



# Predominantly Antibody-Deficient Patients With Non-infectious Complications Have Reduced Naive B, Treg, Th17, and Tfh17 Cells

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**Background:** Patients with predominantly antibody deficiency (PAD) suffer from severe and recurrent infections that require lifelong immunoglobulin replacement and prophylactic antibiotic treatment. Disease incidence is estimated to be 1:25,000 worldwide, and up to 68% of patients develop non-infectious complications (NIC) including autoimmunity, which are difficult to treat, causing high morbidity, and early mortality. Currently, the etiology of NIC is unknown, and there are no diagnostic and prognostic markers to identify patients at risk.

**Objectives:** To identify immune cell markers that associate with NIC in PAD patients.

**Methods:** We developed a standardized 11-color flow cytometry panel that was utilized for in-depth analysis of B and T cells in 62 adult PAD patients and 59 age-matched controls.

**Results:** Nine males had mutations in Bruton's tyrosine kinase (BTK) and were defined as having X-linked agammaglobulinemia. The remaining 53 patients were not genetically defined and were clinically diagnosed with agammaglobulinemia ( $n = 1$ ), common variable immunodeficiency (CVID) ( $n = 32$ ), hypogammaglobulinemia ( $n = 13$ ), IgG subclass deficiency ( $n = 1$ ), and specific polysaccharide antibody deficiency ( $n = 6$ ). Of the 53, 30 (57%) had one or more NICs, 24 patients had reduced B-cell numbers, and 17 had reduced T-cell numbers. Both PAD–NIC and PAD+NIC groups had significantly reduced Ig class-switched memory B cells and naive CD4 and CD8 T-cell numbers. Naive and IgM memory B cells, Treg, Th17, and Tfh17 cells were specifically reduced in the PAD+NIC group. CD21<sup>lo</sup> B cells and Tfh cells were increased in frequencies, but not in absolute numbers in PAD+NIC.

**Conclusion:** The previously reported increased frequencies of CD21<sup>lo</sup> B cells and Tfh cells are the indirect result of reduced naive B-cell and T-cell numbers. Hence, correct interpretation of immunophenotyping of immunodeficiencies is critically dependent on

absolute cell counts. Finally, the defects in naive B- and T-cell numbers suggest a mild combined immunodeficiency in PAD patients with NIC. Together with the reductions in Th17, Treg, and Tfh17 numbers, these key differences could be utilized as biomarkers to support definitive diagnosis and to predict for disease progression.

**Keywords:** predominantly antibody deficiency, common variable immunodeficiency, autoimmunity, X-linked agammaglobulinemia, follicular helper T cells, CD21<sup>lo</sup> B cells, naive T cells, EuroFlow

## INTRODUCTION

Predominantly antibody deficiency (PAD) represents the largest group of primary immunodeficiencies (PIDs) and includes up to 70% of all patients (1–5). The hallmark of PAD is a history of severe and recurrent sinopulmonary infections and poor vaccination responses underpinned by impaired B-cell differentiation and antibody production. As such, these patients require lifelong immunoglobulin replacement therapy (IgRT) and prophylactic antibiotics (3, 4).

The archetypical PAD is agammaglobulinemia (6), with patients lacking all serum Ig isotypes as well as circulating B cells. The majority of patients are boys suffering from X-linked agammaglobulinemia (XLA) as a result of mutations in the gene encoding Bruton's tyrosine kinase (*BTK*) (7, 8), an enzyme pivotal in the development of B cells. Autosomal recessive agammaglobulinemia is typically the result of mutations in other components of the pre-B-cell receptor complex or in B-cell transcription factors (9–15).

In a minority of PAD patients with circulating B cells, monogenic defects underpinning clinical phenotype, and pathophysiology have been identified. Most involve B-cell receptor signaling components or molecules required for T: B cell interactions (2, 5, 15–27). With the advancements in genomics, genetic diagnosis is now feasible for 20–30% of patients (28–30). However, for the remainder, diagnosis is typically made by ways of exclusion of clinical and general immunological characteristics (5).

Common variable immunodeficiency (CVID) is the most well-defined PAD (2, 5) and is defined diagnostically by reduced total serum IgG and IgA and/or IgM levels in the presence of impaired vaccination responses and recurrent bacterial infections (31–35). Less well-defined PADs include unclassified hypogammaglobulinemia (HGG), defined by reduced IgG levels but normal IgM and IgA levels; specific polysaccharide antibody deficiency (SpAD), defined by normal serum Ig isotypes but the absence of specific antibody responses to vaccination; and IgG subclass deficiency (IGSCD), defined by normal total IgG levels but reduced levels of one or more IgG subclasses (2, 5, 36).

In addition to recurrent infections and resulting complications, up to 68% of PAD patients suffer from non-infectious complications (NICs), which typically include

autoimmunity, autoinflammation, gastrointestinal disease, and lymphoid malignancies (3, 37–43). NICs are most frequent in, but not exclusive to, CVID patients (36, 38, 44). The dominant presentation of NIC can overshadow infectious problems and thereby complicate diagnosis of PAD. Furthermore, NICs are often hard to treat, rendering patients at risk of early morbidity, and high mortality (38, 44, 45).

From the early 2000s, immunophenotyping of the B-cell compartment has been utilized to improve PAD diagnosis and has formed the basis of two classification systems: Freiburg (46) and EUROclass (47). In both strategies, CD19<sup>+</sup> B cells are delineated using normally low Ig switched memory B-cell (smB) frequencies (CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup>) and abnormally high proportions of B cells with reduced CD21 expression (CD21<sup>lo</sup> B cells) to distinguish subsets. In addition, to these two cell subsets, the EUROclass classification also uses abnormally high frequencies of transitional B cells (CD19<sup>+</sup>CD27<sup>-</sup>CD38<sup>+</sup>) for further subgrouping. Subsequently, more detailed studies have been applied to define immunophenotypes for subgroups in patients with CVID or PAD (36, 48, 49). Despite the identification of clearly distinct phenotypes, these have thus far provided limited prognostic value to predict clinical progression or disease complications. The strongest association found to date is the expansion of CD21<sup>lo</sup> B cells in patients with splenomegaly (47, 50). In addition, marked reductions in total, Ig switched memory, and marginal zone B cells were found to be associated with splenomegaly (47). Furthermore, the presence of any form of autoimmune disease in CVID patients was associated with expansion of CD21<sup>lo</sup> B cells (46) and significant reductions in plasmablasts (47) and Ig smBs (37). A proportional expansion of transitional B cells was found to be associated with lymphadenopathy, whereas CVID patients with granulomatous disease presented with significant reductions in Ig class-switched memory and marginal zone B cells (47).

Although PAD is by definition a disease resulting from defective antibody production, multiple studies have demonstrated that disturbances in T and natural killer (NK) cell homeostasis likely contribute to the disease etiology and pathophysiology. Specifically, decreased circulating NK-cell numbers in CVID and XLA patients were found to be associated with severe bacterial infections and granulomas (51, 52). Pronounced CD4 T-cell lymphopenia occurs in some CVID patients (44, 53), and reductions in naive CD4 T cells were associated with splenomegaly (53), autoimmunity, and polyclonal lymphoproliferation. Reductions in naive CD8 T-cell numbers have also been associated with autoimmunity in CVID patients (54). In addition, follicular T helper cells

**Abbreviations:** CT, cortisone; CVID, common variable immunodeficiency; HGG, hypogammaglobulinemia; IGSCD, IgG subclass deficiency; IgRT, immunoglobulin replacement therapy; MS, multiple sclerosis; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SpAD, specific polysaccharide antibody deficiency; XLA, X-linked agammaglobulinemia.

