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Peptide-specific T helper cells identified by MHC class II tetramers differentiate into several subtypes upon immunization with CAF01 adjuvanted H56 tuberculosis vaccine formulation

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ABSTRACT

CD4+ T-cell priming is an essential step in vaccination due to the key role of T helper cells in driving both effector and memory immune responses. Here we have characterized in C57BL/6 mice the T helper subtype differentiation among tetramer-specific CD4+ T cells primed by subcutaneous immunization with the tuberculosis vaccine antigen H56 plus the adjuvant CAF01. Peptide-specific population identified by the MHC class II tetramers differentiated into several T helper subtypes upon antigen encounter, and the frequency of subpopulations differed according to their localization. Th1 (CXCR3+T-bet+), Tfh (CXCR5+PD-1+Bcl-6+) and RORγt+ cells were induced in the lymph nodes draining the immunization site (dLN), while Th1 cells were the predominant subtype in the spleen. In addition, CD4+ T cells co-expressing multiple T-cell lineage-specifying transcription factors were also detected. In the lungs, most of the tetramer-binding T cells were RORγt+, while Tfh and Th1 cells were absent. After boosting, a higher frequency of tetramer-binding cells co-expressing the markers CD44 and CD127 was detected compared to primed cells, and cells showed a prevalent Th1 phenotype in both dLN and spleens, while Tfh cells were significantly reduced. In conclusion, these data demonstrate that parenteral immunization with H56 and CAF01 elicits a distribution of antigen-specific CD4+ T cells in both lymphoid tissues and lungs, and gives rise to multiple T helper subtypes, that differ depending on localization and following reactivation.

1. Introduction

T helper cells play a fundamental role in the adaptive immune response upon vaccination, since they are required for the induction of effector as well as memory immune responses [1–3]. Characterization of T-cell priming induced by a vaccination strategy is critical in order to develop optimal prime-boost combinations capable of eliciting the type of immune response required to fight a specific pathogen [4]. Magnitude, quality and localization of primary T helper (Th) cell responses are of fundamental importance for the evaluation of novel vaccine candidates. T-cell priming can be evaluated as a target for improving and modulating the immune response during vaccination [5], and as an early predictor of vaccine immunogenicity [6,7]. CD4+ T-cell priming has been studied to characterize the mechanism of action of a broad range of adjuvants such as alum [8], CpG ODN [9], lipopolysaccharide [10], cholera toxin [11] or its B subunit (CTB) [12].

The analysis of T helper cell subsets allows the characterization of the specialized effector cells elicited by vaccination. Upon antigen encounter, clonally expanded CD4+ T cells differentiate into various functionally defined effector subpopulations, according to the local pro-inflammatory environment, the dose and the route of the vaccine used [13–15]. Besides the Th1, Th2, Th17 and T-reg subtypes, T follicular helper (Tfh) cells have emerged in recent years as another CD4+ T cell subtype essential for promoting the differentiation of activated B cells into memory B cells and long lived plasma-cells [16]. The induction of Tfh cells is therefore a target for vaccines aimed at stimulating a protective antibody-mediated immune response. Tfh cells express many molecules important for their differentiation and function, such as the follicle homing chemokine receptor CXCR5, the transcriptional factor B-cell lymphoma-6 (Bcl6), the programmed cell death-1 (PD-1) [17].

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The *ex vivo* study of primed CD4+ T cells is technically limited due to the very low frequency of antigen-specific T cells. Antigen-specific T cell responses have been detected by functional assays upon *in vitro* antigenic restimulation. Nevertheless, since phenotypic and functional properties of reactivated cells may be altered by this restimulation phase [18], technologies such as the adoptive transfer of TCR-transgenic T cells in recipient mice [9,12,19–22,22] and, more recently, MHC class II tetramers [23] have been developed and employed to study T-cell priming upon vaccination [2]. MHC class II-peptide complex tetramers in particular, have provided an invaluable way of monitoring *ex vivo* T-cell mediated immune responses and quantifying the development of an antigen-dependent response in animal studies [23–26].

In the present study we have identified and characterized, by using the tetramer technology, CD4+ T cells specific for a peptide of the *Mycobacterium tuberculosis* vaccine antigen H56 upon parenteral immunization. H56 is a promising vaccine candidate consisting of 6-kDa early secretory antigenic target (ESAT-6), Ag85B, and the protein Rv2660, that is a component of vaccines currently in clinical trials (ClinicalTrials.gov Identifier numbers: NCT01967134, NCT01865487) [27]. The soluble antigen was here combined with the adjuvant CAF01, a liposome system that has been shown to induce combined Th1 and Th17 responses and promote robust long-lived memory immunity [28–30]; CAF01 has recently been also tested in human studies [31]. The magnitude and quality of the peptide-specific CD4+ T cell response elicited by subcutaneous (SC) immunization with H56 and CAF01 was assessed by employing MHC class II tetramers complexed with an Ag85B-derived peptide, in the C57BL/6 mouse model. The expansion of Ag85B-tetramer binding cells, their subtype differentiation and dissemination towards effector sites were analysed upon priming and subsequent boosting.

2. Materials and methods

2.1. Mice

Eight-weeks old female C57BL/6 mice, purchased from Charles River (Lecco, Italy) were maintained under specific pathogen-free conditions at the University of Siena, and treated according to national guidelines (Decreto Legislativo 26/2014). All animal studies were approved by the Ethics Committee "Comitato Etico Locale dell'Azienda Ospedaliera Universitaria Senese" and the Italian Ministry of Health (number 4/2011, on date 20/07/2011).

2.2. Immunizations and sample collection

Mice were immunized by the SC route with the *M. tuberculosis* fusion protein H56 [27] (10 μg/mouse) mixed with the adjuvant CAF01 [30] (250 μg dimethyldioctadecylammonium (DDA) and 50 μg trehalose dibehenate (TDB)/mouse; Statens Serum Institut, Denmark). SC immunization was performed at the base of the tail in a total volume of 150 μl of TRIS HCl 10 μM. Groups of mice were immunized at day 0 and sacrificed on day 7, or boosted at week 4, and sacrificed 7 days later. Iliac (subiliac, medial and external) lymph nodes, lungs and spleens were collected 7 days after primary or booster immunization and treated as previously described [20].

