Mutagenesis, 2023, 38, 201–215
https://doi.org/10.1093/mutage/gead018
Advance access publication 16 June 2023
Original Manuscript

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Genotoxicity of cytokines at chemotherapy-induced 'storm' concentrations in a model of the human bone marrow

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Abstract

Donor cell leukaemia (DCL) is a complication of haematopoietic stem cell transplantation where donated cells become malignant within the patient's bone marrow. As DCL predominates as acute myeloid leukaemia, we hypothesized that the cytokine storm following chemotherapy played a role in promoting and supporting leukaemogenesis. Cytokines have also been implicated in genotoxicity; thus, we explored a cell line model of the human bone marrow (BM) to secrete myeloid cytokines following drug treatment and their potential to induce micronuclei. HS-5 human stromal cells were exposed to mitoxantrone (MTX) and chlorambucil (CHL) and, for the first time, were profiled for 80 cytokines using an array. Fifty-four cytokines were detected in untreated cells, of which 24 were upregulated and 10 were downregulated by both drugs. FGF-7 was the lowest cytokine to be detected in both untreated and treated cells. Eleven cytokines not detected at baseline were detected following drug exposure. TNFα, IL6, GM-CSF, G-CSF, and TGFβ1 were selected for micronuclei induction. TK6 cells were exposed to these cytokines in isolation and in paired combinations. Only TNFα and TGFβ1 induced micronuclei at healthy concentrations, but all five cytokines induced micronuclei at storm levels, which was further increased when combined in pairs. Of particular concern was that some combinations induced micronuclei at levels above the mitomycin C positive control; however, most combinations were less than the sum of micronuclei induced following exposure to each cytokine in isolation. These data infer a possible role for cytokines through chemotherapy-induced cytokine storm, in the instigation and support of leukaemogenesis in the BM, and implicate the need to evaluate individuals for variability in cytokine secretion as a potential risk factor for complications such as DCL.

Keywords: genotoxicity; cytokines; chemotherapy; induced; storm; concentrations

Introduction

There are about 9900 new leukaemia cases every year in the UK, with about 25% of cases affecting children; these numbers are projected to rise by about 5% between 2014 and 2035 [1]. Improved therapies have increased survival with roughly half of patients surviving beyond 10 years [2]. The mainstays of treatment are systemic therapies such as chemotherapy and various targeted approaches, but haematopoietic stem cell transplantation (HSCT) is considered if these treatments fail. HSCT also utilizes high doses of chemotherapy as a 'conditioning therapy' to clear the bone marrow (BM) of leukaemia and immunosuppress the patient before transplant. Studies show that while the stem cell compartment is depleted in myeloablative conditioning, the microenvironment remains of patient origin [3,4] and suffers long-term damage [5,6]. Despite being considered a 'curative option' HSCT still has significant short- and long-term complications [7,8].

Given that patients currently have longer survival, an increasing concern is the development of new malignancy over the years following genotoxic chemotherapy exposure. Therapy-related leukaemia (TRL) and donor cell leukaemia (DCL) are significant complications post-chemotherapy and HSCT, respectively. TRL is associated with exposure to alkylating agents and topoisomerase inhibitors [9], whereas DCL is a malignancy shown to develop in the donated stem cells despite the donor remaining healthy [10]. This donor cell–derived neoplasm has gained wider attention in the past decade; however, its aetiology and pathogenic mechanism remain unexplained [10,11]. Of interest, both TRL and DCL predominantly follow the myeloid lineage, mainly presenting as acute myeloid leukaemia (AML) and myelodysplasia [12,13]. Cytokines and their cognate receptors are central to directing stem cells to develop into specific lineages; thus, we speculated that cytokines involved in myeloid lineage development would play a role in DCL aetiology and support the development as an AML.

Previous work in our laboratory (K. Okeke, A.D. Thomas, M.E. Conway & H.R. Morse, in preparation) suggests a chemotherapy-induced bystander effect (CIBE) exists. Briefly, BM stromal cells were directly exposed to 22

Received 25 July 2022; accepted 14 June 2023

chemotherapeutic drugs, which included alkylating agents, antibiotics, anti-metabolites, plant alkaloids, and topoisomerase inhibitors at clinically relevant doses, then using a transwell co-culture model, TK6 cells were utilized as bystander cells. Both cytotoxicity and genotoxicity occurred in the TK6 cells either when co-cultured with treated BM cells or when the culture medium was transferred from treated cells to untreated cells. These observations reflect cell communication through secreted factors supporting the idea that cytokines may play a role in CIBE, possibly resulting in DCL.

The role of cytokines in inducing a bystander effect (BE) following radiation exposure is well-described [14,15], and cytokines have also been widely researched following HSCT [16–18]. Following chemotherapy treatment and HSCT conditioning therapy, it is observed that there is a 'cytokine storm' *in vivo* [16,19,20] with cytokines demonstrating variability in very high levels produced, duration of release, and time of peaking post-therapy [16]. Cytokines, including TGF-β1, GM-CSF, IL-4, and IL-13 activate downstream signalling pathways and increase the expression of other cytokines and growth factors, resulting in toxicity through high levels of reactive oxygen species (ROS) when other immune cells, such as macrophages and T cells are activated [21,22]. Indeed, studies into irradiation exposure support the concept that BE occurs through cytokine secretion from activated T cells [23]. While cytokines play a well-described role in the genotoxic BE from irradiation, their role in CIBE and the influence of such high levels produced during cytokine storm post-chemotherapy have not been previously studied.

Not all patients get TRL [24] or DCL [12], inferring that inter-individual differences exist, which would be helpful to identify to monitor at-risk patients. Homeostatic or detoxification mechanisms may influence risk, but cytokine genes are also known to be highly polymorphic, which predicts variations in secretion levels between individuals [25,26]. Thus, given the observation that some cytokines have been shown to influence post-transplant outcomes [27] and have been shown to be genotoxic [28], the levels of cytokines produced could be important. Thus, we hypothesized that cytokines expressed at 'storm' levels from the BM could be a contributing factor to the development of DCL in donated cells that were unexposed to chemotherapeutics. This research, therefore, compared the profile of secreted cytokines in a cell line model of the human BM (HS-5) with and without chemotherapy exposure and determined the capacity of highly secreted and myeloid differentiating cytokines to induce cyto- and genotoxicity at baseline versus storm levels in a model of the haematopoietic compartment (TK6 lymphoblasts). The alkylating agent (nitrogen mustard) chlorambucil (CHL), and the topoisomerase II inhibitor (anthracenedione) mitoxantrone (MTX) were chosen as these are both implicated in TRL [24]. Chlorambucil is a crosslinking agent, which disrupts DNA replication and leads to strand breakage [29], whereas mitoxantrone potently inhibits topoisomerase II, which is responsible for DNA uncoiling, leading to DNA fragmentation. Mitoxantrone can also intercalate into the DNA, resulting in crosslinks and strand breakage [30].

Materials and methods

Chemotherapeutic agents

All reagents used in this research were purchased from Sigma-Aldrich (Dorset, UK) except where otherwise stated. Two chemotherapeutic drugs linked to TRL [31,32] were used at doses equivalent to clinically relevant or *in vivo* observed plasma concentrations [33–36]. CHL (alkylating agent) and MTX (topoisomerase II inhibitor) were assessed for induction of cytokine secretion in the human BM stromal cell line HS-5. Drug stocks were prepared in 100% ethanol at 100× concentrates and frozen at −80°C. CHL was used at 40 μM, which was equivalent to plasma levels measured in mouse pharmacokinetic studies [33,34]. MTX was used at 1.12 μM (500 ng/ml), aligning with levels measured in human plasma following *in vivo* administration [35,36]. Mitomycin C (MMC; 10,000 pg/ml/30 nmol) was used as a positive control in the micronucleus (MN) assay and was dissolved in dimethyl sulphoxide (final concentration 0.01%; Thermo Fisher Scientific).

Cell culture

The human lymphoblastoid cell line TK6 (13051501; ECACC) was utilized for the genotoxicity studies as a model of the donor cells, as TK6 are well described for their accuracy in predicting genotoxicity [37]. TK6 and the human BM stromal cell line HS-5 (CRL-11882; ATCC), were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies) and in high glucose Dulbecco's Modified Eagles (DMEM-HG) culture medium, respectively, supplemented with 10% heat inactivated foetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (termed 'complete culture medium' [CCM]). TK6 cells were maintained in culture between 3×10^5 and 1×10^6 cells/ml, and HS-5 cells were maintained between 4×10^3 and 2.4×10^4 cells/cm^2 (37°C, 5% CO_2). Cells in passages 3–9 (TK6) and 6–10 (HS-5) were used for experiments.

