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A Cytokine-Independent Approach To Identify Antigen-Specific Human Germinal Center T Follicular Helper Cells and Rare Antigen-Specific CD4+ T Cells in Blood

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Detection of Ag-specific CD4+ T cells is central to the study of many human infectious diseases, vaccines, and autoimmune diseases. However, such cells are generally rare and heterogeneous in their cytokine profiles. Identification of Ag-specific germinal center (GC) T follicular helper (Tfh) cells by cytokine production has been particularly problematic. The function of a GC Tfh cell is to selectively help adjacent GC B cells via cognate interaction; thus, GC Tfh cells may be stingly cytokine producers, fundamentally different from Th1 or Th17 cells in the quantities of cytokines produced. Conventional identification of Ag-specific cells by intracellular cytokine staining relies on the ability of the CD4+ T cell to generate substantial amounts of cytokine. To address this problem, we have developed a cytokine-independent activation-induced marker (AIM) methodology to identify Ag-specific GC Tfh cells in human lymphoid tissue. Whereas Group A Streptococcus–specific GC Tfh cells produced minimal detectable cytokines by intracellular cytokine staining, the AIM method identified 85-fold more Ag-specific GC Tfh cells. Intriguingly, these GC Tfh cells consistently expressed programmed death 1 upon activation. AIM also detected non-Tfh cells in lymphoid tissue. As such, we applied AIM for identification of rare Ag-specific CD4+ T cells in human peripheral blood. Dengue, tuberculosis, and pertussis vaccine–specific CD4+ T cells were readily detectable by AIM. In summary, cytokine assays missed 98% of Ag-tissue. As such, we applied AIM for identification of rare Ag-specific CD4+ T cells in human peripheral blood.

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Abbreviations used in this article: AIM, activation-induced marker; aP, acellular pertussis; CTV, Cell Trace Violet; DENV, dengue virus; GC, germinal center; ICS, intracellular cytokine staining; IGRA, IFN-γ release assay; IRB, institutional review board; LCL, lymphoblastoid cell line; LJI, La Jolla Institute for Allergy and Immunology; LTBI, latent tuberculosis infection; mTfh, mantle Tfh; PD-1, programmed death 1; PD-L1, programmed death ligand 1; pMHC, peptide:MHC, SEB, staphylococcal enterotoxin B; Tfh, T follicular helper.

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that conventional cytokine staining missed 98% of human Ag-specific GC Tfh cells. We further determined that AIM is a highly sensitive technique valuable for detecting human CD4+ T cells specific for a range of viral and bacterial Ags.

Materials and Methods

**Human samples**

Fresh tonsils were obtained from pediatric donors undergoing tonsillectomy at Rady Children’s Hospital or the Naval Medical Center. Informed consent was obtained from all donors under protocols approved by the institutional review boards (IRBs) of the University of California, San Diego, the La Jolla Institute for Allergy and Immunology (LJI), and the Naval Medical Center. Tonsillar mononuclear cells were obtained by homogenizing the tissue using a wire mesh, passage through a cell strainer, and isolation via Ficoll density gradient using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). For the dengue virus (DENV) studies, peripheral blood was obtained from the National Blood Center and approved by the IRBs of both LJI and the Medical Faculty, University of Colombo (16). For the *Mycobacterium tuberculosis* studies, healthy control subjects or individuals with latent tuberculosis infection (LTBI) were obtained from the University of California, San Diego Antiviral Research Center. LTBI status was confirmed by a positive IFN-γ release assay (IGRA; Quantiferon-TB Gold In-Tube [Cellsetis] or T-Spot.TB [Oxford Immunotec]), and healthy controls all had a negative IGRA. None of the subjects had received a Bacillus Calmette-Guérin vaccine. For the pertussis studies, individuals who were originally primed with either acellular pertussis (aP) or whole cell pertussis vaccine were from San Diego, California. A subset of these donors was boosted with aP within 3 mo of donation. Informed consent was obtained from all donors and approved by the IRB at LJI. All individuals included in the EBV/CMV studies were assumed to have been exposed to one or both of these viruses.

**PBMC isolation**

PBMCs were isolated by density gradient centrifugation, according to manufacturer’s instructions. Cells were suspended in FBS containing 10% dimethyl sulfoxide and cryopreserved in liquid nitrogen.

**Serology**

DENV seropositivity was determined by dengue IgG ELISA as previously described (17). Flow cytometry–based neutralization assays were performed for further characterization of seropositive donors, as previously described (18). Neutralization assays determined that all DENV donors included in this study have experienced infection with more than one serotype.

