RESEARCH ARTICLE

Early Rise of Blood T Follicular Helper Cell Subsets and Baseline Immunity as Predictors of Persisting Late Functional Antibody Responses to Vaccination in Humans

Fabiana Spensieri[1☯¤a], Emilio Siena[1☯¤a], Erica Borgogni[1¤a], Luisanna Zedda[1¤a], Rocco Cantisani[1¤b], Nico Chiappini[1¤a], Francesca Schiavetti[1¤a], Domenico Rosa[1¤a], Flora Castellino[1¤c], Emanuele Montomoli[2], Caroline L. Bodinham[3], David J. Lewis[3], Duccio Medini[1¤a], Sylvie Bertholet[1¤a]*, Giuseppe Del Giudice[1¤a]

1 Novartis Vaccines & Diagnostics S.r.l., Siena, Italy, 2 Departement of Molecular and Developmental Medicine, University of Siena, & VisMederi S.r.l., Siena, Italy, 3 Surrey Clinical Research Center, University of Surrey, Guildford, United Kingdom

☯ These authors contributed equally to this work.
¤a Current address: GSK Vaccines S.r.l., Siena, Italy
¤b Current address: Laboratory of Immunohematology, University Hospital, Pisa, Italy
¤c Current address: Center for Human Immunology, NHLBI, NIH, Bethesda, MD, United States of America
* sylvie.c.bertholet-girardin@gsk.com

OPEN ACCESS

Abstract

CD4+ T follicular helper cells (T_{FH}) have been identified as the T-cell subset specialized in providing help to B cells for optimal activation and production of high affinity antibody. We recently demonstrated that the expansion of peripheral blood influenza-specific CD4+IL-21+ICOS1+ T helper (T_H) cells, three weeks after vaccination, associated with and predicted the rise of protective neutralizing antibodies to avian H5N1. In this study, healthy adults were vaccinated with plain seasonal trivalent inactivated influenza vaccine (TIIV), MF59®-adjuvanted TIIV (ATIIV), or saline placebo. Frequencies of circulating CD4+ T_{FH}1 ICOS+ T_{FH} cells and H1N1-specific CD4+- IL-21+ICOS+ CXCR5+ T_{FH} and CXCR5- T_H cell subsets were determined at various time points after vaccination and were then correlated with hemagglutination inhibition (HI) titers. All three CD4+ T cell subsets expanded in response to TIIV and ATIIV, and peaked 7 days after vaccination. To demonstrate that these T_{FH} cell subsets correlated with functional antibody titers, we defined an alternative endpoint metric, decorrelated HI (DHI), which removed any correlation between day 28/day 168 and day 0 HI titers, to control for the effect of preexisting immunity to influenza vaccine strains. The numbers of total circulating CD4+ T_{FH}1 ICOS+ cells and of H1N1- specific CD4+IL-21+ICOS+ CXCR5+, measured at day 7, were significantly associated with day 28, and day 28 and 168 DHI titers, respectively. Altogether, our results show that CD4+ T_{FH} subsets may represent valuable biomarkers of vaccine-induced long-term functional immunity.

Trial Registration

ClinicalTrials.gov NCT01771367

Introduction

Protein-based vaccines confer protection against pathogens mainly through the induction of T cell-dependent high affinity functional antibody responses. In this context a specialized subset of T helper cells (T_H), identified as T follicular helper cells (T_{FH}), differentiate and provide help to B cells in the germinal centers (GC) of secondary lymphoid organs, leading to B-cell proliferation and differentiation, and reshaping of the B-cell repertoire and Ig affinity maturation [1–5]. Thus, T_{FH} cells play a critical role in the generation of long-lived humoral responses to antigens [3].

T_{FH} cells were first isolated and identified in human tonsils, and were characterized by the expression of B cell follicle homing chemokine receptor CXCR5 and the inducible costimulatory molecule ICOS [6, 7]. T_{FH} cells efficiently provide help to B cells and promote IgM to IgG immunoglobulin class switching through the production of interleukin-21 (IL-21) [8]. Studies in animal models have shown that, once differentiated and activated, T_{FH} cells can exit GC, developing into memory T_{FH} cells [9–12]. However, the origin of human blood circulating T_{FH} cells remains to be established.

$CD4^+$ T_H cells expressing the chemokine receptor CXCR5 are currently termed blood memory or peripheral T_{FH} cells and are long-lived memory cells [7, 13–15]. Recently, some human studies have contributed to a deeper characterization of blood T_{FH} cells on the basis of the expression of additional chemokine receptors such as CXCR3, CCR6, and CCR7, the costimulatory molecule ICOS, and the immunomodulatory molecule PD-1 [13, 16, 17]. T_{FH} cells defined as $CXCR3^+CCR6^-$ share properties with T_H1 cells (hereafter called $T_{FH}1$ cells), while $CXCR3^-CCR6^-$ and $CXCR3^-CCR6^+$ cells share properties of T_H2 cells ($T_{FH}2$) and of T_H17 cells ($T_{FH}17$), respectively [13]. $T_{FH}2$ and $T_{FH}17$ have a more efficient T helper activity on naive B cells, while $T_{FH}1$ $ICOS^+$ cells have a higher propensity to provide help to memory B cells [17]. In addition, we previously demonstrated that antigen-specific T_{FH} can be identified by flow cytometry by intracelluar staining of IL-21 upon *in vitro* antigen stimulation [18].

The identification of early biomarkers predicting vaccine efficacy may contribute to accelerate the development of novel vaccine candidates. These biomarkers should be easy to test in large clinical trials and have a clear mechanistic relationship with the correlates or surrogates of protection taken as study's endpoints. Recent studies showed that immunization with influenza A/California/2009 (H1N1) vaccine led to an expansion of peripheral T_{FH} subsets in humans [13, 17, 19–21]. Moreover, *ex vivo* frequencies of peripheral $T_{FH}1$ cells at day 7 correlated with the frequency of circulating plasmablasts and with increased levels of neutralizing antibodies to H1N1 at day 21 [13, 17]. In a previous study, we showed that a single dose of an avian H5N1 influenza vaccine induced the expansion of H5N1-specific $CD4^+ICOS^+IL$-21^+ T_H cells in the blood three weeks after vaccination, and that the increased frequency of these cells predicted the protective antibody titers found after the second dose of the vaccine [18].

