An efficient immunoassay for the B cell help function of SARS-CoV-2-specific memory CD4+ T cells

Graphical abstract

Highlights

- Monocyte supplementation enhances antigen-specific T-B coculture assays
- Monocytes potentiate B cell outputs, such as plasma cells and antibodies
- Monocytes promote early T cell priming and support B cell survival via BAFF and SCGF
- Detection of potent SARS-CoV-2-specific memory CD4+ T cell helper functions

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In brief

Ansari et al. describe an efficient T-B cell coculture assay to assess B cell help function of antigen-specific T helper cells in healthy and coronavirus disease 2019 (COVID-19) recovered individuals, based on the inclusion of monocytes. Increased B cell output in this assay may provide extra sensitivity to evaluate immunological responses in different contexts.
An efficient immunoassay for the B cell help function of SARS-CoV-2-specific memory CD4+ T cells

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https://doi.org/10.1016/j.crmeth.2022.100224

SUMMARY
The B cell “help” function of CD4+ T cells is an important mechanism of adaptive immunity. Here, we describe improved antigen-specific T-B cocultures for quantitative measurement of T cell-dependent B cell responses, with as few as ~90 T cells. Utilizing M. tuberculosis (Mtbc), we show that early priming and activation of CD4+ T cells is important for productive interaction between T and B cells and that similar effects are achieved by supplementing cocultures with monocytes. We find that monocytes promote survivability of B cells via BAFF and stem cell growth factor (SCGF)/C-type lectin domain family 11 member A (CLEC11A), but this alone does not fully recapitulate the effects of monocyte supplementation. Importantly, we demonstrate improved activation and immunological output of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-specific memory CD4+ T-B cell cocultures with the inclusion of monocytes. This method may therefore provide a more sensitive assay to evaluate the B cell help quality of memory CD4+ T cells, for example, after vaccination or natural infection.

INTRODUCTION
CD4+ T helper cells play a crucial role in establishing the humoral arm of adaptive immunity. By supporting B cell function and antibody responses, CD4+ T cells play an important role in controlling various infections caused by bacteria, fungus, and virus. The CXCR5-expressing subset of CD4+ T cells known as follicular helper T (Tfh) cells are the specialized subset that provide help to B cells in germinal centers (GCs) (Crotty, 2011). Tfh cells make cognate interactions with B cells and provide essential signals for its survival and differentiation (Ansel et al., 1999; Breitfeld et al., 2000). Defects in GC formation and antibody production are associated with the deficiency in CD4+-T cell-derived signals like CD40L-CD40 interaction and interleukin-21 (IL-21) (Alien et al., 1993; Tangye and Ma, 2020). Clearly, a synchronized and robust cognate interaction between CD4+ T cell and B cell is the basis of productive humoral output in terms of memory B cells, plasma cells, and antibody. Thus, the
extent of help stimulus provided by CD4+ T cells may correlate with the magnitude and quality of humoral response generated after infection or vaccination.

The ex vivo T-B coculture assay is the attempt to recapitulate physiological T-B cognate interactions for measuring the help ability of CD4+ T cell subsets. The most commonly used ex vivo method involves coculturing of CD4+ T cells with B cells in presence of superantigen-like Staphylococcal enterotoxin B (SEB) for 7–12 days. The importance of this method is that it allows cross-linking between CD4+ T cells and B cells through T cell receptor (TCR)-peptide-major histocompatibility complex class II (pMHC-II) interactions, thus resembling the physiological condition of T cells’ engagement with B cells. This method has been widely used to compare the helper potential of CXCR5+ Tfh subsets or the CXCR5- non-Tfh subsets expressing activation markers in various infections and autoimmune diseases (Bentebibel et al., 2013; Caielli et al., 2019; Locci et al., 2013; Morita et al., 2011; Rao et al., 2017). Indeed, superantigen-based method was instrumental in revealing the potential and superiority of Tfh subsets in providing help to B cells. However, the superantigen-based method does not signify the antigen (Ag)-specific cognate interactions between T cells and B cells and thus cannot be utilized for defining the help potential of CD4+ T cell subset in Ag-specific manner. Recently, MHC-class-II-restricted (CD4+) peptides are used to determine the quantity of Ag-specific CD4+ T cells in the peripheral blood mononuclear cells (PBMCs) (Reiss et al., 2017). The stimulation of T-B cultures using peptides was helpful in defining the Ag cell helper function of HIV-specific CD4+ T cells (Del Alcazar et al., 2019). However, the limited use of Ag-specific T-B cultures may be due to the inconsistent detection of plasma cell output and the poor sensitivity in examining other immunologic parameters of the productive T-B cross-talk.

In fact, Ag-specific T-B coculture utilizes the function of B cells both as the Ag-presenting cells (APCs), to activate CD4+ T cells, as well as the recipient of helper signals from activated cognate CD4+ T cells. Thus, the efficiency and success of T-B cultures are completely dependent on the ability of B cells to prime and activate Ag-specific CD4+ T cells. Further, the survivability of B cells in the ex vivo cultures is also crucial, because these cells cannot survive longer in absence of optimal survival signals (Kraus et al., 2004; Schweighoffer et al., 2013). In the peptide-stimulated cultures, it is unlikely that B cells are efficiently performing their APC functions or getting sufficient signals for their optimal survival in absence of B cell receptor (BCR)-mediated signaling. This could also be a probable reason for poor sensitivity of conventional T-B cocultures in producing the consistent Ag-specific responses.

The current pandemic signifies the need of measuring multiple immunologic parameters for establishing the correlates of protection from coronavirus disease 2019 (COVID-19) vaccine or for defining the traits of broad protective immunity in infection. The CD4+ T cells are widely implicated in the less severe outcome of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (Braun et al., 2020; Rydzynski Moderbacher et al., 2020; Tan et al., 2021). Indeed, efficient Tfh responses and the ensuing enhanced plasmablasts and antibody production are associated with mild outcome of COVID-19 (Kuri-Cervantes et al., 2020). Because the T cell help is indispensable for good quality B cell and antibody responses, the qualitative assessment of the helper function of memory CD4+ T cells may serve as an immunologic parameter for defining functional memory responses. Here, we describe an Ag-specific T cell and B cell coculture assay that is highly consistent in measuring the help potential of memory CD4+ T cells. We investigated the role of monocytes in enhancing the efficiency of autologous T-B cultures for Ag-specific analysis. Using M. tuberculosis (Mtbc)-specific settings, we demonstrate that monocytes are superior APCs than B cells in the early stages of cultures to prime and activate Ag-specific CD4+ T cells. Moreover, monocytes also promote B cells survivability in cultures via growth factors like BAFF and stem cell growth factor (SCGF)/C-type lectin domain family 11 member A (CLEC11A). However, enhancing the B cell survivability alone could not replace monocyte supplementation. We further demonstrate the application of monocyte-supplemented T-B cocultures in assessing the helper function of SARS-CoV-2 spike-specific CD4+ T cells in COVID-19 recovered donors. The method described here is highly sensitive and provides quantitative measurement of several immunologic parameters of T-dependent B cell responses.

RESULTS

B cells survival is enhanced by supplementing T-B cocultures with the monocytes

The availability of functionally viable cells is important for autologous T and B cocultures. Thus, to examine the cell survivability, we sorted cells from healthy donors’ PBMCs (Figure S1) and cocultured 60,000 cells of memory CD4+ T cells and CD20+ B cells in 1:1 ratio up to 12 days, without exogenous Ag. As expected, only 3.8% ± 1.3% (mean ± SEM) total cells survived until 12 days (Figures S2A and S2B). Number of live B cells was significantly reduced after 12 days of coculture as compared with 9 days (Figure S2C; p = 0.02). Both naive and memory B cells died gradually until 12 days (Figure S2D). Similarly, substantial decline in live T cells was observed in 12 days than in 9 days (Figure S2E; p = 0.008). Mtbc-specific CD4+ T cells are present in a majority of the healthy subjects (unpublished data). Thus, for Ag-specific stimulations, we used Mtbc-specific CD4+ T cell peptides (Mtbc peptides) (Lindestam Arlehamn et al., 2016). No significant variation in B cell survivability was observed in presence of Mtbc peptides over the unstimulated conditions (unstm) (Figure S3A). However, in Mtbc peptides stimulation, significant increase in live T cell count was observed at day 9 (Figure S3B; p = 0.03). Like total B cells, no significant increase was observed in plasma cells (CD20+CD38+) in presence of Mtbc peptides (Figures S3C and S3D). We then quantified the activated CD4+ T cells in cocultures. We detected a significant number of CD38PD1CD4+ T cells as early as 3 days, which peaked at day 9 (Figures S3E and S3F; p = 0.001). In addition, all of the CD38PD1CD4+ T cells expressed high levels of ICOS as activation-associated co-stimulatory molecule, which showed better detectability than CD38 (data not shown). Therefore, with the maximal activation of T cells without significant loss in the viability of both T and B cells, we concluded 9 days of coculture as the optimal duration for further...
experiments. Also, we utilized ICOS$^+$PD-1$^+$ co-expression to distinctly identify the activated CD4$^+$ T cells in subsequent experiments.

