IMMUNE-RELATED NON-COMMUNICABLE DISEASES IN AFRICA

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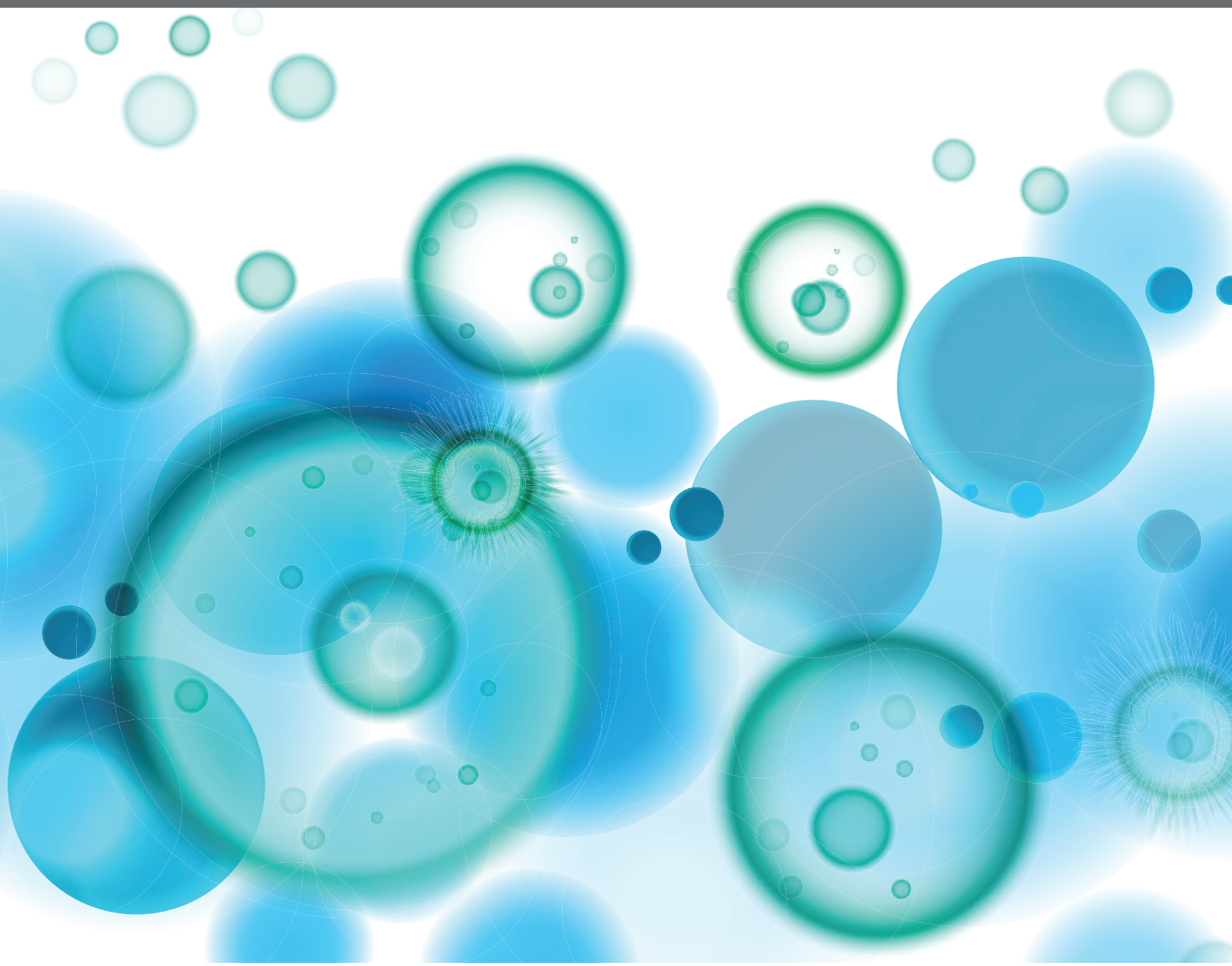

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IMMUNE-RELATED NON-COMMUNICABLE DISEASES IN AFRICA

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EDITORIAL
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Editorial: Immune-Related Non-Communicable Diseases in Africa

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Editorial on the Research Topic

Immune-Related Non-Communicable Diseases in Africa

The African continent has a long-standing tradition of research in communicable diseases. Indeed, several national, regional, and international agencies are investing in understanding the pathogenesis of and immunity to infection, particularly those diseases which are endemic to the continent and responsible for substantial morbidity and mortality, notably HIV/AIDS, tuberculosis, and malaria. More recently, interesting efforts have been made to encourage and support research in the field of neglected tropical diseases. In contrast, with few exceptions African immunologists are less involved in researching other immune-mediated diseases such as allergy, cancer, autoimmunity, inborn errors of immunity (IEI), and auto-inflammation despite an expansion of the burden of these diseases on the African continent, commensurate with the epidemiological transition from infectious diseases to chronic, non-communicable diseases. Public health authorities are reporting a surge of non-communicable diseases. Their particularities in African populations are interesting challenges worthy of investigation.

To dissect the immunological mechanisms underlying several of these immune-mediated non-communicable diseases and highlight their specificities in Africa were the aims of this Research Topic. The frontiers between communicable and non-communicable diseases also require exploration, such as host genetic susceptibility to infection, and the interplay between allergy and parasitic infection.

Ten articles were included in this Research Topic and classified into the following categories: six original and not original research manuscripts (Elhaj Mahmoud et al., Tahiat et al., Engelbrecht et al., Harrison et al., Sibanda et al., Ait Ssi et al.); three case reports (Rais et al., Snen et al., Belaid et al.) and one review (Ghouzlani et al.).

Three original research papers explored immune mechanisms underlying autoimmune and rheumatological conditions. Sibanda et al. investigated systemic sclerosis in Zimbabwe, an autoimmune disease that is rarely reported in Africa. In a large cohort of 240 patients, they showed that specific autoantibodies are biomarkers of the condition and can precede overt disease. Autoantibodies also determine disease phenotypes and vary between racial groups. Elhaj Mahmoud et al. studied the pro-inflammatory activity of Wnt5a in rheumatoid arthritis tissue-derived fibroblast-like synoviocytes. They showed that this activity is enhanced *in vitro* in the presence of SFRP5 through mechanisms involving the inhibition of TCF4 and LRP5 expression. These findings may contribute to the identification of potential therapeutic targets. In another contribution, Harrison et al. described features of the arthropathy rarely observed in children with advanced HIV

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infection, which may be linked to immune suppression where multiple immune aberrations contribute to loss of self-tolerance.

Molecular aspects of IEI were addressed in two original studies and two case reports. Tahiat et al. completed the first systematic evaluation of autoimmunity and autoantibody profiles in Algerian patients with IEIs. Of approximately 300 IEI patients screened for 54 different autoantibodies, 32% had detectable autoantibodies, a significantly higher prevalence than the healthy control group. Predictably, the prevalence of autoantibodies was highest among those patients with diseases of immune dysregulation. An interesting benefit of systematic screening was the identification of several asymptomatic patients with transglutaminase IgA antibodies who on further investigation were shown to have early celiac disease. In Africa, next generation sequencing technologies are not widely available for evaluating patients with suspected IEI. The study of Engelbrecht et al. demonstrated the utility of whole exome sequencing and/or targeted gene panels in 80 patients with suspected IEI. Although molecular diagnoses of IEI were confirmed in 31%, the impact of molecular testing was far greater, influencing the management of two-thirds of the patients. The introduction of routine molecular diagnostic testing is an important priority for African countries. This will improve patient care as shown in this study, increase our understanding of epidemiology of IEI in Africa particularly in many countries with limited or no access to routine immunological diagnostics, and enhance the continent's capacity to contribute to elucidating the molecular pathogenesis of IEI. Novel case reports by Rais et al. and Belaid et al. reveal phenotypic commonality between IPEX and ALPS and expands the spectrum of hyper-IgE syndrome to include selective interleukin-2 receptor common gamma chain defects, respectively.

Immunity to cancer in the African context is addressed in one original research paper and one review, both focusing on the immunological microenvironment of glioma, a common primary brain neoplasm. Ait Ssi et al. analysed the tumour microenvironment using transcriptomic data from patients with glioma. Unsupervised, hierarchical clustering after enrichment analysis enabled the identification of two patient groups, one characterized by immune cells with anti-tumour immune potential and the other enriched by immunosuppressive immune cells. The timely review by Ghouzlani et al. complemented the findings of the Ait-Ssi study and described immune checkpoints and checkpoint inhibitors used by glioma cells to regulate the immune response and evade immune destruction. While these molecules are potential immunotherapeutic targets, a major consideration is the development of immunotherapies that will cross the blood brain barrier in concentrations capable of blocking immune checkpoint inhibitors used by glioma cells to escape the immune response.

Finally, the paper by Snen et al. described the treatment of a complicated case of allergic bronchopulmonary aspergillosis (ABPA) in a patient with underlying asthma. This report draws attention to the difficulty of diagnosing ABPA and the importance of early diagnosis and effective treatment to circumvent the long-term morbidity of the condition.

In conclusion, this Research Topic documented immunological perspectives on a range of non-communicable diseases, demonstrating the potential of African immunologists to contribute to this interesting area of research and clinical practice.

AUTHOR CONTRIBUTIONS

M-RB and BE did edit the Research Topic and did write the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Diagnostic and Predictive Contribution of Autoantibodies Screening in a Large Series of Patients With Primary Immunodeficiencies

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Objectives: To evaluate the diagnostic and predictive contribution of autoantibodies screening in patients with primary immunodeficiencies (PIDs).

Methods: In the present study, PID patients and healthy controls have been screened for 54 different autoantibodies. The results of autoantibodies screening in PID patients were correlated to the presence of autoimmune diseases.

Results: A total of 299 PID patients were included in this study with a predominance of antibody deficiencies (27.8%) followed by immunodeficiencies affecting cellular and humoral immunity (26.1%) and complement deficiencies (22.7%). Autoimmune manifestations were present in 82 (27.4%) patients. Autoimmune cytopenia (10.4%) was the most common autoimmune disease followed by gastrointestinal disorders (10.0%), rheumatologic diseases (3.7%), and endocrine disorders (3.3%).

Autoantibodies were found in 32.4% of PID patients and 15.8% of healthy controls ($P < 0.0005$). Anti-nuclear antibodies (ANA) (10.0%), transglutaminase antibody (TGA) (8.4%), RBC antibodies (6.7%), anti-smooth muscle antibody (ASMA) (5.4%), and ASCA (5.0%) were the most common autoantibodies in our series. Sixty-seven out of the 82 patients with autoimmune manifestations (81.7%) were positive for one or more autoantibodies. Eleven out of the 14 patients (78.6%) with immune thrombocytopenia had positive platelet-bound IgM. The frequencies of ASCA and ANCA among patients with IBD were 47.4% and 21.0% respectively. All patients with celiac disease had TGA-IgA, while six out of the 11 patients with rheumatologic diseases had ANA (54.5%). Almost one third of patients (30/97) with positive autoantibodies had no autoimmune manifestations. ANA, rheumatoid factor, ASMA, anti-phospholipid antibodies and ANCA were often detected while specific AID was absent. Despite the low positive predictive value of TGA-IgA and ASCA for celiac disease and inflammatory bowel disease respectively, screening for these antibodies identified undiagnosed disease in four patients with positive TGA-IgA and two others with positive ASCA.

Conclusion: The present study provides valuable information about the frequency and the diagnostic/predictive value of a large panel of autoantibodies in PIDs. Given the frequent association of some AIDs with certain PIDs, screening for corresponding autoantibodies would be recommended. However, positivity for autoantibodies should be interpreted with caution in patients with PIDs due to their low positive predictive value.

Keywords: primary immunodeficiencies, autoantibody, screening, autoimmune cytopenia, celiac disease, platelet-bound IgM, transglutaminase antibody

INTRODUCTION

Primary immunodeficiencies (PIDs) are a heterogeneous group of genetic disorders that affect distinct components of both humoral and cellular arms of the immune system (1). PIDs are no longer defined by recurrent infections alone. Patients with such disorders are increasingly recognized with features of immune dysregulation, including autoimmunity and inflammation (2–5). In a retrospective study of the French Registry, authors have reported that more than 26% of patients with PIDs developed one or more autoimmune or inflammatory manifestations throughout their lifetime (6). The risk for autoimmune diseases (AID) was at least 10 times higher than in the general population (6). Mechanisms underlying the development of autoimmunity in PIDs include defective T and B cell development and tolerance, defective regulatory T cell (Treg) development or function, increased type I interferon signature and lack of clearance of immune complexes and apoptotic debris (7).

The presence of serum autoantibodies directed to multiple cell surface and intracellular antigens is a serological hallmark of autoimmune diseases and a helpful biomarker for establishing an early and accurate diagnosis. Autoimmunity has been widely studied in PIDs. However, most of published studies were focused on clinical manifestations and only few is known about the clinical relevance of autoantibody testing in such monogenic defects (6, 8). In the present study, a large series of patients with PIDs have been screened for a broad panel of autoantibodies. The study's main objective was to evaluate the diagnostic and predictive contribution of autoantibodies screening in patients with PIDs. A secondary goal was to report the frequency of autoantibodies in different PID categories.

MATERIAL AND METHODS

Patients and Healthy Controls

In the present study, 299 Algerian patients with PIDs have been enrolled in two-year period (January 2018 to January 2020). All patients met the updated criteria of the European Society for Immunodeficiency (ESID) (www.esid.org). Secondary immunodeficiencies were ruled out for each patient. Patients were categorized according to the International Union of Immunological Societies (IUIS), Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity (2019) (1). In addition, the study included 120 healthy subjects (70 children and 50 adults) as a control group. The study was approved by the local ethics Committee and it conforms to the provisions of the World Medical Association's Declaration of Helsinki.

Diagnosis of Autoimmune Diseases

Data on autoimmune manifestations were collected and analyzed for each patient. The diagnosis of AID was based on clinical and complementary paraclinical findings (ex., radiology, endoscopy, colonoscopy and biopsy results) and laboratory tests (ex.,

Coombs test, antinuclear antibodies, and transglutaminase antibody), according to international criteria for a specific disease. For instance, patients who were diagnosed with autoimmune hemolytic anemia (AIHA) presented with anemia that was associated with a positive Coombs test.

Autoantibody Testing

Patients and healthy controls were systematically screened for a broad panel of autoantibodies (54 distinct autoantibodies) that included : anti-nuclear antibodies (ANA), rheumatoid factor (RF), anti-citrullinated protein antibody (ACPA), anti-neutrophil cytoplasmic antibodies (ANCA), anti-phospholipid antibodies (aPL), anti-tissue antibodies (such as anti-smooth muscle antibody (ASMA)), anti-saccharomyces cerevisiae antibody (ASCA), transglutaminase antibody (TGA), red blood cell (RBC) antibodies, platelet-bound antibodies (PA) and anti-neutrophil antibodies (**Table 1**).

ANA screening was performed at a dilution of 1:80 by indirect immunofluorescence (IIF) using HEp-2 cell line as substrate. For positive sera, nuclear and cytoplasmic staining patterns were read by two experts and ANA specificities, including dsDNA, extractable nuclear antigens (ENA), nucleolar antigens and cytoplasmic antigens were identified by enzyme-like immunosorbent assay (ELISA) and/or Immunodot. IIF and ELISA have been used for ANCA screening and identification, respectively. Detection of RBC antibodies was performed by Coombs test. PA-IgG, PA-IgM and PA-IgA were detected by flow cytometry, using a commercial kit (THROMBOCYTEST, BD Biosciences, CA, USA), according to the manufacturer's instructions. Briefly, isolated platelets from EDTA whole blood were incubated with PE-labeled goat Ig anti-human

TABLE 1 | Immunoassays used for antibody testing.

AID	Autoantibody	Immunoassay (manufacturer)
Rheumatologic diseases	ANA (screening)	IIF/HEp-2 (EUROIMMUN, Germany)
	ANA (identification)	ELISA (Bio-Rad, CA, USA)
	dsDNA	Immunodot (D-tek, Belgium)
	ENA : Sm, RNP, Sm/RNP, SSA, SSB, Scl-70, PM/Scl, Ku, CENP-A/B, PCNA, Mi-2 & DFS-70	Immunodot (D-tek, Belgium)
	Nucleolar antigens : PM/Scl, Th/To, Fibrillarin, NOR-90	Immunodot (D-tek, Belgium)
	Cytoplasmic antigens : M2/nPDC, Jo-1, PL-7, PL-12, SRP and Ribosome P0	
	RF	Laser Nephelometry (BN ProSpec, Siemens, Germany)
	ACPA	ELISA (Bio-Rad, CA, USA)
APS	aCL-IgG & aCL-IgM	ELISA (Bio-Rad, CA, USA)
	aβ2GPI-IgG & β2GPI-IgM	ELISA (Bio-Rad, CA, USA)
AAV	ANCA (screening)	IIF/human neutrophils (EUROIMMUN, Germany)
	ANCA (identification)	Immunodot (D-tek, Belgium)
IBD	ASCA-IgA & ASCA-IgG	ELISA (Bio-Rad, CA, USA)
CD	TGA-IgA & TGA-IgG	ELISA (Bio-Rad, CA, USA)
Pernicious anemia	APCA	IIF (EUROIMMUN, Germany)
AIH & PBC	ASMA	IIF (EUROIMMUN, Germany) and Immunodot (D-tek, Belgium)
	anti-LKM 1	IIF (EUROIMMUN, Germany) and Immunodot (D-tek, Belgium)
	anti-LC1	IIF (EUROIMMUN, Germany) and Immunodot (D-tek, Belgium)
	anti-SLA	Immunodot (D-tek, Belgium)
	AMA	IIF (EUROIMMUN, Germany) and Immunodot (D-tek, Belgium)
	anti-gp210	Immunodot (D-tek, Belgium)
	anti-SP100	Immunodot (D-tek, Belgium)
Autoimmune thyroiditis	anti-TPO	ELISA (EUROIMMUN, Germany)
	anti-TG	ELISA (EUROIMMUN, Germany)
Insulin-dependent diabetes mellitus	anti-GAD	ELISA (EUROIMMUN, Germany)
AIHA	RBC antibodies	Coombs test
ITP	PA (IgG, IgM et IgA)	Flow cytometry
Autoimmune neutropenia	anti-neutrophil antibodies (IgG et IgM)	Indirect GIFT- Flow cytometry

AAV, ANCA associated vasculitis; ACPA, anti-citrullinated protein antibody; aCL, anti-cardiolipin antibodies; aβ2GPI, anti-β2 glycoprotein I antibodies; AID, autoimmune disease; AIH, autoimmune hepatitis; AIHA, autoimmune hemolytic anemia; AMA, anti-mitochondrial antibody; ANA, anti-nuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; APCA, anti-parietal cell antibody; APS, anti-phospholipid syndrome; ASCA, anti-saccharomyces cerevisiae antibody; ASMA, anti-smooth muscle antibody; CD, celiac disease; ELISA, enzyme-like immunosorbent assay, GIFT, granulocyte immunofluorescence test; IBD: inflammatory bowel disease; IIF, indirect immunofluorescence, ITP, immune thrombocytopenia; LC, liver cytosol; LKM, liver-kidney microsome; PA, platelet-bound antibodies; PBC, primary biliary cirrhosis; RBC, red blood cell; RF, rheumatoid factor; SLA, soluble liver antigen; TG, thyroglobulin; TGA, anti-transglutaminase antibody; TPO, thyroid peroxidase.

IgG, IgM and IgA to detect platelet-bound antibodies. Binding of the antibodies was measured with a FACSCanto (BD Biosciences, CA, USA). Anti-neutrophil antibodies (IgG and IgM) were detected by indirect granulocyte immunofluorescence test (GIFT) as described previously (9). Briefly, a mixture of fresh anticoagulated blood samples obtained from 10 healthy subjects was used as a source of neutrophils. The rationale for using multiple donors was to obtain a good representation of two common human neutrophil antigens (HNA) alloforms 1 and 2. The mixed blood sample was treated with ammonium chloride-based RBC-lysing reagent. The cells were thoroughly washed, resuspended and incubated at room temperature with the patients' sera for 30 minutes, washed twice and incubated for 15 minutes with FICT-conjugated anti-human IgG and IgM rabbit $F(ab')_2$ antibody fragment.

Statistical Analysis

The comparison of frequencies was performed by chi-square test or Fisher's exact test. The threshold for statistical significance was set to a P value of less than 0.05 in all analyses. The statistical analyses were performed using SPSS version 23.0 (software package (IBM), Chicago, IL, USA).

RESULTS

Demographic Data

The demographic data of PID patients and healthy controls are shown in **Table 2**. Of the 299 patients included in this study, 169 (56.5%) were males and 130 (43.5%) were females. The mean age of patients was 12.8 years (0.1 – 80 years), the mean age at onset of PID symptoms was 4,2 years (0 – 60 years) and the mean disease duration was 5.7 years (0.1 – 53 years). Two hundred three (68%) patients were children and 96 (32%) were adults. Ninety patients (30.1%) were born from consanguineous parents. Diagnosis of PID was confirmed by immunological testing in all patients and genetically in 60 (20%) patients. The distribution of patients according to the classification from the IUIS Expert Committee is shown in **Table 2**. Predominantly antibody deficiencies (27.8%) were the most common, followed by immunodeficiencies affecting cellular and humoral immunity (26.1%) and complement deficiencies (22.7%).

Autoimmune Diseases

Autoimmune manifestations were present in 82 (27.4%) patients, including 45 (55%) males and 37 (45%) females. There was no statistically significant association between having AID and gender (P = 0.72). AIDs was more common in patients with immune dysregulation (70.0%), followed by immunodeficiencies affecting cellular and humoral immunity (33.3%) (P < 0.0005). The lowest frequency of AID was seen in patients with complement deficiencies (10.3%) (**Table 3**). Unlike patients with "classical" severe combined immunodeficiencies (SCIDs), patients with atypical forms of SCID, including Omenn syndrome (OS) (n = 6) and leaky SCID (n = 9) presented a high frequency of AID (14.3% *vs.* 66.7%; P = 0,006). The frequency of autoimmune manifestations in patients with predominantly antibody deficiencies was 24.1%. Such manifestations were more frequent in patients with common variable immunodeficiencies (CVID) compared to patients with other antibody deficiencies (30.9% *vs.* 17.1%), but the difference did not reach level of significance (P = 0.14) (**Tables 3** and **5**).

A wide range of autoimmune manifestations was observed in our series; autoimmune cytopenia (10.4%) was the most common AID followed by gastrointestinal disorders (10.0%), rheumatologic diseases (3.7%), skin manifestations (3.3%) and endocrine disorders (3.3%) (**Table 4**). The distribution of different AIDs with respect to PID categories is shown in **Table 5**. The most striking findings are that autoimmune cytopenia and skin diseases were common in patients with combined immunodeficiencies (CID), especially atypical SCID, while IBD and endocrine disorders predominated in patients with diseases of immune dysregulation. IBD was also common in patients with chronic granulomatous disease (CGD).

TABLE 2 | Demographic data of PID patients and healthy controls.

Category	Number (%)	Gender M/F	Age Mean/years (range)	Disease duration Mean/years (range)
Immunodeficiencies affecting cellular and humoral immunity	78 (26.1)	48/30	3.9 (0.1 – 76)	2.8 (0 – 21)
Combined immunodeficiencies with associated or syndromic features	28 (9.4)	20/8	3.3 (0.2 – 14)	3.0 (0 – 13)
Predominantly antibody deficiencies	83 (27.8)	46/37	19.2 (0.3 – 80)	6.6 (0 – 30)
Diseases of immune dysregulation	20 (6.7)	13/7	4.4 (0.3 – 17)	3.4 (0 – 13)
Congenital defects of phagocyte number or function	9 (3.0)	7/2	4.8 0.1 – 31	0.7 (0 – 2)
Defects in intrinsic and innate immunity	6 (2.0)	2/4	6.7 (2 – 21)	5.7 (1.5 – 17)
Complement deficiencies	68 (22.7)	28/40	26.6 (0.5 – 67)	18.7 (2 – 53)
Other immunodeficiencies	7 (2.3)	5/2	20.1 2 – 65	1.1 (1 – 3)
Healthy controls	120	69/51	17.9 (1 – 58)	/

TABLE 3 | AID and autoantibodies in different PID categories and healthy controls.

Category	AIDs *n(%)*	Autoantibodies *n(%)*
Immunodeficiencies affecting cellular and humoral immunity	26 (33.3)	30 (38.5)
Combined immunodeficiencies with associated or syndromic features	8 (28.6)	14 (50.0)
Predominantly antibody deficiencies	20 (24.1)	17 (20.5)
Diseases of immune dysregulation	14 (70.0)	13 (65.0)
Congenital defects of phagocyte number or function	3 (33.3)	3 (33.3)
Defects in intrinsic and innate immunity	2 (33.3)	2 (33.3)
Complement deficiencies	7 (10.3)	15 (22.1)
Healthy controls	0 (0)	19 (15.8)

AID, autoimmune disease.

TABLE 4 | Main autoimmune manifestations in our series.

AID	Number	Percentage (*n* = 299)
Autoimmune cytopenia	31	10,4
AIHA	17	5,7
ITP	11	3,7
Evans syndrome	3	1,0
Autoimmune neutropenia	3	1,0
Gastrointestinal disorders	30	10.0
IBD	19	6,4
Celiac disease	8	2,7
Pernicious anemia	4	1.3
Rheumatologic disorders	11	3,7
SLE	4	1.3
Endocrine disorders	10	3,3
Insulin-dependent diabetes mellitus	6	2,0
Hashimoto's disease	4	1,3
Skin diseases	10	3,3

AID, autoimmune disease; AIHA, autoimmune hemolytic anemia; IBD: inflammatory bowel disease; ITP, immune thrombocytopenia; SLE, systemic lupus erythematosus.

Autoantibodies

The results of autoantibody testing in PID patients and healthy controls are shown in **Table 3** and **6**. One or more autoantibodies were found in 32.4% of PID patients and 15.8% of healthy individuals ($P < 0.0005$). Among the 97 patients with positive autoantibodies, 58 (60%) were males and 39 (40%) were females. There was no statistically significant association between having autoantibodies and gender ($P = 0.54$). The distribution of patients according to the number of positive autoantibodies showed that 46 patients (47.4%) were positive for 1 autoantibody, 22 patients (22.7%) were positive for 2 autoantibodies, 15 patients (15.5%) had 3 autoantibodies, while 14 patients (14.4%) developed 4 or more autoantibodies. There was a statistically significant association between having autoantibodies and PID category ($P = 0.001$). Serum autoantibodies were more frequent in patients with diseases of immune dysregulation followed by T cell immunodeficiencies, including immunodeficiencies affecting cellular and humoral immunity (38.5%) and CID with associated or syndromic features (50%) (**Table 3**).

The most frequent autoantibodies in our series were: ANA (10.0%), TGA (8.4%), RBC antibodies (6.7%), ASMA (5.4%), RF (5.0%), ASCA (5.0%), ANCA (4.3%), and PA (4.3%). RBC antibodies ($P = 0.002$), PA-IgM ($P = 0.023$), TGA-IgA ($P = 0.021$), anti-ENA antibodies ($P = 0.039$), and RF ($P = 0.048$) were significantly more frequent in PID patients when compared to healthy controls (**Table 6**). The distribution of different autoantibodies with respect to PID categories is shown in **Table 7**. The frequencies of most tested autoantibodies were higher in patients with diseases of immune dysregulation (**Table 7**). However, when considering PID patients with increased susceptibility to infections, and after excluding patients who have defects of phagocyte number or function, and defects in intrinsic and innate immunity due to low numbers, the frequency of RBC antibodies was significantly higher in patients with T cell immunodeficiency ($P = 0.003$). The frequency of PA was also higher in patients with T cell immunodeficiency but the difference did not reach level of significance ($P = 0.26$). There was no statistically significant association between having ANA, ASCA, ANCA and TGA-IgA and PID category. However, it is interesting to note that TGA-IgA (8.8%) was common in patients with complement deficiencies, especially in patients with hereditary angioedema due to C1-inhibitor deficiency (HAE/C1-inhibitor deficiency) (9.1%) (**Table 7**).

Sixty-seven out of the 82 patients (81.7%) with autoimmune manifestations were positive for one or more autoantibodies. All patients with AIHA had positive Coombs test. Twelve out of the 14 patients (85.7%) with immune thrombocytopenia (ITP) had positive PA. Among these patients, six (50%) were positive for PA-IgM, three (25%) for PA-IgM and PA-IgG, two (16.7%) for PA-IgM and PA-IgA and one (8.3%) for PA-IgG. Interestingly, PA-IgM and PA-IgG were present in a patient with autoimmune lymphoproliferative syndrome (ALPS) (due to homozygous mutation in *FAS*) and normal platelet count (188000 per uL). IgM ($n = 2$) and IgG ($n = 1$) anti-neutrophil have been detected by indirect GIFT in three patients with autoimmune neutropenia. The prevalence of ASCA and ANCA among patients with IBD ($n = 19$) were 47.4% and 21.0% respectively. All patients with celiac disease (CD) ($n = 8$) had TGA-IgA, while none of them had TGA-IgG. Among patients with rheumatologic diseases ($n = 11$), six had ANA (54.5%), two were positive for RF (18.2%) while none had ACPA.

Almost one third of patients (31%) with positivity for one or more autoantibodies had no autoimmune manifestations. ANA, RF, ASMA, aPL and ANCA were often detected while specific AID was absent (**Figure 1**). Thus, only 20% of patients with positivity for ANA had rheumatologic disease. Such AID was found in only 13.3% of patients with RF. Similarly, 18.7% of patients with positive ASMA had autoimmune hepatitis (AIH) and none of the eight patients who were positive for aPL had anti-phospholipid syndrome (APS).

Eight out of the 22 patients (36.4%) with positive TGA-IgA had CD. The screening of TGA-IgA in our series identified four earlier undiagnosed CD. The diagnostic was confirmed by a

TABLE 5 | Distribution of major autoimmune diseases among different categories/types of PIDs.

Category/type of PIDs	Number of patients	Autoimmune Cytopenia (%)	IBD (%)	CD (%)	Endocrine disease (%)	Skin (%)
Immunodeficiencies affecting cellular and humoral immunity	78	17.9	2.6	2.6	1.3	10.3
SCID	14	14.3	0	0	0	0
OS	6	50.0	0	0	0	100
Leaky SCID	9	33.3	0	0	0	11.1
bona fide CID	49	12.2	4.1	4.1	2.0	2.0
Combined immunodeficiencies with associated or syndromic features	28	17.9	0	3.6	3.6	0
Wiskott–Aldrich syndrome	6	33.3	0	16.7	0	0
DiGeorge syndrome	4	25.0	0	0	0	0
Ataxia telangiectasia	6	0	0	0	0	0
Hyper-IgE syndrome	7	0	0	0	0	0
Other CID with syndromic features	5	40	0	0	20	0
Predominantly antibody deficiencies	83	8.4	7.2	2.4	1.2	1.2
Agammaglobulinemia	14	0	0	0	0	0
CVID	42	11.9	9.5	4.8	0	2.4
Other antibody deficiencies	27	7.4	7.4	0	3.7	0
Diseases of immune dysregulation	20	20.0	40.0	5.0	20.0	5.0
ALPS (Fas deficiency)	3	100	0	0	0	0
IPEX syndrome	1	100	0	0	100	0
LRBA deficiency	1	0	0	0	0	0
Immune dysregulation with colis	6	0	100	0	0	16.7
Familial hemophagocytic lymphohistiocytosis	3	0	0	0	0	0
Chediak Higashi	2	0	0	0	0	0
Other diseases of immune dysregulation	4	0	50	25	75	0
Congenital defects of phagocyte number or function	9	11.1	22.2	0	11.1	0
CGD	4	0	50.0	0	25.0	0
Congenital neutropenia	3	33.3	0	0	0	0
Other phagocytic defects	2	0	0	0	0	0
Defects of intrinsic and innate immunity	6	0	0	0	16.7	0
STAT1 GOF	2	0	0	0	50	0
MSMD	4	0	0	0	0	0
Complement deficiencies	68	0	0	2.9	0	0
HAE/C1-inhibitor deficiency	55	0	0	3.6	0	0
Other complement deficiencies	13	0	0	0	0	0

ALPS, autoimmune lymphoproliferative syndrome; CD, celiac disease; CGD, chronic granulomatous disease; CID, combined immunodeficiency; CVID, common variable immunodeficiency; GOF, gain-of-function; HAE, hereditary angioedema; IBD, inflammatory bowel disease; IPEX, immune dysfunction, polyendocrinopathy, enteropathy, LRBA, lipopolysaccharide-responsive and beige-like anchor protein; MSMD, mendelian susceptibility to mycobacterial disease; OS, Omenn Syndrome; PID, primary immunodeficiency disease; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription.

biopsy in two patients with CID and CVID and by a "no-biopsy approach" (i.e., TGA-IgA values ≥10 times the upper limit of normal and positive endomysial antibodies (EMA-IgA) in a second serum sample) in two patients with HAE/C1-inhibitor deficiency. Nine out of the 15 patients (60%) with positive ASCA (IgA and or IgG) had IBD. The screening for ASCA identified two undiagnosed IBD in two patients with CGD and CVID.

DISCUSSION

In the present study, a large series of Algerian patients with PIDs were systematically tested for a broad panel of autoantibodies. The results of autoantibodies screening were correlated to the presence of autoimmune manifestations. Although the occurrence of autoimmunity in patients with PIDs has been previously reported, to the best of our knowledge, this is the first report to evaluate the diagnostic and predictive values of autoantibodies for AIDs in the context of PIDs. Moreover this is the first systematic report of autoimmunity and autoantibody profile in patients with PIDs from Africa.

A total of 299 Algerian patients with 60 different PIDs, belonging to 7 different categories of IUIS classification, were included in this study. Autoimmune manifestations were present in 27.4% of patients. A similar prevalence (26.2%) has been reported in a previous study of the French National Primary Immunodeficiencies Registry (6). However, the frequency of AIDs in the Slovenian (22%) and Kuwaiti (20%) studies were lower (8, 10). Many factors, such as ethnicity, environment, cohort size, age of patients, disease duration, and access to hematopoietic stem cell transplantation could have contributed to such differences between series. In our study, AIDs were more common in patients with immune dysregulation (70.0%) followed by immunodeficiencies affecting cellular and humoral immunity (33.3%). Although autoimmunity was less prevalent in patients with complement deficiencies, it is worth to be mentioned that six out of the 55 patients (10.9%) with HAE/

TABLE 6 | Frequencies of different autoantibodies in PID patients and healthy controls.

Autoantibody	PID patients %	Healthy controls %	*P*
Autoantibodies	32.4	15.8	<0.0005
RBC antibodies	6.7	0	0.002
PA	4.3	0	0.024
PA-IgM	4.0	0	0.023
PA-IgG	1.7	0	NS
PA-IgA	0.7	0	NS
aNL	1.0	ND	ND
aNL-IgM	0.7	ND	ND
aNL-IgG	0.3	ND	ND
ASCA	5.0	4.2	NS
ASCA-IgA	4.3	4.2	NS
ASCA-IgG	1.3	1.7	NS
ANCA	4.3	0.8	NS
TGA	8.4	1.7	0.014
TGA-IgA	7.4	1.7	0.021
TGA-IgG	2.3	0.8	NS
APCA	2.0	2.5	NS
ANA	10.0	6.7	NS
anti-dsDNA	1.7	0	NS
anti-ENA	3.7	0	0.039
anti-SSA/Ro	1.3	0	NS
anti-SSB/La	0.7	0	NS
anti-RNP	1.3	0	NS
anti-Sm	0.7	0	NS
anti-Scl70	0.3	0	NS
anti-Ku	0.3	0	NS
anti-DFS70	0.3	0	NS
RF	5.0	0.8	0.048
ACPA	1.0	0.0	NS
ASMA	5.4	0.8	0.051
anti-LKM1	0.3	0.0	NS
anti-GAD	1.7	ND	ND
anti-TPO	2.0	ND	ND
anti-TG	1.3	ND	ND
aPL	2.7	0.8	NS
aCL-IgG	1.0	0	NS
aCL-IgM	1.0	0.8	NS
aβ2GPI-IgG	0	0	NS
aβ2GPI-IgM	1.0	0	NS

ACPA, anti-citrullinated protein antibody; aCL, anti-cardiolipin antibodies; aβ2GPI, anti-β2 glycoprotein I antibodies; ANA, anti-nuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; aNL, anti-neutrophil antibody; APCA, anti-parietal cell antibody; aPL, antiphospholipid antibodies; ASCA, anti-saccharomyces cerevisiae antibody; ASMA, anti-smooth muscle antibody; GAD, anti-glutamic acid decarboxylase, LKM, liver-kidney microsome; ND, not determined; NS: not significant; PA, platelet-bound antibodies; RBC, red blood cell; RF, rheumatoid factor; TG, thyroglobulin; TGA, anti-transglutaminase antibody; TPO, thyroid peroxidase.

C1-inhibitor deficiency (which is the most common complement deficiency in our series) had autoimmune diseases, including two patients (3.6%) with systemic lupus erythematosus and two others (3.6%) with CD.

Autoimmune cytopenia (10.4%) was the most common autoimmune manifestation in our series. This finding is in accordance with the French and the Kuwaiti studies and emphasize the importance of recognizing autoimmune cytopenia as 'warning sign' of immunodeficiency (especially in children) that should trigger an immune evaluation. Furthermore, gastrointestinal disorders (10.0%), rheumatologic diseases (3.7%) and endocrine disorders (3.3%) were also common in our series. This is comparable to the percentages found in the French study (9.5%, 5.0% and 3.2%, respectively) (6, 8). However, skin manifestations, which have been observed in 3.3% of our patients were more frequent in the French registry (5.5%) (6).

In our series, autoantibodies were present in 97 patients (32.4%), they were significantly more frequent than in the control group ($P < 0.0005$). Among patients with positive autoantibodies, 67 (69%) had AIDs while 30 (31%) did not exhibit any autoimmune manifestation. The presence of autoantibodies was a common feature of all types of PIDs. However, their prevalence was significantly higher in patients with defects of immune dysregulation. In our study, all patients with ALPS, lipopolysaccharide-responsive and beige-like anchor protein (LRBA) deficiency and immune dysfunction, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome developed autoantibodies, highlighting the role of B-cell homeostasis (Fas deficiency), Treg (IPEX syndrome) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) surface expression (LRBA deficiency) in the control of autoreactive B cells and autoantibody production. Furthermore, 38.5% of patients with immunodeficiencies affecting cellular and humoral immunity were positive for at least one autoantibody. Although this category is mainly characterized by defects in T-cell development and function (1), aberrant autoantibody production is likely to be related to defects in B-cell tolerance and function. In recombination-activating gene (RAG) deficiency, the presence of a wide spectrum of autoantibodies, both in patients (11) and in animal models (12, 13) has been attributed to defects in central and peripheral B-cell tolerance. Re-expression of RAG proteins in mice allows receptor editing in bone marrow immature B cells and reduce the frequency of self-reactive B-cells (14).

Red blood cell and platelet-bound antibodies were detected in 6.7% and 4.3% of patients respectively. All patients with AIHA had positive Coombs test, while 85.7% of patients with ITP had positive PA. The detection of PA by flow cytometry is an excellent diagnostic tool for ITP, but it is worth noting that PA could be detected in the absence of ITP. Unlike RBC antibodies, most of detected platelet-bound antibodies were IgM. PA-IgM was present in 78.6% of patients with ITP (mostly children), while PA-IgG was detected in only 28.6% of patients. This finding is in accordance with a recent Danish study, in which PA-IgM were significantly more frequent than PA-IgG in children with ITP (63% *vs.* 44%, $P = 0.03$) (15). Schmidt et al have also reported that anti-platelet antibodies were predominantly of the IgM class in children with newly diagnosed ITP (16). In the same study, the authors have noted that IgM responses were present short term without evidence of class-switching to IgG (16). Such responses are likely to be triggered by infections, and molecular mimicry may play a central role in the development of self-directed anti-platelet immunity (17, 18). Furthermore, a pathophysiological role of IgM anti-platelet antibodies in ITP has been demonstrated (16, 19). Unlike IgM anti-erythrocyte autoantibody that promotes anemia through a massive agglutination of RBCs in spleen and

TABLE 7 | Distribution of autoantibodies among different categories/types of PIDs.

Category/type of PIDs	Number of patients	RBC antibodies(%)	PA (%)	ASCA (%)	ANCA (%)	TGA (%)	ANA (%)
Immunodeficiencies affecting cellular and humoral immunity	78	11.5	5.1	2.6	3.8	5.1	11.5
SCID	14	14.3	0	0	0	0	0
OS	6	33.3	16.7	0	0	0	0
Leaky SCID	9	22.2	0	0	11.1	11.1	11.1
bona fide CID	49	6.1	6.1	4.1	4.1	6.1	16.3
Combined immunodeficiencies with associated or syndromic features	28	14.3	7.1	0	0	17.8	7.1
Wiskott–Aldrich syndrome	6	33.3	0	0	0	16.7	0
DiGeorge syndrome	4	25.0	25.0	0	0	0	0
Ataxia telangiectasia	6	0	0	0	0	0	16.7
Hyper-IgE syndrome	7	0	0	0	0	28.6	0
Other CID with syndromic features	5	20	20	0	0	40	20
Predominantly antibody deficiencies	83	2.4	4.8	2.4	1.2	3.6	6.0
Agammaglobulinemia	14	0	0	0	0	0	0
CVID	42	4.8	4.8	4.8	0	7.1	2.4
Other antibody deficiencies	27	0	7.4	0	3.7	0	14.8
Diseases of immune dysregulation	20	20.0	10.0	25.0	30.0	20.0	30.0
ALPS (Fas deficiency)	3	100	66.7	0	66.7	0	33.3
IPEX syndrome	1	100	0	100	0	100	100
LRBA deficiency	1	0	0	0	0	0	0
Immune dysregulation with colis	6	0	0	16.7	50	0	33.3
Familial hemophagocytic lymphohistiocytosis	3	0	0	0	0	0	33.3
Chediak Higashi	2	0	0	50	0	0	50
Other diseases of immune dysregulation	4	0	0	50	25	75	0
Congenital defects of phagocyte number or function	9	11.1	11.1	22.2	0	11.1	11.1
CGD	4	0	0	50.0	0	25.0	25.0
Congenital neutropenia	3	33.3	33.3	0	0	0	0
Other phagocytic defects	2	0	0	0	0	0	0
Defects of intrinsic and innate immunity	6	0	0	16.7	33.3	0	16.7
STAT1 GOF	2	0	0	0	50	0	50
MSMD	4	0	0	25	25	0	0
Complement deficiencies	68	0	0	2.9	1.5	8.8	7.3
HAE/C1-inhibitor deficiency	55	0	0	3.6	1.8	9.1	7.2
Other complement deficiencies	13	0	0	0	0	7.7	7.7

ALPS, autoimmune lymphoproliferative syndrome; ANA, anti-nuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; ASCA, anti-saccharomyces cerevisiae antibody; CD, celiac disease; CGD, chronic granulomatous disease; CID, combined immunodeficiency; CVID, common variable immunodeficiency; GOF, gain-of-function; HAE, hereditary angioedema; IBD, inflammatory bowel disease; IPEX, immune dysfunction, polyendocrinopathy, enteropathy, LRBA, lipopolysaccharide-responsive and beige-like anchor protein; MSMD, mendelian susceptibility to mycobacterial disease; OS, Omenn Syndrome; PA, platelet-bound antibodies; PID, primary immunodeficiency disease; RBC, red blood cell; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription; TGA, anti-transglutaminase antibody.

liver, PA-IgM induces thrombocytopenia through uptake of opsonized platelets (19, 20). In the mouse, it has been reported that IgM autoantibody-mediated thrombocytopenia was macrophage dependent and the Fcα/μR was required for macrophage uptake of opsonized thrombocytes (21). Complement fixation on IgM might also potentially activate phagocytosis involving complement receptors expressed on macrophages (16). Taken all findings together, childhood ITP unlike chronic adult ITP is mainly IgM-mediated. Patients should be tested for both PA-IgM and PA-IgG in order to improve the sensitivity of the flow cytometric test and to determine the prognosis of the ITP (16).

When considering PID patients with increased susceptibility to infections (i.e., after excluding patients with diseases of immune regulation), RBC antibodies and PA were more common in patients with T-cell immunodeficiency. Of 8 RAG-deficient patients, 3 (37.5%) were positive for RBC antibodies or PA and exhibited autoimmune cytopenia. In previous studies, biallelic *RAG1* and *RAG2* mutations have been associated with a wide range of clinical and immunological phenotypes (22). While null mutations result in SCID with the absence of T and B lymphocytes (T− B− NK+ SCID) (23), hypomorphic mutations allowing for residual protein function are associated with atypical forms including OS (24–26), and leaky SCID (23, 27). Autoimmune manifestations are rare in RAG-deficient patients presenting with typical SCID. However, cytopenias, in particular AIHA, have been reported in more than half of patients with hypomorphic mutations in *RAG1* and *RAG2* (28). In our series, none of the two RAG-deficient patients with SCID had autoimmune cytopenia while three out of the 6 patients with hypomorphic mutations (manifesting as OS in 4 patients and leaky SCID in 2 others) presented with autoimmune cytopenias, including AIHA in two patients and ITP in another one.

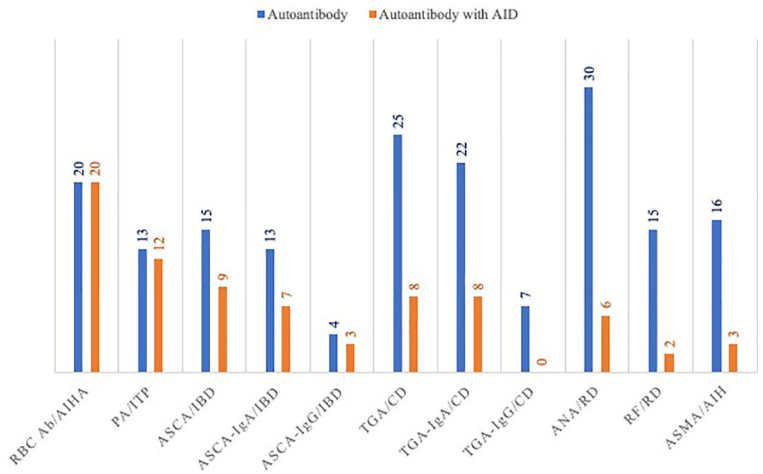

FIGURE 1 | Autoimmune diseases in patients with positive autoantibodies. AIHA, autoimmune hemolytic anemia; ANA, anti-nuclear antibodies; ASCA, anti-saccharomyces cerevisiae antibody; ASMA, anti-smooth muscle antibody; CD, celiac disease; IBD: inflammatory bowel disease; PA, platelet-bound antibodies; RBC Ab, red blood cell antibodies; RD, rheumatoid disease; TGA, anti-transglutaminase antibody.

Inflammatory bowel disease was the most common gastrointestinal disease in our cohort. IBD was more frequent in patients with defects of immune dysregulation (40%), CGD (50%), and CVID (9.5%). The frequencies of ASCA and ANCA among patients with IBD were 47.4% and 21.0%, respectively. ASCA was associated with IBD in 60% of patients. Interestingly, the systematic testing for ASCA identified two undiagnosed IBD, representing 10.5% of total IBD cases. Hence, patients presenting PIDs with a high risk for IBD, such as CGD and CVID, should be tested for ASCA even in the absence of digestive symptoms. In case of positive ASCA, additional investigations (ex., radiology, colonoscopy and biopsy) are necessary to confirm the diagnosis.

One of the most striking results of our study is the high frequency of TGA. We were somewhat surprised to observe that 25 patients (8.4%) were positive for TGA-IgA and/or TGA-IgG (compared to 1.7% in the control group). Among patients with TGA-IgA, only 8 (36.4%) had CD. Despite their low positive predictive value (PPV), TGA-IgA testing identified four earlier undiagnosed CD, representing 50% of total CD cases in our series. Interestingly enough, the screening of HAE patients for TGA-IgA revealed a frequent association of CD with C1-inhibitor deficiency (3.6%). Such association was also reported by a Hungarian study in which 3.1% of patients with HAE had CD (29). Considering the high prevalence of CD among patients with HAE, screening for the former is warranted. In addition, it may help in differential diagnosis as well as in the selection of the most appropriate therapy, which is very important known the similarities between the symptoms of HAE and CD.

In our study, ANA and RF were present in 10% and 5% of patients respectively. However, their PPVs for rheumatologic diseases were very low (20% and 13.3% for ANA and FR, respectively). Similarly, only 18.7% of patients with positive ASMA had AIH and none of the positive patients for aPL had APS. The aberrant autoantibody production in patients with PIDs may be related to several intrinsic and environmental factors (7, 30). Infections would play a central role in triggering autoimmunity and autoantibody production in patients with PIDs. In fact, the presence of autoantibodies such as ANA, RF, ACPA, aPL, ANCA and ASMA has been described as an epiphenomenon in several bacterial and viral infections (ex., *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *HCV*) (30–34).

In conclusion, the present study provides valuable information about the frequency and the diagnostic/predictive value of a large panel of autoantibodies in PIDs. Considering the frequent association of some AIDs with certain PIDs, such as autoimmune cytopenia/Fas and RAG deficiencies, CD/C1-inhibitor deficiency and IBD/CGD, systematic screening for corresponding autoantibodies would be recommended. However, positivity for autoantibodies, especially ANA, RF, ANCA, aPL and ASMA, should be interpreted with caution due to their low positive predictive value.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethics Committee of Rouiba Hospital, Algiers, Algeria. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AT: designed the study, conducted flow cytometry assays and autoantibody testing, collected and analyzed data, performed statistical analysis, and wrote the manuscript. AY: contributed to data analysis and interpretation, and edited the manuscript. HI, ST, AS and AO: contributed to autoantibody testing. SL, RBe, SA, LA, DB, SM, CBo, WD, CBe, AK, HMe, NB, AI, TBM, HB, KB, MK, NC, LS, ZA, ZZ, ZB, OI, HMa, and RBo: provided clinical diagnosis of patients. KD: designed and supervised the study, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Tangye SG, Al-Herz W, Bousfiha A, Chatila T, Cunningham-Rundles C, Etzioni A, et al. Human Inborn Errors of Immunity: 2019 Update on the Classification from the International Union of Immunological Societies Expert Committee. *J Clin Immunol* (2020) 40:24–64. doi: 10.1007/s10875-019-00737-x
2. Goyal R, Bulua A, Nikolov N, Schwartzberg P, Siegel R. Rheumatologic and autoimmune manifestations of primary immunodeficiency disorders. *Curr Opin Rheumatol* (2009) 21:78–84. doi: 10.1097/BOR.0b013e32831cb939
3. Kolhatkar NS, Brahmandam A, Thouvenel CD, Becker-Herman S, Jacobs HM, Schwartz MA, et al. Altered BCR and TLR signals promote enhanced positive selection of autoreactive transitional B cells in Wiskott-Aldrich syndrome. *J Exp Med* (2015) 212:1663–77. doi: 10.1084/jem.20150585
4. Magnani A, Brosselin P, Beaute J, de Vergnes N, Mouy R, Debre M, et al. Inflammatory manifestations in a single-center cohort of patients with chronic granulomatous disease. *J Allergy Clin Immunol* (2014) 134:655–62. doi: 10.1016/j.jaci.2014.04.014
5. Hoyt K, Chatila T, Notarangelo L, Hazen M, Janssen E, Henderson L. The immunologic features of patients with early-onset and polyautoimmunity. *Clin Immunol* (2020) 211:108326. doi: 10.1016/j.clim.2019.108326
6. Fischer A, Provot J, Jais JP, Alcais A, Mahlaoui N. members of the CEREDIH French PID study group. Autoimmune inflammatory manifestations occur frequently in patients with primary immunodeficiencies. *J Allergy Clin Immunol* (2017) 140:1388–93. doi: 10.1016/j.jaci.2016.12.978
7. Grimbacher B, Warnatz K, Yong P, Korganow A, Peter H. The crossroads of autoimmunity and immunodeficiency: lessons from polygenic traits and monogenic defects. *J Allergy Clin Immunol* (2016) 37:3–17. doi: 10.1016/j.jaci.2015.11.004
8. Massaad MJ, Zainal M, Al-Herz W. Frequency and Manifestations of autoimmunity among children registered in the Kuwait National Primary Immunodeficiency Registry. *Front Immunol* (2020) 11:1119. doi: 10.3389/fimmu.2020.01119
9. Sella R, Flomenblit L, Goldstein I, Kaplinsky C. Detection of anti-neutrophil antibodies in autoimmune neutropenia of infancy: a multicenter study. *Isr Med Assoc J* (2010) 12(2):91–6.
10. Blazina Š, Markelj G, Jeverica AK, Toplak N, Bratanič N, Jazbec J, et al. Autoimmune and Inflammatory Manifestations in 247 Patients with Primary Immunodeficiency-a Report from the Slovenian National Registry. *J Clin Immunol* (2016) 36(8):764–73. doi: 10.1007/s10875-016-0330-1
11. Walter JE, Rosen LB, Csomos K, Rosenberg JM, Mathew D, Keszei M, et al. Broad-spectrum antibodies against self-antigens and cytokines in RAG deficiency. *J Clin Invest* (2015) 125(11):4135–48. doi: 10.1172/JCI91162
12. Cassani B, Poliani PL, Marrella V, Schena F, Sauer AV, Ravanini M, et al. Homeostatic expansion of autoreactive immunoglobulinsecreting cells in the Rag2 mouse model of Omenn syndrome. *J Exp Med* (2010) 207(7):1525–40. doi: 10.1084/jem.20091928
13. Walter JE, Rucci F, Patrizi L, Recher M, Regenass S, Paganini T, et al. Expansion of immunoglobulin-secreting cells and defects in B cell tolerance in Rag-dependent immunodeficiency. *J Exp Med* (2010) 207(7):1541–54. doi: 10.1084/jem.20091927
14. Jankovic M, Casellas R, Yannoutsos N, Wardemann H, Nussenzweig MC. RAGs and regulation of autoantibodies. *Annu Rev Immunol* (2004) 22:485–501. doi: 10.1146/annurev.immunol.22.012703.104707
15. Nielsen OH, Tuckuviene R, Nielsen KR, Rosthøj S. Flow cytometric measurement of platelet-associated immunoglobulin in children with newly diagnosed Immune Thrombocytopenia. *Eur J Haematol* (2016) 96(4):397–403. doi: 10.1111/ejh.12605
16. Schmidt DE, Heitink-Polle KMJ, Porcelijn L, van der Schoot CE, Vidarsson G, Bruin MCA, et al. Anti-platelet antibodies in childhood immune thrombocytopenia: Prevalence and prognostic implications. *M J Thromb Haemost* (2020) 18(5):1210–20. doi: 10.1111/jth.14762
17. Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. *Blood* (2009) 113(26):6511–21. doi: 10.1182/blood-2009-01-129155
18. Heitink-Polle KMJ, Nijsten J, Boonacker CWB, de Haas M, Bruin MCA. Clinical and laboratory predictors of chronic immune thrombocytopenia in children: a systematic review and meta-analysis. *Blood* (2014) 124(22):3295–307. doi: 10.1182/blood-2014-04-570127
19. Hoemberg M, Stahl D, Schlenke P, Sibrowski W, Pachmann U, Cassens U. The isotype of autoantibodies influences the phagocytosis of antibody-coated platelets in autoimmune thrombocytopenic purpura. *Scand J Immunol* (2011) 74(5):489–95. doi: 10.1111/j.1365-3083.2011.02600.x
20. Detalle ,L, . Su D, van Rooijen N, Coutelier J-P. Immunoglobulin M antiplatelet autoantibodies from mice immunized with rat platelets induce thrombocytopenia and platelet function impairment. *Exp Biol Med (Maywood)* (2010) 235:1464–71. doi: 10.1258/ebm.2010.010018
21. Legrain S, Su D, Breukel C, Detalle L, Claassens JW, van der Kaa J, et al. Involvement of Fcα/μ Receptor in IgM Anti-Platelet, but Not Anti-Red Blood Cell Autoantibody Pathogenicity in Mice. *J Immunol* (2015) 195(9):4171–5. doi: 10.4049/jimmunol.1500798
22. Niehues T, Perez-Becker R, Schuetz C. More than just SCID–the phenotypic range of combined immunodeficiencies associated with mutations in the recombinase activating genes (RAG) 1 and 2. *Clin Immunol* (2010) 135 (2):183–92. doi: 10.1016/j.clim.2010.01.013
23. Schwarz K, Gauss GH, Ludwig L, Pannicke U, Li Z, Lindner D, et al. RAG mutations in human B cell-negative SCID. *Science* (1996) 274(5284):97–9. doi: 10.1126/science.274.5284.97
24. Villa A, Santagata S, Bozzi F, Giliani S, Frattini A, Imberti L, et al. Partial V(D) J recombination activity leads to Omenn syndrome. *Cell* (1998) 93(5):885–96. doi: 10.1016/s0092-8674(00)81448-8
25. de Saint-Basile G, Le Deist F, de Villartay JP, Cerf-Bensussan N, Journet O, Brousse N, et al. Restricted heterogeneity of T lymphocytes in combined immunodeficiency with hypereosinophilia (Omenn's syndrome). *J Clin Invest* (1991) 87(4):1352–9. doi: 10.1172/JCI115139
26. Rieux-Laucat F, Bahadoran P, Brousse N, Selz F, Fischer A, Le Deist F, et al. Highly restricted human Tcell repertoire in peripheral blood and tissue-infiltrating lymphocytes in Omenn's syndrome. *J Clin Invest* (1998) 102 (2):312–21. doi: 10.1172/JCI332
27. Villa A, Sobacchi C, Notarangelo LD, Bozzi F, Abinun M, Abrahamsen TG, et al. V(D)J recombination defects in lymphocytes due to RAG mutations: severe immunodeficiency with a spectrum of clinical presentations. *Blood* (2001) 97(1):81–8. doi: 10.1182/blood.v97.1.81
28. Delmonte OM, Schuetz C, Notarangelo LD. RAG Deficiency: Two Genes, Many Diseases. *J Clin Immunol* (2018) 38(6):646–55. doi: 10.1007/s10875-018-0537-4
29. Csuka D, Kelemen Z, Czaller I, Molnár K, Füst G, Varga L, et al. Association of celiac disease and hereditary angioedema due to C1- inhibitor deficiency.

Screening patients with hereditary angioedema for celiac disease: is it worth the effort? *Eur J Gastroenterol Hepatol* (2011) 23(3):238–44. doi: 10.1097/MEG.0b013e328343d3b2
30. Jara LJ, Medina G, Saavedra MA. Autoimmune manifestations of infections. *Curr Opin Rheumatol* (2018) 30(4):373–79. doi: 10.1097/BOR.0000000000000505
31. Alam J, Yong CK, Choi Y. Potential role of bacterial infection in autoimmune diseases: a new aspect of molecular mimicry. *Immune Netw* (2014) 14:7–13. doi: 10.4110/in.2014.14.1.7
32. Chen M, Kallenberg CG. The environment, geoepidemiology and ANCA-associated vasculitides. *Autoimmun Rev* (2010) 9:A293–8. doi: 10.1016/j.autrev.2009.10.008
33. Sasaki H, Inagaki M, Shioda M, Nagasaka K. Poncet's disease with high titers of rheumatoid factor and anticitrullinated peptide antibodies mimicking rheumatoid arthritis. *J Infect Chemother* (2015) 21:65–9. doi: 10.1016/j.jiac.2014.07.015
34. Gilman AJ, Le AK, Zhao C, Hoang J, Yasukawa LA, Weber SC, et al. Autoantibodies in chronic hepatitis C virus infection: impact on clinical outcomes and extrahepatic manifestations. *BMJ Open Gastroenterol* (2018) 5 (1):e000203. doi: 10.1136/bmjgast-2018-000203

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Clinical Utility of Whole Exome Sequencing and Targeted Panels for the Identification of Inborn Errors of Immunity in a Resource-Constrained Setting

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Primary immunodeficiency disorders (PIDs) are inborn errors of immunity (IEI) that cause immune system impairment. To date, more than 400 single-gene IEI have been well defined. The advent of next generation sequencing (NGS) technologies has improved clinical diagnosis and allowed for discovery of novel genes and variants associated with IEI. Molecular diagnosis provides clear clinical benefits for patients by altering management, enabling access to certain treatments and facilitates genetic counselling. Here we report on an 8-year experience using two different NGS technologies, namely research-based WES and targeted gene panels, in patients with suspected IEI in the South African healthcare system. A total of 52 patients' had WES only, 26 had a targeted gene panel only, and 2 had both panel and WES. Overall, a molecular diagnosis was achieved in 30% (24/80) of patients. Clinical management was significantly altered in 67% of patients following molecular results. All 24 families with a molecular diagnosis received more accurate genetic counselling and family cascade testing. Results highlight the clinical value of expanded genetic testing in IEI and its relevance to understanding the genetic and clinical spectrum of the IEI-related disorders in Africa. Detection rates under 40% illustrate the complexity and heterogeneity of these disorders, especially in an African population, thus highlighting the need for expanded genomic testing and research to further elucidate this.

Keywords: inborn errors of immunity, South Africa, whole exome sequencing, targeted sequencing, genetic variants

INTRODUCTION

Primary immunodeficiency disorders (PIDs) are a group of genetically heterogeneous inborn errors of immunity (IEI), which lead to a predisposition to the development of autoimmune and inflammatory diseases, lymphoproliferation and infection (1–5). Individually, IEIs are rare, but collectively represent a significant burden of disease (4, 6). It has recently been estimated that close to one million people in Africa suffer from an IEI, with South Africa contributing approximately 42,000 cases (7). However, human immunodeficiency virus (HIV), tuberculosis (TB) and other infectious epidemics in sub-Saharan Africa, limited clinical and laboratory infrastructure for IEIs, as well as lack of awareness for IEIs in these settings, have been major obstacles to diagnosis and treatment (7–10).

The diagnostic workup for a suspected IEI is shifting from only detailed clinical evaluations of the patient, to include a number of laboratory tests which may include targeted gene screening and immunological assays. Given that most IEIs are monogenic, genetic testing has become an important modality for providing an accurate and definitive diagnosis, altering management and aiding genetic counselling (11–13). This said, the identification of a disease-associated variant in a suspected case of IEI can be challenging with many cases having no single causative variant identified and large genotype-phenotype variation reported (14–16).

The identification of genes and genetic variants associated with IEIs has increased exponentially, with International Union of Immunological Societies (IUIS) currently reporting on 406 distinct disorders, with 430 genetic defects (6). Between 2015 and 2017, a total of 85 new genes were associated with IEIs (17). This increase has largely been driven by the use of next generation sequencing (NGS), more specifically whole genome sequencing (WGS), whole exome sequencing (WES) and the more restricted targeted gene panel sequencing (17). WES involves the investigation of all the protein-coding regions of the human genome (exome) allowing identification of around 85% of all disease-associated mutations (18). NGS gene panels are more targeted and analyse only known disease-associated genes for specific diseases. The advantages of panels over WES or WGS include lower cost, simpler analysis, and optimisation of variant detection in the included genes. Disadvantages include the inability to analyse or re-analyse genes not included on the panel (19).

Currently, NGS gene panels are the mainstay diagnostic tools in clinical settings, whereas WES and WGS may be second-line testing options. In South Africa, WES and WGS largely remain research tools because of their cost and the complexity of data interpretation. Factors that limit their provision in clinical service as diagnostic/clinical tests are that they must have acceptable analytical and clinical validity and clinical utility (20), must meet rigorous quality assurance standards, and must ensure rapid turnaround times in order to achieve ethical test outcomes (20). In a developing country, clinical genomic testing remains a considerable logistical challenge, with low volumes of samples, from patients with rare genetic diseases, leaving them to be considered lower priority.

In the interests of patient care, Tygerberg Hospital has sourced clinical genomic tests from specific international genomic testing providers since 2017, and supplemented this with research-based WES/WGS, at no cost to the patient. To date more than 730 diagnostic panels have been ordered across all clinical areas, of which 28 have been ordered on patients with suspected IEIs. WES has been performed on the DNA of 54 patients, two of whom had prior NGS panel testing.

The benefit of using NGS in patients with suspected IEIs has been shown in numerous international studies (11, 16, 21–23) and is likely to be beneficial in African patients. However it is likely that pathogenic variants, and perhaps even genes, may differ in the African population compared to more intensively studied populations. There was therefore a need to assess the clinical utility of gene panel testing and/or WES in a South African cohort. We report on molecular results, diagnoses and alterations in clinical management in patients with suspected IEIs, tested within the South African state healthcare system.

MATERIALS AND METHODS

Patients

The study includes 80 patients and 107 family members recruited over an 8 year period (2013-2020). Most of the patients have received or are currently receiving their medical care at Tygerberg Hospital, a state academic hospital situated in Cape Town that provides tertiary level services to a population of nearly 3 million people in the Western Cape province of South Africa. The immunology service also receives referrals from further afield in South and southern Africa. Three patients included in this study are receiving medical care in the private healthcare sector and one was within the Gauteng state healthcare system. These patients' data were included in this study as the families had either sought care from one of the immunology or genetic healthcare specialists working at Tygerberg Hospital or had received genetic testing through this system.

WES was performed in-house on DNA samples from 54 patients. Patients recruited for WES were children between the ages of 0 and 15 years that have a history of severe, unusual, persistent and/or recurrent TB or were suspected to have mendelian susceptibility to mycobacterial disease (MSMD). Depending on the available family members, genomic analysis of a trio (patient and both parents), duo (patient and one parent) or a single individual was performed. Of note is that not all 107 recruited family members were subjected to WES or genomic analysis as a result of either lack of budget or DNA that did not meet quality metrics for sequencing.

NGS diagnostic panel testing became available in 2017 through an external provider, and 28 gene panel tests were performed. Gene panel testing was offered to patients thought to have any one of a broad range of IEIs. This was done following a clinical and immunological assessment by a rheumatologist/

clinical immunologist, and assessment of the family history by a genetic counsellor. Tygerberg hospital budget funded panel testing for 22 of the 28 patients, and this was performed through Invitae Corporation (San Francisco, California, United States). Panel selection was based on the clinical phenotype and presentation of the patient, with the number of genes available for inclusion increasing from 207 to 407 genes over time. In January 2019, a number of IEI diagnostic gene panel tests were made available free of charge through a pilot sponsored programme for Jeffrey Modell Centers Network (JMCN) patients, in collaboration with Invitae Laboratories. This allowed access to a 207 gene panel for 6 patients who were not eligible for tests through the hospital and could not themselves afford the cost (2, 4, 17). For patients who received NGS panel tests, family member testing was performed, if required for resolution of variants of uncertain significance (VUS) or for carrier testing of relatives.

NGS gene panels soon became the first-line genomic diagnostic test, with patients that had a negative panel result, or an atypical clinical phenotype, then receiving WES if the research budget allowed. Two patients received both panel testing and subsequent WES testing.

Panel Testing

Saliva was collected for each of the patients in saliva kits provided by Invitae. Saliva was collected and stored in Oragene™ assisted saliva collection kits (DNA Genotek, Ottawa, Ontario, Canada) (OG575/ODG575), specifically for young children or patients where active saliva donation may be difficult. Two saliva tubes totalling 0.75ml of saliva were collected per kit. Saliva was subsequently stored at room temperature and shipped to Invitae for sequencing and analysis of 207 genes well established to be associated with IEIs. Genomic DNA obtained from the submitted samples was enriched for targeted regions using a hybridization-based protocol, and sequenced using Illumina technology. All targeted regions were sequenced with ≥50x depth or in cases where this falls lower than the accepted range, are supplemented with additional analysis. Reads were aligned to reference sequence hg19 (GRCh37). Promoters, untranslated regions, and other non-coding regions were not interrogated. More information on Invitae's assays can be found here: (https://www.invitae.com/en/assay/).

Whole Exome Sequencing

DNA was extracted from whole blood of recruited patients and, wherever possible, their parents and additional family members using standard protocols. A total of 5ml of blood was drawn into BD Vacutainer™ EDTA blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States). DNA was extracted using the Macherey-Nagel NucleoSpin XL DNA extraction kit (GmbH & Co. KG, Düren, Germany). Library preparation was conducted using the Ion AmpliSeq™ Exome RDY Kit and the Ion Xpress™ Barcode Adaptors 1–16 Kit (Life Technologies, Carlsbad, California, United States). The DNA template was prepared on the Ion Chef system using the Ion PI™ Hi-Q™ Chef Kit and the Ion PI™ Chip Kit v3. Sequencing was performed at the Central Analytical Facility (CAF) at Stellenbosch University, Stellenbosch, South Africa using the Ion Proton™ (Thermo Fisher, Carlsbad, California, United States). The average coverage for WES was 30X across the entire exome.

Variant calling: Sequences were aligned to the human reference genome, GRCh37 using TMA (version 5.2.1) in the ion-analysis workflow on the Torrent Suite (version 5.2.1). The Variant Caller (version 5.2.1.1) plugin on the Torrent suite was used for base quality score recalibration, indel realignment and variant calling and variant called format (VCF) files were produced. Variant annotation was done using ANNOVAR (5) and variant prioritization was carried out using a custom-designed, in-house method, TAPER™ (21).

Sanger Sequencing: All candidate variants identified through WES were verified using Sanger sequencing. Each amplicon was bi-directionally sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems Inc., Foster City, California, USA.), followed by electrophoresis on an ABI 3130XL Genetic Analyzer (Perkin-Elmer, Applied Biosystems Inc., Foster City, California, USA). All automated DNA sequencing reactions were performed at the Central Analytical Facility (CAF) at Stellenbosch University, Stellenbosch, South Africa.

