Activated integrins identify functional antigen-specific CD8$^+$ T cells within minutes after antigen stimulation

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Immediate β_2-integrin activation upon T cell receptor stimulation is critical for effective interaction between T cells and their targets and may therefore be used for the rapid identification and isolation of functional T cells. We present a simple and sensitive flow cytometry-based assay to assess antigen-specific T cells using fluorescent intercellular adhesion molecule (ICAM)-1 multimers that specifically bind to activated β_2-integrins. The method is compatible with surface and intracellular staining; it is applicable for monitoring of a broad range of virus-, tumor-, and vaccine-specific CD8$^+$ T cells, and for isolating viable antigen-reacting cells. ICAM-1 binding correlates with peptide-MHC multimer binding but, notably, it identifies the fraction of antigen-specific CD8$^+$ T cells with immediate and high functional capability (i.e., expressing high levels of cytotoxic markers and cytokines). Compared with the currently available methods, staining of activated β_2-integrins presents the unique advantage of requiring activation times of only several minutes, therefore delivering functional information nearly reflecting the in vivo situation. Hence, the ICAM-1 assay is most suitable for rapid and precise monitoring of functional antigen-specific T cell responses, including for patient samples in a variety of clinical settings, as well as for the isolation of functional T cells for adoptive cell-transfer immunotherapies.

antigen-specific T cells | ICAM-1 multimers | integrins | flow cytometry cell sorting | monitoring

T cell immunity plays a crucial role in the immune defense against pathogens and tumors. Assessment of T cell frequencies, phenotypes, and functionality is essential to monitor antigen-specific immune responses and to identify correlates of protection after vaccination or during therapy (1). Upon T cell receptor (TCR) engagement by cognate antigens, T lymphocytes up-regulate a number of activation markers and develop multiple effector functions, including proliferation, cytotoxicity, and cytokine production. One method of choice for identification of antigen-specific T cells is the use of fluorescent peptide-MHC multimers (pMHC multimers). While this method has revolutionized our understanding of antigen-specific T cells, it does not provide direct information on their function (2, 3). Therefore, the quality of the T cell response is additionally assessed by up-regulation of activation markers (e.g., CD154/CD137), CD107a surface expression, or cytokine/chemokine production (4–7). However, addressing these functional attributes requires several hours of stimulation with antigens and elaborated protocols.

Another functional property of T lymphocytes that has not yet been used as a monitoring tool for antigen-specific cells is the activation of integrins upon TCR engagement (8). Resting, antigen-experienced T lymphocytes express high levels of membrane-bound β_2-integrins (9, 10); however, these are maintained in a nonadhesive state (11). Following TCR-mediated stimulation, integrin activation occurs within seconds through a process known as "inside-out" signaling that leads to an affinity increase and clustering of membrane-bound integrins. This jointly enhanced avidity is critical for effective interaction between T cells and their targets, a process essential for execution of effector functions (12–15). We aimed to establish a flow cytometry assay that detects integrin activation on antigen-specific T cells. To overcome the problem that the interaction between β_2-integrins and the soluble monomeric or dimeric form of intercellular adhesion molecule 1 (ICAM-1) is very weak and unstable, we produced multimeric ICAM-1–Fc/anti–Fc-fluorochrome complexes by mixing recombinant human ICAM-1–Fc with goat anti-human Fc F(ab′)$_2$ fluorochrome-labeled fragments (hereafter referred to as mICAM-1) (16–18).

In this study, we establish activated integrins as an ideal marker for the rapid, sensitive, and selective identification of CD8$^+$ T cells reacting to a broad range of antigens, like the superantigen staphylococcal enterotoxin B (SEB), as well as peptides from cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza virus (Flu), HIV, yellow fever virus (YFV), and tumor-associated antigens. By comparing the mICAM-1 method with the pMHC

Significance

Assessing antigen-specific T cells is crucial for our understanding of immune reactions against pathogens and tumors, and for evaluating immunotherapies in patients. Existing techniques to evaluate the functionality of T lymphocytes all rely on de novo expression of proteins, typically intracellular cytokines, and therefore require elaborated protocols and reagents. We have established a simple flow cytometry-based method to assess the functionality of CD8$^+$ T cells by identifying immediate changes in the conformation and valency of cell surface integrins that occur within minutes following antigenic stimulation. Because of its robustness, sensitivity, and broad applicability, the assay can be rapidly implemented for the measurement and isolation of functional T cells for basic research and in the clinical setting.

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multimer and intracellular staining assays, we show that mICAM-1 multimers identify the fraction of pMHC$^+$ CD8$^+$ T cells, which predominantly produce cytokines and cytotoxic markers upon antigen stimulation. We also demonstrate that the assay is suitable for the isolation of viable antigen-specific CD8$^+$ T cells that can be readily expanded in vitro while maintaining functionality. Notably, due to its simplicity and robustness, mICAM-1 staining is especially appropriate to quickly assess functional antigen-specific T cells in patient samples.

Results

Antigen-Specific β_2-Integrin Activation on CD8$^+$ T Cells Can Be Visualized by mICAM-1 Binding. We investigated the feasibility of using mICAM-1 complexes as a staining reagent for antigen-specific CD8$^+$ T cells. First, we assessed the kinetics of β_2-integrin activation and mICAM-1 binding to determine the optimal duration for cell stimulation (Fig. 1). Whole blood—the main source of cells used in this study—from one to three selected HLA-A2$^+$ donors (hereafter referred to as A2$^+$donors) was stimulated with antigens as indicated or remained unstimulated. mICAM-1 was added for the final 4 min of activation. Blood cells were harvested after different incubation times and further processed (kinetics are shown in Fig. 1*A* and representative examples in Fig. 1*B*). Unless noted otherwise, the gating strategy to identify CD8$^+$ T cells is shown in *SI Appendix*, Fig. S1. Unstimulated cells showed a very low staining with mICAM-1 (0.01–0.05%), which did not change significantly over time. In contrast, cells stimulated with SEB or viral peptides were readily stained by mICAM-1. Of note, different antigen-specific CD8$^+$ T lymphocytes showed different kinetics of β_2-integrin activation: SEB, NLVPMVATV peptide from CMV pp65, amino acids 495–503 (CMV/NLV), GLCTLVAML peptide from EBV BMLF1, amino acids 259–267 (EBV/GLC), and LLWNGPMAV peptide from YFV NS4B, amino acids 214–222 (YFV/LLW) induced an immediate peak response within only 4 min. With CMV/pp65 or HIV/p17 pools of overlapping peptides for stimulation, maximal staining with mICAM-1 was achieved after 32 min, whereas 1 h was required to detect GILGFVFTL from FLU M1, amino acids 58–66 (Flu/GIL)-specific cells. Following prolonged activation with the antigens (>1 h), β_2-integrin activation decreased again.

Next, we tested the applicability of mICAM-1 staining to detect various frequencies of activated T cells within different cell preparations. We incubated whole blood from nine CMV-seropositive

