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A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles

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Recently activated, but not resting, CD4+ T cells express CD154, providing costimulatory signals to B cells and antigen-presenting cells (APCs). Therefore, *de novo* CD154 expression after stimulation identifies antigen-specific CD4+ T cells. Previous assays were limited by the transient nature of surface CD154 expression; we overcame this by including fluorescently conjugated CD154-specific antibody during stimulation. Our assay is fully compatible with intracellular cytokine staining, and can be used for stimulations as long as 24 h. Notably, it is nonlethal, providing a means to purify viable antigen-specific CD4+ T cells for further analysis. Using this assay, we found that stimulated cells expressing tumor necrosis factor (TNF)-α, interleukin (IL)-2 or interferon (IFN)-γ were predominantly CD154+. Furthermore, some cells expressing none of these cytokines also expressed CD154, suggesting that CD154 marks cells with other effector functions. For vaccine- or pathogen-specific responses, we found substantial heterogeneity in expression of CD154 and cytokines, suggesting previously unrecognized diversity in abilities of responding cells to stimulate APCs through CD40.

CD4+ T-cell responses are commonly recognized by the *ex vivo* expression of various cytokines, such as IFN-γ and IL-2, after a brief stimulation with whole antigen or pools of peptides derived from the antigen using one of a variety of methods. Secreted cytokines can be quantified by ELISA or ELISPOT; however, these assays cannot assess the phenotype of antigen-responsive cells[1]. Alternatively, cytokine production can be measured by flow cytometry using golgi inhibitors (such as monensin or brefeldin A), combined with phenotypic and intracellular staining for cytokines (ICS)[2]. But the fixation precludes sensitive RNA-based assays and is lethal to cells. Cytokine-capture assays were developed to identify cells that secrete cytokine without killing the cells, but are rather laborious and of limited sensitivity[3]. Finally, multimers of major histocompatibility complex (MHC) class II proteins can identify antigen-specific CD4+ T cells; however, this technology is limited by the paucity of available tetramers, requires knowledge of the MHC haplotype of the individual studied and does not necessarily identify functionally responsive cells[4].

Because of the substantial heterogeneity of antigen-specific immune responses[5], a wide variety of functional responses must be measured to identify the totality of a response. Although numerous cytokines can be measured simultaneously using recently developed multiplexing technologies (for example, Luminex, cytokine bead arrays[6], polychromatic flow cytometry[7]), these technologies are not yet widely available, leaving many investigators to examine antigen-specific T-cell responses one cytokine at a time. For these reasons, a marker that represents multiple functional responses of CD4+ T cells is needed.

Here we describe an assay to identify antigen-responsive CD4+ T cells that overcomes many of the limitations of existing assays. The assay detects *de novo* synthesis of a protein fundamental to immune responses, CD40 ligand (also known as CD154). By providing crucial costimulatory signals, the expression of CD154 by antigen-specific T cells identifies an important functional correlate of the cells—specifically, by identifying cells that may provide B-cell help[8,9] or activate APC function[10].

Previous assays for CD154 were limited by the transience of cell-surface CD154 expression[11,12]. A modified assay detects intracellular CD154 (ref. 13); however, this method is lethal to the cells. We reasoned that inclusion of the detection antibody in the culture would overcome these limitations[14], because the antibody would become cell associated whenever CD154 was on the surface; subsequent internalization of CD154 would retain the conjugate, rendering the cell fluorescent. In theory, this method does not immediately identify newly synthesized CD154 *en route* to the cell surface; however, because CD154-specific antibodies are present throughout stimulation, these molecules are eventually labeled when they reach the cell surface. Here, we show the optimization and validation of this assay to identify vaccine- or pathogen-induced CD4+ T cells, and show its utility in sorting live, antigen-specific CD4+ T cells.

RESULTS

Optimization of CD154 detection

Initial experiments investigated the kinetics of CD154 expression to determine the optimal duration of cell stimulation (**Fig. 1**). Cells incubated with fluorescent CD154-specific antibody and monensin (coculture method), and stimulated with *Staphylococcus* enterotoxin B (SEB) or left unstimulated, were removed from culture at various time points, stained with antibodies to CD3 and CD4, then analyzed by flow cytometry. As expected, unstimulated cells did not express CD154 (**Fig. 1a**).