Cytokine array analysis of drug-treated HS-5 cells

A profile of candidate cytokines released by HS-5 with and without drug exposure was performed using the Abcam 80 targets cytokine array (ab133998). We focussed on cytokine secretion on 2- and 3-day post-chemotherapy exposure for MTX and CHL, respectively, as previously unpublished data within our laboratory demonstrated higher respective cytokine expression on these days, which also aligned with maximal genotoxic bystander effects, measured as MN, following 1 h of drug exposure.

Three culture flasks (25 cm^2) were seeded with HS-5 at 1.4×10^6 cells/flask with DMEM-CCM to allow cells to adhere to flasks for 72 h under normal culture conditions. After 72 h, one flask was treated with 40 μM of CHL. After 1 h incubation, this flask was washed with phosphate-buffered saline (PBS) three times and fresh DMEM-CCM was added before incubating for 48 h under standard conditions (37°C, 5% CO_2). Twenty-four hours later, a further flask was treated for 1 h with 1.12 μM of MTX. The flask was washed three times with PBS and replaced with fresh DMEM-CCM before incubating overnight (24 h). During treatment with CHL and MTX, an untreated control flask was also washed three times with PBS and replenished with medium to undergo the same manipulation of treated flasks.

To collect the cytokines only produced within the 24-h period on day 2 (MTX) and day 3 (CHL), all the culture media were removed from the flasks and replaced with 5 ml of fresh DMEM-CCM. One flask with only culture medium (no cells) was also prepared as a negative control to negate any natural cytokines in the FBS, which may bind to the array and distort the data. The four flasks were placed into the incubator for 24 h at 37°C, 5% CO_2, and this medium (conditioned medium) would be collected for cytokine analysis.

The Abcam cytokine array was performed as described in the manufacturer's instructions. Following the addition of the detection buffer, membranes were placed between a plastic sheet and inserted into the Li-cor reader (Bioscience UK Ltd) on a tray and were read simultaneously at 2 min. Utilizing the densitometry data, a positive control normalization factor was determined using the 'positive control IgG spots' on all four array membranes and was used to normalize signal responses for data comparison. Cytokine spots on each array were then similarly corrected for the respective correction factor for a given array and could be compared within and between repeats as described in the manufacturer's instructions.

Each array experiment was repeated three times, and results were analysed using 'Image Studio Lite' and averaged for each cytokine. Background intensities (medium alone) were subtracted from untreated cells to find baseline secretion (cytokines that are typically secreted from the HS-5 cells) and also from each cytokine array spot in drug-treated samples to obtain the absolute changes in cytokine secretion due to drug exposure. Cytokine secretion was also assessed for fold change in secretion as well as to determine which cytokines were only secreted prior to, or following, drug exposure. Five cytokines were selected for further investigation based on the highest absolute expression and/or fold up-regulation in response to both drugs relative to untreated cells. The selection of some cytokines was also based on a possible role in promoting myeloid differentiation.

In vitro micronucleus assay for recombinant candidate cytokines at reference range and cytokine storm levels

A literature search was performed to ascertain the range of cytokine concentrations measured in healthy individuals versus cytokine storm events. A panel of test concentrations were then established to cover these ranges [17,38–44]. Healthy baseline doses of 50, 250, 500, and 1000 pg/ml were used for TNFα and IL-6, and 100, 250, 500, 750, and 1000 pg/ml for GM-CSF, G-CSF, and TGFβ1. Storm levels of 1000, 2000, 3000, and 4000 pg/ml were utilized for all five cytokines. Of note is that what might be deemed 'high' for one person at healthy levels could be deemed 'low' for another at storm levels, and so there are no clear threshold divides due to genetic polymorphism in cytokine genes. Thus, 1000 pg/ml was utilized as a suggested overlap between healthy and storm levels for GM-CSF, G-CSF, and TGFβ1, whereas 2000 pg/ml was considered and overlap for TNFα and IL-6.

TK6 cytotoxicity and genotoxicity assessment

The *in vitro* MN assay was performed according to the Organisation for Economic Co-operation and Development (OECD [45]) guidelines 487, using the 24-h treatment plus 24-h recovery period approach as described by Wilson and colleagues [46]. The OECD requirement of a relative population doubling (RPD) of > 55% ± 5% as a measure of cytotoxicity for the accurate assessment of MN was applied to all samples. Therefore, cell counts were also taken at 24 h pre- and post-treatment using the automated Luna-FLTM cell counter (Labtech International Ltd), and RPD was calculated as described in Fellows *et al*. [47].

TK6 cells were directly exposed to three different treatment approaches: each recombinant cytokine in isolation at healthy reference range doses; each recombinant cytokine at cytokine storm doses; and pairs of cytokines at combinations of 'high' baseline and 'high' storm doses. The combination doses were determined based on data procured from single cytokine exposures. Thus, doses used were 1000 and 4000 for IL-6 and 1000 and 3000 pg/ml for the other four cytokines.

In each MN assay, TK6 was treated with MMC as a positive control and PBS as a negative control. The MMC dose was determined from historical lab data with RPD > 50% and statistically significant MN induction and was used at 30 nM (10 ng/ml; 48).

Briefly, on day 1 (0 h), TK6 were seeded at 1.5×10^5 cells/ml in RPMI-CCM and cultured overnight. On day 2 (24 h), a cell count was determined, and then cells were exposed to recombinant cytokine(s) for 24 h. Cells were then washed, counted, and reseeded into the fresh culture medium for a further 24 h recovery. On day 4 (72 h), cell counts were performed to determine RPD, then 2×10^4 cells were centrifuged at 800 rpm (300 × g) for 8 min onto glass slides using a Cytospin 4 (ThermoFisher Scientific) to assess MN. Slides were air dried and fixed using 90% methanol for 10 min at room temperature before being stained with 12% (w/v) acridine orange in PBS for 1 min. Slides were destained in two washes of fresh PBS (15 and 10 min, respectively). Slides were air-dried and stored in the dark until scoring. MN was analysed using a fluorescent microscope (Nikon Eclipse 80i TE300) with an attached Nikon Digital Sight DSF1 camera Nikon Coolpix 950 camera (Nikon Instruments Europe) at ×40 magnification.

Images were visualized with NIS Elements software, and slides were scored for MN. A total of 2000 cells were scored per treatment. For all concentrations, three biological replicates were performed; data were averaged and presented per 1000 mononucleated cells. RPD was measured relative to the vehicle (PBS) and positive (MMC) control in line with the OECD guideline 487 [45]. None of the cytokine doses produced an RPD < 50%, so all treatments were scored for MN.

Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) to evaluate the significant differences, where $P < 0.05$ was considered significant. Error bars are expressed as mean ± standard deviation (SD) of three independent experiments performed in duplicate. All statistics and graphical illustrations were done using GraphPad Prism software v. 8.2.1 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Two-way ANOVA was used to compare samples for simple effects within rows. Tukey's multiple comparison tests were used to identify pairs with significant differences relative to the negative control in the MN assay or other candidate cytokines in array data.

Results

Profile of cytokine secretion from HS-5 cells

Out of 80 cytokines on the array, 54 cytokines were secreted at baseline (untreated) HS-5 cells (Fig. 1). These 54 cytokines showed positive absorbances on all three repeat membranes after correction for technical positive and negative controls, and when the culture medium alone values were subtracted. FGF-7 was secreted at the lowest levels, which was still positive on all three membranes; for this reason, an absorbance value of 0.01 (average absorbance for FGF-7 was 0.0095) was set as a threshold of 'presence' of the cytokine with anything below this to be considered as not secreted/absent. Fifteen of the 80 cytokines were excluded from the baseline data due to negative absorbances in at least one repeat membrane; these included IL-4, IL-7, TNF-β, EGF, angiogenin, PDGF-BB, BDNF, IGFBP-1, LIGHT, NT-4, PARC, PIGF, TGF-β2, TGF-β3, and HGF. Caution should be assigned for some of these cytokines, however, as some of these were positive in two of the three membranes and had values higher than both the 0.01 threshold, and also the FGF-7 average. These cytokines included: IL-4, IL-7, TNF-β, EGF, angiogenin, PDGF-BB, BDNF, IGFBP-1, PARC, and HGF. The remaining 11 cytokines were not secreted in untreated cells (at least two negative membranes).