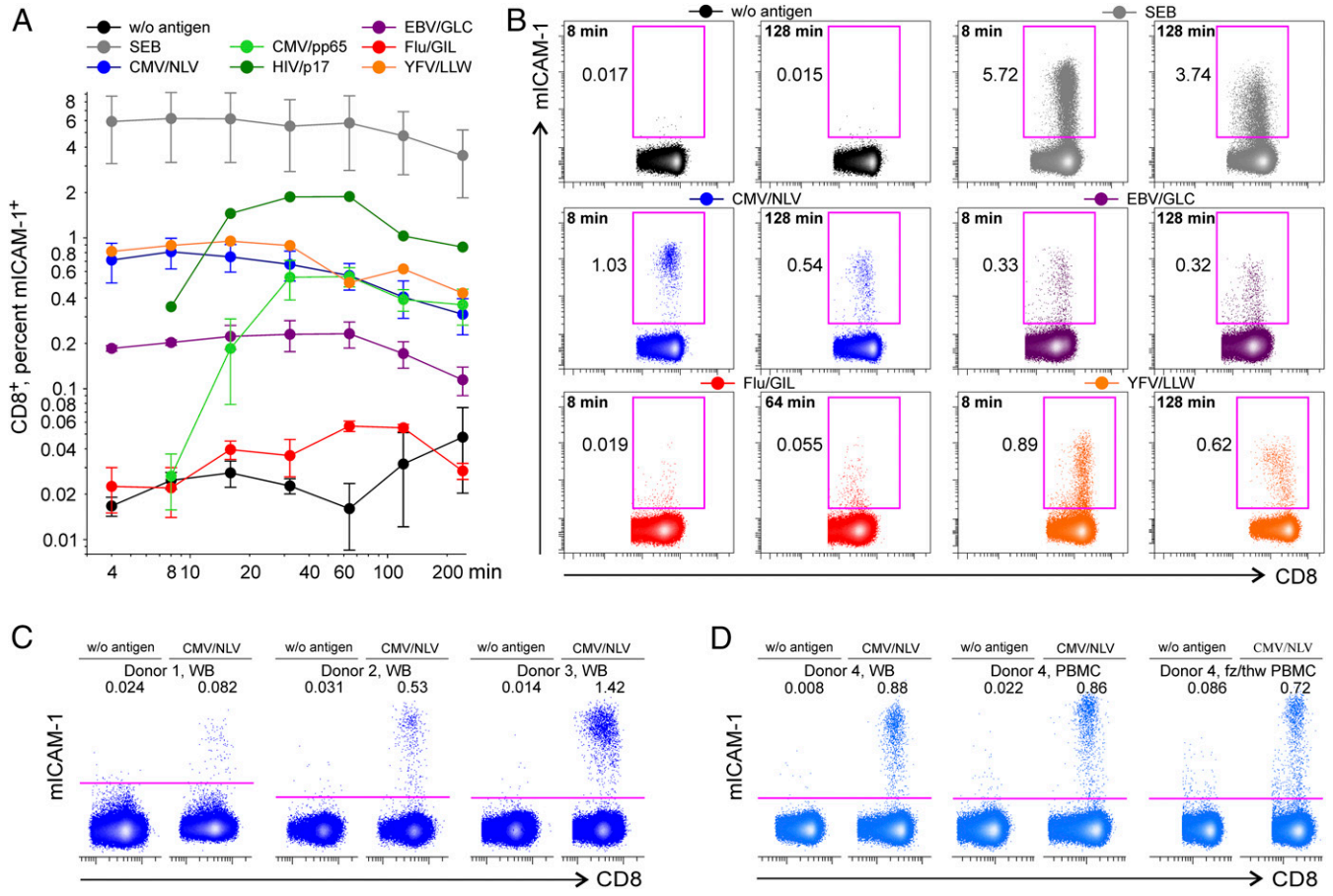

Fig. 1. Flow-cytometry assessment of antigen-specific CD8$^+$ T cells using mICAM-1. (*A*) Time course of activated β_2-integrin staining following incubation of whole blood (WB) without antigen (black, three donors), with SEB (gray, three donors), CMV/NLV peptide (blue, three donors), CMV/pp65 pool of overlapping peptides (light green, three donors), HIV/p17 pool of overlapping peptides (dark green, one HIV-seropositive patient), EBV/GLC peptide (purple, two donors), Flu/GIL peptide (red, two donors), or YFV/LLW peptide (orange, one vaccinated subject) for 4, 8, 16, 32, 64, 128, or 256 min. For the final 4 min of incubation, mICAM-1 was added. Data show percentages of mICAM-1$^+$ cells among total CD8$^+$ T cells; for the antigen-stimulated sample, background from the relevant unstimulated sample was subtracted (mean ± SEM). (*B*) mICAM-1 staining obtained from three representative donors after 8 and 64/128 min of stimulation. (*C*) Examples of mICAM-1 staining of WB from three CMV-seropositive donors with low, intermediate, and high frequencies of CMV-specific cells after 8-min stimulation with CMV/NLV peptide. (*D*) mICAM-1 staining was compared in WB cells, fresh PBMCs and frozen/thawed (fz/thw) PBMCs after 8 min of stimulation with the CMV/NLV peptide. One of two donors is shown. Numbers indicate percentage of mICAM-1$^+$ cells among the CD8$^+$ T cells. w/o, without.

$A2^+$ donors for 8 min in the presence or absence of the NLV peptide. Unstimulated cells showed negligible staining with mICAM-1 (≤0.031%), whereas mICAM-1–stained NLV-activated $CD8^+$ T cells made up 0.08–3.8% of total $CD8^+$ T cells. Examples of mICAM-1 staining for detecting low, intermediate, and high frequencies of CMV-specific T cells are shown in Fig. 1*C*. In addition to whole blood, we stained freshly prepared peripheral blood mononuclear cells (PBMCs) and frozen/thawed PBMCs of two additional donors. The percentages of mICAM-1–stained $CD8^+$ cells were comparable in all three cell sources, albeit slightly reduced in frozen/thawed PBMCs (Figs. 1*D* and 2*C*). Taking these data together, we find that mICAM-1 can be used to detect a wide range of frequencies and specificities of $CD8^+$ T cells in whole blood and in PBMCs at very early time points of activation.

mICAM-1 Binding Reveals a Subset of $pMHC^+$ $CD8^+$ T Cells. To confirm that our method selectively identifies antigen-specific T cells, we stained blood cells with mICAM-1 and pMHC multimers. Cells from the same $A2^+$ donors were processed as in the prior kinetic experiment, but in addition CMV-, EBV-, Flu-, or YFV-specific cells were detected using staining with A2/NLV, A2/GLC, A2/GIL, or A2/LLW multimers, respectively (kinetics and representative examples are shown in Fig. 2 *A* and *B*). Maximal staining with mICAM-1 was achieved within 4–16 min of activation for CMV-, EBV-, and YFV-specific cells, and after 1 h for Flu-specific T cells. Remarkably, the vast majority (90–98%) of $mICAM\text{-}1^+$ cells were pMHC multimer-positive. Conversely, most $A2/NLV^+$ cells bound mICAM-1 (70–94%), whereas approximately half of $A2/GLC^+$ or $A2/LLW^+$ cells and one-quarter of $A2/GIL^+$ cells were $mICAM\text{-}1^+$.

Finally, we stained the cells from 10 prescreened donors with either pMHC multimers or with mICAM-1 after 8–64 min of activation with the virus-derived peptides. We observed close-to-perfect correlation between the frequencies of cells measured with the two assays, particularly for the CMV-specific cells ($r = 0.991$, $P < 0.001$) (Fig. 2*C*). Hence, our results show mICAM-1 staining identifies a subset of $pMHC^+$ antigen-specific T cells, which varies depending on the antigen specificity.

mICAM-1 Binding Identifies Highly Functional Antigen-Specific $CD8^+$ T Cells. Next, we assessed whether mICAM-1 staining provides information about the functionality of antigen-specific $CD8^+$ T cells. We examined cytokine production and mobilization of the degranulation marker CD107a in T cells stimulated with SEB or viral peptides and costained with mICAM-1. Because β_2-integrin activation displays a different kinetic than cytokine production or plasma membrane expression of CD107a, we selected stimulation times of 1 and 2 h, after which both a strong mICAM-1 staining and adequate amounts of cytokines or CD107a can be simultaneously detected. We observed that irrespective of antigens used: (*i*) expression of the functional markers was predominantly confined to the $mICAM\text{-}1^+$ cell fraction; and (*ii*) mainly cells with the strongest mICAM-1 binding costained for the functional markers CD107a, IFN-γ, and TNF (Fig. 3 and *SI Appendix*, Fig. S2). Specifically, $CD8^+$ T cells producing at least one functional marker were for the most part $mICAM\text{-}1^+$ after 1 h (94.7%, as shown in Fig. 3*B*) or 2 h (85.2%, as shown in *SI Appendix*, Fig. S2*D*) of stimulation with CMV/NLV. Conversely, the majority of CMV/NLV-stimulated $mICAM\text{-}1^+$ T cells, and in particular the $mICAM\text{-}1^{hi}$ population, expressed CD107a, IFN-γ, and/or TNF after 1 h (Fig. 3 *A* and *B*) or 2 h of stimulation (*SI Appendix*, Fig. S2 *C* and *D*). When we assessed CMV-specific cells after stimulation with pp65-overlapping peptides, again most cells expressing at least one of the three functional markers were $mICAM\text{-}1^+$ (93.1% after 1 h and 83.8% after 2 h of stimulation) (*SI Appendix*, Fig. S2 *B* and *D*). Similar results were obtained with the other antigen-specificities tested (EBV/GLC, Flu/GIL, or SEB) (*SI Appendix*, Fig. S2).