2.3. Flow cytometric analysis

Cells were incubated for 30 min at 4 °C in Fc-blocking solution (complete medium plus 5 μg/ml of CD16/CD32 mAb [clone 93; eBioscience, CA, USA]) and then stained for 1 h at RT with PE-conjugated I-A(b) *M. tuberculosis* Ag85B precursor 280–294 (FQDAYNAAGGHNAVF), or PE-conjugates human class II-associated invariant chain peptide (PVSKMRMARPLLMQA) tetramers (kindly provided by NIH MHC Tetramer Core Facility, Emory University, Atlanta, GA) together with APC-conjugated anti-CXCR5 (clone 2G8, BD biosciences, CA, USA). Surface staining was performed on ice with FITC-conjugated anti-PD-1 (clone J43), PE-CY7-conjugated anti-CXCR3 (clone CXCR3-73, both from Biolegend, CA, USA), HV500-conjugated anti-CD4 (clone RM4-5), HV450-conjugated anti-CD44 (clone IM7, both from BD biosciences) and PerCp-eFl710-conjugated anti-CD127 (clone SB/199, eBioscience). Samples were labelled with LIVE/DEAD Fixable Near IR Dead Cell Stain Kit according to the manufacturer's instructions (Invitrogen, USA). Intracellular staining for BV605-conjugated anti-T-bet (clone 4b10, Biolegend), PECF594-conjugated anti-Bcl-6 (clone K112-91) and PerCPCY5.5-conjugated anti-RORγt (clone Q31-378, both from BD biosciences) was performed using the Foxp3 staining buffer set (eBioscience) according to the manufacturer instruction. Antibodies and tetramers were titrated for optimal dilution. CD44+ tetramer+ cells were identified using a gating strategy based on live cells, FSC × SSC lymphocyte characteristics, singlets and CD4+ expression; gates were set according to Fluorescence Minus One (FMO) controls. About 10^6 cells were stored for each sample acquired on LSR II flow cytometer (BD biosciences); data analysis was performed using FlowJo software (TreeStar, OR, USA).

2.4. Cytokine assay

IFN-γ, IL-4, IL-10, IL-17A, IL-2 and IL-21 production was assessed in culture supernatants of restimulated iliac lymph nodes cells by Bio-Plex cytokine immunoassay (Bio-Rad). Lymphocytes were cultured with 5 μg/ml of H56 in complete medium for 72 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-17A, IL-2 cytokines were detected in supernatants using the 5-plex assay system, while IL-21 was analysed individually. Cytokine detection was performed following the manufacturer's protocol, and analysed by Luminex 100 (Bio-Rad). Cytokine concentrations were calculated based on standard curve data using Bio-Plex Manager software (version 4.0).

2.5. Statistical analysis

Two-tailed Student's *t*-test was employed to analyse statistical differences between the frequencies of Ag85B-specific CD4+ T cells detected in primed and boosted groups, and between the percentage of T-helper subsets detected in primed and boosted groups. Statistical significance was defined as $P \leq 0.05$. Graphpad 4.0 software was used for analysis.

3. Results

3.1. Primed Ag85B-specific CD4+ T cells differentiate into multiple Th subtypes according to their localization.

The primary antigen-specific CD4+ T-cell response was studied after SC immunization with H56 plus CAF01 adjuvant in lymph nodes draining the immunization site (dLN), spleen and also in the lungs, that are an essential effector site for fighting airways pathogens. CD4+ T cells specific for the immunodominant epitope of Ag85B, that is part of the H56 fusion protein, were identified using $Ag85B_{280-294}$-complexed MHC class II tetramers. Staining specificity of $Ag85B_{280-294}$-complexed MHC class II tetramers was determined using a control tetramer complexed with an unrelated antigen that showed a level of staining below 0.02% (data not shown). Tetramer-positive (Tet+) T cell subtypes were characterized 7 days after priming for the expression of surface markers and transcription factors indicative of Tfh, Th1, and Th17 subtype differentiation. Tfh cell phenotype was identified by assessing

