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SHORT COMMUNICATION

Single-cell profiling of lineage determining transcription factors in antigen-specific CD4+ T cells reveals unexpected complexity in recall responses during immune reconstitution

Chansavath Phetsouphanh[1,3], Yin Xu[1], Mee Ling Munier[1], John J Zaunders[1,2] and Anthony D Kelleher[1,2]

Recent studies of protein and gene expression at the single-cell level have revealed that the memory T-cell compartment is more heterogeneous than previously acknowledged. Identifying different T helper subsets involved in memory responses at the single-cell level is thus necessary to understand the level of heterogeneity within this population. Antigen-specific CD4+ T cells were measured using the CD25/OX40 assay together with a qualitative multiplex single-cell RT-PCR assay. Transcription profiles and subset proportions within the antigen-specific CD4+ T-cell population were dissected. Cytomegalovirus (CMV)-specific CD4+ T-cell responses skewed toward a Th1 response, whereas Tetanus toxoid responses skewed toward a Th2 type response. Fluctuations in CD4+ T-cell subsets were observed within the HIV-Gag-specific response during ongoing antiretroviral therapy. Strong effector responses (Th1) were observed in early treatment, however with ongoing therapy this effector response significantly decreased in combination with an increase in Tregs and circulating Tfh-like BCL-6+ memory cells. The apparent increase in Tcm in peripheral blood after a several weeks of antiretroviral therapy may be due to Tfh-like cell egress from germinal centers into the periphery.
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INTRODUCTION

T cells are indispensible for their role in immune protection against a wide variety of infections. Development of effective vaccines against malaria, tuberculosis and HIV will likely require the generation of potent and long-lasting T-cell responses. Although many promising vaccine formulations capable of eliciting strong T-cell immunity are currently being investigated in humans,[1–3] the correlates of protection still need to be defined.

Antigen (Ag)-specific CD4+ T cells are crucial components of the immune response, particularly to viruses, having been shown to play a role in limiting viral replication and controlling virus related morbidity.[4] As Ag-specific CD4+ T cells are functionally heterogeneous and mediate their function via a variety of mechanisms, a major obstacle in quantifying protective responses has been the limitations of current assays that fail to assess the complexities of these responses.[5]

The most commonly measured characteristic of a T-cell response is its magnitude. This is commonly represented as the frequency of antigen-specific T cells detected or the expression of a particular cytokine, such as IFN-γ. However, measuring the magnitude of a T-cell response by a single parameter does not reflect the functional potential or complexity of the total response.[6] The functional diversity of CD4+ T-cell responses includes the ability of T cells to: proliferate, induce differentiation of other cells, regulate immune responses through mechanisms such as cell to cell contact, secrete chemokines or cytokines, and carry out a range of effector functions, including cytolysis. These functions can occur in complex combinations and can be defined as the 'quality' of the T-cell response. While some insight into the quality of a response can be defined by looking globally at an antigen-specific population, greater insight into the complexity of the response can only be derived from looking at a single-cell level.[7]

Here we measured and assessed the antigen-specific CD4+ T cells using the CD25/OX40 assay together with the qualitative multiplex single-cell RT-PCR assay,[8,9] using different antigens, such as, CMV, Tetanus toxoid (TT) and HIV-Gag; transcription profiles and proportions of subsets within the antigen-specific CD4+ T-cell population were dissected. As expected CMV-specific CD4+ T-cell responses skewed toward a Th1 response, whereas TT responses skewed toward a Th2-type response. Fluctuations in CD4+ T-cell subsets were observed within the HIV-Gag-specific response following initiation of antiretroviral therapy (ART). Surprisingly, these responses were dominated by populations of cells expressing Bcl-6 or Foxp3. These results provide insight into the extent of variability and the

