Establishment of a Healthy Human Range for the Whole Blood “OX40” Assay for the Detection of Antigen-Specific CD4+ T Cells by Flow Cytometry

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Background: Clinical investigation of antigen-specific T cells in potentially immunodeficient patients is an important and often challenging aspect of patient diagnostic work up. Methods for detection of microbial exposure to the T-cell compartment exist but are laborious and time consuming. Recently, a whole blood technique involving flow cytometry and detection of CD25 and OX40 (CD134) expression on the surface of activated CD4+ T cells was shown to be accurate and concordant when compared with more traditional methods of antigen-specific T-cell detection.

Methods: Whole heparinized blood was collected from healthy donors and set up using the “OX40” assay to detect antigen-specific CD4+ T-cell responses to Varicella Zoster Virus, Epstein-Barr Virus (EBV), Cytomegalovirus, Candida albicans, and Streptococcus pneumoniae.

Results: The “OX40” assay technique was clinically validated for routine use in an NHS clinical immunology laboratory by analysis of incubation length (40–50 h), sample transport time (up to 24 h at room temperature), concordance with serology testing, proliferation and interferon-gamma production. In addition, 63 healthy controls (age range 21–78) were tested for responses to generate a healthy control reference range.

Conclusions: The OX40 assay, as presented in this report, represents an economical, rapid, robust whole blood technique to detect antigen-specific T cells, which is suitable for clinical immunology diagnostic laboratory use. © 2014 International Clinical Cytometry Society

Key words: flow cytometry; costimulation/costimulatory; diagnostics; CD4+ T cells

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Detection of previous exposure to a pathogen is an important aspect of clinical investigation (1). Traditionally this has been accomplished via two strategies. First, by detection of the pathogen in the subject, this can be carried out by culturing of patient fluid, or more recently by molecular techniques such as quantitative polymerase chain reaction (2). The second approach has been to analyze components of the immune system for their ability to specifically recognize the pathogen in question, this has most commonly been achieved in the clinical environment by serology testing (3) but now includes limited T-cell function detection methods, for example ELISpot assays, for pathogens such as tuberculosis (4). This second approach has the advantage of not only informing the clinician whether the patient has previously been exposed to the pathogen but also can indicate the degree of specific immune response the patient is capable of mounting.

Serology has been and will continue to be useful in measuring antibody responses to pathogens (3). However, there is an urgent and increasing need for rapid, robust and accurate clinically validated assays capable of measuring T-cell antigen-specific responses, especially in patients unable to produce antibody or who are on immunoglobulin replacement therapy. Previous work by Zaun-ard Malley (Boston, Massachusetts).

Reagents

Phytohemagglutinin (PHA) was obtained from Sigma-Aldrich (Dorset, UK) and used at 15 µg/ml, Cytomegalovirus (CMV), Varicella Zoster Virus (VZV), EBV, Vero lystate, and human foreskin fibroblast (HFF) lystate were all supplied by Source Bioscience (Nottingham, UK) and used at 2 µg/ml. Candida albicans (Candida) lystate was supplied by Oxford Biosystems (Oxford, UK) and used at 2 µg/ml. Whole ethanol killed Streptococcus pneumoniae used at 1 × 10^8 cfu/tube was a kind gift from Richard Malley (Boston, Massachusetts).

OX40 Assay

Heparin from each subject was diluted 1:1 with iscoves modified medium (Sigma-Aldrich) and aliquoted into 500 µl volumes in sterile capped 5 ml polystyrene flow cytometry tubes (BD Bioscience, Oxford, UK). To each relevant sample, 2 µg/ml of antigen or 15 µg/ml of PHA was added. Each assay was run with two negative control tubes which were media only and a cell lystate control (either Vero or HFF, Source Bioscience), run at 2 µg/ml. After incubation at 37°C 5% CO₂ for 42–44 h, samples were vortexed and 100 µl was aliquotted to a new tube. Blood was then stained with the following monoclonal fluorochromes, CD25-FITC, CD134-PE, CD14-PerCP, Viaprobe (7-AAD), CD4-APC, and CD3-PE-Cy7 (BD Bioscience). Samples were incubated for 15 minutes at room temperature before having 1 ml of FACSLyse (BD Bioscience) solution added for 5 minutes. Samples were washed twice with PBS before being fixed with 1% paraformaldehyde and run on a BD FACSCanto II (BD Bioscience). Analysis was carried out using BD FACSdiva software (version 6.1; BD Biosciences).