Variant interpretation: Variants detected by NGS testing were all rigorously subjected to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) variant classification criteria in order to establish pathogenicity (24). Variants that were classified as benign or likely benign were not reported in this study. Variants classified as uncertain, likely pathogenic and pathogenic (LP/P) were included.

Confirmation of diagnosis and reporting: A WES-based molecular diagnosis was made in a patient when a LP/P variant was found in a gene associated with the phenotype, consistent with the known patterns of inheritance associated with that gene, and confirmed on Sanger sequencing. In these cases, a report was issued to the referring clinician and explained to the patient and their family members by a genetic counsellor.

Ethics and Institutional Approvals

NGS gene panels were performed as part of clinical diagnostic investigation of patients suspected to have a broad range of IEIs. Tests were preceded by genetic counselling and subject to informed consent. WES was performed as part of two research studies that aimed at identifying genes and variants associated with a specific sub-group of IEIs known MSMD.

The WES studies were approved by the Health Research Ethics Committee of Stellenbosch University (study numbers: N13/05/075 and N13/05/075(A) and done through the Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University. Patients included in these studies are children between the ages of 0 and 15 years clinically suspected to have MSMD. Such patients were identified by both the National Health Laboratory Services and Desmond Tutu Centre for Tuberculosis and recruited by

a clinical immunologist and a clinical geneticist at Tygerberg Hospital. Written and informed consent was obtained from the parents or legal guardians of eligible patients. Children of age were also asked to assent to the study. The studies adhered to the ethical guidelines as set out in the "Declaration of Helsinki, 2013" (16).

RESULTS

This study includes a total of 80 patients. Patient demographic data can be found in **Table 1**.

A molecular result that explained all or some of the patient's phenotype was made in 24/80 (30%) cases, using panel and/or WES. Of the patients who had panel testing only, a diagnosis was made on 12/26 (46%). For patients that had WES only, 12/52 (23%), had a confirmed molecular diagnosis. Of the two patients who had both panel and WES, a molecular diagnosis that confirmed part of the phenotype was made by WES in one. A total of 35/80 (44%) patients have uncertain results and 21/80 (26,2%) patients had no variants of interest identified.

Genetic Spectrum and Clinical Implications of Molecular Findings

In the 24 probands with a confirmed molecular result, 24 different LP/P variants were identified in 19 genes well known to be associated with IEIs. Clinical and molecular information for each proband can be found in **Table 2**. In 22 probands with an uncertain result, VUSs identified using WES will be subject to further investigation using functional studies, family studies (where possible) and follow-up for re-analysis of variant classification over time. This is because they were identified to potentially be causative of the patients' phenotype, but currently do not meet ACMG-AMP classification criteria to be classified as likely pathogenic or pathogenic (information in **Supplementary Table 1**) (24).

For 24 probands with a confirmed molecular diagnosis, the mode of inheritance was found to be autosomal recessive in 10/24 (41,2%) cases; X-linked recessive in 8/24 (33,3%) cases and autosomal dominant (*de novo* or due to germline mosaicism) in 6/24 (25%) cases. Results are summarised in **Table 3**.

Molecular diagnosis altered management in 67% (12/18) of living patients. Management could have been altered in 29,1% (7/24) of cases with a molecular diagnosis if referral and diagnosis was made sooner and patients had not died. Having a molecular diagnosis also allowed for family cascade testing, identifying 19 families at risk of having another child affected with an IEI.

TABLE 1 | Patient demographic data.

Total number of patients	**80**
Males	48
Females	32
Age (Mean)	6 years 7 months

DISCUSSION

NGS is becoming the gold standard for confirming diagnoses in patients with suspected IEIs (12, 15) with our detection rates being comparable to other published studies (14, 16). This study highlights several important aspects of the clinical utility of NGS testing in patients with IEIs, even in setting where the healthcare services are significantly resource-constrained, and the majority of patients belong to indigenous African populations (populations considered understudied in genetic research) (25).

Our results show that NGS testing yielded a definitive diagnosis in 30% (24/80) of patients tested. Three of these diagnoses were made after the patient died and four died shortly after diagnosis was made. In 67% of the 18 living patients, a molecular diagnosis allowed significant alteration to management that in the long term may reduce morbidity, mortality and costs of care. Significant changes to health management included two patients successfully receiving HSCT and three more being eligible for HSCT as a curative option. It is now known that some patients have variant associated malignancy risks and screening for this has been put into place. Definitive diagnosis has also allowed access to various other clinical screenings for some such as audiological, endocrinological and haematological screening, as well as dedicated ophthalmology screening (e.g. for glaucoma and uveitis). Various diagnosed PIDDs are known to benefit from antibiotic prophylaxis based on risks for bacterial infection, and relevant patients will now appropriately receive this either chronically or intermittently. Diagnosis has also assisted with identifying patients that are required to avoid live vaccines because of known risks for dissemination. One patient is now under observation as a candidate for organ transplant (liver) in adulthood. The remainder of live patients with a confirmed genetic diagnosis largely continued on treatment prescribed pre molecular diagnosis. However, having a confirmed genetic result allows them to potentially be eligible for curative treatments such as gene therapy in the future.

A total of 5 patients with life-threatening IEIs in this cohort died before receiving HSCT as a curative therapy, highlighting the need for rapid diagnosis in IEIs. This issue urgently requires increased awareness of PIDs among primary practitioners and subsequent referral to clinical immunology and genetic services without delay. It also requires early use of NGS testing as part of patient care, where possible in the form of clinical/diagnostic testing (as opposed to research recruitment). In our services, these difficulties are improving over time as awareness increases of the availability of suitable NGS testing, and it becomes more widely understood. These improvements have resulted in part from an active molecular genetic research program in the field of IEIs. In addition, there is hospital buy-in from Tygerberg Hospital to the clinically appropriate use of NGS clinical/diagnostic testing for rare disease diagnosis, but this is not currently the case at national level, and there remains an urgent need for national policy on NGS diagnostic testing for rare diseases.

A positive outcome for all families whether with deceased or living probands is that molecular results allowed for accurate

TABLE 2 | Clinical presentation of patients with genes with pathogenic/likely pathogenic variants.

ID	Sex	Age at diagnosis	Relevant family history	Main clinical features	Panel/ WES	Implicated gene	Variant classification (zygosity)	Accession numbers for pathogenicity scores	Diagnosis	Status	Medical interventions as indicated and/or substantiated by molecular diagnosis
Patients with likely pathogenic/pathogenic variants in genes previously associated with IEI (IUIS criteria)											
001	F	2 years 5 months	None	Recurrent URTI; lymphadenitis; recurrent abscesses of lymph nodes; soft dysmorphism.	Panel[1]	*PIK3R1* c.1425+1G>A (Splice donor)	Pathogenic (heterozygous)	rs587777709/ VCV000372467.8	Autosomal dominant activated PI3K-delta syndrome	Alive	Prophylactic antibiotics. Annual surveillance for malignancy (Lymphoma). Regular surveillance for TB. Monthly Immune replacement therapy for life. Annual audiology screening.
002	M	8 months	Unknown	Recurrent and severe URTI requiring ICU admission in 1st year of life.	Panel[1]	*CD40LG* c.346G>T p.Gly116Cys	Likely pathogenic (hemizygous)	Invitae internal calling.	CD40 Ligand deficiency/X-linked Hyper IgM syndrome	Demised after molecular diagnosis	3 weekly immune replacement therapy. Regular neutrophil screening for indication of GCSF. Cotrimoxazole prophylaxis. Would have qualified for HSCT.
003	M	4 months	Unknown	Persistent hypoglycemia; eczema; recurrent fevers and infections with persistent neutropenia. Massive hepatosplenomegaly.	Panel[1]	*SLC37A4* c.59G>A p.Gly20Asp; c.1047_1059delinsGGCTAT p.Phe349Leufs*52	Pathogenic (compound heterozygous)	rs193302881/ VCV000551776.1 and Invitae internal calling.	Autosomal recessive glycogen storage disease type Ib	Alive	GCSF administration for neutropenia. High calorie diet to prevent hypoglycemic attacks. Regular infection screening. Endocrine surveillance. Organ transplant if needed.
004	M	10 months	Mom is a confirmed carrier	Septicaemia with empyema at 8/12, progressive neurodevelopmental delay and encephalopathy with persistent enteroviral shedding from stool, suspected to be oral Polio vaccine derived strain.	Panel[2,3,4]	*BTK* c.215dupA (p.Asn72Lysfs*13)	Pathogenic (hemizygous)	rs886041148/ VCV000279713.4	X-linked Agammaglobulinemia	Demised after molecular diagnosis	Ig replacement therapy.
005	F	8 months	No	Recurrent URTI; flat diffuse warts; raised IgE levels.	Panel[5]	*DOCK8* c.3803del (p.Phe1268Serfs*3)	Pathogenic (homozygous)	Invitae internal calling.	Autosomal recessive hyper-IgE syndrome with combined immunodeficiency	Alive	Prophylactic antibiotics. Malignancy surveillance of skin lesions. Qualifies for HSCT.

(Continued)

TABLE 2 | Continued

ID	Sex	Age at diagnosis	Relevant family history	Main clinical features	Panel/ WES	Implicated gene	Variant classification (zygosity)	Accession numbers for pathogenicity scores	Diagnosis	Status	Medical interventions as indicated and/or substantiated by molecular diagnosis
006	M	1 year 1 month	Affected maternal uncles (deceased) Mom is a confirmed carrier	Recurrent sepsis and shock; CMV induced LRTI; candida UTI; chronic gastroenteritis; FTT.	Panel[6]	*CD40LG* Deletion (Exon 3)	Pathogenic (hemizygous)	Invitae internal calling.	CD40 Ligand deficiency/X-linked Hyper IgM syndrome	Demised after molecular diagnosis	May have benefited from HSCT. Ig replacement therapy.
007	M	1 year 2 months	None	Progressive paralysis likely due disseminated oral polio vaccine; progressive weakness; nosocomial sepsis; reduced CD4 cells, with normal number of C8, C19 and CD16/CD56 cells.	Panel[6]	*RFX5* c.367_368del (p.Leu124Cysfs*21)	Pathogenic (homozygous)	rs1228361094	Bare Lymphocyte syndrome 2	Demised before molecular diagnosis	May have benefited from HSCT.
008	M	2 months	Yes; brother died at 3 months from severe infection (suspected SCID)	SCID; lung disease; CMV; hepatitis.	Panel[1]	*IL2RG* c.116-1G>A (Splice acceptor)	Pathogenic (hemizygous)	rs104895462/ VCV000004696.4	X-linked severe combined immunodeficiency	Demised after diagnosis	Ig replacement therapy. Did not meet criteria for HSCT due to disseminated persistent CMV infection).
009	F	1 year 8 months	No	PJP in early infancy, hypogammaglobulinemia with normal CD19, later onset neutropenia.	Panel[1]	*MAP3K14* c.1033G>A p.Val345Met	Likely pathogenic (homozygous)	Invitae internal calling	MSMD	Alive	Ig replacement therapy. Prophylactic antibiotics. Live BCG vaccines avoided in sib at birth. Sibling vaccinated once results confirmed to be normal.
010	F	3 years 3 months	None	Boggy tenosynovitis of wrists and ankles; uveitis	Single gene screen	*NOD2* c.1000C>T p.Arg334Trp	Pathogenic (heterozygous)	rs104895462/ VCV000004696.4	Blau Syndrome	Alive	Methotrexate. Regular Follow up for uveitis progression.
011	F	2 years 1 month	None	Recurrent septicaemia, oral ulcers; congenital neutropenia; FTT.	Single gene screen	*ELANE* c.416C>T (p.Pro139Leu)	Pathogenic (heterozygous)	rs137854448/ VCV000016743.4	Severe Congenital Neutropenia	Unknown Patient lost to follow up.	GCSF subcutaneous injections. Monitor for malignancies.
012	M	2 years 2 months	Mom is a confirmed obligate carrier	Agammaglobulinemia; absence of mature B-cells; recurrent pneumonias.	Single gene screen	*BTK* Partial Deletion (Exon 11)	Pathogenic (hemizygous)	Novel; Not reported in population databases; Not reported in literature.	X-linked Agammaglobulinemia	Alive	Ig replacement therapy. Eligible for gene therapy.
013	M	3 months	Yes: brother died from the same condition; further history of CID in cousins. Both parents are carriers	Multi-lobular pneumonia; low IgG; dysmorphism; diarrhoea.	WES	*TTC37* c.4507 C>T p.R1503C	Pathogenic (homozygous)	rs200067423/ VCV000287653.5	Trichohepatoenteric syndrome	Demised before molecular diagnosis	Parenteral nutrition. Ig replacement therapy. Surveillance for liver dysfunction.

(Continued)

TABLE 2 | Continued

ID	Sex	Age at diagnosis	Relevant family history	Main clinical features	Panel/ WES	Implicated gene	Variant classification (zygosity)	Accession numbers for pathogenicity scores	Diagnosis	Status	Medical interventions as indicated and/or substantiated by molecular diagnosis
014	F	9 years and 6 months	Yes, affected sibling	Central nervous system and skin; vasculitis, stroke with hemiparesis, seizures, progressive contractures of interphalangeal joints without bone resorption.	WES	*SAMHD1* c.1681_1682del p.Ser561Phe fs*61)	Pathogenic (homozygous)	Novel; Not reported in population databases; Not reported in literature.	Aicardi-Goutières syndrome-5	Alive	Anticoagulant therapy. Immunomodulation therapy. Surveillance for unusual infections including TB. Surveillance for glaucoma.
015	F	14 years	Yes, affected sibling	Contractures of interphalangeal joints without bone resorption, severe glaucoma.	WES	*SAMHD1* c.1681_1682del p.Ser561Phe fs*61	Pathogenic (homozygous)	Novel; Not reported in population databases; Not reported in literature.	Aicardi-Goutieres syndrome-5	Alive Follow up defaulter	Management of glaucoma. Surveillance for unusual infections including TB.
016	M	2 years 1 month	Unknown	Recurrent pneumonia and recurrent gastroenteritis. Chronic oral herpes lesions.	WES	*TNFRSF13B* c.236 A>G p.Tyr79Cys	Pathogenic (heterozygous)	rs72553876/ VCV000281110.4	Common variable immunodeficiency-2 Normal B and T cells with Low IgG.	Alive	Ig replacement therapy for life.
017	F	9 years 5 months	No	Recurrent bacterial septicemias; pneumonias and herpes *Labialis Molluscum*; encephalitis; intellectual disability.	WES	*LRBA* c.3407 C>T p.Pro1136Leu	Pathogenic (homozygous)	rs113022115/ VCV000287734.2	Common variable immunodeficiency-8 with autoimmunity	Demised before molecular dx. Molecular diagnosis made post mortem	Ig replacement therapy and immune modulation eg. CTLA-4 (Orencia) HSCT would have been indicated.
018	F	3 years 7 months	None	Initial cutaneous BCG abscess and later chronic CNS BCG dissemination, severe hypogammaglobulinemia.	WES	*MAP3K14* c.1033 G>A p.Val345Met Avoid live vaccines.	Likely pathogenic (homozygous)	VCV000843423.2	CVID and Mendelian susceptibility to mycobacterial disease	Alive	TB surveillance. Antibiotic prophylaxis. Ig replacement therapy.
019	M	Birth	Yes; male sibling with SCID died in early infancy prior to HSCT	Asymptomatic severe combined immunodeficiency T-B+NK-, identified on basis of positive family history.	WES	*IL2RG* c.443 T>G p.Leu148Arg	Pathogenic (hemizygous)	Novel; Not reported in population databases; Not reported in literature.	X-linked severe combined immunodeficiency	Alive	HSCT successful. Thriving requiring no further intervention.
020	M	4 months	Yes, brother died in early infancy from persistent diarrhea, thrombocytopenia (probable WAS)	Chronic diarrhoea, eczematous skin rashes, CMV pneumonitis, septic arthritis, Group B Streptococcal Septicaemia. Delayed onset thrombocytopaenia.	WES	*WAS* c.397 G>A p.Glu133Lys	Pathogenic (hemizygous)	VCV000870492.1	Wiskott–Aldrich syndrome	Alive	Immune replacement therapy. Prophylactic antibiotics Surveillance of autoimmunity and thrombocytopenia.

(Continued)

TABLE 2 | Continued

ID	Sex	Age at diagnosis	Relevant family history	Main clinical features	Panel/ WES	Implicated gene	Variant classification (zygosity)	Accession numbers for pathogenicity scores	Diagnosis	Status	Medical interventions as indicated and/or substantiated by molecular diagnosis
											Qualifies for HSCT but no consent.
021	F	6 years 4 months	None	Disseminated verrucae, chronic otitis media, Herpes Keratitis, bacterial pneumonias and suspected pulmonary tuberculosis.	WES	*DOCK8* c.3912del p.N1267fs	Pathogenic (homozygous)	Novel; Not reported in population databases; Not reported in literature.	Autosomal recessive hyper-IgE syndrome with combined immunodeficiency	Alive	Screening for TB, malignancy (cervical and skin) & hepatic disorders. Ig replacement therapy. Qualifies for HSCT.
022	M	2 years 4 months	Yes; affected maternal uncle	Stable neutropenia, lymphopenia, hypogammaglobulinemia, pneumonias & upper respiratory infections in infancy and early childhood with spontaneous gradual improvement.	WES	*MSN* c.511C>T p.Arg171Trp	Pathogenic (hemizygous)	rs1057519074/ VCV000372154.6	Immunodeficiency-50 (Mild phenotype)	Alive	Immune replacement therapy. Prophylactic antibiotics. HSCT not indicated because of mild phenotype.
023	F	5 years 4 months	None	Hypogammaglobulinemia, isolated ACTH deficiency, asymptomatic pulmonary infiltrates and canalicular liver function abnormalities.	WES	*NFKB2* c.1288C>T p.Pro430Ser	Likely pathogenic (heterozygous)	rs202001697/ VCV000474775.4	DAVID syndrome	Alive	Hormone replacement. Ig replacement therapy. Surveillance for Liver disease and other endocrine deficiencies.
024	F	7 years 6 months	None	Recurrent pneumonias with bronchiectasis, skin abscesses, scoliosis	WES	*STAT1* c.802 G>T p.Glu268Ter	Likely pathogenic (heterozygous) VUS (heterozygous)	Novel; Not reported in population databases; Not reported in literature.	Hyper IgE syndrome	Alive	Immune replacement therapy. Prophylactic antibiotics. *ZNF341* c.2167 A>C p.Thr723Pro

Panel[1], PR08100.02: Invitae Primary Immunodeficiency Panel; Panel[2], PR08111.02.1: Add-on Hypogammaglobulinemia Genes; Panel[3], PR08111.02.2: Add-on Common Variable Immunodeficiency Genes; Panel[4], PR08111.02:Invitae Agammaglobulinemia Panel; Panel[5], PR08113.01:Invitae Hyper IgE Syndrome Panel; Panel[6], PR08137.02: Invitae Combined Immunodeficiency (CID) Panel; Panel[7], PR08143.02:Invitae Mendelian Susceptibility to Mycobacterial Disease Panel; Panel[8], PR08112.01: Invitae Common Variable Immunodeficiency Panel; Panel[9], PR08112.01.1: Add-on Genes for Primary Immunodeficiencies That Can Mimic Common Variable Immunodeficiency; Panel[10], PR08114.01: Invitae Hyper IgM Syndrome Panel; Panel[11], PR08114.01.1: Add-on Clinically-overlapping Genes; Panel[12], PR08120.02.01: Add-on Autoimmunity Genes; Panel[13], PR08120.02: Invitae Autoinflammatory Syndromes Panel; Panel[14], PR08113.04: Invitae Hyper IgE Syndrome Panel; M, male; F, female; WES, whole exome sequencing; URTI, upper respiratory tract infections; FTT, failure to thrive; UTI, urinary tract infection; TBM, tuberculosis meningitis; ACTH, adrenocorticotropic hormone; LRTI, lower respiratory tract infections; ACTH, adrenocorticotropic hormone; LRTI, lower respiratory tract infections; CID, Combined Immunodeficiency; GIT, gastrointestinal tract; HSCT, haematopoietic stem cell transplant; GCSF, Granulocyte colony-stimulating factor; CMV, Cytomegalovirus; PJP, Pneumocystis jirovecii pneumonia; DAVID, Deficient anterior pituitary with variable immune deficiency; BCG, Bacillus Calmette Guérin; CNS, central nervous system; VUS, variant of unknown significance; ICU, intensive care unit.

TABLE 3 | Summary of results from probands and family member testing.

PROBANDS	AR IEI	XLR IEI	AD IEI	TOTAL
Probands with LP/P variant on panel	4 (3 homozygous; 1 compound heterozygous)	5 (hemizygous males)	3 (heterozygous)	12
Probands with LP/P variant on WES	6 (homozygous or compound heterozygous)	3 (hemizygous males)	3 (heterozygous)	12
Probands total	10 (homozygous or compound heterozygous)	8 (hemizygous males)	6 (heterozygous)	24
FAMILY MEMBERS	**AR IEI**	**XLR IEI**	**AD IEI**	**TOTAL**
Family members identified as carriers	20 (either through testing or known obligate carrier)	8* (heterozygous females)	0	28
Family member excluded as carriers/affected	n/a	1 (maternal aunt of proband)	6*	7

**All were parents of probands.*

VUS, variant of unknown significance; WES, whole exome sequencing; AR, autosomal recessive; XLR, X-linked recessive; AD, autosomal dominant; LP/P, likely pathogenic/pathogenic; IEI, inborn error of immunity.

genetic counselling around diagnosis and prognosis and allowed for the provision of psychosocial support. The confirmed results allowed parents and interested extended family members to have carrier testing to better understand recurrence risks. Of relevance to testing in resource constrained environments is that familial cascade testing, for probands who had a positive molecular result on an Invitae Panel, is free of charge.

In this cohort, familial testing allowed for 19 sets of parents to know their carrier status and therefore potential reproductive risks. For the 11 sets of parents that are proven or assumed obligate carriers of an autosomal recessive condition, the risk to have another affected child is 25% for each pregnancy. The 8 mothers that were confirmed to be carriers of an X-linked IEI could be informed of the 50% chance of sons being affected and daughters being carriers. One maternal aunt of a proband with an X-linked IEI was excluded as a carrier. The autosomal dominant conditions in this cohort were all seen to be *de novo* with low recurrence risk for future pregnancies (as germline mosaicism cannot be excluded) and no risk to extended family members. Although not relevant to this cohort, genomic testing to identify asymptomatic affected individuals has an important clinical role in allowing early diagnosis to allow for screening and preventative measures to improve health outcomes (13, 22, 26, 27).

Knowing carrier status provides access prenatal diagnosis (PND), with the option of termination of an affected pregnancy, or to prepare for the birth of an affected child, in which case it facilitates optimal neonatal management (e.g. avoid live vaccinations; haplotyping for a HSCT donor) (28, 29). Although the data are not presented, we are aware that several family members have received PND in subsequent pregnancies. The option of preimplantation genetic diagnosis (PGD) to prevent an affected pregnancy is another consideration, but is unfortunately not available within the South African state health sector. PGD must be accessed privately and requires considerable financial means.

Even with NGS-based approaches dramatically improving IEI diagnoses, detection rates remain below 50% (14–16). Proposed reasons include IEIs with a complex mode of inheritance, limitations of certain genomic technologies and cases caused by genes and variants not yet discovered or understood (5, 14–16, 19). Our results have shown the same with a detection rate of 30%. In consanguineous families, this detection rate may be as high as 70%, indicating the clinical utility of these NGS platforms (30). Lower detection rates in a South African cohort may also be due to limitations of testing methods used. TGPs look only at a specific subset of genes which have been clinically associated with disease, while WES looks at 1,2% of the human genome, specifically that which, when translated and transcribed, will be expressed as proteins. TGP and WES share a major limitation in that both methods rely on an exon-capture step upstream of the sequencing. Various approaches to exon capture have been developed and modified, but no single method is able to ensure perfect uniformity of target coverage – put plainly, some regions may sequence better and at a higher vertical coverage than others (26, 31, 32). Coupled to this, WES does not consider the fact that non-coding regions of the genome harbour an estimated 15% of variants which have large effect sizes on disease traits and for this reason, any pathogenic variant which may be in these regions, would not be identified (26).

Given the restrictiveness of both TGP and WES, numerous variants and/or novel genes may have been missed as a possible cause for IEI in the remaining 70% of the patients. Multiple studies have shown that WGS is more powerful than TGP and WES as this technique expands beyond targeted regions, and is not restricted by the capture methods which is a significant limitation in both TGP and WES (26, 33–35). WGS was not used on patients in this study even though it is the better genomic testing option in rare diseases. Going forward, a number of patients in this study with no definitive diagnosis or candidate genetic variants identified, will be subjected to WGS as a continuation of the research projects earlier described. Patients with IEIs not previously recruited will also be considered for WGS, with the hopes of improving understanding of genetic contribution to IEIs in African patients and in increasing diagnosis.

Lower detection rates in this cohort could also be attributed to genomic testing in a population in which the genome is highly diverse, understudied and poorly understood. In African patients, undiscovered variants and their corresponding immune pathways may account for differences in susceptibility and consequent clinical manifestations of disease, making variant calling and interpretation following NGS testing a huge challenge (25, 36). To overcome such limitations, there is an urgent need for more research focusing on African genetic diversity in both health and disease, and how this determines the predominant host specific immune responses (25).

An interesting finding from this study was that detection rates using targeted NGS gene panels were higher than detection rates using broader based WES (46% vs 25%), despite panel testing being more restrictive than WES. This is possibly due to more focused phenotyping of patients selected for panels versus those selected for research-based WES. Patients selected for WES were done so based on history of severe, unusual, recurrent or persistent TB and therefore thought to have MSMD. Using such inclusion criteria in a country where the rates of TB are exceptionally high, may have resulted in including patients with a clinical picture that is attributed to environmental exposures and social circumstances, rather than due to an underlying genetic defect. There also remains the possibility that the underlying genetic defects for susceptibility to mycobacterial disease, such as TB, is different in the African population, making gene and variant prioritisation through current bioinformatic pipelines difficult. Selecting for patients with suspected MSMD remains a challenge in countries, such as South Africa, where TB remains a pandemic and very careful evaluation by immunology and genetics experts is needed to ensure resources are not unnecessarily spent.

This study has highlighted how genomic testing has positively altered clinical and family management in a significant number of patients. Motivation for the government to allocate funds for genomic services and testing going forward requires evidence of clinical and public health utility and this study serves as the first of its kind to show this in South Africa. The challenge remains getting government funding for incorporation of such testing into routine clinical care for patients with suspected IEIs. The rapid decline in NGS sequencing cost and the per-sample cost of a singleton WES (i.e. on the proband only) being comparable to that of targeted panels should convince public and private health funders to consider implementing such testing in funded as well as resource constrained environments. Our findings allow the start of the conversation to mainstream NGS testing for IEIs, with the goal being improved overall patient and family care.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health Research Ethics Committee of Stellenbosch University [N13/05/075 and N13/05/075(A)]. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

CE, MU, ME, MM, CK, and BG conceived the project. BP and AC conducted all laboratory experiments. BG conducted all bioinformatics analysis of WES data. MS, CE, and MU assisted with the genetic counselling. ME, DA, and HC were involved with patient recruitment. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Casanova J-L, Conley ME, Seligman SJ, Abel L, Notarangelo LD. Guidelines for Genetic Studies in Single Patients: Lessons From Primary Immunodeficiencies. *J Exp Med* (2014) 211:2137–49. doi: 10.1084/jem.20140520
2. Modell V, Quinn J, Orange J, Notarangelo LD, Modell F. Primary Immunodeficiencies Worldwide: An Updated Overview From the Jeffrey

Modell Centers Global Network. *Immunol Res* (2016) 64:736–53. doi: 10.1007/s12026-016-8784-z
3. Picard C, Bobby Gaspar H, Al-Herz W, Bousfiha A, Casanova J-L, Chatila T, et al. International Union of Immunological Societies: 2017 Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity. *J Clin Immunol* (2018) 38:96–128. doi: 10.1007/s10875-017-0464-9
4. Quinn J, Modell V, Holle J, Truty R, Aradhya S, Johnson B, et al. Jeffrey's Insights: Jeffrey Modell Foundation's Global Genetic Sequencing Pilot Program to Identify Specific Primary Immunodeficiency Defects to Optimize Disease Management and Treatment. *Immunol Res* (2020) 68:126–34. doi: 10.1007/s12026-020-09131-x
5. Stray-Pedersen A, Sorte HS, Samarakoon P, Gambin T, Chinn IK, Coban Akdemir ZH, et al. Primary Immunodeficiency Diseases: Genomic Approaches Delineate Heterogeneous Mendelian Disorders. *J Allergy Clin Immunol* (2017) 139:232–45. doi: 10.1016/j.jaci.2016.05.042
6. Bousfiha A, Jeddane L, Picard C, Al-Herz W, Ailal F, Chatila T, et al. Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification. *J Clin Immunol* (2020) 40:66–81. doi: 10.1007/s10875-020-00758-x
7. Bousfiha AA, Jeddane L, Ailal F, Benhsaien I, Mahlaoui N, Casanova J-L, et al. Primary Immunodeficiency Diseases Worldwide: More Common Than Generally Thought. *J Clin Immunol* (2013) 33:1–7. doi: 10.1007/s10875-012-9751-7
8. Esser M, Banda E, Moller M, Nortje R. (2015). Available at: http://www.asid.ma/images/pdfs/revue_asid4th.pdf (Accessed January 15, 2021). Primary immunodeficiency disease management in tuberculosis endemic regions – are we aware enough and how does a registry assist.
9. Eley B, Esser M. Investigation and Management of Primary Immunodeficiency in South African Children. *S Afr Med J* (2014) 104:793. doi: 10.7196/SAMJ.8946
10. Ramsay M, de Vries J, Soodyall H, Norris SA, Sankoh O. Ethical Issues in Genomic Research on the African Continent: Experiences and Challenges to Ethics Review Committees. *Hum Genomics* (2014) 8:15. doi: 10.1186/s40246-014-0015-x
11. Heimall JR, Hagin D, Hajjar J, Henrickson SE, Hernandez-Trujillo HS, Tan Y, et al. Use of Genetic Testing for Primary Immunodeficiency Patients. *J Clin Immunol* (2018) 38:320–9. doi: 10.1007/s10875-018-0489-8
12. Esteve-Solé A, Sologuren I, Martínez-Saavedra MT, Deyà-Martínez À, Oleaga-Quintas C, Martinez-Barricarte R, et al. Laboratory Evaluation of the IFN-γ Circuit for the Molecular Diagnosis of Mendelian Susceptibility to Mycobacterial Disease. *Crit Rev Clin Lab Sci* (2018) 55:184–204. doi: 10.1080/10408363.2018.1444580
13. Chinen J, Lawrence M, Dorsey M, Kobrynski LJ. Practical Approach to Genetic Testing for Primary Immunodeficiencies. *Ann Allergy Asthma Immunol* (2019) 123:433–9. doi: 10.1016/j.anai.2019.08.016
14. Rae W, Ward D, Mattocks C, Pengelly RJ, Eren E, Patel SV, et al. Clinical Efficacy of a Next-Generation Sequencing Gene Panel for Primary Immunodeficiency Diagnostics. *Clin Genet* (2018) 93:647–55. doi: 10.1111/cge.13163
15. Al-Mousa H, Abouelhoda M, Monies DM, Al-Tassan N, Al-Ghonaium A, Al-Saud B, et al. Unbiased Targeted Next-Generation Sequencing Molecular Approach for Primary Immunodeficiency Diseases. *J Allergy Clin Immunol* (2016) 137:1780–7. doi: 10.1016/j.jaci.2015.12.1310
16. Gallo V, Dotta L, Giardino G, Cirillo E, Lougaris V, D'Assante R, et al. Diagnostics of Primary Immunodeficiencies Through Next-Generation Sequencing. *Front Immunol* (2016) 7:466. doi: 10.3389/fimmu.2016.00466
17. Modell V, Orange JS, Quinn J, Modell F. Global Report on Primary Immunodeficiencies: 2018 Update From the Jeffrey Modell Centers Network on Disease Classification, Regional Trends, Treatment Modalities, and Physician Reported Outcomes. *Immunol Res* (2018) 66:367–80. doi: 10.1007/s12026-018-8996-5
18. Wang W, Corominas R, Lin GN. De Novo Mutations From Whole Exome Sequencing in Neurodevelopmental and Psychiatric Disorders: From Discovery to Application. *Front Genet* (2019) 10:258. doi: 10.3389/fgene.2019.00258
19. Cifaldi C, Brigida I, Barzaghi F, Zoccolillo M, Ferradini V, Petricone D, et al. Targeted NGS Platforms for Genetic Screening and Gene Discovery in Primary Immunodeficiencies. *Front Immunol* (2019) 10:316. doi: 10.3389/fimmu.2019.00316
20. Burke W. Genetic Tests : Clinical Validity and Clinical Utility. *Curr Protoc Hum Genet Editor Board Jonathan Haines Al* (2014) 81:9.15.1–8. doi: 10.1002/0471142905.hg0915s81
21. Seleman M, Hoyos-Bachiloglu R, Geha RS, Chou J. Uses of Next-Generation Sequencing Technologies for the Diagnosis of Primary Immunodeficiencies. *Front Immunol* (2017) 8:847. doi: 10.3389/fimmu.2017.00847
22. Yska HAF, Elsink K, Kuijpers TW, Frederix GWJ, van Gijn ME, van Montfrans JM. Diagnostic Yield of Next Generation Sequencing in Genetically Undiagnosed Patients With Primary Immunodeficiencies: A Systematic Review. *J Clin Immunol* (2019) 39:577–91. doi: 10.1007/s10875-019-00656-x
23. Shillitoe B, Bangs C, Guzman D, Gennery AR, Longhurst HJ, Slatter M, et al. The United Kingdom Primary Immune Deficiency (UKPID) Registry 2012 to 2017. *Clin Exp Immunol* (2018) 192:284–91. doi: 10.1111/cei.13125
24. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* (2015) 17(5):405–23. doi: 10.1038/gim.2015.30
25. Martin AR, Teferra S, Möller M, Hoal EG, Daly MJ. The Critical Needs and Challenges for Genetic Architecture Studies in Africa. *Curr Opin Genet Dev* (2018) 53:113–20. doi: 10.20944/preprints201806.0201.v1
26. Mazzarotto F, Olivotto I, Walsh R. Advantages and Perils of Clinical Whole-Exome and Whole-Genome Sequencing in Cardiomyopathy. *Cardiovasc Drugs Ther* (2020) 34:241–53. doi: 10.1007/s10557-020-06948-4
27. Glanzmann B, Uren C, de Villiers N, van Coller A, Glashoff RH, Urban M, et al. Primary Immunodeficiency Diseases in a Tuberculosis Endemic Region: Challenges and Opportunities. *Genes Immun* (2019) 20:447–54. doi: 10.1038/s41435-018-0041-0
28. Yadav RM, Gupta M, Dalvi A, Bargir UA, Hule G, Shabrish S, et al. Prenatal Diagnosis for Primary Immunodeficiency Disorders—An Overview of the Indian Scenario. *Front Immunol* (2020) 711:3163. doi: 10.3389/fimmu.2020.612316
29. El Hawary RE, Meshaal SS, Abd Elaziz DS, Elsharkawy MA, Alkady RS, Lotfy S, et al. Genetic Counseling in Primary Immunodeficiency Disorders: An Emerging Experience in Egypt. *Mol Diagn Ther* (2017) 21(6):677–84. doi: 10.1007/s40291-017-0297-5
30. Simon AJ, Golan AC, Lev A, Stauber T, Barel O, Somekh I, et al. Whole Exome Sequencing (WES) Approach for Diagnosing Primary Immunodeficiencies (Pids) in a Highly Consanguineous Community. *Clin Immunol* (2020) 214:1–7. doi: 10.1016/j.clim.2020.108376
31. Samorodnitsky E, Jewell BM, Hagopian R, Miya J, Wing MR, Lyon E, et al. Evaluation of Hybridization Capture Versus Amplicon-Based Methods for Whole-Exome Sequencing. *Hum Mutat* (2015) 36:903–14. doi: 10.1002/humu.22825
32. Chilamakuri CSR, Lorenz S, Madoui M-A, Vodák D, Sun J, Hovig E, et al. Performance Comparison of Four Exome Capture Systems for Deep Sequencing. *BMC Genomics* (2014) 15:449. doi: 10.1186/1471-2164-15-449
33. Manase D, D'Alessandro LC, Manickaraj AK, Al Turki S, Hurles ME, Mital S. High Throughput Exome Coverage of Clinically Relevant Cardiac Genes. *BMC Med Genomics* (2014) 7:67. doi: 10.1186/s12920-014-0067-8
34. Belkadi A, Bolze A, Itan Y, Cobat A, Vincent QB, Antipenko A, et al. Whole-Genome Sequencing is More Powerful Than Whole-Exome Sequencing for Detecting Exome Variants. *Proc Natl Acad Sci USA* (2015) 112:5473–8. doi: 10.1073/pnas.1418631112
35. Barbitoff YA, Polev DE, Glotov AS, Serebryakova EA, Shcherbakova IV, Kiselev AM, et al. Systematic Dissection of Biases in Whole-Exome and Whole-Genome Sequencing Reveals Major Determinants of Coding Sequence Coverage. *Sci Rep* (2020) 10:2057. doi: 10.1038/s41598-020-59026-y
36. Campbell MC, Tishkoff SA. African Genetic Diversity: Implications for Human Demographic History, Modern Human Origins, and Complex Disease Mapping. *Annu Rev Genomics Hum Genet* (2008) 9:403–33. doi: 10.1146/annurev.genom.9.081307.164258

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SFRP5 Enhances Wnt5a Induced-Inflammation in Rheumatoid Arthritis Fibroblast-Like Synoviocytes

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Background: Tissue derived fibroblast-like synoviocytes (td-FLS) are key actors in pannus formation and contribute to joint destruction and inflammation during rheumatoid arthritis (RA). Several members of the Wnt family, including Wnt5a, may contribute to RA td-FLS activation and can potentially serve as therapeutic targets.

Objective: The present work aimed to investigate the expression of Wnt5a signaling elements in RA td-FLS and their potential precursors (fluid derived (fd) FLS and fibrocytes). We also studied the role of Wnt5a in RA td-FLS pro-inflammatory activity and whether the inhibitor SFRP5 could restore Wnt5a-induced synovial dysfunction *in vitro*.

Materials and Methods: The levels of Wnt5a, SFRP5, Wnt5a receptors/coreceptors and Wnt5a pro-inflammatory targets were determined in cultured RA td-FLS, fd-FLS and fibrocytes using qPCR under basal conditions. The expression of pro-inflammatory molecules was assessed after RA td-FLS stimulation with Wnt5a and SFRP5 at different time points.

Results: Our data showed that td-FLS, fd-FLS and fibrocytes from patients with RA expressed similar levels of Wnt5a and a set of Wnt5a receptors/coreceptors. We also demonstrated that Wnt5a stimulated the expression of the pro-inflammatory targets, especially IL1β, IL8 and IL6 in RA td-FLS. Wnt5a-induced inflammation was enhanced in the presence of SFRP5. Furthermore, Wnt5a alone and in conjunction with SFRP5 inhibited the gene expression of TCF4 and the protein levels of the canonical coreceptor LRP5.

Conclusion: Wnt5a pro-inflammatory effect is not inhibited but enhanced by SFRP5 in RA td-FLS. This research highlights the importance of carefully evaluating changes in Wnt5a response in the presence of SFRP5.

Keywords: rheumatoid arthritis, Wnt pathway, FLS, fibrocytes, inflammation

Abbreviations: RA, Rheumatoid Arthritis; FLS, fibroblast-like synoviocytes, SF, Synovial fluid; SFRP, Secreted Frizzled-Related Protein.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects approximately 1% of population worldwide (1). Synovial lining fibroblast-like synoviocytes (also called tissue derived fibroblast-like synoviocytes (td-FLS)) are mesenchymal cells that display many characteristics of fibroblasts including expression of type I and V collagens, fibronectin vimentin, and CD90. During RA, these cells exhibit multipotent pathogenic proprieties (2, 3). Explanations for RA td-FLS aggressiveness include migration of new cells from the blood and bone marrow into the inflamed joint (4).

It is also possible that circulating fibrocytes can migrate into the inflamed joint and then differentiate along a fibroblast-like synoviocyte pathway. Furthermore, synovial fluid (SF) from RA patients has been described to give rise to fluid derived FLS (fd-FLS), which are morphologically similar to td-FLS (5). These cells are also described in osteoarthritis (OA) synovial fluid and exhibit similar pro-inflammatory characteristics to OA td-FLS (6).

The activation of td- FLS during RA results from many alterations in several homeostatic mechanisms and signals including the Wnt pathway. This signaling is involved in many biological processes including embryonic development, cell migration, tissue organization and human diseases (7, 8). Wnt5a, a prototype Wnt, is involved in several inflammatory diseases. It mediates the production of many inflammatory molecules including IL1β, IL6 and IL15 during autoimmune disorders (4, 9). In RA td-FLS, Wnt5a expression is enhanced (4, 9). The signaling pathways induced by Wnt5a are mediated by canonical (β-catenin-dependent) or noncanonical (β-catenin independent) pathways (10). The β-catenin dependent signaling pathway is by far the best-characterized and comprises signal transduction through the accumulation of β-catenin protein as well as specific T-cell factor/lymphoid enhancer factor (LEF/TCF) family transcription factors (11, 12)

Wnt5a binds several receptors, including members of frizzled (Fzd) receptor family, Ror2 and Ryk (9, 13). In addition to Fzd receptors, the canonical Wnt pathway requires transmembrane proteins that belong to a subfamily of low-density lipoprotein (LDL) receptor- related proteins (LRP) such as LRP5 (9, 13). The interaction of Wnt5a with Fzd receptors is antagonized by secreted frizzled -related proteins (SFRP) such as SFRP5. However, a previous study postulated that SFRP could also promote long-term Wnt signaling in certain instances by binding to Wnt proteins and protecting them from degradation (14). Data on the relationship between Wnt5a and SFRP5 in humans are limited and inconsistent (15, 16). Furthermore, the Wnt5a ligand can have disparate effects on cells depending on receptor availability. Therefore, the cellular context dictates the effect of Wnt5a (17). Even though td-FLS perform key functions in RA pathogenesis and joint inflammation, the expression of Wnt5a signaling elements in these cells and their previously described precursors (fd-FLS and fibrocytes) has not been well studied.

We investigated whether the three cell types express Wnt5a, SFRP5, Wnt5a receptors/coreceptors and Wnt5a principal targets. We also studied the involvement of Wnt5a in td-FLS pro-inflammatory activities during RA.

MATERIALS AND METHODS

Patients and Samples

The study included 14 patients with RA. Each patient donated a unique biological sample (peripheral blood, synovial fluid, or synovial tissue). Peripheral blood samples were obtained from five patients with RA. Synovial tissues were obtained from five patients with RA during total joint replacement or synovectomy. Synovial fluid (SF) samples were collected from the knees of four patients with active RA using a standard sterile procedure. The median age of all patients was 57 years old (range 35–79) with a disease duration of 15 years (7–26). RA patients fulfilled the criteria for the ACR/EULAR 2010 (18). RA patient characteristics are detailed in **Table 1**. As a control, 3 synovial membranes from patients with osteoarthritis (OA) were used. These tissues were collected at the time of total knee replacement surgery. The experimental protocols used in this study were approved by the ethics committee of la Rabta hospital, Tunis, Tunisia. Informed consent was obtained from each patient included in this study.

TABLE 1 | RA Patient characteristics.

Donation	Blood (n = 5)	Synovial fluid (n = 4)	Synovial Tissue (n = 5)
Age, years[a]	59 (50-79)	60 (52-79)	50 (35-64)
Disease duration, years[a]	14 (7-26)	18 (12-26)	14 (8-20)
Female sex, n (%)	3/5	2/5	3/5
Site	Peripheral blood	Knee	Wrist (1 synovectomy and 4 total joint replacement)
Volume (ml)	10-12	1-20	
Sample collection	Sterile heparinized tubes	sterile syringe	50 ml Falcon centrifuge tube with medium
ACPA[b] + and RF[c]+, n (%)	5/5	4/4	N/A
CRP[a] mg/l	42.9 (5-164.8)	16.4 (7.6-13.6)	N/A
Medications, n	5/5	4/4	N/A
TNF-inhibitors[d], n	0	0	
Methotrexate, n	5/5	2/4	
Prednisone, n	0	2/4	

[a]Median and range, [b]Anti-citrullinated Protein Antibody, [c]Rheumatoid Factor, [d]TNF inhibitors = Humira, Enbrel, Simponi, Remicade, N/A data at the time of donation is not available.

Td-FLS Isolation and Culture

RA and OA synovial fragments were washed with sterile phosphate-buffered saline (PBS). The tissues were minced and treated overnight with 1.5 mg/ml of collagenase A (Roche®) in Dulbecco's modified Eagle medium (DMEM) (Gibco®) at 37°C. Dissociated cells were then centrifuged at 3500 rpm for 3 min, washed and resuspended in DMEM supplemented with 20% fetal bovine serum (FBS) and 1% penicillin- streptomycin (Gibco®). The cell pellet was seeded in T-75 culture flasks (Nunc®) and cultured at 37°C with 5% CO2. After 72h, non-adherent cells were removed, and adherent cells were kept at 37°C in 5% CO2. The medium was replaced every 3 days. After 10-14 days of culture, adherent cells were trypsinized using trypsin-EDTA (Gibco®) and transferred to new flasks with a density of 5.10^5 cells/flask. Passage 3 cells were morphologically homogenous and were used for experiments.

Fd-FLS Isolation and Culture

RA synovial Fluid was centrifuged at 1000 rpm for 30 min and cell pellets were resuspended in DMEM containing 20% FBS and 1% penicillin-streptomycin in a T-75 flask. After 72h, non-adherent cells were removed by replacing the culture medium with a fresh one, and attached cells were cultured in the above -described culture medium. Passage 3 cells were used for RNA and protein extraction.

Fibrocyte Isolation and Culture

RA Fibrocytes were generated in peripheral blood mononuclear cell (PBMC) cultures. Peripheral blood was mixed with DMEM (2:1), layered over Ficoll/Plaque (Sigma®) and centrifuged at 1800 rpm for 30 min. PBMC were collected, washed then cultured in DMEM supplemented with 20% FBS and 1% penicillin-streptomycin (Gibco®). On the fifth day, DMEM was replaced with a fresh medium. After 14 days, the adherent fibrocytes were trypsinized and transferred in 6 well plates (Nunc®). The viability of the isolated cells was assessed by the trypan blue exclusion method. Fibrocytes were identified based on the previously described criteria: adherent cells with elongated spindle-shaped morphology distinct from lymphocytes or adherent monocytes and oval nuclei (19, 20). Fibrocytes were used on day 17 for RNA and protein extraction.

RA td-FLS Stimulation

RA td-FLS were seeded in 6-well plates at a density of 12×10^4 cells/well and maintained at 5%CO_2 for 24h. Cells were treated with recombinant Wnt5a (300 ng/ml) (R&D systems®) and/or recombinant SFRP5 (R&D systems®) for 4h and 24 h. For the combined Wnt5a/SFRP5 treatment, SFRP5 was added 10 min before Wnt5a stimulation. Td-FLS were collected and used for RNA and/or protein extraction.

RNA Isolation and cDNA Synthesis

Total RNA was extracted using the "PureLink® RNA Mini Kit (Invitrogen®) according to the manufacturer's protocol. Sample concentrations were measured (Nanodrop 2000®). Reverse transcription was realized as follows: 1µl of random primers (Invitrogen®) was added to the RNA solution and incubated 10 min at 70°C followed by 5 min incubation on ice. dNTP, M-MLV, RNase out, 5X buffer and DTT (Invitrogen®) were added to RNA solution, incubated for one hour at 37°C then 10 min at 70°C.

Quantitative PCR

Real-time PCR was performed using ABI7500® qPCR System using the SYBR Green qPCR Kit (Invitrogen®) with the primers listed in **Table 2**. A reaction volume of 20 µl (2.0 µl cDNA) was amplified for 40 cycles after initial denaturation (95°C, 10 min) with the following parameters: 95°C for 15 s, 60°C for 1min. Specificity was checked and reaction concentration was optimized before sample analysis. Samples were run in duplicate or triplicate, and a relative quantification of mRNA level was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous reference. The basal gene expression between RA td-FLS, RA fd-FLS and fibrocytes were analyzed using the ΔCT method; all other data (stimulation experiments) were presented as fold change using the $2^{-\Delta\Delta}$CT method.

TABLE 2 | Primers (Invitrogen®) used for qPCR.

Targets	Forward	Reverse
Wnt5a	CAACTGGCAGGACTTTCTCA	TTCTTTGATGCCTGTCTTCG
SFRP5	CACAAGTTCCCCCTGGACAA	TGTGCTCCATCTCACACTGG
Fzd4	TACCTCACAAAACCCCCATCC	GGCTGTATAAGCCAGCATCAT
Fzd5	GTCACACCCGCTCTACAACA	CACTGAAGGACGGCTGGTAG
Ror2	ATGGAACTGTGTGACGTACCC	GCGAGGCCATCAGCTG
Ryk	TCTACCTGAGCGAGGACGAG	CCACTTGGAATCCCAGCTTA
LRP5	ATGGGCGCCAGAACATCAA	AGATGTCGATGCTGAGGTCGTG
TCF4	CTGCCTTAGGGACGGACAAAG	TGCCAAAGAAGTTGGTCCATTTT
β-catenin	TCTGAGGACAAGCCACAAGATTACA	TGGGCACCAATATCAAGTCCAA
IL6	GTAGCCGCCCCACACAGA	CATGTCTCCTTTCTCAGGGCTG
IL8	ATAAAGACATACTCCAAACCTTTCCAC	AAGCTTTACAATAATTTCTGTGTTGGC
IL1β	AAATACCTGTGGCCTTGGGC	TTTGGGATCTACACTCTCCAGCT
CCL2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
CXCL10	GAGCCTACAGCAGAGGAACC	GAGTCAGAAAGATAAGGCAGC
COX2	CCCATGTCAAAACCGAGGTG	CCGGTGTTGAGCAGTTTTCTC
GAPDH	ACTTCAACAGCGACACCCACTC	TACCAGGAAATGAGCTTGACAAAG

Western Blotting

Total protein extraction was realized using the "Qproteome mammalian protein prep kit" (Qiagen®) according to the manufacturer's protocol. Protein concentrations were measured and 40µg of proteins were loaded into an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into nitrocellulose membranes (Biorad®). Membranes were blocked with 5% nonfat dry milk in PBS-0.5% Tween 20 (PBS-T) at room temperature for 1h, followed by overnight incubation at 4°C with primary antibodies against alpha-1 type I collagen (ColIA1), fibronectin, LRP5, GAPDH or β-actin (R&D systems®). The membranes were washed 3 times with PBS-T and incubated 1h at room temperature with horseradish peroxidase-conjugated secondary antibodies (RnDsystems®). After PBS-T washing, membranes were incubated for 1 min with the enhanced chemiluminescence detection solution (ECL) (Perkin Elmer®) and signals were detected using Biomax MR® Films (Kodak®).

Statistical Analysis

Since sample size is not large enough to verify the normality of the data, significance was determined with a non-parametric Kruskal Wallis test using Graph Pad Prism 6. If the Kruskal Wallis test rejected the null hypothesis a post-hoc test was run for multiple comparisons using XLSTAT. Significance is represented as follows: *p<0.05 and **p<0.01.

RESULTS

Expression of Wnt5a in RA *vs.* OA td-FLS

To determine whether Wnt5a expression is specific to RA FLS, we first cultured td-FLS from patients with RA or OA for 3 passages (**Figures 1A, B**). Then, we analyzed the basal mRNA expression of Wnt5a. The real-time PCR showed that Wnt5a was expressed in RA td-FLS but not in OA td-FLS (**Figure 1C**).

Phenotypic Identification of RA td-FLS Eventual Precursors: RA fd-FLS and RA Fibrocytes

As for td-FLS, successful fd-FLS and fibrocyte cultures were obtained from synovial fluids and blood of patients with RA. After 3 passages, td-FLS and fd-FLS from patients with RA showed similar morphology with a spindle-shaped appearance and few branched cytoplasmic processes (**Figures 2A–D**).

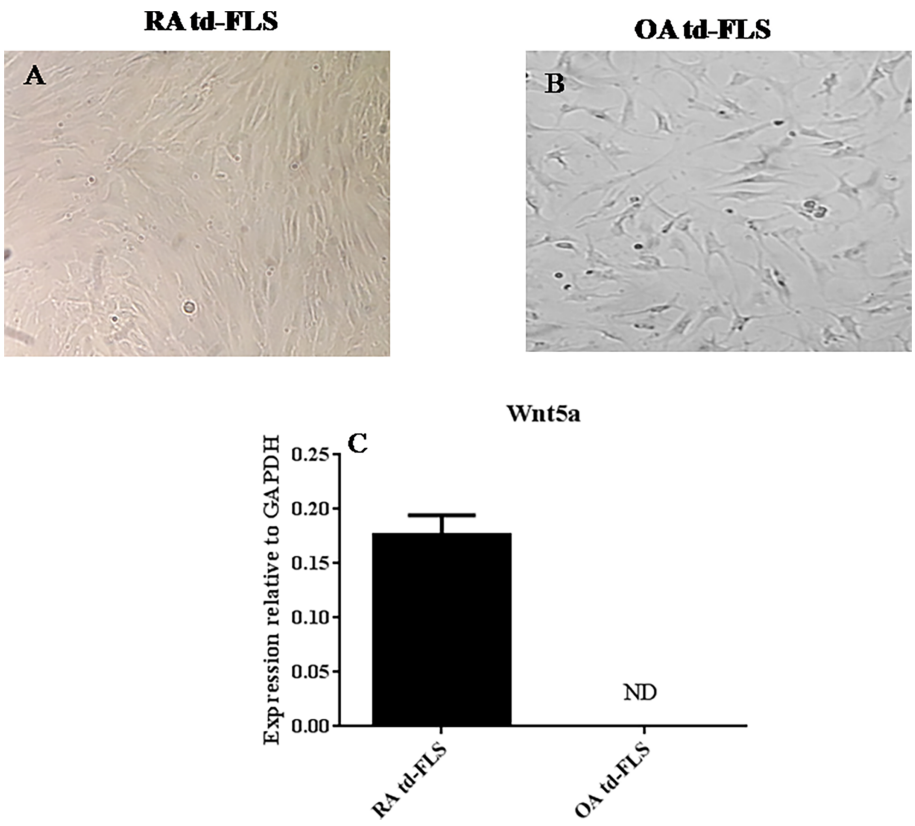

FIGURE 1 | Comparison of Wnt5a expression in td-FLS from patients with RA and OA: Third passage RA td-FLS **(A)**. Third passage OA td-FLS **(B)**. Wnt5a mRNA expression in RA td-FLS (n=3) and OA td-FLS (n=3) **(C)**. Wnt5a was expressed in RA td-FLS but not in OA td-FLS. ND, Not detected. Results are shown as the mean ± SD.

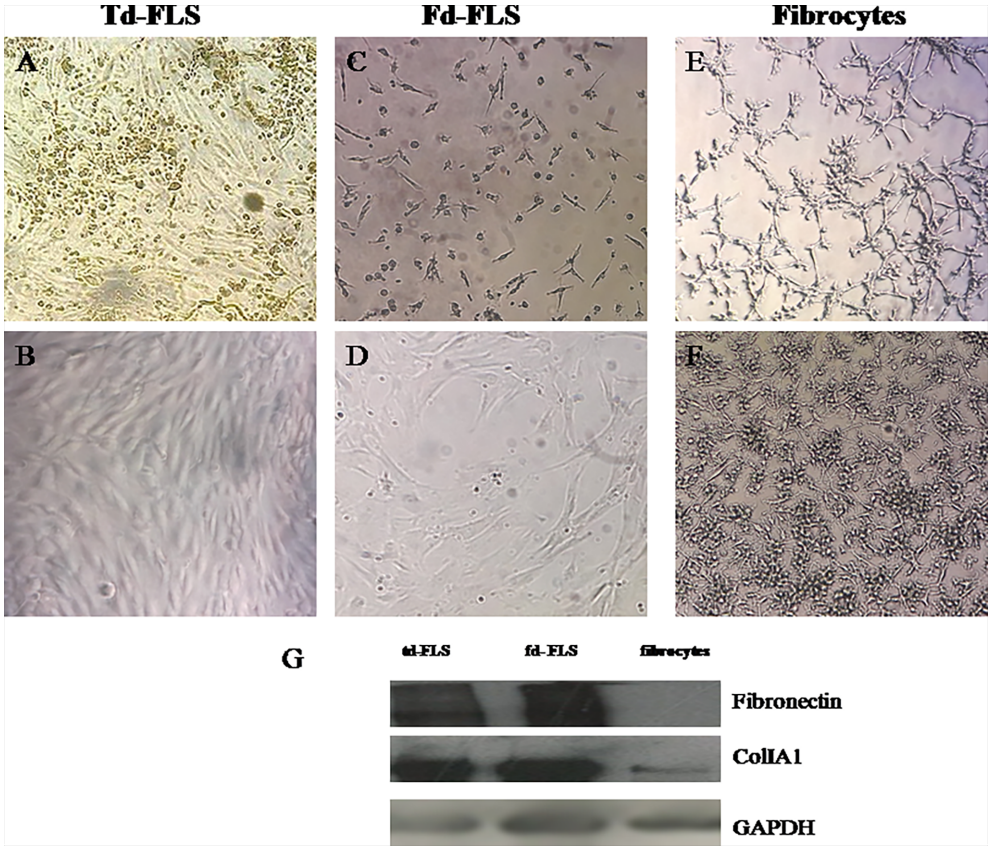

FIGURE 2 | Light microscopic features and mesenchymal marker expression in td-FLS, fd-FLS and fibrocytes from patients with RA: Spindle-shaped adherent cells started to appear in synovial fluid cultures from patients with RA after few days of culture **(C)**. Third passage fd-FLS showed a spindle-shaped appearance **(D)** similar to RA td-FLS **(A, B)**. Fibrocytes from PBMC of patients with RA displayed some features of td-FLS, such as a fibroblast-like morphology. Fibrocytes showed cytoplasmic projections and a slim bipolar-shape **(E, F)** (original magnification ×100). Western blot analysis showed fibronectin, ColIA1 and GAPDH expression in fd-FLS, td-FLS and fibrocytes from patients with RA **(G)**. The Western blots are representative of one experiment out of two.

Cultured fibrocytes had a spindle-shaped morphology with numerous cytoplasmic projections (**Figures 2E, F**). Since fibronectin and collagen I (colI) are mesenchymal markers, we evaluated their expression in RA td/fd-FLS from passage 3 and RA fibrocytes after 17 days of cultures using Western blot. As shown in **Figure 2G**, only RA td-FLS and RA fd-FLS expressed fibronectin. The three cell types expressed ColIA1. The higher levels of ColIA1 were observed in RA FLS (td and fd) compared to fibrocytes.

Expression of Wnt5a and SFRP5 in RA td-FLS, RA fd-FLS and RA Fibrocytes

After obtaining homogenous cultures, we investigated the expression of Wnt5a and SFRP5 genes in td-FLS, fd-FLS and fibrocytes from patients with RA.

No significant difference in Wnt5a transcript level was found between the three cell types (**Figure 3A**). SFRP5 mRNA was detected in RA td-FLS and RA fd-FLS but not in RA fibrocytes (**Figure 3B**).

Expression of Wnt5a Receptors and Coreceptors in RA td-FLS, RA fd-FLS, and RA Fibrocytes

To examine whether RA td-FLS, RA fd-FLS and RA fibrocytes express receptors/coreceptors for Wnt5a, we analyzed the mRNA expression for Fzd4, Fzd5, Ror2, Ryk and LRP5 by qPCR. As shown in **Figure 4**, the Fzd5 mRNA level was significantly higher in RA fibrocytes than in td-FLS and fd-FLS. Therefore, no differential expression in Fzd4, Ror2, Ryk and LRP5 level was found between the three cell types.

Expression of Inflammatory Mediators in RA td-FLS, RA fd-FLS, and RA Fibrocytes

In order to obtain a basic measure of cultured RA td-FLS, fd-FLS and fibrocyte inflammatory profile, we examined the mRNA expression of the inflammatory mediators: IL6, IL1β, IL8, CXCL10, CCL2 and COX2.

We found a significantly higher expression of IL6 in RA fd-FLS compared to td-FLS and fibrocytes (**Figure 5A**). The mRNA

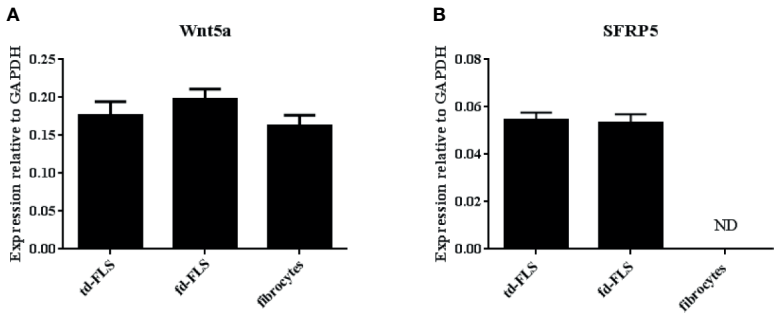

FIGURE 3 | Expression of Wnt5a and SFRP5 in td-FLS, fd-FLS and fibrocytes during RA. Wnt5a gene level profiles in the three cell types by quantitative RT-PCR: No significant differences were found in mRNA levels **(A)**. SFRP5 mRNA expression: SFRP5 was expressed in td-FLS and fd-FLS but not in fibrocytes (n=3-4) **(B)**. ND, Not detected. Results are shown as the mean ± SD.

expression levels of IL1β, IL8, CXCL10 and CCL2 were significantly higher in RA fibrocytes than in td-FLS and fd-FLS (**Figures 5B–E**). Furthermore, IL1β transcript levels were greater in RA fd-FLS when compared to RA td-FLS (**Figure 5B**). No differential COX2 expression levels were found between the three cell types (**Figure 5F**).

Effect of Wnt5a on Inflammatory Mediator Synthesis by Activated RA td-FLS

The above findings prompted us to explore the involvement of Wnt5a in the pro-inflammatory activity of RA td-FLS. The Wnt5a stimulated expression profile of pro-inflammatory mediators in RA td-FLS was analyzed using qPCR after 4h and 24h of stimulation.

As shown in **Figure 6A**, IL1β expression was enhanced robustly, more than 48 times over the untreated cells after 4h of treatment. IL8, IL6, CCL2 and COX2 were also rapidly up-regulated (25.7 fold, 13.7 fold, 8.5 and 7.4 fold respectively). CXCL10 was up-regulated mildly (1.5 fold) after 4 h of treatment. We sought if the inflammatory profile induced by Wnt5a in RA td-FLS was inhibited by the Wnt5a soluble regulator: SFRP5. RA td-FLS cytokine production induced by Wnt5a was enhanced in the presence of SFRP5. The combined Wnt5a/SFRP5 treatment induced a massive expression of IL1β,

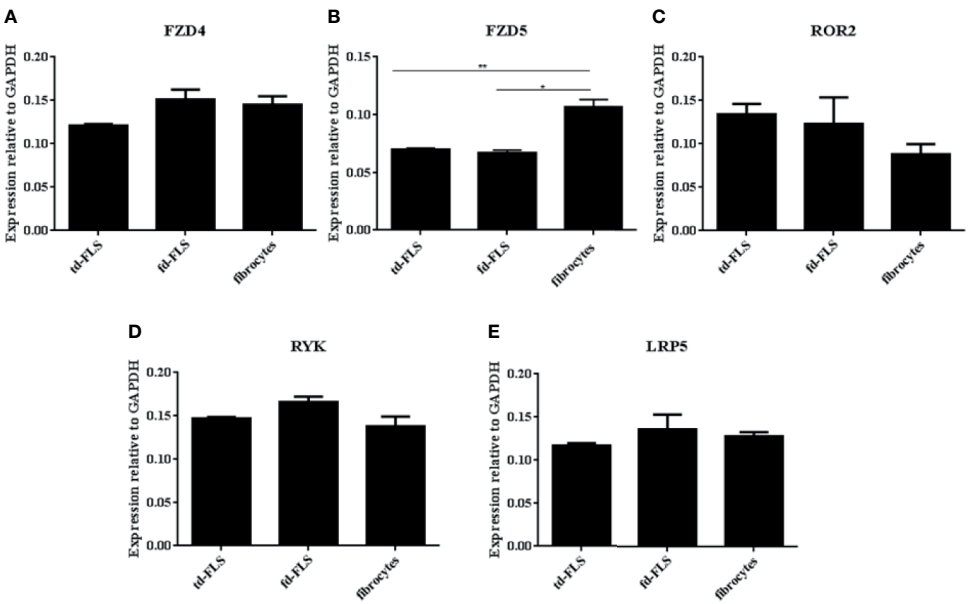

FIGURE 4 | Expression of Wnt5a receptors and coreceptors in td-FLS, fd-FLS and fibrocytes during RA: Relative mRNA levels of Fzd4, Ror2, Ryk and LRP5 in the three cell types: No difference was detected between the cells using qPCR analysis **(A, C–E)**. Fzd5 was highly expressed in fibrocytes compared to td-FLS and fd-FLS (n=3-4) **(B)**. Results are shown as the mean ± SD *Statistically significant difference $p < 0.05$, **statistically significant difference $p < 0.01$, using non-parametric Kruskal Wallis test with a *post hoc* test.

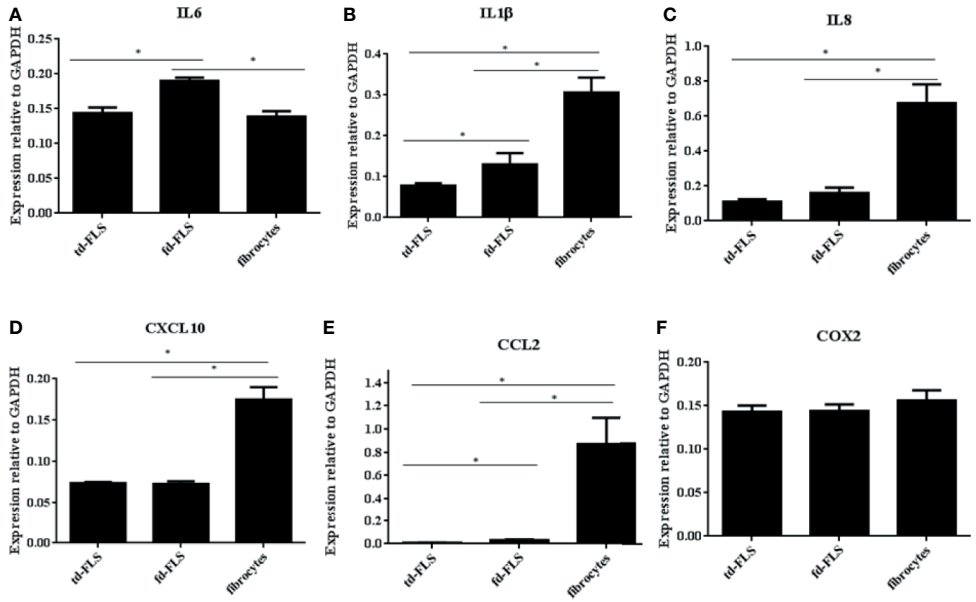

FIGURE 5 | Expression of Wnt5a pro-inflammatory targets in td-FLS, fd-FLS and fibrocytes during RA. IL6 was highly expressed in fd-FLS compared to td-FLS and fibrocytes **(A)**. IL1β and CCL2 were expressed by the three cell types **(B, E)**. The higher mRNA levels for IL1β and CCL2 were found in fibrocytes followed by fd-FLS. IL8 and CXL10 expression profiles were greater in fibrocytes than in td-FLS or fd-FLS **(C, D)**. Relative mRNA levels of COX2 in the three cell types **(F)**: No difference was detected between the cells using qPCR analysis (n=3-4). Results are shown as the mean ± SD.*Statistically significant difference $p < 0.05$ using non-parametric Kruskal Wallis test with a *post hoc* test.

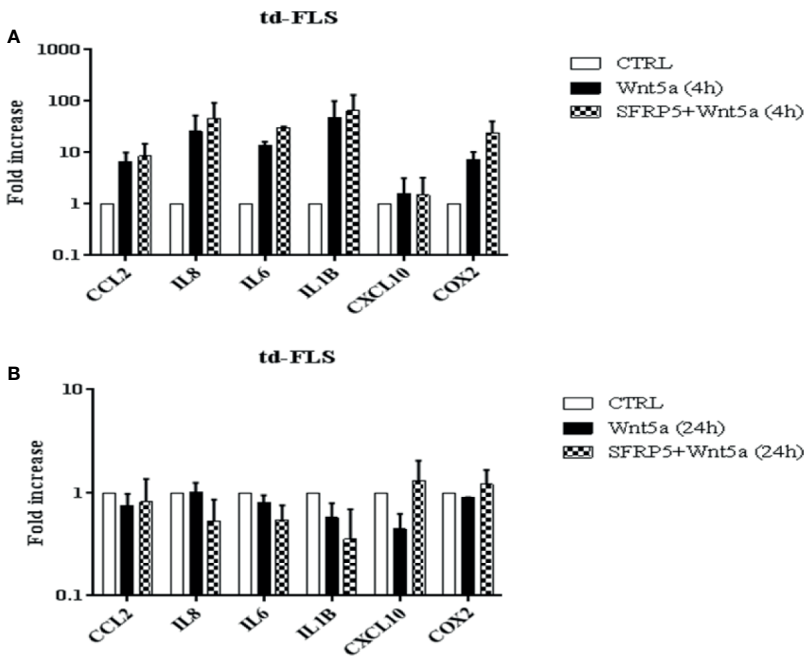

FIGURE 6 | Effect of Wnt5a on the inflammatory response of RA td-FLS. RA td-FLS (passage 3) were stimulated with recombinant Wnt5a (300 ng/ml) in the presence or absence of SFRP5 (1 mg/ml) for 4 h **(A)** and 24h **(B)**. Cytokine expression profiles (CCL2, IL8, IL1β, CXCL10 and COX2) were determined in cell culture using qPCR. Gene expression levels in unstimulated cells were assumed to be 1. Data are means ± SD of two independent experiments each performed in triplicates using at least two different lots of each of the recombinant Wnt proteins.

