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*J Immunol* 2009; 183:2827-2836; Prepublished online 27 July 2009; doi: 10.4049/jimmunol.0803548

http://www.jimmunol.org/content/183/4/2827

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High Levels of Human Antigen-Specific CD4+ T Cells in Peripheral Blood Revealed by Stimulated Coexpression of CD25 and CD134 (OX40)

John J. Zaunders,²,* Mee Ling Munier,* ‡ Nabila Seddiki,* ‡ Sarah Pett,‡ Susanna Ip,* ‡ Michelle Bailey,* ‡ Yin Xu,* ‡ Kai Brown,* Wayne B. Dyer,‡ Min Kim,§ Robert de Rose,¶ Stephen J. Kent,¶ Lele Jiang,* Samuel N. Breit,* Sean Emery,† Anthony L. Cunningham,§ David A. Cooper,* ‡ and Anthony D. Kelleher* ‡

Ag-specific human CD4+ memory T lymphocytes have mostly been studied using assays of proliferation in vitro. Intracellular cytokine and ELISPOT assays quantify effector cell populations but barely detect responses to certain recall Ags that elicit strong proliferative responses, e.g., tetanus toxoid, that comprise non-Th1 CD4+ cells. We have found that culturing whole blood with Ag for 40–48 h induces specific CD4+ T cells to simultaneously express CD25 and CD134. This new technique readily detects responses to well-described CD4+ T cell recall Ags, including preparations of mycobacteria, CMV, HSV-1, influenza, tetanus toxoid, Candida albicans, and streptokinase, as well as HIV-1 peptides, with high specificity. The assay detects much higher levels of Ag-specific cells than intracellular cytokine assays, plus the cells retain viability and can be sorted for in vitro expansion. Furthermore, current in vitro assays for human CD4+ memory T lymphocytes are too labor-intensive and difficult to standardize for routine diagnostic laboratories, whereas the whole-blood CD25+CD134+ assay combines simplicity of setup with a straightforward cell surface flow cytometry readout. In addition to revealing the true extent of Ag-specific human CD4+ memory T lymphocytes, its greatest use will be as a simple in vitro monitor of CD4+ T cell responses to Ags such as tuberculosis infection or vaccines. The Journal of Immunology, 2009, 183: 2827–2836.

Antigen-specific CD4+ T lymphocytes occupy a central role in human immune responses, amply demonstrated by the primary immunodefiencies associated with mutations affecting CD40-CD40L or STAT3 signaling (1, 2) and by HIV-induced acquired immunodeficiency (3). Human Ag-specific CD4+ T cell responses in vivo have been inferred from delayed-type hypersensitivity responses in skin testing and also from proliferation of PBMC after 6–7 days culture, in the presence of recall Ags, using [3H]thymidine incorporation into newly synthesized DNA (4), or by flow cytometry, using CFSE labeling (5, 6). Alternatively, cytokine synthesis and secretion by PBMC in response to Ags have been widely measured by intracellular cytokine

Received for publication October 24, 2008. Accepted for publication June 8, 2009.

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1 J.J.Z., A.D.K., D.A.C., M.K., and A.L.C. are partly supported by program grants from the Australian National Health and Medical Research Council. M.L.M. and A.D.K. are recipients of a Dora Lush postgraduate scholarship and a Practitioner Fellowship, respectively, from the National Health and Medical Research Council. The National Centre in HIV Epidemiology and Clinical Research is supported by the Commonwealth Department of Health and Ageing.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803548

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3 Abbreviations used in this paper: ICC, intracellular cytokine; Flu, influenza A; LPA, lymphoproliferation assay; MAI, Mycobacterium avium; MTB, Mycobacterium tuberculosis; PET, polyester; SEB, staphylococcal enterotoxin B; SKSD, streptokinase; TT, tetanus toxoid.
CD134 assay is not limited by a priori decisions about which effector function or cytokine to detect.

Materials and Methods

Subjects

Healthy adult volunteers were recruited from university and hospital staff. Three individuals were inoculated with vaccinia and provided longitudinal samples, as described elsewhere (12). Samples of whole blood were also obtained from 13 consecutive untreated subjects with established HIV-1 infection (median, 158 CD4 cells/µl; 4.7 log RNA copies/ml). CMV serology was determined as described previously (14). All subjects gave informed written consent.