The change in cytokine secretion due to drug exposure was compared with baseline cytokine secretion levels. Changes in absorbance for secretion levels were not statistically significant for any of the cytokines; however, it was acknowledged that a change from very low to higher levels might be more biologically relevant; for these reasons, absolute changes in absorbance (Fig. 2) as well as fold change in secretion (Fig. 3) were explored.

Out of the 54 baseline-expressed cytokines, 24 cytokines were up-regulated for both drugs following treatment (Fig. 2A). Similar to the baseline data, the highest cytokine expressed by HS-5 following treatment was IL-6, with TNFα and GM-CSF next most highly secreted. FGF-7 again was the lowest cytokine to be both positive in all three membranes and also up-regulated by both drugs. IL-8 was the fourth most highly secreted at baseline but was slightly down-regulated following exposure to the drugs, alongside nine other cytokines, which also had slightly lower secretion following both drug exposures (Fig. 2B). Two cytokines were increased by MTX exposure but not by CHL (ENA-78 and GRO-alpha; Fig. 2C), whereas 18 cytokines were increased by CHL, but decreased by MTX (Fig. 2C).

On reviewing the fold difference in expression, the order of placement of cytokines in the graphs from highest to lowest, differed when absolute secretion data for each cytokine was compared with its respective fold change. Figure 3A demonstrates the descending order of fold change for cytokines which were increased following exposure to both drugs. Although IL-6 was the most highly secreted cytokine, the fold change was the lowest, with both TNFα and GM-CSF similarly placed towards the lower end of fold change. Unsurprisingly, as SCF and SDF-1 had low secretion at baseline, the fold change was more marked following drug treatment, and these were the highest relative changes overall. For all of these cytokines, CHL created a higher fold increase than MTX, reflecting the higher absolute secretion levels post-drug exposure.

The 10 cytokines with decreased secretion following exposure to both drugs are represented as decreased fold change in Figure 3B and presented in decreasing order of change. Nine out of the 10 cytokines showed higher fold reduction with CHL than MTX; only FGF-4 was reduced more by MTX (Fig. 3B). While the order of placement of cytokines also changed relative to the absolute secretion levels, this was not as marked as for the cytokines which were upregulated. The order of placement of cytokines which were upregulated by one drug and down regulated by the other, showed a rearrangement of the placement order, with GRO-alpha and ENA-78 having higher overall secretion levels, but the lowest fold change of this group of cytokines (Fig. 3C). While some of the more highly secreted cytokines had the lowest fold change (e.g. MIP-3α, TIMP-1), and some of the less highly secreted had the highest fold change (e.g. IGFBP-2, Leptin, IL-3), this pattern did not follow for all cytokines. Thus, a consideration of whether absolute levels or fold change might be more biologically significant warrants further investigation.

Out of the 24 upregulated cytokines, the top 10 highest fold change cytokines with CHL treatment were SCF, SDF-1, MIF, TGF-β1, G-CSF, IL-5, GRO, IP-10, VEGF, and MCP-4, whereas the MTX highest were MIF, SCF, GRO, SDF-1, G-CSF, IP-10, TGF-β1, VEGF, RANTES, and MCP-4. Thus,

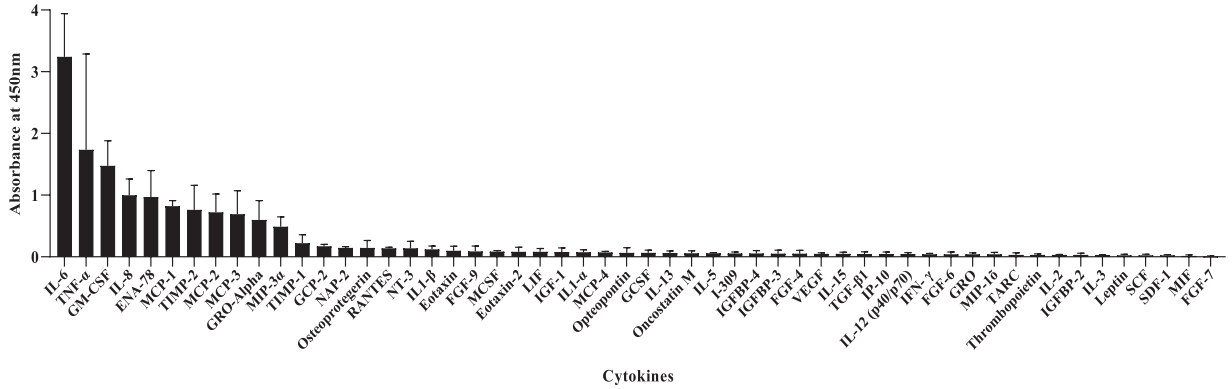

Figure 1. Profile of cytokine secretion by untreated HS-5 cells. Out of 80 cytokines tested, 54 cytokines were secreted in untreated HS-5. Normalization was performed using positive control signals on each array. Cytokines were measured semi-quantitatively utilizing absorbance values of each cytokine spot on the membrane and corrected for background cytokines from the culture medium. Data show mean ± SD (n = 3).