[1]The Kirby Institute for Infection and Immunity in Society, University of New South Wales, Kensington, New South Wales, Australia and [2]Centre for Applied Medical Research, St Vicent's Hospital, Sydney, Australia
[3]Current address: Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford OX1 3SY, UK.
Correspondence: Dr C Phetsouphanh, Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford OX1 3SY, UK.
E-mail: chan.phetsouphanh@ndm.ox.ac.uk
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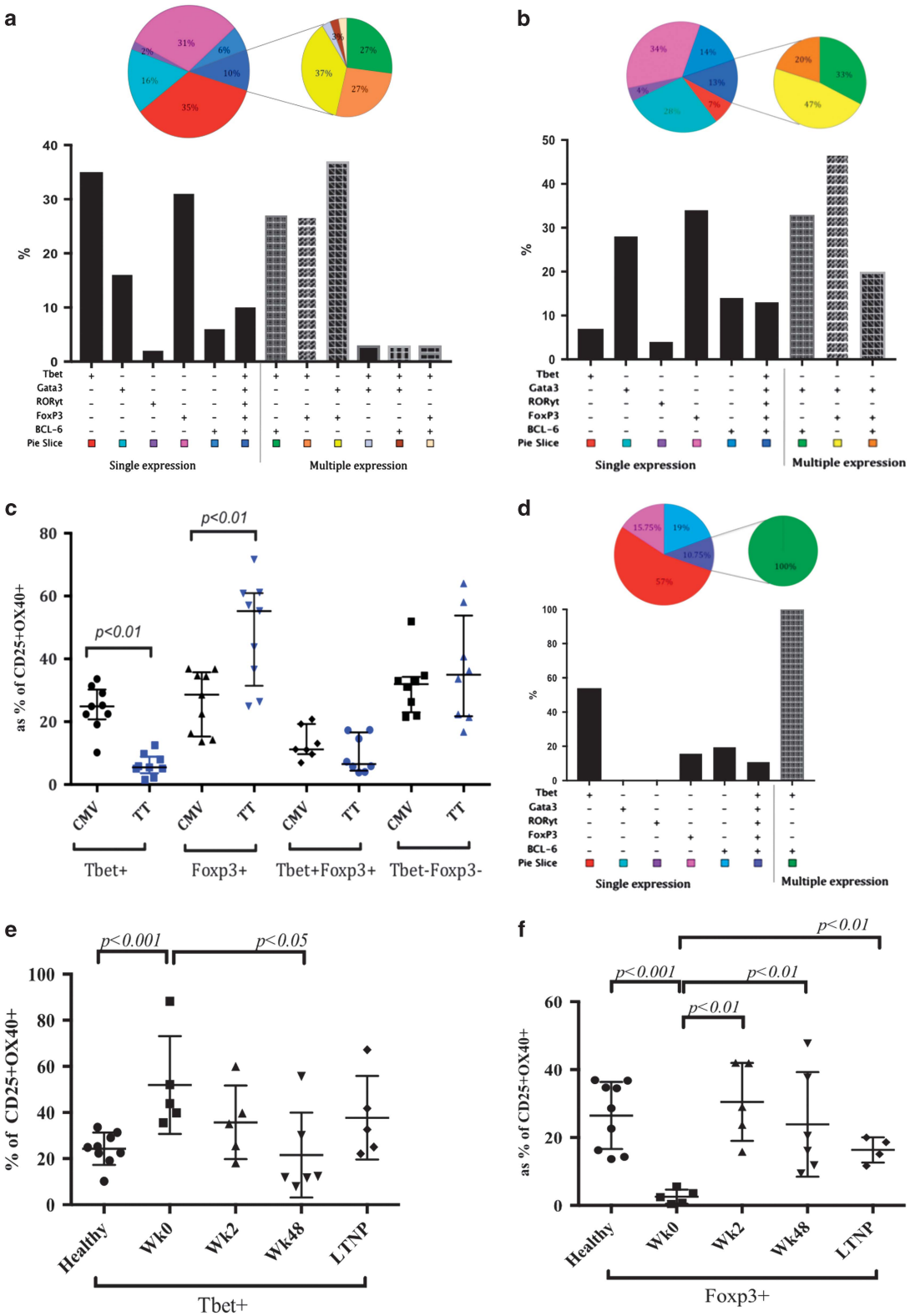

Figure 1 Transcription factor (TF) profiles from CMV- and TT-specific CD4+ T cells in healthy subjects. (**a**) CMV-specific TF profile $n=9$. (**b**) TT-specific TF profile $n=7$. Black columns represent single TF expressed within one cell. Dark blue segment of the pie slice is indicative of single cells that expressed more than one TF. Differing combinations of TF were expressed in these cells; no cell expressed all five TFs. Shaded columns represent combinations of multiple TF expression. Larger pie chart shows column results as percentages. Smaller pie chart represents multiple TF expression as percentages. (**c**) Intracellular TF staining in CMV- and TT-specific CD4+ T cells from healthy controls. (**d**) CMV-specific TF profile in HIV-positive subjects at week 2 post ART (percentages shown as medians). (**e**) Tbet protein expression in CMV-specific CD4+ T cells from healthy, chronically infected ART-treated patients and LTNP. (**f**) FoxP3 protein expression in CMV-specific CD4+ T cells from healthy, chronically infected and LTNP subjects.

dynamics of subpopulations within antigenic T-cell responses measured at the single-cell level, allowing the elucidation of subtle changes to $CD4^+$ T-cell subsets post ART and highlighting the heterogeneity within antigen-specific and *ex vivo* populations not revealed by standard approaches.

RESULTS

CMV and TT-specific cells show different transcription factor profiles

To decipher which T helper subsets are involved in CMV-specific responses, CMV-specific $CD4^+$ T cells were sorted from nine healthy individuals and scRT-PCR was performed. Approximately 90% of cells expressed only one of the six transcription factors (TFs), with the other 10% expressing different combinations of up to three TFs. The transcription profile revealed that the dominant T helper subset responding to CMV was the Th1 subset, with 35% of cells expressing *tbet* (Figure 1a). Interestingly, the next highest proportion of cells expressed *foxp3* (31%), which suggests that there are considerable responses from Treg cells.

A smaller proportion of the CMV-specific cells expressed *gata3* (16%), suggesting involvement of Th2 cells. There were very low proportions of cells expressing *rorc* and *Bcl-6* in CMV-specific cells, although the presence of these TFs suggested small contributions from Th17 and Tfh-like cells. Of the 10% of CMV-specific cells that expressed two or more TFs, the most frequent combination of TF expression was $gata3^+foxp3^+$ (37%), followed by $tbet^+bcl6^+$ (27%) and $tbet^+foxp3^+$ (27%). There were varying but low frequencies of other combinations that included: $tbet^+gata3^+$, $tbet^+gata3^+Bcl\text{-}6^+$ and $tbet^+foxp3^+bcl6^+$ (~3% each).