Reagents

PHA and staphylococcal enterotoxin B (SEB; Sigma-Aldrich) were used at final concentrations of 5 and 1 µg/ml, respectively. CMV and vaccinia lysates, respectively, were prepared and used at a final concentration of 1/250, as described previously (12, 14). Mycobacterial Ag preparations from Mycobacterium tuberculosis (MTB) and Mycobacterium avium (MAI) complex, as well as TT and purified influenza A (Flu), were obtained from Commonwealth Serum Laboratories and used at a final concentration of 5 µg/ml, 5 µg/ml, 2 LF U/ml, and 200 hemagglutinin U/ml, respectively. Candida albicans (Candida) was from Pasteur Sanofi, streptokinese (SKSD) from Sigma-Aldrich, and tuberculin from Serum Statens/H9262.

Candida albicans (Candida) was from Pasteur Sanofi, streptokinese (SKSD) from Sigma-Aldrich, and tuberculin from Serum Statens Institute and used at final concentrations of 25, 5, and 1 µg/ml, respectively. HSV type 1 (F-strain) was prepared from Vero cells, infected for 48 h, by brief sonication, centrifugation, and UV inactivation. Overlapping HIV-1 Gag 15-mer peptides, from the sequence of strain HXB2, were obtained from the National Institutes of Health AIDS reference reagents program. Gag peptides were used as a pool of 123 peptides, at an individual concentration of 2 µg/ml each, as described previously (14).

Lymphoproliferation assays (LPA)

PBMC were isolated as previously described (14), and 1 x 10⁶ PBMC were incubated per well in quadruplicate for 6 days in RPMI 1640 (JRH) containing 10% human AB serum (Cambrex Biosciences), then with 0.5 µg/ml of ³H]thymidine (Amersham Biosciences) for an additional 18 h before harvesting and counting (12, 14).

The CFSE assay of proliferation of CD4⁺ T cells was performed as described previously (14). After 7 days of culture with recall Ags, cells were stained with CD3-PerCP-Cy5.5, CD4-PE-Cy7, CD8-allophycocyanin-Cy7, CD25-allophycocyanin, and CD71-PE (BD Biosciences) and analyzed as described below.

ICC assay

A 6-µl whole-blood ICC assay, using six-color flow cytometry, was used to measure Ag-specific CD4⁺ T cells (11). Briefly, 0.5 ml of sodium heparin-anticoagulated whole blood was cultured with costimulatory Abs CD28 and CD40 in the presence of no additive (control), or polyclonal mitogen SEB, or various Abs for 2 h at 37°C, followed by an additional 4-h incubation with 10 µg/ml brefeldin A (Sigma-Aldrich). Intracellular staining for cytokines and CD40L were performed as described previously (11, 12, 14, 15).

Whole-blood CD25/CD134 assay

Sodium heparin-anticoagulated whole blood (0.25 ml) was mixed with 0.25 ml of IMDM (JRH) in 24-well plates (BD Biosciences). Ags and mitogens were added at the specified concentrations, and cultures were incubated at 37°C for 40–48 h in a humidified atmosphere of 5% CO₂ in air. In some experiments, cultures were performed in 5-ml sterile polystyrene screw-cap jars (Biolabs) with the cap loosely attached during the incubation. Negative control cultures comprised whole blood mixed with IMDM only. Positive control cultures contained either PHA or SEB. Negative and positive control cultures were included in every experiment.

In some experiments, whole-blood samples were cultured with Ag for 48 h, as above, and brefeldin A (10 µg/ml; Sigma-Aldrich) was added to the culture for the final 14 h. In addition to cell surface staining for CD3, CD4, CD25, and CD134 (see below), cells were permeabilized and stained intracellularly for IFN-γ, as described previously (14).

In other experiments, whole-blood samples from two different individuals, with known negative and positive responses to the added Ag, respectively, were cultured in individual wells of 24-well plates in upper and lower chambers separated by 6.5-mm Transwell polyester (PET) membranes (Falcon; BD Biosciences), according to the manufacturer’s directions. At the end of these Transwell cultures, cells from the two different individuals were stained and analyzed separately.

Flow cytometry

At the end of the whole-blood culture, 100 µl was stained with CD3-PerCP-Cy5.5, CD4-PE-Cy7, CD25-allophycocyanin, and CD134-PE (BD Biosciences) for 15 min at room temperature, treated with Optilyse C (Beckman Coulter) according to the manufacturer’s directions, and washed once with 2 ml of PBS (JRH). Cells were resuspended in 0.5 ml of 0.5% paraformaldehyde (Proscitech) in PBS and analyzed on a three-laser LSRII flow cytometer (BD Biosciences) using FACS Diva v4.1 software, as described previously (14). T lymphocytes were first identified using a CD3-PerCP-Cy5.5 vs side scatter gate, followed by gating on CD3⁺CD4⁺ T cells, which were then analyzed for binding of CD25-allophycocyanin and CD134-PE. Gates for CD25⁺ and CD134⁺ cells were based on comparison of negative control (no Ag) and positive control (PHA) cultures to include cells highly expressing CD25 plus positive for CD134 (see Fig. 1a).