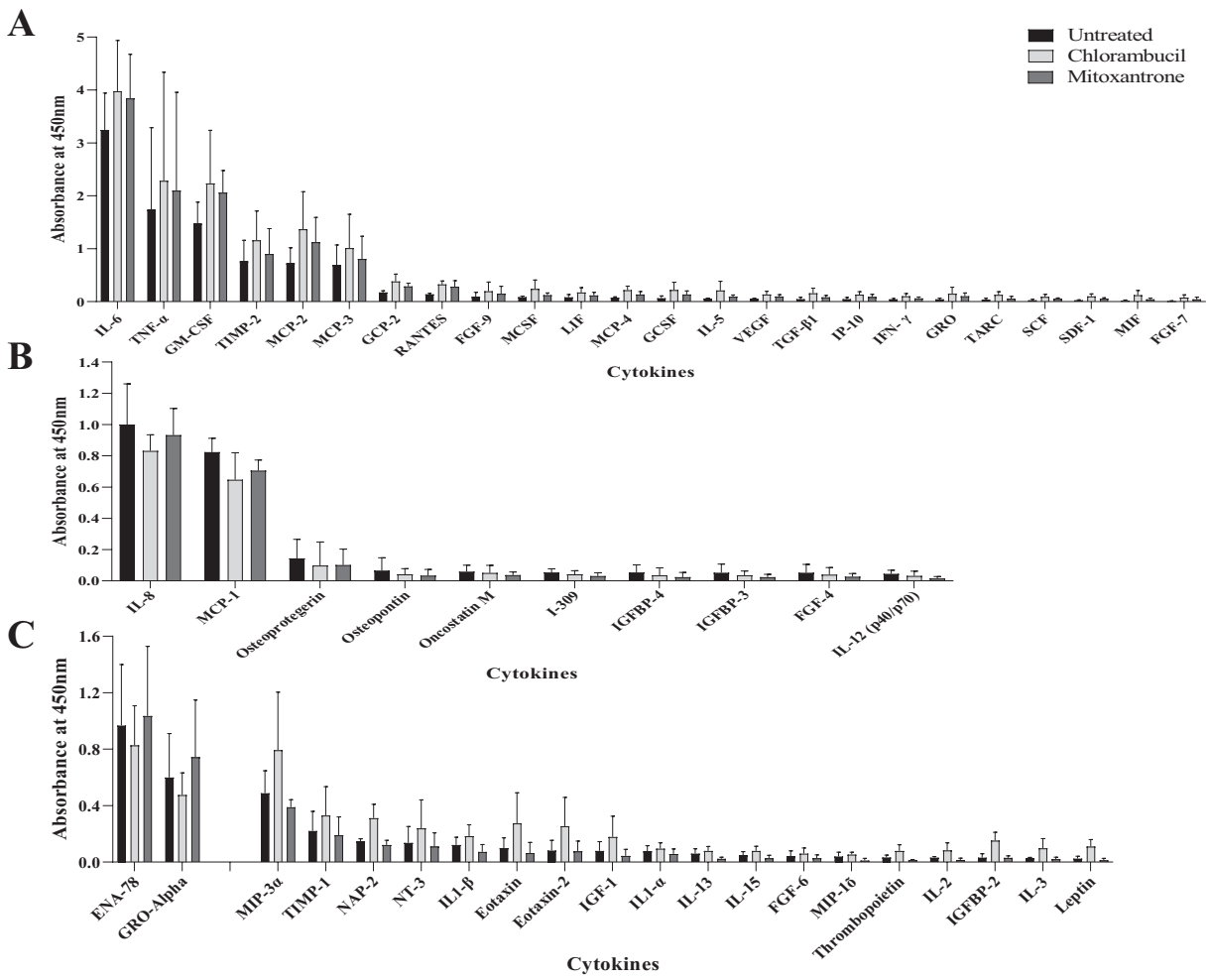

Figure 2. Cytokine secretion at 72 and 48 h from HS-5 cells following 1 h exposure to CHL and MTX, respectively. Cytokine secretion is arranged in the order of magnitude for CHL. (A) Cytokines which were upregulated by both drugs in comparison to untreated HS-5 cells. (B) Cytokines which were downregulated by both drugs relative to untreated HS-5 cells. (C) Cytokines which were upregulated by one drug, but down regulated by the other; ENA-78 and GRO-Alpha were upregulated by MTX, whereas the remaining cytokines were upregulated by CHL. Data show mean ± SD (n = 3). CHL, chlorambucil; MTX, mitoxantrone.

the common cytokines in the top 10 for both drugs were SCF, SDF-1, MIF, TGF-β1, G-CSF, GRO, IP-10, VEGF, and MCP-4.

All 11 cytokines that were deemed to be not secreted by HS-5 cells at baseline were detected following drug exposure. All 11 were more highly upregulated by CHL than MTX and are presented in descending order of secretion in Figure 4, alongside FGF-7 as a comparator cytokine which was the lowest cytokine to be detected on all three membranes at baseline and following drug exposure.

Based on the array data, IL-6, TNFα, GM-CSF, G-CSF, and TGF-β1 were chosen to explore genotoxicity in further detail and were chosen as representatives of high overall secretion or high fold increase, involvement in myeloid differentiation, contribution to cytokine storm *in vivo* and known role in carcinogenesis.

Evaluation of the functional change to the HS-5 following drug exposure

We performed a literature review of all 80 cytokines on the array to explore their functionality in order that a general picture might surface of how the HS-5 cells respond to chemotherapy. The overall response inferred an upregulation of pro-inflammatory cytokines, supported by an increase in chemotactic factors, promotion of angiogenesis, and proliferation. There appeared to be a balance in control of pro- and anti-apoptotic factors, with an overall sway towards promoting apoptosis. These observations align with the clinical picture of a cytokine storm in patients following chemotherapy. Thus, these data support the use of HS-5 as a model of the BM microenvironment.

In vitro MN assay

All treatments were assessed for RPD, and all cytokine treatments demonstrated RPD values of 50% or more; thus, all were scored for MN (Fig. 5). At 'healthy' concentrations, only TNFα showed a dose-dependent decrease in RPD, but remained above the 50% threshold set by the OECD guidelines for scoring MN. All cytokines except TGF-β1 showed some degree of cytotoxicity at cytokine storm concentrations but still remained above the 50% threshold, as might be expected for natural biological molecules.

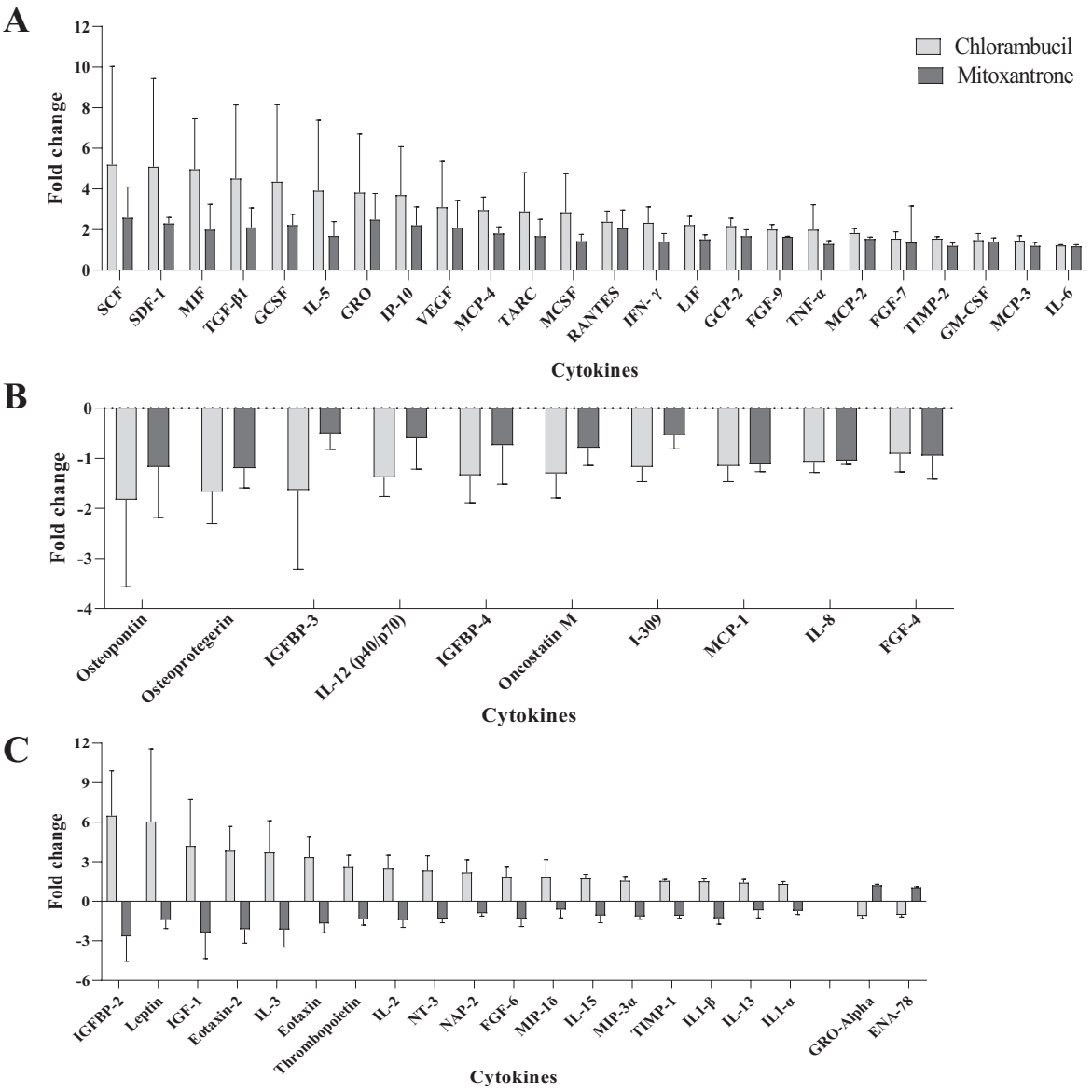

Figure 3. Fold change of cytokine expression in HS-5 cells exposed to CHL and MTX for 1 h relative to untreated cells. Cytokines are arranged in order of magnitude of fold change for CHL. (A) Cytokines which have positive fold change by exposure to both CHL and MTX. (B) Cytokines which have negative fold change common to both drugs. (C) Cytokines which were positive for one drug, but negative fold change for the other. Data show mean ± SD (n = 3). CHL, chlorambucil; MTX, mitoxantrone.

Except for the positive control (MMC), there was no statistically significant increase in MN compared to the negative control (PBS). All MN scoring for the negative control and MMC was consistent with historical laboratory data (control = 7.875 ± 1.78; MMC 36.75 ± 4.94; mean ± SD). As expected, G-CSF did not increase MN at healthy concentrations. GM-CSF had a slight increase in MN for healthy treated samples over the negative control, but the levels were consistent between doses. Similarly, IL-6 only increased the presence of MN towards the higher end of the 'healthy' range, at concentrations >500 pg/ml. However, at concentrations above 1000 pg/ml for TNFα, and at all doses for TGF-β1, MN were non-significantly induced at levels more than twice the negative control, which may infer alignment with previous reports of a role in genotoxicity [49] and carcinogenicity (Fig. 5; [50]).

