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Research paper

Green tea polyphenol "epigallocatechin-3-gallate", differentially induces apoptosis in CLL B-and T-Cells but not in healthy B-and T-Cells in a dose dependant manner

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ABSTRACT

B-cell chronic lymphocytic leukaemia (CLL) is characterized by an accumulation of CD5-positive monoclonal B-cells due in large part to a failure of apoptosis. The ability to study CLL B-cells in vitro has always been a challenge and hampered by the low viability of the CLL B-cells in cell culture systems. In this study, we present a multicellular cell culture system to maintain CLL B-cells viable in culture for 60 h in the presence of a stromal cell feeder layer in combination with a whole white blood cell preparation. Using this optimized system, we tested and showed that the addition of epigallocatechin-3-gallate (EGCG) at concentrations ranging from 25 to 100 μg/ml induced apoptosis in CLL B-cells whilst not affecting healthy control B-cells. Moreover, the results showed that in contrast to healthy controls, T-cells from CLL patients underwent apoptosis in the presence of EGCG. This study demonstrated that the combination of a cell feeder layer with a whole white blood cell preparation maintained B-cell viability *in vitro* over an extended period of time. In addition, the study showed that EGCG differentially induces apoptosis in CLL B-and T-Cells but not in healthy B-and T-Cells in a dose dependent manner.

1. Introduction

Chronic lymphocytic leukaemia is the most common type of leukaemia in Western countries with an annual incidence of approximately 4/100,000 population [1]. The median age at diagnosis is approximately 70 years and it is more common in men. The disease is characterized by a clonal proliferation of CD5-positive B-cells in blood, bone marrow and lymphatic organs. Typically, the clonal B-cells display features consistent with a failure of apoptosis and this is considered a hallmark of malignant CLL B-cells [2,3]. CLL follows a variable clinical course with some patients having indolent disease not requiring treatment for many years while others progress more rapidly. Treatment is reserved for symptomatic disease and there is no defined benefit for treating in the earlier stages of disease. However, an intervention which could delay disease progression for those patients with early stage disease would be valuable, particularly if the side-effect profile was favourable and, in this context, green tea compounds have been studied [4–7].

Epigallocatechin-3-gallate (EGCG), the most predominant and biologically active catechin found in green tea (*Camellia sinessis*) has been postulated as an anti-cancer therapy agent [8,9] with in-vitro studies showing its anti-proliferative and pro-apoptotic capabilities [10–12]. EGCG inhibits cell growth or induces cell death in various tumour cell lines by affecting many different biochemical pathways such as the ability to induce apoptosis in human fibrosarcoma HT-1080 cells [12], suppression of proliferation in human A431 epidermoid carcinoma cells [13] and down-regulation of vascular endothelial growth factor expression in human colon cancer cells [14]. In addition, EGCG can modulate epidermal growth factor receptor and platelet-derived growth factor-BB, which have been both implicated in tumourigenesis [13,15–18]. Alternatively, EGCG can induce apoptosis through various mechanisms, including modulation of the expression of BCL-2 proteins [19–26], the induction of caspase independent apoptosis [11] or by permeabilisation of the lysozomal membrane [27]. In addition, animal models have demonstrated a protective effect of EGCG with oral administration inhibiting prostate cancer cell growth [28].

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In the present study, we developed an *in vitro* model to assay the effect of compounds such as EGCG against CLL patient B-cells over a 60 h period. In doing so, we showed several factors from different cell compartments are required to maintain *in vitro* B-cell viability over an extended period of time. In addition, we demonstrated the apoptosis-inducing effect of EGCG is specific to CLL patient B-cells in the presence of these same cell compartments. However, our data further suggests that CLL impairments may not be limited to the B-cell compartment and that further studies are needed to determine to what degree other white blood cells are impaired.

