Non-classical CD45RBlo memory B-cells are the majority of circulating antigen-specific B-cells following mRNA vaccination and COVID-19 infection.

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Article

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Abstract

Resting memory B-cells can be divided into classical and non-classical groups based on differential expression of markers such as CD27 and CD11c, while activated memory B-cells express a combination of markers, making their ontogeny hard to determine. Here by longitudinal analysis of COVID-19, bacterial sepsis, and BNT162b2 mRNA vaccine recipients by mass cytometry and CITE-seq we describe a three-branch structure of resting B-cell memory consisting of “classical” CD45RB⁺ memory and two branches of CD45RB LO memory further defined by expression of CD23 and CD11c respectively. Stable differences in CD45RB upon activation allowed tracking of activated B-cells and plasmablasts derived from CD45RB⁺ classical and CD45RB LO non-classical memory B-cells. In both COVID-19 patients and mRNA vaccination, CD45RB LO B-cells formed the majority of SARS-CoV2 specific memory B-cells and correlated with serum antibodies while CD45RB⁺ memory was most strongly activated by bacterial Sepsis. These results suggest that diverse non-classical CD45RB LO memory B-cells consisting of branches of CD11c⁺Tbet⁺ and CD23⁺ fractions form a critical part of responses to viral infection and vaccination.

Introduction

Humoral immune memory against SARS-CoV2 forms a critical barrier preventing or limiting the severity of later infections [1]. Humoral immunity relies on a complex interplay of CD4 T-cells such as T-follicular helper cells (Tfh) and antibody-producing B-cells and their memory populations [2].

Circulating memory B-cells in human blood can be broadly divided into two main fractions. The first, classical memory, expresses CD27 and CD21 but lacks expression of CD11c while non-classical memory cells, often referred to as atypical, DN2 or age-associated B-cells (ABCs), are typified by the opposite pattern of CD11c positivity but lacking CD27 and CD21 [3–6]. During acute infection or following recent vaccination, a population of activated memory B-cells has been recognized by their expression of a variety of markers, most notably CD71 [7, 8] and lack of CD21 expression [9, 10]. Since these cells express markers associated with both classical (CD27) and atypical (CD11c, Tbet and FCRL5) [9, 11] B-cells, determining if they are derived from resting classical or atypical memory B-cells has been difficult.

Outside of these main branches, a variety of rare B-cell populations have been described, but there is a lack of consensus on the best markers and nomenclature [3, 12]. Several recent projects have attempted to disentangle this by using large mass cytometry (CyTOF) or flow cytometry antibody panels to screen for the most effective markers of human and mouse memory B-cells [13, 14]. In both cases a glycosylated epitope of CD45RB, recognized by the MEM55 clone, was identified as a key marker of circulating human classical memory B-cells [13, 14]. CD45RB positivity was also described to precede CD27 expression in the classical branch of memory B-cells, characterizing an early form of CD45RB⁺CD27⁻ memory [13, 15]. CD45RB is not expressed by either naïve or germinal center B-cells but
was rather found to be a marker of the transition from a germinal center (GC) to early memory and plasmablast phenotypes and thus has been considered a marker of GC derived memory [16, 17].

Since the GC is the primary site of efficient affinity maturation, GC-derived memory cells play a critical role in humoral immunity to COVID-19 and related vaccination [18, 19]. However, while faster extrafollicular responses are expected to produce less durable immunity there is also an increasing appreciation of the role of CD11c and Tbet expressing atypical B-cells in a range of settings such as viral infections, including COVID-19, and autoimmunity [8, 20–26].

Both the classical and atypical routes of antibody production are largely reliant on T-cell help and T-cell memory is another critical component of protection from viral infections such as COVID-19 [1, 27]. Of B-helper CD4 T-cells, T-follicular helper (Tfh) cells are the best described. Tfh express the characteristic chemokine receptor CXCR5 which allows their trafficking to the B-cell follicle and the GC itself. A circulating form of Tfh, (cTfh) have been identified to be strongly predictive of ongoing germinal center responses [28]. Tfh themselves also contain a variety of subsets identified by chemokine receptors including Tfh1 (CXCR3⁺CCR6⁻), Tfh2 (CXCR3⁻CCR6⁻) and Tfh17 (CXCR3⁻CCR6⁺) alongside activation status determined by increased levels of PD1 and ICOS but reduced CCR7 [28–30].

While Tfh are the main drivers of follicular antibody responses, recently T-peripheral helper (Tph) cells, characterized by high expression of PD1 and lack of CXCR5 have been identified as a major helper of B-cell responses in extrafollicular regions [31, 32]. Tph were originally described in the context of autoimmune diseases such as RA and SLE [32, 33] but have also recently been shown by us and others to be present in high numbers during COVID-19 [34, 35]. In all these cases Tph appear strong predictors of the frequency of atypical B-cells in the blood [33, 34].

This phenotypic complexity, which varies distinctly over time in the different settings of infection and vaccination has made it difficult to resolve the contributions of classical and non-classical memory B-cells and their activated subsets in humans. In this study, by screening large longitudinal cohorts of infection and vaccination by mass cytometry and CITE-seq [36] we found that the majority of blood circulating memory B cells responding to SARS-CoV2 consisted of distinct groups of CD11c⁺ CD45RB⁻ activated and CD23⁺ CD45RBlo resting non-classical B-cells. Use of CD45RB allowed activated B-cells and plasmablasts to be separated into classically (CD45RB⁺) and non-classically (CD45RB⁻) derived fractions with the classical cells strongly responsive to bacterial sepsis while the non-classical cells expanding in both COVID-19 and mRNA vaccination in a response coordinated with Tfh1 and Tph CD4 cells. Therefore, while GC (classical) responses are critical for COVID-19 and mRNA vaccination, our results suggest an important contribution from distinct sets of CD45RB⁻ non-classical B cells, possibly arising from extrafollicular responses, both in the acute and resting memory phases.

Results

Study design and mass cytometry analysis.
To perform an in-depth comparison of different disease state and vaccine responses we examined a large longitudinal cohort of severe COVID-19 and bacterial sepsis patients alongside a healthcare worker cohort of Pfizer mRNA vaccine recipients (Fig. 1A, Table S1). Samples were processed in large batches using sample barcoding and a split panel design for analysis of T cells, non-T cells and total lineage proportions in CD3+ magnetic bead enriched, CD3-depleted, or unenriched cells respectively (Fig. 1A, Table S2). After quality control, removal of batch controls and debarcoding (Figure S1A, B) this gave a total of 632 unique samples from 218 donors split into 26.8, 34.7, and 7.3 million cells in the T-cell, non-T cell and lineage datasets respectively (Fig. 1A). All samples were analyzed in a single combined workflow allowing in-depth comparison of cellular states across our separate cohorts [34, 37, 38].

Analysis of total lineage populations in our ICU cohort showed that B-cells (including plasmablasts) were notably increased at the earliest timepoints in COVID-19 but not Sepsis (Figure S1 C-E). We then performed detailed analysis of B-cells and annotated the clusters based on our previous work and recent studies using large screening approaches for B-cell population analysis [13, 14, 34]. IgM, IgG, and IgA were measured but excluded from clustering to avoid excessive fractionation of memory populations.

For visualization we used a force directed approach (ForceAtlas2) that preserves global structure (relationships between clusters) and was able to lay out cells in a biologically interpretable order (Fig. 1B) [39, 40]. Transitional B-cells were recognized as CD38+CD24+IgD+CD27- and were adjacent to CD38loCD24loIgD+CD27- Naïve B-cells (Fig. 1B, C, D). The CD45RB+CD27- early memory population [13, 15] was visible as a branch emerging from naïve B-cells and terminating in CD45RB+CD27+ classical memory cells. A second branch of CD45RB-CD11c-CD21+IgD- class-switched cells that we identified as having a similar progression from CD27 negative to positive was annotated as CD45RB-CD27- and CD45RB-CD27+ Mem respectively. CD11c+CD21loCXCR5-CD27-IgD- Atypical (AT) naïve B-cells [41] were visible and adjacent to class-switched CD11c+CD21loCXCR5-CD27-IgD- atypical (AT) memory cells.

Previous reports have demonstrated that activated memory B-cells can be defined by expression of CD71, CD27, CD11c and lack of CD21 [7]. Here we found that CD71+CD27+CD11c+Ki67+CD21lo activated B-cells could be clearly divided into two groups using CD45RB, whose expression has been reported as restricted to classical memory B-cells [13–15]. Accordingly, we annotated these cells as CD45RB+ activated memory (CD45RB+ Act) and CD45RB- atypical activated memory (CD45RB- AT Act).

