



Pediatric Burn Survivors Have Long-Term Immune Dysfunction With Diminished Vaccine Response

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OPEN ACCESS

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Specialty section:

This article was submitted to
Inflammation,
a section of the journal
Frontiers in Immunology

Received: 23 April 2020

Accepted: 08 June 2020

Published: 21 July 2020

Citation:

Johnson BZ, McAlister S,
McGuire HM, Palanivelu V,
Stevenson A, Richmond P, Palmer DJ,
Metcalfe J, Prescott SL, Wood FM,
Fazekas de St Groth B, Linden MD,
Fear MW and Fear VS (2020) Pediatric
Burn Survivors Have Long-Term
Immune Dysfunction With Diminished
Vaccine Response.
Front. Immunol. 11:1481.
doi: 10.3389/fimmu.2020.01481

Epidemiological studies have demonstrated that survivors of acute burn trauma are at long-term increased risk of developing a range of morbidities. The mechanisms underlying this increased risk remain unknown. This study aimed to determine whether burn injury leads to sustained immune dysfunction that may underpin long-term morbidity. Plasma and peripheral blood mononuclear cells were collected from 36 pediatric burn survivors >3 years after a non-severe burn injury (<10% total body surface area) and from age/sex-matched non-injured controls. Circulating cytokine and vaccine antibody levels were assessed using multiplex immunoassays and cell profiles compared using a panel of 40 metal-conjugated antibodies and mass cytometry. TNF- α (1.31-fold change from controls), IL-2 (1.18-fold), IL-7 (1.63-fold), and IFN- γ (1.18-fold) were all significantly elevated in the burn cohort. Additionally, burn survivors demonstrated diminished antibody responses to the diphtheria, tetanus, and pertussis vaccine antigens. Comparisons between groups using unsupervised clustering identified differences in proportions of clusters within T-cells, B-cells and myeloid cells. Manual gating confirmed increased memory T-regulatory and central memory CD4+ T-cells, with altered expression of T-cell, B-cell, and dendritic cell markers. Conclusions: This study demonstrates a lasting change to the immune profile of pediatric burn survivors, and highlights the need for further research into post-burn immune suppression and regulation.

Keywords: non-severe burn injury, immunity, vaccination, mass cytometry, acute trauma, systemic

INTRODUCTION

Burns continue to impact the lives of millions of people each year; from new injuries to ongoing recovery, the psychological, physical, and financial burden is persistent. In 2004 the World Health Organization (WHO) estimated that 11 million people globally required medical attention for a burn injury (1). A more recent annual report from the Burns Registry of Australia and New Zealand (BRANZ) recorded 3,295 cases treated at specialized burn clinics across the two countries

(2016–2017), with pediatric cases accounting for 30% of the cohort (2).

Patient outcomes are influenced by the severity of the burn injury (3, 4). Total body surface area (TBSA) involvement is used to classify burns as severe ($\geq 20\%$ TBSA) or non-severe ($< 20\%$ TBSA). Due to their profound local and systemic effects (5, 6), severe burns remain the focus of the majority of burns research. However, the majority of burns (84%) involve a TBSA of $< 10\%$ (2), and it is becoming increasingly apparent that even non-severe burns have a long-term impact on the health of survivors.

Epidemiological studies have found that burn survivors, regardless of severity, are at increased risk for a range of diseases even decades after injury, and typically have a longer length of stay when hospitalized for them. These include cardiovascular diseases (7, 8), nervous disorders (9), musculoskeletal diseases (notably infectious and inflammatory polyarthropathies) (10), cancers (11), diabetes mellitus (12), gastrointestinal diseases (13), and infections (14). Extensive data in the literature support a role for innate and adaptive immune cell dysfunction in the pathogenesis of the diseases that have an elevated incidence in burn survivors (15–19), suggesting immune dysfunction may contribute to post-burn morbidity.

In our laboratory pre-clinical studies in mice, modeling 8% TBSA involvement as a non-severe burn injury (NSBI), have demonstrated changes in innate and adaptive immunity up to 84 days post-injury (14, 20). In pediatric patients with severe burn injury, sustained elevation of circulating cytokines has been observed up to 3 years after the injury (21). In this study we have investigated whether there is an enduring change within the immune compartment of pediatric patients more than 3 years after a non-severe burn injury. We hypothesized that patients would manifest significant changes to the circulating immune profile compared to uninjured controls, reflecting a sustained impact of acute but non-severe burn trauma on the immune system.

MATERIALS AND METHODS

Specimen Collection

Children were recruited at least 3 years after presenting for a non-severe burn injury at Princess Margaret Hospital. They were aged 0–4 years of age at the time of original presentation. Age/sex-matched controls were selected from a pool of healthy donors. All samples were obtained with informed consent of a parent or guardian and the collection was conducted with ethical approval from the Child and Adolescent Health Service WA (approval numbers: 2015219EP; 1111EP; 768EP). All patients recruited had no history of pre-existing illness and were not currently on medication at time of sampling. No patients had visible signs or recent history of acute infection at the time of blood collection. Blood was collected into tubes containing preservative-free heparin, then centrifuged to collect the plasma. The remaining blood was resuspended in RPMI-1640 (Gibco, USA) and the peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (STEMCELL Technologies, Canada), and then cryopreserved in 10% DMSO upon slow freeze for storage.

Multiplex Cytokine Assay

Circulating cytokines were assessed using a customized Milliplex MAP human high sensitivity T cell panel multiplex bead assay (Merck). Cytokines tested were Tumor necrosis factor-alpha (TNF- α), Interleukin-8 (IL-8), Interleukin 7 (IL-7), Interleukin-6 (IL-6), Interleukin-5 (IL-5), Interleukin-2 (IL-2), Interleukin-1beta (IL-1 β), Interleukin-17A (IL-17A), Interleukin-13 (IL-13), Interleukin-12 p70 (IL-12(p70)), Interleukin-10 (IL-10), Interferon-gamma (IFN- γ) and Granulocyte macrophage colony stimulating factor (GM-CSF). Briefly, plasma samples that had not been thawed since the original freeze were thawed, filtered using 0.45 μm syringe filters (Nalgene) and a 50 μl aliquot removed. Standards were prepared for each cytokine and plated in duplicate and assay conducted as per manufacturer's instructions. Each 96-well plate was read on a Luminex 200 instrument. Each sample was assayed in duplicate and the mean value for each cytokine/patient was used for statistical analysis.

Diphtheria-Tetanus-Acellular Pertussis Multiplex Immunoassay

Total IgG concentrations against vaccine antigens pertussis toxin (PT), pertactin (PRN), filamentous hemagglutinin (FHA), fimbriae 2/3 (FIM 2/3), tetanus toxin (TT), and diphtheria toxoid (DT) were measured using an in-house multiplex bead-based immunoassay. PT, PRN, and FHA were kindly provided by GlaxoSmithKline (Belgium). TT was purchased from Sigma-Aldrich while DT and FIM 2/3 was sourced from List biological laboratories (USA). A standard curve was generated using a 10-step 3-fold serial dilution of an in-house reference sera previously quantified against National Institute for Biological Standards and Control reference sera: PT (06/140), TT (TE-3), and DT (10/262). The concentration of FIM 2/3 IgG was previously assigned to 06/140 by an international collaborative study (22). Blanks and two QC samples were included on every plate to calculate% critical variance across all assays, which fell between 6 and 12.7%. Assay specificity was determined using inhibition and interference assays. No cross reactivity was detected (data not shown).

