Immune Dysregulation of Nucleocapsid-Specific CD4+ T-Cell Responses in Neuro-Long-COVID Endotype

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BACKGROUND and AIM

- 10-20% of SARS-CoV-2 infections lead to Long-COVID (LC): multisystemic disorder with multiple and heterogeneous symptoms that last at least 3 months.
- Neurocognitive disturbances (neuroLC) represent a major and health public concern, affecting over 60% of patients.

AIM: to evaluate the contribution of cellular immune dysregulation to neuroLC in the context of SARS-CoV-2- and Herpesvirus-specific immune responses, as well as potential herpesvirus reactivation.

STUDY GROUPS AND CLINICAL SYMPTOMATOLOGY

Table 1. Epidemiological characteristics of study groups.

	neuroLC n=43	LC n=29	Recovered n=28	Uninfected n=32	p-value
Sex, Female, n (%)					
Female	37 (86%)	23 (79%)	25 (89%)	20 (62.5 %)	0.0287
Male	6 (14%)	6 (21%)	3(11%)	12 (37.5%)	
Age, median (IQR)*	49 (43-55.5)	47 (37.5-59)	47 (37-55)	45 (34.5-58.8)	ns
Number of SARS-CoV-2 vaccination, n (%)*					
0 dose	-	-	1 (4%)	15 (47%)	
1 dose	4 (9%)	2 (7%)	-	1 (3%)	
2 dose	16 (37%)	12 (41%)	4 (14%)	1 (3%)	
3 dose	20 (47%)	11 (38%)	18 (64%)	9 (28%)	
4 dose	3 (7%)	3 (10%)	3 (11%)	4 (13%)	
5 dose	-	1 (3%)	2 (7%)	2 (6%)	
Number of SARS-CoV-2 infections, n (%)*					
0	-	-	-	32 (100%)	
1	22 (51%)	20 (69%)	16 (57%)	-	
2	21 (49%)	9 (31%)	2 (7%)	-	
3	-	-	2 (7%)	-	
4	-	-	1 (4%)	-	
NA			7 (25%)	-	
Days from first infection, median (IQR)*	1029 (808-1068)	729 (634.5-1102)	717 (536-1053)	-	ns
SARS-CoV-2 Variant					
Wh1	36 (84%)	21 (72%)	8 (29%)	-	0.0013
Alpha 08/02/2021	3 (7%)	-	1 (4%)	-	
Delta 28/06/2021	3 (7%)	1 (4%)	3 (10%)	-	
Omicron 20/12/2021	1 (2%)	7 (24%)	9 (32%)	-	
NA	-	-	7 (25%)	-	
Days from last infection, median (IQR)*	405 (239-1047)	671 (389-741.5)	608 (471-1014)	-	ns
SARS-CoV-2 Variant					
Wh1	19 (44%)	14 (48%)	5 (18%)	-	
Alpha 08/02/2021	-	-	1 (4%)	-	
Delta 28/06/2021	3 (7%)	1 (4%)	2 (7%)	-	
Omicron 20/12/2021	15 (35%)	14 (48%)	13 (68%)	-	
NA	-	-	7 (25%)	-	
Herpesvirus reactivation, n(%)	-	-	-	-	

* Information at sample timepoint; NA: non-available

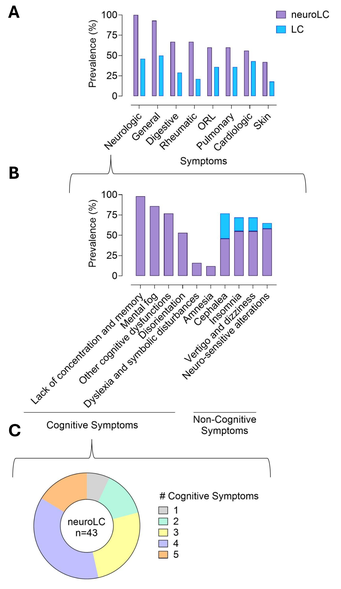


Figure 1. Clinical symptomatology in neuroLC and LC groups. (A) Prevalence of general symptoms categories. **(B)** Prevalence of cognitive and non-cognitive neurological symptoms. **(C)** Distribution of the number of cognitive symptoms in neuroLC group, median 4 (IQR, 3-4).

