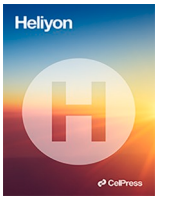
Heliyon 9 (2023) e20590

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Incompetent memory immune response in severe COVID-19 patients under treatment

Shukla Das [a], Gargi Rai [b], Vikas Sood [c], Praveen Kumar Singh [a], Asha Tyagi [d], Rashmi Salhotra [d], Chhavi Gupta [e], Viniita Kumar Jaggi [f], Sajad Ahmad Dar [g], Mohammad Ahmad Ansari [b,*]

[a] *Department of Microbiology, University College of Medical Sciences (University of Delhi) & GTB Hospital, New Delhi-110095, India*
[b] *Multi-disciplinary Research Unit (Department of Health Research), University College of Medical Sciences (University of Delhi) & GTB Hospital, New Delhi-110095, India*
[c] *Department of Biochemistry, Jamia Hamdard, New Delhi-1100, India*
[d] *Department of Anesthesia, University College of Medical Sciences (University of Delhi) & GTB Hospital, New Delhi-110095, India*
[e] *All India Institute of Medical Science, New Delhi, 110029, India; Present Address: Consultant Infectious Diseases, Fortis Hospital, Sector 62, Gautam Buddh Nagar, Noida, Uttar Pradesh-201301, India*
[f] *Department of Surgical Oncology, Division of Gynecological Oncology, Delhi State Cancer Institute (GNCT of Delhi), Dilshad Garden, New Delhi-110095, India*
[g] *Research and Scientific Studies Unit, College of Nursing, Jazan University, Jazan-45142, Saudi Arabia*

ARTICLE INFO

Keywords:
COVID-19
Treg cells
Th17 cells
PBMC
Flow cytometry
SARS-CoV-2

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) associated coronavirus disease 2019 (COVID-19) pandemic has affected millions of people worldwide and declared a Public Health Emergency by the World Health Organization (WHO) on January 30, 2020. Albeit, unprecedented efforts have been made from the scientific community to understand the pathophysiology of COVID-19 disease, the host immune and inflammatory responses are not explored well in the Indian population. Continuous arrival of new variants fascinated the scientists to understand the host immune processes and to eradicate this deadly virus. The aim of this study was to see the helper and cellular host immune responses including memory and activated cell subsets of COVID-19 patients admitted to the intensive care unit (ICU) at different time intervals during the treatment. PBMCs separated from nine patients with SARS-CoV-2 infection were incubated with fluorescent conjugated antibodies and acquired on flow cytometer machine to analyze the T and B cell subsets. The results in COVID-19 patients versus healthy volunteers were as follows: elevated helper T cells (57.4% vs 44.9%); low cytotoxic T cells (42.8% vs 55.6%), and activated T (17.7% vs 21.2%) subsets. Both, TREG (40.15% vs 51.7%) and TH17 (13.2% vs 24.6%) responses were substantially decreased and high expression of TREG markers was observed in these patients compared with controls.

* Corresponding author. Research Scientist-II Room No.-121, First floor, College building, Multi-disciplinary Research Unit (Department of Health Research), University College of Medical Sciences (University of Delhi) & GTB Hospital, Dilshad Garden, New Delhi-110095, India.
E-mail address: ansari.aiims@gmail.com (M.A. Ansari).

https://doi.org/10.1016/j.heliyon.2023.e20590
Received 19 February 2023; Received in revised form 4 September 2023; Accepted 29 September 2023
Available online 30 September 2023

1. Introduction

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), emerged first in Wuhan in December 2019 and has rapidly spread globally [1,2]. It was declared a Public Health Emergency of International Concern by the World Health Organization (WHO) on January 30, 2020 [3]. As of March-2022, a total of 4,30,20,723 laboratory-confirmed cases were identified in India, with 5,21,035 fatalities (1.21%), according to the data from Indian government official reports [4]. Although, the virological, epidemiological, and clinical patterns and management outcomes of COVID-19 patients have been defined [1,5–9], the host immune profile and inflammatory responses are not explored well in the Indian population.

A wide variety of clinical manifestations occurs in COVID-19 patients varying from mild to severe disease causing acute respiratory distress syndrome (ARDS) and death [1,7–9]. Inflammation is alleged to buttress severe COVID-19, due to peaked levels of proinflammatory cytokines such as interleukin-1 beta (IL-1b), and tumor necrosis factor alpha (TNF-a) in severe COVID-19 patients [1]. Albeit, the host response is lively and can oscillate extensively from one day to another. Whether such variability is important to COVID-19 pathogenesis remains unknown.

Fatalities were higher in the elderly and in individuals with co-morbidities [10], however, during the second wave of SARS-CoV-2, the maximum adverse impact was observed in the younger adults in India. Though inflammation and host immune responses are important for the curtailment of the virus, dysregulated response plays a key role in the pathogenesis. Several studies have shown the induced inflammatory response and elevated pro-inflammatory cytokine & chemokine response, which lead to severe COVID-19 manifestations [11,12]. It is well known that $CD8^+$ T cells and NK cells are key subsets in preventing viral replication, lymphopenia and gradual fall in $CD8^+$ T cells can be detrimental in COVID-19 patients [13].

New variants like Delta plus and Omicron, which are highly contagious in nature are currently being reported in several parts of India. The understanding of the host immune process is therefore important to further comprehend the pathophysiology of this viral agent. Very little is known about the impact of different lymphocyte subsets on the immune response of patients with COVID-19 and its consequences. This study was planned to see the inflammatory and immunological parameters and expression of cell surface markers on lymphocyte subsets by flow cytometer in severe COVID-19 patients admitted to the intensive care unit (ICU) of a tertiary care hospital in New Delhi during the first wave.

