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Intracellular CD154 Expression Reflects Antigen-specific CD8$^+$ T Cells but Shows Less Sensitivity than Intracellular Cytokine and MHC Tetramer Staining

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Abstract A recent report showed that analysis of CD154 expression in the presence of the secretion inhibitor Brefeldin A (Bref A) could be used to assess the entire repertoire of antigen-specific CD4$^+$ T helper cells. However, the capacity of intracellular CD154 expression to identify antigen-specific CD8$^+$ T cells has yet to be investigated. In this study, we compared the ability of intracellular CD154 expression to assess antigen-specific CD8$^+$ T cells with that of accepted standard assays, namely intracellular cytokine IFN-γ staining (ICS) and MHC class I tetramer staining. The detection of intracellular CD154 molecules in the presence of Bref A reflected the kinetic trend of antigen-specific CD8$^+$ T cell number, but unfortunately showed less sensitivity than ICS and tetramer staining. However, ICS levels peaked and saturated 8 h after antigenic stimulation in the presence of Bref A and then declined, whereas intracellular CD154 expression peaked by 8 h and maintained the saturated level up to 24 h post-stimulation. Moreover, intracellular CD154 expression in antigen-specific CD8$^+$ T cells developed in the absence of CD4$^+$ T cells changed little, whereas the number of IFN-γ-producing CD8$^+$ T cells decreased abruptly. These results suggest that intracellular CD154 could aid the assessment of antigen-specific CD8$^+$ T cells, but does not have as much ability to identify heterogeneous CD4$^+$ T helper cells. Therefore, the combined analytical techniques of ICS and tetramer staining together with intracellular CD154 assays may be able to provide useful information on the accurate phenotype and functionality of antigen-specific CD8$^+$ T cells.

Keywords: Intracellular CD154, intracellular cytokine staining, MHC class I tetramer, antigen-specific CD8$^+$ T cells

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CD8$^+$ cytotoxic T lymphocytes (CTL) play an important role in host defense against virus-infected cells or cells undergoing malignant growth. Target cell lysis mediated by effector CD8$^+$ T cells occurs *via* perforin- or Fas-dependent pathways [7, 34] and is initiated by T cell receptor (TCR) engagement of a specific peptide (epitope) that is derived from the virus or tumor and bound to the cell-surface class I major histocompatibility (MHC) molecule [1, 8, 21, 22, 32]. CD8$^+$ T cells also elicit effector function by producing antiviral cytokines such as IFN-γ and TNF-α [2, 19, 30, 40]. Therefore, effective CD8$^+$ T cell-mediated immunity is associated with long-term protection against chronic or subsequent exposure to the virus or tumor through stable induction of antigen-specific CD8$^+$ T cell memory [5, 14, 29].

One of the key issues in studies involving clinical trials for therapeutic and prophylactic strategies against viral infection and tumor is the ability to monitor critical parameters of the ongoing *in vivo* antigen-specific CD8$^+$ T cell immunity that is elicited by a vaccine [12, 20, 23, 37]. Traditionally, monitoring of antigen-specific CD8$^+$ T cell responses in an individual receiving vaccine has consisted of *in vitro* cell-culture-based assays such as proliferation, cytokine secretion (ELISA), and *ex vivo* CTL assay [12, 37, 38]. The advent of peptide-MHC multimeric staining or methods that evaluate antigen-specific CD8$^+$ T cells has led to major breakthroughs [1, 15, 24, 26]. The only limitations of such tetramer technology at this time are the somewhat extensive biological process associated with the synthesis of the MHC molecule and the inability to associate certain peptides within the MHC structure. From an immune response point of view, the enumeration of antigen-specific CD8 T cells by tetramer analysis alone is unable to provide any information on the functional activity associated with CD8$^+$ T cells [4, 30, 35]. However,

when used in combination with intracellular staining protocols and rapid stimulation of peripheral blood cells with minimal manipulation, tetramer analysis allows for the potential enumeration of cytokine-secreting $CD8^+$ T cells within the antigen-specific $CD8^+$ T cell population [4, 30, 35]. The intracellular cytokine staining (ICS) protocols, however, face the disadvantages that the detection of intracellular cytokine secretion by flow cytometry is considered suboptimal compared with both that in cells stimulated in culture and cytokine secretion monitored over a longer period of time. Moreover, the required fixation precludes sensitive RNA-based assays and is lethal to cells.

A recent report showed that CD154 (also known as CD40 ligand) expression could be used to assess the entire repertoire of antigen-specific $CD4^+$ T helper cells that have diverse cytokine profiles [11, 16]. CD154, a type II membrane protein of the TNF superfamily, is one of the best-characterized costimulatory molecules [36]. To date, the Th-cell activation marker CD154 has not been used to directly assay for antigen-specific Th cells because it is only transiently expressed in the course of Th-cell activation [3, 17, 39]. However, Frentsch *et al.* [16] showed that expression of CD154 in $CD4^+$ T helper cells can be stabilized intracellularly with the secretion inhibitor Brefeldin A (Bref A) and extracellularly with CD40-specific blocking monoclonal antibody. In another study, Chattopadhyay *et al.* [11] found that stimulated cells expressing TNF-α, IL-2, or IFN-γ were predominantly $CD154^+$. In addition, some cells that do not express cytokines have been reported to express CD154, suggesting that CD154 marks cells with other effector functions [11]. However, the ability of intracellular CD154 expression to identify antigen-specific $CD8^+$ T cells has yet to be investigated. Therefore, we compared intracellular CD154 expression with accepted standard assays, namely ICS and MHC class I tetramer staining, for its ability to assess functional antigen-specific $CD8^+$ T cells after short-term *in vitro* stimulation with a defined antigen.