STUDY DESIGN AND METHODS

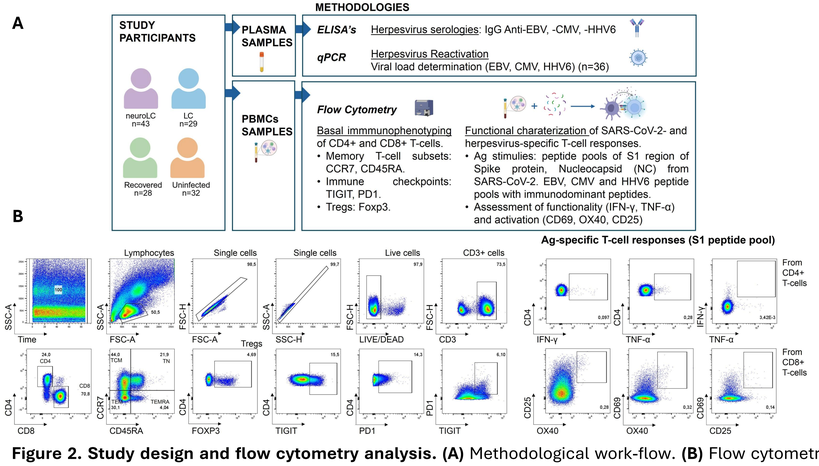


Figure 2. Study design and flow cytometry analysis. (A) Methodological work-flow. **(B)** Flow cytometry gating strategy for basal phenotyping and functional characterization of Ag-specific T-cell responses. The gating strategy is shown for CD4+ T-cells and applied similarly to CD8+ T-cells. All data generated by these methods were analyzed using non-parametric statistical tests, including the Mann-Whitney U test and the Kruskal-Wallis test.

The neuroLC endotype is characterized by increased SARS-CoV-2 nucleocapsid-specific CD69+OX40+ CD4+ T-cells

RESULTS

I. Comparable IgG levels against EBV, CMV and HHV6 across groups

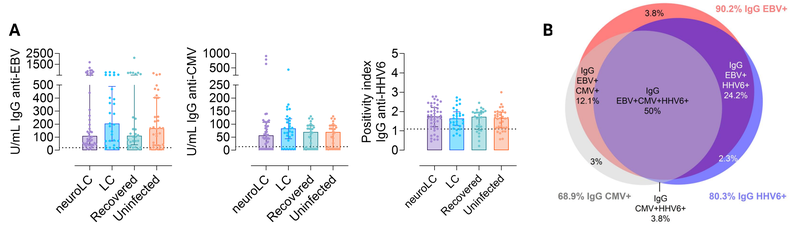


Figure 3. Herpesvirus seroprevalence across study groups. (A) IgG seropositivity for EBV, CMV and HHV6 across study groups. **(B)** Overlap of herpesvirus seropositivity is represented in Venn diagram. Ninety percent of participants were seropositive for at least two herpesviruses. No evidence of herpesvirus reactivation was detected. Bar graphs show the median ± IQR.

II. The LC group exhibits elevated CD8+ TEMRA T-cells compared with Recovered

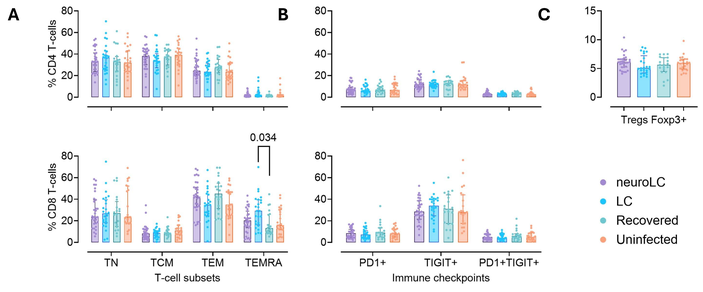


Figure 4. Analysis of CD4+ (top) and CD8+ (bottom) T-cell populations in PBMCs from study groups. (A) T-cell subsets: naïve, TN; central memory, TCM; effector memory, TEM; effector memory re-expressing CD45RA, TEMRA. **(B)** Percentage of CD4+ and CD8+ T-cells expressing immune checkpoint molecules (TIGIT, PD1). **(C)** CD4 regulatory T-cells (Foxp3+). Bar graphs show the median ± IQR.