**Peptides**

Peptides were synthesized by A and A (San Diego) as crude material on a 1-mg scale. Individual peptides were resuspended in DMSO, and equal amounts of each peptide were pooled to construct peptide pools. The pools used in this study were DENV [32 peptides, previously defined epitopes (16)], ESAT-6 [22 peptides, 15-mers overlapping by 10 and optimal epitopes (19)], CFP10 [21 peptides, 15-mers overlapping by 10 and optimal epitopes (19)], EBV/CMV [122 previously defined epitopes (20)], and pertussis [132 previously defined epitopes (21)]. ESAT-6 and CFP10 proteins are specific for the *M. tuberculosis* complex, which includes *M. bovis* but not the *Bacillus* Calmette-Guérin vaccine that has the region encoding ESAT-6 and CFP10 deleted (19). The resulting peptide pools of >100 peptides were then lyophilized and resuspended in DMSO to minimize DMSO concentrations.

**Intracellular cytokine staining**

Cryopreserved tonsillar cells were thawed and cultured in serum-free AIM-V media (Life Technologies, Grand Island, NY) overnight for 18 h. Cells were stimulated with 10 ng/ml heat-inactivated antibiotic-killed Group A *Streptococcus*, strain 5448. Four hours before staining, cells were incubated with 10 μg/ml brefeldin A. As a positive control, cells were stimulated with 25 ng/ml PMA and 1 μg/ml ionomycin in the presence of brefeldin A for 4 h.

**Flow cytometry**

Cells were labeled with fixable viability dye eFluor 780 or eFluor 506 (eBioscience). FACS panels used for the AIM assay are detailed in Supplemental Table I. Anti-human Abs for surface staining, by company as follows: eBioscience (San Diego, CA): CD19 eFluor 780 (clone HIB19), CD14 eFluor 780 (clone 61D3), CD16 eFluor 780 (clone eBioCB16), CD8a eFluor 780 (clone RPA-T8), CD3 AF700 (clone UCHT1), CD4 allophycocyanin, FITC or eFluor 780 (clone RPA-T4), CXC5R5 allophycocyanin (clone MUSU-BEE), CD25 PE and PE-Cy5.7 (clone BC96), PD-1-PE-Cy5.7 (clone HIB19), CD14 V500 (clone M5E2), CD25 FITC (clone M-A251). Intra-cellular cytokine Abs used included: TNF-αF488 (clone MAb11), CD40L PerCP-eFluor 710 (clone 24-31), IFN-γ PE-Cyanine7 (clone 45.B3), IL-21 PE (clone eBio3A3-N2), IL-10 PE (clone JES5-29D7), IL-4–PE-Cy5.7 (clone HIB19), CD14 V500 (clone M5E2), CD25 FITC (clone M-A251). Intra-cellular cytokine Abs used included: TNF-αF488 (clone MAb11), CD40L PerCP-eFluor 710 (clone 24-31), IFN-γ PE-Cyanine7 (clone 45.B3), IL-21 PE (clone eBio3A3-N2), IL-10 PE (clone JES5-29D7), IL-4–PE-Cy5.7 (clone HIB19), CD14 V500 (clone M5E2), CD25 FITC (clone M-A251). Intracellular cytokine Abs used included: TNF-αF488 (clone MAb11), CD40L PerCP-eFluor 710 (clone 24-31), IFN-γ PE-Cyanine7 (clone 45.B3), IL-21 PE (clone eBio3A3-N2), IL-10 PE (clone JES5-29D7), IL-4–PE-Cy5.7 (clone HIB19), CD14 V500 (clone M5E2), CD25 FITC (clone M-A251). Intracellular cytokine Abs used included: TNF-αF488 (clone MAb11), CD40L PerCP-eFluor 710 (clone 24-31), IFN-γ PE-Cyanine7 (clone 45.B3), IL-21 PE (clone eBio3A3-N2), IL-10 PE (clone JES5-29D7), IL-4–PE-Cy5.7 (clone HIB19), CD14 V500 (clone M5E2), CD25 FITC (clone M-A251).

**Cellular proliferation**

Cryopreserved tonsillar cells were thawed and cultured in RPMI 1640 medium containing 10% Human AB serum (Gemini Bio-Products, West Sacramento, CA). As a positive control, cells were stimulated with 100 ng/ml staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL). For Ag-specific proliferation, cells were stimulated with Streptolysin O, which had been previously heat inactivated at 65°C for 20 min (Sigma, St. Louis, MO). Cells were labeled with Cell Trace Violet (CTV; Thermo Scientific, Waltham, MA) and cultured for 96 h in medium supplemented with 4 ng/ml IL-7 (Peprotech, Rocky Hill, NJ). Cells were labeled with fixable viability dye eFluor 780 (eBioscience). Tonsils were sorted using BD FACSAria III to isolate GC Tfh cells (Live CD19+, CD4+, CD8a-, CD4+ CD45RA+CXCR5+PD-1+), mTfh cells (Live CD19+, CD4+, CD16+, CD8a-, CD4+CD45RA+CXCR5+PD-1+), and non-Tfh cells (Live CD19+, CD4+, CD16+, CD8a-, CD4+CD45RA+CXCR5+). Sorted cells were incubated at a 1×10⁶ T cell:1 APC ratio with irradiated autologous lymphoblastoid cell lines (LCLs) created from each donor tonsil as APCs. Cells were cultured for 96 h and acquired on BD Fortessa.