We subsequently analyzed the expression of functional markers on $mICAM\text{-}1^+$ and $mICAM\text{-}1^-$ $pMHC^+$ cells in the same subjects. We examined every combination of the CD107a, IFN-γ,and TNF markers after 1 h of stimulation with viral antigens among the $mICAM\text{-}1^+$ and $mICAM\text{-}1^-$ fractions of $A2/NLV^+$, $A2/GLC^+$, or $A2/GIL^+$ cells. A representative example for $A2/NLV^+$ cells is shown in Fig. 3*C*. Among $A2/NLV^+$ cells, we observed that more than 60% of $mICAM\text{-}1^+$ cells expressed a combination of at least two functional markers after 1 h of activation, whereas most $mICAM\text{-}1^-$ cells were negative for any functional marker (>80%) (Fig. 3 *D* and *E*). Comparable results were obtained for EBV- and

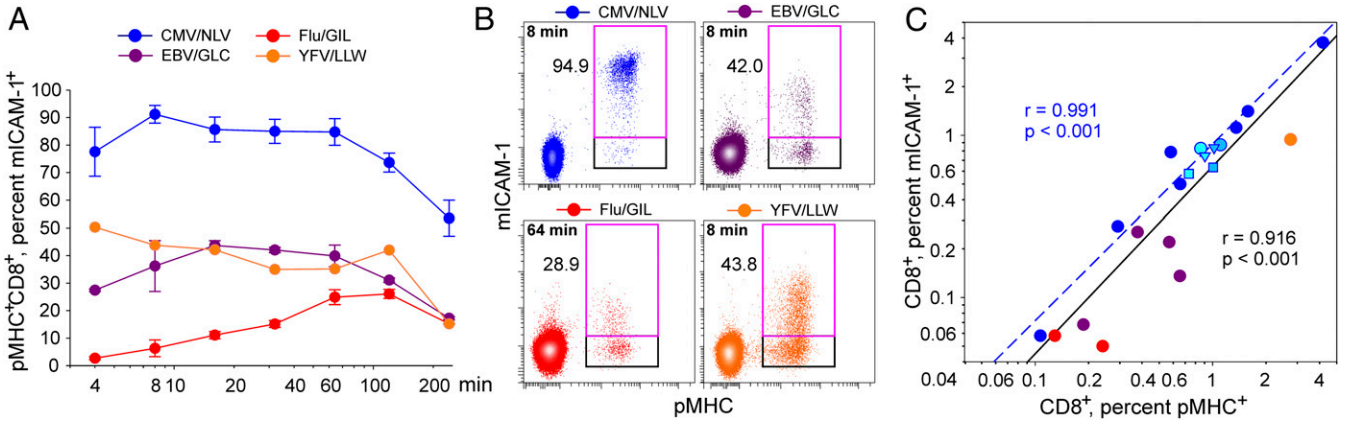

Fig. 2. Comparison of the mICAM-1 and pMHC multimer staining assays. (*A*) Time course of activated β_2-integrin staining after stimulation with CMV/NLV peptide (blue, three donors), EBV/GLC peptide (purple, two donors), Flu/GIL peptide (red, two donors), or YFV/LLW peptide (orange, one vaccinated subject) depicted as percentage (mean ± SEM) of the respective $pMHC^+$ $CD8^+$ cells. WB cells from the same donors as in Fig. 1*A* were stimulated with the indicated peptides for 4, 8, 16, 32, 64, 128, or 256 min. For the final 4 min of incubation A2/NLV, A2/GLC, A2/GIL, or A2/LLW multimers, respectively, together with mICAM-1 were added. (*B*) Responses obtained from three representative donors after 8 or 64 min of incubation. Numbers indicate the percentage of $mICAM\text{-}1^+$ cells among $pMHC^+$ $CD8^+$ T cells. (*C*) Correlation between antigen-specific $CD8^+$ T cell frequencies as detected by pMHC multimer and mICAM-1 stainings. WB cells (circles), or (only for CMV) freshly isolated PBMCs (triangles), or frozen/thawed PBMCs (squares) were either stimulated with the indicated peptides (8 min for CMV, EBV, and YFV and 64 min for Flu) and stained with mICAM-1 or directly stained with the indicated pMHC multimers (in total 10 donors). Different shapes in the same shade of blue indicate the same donor. The antigens are color-coded as in *A*. Dashed blue bold line represents the optimal linear correlation for CMV-specific cells. Bold black line represents the optimal linear correlation across all antigens. p, *P* value; r, correlation coefficient.

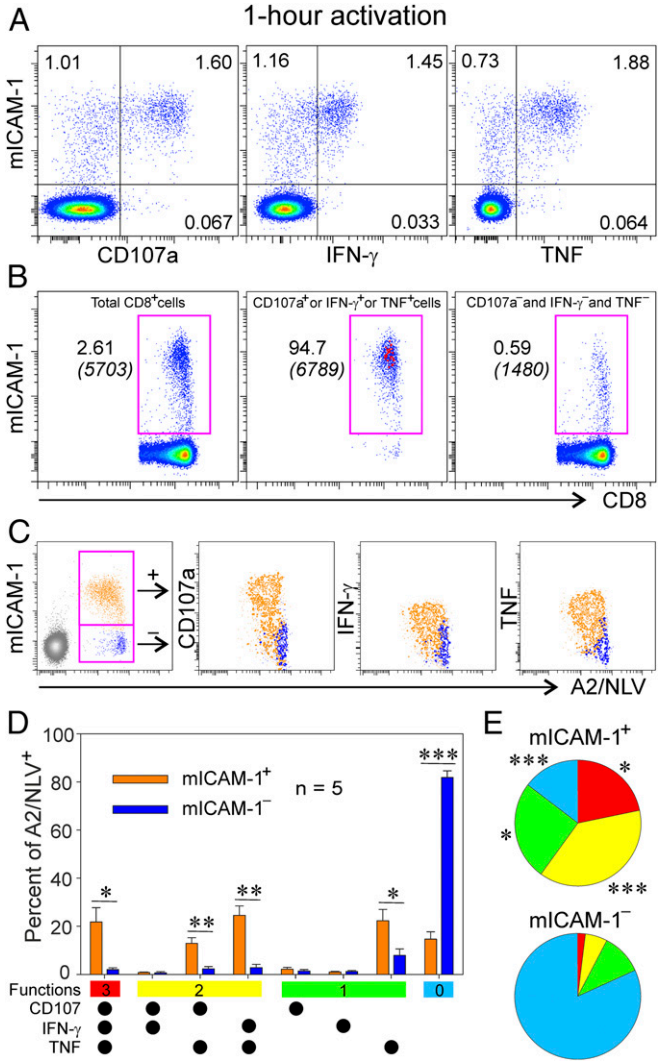

Fig. 3. Functional profile of mICAM-1$^+$ T cells. (*A*) Examples of CD107a, IFN-γ, and TNF expression by CD8$^+$ T cells after stimulation with the CMV/NLV peptide for 1 h and staining with mICAM-1 for the final 4 min. Numbers indicate frequency among CD8$^+$ T cells. (*B*) mICAM-1 staining among total CD8$^+$ T cell population (*Left*), among CD8$^+$ cells producing at least one functional marker (*Center*), or among CD8$^+$ cells producing none of them (*Right*). Cells expressing at least one functional marker were almost exclusively (94.7%) mICAM-1$^+$, whereas cells expressing none of the markers were almost exclusively (99.4%) mICAM-1$^-$. Numbers indicate frequency of ICAM-1$^+$ cells and italic numbers in brackets indicate MFI of mICAM-1. (*C*) mICAM-1 vs. A2/NLV staining on CD8$^+$ T cells from the same donor after 1-h stimulation with the CMV/NLV peptide (final 4 min in the presence of mICAM-1 and A2/NLV). mICAM-1$^+$ (orange) and mICAM-1$^-$ (blue) populations are further displayed according to CD107a, IFN-γ, or TNF expression. (*D*) Mean ± SEM percentages of mICAM-1$^+$ (orange) and mICAM-1$^-$ (blue) A2/NLV$^+$ T cells showing 3, 2, 1 or no functional markers, obtained in five donors. (*E*) The pie charts represent the mean expression of the functional parameters grouped by number of functions (0–3) among the five donors. Most of the mICAM-1$^-$ A2/NLV$^+$ cells did not produce any functional markers. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (mICAM-1$^+$ vs. mICAM-1$^-$ fractions).

