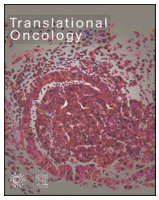
Translational Oncology 46 (2024) 102030

Contents lists available at ScienceDirect

Translational Oncology

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



IL-6 knockdown in a model of the human bone marrow, abrogates DNA damage induction in bystander cells post-chemotherapy induced cytokine release syndrome

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ARTICLE INFO

Keywords:
Cytokine storm
Cytokine release syndrome
Genetic toxicology
Bone marrow
Bystander effect

ABSTRACT

Following infection or exposure to therapeutic agents, an aggressive immune response may result, termed cytokine storm (CS) or cytokine release syndrome. Here the innate immune system becomes uncontrolled, leading to serious consequences including possible death. Patients surviving CS are at greater risk for *de novo* tumorigenesis, but it is unclear if any specific cytokines are directly responsible for this outcome. *De novo* tumorigenesis has been observed in donated cells exposed to CS following haematopoietic stem cell transplant (HSCT).

Modelling HSCT, we firstly demonstrated the release of CS levels from the HS-5 human bone marrow stromal cell line, post-exposure to chemotherapy. We then exposed the TK6 lymphoblast cell line to healthy and storm doses of IL-6 and measured increased genotoxicity via the micronucleus assay. During HSCT, haematopoietic cells are exposed to a complex mix of cytokines, so to determine if IL-6 was integral in a chemotherapy-induced bystander effect, we attempted to inhibit IL-6 from HS-5 cells using resatorvid or siRNA, treated with chlorambucil or mitoxantrone, and then co-cultured with bystander TK6 cells. Whilst resatorvid did not reduce IL-6 and did not reduce micronuclei in the bystander TK6 cells, siRNA inhibition reduced IL-6 to healthy *in vivo* levels, and micronuclei aligned with untreated controls.

Our data suggests that exposure to high IL-6 (in the absence of inflammatory cells) has potential to induce genetic damage and may contribute to *de novo* tumorigenesis post-CS. We suggest that for individuals with a pro-inflammatory profile, anti-IL-6 therapy may be an appropriate intervention to prevent complications post-CS.

Introduction

Cytokine storm (CS), is a serious excessive immune system response associated with viral exposure, sepsis and septic shock. CS can also result from a range of diverse conditions such as familial hemophagocytic lymphohistiocytosis, macrophage activation syndrome and cytokine release syndrome (CRS). CS was first described by Ferrara [1] to describe the extreme inflammation observed during graft-versus-host disease following haematopoietic stem cell transplantation (HSCT), whereas Chatenoud [2] introduced the term "cytokine release syndrome" to describe massive cytokine release in response to therapy. CRS may occur days to weeks after therapy, whereas immediate onset cytokine release is a "cytokine storm". Within this manuscript, we are addressing the CRS that occurs post-chemotherapy exposure, and the associated complication of *de novo* tumorigenesis in donated cells following HSCT.

Intriguingly, these CS/CRS responses may be directly or indirectly linked to both the induction of *de novo* cancer [3–8], as well as both pro- and anti-tumorigenic responses to therapy [9,10]. Evidence in the literature suggests that individuals surviving CS are more at risk of developing *de novo* cancer, than those who haven't experienced CS. Literature supports increased cancer risk following sepsis [3,4], COVID [5,6] and chemotherapy [7,8], with recent evidence supporting an integral role for IL-6 secretion in the CS following COVID infection, suggesting a need for anti-IL-6 therapeutics [11]. These data from sepsis and COVID suggest that cytokines have the capacity to contribute to the induction of *de novo* carcinogenesis, in the absence of genotoxic agents, however whether their role is direct or indirect remains to be elucidated. These observations advance previous knowledge, where the potential mutagenicity of cytokines was mainly attributed to generation of

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https://doi.org/10.1016/j.tranon.2024.102030
Received 3 February 2024; Received in revised form 8 May 2024; Accepted 5 June 2024
Available online 12 June 2024

reactive oxygen species or interaction with enzymes such as DNA topoisomerase II; in his paper, Lazutka [12] highlighted a need to confirm that measured genotoxicity is induced by the cytokine alone and not by other factors. This is a valid point, as various known and unknown factors related to inflammation have been documented as causing mutagenicity [13,14]. This indirect genotoxicity was highlighted by Åkerlund [14], in that conditioned medium from nanoparticle-exposed macrophages induced genotoxicity in bronchial cells within *in vitro* culture, offering parallels with genotoxicity from irradiation-induced bystander effect [15]. The release of cytokines following irradiation are well documented as playing a role in bystander effect.

Our research group has been interested in a phenomenon post-HSCT called "donor cell leukaemia" (DCL), where patients appear to relapse, but are actually developing a "new" leukaemia in the recently donated stem cells. Invariably the donor remains healthy. Suarez-Gonzalez [16] reviewed DCL cases and noted that DCL commonly presents as acute myeloid leukaemia and myelodysplasia. With evidence that the bone marrow (BM) microenvironment remains of patient origin following high dose chemotherapy [17] for HSCT, we speculated that chemotherapy-treated BM released a plethora of cytokines, with capacity to induce genotoxicity in the healthy transplanted cells, and promotion of myeloid differentiation potentially leading to *de novo* leukaemia in donor cells; a chemotherapy-induced bystander effect. However, whether the mechanism of genotoxicity in these donated stem cells originated from the cytokines themselves, remained to be determined.

In a cell line model of the human BM [18] we previously confirmed increased cytokine release following chemotherapy and demonstrated that direct exposure to CS concentrations of selected myeloid cytokines (IL-6, TNFα, TGF-β1, GM-CSF and G-CSF) could induce genotoxicity relative to untreated cells or cells exposed to healthy cytokine levels. Furthermore, when these cytokines were paired, the genotoxicity statistically increased above untreated, and in some cases exceeded our positive genotoxic control, mitomycin C (MMC) [18].

In vivo, cells would be exposed to a complex mixture of cytokines alongside various other released factors, during a CRS response to chemotherapy. As paired cytokines in our *in vitro* assays could exacerbate genotoxicity, we wished to determine if such a complex mixture was more potent, and if any of our five candidate cytokines, played an important role in the generation of genotoxicity.

Here we follow our previous publication [18] by exploring the role of IL-6 secretion following exposure to the alkylating agent chlorambucil, and the topoisomerase II inhibitor mitoxantrone. We show that siRNA reduction of IL-6 to healthy levels, reduced genotoxicity in bystander cells supporting the observations and recommendations of Turnquist [11] of anti-IL-6 therapy for patients suffering from CS/CRS.

Materials and methods

Cell lines and chemotherapeutic reagents

Reagents quoted in this research were sourced from Sigma-Aldrich (UK) except where otherwise stated. All drugs, positive controls and cell lines were as described in Asurappulige [18]. Briefly, chlorambucil (CHL) was used at 4 μM and mitoxantrone (MTX) was used at 1.12 μM (500 ng/ml), considered clinically relevant doses [19–21]. Mitomycin C (MMC; 10,000 pg/ml, 30 nmol in dimethylsulphoxide [0.01 %]; ThermoFisher Scientific, UK) was used as a positive genotoxic control in the micronucleus (MN) assay.