Myeloid cells are implicated in supporting the survivability of lymphoid cells, mainly B cells (Epron et al., 2012; Mueller et al., 2007). Thus, to enhance the cell survivability, we supplemented the cocultures with monocytes that are abundantly available blood myeloid cells. To determine the impact on B cells survivability, we cultured 60,000 CD20$^+$ B cells with CD14$^+$ monocytes (M) for 9 days in various M-to-B ratios, 1:4, 1:2, and 1:1, in absence of any exogenous Ag. Titration of monocytes to B cells revealed that B cells survivability increased significantly at 1:2 ratio compared with B cells alone (Figure 1C; p < 0.0001), with the comparable impact of 1:2 and 1:1 M-to-B ratios (Figure 1C; non-significant [ns]). Thus, we used the M-to-B-cell ratio of 1:2 for further experiments. We then compared the B cells and T cells survivability in the autologous unstimulated cultures, with or without monocytes. We found significantly higher count of live B cells in presence of monocytes (B + M) as compared with B cells alone (Figures 1B and 1C; B alone: 110 ± 43; B + M: 1,644 ± 245; p < 0.0001), which was not prominent in case of B+T (Figure 1C; 501 ± 77; ns). Monocytes enhanced the B cells survivability by 30-fold (median), in contrast to only 9-fold increase in case of T cells (Figure 1D; B + M versus B + T: p = 0.009). Moreover, monocytes enhanced the survivability of both naive (CD27$^-$ B cells) and memory (CD27$^+$ B cells) compartments, with similar impact on both the compartments, in comparison with B + T (Figure 1E; naive: B + M: 68-fold, B + T: 13-fold, p = 0.02; memory: B + M: 24-fold, B + T: 8-fold, p = 0.04). Unlike B cells, T cells survived more efficiently when cocultured with monocytes or B cells (Figures 1F and 1G; T alone: 2,789 ± 330, T + M: 6,122 ± 663, p < 0.0001; T + B: 4,574 ± 463, p = 0.005). Survivability of T cells was comparable in presence of either monocytes or B cells (Figure 1H; ns). Altogether, the data suggest that monocytes supplementation promotes the survivability of B cells in autologous cultures.

Monocytes support in an efficient qualitative assessment of Ag-specific helper T cells

The quantitative measurement of plasma cell differentiation and antibody secretion is a benchmark parameter to determine B cell help quality of CD4$^+$ T cells. Thus, we addressed whether adding monocytes to T-B cocultures only enhances the survivability or it also augments the productivity of T-B interactions in the
**A**  
9 days co-culture  
B+M  
T+B  
T+M  
T+B+M  
Mtb Peptides/ Mtb Lysate  

**End-point analyses**  
- Plasma cell differentiation  
- Secreted Ig quantification  
- T cell activation  

**B**  
Unstimulated CD20  
B+M  
T+B  
T+B+M  
Mtb Peptides  
Mtb Lysate  

**C**  
Mtb Peptides  
Plasma cells (cell count/well)  
Unstimulated  
Mtb Peptides  

**D**  
Mtb Lysate  
Plasma cells (cell count/well)  
Unstimulated  
Mtb Lysate  

**E**  
Plasma cells  
Background subtracted (cell count/well)  
Mtb Peptides  
Mtb Lysate  
p = 0.002  
p = 0.05  
p = 0.06  

**F**  
Plasma cells  
Background subtracted (cell count/well)  
Mtb Peptides  
Mtb Lysate  
p = 0.03  
p = 0.06  

**G**  
Mtb Peptides  
Total IgG (mg/ml)  
Unstimulated  
Mtb Peptides  
p = 0.0001  
p = 0.04  
p = 0.01  

**H**  
Mtb Lysate  
Total IgG (mg/ml)  
Unstimulated  
Mtb Lysate  
p = 0.001  
p = 0.02  
p = 0.01  

**I**  
Unstimulated PD-1  
T+M  
T+B  
T+B+M  
Mtb Peptides  
Mtb Lysate  

**J**  
Mtb Peptides  
ICOS+PD-1 CD4+ T cells (cell count/well)  
Unstimulated  
Mtb Peptides  
p = 0.002  
p = 0.01  

**K**  
Mtb Lysate  
ICOS+PD-1 CD4+ T cells (cell count/well)  
Unstimulated  
Mtb Lysate  
p = 0.004  
p = 0.01  

**L**  
ICOS+PD-1 CD4+ T cells  
Background subtracted (cell count/well)  
Mtb Peptides  
Mtb Lysate  
p = 0.001  
p = 0.05  
p = 0.001  

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antigenic stimulations. We cocultured 60,000 cells of memory CD4+ T cells and CD20+ B cells in absence or presence of 30,000 CD14+ monocytes for 9 days (Figure 2A). Absence of any exogenous Ag (unstm) was used as a control for background response. For Ag-specific stimulation, we used the Mtb whole-cell lysate (Mtb lysate) and above used Mtb peptides. Mtb lysate is the mixture of various Mtb Ags present in the bacterial cell (Brennan, 2003; Jankute et al., 2015), and therefore, they need to be internalized and processed by monocytes or B cells before presentation to T cells. On contrary, Mtb peptides do not need processing and can quickly be presented by APCs. In T-B coculture, the plasma cell count was not significantly different between the Mtb peptide and unstm (Figures 2B and 2C; T + B; unstm: 11 ± 4; Mtb peptides: 155 ± 89; ns). However, stimulation with Mtb lysate induced significantly higher plasma cells over the control (Figures 2B and 2D; T + B; unstm: 108 ± 45; Mtb lysate: 760 ± 227; p = 0.03). Monocytone supplementation to T-B cocultures (T + B + M) significantly enhanced the output of plasma cells in presence of both the Ags, Mtb peptides (Figures 2B and 2C; T + B + M; unstm: 17 ± 7; Mtb peptides: 1,455 ± 396; p = 0.006) and Mtb lysate (Figures 2B and 2D; T + B + M; unstm: 281 ± 95; Mtb lysate: 3,004 ± 926; p = 0.01). In the B + M cultures, in absence of T cells, no significant number of plasma cells was detected in either absence or presence of any Ag (Figures 2B–2D). Similarly, no or minimal plasma cell output in absence of any Ag (unstm) further indicates that the Ag-specific interactions are required for the B cell responses in these cocultures (Figures 2B–2D; T + B and T + B + M). Next, we compared the background subtracted number of plasma cells and found that T + B + M cocultures were highly efficient in plasma cell output compared with T + B cocultures in presence of both the Ags, Mtb peptides (Figure 2E; T + B versus T + B + M; p = 0.005), and Mtb lysate (Figure 2F; T + B versus T + B + M; p = 0.03). Considering the mean output of T + B + M coculture as 100%, we noted that the Ag-specific T + B alone coculture yielded only 10.6% and 25.3% of plasma cell output in response to Mtb peptides and Mtb lysate, respectively. We further measured the level of secreted immunoglobulin G (IgG) in supernatant and found that IgG level in T + B + M was superior than T + B for Mtb peptides (Figure 2G; T + B, 493 ± 251 ng/mL; T + B + M, 3,454 ± 689 ng/mL; p = 0.004) and Mtb lysate (Figure 2H; T + B, 1,268 ± 324 ng/mL; T + B + M, 3,507 ± 786 ng/mL; p = 0.008). We then quantified the activated CD4+ T cells in cocultures. Number of ICOS+PD-1+CD4+ T cells was detected significantly in all the cocultures (T + M, T + B, and T + B + M) in presence of both Mtb peptides and Mtb lysate when compared with respective unstm controls (Figures 2I–2K). Similar to plasma cell differentiation, T + B + M showed enhanced magnitude of activated CD4+ T cells compared with T + B cocultures in presence of Mtb peptides (Figure 2L; T + B, 1,904 ± 635; T + B + M, 5,983 ± 1,263; p = 0.001) and Mtb lysate (Figure 2M; T + B, 2,318 ± 714; T + B + M, 5,247 ± 1,509; p = 0.05). These findings indicate that the enhanced B cell output in these T + B + M cocultures require extensive cross-talk and T-cell dependent help signals. Therefore, we further examined that the efficient interaction between T and B cells was the basis for this productive outcome. ICOS-ICOSL interaction is essential for the efficient cross-talk between T and B cells (Tafuri et al., 2001). Thus, the ICOS-ICOSL interaction was disrupted in Mtb Ag-specific T-B cocultures by incorporating ICOSL-Fc and then the B and T cell output was checked (Figures S4D–S4G). In stimulation with Mtb peptides, blocking the ICOS in T + B + M coculture significantly reduced the CD4+ T cells activation (Figures S4D and S4E; control versus ICOSL-Fc, p = 0.03); consequent to this, we found an almost complete inhibition in plasma cell differentiation (Figures S4F and S4G; control versus ICOSL-Fc, p = 0.001). These results confirmed that the efficient interactions of Ag-specific CD4+ T cells with Ag-presenting B cells lead to a productive B cell output in monocyte-supplemented cocultures.

