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Exposure to a mycovirus containing *Aspergillus Flavus* reproduces acute lymphoblastic leukemia cell surface and genetic markers in cells from patients in remission and not controls

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ABSTRACT

The etiology of acute lymphoblastic leukemia (ALL) remains unknown. A recent "two-hit" model for the occurrence of precursor B cell acute lymphoblastic leukemia propose that this disease arises through a two-step process, including predisposing genetic mutation and exposure to infections. While several genetic mutations are proposed, no infection category has been suggested. We have isolated a certain *Aspergillus Flavus* from residence of an ALL patient. This organism contains mycovirus and does not produce aflatoxin. The supernatant of culture of this mycovirus containing *Aspergillus Flavus* (SAF) was tested on the PBMCs of ALL patients in remission and controls. Cell surface phenotypes and genetic markers were examined. The effects of its combination with Epstein-Barr virus (EBV) was also investigated. For the SAF, positive and negative controls were aflatoxin and culture of *Mycocladus corymbifer,* respectively. Controls for ALL were sickle cell patients undergoing exchange transfusion. Incubation of the PMBCs from ALL patients in remission, or controls, with SAF resulted in re-development of ALL cell surface phenotypes and genetic markers in ALL patients in remission and not controls. These differentiating effects were not seen with aflatoxin or culture of *Mycocladus Corymbifer*. Addition of EBV did not alter effects of SAF. Currently, there are no techniques to discriminately reproduce characteristic leukemic genetic markers and cell surface phenotypes in cells from ALL patients in remission and not controls. These studies may provide a test for recognition of ALL patients in remission and new prospects for the investigation of leukemogenesis.

Abbreviations

MCAF: Mycovirus containing *Aspergillus Flavus*
ALL: Acute Lymphoblastic Leukemia
EBV: Epstein-Barr Virus
GWAS: Genome Wide Association Studies
PBMC: Peripheral Blood Mononuclear Cells
PFU: Plaque-Forming Units
SAF: Supernatant of the culture of mycovirus containing *Aspergillus Flavus*
SCD: Sickle Cell Disease

Introduction

Acute lymphoblastic leukemia (ALL), with an overall incidence of 1.7 per 100,000 population, occurs both in adults and children [1,2]. However, the incidence is much higher in the pediatric age group, amounting to 2–5 per 100,000 population [1]. As such, it constitutes the most common cancer in the pediatric and adolescents, accounting for approximately 25–30% of all malignant disorders in this age group.

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While there has been significant progress in the treatment of ALL, the etiology of this disorder remains unknown. ALL is a heterogeneous disease, consisting of multiple subtypes, each with its own distinctive characteristics, including cellular, immunologic and genetic patterns, gross chromosomal rearrangements, DNA copy number or sequences and structural alterations [1, 3-7]. These result in changes in coding genes and noncoding elements, including noncoding RNA and enhancers [6-8]. Genome-wide association studies (GWAS) have identified multiple gene variant candidates as risk factors for ALL; however, the presence of these genetic findings does not equate the diagnosis of, or definitive susceptibility to, ALL. Currently, standard for the clinical diagnosis of ALL includes morphology, immunophenotyping, cytochemistry, genetic and cytogenetic evaluation. These techniques cannot diagnose ALL patients in a complete remission. By extension, such a diagnostic test may have potential to be used for screening and detection of susceptibility to ALL.

We have isolated a certain *Aspergillus Flavus* from the home of a single patient with ALL, where two members this family had the same disease. Electron microscopy examination of the culture of this organism identified mycovirus particles, in single or aggregate, with or without patent dense cores, within the body of the organism, in the hyphae and its culture supernatant. Supernatant of the culture of this organism was subjected to extensive chemical analysis which revealed lack of production of aflatoxin. The following report describes the effects of the supernatant of the culture of this mycovirus containing *Aspergillus Flavus* (SAF) on the PBMC of ALL patients in remission, long term survivors and controls i.e. patients with sickle cell disease undergoing exchange transfusion. The effect SAF on the PMBCs was analyzed by cell surface phenotypes, genetic markers and transcription factors known to be characteristic of ALL. The effects of co-incubation with Epstein-Barr Virus (EBV) was also explored. These findings may provide a new venue for investigation of leukemogenesis, and by extension, may have potential as a tool for research towards screening for ALL.