Both cell lines were human-derived; TK6 cells (13051501; ECACC, UK) represented the donor stem cells, as they are accurate in predicting genotoxicity [22]. HS-5 cells (CRL-11882; ATCC; from LGC, UK), represented the BM microenvironment. Cells were cultured as described in Asurappulige [18]. For consistency within assays, cells within passages 3–9 (TK6) and 6–10 (HS-5) were selected.

Monoculture cell treatments

HS-5 cells were determined for IL-6 secretion when untreated or following chemotherapy exposure. HS-5 were exposed to CHL or MTX for 1 h, then washed free of drug. After 48 h, conditioned medium was collected over a 24 h period and measured for IL-6 secretion using an in-house ELISA assay. TK6 untreated levels were also measured to determine any contribution of IL-6 when in co-culture.

TK6 cells were exposed to recombinant IL-6 at 'healthy' doses (50–1000 pg/ml) [23,24] or CS/CRS levels (2000–4000 pg/ml) [25,26] as described in Asurappulige [18] and collected to assess viability and genotoxicity (MN).

Co-culture bystander model

Cells were combined in a co-culture bystander model, to assess viability and genotoxicity in TK6 bystander cells by chemotherapy-exposed HS-5, with and without IL-6 inhibition through resatorvid and siRNA approaches.

HS-5 cells were seeded at 7×10^4 cells/well into a 12-well plate with 1 ml of RPMI 1640 supplemented medium. Following 24 h incubation, HS-5 cells were treated with CHL and MTX for 1 h. Each well was washed free of excess drug using PBS and replaced with fresh medium. Forty-eight hours after drug treatments, culture media were replaced in all wells. Subsequently, 0.4 μm polyethylene terephthalate (PET) hanging culture inserts (Merck Millipore, UK) containing 3×10^5 TK6 cells in 1 ml medium were transferred into each well using sterile forceps. After 24 h incubation, aliquots of 20,000 bystander TK6 cells were harvested for the MN assay and culture media were collected for IL-6 ELISA analysis.

The bystander assay was repeated incorporating optimised IL-6 siRNA or resatorvid reagents to perform the inhibition assays; siRNA reagents or resatorvid were added 24 and 2 h respectively prior to drug treatment.

ELISA measurement of IL-6

We developed an in-house IL-6 sandwich ELISA, using paired antibodies from BD Pharmingen (BD Biosciences, UK), and recombinant IL-6 from Abcam (UK), as this is considered a gold standard measurement [27]. Briefly, the capture antibody (purified rat anti-IL6; MQ2–13A5) was coated onto an ELISA plate at 3.5 μg/ml in fresh bicarbonate buffer (pH 9.6) and left for 24 h at 4 °C. Plates were washed with PBS/0.1 % Tween-20 and blocked for 1 h with PBS/1 % bovine serum albumin (BSA). Following a wash step, a standard curve was constructed across the plate using double dilution of recombinant IL-6 with the top standard at 8000 pg/ml. High- and low-quality controls were generated with recombinant IL-6, alongside negative controls of PBS/1 % BSA and culture medium. Standards and controls were performed in duplicate, whereas samples were pipetted in duplicate incorporating three biological repeats. Samples and standards were incubated at room temperature for 2 h. Plates were washed with PBS/0.1 % Tween-20, followed by PBS, then the detection antibody (biotinylated rat anti-human IL6; MQ2–39C3) was added at 1.75 μg/ml in PBS/1 % BSA and incubated for 1 h. After a further wash step, 50 μl per well of 1:1000 dilution of poly-horseradish peroxidase (Fisher Scientific, UK) in PBS/1 % BSA was added and incubated at room temperature for 30 min. The plate was washed a final time, then 100 μl substrate (100 μg/ml tetramethylbenzidine, 0.009 % H_2O_2 in phosphate/citrate buffer) was added to each well and the colour allowed to develop in the penultimate standard before adding 50 μl of 2 M H_2SO_4 to stop the reaction. Colour change relative to cytokine concentration was measured at 450 nm, with blank at 595 nm.

In vitro micronucleus assay

The MN assay was performed as described in Asurappulige [18], according to the Organisation for Economic Co-operation and Development (OECD) 487 guidelines using the 24 h treatment plus 24 h recovery period approach, described by Wilson [28]. An OECD criterion of relative population doubling (RPD) exceeding 55 % ± 5 % was applied as an indicator of cytotoxicity, for samples to be accurately scored for MN. The RPD values were measured in relation to the vehicle (PBS) control as described in Fellows [29]. Representative pictures of micronuclei are presented in Fig. 1.

Chemical inhibition of IL-6 synthesis with resatorvid

Resatorvid (TAK-242; Stratech, UK) functions as a chemical inhibitor of IL-6 and TNFα. Resatorvid dosing was optimised to preserve viability of HS-5 cells and measure IL-6 reduction over time, as to our knowledge, this has not previously been determined in HS-5 or primary mesenchymal stem cells. HS-5 cells were initially seeded in a 6-well plate at a density of 1.4×10^5 cells/well. Following cell adherence (24 h), they were treated with resatorvid (1, 3, 5, 7, 9, and 11 μM) and incubated at 37 °C in a 5 % CO_2 incubator. After 24 h and every 24 h thereafter for 5 days, conditioned media was collected and HS-5 cells were trypsinised, counted, and re-seeded in new media. Cell counting allowed for viability determination and IL-6 secretion was measured by ELISA. Stock resatorvid solutions were stored at −80 °C and working solutions of 3 μM were prepared following the optimisation.

To confirm inhibition of IL-6 secretion from HS-5 cells, they were seeded at 7×10^4 cells in a 12-well plate and allowed to adhere overnight. Cells were treated with 3 μM resatorvid for 2 h, followed by treatment with CHL (4 μM) and MTX (1.12 μM) for 1 hour. Cells were then washed with PBS, fresh complete medium added and incubated for 24 h, after which the supernatant was collected and stored at −80 °C for ELISA. New media was added to each well and collected every 24 h for a total of 5 days (120 h) following chemotherapy treatments.

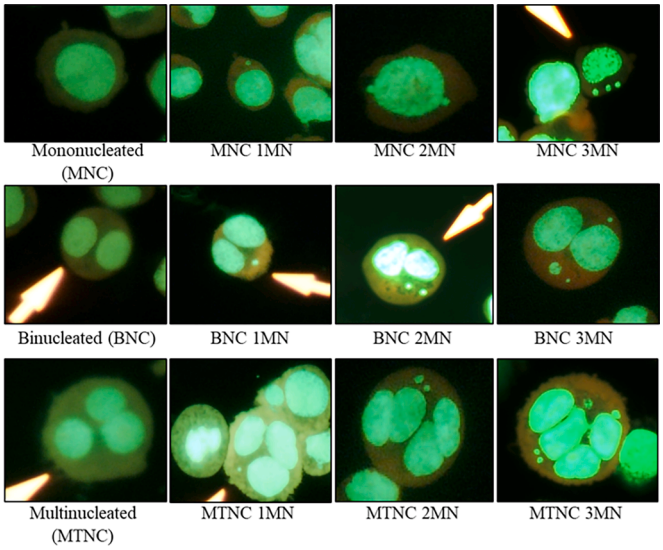

Fig. 1. Visual presentation of micronuclei (MN) for scoring in the micronucleus genotoxicity assay following recombinant cytokine treatments. Micronuclei are formed by nuclear envelope deposition around fragments or lagging chromosomes during mitosis as a result of nuclear damage. Genetic material (main nucleus and MN) are stained green against an orange cytoplasmic background when stained with acridine orange. Different parameters were observed during the MN scoring including, mononucleated cells (MNC), binucleated (BNC) and multinucleated cells (MTNC) with/without micronuclei (MN).