The TF profile of TT-specific responses differed from the CMV-specific response. There was very little *tbet* expression (7%), which indicated minimal Th1 involvement in TT-specific responses (Figure 1b). The second highest response came from Th2-like cells that expressed *gata3* (28%). Surprisingly, cells expressing *foxp3* (34%), were the largest fraction of cells which suggests that recall responses to TT are likely to be modulated or suppressed by these regulatory T cells. There was a low frequency of *rorc* expression (4%) and of *Bcl-6* expression (14%). Thirteen percent of cells expressed two or more TFs in differing combinations that included: $gata3^+Bcl\text{-}6$ (20%), $gata3^+foxp3$ (47%) and $gata3^+foxp3^+Bcl\text{-}6$ (20%).

To confirm results obtained from the qualitative multiplex single-cell RT-PCR assay, intra-nuclear staining was performed using antibodies to TFs Tbet and Foxp3. Antibodies for other human TFs, such as Gata3and Rorγt have not proven sensitive or specific enough for intra-nuclear analyses. peripheral blood mononuclear cell (PBMC) from nine healthy individuals with known CMV and TT responses were studied.

Similar to the findings observed from the analysis of scRT-PCR, CMV-specific T cells as defined by $CD4^+CD25^+OX40^+$, displayed a Th1 dominant phenotype. The median proportion of CMV-specific cells expressing Tbet was found to be significantly higher than in TT-specific cells (24.9% and 5.5%, respectively; $P<0.01$) (Figure 1c). The proportions of cells expressing Foxp3 were consistent with the scRT-PCR results, as high proportions of both CMV and TT-specific cells expressed this TF. However, a greater proportion of TT-specific cells expressed Foxp3 compared to CMV-specific cells (55.2% and 28.6%, respectively; $P<0.01$), again suggesting that a large proportion of responding cells are Tregs. Double positive ($Tbet^+Foxp3^+$) and double negative ($Tbet^-Foxp3^-$) cells were equivalent in both antigen responses and were not significantly different between the two, representing ≈34% of the response.

Reduction of Th1-like CMV-specific $CD4^+$ T cells from HIV-positive subjects post therapy

Cryopreserved PBMC from nine HIV-positive patients with progressive chronic infection taken at an early time after commencing therapy (weeks 2 and 4) were thawed and activated with CMV lysate, and then scRT-PCR was performed. The viability of thawed PBMC was >90%, however this decreased to ⩾70% after antigen activation. In five patient samples, there were high levels of cell death, thus sorting could not be performed. RNA quality from these samples was also reduced, with β-actin signals detected ~5 cycles later than in the healthy counterparts. Sorting efficiency was also slightly lower than in the healthy samples (that is, ~75% compared to ~90%, respectively).

Single-cell TF profiling showed that there was a dominance of *tbet* (57%) expression observed in CMV-specific cells from HIV-positive individuals (Figure 1d). At an early treatment time point (that is, week 2), there were moderate levels of cells expressing *foxp3* (15.75%) and *Bcl-6* (19%). 10.75% of the cells expressed both *tbet* and *Bcl-6*, and this was the only combination observed. This profile was somewhat similar to that seen in CMV responses in healthy controls, but the proportions of cells expressing these two TFs were significantly higher than in healthy controls ($P<0.001$). This may be driven by more frequent sub clinical reactivation of CMV in these late-stage previously untreated patients.

To further investigate changes in the $CD4^+$ T-cell immune response following ART the expression patterns of Tbet and Foxp3 were followed longitudinally, by intra-cellular TF staining at baseline (week 0), week 2 and week 48 post therapy. Healthy HIV-negative subjects, as well as, HIV-positive long-term non-progressor (LTNP) subjects were used as comparators. The proportion of CMV-specific cells expressing Tbet in patients with chronic progressive HIV infection pre-therapy was considerably higher than both healthy controls and LTNP (43.9%, compared to 24.9%, ($P<0.001$); and 32.6%, respectively) (Figure 1e). With ongoing ART treatment, the proportion of Tbet expressing cells decreased at week 2 and this decrease became statistically significant at week 48 (43.9 to 12.5%; $P<0.05$). This ~3.5-fold reduction in Tbet expression in CMV-specific cells was considerably lower than in healthy controls or LTNPs.

The proportion of CMV-specific cells in chronic HIV-infected patients expressing Foxp3 as judged by intracellular staining at baseline was significantly lower than in healthy controls (2.44% and 28.6%, respectively; $P<0.001$) and LTNP (16.85%; $P<0.01$) (Figure 1f). However, at week 2 of ART there was a marked (~12-fold) increase in the proportion of cells expressing Foxp3 to 29% ($P<0.01$). At week 48 the proportion of cells expressing Foxp3 remained unchanged and was comparable to LTNP, remaining significantly higher than baseline (18.3%, $P<0.01$).

