Immunity in young adult survivors of childhood leukemia is more similar to elderly rather than age-matched controls: Role of cytomegalovirus (CMV)

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Abbreviations

Childhood cancer survivor (CCS); cytomegalovirus (CMV); immunoglobulin G (IgG); interleukin-6 (IL-6); soluble CD14 (sCD14); high sensitivity CRP (hsCRP); soluble CD163 (soluble CD163), interquartile range (IQR); lipopolysaccharide (LPS)

Abstract

Many treatment complications which occur late in childhood cancer survivors resemble age-related co-morbidities observed in the elderly. An immune phenotype characterized by increased immune activation, systemic inflammation and accumulation of late-differentiated memory CD57⁺CD28⁻ T cells has been associated with co-morbidities in the elderly. Here we explored if this phenotype was present in young adult leukemia survivors following an average of 19 years from chemotherapy and/or radiotherapy completion, and compared this with that in age-matched controls. We found that markers of systemic inflammation – IL-6 and human C-reactive protein (hCRP) and immune activation – CD38 and HLA-DR on T cells, sCD163 from monocytes and macrophages – were increased in survivors compared to controls. T-cell responses specific to cytomegalovirus (CMV) were also increased in survivors compared to controls while CMV IgG levels in survivors were comparable to levels measured in the elderly (>50 years) and correlated with IL-6, hCRP, sCD163 and CD57⁺CD28⁻ memory T cells. Immune activation and

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inflammation markers correlated poorly with prior chemotherapy and radiotherapy exposure. These data suggest that CMV infection/reactivation is strongly correlated with the immunological phenotype seen in young childhood leukemia survivors and these changes may be associated with the early onset of age-related co-morbidities in this group.

Introduction

Childhood cancer survivors (CCS) have an increased risk of second neoplasms and co-morbidities including cardiovascular disease, endocrine dysfunction, renal insufficiency, abnormal lung function and neurological complications [1, 2]. A study in the St Jude Lifetime cohort also reported that the prevalence of frailty and pre-frailty in young CCS (mean age 35 years) were comparable to that of controls who were aged 65 years and older [3]. In addition, CCS have also been associated with an increased risk of multiple infections [4], increased hospital admissions as well as increased length of hospital stay [5, 6]. Collectively, this suggests that the morbidities experienced in young adult CCS are remarkably similar to the spectrum of diseases seen in the elderly.

The increased risk of co-morbidities in CCS has largely been attributed to chemotherapy- and radiation-induced damage to specific organs [7, 8] and thus
referred to as long-term complications of cancer treatment. Postulated reasons for the development of these co-morbidities include hormone deficiencies as a result of steroid use and damage to the hypothalamus-pituitary axis following cranial radiotherapy; direct chemotherapy-induced organ damage including anthracycline-induced cardiomyopathy and vincristine-induced neuropathy; and reduced physical activity due to poor self-perception (reviewed in [8]). Most studies assessing these long-term complications in CCS have focused predominantly on the association of these co-morbidities with prior exposure to specific classes or intensity of radio- and chemotherapy [2, 9]. To date, the exact cellular and molecular mechanisms contributing to the occurrence of these co-morbidities are largely unknown impeding the development of targeted interventions to mitigate this problem.

Studies in the elderly population have described an association between mortality and the development of age-related co-morbidities with an immune phenotype of aging characterized by increased circulating inflammatory cytokines, an inverted CD4:CD8 ratio, increased T cell senescence, accumulation of terminally differentiated T cells and shortened telomere length [10-12]. Many of these immunologic changes in the elderly have been attributed to a lifetime increase in antigenic burden including cytomegalovirus (CMV) exposure [12]. Whether a similar immune phenotype is associated with the development of co-morbidities in CCS has yet to be demonstrated. We explored if an immune phenotype of aging was present in young adult survivors of childhood leukemia compared to age-matched controls and its association with chemo/radiotherapy and CMV exposure. The immunological changes measured in these survivors were also compared to levels
measured in community-dwelling elderly individuals (>50 years) as well as young adult HIV-infected individuals, a population well described to experience an early onset of age-related co-morbidities similar to CCS (reviewed in [13]).

Results

Eighty-one leukemia survivors and 71 age-matched controls were recruited. The median (IQR) age in survivors and controls were 26 (22-30) and 24 (23-27) years, respectively (Table 1). All survivors were treated following the German Berlin-Frankfurt-Munster (BFM) protocol and the median (IQR) duration from treatment completion to recruitment was 19 (14-22) years. Compared to age-matched controls, survivors had higher BMI 23.5 (20.7-27.8) vs 22.1 (20.4-24.9) kg/m², \(P=0.028\); increased triglycerides 1.10 (0.80-1.55) vs 0.80 (0.60-1.20) mmol/L, \(P<0.001\); fasting glucose 5.1 (4.8-5.8) vs 4.9 (4.7-5.2) mmol/L, \(P=0.002\), and lower high density lipoprotein levels, 1.31 (1.12-1.54) vs 1.54 (1.35-1.80) mmol/L, \(P<0.001\). There was also a greater proportion of smokers/ex-smokers (15% vs 6%, Chi-square=3.90, \(P=0.048\)) among survivors. Five percent of survivors reported a pre-existing comorbidity at recruitment compared to none among the controls (Table 1).

