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Deficient SARS-CoV-2 hybrid immunity during inflammatory bowel disease

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ABSTRACT

Patients with Inflammatory Bowel Disease (IBD) undergoing immunosuppressive therapies face heightened susceptibility to severe COVID-19. An in-depth understanding of systemic inflammation and cellular immune responses after SARS-CoV-2 vaccination and breakthrough infections (BTI) is required for optimizing vaccine strategies in this population.

While the prevalence of high serological responders post- third COVID-19 vaccine dose was lower, and the antibody waning was higher in IBD patients than in healthy donors (HD), IBD patients showed an increase in anti-RBD Wild Type IgG levels and cross-reactive Spike -specific memory B cells following BTI. However, there was no significant enhancement in cellular immune responses against anti-SARS-CoV-2 post-BTI, with responses instead characterized by activation of SARS-CoV-2 specific and also bystander CD8 T cells. These results suggest a complex interaction between chronic inflammation in IBD and the generation of new immune responses, highlighting the need for tailored vaccine regimens and anti-inflammatory therapies to boost cellular immunity against SARS-CoV-2.

1. Introduction

The COVID-19 pandemic, caused by SARS-CoV-2 has emphasized the pivotal role of immune status in eliciting robust immune responses to vaccines and in fighting infections from both the original virus strains and emerging variants of concern (VOCs) [1,2]. IBD patients represents a high-risk population for severe COVID-19, not only due to the inflammatory nature of the disease itself [3] but also because many undergo immune-suppressive therapy [4–7].

This study aims to characterize the unique inflammatory landscape in triple-dose vaccinated patients with IBD, both preceding [8–13] and following breakthrough infections (BTI), in comparison with vaccinated healthy individuals experienced similar BTI [14].

A key objective was to assess whether the previously observed reduced serological response in vaccinated patients with IBD [15–20] could impede the establishment of hybrid immunity following natural infection with VOCs. Here, we outline the serological, humoral, and cellular responses in vaccinated persons with and without IBD who subsequently experienced BTI.

Overall, our findings reveal a distinct pattern: while BTI enhances humoral responses in IBD patients, it does not improve their cellular immunity. The persistent systemic inflammation and activation of both bystander or SARS-CoV-2-specific T cells in these patients underscore the need for targeted booster strategies. Such strategies should

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incorporate the monitoring of inflammatory markers, to ensure that booster doses induce an adequate cellular response extending protection beyond Spike protein-specific immunity.

2. Results

2.1. Inflammatory & Innate profile of IBD patients during BTI

We initially characterized the systemic inflammatory profile of a longitudinal cohort of IBD patients, including individuals who later developed BTI (n = 113, with post-BTI follow-up PBMCs samples available for 24 patients). These IBD patients, who included individuals with either Crohn's Disease (CD, n = 12) or Ulcerative Colitis (UC, n = 12), were treated with various immune-suppressive therapies as per clinical guidelines, including anti-TNF (mainly), anti-α4-β7, anti-IL-12/IL-23, and JAK-inhibitors, see Table 1. For controls, we included 37 vaccinated healthy donors (HD), in the acute phase (1–6 weeks post-BTI), see Table 2, vaccinated with rheumatoid arthritis (RA) patients as an inflammatory disease control group (Table 3), vaccinated HDs, and pre-pandemic HDs.

Using Olink technology, we defined the plasma proteome by measuring the normalized protein expression (NPX) of 92 molecules, Fig. 1A. An inflammatory signature distinctive to inflammatory diseases (RA and IBD) emerged, differing from those observed in acute BTI and HD (vaccinated and unvaccinated), Fig.S1A. Specifically, vaccinated IBD patients with/without BTI (longitudinal patients treated exclusively with anti-TNF) displayed an inflammatory profile that was significantly elevated compared to vaccinated HD, COVID-19 convalescent, BTI HDs, as well as vaccinated RA patients, Fig. 1B, and Fig.S1B. In particular, the concentration of TNF, Caspase 8, MMP-1, MCP-4, and HGF, were elevated in IBD patients compared to vaccinated HDs. Caspase 8 and TNF were significantly increased over levels found in BTI HDs. The TNF signature in IBD patients also differed from that in RA patients, reflecting distinct disease-related inflammatory patterns and responses to anti-inflammatory treatments. Notably, only CXCL9 (a Th1-associated chemokine) was upregulated after BTI in IBD patients, and was associated with the increased concentration of OSM and TNFSF14 compared to BTI HDs. However, BTI did not induce persistent inflammation in IBD patients; rather, it resulted in a down-modulation of Th1/Th17 cytokines (TNF, IL-17 A), typically elevated in this group.

To complement this analysis, we included additional markers of innate immune activation specifically modulated during severe COVID-19, Fig. 2A.

The concentration of pro-inflammatory molecules such as Galectin-9, sCD14, sCD163, LBP, IL-18, IP-10, Calprotectin and MPO were persistently more elevated after BTI in HD in comparison to vaccinated HDs. Similarly, an elevated concentrations of pro-inflammatory molecules were detected after BTI in IBD and further included PF-4 (CXCL-4), and PTX3. Principal Component Analysis (PCA) highlighted the distinct inflammatory signature in IBD patients (Fig. 2B), and these differences were quantified in the Fig. 2C.

The global overview of innate immunological changes after BTI, was summarized in the Fig. 2D and revealed different inflammatory networks in IBD patients such as Calprotectin/IP-10, LBP/Galectin-9, LBP/PF4, and sCD14/sCD163. Although quantitative differences of inflammatory markers were limited after BTI between IBD and HDs, IBD patients exhibited stronger correlations between inflammatory molecules, suggesting heightened interconnectivity within the inflammatory response.

2.2. Humoral response & SARS-CoV-2-binding B cells during BTI in IBD

We investigated the humoral response to SARS-CoV-2 vaccination and BTI in persons living with IBD to assess potential differences in vaccine-elicited immunity, Fig. 3. The humoral response (anti-RBD SARS-CoV-2 wild type IgG) to vaccines and BTI was monitored longitudinally in HDs, Fig. 3A. The median half-life of antibody titer ($t_{1/2}$) was approximately 75 days (red line) after the third dose of vaccine and extended to 150 days (yellow line) after BTI in HD. Comparatively, in IBD patients, the prevalence of high antibody titers following vaccination was lower (40 % at D2m1, n = 31/78, 53 % at D3m1, n = 43/78, visualized in the green area with titer >2000 BAU/mL) in comparison to HDs (100 % response rate at D3m1 [5], n = 56), Fig. 3B and table 1. Notably, anti-RBD IgG titers was enhanced in the majority of patients after BTI (97 % one month after BTI, median day = 29 with day 25–34 as IQR, n = 76/78, $p < 0.0001$). We did not observe any significant difference for the anti-RBD humoral response according to immune-suppressive therapies. The waning of humoral response was faster in IBD after vaccination but not BTI. The decline of anti-SARS-CoV-2 titer was illustrated by $t_{1/2}$ of 30 days before BTI (red line) and extending $t_{1/2}$ to 200 days after BTI (yellow line), Fig. 3C.

To evaluate the quality of the humoral response during hybrid immunity, we assessed cross-reactivity of vaccine-induced antibodies with variants of concern (VOC), Fig. 3D. We measured the neutralization titer (NT) of antibodies detected post-BTI and compared to vaccinated HDs and non-vaccinated COVID-19 convalescents, Fig. 3D. NTs were higher against WT and Delta strains than Omicron BA.1 SARS-CoV-2 strains in all groups, with significant differences including BTI ($p < 0.01$ for Delta and Omicron VOC against WT respectively). As proxy for neutralizing antibodies, we measured the inhibitory effect of serum on the interaction between ACE2 and RBD [21], Fig. 3E. Neutralizing activity against both RBD WT and BA.1 increased over time in BTI HDs for WT RBD ($p < 0.05$) and for BA.1 RBD ($p < 0.01$), though WT RBD neutralization remained stronger than BA.1 RBD in particular at day 1 ($p < 0.0001$). Collectively these results highlight that BTI in HDs and IBD patients elicited a robust and durable humoral response.