Assessment of resatorvid inhibition of HS-5 cells on viability and genotoxicity of bystander TK6

As described above, HS-5 cells were seeded, treated with 3 μM resatorvid for 2 h, then with chemotherapy for 1 h and washed with PBS before incubating with fresh co-culture medium for 48 h at 37 °C in a 5 % CO_2 incubator. The culture medium was then replaced with fresh medium, and a bystander co-culture assay was conducted for 24 h as outlined above. The collected supernatant underwent IL-6 ELISA, and TK6 cells were collected for the MN assay. The remaining TK6 cells were re-seeded into new wells with fresh culture medium to calculate their RPD.

Optimisation of siRNA knockdown of IL-6

The IL-6 knockdown siRNA kit, obtained from ThermoFisher, (UK), comprises Silencer™ Select Negative Control siRNA (N/C) (Cat 4390843), Opti-MEM™ I Reduced Serum Medium (31985062), Lipofectamine™ RNAiMAX Transfection Reagent (13778030), and IL-6 siRNA (4390824). An optimisation study was conducted according to the manufacturer's instructions for all knockdown reagents, leading to the determination of the optimal working concentration (6 pmol) for the knockdown siRNA.

As we have previously shown that peak cytokine secretion and bystander effect result at 2 and 3-days post treatment for MTX and CHL respectively [18], it was crucial to optimise siRNA knockdown and verify longevity of the effect. Thus, optimisation assays were performed over 5 days.

Briefly, one day before transfection, HS-5 cells were seeded at 3.5×10^4 cells per well (24-well plate) with 500 μl of complete medium. Transfection reagents were prepared by mixing either IL-6 siRNA or N/C siRNA at 3, 6 and 12 pmol in 50 μl Opti-MEM and mixed gently. Lipofectamine was gently mixed before use, then 1 μl was added to 50 μl Opti-MEM. The diluted siRNA/Opti-MEM and diluted lipofectamine were combined and incubated for 20 min at room temperature. Then siRNA-lipofectamine mixes were transferred to each well (100 μl per well). Plates were mixed on a rocker for 10 min to evenly distribute the reagents, then plates were placed in the culture incubator for 24 h. Wells were also prepared with HS-5 alone, with lipofectamine or Opti-MEM alone, and lipofectamine with Opti-MEM.

Following overnight incubation, culture medium was collected and replaced every 24 h for 5 days. Collected medium was stored at −80 °C for ELISA analysis of IL-6. Subsequently, 6 pmol was considered the optimised siRNA concentration to reduce IL-6 secretion and used for subsequent experiments. This concentration of siRNA was tested on CHL and MTX treated HS-5 cells. Briefly, HS-5 were seeded and allowed to adhere overnight, then transfected with 6 pmol siRNA or N/C-siRNA as described above. Twenty-four hours after transfection the cells were treated with 4 μM CHL and 1.12 μM MTX ('untreated' control was treated with PBS) for 1 h, then washed and replaced with fresh medium. Every 24 h, culture medium was collected to assess IL-6 secretion by ELISA.

To assess the role of IL-6 in the bystander assay using siRNA, HS-5 cells were seeded, allowed to adhere (24 h), subject to siRNA knockdown (24 h) then drug treatment (1 h), then fresh medium applied as described above. Forty-eight hours after drug treatment, the bystander assay was performed as described above, by adding TK6 cells in a culture insert into the well. Twenty-four hours after co-culture, the TK6 were collected for MN assay and for re-seeding to calculate the RPD, and the culture medium collected to measure IL-6 by ELISA.

Statistical analysis

All statistics and graphical illustrations were performed using GraphPad Prism software v. 8.2.1 for Windows (GraphPad Software, Inc., La Jolla, California, USA). Group comparisons were conducted

through one-way or two-way ANOVA, followed by Dunnett's, Tukey's, or Šídák's multiple comparison tests as recommended by the software. All error bars are expressed as mean ± standard deviation (SD) of three independent biological repeats unless otherwise stated. Statistical significances were performed using analysis of variance and are represented as (*) for $p \leq 0.05$, (**) for $p \leq 0.01$, (***) for $p \leq 0.001$ and (****) for $p \leq 0.0001$.

Results

HS-5 human BM stromal cells were assessed for the secretion of IL-6 at untreated baseline and following exposure to the chemotherapeutic agents CHL and MTX for 1 h. The TK6 human lymphoblast cell line was also assessed for IL-6 secretion as this would form part of our co-culture bystander model. As can be seen in Fig. 2A, HS-5 naturally secretes quite high levels of IL-6 without any stimulus, but can be induced to secrete significantly more IL-6 up to 2–3 days later, following only 1 h exposure to CHL ($p < 0.0001$) and MTX. TK6 showed barely detectable levels of IL-6 within our ELISA assay, suggesting it would contribute very little IL-6 to the co-culture. With the knowledge that HS-5 secreted levels equivalent to high CRS concentrations following drug exposure [18,25,26], we measured the ability for documented healthy and storm levels of IL-6 to directly induce genotoxic events in the TK6 cells. Fig. 2B shows MN induction following recombinant IL-6 treatment of TK6 cells in comparison with negative (PBS) and positive (MMC) controls. Whilst MMC induced a statistical increase in MN ($p < 0.05$) above the PBS control, IL-6 at healthy levels (up to 1000 pg/ml) resulted in a non-significant dose dependent increase in MN which stayed stable up to 3000 pg/ml. However, at 4000 pg/ml MN were markedly, but not statistically, increased above the PBS control. Where data adheres to historical control results [18,30] a result more than twice the negative control, infers caution of possible weak genotoxicants, suggesting doses of 4000 pg/ml and above might be cause for concern. Furthermore, our previous work [18] demonstrated genotoxicity potentiation of IL-6 when combined with other cytokines. Thus, to assess the role of IL-6 as a possible genotoxicant within the complex mix of cytokines released during CRS, we aimed to inhibit IL-6 secretion from HS-5 cells and assess the impact on bystander genotoxicity.