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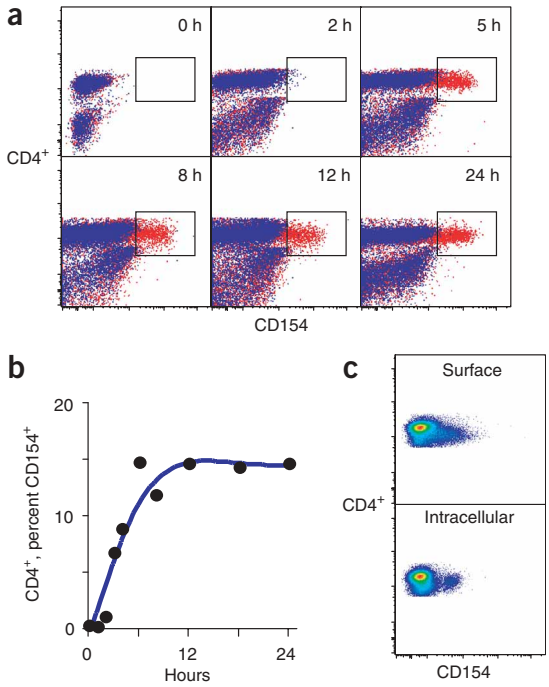

Figure 1 Optimization of CD154 detection. (**a**) Time course of CD154 staining (red) and background fluorescence (blue), as measured by coculture method. Background fluorescence (unstimulated cultures) increased during the first 2 h of coculture, but subsequently stabilized. (**b**) Kinetics of CD154 expression within $CD4^+$ T cells, as measured by coculture method. Peak expression of CD154 first detected with 6 h of stimulation; CD154 expression was detected in 15% of all $CD4^+$ T cells. Maximal levels of CD154 were still observed with 24 h of stimulation. (**c**) CD154 expression measured by surface and intracellular methods. Surface and intracellular methods identified CD154 expression in 6% and 3%, respectively, of $CD4^+$ T cells; in the same sample, the coculture assay identified 15% of the $CD4^+$ T cells. Data are representative of two to four experiments.

We noted that unstimulated cells show a small increase in 'background' CD154 fluorescence that is difficult to explain. This increase does not result from nonspecific (pinocytic) uptake of the antibody, as we did not observe it when we tested isotypic and irrelevant antibodies. But we found that background CD154 only increased during the first 3 h, reaching a plateau thereafter (**Fig. 1a**). In contrast, for the stimulated culture, a population of $CD154^+$ cells could be clearly distinguished from background after this time. CD154 staining in stimulated cells was bright (between 10^3 and 10^4 fluorescence units), and the fluorescently tagged population persisted for more than 24 h (**Fig. 1a**). This suggests that, unlike previous methods, coculture positively identifies cells despite the highly transient nature of the CD154 expression.

We next compared the proportions of $CD154^+$ cells detected by cell-surface staining, intracellular CD154 staining and by our coculture method. Previous reports showed peak expression of cell-surface and intracellular CD154 with 6 h of SEB stimulation[11,12,15]. We observed a similar result for cells assayed by the coculture method (**Fig. 1b**); therefore, subsequent experiments used 6-h stimulations, unless otherwise noted. We found that our method identified a substantially larger fraction of SEB-responding $CD4^+$ T cells (15% of $CD4^+$ cells were $CD154^+$), compared to either the intracellular or surface-staining methods (6% and 3%, respectively). We also found that staining of CD154 by coculture was considerably brighter than surface or intracellular staining (**Fig. 1c**).

Notably, in the coculture assay, no CD154 expression could be detected when brefeldin A was used (data not shown). This proves that our assay detects only *de novo*–synthesized CD154 that can be transported (even transiently) to the cell surface.

