Integrin activation enables simultaneous and sensitive detection of functional virus-specific CD4$^+$ and CD8$^+$ T cells

Anna Schöllhorn
University of Tübingen
Juliane Schuhmacher
University of Tübingen
Luciana Besedovsky
University of Tübingen
Rolf Fendel
University of Tübingen
Anja T.R. Jensen
University of Copenhagen
Stefan Stevanović
University of Tübingen
Tanja Lange
University of Lübeck https://orcid.org/0000-0001-8309-2112
Hans-Georg Rammensee
University of Tübingen https://orcid.org/0000-0003-1614-2647
Jan Born
University of Tübingen https://orcid.org/0000-0002-1847-6248
Cécile Gouttefangeas (✉ cecile.gouttefangeas@uni-tuebingen.de)
University of Tübingen https://orcid.org/0000-0002-6410-2378
Stoyan Dimitrov (✉ stoyan.dimitrov@uni-tuebingen.de)
University of Tübingen https://orcid.org/0000-0002-1184-1441

Method Article

Keywords: beta2-integrin, antigen-specific CD4 and CD8 T cells, SARS-CoV-2, vaccination, flow cytometry, m24 antibody

Posted Date: August 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-67111/v1

Version of Record: A version of this preprint was published at Frontiers in Immunology on August 28th, 2020. See the published version at https://doi.org/10.3389/fimmu.2021.626308.

Abstract

We have previously shown that beta$_2$-integrin conformational change is a very early activation marker that can be detected with fluorescent multimers of its ligand ICAM-1 for a rapid assessment of antigen-specific CD8$^+$ T cells. Here, we describe a modified protocol of this assay for sensitive detection of functional antigen-specific CD4$^+$ T cells using a monoclonal antibody (clone m24) specific for the open, high affinity conformation of the beta$_2$-integrin. Kinetics of beta$_2$-integrin activation were different on CD4$^+$ and CD8$^+$ T cells (several hours vs. few minutes, respectively), however, m24 antibody readily stained both cell types 4-6 hours after antigen stimulation. With this protocol, we were able to monitor CD4$^+$ and CD8$^+$ virus-specific T cells specific for CMV, EBV, HBV, and SARS-CoV-2 in whole blood or cryopreserved PBMCs of infected or vaccinated individuals. By costaining with m24 and CD154 antibodies, we assessed extremely low frequencies of polyfunctional CD4$^+$ T cell responses to SARS-CoV-2 derived peptides. Our novel assay thus allows very sensitive and simultaneous screening of both CD4$^+$ and CD8$^+$ T cell reactivities with versatile applicability in clinical and vaccination studies, and for epitope discovery.

Introduction

One of the first events that can be detected upon T cell receptor (TCR)-mediated stimulation of T cells is the activation of membrane-bound b$_2$-integrins. This activation involves a conformational change (from the bent, inactive form, to the open, high affinity conformation) and a clustering of integrin molecules on the cell membrane. Activated integrins are essential for ligand binding (e.g. binding of the b$_2$-integrin leukocyte function-associated antigen (LFA)-1 to intercellular adhesion molecule (ICAM)-1), for immunological synapse formation, and, as more recently recognized, downstream T cell functions [1-4]. Integrin conformational change is therefore a good correlate to T cell function that can be detected very early, provided that specifically the activated form of the integrins can be detected. Using fluorescent multimers of ICAM-1 (mICAM-1), we have previously shown that CD8$^+$ T lymphocytes can be specifically and very rapidly detected upon antigen-specific T cell stimulation. We observed that binding of mICAM-1 to b$_2$-integrins on CD8$^+$ T cells identifies functional effectors that will later degranulate and produce effector cytokines. In addition to being a quick read-out (approximately after 4 to 20 min stimulation in most cases) based on the use of a single detecting reagent, the assay preserves cell viability and can be used for the enrichment of antigen-responding CD8$^+$ T cells for immediate or post *in vitro* expansion analysis [5, 6].

Here, we describe a modified protocol of this assay, which is likewise taking advantage of b$_2$-integrin activation on T cells but was adapted for sensitive detection of antigen-specific CD4$^+$ effectors. Instead of mICAM-1, we now used a monoclonal antibody (Ab) specific for the open, high affinity conformation of the b$_2$-integrin (Ab clone m24 [7, 8]) and EDTA treatment to disrupt the cell conglomerates, which formed during CD4$^+$ T cell activation. The assay can be used for simultaneous screening of both CD4$^+$ and CD8$^+$

T cell reactivities and should be useful for high throughput detection of antigen-specific T lymphocytes, epitope discovery, and the monitoring of clinical studies. When combined with CD154 staining, it allows sensitive and accurate detection of extremely low frequencies of polyfunctional $CD4^+$ T cells. We give examples of antigen-specific $CD4^+$ and $CD8^+$ T cell detection in whole blood (WB) or cryopreserved PBMCs obtained from CMV- and EBV-infected volunteers and HBV vaccinees. In addition, SARS-CoV-2 directed T cells were readily detected in convalescents who have previously been tested positiv for the virus, as well as in an individual who received a SARS-CoV-2 peptide vaccine.

Results

Antigen-specific $CD4^+$ T cells can be best visualized with an antibody specific for activated β_2-integrins

We have previously shown that antigen-specific $CD8^+$ T cells are successfully detected by staining of activated β_2-integrins with fluorescent mICAM-1 [5]. We therefore investigated whether mICAM-1 binding could also be used as a marker for detection of antigen-specific $CD4^+$ T cells. We selected a healthy donor with a high frequency of *ex vivo* detectable $CD4^+$ T cells specific for the HPTFTSQYRIQGKLE epitope derived from the CMV pp65 protein (CMV/HPT). WB cells were incubated with the CMV/HPT peptide, with Staphylococcus enterotoxin B (SEB), or remained unstimulated for 4 h, and mICAM-1 was added for the final 4 min of activation. Blood cells were further processed for detection of intracellular TNF production (gating strategy is shown in Fig. 1*A*). CMV/HPT-, as well as SEB- stimulated cells, showed only a modest staining for both TNF and activated integrins (Fig. 1*B,* top). This was unexpected since the frequency of CMV/HPT reactive $CD4^+$ T cells was found to be approximately 1.2% in intracellular cytokine staining prescreening experiments in this donor. To check whether the antigen-specific $CD4^+$ T cells had aggregated during the activation, and therefore were excluded from the lymphocyte gate, we treated WB with 4 mM EDTA after the mICAM-1 staining (Fig. 1*B,* bottom). Then, much higher frequencies of CMV/HPT-specific or SEB-reacting TNF^+ $CD4^+$ T cells were detected (approximately 1.2% and 9%, respectively). However, these were mostly mICAM-1^{neg}, most probably because EDTA reversed mICAM-1 binding to activated β_2-integrins by chelating Mg^{2+} and Ca^{2+} ions required for β_2-integrin conformational change and stable interaction with mICAM-1. To confirm that the stimulation leads to aggregation of antigen-specific $CD4^+$ T cells, which can be disrupted by EDTA treatment, we also analyzed the distribution of TNF^+ $CD4^+$ T cells within the lymphocyte gate (FSC-A/SSC-A) or within singlet cells (FSC-A/FSC-H). Without EDTA treatment, 15% of the CMV/HPT-specific and 42% of the SEB-responding TNF^+ cells were within the lymphocyte or singlet gates, whereas EDTA treatment increased these numbers to 84-88% (Fig. S1 *A-D*).

Because mICAM-1 staining was not suitable for labelling activated β_2-integrins on antigen-specific $CD4^+$ T lymphocytes, we next tested staining of the cells with a monoclonal Ab that binds specifically to the extended/open high-affinity, but not to the resting, un-activated, conformation of β_2-integrins (clone m24, referred thereafter as m24 Ab). WB cells from the same donor were stimulated with the CMV/HPT

peptide, with SEB, or remained unstimulated for 4 h, then stained with m24 Ab for 15 min, followed by detection of intracellular TNF. Unstimulated cells showed a very low staining with m24 or anti-TNF Ab. Significant numbers of TNF^+ $CD4^+$ T cells were detected after stimulation with CMV/HPT (0.2%) and SEB (6.1%), most of them being $m24^+$ (Fig. 1*C,* top), however, these were much less than when mICAM-1 and EDTA were used. Addition of EDTA after m24 Ab staining improved TNF detection to expected frequencies (0.94% and 10.5% for CMV/HPT and SEB, respectively, Fig. 1*C,* bottom). The TNF-producing cells were essentially $m24^+$, indicating that once the Ab is bound, EDTA does not reverse the binding, as it does for mICAM-1. Without EDTA treatment, 18% of the CMV/HPT-specific and 50% of the SEB-stimulated TNF^+ cells were within the lymphocyte or singlet gates, whereas EDTA treatment increased these numbers to 74-86% (Fig. S1 *E-H*). When EDTA was added before, or at the time of mICAM-1 or m24 Ab staining, the cells did not stain with either of the reagents ($\leq$ 0.001%). Hence, m24 Ab staining of activated β_2-integrins followed by EDTA treatment can be used to detect antigen-specific $CD4^+$ T cells.

Antigen-specific β_2-integrin activation on $CD8^+$ T cells can be visualized by either mICAM-1 or m24 Ab staining

In our previous work, antigen-specific $CD8^+$ T cells were detected with mICAM-1 complexes (without EDTA) [5]. We therefore tested if staining of activated $CD8^+$ T cells with m24 Ab would be comparable to that obtained with mICAM-1, and to which extent EDTA influences detection. We selected an HLA-A*02$^+$ donor with a high frequency of $CD8^+$ T cells specific for the immunodominant epitope CMV pp65 NLVPMVATV (NLV). WB cells were stimulated with the CMV/NLV peptide for 16 min, then mICAM-1 and HLA-A*02 (referred thereafter as A*02) tetramers refolded with the CMV/NLV peptide were added for the final 4 min of the activation, or m24 Ab and A*02/NLV tetramers for 15 min after the activation. The combination of A*02/NLV and mICAM-1 identified 0.92% A*02/NLV^+ $CD8^+$ T cells (from which nearly all - 0.88% - were mICAM-1^+, Fig. 1*D*, top left), while the combination of A*02/NLV and m24 Ab identified 0.99% A*02/NLV^+ $CD8^+$ T cells (0.95% were $m24^+$, Fig. 1*D*, top right). Treatment with EDTA after mICAM-1 or m24 Ab staining only marginally increased the frequency of $tetramer^+$ cells (1.1%, Fig. 1*D,* bottom). Similar to what we had observed for $CD4^+$ T cells, staining of activated integrins on $CD8^+$ T cells was largely lost when EDTA was used in combination with mICAM-1, but not with m24 Ab. To confirm that the stimulation did not aggregate the antigen-specific $CD8^+$ T cells even without EDTA treatment, we analyzed the distribution of A*02/NLV^+ $CD8^+$ T cells on FSC-A/SSC-A and FSC-A/FSC-H plots (Fig. S2 *B* and *C*, top). Without EDTA treatment, 80-81% of the CMV/NLV^+ cells were within the lymphocyte or singlet gates, whereas EDTA treatment increased slightly these numbers to 91-93% (Fig. S2 *B* and *C*, bottom).