To explore the source and longevity of anti-SARS-CoV-2 antibodies, we analyzed ex vivo SARS-CoV-2 binding B cells after BTI in both HDs and IBD, Fig. 4A. BTI induced a rapid increase of SARS-CoV-2 binding B cells ($Spike^{low}RBD^{+}$ and $Spike^{+}RBD^{-}$, $p < 0.001$ and $p < 0.01$) within 7 days post-infection, Fig. 4B-C. We also identified B cells targeting conserved (public) RBD epitopes and VOC vs WT- specific RBD epitopes, Fig. 4A-C. We observed a expansion of RBD-binding B cells in IBD BTI, Fig. 4D-E. However, $Spike^{+}RBD^{-}$ binding B cells were more frequent than RBD-binding B cells ($p < 0.0001$), Fig. 4F. Moreover, the emergence of new variants during the inclusion time of IBD prompted us to investigate the responsiveness of B cells to VOC RBD such as BA.1 and BA.2. BTI induced the expansion of B cells with cross-reactivity between WT and VOC RBD (BA.2/BA.1), highlighting adaptability to emerging variants (Fig. 4F).

We examined correlations between serology and SARS-CoV-2 binding B cells frequencies after BTI in HDs or IBD patients (Fig.S2A-B). in HDs, positive correlations were observed for $Spike^{+}RBD^{-}$ and RBD-binding B cells (p = 0.0017 and p = 0.0177 respectively), while in IBD, correlation was significant for RBD-binding B cells (p = 0.008).

Finally, to assess the quality and potential duration of the humoral memory response, we evaluated the phenotype of SARS-CoV-2 binding B cells post-BTI (Fig. 4G), comparing HDs and IBD patients to vaccinated controls. We observed differences in response between $Spike^{+}$ RBD^{-} (Spike) and RBD-specific B cells, based on epitope binding (WT vs. VOC RBD; Figs. 4G and S2C). Notably, BTI in both HDs and IBD patients led to a decreased expression of the immature marker CD24, an increased expression of the activation marker CD71, and isotype switching from IgD to IgG on WT RBD-binding B cells (Fig. S2D-E). Most BA.1-specific RBD-binding B cells in IBD displayed an immature phenotype (IgD^{+}), indicating a limited isotype switch (Fig. S2F). We further defined the specific signature of SARS-CoV-2-binding B cells in HDs and IBD patients based on cognate epitopes, vaccination status, and BTI exposure (Fig. 4H). Despite later BTI recruitment in IBD patients, a strong IgD expression on SARS-CoV-2-binding B cells was still observed (Fig. 4I). CD71, a marker of activation, remained persistently expressed on these cells in IBD patients. As with BTI in HDs, VOC RBD-binding B cells in IBD

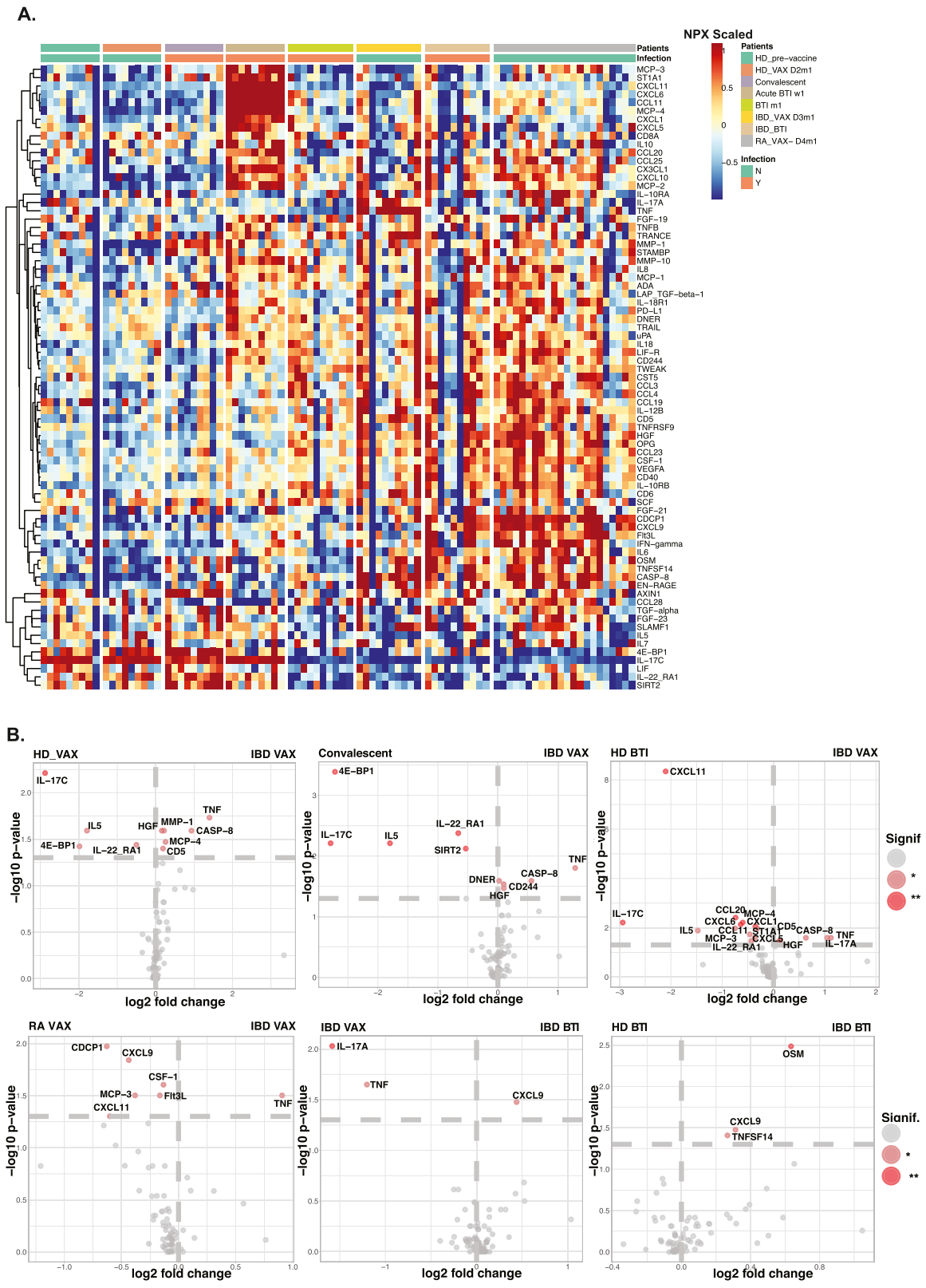

Fig. 1. Inflammatory profile of BTI in IBD.

A. Proteomic signature of BTI by Olink. Quantification of pro-inflammatory molecules in plasma from healthy donors (HD), IBD and rheumatoid arthritis (RA) after seroconversion due to vaccination ($n = 9$ dose 2 and $n = 10$ dose 3 and $n = 22$ dose 4 respectively), or from acute BTI (n = 9), and convalescent COVID-19 patients (n = 9). Plasmas from non-vaccinated HD, before COVID-19 pandemic were used as negative controls (n = 9). NPX (normalized protein expression) values were scaled for each marker. The documented SARS-CoV-2 infection status is indicated by the bar chart on the top.

B. Specific proteomic signature of persons with IBD during vaccination and BTI. Visualization by volcano plots of the significant inflammatory molecules detected in vaccinated HD and IBD before and after BTI. Significance levels from the Mann-Whitney test are indicated: * for $p < 0.05$ and ** for $p < 0.01$.