2. Materials and methods

2.1. Patients

Approval to conduct the study was obtained from the Sir Charles Gairdner Hospital Human Research Ethics Committee (HREC# 2012-045). Peripheral blood samples from 4 patients with untreated CLL were obtained from the Charles Day Tissue Bank (HREC # 2008-108). Peripheral blood samples collected in EDTA vacutainer tubes were processed to remove red blood cells. Briefly, red blood cells were lysed for 10 min in BD Pharm Lyse (BD, USA) then washed twice in Hank's Balance Salt Solution (HBSS) (Thermo Fisher Scientific, USA). White blood cells were cryogenically stored in liquid nitrogen for later use. Control samples were obtained from healthy volunteers following informed consent and processed and stored using the same protocol.

2.2. Cell lines

The HS-5 human bone marrow stromal cell line was used in this study. The cell line was maintained in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, USA) containing 1.5 g/L $NaHCO_3$, 10% Foetal calf serum and 1% Penicillin-Streptomycin in a 37 °C, 5% CO_2 incubator. Collection of HS-5 conditioned media (HS-5CM) was performed by replacing the media with fresh RPMI 1640 (Thermo Fisher Scientific, USA) media containing 1.5 g/L $HaHCO_3$, 10% Foetal calf serum and 1% Penicillin-Streptomycin when the cell line had reached 80% confluency and collecting the media after 24 (24hrHS-5CM), 48 (48hrHS-5CM) and 72 (72hrHS-5CM) hours.

2.3. B-cell isolation

Isolation of patient and healthy control B-cells was carried out using the B-CLL Cell Isolation Kit by Miltenyi Biotec (Germany). Briefly, cryogenically preserved CLL white blood cells (WBC) were thawed rapidly and counted. Up to 1×10^7 B-cells were negatively selected for from 5×10^7 white blood cells by magnetic separation using antibodies against non B-cells (CD2, CD4, CD11b, CD16, CD36, Anti IgE and CD235a). B-cell purity was determined by staining cells for CD3-FITC (clone UCHT1), CD5-PE (clone UCHT2), CD45-PerCP-Cy5.5 (clone HI30), CD19-PE-Cy7 (clone HIB19) and CD20-APC (clone 2H7). Cells were incubated for 30 min at room temperature in the dark, washed in HBSS and cell surface expression was measured using a BD FACSCanto II flow cytometer and DIVA software.

2.4. Apoptosis analysis

HS-5 cells were plated in RPMI 1640 media at a concentration of 1×10^6 cells/well in a 12-well plate or 5×10^5 cells/well in a 24-well plate and grown for 8 h. An equal number of wells were prepared with RPMI 1640 media alone. Following incubation either isolated B-cells or total WBCs were added to the wells at a final whole cell concentration of either 2×10^6 cells/well or 1×10^6 cells/well in a 12-well or 24-well plates respectively. EGCG was added to the wells to give a final concentration of 0, 25, 50, 75 or 100 μg/ml. At various time points, both adherent and non-adherent cells were collected and stained with CD3-APC (clone SK7), CD56-PE-Cy7 (clone B159), CD14-APC-H7 (clone MφP9), CD19-V450 (clone SJ25C1), FITC-Annexin V and Propidium Iodide (PI) for 15 min. Cells were analysed by using a BD FACSCanto II flow cytometer and DIVA software. Lymphocytes were first gated for by size (FSC) and granularity (SSC). Specific lymphocyte populations were identified and gated by expression of CD3 (T cells), CD56 (NK cells), and CD19 (B cells). AnnexinV/PI staining was used to gate the various lymphocyte subpopulations into viable, early apoptotic or late apoptotic cells.

2.5. Statistics

Data was presented as the mean ± standard error of the mean (SEM). Student's *t*-test was used to compare the effect of EGCG at various time points to the untreated control. A p value of <0.05 was regarded as significant.