**AIM assay**

Cryopreserved tonsillar cells were thawed and cultured in serum-free AIM-V media (Life Technologies, Grand Island, NY) overnight for 18 h. Cells were stimulated with 10 ng/ml heat-inactivated antibiotic-killed Group A *Streptococcus*, strain 5448. As a positive control, cells were stimulated with 1 μg/ml SEB (Toxin Technology, Sarasota, FL). Cells were cultured for 18 h and acquired on BD Fortessa. Brefeldin A or monensin or other golgi inhibitors (e.g. Golgi Stop) must not be added to the culture. PBMCs were thawed and cultured in complete RPMI 1640 with 5% human AB serum (Gemini Bio-Products) for 24 h. Cells were stimulated with 2 μg/ml peptide pools or 10 μg/ml PHA.

**ELISPOT assays**

Ex vivo IFN-γ ELISPOT assays were used for *M. tuberculosis* peptide pools as previously described (22). Responses were considered positive if the net spot-forming cells per 10⁶ were ≥20, the stimulation index ≥2, and p ≤ 0.05 (Student’s t test, mean of triplicate values of the response against relevant pools versus the DMSO control). For pertussis, PBMCs were cultured for in vitro expansion by incubating in RPMI 1640 supplemented with 5% human AB serum (Gemini Bioscience), GlutaMAX (Life Technologies), and penicillin/streptomycin (Omega Scientific) at 2 × 10⁵/ml in the presence of individual pertussis Ags; filamentous hemagglutinin (Reagent Proteins), pertactin (Reagent Proteins), formylglycine fixed peptide toxin (‘PT’, Reagent Proteins), and fimbriae 2/3 (List Biological Labs), each at 5 μg/ml. Every 3 d, 10 U/ml IL-2 in media were added to the cultures. After 14 d of culture, responses to peptides were measured by IFN-γ and IL-5 dual ELISPOT as previously described (23). Response had to fulfill all three criteria described earlier to be considered positive.

**Statistics**

Comparisons between groups were made using the Mann–Whitney U test. Prism 5.0 (GraphPad) was used for all of these calculations.
Results

Detection of Ag-specific human GC Tfh cells by intracellular cytokine production

To identify Ag-specific GC Tfh cells within tonsils, we tested for expression of different cytokines by intracellular cytokine staining (ICS). Ag-experienced CD4+ T cells within lymphoid tissue were categorized as GC Tfh (CD4+CD45RA- CXCR5hiPD-1hi), follicular mantle Tfh (mTfh, also known as Tfh or pre-Tfh; CD4+ CD45RA- CXCR5PD-1-), and non-Tfh or effector cells (CD4+ CD45RA- CXCR5-) (Fig. 1A). One ubiquitous pathogen is Group A Streptococcus, which is the causative agent for strep throat. Given the prevalence of strep throat within the pediatric population and ~10–15% asymptomatic carriage rate in healthy children, we reasoned that essentially all individuals have been exposed to this pathogen (24–27). We thus tested for production of IL-21 after stimulation with whole heat-inactivated antibiotic-killed Group A Streptococcus. Very few cells produced detectable IL-21 after stimulation with Group A Streptococcus (median <0.1%, Fig. 1A), and the IL-21 mean fluorescence intensity was close to the limit of detection, which was insufficient to allow for confident identification of Streptococcus-specific GC Tfh cells. This is similar to other studies in which Ag-specific GC Tfh responses have been difficult to identify via IL-21 production (15, 28). Streptococcus-specific TCR stimulation did not increase IL-4 production (data not shown). We then tested other cytokines, including TNF, CD40L, IFN-γ, IL-13, IL-17, and IL-10 (Fig. 1B, 1C). There was minimal IFN-γ, IL-13, IL-17, and IL-10 expression by GC Tfh cells in response to Ag stimulation (Supplemental Fig. 1), consistent with previous observations that GC Tfh cells produced minimal Th1, Th2, or Th17 cytokines upon strong PMA/ionomycin stimulation (8, 29, 30).