IL8, IL6, COX2 and CCL2 (65.5 fold, 45,2 fold, 30.1 fold, 24.2 fold and 8.5 fold respectively).

At 24h of Wnt5a stimulation, IL1β, IL8, IL6 and CCL2 expression declined and dropped under the initial levels found in the untreated cells (**Figure 6B**).

Effect of Wnt5a on the Principal Canonical Wnt Component Expression

Finally, we analyzed whether Wnt5a alone or associated with SFRP5 changed the expression of the principal canonical Wnt elements. We analyzed the gene expression levels of β-catenin and TCF4. We also evaluated the protein expression of the coreceptor LRP5 which is crucial for the canonical Wnt activation.

After Wnt5a stimulation for 4 h and 24 h, no changes in β-catenin expression were observed. The expression of the canonical transcription factor TCF4 decreased after 4h of Wnt5a stimulation and Wnt5a/SFRP5 treatment (**Figure 7A**). Consistent with this, td-FLS treatment with Wnt5a alone or associated with SFRP5 for 24h diminished the protein expression levels of the canonical coreceptor LRP5. It is worth noting that SFRP5 alone did not elicit LRP5 expression (**Figure 7B**).

DISCUSSION

RA is an acquired disorder that results from the concerted actions of different cell types both in the inflamed joint and in the circulation. RA td-FLS have a key role in all principal features of RA including hyperplasia and inflammation (3). Since neither increased proliferation nor inadequate apoptosis are totally responsible for the accumulation of RA td-FLS in the joint and synovial hyperplasia, there is certainly a cell migration phenomenon of progenitors from the blood to the synovium. The paper by Galligan and colleagues suggests that circulating fibrocytes are precursors of td-FLS and their activation in the circulation may be indicative of subsequent synovial inflammation and joint destruction (21). In a previous study, we showed that synovial fluid from RA patients gives rise to fd-FLS that contribute to the progression of RA (22). It is possible that fd-FLS derived from circulating fibrocytes (5).

As td-FLS are important in the pathogenesis of RA, the identification of endogenous molecules that regulate td-FLS function is of paramount importance. The evidence accumulated to date shows that the Wnt signaling pathway plays a critical role in the pathogenesis of RA. In fact, some members of the Wnt pathway, such as Wnt5a, have been identified in the synovial tissue and serum of patients with RA (9). Firstly, we found that Wnt5a expression was restricted to td-FLS from RA patients. Our results are consistent with Sen and colleague's study showing that RA tissues express high gene levels of Wnt5a whereas OA tissues express either much less or almost no detectable Wnt5a mRNA (4). Subsequently, we demonstrated that Wnt5a was expressed in the two eventual precursors of td-FLS: fd-FlS and fibrocytes during RA.

Surprisingly, it has been possible to show greater expression of Wnt inhibitors such as SFRP in blood and synovial tissues during RA (23). Our data, demonstrated that SFRP5 was expressed in RA td-FLS, RA fd-FLS but not in RA fibrocytes. Indeed, as was shown by Kwon and colleagues, both RA td-FLS

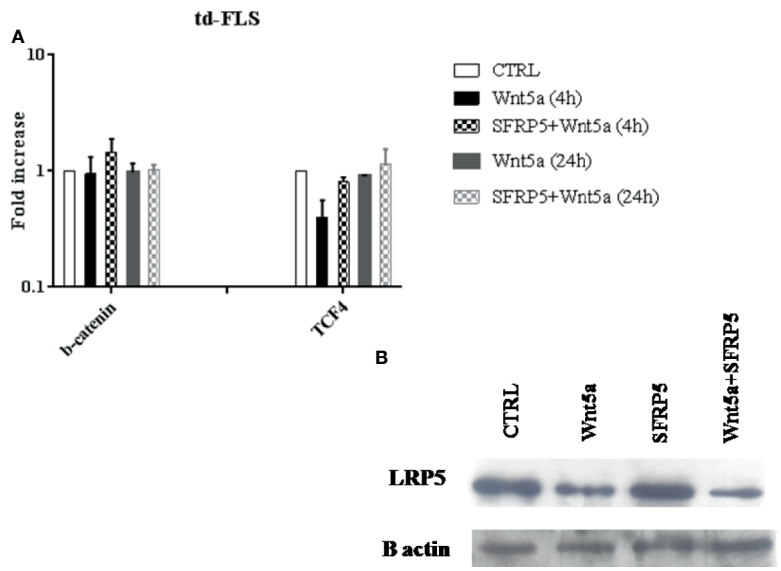

FIGURE 7 | Effect of Wnt5a on the canonical Wnt pathway activation in RA td-FLS. RA td-FLS (passage 3) were stimulated with recombinant Wnt5a (300 ng/ ml) in the presence or absence of SFRP5 (1 mg/ml) for 4 h and/or 24h. Intracellular regulator expression profiles (β- catenin and TCF4) were determined in cell culture using qPCR **(A)**. Gene expression levels in unstimulated cells were assumed to be 1. Results are shown as the mean ± SD of two independent experiments each performed in triplicates using at least two different lots of each of the recombinant Wnt proteins. The protein abundance of LRP5 was studied by Western blot analysis after 24h of stimulation **(B)**. The Western blot is representative of one experiment out of two.

and PBMC express SFRP5 (24). SFRP can prevent Wnt interaction with some Fzd receptors and decrease Wnt signaling. However, in some contexts, SFRP proteins could facilitate Wnt signaling by enhancing Wnt diffusion (14, 23).

Recent reports identified Wnt5a to interact with various members of the Fzd receptor family (Fzd 4, 5, 8) as well as the receptors Ror2 and Ryk (9, 13, 25). Our data revealed that td-FLS, fd-FLS and fibrocytes expressed several Wnt5a receptors: Fzd4, Fzd5, Ror2, Ryk and the coreceptor LRP5 during RA. Interestingly, our study demonstrated that the expression profile of Fzd5 was particularly higher in fibrocytes compared to td-FLS and fd-FLS during RA. A previous report demonstrated that Fzd5 is expressed in RA tissues. Furthermore, Fzd5 levels are higher in RA synovium in comparison to osteoarthritis and normal adult synovium. This Wnt5a receptor plays an important role in FLS-mediated inflammatory response. RA td-FLS treatment with a polyclonal antibody specific for the extracellular domain of Fzd5 blocks IL6 and IL15 expression at both RNA and protein levels (9). Fzd5 plays a pivotal role not only in inflammation but also in cell migration (26). Considering all these results, Fzd5 may constitute one of the receptors involved in fibrocyte migration into the inflamed joint during RA.

Several research lines suggest that the Wnt5a-mediated signaling plays an important role in the induction of cytokines and other pro-inflammatory molecules in many cell types including neutrophils, monocytes, and endothelial cells (27–29). To understand the functional role of Wnt5a *in vitro*, RA td-FLS were stimulated with Wnt5a for 4 h and 24 h.Our data showed that Wnt5a enhanced rapidly the expression of IL1β, IL6, IL8, CCL2, CXCL10 and COX2 in RA td-FLS. According to Sen and colleague's study, the transfection of RA td-FLS with either an antisense Wnt5a vector or a dnWnt5a down-regulates cytokine expression (9). Our data clearly demonstrated a robust induction of IL1β in response to Wnt5a in RA td-FLS. This cytokine plays a key role in invasiveness, inflammation and FLS activation (30). It may promote the formation of osteoclast-like cells that can enlarge channels between the bone marrow and the synovial cavity (9). Wnt5a stimulated the expression of three important chemokines: IL8, CCL2 and CXCL10; it might stimulate leukocyte migration, predominantly neutrophils and fibrocytes (30).

Although the role of the inhibitor SFRP5 was characterized in some cell types, there is little to no evidence about its inflammatory function in RA td-FLS. Surprisingly, we found that RA td-FLS cytokine production induced by Wnt5a was not inhibited but enhanced in the presence of SFRP5. This observation is consistent with a previous report by Yu and colleagues who demonstrated that SFRP5 enhances the pro-inflammatory activity of Wnt5a in macrophages (31). In fact, IL12, IL6 and TNF levels were higher in macrophages treated with Wnt5a associated with SFRP5 than in macrophages stimulated by Wnt5a alone. A similar picture was seen when macrophages are treated with Wnt5a associated with the canonical inhibitors: SFRP1 or Dickkopf 1. These observations indicate that there is, in fact, a direct link between the pro-inflammatory effect of Wnt5a and the inhibition of the canonical Wnt pathway. In our study, we measured the gene expression of TCF4 and β-catenin after cell treatment. We were able to detect a decrease in the canonical transcription factor: TCF4 levels after Wnt5a addition alone or associated with SFRP5. However, we did not observe changes in β-catenin expression after cell stimulation. Our data agree with a previous report by Mikels and Nusse. The authors described that Wnt5a protein treatment has no effect on β-catenin levels but rather inhibits canonical Wnt signaling at the level of TCF transcription (25). Other researchers found that Wnt5a inhibits the canonical Wnt signaling by diminishing the gene expression of Axin2 which is an important element in the canonical cascade (31). In this study, we limited our exploration to an evaluation of the mRNA expression of β-catenin and we did not assess the phosphorylated form of this protein in RA td-FLS after Wnt5a and SFRP5 treatment. It is also believed that Wnt signaling pathways can act in an antagonistic manner. Various noncanonical Wnt signaling mechanisms have been reported to inhibit the β-catenin pathway by increasing β-catenin turnover or decreasing β-catenin/TCF association with DNA (32). It will also be interesting to explore the noncanonical Wnt pathways after cell treatment with Wnt5a and SFRP5.

Finally, we examined the effects of Wnt5a and SFRP5 on the canonical coreceptor LRP5. Our results indicated that the addition of Wnt5a alone and in conjunction with SFRP5 induced a clear drop in LRP5 protein expression. In fact, Wnt5a can activate the canonical and the noncanonical pathways depending on the cell type or the repertory of available receptors (13, 25, 33). A study conducted by Mikels and Nusse showed that Wnt5a activates the canonical Wnt pathway in the presence of both Fzd4 and LRP5. However, the interaction between Wnt5a and Ror2 inhibits the canonical Wnt pathway and activates a noncanonical Wnt signaling (25). The diversity of receptors that interact with Wnt and transduce their signals is matched by an equally diverse set of proteins that modulate Wnt activity in the extracellular space (34). Several molecules, including SFRP, act by forming nonfunctional complexes with some Wnt receptors and then reduce the possibilities of interaction between Wnt molecules and those receptors (35). As previously noted, noncanonical Wnt signaling can inhibit β-catenin transcriptional activity (32). Such a mechanism could be activated by some SFRP, such as SFRP5, *via* blocking particular receptors to enhance the interaction between Wnt and some noncanonical receptors (36). This could be the reason behind the enhanced pro-inflammatory response of Wnt5a when associated with SFRP5. The contradictory results between the studies that used recombinant SFRP5 (31) and the studies based on SFRP5 gene silencing (24) could perhaps be explained by the role of SFRP5 protein in controlling Wnt5a-Wnt receptor interactions.

Certain limitations should be considered in our study. The first limitation was related to the small number of RA patients and samples. While increasing the number of synovial fluid and blood samples was possible, it was difficult to obtain more fresh synovial tissue specimens for td-FLS cultures. Recent advances in

managing patients with RA have resulted in reduced access to synovial tissues for researchers. A second limitation of our study was inherent to the exclusive use of cell cultures, which is known to be unable to recapitulate the full pathogenesis of RA. The third limitation was the absence of an evaluation of Wnt5a and SFRP5 effects on normal or OA td-FLS pro-inflammatory response. The findings may not be similar in non- RA cells, perhaps due to the unique aggressive characteristics of RA FLS and the cellular context. In addition, our study was limited to the evaluation of mRNA expression of the pro-inflammatory targets after Wnt5a and SFRP5 treatment. The evaluation of cytokine secretion in the supernatant remains to be established to firmly establish the importance of the Wnt5 pathway involvement in RA-FLS mediated inflammation.

CONCLUSION

The findings of this study indicate that td-FLS and their eventual precursors: fd-FLS and fibrocytes express Wnt5a signaling elements during RA. The pro-inflammatory effects of Wnt5a on td-FLS are enhanced in the presence of SFRP5. Furthermore, the association of Wnt5a with SFRP5 stimulates RA td-FLS response through mechanisms involving the inhibition of TCF4 and LRP5. We provide Wnt5a signaling a new argument for a potential target of td-FLS-directed RA therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The studies involving human participants were reviewed and approved by La Rabta Hospital ethic committee, Tunis, Tunisia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DM and WK: experiments and writing. NS, AM MM, LA, SJ, SR, and LT: experiments. MK-S, EC, and LL: concept and writing. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Nygaard G, Firestein GS. Restoring Synovial Homeostasis in Rheumatoid Arthritis by Targeting Fibroblast-Like Synoviocytes. *Nat Rev Rheumatol* (2020) 16:316–33. doi: 10.1038/s41584-020-0413-5
2. Vital EM, Emery P. The Development of Targeted Therapies in Rheumatoid Arthritis. *J Autoimmun* (2008) 31:219–27. doi: 10.1016/j.jaut.2008.04.006
3. Filer A. The Fibroblast as a Therapeutic Target in Rheumatoid Arthritis. *Curr Opin Pharmacol* (2013) 13:413–19. doi: 10.1016/j.coph.2013.02.006
4. Sen M, Lauterbach K, El-Gabalawy H, Firestein GS, Corr M, Carson DA. Expression and Function of Wingless and Frizzled Homologs in Rheumatoid Arthritis. *Proc Natl Acad Sci USA* (2000) 97:2791–96. doi: 10.1073/pnas.050574297
5. Stebulis JA, Rossetti RG, Atez FJ, Zurier RB. Fibroblast-Like Synovial Cells Derived From Synovial Fluid. *J Rheumatol* (2005) 32:301–06.
6. Machado CRL, Resende GG, Rafaela BV, Macedo RBV, do Nascimento CV, Branco AS, et al. Fibroblast-Like Synoviocytes From Fluid and Synovial Membrane From Primary Osteoarthritis Demonstrate Similar Production of Interleukin 6, and Metalloproteinases 1 and 3. *Clin Exp Rheumatol* (2018) 37:306–09.
7. Zhao Y, Wang CL, Li RM, Hui TQ, Su YY, Yuan Q, et al. Wnt5a Promotes Inflammatory Responses *via* Nuclear Factor kappaB (NF-KappaB) and Mitogen-Activated Protein Kinase (MAPK) Pathways in Human Dental Pulp Cells. *J Biol Chem* (2014) 289:21028–39. doi: 10.1074/jbc.A113.546523
8. Freese JL, Pino D, Pleasure SJ. Wnt Signaling in Development and Disease. *Neurobiol Dis* (2010) 38:148–53. doi: 10.1016/j.nbd.2009.09.003
9. Sen M, Chamorro M, Reifert J, Corr M, Carson DA. Blockade of Wnt-5A/frizzled 5 Signaling Inhibits Rheumatoid Synoviocyte Activation. *Arthritis Rheumatol* (2001) 44:772–81. doi: 10.1002/1529-0131(200104)44:4<772::AID-ANR133>3.0.CO;2-L
10. Sato A, Yamamoto H, Sakane H, Koyama H, Kikuchi A. Wnt5a Regulates Distinct Signalling Pathways by Binding to Frizzled2. *EMBO J* (2010) 29:41–4. doi: 10.1038/emboj.2009.322
11. Nusse R, Clevers H. Wnt/Beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* (2017) 169:985–99. doi: 10.1016/j.cell.2017.05.016
12. Zarkou V, Galaras A, Giakountis A, Hatzis P. Crosstalk Mechanisms Between the WNT Signaling Pathway and Long Non-Coding RNAs. *Noncoding RNA Res* (2018) 3:42–53. doi: 10.1016/j.ncrna.2018.04.001
13. Rauner M, Stein N, Winzer M, Goettsch C, Zwerina J, Schett G, et al. WNT5A Is Induced by Inflammatory Mediators in Bone Marrow Stromal Cells and Regulates Cytokine and Chemokine Production. *J Bone Miner Res* (2012) 27:575–85. doi: 10.1002/jbmr.1488
14. Sen M. Wnt Signalling in Rheumatoid Arthritis. *Rheumatol (Oxford)* (2005) 44:708–13. doi: 10.1093/rheumatology/keh553
15. Ouchi N, Higuchi A, Ohashi K, Oshima Y, Gokce N, Shibata R, et al. Sfrp5 is an Anti-Inflammatory Adipokine That Modulates Metabolic Dysfunction in Obesity. *Science* (2010) 329:454–57. doi: 10.1126/science.1188280
16. Nanbara H, Wara-aswapati N, Nagasawa T, Yoshida Y, Yashiro R, Bando Y, et al. Modulation of Wnt5a Expression by Periodontopathic Bacteria. *PloS One* (2012) 7:e34434. doi: 10.1371/journal.pone.0034434
17. Zhan T, Rindtorff N, Boutros M. Wnt Signaling in Cancer. *Oncogene* (2017) 36:1461–73. doi: 10.1038/onc.2016.304
18. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Ann Rheum Dis* (2010) 69:1580–88. doi: 10.1136/ard.2010.138461
19. Pilling D, Tucker NM, Gomer RH. Aggregated IgG Inhibits the Differentiation of Human Fibrocytes. *J Leukoc Biol* (2006) 79:1242–51. doi: 10.1189/jlb.0805456

20. Pilling D, Cox N, Vakil V, Verbeek JS, Gomer RH. The Long Pentraxin PTX3 Promotes Fibrocyte Differentiation. *PloS One* (2015) 10:e0119709. doi: 10.1371/journal.pone.0119709
21. Galligan CL, Keystone EC, Fish EN. Fibrocyte and T Cell Interactions Promote Disease Pathogenesis in Rheumatoid Arthritis. *J Autoimmun* (2016) 69:38–50. doi: 10.1016/j.jaut.2016.02.008
22. Elhaj Mahmoud D, Sassi N, Drissi G, Barsaoui M, Zitouna K, Sahli H, et al. sFRP3 and DKK1 Regulate Fibroblast-Like Synoviocytes Markers and Wnt Elements Expression Depending on Cellular Context. *Immunol Invest* (2017) 46:314–28. doi: 10.1080/08820139.2016.1267204
23. Mii Y, Taira M. Secreted Frizzled-related Proteins Enhance the Diffusion of Wnt Ligands and Expand Their Signalling Range. *Development* (2009) 136:4083–88. doi: 10.1242/dev.032524
24. Kwon YJ, Lee SW, Park YB, Lee SK, Park MC. Secreted Frizzled-Related Protein 5 Suppresses Inflammatory Response in Rheumatoid Arthritis Fibroblast-Like Synoviocytes Through Down-Regulation of C-Jun N-Terminal Kinase. *Rheumatology* (2014) 53:1704–11. doi: 10.1093/rheumatology/keu167
25. Mikels AJ, Nusse R. Purified Wnt5a Protein Activates or Inhibits Beta-Catenin-TCF Signaling Depending on Receptor Context. *PloS Biol* (2006) 4: e115. doi: 10.1371/journal.pbio.0040115
26. Brandt MM, van Dijk CGM, Chrifi I, Kool HM, Burgisser PE, Louzao-Martinez L, et al. Endothelial Loss of Fzd5 Stimulates PKC/Ets1-Mediated Transcription of Angpt2 and Flt1. *Angiogenesis* (2018) 21:805–21. doi: 10.1007/s10456-018-9625-6
27. Jung YS, Lee HY, Kim SD, Park JS, Kim JK, Suh PG, et al. Wnt5a Stimulates Chemotactic Migration and Chemokine Production in Human Neutrophils. *Exp Mol Med* (2013) 45:e27. doi: 10.1038/emm.2013.48
28. Kim J, Chang W, Jung Y, Song K, Lee I. Wnt5a Activates THP-1 Monocytic Cells *via* a Beta-Catenin-Independent Pathway Involving JNK and NF-KappaB Activation. *Cytokine* (2012) 60:242–48. doi: 10.1016/j.cyto.2012.06.013
29. Kim J, Kim J, Kim DW, Ha Y, Ihm MH, Kim H, et al. Wnt5a Induces Endothelial Inflammation *via* Beta-Catenin-Independent Signaling. *J Immunol* (2010) 185:1274–82. doi: 10.4049/jimmunol.1000181
30. Suzuki M, Tetsuka T, Yoshida S, Watanabe N, Kobayashi M, Matsui N, et al. The Role of p38 Mitogen-Activated Protein Kinase in IL-6 and IL-8 Production From the TNF-Alpha- or IL-1Beta-Stimulated Rheumatoid Synovial Fibroblasts. *FEBS Lett* (2000) 465:23–7. doi: 10.1016/s0014-5793(99)01717-2
31. Yu CH, Nguyen TT, Irvine KM, Sweet MJ, Frazer IH, Blumenthal A. Recombinant Wnt3a and Wnt5a Elicit Macrophage Cytokine Production and Tolerization to Microbial Stimulation *via* Toll-like Receptor 4. *Eur J Immunol* (2014) 44:1480–90. doi: 10.1002/eji.201343959
32. Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ, Yang Y. Wnt-5a Inhibits the Canonical Wnt Pathway by Promoting GSK-3-Independent Beta-Catenin Degradation. *J Cell Biol* (2003) 162:899–908. doi: 10.1083/jcb.200303158
33. Boucher P, Matz RL, Terrand J. Atherosclerosis: Gone With the Wnt? *Atherosclerosis* (2020) 301:15–22. doi: 10.1016/j.atherosclerosis.2020.03.024
34. Grainger S, Willert K. Mechanisms of Wnt Signaling and Control. *Wiley Interdiscip Rev Syst Biol Med* (2018) 10:e1422. doi: 10.1002/wsbm.1422
35. Cruciat CM, Niehrs C. Secreted and Transmembrane Wnt Inhibitors and Activators. *Cold Spring Harb Perspect Biol* (2013) 5:a015081. doi: 10.1101/cshperspect.a015081
36. Xavier CP, Melikova M, Chuman Y, Üren A, Baljinnyam B, Rubin JS. Secreted Frizzled-Related Protein Potentiation Versus Inhibition of Wnt3a/β-Catenin Signaling. *Cell Signal* (2014) 26:94–101. doi: 10.1016/j.cellsig.2013.09.016

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CASE REPORT
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Case Report: Allergic Bronchopulmonary Aspergillosis Revealing Asthma

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Allergic bronchopulmonary aspergillosis (ABPA) is an immunological pulmonary disorder caused by hypersensitivity to *Aspergillus* which colonizes the airways of patients with asthma and cystic fibrosis. Its diagnosis could be difficult in some cases due to atypical presentations especially when there is no medical history of asthma. Treatment of ABPA is frequently associated to side effects but cumulated drug toxicity due to different molecules is rarely reported. An accurate choice among the different available molecules and effective on ABPA is crucial. We report a case of ABPA in a woman without a known history of asthma. She presented an acute bronchitis with wheezing dyspnea leading to an acute respiratory failure. She was hospitalized in the intensive care unit. The bronchoscopy revealed a complete obstruction of the left primary bronchus by a sticky greenish material. The culture of this material isolated *Aspergillus fumigatus* and that of bronchial aspiration fluid isolated *Pseudomonas aeruginosa*. The diagnosis of ABPA was based on elevated eosinophil count, the presence of specific IgE and IgG against *Aspergillus fumigatus* and left segmental collapse on chest computed tomography. The patient received an inhaled treatment for her asthma and a high dose of oral corticosteroids for ABPA. Her symptoms improved but during the decrease of corticosteroids, the patient presented a relapse. She received itraconazole in addition to corticosteroids. Four months later, she presented a drug-induced hepatitis due to itraconazole which was immediately stopped. During the monitoring of her asthma which was partially controlled, the patient presented an aseptic osteonecrosis of both femoral heads that required surgery. Nine months after itraconazole discontinuation, she presented a second relapse of her ABPA. She received voriconazole for nine months associated with a low dose of systemic corticosteroid therapy with an improvement of her symptoms. After discontinuation of antifungal treatment, there was no relapse for one year follow-up.

Keywords: allergic bronchopulmonary aspergillosis, *Aspergillus fumigatus*, antifungal therapy, drug toxicity, uncontrolled asthma

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INTRODUCTION

Fungal pulmonary infections are rare in immunocompetent patients. These infections can be caused by several pathogens such as *Aspergillus*, *Pneumocytis jirovecii* and *Cryptococcus*. However, even immunocompetent patients can be affected by these pathogens. In fact, they can affect patients with chronic bronchopulmonary pathologies such as asthma, cystic fibrosis and chronic obstructive pulmonary disease (1). The most frequent pathogen associated with fungal pulmonary infections is *Aspergillus*, which is a saprophytic mold isolated abundantly from soil, construction sites and hospitals (1, 2). There are three clinical presentations of pulmonary aspergillosis: chronic pulmonary aspergillosis (CPA), invasive pulmonary aspergillosis (IPA) and allergic bronchopulmonary aspergillosis (ABPA) (3). Depending on the interaction between the pathogen and its host, pulmonary aspergillosis can lead to one of these clinical presentations (4). The estimated worldwide global rate of ABPA among asthmatic adult patients is 2,5% (5, 6), however only some cases are reported in Tunisia (7). ABPA is most often associated with severe uncontrolled asthma (5, 6) and drug toxicity is frequently reported during therapeutic management. We report a case of ABPA in a patient with a medical history of an allergic rhinitis, with no respiratory symptoms and who developed drug toxicity to corticosteroids then to itraconazole. Thus, we emphasize the challenges in diagnosing and treating ABPA due to its atypical clinical presentations and significant drug toxicity associated with its therapeutic management.

CASE PRESENTATION

A 72-year-old woman, non-smoker, consulted in December 2017, an office-based pulmonologist for acute bronchitis with wheezing dyspnea. In her medical history she reported an allergic rhinitis diagnosed in childhood without respiratory symptoms. She was sensitized to some pollen types which were not specified. She is of French origin and living in Tunisia for thirty years. She had no respiratory nor rhinitis symptoms since she came in Tunisia and until she developed a bronchitis in December 2017. This bronchitis was resistant to symptomatic treatment and short-term systemic corticosteroid therapy. The patient was hospitalized in an intensive care unit for an acute respiratory failure due to her bronchitis. A chest X-ray (face and profile, **Figure 1**) showed a left hilo-axillary linear opacity with retraction signs evoking atelectasis. Her blood tests didn't show a biological inflammatory syndrome (CRP= 11mg/l; white blood cells= 9780/mm^3 with an eosinophilic count= 939/mm^3). Chest computed tomography (CT) confirmed the diagnosis of atelectasis and showed segmental collapse of the lingula and posterior segment of the left basal pyramid with no parenchymatous lesion (**Figure 2**). Flexible bronchoscopy revealed a complete obstruction of the left primary bronchus by a sticky greenish material that could be removed (**Figure 3**). Bacterial culture of this material isolated *Pseudomonas aeruginosa* and the patient received consequently an antibiotherapy associating Levofloxacin and Cefpodoxime for two weeks, with partial improvement in respiratory symptoms. The mycological culture isolated *Aspergillus fumigatus*. Aspergillus serology (IgG) was positive at 12 AU/mL (ELISA, Biorad®). Total IgE count was 233 IU/ml (ELFA, Biomérieux®). *Aspergillus fumigatus* specific IgE and *Aspergillus* skin testing were not done. During pulmonary function test, the forced vital capacity (FVC) was at 1,97 l (82%) and forced expiratory volume in one second (FEV1) was at 1,60 l (81%). The diagnosis of ABPA associated to an asthma was established. The patient received a high-dose regimen of oral corticosteroids for five weeks (1 mg/kg/day: 80mg of prednisone for one week then

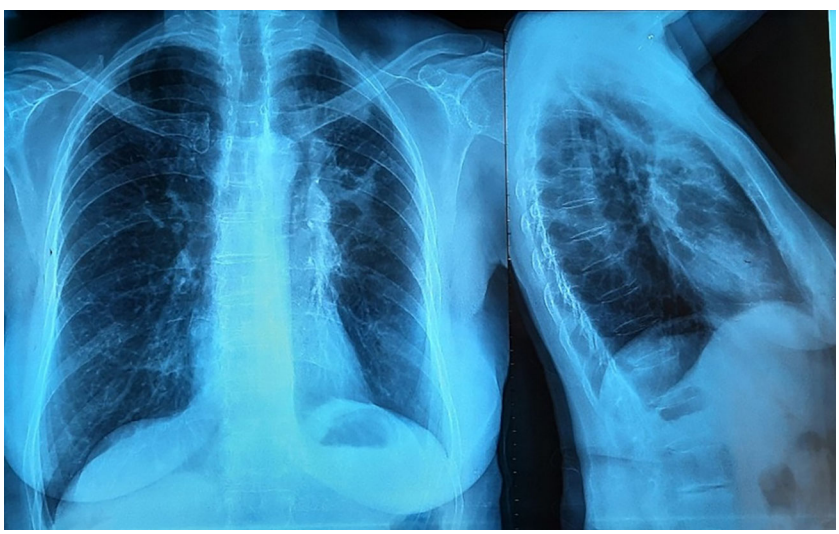

FIGURE 1 | Left hilo-axillary linear opacity associated with retraction signs evoking atelectasis on chest-ray face and profile.

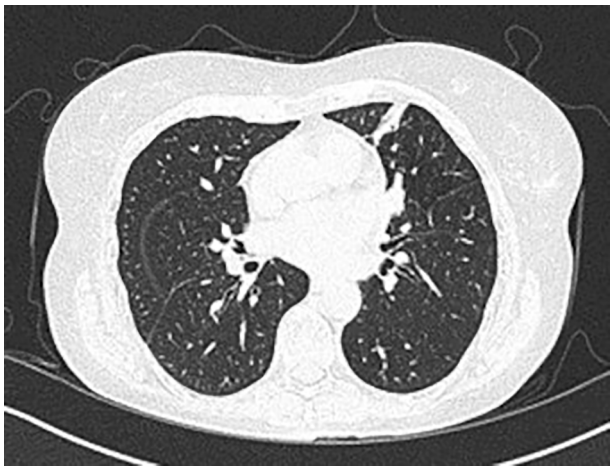

FIGURE 2 | Chest CT scan image showing segmental aerated collapse of the lingula.

40mg for one week then 20mg for one week then 10mg for one week then 5mg for one week) and an inhaled association of long-acting bronchodilator and a high dose of corticosteroids (salmeterol and fluticasone). When searching for mold exposure in the patient's medical history, we discovered her gardening activities and that she had multiple indoor plants at her house. In addition to medical treatment, it was recommended that the patient should remove all the indoor plants. The evolution has been marked by the disappearance of respiratory symptoms and of the atelectasis on chest x-rays and the decrease in the eosinophils' count (282/mm3). However, during the decrease of corticosteroid therapy dosage, she presented a relapse of her respiratory symptoms at the dose of 5mg of prednisone per day. She consulted in March in our department. The itraconazole was prescribed in association with corticosteroid therapy (medium-dose regimen: 0,5 mg/kg/day; 40mg of prednisone for one month then 30mg for one month then 25mg for two weeks then 20mg for two weeks then 15mg for two weeks, then 10mg for two weeks). After four months of anti-fungal treatment, the patient developed a jaundice with an intense deterioration of her general condition. Liver biological tests showed significant hepatic cytolysis (AST= 485 IU/L, ALT= 182 IU/L) and cholestasis (Gamma-GT= 933 IU/L, Alkaline phosphatse= 758 IU/L). After exclusion of the intake of any other treatment associated to drug liver toxicity, the diagnosis of an acute liver failure due to a drug-induced hepatitis associated to the anti-fungal treatment was established. As a result, the itraconazole was immediately stopped. At that time, the titer of *Aspergillus fumigatus* specific IgE was 0.59 KUA/L (FEIA, Thermaoscientific/Phadia®), hence the decision to continue inhaled asthma treatment and to continue monitoring the patient. For the next seven months, the patient's asthma was partially controlled. The patient developed a right hip joint pain and a lameness. The various explorations concluded to an aseptic osteonecrosis of both femoral heads. The patient underwent surgery for her right hip. In May 2019, the patient presented acute bronchitis with mucus produced by coughs. She had persistent respiratory symptoms which were resistant to antibiotherapy and short-term systemic corticosteroid therapy. Chest X-ray showed an atelectasis in the left lower lobe. Flexible bronchoscopy revealed a mucus plug at the left lower lobar bronchus. The middle lobar bronchus had a reduced caliber and was non-catheterizable. Chest CT revealed alveolar opacities associated with bronchiectasis in the posterior and medial segment of the right basal pyramid and lateral segment of the middle lobe (**Figure 4**). During bronchoscopy, the plug has been

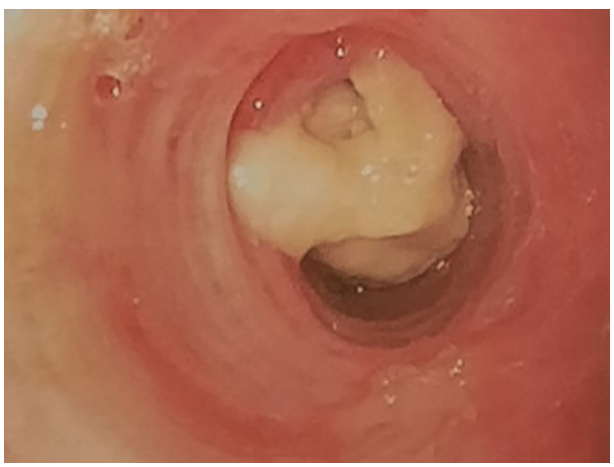

FIGURE 3 | Complete obstruction of the left strain bronchus by sticky greenish material in flexible bronchoscopy.

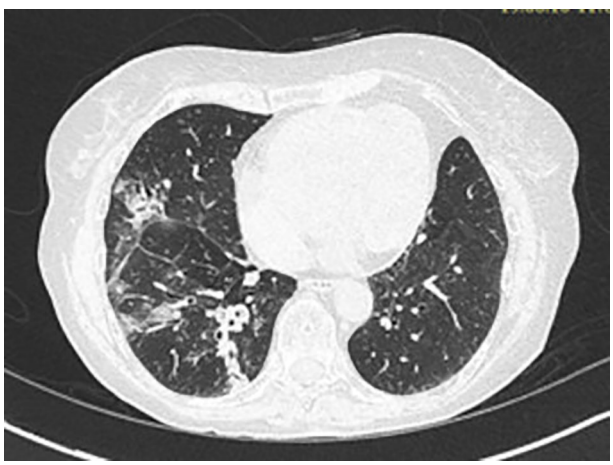

FIGURE 4 | Chest CT scan image showing alveolar opacities associated to bronchiectasis in posterior and medial segment of the right basal pyramid.

removed and cultures done. Bacterial culture of this material was negative. The mycological culture isolated *Aspergillus fumigatus*. In vitro susceptibility testing against voriconazole was performed using E-test (Biomérieux®). This strain was susceptible to Voriconazole (MIC: 0.38µg/mL). Total IgE count was at 301 IU/ml, eosinophilic count was at 159/mm^3. The titer of *Aspergillus fumigatus* specific IgE was at 13.90 KUA/L. Voriconazole was prescribed in addition to a step up in the patient's asthma treatment. The patient received antifungal treatment for nine months in addition to a low dose of systemic corticosteroid therapy (prednisone 10mg/day) and an inhaled long-acting anticholinergic (tiotropium) in association to montelukast and an inhaled association of long-acting bronchodilator and a high dose of corticosteroids. The *Aspergillus fumigatus* specific IgE decreased to 2 KUA/L. The pulmonary function test results improved [FVC=2,38 l (90%) and FEV1 = 1,98 l (96%)]. After discontinuation of antifungal treatment, there was no relapse for one year follow-up.

DISCUSSION

ABPA is caused by hypersensitivity to *Aspergillus fumigatus* (8). It is frequently associated with severe and uncontrolled asthma or cystic fibrosis. The small conidia of *Aspergillus fumigatus* can easily enter the airways. Exposure to large numbers of conidia may cause ABPA (9, 10), but not all asthmatics develop ABPA despite being exposed to the same environmental factors. This means that other factors play a role in the pathogenesis of ABPA (9). In a genetically predisposed individuals, inhaled conidia of *Aspergillus fumigatus* germinate into hyphae with release antigens that activate the innate and adaptive immune responses (Th2 CD4+ T cell responses) of the lung (9, 10).

When treating ABPA, exposure to molds in the patient's environment must be investigated to indicate remediation. This is important to prevent relapse after discontinuation of treatment, which was the case for our patient. The first publication about ABPA as an entity was in 1952, from the United Kingdom (11). A decade later, the second case of ABPA was described in the United States (12, 13). The diagnosis of this disease is still difficult, particularly during atypical clinical presentations which was the case of our patient, who had an allergic rhinitis and no prior asthma history or diagnosis. In fact, up to one third of patients with controlled asthma had a relatively asymptomatic ABPA, and the diagnosis is discovered during routine testing (14). Moreover, some clinical presentations are confusing such as the case reported by Savi and al. and where the diagnosis of ABPA was established in a previously healthy male (15). Also, in a nationwide Japan survey, 19% of patients diagnosed with ABPA had no medical history of asthma (16). ABPA is due to an inflammatory pulmonary disorder which often causes non-specific symptoms such as chronic cough, wheezing and recurrent pulmonary infiltration (8). It may be associated to other symptoms such as fever, weight loss, deterioration of general condition, hemoptysis, chest pain and night sweats (17). Expectoration of yellowish-green lumps of mucus is characteristic of ABPA and can be observed in half of the cases (8) which was the case of our patient. The chest X-ray may be normal in the early stages of the disease and can reveal some abnormalities such as "Tramline shadows" and "finger-in-glove opacities" which are temporary patterns corresponding to bronchial wall edema and thickening; "Toothpaste shadows" which are also transient and indicate mucus plugs within bronchi; "parallel line shadows" which appear when the mucus plugs are expectorated (18, 19). In the case of our patient, the chest X-ray showed a linear opacity due to an atelectasis during the first consultation and during the relapse. The chest CT confirmed the diagnosis of segmental collapse during the first consultation and showed bronchiectasis during the relapse. Flexible bronchoscopy has an important place particularly in patients with atelectasis to rule out malignant etiologies. It also allows bacteriological and mycological samples. Sputum or bronchial fluid cultures are positive for *Aspergillus* in nearly 40–60% of cases (20, 21). The presence of *Aspergillus fumigatus* in the sputum culture is not sufficient to confirm the diagnosis of ABPA as this fungus is human saprophyte and can be present in other pulmonary diseases (9, 10). Sputum cultures can contribute to the diagnosis by isolating *Aspergillus* and by performing *in vitro* antifungal-susceptibility testing and molecular testing for resistance, of the isolated strains (9, 10, 22). In a study involving 13 countries from four continents, 6% of the 2026 isolates of *A. fumigatus* were triazole resistant using molecular tools (23). This resistance prevalence varies in its geographic distribution. For example, in a French study, the prevalence of *Aspergillus fumigatus* azole resistance in patients with cystic fibrosis was detected in 6.8% of cases (24). However, in an English study, the azole resistance prevalence was higher. Resistance to at least one azole antifungal drug was confirmed in 13.2% of included patients among whom 16.2% had cystic fibrosis (TR_{34}/L98H was identified in 27.3% of azole-resistant isolates) (25). Furthermore, the association of *Pseudomonas aeruginosa* and *Aspergillus fumigatus* has been reported in the literature. Both microbes are responsible for considerable morbidity and mortality particularly in patients with cystic fibrosis, among whom the co-infection accelerates the lung disease progression (26). In fact, metabolite exchange and intermicrobial competitions between both germs have been studied to better explain the important morbidity and mortality due to their association (27).

When the diagnosis of ABPA is suspected, some biological investigations are used for the diagnosis and monitoring of ABPA. The relevant tests are eosinophil count, total serum IgE level, serum IgE antibodies specific to *Aspergillus fumigatus* and serum precipitins or specific IgG against *Aspergillus fumigatus* (9, 14). First, blood eosinophil count should be checked and a level over 500 cell/L can help to establish the diagnosis. Our patient had an elevated eosinophilic count (939/mm3). However, high eosinophil counts can be detected in many other diseases and normal levels are reported in patients with ABPA receiving corticosteroids (14). It is known that the pulmonary eosinophilia is far greater than in peripheral blood; thus, a low eosinophil count does not exclude

ABPA (9, 28). The measurement of the serum total IgE level is an accurate and important test for the diagnosis and the follow-up of ABPA (10). Active ABPA is generally excluded when serum IgE is normal (9, 10). For the cut-off value of IgE level that should be used in the diagnosis of ABPA, there is no consensus, and it remains uncertain (9). In addition, the reported IgE values in different units (IU/mL, ng/mL) could lead to false interpretation (9). Some laboratories employ 417 IU/mL as a cut-off value, while others use a value of 1000 IU/mL (29). So, a validation of the IgE cutoff value across all populations is required since it could be influenced by both risk of exposure to *Aspergillus* antigens and ethnicity (9, 18). Despite this, the most sensitive investigation in the diagnosis of ABPA is currently the detection of high levels of serum IgE antibodies specific to *Aspergillus fumigatus* (>0.35 kUA/l). This test is also considered the preferred one for screening asthmatic patients for ABPA (6, 9, 10). When our patient had the first relapse, the serum specific IgE were high with a value of 13.9 KUA/L. Although the detection of IgE antibodies specific to *Aspergillus fumigatus* is useful for the diagnosis, it is less helpful in the follow up of patients (10). In addition, serum precipitins or specific IgG against *Aspergillus fumigatus* are detected in 69–90% of cases of ABPA (9, 30). In our case, the patient had positive specific IgG, but the technics used are not equal. In fact, double gel diffusion technique for the detection of *Aspergillus fumigatus*-specific IgG has a limited sensitivity (27%) in the diagnosis of ABPA, whereas, commercial enzyme immunoassays have a sensitivity exceeding 90% (10, 30).

The diagnosis of ABPA is confirmed when the case presentation meets the criteria established in 2013 by the ABPA Working Group of the International Society for Human and Animal Mycology. If total IgE level are over 1000 IU/mL, two among three criteria are sufficient for establishing the diagnosis of ABPA: positive serum precipitins/*Aspergillus fumigatus* IgG, eosinophil count >500 cell/L, chest CT consistent with ABPA (mucus impaction, tree-in-bud pattern, centrilobular nodules, mosaic attenuation (31); high attenuation mucus, pathognomonic for ABPA (9, 32); segmental, lobar and total lung collapse due to mucus plugs (33–35); central or peripheral bronchiectasis). Patients with a total IgE levels under 1000 IU/mL, who, otherwise, meet all the remaining criteria are also diagnosed with ABPA (9). This is the case for our patient who had a total IgE level under 1000 IU/mL. In the different cases reported in Tunisian patients, total IgE level was also under 1000 IU/mL (7). Patients with uncontrolled asthma and positive skin prick test to *Aspergillus* or IgE sensitization to *Aspergillus* and who have a total IgE levels under 1000 IU/mL, without meeting all the other criteria may be diagnosed with severe asthma with fungal sensitization. A normal total IgE level or a negative screening test in a glucocorticoid-naïve patient potentially excludes the diagnosis of ABPA (9).

Different therapeutics have shown their efficiency in the treatment of ABPA. Glucocorticoids are the first molecules to be used. A randomized trial showed that the medium-dose regimen and high-dose regimen are both effective against ABPA with less side effects for the medium-dose regimen (36). In the medium-dose regimen, prednisolone is prescribed in monotherapy for a total duration of three to five months (0.5 mg/kg/day for two weeks, then on alternate days for eight weeks, then 5 mg less every two weeks) (37). When a patient is on glucocorticoids and still has recurrent exacerbations or worsening pulmonary function test or become glucocorticoid-dependent, antifungal therapy could be added (37, 38). In our case, the patient presented a severe complication of glucocorticoid treatment which is aseptic osteonecrosis of both femoral heads requiring surgery. Itraconazole is usually used with or without glucocorticoids for at least six months, at a dose of 200 mg twice a day (38). It requires frequent liver enzymes level monitoring because of its toxicity (38). In fact, itraconazole can cause liver toxicity which was the case of our patient. Other oral azoles such as voriconzaole and posaconazole are also effective in ABPA and can be used when itraconzaole is toxic or contraindicated (39). However, when there is a drug toxicity due to one molecule of azole, there is a risk of a cross-azole toxicity. So alternative approaches to antifungal treatment, in ABPA, that avoid systemic effects were tested and inhaled amphotericin B has been explored with varying results in uncontrolled studies (40, 41). In our case, inhaled amphotericin B was not available and voriconazole was used without a cross-azole toxicity. It led to remission without relapse after discontinuation of antifungal therapy. Furthermore, omalizumab has also proven its efficacy in ABPA, compared to long-term glucocorticoids and it can be administered even in cases with high level of IgE (42). In the case of acute lung collapse, broncho-alveolar lavage during rigid or flexible bronchoscopy helps the lung re-expansion and significant improvement of ABPA symptoms (33, 43). For patients with thick sputum, chest physiotherapy and nebulized hypertonic saline solution improve the symptoms (44, 45). Patients should be examined every two months with chest radiography and total serum IgE levels until remission (9). Exacerbation is confirmed when the baseline total IgE levels doubles with clinical or radiological deterioration (9). Response to therapy is defined by a minimum of 25% decrease in total IgE levels with clinical and radiological improvement and remission is confirmed when the patient has no exacerbations for at least six months after stopping all therapeutics (9). However, it has not been demonstrated that there are benefits of treating ABPA diagnosed on routine investigation in asymptomatic patients with well controlled asthma. Long-term prognosis of patients with ABPA is still not clear (46). But early detection of the disease and prescription of treatments lead to a good prognosis (47). Untreated patients progress to irreversible lung fibrosis and respiratory failure (48).

CONCLUSION

We report a case of ABPA occurring in a woman with a prior history of atopic rhinitis but without known history of asthma. She was exposed to a high indoor and outdoor fungal load. We emphasize the importance of an early diagnosis in order to prevent long-term morbidity associated with the irreversible changes that occur with untreated ABPA. This case highlights

the challenges of establishing the diagnosis of ABPA and especially the challenges faced during its therapeutic management due to glucocorticoids' and triazoles' significant side effects and drug toxicity. Management of ABPA must include mandatory *Aspergillus* exposure remediation to prevent relapse after discontinuation of treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Diagnostic and therapeutic management: HS, HB, NS, NM, and BL. Immunology and mycology investigation: AK, SJ, SM, SB, and KK. Writing, review and editing: all authors. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Kousha M, Tadi R, Soubani AO. Pulmonary Aspergillosis: A Clinical Review. *Eur Respir Rev* (2011) 20(121):156–74. doi: 10.1183/09059180.00001011
2. Hansen D, Blahout B, Benner D, Popp W. Environmental Sampling of Particulate Matter and Fungal Spores During Demolition of a Building on a Hospital Area. *J Hosp Infect* (2008) 70(3):259–64. doi: 10.1016/j.jhin.2008.07.010
3. Kanj A, Abdallah N, Soubani AO. The Spectrum of Pulmonary Aspergillosis. *Respir Med* (2018) 141:121–31. doi: 10.1016/j.rmed.2018.06.029
4. Kosmidis C, Denning DW. The Clinical Spectrum of Pulmonary Aspergillosis. *Thorax* (2015) 70(3):270–7. doi: 10.1136/thoraxjnl-2014-206291
5. Denning DW, Pleuvry A, Cole DC. Global Burden of Allergic Bronchopulmonary Aspergillosis With Asthma and its Complication Chronic Pulmonary Aspergillosis in Adults. *Med Mycol* (2013) 51(4):361–70. doi: 10.3109/13693786.2012.738312
6. Agarwal R, Aggarwal AN, Gupta D, Jindal SK. Aspergillus Hypersensitivity and Allergic Bronchopulmonary Aspergillosis in Patients With Bronchial Asthma: Systematic Review and Meta-Analysis. *Int J Tuberc Lung Dis* (2009) 13(8):936–44.
7. Fekih L, Boussoffara L, Ben Abdelghaffar H, Fenniche S, Akrout I, Hassene H, et al. L'aspergillose Broncho-Pulmonaire Allergique. A propos de 3 cas. *Tunis Med* (2011) 89(5):491–6.
8. Agarwal R. Allergic Bronchopulmonary Aspergillosis. *Chest* (2009) 135 (3):805–26. doi: 10.1378/chest.08-2586
9. Agarwal R, Chakrabarti A, Shah A, Gupta D, Meis JF, Guleria R, et al. Allergic Bronchopulmonary Aspergillosis: Review of Literature and Proposal of New Diagnostic and Classification Criteria. *Clin Exp Allergy* (2013) 43(8):850–73. doi: 10.1111/cea.12141
10. Agarwal R, Sehgal IS, Dhooria S, Muthu V, Prasad KT, Bal A, et al. Allergic Bronchopulmonary Aspergillosis. *Indian J Med Res* (2020) 151(6):529–59. doi: 10.4103/ijmr.IJMR_1187_19
11. Hinson K, Moon A, Plummer N. Broncho-Pulmonary Aspergillosis. *Thorax* (1952) 7:317–33. doi: 10.1136/thx.7.4.317
12. Patterson R, Golbert TM. Hypersensitivity Disease of the Lung. *Univ Mich Med Cent J* (1968) 34:8–11.
13. Slavin RG, Stanczyk DJ, Lonigro AJ, Broun GO. Allergic Bronchopulmonary Aspergillosis-A North American Rarity. Clinical and Immunologic Characteristics. *Am J Med* (1969) 47(2):306–13. doi: 10.1016/0002-9343(69)90156-9
14. Shah A, Panjabi C. Allergic Aspergillosis of the Respiratory Tract. *Eur Respir Rev* (2014) 23(131):8–29. doi: 10.1183/09059180.00007413
15. Savi D, Valente G, Iacovelli A, Olmati F, Bezzi M, Palange P. Uncommon Presentation of Allergic Bronchopulmonary Aspergillosis During the COVID-19 Lockdown: A Case Report. *BMC Pulm Med* (2020) 20:325. doi: 10.1186/s12890-020-01373-7
16. Oguma T, Taniguchi M, Shimoda T, Kamei K, Matsuse H, Hebisawa A, et al. Allergic Bronchopulmonary Aspergillosis in Japan: A Nationwide Survey. *Allergol Int* (2018) 67:79–84. doi: 10.1016/j.alit.2017.04.011
17. Zhang M, Gao J. Clinical Analysis of 77 Patients With Allergic Bronchopulmonary Aspergillosis in Peking Union Medical College Hospital. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* (2017) 39(3):352–7. doi: 10.3881/j.issn.1000-503X.2017.03.009
18. Shah A, Panjabi C. Allergic Bronchopulmonary Aspergillosis: A Perplexing Clinical Entity. *Allergy Asthma Immunol Res* (2016) 8(4):282–97. doi: 10.4168/aair.2016.8.4.282
19. Agarwal R, Khan A, Garg M, Aggarwal AN, Gupta D. Pictorial Essay: Allergic Bronchopulmonary Aspergillosis. *Indian J Radiol Imag* (2011) 21(4):242–52. doi: 10.4103/0971-3026.90680
20. Agarwal R, Khan A, Aggarwal AN, Varma N, Garg M, Saikia B, et al. Clinical Relevance of Peripheral Blood Eosinophil Count in Allergic Bronchopulmonary Aspergillosis. *J Infect Public Health* (2011) 4(5–6):235–43. doi: 10.1016/j.jiph.2011.08.006
21. Tashiro T, Izumikawa K, Tashiro M, Takazono T, Morinaga Y, Yamamoto K, et al. Diagnostic Significance of Aspergillus Species Isolated From Respiratory Samples in an Adult Pneumology Ward. *Med Mycol* (2011) 49(6):581–7. doi: 10.3109/13693786.2010.548084
22. Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore M, et al. High-Frequency Triazole Resistance Found in Nonculturable Aspergillus Fumigatus From Lungs of Patients With Chronic Fungal Disease. *Clin Infect Dis* (2011) 52:1123–9. doi: 10.1093/cid/cir179
23. Ashu EE, Hagen F, Chowdhary A, Meis JF, Xu J. Global Population Genetic Analysis of. *mSphere* (2017) 2(1):e00019–17. doi: 10.1128/mSphere.00019-17
24. Lavergne R-A, Morio F, Danner-Boucher I, Horeau-Langlard D, David V, Hagen F, et al. One-Year Prospective Survey of Azole Resistance in Aspergillus Fumigatus at a French Cystic Fibrosis Reference Centre: Prevalence and Mechanisms of Resistance. *J Antimicrob Chemother* (2019) 74(7):1884–9. doi: 10.1093/jac/dkz144
25. Abdolrasouli A, Scourfield A, Rhodes J, Shah A, Elborn JS, Fisher MC, et al. High Prevalence of Triazole Resistance in Clinical Aspergillus Fumigatus Isolates in a Specialist Cardiothoracic Centre. *Int J Antimicrob Agents* (2018) 52(5):637–42. doi: 10.1016/j.ijantimicag.2018.08.004
26. Amin R, Dupuis A, Aaron SD, Ratjen F. The Effect of Chronic Infection With Aspergillus Fumigatus on Lung Function and Hospitalization in Patients With Cystic Fibrosis. *Chest* (2010) 137:171–6. doi: 10.1378/chest.09-1103
27. Chatterjee P, Sass G, Swietnicki W and Stevens DA. Review of Potential Pseudomonas Weaponry, Relevant to the Pseudomonas–Aspergillus Interplay, for the Mycology Community. *J Fungi* (2020) 6:81. doi: 10.3390/jof6020081
28. Wark PA, Saltos N, Simpson J, Slater S, Hensley MJ, Gibson PG. Induced Sputum Eosinophils and Neutrophils and Bronchiectasis Severity in Allergic Bronchopulmonary Aspergillosis. *Eur Respir J* (2000) 16(6):1095–101. doi: 10.1034/j.1399-3003.2000.16f13.x
29. Mahdavinia M, Grammer LC. Management of Allergic Bronchopulmonary Aspergillosis: A Review and Update. *Ther Adv Respir Dis* (2012) 6(3):173–87. doi: 10.1177/1753465812443094
30. Agarwal R, Dua D, Choudhary H, Aggarwal AN, Sehgal IS, Dhooria S, et al. Role of Aspergillus Fumigatus-Specific IgG in Diagnosis and Monitoring

Treatment Response in Allergic Bronchopulmonary Aspergillosis. *Mycoses* (2017) 60(1):33–9. doi: 10.1111/myc.12541
31. Kaur M, Sudan DS. Allergic Bronchopulmonary Aspergillosis (ABPA)-The High-Resolution Computed Tomography (HRCT) Chest Imaging Scenario. *J Clin Diagn Res* (2014) 8(6):RC05–7. doi: 10.7860/JCDR/2014/8255.4423
32. Phuyal S, Garg MK, Agarwal R, Gupta P, Chakrabarti A, Sandhu MS, et al. High-Attenuation Mucus Impaction in Patients With Allergic Bronchopulmonary Aspergillosis: Objective Criteria on High-Resolution Computed Tomography and Correlation With Serologic Parameters. *Curr Probl Diagn Radiol* (2016) 45(3):168–73. doi: 10.1067/j.cpradiol.2015.07.006
33. Kumar R, Poongadan MN, Singh M. Allergic Bronchopulmonary Aspergillosis Presenting as Lobar or Total Lung Collapse. *Pneumonol Alergol Pol* (2015) 83(2):144–50. doi: 10.5603/PiAP.2015.0023
34. Shah A, Behera S, Panjabi C. Middle Lobe Syndrome: A Rare Presentation of Allergic Bronchopulmonary Aspergillosis. *Eur Ann Allergy Clin Immunol* (2014) 46(4):147–51. doi: 10.1136/bcr-2016-214670
35. Ghosh K, Sanders BE. Allergic Bronchopulmonary Aspergillosis Causing Total Lung Collapse. *BMJ Case Rep* (2012) 2012:1–2. doi: 10.1136/bcr.12.2011.5349
36. Agarwal R, Aggarwal AN, Dhooria S, Sehgal IS, Garg M, Saikia B, et al. A Randomised Trial of Glucocorticoids in Acute-Stage Allergic Bronchopulmonary Aspergillosis Complicating Asthma. *Eur Respir J* (2016) 47(2):490–8. doi: 10.1183/13993003.01816-2015
37. Agarwal R, Sehgal IS, Dhooria S, Aggarwal AN. Developments in the Diagnosis and Treatment of Allergic Bronchopulmonary Aspergillosis. *Expert Rev Respir Med* (2016) 10(12):1317–34. doi: 10.1080/17476348.2016.1249853
38. Stevens DA, Schwartz HJ, Lee JY, Moskovitz BI, Jerome DC, Catanzaro C, et al. A Randomized Trial of Itraconazole in Allergic Bronchopulmonary Aspergillosis. *N Engl J Med* (2000) 342(11):756–62. doi: 10.1056/NEJM200003163421102
39. Chishimba L, Niven RM, Cooley J, Denning DW. Voriconazole and Posaconazole Improve Asthma Severity in Allergic Bronchopulmonary Aspergillosis and Severe Asthma With Fungal Sensitization. *J Asthma* (2012) 49(4):423–33. doi: 10.3109/02770903.2012.662568
40. Chishimba L, Langridge P, Powell G, M Niven R, W Denning D. Efficacy and Safety of Nebulised Amphotericin B (NAB) in Severe Asthma With Fungal Sensitisation (SAFS) and Allergic Bronchopulmonary Aspergillosis (ABPA). *J Asthma* (2015) 52:289–95. doi: 10.3109/02770903.2014.958853
41. Casciaro R, Naselli A, Cresta F, Ros M, Castagnola E, Minicucci L. Role of Nebulized Amphotericin B in the Management of Allergic Bronchopulmonary Aspergillosis in Cystic Fibrosis: Case Report and Review of Literature. *J Chemother* (2015) 27:307–11. doi: 10.1179/1973947814Y.0000000194
42. Voskamp AL, Gillman A, Symons K, Sandrini A, Rolland JM, O'Hehir RE, et al. Clinical Efficacy and Immunologic Effects of Omalizumab in Allergic Bronchopulmonary Aspergillosis. *J Allergy Clin Immunol Pract* (2015) 3 (2):192–9. doi: 10.1016/j.jaip.2014.12.008
43. Khalil KF. Therapeutic Bronchoalveolar Lavage With Conventional Treatment in Allergic Bronchopulmonary Aspergillosis. *J Coll Physicians Surg Pak* (2015) 25(5):359–62.
44. Kellett F, Redfern J, Niven RM. Evaluation of Nebulised Hypertonic Saline (7%) as an Adjunct to Physiotherapy in Patients With Stable Bronchiectasis. *Respir Med* (2005) 99(1):27–31. doi: 10.1016/j.rmed.2004.05.006
45. Murray MP, Pentland JL, Hill AT. A Randomised Crossover Trial of Chest Physiotherapy in non-Cystic Fibrosis Bronchiectasis. *Eur Respir J* (2009) 34 (5):1086–92. doi: 10.1183/09031936.00055509
46. Vlahakis NE, Aksamit TR. Diagnosis and Treatment of Allergic Bronchopulmonary Aspergillosis. *Mayo Clin Proc* (2001) 76(9):930–8. doi: 10.4065/76.9.930
47. Knutsen AP, Slavin RG. Allergic Bronchopulmonary Aspergillosis in Asthma and Cystic Fibrosis. *Clin Dev Immunol* (2011) 2011:843763. doi: 10.1155/2011/843763
48. Vaughan LM. Allergic Bronchopulmonary Aspergillosis. *Clin Pharm* (1993) 12(1):24–33.

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frontiers in Immunology

CASE REPORT
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Case Report: Interleukin-2 Receptor Common Gamma Chain Defect Presented as a Hyper-IgE Syndrome

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X-linked severe combined immunodeficiency (X-SCID) is caused by mutations of *IL2RG*, the gene encoding the interleukin common gamma chain (IL-2Rγ or γc) of cytokine receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Hypomorphic mutations of *IL2RG* may cause combined immunodeficiencies with atypical clinical and immunological presentations. Here, we report a clinical, immunological, and functional characterization of a missense mutation in exon 1 (c.115G>A; p. Asp39Asn) of *IL2RG* in a 7-year-old boy. The patient suffered from recurrent sinopulmonary infections and refractory eczema. His total lymphocyte counts have remained normal despite skewed T cell subsets, with a pronounced serum IgE elevation. Surface expression of IL-2Rγ was reduced on his lymphocytes. Signal transducer and activator of transcription (STAT) phosphorylation in response to IL-2, IL-4, and IL-7 showed a partially preserved receptor function. T-cell proliferation in response to mitogens and anti-CD3/anti-CD28 monoclonal antibodies was significantly reduced. Further analysis revealed a decreased percentage of $CD4^+$ T cells capable of secreting IFN-γ, but not IL-4 or IL-17. Studies on the functional consequences of IL-2Rγ variants are important to get more insight into the pathogenesis of atypical phenotypes which may lay the ground for novel therapeutic strategies.

Keywords: Interleukin-2 receptor gamma, combined immunodeficiency, hypomorphic mutations, hyper-IgE, inborn error of immunity

Abbreviations: FOXP3, forkhead box P3; HIES, Hyper-IgE syndrome; IFN, Interferon; IL, Interleukin; IL2RG, Interleukin 2 receptor gamma; JAK3, Janus kinase 3; γC, common gamma chain; NK, Natural Killer; PBMC, Peripheral Blood Mononuclear cells; PHA, phytohemagglutinin; PMA, Phorbol 12-myristate 13-acetate; STAT, Signal transducer and activator of transcription.; SCID, Severe combined immunodeficiency; Th, Helper T lymphocyte.

INTRODUCTION

X-linked severe combined immunodeficiency (X-SCID) is a life-threatening inborn error of immunity, accounts for approximately half of all cases of SCID (1–5). Most infants with SCID die within their first year of life in the absence of immune reconstitution *via* hematopoietic stem cell transplantation, due to severe and recurrent infections that begin in the first months of life, frequently associated with diarrhea and growth retardation. The infections may be viral, bacterial, and/or fungal, and vaccination with BCG may lead to disseminated infection (6).

X-SCID is caused by hemizygous pathogenic variants of the interleukin 2 receptor gamma (*IL2RG*) gene, organized in eight exons that encode the common γ chain, (IL-2Rγ or γc, also known as CD132), which is a part of the IL-2 high-affinity receptor and several interleukin receptors, including those for IL-4, IL-7, IL-9, IL-15 and IL-21 (7, 8). The γc protein is expressed on the surface of lymphoid, myeloid, and hematopoietic progenitor cells. The extracellular domain of the chain is encoded by exons 1–5, followed by a transmembrane domain encoded by exon 6, while the two last exons encode the intracellular portion which can cooperate with the Janus kinase family member 3 (JAK3) (9, 10), a signaling kinase that interacts with other JAK and STAT proteins in complex signal transduction through common gamma chain of cytokine receptor subfamily (11). These receptors' engagement is crucial to lymphocyte activation, proliferation, and function. Impaired signaling downstream of IL-7 (12) and IL-21 (13–17) explains, at least in part, the absence of T cells and impaired B-cell function, respectively, of X-SCID patients. Altogether, the absence of normal IL-4, IL-7, IL-9, IL-15, and IL-21 signaling leads to the almost complete absence of T and NK cells, with nonfunctional B cells observed in the typical $T^-B^+NK^-$ X-SCID patients carrying amorphic mutations of *IL2RG* (4, 6). On the other hand, atypical cases of X-SCID have been described in male patients carrying hypomorphic mutations of *IL2RG*, resulting in either a residual expression of γc protein with a weaker affinity for IL-2, or a weaker interaction of γc with the downstream kinase JAK3 leading to an impaired signaling (18–21).

The pathogenic genetic variations causing X-SCID are found throughout the *IL2RG* sequence, with missense mutations being the most common ones, followed by nonsense mutations and insertions/deletions (7). Mutations resulting in a complete absence of IL-2Rγ expression are likely to result in the classical X-SCID phenotype. On the other hand, missense mutations in the IL2RG locus have been mainly associated with less severe phenotypes referred to as "leaky or atypical X-SCID" (22–24). Furthermore, some atypical X-SCID cases with hypomorphic mutations may display, later in life, chronic lung diseases, warts, and recurrent respiratory and gastrointestinal tract infections, as well as other atypical clinical manifestations (25).

We report the case of a male child with an atypical clinical presentation carrying to a missense mutation of *IL2RG* gene, with normal T, B, and NK cell counts, high serum IgE levels, persistent eczema, and recurrent sinopulmonary infections. We performed a deep immunophenotyping and functional tests to better characterize the impact of this *IL2RG* variation. This report adds to the ever-growing knowledge on atypical X-SCID disorders and contributes to a better understanding of the clinical variability associated with the *IL2RG* gene defect.

RESULTS

Case Presentation

The patient, a 7-year-old Algerian male born to unrelated healthy parents, was admitted to the department of pediatrics at Beni Messous University Hospital due to recurrent sinopulmonary infections and treatment-resistant eczema. He was born at term by cesarean section with a birth weight of 3400g and received all the routine vaccinations with no noticeable complications. Besides moderate growth retardation, his mental and psychomotor development was normal. Recurrent upper and lower respiratory tract infections began at four years of age, requiring antibiotic therapy and hospitalization. In addition, the patient had several episodes of ear, nose, and throat (ENT) infections with a marked increase of C-reactive protein (CRP) concentration. He suffered from severe eczema since the age of 4 years, refractory to topical corticosteroids; one year later, he also developed a cutaneous leishmaniasis treated successfully with pentavalent antimonial salts (meglumine antimoniate) by parenteral administration. Investigation of his family history (**Figure 1A**) revealed that two of his maternal uncles displayed similar clinical manifestations, with one of them who has been treated for a celiac disease and who died of infectious complications at 22 years of age, while the other one, who was treated for bronchiectasis and severe refractory eczema, died at the age 28 years.

The patient who was underweight exhibited during the first physical examination, at the age of 6 years, a ringworm of the scalp accompanied by hair loss (**Figure 1C**), and an eczema present on his ears, elbow folds, and bursae. His first blood test (**Table 1**) showed a high white blood cell (WBC) count 13800/mm3 (neutrophils 57%, lymphocytes 21%, monocytes 6.5%, eosinophils 15.5%), and normal liver and kidney function tests. Measurement of serum immunoglobulin concentrations showed a pan-hypergammaglobulinemia: IgG 1755 mg/dL (normal or elevated IgG subclasses), IgA 219 mg/dL, IgM 145 mg/dL, and IgE 31120 IU/mL. A flow cytometry analysis of the different lymphocyte subsets (**Supplementary Figure S1**) revealed normal numbers and frequencies of $CD3^+$ T cells 84% (absolute count 2434/mm^3), $CD3^+CD8^+$ T cells 61.5% (1782/mm^3), $CD19^+$ B cells 9.5% (275/mm^3), and $CD56^+$NK cells 6.5% (188/mm^3). However, the patient had low numbers of CD3+CD4+ T cells 15.5% (449/mm^3), with inverted $CD4^+/CD8^+$ ratio (0.25).