(Tfh), regulatory T cells (Treg), and Th17 cell disturbances have been identified in PAD patients. In particular, XLA patients who lack B-cell follicles have reduced numbers of circulating Tfh cells (54), whereas in CVID patients with autoimmunity and/or splenomegaly, increased proportions of circulating Tfh have been observed (37, 55–57). Furthermore, reduced absolute numbers (54) and frequencies of Treg (58, 59) have been described in CVID patients with autoimmunity and/or splenomegaly, and these reductions are associated with the expansion of CD21<sup>lo</sup> B cells (59). Th17 cells were also decreased in number and frequency in CVID, paralleled by expansions of CD21<sup>lo</sup> B cells and activated CD4 T cells with no link to clinical manifestations (60). Finally, interleukin (IL)-2 and interferon gamma (IFN- $\gamma$ ) production by CD4 T cells was higher in patients with hepatomegaly, and chemokine receptor CCR5 known to be expressed on Th1 cells was shown to be higher on CD4 T cells in patients with granulomas (61), implying Th1 bias in these patients.

The many published abnormalities in circulating B and T cells, with several potentially correlating with specific clinical phenotypes, stress the need for high-quality and reproducible immunophenotyping of circulating lymphocytes in PAD patients. The EuroFlow consortium has developed standards for instrument setup and sample preparation (62) and multicolor panels to examine PAD patients (63, 64). We here utilized this expertise to develop a compact 11-color flow cytometry panel that was applied to our cohort of 62 adult PAD patients to identify abnormalities in the B- and T-cell compartments, specifically aiming to discriminate between patients with and without NICs.

## MATERIALS AND METHODS

### Patients

From November 2015 to June 2019, 62 adult patients with a clinical diagnosis of PAD were enrolled in a low-risk research study to examine their blood leukocyte subsets (projects Alfred Health 109/15 and Monash University CF15/771-2015-0344). All patients consented to the collection of their medical information and a donation of 40 ml of blood. In parallel, 59 adult healthy controls were enrolled in a low-risk reference value study (Monash University project 2016-0289) and consented to collection of basic demographics (age, sex, and history of immunological and hematological diseases) and donation of 40 ml of blood. The study was conducted according to the principles of the Declaration of Helsinki and was approved by local human research ethics committees.

### Assessment of Absolute Numbers of Leukocyte and Lymphocyte Subsets

Absolute numbers of leukocytes were determined using a lyse-no-wash method within 24 h of blood sampling in Vacutainers containing EDTA (BD Biosciences). Fifty microliters of whole blood was added to a TruCount tube (BD Biosciences) together with an antibody cocktail of 20  $\mu$ l to stain CD3, CD4, CD8, CD16, CD45, and CD56 (**Supplementary Figure 1** and **Supplementary Tables 1, 2, 5**). Following incubation for 15 min

at room temperature, 500  $\mu$ l of 0.155 M NH<sub>4</sub>Cl was added to lyse red blood cells for 15 min. Subsequently, the mixture was stored in the dark at 4°C prior to acquisition on a flow cytometer within 2 h.

### Design of Three Multicolor Tubes for Staining B- and T-Cell Subsets

The design of the tubes was based on the EuroFlow PID antibody panels (64), which had undergone stringent testing and optimization in a multi-laboratory setting. In contrast to the EuroFlow PID initiative, we undertook a research study of adult patients with the intent to examine all cell subsets in all enrolled individuals. Hence, the PID orientation tube (PIDOT) (63, 65) was not included, as the data obtained would be redundant with subsequent lineage-specific analysis. In addition, we had access to 11 fluorescent parameters. This enabled us to merge the EuroFlow pre-GC and post-GC tubes, which share six of the eight markers into one B-cell tube with 12 markers in 10 fluorescent channels (**Supplementary Figure 2** and **Supplementary Tables 1–3**). For eight of the 12 markers, the same reagents were used as in the eight-color EuroFlow panel (64). The same antibody clones were used for CD5, CD21, and CD38, with a different fluorescent label; and for CD19, different clone (SJ25C1) and fluorochrome were used.

Similarly, the EuroFlow SCID/RTE and T-cell tubes were combined into one 11-parameter T-effector tube. Of the 11 markers, seven were identical to those of the EuroFlow protocol (64). For CD3 and HLA-DR, the same clones were used on different fluorochromes, and for CCR7 (CD197), a different clone (G043H7) and another fluorochrome were used. CD45RA was newly inserted in this tube, as this is present in the EuroFlow PIDOT, whereas CD62L was not included owing to redundancy with the CCR7 marker (**Supplementary Figure 3** and **Supplementary Tables 1–3**).

Finally, an 11-color Th-subset tube was designed based on our previous work (66) with the objective to use membrane markers to distinguish Treg (67, 68), helper T-cell subsets (Th) (69, 70), and Tfh (71, 72), and their subsets (73) (**Supplementary Figure 4** and **Supplementary Tables 1–3**). This resulted in a panel of three tubes (B cell, T effector, and T helper) in addition to the TruCount analysis (**Supplementary Table 1**), and these were run for all patients and controls enrolled in the study.

### Sample Preparation for B-Cell and T-Cell Subset Tubes

To gain detailed insight into the composition of the lymphocyte compartment, including robust identification of small cell populations such as plasma cell subsets, standard operating procedures (SOPs) from the EuroFlow consortium were adopted for acquisition of high cell numbers ( $1\text{--}5 \times 10^6$  total nucleated cells) (74–76). The bulk-lysis-stain technique was performed (62, 74). Briefly, samples (up to 2 ml) were diluted in a total volume of 50 ml of an NH<sub>4</sub>Cl hypotonic solution, gently mixed, and incubated for 15 min at room temperature on a roller. Then, nucleated cells were centrifuged and washed twice in phosphate-buffered saline (PBS) containing 0.5% bovine

**TABLE 1** | Demographics, clinical details, and diagnostic results of the patients in this study.

	Healthy controls (n = 59)	All patients (n = 62)	PAD-NIC (n = 23)	PAD+NIC (n = 30)	XLA (n = 9)
<b>DEMOGRAPHICS</b>					
Median age (years; range)					
At inclusion	30 (20–71)	43 (18–82)	46 (19–73)	44 (23–82)	24 (18–59)
At diagnosis	N/A	35 (2 months to 74 years)	45 (18–73)	36 (12–74)	9 (2 months to 13 years)
Female sex (%)	33 (58%)	34 (55%)	14 (61%)	20 (67%)	0
Clinical diagnosis					
Agammaglobulinemia (%)	0	10 (16%)	1 (4%)	0	9 (100%)
CVID (%)	0	32 (52%)	9 (39%)	23 (77%)	0
HGG (%)	0	13 (21%)	7 (30%)	6 (20%)	0
IGSCD (%)	0	1 (2%)	1 (4%)	0	0
SpAD (%)	0	6 (9%)	5 (22%)	1 (3%)	0
<b>IMMUNOLOGICAL PRESENTATION</b>					
Decreased serum immunoglobulin levels					
IgG (%)	N/A	40/54 (74%)	14/23 (61%)	26/30 (87%)	0/1 <sup>#</sup>
IgA (%)	N/A	46/61 (75%)	15/23 (65%)	24/30 (80%)	8/8 (100%)
IgM (%)	N/A	34/61 (56%)	11/23 (48%)	15/30 (50%)	8/8 (100%)
Impaired vaccination responses (%)	N/A	25/30 (83%)	12/16 (75%)	12/14 (86%)	N/A <sup>#</sup>
Reduced cell numbers					
B cells (%)	N/A	24 (39%)	3 (13%)	12 (40%)	9 (100%)
T cells (%)	N/A	17 (27%)	5 (22%)	11 (37%)	2 (22%)
<b>TREATMENT</b>					
IgRT at sampling (%)	N/A	46 (74%)	11 (48%)	25 (83%)	9 (100%)
IgRT started after inclusion (%)	N/A	12 (19%)	7 (30%)	5 (17%)	N/A
Immunomodulators* (%)	N/A	8 (13%)	3 (13%)	4 (13%)	1 (11%)

Reference ranges: IgG, 6.1–16.2 g/L; IgA, 0.85–4.99 g/L; IgM, 0.35–2.42 g/L; B cells, 97–614 cells/ $\mu$ L; T cells, 830–2,430 cells/ $\mu$ L.

(B- and T-cell reference ranges were derived from the 5th and 95th percentiles of our healthy controls).

<sup>#</sup>Serum IgG levels and vaccination responses not assessed owing to historic nature of disease.

\*On immunomodulators within 6 months prior to blood sampling.