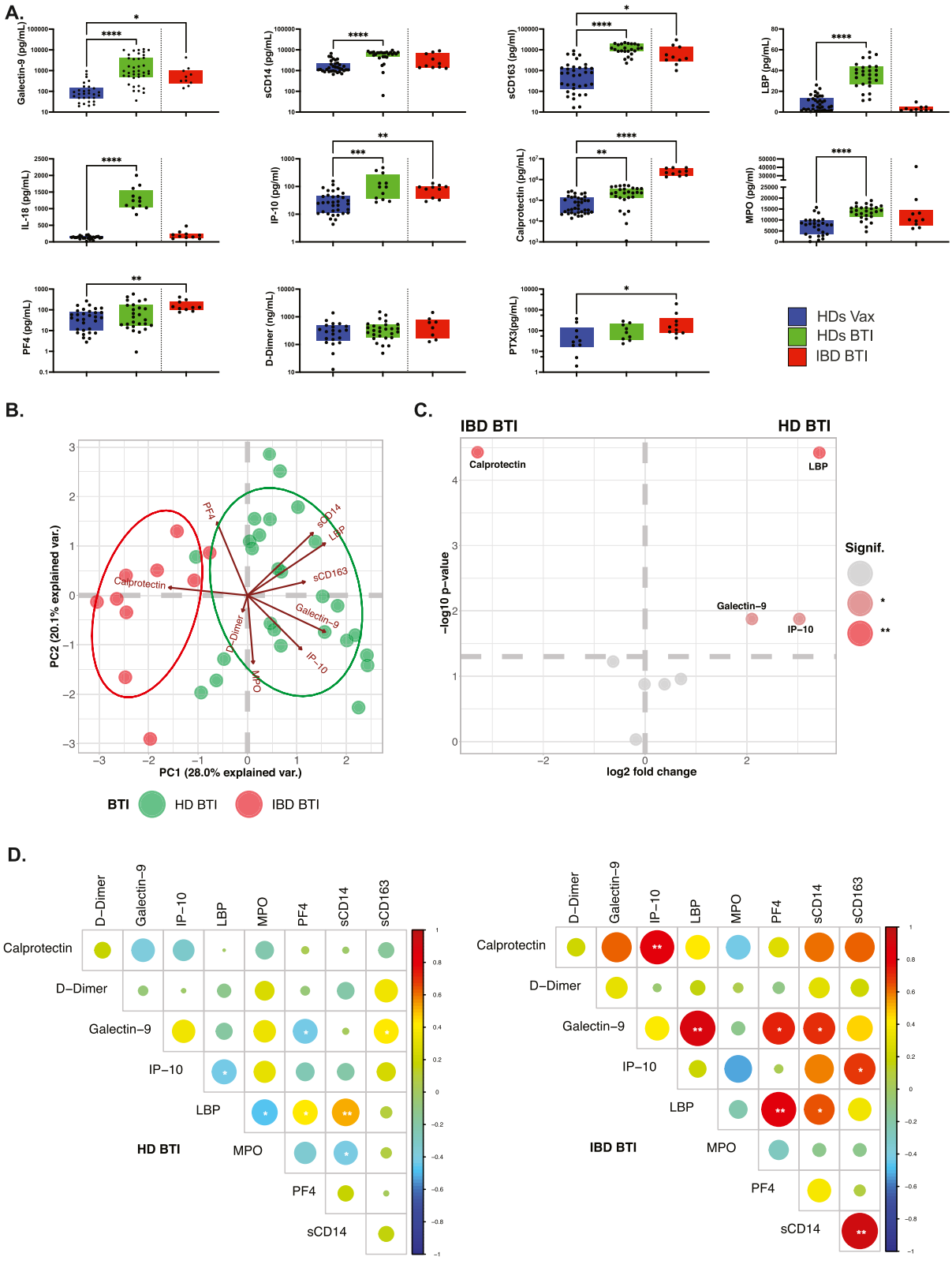

Fig. 2. Inflammatory profile of BTI in persons with IBD.

patients were predominantly immature. Notably, IBD patients exhibited a higher proportion of Spike^{+} over RBD-binding B cells, characterized by significant IgG ($p < 0.0001$) and CD71 ($p < 0.0001$) expression compared to HDs post-BTI (Fig. S2G). Overall, these findings provide a comprehensive view of the humoral response and B cell dynamics in IBD patients post-BTI, demonstrating both quantitative and qualitative distinctions in immune responses compared to HDs.

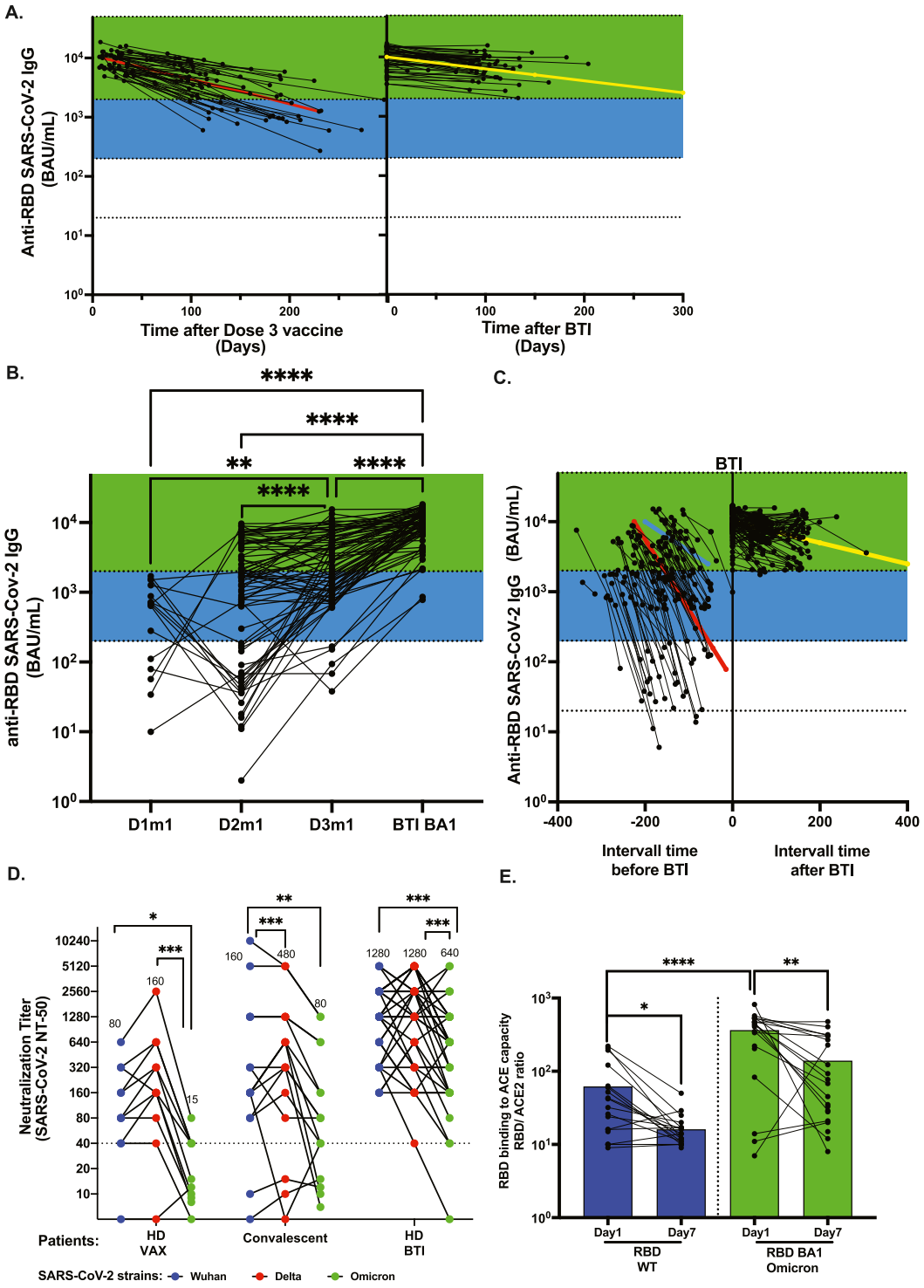

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Fig. 3. Humoral profile of BTI in persons with IBD

A. Time-evolution of serological response in HD. XY plots show anti-SARS-CoV-2 RBD IgG titer trajectories with time (days), left: vaccinated HD after D3 ($n = 56$), right: vaccinated HD after BTI ($n = 57$). Slopes corresponding to $T_{1/2}$ of 75 days (red) are shown in triple dose vaccinated HD and $T_{1/2}$ of 150 days (yellow) after BTI as indicative values.

B. Longitudinal follow-up of anti-RBD IgG titer during mRNA vaccination and BTI in IBD. Quantification of anti-SARS-CoV-2 RBD IgG antibodies after COVID-19 mRNA vaccination and Omicron (BA1) BTI ($n = 78$). Dotted lines demarcate ranges (>2000, 200–2000, 20–200 BAU/mL), to visualize patients with good (green), low (blue), and non-humoral responsiveness respectively. Wilcoxon matched pair signed rank test, and two-tailed *P* values are indicated for longitudinal paired BTI comparisons with **, ****, denoting $p < 0.01$ and $p < 0.0001$ respectively.

C. Time-evolution of serological response in IBD. XY plots show anti-SARS-CoV-2 RBD IgG titer trajectories with time (days), left: vaccinated IBD before BTI, right: vaccinated IBD after BTI. Slopes corresponding to $T_{1/2}$ of 30 (red), 60 days (blue) are shown in vaccinated IBD, and $T_{1/2}$ of 200 days (yellow) after BTI as indicative values.