3. Results

3.1. HS-5 cells improves survival of isolated B-cells in vitro

Prior to evaluating the effect of EGCG on the in vitro survival of B-cells, the study required the maintenance of a B-cell population for at least 48 h. Initial culturing of isolated healthy and CLL patient derived B-cells in RPMI 1640 culture media observed rapid cell death with a loss of 90% of viable cells within 48 h (Fig. 1A). Replacement of RPMI 1640 culture media with HS-5 conditioned media (HS-5CM) improved survival over 48 h, with 45% of healthy controls B-cells (Fig. 1B) and 35% of CLL B-cells (Fig. 1C) surviving. Similarly survival of healthy B-cells was improved to 45% after culturing B-cells in the presence of HS-5 cells feeder layer separated by a Transwell membrane (Fig. 1D). However, direct co-culturing without Transwell membranes of healthy B-cells with the HS-5 cells feeder layer further improved survival, with 55% of B-cells viable after 48 h incubation with 0.5×10^6 HS-5 cells and 70% viable with 1.0×10^6 HS-5 cells (Fig. 1D).

3.2. EGCG induces apoptosis in isolated CLL B-cells and healthy control B-cells

Isolated B-cells, co-cultured with 0.5×10^6 HS-5 cells were exposed to various concentrations of EGCG (0, 25, 50, 75 or 100 μg/ml) for 60 h with apoptosis measured by Annexin V/PI staining. Approximately 40% of isolated healthy B-cells underwent apoptosis over 60 h when not exposed to EGCG. This increased in a dose dependent manner to 70% cell death at a concentration of 100 μg/ml EGCG (Fig. 2A). Approximately 25% of CLL B-cells underwent apoptosis over 60 h in the absence of EGCG and this increased in dose-dependent manner to 65% at 100 μg/ml EGCG (Fig. 2B).

3.3. Significant survival improvement of B-cells in the presence of both HS-5 feeder layer and whole white blood cell preparations

Although HS-5 feeder layer cells improved the survival of both CLL and healthy isolated B-cells, a significant proportion of cells died in culture over a 60 h period. We postulated that to maintain a higher rate of survival of B-cells *in vitro*, they require similar micro-environmental interactions as experienced *in vivo*. To investigate this, whole white blood cell preparations (whole blood following red blood cell lysis) were cultured *in vitro* for 60 h with or without HS-5 cells feeder layer. In the absence of an HS-5 feeder layer (RPMI 1640 only), healthy control B-cells and CLL B-cells showed a significant reduction in viability, whilst in the presence of an HS-