Materials and Methods

Mice

C57BL/6 mice were purchased from Damul Sci. Inc. (Daejeon, Korea), housed in our pathogen-free facility, and used at 6–9 weeks of age. OT-I mice, which are transgenic for a $V_{\alpha 2}/V_{\beta 5}$ TCR that recognizes the H-$2K^b$-restricted 257-264 (SIINFEKL) peptide of chicken ovalbumin (OVA), and B6.PL-$Thy1^a$/Cy(B6.PL) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). The investigators adhere to the guidelines set by the Committee on the Care of Laboratory Animal Resources, Chonbuk National University. The animal facility of the Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Viruses

Recombinant vaccinia viruses expressing SIINFEKL ($VVOVA_{257\text{-}264}$) and the immunodominant SSIEFARL ($gB_{498\text{-}505}$) peptide of herpes simplex virus ($VVgB_{498\text{-}505}$) were kindly provided by Dr. B. Moss (NIH, Bethesda, WA, U.S.A.) and propagated in African green monkey kidney CV-1 (ATCC CCL70) cell lines, using DMEM supplemented with 2.5% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml). The cultures were incubated at 37°C in a humidified CO_2 incubator. The virus stocks were concentrated, titrated, and stored in aliquots at –80°C until needed.

Antibodies and Peptides

The following monoclonal antibodies (MAb) were obtained from eBioscience (San Diego, CA, U.S.A.) and used for FACS analysis and other experiments: fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (clone Ly-2), anti-CD3 (clone 145-2C11), phycoerythrin (PE)-conjugated anti-IFN-g (clone XMG 1.2), anti-CD154 (clone MR1), and phycoerythrin-Cy5 (PE-Cy5)-conjugated anti-CD90.2 (clone 53-2.1). PE-conjugated OVA-specific MHC I tetramer, K^b/OVAp-Tet, as described elsewhere [10], was kindly provided by Dr. Jun Chang (Ehwa Univ., Seoul, Korea). The SIINFEKL ($OVA_{257\text{-}264}$) and SSIEFARL ($gB_{498\text{-}505}$) peptides were chemically synthesized at Peptron Inc. (Daejeon, Korea).

In Vitro Stimulation of Antigen-specific $CD8^+$ T Cells

For *in vitro* stimulation, single-cell suspensions of splenocytes isolated from either C57BL/6 or B.PL-$Thy1^a$/Cy mice were depleted of red blood cells by NH_4Cl lysis. At a density of 5×10^6 cells/ml, the cells were then incubated with either SIINFEKL or SSIEFARL peptide (2 μg/ml) in RPMI media supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C for the indicated time (2, 4, 6, 8, 12, 24, and 48 h). After the incubation, the cells were washed twice with PBS containing 1% BSA and 0.05% NaN_3 to remove any unbound peptide. The number of intracellular CD154, INF-γ, and K^b/OVAp tetramer-positive $CD8^+$ T cells was determined by flow cytometry.

Adoptive Transfer and Immunization

Kinetic analysis of intracellular CD154, IFN-γ, and K^b/OVAp tetramer-positive $CD8^+$ T cells after immunization was assessed by adoptive transfer of OT-I $CD8^+$ T cells (Thy1.2) into B6.PL-$Thy1^a$/Cy (Thy1.1) mice. Single-cell suspensions of splenocytes isolated from OT-I mice were prepared, and 2×10^6 SIINFEKL-specific $CD8^+$ T cells were injected intravenously into B6.PL-$Thy1^a$/Cy mice. The day after adoptive transfer, the recipient mice were immunized intramuscularly with $VVOVA_{257\text{-}264}$ (1×10^6 pfu/mouse). The number of intracellular CD154, IFN-γ, and K^b/OVAp

tetramer-positive CD8$^+$ T cells was subsequently determined by flow cytometry on the indicated days post-immunization.

Depletion of CD4$^+$ T Cells

To explore the expression of intracellular CD154 and IFN-γ in defective CD8$^+$ T cells, antigen-specific CD8$^+$ T cells that developed in the absence of CD4 T cell help were used. *In vivo* depletion of CD4$^+$ T cells was accomplished by treatment with the anti-CD4 MAb, GK1.5. The recipients, B6.PL-Thy1^a/Cy mice, were immunized with VVOVA$_{257\text{-}264}$ (day 0) and were given intraperitoneal injections of purified MAb (300 μg/mouse) two days before, at the time of (day 0), and two days after immunization. Purified rat IgG was used as a control treatment in all cases. The efficacy of GK1.5 MAb treatment was determined by comparing flow cytometry before immunization and at the completion of the experiment using a MAb that does not cross-compete with GK1.5 for binding (RM4-5; BD Pharmingen, San Diego, CA, U.S.A.); GK1.5 MAb treatment consistently resulted in >98% reduction in the number of splenic CD4$^+$ T cells.

Intracellular CD154 and IFN-γ Staining

We used intracellular CD154 and IFN-γ staining to identify antigen-specific CD8$^+$ T cells, as previously described [11, 16]. In brief, 10^6 freshly explanted splenocytes per well were stimulated with the indicated peptide (SIINFEKL or SSIEFARL) in U-bottom 96-well plates. Bref A (2 μg/ml) was added for the duration of the culture period to facilitate intracellular CD154/IFN-γ accumulation. After the stimulation period, cells were washed twice with PBS containing 1% BSA, 0.05% NaN_3, and 2 μg/ml Bref A. Cells were subsequently incubated with FITC-conjugated anti-CD8 for surface staining, followed by fixation with PBS containing 10% formaldehyde. The surface-stained cells were then permeabilized with PBS containing 0.5% saponin at room temperature for 30 min. Again, the cells were washed and resuspended in PBS containing 1% BSA, 0.05% NaN_3, and 0.5% saponin. The cells were stained intracellularly by incubation with PE-conjugated anti-CD154 and anti-IFN-γ for 30 min at room temperature. After several washes, the intracellular CD154 and IFN-γ molecules were determined by flow cytometry.

MHC Class I Tetramer Staining

CD8$^+$ T cells specific for SIINFEKL (OVA$_{257\text{-}264}$) were also determined by MHC class I tetramer (K^b/OVAp-Tet) staining. The single-cell suspension of splenocytes isolated from the recipients was washed twice and suspended in PBS containing 1% BSA and 0.05% NaN_3 (FACS buffer). The cells were divided into aliquots of 3×10^6 cells/well in a 96-well round-bottom plate. Aliquots (2 μl) of PE-conjugated K^b/OVAp-Tet and FITC-conjugated anti-CD8 MAb were then added to the cells. The plates were vortexed and incubated at room temperature for 30 min in the dark. In some cases, the cells were also stained with PE-Cy5-conjugated anti-CD90.2 (Thy1.2) to distinguish endogenous cells from exogenous cells. After two washes with buffer, the cells were resuspended in PBS containing 10% formaldehyde for fixation and examined by flow cytometry.

