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Research paper

# Identification of murine antigen-specific T follicular helper cells using an activation-induced marker assay

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ABSTRACT

Protective antibody (Ab) responses induced by natural infection or vaccination play a central role in defense against invasive pathogens. Germinal centers (GCs) are the sites of Ab affinity maturation and T follicular helper (Tfh) cells are a critical factor for driving GC formation and B cell selection. Therefore characterization of antigen (Ag)-specific Tfh cells is increasingly essential to define the mechanistic basis of protective antibody responses. However, since Tfh are weak producers of cytokines it is difficult to detect Ag-specific Tfh cells using conventional intracellular cytokine staining (ICS). Here, we report an assay identifying mouse Ag-specific Tfh cells by assessing the upregulation of surface activation-induced markers (AIM). Murine lymph node (LN)-derived Tfh cells largely retained CXCR5 and PD-1 expression following 18-hour cell culture. After influenza infection or influenza hemagglutinin (HA) protein vaccination of mice, stimulation of lymph node cell suspensions with peptide pools or whole protein drove upregulation of CD25, OX40 (CD134), ICOS (CD278) and CD154 on Tfh cells. Upregulation of either CD154 or CD25/OX40 proved a sensitive method for delineating HA-specific Tfh cells. This assay provides the opportunity to quantify antigen-specific Tfh cells in mice without the need for transgenic models or MHC-II tetramer reagents restricted to specific epitopes.

## 1. Introduction

Vaccination is widely acknowledged as one of the most cost-effective preventive measures for fighting infectious diseases. Although immunogens can induce both antibody and cell-mediated immune responses, most current licensed vaccines confer protection against subsequent infection by eliciting protective antibodies. Generation of protective antibodies with high affinity requires co-ordinated activation and differentiation of antigen-specific B and T lymphocytes, a process which takes place in secondary lymphoid organs (SLO) (Victora and Nussenzweig, 2012).

T follicular helper (Tfh) cells are a subset of CD4+ T cells which localize in the germinal centres (GCs) of SLO (Crotty, 2011; Linterman and Hill, 2016; Qi, 2016; Vinuesa et al., 2016). These specialized cells are crucial for the formation of GC, affinity maturation and the maintenance of B cell memory (Gatto and Brink, 2010; Victora et al., 2010; Victora and Nussenzweig, 2012; Liu et al., 2015). BCL6 is the master transcription factor for Tfh differentiation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), and distinguishing phenotypic markers of Tfh cells include the high expression of CXCR5, PD-1, and ICOS. IL-21, IL-4, and CD40L are crucial effector molecules produced by Tfh cells to help GC B cell proliferation and differentiation (Vinuesa et al., 2016).

Interactions between GC B cells and Tfh are mediated via TCR recognition of cognate peptides presented by B cells upon MHC class II (Tangye et al., 2015), which leads to the activation and proliferation of antigen-specific Tfh cells. Classical intracellular cytokine staining (ICS) assays have been extensively employed to identify Ag-specific CD4+ T cells in blood (Phetsouphanh et al., 2015). Typically, ICS assays have measured IFN-γ, IL-2 and TNF-α, which are produced in sufficient amounts to be readily detected by flow cytometry. However, analogous detection of Ag-specific Tfh cells via ICS is difficult because Tfh cells mainly provide contact dependent, selective help to GC B cells as opposed to wholesale cytokine secretion (Dan et al., 2016). Recently, an alternative to ICS assays was developed; this technique identifies Ag-specific Tfh based on the upregulation of surface activation markers,

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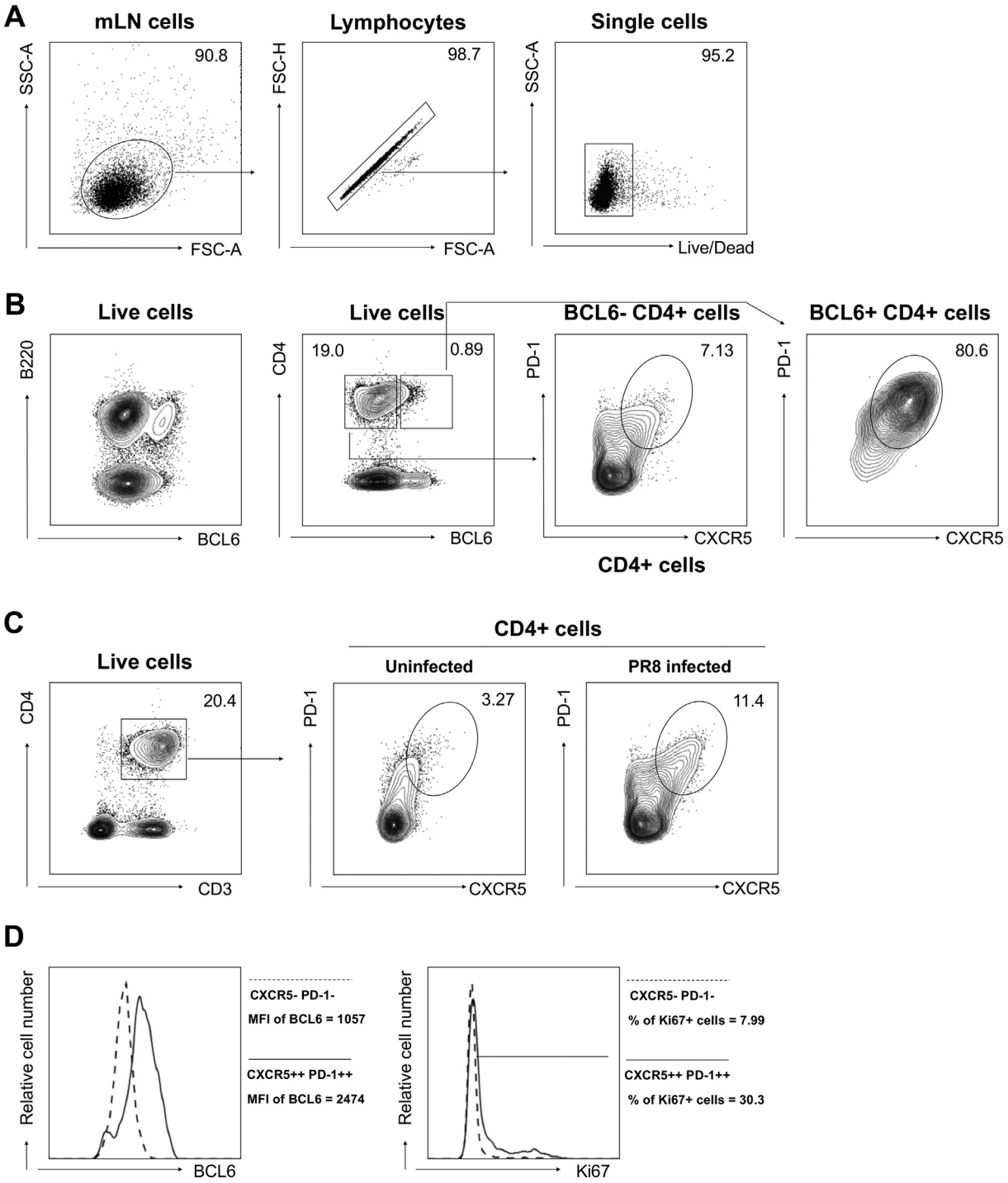