Flu-specific responses (*SI Appendix*, Fig. S3 *B* and *D*). After 2-h activation with the various peptides, most mICAM-1$^+$ cells produced at least two functional markers (60–80%), whereas cells expressing no functional marker dominated within the mICAM-1$^-$ fraction (45–75%) (*SI Appendix*, Fig. S3 *A*, *C*, and *E*). Thus, mICAM-1 staining identifies highly polyfunctional CD8$^+$ T cells within pMHC$^+$ cells.

mICAM-1 Binding Identifies Functional T Cells of Various Differentiation Stages. Our data so far indicate that mICAM-1 staining detects the fraction of antigen-specific CD8$^+$ T cells with immediate function (Figs. 2 and 3). To further characterize these cells, we used surface phenotypic (CD27, CD28, and CD45RA) and intracellular cytotoxic [granzyme B (GrB) and perforin (Perf)] markers to stain SEB-stimulated and virus-specific mICAM-1$^+$ CD8$^+$ cells (19). Approximately 64% of the mICAM-1$^+$ SEB-stimulated CD8$^+$ T cells displayed an intermediate (CD27$^+$CD28$^-$CD45RA$^{+/-}$) or late (CD27$^-$CD28$^-$CD45RA$^{+/-}$) differentiation phenotype (Fig. 4*A* and *SI Appendix*, Fig. S4*A*). Furthermore, most of them expressed both cytotoxic factors (GrB$^+$Perf$^+$) (*SI Appendix*, Fig. S5 *A–C*), indicating that they were antigen-experienced cytotoxic effector cells.

To characterize virus-specific CD8$^+$ T cells, blood cells from A2$^+$ donors were stimulated with CMV/NLV, EBV/GLC (for 8 min), or Flu/GIL (for 1 h) peptides and stained with mICAM-1, as well as A2/NLV, A2/GLC, or A2/GIL multimers. As shown previously (19), pMHC$^+$ CD8$^+$ cells varied in their differentiation phenotype between the different virus specificities. Within the mICAM-1$^+$ fraction, early-differentiated (CD27$^+$CD28$^+$CD45RA$^-$) CMV-specific CD8$^+$ T cells were significantly diminished and those of the intermediate (CD27$^+$CD28$^-$CD45RA$^-$) phenotype were enriched. Differentiation stages of EBV- and Flu-specific cells showed a similar distribution within the mICAM-1$^+$ and mICAM-1$^-$ fractions (Fig. 4 *B–D* and *SI Appendix*, Fig. S4 *B–D*). For the cytotoxic markers, we found that mICAM-1$^+$ cells

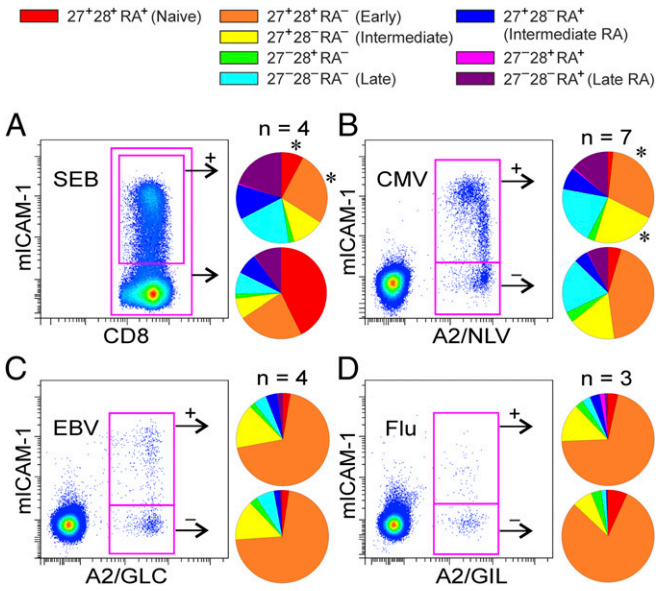

Fig. 4. Expression of differentiation markers according to β$_2$-integrin activation. (*A*) mICAM-1 staining after stimulation of WB cells with SEB from one representative subject is presented as a density plot (*Left*). Total CD8$^+$ T cells and the mICAM-1$^+$ fraction were analyzed for CD27, CD28, and CD45RA expression (a representative example is shown in *SI Appendix*, Fig. S4*A*); mean results are depicted as pie charts (*Right*). (*B–D*) mICAM-1 staining on CD8$^+$ T cells after stimulation of WB from selected A2$^+$ donors with CMV/NLV (*B*), EBV/GLC (*C*), or Flu/GIL peptides (*D*) (*Left*). Virus-specific cells were selected using staining with A2/NLV, A2/GLC, or A2/GIL multimers, and mICAM-1$^+$ and mICAM-1$^-$ fractions were then analyzed for CD27, CD28, and CD45RA expression (representative density plots are shown in *SI Appendix*, Fig. S4 *B–D*) and are depicted as pie charts (*Right*). All possible combinations of CD27, CD28, or CD45RA are shown, with each pie chart representing the mean percentage of each subset among the indicated number of subjects. Cells were stimulated for 4 min (SEB, CMV/NLV, or EBV/GLC) or for 1 h (Flu/GIL), followed by 4-min staining with mICAM-1 and relevant A2/pMHC multimers. *$P < 0.05$ (mICAM-1$^+$ vs. mICAM-1$^-$ fractions).

often expressed increased levels of GrB or Perf compared with mICAM-1$^-$ cells (*SI Appendix*, Fig. S5 *D–F*). Inversely, GrB$^-$Perf$^-$ cells contained a minority of mICAM-1$^+$ cells (2–20%). Hence, mICAM-1 staining identifies cells with strong effector function among memory and effector T cells at different stages of differentiation.

mICAM-1 Staining Allows Rapid Isolation of Viable Antigen-Specific CD8$^+$ T Cells with Immediate Effector Functions. We then tested whether mICAM-1 staining can be used for isolation of pure population of functional antigen-specific T cells. Fresh PBMCs from a CMV-seropositive A2$^+$ donor were activated with the NLV peptide and stained with mICAM-1 and anti-CD8 Ab (Fig. 5*A*). Highly enriched fractions of mICAM-1$^+$ CD8$^+$ and mICAM-1$^-$ CD8$^+$ cells were obtained by flow-cytometry sorting (purity of >90% and 99%, respectively) (Fig. 5*B*). Staining of the sorted mICAM-1$^+$ cells with the A2/NLV multimers revealed that they were mostly (87%) CMV-specific (Fig. 5*C*). We restimulated the two fractions with the NLV peptide for 5 h and measured CD107a expression and cytokine production by intracellular staining. mICAM-1$^+$ cells were highly enriched for these functional markers; inversely, mICAM-1$^-$ sorted cells neither expressed CD107a nor produced relevant amounts of cytokines (Fig. 5*D*).

In a second experiment, we again sorted mICAM-1$^+$ and mICAM-1$^-$ cell populations from the same donor with similar purities and cultured them in vitro in the presence of anti-CD3