However, at storm levels, all cytokines were able to induce MN at more than twice the untreated control, with most treatments resulting in at least 3-fold induction of MN. TNFα at 3000 and 4000 pg/ml showed a 3-fold increase in MN over the negative control in TK6. A 3-fold increase in MN was also apparent at 4000 pg/ml of IL-6. Both 2000 and 3000 pg/ml of GM-CSF induced MN at levels 3-fold above the negative control, and all concentrations above 2000 pg/ml of G-CSF were more than 2-fold higher, with 2000 pg/ml at 3-fold higher. Intriguingly, as doses escalated for G-CSF, MN numbers decreased, despite an overall improvement in RPD at these doses compared with lower doses. Contrastingly, TGF-β1 approached a 2-fold increase at 1000 pg/ml but was more than 3-fold higher than the negative control at all doses above 2000 pg/ml.

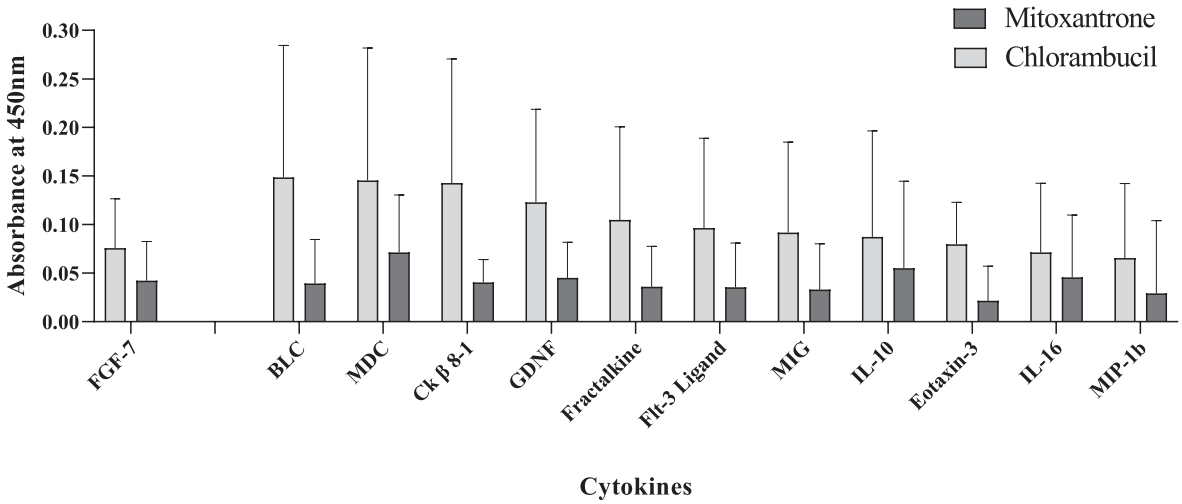

Figure 4. Detection of cytokines secreted only in response to CHL and MTX drug exposure. Cytokines were identified for being absent in untreated HS-5 (negative absorbance values on untreated membranes) but then detected following drug exposure. Out of 80 cytokines, 11 cytokines were not secreted from untreated HS-5 cells however, all were detected following drug exposure. FGF-7 as the lowest detected cytokine both in untreated and drug treated HS-5 cells is presented for comparison of relative secretion levels. Data are presented as mean ± SD (n = 3).

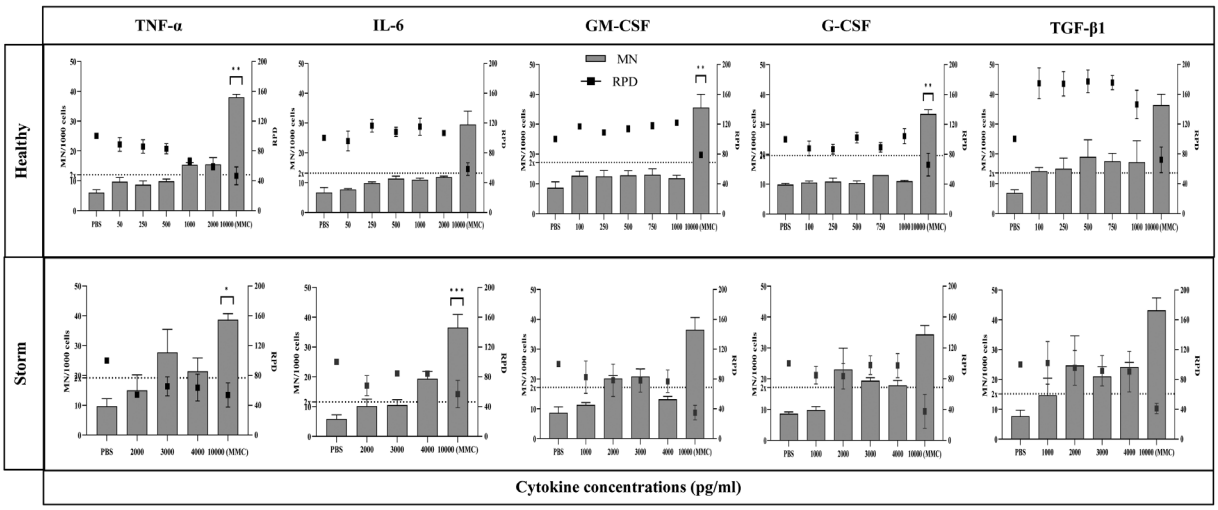

Figure 5. The induction of micronuclei in TK6 cells due to direct treatment of cytokines at healthy and storm plasma concentrations. TK6 cells were cultured in the presence of each cytokine for 24 h. After a 24-h recovery period, cells were harvested and evaluated for relative population doubling (RPD) and chromosomal damage by scoring the number of MN present. Mitomycin C (MMC; 10,000 pg/ml) was the positive control, and PBS was the negative control. Controls were used for all experimental repeats. Data show the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 (for MMC vs. PBS).

While these individual treatments of cytokines might suggest candidate cytokines (and concentrations) that may play a role in genotoxicity related to DCL, it is noted that *in vivo*, patients are exposed to drugs that induce a wide variety and intensity of cytokine response. This cytokine response can vary depending on the genetic polymorphisms within their respective genes, with some cytokines expressed at the higher end, and others at the lower end, of the range depending on what polymorphisms are carried by the individual [26]. With the knowledge that cytokines actively interact with each other, it was of relevance to explore if combinations of cytokines potentiated the observed increase in MN. Thus, the five candidate cytokines were paired at doses towards the higher ends of the healthy and storm concentrations. The healthy doses were chosen to overlap with the lower end of the storm range, and the higher doses were mainly chosen to represent 'average' (but at least 2-fold higher) MN than the negative control, when used in isolation.

In combination, all cytokine concentrations did not adversely affect the RPD and TK6 cultures were close to, or at, 100% RPD, with some exceeding this value (Fig. 6). Each graph shows the candidate cytokine MN values from isolated exposure (data from Fig. 5) alongside the MMC control as a point of reference when considering the MN induced following combined exposure. It is apparent that every combined pair induced MN at more than twice the value of the PBS control, and surprisingly, some combinations even exceeded the value of the MMC positive control. When reviewing the combinations that were higher than the MMC control, all five of the candidate cytokines featured in at least one combination. Overall, the highest MN per 1000 cells was induced by a combination of low GM-CSF with high G-CSF (47 MN per