Attempts to optimise resartovid inhibition of IL-6 synthesis in HS-5 cells are shown in Fig. 3, demonstrating that doses of 9 μM and above significantly reduced the cell viability below the untreated control (Fig. 3A; $p < 0.01$). This lowered viability is reflected in the reduced quantity of IL-6 measured in these samples, so analysis of IL-6 at doses of >9 μM were not considered relevant (Fig. 3B). However, in comparison with untreated cells, resatorvid was unconvincing in reducing IL-6 secretion at doses between 1 and 7 μM, and in some cases appeared to slightly increase IL-6 secretion. There did appear to be some decreased secretion around days 2–3, at 3 and 5 μM doses, which are the days of interest for our CRS of HS-5 following drug treatment. Thus, as 3 μM was the lowest reduction of IL-6 at 72 h, we tested this dose on HS-5 cells, with and without chemotherapy treatment (Fig. 3C). The data showed a clear increase in IL-6 secretion over 5 days, in the presence of CHL with a peak at day 3, which aligns with previous unpublished data from our laboratory ($p < 0.05$). Whilst MTX increases IL-6 secretion, this was not as notable as for CHL. In line with our optimisation studies, there was no difference in IL-6 secretion in untreated controls with and without resatorvid, but the inhibitor markedly reduced the IL-6 secretion post-CHL treatment to produce levels close to the untreated controls (around 4000 pg/ml). This suggests that resatorvid may only be helpful in extreme IL-6 release, but may be more limited in pro-inflammatory profiles, or CRS/CS around 4000 pg/ml.

We tested the capacity for chemical inhibition of IL-6 using resatorvid, on bystander effects in a co-culture of HS-5 and TK6. HS-5 were cultured with resatorvid, and bystander was measured in the subsequently co-cultured TK6 (Fig. 4).

With the knowledge from Fig. 2, that TK6 produce little to no IL-6 secretion, Fig. 4A (without resatorvid) suggests that TK6 has the capacity to take up IL-6 from HS-5 which is secreted into the medium,

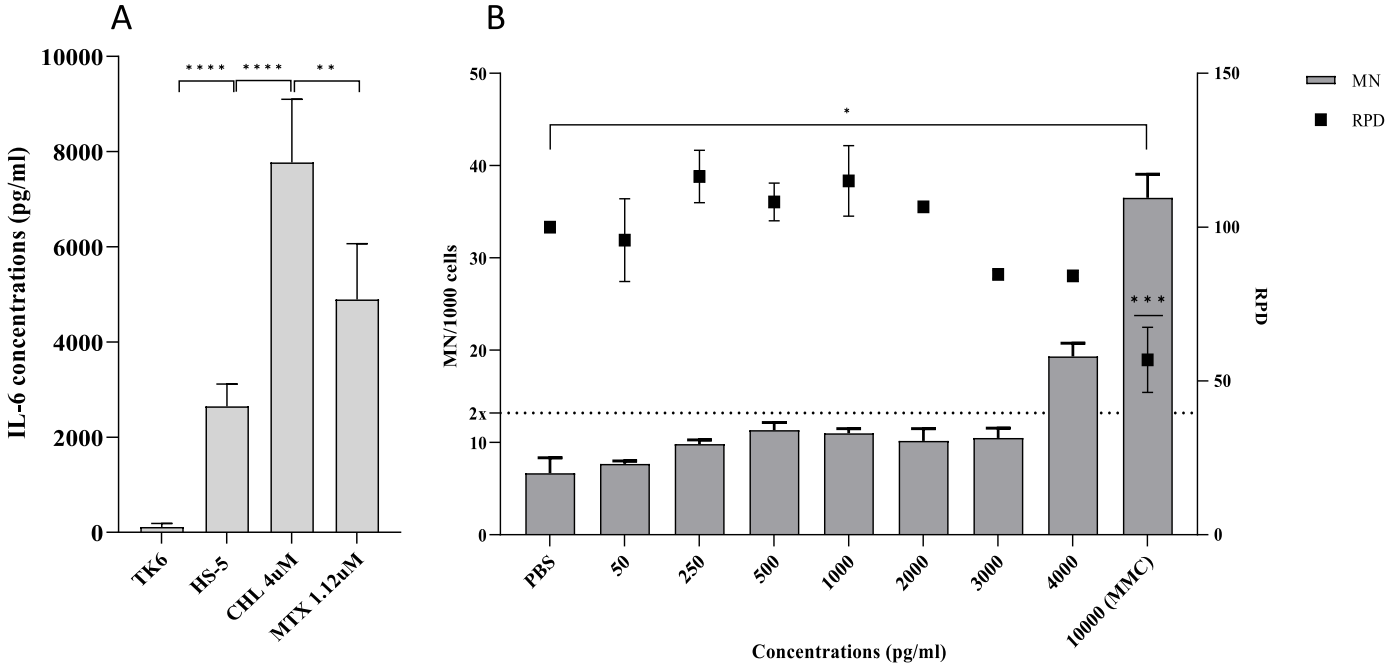

Fig. 2. Detection of IL-6 expression from cell line models and micronuclei induction due to direct IL-6 recombinant treatment. (A) Demonstrates the IL-6 secretion from untreated TK6 and HS-5, and from HS-5 72 h after treatment with CHL (4 μM) and MTX (1.12 μM) for 1 h. (B) TK6 cells were cultured in the presence of recombinant IL-6 for 24 h, at doses aligned with both healthy and storm plasma levels. After a 24-hour recovery period, cells were assessed for relative population doubling (RPD) and micronuclei (MN) scoring. Mitomycin C (MMC; 10,000 pg/ml) served as the positive control, while PBS served as the negative control. The presented data is expressed as mean ± SD ($n = 3$), and statistical significance is indicated by *$p \leq 0.05$, **$p \leq 0.01$, and ****$p \leq 0.0001$, determined by two-way ANOVA with Tukey's multiple comparisons test.