Relationship between CD154 and cytokine expression

After coculturing SEB-stimulated peripheral blood mononuclear cells (PBMCs) with CD154-specific antibody and monensin, we analyzed synthesis of various cytokines by ICS and polychromatic flow cytometry. As shown in **Figure 2**, cytokine measurement by ICS is compatible with the coculture assay for CD154. The vast majority of cells producing at least one cytokine also express CD154 (**Fig. 2**), confirming that CD154 identifies the majority of responding cells, regardless of function. The assay also identifies cells expressing CD154 but lacking expression of the cytokines measured (**Fig. 2**); these cells probably show other effector functions (for example, secretion of MIP1β[16] or other cytokines). Thus, CD154 is a surrogate marker for multiple, diverse T-cell functions.

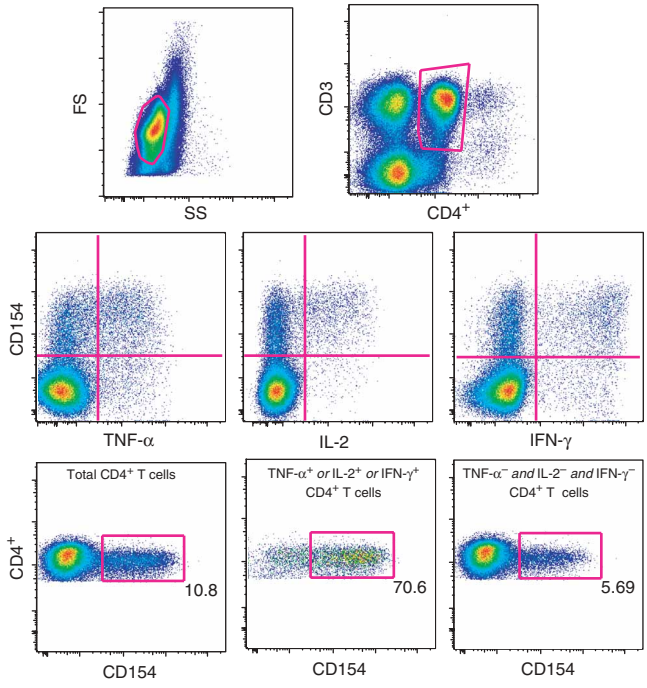

Figure 2 Relationship between CD154 and cytokine expression. Top panels, gating strategy used to identify lymphocytes (based on forward-scatter and side-scatter properties) and $CD4^+$ T cells (based on coexpression of CD3 and CD4). Those cells that have downregulated CD3 expression (as a result of SEB stimulation) are included in the analysis. Middle panels, measurement of CD154 by coculture is compatible with ICS staining for TNF-α, IL-2 and IFN-γ. Lower panels, CD3 and CD154 expression within total $CD4^+$ T-cell population (lower left), cells producing at least one of the cytokines measured (lower middle), or cells producing none of the cytokines measured (lower right). In this sample, approximately 11% of cells $CD3^+CD4^+$ T cells expressed CD154 after SEB stimulation. Cells expressing at least one cytokine were mostly (70.6%) $CD154^+$, whereas cells expressing none of the measured cytokines were mostly (but not exclusively) $CD154^-$. Data are representative of 10 experiments.