The sensitivity of monocyte-supplemented T-B coculture was then measured in the Mtb peptides stimulated conditions (T + B and T + B + M), seeded with the serially diluted CD4+ T cells. We first quantified the number of Ag-specific CD4+ T cells in these serially diluted total CD4+ T cells. In Mtb peptides stimulation for 2 days, specified numbers of CD4+ T cells were cocultured with B + M as APCs and analyzed by activation induced marker (AIM) assay (Dan et al., 2016). Mtb peptides stimulation showed a significant detection of Ag-specific (OX40+CD25+) CD4+ T cells as low as 38 ± 12 (−25–50 cells) in 15,000 CD4+ T cells (Figures S5A and S5B; unstm versus Mtb peptides, p = 0.03). Clearly, serially diluting the total CD4+ T cells from 60,000 to 3,750 also showed a linear reduction in the number of Ag-specific CD4+ T cells. Moreover, activated CD4+ T cells
**Figure A**

9 days co-culture of T+B with SARS-CoV-2 (CoV) Spike Peptides/Protein leads to end-point analyses including:
- Plasma cell differentiation
- Secreted Ig quantification
- Antibody secreting cell (ASCs)

**Figure B**

Table showing CD20 and CD38 expression for Unstimulated, CoV-S Peptides, and CoV-S protein conditions:
- T+B: 8.91, 10.4
- T+B+M: 53.7, 74.2, 38.0, 68.8

**Figure C**

Graphs showing plasma cells (cell count/well) for CoV-S Peptides:
- T+B: p=0.0008
- T+B+M: p=0.01

**Figure D**

Graph showing CoV-S protein levels:
- T+B: p=0.005
- T+B+M: p=0.007

**Figure E**

Graphs showing total IgG (ng/mL) and IgG specific Ig (ng/mL) for T+B and T+B+M conditions:
- Total IgG: p=0.002
- IgG specific Ig: p=0.003

**Figure J**

Images showing total ASCs, IgM ASCs, and IgG ASCs for T+B and T+B+M conditions:
- Total ASCs: p=0.0009
- IgM ASCs: p=0.002
- IgG ASCs: p=0.002

**Figure M**

Graphs showing CoV-S specific IgM ASCs and IgG ASCs (spot count/well) for T+B and T+B+M conditions:
- CoV-S specific IgM ASCs: p=0.0003
- CoV-S specific IgG ASCs: p=0.003

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could be detected in the 9 days T + B + M cocultures seeded with 38 ± 12 Ag-specific CD4+ T cells, but not in the T-B alone cocultures, which requires minimum 266 ± 12 CD4+ T cells to yield a fairly detectable T cell activation (Figures SSC and SSD). Furthermore, a detectable plasma cell output was obtained with 50,000 CD4+ T cells containing 119 ± 29 (~90–150) Ag-specific cells in the T + B + M cocultures (Figures SSE and SSF). As seen above, T-B-alone cultures failed to yield any detectable plasma cell output in the tested conditions. Thus, Mtb-specific T + B + M cocultures showed a sensitivity of ~25–50 and ~90–150 Ag-specific T cells for the detection of T cell activation and measuring plasma cell output, respectively. Hence, the data from autologous Ag-specific cultures using the Mtb Ags clearly suggest that monocyte supplementation to T-B cocultures increases the productivity of efficient T-B interactions and enhances the assay sensitivity in detecting the plasma cell output and T cell activation.

**Qualitative evaluation of helper functions of SARS-CoV-2-specific memory CD4+ T cells using monocyte-supplemented T-B cocultures**

Several studies have highlighted the crucial role of CD4+ T cells in protective response to SARS-CoV-2. Others and we have shown that immune memory in terms of CD4+ T cells and B cells is persistent after several months in COVID-19 recovered subjects (Ansari et al., 2021; Cohen et al., 2021; Dan et al., 2021; Rodda et al., 2021). However, the helper quality of memory CD4+ T cells in COVID-19 or in response to vaccination is not studied. Thus, we investigated the monocyte-supplemented T-B coculture assay in evaluating the helper functions of CD4+ T cells in COVID-19 recovered subjects. We cocultured 50,000 memory CD4+ T cells and CD20+ B cells in absence or presence of 30,000 CD4+ monocyes for 9 days (Figure 3A). Unstim condition was used as a control for background response. For Ag-specific stimulation, we used the SARS-CoV-2 spike peptides (CoV-S peptides) and SARS-CoV-2 full-length spike protein (CoV-S protein). We detected plasma cells output in T + B coculture in presence of CoV-S peptides (Figures 3B and 3C; T + B; unstm: 22 ± 9; CoV-S peptides: 911 ± 235; p = 0.003), but not in presence of CoV-S protein (Figures 3B and 3D; T + B; unstm: 15 ± 4; CoV-S protein: 78 ± 30; ns). However, T + B + M coculture allowed the detection of significantly higher output of plasma cells in presence of both CoV-S peptides (Figures 3B and 3C; T + B + M; unstm: 43 ± 13; CoV-S peptides: 2,477 ± 511; p = 0.0009) and CoV-S protein (Figures 3B and 3D; T + B + M; unstm: 52 ± 14; CoV-S protein: 1,182 ± 330; p = 0.005). The T + B + M coculture significantly enhanced the magnitude of plasma cell differentiation compared with T + B coculture in presence of both CoV-S peptides (Figure 3E; p = 0.01) and CoV-S protein (Figure 3F; p = 0.007). In reference to the monocyte-supplemented coculture, the conventionally performed Ag-specific T-B coculture showed a limited output of 36.8% and 6.6% of plasma cells in response to CoV-S peptides and CoV-S protein, respectively. The higher IgG levels in response to CoV-S peptides (Figure 3G; p = 0.002) and CoV-S protein (Figure 3H; p = 0.003) further substantiated the superior plasma cell output in T + B + M conditions. Monocyte supplementation to T-B coculture also enhanced the CoV-S-protein-specific IgG level by ~20-fold higher than the T + B cocultures (Figure 3I; p = 0.003). The quantitative analyses of antibody-secreting cells (ASCs) (Figures 3J and 3K) corroborated with our findings of higher plasma cell output and IgG production, as total IgM and IgG ASCs were significantly increased in T + B + M cocultures (Figure 3L; IgM ASCs: p = 0.0009; IgG ASCs: p = 0.002). Similarly, CoV-S-specific IgM and IgG ASCs were also enhanced in T + B + M cocultures by 4- and 5-fold, respectively, over the T + B cocultures alone (Figure 3M; T + B versus T + B + M; IgM ASCs: p = 0.005; IgG ASCs: p = 0.03). Altogether, the monocyte-supplemented T-B coculture was superior to the T-B alone coculture in detecting the T-dependent plasma cell differentiation and antibody generation in response to SARS-CoV-2 virus Ags.

We next examined the level of activated CD4+ T cells in these T + B + M cocultures. In T + B cocultures, a significant number of ICOS+PD-1+CD4+ T cells were detected in presence of CoV-S peptides (Figures 4B and 4C; unstm: 576 ± 212; CoV-S peptides: 5,316 ± 1,121; p = 0.001), but not in case of CoV-S protein (Figures 4B and 4D; unstm: 383 ± 128; CoV-S protein: 1,901 ± 850; ns). The T + B + M cocultures showed significantly higher number of activated T cells in presence of both CoV-S peptides (Figures 4B and 4C; unstm: 2,511 ± 745; CoV-S peptides: 18,122 ± 3,310; p = 0.0004) and CoV-S protein (Figures 4B and 4D; unstm: 2,407 ± 755; CoV-S protein, 9,751 ± 2,945; p = 0.02). Like plasma cell output, consistently higher magnitude
9 days co-culture
SARS-CoV-2 (CoV)
Spike - Peptides/Protein

End-point analyses
- T cell activation
- IL-21 secreting cells
- Secreted cytokines

B
Unstimulated
CoV-S Peptides
CoV-S protein

C
ICOS

D

E
ICOS PD-1+CD4+ T cells
Background subtracted (cell count/well)

F
ICOS PD-1+CD4+ T cells
Background subtracted (cell count/well)

G
IL-21 SFCs
Unstimulated
CoV-S Peptides

H
CoV-S Peptides

I
IL-2
IFN-γ
IL-4
IL-13
IL-15
IL-17
Cytokines (pg/mL)
sCD40L
IL-21
IL-10
IL-1β
TNF-α
IL-6

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of activated CD4+ T cells was observed in T + B + M cocultures than the T + B cultures (Figures 4E and 4F; CoV-S peptides: p = 0.001; CoV-S protein: p = 0.04). The IL-21 cytokine secreted by CD4+ T cells is an important factor for the T cell-dependent plasma cell differentiation (Bryant et al., 2007; Ettinger et al., 2005; Kuchen et al., 2007). Thus, we compared the magnitude of IL-21-secreting cells in T + B + M or T + B alone cocultures, after restimulation with CoV-S peptides (Figure 4G). As expected, the number of IL-21-secreting cells was significantly ~4-fold higher in T + B + M cocultures over the T + B alone cultures (Figure 4H; T + B: 48 ± 14; T + B + M: 178 ± 41; p = 0.006). We further quantified the secreted factors in T + B + M or T + B alone cocultures in CoV-S protein stimulation. We detected significantly higher levels of cytokines IL-2, IL-4, IL-10, and IL-15 in T + B + M conditions (Figure 4I). The majority of these cytokines may help in the B cell proliferation, differentiation, and isotype switching (Vazquez et al., 2015). However, the secreted IL-21 was below the limit of quantification in the cytokine multiplex assay. In T + B + M, we also detected higher levels of pro-inflammatory cytokines IL-1β, tumor necrosis factor alpha (TNF-α), and IL-6 (Figure 4I). Thus, we confirmed that monocyte supplementation to Ag-specific T-B cocultures enables the activation of functionally potent SARS-CoV-2-specific memory CD4+ T cells, which deliver the appropriate help signals to B cells. To further confirm that the output from CoV-S-specific cocultures is a result of cognate interaction between Ag-specific CD4+ T cells and B cells and not the outcome of a polyclonal activation, we tested the setup using pre-pandemic PBMCs from healthy donors. We did not witness any significant induction of plasma cell differentiation and CD4+ T cells activation in T + B or T + B + M cocultures in presence of SARS-CoV-2 Ags (Figures S6A–S6D). Altogether, the results clearly suggest that the monocyte supplementation to the T-B coculture enhances the sensitivity for efficient qualitative assessment of the SARS-CoV-2-specific memory CD4+ T cells.

