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Impaired immune function in children and adults with Fanconi anemia

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Abstract

Background—Fanconi anemia (FA) is a rare genetic disorder characterized by genome instability, bone marrow failure, and cancer predisposition. Previously, small studies have reported heterogeneous immune dysfunction in FA.

Procedure—We performed a detailed immunologic assessment in a large FA cohort who have not undergone bone marrow transplantation or developed malignancies. Comprehensive quantitative and functional immunologic assessment of 29 FA individuals was compared to healthy age-matched controls.

Results—Compared to non-FA persons of similar ages, FA individuals showed lower absolute total B cells ($P < 0.001$), lower memory B cells ($P < 0.001$), and decreased IgM ($P < 0.001$) but normal IgG. NK cells ($P < 0.001$) and NK cytotoxicity ($P < 0.001$) were decreased. CD4⁺ T cells were decreased ($P = 0.022$), while CD8⁺ T cell and absolute T-cell numbers were comparable. Cytotoxic T cells ($P < 0.003$), and antigen proliferation response to tetanus ($P = 0.019$) and candida ($P = 0.019$), were diminished in FA. Phytohemagglutinin responses and plasma cytokines were normal. Within FA subjects, adults and older children (> 10 years) exhibited higher CD8⁺ T cells than younger children ($P = 0.004$). Documented atypical infections were infrequent, although oral human papilloma virus (HPV) prevalence was higher (31% positive) in FA.

Conclusions—Overall, these results demonstrate a high rate of significant humoral and cellular immune dysfunction. Continued longitudinal study of immune function is critical to understand evolution with age, bone marrow failure, and cancer development.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Keywords

bone marrow failure; Fanconi anemia; immune response

1 INTRODUCTION

Fanconi anemia (FA) is a rare genetic disorder characterized by genome instability, progressive bone marrow failure (BMF) and predisposition to malignancy, predominantly acute myeloid leukemia, and gynecological and head and neck squamous cell carcinomas.¹ The FA phenotype is the result of mutations in one of 20 known genes that function in the DNA interstrand cross-link repair pathway,^{2,3} leading to aberrant correction of DNA breaks. Heterogeneous abnormalities in the immune function of individuals with FA have been reported in several studies.^{4,5} We have previously reported in a small series that individuals with FA have decreased natural killer (NK) cells and NK function, perforin and granzyme levels, CD19⁺ B cells, cytotoxic lymphocyte (CTL) function, and tetanus responses.⁶ Justo et al. also observed a decrease in NK CD56dimCD16⁺ and CD8⁺ T cells in FA compared to non-FA controls.⁵ Giri et al. found that FA adults, but not children, had significantly reduced IgG, IgA, and IgM; total lymphocytes; and CD4T cells compared with their adult relatives ($P < 0.001$).⁴ Consistent with our previous study, Giri et al. found that both children and adults with FA had fewer B and NK cells compared with controls.

Better understanding of immune function in individuals with FA is critical to advancing our comprehension of differences in viral susceptibility and/or response, as well as immune surveillance with regard to neoplastic cells in this population with known predisposition to DNA damage and malignancy. The effects of age or disease status must also be explored. We present a comprehensive assessment of immune function in a cohort of young children and adults with FA, compared to healthy age-matched controls.

2 METHODS

2.1 Study participants and characteristics

Patients with a confirmed diagnosis of FA (by diepoxybutane testing) followed at the Fanconi Anemia Comprehensive Care Center (FACCC) at Cincinnati Children's Hospital Medical Center (CCHMC) were invited to participate in the study if they had no history of cancer and had not yet received a bone marrow transplant ($N = 25$). Individuals with confirmed diagnosis of FA participating in the Fanconi Anemia Research Fund-sponsored Adult or Family Meetings at Camp Sunshine were also offered the opportunity to participate ($n = 4$). Informed consent was obtained from participants who were at least 18 years of age. Parental permission was obtained for children less than 18 years of age. The CCHMC Institutional Review Board approved the study. Medical records were reviewed for clinical infectious history. BMF status was defined as follows: normal/mild: normal hemoglobin for age or >10 gm/dl, absolute neutrophil count $>1.0 \times 10^9/l$, platelets $>100 \times 10^9/l$; moderate: one or more of the following hemoglobin 8–10 g/dl, absolute neutrophil count $<1.0 \times 10^9/l$, platelets $>20 \times 10^9/l$; severe: transfusion dependent or hemoglobin <8 gm/dl, absolute neutrophil count $<0.5 \times 10^9/l$, platelets $<20 \times 10^9/l$.