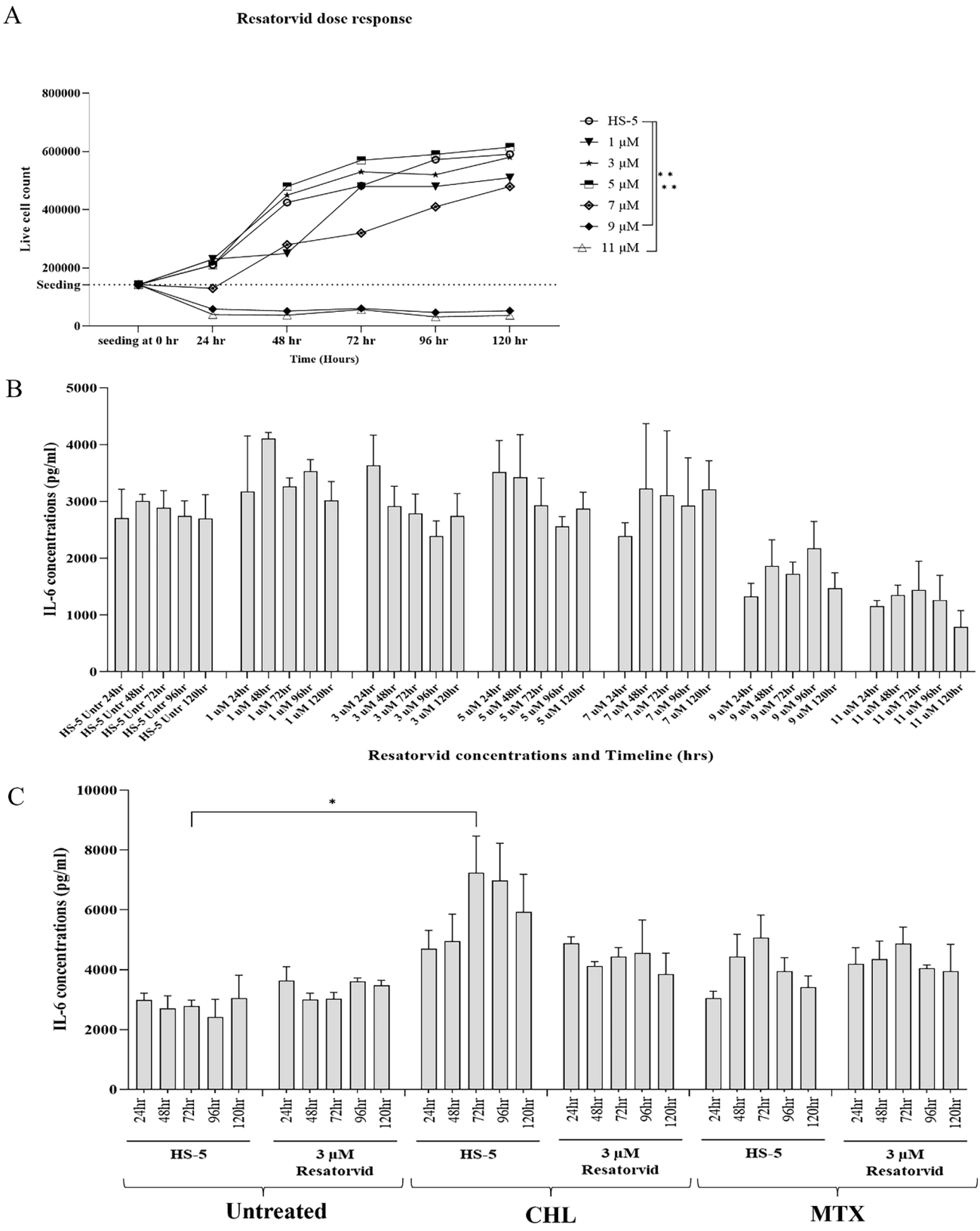

Fig. 3. HS-5 viability and IL-6 expression following exposure to different dosages of resatorvid over 5 days. HS-5 cells were seeded and treated with range of resatorvid doses over 5 days (120 h). (A) HS-5 live cell counts were taken in every 24 h and (B) IL-6 secretion from HS-5 cells post-exposure to doses of resatorvid. (C) IL-6 expression from HS-5 cells exposed to 3 µM resatorvid with and without chemotherapy. Culture media were collected every 24 h following drug treatment and IL-6 level was analysed using ELISA. Data shows the mean ± SD ($n = 3$) and significant differences shown as $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ as determined by two-way ANOVA, Tukey's multiple comparisons test.

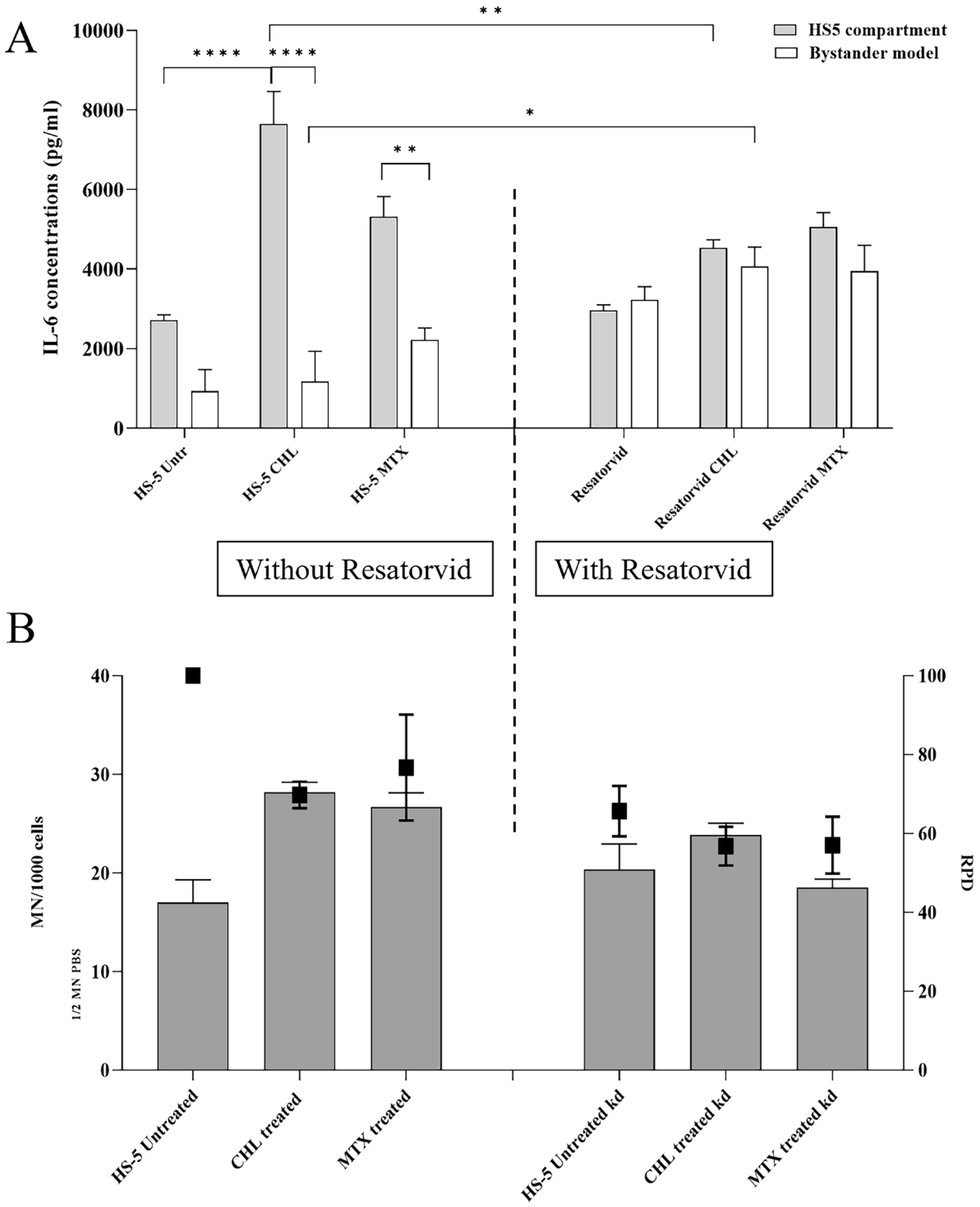

Fig. 4. Resatorvid treated HS-5 co-cultured with bystander TK6 cells. HS-5 cells were treated with resatorvid and chemotherapy, then co-cultured with TK6 bystander cells, separated by a culture insert. Conditioned media were collected on the subsequent day. (A) IL-6 expression from resatorvid treated HS-5 in isolation (grey bars) and from TK6 co-culture (white bars). (B) MN induction and RPD evaluation in bystander TK6 cells after co-culture. Data shows the mean ± SD ($n = 3$) and significant differences are shown as *$p \leq 0.05$, **$p \leq 0.01$ and ****$p \leq 0.0001$ as determined by two-way ANOVA, Šídák's multiple comparisons test.