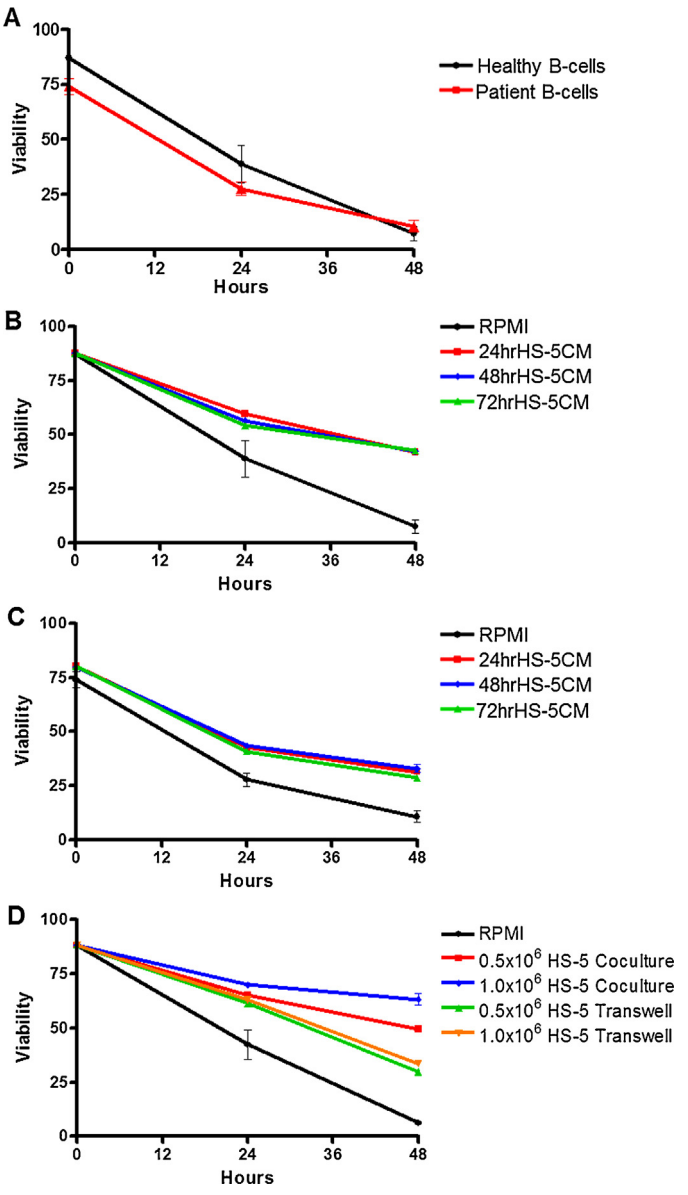

Fig. 1. HS-5 cell-B-cell interaction improves in vitro survival of isolated B-cells. Isolated B-cells from healthy controls and CLL patients were cultured in-vitro for 48 h. At various time-points, cells were collected and stained with AnnexinV/PI for analysis by flow cytometry. Pooled data showing viability of healthy (n = 4) and patient (n = 5) B-cells over 48 h in RPMI media alone (A), healthy (n = 2) B-cells in conditioned media (B), patient (n = 3) B-cells in conditioned media (C) and healthy (n = 2) B-cells co-cultured with HS5 cells (D). Pooled data is shown as mean ± SEM.

5 feeder layer, healthy control B-cells and CLL B-cells maintained their viability over the 60 h period (Fig. 3A and B).

3.4. EGCG differentially induces apoptosis in CLL B- and T-cells but not in healthy control B-and T-cells in whole white blood cell preparations

Whole white blood cells were cultured in the presence of HS-5 cells for 60 h with increasing doses of EGCG (0, 25, 50, 75 or 100 μg/ml). In healthy control B- and T-cells, there was minimal effect of increasing doses of EGCG on the induction of apoptosis (Fig. 4A and C). In contrast, in CLL B- and T-cells, increasing doses of EGCG resulted in apoptosis induction in a dose dependent manner, with approximately 90% of B-cells and virtually all T-cells entering apoptosis at the highest concentration of 100 μg/ml (Fig. 4B and D).

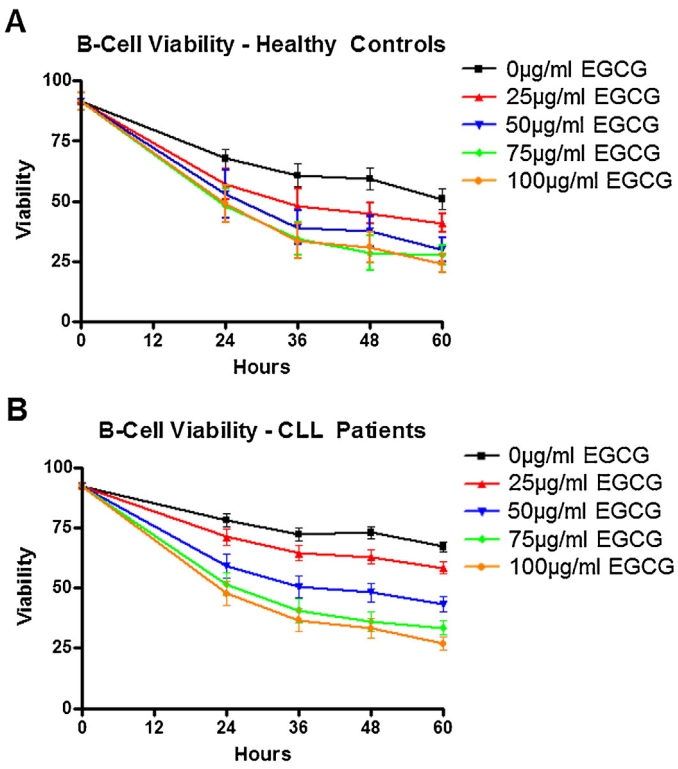

Fig. 2. EGCG induces in vitro apoptosis in both CLL patient and Healthy control B-cells. Isolated B-cell from healthy controls and CLL patients were co-cultured with HS-5 cells and exposed to various concentrations of EGCG for 60 h. At various time-points cells were collected and stained with PI/Annexin V for flow cytometric analysis of viability. Pooled data showing viability of healthy control B-cells (A: n = 2) and patient control B cells (B: n = 8). Pooled data is shown as mean ± SEM.