Reduction of Th1-like HIV-Gag-specific $CD4^+$ T cells and expansion of regulatory subsets in HIV-positive subjects post ART

The Gag-specific TF profile was similar to the pattern observed from CMV-specific cells. There were a high proportion of Gag-specific cells expressing messenger transcripts for *tbet* (46.5%) in untreated HIV-positive individuals during chronic progressive infection (Figure 2a). There were moderate proportions of Gag-specific cells expressing *foxp3* (18%) and *Bcl-6* (16.5%), which suggests that at early time points (weeks 2–4), when viral load is still relatively high, there is a Th1-biased response against the virus. A considerable percentage of cells expressed two or more TFs (that is, 19%). Interestingly, the only combination observed was $tbet^+Bcl\text{-}6^+$.

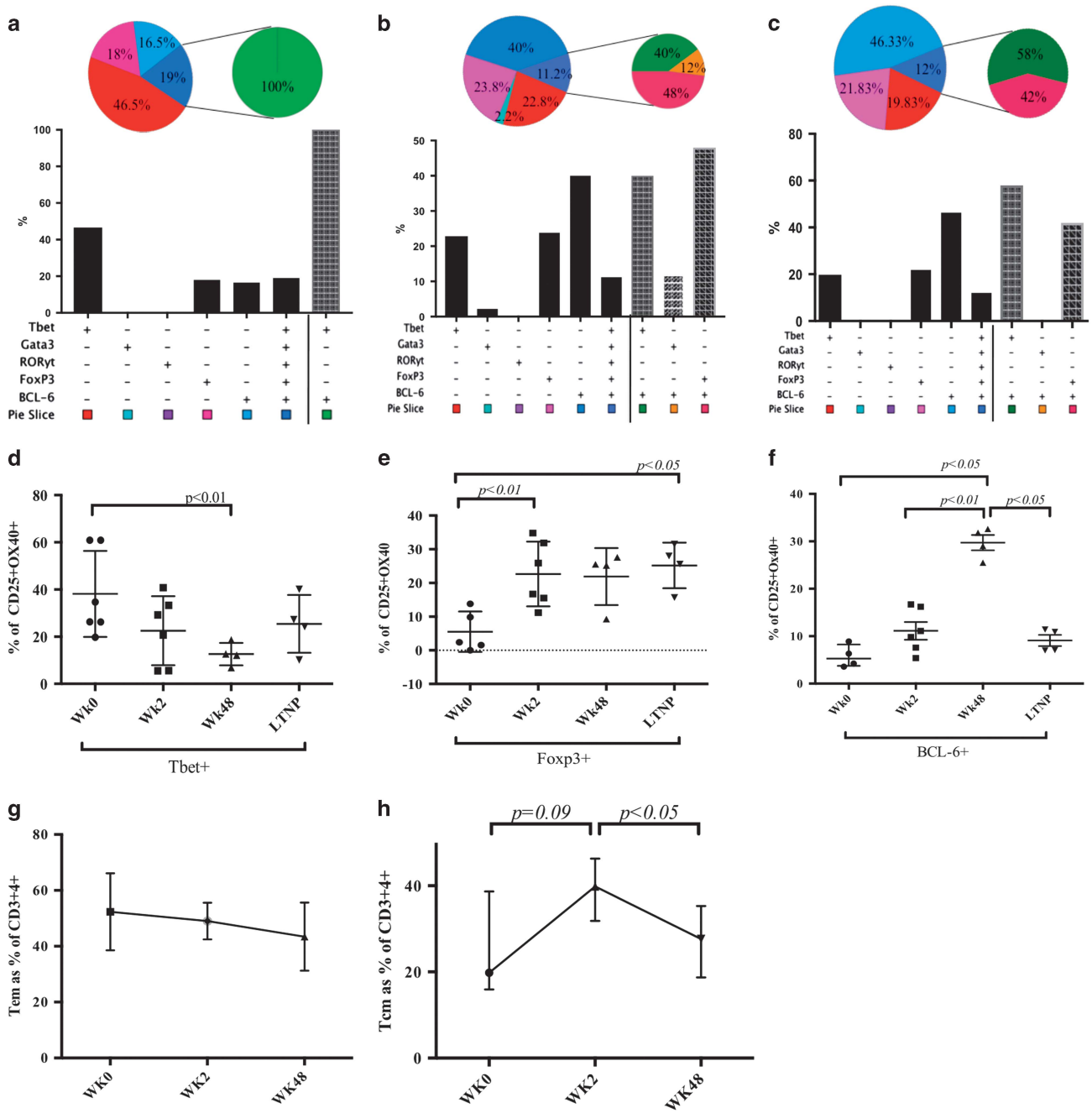

Figure 2 Transcription factor (TF)profiles from Gag-specific CD4+ T cells in HIV-positive subjects in late treatment and LTNP. (**a**) Gag-specific TF profile in chronic early treatment week 2 $n=6$. (**b**) Gag-specific TF profile in chronic late treatment week 48 $n=4$. (**c**) Gag-specific TF profile LTNP $n=6$. Longitudinal TF expression in chronic subjects compared to LTNP: Tbet (**d**), Foxp3 (**e**) and BCL-6 (**f**). (**g**) Effector memory (Tem) percentages post ART at week 2 and week 48 in chronic HIV-infected subjects. (**h**) Central memory (Tcm) dynamics post ART.