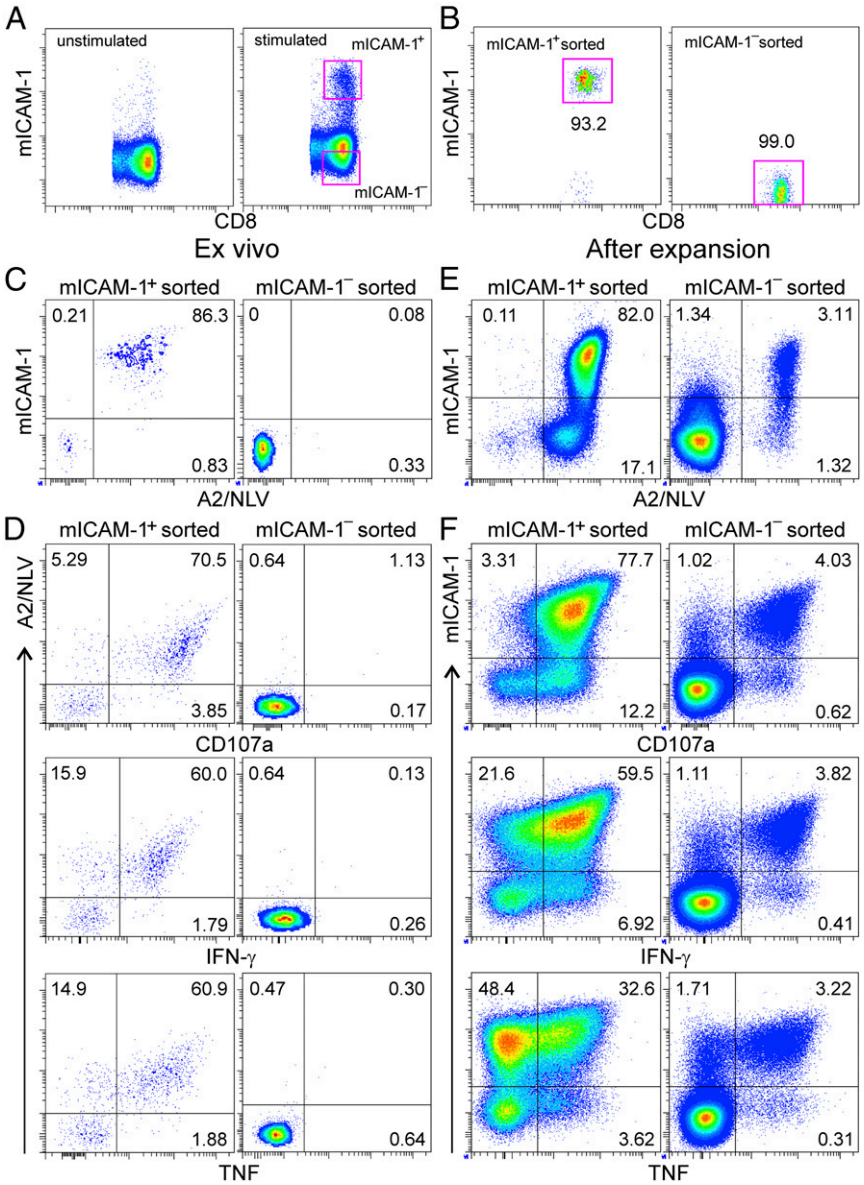

Fig. 5. Functional characterization of sorted mICAM-1$^+$ CD8$^+$ T cells. PBMCs from a CMV-seropositive A2$^+$ donor were cultured without or with the NLV peptide; the last 4 min in the presence of mICAM-1. (*A*) Sorting gates for mICAM-1$^+$ CD8$^+$ and mICAM-1$^-$ CD8$^+$ cells and (*B*) immediate postsorting analysis are shown. Isolated mICAM-1$^+$ and mICAM-1$^-$ fractions were then analyzed immediately (ex vivo) or expanded for 10 d in the presence of anti-CD3 Ab, IL-2, and IL-15 and then analyzed. (*C* and *D*) mICAM-1$^+$ sorted (*Left*) and mICAM-1$^-$ sorted (*Right*) fractions were stained with A2/NLV multimers and analyzed (*C*) or stained with A2/NLV multimers, stimulated for 5 h, stained with mICAM-1, and analyzed for functional markers (*D*). (*E* and *F*) Following in vitro expansion for 10 d, mICAM-1$^+$ sorted (*Left*) and mICAM-1$^-$ sorted (*Right*) fractions were stained with mICAM-1 and A2/NLV multimers after an 8-min stimulation with the NLV peptide (*E*) or stimulated for 5 h, stained with mICAM-1, and analyzed for functional markers (*F*). Numbers indicate the frequency among the sorted CD8$^+$ T cells.

antibody, IL-2, and IL-15. Both fractions expanded equally well (~500-fold) after 10 d. A short, 8-min reactivation of the expanded mICAM-1$^+$ and mICAM-1$^-$ bulk cells with the NLV peptide and costaining with A2/NLV and ICAM-1 multimers revealed that sorted mICAM-1$^+$ cells were almost exclusively (99%) CMV-specific and the majority were mICAM-1$^+$ (82%). In contrast, a small proportion (4.4%) of sorted mICAM-1$^-$ cells was CMV-specific (Fig. 5*E*). Upon restimulation for 5 h in the presence of the NLV peptide, we found mICAM-1$^+$ cells to be highly functional, with a 10- to 20-fold enrichment in the expression of CD107a, IFN-γ, and TNF compared with the mICAM-1$^-$ sorted population (Fig. 5*F*). Similar results, obtained from a second donor are shown in *SI Appendix*, Fig. S6. Hence, mICAM-1 staining is suitable for isolating viable, functional subsets of antigen-specific cells.

Finally, we tested whether the ICAM-1$^-$ antigen-specific cells are nonfunctional or just delayed in their functionality. We first sorted mICAM-1$^+$ pMHC$^+$ and mICAM-1$^-$ pMHC$^+$ CD8 cells from a CMV-seropositive and a YFV-vaccinated donor after optimal stimulation with specific peptides. The mICAM-1$^+$ (A2/NLV$^+$ or A2/LLW$^+$) and mICAM-1$^-$ (A2/NLV$^+$ or A2/LLW$^+$) fractions were then additionally cultured for 1 or 5 h and the functional markers CD107a, IFN-γ, and TNF were analyzed (Fig. 6). Already 1 h after the stimulation, the majority (90%) of mICAM-1$^+$ CMV-specific cells and ~50% of the YFV-specific cells expressed functional markers (Fig. 6 *C* and *E*, respectively). As expected, a very small amount of CD107a and cytokines were expressed in the mICAM-1$^-$ sorted cells. After 5 h, a further increase of the functional markers in the mICAM-1$^+$ fraction

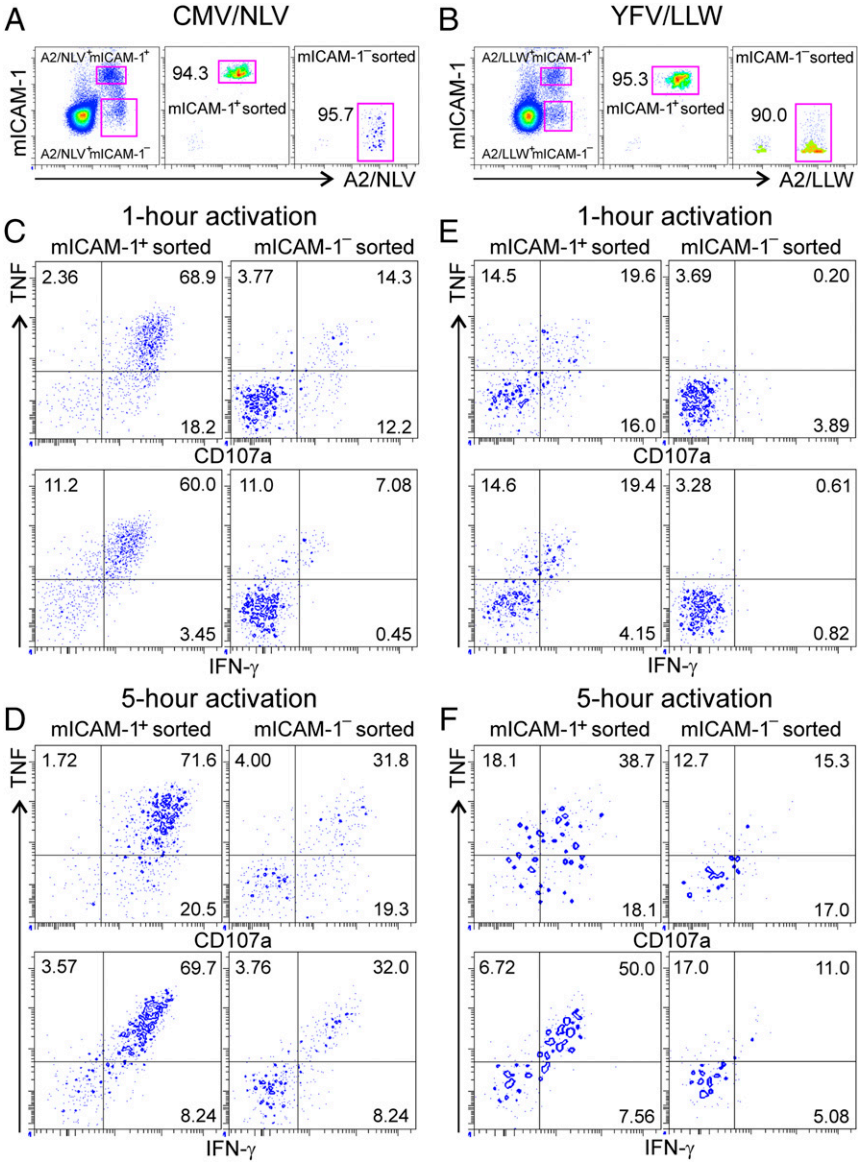

Fig. 6. Functional characterization of sorted CMV A2/NLV- or YFV A2/LLW-specific CD8$^+$ T cells according to β_2-integrin activation status. PBMCs from a CMV-seropositive (*Left* panels) or a YFV-vaccinated A2$^+$ donor (*Right* panels) were stimulated with the NLV or LLW peptides in the presence of A2/NLV or A2/LLW multimers and mICAM-1. (*A* and *B*) Sorting gates and immediate postsorting analysis for mICAM-1$^+$ and mICAM-1$^-$ of A2/NLV$^+$ CD8$^+$ (*A*) or A2/LLW$^+$ CD8$^+$ cells (*B*) are shown. (*C*–*F*) mICAM-1$^+$ A2/NLV$^+$ sorted and mICAM-1$^-$ A2/NLV$^+$ sorted fractions (*C* and *D*) or mICAM-1$^+$ A2/LLW$^+$ sorted and mICAM-1$^-$ A2/LLW$^+$ sorted fractions (*E* and *F*) were stimulated for 1 (*C* and *E*) or 5 h (*D* and *F*) and analyzed for functional markers. Numbers indicate the frequency among the sorted pMHC$^+$ CD8$^+$ T cells. Note, that the 1-h stimulation was in fact 2 h, because it took 60 min from the beginning of the first stimulation—for the sorting—until the time the sorted cells were placed in the incubator for additional 1-h reactivation.

was observed, particularly within the YFV-specific cells. Of note, functional cells were also increased within mICAM-1$^-$ sorted fractions at this time, although their percentages and mean fluorescent intensities (MFIs) were much lower than in the sorted mICAM-1$^+$ fractions (Fig. 6 *D* and *F* and *SI Appendix*, Fig. S7 *B* and *D*). The majority of the CD107a- and cytokine-producing cells within the mICAM-1$^-$ sorted cells then also stained for mICAM-1, suggesting a delayed activation in β_2-integrins coinciding with a delayed and lower production of functional markers (*SI Appendix*, Fig. S7).

mICAM-1 Staining for Monitoring Clinical Samples. Finally, we examined the usefulness of our assay to monitor antigen-specific CD8$^+$ T cells in cell samples obtained following vaccination of a healthy subject against yellow fever or from tumor-positive or HIV$^+$ patients.