2.2 Health survey

Health surveys were administered by using the Research Electronic Data Capture system.⁷ The survey collected demographic, socioeconomic, clinical, sexual history, lifestyle, and environmental exposure information. Participants aged 15 years and older were asked to complete surveys for themselves unless they were physically or mentally unable to do so, in which case a parent or guardian was asked to complete the survey on their behalf. For younger children, particularly those less than 12 years old, a parent/guardian was asked to complete the survey for participating children.

2.3 Blood collection and processing

Approximately 90% of blood samples were collected at the FACCC and immediately processed (N = 25). Participants recruited at the Fanconi Anemia Research Fund-sponsored Family Meeting at Camp Sunshine and Adult Meeting in Baltimore Maryland were given a kit with blood tubes, physician orders, and a prepaid mailing label with all samples arriving within the 24-hr time limit for testing.

2.4 Immune assays

Participant peripheral blood samples were tested for lymphocyte subsets, B-cell panel, and immunoglobulin levels (IgG, IgM, IgE, and IgA) along with tetanus and diphtheria titers. In addition, NK function, CTL function, intracellular and plasma cytokines, T-cell receptor (TCR)-alpha/beta, and TCR-gamma/delta populations, as well as antigen and mitogen stimulation, were tested. Immunoglobulin levels (IgG, IgM, IgE, and IgA) levels were determined by standard methods in the CCHMC Clinical Laboratory. Tetanus and diphtheria titers were determined by Quantitative Multiplex Bead Assay at ARUP Laboratories. Remaining assays were performed at CCHMC Diagnostic Immunology Laboratory as previously described.⁶ Results were interpreted with respect to age-appropriate reference ranges established in the laboratories.

Analysis of intracellular cytokines was performed as mentioned in previously published studies.⁸ In short, heparinized peripheral blood samples were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A. The cells were then stained with cell surface monoclonal antibodies for phenotyping, fixed, permeabilized, and stained with monoclonal antibodies to the cytokines interferon (IFN)- γ , TNF- α , and IL-4. The cells were analyzed by flow cytometry on a FACS Canto II (BD Biosciences).

Plasma cytokines were analyzed using the Human Cytokine Magnetic 10-plex Panel from Life Technologies as previously published.⁹ In short, this panel measures the plasma concentration of the following cytokines: interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, IFN- γ , and TNF- α using beads of defined spectral properties conjugated to analyte-specific capture antibodies.

2.5 Immunophenotypic analysis of B-cell maturation in bone marrow

Bone marrow aspirates were processed for flow cytometric immunophenotyping using a standardized approach.¹⁰ Briefly, an aliquot of the sample was incubated with saturating amounts of relevant fluorescently conjugated mouse anti-human monoclonal antibodies,

followed by red cell lysis and fixation. Using a FACSCanto II flow cytometer (BD Biosciences), $0.5\text{--}1.0 \times 10^6$ events were acquired from each sample and data were analyzed with FCS Express v4.0 software (De Novo Software). Antibodies (BD Biosciences) to the following antigens were used to specifically profile the three stages of normal B-cell maturation clustered populations based on fluorescent intensities and gated CD45 and orthogonal light-scattering properties: CD10 phycoerythrin cyanine 7 (PE-Cy7), CD19 peridinin chlorophyll protein (PerCP), CD20 PerCP, CD34 phycoerythrin (PE), and CD38 PerCP and CD45 V450 (BD Horizon™). Late-stage and mature B cells included CD45^{bright} CD34^{neg} CD10^{dim-mod} CD19^{mod-bright} CD20^{bright} CD38^{mod}; intermediate precursor B cells: CD45^{mod} CD34^{neg} CD10^{mod-bright} CD19^{bright} CD20^{dim} CD38^{bright}; and early-stage precursor B cells: CD45^{dim} CD34^{mod-bright} CD10^{bright} CD19^{mod} CD20^{neg} CD38^{bright}. Percentages of these B-cell stages were calculated based on total events divided by events in each B-cell stage and multiplied by 100.