As a comparator group, community-dwelling elderly (n=27, median (IQR) age=61 (55-71) years) and HIV-infected individuals on stable antiretroviral treatment (n=30, median (IQR) age = 33 (31-34) years, median (IQR) CD4⁺ T cell counts = 541 (477-717) cells/µl, HIV RNA <50 copies/ml in all) were recruited (Table 1). The detailed
biochemical laboratory results for the cohort are shown in Supporting Information table 1.

**Immune activation and systemic inflammation are increased in young adult CCS**

We first compared the levels of immune activation in the adaptive immune compartment as measured by the co-expression of CD38 and HLA-DR on T cells in whole blood. The proportion of activated, CD38^+HLA-DR^+ CD4^+ and CD8^+ T cells were significantly higher in survivors compared to age-matched controls (P<0.0001 for both comparisons, Figure 1A - B, respectively) and notably comparable to the levels measured in the community-dwelling elderly individuals in the CD4^+ T cell subset (P=0.6882) but lower for CD8^+ T cells (P=0.0001). The activation levels were significantly lower for both T cell subsets in the survivors compared to HIV-infected individuals (P=0.0003 for CD4^+ T cells and P<0.0001 for CD8^+ T cells).

A similar profile was seen in the innate immune compartment. The proportion of activated NK cells (co-expressing CD38 and HLA-DR) were significantly higher in survivors compared to controls (P<0.0001; Figure 1C) but lower when compared to HIV-infected (P<0.0001) and elderly individuals (P=0.0099). Soluble CD163, a marker of monocyte/macrophage activation following exposure to inflammatory cytokines was also significantly higher in survivors compared to controls (P<0.0001,
Figure 1D). Conversely, the lipopolysaccharide (LPS)-binding receptor, sCD14 which is shed from monocytes following activation with LPS, was not different in survivors compared to controls ($P=0.6724$, Figure 1E) and levels in survivors were significantly lower compared to HIV-infected individuals ($P<0.0001$) and community-dwelling elderly individuals ($P=0.0080$).

We also measured levels of IL-6 and hs-CRP which are markers of systemic inflammation. The median levels of IL-6 and hsCRP were significantly higher in survivors compared to controls ($P=0.0117$ and $P<0.0001$, respectively; Figure 2A - B). The CD16$^+$ monocyte subsets have an increased capacity to produce pro-inflammatory cytokines and has been used as a surrogate to measure inflammation in HIV-infected and uninfected individuals [14]. Young adult leukemia survivors had marginally higher proportions of CD16$^+$ monocytes compared to age-matched controls but this did not reach statistical significance ($P=0.1093$; Figure 2C). The proportion of CD16$^+$ monocytes in survivors were comparable to levels measured in the elderly ($P=0.2258$) and surprisingly higher compared to levels measured in HIV-infected individuals ($P=0.0003$). There was no difference in the proportion of individuals who experienced elevated D-dimer levels (>200ng/ml) in survivors compared to age-matched controls (11.1% vs 8.7%, Chi-square=0.230, $P=0.632$) (Figure 2D).

We next compared markers of late-differentiated memory T cells, CD57$^+$, CD28$^-$ and the combination of CD57$^+$CD28$^-$ on CD4$^+$ and CD8$^+$ T cells. There was a wide variation in the proportion of T cells expressing these markers in both survivors and
age-matched controls. The loss of CD28 expression on T cells (denoted by CD28\(^{-}\)) is a marker of well differentiated memory T cells with shortened telomeres. The proportion of CD28\(^{-}\) T cells in survivors compared to controls were higher on CD4\(^{+}\) and CD8\(^{+}\) T cells (Figure 3A, D) but this did not reach statistical significance (\(P=0.0715\) and \(P=0.0962\), respectively). CD57\(^{+}\) identify cells with reduced proliferative capacity [15]. We found significantly higher proportions of CD57\(^{+}\) cells in survivors compared to age-matched controls in CD4\(^{+}\) T cells (\(P=0.0115\), Figure 3A) but not CD8\(^{+}\) T cells (\(P=0.0869\), Figure 3E). When the T cell phenotype CD57\(^{+}\)CD28\(^{-}\), a marker of late-differentiated memory T cell were compared, levels in survivors were significantly higher compared to controls in the CD4\(^{+}\) T cell subset (\(P=0.0479\), Figure 3C) but not different in the CD8\(^{+}\) T cell subset (\(P=0.1617\), Figure 3F).