Materials and methods

Ethics statement and IRB approval

The study was approved by the Institutional Review Board a at St. Joseph's Hospital, Tampa Florida, FWA#00,006,065, IRB #1422, and informed written consent from all parents of participants under 18 and from all participants over 18 years of age was obtained, along with written assent from those between ages 12–17. All described experiments comply with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Patients and control cohorts

Twenty-five patients with ALL,14 males and 11 females, ages 5 to 23, including nineteen Whites, five Hispanics and one patient of Asian descendancy participated in the study. Twenty patients were in their first remission and on chemotherapy and five were long term survivors and off therapy ranging from 1 to 4 years. All ALL patients had B-cell phenotype disease. Consecutive patients were offered to participate in the study and all of those who accepted were enrolled. No bias of any kind, including selection based on risk factors or prognosis was made. Controls were patients with Sickle Cell Disease (SCD) undergoing manual partial exchange transfusion (23 patients, 14 females and 9 males). Patients with sickle cell disease were selected as controls because of the requirement for periodical partial exchange transfusion and thus ease of availability of blood. In a preliminary evaluation, race did not affect or alter the results. For this group, only the first discarded blood drawing was used. In separate plasma antibodies studies, there were no differences between healthy normal individuals and patients with sickle cell disease [9].

Cultures and controls

Aspergillus Flavus (AF) was isolated from home of a patient with ALL. This was done as a part of surveillance of home environment of the residence of a single patient, where there were two family members with ALL. Parents were given a petri dish of Sabouraud Dextrose Agar media to open in the bedroom of the patient for 10 min and return it for fungal evaluation. The sample was obtained after the second member of the family was diagnosed with ALL. The patient's ALL was in remission and there was no prior history of fungal infection or other unique characteristics. One axenic isolate was subsequently cultured in a glass bottle with an underlayer of 1% solid agarose (Aneresco, Solon, Ohio) and overlayer of 3.5 gm% Czapek-Dox broth (Difco, Becton Dickenson, Sparks, MD). Only one culture from the residence was obtained and used throughout the study. The culture was incubated at 28 °C in air with removal of supernatant and replacement of Czapek-Dox broth every approximately 4 weeks. The supernatant of the culture of *Aspergillus Flavus* (SAF) was filtered through a 0.45-micron filter (Corning Inc, Corning, NY) and adjusted to approximately 3 mg/ml protein before use. *Mycocladus Corymbifera*, was used as a negative control, and aflatoxin producing *Aspergillus Flavus* (Aflatoxin) and subsequently aflatoxin was used as a positive control. *Mycocladus Corymbifera* was cultured and its supernatant processed in an identical fashion as that of mycovirus containing *Aspergillus Flavus*.

To evaluate if our isolated AF contains mycovirus, the entire culture, including fungal growth and supernatant, were analyzed for viral contents by electron macroscopy (EM). This investigation was done with the knowledge that mycoviruses can suppress aflatoxin production.

Electron microscopy

A sample of *Aspergillus Flavus* culture was collected in 2.5% Glutaraldehyde in phosphate buffer. Raw suspension and filtered (0.1 μm) suspension fluid of sample were prepared for electron microscopy by applying approximately 2 μl of each onto a formvar coated grid, allowed to settle for 2 min and to dry after removal of excess liquid by wicking action of filter paper. The grids were subsequently stained negatively with 1% aqueous uranyl acetate for 1 minute. Again, excess liquid was wicked away with filter paper with grid allowed to dry completely before imaging in JEOL 1400 Transmission Electron Microscope (TEM). The solid component of the sample were classified as gel, agar, filamentous and sheet conformations. These were treated as fixed tissue and processed into polymer resin for eventual imaging by TEM. The standard protocol for osmication (1% aqueous osmium tetraoxide 1 hr) dehydration (graded ethanol series to 100% 10 min. ea.), resin infiltration and embedding (increasing concentration of resin in acetone with immersion in pure resin 1 hr. to overnight) was applied to the above solids. After polymerization (overnight at 70 70 7070 70 degrees Celsius), ultrathin section (80–90 nm) were collected onto copper grids, allowed to dry and contrast enhanced for 10 min in 1% aqueous uranyl acetate, rinsed well in DI water, and allowed to dry prior to examination by TEM. Ultramicrotomy sections were 100 nm or less.

Protein purification

For protein purification, SAF was buffer exchanged into 50 mM Tris (pH 8.0) containing 1 mM EDTA and 1 mM DTT via HiPrep 26/10 Desalting column (GE Healthcare), before loaded onto a HiTrap Q HP column (GE Healthcare). Target protein was purified by a 20-column volume linear gradient of 0–500 mM NaCl. Fractions containing target protein and SAF were concentrated and loaded onto a Superdex 200 10/300 GL (GE Healthercare) which was equilibrated with 50 mM Tris (pH 7.5) containing 300 mM NaCl and 1 mM EDTA. Fractions were assessed by SDS-PAGE and the corresponding band was confirmed by Mass Spectrometry.