PAD, predominantly antibody deficiency; NIC, non-infectious complications; XLA, X-linked agammaglobulinemia; CVID, common variable immunodeficiency; HGG, hypogammaglobulinemia; IGSCD, IgG subclass deficiency; SpAD, specific polysaccharide antibody deficiency; IgRT, immunoglobulin replacement therapy; IGSCD, IgG subclass deficiency. A subset of patients has been reported in a recent publication (77).

serum albumin (BSA). Subsequently, the surface membrane markers on nucleated cells were stained with the corresponding antibody mixtures.

## Flow Cytometer Setup

All flow cytometry was performed across three instruments in our flow core facility that contained either four lasers (BD LSRII and BD LSRFortessa) or five lasers (BD LSRFortessa X-20) with a nearly identical setup for the shared four lasers (Supplementary Table 4). Instrument setup and calibration were performed using EuroFlow SOPs as previously described in detail (Supplementary Table 5) (62), with in-house optimization for the additional three fluorescent channels (V610, V710, and YG610).

## Data Analysis and Statistics

All data were analyzed with FACS DIVA v8.0.1 (BD Biosciences) and FlowJo v10 software packages (FlowJo, LLC). Reference ranges were defined as being within the 5th and 95th percentiles of absolute cell numbers from our 59 adult controls. Statistical

analysis for multiple-group comparison was performed with the non-parametric Kruskal-Wallis test. If significant, pairwise comparisons were made with the non-parametric Mann-Whitney *U* test. Statistical analysis of sampling distributions was assessed with the chi-square test. For all tests,  $p < 0.05$  was considered significant.

## RESULTS

### Clinical and Immunological Features of Predominantly Antibody Deficiency Patients

Sixty-two PAD patients were recruited in a prospective research study from a teaching hospital in Melbourne, Australia. Median age of the patients was 43 years (range, 18–82 years), and 34 were female (Table 1). CVID was the most common clinical diagnosis in 52% of all patients, followed by 21% with HGG, 16% with agammaglobulinemia, 9% with SpAD, and 2% with IGSCD. Of the 10 patients diagnosed with agammaglobulinemia,

**TABLE 2** | Complications in patients with predominantly antibody deficiency.

	All patients (n = 62)	PAD–NIC (n = 23)	PAD+NIC (n = 30)	XLA (n = 9)
<b>INFECTIOUS COMPLICATIONS</b>				
URTI (%)	49 (79%)	17 (74%)	24 (80%)	8 (89%)
Sinusitis (%)	48 (77%)	16 (70%)	24 (80%)	8 (89%)
Otitis (%)	20 (32%)	7 (30%)	7 (23%)	6 (67%)
LRTI (%)	49 (79%)	17 (74%)	25 (83%)	8 (89%)
Bronchitis (%)	8 (13%)	4 (17%)	4 (13%)	0
Pneumonia (%)	38 (61%)	12 (52%)	20 (67%)	7 (78%)
Bronchiectasis (%)	17 (27%)	4 (17%)	8 (27%)	5 (56%)
Gastrointestinal (%)	4 (6%)	0	4 (13%)	0
Giardia (%)	4 (6%)	0	4 (13%)	0
Other sites*	8 (13%)	1 (4%)	6 (20%)	1 (11%)
<b>NON-INFECTIOUS COMPLICATIONS</b>				
GLILD (%)	1 (2%)	0	1 (3%)	0
Autoimmunity (total) (%)	18 (29%)	0	18 (60%)	0
Musculoskeletal (%)	5 (8%)	0	5 (17%)	0
Cytopenia (%)	6 (10%)	0	6 (20%)	0
Endocrine (%)	3 (5%)	0	3 (10%)	0
Splenomegaly (%)	4 (6%)	0	4 (13%)	0
Lymphadenopathy (%)	1 (2%)	0	1 (3%)	0
Gastrointestinal disease total (%)	11 (18%)	0	11 (37%)	0
Enteropathy (%)	10 (16%)	0	10 (33%)	0
Colitis (%)	2 (3%)	0	2 (7%)	0
Granulomatous disease (%)	3 (5%)	0	3 (10%)	0
Malignancy (%)	2 (3%)	0	2 (7%)	0
Solid Organ (%)	2 (3%)	0	2 (7%)	0
Hematological (%)	0	0	0	0 <sup>#</sup>

PAD, predominantly antibody deficiency; PAD–NIC, PAD without non-infectious complications; PAD+NIC, PAD with non-infectious complications; XLA, X-linked agammaglobulinemia; GLILD, granulomatous-lymphocytic interstitial lung disease.

\*Other includes osteomyelitis, pertussis, prostatitis, and systemic viral infection.

<sup>#</sup>One XLA patient developed an acute precursor-B-cell leukemia 6 months after inclusion in this study (78).

nine were male and genetically confirmed to have XLA (**Table 1** and **Supplementary Tables 6, 7**). The other 53 patients did not undergo any genetic testing.

All patients presented with infectious manifestations (**Table 2** and **Supplementary Table 6**), and these were generally confined to the respiratory tract: sinusitis (77%), pneumonia (61%), and otitis (32%) with frequent complications of bronchiectasis (27%). Infections of the gastrointestinal tract and other sites, such as the prostate and bone, were less frequently involved (19%). Of all 62 patients, 30 (48%) presented with at least one NIC, with autoimmunity being the most frequent (29%), followed by gastrointestinal (18%), and granulomatous diseases (5%; **Table 2**). At the time of inclusion in the study, 46 (74%) patients were treated with IgRT. A further 12 (19%) newly diagnosed patients commenced IgRT directly after inclusion. Eight patients (13%) had been prescribed immunomodulators within 6 months prior to inclusion (**Table 1**). Three patients were treated with immunomodulators for asthma or for IgRT tolerability, which were deemed unrelated to their PAD, and thus, these patients were defined as PAD–NIC.

Serum IgG levels prior to commencement of IgRT were obtained from medical records of 47 patients (**Table 1**), and in 40/47 (85%), these were below the normal range. Serum IgA and IgM concentrations were available for 61 patients, and of these, 46 (75%) had reduced IgA and 34 (56%) had reduced IgM. The results of vaccination responses in most cases to polysaccharide pneumococcal vaccine were documented for 30 patients and impaired in 25 (83%). B- and T-cell numbers were below the normal range (<5th percentile of our healthy control cohort) for 39 and 27% of all patients, respectively. A total of 11% of patients had a reduction of both B- and T-cell numbers (**Table 1**). B-cell numbers were below the normal range for all nine XLA patients, with two patients (22%) also having reduced T-cell numbers.

For further immunological analysis, the PAD patient cohort was divided into three groups: 9 patients with genetically diagnosed XLA, 23 PAD patients without NICs (PAD–NIC), and 30 with NICs (PAD+NIC). The nine XLA patients presented with infectious complications only, and these included respiratory infections (89%). Five XLA patients (56%) had evidence of bronchiectasis; this incidence was 2-fold higher than in other PAD groups (17% in PAD–NIC; 27% in PAD+NIC). Of the 32 CVID patients, 23 (72%) presented with one or more NICs (**Table 2**), whereas only a minority of patients diagnosed with HGG or SpAD presented with NIC. By definition, PAD+NIC patients displayed a more severe and complex clinical phenotype (**Table 2**) and were diagnosed at a younger age than were PAD–NIC patients (median age at diagnosis: 3 vs. 45 years).

All classifications of patients were undertaken alongside those of 59 healthy controls (median age 30 years and 55% females). The healthy controls and the non-XLA PAD patients were classified according to the Freiburg and EUROclass definitions (47, 79) on the basis of their B-cell phenotypes (**Table 3**). According to the Freiburg classification, 95% of controls had normal frequencies of smBs and no increases in CD21<sup>lo</sup> B cells (group II), with the remaining 5% having low smB (Ib). The majority of all PAD patients (29; 55%) had reduced smB frequencies (Ia/Ib;  $p < 0.0001$  vs. controls). Seven PAD patients had increased frequencies of CD21<sup>lo</sup> B cells (Ia), and the majority of these patients ( $n = 5$ ) were in the PAD+NIC group. According to the EUROclass scheme, all controls had normal smB and CD21<sup>lo</sup> B-cell frequencies (**Table 3**). Of all PAD patients, 12 (22%) had reduced smB frequencies and 13 (25%) had increased CD21<sup>lo</sup> B-cell frequencies. Slightly more PAD+NIC patients had reduced smB and increased CD21<sup>lo</sup> B cells than had PAD–NIC, but these differences were not significant (CD21<sup>lo</sup> expansion,  $p = 0.06$ ).