evidenced by the significant reduction in IL-6 measured in co-culture compared with HS-5 alone following CHL ($p < 0.0001$) and MTX ($p < 0.01$). However, when HS-5 cells were treated with resatorvid, higher IL-6 levels were measured in all co-cultures relative to without resatorvid (CHL without vs with resatorvid $p < 0.05$); with untreated, CHL and MTX treated co-cultures all higher than HS-5 untreated without resatorvid (Figs. 2A and 4A). Here, the data hints that either the pre-treatment of HS-5 with resatorvid slightly increases the IL-6 released from HS-5 cells, and/or that it somehow reduces the capacity for TK6 to take up IL-6. When the MN assay was performed (Fig. 4B), there is a non-significant increase in micronuclei and a slight reduction in RPD following treatment with CHL and MTX in non-resatorvid co-cultures, but in resatorvid treated cultures, MN were slightly raised in untreated controls, and only a slight reduction in MN in drug treated cultures.

None of these data were significantly different and were in line with data presented in Figs. 3C and 2B, where 4000 pg/ml IL-6 produces around 20 MN per 1000 nucleated cells. As IL-6 measurements in resatorvid co-cultures are slightly higher than IL-6 in untreated HS-5 without resatorvid, it is unsurprising that MN are similar in number.

We then attempted to reduce the IL-6 secretion using siRNA knockdown approaches. Fig. 5A demonstrates a stable reduction of IL-6 secretion using 6 pmol siRNA specific for IL-6 across a 5-day incubation period. Using this concentration of siRNA, secretion of IL-6 from HS-5 cells was reduced from around 3000 pg/ml in untreated and mock to around 1000 pg/ml (Fig. 5B), which according to the literature are representative of CRS/CS [25,26] and healthy levels [23,24] respectively. To ensure that this optimised knockdown was equally efficient following chemotherapy treatment, we tested 6 pmol of IL-6 siRNA following CHL and MTX treatment. Fig. 5B shows that both drug treatments showed reduced IL-6 secretion following siRNA knockdown, with CHL significantly reduced on day 3 from about 7000 pg/ml (no knockdown; $p < 0.01$), and non-significantly reduced from ~5000 pg/ml (mock) and ~4000 pg/ml (N/C-siRNA) to ~2000 pg/ml (+k/d). Knockdown following MTX showed a non-significant reduction of IL-6 in both the N/C and siRNA knockdown, in comparison to untreated and mock samples. Whilst the reduction from 5000 (mock) and 4000 pg/ml (N/C-siRNA) to 2000 pg/ml siRNA following CHL exposure was not statistically significantly different, these IL-6 levels represent a reduction from CS/CRS to healthy levels and may prove to be biologically relevant, as inferred by the MN data below.

SiRNA knockdown of IL-6 was performed in the bystander assay to reduce/remove IL-6 from the cytokine mix secreted from HS-5, to which TK6 would be exposed. This was done to determine the role of IL-6 in bystander genotoxicity in TK6 cells.

Fig. 6A shows the measured IL-6 from HS-5 alone versus the HS-5/TK6 co-culture, with and without knockdown. IL-6 levels are lower in all co-cultures compared to HS-5 alone, inferring IL-6 take-up by TK6 cells. The data shows that IL-6 was successfully knocked down in all HS-5 monocultures (CHL -k/d vs +k/d; $p < 0.0001$), inferring that TK6 would be exposed to lower IL-6 when co-cultured with siRNA treated HS-5 +/- drugs in comparison to their respective treatments without knockdown. Cells transfected with N/C-siRNA and then treated with CHL showed a non-significant decrease in IL-6 at 72 h (Fig. 5B) in the HS-5 compartment alone but maintained CS/CRS levels at around 4000 pg/ml. However, the levels of IL-6 in co-culture were the same as for the -k/d control (data not shown).

Whilst IL-6 levels in HS-5 monocultures and co-culture with resatorvid remained high at ≥3000 pg/ml; in contrast following siRNA knockdown, HS-5 monocultures remained <2000 pg/ml, with co-cultures <~1300 pg/ml, suggesting that siRNA was more successful at reducing IL-6 secretion from HS-5. Furthermore, TK6 bystander cells in co-culture with resatorvid-treated HS-5 would be exposed to higher IL-6