Purified EBV was purchased and the titer was adjusted to 2×10^6

PFU/ml. Another source of EBV was Type 2 Burkitt's lymphoma Jijoye cell line (CCL87) (American Type Culture Collection (ATCC), Monassas, Manassas,VA). Owl monkey B-lymphoblast cell line (OML, clone 13C, CRL- 2312 ATCC), which was used as a negative control for CCL87 and EBV, was cultured as required. Supernatant of the culture of *Mycocladus Corymbifera,* was used as controls for SAF.

Using Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden), the peripheral blood mononuclear cells (PBMC) were isolated from the whole blood by density gradient centrifugation at 400 x g for 20 min at 18 °C, utilizing standard technique. PBMC from patients and controls were incubated at 10^6/ml with media, aflatoxin, SAF alone or in combination with patients' plasma or EBV/ culture supernatant of CCL-87 or CRL2312 at 1:1 ratio for a total volume to equal 10 ml at 37 °C with 5% CO^2 for 72 h or as described.

For cell surface marker investigations, initially a time exposure study at 1, 2, 3, 4, 5, 6, 24, 48 and 72-hour incubation with SAF was performed. For further studies, the 72-hour incubation was selected. Briefly, cell suspension from each post treatment specimen was centrifuged and cell pellets were incubated with anti-CD10-PE (Clone HI 10A), anti-CD-1^9-APC (Clone HIB19), anti CD45-APC—Cy7 (Clone2D1), anti-CD-117-PerCP-Cy5.5 (Clone 104D2) (all from BD Biosciences), for 45 min at 4 °C. Cells were washed and re-suspended in stain buffer (BSA). Acquisition and analysis were carried out on BD FACS Canto II using FACS Diva v 6.1.2. Samples were gated on the bases of forward and side scatter. Dead cells were excluded by setting appropriate threshold values. The percentage of cells positive for co-expression of CD10/CD19, CD34/CD19 and CD34/CD117 was recorded.

For microarray studies, five micrograms of purified total RNA was reverse-transcribed and labeled with biotin. The final cRNA obtained was hybridized to the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Thermo Fisher Scientific, Santa Clara, CA). Arrays were washed and stained using the Affymetrix Fluidics Station 450 and then scanned using Affymetrix GeneArray scanner. Array quality control was performed using Affymetrix Expression Console. Signal intensities were transformed to log2 base and imported to Partek Genomics Suite (Partek Genomics Suite, St. Louis, MO) software to conduct statistical analysis.

Statistical analysis

Cell surface phenotype study results were expressed as mean ± standard deviation. Student *t*-test was used to compare mean values. A p value of ≤ 0.05 was considered as statistically significant.

Results

Electron microscopic examination of the culture of the isolated *Aspergillus Flavus* revealed intracellular and extracellular existence of viral particles measuring 30–50 nm in single or aggregate form.

Chemical evaluation of SAF by SDS-PAGE, confirmed by mass spectrometric analysis, was compatible with a pattern known for *Aspergillus* species, but no aflatoxin was found. The probable major constituent proteins giving the maximum hits with the queries are shown in **Table 1**.

Flow cytometric analysis of PMBCs, cultured with supernatant of mycovirus containing *Aspergillus Flavus* (SAF) revealed that, unlike the controls, cells from ALL patients in remission and long-term survivors acquire ALL-like cell surface markers, i.e. CD10/CD19, CD19/CD34, CD34/CD117 $P = 0.0001$ (**Fig. 1**). The transition was gradual and achieved a plateau after 24 h (**Fig. 2**). Exposure to EBV alone produced a similar pattern and results. Addition of EBV to SAF however, did not change either the pattern or timing of the changes noted in the cell surface phenotypes of PBMC from ALL patients in remission/long term survivors as was seen with the SAF alone ($P>0.05$). There were no significant changes in the cell surface phenotype of PBMC from patients with sickle cell disease, which were used as control, with SAF, EBV or their combination as compared to the plain media ($P>0.05$) **(Fig. 2)**.

Table 1
Probable major constituents of mycovirus containing Aspergillus Flavus used in the experiments, as assessed by SDS-PAGE and confirmed.

	Name of Protein	Accession Number	M.W. (kDa)
1	Probable dipeptidyl peptidase 5	B8NBM3	80
2	Catalase B	Q877A8	80
3	Probable acetylxylan esterase A	A9JPE6	33
4	Probable beta-glucosidase A	B8NRX2	93
5	probable leucine aminopeptidase 2	Q2ULM2	54
6	probable beta-galactosidase A	B8N6V7	110
7	Probable dipeptidyl peptidase 4	B8N970	87
8	Probable glucan endo 1,3-beta-glucosidase C	Q2UUZ1	47
9	Glucoamylase	P36914	65
10	Probable mannosyl-oligosaccharide alpha-1,2-mannosidase 1B	B8N417	57
11	Probable beta-glucosidase M	B8N5S6	82
12	Probable glucan endo 1,6-beta-glucosidase B	B8NBJ4	45
13	Probable beta-glucosidase G	O75071	88
14	probable alpha-galactosidase A	B8MWJ5	59
15	probable alpha-galactosidase C	B8NWY6	83