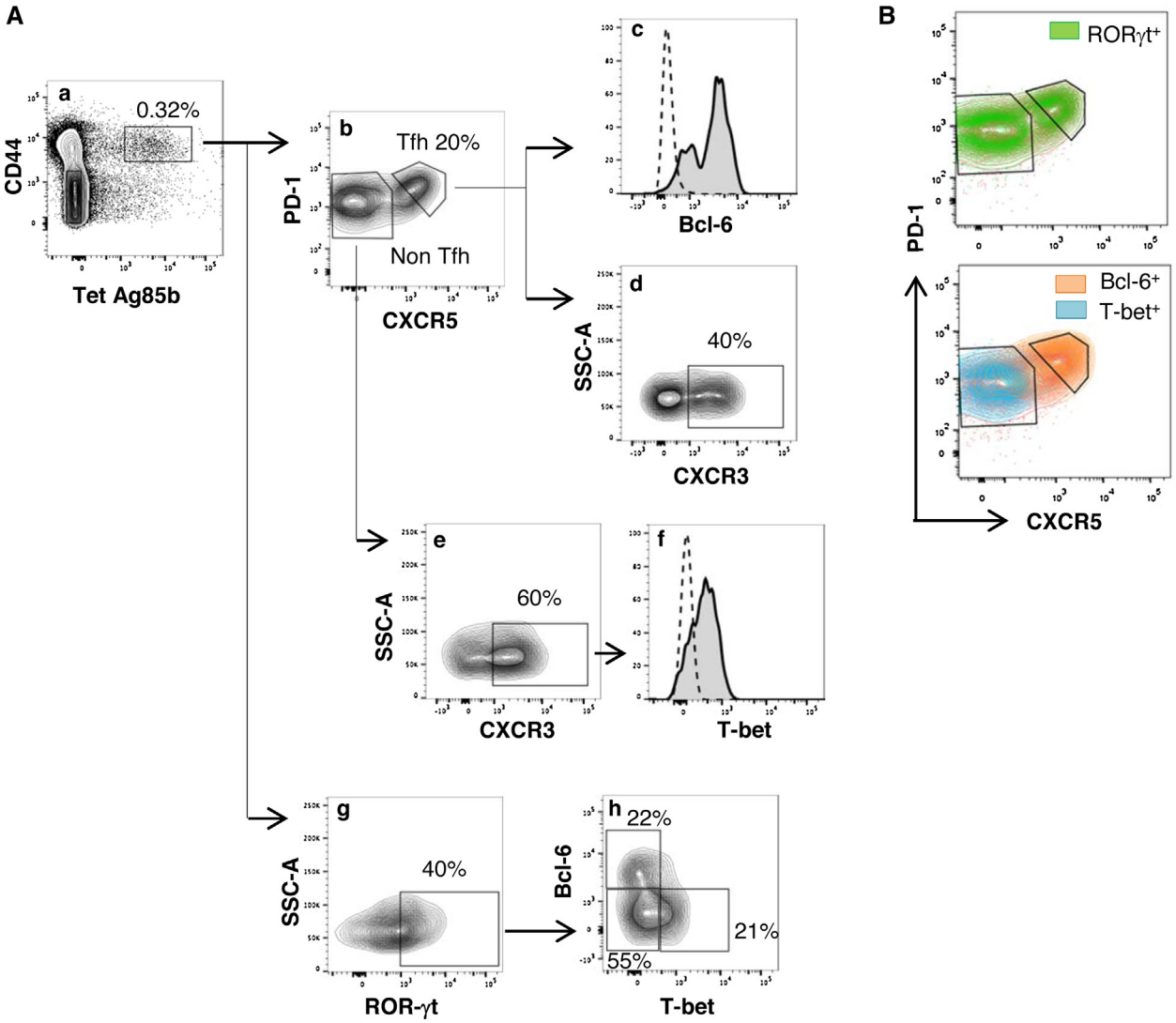

Fig. 1. Characterization of Ag85B-specific CD4⁺ T cell subpopulations in lymph nodes draining the immunization site. C57BL/6 mice were primed with H56 and CAF01 by the SC route, and sacrificed 7 days later. Lymphocytes from dLN were collected, stained with $Ag85B_{280–294}$ MHC class II tetramer, and analysed for the expression of surface markers and intracellular transcription factors for identifying different subpopulations. (A) Detection of Tet⁺ T cells as $CD44^{high}$ Tet-Ag85B⁺ cells gated on live CD4⁺ lymphocytes (a); percentage of Tet⁺ T cells respect to CD4⁺ T cells is reported. Gated Tet⁺ T cells were assessed for the expression of CXCR5 and PD-1 cells (b), and double-positive cells (indicated as Tfh, with frequency respect to Tet⁺ T cells) were analysed for Bcl-6 expression (c, filled histogram; in overaly Bcl-6 expression assessed in $CD4^+CD44^-$ $tetramer^-$ cells, open histogram). Both Tfh and non-Tfh cells were assessed for the expression of CXCR3 (d and e, percentages reported are referred to Tfh and non-Tfh cells respectively), and non-Tfh $CXCR3^+$ cells were analysed for the expression of T-bet (f, filled histogram, in overlay its expression assessed in $CD4^+CD44^-$ $tetramer^-$ cells, open histogram). Tet⁺ T cells were tested for RORγt expression (g, percentage reported is referred to Tet⁺ T cells), and gated positive cells were analysed for the expression of Bcl-6 *versus* T-bet (h, percentages reported in each quadrant are referred to RORγt⁺ cells). (B) RORγt (green), Bcl-6 (orange), and T-bet (blue) expression across CXCR5 *versus* PD-1 dot plot analysis. Dot plots represent a single animal from 2 independent experiments with 5 mice each; about 10^6 cells were stored for each sample.

the expression of PD-1 and CXCR5 together with the intracellular expression of Bcl-6 [33], Th1 cells were detected by the expression of CXCR3 and T-bet [34], while Th17 by RORγt [35]. Antigen-specific CD4⁺ T cells were not detected using MHC class II tetramers specific for the ESAT6 peptide (QQWNFAGIEAAASA, data not shown), further confirming the immunodominance of the selected Ag85B epitope, as shown also in previous studies [32].

3.2. T helper subpopulations in dLN

In dLN Tet⁺ T cells expanded (0.32% of total CD4⁺ T cells) and differentiated into several T helper cell subpopulations (Fig. 1A). About 20% of Tet⁺ T cells expressed the Tfh surface markers PD-1 and CXCR5 (Fig. 1A, panel b) and these gated cells expressed at high levels the specific Bcl-6 transcription factor (Fig. 1A, panel c). About 40% of gated Tfh cells expressed CXCR3, a marker preferentially maintained by cells committed to the Th1 cell pathway [36] (Fig. 1A, panel d), but they did not express the Th1 master regulator factor T-bet (data not shown). It has been previously reported that circulating $CD4^+CXCR5^+$ T cells can express CXCR3 [37], here we show that also lymph node resident Tfh cells can express this chemokine receptor. Among non-Tfh gated cells (Fig. 1A, panel b) 60% expressed CXCR3 (Fig. 1A, panel e), and most of them were T-bet-positive (Fig. 1A, panel f) indicating the differentiation into Th1 cells. $CXCR3^+$T-bet^+ Th1 cells thus made up about 27% of the Tet⁺ T cells.

About 40% of Tet⁺ T cells expressed the Th17 master regulator factor RORγt (Fig. 1A, panel g), and interestingly, many of gated RORγt⁺ cells co-expressed other master regulator factors, such as Bcl-6 (22%) or T-bet (21%), while about 55% were positive only for RORγt (Fig. 1A, panel h). This analysis shows the presence of "transitional" phenotypes in which the co-expression of more than one "master" transcription factor can be due to a transient status of cells that were redirecting their phenotype and