Flow Cytometric Analysis

The absolute count of CD8$^+$ T cells specific for SIINFEKL (OVA$_{257\text{-}264}$) or SSIEFARL (gB$_{498\text{-}505}$) was determined by three-color flow cytometric analysis of cells positive for intracellular CD154, IFN-γ, and K^b/OVAp-Tet. After staining, cells were washed in FACS buffer once, adjusted to 500 μl in PBS, and stored at 4°C until analysis. Labeled cells were analyzed in real time using a FACSCalibur equipped with the CellQuest program (Becton-Dickinson, Mountain View, CA, U.S.A.) and WinMDI 2.8 software.

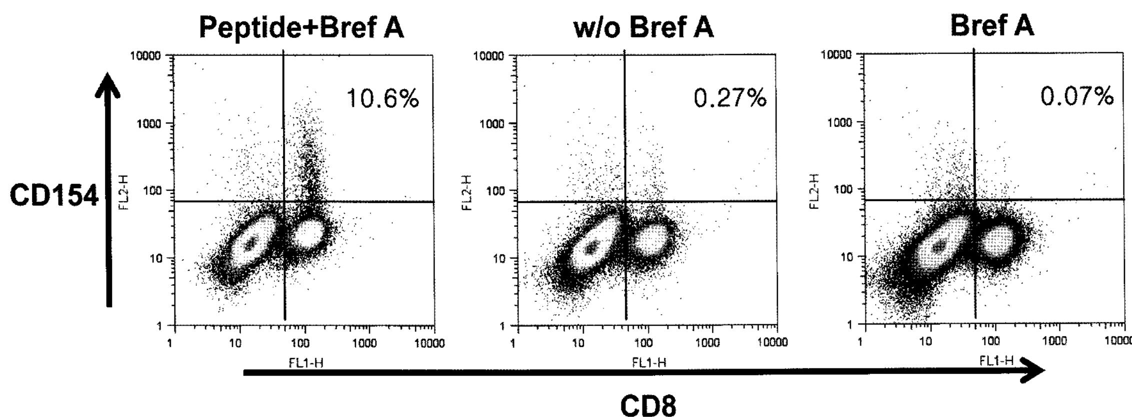

Fig. 1. Detection of intracellular CD154 expression in CD8$^+$ T cells stimulated in the presence of Bref A.
Single-cell suspension of splenocytes obtained from OT-I mice was stimulated with OVA$_{257\text{-}264}$ (SIINFEKL) peptide in the presence or absence of Bref A (2 μg/ml) for 6–8 h. Following stimulation, intracellular CD154 molecules were detected by intracytometric assessment.

RESULTS

Detection of Intracellular CD154 Molecules in Stimulated CD8⁺ T Cells

Prior to testing the ability of CD154 expression to identify antigen-specific $CD8^+$ T cells, the surface expression of CD154, which is degraded after interaction with its ligand CD40 on antigen-presenting cells or B cells, must be circumvented in order to detect intracellular CD154 expression in stimulated $CD8^+$ T cells [39]. Thus, single-cell suspensions of splenocytes obtained from OT-I mice were stimulated with SIINFEKL peptide (2 μg/ml) in the presence of Bref A to block the transport of proteins to the cell surface and inhibit surface CD154 expression [25]. As shown in Fig. 1, CD154 was detected intracellularly in the presence of Bref A in cultures, whereas the staining intensity of intracellular CD154 was reduced in the absence of Bref A. Moreover, nonspecific expression of intracellular CD154 molecules was not observed, as shown in populations stimulated without the cognate peptide.

Comparison of Intracellular CD154 and IFN-γ Expression in CD8⁺ T Cells Stimulated with Cognate Peptides

To compare intracellular CD154 expression with IFN-γ expression from peptide-stimulated $CD8^+$ T cells, intracellular CD154 and IFN-γ expression levels in $CD8^+$ T cells were assessed at the indicated time (2, 4, 6, 8, 12, and 24 h) after stimulation of splenocytes obtained from C57BL/6 mice immunized with either $VVOVA_{257\text{-}264}$ or $VVgB_{498\text{-}505}$. The intracellular CD154 expression peaked 8 h after stimulation (Fig. 2A), and both antigenic peptides followed a similar pattern. Around 0.6% of total splenocytes isolated from C57BL/6 mice immunized with either $VVOVA_{257\text{-}264}$ or $VVgB_{498\text{-}505}$ were intracellular CD154-positive $CD8^+$ T cells by peptide stimulation at 8 h (Figs. 2B and 2C). This saturated percent of intracellular CD154-positive $CD8^+$ T cells was maintained up to 24 h post-stimulation, indicating that intracellular CD154-positive $CD8^+$ T cells could be detected at the 8-h time point after stimulation. Therefore, we used this stimulation time point (8 h) to identify