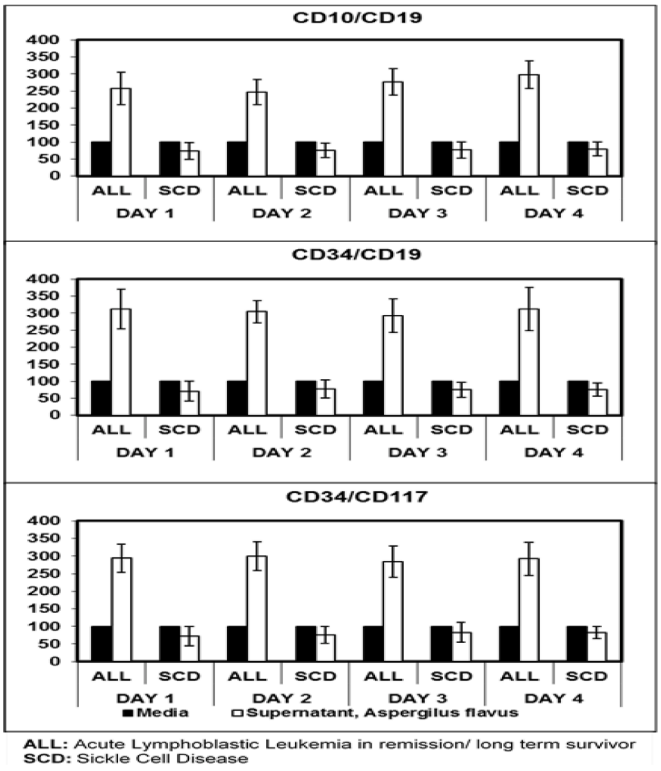

Fig. 1. Exposure of peripheral blood mononuclear cells (PBMC) from patients with acute lymphoblastic leukemia in remission/long term survivors and controls to the supernatant of mycovirus containing Aspergillus Flavus (SAF) collected from home of a patient with this disease, or plain media versus controls. Controls consisted of patients with sickle cell disease (SCD) undergoing exchange transfusion. PBMC were exposed for one to four days to SAF and samples were evaluated for cell surface phenotypes, using flow cytometry, every 24 h for four days. Samples were tested for CD10/CD19, CD34/CD19 and CD34/ CD117. Results are recorded as percentage of control. There was a significant appearance of cell surface phenotypes, characteristic of ALL, in the PBMC from ALL patients exposed to SAF as compared to controls $P<0.0001$. There were no differences between 24 h to 96 h evaluations.

The findings remained statistically unaffected at 48, 72 and 96 h (**Fig. 3**). Addition of autologous plasma to SAF reduced its effects on the development of ALL cell surface markers (**Fig. 3**). Unlike SAF, aflatoxin indiscriminately induced cell surface phenotypes, both in PBMC of