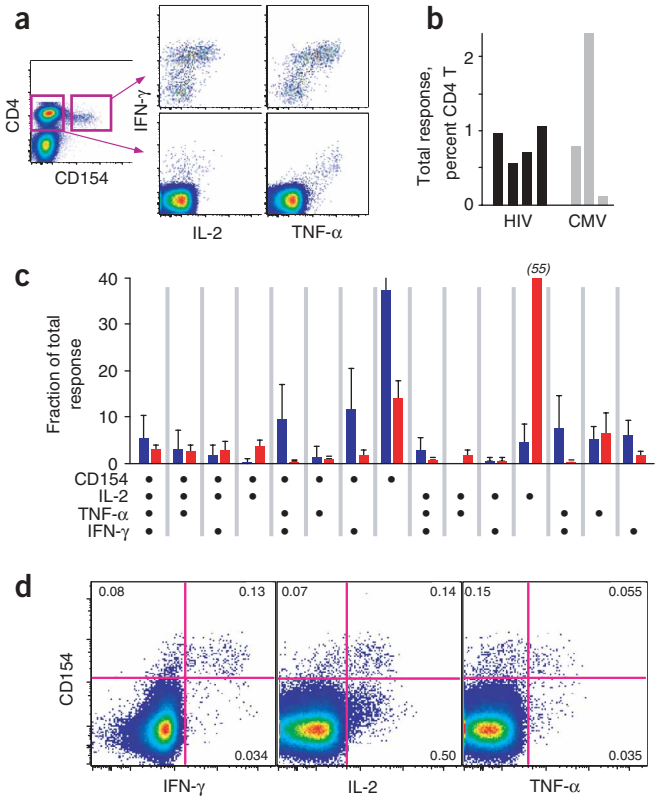

Figure 3 Characterization of antigen-specific T cells. $CD4^+$ T cells from vaccinated ($n = 4$) or CMV-infected individuals ($n = 3$) were stimulated with antigenic peptides. (**a**) Examples of CD154, IFN-γ, IL-2 and TNF-α expression after stimulation with a dominant peptide from CMV pp65. Most of the cytokine expression is observed within the $CD154^+$ population. (**b**) The magnitude of the total HIV- or CMV-specific response (as a fraction of $CD4^+$ T-cell numbers) is charted for each study participant. (**c**) The fraction of the total antigen response that shows each CD154-cytokine phenotype is charted. The total antigen response is defined as the number of $CD4^+$ T cells expressing any combination of CD154, IFN-γ, IL-2 or TNF-α. Bars represent averages for the individuals studied. Stimulation with CMV peptide (blue bars) induced a broad variety of CD154-cytokine phenotypes, most of which were $CD154^+$. Stimulation with HIV-envA (red bars) induced more $IL\text{-}2^+$ cells than CMV stimulation; the majority of these cells did not express CD154. Within most cytokine phenotypes, there are both $CD154^+$ and $CD154^-$ cells, suggesting that cells producing the same cytokines can differ in their ability to stimulate through the CD40 pathway. (**d**) Example responses from one of the HIV-envA–stimulated samples from a vaccinee. Graphs show data gated on $CD4^+$ T cells; the numbers in the quadrants are the percentage of cells in the stimulated sample minus the percentage in the costimulated control sample for the same gate (that is, background-subtracted, antigen-specific responses).

Functional profile after vaccination or infection

We next investigated whether CD154 measurements could provide qualitative information about $CD4^+$ T-cell responses, beyond what could be obtained by cytokine measurements alone. To do this, we examined every combination of cytokine and CD154 phenotypes after stimulation with peptides from HIV envelope A (HIV-envA) for vaccinated adults, or from cytomegalovirus (CMV) for naturally infected adults (**Fig. 3a**,**b**).

In CMV-stimulated cells, we observed a wide variety of CD154 responses with and without cytokines; 13 different phenotypes each represented a small fraction (no more than 20%) of the overall response (**Fig. 3c**). Regardless of cytokine phenotype, $CD154^+$ cells dominated the CMV response, with cells expressing CD154 representing more than 70% of responsive cells. But CD154 was not expressed on the remainder of responsive, cytokine-expressing cells. Thus, within most of the cytokine phenotypes, there are both $CD154^+$ and $CD154^-$ cells, suggesting that natural CMV infection induces a polyfunctional T-cell response characterized by a variety of cytokine-expressing cell types with differences in their ability to provide CD154-mediated help.

Compared to CMV-specific cytokine responses, the HIV-envA–specific responses (12 weeks after immunization) contained many more IL-2–producing cells (**Fig. 3c**). In fact, cells producing only IL-2 ($IFN\text{-}\gamma^-$ $IL\text{-}2^+$ $TNF\text{-}\alpha^-$) dominated the response. Notably, most of these cells did not express CD154, suggesting that at this early time point, the vaccine-elicited cells may not provide CD154-mediated costimulation. These results also indicate that, for some antigen-specific responses, CD154 may not identify the complete spectrum of cytokine-producing cells (**Fig. 3d**), and thereby show heterogeneity in the antigen-specific T cells to stimulate APCs.