The goal of the present study was to identify, in human peripheral blood, early T_{FH} cells subset(s) predicting not only the rise but also the long term persistence of functional antibody titers after seasonal influenza vaccination. For this purpose, we had access to human PBMCs collected in the framework of the European Innovative Medicine Initiative funded public-private project BIOVACSAFE [22]. PBMCs from healthy subjects immunized with one dose of seasonal adjuvanted or non-adjuvanted trivalent inactivated influenza vaccine (ATIIV and TIIV, respectively) were analyzed both directly *ex vivo* or after *in vitro* antigen stimulation. Frequencies of T_{FH} cells were determined and then correlated with HI antibody titers measured at days 28 and 168 post-vaccination. Both antigen-specific $CD4^+IL$-$21^+ICOS^+CXCR5^+$ T_{FH} cells and *ex vivo* $T_{FH}1$ $ICOS^+$ cells expanded seven days after vaccination and returned to baseline levels by day 28. After accounting for the effect of baseline HI titers, we showed that the

magnitude of the response of these T_{FH} cell subsets correlated with functional antibody responses measured up to 6 months after vaccination.

Materials and Methods

Clinical samples and vaccines

The study received ethical approval from London—Surrey Borders Research Ethics Committee (REC Ref: 13/LO/0044), and was registered on ClinicalTrials.gov prior to enrolment (NCT01771367). Forty-nine healthy adults (18–43 years old) were enrolled at the Surrey Clinical Research Centre, University of Surrey, Guildford, UK, as part of the BIOVACSAFE Consortium-funded clinical trial protocol CRC305C sponsored by the University of Surrey. The study was a partial-blind (participant and laboratory), randomised, placebo controlled exploratory study. The full study protocol is described in supplementary document S1 Text. All participants provided written informed consent. In the 2012-2013 winter season, 49 participants were randomized to allow 48 to complete with the full required reportable data in three arms and received one dose of A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010-like TIIV (Agrippal®; n=21), ATIIV (Fluad®, n=20), or saline placebo (n=8). Recruitment for the study began on February 7th 2013 and ended with the last follow-up visit on November 25th 2013. See CONSORT diagram and checklist (Fig 1 and S2 Text, respectively). PBMCs were collected from each group at baseline, day 7 and day 28 after immunization, and analyzed for plasmablasts and CD4$^+$ T cell responses. Sera were collected at day 0, day 7, day 28 and day 168 after immunization for the analyses of antibody responses.

Hemagglutination Inhibition Assay (HI)

HI titers were measured for A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), and B/Wisconsin/1/2010-like vaccine strains (Novartis Vaccines & Diagnostics) as described elsewhere [23, 24].

Polychromatic flow cytometry analyses

Frozen PBMCs from vaccinated participants were thawed and then analyzed by flow cytometry both *ex vivo* and after 18 h of stimulation at 37°C with anti-CD28 and anti-CD49d (1 μg/ml each, BD Biosciences), A/California/7/2009 (H1N1) subunit vaccine antigen (1 μg/ml, Novartis Vaccines & Diagnostics), or *Staphylococcus* enterotoxin B (SEB) (1 μg/ml, Sigma), in the presence of Brefeldin A (5 μg/ml, Sigma) as previously described [18, 25]. Cells were stained *ex vivo* with Live/Dead Yellow (Invitrogen), fluorochrome-conjugated antibodies: CD3-PE-Texas Red (SK7), CD4-APC-Horizon 7 (SK3), ICOS-PE (ISA-3), CXCR5-FITC (RF8B2), CXCR3-PE-Cy5 (1C6), CCR6-Brilliant Violet 421 (11A9), PD1-Brilliant Violet 785 (EH12.2H7), CD19-APC (SJ25C1), CD20-PerCP-Cy5.5 (L27), CD27-PE (L128), CD38-Alexa fluor 700 (HIT2) (BD Biosciences), CD8-Horizon V500 (RPA-T8) (Biolegend), CD45RA-PE-Cy7 (HI100) (eBioscience). H1N1-specific CD4$^+$ T cells were analyzed for intracellular production of IL-21 with anti-IL-21-APC (3A3-N2.1) (BD Biosciences) [18, 25]. Stained cells were acquired on a BD LSR Fortessa special order flow cytometer (BD Biosciences).

Analysis of H1N1-specific CD4$^+$IL-21$^+$ ICOS$^+$ T_H cells

Frequency, phenotype, and cytokine profile of H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$ T_H cells were determined by polychromatic flow cytometry following the gating strategy described in S1C Fig. The response to medium was subtracted for each subject at each time point. Data are
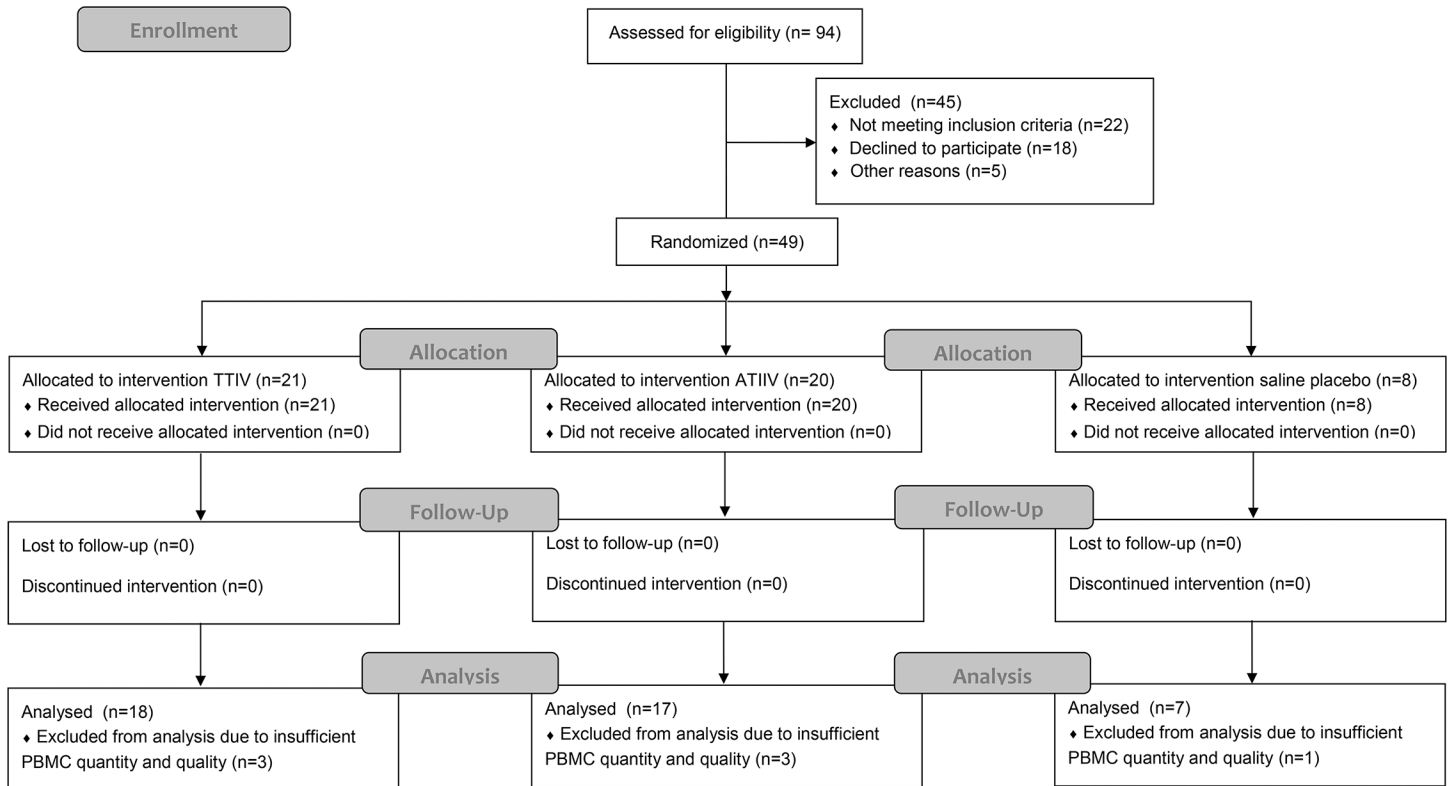
Fig 1. CONSORT Flow diagram.