Comparison of these two populations revealed that CD45RB- AT Act has significantly higher expression of CD11c and Ki67 while CD45RB+ Act has higher expression of CD21, CXCR5, CD27, CD95 (Fig. 1C, S2A). However, while these differences were statistically significant in this large dataset, they were also subtle, leaving CD45RB as the only non-overlapping marker allowing reliable separation of these cells. The activated phenotype of CD45RB+ Act was confirmed by direct comparison with CD45RB+CD27+ memory by its significantly lower expression of CD21, CXCR5, CXCR4 and CD24 alongside significantly increased CD11c, CD39, CD71, CD38, CD95, Ki67 and CD19 (Figure S1A). Analysis of Isotypes also showed that AT memory cells had a high level of IgG while CD45RB+ classical memory groups had a mixture of IgA and IgG (Fig. 1D).
CD45RB<sup>lo</sup> activated B-cells respond to COVID-19 and vaccination while CD45RB<sup>hi</sup> activated B-cells expand during bacterial Sepsis.

We then examined changes to the proportions of B-cells in our longitudinal ICU cohort (Fig. 2A, Table S1). Most B-cells in age matched healthy donors were found in naïve and classical memory (CD27<sup>+</sup>CD45RB<sup>+</sup>) groups with atypical cells, activated B-cells and plasmablasts comparatively rare (Figure S2B). During COVID-19 and Sepsis, CD45RB<sup>−</sup> AT Act B-cells were significantly expanded at all measured time points in comparison to healthy controls (Fig. 2B). In contrast CD45RB<sup>+</sup> Act B-cells were significantly expanded in Sepsis in comparison to either COVID-19 or healthy controls suggesting that this population is responsive to bacterial, but not viral infection (Fig. 2B). Both CD45RB<sup>lo</sup> and CD45RB<sup>hi</sup> plasmablasts were expanded in both COVID-19 and Sepsis with an initial skew to COVID-19 at days 1–4 after ICU admission but greater proportions of these cells in Sepsis from day 5 onwards (Fig. 2B). A broad shift in loss of CD45RB<sup>hi</sup> memory cells but gain of CD45RB<sup>lo</sup> cells was observed in non-activated memory groups with both CD45RB<sup>+</sup>CD27<sup>+</sup> and CD45RB<sup>+</sup>CD27<sup>−</sup> cells reduced in COVID-19 and Sepsis while the CD45RB<sup>−</sup>CD27<sup>−</sup> group being expanded most strongly in COVID-19 (Figure S2B). Naïve cells were unaffected while transitional B-cells suffered an increasing loss over time (Figure S2B). We also examined a cohort of moderate COVID-19 patients and found that these trends were broadly similar with CD45RB<sup>−</sup> AT Act B-cells increased while CD45RB<sup>+</sup> Act B-cells were not (Figure S2C).

We then examined a longitudinal cohort of healthcare worker BNT16b2 mRNA vaccine recipients (Fig. 2C). Analysis of B-cells during mRNA vaccination revealed that by day 12–21 post-primary vaccination a significant expansion of CD45RB<sup>lo</sup> but not CD45RB<sup>hi</sup> plasmablasts was observed (Fig. 2D). Following secondary vaccination this increase of CD45RB<sup>lo</sup> plasmablasts was sustained. At this time point a significant increase in CD45RB<sup>−</sup> AT Act was also observed while CD45RB<sup>+</sup> Act had no clear response. By three months after the secondary vaccination, these increases in activated cells had resolved. However, following tertiary vaccination CD45RB<sup>−</sup> AT Act B-cells showed a significant expansion while no other B-cell subset responded (Fig. 2D) suggesting that although this population is rare, it is highly responsive to mRNA vaccination.

Overall, we saw significant increases in CD45RB<sup>−</sup> activated atypical B-cells following both COVID-19 infection and vaccination and that CD45RB<sup>lo</sup> subsets of memory, atypical memory and plasmablasts seemed to have a closer relationship to COVID-19 and vaccination while CD45RB<sup>+</sup> activated memory cells were only expanded during bacterial Sepsis.

**Tph cells are predominant during early COVID-19, but Tph and Tfh responses are more balanced during vaccination.**

Using the same analysis approach as for B-cells we examined the CD4 T cell compartment in the same samples (Fig. 3A). We defined Tph as CD45RA<sup>−</sup>CD45R0<sup>+</sup> PD1<sup>+</sup>ICOS<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup>Tbet<sup>lo</sup>CXCR5<sup>−</sup> (Figure S3A) and further divided them based on expression of the proliferation marker Ki67 into Tph and
Ki67^Tph. Both Tph and Ki67^Tph were expanded in the blood of COVID-19 and Sepsis patients over time (Fig. 3B). The Ki67^Tph subgroup was most associated with COVID-19 while bacterial Sepsis had a significantly higher proportion of the less activated Tph. Proliferating effector memory (Ki67^ EM) Th1 cells were also increased in both settings, again to a significantly higher degree in COVID-19 than Sepsis (Fig. 3B).

We saw that overall circulating Tfh (cTfh) were slightly but significantly reduced in the blood of COVID-19 patients (Fig. 3B). Since cTfh themselves are known to harbor a range of subpopulations we clustered these cells to fully dissect Tfh subgroups. We split cTfh into the large central memory (CM)-like group of PD1 negative cells and subgroups based on expression of CCR4, CCR6, CD161, CXCR3 and Tbet. PD1, ICOS, and Tox were then used to define more activated cells and Ki67 used to define the most highly activated proliferating groups (Figure S3C).

PD1^CXCR3^Tbet^ Tfh1 were reduced as a proportion of Tfh while their rarer proliferating subgroup Tfh1 Ki67^PD1^ was increased, with the overall result that Tfh1 were reduced but those that remained were highly activated (Fig. 3C). CCR6^CD161^ Tfh17 were significantly increased in Sepsis but not COVID-19 while CCR6^CXCR3^ Tfh2 were increased over time in both COVID-19 and Sepsis (Figure S3D). Taken together, there was a slight decrease in cTfh, but fine analysis of subgroups revealed a more complex picture with shifts within Tfh towards Tfh17 in Sepsis and Tfh1 being proportionally reduced but highly activated (Fig. 3C, S3D).

During vaccination, analysis of CD4 T-cells revealed a significant expansion of Ki67^ Tph at the same time points (post-primary and post-secondary) where we observed expansion of CD45RB^ B-cells (Fig. 3D). The post-secondary time point also saw expansion of proliferating Th1 similar to that seen in the initial trials of BNT162b1[42]. In contrast to COVID-19, overall cTfh were slightly increased during vaccination (Fig. 3D). Close inspection of Tfh sub-phenotypes revealed a significant expansion of proliferating Tfh1 (Fig. 3E).

Overall, expansion or activation of Tph and Tfh1 was not an exclusive property of either infection or vaccination, but vaccination shows a balanced response of Tph and Tfh1 while COVID-19 is more strongly skewed to a Tph response. Tph and Tfh1 are also expanded in Sepsis but with greater Th17 and Tfh17 responses than that seen in either COVID-19 or vaccination.

**Monocyte interferon signature seen in COVID-19 and vaccination but not bacterial sepsis.**

Previously, atypical B-cell activation has been associated with both Tph, Th1 and Tfh1 which are themselves induced by early type 1 interferon and its effects on monocytes and dendritic cells [43]. Analysis of classical monocytes in our dataset (Figure S4A) demonstrated that at day 1 following ICU admission over 90% of monocytes express the interferon signature protein CD169 (Siglec-1) which rapidly drops to baseline levels within 5 days (Figure S4B). No clear increase in CD169 expressing monocytes was seen in Sepsis despite these cells also being driven into a characteristic HLA-DR^lo state
shared by COVID-19 and Sepsis [44, 45], supporting previous results that CD169 is an effective separator of viral and bacterial inflammation [46].

During vaccination, both CD169 expressing HLA-DR$^{\text{hi}}$ and HLA-DR$^{\text{lo}}$ monocytes showed a similar increase 2 days after vaccination, preceding changes to CD4 and B-cells (Figure S4C). This response, although much lower in magnitude than COVID-19, suggests an early role for interferon signaling in monocytes in driving the similarity in CD4 and B-cell responses.

**CITE-seq analysis of B-cell memory confirms phenotype of CD45RB$^+$ and CD45RB$^-$ Activated memory groups.**

Having established the broad changes to the immune system in these large cohorts, and the importance of CD45RB as a marker, which is not recoverable by mRNA, we then performed CITE-seq to further explore B cell populations of interest (Fig. 4A). We first hashtagged PBMCs from 3 sepsis, 3 COVID-19, 3 post-secondary and 3 post-tertiary vaccine timepoints. Cells were stained with fluorescent TotalSeqC antibodies and double stained with SARS-CoV2 spike tetramer and then IgD$^-$ B-cells were sorted and sequenced via the chromium 10x platform to obtain information on antigen specificity, surface protein, mRNA and BCR sequences.