The multiplex immunoassay was carried out as per van Gageldonk et al. (23), with minor modifications. In brief, BioPlex[®] COOH-microspheres (6.25 \times 106) were conjugated with optimized concentrations of antigen in 1 x PBS pH 7.2 (Life Technologies, AUS) as follows: PT 10 $\mu\text{g}/\text{ml}$, PRN 75 $\mu\text{g}/\text{mL}$, FHA 25 $\mu\text{g}/\text{ml}$, FIM 2/3 100 $\mu\text{g}/\text{ml}$, TT 100 $\mu\text{g}/\text{mL}$, and DT 100 $\mu\text{g}/\text{ml}$. Samples were diluted in PBS containing 3% bovine serum albumin (BSA) and 0.05% Tween 20 (Sigma-Aldrich). MultiScreen Filter Plates (Merck) were pre-wet with 50 μl PBS containing 0.05% Tween 20 (PBS.T) and the liquid removed by vacuum manifold (2–5 mmHg). Diluted plasma samples were mixed with bead-mix (25 μl ; PBS containing 4000 beads/region) in individual wells and incubated on a plate shaker (500 rpm) protected from light for 30 min. Plates were washed twice with 100 μl of 0.05% PBS.T before the addition of 100 μl 1:200 RPE-conjugated goat-anti human IgG Fc secondary antibody (Jackson ImmunoResearch Laboratories Inc.) and incubated as above for a further 30-min. Following washing, the beads were resuspended in 125 μl 0.05% PBS.T and read using a bioplex-200 machine. Antigen-specific IgG concentrations (mIU/mL) were

determined using a 5-PL linear curve generated with Bioplex Manager software version 5.0.

Immunophenotyping by Mass Cytometry

All reagents used for mass cytometry were prepared in plastics that had not been exposed to detergents, to avoid barium contamination. Stain buffer was prepared as 0.1% bovine serum albumin (Sigma-Aldrich, Australia), 2 mM EDTA (Sigma-Aldrich), and 0.05% sodium azide (Sigma-Aldrich) dissolved in calcium/magnesium-free phosphate buffered saline (PBS; Gibco) and adjusted to pH 7.4. 4% paraformaldehyde (PFA) prepared fresh each day by dissolving PFA (Sigma-Aldrich) in PBS and adjusting pH to 7.4.

Metal-labeled antibodies (**Table 1**) were validated, pre-tittered, and supplied in per-test amounts by the Ramaciotti Facility for Human Systems Biology Reagent Bank. Reagent bank antibodies were either purchased from Fluidigm in pre-conjugated form or unlabeled antibodies were purchased in a carrier-protein-free format and conjugated at the Ramaciotti Facility with the indicated metal isotope using the MaxPAR conjugation kit (Fluidigm, South San Francisco, CA) according to the manufacturer's protocol. Four Element EQ Beads, Maxpar water, Cell-ID cisplatin, and Cell-ID DNA intercalator were purchased from Fluidigm.

Cryopreserved PBMCs were thawed rapidly then transferred into warm RPMI + 10% heat-inactivated fetal calf serum (FCS) (Bovogen, French origin) + Pierce universal nuclease (ThermoFisher, Australia). Two million live PBMCs were stained as previously described (24), with amendments: 100 μ L of the first surface antibody cocktail containing an individual barcoding reagent (either Pd104-CD45 for patients or Pd108-CD45 for controls, **Table 1a**) was added. Samples were incubated with stain at room temperature for 30 min. Cells were washed, then patient and control pairs were combined into a single tube and washed again. The remainder of the surface staining antibodies (**Table 1b**) were added, 100 μ L per sample, and incubated on ice for 30 min. Cells were washed twice, then permeabilized with FoxP3 Fix/Perm buffer (ThermoFisher) and incubated with the intracellular antibodies (**Table 1c**) on ice for 30 min. Cells were fixed overnight at 4°C in 4% PFA containing 0.125 μ M DNA intercalator. After washing, cells were resuspended at 8.5×10^5 cells/mL in a 1:10 suspension of EQ beads and Maxpar water prior to data acquisition on a Fluidigm Helios mass cytometer.

Data were normalized using CyTOF Software (v6.7.1014, normalization passport EQ-P13H2303_ver2) (25, 26) and gated using Flowjo (v10.4.2). An overview of analysis is outlined in **Figure 1**. Files from each sample were cleaned by gating on Ir191_DNA, Ir193_DNA, event length, and bead-specific Ce140 to remove debris and non-cellular events. Patient and control events were debarcoded and the exported files further cleaned to remove dead cells based on high cisplatin staining (**Supplementary Figure 1**). T-cells (CD3+ CD19-), B-cells (CD19+ CD3-), and other cell lineages (CD3- CD19-) were gated and exported as individual files for use in downstream analysis. The CAPX data analysis pipeline (v2.5, Sydney Cytometry) (27) was then used to down-sample, transform (arcsine), cluster (FlowSOM) (28), and perform

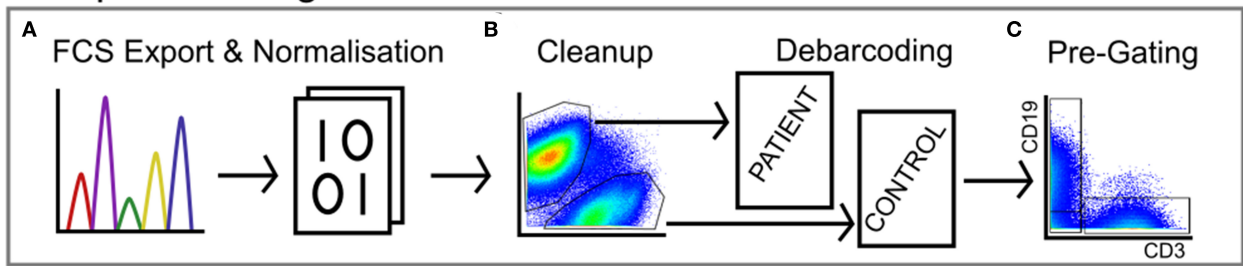
TABLE 1 | Antibody cocktails for immunophenotyping PBMCs by mass cytometry.

	Marker	mAb	Isotope label
a) Surface stain 1	CD45*	HI30	Pd_104
	CD45*	HI30	Pd_108
	CCR2	K036C2	Eu_151
	CD183 (CXCR3)	REA232	Dy_163
	CD184 (CXCR4)	12G5	Lu_175
	CCR7	G043H7	Tb_159
b) Surface stain 2	IgD	IA6-2	Y_89
	CD11c	Bu15	In_115
	CD19	HIB19	Nd_142
	CD56	NCAM16.2	Nd_143
	CD4	RPA-T4	Nd_145
	CD8a	RPA-T8	Nd_146
	CD20	2H7	Sm_147
	CD16	3G8	Nd_148
	CD25	M-A251	Sm_149
	CD275 (ICOSL)	MIH12	Nd_150
	CD45RO	UCHL1	Gd_152
	CD68	Y1/82A	Eu_153
	CD31	WM59	Gd_155
	CD86	IT2.2	Gd_156
	CD123	6H6	Dy_161
	CCR6	11A9	Pr_141
	CX3CR1	2A9-1	Er_164
	CD61	VI-PL2	Ho_165
	CD34	581	Er_166
	CD27	M-T271	Er_167
	CD45RA	HI100	Tm_169
	CD3	UCHT1	Er_170
CD38	HIT2	Yb_172	
CD14	M5E2	Yb_173	
HLA-DR	G46-6	Yb_174	
CCR5	HEK/1/85a	Nd_144	
CD127	A019D5	Lu_176	
CD11b	ICRF44	Bi_209	
CCR4	L291H4	Gd_158	
c) Intra-cellular stain	IDO-1	700838	Gd_154
	T-bet	4B10	Gd_160
	FoxP3	PCH101	Er_162
	Ki67	B56	Er_168
	Arginase I	14D2C43	Yb_171