2.6 Oral sample collection and human papilloma virus testing

Oral-rinse and gargle samples were collected in 15 ml saline and processed as previously described.¹¹ DNA was isolated using the 5 Prime ArchivePure DNA kit (5 Prime Inc., Gaithersburg, MD) according to the manufacturer's protocol, and The Linear Array HPV Genotyping Test (Roche Molecular Diagnostics, Indianapolis, IN¹²) was used to detect 37 individual human papilloma virus (HPV) types including high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, and Subtype 82 W13B) and low-risk HPV types (6, 11, 40, 42, 54, 55, 61, 62, 64, 72, 81, 83, 84, and 89) in the Laboratory of Darron Brown at Indiana University as previously described.¹¹

2.7 Statistical analysis

We compared the immune assays of participants with FA with reference values to assess whether the immune function of FA participants was impaired. Reference values were from normal non-FA individuals tested in our CLIA-certified Diagnostic Immunology Laboratory. For IgG, IgM, *Candida albicans*, tetanus, phytohemagglutinin (PHA), CTL lytic units, and NK lytic units, the 5th and 95th percentiles were available from the non-FA controls of various ages. By comparing FA data with the 5th and 95th percentiles, we categorized the immune measures of the FA patients into low, normal, and high groups. To test whether one particular immune function was lower in FA compared with non-FA, we performed an exact binomial test, in which the proportion of FA patients who had "low" immune measures was compared with 5%, which is the expected proportion of "low" from the non-FA population. For total B cells, memory B cells, total T cells, CD8⁺ and CD4⁺ T cells, and NK cells, mean and standard deviation of the non-FA were available. Using age-specific references to account for immune function changes in response to growth and development, we calculated age-specific z scores as (immune measure (FA) – mean (non-FA))/SD (non-FA). We then conducted one-sided one sample *t*-test to test whether the z scores of the FA patients were lower than 0, which are the expected mean z scores of the non-FA population. We performed age-based comparisons, in which age was dichotomized into <10 years versus ≥10 years groups, or <18 years (pediatric) versus ≥18 years (adult) groups. Ten years of age was selected based on the average age of bone marrow transplant at CCHMC over the last 5

years. All statistical analyses were performed using SAS, version 9.3. A P value ≤ 0.05 was used to indicate statistical significance.

3 RESULTS

3.1 Study population

Baseline demographics and disease characteristics for the entire cohort of 29 patients as well as age group specific data are described in Table 1. Twenty-nine individuals with FA completed the baseline survey and provided blood for immune testing. The median age for all participants was 12 years (range 2–47). Twelve of the 29 subjects were under the age of 10 years and 22 participants were 18 years or younger. None of the participants had a history of cancer or had received a bone marrow transplant at the time of enrollment. No participants had severe BMF at baseline. Additionally, 52% were male, 41% were complementation group A, and 34% had other complementation groups, while 24% did not yet know their complementation group. There were no statistically significant differences in demographics between the younger and the older groups. Overall, there were considerable differences in the immune parameters observed across participants with FA with most being significantly reduced after considering typical differences related to normal growth and development by age (Figs. 1 and 2). Across the immune parameters studied, however, there were no differences observed by marrow failure status.

3.2 T lymphocytes

Subjects with FA had fewer absolute $CD4^+$ T cells ($z = -1.41$; $P < 0.001$) than the non-FA population, but absolute $CD3^+$ total T cells ($z = -0.02$; $P = 0.47$) and $CD8^+$ T cells ($z = 0.49$; $P = 0.98$) did not significantly differ (Fig. 1 and Table 2). In general, $CD8^+$ T cells did not statistically differ from normal subjects ($P = 0.98$), a significant difference was observed between individuals with FA who were less than 10 years old and those that were older than 10 years ($P = 0.004$). Older subjects tended to have greater absolute $CD8^+$ T cells than normal subjects, while younger children commonly were similar to normal children or were more likely to have lower $CD8^+$ values (Fig. 1). T-cell proliferation in response to antigen was reduced in individuals with FA in response to *C. albicans* (19% abnormal; $P = 0.019$) and tetanus (19% abnormal; $P = 0.019$), but not to PHA ($P = 0.64$). CTL function was also significantly reduced in those with FA (24% abnormal; $P = 0.003$) (Fig. 2 and Table 3).

3.3 B lymphocytes

Absolute $CD19^+$ B cells ($z = -1.41$; $P < 0.001$) and $CD19^+/CD27^+$ memory B cells ($z = -0.98$; $P < 0.001$) numbers were significantly reduced (Fig. 1 and Table 2). Concomitant immunophenotypic examination of 15 available marrow samples revealed a strong positive correlation ($r = 0.96$) between the mature marrow B-cell population and $CD19^+$ B cells, suggesting a similarity between peripheral blood and bone marrow populations. IgM levels were significantly diminished in FA individuals ($P < 0.001$), whereas IgG levels were not statistically lower (Fig. 2 and Table 3).

