $T_{transf} = 0$ h).

2.4 MN scoring

The air-dried slides were stained with 5% Giemsa and, using an optical microscope equipped with a 40× objective (400× final magnification), we counted 1000 cells per culture for the presence of MN for each experimental point. In MMC-treated cultures we analysed MN in binucleated cells, according to standard criteria for the CBMN test [25,28], while in the case of VCR, we performed the MN scoring in mononucleated cells. It is in fact well known that this drug, at certain doses, is able to cause a multitude of mononucleated cells with MN rather than induce MN in binucleates [29,30]. MN frequency was then expressed as the average number of MN per 1000 scored cells.

Where, due to a marked cytotoxicity, the number of cells was not sufficient to allow correct MN analysis, we assessed the damage in terms of toxicity and/or cell cycle delay calculating the cell proliferation index (CPI) according to the following formula: (M + 2B + 4P)/(M + B + P), where M, B and P were the number of cells that had still not entered the first mitosis (M, mononucleated) and cells that had divided once (B, binucleated) and twice (P, plurinucleated; the latter cells comprise both tri- and tetranucleated), respectively. (M+B+P) represents a total of at least 1000 scored cells.

2.5 FISH analysis in cell mixing

In the cell mixing experiments, DC and RC, containing whole blood from a female and a male donor both with the same blood group, were set up as described above (see paragraph 2.2). Treatment of DC was performed with a dose of each mutagen able to induce a clear bystander response (MMC 12 μM and VCR 0.8 μM). At the end of treatment, DC were washed as previously described and the cell pellet was re-suspended in 2.5 ml of complete preheated fresh medium and then mixed with 2.5 ml of cell suspension from the respective RC. To distinguish between DC and RC we used a fluorescent DNA probe recognizing the Y chromosome. Slides were processed for FISH using the FITC-conjugated LPE 0YqG Satellite Enumeration DNA probe (Cytocell Aquarius, Cambridge, UK) according to the manufacturer's instructions. Cells were then counterstained applying 10 μl of propidium iodide (PI, 0.15 μM in antifade) on each slide. Determination of MN frequencies was performed, scoring, for each experimental point, 1000 cells per culture under a 40x or 100x objective (400x or 1000× magnification) of a Nikon-Optiphot-2 fluorescence microscope equipped with the proper filters for FITC and PI visualization. MN were scored in cells containing the green-yellow spot due to the presence of the Y chromosome (recipient cells) which allowed us to clearly distinguish male donor cells in the mixed population.

2.6 Fluorescence MN analysis with a pancentromeric DNA probe to identify MN origin

Once again, treatment of DC was performed with a representative dose of MMC (12 μM) and VCR (0.8 μM) and after three washes the CM was transferred to RC at time T_{transf} = 1.5 h. Slides were then processed for FISH with the Human IDetect™ PanCentromeric GREEN DNA probe (Li

StarFish, Milan, Italy) according to the manufacturer's instructions. Cells were counterstained applying 10 μl of propidium iodide (PI, 0.15 μM in antifade) on each slide. MN analysis was performed under a 60x objective (600x magnification) of a Nikon-Optiphot-2 fluorescence microscope equipped with the proper filters for FITC and PI visualization analysing at least 50 MN per culture. The percentage of C+ centromere positive (C+) or centromere negative (C-) MN was determined as the ratio of MN showing, or not, a green-yellow fluorescent spot to the total MN analysed.

2.7 Involvement of ROS

Treatment of DC was performed with a representative dose of MMC (12 μM) and VCR (0.8 μM) for the established time length and transfer of the conditioned medium was performed at time T_{transf} = 1.5 h after the washing procedure. To assess the involvement of ROS, two scavenger molecules acting as antioxidants via different mechanisms were used. DMSO was added to RC immediately before medium transfer, at a final concentration of 10 μM as a scavenger of HO• radicals [23]. Curcumin (CUR, Sigma-Aldrich, Milan, Italy), a natural compound with intracellular scavenger ability for H_2O_2, HO•, ROO• [31], was added to RC, at a final concentration of 10 μM and 100 μM, 1 h prior incubation of RC with the conditioned medium. Immediately before medium transfer from DC, RC were washed three times to remove the excess of CUR. Slides were processed by the Giemsa staining protocol.

2.8 Identification of soluble proteins in conditioned medium by western blot

DC set up, treatment and washing were performed as described in 2.1 with the exception that mutagen removal was accomplished in a DMEM serum-free medium (Lonza, Milan, Italy). After donor cells were allowed to produce the conditioned medium for 1.5 h, DC were centrifuged to precipitate the cells and a final volume of 14 ml of conditioned medium was transferred into ultracentrifuge tubes and cooled for 30 min. According to the protocol described elsewhere [32], proteins were precipitated on ice for 120 min in trichloroacetic acid (Panreac, Milan, Italy) and sodium lauroyl sarcosinate (AppliChem, Milan, Italy), centrifuged at 4°C for 10 min at 10000 g