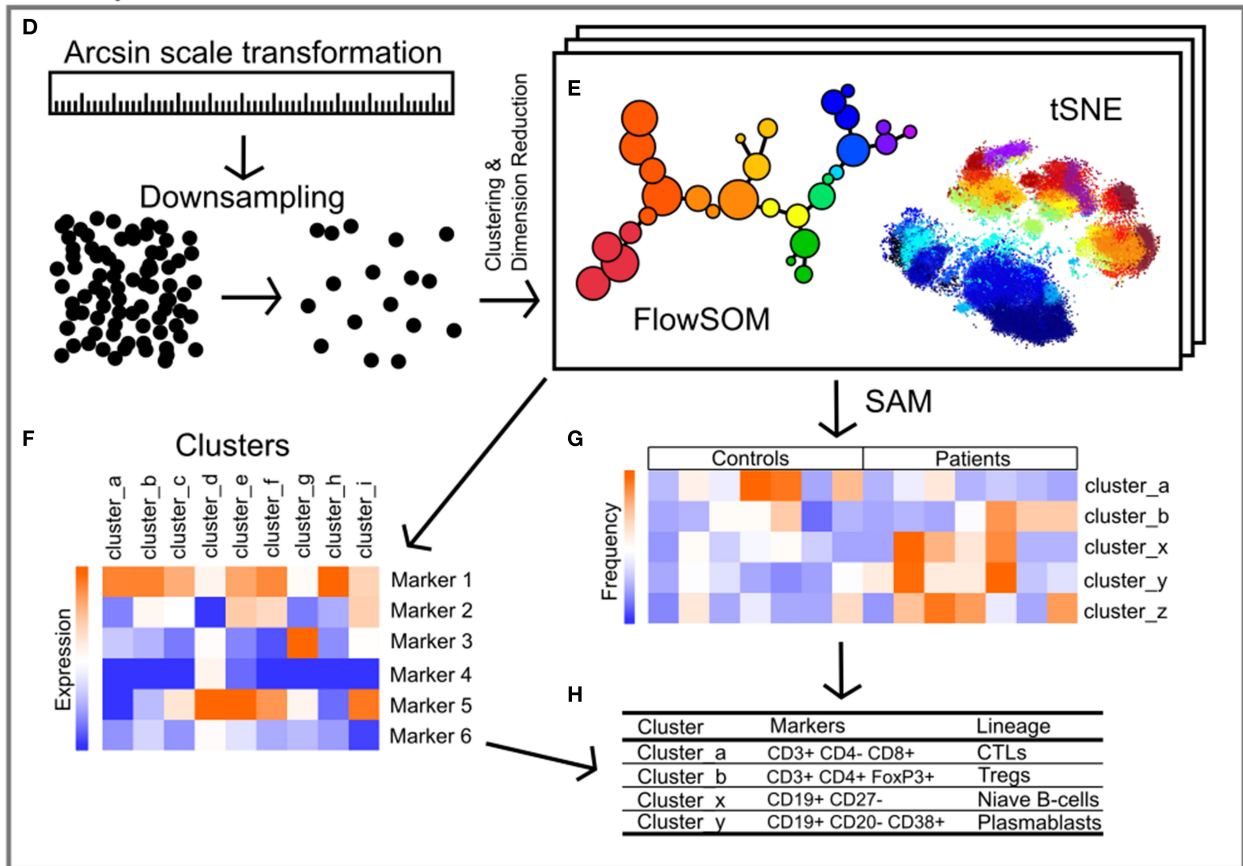
*CD45 is on two isotopes in order to barcode patients (Pd_104) and controls (Pd_108). CD45 stains all leukocytes. mAb, monoclonal antibody.

dimensionality reduction (tSNE) on the 3 gated populations for each subject. Events were clustered using all markers in each file except CD45, DNA-intercalator, cisplatin, and CD61. B-cells, T-cells, and "other cells" were analyzed with a range of cluster numbers between 10 and 90 to ensure cluster separation on meaningful markers. The intent was to identify the maximum number of clusters without artificially separating events that were biologically similar. A hierarchical

Pre-processing



Unsupervised



Supervised

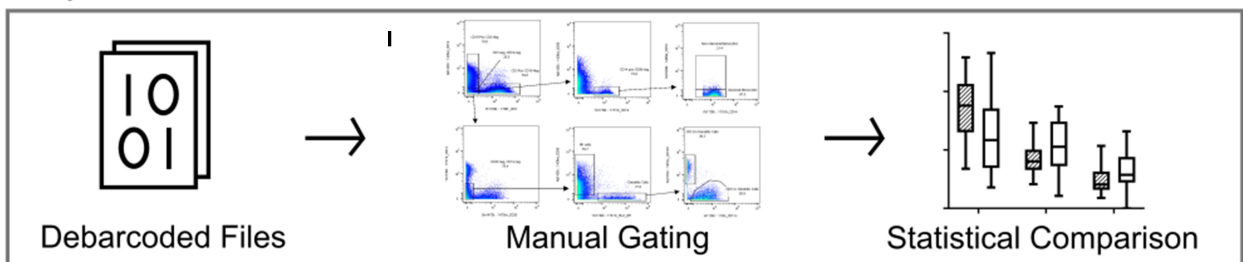


FIGURE 1 | Pipeline for the analysis of mass cytometry data. **(A)** Raw files acquired from the Helios mass cytometer were normalized based on the signal intensity of bead-specific isotopes, and exported as FCS files. **(B)** Files were manually cleaned to identify intact cells based on Ir_191 DNA staining, beads removed based on

(Continued)

FIGURE 1 | bead specific Ce_140, and doublets excluded using Ir_191 DNA staining vs. event length. Patient and control samples were debarcoded and exported into separate files based on staining with CD45-Pd_104 (patients) and CD45-Pd_108 (controls). Live cells were identified based on cisplatin stain intensity. **(C)** Events were pre-gated on CD3 vs. CD19 to export files containing T-cells (CD3+ CD19-), B-cells (CD19+ CD3-) and non-T-non-B cells (CD3- CD19-, i.e., monocytes, dendritic cells, NK cells). **(D)** The signal intensity of each marker was transformed by arcsin scaling and events from each individual were downsampled then concatenated for clustering analysis. **(E)** T-cells, B-cells, and non-T-non-B-cells were clustered separately by FlowSOM and visualized by t-stochastic neighbor embedding (tSNE). This was repeated multiple times with cluster numbers ranging from 40 to 90, in order to determine the most appropriate number of clusters **(F)** based on marker expression. **(G)** Significance analysis of microarrays (SAM) was used to identify clusters with different frequencies between patients and controls **(H)**, and the lineage of these clusters was determined based on the expression of markers **(F)**. **(I)** Binary gating was employed to manually investigate previously characterized PBMC subpopulations, and to further investigate events corresponding to clusters identified in unsupervised analysis.

gating strategy (**Figure 2**) was also implemented to investigate previously described PBMC subpopulations (29) and populations identified during unsupervised clustering.

Statistics

For each sample, the mean of duplicate runs of the cytokine and vaccine antibody assays were used for Mann-Whitney comparisons of burn survivors vs. controls. Multiple Experiment Viewer (v4.9.0, TM4) (30) was used to visualize immune subset frequencies as heatmaps. Each cell in the heatmap represented the percentage of the cluster contributed by an individual subject. Significance analysis of microarrays (SAM), a technique used for large datasets (31), was implemented to identify clusters with a different frequency between paired patients and controls. Wilcoxon signed-rank test was performed on paired data from supervised gating and *p*-values adjusted for false-discovery rate. Graphs produced using GraphPad Prism (v8.0.1).