2. Materials and Methods

2.1. Patients and sample collection

The study included nine patients with COVID-19 disease admitted to the intensive care unit (ICU) of the tertiary care hospital between October to December 2020 and five healthy volunteers as controls. All the patients were diagnosed with SARS-CoV-2 and confirmed by RT-PCR as per the ICMR guideline performed at the dedicated COVID-19 testing laboratory of the hospital. The study was approved by the Institutional Ethics Committee (IEC-HR) with reference number GTBHEC 2021/P-149. The study population including healthy volunteers was unvaccinated as COVID-19 vaccination launch was still awaited in India and with no history of previous SARS-CoV-2 infection. The criteria used for admitting COVID-19 patients to ICU, was as per CLINICAL MANAGEMENT PROTOCOL: COVID-19, MoH&FW (EMR division), Govt. of India [14]. Detailed history, clinical findings and relevant laboratory investigations of patients were collected at the time of enrollment in a pre-designed case record form.

Five ml venous blood sample was collected from the patients and age matched healthy controls in EDTA vials for preparation of peripheral blood mononuclear cells (PBMCs) which was used for screening of molecular markers, and also in plain vial for serum separation. The samples were collected on the day of admission (zero day), followed by 3rd day and 6th day of ICU admission, after getting the informed written consent. Serum was used for the detection of IL6 and ferritin by chemiluminescence immunoassay (CLIA) via IMMULITE®2000 XPI analyzer (Siemens).

PBMCs separation: PBMCs were isolated from peripheral venous blood by the Ficoll-Plaque density gradient method. Briefly, whole blood was diluted with RPMI-1640 (Gibco, Life Technologies), gently overlayered on Ficoll reagent and centrifuge at 1000rpm for 20 min at room temperature. The cloudy interface was collected carefully, taken in a fresh tube and washed twice with ice-cold phosphate buffer saline (PBS; HiMedia, India). The viability of the cells was confirmed by Trypan Blue dead cell exclusion assay under a light microscope using Neubauer Chamber.

Staining with fluorochrome-labeled monoclonal antibodies and flow cytometry: PBMCs were stained immediately with four

Table-1
Details of fluorochrome dyes allocated with anti-human monoclonal antibodies against cell surface markers to study different lymphocyte subsets in COVID-19 patients.

Panel 1 (Treg markers)	Panel 2 (Treg markers)	Panel 3 (Th17 markers)	Panel 4 (Memory T&B)
CD3-APC Cy7	CD3-APC Cy7	CD3-APC Cy7	CD4-FITC
CD4-APC	CD4-APC	CD4-APC	CD8-APC H7
CD25-PE	CD25-PE	IL23R-FITC	CD20-PE Cy7
GITR-AF488	CCR4-PE Cy7	CD161-PE	CD45RO-APC
CD122-BV510	CCR7-BV510	HLADR-PE Cy7	
CD127-PE Cy7		CD45RO-BV510	
CD152-BV421		CCR5-BV421	

panels of fluorochrome-conjugated anti-human monoclonal antibodies and Th17, Treg, T and B immune cells were studied by flow cytometer. Firstly, PBMCs were incubated with 100 μl of Fc block reagent at room temperature for 10 min and then $1x10^6$ cells were re-suspended in 25 μl of ice-cold staining buffer and incubated with anti-human monoclonal antibodies, CD3, CD4, CD25, GITR, CD122, CD127, CD152, CCR4, CCR7, IL23R, CD161, HLA-DR, CD45RO, CCR5, CD8 and CD20 (BD Biosciences, CA, US) at room temperature for 20 min in dark (Table-1). Cells were washed with PBS buffer and resuspended in PBS with 2% paraformaldehyde. Samples were acquired on FACS Aria III (BD Biosciences, USA) immediately or within 24 h of staining and at least 30,000 events were recorded for each sample. Area versus height data was recorded on FSC and SSC scale for doublets discrimination. Single positive control and unstained control were used to analyze the data and to avoid the noise population. Briefly, PBMC cells were targeted, followed by singlet selection and then helper T cells ($CD3^+CD4^+$) were gated from total T cells ($CD3^+$). Data were analyzed on FlowJo software (BD Biosciences, CA, US) and the results were presented as percentage positivity.

Data Analysis: The data is described as mean value and range. All the analyses and bar and line diagrams were generated on MS Excel version 19 software. Figures for flow cytometry were plotted on FlowJo_v10 software (BD Biosciences, CA, USA).

3. Results

3.1. Demographics and baseline characteristics of COVID-19 patients

A total of nine patients with COVID-19 disease confirmed by RT-PCR, admitted to the intensive care unit (ICU) during the study period were enrolled in this study. All patients had severe COVID-19 illness, six patients were mechanically ventilated and three others were managed with either non-invasive ventilation or high flow nasal oxygen therapy. The demographics and baseline characteristics of these patients are shown in Table-2 & −3 and Figure-1A. With a median age of 53 years (45–76), 44% of patients were male. Diabetes mellitus was the most common comorbidity seen in 66.6% of patients followed by hypertension (55%). Fever, breathlessness, cough, diarrhea, and nausea were the most frequent presenting symptoms.

Parameters of routine assessments and laboratory investigations were significantly deranged in all patients and were categorized as severe COVID-19 patients, with high respiration rates during the ICU stay as shown in Table-3. Routine investigations showed total leukocyte count (TLC), liver function test, kidney function test and serum electrolytes were raised in all these patients at different time points i.e., zero day, 3rd day & 6th day, as shown in Table-3 and Figure-1A. Levels of urea, ferritin and interleukin-6 were significantly high in these patients. All patients received systemic corticosteroids, Remdesivir and low molecular weight heparin, as per the institutional management protocol. The stay in ICU was for a median of 18 days (14–23) and all patients succumbed to illness.

3.2. Immune status of $CD4^+$ and $CD8^+$ T lymphocyte subsets in patients of COVID-19

Flow cytometer analysis revealed the changes in the numbers of total $CD3^+$ T cells, $CD3^+CD4^+$ helper T cells, $CD3^+CD8^+$ cytotoxic T cells and $CD20^+$ B cells in COVID-19 patients as shown in Table-4, Figure-1B, 1C & 2(A-D). Analysis of $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets demonstrated a significant elevation in helper T cells and remained increased during the treatment in severe COVID-19 patients compared to healthy controls and cytotoxic T cells significantly diminished. This resulted in raised CD4+/CD8+ ratio in COVID-19 patients and became more prominent during the course of treatment as compared to healthy controls.