In the adjusted analysis controlling for age, sex, BMI and smoking status, markers of systemic inflammation (IL-6, hsCRP) and immune activation (CD4\(^{+}\) and CD8\(^{+}\) T cell activation, NK cell activation, sCD163) all remained significantly higher in survivors compared to controls. However, the proportion of memory cells expressing markers of late-differentiation (CD28\(^{-}\), CD57\(^{+}\) and CD28\(^{-}\)CD57\(^{+}\)) on both CD4\(^{+}\) and CD8\(^{+}\) T cells were no longer significantly different in the two groups (Table 2).

**Young adult CCS have higher CMV IgG levels and increased CMV-specific CD8\(^{+}\) T cell responses**

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Given the role of CMV in driving immunologic aging in the general population [16, 17], we also measured CMV IgG levels in the study participants. Interestingly, the level of CMV IgG in survivors were comparable to levels measured in community-dwelling elderly (P=0.3907) and in HIV-infected individuals (P=0.0874) and significantly higher compared to age-matched controls (P=0.0088; Figure 4A). In a subset of survivors and controls, we also measured CMV-specific responses in CD8+ T cells using the QuantiFERON-CMV assay. We found that IFN-γ production following stimulation with CMV peptides were significantly higher in CMV seropositive survivors vs controls (P=0.0234, Figure 4B). Both survivors and controls who were CMV seronegative had undetectable IFN-γ following incubation with CMV peptides. The amount of IFN-γ produced following CMV peptide stimulation were also strongly correlated with the proportion of CD4+CD57+CD28- (r=0.801, P<0.0001, Figure 4C) and CD8+CD57+CD28- T cells (r=0.683, P=0.0020; Figure 4D) and remained significant even when CMV-seronegative survivors were omitted from the analysis. This association was however not significant for the other markers of immune activation and inflammation (data not shown).

**CMV IgG levels are consistently correlated with markers of immunologic aging**

We next assessed correlations between markers of immune activation, inflammation and late-differentiated memory T cells with exposure to anthracycline, alkylating agent and radiotherapy (measured as cumulative doses) to explore if prior treatment exposure influenced the accumulation of these cells in young leukemia survivors (Table 3). We also measured the correlation of these immunologic markers with CMV IgG levels. We found significant correlations between multiple markers of
inflammation, immune activation and late-differentiated memory T cells with CMV IgG levels but only a limited number of immunological markers were significantly correlated with prior treatment exposure. Specifically, levels of CMV IgG were significantly correlated with hs-CRP, IL-6, sCD163, and CD28 CD57+ on both CD4+ and CD8+ T cells, implying that CMV infection/reactivation may be a strong driver of these immunological changes seen in young leukemia survivors. The proportion of CD28'CD57+ on CD4+ T cells was also correlated with prior anthracycline exposure, while radiotherapy exposure was correlated with sCD163. Prior exposure to alkylating agents was not correlated with any of the measured immunological changes in young adult leukemia survivors.

To further interrogate the association between CMV infection/exposure with the accumulation of immune activation and late-differentiated memory T cell markers, we analysed the markers according to CMV-seropositive status in the cohort independent of prior cancer exposure (Supporting Information Table 2). In this analysis, with the exception of CD16+ monocytes, all the other markers of activation, late-differentiated memory T cells and inflammation were significantly different in the two groups.

