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Immunologic Consequences of Chemotherapy for Acute Myeloid Leukemia

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Abstract

There are few data characterizing the immunologic consequences of chemotherapy for AML and almost nothing is known about the effects of chemotherapy in a pediatric AML cohort. We identified T cell subsets, B cell subsets and used ELISPOT analyses to define the function of T cells and B cells in seven pediatric patients with AML on chemotherapy. The data demonstrate that the effects of chemotherapy disproportionately target the B cell and depletion of B cells is associated with impaired responses to the inactivated influenza vaccine. Diminished T cell numbers were also observed although the magnitude of the effect was less than what was seen for B cells. Furthermore, measures of T cell function were largely intact. We conclude that humoral immunity is significantly affected by chemotherapy for AML.

Introduction

Acute myeloid leukemia (AML) occurs in approximately 1000 children per year in the United States ¹. The incidence of pediatric AML is estimated to be approximately 6 cases per million per year with the peak incidence around two years of age ²⁻⁴. Most children who develop AML have no recognized predisposition although a number of inherited conditions are associated with an increased risk of AML ⁵⁻⁸. The pathogenesis of AML is diverse and includes a series of mutations that alter either proliferation, differentiation, or apoptosis of

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myeloid precursor cells⁹⁻¹². The prognosis of AML is related to the cellular phenotype, the specific genotype, cytogenetics, the response to therapy, and host factors¹³.

AML comprises a minority of all pediatric leukemia cases in the United States although it is disproportionately associated with mortality. A survival rate of 60% and rates of complete remission as high as 80 to 90% are now reported¹⁴. The success rate today reflects the current chemotherapy which is far more intensive than in previous decades. The most favorable outcomes require the use of high cumulative doses of anthracyclines and cytarabine¹⁴. Most of the mortality occurs close to the time of diagnosis and causes of death include bleeding, leukostasis, tumor lysis syndrome, and infection¹⁵⁻¹⁹. Infectious complications during the aggressive therapy remain a major cause of morbidity and mortality and streptococcal infections occur at high rates in patients undergoing chemotherapy¹⁶. A high rate of relapse also contributes to mortality. In addition, RSV infections can occur and while the rate of RSV has been found to be low, the mortality associated with RSV infection is as high as 10%²⁰. The high rates of infection and the increased mortality associated with infection has been attributed to the effects of chemotherapy, however, in some cases the malignant cells may themselves be immunosuppressive. The production of indoleamine 2,3 dioxygenase catalyzes the conversion of tryptophan to kynurenine and this enzyme can be expressed in the malignant cells²¹. The impact of this pathway is to drive the induction of regulatory T cells which are themselves suppressive. In spite of clinical evidence suggesting that patients with AML have high rates of infection, there have been few studies of immunologic function in pediatric patients with AML. This study was designed to characterize immunologic function in a small cohort of pediatric patients with AML. The study identified significant immune deficiency in this cohort, which doubtless contributes to their predisposition to infection.

Methods

Subjects

This study was approved by the Institutional Review Board at The Children's Hospital of Philadelphia. Subjects were recruited from inpatient and outpatient venues and were enrolled in the study from 2005-2010. Pediatric controls were recruited by advertising from well child visits and elective surgical procedures. Due to blood volume requirements, the pediatric control samples were limited to one or two assays per sample (achieving a sample size of 16-38 for each assay), while the eight AML patients had all assays performed from each blood sample. Subjects were tested at baseline (day of vaccination) and two, four, and twelve months after vaccination. Three AML patients were previously reported as were a small subset of the controls^{22, 23}. Figure 1 demonstrates the tie lines of the AML patients. Pediatric controls have only post-vaccine values.

Immunologic Assays

Flow cytometry was used to define T cell subsets and B cell subsets. T cell analyses used fixed samples and were based on approximately 200,000-500,000 total events. Samples were run on an LSR II (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR). CD4 Naïve cells were defined as CD45RA+CD31+, CD4 Central Memory T cells were defined as CD45RO+CCR7+, CD4 Effector Memory T cells were defined as CD45RO+CCR7-, and CD4 Reverted Memory T cells were defined as CD45RA+/CD31-/CCR7+^{24, 25}. CD8 Naïve cells were defined as CD45RA+CD31+, CD8 Central Memory T cells were defined as CD27+CD45RO+CCR7+, CD8 Effector Memory T cells were defined as CD45RO+/CD27-/CCR7-, and CD8 Reverted Memory T cells were defined as CD45RA+/CD31-/CCR7+²⁶⁻²⁸.