expressed as number of antigen-specific $CD4^+$ T cells per million of total $CD4^+$ T cells. Data were analyzed using FlowJo (version 9.6, Tree Star) [18].

Statistical analyses

Median HI titers and frequencies of $CD4^+$ T cells measured across different time points were compared using Wilcoxon's signed rank test. Analyses were performed with SAS JMP 8.0.1 software. Associations between day 7 cellular responses and day 28 and 168 functional antibody responses were evaluated using the Pearson product-moment correlation metric. Data from TIIV and ATIIV vaccine cohorts were merged and analyzed as a single dataset. The potential effect of vaccine formulation as well as the negative correlation between HI responses to vaccination and baseline titers were removed, for each antigen, by fitting a mixed model and obtaining the residuals, based on a method adapted from Bucasas *et al.* [26]. The R package *lme4* was used to perform a linear mixed effects analysis of the relationship between day 28, or day 168, HI titers fold-increase and baseline HI titers [27]. The baseline HI titer was used as only fixed effect, while random slopes and intercepts for the two vaccine cohorts were included as random effects. The model was formulated as follows: $HI_{DayX/Day0} \sim HI_{Day0} + (1 + HI_{Day0}|$ Cohort) $+ \varepsilon$. Where $HI_{DayX/Day0}$ represents the HI fold-increase measured either at day 28 or 168 post-vaccination, HI_{Day0} corresponds to the baseline HI titer, Cohort is a 2-level factor indicating which vaccine was used and ε is the residual error. Given the lack of antigen specificity, cell responses derived from the *ex vivo* staining were correlated to the maximun response observed across the three strains represented in the vaccine, while H1N1-specific T_{FH} cells were correlated to H1N1-specific HI responses.

Results

Of the participants who were randomised, PBMCs of sufficient quantity and quality were obtained for 18 participants who received TIIV, 17 participants who received ATIIV and 7 who received placebo. Therefore the results reported below are only for 42 participants.

Influenza vaccination induces fast and long-lasting functional antibody responses

In this study, 42 healthy adults received a single intramuscular immunization with 2012-2013 TIIV, ATIIV, or saline placebo. B-cell responses were characterized as (i) frequencies of blood $CD19^+CD20^-CD38^+$ plasmablasts measured by flow cytometry at day 0, 7 and 28, and (ii) as vaccine-specific HI antibody titers at day 0, 7, 28, and 168.

Consistent with previous studies [28–30], TIIV and ATIIV increased the frequencies of plasmablasts at day 7 compared to placebo (Fig 2A and S1A Fig for subset identification). HI geometric mean antibody titers (GMT) against the three influenza virus strains significantly increased 7 days after immunization compared to placebo. HI antibody titers peaked at day 28, and persisted up to six months later at titers which were significantly higher than placebo for the H3N2 and B strains following ATIIV (Fig 2B and 2C). These higher titers at 6 months post-ATIIV administration paralleled seroprotection rates which were higher after ATIIV than TIIV (S1 Table).

Circulating $T_{FH}1$ cells expressing ICOS expand after both TIIV and ATIIV influenza vaccination

The number of $CD4^+ICOS^+$ T_H cells expressing CXCR5 ($CXCR5^+ICOS^+$) increased at day 7 after vaccination with TIIV and ATIIV and returned to baseline levels by day 28 (Fig 3A). Vaccination did not affect the number of $CXCR5^+ICOS^-$ nor of $CXCR5^-ICOS^+$ $CD4^+$ T_H cells (S2 Fig), in agreement with previous observations. [17]. We then determined the expression of ICOS in different T_{FH} subsets in PBMC at day 0, 7, and 28 after administration of TIIV or ATIIV (S1B Fig for subset identification) [17]. The number of $CD4^+$ $T_{FH}1$ $ICOS^+$ and $PD-1^+$ cells peaked at day 7 after vaccination with TIIV and ATIIV compared to day 0, returned to baseline levels by day 28, and were also significantly higher compared to saline placebo controls (Fig 3B and 3C). Vaccination did not modify the frequencies of $ICOS^+$ cells in the $T_{FH}17$ and $T_{FH}2$ cell subsets (S1B and S3 Figs). No changes in numbers of $CD4^+$ T_{FH} cells were observed in the placebo controls at any time point. Thus, these data show that peripheral blood $T_{FH}1$ cells, expressing ICOS and PD-1, transiently expand at day 7 after TIIV, as shown previously [17], and also after ATIIV.

