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

Dimitrov, Stoyan; Gouttefangeas, Cécile; Besedovsky, Luciana; Jensen, Anja T R; Chandran, P Anoop; Rusch, Elisa; Businger, Ramona; Schindler, Michael; Lange, Tanja; Born, Jan; Rammensee, Hans-Georg

Published in:
Proceedings of the National Academy of Sciences of the United States of America

DOI:
10.1073/pnas.1720714115

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY-NC-ND

Citation for published version (APA):
Dimitrov, S., Gouttefangeas, C., Besedovsky, L., Jensen, A. T. R., Chandran, P. A., Rusch, E., ... Rammensee, H-G. (2018). Activated integrins identify functional antigen-specific CD8 T cells within minutes after antigen stimulation. Proceedings of the National Academy of Sciences of the United States of America, 115(24), E5536-E5545. https://doi.org/10.1073/pnas.1720714115

Download date: 07. Mar. 2020
Activated integrins identify functional antigen-specific CD8+ T cells within minutes after antigen stimulation

Stoyan Dimitrov1,2,3, Cécile Gouttefangeas1,4, Luciana Besedovsky5, Anja T. R. Jensen6, P. Anoop Chandran7, Elisa Rusch8, Ramona Businger1, Michael Schindler1, Tanja Lange6,2, Jan Born9,2, and Hans-Georg Rammensee4,6,1,2

1Institute of Medical Psychology and Behavioral Neurobiology, University of Tübingen, 72076 Tübingen, Germany; 2German Center for Diabetes Research (DZD), 72076 Tübingen, Germany; 3Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich, University of Tübingen, 72076 Tübingen, Germany; 4Department of Immunology, Institute of Cell Biology, University of Tübingen, 72076 Tübingen, Germany; 5Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark; 6Institute of Medical Virology and Epidemiology of Viral Diseases, University Hospital Tübingen, 72076 Tübingen, Germany; 7Clinic for Rheumatology and Clinical Immunology, University of Lübeck, 23562 Lübeck, Germany; and 8Partner Site Tübingen, German Cancer Consortium (DKTK), 72076 Tübingen, Germany

Edited by Rafi Ahmed, Emory University, Atlanta, GA, and approved May 4, 2018 (received for review November 28, 2017)

Immediate β2-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 β2-integrins. The method is compatible with surface and intracellular staining; it is applicable for monitoring 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 β2-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

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 β2-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 β2-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 (Fab′)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...
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 β₂-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 β₂-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. 1A and representative examples in Fig. 1B). 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 β₂-integrin activation: SEB, NLVPVMVATV 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), β₂-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 β₂-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.

Dimitrov et al.
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 0.8–3.8% 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. 1C. 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. 1D and 2C). 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. 2A 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-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-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. 2C). Hence, our results show that 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 CD107α in T cells stimulated with SEB or viral peptides and costained with mICAM-1. Because β2-integrin activation displays a different kinetic than cytokine production or plasma membrane expression of CD107α, we selected stimulation times of 1 and 2 h, after which both a strong mICAM-1 staining and adequate amounts of cytokines or CD107α can be simultaneously detected. We observed that irrespective of antigens used: (i) expression of the functional markers was predominantly confined to the mICAM-1+ cell fraction; and (ii) mainly cells with the strongest mICAM-1 binding costained for the functional markers CD107α, 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-1+ after 1 h (94.7%, as shown in Fig. 3B) or 2 h (85.2%, as shown in SI Appendix, Fig. S2D) of stimulation with CMV/NLV. Conversely, the majority of CMV/NLV-stimulated mICAM-1+ T cells, and in particular the mICAM-1hi population, expressed CD107α, IFN-γ, and/or TNF after 1 h (Fig. 3A and B) or 2 h of stimulation (SI Appendix, Fig. S2C and D). When we assessed CMV-specific cells after stimulation with p65-overlapping peptides, again most cells expressing at least one of the three functional markers were mICAM-1+ (93.1% after 1 h and 83.8% after 2 h of stimulation) (SI Appendix, Fig. S2B 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-1hi and mICAM-1lo pMHC+ cells in the same subjects. We examined every combination of the CD107α, IFN-γ, and TNF markers after 1 h of stimulation with viral antigens among the mICAM-1hi and mICAM-1lo fractions of A2/NLV+, A2/GLC+, or A2/GIL+ cells. A representative example for A2/NLV+ cells is shown in Fig. 3C. Among A2/NLV+ cells, we observed that more than 60% of mICAM-1hi cells expressed a combination of at least two functional markers after 1 h of activation, whereas most mICAM-1lo cells were negative for any functional marker (>80%) (Fig. 3D 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 β2-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+ T cells. WB cells from the same donors as in Fig. 1A 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-1+ cells at the indicated time point. (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.
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. 4A and SI Appendix, Fig. S4A). 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. 4B–D and SI Appendix, Fig. S4 B–D). For the cytotoxic markers, we found that mICAM-1+ cells