Next, we assessed whether m24 Ab staining indeed identifies the same antigen-specific T cells as mICAM-1 staining. WB cells from the same donor were stimulated with the CMV/NLV peptide, with SEB, or remained unstimulated for 16 min, then the cells were stained with mICAM-1 and m24 Ab. Double stainings confirmed that both mICAM-1 and m24 Ab essentially identify the same cells (Fig. 1*E*). Hence,

antigen-specific β_2-integrin activation on singlet $CD8^+$ T lymphocytes can be visualized by either mICAM-1 (without EDTA) or by m24 Ab with or without EDTA treatment, but for simultaneous detection of $CD4^+$ and $CD8^+$ T cells, m24 Ab and EDTA treatment should be combined.

Kinetics of β_2-integrin activation and m24 Ab binding is different for $CD4^+$ and $CD8^+$ T cells

To determine the optimal duration for cell stimulation, we performed time courses of β_2-integrin activation and m24 Ab binding on various antigen-specific $CD4^+$ and $CD8^+$ T cells. WB from one to four selected donors was stimulated with various antigens as indicated or remained unstimulated. Blood cells were harvested after different incubation times, followed by m24 Ab staining plus EDTA treatment (kinetics are shown as mean±SEM % $m24^+$ in Fig. 2*A* and representative examples in Fig. 2 *B-E*). As previously observed with mICAM-1 staining [5], responding CMV/NLV^+ $CD8^+$ T cells were very rapidly detected (immediate peak response of m24 Ab staining within only 4-16 min, Fig. 2*A*). Following prolonged activation with the CMV/NLV epitope (>1 h), percentage of $m24^+$ cells diminished only slightly (1.45% vs. 1.37% after 16 min or 4 h of stimulation, respectively, with a more substantial decrease found in the intensity, Fig. 2*D*), in contrast to the strong decline if using mICAM-1 [5]. Antigen-specific $CD4^+$ T lymphocytes showed different kinetics of β_2-integrin activation. A short-term activation with the CMV/HPT epitope in one donor induced only a weak m24 Ab staining on $CD4^+$ T cells, with a peak response after 4-6 h of stimulation (Fig. 2 *A* and *B).* Reactivity to overlapping 15mer peptides (HBV/Env) in two HBV-vaccinated donors also reached maximum between 4 and 6 h (Fig. 2 *A* and *C*). Finally, the percentage of $m24^+$ $CD4^+$ T cells strongly increased and peaked after 4-6 h of SEB stimulation for all 4 donors tested (Fig. 2 *A-C*). For $CD8^+$ T cells, m24 Ab staining peaked at 4 min in one case, while for the other two donors, it reached maximum at 4 h, but with decreased intensity (Fig. 2 *A* and *D*). All unstimulated cells showed a weak staining with m24 Ab (0.01 to 0.05%, Fig. 1 *B-D*).

To confirm that m24 Ab selectively identifies antigen-specific T cells, we co-stained cells from the CMV-reactive HLA-$A*02^+$ donor with A*02/NLV tetramers and m24 Ab. The majority of NLV-specific $CD8^+$ T cells could be detected by m24 Ab binding, with maximal staining achieved within 4-16 min of activation (Fig. 2 *A* and *E*, 97% of double-stained cells after 16 min of stimulation). For the HLA-$DRB1*11^+$ donor, co-staining with CMV/HPT tetramers and m24 Ab was not feasible because of strong TCR downregulation after peptide stimulation, however, we found comparable frequencies of CMV-specific cells with both methods (2% DRB1*11/HPT^+ (Fig. 2*F*) vs. 1.76% $m24^+$ $CD4^+$ T cells at the max. of response after 6 h of stimulation with the CMV/HPT peptide). Hence, m24 Ab identifies antigen-responding $CD4^+$ T cells, but in contrast to $CD8^+$ T cells, several hours of stimulation are required to activate β_2-integrins.

m24 Ab binding identifies functional antigen-specific $CD4^+$ T cells

We then tested whether m24 Ab binding correlates with functionality of antigen-specific $CD4^+$ T cells. WB from the DRB1*11^+ donor was stimulated with the CMV/HPT peptide, with SEB, or remained unstimulated. Blood cells were harvested after 1, 2, 3, 4 or 6 h, followed by incubation with m24 Ab plus EDTA treatment, and staining for intracellular CD154 and cytokines (TNF, IFN-γ, and IL-2). Results of the CMV/HPT stimulation are shown in Fig. 3*A*, and density plots after 6 h incubation without or with CMV/HPT in Fig. 3*B* top and middle, respectively. m24 Ab binding was the earliest event to be detected (after 1-2 h of stimulation, Fig. 3*A* opened bars), and upregulation of CD154 and cytokines was exclusively confined to the $m24^+$ cell subset (positive ($marker^+$ $m24^+$ cells) vs negative ($marker^+$ $m24^{neg}$ cells) colored bars, Fig. 3*A*). Also, almost all $m24^+$ cells (85%) expressed CD154, TNF or IFN-g at 6 h. Moreover, cytokine producing cells were mainly $m24^+$ $CD154^+$. Only 15%, 12%, and 1% of the TNF, IFN-g or IL-2 producing cells did not express CD154, and virtually none were $m24^{neg}$ (Fig. 3*B* bottom). Some single stained $m24^+$ or $CD154^+$ events, but no double stained $m24^+$ $CD154^+$ were observed in unstimulated cells (Fig. 3*B* top, left). Comparable results were obtained for the SEB-stimulated cells in this individual, with a strong association between m24 Ab staining and TNF or IFN-g production; still, a fraction of the $CD154^+$ or IL-2^+ cells were $m24^{neg}$, however, we found that $CD154^+$ $m24^{neg}$ cells were predominantly $cytokine^{neg}$ (Fig. S3 *A* and *B*).

To address specificities against further viruses, WB from two HBV-vaccinated donors were stimulated with HBV/Env overlapping peptides, with SEB (one donor only), or remained unstimulated. Cells were harvested after 1, 2, 3, 4, or 6 h, followed by m24 Ab staining, EDTA treatment, and staining for intracellular CD154 and cytokines. For the first donor, time course of HBV/Env stimulation is shown in Fig. 3*C* and density plots after 6 h of incubation without or with HBV peptides are displayed in Fig. 3*D* top and middle, respectively. Frequencies of HBV-specific cells were low (approximately 0.1% within the $CD4^+$ T cell subset), but readily detectable by m24 Ab staining. Similar to what we observed with CMV-specific cells, CD154 expression and cytokine production were essentially confined to the $m24^+$ cell fraction (positive ($marker^+$ $m24^+$ cells) vs negative ($marker^+$ $m24^{neg}$ cells) colored bars in Fig. 3*C*). However, a significant proportion of $m24^+$ cells (about 50%) did not upregulate CD154 or cytokines even after 6 h. Some $m24^+$ cells negative for the functional markers were also present in the unstimulated control. When we analyzed single and double $m24^+$ and $CD154^+$ cells, we found that cytokine-producing cells were mainly $m24^+$ $CD154^+$. Only 6%, 0% and 0% of the TNF, IFN-g, or IL-2 producing $CD4^+$ T cells, respectively, were $CD154^{neg}$, while 6%, 9% and 7% were $m24^{neg}$ (Fig. 3*D* bottom). Some single stained $m24^+$ or $CD154^+$, but no double stained $m24^+$ $CD154^+$ events were observed in unstimulated cells (Fig. 3*D* top, left). After SEB stimulation, a strong association between m24 Ab staining and TNF, IFN-g, and IL-2 production was observed. Again, a significant proportion of the $CD154^+$ were $m24^{neg}$, however, those were predominantly also $cytokine^{neg}$ (Fig. S3 *C* and *D*).

Similar observations were made for a second HBV vaccinee (Fig. S4). Although some background staining with m24 or CD154 Abs was observed in the unstimulated test, the combination of both markers (0.001% of $m24^+$ $CD154^+$ cells) allowed to distinctly detect HBV-specific cells even at very low

frequencies (Fig. S4*B*: 0.011%, 0.008%, 0.001%, and 0.004% for CD154, TNF, IFN-g, and IL-2 dot plots, respectively).

In conclusion, m24 Ab staining is suitable for detecting functional antigen-specific $CD4^+$ T cells, and the combination of this marker with CD154 can be used for identification of extremely low frequencies of functional antigen-specific T cells.

Monitoring of SARS-CoV-2 specific T cell immunity

Next, we examined the feasibility of our assay to monitor SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells. Blood was obtained from CoV-2 convalescents (n=3) and unexposed healthy donors (n=4), as confirmed by antibody ELISA on the day of blood withdrawal. In accordance with our previous results for CMV- and HBV-specific $CD4^+$ T cells, there were no $m24^+$ cells producing CD154 or cytokines without peptide-specific stimulation (0-0.001%, exemplary donor CoV-2 3 is shown in Fig. 4*A* top, pink frames). Addition of overlapping peptides derived from the membrane (M), nucleocapsid (N), or spike (S) proteins induced a clear $CD4^+$ T cell response in previously exposed individuals, detectable by co-staining with m24 and CD154 Ab, even at very low frequencies (down to 0.005-0.006%, M-, N-, and S-specific $CD4^+$ T cell responses from donor CoV-2 3 occurring at frequencies of 0.050%, 0.006%, and 0.014% are shown in Fig. 4*A*, pink frames). All three volunteers responded to the three proteins, albeit at a different intensity. Strongest responses were directed at the protein M (up to approximately 0.05% of the $CD4^+$ T cells in CoV-2 2 and 3, Fig. 4*B*). CoV-2 1 responded weakly to protein M (0.005%), but strongly to proteins N and S (both 0.009%). Cytokine production (TNF, IFN-g, and IL-2) was observed within $m24^+$ cells. T cell activation was virtually not detected in the four unexposed donors (0-0.003%), except for one of them (UD 2) who interestingly showed a distinct, but very low (possibly cross-reactive) response to the protein S only (0.005% $m24^+$ cells expressing either CD154, TNF or IFN-γ, Fig. 4*B*).

Anti-SARS-CoV-2 $CD8^+$ T cell reactivity was also assessed, either simultaneously to the $CD4^+$ T cell measurement (m24 Ab staining after 4 h of stimulation) or in an independent test after 1 h of stimulation only, followed by surface detection of activated integrins with mICAM-1. There were no $m24^+$ cells producing cytokines without antigen-specific stimulation (0-0.001%, shown for $CD8^+$ T cells from CoV-2 1 in Fig. 5*A*, top left). Interestingly, only protein N was recognized in two out of three convalescents, and $m24^+$ cells produced TNF (0.017% and 0.006% for CoV-2 1 and CoV-2 3, respectively) and IFN-γ (0.023% and 0.011%) (Fig. 5*B*, left), but no IL-2 (≤ 0.001%). None of the four unexposed volunteers reacted to any of the CoV-2 peptides (0-0.002%), except again for UD 2 who had a response to the N-derived peptide pool (0.010% $m24^+$ $CD8^+$ T cells expressing either TNF or IFN-γ). When mICAM-1 staining was applied for detection, the background was stronger (0-0.006%, CoV-2 1 is shown in Fig. 5*A*, right), which did not allow for a sensitive assessment of specific $CD8^+$ T cells. As a result, only the response of CoV-2 1 directed at protein N was clearly detected (0.011%; Fig. 5*B*, right).