RESULTS

Sample Demographics

Blood was collected from 36 pediatric burn survivors aged 4–8 years old and compared to 36 age- and sex-matched uninjured controls. Mean burn size was $3.95 \pm 3.1\%$ TBSA, mean age at time of injury was 22 months \pm 9 months and mean age at time of sample collection was 6.1 years \pm 1.1 years. 17 female and 19 male burn survivors were recruited (47%:53% respectively). Age at time of injury, total body surface area, etiology of injury, and time between injury and sample collection are detailed in **Table 2**.

The Australian National Immunisation Program recommends all children receive primary diphtheria-tetanus-acellular pertussis (DTPa) vaccinations at 6–8 weeks, 4 months and 6 months of age, followed by booster doses at 18 months and 4 years. Vaccination status was verified using the Australian Immunisation Register. Records were available for 35 of the burn survivors and 27 of the controls, confirming they had completed all DTPa vaccinations according to the Australian schedule. This included receiving the vaccination at 4 years of age, which was after the burn injury for all the burn patients. Only individuals with vaccination status records were included in the vaccine-specific antibody analysis, and the others were excluded as we could not confirm vaccination status.

Cytokine Profiling

Plasma was isolated from each blood sample and tested for TNF- α , IL-8, IL-7, IL-6, IL-5, IL-2, IL-1 β , IL-17A, IL-13,

IL-12(p70), IL-10, IFN- γ , and GM-CSF. Of the 13 cytokines analyzed, four were found to be significantly elevated in the burn survivors (**Figure 3A**). The inflammatory cytokine TNF- α was measured at a 1.31-fold concentration greater in patients compared to controls (mean \pm SE controls, mean \pm SE burn; *p* < 0.01). B cell and T cell modulating cytokines were also significantly increased in the burn group. Notably, IL-7 was 1.63-fold higher (*p* < 0.01), whilst IL-2 (mean \pm SE con v burn) and IFN- γ (mean \pm SE con v burn) both showed a 1.18-fold increase (*p* < 0.05). The elevation of these cytokines in the patient cohort suggests a sustained pro-inflammatory milieu may be present for many years after the initial acute trauma.

Vaccine Antibodies

Antibody responses to DTPa antigens, were compared between control and burn groups in individuals who had completed the DTPa vaccination protocol according to the Australian schedule. Burn survivors showed a diminished IgG response to pertussis toxin burn mean \pm SE and control mean \pm SE (0.48-fold reduction, *p* < 0.05). Similarly, pertactin IgG response was significantly decreased burn mean \pm SE and control mean \pm SE (0.46-fold reduction, *p* < 0.01) (**Figure 3B**). In addition, for pertussis (PT IgG \geq 5 IU/mL) 31% of the patient cohort was below the seropositive cut-off, compared to 15% of the controls (**Figures 3C,D**). A significantly diminished response in the burn group was also observed for tetanus specific IgG, burn mean \pm SE and control mean \pm SE (0.48-fold, *p* < 0.01). While diphtheria toxoid IgG concentrations were comparable between groups, 11% of the burn cohort were below the threshold of long-term seroprotection against diphtheria (DT IgG \geq 0.1 IU/mL) compared with none of the controls (**Figures 3C,D**). This decreased response to vaccine antigens in the patient cohort, observed despite the administration of a vaccine post-injury, suggests that the acute trauma may reduce the ability to respond to vaccination, mediated by a sustained systemic change, since the vaccine was administered in many cases over a year after the injury.

Immunophenotyping by Mass Cytometry

Of the 36 patients recruited, sufficient PBMCs were obtained from only 29 due to the small volume of blood collected. Of these 29, seven were excluded due to poor sample quality resulting from low cell viability, and two additional sample pairs were excluded as the barcoding step failed. Of the 20 remaining pairs,

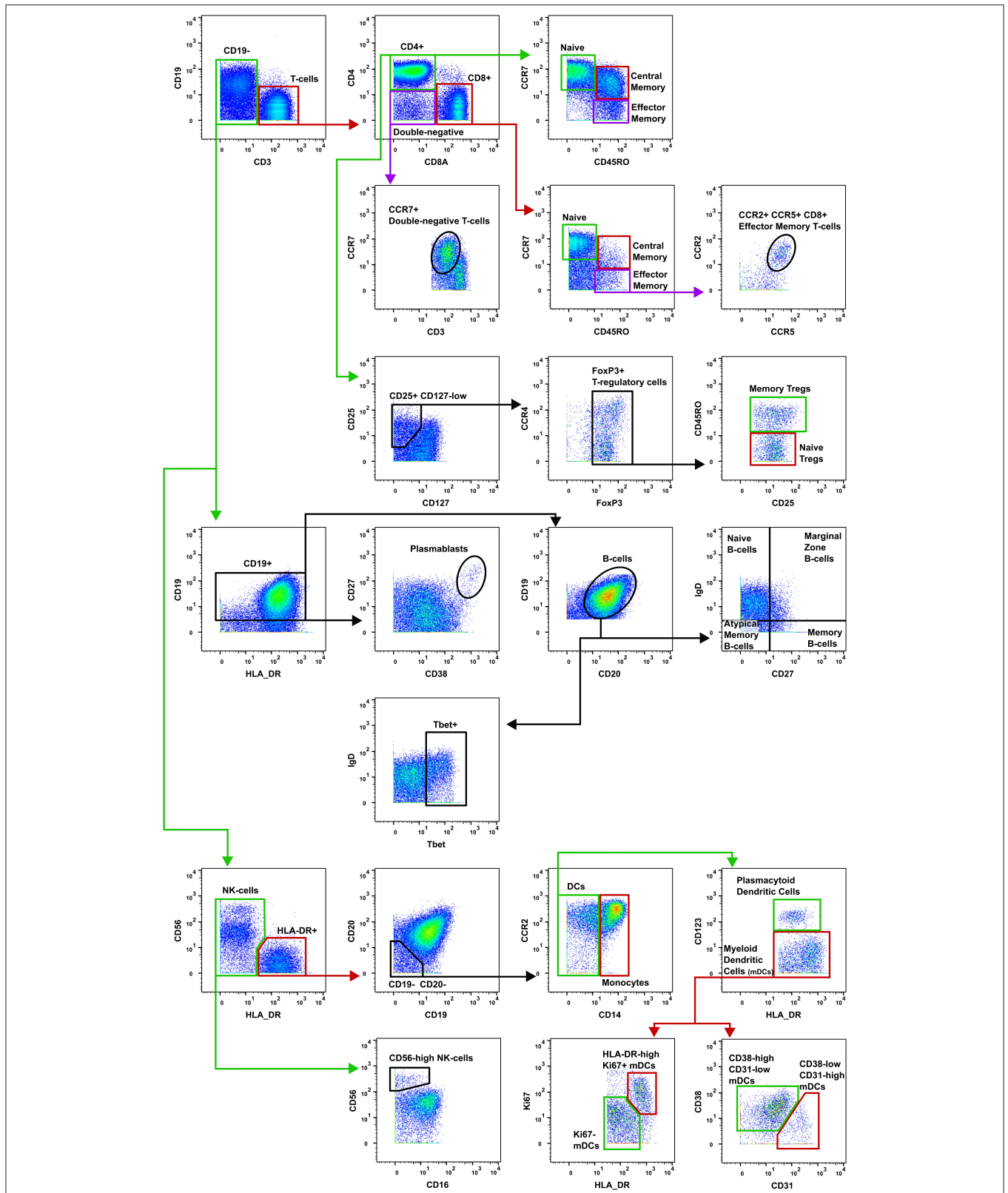


FIGURE 2 | Gating strategy for T-cell subsets. The gating strategy used to quantify the frequency of T-cell subsets in burn patients and controls. CD, cluster of differentiation; Tregs, T-regulatory cells; NK, natural killer; DCs, dendritic cells; mDCs, myeloid dendritic cells.