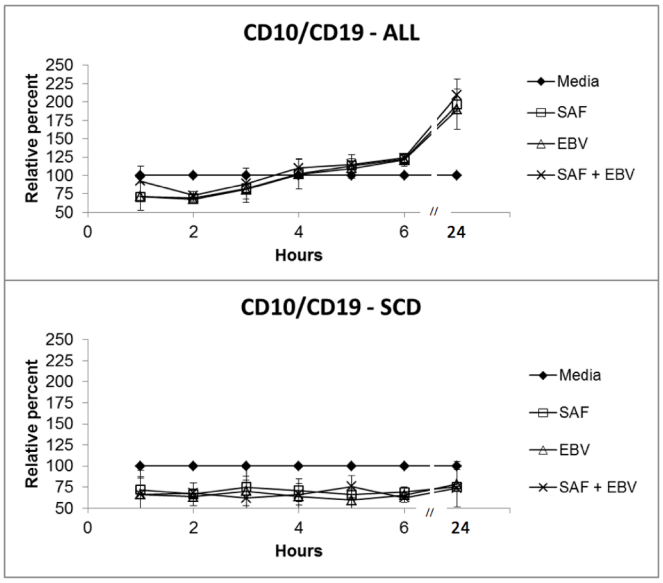

Fig. 2. Timeline evaluation of the effects of the supernatant of Aspergillus Flavus (SAF) with and without purified Epstein Barr Virus (EBV) on the peripheral blood mononuclear cells (PBMC) from patients with acute lymphoblastic leukemia in remission / long term survivors (ALL). Patients with sickle cell disease (SCD) were used as controls. PBMC were exposed to culture media, SAF, EBV or the combination of SAF and EBV for 1- 24 h. Samples were tested for cell surface phenotypes using flow cytometry, hourly for 6 h and then, at 24 h. On each occasion, CD10/CD19, CD34/CD19 and CD34/ CD117 cell surface markers were tested, and results expressed as percent of control. The effects on change of surface markers expressions for all above tested combination phenotypes was similar and therefore, only the results of CD10/CD19 are shown. Upper panel shows the effects of SAF on cell surface phenotype of PBMC from ALL patients in remission, which was gradual and reached significance at 24 h. Exposure to EBV alone had a similar pattern and effects. Addition of EBV to SAF did not change the pattern or timing of the change in cell surface phenotypes of PBMC of ALL patients in remission. As shown in the lower panel, there were no statistically significant changes ($P>0.05$) in the cell surface phenotype of PBMC from patients with sickle cell disease, which were used as control, with SAF, EBV or their combination as compared to the plain media.

normal controls as well as those from patients with ALL in remission $P<0.0001$ (Fig. 3). Culture media, patient's plasma alone, supernatant of CRL-2312, Avian leukosis virus and supernatant of the culture of *Mycocladus Corymbifera*, used as controls, had no significant effects on the cell surface phenotypes of PBMC of either ALL patients in remission or controls ($p \geq 0.05$) (Fig. 3).

Similar to the effects noted in the above timing studies, incubation of PBMCs from ALL patients and controls with EBV or CCL87 produced similar results to that of SAF at 24, 48, 72 and 96 h of incubation ($P = 0.0001$). In studies of incubation form 24 to 96 h, the addition of CCL-87 to the SAF did not have any additive effects on the development of cell surface phenotypes in PBMCs from ALL patients in remission as compared to the SAF alone ($P>0.05$) **(Fig. 3)**. Control i.e., CRL-2312 had no effects on the development of the cell surface phenotypes in PMBCs from ALL patients in remission or controls **(Fig. 3)**.

DNA microarray analysis of PBMC, before and after exposure to the supernatant of the culture of the mycovirus containing *Aspergillus flavus* revealed significant changes in the cells from ALL patients in remission as compared to their pre-exposure and control counterparts. Examples of upregulations included JAK1 (x 12.87-fold), JAK2 (x 1.5 -fold), JAK3 (x 2.73 -fold), IKZF1 (x 10.12 -fold), MCL1 (x 59.37-fold), MYC (x14.19 -fold) HDAC 1 (x26.39 -fold) and downregulation of PAX 5 (−3.05 -fold). Using the Western blot technique, in a limited study, immunoblotting analysis post incubation with SAF, showed a significant robust activation of transcription factor NF- ϰB p65 in ALL patients while there were no changes in controls.

Discussion

Acute lymphoblastic leukemia occurs in all age groups and is the most common childhood cancer, constituting 72% of all leukemias in children [1]. In addition, the clinical distinction between diffuse lymphoblastic lymphoma and ALL in the pediatric age group is arbitrary and based on the location of the disease. Acute lymphoblastic leukemia occurs in adults, albeit with much lower frequency [2]. The etiology of ALL remains elusive and currently there are no methods to universally predict susceptibility to this disease and means to prevent it [9]. While a large and diverse number of causes and risk factors for ALL have been suggested [10-36], none can be proven with any degree of certainty or be invariably applied to all cases. A recent revised two-hit model for the occurrence of precursor B cell acute lymphoblastic leukemia propose that this disease arises through a two-step process [37]. The first step is a predisposing genetic mutation and the second, involves exposure to infections [37]. Based on this proposal, the process of development of ALL is initiated in utero by fusion gene formation or hyperdiploidy, and production of pre-leukemic clone. The first step is estimated to be found in 5% of the newborns, but only one in a 100 of the predisposed go on to develop the disease. It is postulated that exposure to infections early in life are protective, but in their absence, in a small fraction of the population, interactions later in life trigger the critical secondary cellular mutations. Although the exact sequence of the events cannot be proven and multiple alternatives may exist, genetic predisposition with random exposure to an infection can be plausible. While a number of genetic mutations are proposed, no infectious agent or category has been suggested. Our findings introduce a certain organism i.e. mycovirus containing *Aspergillus flavus*. While *Aspergillus flavus* is widespread in nature, it may not always contain the certain mycovirus.

