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T-cell dysfunction as a potential contributing factor in post-COVID-19 mucormycosis

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Abstract

Background: The second wave of COVID-19 in India was followed by large number of mucormycosis cases. Indiscriminate use of immunosuppressive drugs, underlying diseases such as diabetes, cancers, or autoimmune diseases was thought to be the cause. However, the mortality was not as high as that seen in non-COVID mucormycosis.

Objective: To study the detailed characteristics of T-cells for evaluating the underlying differences in the T-cell immune dysfunction in post-COVID and non-COVID mucor patients.

Material and method: The study included histopathologically confirmed cases of mucor (13 post-COVID, 13 non-COVID) and 15 healthy individuals (HI). Expression of T-cell activation (CD44, HLADR, CD69, CD38) and exhaustion (CTLA, PD-1, LAG-3 and TIM-3) markers was evaluated by flow cytometry.

Results: All cases showed significant depletion of T-cells compared to HI. Both post-COVID and non-COVID groups showed increased activation and exhaustion as compared to HI. Non-COVID mucor group showed significant activation of CD4+ T cells for HLADR and CD38 ($p = .025$, $p = .054$) and marked T-cell exhaustion in form of expression of LAG-3 on both CD4+ T and CD8+ T cells in comparison with post-COVID patients ($p = .011$, $p = .036$). Additionally, co-expression of PD-1 & LAG-3 and LAG-3 & TIM-3 on CD8+ T cells was statistically significant in non-COVID mucor patients ($p = .016$, $p = .027$).

Conclusion: Immunosuppression in non-COVID mucor showed pronounced exhaustion of T-cells in comparison to post-COVID mucor cases implicating T-cell immune dysfunction is much more severe in non-COVID mucor which are in a state of continuous activation followed by extreme exhaustion leading to poorer outcome.

KEYWORDS
activation, exhaustion, mucormycosis, non-COVID, post-COVID, T cells

1 | INTRODUCTION

Mucormycosis is an invasive fungal disease caused by inhalation of sporangiospores of order mucorales. It is distributed worldwide and associated with the increased morbidity and mortality.[1] The exact prevalence of mucor in India is unknown but the estimated disease burden is approximately 70 times higher than the global prevalence.[2] The most common causative agent is *Rhizopus arrhizus* (also known as *oryzae*) and less common are *Lichtheimia*, *Apophysomyces*, *Rhizomucor* and *Cunninghamella*.[2]

The current epidemiological data shows recent surge in disease incidence due to COVID-19 pandemic. Though the rise in cases was

pan global but largest numbers of cases were reported from India.[3,4] The contributing factors were thought to be indiscriminate use of immunosuppressant such as steroids, tocilizumab and eculizumab in severe COVID-19 patients who had underlying chronic diseases such as diabetes mellitus, tuberculosis, haematological malignancies and autoimmune diseases.[5–9] During the course of treatment, alteration in immune defence system led to increase in the susceptibility of mucor.[10]

Marked lymphopenia is observed in severe COVID infection along with strong activation of CD8+ T-cells and minimal CD4+ T cell activation characterised expression of activation markers such as CD38, CD69, CD39, CD57 and HLA–DR and CD57.[11] Various studies have also shown terminally differentiated or exhausted T cells with expression of cytotoxic T lymphocyte antigen-4 (CTLA), lymphocyte activation gene-3 protein (LAG-3), Programmed cell death domain-1 (PD-1) and T-cell immunoglobulin domain and mucin domain-containing protein-3 (TIM-3) have been demonstrated in COVID-19 in various studies compared with healthy individuals (HI).[12]

Immune response to fungal infection involves both adaptive and innate immunity. Invasive mucormycosis (IM) mucorales specific T-cell predominate throughout the course of infection and produces various cytokines including interferon gamma (IFN-γ) that directly invade the mucorales hyphae.[13] Th1 and Th17 are the predominant CD4+ T Cells involved in the antifungal immune response. Priming of CD4+ T cells are done by dendritic cells and thus help to differentiate in to Th1 and Th17. Th1 cell then secrete IFN-γ and IFN-α which activates the innate immunity cells such as neutrophils, macrophages and dendritic cells.[14,15]

Macrophages and neutrophils play the role of primary immune defence system against the fungal spore. An insufficient immune defence, allows spore germination and growth to establish full blown infection.[16]

The altered cell mediated immune response causes lymphopenia with reduction of CD4+ T & CD8+ T cells and plays the critical link in pathogenesis of mucormycosis in COVID-19 patients.[17,18] However, there were few differences which were noted between the COVID related mucor (post-COVID mucor) and non-COVID related mucor (non-COVID mucor). Firstly, the extent of mucor which was seen after COVID-19 was overwhelming with a total case load of more than 300 at our centre within a span of 3 months, but the mortality rate was not as high as that seen in non-COVID mucor.[19] This led us to hypothesize that there might be some underlying differences in the immune dysregulation between post-COVID mucor and non-COVID mucor patient. This study was aimed to evaluate the characteristics of T-cell subsets (CD4+ T & CD8+ T cells) and differences in T-cell dysfunction between post-COVID mucor and non-COVID mucor.