3.4 NK cells

Absolute CD16⁺ NK cells were also significantly diminished in FA ($z = 0.92$; $P < 0.001$) (Figs. 1 and 2). Importantly, 16% of FA individuals with normal levels of NK cells had reduced NK-cell function ($P = 0.07$; Fig. 2 and Table 3), suggesting that function may be impaired even in those with normal NK levels. Analysis of intracellular and plasma cytokines (IL1b, IL2, IL4, IL6, IL8, IL10, IFN- γ , TNF- α , and GM-CSF) did not reveal significant differences between our FA population and controls.

3.5 Infections

Review of clinical records did not reveal record of any atypical infections such as *Pneumocystis jiroveci* or disseminated or invasive bacterial, viral, or fungal infections requiring hospital admission or intensive care. One young adult FA female was identified with complete absence of B cells and a 1-year history of progressive extensive bilateral lower leg ulceration and found to have infection with *Flexispira* and *Helicobacter* species. Infections with these organisms leading to similar wound presentations have been documented in individuals with X-linked agammaglobulinemia.¹³

All 29 of these individuals were tested for 37 oral HPV types and nine of 29 (31%) were positive for at least one HPV strain, including high-risk types such as HPV16 and HPV18, but also less common types such as HPV84. The median age of those testing positive for oral HPV was 12 years (range 3–34 years). Only three of the nine (33%) HPV-positive participants had previously been vaccinated for HPV by self-report and two reported a history of sexual exposure. Another participant reporting previous sexual history had not yet been vaccinated at the time of sampling.

4 DISCUSSION

Immune dysfunction is known to occur in individuals with FA, but has not been well characterized, especially in those with less severe BMF. Our study examined quantitative and functional aspects of immune function in a relatively young cohort of individuals with FA without advanced marrow failure or history of malignancy, and used more detailed testing than prior studies including the use of age-specific references to control for immune function variations in response to growth and development. Consistent with our previous work, we confirm decreases in number and function of B and NK cells despite overall less severe marrow disease.⁶ We also report a previously unreported decrease in memory B-cell numbers in FA individuals. Moreover, our cohort demonstrates quantitative changes in CD4⁺ T cells, as well as abnormalities in T-cell function, demonstrated by reduced antigen responses and reduced CTL function.

Previous studies have reported decreased numbers of B cells^{6,14} as well as both normal^{6,15} or decreased immunoglobulins^{16,17} in individuals with FA. Recently, Giri et al. demonstrated normal immunoglobulins in a small cohort of younger FA individuals, but decreased IgG and IgM in older FA individuals, several of whom had malignancy.⁴ Our cohort demonstrates not only decreased total B-cell numbers, but also a novel quantitative defect in CD19⁺CD27⁺ memory B cells. In contrast to the report of Giri et al., our younger

cohort demonstrates decreased IgM in both children and adults with FA but no decrease in IgG. Quantitatively normal total immunoglobulin levels, however, do not address potential differences in immunoglobulin diversity, particularly in the memory B-cell compartment.

We did not detect differences in absolute T-cell numbers (CD3⁺) in FA regardless of age, in contrast to the lower T-cell numbers observed in adults with FA reported by Giri et al. This difference may reflect the younger age of our cohort. Interestingly, we observed a significant reduction in CD4⁺ T cells in FA individuals. We also detected an association between absolute CD8 T cells and age even after z-transformation of the CD8 measurement. Lower age-specific CD8 z scores were observed in FA subjects younger than 10 years, indicating a skewing of the CD4/CD8 ratio in older FA individuals. This association may suggest that age could serve as a proxy for an unknown disease variable. Indeed, as the older individuals in the study by definition have not progressed to marrow failure or need for hematopoietic stem cell transplant, they may represent a different disease phenotype.

Literature reports of T-cell responses in FA individuals have been heterogeneous. Some studies show poor response to mitogens (PHA and pokeweed) as well as antigens such as tetanus toxoid, mycobacterium derivative, or cytomegalovirus antigens in FA individuals or their family members.^{6,18,19} Others have demonstrated normal proliferation to antigens and mitogens.^{4,20} Our current cohort confirms our previous findings of a decreased response to tetanus toxoid and a normal response to PHA; however, we also demonstrate a poor response to candida. Additionally, we report evidence of more global T-cell dysfunction, as reflected in a decrease in cytotoxic T-cell responses. Given that this *in vitro* assay measures cytotoxic activity toward EBV, our data collectively suggest that individuals with FA may develop an increased vulnerability to some infections, including viral infections.