(Beckman Coulter Inc., Milan, Italy), washed two times in cooled tetrahydrofuran (AppliChem, Milan, Italy) and finally re-centrifuged at 4°C for 10 min at 10000 g. The protein pellet obtained was dried, re-suspended in a minimum volume of sterile water and then tested with Pierce BCA assay (Thermo Fisher Scientific Inc., Milan, Italy) for protein quantification. 10 μg of proteins from each sample were mixed with an equal volume of loading buffer containing 5% β-Mercaptoethanol (BioRad Laboratories, Milan, Italy). The samples were heat denatured before loading, boiling them for 10 min. Protein electrophoresis was conducted on acrylamide/bisacrylamide gel (15% separating gel, 5% stacking gel) in cell Mini-Protean (BioRad Laboratories, Milan, Italy) for about 1 h with constant voltage (110 V). The separated proteins were then transferred onto a nitrocellulose membrane (BioRad Laboratories, Milan, Italy) for 60 minutes at 200 mA. We applied reversible Ponceau staining to check equal loading of samples. Immunodetection was performed employing anti TGF-β and anti IL-32 antibody (D.B.A. ITALIA, Milan, Italy). Detection was obtained using Anti-Rabbit IgG (H+L) Antibody, Peroxidase labeled (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and BM Chemiluminescence Blotting Substrate (Roche, Milan, Italy). Acquisition and image analysis were carried out with a Chemi-Doc apparatus (BioRad Laboratories, Milan, Italy); molecular weight of the bands was identified referring to Thermo Scientific Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific Inc., Milan, Italy). Quantification of protein content was accomplished using ImageJ® software (version 1.46, download at http://imagej.nih.gov/ij/).

2.9 Statistical analysis

The statistical analysis of the data was performed by the STATGRAPHICS Plus version 5.1 software package (Statistical Graphics Corporation, 2001, Rockville, MD, USA). Analysis of variance (ANOVA), T-test or χ_2-test were conducted to assess the level of significance of the observed responses. Multiple or pairwise comparisons among mutagen doses vs. control or between various experimental points were carried out with the Dunnett's test or χ_2-test. Regression analysis

was also performed to identify the existence of a linear correlation between MN frequencies and treatment dose in the temporal kinetic experiments.

3. Results

3.1 Temporal kinetic analysis

To elucidate the optimal time required for the manifestation of a bystander response in human peripheral lymphocytes under our experimental conditions, we examined the MN frequencies in MMCtreated RC performing the medium transfer at four different times, assuming that the effect was present and there was no mutagen residual in the transferred medium. As expected, the cytogenetic analysis performed on DC (donor cultures) revealed the presence of direct damage induced by MMC. At MMC 6 μM we observed a 6-fold increase (29.75±4.97) in the spontaneous MN rate (5.00±0.49), while, compared to control cultures, the doses of 12 μM and 15 μM caused a marked and significant ($p < 0.05$) cytotoxic effect assessed as a decrease in the cell proliferation index (CPI) up to 32.17% and 38.34% of the control value, respectively (data not shown). ANOVA analysis showed that MN frequencies obtained in RC (Fig. 2) were significantly dependent both on medium transfer time and treatment doses ($p < 0.01$). Dunnett's test showed that at time T_{transf} = 1 h MN frequencies did not differ from the control at any dose, while at time T_{transf} = 1.5 h, MN frequencies were significantly higher than untreated cultures (8.75±0.85) for all MMC concentrations: 6 μM, 15.75±1.25, $p < 0.05$; 12 μM, 20.25±1.03, $p < 0.01$; 15 μM, 28.50±2.22, $p < 0.01$). At T_{transf} = 2 h and 2.5 h, statistical significance was maintained for 12 μM and 15 μM ($p < 0.05$). Regression analysis highlighted a strong association between the two variables with the exception of T_{transf} = 1 h. As the best dose-effect relationship was obtained at T_{transf} = 1.5 h (T_{transf} = 1.1794 × MMC + 8.7076, $R^2 = 0.94125$) (data not shown), this was considered the most suitable time for detecting a bystander response in subsequent experiments with VCR.

3.2 Induction of bystander effect by CM transfer

Table 1 reports the MN frequencies induced by the two mutagens in both DC and WDC (washing

donor cultures) that allow us to verify the effectiveness of the treatment. DC receiving 6 μM of MMC showed an increase in MN frequency (46.00±6.01) with respect to untreated cultures (5.75±0.88), while a strong decrease in CPI was observed at MMC 12 μM (reduction of ~76%) and 15 μM (reduction of ~90%) that prevented us from scoring MN frequencies correctly. In the case of the spindle poison VCR, the 2 h treatment of DC produced a clear dose-dependent increase of MN frequencies ranging from 32.75±0.53 (0.2 μM) up to a maximum of 86.5±6.36 (1.6 μM), which is 31.5-fold the value of untreated cultures (2.75±0.88). Elevated MN levels or consistent cytostatic effects were also observed in WDC treated with both mutagens. Fig. 3 displays the comparison between MN frequencies of RC (recipient cultures) and WCR (washing recipient cultures) of the mutagens that is relevant for identification of the bystander effect. In the case of the reference mutagen MMC, WRC showed MN levels not significantly different from those of untreated cultures at each dose tested. By contrast , we observed a different trend in RC: cultures receiving CM harvested from DC 6 μM, 12 μM or 15 μM showed approximately a 3-fold (14.5±1.41), 4.5-fold (22±2.12) or 6-fold (28.25±5.13) increase of the spontaneous frequency (4.75±1.59), respectively (Fig. 3A). Also for VCR, MN frequencies of WRC did not differ significantly from the mean of the untreated cultures. In RC, with the exception of 0.2 μM, we found a dose-dependent increase in the MN rate (Fig. 3B) with respect to the untreated cultures (2.75±0.88) suggesting the induction of a bystander response (0.4 μM, 27.25±2.30; 0.8 μM, 46.25±1.94; 1.6 μM, 98.25±7.25).