2 | MATERIAL AND METHODS

2.1 | Study population

The study included thirteen patients each of histopathologically proven post-COVID mucor and non-COVID mucor respectively. Fifteen age and sex matched healthy individual (HI) were also included as control. Written informed consent was taken from each participant. Clinical details, history of underlying chronic disease (diabetes, hypertension, renal diseases, malignancy, autoimmune diseases) and drug history (corticosteroid intake and other immunosuppressant therapy) were noted from hospital records. Haematological and biochemical parameters including complete hemogram, serum ferritin, C-reactive protein and lactate dehydrogenase (LDH) were recorded. Ethical approval was taken by the Institutional Ethics Committee.

2.2 | Flow cytometry assay

Three millilitres of peripheral blood sample were collected in ethylene diamine tetra acetic acid (EDTA) anticoagulant from patients and HI. Sample was prepared by stain-lyse-wash protocol. The panel and clone of antibody fluorochrome used was as follows: (i) Blank tube—without any antibody (ii) T-Cell activation tube—CD3 PerCP-Cy5.5 (SP34-2)/CD4 PE-Cy7 (SK3)/CD8 APC-H7 (SK1)/Fixable viability dye 520 (FVS)/CD44 APC (G44-26)/CD69 BV421 (FN50)/HLADR BV480 (G46-6)/CD38 PE (HIT2) (iii) T-cell exhaustion tube—CD3 PerCP-Cy5.5 (SP34-2)/CD4 PE-Cy7 (SK3)/CD8 APC-H7 (SK1)/Fixable viability dye 520 (FVS)/CTLA APC (BN13)/PD-1 BV421 (MIH4)/LAG-3 BV480 (T47-530)/TIM-3 PE (7D3). The antibodies used were procured from Becton Dickinson (BD) Biosciences, San Jose, California, USA. Dead cells were excluded by staining with Fixable viability stain. Acquisition of cells was done on FACS Canto II flow cytometer, BD Biosciences and FACS Diva version 8 software was used for analysis of data. A minimum of 10,000 CD3+ T cells were acquired in all cases. The gating strategy has been depicted in Figure 1. The mean florescence intensity (MFI) of activation and exhaustion markers on CD4+ T and CD8+ T cells in post-COVID and non-COVID mucor cases has been shown in Figure 2A,B,C.

2.3 | Statistical analysis

The data was described as mean ± SD. The mean fluorescence intensity (MFI) was used to compared expression of antibodies. The continuous variable data of three groups were compared by one-way analysis of variance (ANOVA) and multiple group comparison were done with Tukey–Kramer Post Hoc analysis. Comparison of categorical variables was done by Chi-square test and continuous variables was done by independent sample *T* test. p value <.05 was taken as significant. All the data were analysed with IBM SPSS Statistics for Windows, Version 24.0, Armonk, NY.

3 | RESULTS

3.1 | Demographic and clinical characteristics of study population

Study population included thirteen cases each of post-COVID and non-COVID mucor and 15 HI as control. Rhino-orbital-cerebral