**Fig. 1.** Identification of Tfh cells in mouse mediastinal lymph node (mLN).
(A) Gating strategy to identify live lymphocytes in the mLN. Lymphocytes were identified by forward scatter area (FSC-A) and side-scatter area (SSC-A). Doublets were excluded by gating on single cells as determined by FSC-A versus FSC-H, and live cells were identified by viability dye exclusion. For each step, the parental population is indicated above the plot. (B) BCL6 expression among B220 + B cells was used as a gating guide to identify BCL6- and BCL6 + CD4 + T cells. Subsequent plots show the representative expression of CXCR5 and PD-1 within the CD4 + BCL6- and CD4 + BCL6 + T cell populations, which was used to generate a CXCR5 + + PD-1 + + gate to identify Tfh cells. Numbers indicate the proportion of the parent gate within the CXCR5 + + PD-1 + + gate. (C) Using the Tfh surface marker gate determined in B, Tfh cells were identified within the CD4 + T cell population of naïve versus influenza infected mice (at day 14 post-infection). (D) Representative histograms depicting the expression of BCL6 (median fluorescent intensity, MFI) and Ki67 (% positive) among Tfh and non-Tfh cells from influenza-infected mice.

such as CD25, PD-L1 (CD274) and OX40 (CD134) (Zaunders et al., 2009; Keoshkerian et al., 2012; Dan et al., 2016; Havenar-Daughton et al., 2016; Reiss et al., 2017; Bowyer et al., 2018). The CD25/OX40 assay was initially used to identify human Ag-specific CD4 T cells in peripheral blood (Zaunders et al., 2009), and has subsequently been extended to both human and macaque lymph node samples (Dan et al., 2016; Havenar-Daughton et al., 2016). Compared with ICS, the AIM assay identified 85-fold or 10-fold more Ag-specific Tfh cells in humans (Dan et al., 2016) or macaques (Havenar-Daughton et al., 2016), respectively, highlighting the increased sensitivity of this approach. Here,