D. Neutralization of Wuhan, Delta, and Omicron viral replication by anti-SARS-CoV-2 antibodies induced after acute BTI (Day 7 after inclusion). Plasma samples from healthy donors were vaccinated with 2 doses ($n = 10$), non-vaccinated convalescent COVID-19 patients ($n = 11$), and Omicron BTI ($n = 15$). The dotted line indicates the neutralization assay's first serum dilution (1:40). The samples that did not show any inhibition of viral infection are presented as 5 for plotting purposes. Statistical analysis was performed using the Friedman test with Dunn's multiple comparisons test with* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

E. Longitudinal follow-up of binding activity from serum of HD during acute BTI. Inhibition of ACE2-binding to wild type (Wuhan) and Omicron RBD by competition with serum from Omicron BTI, collected one day and one week after inclusion. Mann Whitney test and two-tailed *P* values are indicated for WT and Omicron RBD comparisons with *, **and **** denoting $p < 0.05$, $p < 0.01$ and $p < 0.0001$ respectively.

2.3. T_{FH} profile after BTI

To investigate the effect of BTI on the humoral response, we sought to identify signals delivered by T_{FH} to B cells during acute infection. T_{FH} cells, characterized by CXCR5 expression, were subdivided into Follicular Regulatory CD4 T cells (T_{FR}, defined as $CD25^{high}CD127^{low}$), T_{FH1} (expressing only CXCR3), T_{FH17} (expressing only CCR6), and $T_{FH1/17}$ (expressing both CXCR3/CCR6), Fig.S3A. The activation levels were also assessed via PD-1, HLA-DR and CD38 expression. In IBD patients, T_{FH} frequencies among Total CD4 T cells after BTI were lower compared to vaccinated ($p < 0.01$) and BTI HDs ($p < 0.05$). Increased activation was detected after BTI, as indicated by PD-1 engagement ($p < 0.01$ vs HD3m1), with a similar profile after BTI in IBD, Fig. S3B. Although no significant change was noted in T_{FR} (not shown), BTI altered the T_{FH} distribution, affecting the balance between T_{FH1} and T_{FH17} frequencies ($p < 0.01$ and $p < 0.0001$ vs. HD3m1 respectively), as well as $T_{FH1/17}$ frequencies ($p < 0.001$ vs HD3m1). A positive correlation was established between peripheral T_{FH} CD4 cells frequency and both SARS-CoV-2 RBD-binding B cells ($p = 0.009$), or anti-SARS-CoV-2 RBD IgG titer ($p = 0.007$), supporting the co-regulation of humoral and cellular immunity, Fig. S3C.

Next, we explored whether phenotypic changes in T_{FH} influence their functions after BTI. FACS-sorted T_{FH} and non-T_{FH} CD4 T cells were polyclonally stimulated, and supernatants were collected after 48 h, Fig. S3D (and data not shown). Despite BTI, cytokine profiles remained distinct between T_{FH} and non-T_{FH} CD4 cells, with a Th17 profile confirmed in T_{FH} post-BTI compared to vaccinated HDs (Fig. S3E). We sought to determine whether the altered functions of T_{FH} might reflect transcriptional profile changes. The ex vivo mRNA expression of T_{FH} CD4 cells was analyzed from the same cohort, Fig.S3F. Several transcription factors, including TBX21(T-bet), BCL-6, IRF-4, and cMAF were minimally expressed in T_{FH} after BTI.

Future studies should explore the impact of these T_{FH} subsets on antibodies generation (affinity, Isotypes switching) following BTI.

2.4. T cell phenotypes in IBD after BTI

To accurately assess the frequency and phenotype of virus-specific CD8 T cells ex vivo, we used peptide-HLA multimers combined with high parameter flow cytometry to characterize SARS-CoV-2 specific CD8 T cells, Fig. 5A and Fig.S4A. We tested previously described immunodominant peptides in convalescent and early acute BTI HDs for late acute BTI HDs and IBD, covering multiple HLA alleles (A*01:01, A*02:01, A*11:01, A*24:02, and B*07:02). The median frequencies of SARS-CoV-2 Spike and non-Spike-specific multimer CD8 T cells after BTI were similar in HD and IBD. The frequency of SARS-CoV-2 Spike multimer CD8 T cells was significantly higher than non-Spike-specific multimer CD8 T cells in HD BTI (adjusted $p < 0.05$), but this distinction was not observed in IBD BTI. In terms of other viruses, CMV-specific multimer CD8 T cells showed similar frequencies between HD and IBD BTI, whereas EBV-specific multimer CD8 T cells differed significantly ($p < 0.0001$). We analyzed virus-specific T cells both classically and using an unbiased approach, Fig.S4A and Fig. 5B. UMAP visualization demonstrated a distinct distribution of virus-specific CD8 T cells between HD and IBD BTI. Clusters were automatically identified by phenograph (Fig. 5B-C), and quantified for each virus type, Fig. 5D. SARS-CoV-2 Spike and non-Spike-specific multimer CD8 T cells in IBD showed a more activated (CD38) and exhausted (CD244, TIM-3) phenotype (Cluster 2/4) compared to HD BTI, where Spike-specific multimer CD8 T cells displayed a long-term memory marker (CD127 in Cluster 6). Additionally, control virus-specific CD8 T cells exhibited phenotypic differences between HD and IBD BTI. To identify phenotypic markers distinguishing each virus-specific CD8 T cells after BTI in HD and IBD, we quantified the expression of individual markers, (Fig. 5E). We identified distinct signatures for CMV-, EBV-, Spike-, and MNO-specific CD8 T cells, Fig.S4B, further confirming the differences in phenotype between SARS-CoV-2-specific CD8 T cells in HD BTI and IBD BTI, particularly in the expression of CD38, CD244, TIM-3, and independently of HLA restriction, Fig. 5F and Fig.S4C. Spike- and MNO-specific CD8 T cells in IBD BTI were characterized by increased expression of the inhibitory receptor TIM-3 and a generally activated phenotype (HLA-DR, CD38, PD-1), Fig. 5G. This heightened activation was also observed on CMV- and EBV-specific CD8 T cells in IBD BTI, Fig. S4D.

2.5. Cellular response in IBD after BTI

The next question was to investigate whether the activated phenotype of SARS-CoV-2 specific T cells affect their functional capabilities. We performed an in vitro assessment using AIM/ICS assay, to identify vaccine- and BTI-elicited Spike-specific T cells, Figure Fig. S5A. The functionality of these cells was evaluated based on the upregulation of CD137 and CD154 (for CD4 T cells) or secretion of IFNγ/TNF (for CD8 T cells). No significant difference was observed between Crohn's disease and Ulcerative colitis patients regarding the detection of SARS-CoV-2 specific T cells. However, the functionality of Spike-specific T cells was notably reduced in IBD patients after BTI for both CD4 and CD8 T cell responses ($p < 0.001$ or $p < 0.0001$ respectively). We extended our analysis to non-Spike T cell responses post-BTI, Fig. S5B. While both Spike- and MNO-specific T cell specific T cell responses were concomitantly present in HDs following BTI, this combined response was not observed in IBD BTI. To further elucidate differences in the quality of the anti-SARS-CoV-2 cellular response, we assess T cells polyfunctionality in HD and IBD patients after BTI Fig. S5C-D. In HDs, Spike SARS-CoV-2-