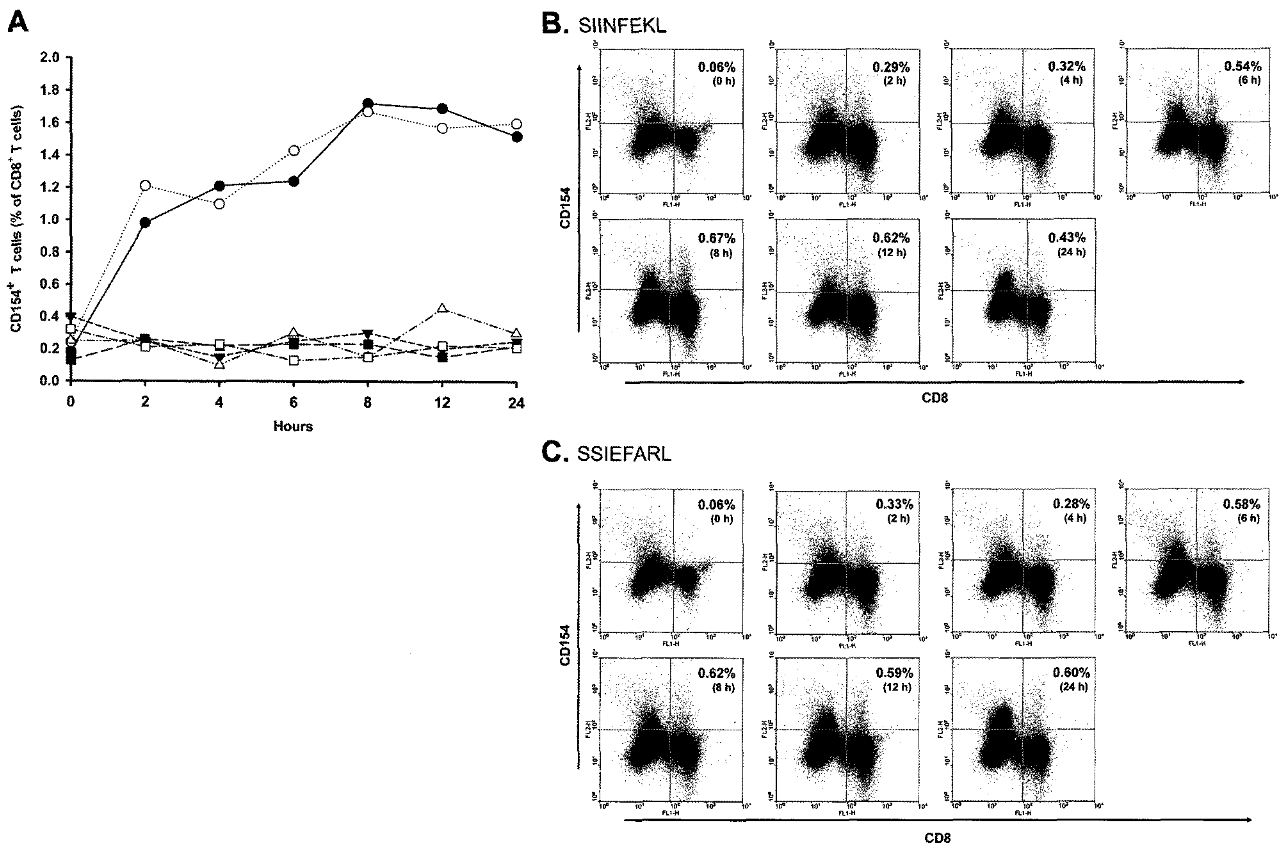

Fig. 2. Kinetics of intracellular CD154 expression in $CD8^+$ T cells stimulated with cognate peptides.

Single-cell suspension of splenocytes obtained from C57BL/6 mice immunized with either $VVOVA_{257\text{-}264}$ or $VVgB_{498\text{-}505}$ (10^6 pfu/mouse) was stimulated with SIINFEKL and SSIEFARL peptides (2 μg/ml) in the presence of Bref A. **A.** Kinetic frequency of intracellular $CD154^+$ T cells in stimulated $CD8^+$ T cell population at different time points after stimulation. ●, $VVOVA_{257\text{-}264}$ immunized and SIINFEKL stimulation; ○, $VVgB_{498\text{-}505}$ immunized and SSIEFARL stimulation; ▼, nonimmunized and SIINFEKL stimulation; △, nonimmunized and SSIEFARL stimulation; ■ , $VVOVA_{257\text{-}264}$ immunized and SSIEFARL stimulation; □, $VVgB_{498\text{-}505}$ immunized and SIINFEKL stimulation. **B.** Time course of intracellular CD154 staining for $CD8^+$ T cells stimulated with SIINFEKL peptide. **C.** Time course of intracellular CD154 staining for $CD8^+$ T cells stimulated with SSIEFARL peptide. The percent values in the dot-plots represent the proportion of total splenocytes stimulated with cognate peptides. Data are one representative of three experiments.