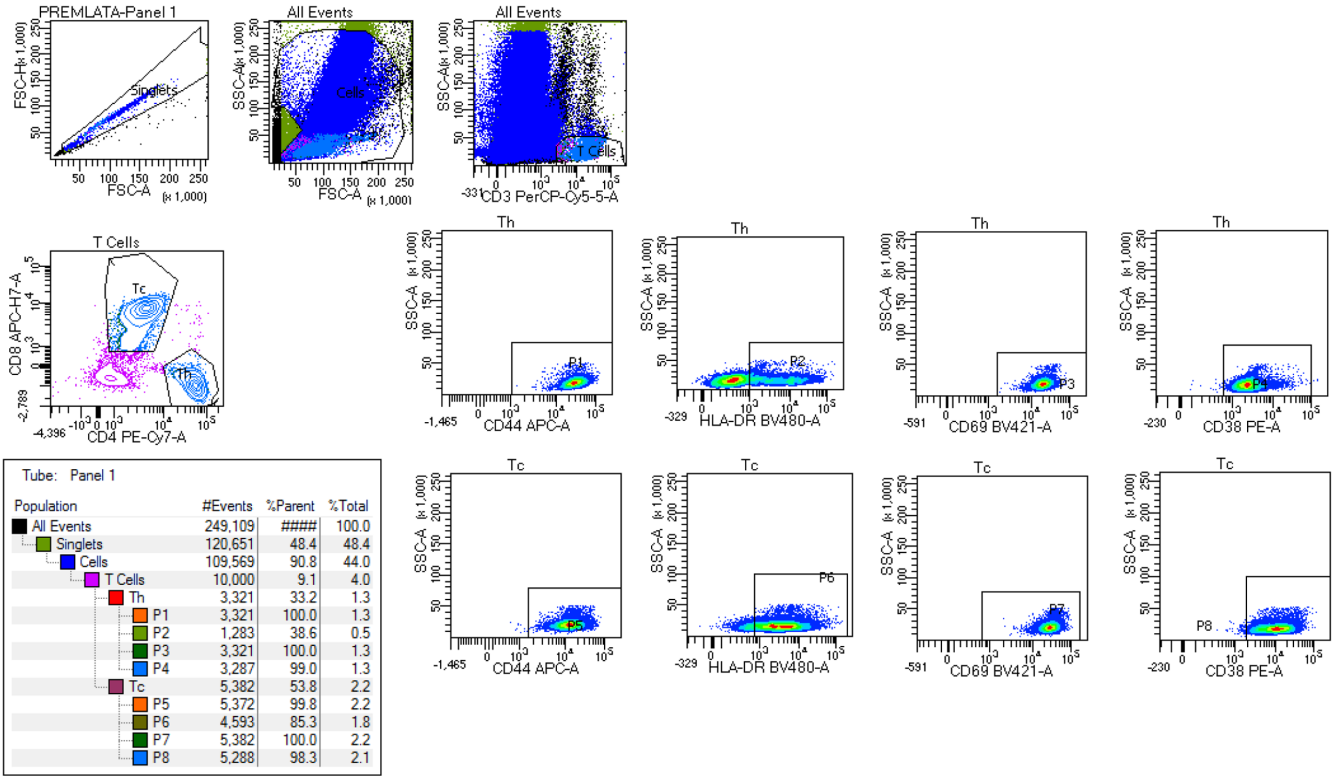


FIGURE 1 Flow plot showing sequential gating strategy; singlets followed by viable cells followed by T cells (SSC vs CD3), followed by CD4+ T and CD8+ T cells (CD4+ vs CD8+), followed by expression of various markers on CD4+ T and CD8+ T cells.

mucor (ROCM) was most common followed by pulmonary mucor in both post-COVID and non-COVID mucor groups. 69.23% (9/13) of post-COVID mucor patients had underlying diabetes mellitus and history of corticosteroid intake during the course of hospitalisation and the average duration of development of mucor was 15–20 days. Among the non-COVID mucor patients, 46.15% (6/13) had underlying diabetes mellitus, 15.38% (2/13) had chronic renal disease and 5.38% (1/13) had breast cancer.

Mild anaemia was observed in non-COVID mucor group as compared to post COVID mucor group and control ($p = .001$). Neutrophilic leukocytosis was present in post-COVID mucor patients ($p < .010$), however no significance difference was seen in platelet count and absolute lymphocyte counts (ALC). Among the biochemical parameters, serum inflammatory markers, for example serum ferritin, C-reactive protein and LDH were significantly higher in patients' group as compared with HI. Demographic and clinical characteristics all three groups are compiled in Table 1.

3.2 | Immunophenotyping characteristics of T cells

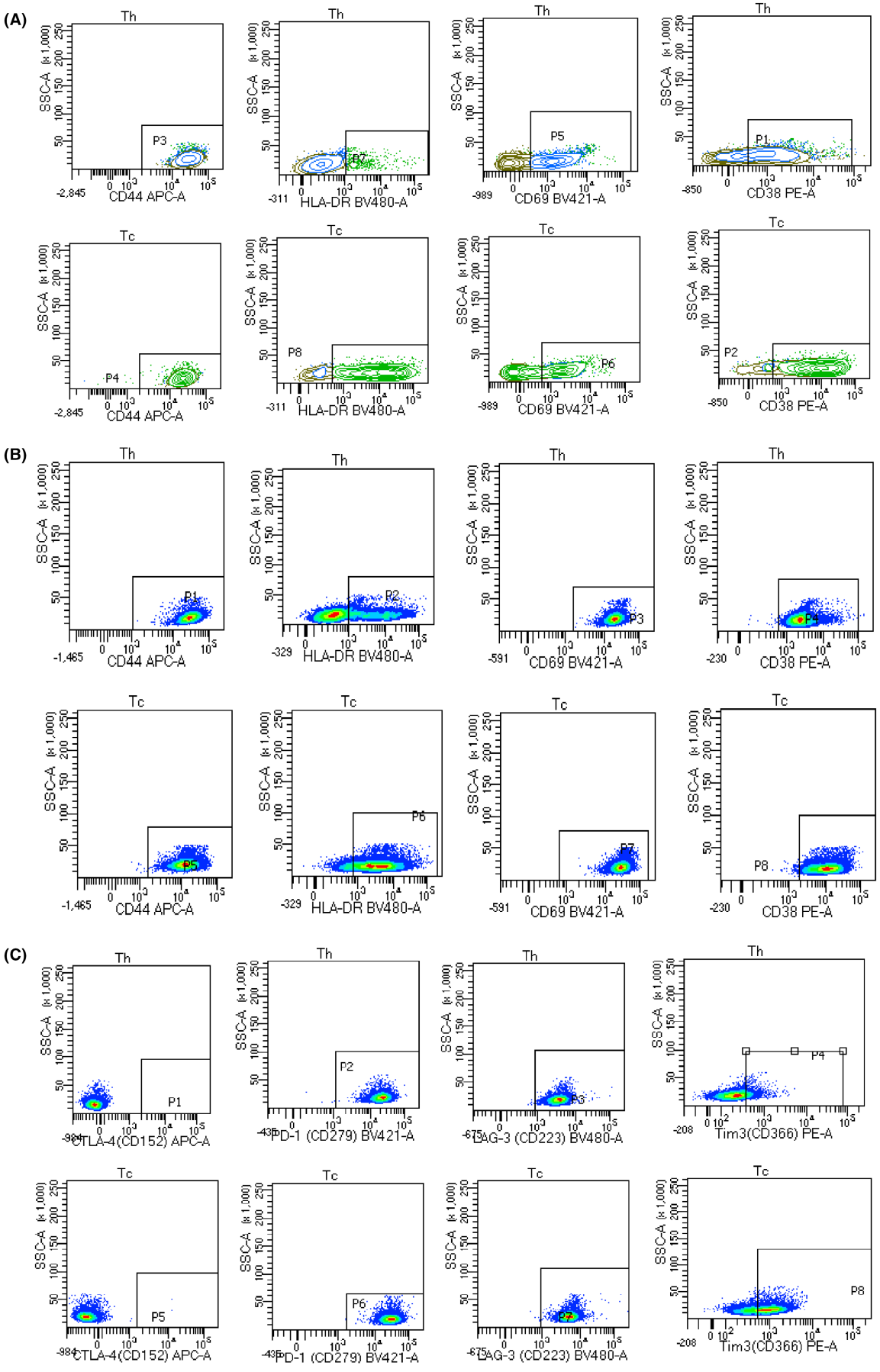
Significant depletion of T cells was observed in both non-COVID and post-COVID mucor groups as compared to HI ($p < .001$) with marked reduction in non-COVID mucor ($P = 0.003$). However, the ratio of CD4+ T and CD8+ T cells was not altered in any group (Figures 3 and 4).