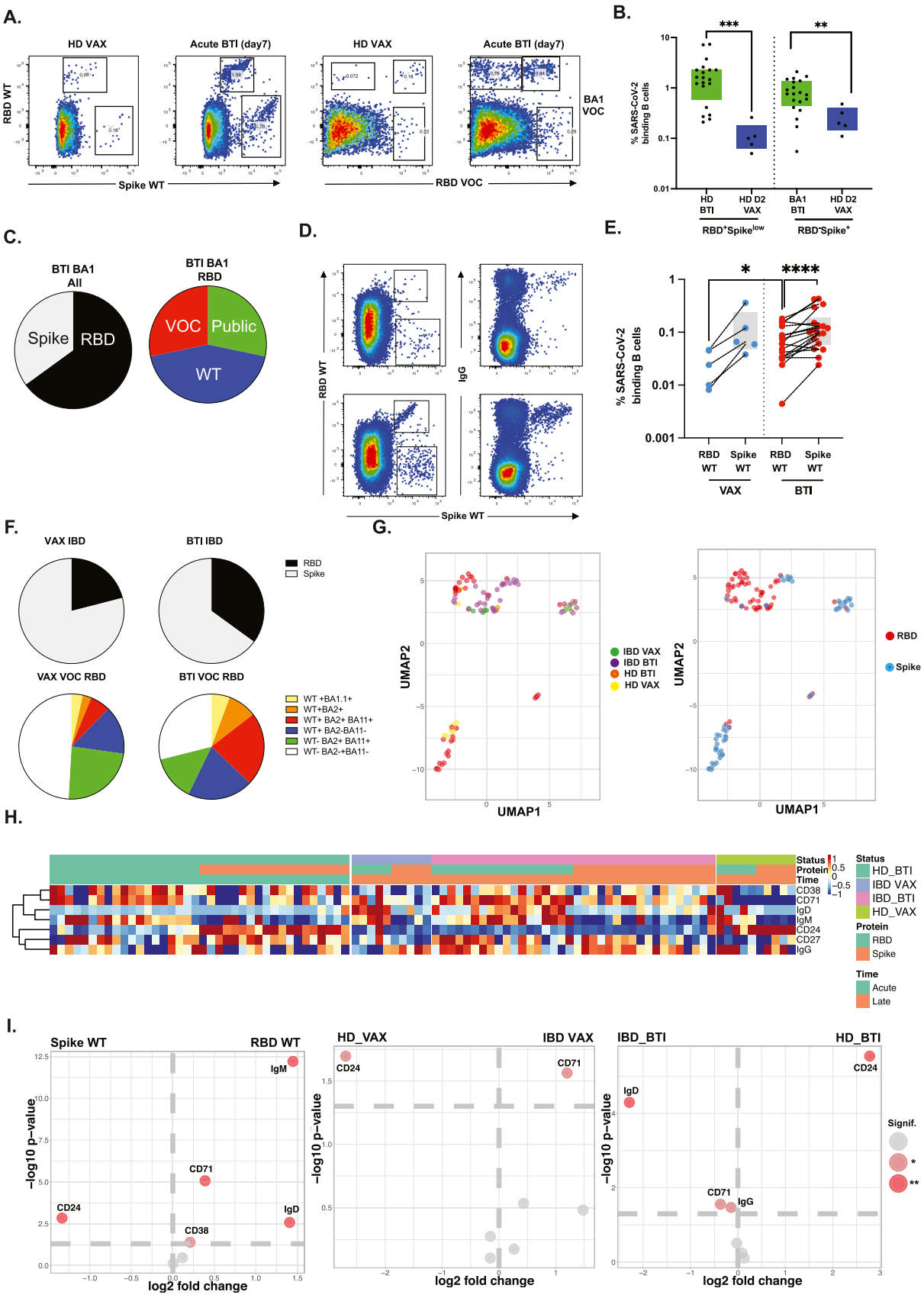

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Fig. 4. SARS-CoV-2-binding B cells after BTI in persons with IBD

A. SARS-CoV-2-binding B cells during acute BTI. Dot plots represented SARS-CoV-2 Binding B cells before and after BTI (day7 post-inclusion) for HD, infected by VOC Omicron. SARS-CoV-2 Binding B cells were identified as $RBD^{+}Spike^{low}$ (RBD) and $RBD^{-}Spike^{+}$(Spike) with wild type probes for RBD and total Spike (Wuhan), left. SARS-CoV-2 Binding B cells were identified as $RBD\text{-}WT^{+}$ $RBD\text{-}VOC^{+}$ (public RBD), $RBD\text{-}WT^{+}$ $RBD\text{-}VOC^{-}$ (WT RBD) and $RBD\text{-}WT^{-}$ $RBD\text{-}VOC^{+}$ (VOC RBD) with wild and VOC (BA.1) probes for RBD, right. Cells were gated on total live B cells.

B. Frequency of SARS-CoV-2-binding B cells during acute BTI. Quantification of $RBD^{+}Spike^{low}$, and $RBD^{-}Spike^{+}$ binding B cells in HD after vaccination (6 months post Dose 2, blue) and BTI (Day 7 post-inclusion, green). Mann Whitney test, two-tailed *P* values <0.01 (**), and < 0.0001 (***).

C. Distribution of SARS-CoV-2-binding B cells during acute BTI. Relative proportion of $RBD^{+}Spike^{low}$ (RBD, black) and $RBD^{-}Spike^{+}$ (Spike, grey) from total SARS-CoV-2 Binding B cells in BA.1 BTI (Day 7), left. Relative proportion of public RBD (green), WT RBD (blue), and VOC RBD (red) from total SARS-CoV-2 RBD Binding B cells in BA.1 BTI (Day 7), right.

D. SARS-CoV-2-binding B cells during acute BTI in IBD patients. Dot plots represented SARS-CoV-2 Binding B cells before (top) and after BTI (bottom) for the same individual with IBD. SARS-CoV-2 Binding B cells were identified as $RBD^{+}Spike^{low}$ (RBD) and $RBD^{-}Spike^{+}$(Spike) with wild type probes for RBD and total Spike (Wuhan), left. SARS-CoV-2 Binding B cells expressing surface IgG were identified as memory, right. Cells are gated on total live B cells.

E. Frequency of SARS-CoV-2-binding B cells after BTI in IBD patients. Quantification of RBD and Spike -binding B cells in IBD after vaccination ($n = 6$) and BTI ($n = 18$). Mann Whitney test for unpaired comparison of RBD, two-tailed *P* values <0.05 (*). Wilcoxon matched pair signed rank test for SARS-CoV-2-binding B cells after BTI, with **** denoting $p < 0.0001$.

E. Distribution of SARS-CoV-2-binding B cells during BTI in IBD patients. Relative proportion of $RBD^{+}Spike^{low}$ (RBD, black) and $RBD^{-}Spike^{+}$ (Spike, grey) from total SARS-CoV-2 Binding B cells after vaccination (Dose 3 m3) and BA.1 BTI in IBD, top. Relative proportion of public RBD (yellow, orange, red), WT RBD (blue), and VOC RBD (green, white) from total SARS-CoV-2 RBD Binding B cells in vaccinated and after BA.1 BTI IBD, bottom.

F. High-Dimensional Analysis of SARS-CoV-2-binding B cells during BTI. UMAP visualization of SARS-CoV-2-binding B cell phenotype by flow cytometry. IBD were distinguished from HD by vaccination and infection status (left). The epitope specificity of SARS-CoV-2-binding B cells (RBD and Spike) was identified for vaccinated or BTI HD and IBD, right.

G. Phenotype of SARS-CoV-2-binding B cells after BTI. Heat plot represents the scaled frequency of markers expressed by SARS-CoV-2-binding B cells according to immune status (vaccination vs BTI) in HD or IBD and to recognized protein (RBD, Spike).

H. Specific signature of SARS-CoV-2-binding B cells in IBD patients. Volcano plots represent significant fold changes in the phenotype of SARS-CoV-2 Spike- or RBD-binding B cells (left), after vaccination (middle) or after BTI (right). Mann Whitney test, two-tailed *P* values <0.05 (*), values <0.01 (**).

specific CD4 T cell polyfunctionality differed from that of MNO SARS-CoV-2-specific CD4 T cells ($p = 0.021$). For IBD patients, we compared the polyfunctionality of SARS-CoV-2-specific CD4 T cells before and after BTI for Spike and examined only after BTI for non-Spike epitopes, Fig. S5C. We observed no significant changes in Spike SARS-CoV-2-specific CD4 T cell polyfunctionality before and after BTI. A similar analysis for CD8 T cells indicated that Spike SARS-CoV-2-specific CD8 T cell polyfunctionality was not significantly enhanced after BTI in IBD patients, Fig. S5D.

3. Discussion

The COVID-19 pandemic has highlighted the increased vulnerability of patients with co-morbidities to severe disease outcomes. This study aimed to determine whether persons with IBD, receiving stable medication, could mount a robust immune response following a standard COVID-19 vaccine regimen and subsequent exposure to BTI with VOC. Here, we describe persistent inflammation and chronic activation of SARS-CoV-2 specific B and T cells in vaccinated IBD patients. While BTI enhanced humoral responses, there was no comparable enhancement in anti-Spike cellular responses.

Leveraging cutting-edge proteomics, we delineated an inflammatory signature in triple-dose vaccinated IBD patients, indicating potential inadequate regulation of the tumor necrosis factor (TNF) pathway by standard of care IBD therapies [22]. This distinct inflammatory signature, which includes elevated concentration of TNF, Caspase-8, and HGF, differs from that observed in HD or patients with rheumatoid arthritis, as well as from responses to SARS-CoV-2 infection in convalescent or BTI cases. Interestingly, BTI in IBD patients led to decreased TNF and IL-17 A levels [23], alongside increased CXCL9, .a Th1 chemokine associated with UC disease activity and pro-inflammatory cells recruitment [24]. Elevated Oncostatin (OSM), a predictive factor of anti-TNF-α therapy response [25], was linked to reduced peripheral TNF levels. Among other pro-inflammatory markers elevated post-BTI in persons with IBD, sCD14, MPO, and LBP were comparable to HD levels six weeks post-BTI. Moreover, Calprotectin, both fecal [26] and blood-derived [27,28], correlates with disease activity, including relapses in UC. Additionally, the level of PF4 (CXCL4) was elevated in vaccinated IBD individuals [29] and further increased after BTI, suggesting a potential exacerbation of the threefold risk of thrombosis observed in patients with active IBD (versus healthy individuals) [30] following BTI.