Viable isolation of $CD4^+CD154^+$ T cells

We tested whether stimulated cells, stained by the CD154 coculture method and purified by flow cytometry, would be compatible with assays that required live cells. After 5 h of SEB stimulation, we obtained pure $CD4^+CD154^+$ or $CD4^+CD154^-$ fractions by sorting (purity of >99% and 95%, respectively; **Fig. 4a**). We then cultured the purified cells for an additional 30 h, during which we measured the kinetics of cytokine expression. We assayed the cytokines released during this culture period using a flow cytometry–based assay of cell culture supernatants (cytokine bead array).

Cells sorted based on the CD154 coculture assay retained the ability to secrete multiple cytokines (**Fig. 4b**). Notably, CD154 expression at 5 h identified cells destined to make the T helper type 2 (T_H2) cytokines IL-5 and IL-10 more than 24 h later. Overall, $CD154^+$ cells were highly enriched (10- to 40-fold) for cytokine production (**Fig. 4c**). Thus, CD154 expression provides a means to identify the vast majority of antigen-responsive $CD4^+$ T cells, regardless of the type or kinetics of the cytokines expressed.

Indeed, our assay conditions do not affect the proliferative capacity of $CD4^+$ T cells for extended culture after assay. Together with the cytokine experiments, this shows that our assay can be used to isolate diverse populations of responsive, fully viable antigen-specific $CD4^+$ T cells with a broad spectrum of functions.

DISCUSSION

CD154 expression has a fundamental role in the interaction between $CD4^+$ T cells and APCs[8–10,12,17,19]. The biologic importance of CD154 (refs. 10,15,17,19), coupled with its exclusive expression on activated cells[11,12,15], make this protein a potentially useful correlate of antigen-specific immune responses. Here we present a straightforward method to detect CD154 expression in living cells. This coculture technique successfully identifies antigen-specific $CD8^+$ T cells (using CD107, ref. 14), and for greater-sensitivity measurements of chemokine receptors[21].

Compared with previous methods to detect CD154, and other techniques to measure antigen-responsive cells, our assay has a number of advantages, including identification of broadly functional cells, high sensitivity, ease of use, compatibility with other assay formats (for example, ICS), and, most importantly, preservation of

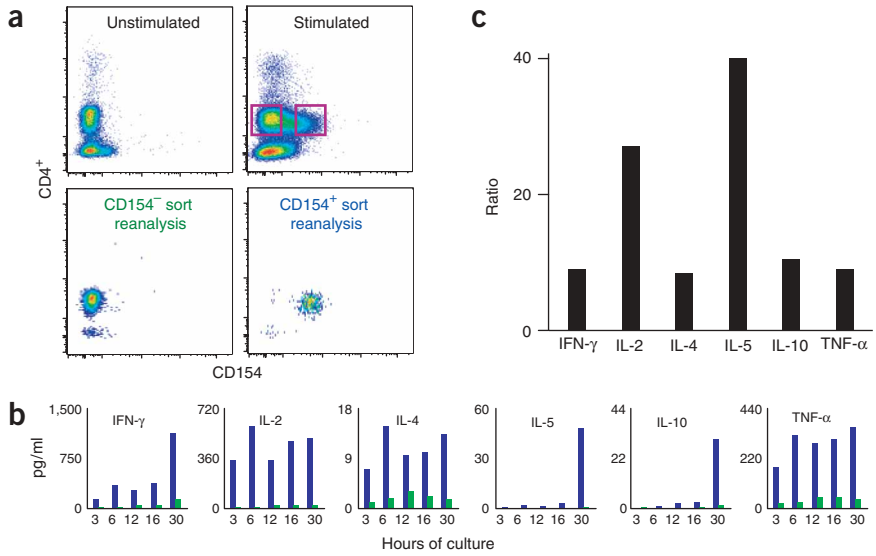

Figure 4 Viable isolation of CD4⁺CD154⁺ T cells. (**a**) Sorting gates for SEB-stimulated CD4⁺CD154⁻ and CD4⁺CD154⁺ cells (top right) were based on expression of CD154 in unstimulated culture (top left). Post-sorting analysis showed that both CD154⁻ (bottom left) and CD154⁺ (bottom right) were pure with respect to CD154 subsets. (**b**) Kinetics of cytokine secretion by CD154⁺ (blue bars) cells and CD154⁻ cells (green bars). CD154⁺ fractions were highly enriched for production of each cytokine measured. Moreover, CD154 expression preferentially identified even cells producing cytokines with delayed kinetics, such as IL-5 and IL-10, or those with low levels of expression (IL-4). (**c**) Ratio of cytokine production in CD154⁺ to CD154⁻ cells after 30 h in culture. The CD154⁺ fraction was enriched for cytokine-secreting cells by 10- to 40-fold. In addition, assay of CD154 (followed by sorting) did not affect the viability of the cells, as they continued to show broadly elicited functions after 30 h in culture.