3.3 Induction of bystander effect by the cell-mixing approach

In order to confirm the results obtained with MMC and VCR in medium transfer experiments, we used a cell-mixing approach, in which treated and untreated cultures representing two different cell populations were mixed together and then analysed for MN frequencies. MN scoring was performed as shown in Fig. 4A, distinguishing between donor and recipient cells thanks to the signal of the Y chromosome probe (its presence indicates that the cells belong to RC). The results of cytogenetic analysis carried out on cell-mixed cultures treated with a representative dose of MMC (12 μM) or VCR (0.8 μM) is shown in Fig. 4B. MN of MMC co-cultures increased significantly (*p*

< 0.01) to 43.25±2.39 in recipient cells with respect to 10.00±0.91 of untreated cultures. The induction of bystander effect was also present in VCR co-cultures where we detected a significantly higher ($p < 0.01$) MN frequency of 135.50±3.30 in recipient cells as compared to that of the control cultures (11.00±1.58).

3.4 Fluorescence analysis of MN induced by the conditioned media from MMC and VCR

For insight into the possible mechanism generating the genotoxic damage (*i.e.* MN formation) in the chemical-induced bystander effect, we compared the type of micronuclei induced directly by the mutagens in DC and those induced in RC by the bystander response (Fig. 5). MN are formed in anaphase from a lagging acentric fragment (DNA breakage) or a whole chromosome (chromosome missegregation event). We therefore performed a fluorescent in situ hybridization with a pancentromeric DNA probe recognizing the centromere of all human chromosomes to discriminate between MN containing a chromosome fragment (C- MN) or a whole chromosome (C+ MN) and thus, between MN generated by clastogenic or aneuploidogenic mechanisms. While control cultures showed almost equal proportions of C+ and C- MN (data not shown), in MMC 12 μM treated cultures and in the corresponding RC, only a minor fraction of MN contained the hybridization signals. This indicates the prevalence of a clastogenic damage which occurred to the same extent in both DC and RC (90% and 82% of C- MN, respectively). MN induced in DC and RC by VCR 0.8 μM were prevalently of the centromere-positive type (DC: 82% of C+ MN and 18% of C- MN, RC: 66% of C+MN and 34 % of CMN). However, the ratio of C+ MN to C- MN between DC and RC was statistically different, due to an increase in the CM-induced C- MN.

3.5 Involvement of ROS in chemical-induced bystander effect

To better elucidate the origin of MN observed in RC, we tested the hypothesis of the presence of ROS in the CM, possibly implicated in the response. DMSO or curcumin (CUR) pre-treated RC were exposed to CM from MMC 12 μM (Fig. 6A) and VCR 0.8 μM (Fig. 6B) and then analysed for changes in MN frequencies, after checking the absence of genotoxic or cytotoxic damage linked to the direct action of the two scavengers (data not shown). In particular, MMC 12 μM RC pre-treated

with DMSO 10 μM did not show a decrease in MN frequencies in comparison to the respective control (*i.e.* MMC 12 μM RC not exposed to the scavenger) (31.50±4.27 vs. 30.00±5.44, respectively). Conversely, MN levels of RC incubated with the VCR 0.8 μM CM decreased significantly ($p < 0.01$) when pre-treated with DMSO 10 μM (25.75±3.11) as compared to the respective non pre-treated RC (56.25±5.44). When MMC 12 μM RC were pre-treated with CUR 10 μM or CUR 100 μM, we did not detect any difference with the corresponding non pre-treated cultures. On the contrary, CUR pre-treated cultures incubated with VCR 0.8 μM CM exhibited a dose-dependent decrease in MN frequencies (45.00±2.58 at 10 μM, $p < 0.05$ and 33.75±3.87 at 100 μM, $p < 0.01$).

3.6 Western blot analysis of proteins from the conditioned medium

The involvement of soluble mediators was investigated by analysing the protein content of conditioned medium produced by MMC 12 μM and VCR 0.8 μM DC after 1.5 h. On the basis of preliminary results obtained with SDS-page, which allowed us to detect the presence and the molecular weight of the proteins secreted in conditioned medium samples, we analysed the cytokines IL-32 and TGF-β by western blot. In MMC conditioned medium, while IL-32 did not vary, TGF-β level was significantly decreased as compared to that from untreated cultures (0.86-fold less than control, $p < 0.01$). On the other hand, conditioned medium from VCR-treated cultures showed significantly increased levels of both IL-32 and TGF-β (1.47-fold, $p < 0.05$ and 1.34-fold, $p < 0.05$) (see Fig. 7).