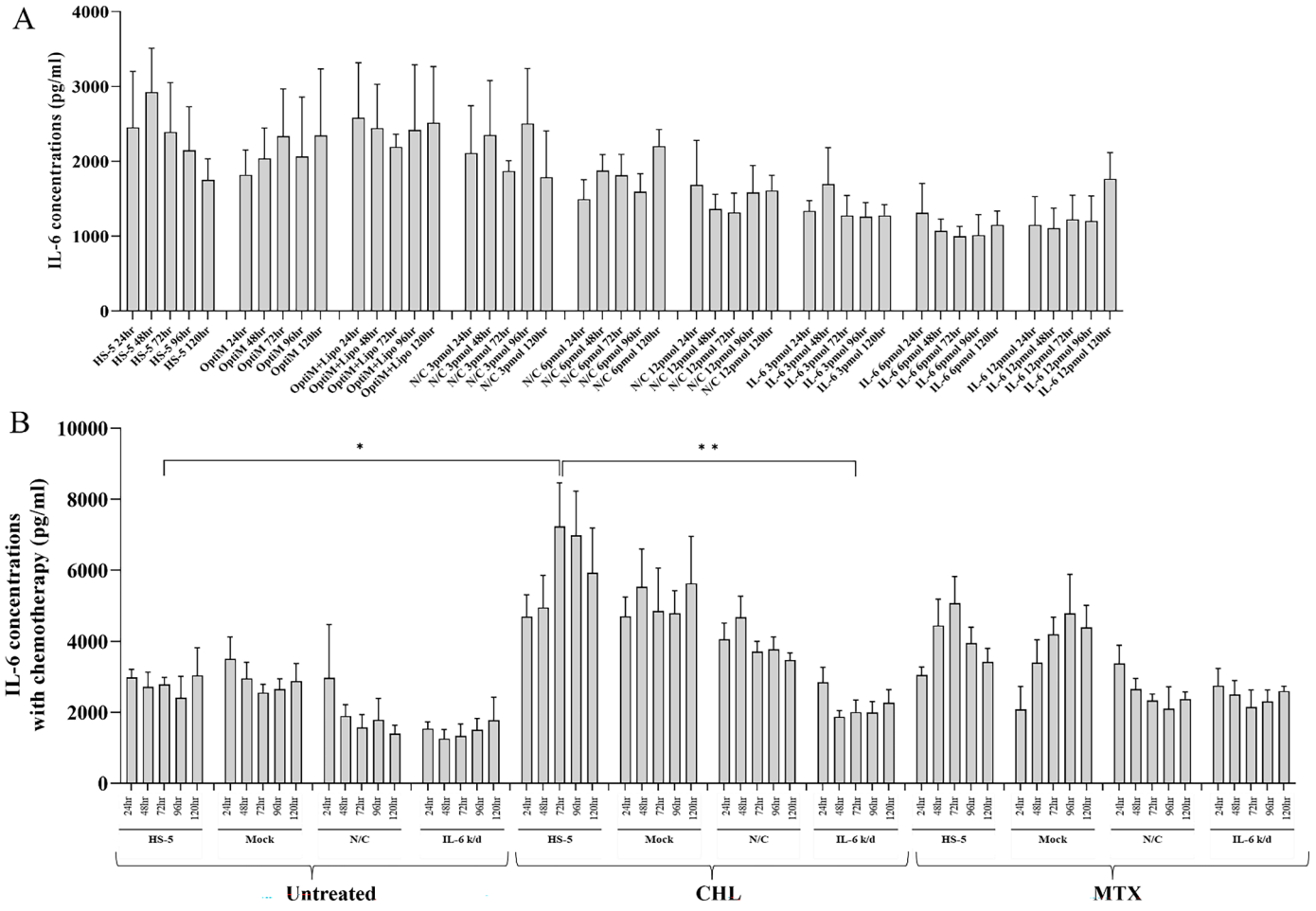

Fig. 5. Optimisation of IL-6 knockdown in HS-5 cells transfected with IL-6 siRNA without and with chemotherapies. (A) Cells were treated with three different siRNA concentrations (3, 6, 12 pmol) alongside the IL-6 siRNA N/C. Separate HS-5 samples were treated with knockdown reagents (OptiM and Lipo) to test their effect on HS-5 cells. Culture media was collected every 24 h over 5 days and analysed using IL-6 ELISA. (B) After the optimum siRNA concentration was confirmed (6 pmol), untreated and drug treated HS-5 are represented as control (non-transfected) HS-5, mock knockdown, negative control siRNA (N/C) and IL-6 siRNA, and measured for IL-6 secretion. Data shows the mean ± SD ($n = 3$) and significant differences shown as $*p \leq 0.05$ and $**p \leq 0.01$, as determined by one-way ANOVA, Tukey's multiple comparisons test.

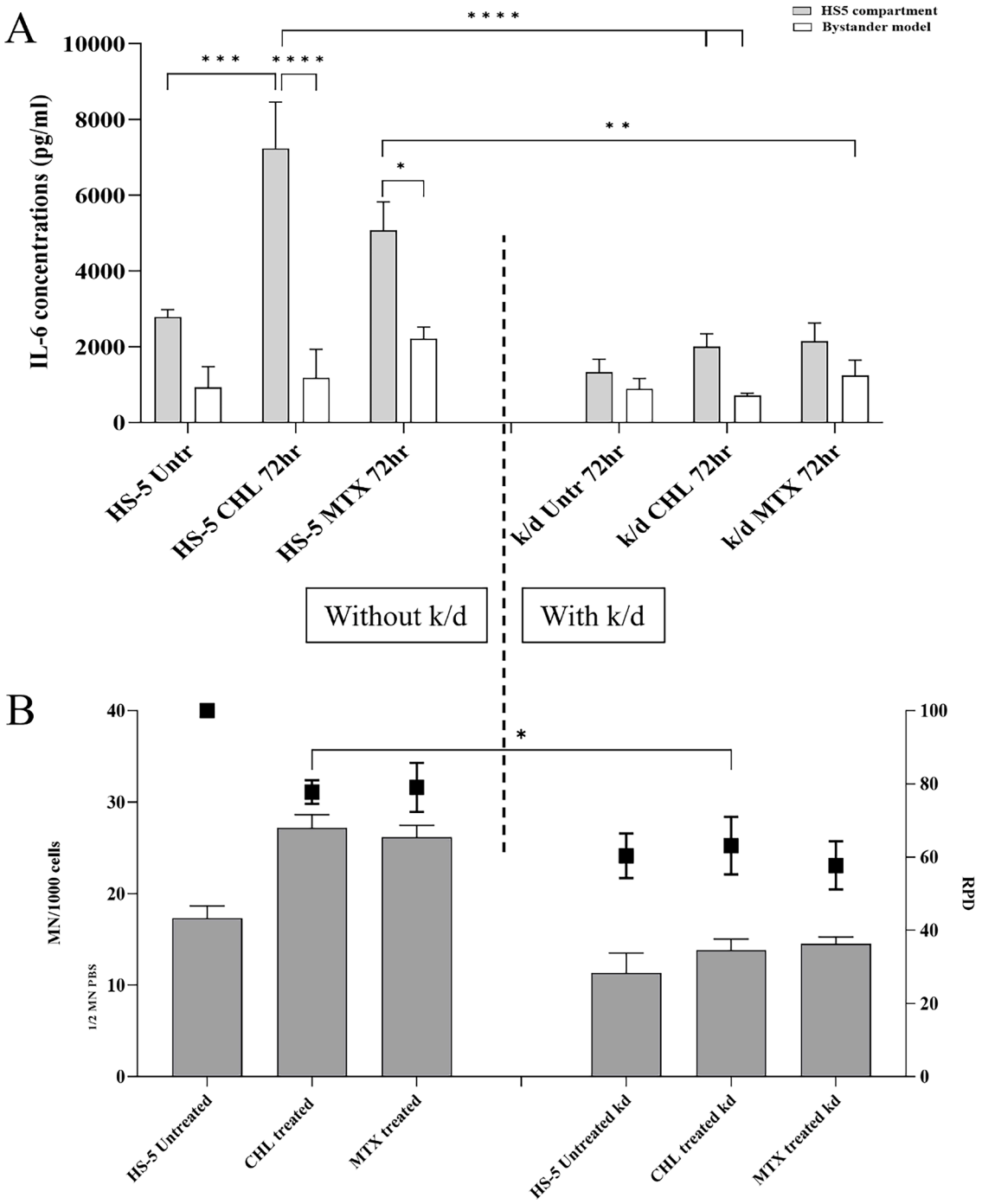

Fig. 6. IL-6 siRNA transfected HS-5 co-cultured with TK6 bystander cells. HS-5 cells were transfected with IL-6 siRNA, then treated with CHL (4 μM) and MTX (1.12 μM). TK6 bystander cells were co-cultured with HS-5 cells, 48 h after drug treatments. (A) IL-6 secretion from IL-6 siRNA HS-5 alone (grey bars) vs media from the co-culture with TK6 (white bars) analysed by ELISA. (B) Micronuclei in TK6 cells co-cultured with IL-6 siRNA transfected HS-5. TK6 cells were harvested after 24 h and evaluated for relative population doubling (RPD) and MN score. Data shows the mean ± SD ($n = 3$) and significant differences are shown as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$ using two-way ANOVA, Tukey's multiple comparisons test (ELISA) and Dunnett's multiple comparisons test (MN).

levels, than their counterparts co-cultured with siRNA-treated HS-5.