Vaccination of a naïve A2$^+$ subject with YFV elicited a strong CD8$^+$ T cell response detectable ex vivo by mICAM-1 staining after 8-min activation with the immunodominant LLW peptide. LLW-specific CD8$^+$ T cells increased from 0.01% before vaccination to 1.16% 2 wk after vaccination, and decreased thereafter to 0.52% 5 wk after vaccination. In accordance with our previous results with CMV-, Flu-, and EBV-specific cells, costaining with A2/LLW multimers revealed that mICAM-1 staining was confined to A2/LLW$^+$ cells and that only a portion of the A2/LLW$^+$ cells (38–44%) were mICAM-1$^+$ (Fig. 7*A*); mICAM-1$^+$ A2/LLW$^+$ cells at week 2 postvaccination had an early-differentiated phenotype (CD27$^+$CD28$^+$CD45RA$^-$) and were highly enriched for the expression of functional and cytotoxic markers (*SI Appendix*, Fig. S8).

We next analyzed PBMC CD8$^+$ T cells obtained from three prostate cancer patients who had received experimental peptide-vaccination (20). T cells were first expanded in vitro using the relevant tumor peptide [i.e., prostate-specific membrane antigen, ALFDIESKV peptide from PSMA, amino acids 711–719 (PSMA/ALF), A2 restricted], then restimulated with the same peptide for 4 min, followed by costaining with mICAM-1 and A2/ALF multimers for an additional 4 min. Patient 1 showed higher frequencies of A2/ALF$^+$ cells than patients 2 and 3 (43.4% vs. 26.8% and 10.4%, respectively). In all individuals, a fraction of these cells was mICAM-1$^+$ (6% vs. 20% vs. 19%), suggesting the assay cannot only be used to identify tumor-antigen–specific T cells in patients, but also to assess their functionality (Fig. 7*B*).

Finally, we evaluated the utility of mICAM-1 staining to monitor HIV-specific responses in HIV-seropositive patients. Whole-blood T cells from two recently diagnosed patients were stimulated ex vivo for 28 min with p17 or Pol overlapping peptides, followed by 4-min staining with mICAM-1. A strong response was detected in patient 1 (1.9% and 0.6% of the CD8$^+$ T cell subsets for p17 and Pol, respectively), who had a viral load of 318 copies per milliliter. In contrast, a negligible percent of HIV-specific mICAM-1$^+$ CD8$^+$ cells was detected in patient 2 despite a very high viral load of >50,000,000 copies per milliliter, strongly suggesting a very recent infection or an ineffective immune response (Fig. 7*C*) (21). Confirming the mICAM-1 staining results, high frequencies of HIV-specific CD8$^+$ T cells, mainly producing IFN-γ, were detected in patient 1 after 4 h of stimulation with peptides (1.92% and 0.95% IFN-γ$^+$ and 0.092% and 0.033% TNF$^+$ cells among the CD8$^+$ fraction following stimulation with p17 and Pol, respectively), whereas only negligible cytokine production was detected in patient 2 (0.038% and 0.007% IFN-γ$^+$, and 0% and 0% TNF$^+$ cells among the CD8$^+$ fraction following stimulation with p17 and Pol, respectively). Further analyses in a third HIV-seropositive patient (viral load of 17,100 copies per milliliter) showed that HIV-specific mICAM-1$^+$ cells had an intermediate-differentiated phenotype (CD27$^+$CD28$^-$ CD45RA$^-$) and were enriched in cytotoxic markers and cytokine-producing cells (*SI Appendix*, Fig. S9). Altogether, these data indicate mICAM-1 staining is a fast and simple method to monitor functional CD8$^+$ T cell responses in a variety of clinical samples.

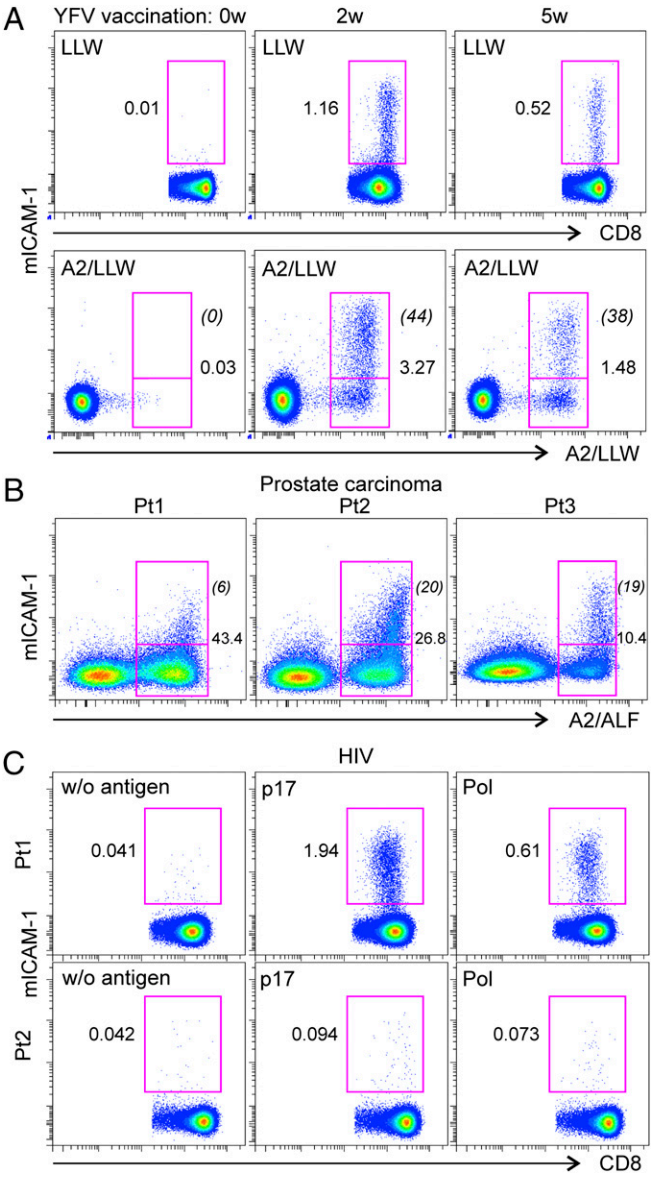

Fig. 7. mICAM-1 staining of virus- or tumor antigen-specific CD8$^+$ T cells. (*A*) YFV vaccination elicits a strong in vivo expansion of LLW-specific CD8$^+$ T cells as measured by mICAM-1 staining. WB cells from an A2$^+$ vaccinee at baseline (*Upper Left*), week 2 (*Upper Center*), and week 5 (*Upper Right*) after YFV-vaccination were stimulated with the LLW peptide for 8 min (final 4 min in the presence of mICAM-1). Costaining with A2/LLW multimers revealed mICAM-1$^+$ staining to be specific for the A2/LLW$^+$ cells (*Lower*). Regular numbers indicate frequency among CD8$^+$ T cells, italic numbers in brackets indicate frequency of mICAM-1$^+$ cells among A2/LLW$^+$ cells. (*B*) PBMCs from three A2$^+$ prostate cancer patients (Pt) who had received experimental peptide vaccination were expanded for 12 d in the presence of PSMA/ALF peptide, restimulated for 4 min with the peptide and stained for 4 min with A2/ALF multimers and mICAM-1. (*C*) Blood cells from two HIV$^+$ patients with a viral load of 318 (*Upper*) or 50,000,000 (*Lower*) copies per milliliter were incubated without (*Left*) or in the presence of peptide pools derived from p17 (*Center*) or Pol (*Right*) proteins for 32 min.