Overall, our patient cohort is diverse in clinical and immunological presentations, in line with previously reported cohorts of adult PAD (38, 44). Importantly, PAD–NIC and PAD+NIC groups are seemingly different in their immunological profiles. With almost equally large PAD–NIC and PAD+NIC groups and a substantial group of genetically diagnosed XLA patients, this cohort is well-suited to examine immunological differences that associate with the presence of NICs.

**TABLE 3** | Classification of PAD patients according to the Freiburg and EUROclass definitions.

	Healthy controls (n = 59)	All PAD <sup>a</sup> (n = 53)	PAD-NIC (n = 23)	PAD+NIC (n = 30)
<b>FREIBURG</b>				
Ia (smB-21lo)	0	7 (13%)	2 (9%)	5 (16%)
Ib (smB-21norm)	3 (5%)	22 (42%)	8 (36%)	14 (47%)
II (smB+21lo or norm)	56 (95%)	24 (45%)	13 (55%)	11 (37%)
<b>EUROCLASS</b>				
B-	0	3 (6%)	2 (9%)	1 (3%)
B+smB-21normTrnorm	0	5 (9%)	3 (13%)	2 (7%)
B+smB-21normTrhi	0	1 (2%)	0	1 (3%)
B+smB-21loTrnorm	0	5 (9%)	0	5 (17%)
B+smB-21loTrhi	0	1 (2%)	0	1 (3%)
B+smB+21norm	59 (100%)	31 (58%)	15 (65%)	16 (53%)
B+smB+21lo	0	7 (14%)	3 (13%)	4 (14%)

PAD, predominantly antibody deficiency; NIC, non-infectious complications; smBs, switched memory B cells, 21, CD21; Tr, transitional B cells.

Freiburg classification undertaken on patients with  $\geq 1\%$  B cells in lymphocyte gate; definitions (79): smB-,  $<0.4\%$  CD28<sup>+</sup>IgD<sup>-</sup> B cells within lymphocytes; 21lo,  $\geq 20\%$  B cells are CD21<sup>lo</sup>.

EUROclass definitions (47): B-,  $<1\%$  of lymphocytes; smB-,  $<2\%$  of B cells; 21<sup>lo</sup>,  $\geq 10\%$  B cells are CD21<sup>lo</sup>; Trhi,  $>9\%$  of B cells are transitional (CD38<sup>hi</sup>CD27<sup>-</sup>).

<sup>a</sup>Excluding patients with a clinical diagnosis of X-linked agammaglobulinemia (XLA), as these were all B cell negative.

## Reduced Numbers of Lymphocytes in Predominantly Antibody Deficiency Patients

As a large fraction of the non-XLA PAD patients had B- and/or T-cell numbers below the normal range of controls (Table 1), we first examined the numbers of leukocyte and lymphocyte subsets in our patient groups (Supplementary Figures 1, 5 and Supplementary Table 3). Circulating numbers of granulocytes and monocytes in both PAD-NIC and PAD+NIC were similar to those of controls, whereas these were significantly increased in XLA patients (Supplementary Figures 5A,B). In contrast, total lymphocytes, and NK cells were significantly reduced in both PAD groups as compared with controls, whereas these were normal in the XLA group (Supplementary Figures 5C,D).

## Reduced Total, Naive, and IgM Memory B Cells in Predominantly Antibody Deficiency Patients With Non-infectious Complications

Further detailed analysis of the B-cell compartment was restricted to the 59 controls and the 53 non-XLA PAD patients, as B cells were completely absent in the XLA patients (Figure 1A). Numbers of circulating B cells were significantly reduced in the PAD+NIC, but not in the PAD-NIC group (Figure 1B). Within the peripheral B-cell compartment, frequencies of IgM memory, Ig switched memory, and plasmablasts were significantly lower in both PAD-NIC and PAD+NIC compared with controls (Figure 1C). Frequencies of naive B cells were increased in both patient groups.

However, when expressed as absolute numbers, these were actually normal in PAD-NIC and significantly reduced in PAD+NIC (Figures 1C,D and Supplementary Figure 6A). IgG and IgA smBs remained reduced in terms of absolute numbers in both PAD groups. In contrast, absolute numbers of IgM memory B cells were normal in PAD-NIC whereas reduced in PAD+NIC (Figures 1C,D and Supplementary Figures 6A,B). Finally, CD21<sup>lo</sup> B-cell frequencies were increased in the PAD+NIC group, but the absolute numbers of circulating CD21<sup>lo</sup> B cells were normal (Figure 1C).

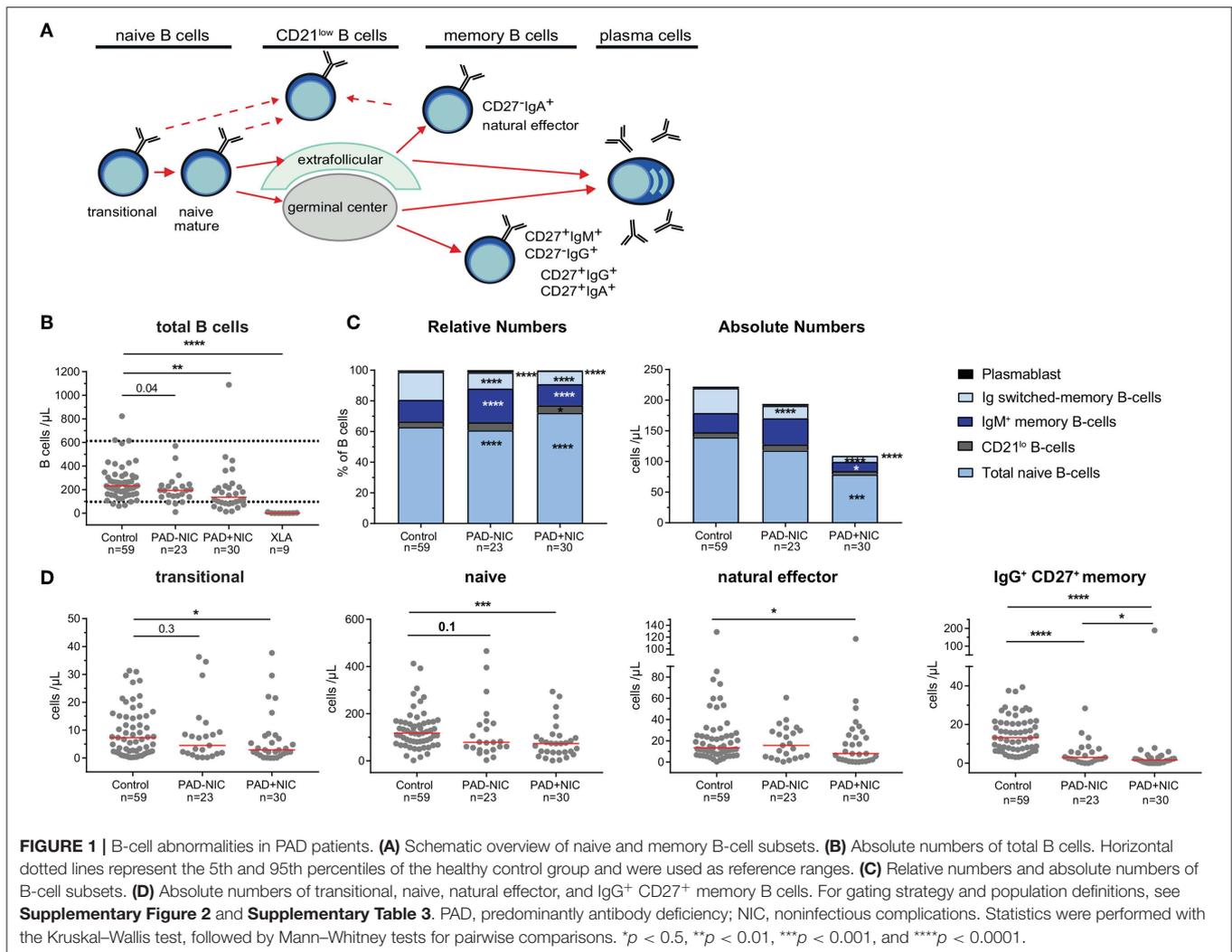
Taken together, both PAD-NIC and PAD+NIC have severely reduced numbers of Ig smBs. In addition, as a group, PAD+NIC patients have reduced numbers of circulating total, naive, and IgM memory B cells.