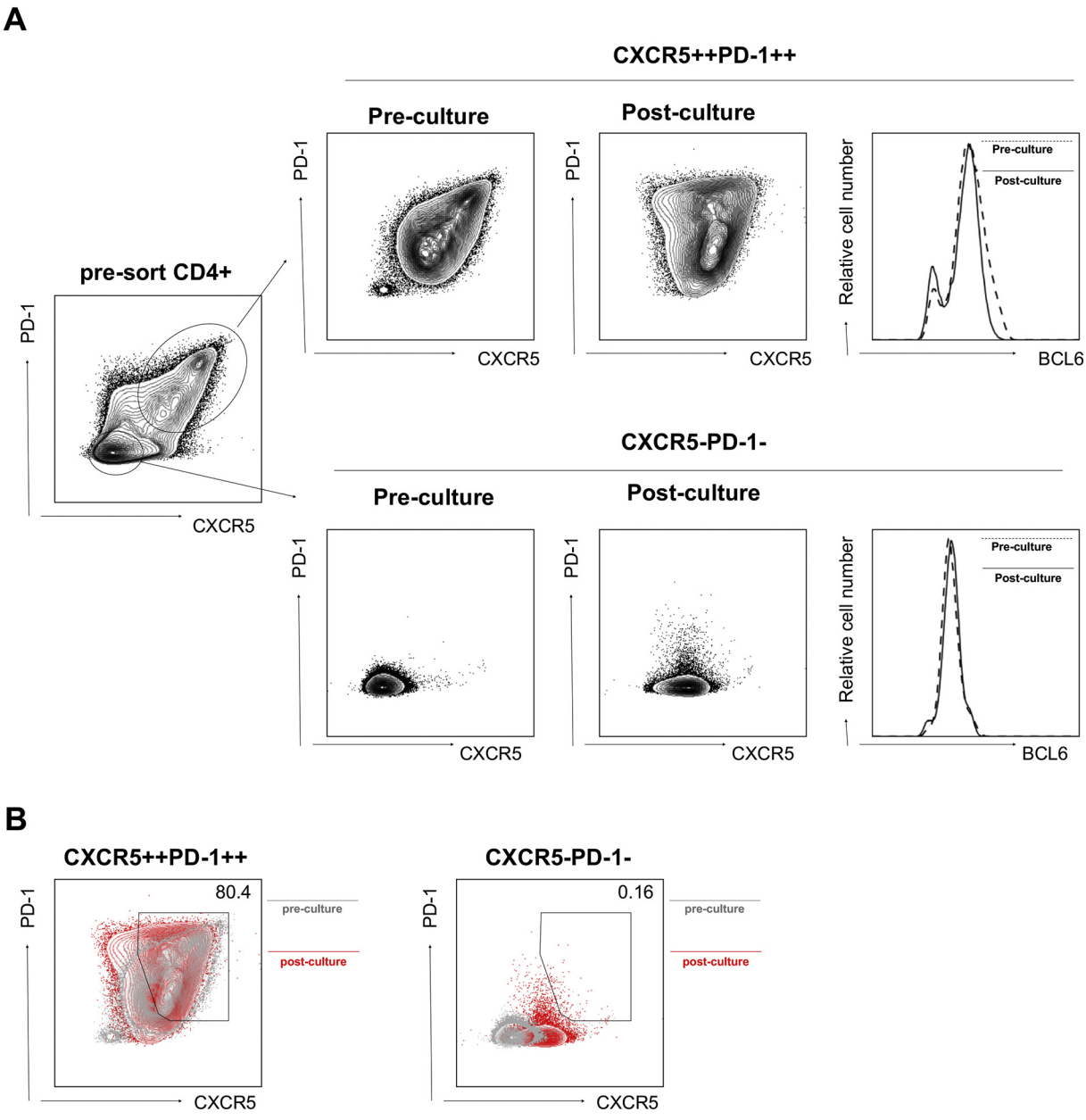


**Fig. 2.** Confirmation of Tfh phenotype following tissue culture.
(A) Tfh (CD4 + CXCR5 + + PD-1 + +, upper row) and non-Tfh (CD4 + CXCR5-PD-1-, lower row) cells were sorted from the mLN cells of influenza-infected mice (day 14 post-infection). Immediately post-sort, both populations were stained for intracellular BCL6 expression and analyzed for BCL6, CXCR5 and PD-1 expression (pre-culture plots). Tfh and non-Tfh cells were cultured separately for 18 h, and stained for expression of CXCR5, PD-1 and BCL6 (post-culture plots). Histograms show a comparison of BCL6 expression in each population before (dashed line) and after (solid line) culture. Results are representative of two independent experiments. (B) A gate to identify Tfh cells following cell culture using CXCR5 and PD-1 expression was determined by comparing the phenotype of Tfh and non-Tfh cells pre- (grey) and post-culture (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

we present an AIM assay to identify mouse Ag-specific Tfh cells, validated in the context of influenza infection and vaccination.

## 2. Materials and methods

### 2.1. Mouse infection and immunization

Mouse studies and related experimental procedures were approved by the University of Melbourne Animal Ethics Committee (#1714193). Female C57BL/6 mice (6–8 weeks old) were anesthetized by isoflurane inhalation prior to infection or immunization. For intranasal infections, mice were instilled with 50 µL of 50 $TCID_{50}$ of A/Puerto Rico/8/34 (PR8). For intramuscular vaccinations, 5 µg of PR8 HA protein with Addavax (1:1 ratio; InvivoGen) were injected into both hind quadriceps using a 29G needle. 14 days post infection or vaccination, draining lymph nodes (mediastinal lymph nodes (mLN) for infected animals, inguinal lymph nodes (inLN) and iliac lymph nodes (ilLN) for vaccinated animals) and spleen were collected.

### 2.2. Antigen stimulations and cell culture

Draining lymph nodes from flu-infected or HA-vaccinated mice were mashed into single cell suspensions and either pooled or cultured individually in RPMI 1640 supplemented with 10% fetal calf serum and penicillin/streptomycin (RF10). To identify antigen-specific Tfh cells using peptide stimulation, cell suspensions were stimulated for 18 h in

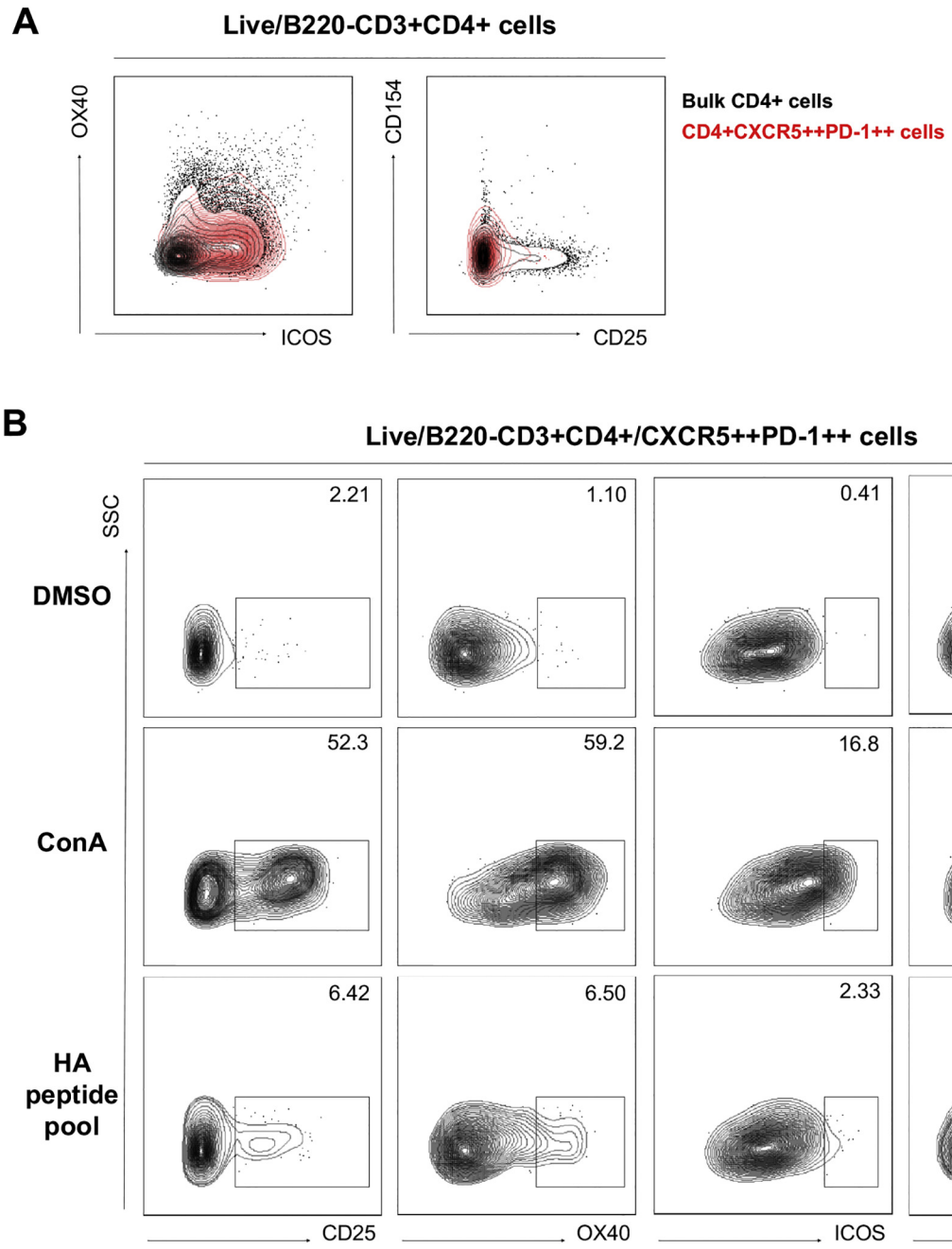


**Fig. 3.** Activation-induced marker expression following mitogenic or antigen-specific stimulation.
(A) Expression of ICOS, OX40, CD25 and CD154 in unstimulated CD4+ (black) and Tfh cells (red) following culture. (B) Representative expression of CD25, OX40, ICOS and CD154 expression on Tfh cells after 18 h of stimulation with DMSO, ConA or HA peptide pool. Gates for ICOS and OX40 indicate ICOS++ and OX40++ cells, as determined by baseline ICOS and OX40 expression. Cells were derived from pooled mLN samples of n = 10 influenza-infected mice. Results are representative of 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

RF10 with a PR8 HA peptide pool (2 μg/peptide/mL, 93 peptides of 17mers overlapping by 11 amino acids, BEI resources), Concanavalin A (ConA, 5 μg/mL, Sigma) or a vehicle (DMSO) control. Cells were cultured in a 48-well plate in 500 μl at a concentration ranging from 2 to 8 million cells/ml. At the time of stimulation, anti-CD154 BV650 mAb (MR1; BD Biosciences) was added to all culture conditions. For kinetics experiments, mLN single cell suspensions from 5 mice were pooled together and 2 million cells were seeded into each well. For other experiments, LN single cell suspensions from individual mice were analyzed.