Discussion

To our knowledge, this is the first study to characterize the presence of an immune phenotype of aging in young adult leukemia survivors. We found markers of
systemic inflammation (IL-6, hsCRP) and immune activation in the innate (sCD163, NK cell activation) and adaptive immune (CD4⁺ and CD8⁺ T cell activation) compartments to be significantly higher in young leukemia survivors compared to controls even after adjusting for age, sex, BMI and smoking status. Most of the markers of immune activation and inflammation were also correlated with one another (Supporting Information table 3), consistent with previously described models of aging where systemic inflammation, immune activation and the accumulation of late-differentiated memory T cells are suggested to work in concert to drive the development of age-related complications [18]. Most of the immunological markers measured in young leukemia survivors were however lower compared to levels in young HIV-infected individuals on stable cART, a population well described to experience early onset of age-related complications. CMV IgG levels in survivors were comparable to levels measured in the elderly. Consistent with serology results, we also found an increase in the magnitude of CMV-specific CD8⁺ T cell responses in survivors compared to controls which correlated with the proportion of late-differentiated memory CD4⁺ and CD8⁺ T cells. Additionally, many of the markers of immune activation and inflammation were significantly correlated with CMV IgG levels but correlated poorly with prior exposure to chemotherapy and radiotherapy implying that CMV infection/reactivation may be strongly associated with the immunologic aging process in childhood leukemia survivors.
Low-grade systemic inflammation is well characterized in the elderly and has been found to be associated with the development of multiple age-related co-morbidities including cardiovascular disease [19, 20], neurocognitive impairment [10, 21], metabolic dysfunction [22] as well as mortality [23]. Low-grade systemic inflammation has also been associated with frailty [24] and sarcopenia [25] which characterize functional decline in elderly individuals. We found levels of IL-6 and hsCRP to be significantly higher in young adult leukemia survivors compared to age-matched controls. Although levels in survivors were lower compared to levels measured in the community-dwelling elderly, these elevations could potentially be signs of early pathological changes that eventually lead to the development of age-related co-morbidities and physical dysfunction reported to occur at a younger age in childhood cancer survivors [2, 3]. In HIV-infected individuals who also experience age-related co-morbidities (also known as serious non-AIDS events) at a younger age, increased IL-6 levels have been shown to precede the occurrence of these co-morbidities and predict mortality [26]. Whether a similar association is true in childhood cancer survivors is currently unknown and should be explored in larger prospective studies. A recent small study (n=27 cases and n=20 controls, median age=20 years) in adult survivors of childhood ALL did not find differences in markers of systemic inflammation (TNF-α, IL-6 and hsCRP) in cases and controls [27]. This could potentially be due to the small sample numbers. Alternatively, the influence of CMV-driven immune activation may have been more subtle in this Polish-based study. The prevalence of CMV seropositivity in the cohort was however not reported to help confirm this. The study however did find an increased proportion of
the inflammatory monocyte subset, CD14^{++}\text{CD}16^+ and increased endothelial activation markers in cases compared to controls. We did not measure endothelial activation in our cohort but found a marginal increase in CD16^+ monocytes in survivors compared to controls that was trending towards statistical significance in the adjusted analysis.

We also found sCD163, a marker of monocyte/macrophage activation was significantly higher in survivors compared to age-matched controls. Elevated sCD163 has previously been shown to be associated with the development of atherosclerotic plaque in both HIV-infected and uninfected individuals [28, 29] and with the development of insulin resistance and type 2 diabetes mellitus in the general population [30]. Together with a background of increased systemic inflammation, young adult leukemia survivors seem to experience an immunological milieu that is conducive for the development of atherosclerosis and metabolic dysfunction and may explain the excess cardiovascular morbidity reported in this population [31, 32]. Of note, these markers remained elevated compared to controls despite adjusting for lifestyle factors and even after excluding individuals who reported pre-existing co-morbidities at recruitment (data not shown) albeit this made up only 5% of the survivors.

We did not find a significant difference in the levels of the LPS-binding receptor sCD14 in young adult leukemia survivors compared to age-matched controls despite other markers of monocyte activation and inflammation being significantly different in
the two groups. In HIV-infected individuals, sCD14 levels are elevated due to persistent microbial translocation as a result of HIV-induced damage to the gut-associated lymphoid tissue (GALT) which recovers poorly despite treatment [33]. Similarly, low-level endotoxemia has also been described in the elderly and elevated sCD14 levels have been associated with mortality and increased cardiovascular disease in both populations [34, 35]. We also found levels of sCD14 significantly higher in HIV-infected individuals and in community-dwelling elderly individuals compared to young leukemia survivors and controls. In a subset of survivors and controls, we measured levels of citrulline, a previously described surrogate for gut epithelial damage following radiation and chemotherapy in cancer patients [36] but did not find a difference in the two groups (Supporting Information table 1). Levels in survivors were also not correlated with sCD14 levels (data not shown), implying that microbial translocation secondary to persistent chemo/radiotherapy-induced damage to the gut epithelial tissue is unlikely to be a factor driving the phenotype of increased inflammation and immune activation seen in young adult leukaemia survivors.

Increased immune activation and inflammation both in the elderly and HIV-infected individuals have been shown to be associated with latent CMV infection/reactivation [37]. The carriage of CMV drives the activation, proliferation and differentiation of both CD4+ and CD8+ T cells leading to the accumulation of highly differentiated memory T cells characterized by the loss of CD28 and the expression of CD57+ which are pro-inflammatory [38]. Additionally, increased CMV-specific T cells and
CMV IgG levels have also been associated with age-related co-morbidities in HIV-infected individuals [39]; both enhanced [40] and reduced vaccine responses [41] and increased mortality in the elderly [11, 12, 16]. We found highly variable CMV IgG levels in both survivors and controls though median levels in young leukemia survivors were similar to that seen in the elderly and HIV-infected individuals. Survivors also demonstrated an increased CMV-specific CD8\(^+\) T cell response in QuantiFERON CMV assays compared to age-matched controls, implying an increased magnitude of CMV-specific CD8\(^+\) T cells. Although the proportion of terminally differentiated memory T cells were not significantly different in survivors and controls after adjusting for demographic and lifestyle factors, the accumulation of these cells were found to be significantly correlated with the degree of IFN-\(\gamma\) production following CMV-specific stimulation of T cells, CMV IgG levels and markers of inflammation. When analysed by CMV-seropositivity, many of the immunological markers were also significantly higher in CMV-seropositive compared to seronegative individuals. Taken together, the immune phenotype seen in young leukemia survivors resembles that seen in individuals with a high CMV burden. Prior studies have shown that CMV-specific T cells may be preferentially expanded during reconstitution of T cells following chemotherapy [42] and post-transplant [43]. It is therefore conceivable that survivors of childhood leukemia may have acquired CMV earlier and accumulated a larger CMV-specific immunity over time compared to age-matched controls though this needs to be confirmed in further studies. Additionally, CMV predominantly affects the vascular endothelium and extensive vascular dysfunction has been reported to occur even in adolescent survivors of childhood.
cancer with no pre-existing co-morbidities [44, 45]. Therefore the potential role of CMV in driving vascular pathologies in CCS needs to be explored further especially given recent findings that several interventions may help reduce CMV-driven immune activation [46, 47].