We next tested $CD4^+$ T cell reactivity in the blood of a healthy volunteer who had been immunized approximately 6 weeks before with a cocktail of synthetic peptides containing HLA-class II SARS-CoV-2 sequences derived from the nucleocapsid (N) protein (IGYYRRATRRIRGGD, IGY and ASAFFGMSRIGMEVT, ASA) and from the envelope (Env) protein (FYVYSRVKNLNSSRV, FYV), as well as one recall CMV pp65 peptide (YQEFFWDANDIYRIF, YQE) [9]. Virtually no $m24^+$ cells producing CD154 or cytokines were detected when cells were left unstimulated for 6 h (0-0.001%, Fig. 6*A*). CMV/YQE stimulation caused a strong expression of CD154 (0.418%), TNF (0.312%), IFN-γ (0.382%), and IL-2 (0.253%) within $m24^+$ cells (Fig. 6*B*). The three SARS-CoV-2 peptides used for vaccination induced variable responses, with 0.094%, 0.060% and 0.012% of $m24^+$ $CD154^+$ $CD4^+$ T cells, for SARS-CoV-2 N/IGY, N/ASA and Env/FYV, respectively, as well as production of all three cytokines (Fig. 6*C-E*). These results are coherent with those obtained by IFN-γ ELISpot approximately 3 weeks after immunization [9].

m24 Ab stainings of antigen-specific $CD4^+$ and $CD8^+$ T cells in frozen/thawed PBMCs

We have also tested our method for detection of T cells within cryopreserved PBMC samples. All PBMCs were thawed and rested overnight at 37 °C and 7.5% CO_2. Then $2x10^6$ cells were stimulated in 1 ml TCM. Two donors, with high (approximately 2% of DRB1*11/HPT tetramer$^+$) and low (approximately 0.05% $CD154^+$ against HBV/Env) frequencies of specific $CD4^+$ T cells were selected (experiments with WB of the same donors were reported in Fig. 2 and 3). PBMCs were stimulated with the CMV/HPT peptide (first donor), with HBV/Env overlapping peptides (second donor) or remained unstimulated for 6 h, followed by incubation with m24 Ab plus EDTA treatment, and staining for intracellular CD154 and cytokines (TNF, IFN-γ, and IL-2).

For the first donor, b_2-integrin activation (m24 Ab binding) and functionality (CD154, TNF, IFN-g and IL-2 production) were stronger in PBMCs than in WB (1.90% vs 1.36% of $m24^+$ $CD154^+$ cells, respectively; compare Fig. S5*B* and Fig. 3*B*). Similar to what we observed with WB (Fig. 3*A*), upregulation of CD154 and cytokines was exclusively confined to the $m24^+$ cell subset and almost all $m24^+$ cells (93%) expressed CD154, TNF or IFN-g (Fig. S5*B*). Moreover, cytokine producing cells were mainly $m24^+$ $CD154^+$. Only 17%, 12%, and 1% of the TNF, IFN-g or IL-2 producing cells did not express CD154, and virtually none were $m24^{neg}$ (Fig. S5*B* bottom). Similar observations were made for the second donor, with a more robust response in PBMCs than in WB (0.058% vs 0.041% of $m24^+$ $CD154^+$ cells for PBMCs and WB, respectively; compare Fig. S5*C* and Fig. 3*D*). Again, some single stained $m24^+$ or $CD154^+$ events were observed in unstimulated cells, but virtually no double-stained $m24^+$ $CD154^+$ events were detected (Fig. S5*C* top, left). Cytokine-producing cells were mainly $m24^+$ $CD154^+$. Virtually none of the TNF, IFN-g, or IL-2 producing $CD4^+$ T cells, were $CD154^{neg}$, while only 1%, 0% and 8%, were $m24^{neg}$, respectively (Fig. S5*C* bottom). Thus, m24 Ab staining together with the expression of CD154 allows the reliable identification of low frequency, polyfunctional $CD4^+$ T cells in cryopreserved PBMCs.

We applied the m24 Ab assay for simultaneous detection of antigen-specific $CD4^+$ and $CD8^+$ T cells within PBMCs. Cells of one pre-screened donor (with a known low $CD4^+$ T cell reactivity against a HLA-class II peptide mix containing CMV, EBV and Flu-derived epitopes, and a low frequency of $CD8^+$ T cells against one HLA-A*02 restricted EBV-derived epitope) were thawed, rested, then stimulated with all peptides in pool for 4 h. Activation was then measured with m24 Ab combined to intracellular Abs. A background staining was observed with single m24 or single CD154 Abs on unstimulated $CD4^+$ T cells, however the combination of both markers allowed to readily detect approximately 0.05% of reactive cells (Fig. 7*A*). Combination of m24 Ab with TNF and IFN-g (but not with CD154 or IL-2) identified sensitively EBV-specific $CD8^+$ T cells (Fig. 7*B*).

Optimization of m24 Ab and ICAM-1 stainings of $CD8^+$ T cells within frozen/thawed PBMCs after short-term stimulation

The short-term stimulation in contrast to the 4-6 h protocol allows faster and simpler evaluation of functional $CD8^+$ T cells by surface detection of activated β_2-integrins alone or together with tetramers [5]. Hence, we optimized the detection of activated β_2-integrins on antigen-stimulated cryopreserved $CD8^+$ T cells after short-term stimulation. PBMCs from a HLA-A*02^+ donor with a high frequency (approximately 1.2%) of A*02/NLV tetramer$^+$ $CD8^+$ T cells were thawed and rested overnight at 37 °C and 7.5% CO_2. $2x10^6$ cells (in 1 ml or 0.2 ml TCM) were subsequently stimulated for 16 min with the CMV/NLV peptide and stained with either m24 Ab (with EDTA) or mICAM-1 (without EDTA); some stains were also performed in combination with CMV/NLV tetramers. Activation of specific $CD8^+$ T cells was much stronger for the $2x10^6$ cells/ml concentration, as seen for m24 Ab and mICAM-1 stainings (m24 Ab fluorescence intensity was weaker than that of mICAM-1 multimers, Fig. S6 and S8). Indeed, antigen-stimulated $CD8^+$ T cells aggregated heavily at the higher cell concentration ($1x10^7$ cells/ml) and were consequently lost from the FSC-A/SSC-A lymphocyte gate (Fig. S7 and S9). This aggregation depended on the presence of the CMV/NLV peptide during the stimulation, but not on that of m24 Ab or mICAM-1 (Figs. S 6*E*-9*E*).

Finally, we assessed the ability of m24 Ab or mICAM-1 to detect rare antigen-specific $CD8^+$ T cells using the optimal concentration of PBMCs ($2x10^6$ cells in 1 ml). An HLA-A*02^+ donor with approximately 0.04% of A*02/GLC tetramer$^+$ $CD8^+$ T cells was chosen. Staining with m24 Ab detected 0.022% while staining with mICAM-1 detected 0.017% EBV/GLC-specific cells (Fig. S10). However, m24 Ab staining had a lower intensity than that of mICAM-1, and more cells were observed in the control (0.012% $m24^+$ vs 0.004% mICAM-1^+), making mICAM-1 a superior reagent when $CD8^+$ T cells alone shall be detected without additional markers.

Discussion

We describe a protocol for sensitive assessment of virus-specific $CD4^+$ T cells, which, comparable to our prior mICAM-1 assay for detection of $CD8^+$ T cells, takes advantage of the conformational change of membrane-bound β_2-integrins after activation. This modified method allows simultaneous detection of very low numbers of effector $CD4^+$ and $CD8^+$ T cells in WB or cryopreserved PBMCs specific for a range of viruses, i.e. CMV, EBV, HBV, and SARS-CoV-2.

While the activation of β_2-integrins on $CD8^+$ T cells occurs within several minutes of peptide stimulation [5], we show that several hours are needed for $CD4^+$ T cells. This might reflect functional differences in the integrin activation pathways of the two T cell subsets, and might explain their distinct patterns of immunological synapse formation (transient vs. stable) and/or main functions (serial killing of numerous target cells vs. cytokine production) [10]. EDTA treatment was specifically required to visualize singlet responding $CD4^+$ T cells, suggesting intense cell clustering between T helper and HLA-class II^+ antigen-presenting cells. Although EDTA is needed for disrupting the cell aggregates, it reversed the binding of mICAM-1 to the activated β_2-integrins. Therefore, we used an Ab specific for the open, high-affinity conformation of b_2-integrin (clone m24), whose binding was not affected by subsequent EDTA treatment. Similar to the detection with mICAM-1 [5], staining of β_2-integrin activation with m24 Ab on $CD8^+$ T cells was maximal after 4-16 min of stimulation with peptides. However, in contrast to the mICAM-1, m24 Ab binding on antigen-specific $CD8^+$ T cells diminished only slightly following prolonged activation (4-6 h). Therefore, m24 Ab staining can be used for simultaneous screening of both $CD4^+$ and $CD8^+$ T cell reactivities. However, since the fluorescence intensity of m24 Ab is generally dimmer than that of mICAM-1, we recommend to perform staining after a short-term stimulation with mICAM-1 (without EDTA treatment) when only antigen-specific $CD8^+$ T cells are assessed.

With the aim to use the assay in clinical samples, it was important to optimize the protocol for the detection of T cells not only in WB, but also in cryopreserved PBMCs. Using $2x10^6$ PBMCs in 1 ml medium (the concentration can be upscaled by decreasing the volume if needed), frequencies of antigen-specific $CD4^+$ T cells were higher than when using WB, possibly reflecting different cell interaction conditions in the two cell sources. We also optimized the detection of $CD8^+$ T cells after short-term stimulation of cryopreserved PBMCs. In contrast to the 4-6 h protocol, the cell concentration (we used $2x10^6$ cells in 1 ml or 0.2 ml medium) influenced the detection of antigen-specific T cells. Antigen-specific cells aggregated heavily in the presence of activation peptide when the higher PBMC concentration was used (regardless of absence or presence of EDTA), therefore the lower PBMC concentration should be used for short-term stimulation (the volume can be increased keeping the same concentration if needed). For stimulation of $CD4^+$ and $CD8^+$ T cells in the 4-6 h protocol upscaling of cell concentration is possible without major problems of T cell aggregation.

Because m24 Ab staining on $CD4^+$ vs $CD8^+$ T cells was dimmer, some $m24^+$ cells were present in the unstimulated control, making it difficult to assess low frequencies of antigen-specific $CD4^+$ T cells with m24 Ab alone. The combination of m24 and CD154 Ab gave remarkable low background on

unstimulated samples (≤ 0.002% of $CD4^+$ T cells), allowing the reliable detection of antigen-specific $CD4^+$ T cells at extremely low frequencies (down to 0.004%). This is far below the lower detection limit of standard flow cytometry assays and in the range of what can be resolved with combinatorial tetramer staining [6, 11].