Monocytes act as superior antigen-presenting cells for initial priming and activation of Ag-specific CD4+ T cells

We next examined why adding monocytes enhanced the activation of CD4+ T cells and augmented the productivity of T-B cross-talk, while B cells alone were not sufficient. Among the classical APCs, CD14+ monocytes in healthy donor’s PBMCs are present in the proportion of 14%–23% (Figures S7A and S7B; 18% ± 1%), which is higher than CD20+ B cells (Figures S7A and S7B; 9%–11%). Monocytes represent relatively more homogeneous population compared with CD20+ B cells, which showed 70% naive, 10% unswitched, and 14% of switched memory B cells (Figures S7A and S7B). Though the monocytes and CD20+ B cell subsets constitutively express similar level of human leukocyte Ag-DR isotype (HLA-DR), they differ in the expression of co-stimulatory molecules, CD80 and CD86 (Figures S7C and S7D). Nearly all of the monocytes constitutively express CD86, but not CD80, whereas none of the B cell subsets express CD86 (Figures S7C and S7D). Roughly a half (55% ± 3%; data not shown) of the memory B cells express CD80. Thus, CD20+ B cells have less potential to provide co-stimulatory signals than CD14+ monocytes during the initial priming of memory T cells. To determine which one of monocytes or B cells is superior in initial priming of memory T cells, we analyzed the cocultures of T + M, T + B, or T + B + M after 2 days of Mtb Ags stimulation (Figure 5A). We were able to detect AIM+ CD4+ T cells in all the cocultures (T + M, T + B, and T + B + M) in presence of both Ags, Mtb peptides (Figures 5B and 5C) and Mtb lysate (Figures 5B and 5D). In response to both the Mtb Ags, the magnitude of background subtracted AIM+ CD4+ T cells was more robustly detected in T + B + M cocultures than the T + B alone cultures (Figures 5E and 5F). We further examined the expression of CD40L molecule on the AIM+ CD4+ T cells, which was detected in all the Mtb Ags stimulated cocultures (Figure 5G). CD40L expressed on activated CD4+ T cells functions as a co-stimulatory molecule to deliver constant survival and proliferation signals to the APCs like B cells (Elgueta et al., 2009; Garside et al., 1998; Xu et al., 1994). We found that cocultures containing monocytes (T + M and T + B + M) induced the CD40L expression (CD40L+ cells) in AIM+ CD4+ T cells almost 2-fold higher than the T + B alone cocultures in presence of both the Mtb Ags (Figures 5H and 5I). The magnitudes of AIM+ CD4+ T cells and CD40L+ cells in the cocultures of T + M and T + B + M also indicated that the presence of B cells in T + M coculture did not bring significant favor for priming and activation of CD4+ T cells at the early stages. As expected, T cell culture with either monocyte or B cells did not induce the expression of PD1/ICOS at early time points (data not shown). Hence, these results suggest that the presence of monocytes in T-B cocultures contributed to an initial priming and activation of Ag-specific T cells during the early stages of coculture, superior to the B cells. The early efficient priming by monocytes seems to help in the T cell-dependent plasma cell differentiation and CD4+ T cells activation in T + B or T + B + M cocultures in presence of SARS-CoV-2 Ags (Figures S6A–S6D). Altogether, the results clearly suggest that the monocyte supplementation to the T-B coculture enhances the sensitivity for efficient qualitative assessment of the SARS-CoV-2-specific memory CD4+ T cells.

Figure 4. Enhanced activation and functional potential of SARS-CoV-2-specific memory CD4+ T cells in monocytes-supplemented T-B cocultures

(A) Scheme for the analysis of SARS-CoV-2-specific memory CD4+ T cells in T-B coculture.

(B) Flow cytometric contour plots show the analysis of activated (ICOS+PD-1+) CD4+ T cells in T + B and T + B + M cocultures in unstim and CoV-S-peptides- or CoV-S-protein-stimulated conditions.

(C and D) Line graphs show the activated T cell count in T + B and T + B + M cocultures stimulated with (C) CoV-S peptides and (D) CoV-S protein. Line connecting gray (unstim) and red (Ag-stimulated) open squares represent the same donor.

(E and F) Background subtracted activated T cell count in (E) CoV-S-peptides- and (F) CoV-S-protein-stimulated condition.

(G) ELISpot well images show the IL-21 spot-forming cells (SFCs) detected in CoV-S-peptides-stimulated cocultures.

(H) Spot count of CoV-S-specific IL-21 SFCs in cocultures as mentioned in (G).

(I) Quantification of secreted cytokines in supernatant of cocultures in the CoV-S-protein-stimulated condition. Dotted line represents the lowest limit of quantification (LLOQ).

Data are represented as mean ± SEM, with each dot representing one donor. Data represent the pool of two independent experiments. Statistics are as follows: (C–F and H) two-tailed paired t test and (I) multiple t test corrected using the Bonferroni-Sidak method. See also Figure S8.
endow the T cells with strong potential of cognate interactions with B cells and to deliver the vital helper signals in the successive stages of coculture.

Monocytes-driven enhanced B cell survival is largely mediated by BAFF and SCGF-β

In addition to the efficient early priming of T cells, monocytes also enhanced the B cells survivability. Thus, in unstimulated autologous cultures, we measured the chemokines, cytokines, and growth factors secreted in the supernatant of B + M coculture and compared it with B cell-alone condition. We detected significantly higher levels of chemokines CXCL1, CCL2, and CXCL8; cytokines IL-1α, IL-6, TNF-α, interferon (IFN)-γ, and IFN-α2; and growth factors granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), and BAFF. These findings highlight the critical role of monocytes in promoting B cell survival through the secretion of these pro-survival factors.
TRAIL, LIF, MIF, and SCGF-β in B + M coculture compared with B alone (Figures 6A and 6B; Figure S8). Thus, we investigated the role of survival factors IL-6, SCGF-β, and MIF that were detected in substantial concentration in B + M coculture. In addition, we also examined the implication of BAFF that promotes survival and proliferation of mature B cells (Batten et al., 2000; Fu 2004).
et al., 2009; Schweighoffer et al., 2013). We cultured CD20+ B cells with CD14+ monocytes (M) for 6 days in 1:2 ratio (M to B) in unstimulated conditions either in absence of any antibody, isotype antibody, or neutralizing antibodies against BAFF, IL-6, SCGF-β, and MIF. Blocking the BAFF molecules by antibody (±BAFF) significantly reduced the live B cell numbers. Although blocking of IL-6 showed a decline in B cell viability, the SCGF-β blocking was more efficient in reducing the B cell survival (Figure 6D; count: isotype, 2,889 ± 125 versus ±BAFF: 1,767 ± 92, p < 0.0001; ±SCGF-β: 1,776 ± 149, p < 0.0001; ±IL-6: 2,140 ± 166, p = 0.0001). Besides, blocking of secreted MIF showed a marginal reduction in the count of live B cells (Figures S8B and S8C; isotype, 2,645 ± 85 versus ±MIF, 2,325 ± 124; p = 0.01). Thus, the blocking of BAFF and SCGF-β was equally superior in reducing the monocyte-dependent enhanced B cell survival. Interestingly, a significant synergistic effect on the reduction in B cell survival was observed in SCGF-β + BAFF combined blocking (Figure 6D; count: isotype versus ±BAFF + ±SCGF-β: 1,268 ± 124; p < 0.0001). Thus, blocking BAFF was able to reduce the survival of B cells to 56% ± 3%, which was further reduced to 38% ± 5% in the combination blocking in presence of ±SCGF-β (Figure 6E; isotype versus ±BAFF + ±SCGF-β; p < 0.0001).

Altogether, these results suggest that monocytes-mediated enhanced B cell survival is majorly supported by BAFF and newly described growth factor SCGF-β/CLEC11A.