With ongoing therapy (week 48) the transcription profile of HIV-Gag-specific CD4+ T cells skewed toward a regulatory type response (Figure 2b). *Tbet*+ cells decreased to ~22.8%. The proportion of *foxp3*+ regulatory T cells increased (from 16.9 to 23.7%), as did *Bcl-6*+ cells (16.6% and 40%, respectively). A very small percentage of *gata3*+ cells were observed (2.2%). 11.2% of cells expressed multiple TFs, but always in combination with *bcl-6*, with the proportions in decreasing order being *foxp3*+*Bcl-6*+ (48%), *tbet*+*Bcl-6*+ (40%) and *gata3*+*Bcl-6*+ (12%). Surprisingly, within the LTNP cohort, the TF profile was similar to that observed in chronically infected subjects at week 48 post ART. Among Gag-specific cells 19.83% were *tbet*+, 21.83% were *foxp3* and 46.33% were *Bcl-6*+ (Figure 2c).

To confirm the scRT-PCR results, intracellular TF protein staining was performed. There was a general reduction of Tbet+ expression in Gag-specific cells post therapy (Figure 2d). At week 48 there was a significant ~2.5-fold reduction in Tbet expression ($P<0.01$) from baseline. The proportion of Gag-specific cells expressing Foxp3 markedly increased (~9-fold) at week 2 from baseline (21.3% and 2.38%, respectively, $P<0.01$). Foxp3 expression stabilized at week 48,

at levels comparable to LTNP. At baseline the proportion of cells expressing Foxp3 levels was significantly lower (~10-fold) than in LTNP (2.38% and 26.8%, respectively; $P<0.05$) (Figure 2e). The same was observed with Bcl-6 expression. The proportion of cells expressing Bcl-6 increased at week 2 post therapy and continued to increase at week 48 (>3-fold) (Figure 2f). Interestingly, the proportion of Gag-specific cells expressing BCL-6 at week 48 in the ART-treated patients was much higher than the levels observed in LTNPs.

Changes in bulk effector/memory populations post therapy

These results are consistent with the fluctuations in memory $CD4^+$ T cells subsets post therapy. There was a reduction of effector memory cells (Tem: $CD45RO^+CD62L^-$), an increase of central memory (Tcm: $CD45RO^+CD62L^+$) after just 2 weeks of ART that remained higher at week 48 compared to baseline (week 0 vs week 2; $P=0.09$ and week 2 vs week 48; $P<0.05$) (Figures 2g and h). This suggests that in this late stage of treated infection the profile of immune responses is distorted by recirculation of HIV-experienced lymphoid resident cells into the periphery. However, peripheral Tfh cells express low levels of BCL-6 *ex vivo* (Supplementary Figure 1B), as opposed to Tbet that is highly expressed in *ex vivo* non-Tfh. CXCR5 expression did not correlate with Bcl-6 in the periphery and the cells require antigenic stimulation in order to upregulate this TF as observed in the OX40 assay (data not shown).

DISCUSSION

Recent studies of protein and gene expression at the single-cell level have revealed that the memory T-cell compartment is more heterogeneous than previously acknowledged.[10,11] It is important to go beyond the Tcm and Tem or Th1 and Th2 dichotomies when investigating Ag-specific responses. Other models of human memory differentiation, including more recently described subsets should also be considered. Integration of these T helper subsets in defining memory responses will have potential implications in our understanding of immune responses, the determinants of disease outcomes and in designing therapeutic approaches. It is becoming apparent that single cells with a given flow cytometric phenotype are not created equal, as they can differ in gene expression or *in vivo* function.[12] Thus, it is crucial to identify the different T helper subsets involved in memory responses at the single-cell level, in order to understand the level of heterogeneity within this population so that future therapies can target the appropriate $CD4^+$ T-cell responses that prove to be critical in determining disease outcomes.

As expected in healthy controls CMV-specific $CD4^+$ T cells displayed a Th1-like dominant response, with a high percentage of cells expressing only *tbet*, and TT-specific cells displayed a Th2-like response, with *Gata3* being expressed by a high proportion of these cells. However, the populations mediating both responses contained large subpopulations expressing *foxp3*, suggesting that antigen-specific Tregs are involved in recall responses to CMV and TT.[13,14] This phenomenon has now also been seen in Celiac disease.[15] As Tregs are known for their immuno-suppressive effects, it is possible that these antigen-specific Tregs are required to control or dampen the recall response from their outset. Importantly, these results from molecular profiling of single cells were concordant with data derived from intra-cellular staining for these TFs in antigen-specific T cells detected by CD25/OX40 co-expression.

In HIV-positive subjects with chronic untreated progressive infection, the TF profile was more restricted and did not display global expression of all five master regulators. For both CMV- and Gag-specific responses, there were higher proportions of cells expressing *tbet*, indicating a Th1 dominant response at the early treatment time point (week 2). Further the proportion of the responses represented by *foxp3*$^+$ cells was markedly smaller pre-therapy, but rapidly increased following initiation of therapy. This increase within the CMV response was to levels seen in healthy controls, suggesting normalization of antigen-specific Treg numbers.