4. Discussion

The present work aimed to investigate the ability of a spindle poison such as VCR to induce bystander effect in stimulated human peripheral blood lymphocytes. Peripheral T-lymphocytes play a very important role in the immune defences, modulating immune responses to pathogens and tumours. These cells are commonly used also in cytogenetic studies for biological monitoring of human populations, as they can reach every part of the body and percept and record the genotoxic

insults [28]. Peripheral lymphocytes exposed to medium harvested from X-rays irradiated, bleomycin or neocarzinostatin treated lymphocytes, manifested loss in cell viability, increased induction of apoptosis, telomere shortening, increase in ROS levels and MN induction [23, 33]. Our results not only confirm these findings but also provide fresh information, as they indicate that compounds with distinct mechanisms of action (i.e. clastogenic or aneugenic) can induce bystander effects with a dosedependent trend. In fact, in medium transfer experiments, we detected a clear response in RC, not attributable to the presence of mutagen residues, for both MMC and VCR. The fact that also aneugens are able to induce a bystander response offers an important novelty in the field of chemical-induced bystander effect. Several studies in the past have demonstrated that non-targeted effects of ionizing radiation can also be triggered by cytoplasm irradiation, implying that the nucleus is not the only target [34,35]. Our findings show therefore that also for chemical mutagens the induction of bystander effect can be triggered by a DNA non-damaging agent such as VCR. The results of cell mixing experiments, where we used two donors, the response to of which chemical mutagen treatment was repeatedly assessed at our lab and deemed to be absolutely comparable, confirmed the chemical-induced bystander response of peripheral lymphocytes, with an observed effect exceeding twice that obtained in CM transfer experiments. In fact, short half-lived mediators (i.e. ROS) and/or molecules released by DC over longer times than those we used, might be lost. In support of this, we showed that the transfer of CM from MMC-treated cultures after the optimal time (1.5 h) was related to a decrease of the bystander response. This is probably attributable to reduced secretion, natural decay or partial inactivation by serum components of the bystander mediators, as well as to protective mechanisms triggered by DC. The continued exposure to the complete pattern of molecules released in cell mixing experiments suggests a cumulative effect of these effectors/mediators. In agreement with our findings, conditioned medium from actinomycin-D treated cultures seem to release bystander signaling molecules following a precise temporal kinetics whose effects can be cumulative in CH V79 cells [22]. However, as an inter-

variability in the bystander response elicited by ionizing radiation has been demonstrated in lymphocytes of healthy controls [36], the possibility of an intrinsic high sensitivity of the recipient donor cannot be completely ruled out.

In our study, damage observed in both DC and RC was of the same type for each mutagen: increased frequency of MN in binucleated and in mononucleated cells for the clastogenic agent MMC and the spindle poison VCR, respectively. This may be due to the production by DC of signalling molecules specific for the type of damage (*i.e.* chromosome breakage or missegregation) which is then reproduced in the corresponding RC. Many studies have shown ROS as a fundamental component of the bystander signalling [22,33]. Here we showed that the MMC-induced bystander effect was not affected by the presence of scavengers like DMSO or CUR, whereas VCR bystander cells showed a progressive decrease in MN frequencies when pre-treated with these antioxidants. This different response could be at least partially explained by a free radical production dependent on the type of genotoxic insult received, as supported by the results obtained with fluorescence analysis of MN content. VCR induced an increase of C- MN in recipient cells as compared to donor cells, suggesting that cellular molecules with clastogenic activity were present, while this does not occur in RC treated with MMC. Cytokines are known to be involved in bystander effect via the activation of several signaling pathways in the cells. Although the role of IL-32, a pro-inflammatory cytokine produced in human peripheral lymphocytes after mitogen stimulation [37], is still under investigation, there is evidence that it is able to induce other cytokines such as TNF-α and IL-8, and to activate the signal pathways of NF-κB and p38 MAPK [38]. However, we observed an increased level of IL-32 only in the CM from VCR treated cultures. The dysfunction of the spindle apparatus caused by VCR forced damaged cells to enter an aberrant division via mitotic exit, the main mechanism responsible for MN formation in mononucleated cells, while the induction of MN in binucleated cells by spindle poisons leads preferentially to cell death [39]. Given this, the increased IL-32 production could represent a survival signal of VCR-treated donor cells in restoring proliferation of recipient cells through the anti-apoptotic NF-κB and

p38 MAPK cascade. TGF-β is implied in cellular differentiation, proliferation, apoptosis and in radiation-induced bystander response. In mammalian cells, the TGF-β target genes are involved in the regulation of cell growth or in the alteration of intracellular levels of ROS and RNS [40,41]. The upregulation of ROS in bystander cells has been linked to the redox activation of TGF-β1 cytokine, which is able to increase the intracellular production of H_2O_2 and ROO• [42]. Therefore, the observed increased TGF-β level in the CM from VCR DC highlights the involvement of ROS (or other free radicals) in determining the bystander effect. In this view, also CM from actinomycin-treated V79 fibroblasts induced apoptosis in bystander cells via ROS generation [22]. On the other hand, a study conducted on peripheral blood lymphocytes from patients with multiple sclerosis showed that decreased levels of TGF-β were associated with increased synthesis of pro-inflammatory cytokines such as IL-2, IFN-7 and TNF [43]. Thus, an increase in other pro-inflammatory cytokines, as well as the insensitiveness to ROS scavengers we detected, may be related to the reduced concentration of TGF-β observed in the case of MMC. In this view, other authors showed that the bystander effect induced by MMC in human lymphoblastoid cells involved the MAPK cell signalling pathway [21].

In conclusion, the results of this study demonstrate that VCR, a classical spindle poison, can induce the bystander effect in human peripheral lymphocytes, confirming, as for ionizing radiation, that the DNA is not the unique target. Notably, recipient cells manifest the damage in response to a precise signaling of treated cells, which is dependent on the type of mutagen. Cell exposure to a damaging agent inevitably alters the profile of signalling molecules (identified here as changes in ROS and cytokines levels) that is able to activate a series of important modifications in neighbouring cells. However, we should also take into account the possibility of a protective response *per se* where bystander cells are activated to counteract the adverse conditions they are exposed to, as already demonstrated in the case of ionizing radiation exposure [44-46].