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.
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. 5A).

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. 5B). Staining of the sorted mICAM-1+ cells with the A2/NLV multimers revealed that they were mostly (87%) CMV-specific (Fig. 5C). 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. 5D).

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.](https://www.pnas.org/cgi/doi/10.1073/pnas.1720714115)
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. 5E). 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. 5F). 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. 6C 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 was observed. The results are summarized in Fig. 6.

Fig. 6. Functional characterization of sorted CMV A2/NLV− or YFV A2/LLW-specific CD8+ T cells according to β2-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 β2-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. 7A); mICAM-1+ A2/LLW+ cells at week 2 postvaccination had an early-differentiated phenotype (CD27+ CD28–integrins) 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, ALF-DIESKV 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. 7B).

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. 7C) (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-α 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-α 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 cytokine 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.

**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 β2-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+ T 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 effecter 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 β2-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/Po 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 found 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) were 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 β2-integrins reveals highly effective, functional antigen-specific T cells. Similarly, mICAM-1+ T cells showed enriched coexpression of the two cytotoxic molecules GrB and Perp, with the phenotype of mICAM-1+ T cells, according to surface expression of CD27, CD28, and CD45RA, ranging 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 β2-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 bifpecific 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 β2-integrin activation as a hallmark of immediate T cell functionality, which is detectable at the very early stages of activation. Staining of β2-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 CMV-seropositive, HLA-A2* subjects (YFV19, Standard; A2/PSMA and 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 samples were also obtained by coincubation of PBMCs recently diagnosed with 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 cell/μm3, 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: NLVP/MAVT 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), LLNGPM/GAV peptide from YFV N54B, amino acids 214–222 (YFV/LIL), and ALFDIESKV peptide from YFV NS4B, amino acids 711–719 (YFV/A2/LLW). All peptides were synthesized and desalted as previously described (28) and were kindly provided by S. Stevanovic, University of Tübingen, Tübingen, Germany, except for YFV/LIL (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 DM/SO, and aliquoted and kept frozen at −20 °C until further use.

We produced biotinylated phLA-A*0201 multimers (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 (Thermo-fisher) 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 multimers or complexes thereof have been previously used to monitor changes in β2-integrin affinity induced by interaction 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 coinubating 200 μg/mL recombinant human ICAM-1–Fc (produced and purified as previously described (31)) with polyconal anti-human Fc/FITC (Fab′)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. S10A), or with anti-human Fc–PE Fab′)2 fragments (Jackson ImmunoResearch) at a 3.4

Dimitrov et al.
(ICAM-1–Fc)1 (PE of anti-Fc–PE fragments) or 1:7 molar ratios (SI Appendix, Fig. S10B) at 4 °C for 3 h. We used multimeric ICAM-1 complexes (mICAM-1) at 10 μg/mL for 3 or 20 min at 4 °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-1-working concentration, we stimulated 380 μL of fresh whole blood from a CMV A2/NLV multimer2 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 25–0.078 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 for the assays. We isolated PBMCs by using Biocoll (Biochrom) gradient centrifugation. PBMCs were either used immediately or frozen in aliquots without requiring knowledge of epitope specificities.