cell viability. In addition, our assay can be used for stimulation times of at least 24 h, making it useful when highly synchronized responses are not possible (for example, in assays requiring viral delivery of antigen for stimulation of CD4 T cells, such as vaccinia-based assays).

Monensin is commonly used in ICS assays to disrupt the maturation of cytokine processing, and thereby prevent their secretion[14]. But this pathway does not seem to be required for the maturation and surface expression of CD154. Instead, in the setting of our assay, it is probable that the inhibitory effect of monensin on sodium-proton antiports neutralizes acidic intracellular compartments to preserve CD154-antibody complexes. In contrast, brefeldin A completely inhibited surface expression of CD154, suggesting that the golgi pathways disrupted by brefeldin A are required for CD154 maturation. Inclusion of monensin was required to prevent endosomal degradation of protease-sensitive fluorochromes (such as the phycobiliproteins phycoerythrin, allophycocyanin and their derivatives) and acid-sensitive fluorochromes (such as fluorescein derivatives). Monensin was also required even when we used protease- and acid-resistant fluorochromes (such as Alexa 680), presumably to prevent the release of the free fluorochrome after proteolysis of the antibody had occurred. Because we could efficiently detect CD154 even with low concentrations of monensin, investigators may wish to titrate both monensin and CD154-specific antibody to suit their particular systems.

To validate the CD154 assay, we studied the relationship between CD154 expression and cytokine production after stimulation with SEB or specific antigens. Using polychromatic flow cytometry, we showed that our method for CD154 detection is compatible with measurement of IL-2, IFN-γ and TNF-α by ICS. These experiments also showed the specificity of CD154 as a marker for cytokine-producing cells; cells expressing at least one cytokine were predominantly CD154⁺. Notably, some cells lacking expression of all three cytokines were also CD154⁺, suggesting that CD154 identifies cells with other effector functions. This is consistent with our published findings that (particularly for some antigens), the dominant CD4⁺ T-cell response may not include the production of IL-2, IFN-γ or TNF-α[16].

Indeed, our sorting experiment shows that at least some of these CD154⁺ cells will secrete IL-4, IL-5 and IL-10 with longer stimulation periods. Taken together, these results indicate that CD154 is a useful marker for describing a variety of CD4⁺ T-cell cytokine responses. Thus, coculture-based measurements of CD154 may be used as a preliminary screen for tracking immune responses during disease or with vaccination.

Finally, the coupling of CD154 synthesis to multiplexed measurements of cytokine expression provides a new level of detail for the study of CD4⁺ T-cell responses. Using this approach, we found that natural CMV infection elicited a broad cytokine response, consisting mostly of cytokine-producing cells capable of CD154-mediated help. In contrast, cells unable to provide CD154-mediated costimulation (and producing only IL-2) dominated the DNA vaccine response at an early time point after vaccination. The ability to discriminate antigen-specific T cells that can or cannot provide APC stimulation may be an important tool for identifying correlates of vaccine efficacy or disease morbidity.

METHODS

Cell stimulation. For studies of healthy individuals, we isolated fresh PBMCs from whole blood by density-gradient centrifugation. We resuspended cells to a concentration of 2×10^6 cells/200 μl in RPMI media supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco). We treated cultures of stimulated cells with 1 μg/ml SEB (Sigma). We performed antigen-specific stimulations on cryopreserved PBMCs from three individuals with natural CMV infection and four individuals who had received a DNA vaccine encoding HIV-envA. Viability after thawing (measured by ethidium bromide–Acridine orange; Molecular Probes) was consistently above 85%. We stimulated cells with 2.5 μg/ml of overlapping peptides covering HIV-envA or with 5 μg/ml CMV pp65 peptide (QEFFWDANDIYRIFA), and plated them onto 96-well plates for culture at 37 °C.