As an actual example, we evaluated $CD4^+$ and $CD8^+$ antigen-specific T cells in SARS-CoV-2 convalescents in comparison to unexposed healthy donors. $CD4^+$ T cells specific for spike, nucleocapsid and membrane proteins were detected in all three convalescents, whereas $CD8^+$ T cells were detected only towards the nucleocapsid protein. In line with other recent reports, we also observed in one unexposed donor very low frequencies of $CD4^+$ and $CD8^+$ T cells against spike and nucleocapsid proteins, respectively, probably due to cross-reactive cells primed against other SARS viruses [12-14]. We also used m24 Ab staining to confirm the induction of anti-vaccine $CD4^+$ T cells after experimental immunization of a healthy volunteer with synthetic peptides from the spike and nucleocapsid proteins [9]. Importantly, the results matched those of a previous IFN-γ ELISpot, and our method allowed to reliably detect the weakest anti-peptide response (0.012% $m24^+$ $CD154^+$ $CD4^+$ T cells vs 29 spots/ 300,000 PBMCs against CoV-2/FYV peptide).

m24 Ab identifies functional cells, i.e., those that produce cytokines upon stimulation. Interestingly, different $CD4^+$ antigen specificities showed unique cytokine profiles, which were also distinct in post-infection vs vaccination conditions (Fig. 8). Chronically CMV-infected individuals showed a prevalent TNF and IFN-γ production pattern, with some IL-2, while CMV peptide boost vaccination was predominated by a triple cytokine production with higher IL-2 levels. HBV vaccination selects for a TNF dominant memory cell pool that was almost equally distributed in TNF production alone, together with IL-2, or with both IL-2 and IFN-γ. SARS-CoV-2 convalescents had a more heterogeneous functional profile of either single, double or triple cytokine-producing cells, possibly reflecting a peculiar immune response to the virus, and/or a recent infection. Only the response to membrane protein was dominated by triple positive cells. Finally, the vaccination response to one of the three CoV-2 peptides (CoV-2/IGY) was not only the strongest, but also characterized by the highest proportion of triple cytokine positive cells. Thus, *ex vivo* costaining for activated integrins and CD154 on very low frequencies of effector/memory $CD4^+$ T cells allows to gain precise information about the polyfunctionality of the immune response. This is of importance since prior findings showed that the magnitude of the vaccine-induced polyfunctionality highly correlates with protection against pathogens [15-17]. In conclusion, we present a cytometry-based assay for the identification of antigen-specific $CD4^+$ and $CD8^+$ T cells. For $CD4^+$ T cells in particular, costaining of b_2-integrins with the activation marker CD154 allows detection of extremely low frequencies of responding polyfunctional cells. The assay can be used for highly sensitive screening of T cell reactivities, for example in epitope mapping studies or in translational approaches.

Materials And Methods

Study subjects and blood samples

For the studies with healthy individuals, either mononuclear cell blood concentrates or Na-heparinized blood, were obtained. We selected one DRB1*11:01$^+$ person with detectable CMV HLA-DRB1*11 multimer$^+$ CD4$^+$ T cells, two HLA-A*02$^+$ people with detectable CMV A*02/NLV multimer$^+$ CD8$^+$ T cells, one HLA-A*02$^+$ person with detectable EBV A*02/GLC multimer$^+$ CD8$^+$ T cells, one with detectable EBV A*02/YVL multimer$^+$ CD8$^+$ T cells and a CD4$^+$ T cell reactivity to an in house-made, nine HLA-class II peptide-pool containing CMV, EBV and Flu-derived epitopes. Two individuals vaccinated with hepatitis B vaccine (Engerix-B, GlaxoSmithKline, 20 µg HBs) approximately 10 years ago were also included. PBMCs were isolated by cell density centrifugation (Biocoll, Biochrom AG), washed twice in PBS w/o Ca2$^+$/Mg$^+$ and frozen in aliquots in 90% heat-inactivated fetal calf serum (ThermoFisher) + 10% DMSO (Merck).

Na-heparinized blood was also obtained from three convalescent persons who had been recently diagnosed with SARS-CoV-2 infection (either by PCR testing after nasopharyngeal swab or by serological Ab testing) and additionally from four uninfected control donors. Na-heparin plasma was isolated by centrifugation. Anti-CoV-2 Ab testing was performed with the SARS-CoV-2 ELISA from EUROIMMUN. Tests were specifically detecting spike protein domain 1 (S1)-specific IgG and nucleocapsid (N)-specific IgG. ELISA was performed according to manufacturer's instructions. All three individuals had developed IgG against S1 and/or N at the time of T cell testing. The unexposed control donor sera shown in Fig. 4 and 5 did not show any IgG reaction (data not shown). All studies were approved by the Ethics Committee of the University of Tübingen (approvals 156/2012B01, 713/2018BO2, and 256/2020BO2), and participants gave written informed consent.

The self-experimenting healthy volunteer vaccinated with SARS-CoV-2 derived peptides has been presented in details elsewhere [9]. Briefly, the donor received CMV- and CoV-2-derived synthetic peptides together with the adjuvant XS15. The vaccine contained among others the four HLA-class II peptides, CMV/YQE (which had been already applied in 2017), as well as CoV/IGY, CoV/ASA, and CoV/FYV (see next section). According to the results of an IFN-g ELISpot experiment performed approximately three weeks post-vaccination, all four peptides had induced a T cell response in vivo. For the experiment shown in Fig. 6, blood was obtained approximately five weeks post-first vaccination.

Peptides, stimuli and pMHC tetramers

For antigen-specific stimulation, we used the following synthetic peptides, representing known T cell epitopes derived from virus-associated antigens: For CD4$^+$ T cell stimulation: HLA-DRB1*11:01-restricted HPTFTSQYRIQGKLE peptide from CMV pp65, aa 366-380 (CMV/HPT); a mix of 9 HLA-class II binders from various proteins of CMV (n=2), EBV (n=6) and Flu (n=1) viruses. For CD8$^+$ T cell stimulation: HLA-A*02-restricted NLVPMVATV peptide from CMV pp65, aa 495-503 (CMV/NLV); HLA-A*02-restricted GLCTLVAML peptide from EBV BMLF1, aa 259-267 (EBV/GLC); and HLA-A*02-restricted YVLDHLIVV peptide from EBV BRLF1, aa 109-117 (EBV/YVL). HLA-class II peptides used for vaccinations were CMV pp65 aa 510-524 YQEFFWDANDIYRIF (CMV/YQE), SARS-CoV-2 N aa 83-98 IGYYRRATRRIRGGD (CoV-

2/IGY) and aa 311-326 ASAFFGMSRIGMEVT (CoV-2/ASA), and SARS-CoV-2 Env aa 56–70 FYVYSRVKNLNSSRV (CoV-2/FYV). All peptides were synthesized and dissolved as previously described (purity ≥ 80%) [18]. We also used pools of 15-mer peptides overlapping by eleven amino acids and spanning the entire HBV/Env, SARS-CoV-2/Prot N or SARS-CoV-2/Prot M proteins or covering the immunodominant sequence domains of SARS-CoV-2/Prot S for activation. HBV/Env peptide pools (JPT) were dissolved in 100% DMSO, while all SARS-CoV-2 pools (Miltenyi) in 10% DMSO, aliquoted, and kept at -80 °C until further use. SEB (Sigma-Aldrich) was solved in PBS w/o $Ca2^{+}/Mg^{+}$ and frozen at -20°C in 1 mg/ml aliquots.

We produced biotinylated pHLA-A*02:01 monomers (CMV A*02/NLV) in-house by conventional refolding, as previously described [19]. We generated fluorescent pHLA-A*02:01 tetramers by coincubating streptavidin-PE or -APC (Biolegend and Thermofisher) at a 4 (streptavidin):1 (pHLA-A2 monomer) molar ratio. Multimers were aliquoted and stored at -80°C in a TBS buffer containing 16% glycerol [19]. PE-labeled DRB1*11:01 CMV/HPT tetramer was kindly provided by NIH Tetramer Core Facility and stored at 4°C

Production of human ICAM-1 multimers

Soluble fluorescent ICAM-1 complexes were produced as described previously [5]. Shortly, we generated fluorescent ICAM-1-Fc/anti-Fc multimeric complexes by co-incubating 200 µg/ml recombinant human ICAM-1-Fc (produced and purified as previously described [20] or bought from Biolegend (cat. number 552906) with 3.41 x diluted polyclonal anti-human Fc-PE $F(ab')_2$ fragments (Jackson ImmunoResearch) at 4 °C for 24 h while slightly rotating (15 RPM). For example, to prepare 100 µl of 200 µg/ml mICAM-1 using ICAM-1-Fc from Biolegend, we mixed 24.4 µl ICAM-1-Fc (4.1 x dilution, final concentration 200 µg/ml) with 29.3 µl Fc-PE $F(ab')_2$ fragments (3.41 x dilution) and 46.3 µl sterile PBS. The stock solutions of the anti-human Fc-PE $F(ab')_2$ fragments - which came lyophilized - were prepared according to the manufacturer recommendation (diluted in 1 ml Milli-Q water). In our experience, the multimeric ICAM-1 complexes (mICAM-1) are stable for at least 2 months when stored at 4 °C. For longer storage, mICAM-1 can be kept at -80 °C, but not at -20 °C, since this increases significantly the background staining.

Cell stimulation, m24 Ab or mICAM-1 stainings

We used fresh heparinized blood or frozen PBMCs for the assays. WB was used within 2 h after collection. Cryopreserved PBMCs were thawed, washed, and rested overnight in T cell medium (TCM) [IMDM (Lonza10% heat-inactivated human serum and 100 U/ml penicillin/ 100 µg/ml streptomycin (Sigma-Aldrich), and 50 µM β-mercaptoethanol (Merck)] containing 1 µg/ml DNAse I (Sigma-Aldrich), then washed and counted on Neubauer chamber before stimulation and staining.

We stimulated WB (up to 1 ml per test) or PBMCs, $2x10^6$ cells/ml (1000 µl/test) or $1x10^7$ cells/ml (200 µl/test) in TCM, for the indicated times at 37 °C in a water bath or incubator in 5 ml or 15 ml Falcon tubes (BD Biosciences) with the following peptides: 4 µg/ml of the single peptides CMV/HPT, CMV/HQE, CoV/IGY, CoV/ASA, CoV/FYV, EBV/GLC, a pool of EBV/YVL and 9 HLA-class II binders with 4 µg/ml per peptide; 2 µg/ml of the peptide pools HBV/Env, SARS-CoV-2/ S, CoV-2/ N, CoV-2/M, or 4 µg/ml SEB.

After stimulation, we left the tubes at RT for 1 to 4 min and stained the cells at RT with a pre-titrated amount (1:80 to 1:200) of the m24 Ab clone (Biolegend) labelled with PE. After 15 min incubation, EDTA was added for 10 min more at RT at a final concentration of 4 mM. Whenever indicated, the cells were also stained with HLA-A*02/ pMHC multimers (0.6 µg/ml).

For alternative staining with mICAM-1 after stimulation, we incubated the cells with mICAM-1 for 4 min at 37 °C and left the tubes at RT for 5 min.. Whenever indicated, the cells were incubated with EDTA for 10 min at RT at a final concentration of 4 mM or also stained with pA*02 multimers (0.6 µg/ml).

After m24 Ab or mICAM-1 staining, we fixed the samples and lysed erythrocytes with the FACS-Lysing solution (BD Biosciences) for WB or fixed the samples with 1.5% Fix solution (Polysciences) for PBMCs, followed by washing with PBS/0.5% bovine serum albumin (BSA)/0.1% sodium azide. After centrifugation, we stained the cells for surface markers in order to identify $CD4^+$ or $CD8^+$ T cells with antibodies (Ab) CD3-PerCP-Cy5.5, CD4-BV421 and CD8-BV605/APC in PBS/0.5% BSA/0.1% sodium azide for 15 min at RT. Cells were washed once and fixed in PBS/0.5% BSA/0.5% formaldehyde. All antibodies used were from Biolegend.