Clustering yielded a similar population structure that we had seen in the IgD$^-$ compartment of the CyTOF dataset consisting of CD45RB$^-$ AT memory, Activated AT memory B-cells (that we further divided into early Act AT memory and Act AT memory), a CD45RB$^{\text{lo}}$ memory group, and CD45RB$^+$ Act memory and CD45RB$^+$ memory (Fig. 4B). We could not clearly separate CD27$^{+/-}$ compartments of CD45RB$^+$ and CD45RB$^{\text{lo}}$ memory on either mRNA or CD27 staining suggesting these are transcriptionally similar groups. As before, the Activated memory groups co-express CD27, CD71 (TFRC) and CD11c (ITGAX) at both protein and RNA levels alongside activation markers such as FAS, S100A10 and ANXA4 (Fig. 4B, C, D, S5A, S5B, S5D). Separate groups of CD45RB$^{\text{lo}}$ and CD45RB$^{\text{hi}}$ plasmablasts were also resolved. We also confirmed the identification of the Atypical (AT) groups through their expression of characteristic markers such as TBX21 (Tbet), ZEB2 and FCRL5 (Fig. 4B, E).

**CD45RB$^{\text{lo}}$ resting memory cells are characterized by expression of CD23 and IL4R.**

In addition to further analysis of atypical cells we found that the CD11c$^-$$^{\text{CD45RB}^{\text{lo}}}$ memory cells had a broadly resting phenotype (lack of activation markers) shared with CD45RB$^+$ Memory cells, but that differential gene analysis showed enriched expression of FCER2 (CD23), IL4R, STAG3, IL13RA1 and IGHE (Fig. 4D, E, S5B, S5D). Several groups have recently described a similar signature in both CD27$^-$IgD$^-$ [47] or CD27$^+$IgD$^-$ memory B-cells in the context of allergy [48, 49]. All three studies and our own data display a striking similarity of phenotype between these cells.

**CD45RB$^{\text{lo}}$ phenotypes form the majority of SARS-CoV2 reactive B-cells during early COVID-19 or post-secondary and tertiary vaccination.**
We then analyzed antigen specificity in the CITE-seq data by identification of memory B-cells that dual bound to the spike tetramers used (Fig. 5A, B). While CD45RB⁺ B-cells made up the majority of SARS-CoV2 spike negative memory B-cells, we saw that in both COVID-19 and vaccination, SARS-CoV2 spike positive cells were strongly concentrated into the CD45RB⁻ clusters, particularly the AT early Act memory and AT Act memory groups (Fig. 5C, D), supporting our finding of expanded CD45RB⁺ AT Act in the CyTOF dataset (Fig. 2B, C).

**B cell receptor analysis shows clonal overlap between atypical subsets and CD45RB⁻ plasmablasts.**

We then examined levels of mutation in BCR sequences in vaccine samples and found that all groups harbored mutations in their Spike non-binding populations, with Act Memory cells having the highest levels (Fig. 5E). Examination of spike binding B-cells showed that both the AT early Act memory and the AT Act memory groups had low levels of mutation after the second vaccination but by post-3rd vaccine there was a clear increase (Fig. 5E). Examination of individual clone trees also demonstrated that single clones could be found to gain mutations over time and that the same clone could be detected in the AT like groups, plasmablasts and CD45RB⁻ memory (Fig. 5F). A broader look at clonal overlaps between all spike positive clones revealed strong clonal sharing between the AT early Act mem, AT Act mem and plasmablasts (Fig. 5G). Examining all B-cells showed that while AT Act memory had a strong overlap with plasmablasts in COVID-19 and vaccination, the CD45RB⁺ Act memory population had the strongest overlap with plasmablasts in Sepsis (Figure S6B), supporting the Sepsis-specific expansion of CD45RB⁺ Act mem seen in the CyTOF dataset (Fig. 2B). Together these results suggest that CD45RB⁻ atypical-like cells are the primary source of plasmablasts in COVID-19 but in Sepsis CD45RB⁺⁺ memory cells were the primary source of plasmablasts and that it is possible to track the plasmablast progeny of different memory B-cell subgroups.

RNA velocity analysis [50] of vaccine and COVID-19 samples also showed that the less activated CD45RB⁺ memory and AT memory groups appeared to flow through the corresponding activated memory groups and terminate in plasmablasts as expected (Figure S6C).

**CD45RB⁻ Atypical and CD23⁺ memory cells are a large proportion of SARS-CoV2 specific cells during acute or resting time points.**

To further confirm the accuracy of these results, we used mRNA-based anchor transfer [51] of previously published CITE-seq datasets onto our cluster identities (Fig. 5I, S6D-G). These datasets included peripheral blood BNT162b2 mRNA vaccine recipients following secondary, pre-, and post-tertiary vaccination and a mixed dataset of peripheral blood and tonsil cells from mRNA vaccine recipients [52]. We excluded naïve or GC-like cells since they were not in our dataset. Comparison of the expression patterns of the differentially expressed genes from our dataset (Fig. 4B) and the PBMC dataset (Figure S6E) confirmed that the transfer was effective although analysis of plasmablasts was limited due to a low frequency of plasmablasts in the transferred data.
Similar to our initial findings, CD45RB⁺ memory dominated wild-type (wt) Spike-negative memory B cells but wtSpike, wtRBD and B.1.1.529-Spike positive B-cells had a larger proportion of CD45RBloff memory cells (Fig. 5H). The AT like groups could be observed shortly after post-secondary and post tertiary vaccinations but, interestingly, at the resting time point 6 months post-secondary vaccination, the resting CD45RBloff mem population made up a significant proportion of tetramer positive memory cells. This high proportion remained after the third vaccination alongside expansion of the CD45RBloff AT Act groups (Fig. 5H) as also seen in our CITE-seq and CyTOF datasets (Fig. 2D, Fig. 5D). While our own CITE-seq data had a higher proportion of activated cells, possibly due to differences in time of blood collection after vaccination, the pattern of the result was similar, and it was notable that the majority of SARS-CoV2 binding B-cells lacked CD45RB expression following the third vaccination.

While the first transferred PBMC dataset did not include CD45RB staining, the second matched tonsil and PBMC dataset included it, allowing clearer confirmation that mRNA-based anchor transfer was able to accurately identify the same CD45RBloff atypical and CD23⁺ groups cells that we had previously observed (Fig. 5I, S6D-G). Since this dataset was derived from resting timepoints long after vaccination and AT cells are expected to be low in lymphoid organs outside the spleen [53], only the CD45RB⁺ mem and CD45RBloff mem were observed in significant numbers. Again, comparison of SARS-CoV2 binding and non-binding cells demonstrated that CD45RBloff cells were strongly enriched in spike or RBD binding fractions and were often the majority of antigen specific cells in both the blood and tonsils (Fig. 5J). Overall, the analysis of our and other CITE-seq datasets supported the findings of the CyTOF analysis and suggested that further close analysis of the pre- and post-3rd vaccination time point was warranted.

**CyTOF analysis of tetramer specific B-cells**

We then adapted our B-cell CyTOF panel by incorporating tetramers for Spike, wtRBD and Omicron RBD (Fig. 6A) alongside additional markers such as Tbet and CD23 (Figure S7A, table S1). We concentrated on a smaller confirmatory subset of COVID-19 samples and a larger group of pre- and post-tertiary vaccination samples to examine the distribution of virus specific B-cells at both resting and acute time points. Initially we used the expanded marker panel to confirm that, as predicted AT and Act AT memory B-cells express high levels of Tbet, FCRL5 and CD20 (Figure S7A). We also observed that CD45RB⁺ Act expressed intermediate levels of Tbet, FCRL5 and CD20 both lower than AT cells but significantly higher than CD45RB⁺CD27⁺ memory cells.

CD45RB⁺CD27⁺ memory cells had significantly higher (although still low) expression of Tbet and CXCR3 than CD45RB⁺CD27⁺ memory cells. CD23 expression was seen on CD45RBloff memory B-cells confirming that these are the same cells seen in the CITE-seq datasets (Figure S7A). CD23 levels decreased as these cells became CD27 positive which, alongside a decrease in CXCR4 and increased CD95 (Figure S2A) suggests that CD27 may act as a maturation marker in a similar manner to CD45RB⁺ memory cells described previously [13]. BLIMP1 was restricted to plasmablasts with CD45RBloff plasmablasts having
lower BLIMP1 but higher Tbet and HLA-DR expression than their CD45RB<sub>hi</sub> counterparts suggesting a less mature phenotype with a recent switch from Tbet expressing cells (Figure S2A, S7A).