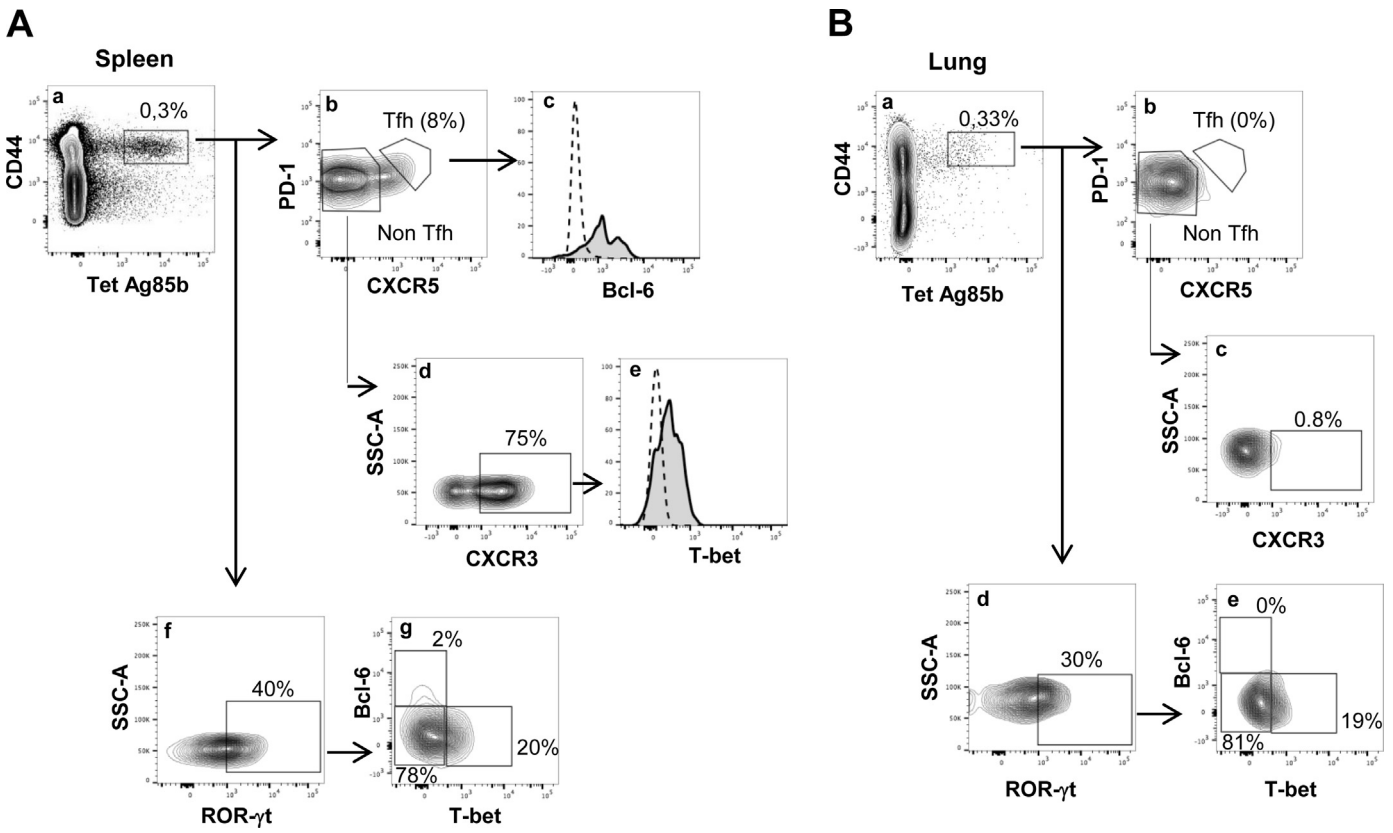

Fig. 2. Characterization of Ag85B-specific CD4⁺ T cell subpopulations in spleen and lungs. C57BL/6 mice were primed with H56 and CAF01 by the SC route, and sacrificed 7 days later. Lymphocytes from SPL (A) and lungs (B) were collected, stained with $Ag85B_{280–294}$ MHC class II tetramer, and analysed for the expression of surface markers and intracellular transcription factors for identifying different subpopulations. (A) Detection of Tet⁺ T cells as $CD44^{high}$ Tet-Ag85B⁺ cells gated on live CD4⁺ lymphocytes (a); percentage of Tet⁺ T cells respect to CD4⁺ T cells is reported. Gated Tet⁺ T cells were assessed for the expression of CXCR5 and PD-1 (b) and double-positive cells (indicated as Tfh, with frequency respect to Tet⁺ T cells in brackets) were analysed for Bcl-6 expression (c, filled histogram; in overlay Bcl-6 expression assessed in $CD4^+CD44^-$ tetramer⁻ cells, open histogram). Non-Tfh cells were analysed for the expression of CXCR3 (panel d, percentage reported is referred to non-Tfh cells), and gated CXCR3⁺ cells were analysed for the expression of T-bet (e, filled histogram, in overlay its expression assessed in $CD4^+$ $CD44^-$ tetramer⁻ cells, open histogram). Tet⁺ T cells were tested for RORγt expression (f, percentage reported is referred to Tet⁺ T cells), and gated positive cells were analysed for the expression of Bcl-6 *versus* T-bet (g, percentages reported in each quadrant are referred to RORγt⁺ cells). (B) Same analysis described in (A), performed in the lungs. Percentages of positive cells are reported in each dot plot. Dot plots shown represent a single animal from 2 independent experiments with 5 mice each; about 10^6 cells were stored for each sample.

function towards one specific subtype. Cells expressing RORγt were equally distributed between CXCR5 and PD-1 positive or negative subpopulations, while Bcl-6 and T-bet showed a clear separation between these two populations (Fig. 1B). The study of T helper subtypes performed within dLN highlights the CD4⁺ T cell plasticity, and the close relationship between different Th cell lineages [38,39].

3.3. *T helper subpopulations in spleen and lungs*

At the same time point, the profile of primed Tet⁺ T cells was analysed into spleen and lungs. The subpopulations detected in dLN were observed also in the spleen but with different frequencies (Fig. 2A). Tfh cells ($PD\text{-}1^+CXCR5^+$ expressing Bcl-6) were about 8% of Tet⁺ T cells, while the frequency of $CXCR3^+$ T-bet^+ Th1 cells was around 42% (Fig. 2A, panels b, d, e). About 40% expressed RORγt (Fig. 2A, panel f), of these cells, 20% co-expressed T-bet, while about 78% were positive only for RORγt and only 2% co-expressed RORγt and Bcl-6 (Fig. 2A, panel g).

The analysis performed in the lungs showed the absence of both Tfh ($CXCR5^+PD\text{-}1^+$) and Th1 ($CXCR5^-PD\text{-}1^-CXCR3^+$) cells (Fig. 2B, panels b and c). About 30% of Tet⁺ T cells expressed RORγt, and most of them (81%) did not co-expressed other transcription factors (Fig. 2B, panels d and e). These data show a prevalence of Th1 cells in the spleen and RORγt-positive cells in lungs.