Our data confirm low NK-cell numbers in FA individuals, as seen in our previous report and reported by Giri et al.,⁴ and complement our previous finding of reduced perforin and granzyme B expression in NK cells by reporting decreased NK-cell function as evaluated by lytic units, even in FA individuals with normal numbers of NK cells. Importantly, reduced NK-cell activity may comprised T cell and CTL function and critically alter immune surveillance with regard to neoplastic cells in a population with known predisposition to DNA damage and malignancy, as well as may lead to greater susceptibility to viral infection that may be reflected in the increased prevalence of oral HPV infection seen in this cohort and reported previously by our group.¹¹ However, higher rates of infections with viruses such as HSV, CMV, or EBV and atypical bacterial infections were not observed. In particular, we saw no instances of *P. jiroveci* pneumonia to support need for routine prophylaxis. This suggests the phenotype of progressive immune deficiency may differ among individuals with FA or is perhaps more specific for viruses such as HPV and not globally profound.

Taken together, compromised humoral and cellular immune dysfunction may facilitate the HPV as a potential driver of malignancy in this high-risk population,²¹ such that early identification of deficiencies in immunological integrity may allow for modification of immunization strategies. Additional studies of immune function and HPV may help to clarify the still controversial role of HPV in FA-associated squamous cell carcinoma.

In summary, our data support immune dysfunction in all tested compartments in individuals with FA, even in those with mild BMF. Continued prospective longitudinal studies are needed to look more closely at longitudinal changes over time in immune function of individuals with FA. This will be critical to advance our understanding of the interplay of age and immune function with respect to chronic inflammation, progression of marrow failure, viral susceptibility, vaccine response, and immune surveillance of malignancy.

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Abbreviations

BMF	bone marrow failure
CCHMC	Cincinnati Children's Hospital Medical Center
CTL	cytotoxic lymphocyte
FA	Fanconi anemia
FACCC	Fanconi Anemia Comprehensive Care Center
HPV	human papilloma virus
IFN	interferon
IL	interleukin
NK	natural killer
PHA	phytohemagglutinin
TCR	T-cell receptor

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FIGURE 1. Quantitative immune assays

Age-specific z scores of each of the quantitative immune assays were shown as dots for the FA individuals. The lines showed the regression of the immune assay with age in the young and old age groups. The mean z scores of all the FA individuals were presented, and P values indicated the differences between FA to the non-FA population

FIGURE 2. Functional immune assays

Six immune assays for each of the FA individuals were categorized into high, normal, and low and shown as dots. The proportions of low in the FA patients were presented, and P values indicated the differences in the low% between FA and the non-FA population

TABLE 1

FA study population

Characteristics	All subjects (N = 29)	<10 years (N = 12)	10 years (N = 17)	P-value
Median age (IQR)	12 (6–18)	4(3–6)	17 (13–20)	<0.001
Personal history of cancer	0%	0%	0%	–
Bone marrow failure status				0.72
Normal	12%	20%	7%	
Mild	36%	30%	40%	
Moderate	52%	50%	53%	
Male sex	52%	58%	47%	0.71
Complementation group				0.10
A	41%	25%	53%	
Non-A	34%	58%	18%	
Unknown	24%	17%	29%	

Age was shown as median (IQR) and compared using Wilcoxon rank sum test; all other characteristics were shown as proportions and tested using a Fisher's exact test.

TABLE 2

Quantitative immune assays

Immune assay	FA mean z-scores (SD)	P-value
CD19 ⁺ total B cells	−1.41 (1.92)	< 0.001
CD19 ⁺ CD27 ⁺ memory B cells	−0.98 (0.95)	< 0.001
CD3 ⁺ total T cells	−0.02 (1.02)	0.47
CD8 ⁺ T cells	0.49 (1.18)	0.98
CD4 ⁺ T cells	−0.31 (0.79)	0.022
CD16 ⁺ NK cells	−0.92 (0.89)	< 0.001

Forzscores, the H_0 is ≤ 0 ; a P -value < 0.05 (in bold) indicates that the z score is lower than the expected population mean of 0.

TABLE 3

Functional immune assays in FA individuals

Immune assay	N	% abnormal in FA	P-value
IgG	29	10	0.18
IgM	28	57	<0.001
<i>Candida albicans</i>	21	19	0.019
Tetanus	21	19	0.019
Phytohemagglutinin	20	5	0.64
CTL lytic units	21	24	0.003
NK lytic units	19	16	0.07

Data are shown as proportions of subjects who had low immune function comparing with clinical cutoff values (5% percentiles of a normal population). A *P*-value < 0.05 (in bold) indicates that the proportion of subjects with low measures is higher than 5%, which is expected to be observed in a normal population; NK lytic units are measured in those (N = 19) with normal NK cell numbers.