Basal levels of CD40L were high and heterogenous from donor to donor, making identification of Ag-specific cells based on CD40L alone infeasible. Intracellular staining for TNF and CD40L upregulation identified Streptococcus-specific GC Tfh cells better than any other cytokine or combination of cytokine plus intracellular CD40L (Fig. 1B). Nevertheless, only 0.053% of GC Tfh cells were Streptococcus specific based on TNF and CD40L upregulation. These data prompted us to conclude that cytokine production may significantly underestimate Ag-specific GC Tfh cell frequencies and prompted us to explore cytokine-independent approaches to identify Ag-specific human GC Tfh cells.

Detection of Ag-specific GC Tfh cells by cellular proliferation

We next sought to identify Ag-specific GC Tfh cells by proliferation. GC Tfh cells are not end-stage differentiated cells. GC Tfh cells can migrate within and between GCs (31), proliferate (32–34), and become memory cells (1, 33, 35–37). Maintenance in the GC state requires TCR triggering by surrounding GC B cells, as well as costimulation by other activation markers. We therefore assessed GC Tfh proliferation after a 96-h culture of CTV-labeled tonsillar cells in the presence or absence of SEB. We were able to identify SEB-responsive cells based on proliferative responses within the GC Tfh (CD4+CD45RA- CXCR5hiPD-1hi), mTfh (CD4+CD45RA- CXCR5PD-1-), and non-Tfh populations (CD4+CD45RA- CXCR5-) (Fig. 2A). Interestingly, proliferating SEB-responsive cells upregulated CD25 and OX40 and CD25 in vitro, we sought to determine whether the proliferating SEB-responsive cells identified as CXCR5hiPD-1hi after proliferation were truly GC Tfh cells. To establish that we were observing proliferating GC Tfh cells after 96 h in culture, separate GC Tfh, mTfh, and non-Tfh cell cultures were required. We created autologous LCLs for each tonsil donor as APCs. GC Tfh, mTfh, and non-Tfh cells were FACs sorted from donors and cultured with autologous LCL in the presence or absence of SEB. Proliferating GC Tfh cells highly upregulated both CD25 and OX40 in response to SEB, a potent TCR stimulus (Fig. 2C). CD25 and OX40 were also upregulated on SEB-responsive mTfh and non-Tfh cells (Fig. 2C). Thus, Ag-specific GC Tfh could potentially be identified by proliferation in response to Ag. We observed extensive SEB-responsive proliferation in the GC Tfh sorted populations, as the LCLs had much improved Ag-presenting capacity over primary B cells. Moreover, small quantities of IL-7 were added to enhance survival during culture, at a concentration that does not promote T cell proliferation. Subsequent CTV proliferation experiments with whole Group A Streptococcus, heat-inactivated Streptolysin O (a serodiagnostic marker for recent Group A Streptococcus infection), or diphtheria CRM197 were unable to consistently identify GC Tfh cells by proliferation because of variable low-level non-specific proliferation (data not shown).

Detection of Ag-specific GC Tfh cells by activation marker induction

Tfh help is the primary positive selection step in a GC (1, 2). Tfh cells selectively provide help to the B cells with the most antigenic peptides, which are the high-affinity B cells that have bound and endocytosed the most Ag (44, 45). The amount of help provided by the Tfh cell directly translates to the number of cell divisions and mutations a GC B cell will undergo in the dark zone in a single selection cycle (46, 47). One of the functional challenges for Tfh cells in GCs is that they are constantly exposed to Ag. They must therefore be able to distinguish between GC B cells with modest differences in their numbers of p:MHC and respond by providing help preferentially to the cognate B cells presenting more p:MHC complexes. Thus, we hypothesized that although GC Tfh cells retain sensitive TCR signaling, the outcome of the TCR detection of cognate Ag is not reflected by cytokine production, as GC Tfh cells may produce only infinitesimal quantities of cytokine necessary to signal the GC B cell in synaptic contact. We therefore explored whether changes in expression of proteins other than cytokines might be more consistent indicators of TCR signaling by GC Tfh cells. Based on our observation that proliferating GC Tfh cells upregulated OX40 and CD25 in vitro, we sought to determine whether CD25 and OX40 were consistently upregulated on Ag-specific GC Tfh cells in response to TCR stimulation at more proximal time points. After an 18-h SEB stimulation, we observed a clear and robust population of CD25OX40 GC Tfh cells, consistent with the expected frequency of cells expressing SEB-reactive TCRβ-chains (Fig. 3B). Each tonsil varied in the frequency of CD25OX40 GC Tfh cells observed after 18 h in vitro in the absence of exogenous Ag, with a mean of 4.1%. We speculated that the CD25OX40 GC Tfh cells observed in the absence of exogenous Ag were responding to Ag presented by GC B cells and other APCs in the in vitro culture, as each tonsil contains Ag (Supplemental Fig. 3A). SEB stimulation of GC Tfh cells from
14 donors yielded a mean frequency rate of 44% CD25+OX40+ SEB-responsive GC Tfh cells (Fig 3C) [At a concentration of 1 μg/ml, SEB stimulates proliferation of lower-affinity TCR Vβ families (48)]. Thus, CD25 and OX40 were reproducible indicators of GC Tfh cell TCR stimulation.