3.3 | Both helper and cytotoxic T cells showed increased expression of all activation markers

(CD44, HLA-DR, CD69 and CD38). On post-hoc analysis, both non-COVID mucor and post-COVID mucor group showed increased expression of HLADR, CD69 and CD38 on both CD4+ T and CD8+ T cells when compared with HI. Except for increased expression of HLADR and CD38 on CD4+ T cells in non-COVID mucor group, none of the marker show statistically significant difference between non-COVID mucor and post-COVID mucor group ($p = .025$, $p = .054$).

We further analysed the expression of exhaustion markers (CTLA, PD-1, LAG-3 and TIM-3) on both T-cell subset. Expression of PD-1 on CD4+ T cells and LAG-3 on both CD4 and CD8+ T cells was significantly increased when compared with HI ($p = .011$, $p = .003$ and $p = .012$). Though on post-hoc analysis, expression of only LAG-3 on both CD4 and CD8+ T was found to be significantly increased in non-COVID mucor group when compared with post COVID mucor group ($p = 0.011$, $p = .036$). The MFI of expression of activation and exhaustion markers are summarised in Table 2.

CTLA was expressed in minority of CD4+ T cells (7.6% patients) and CD8+ T cells (23.1% patients) in non-COVID mucor group. None of the HI or post-COVID mucor patients showed expression of CTLA. Expression of PD-1 was observed in majority of patients in both post-COVID and non-COVID mucor group along with expression in HI (80% of CD4+ T cells and 93.3% of CD8+ T cells) though


(A)
Th
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SSC-A (x 1,000)
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HLA-DR BV480-A
CD69 BV421-A
CD38 PE-A
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SSC-A (x 1,000)
CD44 APC-A
HLA-DR BV480-A
CD69 BV421-A
CD38 PE-A
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P4
P5
P6
P7
P8
(C)
Th
Tc
SSC-A (x 1,000)
CTLA-4(CD152) APC-A
PD-1 (CD279) BV421-A
LAG-3 (CD223) BV480-A
Tim3(CD366) PE-A
P1
P2
P3
P4
P5
P6
P8

FIGURE 2 A. Frequency of expression of activation markers (CD44, HLADR, CD69 and CD38) on CD4+ T (helper) and CD8+ T (cytotoxic) in a post-COVID patients, 2B. Frequency of expression of activation markers CD44, HLADR, CD69 and CD38 on CD4+ T (helper) and CD8+ T (cytotoxic) in non-COVID patients, 2C. Frequency of expression of exhaustion markers (CTLA, PD-1, LAG-3, TIM-3) on CD4+ T (helper) and CD8+ T (cytotoxic) in non-COVID patients.