TABLE 2 | Details of burn injury population including age at time of injury, TBSA and etiology of the burn.

Age at burn/months	TBSA (%)	Cause of burn	Time since burn to sample collection/months
22	1	Frictional	65
13	7	Scald	59
15	3	Scald	54
18	3	Scald	51
24	<10	Scald	54
37	3	Scald	36
18	2	Frictional	44
25	2.50	Scald	52
25	8	Scald	49
18	1.5	Scald	50
16	<10	Scald	49
12	6	Scald	44
18	9	Scald	63
12	7	Scald	60
19	5	Scald	59
12	2	Chemical	65
7	1	Electrical	63
12	<2	Scald	66
18	1	Contact	62
6	<10	Sun burn	67
12	8	Scald	65
18	9	Scald	54
18	2	Thermal	55
12	1	Scald	51
18	<5	Chemical	57
12	5	Contact	53
10	<1	Contact	66
30	2	Contact	51
42	<2	Frictional	36
24	<1	Frictional	48
16	<2	Contact	55
14	3	Contact	67
41	<1	Frictional	50
38	10	Contact	59
38	1	Scald	61
36	2.50	Scald	62
36	9	Scald	54
30	<5	Frictional	63
22	1.50	Contact	60
36	1	Frictional	64
30	<1	Scald	67
36	2-3	Scald	64

13 were males and 7 were females, with a mean age of 6.3 years at time of sample collection.

Unsupervised analysis on pre-gated T-cells (CD3+), B-cells (CD19+), and all other cells (CD3-CD19-) using the CAPX pipeline (27) was used to identify 50 T-cell clusters (**Supplementary Figure 2**), 20 B-cell clusters

(**Supplementary Figure 3**), and 10 non-T non-B clusters (**Supplementary Figure 4**). Analysis of the data using t-distributed stochastic neighbor embedding (t-SNE) did not demonstrate any apparent differences between patients and controls (**Supplementary Figures 2-4**). However, analysis using significance analysis of microarrays (SAM) indicated that four T-cell clusters (**Figure 4A**), four B-cell clusters (**Figure 4B**), and one non-T non-B cluster (**Figure 4C**) differed in frequency between burn survivors and controls (SAM test delta adjusted so that the type 1 error rate was 0%).

Supervised analysis using a curated gating strategy informed by the unsupervised analysis (whereby markers present on clustered populations were used to focus investigation) identified a difference in the frequencies of several T-cell subpopulations. However, there were no changes in frequency of B-cell, NK, or myeloid cell populations (**Figures 5A-C**). There was a significant increase in the frequency of central memory (CM) CD4+ T-cells (CD3+ CD4+ CD45RO+ CCR7+; 1.42-fold, $p < 0.05$) in the burn group compared to controls. There were also changes in the frequency of memory T-regulatory cells (32) (Tregs; CD3+ CD4+ CD25+ CD127-low FoxP3+ CD45RO+; 1.69-fold, $p < 0.05$) in the burn group compared to controls. In addition, there was a trend toward a higher frequency of CCR7+ double-negative (CD4- CD8-) T-cells in the burn group that failed to reach statistical significance (1.42-fold $p \approx 0.06$) (**Figure 5B**).

Several markers that were expressed by the clusters highlighted by SAM analysis of the FlowSOM data were investigated by geometric mean signal intensity on manually gated subpopulations corresponding with those identified by unsupervised analysis (**Figures 5D-G**). Interestingly, there was a significant increase in mean expression of the chemokine receptor CXCR4 on T-cells, B-cells, and mDCs in burn survivors compared to controls (1.67-fold, $p < 0.01$; 1.81-fold, $p < 0.01$; and 1.52-fold, $p < 0.05$, respectively). There was also a significant 0.73-fold decrease ($p < 0.05$) in the expression of CXCR3 on B-cells and CCR7+ DN T-cells (33, 34) in burn survivors, and a trend toward lower Tbet expression in B-cells and higher HLA-DR expression by mDCs.

DISCUSSION

Burns patients have a lifelong increase in the likelihood of developing a range of chronic inflammatory conditions (9-14, 35). The possibility of immune disruption in acute burn injuries, and more specifically severe burn trauma, has been extensively investigated. It has been established that burn injury shifts hematopoiesis to increased production of myeloid cells in the acute response to severe injury (36, 37), and there is a transient increase in circulating DC frequency after the sudden drop seen soon after severe burns (38). In cases involving sepsis, DCs fail to regain normal numbers in the circulation in the weeks following injury. Severe burn injury has also been found to abrogate proinflammatory DC responses and to disrupt DC-mediated T-cell priming, increasing the risk of infection for at least 5 days following injury (39). It is clear that