Discussion

We introduce a fast and straightforward flow-cytometry method for the assessment and isolation of live antigen-specific CD8$^+$ T cells, based on the staining of activated β_2-integrins with ICAM-1

multimers. Our assay is unique because it identifies cells by detecting immediate changes in the conformation and clustering of a surface molecule, rather than the up-regulation or production of a protein that takes significantly longer after stimulation. Hence, compared with current methods used to detect functional antigen-specific $CD8^+$ T cells, our assay presents the particular advantage of requiring activation times of only several minutes, and therefore of delivering functional information nearly reflecting the in vivo situation. In contrast, existing techniques to enumerate functional $CD8^+$ T cells [e.g., intracellular-cytokine staining (22), CD107a mobilization (4), and CD137 activation (7)] required to culture the cells for 6–24 h, and—in most cases—to block cellular transport processes and to permeabilize and kill the cells. While prolonged incubation times or suboptimal culture conditions might increase intrinsic background (23), we found that unstimulated $CD8^+$ cells showed a very low staining with mICAM-1 (0.01–0.04%), allowing detection of very low frequencies of antigen-specific cells ex vivo. Another popular assay used to identify antigen-specific T cells is the direct staining with pMHC multimers; the method is fast and robust, but cannot be used to detect functional cells, relies on preexisting knowledge about T cell epitopes, and requires elaborate and costly reagents (24).

Our methodology is applicable for whole blood, freshly isolated or frozen PBMCs, and cultured T cells. It enables the monitoring of effector cells that respond to a broad range and format of antigenic stimuli, including virus- and tumor-specific $CD8^+$ T cells in infectious diseases, cancers, or following prophylactic or therapeutic vaccination. We detected a strong, immediate (within minutes) activation of β_2-integrins following activation with SEB as well as CMV, EBV, YFV, and PSMA 9-mer immunodominant epitopes. One hour of stimulation was needed to detect activated integrins after incubation with a Flu 9-mer immunodominant epitope, which might suggest a lower binding affinity of this peptide to the TCR or a delayed kinetic of integrin activation for the Flu-specific cells. About 30 min of incubation was required to reach optimal staining when CMV/pp65, HIV/p17, or HIV/Pol pools of 15-mer overlapping peptides were used for activation. Because MHC class I molecules have a closed binding groove, which restricts the length of bound peptides to about 8–10 residues (25), 15-mer peptides might require partial processing (i.e., by antigen-presenting cells in the blood culture).

It was important to compare the assay with available flow-cytometry techniques. Using different antigens, we showed a high correlation between mICAM-1 and pMHC stainings. Interestingly, only a fraction of the $pMHC^+$ T cells was also stained with mICAM-1 (between 25% for Flu and 90% for CMV), suggesting that the remaining $pMHC^+$ T cells were nonfunctional at this time. Sorting of mICAM-1^- and mICAM-1^+ $pMHC^+$ cells for two model antigens demonstrated that functional cells (producing cytokines and/or expressing CD107a) are indeed those with early integrin activation. We also observed that (multi)functional $CD8^+$ T cells with strong cytokine responses and high CD107a expression were detectable exclusively within the mICAM-1^+ bright fraction, demonstrating that a robust activation of β_2-integrins reveals highly effective, functional antigen-specific T cells. Similarly, mICAM-1^+ T cells showed enriched coexpression of the two cytotoxic molecules GrB and Perf. The phenotype of mICAM-1^+ T cells, according to surface expression of CD27, CD28, and CD45RA, ranged from early to late stages of differentiation and were in line with previously reported differentiation patterns of CMV-, EBV-, Flu-, YFV-, and HIV-specific $CD8^+$ T cells (19). Hence, activated β_2-integrins mark all T cells with immediate, strong effector function, irrespective of their differentiation stages. Our preliminary experiments indicate that this is also the case for $CD4^+$ T cells.

Our method preserves cell viability and allows fast and easy isolation of functional cytotoxic T cells, which puts it in favorable contrast to time-consuming and elaborate bispecific antibody capture systems (26). The sorting experiments on antigen-specific cells show that the mICAM-1^+ fraction expresses functional markers, greatly expands in culture, and that sorted cells retain their functional properties after in vitro expansion. Thus, the assay should allow the isolation of highly functional $CD8^+$ T lymphocytes for further gene or protein analysis, as well as for adoptive transfer (e.g., in clinical settings), because the procedure and the required reagents accord with GMP standards.

Taken together, our results indicate β_2-integrin activation as a hallmark of immediate T cell functionality, which is detectable at the very early stages of activation. Staining of β_2-integrin activation is therefore a powerful tool for monitoring and isolating functional antigen-specific T cells. Because this method is fast, robust, and versatile, it could also be rapidly implemented for the measurement of T cells in patient samples in a variety of clinical settings, for example, during standard or experimental therapy.

Materials and Methods

Study Subjects and Blood Samples. For the studies in healthy individuals, we selected nine CMV-seropositive, HLA-$A2^+$ subjects with a variable frequency of CMV A2/NLV $multimer^+$ $CD8^+$ T cells (age range 18–30 y). Additionally, those individuals with detectable levels of EBV A2/GLC$^+$ or Flu A2/GIL$^+$ $CD8^+$ T cells were used for the assays involving Flu- and EBV-specific $CD8^+$ T cells. To identify HLA-A2 positivity, we stained with anti–HLA-A2-APC antibody (clone BB7.2; Biolegend).

Another two healthy HLA-$A2^+$ individuals were subcutaneously vaccinated with a single dose of yellow fever vaccine (YF-17D, Stamaril; Aventis Pasteur MSD) and heparinized blood samples were obtained before and after vaccination. Cancer patients were taking part in an experimental phase I/II peptide vaccination study (20). Postvaccination PBMC samples were accordingly selected (after the 11th, 17th, or 16th vaccinations for patient 1, patient 2, and patient 3, respectively), thawed, and stimulated for 12 d in the presence of the relevant peptide and IL-2 before mICAM-1 staining (27). Blood was also obtained from three recently diagnosed, therapy-naive HIV-infected patients (HLA not known). The viral loads were 318, 50,000,000, and 17,100 copies per milliliter, and the CD4 cell counts were 727, 571, and 355 cells/mm^3, respectively. The precise infection time was not known.

All studies were approved by the Ethics Committee of the University of Tübingen, and participants gave written informed consent.

Peptides and pMHC Multimers. For antigen-specific stimulation, we used the following synthetic peptides, representing known immunodominant HLA-A*02-restricted $CD8^+$ epitopes derived from virus or tumor-associated antigens: NLVPMVATV peptide from CMV pp65, amino acids 495–503 (CMV/NLV), GLCTLVAML peptide from EBV BMLF1, amino acids 259–267 (EBV/GLC), GILGFVFTL from FLU M1, amino acids 58–66 (Flu/GIL), LLWNGPMAV peptide from YFV NS4B, amino acids 214–222 (YFV/LLW), and ALFDIESKV peptide from PSMA, aa 711–719 (PSMA/ALF). All peptides were synthesized and dissolved as previously described (28) and were kindly provided by S. Stevanović, University of Tübingen, Tübingen, Germany, except for YFV/LLW (JPT Peptide Technologies). We also used for activation pools of 15-mer peptides overlapping by 11 amino acids and spanning the entire CMV/pp65, HIV/p17, or HIV/Pol proteins. All peptide pools were obtained from JPT, dissolved in DMSO, and aliquoted and kept frozen at −20 °C until further use.

We produced biotinylated pHLA-A*0201 monomers (CMV A2/NLV, EBV A2/GLC, Flu A2/GIL, YFV A2/LLW, and PSMA A2/ALF) in-house by conventional refolding, as previously described (29). We generated fluorescent pHLA-A*0201 multimers by coincubating streptavidin-PE or -APC (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 (29). The final concentration of azide was 0.035%.