**CyTOF analysis confirms that CD45RB<sub>lo</sub> groups are the main SARS-CoV2 reactive memory B-cells pre and post tertiary vaccination.**

To examine the distribution of the SARS-CoV2 antigen specific memory populations, we first gated tetramer positive B-cells (Fig. 6A) and confirmed that most spike positive B-cells pre and post vaccination were class-switched memory (IgD negative non-plasmablasts) (Fig. 6B).

Proportional analysis of memory subsets demonstrated that CD45RB<sup>+</sup> memory cells were dominant among spike negative cells while most spike positive cells fell into the CD45RB<sub>lo</sub> memory groups (Fig. 6C, D). As expected, the distribution of spike positive subsets changed across time with resting cells making up the majority of antigen specific memory prior to the third vaccination with both CD45RB<sup>+</sup> and CD45RB<sub>lo</sub> CD27<sup>+</sup> and CD27<sup>-</sup> subgroups being enriched (Fig. 6C, D). Most SARS-CoV2 specific cells expanding post vaccination were found in the AT Mem, CD45RB<sup>-</sup> AT Act and CD45RB<sup>+</sup> Act groups while CD45RB<sup>-</sup>CD27<sup>-</sup> mem were reduced as a proportion of total memory groups (Fig. 6C). wtSpike, wtRBD and B1.1.529-RBD all showed a similar distribution although both RBDs had a slightly higher proportion of CD45RB<sup>+</sup> cells, particularly pre-third vaccination (Fig. 6D). This data, along with our CITE-seq analysis (Fig. 5D, H) suggests resting and recently vaccinated time points are distinguished by the shift from CD45RB<sup>+</sup> and CD45RB<sub>lo</sub> resting populations to activated and atypical phenotypes seen shortly after vaccination.

Examination of our COVID-19 cohort also showed a strong shift in phenotypes with CD45RB- AT Act being the dominant antigen specific populations at day 1 post ICU admission (Fig. 6E). By day 7 the same donors had begun have a more balanced distribution with non-AT cells beginning to recover as a proportion of antigen specific cells, mirroring the shifts in non-antigen specific B cells seen over time in the large cohort (Fig. 2B).

**Serum antibody levels are significantly correlated with SARS-CoV2 reactive CD45RB<sub>lo</sub> Atypical B-cells but not classical memory B-cells.**

We then examined levels of SARS-CoV2 specific antibodies in the blood of the same vaccinated cohort. As expected, levels of IgG recognizing the RBD, S1 and S2 subunits of spike protein strongly increased following the third vaccination while a lack of anti-nucleocapsid antibodies confirmed an absence of asymptomatic infections (Fig. 6F). We then examined the correlation between serum antibodies and proportions of antigen specific B-cells in the same patients. Positive correlations were observed between antibody levels and antigen specific B-cells but only the AT mem and CD45RB- At Act populations reached significance (Fig. 6G, H, Figure S7B, C). Since the CD45RB<sub>lo</sub>CD23<sup>+</sup> subset of B-cells has previously been associated with sequential switching to IgE production [48, 49], we also assessed levels of SARS-CoV2 spike reactive IgE but were unable to detect spike reactive IgE by ELISA (limit of detection...
2.5ng/ml) either pre or post vaccination despite substantial increases in spike reactive IgG within the same donors (Fig. 6F). This suggests that while these cells may be primed to switch to IgE production in some settings, they do not appear to do so during mRNA vaccination. Indeed, while IgE transcripts were enriched in this subset the majority (84.7%) of the cells lacked its expression (Figure S5C).

**In vitro tracking of B-cell subsets confirms that CD45RB is a stable marker of cellular origin and that all B-cells can upregulate atypical makers in a T-cell dependent manner.**

Together our results suggested that heterologous CD45RBlo memory B-cells may have a key role in the antibody responses to both vaccination and SARS-CoV2 infection. While CD45RB has previously been considered to be a stable marker of classical memory B-cells [13, 15–17] recent results have also demonstrated that classical memory B-cells can switch to a Tbet expressing phenotype, making these boundaries less clear [52, 54]. While relationships between resting and activated populations can be implied by their similar phenotypes, RNA velocity and clonal analysis, a more direct way to show this is through direct tracking of the fate of sorted CD45RBlo or high memory groups upon further stimulation.

Therefore, we sorted IgD+ Naïve, CD45RB−CD27+ (CD45RBloCD27+CD11c−IgD−), CD45RB+CD27+ (CD45RBhiCD27+CD11c−IgD−), CD45RB+ Act (CD45RBhiCD27+CD11c+IgD−) and CD45RB− AT Act (CD45RBloCD27+CD11c+IgD−) CD19+ B-cells with the aim of tracing their phenotypes after *in vitro* stimulation (Fig. 7A, S7D). To provide relatively physiological conditions, we added the sorted B-cell subsets into B-cell depleted PBMCs and then stimulated them indirectly via anti-CD3 driven T-cell activation and cytokine supplementation and used CyTOF to readout the resulting phenotypes.

We broadly divided the resulting cells into Naïve, activated B-cells and plasmablasts using markers such as IgD, CD20 and BLIMP1 (Fig. 7B-D) and determined that all sorted B-cell subsets could differentiate into plasmablasts (Fig. 7E). On examination of activated memory B-cells derived from any of these initial populations we found that all sorted subsets were capable of upregulating CD11c and Tbet to a similar degree (Fig. 7F). However, CD45RB was neither lost by initially CD45RB+ cells nor gained by initially CD45RBlo cells prior to plasmablast differentiation, while CD27 was also slightly lower (Fig. 7F). This difference could not be explained by lower proliferation or activation since levels of Ki67, CD71 and active RNA and protein production measured by incorporation of BrU and puromycin [55] were either not different or higher for AT Mem derived cells (Figure S7E) as also observed previously [13]. When examining CD45RB expression in plasmablasts we again saw that AT mem derived cells had significantly lower CD45RB expression (Fig. 7F) although it was upregulated in comparison to activated B-cells. Together with the different levels of HLA-DR, BLIMP-1 and Tbet between these populations, we interpreted these findings as indicating that CD45RBlo plasmablasts are a less mature intermediate derived from CD45RBlo memory cells but may later switch to a more mature CD45RB+ phenotype.

**Discussion**
Here we used multimodal analysis of longitudinal COVID-19, Sepsis, and mRNA vaccination cohorts to dissect the cellular immune response in these settings, with a focus on a deep analysis of B cells. Several reports have already described clear increases in classical, atypical, and activated memory following mRNA vaccination and during COVID-19 [8, 20–24, 26]. However, we have demonstrated that the stable absence of CD45RB in non-classical B-cells can be used to better demarcate activated and plasmablast B-cells derived from initially CD45RB\textsubscript{lo} non-classical B-cells. We found that while they are a rarer subset, CD45RB\textsubscript{lo} activated memory cells with an atypical phenotype had a stronger response to COVID-19 and mRNA vaccination than CD45RB\textsuperscript{+} activated memory cells which were primarily increased in Sepsis. Both phenotypic and clonal relationships suggest that these activated cells were at an intermediate stage between CD45RB\textsubscript{lo} or CD45RB\textsubscript{hi} resting memory B-cells and terminated in matching groups of CD45RB\textsubscript{lo} or CD45RB\textsubscript{hi} plasmablasts.

While atypical memory cells are detectable in blood, it is a diverse group and many of the markers used to identify it may also catch B-cells recently activated in the presence of interferon or IL21 [56]. Several recent reports identified some degree of identity switching, as classical memory B-cells were seen to gain atypical-like activation signatures defined by Tbet and FCRL5 upon reactivation [52, 54]. Here we also support this conclusion demonstrating that essentially all B-cells can gain Tbet expression upon re-stimulation. However, we also suggest that while both CD45RB\textsubscript{lo} and CD45RB\textsubscript{hi} activated B-cells express CD11c and Tbet, the former may be the product of atypical memory cells while the latter may derive from interferon-driven activation of classical memory B-cells.