In some experiments, CD134-FTC was used, as indicated. A minimum of 50,000 events were analyzed and compensation checked as described previously (14).

Statistical analysis

Results for subject groups were expressed as medians and compared by nonparametric Mann-Whitney U test, using Statview v5.0 for Macintosh (Abacus Concepts). A two-sided p < 0.05 was considered statistically significant.

Results

Characterization of CD25⁺CD134⁺CD4⁺ T cells in whole-blood cultures

CD4⁺ T lymphocytes in peripheral blood that are CD25⁺ CD127dim can be readily identified and represent Foxp3⁺ T regulatory cells (16), but these cells are CD134⁻. Therefore, background coexpression of CD25 and CD134 (OX40) on CD4⁺ T cells in peripheral blood is extremely low, as shown in a representative, unstimulated 44-h culture of whole blood in Fig. 1a, left histogram. Overall, in unstimulated cultures from 31 separate experiments, the mean plus three times the SD was calculated as 0.03% of CD4⁺ T cells.

In contrast, in whole-blood cultures to which polyclonal mitogen PHA or SEB were added, high levels of CD25⁺CD134⁺CD4⁺ T cells were detected. Representative histograms showing the response of CD4⁺ T cells to PHA and SEB are shown in Fig. 1a, middle and right histograms. Overall, for 17 healthy adult controls, the mean percentage of CD25⁺CD134⁺CD4⁺ T cells in response to PHA was 54% (SD, 13%) and 14% (SD, 4.4%) in response to SEB.

Moreover, CD25⁺CD134⁺CD4⁺ T cells were found after 40–48 h culture of whole blood with known CD4⁺ T cell recall Ags. Representative histograms showing various responses in healthy adult controls to the standard recall Ags MTB, CMV lysate, TT, SKSD, Flu, and Candida are shown in Fig. 1b. Differences between duplicate cultures were generally <10%, and in 12 replicates, the CV was 11% (data not shown). In addition to the CD25⁺CD134⁺CD4⁺ T cells found in Ag-responsive whole-blood cultures, we also observed some single-positive CD25⁺CD134⁻ and CD25⁻CD134⁺CD4⁺ T cells (Fig. 1b), which may be due to bystander activation, because the CD25⁺CD134⁺CD4⁺
T cells could be also induced by addition of exogenous IL-2 (supplemental Fig. 1). Neither of the single-positive cell populations were enriched for IFN-γ/H9253/H11001 cells (see below), nor did they proliferate when purified by cell sorting and further cultured with Ag and IL-2, in contrast to the double-positive CD25+/CD134+CD4+ T cells (see below).

The kinetics of the development of CD25+/CD134+CD4+ T cells in whole-blood cultures is shown in Fig. 1c. The results show that CD4+ T cell responses were optimally detected at 48 h. It should be noted that CD4+ T cells begin to proliferate at 48 h in response to PHA, as detected by the CFSE LPA (see below), so 40–48 h was chosen as the best time point at which to measure CD25+/CD134+CD4+ T cells to reflect the number of precursor cells present in whole blood.

Comparison of the CD25/CD134 assay with standard [3H]thymidine and CFSE proliferation assays

Recall responses to CMV, TT, MTB, and MAI were compared in the whole-blood assay with the two different proliferation assays (Table I). Representative flow cytometry histograms for one of the three individuals are shown in Fig. 2a, comparing the whole-blood assay with the CFSE assay of CD4+ T cell proliferation. Note in
Table I. Direct comparison of whole blood CD4+ CD25+ CD134+ assay, CFSE assay of CD4+ proliferation, and LPA

<table>
<thead>
<tr>
<th>Subject</th>
<th>Ag</th>
<th>CD25+ CD134+ % of CD4</th>
<th>CFSE Proliferation % of CD4</th>
<th>LPA Stimulation Index</th>
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Table I that two individuals were negative for responses to CMV across all assays, while a third individual was positive across all three assays. We analyzed CMV responses from 17 healthy adults (Fig. 2b). The 13 individuals who were CMV seropositive had a median CD25+ CD134+ CD4+ T cell response (interquartile range) of 4.0% (3.0–5.3), which was significantly higher than the median for the 4 seronegative individuals (0.05% (0.025–0.075); p = 0.003).

Furthermore, we tested five different peptides representing common epitopes from CMV pp65 for CD4+ T cells (K. Brown, N. Seddiki, and A. D. Kelleher, manuscript in preparation). Healthy adult controls C002 and C007 had CD25+ CD134+ CD4+ T cell responses to peptides 1 and 4, respectively (Fig. 2c, upper panels). The corresponding peptides elicited proliferation responses in vitro using the CFSE assay (Fig. 2c, lower panels), indicating the fine specificity of the CD25/CD134 assay at the epitope level.