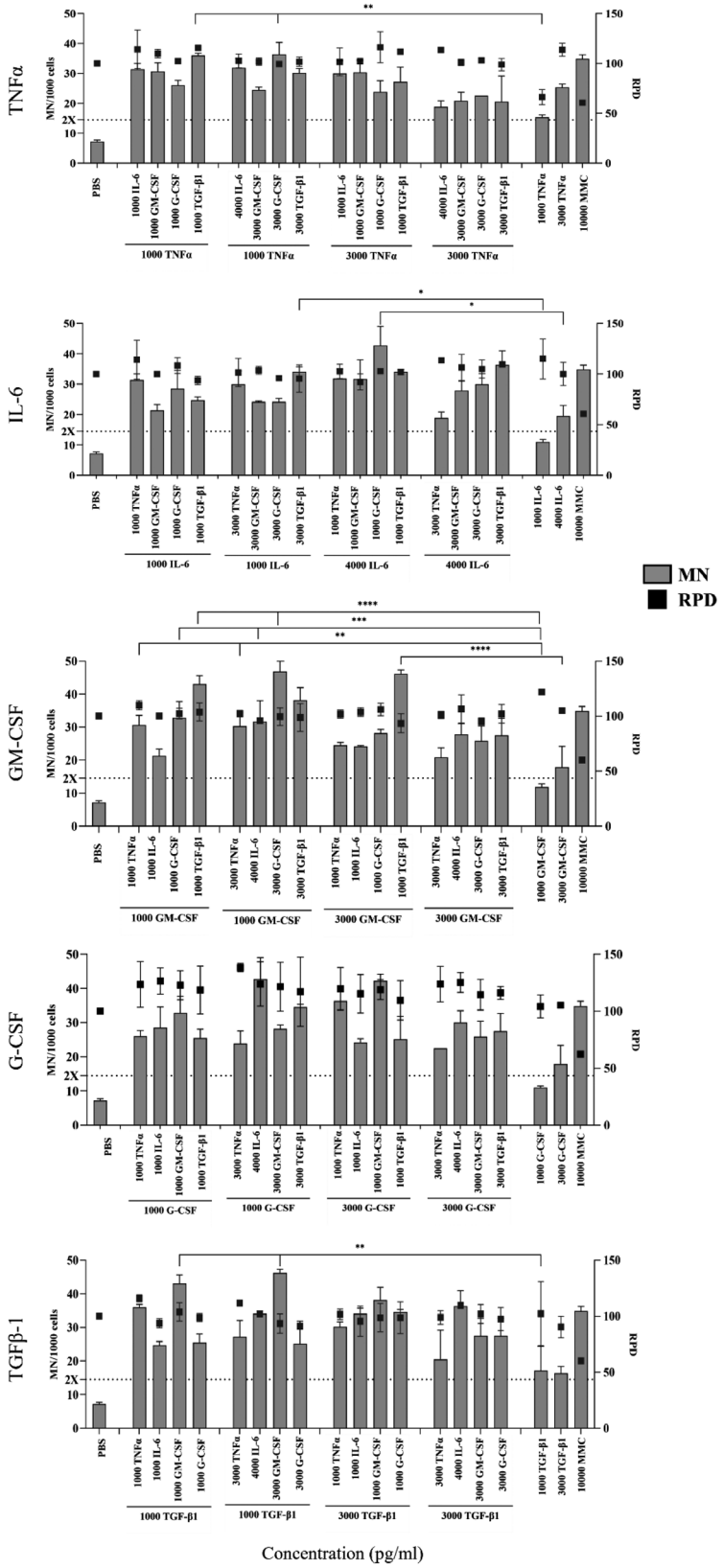

Figure 6. The induction of micronuclei in TK6 cells following paired cytokine combination treatments. TK6 cells were cultured with two cytokines at different concentrations for 24 h. After a 24-h recovery period, cells were harvested and evaluated for the RPD and number of MN present. MMC (10,000 pg/ml) was the positive control and PBS was the negative control for all experimental repeats. Data are presented as mean ± SD ($n = 3$) for all the concentrations analysed and significant differences shown as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$.

1000 cells), closely followed by combinations of high GM-CSF with low TGF-β1 (46 MN), high IL-6 with low G-CSF (43 MN), and low GM-CSF with low TGF-β1 (43 MN). This is in comparison to an average MN count of 34.8 per 1000 cells for MMC.

All combinations except most G-CSF combinations significantly raised MN above the PBS control (range: $P < 0.05$–0.0001); the only exceptions were high-dose TNFα in combination with high dose of all four of the cytokines (all of which reduced MN below TNFα at 3000 pg/ml in isolation). The only G-CSF combinations that were significantly increased over the PBS control were 1000 pg/ml G-CSF with 4000 pg/ml IL6 ($P < 0.05$), and 3000 pg/ml G-CSF with 1000 pg/ml GM-CSF ($P < 0.01$). However, of more importance was the ability of combinations to significantly raise the MN over the counts for the cytokine in isolation. Here, only combinations with TNFα, IL-6, GM-CSF, and TGFβ1 were statistically significantly raised above their MN counts in isolation (Fig. 6). GM-CSF and IL-6 at both doses induced statistically significant changes, whereas TGFβ1 only achieved this at a lower dose in combination with GM-CSF at 1000 and 3000 pg/ml ($P < 0.01$). Similar to TGFβ1, TNFα only statistically raised MN at lower dose in combination with low-dose TGFβ1 ($P < 0.01$) and high-dose G-CSF ($P < 0.01$).

We analysed the capacity for the cytokines to be protective, additive or to synergize MN induction when used in combination. In general, combinations of cytokines were less than the sum of MN for each cytokine in the pair when used in isolation. However, a few combinations were either additive or synergized the MN induction. Combinations that were additive were low G-CSF with low TNFα, and also a combination of high IL-6 with either low GM-CSF or high TGF-β1. Intriguingly, no synergy occurred when two cytokines were combined at high doses for both. The majority of synergistic combinations occurred where low dose was combined with low dose (e.g. low TNFα with low IL-6, GM-CSF and TGF-β1 and also low GM-CSF with low G-CSF, TGF-β1 among others); however, there was some synergy between low with high dose (e.g. low TNFα with high G-CSF; low IL-6 with high TGF-β1; low GM-CSF with high G-CSF and TGF-β1 among others).

It is of intrigue that G-CSF frequently features in these analyses as this cytokine is used to mobilize stem cells out of donors' BMs and has been controversially speculated to be a risk for subsequent development of leukaemia in donors [51]. This observation warrants the exploration of donated cells being 'primed' for genotoxic events by exposure to G-CSF. Where typically TNFα and TGF-β1 have been noted as risks for genotoxicity and carcinogenesis, it is clear that particularly GM-CSF and G-CSF might play a role.

Discussion

The role of cytokines in leukaemia and the bone marrow microenvironment

Leukaemia is a malignancy of the BM, resulting in reduced production of normal blood cells, accumulation of abnormal white blood cells due to uncontrolled proliferation, lack of differentiation, and apoptosis in the BM due to haematopoietic dysregulation and immune suppression. However, the treatment regimens have greatly improved the prognosis of leukaemia in the last few decades [2]. In general, HSCT is considered a curative, but last resort therapy as although it is considered a routine procedure, it can be life-threatening due to multiple complications, including DCL. The aetiology of DCL is currently unknown, but putative theories include; donor genetic predisposition (occult leukaemia/preleukaemic potential), viral integration, oncogenic activation, residual effects of therapy, and defective BM microenvironment niche [10].

The research presented here hypothesizes that DCL might result from a bystander effect through intercellular communication between the BM mesenchymal stromal cells and incoming donated stem cells, with cytokines playing a key role. The seminal 'seed and soil hypothesis' can be proposed as a model for DCL. Optimal development of engrafted HSCs (seeds) occurs when the seed and the soil (BM niche) are in equilibrium. The soil plays an essential role in the maintenance of the seed by regulating quiescence, self-renewal, proliferation, and differentiation [52]. Signals identified that mediate bystander communication between soil and seed are cytokines, chemokines, ROS, nitric oxide, and micro RNAs, which can be transferred between cells via gap junctions or extracellular medium [14,53]. However, recent research demonstrates that chemotherapy exposure can produce long-term functional [5] and genotoxic [6] damage to the BM microenvironment, disrupting this equilibrium and providing potential for tumourigenic growth. Indeed, research by Gynn *et al.* [54] supported our observations of increased genotoxicity in the BM microenvironment [6], but also showed that trans-well culture of the HL-60 leukaemic cell line with primary mesenchymal (MSC) stem cells exposed to cytarabine *in vitro* resulted in the protection of the HL-60 and sensitization of the MSC to the genotoxic effects of cytarabine. As DCL results from normal unexposed haematopoietic cells, it remains to be confirmed if the protection afforded to HL-60 cells by MSC would also be observed with HSC. However, the BM utilized by Gynn [54] was from patients under haematological investigation, which may have direct relevance to our model, as van den Berk noted disruption of signalling within the niche under the influence of leukaemic cells [55], which may persist post-myeloablation. Furthermore, work by Odagiri *et al.* [56] and Umegaki and Fenech [57] support the increased sensitivity to genotoxicity of myeloid cells compared with erythroid cells, the latter of which is typically evaluated in genotoxicity testing; this suggests a further explanation for the observation of DCL as AML. Alongside the knowledge that HSCT typically utilizes high-dose chemotherapy as conditioning therapy, and that the BM microenvironment remains of recipient origin following HSCT [3,4] supports the idea that aberrant bystander signalling could play a role in the development and/or support of DCL.