Further within the Gag-specific population, the proportion of $Bcl\text{-}6^+$ cells increased markedly with ART to levels in excess of those seen in LTNPs. The expansion of Tfh cells in lymph nodes during chronic HIV infection has been well documented,[16–18] the increase of Tfh cells correlated with hypergammaglobulemia and hyperplastic germinal centers.[19] The striking increase in HIV-Gag-specific $Bcl\text{-}6^+$ Tfh-like cells within the periphery may be due to the recirculation of antigen experienced germinal center Tfh or Tfh precursors from secondary lymphoid tissues. Tfh cells have been shown to have a central memory phenotype expressing both CD62L and CCR7.[16,20] An increase of Tcm in peripheral blood after a few weeks of ART has been described by Kelleher *et al.*,[21] but prior to this more sophisticated analysis the nature of these cells had not been clear. These data strongly suggest that the increase in these cells may be due egress of TFh or pre-Tfh from lymphoid follicles into the periphery.

It is noteworthy that in the dual TF-positive populations within the Gag-specific response, all cells expressed Bcl-6 in combination with Tbet, Foxp3 or Gata3. This suggests even a greater dominance of Tfh like cells in the Gag-specific response and that these previously sequestered cells are circulating with greater frequency as antigen is reduced in lymphoid tissue by effective antiretroviral therapy. Cells likely to have previously resided in the lymph node make up a large proportion of the Gag-specific response in the periphery post therapy. However, even in untreated LTNP who are controlling viremia, a similar bias toward $Bcl\text{-}6^+$ cells is seen within the Gag response suggesting this is not only a post therapy phenomenon but also a characteristic of responses to Gag, perhaps because lymphoid tissue, and in particular Tfh cells represents a substantial portion of the HIV reservoir. This is reinforced by the observation that in cells that express two of these TFs, one of these is always Bcl-6.

Tbet and Bcl-6 have been suggested to collaborate during the Th1-Tfh transitional state. There is increasing evidence suggesting that Tbet and Bcl-6 may play collaborative roles in mounting a Th1-type response.[22] These two TFs can be co-expressed during early Th1 development and in a small subset of Tfh cells. Tbet can directly interact with Bcl-6, targeting it to Tbet-binding elements where the Tbet–Bcl-6 complex can repress Th1 genes.[23] This complex has also been identified in T cells within germinal centers during Th1-polarizing infections.[24]

Further it is recognized that $FoxP3^+$ Treg cells can co-opt the Tfh differentiation pathway in the development of Follicular Tregs.[25] This suggests that the Gag-specific T-cell response is biased toward a follicular phenotype, again perhaps due to the location of the antigen, as HIV is known to accumulate on FDC and within $CD4^+$ T cells. This predisposition biases the immune response and may limit its ability to respond to the virus in areas outside the follicle. This bias may extend beyond the Gag response as in the HIV-infected patients the only co-expression among the TFs studied at week 0 (pre-therapy) is between Tbet and Bcl-6.

Co-expression of these lineage-determining TFs was observed in a smaller percentage of the CMV- and TT-specific populations in healthy controls (~10–13%) expressed $\geqslant 2$ TFs. The diversity of combinations of the TFs in these cells is greater than that seen in the Gag-specific responses and the expression of Bcl-6 within these combinations is not ubiquitous. As described above, these cells

Table 1 CD4 T-cell count, plasma VL of HIV-positive subjects

Patient ID	*CD4+ T cells (Count per μl) week 2*	*Plasma VL (log_{10} copies per ml) week 2*	*CD4+ T cells (count per μl) week 48*	*Plasma VL (log_{10} copies per ml) week 48*	*CD4+ T cells (count per μl) LTNP*	*Plasma VL (log_{10} copies per ml) LTNP*
1	126	4.12	390	1.7		
2	143	3.88	255	1.7		
3	160	5.83	315	1.7		
4	104	4.98	288	1.7		
5	102	5.82	252	1.7		
6	88	3.99	336	1.7		
7	77	5.56	255	1.7		
8	48	5.0	231	1.7		
9	104	5.84	315	1.7		
10					756	1.7
11					1260	2.26
12					1134	1.7
13					891	1.7
14					980	1.7
Mean	106	5.00	293	1.7	1004	1.81

Abbreviations: LTNP, long-term non-progressor; VL, viral load.
Patients 1–9 are chronic HIV-1-infected subjects at early and late treatment time points. Patients 10–14 are LTNP subjects.

are likely to be poly-functional cells that have the ability to express cross-lineage cytokines or may be in the transition phase of memory commitment. Co-expression of lineage-specifying TFs in certain combinations have the ability to collaborate and 'interplay' with each other to produce subset-specific functions.[22,23]

Foxp3 and Tbet can be co-expressed together in other scenarios. Cells that express both TFs are regarded as having a Treg-like phenotype or to be a Th1-Treg intermediate. It has been shown that Tregs have the ability to upregulate expression of Tbet during type-1 immune responses. Tbet promotes the expression of CXCR3 on Tregs, and Tbet$^+$ Treg cells accumulate at sites of Th1-mediated inflammation.[26] The presence of these cells *in vivo*, has also been examined in the context of airway hyper-reactivity. In that scenario, the antigen-specific Treg cells arose from naive CD4$^+$CD25$^-$ T cells during T helper 1-polarized response, were able to produce IL-10 and IFNγ, and had the potential to inhibit the development of airway hyper-reactivity.[27]