When the MN assay was performed on these bystander cells, even the reduced IL-6 in untreated controls, resulted in lower MN measured, with a significant reduction in MN relative to the -k/d control for CHL treated co-cultures ($p < 0.05$). Where the -k/d control had levels of ~7000 pg/ml and N/C-siRNA had 4000 pg/ml IL-6, we noted that MN levels for N/C-siRNA were non-significantly reduced relative to the -k/d control, from 28 to 25 per 1000 cells, which appears to be reflective of exposure to IL-6 at CS/CRS levels [25,26] (concentrations of ≥4000 pg/ml; Fig. 2). Indeed, both -k/d and N/C-siRNA produced MN levels slightly higher than TK6 directly treated with 4000 pg/ml recombinant IL-6 (20 MN per 1000 cells; Fig. 2). These data futher align with the MN data in Fig. 2B, where TK6 exposed to recombinant IL-6 at doses of <2000 pg/ml (as for CHL and MTX with siRNA knockdown) produced about 10 MN per 1000 cells, whereas 4000 pg/ml recombinant IL6 (as for CHL and MTX with resatorvid) produced around 20 MN per 1000 cells (see Fig. 4B). These data infer that if IL-6 remains below around 1000 – 2000 pg/ml, MN are not likely to be an issue in bystander cells, whereas levels

of 4000 pg/ml and above may play a role in possible *de novo* mutagenesis and supports a role for IL-6 in producing these MN.

Discussion

The HS-5 cell line was derived from normal human BM and has been described to secrete high levels of various cytokines including IL-6 [18,31], as well as successfully used to represent the human BM for *in vitro* models of genotoxicity and cancer propagation [30,32]. Here we have advanced our previous work on cytokine secretion from HS-5 as a model of CRS [18] to determine if cytokines can produce genotoxicity in cells, and if they might play a direct role in a chemotherapy-induced bystander effect, to offer a possible explanation of DCL. The TK6 cell line is well-described in genotoxicity studies for being accurate in measuring genetic events, due to its p53 competence [22] and has been previously used to measure genotoxicity in complex 3D models of the human BM [30]. Here TK6 was used as a model of the incoming donor cells.

Measurement of baseline IL-6 secretion from HS-5 was in-line with previous measures of 2000 pg/ml [31]. This manuscript represents the first time that IL-6 has been measured in TK6 and was recorded to secrete low levels at mean 120 pg/ml whereas HS-5 was around 2600 pg/ml, demonstrating that the majority of IL-6 originated from the HS-5 in this co-culture model. Whilst both drugs increased the IL-6 secretion as expected, this was most notable with CHL. Both drugs lifted the IL-6 into what might be considered CRS measurements of >2000–3000 pg/ml [25,26], where healthy levels are below this, often in the range of 10–20 pg/ml [23,24]. CHL produced levels of around 8000 pg/ml (Fig. 2A), which is still lower than measures of 80,000 – 100,000 following mixed lymphocyte culture and irradiation respectively [33]. Within the MN assay of TK6 treated directly with recombinant IL-6, an increase of more than twice the control was observed for 4000 pg/ml, inferring IL-6 in isolation might be considered a weak genotoxin above this threshold (Fig. 2B). Of note is that our previous work showed that combining 4000 pg/ml IL-6 with CRS cytokines such as TGF-β1 (3000 pg/ml) and G-CSF (1000 pg/ml) exceeded the positive genotoxic control in the MN assay, suggesting that combination cytokines have capacity to directly induce genotoxicity [18].

In considering the BM microenvironment post-HSCT, incoming stem cells might be bathed in a complex mixture of cytokines secreted days to weeks post-conditioning therapy, inferring a bystander effect on donated cells from BM-derived cytokines. To ascertain if IL-6 was integral in this effect, we attempted to optimise the chemical inhibitor resatorvid and an IL-6 specific siRNA. Resatorvid is a selective antagonist of the Toll-like receptor 4 (TLR4), downregulating inflammatory responses leading to a reduction in the secretion of IL-6 and IL-12 from BM macrophages [34] but is also documented as inhibiting TNFα [35]. In our hands, resatorvid was only a convincing inhibitor of IL-6 at the very high levels induced following CHL treatment but had little effect on baseline HS-5 secretion at any non-toxic dose (1 – 7 μM). Resatorvid was incapable of reducing HS-5 derived IL-6 below around 4000 pg/ml, inferring that *in vivo* CRS up to these levels are unlikely to benefit from similar treatment, but if proven to be clinically safe might be beneficial where CRS IL-6 measures 8000 pg/ml and above. Unsurprisingly, the minimal reduction in IL-6 in co-culture under resatorvid inhibition, only slightly reduced MN induction in bystander cells (Fig. 4B), in-line with levels of 4000 pg/ml IL-6 having capacity to induce MN at around 20 per 1000 cells (Fig. 2B). Intriguingly, in co-culture, IL-6 levels were higher in the conditioned medium, but it is not clear why this occurred. Stability of resatorvid has been shown in various skin models and despite its high molecular weight, is found to easily penetrate the cells and be stable for up to 9 days [36,37]. It is therefore of intrigue if resatorvid might be able to elute from HS-5 and impact on TK6, to somehow prevent IL-6 uptake, or alternatively promote IL-6 secretion from HS-5 when in co-culture. Optimisation assays at some resatorvid concentrations and timepoints did suggest a slight increase in IL-6 from HS-5, suggesting an alternative route for IL-6 secretion in stromal cells, but this outcome remains to be determined.

Conversely, siRNA stably knocked down IL-6 over a 5-day period in both untreated and treated cells, with levels ranging from 1000 – 2000 pg/ml. This was further demonstrated in the co-culture model where all IL-6 measures were below ~1300 pg/ml, and MN levels in drug-exposed co-cultures were aligned with the untreated control. These data strongly infer an integral role for IL-6 in inducing genotoxic events in this bystander model as the other cytokines measured in Asurappulige [18] should all be present within the co-culture. It is interesting to note that there appears to be a threshold of toxicity; IL-6 doses up to 3000 pg/ml directly exposed to TK6 did not notably raise MN above the control, but doses of 4000 pg/ml and above appear to have some genotoxic potential.

Turnquist [11] highlighted the integral role that IL-6 has in *de novo* tumorigenesis and CS following COVID-19, raising interest in the use of the anti-IL-6 cancer therapeutics to address cytokine-related complications. As previously noted, there was a need to clearly show that cytokines directly produce genotoxic events [12] and to demonstrate that selective inhibition would abrogate them; here we have supported this proposal for IL-6. Furthermore, it is well-documented that polymorphic variability in cytokine genes leads some individuals to be more pro-inflammatory and thus at greater risk for these complications [38]. It may therefore be possible using genetic testing, to predict who is at risk for adverse effects from CS/CRS and intervene with clinically proven anti-IL-6 therapeutics.

Funding statement

This work was solely funded through a PhD studentship for HSHA, provided by this author's family.

CRediT authorship contribution statement

Harshini S.H. Asurappulige: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Michael R. Ladomery:** Supervision, Project administration, Methodology, Investigation, Conceptualization. **H. Ruth Morse:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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