Acknowledgements

The authors express their appreciation to Alessandro Corti for his assistance in western blot analysis and Andrea Oliveri for his assistance in revision of the English language.

Conflict of interest statement

None declared.

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Legend to figures

Fig. 1 Schematic representation of the protocol adopted in medium transfer experiments with the mutagens mitomycin c (MMC) and vincristine (VCR). The *donor cultures* (DC) and *recipient cultures* (RC) were incubated at 37°C (0 h) up to 72 h. At 24 h DC were treated for 1 h (MMC) and 2 h (VCR). After the treatment, the mutagen was washed away and the *conditioned medium* (CM) was transferred to the corresponding RC at $T_{transf} > 0$ h (1.0, 1.5, 2.0 and 2.5 for the temporal kinetic analysis, and 1.5 h for the bystander experiments). RC were then analysed for the induction of a

bystander response. Cyt B was added to all cultures at 44 h. After cell harvesting (72 h), slides were set up for micronuclei analysis. A parallel series of cultures called respectively *washing donor cultures* (WDC) and *washing recipient cultures* (WRC) were also set up, which underwent the same procedures described for DC and RC with the difference that WRC received the medium from WDC immediately after the washings (T_{transf} = 0 h).

Fig. 2 Kinetic of bystander effect induction. MN frequencies in MMC-treated recipient cultures as a function of the medium transfer time (T_{transf}). Points are the mean ± SE of two independent experiments. T_{transf} = 1.5 h represents the time at which bystander effect occurred at all the tested doses. At this time we also observed the highest MN induction.

Fig. 3 Bystander effect induction by mutagens. MN frequencies obtained in WRC and RC for different concentrations of MMC (A), VCR (B). Bars are the mean ± SE of at least two independent experiments. Asterisks denote a significant difference (* $p < 0.05$, ** $p < 0.01$) vs. the respective untreated cultures.

Fig. 4 Co-culture experiments. (A) Photos show the mixed cultures of untreated recipient cells (R) showing the signal (green-yellow fluorescent spot in the red PI counterstained nucleus) of the probe for the Y chromosome (F) close to treated donor cells (D) not showing the FISH signal. Panel a) or b) show a MN in a binucleated or mononucleated cell, respectively (600x final magnification). (B) The graph shows MN frequencies in untreated (0) and MMC 12 μM binucleated recipient cells or untreated (0) and VCR 0.8 μM mononucleated recipient cells. Bars are the mean ± SE of at least two independent experiments. Asterisks denote a significant difference (* $p < 0.05$, ** $p < 0.01$, T-test) vs. the respective untreated cultures.

Fig. 5 Fluorescence analysis of MN using a DNA pancentromeric probe. Type of MN (C- MN and C+ MN) induced directly by the mutagen in DC and via bystander response in RC. (A) Panels show photos of binucleated (a and b) and mononucleated (panels c and d) cells with MN showing or not the green-yellow fluorescent spot (this indicates the presence of a centromere) in the red PI counterstained MN, respectively (600x final magnification). (B) Comparison between the values

obtained for MMC 12 μM or VCR 0.8 μM in donor and recipient binucleated cells (on the left) or mononucleated cells (on the right). Asterisk denotes a significant difference (* $p < 0.05$, χ^2 test: RC vs. DC). Data are representative of at least two independent experiments.

Fig. 6 Involvement of ROS in the bystander response. Effect of pre-treatment with DMSO or curcumin (CUR, 10 and 100 μM) of RC on MN frequencies for (A) MMC 12 μM and (B) VCR 0.8 μM. Bars are the mean ± SE of at least two independent experiments; asterisks denote a significant difference (* $p < 0.05$, ** $p < 0.01$, χ^2 test) between untreated RC vs. the respective pre-treated RC.

Fig. 7 Western blot analysis of the conditioned medium. (A) Expression of IL-32 and TGF-β secreted by untreated (CTRL), MMC 12 μM and VCR 0.8 μM treated donor cultures. We applied the reversible Ponceau staining followed by densitometric analysis to check equal loading of samples. Relative density of IL-32 (B) and TGF-β (C) obtained from conditioned media produced by MMC 12 μM, VCR 0.8 μM or by control cultures (0 μM, CTRL). Asterisks denote a significant difference (* $p < 0.05$, ** $p < 0.01$, T-test) vs. the respective untreated cultures. Data are representative of two independent experiments.

Table 1. MN frequencies in donor cultures (DC) and washing donor cultures (WDC) treated with MMC or VCR. Values are the mean±S.E. of two independent experiments

MMC			VCR		
Dose (μM)	Culture type		Dose (μM)	Culture type	
	DC	WDC		DC	WDC
0	5.75±0.88	7.00±1.41	0.0	2.75±0.88	3.25± 0.88
6	46.00±6.01	63.25±6.89	0.2	32.75±0.53	37.25± 3.61
12	n.a.[a]	n.a.[c]	0.4	46.25±1.24	40.75± 2.65
15	n.a.[b]	n.a.[d]	0.8	69.25±0.53	71.25± 2.30
			1.6	86.50±6.36	104.50±12.37

n.a., not analyzed: [a]CPI value = 1.22±0.02; [b]CPI value = 1.07±0.02; [c]CPI value = 1.15±0.03; [d]CPI value = 1.04±0.04.