For MHC-class II tetramer staining (Fig. 2), PBMCs were incubated with 18 µg/ml DRB1*11:01 CMV/HPT tetramer for 3 h at 37°C diluted in 200 µl PBS/0.5% BSA (Sigma-Aldrich), washed once, then Ab CD4-FITC (in-house production), CD8-PE-Cy7 (Beckman Coulter) and Zombie Aqua (Biolegend) were added in 50 µl PBS/0.5% BSA for 15 min more. Cells were washed once with PBS/0.5% BSA/0.1% sodium azide and fixed in PBS/0.5% BSA/1 % formaldehyde.

m24 Ab combined to intracellular cytokine staining

For the assessment of intracellular CD154 and cytokine expression, we stimulated up to 1 ml WB or PBMCs at 37°C ($2x10^6$ or $1x10^7$ cells/ml in TCM) for the indicated times and with the indicated peptides and concentrations in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich) and 5 µg/ml monensin (BD Biosciences). After stimulation, we left the tubes at RT for 1 to 4 min, then extra-cellular staining with m24-PE Ab, together with Zombie aqua staining when indicated, was performed for 15 min at RT, followed by incubation with 4 mM EDTA for 10 min at RT. Samples were fixed and erythrocytes lysed with FACS-Lysing solution, followed by washing with PBS/0.5% BSA/0.1% sodium azide. CD3-PerCP-Cy5.5, CD4-BV421, and CD8-APC-Cy7 Abs were added in PBS/0.5% BSA/0.1% sodium azide for 15 min at RT. After one washing step in PBS/0.5% BSA/0.1% sodium azide, cells were then permeabilized for 20 min at

RT with FACS-Perm2 solution (BD Biosciences), washed with PBS/0.5% BSA/0.1% sodium azide, followed by intracellular staining with IFN-g-FITC, IL-2-PE-Cy7, TNF-BV605 and CD154-APC Abs in PBS/0.5% BSA/0.1% sodium azide for 30 min at RT. Cells were washed once with PBS/0.5% BSA/0.1% sodium azide and fixed in PBS/0.5% BSA/0.5 % formaldehyde. All antibodies used were purchased from Biolegend.

Flow cytometry and data analysis

We acquired the data on a LSRFortessa (BD Biosciences), analyses were performed with the softwares FACS DiVa version 8.0 or Flow Jo 10.6.2. We collected between 50,000 and 400,000 $CD4^+$ or $CD8^+$ events for the antigen-specific assays. Gating strategies are shown in Fig. 1, and in Supplementary Figs. S1, S2, and S5-S9. Results are presented as % of cells within the parent populations.

Declarations

Acknowledgements

We thank S. Heidu for the production of HLA-class I tetramers and excellent technical assistance. The HLA-class II (DRB*11/CMV HPT) multimer was kindly provided by the NIH Tetramer Core Facility. The MR1 tetramer technology was developed jointly by Dr. James McCluskey, Dr. Jamie Rossjohn, and Dr. David Fairlie, and the material was produced by the NIH Tetramer Core Facility as permitted to be distributed by the University of Melbourne. CG, AS and HGR are supported by the Deutsche Forschungsgemeinschaft, Collaborative Research Center 1399. CG, SS and HGR receive support from the Deutsche Forschungsgemeinschaft under Germany's Excellence Strategy - EXC 2180 – 390900677 (CG und HGR sind gefördert durch die Deutsche Forschungsgemeinschaft (DFG) im Rahmen der Exzellenzstrategie des Bundes und der Länder - EXC 2180 – 390900677). LB, TL, JB and SD are supported by grants from the German Research Foundation (TR-SFB 654, “Plasticity and Sleep”).

Author Contributions

AS, JS, LB, TL, HGR, JB, CG, and SD conceived the study and designed the experiments; AS, RF, CG, and SD conducted the experiments; AS, JS, CG, and SD performed the data analysis and prepared the figures; ATRJ, and SS provided critical reagents; AS, JS, CG, and SD wrote the manuscript; CG and SD supervised the study.

Competing financial interest

The authors declare no competing financial interests.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Verma, N.K. and D. Kelleher, *Not Just an Adhesion Molecule: LFA-1 Contact Tunes the T Lymphocyte Program.* J Immunol, 2017. **199**(4): p. 1213-1221.
2. Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells.* Nature, 1998. **395**(6697): p. 82-6.
3. Long, E.O., *ICAM-1: getting a grip on leukocyte adhesion.* J Immunol, 2011. **186**(9): p. 5021-3.
4. Fooksman, D.R., et al., *Functional anatomy of T cell activation and synapse formation.* Annu Rev Immunol, 2010. **28**: p. 79-105.
5. Dimitrov, S., et al., *Activated integrins identify functional antigen-specific CD8(+) T cells within minutes after antigen stimulation.* Proc Natl Acad Sci U S A, 2018. **115**(24): p. E5536-e5545.
6. Gouttefangeas, C., J. Schuhmacher, and S. Dimitrov, *Adhering to adhesion: assessing integrin conformation to monitor T cells.* Cancer Immunol Immunother, 2019. **68**(11): p. 1855-1863.
7. Yang, W., et al., *Activation of integrin β-subunit I-like domains by one-turn C-terminal α-helix deletions.* Proceedings of the National Academy of Sciences, 2004. **101**(8): p. 2333-2338.
8. Comrie, W.A., A. Babich, and J.K. Burkhardt, *F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapseActin flow sustains LFA-1 activation at the IS.* The Journal of cell biology, 2015. **208**(4): p. 475-491.
9. Rammensee, H.-G., et al., *Designing a therapeutic SARS-CoV-2 T-cell-inducing vaccine for high-risk patient groups.* PREPRINT (Version 1) available at Research Square. DOI: 10.21203/rs.3.rs-27316/v1, 2020.
10. Ortega-Carrion, A. and M. Vicente-Manzanares, *Concerning immune synapses: a spatiotemporal timeline.* F1000Res, 2016. **5**.
11. Hadrup, S.R., et al., *Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers.* Nat Methods, 2009. **6**(7): p. 520-6.
12. Nelde, A., et al., *SARS-CoV-2 T-cell epitopes define heterologous and COVID-19-induced T-cell recognition.* PREPRINT (Version 1) available at Research Square. DOI: 10.21203/rs.3.rs-35331/v1 2020.
13. Braun, J., et al., *SARS-CoV-2-reactive T cells in healthy donors and patients with COVID-19.* Nature, 2020.
14. Le Bert, N., et al., *SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls.* Nature, 2020.

15. Precopio, M.L., et al., *Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses.* J Exp Med, 2007. **204**(6): p. 1405-16.
16. Darrah, P.A., et al., *Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major.* Nat Med, 2007. **13**(7): p. 843-50.
17. Seder, R.A., P.A. Darrah, and M. Roederer, *T-cell quality in memory and protection: implications for vaccine design.* Nat Rev Immunol, 2008. **8**(4): p. 247-58.
18. Löffler, M.W., et al., *A Non-interventional Clinical Trial Assessing Immune Responses After Radiofrequency Ablation of Liver Metastases From Colorectal Cancer.* Front Immunol, 2019. **10**: p. 2526.
19. Hadrup, S.R., et al., *Cryopreservation of MHC multimers: Recommendations for quality assurance in detection of antigen specific T cells.* Cytometry A, 2015. **87**(1): p. 37-48.
20. Bengtsson, A., et al., *Transfected HEK293 cells expressing functional recombinant intercellular adhesion molecule 1 (ICAM-1)–a receptor associated with severe Plasmodium falciparum malaria.* PLoS One, 2013. **8**(7): p. e69999.

Figures

Figure 1

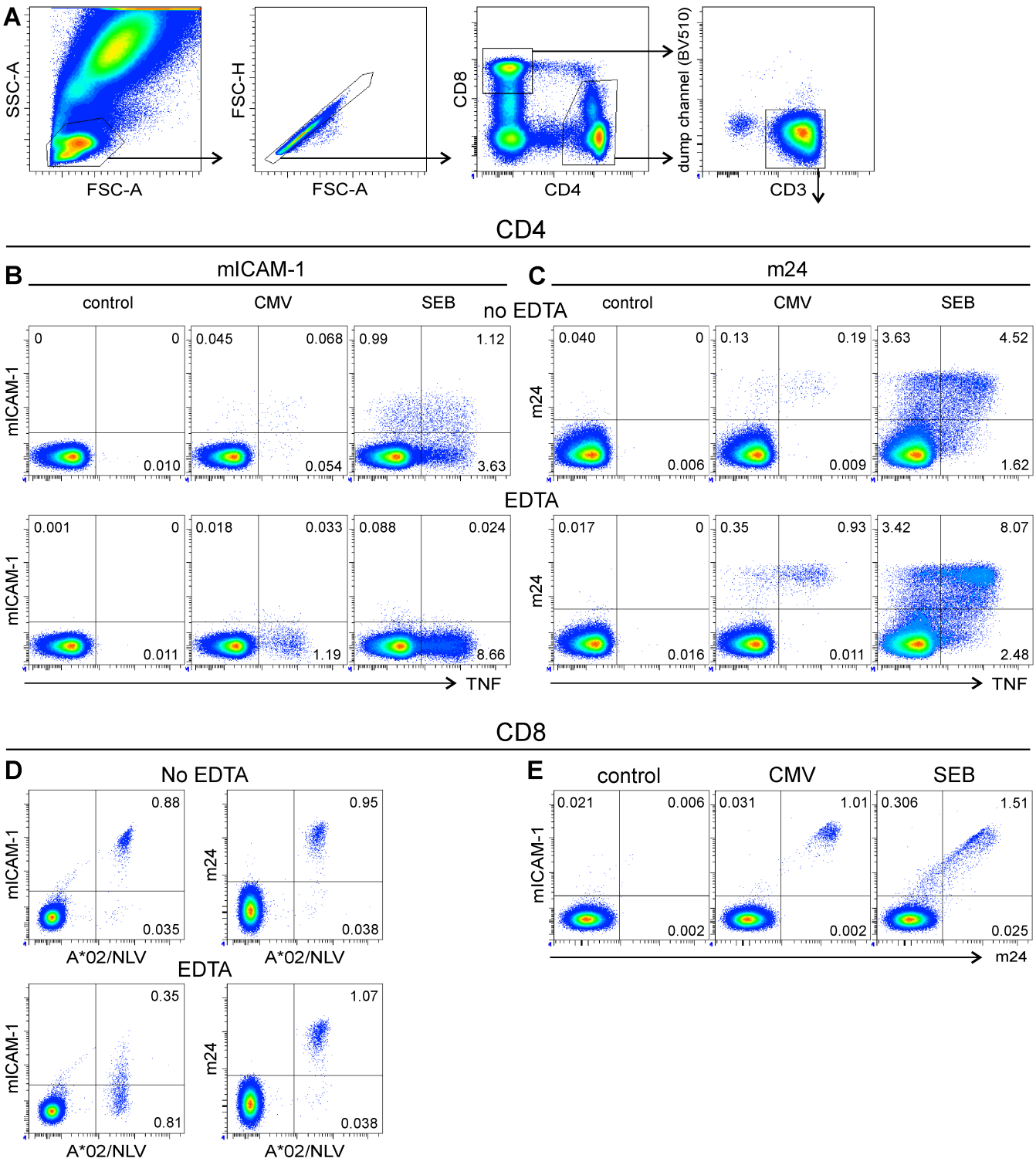

Figure 1

Assessment of antigen-specific CD4+ and CD8+ WB T cells by staining of activated β2-integrins. (A) Gating strategy. From left to right, the lymphocyte gate, the FSC-A/H duplet exclusion, the gating of CD4+ or CD8+ and CD3+ events. (B, C) WB from a donor having a large number of CMV/HPT-specific CD4+ T cells was cultured without stimulus (left, control), with the CMV/HPT peptide (middle) or with SEB (right) in the presence of brefeldin A and monensin. After 4 h, cells were either stained with mICAM-1 for 4 min