Strikingly, the majority of tetramer-positive cells following both COVID-19 and mRNA vaccination were in the CD45RB\textsubscript{lo} compartment of B-cell memory, and the question arises as to what is the source of these cells? At the resting time-point before the 3rd dose of mRNA vaccines, most CD45RB\textsubscript{lo} SARS-CoV2 reactive B cells in both the blood and tonsils did not express atypical markers, but rather a signature characterized by CD23 and IL4R that has recently been described in both CD27\textsuperscript{−} [47] and CD27\textsuperscript{+} memory cells in the context of allergy [48, 49]. The observed CD23\textsuperscript{+}IL4R\textsuperscript{+}CD45RB\textsubscript{lo} B-cells had little in common with the interferon driven signature seen in atypical B-cells, lacking markers such as ZEB2, CD11c and Tbet [57]. However, we demonstrated that during \textit{in vitro} culture that both these and classical memory B-cells were capable of upregulating Tbet and CD11c. While initially CD45RB\textsubscript{lo} and CD45RB\textsubscript{hi} cells could still be separated on the basis of CD45RB, the CD11c\textsuperscript{+} (AT mem) and CD11c\textsuperscript{−} CD45RB\textsubscript{lo} memory cells were essentially indistinguishable after culture. Since both in our own and other datasets we saw that CD45RB\textsubscript{lo} non-atypical cells were a significant reservoir of resting SARS-CoV2 memory cells prior to 3rd vaccination, this raises the possibility that they may be one source of the activated CD45RB\textsubscript{lo} Tbet positive cells seen following vaccination. Another non-mutually exclusive possibility is that the cells are also derived from rare but still detectable AT memory cells that may expand rapidly upon revaccination.

Thirdly, naïve B-cells are also a potential source of CD45RB\textsubscript{lo} activated cells, however in this case we also saw clonal lineages across vaccination time points and a clear increase of variable gene mutations in the later vaccination suggesting that memory cells are a likely source. While increased mutations over
time could be an indicator of reentry into secondary GCs, SHM is not completely exclusive to GCs [58, 59] and Tbet expressing B-cells have previously been reported to have lower mutations than GC derived B-cells but higher than naïve cells [60]. Here we also observed that the CD45RB<sup>lo</sup> activated subset of B-cells had a lower level of mutation than CD45RB<sup>+</sup> activated B-cells, another potential indicator of different origins.

Due to the recent discovery of CD45RB<sup>lo</sup>CD23<sup>+</sup>IL4R<sup>+</sup> memory B-cells very little is known about their origin [47–49]. However, a recent preprint suggests that a murine equivalent to these cells is observable and may be partially independent of germinal centers [49]. The recent descriptions of these cells have focused on their presence in allergy and ability to switch to IgE production [48, 49], but here we saw no evidence of SARS-CoV2 reactive IgE after vaccination.

The balance between Tfh and Tph may also have a key role in determining B-cell fates. A recent study demonstrated that during severe, but not mild, COVID-19 there is an overall decrease in cTfh and a delay in the development of antigen specific Tfh [61]. Together with previous findings demonstrating suppressed GC in deceased COVID-19 patients, this indicates that some suppression of Tfh may occur during severe acute COVID-19 [62, 63]. However, studies of convalescent patients also show a clear increase in antigen specific Tfh and functional GCs underlining that this may be a temporary phenomenon specific to severe disease [18, 64–66]. More specifically the Tfh1 subset has also been observed to be functional in COVID-19 and associated with atypical phenotypes of B-cells [67–69]. Tph have also been shown to increase in COVID-19 [34, 35]. Previous reports have suggested that atypical cells can be induced by either IFNγ or IL-21, both of which may be sourced from either Tph or Tfh1 [70–72]. As a result, both Tph and Tfh1 are credible sources of B-cell help in this context and here we saw that all these previously reported phenomena were observable in a time and context dependent manner. While severe COVID-19 was characterized by an expansion in Tph but relatively weak response from Tfh, mRNA vaccination showed a more balanced response with both Tph and Tfh1 expanded at the same time points suggesting that multiple branches of T-cell help can be active simultaneously.

In conclusion, using multimodal analysis of large longitudinal cohorts from several settings we have been able to provide a comprehensive structure of circulating B-cells in human blood. The stable difference in the expression of CD45RB made it possible to track B-cells derived from three branches of memory CD27<sup>+</sup>CD45RB<sup>+</sup> “classical memory”, CD23<sup>+</sup>CD45RB<sup>lo</sup> “non-classical memory” and Tbet<sup>+</sup>CD45RB<sup>lo</sup> “atypical memory” through matching stages of activated and plasmablast differentiation. Importantly most SARS-CoV2 binding B-cells following mRNA vaccination COVID-19 fell into the CD45RB<sup>lo</sup> branch of memory/plasmablast differentiation suggesting that this non-classical route of B-cell activation may have a key role in the response to viral infections. Understanding this balance in both infection and vaccination will be critical to the development of new treatments and increasing the efficacy of vaccines to emerging and existing threats from infectious disease.

Declarations
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Investigation: DP, JT, JNS.

Methodology: JBW, DP, DO.

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Writing – original draft: JBW, DP.

Writing – review & editing: All authors

Declaration of interests

The authors declare no competing interests.

Data availability
Sequencing data has been deposited at NCBI's Gene Expression Omnibus (GEO). GSE247488

References


Methods

Human participants recruitment and sampling ICU Cohort

PBMC and plasma samples were collected longitudinally from cohorts of moderate COVID-19 (non-Intensive care) and severe (Intensive care) COVID-19 and severe (Intensive care) Sepsis patients, and healthy age-matched healthy controls (Table 1). Hospitalized cases diagnosed as COVID-19 were enrolled by physicians using clinical manifestation and PCR test results. Sepsis patients were enrolled by physicians using clinical manifestation based on sepsis-3 criteria. Age-matched control subjects were collected at Osaka University Hospital and Osaka General Medical Center. Patients with COVID-19 were further categorised by the WHO ten-point clinical progression scale, (Table 1) [73].
Additionally, PBMC and plasma samples were collected from a cohort of healthy donors during a vaccination time-course with the Pfizer BioNTech BNT162b2 SARS-CoV-2 vaccine (BNT162b2) (Table 1). Samples were collected from August 2020 to May 2021 at Osaka University Hospital and Osaka General Medical Center. This study followed the principles of the Declaration of Helsinki and was approved by the institutional review board of Osaka University Hospital (permit nos. 907 and 885 [Osaka University Critical Care Consortium Novel Omix Project; Occonomix Project]). Informed consent was obtained from the patients or their relatives and the healthy volunteers for the collection of all blood samples.

**Peripheral blood sample processing**

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples using Ficoll-Paque density gradient centrifugation, resuspended at 1-2E6 cells/ml Cellbanker 1 (Takara Bio) before being subject to controlled freezing at -80°C in a CoolCell device (Corning) and later stored in gas-phase liquid nitrogen.

**Mass cytometry antibody production.**

We obtained Indium-113 and -115 and gadolinium-157 (Trace Sciences) for conjugation to antibodies. X8 polymer MaxPar kits (Standard Biotools) were used to conjugate Indium and lanthanide isotopes, while MCP9 polymer kits were used to conjugate Cadmium isotopes according to the manufacturer’s instructions. Platinum-labeled antibodies were conjugated with cisplatin as previously described [74]. Conjugated antibodies were stored in a PBS-based antibody stabilizer or HRP-protector stabilizer for cadmium labeling (Candor Biosciences). All antibodies were titrated using control PBMCs to obtain optimal staining concentrations. Antibody staining panels were either prepared fresh, or prepared in bulk and stored as aliquots at -80°C.

**CD45 barcoding and cell staining for mass cytometry.**

Samples were measured across 21 experimental runs. The 21st run used an updated panel for B cells that included tetramers for SARS-CoV-2 spike protein.

Frozen PBMC samples were defrosted in a 37°C water bath for 2 minutes and poured into a 15-ml tube and 5 ml of prewarmed RPMI containing 10% FCS and 20 IU/ml Pierce Universal Nuclease for Cell Lysis was added. Samples were then washed with the same buffer without resuspending, then resuspended in 2 mL of CyFACS buffer, and live cells counted by Acridine Orange/Propidium Iodide staining on a LUNA-FL fluorescence cell counter (Logos Biosystems). In each experiment, up to 4E6 viable cells per sample were labelled with a seven choose-three pattern of anti-CD45 barcodes (89Y, 113In, 115In, 194Pt, 195Pt, 196Pt, and 198Pt) to give a combination of up to 35 barcoded samples per experiment, including a spike in control used to monitor batch effect (Figure S1B) [75]. Barcoding antibody aliquots in CyFACS were prepared in bulk and stored at -80°C. Samples were incubated with CD45 barcodes together with FC-block and anti-CXCR5 biotin (in run 21 a direct anti-CXCR5 antibody was used to avoid interference with streptavidin-biotin B cell tetramer conjugates) in CyFACS buffer (PBS with 0.1% BSA and 2 mM EDTA) for
30 min at room temperature (RT), and then washed once by adding 5 mL CyFACS buffer, centrifugation at 400g and the supernatant was poured off. The barcoded cells were resuspended in 700 μl CyFACS buffer, pooled and filtered through a 70 μm filter (Miltenyi) to remove dead cell debris. At this stage 5-10% cells were transferred to a 15 mL tube (Lineage stain tube, total PBMCs used for lineage proportions across CD45+ PBMCs). The remainder of the cells were separated into a CD3+ fraction (T stain tube) and CD3-depleted fraction (non-T stain tube) using a magnetic bead-based CD3 positive selection kit (StemCell). Cells in each tube were stained with separate surface and intracellular panels for T cells (CD4, Tfh, Treg, CD8 and γδT), non-T cells (Monocytes, DCs, B and NK cells), and total lineage proportions (panel information Table S2). Premixed and frozen master mixes were used to reduce batch effects. For the 21st experiment, only the non-T stain tube was stained using panel for B cells.