Samples were gated to acquire 30,000 CD3 positive events, which were then negatively gated for CD14 and 7AAD. Quantification of CD3+CD4+CD25+CD134+ events were calculated by setting gate coordinates on the media only sample to equal 0.1% CD3+CD4+CD25+CD134+ events as a percentage of all CD3+CD4+ events. These coordinates were applied to subsequent antigen tubes to produce a percentage value of CD25+CD134+ double positive events.

CFSE Proliferation Assay

Peripheral blood mononuclear cells (PBMCs) were purified using a lymphoprep gradient (Axis-Shield, Oslo, Norway). PBMCs were counted and diluted to 1 × 10⁶/ml in RPMI (Sigma-Aldrich) before being stained with...
1 μM/ml of CFSE for 15 minutes at 37°C. After incubation, cells were quenched with 10 ml of ice cold RPMI (Sigma-Aldrich) before being spun at 350g for 5 min. This wash step was repeated before adding 200 μl of cells/well. Various antigens were added to wells at concentrations ranging from 0.2 to 1 μg/ml, cells were then incubated for 5 days at 37°C 5% CO2. After this time, cells were washed in 1 ml of PBS at 350g for 5 min before being incubated for 15 minutes at room temperature with CD3-PE-CY7 and CD4-APC (BD Bioscience). After a final wash, cells were acquired on a BD FACs-canto II and analyzed. Cells were first gated using CD3 and then by forward/side scatter to capture T cells that showed “blast-like” appearance. Proliferating cells were measured by a decrease in CFSE fluorescence intensity compared to unstimulated control.

Serological ELISAs for CMV/EBV/VZV

Patient serology to EBV, CMV, VZV, and Candida [immunoglobulin G (IgG)] were measured according to manufacturers instructions (Diasorin, Vercelli, Italy).

S. pneumoniae Serotype Testing

The quantification of specific IgG to 12 S. pneumoniae serotypes was performed using a multiplex assay. Conjugation of pneumococcal polysaccharide to beads and performing the multiplex assay follows techniques described previously (9–11). Briefly, serotypes 1, 3, 4, 5, 7F, 9V, 18C, 19A, 6B, 14, 19F, and 23F (Statens Serum Institut, Copenhagen, Denmark) were conjugated and coupled onto activated carboxylated beads (Bio-Rad, Hemel Hempstead, UK). Samples were acquired and analyzed on a Bio-Plex-200 machine using Bio-Plex Manager 6.0 software (Bio-Rad) and the quantity of serotype-specific IgG in μg/ml was determined by comparing the mean fluorescent intensity (FI) to a Log-SP1 standard curve generated from FI against expected IgG concentration for 89-sf (Food and Drug Administration, MD).

Interferon-Gamma Production From Whole Blood Cultures

Whole blood stimulated by the OX40 assay (as described previously) was removed from the incubator after 42–44 hrs incubation. 200 μl of cell free supernatant was removed from the cultures and immediately stored at -80°C until required. Interferon-gamma (IFN-γ) concentrations were measured by Luminex assay using a Bio-Plex-200 machine and a human IFN-γ kit supplied by Bio-Rad. Analysis was carried out using Bio-Plex Manager 6.0 software (Bio-Rad).