3.4. *Effect of booster immunization on Ag85B-specific CD4⁺ T cells*

The response of primed Ag85B-specific CD4⁺ T cells and the persistence of Th subpopulations were studied upon booster immunization with H56 and CAF01 in dLN, spleen and lungs 7 days after boosting. A significant increase in the percentage of Tet⁺ T cells was observed in all investigated organs indicative of the recall response, and the frequencies of Tet⁺ T cells increased to 0.56%, 1% and 2.3% in dLN, spleen and lungs, respectively ($P < 0.001$, $P < 0.01$ and $P < 0.05$ respect to primed groups; Fig. 3A). The amount of Tet⁺ T cells after booster immunization was about $40{,}000 \pm 8{,}000$ cells in dLN, $42{,}000 \pm 10{,}000$ in lungs, and $183{,}000 \pm 54{,}000$ in the spleens (mean value ± SEM of 15 mice), and the fold-increase respect to the amount generated by the primary immunization was 7.2, 4.2 and 2.6 in lungs, spleen and dLN, respectively (data not shown). CD127 expression, that is believed to be indicative of memory, was analysed in Tet⁺ T-cells. After booster immunization the frequency of Tet⁺ $CD44^{hi}CD127^+$ antigen-experienced T cells increased respect to primed cells (Fig. 3B). The frequency, as well as the absolute number, of $PD\text{-}1^+CXCR5^+$ Tfh cells detected upon priming, significantly decreased following booster immunization both in dLN and spleens (Fig. 4A), while the percentage of Th1 and Th17 cells were steadily maintained in all organs (Fig. 4A). The analysis of functional activity of T cells collected from dLN correlated with the phenotype observed, as shown by the production of IFN-γ and IL-17, while

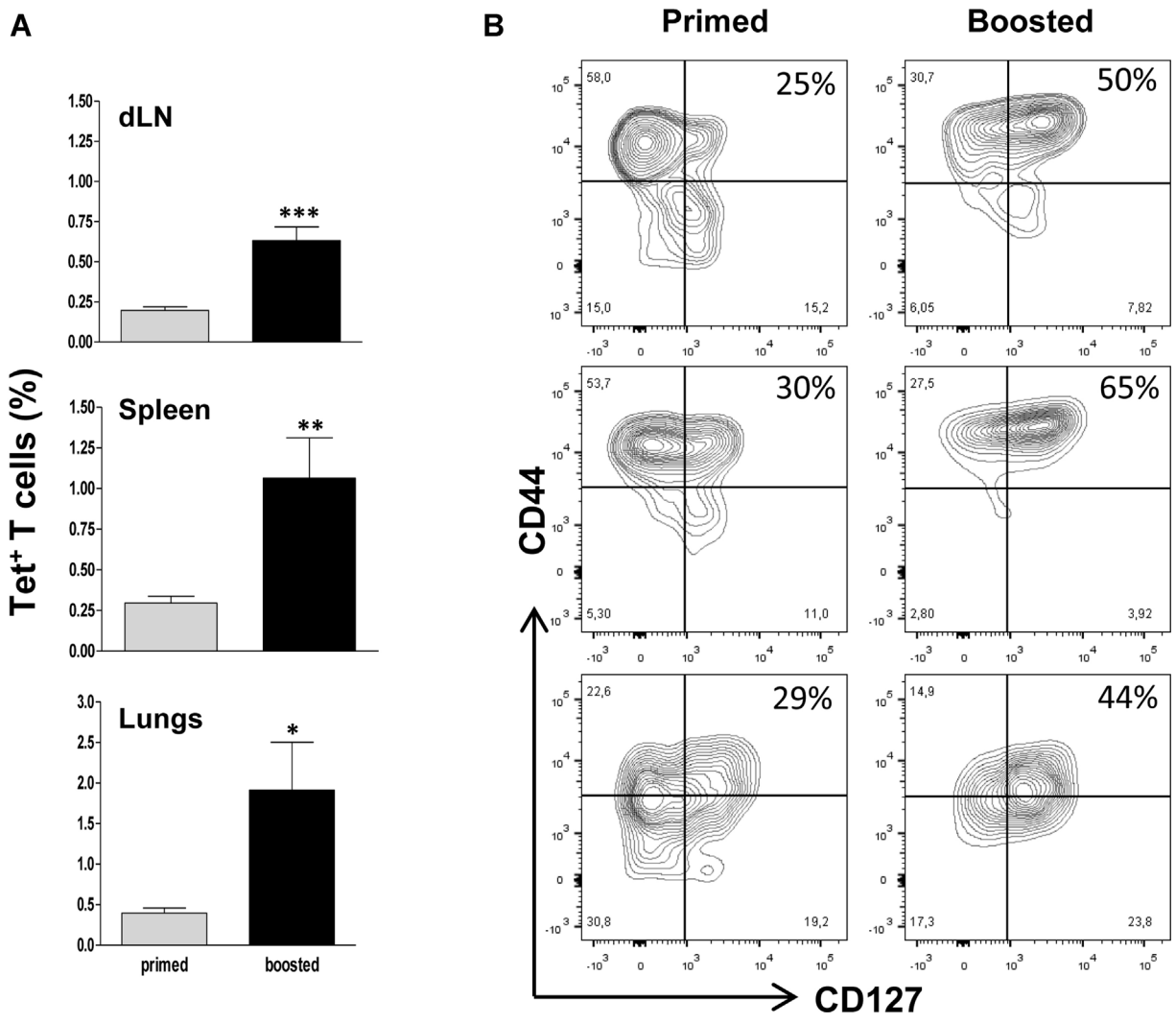

Fig. 3. Frequency of Ag85B-specific CD4$^+$ T cells following booster immunization. C57BL/6 mice were primed with H56 and CAF01 and boosted 4 weeks later. Lymphocytes from dLN, SPL and lungs, collected 7 days after primary or booster immunization, were stained with $Ag85B_{280–294}$ MHC class II tetramers and surface marker antibodies. (A) Frequencies of Tet$^+$ T cells respect to CD4$^+$ cells following primary (gray) or booster immunization (black). Data are reported as the mean ± SEM of 15 mice from three independent experiments. Two-tailed Student's *t*-test was used for comparing values. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. (B) CD44 *versus* CD127 expression on CD4$^+$ tetramer-Ag85B$^+$ gated cells following primary (left) or booster (right) immunization in dLN (top panels), spleen (middle panels) and lungs (bottom panels). Percentages of CD44 and CD127 double positive cells respect to CD4$^+$ tetramer-Ag85B$^+$ cells are shown. Dot plots represent a single animal from 2 independent experiments with 5 mice each; about 10^6 cells were stored for each sample.

the release of IL-2 was indicative of the proliferative response and activation program of Tet$^+$ T cells (Fig. 4B).