Antigen-specific $CD4^+IL-21^+ICOS^+$ T_H cells expand at day 7 after vaccination and show a mixed $CXCR5^+/CXCR5^-$ phenotype

We previously showed that antigen-specific blood $CD4^+IL-21^+ICOS^+$ T_H cells expand three weeks after influenza vaccination [17]. In the present study, we extended these observations by measuring the frequency of circulating H1N1-specific $CD4^+IL-21^+ICOS^+$ T_H cells as early as 7 days after vaccination. PBMCs were stimulated overnight with A/California/7/2009 (H1N1) subunit antigen, and IL-21-producing cells were identified by flow cytometry (S1C Fig for subsets identification).

H1N1-specific $CD4^+IL-21^+ICOS^+$ T_H cells were already detectable on day 0 (mean values of 51 ± 25, 37 ± 7, and 53 ± 14 cells/10^6 $CD4^+$ T cells in the placebo, TIIV, and ATIIV groups, respectively) suggesting that most of the participants had memory T cells due to prior

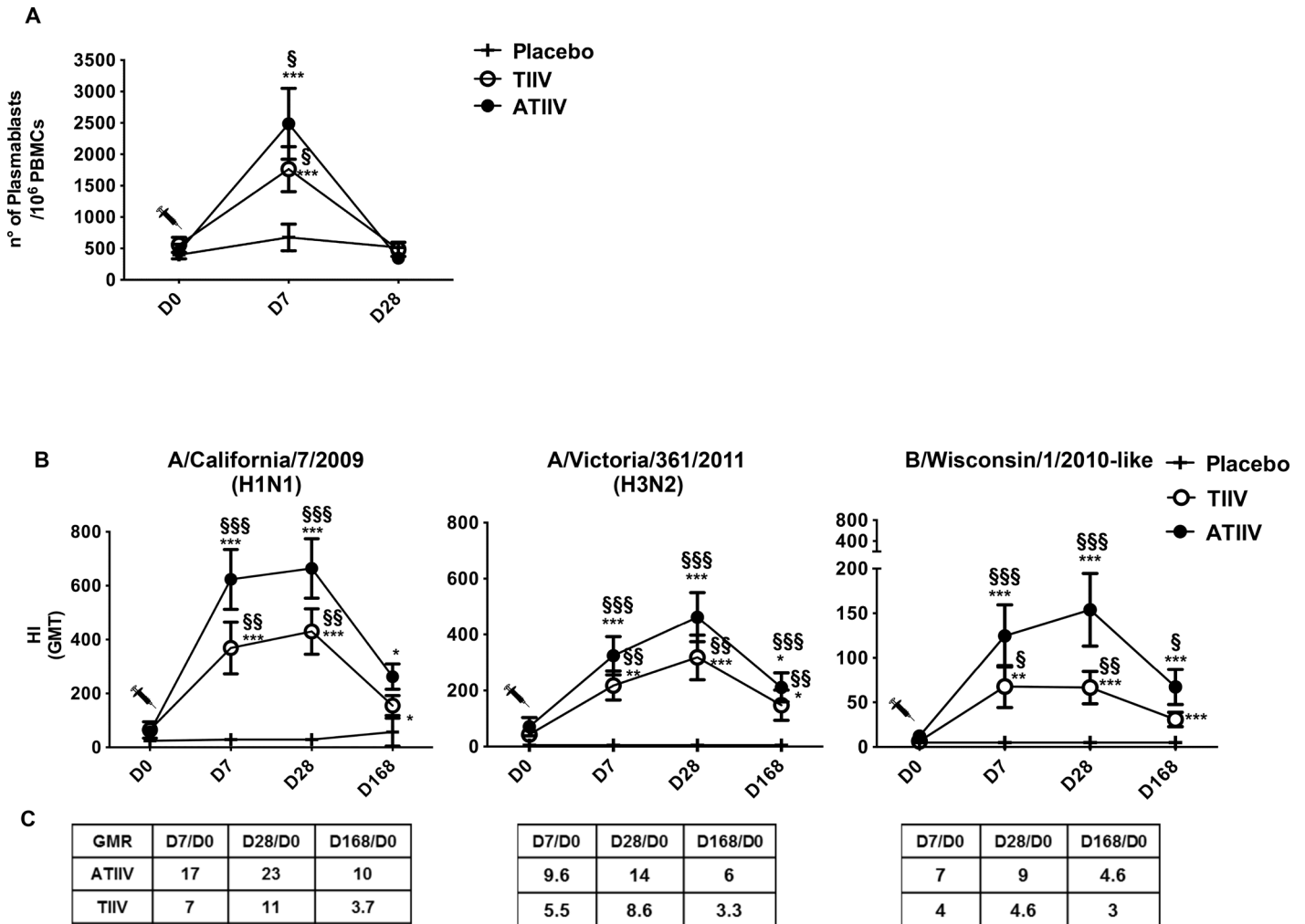**Fig 2. B-cell and functional antibody responses after seasonal influenza vaccination.** (**A**) Absolute number of plasmablasts ($CD19^+CD20^- CD38^+$) in 10^6 live PBMCs acquired. (**B**) HI Geometric mean titers (GMT) for A H1N1 and H3N2, and B influenza strains at baseline (D0), 7 days (D7), 28 days (D28), and 168 days (D168) after a single dose of influenza vaccine. Data show three cohorts: saline placebo (n=7), TIIV (n=18), and ATIIV (n=17). (**C**) Geometric mean ratio (GMR) for all vaccine strains. Non-parametric Wilcoxon's signed rank test was used for statistical analyses: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to day 0; §$p < 0.05$, §§$p < 0.01$ and §§§$p < 0.001$ compared to saline placebo.

vaccination or to natural exposure to circulating influenza viruses (Fig 4). Following vaccination with TIIV and ATIIV, the number of H1N1-specific $CD4^+IL-21^+ICOS^+$ T_H cells significantly increased at day 7 compared to day 0, and decreased by day 28 in both groups, although remaining significantly higher than baseline in the ATIIV group (Fig 4). Finally, the increased frequency of $CD4^+IL-21^+ICOS^+$ T_H cells at day 7 in response to ATIIV was significantly higher than in placebo controls, and persisted up to four weeks after vaccination.