Subject C003 had a very large in vitro response to MTB in all three assays and also had a 30-mm Mantoux skin test result, consistent with having a significant proportion of CD4+ T cells specific for MTB Ags, both in vivo and in vitro. A similarly large MTB response was also observed in one other individual (C006; see Fig. 1b) who had recently worked as a physician in a region with high prevalence of tuberculosis.

CD4+ T cells from subject C002 exhibited a vigorous proliferative response to CMV and had 3.5% CD25+ CD134+ CD4+ T cells (Table I). By comparison, using the ICC assay, subject C002 had a CMV-specific CD4+ T cell response, typically with 0.2% IFN-γ+ single-positive, 0.3% IFN-γ+ IL-2+ double-positive, and 0.1% IL-2+ single-positive CD4+ T cells, as well as a similarly low-level response using the intracellular CD40L assay (15) (supplemental Fig. 2). We closely examined the kinetics of this CMV response using the CFSE assay. Between days 3 and 4, ~6% of CD4+ T cells had divided, with 2- to 3-fold further increases each day thereafter (Fig. 2d). In the same PBMC cultures at 48 h, there were 2.1% CD25+ CD134+ CD4+ T cells (Fig. 2e, left histogram). By using the cell divisions observed in the PHA culture at day 3 as a guide (Fig. 2e, middle histogram), we estimated that in the CMV culture at day 4 (Fig. 2e, right histogram), 1.2% of CD4+ T cells had divided once, 0.7% divided twice, and 0.2% divided three times, with a total precursor frequency of 2.1%, consistent with the CD25/CD134 assay results. Similarly, for subject C001, the response of CD4+ T cells to TT showed ~0.1% of responsive cells by ICC, compared with a CD25+ CD134+ response of 1.4% (Table I). Furthermore, 4.2% of C001 CD4+ T cells had undergone one to four divisions in the CFSE assay at day 6, consistent with a precursor frequency of 1.5% (data not shown).

Apoptosis, cytokine synthesis, and bystander activation

We studied whether apoptosis of bystander CD4+ T cells may have increased the observed level of Ag-specific cells in the cultures. The results show that in cultures of CFSE-labeled PBMC, the level of apoptosis at 24–48 h was extremely low (Fig. 3a, left and middle histograms). Instead, significant apoptosis was observed in Ag-specific cells after they had divided many times at day 6 (Fig. 3a, right histogram), suggesting that the levels of Ag-specific CD4+ T cells were not increased by apoptosis of bystander cells.

To corroborate the Ag specificity of the whole-blood assay, we analyzed concurrent cytokine responses in CD25+ CD134+ cells by adding brefeldin A for the last 14 h of culture and measuring intracellular IFN-γ levels. The results show that a large proportion of CD25+ CD134+ CD4+ T cells in CMV responses was IFN-γ+ (Fig. 3b). Conversely, the majority of IFN-γ+ CD4+ T cells was CD25+ CD134+ (Fig. 3c). In contrast, single-positive CD25+ CD134+ or CD25− CD134+ CD4+ T cells did not appear to be significantly enriched for IFN-γ+ CD4+ T cells (Fig. 3b).

Furthermore, whole blood from pairs of donors, with respective positive and negative results to added Ag, were cultured separated by PET membranes in Transwells. Fig. 3d summarizes the results from seven responsive/nonresponsive donor pairs in three independent experiments. The results show that while the Ag-responsive cultures showed on average 2.1% CD25+ CD134+ CD4+ T cells, there was, on average, only 0.12% CD25+ CD134+ CD4+ T cells in the nonresponsive “bystander” cultures in the same wells but separated by the membrane (see also supplemental Fig. 1).

We also cultured whole blood in the presence of rIL-2, added at a concentration of 10 U/ml for the final 24 h of the culture, in the absence of any added Ag, and did not observe any appearance of bystander CD25+ CD134+ CD4+ T cells. However, it should be noted that addition of exogenous IL-2 alone, in the absence of any recall Ag, did induce a 2-fold increase in single-positive CD25+ CD134+ CD4+ T cells in whole-blood cultures (supplemental Fig. 1), as well as detectable proliferation in cultures of CFSE-labeled PBMC (19).

Monitoring of new CD4+ T cell responses after immunization

We followed the development of CD4+ T cell responses to vaccinia, after inoculation of naive recipients (12), using the whole-blood CD25/CD134 assay. Representative histograms are shown in Fig. 4a. Longitudinal vaccinia-specific responses, as measured by the whole-blood assay, for three different vaccine-naive individuals, are summarized in Fig. 4b. There was a similar pattern of development of responsiveness in all vaccinees, demonstrating that the assay can be used to study the dynamics of the CD4+ T cell vaccine response.