Inflammatory disease involving cytokine storm has also been associated with increased risk of cancer. For example, Liu *et al.* [58] noted a highly significant increased risk for a small number of cancers following sepsis, of which AML, CML, and myelodysplasia were particularly noted alongside colon, rectum, cervix, liver, and lung. Furthermore, TNFα has been associated with systemic genotoxicity in mice *in vivo* [49] which was potentiated by IL-1β and decreased by IL-10. While MN collapse at interphase [59,60] can lead to the cGAS-STING inflammatory response and release of interferons [61], which might be speculated to compound

the problem, interferon-α has been shown to reduce MN induction in gamma-irradiated *in vivo* mouse models [62]. Thus, the combination of pro- versus anti-inflammatory mediators presents a complex picture. However, in alignment with our hypothesis, Chinnadurai [63] demonstrated a bleomycin and neocarzinostatin-induced bystander effect (measured as MN), which was higher in undifferentiated BM MSC and peripheral blood than in fibroblasts and adenocarcinoma cells, and this was partially abrogated by ROS scavenging.

Validation of HS-5 cells as a model of the BM

Cytokine expression from untreated and drug-exposed HS-5, as a possible mechanism of CIBE was detected using a commercial cytokine array. The kit was chosen to screen 80 cytokines simultaneously, which cover documented BM-expressed cytokines (cytokine storm) from the literature, as well as noted genotoxic and myeloid-focused cytokines [16,19,20,64–66]. We have previously utilized HS-5 in a model of the BM [48], and these have been shown to ably support HSC [67] and TK6 cells [48]. Data procured for cytokines secreted in untreated (baseline) cells in our study (Fig. 1) align with those listed in the literature and validate the use of HS-5 to represent the mesenchymal features of the BM [68]. FGF-7, as the cytokine showing the lowest levels of secretion, which was also detected on all three repeat membranes, was set as an arbitrary threshold for the limit of detection of the array; this cytokine was used as a marker when changes in secretion were measured following drug exposure and also allowed for the assignment of presence versus absence of the cytokines. The understanding that the ability to induce a biologically relevant bystander effect required either the presence or upregulation of candidates, allowed us to assess our data both as absolute and fold change, as well as to identify the cytokines that were only detected following drug exposure. Of interest was that while some cytokines were universally up or down regulated by both drugs, and others were upregulated by CHL, but downregulated by MTX (and *vice versa*), of those cytokines that were not detected at baseline, all of these were detected following drug exposure. Thus, there were no cytokines on the array, which were never detected from the HS-5 cells.

Little is known about changes in either the cytokine secretion or release of other bystander mediators from the BM microenvironment following chemotherapy exposure, but some knowledge is available on how diseased BM differs from healthy BM, and cytokine secretion following irradiation is also well described [69,70]. Stromal cell layers from AML patients have been shown to produce 2-fold higher levels of IL-6 and 22-fold higher levels of TNFα [71]. Indeed Sanchez-Correa and colleagues assert that prognosis for AML can be predicted by measuring patient cytokine levels, with high IL-6 and TNFα, alongside low IL-10, indicating a poorer outcome [72]. Furthermore, in response to radiation, BM fibroblasts and macrophages continuously secrete soluble signals such as growth factors, cytokines (IL-1, IL-6, and TNFα), chemokines, ROS/reactive nitrogen species (RNS), and TGF-β1 (22), which promote survival and proliferation of AML cells *in vitro* [15]. Results of cytokine response post-HSCT [73] also showed high levels of IL-6 in children with complications. As DCL predominates as AML or myelodysplastic syndrome, it is therefore important to focus on cytokines associated with the myeloid progenitor lineage (e.g. IL-1, 3, 6, GM-CSF, and SCF). Of note is that HS-5 cells expressed relatively high levels of IL-6 and GM-CSF (treated and untreated) and showed the highest fold change in SCF following CHL and MTX treatment, supporting the use of HS-5 for these studies.

While HS-5 has acted as a model for CIBE in our experiments and clearly only reflects the cytokine profile of the donor of origin, the profile measured within our data reflects that seen in clinical settings following toxic insult as described above. However, HS-5 only reflects one person's profile, a white 30-year-old male with normal BM; so it is clearly relevant to review changes to cytokine levels in a range of leukaemic patients, as well as further modifications to expression following chemo- and radiotherapy. The ability to currently explore the range of profiles *in vitro* is hampered by the lack of BM mesenchymal stem cell lines and requires procurement of patient samples for *ex vivo* analysis, but HS-5 has been shown to represent a good model for BM studies to date [74,75].

Cytokine storm, the role of chemotherapy, and inter-individuality

TNF-α, IFN-γ, IL-1β, 2, 4, 6, 8, and so on are key cytokines involved in the cytokine storm after a surge of the immune system following either toxic insult or transplantation [5,16,19,20]. Within our data, IFN-γ had low secretion (CHL: 0.1 and MTX: 0.06) but was 2.34-fold upregulated with CHL and 1.42-fold upregulated with MTX. Furthermore, TNF-α showed the second highest secretion for both drugs but also had a 2-fold increase with CHL and 1.3-fold increase with MTX. IL-1β was also upregulated by CHL, but only by about 1.5-fold, whereas it was downregulated by MTX by 1.3-fold. Similar to IL1β, IL-2 was upregulated by CHL but downregulated by MTX. MTX has been shown to induce a TH2 cytokine profile in multiple sclerosis patients [76], but elsewhere has been shown to be highly immunosuppressive [77], which may explain the overall lower response with MTX. We are mindful that CHL may have been used at a 10-fold dose higher than was (human) clinically relevant, as pharmacokinetic studies in mice reveal plasma levels of CHL of 40 μM [33,34], whereas subsequent literature searching suggests that plasma levels in patients may be closer to 4 μM [78,79]. While this highlights disparity following the dosing of rodents, which may be important in toxicity testing extrapolations between rodent and man, further research is required to explore if a 10-fold lower dose of CHL would have significantly altered the data presented here.

Nevertheless, while aiming to understand patients at risk for post-chemotherapy complications through cytokines, as discussed here, we need to remember the complexity of the picture, in that, there can be considerable inter-individual differences in metabolism leading to final plasma concentrations [80], as well as genetic polymorphism in cytokine genes resulting in inter-individual secretion profile differences [26,81] in response to these drugs. Thus, our study covered a range of concentrations recorded in the literature for both healthy and storm levels in humans. However, overall, the picture of cytokine secretion seems to promote a pro-inflammatory and chemotactic environment within our model, which aligns with the clinically observed

cytokine storm. Cytokines detected post-drug exposure included IL-16, Eotaxin-3, Fractalkine, MIG, and BLC, which support the overall chemotaxis required to home the incoming stem cells, as well as Flt3 ligand, GDNF, MDC, and MIP-1b, which support inflammation, cellular survival, and differentiation.

Genotoxicity of candidate cytokines

Following the acquisition of these secretion data, three criteria were considered when selecting candidate cytokines out of the 80 on the array as contributing to the development of DCL:

1. Cytokines which promote myeloid lineage differentiation (IL-1 [family], IL-3, IL-6, GM-CSF, G-CSF, and SCF)
2. Cytokine storm candidates (TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, and IL-8)
3. Cytokines previously implicated in malignancy or mutagenesis (GM-CSF, IFN-γ, TNF-α, IL-6, G-CSF, IL-3, and TGF-β1).

By considering all the results from the array and documented literature, IL-6, GM-CSF, and TNF-α were selected from the absolute change data and GCSF and TGF-β1 were selected from the fold-change data for future genotoxic experimentation in this research.

Current literature on the genotoxicity of cytokines tends to focus on the role of the immune system, the inflammatory processes, and the role of neighbouring cells, such as macrophages, as a potent source of ROS production [28,82]. Clearly, within *in vivo* studies, or *in vitro* studies utilizing complex mixtures of immune cells as would be expected for cytokine research, the indirect activity of released molecules such as ROS cannot be ignored and likely plays an important role in genotoxic endpoints. However, some studies state that some cytokines have the ability to induce genotoxicity directly [83,84]. Our model addresses this point, by directly exposing TK6 cells to the candidate cytokines in the absence of an immune cell environment, to explore a possible direct genotoxic activity of these candidates if they were overexpressed and/or induced during cytokine storm following chemotherapy exposure. It must be remembered that TK6 cells are B lymphoblastoid in nature and might be expected to also secrete cytokines. However, extensive literature searching has uncovered limited information on this, and to date, secretion of IFNγ, IL-29, LIF, and TNFSF4 from TK6 cells has been confirmed [85].