**Enhancing B cell survival alone is not sufficient to replace the monocyte supplementation in Ag-specific T-B coculture**

We next examined whether monocytes-mediated increased B cell survival is driving the enhanced output of T-B collaborations. We thus tested Toll-like receptor (TLR) stimulus for increasing the B cell viability. As expected, in absence of Ag stimulation, R848 promoted the B cells survival and induced robust plasma cell differentiation (Figures S9A–S9C). However, in Ag-specific cocultures, R848 showed no beneficial impact on plasma cell output or T cell activation over the T-B-alone cocultures (Figures S9D–S9G). R848 induces the polyclonal stimulation of B cells, and thus, it could not replace monocyte supplementation for defining the Ag-specific output in cocultures. Next, we exogenously added BAFF to recapitulate the monocytes-dependent enhanced B cell viability. We titrated BAFF and found that 10 ng/mL concentration was sufficient to significantly enhance the B cell viability up to 63% and the mean survival of 46% ± 4% (Figures S10A and S10B; Figures 7A and 7B). Like BAFF, we also examined the potential of recombinant SCGF alone in supporting the B cell survival. Increasing concentrations of SCGF showed a maximum enhancement in B cell survival at 10 ng/mL up to 28% and the mean survival of 11% ± 2% (Figures S10C and S10D; Figures 7A and 7B). Interestingly, BAFF in conjunction with SCGF showed an additive effect on the B cell survival up to 78% as compared with the monocytes-supplemented cultures (Figure 7C; SCGF: 11% ± 2%; BAFF: 46% ± 4%; BAFF + SCGF: 58% ± 3.5%; p < 0.0001). Subsequently, we tested BAFF + SCGF as the exogenously provided survival factors to examine whether enhanced B cell survival could replace the need of monocytes. In Mtbb-specific cocultures, exogenous addition of BAFF or BAFF + SCGF to T-B coculture was not effective in promoting any plasma cell differentiation (Figures 7D and 7E) but showed significant response in T cell activation (Figures 7F and 7G). However, unlike T + B + M cocultures, addition of survival factors was not of any significant benefit over T + B alone for both plasma cell and T cell output (Figures 7H and 7I). We finally speculated that the exogenous supplementation of survival factors might not be substituting for monocytes-mediated priming of Ag-specific CD4+ T cells at early stages of coculture. Thus, we analyzed the priming of T cells in BAFF and BAFF + SCGF supplemented cocultures after 2 days of Mtbb peptides stimulation. As expected, we found no significant increase in AIM+ CD4+ T cells in presence of BAFF or BAFF + SCGF over T + B alone (Figures 7J and 7K). Besides, monocyte supplementation (T + B + M) showed significantly heightened AIM+ CD4+ T cells (Figures 7J and 7K). Hence, these data suggest that monocyte-promoted B cell survivability is not the primary factor, but monocyte-mediated early priming of T cells is essential for the enhanced output of T-B cocultures.

**DISCUSSION**

The availability of T-B cocultures is instrumental for functional assessment of CD4+ T cells in inducing T-dependent B cell responses. In this study, we demonstrated a method to examine the quality of CD4+ T helper cells in an Ag-specific manner with a greater sensitivity than conventionally used coculture assay. The striking finding is the implication of autologous monocytes in facilitating collaboration between T cells and B cells. In fact, monocytes provided two crucial signals in the T-B cocultures: early priming of CD4+ T cells for better T-B cognate interactions and the survival signals to B cells that were mainly mediated by BAFF and SCGF/CLEC11A.

The robust presence of Mtbb-specific T cells in healthy blood donors provided an opportunity to optimize the Ag-specific T-B cocultures. The conventional T-B cocultures showed poor survivability of T and B cells and the inefficient plasma cell output. It seems that B cells presenting CD4+ peptides do not survive long enough until the Mtbb-specific CD4+ T cells expand and become armed to deliver help signals to B cells. Clearly, conventional settings were lacking essential signals for the survival and efficient cross-talk between T and B cells. The cognate T-B interactions at the T-B border or in the GCs are supported by various signals from the myeloid and stromal cells (Pereira et al., 2010; Victoria and Nussenzweig, 2012). Indeed, monocyte supplementation in cocultures supported B cells to survive more profoundly than with T cells alone. That survival of naive B cells was more prominent than the memory B cells may be due to the intrinsic capability of memory B cells in higher expression of anti-apoptotic genes (Good et al., 2009; Tangye et al., 2003). The monocyte-supplementation effect was clearly visible in Mtbb-specific conditions, which provided an opportunity to detect 10-fold higher B cell output than the conventional settings. In contrast to peptides, Mtbb lysate stimulation showed higher expansion of Mtbb-specific CD4+ T cells, plasma cells, and the secreted antibodies. It could be due to the presence of various Ags in the lysate leading to larger breadth of expansion in specific B and T cells (Cooper, 2009).
In fact, conventional T-B cocultures showed limited 10%–25% of the total B cell output generated with monocyte-supplemented cocultures. The monocyte-supplemented cocultures demonstrated higher sensitivity than T-B-alone cultures in yielding the B cell responses with as low as 90–150 Ag-specific T cells. This is indeed important for the human studies, where there is always a scarcity of sample volume for multiple cellular analyses. These findings also establish that monocyte supplementation significantly enhanced the sensitivity and provided consistent detection of T cell activation, plasma cell differentiation, and antibody response in the presence of both Mtb peptides and lysate Ags. Importantly, monocytes-mediated enhanced B cell response was largely dependent on the efficient interaction between CD4⁺ T cells and B cells, as the absence of CD4⁺ T cells or absence of Mtb Ags failed to yield any plasma cell or antibody output from the cultured B cells. It was further evident by the observation that inhibiting efficient cross-talk between T cell and B cell via blocking ICOS-ICOSL interaction completely abolished the plasma cell output in Ag-stimulated cocultures.

The prominent association of CD4⁺ T cells in robust response to SARS-CoV-2 is widely recognized (Lipsitch et al., 2020; Sette and Crotty, 2021). Many studies showed persistent memory CD4⁺ T cells in COVID-19 recovered donors, mostly targeted toward the spike protein (Ansari et al., 2021; Braun et al., 2020; Grifoni et al., 2020; Le Bert et al., 2020; Low et al., 2021; Weiskopf et al., 2020). However, the qualitative evaluation of SARS-CoV-2-specific CD4⁺ T cells in providing help to B cells has never been attempted. We observed plasma cell differentiation, IgG production, and ASCs in CoV-S-peptides-stimulated
T-B cocultures. In these T-B cocultures, we also detected a corresponding expansion of CoV-S-specific CD4+ T cells, which can secrete IL-21 to help B cells for their differentiation and functions. However, CoV-S-protein-stimulated conventional T-B cocultures showed no plasma cell differentiation or antibody secretion, which coincided with the failure in generation of ICOS+PD1+ CD4+ T cells. The observed differences in CoV-S peptides and CoV-S protein could be partly explained by the way these Ags are processed and presented to prime the specific CD4+ T cells. CoV-S protein needs to be internalized, processed, and presented by spike-specific B cells. In contrast, CoV-S peptides could be directly presented on MHC class II of spike-specific or non-specific B cells, leading to vigorous priming of the specific CD4+ T cells. However, later condition of peptide stimulation also leads to prominent non-specific B cell expansion as evident with the total IgG and total ASCs responses. It should not be a concern, as these non-specific responses could be easily excluded by employing the Ag-specific analyses of the assay readouts. Certainly, monocyte-supplemented T-B cocultures overcome these limitations by consistently enhancing the plasma cell output and antibody production in cocultures stimulated with CoV-S protein and peptides. Moreover, the improved sensitivity in the B cell output in monoocyte-supplemented settings was accompanied by the enhanced magnitude of functional activated CD4+ T cells, which were associated with enhanced level of TCR-directed effector cytokines like IL-2, IL-4, and IL-10 (Geginat et al., 2001; Rochman et al., 2009). Monocytes also provided crucial cytokines like IL-15 that seem to promote the survival and proliferation of T cells in these spike-specific cocultures (Dooms et al., 1998; Geginat et al., 2001). These observations also highlighted the fact that these COVID-19 recovered subjects harbor spike-specific memory CD4+ T cells that are capable of inducing B cell differentiation into ASCs and produce IgG during any recall response. The cocultures of memory cells from pre-pandemic unexposed subjects failed to yield any T cell and B cell output. These control experiments further provided evidence that the presence of Ag-specific T cells is essential for any T cell or B cell output in these monocyte-supplemented cocultures.

Monocytes and B cells act as APCs to prime and activate the memory CD4+ T cells (Hong et al., 2018; Hua and Hou, 2020; Randolph et al., 2008; Tacke et al., 2006). However, they differ in their abilities to deliver the secondary activation signals, as evident by the constitutive expression of CD86 on all classical monocytes, but not on the B cells. In fact, signals from activated T cells like IL-21 are essential for inducing the co-stimulatory molecules, and therefore, only activated T cells can deliver their effector functions to B cells. Thus, pre-activation of Ag-specific CD4+ T cells is necessary in T-B cocultures to establish the cognate adhesion and activation of the B cells. It was clearly evident in the absence of monocytes supplementation, where T-B cocultures showed poor activation of Mtb-specific T cells at both early and later stages of cocultures. Although B cells do not provide efficient signals to prime and activate CD4+ T cells in early coculture period, they could receive T-dependent helper signals from the optimally activated CD4+ T cells in the successive stages of cocultures.