We next tested whether Streptococcus-specific GC Tfh cells could be detected by CD25 and OX40 expression after stimulation with Group A Streptococcus Ag. Upon stimulation, we observed a robust Streptococcus-specific GC Tfh cell population based on CD25+OX40+ coexpression (Fig. 3D). This activation-induced marker (AIM) assay detected Streptococcus-specific GC Tfh, mTfh, and non-Tfh cells. Representative FACS plots after 18-h culture of tonsil cells with 10 μg/ml Group A Streptococcus or PMA/ionomycin, as a positive control. Limit of detection denoted by the gray dotted line. Data are from nine donors. The response by Ag-specific cells was background subtracted for each donor. (C) Median intracellular cytokine production of IL-4+IL-13+, IL-10+, and IL-17+ by Streptococcus-specific GC Tfh, mTfh, and non-Tfh cells. Limit of detection denoted by the gray dotted line. Data are from 19 donors.

85-fold improvement in sensitivity for quantifying Streptococcus-specific GC Tfh cells. Thus, cytokine-dependent identification missed >98% of Ag-specific GC Tfh cells.

In a separate study, RNAseq analysis of rhesus macaque (Macaca mulatta) lymph node GC Tfh cells revealed that PD-L1 was robustly induced upon TCR stimulation (49). We therefore examined whether PD-L1 was upregulated on Ag-specific GC Tfh cells upon Streptococcus Ag stimulation or SEB stimulation. Similar to CD25+OX40+ coexpression, we observed a population of CD25+PD-L1+ GC Tfh cells after either Group A Streptococcus or SEB stimulation (Fig. 3E). PD-L1 expression was present on 95.7% of Streptococcus-specific GC Tfh cells expressing CD25+OX40+ (Fig. 3E). PD-L1 thus functions as a TCR signaling output after Ag-specific GC Tfh cells stimulation, and PD-L1, OX40, and CD25 are interchangeable for identification of responding GC Tfh cells.

After establishing a highly sensitive method to detect Ag-specific GC Tfh cells, we next wanted to assess the ability of the AIM assay to identify Streptococcus-specific mTfh and non-Tfh. As with GC Tfh cells, we found CD25, OX40, and PD-L1 expression on Streptococcus-specific mTfh and non-Tfh cells upon stimulation.
with Group A *Streptococcus* Ag (Fig. 4). *Streptococcus*-specific non-Tfh cells or effector cells (CD4+CD45RA2CXCR52) could be identified by the AIM assay as either CD25+OX40+ (Fig. 4A, 4C) or CD25+PD-L1+ (Fig. 4B, 4D). Thus, the cell-surface proteins CD25, OX40, and PD-L1 appear to be activation markers expressed by Ag-specific human CD4+ T cells, independent of whether the cells were GC Tfh cells. Therefore, the AIM assay may be of practical utility for identifying Ag-specific CD4+ T cells in lymphoid tissue, independent of abundant production of a given cytokine.

**AIM detects Ag-specific CD4 T cells in human peripheral blood**

We identified CD25, OX40, and PD-L1 as TCR activation-dependent markers of Ag-specific human GC Tfh cells. Independently, CD25+OX40+ coexpression was explored for detection of memory CD4+ T cells to EBV/CMV and *Mycobacterium tuberculosis* in human peripheral blood (50, 51). Those studies frequently used ∼48-h stimulation periods, which may allow sufficient time for significant T cell proliferation or death, thus skewing the quantitation of responding T cells. In addition, 50% of LTBI cases, which are known to respond to *M. tuberculosis* Ags in the QuantiFERON-TB Gold clinical test to determine previous exposure to *M. tuberculosis* (36). Healthy control subjects (QuantiFERON-TB Gold negative) were compared with individuals with LTBI who are by definition QuantiFERON-TB Gold+ in an IFN-γ ELISPOT assay, PBMCs from most LTBI individuals produced IFN-γ in response to both ESAT-6 and CFP10 (Fig. 6A). In the AIM assay, PBMCs from LTBI individuals had a 14.5-fold increase in the percentage of CD25+OX40+ memory CD4+ T cells after stimulation with ESAT-6, and a nearly 40-fold increase after stimulation with CFP10 compared with healthy control subjects (p = 0.027 and p < 0.0001, respectively) (Fig. 6B). Of interest, 100% of LTBI donors were detected as positive by AIM using CFP10, in contrast with ELISPOT (Fig. 6B). Identification of *M. tuberculosis*-specific and EBV/CMV-specific CD4+ T cells by AIM was specific for Ag-experienced CD4+ T cells (Figs. 5A, 6B). Notably, background noise in the AIM assay was very low when using PBMCs (Figs. 5A, 6B), in contrast with Ag-containing tonsillar tissue (Figs. 2B, 3B), consistent with the background signal in tonsils being due to an Ag-specific CD4+ T cell responding to Ags in tonsil, given that tonsils are a sentinel tissue. There was also undetectable bystander activation in the PBMCs, as there was no upregulation of CD25+OX40+ on CD8+ T cells or naive CD4+ T cells (Figs. 5A, 7, 8A).