TABLE 1 Demography, clinical characteristics and laboratory parameters of study groups

Variables (mean)	Post-COVID mucormycosis (n = 13)	Non-COVID mucormycosis (n = 13)	p-value
Clinical characteristics			
Age (years)	46.08	43.9	.467
Male:female ratio	10:2	8:4	.352
Rhino-orbital-cerebral-mucor (ROCM)	10	9	.658
Pulmonary mucor	2	3	
Smoking	4	2	.137
Underlying diseases	9	8	.680
History of steroid intake	9	2	.005
Laboratory parameters			
Haemoglobin (g/dl)	11.9	9.7	.961
Total Leukocyte count ($\times 10^9$/L)	11.52	7.72	.139
Platelet count ($\times 10^9$/L)	216.23	255.52	**.018**
Absolute neutrophil count (×10/L)	7.49	5.86	.640
Absolute lymphocyte count (×10/L)	3.45	1.67	**.023**
Serum ferritin (ng/ml)	1029.7	432.1	**.470**
Serum CRP (mg/dl)	136.8	52.0	.001
Serum LDH (U/L)	1032.7	465.1	**.035**

Statistically significant values are shown in bold.

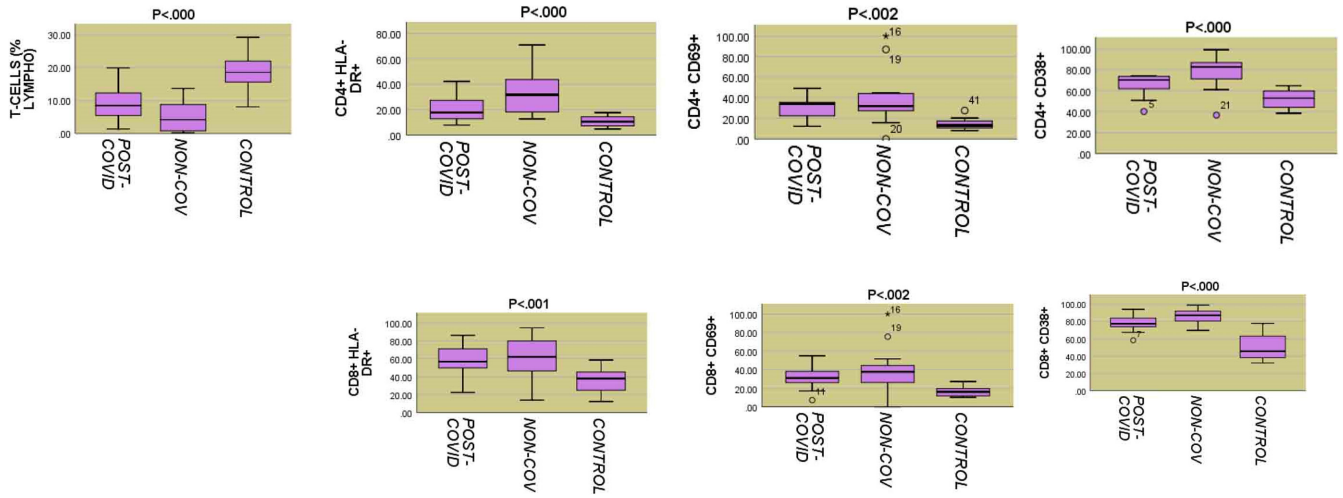

FIGURE 3 Box plot showing comparison of post-COVID, non-COVID and control group for activation markers (HLADR, CD69 and CD38)

the expression in HI was dim as compared with disease group. Significantly increased expression of LAG-1 was noted on CD8+ T (61.5% patients) cells in non-COVID mucor as compared with post-COVID mucor patient (p = .016). Similarly, expression of TIM-3 was observed in both CD4+ T cells (100% patients) and CD8+ T cells (69.3% patients) in non-COVID mucor patients when compared with T cells (CD4+ [7.6%] and CD8+ [53.8%], respectively), in post-COVID mucor patients (p = .001, p = .420).

We also analysed simultaneous expression of different exhaustion markers in all groups and found that co-expression of PD-1 and LAG-3 on both CD4+ and CD8+ T cells was seen in significant number of non-COVID mucor and post-COVID mucor patients in comparison to HI (p = .002, p = .001). However, between non-COVID mucor and post-COVID, co-expression on only CD8+ T cells was found to be significant (p = .016). Additionally, co-expression of PD-1 and CTLA was seen in significant number of non-COVID