There was a very strong correlation between results from the CD25/CD134 assay and results from IFN-γ+ ICC assays (Fig. 4c) performed on the same longitudinal samples (reported in detail elsewhere (12)). The whole-blood assay appeared to detect 8-fold more Ag-specific cells, and this higher number of Ag-specific CD4+ T cells is much closer to the number of presumptive Ag-specific CD4+ T cells identified by immunophenotyping (12). This is also consistent with our previous observation that the ICC results during primary HIV-1 and primary CMV infections were ~10-fold lower than presumptive Ag-specific CD4+ T cells by immunophenotyping (11).

We also monitored responses to vaccination with SIV-derived peptides in pigtail macaques, with an overall correlation between the whole-blood assay and IFN-γ+ ICC of r2 = 0.20, p = 0.008, as reported elsewhere (20). Similarly, we used CD25+ CD134+...
expression to detect Ag-specific CD4+ T cells in cultures of mouse splenocytes, following a standard immunization protocol with methyl-BSA (data not shown).

Isolation and in vitro expansion of CD25+CD134+ responding CD4+ T cells

CFSE-labeled PBMC were cultured with CMV lysate for 48 h, and then, CD25+CD134+, CD25-CD134-, and CD25+CD134- fractions of CD4+ T cells were purified by cell sorting (Fig. 5a). The cells were then added to wells containing autologous adherent cells and cultured for an additional 5 days with CMV lysate and 20 U/ml IL-2. We found that 2 days after cell sorting (day 4 after initial exposure to Ag in culture), CD25+CD134+ cells formed clusters of blast cells around adherent cells, whereas CD25+CD134- and CD25-CD134- cells remained as small, quiescent cells (Fig. 5b).

Over the next 5 days, CD4+ T cells in cultures of unfractionated CFSE-labeled PBMC proliferated in response to CMV lysate (Fig. 5a, upper right histogram). In the cultures of the sorted cell populations, only the CD25+CD134+ cells proliferated over the next 5 days, whereas CD25+CD134- cells mostly maintained CD25 expression without proliferation, and CD25-CD134- cells showed minimal proliferation and no significant expansion of cell numbers (Fig. 5a, lower histograms).
Measurement of opportunistic pathogen- and HIV-specific CD25+CD134+CD4+ T cells in chronic HIV-1 infection

HIV-infected individuals with advanced disease usually have reduced PBMC proliferative responses to recall Ags, and particularly HIV-1 Ags (21, 22), but at the same time have low but detectable HIV-specific responses by ICC (23). Therefore, we used the CD25/CD134 assay of Ag-specific CD4+ T cells to reassess the level of Ag-specific CD4+ T cells in untreated chronic HIV-1 infection. Samples of whole blood from 17 healthy adult controls and 13 consecutive untreated subjects with established HIV-1 infection were examined.

**Figure 3.** Apoptosis and bystander activation in whole blood cultures. a, Histograms from C002 as in Fig. 2d, showing the level of apoptosis of CD4+ T cells in PBMC cultures containing CMV lysate at various times, as measured by intracellular staining for activated caspase-3, compared with cell divisions in CFSEdim proliferating CD4+ T cells. b, Histograms from donor C008 showing subsets of CD4+ T cells according to cell surface CD25 and CD134 coexpression after incubation of whole blood with CMV for 48 h, including incubation for the last 14 h with brefeldin A. The respective subsets are then shown with their concurrent intracellular IFN-γ levels. Results are representative of three experiments. c, As for b, histograms from donor C008 showing intracellular IFN-γ+ CD4 T cells, after incubation of whole blood with CMV for 48 h, including incubation for the last 14 h with brefeldin A. The intracellular IFN-γ+ CD4 T cells are then shown with their cell surface CD25 and CD134 coexpression. d, Comparison of CD25+CD134+CD4+ T cells in whole-blood cultures separated by PET membranes in Transwells. In each culture, whole blood from two different donors were added to either upper or lower wells, respectively. Only one donor in each pair was responsive to the added Ag, and the other nonresponsive donor blood was the bystander. The results summarize 14 pairings, in three separate experiments, for five different donors (C002, C007, C001, C009, and C010), using CMV lysate or CMV peptides nos. 1 and 4, as described in Fig. 2c. As shown in the figure, the two groups of results were significantly different by Mann-Whitney U test.
infection were incubated with antigenic preparations from common opportunistic pathogens, including lysates of CMV, MTB, and MAI, UV-inactivated HSV-1, as well as a pool of overlapping 15-mer peptides covering HIV-1 Gag.