To identify antigen-specific Tfh cells using protein stimulation, freshly isolated LN single cell suspensions were either cultured alone in 96-well U-bottom plate in 200 μL RF10 or labelled with CellTrace Yellow (Thermo Fisher) and co-cultured with unlabeled splenocytes (1:10 ratio of splenocytes:LN cells) in a 48-well plate in 500 μL RF10. Cells were stimulated with HA protein (5 μg/mL), or a negative protein control (BSA, 5 μg/mL). At the time of stimulation, anti-CD154 BV650 mAb (MR1; BD Biosciences) was added to all culture conditions.

### 2.3. Antibodies and flow cytometry

For detection of Tfh cells ex vivo, freshly isolated LN cell suspensions were stained with the following panel: Live/dead Red (Thermo Fisher), B220 BV605 (RA3-6B2; BD Biosciences), CD3 BV510 (145-

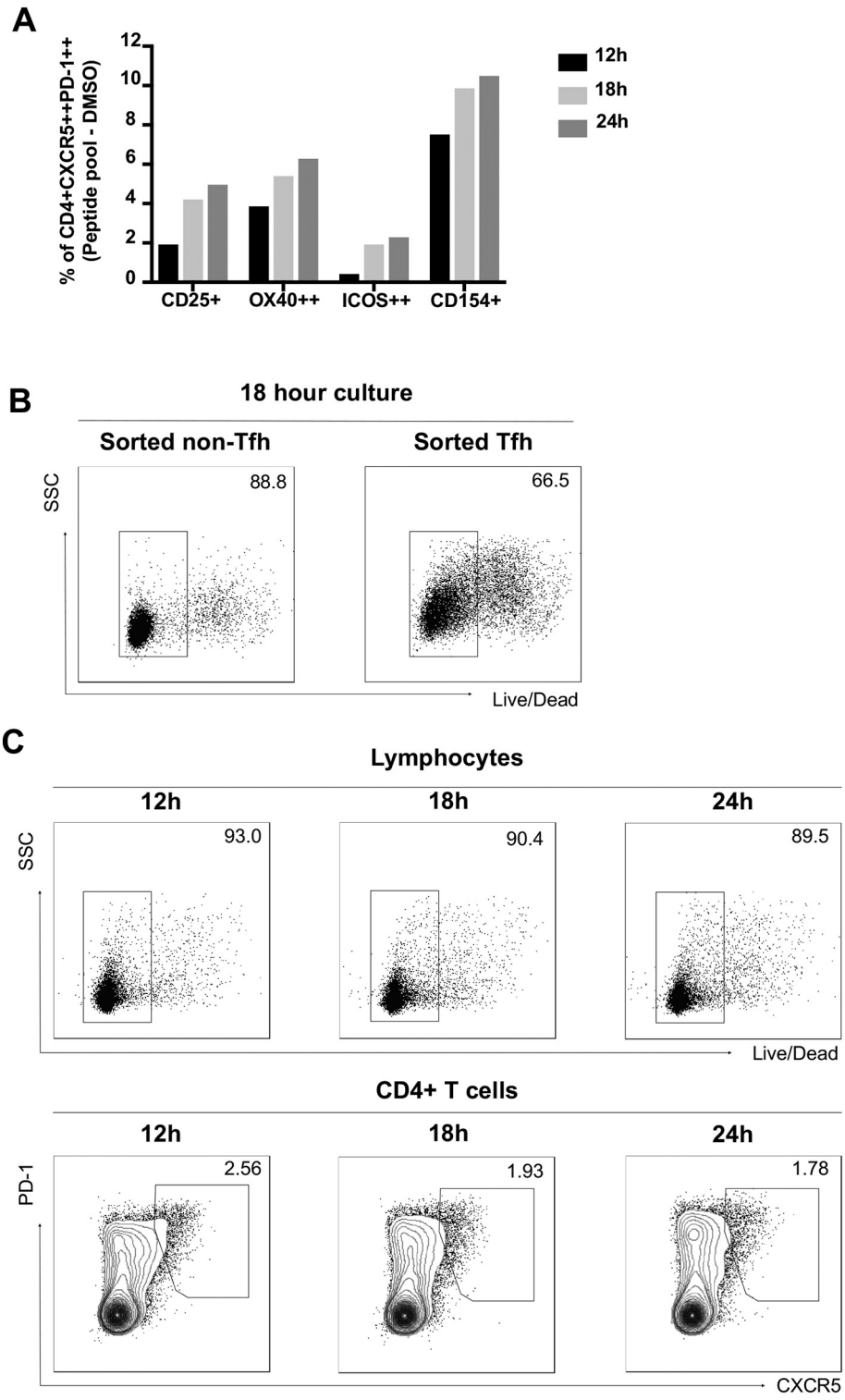


**Fig. 4.** Kinetics of Tfh cell activation marker expression and Tfh cell viability in cell culture.
(A) Kinetics of CD25, OX40, ICOS and CD154 upregulation on Tfh cells after 12 h, 18 h or 24 h of stimulation with HA peptide pool. Data is background subtracted based on the DMSO control. (B) Viability of sorted non-Tfh (CD4 + CXCR5-PD-1-) and Tfh (CD4 + CXCR5+ +PD-1+ +) cells from influenza-infected mice after culture for 18 h. (C) Plots indicate the viability of the bulk mLN lymphocyte population and the proportion of Tfh cells (within the CD4+ T cell population) after 12, 18 or 24 h of cell culture in influenza-infected mice.

2C11; BioLegend), CD4 BUV737 (RM4-5; BD Biosciences), CXCR5 BV421 (L138D7; BioLegend), PD-1 BV786 (29F.1A12; BioLegend), BCL6 AF647 (IG191E/A8; BioLegend), Ki67 BUV395 (B56; BD Biosciences). For BCL6 and Ki67 staining, cells were fixed, permeabilized, and stained using the BD Transcription Factor Buffer kit (BD Biosciences) according to the manufacturer's instructions.