Most observational studies have found an association between prior chemotherapy and radiotherapy exposure with late-effects in childhood cancer survivors [2, 48] however not all studies have found an association [45]. We found only a limited number of markers of immune activation and inflammation to be correlated with prior anthracycline and radiotherapy exposure though this lack of association could be due to the small number of survivors in this cohort. Anthracycline exposure in childhood cancer survivors has most commonly been associated with cardiotoxicity and cranial radiotherapy on the other hand, has been associated with growth hormone deficiency, neurocognitive impairment and endocrine dysfunction (reviewed in [49]). No studies to date have reported an association between chemo- and radiotherapy exposure with long-term immunological changes in leukemia survivors. It is currently unclear how these factors may be related though it is plausible that the correlations we found are due to an indirect association with specific chemo/radiotherapy-induced organ damage that were not assessed in this study.

There were several important limitations in this study. Firstly, although we showed that markers of immune activation and inflammation were significantly higher in
leukemia survivors compared to age-matched controls, we have not shown an
association with clinical outcomes including age-related co-morbidities and functional
impairment in survivors. Given the young age of the survivors, <5% presented with
pre-existing comorbidities. Secondly, we cannot be certain that the immunological
changes we found in survivors are not due to an undescribed residual effect from
their previous haematological cancer diagnosis. However, when analysis was
restricted to ALL survivors, changes in monocyte/macrophage subsets (non-
lymphoid lineage) persisted (data not shown) which somewhat implies that this may
be a generalised phenomenon. Nevertheless, characterisation of similar immune
markers in non-hematological childhood cancer survivors should be conducted to
confirm this. Thirdly, although we also found a correlation between many of the
measured immunological markers with CMV IgG, we cannot exclude the potential
influence of other chronic viral infections and this should be explored further.
Additionally, this is a cross-sectional study and we therefore cannot make any
conclusions regarding the causality of these immunologic changes. Finally, though
our data implies that there is a greater proportion of activated and terminally
differentiated memory T cells which accumulate with age in leukemia survivors
compared to age-matched controls, the age-range in our cohort was too narrow to
demonstrate differences in the trajectory in these parameters (interquartile range:
22-30 years) and should be confirmed in studies with participants from a wider age
range.
In conclusion, we have shown that young childhood leukemia survivors experience an immune phenotype of increased immune activation and systemic inflammation similar to that seen in CMV seropositive elderly. Young adult survivors of childhood leukemia had increased CMV-specific responses compared to age-matched controls. CMV IgG levels were also comparable to elderly individuals and strongly correlated with markers of immune activation, inflammation and late-differentiated memory T cells. We hypothesize that the development of age-related co-morbidities in childhood cancer survivors may in part be associated with this immunological signature of aging which is closely linked to CMV infection/reactivation.

Materials and methods

Patients

We recruited acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) survivors who returned to the Pediatric Oncology Unit, University Malaya Medical Centre, Malaysia for their routine annual follow-up. The inclusion criteria were 1) individuals aged 18-35 years; 2) at least 5 years since completion of leukemia treatment; 3) no history of bone marrow transplant; 4) no acute illness and not pregnant at the point of recruitment and 5) did not receive a vaccination in the 6 months prior to recruitment. Age-matched controls were recruited amongst hospital staff.
Comparator groups consisting of community-dwelling elderly (age >50 years) and HIV-infected individuals on stable antiretroviral therapy (age range 18-35 years) were recruited from an on-going aging study in our centre. The study protocol was approved by the hospital institutional review board (MEC 2014/1093.65) and all participants provided written informed consent.

**Data collection**

A standardized questionnaire was used to collect details on demographic and smoking history. Height and weight measurements as well as biochemical screening were performed in all participants while a detailed history of cancer treatment was obtained from medical records by two independent researchers to ensure accuracy.