This study confirms that cumulative doses of vaccines enhance humoral immunity in IBD patients following BTI, generating a robust and broad neutralizing antibody response. However, the durability of this humoral response and the potential for antibody decay warrant ongoing monitoring. To address this concern, we conducted a comprehensive phenotyping of SARS-CoV-2 binding B cells and evaluated the quality of the memory B cell response.

Notably, epitope targeting showed a shift in IBD patients, with a predominance of Spike-binding (non-RBD) B cells pre-BTI that shifted to RBD-binding B cells post-BTI. This was accompanied by persistent expression of the activation marker CD71 on B cells, which may reflect hyperactivation [31] and could potentially hinder optimal vaccine responsiveness.

B cells are influenced by the activity of T follicular helper cells, and our investigation aimed to understand how BTI modulate this CD4 T cell subset in both HD and IBD patients. While the peripheral frequency showed slight modulation after BTI in IBD, the notable characteristic of T_{FH} after Omicron BTI was the deviation towards Th17 immunity [32] at transcriptional and functional (secreted cytokines) levels.

Another crucial aspect of SARS-CoV-2 immunity is cellular immunity [33,34]. We examined whether robust cellular immunity could be generated despite chronic inflammation in persons with IBD [35]. Quantitative analysis did not reveal significant differences in the frequencies of Spike- and MNO-specific CD8 T cells detected via HLA-Class I restricted multimers. However, qualitative phenotyping analysis revealed an activated profile of Spike- and MNO-specific CD8 T cells (expressing HLA-DR, CD38), acquisition of inhibitory receptors (TIM-3, PD-1, likely due to recent TCR activation), and memory markers (CD45RO, CD95). Samples were adjusted for HLA, therapies and interval time after BTI, indicating that these parameters did not influence the phenotype of SARS-CoV-2 specific CD8 T cells. This activated signature was also detected on CMV- and EBV-specific CD8 T cells. We assessed the impact of this activation on the functionality of SARS-CoV-2 specific T cells and found that cellular immunity was not enhanced after BTI in IBD. Neither the frequency of Spike- and MNO SARS-CoV-2- specific CD8 T cells nor the polyfunctionality of T cells increased after BTI in IBD.

The common presenting symptoms of natural COVID-19 infection in IBD patients mirrored those in the general population [36]. Even so, IBD patients had significantly increased gastrointestinal symptoms,

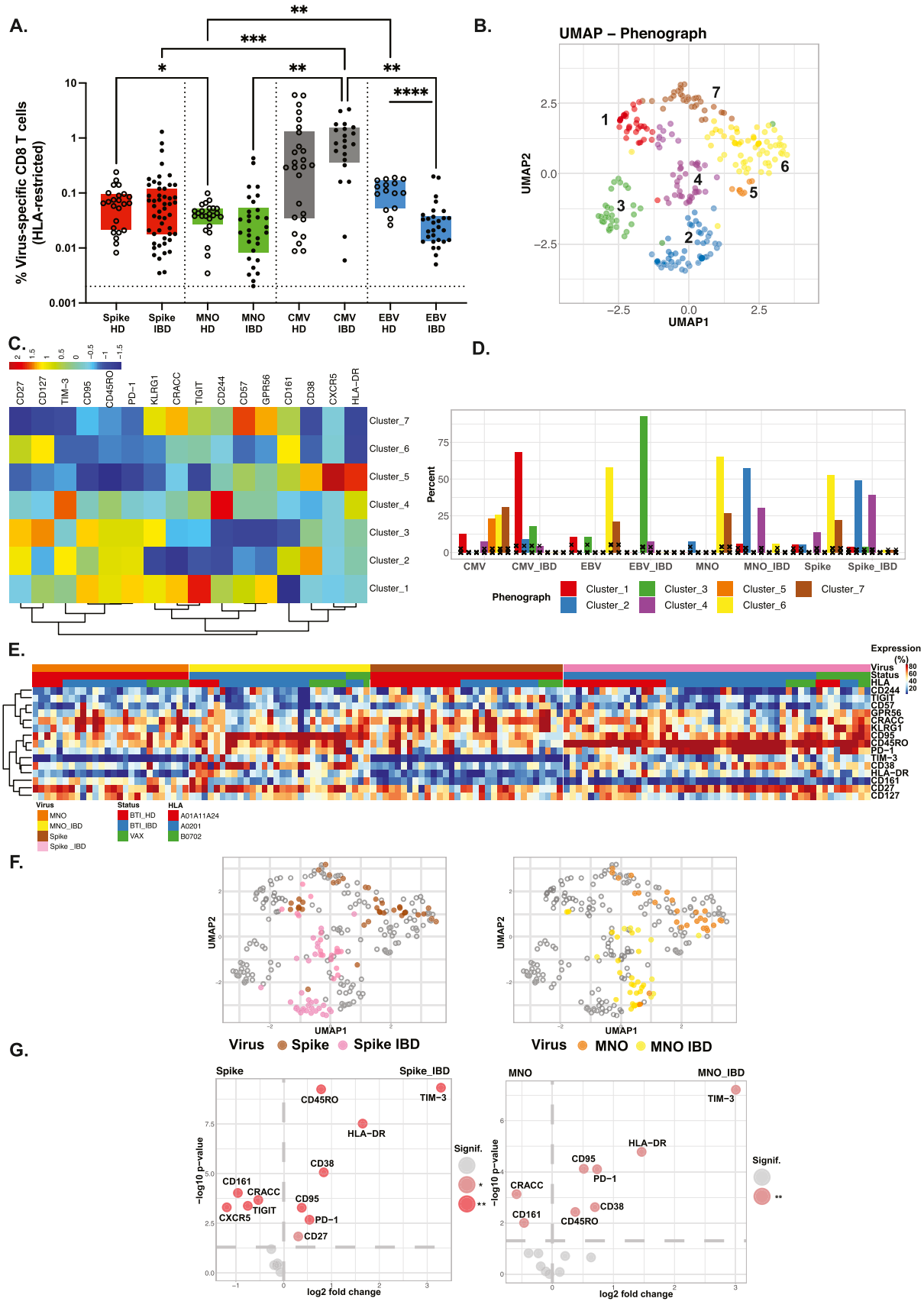

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Fig. 5. SARS-CoV-2-specific T cells after BTI in persons with IBD
A. Frequency of SARS-CoV-2-specific CD8 T cells after BTI in IBD. Frequency of virus-specific multimer CD8 T cells after Dose 3 of the mRNA vaccine in HD and IBD or BTI (1–2 months post-BTI) or (including up to three different HLA per donor among HLA*A0101, HLA*A0201, HLA*A2402, and HLA*B0702). Mann Whitney test, two-tailed P values <0.05 (*), <0.01 (**), <0.001 (***), <0.0001 (****).
B. Identification of clusters derived from Virus-specific CD8 T cells after BTI in IBD. UMAP visualization of virus-specific CD8 T cells phenotype defined by flow cytometry from PBMCs of HD and IBD patients after BTI. Clusters were automatically identified by phenograph.
C. Characterization of clusters derived from Virus-specific CD8 T cells after BTI in IBD. Normalized expression of markers' frequencies was represented in a cold-hot heatmap. The protein markers were automatically clustered.
D. Distribution of clusters derived from Virus-specific CD8 T cells after BTI in IBD. The frequencies of cluster that were automatically identified (see E) were detailed for each viral specificity according to patients' status (HD/IBD).
E. Phenotype of virus-specific T cells after BTI. Heat plot represents the frequency of markers expressed by SARS-CoV-2-specific multimer CD8 T cells (MNO, Spike) according to immune status (vaccination vs BTI) in HD or IBD depending on HLA-restriction.
F. Distribution of SARS-CoV-2-specific T cells after BTI in IBD. UMAP visualization of SARS-CoV-2-specific CD8 T cells phenotype defined by flow cytometry after BTI in HD or IBD.
G. Signature of SARS-CoV-2-specific CD8 T cells after BTI in IBD. Volcano plots represent the significant modulation of protein expression at the surface of SARS-CoV-2-specific CD8 T cells from HD and IBD after BTI. Mann Whitney test, two-tailed P values <0.05 (*), values <0.01 (**).

including abdominal pain, diarrhea, nausea and vomiting [37,38]. However, the proportion of gastrointestinal manifestations may have been confounded by active disease at the time of COVID-19 [38,39]. Moreover, there were no evident changes in IBD activity up to 7 months post-COVID-19 after natural infection [39].