The viability of NK-cells from healthy controls was mildly reduced over the 60 h period, particularly at the highest EGCG concentration of 100 μg/ml (Fig. 4E). In contrast, NK-cells from CLL patients could not be maintained using this system (Fig. 4F). A detailed statistical analysis of the summary data presented in Fig. 4 is shown in Table 1.

4. Discussion

The primary aim of this study was to develop an *in vitro* model to investigate the effect of compounds such as EGCG on the viability of B-cells from CLL patients. Previous studies investigating the effect of green tea extracts on leukaemic cells typically represent viability as a percentage of control [10,29]. Unfortunately due to the spontaneous apoptosis of B-cells in culture, this model is not suitable for long term studies due to the loss of total B-cell numbers. The phenomenon of spontaneous apoptosis is interesting, as *in vivo* these B-cells are long lived and resistant to programmed cell death. Several studies have indicated that exogenous factors or interactions from various cell types have the capacity to prolong their survival. These include mesenchymal bone marrow stromal cells, nurse-like-cells [30] and follicular dendritic cells [31].

Similar to studies by Ghamlouch et al. [32] and Collins et al. [33] the B-cells from the CLL patients used in this study underwent apoptosis rapidly in culture. However, this effect was not limited to the CLL patient B-cells, as healthy B-cells also died at a similar rate. This data would indicate that it is not the malignant status of the B-cells that determines *in vitro* death, but rather an inherent B-cell trait in this environment. As previously mentioned, bone marrow stromal cells have been used to maintain the survival of B-cells from CLL patients [34–36]. Indeed in our study survival was shown

Table 1
Statistical analysis of the effect of EGCG on the viability of B-cells, T-cells and NK-cells from healthy volunteers and CLL patients.

Time	EGCG Concentration	B-Cells		T-Cells		NK Cells	
		Healthy	CLL	Healthy	CLL	Healthy	CLL
6 h	25 μg/ml	0.1313	0.4416	0.5265	0.4501	0.6306	0.2593
	50 μg/ml	0.1153	0.5272	0.315	0.0946	0.3247	**0.0455**
	75 μg/ml	**0.0170**	0.5041	0.5872	**0.0171**	0.2293	0.0683
	100 μg/ml	0.0658	0.7776	0.1482	**0.0190**	0.2673	**0.0253**
2 h	25 μg/ml	0.7296	**0.0054**	0.0745	0.3644	0.5051	0.1454
	50 μg/ml	**0.0051**	0.5625	0.6365	0.0579	0.3468	0.1081
	75 μg/ml	0.2775	0.3699	0.1939	0.0753	0.334	0.1748
	100 μg/ml	**0.0274**	**0.0003**	0.0991	**0.0096**	0.2598	**0.0415**
24 h	25 μg/ml	0.9322	0.0755	0.3917	0.0741	0.9601	0.1641
	50 μg/ml	0.7741	**0.0052**	0.1917	**0.0090**	0.8369	**0.0455**
	75 μg/ml	0.2356	**0.0029**	0.0369	**0.0065**	0.8038	0.0501
	100 μg/ml	0.245	**0.0004**	0.0675	**0.0008**	0.6789	**0.0226**
36 h	25 μg/ml	0.224	0.0882	0.767	**0.0340**	0.9366	0.0651
	50 μg/ml	0.4736	**0.0062**	0.5597	**0.0107**	0.9811	**0.0125**
	75 μg/ml	0.3804	**0.0010**	0.2536	**0.0047**	0.6983	**0.0046**
	100 μg/ml	0.2031	**0.0002**	0.2269	**0.0004**	0.6462	**0.0040**
48 h	25 μg/ml	0.9427	**0.0088**	0.0815	**0.0146**	0.7996	0.0629
	50 μg/ml	0.28	**0.0022**	**0.0222**	**0.0023**	0.6172	**0.0100**
	75 μg/ml	0.1372	**0.0025**	**0.0209**	**0.0018**	0.3863	**0.0073**
	100 μg/ml	0.0262	**<0.0001**	**0.0020**	**0.0004**	0.2443	**0.0068**
60 h	25 μg/ml	0.686	**0.0213**	0.3746	**0.0011**	0.8853	**0.0196**
	50 μg/ml	0.3695	**0.0153**	0.0676	**0.0015**	0.6114	**0.0109**
	75 μg/ml	0.0597	**0.0016**	**0.0449**	**0.0005**	0.3812	**0.0061**
	100 μg/ml	**0.0217**	**<0.0001**	**0.0029**	**<0.0001**	0.2744	**0.0044**