**Immunophenotyping**

Immunophenotyping was performed on whole blood using two panels of 8-color flow cytometry. Briefly, 100 µl of whole blood was stained with antibody master mixes, incubated in the dark for 15 minutes and followed by red blood cell lysis using 1:10 BD FACS lysing solution (BD Biosciences, San Jose, CA). Surface staining was performed using the following monoclonal antibodies: CD3-PerCP-Cy5.5, CD4-PE-Cy7, CD8-APC-H7, CD28-APC, CD57-FITC, HLA-DR-BV421 and CD38-PE for the T-cell panel and CD3-PerCP-Cy5.5, CD16-PE-Cy7, CD56-APC, CD14-FITC, HLA-DR-BV421 and CD38-PE for the NK cell/monocyte panel (all reagents from BD).
Samples were acquired on a BD Canto II (BD Biosciences) and analysed using the FACS Diva software (version 6.0). For the T cell panel, following gating to exclude doublets, CD3+ cells were selected and sequentially gated for CD4+ and CD8+ T cells. The proportion of activated cells were defined by the co-expression of CD38+HLA-DR+ while late differentiated memory T cells were identified by T cells expressing CD57+, the loss of the co-stimulatory molecule CD28 and both CD57+CD28− cells. For the NK cell/monocyte panel, NK cells were defined as CD3− CD56+ cells. Monocyte subsets were gated to identify the classical monocyte subset, CD14++CD16− and the inflammatory monocyte subset CD14variableCD16+ (sum of CD14++CD16+ and CD14+CD16++) as previously described [50]. The detailed gating strategy is shown in Supporting Information figure 1.

Cytokine and inflammatory markers

Whole blood was collected and processed within 4 hours of collection. The systemic inflammatory cytokine interleukin-6 (IL-6) and monocyte/macrophage activation markers, soluble (s)CD163 and sCD14 were measured by enzyme-linked immunosorbent assay (ELISA) in plasma samples using the Quantikine HS IL-6 (R&D Systems, Minneapolis, USA), Macro163 (Trillium Diagnostics, Maine USA) and Quantikine sCD14 (R&D systems) kits, respectively. All assays were performed according to the manufacturer’s instructions and assayed in duplicate.
**CMV serology and CMV-specific CD8+ T cell immunity**

CMV serology was performed using the Roche Cobas CMV IgG kit (Roche Diagnostics, Sandhofen) by the hospital's diagnostic laboratory. The magnitude of CMV-specific CD8+ T cell responses were measured in a subset of survivors (n=19) and controls (n=15) using the QuantiFERON® CMV assay (Cellestis, Chadstone, Australia) which measures IFN-γ production after stimulation with previously defined CMV CD8+ T cell epitopes [51]. The assay was performed according to the manufacturer's instructions.

**Statistical analysis**

Immune activation, inflammation and late differentiation markers in the survivors were compared to the other groups using Mann-Whitney U where adjustments for multiple comparisons were made using the Benjamini-Hochberg method while the association of categorical variables were assessed using Pearson's Chi-square. Correlations were assessed using Spearman's correlation. Multivariate logistic regression was used to assess if markers of immune activation, late-differentiated memory cells and inflammation were significantly different in survivors compared to controls after adjusting for age, sex, smoking status and BMI. A P-value of <0.05 was considered significant in this analysis. All statistical analyses were performed using SPSS (version 22, IBM).
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Conflict of interest

The authors declare no commercial or financial conflict of interest.

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reconstitution and results in defects in the underlying TCRbeta repertoire. 


**Figure legends**

**Figure 1.** Immune activation markers in young adult leukemia survivors compared to age-matched controls, elderly and HIV-infected individuals. (A-C) The proportion of cells expressing the activations markers CD38 and HLA-DR on (A) CD4+ T cells, (B) CD8+ T cells and (C) NK cells were measured by flow cytometry in whole blood. (D, E) The soluble monocyte/macrophage activation markers, (D) sCD163 and (E) sCD14 were measured by ELISA in plasma samples of survivors (n=81), age-matched controls (n=71), the elderly (n=27) and HIV-infected individuals (n=30). Data shown as median ± interquartile range. * denotes significant *P*-values calculated by the Mann-Whitney *U* test after Benjamini-Hochberg correction.
**Figure 2.** Markers of systemic inflammation in young adult leukemia survivors compared to age-matched controls, elderly and HIV-infected individuals. (A-C) Systemic inflammation was measured by levels of (A) interleukin-6 (IL-6), (B) highly sensitive C-reactive protein (hs-CRP) levels in plasma by ELISA and (C) the proportion of inflammatory CD16+ monocytes in whole blood by flow cytometry. (D) The coagulation profile was evaluated by the proportion of individuals with D-dimer levels higher than 200 ng/ml. Markers were measured in survivors (n=81), age-matched controls (n=71), the elderly (n=27) and HIV-infected individuals (n=30). Data shown as median ± interquartile range. * denotes significant P-values calculated by the Mann-Whitney U test after Benjamini-Hochberg correction. Pearson’s Chi-square was used to assess the difference in proportion with positive D-dimer tests between survivors and age-matched controls.