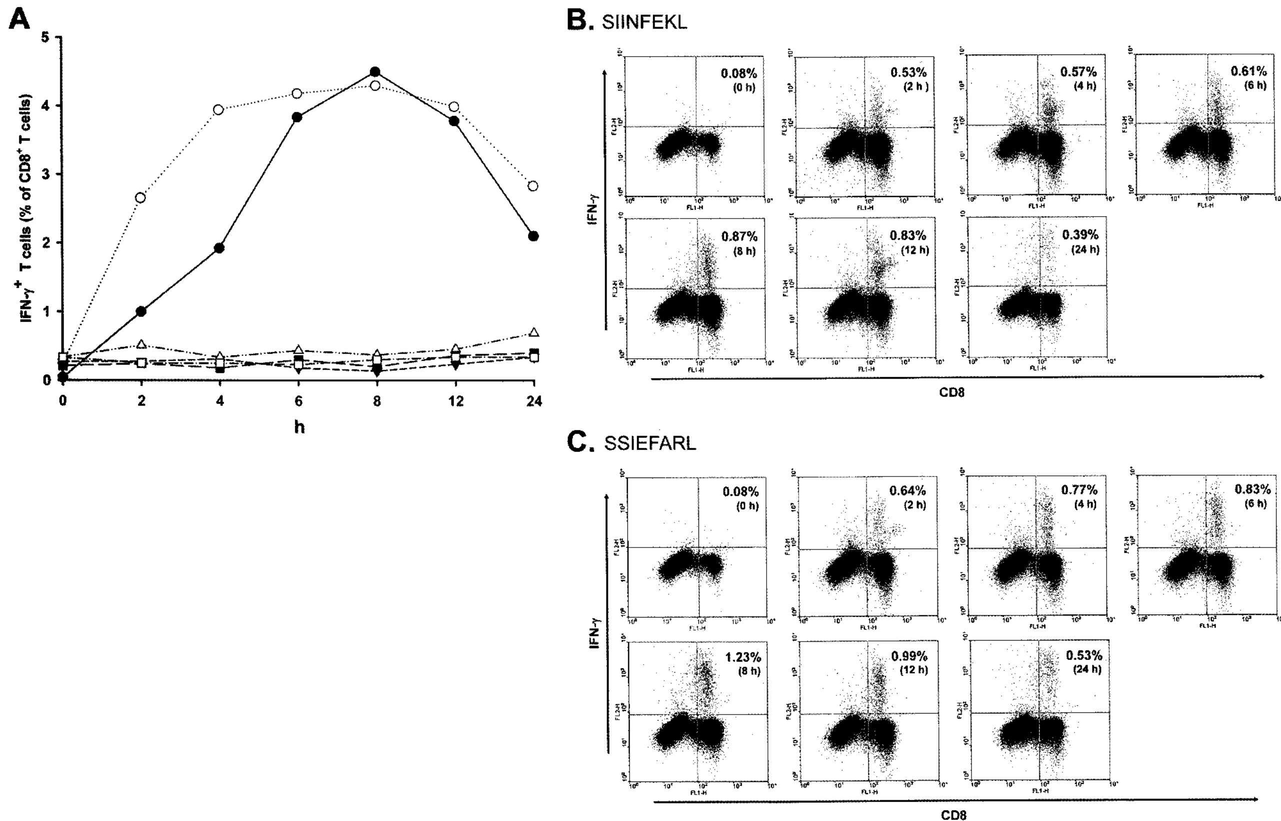

Fig. 3. Kinetics of intracellular cytokine IFN-γ expression in $CD8^+$ T cells stimulated with cognate peptides.
Single-cell suspension of splenocytes obtained from C57BL/6 mice immunized with either $VVOVA_{257\text{-}264}$ or $VVgB_{498\text{-}505}$ (10^6 pfu/mouse) was stimulated with SIINFEKL and SSIEFARL peptides (2 μg/ml) in the presence of Bref A. **A.** Kinetic frequency of intracellular IFN-γ^+ T cells in stimulated $CD8^+$ T cells at different time points after stimulation. ●, $VVOVA_{257\text{-}264}$ immunized and SIINFEKL stimulation; ○, $VVgB_{498\text{-}505}$ immunized and SSIEFARL stimulation; ▼, nonimmunized and SIINFEKL stimulation; △, nonimmunized and SSIEFARL stimulation; ■, $VVOVA_{257\text{-}264}$ immunized and SSIEFARL stimulation; □, $VVgB_{498\text{-}505}$ immunized and SIINFEKL stimulation. **B.** Time course of intracellular cytokine IFN-γ staining for $CD8^+$ T cells stimulated with SIINFEKL peptide. **C.** Time course of intracellular cytokine IFN-γ staining for $CD8^+$ T cells stimulated with SSIEFARL peptide. The percent values in the dot-plots represent the proportion of total splenocytes stimulated with cognate peptides. Data are one representative of three experiments.

antigen-specific $CD8^+$ T cells positive for intracellular CD154 molecules in the following experiments.

When we assessed intracellular IFN-γ expression in $CD8^+$ T cells stimulated with antigenic peptides, this expression by $CD8^+$ T cells in the presence of Bref A showed a different pattern from that of intracellular CD154 expression (Fig. 3A). Intracellular IFN-γ-positive $CD8^+$ T cells detected by flow cytometry plateaued 6–8 h after stimulation. However, contrary to CD154 expression, this saturated level of $CD8^+$ T cells expressing intracellular IFN-γ began to decline at later time points (Fig. 3A). Moreover, the percent of intracellular IFN-γ-positive cells in $CD8^+$ T cells was about 2-fold higher than that of intracellular CD154-positive cells. Thus, around 0.8%–1.2% of total splenocytes obtained from animals immunized with $VVOVA_{257\text{-}264}$ and $VVgB_{498\text{-}505}$ were intracellular IFN-γ-positive $CD8^+$ T cells 8 h after stimulation, but the number of intracellular IFN-γ^+CD8^+ T cells declined to around 0.3%–0.5% at 24 h after stimulation (Figs. 3B and 3C). Therefore, intracellular IFN-γ expression in $CD8^+$ T cells is exhausted by prolonged stimulation, suggesting that there is a narrow window for the optimal stimulation time point for intracellular IFN-γ staining to identify antigen-specific $CD8^+$ T cells.

Intracellular CD154, IFN-γ, and MHC Class I Tetramer Staining to Identify Antigen-specific $CD8^+$ T Cells Adoptively Transferred into Recipients

Next, we aimed to compare the suitability of intracellular CD154, IFN-γ, and MHC class I tetramer staining to identify antigen-specific $CD8^+$ T cells. To test this, we used a model with a high frequency of antigen-specific $CD8^+$ T cells, *i.e.*, adoptive transfer of $CD8^+$ T cells isolated from transgenic mice. SIINFEKL-specific $CD8^+$ T cells obtained from OT-I mice (CD90.2) were adoptively transferred intravenously into B6.PL-Thy1a/Cy mice (CD90.1), and the recipients were subsequently immunized with $VVOVA_{257\text{-}264}$. The $CD8^+$ T cells specific for SIINFEKL peptide were kinetically identified by staining for intracellular CD154, IFN-γ, and MHC class I tetramer, K^b/OVAp-Tet,