The data points represent p values for the comparison of the indicated time point and EGCG concentration with time point 0/EGCC concentration 0 μg/ml. Significance determined by Student's T test with p values < 0.05 shown in bold.

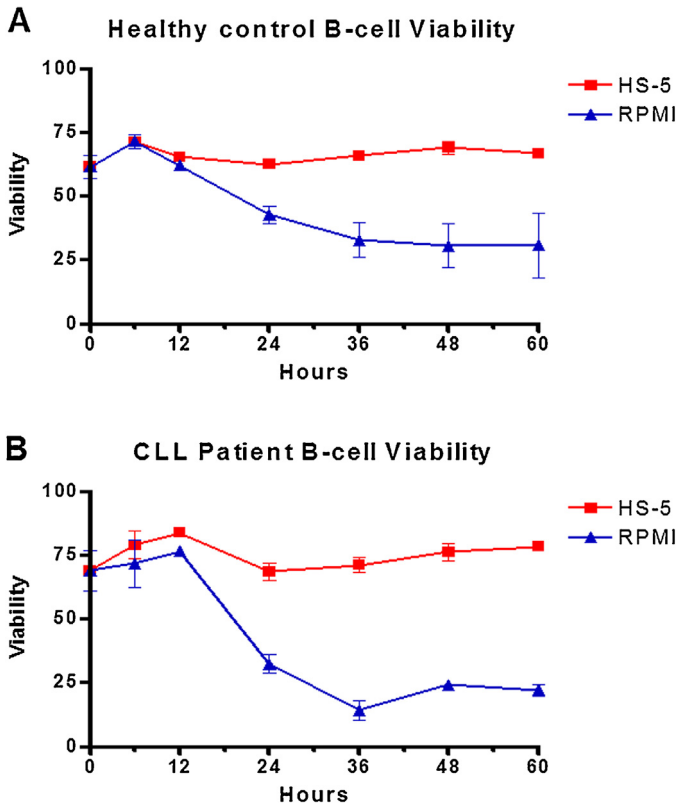

Fig. 3. In vitro survival of B-cells is improved in the presence of autologous white blood cells. White blood cells from CLL patients and healthy controls were cultured in vitro for 60 h in the presence or absence of HS-5 cells. At various time-points cells were collected and stained with CD19, Annexin V and PI for flow cytometric analysis of apoptosis of CD19+ B-cells. Pooled data showing viability of healthy (A: n = 6) and patient (B: n = 6) B-cells with or without co-culturing with HS5 cells. Pooled data is shown as mean ± SEM.

to improve, but the improvement was not as dramatic as seen in the previously published studies. Culturing of either healthy B-cells or CLL B-cells with HS-5 conditioned media resulted in improvements in survival from 10% to 35–50% at 48 h. In contrast, Schulz et al. [35] observed 85% viability of patient B-cells at 168 h. It should be noted that in the study by Schulz et al., the viability is shown to be at 100% at time 0, but it is not clear if this represents data normalized to a control, which could potentially explain the difference from our study. Regardless, the greatest survival increase in B-cells was observed when B-cells were co-cultured directly with HS-5 cells whilst the use of a transwell membrane yielded survival results similar to using conditioned media alone. This would indicate a direct interaction between the stromal cells and the B-cells aiding survival. This could be mediated by receptor crosstalk between the two cell types or the HS-5 cells could be acting similar to "nurse-like cells" [30].