**Figure 3.** Markers of late-differentiated memory CD4+ and CD8+ T cells in young adult leukemia survivors compared to age-matched controls, elderly and HIV-infected individuals. The markers of late-differentiated memory T cells were measured by flow cytometry in whole blood and include the proportion of (A-C) CD4+ cells with (A) CD28+, (B) CD57+, (C) CD28CD57+ and (D-F) CD8+ cells with (D) CD28+, (E) CD57+ (F) CD28CD57+ expression phenotypes. Markers were measured in survivors (n=81), age-matched controls (n=71), the elderly (n=27) and HIV-infected individuals (n=30). Data shown as median ± interquartile range. *
denotes significant $P$-values calculated by the Mann-Whitney $U$ test after Benjamini-Hochberg correction.

**Figure 4.** CMV IgG levels and CMV-specific responses in leukemia survivors compared to age-matched controls, elderly and HIV-infected individuals. (A) The levels of CMV IgG were measured in plasma samples of leukemia survivors (n=81), age-matched controls (n=71), the elderly (n=27) and HIV-infected individuals (n=30). (B) IFN-$\gamma$ production following CMV peptide stimulation in CD8$^+$ T cells using the QuantiFERON-CMV assay were measured in survivors (n=19) compared to controls (n=15). Data shown as median ± interquartile range. (C, D) Correlations of (C) CD4$^+$ and (D) CD8$^+$ T cells expressing the markers of late-differentiated memory cells CD28$^-$CD57$^+$ with IFN-$\gamma$ production in CMV-seropositive survivors and controls. Dotted line indicates 95% confidence interval. $P$-values were calculated by the Mann-Whitney $U$ test with Benjamini-Hochberg correction for group comparisons and Spearman’s ($\rho$) rank test to determine correlations. * denotes significant $P$-values.
### Table 1. Clinical and demographic characteristics of study participants

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Survivors</th>
<th>Controls</th>
<th>Elderly</th>
<th>HIV+</th>
</tr>
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<tbody>
<tr>
<td>Number in each group</td>
<td></td>
<td>81</td>
<td>71</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td><strong>Sex, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>34 (42%)</td>
<td>31 (44%)</td>
<td>14 (52%)</td>
<td>27 (90%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>47 (58%)</td>
<td>40 (56%)</td>
<td>13 (48%)</td>
<td>3 (10%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age at recruitment, years</strong></td>
<td></td>
<td>26 (22-30)</td>
<td>24 (23-27)</td>
<td>61 (55-71)</td>
<td>33 (31-34)</td>
</tr>
<tr>
<td><strong>Age at diagnosis, years</strong></td>
<td></td>
<td>5 (3-9)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td></td>
<td>23.5 (20.7-27.8)</td>
<td>22.1 (20.4-24.9)</td>
<td>24.7 (23.0-29.1)</td>
<td>21.6 (19.7-24.2)</td>
</tr>
<tr>
<td><strong>Diagnosis, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ALL</td>
<td>71 (92%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AML</td>
<td>6 (8%)</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
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<tr>
<td><strong>Chemotherapy received</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Antracyclines, n (%)</td>
<td>63 (78%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Antracycline cumulative dosage, mg/m²</td>
<td>240 (120-240)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Alkylating agents, n (%)</td>
<td>63 (78%)</td>
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<td>NA</td>
<td>NA</td>
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<tr>
<td>Alkylating agent cumulative dosage, mg/m²</td>
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<td>NA</td>
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<td>NA</td>
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<tr>
<td>Anthracyclines and alkylating agents, n (%)</td>
<td>60 (74%)</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Radiotherapy received, n (%)</td>
<td>42 (52%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Radiotherapy dosage, Gy</td>
<td>12 (0-18)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td><strong>Smoking status, n (%)</strong></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Smoker</td>
<td>11 (14%)</td>
<td>2 (3%)</td>
<td>4 (15%)</td>
<td>4 (13%)</td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>1 (1%)</td>
<td>2 (3%)</td>
<td>2 (7%)</td>
<td>7 (23%)</td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>63 (78%)</td>
<td>66 (93%)</td>
<td>21 (78%)</td>
<td>19 (63%)</td>
<td></td>
</tr>
<tr>
<td>Not available</td>
<td>6 (7%)</td>
<td>1 (1%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Second neoplasms, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-existing co-morbidities, n (%)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>1 (1%)</td>
<td>0</td>
<td>7 (26%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>3 (4%)</td>
<td>0</td>
<td>14 (52%)</td>
<td>5 (17%)</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>0</td>
<td>0</td>
<td>1 (4%)</td>
<td>1 (3%)</td>
<td></td>
</tr>
</tbody>
</table>