As shown in Fig. 6, recall responses to CMV, MAI, and HSV-1 were readily detected in healthy adult controls, with some individual responses comprising >10% of CD4 T cells. In the cohort of HIV-infected subjects, responses to CMV, MAI, and HSV-1 were also detected at high levels, up to 18% of CD4 T cells. Responses to HIV-1 Gag peptides were very low in healthy adult controls (median, 0.10%), but in the HIV-infected subjects, responding cells were a median 0.80% of CD4 T cells (p < 0.001 compared with controls), with 6 of 13 subjects having much higher responses, ranging from 1.70 to 4.80% of CD4 T cells.

Discussion
Genetic studies in mice have shown that CD134-CD134L (OX40-OX40L) interactions are crucial for the generation of memory T cells, promoting the survival of effector T cells (24). Consistent with previous observations that CD134 is expressed after TCR engagement, peaking 24–48 h after (24), we have found that there is very little expression of CD134 on unstimulated CD4 T cells in human blood (Ref. 16 and this study). Similarly, after T cell stimulation, up-regulation of the high-affinity IL-2R is important in control of proliferation, differentiation, and apoptosis of CD4 T cells (25). The high-affinity IL-2R is generated by expression of CD25, which is normally only found on a small minority population of CD25 CD4 T regulatory cells in human blood (26), that are also identified as CD127dim and Foxp3 ex vivo (16). Altogether, <0.03% of CD4 T cells in peripheral blood expressed both CD25 and CD134, making it possible to detect specific simultaneous induction of these two cell surface markers following exposure to recall Ags. Additionally, it was observed that the induced level of cell surface expression of CD25 on the Ag-responsive CD4 T cells was much higher than the modest level of CD25 expressed on T regulatory cells (Fig. 1a). These results agree with recent reports that described coexpression of CD25 and CD134 after Ag stimulation of cloned CD4 T cells from PBMC in the settings of autoimmunity (27) or HIV infection (18) and single up-regulation of CD134 in recall responses to MTB Ags (28).

Combining detection of this induced coexpression of CD25 and CD134 with whole-blood culture affords an extremely simple assay that has allowed us to visualize responses to a diverse range of Ags, including viral Ags (derived from CMV, HSV-1, Flu, and HIV-1), bacterial Ags (MTB, MAI, SKSD, and TT), and a fungal Ag (Candida). In all cases, the responses were highly correlated with the current gold standard assay, the LPA, using either [3H]thymidine or CFSE. In another series of experiments following inoculation with vaccinia, the whole-blood CD25/CD134

![FIGURE 4. Appearance of CD25 CD134 CD4 T cells in response to vaccinia inoculation.](http://www.jimmunol.org/)
assay was also highly correlated with, but significantly more sensitive at detecting Ag-specific cells than, the ICC assay detecting IFN-γ. Also, cell sorting directly demonstrated that in PBMC cultured with CMV lysate, the purified CD25+CD134+CD4+ T cell population contained the cells that subsequently proliferated. These results therefore also suggest that this simple means of identifying Ag-specific CD4+ T cells allows separation of live cells for molecular studies and potentially for therapeutic studies.

Surprisingly, we have found, using this assay, that Ag-responsive cells can represent a high proportion of circulating CD4+ T cells. Although it might be argued that some of these responsive cells are bystanders to Ag-specific cells, several observations argue against this interpretation. First, we carefully followed the initial proliferation of cells in CFSE cultures and consistently found a relatively high proportion of cells proliferating in response to recall Ags, more consistent with the CD25/CD134 assay results than with the lower responses seen in ICC assays, particularly for Ag-specific responses best characterized by proliferation assays, such as TT and SKSD. Second, sorted CD25+CD134+CD4+ T cells clearly proliferated when provided with adherent cells, Ag, and exogenous IL-2. Finally, we saw little or no induction of CD25+CD134+CD4+ cells in “bystander” cultures separated from a recall response by a permeable membrane, nor when exogenous IL-2 was added to whole-blood cultures (supplemental Fig. 1). It should be noted that in one study investigating the total response to CMV, 10% of memory CD4+ T cells responded to all available antigenic peptides (29). Furthermore, we had previously shown that CMV-seropositive healthy adults had on average 5% more CD4 with a CTL phenotype, which included CMV-specific cells (14).