We performed a test of AIM specificity using an *M. tuberculosis*-specific class II tetramer (HLA DRB5*01:01 CFP1052–66 tetramer).
The HLA DRB5*01:01 LTBI donor selected was found to have a very strong *M. tuberculosis*–specific CD4+ T cell response by AIM (17%; Fig. 6B) and was also EBV/CMV+ (4% EBV/CMV+ CD4+ T cells; Fig. 5A). When PBMCs from the HLA DRB5*01:01 *M. tuberculosis* EBV/CMV+ donor were stimulated with the EBV/CMV peptide pool and then stimulated with the *M. tuberculosis* tetramer, the *M. tuberculosis*–tetramer–specific CD4+ T cells showed no evidence of bystander activation (Fig. 5B, 5C). Thus, the AIM method specifically detects Ag-specific CD4+ T cells.

We then sought to assess the applicability of AIM for identifying DENV-specific CD4+ T cells or rare pertussis vaccine–specific memory CD4+ T cells in peripheral blood. We first focused on identifying DENV-specific CD4+ T cell responses using PBMCs from healthy individuals from a highly endemic area who were either seropositive (DENV+) or seronegative (DENV−) for prior DENV infection. High frequencies of DENV-specific CD4+ T cells have been correlated with a decreased likelihood of developing DENV hemorrhagic fever (16, 52). We were able to discern DENV-specific memory CD4+ T cell responses using the AIM assay (Fig. 7). Using PBMCs from five DENV+ individuals bearing the HLA DRB1*04:01 allele, we were able to detect 0.08–0.94% DENV-specific memory CD4+ T cells after stimulation with dengue DRB1*04:01-restricted epitopes after 24 h in culture, *p* = 0.016 (16). CD8+ T cells did not respond in the AIM assay to the dengue class II peptides, as expected (Fig. 7).

Pertussis-specific CD4+ T cells have been detected in most individuals only after 14-d expansion of cells in vitro followed by cytokine ELISPOT. We examined whether pertussis-specific CD4+ T cells could be detected directly ex vivo by the AIM assay, as a stringent test of assay sensitivity. Ten donors were tested, and
pertussis-specific CD4+ T cells in all 10 donors were detected by AIM (CD25+OX40+: mean = 0.97, range 0.45–2.32%, Fig. 8A; CD25+PD-L1+: mean = 0.36, range 0.0134–2.13%, Fig. 8B). These donors were healthy individuals who had no recent pertussis immunizations. Pertussis-specific CD4+ T cells were memory cells (Fig. 8C) and were found to predominantly (80%) have an effector memory phenotype (CD45RA-2 CCR7-2) (Fig. 8C). Two donors received an aP vaccine boost within a 3-mo period from their pre-aP boost PBMC specimen. The AIM assay detected an increase in pertussis-specific CD4+ T cell responses after the aP vaccination (Fig. 8D). In comparison with a 14-d restimulation ELISPOT assay, the 24-h AIM assay followed the same data trends as the 14-d restimulation ELISPOT assay (Fig. 8D). The AIM assay was simpler and shorter, and allowed for extensive phenotypic cellular characterization at the single-cell level by multiparameter flow cytometry.

Discussion

We have shown that it is extremely difficult to quantify Ag-specific GC Tfh cells within human tonsils using the traditional ICS method because GC Tfh cells are inherently stingy cytokine producers and make little to no detectable cytokine upon Ag stimulation. How-
ever, we determined that Streptococcus-specific GC Tfh cells do retain sensitive TCR activation in response to Ag, resulting in up-regulation of CD25, OX40, and PD-L1. We exploited this biology to identify Ag-specific GC Tfh cells with 85-fold greater sensitivity than ICS. Furthermore, we observed that the utility of this approach is not limited to GC Tfh cells but can be extrapolated to other CD4+ T cells within human lymphoid tissue and peripheral blood.