B cell subsets were analyzed using fresh samples and stained using a protocol previously described^{29, 30}. Samples were run on a FACSCalibur and analyzed with CellQuest software (Version 5.2.1, Becton Dickinson, San Jose, CA). The absolute B cell count was obtained by multiplying the absolute lymphocyte count by the CD19+ fraction. We defined non-switched memory B cells as CD27+IgM+, switched memory B cells as CD27+IgM- and naïve B cells as CD27-IgM-.

An influenza protein mixture (Protein Sciences, Meriden, CT), based on the year-specific vaccine, was used as specific antigen (at 5 µg/mL) in a standard -interferon T cell ELISPOT assay³¹. This assay examined a range of epitopes, and was HLA-dependent. CD8 depletion was used to define CD4 responses. PMA and ionomycin (combined at 5 µg/mL each) were used to measure global responses. The B cell ELISPOT defined the frequency of memory B cells activated by influenza to produce antibody and total IgG-producing B cells³². PBMC were stimulated for 6 days with pokeweed mitogen at 1:100,000, *Staphylococcus aureus* (SAC) at 1:10,000 and CpG-2006 at 6 g/ml (Sigma Aldrich St. Louis, MO). Subsequently, cells were treated for six hours with either the influenza protein cocktail described above (at 0.5 µg/mL) or anti-IgG. ImmunoSpot (CTL, version 4) software was used to quantitate spots. Proliferation assays utilized carboxyfluorescein succinimidyl ester (CFSE)-loaded cells stimulated for five days with season-specific influenza virus. Flow cytometry (FlowJo) was used to define the divided cells.

The assessment of the influenza vaccine response utilized a standard hemagglutination inhibition (HAI) assay optimized for the vaccine administered each year³³.

Statistical analysis

The independent t-test or the non-parametric two-tailed Wilcoxon rank sum test (as appropriate) was used for the comparisons of individual variables between groups. Due to the study design and the small number of cases, corrections for multiple comparisons were not performed. Significance was set at $p < 0.05$.

Results

Eight subjects with AML were recruited for this study between 2005-2010. One subject was vaccinated prior to diagnosis and was therefore excluded from analysis. The mean age was seven years and the majority of the subjects were male (Table 1). The subjects were studied at four time points (baseline, 2 months, 4 months, 12 months) and the influenza vaccine was given at baseline to assess the ability to respond to vaccines. The subjects were enrolled during the winter months and were heterogeneous with respect to the aggregate time on chemotherapy at the time of enrollment. Patients were either on active chemotherapy ($n=7$) or within two months ($n=1$) of completing chemotherapy at the time of enrollment. Two patients had relapsed at the time of vaccination and two patients received a bone marrow transplant after all study time points were completed. One patient received reduced intensity chemotherapy due to Down syndrome and one patient received Mylotarg (gemtuzumab) in addition to standard chemotherapy.

We recruited age-matched pediatric controls for comparison purposes (Table 1). There is a growing appreciation of the importance of immunologic function as a prognostic factor and we hypothesized that immunologic function on the day of vaccination would predict vaccine responses³⁴. Baseline immunologic function has been shown in other settings to predict influenza vaccine responses³⁵. We compared baseline (day of vaccination) lymphocyte, B cell and T cell counts. The absolute lymphocyte count in the patients was approximately half of that in the control group ($p=0.0012$). Surprisingly, the major effect was on B cells, where the patients had less than 10% of the CD19 B cells compared to the controls ($p=0.0001$). T

cells were more preserved in these patients on chemotherapy, with patients having approximately half of the CD3 T cells compared to controls ($p=0.0200$) (Figure 2).

To better characterize the immunologic consequences of chemotherapy for AML, we defined T cell and B cell subsets across the one year time frame of the study. At baseline, among CD4 T cells, the most affected subset was the Regulatory T cells ($p=0.0254$). Among CD8 T cells, the Reverted Memory subset ($p=0.0288$) was most affected by the chemotherapeutic regimen (Figure 3). These effects begin to reverse off of chemotherapy at the one year time point although the effects differ between subsets.