Further characterization of the lymphocyte subpopulations (**Table 1**) showed a substantial shift towards a memory phenotype (**Supplementary Figure S2**) with an increased percentage of CD45RO+ CD4+ and CD45RO+ CD8+T cells, low percentages of naïve T cells (CCR7+CD45RA+), and high percentages of CD4+ and CD8+ effector memory T cells (TEM) (CCR7-CD45RA-) (**Supplementary Figure S3**). By contrast, the frequency of $CD4^+$ T cells displaying the phenotypical characteristics of recent thymic emigrants (RTE) ($CD4^+CD45RA^+CD31^+$) was in the normal range (**Supplementary Figure S4**). similarly, the evaluation of peripheral

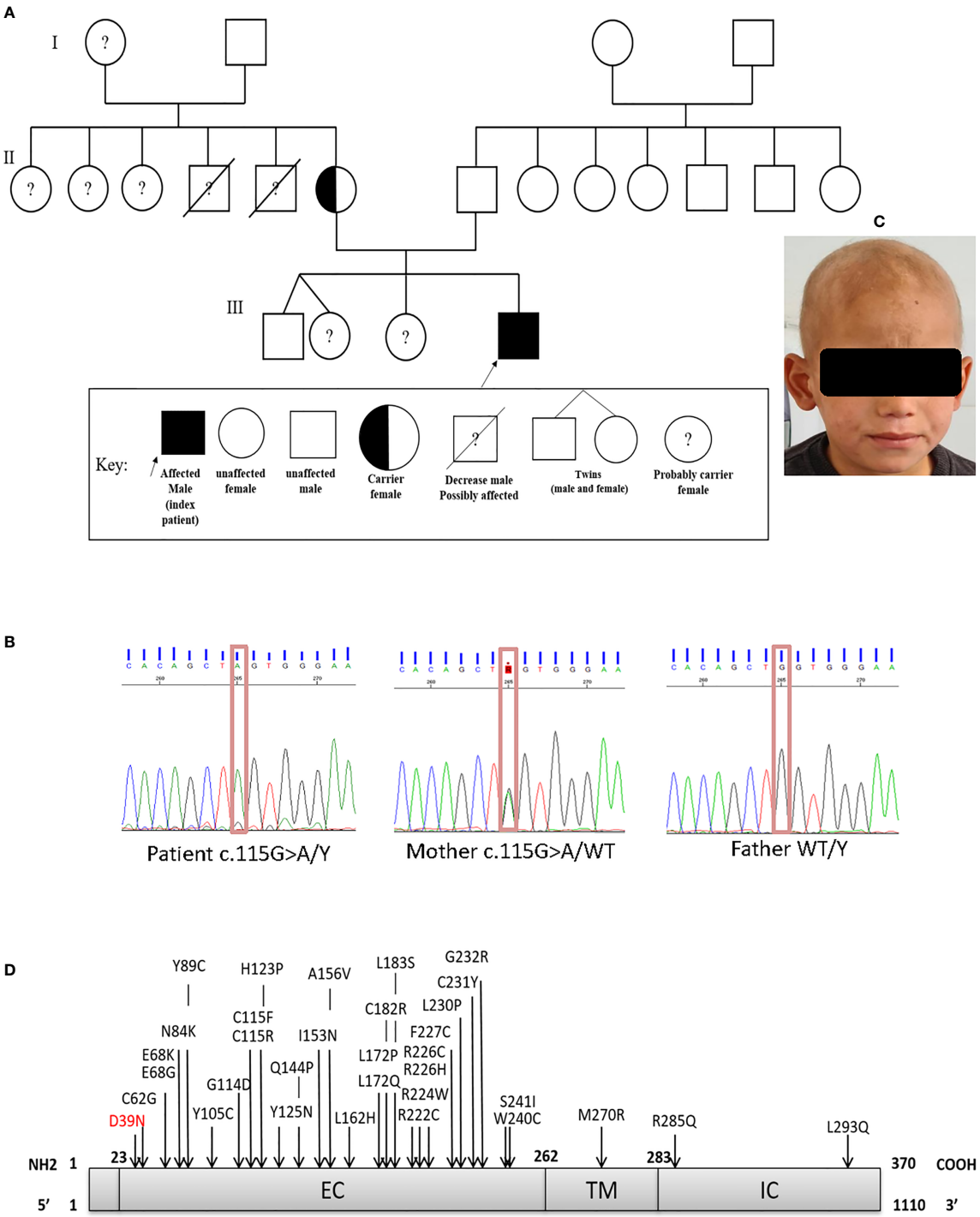

FIGURE 1 | **(A)** Family pedigree of the patient. **(B)** Sanger sequencing confirmed the presence of the mutation in exon 1 (c.115G>A; p.Asp39Asn). The heterozygous (mother) and WT states. **(C)** Scarring alopecia, hypotrichosis with ringworm of the scalp. **(D)** Schematic diagram of the IL-2RG protein, with its extracellular (EC), transmembrane (TM), intracellular (IC) domains. In red the mutation carried by the patient and in black the positions affected by the mutations already described in the literature.

B lymphocytes demonstrated a normal distribution of B cell subsets (**Supplementary Figure S5**). In fact, despite an expansion of $CD19^{+}CD21^{-/low}$ B cell subset, the Percentages of naïve B cells ($CD19^{+}IgD^{+}CD27^{-}$), switched and unswitched memory B cells ($CD19^{-}CD27^{+}IgD^{-}$ and $CD19^{+}CD27^{+}IgD^{+}$, respectively), transitional B cells ($CD19^{+}CD24^{++}CD38^{++}$), as well as plasmablasts ($CD19^{+}CD24^{-}CD38^{++}$) did not significantly differ from values observed in healthy age-matched individuals.

TABLE 1 | Immunologic characteristics of the patient with hypomorphic/atypical X-SCID.

Parameters	Result	Normal range
Complete blood count		
White blood cells (cells/µl)	***13800***	4400 – 9500
Lymphocytes (cells/µl)	2898	1900 – 3700
Neutrophils (cells/µ)	***7866***	2600 – 6300
Eosinophils (cells/µl)	***2139***	0 – 200
monocytes (cells/µl)	897	300 – 900
Hemoglobin (g/dl)	12.5	12 – 17
Platelet ($\times 10^3$ cells/µl)	***489***	150 – 450
Immunoglobulin levels		
IgG (mg/dl)	***1755***	680 – 1180
IgG_1 (mg/dl)	***1312.4***	288 – 918
IgG_2 (mg/dl)	144	44 – 375
IgG_3 (mg/dl)	***160.1***	15.5 – 85.3
IgG_4 (mg/dl)	44.6	0.4 – 99.2
IgA (mg/dl)	***219***	70 – 190
IgM (mg/dl)	***145***	32 – 98
IgE (IU/ml)	***31120***	<45
Lymphocyte subsets		
$CD3^+$ T cells/µl	2434	1200 – 2600
αβT cells (TCRαβ+TCRγδ–/CD3+)/T cells%	***83***	85 – 95
γδT cells (TCRαβ–TCRγδ+/CD3+)/T cells%	17	7 – 20
$CD4^+$ T cells/µl	***449***	650 – 1500
$CD4_{memory}CD45RO^+$ T cells/$CD4^+$ T cells%	***65.5***	13 – 30
$CD4_{Naive}CD45RA^+CCR7^+$ T cells/$CD4^+$ T cells%	20.13	15.5 – 59.4
$CD4_{CM}CD45RA^-CCR7^+$ T cells/$CD4^+$ T cells%	19.32	12.2 – 26.2
$CD4_{EM}CD45RA^-CCR7^-$ T cells/$CD4^+$ T cells%	***54.31***	10.6 – 34.2
$CD4_{TEMRA}CD45RA^+CCR7^-$ T cells/$CD4^+$ T cells%	6.24	4.5-43.6
$CD4_{RTE}CD45RA^+CD31^+$/$CD4^+$ T cells%	24.5	19.4 – 60.9
$CD8^+$ T cells/µl	***1782***	370 – 1100
$CD8_{memory}CD45RO^+$ T cells/$CD8^+$ T cells%	***82***	8 – 37
$CD8_{Naive}CD45RA^+CCR7^+$ T cells/$CD8^+$ T cells%	***1.6***	5.5 – 39.7
$CD8_{CM}CD45RA^-CCR7^+$ T cells/$CD8^+$ T cells%	***0.25***	1.2 – 3.8
$CD8_{EM}CD45RA^-CCR7^-$ T cells/$CD8^+$ T cells%	***91.98***	20.1– 44.7
$CD8_{TEMRA}CD45RA^+CCR7^-$ T cells/$CD8^+$ T cells%	***6.17***	21.5 – 61
$CD4^+/CD8^+$ Ratio	***0.25***	1.5 – 2.9
DN T cells (CD4–CD8–/CD3+TCRαβ+)	0.35	0.18 – 2.81
Regulatory T cells (CD25+IL7R–/CD3+CD4+)	11.52	4 – 14
$CD19^+$B cells/µl	275	270 – 860
B_{naive} ($CD27^-IgD^+$)/$CD19^+$ B cells %	***68.11***	69.4 – 80.4
$B_{switched\ memory}$ ($CD27^+IgD^-$)/$CD19^+$ B cells %	8.09	5.2 – 12.1
$B_{unswitched\ memory}$ ($CD27^+IgD^+$)/$CD19^+$ B cells %	***7.13***	7.5 – 12.4
$B_{transitional}$ ($CD24^{++}CD38^{++}$)/$CD19^+$ B cells %	***4.3***	4.5 – 9.2
$B_{plasmablast}$ ($CD24^-CD38^{++}$)/$CD19^+$ B cells %	2.43	0.7 – 3.5
$B_{CD21(-/low)}$ ($CD21^{low}CD38^{low}$)/$CD19^+$ B cells %	***17.5***	0.9 – 3.5
$CD3^-CD16^+CD56^+$ NK cells/µl	188	100 – 480

Values in boldface and italics are abnormal.
CM, Central memory; EM, effector memory; TEMRA, Terminally differentiated T cells; RTE, Recent Thymic Emigrant, DN, Double negative.

Given the presence of refractory eczema associated with high serum IgE concentrations, hypereosinophilia, and recurrent bacterial infections, we initially suspected a hyper-IgE syndrome. Therefore, we used a scoring system which the NIH has developed to help in the diagnosis of these patients based on both the immunologic and clinical features of the syndrome. Indeed, a score of >40 points makes the diagnosis of AD-HIES highly probable and unlikely with a score of < 20 points (26). When the scoring criteria were applied to our patient, the overall clinical and immunological presentation was between STAT3-HIES and DOCK8-CID with 39 points in the NIH-HIES score. Therefore, IL-6-induced expression of intracellular phospho-STAT3 (pSTAT3) was determined in CD4+T-cells by whole blood flow cytometric analysis, which included assessment of surface IL-6RA and gp130 expression. No difference for pSTAT3 expression was detected between patient and healthy control (**Figure 2B**).

In order to identify the molecular defect behind the pathology, whole-exome sequencing (WES) identified the presence of a hemizygous missense mutation (c.115G>A) in exon 1 of the *IL2RG* gene. The presence of the missense mutation was further confirmed by Sanger sequencing (**Figure 1B**). This substitution resulted in an amino acid change from Aspartic Acid to Asparagine at position 39 (p.Asp39Asn) of the mature protein. Bioinformatic analysis predicted that the p.Asp39Asn mutation was predicted to be probably pathogenic and affected a highly conserved amino-acid residue (SIFT-deleterious; PolyPhen2-possibly damaging), (**Supplementary Figure S6**). The high CADD score of 24.2 (sensitivity: 0.41; specificity: 0.98) also suggested a probably

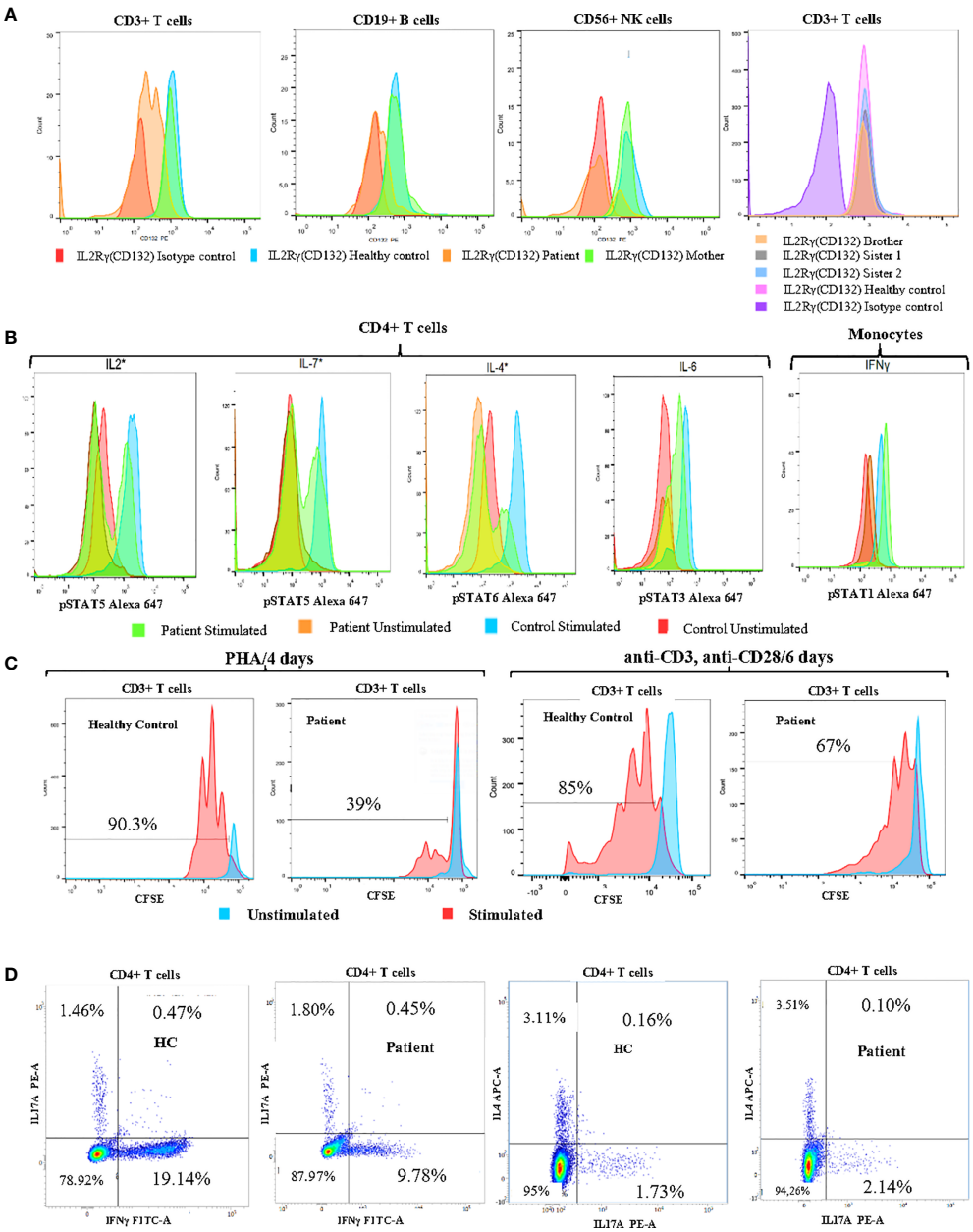

FIGURE 2 | Immune functional and proliferation assays in subsets of lymphocytes. **(A)** Flow cytometry analysis of CD132 (γc) expression on T, B and NK cells of the patient, his mother, and healthy control, and screening of siblings for CD132 expression on T cells. **(B)** Histogram of CD4+ T cell and monocyte response with STAT phosphorylation to several γc dependent (IL-2, IL-4, IL-7) and independent (IL-6, IFN-γ) cytokines in whole blood of patient. **(C)** Analysis of proliferation of healthy control (HC) and patient CFSE labeled cells with the indicated stimuli: 4 days of culture with Phytohemaglutinin (PHA) and 6 days with anti-CD3/anti-CD28 monoclonal antibodies. **(D)** The percentages of IL-17A, IFNγ and IL-4-producing CD4+ T cells from patient and age-matched control using PMA/Ionomycin stimulation in the presence of monensin for 5 hours. *Gamma chain dependent cytokines; the others are gamma chain independent.

damaging effect of the variant. No additional mutations were found in any other known disease-causing genes (**Supplementary Figure S7**).

We also found that the patient's mother was a heterozygous carrier of this variant. An immunostaining with anti-CD132 and flow cytometry analysis showed a significant reduction of γc surface expressions on T, B and NK cells of the patient when compared to his parents, siblings, or healthy control (**Figure 2A**).

In order to study the impact of Asp39Asn amino acid substitution on immune cell signaling, we measured STAT phosphorylation in CD4+ T cells and monocytes. The mutation was associated with defective phosphorylation of STAT5, STAT6, and STAT5 upon the stimulation of CD4+ T cell with IL-2, IL-4, and IL7, respectively. However, γc independent signaling in monocytes (IFN-γ induced pSTAT1)

was not affected (**Figure 2B**). Evaluation of CD3+ T cell proliferation through carboxyfluorescein succinimidyl ester (CFSE) staining revealed a significant defect of mitogen phytohemagglutinin (PHA)-induced cell proliferation compared to healthy control (39% *vs.* 90.3%). Similarly, the activation of CD3+ T cells of the patient with anti-CD3 and anti-CD28-coated beads led to a reduced proliferation compared to healthy control (67% *vs.* 85%) (**Figure 2C**). Taken together, these data suggest that the patient has a "leaky" form of SCID.

To better characterize the immunologic defects associated with the patient's mutation, the percentages of CD4+IFNγ+ (Th1), CD4 +IL4+ (Th2), and CD4+IL-17A+ (Th17) cells were assessed in PBMCs by flow cytometry. The patient exhibited a significant reduction in the proportion of IFNγ producing CD4+ T cells (Th1) and normal CD4+IL4+(Th2) and CD4+IL17A+(Th17) frequencies when compared to an age-matched control subject (**Figure 2D**). CD4+CD25^{high}CD127$^{low/-}$FoxP3+ Regulatory T-cells represent 11,52% of CD4+ T-cells. Based on these findings, we have recommended allogeneic hematopoietic stem cell transplantation as a definitive treatment. Since, the patient regularly performed immunoglobulin replacement therapy and received prophylactic antimicrobial (co-trimoxazole, sulfamethoxazole, and itraconazole). He is checked for signs of malignant disease on a regular basis, but so far, no lymphoma or other tumors have been noted.

DISCUSSION

This report describes the case of a seven-year-old Algerian patient carrying a hemizygous missense (c.115G>A) variant of *IL2RG* gene, and results in replacement of aspartic acid by asparagine at position 39 (p.Asp39Asn) in the extracellular domain of the protein (**Figure 1D**). As far as we are aware, only a single other case carrying a same mutation has been reported in the literature (27, 28).

Our patient displayed unusual clinical and immunological features with persistent eczema and ringworm of the scalp, associated with recurrent sinopulmonary infections, high IgE levels, and peripheral blood eosinophilia, which initially suggested a hyper-IgE syndrome. A reported case (27, 28) with the same amino acid substitution and a more severe clinical phenotype (**Table 2**) was mainly characterized by repetitive infections and protracted diarrhea starting around nine months of age without a history of eczema. Given the different clinical and immunological manifestations, it seems reasonable to hypothesize that other factors, such as epigenetic, environmental, and ethnic contributions, could affect the disease's evolution (29). These findings emphasize the issues that might be involved in the relationship between the environment and the genome in multifactorial disorders, in which numerous environmental factors are included (30). As reported by Dworkin et al,

TABLE 2 | Characteristics of reported patient carrying the IL2RG$^{115G>A}$ missense mutation.

	DiSanto et al. (16), de saint basile et al. (17)	Belaid et al.
Patient (age, ethnicity)	5 1/2-year-old Portuguese male	7-year-old Algerian male
Consanguinity	No	No
Medical history	protracted diarrhea, otitis media	Persistent eczema, recurrent infections, cutaneous leishmaniasis
Family history	3 maternal uncles and 2 older brothers died of bronchopneumonitis with bronchiectasis, skin infections, protracted diarrhea, and failure to thrive	2 maternal uncles died of recurrent infections with bronchiectasis.
Onset	9-month-old	4-year-old
Immunological findings		
Thymic shadow	extreme thymic atrophy with rare Hassal's corpuscles	n.a
Blood count	Normal	Normal
Immunophenotype	T^{low}, B^+, NK^+	T^+, B^+, NK^+
γδ T cells	Normal	Normal
Microbiological examination	Poliovirus, Bordetella pertussis	leishmania infantum
Extended immunophenotyping	T cells skewed to the memory phenotype	T cells skewed to the memory phenotype, profound decreased of naïve T cells, normal distribution of B cells, reduced Th1.
Immunoglobulin levels	Normal with elevated IgE levels	Hypergammaglobulinemia with extremely high IgE levels.
TCR-Vβ repertoire	limited TCRβ heterogeneity and diminished functional activity	n.a
Lymphocyte proliferation	Reduced	Reduced
IL2RG expression	n.a	Severely reduced (by FCM)
IL21/STAT3 phosphorylation	n.a	n.a
IL2 &IL7/STAT5 phosphorylation	n.a	Partially defective
IL4/STAT6 phosphorylation	n.a	Partially defective
TRECs	n.a	n.a

n.a, not assessed; FCM, flow cytometry; TRECs, T-cell receptor excision circles.
Both Disanto et al, and de Saint Basile et al. had reported the same case.

attempting to understand the molecular interaction of genetic background effects using model organisms, found that were partly associated with differentially expressed genes where quantitative transcription level differences correlated with variation for the phenotype (31). Furthermore, Lachance et al. studied genetic background effects on an X-linked gene in Drosophila melanogaster leading to wing defects. The natural variant was placed into multiple backgrounds, then they assessed penetrance and expressivity of wing defects. They found significant complex interactions that were affected by the genetic background (32).

Typically, patients with genetic mutations of the *IL2RG* gene are classically characterized by the absence or severe reduction of T and NK cell numbers, as well as the presence of non-functional B cells (33), these mutations are mainly localized within the exons 3-5 (34, 35). Missense mutations are the most common pathogenic changes observed, followed by nonsense variants and insertions/deletions (35). On the other hand, atypical clinical presentations of X-SCID have been also described in patients carrying missense mutations of *IL2RG* resulting in the expression of lower amounts of γc protein with conserved binding affinity for IL-2, or in its reduced interaction with JAK3, and thus impairing T cell activation (9, 36). However, such mutation requires assessing a cell line from the affected patient to evaluate γc cell surface expression and/or IL2RG mRNA transcripts as a direct proof if the mutation is deleterious. In the present case, an accurate diagnosis of atypical X-SCID was initially compromised because of the unusual clinical presentation, with almost normal development and growth, a low number of infections during the first 3 years of life. Moreover, the most first-line immunological investigations showed normal percentages and numbers of total T, NK, and B cells, but CD4+ T cell counts decreased while CD8+ T cell counts were expanded, combined with a polyclonal hypergammaglobulinemia; hence, these findings were initially inconsistent with X-SCID. Atypical cases with normal numbers of T and NK cells are very unusual and were previously reported in only few cases with *IL2RG* mutations (37–41), and some *IL2RG* mutations can lead to functional lymphocyte abnormalities rather than cell development defects, as some patients with normal lymphocyte differentiation and normal thymus biopsies were reported (37). Such phenotype can be a direct consequence of a residual γc expression providing sufficient signal for normal T and NK cell development. This is in accordance with the preserved signaling by the IL-7R and IL-15R, as it has been shown before by Smyth et al. that signal transduction by the IL-7R is crucial for T-cell development but is dispensable for NK cell development, whereas adequate signaling *via* the IL-15R is essential for NK cell but not for T-cell development (42, 43). This mechanism corroborates with our findings, suggesting that the amount of γc required for the correct signaling of various signaling pathways is different.

In addition to the aforementioned findings, the T lymphocyte differentiation and maturation skewed toward memory phenotype ($CD45RO^+$), combined with increased counts of total $CD8^+$ T cells as well as expansion of $CD45^-CCR7^-$ effector memory for both $CD4^+$ and $CD8^+$ T cells. This condition probably is in consistence with persistent viral infection (44), although no virological confirmation was possible. On other hand, thymic stromal lymphopoietin (TSLP) is another cytokine that is not a member of the γc family but has overlapping functions with IL-7 (45). Indeed, whereas the IL-7 receptor contains IL-7Rα and γc, the TSLP receptor consists of IL-7Rα and TSLPR, which is closely related to γc (46, 47). *IL2RG*$^{-/-}$ mice treated with recombinant TSLP, which cannot respond to IL-7 or other γc family cytokines, lead to a partial increase in CD8+ T cell numbers (48). Moreover, TSLP promotes the survival of CD8+ T cells in both normal and lymphogenic conditions (49).

In addition, our patient displayed moderately reduced capacity of T cells to proliferate in response to PHA or anti-CD3/anti-CD28 stimulation, reflecting disturbed IL-2 signaling. Indeed, IL-2 is key growth factor required for T cell expansion and promotes the proliferation and survival of activated T cells (50). Thus, it influences effector T cell differentiation and promotes fate decisions in activated T cells (51).

Furthermore, our patient had normal percentages of switched memory B cells, transitional B cells, and plasmablasts, but negligible reduction of naïve B cells, and unswitched memory B cells. There may exist a threshold level of γc expression necessary for normal B cell function, consequently, the collaborative IL-4 and IL-21 signaling might be sufficient for humoral responses (52–55). In contrast, we found signs of misguided enhanced B cell activity reflected by a large $CD21^{low}CD38^{low}$ B cell population, in addition to a polyclonal hypergammaglobulinemia. $CD21^{low}CD38^{low}$ B cells are a distinct B cell population that is mainly associated with manifestations of chronic immune activation, lymphoproliferation, and autoimmunity (56–58). Interestingly, in our case there was no evidence of conditions considered to be autoimmune or lymphoproliferation. This is in consistence with normal numbers of regulatory T cells (T reg) in indexed patient, although IL-2 is also critical for the development of Tregs in the thymus and for their maintenance and function in the periphery (59). In addition, high IgG1 and IgG3 levels were also observed which can recognize protein and viral antigens (60, 61). In fact, viral infections in general lead to IgG antibodies of the IgG1 and IgG3 subclasses, with IgG3 antibodies appearing first in the course of the infection (60, 61).

In the case reported by DiSanto et al., with the same mutation as the present patient, a reduced capacity to splice a correct-sized transcript, leading to the production of a nonfunctional transcript containing an insertion of 27 bp, and a reduced amount of a normal sized transcript containing a single amino acid substitution has been shown (28). Thus, they demonstrated that splicing of exons 1 and 2 normally generates the codon GAT, but with the base change, the resultant codon becomes AAT, and the point mutation (D39N) appeared not to impair IL-2 binding or its subsequent endocytosis (28). Therefore, a residual expression of *IL2RG* transcripts with normal length may account for the limited expression of γc detectable at the cell surface. Unfortunately, the size of the transcripts and γc protein were not assessed in the current work. However, our data showed that γc expression and STAT5/STAT6 phosphorylation were reduced but not completely abolished. These findings are consistent with a moderate phenotype suggesting that this mutation (p.Asp39Asn) is hypomorphic.

Interestingly, our patient developed atopic dermatitis-like skin lesions and alopecia associated to IgE hyperproduction (31,120 IU/

ml) and hypereosinophilia (2139 cells/μl), such conditions were surprisingly rare in reviewed patients carrying *IL2RG* hypomorphic mutations. Indeed, only four patients, suffering from eczema or other skin rashes, were reported in the literature (15, 62–64). Milner et al. demonstrated that reduced T-cell activation caused by a weak signaling has been associated with a skew towards the development of T helper type 2 cells (Th2 cells) (62), this may suggest its profile as a default differentiation pathway partly underlying the features of skin lesions in these patients. This is a typical finding in patients with Omenn syndrome in whom expanded T-cell clones were consistently found to be predominantly of TH2 type (65), and to secrete IL-4/IL-13, IL-5 and IL-9, which promote immunoglobulin class-switching to IgE, activates eosinophils, and activates mast cells, respectively (66–69). These results are consistent with our patient, who showed normal percentage of IL-4 producing T cells and low proportions of IFN-γ producing T cells compared to age-matched control subject (**Figure 2D**), suggesting that Th2-type cytokines are central in the pathogenesis of this hyper-IgE production. Moreover, previous studies have also demonstrated that B cells from X-SCID patients with decreased expression of γc can respond to IL-4 *via* a type II IL-4R complex composed of IL-4Rα/IL-13R chains (70–72).

In conclusion, X-SCID is an inborn error of immunity that manifests as different clinical phenotypes, from milder to severe disease. Most of the attention focused on the possible relationship between mutations of the *IL2RG* gene and clinical/immunological features. Further investigations are required to achieve a better classification of the disease. We believe that unusual clinical and laboratory observations may be very useful to unravel complex diseases and help find novel gene-function relationship laying the ground for novel targeted therapeutic approaches.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'éthique du CHU Beni Messous. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BB drafted the manuscript and contributed in collecting and analyzing the data. LM designed and performed experiments, analyzed the data, and reviewed the manuscript critically. AM, YK, and NB, cared for the patient and provided clinical data. AP, MM, and JC reviewed the manuscript critically and suggested changes to the final version and did the genetic testing. RD reviewed the manuscript and conceptualized the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.696350/full#supplementary-material

REFERENCES

1. Stephan JL, Vlekova V, Deist F, Blanche S, Donadieu J, Saint-Basile G, et al. Severe Combined Immunodeficiency: A Retrospective Single-Center Study of Clinical Presentation and Outcome in 117 Patients. *J Pediatr* (1993) 123:564–72. doi: 10.1016/S0022-3476(05)80951-5
2. Haddad E, Landais P, Friedrich W, Gerritsen B, Cavazzana-Calvo M, Morgan G, et al. Long-Term Immune Reconstitution and Outcome After HLA-Nonidentical T- Cell-Depleted Bone Marrow Transplantation for Severe Combined Immunodeficiency: A European Retrospective Study of 116 Patients. *Blood* (1998) 91:3646–53. doi: 10.1182/blood.V91.10.3646
3. Bertrand Y, Landais P, Friedrich W, Gerritsen B, Morgan G, Fasth A, et al. Influence of Severe Combined Immunodeficiency Phenotype on the Outcome of HLA Non-Identical, T-Cell- Depleted bone Marrow Transplantation. *J Pediatr* (1999) 134:740–8. doi: 10.1016/s0022-3476(99)70291-x
4. Antoine C, Müller S, Cant A, Cavazzana-Calvo M, Veys P, Vossen J, et al. Long-Term Survival and Transplantation of Haemopoietic Stem Cells for Immunodeficiencies: Report of the European Experience 1968-99. *Lancet* (2003) 361:553–60. doi: 10.1016/S0140-6736(03)12513-5
5. AL Dossari D, Kamal D. Clinical Practice: Clinical Practice. *J Gastroenterol Hepatol* (2015) 30:13–26. doi: 10.1111/jgh.13088
6. Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, et al. Interleukin-2 Receptor γ Chain Mutation Results in X-Linked Severe Combined Immunodeficiency in Humans. *Cell* (1993) 73:147–57. doi: 10.1016/0092-8674(93)90167-O
7. Puck JM, Deschenes SM, Porter JC, Dutra AS, Brown CJ, Willard HF, et al. The Interleukin-2 Receptor γ Chain Maps to Xq13.1 and Is Mutated in X-

linked Severe Combined Immunodeficiency, SCIDX1. *Hum Mol Genet* (1993) 2:1099–104. doi: 10.1093/hmg/2.8.1099
8. Yrina R, Spolski R, WJL. New Insights Into the Regulation of T Cells by γc Family Cytokines. *Bone* (2008) 23:1–7. doi: 10.1038/nri2580.New
9. Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, et al. Interaction of IL-2Rβ and γc Chains With Jak1 and Jak3: Implications for XSCID and XCID. *Science (80-)* (1994) 266:1042–5. doi: 10.1126/science.7973658
10. Miyazaki T, Kawahara A, Fujii H, Nakagawa Y, Minami Y, Liu ZJ, et al. Functional Activation of Jak1 and Jak3 by Selective Association With IL-2 Receptor Subunits. *Science (80-)* (1994) 266:1045–7. doi: 10.1126/science.7973659
11. Puck JM, Pepper AE, Henthorn PS, Candotti F, Isakov J, Whitwam T, et al. Mutation Analysis of IL2RG in Human X-Linked Severe Combined Immunodeficiency. *Blood* (1997) 89:1968–77. doi: 10.1182/blood.V89.6.1968
12. Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL7R Expression in T-B+NK+ Severe Combined Immunodeficiency. *Nat Genet* (1998) 20:394–7. doi: 10.1038/3877
13. Kotlarz D, Ziętara N, Uzel G, Weidemann T, Braun CJ, Diestelhorst J, et al. Loss-of-Function Mutations in the IL-21 Receptor Gene Cause a Primary Immunodeficiency Syndrome. *J Exp Med* (2013) 210:433–43. doi: 10.1084/jem.20111229
14. Erman B, Bilic I, Hirschmugl T, Salzer E, Çagdas D, Esenboga S, et al. Combined Immunodeficiency With CD4 Lymphopenia and Sclerosing Cholangitis Caused by a Novel Loss-of-Function Mutation Affecting IL21R. *Haematologica* (2015) 100:e216–9. doi: 10.3324/haematol.2014.120980
15. Stepensky P, Keller B, Abuzaitoun O, Shaag A, Yaacov B, Unger S, et al. Extending the Clinical and Immunological Phenotype of Human interleukin-21 Receptor Deficiency. *Haematologica* (2015) 100:63–7. doi: 10.3324/haematol.2014.112508
16. Béziat V, Li J, Lin JX, Ma CS, Li P, Bousfiha A, et al. A Recessive Form of hyper-IgE Syndrome by Disruption of ZNF341-Dependent STAT3 Transcription and Activity. *Sci Immunol* (2018) 3:1–19. doi: 10.1126/sciimmunol.aat4956
17. Berglund LJ, Avery DT, Ma CS, Moens L, Deenick EK, Bustamante J, et al. Il-21 Signalling *Via* STAT3 Primes Human Näive B Cells to Respond to IL-2 to Enhance Their Differentiation Into Plasmablasts. *Blood* (2013) 122:3940–50. doi: 10.1182/blood-2013-06-506865
18. Fischer A. Severe Combined Immunodeficiencies (SCID). *Clin Exp Immunol* (2000) 122:143–9. doi: 10.1046/j.1365-2249.2000.01359.x
19. Roifman CM, Somech R, Kavadas F, Pires L, Nahum A, Dalal I, et al. Defining Combined Immunodeficiency. *J Allergy Clin Immunol* (2012) 130:177–83. doi: 10.1016/j.jaci.2012.04.029
20. Okuno Y, Hoshino A, Muramatsu H, Kawashima N, Wang X, Yoshida K, et al. Late-Onset Combined Immunodeficiency With a Novel IL2rg Mutation and Probable Revertant Somatic Mosaicism. *J Clin Immunol* (2015) 35:610–4. doi: 10.1007/s10875-015-0202-0
21. Stevens M, Frobisher C, Hawkins M, Jenney M, Lancashire E, Reulen R, et al. The British Childhood Cancer Survivor Study: Objectives, Methods, Population Structure, Response Rates and Initial Descriptive Information. *Pediatr Blood Cancer* (2008) 50:1018–25. doi: 10.1002/pbc
22. Fuchs S, Rensing-Ehl A, Erlacher M, Vraetz T, Hartjes L, Janda A, et al. Patients With T+/low Nk+ IL-2 Receptor γ Chain Deficiency Have Differentially-Impaired Cytokine Signaling Resulting in Severe Combined Immunodeficiency. *Eur J Immunol* (2014) 44:3129–40. doi: 10.1002/eji.201444689
23. Felgentreff K, Perez-Becker R, Speckmann C, Schwarz K, Kalwak K, Markelj G, et al. Clinical and Immunological Manifestations of Patients With Atypical Severe Combined Immunodeficiency. *Clin Immunol* (2011) 141:73–82. doi: 10.1016/j.clim.2011.05.007
24. Disanto JP, Rieux-Laucat F, Dautry-Varsat A, Fischer A, De Saint Basile G. Defective Human Interleukin 2 Receptor γ Chain in An Atypical X Chromosome-Linked Severe Combined Immunodeficiency With Peripheral T Cells. *Proc Natl Acad Sci USA* (1994) 91:9466–70. doi: 10.1073/pnas.91.20.9466
25. Allenspach E, Rawlings DJ, Scharenberg AM. *X-Linked Severe Combined Immunodeficiency* (1993). Seattle: University of Washington. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20301584 (Accessed April 15, 2021).
26. Yong PFK, Freeman AF, Engelhardt KR, Holland S, Puck JM, Grimbacher B. An Update on the Hyper- IgE syndromes. *Arthritis Res Ther* (2012) 14:1–10. doi: 10.1186/ar4069
27. De Saint-Basile G, Le Deist F, Caniglia M, Lebranchu Y, Griscelli C, Fischer A. Genetic Study of a New X-Linked Recessive Immunodeficiency Syndrome. *J Clin Invest* (1992) 89:861–6. doi: 10.1172/JCI115665
28. Disanto JP, Rieux-laucat F, Dautry-varsatt A, Fischer A. Basile GDE Saint. Chromosome-Linked Peripheral Cells. *Sci York* (1994) 91:9466–70. doi: 10.1073/pnas.91.20.9466
29. Kuijpers TW, van Leeuwen EMM, Barendregt BH, Klarenbeek P, aan de Kerk DJ, Baars PA, et al. Van Der Burg M. A Reversion of An IL2RG Mutation in Combined Immunodeficiency Providing Competitive Advantage to the Majority of CD8+ T Cells. *Haematologica* (2013) 98:1030–8. doi: 10.3324/haematol.2012.077511
30. Weatherall DJ. Phenotype-Genotype Relationships in Monogenic Disease: Lessons From the Thalassaemias. *Nat Rev Genet* (2001) 2:245–55. doi: 10.1038/35066048
31. Dworkin I, Kennerly E, Tack D, Hutchinson J, Brown J, Mahaffey J, et al. Genomic Consequences of Background Effects on Scalloped Mutant Expressivity in the Wing of Drosophila Melamogaster. *Genetics* (2009) 181:1065–76. doi: 10.1534/genetics.108.096453
32. Lachance J, Jung L, True JR. Genetic Background and GxE Interactions Modulate the Penetrance of a Naturally Occurring Wing Mutation in Drosophila Melanogaster. *G3 Genes Genomes Genet* (2013) 3:1893–901. doi: 10.1534/g3.113.007831
33. Blanco E, Izotova N, Booth C, Thrasher AJ. Immune Reconstitution After Gene Therapy Approaches in Patients With X-Linked Severe Combined Immunodeficiency Disease. *Front Immunol* (2020) 11:608653. doi: 10.3389/fimmu.2020.608653
34. Puck JM. IL2RGbase : A Database of γc-Chain Defects Causing Human X-SCID. *Trends Immunol Today* (1996) 17:507–11. doi: 10.1016/0167-5699(96)30062-5
35. Villa A, Moshous D, de Villartay JP, Notarangelo LD, Candotti F. Severe Combined Immunodeficiencies. In *Stiehm's Immune Deficiencies* Elsevier Inc. (2014) pp. 87–141. doi: 10.1016/B978-0-12-405546-9.00004-2
36. Schmalstieg FC, Leonard WJ, Noguchi M, Berg M, Rudloff HE, Denney RM, et al. Missense Mutation in Exon 7 of the Common γ Chain Gene Causes a Moderate Form of X-linked Combined Immunodeficiency. *J Clin Invest* (1995) 95:1169–73. doi: 10.1172/JCI117765
37. Sharfe N, Shahar M, Roifman CM. An Interleukin-2 Receptor γ Chain Mutation With Normal Thymus Morphology. *J Clin Invest* (1997) 100:3036–43. doi: 10.1172/JCI119858
38. Illig D, Navratil M, Kelečić J, Conca R, Hojsak I, Jadrešin O, et al. Alternative Splicing Rescues Loss of Common Gamma Chain Function and Results in IL-21R-like Deficiency. *J Clin Immunol* (2019) 39:207–15. doi: 10.1007/s10875-019-00606-7
39. Stepensky P, Keller B, Shamriz O, von Spee-Mayer C, Friedmann D, Shadur B, et al. T+ NK+ Il-2 Receptor γ Chain Mutation: A Challenging Diagnosis of Atypical Severe Combined Immunodeficiency. *J Clin Immunol* (2018) 38:527–36. doi: 10.1007/s10875-018-0514-y
40. Tuovinen EA, Grönholm J, Öhman T, Pöysti S, Toivonen R, Kreutzman A, et al. Novel Hemizygous IL2RG P. (Pro58Ser) Mutation Impairs IL-2 Receptor Complex Expression on Lymphocytes Causing X-Linked Combined Immunodeficiency. *J Clin Immunol* (2020) 40:503–14. doi: 10.1007/s10875-020-00745-2
41. Somech R, Roifman CM. Mutation Analysis Should be Performed to Rule Out γc Deficiency in Children With Functional Severe Combined Immune Deficiency Despite Apparently Normal Immunologic Tests. *J Pediatr* (2005) 147:555–7. doi: 10.1016/j.jpeds.2005.05.010
42. Smyth CM, Ginn SL, Deakin CT, Logan GJ, Alexander IE. Limiting γc Expression Differentially Affects Signaling *Via* the Interleukin (IL)-7 and IL-15 Receptors. *Blood* (2007) 110:91–8. doi: 10.1182/blood-2006-11-055442
43. Waickman AT, Park JY, Park JH. The Common γ-Chain Cytokine Receptor: Tricks-and-treats for T Cells. *Cell Mol Life Sci* (2016) 73:253–69. doi: 10.1007/s00018-015-2062-4
44. Klenerman P, Oxenius A. T Cell Responses to Cytomegalovirus. *Nat Rev Immunol* (2016) 16:367–77. doi: 10.1038/nri.2016.38
45. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt RDW, et al. Tslp: An Epithelial Cell Cytokine That Regulates T Cell Differentiation by Conditioning Dendritic Cell Maturation. *Annu Rev Immunol* (2007) 25:193–219. doi: 10.1146/annurev.immunol.25.022106.141718

46. Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, et al. Cloning of a Receptor Subunit Required for Signaling by Thymic Stromal Lymphopoietin. *Nat Immunol* (2000) 1:59–64. doi: 10.1038/76923
47. Park LS, Martin U, Garka K, Gliniak B, Di Santo JP, Muller W, et al. Cloning of the Murine Thymic Stromal Lymphopoietin (TSLP) Receptor: Formation of a Functional Heteromeric Complex Requires Interleukin 7 Receptor. *J Exp Med* (2000) 192:659–69. doi: 10.1084/jem.192.5.659
48. Al-Shami A, Spolski R, Kelly J, Fry T, Schwartzberg PL, Pandey A, et al. A Role for Thymic Stromal Lymphopoietin in CD4+ T Cell Development. *J Exp Med* (2004) 200:159–68. doi: 10.1084/jem.20031975
49. Rochman Y, Leonard WJ. The Role of Thymic Stromal Lymphopoietin in CD8 + T Cell Homeostasis. *J Immunol* (2008) 181:7699–705. doi: 10.4049/jimmunol.181.11.7699
50. Rochman Y, Spolski R, Leonard WJ. New Insights Into the Regulation of T Cells by γc Family Cytokines. *Nat Rev Immunol* (2009) 9:480–90. doi: 10.1038/nri2580
51. Ursini MV, Gaetaniello L, Ambrosio R, Matrecano E, Apicella AJ, Salerno MC, et al. Atypical X-Linked SCID Phenotype Associated With Growth Hormone Hyporesponsiveness. *Clin Exp Immunol* (2002) 129:502–9. doi: 10.1046/j.1365-2249.2002.01823.x
52. Weinstein JS, Herman EI, Lainez B, Licona-Limón P, Esplugues E, Flavell R, et al. T FH Cells Progressively Differentiate to Regulate the Germinal Center Response. *Nat Immunol* (2016) 17:1197–205. doi: 10.1038/ni.3554
53. McGuire HM, Vogelzang A, Warren J, Loetsch C, Natividad KD, Chan TD, et al. IL-21 and IL-4 Collaborate To Shape T-Dependent Antibody Responses. *J Immunol* (2015) 195:5123–35. doi: 10.4049/jimmunol.1501463
54. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. Il-21 Acts Directly on B Cells to Regulate Bcl-6 Expression and Germinal Center Responses. *J Exp Med* (2010) 207:353–63. doi: 10.1084/jem.20091738
55. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. Il-21 Regulates Germinal Center B Cell Differentiation and Proliferation Through a B Cell-Intrinsic Mechanism. *J Exp Med* (2010) 207:365–78. doi: 10.1084/jem.20091777
56. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement Receptor 2/CD21- Human Naive B Cells Contain Mostly Autoreactive Unresponsive Clones. *Blood* (2010) 115:5026–36. doi: 10.1182/blood-2009-09-243071
57. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass Trial: Defining Subgroups in Common Variable Immunodeficiency. *Blood* (2008) 111:77–85. doi: 10.1182/blood-2007-06-091744
58. Mouillot G, Carmagnat M, Gérard L, Garnier JL, Fieschi C, Vince N, et al. B-Cell and T-Cell Phenotypes in CVID Patients Correlate With the Clinical Phenotype of the Disease. *J Clin Immunol* (2010) 30:746–55. doi: 10.1007/s10875-010-9424-3
59. Nelson BH. Il-2, Regulatory T Cells, and Tolerance. *J Immunol* (2004) 172:3983–8. doi: 10.4049/jimmunol.172.7.3983
60. Upton J. Immunodeficiencies With Hypergammaglobulinemia: A Review. *LymphoSign J* (2015) 2:57–73. doi: 10.14785/lpsn-2014-0019
61. Vidarsson G, Dekkers G, Rispens T. Igg Subclasses and Allotypes: From Structure to Effector Functions. *Front Immunol* (2014) 5:520. doi: 10.3389/fimmu.2014.00520
62. Milner JD, Fazilleau N, McHeyzer-Williams M, Paul W. Cutting Edge: Lack of High Affinity Competition for Peptide in Polyclonal $CD4^+$ Responses Unmasks IL-4 Production. *J Immunol* (2010) 184:6569–73. doi: 10.4049/jimmunol.1000674
63. Brooks EG, Schmalstieg FC, Wirt DP, Rosenblatt HM, Adkins LT, Lookingbill DP, et al. A Novel X-Linked Combined Immunodeficiency Disease. *J Clin Invest* (1990) 86:1623–31. doi: 10.1172/JCI114884
64. Wada T, Yasui M, Toma T, Nakayama Y, Nishida M, Shimizu M, et al. Detection of T Lymphocytes With a Second-Site Mutation in Skin Lesions of Atypical X-linked Severe Combined Immunodeficiency Mimicking Omenn Syndrome. *Blood* (2008) 112:1872–5. doi: 10.1182/blood-2008-04-149708
65. Schandené L, Ferster A, Mascart-Lemone F, Crusiaux A, Gérard C, Marchant A, et al. T Helper Type 2-Like Cells and Therapeutic Effects of Interferon-γ in Combined Immunodeficiency With Hypereosinophilia (Omenn's Syndrome). *Eur J Immunol* (1993) 23:56–60. doi: 10.1002/eji.1830230110
66. Larché M, Akdis CA, Valenta R. Immunological Mechanisms of Allergen-Specific Immunotherapy. *Nat Rev Immunol* (2006) 6:761–71. doi: 10.1038/nri1934
67. Akdis CA, Akdis M. Mechanisms of Allergen-Specific Immunotherapy. *J Allergy Clin Immunol* (2011) 127:18–27. doi: 10.1016/j.jaci.2010.11.030
68. Geha RS, Jabara HH, Brodeur SR. The Regulation of Immunoglobulin E Class-Switch Recombination. *Nat Rev Immunol* (2003) 3:721–32. doi: 10.1038/nri1181
69. Gould HJ, Sutton BJ. Ige in Allergy and Asthma Today. *Nat Rev Immunol* (2008) 8:205–17. doi: 10.1038/nri2273
70. Matthews DJ, Clark PA, Herbert J, Morgan G, Armitage RJ, Kinnon C, et al. Function of the Interleukin-2 (IL-2) Receptor γ-Chain in Biologic Responses of X-Linked Severe Combined Immunodeficient B Cells to IL-2, Il-4, IL-13, and IL-15. *Blood* (1995) 85:38–42. doi: 10.1182/blood.v85.1.38.bloodjournal85138
71. Matthews DJ, Hibbert L, Friedrich K, Minty A, Callard RE. X-Scid B Cell Responses to Interleukin-4 and Interleukin-13 Are Mediated by a Receptor Complex That Includes the Interleukin-4 Receptor α Chain (p140) But Not the γc Chain. *Eur J Immunol* (1997) 27:116–21. doi: 10.1002/eji.1830270118
72. Lin JX, Migone TS, Tseng M, Friedmann M, Weatherbee JA, Zhou L, et al. The Role of Shared Receptor Motifs and Common Stat Proteins in the Generation of Cytokine Pleiotropy and Redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* (1995) 2:331–9. doi: 10.1016/1074-7613(95)90141-8

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REVIEW
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Immune Checkpoint Inhibitors in Human Glioma Microenvironment

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Gliomas are the most common primary brain tumors in adults. Despite the fact that they are relatively rare, they cause significant morbidity and mortality. High-grade gliomas or glioblastomas are rapidly progressing tumors with a very poor prognosis. The presence of an intrinsic immune system in the central nervous system is now more accepted. During the last decade, there has been no major progress in glioma therapy. The lack of effective treatment for gliomas can be explained by the strategies that cancer cells use to escape the immune system. This being said, immunotherapy, which involves blockade of immune checkpoint inhibitors, has improved patients' survival in different cancer types. This novel cancer therapy appears to be one of the most promising approaches. In the present study, we will start with a review of the general concept of immune response within the brain and glioma microenvironment. Then, we will try to decipher the role of various immune checkpoint inhibitors within the glioma microenvironment. Finally, we will discuss some promising therapeutic pathways, including immune checkpoint blockade and the body's effective anti-glioma immune response.

Keywords: Glioma, immune response, immune checkpoint, immunotherapy, Glioblastoma

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INTRODUCTION

The immune system is made up of several cell types, which defend the body against possible pathogens (1).

Gliomas or glial tumors are the most common primary brain tumors and they account for 81% of all malignant ones. Although they are relatively rare, gliomas cause significant morbidity and mortality. Glioblastoma is the most aggressive and common (45%) of all 6 glioma types and grades, and presents with a median survival of around 15 months (2).

For more than 10 years of research there has been no significant progress in Glioma treatment until now (3) and the lack of effective treatment for glioma can be explained by the many strategies that cancer cells use to escape the immune system (4).

Immunotherapy is an immunological treatment which uses the host's immune system to recognize and eliminate cancer cells. Indeed, this type of treatment has been shown to be fairly effective against various types of cancer (3), especially with the blockade of inhibitory immune checkpoint molecules (5). These immune checkpoints control the interactions between T cells and cancer cells through the inhibition or activation of T cells. This process occurs according to the organism's needs and action of the tumor (3). Moreover, immunotherapy, which acts on immune

checkpoint inhibitor's blockade, has improved patients' survival in different types of cancers. This new hope of cancer therapy remains one of the most promising approaches for the effective activation of therapeutic antitumor immunity (6).

In recent years, these observations have raised the curiosity of researchers who have taken a great interest in immune checkpoint blockade in glioma. Previous studies have shown that the combination of anti-PD-1 and anti- CTLA-4 blocking Abs, does not improve the overall survival [(7), p. 143].

In addition, no obvious benefit of neoadjuvant nivolumab was obtained with resectable glioblastoma (GBM), and presented with a median overall survival of just 7.3 months (8). Similarly, a phase III trial comparing nivolumab (anti-PD-1 blocking Ab) to bevacizumab (anti-VEGF blocking Ab) on patients with recurrent GBM failed to substantiate the benefit of nivolumab, and conferred a similar median overall survival (mOS, 9.8 vs 10.0 months) (9).

The present review aims at describing the immune response within the glioma microenvironment, and discussing the involvement of various immune checkpoint inhibitor molecules used by glioma cells in order to escape the immune response. It will also report some potential therapeutic pathways which involve immune checkpoints blockade.

IMMUNE RESPONSE WITHIN THE BRAIN

It is becoming more and more accepted that there exists an intrinsic immune system which is present and functional in the central nervous system (CNS) (10). However, in the 20th century, the brain was defined as a privileged organ, which meant that the brain and meninges were devoid of lymphatics (11). It was initially presumed that the physiological characteristics of the CNS, absence of antigen-presenting cells (dendritic cells) and presence of the blood brain barrier (BBB) were the causes of the lack of immune surveillance in the brain. Previous studies have shown that upon infiltration of bacteria and viruses, the immune system response could not be recognized or established (12). Hence, when Lowenstein et al. transplanted skin grafts into the brain of non-immunized animals, they found that it did not elicit an immune response (13). These studies led to the belief that the brain is a privileged organ (11, 13).

However, in October 2015, a study conducted by Louveau et al. on mice showed that the brain, like every other tissue, is connected to the peripheral immune system (10).

Through the use of novel techniques, such as staining mice's meninges with immunohistochemistry, they were able to highlight that endothelial cells, T cells and MHC II-expressing cells were the most present near the dural sinuses. Upon resection of the deep cervical lymph nodes, there was an accumulation of meningeal T-cells due to an inability of T-cells to drain from the meningeal space (14). Hence, the suggestion that the primary route of drainage is from the meningeal vessels (15). The basis of this new theory was a report by Aspelund et al. where they discovered that the Schlemm's canal in the eye acted like a lymphatic vessel (16). This led other scientists to hypothesize that similar vessels may also be present within the brain and to question the extent of the brain's immune privilege (10).

Innate immunity-related molecules like cytokines, toll-like receptors and the major histocompatibility complex are expressed in the brain and they influence the generation of an efficient immune microenvironment (17). These cellular constituents elicit an immune response which further supports the idea that the brain does in fact have immune surveillance. Microglia cells are the most predominant and make up 80% of the immune cells in the brain (18). Others include dendritic cells, B-cells, and T-cells, of which B-cells are the most abundant (15). The movement of immune cells and fluid from the CSF is made possible by the lymphatic system lining the dural sinuses (10). Cytokines are signaling proteins and are mostly secreted by immune cells. They can be described as pro-inflammatory or anti-inflammatory (19). It was originally thought that cytokines could not pass through the BBB *via* membrane diffusion as they were too large and hydrophobic. However, cytokines have overcome this by using saturable transport systems or by passing through the disrupted parts of the BBB (20).

Chemokines are low molecular weight proteins that are involved in direct cell migration. They attract leukocytes to the site of infection to allow the mediation of acute and chronic inflammation (21).

In normal circumstances, however, their expression is diminished. Homeostatic chemokines are involved with maintaining leukocyte composition in preparation for an immune response to an insult. Still, inflammatory chemokines are produced during infections or in response to an inflammatory stimulus (22). The immune response in the brain progresses at a much slower rate as opposed to that in the peripheral tissue (14).

Microglia are the tissue resident macrophages of the brain and are involved in innate immunity and infection. They are the largest source of inflammatory mediators in the brain and are derived from hematopoietic precursor cells of the yolk sac and are defined as CD11b+/CD45 low (23).

Microglia are incredibly important for the regulation of angiogenesis and vascularization, which plays a key role in tumor development. In pathological states, injury serves as an example; there is microglia-mediated neuronal injury and glial cell injury through the production of proinflammatory factors like cytokines and chemokines (20). Following this activation, inflammatory molecules are released, which, in turn, activates astrocytes and cells of the immune system. In this disease state, the activated cytokines and the chemokines are essential in maintaining the immune surveillance (22).

Cancer cells are capable of avoiding recognition and cancer immune-editing can be conceptualized into three phases : Elimination, Equilibrium and Escape (17, 24). However, when the tumor cells escape immune recognition, they progress to a clinical stage of cancer and mark the escape stage of immune-editing. Tumors are able to escape either because of tumor induced immunosuppression or because of immune system deterioration (17).

GLIOMA MICROENVIRONMENT

Primary tumors of the central nervous system account for only 2% of all tumors. Despite their low incidence, they are highly prevalent in small children, adolescents and young adults with relatively high mortality and morbidity (25). Gliomas are the most common primary central nervous system (CNS) tumors (26), and are classified according to grades (I to IV) of the World Health Organization (WHO) (27). Thus, gliomas are divided into two groups according to the malignancy of the tumor: tumors of low grades (grades I and II), which have slow growth, and high-grade gliomas (III and IV), which strongly infiltrate the brain parenchyma (28).

To date, glioblastoma is the most aggressive glioma and the deadliest of all (29). Even with the current treatments, namely surgical resection, radiotherapy, and chemotherapy (30), it is still an incurable disease with a fairly poor survival rate, ranging between 12 to 15 months,. GBM manages to escape the immune system in a deadly symbiotic collaboration. Furthermore, it can also come from several cell types, not just glial cells. It is mainly present in adults aged 64 years and older, but can also occur in children, with a higher incidence in men compared to women. Gliomas can either be primary (precursor), or secondary (when a low-grade glioma is transformed) (30, 31). Studies have shown that patients with an isocitrate dehydrogenase (IDH) mutation have a longer survival and respond to chemotherapy and radiotherapy well unlike those who do not have the mutation (32).

Tumor-infiltrating immune cells are cells that have left the bloodstream to enter the tumor microenvironment. Their function may change throughout tumor progression, depending on the type of cells and their functional interactions. Indeed, immune cells may play a key role in tumor suppression or in tumor growth support, with specific effects on patient behavior (33).

Tumor-Associated Macrophages (TAM)

They represent microglia which are intrinsic to the brain, and act by creating supporting stroma for the expansion and invasion of neoplastic cells. TAMs that are recruited into the tumor microenvironment of gliomas can release growth factors and cytokines in response to cancer cell activity (34). Thus, TAM infiltrates gliomas in moderate numbers and often exhibit an immunosuppressive phenotype and functional behavior (33).

Natural Killer Cells (NK)

NK cells are the prototypes of innate lymphoid cells. They are characterized by large granular lymphocytes containing perforins and granzymes and have a destructive function. These cells are able to kill tumor cells using soluble molecules of the tumor necrosis factor (TNF) family (35). Additionally, NK cells have also been identified in primary and metastatic brain neoplasms, where they have a key role in suppressing brain tumors (36). The level of tumor infiltration by NK cells tends to remain low and their functionality often affected by factors released by tumors or other immunosuppressive cells (37, 38).

Dendritic Cells (DC)

DC are professional antigen-presenting cells that are found upon recognition of the pathogen at the site of inflammation. In cancers, in addition to antigen presentation, mature dendritic cells (mDC) release cytokines and chemokines to induce tumor-specific T cell activation (39). CD11c+ DCs were studied extensively in GL261 mouse glioma model and showed little or no co-stimulatory molecules in addition to being unable to stimulate T cells. However, these cells favored the development of regulatory T cells (Treg). Analysis of the peripheral blood in glioma patients showed a decrease in numbers compared to healthy patients, suggesting that these cells may have been implicated in tumor pathogenesis (33).

Usually, high levels of cytotoxic T cell-directed human glioma cells (CTL) are associated with increased antitumor activity, whereas high levels of helper T cells (particularly Th17) are thought to be associated with the role of promoting tumor development. Treg cells are a subset of CD4 T cells that express CD25 and FoxP3 (40). These play an important role in the regulation of the immune response by suppressing the proliferation of other T cells presented in the tumor microenvironment, through mechanisms directly dependent on cell contact or indirectly by the secretion of IL-10 and TGFβ (41, 42). In the tumor microenvironment, the production of specific chemokines and cytokines appears to be associated with preferential recruitment of Treg and subsequently poor prognosis (43) (**Figure 1**).

IMMUNE CHECKPOINT INHIBITORS IN GLIOMA THERAPY

Immune Checkpoints in Cancer

To escape immune surveillance, cancer cells have developed several mechanisms that induce a state of immune tolerance and evade immune destruction (44). One of the mechanisms is the use of the inhibitory and costimulatory receptors, called "immune checkpoints" (45). Clinical cancer treatment has become directed towards targeting T cell inhibitory receptors by using immune checkpoint inhibitors (ICI) (46, 47).

Each tumor has a specific dynamic interaction of immune checkpoints, which highlights the importance of having a better understanding of the tumor-immune interactions in a hope to achieve and design a rational combination therapy specific to each tumor (48). Cancer immunotherapy differs from chemotherapy in that it aims to enhance the immune response in different stages of tumor progression and, in so doing, reducing patients' clinical poor outcomes. Chemotherapy, on the other hand, destroys cancer cells directly (**Figure 2**) (49).

In recent years, inhibitors of CTLA-4 have shown remarkable success in cancer immunotherapy. Tremelimumab is a fully human monoclonal antibody to CTLA4 that has shown beneficial responses in clinical trials against different tumors, especially when combined with PD-1/PD-L1 blockade. However, in the case of glioblastoma, several studies reported that anti-

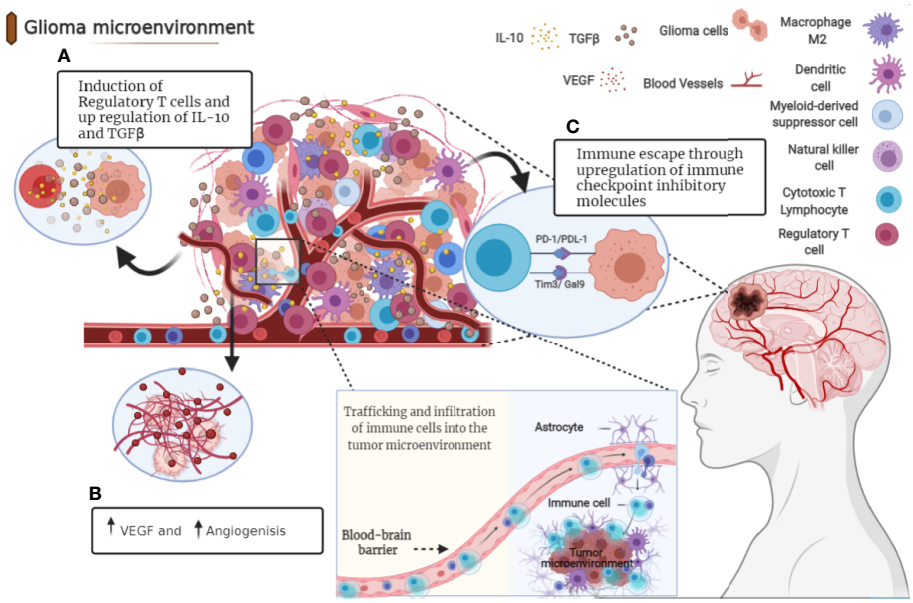

FIGURE 1 | Immunosuppressive microenvironment of glioma. Tumor cells release molecules which contribute to multiple unique immunosuppression mediated by various cellular players in glioma microenvironment. **(A)** After recruitment to the tumor site, Tregs directly suppress the activity of cytolytic T cells and induce their apoptosis through secretion of various types of cytokines including IL-10 and TGFβ. **(B)** Angiogenesis is a pathologic hallmark of glioblastoma mainly mediated by vascular endothelial growth factor (VEGF). **(C)** Immune checkpoints suppress T cell function in glioma microenvironment through distinct mechanisms.

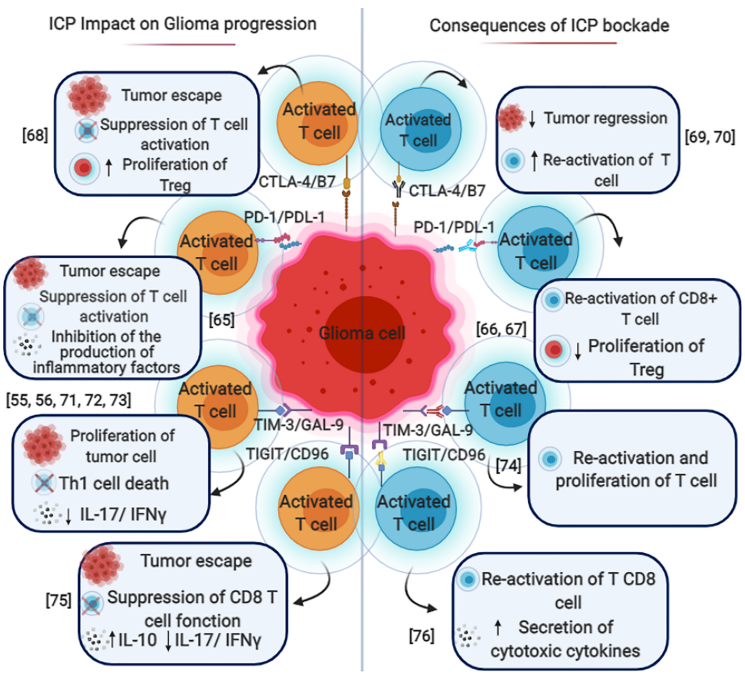

FIGURE 2 | Immune checkpoint blockade in gliomas. The mechanisms by which various immune checkpoints promote each other and contribute to the immunosuppressive microenvironment in gliomas. PD-1/PD-L1, CTLA-4/B7, TIM3/GAL9, and TIGIT/CD96 expressed on different types of immune cells such as T cells (CD4 and CD8) Dendritic cells (DC), Natural killer cells (NK) B cells. These pathways could induce FoxP3 expression and promote tumor escape, cytotoxic cell inhibition and Treg conversion with the help of TGF-β and IL-10. The blockade of these immune checkpoint molecules through mono or combined therapy could be used as a potential therapeutic for glioma and especially glioblastoma.

CTLA-4 and/or anti-PD-1 antibodies exhibit no survival benefit compared to standard chemotherapy (50–53).

However, resistance to ICIs has become a common clinical phenotype that we currently do not have much knowledge about. Collaborative efforts are needed for a deeper understanding of biology to prevent, overcome or reverse this resistance (54).

The successful preclinical trials and the very positive results obtained with other tumors promoted the utilization of immune checkpoint inhibitors in GBM. Indeed, the survey of the NIH Clinical Trials Database (https://www.clinicaltrials.gov) performed on July 2018 showed registered trials of malignant glioma. (**Table 1**).

Due to the recent COVID-19 outbreak, oncologists are wondering about the risk of administering ICIs to patients. The concern was mainly regarding the overlap between the possible pneumological toxicity from anti-PD-1/PD-L1 agents, which could be life threatening in the case of coronavirus-related interstitial pneumonia. The overall incidence rate of ICI-related pneumonitis ranges from 2.5–5% with anti-PD-1/PD-L1 monotherapy to 7–10% with anti-CTLA-4/anti-PD-1 combination therapy (55).

Immune Checkpoints in Glioma

Glioma cells secrete different types of chemokines, cytokines and growth factors that enhance infiltration of various cells such as astrocytes, pericytes, endothelial cells, circulating progenitor cells, and a range of immune cells including microglia, peripheral macrophages, myeloid-derived suppressor cells (MDSC), CD4+ T cells as well as Treg cells into the tumor (56–59). However, identification of these factors may facilitate the improvement of glioma immunotherapy as immunomodulatory and immune evasion mechanisms used by glioma cells.

Glioma cells express the ligands which recognize and bind to partner proteins (receptor) on the surface of immune cells. Subsequent preclinical research showed their important role in the maintenance of peripheral immune tolerance and control overreaction to inflammatory responses. In fact, in glioma case different immune checkpoint molecules have been described such as CTLA-4, PD-1, TIM-3, and LAG-3; each of these receptors has corresponding ligands (60).

PD-1/PD-L1

In 2014, the FDA approved immune checkpoint PD-1 targeting. The anti-PD-1 and anti-PD-L1 mAbs act to block distinct inhibitory signals that unleash T cells to have the ability to eradicate tumors (46, 61–63). In the case of GBM, PD-1 is expressed on T cells, B cells, tumor associated macrophages (TAMs), MDSCs, and NK cells (64). Immunotherapy is used to target the PD-1/PD-L1 pathway (**Figure 3B**) to trigger an antitumor immune response (65–67). The immunosuppressive tumors can then be resected, followed by a continuation of immunotherapy to enhance the functions of the TILs (64, 68).

The BBB is a factor that requires attention, unfortunately numerous drugs that have been tested in clinical trials for GBM patients have failed due to the lack of successful drug delivery across the BBB (69), thus affecting their therapeutic efficacy on intracranial tumors. However, despite recent studies showing that anti-PD-1 antibodies cannot cross the BBB, it is in fact not entirely true. Anti-PD-1 have a mechanism which enables them to bind irreversibly to PD1 or CTLA-4 on peripheral lymphocytes and ultimately penetrate the BBB. Once they have passed the BBB, they can bind TILs which occupy the intracranial tumors (70).

In a previous study, GBM tumor-bearing mice were treated with anti-PD-1 antibody or with a combination of anti-PD-1 and anti-CTLA-4 antibodies. Significant improvement in survival was noted in WT and CD73−/− mice treated with a combination of anti-PD-1 compared to controls (48). Furthermore, GBM patients who received anti-PD-1 therapy showed a persistence of immunosuppressive CD73 high myeloid subsets. Benefits of therapy by immune checkpoint inhibitors in a CD73−/− mouse model should be explored further (48).

TIM3/GAL9

T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) is an inhibitory receptor expressed on the surface of T cells and plays a key role in the inhibition of T cell responses against tumors (71) (**Figure 3C**). Galectin-9 has been identified as a ligand for Tim-3, and upon binding, it results in the apoptosis of T cells and a negative regulation of T cell immunity (72–75).

A recent study investigated the expression of Tim-3 and galectin-9 in glioma tissues and showed that there is an association between the expression of these immune checkpoint receptors and the malignancy of gliomas (50). The expression level of Tim-3 on healthy PBMCs was low and the expression of galectin-9 on non-cancerous brain tissues also followed a similar pattern. However, Tim-3 and galectin-9 were highly expressed in TILs and glioma tissues (50).

Yuan et al. demonstrated that Gal9 was highly expressed in the core than in the periphery of tumors in GBM patients, and that those with high expression of Gal9 had significantly shorter survival than those with low expression (76). This suggests that Gal9 is closely related to glioma patient's prognosis and plays a key role in the malignant progression of GBM (76, 77).

CTLA4

In 2011, the FDA approved an immune checkpoint agent, ipilimumab, a monoclonal antibody (mAbs) that targeted the checkpoint molecule CTLA4. This was based on a randomized phase III clinical trial that demonstrated an improved survival rate with durable clinical response for patients with advanced melanoma (46). As shown in **Figure 3A**; CTLA-4 suppresses antigen-specific T-cell activation and is expressed on activated T cells and $CD4^{+}Foxp3^{+}$ Tregs (78, 79).

A higher expression of CTLA-4 was observed in more severe grades of glioma, and this indicates that it is linked to a worse prognosis (80, 81). It was also found that CTLA-4 significantly correlates with PD-1, CD40, and ICOS (53). Besides, it was tightly associated with CXCL12, CXCR3, CXCR6, and TIGIT (a new promising immune checkpoint-related protein). The combination of these molecules can potentially enhance the efficacy of CTLA-4 blockade in cancer immunotherapy (53). Furthermore, it has been observed that the CTLA-4 antibodies do not cross the BBB. To solve that, Galstyan et al. attempted to

TABLE 1 | Current clinical trials involving immune checkpoint blockade in human glioma.