(B) or with m24 Ab for 15 min (C), without (top), or with EDTA addition (bottom) after the staining. Cells were subsequently labeled with CD3, CD4, and CD8 Abs, and for intracellular expression of TNF to identify CMV-specific CD4+ T cells. Numbers indicate frequencies among CD4+ T cells in %. (D) WB from a second selected HLA-A*02+ donor was incubated with the NLV peptide for 16 min, then either not treated (top) or treated with EDTA after the staining with mICAM-1 (left) or m24 Ab (right). Cells were simultaneously stained with A*02/NLV tetramers and thereafter with Abs to identify CMV-specific CD8+ T cells. (E) WB from the same donor was cultured without (left), with the NLV peptide (middle), or with SEB (right) for 16 min, then stained with mICAM-1 and m24 Ab and Abs (no EDTA treatment). Numbers indicate frequencies among CD8+ T cells in %.

Figure 2

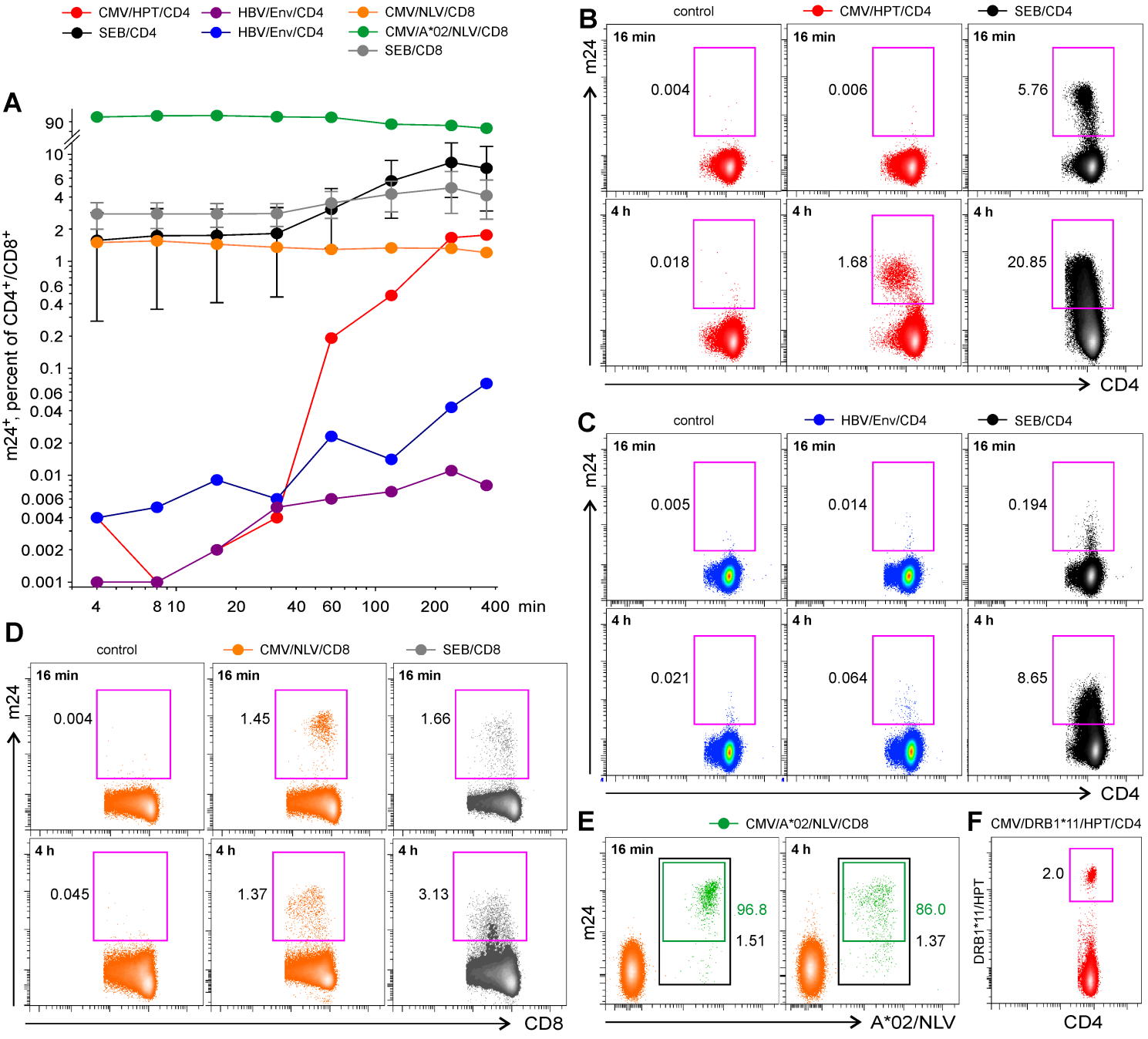

Figure 2

Flow-cytometry assessment of antigen-specific WB T cells by staining of activated β2-integrins and m24 Ab. Cells were stained with m24 Ab, treated with EDTA afterwards, then stained with CD3, CD4, and CD8 Abs, gating strategy is displayed in Fig.1A. Percentages of m24+ cells among total CD4+ or CD8+ T cells are indicated. (A) Time course of m24 Ab staining following incubation of WB cells with SEB (black, four donors for CD4+ T cells; gray, three donors for CD8+ T cells), CMV/HPT peptide (red, one donor, CD4+ T cells), CMV/NLV peptide (orange, one donor, CD8+ T cells; green, the same donor, but additionally stained with A*02/NLV multimers), or HBV/Env pool of overlapping peptides (blue and purple, 2 donors, CD4+ T cells) for 4, 8, 16, 32, 60, 120, 240, or 360 min. Background from the relevant unstimulated sample was subtracted (mean±SEM). (B) Examples of m24 Ab staining obtained after 16 min or 240 min, i.e. 4 h without stimulation (control), in the presence of matched peptides, or after stimulation with SEB; (C) One of the two HBV-vaccinated donors; (D, E) The donor with CMV-specific CD8+ T cells; in (E), combined m24 Ab and CMV/NLV tetramer stainings are shown, and percentages of A*2/NLV+ within the CD8+ subset and of m24+ within the A*2/NLV+ cells are indicated in black and green, respectively. (F) DRB1*11/HPT multimer staining obtained from the same donor as in (B).

Figure 3

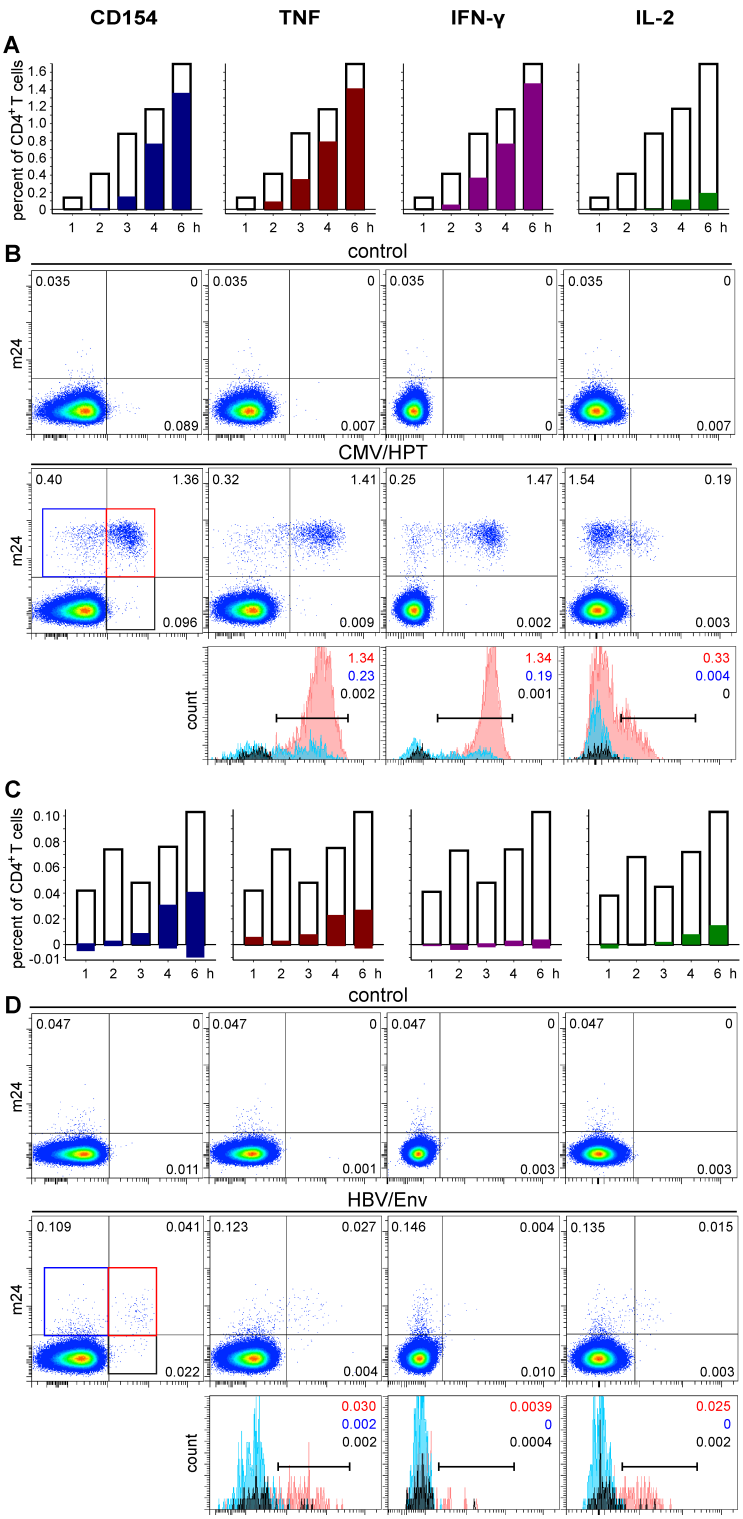

Figure 3

Functional profile of CMV- and HBV-specific m24+ CD4+ WB T cells. (A, B) WB of a DRB1*11+ donor stimulated with the CMV/HPT peptide. (C, D) WB from another donor who was vaccinated approximately 10 years ago against HBV. Cell stimulation was performed with HBV/Env overlapping peptides. The results of the CMV/HPT peptide (A) and HBV/Env overlapping peptides (C) stimulations are shown as graphs (at the indicated times and after subtraction of the background assessed in the control test;

frequencies within CD4+ T cells are shown); empty bars represent total m24+ cells, positive colored bars (blue, CD154; red, TNF; purple, IFN-γ; green, IL-2) represent marker+ m24+ CD4+ T cells, while negative colored bars are marker+m24- CD4+ T cells. (B, D) m24 Ab staining obtained after 6 h without stimulation (control) or in the presence of the matched peptides (CMV/HPT, or HBV/Env). Numbers on density plots indicate frequencies among CD4+ T cells. CD154+ m24neg, CD154neg m24+, and CD154+ m24+ subsets were gated (black, blue and red frames, respectively) and further displayed as histograms according to TNF, IFN-γ, and IL-2 expression. Numbers on histograms indicate frequencies of the respective color-coded population among CD4+ T cells (markers are shown). Gating strategy is as in Fig. 1A.