To understand the effect on the B cell subsets, we compared naïve (IgM+ CD27-), non-switched memory (IgM+ CD27+), and switched memory B cells (IgM-CD27+) in patients and controls. All three subsets were dramatically reduced in patients at baseline compared to controls with all three p values $=0.0001$ (Figure 4). To examine whether one subset was affected disproportionately, we analyzed the data according to the fraction of each subset within the B cell compartment (Figure 4). Analyzed in this manner, the predominant effect appeared to be in the naïve B cell subset ($p=0.0100$). To define functional consequences of this decrement in B cells, we utilized a B cell ELISPOT. We measured total IgG producing B cells as well as influenza-responsive B cells. At baseline, total IgG producing cells were approximately 10% of controls, consistent with the decrement in switched memory B cells identified by flow cytometry although this did not quite reach statistical significance ($p=0.0590$). We compared baseline and two month post-vaccine influenza antibody production in the AML patients using the B cell ELISPOT and found that the patients had no increase in influenza-specific antibody production after vaccination. In fact the number of spots declined slightly (1.67 spots/10,000 cells to 0.83 spots/10,000 cells). At the one year time point, all B cell subsets examined had rebounded.

To examine effects on T cell function, we measured global responses using proliferation in response to PHA and -interferon production after PMA and ionomycin (Figure 5). T cell proliferation was unaffected by chemotherapy when measured as the percent of dividing cells. We also analyzed the Proliferation Index, a measure of the number of cell divisions a responding cell underwent. The patients had a mean Proliferation Index of 1.37 ± 0.99 and the controls had a mean Proliferation Index of 1.70 ± 0.57 , a difference that was not significant. Although - interferon production after stimulation was reduced in patients compared to controls, it was not statistically different. We also compared CD4 ELISPOT responses to influenza at baseline and at 2 months after vaccination and saw no increase after vaccination (data not shown).

To define the effect of chemotherapy on a practical measure of immunologic function, we assessed the humoral response to the seasonal influenza vaccine. The response to vaccination was extremely low and no patient had a four fold response. The post-vaccine titers in the AML patients were well below what was seen in a vaccinated control population (Figure 6). For all three serotypes, the differences between the post-vaccine titers in patients and controls were significantly different with $p<0.01$.

Discussion

This study is to our knowledge the first in depth assessment of immunologic function in a cohort of pediatric AML patients on chemotherapy. One study of adult AML patients suggested that cytarabine and daunorubicin therapy specifically depleted blast cells but left the bone marrow lymphocytes intact³⁶. Therefore, this study provides an important perspective on the immunologic effects of AML chemotherapy. This study documents a significant and medically important effect of chemotherapy on B cells. Both anthracyclines

and cytarabine are known to induce apoptosis of B cells and are used for B cell lymphomas³⁷⁻³⁹. Anthracyclines are intercalating agents but have additional effects such as topoisomerase II inhibition and ceramide generation that might account for the tissue-specific effects⁴⁰⁻⁴². Similarly, cytarabine has been known since 1976 to affect cycling and non-cycling cells alike⁴³. The mechanism of its effect in non-cycling cells appears to relate to the inhibition of RNA synthesis⁴⁴. In mice, doxorubicin and daunorubicin administration led to higher levels of apoptosis in B cells compared to T cells suggesting that chemotherapy effects could be cell type specific⁴².

In our study, the effect of chemotherapy on the B cell compartment was dramatic and all subsets were quantitatively affected. The naïve B cell subset appeared to be more significantly affected than either of the memory subsets and this subset is quantitatively the most significant in children. The non-switched memory B cell subset may represent a bloodstream stage comparable to marginal zone B cells⁴⁵ and marginal zone B cells have been previously implicated in poor vaccine responses⁴⁶⁻⁴⁸. The impact of our study is the clear clinically relevant finding of a marked depletion of antibody-competent B cells and a concomitant failure to respond to influenza vaccination. AML is associated with a significant risk of invasive bacterial infections. Less than 5% of patients in one study had no episodes of infection during chemotherapy. Among patients with blood stream infections, gram positive organisms were the most common and even occurred in the absence of neutropenia¹⁸. The lymphocyte count has been shown to be a prognostic indicator in AML and our data in concert with that finding suggests that impaired humoral responses might contribute to survival⁴⁹.