Clinical trial	Title of the study	Study population	Phase	Intervention	Study design	Date
NCT01670890	Efficacy and Safety of TMZ Plus CDDP in the Patients With Recurrent Malignant Gliomas	Malignant Gliomas	Phase I	Drug: Temozolomide Drug: Temozolomide plus neoadjuvant CDDP	Allocation: Non-Randomized Intervention Model: Parallel Masking: None (Open Label). Primary Purpose: Treatment Assignment	August 2012
NCT03011671	Study of Acetazolamide With Temozolomide in Adults With Newly Diagnosed or Recurrent Malignant Glioma	Malignant Glioma of Brain	Phase I	Drug: Acetazolamide and Tolomozomide	Allocation: N/A Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	October 3, 2018
NCT03973879	Combination of PVSRIPO and Atezolizumab for Adults With Recurrent Malignant Glioma	Malignant Glioma	Phase I Phase II	Biological: PVSRIPO Drug: Atezolizumab	Allocation: N/A Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	February 2020
NCT00953121	Bevacizumab Plus Irinotecan Plus Carboplatin for Recurrent Malignant Glioma (MG)	Malignant Glioma	Phase II	Drug: bevacizumab and CPT-11 and Carboplatin	•Allocation: NonRandomized Intervention Model: Parallel Assignment Masking: None (Open Label) Primary Purpose: Treatment	September 2009
NCT02313272	Hypofractionated Stereotactic Irradiation (HFSRT) With Pembrolizumab and Bevacizumab for Recurrent High Grade Gliomas	Malignant Glioma	Phase I	Radiation: Hypofractionated Stereotactic Irradiation (HFSRT) Drug: Pembrolizumab Drug: Bevacizumab	Allocation: N/A Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	May 5, 2015
NCT02829931	Hypofractionated Stereotactic Irradiation With Nivolumab, Ipilimumab and Bevacizumab in Patients With Recurrent High Grade Gliomas	Malignant Glioma	Phase I	Radiation: Hypofractionated Stereotactic Irradiation Drug: Nivolumab Drug: Bevacizumab Drug: Ipilimumab	Allocation: N/A Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	August 22, 2016
NCT01891747	A Phase I Study of High-dose L-methylfolate in Combination With Temozolomide and Bevacizumab in Recurrent High Grade Glioma	Malignant Glioma	Phase I	Drug: Bevacizumab Drug: Temozolomide Dietary Supplement: Vitamin C	Allocation: N/A Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	July 2013
NCT00271609	Bevacizumab for Recurrent Malignant Glioma	Recurrent High-Grade Gliomas Malignant Gliomas	Phase II	Drug: Bevacizumab	Allocation: Randomized Intervention Model: Parallel Assignment Masking: None (Open Label) Primary Purpose: Treatment	December 2005
NCT02590263	Study Evaluating ABT-414 in Japanese Subjects With Malignant Glioma	Malignant Glioma Glioblastoma Multiforme	Phase I Phase II	Radiation: Whole Brain Radiation Drug: Temozolomide Drug: ABT-414	Allocation: NonRandomized Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	August 24, 2015
NCT00782756	Bevacizumab, Temozolomide and Hypofractionated Radiotherapy for Patients With Newly Diagnosed Malignant Glioma	Brain Cancer Malignant Glioma	Phase II	Other: radiotherapy (RT) in combination with temozolomide and bevacizumab	Allocation: N/A Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	October 28, 2008
NCT01738646	Ph II SAHA and Bevacizumab for Recurrent Malignant Glioma Patients	Recurrent Glioblastoma Multiforme Malignant Glioma Adult Brain Tumor	Phase II	Drug: Vorinostat Drug: Bevacizumab	Allocation: N/A Intervention Model: Single Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	January 2013

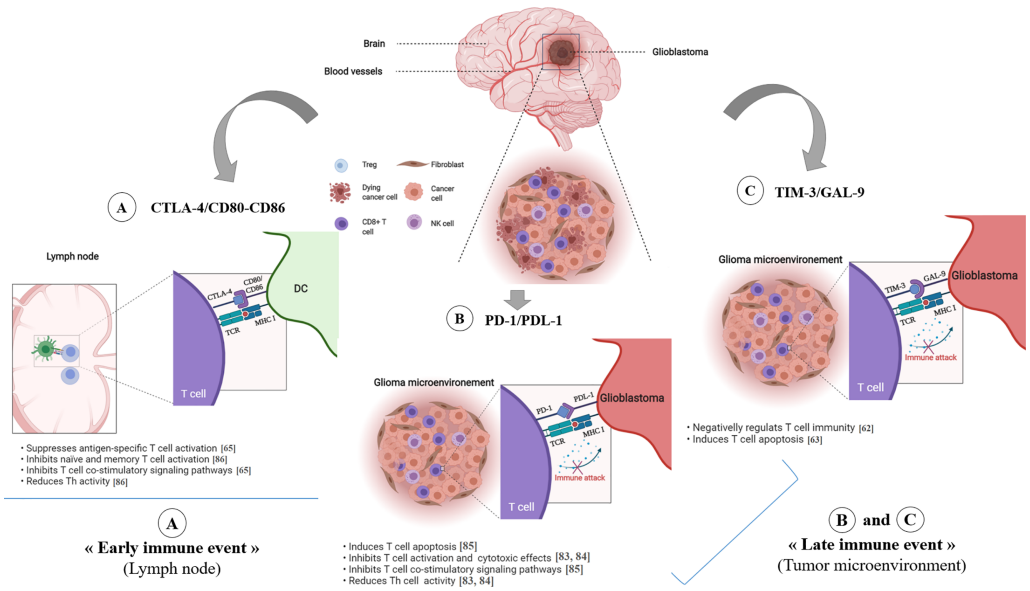

FIGURE 3 | Immune checkpoint inhibitors in glioblastoma. The CTLA-4 immune checkpoint **(A)** operates early during the priming phase of the immune response. CTLA-4 preferentially binds to CD80/CD86 on the surface of APCs, thus leading to decreased T-cell activation and proliferation in the context of tumor antigen presentation. The T cell-expressed inhibitory PD-1 receptor interacts with PD-L1 **(B)**, which is expressed on tumor cells. Engagement of PD-1 and PDL-1, in the context of tumor antigen- presentation by MHC class I molecules, induces T cell apoptosis, inhibits T cell activation/cytotoxicity, promotes Tregs proliferation and blocks the production of inflammatory mediators, resulting in T cell inactivity. TIM-3/GAL-9 pathway **(C)** negatively regulates T cell immunity and induces T cell apoptosis. *ICP, Immune Checkpoint.

combine nanotechnology and immunotherapy. They delivered nanoscale immunoconjugate (NIC) drugs across the BBB to treat GBM (82). They used a versatile drug carrier and poly (β-L-malic acid) (PMLA),a natural polymer obtained from the slime mold *Physarum polycephalum*, to deliver covalently conjugated CTLA-4 and PD-1 antibodies to brain tumor cells. This resulted in a local immune system activation and a prolonged survival of intracranial GBM in GL261-bearing mice (82). Currently, clinical trials of anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) are being performed in patients with glioma, testing the safety, toxicities, and efficacy (78).

LAG3

Lymphocyte activation gene-3 (LAG3), also known as CD223, is a potential cancer immunotherapeutic target because of its negative regulatory role on T cells (83). It is expressed on activated human T and NK cells, and is an activation marker for $CD4^+$ and $CD8^+$ T cells (83). Mair et al. have shown that LAG-3^+ TILs are rarely observed in IDH-wt and absent in IDH-mt glioma (84). However, these cells are more present in an active inflammatory microenvironment but according to LAG-3^+ TIL infiltration; there was no difference in overall survival (84).

TIGIT/CD96

T-cell immunoglobulin and ITIM domain (TIGIT) and CD96 are co-inhibitory receptors. TIGIT is expressed on conventional αβ T cells upon activation, memory T cells, regulatory T cells (Treg), both follicular helper T cells (TFH) and follicular regulatory T cells (TFR), NKT and NK cells (85). However the expression of CD96 has been reported primarily on conventional αβ and γδ T cells, NK cells, and NKT cells (85). Hung et al. have found high levels of TIGIT expression on $CD8^+$ and $CD4^+$ TILs in glioma patients. They have also shown that anti-TIGIT therapy alone had no significant effect on the survival rate in the GBM mouse model (86). However, combination therapy using anti-TIGIT and anti-PD-1 showed a significant increase in survival (87, 88); this was carried out through modulation of both the T cell and myeloid compartments (86). Additionally, elevated frequencies of CD8 + and CD4 + T cells with double expression of IFNγ and TNFα have also been reported during combination therapy, compared to monotherapy and control groups (86).

Zhang et al. showed that high expression of *CD96* was present in the malignant molecule phenotype, including IDH wild type and mesenchymal subtype. They also stressed that it had a positive association with inflammatory activities (89). Indeed, *CD96* showed a high concordance with immune checkpoints such as *PD-1*, *CTLA-4*, *TIGIT*, *TIM-3*, *NR2F6*, and *GITR*, which would suggest a potential synergism (89). In addition to that, they discovered that higher *CD96* expression predicted worse survival rates in glioma and GBM patients overall. This implied that *CD96* blockade may significantly improve the prognosis of glioma patients (89) (**Figure 2**).

CONCLUSION

In the present review, we managed to collect further evidence which demonstrates that the immune system is involved in

glioma physiopathology and describes the general concept of immune response within the glioma microenvironment.

The immune cells are highly inhibited in the glioma microenvironment through various mechanisms, including immune checkpoint inhibitors. Undoubtedly, the discovery of immune checkpoints such as CTLA-4 and PD-1 played a key role in the development of cancer immunotherapy. Although these molecules were originally discovered as molecules with a role in the activation and apoptosis of T cells, subsequent preclinical studies showed their important role in the maintenance of peripheral immune tolerance. In addition, several predictive glioma biomarker studies are completed and many are underway. Indeed, the clinical validation of the identified biomarkers is necessary. Lastly, investigations in glioma immunotherapy should decipher adequate ways to facilitate BBB crossing of these therapeutic molecules in order to potentially benefit from current and future therapies. Integrated approaches should also be developed to identify patient-specific choices for checkpoint monotherapies or combination therapies.

AUTHOR CONTRIBUTIONS

AG, SK, MT, KR, and SR wrote the review manuscript sections. AB wrote the review manuscript and supervised the study. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. OMS. *Rapport Sur La Situation Mondiale Des Maladies non Transmissibles 2014*. WHO. Available at: http://www.who.int/nmh/publications/ncd-status-report-2014/fr/ (Accessed consulté le juin 24, 2020).
2. Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE, et al. The Epidemiology of Glioma in Adults: A "State of the Science" Review. *Neuro-Oncol* (2014) 16(7):896–913. doi: 10.1093/neuonc/nou087
3. Yeo AT, Charest A. Immune Checkpoint Blockade Biology in Mouse Models of Glioblastom. *J Cell Biochem* (2017) 118(9):2516–2527. doi: 10.1002/jcb.25948
4. Chouaib S, Hage FE, Benlalam H, Mami-Chouaib F. Immunothérapie Du Cancer: Espoirs Et Réalités. *médecine/sciences* (2006) 22(8–9):755–9. doi: 10.1051/medsci/20062289755
5. Migliorini D, Dietrich P-Y. Progrès Dans L'immunothérapie Des Tumeurs Cérébrales: Difficultés Et Perspectives. *Rev Médicale Suisse* (2016) 12(516):828–831. doi: 10.1159/000436986
6. Pardoll DM. The Blockade of Immune Checkpoints in Cancer Immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–264. doi: 10.1038/nrc3239
7. Omuro A, Vlahovic G, Lim M, Sahebjam S, Baehring J, Cloughesy T, et al. Nivolumab With or Without Ipilimumab in Patients With Recurrent Glioblastoma: Results From Exploratory Phase I Cohorts of CheckMate 143. *Neuro-Oncol* (2018) 20(5):674–686. doi: 10.1093/neuonc/nox208
8. Schalper KA, Rodriguez-Ruiz ME, Diez-Valle R, López-Janeiro A, Porciuncula A, Idoate MA, et al. Neoadjuvant Nivolumab Modifies the Tumor Immune Microenvironment in Resectable Glioblastoma. *Nat Med* (2019) 25:470–476. doi: 10.1038/s41591-018-0339-5
9. Filley AC, Henriquez M, Dey M. Recurrent Glioma Clinical Trial, CheckMate-143: The Game is Not Over Yet. *Oncotarget* (2017) 8(53):91779–91794. doi: 10.18632/oncotarget.21586
10. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and Functional Features of Central Nervous System Lymphatic Vessels. *Nature* (2015) 523(7560):337–41. doi: 10.1038/nature14432
11. Louveau A, Harris TH, Kipnis J. Revisiting the Concept of CNS Immune Privilege. *Trends Immunol* (2015) 36(10):569–577. doi: 10.1016/j.it.2015.08.006
12. Murlidharan G, Samulski RJ, Asokan A. Biology of Adeno-Associated Viral Vectors in the Central Nervous System. *Front Mol Neurosci* (2014) 7:76. doi: 10.3389/fnmol.2014.00076
13. Lowenstein PR, Castro MG. Inflammation and Adaptive Immune Responses to Adenoviral Vectors Injected Into the Brain: Peculiarities, Mechanisms, and Consequences. *Gene Ther* (2003) 10(11):946–54. doi: 10.1038/sj.gt.3302048
14. Dantzer R. Neuroimmune Interactions: From the Brain to the Immune System and Vice Vers. *Physiol Rev* (2017) 98(1):477–504. doi: 10.1152/physrev.00039.2016
15. Bakker ENTP, Bacskai BJ, Arbel-Ornath M, Aldea R, Bedussi B, Morris AWJ, et al. Lymphatic Clearance of the Brain: Perivascular, Paravascular and Significance for Neurodegenerative Disease. *Cell Mol Neurobiol* (2016) 36(2):181–194. doi: 10.1007/s10571-015-0273-8
16. Aspelund A, Tammela T, Antila S, Nurmi H, Leppänen VM, Zarkada G, et al. The Schlemm's Canal is a VEGF-C/VEGFR-3–Responsive Lymphatic-Like Vessel. *J Clin Invest* (2014) 124(9):3975–3986. doi: 10.1172/JCI75395
17. Teng MWL, Galon J, Fridman W-H, Smyth MJ. From Mice to Humans: Developments in Cancer Immunoediting. *J Clin Invest* (2015) 125(9):3338–3346. doi: 10.1172/JCI80004
18. Morimoto K, Nakajima K. Role of the Immune System in the Development of the Central Nervous Syste. *Front Neurosci* (2019) 13:916. doi: 10.3389/fnins.2019.00916
19. Gorby C, Martinez-Fabregas J, Wilmes S, Moraga I. Mapping Determinants of Cytokine Signaling via Protein Engineerin. *Front Immunol* (2018) 9:2143. doi: 10.3389/fimmu.2018.02143
20. Kennedy RH, Silver R. Neuroimmune Signaling: Cytokines and the Central Nervous Syste. In: DW Pfaff, ND Volkow, editors. *Neuroscience in the 21st Century: From Basic to Clinica*. New York: NY: Springer (2016). p. p601–641 doi: 10.1007/978-1-4939-3474-4_174
21. Zhao B, Yin Q, Fei Y, Zhu J, Qiu Y, Fang W, et al. Research Progress of Mechanisms for Tight Junction Damage on Blood–Brain Barrier Inflammation. *Arch Physiol Biochem* (2020), 1–12. doi: 10.1080/13813455.2020.1784952
22. Ramesh G, MacLean AG, Philipp MT. Cytokines and Chemokines at the Crossroads of Neuroinflammation, Neurodegeneration, and Neuropathic Pai. *Mediators Inflammation* (2013) 2013:480739. doi: 10.1155/2013/480739
23. Lenz KM, Nelson LH. Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Functio. *Front Immunol* (2018) 9:698. doi: 10.3389/fimmu.2018.00698
24. Gonzalez H, Hagerling C, Werb Z. Roles of the Immune System in Cancer: From Tumor Initiation to Metastatic Progression. *Genes Dev* (2018) 32(19–20):1267. doi: 10.1101/gad.314617.118
25. Buckner JC, Brown PD, O'Neill BP, Meyer FB, Wetmore CJ, Uhm JH. Central Nervous System Tumors. *Mayo Clin Proc* (2007) 82(10):1271–1286. doi: 10.4065/82.10.1271
26. Ilkhanizadeh S, Lau J, Huang M, Foster DJ, Wong R, Frantz A, et al. Glial Progenitors as Targets for Transformation in Glioma. *Adv Cancer Res* (2014) 121:1–65. doi: 10.1016/B978-0-12-800249-0.00001-9
27. Louis DN, Perry A, Reifenberger G, Deimling AV, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of

Tumors of the Central Nervous System: A Summary. *Acta Neuropathol (Berl.)* (2016) 131(6):803–820. doi: 10.1007/s00401-016-1545-1
28. Gieryng A, Pszczolkowska D, Walentynowicz KA, Rajan WD, Kaminska B. Immune Microenvironment of Gliomas. *Lab Invest* (2017) 97(5):498–518. doi: 10.1038/labinvest.2017.19
29. Charles NA, Holland EC, Gilbertson R, Glass R, Kettenmann H. The Brain Tumor Microenvironment. *Glia* (2011) 59(8):1169–1180. doi: 10.1002/glia.21136
30. Manini I, Caponnetto F, Bartolini A, Ius T, Mariuzzi L, Di Loreto C, et al. Role of Microenvironment in Glioma Invasion: What We Learned From In Vitro Model. *Int J Mol Sci* (2018) 19(1):147. doi: 10.3390/ijms19010147
31. Davis ME. Glioblastoma: Overview of Disease and Treatmen. *Clin J Oncol Nurs* (2016) 20(5):S2–S8. doi: 10.1188/16.CJON.S1.2-8
32. SongTao Q, Lei Y, Si G, YanQing D, HuiXia H, XueLin Z, et al. IDH Mutations Predict Longer Survival and Response to Temozolomide in Secondary Glioblastoma. *Cancer Sci* (2012) 103(2):269–273. doi: 10.1111/j.1349-7006.2011.02134.x
33. Domingues P, González-Tablas M, Otero A, Pascual D, Miranda D, Ruiz L, et al. Tumor Infiltrating Immune Cells in Gliomas and Meningiomas. *Brain Behav Immun* (2016) 53:1–15. doi: 10.1016/j.bbi.2015.07.019
34. Hambardzumyan D, Gutmann DH, Kettenmann H. The Role of Microglia and Macrophages in Glioma Maintenance and Progression. *Nat Neurosci* (2016) 19(1):20–7. doi: 10.1038/nn.4185
35. Morvan MG, Lanier LL. NK Cells and Cancer: You can Teach Innate Cells New Tricks. *Nat Rev Cancer* (2016) 16(1):7–19. doi: 10.1038/nrc.2015.5
36. Alizadeh BH, Foroumadi A, Emami S, Khoobi M, Panah F, Ardestani SK, et al. Isochaihulactone Analogues: Synthesis and Anti-Proliferative Activity of Novel Dibenzylbutyrolactones. *Eur J Med Chem* (2010) 45(12):5979–5984. doi: 10.1016/j.ejmech.2010.09.064
37. Lines JL, Pantazi E, Mak J, Sempere LF, Wang L, O'Connell S, et al. VISTA Is an Immune Checkpoint Molecule for Human T Cell. *Cancer Res* (2014) 74 (7):1924–1932. doi: 10.1158/0008-5472.CAN-13-1504
38. Kmiecik J, Poli A, Brons NHC, Waha A, Eide GE, Enger PØ, Zimmer J, et al. Elevated CD3+ and CD8+ Tumor-Infiltrating Immune Cells Correlate With Prolonged Survival in Glioblastoma Patients Despite Integrated Immunosuppressive Mechanisms in the Tumor Microenvironment and at the Systemic Level. *J Neuroimmunol* (2013) 264(1):71–83. doi: 10.1016/j.jneuroim.2013.08.013
39. Kim JW, Kane JR, Panek WK, Young JS, Rashidi A, Yu D, et al. A Dendritic Cell-Targeted Adenoviral Vector Facilitates Adaptive Immune Response Against Human Glioma Antigen (CMV-IE) and Prolongs Survival in a Human Glioma Tumor Mode. *Neurotherapeutics* (2018) 15(4):1127–1138. doi: 10.1007/s13311-018-0650-3
40. Dunn GP, Dunn IF, Curry WT. Focus on TILs: Prognostic Significance of Tumor Infiltrating Lymphocytes in Human Glioma. *Cancer Immun Arch* (2007) 7(1):12. doi: 10.2217/cns-2017-0037
41. Hamilton A, Sibson NR. Role of the Systemic Immune System in Brain Metastasis. *Mol Cell Neurosci* (2013) 53:42–51. doi: 10.1016/j.mcn.2012.10.004
42. Ghouzlani A, Kandoussi S, Rafii S, Lakhdar A, Badou A. High Expression Levels of Foxp3 and VISTA in Advanced Human Gliomas and Impact on Patient's Prognosis. *Arch Clin Biomed Res* (2020) 4(6):691–703.
43. Biswas SK, Mantovani A. Macrophage Plasticity and Interaction With Lymphocyte Subsets: Cancer as a Paradigm. *Nat Immunol* (2010) 11 (10):889–96. doi: 10.1038/ni.1937
44. Drake CG, Jaffee E, Pardoll DM. Mechanisms of Immune Evasion by Tumors. *Adv Immunol* (2006) 90:51–81. doi: 10.1016/S0065-2776(06)90002-9
45. Armand P. Immune Checkpoint Blockade in Hematologic Malignancies. *Blood* (2015) 125(22):3393–400. doi: 10.1182/blood-2015-02-567453.
46. Sharma P, Allison JP. Dissecting the Mechanisms of Immune Checkpoint Therapy. *Nat Rev Immunol* (2020) 20(2):75–76. doi: 10.1038/s41577-020-0275-8
47. Chraa D, Naim A, Olive D, Badou A. T Lymphocyte Subsets in Cancer Immunity: Friends or Foes. *J Leukoc Biol* (2019) 105(2):243–55. doi: 10.1002/JLB.MR0318-097R
48. Goswami S, Walle T, Cornish AE, Basu S, Anandhan S, Fernandez I, et al. Immune Profiling of Human Tumors Identifies CD73 as a Combinatorial Target in Glioblastoma. *Nat Med* (2020) 26(1):39–46. doi: 10.1038/s41591-019-0694-x
49. Singh S, Hassan D, Aldawsari HM, Molugulu N, Shukla R, Kesharwani P. Immune Checkpoint Inhibitors: A Promising Anticancer Therapy. *Drug Discovery Today* (2020) 25(1):223–229. doi: 10.1016/j.drudis.2019.11.003
50. Liu Z, Han H, He X, Li S, Wu C, Yu C, et al. Expression of the Galectin-9-Tim-3 Pathway in Glioma Tissues is Associated With the Clinical Manifestations of Glioma. *Oncol Lett* (2016) 11(3):1829–1834. doi: 10.3892/ol.2016.4142
51. Harris-Bookman S, Mathios D, Martin AM, Xia Y, Kim E, Xu H, et al. Expression of LAG-3 and Efficacy of Combination Treatment With Anti-LAG-3 and Anti-PD-1 Monoclonal Antibodies in Glioblastoma. *Int J Cancer* (2018) 143(12):3201–3208. doi: 10.1002/ijc.31661
52. Lamrani M, Sassi N, Paul C, Yousfi N, Boucher JL, Gauthier N, et al. Tlr4/Ifnγ Pathways Induce Tumor Regression via NOS II-Dependent NO and ROS Production in Murine Breast Cancer Models. *OncoImmunology* (2016) 5(5): e1123369. doi: 10.1080/2162402X.2015.1123369
53. Liu F, Huang J, Liu X, Cheng Q, Luo C, Liu Z. CTLA-4 Correlates With Immune and Clinical Characteristics of Glioma. *Cancer Cell Int* (2020) 20 (1):7. doi: 10.1186/s12935-019-1085-6
54. Schoenfeld AJ, Hellmann MD. Acquired Resistance to Immune Checkpoint Inhibitor. *Cancer Cell* (2020) 37(4):443–455. doi: 10.1016/j.ccell.2020.03.017
55. Bersanelli M. Controversies About COVID-19 and Anticancer Treatment With Immune Checkpoint Inhibitors. *Immunotherapy* (2020) 12(5):269–273. doi: 10.2217/imt-2020-0067
56. Fecci PE, Mitchell DA, Whitesides JF, Xie W, Friedman AH, Archer GE, et al. Increased Regulatory T-Cell Fraction Amidst a Diminished CD4 Compartment Explains Cellular Immune Defects in Patients With Malignant Glioma. *Cancer Res* (2006) 66:3294–302. doi: 10.1158/0008-5472.CAN-05-3773
57. Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, Ahmadi R, et al. Effector T-Cell Infiltration Positively Impacts Survival of Glioblastoma Patients and is Impaired by Tumor-Derived TGF-β. *Clin Cancer Res* (2011) 17:4296–308. doi: 10.1158/1078-0432.CCR-10-2557
58. Alexiou GA, Vartholomatos G, Karamoutsios A, Batistatou A, Kyritsis AP, Voulgaris V. Circulating Progenitor Cells: A Comparison of Patients With Glioblastoma or Meningioma. *Acta Neurol Belg* (2013) 113:7–11 doi: 10.1007/s13760-012-0097-y
59. Wainwright DA, Dey M, Chang A, Lesniak MS. Targeting Tregs in Malignant Brain Cancer: Overcoming IDO. *Front Immunol* (2013) 4:116. doi: 10.3389/fimmu.2013.00116
60. Kaminska B, Ciechomska IA, Cyranowski S. Chapter 3 - Autophagy in Brain Tumor Immune Evasion and Responses to Immunotherapy. In: S Chouaib, editor. *Sensitizing Agent-Canc Resistant-Cell Mediated Immtherap, Autophagy in Immune Response: Impact on Cancer Immunotherap*. Academic Press (2020) pp. 29–52. doi: 0.1016/B978-0-12-819609-0.00003-1
61. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott M, Gutierrez M, et al. P. PD-1 Blockade With Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. *N Engl J Med* (2015) 372(4):311–9. doi: 10.1056/NEJMoa1411087
62. Baruch K, Deczkowska A, Rosenzweig N, Tsitsou-Kampeli A, Sharif A, Matcovitch O, et al. PD-1 Immune Checkpoint Blockade Reduces Pathology and Improves Memory in Mouse Models of Alzheimer's Disease. *Nat Med* (2016) 22. doi: 10.1038/nm.4022
63. Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, et al. Association of PD-1, PD-1 Ligands, and Other Features of the Tumor Immune Microenvironment with Response to Anti-PD-1 Therapy. *J Am Assoc Cancer Res* (2014) 20(19):5064–74. doi: 10.1158/1078-0432.CCR-13-3271
64. Shu C, Li Q. Current Advances in PD-1/PD-L1 Axis-Related Tumour-Infiltrating Immune Cells and Therapeutic Regimens in Glioblastoma. *Crit Rev Oncol Hematol* (2020) 151:102965. doi: 10.1016/j.critrevonc.2020.102965
65. Boussiotis VA, Charest A. Immunotherapies for Malignant Glioma. *Oncogene* (2018) 37(9):1121–41. doi: 10.1038/s41388-017-0024-z
66. Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH, et al. Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Mode. *Cancer Immunol Res* (2016) 4(2):124–35. doi: 10.1158/2326-6066.CIR-15-0151
67. Zeng J, See AP, Phallen J, Jackson CM, Belcaid Z, Ruzevick J, et al. Anti-PD-1 Blockade and Stereotactic Radiation Produce Long-Term Survival in Mice With Intracranial Gliomas. *Int J Radiat Oncol Biol Phys* (2013) 86(2):343–9. doi: 10.1016/j.ijrobp.2012.12.025

68. Ghouzlani A, Rafii S, Karkouri M, Karkouri A, Badou A. The Promising IgSF11 Immune Checkpoint Is Highly Expressed in Advanced Human Gliomas and Associates to Poor Prognosis. *Front Oncol* (2021) 10:608609. doi: 10.3389/fonc.2020.608609
69. van Tellingen O, Yetkin-Arik B, de Gooijer MC, Wesseling P, Wurdinger T, de Vries HE. Overcoming the Blood–Brain Tumor Barrier for Effective Glioblastoma Treatment. *Drug Resist Updat* (2015) 19:1–12. doi: 10.1016/j.drup.2015.02.002
70. van Bussel MTJ, Beijnen JH, Brandsma D. Intracranial Antitumor Responses of Nivolumab and Ipilimumab: A Pharmacodynamic and Pharmacokinetic Perspective, a Scoping Systematic Review. *BMC Cancer* (2019) 19(1):519. doi: 10.1186/s12885-019-5741-y
71. Koguchi K, Anderson DE, Yang L, O'Connor KC, Kuchroo VK, Hafler DA. Dysregulated T Cell Expression of TIM3 in Multiple Sclerosis. *J Exp Med* (2006) 203(6):1413–1418. doi: 10.1084/jem.20060210
72. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 Ligand Galectin-9 Negatively Regulates T Helper Type 1 Immunity. *Nat Immunol* (2005) 6(12):1245–1252. doi: 10.1038/ni1271
73. Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, et al. TIM-3 is Expressed on Activated Human CD4+ T Cells and Regulates Th1 and Th17 Cytokines. *Eur J Immunol* (2009) 39:2492–501. doi: 10.1002/eji.200939274
74. Romani M, Pistillo MP, Carosio R, Morabito A, Banelli B. Immune Checkpoints and Innovative Therapies in Glioblastom. *Front Oncol* (2018) 8.
75. Du W, et al. TIM-3 as a Target for Cancer Immunotherapy and Mechanisms of Actio. *Int J Mol Sci* (2017) 18:645.
76. Yuan F, Ming H, Wang Y, Yang Y, Yi L, Li T, et al. Molecular and Clinical Characterization of Galectin-9 in Glioma Through 1,027 Samples. *J Cell Physiol* (2020) 235(5):4326–4334. doi: 10.1002/jcp.29309
77. Zhang Q, Luan H, Wang L, He F, Zhou H, Xu X, et al. Galectin-9 Ameliorates Anti-GBM Glomerulonephritis by Inhibiting Th1 and Th17 Immune Responses in Mice. *Am J Physiol Renal Physiol* (2014) 306:F822–32. doi: 10.1152/ajprenal.00294.2013
78. Huang J, Liu F, Liu Z, Tang H, Wu H, Gong Q, et al. Immune Checkpoint in Glioblastoma: Promising and Challengin. *Front Pharmacol* (2017) 8:242. doi: 10.3389/fphar.2017.00242
79. Topalian SL, Sharpe AH. Balance and Imbalance in the Immune System: Life on the Edge. *Immunity* (2014) 41(5):682–4. doi: 10.1016/j.immuni.2014.11.005
80. Peggs KS, Quezada SA, Korman AJ, Allison JP. Principles and Use of Anti-CTLA4 Antibody in Human Cancer Immunotherapy. *Curr Opin Immunol* (2006) 18:206–13. doi: 10.1016/j.coi.2006.01.011
81. Fecci PE, Ochiai H, Mitchell DA, Grossi PM, Sweeney AE, Archer GE, et al. Systemic CTLA-4 Blockade Ameliorates Glioma-Induced Changes to the CD4 + T Cell Compartment Without Affecting Regulatory T-Cell Function. *Clin Cancer Res* (2007) 13(7):2158–67.
82. Galstyan A, Markman JL, Shatalova ES, Chiechi A, Korman AJ, Patil R, et al. Blood–brain Barrier Permeable Nano Immunoconjugates Induce Local Immune Responses for Glioma Therapy. *Nat Commun* (2019) 10(1):1–13. doi: 10.1038/s41467-019-11719-3
83. Andrews LP, Marciscano AE, Drake CG, Vignali DAA. LAG3 (CD223) as a Cancer Immunotherapy Target. *Immunol Rev* (2017) 276(1):80–96. doi: 10.1111/imr.12519
84. Mair M, Kiesel B, Feldmann K, Widhalm G, Dieckmann K, Woehrer A, et al. Lymphocyte-Activation Gene 3 (LAG-3) Expression in the Inflammatory Microenvironment of Glioma. *J Clin Oncol* (2020) 38(15_suppl):2553–2553. doi: 10.1200/JCO.2020.38.15_suppl.2553
85. Dougall WC, Kurtulus S, Smyth MJ, Anderson AC. TIGIT and CD96: New Checkpoint Receptor Targets for Cancer Immunotherapy. *Immunol Rev* (2017) 276(1):112–120. doi: 10.1111/imr.12518
86. Hung AL, Maxwell R, Theodros D, Belcaid Z, Mathios D, Luksik AS, et al. TIGIT and PD-1 Dual Checkpoint Blockade Enhances Antitumor Immunity and Survival in GBM. *OncoImmunology* (2018) 7(8):e1466769. doi: 10.1080/2162402X.2018.1466769
87. Marin-Acevedo JA, Dholaria B, Soyano AE, Knutson KL, Chumsri S, Lou Y. Next Generation of Immune Checkpoint Therapy in Cancer: New Developments and Challenges. *J Hematol Oncol* (2018) 11:39.
88. Chauvin J-M, Pagliano O, Fourcade J, Sun Z, Wang H, Sander C, et al. TIGIT and PD-1 Impair Tumor Antigen–Specific $CD8^+$ T Cells in Melanoma Patients. *J Clin Invest* (2015) 125:2046–58.
89. Zhang Q, Zhong H, Fan Y, Liu Q, Song J, Yao S, et al. Immune and Clinical Features of CD96 Expression in Glioma by in Silico Analysi. *Front Bioeng Biotechnol* (2020) 8:592. doi: 10.3389/fbioe.2020.00592

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Clinical Features of HIV Arthropathy in Children: A Case Series and Literature Review

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Background: HIV infection has been associated with a non-erosive inflammatory arthritis in children, although few published reports exist. This study describes the clinical, laboratory and imaging features of this noncommunicable disease in a series of HIV-infected children in South Africa.

Methods: A database search was conducted to identify HIV-infected children enrolled in a Paediatric Rheumatology service in Cape Town, South Africa between 1 January 2010 and 31 December 2020. Retrospective data were collected from individuals classified with HIV arthropathy, based on a predefined checklist. Demographic, clinical, laboratory, sonographic, therapeutic, and outcomes data were extracted by chart review. Descriptive statistical analysis was performed using R (v4.0.3).

Results: Eleven cases of HIV arthropathy were included in the analysis. Cases predominantly presented in older boys with low CD4+ counts. Median age at arthritis onset was 10.3 years (IQR 6.9 – 11.6) and the male-female ratio was 3.0. The median absolute CD4+ count was 389 cells/uL (IQR 322 – 449). The clinical presentation was variable, with both oligoarthritis and polyarthritis being common. Elevated acute phase reactants were the most consistent laboratory feature, with a median ESR of 126 mL/h (IQR 67 – 136) and median CRP of 36 mg/L (IQR 25 – 68). Ultrasonography demonstrated joint effusions and synovial hypertrophy. Response to therapy was slower than has generally been described in adults, with almost all cases requiring more than one immunosuppressive agent. Five children were discharged in established remission after discontinuing immunotherapy, however outcomes data were incomplete for the remaining six cases.

Conclusions: In this case series, HIV arthropathy was associated with advanced immunosuppression. Therapeutic modalities included immunomodulators and antiretroviral therapy, which consistently induced disease remission although data were limited by a high rate of attrition. Prospective studies are needed to define and understand this HIV-associated noncommunicable disease.

Keywords: paediatric HIV, inflammatory arthritis, Africa, autoimmunity, non-communicable disease, musculoskeletal manifestations

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INTRODUCTION

Autoimmune disease has been reported in the context of human immunodeficiency virus (HIV) infection since the origin of the HIV pandemic. The spectrum of HIV-associated rheumatic disease has evolved over time, likely reflecting the effects of antiretroviral therapy (ART) on survival and immunological function (1–4). Variable prevalence rates (11 - 72%) have been reported for musculoskeletal disorders in HIV-infected adults (5–8). However, data are scarce for the paediatric population.

South Africa is positioned at the epicentre of the global HIV pandemic, with a prevalence of 19% in adults and approximately 340,000 HIV-infected children (9, 10). The number of new paediatric HIV infections decreased almost five-fold from 47,000 in 2010 to 10,000 in 2020, reflecting reduced vertical transmission. However, a considerable treatment gap remains in the paediatric population, with only 63% of HIV-infected children receiving ART and only 46% of those on ART achieving viral suppression (11). Despite this, mortality in HIV-infected children has declined dramatically (12–14). As life expectancy in this high-risk group increases, a greater number of children may present with rheumatic sequelae of HIV infection and therapy.

HIV infection disrupts the immunological milieu, giving rise to a broad spectrum of inflammatory and immune-mediated syndromes (1–3). In the setting of advanced HIV infection, multiple immune aberrations contribute to loss of self-tolerance. Proposed mechanisms include the destruction of CD4+ T-lymphocyte populations, autoreactive CD8+ T-lymphocyte responses, T-regulatory cell dysfunction, exposure of autoantigens, B-lymphocyte activation, generation of autoantibodies and immune complexes, molecular mimicry, and direct viral invasion of immunologically active tissue (1, 15–20). A combination of these mechanisms have been implicated in the diverse immune-mediated non-communicable sequelae of HIV infection. Conversely, the balance of immune activation and deficiency induced by HIV infection appears to be protective against certain autoimmune phenomena, including rheumatic heart disease and systemic lupus erythematosus (SLE) (2, 21, 22).

Inflammatory arthritis appears to be associated with HIV infection, although the existing literature is limited by conflicting definitions of disease, inconsistent application of classification systems, and variable availability of relevant diagnostic tests and expertise (2). The spondyloarthropathies have been extensively investigated in relation to HIV infection (23–26). These conditions are strongly associated with the human leucocyte antigen (HLA)-B27 allele. This HLA haplotype is extremely uncommon in Black African populations (27, 28), and resultantly the spondyloarthropathies were rarely reported in Africa prior to the HIV pandemic. The prevalence of these conditions in sub-Saharan Africa appears to have increased dramatically in tandem with the HIV pandemic (29–31). HIV-related immunosuppression can reduce disease activity and even induce remission in patients with established rheumatoid arthritis (RA) (32). Early reports describing this phenomenon led to the conviction that HIV and RA were mutually exclusive disorders. However, this position has since shifted in the face of a growing body of evidence indicating that HIV-positive individuals can develop an entity clinically indistinguishable from RA (8, 33–35), with a reported prevalence of 0.1 - 5.0% (31, 36–38). Most cases present in immunocompetent HIV-infected individuals (34, 39).

HIV arthropathy has been defined as a self-limiting, asymmetrical, nonerosive, oligo- or polyarthritis that predominantly involves the lower limbs and typically resolves within six weeks (1, 2, 40). Some authors have suggested that rheumatoid factor (RF) and HLA-B27 negativity be required for its diagnosis (41, 42), in order to differentiate the entity from RA or spondyloarthropathies. Reveille and colleagues define adult-onset HIV arthropathy as "arthritis of the large joints, lasting less than six weeks, in the absence of either HLA B27 positivity, or radiological changes … distinct from any other recognized rheumatological entity, with no discerned infective triggers" (41). A prevalence of 0.4 – 13.8% has been reported in adults (2, 37, 43–47), with higher rates reported in studies conducted in predominantly African populations (31, 44, 48). HIV arthropathy is generally described in the setting of advanced HIV (8, 49). When performed, synovial fluid analysis reveals sterile inflammation, with nonspecific features of chronic synovitis on biopsy (49, 50). Tubulo-reticular inclusions, HIV-associated p24 antigen, and HIV DNA-containing dendritic cells have been identified in the synovial tissue of affected individuals, suggesting a direct viral aetiology (40, 49, 51, 52). It has been proposed that HIV arthropathy may represent a type of reactive arthritis triggered by the virus itself (2, 53), however the precise pathophysiology has yet to be described.

The optimal management of HIV arthropathy has not been completely defined. In the current understanding of the disease, therapy is primarily directed at the underlying cause. Therefore, effective ART and immune reconstitution are the cornerstones of management. Specific therapy may also be directed against the inflammatory component of HIV arthropathy. Chloroquine is frequently used and has been shown to have beneficial anti-viral activity in addition to its anti-inflammatory effects (54–56). Nonsteroidal anti-inflammatories are routinely used for symptomatic control. Systemic corticosteroids may be used, although the duration of therapy should be limited to reduce toxicity. Similarly, intra-articular corticosteroids are considered safe and efficacious. The role of other immunomodulator therapies, including methotrexate, sulfasalazine, and biologic agents, remains undefined.

There are few published reports on HIV arthropathy in children. A case series from Durban, South Africa described 35 HIV-infected children with arthritis (50). This series reported a

Abbreviations: ALC, Absolute lymphocyte count; ANC, Absolute neutrophil count; ART, Antiretroviral therapy; CLQ, Chloroquine; CMV, Cytomegalovirus; CRP, C-reactive protein; DIPJ, Distal interphalangeal joint; ESR, Erythrocyte sedimentation rate; Hb, Haemoglobin; HIV, Human immunodeficiency virus; HLA, Human leucocyte antigen; HREC, Human Research Ethics Committee; HSV, Herpes simplex virus; IPJ, Interphalangeal joint; IQR, Interquartile range; IRIS, Immune reconstitution inflammatory syndrome; LAD, Lymphadenopathy; MCPJ, Metacarpophalangeal joint; MTX, Methotrexate; NSAID, Nonsteroidal anti-inflammatory drug; PIPJ, Proximal interphalangeal joint; RA, Rheumatoid arthritis; SLE, Systemic lupus erythematosus; TB, Tuberculosis; uL, Microlitre; UWFA, Underweight-for-age; VPS, Ventriculoperitoneal shunt; VZV, Varicella zoster virus; WCC, White cell count; WFA, Weight-for-age.

male predominance, and a mean age of onset of 5.5 years. The most common pattern of articular involvement was asymmetrical polyarthritis, and RF and HLA-B27 were negative in almost all cases. Another report from the same centre described seven HIV-infected children with both arthritis and uveitis, in whom no cause other than HIV could be found (57). Two isolated case reports of paediatric HIV-associated arthritis have been published in India (58, 59). Arthritis was the presenting feature of HIV infection in both cases. A case series of musculoskeletal manifestations in HIV-infected children in India reported 3 cases of inflammatory arthritis, all of which occurred in older girls with advanced immunosuppression (60). Another report from India described a single case of HIV arthropathy, and three cases of HLA-B27-associated arthritis in HIV-infected children (61). A study of juvenile idiopathic arthritis from Zambia reported 7 cases of arthritis in HIV-infected children, but did not provide details of subgroup analysis (62). A large South African study which screened perinatally HIV-infected adolescents and HIV-uninfected controls for musculoskeletal disorders reported a low prevalence of musculoskeletal abnormalities, which were associated with advanced immunosuppression and longer duration of ART (63). A pre-ART study conducted among 40 HIV-infected children in the United States found no clinically significant rheumatological manifestations, although high rates of hypergammaglobulinemia, autoantibody positivity, and the presence of circulating immune complexes were reported (64). A Mexican study of 26 HIV-infected children described several cases of Raynaud's phenomenon and peripheral vasculitis, and one child with septic arthritis (65). Although no cases of inflammatory arthritis were observed in the North American children, arthralgia was commonly reported in both studies (64, 65).

There is very limited published data on paediatric HIV arthropathy, both globally and in sub-Saharan Africa. This study aimed to describe features of HIV arthropathy in a case series of children who presented to a Paediatric Rheumatology centre in Cape Town, South Africa.

MATERIALS AND METHODS

Aims, Design, and Setting

This study was conducted at two large referral centres in Cape Town, South Africa. The study was approved by the Human Research Ethics Committee of the University of Cape Town (HREC 011/2021). The aim of this study was to describe HIV arthropathy in terms of clinical presentation, laboratory characteristics, sonographic features, therapy, and outcomes in a case series of HIV-infected children in South Africa. Relevant data were collected by retrospective review of medical records.

Participants

All HIV-infected paediatric patients enrolled in the Paediatric Rheumatology clinics at either Red Cross War Memorial Children's Hospital or Groote Schuur Hospital between 1 January 2010 and 31 December 2020 were retrospectively identified by searching the digital databases of the two centres, using the keywords *human immunodeficiency disease/HIV* and *retroviral disease/RVD*. Chart review was undertaken to identify those patients classified as having HIV arthropathy, using a predefined checklist adapted from Reveille and colleagues' definition (41). HIV-infected children with other rheumatic disorders were excluded from the analysis.

Data Collection

Demographic, clinical, and laboratory data were extracted by review of participant details, case notes and laboratory results. Clinical manifestations, laboratory results, and medical therapies were captured at initial presentation and subsequent follow up. Radiographic and ultrasonographic data were extracted by review of saved images and reports for existing studies. Therapeutic modalities were analysed as binary variables, with positivity conferred by a record of an agent having been previously prescribed. Response to therapy was measured by extrapolating clinical data recorded at outpatient follow up consultations, such as the number of active involved joints, and linear scales of patient/parent-rated functional limitation and clinician-rated functional limitation, relative to dates of starting or stopping therapies, or changing doses.

Statistical Analysis

Characterisation of demographics and clinical presentation was summarised using descriptive statistics. Demographic, clinical and laboratory variables were expressed as medians with interquartile ranges, as the frequency distributions for these data were skewed. Treatment and outcomes data were summarized using line graphs, in which duration of a specific therapy was plotted against a measure of disease activity. Data were analysed through statistical package R version 4.0.3.

RESULTS

An initial search identified 22 HIV-infected patients, from a total of 900 children enrolled in the Paediatric Rheumatology clinics. Eleven patients were excluded, due to alternative diagnoses (sarcoidosis, n=1; systemic sclerosis, n=1; uveitis, n=3; Osgood-Schlatter, n=2; vasculopathy, n=1; pernio, n=1; cellulitis, n=1) or incomplete data (n=1). A total of 11 cases of paediatric-onset HIV arthropathy were included in the analysis.

Clinical Presentation

Most HIV arthropathy cases presented in older children, with a median age at arthritis onset of 10.3 years (IQR 6.9 – 11.6). There was a significant male predominance (male-female ratio 3.0). Although the median age at HIV diagnosis was 7.6 years (IQR 5.6 – 10.6), it was considered likely that most of these patients acquired HIV infection *via* vertical transmission. Most cases presented in the setting of advanced HIV infection, with a median absolute CD4+ count of 389 cells/uL (IQR 322 – 449) and median CD4+ proportion of 19.5% (IQR 14.8 – 25.0) at presentation, although there was one outlier with an absolute CD4+ count of 2364 cells/uL (CD4+ proportion 49.0%).

Arthritis was the presenting feature of HIV infection in 4 cases. Six children (55%) were ART naïve at the time of presentation with arthritis. The remaining five children had all defaulted ART for a period of more than six months prior to presentation.

Clinical features are summarized in **Table 1**. The pattern of articular involvement was variable, with six cases of oligoarthritis and five cases of polyarthritis. The median number of involved joints was 4 (IQR 3- 6). The distribution of joint involvement is shown in **Figure 1**. All eleven children had large joint involvement (wrists, n=8; knees, n=7; ankles, n=5; hips, n=2; elbows, n=2; shoulders, n=1), which was asymmetrical in nine cases. In addition, four children had asymmetrical small joint involvement (finger interphalangeal, n=3; toe interphalangeal, n=1; metacarpophalangeal, n=2; midtarsal, n=1) and one child had symmetrical involvement of the subtalar and midtarsal joints. Enthesitis was found in four patients, and one child had dactylitis. Associated clinical features included lymphadenopathy (n=5), hepatomegaly (n=4), splenomegaly (n=2), and parotidomegaly (n=2). Seven children had associated skin rashes, which were classified as papular pruritic eruption (n=4), psoriasis (n=1), erythema nodosum (n=1), and scabies (n=1). Two children had uveitis. The nutritional status of these patients was generally poor, with 89% being classified as underweight-for-age and all being stunted. Approximately half (54.5%) had a personal history of previous tuberculosis (TB), of which four cases were pulmonary and one case was intra-abdominal. There was also one case of TB monoarthritis, involving the left knee, in a child with pre-existing HIV arthropathy involving both wrists and the interphalangeal joint of the left great toe. Other opportunistic infections included oral candidiasis, mucocutaneous herpes simplex ulcers, varicella zoster meningitis, parvovirus B19 aplastic anaemia, and cytomegalovirus gastroenteritis.

TABLE 1 | Clinical presentation.

	CD4 count at HIV diagnosis, cells/uL (%)	Pattern of articular involvement (number of active joints)	Associated rheumatic features	Associated clinical features	Opportunistic infections	Comorbidities	Nutritional status
1	431 (20)	Polyarthritis (6)	Erythema nodosum	LAD, hepatomegaly	Pulmonary TB	–	Mild UWFA Mild stunting
2	752 (29)	Oligoarthritis (3)	Tenosynovitis	Splenomegaly, PPE	–	–	Normal WFA Mild stunting
3	444 (15)	Oligoarthritis (4)	Enthesitis	PPE	Oral candidiasis	–	Moderate UWFA Mild stunting
4	453 (15)	Oligoarthritis (2)	–	LAD, hepatomegaly, parotidomegaly, PPE	–	–	Moderate UWFA Mild stunting
5	161 (11)	Oligoarthritis (4)	–	LAD, HSM	Abdominal TB, parvovirus B19 aplastic anaemia	Severe acute malnutrition	Severe UWFA Moderate stunting
6	375 (19.5)	Polyarthritis (10)	Enthesitis, myalgia	Oral ulcers, clubbing, scabies	Pulmonary TB	Post-TB bronchiectasis	*
7	237 (18)	Oligoarthritis (3)	Dactylitis, myalgia	LAD, PPE	TB monoarthritis, CMV enteritis	–	Severe UWFA Severe stunting
8	389 (21)	Oligoarthritis (2)	Enthesitis, uveitis	Bilateral cataracts, hepatomegaly	Pulmonary TB, VZV meningitis	–	Severe UWFA Severe stunting
9	2364 (49)	Polyarthritis (6)	–	LAD	Chronic HSV ulcers	Severe acute malnutrition	Severe UWFA Severe stunting
10	282 (14)	Polyarthritis (8)	Enthesitis	–	–	Obstructive hydrocephalus	Severe UWFA Severe stunting
11	361 (34)	Polyarthritis (5)	Psoriasis	Parotidomegaly	Pulmonary TB	–	*

Rt, right; Lt, left; LAD, lymphadenopathy; UWFA, underweight-for-age; WFA, weight-for-age; MCPJ, metacarpophalangeal joint; IPJ, interphalangeal joint; PIPJ, proximal interphalangeal joint; DIPJ, distal interphalangeal joint; TB, tuberculosis; HSV, herpes simplex virus; CMV, cytomegalovirus; VZV, varicella zoster virus.
**Insufficient data.*

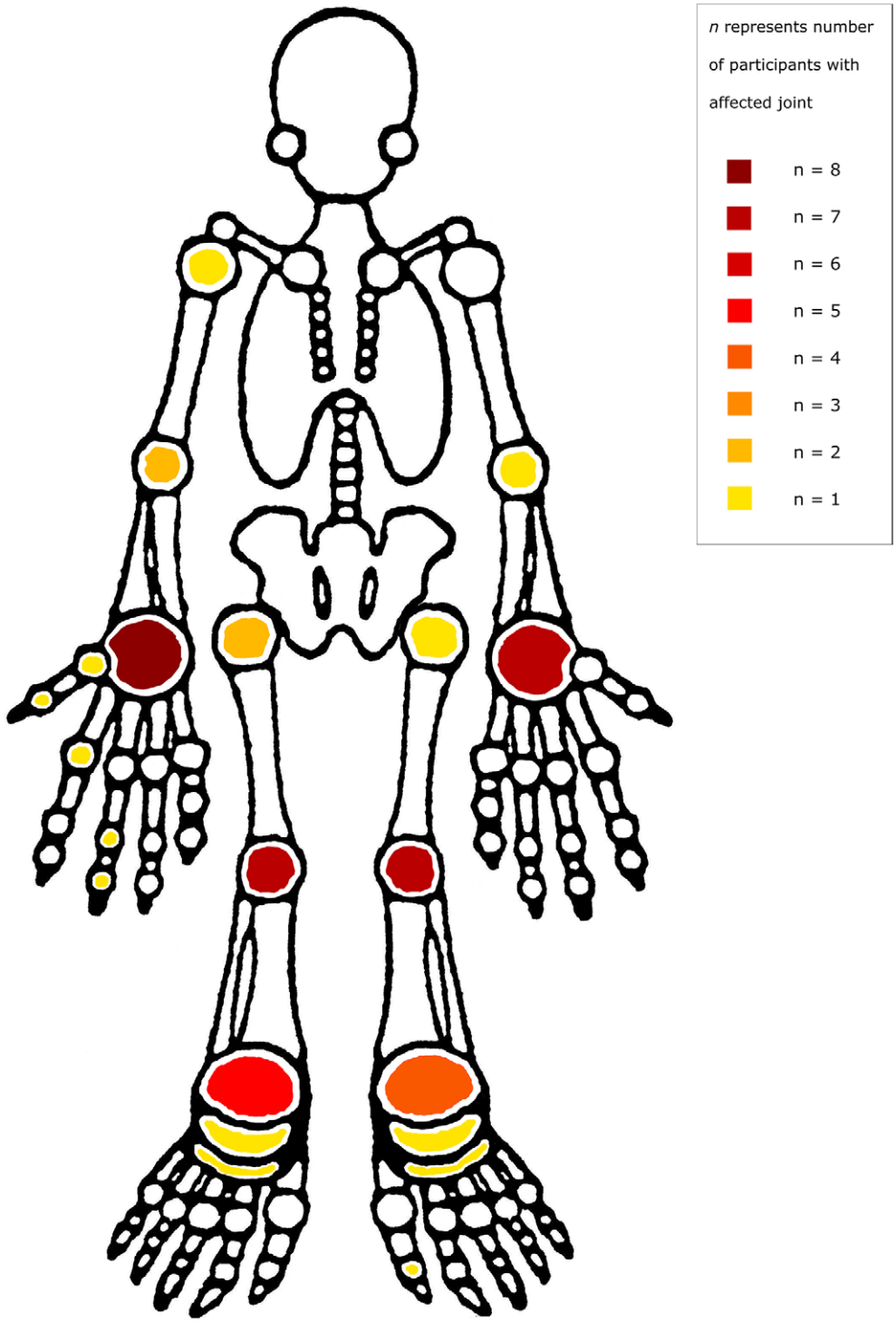

FIGURE 1 | Distribution of joint involvement.

Laboratory and Imaging Characteristics

Laboratory features are summarised in **Table 2**. Synovial aspirate was performed in 3/11 cases. The typical findings were of a lymphocyte-predominant, exudative effusion with negative bacterial and mycobacterial analyses. Synovial biopsy was performed in 4/11 cases. Histological analyses demonstrated nonspecific subacute and chronic synovitis. All microbiological analyses were negative (Ziehl-Neelson, Auramine, Gram, Brown-Brenn, and periodic

acid-Schiff stains; CMV immunohistochemistry; bacterial and mycobacterial cultures), except for a repeat synovial biopsy sample which cultured Micrococcus species, which was interpreted as a contaminant. Autoimmune serological data are presented in **Table 3**.

Joint ultrasound data were available for 7/11 cases (63.6%); findings included joint effusions (6/7) and synovial hypertrophy (4/7). Joint plain film radiographic data were available for 3/11 cases (27.3%); two X-rays were normal, and one showed a joint effusion.

Treatment and Outcome

Immune-directed therapeutic modalities are summarized in **Table 4**. Non-steroidal anti-inflammatory drugs (NSAIDs) were used in all cases. Oral steroids were used in seven children. Intra-articular steroids were used in three of the remaining four children, and the fourth child never returned for follow-up after initial presentation. Two children required intravenous pulse steroid therapy to control severe disease flares. Clinical responses to chloroquine, methotrexate, and prednisone therapy are illustrated in **Figures 2–4**. Chloroquine was either withheld or stopped in four children, due to possible or confirmed TB infection, and methotrexate was stopped in one child with active TB infection.

Five children were discharged in established remission after discontinuing immunotherapy. Two children were lost to follow-up immediately after presentation. Another four children were lost to follow-up after longer periods of routine assessment, however all four were in remission at last assessment.

ART was initiated in all eleven children, at a median age of 7.4 years (IQR 6.3 – 10.1). Viral suppression (defined as a single recorded viral load <100 copies/uL) was achieved in 9/11 children (82%), with a median time to viral suppression of 45.5 months (IQR 9.3 – 124.9). Over the study period, the mean change in absolute CD4+ count was -57.1 cells/uL and the mean change in CD4+ proportion was +3.0%. Five children were changed to a second-line ART regimen during the study period.

TABLE 2 | Laboratory features.

	n/N (%)
Leucocytosis (WCC > 11.0 x10^9 cells/L)	2/11 (18.2)
Leucoopenia (WCC <4.0 x10^9 cells/L)	1/11 (9.1)
Neutropenia (ANC < 1.5 x10^9 cells/L)	2/10 (20.0)
Lymphopenia (ALC < 1.4 x10^9 cells/L)	3/9 (33.3)
Anaemia (Hb < 11.0 g/dL)	6/11 (54.5)
Thrombocytosis (Platelet count > 400 x10^9 cells/L)	5/11 (45.5)
CRP > 10 mg/L	11/11 (100)
ESR > 30 mL/h	10/11 (90.9)
	Median (IQR)
WCC, x10^9 cells/L	8.8 (8.0 – 9.2)
ANC, x10^9 cells/L	4.2 (3.4 – 5.6)
ALC, x10^9 cells/L	1.9 (1.3 – 2.1)
Hb, g/dL	10.7 (9.9 – 11.9)
Platelet count, x10^9 cells/L	361 (335 – 503)
CRP, mg/L	36 (25 – 68)
ESR, mL/h	126 (67 – 136)

WCC, white cell count; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; Hb, haemoglobin; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n, number; N, sample; IQR, interquartile range.

TABLE 3 | Autoantibodies.

	n tested (%)	n positive
ANA	6/11 (54.5)	0
Anti-dsDNA	3/11 (27.3)	0
RF	6/11 (54.5)	0
HLA-B27	3/11 (27.3)	1
ASO	3/11 (27.3)	0
Anti-DNase B	3/11 (27.3)	0

ANA, antinuclear antibodies; anti-dsDNA, anti-double stranded deoxyribonucleic acid antibodies; RF, rheumatoid factor; HLA-B27, human leukocyte antigen B27; ASO, antistreptolysin O; anti-DNase B, anti-deoxyribonuclease B.

TABLE 4 | Therapeutic modalities.

	n (%)	Duration at remission, in months Median (IQR)	Total duration, in months Median (IQR)
NSAID	11 (100.0)	–	–
CLQ	9 (81.8)	12.0 (9.1 – 18.9)	22.8 (18.0 – 48.1)
MTX	5 (45.5)	15.0 (11.5 – 20.0)	27.7 (11.7 – 30.1)
Leflunomide	1 (9.1)	–	–
Oral prednisone	7 (63.6)	13.8 (12.4 – 18.7)	22.0 (18.0 – 32.9)
Intravenous steroid therapy	2 (18.2)	–	–
Intraarticular steroid therapy	4 (36.4)	–	–

NSAID, nonsteroidal anti-inflammatory drug; CLQ, chloroquine; MTX, methotrexate; n, number; IQR, interquartile range.

DISCUSSION

A plethora of research efforts have attempted to explore and address the diverse biopsychosocial challenges which shape the lives of children living with HIV/AIDS. However, musculoskeletal manifestations of HIV infection have not been prioritized and remain poorly defined. Rheumatological disorders in HIV-infected children appear to be relatively uncommon, and paediatric practice has been shaped by data generated in adult populations.

While the number of children with advanced HIV/AIDS has declined substantively in the current era of universal ART, significant treatment gaps continue to affect children living with HIV. A recent study integrating paediatric HIV data sources in South Africa reported that approximately one quarter of HIV-infected children remain undiagnosed, compared to a corresponding estimate of one tenth of adults (66). ART coverage in children was estimated to be only 51.2%. This data suggests that the dramatic reductions in AIDS-related under-five mortality have largely been driven by preventive

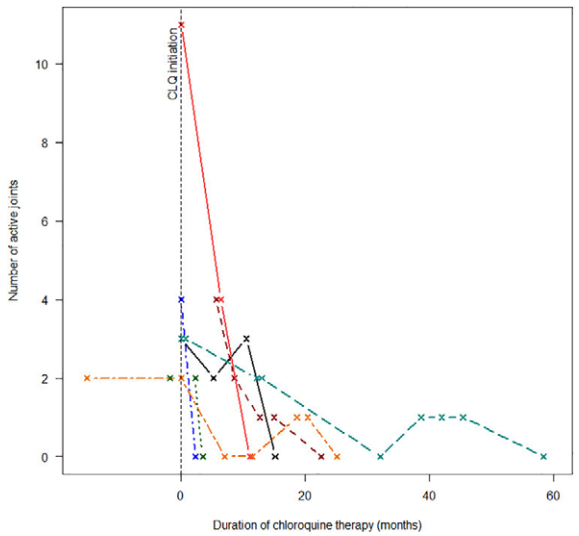

FIGURE 2 | Response to chloroquine therapy.

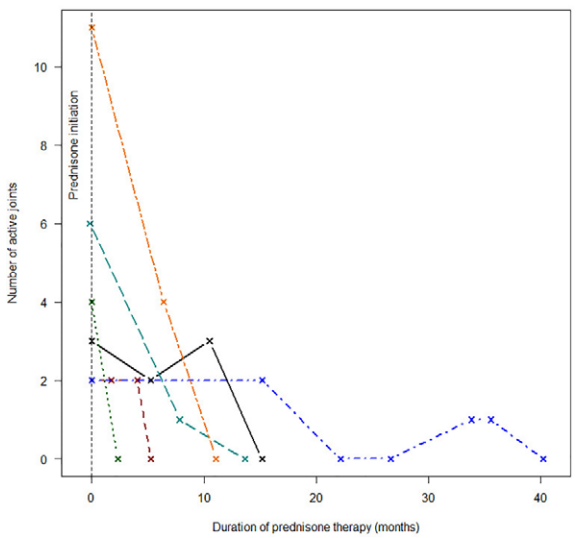

FIGURE 4 | Response to prednisone therapy.

strategies, such as prevention of mother-to-child transmission, rather than effective management of existing paediatric HIV cases. In this context, the effective recognition and management of both communicable and non-communicable sequelae of paediatric AIDS remains imperative. This report adds to the small body of literature on paediatric HIV arthropathy, which has emerged as a clinical entity distinct from other rheumatic disorders of childhood. As in adults, paediatric HIV arthropathy occurs in the setting of advanced immunosuppression (8, 49). Additionally, it appears to predominantly affect older children (50, 60). In this case series, the median age of arthritis onset was 10.3 years (IQR 6.9 – 11.6), which is similar to existing literature. Most children had advanced immunosuppression at the time of presentation, with a median CD4+ proportion of 19.5% (IQR 14.8 – 25.0).

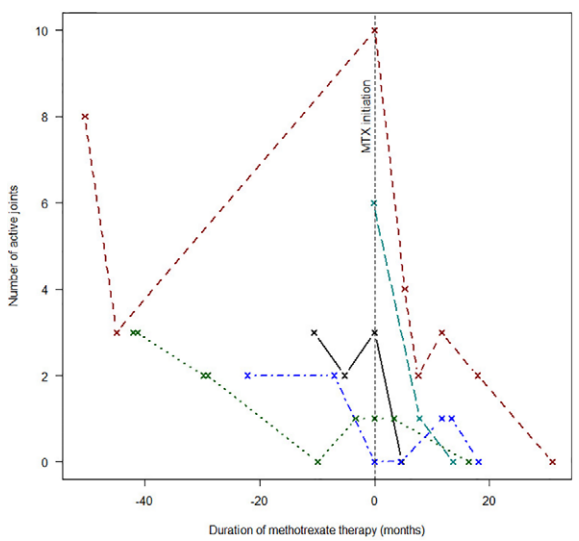

FIGURE 3 | Response to methotrexate therapy.

A high male-female ratio was evident in this case series. This finding is consistent with reports of a male predominance in both children and adults with HIV arthropathy (23, 50, 67) and contrasts with reports of older children with advanced HIV disease without arthropathy (68–70). The mechanisms contributing to the observed sex difference remain unclear. Biological sex has a profound effect on the immune dysregulation that characterises HIV infection. Numerous studies in children and adults have demonstrated lower levels of viraemia in females (71–74). Women are over-represented amongst HIV-infected individuals with the capacity to spontaneously suppress viraemia without ART, known as elite controllers. Across the literature, approximately 80% of adult elite controllers and 90% of paediatric elite controllers are female (71, 75–78). Sex differences in other non-communicable HIV-associated comorbidities, such as cardiovascular disease, neurocognitive disorders and malignancies, have been linked to substantial differences in immune activation and regulation between males and females living with HIV (79–81).

In comparison with adults, most untreated HIV-infected children follow a rapid course of disease progression. At one year of life, 35–50% of untreated, perinatally infected infants will have progressed to AIDS or died (71, 82–85), although it should be noted that outcomes vary widely across different settings, with environmental exposures playing a significant role in early life. By comparison, the median survival time in untreated adults is approximately 10 years (86). It is therefore surprising that a significant proportion of children (5-10%) exhibit the immunological phenomenon of non-progression, which is extremely uncommon in adults (75, 87). Non-progressors are

ART-naïve HIV-infected individuals who do not experience immunosuppression but maintain normal CD4+ counts despite ongoing viraemia (75, 88). Most of the children in this series experienced long delays to ART initiation, with median age at ART initiation being 7.4 years (IQR 6.3 – 10.1). This suggests that delay to ART initiation and subsequent HIV progression may be a risk factor for HIV arthropathy in children. Conversely, at least some of these children may have been transient non-progressors, facilitating their survival into later childhood. HLA haplotype-encoding genes influence the rate of disease progression in HIV infection (88–91), with certain HLA haplotypes being recognized as protective. However, several such alleles are also implicated in the pathogenesis of specific rheumatic syndromes. In a large case-control study based in Zambia, López-Larrea and colleagues demonstrated that the presence of the HLA-B*5703 allele was independently associated with both HIV non-progression and the development of spondyloarthropathy (92). Other reports have demonstrated high frequencies of HLA haplotypes associated with spontaneous HIV control in individuals with psoriasis and spondyloarthropathy (93, 94). An intriguing possibility in the pathogenesis of HIV arthropathy is the notion that an as-of-yet unknown factor may be partially protective against HIV progression and confer an increased risk of inflammatory arthritis.

A variable pattern of articular involvement was observed in this case series, with six children presenting with oligoarthritis and five having polyarthritis. This differs from reports of adult-onset HIV arthropathy, in which asymmetrical oligoarthritis predominates, although symmetrical polyarthritis and monoarthritis have also been described (23, 95). Polyarthritis was the commonest presentation in the largest existing report of arthritis in HIV-infected children (50). Most of the children in this case series (9/11) had symmetrical involvement of the large joints, particularly wrists and knees. Existing literature has reported preferential involvement of the lower limbs, particularly knees and ankles, in both adults and children (23, 49, 50). Enthesitis (3/11) was less prevalent than reported elsewhere (50, 96), and only one case was associated with psoriasis. Other observed clinical features (such as papular pruritic eruption, stunting, lymphadenopathy, hepatosplenomegaly and parotidomegaly) likely reflect the advanced stage of HIV infection in which arthropathy presents, rather than a true association. Opportunistic infections were common in this case series, particularly TB (6/11) including two extrapulmonary cases.

The most consistent laboratory finding was elevated acute phase reactants, which were present in more than 90% of cases. ESR was usually elevated to a greater extent than CRP. The median ESR was 126 mL/h (IQR 67 – 136), compared to a median CRP of 36 mg/L (IQR 25 – 68). Joint ultrasound data were available for seven cases (63.6%) in this series. Prominent sonographic features included joint effusion (6/7) and synovial hypertrophy (4/7). Synovial hypertrophy was distinctive; all four cases were described as having abundant or exuberant hypertrophy, relative to findings in patients with juvenile idiopathic arthritis (**Figures 5–7**). Published evidence on the sonographic features of HIV arthropathy is scarce for adults and children alike. However, several reports have highlighted the radiographic features of joint effusions, juxta-articular osteopenia, joint space narrowing, and marginal erosions in adults with HIV-associated arthritides (95, 97, 98). Findings on plain radiograph are generally similar to those seen in patients with RA, with the exception of periosteal reaction and proliferative bone formation, which are more specific to HIV arthropathy (97, 99).

Approximately half of the children in this case series (45.5%) were discharged in established remission after discontinuing immunotherapy. The remaining six children were lost to follow up; four of them were in remission at last assessment. Two children had no follow up data, having been lost to follow up immediately after initial presentation. Notably, all cases with reliable follow-up data had joint disease persisting beyond 6 weeks, which contrasts with Reveille and colleagues' classic definition of adult-onset HIV arthropathy as a self-limiting condition (41). This phenomenon has been observed in some adults, who develop a chronic arthropathy associated with functional impairments (100).