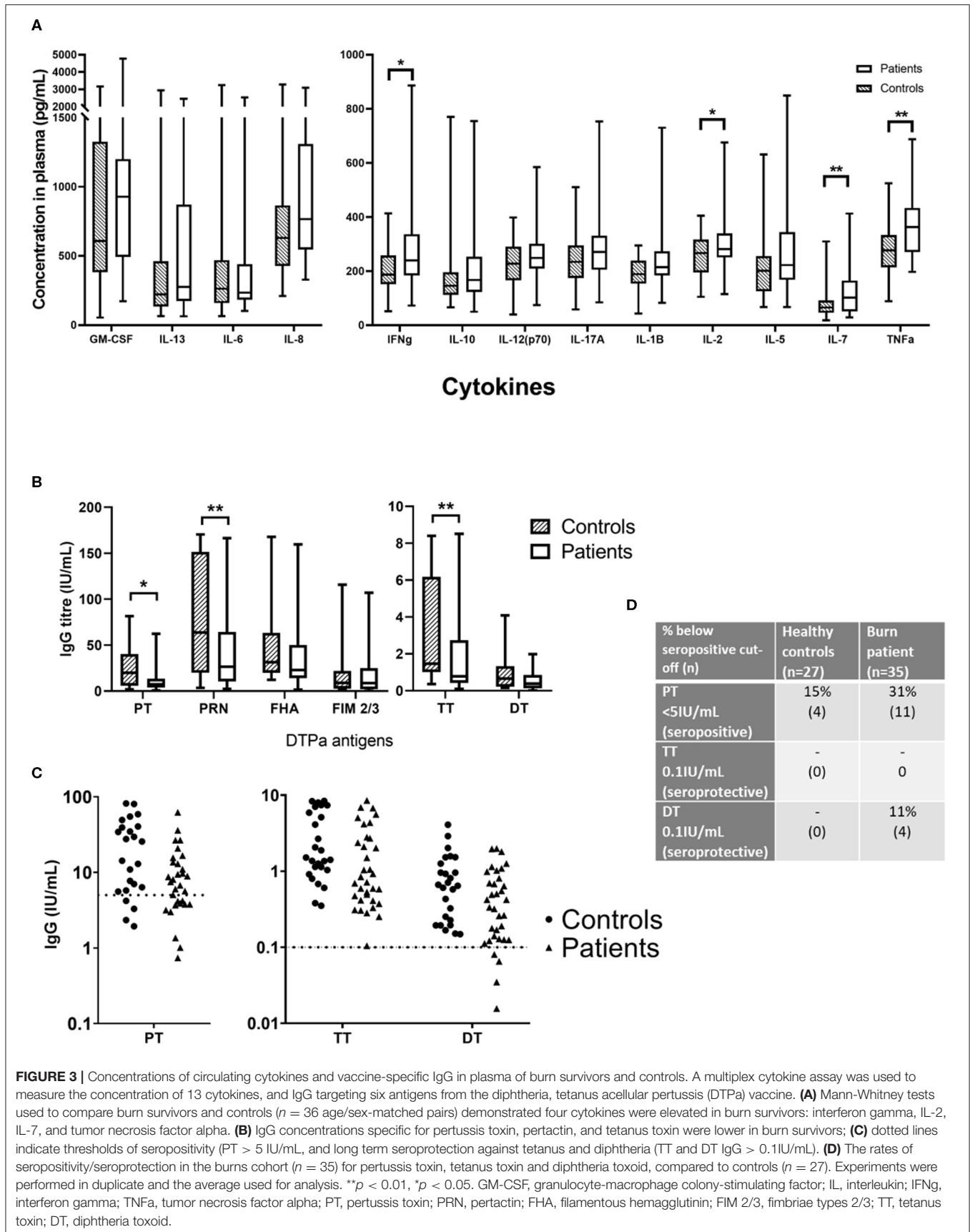


FIGURE 3 | Concentrations of circulating cytokines and vaccine-specific IgG in plasma of burn survivors and controls. A multiplex cytokine assay was used to measure the concentration of 13 cytokines, and IgG targeting six antigens from the diphtheria, tetanus acellular pertussis (DTPa) vaccine. **(A)** Mann-Whitney tests used to compare burn survivors and controls ($n = 36$ age/sex-matched pairs) demonstrated four cytokines were elevated in burn survivors: interferon gamma, IL-2, IL-7, and tumor necrosis factor alpha. **(B)** IgG concentrations specific for pertussis toxin, pertactin, and tetanus toxin were lower in burn survivors; **(C)** dotted lines indicate thresholds of seropositivity (PT > 5 IU/mL, and long term seroprotection against tetanus and diphtheria (TT and DT IgG > 0.1IU/mL). **(D)** The rates of seropositivity/seroprotection in the burns cohort ($n = 35$) for pertussis toxin, tetanus toxin and diphtheria toxoid, compared to controls ($n = 27$). Experiments were performed in duplicate and the average used for analysis. $**p < 0.01$, $*p < 0.05$. GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN γ , interferon gamma; TNFa, tumor necrosis factor alpha; PT, pertussis toxin; PRN, pertactin; FHA, filamentous hemagglutinin; FIM 2/3, fimbriae types 2/3; TT, tetanus toxin; DT, diphtheria toxoid.

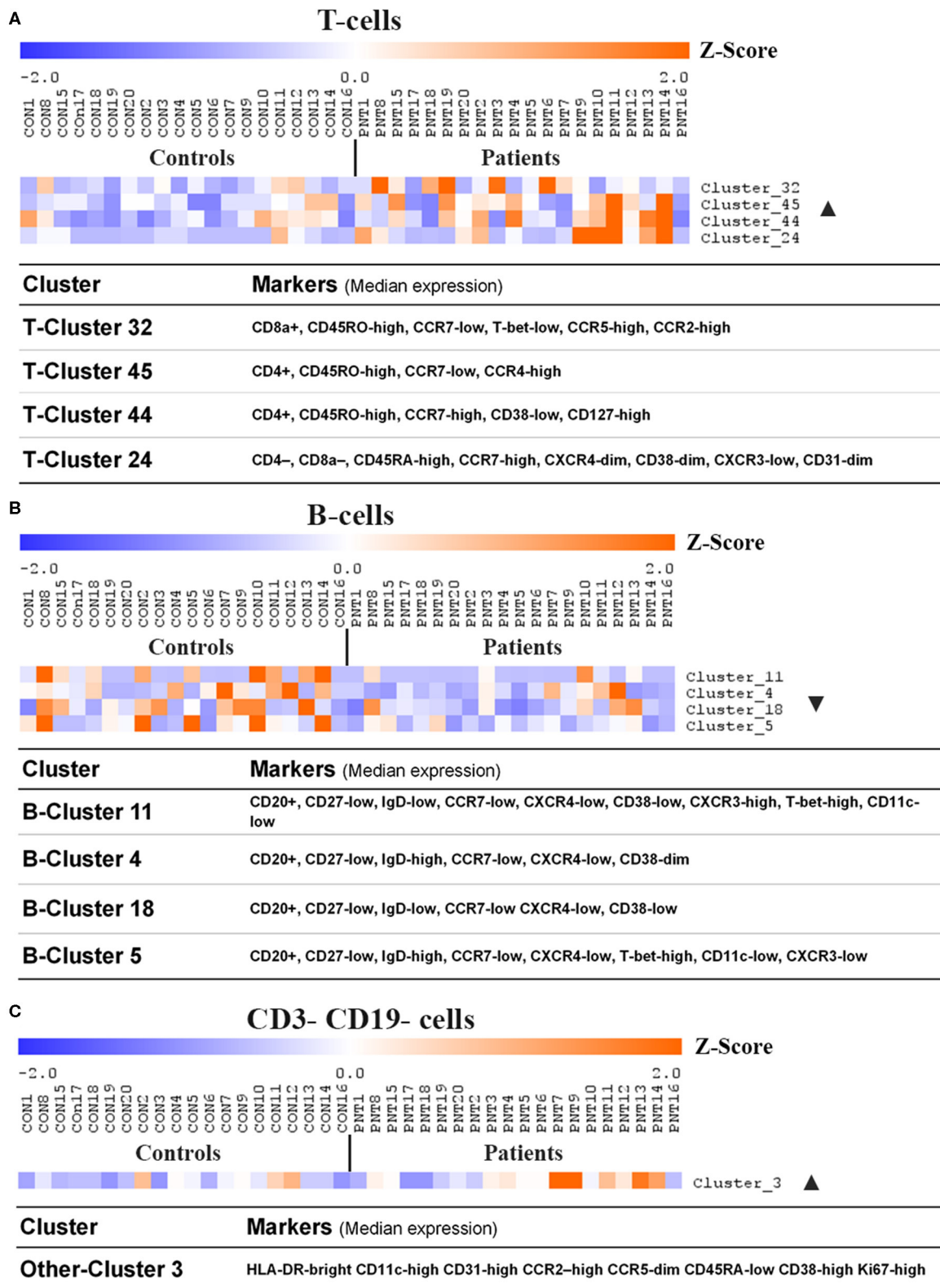
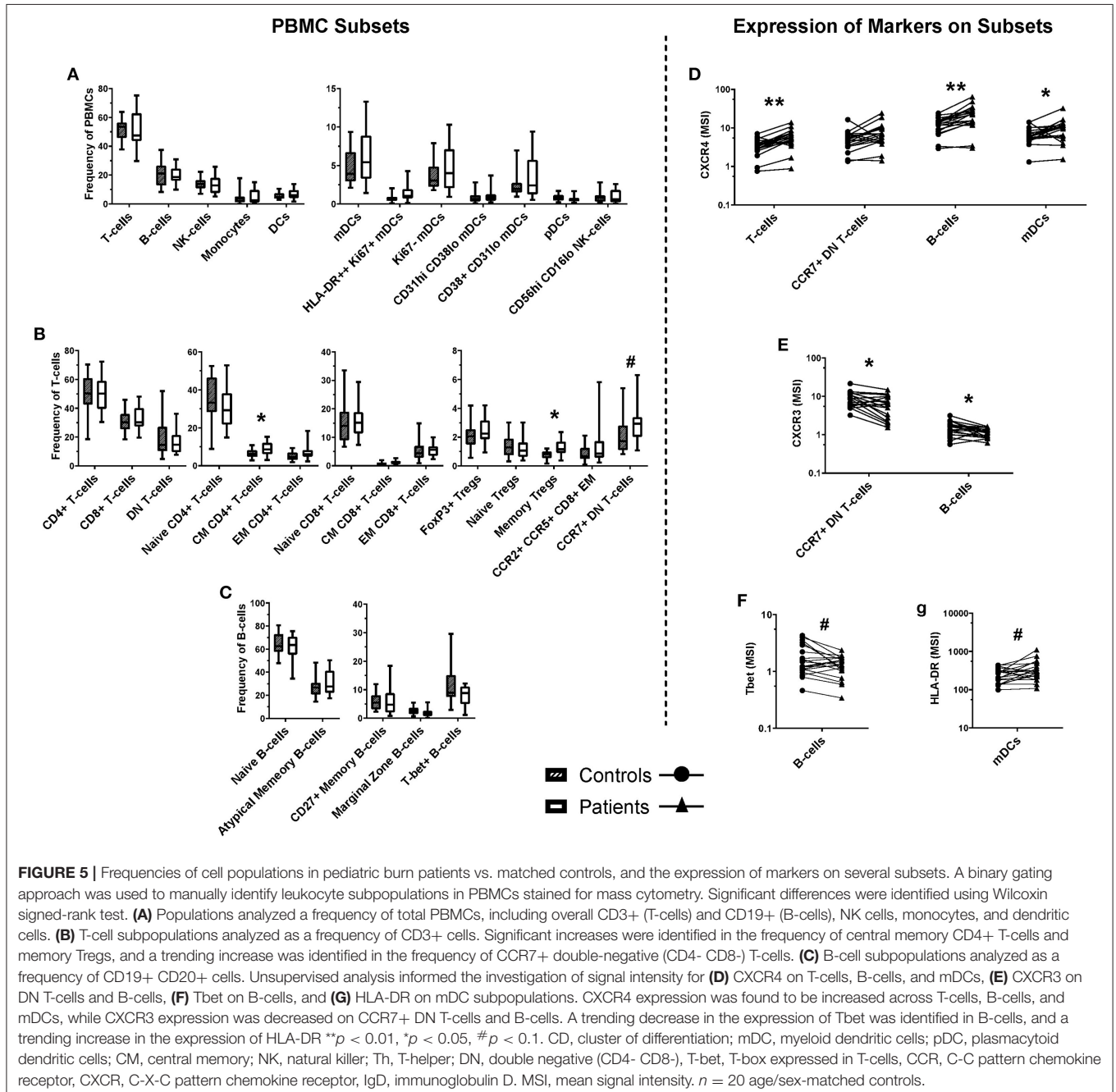