Upon TCR triggering of an Ag-experienced CD4+ T cell, co-stimulatory molecules and coinhibitory molecules are upregulated (53). The interplay of costimulatory and coinhibitory molecules and which signals dominate are part of Ag-specific CD4+ T cell development (54). OX40 is one such receptor, upregulated on Ag-experienced CD4+ T cells as early 1–4 h upon TCR stimulation, depending on the type of CD4+ T cell and the strength of the p: MHC II complex (3). OX40 on Ag-specific CD4+ T cells interacts with OX40L on APCs to promote CD4+ T cell survival (3). Similarly, CD25 is another activation marker quickly upregulated upon TCR stimulation and sustained after expression of IL-2 in an autocrine or paracrine manner (55). For GC Tfh cells, OX40 up-regulation upon Ag stimulation by GC B cells in the follicle was not unexpected as OX40:OX40L constitutes part of the GC Tfh: GC B cell cognate interaction (1). However, CD25 upregulation on GC Tfh cells within the GC follicle is counterintuitive because IL-2 is known to inhibit Tfh differentiation (40, 41). GC Tfh cells expressed low amounts of CD25 directly ex vivo (Supplemental Fig. 2C), even though most GC Tfh cells experience frequent TCR stimulation (56, 57). Thus, a negative feedback mechanism to limit CD25 expression appears to be engaged in vivo but not in vitro. An alternative explanation is that stimulation with a small amount of Ag in vitro is supraphysiologic and does not recapitulate what is seen in vivo, permitting CD25 upregulation. Many receptors that inhibit GC Tfh cells are expressed by GC Tfh cells (58, 59), pointing to a central role of inhibitory pathways in maintaining appropriate Tfh biology in GCs.

PD-L1 upregulation on Streptococcus-specific GC Tfh was also unexpected. A role of PD-L1 on GC Tfh cells has not been described. PD-L1 expression is not restricted to myeloid cells and non-hematopoietic cells; PD-L1 expression has previously been observed on both CD4+ and CD8+ T cells in certain conditions (60). PD-L1 has been found on activated CD4+ T cells in rheumatoid arthritis patients (61, 62). For GC Tfh cells, interpretation of the function of PD-L1 is complicated because these cells also express PD-1. The PD-1/PD-L1 axis is involved in GC Tfh interactions with GC B cells, with PD-1 highly expressed by GC Tfh and PD-L1 expressed by GC B cells (63). High PD-1 expression prevents GC Tfh cell proliferation, allowing the GC to function properly, as the purpose of the GC is to drive GC B cell proliferation and selection while maintaining a relatively constant number of GC Tfh cells (34, 58, 64). Expression of PD-L1 by an activated GC Tfh cell may inhibit neighboring PD-1+ GC Tfh cells in a bystander manner, as an additional mechanism to limit excessive GC Tfh cell activation and/or proliferation.

The AIM method exhibited greatly increased sensitivity for detecting Streptococcus-specific GC Tfh cells in comparison with ICS. IL-21 protein production was rarely detected by ICS of GC Tfh cells after Ag stimulation. Detection of IL-21 protein from Ag-specific human GC Tfh cells within lymphoid tissue has been difficult, with perhaps only one reported success (15). In macaque studies, there has been little to no detection by ICS of IL-21 protein induction in Ag-specific GC Tfh cells (28, 65). IL-21 RNA can be detected in stimulated human GC Tfh cells, and an IL-21 fluorescent reporter mouse detects shifts in fluorescent reporter protein expression after Ag stimulation in vivo (56), consistent with Il21 mRNA induction. Thus, it appears that GC Tfh cells are intrinsically stingy producers of IL-21 protein. Note that...
this cytokine biology of GC Tfh cells within lymphoid tissue
does not extend to circulating resting memory Tfh cells or
circulating recently activated Tfh-like cells in blood (9, 11, 66, 67),
which are not GC Tfh cells. GC Tfh cells are not in peripheral
blood. CD4+ T cells in peripheral blood much more readily
produce cytokine than do bona fide GC Tfh cells in lymphoid
tissues. Peripheral Tfh cells consist of multiple populations,
but the vast majority (∼99%) (9) can be categorized as resting
central memory Tfh cells (based on their resting phenotype),
and a small population (∼1–5%) can be characterized as re-
cently activated Tfh-like cells, based on their activated phe-
notype (ICOS+PD-1hiKi67+, but no detectable Bcl6 protein, or
Maf, or CD200). A significant fraction of the resting central
memory Tfh cells circulating through blood readily produce cyto-
kines, including IL-21, as shown by multiple laboratories. GC Tfh
cells are quite different, as they are more differentiated cells, in a
very different environment, and have much more restricted cytokine
production.