4. Discussion

The present work explores the antigen-specific CD4$^+$ T proliferation and differentiation into T helper suptypes following parenteral immunization with the vaccine formulation H56 and CAF01. Our data highlight that the peptide-specific population, identified by MHC class II tetramers, produced different effector cell patterns upon antigen encounter, and that the frequency of subpopulations differed according to their localization. In particular, Tfh, Th1 and RORγt$^+$ cells were all induced into dLN together with "transitional" subtypes that co-expressed multiple helper T cell lineage-specifying transcription factors. Tfh cells were largely harboured within LN draining the immunization site, while Th1 cells were predominant in the spleen, and RORγt$^+$ cells in the lungs. Therefore, our results show that primed T helper cells that recognized the same peptide-MCH class II complex, did not differentiate into a single polarized phenotype but in more subtypes, that showed a different fate upon reactivation. Although the cytokine milieu it is known to play an important role in determining the types of effector cells generated, recent evidence has demonstrated that signals received through the TCR are also important for effector cell differentiation [26]. The interclonal and intraclonal functional heterogeneity of CD4$^+$ T cells primed by pathogens or vaccines has also been recently demonstrated by TCR deep sequencing [40], thus supporting the one-cell multiple fates model of helper T cells differentiation. The CD4$^+$ T-cell response that we have tracked using Ag85B epitope-specific tetramers, can be considered representative of the response towards the whole vaccine, since it has been previously shown to be an immunodominant peptide [32] and the staining with tetramers specific for other epitopes included in H56, did not identify any population.

Generation of Tfh cells is essential for the efficacy of vaccines in which antibody response is strongly required. Ag85B-specific Tfh cells were detected upon priming within dLN, and with a lower frequency in the spleen. Whether Tfh cells establish a persistent local memory compartment is still poorly understood, as it is not clear if Tfh are terminally differentiated cells or can give rise to

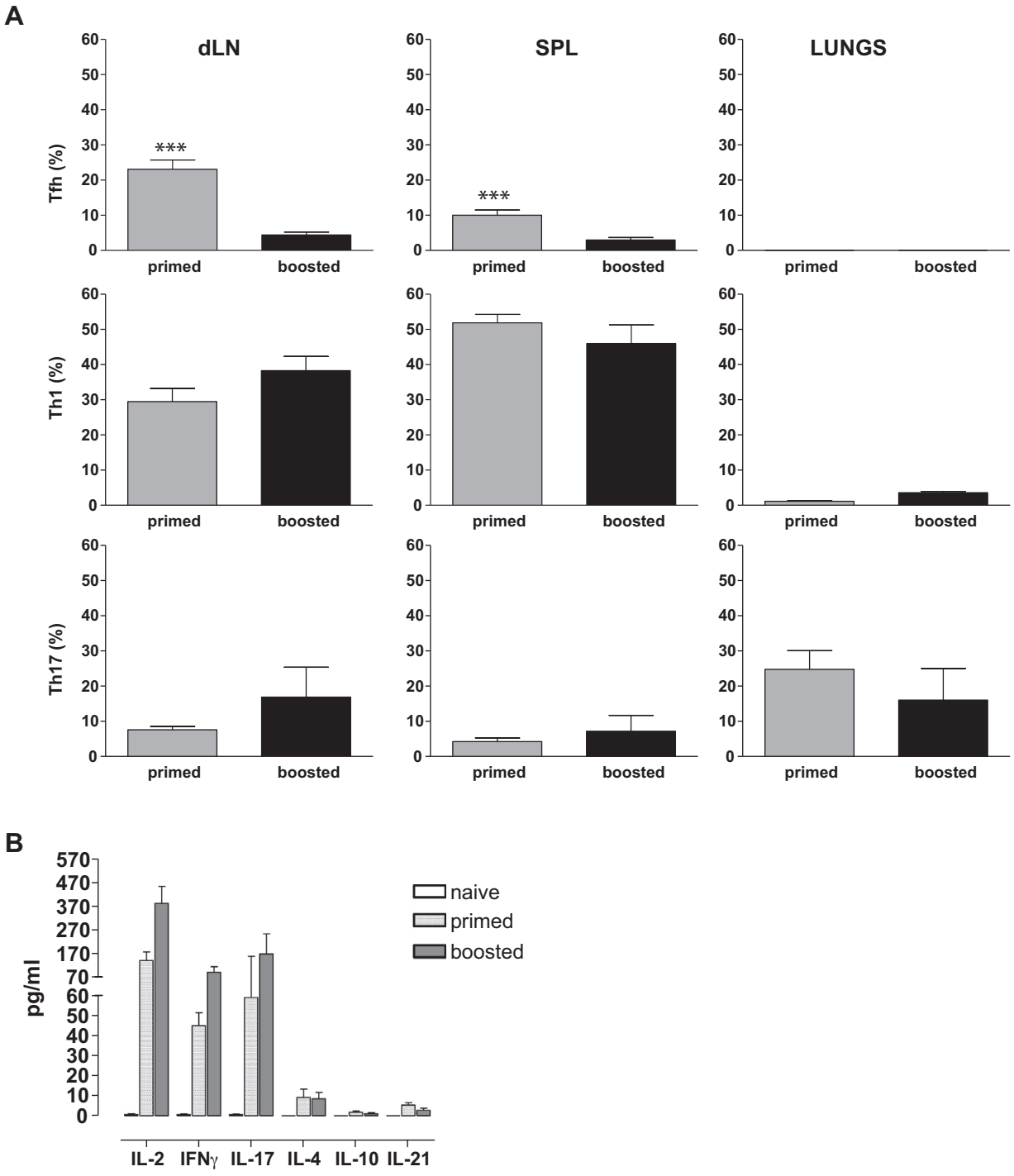

Fig. 4. Ag85B-specific CD4$^+$ T cells upon booster immunization. Groups of C57BL/6 mice were primed with H56 and CAF01 and boosted 4 weeks later. Lymphocytes from dLN, SPL and lungs were collected 7 days after primary or booster immunization and stained with $Ag85B_{280–294}$ MHC class II tetramers, surface and intracellular antibodies. A. Frequencies of Tfh (CXCR5$^+$PD-1$^+$), Th1 (CXCR5$^-$PD-1$^-$CXCR3$^+$), and Th17 (Bcl-6$^-$T-bet$^-$RORγt$^+$) cells respect to Tet$^+$ T cells in dLN, SPL and lungs following primary (gray) or booster (black) immunization. Bars represent the mean frequencies ± SEM of 10 mice from two independent experiments. Frequencies of different subpopulations were compared using the two-tailed Student's *t*-test. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. (B) IL-2, IFNγ, IL-17, IL-4, IL-10 and IL-21 concentrations assessed by Bio-Plex immunoassay in culture supernatants of lymphocytes restimulated with H56 for 72 h, collected from dLN of naive (white), primed (dotted) or boosted (gray) mice.