Further, an elevated percentage of $CD3^+CD4^+CD45RA+$ naïve helper T cells (T_N) was observed in COVID-19 patients compared to healthy controls. Over the time during treatment T_N cells became inflated but the expression of CCR7 and CCR4 remained significantly low throughout the treatment as was received in ICU. Furthermore, analysis of CD45RO expression (T_{EM}) on $CD3^+$ cells, CD4+cells and $CD8^+$ T cells revealed the initial heightened memory response, up to 7–10 days of ICU stay in severe COVID-19 patients and later fell below the normal range, Figure-2B, 2C & 3A. In SARS-CoV-2 infected patients, evaluation of T cell activation showed statistically lower activation compared to healthy controls as indicated by the percentage of HLA-DR + $CD8^+$ T cells. The decline in $CD3^+$CD4+HLADR + helper T cells and $CD3^+$CD8+HLADR + cytotoxic T cells in severe COVID-19 patients was consistent and remained throughout their stay in ICU, shown in Figure-3B.

3.3. TREG and TH17 subsets in patients of COVID-19

Compared with healthy controls, in SARS-CoV-2 infection, there was a significant decline in $CD3^+CD4^+CD25^{hi}CD127^{lo}$ TREG cells in patients at all time points. Additionally, significantly higher expression of cytotoxic T lymphocyte associated protein-4 (CTLA-4 or

Table-2
Details of subjects recruited under this study.

CRITERIA	CONTROL GROUP	PATIENT GROUP
Number of Subjects	5	9
Median Age (year)	55 (50–60)	53 (45–76)
Gender (M/F)	60%/40%	56%/44%
Comorbidity: Diabetes	Nil	66.6%
Comorbidity: Hypertension	Nil	55%
SARS-CoV-2 infection	No previous history	No previous history
COVID vaccination	Not vaccinated	Not vaccinated