Taken together these data suggest bulk antigen-specific subsets contain a diversity of T helper cell types that are involved in recall memory responses. Healthy controls showed a more global expression of lineage defining TFs compared to HIV-infected subjects in whom the Gag responses appear biased to the expression of *bcl6*, suggesting that the CD4$^+$ T-cell responses are primarily generated in the lymphoid follicles and that this bias becomes obvious in the peripheral blood post therapy. Previous studies have shown that HIV-specific interferon-γ mediated CD4$^+$ T-cell responses persist into late stage disease. While the data presented here are consistent with the reported literature of a Th1 bias response, it challenges and extends this understanding, showing that the reality is more complex and that Tfh-like cells contribute markedly to this response.[28] The data suggest that responses measured in the periphery by standard assays may not accurately reflect responses in the tissue, but that perturbations of the steady state system may reveal tissue response by altering trafficking. Similar observations have been made in autoimmune disease. Therapeutic vaccines and other forms of immunotherapy are being explored as components of interventions to clear the reservoir as part of 'HIV cure strategies'. Understanding the exact nature of these responses and the relationship between tissue and peripheral responses has implications for the design of these immune-modulators and how their immunological effects are monitored in clinical studies. Further the observation that Tfh-like cells dominate the response in LTNP may provide an indication of the type of immune response required for control of virus in tissues.

METHODS

Samples

PBMCs were extracted from anti-coagulated whole blood of healthy volunteers, by Ficoll density centrifugation, for the purpose of development, optimization and validation of assays. PBMCs were also obtained from 10 subjects with established late stage, chronic, HIV-1 infection before starting ART (median CD4$^+$ T-cell count: 80 cells per μl; plasma viral load 5.0 log_{10} RNA copies per ml) who were followed for 48 weeks after commencing ART to study immune reconstitution (Table 1). In addition, longitudinal samples and clinical data from LTNPs were collected at various sites across New South Wales; samples were processed and stored at St Vincent's Centre of Applied Medical Research, Darlinghurst. Eligible subjects were HIV-positive, asymptomatic and diagnosed at least 8 years previous to enrolment, treatment naive, and had an absolute CD4$^+$ T-cell count $\geqslant$500 cells per μl. All participants gave informed written informed consent. The St Vincent's Research Ethics Committee (EC00140) approval numbers for these studies are: Healthy volunteers (13/094); chronic HIV-1 cohort (08/052); and LTNP (approval number: HREC/12/SVH/298, SVH 12/217).

CD25/OX40 assay

PBMCs were cultured in 0.5 ml of IMDM (JRH, Lenexa, KS, USA) supplemented with 10% heat-inactivated Human AB serum (Lonza, NJ, USA) in 24-well plates (BD Biosciences, North Ryde, NSW, Australia). Individual cultures were stimulated with CMV lysate (grade III; Meridian Life Science, Memphis, TN, USA) at a final concentration of 2 μg ml^{-1}; TT (Commonwealth Serum Laboratories, Melbourne, VIC, Australia) at a final concentration of 2 LFU per ml); or HIV Clade B gag pool of 123 15mer overlapping peptides (NIH AIDS Research and Reference Reagent Program) used at a final concentration of 2 μg ml^{-1} for each peptide. Cultures were incubated at 37 °C for 48 h in a humidified atmosphere with 5% CO_2 in air. Negative control cultures comprised PBMCs mixed with IMDM with 10% heat inactivated AB serum only, while SEB (5 μg ml^{-1}) was used for positive control cultures.[9]

TF staining and gating strategies

After 48 h incubation as above, $CD3^+CD4^+$ and $CD3^+CD8^+$ populations were gated from the lymphocyte subset defined by FSC vs SSC; doublets were excluded from analyses and single-cell sorting. $CD3^+CD8^+$ were used to set the quadrants for TF analysis, as $CD3^+CD8^+$ cells express Tbet, but not Foxp3. $CD3^+CD4^+$ cells were divided into antigen-specific cells using $CD25^+CD134$ $(OX40)^+$ (quadrant 2) and these cells were then analyzed for Tbet and Foxp3 expression (Supplementary Figure 1A).

Single-cell RT-PCR

A number of 186 single cells were sorted by FACSAria into individual wells in 3×96-well PCR plates. Before first round RT pre-amplification, 12 primer pairs and Superscript III/Taq polymerase enzymes were added. Lysis of the cells was achieved by differential osmolality between the cells and the PCR buffer, and heating to 51 °C prior to the RT step. Twenty-two cycles of multiplex pre-amplification was necessary to ensure that the low-level mRNA transcripts within each cell were detectable in real-time PCR. The first round product was diluted and split into aliquots for second round nested real-time PCR in a 384-well plate for subsequent detection of each individual TF *(tbx21*, *gata3*, *rorc*, *foxp3* and *Bcl-6)*, β-actin was used as the reference gene. For further details please refer to Phetsouphanh *et al.*[8]

Statistics

Wilcoxon paired *t*-test was used to analyze statistical data employing the Prism 5.0a (GraphicPad, La Jolla, CA, USA) software. For unpaired samples, the Mann–Whitney *U*-test was used. *P*-values <0.05 were considered significant. SPICE software (NIAID, NIH, Bethesda, MD, USA) was used for pie charts.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