For Ag-specific Tfh identification, cells were cultured as described above and then stained with the following panel: Live/dead Blue (Thermo Fisher), B220 BV605 (RA3-6B2; BD), CD3 BV510 (145-2C11; BioLegend), CD4 BUV737 (RM4-5; BD), CXCR5 BV421 (L138D7;

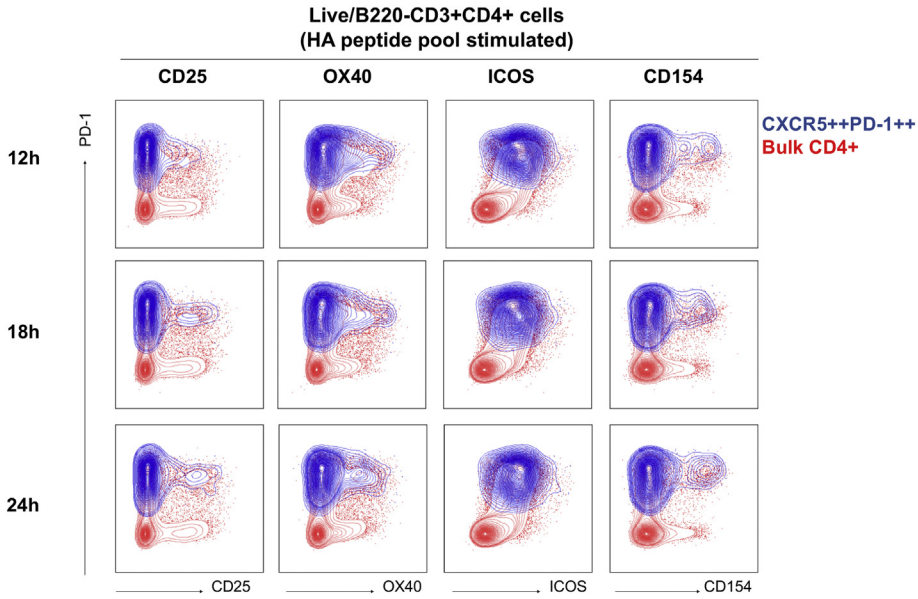


**Fig. 5.** Comparison of PD-1 and activation marker expression on HA-stimulated Tfh cells over time. mLN-derived cells were stimulated with HA peptide pool for 12, 18 or 24 h. Plots show bulk CD4 + T cells (red) or CXCR5 + +PD-1 + + Tfh cells (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BioLegend), PD-1 BV786 (29F.1A12; BioLegend), CD25 BB515 (PC61; BD), OX40 PeCy7 (OX-86; BioLegend), ICOS PerCP-eFluor710 (15F9; Thermo Fisher), BCL6 AF647 (IG191E/A8; BioLegend).

All samples were acquired on a BD LSR Fortessa using BD FACS Diva and data was analyzed in FlowJo v10.

### 2.4. Cell sorting and culture

For cell sorting, freshly isolated mediastinal lymph nodes (mLN) from influenza infected mice were mashed into single cell suspensions, pooled together and stained with the following panel: Live/dead Red (Thermo Fisher), B220 BV605 (RA3-6B2; BD), CD3 BV510 (145-2C11; BioLegend), CD4 FITC (RM4-5; BioLegend), CXCR5 BV421 (L138D7; BioLegend), PD-1 BV786 (29F.1A12; BioLegend). Cells were sorted on a BD Aria III. After sorting, a portion of the sorted cells were intracellularly stained with BCL6 AF647 (IG191E/A8; BioLegend) and acquired on a BD LSR Fortessa. The remaining cells were cultured for 18 h in RF10 and then stained with the aforementioned panel of antibodies and acquired on a BD LSR Fortessa.

### 2.5. Statistical analysis

Data is presented as mean ± standard deviation and produced using GraphPad Prism version 7 (GraphPad Software, La Jolla California USA).

## 3. Results

### 3.1. Identification of Tfh cells in murine lymph node suspensions

BCL6 expression is the canonical marker to distinguish Tfh cells from other CD4 + T cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). However, many studies use high expression of CXCR5 and PD-1 as surrogate surface markers for the Tfh population in murine lymphoid tissues (Meli and King, 2015). We first validated a flow cytometric panel to allow Tfh identification in mice experimentally infected with influenza. C57BL/6 mice were intranasally inoculated with a sublethal dose of influenza virus and 14 days post-infection, lung-draining mediastinal lymph nodes (mLN) were harvested, stained with a panel of antibodies, and analyzed by flow cytometry (Fig. 1A). Using the BCL6 + GC B cell population (B220 + CD3- cells) as a guide, we identified BCL6 + CD4 + T cells (Fig. 1B). The majority (over 80%) of BCL6 + CD4 + T cells were CXCR5 + +PD-1 + +, enabling the definition of a gated population that were predominantly Tfh cells (Fig. 1C). In naive mice, this population was relatively infrequent compared to influenza-exposed mice (Fig. 1C). Relative to non-Tfh cells (CD4 + CXCR5-PD-1-), Tfh cells (CD4 + CXCR5 + +PD-1 + +) displayed elevated expression of both BCL6 and the proliferation marker Ki67 (Fig. 1D). We therefore used this analysis to set the CXCR5/PD-1 gate used for subsequent identification and sorting of murine Tfh cells ("ex vivo Tfh gate").

### 3.2. Phenotype of Tfh cells following cell culture

Determining the antigen specificity of T cells often requires re-stimulation in vitro, which could cause changes to Tfh surface phenotype. We therefore assessed the stability of CXCR5 and PD1 expression on Tfh and non-Tfh CD4 + T cell populations following culture. Using the ex vivo Tfh gate, Tfh and non-Tfh cells were sorted from pooled mLN cell suspensions of influenza-infected mice at day 14 post-infection and were analyzed immediately post-sort, or after 18 h of culture. The Tfh cells exhibited some downregulation of PD-1 expression during culture (Fig. 2A). We also observed minor changes in CXCR5 expression after culture, with a degree of downregulation observed in Tfh cells, and a minor upregulation in non-Tfh cells (Fig. 2A). There was a minor loss of BCL6 expression in the Tfh population, but no change in the non-Tfh cells (Fig. 2A). Despite the slight changes in PD-1 and CXCR5 expression, clear differentiation of non-Tfh and Tfh populations using PD-1 and CXCR5 remained possible. A new PD1 + +CXCR5 + + gate was generated to selectively identify Tfh cells post-culture ("post-culture" Tfh gate, Fig. 2B), which was applied to all antigen stimulation experiments.