Production of Human ICAM-1 Multimers. Soluble fluorescent ICAM-1 monomers or complexes thereof have been previously used to monitor changes in β_2-integrin affinity induced by activation with chemokines (16), activating antibodies (30), or using unspecific stimulation in human T cells (18). For our studies, we generated fluorescent ICAM-1–Fc/anti-Fc multimeric complexes by coincubating 200 μg/mL recombinant human ICAM-1-Fc [produced and purified as previously described (31)] with polyclonal anti-human Fc-FITC $F(ab')_2$ fragments (Jackson ImmunoResearch) at a 1 (ICAM-1–Fc):2 (IgG part of anti-Fc–FITC fragments) or 1:4 molar ratios (*SI Appendix*, Fig. S10*A*), or with anti-human Fc-PE $F(ab')_2$ fragments (Jackson ImmunoResearch) at a 3.4

(ICAM-1–Fc):1 (PE of anti–Fc-PE fragments) or 1.7:1 molar ratios (*SI Appendix*, Fig. S10*B*) at 4 °C for 3 h. We used multimeric ICAM-1 complexes (mICAM-1) either immediately or stored them in aliquots at −20 °C, for no more than 2 wk until use. A control solution replacing ICAM-1–Fc with PBS, thus containing only the fluorescent anti-Fc fragments at the same concentration as in the multimeric complexes was used as a negative control in the titration experiments to assess the unspecific binding (*SI Appendix*, Fig. S10, *Bottom* panels). To establish the optimal mICAM-1working concentration, we stimulated 380 μL of fresh whole blood from a CMV A2/NLV multimer$^+$ donor with NLV peptide for 8 min. In the final 4 min of activation, A2/NLV multimers (0.6 μg/mL) and a decreasing concentration of mICAM-1 (in the range of 25–0.78 μg/mL ICAM-1–Fc) were added between min 4 and min 8. A shorter period of incubation with mICAM-1 (1 or 2 min) yielded a similar staining but we opted for 4 min to stain simultaneously with pMHC multimers. The amount of mICAM-1 that resulted in a maximal percentage of positive cells (>60%) with a low background staining (<0.05%) was chosen for further experiments (6.25 μg/mL ICAM-1–Fc for ICAM-1–Fc/anti–Fc-FITC and 3.13 μg/mL ICAM-1–Fc for ICAM-1–Fc/anti–Fc-PE) (*SI Appendix*, Fig. S10 *A* and *B*, respectively).

Cell Stimulation and mICAM-1 Staining. We used fresh heparinized blood or PBMCs for the assays. We isolated PBMCs by using Biocoll (Biochrom) gradient centrifugation. Cells were washed with T cell medium (TCM): IMDM (Lonza) containing 10% autologous plasma and 1% penicillin/streptomycin (Sigma-Aldrich). PBMCs were either used immediately or frozen in aliquots in freezing solution [heat-inactivated FCS (Biochrom) containing 10% DMSO (Sigma-Aldrich)] and stored in liquid nitrogen until use. Cryopreserved PBMCs were thawed, washed in TCM, and rested overnight at 37 °C before use.

We stimulated whole blood (380 μL per test) for the indicated times at 37 °C in a water bath in 5-mL Falcon tubes (BD Biosciences) with the following peptides: 4 μg/mL CMV/NLV, 4 μg/mL EBV/GLC, 4 μg/mL Flu/GIL, 4 μg/mL YFV/LLW, 2 μg/mL PSMA/ALF, peptide pools: 2 μg/mL CMV/pp65, 2 μg/mL HIV/p17, 2 μg/mL HIV/Pol, or 4 μg/mL SEB (Sigma-Aldrich). For the final 4 min of incubation, we added mICAM-1/FITC or mICAM-1/PE multimers and A2/peptide multimers (0.6 μg/mL) as indicated. Immediately after stimulation, we fixed the samples and lysed erythrocytes with FACS-Lysing solution (BD Biosciences) containing 1 mM Ca^{2+} and 2 mM Mg^{2+} for 5 min, followed by washing with PBS/0.5% albumin/0.1% sodium azide. After centrifugation, we stained the cells for surface markers to identify CD8$^+$ T cells: CD3-BV510, CD8-BV605, CD14-APC-Cy7, and CD19-APC-Cy7 (dump channel) for 15 min at room temperature. All antibodies used were from by Biolegend. A similar protocol was applied for PBMCs, stimulating 2×10^6 cells/mL in TCM (380 μL per test).

mICAM-1 and Intracellular Cytokine Staining. For the assessment of intracellular cytokines and CD107a membrane expression, we stimulated whole blood either for 1 or 2 h with the indicated peptides and concentrations in the presence of 5 μg/mL brefeldin A (Sigma-Aldrich), 5 μg/mL monensin (Sigma-Aldrich), and pretitrated amount of CD107-PE-Cy7. For the final 4 min of incubation, we added mICAM-1 and pMHC multimers as indicated. Immediately after stimulation, we fixed the samples and lysed erythrocytes. The cells were stained for surface markers to identify CD8$^+$ T cells: CD8-APC, CD3-AF700, CD14-APC-Cy7, and CD19-APC-Cy7. Cells were then permeabilized with FACS-Perm2 solution (BD Biosciences) followed by staining for intracellular markers IFN-γ–PerCP–Cy5.5 and TNF-BV605. All antibodies used were purchased from Biolegend.

mICAM-1 and Differentiation Markers, Intracellular GrB, and Perf Staining. For surface markers, GrB, and perforin expression, we stimulated whole blood for 8 min (1 h for the detection of Flu-specific cells) with the indicated antigens. In the final 4 min of incubation we added mICAM-1, and A2/peptide multimers as indicated. Immediately after the stimulation period, we fixed the samples and lysed erythrocytes for 20 min. The cells were stained for surface markers to identify CD8$^+$ T cells: CD3-BV510, CD8-BV605, CD14-APC-Cy7, and CD19-APC-Cy7 for 15 min at room temperature. For assessment of cell differentiation, the cells were also stained for the following markers: CD28-BV421, CD27-APC, and CD45RA-AF700. Alternatively, cells were permeabilized with FACS-Perm2 solution (BD Biosciences), followed by staining with perforin-BV421 and GrB-AF647 antibodies for 15 min (Biolegend).

T Cell Sorting and Expansion. PBMCs were isolated by using BD Vacutainer cell preparation tubes (BD Biosciences). Cells washed in PBS containing 0.5% albumin, 1 mM Ca^{2+}, and 2 mM Mg^{2+} (5×10^6 cells/mL, 400 μL per test) were activated with 4 μg/mL peptide for 16 min (centrifuged prior the activation at $1{,}000 \times g$ for 1 min) followed by 4 additional minutes of staining with ICAM-1 multimers, 4 min of staining with A2/NLV or A2/LLW for the pMHC sorting experiments (Fig. 6 and *SI Appendix*, Fig. S7), and 4 min with CD8-APC (Biolegend) at 37 °C. The sortings for the cell expansion experiments were performed under the same conditions, except that the cells were activated only for 8 min with peptide and not spun down before the activation. Then the cells were diluted 1:1 with PBS, and immediately sorted on a BD FACSJazz (BD Biosciences) under sterile conditions. For cell expansion, isolated mICAM-1$^+$ CD8$^+$ and mICAM-1$^-$ CD8$^+$ cells were incubated with 150,000 irradiated (60 Gy) fresh PBMCs from three donors per well of a 96-well plate in TCM supplemented with 150 U/mL IL-2 (Proleukin; Novartis), 5 ng/mL IL-15 (R&D), and 30 ng/mL anti-CD3 antibody (Miltenyi Biotec), and incubated for 10 d at 37 °C. Medium was changed every third day and the cultures were split when necessary. Anti-CD3 antibody was present only in the initial medium and for the final 3 d of stimulation no cytokines were added. We isolated 4,000–15,000 mICAM-1$^+$ CD8$^+$ or mICAM-1$^-$ CD8$^+$ cells, and they expanded to 2×10^6 in the 10 d of incubation.

In Vitro Restimulation of the Sorted (and Expanded) CD8$^+$ T Cells. For mICAM-1$^+$ CD8$^+$ and mICAM-1$^-$ CD8$^+$ sorted cells, we first stained with A2/NLV multimers and then restimulated the freshly isolated or expanded fractions with the CMV/NLV peptides (4 μg/mL) for 5 h in the presence of 10 μg/mL brefeldin A (Sigma-Aldrich), 1:1,500 diluted GolgiStop (BD Biosciences), and CD107-PE-Cy7 (Biolegend). For mICAM-1$^+$ pMHC$^+$ and mICAM-1$^-$ pMHC$^+$ sorted cells, the cells were treated as above, except that no CMV/NLV or YFV/LLW peptides or multimers were added. For antibody staining and analysis of intracellular IFN-γ and TNF, the cells were treated as described above.

Flow Cytometry and Data Analysis. All antibodies were used at pretested optimal concentrations. We acquired the data on a LSRFortessa (BD Biosciences) and analyzed it using FACS DiVa v8.0. We collected at least 50,000 CD8$^+$ events for the antigen-specific assays. Results are presented as percentage of cells within the parent populations or as MFI. Statistical analyses were based on paired two-side *t* tests and Pearson correlation analysis using IBM SPSS Statistics 22.

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