1 Abdula S, Sallim N, Tanner M. Randomized, controlled trial of the long term safety, immnogenicity and efficacy of RTS,S/AS02D malaria vaccine in infants living in a malaria-endemic region. *Malar J* 2013; **12**: 1–11.
2 Delogu G, Manganelli R, Brennan MJ. Critical research concepts in tuberculosis vaccine development. *Clin Microbiol Infect* 2013; **20**: 59–65.
3 Plotkin SA. The RV144 Thai HIV vaccine trial. *Hum Vaccin* 2010; **6**: 159.
4 Klenerman P, Hill A. T cells and viral persistence: lessons from diverse infections. *Nat Immunol* 2005; **6**: 873–879.
5 Swain SL, McKinstry KK, Strutt TM. Expanding roles for $CD4^{(+)}$ T cells in immunity to viruses. *Nat Rev Immunol* 2012; **12**: 136–148.
6 De Rosa SC, Lu FX, Yu J, Perfetto SP, Falloon J, Moser S *et al.* Vaccination in humans generates broad T cell cytokine responses. *J Immunol* 2004; **173**: 5372–5380.
7 Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 2008; **8**: 247–258.
8 Phetsouphanh C, Xu Y, Amin J, Seddiki N, Procopio F, Sekaly R *et al.* Characterization of transcription factor phenotypes within antigen-specific $CD4^+$ T cells using qualitative multiplex single-cell RT-PCR. *PLoS ONE* 2013; **8**: e74946.
9 Zaunders JJ, Munier ML, Seddiki N, Pett S, Ip S, Bailey M *et al.* High levels of human antigen-specific $CD4^+$ T cells in peripheral blood revealed by stimulated coexpression of CD25 and CD134 (OX40). *J Immunol* 2009; **183**: 2827–2836.
10 Dominguez MH, Chattopadhyay PK, Ma S, Lamoreaux L, McDavid A, Gottardo R *et al.* Highly multiplexed quantitation of gene expression on single cells. *J Immunol Methods* 2013; **391**: 133–145.
11 Mahnke YD, Greenwald JH, DerSimonian R, Roby G, Antonelli LR, Sher A *et al.* Selective expansion of polyfunctional pathogen-specific $CD4^{(+)}$ T cells in HIV-1-infected patients with immune reconstitution inflammatory syndrome. *Blood* 2012; **119**: 3105–3112.
12 Gerlach C, Perie L, van Rooji N, van Heijst JW, Velds A *et al.* Heterogeneous differentiation patterns of individual $CD8^+$ T cells. *Science* 2013; **340**: 635–639.
13 Li S, Gowans EJ, Chougnet C, Plebanski M, Dittmer U. Natural regulatory T cells and persistent viral infection. *J Virol* 2008; **82**: 21–30.
14 Vukmanovic-Stejic M, Agius E, Booth N, Dunne PJ, Lacy KE, Reed JR *et al.* The kinetics of $CD4^+Foxp3^+$ T cell accumulation during a human cutaneous
antigen-specific memory response *in vivo. J Clin Invest* 2008; **118**: 3639–3650.
15 Cook L, Munier CM, Seddiki N, van Bockel D, Ontiveros N, Hardy MY *et al.* Circulating gluten-specific $FOXP3^+CD39^+$ regulatory T cells have impaired suppressive function in patients with celiac disease. *J Allergy Clin Immunol* 2017; **8**: 1–28.
16 Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G *et al.* Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* 2012; **122**: 3271–3280.
17 Xu Y, Weatherall C, Bailey M, Alcantara S, De Rose R, Estaquier J *et al.* Simian immunodeficiency virus infects follicular helper CD4 T cells in lymphoid tissues during pathogenic infection of Pigtail Macaques. *J Virol* 2013; **87**: 3760–3773.
18 Colineau L, Rouers A, Yamamoto T, Xu Y, Urrutia A, Pham HP *et al.* HIV-infected spleens present altered follicuar helper T cell (Tfh) subsets and skewed B cell maturation. *PLoS ONE* 2015; **10**: e0140978.
19 Onabajo OO, Mattapallil JJ. Expansion or depletion of T follicular helper cells during HIV infection: consequences for B cell responses. *Curr HIV Res* 2013; **11**: 595–600.
20 Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC *et al.* CD4 T follicular helper cell dynamics during SIV infection. *J Clin Invest* 2012; **122**: 3281–3294.
21 Kelleher AD, Carr A, Zaunders J, Cooper DA. Alterations in the immune response of HIV-infected subjects treated with a HIV-specific protease Inhibitor: Ritonavir. *J Infect Dis* 1996; **173**: 321–329.
22 Oestreich KJ, Huang AC, Weinmann AS. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *J Exp Med* 2011; **208**: 1001–1013.
23 Evans CM, Jenner RG. Transcription factor interplay in T helper cell differentiation. *Brief Funct Genomics* 2013; **12**: 499–511.
24 Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* 2011; **35**: 583–595.
25 Linterman MA, Liston A, Vinuesa CG. T-follicular helper cell differentiation and the co-option of this pathway by non-helper cells. *Immunol Rev* 2012; **247**: 143–159.
26 Koch MA, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 2009; **10**: 595–602.
27 Stock P, Berry G, Freeman GJ, Dekruyff RH, Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol* 2004; **5**: 1149–1156.
28 Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* 2011; **208**: 987–999.

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