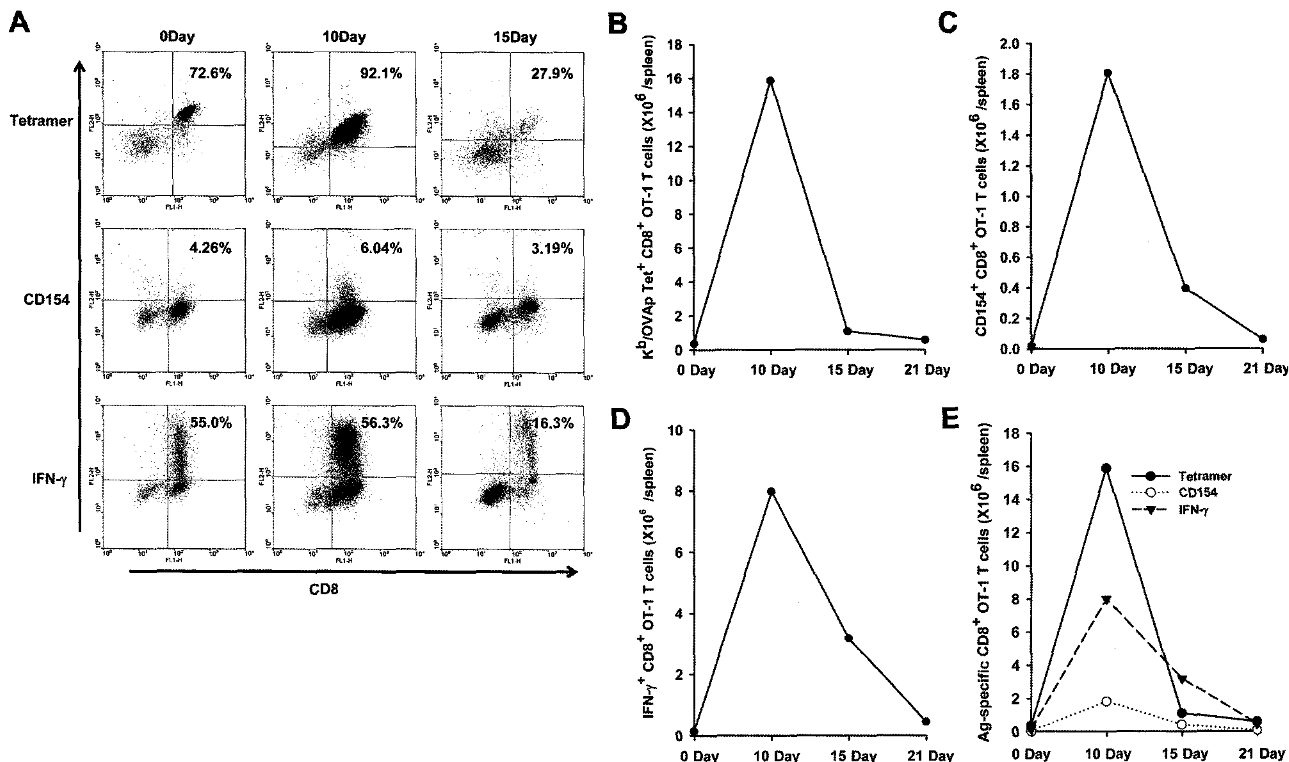

Fig. 4. Identification of antigen-specific CD8$^+$ T cells adoptively transferred into recipients by intracellular CD154, IFN-γ, and MHC class I tetramer staining.

The SIINFEKL-specific CD8$^+$ T cells obtained from OT-I mice (CD90.2) were adoptively transferred intravenously into B6.PL-Thy1^a/Cy mice (CD90.1), and the recipients were subsequently immunized with VVOVA$_{257\text{-}264}$ (10^6 pfu/mouse). CD8$^+$ T cells specific for SIINFEKL peptide were identified by staining of intracellular CD154, IFN-γ, and MHC class I tetramer, K^b/OVAp-Tet, on the indicated days post-immunization (0, 5, 10, 15, and 21 days post-immunization). **A.** Time course of SIINFEKL-specific CD8$^+$ T cells identified by three assays. The percent values in the dot-plots represent the proportion of CD8$^+$ T cells gated with CD90.2$^+$ cells. **B.** Total frequency of K^b/OVAp-Tet$^+$CD8$^+$ T cells per spleen at different days post-immunization. **C.** Total frequency of intracellular CD154$^+$CD8$^+$ T cells per spleen at different days post-immunization. **D.** Total frequency of intracellular cytokine IFN-γ$^+$CD8$^+$ T cells per spleen at different days post-immunization. **E.** Total combined frequencies of antigen-specific CD8$^+$ T cells identified by three assays. Data are one representative of three experiments.

on the indicated days post-immunization (0, 5, 10, 15, and 21 days post-immunization). As shown in Fig. 4A, all three assays for identifying antigen-specific CD8$^+$ T cells showed a similar pattern, peaking at 10 days post-immunization. However, each assay displayed a different magnitude for detecting antigen-specific CD8$^+$ T cells. At 10 days post-immunization, over 90% of CD8$^+$ T cells gated with CD90.2$^+$ cells were K^b/OVAp tetramer-positive, whereas the percent of intracellular IFN-γ-positive cells was around 50%. This indicates that not all antigen-specific CD8$^+$ T cells detected by tetramer staining produce IFN-γ in response to antigenic stimulation. The percent of CD8$^+$ T cells producing intracellular CD154 molecule was much less than that of intracellular IFN-γ and MHC class I tetramer staining. Moreover, the same patterns were observed when total numbers of intracellular CD154$^+$, IFN-γ$^+$, and K^b/OVAp-Tet$^+$CD8$^+$ T cells were calculated per spleen (Figs. 4B, 4C, and 4D). The differences between intracellular CD154 staining and intracellular IFN-γ and tetramer staining in identifying antigen-specific CD8$^+$ T cells become markedly clear at 10 days post-immunization. These results indicate that the intracellular CD154 staining assay could reflect the kinetic trend of antigen-specific CD8$^+$ T cell number after immunization, but is less sensitive than accepted standard assays, namely intracellular IFN-γ and tetramer staining, for identifying antigen-specific CD8$^+$ T cells.

Intracellular CD154 Expression of CD8$^+$ T Cells Generated in CD4 T Cell-depleted Animals

CD4$^+$ T cells play a critical role in the maintenance of functional CD8$^+$ T cell responses in murine models [31, 33]. Thus, transient CD4$^+$ T cell depletion at the time of immunization can induce the exhaustion of specific CD8$^+$ T cells as well as the appearance of circulating nonfunctional CD8$^+$ T cell clones. To compare intracellular expression of CD154 and IFN-γ in CD8$^+$ T cells developed in CD4$^+$ T cell-depleted animals, we depleted CD4$^+$ T cells by injecting mice with purified anti-CD4 MAb (clone GK1.5) two days before (day –2), at the time of (day 0), and two days after