Figure 4

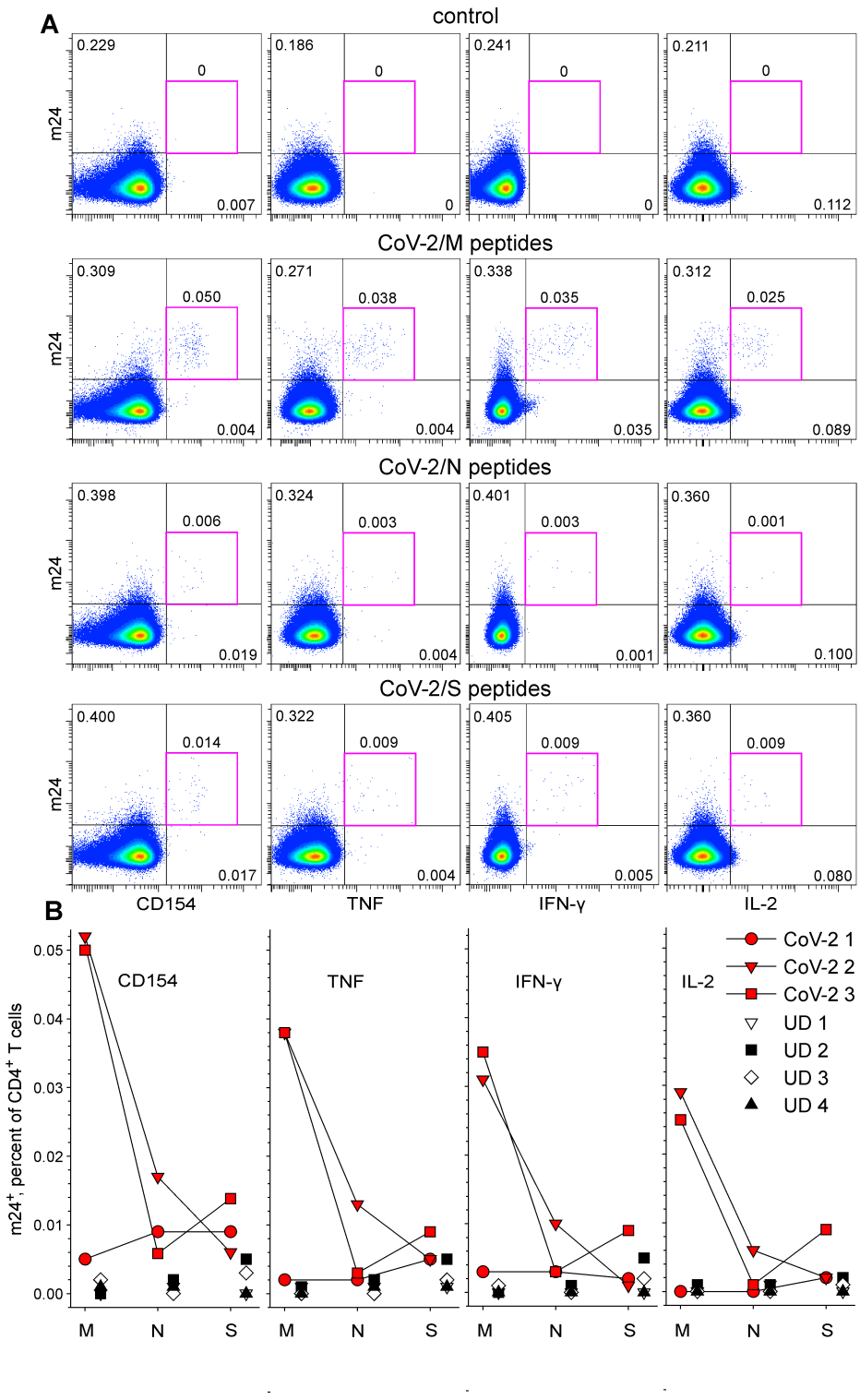

Figure 4

Functional profile of SARS-CoV-2-specific m24+ CD4+ WB T cells. m24 Ab staining in combination with CD154, TNF, IFN-γ or IL-2 expression after stimulation with pools of CoV-2 M, N, and S-derived overlapping peptides for 4 h. (A) Examples of m24 Ab staining obtained from CoV-2 3 without stimulation (control) or in the presence of the M, N or S peptides. Numbers indicate frequencies among CD4+ T cells in %. (B) The results for marker+ m24+ CD4+ T cells obtained from SARS CoV-2 convalescents (n=3, CoV-

2 1 to 3) and unexposed healthy controls (n=4, UD 1 to 4) are shown as graphs (after subtraction of background assessed in the control test). Gating strategy is as in Fig. 1A.

Figure 5

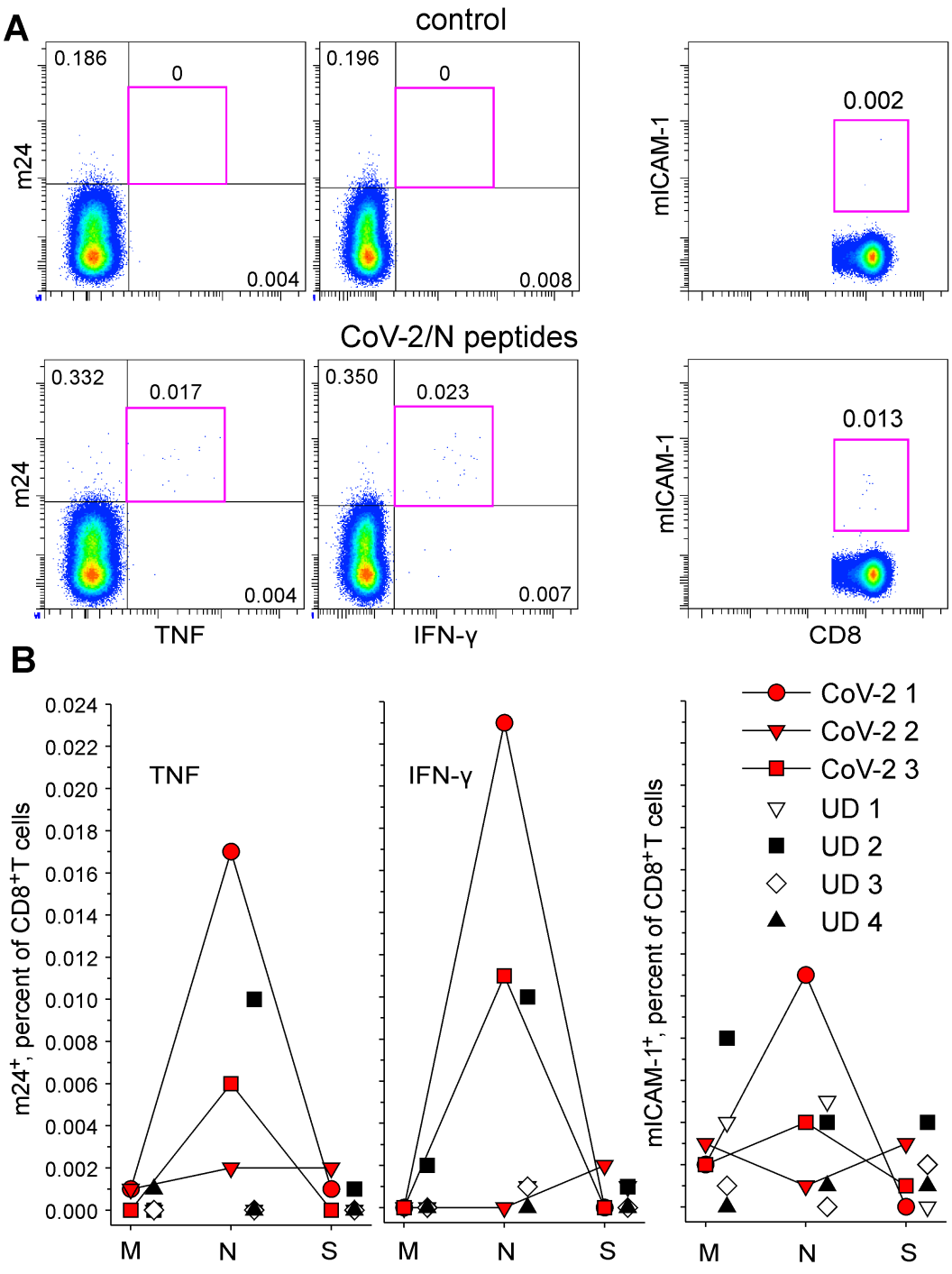

Figure 5

Functional profile of SARS-CoV-2-specific m24+ or mICAM-1+ CD8+ WB T cells. m24 Ab staining in combination with TNF or IFN-γ expression (left) after 4 h, or mICAM-1 staining (right) after 1 h of stimulation with pools of SARS-CoV-2 M, N, and S-derived overlapping peptides. (A) Examples of m24 Ab

or mICAM-1 staining from CoV-2 1 obtained without stimulation (control) or in the presence of N peptides. Numbers indicate frequencies among CD8+ T cells in %. (B) The results for marker+ m24+ or mICAM-1+ CD8+ T cells obtained from SARS CoV-2 convalescents (n=3, CoV-2 1 to 3) and unexposed healthy donors (n=4, UD 1 to 4) are shown as graphs (after subtraction of background assessed in the control test). Gating strategy as in Fig. 1A.