FIGURE 4 | Clusters and cell lineages with disparate frequencies between burn survivors and controls determined by unsupervised clustering analysis. FlowSOM clustering was undertaken on paired data from patients and matched controls. Data was pre-gated on CD3+ and CD19+ to analyse **(A)** T-cell subpopulations, **(B)** *(Continued)*

FIGURE 4 | B-cell subpopulations, and **(C)** CD3- CD19- subpopulations, respectively. Significance analysis of microarrays (SAM) was used to identify clusters with disproportionate frequencies between patients and controls, which are shown here. The frequency of events in a cluster from each individual has been normalized per-cluster (rows) and presented as a z-score. Positive and negative markers for identifying lineages were determined using median expression values of markers in each cluster. Ordered by age of pairing. $n = 20$ age/sex-matched pairs. CD, cluster of differentiation; ▲, increased frequency in patients compared to controls; ▼, decreased frequency in patients compared to controls.



burn injuries, specifically severe burn injuries, result in acute immune dysregulation. Current research has understandably focused on improving survival for those worst impacted by burn trauma. What remains unclear is whether these changes persist,

and to what extent they manifest in survivors of non-severe burn injuries.

In this study we performed a comprehensive analysis of immune parameters in children who had suffered a burn

to <10% of total body surface area at least 3 years prior, compared to age- and sex-matched controls. We noted multiple abnormalities in the patient cohort, including increased plasma cytokines, decreased vaccine responses, and a number of changes in immune cell populations and the expression of immune molecules by those populations.

Circulating concentrations of TNF- α , IL-7, IL-2, and IFN- γ were all elevated in the patient cohort. DTPa-specific antibodies were lower resulting in diminished rates of seroprotection to diphtheria and pertussis seropositivity amongst the burn survivors. The frequency of memory T-cell subsets—central memory CD4+ T-cells and memory Tregs—was higher for burn survivors, and expression of CXCR4 across B-cells, T-cells, and myeloid dendritic cells was increased. CXCR3 expression on B-cells and a subset of double-negative T-cells was lower in patients than controls. The increase in central memory CD4+ T-cells and memory Tregs is consistent with the findings in a recent report that used mass cytometry to investigate blood immune subsets in complex regional pain syndrome, another condition in which inflammation persists long after the original injury (40).

Elevated plasma levels of TNF- α are associated with inflammation and are a risk factor for cardiovascular disease (41). TNF- α is also implicated in the development of diabetes mellitus and inflammatory polyarthropathies (42–44). A sustained elevation of TNF- α suggests that burn survivors have a chronic inflammatory condition. This would typically be driven by macrophages, which have been shown to persist in scar tissue many weeks after injury (45). However, TNF- α secreting M1 macrophages are generally replaced by M2 macrophages after several weeks of healing (46, 47). An alternative source of TNF- α (and IL-2 and IFN- γ) in the scar/skin microenvironment is tissue resident memory T-cells (Trm) (48). It is unknown whether a distinct population of Trm persists in burn scars or other parts of the dermis, but they have previously been associated with chronic inflammatory diseases and pose an interesting avenue for further research (48).

IL-7 is produced by stromal cells in the bone marrow, thymus, and lymph nodes, all hematopoietic tissues, and cells found in the skin, including keratinocytes and fibroblasts (49, 50). IL-7 is necessary for lymphoid proliferation and maturation, and acts to maintain peripheral homeostasis of T-cells (51). Exposure to IL-7 has been associated with increased expression of CXCR4 in mature T-cells, which may explain the significantly increased expression of CXCR4 on T-cell subsets in burn survivors. IL-7 is known to protect against apoptosis in T-cells via increased expression of Bcl2 (52), which contributes to T-cell survival. This may have an impact on the T-cell frequencies observed in burn survivors, particularly (central) memory T-cells, as IL-7 supports the transition from effector phenotypes to long-term memory (53).

CXCR4 is involved in the regulation of hematopoiesis, bone marrow homing, and sequestering progenitor cells in the bone marrow (54). We did not identify any differences in the frequency of circulating progenitor cells, and there was no significant decrease in the frequency of any PBMC subset despite an increase in CXCR4 expression across B-, T-, and myeloid cells. This study did not consider changes to the bone marrow niche, which may

be disrupted by aberrant CXCR4 expression, or the expression of the CXCR4 ligand CXCL12, which is constitutively expressed by bone marrow stromal cells (55).

Dendritic cells have been shown to downregulate MHC-II expression in response to IL-7 (56). However, our data show a trend toward increased expression of HLA-DR in burn survivors at 3 years post-injury. Decreased expression of MHC-II on DCs has been reported in studies using mouse models of burn injury (20) at 3 months post-injury; the duration of this reduction is unknown, though the data presented here suggests it does not persist for 3 years. Elevated IL-7 concentrations could potentially be contributing to low seroprotection rates in burn survivors via DC MHC-II downregulation at the time of vaccination, although further studies will be required to confirm such a mechanism.