Statistics

Statistical analysis was performed using Microsoft Excel and Prism GraphPad version 5 Software (GraphPad Prism, San Diego, CA). Reference ranges were generated by calculating the median, 5th and 95th percentile for each pathogen. For 2 group comparisons, nonparametric Mann-Whitney tests were carried out. Correlation analysis was made using a Spearman Rank test. For all analyses, P < 0.05 was considered to be statistically significant.

Concordance was calculated as the percentage of the true positives and true negatives generated by the assay compared to the total number of results generated (true positives + true negatives + false positives + false negatives). Sensitivity values were calculated as the percentage of the true positive results generated by the assay when compared to the total number of true positives + false negatives. Specificity values were calculated as the percentage of the true negatives generated by the assay when compared to the total number of true negatives + false positives.

RESULTS

A CD3+ Gate Followed by a Negative Gate to Remove Dying/Dead Cells and CD14+ Cells Is Essential to Capture All Relevant Cells While Eliminating Contaminants

Initial development of the “OX40” assay (5) used a four-color system that gated on lymphocytes using forward and side scatter, followed by identification of CD3+CD4+ cells before measuring CD25 and CD134 expression (Fig. 1A). A concern with this original strategy was that using an initial forward/side scatter gate when examining activated cells could lead to some cells that showed increased granularity from their activation being overlooked from analysis. This is highlighted in Figure 1Aii with analysis of a PHA stimulated sample that has been shown with an overly expanded initial gate to show potentially missed events. The importance of using an expanded gate is further shown in Figure 1B where the same sample stimulated with CMV lysate was analyzed with a “compact” lymphocyte gate and an “expanded” lymphocyte gate. Figure 1Bii demonstrates CD25+CD134+ CMV-specific T cells were present in the forward/side scatter plot outside the “compact” lymphocyte gate (Fig. 1Bi), leading to different CD25+CD134+ T-cell percentages (4.6 vs 5.3%).

A concern with using an “expanded” gating strategy was the potential inclusion of either dying cells that would be aberrantly upregulating cell markers or CD14 monocytes that possess large amounts of FC receptors on their surface, leading to nonspecific monoclonal antibody binding. To confront these two potential issues, it was decided that initial gating with a comprehensive CD3+ gate should be sufficiently inclusive to include all true T cells followed by an additional negative selection gate using CD14 and the viability marker 7-AAD to help minimize nonspecific cell contamination (Fig. 2A).

Detection and quantification of antigen-specific T cells was measured against viral (VZV, EBV, and CMV), fungal (Candida), and bacterial (S. pneumoniae) antigens. These encompassed both pathogens that inhabit perinatally and those that are associated with an isolated infection (Fig. 2B). As a further control measure to test the specificity of the response, individuals were tested against lysates of two established cell lines used to propagate the VZV and CMV antigen used in the experiments (Fig. 2B). No individual tested showed a response to either cell line that was >0.1% above the unstimulated control, in addition, increasing concentrations of cell
Fig. 1. (A) Original gating strategy from Zaunder et al. (5) on healthy control blood that has been unstimulated or stimulated with PHA. Initial gating of cells made by gating lymphocyte population using forward and side scatter, PHA stimulated lymphocytes show increased granularity and size requiring a bigger gate to capture lymphocyte population. Black events within gates are CD3\(^+\)CD4\(^+\)CD25\(^+\)OX40\(^+\) cells. (B) Comparison of different sized initial lymphocyte gates using forward/side scatter on collection of CD3\(^+\)CD4\(^+\)CD25\(^+\)OX40\(^+\) cells. The gating strategy on a healthy control sample stimulated with CMV was analyzed, first using a compact lymphocyte gate (i) and second using an expanded lymphocyte gate (ii). Black events within gates are CD3\(^+\)CD4\(^+\)CD25\(^+\)OX40\(^+\) cells. Percentage CD25\(^+\)OX40\(^+\) cells as a proportion of all CD3\(^+\)CD4\(^+\) cells is marked in the quadrant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
FIG. 2. New proposed gating strategy for the detection of CD14-7AAD-CD4-CD25-CD4+T cells. (A) A healthy control sample was left unstimulated or stimulated with PHA. Cells were first gated as CD3+ before being gated as CD14− and 7AAD−. These cells were then selected according to CD4 expression and then analyzed for CD25 and OX40 expression. Black cells within gates are CD3+CD14−7AAD−CD4-CD25-CD4+T cells. (B) Representative plots of healthy control whole blood stimulated with various pathogens using the strategy outlined in (A). Percentage CD25+OX40+ cells as a proportion of all CD3+ CD4+ cells is marked in the quadrant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
line lysate did not increase the level of CD25+CD134+T cells (n = 63, data not shown).