Furthermore, the enhanced survivability of B cells in the presence of monocytes helped them to remain available for interaction and cognate T cell engagement. In ex vivo settings, the specific requirement to keep surviving B cells without any Ag is not fully understood. However, B cells survive the ex vivo cultures in the presence of stimulation with synthetic ligands, like TLR7/8 and TLR9 agonists (Auladell et al., 2019; den Hartog et al., 2018; Franke et al., 2020; Jahnnatz et al., 2013). However, as shown here, incorporation of TLR stimulus is not an option for examining the Ag-specific cocultures, because it induces polyclonal stimulation of B cells even in the absence of any Ag. In fact, enhanced B cell survival was associated with the accumulated monocytes in tumor microenvironment (Epron et al., 2012). Our study clearly demonstrates that monocyte supplementation provides crucial signals for the enhanced survivability of B cells, which is mainly mediated by growth factors BAFF and SCGF. Although IL-6 has been shown to induce the growth-responsive genes in activated B cells (Tozato et al., 1988), it was not superior than BAFF and SCGF in enhancing the B cell survivalvability. Similarly, MIF that seems to impact the B cell survivability was not superior than BAFF and SCGF alone was not efficient in providing the survival signals. It seems that SCGF promotes the B cell survival via CD74/CD44 signaling axis like BAFF (Craxton et al., 2001). Our data suggest that human monocytes are also capable of secreting this growth factor. It is plausible that SCGF is promoting the B cell survival via ERK pathway like BAFF (Craxton et al., 2005) or via Wnt-mediated activation through integrin receptors (Shen et al., 2019). The SCGF showed substantial synergy with BAFF in effectively regulating the B cells survival. However, unlike BAFF, SCGF alone was not efficient in providing survival signals. It seems that SCGF promotes the B cell survival in consort with other growth factors and/or a parallel signaling axis. Thus, in-depth studies are warranted to establish the role
of this newly described growth factor in B cell survival and function. However, as demonstrated here, merely providing the survival signals through B cell growth factors like BAFF and SCGF could not substitute for the monocyte supplementation. Certainly, survivability is not the primary factor, and monocyte-mediated early T cell priming is also critical in enhancing the output of Ag-specific cocultures. Further investigations may delineate additional mechanisms operated by monocytes in promoting the output of T and B cell collaboration.

In summary, our study described an efficient method for qualitative evaluation of memory CD4+ T cells in an Ag-specific manner. We demonstrate that monocytes contributed to enhancing the propensity of efficient T-B interactions by crucially providing the early activation signals to the T cells. In addition, we provide the mechanistic details by which monocytes are supporting the optimal survivability of B cells in the cocultures. Although effective COVID-19 vaccines have been rolled out, the trials for more broadly protective vaccines will continue. This is an unprecedented situation that demands for multiple immunologic parameters for the evaluation of vaccines in early phase of trials as well as after the roll out. Because the induction of humoral response depends on the CD4+ T cells, it is important to utilize B cell help function of CD4+ T cells as a correlate of protective adaptive immunity. The higher sensitivity and the ease of using various types of Ag overcome the existing limitations in widespread use of Ag-specific T-B coculture assays. Thus, the hereby described method provides an opportunity for the assessment of vaccine-induced functional memory responses as well as for studying the mechanism of T-B cross-talk in various infections.

Limitations of the study
There are limitations inherent to this assay. Although monocyte supplementation to the coculture system can enhance the sensitivity and consistency in T and B cell output, the inter-individual variability in Ag-specific T cell and B cell repertoires may influence the assay readouts. The human inter-individual heterogeneity in immune response is an inherent biological variation. Thus, it may be appropriate to apply this assay after confirming the detectable humoral and Ag-specific T cell response. Also, for comparative study, it may be helpful to select the subjects with comparable frequency of Ag-specific CD4+ T cells. Moreover, functional deficiency in an individual’s monocytes, like for T cell priming, may add into variable outcome in this assay. However, any such functional inefficiency may also be reflected in the individual’s capability of mounting an immune response, as well as in the related assay readouts. Therefore, the qualitative assessment of CD4+ T cells should be performed subsequent to the quantitative measurement of Ag-specific T cells and humoral responses. Another limitation is the detailed investigation of basic assay performance characteristics, which needs to be established in future studies in the larger sample size from various infection and vaccination models.

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at [https://doi.org/10.1016/j.crnmeth.2022.100224](https://doi.org/10.1016/j.crnmeth.2022.100224).

ACKNOWLEDGMENTS
We are thankful to the participants for generous support in this study. Mtb H37Rv lysate (NR-14822A) was obtained through BEI Resources, NIAID, NIH. This work was financially supported by SERB, DST grant (IP/A2020/000077), and DBT grant (BT/PR30223/MED/2018) to N.G. and NII core grant. Further support was provided from NIH contract 7SN9301900805(to A. Sette and D.W.) and NIH grant U01 (U01AI141995-03) to A. Sette.

AUTHOR CONTRIBUTIONS
Conceptualization and supervision, N.G.; formal analysis, A.A. and N.G.; investigation, A.A. and S.S.; enrollment and sample collection, B.P.J., A. Sharma, and P.C.; resources (peptide pools), A. Sette and D.W.; writing, A.A. and N.G.; funding acquisition, N.G. and A. Sette.

DECLARATION OF INTERESTS
N.G. and A.A. are listed as inventors on patent application no. 202111003148, submitted by NII, which covers the use of described method for vaccine evaluation and adjuvant testing purposes. A. Sette is listed as inventor on patent application no. 63/012,902, submitted by LIJ, which covers the use of the peptide megapools for therapeutic and diagnostic purposes. Other authors declare no competing interests.

Received: August 23, 2021
Revised: November 27, 2021
Accepted: April 28, 2022
Published: May 10, 2022

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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| CD4, Alexa Fluor 700 | BioLegend | Cat# 300526; Clone RPA-T4; RRID: AB_493743 |
| CD45RO, FITC        | BD Biosciences | Cat# 555492; Clone UCHL1; RRID: AB_395883 |
| CD14, PE            | BD Biosciences | Cat# 562691; Clone M6P9; RRID: AB_2737725 |
| CD20, PE Cy7        | BioLegend | Cat# 302312; Clone 2H7; RRID: AB_314260 |
| CD3, APC Cy7        | BioLegend | Cat# 300318; Clone HIT3a; RRID: AB_314054 |
| CXCR5, Alexa Fluor 647 | BD Biosciences | Cat# 558113; Clone RF8B2; RRID: AB_2737606 |
| ICOS, FITC          | eBioscience | Cat# 11-9949-82; Clone C938.4A; RRID: AB_465458 |
| PD-1, PE            | BioLegend | Cat# 329906; Clone EH12.2H7; RRID: AB_940483 |
| CD19, Brilliant Violet 785 | BioLegend | Cat# 302240; Clone HIB19; RRID: AB_2563442 |
| CD27, PE Dazzle 594 | BioLegend | Cat# 56422; Clone M-T271; RRID: AB_2564101 |
| CD38, PE-Cy5        | BD Biosciences | Cat# 555461; Clone HIT2; RRID: AB_395854 |
| CD14, BV786         | BD Biosciences | Cat# 563698; Clone M5E2; RRID: AB_2744287 |
| CD20, APC eFluor780 | eBioscience | Cat# 47-0209-42; Clone 2H7; RRID: AB_1272038 |
| IgD, FITC           | BioLegend | Cat# 348206; Clone IA6-2; RRID: AB_10612567 |
| IgM, APC            | BioLegend | Cat# 314510; Clone M-HM-88; RRID: AB_493011 |
| HLADR, BV650        | BioLegend | Cat# 307650; Clone L243; RRID: AB_2563828 |
| CD80, PE            | BD Biosciences | Cat# 557227; Clone L307.4; RRID: AB_396606 |
| CD86, PE Cy7        | BD Biosciences | Cat# 561128; Clone 2331(FUN-1); RRID: AB_10563077 |
| CD3, Brilliant Violet 650 | BD Biosciences | Cat# 563852; Clone UCHT1; RRID: AB_2744391 |
| OX40, PE Cy7        | BioLegend | Cat# 350012; Clone Ber-Act35; RRID: AB_10901161 |
| CD25, FITC          | BD Biosciences | Cat# 555431; Clone M-A251; RRID: AB_395825 |
| CD40L, APC          | BioLegend | Cat# 310810; Clone 24-31; RRID: AB_314833 |
| Goat anti-BAFF, purified | R&D Systems | Cat# AF124; RRID: AB_354690 |
| SCGF, purified      | R&D Systems | Cat# MAB1904; Clone 239029; RRID: AB_2083138 |
| IL-6, purified      | BioLegend | Cat# 501125; Clone MQ2-13A5; RRID: AB_2810625 |
| MIF, purified       | BioLegend | Cat# 525502; Clone 10C3; RRID: AB_2563134 |
| SARS-CoV-2 IgG      | ter Meulen et al., 2006 | Clone CR3022; RRID: AB_2848080 |
| Goat anti-human IgG HRP | Southern Biotech | Cat# 2014-05; RRID: AB_2795580 |
| Goat anti-human Ig Fab | Southern Biotech | Cat# 2085-01; RRID: AB_2795785 |

#### Chemicals, peptides, and recombinant proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Ficoll-Paque PLUS   | Cytiva Life Sciences | Cat# 17144003 |
| DMSO                | Sigma  | Cat# D2650 |
| FBS                 | Gibco  | Cat# 10270-106 |
| RPMI 1640           | Cytiva Life Sciences | Cat# SH30027 |
| RPMI 1640 + GlutaMAX-I (1X) | Gibco | Cat# 61870-036 |
| Dnase I             | StemCell | Cat# 07900 |
| PI/AO stain         | Logos Biosystems | Cat# F23001 |
| AIM-V medium (1X)   | Gibco  | Cat# 12055-091 |
| Mtb peptides        | Arlehamn et al., 2016 | N/A |
| CoV-S peptides      | Grifoni et al. (2020) | N/A |
| CoV-S protein       | Native Antigen, UK | Cat# REC31868 |
| Mtb lysate          | BEI Resources | Cat# NR-14822 |
| Staphylococcal enterotoxin B | Sigma-Aldrich | Cat# S4881 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Nimesh Gupta (nimesh.gupta@nii.ac.in).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The published article includes all the dataset generated during this study. This paper does not report original code. Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study approval
The study was approved by the Institutional Review Boards of National Institute of Immunology (NII) and All India Institute of Medical Sciences (AIIMS), New Delhi, India. Informed consent was obtained from all the donors/subjects during the enrolment.