other helper T cell subsets once the germinal center reaction is resolved [41]. Our data show the presence of antigen-experienced CD44^{high}CD127$^+$ Tet$^+$ T cells within dLN after booster immunization, nevertheless we observed a reduction of CXCR5 and PD-1 double-positive cells, while CXCR3$^+$ Th1 cells were steadily maintained. Recent studies show that Tfh cells give rise to memory T cells with unexpected plasticity that allow them to re-differentiate also into conventional helper T cells [41]. This supports the idea that Tfh cells are not terminally differentiated, and maintain a close relationship with other Th cell lineages. The developmental plasticity model of Th differentiation is also supported by the transcription factors analysis that we performed among Tet$^+$ T cells after priming. The analysis clearly showed not only the presence of "defined" Th subpopulations, such as Tfh and Th1, but also of transitional phenotypes, in which the co-expression of more than one "master" transcription factor was observed. We detected CXCR5$^+$PD-1$^+$ cells

that expressed Bcl-6 together with RORγt, or CXCR5$^-$ PD-1$^-$ CXCR3$^+$ cells positive for both RORγt and T-bet. While the co-expression of RORγt and T-bet has been frequently reported [39], more strikingly is the expression of RORγt within CXCR5$^+$PD-1$^+$Bcl-6$^+$ cells. In general, there is a high degree of uncertainty as to whether Th17 cells represent a stable, terminally differentiated Th lineage. Some studies have highlighted that Th17 cells can provide help to B cells and promote GC formation including isotype switching [42], or that intestinal Th17 cells acquired a Tfh phenotype within Peyer's patches and induced the development of IgA-producing germinal center B cells [43]. Taken together, these data suggest that the concomitant expression of RORγt and Bcl-6 lineage-specifying transcription factors can be due to a transient status of cells that were redirecting their phenotype and function towards one specific subtype, in accordance with a plastic, more than defined, model of T cell differentiation [39].

Following parenteral immunization, primed and boosted Ag85B-specific CD4$^+$ T cells were detected also in the lungs. We can speculate that most of reactivated cells exit the dLN to disseminate into lungs, but also that lung tissue-resident memory cells, generated by primary immunization, were reactivated *in situ* by booster immunization. It has been demonstrated that it is technically complex to distinguish parenchyma-associated from lung blood vasculature circulating cells [44]; therefore we are currently working for characterizing the actual localization of these cells (unpublished data). The phenotypic analysis of Tet$^+$ T cells associated with the lungs showed characteristics different from cells detected in the lymphoid compartments, as shown by the absence of Tfh and Th1 cells. Since CXCR3 affects trafficking of effector T cells to the lungs upon intranasal infection [45,46], the low expression of CXCR3 on Tet$^+$ T cells could mean that cells were already resident in the lungs. Nevertheless, it is also possible that other chemokines can mediate Tet$^+$ T cells migration to the lungs, following a parenteral immunization. Interestingly, Ag85B-specific CD4$^+$ T cells detected in the lungs expressed prevalently RORγt as single master transcription factor in line with pre-clinical data obtained with CAF01 adjuvant that observed the induction of Th17 cells [29]. Th17 cells play an important role in protective immunity against several pathogens, and in such cases it would be highly relevant and beneficial to induce Th17 responses.

In conclusion, our results show that T helper cells that recognize the same peptide-MCH class II complex differentiate into more subsets, with different frequencies according to their localization. Tfh cells were elicited and harboured within LN draining the immunization site, while Th1 were predominant in the spleen, and RORγt$^+$ cells in the lungs. T helper cells also displayed a high degree of plasticity, as shown by both the presence of "transitional" subtypes that co-expressed multiple "master regulator" factors, and phenotypes of reactivated memory cells that were partially different from those observed upon priming. These data provide relevant knowledge about CD4$^+$ T cell responses elicited by the vaccine formulation H56 and CAF01 and on the role of the priming event in affecting the cellular response in the context of a vaccination strategy.