Since the mycovirus containing *Aspergillus flavus* was collected from the residence of the patient when a full remission was achieved several months post diagnosis, its existence at the time of diagnosis cannot be ascertained. The development of cell surface phenotypes and genetic changes, characteristic of ALL, upon exposure to a mycovirus containing *Aspergillus Flavus* in the PMBCs from ALL patients, and not in controls, may indicate their predisposition to this disease and may highlight their distinctive biology. This may be partly due to genetic or epigenetic factors in ALL cells which may be absent or biologically silenced in controls. In support of the validity of changes in the cell surface phenotypes, post exposure to SAF in PBMC from ALL patients in remission, there were up-regulation of genetic markers typical of ALL, including, but not limited to, JAK1, JAK2, JAK3, IKZF1, MCL1, and down-regulation of PAX-5. Likewise, post incubation with SAF, there was a significant activation of NF-ϰB p65 transcription in ALL patients and not controls. This data is in line with our previous findings which reveal that the plasma of patients with ALL react to the supernatant of the culture of the same mycovirus containing *Aspergillus flavus* used in the present study. In that study [9] only plasma of patients were utilized using ELISA technique for the detection of antibody to mycovirus containing *Aspergillus flavus*.

Infectious agents have been implicated to cause childhood acute leukemias, an assumption without availability of a consistent agent [33-37]. The association of EBV and Burkett's lymphoma in the endemic area of Eastern Africa is strong. However, lack of universal application of the finding, characteristic 8;14 chromosomal translocation resulting in constitutive activation of c-Myc oncogene, variation in viral gene expression in subgroups of patients, effects of EBV oncoproteins, p53 mutations and a host of other factors complicate this association [34]. Research data regarding the exposure to EBV in the first two years of life, resulting in serological response are available. HIV and other viral agents have also been implicated in the etiology of ALL [35-37]. In our studies, direct effects of EBV on cell surface phenotypes of PBMCs of ALL patients, and not controls, is of interest. In view of existing literature