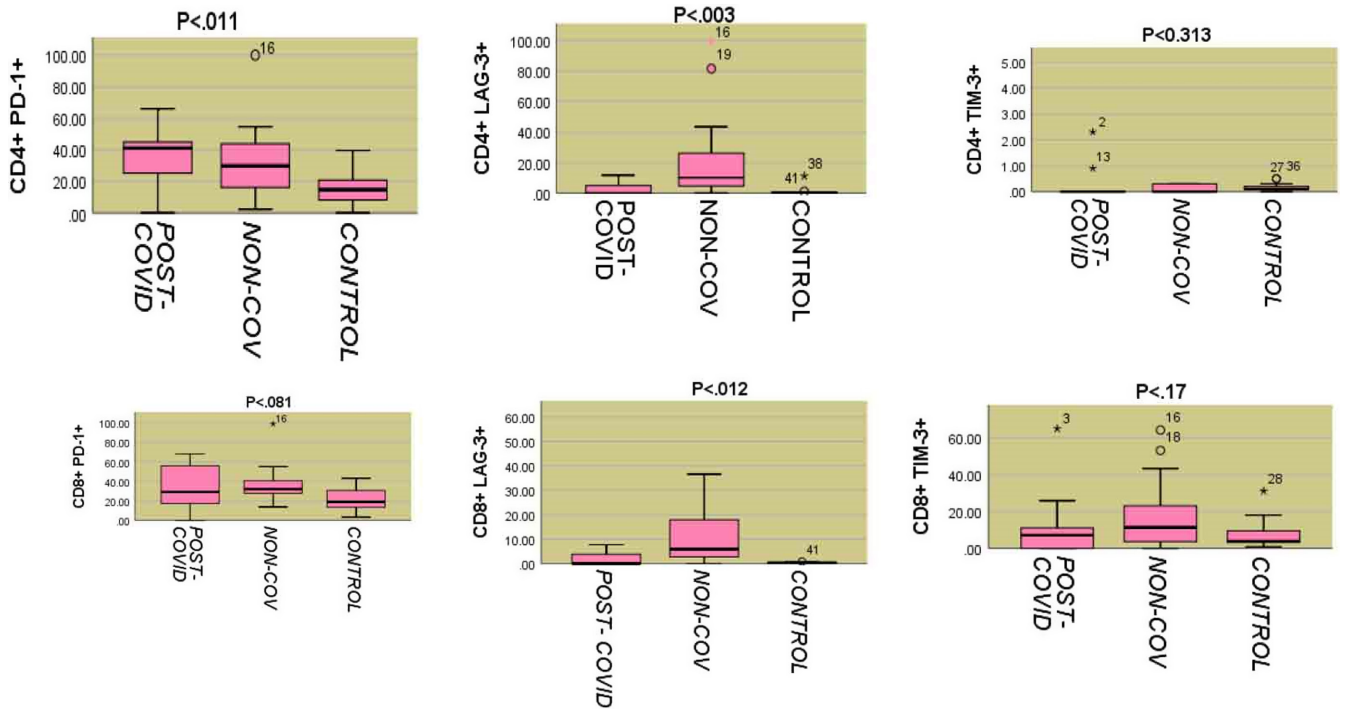

FIGURE 4 Box plot showing comparison of post-COVID, non-COVID and control group for exhaustion markers (PD-1, LAG-3, TIM-3)

and post-COVID cases, but intergroup difference was not significant (p = .031, p = .66). Co-expression of LAG-3 and TIM-3 on CD8+ T cells was statistically significant in non-COVID mucor patients (p = .027). Frequency of co-expression of exhaustion markers is summarised in Table 3.

4 | DISCUSSION

The natural course of post-COVID mucor was variable, ranging from localised behaviour to aggressive disseminated disease. The most common mucorale at our centre was *Rhizopus oryzae*, followed by *R. microspores*.[19] The potent contributing factors for mucor in severe COVID were thought to be underlying diseases, non-judicial steroid intake, mechanical ventilation system, hyperferritinemia, longer duration of hospital stays, improper air filtration and contaminated IV cannulas which are common for any opportunistic mucormycosis infection. Even with these contusive environment prognoses in post-COVID mucor was good with amphotericin B and itraconazole in majority of patients.[19,20]

Severe COVID-19 is charactered by peripheral neutrophilia.[21] Neutrophils are potent in providing immunity against the mucorales by generation of oxidative metabolite which could be a reason for better prognosis in post-COVID mucor. On the contrary, severe COVID is characterised by lymphopenia owing to sequestration of lymphocyte in lungs. Persistent viral stimulation of T cells leads to activation followed by exhaustion of T cells. This immune dysregulation may lead to opportunistic fungal infections.[22,23] However, incidence of candida and aspergillosis is far more common than mucormycosis in ICU setting.[24] Lymphopenia as such does not increase susceptibility to mucorale as seen in an autopsy study of mucormycosis in HIV patients.[25] Several factors such as hyperferritinemia, diabetic ketoacidosis and endothelitis have been considered to be contributing factors in post-COVID mucor development. We observed lymphopenia with reduction of total number T lymphocytes in both post-COVID and non-COVID mucor patients with reduction in both CD4+ and CD8+ T cells compared with HI as seen in previous studies.[26,27] However, the ratio of CD4+ T cell and CD8+ T cells was unaltered. We further evaluated the dysfunction of T cells in both groups to find any difference which can lead us to understand the better prognosis of post-COVID mucor in comparison to non-COVID mucor.

T-cell activation is the phenomenon that plays an important role in immune response against foreign antigen and is initiated by interaction between the antigens specific T-cells and antigen presenting cells (APC). Impaired T-cell activation causes infectious pathology while unregulated T-cell activation can cause autoimmunity.[28] Severe COVID-19 is characterised by hyperactivation of both CD4+ and CD8+ T cells and show expression of CD38, CD69 and HLADR.[12] In our study also, we found pronounced expression HLA-DR, CD69 and CD38 in patient group and more so in non-COVID mucor patients on both CD4+ and CD8+ T cells as compared with HI (MFI-35.56, 38.66 and 77.22 in CD4+ T and 62.06, 39.93 and 85.34 in CD8+ T cells, respectively). CD44 is not a marker of recent T-cell receptor (TCR) activation, and this can explain the expression in normal HI in our study.[29] CD4+ T cells play important role in clearance of mucor, and few studies have demonstrated reduction in CD4+ and CD8+ T cells but we did not find any study on T-cell activation pattern in mucor cases in literature.[16] HLADR expression on CD4+ T cells was significantly higher in non-COVID mucor and it can be used as diagnostic purposes.