## Reduced Naive CD4 and CD8 T Cells in Predominantly Antibody Deficiency Patients

In addition to the lymphocyte, NK-cell, and B-cell abnormalities, the PAD-NIC and PAD+NIC groups had significantly lower numbers of circulating T cells than had controls. These concerned all three major lineages: TCR $\gamma\delta$  T cells in PAD-NIC and both CD4 and CD8 T cells in both PAD groups. In contrast, only two XLA patients had reduced total T-cell numbers (Figure 2A).

To examine the nature of the T-cell abnormalities, we delineated the CD4 and CD8 lineages into CCR7<sup>+</sup>CD45RO<sup>-</sup> naive (Tn), CCR7<sup>+</sup>CD45RO<sup>+</sup> central memory (Tcm), CCR7<sup>-</sup>CD45RO<sup>+</sup> effector memory (TemRO), and CCR7<sup>-</sup>CD45RO<sup>-</sup> effector memory (TemRA) T cells (Supplementary Figure 3 and Supplementary Table 3). Frequencies and absolute numbers of naive CD4 and CD8 T cells were reduced in both PAD-NIC and PAD+NIC, but not in XLA patients (Figures 2B,C). To examine the association between naive CD4 and naive CD8 T-cell counts, we performed correlation analysis of these in both the healthy control and the patient cohorts. This revealed a reasonable positive correlation for controls ( $r = 0.32$ ;  $p < 0.05$ ) and strong correlation for total group of 53 non-XLA PAD patients ( $r = 0.63$ ;  $p < 0.0001$ ; Figure 3A). As PAD+NIC patients also had reduced naive B cells, we examined their association with naive CD4 and CD8 T cells. No correlations were found in the control group, but naive B-cell numbers were positively correlated with naive CD4 T cells ( $r = 0.53$ ;  $p < 0.0001$ ) and naive CD8 cells ( $r = 0.48$ ;  $p < 0.0001$ ; Figures 3B,C).

PAD+NIC had increased frequencies of CD4 Tcm and TemRO, but as a result of the reduced total CD4 T cells, these were normal in terms of numbers (Figure 2B). Early-, intermediate-, and late-differentiated TemRO and TemRA cells were distinguished by the progressive loss of CD27 and/or CD28 (80) (Supplementary Figure 3). Within the CD4 TemRO compartment, numbers of early-differentiated cells were increased in PAD-NIC and XLA whereas reduced in PAD+NIC. Intermediate-TemRO cell numbers were reduced in PAD-NIC, and late-TemRO numbers were increased in XLA (Supplementary Figure 7A). Intermediate-TemRA



numbers were reduced in PAD+NIC, but proportions were unchanged (**Supplementary Figure 7B**). CD8 TemRO numbers were not different between patients and controls (**Supplementary Figure 7C**), whereas CD8 TemRA early and intermediate cell subsets were significantly reduced in all three patient groups (**Supplementary Figure 7D**).

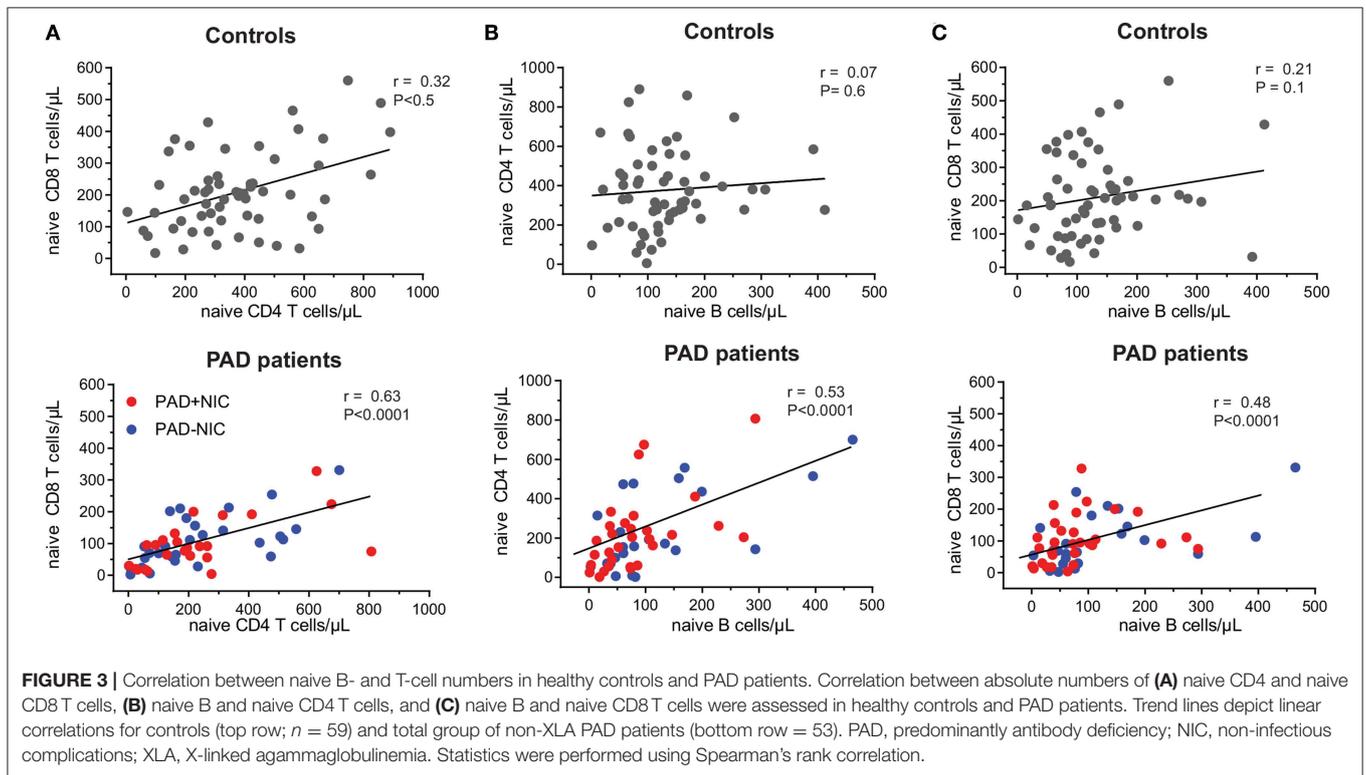
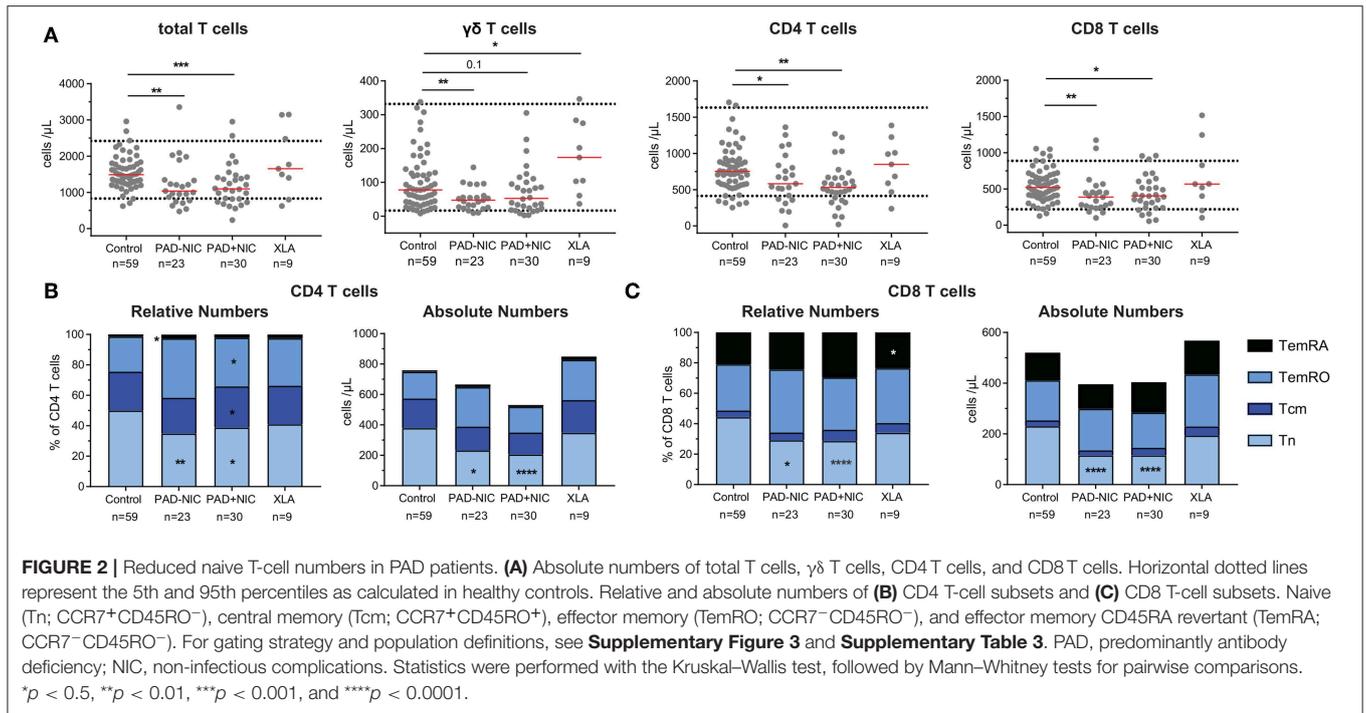
Overall, both PAD–NIC and PAD+NIC patients exhibit a marked reduction in circulating T cells, mainly as a result of reductions in the naive CD4 and CD8 T-cell subsets.