Figure 1

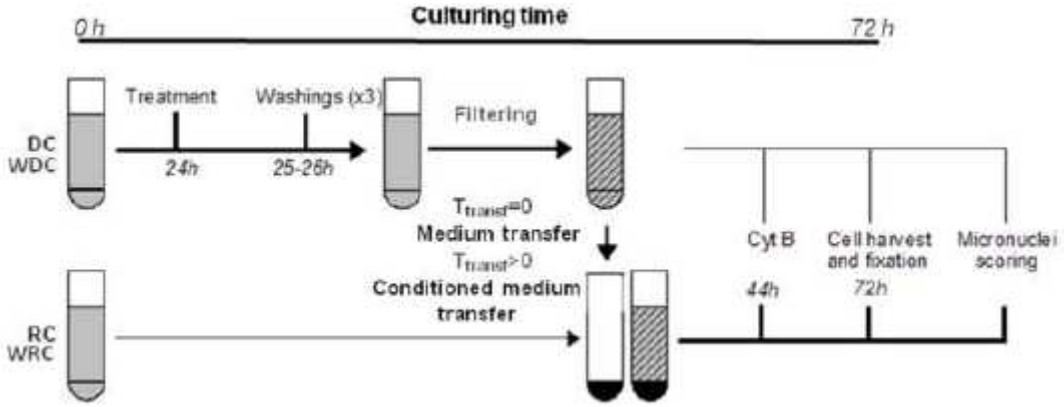

Culturing time
0 h
72 h
Treatment
Washings (x3)
Filtering
24h
25-26h
DC
WDC
T_{transf}=0
Medium transfer
T_{transf}>0
Conditioned medium
transfer
RC
WRC
Cyt B
Cell harvest
and fixation
Micronuclei
scoring
44h
72h

Figure 2

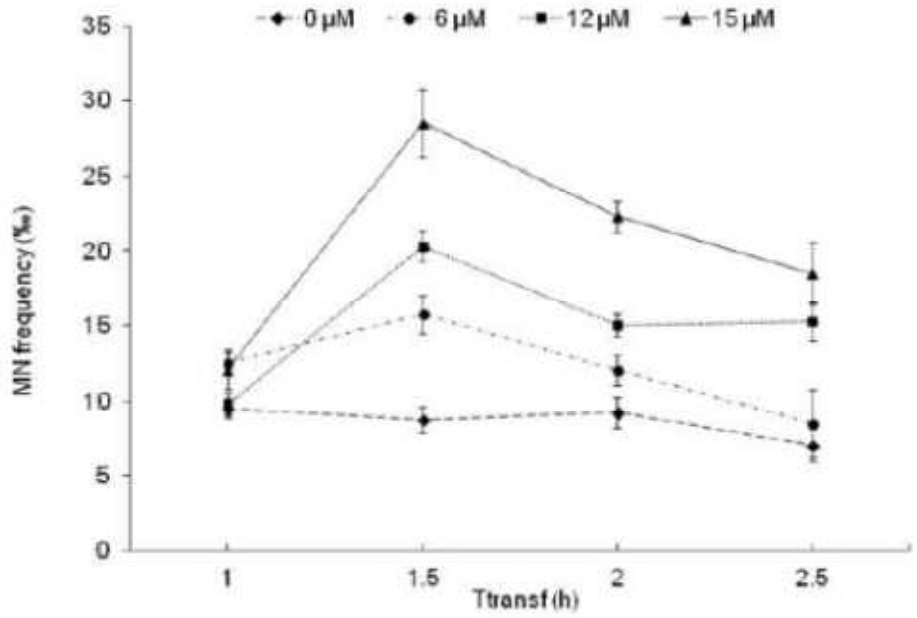

0 µM
6 µM
12 µM
15 µM
MN frequency (‰)
35
30
25
20
15
10
5
0
1
1.5
2
2.5
Ttransf (h)

Figure 3

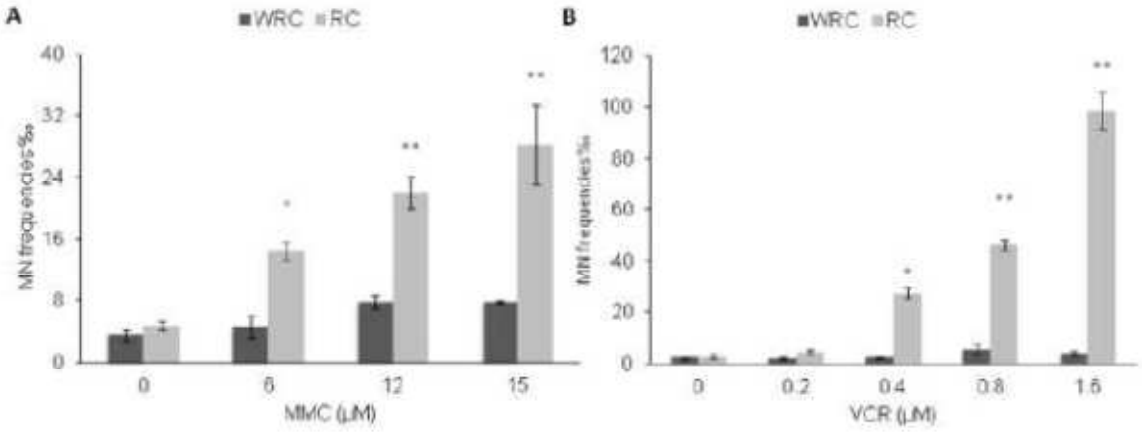

A
WRC
RC
MN frequencies‰
40
32
24
16
8
0
0
6
12
15
MMC (μM)
B
WRC
RC
MN frequencies‰
120
100
80
60
40
20
0
0
0.2
0.4
0.8
1.6
VCR (μM)