### 3.3. Activation-induced marker expression to delineate Ag-specific Tfh cells

We next assessed whether previously reported activation markers were induced on murine Tfh cells following antigen stimulation. mLN cell suspensions from influenza-infected mice at day 14 post-infection were stimulated with DMSO, a HA peptide pool or ConA for 18 h. Due to the reported transient upregulation of CD40L during T cell activation (Chattopadhyay et al., 2005; Frentsch et al., 2005), anti-CD40L (CD154) antibody was added to the culture media for the duration of the stimulation. Cells were stained for activation markers CD25, OX40 and ICOS and compared to DMSO unstimulated controls. Baseline expression (stimulation with DMSO) of ICOS and OX40 upon Tfh was high, with low levels of CD25 and CD154 observed (Fig. 3A). As a

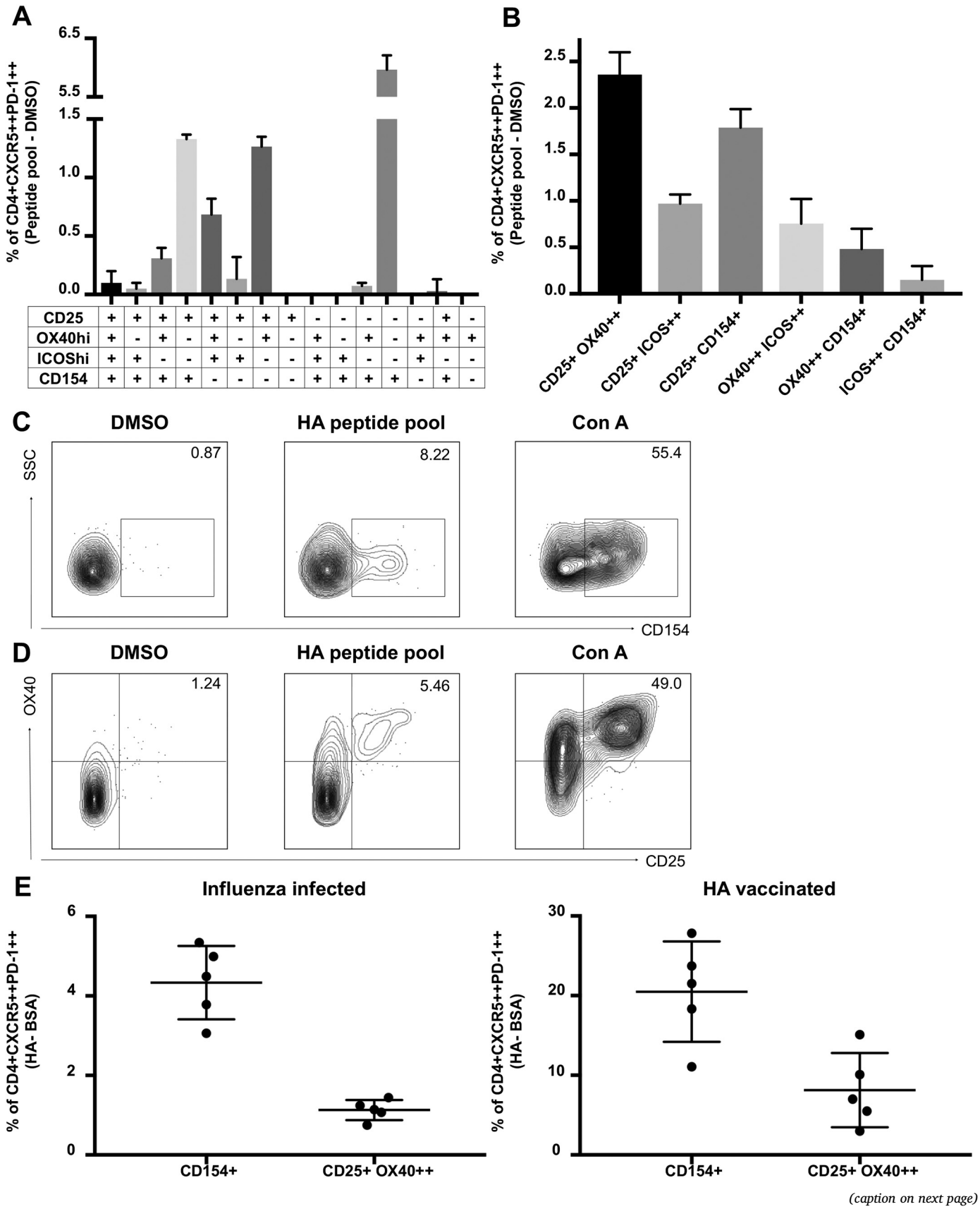


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**Fig. 6.** Comparison of activation markers for identifying Ag-specific Tfh cells.
(A) mLN-derived cells were stimulated with DMSO, HA peptide pool or ConA for 18 h. Boolean gating was used to identify the phenotypes of antigen-specific Tfh cells expressing any combination of CD25, OX40, ICOS or CD154. Error bars indicate mean ± SD from 2 replicates. (B) Boolean-gated populations were combined to determine the combination of two activation markers that best identified antigen-specific Tfh cells. Error bars indicate mean ± SD from 2 replicates. (C) Representative plots of CD154+ expression on Tfh cells after DMSO, HA peptide pool or ConA stimulation for 18 h. (D) Representative plots identifying CD25 + OX40++ Tfh cells after DMSO, HA peptide pool or ConA stimulation for 18 h. All results are representative of 3 independent experiments. (E) Quantification of Ag-specific Tfh cells measured by the proportion of CD154+ or CD25 + OX40++ cells after background subtraction in individual influenza infected or HA vaccinated mice. N = 5 mice in each group. All results are representative of 2 independent experiments.

result, we assessed activation defined by CD25 or CD154 expression, or the proportion of cells with an OX40++ or ICOS++ phenotype. Stimulation with ConA confirmed that all four markers were upregulated upon Tfh cell activation. Stimulation with the HA peptide pool similarly resulted in the upregulation of the activation markers (Fig. 3B), confirming that these markers have utility for identifying Ag-specific Tfh populations in mice.