The perceived risk of vaccine related adverse events and flare of IBD was a main reason of SARS-CoV-2 vaccine hesitancy for IBD patients. Meta-analysis in the general population revealed that around one-third of participants observed changes in gastro-intestinal activity following any doses of SARS-CoV-2 vaccines [40]. This side effect was mainly observed after the two first doses and limited to less than three days. Similar methodology confirmed the safety of SARS-CoV-2 vaccines in IBD patients [41,42]. Lastly, a recent study has associated IBD flares with long COVID-19 in IBD patients that had received two least two vaccinations [43] but this topic will need further study and follow-up.

Despite the notable strengths of this study, such as comprehensive high-dimensional analysis of the SARS-CoV-2-specific adaptive immune response following vaccination and natural SARS-CoV-2 infection in both IBD and HD populations, several limitations need to be considered. First, the causal relationship between systemic inflammation and activation of T cells has been well documented in chronic viral infections like HIV but remains underexplored in IBD [44,45]. Second, while the sample size is substantial, particularly for a longitudinal study employing in-depth immune profiling of persons with IBD, it remains relatively modest compared to clinical trials and the complexity of the disease. Third, several therapeutic approaches exist for IBD, but we restricted our study to primarily TNF inhibitors, leaving other IBD therapies unexamined [8,46]. The role of biologic drugs needs to be distinguished from the disease per se [47]. Next, the absence of severe COVID-19 in our IBD cohort, and therefore, the lack of systemic inflammation limits certain comparisons. Moreover, the comparison of variant-specific immune memory induced by vaccination versus infection is constrained by circulating strains (Delta/Omicron for HD vs Omicron variants for IBD). Finally, only the durability of anti-SARS-CoV-2 humoral but not the cellular immune response has been documented here. It is crucial to understand whether the activated effector profile of SARS-CoV-2-specific T cells contribute to the waning of "protective" immunity or the accelerated decay of the humoral response.

In summary, our findings reveal an activated but deficient hybrid immunity in IBD patients. Despite stable immunosuppressive therapy, which prevents further innate activation and the cytokine storm associated with severe forms of the disease, the chronically activated SARS-CoV-2 specific B and T cells may compromise the robustness of hybrid immunity. The durability of the immune response requires further investigation, and it will be interesting to evaluate whether alternative anti-inflammatory therapies might enhance vaccine efficacy in IBD patients [36].

4. Methods

4.1. Patients, and vaccinations

We enrolled vaccinated patients with IBD including patients suffering from BTI ($n = 48$ and $n = 24$ with longitudinal BTI follow-up). IBD patients had either Crohn's Disease (CD, n = 24) or Ulcerative Colitis (UC, n = 24), see Table 1. BTI was diagnosed with positive SARS-CoV-2 RT-PCR test, with or without positive Omicron or Delta variant PCR on oro-nasopharyngeal specimens (i.e., verified or suspected Omicron/Delta variant cases, respectively). 37 vaccinated healthy donors (HD) served as controls, these were sampled in the early acute phase (Day 7–14) after BTI, and in the late acute phase (6 weeks) after infection (NCT04320732 and NCT04381819), Table 2 [48]. HD were divided according to BTI with Omicron BA.1 or Delta VOC. The inflammatory response in triple dose vaccinated IBD was compared to the serological signature observed in non-vaccinated COVID-19 convalescents ($n = 9$), double dose vaccinated patients after BTI ($n = 37$), and with four dose vaccinated Rheumatoid Arthritis patients (RA) as a control of inflammatory disease, Table 3.

HDs vaccinated or collected pre-COVID-19 time were used as negative controls.

In an observational study, we performed a longitudinal follow-up of persons with IBD to measure vaccination responsiveness. Initially, we chose a cut-off of 2000 BAU/mL, 1 month after previous mRNA vaccine doses (after standardization allowed WHO units) to identify vaccine-high responders. IBD received only mRNA vaccines for the third vaccination including BNT162b2 (Pfizer-BioNTech) or mRNA-1273 (Moderna). PBMC samples were collected longitudinally from IBD one month after dose 3 of COVID-19 vaccine or BTI, (n = 24), and from HD after BTI (n = 37).

See Supplementary Table S1 for cohort details.

4.2. Sample preparation and HLA typing

IBD and HDs were pre-typed by flow cytometry for A02, A24, and B07 expression. Frozen PBMCs from individuals positive for specific HLA were subsequently stained with the corresponding Dextramers/Tetramers Class I restricted (HLA-A*01:01, HLA-A*02:01, HLA-A*24:02, and HLA-B*07:02) as described [14]. The non-SARS-CoV-2 related viruses were used as an internal control for the same individuals (CMV, EBV, Influenza, see Supplementary Methods).

4.3. ELISA

The plasma of IBD and HD were collected after D2 or BTI and cryopreserved. The following ELISA kits were used according to manufacturer protocols. From R&D Systems: Human CD14 DuoSet ELISA (DY383), Human CD163 DuoSet ELISA (DY1607), Human LBP DuoSet

ELISA (DY870–05), Human Galectin-9 DuoSet ELISA (DY2045), Human GDF-15 Quantikine ELISA Kit (DGD150), Human CXCL4/PF4 Quantikine ELISA (Kit DPF40), Human IFN-alpha (41100); from Ebioscience: Human MPO Instant ELISA Kit (BMS2038INST); from Thermo Scientific: Invitrogen novex IP 10 Human ELISA Kit (KAC2361); from Abcam Human C-Reactive Protein/CRP (Ab99995); from MyBioSource: Human zonulin ELISA Kit (MBS706368); from Meso Scale diagnostics: human Calprotectin (F21YB-3).

4.4. Olink® Target 96 inflammatory panel

The systemic inflammation of IBD, after D3 or BTI and controls such as vaccinated HD, BTI, or severe COVID-19 patients was determined by Olink Proteomic Proximity Extension Assay (PEA) technology. The Olink® Target 96 inflammatory panel was used on an Olink® Signature Q100 instrument according to the manufacturer's instructions.

4.5. Flow cytometry

Cryo-preserved PBMCs were enriched for live cells by magnetic depletion of dead cells (Dead cells removal microbeads, Miltenyi) in the presence of Citrate buffer and stained with antibody panels to quantify and characterize the phenotype of specific T cell responses directed against Spike peptides (two million cells per sample) and B cell responses to RBD or Spike protein (one million cells per sample). Cells were acquired on a BD FACSymphony (BD Biosciences) or Attune NxT (ThermoFisher). Statistical significance was set with thresholds as: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4.6. In vitro stimulation assays

Thawed cells were stimulated for 16 h with SARS-CoV-2 PepTivator Spike protein peptides consisting of 15-mer sequences with 11 amino acid overlaps (Wuhan-Hu-1, i.e. wild type WT. Miltenyi Biotec) as described [14], see Methods for details. For non-Spike (WT) responses cells were stimulated with Nucleoprotein (PepTivator SARS-CoV-2 Prot N) and Membrane protein (PepTivator SARS-CoV-2 Prot M), consisting of 15-mer sequences with 11 amino acid overlaps in addition to the 4 ORF1ab/Orf3a peptides in Supplementary Table S2. i.e. stimulated with M + N + O. Alternatively, cells were stimulated with 88 pooled WT immunodominant oligopeptides from the whole proteome (PepTivator SARS-CoV-2 Select, Miltenyi Biotec) consisting of peptides from structural proteins (S, M, N) as well as non-structural proteins (O).

4.7. Detection of specific memory CD8 T cells

Antigen-specific CD8 T cells were detected by peptide: HLA multimers as described [14] (see Supplementary Methods and Table S2 for an overview). The peptides are referenced individually in the Supplementary Section.