Human blood samples
For Mycobacterium tuberculosis (Mtb) specific assays, peripheral blood buffy coats from healthy adult donors were collected from blood bank at the AIIMS, New Delhi, India. For SARS-CoV-2 specific assays, peripheral blood from COVID-19 recovered adult subjects were collected at AIIMS, New Delhi, India. The characteristics of all participants is provided in the Table S1.

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| R848                | InvivoGen | Cat# tlr-r848 |
| Recombinant BAFF    | R&D Systems | Cat# 2149-BF |
| Recombinant SCGF    | R&D Systems | Cat# 1904-SC |
| Recombinant ICOSL-Fc | R&D Systems | Cat# 165-B7 |
| Skimmed milk powder | HiMedia  | Cat# GRM1254 |
| Tween-20            | Sigma-Aldrich | Cat# P1379 |
| 10X PBS             | Gibco   | Cat# 14200-075 |
| OPD tablet, 10mg     | Sigma   | Cat# P8287 |
| Hydrochloride acid   | Merck Millipore | Cat# 1.00317 |
| Fixable Viability Dye eFluor 506 | eBioscience | Cat# 65-0866-14 |
| Foxp3/Transcription Factor Staining Buffer Set | eBioscience | Cat# 00-5523-00 |
| Ethanol             | Merck Millipore | Cat# 1.00983 |

### Critical commercial assays

| Assay                          | SOURCE | IDENTIFIER |
|-------------------------------|--------|------------|
| Bio-Plex Pro Human cytokine panel 14-plex | Bio-Rad | Cat# 12011038 |
| Bio-Plex Pro Human Th17 3-plex | Bio-Rad | Cat# 17003714 |
| Bio-Plex Pro Human cytokine screening 48-plex | Bio-Rad | Cat# 12007283 |
| U-bottom 96-well plate         | Thermo Scientific | Cat# 163320 |
| Maxisorp ELISA 96-well plate   | Thermo Scientific | Cat# 442404 |
| Human IgG/IgA/IgM FluorSpot kit | Mabtech | Cat# FS-050617-2 |
| Human IL-21 ELISpot kit        | Mabtech | Cat# 3540-2A |

### Software and algorithms

| Software or algorithm | Source or URL |
|-----------------------|---------------|
| FlowJo v10            | FlowJo, LLC https://www.flowjo.com/ |
| GraphPad Prism 8       | GraphPad software https://www.graphpad.com/ |
| Bio-Plex Manager 6.2   | Bio-Rad Laboratories, Inc. https://www.bio-rad.com/ |
| AID EliSpot 7.0 software | AID GmbH https://www.elispot.com/ |
PBMCs isolation and cell sorting
All peripheral blood samples were collected in CPD blood bags for healthy blood donors and K3 EDTA vacutainers for COVID-19 recovered subjects. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare Life Sciences) density gradient medium and cryopreserved in multiple aliquots in FBS (Gibco) containing 10% DMSO (Thermo-Fisher). PBMCs were revived in pre-warmed HyClone RPMI 1640 (Cytiva Life Sciences) and treated with Dnase I (StemCell) at 500 U/mL for 15 min at 37°C. For all the autologous assays, PBMCs after revival were surface stained with antibody cocktail for 15–20 min at 4°C in dark: fixable viability dye efluor 506, anti-CD4 AF700 (RPA-T4), anti-CD45RO FITC (UCHL1), anti-CD14 PE (M6P9), anti-CD20 PE-Cy7 (2H7). Memory CD4+ T cells were sorted from PBMCs as live CD14-CD20-CD4+CD45RO+ cells. CD14+ monocytes and CD20+ B cells were sorted from PBMCs as live CD14+CD20+CD45RO+ cells. CD14+ monocytes and CD20+ B cells were sorted from PBMCs as live CD14+CD20+CD45RO+ cells. All the cells, T cells, B cells and monocytes were sorted in RPMI 1640 (Gibco) medium containing 50% FBS on a BD FACSAria Fusion flow cytometer (BD Biosciences) at intermediate flow rate, using a 70 μm nozzle. The full FACS sorting scheme is shown in Figure S1. Samples with the purity of all the cell type ≥ 95% were included for the assays.

Monocyte supplemented CD4+ T cell and B cell (T-B) cocultures
FACS sorted memory CD4+ T cells were cocultured with autologous CD20+ B cells (60,000 cells each per well) in 1:1 ratio (T to B) in absence (T + B) or presence of autologous CD14+ monocytes (T + B + M) (30,000 cells per well) for 9 days or as mentioned in the respective figure legend. For T cell + Monocytes (T + M) or B cell + Monocytes (B + M) cocultures, memory CD4+ T cells or B cells (60,000 cells each per well) were cocultured with autologous monocytes (30,000 cells per well). Cells were seeded in U-bottom 96-well plates (Nunc, Thermo) in 200 μL per well in AIM-V serum-free medium (Gibco) with no exogenous stimulation (UNSTM control) or stimulation with M. tuberculosis specific pool of CD4+ T cell peptides (Mtb peptides, 1 μg/mL) or M. tuberculosis strain H37Rv whole cell lysate (Mtb lysate, 10 μg/mL) (BEI Resources). The culture medium remains unchanged throughout the duration of cocultures. In some of the experiments, 1 μg/mL of R848 (InvivoGen), 10 ng/mL of recombinant BAFF (#2149-BF, R&D Systems) with or without 100 ng/mL of recombinant SCGF (#1904-SC, R&D Systems) was added to Mtb peptides stimulated T-B coculture. For blocking of ICOS-ICOS-L interaction, 5 μg/mL of recombinant ICOSL-Fc (#165-B7, R&D Systems) was added to Mtb peptides stimulated T + B and T + B + M cocultures. For T-B cocultures in COVID-19 recovered and pre-pandemic subjects, cells were kept UNSTM or stimulated with SARS-CoV-2 spike specific pool of CD4+ T cell peptides (CoV-S peptides, 1 μg/mL) or SARS-CoV-2 full length Spike protein (CoV-S protein, 10 μg/mL) (Native Antigen, UK). After 9 days of coculture, total cell counting in each well was performed by LUNA-FL automatic cell counter, and the phenotype and cell count of activated CD4+ T cells and plasma cells were determined by flow cytometry. Simultaneously, coculture supernatants were stored at –80°C for the measurement of secreted IgG concentration by ELISA or cytokine concentration by bead-based multiplex ELISA. In some experiments, after 9 days of coculture, IgM and IgG ASCs or IL-21 SFCs were measured by FluoroSpot and ELISpot, respectively.

T cell and B cell survival assay
FACS sorted CD20+ B cells or memory CD4+ T cells (60,000 cells each per well) were cultured either alone or cocultured with autologous CD14+ monocytes (30,000 cells per well). Cells were seeded in U-bottom 96-well plates (Nunc, Thermo) in 200 μL per well in AIM-V serum-free medium (Gibco) with no exogenous stimulation (UNSTM) for 9 days. After 9 days of incubation, cells were analyzed for the count of live B cells and live T cells by flow cytometry. At the same time, cell culture supernatants were stored at –80°C for the measurement of cytokine concentration by bead-based multiplex ELISA. For the titration of monococyte to B cell ratio, B cells (60,000 cells per well) were cocultured with autologous monocytes in various M to B ratios: 1:4, 1:2 and 1:1 in UNSTM control. For the blocking experiments, sorted CD20+ B cells (60,000 cells per well) were cultured either alone (B alone) or with autologous CD14+ monocytes (B + M) (30,000 cells per well) in UNSTM condition for 6 days. Soluble growth factors/cytokines in B + M cocultures were blocked using 10 μg/mL of following neutralizing antibodies: purified anti-BAFF (#AF124) and anti-SCGF-β (MAB1904) (R&D Systems), purified anti-IL-6 (MQ2-13A5) and anti-MIF (10C3) (BioLegend). For isotype control, 10 μg/mL purified mouse antibody IgG (4G2) was used in B + M cocultures. After 6 days of incubation, cells were analyzed for the count of live B cells by flow cytometry. In some B cell survival assay, B cells were supplemented with either the recombinant BAFF (#2149-BF, R&D Systems) or SCGF (#1904-SC, R&D Systems) at various concentrations.