Previously, EGCG has been shown to inhibit tumour cell growth as well as induce apoptosis in various cell lines. In our study, using the HS-5 co-culture system, administration of EGCG to isolated B-cells induced a dose dependent apoptosis irrespective of whether the B-cells were from a healthy individual or a CLL patient. Whilst the effect of EGCG on CLL B-cells has been previously shown by Lee et al. [11], the effect on healthy cells is a novel observation. This data would indicate that whilst HS-5 cells offer a protection of B-cells from spontaneous apoptosis, EGCG either reverses the protection or activates an alternative pathway leading to apoptosis. Interestingly, it should be noted that EGCG did not induce apoptosis in the HS-5 cells themselves (data not shown).

As the data showed, even culturing B-cells with the HS-5 cells only allowed for apoptotic free survival of approximately 60% of the cells. This data would indicate that further pro-survival factors are required to maintain B-cell viability. The most logical source of these other factors would be cells contained within the B-cells normal microenvironment. Indeed, when B-cells are left unsorted and cultured in vitro, survival is improved by the presence of the other white blood cells. Improved survival was seen for both healthy and CLL B-cells. This data agrees with previous studies showing that cytokines such as IL-4 (produced by T-cells) and BAFF (produced

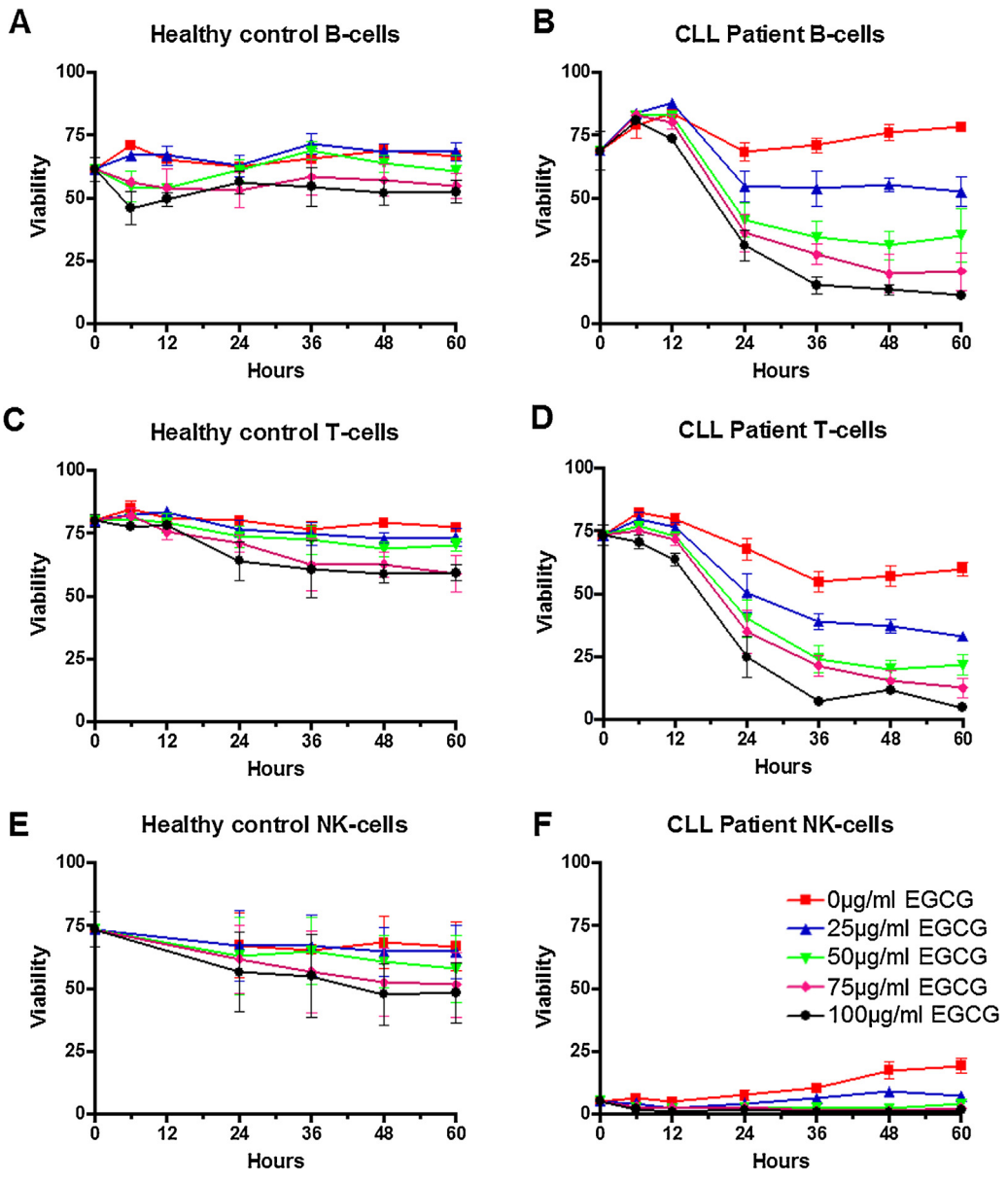

Fig. 4. White blood cells from healthy controls and CLL patients were co-cultured with HS-5 cells and exposed to various concentrations of EGCG for 60 h. At various time-points cells were collected and stained for analysis by flow cytometry. Leukocytes were gated by size (FSC) and granularity (SSC) as per Fig. 3A. Pooled data showing viability of healthy control and CLL patient B-cells (Fig. 4A, B), T-cells (Fig. 4C, D) and NK-cells (Fig. 4E, F). Pooled data is shown as mean ± SEM.

by nurse-like cells) have the capacity to improve survival significantly [32]. Interestingly, by culturing the complete white blood cells populations with the HS-5 feeder layer, apoptosis was arrested for both healthy and CLL B-cells indicating that both the white blood cell populations and the HS-5 feeder layer produce different factors which complement each other to maintain overall cell survival.