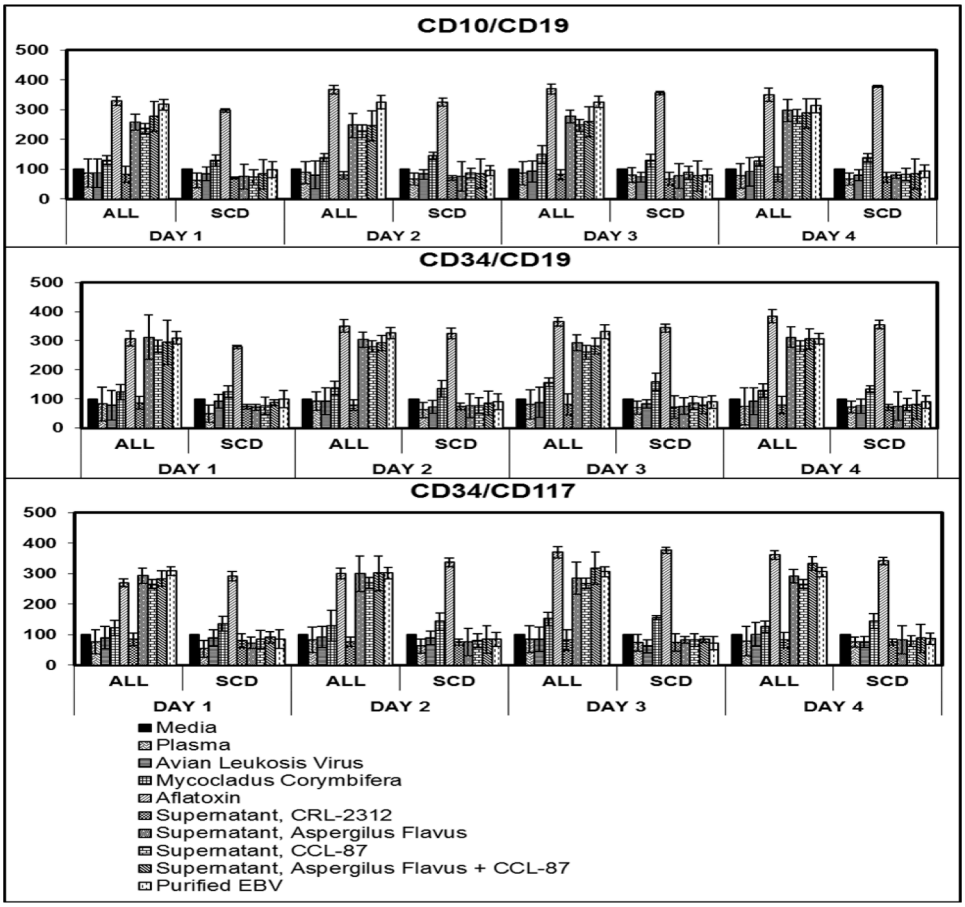

Fig. 3. Exposure of peripheral blood mononuclear cells (PBMC) from patients with acute lymphoblastic leukemia in remission / long term survivors(ALL) and sickle cell disease (SCD) to the culture media, plasma, avian leucosis virus, *Mycocladus Corymbifera*, aflatoxin, supernatant of CRL-2312 cell line culture, supernatant of culture of *Aspergillus Flavus* obtained from a home of a patient with acute lymphoblastic leukemia, Supernatant of CCL87 culture which contains EBV, supernatant of culture of *Aspergillus Flavus* co-cultured with purified Epstein Barr Virus (EBV) and purified EBV alone. Samples were analyzed for cell surface phenotype using flow cytometry every 24 h for four days. Each day, samples were tested for CD10/CD19, CD34/CD19 and CD34/CD117. Results were recorded as percentage of control. Aflatoxin indiscriminately induced abnormal cell surface phenotypes, both, in PBMC of ALL patients as well those of sickle cell disease controls. Supernatant of culture of *Aspergillus Flavus*, with and without EBV, and CCL87 or EBV alone induced positive cell surface phenotypes in PBMC of ALL patients in remission and not controls, distinguishing these two groups ($p<0.0001$). Other agents used had no effects either on PBMC of ALL patients in remission or controls.

regarding EBV and malignant disorders, interaction of these organisms adds to the complexities of the subject and needs to be explored. The observation that addition of EBV sources to SAF does not change the results is of interest. The *Aspergillus Flavus* used in our studies contains mycovirus particles. To what extent this, singularly or in combination with its host, affects the alterations seen in the described studies needs to be further explored. The role of genetic factors and their variations in acute leukemias are well recognized [7,8,37]. Mice with monoallelic loss of the B-cell transcription factor PAX5 are genetically predisposed to B-cell ALL, if exposed to pathogens [37]. In our studies, upon exposure of PBMCs to SAF, PAX5 was downregulated.

Carcinogenic effects of fungi and aflatoxin have long been recognized [38-41]. Published reports of mycotoxin and aflatoxin producing strains of fungi, including *Aspergillus Flavus* in residences of leukemia patients, including ALL, are available[38-41]. In some of the prior reports however, carcinogenic impacts of fungi were attributed to mycotoxins or their immunosuppressive effects [38-40], and not direct effect as described in our studies. Of course this cannot be taken as proof that *Aspergillus flavus* is a direct etiological factor in ALL. While significant data regarding the carcinogenic effects of various mycotoxins is published, mycobiome has only rarely been implicated in direct tumorigenesis such as pancreatic oncogenesis. No data pertaining to the effects of fungal organisms infected with a mycovirus, is readily available.

In a published report in which sera from 36 cancer patients were tested against an aflatoxin producing *Aspergillus Flavus* isolated from a leukemia-associated home, utilizing a modified microimmunodiffiusion, 30% of cancer patients, 15 of whom had leukemia or lymphoid malignancy, and only 6% of controls had yielded a precipitation band [39]. A published report describes four leukemic patients, from three families, in a house where mycotoxin-producing fungi was isolated [38]. The authors attributed leukemogenesis to the immune depressive effects of mycotoxins [38]. In a study of fungal isolates from a house where a husband and wife had developed acute myelomonocytic and undifferentiated leukemia, respectively, the extract of three fungal isolates had a depressive effect on a phytohemagglutinin skin test in guinea pigs as compared to fungal extracts from a control residence [40]. Report of the occurrence of multiple cases of leukemia in a single dwelling is also available [41]. It is of interest that in our study, mycovirus containing *Aspergillus Flavus* species was isolated from home of a patient with ALL.

Mycotoxins, including aflatoxins, ochratoxin A, fumonisins, certain trichothecenes, and zearalenone, produced by fungal agents are known to be carcinogens. In the experiments described here, aflatoxin, which was used as a positive control, indiscriminately induced abnormal cell surface phenotypes in both, PBMC from ALL patients in remission and controls. It is of interest that the *Aspergillus Flavus* utilized in the present studies did not produce any aflatoxin. Loss of aflatoxin production is reported to be associated with *Aspergillus Flavus* infected by mycoviruses [42-44]. This appears to be the case in the mycovirus containing *Aspergillus Flavus* isolate utilized in our studies. In electron microscopy examination, particles consistent with mycovirus were identified in the *Aspergillus Flavus* used in the above experiments. Some mycotoxins, such as Patulin and Gliotoxin, a toxic epipolythiodioxopiperazine (ETP) metabolite with significant immunosuppressive activity, can cause apoptosis in PBMC and possess selective in vitro cytotoxicity [45], while others have suppressive effects on the immune response [46]. The studies outlined in this report, for the first time, reveal direct cellular markers and genetic changes, characteristic of ALL, upon exposure of PBMCs form ALL patients in complete remission to the supernatant of the culture of mycovirus containing *Aspergillus Flavus*. These effects were not seen in PBMCs of controls. Gliotoxin, in vivo, is shown to inhibit transcription of NF-ϰB in response to a variety of stimuli in T and B cells [47]. In high concentrations, this agent was reported to prevent NF- ϰB

DNA binding in vitro [47]. Preliminary observations from our studies suggest that SAF, when incubated with PBMC of patients with ALL in remission, results in a significant activation of transcription factor NF-ϰB p65 in ALL patients in remission and not in controls. Presence of NF-ϰB p65 (Rel A) is required for protection from TNA-α. It is of interest that constitutively activated NF- ϰB complexes have been previously reported in the majority (39/42) of ALL patients without subtype restriction [48].