Data shown are median (interquartile range, IQR) or n (%). NA indicates not applicable. *Variables that are significantly different (P<0.05 on Mann Whitney or Chi-square tests) in survivors compared to controls.
Table 2. Multivariate logistic regression analysis comparing markers of immune activation and inflammation in young leukemia survivors compared to controls after adjusting for age, sex, BMI and smoking status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient (b)</th>
<th>Adjusted OR* (95% Confidence Interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺ T cell activation</td>
<td>0.52</td>
<td>1.69 (1.30, 2.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD4⁺CD57⁺ T cells</td>
<td>0.06</td>
<td>1.06 (0.97, 1.17)</td>
<td>0.215</td>
</tr>
<tr>
<td>CD4⁺CD28⁻ T cells</td>
<td>0.01</td>
<td>1.01 (0.91, 1.11)</td>
<td>0.898</td>
</tr>
<tr>
<td>CD4⁺CD57⁺CD28⁻ T cells</td>
<td>0.01</td>
<td>1.01 (0.90, 1.14)</td>
<td>0.855</td>
</tr>
<tr>
<td>CD8⁺ T cell activation</td>
<td>0.10</td>
<td>1.11 (1.05, 1.17)</td>
<td>0.001</td>
</tr>
<tr>
<td>CD8⁺CD57⁺ T cells</td>
<td>0.01</td>
<td>1.01 (0.99, 1.04)</td>
<td>0.314</td>
</tr>
<tr>
<td>CD8⁺CD28⁻ T cells</td>
<td>0.01</td>
<td>1.01 (0.99, 1.04)</td>
<td>0.357</td>
</tr>
<tr>
<td>CD8⁺CD57⁺CD28⁻ T cells</td>
<td>0.01</td>
<td>1.01 (0.98, 1.04)</td>
<td>0.505</td>
</tr>
<tr>
<td>NK cell activation</td>
<td>0.03</td>
<td>1.03 (1.01, 1.06)</td>
<td>0.017</td>
</tr>
<tr>
<td>sCD163</td>
<td>0.002</td>
<td>1.002 (1.001, 1.003)</td>
<td>0.002</td>
</tr>
<tr>
<td>sCD14</td>
<td>0.00</td>
<td>1.00 (1.00, 1.00)</td>
<td>0.593</td>
</tr>
<tr>
<td>CD16⁺ monocyte</td>
<td>0.09</td>
<td>1.09 (0.99, 1.21)</td>
<td>0.080</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.54</td>
<td>1.72 (1.04, 2.84)</td>
<td>0.036</td>
</tr>
<tr>
<td>hsCRP</td>
<td>2.48</td>
<td>11.92 (1.56, 90.99)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

OR indicates odd ratio; T cell activation represents cells co-expressing CD38⁺HLA-DR⁺. Late-differentiated memory T cells represent cells with the phenotype CD28⁻, CD57⁺ and CD28⁻CD57⁺.

*Adjusting variables (age, sex, BMI and smoking status) were initially checked for collinearity using linear regression and all variables had a variance inflation factor (VIF) of <10.

Table 3. Correlations between immunological markers with treatment exposure and cytomegalovirus (CMV) IgG levels in the survivors

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Anthracyline (n=81)</th>
<th>Alkylating agent (n=81)</th>
<th>Radiotherapy (n=81)</th>
<th>CMV IgG levels (n=77)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺ T cell activation</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CD8⁺ late-differentiated memory T cell</td>
<td>~0.288, P=0.010</td>
<td>ns</td>
<td>ns</td>
<td>~0.550, P=0.001</td>
</tr>
<tr>
<td>CD8⁺ T cell activation</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CD8⁺ late-differentiated memory T cell</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>~0.291, P=0.010</td>
</tr>
<tr>
<td>NK cell activation</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>sCD163</td>
<td>ns</td>
<td>ns</td>
<td>~0.269, P=0.043</td>
<td>~0.295, P=0.012</td>
</tr>
<tr>
<td>sCD14</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CD16⁺ monocyte</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>~0.296, P=0.012</td>
</tr>
<tr>
<td>hsCRP</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>~0.229, P=0.047</td>
</tr>
</tbody>
</table>

ns indicates not significant; r, Spearman’s correlation coefficient.

Only significant Spearman’s correlations are shown (P<0.05).

Non-exposure to specific treatment were assigned a value of 0 in the correlation analysis.
An immunologic aging phenotype characterized by increased systemic inflammation and immune activation was observed in young adult survivors of childhood leukemia compared to age-matched controls. Cytomegalovirus (CMV) IgG levels in survivors were comparable to the elderly and CMV-specific T cells correlated with markers of immune activation, inflammation and late-differentiated memory T cells.