Cells were stained with the respective surface stain antibody cocktail for 45 min at RT. The cells were then washed twice in CyFACS buffer (400g, 5 min, RT), stained using 500 nM Zirconium based viability stain [76] in PBS for 5 min at RT, washed, and then fixed and permeabilized using the Foxp3 Transcription Factor Staining Buffer Set according to the manufacturer’s protocol (eBioscience). The cells were subsequently stained with the respective intracellular antibody cocktail for 45 min at 4°C and then washed twice (centrifugation at 800g, 5 min, 4°C) in CyFACS buffer and once in PBS. The cells were then fixed overnight in 2% formaldehyde (Thermo) in PBS containing 1 μM DNA Cell-ID Intercalator-103Rh (Standard Biotools).

**Preparation of B cell tetramers for mass cytometry staining**

B cell tetramers were prepared in a similar way to previous reports [77, 78]. Briefly, biotin-labelled SARS-CoV-2 wild-type full-length Spike (Miltenyi), wild-type RBD domain or B.1.1.529/OmicronRBD (Acro Biosystems) that had been reconstituted at 100-200 μg/mL in PBS10 were mixed with Streptavidin (SA)-FITC, SA-PE or SA-APC (Biolegend) respectively in three steps separated by 15 minutes to give a final 4:1 molar ratio of protein:SA and total incubation time of RT for 45 min. Tetramer staining mix was prepared by adding each tetramer to CyFACS with 2.5 μM Biotin to saturate unbound streptavidin and minimise probe cross-reactivity. Following barcoding and mixing of cells, they were stained with the three tetramers (wtSpike-FITC, wtRBD-PE and B.1.1.529/OmicronRBD-APC) at RT for 45 min in a total volume of 550 μl. Then cells were washed three times in CyFACS buffer (400g, 5 min, RT) following which the staining protocol was identical to that for experiments 1-20 but using the B-cell staining panel (Table S2).

**Mass cytometry data acquisition**

Cells were washed (800g, 5 min, 4°C) once in 5 mL CyFACS buffer and twice in 2 mL Cell Acquisition Buffer (CAS, Standard Biotools). The cells were diluted to 1.1E6 cells/mL in CAS with 15% EQ Four Element Calibration Beads (Standard Biotools) and passed through a 35-μm filter immediately before collection. Cells were run at 200 to 500 cells/s on a Helios mass cytometer (Standard Biotools), or CyTOF XT. Flow Cytometry Standard (FCS) files were normalized to EQ beads and concatenated using Standard Biotools software before export.

**B cell in vitro plasmablast differentiation assay**
The B cell in vitro plasmablast differentiation assay was performed using frozen PBMC from healthy Leukopak donors (StemCell). For each assay, ~1E8 cells in liquid nitrogen were thawed as above except 50 mL tubes were used for the initial thawing and washing step. 10% of PBMC was set aside for sorting of non-B cells while 1% of PBMC was set aside on ice for the whole PBMC control. The remainder was enriched for B cells using the EasySep™ Human B Cell Isolation Kit (StemCell). Enriched B cells were stained with a panel of fluorescently labeled antibodies (Table S1) and total PBMC were stained with anti-CD19 and anti-IgD to allow sorting of non-B cells (CD19-IgD-). Cells were stained for 30 min at RT and washed twice in 5 mL PBS + 10% FCS (PBS10), then resuspended in 1-2 mL of PBS10 and sorted at 4°C on a BD FACSymphony S6 cell sorter (BD). B cells were sorted into six different populations (Figure S7D) after which non-B cells were sorted. Sorted cells were transferred from 5 mL FACS tubes into 15 mL tubes and spun down. Up to 18 conditions were included in the B cell assay. The base media for the B cell assay (RPMI + 10% FBS + Pen/strep (cRPMI) with 1 μg/mL anti-CD3 antibody (Biolegend), 10 U/mL IL2 (Immunace35, Shinogi) and 10 ng/mL IL-1β (Peprotech). For further stimulation of B cells, some conditions were supplemented with a cocktail of 10 ng/mL IL-10, 10 ng/mL IL-4 and 50 ng/mL IL-21 (Peprotech) added on day 4 of the assay. To prepare the assay, in 96-well U bottom plates (Corning #163320), 100 μl of media (containing 2x concentration of stimulants) was added to wells followed by 100 μl of cells in cRPMI. For the whole PBMC condition, 1E5 of the whole PBMC (100 μl at 1E6 cells/mL) was added to respective wells. For B cell assay conditions, sorted non-B cells were added to each well (9E4 cells in 50 μl) followed by sorted B cells (1E4 cells in 50 μl) yielding an approximately physiological ratio of 10% B cells to 90% non-B cells in 200 μl per well. Plates were incubated at 37°C 5% CO₂ for six days. On the fourth day, 50 μl of media was aspirated from each well and replaced with 50 μl cRPMI or cRPMI with 4x cocktail for the day 4 cocktail condition.

On day 6 samples were stained and measured by CyTOF with the following modifications. Cells were pipetted out of wells into 15 mL tubes, spun down and first labeled with a six choose-three pattern of anti-CD45 barcodes (89Y, 113In, 115In, 194Pt, 195Pt, and 196Pt) to give a combination of up to 18 barcoded samples per experiment with 198Pt to be used for dead cell staining. To allow incorporation of puromycin, IdU and BrU into cellular macromolecules [55], the staining protocol included a 30 min pre-stain at 37°C in cRPMI at which time cells were also stained for chemokine receptors (CCR7, CXCR3, CXCR5) (Table S2). Cells were then stained with surface and intracellular panels. B cell assay samples were run on a CyTOF XT using suspension mode in CAS+ buffer with 10% 6-element EQ beads (Standard Biotools). Flow Cytometry Standard (FCS) files were normalized to EQ beads and concatenated using Standard Biotools software before export.

Mass cytometry data analysis

FCS files were uploaded to Cytobank software (Beckman Coulter) and gated as positive for DNA, negative for EQ beads, below a threshold on the Zr live/dead stain (or 198Pt negative in the B-cell assay), with normal ion cloud Gaussian parameters (Figure S1A), before being manually debarcoded based on the CD45 barcodes (Figure S1A, B) and exported as FCS files.
Firstly, FCS files were imported into R (v.4.3.0) via flowCore (v.2.12.2) and batch corrected (SOM size 10 by 10, arcsinh 5 transformed) with cyCombine (v.0.2.15)[79]. Spike in samples in every experiment were used to confirm the accuracy of batch correction. In the T stain tube, CCR2, which was not in the panel for experiments 1 and 2, was imputed onto these two batches using the salvage_problematic() function of cyCombine. In the Lineage stain tube, CD33, CD95, CXCR4, CD27, IgD, CD56, Helios, Granzyme B, TCRγδ, CD38, HLA-DR were imputed from experiments 2-20 onto experiment 1 using the CyTOF 2 panels vignette of cyCombine.

Following batch correction samples were exported as FCS files and then re-imported to R and converted to single cell experiment format (SCE) and analysed with CATALYST (v.1.24.0) [37, 38] as previously described [34]. Briefly, data was then compensated using a compensation matrix derived from a compensation panel of single-metal-labeled compensation beads. Data was clustered using flowSOM (using a 10 by 10 SOM and subsequent meta-clustering) using a multi-step process. The first step employed a limited set of markers optimised to separate into the main immune lineages. Secondly, each lineage population was sub-clustered to obtain fine cell sub-types. Detailed cell type annotation was based on prior knowledge combined with analysis of heatmaps and UMAPs (downsampling to 1000 cells per sample and 40 nearest neighbours). In the case of CD4 cells, CXCR5^+Foxp3^- Tfh were also handled as a subcluster to allow their in-depth analysis.

Non-T stain tube data for experiments 1-20 was clustered to obtain B cells, NK cells and Myeloid & DCs. For B cells, the panel for experiments 1-20, which was designed for clustering all CD3- cells, was different from that of experiment 21, which was focussed specifically on B cells and tetramers. However, we sub-clustered the B cell data from experiments 1-21 together using only shared markers after batch correction with cyCombine using the CyTOF 2-panels vignette. For simplicity we merged the CD73+ or CD73- Naive and CD73+ or CD73- Classical B cell types previously identified by Glass et al [13]. To calculate isotype proportions, the B cell SCE was reclustered using only IgG, IgA, IgD and IgM and isotype proportion per phenotypic cluster was calculated.