Figure 4

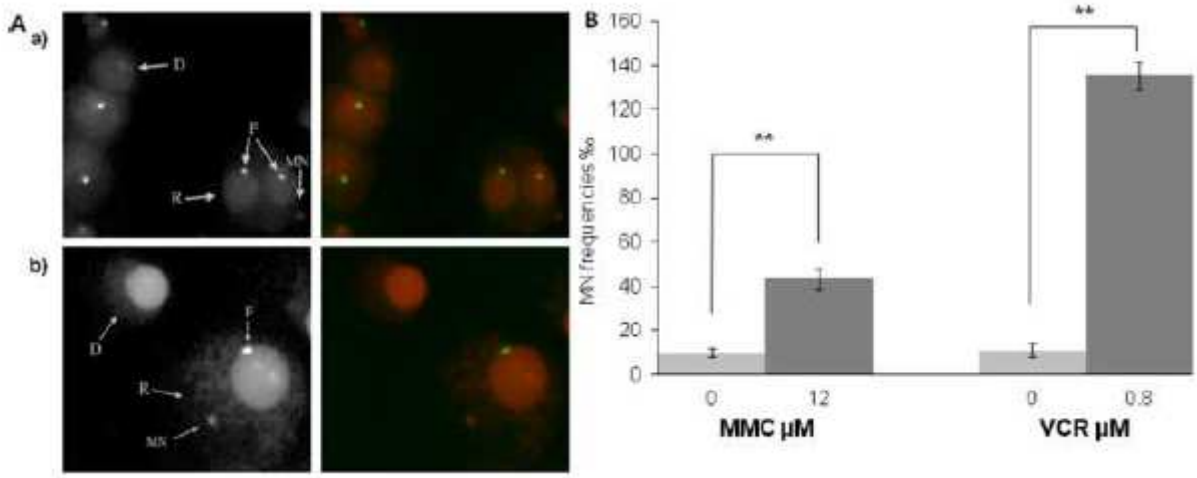

A
a)
D
F
MN
R
b)
D
F
R
MN
B
MN frequencies ‰
160
140
120
100
80
60
40
20
0
**
**
0
12
0
0.8
MMC µM
VCR µM

Figure 5

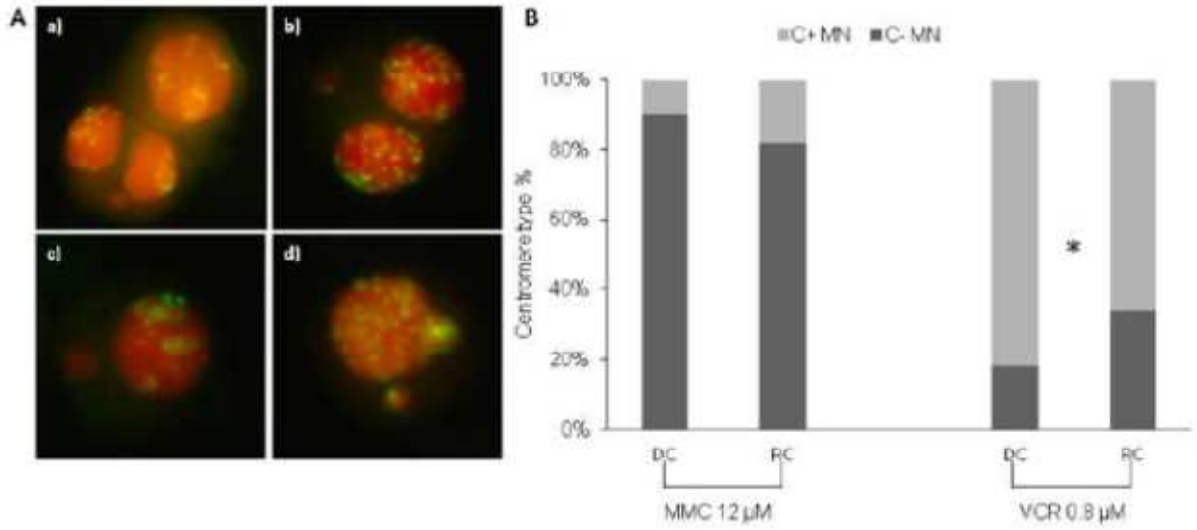

A
a)
b)
c)
d)
B
C+ MN
C- MN
100%
80%
60%
40%
20%
0%
Centromere type %
*
DC
RC
DC
RC
MMC 12 µM
VCR 0.8 µM