Flow cytometric analyses
Phenotypic analysis and cell count measurement of T cells and B cells at different time points of T-B cocultures were performed by flow cytometry. Briefly, all the cells were surface stained with antibody cocktail in U-bottom 96-well plates (Nunc, Thermo) in FACS buffer (PBS containing 2% FBS) for 30–40 min at 4°C in dark. Antibody cocktail contained the following antibodies: fixable viability dye efluor 506, anti-CD3 APC-Cy7 (HIT3a), anti-CD4 AF700 (RPA-T4), anti-CXCR5 AF647 (RF6B2), anti-ICOS FITC (C989.4A), anti-PD-1 PE (EH12.2H7), anti-CD19 BV786 (HB19), anti-CD27 PE-Dazzle594 (M-T271), anti-CD20 PE-Cy7 (2H7), anti-CD38 PE-Cy5 (HIT2). Following the surface staining, cells were washed with FACS buffer and re-suspended in 200 μL FACS buffer. The full gating scheme for analysis of plasma cells and activated CD4+ T cells is shown in Figure S1. All the samples were acquired on BD LSR.
Fortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo 10.3.0. Phenotyping of monocytes and B cells were performed immediately after reviving PBMCs from healthy donors. PBMCs were surface stained in FACS buffer with following antibody cocktail: fixable viability dye efluor 506, anti-CD4 AF700 (RPA-T4), anti-CD14 BV786 (M5E2), anti-CD20 APC-efluor780 (2H7), anti-CD27 PE-Dazzle594 (MT-271), anti-IgD FITC (IA6-2), anti-IgM APC (MHM-88), anti-HLADR BV650 (L243), anti-CD80 PE (L307.4), anti-CD86 PE-Cy7 (2331(FUN-1)) Gating strategy for phenotyping of monocytes and B cell subsets is shown in Figure S5.

**Activation induced marker (AIM) assay**

Early stage priming and TCR-downstream activation of Ag-specific memory CD4+ T cells was measured by AIM assay. In this assay, activated CD4+ T cells were detected by phenotyping concurrent expression of OX40 and CD25. Healthy donor PBMCs were revived and surface stained for FACS sorting of memory CD4+ T cells, CD20+ B cells and CD14+ monocytes as mentioned previously, with the following antibody cocktail: fixable viability dye efluor 506, anti-CD4 AF700 (RPA-T4), anti-CD45RO FITC (UCHL1), anti-CD14 BV786 (M5E2), anti-CD20 APC-efluor780 (2H7). Sorted memory CD4+ T cells (60,000 cells per well) or cocultured with either CD14+ monocytes (30,000 cells per well) or CD20+ B cells (60,000 cells per well) or both monocytes and B cells in autologous manner. Cells were seeded in U-bottom 96-well plates (Nunc, Thermo) in 200μL well in AIM-V serum-free medium for 2 days. Cells were kept either UNSTM (no exogenous stimulation) or stimulated with Mtb peptides (1 μg/mL) or Mtb lysate (10 μg/mL) for stimulation of antigen specific CD4+ T cells. In some experiments, Staphylococcal enterotoxin B (Sigma-Aldrich) was used for positive control stimulation. After incubation, cells were stained with the following antibody cocktail: fixable viability dye efluor 506, anti-CD3 BV650 (UCHT1), anti-CD4 AF700 (RPA-T4), anti-CD27 PE-Dazzle594 (MT-271), anti-IgD FITC (M-A251) and anti-CD20 APC-efluor780 (2H7). Following the surface staining, cells were washed with FACS buffer, fixed and permeabilized with Foxp3/Transcription factor staining buffer set (bioscience) followed by intracellular staining with anti-CD40L APC (24–31). After staining cells were washed and re-suspended in 200μL FACS buffer. Samples were acquired on BD LSR Fortessa flow cytometer. Data were analyzed using FlowJo 10.3.0.

**Enzyme-linked immunosorbent assay (ELISA)**

We used in-house developed indirect ELISA for measurement of SARS-CoV-2 spike specific IgG antibody in the coculture supernatants as described previously (Ansari et al., 2021). Maxisorp ELISA 96-well plates (Nunc, Thermo) were coated with 50μL/well SARS-CoV-2 full length Spike protein (CoV-S protein, 1 μg/mL) (Native Antigen, UK) in PBS for overnight at 4°C. After wash, the plates were blocked with blocking buffer (PBS containing 3% Skim milk, 0.05% Tween 20) and incubated at room temperature (RT) for 2 h. After 5× washing, 50μL/well neat cleared supernatants were added in duplicates and incubated at RT for 1.5 h. After 5× washing, Goat anti-human IgG conjugated with HRP (Southern Biotech) was added and plates were incubation at RT for 1 h. For calculating the concentration of SARS-CoV-2 spike specific IgG antibody, purified SARS-CoV-2 neutralizing antibody (CR3022) (ter Meulen et al., 2006) was used for plotting the standard. For measurement of the total IgG, we used another in-house developed sandwich ELISA. Plates were coated with 100μL/well purified Goat anti-human Ig Fab unlabelled (0.5 μg/mL) (Southern Biotech). Samples were appropriately diluted in diluent buffer (PBS containing 1% Skim milk, 0.05% Tween 20) and the concentration of human IgG was calculated by plotting standard with the purified human IgG. Goat anti-human IgG Fc-HRP (Southern Biotech) was used to detect the bound human IgG. The reaction was developed by adding 100μL/well OPD peroxidase substrate (Sigma) for 10 min in dark at RT. The reaction was stopped by adding 50μL/well of 2N hydrochloric acid (HCl), followed by optical density (OD) measurement at 492 nm using MultiskanGO ELISA reader (Thermo-Fisher).

**Bead-based multiplex cytokine immunoassay**

Bio-Plex Pro Human Cytokine Panel 14-Plex and Pro Human Th17 3-plex set (BIO-RAD) was used to quantitate the concentration of soluble cytokines in supernatants from 9 days cocultures, T + B and T + B + M wells stimulated with CoV-S protein. Bio-Plex Pro Human Cytokine Screening 48-Plex Panel was used to measure the quantity of secreted factors in supernatants from 9 days cocultures, B + M and B alone wells kept UNSTM. The cell free supernatants were stored at –80°C until thawed for the quantifications of secreted factors. The multiplex cytokine immunoassays were performed as per the manufacturer’s instructions.

**FluoroSpot/ELISpot assay**

SARS CoV-2 spike specific antibody secreting cells (ASCs) were detected by dual-fluorochrome FluoroSpot assay. Multiscreen IPFL FluoroSpot plate (Mabtech) was charged with 35% ethanol and coating was done with SARS-CoV-2 full length Spike protein (CoV-S protein, 5 μg/mL) (Native Antigen, UK) for overnight at 4°C. For detection of total ASCs, plates were coated with anti-human capture mAbs MT91/145 (for IgG, 15 μg/mL) and MT11/12 (for IgM, 15 μg/mL) (Mabtech). Plates were washed and blocked with AIM-V medium containing 10% FBS for at least 30 min at RT. After 9 days of coculture, cells from CoV-S peptides stimulated wells were seeded at 4/5th dilution for SARS-CoV-2 spike specific ASCs and 1/5th dilution for total ASCs. Cells were incubated at 37°C for 16 h. Cells were discarded and plates were washed 5 times with PBS. For detection of AIM spots, anti-human IgG-550 (MT78/145) and IgM-640 (MT22) detection antibodies (Mabtech) were added and incubated for 2 h at RT in dark. Plates were washed 5 times with PBS Fluorescence enhancer-II (Mabtech) and incubated for 15 min at RT. ASC spots were read on AID vSpot Spectrum Elispot/Fluorospot reader system using AID Elispot software version 7.x. ASC counts were calculated by multiplying the detected counts with dilution factor. IL-21 secreting cells were detected by human IL-21 ELISpot (3540; Mabtech). ELISpot plate (MSIPS4510; Millipore) was
charged with 35% ethanol and coated with 10 μg/mL of coating mAb MT216G (Mabtech), followed by overnight incubation at 4°C. Plates were washed and blocked as mentioned above. After 9 days of coculture, cells from CoV-S peptides stimulated wells were seeded either UNSTM or stimulated with CoV-S peptides (1 μg/mL). Cells were incubated for 24 h at 37°C. Plates were washed 5 times with PBS and incubated with 0.25 μg/mL detection mAb MT21.3m-biotin (Mabtech) for 2 h at RT. After incubation, plates were developed as per the manufacturer’s instructions. IL-21 spot forming cells (SFCs) were read as stated above. SFC counts were calculated by multiplying the detected counts with dilution factor.

QUANTIFICATION AND STATISTICAL ANALYSIS

In all the experiments, data are represented as the mean ± SEM. The significance of the differences between the groups was analyzed with two-tailed paired t-test, two-tailed Mann-Whitney test, two-tailed Wilcoxon matched-pairs signed rank test, one-way ANOVA followed by Bonferroni’s multiple comparisons test, multiple t tests corrected using specified methods. p values <0.05 were considered statistically significant. The specific p value is depicted in the respective figure panel. Statistical analyses and data visualization were performed with the GraphPad Prism software version v8.4.0.