Differential abundance testing among clusters was performed with diffcyt (v.1.20.0) in DA mode using edgeR (v.3.42.4) and default settings. Prior to plotting, clusters with 0% abundance were set to 0.01% for display on Log scaled plots. Expression levels of markers were tested for statistical significance using Kruskal-Wallis followed by Dunn's with Holm adjustment with rstatix (v. 0.7.2).

**ForceAtlas2 Visualization**

For visualisation of B-cells by forceatlas2, 5000 cells per cluster were used to generate a KNN edge matrix in vortex software [80], the resulting exported XML file containing edge and node lists was imported into Gephi (v.0.10.1) network analysis software for layout by the Forcealtas2 algorithm using, Tolerance = 1, Approximate repulsion = 1.2, scaling =1 , gravity =4 and dissuade hubs ON [39].

**CITE-seq**
CITE-seq was performed on 12 samples: three COVID-19 day 1 samples, three Sepsis day 1 samples, and samples from three vaccine cohort donors after their second and third vaccine dose. PBMC for each sample were thawed as above and cells were barcoded with TotalSeq-C Hashtag antibodies. Cell pellets were resuspended in a total of 100 μl barcode mix (0.5 μl hashtag antibody in PBS10) and stained for 30 minutes at 4°C. Cells were then washed twice in 5 mL PBS10 (400g, 5 min, 4°C), resuspended in 1 mL PBS10 and then mixed into a single tube and centrifuged (400g, 10 min, 4°C).

The cell pellet was resuspended in a total of 200 μl staining mastermix containing fluorescently labelled sorting antibodies, TotalSeqC antibodies and two freshly prepared SARS-CoV-2 wild-type full-length Spike tetramers (Table S2) and stained for 30 minutes at 4°C. Cells were then washed twice in 5 mL PBS10 (400g, 5 min, 4°C) and sorted on a FACSaria III (BD) into 1.5 mL tubes. IgD- B cells (CD19+IgD-CD4-CD14-) (post sort viability >95%) were resuspended at 700 cells per μl and submitted to the Osaka University Biken NGS Core for library preparation and sequencing.

**Single-cell RNA library construction and sequencing**

Single-cell suspensions were processed with a 10x Genomics Chromium Controller using the Chromium Next GEM Single Cell 5’ Kit v2, Chromium Next GEM Chip K Single Cell Kit and Dual Index Kit TT Set A according to the manufacturer’s instructions. Library preparation and sequencing was done by loading approximately 16,500 live cells per well on the Chromium controller to generate 10,000 single-cell gel-bead emulsions (5 wells total). Oil-encapsulated single cells and barcoded beads (GEMs) were reverse-transcribed on a Veriti Thermal Cycler (Thermo). The resulting cDNA tagged with a cell barcode and unique molecular index (UMI) was amplified to generate single-cell libraries, which were quantified with an Agilent Bioanalyzer High Sensitivity DNA assay (Agilent). The amplified cDNA was enzymatically fragmented, end-repaired, and polyA tagged followed by cleanup and size selection using SPRIselect magnetic beads (Beckman-Coulter). Next, Illumina sequencing adapters were ligated to the size-selected fragments followed by another round of cleanup. Finally, sample indices were selected and amplified, followed by a double-sided size selection using SPRIselect magnetic beads.

For VDJ repertoire profiling, full-length VDJ regions were enriched from amplified cDNA by PCR amplification with primers specific to the Ig constant regions using the Chromium Single Cell Human BCR Amplification Kit and enriched VDJ segments were used for VDJ-library construction. After final library quality assessment using an Agilent Bioanalyzer High Sensitivity DNA assay, samples were sequenced on an Illumina NovaSeq 6000 as paired-end mode (read1: 28bp; read2: 91bp).

**Preprocessing of single-cell RNA sequencing data**

Data was pre-processed using the CellRanger multi pipeline (version 7.0.0). Analysis was done in R (v.4.3.0) using the Seurat package (v.4.3.0). Since samples were split across multiple GEM wells, a well ID was first added to the barcodes followed by sample assignment based on Hashtags using the HTODemux() function. Data was then sent through the following QC pipeline. The data was filtered for hashtag singlets and the following QC metrics (500 > sample > 8000 mRNA features per cell, < 10%
mitochondrial reads, > 5% ribosomal reads and < 3000 ADT counts per cell). Ribosomal and mitochondrial genes were then removed along with the MALAT1 gene. Data was then normalised according to recommended settings with RNA (method = LogNormalize, scale.factor = 10000) and ADT (method = CLR, margin = 2). Cell cycle was then assigned using CellCycleScoring and data was scaled regressing out the %mitochondrial and cell cycle difference (S score minus G2M score) from the RNA data. This resulted in a Seurat object with 28491 QC-passed cells (10341 for COVID-19, 2296 for Sepsis and 15854 for Vaccine samples).

**Analysis of single-cell RNA sequencing data**

FindVariableFeatures() was used to identify the list of top 3000 variable genes. IGHV, IGKV and IGLV immunoglobulin genes were removed from this list. Seurat (v.4.3.0) multimodal clustering of the GEX (mRNA) and ADT data was performed. Principal component analysis (PCA) was run on the GEX data using the variable features and another PCA was run on all ADT features. FindMultiModalNeighbors() was then run using PCs 1-6 of GEX and PCs 1-4 of ADT at k=50 nearest neighbours. The wnnUMAP was made and data was clustered using FindClusters with SLM algorithm and resolution = 2. The 24 resulting clusters were then manually merged and annotated to give clusters shown (Figure 4B). SARS-CoV-2 wtSpike tetramer positive cells (total: 1042) were annotated as ≥ 0.2 in both the PE and APC ADT channels (Figure 5B). Differential expression between populations was performed with FindMarkers() at default settings.

**Anchor transfer**

Anchor transfer of the datasets [52] was done with Seurat (v.4.3.0) in R (v.4.3.0) using a supervised PCA made from the wnn graph, and k.filter set to NA. IgD+ cells were removed from the query data, as the reference was sorted for IgD- cells (RNA cutoff: 0.1, protein cutoff: 0.5).

**RNA velocity analysis**

Count matrices of unspliced and spliced RNA was generated using velocyto (v.0.17.17) alignment to hg38 in zsh (v.5.9) and merged with loompy (v.3.0.6) in python (3.9.16). RNA velocity was calculated using the dynamic model in scVelo (v. 0.2.5) with variable features pre-defined in the seurat analysis, 50 principal components, 50 neighbors, and 10 shared counts. scVelo was run in Jupyter notebook (v.7.0.0) using the following dependencies: anndata (v.0.9.1), scanpy (v.1.9.3), numpy (v.1.21.1), scipy (v.1.10.1), pandas (v.1.5.3), scikit-learn (v.1.2.2), matplotlib (v.3.7.1), python-igraph (v.0.10.6), louvain (v.0.8.0), pybind11 (v.2.11.1), hnswlib (v.0.6.2), ipython (v.8.14.0), tqdm (v.4.65.0), and ipywidgets (v.8.0.7). For use in python, the seurat object was converted to h5ad using SeuratDisk (v.0.0.0.9020) in R (v.4.3.0).

**Analysis of BCR data**

The Immcantation [81] and scRepertoire (v. 1.11.0) [82] workflows were used for analysis of BCR data. In scRepertoire the 'strict' definition of clonotype was used for all analyses. The Immcantation workflow starts by running igblast (version 1.21.0) using Python scripts provided by Immcantation to give the
“filtered_contig_igblast_db-pass.tsv files”. Given that the data spanned 5 GEM wells, a well ID was appended to the cell_id and sequence_id columns, after which the data was concatenated, therefore allowing cells found in both the GEX and BCR datasets to be matched by their cell barcode.

Briefly, data was filtered for productive BCRs, and cells with none or multiple heavy chains were removed (yielding 21300 cells with a single heavy chain). Then clonotyping was done with Scoper using an automatically-determined threshold. For calculating clonal overlap between Seurat clusters, clonotyping results were filtered for IgH and then metadata columns from the seurat object were attached giving 15103 cells found in both datasets (6692 for COVID-19, 1538 for Sepsis and 6873 for Vaccine), of which 460 were spike positive.