Figure 6

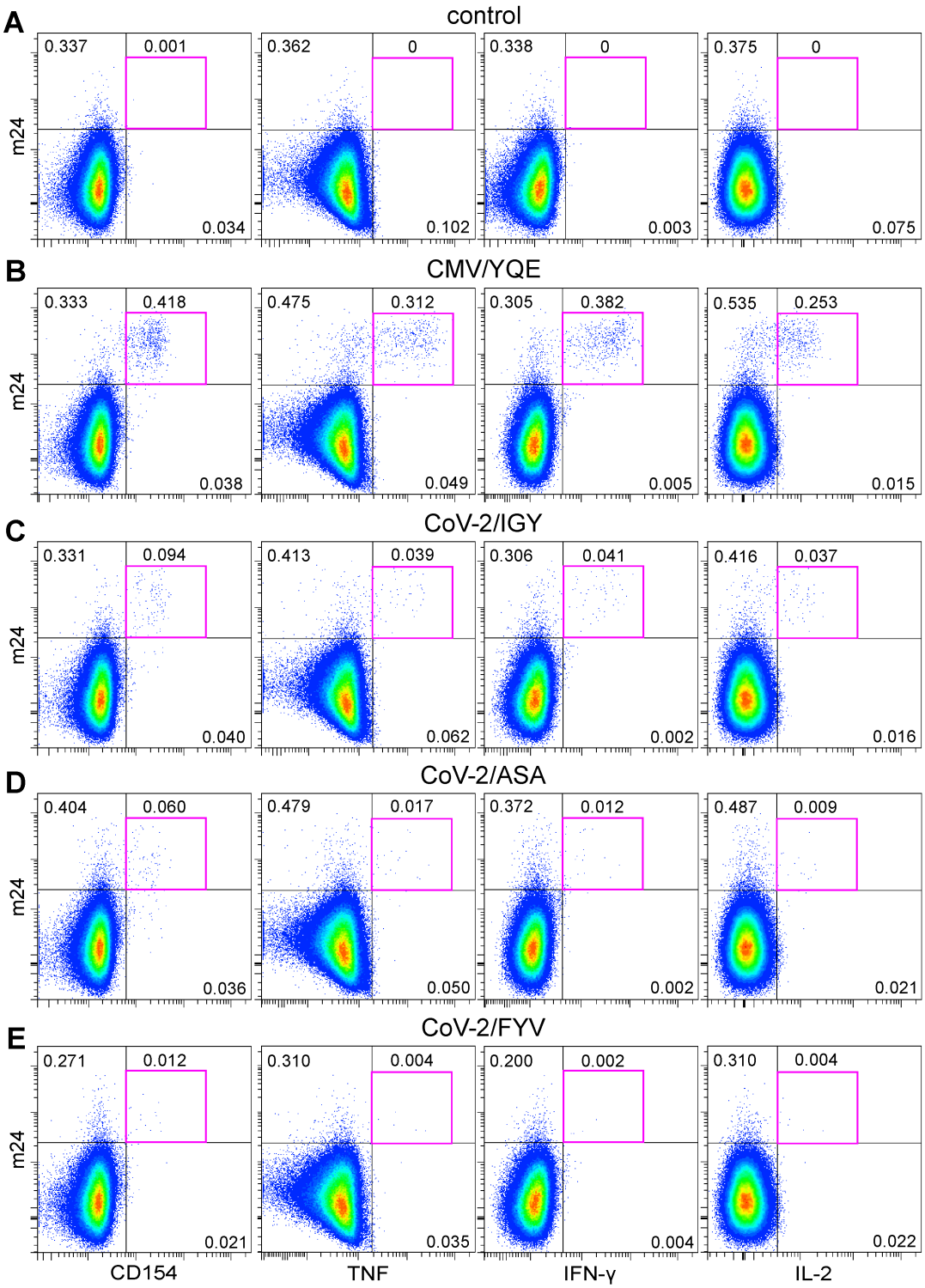

Figure 6

Functional profile of CMV- and SARS-CoV-2-specific CD4+ WB T cells after peptide vaccination. (A-E) m24 Ab staining in combination with CD154, TNF, IFN-γ or IL-2 expression obtained from a donor vaccinated with one CMV and three SARS-CoV-2 MHC-class II peptides without stimulation (control) (A), with CMV/YQE (B), CoV-2/IGY (C), CoV-2/ASA (D) or CoV-2/FYV peptides (E) for 6 h stimulation. Numbers indicate frequencies among CD4+ T cells in %. Gating strategy is as in Fig. 1A.

Figure 7

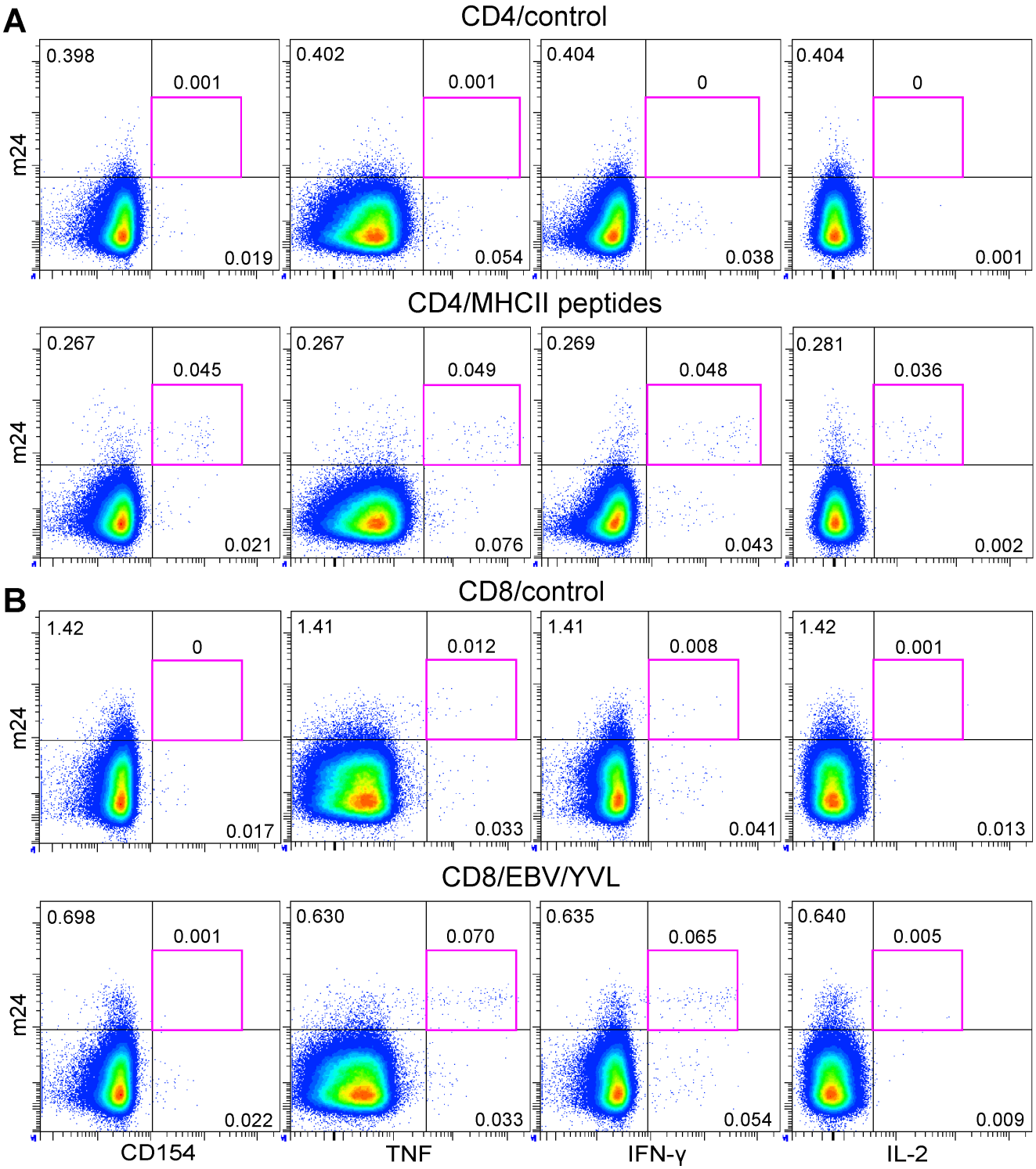

Figure 7

Application of the m24 Ab assay for simultaneous detection of antigen-specific CD4+ and CD8+ T cells within cryopreserved PBMCs. PBMCs of a pre-selected healthy donor were thawed and rested overnight before a 4 h stimulation with a pool of HLA-class II and HLA-A*02 peptides. (A) m24 Ab staining and CD154, TNF, IFN-γ and IL-2 expression for unstimulated (control) or MHC-class II peptide-stimulated CD4+ T cells (mix of n=9 virus-derived peptides). (B) The same stainings for CD8+ T cells without stimulation (control) or after stimulation with a HLA-A*02 epitope (YVLDHLIVV, EBV/YVL). Gating strategy is similar to that of Fig. 1A, with Zombie Aqua included as a live/dead cell marker. See also Fig. S5A.

Figure 8

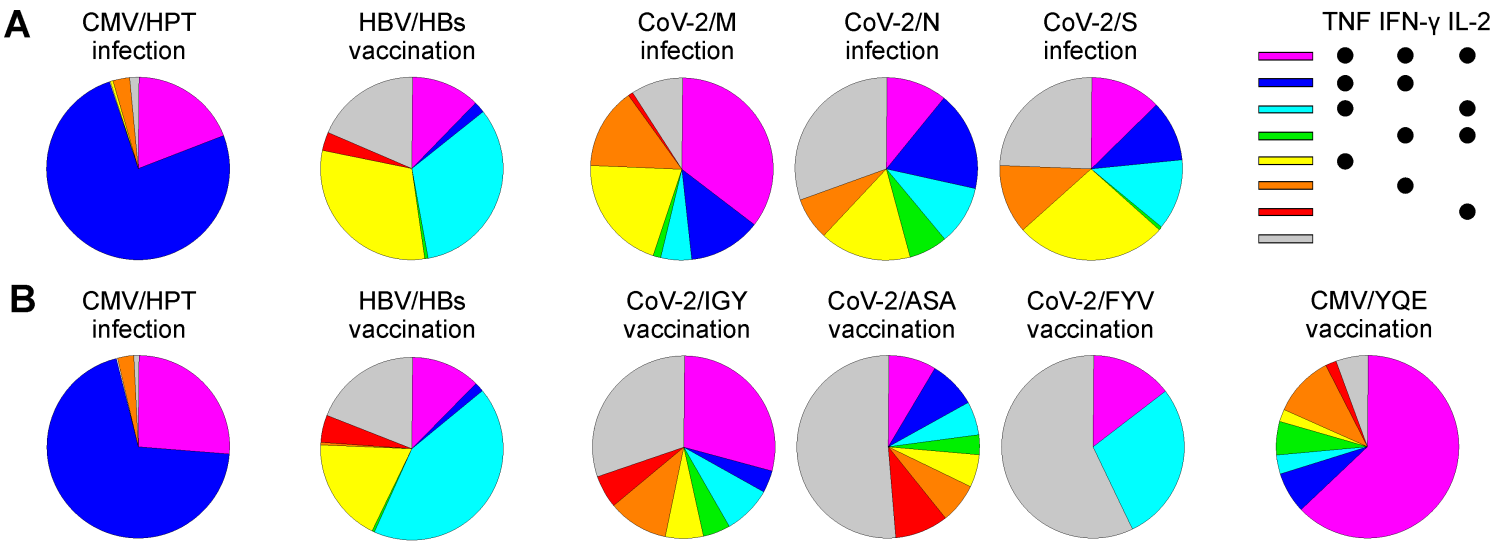

Figure 8

Polyfunctional profile of CD154+ m24+ CD4+ WB T cells for various viruses and challenge conditions. The pie charts represent the distribution of the cytokine production patterns from the donors and antigens tested in the study after CMV peptide vaccination (n=1), CMV infection (n=1), CoV-2 infection (n=3, mean is represented), CoV-2 peptide vaccination (n=1) or HBV vaccination (n=2, mean is represented) after 4 h (A) or 6 h (B) of stimulation with the respective peptides. Samples of the CMV infection and HBV vaccination were stimulated for 4 and 6 h for comparative purposes.

Supplementary Files

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

- SupplementaryFigures.docx