**OX40 Assay Results Are Concordant With Antigen-Specific T-Cell Proliferation Assays**

To determine the accuracy of the “OX40” assay, parallel assays were setup on the same healthy controls to measure VZV, EBV, CMV, and Candida status (n = 15) by both the OX40 assay and an antigen-specific CD4+ T-cell proliferation assay using CFSE. Although this assay is measuring proliferation rather than activation of CD4+ T cells in response to the antigen like the OX40 assay, it was preferred to other techniques such as intracellular cytokine staining or Elispot since it captures the entire antigen-specific population rather than one particular cohort such as IFN-γ+CD4+ T cells for example. There was 100% concordance between results generated by the two different assays with all antigens measured, in all individuals both in either detecting the serologically confirmed presence or absence of a particular pathogen. Correlative analysis of levels of proliferating antigen-specific CD4+ T cells vs OX40+CD25+CD4+ T cells for each individual against the selected pathogens showed variable results with significant correlations seen in CMV, EBV, and VZV responses (r = 0.93, 0.82, and 0.68, respectively, P < 0.001 for all results, data not shown) but not candida (r = 0.04, data not shown). Figures 3A–3E show the representative OX40 and CFSE proliferation plots of a healthy control that had confirmed VZV+EBV+CMV− serology.

**The 40–50 h Incubation Is Suitable for Consistant Measurements**

For use in a clinical environment, incubation times must be pragmatic and reproducible with regard to results. To assess incubation timing on individual response variation, time course experiments were performed, measuring PHA and CMV responses in five CMV positive individuals with stimulation times between 40 and 50 h (Fig. 4). The highest variance seen between a 40-h and a 50-h incubation sample in any one individual was 8.5 and 0.9% for PHA and CMV, respectively (Figs. 4A and 4B). Variance for all samples between 40 and 50 h incubation in each individual gave a mean coefficient of variation (CV%) of 9.1 and 12.9% for PHA and CMV, respectively. These CV% values are within an acceptable
Fig. 4. Differences in antigen-specific CD4+ T-cell responses depending on incubation time with antigen. Five healthy control whole blood samples were either stimulated with PHA (A) or CMV (B). Samples were stained and processed for detection of CD25+OX40+ CD4+ T cells at 2-h intervals from 40 to 50 h poststimulation. (C) IFN-gamma production from whole blood samples after stimulation with either EBV or CMV. Whole blood from healthy controls was incubated for 42–44 h with either CMV or EBV lysate. Samples were grouped as either both serologically and OX40 assay negative or both positive. Correlations between the amount of IFN-gamma released after each stimulation and OX40 assay result generated in that same individual were also carried out. Identical results (individuals producing 0% OX40 result and 0 pg/ml) are overlaid on the graph.
CV% for flow cytometric assays on live blood cells indicating that clinical testing using this assay could allow for some flexibility in the incubation times of samples without fear of producing significantly different results. For the remainder of the work in this study, all samples were incubated for between 42 and 44 h as this suited the work flow of the clinical laboratory.