TABLE 2 Mean Florescence intensity (MFI) of activation and exhaustion markers on $CD4^+$ T and $CD8^+$ T cells.

Variables	Post-COVID mucormycosis ($n = 13$)	Non-COVID mucormycosis ($n = 13$)	Healthy Individuals ($n = 15$)	*p*-value	*p*-value between post-COVID and Non-COVID
Total events (million)	3.29	10.51	4.05	**.010**	**.003**
T-cells (% of total viable cells)	9.21	4.95	18.33	**.001**	.113
$CD4^+$ (%T cells)	40.40	42.25	48.26	.361	.948
$CD8^+$ (%T cells)	52.64	41.88	40.71	.139	.241
Activation marker					
$CD4^+$ $CD44^+$(% $CD4^+$)	99.99	99.95	99.84	.403	.946
$CD4^+$ $HLA\text{-}DR^+$ (% $CD4^+$)	21.50	35.56	10.66	**.001**	**.025**
$CD4^+$ $CD69^+$ (% $CD4^+$)	31.35	38.66	14.68	**.002**	.519
$CD4^+CD38^+$ (% $CD4^+$)	66.06	77.22	51.74	**.001**	**.054**
$CD8^+$ $CD44^+$ (% $CD8^+$)	99.95	99.33	97.34	.130	.893
$CD8^+$ $HLA\text{-}DR^+$ (% $CD8^+$)	58.97	62.06	35.7	**.001**	.904
$CD8^+$ $CD69^+$ (% $CD8^+$)	32.64	39.93	16.87	**.003**	.514
$CD8^+$ $CD38^+$ (% $CD8^+$)	78.27	85.34	50.62	**.001**	.285
Exhaustion marker					
$CD4^+$ $CTLA^+$ (% $CD4^+$)	0.15	1.53	00	.349	.430
$CD4^+$ $PD\text{-}1^+$(% $CD4^+$)	35.62	33.53	15.30	**.011**	.957
$CD4^+$ $LAG\text{-}3^+$ (% $CD4^+$)	2.20	24.16	1.06	**.003**	**.011**
$CD4^+$ $TIM\text{-}3^+$ (% $CD4^+$)	2.44	4.03	0.166	.313	.817
$CD8^+$ $CTLA^+$ (% $CD8^+$)	0.231	9.01	0.087	.063	.104
$CD8^+$ $PD\text{-}1^+$(% $CD8^+$)	33.69	38.38	22.22	.081	.806
$CD8^+$ $LAG\text{-}3^+$ (% $CD8^+$)	2.03	20.24	0.38	**.012**	**.036**
$CD8^+$ $TIM\text{-}3^+$ (% $CD8^+$)	11.75	19.07	7.36	.175	.491

Statistically significant values are shown in bold.

TABLE 3 Frequency of expression of exhaustion markers in all three subgroups

Variables	Post-COVID mucormycosis ($n = 13$)	Non-COVID mucormycosis ($n = 13$)	Healthy individuals ($n = 15$)	*p*-Value	*p*-value between post-COVID and Non-COVID
$CD4^+$ $LAG\text{-}3^+$	4/13 (30.7%)	9/13 (69.2%)	0/15 (0%)	**.001**	.050
$CD4^+$ $TIM\text{-}3^+$	1/13 (7.6%)	13/13 (100%)	0/15 (0%)	**.001**	**.001**
$CD8^+$ $CTLA^+$	0/13 (0%)	3/13 (23.1%)	0/15 (0%)	**.031**	.066
$CD8^+$ $PD\text{-}1^+$	12/13 (92.3%)	13/13 (100%)	14/15 (93%)	.08	.308
$CD8^+$ $LAG\text{-}3^+$	2/13 (15.3.%)	8/13 (61.5%)	0/15 (0%)	**.001**	**.016**
$CD8^+$ $TIM\text{-}3^+$	7/13 (53.8%)	9/13 (69.2%)	4/15 (26.2%)	.07	.420
$CD4^+$ $PD\text{-}1^+$ $LAG\text{-}3^+$	4/13 (30.8%)	8/13 (61.5%)	0/15 (0%)	**.002**	.116
$CD4^+$ $LAG\text{-}3^+$ $TIM\text{-}3^+$	0/13 (0%)	2/13 (26.7%)	0/15 (0%)	.104	.141
$CD8^+$ $PD\text{-}1^+$ $LAG\text{-}3^+$	2/13 (15.4%)	8/13 (61.5%)	0/15 (0%)	**.001**	**.016**
$CD8^+$ $PD\text{-}1^+$ $TIM\text{-}3^+$	6/13 (46.2%)	9/13 (69.2%)	4/15 (26.2%)	.079	.234
$CD8^+$ $PD\text{-}1^+CTLA^+$	0/13 (0%)	3/13 (23.1%)	0/15 (0%)	**.031**	.066
$CD8^+LAG\text{-}3^+TIM\text{-}3^+$	1/13 (7.7%)	6/13 (46.2%)	0/15 (0%)	**.003**	**.027**

Statistically significant values are shown in bold.