## Altered Treg and Th Cell Composition in Predominantly Antibody Deficiency Patients With Non-infectious Complications

To analyze whether Th numbers were altered in PAD patients with and without NIC, we delineated Treg, Th, Tfh, and their subsets (**Supplementary Table 3** and **Supplementary Figure 4**). Total Treg numbers and their respective naive and memory

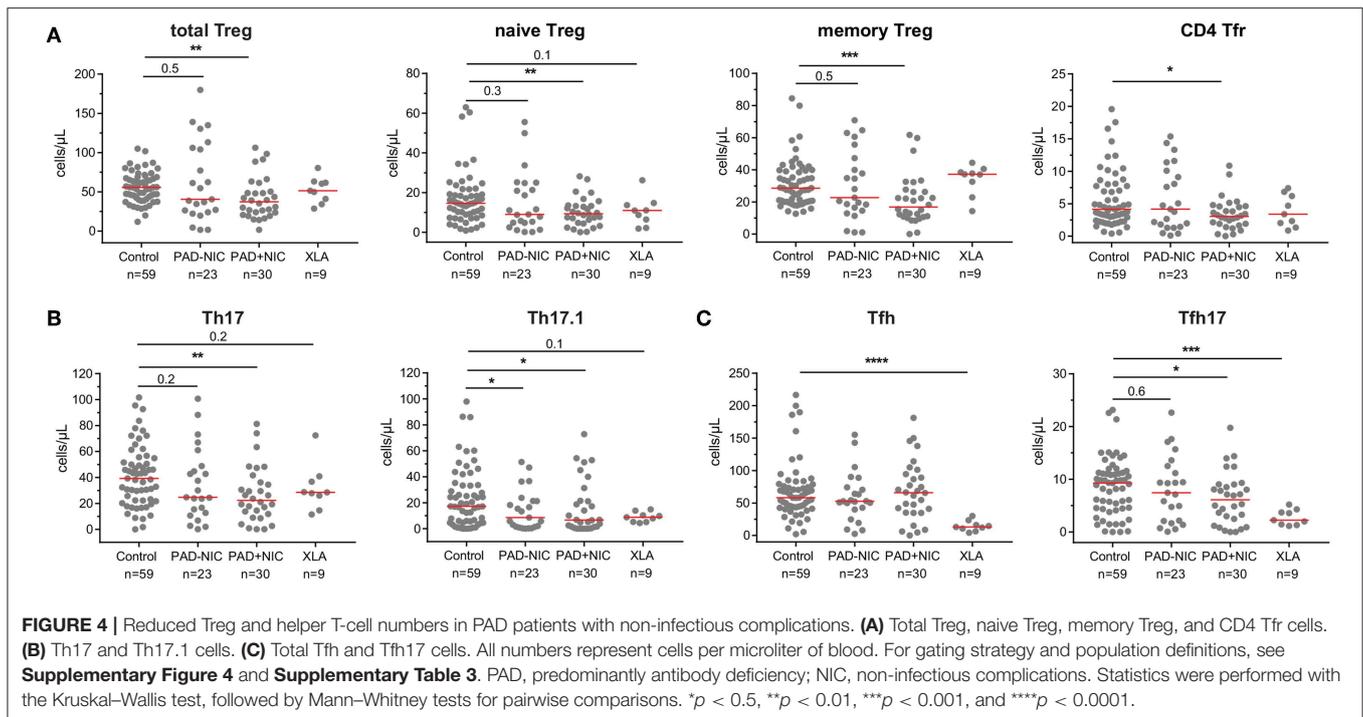
subsets were significantly lower in PAD+NIC than in controls, whereas these were not changed in PAD–NIC and XLA patients (**Figure 4A**). The subset of follicular regulatory T(fr) cells was also specifically decreased in PAD+NIC patients (**Figure 4A**). Within the Th cells, four subsets were defined (i.e., Th1, Th2, Th17, and Th17.1), which are each associated with responses to distinct types of pathogens. No alterations were observed for Th1 (bacterial and viral pathogens) or Th2 cells (extracellular pathogens; data not shown). In contrast, Th17-cell numbers (bacterial and fungal pathogens) were specifically reduced only in PAD+NIC as compared with controls (*p* = 0.005). Finally, numbers of Th17.1 cells (IL-17 and IFN- $\gamma$  double producers) were reduced in both PAD–NIC and PAD+NIC, but not in XLA patients (**Figure 4B**).

Finally, we enumerated Tfh numbers, as these are critical for providing help to B cells in germinal center responses. Absolute numbers of Tfh cells were not different between controls, PAD–NIC, and PAD+NIC, whereas these were significantly reduced in XLA patients (*p*  $\leq$  0.0001). Within the total Tfh population, a similar distinction of four subsets was made as for the Th



subsets: Tfh1, Tfh2, Tfh17.1, and Tfh17 (73). Of these, only Tfh17 was significantly reduced in the PAD+NIC and XLA groups as compared with controls (**Figure 4C**).

Taken together, the CD4 T-cell compartment was most severely affected in PAD+NIC patients with significantly reduced Treg, Th17, Th17.1, and Tfh17 numbers.