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References

[1] Siegrist CA. Vaccine immunology. In: Plotkin AA, Orenstein WA, Offit PA, editors. Vaccines. Philadelphia: Elsevier Inc.; 2008. p. 17–36.
[2] Ciabattini A, Pettini E, Medaglini D. CD4(+) T cell priming as biomarker to study immune response to preventive vaccines. Front Immunol 2013;4:421.
[3] Huster KM, Busch V, Schiemann M, Linkemann K, Kerksiek KM, Wagner H, et al. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. Proc Natl Acad Sci U S A 2004;101(15):5610–5.
[4] Woodland DL. Jump-starting the immune system: prime-boosting comes of age. Trends Immunol 2004;25(2):98–104.
[5] Fiorino F, Pettini E, Pozzi G, Medaglini D, Ciabattini A. Prime-boost strategies in mucosal immunization affect local IgA production and the type of Th response. Front Immunol 2013;4:128.
[6] Galli G, Medini D, Gorgogni E, Zedda L, Bardelli M, Malzone C, et al. Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels. Proc Natl Acad Sci U S A 2009;106(10):3877–82.
[7] Nayak JL, Fitzgerals TF, Richards KA, Yang H, Treanor JJ, Sant AJ. CD4+ T-cell expansion predicts neutralizing antibody responses to monovalent, inactivated 2009 pandemic influenza A(H1N1) virus subtype H1N1 vaccine. J Infect Dis 2013;207(2):297–305.
[8] McKee AS, Burchill MA, Munks MW, Jin L, Kappler JW, Friedman RS, et al. Host DNA released in response to aluminum adjuvant enhances MHC class II-mediated antigen presentation and prolongs CD4 T-cell interactions with dendritic cells. Proc Natl Acad Sci U S A 2013;110(12):E1122–31.
[9] Ciabattini A, Pettini E, Fiorino F, Prota G, Pozzi G, Medaglini D. Distribution of primed T cells and antigen-loaded antigen presenting cells following intranasal immunization in mice. PLoS ONE 2011;6(4):e19346.
[10] McAleer JP, Vella AT. Educating CD4 T cells with vaccine adjuvants: lessons from lipopolysaccharide. Trends Immunol 2010;31(11):429–35.
[11] Marks EM, Helgeby A, Andersson JO, Schön K, Lycke NY. CD4+ T-cell immunity in the female genital tract is critically dependent on local mucosal immunization. Eur J Immunol 2011;41(9):2642–53.
[12] Pettini E, Ciabattini A, Pozzi G, Medaglini D. Adoptive transfer of transgenic T cells to study mucosal adjuvants. Methods 2009;49(4):340–5.
[13] Jelley-Gibbs DM, Strutt TM, McKinstry KK, Swain SL. Influencing the fates of CD4 T cells on the path to memory: lessons from influenza. Immunol Cell Biol 2008;86(4):343–52.
[14] Lanzavecchia A, Sallusto F. Understanding the generation and function of memory T cell subsets. Curr Opin Immunol 2005;17(3):326–32.
[15] Tubo NJ, Jenkins MK. TCR signal quantity and quality in CD4 T cell differentiation. Trends Immunol 2014;35(12):591–6.
[16] Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol 2011; 29:621–63.
[17] Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J Immunol 2007;179(8):5099–108.
[18] Bacher P, Scheffold A. Flow-cytometric analysis of rare antigen-specific T cells. Cytometry A 2013;83(8):692–701.
[19] Kearney ER, Pape KA, Loh DY, Jenkins MK. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity 1994;1(4):327–39.
[20] Ciabattini A, Pettini E, Andersen P, Pozzi G, Medaglini D. Primary activation of antigen-specific naive CD4+ and CD8+ T cells following intranasal vaccination with recombinant bacteria. Infect Immun 2008;76(12):5817–25.
[21] Medaglini D, Ciabattini A, Cuppone AM, Costa C, Ricci S, Costalonga M, et al. In vivo activation of naive CD4+ T cells in nasal mucosa-associated lymphoid tissue following intranasal immunization with recombinant *Streptococcus gordonii*. Infect Immun 2006;74(5):2760–6.
[22] Pettini E, Prota G, Ciabattini A, Boianelli A, Fiorino F, Pozzi G, et al. Vaginal immunization to elicit primary T-cell activation and dissemination. PLoS ONE 2013;8(12):e80545.
[23] McHeyezer-Williams MG, Altman JD, Davis MM. Enumeration and characterization of memory cells in the TH compartment. Immunol Rev 1996;150: 5–21.
[24] Crawford F, Kozono H, White J, Marrack P, Kappler J. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. Immunity 1998;8(6):675–82.
[25] 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(4):583–95.
[26] Tubo NJ, Pagan AJ, Taylor JJ, Nelson RW, Linehan JL, Ertelt JM, et al. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell 2013;153(4):785–96.
[27] Aagaard C, Hoang T, Dietrich J, Cardona PJ, Izzo A, Dolganov G, et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat Med 2011;17(2):189–94.
[28] Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, et al. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 2009;182(12):8047–55.
[29] Lindenstrom T, Woodworth J, Dietrich J, Aagaard C, Andersen P, Agger EM. Vaccine-induced Th17 cells are maintained long-term postvaccination

as a distinct and phenotypically stable memory subset. Infect Immun 2012;80(10):3533–44.
[30] Agger EM, Rosenkrands I, Hansen J, Brahimi K, Vandahl BS, Aagaard C, et al. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PLoS ONE 2008;3(9):e3116.
[31] van Dissel JT, Joosten SA, Hoff ST, Soonawala D, Prins C, Hokey DA, et al. A novel liposomal adjuvant system CAF01, promotes long-lived *Mycobacterium tuberculosis*-specific T-cell responses in human. Vaccine 2014;32:7098–107.
[32] Bennekov T, Dietrich J, Rosenkrands I, Stryhn A, Doherty TM, Andersen P. Alteration of epitope recognition pattern in Ag85B and ESAT-6 has a profound influence on vaccine-induced protection against *Mycobacterium tuberculosis*. Eur J Immunol 2006;36(12):3346–55.
[33] Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. Science 2009;325(5943):1001–5.
[34] Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor T-bet, directs Th1 lineage commitment. Cell 2000; 100(6):655–69.
[35] Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126(6):1121–33.
[36] Groom JR, Luster AD. CXCR3 in T cell function. Exp Cell Res 2011;317:620–31.
[37] Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. Sci Transl Med 2013;5(176):176ra32.
[38] Evans CM, Jenner RG. Transcription factor interplay in T helper cell differentiation. Brief Funct Genomics 2013;12(6):499–511.
[39] Oestreich KJ, Weinmann AS. Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. Nat Rev Immunol 2012;12(11):799–804.
[40] Becattini S, Latorre D, Mele F, Foglierini M, De Gregorio C, Cassotta A, et al. T cell immunity. Functional heterogeneity of human memory CD4+ T cell clones primed by pathogens or vaccines. Science 2015;347(6220):400–6.
[41] Luthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. Nat Immunol 2012;13(5):491–8.
[42] Mitsdoerffer M, Lee Y, Jager A, Kim HJ, Korn T, Kolls JK, et al. Proinflammatory T helper type 17 cells are effective B-cell helpers. Proc Natl Acad Sci U S A 2010;107(32):14292–7.
[43] Hirota K, Turner JE, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. Nat Immunol 2013;14(4):372–9.
[44] Anderson KG, Sung H, Skon CN, Lefrancois L, Deisinger A, Vezys V, et al. Cutting edge: intravascular staining redefines lung CD8 T cell responses. J Immunol 2012;189(6):2702–6.
[45] Kohlmeier JE, Cookenham T, Miller SC, Roberts AD, Christensen JP, Thomsen AR, et al. CXCR3 directs antigen-specific effector CD4+ T cell migration to the lung during parainfluenza virus infection. J Immunol 2009;183(7):4378–84.
[46] Sakai S, Kauffman KD, Schenkel JM, McBerry CC, Mayer-Barber KD, Masopust D, et al. Cutting edge: control of *Mycobacterium tuberculosis* infection by a subset of lung parenchyma-homing CD4 T cells. J Immunol 2014;192(7):2965–9.