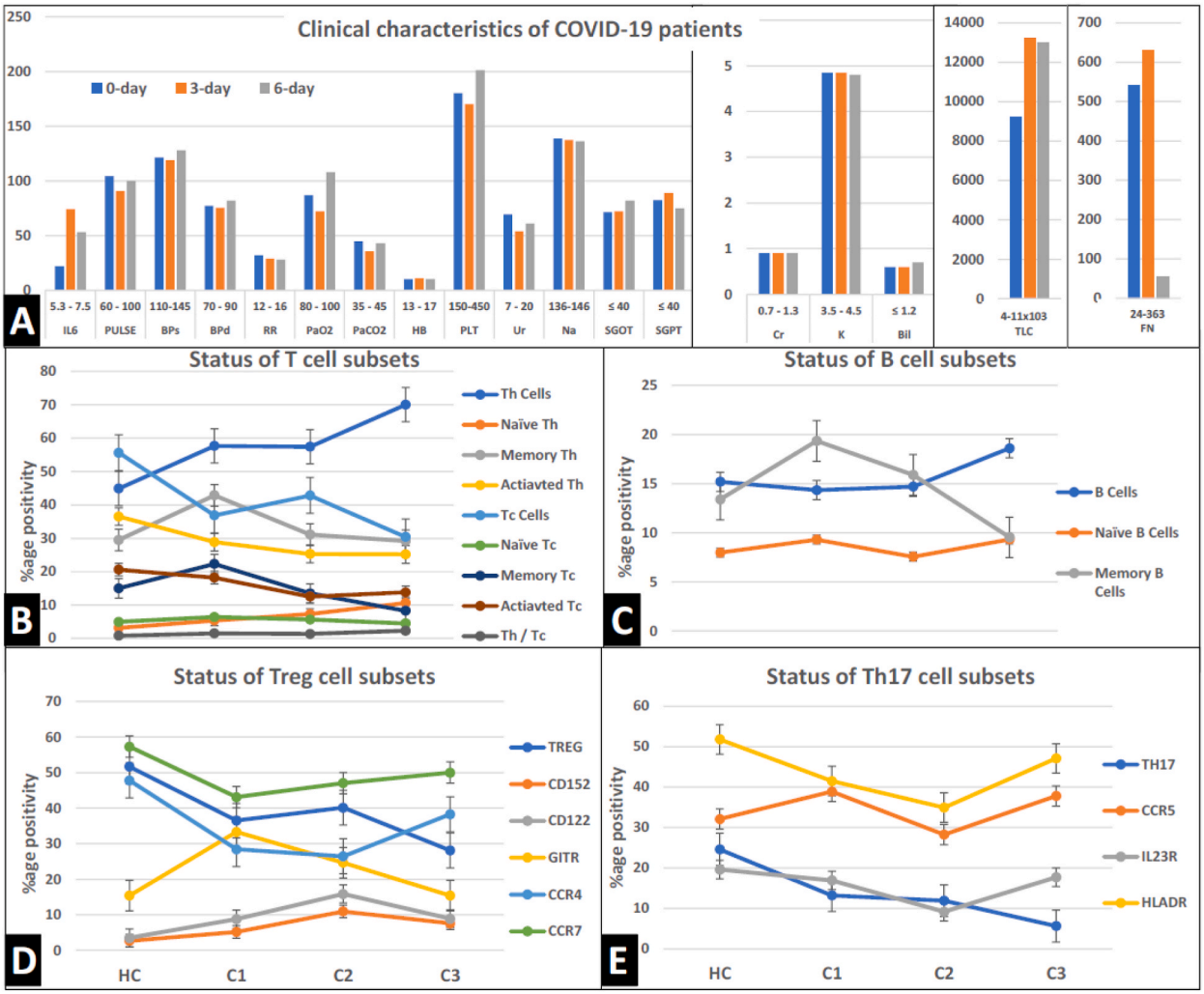


Figure-1. Diagram showing the clinical characteristics and lymphocyte subsets in severe COVID-19 patients. [1-A] Bar diagram represents the various clinical parameters on the day of admission (zero day), followed by 3rd day and 6th day of ICU admission during treatment. **[1-B]** Line diagram represents the naïve, memory and activated cell subsets of Helper & Cytotoxic T cells in COVID-19 patients with respect to healthy volunteers at different time point of ICU treatment. **[1-C]** Line diagram represents the naïve and memory B cell subsets. **[1-D]** Line diagram represents the Treg cells and molecular markers, CD122, CD152, GITR, CCR4 & CCR7 expressed on Treg cells in COVID-19 patients compared to controls at all points of ICU treatment. **[1-E]** Line diagram represents the Th17 cells and molecular markers, IL23R & HLA-DR expressed on Th17 cells in patients compared to controls during ICU treatment.

CD152), CD122 and GITR was observed in COVID-19 patients, and reduced expression of CCR4 and CCR7 on regulatory T cells, shown in Table-4 and Figure-1D & 3A. Again, $CD3^{+}CD4^{+}CD161+$ TH17 cells were reported as significantly reduced in severe COVID-19 patients versus controls. IL23R and HLA-DR expressing TH17 cells were recorded in low numbers in COVID-19 patients compared to controls and remained low at all points of the study as shown in Figure-1E & 3B.

3.4. Immune status of B lymphocyte subsets in patients of COVID-19

In COVID-19 patients, no significant change was recorded in the numbers of $CD20^{+}$ B cells during treatment. Further, analysis of CD45RA and CD45RO expression showed that numbers of CD45RA + $CD20^{+}$ naïve B cells remained relatively unaltered, albeit higher numbers of CD45RO + $CD20^{+}$ memory B cells were recorded in COVID-19 patients and later during the treatment memory B cells declined below the normal range as shown in Figure-1C & 2D.

4. Discussion

SARS-CoV-2, a member of the family coronavirus, responsible for a global pandemic started from Wuhan in December-2019 and

Table-3
Demographic and baseline characteristics of COVID-19 patients at different time points.

Clinical parameters	Day-zero	Day-3rd	Day-6th
Pulse (beats/min)	104.5 (92–112)	90.5 (84–108)	100 (85–102)
Systolic Blood Pressure(mmHg)	121.5 (71–198)	119 (108–148)	128 (120–152)
Diastolic Blood Pressure (mmHg)	77 (59–110)	75.5 (55–86)	82 (68–88)
Respiration Rate	32 (26–42)	27 (24–34)	28 (24–30)
Partial Pressure of Oxygen (mmHg)	87 (61–140)	72 (56.5–91)	108 (58–115)
Partial Pressure of Carbon Dioxide (mmHg)	44.6 (32–60.6)	35.85 (32.2–37)	42.9 (28–50.8)
Hemoglobin	10.25 (8.8–13)	11 (9.4–13.3)	10.3 (9.9–12.7)
TLC ($x10^6$/L)	9200 (5000–12700)	13200 (6900–22600)	13000 (6900–20400)
Platelet ($x10^9$/L)	180 (87–280)	170 (117–340)	201 (167–392)
Urea (mg)	69.5 (33–156)	54 (49–138)	61 (44–75)
Creatinine (mg/dl)	0.9 (0.7–2.4)	0.9 (0.6–1.7)	0.9 (0.6–1.8)
Sodium (mmol/L)	138.5 (129–141)	137.5 (121–141)	136 (136–140)
Potassium (mmol/L)	4.85 (4.4–5.7)	4.85 (4.6–5.5)	4.8 (4–5.5)
SGOT	71.5 (43–133)	75.5 (70–261)	82 (40–261)
SGPT	82.5 (53–254)	89 (77–284)	75 (75–284)
Bilirubin (mg/dL)	0.6 (0.4–0.8)	0.6 (0.3–1.3)	0.7 (0.4–1.1)
IL6 (pg/ml)	21.9 (5.6–52.9)	74.2 (31.4–239)	53.2 (38.1–85.8)
Ferritin (ng/ml)	541.3 (211–654)	630.9 (323–855)	253 (223–543)

Data is presented as mean value and range.

Table-4
Percentage positivity of different T and B cell subsets of COVID-19 patients at different time point of treatment.

Molecular Markers	Healthy controls	Day-zero	Day-3rd	Day-6th
$CD3^+$ T cells	74.1	68.6	71.4	69
$CD3^+CD45RA$ + Naïve T cells	8.4	4.8	4.9	6.2
$CD3^+CD45RO$ + Memory T cells	25.8	34.45	21.5	23.5
CD3+HLADR + Activated T cells	21.2	17.7	15	15.6
$CD3^+CD4^+$ Helper T cells	44.9	57.65	57.4	70
$CD3^+CD4^+CD45RA$ + Naïve helper T cells	3.16	5.37	7.3	10.63
$CD3^+CD4^+CD45RO$ + Memory helper T cells	29.5	42.85	31.1	29.2
$CD3^+CD4$+HLADR + Activated helper T cells	36.5	28.85	25.3	25.2
$CD3^+CD8^+$ Cytotoxic T cells	55.6	36.85	42.8	30.4
$CD3^+CD8^+CD45RA$ + Naïve cytotoxic T cells	4.97	6.41	5.655	4.49
$CD3^+CD8^+CD45RO$ + Memory cytotoxic T cells	15	22.3	13.5	8.27
$CD3^+CD8$+HLADR + Activated cytotoxic T cells	20.6	18.2	12.5	13.8
T Helper/T Cytotoxic ratio	0.81	1.51	1.34	2.30
$CD20^+$ B cells	15.2	14.35	14.7	18.6
CD20+CD45RA + Naïve B cells	7.99	9.305	7.57	9.33
CD20+CD45RO + Memory B cells	13.4	19.35	15.9	9.56
$CD3^+CD4^+CD25^{hi}CD127^{lo}$ Tregs	51.7	36.5	40.15	28.1
CD152 expression on $CD25^+$ Helper T cells	2.7	5.18	10.95	7.6
CD122 expression on $CD25^+$ Helper T cells	3.48	8.77	15.85	8.9
GITR expression on $CD25^+$ Helper T cells	15.4	33.3	24.65	15.4
CCR4 expression on $CD25^+$ Helper T cells	47.8	28.45	26.45	38.3
CCR7 expression on $CD25^+$ Helper T cells	57.3	43.1	47.05	50
$CD3^+CD4^+CD161$+ TH17 cells	24.6	13.2	11.9	5.63
IL23R expression on TH17 cells	19.6	16.9	9.165	17.7
$CD3^+CD4^+CD161$+HLADR + Activated TH17 cells	51.8	41.5	34.95	47.1