## Misinterpretation of Relative Numbers in the Context of Reduced Absolute Cell Numbers

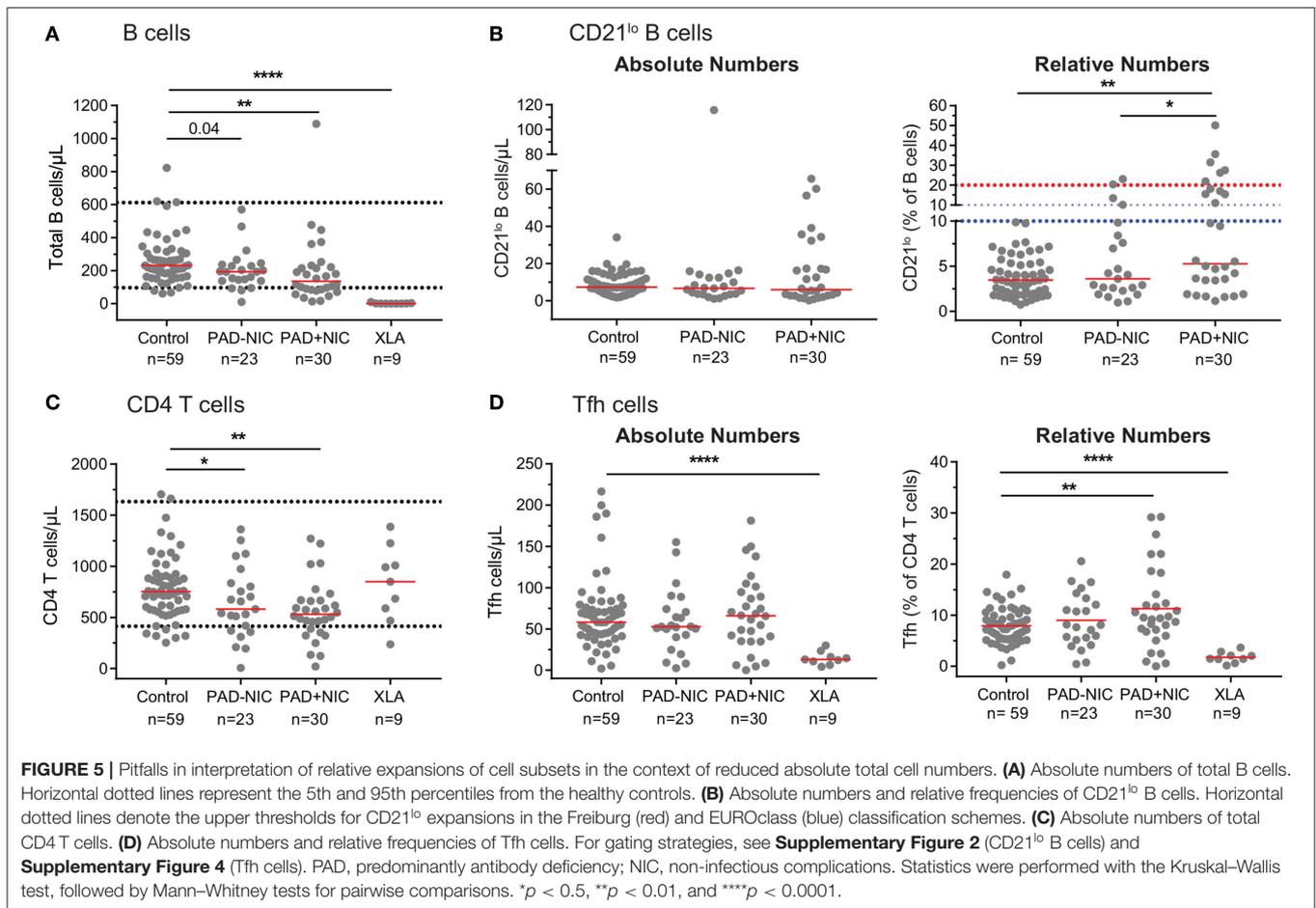
Similar to previous reports, we observed in our cohort that PAD+NIC patients had a significantly increased proportion of CD21<sup>lo</sup> B cells (**Figures 1B, 5**). However, when expressed as absolute numbers of cells per microliter blood, CD21<sup>lo</sup> B cells were normal in our cohort (**Figure 5B**). In fact, the increased proportion of CD21<sup>lo</sup> B cells was the result of a reduction in total B-cell numbers in the PAD+NIC group. Thus, the increased proportion of CD21<sup>lo</sup> B cells did not reflect an increase in this subset but rather a decrease of other, mainly naive B cells. In parallel, we confirmed previous reports (37, 55, 56, 81) of increased proportions of Tfh in the PAD+NIC group (**Figure 5**). However, absolute numbers of Tfh were normal in the context of reduced total CD4 T cells (**Figures 5C,D**). Thus, the relative expansion does not reflect an abnormality in Tfh but rather is the result of abnormally low total T cells, mainly due to the reduction in the naive subset.

## DISCUSSION

Here we present the results of a standardized approach for extensive immunophenotyping of the B- and T-cell compartments in patients with PAD. Irrespective of the presence of NIC, PAD patients show reduced Ig smBs, Th17.1 cells, and naive CD4 and CD8 T cells. In addition, the group of patients with NICs has severe reductions in total B-cell, Th17, Treg, and Tfh17 numbers. Finally, expansions of CD21<sup>lo</sup> B-cell and Tfh-cell frequencies are the product of reduced total B-cell and

T-cell numbers, and not of absolute increases. Thus, our results demonstrate the importance of structured immunophenotypic analysis with the inclusion of absolute cell numbers to delineate affected cell types in PAD patients with NICs.

We here examined a cohort of 62 PAD patients. These included nine genetically diagnosed XLA patients, who were all diagnosed in early childhood and, prior to inclusion into our study, suffered from infectious complications only. Although the XLA group was by far the smallest, it does represent a homogenous group of patients with a B-cell intrinsic defect to be used as patient controls for our PAD–NIC and PAD+NIC immunophenotyping. The vast majority of the other PAD patients were diagnosed with CVID (32; 52%) or HGG (13; 21%). This is in line with previous reports from teaching hospitals and is most likely reflective of the skewed population seen in tertiary care (36, 38, 44, 49). In our cohort, the most frequent infections were of the respiratory tract (13%, upper respiratory tract infection [URTI] only; 21% lower respiratory tract infection [LRTI] only; 66% URTI plus LRTI), with the most prevalent NIC being autoimmunity (60%) (38, 44, 82–85), which is reflective of other clinical studies. Furthermore, the significantly higher incidence of NIC in patients with a CVID diagnosis (72%) vs. all other non-XLA PAD (35%;  $p = 0.01$ ) is similar to that of previous reports (36, 38, 44, 83). In terms of patient numbers, it falls short of multi-institute cohort analyses of clinical and basic immunological features (38, 44, 83, 86). However, our cohort of 62 patients relates well to other immunophenotyping studies (37, 46, 48, 51–53, 79, 87) and is larger than most studies (37, 46, 55, 58–61, 79). Moreover, the division into almost equally sized groups of PAD–NIC ( $n = 23$ ) and PAD+NIC ( $n = 30$ ) and a genetically defined XLA patient control group ( $n = 9$ )



is well-suited for examination of common and NIC-associated abnormalities. The high prevalence of NICs in adult PAD patients in our study likely associates with later onset of symptoms (86) and delayed diagnosis in these patients (38, 44). The clinical and immunological presentations of our patient cohort are diverse, in line with previously published studies. Thus, this cohort is highly representative for the in-depth analysis of immunological differences that segregate with the presence of NICs.

In our study, reduced B-cell numbers segregated with the presence of NICs. Further in-depth examination of the B-cell compartment revealed significant reductions in Ig smBs and plasmablasts in both PAD groups, which was in line with a recent PAD study (48). However, unlike previous reports (37, 47), in our cohort, these abnormalities did not segregate with NICs such as splenomegaly, granulomatous disease, and autoimmunity. In contrast, we observed specific reductions in serum IgM levels and IgM memory B-cell numbers in PAD+NIC patients. As IgM has a role in preventing autoimmunity by promoting phagocytic clearance of cell debris including autoantigen (88, 89), it is possible that this defect contributes to the autoimmune pathophysiology in patients with PAD+NIC.

In addition to the reduced memory B cells, naive B cells were significantly reduced in the PAD+NIC group. As naive B cells form the largest proportion of total B cells, this reduction is

mostly responsible for the reduced total B-cell numbers. The cause of reduced B-cell numbers is unclear and could be related to either reduced production from bone marrow precursors or reduced survival in the periphery. Gene defects underlying either of these processes have been reported in patients with an antibody deficiency syndrome (2). Importantly, the reductions in naive B cells correlated with reduced naive CD4 and CD8 T cells (see *Discussion* below) and could be part of a more general lymphocyte production and/or survival defect that contributes to disease pathology. Finally, the reduction in naive B-cell numbers highlights the importance of measuring absolute cell numbers, as this will affect proportions of other cell subsets (e.g., CD21<sup>lo</sup> B cells; see below) and lead to misinterpretation of indirect findings.