There were modest effects on the T cell compartment and the effect of chemotherapy did not appear to target the more proliferative subsets. The role of various T cell subsets in the defense against viral infections is becoming clearer over time and the report of increased RSV mortality in an AML cohort prompted us to evaluate T cells in detail²⁰. CD8 central memory cells are long-lived and have a high proliferative potential and a broad repertoire^{50,51}. Reverted memory T cells express CD45RA and also have a long life span but have limited proliferative potential⁵². The one actively cycling CD8 subset is the effector memory subset and we had hypothesized that this subset might be disproportionately affected, however, T cell subsets were less impacted overall than expected and effector memory Cd8 T cells were not affected more than the others⁵³.

This study provides important insights in the immunologic fallout from the intensive AML chemotherapy that has led to improved survival. Although it represents significant new information, this study is limited by the small number of patients examined and because patients were not enrolled synchronously. We have made the assumption that the deficiencies observed in this cohort relate to the chemotherapy, however, it is formally possible that the tumor itself is responsible for some of the observed effects. We believe that the identification of the immune deficits are of importance even if the mechanism has not been firmly established. Additional studies evaluating clinical correlates of the immune deficits identified here will be required to clarify the medical relevance. Further studies will also be required to define the longevity of the effects described here. In summary, this study documents a significant decrement in B cells in patients with AML. A correlate of the finding was a compromised ability to respond to the influenza vaccine. T cell effects were less significant and T cell proliferation was not impacted by chemotherapy. This may explain why viral reactivation and overwhelming viral infections are not commonly seen.

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Figure 1.

The timing of the vaccination relative to the chemotherapy window is indicated for each patient. Vertical black arrows indicate the time of vaccination. The chemotherapy protocols are listed within each time line. The lighter colored time lines indicate patients who relapsed.

Figure 2.

To being to analyze the effects of chemotherapy, we defined the mean lymphocyte count and the total T cells (CD3) and B cells (CD19) in patients at baseline (the day of vaccination) and controls. The bars indicate the mean with the standard deviation shown as an error bar. The asterisks indicate significance.

Figure 3.

The absolute counts of various T cell subsets at baseline (the day of vaccination) are given in this figure with the standard deviation shown as an error bar. The asterisks indicate significant differences between patients and controls. The bottom panels demonstrate the changes over time in the AML group and the y-axis is given as a log scale to facilitate comparisons.

Figure 4.

The absolute counts and percentages of the B cell subsets at baseline (the day of vaccination) are displayed in the upper two panels with the error bars representing the standard deviation. The asterisks indicate significance. All three subsets are quantitatively diminished compared to controls: IgM+CD27-(Naïve), IgM+CD27+ (Non-switched Memory), and IgM-CD27+(Switched Memory) with p values <0.001. Nevertheless, the effect on the B cell compartment is most dramatic in the Naïve B cell subset and expressed as a percentage, only the Naïve B cell subset is different in patients compared to controls with p=0.02. The third panel demonstrates the changes in the B cell subsets over time in the AML cohort and the bottom panel demonstrates the total IgG production in the AML patients over time.

Figure 5.

The upper graph displays two types of baseline data, the percentage of CD3 cells that divided in response to PHA at five days and the number of cells/million mononuclear cells producing - interferon after PMA and ionomycin stimulation. The error bars represent the standard deviation and the columns are the mean for each cohort. The differences are not statistically significant. The bottom two panels represent the patient data displayed according to the time point.

Figure 6.

Hemagglutinin inhibition titers are displayed on the y-axis for H1N1, H3N2 and influenza B serotypes across time points for the patients. The post-vaccine control HAI titers are shown to the right. The AML patients do not have a significant change in titer between baseline and two months. The AML patients have post vaccine titers lower than the post-vaccine controls. The difference is significant for Influenza B only.

Table 1

Demographic Comparison

	AML Subjects	Control Subjects
Age	7.4 ± 6.1	8.5 ± 4.6
Male Gender	85%	62%