Data is presented as mean value.

spread worldwide [1]. To understand the immune response patterns of patients infected with SARS-CoV-2, we studied 9 cases of severe COVID-19, hospitalized in the ICU of COVID dedicated tertiary care hospital in Delhi. For immune profiling of T cells and B cells, we performed flow cytometry to observe the sequential immune changes during the infection. Additionally, clinical data was retrieved to understand the association between immune responses to SARS-CoV-2 and disease pathogenesis. Inflammatory biomarkers like interleukin-6, and CRP levels were found raised in severe COVID-19 patients. The immune responses as observed in COVID-19 patients with the myriad of cytokines and chemokines, lead to fatal cytokine storms and mortality [15]. The host immune response is critically regulated by immune cells like $CD3^+$ T cell, $CD4^+$ helper cell, $CD8^+$ cytotoxic cell, $CD20^+$ B cell lymphocytes, and their subsets. These cells constitute the host humoral and cell-mediated immunity against infections including viral agents. Dysregulation in the phenotypes of these lymphocytes results in the pathogenesis of COVID-19 disease [16,17]. SARS-CoV-2 causes a rapid decline in the T cell population which results in lymphopenia and disease progression, hence the understanding of the T cell response to SARS-CoV-2 is critical to give insight regarding the management of severely ill COVID-19 patients. The participation of T cells in establishing long-lasting protective immunity against reinfection and the relevance of cross-reactive cellular immunity in future outbreaks are