### 3.4. Kinetics of Ag-specific Tfh activation and viability

To optimise the detection of Ag-specific Tfh, we next determined the kinetics of Tfh cell activation marker expression following HA peptide stimulation. Overall, the expression of all four markers increased as the stimulation time increased from 12 to 18 to 24 h (Fig. 4A). We noted, however, that recovery of sufficient Tfh cells for analysis was a limiting variable following cell culture. Analysis of sort-purified Tfh and non-Tfh CD4+ T cells confirmed that Tfh cells died more readily than non-Tfh cells during culture (Fig. 4B). Thus, although the viability of the bulk population of mLN lymphocytes changed little from 12 h to 24 h of culture (93% live cells at 12 h versus 89.5% at 24 h), approximately 30% of the Tfh population was lost during this time period (2.56% of bulk CD4+ T cells at 12 h versus 1.78% at 24 h, Fig. 4C). We therefore selected a stimulation time of 18 h in order to achieve a balance between Tfh cell loss and upregulation of surface activation markers.

### 3.5. Maintenance of antigen-specific Tfh phenotype over time

Given the changes in sort-purified Tfh surface phenotype after 18 h of cell culture (Fig. 2A), and the progressive loss of Tfh cells in MLN cultures from 12 to 24 h (Fig. 4C), we sought to determine whether HA-specific Tfh cells progressively downregulated PD-1 expression over this time period. A comparison of PD-1 and activation marker expression among Tfh cells at 12, 18 and 24 hours post-HA stimulation revealed that antigen-specific Tfh cells consistently maintained a high level of PD-1 expression, regardless of the timepoint examined (Fig. 5). This data suggests that antigen-specific Tfh cells do not exhibit preferential loss of PD-1 expression over time, and that their loss from bulk MLN cultures is largely attributable to cell death.

### 3.6. CD154 and CD25/OX40 best identify Ag-specific Tfh cells

While all four markers examined demonstrated dynamic expression upon Tfh in response to stimulation, we used Boolean gating to identify combinations of markers that most robustly identified Ag-specific Tfh cells (Fig. 6A). Considering all 15 four-marker combinations, CD154 single-positive cells represented the highest proportion of influenza-specific Tfh cells following infection (Fig. 6A). When analyzed using combinations of two markers, the CD25 + OX40++ phenotype identified the highest proportion of Ag-specific cells (Fig. 6B). We therefore focused on CD154+ cells and CD25 + OX40++ cells. CD154 upregulation alone detected approximately 60% of the total influenza-specific Tfh identified by AIM expression, while the CD25 + OX40++ phenotype identified 23% of total Ag-specific cells. We therefore conclude that detection of CD154 with a monoclonal antibody during cell culture (Fig. 6C) and/or quantification of CD25 + OX40++ cells following 18 h of peptide stimulation (Fig. 6D) provide the greatest sensitivity for identifying Ag-specific Tfh cells in mice.

Using this assay, we found that a mean of 4.32% (3.06–5.34, ± 0.92) of Tfh cells identified by CD154+ or 1.13% (0.75–1.44, ± 0.25) of Tfh cells identified by CD25 + OX40++ are HA-specific following influenza infection across individual mice (Fig. 6E). In comparison, HA vaccination results in a mean of 20.49% (11.07–20.84, ± 6.30) HA-specific Tfh cells measured by CD154+ or 8.14% (3.00–15.10, ± 4.66) HA-specific Tfh cells measured by CD25 + OX40++ (Fig. 6E). The assay was reliable and reproducible across different experiments, using either pooled LN suspensions or LN suspensions from individual mice.

### 3.7. Detection of Ag-specific Tfh cells following whole protein stimulation

In situations where peptide pools are impractical to obtain or produce, whole proteins may be required as the antigen source for Tfh re-stimulation. While Ag-specific Tfh cells can be readily identified following peptide stimulation in 500 µL RF10 using 48-well plates, we found that whole proteins elicited relatively poor responses under the same conditions. Therefore, we modified the assay to detect Ag-specific Tfh cells following whole protein stimulation. We found that using 96-well U-bottom plates and a culture volume of 200 µL RF10 induced robust upregulation of AIM markers with LN suspensions from both influenza-infected and HA-vaccinated mice (Fig. 7A). Stimulation with whole HA protein facilitated the identification of a mean of 4.66% (3.21–6.49, ± 1.31) or 3.83% (2.60–5.10, ± 1.11) of HA-specific Tfh cells based upon CD154+ or CD25 + OX40++ upregulation respectively (Fig. 7B). By comparison, following HA vaccination we observed a mean of 3.17% (0.66–7.60, ± 2.94) HA-specific Tfh cells measured by CD154+ or 23.29% (16.77–28.90, ± 4.33) HA-specific Tfh cells measured by CD25 + OX40++ (Fig. 7B). As an alternative, we obtained similar results by co-culturing LN suspensions with autologous splenocytes (LN cells: splenocytes = 10:1) in a 48-well plate in 500 µl RF10 (Fig. 7C). Using this method, 4.01% (detected by CD154+) or 3.10% (detected by CD25 + OX40++) of Tfh cells were HA-specific for influenza-infected mice and 1.22% (detected by CD154+) or 5.84% (detected by CD25 + OX40++) of Tfh cells are HA-specific for HA-vaccinated mice (Fig. 7C). Notably, the discrepancy between the CD154+ and CD25 + OX40++ readouts suggest that the CD25 + OX40++ phenotype may be a more robust marker of Ag-specific Tfh following re-stimulation with whole protein. Taken together, these data show that Ag-specific Tfh cells could be detected following whole protein stimulation either using LN suspensions alone in 96-well U-bottom plates or co-culture of LN suspensions with splenocytes in 48-well plates.

## 4. Discussion

Characterization of Ag-specific Tfh cells can greatly assist providing an immunological rationale for the design of novel vaccines against infectious pathogens. However, it is difficult to detect Ag-specific GC Tfh cells by conventional ICS, and methods to study Ag-specific Tfh in mice are lacking. The use of CD25/OX40 for identification of human and macaque Ag-specific Tfh cells has been reported (Dan et al., 2016; Havenar-Daughton et al., 2016), but not validated in mice. Considering the crucial role of CD154 plays in the interaction between Tfh and GC B