The complete abrogation of both CLL and healthy B-cell death using the combination of HS-5 feeder layer cells and white blood populations provides an excellent model for testing compounds such as EGCG. Similar to studies using various cancer cell lines [11,12,15,17,18], EGCG was capable of inducing a dose dependent cell death in primary CLL B-cells. Interestingly, in contrast to earlier results, when white blood cells are present, B-cells from healthy donors were not significantly affected. This would indicate that the survival factors produced by one or several of the other white blood cell types protect healthy B-cells but not CLL B-cells from EGCG-induced apoptosis. Furthermore whilst the healthy T-cells do not appear to be significantly affected by the presence of EGCG, the patient T-cells undergo apoptosis in a dose dependent manner.

Interestingly, NK cells showed the greatest difference between healthy samples and CLL samples. The NK cells from healthy individuals showed initial viability similar to the other observed cells. In contrast, the CLL NK cells were observed to have an inherently low viability even at time 0. This low viability has been previously observed by MacFarlane et al. [37] and could partially explain why previous studies show a low functional activity in CLL NK cells [38,39].

In conclusion, these studies demonstrate a robust *in vitro* model for investigating the effect of compounds on CLL B-cells. Specifically, we showed that long term culturing of CLL B-cells requires both a feeder layer cell line (such as the bone marrow stromal cell line HS-5) as well as other white blood cell populations. In this system, EGCG selectively induces apoptosis in a dose-dependent manner in CLL B-cells and suggests these cells have an intrinsic susceptibility to this compound as compared to healthy B-cells. The

finding that EGCG also selectively induces apoptosis in the T-cells from CLL patients raises the possibility that the apoptosis seen in CLL B-cells may be at least partially mediated through loss of T-cell support. This was an unexpected finding and warrants further investigation.

Contributions

Cornwall performed the experiments, collected the data, and drafted the manuscript. Joske provided the study material and patients and critically revised the manuscript. Cull provided the study material and patients, analysed the data and critically revised the manuscript. Ghassemifar designed the research, analysed and interpreted the data and revised the manuscript.

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