Concordance of OX40 Assay Response in Healthy Individuals With Serological Analysis

To further confirm the validity of the “OX40” assay for detecting previous antigen exposure, serological analysis in a group of healthy controls was compared with their response against the same pathogens on the “OX40” assay. A cohort of healthy controls was used to test antigen-specific T-cell responses to VZV, EBV, and CMV. Table 1 shows the amount of true positives, false positives, true negatives, false negatives, overall concordance and sensitivity and specificity in the OX40 assay compared with serology using set cutoff percentage values for positivity within each antigen test, using the serological result as the gold standard. CMV was most concordant with 100% concordance to serology, this included both seronegative and seropositive individuals. VZV testing produced 1 discordant result, which was found in a 22 year old female who had contracted chicken pox at the age of 18 months. While serology for VZV was negative, the “OX40” assay response was 0.4%. The median level of VZV response for all healthy controls tested below the age of 25 was 1.3% (n = 6, data not shown), indicating that this individual produced a relatively low VZV-specific response. EBV concordance was 94.5% with 1 false positive and 2 false negatives. No specific data regarding the infection of these patients could give a reason for these discrepancies. Using these results, OX40 assay cutoffs for positivity could be determined using the lowest recorded responses seen for each antigen (Table 1). As expected, sensitivity and specificity values for each antigen test gave similar results to the concordance values within each antigen, CMV producing the highest values (100% sensitivity and specificity) and EBV the lowest (96% sensitivity and 88% specificity) (Table 1). Serology testing against Candida and S. pneumoniae was also carried out. Since these are organisms that inhabit the human body consistently from a young age, every individual tested produced a serological response. These data were used for all subsequent OX40 assays in new individuals to determine pathogen exposure positivity without carrying out serology.

OX40 Assay Responses to CMV and EBV Correlate With Production of IFN-γ in Whole Blood Samples

To further validate the accuracy of the OX40 assay, a comparison of IFN-γ production was made in CMV+ vs CMV− individuals and EBV+ vs EBV− individuals as determined by both the OX40 assay and serology. A significant difference in IFN-γ production was seen between both CMV+ vs CMV− and EBV+ vs EBV− individuals (Fig. 4C). Correlation analysis of the relative frequency of OX40+CD25+ T cells and the amount of IFN-γ produced for each individual showed significant correlation for both EBV and CMV (CMV r = 0.97, P < 0.0001, EBV r = 0.8, P < 0.0001 Fig. 4C). These data confirm that OX40+ CD25+ CD4+ T cells can be considered to be activated antigen-specific T cells responding to the specific antigen stimulus.

Establishment of a Healthy Human Range for OX40 Responses to PHA, VZV, EBV, CMV, Candida, and S. pneumoniae

A total of 63 healthy controls with an age range of 21–78 (median 40 years) were recruited and T-cell antigen specificity to a range of commonly encountered pathogens determined. Data for each respective pathogen response were only included for individuals that showed an OX40 response that was higher than the cutoffs as determined by serological analysis and stated in Table 1. Reference ranges were calculated as the 5th and 95th percentiles (Fig. 5). The data showed that PHA stimulation produced the widest normal range (3.8–54.1%) and EBV the narrowest (0.2–3.9%). Gender difference analysis of all antigen reference ranges showed no significant differences (data not shown). Correlation analysis of age with specific antigen responses showed a weak positive trend for increased CMV responses with increasing age (r² = 0.29, P = 0.006). No other antigen showed a correlation with age. For these reasons, age-specific reference ranges were not calculated at this point in time.

OX40 Assay EBV-Specific Responses in Patients With an XIAP Mutation Are Elevated Compared to the Reference Range

In order to test the potential clinical utility of having a healthy control reference range for specific pathogens, a pathogen-specific response was measured in patients
with a diagnosed immunodeficiency. Mutations in XIAP result in affected individuals suffering from recurrent EBV infection (12). EBV-specific responses were investigated in two siblings (Patients 1 and 2) with a characterized XIAP mutation (8). The amount of OX40\(^{+}\)CD25\(^{+}\)CD4\(^{+}\) T cells generated in both siblings in response to EBV was dramatically raised (23.3 and 20.6% Fig. 6A) compared with the reference range (95th percentile for EBV = 3.9%) whilst PHA responses and candida responses were within the reference range generated (95th percentile for PHA = 54.1%, 95th percentile for Candida = 5.0%; Fig. 6B). Specific comparisons of the patient data with age/sex-matched individuals data from the reference range cohort also showed no distinct differences in PHA and candida responses (Fig. 6B). Further confirmation of a large EBV-specific response in Patient 1 was carried out using a CFSE proliferation assay again showing a raised response of 38.2% [mean EBV response by CFSE proliferation in healthy cohort = 6.3% (data not shown); Fig. 6A].