Somatic Hypermutation (SHM) on heavy chain data was then calculated using observedMutations(..., regionDefinition = IMGT_V) (Shazam) (IMGT database updated May 2023), yielding 20687 cells mapped to germline of which 14659 were also found in the GEX data (453 spike positive). Dowser (v.2.0.0) was then used to format and plot the germline-mapped cells as phylogenetic clone trees (build = “pml”) with metadata attached from the Seurat object.

Clonal overlap between Seurat clusters was calculated as the Jaccard index, which is the ratio of the number of intersecting clonotypes to the union count of clonotypes between two clusters. The raw count of intersecting clonotypes between pairs of clusters is also written on the heatmaps. IgH gene usage heatmaps by Seurat cluster were calculated using vizGenes() function in the scReptore pipeline.

**Analysis of plasma antibody levels by Luminex and ELISA**

During preparation of PBMC, plasma samples were also collected and stored at -80°C. Serum titers of SARS-CoV-2 antibodies were assessed using the Bio-Plex Pro Human IgG SARS-CoV-2 N/RBD/S1/S2 4-Plex Panel kit (BioRad) according to the manufacturer’s directions on a BioPlex-200 machine (BioRad). Freshly thawed plasma samples were centrifuged (1000g, 10 min, 4°), diluted 1:1000 (4 μl into 196 μl then 5 μl in 95 μl), and run in duplicate. Background subtracted MFI duplicates were averaged and spearman correlations with cellular proportions from the corresponding PBMC samples were calculated with Corrplot v.0.92.

An Anti-SARS-CoV-2 SPIKE IgE ELISA kit (Matriks Biotech) was run according to the manufacturer’s directions using 1:100 and 1:500 dilution of each plasma sample. The same samples were run as for measuring SARS-CoV-2 IgG antibodies.

**Figures**
Figure 1

Mass cytometry analysis of B-cells reveals distinct groups of activated and resting memory B cell populations.

(A) Overview of CyTOF study design.
(B) ForceAtlas2 visualization of B cell FlowSOM clusters from merged analysis of dataset.

(C) Expression of indicated protein markers on ForceAtlas2 map.

(D) Heatmap of median scaled expression of type markers across merged B cell FlowSOM clusters and donut plots of proportions of isotypes for each B cell cluster. Derived from a total of 9.81 million B-cells, with 632 samples from 218 individuals. Boxplots of selected markers shown in Figure S2A.
Figure 2

**CD45RB<sub>lo</sub>** activated B-cells respond to COVID-19 and vaccination but **CD45RB<sub>hi</sub>** activated B-cells expand during bacterial sepsis.

(A) Schematic of COVID-19 and Sepsis cohort.

(B) Proportions of B cell clusters in COVID-19 and Sepsis ICU patients across time compared to age-matched healthy controls (n = indicated in 2A). Other B-cell populations shown in Figure S2B

(C) Schematic of SARS-CoV2 mRNA vaccination cohort.

(D) Proportions of B cell clusters in SARS-CoV2 mRNA vaccination cohort (n = indicated in 2C). Significance testing in comparison to with pre-first except when indicated.

Adjusted p values from EdgeR. Single symbols P ≤ 0.05, double P ≤ 0.01, triple P ≤ 0.001, quadruple P ≤ 0.0001
Figure 3

Tph cells are predominant during COVID-19, but Tph and Tfh1 responses are balanced during vaccination.

(A) UMAP and Heatmap of markers of CD4 T cell FlowSOM clusters. Boxplots of selected markers shown in Figure S3A.
(B and C) Proportions of CD4 (B) and Tfh (C) cell clusters in COVID-19 and Sepsis ICU patients across time compared to age-matched healthy controls (n = indicated in Figure 2A). Other CD4 populations seen in figure S3B. Further analysis of Tfh is shown in figure S3C-D.

* COVID-19 vs healthy, †Sepsis vs healthy, ‡COVID-19 vs Sepsis.

(D and E) Proportions of CD4 (D) and Tfh (E) clusters in SARS-CoV2 mRNA vaccination cohort (n = indicated in Figure 2C). Significance indicate comparison with the pre-first timepoint.

Adjusted p values from EdgeR. Single symbols P ≤ 0.05, double P ≤ 0.01, triple P ≤ 0.001, quadruple P ≤ 0.0001
Figure 4

CITE-seq analysis of memory B-cells and plasmablasts in COVID-19, Sepsis, and vaccination.

(A) Schematic of CITE-seq experiment.
(B) Weighted nearest neighbor (wnn) UMAP of IgD− memory B cells in CITE-seq colored according to SLM clusters. Dot plot of expression of selected differentially expressed mRNA.

(C) Single cell Violin plots of Antibody Derived Tag (adt) protein markers. Selected other mRNA and protein markers shown in Figure S5A and E.

(D) Expression of indicated markers displayed on wnnUMAP. Other markers are shown in Figure S5B. (E) Volcano plots of differentially expressed genes between indicated populations. Other comparisons shown in figure S5D.
**Figure 5**

CD45RB<sup>lo</sup> B-cells form the majority of SARS-CoV2 reactive B-cells during early COVID-19 and post-secondary and tertiary vaccination.

(A) wnnUMAP of memory B cells highlighted by SARS-CoV2 Spike tetramer binding cells.
(B) Scatterplot of staining intensity for both tetramers with spike positive cells highlighted (> 0.2 in each channel).

(C) Proportions of B memory clusters for Spike-negative and Spike-positive cells for COVID-19 samples (n = 3). Proportions of all memory and plasmablasts shown in Figure S6A.

(D) Proportions of B memory clusters for Spike-negative and Spike-positive B-cells from SARS-CoV2 mRNA vaccination samples (6 samples from 3 donors).

(E) Variable region mutation frequency across B memory clusters for Spike-negative, and Spike-positive cells following the 2nd and 3rd vaccine doses. Spike negatives are pooled from both time points.

(F) Top two spike binding clonal lineage trees.

(G) Heatmaps of Jaccard index of BCRs between SARS-CoV2 spike positive B cell clusters from all conditions. Numbers displayed on map are clone numbers.

(H) Proportions of memory B cells from Zurbuchen et al [52] PBMC vaccine dataset anchor transferred onto our cluster identities. UMAP and marker dotplots in Figure S6D and E.

(I) Single cell Violin plots of indicated markers derived from memory B cells from Zurbuchen et al [52] PBMC and Tonsil vaccine dataset anchor transferred onto our cluster identities.

(J) Proportions of memory B cells from Zurbuchen et al [52] PBMC and tonsil vaccine dataset anchor transferred onto our cluster identities. UMAP and marker dotplots in Figure S6F and G.
Figure 6

Analysis of tetramer reactive B-cells by CyTOF confirms that CD45RB\textsuperscript{lo} groups are the main SARS-CoV2 reactive memory B-cells pre and post tertiary vaccination.

(A) Gating of SARS-CoV2 tetramer-bound B-cells. Cells past the indicated thresholds were considered positive.
(B) Percent wtSpike positive cells per total B-cell of the indicated cell type (Naive, Memory or Plasmablasts) per total B cells. (n = 14).

(C) Percent wtSpike positive, wtRBD positive and B1.1.529-RBD positive cells of the indicated cell type per total B memory cells (excluding Naive and Plasmablasts). (n = 14).

(D) Median proportions of B memory clusters of wtSpike negative and indicated tetramer positive cells pre and post third vaccination (n = 14).

(E) Median proportions of B memory clusters of wtSpike negative and indicated tetramer positive cells for COVID-19 (n = 3)

(F) MFI of plasma levels of indicated antibodies in vaccine samples Pre and post 3rd vaccination. (n = 14). Antibody correlations in Figure S7B.

(G) Heatmaps of Spearman rank correlation coefficients of % of tetramer positive cells of B memory cells correlated with plasma MFI of indicated SARS-CoV2 antibodies. Other correlation in Figure S7C.

(H) Scatterplots of selected data from (G).

Significance *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 by Paired Wilcoxon (A-F) or Spearman's rank correlation (G-H)
Figure 7

In vitro tracking of B-cell subsets confirms that CD45RB is a stable marker of cellular origin and that all B-cells can upregulate atypical makers in a T-cell dependent manner.

(A) Schematic diagram of in vitro assay. Gating strategy for sorting in Figure S7D.
(B) UMAP of B cells from in vitro assay colored by cell cluster.

(C) UMAP with scaled expression of indicated markers.

(D) Heatmap of median scaled expression of all markers used.

(E) Proportion across non-Naive B cells (Activated or Plasmablast) for assay input B cell type. (n = 4).

(F) Median expression of markers expressed by Activated B-cells and Plasmablasts derived from indicated input B cell. P values from, comparison with AT Mem shown (n = 4). Other markers in Figure S7E.

Significance in E & F in comparison to AT Mem *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 ****P ≤ 0.001 from one way ANOVA with Holm adjustment.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

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