III. Stronger CD4+ T-cell responses to SARS-CoV-2 antigens in neuroLC compared with herpesvirus

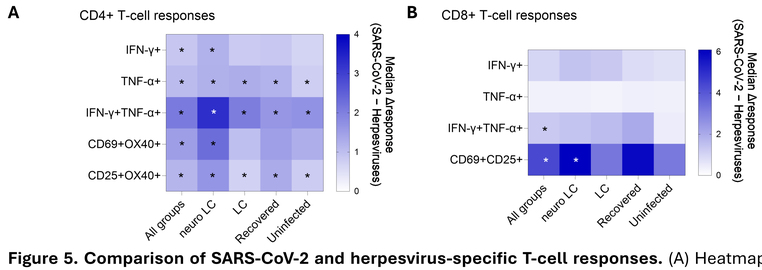


Figure 5. Comparison of SARS-CoV-2 and herpesvirus-specific T-cell responses. (A) Heatmap of stimulation index (SI) median differences in CD4$^+$ T-cell responses between SARS-CoV-2 and herpesvirus. (B) Heatmap of median differences in CD8$^+$ T-cell responses.

IV. Increased CD69+OX40+ NC-specific CD4+ T-cells in neuroLC

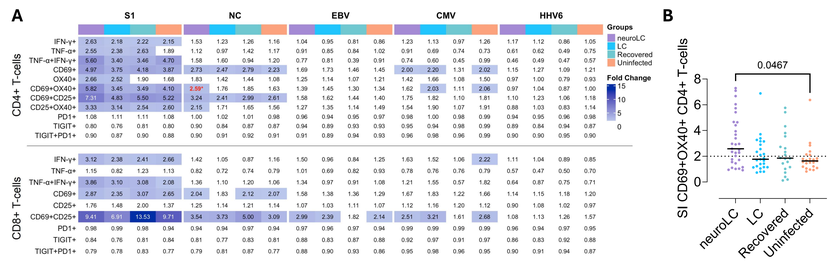


Figure 6. Antigen-specific CD4+ and CD8+ T-cell responses across study groups. (A) Heatmaps showing the median (SI) of cytokines (IFN, TNF), activation induced markers (CD69, OX40, CD25) and immune checkpoints (TIGIT, PD1) following stimulation with SARS-CoV-2 peptide pools (S1, NC) and herpesvirus peptide pools (EBV, CMV, HHV6). **(B)** Dot plot showing CD69+OX40+ CD4+ T-cell responses under NC stimulation. Positive responses are defined as SI >2 and are indicated by the dashed line. The solid line represents the median.

V. Unsupervised analyses confirm alterations in CD69+OX40+ NC-specific CD4+ T-cells in neuroLC associated to the presence of two differential clusters

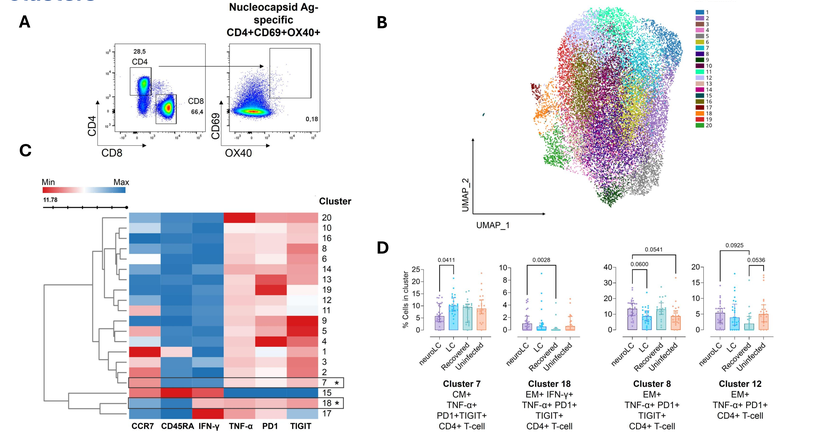


Figure 7. Unsupervised analysis of NC-specific CD69+OX40+ CD4+ T-cells. (A) Gating representation of NC-specific CD69+OX40+ CD4+ T-cells. **(B)** UMAP plot showing cellular clusters identified using the FlowSOM algorithm. **(C)** Heatmap of Z-score-normalized median fluorescence intensity for each marker across clusters. **(D)** Bar plots showing comparisons between groups within each cluster, indicating significant differences ($p<0.05$) or trends ($p< 0.1$). Bar graphs show the median ± IQR.

CONCLUSIONS

- Despite high herpesvirus seropositivity, no evidence of herpesvirus reactivation or virus specific T-cell responses was associated with neuroLC, although samples were collected two years post-infection.
- Different immune dysregulation signatures were observed according to Long-COVID endotype: **i)** LC group exhibited elevated TEMRA CD8+ T-cells; **ii)** neuroLC group showed elevated NC-CD69+OX40+ CD4+ T-cell responses.
- These data reveal **overall immune perturbations in Long-COVID,** suggesting **nucleocapsid antigen** persistence as a potential contributor to neuroLC immunopathogenesis.

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