**DISCUSSION**

Clinical investigations into the presence and extent of antigen-specific T-cell immunity are becoming more important and the need for antigen-specific functional CD4 T-cell assays that can be performed in routine diagnostic clinical laboratories is clear (1). In this report, the OX40 antigen-specific functional CD4\(^{+}\) T-cell assay was clinically validated for use in a routine clinical immunology laboratory and the validated assay was used to establish a healthy control normal response range for antigen-specific T-cell responses to five common pathogens.

OX40 is a member of the TNF superfamily and is considered a later costimulatory marker, important in the activation and survival of T cells as they undergo differentiation from naïve to effector memory status from 1

**Effects of Length of Blood Storage Prior to Assay Setup**

To determine the optimum time between venepuncture and “setting up” of the OX40 assay, five CMV and EBV seropositive healthy controls were analyzed for their antigen-specific T-cell responses to PHA, CMV and EBV. Blood was used at 6 h of venepuncture, 24 h postvenepuncture or 48 h postvenepuncture. In all experiments blood was incubated with antigen for 42–44 h. Analysis of antigen-specific responses showed that all responses against all antigens (except for one control against EBV) were above/on the lower limit of the reference range after 24 h (Supporting Information Fig. 1).

However, the mean CV\% produced over the three time points measured for each individual, in all 3 antigen stimulations used, was increased compared to typical interassay variation for flow cytometric data, with EBV responses showing the highest variance (PHA = 33.5 CV\%, CMV = 34.3 CV\% and EBV = 84.2 CV\%; Supporting Information Fig. 1). This could also in part be due to EBV responses showing the smallest numerical values which can skew CVs to higher values. One control who showed a consistently low PHA response during the generation of the PHA normal range was also tested and this proved to be consistently low, staying below the normal range cutoff (Supporting Information Fig. 1). These results suggest that samples can be left for up to 24 h at room temperature before assay set up if assessing antigen specificity only but not for accurate quantification of antigen-specific response.

**Fig. 5.** Establishment of healthy control normal ranges for antigen-specific CD4\(^{+}\) T cell responses to common pathogens using the OX40 assay. A cohort of 63 health controls was used to generate a normal response range. Samples were all incubated for 42–44 h and data was only used for individuals that showed a positive response to the pathogen as determined by serology (Table 1). Median values and normal range high and low cut-off values, defined as the 5th and 95th percentiles are shown for each plot.
Fig. 6. Investigations into EBV-specific CD4+ T cell responses in patients with XIAP defects. (A) Whole blood from two patients with a characterized XIAP defect was stimulated with either PHA, EBV, or Candida for 42–44 h before being run on the OX40 assay. Patient 1 was also tested for CD4+ proliferation in response to PHA or EBV. (B) Comparison of % CD25+CD134+ CD4+ T cells generated in response to PHA, EBV, or Candida in XIAP patients and in age/sex-matched healthy controls. Dotted line indicates the 95th percentile value from the reference range generated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
to 2 days after antigen recognition (13). There is a concern that this assay could be detecting naïve CD4+ T cells in individuals that are specific for the stimulating antigen rather than pre-existing CD4+ T-cell memory. However, this is unlikely for two reasons. First, strong concordance data with serology from this report support the idea that only memory cells are recognising antigen and reacting with upregulation of OX40. Second, incubation of CMV seronegative individuals blood with CMV antigen does not induce OX40 upregulation for at least 8 days (R. Sadler, unpublished observations), suggesting that T-cell antigen-specific responses described in this report were due to pre-existing effector memory T cells. Another potential issue is that the assay quantifies proliferated antigen-specific T cells—exaggerating the true memory compartment of the individual to that antigen. Previous work reported that proliferation of memory T cells began 48 h post second antigen exposure (5), indicating that incubations of approximately 48 h or below were suitable for detecting baseline CD4+ T-cell memory levels.