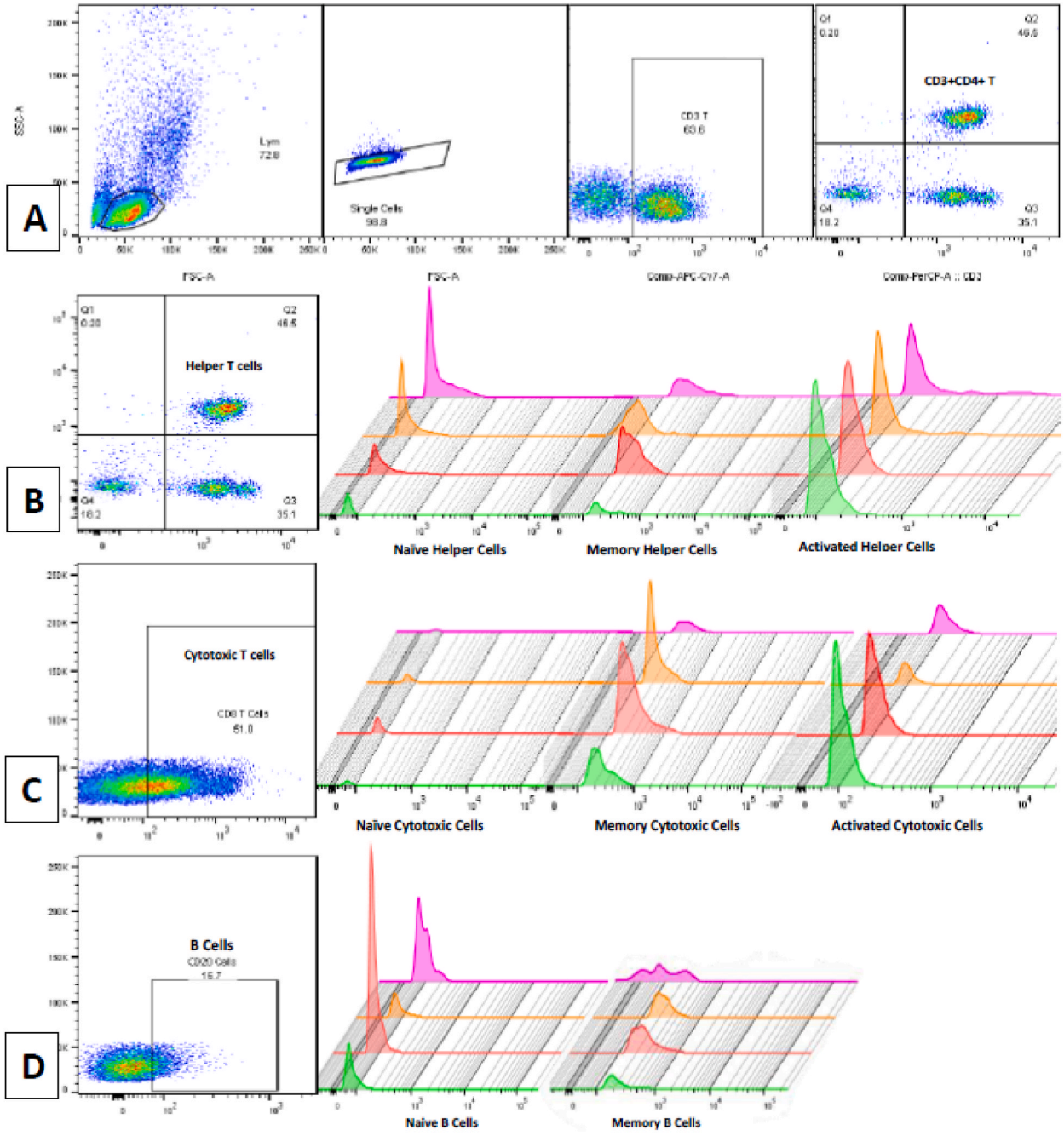


Figure-2. Flow cytometer diagram represents the T and B cell subsets on FlowJo software. [2-A] Gating strategy to select the $CD3^{+}CD4^{+}$ helper T cells after getting the singlets. **[2-B]** Staggered histogram plot was created to represent the data of naïve, memory and activated Helper T cells at the different time points of ICU treatment, with respect to healthy controls. **[2-C]** Staggered histogram plot was created to represent the data of naïve, memory and activated Cytotoxic T cells in COVID-19 patients. **[2-D]** Staggered histogram plot was created to represent the data of naïve and memory B cells. Green plot represents to healthy controls (HC); red plot to COVID-19 patients on zero day of ICU admission; orange plot to 3rd day of admission and pink plot to 6th day of admission.

other important aspects of the T cell response that need to be explored [3].

The helper T cells are the key component of the host immune response in any disease condition. Further, the differentiation of naïve helper T cell population into effector and memory subsets is one of the most fundamental facets of T cell-mediated immunity. Thereby the balance between naïve and memory $CD4^{+}$ T cells is crucial for maintaining an efficient immune response. Our study observed the $CD3^{+}CD4^{+}$ helper T cells, $CD3^{+}CD8^{+}$ cytotoxic T cells, $CD20^{+}$ B cells, and their subsets with further HLA-DR + activation, and

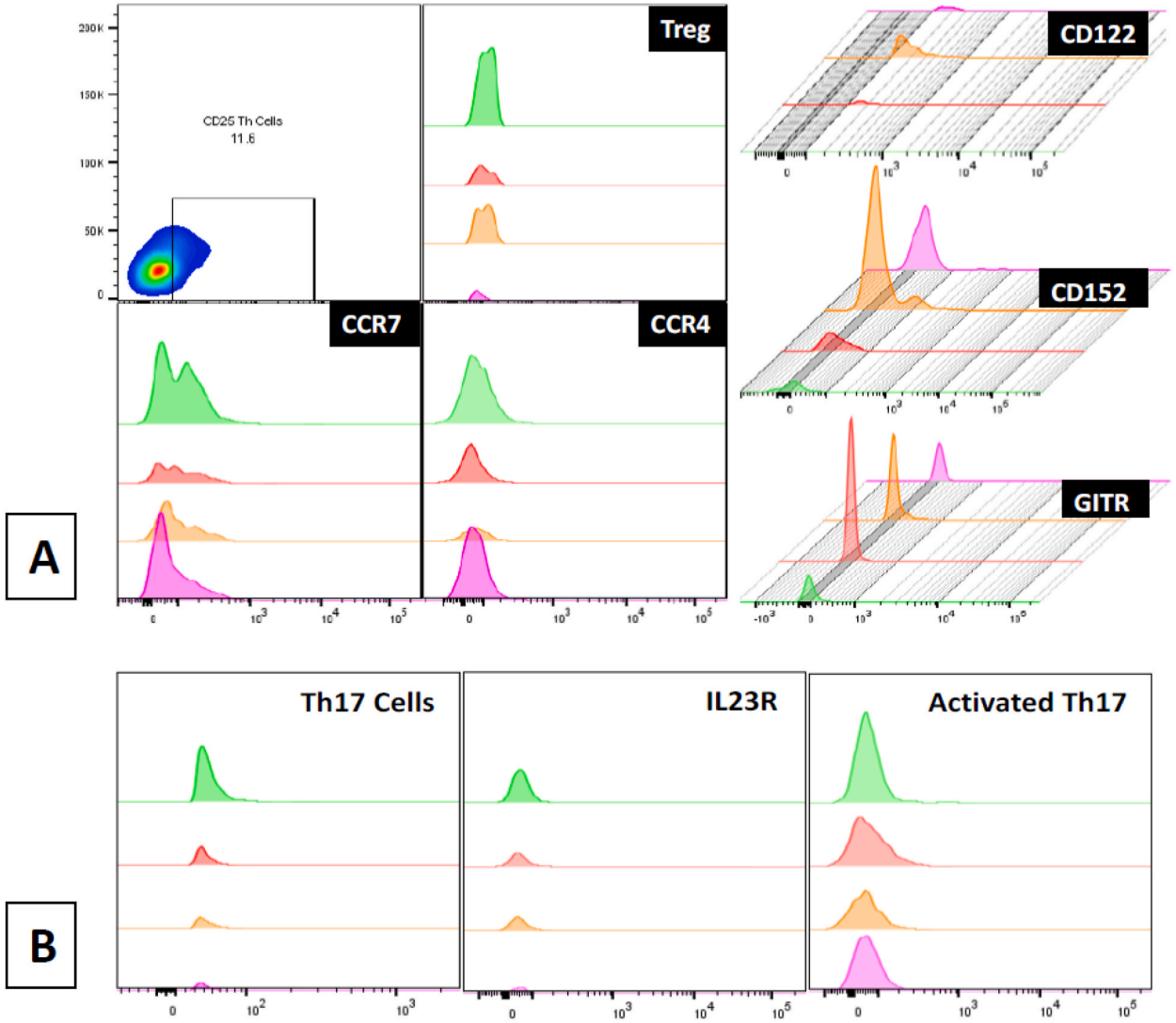


Figure-3. Flow cytometer diagram showing Treg and Th17 cells and their subsets on FlowJo software. [3-A] Half offset histogram plot represents Treg cells and subsets having expression of CCR4 and CCR7 at different time points of ICU treatment. Staggered histogram plots were generated for CD122, CD152 & GITR positive Treg subsets for better data presentation. [3-B] Half offset histogram plots were generated for Th17 cells, activated Th17 cells and IL23R positive subset.
Green plot represents to healthy controls (HC); red plot to COVID-19 patients on zero day of ICU admission; orange plot to 3rd day of admission and pink plot to 6th day of admission.