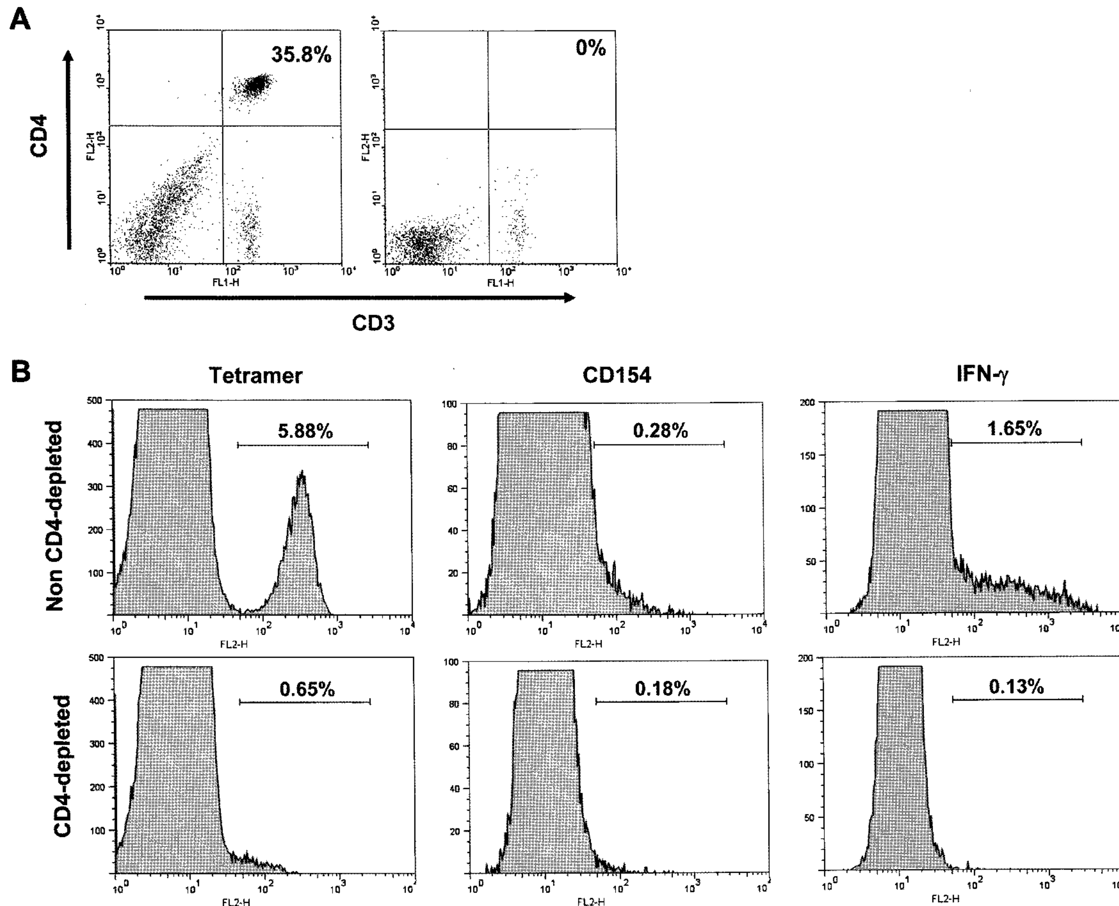

Fig. 5. Intracellular CD154 expression of $CD8^+$ T cells generated in CD4 T cell-depleted animals.
C57BL/6 mice were injected intraperitoneally with anti-CD4 MAb, GK1.5 (300 μg/mouse) two days before (day −2), at the time of (day 0), and two days after (day 2) immunization, and immunized with $VVOVA_{257-264}$ (10^6 pfu/mouse) at day 0. Purified rat IgG was used as a control treatment in all cases. **A**. The efficacy of CD4 T cell-depletion with GK1.5 MAb treatment was assessed by flow cytometric analysis at the time of immunization. This treatment consistently resulted in >98% reduction of $CD4^+$ T cells. **B**. Antigen-specific $CD8^+$ T cells identified by intracellular CD154, IFN-γ, and MHC class I tetramer staining in both CD4 T cell-depleted and nondepleted animals. The CD4 T cell-depleted mice were re-injected with $VVOVA_{257-264}$ 15 days post-immunization. Five days later, intracellular CD154, IFN-γ, and MHC class I tetramer staining were performed. The percent values in histograms represent the proportion of gated $CD8^+$ T cells.

(day 2) immunization. This treatment resulted in >98% reduction in the number of peripheral $CD4^+$ T cells at the time of $VVOVA_{257-264}$ immunization, as determined by flow cytometry (Fig. 5A). When antigen-specific $CD8^+$ T cells were enumerated 5 days after recall response following re-injection of $VVOVA_{257-264}$ 15 days post-immunization, a reduced number of intracellular CD154, IFN-γ, and K^b/OVAp-Tet-positive $CD8^+$ T cells was observed in CD4-depleted animals, as shown in Fig. 5B. In particular, K^b/OVAp-Tet and intracellular IFN-γ-positive cells were greatly reduced to one-tenth of that in animals whose $CD4^+$ T cells were not depleted. However, intracellular $CD154^+CD8^+$ T cells in CD4 T cell-depleted mice were observed in half of that in animals whose CD4 T cells

Table 1. Summary of antigen-specific $CD8^+$ T cells identified by intracellular CD154, IFN-γ, and MHC class I tetramer staining in $CD4^+$ T cell-depleted and nondepleted animals.

	Non CD4-depleted		CD4-depleted	
	Total cell number (×10^4 cells/spleen)	% of Tet^+ $CD8^+$	Total cell number (×10^4 cells /spleen)	% of Tet^+ $CD8^+$
K^b/OVAp-Tet^+CD8^+	75.3	–	2.26	–
$CD154^+$ $CD8^+$	7.26	9.63%	1.11	49.0%
IFN-γ^+ $CD8^+$	31.3	41.5%	0.55	24.4%

were not depleted. This indicates that intracellular CD154 expression was not affected by CD4 T cell depletion in the same way as intracellular IFN-γ expression in the functional differentiation of antigen-specific CD8$^+$ T cells. This observation became clearer when the absolute splenic number of antigen-specific CD8$^+$ T cells determined by intracellular CD154, IFN-γ, and MHC class I tetramer staining was calculated (Table 1). Antigen-specific CD8$^+$ T cells expressing intracellular CD154 and IFN-γ were observed in 9.63% and 41.5% of K^b/OVAp-Tet$^+$CD8$^+$ T cells developed in non-CD4 T cell-depleted animals, respectively. However, in CD4$^+$ T cell-depleted animals, 49.0% of K^b/OVAp-Tet$^+$CD8$^+$ T cells expressed intracellular CD154 molecules, whereas IFN-γ-expressing K^b/OVAp-Tet$^+$CD8$^+$ T cells were reduced in 24.4%. This observation indicates that the detection of intracellular CD154 expression is not affected by the functionality of CD8$^+$ T cells as much as intracellular IFN-γ expression.