Our studies were approved by the Institutional Review Board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and we obtained informed consent from all participants.

Staining of CD154, cell-surface and intracellular antigens. We used three different approaches to study CD154 expression: staining for cell-surface CD154, staining for intracellular CD154 and coculture. We used the following antibodies (we produced fluorescent antibodies in-house, unless otherwise indicated): Cyanin-7–allophycocyanin (Cy7APC)-conjugated CD3-specific antibody (BD Pharmingen), Cascade Blue–conjugated CD4-specific antibody, FITC-conjugated CD8-specific antibody and phycoerythrin-conjugated CD154-specific antibody (BD Pharmingen) or allophycocyanin-conjugated

CD154-specific antibody. For CD154-specific antibody, we compared three commercially available conjugates and found that the two best were both conjugates of the TRAP-1 clone; of these, the PharMingen phycoerythrin-conjugated CD154-specific antibody gave the best signal.

We performed cell-surface analysis by staining after completion of the stimulation period. We separately titrated the conjugated CD154-specific antibody for each condition (intracellular, surface-staining or coculture) to determine the optimal concentration for use. For intracellular analysis of CD154, we stained cells with surface antibodies (to CD3, CD4, CD8) then fixed and permeabilized (CytoPerm Kit, BD Pharmingen) them for CD154 staining. We treated cells studied by the coculture method with CD154-specific antibody and 2 μM monensin (BD Pharmingen) during the stimulation and then stained for either surface or intracellular, or both, cytokine markers after stimulation.

We performed ICS by surface staining (with antibodies to CD3, CD4, CD8), fixation and permeabilization of cocultured cells (as described above). We stained ICS samples with the following combination of antibodies: TNF-α–specific Cy7PE, IFN-γ–specific Alexa 680, and IL-2–specific APC.

Assay validation and optimization. We tested reproducibility by repeatedly analyzing parallel aliquots of PBMC samples over multiple days ($n = 6$). The fraction of cells that were $CD154^+$ after SEB stimulation had a coefficient of variation of $<20\%$. To show the specificity of the assay, we stained SEB-stimulated $CD4^+$ cells with antibodies to the variable (V) regions of the T-cell receptor β chain that are not stimulated by SEB (Vβ1, 2, 13.6). We found that $<1.8\%$ of the nonresponsive Vβs expressed CD154 (data not shown), confirming that CD154 is not upregulated on 'bystander' cells (that is, those that are not specifically stimulated).

We determined that our assay conditions do not affect cell viability or the ability of cells to proliferate. We labeled PBMCs with carboxyfluorescein diacetate, succinimidyl ester (CFSE) and then treated them for 6 or 18 h with 0 μM, 0.7 μM or 2.0 μM (1×) monensin. We washed cells and resuspended them in media containing SEB, so that proliferation (as indicated by dilution of CFSE) could be measured after a 5-d culture period. Samples treated for 6 h showed no effects on proliferation by monensin. With 18 h of monensin treatment, we found a slight reduction of proliferation in samples treated with the highest concentration of monensin, but no effect at lower concentrations. The ability to quantify CD154 was not decreased at 0.7 μM monensin.

Flow cytometry and data analysis. Flow cytometry experiments consisted of four- to six-color panels. We acquired data on an LSRII flow cytometer (BD Biosciences), and analyzed it in FlowJo version 6.1.1 (Tree Star). We performed cell-sorting experiments on a DiVa flow cytometer (BD Biosciences). We collected at least 1,000,000 events for the antigen-specific assays.

Cytokine analysis by cytometric bead arrays. We plated $CD154^+$ and $CD154^-$ cell fractions purified by cell sorting into 96-well plates, with each well receiving 96,000 cells in 200 μl RPMI media. We cultured cells at 37 °C for up to 30 h. At 6 and 30 h after sorting, we centrifuged cells, and drew off the supernatant for analysis by Cytometric Bead Array kit (BD Biosciences).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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