The small size of this series limited statistical power. Participants were recruited from a single clinical service, based at two referral centres in Cape Town, South Africa. Health

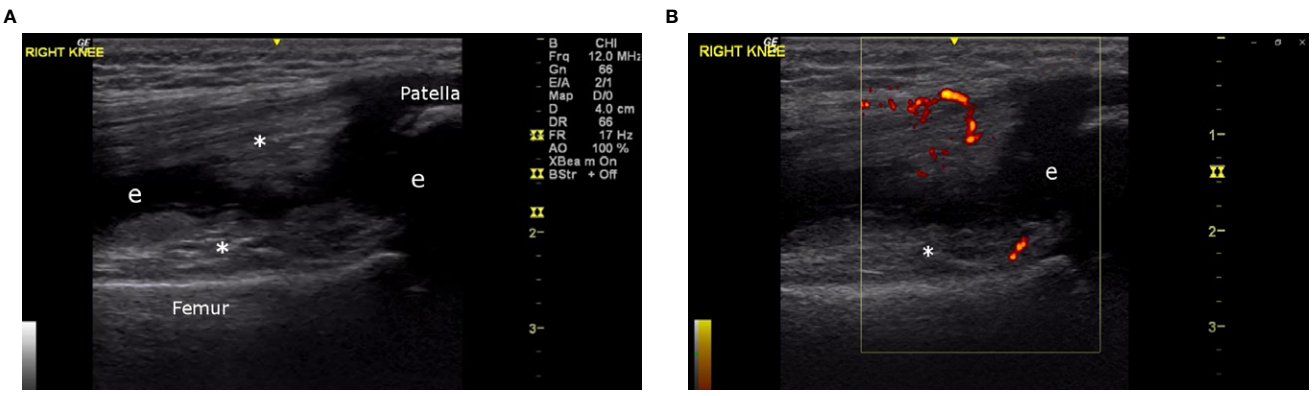

FIGURE 5 | **(A)** Right knee, superior longitudinal view, greyscale (6 year old male, Case 2), demonstrating abundant proliferative synovitis (*) and effusion (e). **(B)** Right knee, superior longitudinal view, color Doppler (6 year old male, Case 2), demonstrating proliferative synovitis with intense 3+ Doppler signal (D).

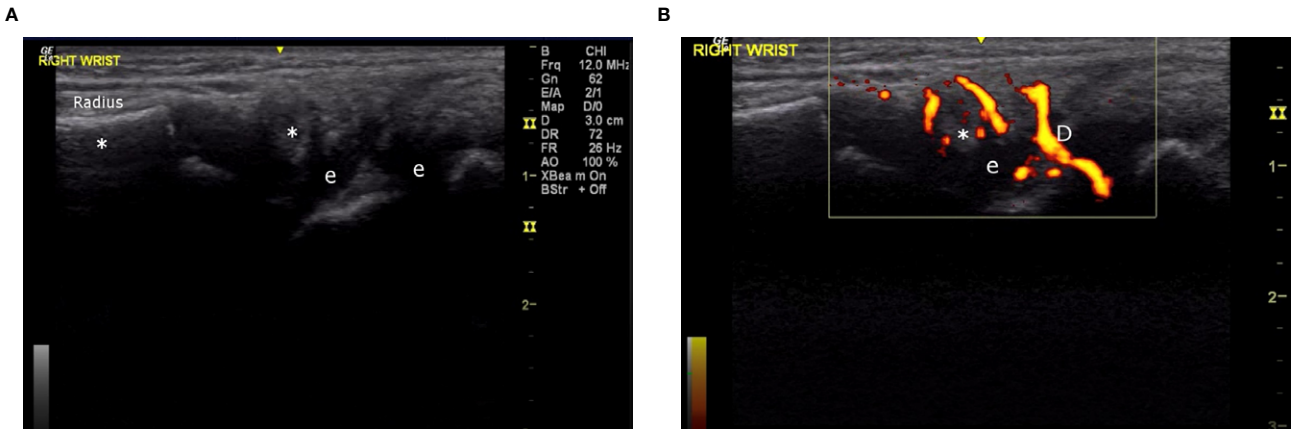

FIGURE 6 | **(A)** Right wrist, 4th compartment, longitudinal view, greyscale (6 year old male, Case 2), demonstrating proliferative synovitis (*) and effusion (e). **(B)** Right wrist, 4th compartment, longitudinal view, color Doppler (6 year old male, Case 2), demonstrating intense 3+ Doppler signal (D) in proliferative synovium.

systems in this region differ considerably from the rest of the continent, potentially limiting the generalisability of these findings to other settings and populations. The study was limited by a retrospective design and the availability of data in existing case files, laboratory records, and imaging reports. Sonographic data were only available for approximately two thirds of participants. Number of active joints was used as an objective surrogate marker of disease activity, as physician- and parent/patient-rated severity scores were not available for every follow-up encounter. It was not possible to assess a possible correlation between arthropathy disease activity and HIV viral load, as the timeframes over which viral load data and serial rheumatological assessment data were available differed in 7/11 cases. This study was further limited by a high rate of attrition. The study describes a series of highly vulnerable children living with a chronic health condition. Many of these children endure adverse social conditions, including poverty, parental and sibling mortality, household substance abuse, and inconsistent primary caregiver relationships, which may have contributed to loss to follow-up. Additionally, these chronically ill children have a significant risk of death, which may have contributed to attrition. In the comparable setting of paediatric ART clinics, several reviews have described high attrition rates (101–105).

A large prospective study would be beneficial to better define the characteristics, diagnosis, and optimal management of HIV arthropathy in children. Translational research is needed to investigate disease pathogenesis and identify biomarkers that could aid diagnosis in under-resourced environments without access to specialist assessment.

This study contributes to the small body of evidence on paediatric HIV arthropathy. Cases predominantly presented in older boys with advanced immunosuppression. The clinical presentation was variable, with both oligoarthritis and polyarthritis being common. Response to therapy was slower

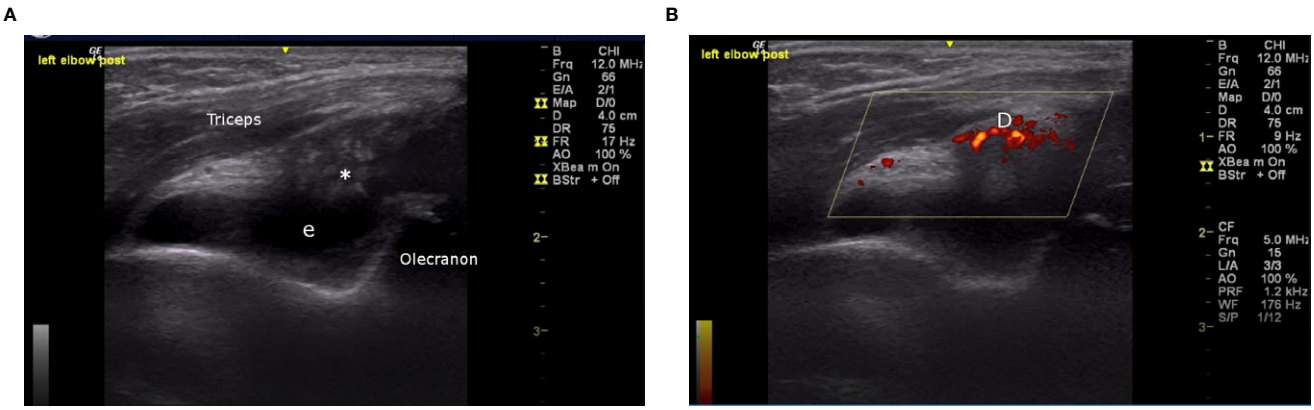

FIGURE 7 | **(A)** Left elbow, posterior longitudinal view, greyscale (10 year old male, Case 6), demonstrating dense proliferative synovitis (*) in olecranon fossa with effusion (e). **(B)** Left elbow, posterior longitudinal view, color Doppler (10 year old male, Case 6), demonstrating intense Doppler signal (D) in olecranon fossa.

than has generally been described in adults, although fair outcomes were obtained with immunomodulating and antiretroviral therapy. Prospective and translational research is required to improve our understanding of this understudied disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee Faculty of Health Sciences University of Cape Town. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors (MH, NB, and CS) contributed to the conception and design of this study. The data were collected by MH and CS. Data analysis was performed by MH, with input from CS. All authors interpreted the data. MH and CS drafted this work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.677984/full#supplementary-material

REFERENCES

1. Patel N, Patel N, Espinoza LR. HIV Infection and Rheumatic Diseases: The Changing Spectrum of Clinical Enigma. *Rheum Dis Clin North Am* (2009) 35(1):139–61. doi: 10.1016/j.rdc.2009.03.007
2. Fox C, Walker-Bone K. Evolving Spectrum of HIV-Associated Rheumatic Syndromes. *Best Pr Res Clin Rheumatol* (2015) 29(2):244–58. doi: 10.1016/j.berh.2015.04.019
3. Maganti RM, Reveille JD, Williams FM. The Changing Spectrum of Rheumatic Disease in HIV Infection. *Nat Clin Pract* (2008) 4(8):428–38. doi: 10.1038/ncprheum0836
4. Lau C-S, Li P. The Effects of AIDS on the Prevalence of Rheumatic Diseases. *Nat Epidemiol.* (2016) 13(8). doi: 10.1038/nrrheum.2016.196
5. Buskila D, Gladman DD, Langevitz P, Bookman AAM, Fanning M, Salit IE. Rheumatologic Manifestations of Infection With the Human Immunodeficiency Virus (HIV). *Clin Exp Rheumatol* (1990) 8(6):567–73.
6. Fernandez SM, Cardenal A, Balsa A, Quiralte J, del Arco A, Peña JM, et al. Rheumatic Manifestations in 556 Patients With Human Immunodeficiency Virus Infection. *Semin Arthritis Rheum* (1991) 21(1):30–9. doi: 10.1016/0049-0172(91)90054-4
7. Medina F, Pérez-Saleme L, Moreno J. Rheumatic Manifestations of Human Immunodeficiency Virus Infection. *Infect Dis Clin North Am* (2006) 20 (4):891–912. doi: 10.1016/j.idc.2006.09.002
8. Berman A, Espinoza L, Diaz J, Aguilar J, Rolando T, Vasey F, et al. Rheumatic Manifestations of Human Immunodeficiency Virus Infection. *Am J Med* (1988) 85(1):59–64. doi: 10.1016/0002-9343(88)90503-7
9. United Nations Joint Programme on HIV/AIDS. *UNAIDS Data 2020*. Geneva, Switzerland: UNAIDS (2020) p. 80–1.
10. Hall K, Sambu W. *Demography of South Africa's children*. Cape Town, South Africa: Children's Institute (2018).
11. United Nations Joint Programme on HIV/AIDS. *UNAIDS Data 2019*. Geneva, Switzerland: UNAIDS (2019) p. 62–3.
12. Ndirangu J, Newell M-L, Tanser F, Herbst AJ, Bland R. Decline in Early Life Mortality in a High HIV Prevalence Rural Area of South Africa: Evidence of HIV Prevention or Treatment Impact? *AIDS* (2014) 24(4):593–602. doi: 10.1097/QAD.0b013e328335cff5
13. Zanoni BC, Phungula T, Zanoni HM, France H, Feeney ME. Risk Factors Associated With Increased Mortality Among HIV-Infected Children Initiating Antiretroviral Therapy in South Africa. *PloS One* (2011) 6(7):4–9. doi: 10.1371/journal.pone.0022706
14. Violari A, Cotton MF, Gibb DM, Babiker AG, Steyn J, Madhi S, et al. Early Antiretroviral Therapy and Mortality Among HIV-Infected Infants. *N Engl J Med* (2008) 359(21):2233–44. doi: 10.1056/NEJMoa0800971
15. Sekigawa I, Ogasawara H, Kaneko H, Hishikawa T, Hashimoto H. Retroviruses and Autoimmunity. *Intern Med* (2001) 40(2):80–6. doi: 10.2169/internalmedicine.40.80
16. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, et al. Depletion of Regulatory T Cells in HIV Infection Is Associated With Immune Activation. *J Immunol* (2005) 174:4407–14. doi: 10.4049/jimmunol.174.7.4407
17. Hong YHX, Luo JXJ. Autoimmunity and Dysmetabolism of Human Acquired Immunodeficiency Syndrome. *Immunol Res* (2016) 64(3):641–52. doi: 10.1007/s12026-015-8767-5
18. Weyand C, Goronzy J. HIV Infection and Rheumatic Diseases: Autoimmune Mechanisms in Immunodeficient Hosts. *Z Rheumatol* (1992) 51(2):55–64. doi: 10.1136/ard.51.2.253
19. Zandman-Goddard G, Shoenfeld Y. HIV and Autoimmunity. *Autoimmun Rev* (2002) 1(6):329–37. doi: 10.1016/S1568-9972(02)00086-1
20. Li Z, Nardi MA, Karpatkin S. Role of Molecular Mimicry to HIV-1 Peptides in HIV-1–Related Immunologic Thrombocytopenia. *Blood* (2005) 106 (2):572–6. doi: 10.1182/blood-2005-01-0243
21. Gleason B, Mirembe G, Namuyonga J, Okello E, Lwabi P, Lubega I, et al. Prevalence of Latent Rheumatic Heart Disease Among HIV-Infected Children in Kampala, Uganda. *J Acquir Immune Defic Syndr* (2016) 71 (2):196–9. doi: 10.1097/QAI.0000000000000827
22. Hovis IW, Namuyonga J, Kisitu GP, Ndagire E, Okello E, Longenecker CT, et al. Decreased Prevalence of Rheumatic Heart Disease Confirmed Among HIV Positive Youth. *Pediatr Infect Dis J* (2020) 38(4):406–9. doi: 10.1097/INF.0000000000002161
23. Adizie T, Moots R, Hodkinson B, French N, Adebajo A. Inflammatory Arthritis in HIV Positive Patients: A Practical Guide. *BMC Infect Dis* (2016) 16(100). doi: 10.1186/s12879-016-1389-2

24. Tikly M, Njobvu P, McGill P. Spondyloarthritis in Sub-Saharan Africa. *Curr Rheumatol Rep* (2014) 16(421). doi: 10.1007/s11926-014-0421-z
25. Njobvu P, McGill P, Kerr H, Jellis J, Pobee J. Spondyloarthropathy and Human Immunodeficiency Virus Infection in Zambia. *J Rheumatol* (1998) 25(8):1553–9.
26. Nguyen B, Reveille JD. Rheumatic Manifestations Associated With HIV in the Highly Active Antiretroviral Therapy Era. *Curr Opin Rheumatol* (2009) 21(4):404–10. doi: 10.1097/BOR.0b013e32832c9d04
27. Díaz-Peña R, Ouédraogo D-D, Vazquez AL, Sawadogo S, López-Larrea C. Ankylosing Spondylitis in Three Sub-Saharan Populations: HLA-B27 and HLA-B14 Contribution. *Tissue Antigens* (2012) 80:14–6. doi: 10.1111/j.1399-0039.2012.01879.x
28. Tshabalala M, Mellet J, Pepper MS. Human Leukocyte Antigen Diversity: A Southern African Perspective. *J Immunol Res* (2015) 746151. doi: 10.1155/2015/746151
29. Njobvu P, Gill P. Psoriatic Arthritis and Human Immunodeficiency Virus Infection in Zambia. *J Rheumatol* (2000) 27(7):1699–702.
30. Bileckot R, Mouaya A, Makuwa M. Prevalence and Clinical Presentations of Arthritis in HIV-Positive Patients Seen at a Rheumatology Department in Congo-Brazzaville. *Rev Rhum Engl Ed* (1998) 65(10):549–54.
31. Stein C, Davis P. Arthritis Associated With HIV Infection in Zimbabwe. *J Rheumatol* (1996) 23(3):506–11.
32. Bijlsma J, Derksen R, Huber-Bruning O, Borleffs J. Does AIDS "Cure" Rheumatoid Arthritis? *Ann Rheum Dis* (1988) 47(4):350–1. doi: 10.1136/ard.47.4.350-b
33. Yao Q, Frank M, Glynn M, Altman RD, Altman RD. Rheumatic Manifestations in HIV-1 Infected Inpatients and Literature Review. *Clin Exp Rheumatol* (2008) 26:799–806.
34. Lordache L, Launay O, Bouchaud O, Jeantils V, Goujard C, Boue F, et al. Autoimmune Diseases in HIV-Infected Patients: 52 Cases and Literature Review. *Autoimmun Rev2* (2014) 13(8):850–7. doi: 10.1016/j.autrev.2014.04.005
35. Zhang X, Li H, Li T, Zhang F, Han Y. Distinctive Rheumatic Manifestations in 98 Patients With Human Immunodeficiency Virus Infection in China. *J Rheumatol* (2007) 34(8):1760–4.
36. Ouédraogo D, Ntsiba H, Tiendrébéogo ZJ, Tiéno H, Bokossa L, Kaboré F, et al. Clinical Spectrum of Rheumatologic Diseases in a Department of Rheumatology in Ouagadougou (Burkina Faso). *Clin Rheumatol* (2014) 33(3):385–9. doi: 10.1007/s10067-013-2455-4
37. Marquez J, Restrepo CS, Candia L, Berman A, Espinoza LR. Human Immunodeficiency Virus-Associated Rheumatic Disorders in the HAART Era. *J Rheumatol* (2004) 31(4):741–6.
38. Calabrese L, Kirchner E, Shrestha R. Rheumatic Complications of Human Immunodeficiency Virus Infection in the Era of Highly Active Antiretroviral Therapy: Emergence of a New Syndrome of Immune Reconstitution and Changing Patterns of Disease. *Eur PMC* (2005) 35(3):166–74. doi: 10.1016/j.semarthrit.2005.03.007
39. Azeroual A, Harmouche H, Benjilali L, Mezalek Z, Adnaoui M, Aouni M, et al. Rheumatoid Arthritis Associated to HIV Infection. *Eur J Intern Med* (2008) 19(6):e34–5. doi: 10.1016/j.ejim.2007.09.020
40. Mody G, Parke F, Reveille J. Articular Manifestations of Human Immunodeficiency Virus Infection. *Best Pr Res Clin Rheumatol* (2003) 17(2):265–87. doi: 10.1016/S1521-6942(03)00003-2
41. Reveille JD, Conant MA, Duvic M. Human Immunodeficiency Virus-Associated Psoriasis, Psoriatic Arthritis, and Reiter's Syndrome: A Disease Continuum? *1Arthritis Rheum* (1990) 33(10):1574–8. doi: 10.1002/art.1780331016
42. Mody G, Patel N. Articular syndromes in association with HIV infection. *CME* (2011) 29(8):320–1. doi: 10.1007/978-0-387-79061-9_5137
43. Berman A, Reboredo G, Spindler A, Lasala M, Lopez H, Espinoza L. Rheumatic Manifestations in Populations at Risk for HIV Infection: The Added Effect of HIV. *J Rheumatol* (1991) 18(10):1564–7.
44. Ekwom PE, Oyoo GO, Amayo EO, Muriithi IM. Prevalence and Characteristics of Articular Manifestations in Human Immunodeficiency Virus Infection. *East Afr Med J* (2010) 87(10):408–14.
45. Medina-Rodriguez F, Guzman C, Jara L, Hermida C, Alboukrek D, Cervera H, et al. Rheumatic Manifestations in Human Immunodeficiency Virus Positive and Negative Individuals: A Study of Two Populations With Similar Risk Factors. *J Rheumatol* (1993) 20(11):1880–4.
46. Lawson E, Walker-Bone K. The Changing Spectrum of Rheumatic Disease in HIV Infection. *Br Med Bull* (2012) 103:203–21. doi: 10.1093/bmb/lds022
47. Berman A, Cahn P, Perez H, Spindler A, Lucero E, Paz S, et al. Human Immunodeficiency Virus Infection Associated Arthritis: Clinical Characteristics. *J Rheumatol* (1999) 26(5):1158–62.
48. Blanche P, Taelman H, Saraux A, Bogaerts J, Clerinx J, Batungwanayo J, et al. Acute Arthritis and Human Immunodeficiency Virus Infection in Rwanda. *Eur PMC* (1993) 20(12):2123–7.
49. Rynes R, Goldenberg D, DiGiacomo R, Olson R, Hussain M, Veazey J. Acquired Immunodeficiency Syndrome-Associated Arthritis. *Am J Med* (1988) 84(5):810–6. doi: 10.1016/0002-9343(88)90057-5
50. Chinniah K, Mody GM, Bhimma R, Adhikari M. Arthritis in Association With Human Immunodeficiency Virus Infection in Black African Children: Causal or Coincidental? *Rheumatology* (2005) 44:915–20. doi: 10.1093/rheumatology/keh636
51. Espinoza L, Aguilar J, Espinoza C, Berman A, Gutierrez F, Vasey F, et al. HIV Associated Arthropathy: HIV Antigen Demonstration in the Synovial Membrane. *J Rheumatol* (1990) 17(9):1195–201.
52. Hughes R, Macatonia S, Rowe I, Keat A, Knight S. The Detection of Human Immunodeficiency Virus DNA in Dendritic Cells From the Joints of Patients With Aseptic Arthritis. *Br J Rheumatol* (1990) 29:166–70. doi: 10.1093/rheumatology/29.3.166
53. Hochberg M, Fox R, Nelson K, Saah A. HIV Infection Is Not Associated With Reiter's Syndrome: Data From the John Hopkins Multicenter AIDS Cohort Study. *AIDS* (1990) 4(11):1149–51. doi: 10.1097/00002030-199011000-00016
54. Romanelli F, Smith K, Hoven A. Chloroquine and Hydroxychloroquine as Inhibitors of Human Immunodeficiency Virus (HIV-1) Activity. *Curr Pharm Des* (2004) 10(21):2643–8. doi: 10.2174/1381612043383791
55. Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: An old drug against today's diseases? *Lancet* (2003) 3(November):722–7. doi: 10.1016/S1473-3099(03)00806-5
56. Devaux CA, Rolain J, Colson P, Raoult D. New Insights on the Antiviral Effects of Chloroquine Against Coronavirus: What to Expect for COVID-19? *Int J Antimicrob Agents.* (2020) 55. doi: 10.1016/j.ijantimicag.2020.105938
57. Zaborowski AG, Parbhoo D, Chinniah K, Visser L. Uveitis in Children With Human Immunodeficiency Virus–Associated Arthritis. *J Am Assoc Pediatr Ophthalmol Strabismus* (2008) 12(6):608–10. doi: 10.1016/j.jaapos.2008.04.011
58. Lakshmanan P, Shah I. Human Immunodeficiency Virus Polyarthropathy. *J Fam Med Prim Care* (2016) 5(3):725–6. doi: 10.4103/2249-4863.197289
59. Zaki SA, Tilak L, Shanbag P. Arthritis as the Presenting Feature of HIV Infection in a Child. *J Infect* (2009) April):391–2. doi: 10.1016/j.jinf.2009.03.002
60. Chakladar D, Mondal RK, Sabui TK, Bhowmik S, Biswas T. Musculoskeletal Manifestations in Pediatric Patients Infected With Human Immunodeficiency Virus: Developing Country Perspective. *Eur J Rheumatol* (2019) 6(1):7–11. doi: 10.5152/eurjrheum.2018.18045
61. Suri D, Sharma A, Bhattad S, Rawat A, Arora S, Minz RW, et al. Arthritis in Childhood Human Immunodeficiency Virus Infection Predominantly Associated With Human Leukocyte Antigen B27. *Int J Rheum Dis* (2016) 19(10):1018–23. doi: 10.1111/1756-185X.12947
62. Chipeta J, Njobvu P, Wa-Somwe S, Chintu C, McGill PE, Bucala R. Clinical Patterns of Juvenile Idiopathic Arthritis in Zambia. *Pediatr Rheumatol* (2013) 11(33). doi: 10.1186/1546-0096-11-33
63. Mahtab S, Scott C, Chris Machemedze T, Joubert S, Asafu-Agyei NA, Myer L, Zar HJ. Musculoskeletal conditions among perinatally HIV-infected adolescents in the Cape Town Adolescent Antiretroviral cohort. *In: Proc 25th Eur Paediatric Rheumatol Congress (PReS 2018) Lisbon Portugal* (2018), 16:50–1. doi: 10.1186/s12969-018-0265-6
64. Schuval S, Bonagura V, Ilowite N. Rheumatologic Manifestations of Pediatric Human Immunodeficiency Virus Infection. *J Rheumatol* (1993) 20(9):1578–82.
65. Martínez-Rojano H, Hernández EJ, Guevara GL, Gorbea-Robles M del C. Rheumatologic Manifestations of Pediatric HIV Infection. *AIDS Patient Care STDS* (2016) 15(10):519–26. doi: 10.1089/108729101753205685

66. Johnson L, Dorrington R, Moolla H. Progress Towards the 2020 Targets for HIV Diagnosis and Antiretroviral Treatment in South Africa. *S Afr J HIV Med* (2017) 18(1):a694. doi: 10.4102/sajhivmed.v18i1.694
67. Aimes S. Management of Inflammatory Arthritis in Patients With Comorbid HIV Infection [Internet](2016). Available at: https://www.rheumatologyadvisor.com/home/topics/rheumatoid-arthritis/management-of-inflammatory-arthritis-in-patients-with-comorbid-hiv-infection/ (Accessed 2021 Jan 25).
68. Ferrand RA, Luethy R, Bwakura F, Mujuru H, Miller RF, Corbett EL. HIV Infection Presenting in Older Children and Adolescents: A Case Series From Harare, Zimbabwe. *Clin Infect Dis* (2007) 44(6):874–8. doi: 10.1086/511878
69. Kranzer K, Bradley J, Musaazi J, Nyathi M, Gunguwo H, Ndebele W, et al. Loss to follow-up among children and adolescents growing up with HIV infection: Age really matters. *J Int AIDS Soc* (2017) 20(1). doi: 10.7448/IAS.20.1.21737
70. Birhan TY, Gezie LD, Teshome DF, Sisay MM. Predictors of CD4 Count Changes Over Time Among Children Who Initiated Highly Active Antiretroviral Therapy in Ethiopia. *Trop Med Health* (2020) 48(1). doi: 10.1186/s41182-020-00224-9
71. Scully EP. Sex Differences in HIV Infection. *Curr HIV/AIDS Rep* (2018) 15 (2):136–46. doi: 10.1007/s11904-018-0383-2
72. Farzadegan H, Hoover D, Astemborski J, Lyles C, Vlahov D. Sex Differences in HIV-1 Viral Load and Progression to AIDS. *Lancet* (1998) 352:1510–3. doi: 10.1016/S0140-6736(98)02372-1
73. Evans JS, Nims T, Cooley J, Bradley W, Jagodzinski L, Zhou S, et al. Serum Levels of Virus Burden in Early-Stage Human Immunodeficiency Virus Type 1 Disease in Women. *J Infect Dis* (1997) 175(4):795–800. doi: 10.1086/513973
74. Touloumi G, Pantazis N, Babiker A, Walker S, Katsarou O, Karafoulidou A, et al. Differences in HIV RNA Levels Before the Initiation of Antiretroviral Therapy Among 1864 Individuals With Known HIV-1 Seroconversion Dates. *AIDS2* (2004) 18(12):1697–705. doi: 10.1097/01.aids.0000131395.14339.f5
75. Vieira VA, Zuidewind P, Muenchhoff M, Roider J, Millar J, Clapson M, et al. Strong Sex Bias in Elite Control of Paediatric HIV Infection. *Aids* (2019) 33 (1):67–75. doi: 10.1097/QAD.0000000000002043
76. Crowell TA, Gebo KA, Blankson JN, Korthuis PT, Yehia BR, Rutstein RM, et al. Hospitalization Rates and Reasons Among HIV Elite Controllers and Persons With Medically Controlled HIV Infection. *J Infect Dis* (2015) 211 (11):1692–702. doi: 10.1093/infdis/jiu809
77. Madec Y, Boufassa F, Porter K, Meyer L. Spontaneous Control of Viral Load and CD4 Cell Count Progression Among HIV-1 Seroconverters. *AIDS* (2005) 19(17):2001–7. doi: 10.1097/01.aids.0000194134.28135.cd
78. de Azevedo SSD, Caetano DG, Côrtes FH, Teixeira SLM, Santos Silva K, Hoagland B, et al. Highly divergent patterns of genetic diversity and evolution in proviral quasispecies from HIV controllers. *Retrovirology* (2017) 14(29). doi: 10.1186/s12977-017-0354-5
79. Raghavan A, Rimmelin DE, Fitch KV, Zanni MV. Sex Differences in Select non-Communicable HIV-Associated Comorbidities: Exploring the Role of Systemic Immune Activation/Inflammation. *Curr HIV/AIDS Rep* (2017) 14 (6):220–8. doi: 10.1007/s11904-017-0366-8
80. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, et al. Sex Differences in the Toll-Like Receptor-Mediated Response of Plasmacytoid Dendritic Cells to HIV-1. *Nat Med* (2009) 15(8):955–9. doi: 10.1038/nm.2004
81. Chang JJ, Woods M, Lindsay RJ, Doyle EH, Griesbeck M, Chan ES, et al. Higher Expression of Several Interferon-Stimulated Genes in HIV-1-Infected Females After Adjusting for the Level of Viral Replication. *J Infect Dis* (2013) 208(5):830–8. doi: 10.1093/infdis/jit262
82. Newell ML, Coovadia H, Cortina-Borja M, Rollins N, Gaillard P, Dabis F. Mortality of Infected and Uninfected Infants Born to HIV-Infected Mothers in Africa: A Pooled Analysis. *Lancet* (2004) 364(9441):1236–43. doi: 10.1016/S0140-6736(04)17140-7
83. Little K, Thorne C, Luo C, Bunders M, Ngongo N, McDermott P, et al. Disease Progression in Children With Vertically-Acquired HIV Infection in Sub-Saharan Africa: Reviewing the Need for HIV Treatment. *Curr HIV Res* (2007) 5(2):139–53. doi: 10.2174/157016207780077002
84. Spira R, Lepage P, Msellati P, Van De Perre P, Leroy V, Simonon A, et al. Natural history of human immunodeficiency virus type 1 infection in children: A five-year prospective study in Rwanda. *Pediatrics* (1999) 104 (5). doi: 10.1542/peds.104.5.e56
85. Jean S, Pape J, Verdier R, Reed G, Hutto C, Johnson W, et al. The Natural History of Human Immunodeficiency Virus 1 Infection in Haitian Infants. *Pediatr Infect Dis J* (1999) 18(1):58–63. doi: 10.1097/00006454-199901000-00014
86. Collaborative Group on AIDS Incubation and HIV Survival including the CASCADE EU Concerted Action. Time from HIV-1 seroconversion to AIDS and death before widespread use of highly-active antiretroviral therapy: A collaborative re-analysis. *Lancet* (2000) 355(9210):1131–7. doi: 10.1016/S0140-6736(00)02061-4
87. Paul ME, Mao C, Charurat M, Serchuck L, Foca M, Hayani K, et al. Predictors of Immunologic Long-Term Nonprogression in HIV-Infected Children: Implications for Initiating Therapy. *J Allergy Clin Immunol* (2005) 115(4):848–55. doi: 10.1016/j.jaci.2004.11.054
88. Goulder PJ, Lewin SR, Leitman EM. Paediatric HIV Infection: The Potential for Cure. *Nat Rev Immunol* (2016) 16(4):259–71. doi: 10.1038/nri.2016.19
89. Kumar P. Long Term non-Progressor HIV Infection. *Indian J Med Res* (2013) 138(3):291–3.
90. Limou S, Le Clerc S, Coulonges C, Carpentier W, Dina C, Delaneau O, et al. Genomewide Association Study of an AIDS-Nonprogression Cohort Emphasizes the Role Played by HLA Genes (ANRS Genomewide Association Study 02). *J Infect Dis* (2009) 199(3):419–26. doi: 10.1086/596067
91. den Uyl D, van der Horst-Bruinsma I, van Agtmael M. Progression of HIV to AIDS: A Protective Role for HLA-B27? *AIDS Rev* (2004) 6:89–96.
92. López-Larrea C, Njobvu PD, González S, Blanco-Gelaz MA, Martínez-Borra J, López-Vázquez A. The HLA-B*5703 Allele Confers Susceptibility to the Development of Spondylarthropathies in Zambian Human Immunodeficiency Virus-Infected Patients With Slow Progression to Acquired Immunodeficiency Syndrome. *Arthritis Rheum* (2005) 52(1):275–9. doi: 10.1002/art.20722
93. Chen H, Hayashi G, Lai OY, Dilthey A, Kuebler PJ, Wong TV, et al. Psoriasis patients Are enriched for genetic variants that protect against HIV-1 disease. *PloS Genet* (2012) 8(2). doi: 10.1371/journal.pgen.1002514
94. Neumann-Haefelin C. HLA-B27-Mediated Protection in HIV and Hepatitis C Virus Infection and Pathogenesis in Spondyloarthritis: Two Sides of the Same Coin? *Curr Opin Rheumatol* (2013) 25(4):426–33. doi: 10.1097/BOR.0b013e328362018f
95. Plate A, Boyle B. Musculoskeletal Manifestations of HIV Infection. *AIDS Read* (2003) 13(2):69–76.
96. Mahajan A, Tandon V, Verma S. Rheumatological Manifestations in HIV Infection. *J Indian Acad Clin Med* (2006) 7(2):136–44.
97. Restrepo CS, Lemos DF, Gordillo H, Odero R, Varghese T, Tiemann W, et al. Imaging Findings in Musculoskeletal Complications of AIDS. *Radiographics* (2004) 24(4):1029–49. doi: 10.1148/rg.244035151
98. Rosenberg Z, Norman A, Solomon G. Arthritis Associated With HIV Infection: Radiographic Manifestations. *Radiology* (1989) 173(1):171–6. doi: 10.1148/radiology.173.1.2781004
99. Allroggen A, Frese A, Rahmann A, Gaubitz M, Husstedt I, Evers S. HIV Associated Arthritis: Case Report and Review of the Literature. *Eur J Med Res* (2005) 10(7):305–8.
100. Reveille J, Williams M. Rheumatologic Complications of HIV Infection. *Best Pract Res Clin Rheumatol* (2006) 20(6):1159–79. doi: 10.1016/j.berh.2006.08.015
101. KIDS-ART-LINC Collaboration. Low Risk of Death, But Substantial Program Attrition in Pediatric HIV Treatment Cohorts in Sub-Saharan Africa. *J Acquir Immune Defic Syndr* (2008) 49(5):523–31. doi: 10.1097/QAI.0b013e31818aadce
102. Carlucci JG, Liu Y, Clouse K, Vermund SH. Attrition of HIV-Positive Children From HIV Services in Low and Middle-Income Countries. *AIDS* (2019) 33(15):2375–86. doi: 10.1097/QAD.0000000000002366
103. Abuogi LL, Smith C, Mcfarland EJ. Retention of HIV-Infected Children in the First 12 Months of Anti-Retroviral Therapy and Predictors of Attrition in Resource Limited Settings : A Systematic Review. *PloS One* (2016) 11(6): e0156506. doi: 10.1371/journal.pone.0156506
104. Lamb MR, Fayorsey R, Nuwagaba-biribonwoha H, Viola V, Mutabazi V, Alwar T, et al. High Attrition Before and After ART Initiation Among Youth (15–24 Years of Age) Enrolled in HIV Care. *AIDS* (2014) 28(4):559–68. doi: 10.1097/QAD.0000000000000054
105. Fox MP, Shearer K, Maskew M, Meyer-rath G, Clouse K, Sanne I. Attrition Through Multiple Stages of Pre-Treatment and ART HIV Care in South Africa. *PloS One* (2014) 9(10):e110252. doi: 10.1371/journal.pone.0110252

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CASE REPORT
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Case Report: *FOXP3* Mutation in a Patient Presenting With ALPS

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ALPS and IPEX are two well-characterized inborn errors of immunity with immune dysregulation, considered as two master models of monogenic auto-immune diseases. Thus, with autoimmunity as their primary clinical manifestation, these two entities may show clinical overlap. Traditionally, immunological biomarkers are used to establish an accurate differential diagnosis. Herein, we describe a patient who presented with clinical features and biomarkers fulfilling the diagnostic criteria of ALPS. Severe apoptotic defect was also shown in the patient's cell lines and PHA-activated peripheral blood lymphocytes. Sanger sequencing of the *FAS* gene did not reveal any causal mutation. NGS screening revealed a novel deleterious variant located in the N terminal repressor domain of *FOXP3* but no mutations in the FAS pathway-related genes. TEMRA cells (terminally differentiated effector memory cells re-expressing CD45RA) and PD1 expression were increased arguing in favor of T-cell exhaustion, which could be induced by unrestrained activation of T effector cells because of Treg deficiency. Moreover, defective FOXP3 observed in the patient could intrinsically induce increased proliferation and resistance to apoptosis in T effector cells. This observation expands the spectrum of FOXP3 deficiency and underscores the role of NGS in detecting mutations that induce overlapping phenotypes among inborn errors of immunity with immune dysregulation. In addition, these findings suggest a potential link between FOXP3 and FAS pathways.

Keywords: FOXP3, IPEX, ALPS, NGS, inborn errors of immunity

INTRODUCTION

Primary immune dysregulation diseases are a group of monogenic disorders characterized by defective immune regulatory pathways (1–3). Their clinical presentation mainly comprises organ-specific autoimmunity, hyperinflammation, and nonmalignant lymphoproliferation. New sequencing technologies have improved the understanding of this ever-expanding group of disorders but they still often pose a diagnostic challenge because of their variable and complex phenotypic expressions and the overlap of symptoms between different entities (1, 4). Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) and autoimmune lymphoproliferative syndrome (ALPS) are archetypes of inborn errors of immunity (IEI) with immune dysregulation and have gained attention as models of monogenic autoimmunity.

IPEX syndrome is secondary to mutations in the Forkhead Box Protein 3 (FOXP3) gene, a transcription factor essential for regulatory T cell differentiation and function. Its clinical expression encompasses various combinations of autoimmune manifestations appearing early in life, with a characteristic triad comprising enteropathy, dermatitis, and autoimmune endocrinopathy, usually, type 1 diabetes (5).

ALPS represents a distinct pathologic mechanism for loss of immune tolerance caused by a defect in the Fas-FasL pathway. Noninfectious and nonmalignant lymphoproliferation associated with autoimmune cytopenias and a greatly increased lifetime risk of lymphoma constitute the major features of ALPS. The marking immunological phenotype remains an expansion of autoreactive double-negative T (DNT) cells (6), a subset of CD3+ cells bearing α/β T cell receptor (TCR), and negative for both CD4 and CD8 co-receptors. They massively accumulate in patients with ALPS but are also reported in other inflammatory and autoimmune conditions (7, 8). A chronic non-malignant lymphadenopathy, an elevation of peripheral DNT cells, and a defective apoptosis assay were first proposed as a required triad of criteria to establish the diagnosis of ALPS (9). Later on, serum sFasL, IL-10, and vitamin B12 were shown to be elevated in ALPS because of *FAS* mutations (ALPS-FAS), and their association to high DNT cells rose as an accurate predictor for the presence of germline or somatic *FAS* mutation (10, 11). Consequently, these biomarkers were included in the revised diagnostic criteria of ALPS to facilitate the patients' identification particularly in settings where no access to advanced genetic analysis or functional testing are available (12).

The functional apoptosis test allows to detect a defective response upon Fas stimulation which results in abnormal cell survival and is sufficient for definitive ALPS diagnosis provided that required criteria are fulfilled (12). Therefore, defective Fas driven apoptosis is considered specific to ALPS and prompts molecular testing when a patient has suggestive clinical and laboratory features.

Despite the establishment of well-characterized ALPS diagnostic guidelines, phenotypic overlap with other primary immune dysregulation conditions still poses a diagnostic challenge (13). Indeed, previous reports have shown that LRBA deficiency, *STAT3* GOF mutations and ITK deficiency may be misdiagnosed as ALPS and may share with it some clinical and immunological features (13–17).

Herein, we describe a patient bearing a novel *FOXP3* mutation in the N terminal repressor domain of the protein who fulfills the current criteria for definitive diagnosis for ALPS (12, 18).

CASE DESCRIPTION

Born to a non-consanguineous marriage, the patient had a family history of infant deaths without clearly identified causes. His clinical manifestations started at the age of 2 months and were marked by generalized squamous dermatitis and multiple adenopathies without hepatosplenomegaly. Langerhans cell histiocytosis diagnosis was initially proposed based on the presence of multiple CD1a+PS100+ cells in a retroauricular lymph node biopsy, then ruled out because of the atypical cutaneous manifestation and the absence of histiocytic proliferation. The dermatitis significantly improved after the use of topical corticosteroids and evolved to skin xerosis. At the age of 10 months, the clinical course was marked by the occurrence of a severe prolonged diarrhea episode with edema and hypoproteinemia requiring hospitalization. Etiological investigations, including coeliac disease autoantibodies screening, sweat test, and RAST test to cow milk proteins were all negative. The diarrhea resolved under symptomatic treatment with exclusion of cow milk and did not relapse. Interestingly, during this episode, biological investigations revealed hemolytic anemia with positive direct Coombs test (AIHA). The patient received oral prednisone at 2 mg/kg/day during 1 month followed by a progressive decrease during 3 months with a stabilization of the hemoglobin rate. At the age of 14 months, he presented with an Evans syndrome as revealed by epistaxis and purpura. The patient's platelet level was very low with a normal platelet size, and his anemia was critical, requiring transfusion. He underwent corticosteroid treatment (prednisone, 2 mg/kg/day) and received intravenous immunoglobulins (1 g/kg) with a poor response. During hospitalization, he also experienced a resolutive episode of pneumonia. The clinical picture was further worsened by multiple relapses of Evans syndrome, which required full-dose corticosteroid (prednisone, 2 mg/kg) and two hospitalizations at age 18 months and 3 years (**Figure 1**). Following the initial period of recurrent corticosteroid-dependent cytopenias, he continued to receive full dose of corticosteroids, allowing full remission at the age of 4 years. Interestingly, corticosteroid has been decreased then stopped for 3 years. His follow-up at age 7 years showed a favorable outcome. His condition is now characterized by the persistence of chronic adenopathies with no histological signs of malignancy and skin xerosis. During this follow-up, no gastro-intestinal manifestations, no endocrinopathies, or other auto-immune manifestations were recorded with the exception of a recent episode of thrombocytopenia. His infectious course was

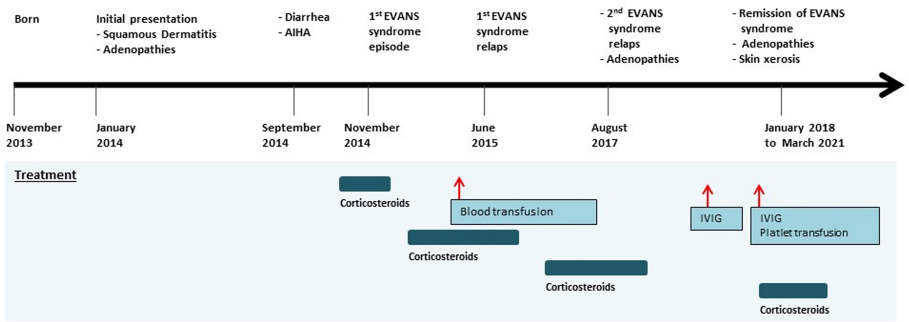

FIGURE 1 | Timeline of the clinical events and treatment strategies. AIHA, autoimmune hemolytic anemia; IVIG, intravenous immunoglobulin.

characterized by the occurrence of recurrent episodes of otitis media.

TIMELINE

The timeline is presented in **Figure 1**.

Diagnostic Assessment

The diagnosis of IEI was suspected given the patient's presentation. Preliminary immunological investigations showed polyclonal hypergammaglobulinemia (IgG: 15.51 g/L), elevated IgE, and hypereosinophilia. The standard immunophenotyping of lymphocyte subpopulations (T, B, and NK cells) assessed by flow cytometry was within the normal range. Lymphoproliferative responses to mitogens and antigens were normal. Recurrent Evans syndrome associated with chronic lymphoproliferations prompted the evaluation of ALPS criteria. Double negative T cells CD3+TCRα/β+CD4−CD8− percentage was increased, reaching 5.1%. Soluble FasL and IL-10 plasma levels were high. Apoptosis functional assay was performed on the patient's EBV-transformed B-cells and showed severe resistance to Fas-mediated cell death in comparison to a healthy control, as confirmed in two separate experiments (**Figure 2A**). It has also been performed on the patient's PHA-activated peripheral blood lymphocytes and confirmed the resistance to apoptosis as compared with healthy control (**Figure 2A**). This defect of apoptosis is persistent at age 7 years. All biological and immunological investigations are summarized in **Table 1**.

The criteria for definitive ALPS diagnosis were fulfilled. Indeed, the two required criteria (i.e., expanded double negative T cells and chronic lymphadenopathy) were present together with one accessory primary criterion, namely, defective lymphocyte apoptosis in two separate assays and two accessory secondary criteria, namely, autoimmune cytopenia with elevated polyclonal IgG and elevated plasma sFasL levels (>200 pg/ml) and IL-10 levels (>20 pg/ml). The resistance to Fas-driven apoptosis argued in favor of a defect in the Fas pathway. The *FAS* gene was screened by Sanger sequencing for mutations in all exons and intron-exon junctions, because FAS deficiency accounts for approximately 65% of all ALPS cases. Surprisingly, no *FAS* germline mutation was found. Somatic *FAS* mutations were excluded because their presence does not fit with the defective apoptosis assay.

Targeted NGS, using the PID v2 gene panel (including all known ALPS genes) and an Ion Torrent S5 sequencer identified a novel missense mutation c.224C>T (p.Pro75Leu) located in *FOXP3*. No variants in other genes were found, which might have contributed to the patient's phenotype. Sanger sequencing confirmed the mutation using forward and reverse specific primers (5′CCATGAGCCTCAGTTTCCAT and 5′ CACCTTT GACCCCCAGAGTA). The patient's mother was heterozygous for the same mutation (**Figure 2B**). This mutation is located in the repressor domain of FOXP3 and is not reported in 1000G or ExAC databases. The affected residue is conserved among vertebrates (**Figure 2C**), and the mutation is predicted to be deleterious in multiple *in silico* prediction tools (probably damaging in Polyphen2, damaging in SIFT and CADD score of 24).

To assess the consequences of the *FOXP3* mutation, FOXP3 expression was performed in patient's PBMCs as compared to healthy control, after staining with anti-CD4/APC, anti-CD25/BB-515 and anti-Foxp3/PE with or without activation for 72 h using anti-CD3/anti-CD28 monoclonal antibodies. FOXP3+ CD25+ cells were absent in patient's unstimulated cells (0.1% of CD4+) but were induced after activation (27.8%) (**Figure 2D**).

The search for the presence of autoantibodies using an extensive set of antigens displayed negative results except for anti-cardiolipin IgG (**Table 1**).

To decipher the potential link between FOXP3 defect and the ALPS phenotype, and based on the hypothesis that Treg deficiency would induce an unrestrained activation of T effector cells that are resistant to Fas driven apoptosis (19), we performed an immunophenotyping of TEMRA cells. These cells have been defined as the latest stage of T-cell differentiation which accumulate through successive rounds of antigen encounters (20, 21) and could be induced by persistent re-stimulation of individual clones (22). We found increased percentage of CD4+ TEMRA cells in the patient reaching 24%

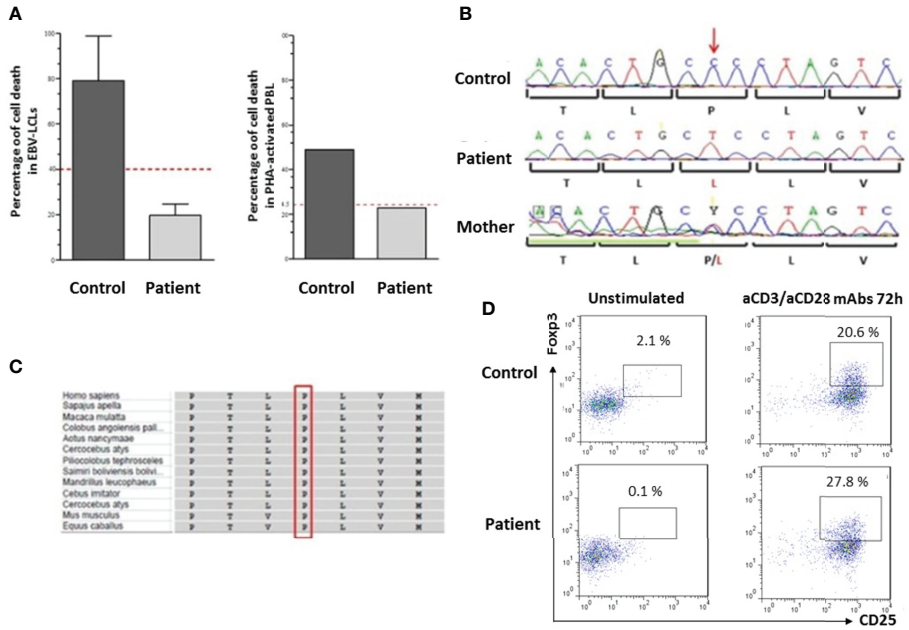

FIGURE 2 | Genetic and functional investigations **(A)** Apoptosis assay: The IPEX patient exhibits a significantly altered apoptosis. Fas-induced apoptosis in EBV-lymphoblastoid cell lines (EBV-LCL) or PHA-activated peripheral blood lymphocytes of the patient and a healthy control were stimulated for 24 h with or without APO-1-3 in the presence of protein A. Subsequently apoptosis was measured by flow cytometric detection of Annexin V-PE and 7AAD. For apoptosis in EBV-LCL, data represent two separate experiments and the T bars indicate the standard deviation. The dotted red line represents 50% of the cell death observed in the control. **(B)** Genomic DNA sequence analysis of *FOXP3* gene showing a missens hemizygous mutation p.Pro75Leu in the patient, the mother was heterozygous for the mutation. **(C)** Multiple protein alignment showing the position of the conserved proline 75 residue in different species using the NCBI Multiple Sequence Alignment Viewer, Version 1.19.1. **(D)** FOXP3 expression in PBMCs after staining with anti-CD4/APC, anti-CD25/BB-515 and anti-Foxp3/PE as compared to healthy control. Treg cells were absent in patient's unstimulated cells and induced after activation with anti-CD3 (aCD3)/anti-CD28 (aCD28) mAb during 72 h.

(normal range for the age, 4–15%) (**Table 1** and **Figure 3A**). Moreover, the patient showed high PD1 expression by 10.9% on CD4+T cells, one of the hallmarks of T cell exhaustion (23), in comparison to healthy controls (**Figure 3B**).

DISCUSSION

The classical IPEX presentation consists of a triad of symptoms, comprising diarrhea, autoimmune endocrinopathies and dermatitis (5, 24, 25). The most consistent clinical finding, often inaugural in this disease, is the exudative enteropathy (24). It classically begins very early, during the first few months of life, characterized by its persistence, and usually complicates with malabsorption and failure to thrive (5, 24, 25). The patient we report did first present with dermatitis and multiple adenopathies. Unlike in the IPEX classical presentation, this patient had a single limited episode of severe diarrhea at age ten months. Shortly after, he developed Evans syndrome associated with bleeding complications. Autoimmune cytopenias are a characteristic feature of ALPS. However, they are also observed in up to 26% of IPEX patients (25, 26). Moreover, the patient did not develop autoimmune endocrinopathies, which are frequently reported in IPEX patients and consist most commonly in type 1 diabetes mellitus occurring in the first year of life (5, 24, 25). It has been reported that in the absence of clinical disease, anti-glutamic acid decarboxylase antibodies or anti-islet cell antibodies may be positive (5). The screening of this patient's serum for autoantibodies associated with endocrinopathies was negative, and no endocrine disorders were recorded during the follow-up. The patient's clinical presentation was also marked by the occurrence of infectious manifestations consisting of recurrent media otitis and one episode of resolving pneumonia. This contrasts with the classical severe infections observed in IPEX patients, which may be life-threatening sepsis (5, 24, 25). Thus, the IPEX clinical presentation in this patient is unusual with mild or absent features of the characteristic IPEX triad and the predominance of an Evans syndrome. In this context, the persistence of benign adenopathies and hypergammaglobulinemia shaped a clinical picture more reminiscent of ALPS, the most typical IEI associated with autoimmune cytopenias (27). Moreover, the immunohistological pattern of sinus histiocytosis with S100 positive cells observed in the patient's lymph node biopsy has not only been reported in ALPS (6) but also in a single description of IPEX patient (28).

The patient's laboratory investigations showed hypereosinophilia and elevated IgE. The increase of these two parameters is common in IPEX patients. Although they are not usually characteristic of ALPS classical presentation, their increase was reported in patients with ALPS (29–31).

TABLE 1 | Results of the immunological investigations.

Immunological Investigation	Results	Reference Values	Unit
Absolute leucocyte count	21.7	6.4–12.00	10^9/L
Absolute lymphocyte count	7.1	3.60–8.90	10^9/L
Absolute eosinophil count	9.8	<0.5	10^9/L
Flow cytometry lymphocyte phenotyping			
T CD3+	91	53–75	% of Lc
T CD4+	52	32–51	% of Lc
T CD8+	25	14–30	% of Lc
TCR α/β+ CD4-CD8- (DNT cells)	**5↑**	<1.5	% of CD3+ Lc
CD25+FOXP3+ (Treg cells)	**0.11↓**	–	% of CD4+ Lc
CD45RA+CCR7- (TEMRA cells)	**24↑**	4–15	% of CD4+ Lc
Lymphocyte proliferation tests			
PHA	108307	Control: 98849	cpm
Anti-CD3	112209	Control: 80842	cpm
Immunoglobulins			
IgG	**15.51↑**	3.4–6.2	g/L
IgA	1.12	0.33–1.22	g/L
IgM	**0.31↓**	0.48–1.43	g/L
IgE	**6046↑**	<144	KU/l
ALPS parameters			
sFasL:	**1.4↑**	≤0.2	ng/ml
IL-10 (tested twice):	**69.4↑ ; 24↑**	≤20	pg/ml
CD95:	67	–	% of Lc
Apoptosis functional assay in EBV-LCL:	**19.6↓**	Control: 79; 4	% of cell death
Apoptosis functional assay in PHA-PBL:	**23↓**	Control: 49	% of cell death
Vitamin B12 (tested twice):	1061; 564	345–1154	pg/ml
Autoantibodies			
Anti-nuclear antibodies	Negative	<1:80	Titer
Anti-DNA	Negative	<1:10	Titer
Anti-smooth muscle	Negative	<1:100	Titer
Anti-mitochondrial antibodies	Negative	<1:100	Titer
Anti-cardiolipin IgM	Negative	<12	PL U/ml
Anti-cardiolipin IgG	**20,81↑**	<12	PL U/ml
Anti-β2 glycoprotein 1 IgM	Negative	<5	PL U/ml
Anti-β2 glycoprotein 1 IgG	Negative	<5	PL U/ml
Rheumatoid factor	Negative	15	U/ml
Anti-cyclic citrullinated peptides	Negative	5	U/ml
Anti-TSH receptor	Negative	5	IU/ml
Anti- thyroperoxidase antibodies	Negative	<50	U/ml
Anti-intrinsic factor antibodies	Negative	–	NA
Autoantibodies against parietal cell antigens	Negative	–	NA
Anti-enzyme tissue transgulataminase IgA	Negative	<4	U/ml
Anti-deamidated gliadin peptide	Negative	<10	IU/ml
Anti-glutamic acid decarboxylase	Negative	<10	IU/ml

Altered parameters are in bold. : ↑: increased; ↓: reduced. SI, Stimulation Index; NA, not applicable; DNT, cells: double negative t cells; Treg cells, T regulatory cells; TEMRA cells, terminally differentiated effector memory cells reexpressing CD45RA; PHA, phytohemagglutinin. Lymphocyte proliferation, expressed as radioactive counts per minutes (cpm) measured by 3H thymidine incorporation into DNA from the patient and a healthy control studied the same day. EBV-LCL, EBV-lymphoblastoid cell lines; PHA-PBL, PHA-activated peripheral blood lymphocytes; anti-TSH receptor, anti-thyroid stimulating hormone receptor.

Besides these overlapping features, the most striking finding in this observation is the presence of almost all ALPS biomarkers.

To our best knowledge, there are no published reports of IPEX associated with elevated ALPS biomarkers and a defective Fas apoptosis assay. Elevated DNT cells were initially considered as a hallmark of ALPS (9) and are still a required criterion for the diagnosis (12). This biomarker specificity has been challenged because other IEIs have displayed elevated DNT cells, such as LRBA deficiency, STAT3 GOF disease and CVID (13, 16). Elevation of DNT cells has also been reported in patients with autoimmune diseases (8, 32). However, when they exceed 4%, they are considered as highly predictive of *FAS* mutations (11). Nevertheless, high percentages of this subset, exceeding this threshold, have been recorded in LRBA deficiency, reaching 10% and in *STAT3* GOF disease reaching 20% (13, 16). In our patient, DNT cells reached 5% and were associated with other ALPS biomarkers, including elevation of IL-10 and sFasL and a defective Fas-induced apoptosis. Interestingly, in a recent review of key diagnostic markers of ALPS, the combination of elevated DNTs and an abnormal *in vitro* apoptosis functional test was considered the most useful in identifying all types of ALPS patients (33). The combination of an abnormal *in vitro* apoptosis functional test and elevated sFasL was a predictive marker for ALPS-FAS group classification (33).

The *FOXP3* mutation identified changed the diagnosis from ALPS to IPEX, clearly showing that NGS use is critical to unmask

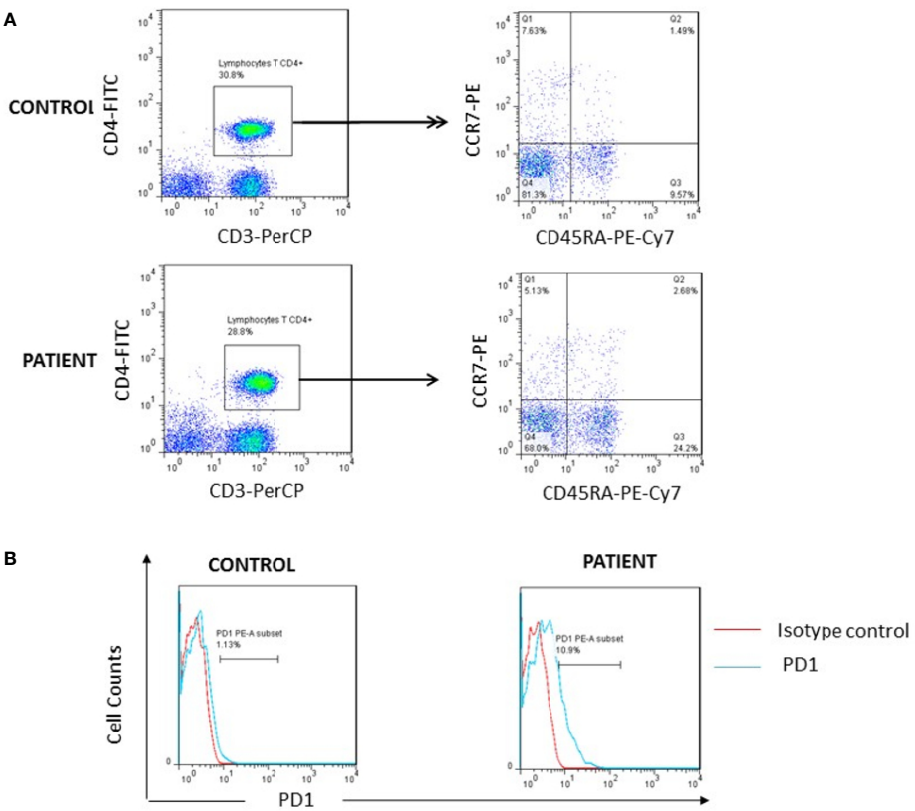

FIGURE 3 | Investigation of T cell exhaustion. **(A)** High percentage of TEMRA cells (Q3) was found in patient after whole blood staining with anti-CD3/PerCP, anti-CD4/FITC, anti-CD45RA/PE-Cy7 and anti-CCR7/PE as compared to one representative healthy control. **(B)** Increased surface expression of PD1 on the patient's CD4+ lymphocytes as compared to one representative control..

atypical presentations of IPEX and is reshaping the clinical and immunological spectrum of this rare and probably under-diagnosed disease (34). No less than 70 pathological variants have been identified in the *FOXP3* gene (5, 35). Interestingly, some mutations were more frequently associated with a mild or atypical presentation (34), such as reported here with currently a favorable outcome. This contrasts with the classical outcome of IPEX syndrome, generally associated with a poor prognosis (24). The mutation we report resides in the N terminal proline-rich domain, thought to mediate the repressive activity of FOXP3 (36). On another hand, the mutation is located in exon 3, which is the second coding exon (the first being a non-coding one) of a total of 12 exons. It is worth mentioning that some of these exons could be alternatively spliced leading to naturally occurring FOXP3 isoforms and may be associated with slower kinetics of FOXP3 export (37, 38). The mutation we identified was associated with the absence of CD4+CD25+FOXP3+ cells. Since steroid administration may reduce FOXP3 expression, the latter was assessed after activation and found to be restored. This finding is in accordance with the study of Gambineri et al. (39), showing that some FOXP3 mutations may not hinder protein expression after activation despite initial very low levels of Treg. The assessment of the correlation between restored FOXP3 expression and residual Treg function must be further investigated and may explain the moderate clinical phenotype of the patient.

The defect of apoptosis assay observed in the patient is somewhat intriguing. An altered functional apoptosis assay was previously observed in other immune regulation disorders, such as STAT3 GOF disease and LRBA deficiency (14, 16). Resistance to apoptosis in STAT3 GOF disease appears to be secondary to a disturbance in the balance of BCL2 family proteins, which are critical intrinsic regulators of the apoptotic pathway (16). The compromised Fas triggered apoptosis described in LRBA deficiency is suggested to be due to an altered autophagy. Indeed, autophagy is significantly reduced in LRBA deficient cells and has been shown to facilitate Fas-mediated apoptosis (14).

The Treg deficiency in the patient could result in unrestrained activation of T effector cells. The latter would become exhausted and thus resistant to apoptosis (19). Consistent with this hypothesis, we found increased CD4+TEMRA cells and PD1 expression in patient as compared with healthy controls.

On the other hand, defective FOXP3 observed in the patient could intrinsically induce increased proliferation and resistance to apoptosis in T effector cells, as herein reported. There is

evidence in the literature that the amount of FOXP3 expressed within a cell controls the balance between life and death. Indeed, it has been shown that T cells from mice and humans that lack functional FOXP3 are hyper-responsive to TCR stimulation, and those from mice that overexpress FOXP3 are hypo-responsive to TCR-mediated stimulation as manifested by the reduction in proliferative capacity. Consequently, defect in cell cycle progression after TCR engagement may result in altered apoptosis (40).

In summary, this case expands the clinical spectrum of Foxp3 deficiency and suggests a potential link between FOXP3 and the FAS apoptotic pathway that requires further investigation.

PATIENT PERSPECTIVE

The patient did adhere to the treatment proposed. He and his family were satisfied by the relative improvement of his clinical condition.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/clinvar/variation/1098427/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'éthique biomédicale de l'Institut Pasteur de Tunis. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AR, NM, NA and IB-M performed immunological investigations and interpretation of results. MS performed the screening of autoantibodies and interpretation of results. FF and MB were the clinicians in charge of patient care and management. MFA and RG performed targeted NGS and interpretation of results. IB-M, M-RB, and RG contributed to conception and design of the study. AR and IB-M wrote the initial manuscript draft. AR, NM, MA, RG, IB-M and M-RB reviewed the manuscript and contributed to the final draft. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Chandrakasan S, Chandra S, Davila Saldana BJ, Torgerson TR, Buchbinder D. Primary Immune Regulatory Disorders for the Pediatric Hematologist and Oncologist: A Case-Based Review. *Pediatr Blood Cancer* (2019) 66(5):e27619. doi: 10.1002/pbc.27619
2. Cepika AM, Sato Y, Liu JM, Uyeda MJ, Bacchetta R, Roncarolo MG. Tregopathies: Monogenic Diseases Resulting in Regulatory T-Cell Deficiency. *J Allergy Clin Immunol* (2018) 142(6):1679–95. doi: 10.1016/j.jaci.2018.10.026
3. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS Phenotypic Classification for Primary Immunodeficiencies. *J Clin Immunol* (2018) 38(1):129–43. doi: 10.1007/s10875-017-0465-8
4. Takagi M, Hoshino A, Yoshida K, Ueno H, Imai K, Piao J, et al. Genetic Heterogeneity of Uncharacterized Childhood Autoimmune Diseases With Lymphoproliferation. *Pediatr Blood Cancer* (2018) 65(2). doi: 10.1002/pbc.26831
5. Gambineri E, Ciullini Mannurita S, Hagin D, Vignoli M, Anover-Sombke S, DeBoer S, et al. Clinical, Immunological, and Molecular Heterogeneity of 173 Patients With the Phenotype of Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) Syndrome. *Front Immunol* (2018) 9:2411. doi: 10.3389/fimmu.2018.02411
6. Matson DR, Yang DT. Autoimmune Lymphoproliferative Syndrome: An Overview. *Arch Pathol Lab Med* (2020) 144(2):245–51. doi: 10.5858/arpa.2018-0190-RS
7. Crispín JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded Double Negative T Cells in Patients With Systemic Lupus Erythematosus Produce IL-17 and Infiltrate the Kidneys. *J Immunol (Baltimore Md: 1950)* (2008) 181(12):8761–6. doi: 10.4049/jimmunol.181.12.8761
8. Tarbox JA, Keppel MP, Topcagic N, Mackin C, Ben Abdallah M, Baszis KW, et al. Elevated Double Negative T Cells in Pediatric Autoimmunity. *J Clin Immunol* (2014) 34(5):594–9. doi: 10.1007/s10875-014-0038-z
9. Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. An Inherited Disorder of Lymphocyte Apoptosis: The Autoimmune Lymphoproliferative Syndrome. *Ann Internal Med* (1999) 130(7):591–601. doi: 10.7326/0003-4819-130-7-199904060-00020
10. Magerus-Chatinet A, Stolzenberg MC, Loffredo MS, Neven B, Schaffner C, Ducrot N, et al. FAS-L, IL-10, and Double-Negative CD4- CD8- TCR Alpha/Beta+ T Cells Are Reliable Markers of Autoimmune Lymphoproliferative Syndrome (ALPS) Associated With FAS Loss of Function. *Blood* (2009) 113(13):3027–30. doi: 10.1182/blood-2008-09-179630
11. Caminha I, Fleisher TA, Hornung RL, Dale JK, Niemela JE, Price S, et al. Using Biomarkers to Predict the Presence of FAS Mutations in Patients With Features of the Autoimmune Lymphoproliferative Syndrome. *J Allergy Clin Immunol* (2010) 125(4):946–9.e6. doi: 10.1016/j.jaci.2009.12.983
12. Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Lenardo MJ, et al. Revised Diagnostic Criteria and Classification for the Autoimmune Lymphoproliferative Syndrome (ALPS): Report From the 2009 NIH International Workshop. *Blood* (2010) 116(14):e35–40. doi: 10.1182/blood-2010-04-280347
13. Cagdas D, Halaçlı SO, Tan Ç, Lo B, Çetinkaya PG, Esenboğa S, et al. A Spectrum of Clinical Findings From ALPS to CVID: Several Novel LRBA Defects. *J Clin Immunol* (2019) 39(7):726–38. doi: 10.1007/s10875-019-00677-6
14. Revel-Vilk S, Fischer U, Keller B, Nabhani S, Gámez-Díaz L, Rensing-Ehl A, et al. Autoimmune Lymphoproliferative Syndrome-Like Disease in Patients With LRBA Mutation. *Clin Immunol (Orlando Fla)* (2015) 159(1):84–92. doi: 10.1016/j.clim.2015.04.007
15. Oliveira JB. The Expanding Spectrum of the Autoimmune Lymphoproliferative Syndromes. *Curr Opin Pediatr* (2013) 25(6):722–9. doi: 10.1097/MOP.0000000000000032
16. Nabhani S, Schipp C, Miskin H, Levin C, Postovsky S, Dujovny T, et al. STAT3 Gain-of-Function Mutations Associated With Autoimmune Lymphoproliferative Syndrome Like Disease Deregulate Lymphocyte Apoptosis and can be Targeted by BH3 Mimetic Compounds. *Clin Immunol (Orlando Fla)* (2017) 181:32–42. doi: 10.1016/j.clim.2017.05.021

17. Wallace JG, Alosaimi MF, Khayat CD, Jaber F, Almutairi A, Beaussant-Cohen S, et al. ITK Deficiency Presenting as Autoimmune Lymphoproliferative Syndrome. *J Allergy Clin Immunol* (2021) 147(2):743–5.e1. doi: 10.1016/j.jaci.2020.06.019
18. Seidel MG, Kindle G, Gathmann B, Quinti I, Buckland M, van Montfrans J, et al. The European Society for Immunodeficiencies (ESID) Registry Working Definitions for the Clinical Diagnosis of Inborn Errors of Immunity. *J Allergy Clin Immunol In Pract* (2019) 7(6):1763–70. doi: 10.1016/j.jaip.2019.02.004
19. Gupta S, Gollapudi S. CD95-Mediated Apoptosis in Naïve, Central and Effector Memory Subsets of CD4+ and CD8+ T Cells in Aged Humans. *Exp Gerontol* (2008) 43(4):266–74. doi: 10.1016/j.exger.2007.12.006
20. Weekes MP, Wills MR, Mynard K, Hicks R, Sissons JG, Carmichael AJ. Large Clonal Expansions of Human Virus-Specific Memory Cytotoxic T Lymphocytes Within the CD57+ CD28- CD8+ T-Cell Population. *Immunology* (1999) 98(3):443–9. doi: 10.1046/j.1365-2567.1999.00901.x
21. Pangrazzi L, Naismith E, Meryk A, Keller M, Jenewein B, Trieb K, et al. Increased IL-15 Production and Accumulation of Highly Differentiated CD8 (+) Effector/Memory T Cells in the Bone Marrow of Persons With Cytomegalovirus. *Front Immunol* (2017) 8:715. doi: 10.3389/fimmu.2017.00715
22. Larbi A, Fulop T. From "Truly Naïve" to "Exhausted Senescent" T Cells: When Markers Predict Functionality. *Cytomet Part A: J Int Soc Anal Cytol* (2014) 85(1):25–35. doi: 10.1002/cyto.a.22351
23. Wherry EJ. T Cell Exhaustion. *Nat Immunol* (2011) 12(6):492–9. doi: 10.1038/ni.2035
24. Tan QKG, Louie R, Sleasman JW. IPEX Syndrome. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Mirzaa G, et al., editors. *GeneReviews® [Internet]*. Seattle (WA): University of Washington, Seattle; 1993-2021 (2004). Available at: https://www.ncbi.nlm.nih.gov/books/NBK1118/.
25. Jamee M, Zaki-Dizaji M, Lo B, Abolhassani H, Aghamahdi F, Mosavian M, et al. Clinical, Immunological, and Genetic Features in Patients With Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) and IPEX-Like Syndrome. *J Allergy Clin Immunol In Pract* (2020) 8(8):2747–60.e7. doi: 10.1016/j.jaip.2020.04.070
26. Barzaghi F, Amaya Hernandez LC, Neven B, Ricci S, Kucuk ZY, Bleesing JJ, et al. Long-Term Follow-Up of IPEX Syndrome Patients After Different Therapeutic Strategies: An International Multicenter Retrospective Study. *J Allergy Clin Immunol* (2018) 141(3):1036–49.e5. doi: 10.1016/j.jaci.2017.10.041
27. Seidel MG. Autoimmune and Other Cytopenias in Primary Immunodeficiencies: Pathomechanisms, Novel Differential Diagnoses, and Treatment. *Blood* (2014) 124(15):2337–44. doi: 10.1182/blood-2014-06-583260
28. Sayar E, Uygun DF, Islek A, Hazar-Sayar E, Akkaya B, Vignoli M, et al. Langerhans Cell Histiocytosis in IPEX Syndrome: Possible Role for Natural Regulatory T Cells? *Pediatr Allergy Immunol: Off Publ Eur Soc Pediatr Allergy Immunol* (2014) 25(6):601–3. doi: 10.1111/pai.12219
29. Butt D, Chan TD, Bourne K, Hermes JR, Nguyen A, Statham A, et al. FAS Inactivation Releases Unconventional Germinal Center B Cells That Escape Antigen Control and Drive IgE and Autoantibody Production. *Immunity* (2015) 42(5):890–902. doi: 10.1016/j.immuni.2015.04.010
30. Kim YJ, Dale JK, Noel P, Brown MR, Nutman TB, Straus SE, et al. Eosinophilia Is Associated With a Higher Mortality Rate Among Patients With Autoimmune Lymphoproliferative Syndrome. *Am J Hematol* (2007) 82(7):615–24. doi: 10.1002/ajh.20851
31. Price S, Shaw PA, Seitz A, Joshi G, Davis J, Niemela JE, et al. Natural History of Autoimmune Lymphoproliferative Syndrome Associated With FAS Gene Mutations. *Blood* (2014) 123(13):1989–99. doi: 10.1182/blood-2013-10-535393
32. El-Sayed ZA, El-Owaidy RH. Alpha Beta Double Negative T Cells in Children With Systemic Lupus Erythematosus: The Relation to Disease Activity and Characteristics. *Mod Rheumatol* (2018) 28:(4):654–60. doi: 10.1080/14397595.2017.1377146
33. Molnár E, Radwan N, Kovács G, Andrikovics H, Henriquez F, Zarafov A, et al. Key Diagnostic Markers for Autoimmune Lymphoproliferative Syndrome With Molecular Genetic Diagnosis. *Blood* (2020) 136(17):1933–45. doi: 10.1182/blood.2020005486
34. Consonni F, Ciullini Mannurita S, Gambineri E. Atypical Presentations of IPEX: Expect the Unexpected. *Front Pediatr* (2021) 9:643094. doi: 10.3389/fped.2021.643094
35. Bacchetta R, Barzaghi F, Roncarolo MG. From IPEX Syndrome to FOXP3 Mutation: A Lesson on Immune Dysregulation. *Ann New York Acad Sci* (2018) 1417(1):5–22. doi: 10.1111/nyas.13011
36. Deng G, Xiao Y, Zhou Z, Nagai Y, Zhang H, Li B, et al. Molecular and Biological Role of the FOXP3 N-Terminal Domain in Immune Regulation by T Regulatory/Suppressor Cells. *Exp Mol Pathol* (2012) 93(3):334–8. doi: 10.1016/j.yexmp.2012.09.013
37. Magg T, Mannert J, Ellwart JW, Schmid I, Albert MH. Subcellular Localization of FOXP3 in Human Regulatory and Nonregulatory T Cells. *Eur J Immunol* (2012) 42(6):1627–38. doi: 10.1002/eji.201141838
38. Mailer RKW. Alternative Splicing of FOXP3-Virtue and Vice. *Front Immunol* (2018) 9:530. doi: 10.3389/fimmu.2018.00530
39. Gambineri E, Perroni L, Passerini L, Bianchi L, Doglioni C, Meschi F, et al. Clinical and Molecular Profile of a New Series of Patients With Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome: Inconsistent Correlation Between Forkhead Box Protein 3 Expression and Disease Severity. *J Allergy Clin Immunol* (2008) 122(6):1105–12 e1. doi: 10.1016/j.jaci.2008.09.027
40. Kasprowicz DJ, Droin N, Soper DM, Ramsdell F, Green DR, Ziegler SF. Dynamic Regulation of FoxP3 Expression Controls the Balance Between CD4+ T Cell Activation and Cell Death. *Eur J Immunol* (2005) 35(12):3424–32. doi: 10.1002/eji.200526339

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Prognostic Gene Expression Signature in Patients With Distinct Glioma Grades

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Background: Glioma is the most common type of primary brain tumor in adults. Patients with the most malignant form have an overall survival time of <16 months. Although considerable progress has been made in defining the adapted therapeutic strategies, measures to counteract tumor escape have not kept pace, due to the developed resistance of malignant glioma. In fact, identifying the nature and role of distinct tumor-infiltrating immune cells in glioma patients would decipher potential mechanisms behind therapy failure.