CD45RO + memory subsets in order to delineate the underlying mechanism and pathogenesis of COVID-19 disease in patients with severe manifestations. We enrolled nine COVID-19 patients admitted to ICU, at all the time points, the T cell response was represented by a reduced $CD8^+$ cytotoxic T cells, and increased numbers of $CD4^+$ helper T cells, indicating an impairment of the protective immune response during SARS-CoV-2 infection in these patients. An increase in the CD4+/CD8+ ratio indicated a poor treatment response in severe COVID-19 patients, while no significant alteration was seen in B cells of COVID-19 patients. These results are in agreement with previous studies. Cui et al. [18], reported reduced $CD8^+$ T cells in 87% patients of SARS, while Mazzoni et al. [19] found the increased $CD4^+$ T cells in COVID-19 patients. Similarly, several others have reported reduced $CD8^+$ cells in severe COVID-19 illness compared to healthy controls suggesting an underlying uncontrolled inflammatory response in such viral infections [20–22]. Lymphopenia is commonly triggered by virus attachment or indirectly by immune grievance from inflammatory mediators. Further to explore the role of $CD4^+$ and $CD8^+$ T subsets in the pathogenesis of severe COVID-19, the expression of naïve (CD45RA), memory (CD45RO) and activation (HLA-DR) markers was analyzed. Additionally, Th17 and Treg associated markers which are largely unidentified, were investigated in SARS-CoV-2 infected patients. The activated cell subsets; CD3+HLADR+, CD4+HLADR+ and CD8+HLADR + cells were exclusively low and further declined during the course of illness in COVID-19 patients, while memory subsets; $CD3^+$CD45RO+, $CD4^+$CD45RO+ and $CD8^+$CD45RO+ were high during early treatment during early course of illness and up to 10 days however during

the later course of illness numbers fell below the normal range of healthy controls. Similar results were reported by Qin et al. and Zhou et al. with reduced memory cells and activated T cells, respectively, in severe COVID-19 patients in China [23,24]. In contrast, few studies also reported the induced activation of helper and cytotoxic T cells in COVID-19 patients compared to controls [23]. A memory B cell response (CD20+CD45RO+) was also achieved within a week, sustained up to 2 weeks maximum, and then declined in severe COVID-19 patients. Both, B & T cell memory responses could not be maintained, conceivably due to excessive reduction of $CD8^+$ T cells in severe COVID-19 cases during the 18 days of ICU stay. And with the persistence of COVID-19 disease, $CD8^+$ T cells continued to decline compared to healthy controls. Henceforth, results indicated that the declined host activation response with a temporary early induced memory response in SARS-CoV-2 infected ICU patients is indicative of a poor outcome.

Th17 and Treg balance play a critical role in maintaining the immune homeostasis, and the equilibrium of proinflammatory and suppressive host immune responses [25]. In our study we observed a low $CD4^+CD25^+$ regulatory T cells (Tregs) and proinflammatory helper T17 (Th17) cells in severe COVID-19 patients compared to controls [23]. Furthermore, analysis of surface molecules confirmed, that although the number of Tregs decreases in severe COVID-19 patients and during treatment, their functional suppressive potential is high with high expression of CD152 (CTLA4), CD122 & GITR. $CD25^+$ helper T cells expressing CD122 were responsible for IL2 signal transduction and activation of NK, B, and T cells [25]. Likewise, pro-inflammatory $CD4^+$CD161+Th17 response was excessively low and became more prominent with the ICU stay. Also, the expression of molecular markers, IL23R and HLA-DR on Th17 cells was decreased which indicated that Th17 cells were functionally exhausted. Low IL23R expression results in absence of IL23/IL23R induced Th17 response and poor expression of HLA-DR indicating the inability to mount a Th17 response. This dysregulated Th17/Treg balance paved the way to COVID-19 disease progression, in severe COVID-19 patients. Though patients infected with SARS-CoV-2 have initially high effector memory helper (T_{EM}) response but the study of expression of other molecules like, CCR7, CD45RA indicated the increased proportion of naïve helper T cells (T_N) in severe COVID-19 patients and reduced CCR7 expressing central memory T cells (T_{CM}) compared with HCs, in SARS-CoV-2 infection.

The older age group is vulnerable and at higher risk between immune-senescence and poor lymphopoiesis due to higher IL6 production. Cui et al. observed significant peripheral depletion of T cell; HLA-DR+, CD45RO+ and effector T cell subsets. The recovery of T cell population is a cardinal component for harnessing recovery in patients with severe COVID-19 disease. The memory T and B cells were not so profound in severe COVID-19 patients.

There are limitations in our study which might make some potential prejudice. It was a single center and small sample study of patients admitted to ICU unit of the hospital. Secondly, patients with secondary infections might affect the immune response of COVID-19 patients. Hence, data from a larger cohort of patients would be beneficial to assess the sequential change in the immune responses after infection with SARS-CoV-2. However, our study gave numerous novel information about host immune response in COVID-19 patients that SARS-CoV-2 might act on lymphocytes, particularly T lymphocytes, inducing a cytokine storm during early infection and a sequence of immune responses which eventually damage the host organs. Therefore, early screening of these specific parameters during critical illness is supportive in the diagnosis and treatment of COVID-19.

The relationship of disease severity in COVID-19 patients is multi-factorial. It appears that the adaptive immunity during SARS-CoV-2 infection is dysregulated with high CD4:CD8 ratio, suggesting poor effector T cell response. The high IL6 levels with reduced T & B cell response and associated lymphopenia are a deadly progression of the COVID-19 disease. Excessive cytokine levels lead to severe inflammation, lung injury, ARDS and hence, play a role in progression of the disease.

Ethical statement

Study has been approved from Institutional Ethical Committee and the reference number is GTBHEC 2021/P-149.