We further characterized H1N1-specific $CD4^+IL-21^+ICOS^+$ T_H cells at day 7 based on the level of CXCR5 expression. Due to the intrinsic variability of the single participants and due to the limited number of participants tested, we investigated this parameter by pooling together the results from the TIIV and ATIIV groups. At baseline, H1N1-specific $CD4^+IL-21^+ICOS^+$ $CXCR5^-$ T_H cells were more abundant than $CXCR5^+$ T_{FH} cells with 40 ± 7 cells/ 10^6 $CD4^+$ T cells and 6 ± 1 cells/ 10^6 $CD4^+$ T cells, respectively (Fig 5), in agreement with our previous results (18). Both subsets expanded at day 7, to 180 ± 44 $CD4^+IL-21^+ICOS^+CXCR5^-$ T_H cells/
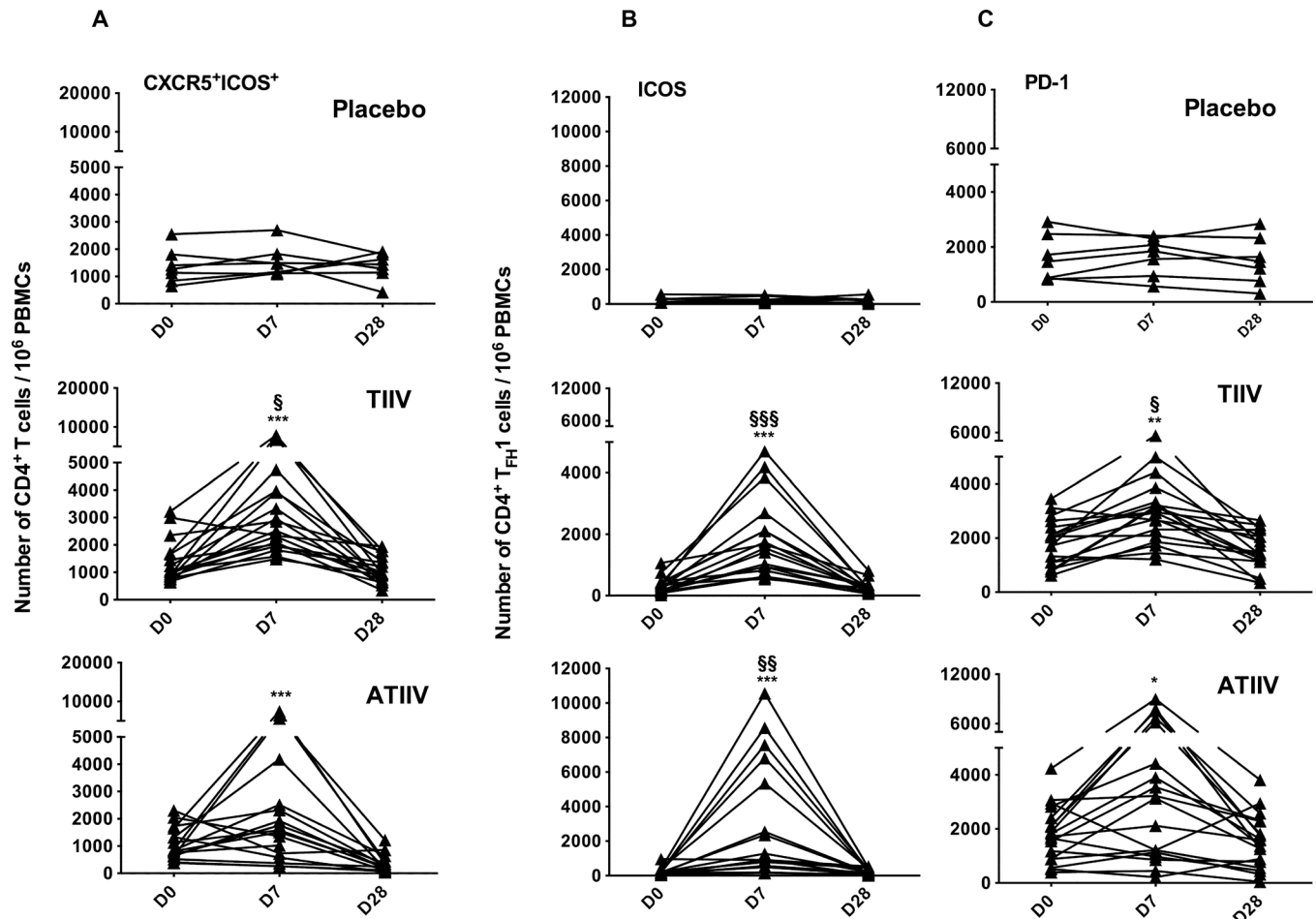
Fig 3. Expansion of ICOS$^+$ and PD-1$^+$ T$_{FH}$1 cells after TIIV and ATIIV vaccination. (**A**) Number of CD4$^+$ T cells expressing CXCR5 and ICOS in human PBMCs after seasonal influenza vaccination. (**B** and **C**) Number of T$_{FH}$1 cells expressing ICOS and PD-1. Data show three cohorts: saline placebo (n=7), TIIV (n=18) and ATIIV (n=17) at baseline (D0), day 7 (D7) and day 28 (D28) after a single dose of influenza vaccine. Data are shown for each participant and expressed as number of cells in 10^6 live PBMCs acquired. Non-parametric Wilcoxon's signed rank test was used for statistical analyses: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to day 0; $^§p < 0.05$, $^{§§}p < 0.01$ and $^{§§§}p < 0.001$ compared to saline placebo.

doi:10.1371/journal.pone.0157066.g003

10^6 CD4$^+$ T cells and 21 ± 5 CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T$_{FH}$ cells/ 10^6 CD4$^+$ T cells, respectively. At day 28, the frequency of H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T$_{FH}$ cells had returned to baseline levels, while CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^-$ T$_H$ cells were still high (80 ± 16 cells/ 10^6 CD4$^+$ T cells). In the placebo group, the frequency of H1N1-specific CD4$^+$IL-21$^+$ ICOS$^+$ T$_H$ cells CXCR5$^-$ or CXCR5$^+$ did not change over time, suggesting that the expansion of these subsets in TIIV and ATIIV groups was specifically driven by the vaccines.