The increased number of Ag-responsive CD4+ T cells detected by the CD25/CD134 assay compared with ICC were also consistent with our previous results showing unexpectedly high levels of circulating CD4+ T cells with the phenotype of highly activated CD38highCCR5+ effector cells, which we concluded were Ag-specific, during a primary response to either vaccinia (12) or HIV-1 infection (11). Similar results have recently been described for unexpectedly high levels of CD8+ T cells specific for vaccinia during the primary response following inoculation, using a similar methodology relying upon CD38, Ki-67, and low Bcl-2 expression.
HIV-1 infection \( (n = 17) \) and subjects with untreated asymptomatic chronic HIV-1 infection \( (n = 13) \), in response to pathogen-derived recall Ags, as well as to a pool of 123 overlapping peptides covering the consensus Gag sequence for clade B HIV-1. Box plots show 10th, 25th, median, 75th, and 90th percentiles for each subject group. The difference in response to Gag peptides between the two subject groups was significantly different by Mann-Whitney \( U \) test, \( p < 0.001 \).

(13). It should be noted that very early in the primary response to vaccinia, these highly activated effector cells were detected in the current study in the CD25/CD134 assay but did not proliferate in a standard LPA until 1 wk later when Ag-specific CD4\(^+\) T cells had acquired a resting phenotype, as described in the previous report (12). Similarly, we readily detected HIV-1 Gag-responsive CD4\(^+\) cells in late chronic HIV-1 infection using the CD25/CD134 assay. At this phase of HIV-1 infection, it is well-described that proliferative responses are rarely detected (22). Also, the level of HIV-specific CD4\(^+\) T cells in chronic infection are \( \sim 10 \) times lower using ICC (23) compared with the CD25/CD134 whole-blood assay. In addition to detecting the highly activated cells that don’t proliferate in vitro and are characteristic of untreated HIV-1 infection, it is possible that we were also detecting cells that produced neither IFN-\( \gamma \) nor IL-2. Such cells might include Th17 cells (30) or T regulatory cells (N. Seddiki, K. Brown, J. Zaunders, and A. D. Kelleher, unpublished results) that are not enumerated by multiparameter ICC assays that make a priori assumptions about the cytokines produced in Ag specific responses.

Furthermore, the simplicity of the CD25/CD134 assay affords a number of advantages over current assays, avoiding the inherent variability of using pooled human serum, nor the need for either PBMC isolation or radionuclides. Only cell surface staining is required, avoiding intracellular staining and reducing the complexity of flow cytometric analysis associated with ICC assays. An important application of this assay may be as a simple in vitro test for recall responses to Ags from common pathogens with low immunogenicity such as hepatitis C virus, and responses to autoantigens, as has been described for T cell clones associated with type 1 diabetes (27).

We are also investigating application of the CD25/CD134 assay for recall responses to Ags from common pathogens with low immunogenicity such as hepatitis C virus, and responses to autoantigens, as has been described for T cell clones associated with type 1 diabetes (27). Similarly, it may also be possible to study CD4\(^+\) T cell responses to tumor Ags, particularly following the recently described efficacy of infusion with a melanoma-specific CD4\(^+\) T cell clone (32). Furthermore, we found that the assay can be applied to study of nonhuman primate whole blood as well as to mouse spleen cells.

This methodology for identifying Ag-specific T cells is not dependent on permeabilization of the membrane as required for ICC assays, thereby allowing for live cell sorting of Ag-specific CD4\(^+\) T cells, in turn allowing the functional and molecular characterization of the Ag-specific CD4\(^+\) T cell response. Avoiding selection of cells on the basis of production of particular cytokines potentially allows for the unbiased characterization of the global CD4\(^+\) T cell response and therefore has the potential to provide new insights into the nature of these responses.

In summary, the CD25/CD134 assay has several advantages over current methods, including ease of culture setup, simplicity of preparation for flow cytometric analysis, and sensitivity and coverage of a very broad range of responses, from Ag-specific CD4\(^+\) T cells to polyclonal responses to mitogens. This assay potentially can add much information regarding Ag-specific CD4\(^+\) T cells in humans that is currently lacking.

**Acknowledgments**

We thank the subjects for their cooperation in providing samples as well as the National Institutes of Health Reference Reagents Program for provision of HIV-1 overlapping Gag peptides.

**Disclosures**

J.Z. and A.K., in conjunction with St Vincent’s Hospital, have lodged a patent application for the whole blood CD25/CD134 assay.

**References**

to vaccinia virus and precede development of interleukin-2

Miller, J. D., R. G. van der Most, R. S. Akondy, J. Glidewell, S. Albot,
Human effector and memory CD8+ T cell responses to smallpox and yellow fever

Zaunders, J. J., W. B. Dyer, B. Wang, M. L. Munier, M. Miranda-Saksena,
R. Newton, J. Moore, C. R. Mackay, D. A. Cooper, N. K. Saksena, and
A. D. Kelleher. 2004. Identification of circulating antigen-specific CD4+ T
lymphocytes with a CCR5+ cytotoxic phenotype in an HIV-1 long-term nonpro-
gressor and in CMV infection. Blood 103: 2238–2247.