Figure 6

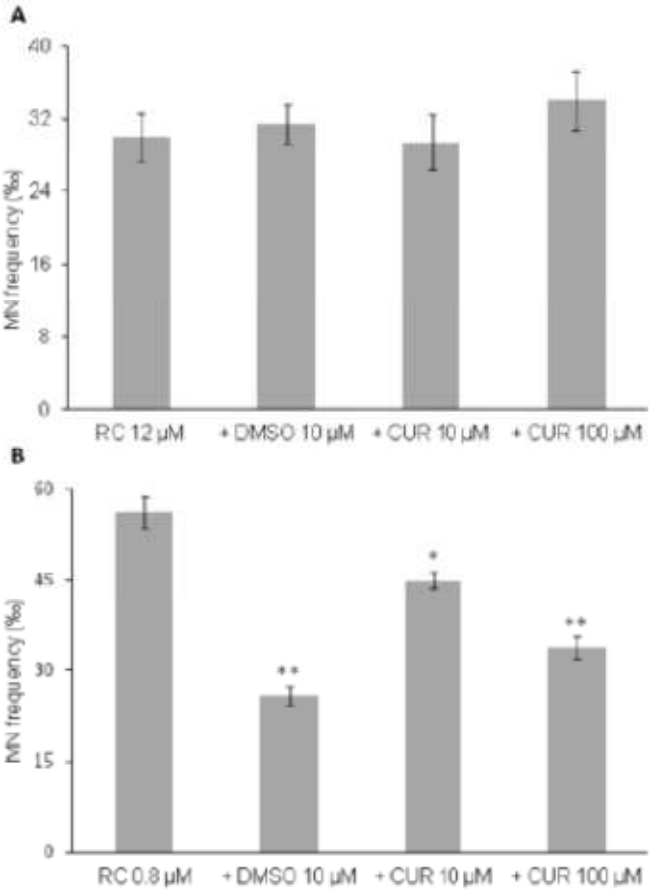
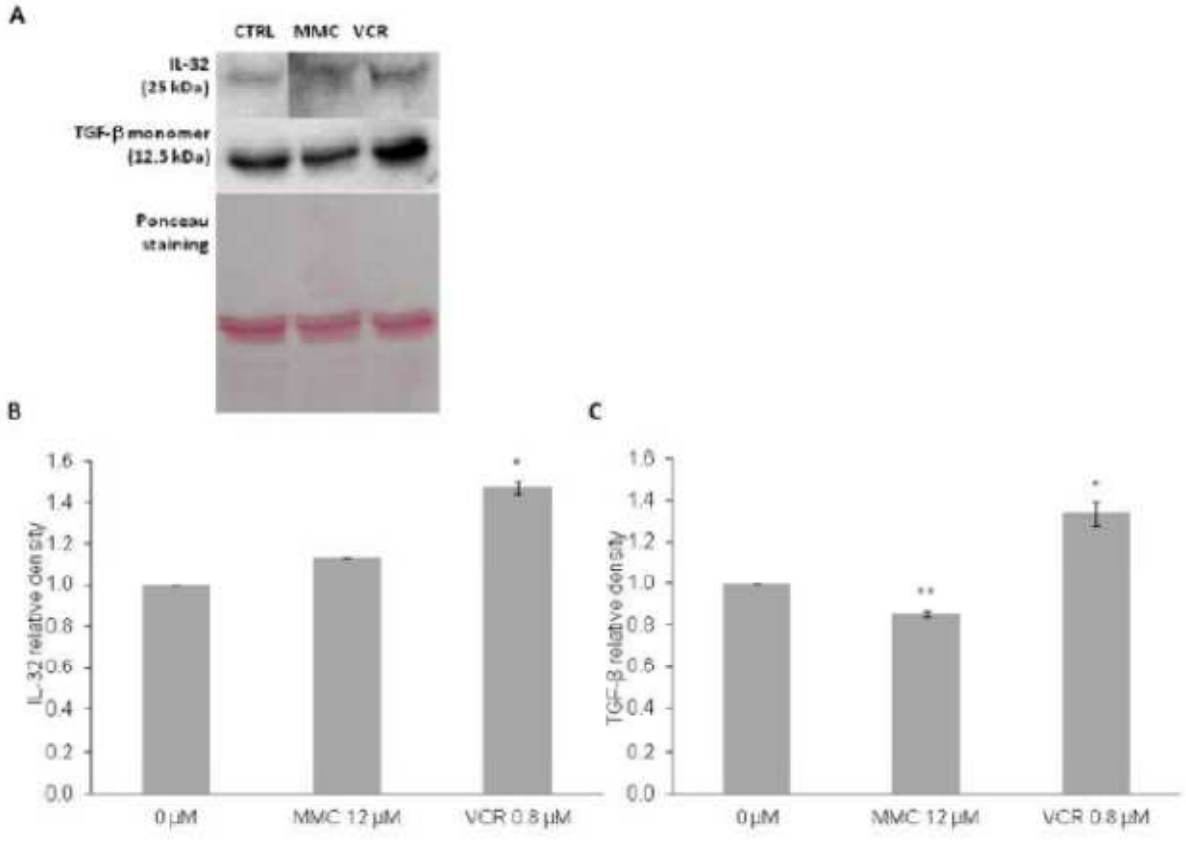

A
40
32
24
16
8
0
MN frequency (‰)
RC 12 µM
+ DMSO 10 µM
+ CUR 10 µM
+ CUR 100 µM
B
60
45
30
15
0
MN frequency (‰)
**
*
**
RC 0.8 µM
+ DMSO 10 µM
+ CUR 10 µM
+ CUR 100 µM

Figure 7


A
CTRL
MMC
VCR
IL-32
(25 kDa)
TGF-β monomer
(12.5 kDa)
Ponceau
staining
B
IL-32 relative density
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
0 µM
MMC 12 µM
VCR 0.8 µM
C
TGF-β relative density
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
0 µM
MMC 12 µM
VCR 0.8 µM