Methods: We integrated into our study glioma transcriptomic datasets from the Cancer Genome Atlas (TCGA) cohort (154 GBM and 516 LGG patients). LM22 immune signature was built using CIBERSORT. Hierarchical clustering and UMAP dimensional reduction algorithms were applied to identify clusters among glioma patients either in an unsupervised or supervised way. Furthermore, differential gene expression (DGE) has been performed to unravel the top expressed genes among the identified clusters. Besides, we used the least absolute shrinkage and selection operator (LASSO) and Cox regression algorithm to set up the most valuable prognostic factor.

Results: Our study revealed, following gene enrichment analysis, the presence of two distinct groups of patients. The first group, defined as cluster 1, was characterized by the presence of immune cells known to exert efficient antitumoral immune response and was associated with better patient survival, whereas the second group, cluster 2, which exhibited a poor survival, was enriched with cells and molecules, known to set an immunosuppressive pro-tumoral microenvironment. Interestingly, we revealed that gene expression signatures were also consistent with each immune cluster function. A strong presence of activated NK cells was revealed in cluster 1. In contrast, potent immunosuppressive components such as regulatory T cells, neutrophils, and M0/M1/M2 macrophages were detected in cluster 2, where, in addition, inhibitory immune checkpoints, such as PD-1, CTLA-4, and TIM-3, were also significantly upregulated. Finally, Cox regression analysis further corroborated that tumor-infiltrating cells from cluster 2 exerted a significant impact on patient prognosis.

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Conclusion: Our work brings to light the tight implication of immune components on glioma patient prognosis. This would contribute to potentially developing better immune-based therapeutic approaches.

Keywords: glioma, tumour-infiltrating immune cell, biomarker, immune response, prognosis

INTRODUCTION

Glioma is the most common type of primary brain tumors in adults, with the most aggressive form known as glioblastoma. Glioma patients are known to be extremely resistant to chemotherapy. Indeed, such a trait is the leading cause of cancer-related mortality and morbidity (1, 2). Patients with glioma have a median overall survival of <16 months (3). The World Health Organization (WHO) classified gliomas into two groups, low-grade glioma (LGG) assembling grades I and II, and high-grade glioma (HGG) of grades III and IV (4, 5). Furthermore, based on histological and molecular features, glioma patients can be stratified into oligodendroglioma or astrocytoma (LGG) and glioblastoma (HGG) with clinically relevant molecular subtypes including mesenchymal, proneural, neural, and classical. Nevertheless, these features can be associated with tumor niche (stromal cells, reactive astrocytes, tumor cells, and immune cells), patient survival, and prognosis (6, 7).

Previous studies on glioma have provided an overview regarding tumor initiation and development (8, 9). As a matter of fact, a variety of chemoattractant and inflammatory cytokines secreted by either cancer or immune cells play a pivotal role in glioma tumor progression by attracting Treg cells into the tumor microenvironment, thus inhibiting NK cell activity and hampering the activation of effector T cells. Moreover, it leads to a plasticity phenomenon that leads to the conversion of tumor-associated macrophages (TAMs) from M1 state to an anti-inflammatory M2 phenotype and T cell transition from Th1 toward a Th2 phenotype which are often associated with poor outcome (6, 10–12). Hence, the cross talk between tumor and the immune cells results in the establishment of an immunosuppressive tumor microenvironment, which promotes tumor escape and cancer progression.

Over the past few years, several immunotherapeutic approaches have been developed (13); however, clinical trials testing vaccines, cell adoptive transfer, and immune checkpoint inhibitors (ICIs) point to a lack of efficacy (14–16).

This failure has shifted the focus toward a more thorough understanding of the distinct components of the glioma microenvironment (17–19). Glioblastomas are very low-immunogenicity tumors (16), enriched with immune-suppressive T cells mainly embedded in the central nervous system (20). As a matter of fact, future treatment strategies should be designed based on an improved understanding of key immune cell interactions with glioma cells.

Recently, computational analyses of RNA-seq data have demonstrated that a glioblastoma microenvironment lacking central memory CD4 T cells or natural killer (NK) cells is further correlated with better prognosis, and the expression levels of *ICOS* and *TNFSF14* were negatively associated with clinical outcome (21). It has also been reported that the glioblastoma microenvironment contains hub genes (CCT3, OLIG2, PSMB9, TRIM21) that were associated with distinct immune cell infiltration characterized by the expression of immune checkpoints and are further involved in cancer development and progression in patients with high-grade glioblastoma (22–24). In low-grade glioma, the expression level of transforming growth factor beta 1 (*TGF-β*) and programmed cell death ligand 1 (*PDL1*) was positively correlated with immune risk score, and prognostic hub genes that were positively correlated with immune cell infiltration have also been revealed (25, 26). Notably, T follicular helper (TFH) cells, activated NK cells, and macrophages have been demonstrated to be independent predictors for malignant transformation in low-grade glioma (27). Furthermore, it has been suggested that the expressions of JAK3, IL2Rß, and CD3ε are further associated with the presence of B cell memory and CD8 T cells, which adversely impact immune cell response in the tumor microenvironment (28). As a matter of fact, Qiu et al. recently demonstrated, through their PROMISE model, differences in immune cell abundance between risk groups (29). Although innate immune cells such CD8 T cells were upregulated and further associated with good prognosis, a downregulation of TFH cells was observed in the high-risk group of glioma patients (29). Zhong et al. demonstrated that resting NK cells, CD8+ T cells, TFH cells, gamma delta T cells, and M0 macrophages were negatively related to prognosis. However, their proportion was significantly related to patient's age and sex (30).

Nevertheless, these studies assessed low-grade glioma or glioblastoma patients individually and did not consider both subtypes as a whole to identify striking resemblance and active involvement of immune genes in glioma patients.

Here we assessed the presence of two distinct groups of glioma patients. The first group, defined as cluster 1, was associated with better patient survival, whereas the second group, cluster 2, exhibited a poor survival. Interestingly, a strong presence of activated NK cells was revealed in cluster 1. In contrast, potent immunosuppressive components such as regulatory T cells, neutrophils, and M0/M1/M2 macrophages were detected in cluster 2, where inhibitory immune checkpoints, such as cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), lymphocyte-activation gene 3 (*LAG3*), and T cell immunoglobulin mucin 3 (*TIM3*), were also significantly upregulated. Finally, Cox regression analysis further corroborated that tumor-infiltrating cells from cluster 2 exerted a significant impact on patient prognosis. Our results pinpoint that the immune response type impacts the clinical outcome of glioma patients.

MATERIALS AND METHODS

Data Analysis From TCGA and Independent Datasets

The workflow of our study is shown in **Figure 1**. Molecular data, including mRNA expression of LGG (n = 516) and GBM (n = 154) patients, were downloaded from The Cancer Genome Atlas (TCGA) data portal (https://www.cbioportal.org/). Clinical data related to glioma patients were downloaded altogether with matrix mRNA gene expression.

Quantification of Immune Signature Enrichment Levels in Glioma Patients

We analyzed 22 immune signatures (e.g., CD8+ T cells, CD4 naive T cells, Treg cells, naïve B cells, memory B cells, plasma cells, CD4 memory resting T cells, CD4 memory activated T cells, follicular helper T cells, gamma delta T cells, resting NK cells, activated NK cells, monocytes, macrophages M0/M1/M2, resting dendritic cells, activated dendritic cells, resting mast cells, activated mast cells, eosinophils, neutrophils). The enrichment level of this immune signature in glioma samples was quantified as the mean expression level of immune signature marker genes. We, therefore, used CIBERSORT (https://cibersort.stanford.edu/index.php) which estimates the relative fraction of each cell type in the signature matrix, such that the sum of all fractions is equal to 1 for a given mixture. The absolute immune fraction score was estimated by the median expression level of all genes in the signature matrix divided by the median expression level of all genes in the mixture.

Hierarchical Clustering

To compare distinct immune cell infiltration genes in glioma patients, unsupervised hierarchical clustering was performed to group the 658 patients into two subgroups termed cluster 1 (n = 293) and cluster 2 (n = 365).

Global Gene Expression Analysis

Differential gene expression (DGE) analysis with RNA-seq data was performed using the R package.

An average raw read count for each gene >1 was applied to determine candidate genes that were reasonably expressed. The expression fold change (FC) denotes upregulation or downregulation according to the FC value. Subsequently, log FC, log CPM, p-value, and the corresponding false discovery rate (FDR) were all reported by the R package. FDR < 0.05, log CPM > 1, and |log FC| > 2 were set as inclusion criteria for DEG selection.

Association of Immune Signature Enrichment Levels With Various Molecular Features

R Programming Environment (version 3.2.5) and Bioconductor were used for preprocessing and data analysis. For data filtering and quality control, the following methods were used. The median rank scores were identified in the dataset, and quartile normalization was performed. Data were visualized as Uniform Manifold Approximation and Projection (UMAP) which engage different parameters such as the distance between points and number of neighbors for a better reduced dimensional representation. We identified the upregulated genes that were significantly associated with immune signature enrichment levels (ISELs) using the Mann–Whitney U test.

Gene-Set Enrichment Analysis

We used FunRich to identify the KEGG pathways that were significantly associated with the genes having an important

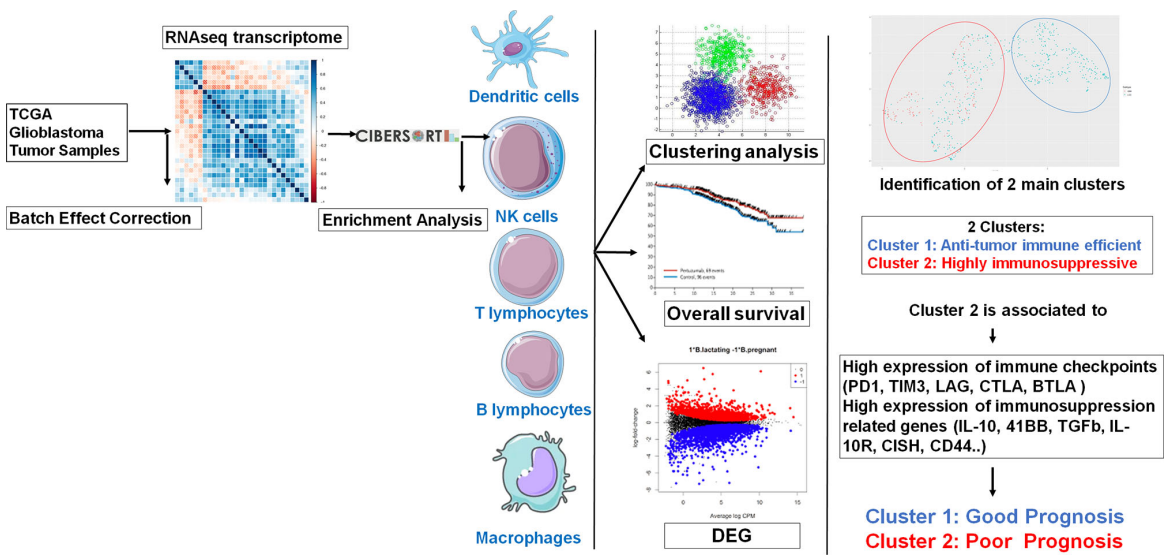

FIGURE 1 | Graphical abstract.

expression correlation with the immune signature using a threshold of FDR < 0.05.

Survival Analysis

We compared the overall survival (OS) between clusters and in the glioma cohort. We used Kaplan–Meier survival curves to show the survival time differences and the log-rank test to assess the significance of survival time differences. Statistical analyses were carried out with GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Single comparisons between two groups were performed with Student's t-test.

Univariate and multivariate Cox regression analyses were used on tumor-infiltrating immune gene signature and clinical parameters, including IDH status and age in TCGA cohorts.

Cox regression analyses were used to evaluate the prognostic effect of the immune signature on glioma patient prognosis and were performed using the R Programming Environment (version 3.2.5) and Bioconductor. To evaluate the difference between groups, the hazard ratios HR ≥ 1 and p-value of ≤0.05 were considered significant.

RESULTS

Independent Immunostimulatory and Immunosuppressive Profiles of Glioma Patients

To identify the role of distinct tumor-infiltrating immune cells in glioma patients, a transcriptomic dataset from the Cancer Genome Atlas (TCGA) cohort (153 GBM and 512 LGG patients) was analyzed in this study. A pretreatment step of glioma transcriptomic data was carried out by emerging LGG and HGG databases, followed by a harmonization step. The unsupervised hierarchical clustering after the enrichment analysis enabled us to demonstrate the presence of two distinct clusters (groups of patients). The first group defined as cluster 1 was characterized by an immunostimulatory profile gathering monocytes, naïve B cells, plasma cells, naïve CD4+ T cells, activated mast cells, NK cells, and T follicular helper cells, whereas the second group (cluster 2) was enriched with immunosuppressive immune cells such as neutrophils, M0/M1/M2 macrophages, regulatory T cells (Tregs), resting mast cells, resting NK cells, resting CD4 memory T cells, and memory B cells (**Figure 2A**).

We further integrated clinicopathological parameters such as IDH status and glioma subtypes (LGG and GBM) to this hierarchical clustering, to assess whether the two clusters were correlated with these parameters. As shown in the hit map (**Figures 2B, C**), cluster 1 contains mainly LGG patients with only six GBM patients inside. However, the majority of GBM patients were grouped in (cluster 2). Moreover, a greater part of LGG patients with IDH mutant and 1p19q codeletion (codel) were gathering in cluster 1. Further, LGG patients with IDH wild-type or IDH mutant with 1p19q non-codeletion (non-codel) were likewise distributed within two clusters.

In order to confirm these results, we used high-dimension reduction algorithm that is based on manifold learning and nonlinear dimensionality reduction, used here as an effective preprocessing step to boost the performance of clustering. These results were confirmed either by increasing the distance between points (near neighbors) or by adding UMAP parameters (Canberra, Cosine, Dice) that control the balance of local *versus* global structure in our data. Indeed, we could observe the same clusters, which clearly suggest that this clustering depends on patients' immune profiles rather than clinicopathological parameters (**Figures S1A, B** and **Figures S2A, B**).

Difference in Immune Cell Abundance Within the Clustered Patients

To confirm that unsupervised clustering could mostly be driven by the immune enrichment profile in patients with gliomas associated with the tumor microenvironment, we used this time UMAP to score the intensity of each infiltrated immune cell population within the clustered patients (**Figure 3**). As a matter of fact, we observed more naive B cells, plasma cells, naive CD4 T cells, TFH cells, monocytes, activated mast cells, and activated NK cells in cluster 1 than in cluster 2. When compared to cluster 1, cluster 2 seems to gather more less memory B cells, CD4 memory resting T cells, CD4 memory activated T cells, regulatory T cells, resting NK cells, M0/M1/M2 macrophages, resting dendritic cells, resting mast cells, and neutrophils. According to the Wilcoxon test, there were statistically significant differences in the immune cell subtypes between the two clusters ($p < 0.05$; FC > 2) (**Figure 4**), which confirm the immunosuppressive trait of cluster 2 relative to cluster 1. Indeed, these results highlight the weight of the immune profile on patients.

Association of Immune Checkpoints Overexpression With Immunosuppressive Status

Considering the critical role of the immune checkpoint inhibitors such as programmed-death receptor-1 (*PD-1*) and cytotoxic T lymphocyte antigen-4 (*CTLA-4*), not only in the negative regulation of T-cell activity but also in modulating T-cell migration into tissues (31), we aimed to compare the expression level of immune checkpoints between cluster 1 and cluster 2. As illustrated in **Figure 4**, *PDL-1*, *CTLA-4*, *TIM-3*, *LAG-3*, band T lymphocyte-associated (*BTLA*), inducible T cell costimulator (*ICOS*), inducible T cell costimulator ligand (*ICOSL*), and GATA-binding protein 3 (*GATA-3*) expressions were significantly higher in cluster 2 than in cluster 1 (p value <0.05; FC > 2).

Furthermore, a significant correlation between the expressions of *PD-1*, *BTLA*, and *ICOS* has been observed. In addition, our results showed that infiltration of immune cells such as CD8 T cells was associated with the expression of *GATA3*. Activated CD4 memory T cells and macrophages (M1/M2) were importantly correlated with expressions of *BTLA* and *ICOS [r (-1 to +1), p<0.05]*. Moreover, regulatory T cells were highly correlated with CD4 memory T cells, resting NK cells, macrophages (M0), and resting mast cells. Likewise, activated NK cells were associated with plasma cells, B

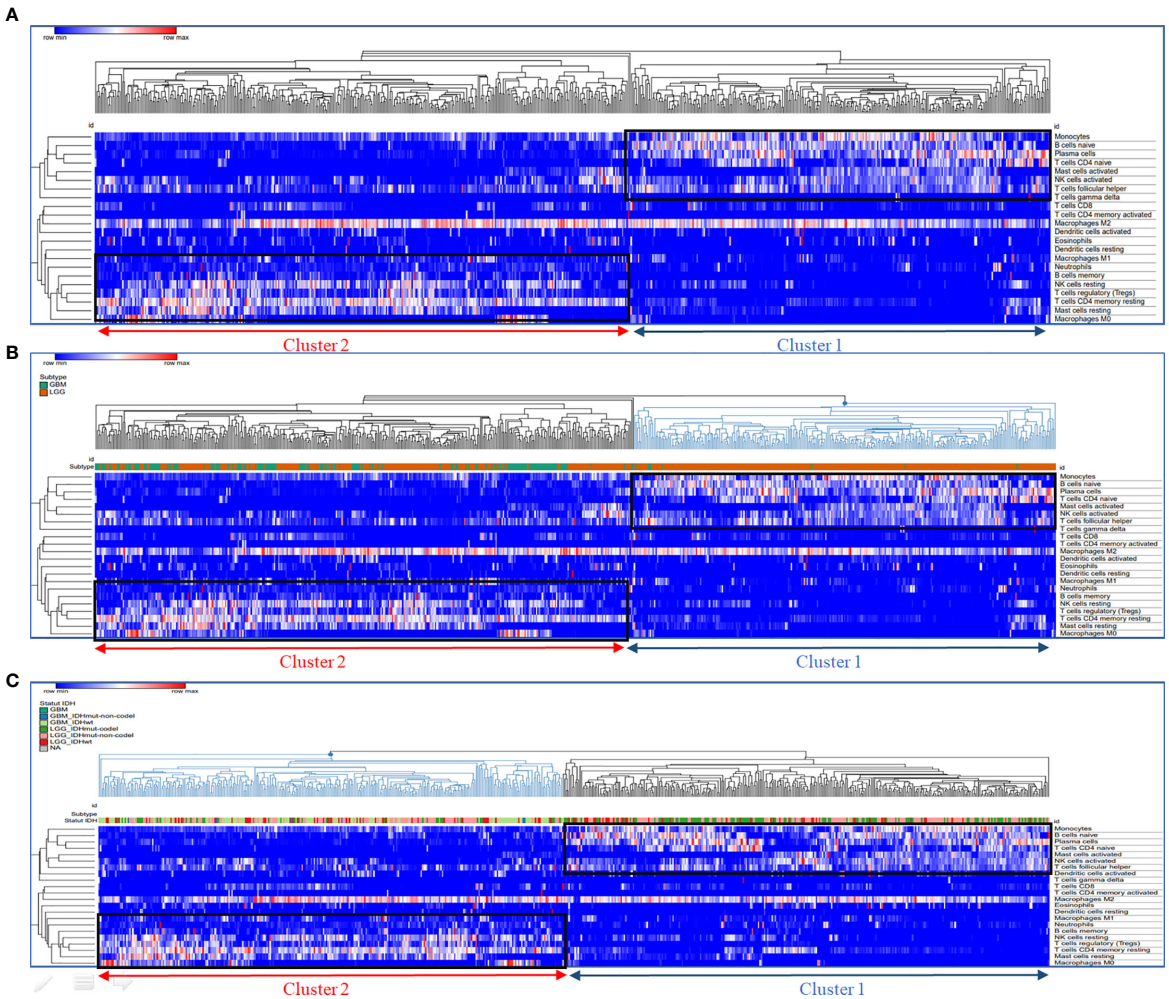

FIGURE 2 | Segregation of enriched immune cell subsets by unsupervised hierarchical clustering. Hierarchical clustering of immune cell frequency in glioblastoma samples. RNA sequencing (RNA-Seq) gene expression data was analyzed with CIBERSORT to quantify the amount of different immune cells in the TCGA glioblastoma samples. Hierarchical clustering of these quantities after normalization is shown in the heat map. **(A)** Hierarchical clustering of immune cell frequency in glioblastoma samples, **(B)** Glioma Subtype groups (GBM and LGG) localisation in cluster 1 and 2; **(C)** Glioma IDH mutation status localisation in cluster 1 and 2. The cluster of samples enriched in immunosuppressive immune cells are represented in cluster 2 (red); the cluster of samples enriched in activated immune cells are represented in cluster 1 (blue). Hierarchical clustering was performed using Euclidean distance and complete linkage methods.

cells naïve, follicular helper T cells, eosinophils, and activated mast cells (**Figure 5** and **Figure S3**).

Our results suggest that NK cells, plasma cells, naïve B cells, follicular helper T cells, eosinophils, and activated mast cells cooperatively exhibit a balance of inhibitory and activating signals in cluster 1 where the cells expressed fewer immune checkpoints.

Relevant Activator and Inhibitor Markers to Guide Characterization of Glioma Clusters

To further deepen our investigation on the identified clusters, we performed differential expression gene (DEG) analysis based on 10,000-gene fold change expression within each cluster. As a matter of fact, we used as a fold change cutoff FC > 0.5 and FC < -0.5; all downregulated genes and upregulated genes are shown in the volcano plot (**Figure 6**).

Our results demonstrated that, on the one hand, genes such as neural cell adhesion molecule 1 (NCAM1), neural cell adhesion molecule 2 (NCAM2), MHC class I polypeptide-related sequence B (MICB), killer cell lectin-like receptor K1 (*NKG2D*), and interleukin 12 (IL-12) described as markers of activation were exclusively upregulated in cluster 1. On the other hand, the expressions of genes such as *TGFβ*, *IL-10*, interleukin 6 (*IL-6*), *GATA3*, regenerating family member 1 (*REG-1*), RAR related orphan receptor C (*RORC*), *LAG3*, *CTLA4*, cluster of differentiation 276 (*CD276*), cytokine-inducible SH2-containing protein (CISH), and cluster differentiation (*CD44*), further associated with the inhibition of T cell effector activity,

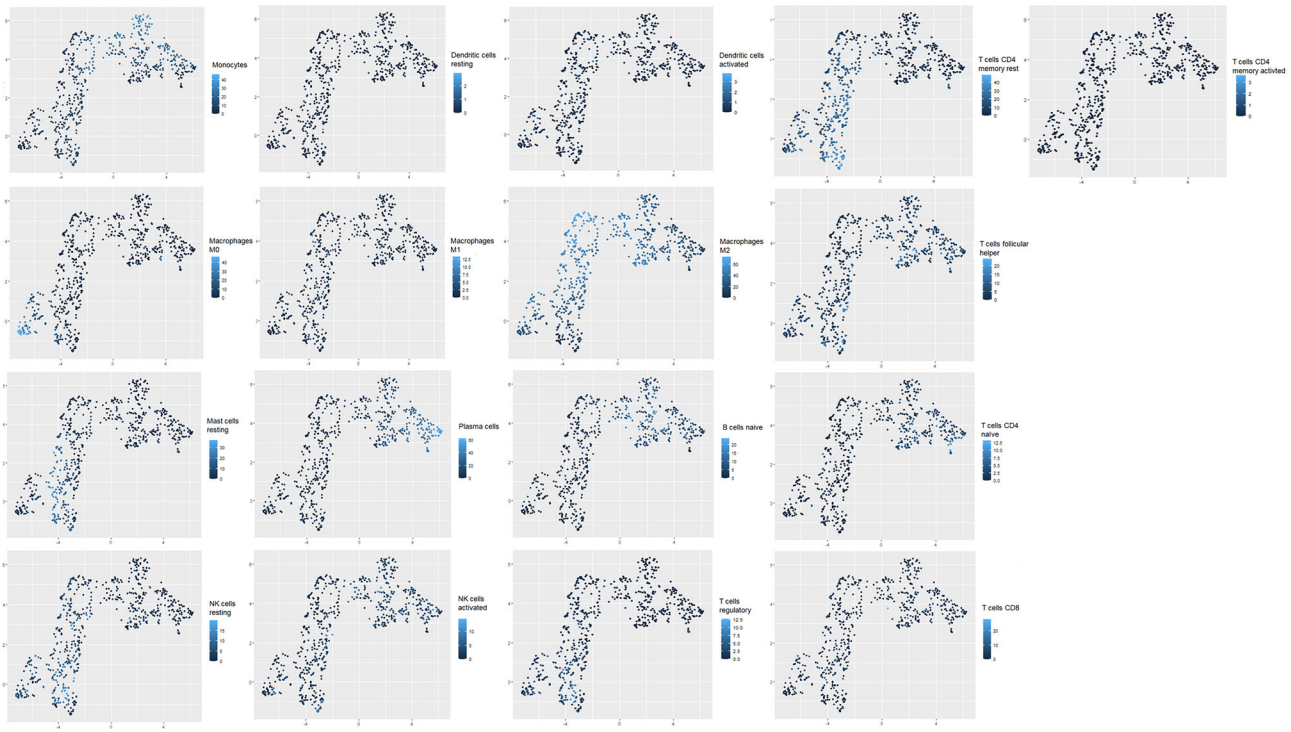

FIGURE 3 | Uniform Manifold Approximation and Projection (UMAP) plots of transcriptomic profiles from glioblastoma datasets. Clustering of tumor-infiltrating immune cell populations (T regulatory cells, dendritic cells, macrophages M0, M1, and M2, CD8 T cells, activated CD4 T cells, activated NK cells, resting NK cells, B cells, T follicular helper cells); intensity of immune cell frequency within the clustered patients (cluster 1 and cluster 2). Light blue reflects a high enrichment intensity, and dark blue is associated with low infiltration intensity.

were upregulated in cluster 2, where immunosuppressive populations are strongly enriched, as we previously demonstrated. Indeed, we could clearly observe the consistent link between the upregulated genes in each immune cluster component.

Immune Cell-Dependent Prognostic Drivers of Glioma Patients' Survival

To assess whether the difference in immune components and other upregulated genes within cluster 1 and cluster 2 would reliably affect patient prognosis, we performed Kaplan–Meier (K-M) analysis between the two clusters. As a matter of fact, cluster 2 was strikingly associated with poor survival (at 50% of overall survival (OS), the survival median was at roughly 25 months) ($p < 0.0001$), which fits our results (**Figure 7**).

Then, we conducted a univariate Cox regression analysis based on clinical features and tumor-infiltrating immune cell parameters of cluster 2 patients for assessing prognostic factors that independently influence glioma patient survival (**Table 1**).

However, we have demonstrated by multivariate Cox regression analysis that regulatory T cells (Treg), neutrophils, and macrophages M0/M1/M2 are key drivers of cluster 2 patient survival, since the Ex coefficients were respectively $7.662e^{+00}$, $5.288e^{+01}$, $1.974e^{+04}$, $1.974e^{+04}$, and $1.765e^{+00}$; $p < 2\ e^{-6}$; and hazard ratio (HR) ≥ 1 (**Table 1**). These results might shed light on the importance of immune infiltrate when establishing patient diagnosis, a key feature that might influence glioma patient overall survival.

DISCUSSION

The tumor microenvironment plays an important role in glioma pathogenesis. Here, we provided the immune signature, which might impact glioma patient prognosis. Using LM22 CIBERSORT, followed by unsupervised hierarchical clustering and UMAP dimensional reduction algorithms, we identified two clusters in glioma patients. These clusters were associated with immune infiltration character which is basically related to some specific expressed genes inside the tumor microenvironment and further associated with either good or poor prognosis. Thus, even though clinico-pathological parameters were added to the unsupervised clustering, those two groups of patients were identified independently of other prognostic parameters such as IDH status and glioma subtype. Then, we employed different methods to confirm our unsupervised clustering. Indeed, our data highlight the strength of the defined immune cells shown in terms of hierarchically clustering our patients.

As a matter of fact, we found that cluster 1 was highly correlated with naive B cell, plasma cell, naive CD4 T cell, T follicular helper cell, NK cell, and monocyte infiltration. It has been previously reported that B cell infiltration in melanoma,

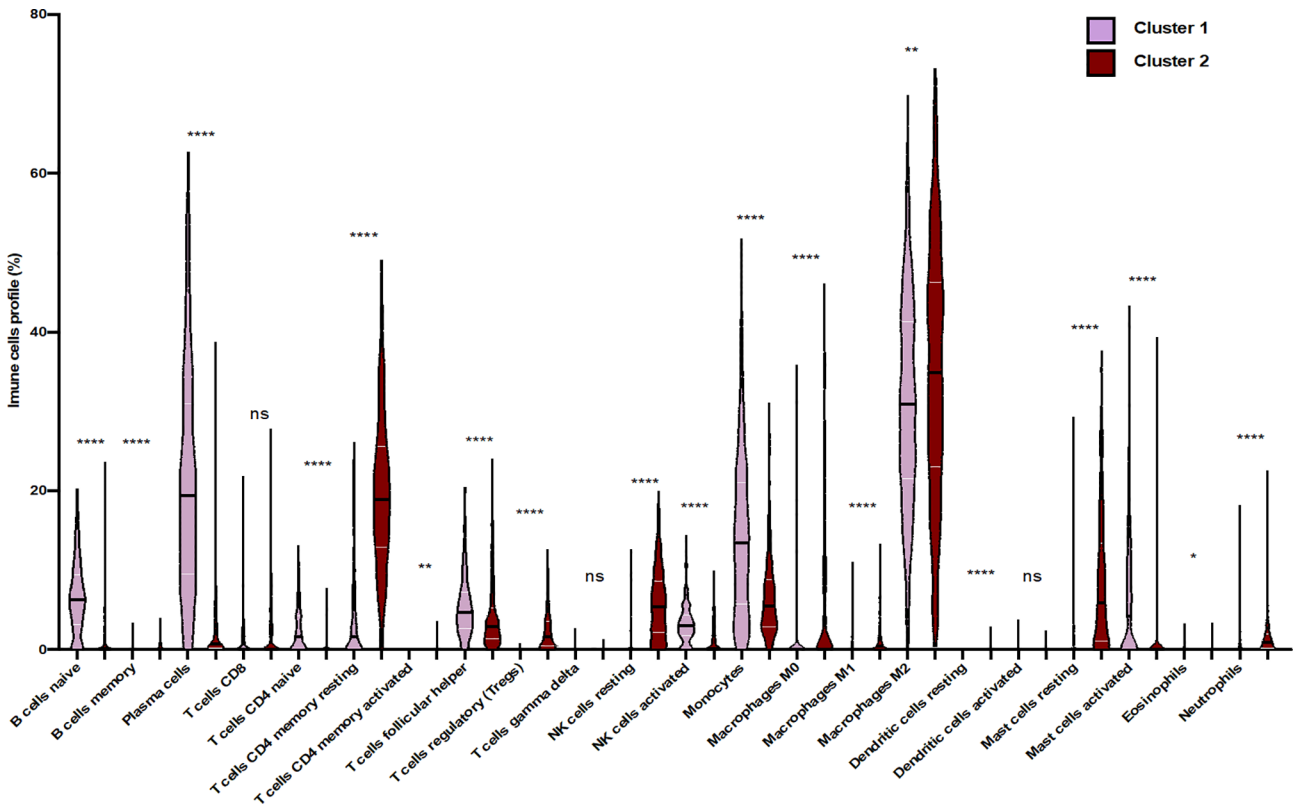

FIGURE 4 | Differential distribution of immune cell proportion within the two clusters of glioma patients. Violin plots present the distribution of each immune subset. Violin plots show median values (black horizontal lines) in glioblastoma data. The Wilcoxon test shows statistically significant differences in the immune cell subtypes between the two clusters (*p < 0.05, **p < 0.01, ****p < 0.0001, ns, statistically no significant).

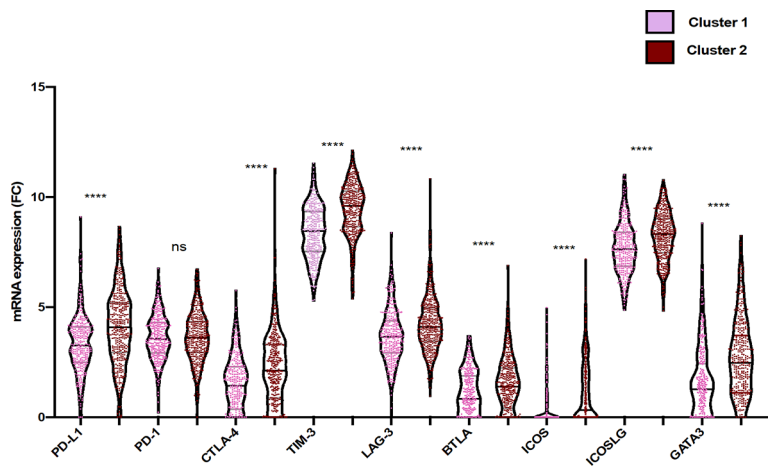

FIGURE 5 | Differential gene expression of immune checkpoint within the two clusters of glioma patients. Violin plots represent the relative mRNA gene expression in the two clusters (pink: cluster 1; red: cluster 2). Each dot represents a sample. Violin plots show median values (black horizontal lines) in glioblastoma data. The two-tailed independent test was applied. p values of differences between groups are shown above (p < 0.05, ****p < 0.0001, ns, statistically no significant).

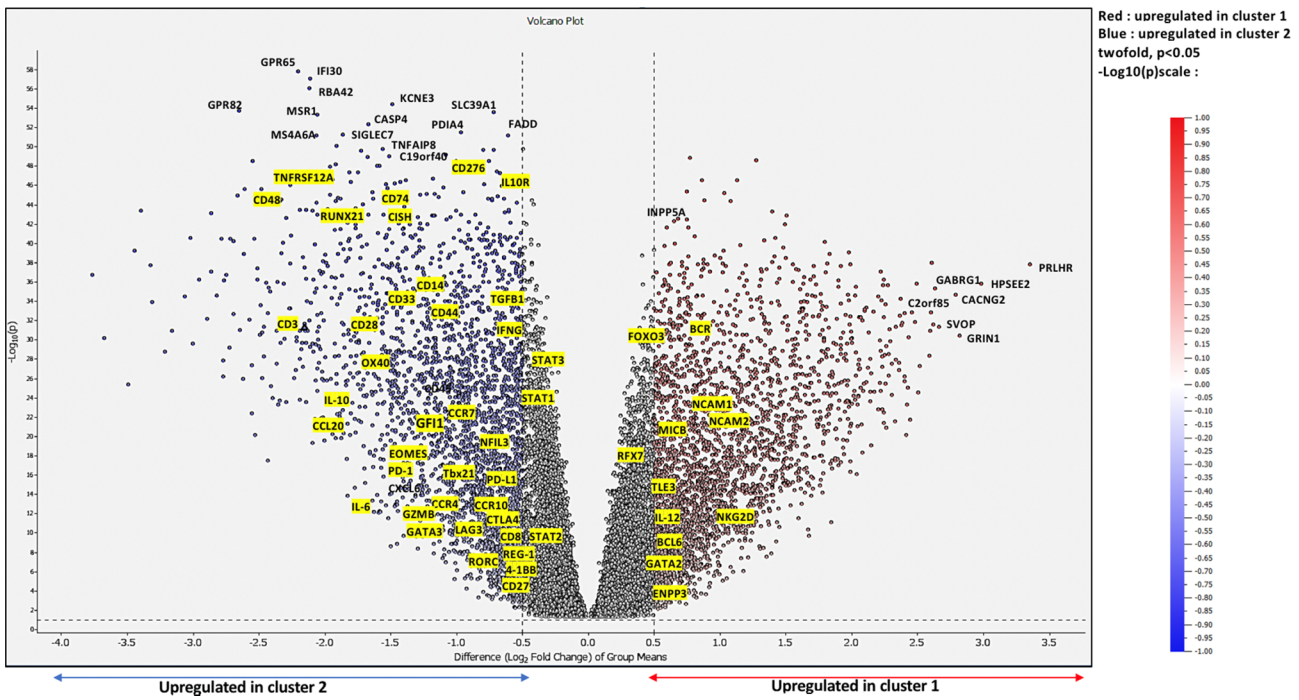

FIGURE 6 | Differentially expressed genes at a Bonferroni-corrected p-value. Volcano plot showing upregulated genes in cluster 1 shown in red *vs.* upregulated genes in cluster 2 shown in blue while non-DE genes are in black.

breast, and lung cancers was positively correlated with a better survival (32, 33). Indeed, naive B cells and plasma cells can enhance an efficient immune response by activating cytotoxic T cell response (34). Furthermore, B cells have been described as a central player in the response to immune checkpoint blockade in patients with metastatic melanoma and renal cell carcinoma, through the activation of T cells and the generation of IgG subclasses of antibodies (35–37). Moreover, combining radiation, PD-L1 inhibition, and B cell-based vaccine that consists of *4-1BBL*+B cell activation promoted glioma cell regression by 80% in mice (38).

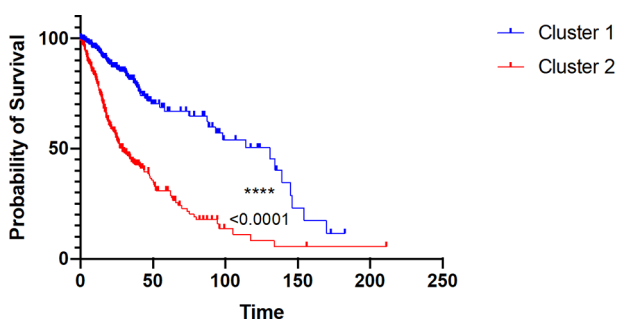

FIGURE 7 | Kaplan–Meier survival probability of the two clusters of glioma patients. Red curve represents overall survival (OS) of cluster 2 patients. Blue curve represents overall survival (OS) of cluster 1 patients, with p value < 0.0001.

Besides, the presence of cytotoxic immune cells such as NK cells is associated with a good prognosis in glioma patients (39). Considering the role of NK cells in glioma immunosurveillance, many clinical trials focus on CAR-NK cells for the treatment of glioblastoma patients (40, 41).

Naïve CD4 T cells can proliferate and differentiate into an effector ThCTL subpopulation which contributes to an effective antitumor response through antigen recognition of peptides presented by MHC class II molecules following CTLA4 blockade (42). Yet, it has been reported that naïve T cells are missing in the glioblastoma microenvironment due to their sequestration in the bone marrow (43). Our data consolidate the thought that cluster 1 is enriched by antitumoral cell subsets with immunostimulatory characteristics.

Our results not only showed that cluster 2 was poorly enriched in activated and cytotoxic T cells but also underlined the high prevalence of M0 and M1 macrophages and neutrophils. Moreover, Treg cells and M2 macrophages were highly enriched in this group of glioma patients. Moreover, Treg accumulation in the tumor microenvironment was associated with antitumor T cell repression (44), poor prognosis (45), and resistance to vascular endothelial growth factor (*VEGF*) therapy in glioblastoma (46). The predominance of M2 macrophages in the glioma microenvironment has been described to be associated with poor survival and resistance to radiotherapy,

TABLE 1 | Multivariate analysis of the mean tumor-infiltrating immune cells in cluster 2 and glioma clinicopathologic features (IDH status, age).

TIICs and clinicopathologic parametres	Ex (-coefficient)	Ex (coefficient)	(95% CI)	
			Lower	Upper
IDH status	2.535e + 00	3.945e - 01	0.33134	4.698e - 01
Age	1.179e + 00	8.480e - 01	0.63580	1.131^{e} + 03
Regulatory T cells	1.305e - 01	7.662e + 00	0.00735	7.988^{e} + 03
Neutrophils	1.891e - 02	5.288e + 01	0.15337	1.823^{e} + 04
Macrophages M0	3.537e - 02	1.974e + 04	6.53557	1.223^{e} + 02
Macrophages M1	5.065e - 5	1.974e + 04	16.30275	2.391^{e} + 07
Macrophages M2	5.666e - 01	1.765e + 00	0.66034	4.718e + 00

Concordance: 0.786 (se = 0.018) p-value<2e-6.
Likelihood ratio: 154.8 p-value<2e-6.
Wald test: 158.8 p-value<2e-6.
Score (log-rank test):179.8 p-value<2e-6.
e = Exponential, i.e., e-01 = 10^{-1}.

whereas M1 macrophages have been reported to be less abundant in high-grade glioma and expression of M1 marker C–C motif chemokine ligand 3 (*CCL3*) was further associated with a good survival (47, 48). Moreover, clinical data suggest that neutrophils were associated with poor prognosis and tumor progression in glioma patients (49, 50). Altogether, these studies and our results come to strengthen the hypothesis that the immune infiltrate components should be taken into consideration when establishing patients' diagnosis. These data show that cluster 2 is infiltrated by pro-tumoral cells with immunosuppressive properties.

According to the results of patients responding to anti-PD-1/anti-PD-L1 immunotherapy, Mellman and colleagues described two categories of microenvironment immune profile. The first is "immune inflamed tumor" that exhibits a preexisting antitumor immune response and provides a more favorable environment for T cell activation and renewal. The second profile is named "non-inflamed tumors" where cells are in a pausing state either in the parenchyma or in the tumor stroma and release more immunosuppressive cytokines (51). Our observations demonstrated that the immune components of cluster 1 patients presented similar features to "immune inflamed tumor" such as the abundance of *IL-12*, *MICB*, *NCAM1*, *NCAM2*, and *NKG2D* while the second group (cluster 2) seemed to exhibit some of the "non-inflamed tumors" characteristics such as the expressions of *TIM3*, *PDL1*, *IL-10*, and *TGF-β*.

Differential expression genes revealed the top genes that are, according to the literature, distinctively associated with an antitumor immune response in cluster 1 (*MICB*, *NKG2D*, *NCAM1*, *NCAM2*, and *IL-12*). As a matter of fact, *MICB* overexpression by tumor cells activates cytotoxic lymphocyte functions such as lysis and elimination of cancer cells through *NKG2D* receptor activation (52, 53). Furthermore, *NKG2D* receptor and *NCAM* upregulation triggers cytotoxic effector functions and provides costimulatory signals for NK cells, CD8 T cells, and γδT cells (54–56). Moreover, *IL-12*, which plays a key role in changing tumor microenvironment composition from one that contains less differentiated Th0 cells to one that has more inflammatory Th1 cells, was also upregulated (57). Besides, *TLE3* upregulation may also induce cell cycle arrest and tumor growth suppression *via* inhibition of *MAPK* and *AKT* pathways (58, 59). On the other hand, *GATA2* upregulation induces cell surface *PDL-1* and *PDL-2* expression in brain tumors, and its inhibition has been reported to stimulate chemotherapy-mediated apoptosis in human AML cells overexpressing *GATA2* (60, 61). Further, *BCL6* overexpression has pro-survival and proliferative activities of glioma cells; its expression in monocytes/macrophage lineage reduces the antitumor immune response and increases the immunosuppressive microenvironment. It has been demonstrated recently that *BCL6* deletion in Treg cells significantly inhibited tumor progression (62–64).

Most interestingly, upregulated genes in cluster 2 (*TGFβ*, *IL-10*, *IL-6*, *GATA3*, *REG-1*, *RORC*, *LAG3*, *CTLA4*, *CD276*, *CISH*, and *CD44*) were related to an activated regulatory T-cell phenotype. Indeed, transcription factors such as *GATA3* and *RORC* are further related to the differentiation of CD4 T cells into Th17 that plays a pivotal role in regulatory T cell generation and recruitment (61–64). TGF-β and IL-10 are anti-inflammatory, which, produced by many cell types, play a crucial role in inhibiting the antitumor immune response in glioma (65–67) by activating the polarization of M0 into M1 or M2 macrophages (68–70). Strikingly, *CD276* and nuclear factor interleukin 3-regulated (NFIL3) overexpression is capable of inducing T cell immunosuppression (71, 72). Moreover, the co-expression of PD-*1* with other inhibitory receptors, such as *PDL-1*, *TIM-3*, *LAG3*, and *CTLA-4*, induces effector T cell exhaustion (73, 74). It has also been reported that the PD-1/PDL-1 pathway is significantly correlated with the most aggressive histological subtype of glioma (75, 76). This could reflect the importance of the complex mechanisms established by the tumor microenvironment to suppress the antitumor immune response.

Our survival results highlight the importance of immune parameters in the outcomes of glioma patients and could further be considered as the most suitable predictive feature when adopting new treatment strategies. So far, clinicopathological and genetic features could not be used as predictive parameters in glioma patients' survival. In fact, glioma patients who have been grouped based on similar clinical characteristics could still present contradictory outcomes (25, 77).

Cox regression results indicated that macrophages M0/M1/M2, neutrophils, and Treg cells might have a detrimental impact in cluster 2 patients' survival. Our findings shed light on the importance of immune infiltration on glioma patients' survival. Differential expression of the immune checkpoint inhibitors between cluster 1 and cluster 2 might occur due the presence of macrophages and Treg cells. Indeed, cluster 2 exhibited a high expression of immune checkpoints and was mainly enriched with macrophages M0/M1/M2, neutrophils, and regulatory T cells. In fact, these cells could be involved in the upregulation of checkpoint expression and thus the inhibition of cytotoxic T cell activities (cluster 2). Therefore, understanding the signaling pathways of these cell subsets and their interaction with immune checkpoints would provide more information to define best-suited treatment strategies for glioma patients.

Over the past decade, it has been strongly believed that glioma patients exhibit resistance to adjuvant therapy. As a matter of fact, several resistance mechanisms limit the efficiency of classical treatments (78). Moreover, glioblastoma bears characteristics that contribute to significant therapeutic resistance by preventing adequate control of the entire tumor mass by drugs. Glioblastoma therefore facilitates escape mechanisms by mediating blood–brain barrier junction proteins dysfunction (79). Understanding the different interactions that occur inside the tumor microenvironment is crucial to conceive reliable therapeutic approaches.

Our findings shed light on the importance of using some immune markers to further stratify high- and low-risk glioma patients in order to suggest the adequate treatment for each group. As a matter of fact, Treg and NK cell infiltration rates found within cluster 2 in our study further indicate that this group would probably respond to NK activation treatment or to Treg cell targeting.

Our study comes with important findings which should be further corroborated. As a matter of fact, we have used CIBERSORT to identify critical immune infiltration subtypes. However, given that LM22 involves a limited set of genes and that we still lack evidence for a direct role of each cell subtype within glioma TME, a deepened characterization of immune infiltrates in glioblastoma might be necessary to further strengthen our findings.

CONCLUSION

Our work brings to light the tight implication of immune components on glioma patient prognosis. Moreover, characterizing immune subsets in the tumor microenvironment could also provide predictive factors that would contribute to potentially developing better immune-based therapeutic approaches.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SA analyzed the data and wrote the manuscript. DC analyzed the data and wrote and revised the manuscript. KA analyzed the data and revised the manuscript. SS and DO contributed to the conception and the design of the study. AB analyzed the data, wrote and revised the manuscript, and supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.685213/full#supplementary-material

Supplementary Figure 1 | Clustering according to nearest neighbor. **(A, B)** distance between point based on IDH status and glioma subtype.

Supplementary Figure 2 | Clustering according to Umap parameters. Correlation clustering based on similarity coefficients.

Supplementary Figure 3 | Correlation matrix of 22 immune subsets and 7 immune checkpoints in Glioma patients. The numbers in the scale represent pearson correlation coefficient. Red cycle represents a negative correlation, blue cycle represents a positive correlation, and white boxes represent no correlation between two kinds of cells or checkpoint inhibitors.

REFERENCES

1. Rabé M, Dumont S, Álvarez-Arenas A, Janati H, Belmonte-Beitia J, Calvo GF, et al. Identification of a Transient State During the Acquisition of Temozolomide Resistance in Glioblastoma. *Cell Death Dis* (2020) 11:1–14. doi: 10.1038/s41419-019-2200-2
2. Chen R, Smith-Cohn M, Cohen AL, Colman H. Glioma Subclassifications and Their Clinical Significance. *Neurotherapeutics* (2017) 14:284–97. doi: 10.1007/s13311-017-0519-x
3. Walid M. Prognostic Factors for Long-Term Survival After Glioblastoma. *Permanente J* (2008) 12:45. doi: 10.7812/tpp/08-027
4. Masui K, Mischel PS, Reifenberger G. Molecular Classification of Gliomas. In: *Handbook of Clinical Neurology*. Elsevier B.V. (2016). p. 97–120. doi: 10.1016/B978-0-12-802997-8.00006-2
5. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: A Summary. *Acta Neuropathol* (2016) 131:803–20. doi: 10.1007/s00401-016-1545-1

6. Al-kharboosh R, ReFaey K, Lara-Velazquez M, Grewal SS, Imitola J, Quiñones-Hinojosa A. Inflammatory Mediators in Glioma Microenvironment Play a Dual Role in Gliomagenesis and Mesenchymal Stem Cell Homing: Implication for Cellular Therapy. *Mayo Clinic Proc: Innovations Qual Outcomes* (2020) 4:443–59. doi: 10.1016/j.mayocpiqo.2020.04.006
7. Buerki RA, Chheda ZS, Okada H. Immunotherapy of Primary Brain Tumors: Facts and Hopes. *Clin Cancer Res* (2018) 24:5198–205. doi: 10.1158/1078-0432.CCR-17-2769
8. Bastola S, Pavlyukov MS, Yamashita D, Ghosh S, Cho H, Kagaya N, et al. Glioma-Initiating Cells at Tumor Edge Gain Signals From Tumor Core Cells to Promote Their Malignancy. *Nat Commun* (2020) 11:1–17. doi: 10.1038/s41467-020-18189-y
9. Azzarelli R, Simons BD, Philpott A. The Developmental Origin of Brain Tumours: A Cellular and Molecular Framework. *Dev (Cambridge)* (2018) 145:1–9. doi: 10.1242/dev.162693
10. Gieryng A, Pszczolkowska D, Walentynowicz KA, Rajan WD, Kaminska B. Immune Microenvironment of Gliomas. *Lab Invest* (2017) 97:498–518. doi: 10.1038/labinvest.2017.19
11. Clavreul A, Menei P. Mesenchymal Stromal-Like Cells in the Glioma Microenvironment: What Are These Cells? *Cancers* (2020) 12:1–12. doi: 10.3390/cancers12092628
12. Shimato S, Maier LM, Maier R, Bruce JN, Anderson RCE, Anderson DE. Profound Tumor-Specific Th2 Bias in Patients With Malignant Glioma. *BMC Cancer* (2012) 12:561. doi: 10.1186/1471-2407-12-561
13. Hanaei S, Afshari K, Hirbod-Mobarakeh A, Mohajer B, Amir Dastmalchi D, Rezaei N. Therapeutic Efficacy of Specific Immunotherapy for Glioma: A Systematic Review and Meta-Analysis. *Rev Neurosci* (2018) 29:443–61. doi: 10.1515/revneuro-2017-0057
14. Yan Y, Zeng S, Gong Z, Xu Z. Clinical Implication of Cellular Vaccine in Glioma: Current Advances and Future Prospects. *J Exp Clin Cancer Res* (2020) 39:1–18. doi: 10.1186/s13046-020-01778-6
15. Goff SL, Morgan RA, Yang JC, Sherry RM, Robbins PF, Restifo NP, et al. Pilot Trial of Adoptive Transfer of Chimeric Antigen Receptor-Transduced T Cells Targeting Egfrviii in Patients With Glioblastoma. *J Immunother* (2019) 42:126–35. doi: 10.1097/CJI.0000000000000260
16. Weenink B, French PJ, Smitt PAES, Debets R, Geurts M. Immunotherapy in Glioblastoma: Current Shortcomings and Future Perspectives. *Cancers* (2020) 12:3–751. doi: 10.3390/cancers12030751
17. Montoya ML, Kasahara N, Okada H. Introduction to Immunotherapy for Brain Tumor Patients: Challenges and Future Perspectives. *Neuro-Oncol Pract* (2020) 7:465–76. doi: 10.1093/nop/npaa007
18. Khamis ZI, Al-Akkary N, Hua T, Draughon SA, Li Y, Sang Q-XA. Clinical Investigations of Immunotherapy for Human Primary Brain Tumors. *Neuroimmunol Neuroinflamm* (2020) 2020:1–14. doi: 10.20517/2347-8659.2020.43
19. Sampson JH, Gunn MD, Fecci PE, Ashley DM. Brain Immunology and Immunotherapy in Brain Tumours. *Nat Rev Cancer* (2020) 20:12–25. doi: 10.1038/s41568-019-0224-7
20. Desland FA, Hormigo A. The CNS and the Brain Tumor Microenvironment: Implications for Glioblastoma Immunotherapy. *Int J Mol Sci* (2020) 21:1–19. doi: 10.3390/ijms21197358
21. Wu S, Yang W, Zhang H, Ren Y, Fang Z, Yuan C, et al. The Prognostic Landscape of Tumor-Infiltrating Immune Cells and Immune Checkpoints in Glioblastoma. *Technol Cancer Res Treat* (2019) 18:1–10. doi: 10.1177/1533033819869949
22. Huang S, Song Z, Zhang T, He X, Huang K, Zhang Q, et al. Identification of Immune Cell Infiltration and Immune-Related Genes in the Tumor Microenvironment of Glioblastomas. *Front Immunol* (2020) 11:585034. doi: 10.3389/fimmu.2020.585034
23. Tang G, Yin W. Development of an Immune Infiltration-Related Prognostic Scoring System Based on the Genomic Landscape Analysis of Glioblastoma Multiforme. *Front Oncol* (2020) 10:154. doi: 10.3389/fonc.2020.00154
24. Hu L, Han Z, Cheng X, Wang S, Feng Y, Lin Z. Expression Profile Analysis Identifies a Novel Seven Immune-Related Gene Signature to Improve Prognosis Prediction of Glioblastoma. *Front Genet* (2021) 12:638458. doi: 10.3389/fgene.2021.638458
25. Song LR, Weng JC, Li CB, Huo XL, Li H, Hao SY, et al. Prognostic and Predictive Value of an Immune Infiltration Signature in Diffuse Lower-Grade Gliomas. *JCI Insight* (2020) 5:1–8. doi: 10.1172/jci.insight.133811
26. Su J, Long W, Ma Q, Xiao K, Li Y, Xiao Q, et al. Identification of a Tumor Microenvironment-Related Eight-Gene Signature for Predicting Prognosis in Lower-Grade Gliomas. *Front Genet* (2019) 10:1143. doi: 10.3389/fgene.2019.01143
27. Lu J, Li H, Chen Z, Fan L, Feng S, Cai X, et al. Identification of 3 Subpopulations of Tumor-Infiltrating Immune Cells for Malignant Transformation of Low-Grade Glioma. *Cancer Cell Int* (2019) 19:1–13. doi: 10.1186/s12935-019-0972-1
28. Zhang B, Guan F, Dai B, Zhu G, Mao B, Liang X, et al. Prognostic Value and Immune Infiltration of Novel Biomarkers in Glioma: Evidence From a Comprehensive Analysis of Tumor Microenvironment. *In Rev* (2020) 1–15. doi: 10.21203/rs.3.rs-125889/v1
29. Qiu H, Li Y, Cheng S, He C, Li J, Li J. Frontiers | A Prognostic Microenvironment-Related Immune Signature *via* ESTIMATE (PROMISE Model) Predicts Overall Survival of Patients With Glioma. *Oncology*. doi: 10.3389/fonc.2020.580263
30. Zhong Q-Y, Fan E-X, Feng G-Y, Chen Q-Y, Gou X-X, Yue G-J, et al. A Gene Expression-Based Study on Immune Cell Subtypes and Glioma Prognosis. *BMC Cancer* (2019) 19:1116. doi: 10.1186/s12885-019-6324-7
31. Brunner-Weinzierl MC, Rudd CE. CTLA-4 and PD-1 Control of T-Cell Motility and Migration: Implications for Tumor Immunotherapy. *Front Immunol* (2018) 9:2737. doi: 10.3389/fimmu.2018.02737
32. Iglesia MD, Parker JS, Hoadley KA, Serody JS, Perou CM, Vincent BG. Genomic Analysis of Immune Cell Infiltrates Across 11 Tumor Types. *J Natl Cancer Inst* (2016) 108:1–11. doi: 10.1093/jnci/djw144
33. Liao P, Han S, Qu H. Expression, Prognosis, and Immune Infiltrates Analyses of E2Fs in Human Brain and CNS Cancer. *BioMed Res Int* (2020) 2020:1–15. doi: 10.1155/2020/6281635
34. Largeot A, Pagano G, Gonder S, Moussay E, Paggetti J. The B-Side of Cancer Immunity: The Underrated Tune. *Cells* (2019) 8:449. doi: 10.3390/cells8050449
35. Diem S, Fässler M, Bomze D, Ali OH, Berner F, Niederer R, et al. Immunoglobulin G and Subclasses as Potential Biomarkers in Metastatic Melanoma Patients Starting Checkpoint Inhibitor Treatment. *J Immunother* (2019) 42:89–93. doi: 10.1097/CJI.0000000000000255
36. Helmink BA, Reddy SM, Gao J, Zhang S, Basar R, Thakur R, et al. B Cells and Tertiary Lymphoid Structures Promote Immunotherapy Response. *Nature* (2020) 577:549–55. doi: 10.1038/s41586-019-1922-8
37. Hollern DP, Xu N, Thennavan A, Glodowski C, Garcia-Recio S, Mott KR, et al. B Cells and T Follicular Helper Cells Mediate Response to Checkpoint Inhibitors in High Mutation Burden Mouse Models of Breast Cancer. *Cell* (2019) 179:1191–1206.e21. doi: 10.1016/j.cell.2019.10.028
38. Lee-Chang C, Miska J, Hou D, Rashidi A, Zhang P, Burga RA, et al. Activation of 4-1BBL+ B Cells With CD40 Agonism and Ifnγ Elicits Potent Immunity Against Glioblastoma. *J Exp Med* (2021) 218:1–21. doi: 10.1084/JEM.20200913
39. Ren F, Zhao Q, Huang L, Zheng Y, Li L, He Q, et al. The R132H Mutation in IDH1 Promotes the Recruitment of NK Cells Through CX3CL1/CX3CR1 Chemotaxis and Is Correlated With a Better Prognosis in Gliomas. *Immunol Cell Biol* (2019) 97:457–69. doi: 10.1111/imcb.12225
40. Burger MC, Zhang C, Harter PN, Romanski A, Strassheimer F, Senft C, et al. CAR-Engineered NK Cells for the Treatment of Glioblastoma: Turning Innate Effectors Into Precision Tools for Cancer Immunotherapy. *Front Immunol* (2019) 10:2683. doi: 10.3389/fimmu.2019.02683
41. Holl EK, Frazier VN, Landa K, Beasley GM, Hwang ES, Nair SK. Examining Peripheral and Tumor Cellular Immunome in Patients With Cancer. *Front Immunol* (2019) 10:1767. doi: 10.3389/fimmu.2019.01767
42. Pluhar GE, Pennell CA, Olin MR. CD8+ T Cell–Independent Immune-Mediated Mechanisms of Anti-Tumor Activity. *Crit Rev Immunol* (2015) 35:153–72. doi: 10.1615/CritRevImmunol.2015013607
43. Schneider JR, Kwan K, Boockvar JA. Missing Naïve T Cells in the Setting of Glioblastoma Are Found to Be Sequestered in the Bone Marrow. *Neurosurgery* (2019) 85:E104–5. doi: 10.1093/neuros/nyz167
44. Dowling MR, Kan A, Heinzel S, Marchingo JM, Hodgkin PD, Hawkins ED. Regulatory T Cells Suppress Effector T Cell Proliferation by Limiting Division Destiny. *Front Immunol* (2018) 9:2461. doi: 10.3389/fimmu.2018.02461
45. Sayour EJ, McLendon P, McLendon R, De Leon G, Reynolds R, Kresak J, et al. Increased Proportion of FoxP3+ Regulatory T Cells in Tumor Infiltrating Lymphocytes is Associated With Tumor Recurrence and Reduced Survival in Patients With Glioblastoma. *Cancer Immunol Immunother* (2015) 64:419–27. doi: 10.1007/s00262-014-1651-7

46. Long Y, Tao H, Karachi A, Grippin AJ, Jin L, Chang Y, et al. Dysregulation of Glutamate Transport Enhances Treg Function That Promotes VEGF Blockade Resistance in Glioblastoma. *Cancer Res* (2020) 80:499–509. doi: 10.1158/0008-5472.CAN-19-1577
47. Vidyarthi A, Agnihotri T, Khan N, Singh S, Tewari MK, Radotra BD, et al. Predominance of M2 Macrophages in Gliomas Leads to the Suppression of Local and Systemic Immunity. *Cancer Immunol Immunother* (2019) 68:1995–2004. doi: 10.1007/s00262-019-02423-8
48. Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L, et al. Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates With Immunological Changes in the Microenvironment. *Cancer Cell* (2017) 32:42–56.e6. doi: 10.1016/j.ccell.2017.06.003
49. Rahbar A, Cederarv M, Wolmer-Solberg N, Tammik C, Stragliotto G, Peredo I, et al. Enhanced Neutrophil Activity Is Associated With Shorter Time to Tumor Progression in Glioblastoma Patients. *OncoImmunology* (2016) 5:1–8. doi: 10.1080/2162402X.2015.1075693
50. Massara M, Persico P, Bonavita O, Poeta VM, Locati M, Simonelli M, et al. Neutrophils in Gliomas. *Front Immunol* (2017) 8:1349. doi: 10.3389/fimmu.2017.01349
51. Chen DS, Mellman I. Elements of Cancer Immunity and the Cancer-Immune Set Point. *Nature* (2017) 541:321–30. doi: 10.1038/nature21349
52. Le Trinh T, Kandell WM, Donatelli SS, Tu N, Tejera MM, Gilvary DL, et al. Immune Evasion by Tgfβ-Induced miR-183 Repression of MICA/B Expression in Human Lung Tumor Cells. *OncoImmunology* (2019) 8:1–11. doi: 10.1080/2162402X.2018.1557372
53. De Andrade LF, En Tay R, Pan D, Luoma AM, Ito Y, Badrinath S, et al. Antibody-Mediated Inhibition of MICA and MICB Shedding Promotes NK Cell-Driven Tumor Immunity. *Science* (2018) 359:1537–42. doi: 10.1126/science.aao0505
54. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK Cells and T Cells by NKG2D, a Receptor for Stress- Inducible MICA. *Science* (1999) 285:727–9. doi: 10.1126/science.285.5428.727
55. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8αβ T Cell by NKG2D *via* Engagement by MIC Induced on Virus-Infected Cells. *Nat Immunol* (2001) 2:255–60. doi: 10.1038/85321
56. Chraa D, Naim A, Olive D, Badou A. T Lymphocyte Subsets in Cancer Immunity: Friends or Foes. *J Leukoc Biol* (2019) 105:243–55. doi: 10.1002/JLB.MR0318-097R
57. Powell MD, Read KA, Sreekumar BK, Jones DM, Oestreich KJ. IL-12 Signaling Drives the Differentiation and Function of a TH1-Derived TFH1-Like Cell Population. *Sci Rep* (2019) 9:1–12. doi: 10.1038/s41598-019-50614-1
58. Xu L, Chen Y, Dutra-Clarke M, Mayakonda A, Hazawa M, Savinoff SE, et al. BCL6 Promotes Glioma and Serves as a Therapeutic Target. *Proc Natl Acad Sci USA* (2017) 114:15. doi: 10.1073/pnas.1609758114
59. Peng LN, Deng XY, Gan XX, Zhang JH, Ren GH, Shen F, et al. Targeting of TLE3 by Mir–3677 in Human Breast Cancer Promotes Cell Proliferation, Migration and Invasion. *Oncol Lett* (2020) 19:1409–17. doi: 10.3892/ol.2019.11241
60. Fu Y, Liu CJ, Kobayashi DK, Johanns TM, Bowman-Kirigin JA, Schaettler MO, et al. GATA2 Regulates Constitutive PD-L1 and PD-L2 Expression in Brain Tumors. *Sci Rep* (2020) 10:1–12. doi: 10.1038/s41598-020-65915-z
61. Wang D, Yang L, Yu W, Wu Q, Lian J, Li F, et al. Colorectal Cancer Cell-Derived CCL20 Recruits Regulatory T Cells to Promote Chemoresistance *via* FOXO1/CEBPB/NF-κb Signaling. *J ImmunoTher Cancer* (2019) 7:1–15. doi: 10.1186/s40425-019-0701-2
62. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal Developmental Pathways for the Generation of Pathogenic Effector TH17 and Regulatory T Cells. *Nature* (2006) 441:235–8. doi: 10.1038/nature04753
63. Miller SA, Weinmann AS. Common Themes Emerge in the Transcriptional Control of T Helper and Developmental Cell Fate Decisions Regulated by the T-Box, GATA and ROR Families. *Immunology* (2009) 126:306–15. doi: 10.1111/j.1365-2567.2008.03040.x
64. Hartigan-O'Connor DJ, Hirao LA, McCune JM, Dandekar S. Th17 Cells and Regulatory T Cells in Elite Control Over HIV and SIV. *Curr Opin HIV AIDS* (2011) 6:221–7. doi: 10.1097/COH.0b013e32834577b3
65. Qi L, Yu H, Zhang Y, Zhao D, Lv P, Zhong Y, et al. IL-10 Secreted by M2 Macrophage Promoted Tumorigenesis Through Interaction With JAK2 in Glioma. *Oncotarget* (2016) 7:71673–85. doi: 10.18632/oncotarget.12317
66. Liu Z, Kuang W, Zhou Q, Zhang Y. TGF-β1 Secreted by M2 Phenotype Macrophages Enhances the Stemness and Migration of Glioma Cells *via* the SMAD2/3 Signalling Pathway. *Int J Mol Med* (2018) 42:3395–403. doi: 10.3892/ijmm.2018.3923
67. Humphries W, Wei J, Sampson JH, Heimberger AB. The Role of Tregs in Glioma-Mediated Immunosuppression: Potential Target for Intervention. *Neurosurg Clinics North America* (2010) 21:125–37. doi: 10.1016/j.nec.2009.08.012
68. Hosokawa H, Tanaka T, Endo Y, Kato M, Shinoda K, Suzuki A, et al. Akt1-Mediated Gata3 Phosphorylation Controls the Repression of IFNÎ 3 in Memory-Type Th2 Cells. *Nat Commun* (2016) 7:1–12. doi: 10.1038/ncomms11289
69. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage Polarization: Different Gene Signatures in M1(Lps+) *vs.* Classically and M2(LPS-) *vs.* Alternatively Activated Macrophages. *Front Immunol* (2019) 10:1084. doi: 10.3389/fimmu.2019.01084
70. Kanhere A, Hertweck A, Bhatia U, Gökmen MR, Perucha E, Jackson I, et al. Jenner RG. T-Bet and GATA3 Orchestrate Th1 and Th2 Differentiation Through Lineage-Specific Targeting of Distal Regulatory Elements. *Nat Commun* (2012) 3:1–9. doi: 10.1038/ncomms2260
71. Zhu C, Sakuishi K, Xiao S, Sun Z, Zaghouani S, Gu G, et al. An IL-27/NFIL3 Signalling Axis Drives Tim-3 and IL-10 Expression and T-Cell Dysfunction. *Nat Commun* (2015) 6:1–12. doi: 10.1038/ncomms7072
72. Yang S, Wei W, Zhao Q. B7-H3, a Checkpoint Molecule, as a Target for Cancer Immunotherapy. *Int J Biol Sci* (2020) 16:1767–73. doi: 10.7150/ijbs.41105
73. Okoye IS, Houghton M, Tyrrell L, Barakat K, Elahi S. Coinhibitory Receptor Expression and Immune Checkpoint Blockade: Maintaining a Balance in CD8 + T Cell Responses to Chronic Viral Infections and Cancer. *Front Immunol* (2017) 8:1215. doi: 10.3389/fimmu.2017.01215
74. Verdon DJ, Mulazzani M, Jenkins MR. Cellular and Molecular Mechanisms of CD8+ T Cell Differentiation, Dysfunction and Exhaustion. *Int J Mol Sci* (2020) 21:1–28. doi: 10.3390/ijms21197357
75. Ghouzlani A, Rafii S, Karkouri M, Lakhdar A, Badou A. The Promising IgSF11 Immune Checkpoint Is Highly Expressed in Advanced Human Gliomas and Associates to Poor Prognosis. *Front Oncol* (2021) 10:608609. doi: 10.3389/fonc.2020.608609
76. Ghouzlani A, Kandoussi S, Tall M, Reddy KP, Rafii S, Badou A. Immune Checkpoint Inhibitors in Human Glioma Microenvironment. *Front Immunol* (2021) 12:679425. doi: 10.3389/fimmu.2021.679425
77. Poon MTC, Sudlow CLM, Figueroa JD, Brennan PM. Longer-Term (≥ 2 Years) Survival in Patients With Glioblastoma in Population-Based Studies Pre- and Post-2005: A Systematic Review and Meta-Analysis. *Sci Rep* (2020) 10:11622. doi: 10.1038/s41598-020-68011-4
78. Auffinger B, Spencer D, Pytel P, Ahmed AU, Lesniak MS. The Role of Glioma Stem Cells in Chemotherapy Resistance and Glioblastoma Multiforme Recurrence. *Expert Rev Neurother* (2015) 15:741–52. doi: 10.1586/14737175.2015.1051968
79. Noch EK, Ramakrishna R, Magge R. Challenges in the Treatment of Glioblastoma: Multisystem Mechanisms of Therapeutic Resistance. *World Neurosurg* (2018) 116:505–17. doi: 10.1016/j.wneu.2018.04.022

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Systemic Sclerosis in Zimbabwe: Autoantibody Biomarkers, Clinical, and Laboratory Correlates

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Introduction: Systemic sclerosis (SScl) is an autoimmune disease whose prevalence is rarely reported in Africa. Autoantibodies are the biomarkers of the condition, precede overt disease and determine disease phenotypes. SSc specific autoantibodies also vary between racial groupings. Objective: To investigate the clinical and laboratory characteristics of Zimbabwean patients who were reactive SSc specific autoantibodies.