Frequencies of T$_{FH}$ cells at day 7 correlate with functional antibody responses after influenza vaccination

We further asked whether the expansion, at day 7, of total CD4$^+$ T$_{FH}$1 ICOS$^+$ cells or H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T$_{FH}$ cells correlated with the rise of HI titers measured at day 28 and 168 post-immunization. In contrast to our previous study in which the vaccinees had no appreciable preexposure to avian A/Vietnam/1194/2004 (H5N1) influenza strain [18], most participants in this study had detectable levels of preexsisitng antibodies specific to
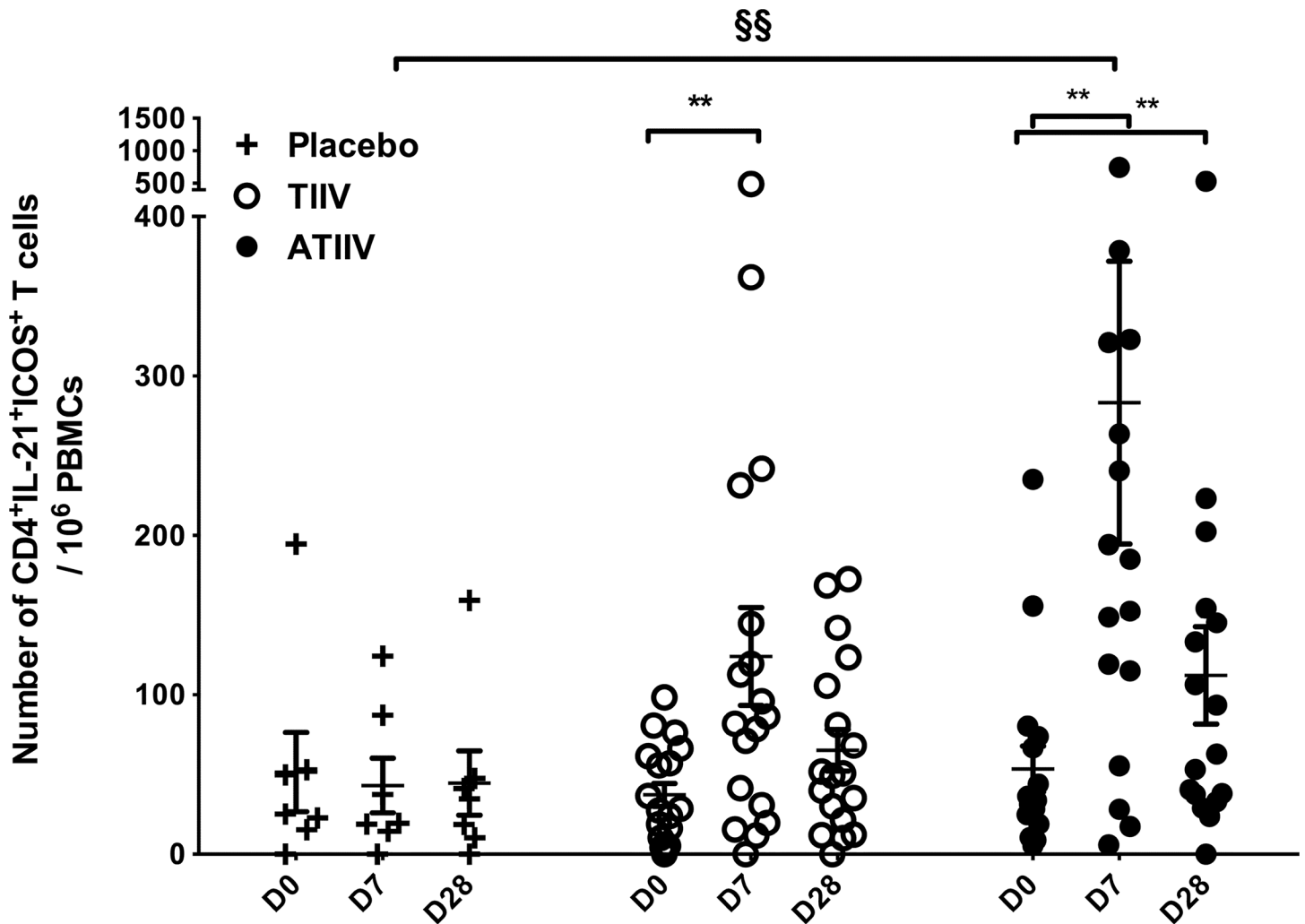
Fig 4. H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells expand 7 days after seasonal influenza vaccination. Numbers of CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells in PBMCs stimulated overnight with A/California/7/2009 (H1N1) antigen. Data show three cohorts: saline placebo (n=7), TIIV (n=18) and ATIIV (n=17) at baseline (D0), day 7 (D7) and day 28 (D28) after a single dose of influenza vaccine. Data are shown for each subject and expressed as number of cells in 10^6 live CD4$^+$ T cells acquired; mean ± SEM is shown. Non-parametric Wilcoxon's signed rank test was used for statistical analyses: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to day 0; $^\S p < 0.05$, $^{\S\S}p < 0.01$ and $^{\S\S\S}p < 0.001$ compared to saline placebo.

doi:10.1371/journal.pone.0157066.g004

one or more strains present in the 2012-2013 seasonal influenza vaccine. Baseline HI titers were inversely associated with HI titers measured at day 28 and 168 after vaccination and reached statistical significance for A/California/7/2009 (H1N1) and A/Victoria/361/2011 (H3N2) strains (S4 Fig). Therefore, in order to determine correlates of vaccine immunogenicity that were independent from vaccinees' baseline preexisting immunity, we defined an alternative endpoint metric: decorrelated hemagglutination inhibition (DHI), which removed any linear correlation between the antibody expansion after vaccination and day 0 HI titers (S5 Fig). Total CD4$^+$ T$_{FH}$1 ICOS$^+$ cells were correlated to the maximun HI response observed across the three strains represented in the vaccine, while H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T$_{FH}$ cells were correlated to H1N1-specific HI titers.