Author contribution

Shukla Das: Conceived the study, interpreted the data & wrote the paper. Gargi Rai: Designed the experiments; Performed the experiments; Contributed reagents, materials, analysis of data. Mohammad Ahmad Ansari: Designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Vikas Sood: Conceived; Analyzed and interpreted the data. Praveen Kumar Singh: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Asha Tyagi: Interpreted the data; Contributed materials, Wrote the paper. Rashmi Salhotra: Interpreted the data; Contributed materials. Chhavi Gupta: Interpreted the data; Wrote the paper. Sajad Ahmad Dar: Designed the experiments; Interpreted the data; Contributed analysis tools. Viniita Kumar Jaggi: Interpreted the data; Contributed reagents, analysis tools or data.

Financial interest

Financial interest The authors declare no financial support from any organization or institute.

Declaration of competing interest

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

Acknowledgement

The authors acknowledge the Multi-disciplinary Research Unit - University College Medical Sciences established by Department of Health Research (DHR), Ministry of Health and Family Welfare, Gov. of India.

References

[1] C. Huang, Y. Wang, X. Li, et al., Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China, Lancet 395 (2020) 497–506.
[2] National Health Commission of the People's Republic of China, Update on the novel coronavirus pneumonia outbreak (Feb 16, 2020), Available at: http://www.nhc.gov.cn/xcs/yqtb/202002/18546da875d74445bb537ab014e7a1c6.shtml.
[3] A public health emergency of international concern over the global outbreak of novel coronavirus declared by WHO, Available at: https://www.who.int/dg/speeches/detail/whodirector-general-s-statement-on-ihr-emergency-committee-on-novel-coronavirus-(2019-ncov.
[4] Indian government official reports on novel coronavirus outbreak available at #IndiaFightsCorona COVID-19 in India, Vaccination, Dashboard, Corona Virus Tracker | mygov.in.
[5] J.F. Chan, et al., Genomic characterization of the 2019 novel human pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan, Emerg. Microbes Infect. 9 (2020) 221–236.
[6] J.F. Chan, et al., A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster, Lancet 395 (2020) 514–523.
[7] D. Wang, et al., Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China, JAMA 323 (2020) 1061–1069.
[8] Z. Xu, et al., Pathological findings of COVID-19 associated with acute respiratory distress syndrome, Lancet Respir. Med. 8 (2020) 420–422.
[9] N. Zhu, et al., A novel coronavirus from patients with pneumonia in China, 2019, N. Engl. J. Med. 382 (2020) 727–733.
[10] N. Chen, M. Zhou, X. Dong, et al., Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study, Lancet 395 (2020) 507–513.
[11] C.K. Wong, C.W. Lam, A.K. Wu, et al., Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome, Clin. Exp. Immunol. 136 (1) (2004) 95–103.
[12] C.K. Min, S. Cheon, N.Y. Ha, et al., Comparative and kinetic analysis of viral shedding and immunological responses in MERS patients representing a broad spectrum of disease severity, Sci. Rep. 6 (2016), 25359.
[13] M.E. Schmidt, S.M. Varga, The CD8 T cell response to respiratory virus infections, J Front. in Immunol. 9 (2018) 678.
[14] Clinical management protocol: COVID-19 of Ministry of health & family Welfare, Gov, of India available at: https://www.mohfw.gov.in.
[15] R. Channappanavar, S. Perlman, Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology, Semin. Immunopathol. 39 (2017) 529–539.
[16] M.H. Chan, V.W. Wong, C.K. Wong, et al., Serum LD1 isoenzyme and blood lymphocyte subsets as prognostic indicators for severe acute respiratory syndrome, J. Intern. Med. 255 (2004) 512–518.
[17] Su R, Li Z, Wang Y, et al. Imbalance between Th17 and regulatory T cells in patients with systemic lupus erythematosus combined EBV/CMV viraemia [published online ahead of print 20 November 2019]. Clin. Exp. Rheumatol.. .
[18] W. Cui, Y. Fan, W. Wu, F. Zhang, J.Y. Wang, A.P. Ni, Expression of lymphocytes and lymphocyte subsets in patients with severe acute respiratory syndrome, Clin. Infect. Dis. 37 (2003) 857–859.
[19] A. Mazzoni, et al., Impaired immune cell cytotoxicity in severe COVID-19 is IL-6 dependent, J. Clin. Invest. (2020), https://doi.org/10.1172/JCI138554.
[20] G. Chen, D. Wu, W. Guo, Y. Cao, D. Huang, H. Wang, et al., Clinical and immunological features of severe and moderate coronavirus disease 2019, J. Clin. Invest. 130 (2020) 2620–2629, https://doi.org/10.1172/JCI137244.
[21] M. Zheng, Y. Gao, G. Wang, G. Song, S. Liu, D. Sun, et al., Functional exhaustion of antiviral lymphocytes in COVID-19 patients, Cell. Mol. Immunol. 17 (2020) 533–535, https://doi.org/10.1038/s41423-020-0402-2.
[22] S. Wan, Q. Yi, S. Fan, J. Lv, X. Zhang, L. Guo, et al., Characteristics of lymphocyte subsets and cytokines in peripheral blood of 123 hospitalized patients with 2019 novel coronavirus pneumonia (NCP), medRxiv (2020), https://doi.org/10.1101/2020.02.10.20021832.
[23] C. Qin, L. Zhou, Z. Hu, S. Zhang, S. Yang, Y. Tao, Dysregulation of immune response in patients with COVID-19 in Wuhan, China, Clin. Infect. Dis. (2020), https://doi.org/10.2139/ssrn.3541136 [Epub ahead of print].
[24] Y. Zhou, B. Fu, X. Zheng, D. Wang, C. Zhao, Y. Qi, et al., Pathogenic T cells and inflammatory monocytes incite inflammatory storm in severe COVID-19 patients, Natl. Sci. Rev. (2020), https://doi.org/10.1093/nsr/nwaa041 nwaa041.
[25] A.H. Banham, Cell-surface IL-7 receptor expression facilitates the purification of FOXP3(+) regulatory T cells, Trends Immunol. 27 (2006) 541–544.