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 (0.6 μg/mL) for the pMHC staining, 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 (Mitenyi 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 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.

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.

ACKNOWLEDGMENTS. We thank S. Stevanovic for providing synthetic peptides; S. Heidu, J. Lehnholz, K. Witte, and E.-M. Schmidt for excellent technical assistance and advice; M. Szczechon, J. C. P. Santiago, M. Esen, M. Buhl, and G. Marasca for sampling blood from the subjects and patients; all subjects and patients who participated in this study; and M. Hallschmid and R. Littwin for critical reading and proofreading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft Grants SFB 654 (to T.L. and H.-G.R.) and SFB 685 (to C.G. and H.-G.R.); grants from the German Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD; 01GI0925) (to S.D. and J.B.); and European Research Council Grant AdG 339894, MUTAEDITING (to H.-G.R.).
16. Chan JR, Hyduk SJ, Cybulsky MI (2003) Detecting rapid and transient upregulation of leukocyte integrin affinity induced by chemokines and chemoattractants. *J Immunol Methods* 273:43–52.

17. Tominaga Y, et al. (1998) Affinity and kinetic analysis of the molecular interaction of ICAM-1 and leukocyte function-associated antigen-1. *J Immunol* 161:4016–4022.

18. Konstandin MH, et al. (2007) A sensitive assay for the quantification of integrin-mediated adhesiveness of human stem cells and leukocyte subpopulations in whole blood. *J Immunol Methods* 327:30–39.

19. Appay V, van Lier RA, Sallusto F, Roederer M (2008) Phenotype and function of human T lymphocyte subsets: Consensus and issues. *Cytometry A* 73:975–983.

20. Feyerabend S, et al. (2009) Novel multi-peptide vaccination in Hla-A2+ hormone sensitive patients with biochemical relapse of prostate cancer. *Prostate* 69:917–927.

21. Streeck H, Nixon DF (2010) T cell immunity in acute HIV-1 infection. *J Infect Dis* 202(Suppl 2):S302–S308.

22. McNeil LK, et al. (2013) A harmonized approach to intracellular cytokine staining gating: Results from an international multiconsortia proficiency panel conducted by the Cancer Immunotherapy Consortium (CIC/Cori). *Cytometry A* 83:728–738.

23. Welters MJ, et al. (2012) Harmonization of the intracellular cytokine staining assay. *Cancer Immunol Immunother* 61:967–978.

24. Davis MM, Altman JD, Newell EW (2011) Interrogating the repertoire: Broadening the scope of peptide-MHC multimer analysis. *Nat Rev Immunol* 11:551–558.

25. Mohan JF, Unanue ER (2012) Unconventional recognition of peptides by T cells and the implications for autoimmunity. *Nat Rev Immunol* 12:721–728.

26. Brosterhus H, et al. (1999) Enrichment and detection of live antigen-specific CD4+ and CD8+ T cells based on cytokine secretion. *Eur J Immunol* 29:4053–4059.

27. Widenmeyer M, et al. (2012) Promiscuous survivin peptide induces robust CD4+ T-cell responses in the majority of vaccinated cancer patients. *Int J Cancer* 131:140–149.

28. Peper JK, Stevanovic S (2015) A combined approach of human leukocyte antigen ligandomics and immunogenicity analysis to improve peptide-based cancer immunotherapy. *Cancer Immunol Immunother* 64:1295–1303.

29. Hadrup SR, et al. (2015) Cryopreservation of MHC multimers: Recommendations for quality assurance in detection of antigen specific T cells. *Cytometry A* 87:37–48.

30. Tang RH, Trng E, Law SK, Tan SM (2005) Epitope mapping of monoclonal antibody to integrin alphaL beta2 hybrid domain suggests different requirements of affinity states for intercellular adhesion molecules (ICAM)-1 and ICAM-3 binding. *J Biol Chem* 280:29208–29216.

31. Bengtsson A, et al. (2013) Transfected HEK293 cells expressing functional recombinant intercellular adhesion molecule 1 (ICAM-1)—A receptor associated with severe Plasmodium falciparum malaria. *PloS One* 8:e69999.