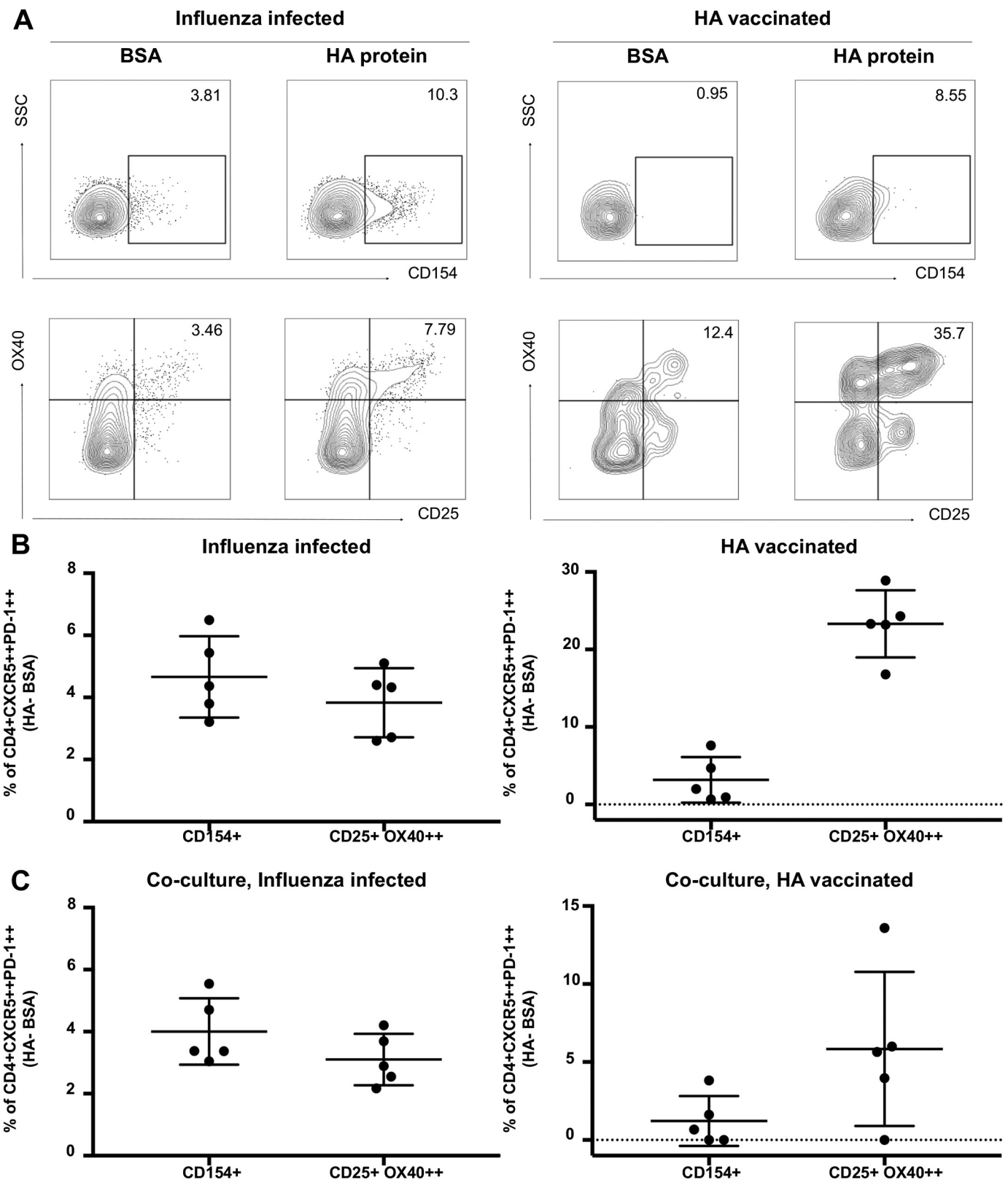


**Fig. 7.** Modification of assay for whole protein stimulation.

Draining lymph nodes were harvested from mice 14 days post-infection or HA vaccination. (A) LN suspensions were cultured in 200uL of RF10 in 96-well U-bottom plates for 18 h and stimulated with 5 μg/mL HA protein or BSA. Plots show representative CD154 or CD25/OX40 staining following stimulation in individual influenza infected or HA vaccinated mice (representative of n = 5). (B) Quantification of Ag-specific Tfh cells measured by the proportion of CD154+ or CD25 + OX40++ cells after background subtraction. N = 5 mice in each group. (C) LN suspensions were co-cultured with autologous splenocytes (LN cells: splenocytes = 10:1) in 500 μL RF10 and stimulated with HA protein or BSA. Graph shows proportion of Ag-specific Tfh as defined by CD154+ or CD25 + OX40++ cells. N = 5 mice in each group.

cells, we also tested it as a candidate activation marker. In this study, we adapted an AIM assay to identify mouse Ag-specific Tfh cells and confirmed that these cells were best detected by quantifying the upregulation of CD154 and CD25/OX40.

Previous studies have highlighted that GC Tfh cells are highly susceptible to apoptosis due to expression of Fas (CD95) (Breitfeld et al., 2000; Marinova et al., 2006; Bentebibel et al., 2011). Once removed from the GC environment, cell culture of this population can be challenging. A primary consideration in the development of a murine AIM assay was therefore ensuring the viability and recovery of Tfh cells following extended cell culture. Although the upregulation of all activation markers studied was greatest at 24 h post stimulation, the Tfh population was prone to cell death and continually declined as the cell culture time increased. Thus, an 18 h stimulation presented the optimal balance of sensitivity and cell viability. While we cannot fully exclude the possibility that a small proportion of antigen-specific Tfh cells downregulate CXCR5 and PD-1 to such an extent that they are no longer captured in the post-culture Tfh gate, the antigen-specific Tfh population maintained high levels of PD-1 expression with no observable downregulation throughout 12–24 h of cell culture and HA peptide stimulation.

Transgenic mice (such as OT II mice) are a powerful tool to study the Ag-specific Tfh cell response to model antigens (such as OVA). Similarly, MHC-II tetramers are insightful reagents to identify Ag-specific Tfh cells in wild-type mice for antigens with defined CD4 T cell epitopes. However there is a critical need for a robust assay to assess Tfh responses to complex antigens in mice, not restricted by a given MHC haplotype. Our AIM-based method for identifying Ag-specific Tfh cells provides a starting point to broaden investigations into Tfh activity and phenotype in mice. In particular, Ag-specific Tfh cells could be sorted for downstream applications, such as TCR sequencing or adoptive transfer experiments. We demonstrated that Ag-specific Tfh cells could restimulated with wither peptide or whole proteins, with comparable frequencies of HA-specific Tfh detected after infection using either approach. The ability to use whole protein stimulation is a tremendous asset that reduces experimental complexity and extends the utility of this our approach to situations where peptide pools are unavailable due to size or cost considerations. In summary, we developed a sensitive assay for identifying mouse Ag-specific Tfh cells using CD154 or CD25/OX40 upregulation that preserves both cell viability and Tfh phenotype.

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