IFN- γ is secreted by CD4+ T-cells, CD8+ effector T-cells, macrophages, and NK cells. Although there were no significant differences in the frequencies of CD8+ T-cells or NK cells between burn survivors and controls (macrophages were not assessed in this study) there may have been changes in cell phenotype. Potentially there is a skew in CD8+ T cells or NK cells toward cytokine secreting rather than cytotoxic cells that contributes to the elevated levels of IFN- γ in burn survivors. The expression of IFN- γ in response to stimulation was not assessed for this study, but should be considered for future work, along with cytotoxicity markers including Granzyme B and Perforin. The functional responses of these cells *ex vivo* or after stimulations with antigen may highlight changes in the burn survivor's immune system.

Of note, IFN- γ is a regulator of DC maturation (57), which is associated with the increased expression of CXCR4 on plasmacytoid DCs (58, 59), which are potent type I IFN producers. Interestingly in this burn patient cohort we found increased CXCR4 expression on the myeloid DC population. In keeping with recent findings of increased viral infection and cancer in burn patients (11, 14), studies indicate prolonged type I IFN production is linked with immune cell dysfunction in both viral infection and cancer (60). The implications for post-burn pathophysiology are unclear—however increased CXCR4 expression by circulating DCs may reflect an overall increase in DC maturation, particularly in secondary lymphoid tissues where DCs drive T-cell responses through antigen presentation and co-stimulatory activation.

IFN- γ has also been shown to directly upregulate the expression of the immune checkpoint molecule PD-L1 that contributes to immune tolerance (61, 62). It is likely that the long-term increase in IFN- γ titres in burn survivors is associated with an increase in expression of PD-1/PD-L1, similar to the increase in PD-1/PD-L1 across B- and T-cells in patients with sepsis or severe burn injury (63). Indeed, the multiorgan failure that is a major cause of morbidity in sepsis, and is associated with increased risk of infection and ineffective adaptive immunity, may be due to aberrant expression of checkpoint molecules leading to impaired immune responses, particularly in T-cells (64). Similar increases in PD-1/PD-L1 have been observed in patients undergoing surgery with systemic inflammatory response syndrome (65).

T-bet expression in B-cells is associated with antigen experience and IgG2a/c class-switching (66). Alternatively,

CXCR3 expression in B-cells is associated with IgG1 co-expression (67), and is involved in lymphoid follicular homing and this may contribute to reduced antibody isotype switching (33). We observed a decrease in CXCR3 expression on B-cells in burn survivors, with a trend for decreased T-bet expression, and this may contribute to the reduced DTPa IgG concentrations, and decreased seroprotection/seropositivity rates. Alternatively, an immunosuppressive environment may drive the difference between T- and B-cell phenotypes in patients and controls: the dominant function of IL-2 is to support the differentiation, survival and function of regulatory T-cells (68). The increased frequency of memory Tregs in burn survivors may reflect increased availability of IL-2. Additionally, IL-2 inhibits the development of T follicular helper cells which have a role in the regulation of B-cell proliferation and class-switching (69, 70). Immunosuppression and tolerance have an important role in the pathogenesis and progression of cancer and infection (15, 71), and this may further contribute to the severity of post-burn morbidities.

There are observed similarities with the changes observed in this study and those observed with severe burn injury and other severe pathologies such as sepsis. Multiorgan failure due to pronounced systemic inflammation is a major cause of morbidity in sepsis, however these patients are also observed to be at increased risk of infection and demonstrate ineffective adaptive immunity (64). Other studies of the impact of burn injury have shown sustained elevated cytokine levels, with IL-1 α , IL-7 and IFN- γ all shown to be elevated for up to 1–2 months post-injury in pediatric patients (final follow-up (5, 72). In the long-term, widespread elevation of cytokines has been observed up to 3 years post-severe burn injury in children (21), with only IL-12p70 and MIP1 β not showing sustained elevation. These studies also demonstrate long-term clinical impacts of the burn on metabolism and physical function, supporting a likely link of this hyperinflammation to pathology.

Our study did not include PD-1 or PD-L1 in our marker panel, so we cannot draw direct conclusions regarding long-term immune checkpoint dysfunction in survivors of NSBI. However, evidence exists that demonstrates a severe burn injury, in conjunction with bacterial infection, can lead to increased PD-L1 expression in a mouse model, with improved survival at 7 days following anti-PD-L1 therapy (73). Therefore, further studies to examine immune checkpoint in NSBI are warranted.

Our approach provides a broad snapshot of the immune system in pediatric burn survivors. Whilst many of the changes observed were subtle between the two groups, given the epidemiological, patient and animal study evidence for sustained impacts of burn injury (7–14, 20, 21), we believe it is likely these subtle immune changes, magnified over time, contribute to the increase in susceptibility to disease. However, there are limitations to these findings. The scope of the study was restricted to the circulating components of the immune system and cannot provide any insight into the constituents of different tissues. Therefore, we cannot draw any conclusions regarding differences between patients and controls that may exist in the bone marrow, lymphoid organs, skin, and other tissues—e.g., it may be more informative to investigate immune memory in the bone marrow (74). The relatively small sample size is also a key limitation

of this study and further patient recruitment will be important to validate findings from this cohort. Nevertheless, this study is comparable to those of others that have also identified changes in PBMC profiles associated with sustained pathology (40) and provides new insight into the possible consequences of acute burn injury and an important basis for further research. Most importantly, whilst these experiments provide observations of changes in these cell populations, functional assays will be critical to understand the potential clinical consequences of the observed disparity between groups.

In conclusion, this study provides evidence of an enduring change to the circulating components of the immune system in pediatric burn survivors at least 3 years after a non-severe burn injury. Burn survivors appeared to have a more limited response to the DTPa vaccine booster (administered at 4 years of age), and significant changes to T-cell lineages, coupled with disparate expression of surface proteins and transcription factors in T-, B- and dendritic cells. This suggests an ongoing impact of burn trauma on the immune system. These changes hint at a mechanism that may drive the rates of post-burn infections and other diseases controlled by the adaptive immune response. Further work to unravel the link between this disparity and the secondary morbidities observed in burn survivors will be important to understanding the systemic impacts of burn trauma, and the development of therapeutic pathways to reduce the incidence of morbidity in children who recover from a burn.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Debarcoded files are uploaded to Flow Repository <http://flowrepository.org/id/FR-FCM-Z2XE>.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Child and Adolescent Health Service Ethics committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BJ, HM, AS, ML, FW, MF, and VF: study concept and design. FW, PR, VP, SP, DP, and JM: patient recruitment and clinical expertise. BJ, HM, SM, and VP: experimental work. BJ, HM, ML, MF, and BF: data analysis and interpretation. All authors: manuscript preparation and revision.

FUNDING

This work was funded through a grant provided by the Western Australian Department of Health Telethon-Perth Children's Hospital Research Fund and funding from the Fiona Wood Foundation. MF was supported by the Stan Perron Centre for Excellence for Childhood Burns and Perth Children's Hospital Foundation.

ACKNOWLEDGMENTS

We acknowledge the facilities and the Scientific and Technical Assistance of Microscopy Australia at the Centre for Microscopy, Characterization & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments, and furthermore the support of Andrea Holme and Irma Larma. Thanks to Felix Marsh-Wakefield and Thomas Ashhurst at the University of Sydney for their advice on analyzing mass cytometry datasets. We would

also like to thank all the support staff at Sydney Cytometry and the Ramaciotti Facility for Human Systems Biology for their assistance with the mass cytometry studies. ML and HM are ISAC Marylou Ingram Scholars.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01481/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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