Additional optimization and clinical validation of the OX40 assay in the study has shown that a different gating strategy increases confidence in the results generated by the assay. This is particularly important since the level of detection of this assay appeared to be ±0.5% in some cases ((5) and this study). Comparisons with a CFSE proliferation assay, an already established T-cell function assay used in clinical immunology laboratories, showed 100% qualitative concordance between the assays in all antigens measured but did not show quantitative correlation when candida responses were analyzed. This was the only antigen measured that did not quantitatively correlate between the two assays and interestingly it was also the only pathogen that can be considered a commensal of humans. This universal exposure of the body to candida could explain the differences seen in proliferation and activation of exposed CD4+ T cells. Crucially, for the purposes of logistics of sample processing in the laboratory, studies show that an antigen incubation window can be used between 40 and 50 h, allowing some flexibility when performing the assay. Sample transportation analysis revealed that by delaying assay setup, an accurate qualitative result of exposure to pathogen could still be obtained as long as samples were processed within 24 h. However, quantifiable levels of antigen-specific response were not concordant between stored blood and blood that was <6 h old, indicating that comparison with this healthy control range can only be made when blood is processed within 6 h of venepuncture. In addition, only CMV and EBV responses were analyzed and investigations into the other pathogens chosen here should be carried out.

Antigen-specific T-cell responses are currently investigated in a number of ways. MHC-tetramer staining is considered the gold standard for detecting antigen-specific T cells. This technique is predominantly used in CD8+ T-cell work and while highly specific and informative, paradoxically the MHC restrictive nature of this technique severely limits who can be measured. In addition, the complex labor required and high consumable costs, together with the paucity of available MHC tetramers makes it difficult to use the technique in routine NHS laboratories. Measurement by antigen-specific CFSE or ELISpot are both possible but require PBMC sorting and extensive optimization to become interpretable. The OX40 assay is a whole blood assay with a minimal setup time before incubation, it allows for both qualitative and quantitative analysis of antigen-specific T cells. Qualitative analysis can be useful in patients with dysgammaglobulinemia or who are on IVIG and therefore are not able to be measured by more established serological methods. Investigations into EBV-specific CD4+ T-cell responses in two patients with a mutation in XIAP revealed grossly abnormal levels of antigen-specific OX40+ CD25+ CD4+ T cells. These raised levels could be a result of the T-cell compartment having to dedicate greater levels of resource to a particular pathogen to compensate for a functional failing in another component of the immune system such as the NK cells or CD8+ T cells. These data support the notion that quantifying levels of antigen-specific CD4+ T cells using the OX40 assay against particular pathogens can be useful in investigating patients with suspected immunodeficiency.

The OX40 assay can only be used to detect antigen-specific CD4+ T-cell responses, not CD8+ T cells. Analysis of CD8+ T cells does not show the same kinetics or accuracy with regards to CD25 and CD134 expression as CD4+ T cells (R. Sadler unpublished observations). Further work needs to be carried out to identify a similar technique to the OX40 assay that can work for CD8+ T cells. Previous studies phenotypically analysing vaccinia tetramer positive CD8 T cells in vaccinated individuals reveal many candidate markers such as CD38 and HLA-DR (14,15).

In summary, this study shows clinical validation and establishment of a healthy human range for the detection of antigen-specific CD4+ T-cell responses to a number of common pathogens using the OX40 assay. This allows quick, cheap, whole blood analysis of patient T-cell immunity to aid in immune profiling of patients.

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