4.8. Detection of SARS-CoV-2 specific memory B cells

Spike-specific B cells were detected using either sequential staining of biotinylated Recombinant WT SARS-CoV-2 Spike-Trimer and RBD (HEK) (Miltenyi) conjugated with streptavidin-PE or streptavidin-BV786 respectively were combined with probes already conjugated with Alexa Fluor 647 for Spike RBD B1.1, 529 (AFR11056, Gly339Asp, Ser371Leu, Ser373Pro, Ser375Phe, Lys417Asn, Asn440Lys, Gly446Ser, Ser477Asn, Thr478Lys, Glu484Ala, Gln493Arg, Gly496Ser, Gln498Arg, Asn501Tyr, Tyr505His, R&D Systems) and conjugated with Alexa Fluor 488 for Full-length spike B1.1, 529 protein (AFG11061, Ala67Val, His69del, Val70del, Thr95Ile, Gly142Asp, Val143del, Tyr144del, Tyr145del, Asn211del, Leu212Ile, ins214Glu-Pro-Glu, Gly339Asp, Ser371Leu, Ser373Pro, Ser375Phe, Lys417Asn, Asn440Lys, Gly446Ser, Ser477Asn, Thr478Lys, Glu484Ala, Gln493Arg, Gly496Ser, Gln498Arg, Asn501Tyr, Tyr505His, Thr547Lys, Asp614Gly, His655Tyr, Asn679Lys, Pro681His, Asn764Lys, Asp796Tyr, Asn856Lys, Gln954His, Asn969Lys, Leu981Phe) (Arg682Ser, Arg685Ser, Lys986Pro, Val987Pro, R&D Systems,). 2×10^6 cryo-preserved PBMC samples were transferred in a 96-well U-bottom plate. Cells were first stained with Fc block (BD Biosciences) for 15 min at room temperature. Cells were then washed and stained with probe master mix containing 100 ng spike-A488, and 25 ng RBD-A647 for 1 h at 4C. Following incubation with antigen probes, cells were washed twice and stained with Blue Live Dead (Thermo Fischer) for 10 min at room temperature. Cells were washed again and stained with antibodies according to manufacturer protocols: BUV805-Mouse Anti-Human CD7, clone M-T701 BD Biosciences, BUV805-Mouse Anti-Human CD14, clone M5E2, BD Biosciences, BV711-Mouse Anti-Human CD19, clone HIB19, BD Biosciences, BUV395-Mouse Anti-Human CD20, clone 2H7, BD Biosciences, BUV737-Mouse Anti-Human CD21, clone B-ly4, BD Biosciences, BUV615-Mouse Anti-Human CD24, clone ML5, BD Biosciences, A700-Mouse Anti-Human CD27, clone L128, BD Biosciences, PE-CF594-Mouse Anti-Human CD38, clone HIT2, BD Biosciences, PE-Cy-7-Mouse Anti-Human CD71, clone CY1G4, BD Biosciences, BV605-Mouse Anti-Human IgD, clone IA6–2, BD Biosciences, PerCP-Cy5.5 Mouse Anti-Human IgM, clone MHM-88, Biolegend, BV421-Mouse Anti-Human IgG, clone G18–145, BD Biosciences, APC-H7-Mouse Anti-Human HLA-DR, clone L243, Biolegend, and BV480-Rat Anti-Human CXCR5, clone RF8B2, BD Biosciences for 30 min on ice. Cells stained with the Spike Trimer were fixed with the transcription factor buffer (Thermo Fischer) and intra-cellularly stained for IRF4 (eFluor660, clone 3E4, Thermo Fischer) and Blimp-1 (PE-CF594, clone 6D3, BD Biosciences). Cells stained with RBD, and full Spike probes, were fixed overnight in 1 % PFA. Samples were acquired on BD FACSymphony.

4.9. Serology

A multiplexed bead-based flow cytometry assay referred to as microsphere affinity proteomics (MAP), was adapted for the detection of WT and VOC SARS-CoV-2 Spike and the receptor-binding domain (RBD) antibodies or anti-viral IgG antibodies (anti-EBV, anti-FLU, anti-Rhinovirus) as described [49–52].

4.10. Measurement of neutralizing antibodies

Vero E6 cells were added into 96-well plates (Costar 3595, Corning Incorporated) in 1×10^4 cells/well. The next day, titrated amounts of sera that had been inactivated at 56C for 30 min were mixed with TCID100 of SARS-CoV-2 viruses (either Human 2019-nCOV strain 2019-mCoV/Italy-INM1 – provided by the European Virus Archive GLOBAL (EVA-GLOBAL that has received funding from the European UnioWs Horizon 2020 program under grant agreement No $71029), SARS-CoV-2/Norway/11421/2021 (Delta/B.1.617.2), or SARS-CoV-2/Norway/29450/2021 (Omicron/B.1.1.529) in triplicates. Following 1.5 h of incubation, the mixtures were added to the cells and incubated for 4 days at 37.C in a 5 % CO2 humidified atmosphere. Next, the plates were washed with PBS and fixed with acetone/PBS for 30 min. The plates were air-dried, washed, and incubated with rabbit anti-SARS-CoV-2 nucleocapsid antibody (cat. 40,143-R004, Sino Biological) overnight at 4OC. Plates were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG Fc antibody (cat. SSA003, Sino Biological) for 1 h at room temperature, developed with TMB Substrate Solution (cat. N301, ThermoFisher), stopped with 1 M of hydrochloric acid, and read with an EnVision 2104 Multilabel Reader (Perkin Elmer). The neutralization titer was determined as the highest plasma dilution that neutralized more than 50 % of the virus. The assay has been validated by comparison with other laboratories.

Sample availability

The patient samples are not available on request due to restricting ethical and legal approvals.

Ethics statement

The study (NCT04320732 and NCT04381819) was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (reference numbers 124,170 and 106,624). The participants provided their written informed consent before inclusion.

Statistics

Comparative analyses of frequencies of cell subsets and marker expression are presented by GraphPad Prism version 9.3 with violin plots and dashed lines to indicate the median and interquartile range showing all data points, and the difference between the control and test group was tested using Mann-Whitney *U* test for unpaired data and Wilcoxon test on paired samples for the comparison between unstimulated and peptides stimulated samples. Tests were two-sided. Values of $p < 0.05$ were considered statistically significant. Correlations were calculated with Pearson's test. A correlation matrix was calculated comparing phenotypic and serological marker variables in a pairwise fashion, using the corr. Test function from the psych CRAN package; the corrplot package was subsequently used to graphically display the correlation matrix. The resulting *P* values were adjusted for multiple testing using the Bonferroni method. Pearson's correlation coefficients were indicated by a heat scale whereby the red color shows a positive linear correlation, and the blue color shows a negative linear correlation. The volcano plots and the correlation matrix were integrated as a package in CYTOGRAPHER®, ImmunoScape cloud-based analytical software. Simplified presentation of incredible complex valuations (SPICE) software was used to determine the polyfunctionality of antigen-specific T cells. A permutation test from SPICE software was used to compare the distribution of cell populations for each pair of pie charts. Values of $p < 0.05$ were considered statistically significant.

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A. Acute plasma signature of BTI in HD and IBD. Quantification of pro-inflammatory molecules in plasma from BTI was assessed by Elisa for the indicated markers for HD ($n = 16$, 6 weeks) and for IBD patients (n = 10, 6 weeks). Mann Whitney test and two-tailed *P* values are indicated for BTI and vaccinated HDs comparisons with *, **, ***, and **** denoting $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ respectively.

B. Specific inflammatory signature of IBD patients after BTI. Visualization by principal component analysis (PCA) of innate molecules detected in plasma of HD and IBD patients after BTI. Ellipses were automatically generated to delineate BTI based on clinical status (HD vs. IBD).

C. Specific innate signature of IBD patients after BTI. Visualization by volcano plots of the significant inflammatory molecules detected in vaccinated HD and IBD patients after BTI. Significance levels from the Mann-Whitney test are indicated: * for $p < 0.05$ and ** for $p < 0.01$.

D. Inflammatory networks after BTI. The correlogram described the significant correlation between the pro-inflammatory molecules after BTI in HD and IBD patients. Pearson correlations with FDR are indicated with * for $p < 0.05$ and ** for $p < 0.01$.

CRediT authorship contribution statement

Amin Alirezaylavasani: Methodology, Formal analysis, Data curation. **Ingrid Marie Egner:** Project administration, Investigation. **Børresdatter Dahl:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Adity Chopra:** Investigation, Data curation. **Taissa de Matos Kasahara:** Methodology, Investigation, Formal analysis. **Guro Løvik Goll:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Jørgen Jahnsen:** Investigation. **Gunnveig Grødeland:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Data curation. **John Torgils Vaage:** Project administration, Funding acquisition. **Fridtjof Lund-Johansen:** Writing – review & editing, Validation, Resources, Methodology, Data curation. **Jan Cato Holter:** Resources, Investigation, Funding acquisition. **Bente Halvorsen:** Supervision, Methodology, Funding acquisition. **Kristin Kaasen Jørgensen:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Ludvig A. Munthe:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Hassen Kared:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Formal analysis, Data curation, Conceptualization.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2024.110404.

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