Exhausted T cells are effector T cells which on exposure to persistent antigen, gets activated and becomes dysfunctional with reduced ability to produce cytokines. These cells are distinguished by expression of several co-inhibitory molecules such as PD-1, TIM-3, LAG-3 and CTLA. The function of these co-inhibitory receptors, are required for homeostasis of lymphocytes suggesting it to be novel target for treatment in tumour and infection.[30,31] This phenomenon has also been reported in various chronic diseases in human such as HIV,

Hepatitis-B, Hepatitis-C and in some malignancies.[32–34] Upregulation of these receptor in severe COVID-19 infection has been studied in recent literature though we did not find any literature in mucormycosis.

Expression of CTLA-4 is seen on activated T cells and T-regulatory cells (Treg), and it competes with CD28 receptors for binding to B7 ligands on APCs. It causes anergy of APC by sequestration of B7 ligands.[35] In our study, only 23.1% cases in non-COVID mucor showed co-expression of CTLA and PD-1 on CD8+ T cells. None of the HI or post-COVID patient showed expression of CTLA implicating it to be marker of extreme exhaustion. In comparison, PD-1 expression was seen on both helper and cytotoxic T cells in patient as well as in HI, though the mean florescent intensity was more in patients. PD-1 is expressed on T cell, B cell, NK cell and (T regulatory cells) Treg. PD-1 upon binding with its ligands inhibit proliferation, cytokine secretion and cytotoxic ability of effector immune cells and leads to immune dysfunction.

Similar to CTLA, LAG-3, was expressed in non-COVID mucor in comparison with post-COVID mucor and HI on both CD4+ T and CD8+ T ($p = .011$, $p = .036$). It regulates the immune response via directly inhibiting the activation and proliferation of T-cells, promoting inhibitory action of T-reg and regulating the function of APC'S. Similar to other exhaustion markers like CTLA & PD-1, LAG-3 is not expressed by the naive T-cell, but its expression can be induced in CD4+ & CD8+ by antigenic stimulation.[36] Continuous stimulations by antigen such as viruses, bacterial and parasites causes the sustained and increased expression of LAG-3 in both CD4+ & CD8+ cells.[37,38] LAG-3 works synergistically with PD-1 to suppress autoimmunity and antitumor immunity.[39] It has been observed that in ovarian epithelial tumour, 80% LAG-3+ tumour infiltrating lymphocytes (TIL) also showed synergistic expression of PD-1.[40] we also found co-expression of PD-1 and LAG-3 in our non-COVID mucor cases.

TIM-3 acts through its ligand galactin-9 which is seen to be increased in severe COVID-19 infection, hepatitis B and HIV.[41,42] Overexpression of TIM-3 have been seen CD4+ T cells in critical COVID patients.[43] We observed TIM-3 expression on CD4+ T cells of all non-COVID mucor whereas TIM-3 on CD8+ T cells showed expression in both patient and control group.

By far anti PD-1 immunotherapy is most studied and approved against various viral infections and malignancy. Check-point inhibition of PD-1/PD-L1 pathway have shown improved outcomes in immunosuppressed mice affected with invasive mucormycosis in even without concomitant antifungal drugs.[44] Both LAG-3 and TIM-3 along with PD-1 can be potential target for immunotherapy in non-COVID mucormycosis.

5 | CONCLUSION

Immunosuppression in post-COVID mucor show less pronounced exhaustion of T cells in comparison with non-COVID mucormycosis cases which are in a state of continuous activation followed by extreme exhaustion represented by marked co-expression of PD-1/LAG-3/TIM-3 on both CD4+ T and CD8+ T cells leading to poorer outcome. Targeted therapy against these immune checkpoints may be helpful in invasive mucormycosis.

AUTHOR CONTRIBUTIONS

Himanshu Dandu: Conceptualization; Methodology; Writing—review & editing; Formal analysis; Writing—original draft; Validation; Software. Manish Kumar: Data curation; Investigation; Writing—original draft. Hardeep Singh Malhotra: Conceptualization; Writing—original draft. Naveen Kumar: Data curation. Neeraj Kumar: Writing—review & editing. Prashant Gupta: Writing—review & editing; Investigation. Bipin Puri: Resources; Writing—review & editing; Project administration. Geeta Yadav: Conceptualization; Methodology; Investigation; Supervision; Formal analysis; Writing—original draft; Writing—review & editing; Validation.

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CONFLICT OF INTEREST

There is no conflict of interest for the authors participating in the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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