As with ICS, the AIM technique is also subject to the potential
concern of possible bystander activation. We have demonstrated a
lack of bystander activation by several independent tests. Class II
peptide pools DENV specific for HLA DRB1*04:01 did not induce
CD25+OX40+ expression on CD8+ T cells. Second, minimal
background was observed in PBMCs. Finally, we demonstrated a
lack of M. tuberculosis-tetramer+ cells among the CD25+OX40+
CD4+ T cells stimulated with an unrelated Ag.

Although identification of Ag-specific GC Tfh cells by CD25,
OX40, and PD-L1 coexpression is novel, CD25+OX40+ coex-
pression has previously been studied in peripheral blood (50, 51,
68, 69). We show in this study that DENV-specific and pertussis-
specific memory CD4+ T cells are sensitively detected in pe-
ripheral blood by this approach, and we show controls for the
specificity of the methodology. For DENV infections, the AIM
assay could potentially be useful in detecting Ag-specific re-
sponses irrespective of the functional cytokine specificity. Indeed
it has been reported that severe DENV infection–associated pa-

**FIGURE 8.** Detection of pertussis-specific CD4+ T cells in peripheral blood using a pertussis peptide megapool. (A) AIM+ (CD25+OX40+) memory
CD4+ T cells (CD45RA−CCR7−CD4+), naive CD4+ T cells (CD45RA−CCR7−CD4+), memory CD8+ T cells (CD45RA−CCR7−CD8+), and naive CD8+
T cells (CD45RA−CCR7−CD8+) were quantified. ****p < 0.0001. (B) CD25+PD-L1+ memory CD4+ T cells were quantified (****p < 0.0001) in 10
individuals. (C) Quantification of AIM+ (CD25+OX40+) pertussis-specific within CD4+ T cell subsets. Pertussis-specific cells were predominantly in the
CD45RA−CCR7− (***p = 0.0079). (D) Comparison of AIM+ (CD25+OX40+) pertussis-specific and ELISPOT pertussis-specific responses in individuals
before and after aP booster vaccination.
thology is linked to complex patterns of cytokine production, and the AIM assay will ensure detection regardless of the effector specificity of the DENV-specific T cells (52). In addition, given the resurgence of pertussis in the United States, the AIM assay could potentially identify children with waning vaccine responses who may warrant more frequent booster vaccinations via ex vivo analysis of their pertussis-specific CD4+ T cell responses (70–72).

In summary, Ag-specific GC Tfh, mTfh, and non-Tfh cells can be detected with great sensitivity within secondary lymphoid tissue using AIM. This method will likely be valuable for understanding GC biology as it applies to infections, cancer, and autoimmune disease. The wide applicability of this assay also makes this ideal for detecting rare ex vivo human Ag-specific CD4+ T cell responses. Moreover, because AIM is a live cell assay, it is well suited in combination for downstream applications.

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Disclosures
The authors have no financial conflicts of interest.

References
SUPPLEMENTARY FIGURE 1. Cytokine production by GC Tfh cells is low. This figure shows representative plots of the data in Figure 1. Representative FACS plots of intracellular cytokine production of IL-4⁺IL-13⁻, IL-10⁺, and IL-17⁺ by Strep-specific GC Tfh, mTfh, and non-Tfh cells, normalized to unstimulated cells per each donor. Tonsil cells were stimulated with 10μg/mL Strep or PMA/Ionomycin, as a positive control.
SUPPLEMENTARY FIGURE 2. Fresh tonsillar cells were stained for CD25 directly ex vivo, without re-stimulation. (A). Representative FACS plot of CXCR5 and CD25 on all CD4⁺ T cells. (B). Representative FACS plots of PD-1 and CXCR5 on all CD4⁺ T cells and % CD25 expression on the CXCR5⁺PD-1⁺, CXCR5⁺PD-1⁺, and CXCR5⁺PD-1⁻ subsets. (C). Quantification of % CD25 expression on the CXCR5⁺PD-1⁺, CXCR5⁺PD-1⁺, and CXCR5⁺PD-1⁻ subsets of CD4⁺ T cells. % CD25 (*, p=0.0195, ** p=0.0039). Data are from 10 independent donor samples. (D). In separate stains, intracellular staining for Foxp3 was performed. Representative FACS plots of CD25 and FoxP3; CXCR5 and CD25; and CXCR5 and FoxP3 on all CD4⁺ T cells.
SUPPLEMENTARY FIGURE 3. Representative flow cytometry plots of the GC Tfh cells from two different donor samples stained for CD25^+OX40^+ expression by GC Tfh cells directly ex vivo and after 18 hour stimulation.
**SUPPLEMENTARY TABLE 1.** Flow cytometry antibodies utilized in the AIM assay for tonsils.

**AIM Panel for Tonsils**

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**AIM Panel for PBMCs**

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