Genotoxic analysis of human cytokines has shown that IFNα/β/γ, TNFα, and IL-2 can induce chromosome aberrations, MN, and sister chromatid exchanges (SCE) in cultures [21,28]. Furthermore, there is currently controversy over the use of G-CSF in mobilizing HSC from donors for subsequent transplantation, with some asserting that G-CSF has the capacity to induce haematological malignancy in the donor [51], whereas others assert its safety [86]; however, the topic is still widely debated due to flaws in testing and study design [87]. We tested the direct genotoxicity of the five candidate cytokines using the *in vitro* MN assay, which is a well-established technique to detect clastogenic and aneugenic chromosomal mutations transmitted to daughter cells during cell division [88]. MN scoring is used as a direct measure of DNA lesions and genotoxicity of chemicals [89,90], as well as representing a reliable biomarker for cancers and other degenerative diseases [91].

Utilizing recorded concentrations from healthy individuals, our data supported the expected safety of IL-6, G-CSF, and GM-CSF, whereas TNFα and TGFβ1 had the capacity to non-significantly increase MN levels more than twice the untreated control, in line with recorded observations of their known links with genotoxicity [49] and carcinogenicity [50]. The good viability of the cells in the presence of these outcomes—while expected for biological molecules—would be of concern as this might promote the inheritance of genotoxic/mutagenic lesions in daughter cells, in view of the observation that all five cytokines produced MN at more than twice the untreated control at recorded cytokine storm levels (Fig. 5).

As stated in the literature, GM-CSF and G-CSF are key cytokines for myeloid lineage development and do not induce MN at their baseline levels. However, at the highest storm levels (2000–4000 pg/ml) both tend to increase the MN expression about 3-fold above the PBS control. This is an interesting factor to consider since both GM-CSF and G-CSF play a major role during HSCT, and stimulation with recombinant G-CSF alone or in combination with GM-CSF dramatically increases the release of HSCs from the BM to the peripheral blood in donors for BM transplantation [8,92,93]. This contributes to the HSC mobilization debate, and queries how these cytokines might affect the donor if both G-CSF and GM-CSF can cause MN at higher dosage/storm levels. Petros *et al*. studied the pharmacokinetics of Filgrastim (recombinant G-CSF) in 21 patients receiving 14-day continuous I.V infusion, and in 10 patients receiving daily 4-h infusions [94]. In the 10 patients, the C_{max} ranged between 68.6 and 1635 ng/ml, which ranges approximately 17–400 times more than the highest storm dose used within our study. They noted severe toxicities in some of the patients associated with these plasma concentrations, and in combination with our data offer insights into the current controversy over the use of high-dose G-CSF in donors.

Of note, TGF-β1 at both baseline (100–1000 pg/ml) and storm levels (2000–4000 pg/ml) showed 2- to 3-fold increase in MN formation compared to the PBS control despite little evidence of any cytotoxicity. A recent meta-analysis of *in vivo* cancer cases supports these fold increases, demonstrating an increase of 2.3-fold or more of lymphocyte MN in cancer cases versus controls [91]. TGF-β1 is a key candidate involved in controlling cellular apoptosis and proliferation; thus, if TGF-β1 can be potentially genotoxic to the cells at even the lowest level (1000 pg/ml), there is a high risk for continuous proliferation of genotoxic (mutated) cells in the system after exposure to chemotherapy. Furthermore, the literature highlights post-transplant complications and has correlated transplant rejection with TGF-β1 polymorphism [25,26,81], indicating the need for further study of TGF-β1 secretion related to genetic profiles for individuals.

Within a 'cytokine storm' (also known as 'cytokine release syndrome'; [5,20]), cytokines are in a complex mixture and may inhibit or synergize each other with respect to functional activity and/or genotoxicity. Therefore, it was important to assess cytokines in combination. Figure 6 demonstrates increased MN in TK6 following exposure to paired cytokines at high baseline and high storm dosage levels. In comparison to the single treatments, cocktail treatment further induced MN for every concentration used, with the notable exception

that at 3000 pg/ml TNFα, the addition of the other four cytokines reduced the MN count below that of TNFα in isolation. Here, the data are complex to interpret. We noted that four of the five cytokines significantly raised the MN above the untreated control and were also more genotoxic (and had better RPD) than the genotoxic positive control MMC for certain combinations. Furthermore, MN counts for IL-6, GM-CSF, and TGFβ1 in isolation, could be statistically increased in certain combinations, but the overall picture was that most combinations were less than additive for MN induction. Cytokines with the most synergistic events were GM-CSF, G-CSF, and TGFβ1, all of which had six synergistic events out of the 16 paired events. TNFα and IL-6 both had four synergistic events each. Further to this, 2 of 16 events were additive for IL-6 (4000 pg/ml IL-6 with both 1000 pg/ml GM-CSF and 3000 pg/ml TGFβ1), whereas TNFα, G-CSF, and TGFβ1 all had one additive event each. Thus, there is a balance of clearly strongly genotoxic events (higher than MMC), with an overall picture that the MN is collectively 'reduced' (not additive) when cytokines were used in combination. To further complicate this observation, recent studies have demonstrated the role of the cGAS-STING pathway [61], where disruption of the MN membrane at interphase [59] leads to the release of DNA fragments and induction of interferon release, which may further contribute to overall genotoxicity [60] if interferons are shown to further potentiate the observations seen here. There is limited literature on cytokine secretion from TK6 cells, but Glover *et al.* [85] detected IFN-γ release 24 h after exposure to UVC light and 12-O-tetradecanoylphorbol-13-acetate (TPA). Interferons have been shown to cause genotoxicity [28], so this may have contributed to the observed genotoxicity, although our data suggest that overall, cytokine combinations are less than additive. It is possible that more complex combinations would, therefore, further complicate the picture, and it is likely that *in vivo*, detoxification processes might counteract some of the MN events observed; otherwise, all cytokine storm patients might be expected to develop further malignancy which is not the case [12,24]. Nevertheless, as mentioned previously, in complex multicellular environments, we cannot ignore the contribution of immune cells and the production of ROS, as well as inter-individual differences in overall cytokine secretion, which clearly plays a role. Furthermore, the BM microenvironment has been shown to be low in glutathione levels [95], but levels can be induced following chemotherapy dependent on polymorphic status [96]. The BM is also metabolically competent, and these functions might be expected to abrogate MN events *in vivo* [97]. These complexities and observations, coupled with clinical outlooks for patients, support the recent advances in 3D multicellular models to better reiterate the *in vivo* microenvironment [48,98].

Finally, it should be noted that within these data, we have focussed on the induction of MN as a genotoxic endpoint, as these were the main events we observed within an *in vitro* model of CIBE. It remains to be seen if cytokines have the capacity to induce other genotoxic endpoints and the contribution this might make to an individual's quality of life.

Conclusions

Currently, it is unknown why DCL occurs, what pathways are involved, and most importantly, why some people get DCL but others do not. Here, we have explored the possibility that cytokines released from chemotherapy-exposed BM cells may play a role in the aetiology of DCL.

For the first time, we have performed a wide profile of cytokine secretion from HS-5 cells as a model of healthy versus chemotherapy-treated BM cells, reiterating an increase in cytokine secretion in response to chemotherapy. Given that the most highly secreted cytokines from the BM cells, both at baseline and following chemotherapy, promote myeloid differentiation, it may be reasonable to suggest that cytokines could influence the occurrence of DCL as predominantly AML. Furthermore, we have shown that direct exposure to the evaluated myeloid cytokines in isolation at storm doses, as well as combinations of high 'healthy' doses can induce MN at levels close to, or exceeding, known genotoxic compounds. While these data may infer a role in the promotion of leukaemogenesis following HSCT, the occurrence of DCL is currently not considered to be as frequent as the presence of cytokine storm, suggesting that inter-individual levels of secretion coupled with other detoxification processes need to be fully explored to predict risk.

Acknowledgements

The authors gratefully acknowledge family funding to support HSHA through PhD research; thanks to Deepal Dewapura Dewage and Mr. & Mrs. Hemasiri. Sincere thanks to Dr Victoria Davenport for the helpful suggestions and ideas during the preparation of this manuscript and to Mr. David Corry and Mrs. Alison Halliday for technical assistance during the execution of these data.

Conflict of interest statement: None declared.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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