The number of CD4$^+$ T$_{FH}$1 ICOS$^+$ cells measured at day 7 after immunization was weakly correlated with DHI responses at day 28 (R = 0.35; n = 35; p = 0.04), but not at day 168 (R = 0.35; n = 28; p = 0.07) after vaccination (Table 1 and Fig 6). In contrast, the number of
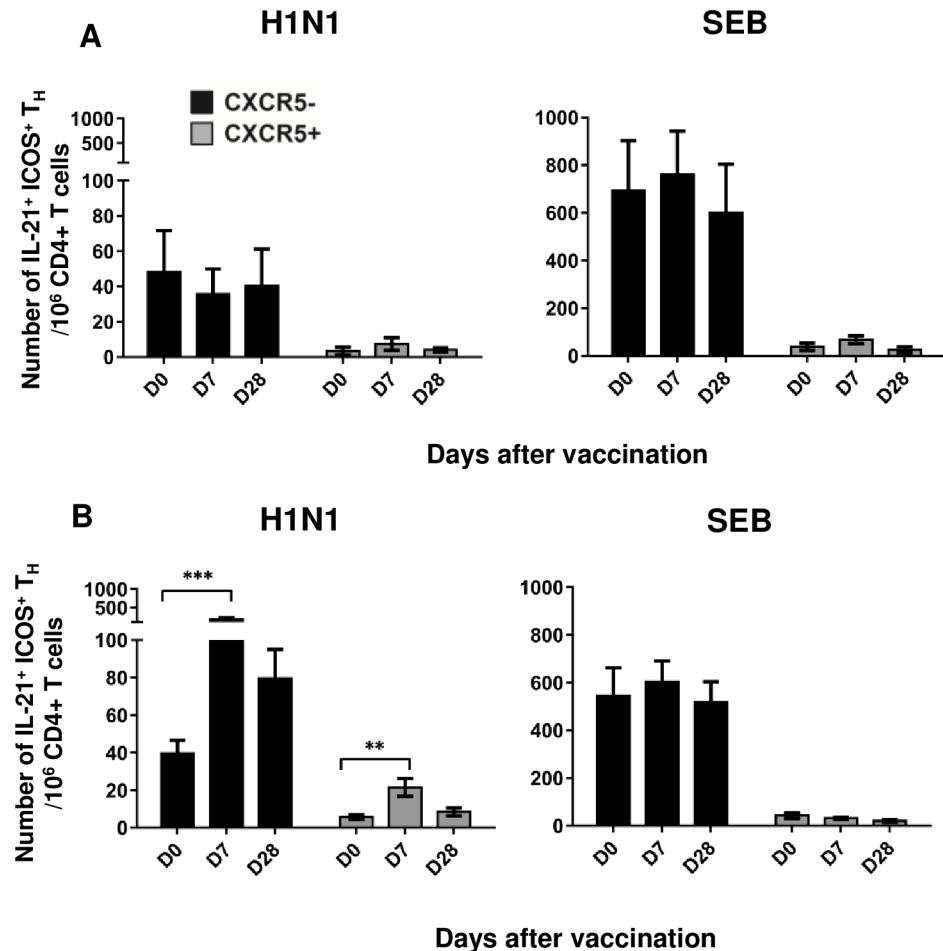
Fig 5. H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells subsets expressing or not CXCR5 expand after influenza vaccination. Number of CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells, showing a CXCR5$^+$ (black) or CXCR5$^-$ (gray) phenotype, in vaccinated participants after overnight stimulation with A/California/7/2009 (H1N1) antigen or SEB. Data show saline placebo (n=7), and merged TIIV (n=18) and ATIIV (n=17) cohorts at baseline (D0), day 7 (D7) and day 28 (D28) after a single dose of influenza vaccine. Data are expressed as number of cells in 10^6 live CD4$^+$ T cells; mean ± SEM is shown. Non-parametric Wilcoxon's signed rank test was used for statistical analyses: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to day 0.

doi:10.1371/journal.pone.0157066.g005

H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T$_{FH}$ cells, measured at day 7, was significantly correlated with both day 28 (R = 0.41; n = 35; p = 0.01) and day 168 (R = 0.43; n = 28; p = 0.02) DHI responses (Table 1 and Fig 7). H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$ and CD4$^+$IL-21$^+$ICOS$^+$ CXCR5$^-$ T$_H$ cells were not significantly associated with DHI responses at any time point. Interestingly, plasmablasts did not show any significant association with DHI responses at any time point (Table 1), while the frequency of plasmablasts observed at day 7 post-vaccination was negatively correlated with day 0 HI titers (R = -0.46; n = 31; p = 0.01) (S6 Fig), suggesting that plasmablast responses to vaccination were also affected by the preexisting immunity status of the vaccinees.

In conclusion, our data suggest that the frequencies of both *ex vivo* T$_{FH}$1 ICOS$^+$ cells and H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T$_{FH}$ cells is associated with vaccine-induced functional antibody responses, independently of the vaccinees' pre-immune status.

Table 1. Correlation between cell subsets and DHI titers.

[a]Predictor	Pearson R	
Day7	DHI Day28/Day0	DHI Day168/Day0
Plasmablasts	[b]0.20	[b]0.23
CD4$^+$T$_{FH}$1 ICOS$^+$ cells	[b]0.35*	[b]0.35
H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells	[c]0.23	[c]0.30
H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T$_{FH}$ cells	[c]0.41*	[c]0.43*
H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^-$ T$_H$ cells	[c]0.20	[c]0.25

Levels of correlation between cell frequencies, measured at day 7, and DHI responses measured 28 and 168 days after vaccination.

* $p \leq 0.05$.

[a] All predictors are expressed as number of cells / 10^6 PBMCs

[b] Maximum DHI response observed across A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010-like vaccine strains.

[c] A/California/7/2009 (H1N1)-specific DHI responses

doi:10.1371/journal.pone.0157066.t001

Discussion

In the present study, we have shown that various subpopulations of T cells sharing phenotypic and functional properties with T$_{FH}$ cells are present in the peripheral blood after vaccination with adjuvanted or non-adjuvanted influenza vaccines. The frequency of these blood T cell subpopulations peaks 7 days post-vaccination, then declines to baseline levels, although some (i.e. H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^-$ T$_H$ cells) persist for at least four weeks. Frequencies of some T$_{FH}$ cell subsets, either total or antigen-specific, predicted HI titers, not only four weeks after vaccination, but also six months later.

T$_{FH}$ cells are acquiring an increasing interest because of their ability to provide help to B cells in the GC of secondary lymphoid organs, leading to B-cell differentiation, reshaping of the B-cell repertoire, and Ig affinity maturation [1–5]. The role of T$_{FH}$ cells in the generation of long-lived humoral responses to antigens gives them an ideal profile as potential early biomarkers of effective take of vaccines and possibly of long-lasting protective antibody responses. Peripheral T$_{FH}$ subsets in whole blood or fresh human PBMCs of healthy participants have been identified by *ex vivo* staining following vaccination against influenza, describing memory CD4$^+$CXCR5$^+$ T$_H$ cells as the most abundant population [13, 17, 31, 32]. In this study performed on frozen PBMCs, a peak expansion of CD4$^+$CXCR5$^+$ T$_{FH}$1 cells expressing ICOS and PD-1 was detected 7 days after TIIV vaccination, while T$_{FH}$2 and T$_{FH}$17 cell subsets were not modulated, confirming previous observations by Bentebibel et al. [17]. Similar responses were also observed after ATIIV vaccination, suggesting that the presence of the adjuvant did not alter the profile of these T$_{FH}$ subsets.