In limited studies, when cultures of *AF* with and without EBV were irradiated, this significantly increased co-expression of CD10/CD19, which is one of the characteristic features of ALL (article in preparation). This was seen only in the PBMC from ALL patients in remission, and not controls. The mechanism of this interaction is not clear.

Lack of any stimulatory effects of culture media, plasma, Avian Leukosis virus, *Mycocladus Corymbifera,* etc., in the induction of ALL cell surface phenotype such as co-expression of CD10 and CD19 and others on the PBMC of patients with ALL in remission and normal controls, may indicate that the results obtained with SAF, with or without EBV, are not due to nonspecific stimulation by the media, a random viral or fungal agent.

The discriminative induction of cell surface phenotypes and genetic changes, seen in PBMC of ALL patients in remission and long- term survivors of this disease, and not controls, may be of significance and may have potential for providing a test for susceptibility to ALL. Furthermore, these studies may provide a mean to better understand the effects of AF, with and without EBV, in leukemogenesis. Most recently, utilizing plasma cfDNA bisulfate sequencing targeting over 100,000 methylation regions, it is reported that depending on the stage of the disease, this methodology can detect 50 type of cancers [49]. The study emphasizes the value of early detection in malignancies. Currently, effective screening for ALL is not available. Limited attempts to screen for this disease has been concentrated on genetic markers [50]. Further studies are needed to examine if SAF can provide a tool for screening for susceptibility to ALL, for possible prevention of the disease and if genetic and epigenetics may have a role in the observed effects.

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Lay summary

The cause of acute lymphoblastic leukemia (ALL) is unknown. A highly publicized “two-hit” model suggests genetic background and infection. While several genes have been identified, no infection is suggested. We have isolated a special type of *Aspergillus Flavus (*AF*),* a fungus, from an ALL patient’s home. This organism contains virus and unlike most AF does not produce a cancer producing substance called aflatoxin. When blood cells from ALL patients in remission (no active disease) were exposed to culture of this virus containing AF, they redeveloped markers and genetic changes known to be characteristic of active leukemia. This did not happen with controls. The findings are unprecedented and This data may add significantly to the knowledge of how leukemia is produced.

Precis for use in the table of contents

- A mycovirus containing *Aspergillus Flavus* (MCAF) was cultured from the home of a patient with acute lymphoblastic leukemia (ALL). This organism does not produce aflatoxin.
- Exposure to the supernatant of the culture of MCAF reproduced ALL surface and genetic markers in the peripheral blood mononuclear cells from ALL patients in complete remission and not controls.

Data availability: Data available post publication per request.

Patents

Studies described are the subject of patents in the United States, European Union, Canada, Japan, Russia and elsewhere.

Ethics

All described experiments comply with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The work presented meets the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals regarding human populations including sex, age and ethnicity.

Ethics statement and irb approval

The study was approved by the Institutional Review Board (IRB) of St. Joseph’s Hospital of Tampa, Florid a, FWA# 00,006,065, IRB #1422 and informed written consent from all parents of participants under 18 and from all participants over 18 years of age was obtained, along with written assent from those between ages 12–17. All described experiments comply with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Data availability

The data that support the findings of this study are available on request from the corresponding author. Patients’ name and data are not publicly available due to privacy and ethical restrictions.

Author statement

This manuscript has been read and approved by all the authors. Each author meets requirements for authorship and believes that the manuscript represents their work.

Author contributions

The original idea, collection and isolation of *aspergillus flavus*, design of the study and preliminary laboratory work are those of Dr. Cameron Tebbi who also supervised conduct of the project. Dr. Aruna Badiga performed flow cytometry, co-incubation with EBV studies, FPLC separation, collection and analysis of the data, Dr. Eva Sahakian supervised confirmatory studies and assisted with preparation of the manuscript. John J. Powers, BSc performed confirmatory studies, Alex N. Achille, BSc assisted with confirmatory studies. Dr. Samuil Patel assisted with the patient recruitment and preparation of samples for confirmatory studies. Felicia Migone performed the initial technical work

Declaration of Competing Interest

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

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