Materials and Method: 240 patients, 173 of them female with SSc specific autoantibodies were included. Autoantibodies were detected by indirect immunofluorescence microscopy and immunoblotting using a panel of 13 SScl (Euroimmun Ag., Germany). Demographic, clinical and laboratory parameters relevant to the monitoring of SScl were captured. These included pulmonary function tests, hematology, clinical chemistry, serology and thyroid function tests. Allergy skin prick tests (SPT) to inhalant and food allergen sources were conducted when indicated.

Results: All the 240 patients (median age was 36 years) expressed SSc specific autoantibodies. 86% were Black, 11% White and 3% Asian and a fifth (20%) were younger than 16 years. Eleven (4.6%) fulfilled the ACR/EULAR classification of SSc. Clinically they had limited cutaneous (n=6), diffuse cutaneous (n=3) and SScl/inflammatory myopathy overlap (n=2). The most frequently detected antibodies anti-RNA polymerase III (RNAP) 55%, anti-Th/To (28%) anti-RNAP 11 (22%), anti-CENPB (18%) and anti-Scl-70/ATA (13%). Racial variations in the expression of these antibodies were apparent between Black, White and Asian patients. The majority (95%), who did not fulfil the ARA/EULAR criteria were symptomatic. Raynaud's Phenomenon was documented in 24%. Respiratory symptoms included coughing, dyspnea and wheezing. There was a restrictive ventilatory defect with increased FEV1/FVC ratio. Pruritus, urticaria and skin depigmentation were the main cutaneous features while constipation, bloating, Gastroesophageal reflux disease (GERD) and abdominal pain dominated GI symptoms. Mean blood pressure readings while normal varied with biomarkers. Haematology and biochemistry parameters were within normal reference ranges.

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Conclusion: The expression of SSc specific autoantibodies is common and associated with known SSc symptoms. The types and frequency of autoantibodies varied with racial groupings. A fifth of the patients were children below the age of 16 years.

Keywords: systemic sclerosis, autoantibodies, clinical, laboratory, respiratory, cutaneous, Zimbabwe

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem autoimmune condition that characterized by cutaneous changes, microvascular abnormalities, visceral fibrosis and internal organ dysfunction (1). SSc specific autoantibodies are detected in over 95% of SSc patients and some are included in the international classification criteria for the condition. The diffuse cutaneous (dcSSc) or limited cutaneous (lcSSc) variants are described on the basis of the extent of skin involvement (2). The variants are respectively associated with the SSc specific autoantibodies, anti-topoisomerase I antibodies (ATA) (also known as anti-Scl-70) and anti-centromere antibodies (ACA) whose detection is mutually exclusive. Some patients with anti-RNA polymerase (RNAP) antibodies have the dcSSc phenotype. The skin changes that are universal in advanced disease may be subtle in early disease. Autoantibodies are detectable at all stages and may temporally correlate with the presence of Raynaud's Phenomenon (RP) a recognized early feature and precursor of SSc. Autoantibodies are diagnostic and predict organ involvement, disease phenotypes and clinical outcomes (3–6). A limited number of autoantibodies are included in the ARA/EULAR classification criteria (7). This SSc classification describes cutaneous changes in symptomatic patients with detectable (anti-ATA, anti-ACA, anti-RNA polymerase III) but does not adequately address non-cutaneous respiratory, gastrointestinal or endocrine symptoms that are early disease features (8).

Pathology

The pathogenesis of SSc involves interplay between an exposure to unidentified trigger, the processing of such an antigenic trigger by genetically predisposed individuals and an immune response that triggers autoreactivity. The systemic immune response culminates in multi-system endothelial cell proliferation, smooth muscle hypertrophy, myofibroblast activation and extracellular matrix synthesis. Host tissue specific autoantibodies that can precede ARA/EULAR classifiable disease by many years are produced in this immune response cascade (4).

Clinical Presentation

Early SSc manifestations are non-specific, and depending on affected organs the symptoms can be variously confused with asthma, dermatitis, rheumatic disease or food allergy. Raynaud's Phenomenon (RP) is one of the early features and, in the presence of SSc-specific autoantibodies predicts the development of SSc in patients (9). The types of autoantibodies detected influences the disease phenotypes and the type and extent of organ involvement. Clinical phenotypes vary with the types of autoantibodies. Some autoantibodies have been associated with malignant conditions.

SSc specific autoantibodies differ with the genetic backgrounds, and (10) are likely to be different in Africans (11). Black ethnicity is associated with a greater frequency of SSc, (12–15) a younger age of onset and more severe disease (16). There are no comparable regional data addressing laboratory and clinical features of patients with detectable SSc specific antibodies. The objective of this study was to profile the clinical and laboratory characteristics of patients with detectable systemic sclerosis specific antibodies.

PATIENTS AND METHODS

Study Design

This was a single center observational study. A review of clinical and laboratory records of patients who had attended the Asthma, Allergy and Immune Dysfunction Clinic, the only clinical immunology referral clinic in Zimbabwe was undertaken.

Study Population

We included 240 patients that had been referred to the Asthma, Allergy and Immune Dysfunction Clinic in Harare, Zimbabwe between 2013 and 2018 (**Table 1**). The commonest reasons for referral was the evaluation of respiratory, gastrointestinal, cutaneous or other symptoms. Patients with detectable SSc specific and associated serum autoantibodies were included. HIV antibody positive patients were excluded since some can aberrantly produce non-specific autoantibodies.

Clinical and Laboratory Investigations

Demographic and routine clinical records, temperature and blood pressure and body mass index (BMI) records were documented. A detailed history and clinical evaluation was performed. Skin thickness was evaluated by palpation and pinching of the face, upper arms, forearms and hands. Capillaroscopy was not conducted for lack of requisite equipment. The respiratory system was assessed using a detailed history, the Asthma Control Test (ACT), lung function testing and both skin prick testing and immunoblot assays to common aeroallergens. Similarly, suspected food allergy was excluded based on the history of exposure and relevant testing.

Autoantibody Determination

Line Immunoblot Assay

Patient sera were analysed using the SSc [Nucleoli] Profile EuroLine [IgG]; Euroimmun, Catalogue Number DL 1532-

TABLE 1 | Demographic profile of patients.

	Combined data	Under 16 yrs.	Over 16 yrs.	Female: Male (F:M) ratio
All patients (n=240)	240	49 (20%)	191 (80%)	
Age range (years)	<1 - 94	<1 - 16	17 - 94	
Median age (years)	36	7.5 (2.73-12.38)	41.9 (39.8-44.0)	
Proportion females	173 (72.1%)	25 (46.5%)	152 (77.6%)	2.4:1
Proportion males	67 (27.9%)	23 (53.5%)	44 (22.4%)	
Black patients (n=203)	203	46 (20.7%)	161 (79.3%)	3.5:1
Age range	0-84	<1-16 years	17-84	
Median age (years)	35	7.56 (2.73-12.38)	40	
White patients (n=27)	27	1 (3.7%)	26 (96.3%)	3:1
Age range (years)	15-94	15	17-94	
Median age (years)	46	15	47	
Asian patients (n=10)	10	2 (20%)	8 (80%)	9:1
Age range (years)	1-60	1-4	22-60	
Median age (years)	31.5	2.5	33.5	

Age, race, and gender distribution of patients with detectable systemic sclerosis-specific autoantibodies.

1601G, a fixed immunoblot panel containing 13 autoantigens that detect SSc specific IgG autoantibodies. The 13 immunoblot panel included ATA, CENP-A, CENP-B, RP11, RP155, NOR 90, fibrillarin (or anti-U3RNP), Th/To, PM-Scl100, PMScl-75, Ku, PDGFR and Ro-52. The patients had been screened using the Ig G antibodies against nuclear antigens (ANA) immunoblot panel from the same manufacturer (Euroline Cat # DL 1590-6401-3G) that includes ATA, CENPB, PM-Scl100 and Ro-52 as well as non-SSc specific AMA-M2, SS-A, SS-B, dsDNA, nucleosomes, Sm, RNP/Sm, PCNA, histones, Rib-P-protein and Jo1. The patients were tested using either the SSc profile or the ANA profile. In some cases samples were tested with both panels. Testing was in accordance with the manufacturer's instructions. Briefly, immunoblot test strips impregnated with different ENA specificities were incubated with a dilution of 15 microliters of serum in 1.5 ml (1:101) of sample buffer. Autoreactivity was detected by incubating the strips with alkaline phosphatase labelled anti-human IgG antisera. The addition of the NBT/BCIP substrate elicited a color reaction, that was evaluated using the manufacturer's software (EUROlabScan) to report positive, borderline or negative reactivity. Positive control bands are included in each strip.

Immunofluorescence

Immunofluorescence examination was conducted in 16% of patients with the aim of correlating this standard method of SSc diagnosis with the line blot immunoassays. Patient sera were stained using the IIFT mosaic basic profile 3A slides (Cat. FA 1802-2010-3) and examined using the EuroStar III Plus immunofluorescence microscope (Euroimmun Ag, Germany). Reagents were from the same manufacturer.

Clinical Laboratory Measurements

Other laboratory investigations were requested as indicated in the course of routine clinical care, and not all 240 patients had all the tests. The parameters measured included but were not limited to complete blood cell counts, erythrocyte sedimentation rate (ESR), C-Reactive Protein (CRP), urinalysis, electrolytes, urea and creatinine, liver enzymes, Complement C3, C4, creatinine kinase (CK), serum IgG, IgA, IgM and IgE immunoglobulin levels, T lymphocyte subsets and thyroid function tests (TSH, T3, T4). Laboratory testing was conducted in an ISO 15189 accredited in-house clinical laboratory. T lymphocyte subset enumeration was performed using a FACSCalibur (Becton Dickinson) dual laser, four colour flow cytometer. Thyroid function parameters were measured using the ELISA technique, different commercial kits were used in the study period.

Statistical Analysis

The one-way ANOVA test was used to compare the means in the different subgroups. The null hypothesis was that the means of the different variables was the same for the different groups of data. A p-value <0.05 was considered significant. The StatPlus (version 6.1) software was used.

Ethics Statement

Institutional approval was obtained and ethical approval to conduct the study was granted by the Medical Research Council of Zimbabwe (MRCZ/B/1479).

RESULTS

Patient Demographic Profile, Ages Gender, and Race

During the period 2013-2018, 4335 patients were referred to the Clinic. Amongst these, 240 had detectable SSc specific autoantibodies and were selected for further investigation. The age distribution of the population was bimodal, 20% were under the age of 16 years, mean age 7.56 (95% CI 2.73-12.38) years. The mean age of older patients was 41.93 (95% CI 39.79-44.07) years. The racial groupings were Black Africans (84.6%), White (11.3%)

and Asians (4.1%). The ancestry of Blacks was Zimbabwean, Whites were European, mainly British and Asians were of the Indian subcontinent. The median age was 36 years, when stratified into racial subgroups, the median age was lowest in Asians (31.5 years), while Black (35 years) were younger than White patients (46 yrs.). Autoantibody positive patients <16 years were Black (45/48) or Asian (2/48), one was White. There was a female predominance amongst the adults, the Female to Male (F:M) was 3.5:1. In the <16-year age group the gender proportion was F:M= 1.2:1.

Vital Signs and Baseline Laboratory Findings

Blood Pressure (BP)

The mean systolic BP for all the groups was normal, 121.66 (95% CI 114.76-128.56) mmHg. There were no significant differences between the autoantibody subgroups. Although mean diastolic BP readings were also normal 75.37 (CI 71.06-79.68) mmHg, there were significant differences between autoantibody subgroups, being higher in the anti-Th/To (78 mmHg, 95% CI=76-81) than the anti-CENPB (72 mmHg 95%CI=68-76 mmHg) groups. (p=0.015). The mean BP readings amongst three race groups were comparable to the overall means in all autoantibody subgroups.

Laboratory Investigations

Haematology

Although mean hematology parameters were within normal reference ranges, there were variations that depended on the types of autoantibodies detected. The hemoglobin (Hb) concentration was higher in anti-RP11 (14.01 g/L, 95% CI=13.6-14.4 g/L) compared to the anti-RP155 (13.4g/L, 95% CI=13.1-13.6) (p=0.016) anti-CENPB (13.15, 95%CI=12.7-13.6 g/L) p=0.006, and anti-ATA (13.1 g/L, 95%CI= 12.4-13.9), p=0.019 positive patients.

The overall erythrocyte sedimentation rate (ESR) was elevated (50.6 mm/h) when all patients were pooled. The ESR however varied with the types of autoantibodies and was significantly higher in anti-CENPB (56.58, mm/h, 95% CI=50.8-62) than anti-NOR90 (41.9 mm/h 95% CI=32.9-50.8), p=0.008 and anti-Ku (41.96 mm/h, 95%CI=33.7-50.2 mm/h), p=0.005 groups. The C-Reactive protein (CRP) was positive in 20% of the patients.

Lymphocyte Subsets

The median total lymphocyte percentage was 35.37% (95% CI=31.91-38.42) with no significant inter-group variations. The mean absolute and percentage CD4+ and CD8+ T lymphocyte counts, while within normal ranges also varied with autoantibody types. The CD4+ T lymphocyte percentage was highest in the anti-ATA (47.33%, 95% CI= 41.97-52.70) and lowest in the anti-CENPB (41.72% CI 37.94-45.50) and anti-RP11 (41.56%, 95%CI 36.98-46.146) antibody positive patients. The mean CD8+ T lymphocyte percentage was 27.56%, (95% CI=22.79-32.66), being highest in the anti-CENPB (28.6%, 95% CI= 24.28-32.92) and lowest in the anti-ATA subgroups (23.7%, 95%CI=19.00-28.41). Values for the other subgroups were not statistically different.

Complement

Complement protein concentrations differed in tandem with SSc specific autoantibody types. Mean C3 complement values (0.126 g/L) were significantly higher in the anti-PMScl100 (p=0.017)), than the anti-CENPB or anti-RP155 groups (p=0.005).

The mean C4 complement levels were highest in the anti-ATA (0.045 g/L) and lowest (0.026 g/L) in the anti-CENPB positive subgroups (p=0.003).

Immunoglobin Isotypes

Four immunoglobulin isotypes (IgA, IgM, IgG and IgE) were measured. The quantities of total IgG and IgA serum antibodies varied depending on the SSc specific autoantibody profile.

Patients who were anti-ATA and anti-PMScl 100 autoantibody positive had the highest total IgG antibody levels. The IgG antibody concentrations were highest in the anti-ATA, 1.717 g/L (95% CI=1,316-2,118) and anti-PMScl 100, 1,581 g/L (95%CI= 1,310 1,851) positive patients. The values anti-fibrillarin (1.221 g/) and anti-Ku (1.217 g/L) anti-Ku, anti-PMScl751,310 g/L anti-RP155 (1,347 g/L) and anti-Th/To 1,301 g/L positive patients were significantly lower (**Table 2**).

The mean IgA concentration for all the patients was 0.270 g/L. There were inter-group variations and the mean concentration of IgA was highest in the anti-ATA, (0.335 g/L) anti-fibrillarin (0.335 g/L) and anti-Ro52 (0.316 g/L) positive patients. The lowest values were obtained in anti-PMScl75 (0.234 g/L) positive patients (**Table 2**). IgM levels were comparable across the autoantibody subgroups. The mean IgE antibody levels of 164 kU/L (95% CI= -127-455) was comparable across all autoantibody subgroups.

Thyroid Function

Abnormal thyroid function (hypothyroidism or hyperthyroidism) was the commonest endocrine abnormalities occurring in 7% (17/240). Diabetes was diagnosed in 5% and parathyroid adenomas in three.

Thyroid hormone levels varied with types of autoantibodies.

Mean free thyroxine (fT4) levels for all the patients were 2.53 ng/dL. The concentrations varied with the types of SSc specific autoantibodies. The concentration of fT4 was highest in anti-ATA (3.36 ng/dL) and anti-PMScl100 (3.02ng/dL) positive patients. These values were significantly higher than in anti-fibrillarin (1.51 ng/dL), anti-Ku (1.05 ng/dL), anti-PMScl 75 (1.23 ng/dL) and anti-Ro-52 (1.32 ng/dL) positive patients (**Table 3**).

The mean free triiodothyronine (fT3) levels were 2.65 pg/mL. The highest fT3 concentrations were in anti-PMScl 100 positive patients (3.25 pg/mL, 95% CI 1.79-4.70). The lowest fT3 concentrations were in anti-PMScl75 positive patients. (1.99 pg/mL, CI 1.61-42.37) (p=0.02).

There were no statistically significant differences in the levels of Thyroid Stimulating Hormone (TSH) between the groups. The mean TSH levels were 2.58 mIU/mL (95% CI 1.22-4.03).

Detection of SSc Specific Autoantibodies

Line Immunoblot Assays

The two immunoblot panels that include SSc specific and associated autoantibodies were used. None of the patients (0%) had detectable anti-CENP-A. The remaining detectable

TABLE 2 | Comparison of total serum IgA, IgM, IgG antibody levels, complement C3, C4 and lymphocyte subset values in patients with different SSc-specific autoantibodies.

	Autoantibody	Mean g/L	95%Confidence Interval		p-value
IgG antibody	**PMScl-100**	**1,581**	**1,310**	**1,851**	
	a. Fibrillarin	1,221	1,129	1,313	0.001
	b. Ku	1,217	1,023	1,411	0.008
	c. NOR90	1,276	1,071	1,480	0.017
	d. PMScl-75	1,310	1,220	1,401	0.009
	e. Th/To	1,301	1,196	1,407	0.003
	ATA (ATA)	**1,717**	**1,316**	**2,118**	
	a. Fibrillarin	1,221	1,129	1,313	0.005
	b. Ku	1,217	1,023	1,411	0.002
	c. NOR90	1,276	1,071	1,480	0.004
	d. PMScl-75	1,310	1,220	1,401	0.002
	e. RP155	1,347	1,269	1,425	0.003
	f. Th/To	1,301	1,196	1,407	0.001
IgA antibody	**Fibrillarin**	**0.335**	**0.267**	**0.404**	0.005
	a. PMScl-75	0.234	0.195	0.273	
	ATA	**0.336**	**0.270**	**0.401**	0.005
	a. PMScl-75	0.234	0.195	0.273	
	Ro-52	**0.316**	**0.268**	**0.365**	0.013
	a. PMScl-75	0.234	0.195	0.273	
Complement (C3)		0.126 g/L.			
	PMScl 100	**0.136**	**0.089**	**0.183**	0.017
	a. CENPB	0.128	0.111	0.145	
	Th/To	**0.136**	**0.119**	**0.153**	0.023
	a. Ro-52	0.109	0.092	0.126	
Complement C4					
	ATA	0.045 g/L			0.003
	a. CENPB	0.026 g/L			
Total Lymphocyte %		35.37%	31.91	38.42	
CD4+ T lymphocytes %		%			
	ATA	**47.33**	**41.96**	**52.70**	
	a. anti-RP11	41.56	36.98	46.15	
	b. CENPB	41.72	37.94	45.50	
	Th/To	**45.45**	**42.47**	**48.43**	
	a. CENPB	41.72	37.94	45.50	
CD8+ T lymphocytes %		27.56%	22.79	32.66	
	CENPB	**28.6**	**24.28**	**32.92**	
	a. ATA	23.7	19.00	24.41	

The levels of IgA, IgG, complement and CD4+ T lymphocytes were highest in anti-ATA and anti-PMScl100 seropositive patients.
The bold values were used as a comparator in the statistical analyses. The p-values are a comparison of the frequency of autoantibody detection relative to the bold values.

TABLE 3 | Immunofluorescence staining patterns and immunoblot reactivity clinical characteristics of patients fulfilling the ACR/EULAR criteria for the diagnosis of systemic sclerosis.

Case Number	Lab ref	Gender, Age	Immunofluorescence staining pattern	Immunoblot result	Clinical classification
10695	5016	Female, 54 yrs.	positive	RP11, PM-Scl75	overlap
		Female, 35 yrs.	cytoplasmic, nucleolar,	RNAP 155	Diffuse
17170	4139	Female, 39 yrs.	positive, nucleolar use slide	Not done	diffuse
9818	2620	Male, 51 yrs.	speckled,	CENPB, RP11, RP155	limited
10101	5329	Female, 39 yrs.	positive, nucleolar, titer 1:1280. use slide	fibrillarin, AMA-M2, Ro-52, SS-A	Diffuse
12193	7208, 7715	Female, 45 yrs.	Positive, nucleolar	RP11, Th/To	limited
12634	5016	Female, 60 yrs.	Granular, speckled	RP11, RP155, PMScl 75,	overlap
13271	112974	Female, 45yrs	speckled, titer 1:640	Ro-52	limited
13908	289/114361	Male, 55 yrs.	Positive, nucleolar	CENPB, Th/To, PMScl 100	limited
17132	4973	Female, 42 yrs.	homogenous, positive	Scl-70, CENPB Neg	limited
17320	4648	Male 47 yrs.	Positive, speckled	CENP-B	limited
23190	11204	Female, 35 yrs.	nucleolar staining, Titer, 1:960	RNAP 155, Fibrillarin, NOR90, SSA	Diffuse

autoantibodies were specific for anti-RNAP III 155 kD (RP155) 54.6%, anti-RNAP III, 11 kD (RP11) 21.6%. Other autoantibodies were anti-Th/To (27.9%) anti-CENPB (18%), anti-ATA/ATA (13%) anti-PMScl75 (17.9%), anti-NOR90 (12%) and anti-PMScl100 (10.4%). The SSc associated anti-Ku antibody was detected in 12% (**Figure 1**). There was infrequent reactivity to SLE specific autoantibodies anti-dsDNA (5.8%), anti-Rib-P-protein (5.4%) and anti-PCNA (4%). The Sjogren's syndrome associated anti-SS-A was detected in 5.4% while the inflammatory myopathy associated anti-Jo-1 was detected in 2.1%.

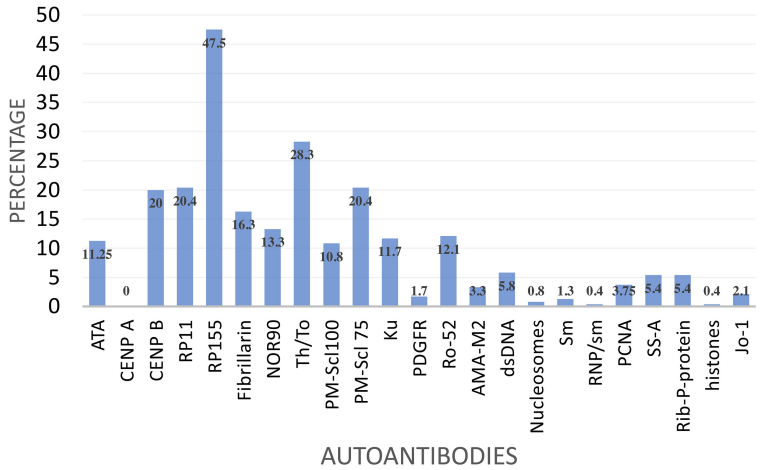

FIGURE 1 | Frequency of reactivity to a panel of selected autoantibodies by SSc specific autoantibody reactive patients.

Immunofluorescence Reactivity

Reactivity to autoantigens was investigated by immunofluorescent (IF) microscopy of Hep-2 cells. The characteristic speckled, threads, nucleolar and centromere staining patterns were observed in some of the patients. Staining patterns that could not be categorized were classified as positive IFT.

Eleven (4.6%) fulfilled the ACR/EULAR criteria for SSc. Six had lcSSc, three dcSSc and three had symptoms suggestive of an overlap between SSc and inflammatory myopathies. One, RNAP 155, fibrillarin, NOR90 and SS-A positive female patient with rapidly progressive dcSSc succumbed, aged 35 years, within 15 months of the diagnosis of SSc. Results for the eleven ACR/EULAR consistent patients are presented in **Table 3**.

Co-Occurrence of the Major Autoantibodies

The co-occurrence of autoantibodies was common, however the expression of (i) anti-ATA and anti-CENPB and (ii) anti-ATA and anti-RNAP11 was mutually exclusive. anti-RNAP155 and anti-ATA were co-associated in 4/31 (13%), anti-CENP-B and anti-RNAP155 in 8/44 (18%) and anti-RNAP11 and anti-CENPB in 5/44 (11%) patients. The RNA polymerase III autoantibodies were infrequently co-associated, anti-RP11 and anti-RP155 were co-associated in 12.9% (31/240). The two PMScl exosomes, (PMScl-100 and PMScl-75) were only co-associated in 3/61 (4.9%).

Autoantibodies in Different Racial Groups

There were racial differences in the frequency of autoantibody detection (**Figure 2**). Anti-ATA was detected in 48% (13/27) White compared to 5% (10/203) Blacks and 0% Asians. Anti-RNAP 11 detection was highest in White (17%) compared to Black (9%) or Asian (5%) patients. Anti-NOR90 was twice more frequent in Black (13%) than in White (6%) or Asian (5%) patients. Anti-PMScl100 and anti-PMScl75 were more frequent in Asian patients. The detection rate for anti-CENPB, anti-RP155, anti-Th/To and anti-Ku was comparable across the racial subgroups. The most frequently detected autoantibodies amongst the 27 White patients were anti-RP11, anti-RP155, anti-Th/To, anti-ATA and anti-CENP-B. None of the White patients had detectable anti-PM-Scl-100 antibodies.

Organ Involvement

The symptoms were referrable to multiple organs predominantly the respiratory (92%), cutaneous (87%), musculoskeletal (51%) and gastrointestinal (47%). Symptoms of Raynaud's vasculopathy were reported by 24% of the patients. The type and frequency of involvement of different organs is summarized in **Table 4**.

Respiratory Tract

Respiratory symptoms were dominant (92%) and included dyspnea, a non-productive dry cough, wheezing, chest pain and chest tightness (6%). Bi-basal velcro sounds were often heard on auscultation. The Asthma Control Test (ACT), a subjective symptom assessment tool gave an average score of 16 (range 6 to 25) amongst those with respiratory symptoms. Nebulization with beta-2 agonist bronchodilators infrequently demonstrated significant changes in FVC, FEV1 or FEF25-75. The values were often depressed following bronchodilator inhalation. Bronchodilator reversibility was only observed in a subset of patients with a positive family history of atopy, reactivity to seasonally relevant inhalant allergens and a clinical diagnosis of asthma (17.5%) and/or allergic rhinitis (11.5%). Pulmonary function parameters varied with the types of autoantibodies detected (**Table 5**).

The FEV_1 varied with the types of detectable autoantibodies. The mean FEV_1 for all the patients (79.96%) was in the lower

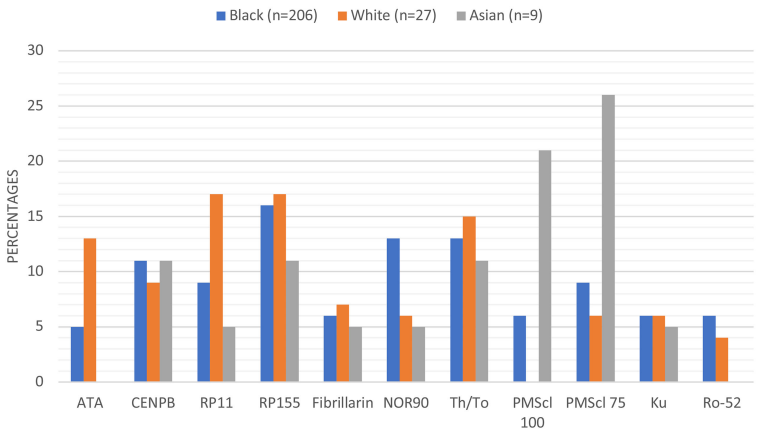

FIGURE 2 | Proportions and types of autoantibodies detected in different racial groups. Anti-ATA and anti-Ro-52 were not detected in any of the Asian patients and anti-PMScl-100 was not detected in any White patient.

limit of normal values. The highest FEV1 volumes were documented in anti-PMScl-100 (87.7%) and anti-Th/To (85.1%) autoantibody positive patients. The FEV1 values for the anti-PMScl-75 (73.6%) p=0.017 and anti-Ku (70.9%) positive patients were significantly lower than the group means and the anti-PMScl100 values.

The FVC values were decreased (mean 74%).

The FVC values varied with the types of detectable SSc specific autoantibodies. FVC% values for anti-PMScl100 (82.35%, CI 73.17-91.53) and anti-Th/To (78.66%, CI 72.69-84.63) positive patients were within were within normal (80-120%) or near-normal ranges and higher than the group means. In contrast the FVC% values for the anti-Ku (65.7%), p=0.013, anti-PMScl75 (68.7%) (p=0.014) and anti-Ro52 (69.86%) positive patients were significantly lower than the normal ranges.

The mean FEV1/FVC ratio for all the patients combined was (107.6%, 95%CI 104.19-111.91) significantly greater than predicted for age, gender, ethnicity, and BMI suggesting a predominantly restrictive pulmonary defect. The severity of the restrictive changes varied with the types of autoantibodies detected. The highest was FEV_1/FVC ratios were in anti-Ro-52 (112; 108.61-115.39), anti-CENPB (111.07, CI=106.37-115.77) and anti-Ku (109.19, CI=105.70-112.68) positive patients. High FEV_1/FVC ratios were also documented in anti-NOR90 (105%), anti-RP155 (106.5%) and anti-Th/To (108.8%) positive patients. When the FEV_1/FVC ratio of the non-SSc specific anti-Ro-52 antibody positive patients was compared with that of patients with the SSc specific anti-NOR90, anti-RNAP155 and anti-Th/To groups the differences were significant (p=0.01).

The mean PEFR for all the patients was 87% of predicted values. There were differences in PEFR values depending on detectable autoantibodies. The mean PEFR of 107.6% for the anti-PMScl-75 positive patients was significantly higher than in the anti-Th/To (89.7%), p=0.006.

Skin Involvement and Tissue Swelling

Cutaneous symptoms were present in 87% (209/240) of patients in all age groups. The most frequent was a predominantly acral pruritic rash of different morphological descriptions (76%), urticaria (38%), dermatitis/eczema (30%), hypo/hyperpigmentation (23%) and a psoriasiform rash (7%) (**Figure 3**). The pruritus was often antihistamine refractory. Morphoea and various dermatoses including a pruritic rash with a psoriasiform morphology were frequent findings in patients of all ages including a 2-year-old (**Figure 4**). Hair thinning, hair loss and alopecia were common (17%) (**Figure 4**). Keratinization, digital ulceration, sclerodactyly and calcinosis were infrequent, being observed in the subgroup that fulfilled the ARA/EULAR criteria for SSc.

Subcutaneous tissue swelling was observed in 41% (99/240). This included angioedema (12%) variously involving the lips, eye lids or hands (6.7%). Skin thickening was observed in 5% of the patients. Puffy fingers (**Figure 5**) were observed in 5% and were most prominent in pediatric groups.

Gastrointestinal Tract

The most frequent gastrointestinal symptoms were nausea (51%), constipation (48%) abdominal and/or epigastric pain (47%), bloating (35%) and gastro-esophageal reflux (30%) and constipation. The symptoms were associated with the presence of defined SSc specific autoantibodies. Constipation was associated with anti-ATA (p=0.033), anti-NOR90 (p=0.039) and anti-Ku (p=0.026). Diarrhea was associated with anti-ATA (p=0.001) and anti-PM-Scl75 (p=0.005), abdominal pain with anti-RP 155 (p=0.023), GERD with anti-fibrillarin (p=0.011) and bloating with anti-Th/To (p=0.011). The most common symptom, nausea and other poorly localizable symptoms (e.g. heartburn, belching, dyspepsia and melena) showed no significant associations with specific autoantibodies.

TABLE 4 | Frequency and types of vascular, respiratory, gastrointestinal, cutaneous and musculoskeletal symptoms reported by patients with SSc-specific autoantibodies.

Raynaud's Phenomenon	Raynaud's Vasculopathy	N 57	% 24%
Respiratory symptoms			
	cough	174	75.5%
	Dyspnea/tightness of the chest	162	67%
	wheeze	59	24.60%
	nasal discharge	59	24.6%
	sneezing	54	22.50%
	Nasal blockage	47	19.60%
	asthma	42	17.50%
	chest pain	18	7.50%
	Rhinorrhea	37	15.4%
	Posterior nasal drip	34	14%
	Allergic rhinitis	27	11.5%
Gastrointestinal			
	constipation	114	47.5%
	Abdominal pain	113	47%
	bloating	84	35%
	Gastro-esophageal Reflux	72	30%
	Heartburn	68	28.3%
	Dysphagia/esophageal dysmotility	42	17.5%
	Peptic ulcers	38	15.8%
	Vomiting	37	15.4%
	Diarrhea	26	10.8%
	Inflammatory bowel disease/syndrome	23	9.6%
Cutaneous			
	urticaria and pruritus	195	81%
	Skin hypo/hyperpigmentation	51	21.3%
	Alopecia or hair thinning	40	16.7%
	psoriasiform rash (morphea)?	37	15%
	Alopecia or hair thinning	40	16.7%
	eczema	39	16.3%
	perioral or periorbital hyperpigmentation	12	5%
	cutaneous blisters or skin ulcers	11	4.6%
	Classical morphoea	7	2.9%
	Calcinosis, teleangiectasia, skin keratinization	8	4.5%
	fingertip ulcers	5	2.1%
Tissue swelling			
	Angioedema (19) face (23), lips (17), eyelids (16)	19	12%
	Hands or feet (including palms and soles)	34	14%
	Puffy fingers	24	10%
	Generalized swelling	10	4.2%
	Other swelling Ear lobe (5), scalp (3), torso (2)	11	4.6%
Musculoskeletal			
	joint pain unspecified (93), knee (57), finger (41), ankle (24), wrist (20), hip (16), feet (12), elbow (12)	93	39%
	Muscle cramps	13	5.4%
	Madonna fingers	3	1.3%
	Jaw movement limitation	3	1.25%

Musculoskeletal involvement was documented in 51% (122/240) of the patients. Joint pains (39%) involving knees (24%), fingers (17%), ankles (10%) and wrists (8%) were dominant. Muscle cramps were reported by 5%. Madonna fingers were seen in three patients. Neurological and psychiatric conditions were reported by 54% (130/240) with headaches (31%) being dominant. The carpal tunnel syndrome was reported by 3%.

Ocular symptoms were reported in 46% (110/240) of the patients (**Supplementary Table 3**). These reported Itchy (35%), inflamed (25%) or red (18%) eyes. The ocular sensations were described as sand grain (13%), gritty (7%), dry (3%) or dry like sandpaper. Allergic conjunctivitis (7.5%) was diagnosed in a subgroup with a positive family history of atopy and SPT or immunoblot confirmed sensitization to aeroallergens. Eye discharge or sticking of the eyelids was in 6% and tearing in 4.5%. Photosensitivity and photophobia were commonly reported (**Supplementary Table 2**).

Neurological Symptoms

The dominant neurological symptoms were headaches (31%), the carpal tunnel syndrome was infrequent (3%) and only diagnosed in patients with the dc-SSc associated autoantibodies.

Cardiovascular symptoms were infrequent, being reported in 42/240 (17.5%) of the patients (**Supplementary Table 3**). Retrosternal or pericardial pain (8%) palpitation (7%) and hypertension (3%) were dominant. Cardiomegaly,

TABLE 5 | Variation of pulmonary function test results FEV1 and FVC depending on the types of serum autoantibodies.

Lung Function Test		*Mean*	*95% Confidence interval*		*p-value*
FEV_1					
	PMScl-100	*87.7*	*79.2*	*96.2*	
	⟹ PMScl-75	73.6	65.5	81.6	0.017
	⟹ Ku	70.9	61.3	80.5	0.011
	Th/To	85.1	78.9	91.4	
	⟹ PMScl-75	73.6	65.5	81.6	0.017
	⟹ Ku	70.9	61.3	80.5	0.011
FVC					
	PMScl 100	82.352	73.2	91.5	
	⟹ Ro-52	69.8	64.9	74.8	0.019
	⟹ PMScl 75	68.7	61	76.4	0.014
	⟹ Ku	65.7	56.4	75	0.007
	Th/To	78.7	72.7	84.6	0.013
	⟹ Ku	65.7	56.4	75	
FEV1/FVC ratio		%			
	Ro-52	112	108.6	115.4	
	⟹ NOR90	104.9	99.79	109.9	0.02
	⟹ RP155	106.5	104.1	108.8	0.01
	⟹ Th/To	106.8	103.8	109.7	0.02
PEFR					
	PMScl 75	107.6	103.3	111.8	
	⟹ §	89.7	78.7	100.8	0.006
	⟹ Ro	82.7	74	91.5	0.001
	⟹ ATA	81.2	62.4	100.1	0.003
	⟹ NOR90	77.6	62.6	92.6	0.001
	⟹ PMScl100	76	67.5	84.5	0.0001
	⟹ RP155	75	69.4	80.6	0.002
	⟹ RP11	74	63.4	84.6	0.018
	⟹ Ku	73.8	64.7	82.9	0.0001
	⟹ Fibrillarin	71.9	63.5	80.4	0.009

Patients with detectable anti-PMScl-100 and anti-Th/To had significantly higher FEV_1 and FVC values than those who were anti-PMScl-75, anti-Ku or anti-Ro-52 positive. The highest PEFR values were recorded in anti-PMScl75 positive patients.

cardiomyopathy, pericardial effusion, chronic cardiac failure, and a history of myocardial infarction were diagnosed or reported in two patients each, while pulmonary embolism, arrythmia, ventricular septal defect and tricuspid regurgitation in one patient each.

The systemic, endocrine, gynecological, renal, hematological, immunological and malignant conditions detected in patients expressing SSc specific autoantibodies are summarized in **Supplementary Table 2**.

Raynaud's disease (24%) was the dominant vascular abnormality. Lymphadenopathy was recorded in 7% of the patients. Non-specific systemic features included tiredness (7%), exhaustion, feeling drained, sweating, fever and chills. Menstrual irregularities were the most frequent gynecological conditions, one (28-year-old) reported premature menopause.

Renal diseases affected 7% of the patients, malignant hypertension being the most severe and the remainder being documented after urinalysis findings. Cortical kidney cysts were detected in two patients. Current or prior diagnoses of malignancy were documented in 2.5% (6/240) of the patients and involved cervical and breast cancer. Other malignant conditions were Kaposi's sarcoma and non-Hodgkin's lymphoma.

The co-existing autoimmune conditions were Sjogren's syndrome (n=1), Myeloperoxidase (MPO) positive vasculitis (n=1) and myasthenia gravis (n=1). Patch test reactivity to epoxy resin, thiuram mix, potassium dichromate and paraben esters that was confirmed in four SSc-specific antibody positive patients were the only potential associations of SSc autoantibody production with an occupational allergen. Anemia and von Willebrand's disease were the only hematological abnormalities. Current infection with tropical diseases was infrequent, two had *Schistosoma haematobium*.

DISCUSSION

This study describes the clinical and laboratory findings in symptomatic Zimbabwean patients who were referred for the evaluation of often non-specific skin, respiratory or gastrointestinal tract, ocular and musculoskeletal ailments. All were reactive to extractable SSc specific cytoplasmic or nuclear antigens. Although a majority of the patients did not fulfil ARA/EULAR criteria for SSc, their symptoms were consistent with those recognized in SSc. A sub-sample of the patients was offered immunofluorescence testing to investigate concordance with standard SSc laboratory diagnostic criteria. The results of the comparison were concordant. The autoantibody profiles differed with the racial groups. An association between the presence of

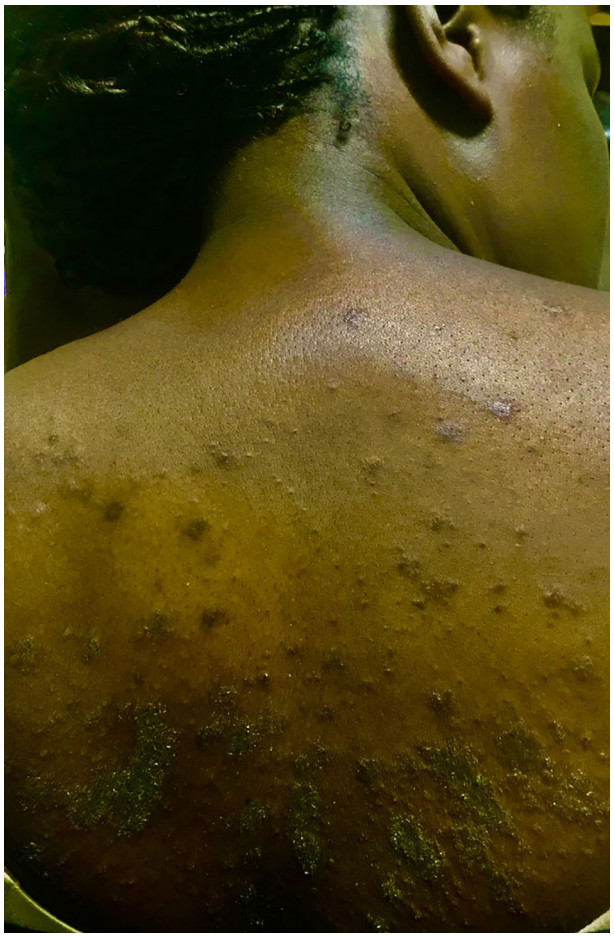

FIGURE 3 | Psoriasiform dermatosis in a patients with SSc specific autoantibodies.

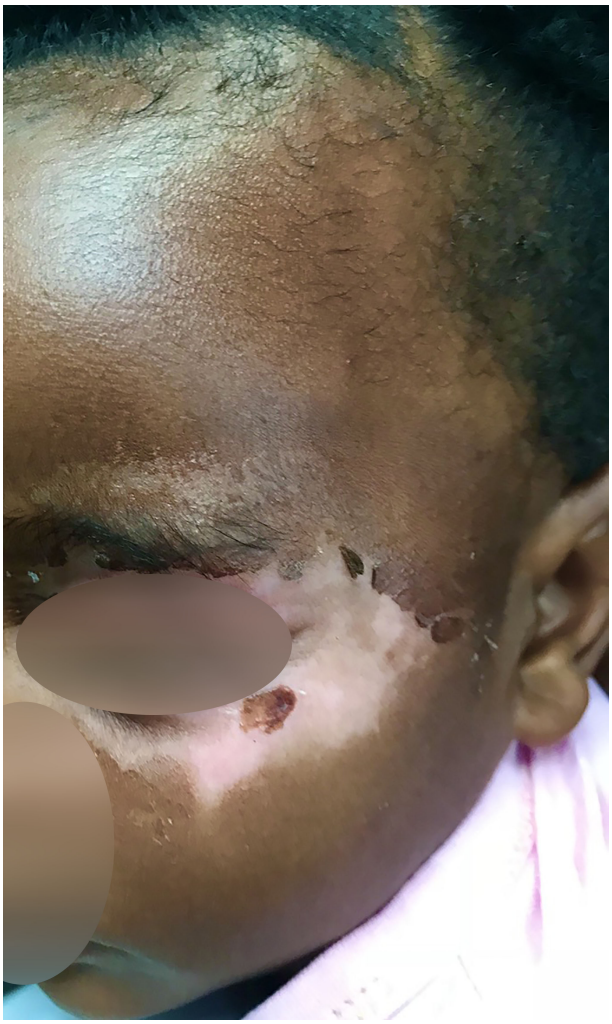

FIGURE 4 | Figure shows skin changes and alopecia in a child with detectable SSc specific autoantibodies.

autoantibodies and disease phenotypes was observed with respect to the respiratory and gastrointestinal tracts and thyroid abnormalities.

Patients

The overall female predominance amongst adult patients (F:M=3.5:1) is comparable to other studies (17, 18). The median adult ages differed significantly along racial subgroups being lowest in Asians (31.5 years), Blacks (35 years) and highest in White (48 years) patients. This difference could explain the recognized earlier onset of SSc in Asian and Black patients. A remarkable finding of this study was that 20% of autoantibody positive patients were <16 years old (mean age 7.56, 95%CI 2.7-12). These were Black (45/48), Asian (2/48) and White (1/48). The racial differences suggest that the presumed rarity of SSc in children could be due to study biases that predominantly report findings in White but not Black patients. To our knowledge, this number of Black pediatric patients with symptoms suggestive of SSc and detectable SSc specific autoantibody reactivity has not been previously reported.

Blood Pressure

Overall systolic and diastolic BP readings were within normal ranges. However, the diastolic pressure readings varied with the types of detected autoantibodies. Diastolic BP was highest in the anti-ATA group and lowest in the anti-PMScl 75 group. The differences in BP could suggest that the types of antibodies produced influence disease phenotypes, skewing patients to towards either the dcSSc or lcSSc phenotypes. The diastolic BP differences between two lcSSc associated anti-Th/To (78 mmHg, 95%CI=76-81) and anti-CENPB (72 mmHg 95%CI=68-76 mmHg) subgroups, (p=0.015) could indicate the recognized higher frequency of renal crisis in association with Th/To but not anti-CENPB as previously reported (19).

FIGURE 5 | Puffy fingers and cuticle hypo pigmentation in a child with detectable SSc specific autoantibodies.

Detection of SSc Specific Autoantibodies in Different Racial Subgroups

The detection of autoantibodies varied with race, anti-ATA and anti-RP11 were more frequently detected in White than Black patients, while anti-PM-Scl 100 was detected in 21% Asian and 6% Black and none (0%) White patients (**Table 3**). The frequency of the lsSSc associated anti-CENPB, anti-RP155, anti-Th/To and anti-Ku was comparable across racial groups. The racial variations that reflect genetic differences, suggest that autoantibodies influence disease phenotypes. The different types of autoantibodies detected in racial groups, makes the targeted tailoring of SSc test panels for defined population groups, one size does not fit all. The textbook focus on anti-ATA and anti-ACA and more recently anti-polymerase III antibodies as prime biomarkers for SSc likely underestimates the prevalence of SSc specific antibodies amongst Black patients, who overwhelmingly expressed anti-RNAP155 in this study.

SSc Diagnosis

The ARA/EULAR criteria for the diagnosis of SSc are internationally accepted. Although only 5%, fulfilled the ARA/EULAR criteria for SSc, all the patients had symptoms that are recognized in established SSc. Raynaud's Phenomenon (RP) an early precursor and predictor of progression to overt SSc was documented in 24%. This figure may underestimate the true prevalence, since cutaneous manifestations of RP in African patients are not obvious and have to be pro-actively solicited. They are affected by the skin colour as well as the climatic conditions. Patients often reported cold hands or feet in colder months of the year. While it is recognized that the mere presence of SSc specific autoantibodies is *per se* not diagnostic of SSc, these are associated with early symptoms that predate overt SSc (5, 15). The purpose of making a diagnosis is to inform the clinical management of patients. In that vein, a combination of the clinical symptoms and the results of detailed physical examination, complemented by relevant laboratory or imaging test results should be prioritized over the fulfilment of international criteria when making a diagnosis. Nevertheless, due to the non-fulfilment of these standard criteria, we avoided labelling all patients as SSc, preferring to describe them as possible early SSc. Nail fold capillaroscopy, a key component for the fulfilment of the Le Roy and Medsger (20) and VEDOSS (21) criteria for early SSc could not be performed. Longitudinal follow-up studies to establish whether or not these patients have SSc and to monitor any disease progression particularly in the pediatric population are recommended.

Laboratory Findings

Laboratory tests were requested and individualized for the routine clinical care of the patients, therefore patients did not have uniform panels of laboratory tests. The elevation of the inflammatory marker ESR, positivity of CRP in 20% and the presence of reactive lymphadenopathy (16%) indicates ongoing inflammatory activity in a proportion of this patient population. The presence and persistence of inflammation in SSc is recognized and has been previously reported (22, 23). Although the patients had GIT symptoms, anemia, a feature of advanced SSc was not observed (24). Anemia is considered less likely in early SSc. The higher mean IgE levels reported in this study could be explained by the inclusion of patients with co-existing allergic asthma and/or rhinitis.

Immune Responses

The innate, cellular, and humoral responses to SSc autoantigens varied significantly with the types of autoantibodies (**Table 6**). Higher levels of Complement (C3), IgA and IgG as well as CD4+ T lymphocytes were more frequent in patients with dc-SSc specific autoantibodies (ATA, PMScl 100 and anti-RP11) than in those with lc-SSc associated autoantibodies (CENPB, Ku and Th/To) who also had lower C3 complement, IgA, IgG antibody levels and CD4+ T lymphocyte percentages. The implication is that immune responses to target autoantigens influence disease phenotypes. Interestingly, the CD8+ lymphocyte percentages were, in contrast, higher in anti-CENPB than anti-ATA positive patients.

Thyroid Function

The frequency of thyroid abnormalities in this cohort was higher than in the background population suggesting it could be

TABLE 6 | Comparison of thyroxine (T3) and triiodothyronine (T4) hormone levels in patients with different types of autoantibodies.

Thyroxine (T4)		Normal range: 0.7-1.8 ng/dL	95% Confidence Interval		p-value
	ATA	**3.37**	**0.77**	**5.97**	
	a. fibrillarin	1.51	0.82	2.19	0.025
	b. Ku	1.05	0.79	1.32	0.024
	c. PMScl-75	1.23	1.04	1.43	0.010
	d. Ro-52	1.32	1.05	1.59	0.017
	PMScl 100	**3.02**	**1.07**	**4.97**	**0.022**
	PMScl75	1.23	1.04	1.43	
Triiodothyronine (T3)		***Normal range: 2.3 - 4.1 pg/mL***			
	PMScl 100	**3.25**	**1.79**	**4.71**	**0.020**
	PMScl 75	1.99	1.61	2.37	
Thyroid Stimulating Hormone (TSH)	Mean for all patients	2.58 mIU/mL	1.22	4.03	

Autoantibodies that are associated with diffuse cutaneous SSc (anti-ATA, anti-PMScl100) were associated with significantly higher hormone levels.
The bold values were used as a comparator in the statistical analyses. The p-values are a comparison of the frequency of autoantibody detection relative to the bold values.

attributable to the autoantibodies. Abnormalities of thyroid function varied with types of SSc autoantibodies. The dc-SSc associated autoantibodies (ATA and PMScl 100) correlated with higher levels of fT3 and fT4, while the lc-SSc associated autoantibodes correlated with lower fT3 and fT4 levels. An association between anti-ATA and thyroid dysfunction is recognized (25). There was a dichotomous relationship between the two PMScl autoantibodies and thyroid disease. Anti-PMScl 100 antibody positive patients had higher fT4 while anti-PMScl75 positive had lower fT3 levels suggesting different effects of the two PMScl antibodies on thyroid function.

Cutaneous Disease

Skin involvement in early stages of SSc consists of different morphological features. The anatomical distribution of the dermatoses tended to be acral with limited or transient flexural involvement. Care providers should consider SSc as a differential diagnosis in managing these frequently atypical dermatoses. The dominant cutaneous symptoms were non-specific pruritus (54%), urticaria (28%) and dermatitis (10%) that are not considered hallmarks of SSc. The characteristic finger-tip ulcers, calcinosis, keratinization of the skin and the classic Madonna were infrequent, being observed amongst those fulfilling the ARA/EULAR criteria. Non-pitting edema of the hands or feet and puffy fingers were prominent in the younger patients.

Respiratory Tract Disease

The predominance (92%) of patients with respiratory symptoms may have been influenced by a referral bias to the Asthma and Allergy Clinic. However, most of the autoantibody positive patients did not have allergic asthma or rhinitis. Lung volume abnormalities were bronchodilator refractory, and the spirometry pattern was predominantly restrictive, contrary to the obstructive, bronchodilator reversible pattern that characterizes asthma. Taken together, with findings of Velcro crackles on auscultation, the spirometry findings suggest fibrotic pulmonary disease, a feature of pulmonary SSc (18, 26). The significant association of the presence of anti-Ku, anti-PMScl-75 and anti-Ro-52 with FVC reductions supports the association of anti-Ku and anti-PMScl75 with ILD as reported by others (27, 28).

Gastrointestinal Tract

The significant association of constipation, diarrhea, abdominal pain, GERD and bloating respectively with anti-ATA, anti-NOR90, anti-Ku anti-PM-Scl75, anti-RP 155, anti-fibrillarin and anti-Th/To suggests that these autoantibodies influence the symptom manifestations. Since the frequency of the antibodies differs with racial groups, these could also explain racial differences in the types and severity of GIT symptoms.

Neurological and Psychiatric Conditions

There was an association between autoantibodies with central and peripheral nervous system symptoms that are recognized in SSc. The carpal tunnel syndrome was only reported in anti-fibrillarin and anti-ATA positive patients. Psychiatric conditions were infrequent, anxiety featured prominently.

Cardiovascular was infrequent as expected in an early SSc cohort. Renal involvement was limited. There were no significant renal abnormalities and observed hematuria and kidney cysts could reflect their prevalence in the background population.

Shortcomings

The main shortcoming is that this paper was not prospective and collected data were not standardized. The absence of a prospectively administered standardized questionnaire poses a risk under reporting the symptoms that were not solicited. In mitigation, all patients were attended to by one of us only (ES) who obtained case histories, examined all the patients and requested the laboratory and imaging tests. Therefore, notwithstanding the shortcomings, any inherent bias is therefore likely to be uniform. While the presence of antibodies suggests SSc, only 5% of the patients fulfilled ARA/EULAR criteria that are designed for the inclusion of patients in clinical trials. These criteria may not be appropriate for early SSc. We suspect our antibody positive symptomatic patients 24% of whom had RP could have early SSc that could not be confirmed or excluded for lack of requisite equipment. The study describes the clinical presentations and laboratory findings that included SSc specific autoantibody reactivity who may or may not have SSc. The lack of access to nail-fold capillaroscopy limited our

ability to confirm early SSc, longitudinal studies if funded will help answer that question.

CONCLUSION

This report covers the clinical history, clinical findings and laboratory investigations conducted on 240 Zimbabwean patients with detectable SSc specific antibodies, 20% of them being younger than 16 years. The observed cutaneous, respiratory, gastrointestinal, musculoskeletal and endocrine aberrations are consistent with findings reported in patients fulfilling the ARA/EULAR criteria. A tendency of alignment of respiratory, gastrointestinal, laboratory and endocrine findings with either dc-SSc or lc-SSc specific autoantibodies was observed. The findings suggest a decisive influence of innate, humoral and adaptive immune responses on disease phenotypes. Racial differences in autoantibody presence and the dominance of anti-RNAP antibodies amongst Black patients, should inform the tailoring of autoantibody test panels to specific populations. We conclude that the presence of SSc specific autoantibodies is common, albeit infrequently detected in Zimbabwe. A fifth of our patients were under 16 years old and presented with non-specific symptoms that should be investigated with a higher index of suspicion in this and similar settings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Research Council of Zimbabwe (MRCZ). Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ES formulated the research idea, performed clinical evaluation of all the patients, supervised the laboratory testing, reviewed and revised the manuscript. YD wrote the initial draft of the manuscript, searched for all the references. MC participated in data collection. TM critically reviewed the manuscript and approved submission. FM critically reviewed the submission of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.679531/full#supplementary-material

Supplementary Table 1 | Blood Pressure measurement.

Supplementary Table 2 | Comparison total Immunoglobulin IgA, IgG, IgM concentrations in patients with different SSc specific autoantibodies.

REFERENCES

1. Kayser C, Fritzler MJ. Autoantibodies in Systemic Sclerosis: Unanswered Questions. (2015) 6:167. doi: 10.3389/fimmu.2015.00167
2. Allcock R, Forrest I, Corris PA, Crook PR, Griffiths I, et al. A Study of the Prevalence of Systemic Sclerosis in Northeast England. (2004) 43(5):596–602. doi: 10.1093/rheumatology/keh124
3. Avouac J, Sordet C, Depinay C, Ardizonne M, Vacher-Lavenu MC, Sibilia J, et al. Systemic Sclerosis–Associated Sjögren's Syndrome and Relationship to the Limited Cutaneous Subtype: Results of a Prospective Study of Sicca Syndrome in 133 Consecutive Patients. (2006) 54(7):2243–9.
4. Burbelo PD, Gordon SM, Waldman M, Edison JD, Little DJ, Stitt RS, et al. Autoantibodies are Present Before the Clinical Diagnosis of Systemic Sclerosis. (2019) 14(3):e0214202. doi: 10.1371/journal.pone.0214202
5. Colaci M, Giuggioli D, Sebastiani M, Manfredi A, Vacchi C, Spagnolo P, et al. Lung Cancer in Scleroderma: Results From an Italian Rheumatologic Center and Review of the Literature. (2013) 12(3):374–9. doi: 10.1016/j.autrev.2012.06.003
6. Derk CT, Sakkas LI, Rasheed M, Artlett C, Jimenez SA. Autoantibodies in Patients With Systemic Sclerosis and Cancer: A Case-Control Study. (2003) 30(9):1994–6.
7. Van Den Hoogen F, Khanna F, Fransen D, Johnson J, Baron SR, Tyndall M, et al. 2013 Classification Criteria for Systemic Sclerosis: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. (2013) 65(11):2737–47. doi: 10.1136/annrheumdis-2013-204424
8. Derk CT, Artlett CM, Jimenez SA. Morbidity and Mortality of Patients Diagnosed With Systemic Sclerosis After the Age of 75: A Nested Case-Control Study. (2006) 25(6):831–4. doi: 10.1007/s10067-005-0177-y
9. Pontifex EK, Hill CL, Roberts-Thomson PJ. Risk Factors for Lung Cancer in Patients With Scleroderma: A Nested Case–Control Study. (2007) 66(4):551–3.
10. Skare TL, Fonseca AE, Luciano AC, Azevedo PM. Autoantibodies in Scleroderma and Their Association With the Clinical Profile of the Disease: A Study of 66 Patients From Southern Brazil. (2011) 86:1075–81. doi: 10.1590/s0365-05962011000600003
11. Barnes J, Mayes MD. Epidemiology of Systemic Sclerosis: Incidence, Prevalence, Survival, Risk Factors, Malignancy, and Environmental Triggers. *J.C.o.i.r.* (2012) 24(2):165–70. doi: 10.1097/BOR.0b013e32834ff2e8

12. Nietert PJ, Mitchell HC, Bolster MB, Shaftman SR, Tilley BC, Silver RM, et al. Racial Variation in Clinical and Immunological Manifestations of Systemic Sclerosis. (2006) 33(2):263–8.
13. Steen V, Domsic RT, Lucas M, Fertig N, Medsger TAJr. A Clinical and Serologic Comparison of African American and Caucasian Patients With Systemic Sclerosis. (2012) 64(9):2986–94. doi: 10.1002/art.34482
14. Gelber AC, Manno RL, Shah AA, Woods A, Le EN, Boin F, et al. Race and Association With Disease Manifestations and Mortality in Scleroderma: A 20-Year Experience at the Johns Hopkins Scleroderma Center and Review of the Literature. (2013) 92(4):191–205. doi: 10.1097/MD.0b013e31829be125
15. McFarlane IM, et al. Gastrointestinal Manifestations of Systemic Sclerosis. (2018) 8(1):.
16. Mayes MD, Lacey JVJr, Beebe-Dimmer J, Gillespie BW, Cooper B, McFarlane TJ, et al. Prevalence, Incidence, Survival, and Disease Characteristics of Systemic Sclerosis in a Large US Population. (2003) 48 (8):2246–55. doi: 10.1002/art.11073
17. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. *N Engl J Med* (2009) 360 (19):1989–2003. doi: 10.1056/NEJMra0806188
18. Elsayed AM, Elhefnya AM, Abogabala MM, Mobashera SA, Abdelzahera A, Alhassaneina KF, et al. Progressive Systemic Sclerosis in Egyptian Patients: Clinical Characteristics and Nail Fold Capillaroscopy. *Egyptian Rheumatologist* (2019) 41(1):47–51. doi: 10.1016/j.ejr.2018.01.009
19. Mitri GM, et al. A Comparison Between Anti-Th/To- and Anticentromere Antibody-Positive Systemic Sclerosis Patients With Limited Cutaneous Involvement. *Arthritis Rheum* (2003) 48(1):203–9. doi: 10.1002/art.10760
20. LeROY EC, Medsger TA. Criteria for the Classification of Early Systemic Sclerosis. *J.T.J.o.r.* (2001) 28(7):1573–6.
21. Muangchan C, et al. Association of C-Reactive Protein With High Disease Activity in Systemic Sclerosis: Results From the Canadian Scleroderma Research Group. (2012) 64(9):1405–14. doi: 10.1002/acr.21716
22. Bellando-Randone S, Matucci-Cerinic M. Very Early Systemic Sclerosis and Pre-Systemic Sclerosis: Definition, Recognition, Clinical Relevance and Future Directions. *J.C.r.r.* (2017) 19(10):1–6. doi: 10.1007/s11926-017-0684-2
23. Mitev A, Christ L, Feldmann D, Binder M, Möller K, Kanne A-M, et al. Inflammatory Stays Inflammatory: A Subgroup of Systemic Sclerosis Characterized by High Morbidity and Inflammatory Resistance to Cyclophosphamide. *Arthritis Res Ther* (2019) 21(1):1–10. doi: 10.1186/s13075-019-2057-x
24. Westerman MP, Martinez RC, Medsger TA Jr, Totten RS, Rodnan GP. Anemia and Scleroderma: Frequency, Causes, and Marrow Findings. (1968) 122(1):39–42. doi: 10.1001/archinte.1968.00300060041007
25. Ugurlu S, et al. Anti-Scl-70 Antibodies in Autoimmune Hypothyroidism. *J Int Med Res* (2008) 36(1):152–6. doi: 10.1177/147323000803600119
26. Sumphao-Ngern P, Foocharoen C, Boonsawat W, Mahakkanukrauh A, Suwannaroj S, Sae-Oue U, et al. Causes and Prevalence of Inadequate Pulmonary Function Testing Among Patients With Systemic Sclerosis. (2015) 11(6):1255. doi: 10.5114/aoms.2015.56352
27. Cavazzana I, Fredi M, Taraborelli M, Quinzanini M, Tincani A, Franceschini F, et al. A Subset of Systemic Sclerosis But Not of Systemic Lupus Erythematosus is Defined by Isolated Anti-Ku Autoantibodies. (2013) 31(2 suppl 76):118–21.
28. Hanke K, Brückner CS, Dähnrich C, Huscher D, Komorowski L, Meyer W, et al. Antibodies Against PM/Scl-75 and PM/Scl-100 Are Independent Markers for Different Subsets of Systemic Sclerosis Patients. *Arthritis Res Ther* (2009) 11(1):1–9. doi: 10.1186/ar2614

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