We here show that the expansion of CD21<sup>lo</sup> B cells, which is reportedly associated with autoimmunity and splenomegaly in CVID patients (46, 47), was only observed in PAD+NIC patients when presented as frequency of total B cells, but not in terms of absolute cell numbers. Expansion of this B-cell subset has also been identified in numerous chronic infections and autoimmune and granulomatous diseases. Specifically, increased frequencies and numbers of CD21<sup>lo</sup> B cells have been observed in Crohn's disease (90), Sjögren's syndrome (91), and poor HIV controllers (92). In addition, increased CD21<sup>lo</sup> B-cell frequencies have also

been identified in rheumatoid arthritis (RA) (93, 94), scleroderma (94), systemic lupus erythematosus (SLE) (95), and multiple sclerosis (MS) (96). Similar to PAD+NIC, absolute numbers of CD21<sup>lo</sup> B cells are normal in RA (93), and the increased frequencies of CD21<sup>lo</sup> B cells were the result of reduced naive and memory B-cell numbers (93). This segregates PAD+NIC and RA from Crohn's disease, Sjögren's syndrome, and HIV in which the absolute numbers of CD21<sup>lo</sup> B cell are increased and might be indicative of distinct pathophysiologies.

Total CD4 and CD8 T-cell numbers were significantly lower in both PAD-NIC and PAD+NIC groups as a result of a reduction in the naive subsets. In contrast to other studies where naive T-cell deficiencies associated with NICs such as splenomegaly (53, 54), this link was not observed in our study. In addition, as memory T-cell numbers were unaffected, we postulate that decreased total CD4 and CD8 T-cell numbers are as a result of smaller numbers of naive T cells. Although PAD has long been considered to result from disturbances in B-cell homeostasis, it is becoming more evident that defective T-cell responses also play a role in disease pathogenesis. In particular, reductions in naive CD4 and CD8 T cells will likely impair immunity in primary infection and vaccination, thus rendering patients more prone to severe and often lethal infections, as shown in studies of aging and HIV infection (97–100).

Further dissection of the CD4 T-cell compartment in our cohort revealed abnormalities in Treg, Th, and Tfh cells. Treg-cell numbers were specifically low in PAD+NIC patients, in line with previous reports (54, 58, 59). This concerned both thymus-derived, naive Treg, and peripherally induced memory Treg. It is very tempting to speculate that the Treg deficiency contributes to the autoimmune pathology, similar to what has been proposed for patients with type 1 diabetes, MS, and SLE (101–106). Still, it remains unclear what causes the reduction and how this contributes to NIC. Potentially, the reduction is related to the reduced total naive T cells and might be reflective of either impaired production from the thymus, or increased maturation into effector memory cells as a result of the high infectious pressure in PAD patients.

Although the precise role of Th17.1 cells in immunity has not yet been fully elucidated, decreased Th17.1 counts have been linked to increased susceptibility to bacterial infection in other immunocompromised patients, including those with hyper-IgE syndrome and HIV/AIDS (107–112). In addition to the function of Th17 cells in controlling infections, these cells have been shown to promote antibody production by B cells (60). Thus, reductions in these cell numbers might contribute to the impaired antibody responses in PAD patients.

In line with previous reports, we observed increased frequencies of Tfh cells in patients with NIC (37, 55, 57). However, we also determined the absolute Tfh numbers and found that these were similar to those of controls. Thus, rather than a role of increased Tfh cells in CVID and autoimmunity (37, 55, 57, 113–115), it might be an altered distribution of Tfh subsets that contributes to disease pathology. Importantly, in PAD+NIC patients, the most potent B-cell helpers, Tfh17 cells, are significantly reduced. This could potentially link the

inefficient antibody responses to pathological B cell responses, driven by, for example, the other Tfh cell subsets.

In our study, we utilized membrane markers only for the delineation of Th, Tfh, and Treg cells. In literature, multiple phenotypic definitions have been used to assess Th cells (cytoplasmic IFN- $\gamma$ , IL-4, IL-10, and IL-17), Tfh cells (CD4<sup>+</sup>CXCR5<sup>+</sup>, CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>, or CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>), and Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo/-</sup> or CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) (71, 72, 116, 117). Although intracellular FoxP3 and cytokine expression represents the gold standard protocols for the delineation of Treg and Th subsets, respectively, these protocols are laborious and not easily amenable to high-throughput analyses. The surface markers utilized to define Treg and Th subsets in our panel have previously been demonstrated to correlate well with intracellular FoxP3 (116) and cytokine expression (117, 118). Therefore, we are convinced that the gating strategies we applied were highly specific. Without the need for *in vitro* activation and/or cytoplasmic staining, our protocol is more straightforward, making it quicker and more easily scalable for adoption in diagnostic laboratories.

Here, we have identified B- and T-cell biomarkers associated with NICs in patients with PAD. Humoral and cellular deficiencies have previously been associated with aging contributing to susceptibility to infection and NIC development including autoimmunity and cancer. This is particularly the case for declining numbers of naive B and T cells (74, 119–121). Thus, it could be suggested that PAD-NIC represent a precursor group whom with aging will develop NICs. However, the lower median age at diagnosis of the PAD+NIC group (25 vs. 45 years in PAD-NIC) suggests that it is more likely that the PAD-NIC and PAD+NIC patients suffer from distinct pathophysiologies.

CVID demonstrates intrinsic clinical, immunological, and genomic heterogeneity complicating diagnosis resulting in decreased overall survival rates (82, 122, 123). Over the past 5 years, criteria utilized to define CVID have been expanded to include total and naive CD4 T-cell quantification, in addition to the assessment of B-cell subsets and clinical parameters of disease. These updated criteria enable the exclusion of patients with combined immunodeficiency (CID) on the basis of demonstration of severe reductions in naive CD4 T cells. It was suggested that reduced proportions of naive CD4 T cells (<10% CD4 T cells) rather than decreased CD4 counts (<200 cells/ $\mu$ l) are more sensitive in the definition of CID, further highlighting the validity of simultaneously quantifying absolute and relative numbers in patient diagnostics and prognostication protocols. Utilizing the updated criteria, the authors redefined previously published patients on the basis of the updated criteria. Here, 2% patients previously defined as CVID were redefined as CID (122–124). Application of these updated criteria to our CVID group would redefine five (16%) patients as CID. The distinction between CVID and CID is extremely important, as a higher incidence of pneumonia, lymphoma, granulomas, autoimmunity, and enteropathy (2, 5, 123), in addition to lower B-cell and naive CD4 T-cell numbers, accounts for the lower 5-year survival rate in CID patients (123). Thus, unlike PAD patients where IgRT is sufficient for disease

management, some patients with CID will require life-saving stem cell transplantation. Hence, the utilization of our panel would aid in the identification and correct classification of patients who progress to CID, as well as PAD patients at risk of developing NICs. We do realize that the differences in cellular immunophenotypes observed in PAD patients with and without NIC will need to be verified in independent international PAD cohorts. Ideally, these markers should be analyzed longitudinally in early-diagnosed patients without NIC to validate if abnormalities will predict disease progression and development of NIC. Therefore, the immunophenotypic defects outlined here could guide genomic analysis of patients, to enable precise diagnosis and predictive prognostic information, as well as guide targeted patient treatment with the potential to limit diagnostic delay, as well as reduce the incidence of early mortality and high morbidity associated with NICs in these patients.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee Alfred Health. The patients/participants provided their written informed consent to participate in this study.

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## AUTHOR CONTRIBUTIONS

MZ, RO'H, and JB conceptualized the study and designed the experiments. EE, JB, PA, RS, PC, JC, FH-L, and MZ collected and interpreted the data. EE and PA performed the experiments. EE and MZ wrote the manuscript. All authors critically read, commented on, and approved the final version of the manuscript.

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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.2019.02593/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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