Discussion

The expression of CD154 on CD4$^+$ and CD8$^+$ T cells is important for CD4$^+$ and CD8$^+$ T cell priming, expansion, and maturation into effector cells capable of cytokine production and lytic activity [36]. Numerous reports on the biological importance of CD154, coupled with its exclusive expression on activated cells, make this protein a potentially useful correlate of antigen-specific immune responses [36]. It has recently been reported that CD154 expression can be used to assess the entire repertoire of antigen-specific CD4$^+$ T helper cells with diverse cytokine profiles [11, 16]. The Th-cell activation marker CD154 has not previously been used to directly detect antigen-specific T cells because it is only transiently expressed in the course of T cell activation [3, 17, 39]. However, the secretion inhibitor Bref A and CD40-specific blocking antibody allowed detection of intracellular CD154 molecules to identify antigen-specific CD4$^+$ T helper cells induced by vaccines and pathogens. This assay demonstrated the utility of CD154 in sorting live antigen-specific CD4$^+$ T helper cells that produce diverse cytokines [11, 16]. Similarly, we tested the utility of intracellular CD154 expression in identifying antigen-specific CD8$^+$ T cells. Unfortunately, the detection of intracellular CD154 using secretion inhibitor Bref A reflected antigen-specific CD8$^+$ T cells, but with less sensitivity than previously accepted standard assays such as intracellular IFN-γ and MHC class I tetramer staining. However, the interesting point was that the intracellular CD154 expression in antigen-specific CD8$^+$ T cells, developed in the absence of CD4$^+$ T cells, was less affected than IFN-γ expression following brief antigenic stimulation; the number of IFN-γ-producing CD8$^+$ T cells was dramatically reduced in the CD4$^+$ T cell-depleted environment.

A desired objective of clinical vaccine trials against intracellular parasites and cancer is to induce vaccine- and pathogen-specific responses mediated by CD8$^+$ T cells. Therefore, it is essential to measure the frequency of CD8$^+$ T cells response before and after vaccination, and to correlate it with the clinical outcome. To achieve this, methods for measuring immunological end points have been optimized, standardized, and quality controlled [12, 37, 38]. The physical presence of antigen-specific CD8$^+$ T cells can be monitored by MHC class I tetramer or qPCR clonotype analysis [1, 13, 24, 26]. Neither of these techniques, however, provides any indications as to the functionality of the cells detected. However, when MHC class I tetramer staining is used in combination with intracellular IFN-γ staining and CFSE-based proliferation assays, the analysis provides the number and functionality of target CD8$^+$ T cells within the antigen-specific CD8$^+$ T cell population [4, 30, 35]. Moreover, measuring the exposure of CD107 a and b, present in the membrane of cytotoxic granules, on the cell surface of antigen-specific CD8$^+$ T cells following TCR engagement can provide information about cytotoxic ability [8]. As demonstrated in the present study, however, the sensitivity of the intracellular CD154 assay to detect antigen-specific CD8$^+$ T cells was less than that of MHC class I tetramer and intracellular IFN-γ staining, although the detection of intracellular CD154 using the secretion inhibitor Bref A can help identify antigen-specific CD8$^+$ T cells. In particular, the detection level in the intracellular CD154 assay was around one-tenth of both MHC class I tetramer and intracellular IFN-γ staining when used to identify the number of antigen-specific CD8$^+$ T cells in an adoptive transfer model. Conceivably, less sensitivity in the intracellular CD154 assay in detecting antigen-specific CD8$^+$ T cells may be caused by differences in CD154 expression levels between activated CD4$^+$ and CD8$^+$ T cells [18, 27, 28]. Therefore, the intracellular CD154 assay can at least be a useful method in the assessment and isolation of heterogeneous CD4$^+$ T helper cells specific for a defined antigen.

However, in attempts to detect antigen-specific CD8$^+$ T cells by intracellular CD154 staining, we found two interesting results that differed from MHC class I tetramer and intracellular IFN-γ staining. First, intracellular CD154 expression peaked by 8 h following antigenic stimulation in the presence of secretion inhibitor Bref A, and the saturated level of CD154 expression was maintained up to 24 h. In contrast, intracellular IFN-γ expression saturated at 8 h after antigenic stimulation and then declined. The decline in IFN-γ levels after saturation in activated CD8$^+$ T cells may be caused by the exhaustion of antigen-specific CD8$^+$ T cells during prolonged antigenic stimulation and/or the secretion of IFN-γ, overcoming the threshold barrier blocked by secretion inhibitor Bref A. These results indicate that intracellular IFN-γ staining to identify antigen-specific

CD8$^+$ T cells requires optimal antigenic stimulation conditions. In contrast, the intracellular CD154 assay is easily optimized, because intracellular CD154 molecules are maintained inside activated CD8$^+$ T cells. The second interesting finding is that the expression of intracellular CD154 in antigen-specific CD8$^+$ T cells developed in the absence of CD4$^+$ T cell help was comparable to IFN-γ expression in CD8$^+$ T cells developed in non-CD4 T cell-depleted animals. In contrast, the number of CD8$^+$ T cells producing IFN-γ decreased abruptly within antigen-specific CD8$^+$ T cells developed in the absence of CD4$^+$ T cells. This suggests that intracellular CD154 expression is not as easily influenced by the maturation state of antigen-specific CD8$^+$ T cells as IFN-γ expression. This finding is also supported by the finding that antigen-specific CD4$^+$ T helper cells expressing no cytokines had a CD154$^+$ phenotype [11]. Therefore, intracellular CD154 staining seems to provide information on the number, but not the functionality, of antigen-specific CD8$^+$ T cells, similar to MHC class I tetramer staining.

The intracellular CD154 assay to identify antigen-specific CD4$^+$ T helper cells producing diverse cytokines has several advantages, including identification of broadly functional cells, high sensitivity, ease of use, compatibility with other assay formats such as intracellular cytokine staining, and, most importantly, preservation of cell viability [11, 16]. Moreover, the intracellular CD154 assay can be used in conjunction with prolonged stimulation time (24 h), making it useful when highly synchronized responses are not possible [11, 16]. Similarly, the application of intracellular CD154 assay to detect antigen-specific CD8$^+$ T cells has comparable advantages, but unfortunately showed low sensitivity. Therefore, intracellular CD154 staining does not provide enough information to identify antigen-specific CD8$^+$ T cells because of the low sensitivity compared with previously accepted standard assays such as intracellular IFN-γ and MHC class I tetramer staining. Presumably, combined analytical techniques, including intracellular cytokine, MHC multimeric, CD107 a/b staining, and T cell recognition of APCs by protein transfer (TRAP) assay, [6] together with intracellular CD154 staining, may provide information on the accurate phenotype and functionality of antigen-specific CD8$^+$ T cells.

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