1: 1–6.

Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay,
interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory

Seddiki, N., B. Santner-Nanan, S. G. Tangye, S. I. Alexander, M. Solomon,
S. Lee, R. Nanan, and B. Fazekas de Saint Groth. 2006. Persistence of naive

2006. OX40 ligation of CD4+ T cells enhances virus-specific CD8+ T cell mem-
ory responses independently of IL-2 and CD4+ T regulatory cell inhibition. J.

A culture amplified multi-parametric intracellular cytokine assay (CAMP-ICC)
345: 1–16.

De Rose, R., R. Mason, L. Lob, V. Peut, M. Z. Smith, C. S. Fernandez,
genicity and efficacy of peptide-pulsed cellular immunotherapy in macaques.

Clerici, M., N. I. Stocks, R. A. Zajac, R. N. Boswell, D. R. Lucey, C. S. Via,
and G. M. Shearer. 1989. Detection of three distinct patterns of T helper cell dys-
function in asymptomatic, human immunodeficiency virus-seropositive patients:
independence of CD4+ cell numbers and clinical staging. J. Clin. Invest. 84:
1892–1899.


Picher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino,
and L. J. Pocker. 1999. HIV-1-specific CD4+ T cells are detectable in most
individuals with active HIV-1 infection, but decline with prolonged viral sup-

Crott, M. 2003. Co-stimulatory members of the TNFR family: keys to effective

Lenardo, M., K. M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang,
17: 221–253.

Shevach, E. M. 2002. CD4+CD25+ suppressor T cells: more questions than

Endl, J., S. Rosinger, B. Schwarz, S. O. Friedrich, G. Rothe, W. Karges,
M. Schlosser, T. Eiermann, D. J. Schendel, and B. O. Boehm. 2006. Coexpres-
sion of CD25 and OX40 (CD134) receptors delineates autoreactive T cells in type

Skoberne, M., T. Malovrh, A. Skralovnik-Stern, and V. Kostnik. 2000. Human
peripheral blood lymphocytes sensitised to PPD respond to in vitro stimulation
with increased expression of CD69 and CD134 activation antigens and produc-
tion of Th-1 type cytokines. Pflugers Arch. 440(5 Suppl.): R58–R60.

Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti,
human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory

antigen-specific CD4+ T cells enhances virus-specific CD8+ T cell mem-
ory responses independently of IL-2 and CD4+ T regulatory cell inhibition. J.

De Rose, R., R. Mason, L. Lob, V. Peut, M. Z. Smith, C. S. Fernandez,
genicity and efficacy of peptide-pulsed cellular immunotherapy in macaques.

Clerici, M., N. I. Stocks, R. A. Zajac, R. N. Boswell, D. R. Lucey, C. S. Via,
and G. M. Shearer. 1989. Detection of three distinct patterns of T helper cell dys-
function in asymptomatic, human immunodeficiency virus-seropositive patients:
independence of CD4+ cell numbers and clinical staging. J. Clin. Invest. 84:
1892–1899.


Picher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino,
and L. J. Pocker. 1999. HIV-1-specific CD4+ T cells are detectable in most
individuals with active HIV-1 infection, but decline with prolonged viral sup-

Crott, M. 2003. Co-stimulatory members of the TNFR family: keys to effective

Lenardo, M., K. M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang,
17: 221–253.

Shevach, E. M. 2002. CD4+CD25+ suppressor T cells: more questions than

Endl, J., S. Rosinger, B. Schwarz, S. O. Friedrich, G. Rothe, W. Karges,
M. Schlosser, T. Eiermann, D. J. Schendel, and B. O. Boehm. 2006. Coexpres-
sion of CD25 and OX40 (CD134) receptors delineates autoreactive T cells in type

Skoberne, M., T. Malovrh, A. Skralovnik-Stern, and V. Kostnik. 2000. Human
peripheral blood lymphocytes sensitised to PPD respond to in vitro stimulation
with increased expression of CD69 and CD134 activation antigens and produc-
tion of Th-1 type cytokines. Pflugers Arch. 440(5 Suppl.): R58–R60.

Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti,
human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory

Bettelli, E., T. Korn, and V. K. Kuchroo. 2007. Th1?: the third member of the


Hunder, N. N., H. Wallen, J. Cao, D. W. Hendricks, J. Z. Reilly, R. Rodmyre,
A. Jungbluth, S. Gnatic, J. A. Thompson, and C. Yee. 2008. Treatment of met-
astatic melanoma with autologous CD4+ T cell against NY-ESO-1. N. Engl.