We recently reported that H5N1-specific CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells expanded 21 days after vaccination, showed a CXCR5$^-$ phenotype, and positively correlated with the H5N1-specific antibody response after the second vaccine dose [18]. Here, we extended these observations by showing that the frequency of CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells, specific for the seasonal influenza A/California/7/2009 (H1N1) antigen, increased seven days after influenza vaccination and decreased by day 28. Both CXCR5$^-$ and CXCR5$^+$ subsets expanded seven days after influenza vaccination, while at day 28, most CD4$^+$IL-21$^+$ICOS$^+$ T$_H$ cells were CXCR5$^-$, suggesting that antigen-specific memory T$_{FH}$ cells might be in a resting state in the periphery and transiently express CXCR5 homing receptor upon antigen encounter.
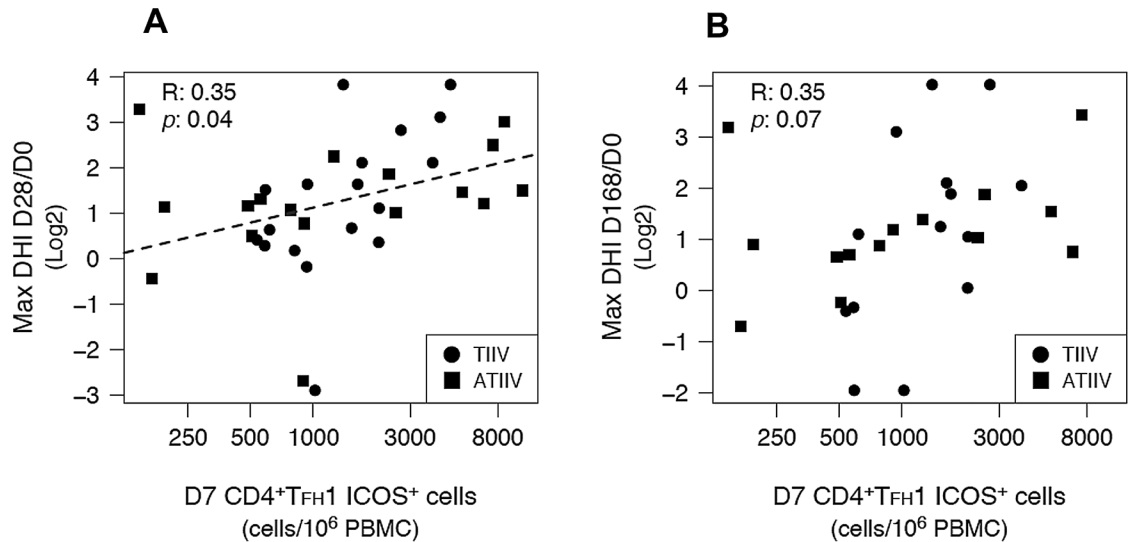
Fig 6. $T_{FH}1$ ICOS$^+$ cells predict functional antibody responses. Correlations between the number of total circulating CD4$^+$ $T_{FH}1$ ICOS$^+$ cells and the maximum DHI responses observed across the three influenza strains represented in the vaccine, measured at (A) day 28 and (B) day 168 after immunizzation. Dashed lines represent the least squares regressions fit to the data. R: Pearson product-moment correlation coefficient. p: correlation-associated p value.

doi:10.1371/journal.pone.0157066.g006

A number of independent studies have reported that preexisting antigen-specific antibodies affect responsiveness to influenza vaccination, with higher baseline levels associated with lower HI responses [33]. Therefore, we defined a new endpoint metric, DHI, that decorralated antibody expansion after vaccination from day 0 HI titers. This new endpoint enabled us to determine cellular correlates of vaccine immunogenicity that were independent from the vaccinees' preexisting immunity to influenza vaccine strains. With this approach, we showed that
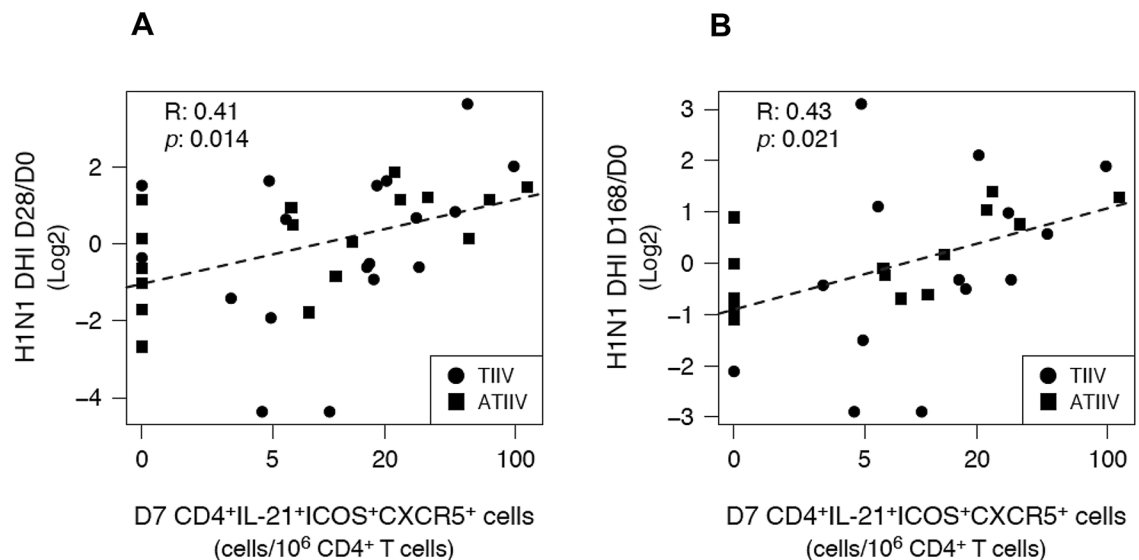
Fig 7. H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T_{FH} cells predict functional antibody responses. Correlations between the number of H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T_{FH} cells and H1N1-specific DHI responses measured at (A) day 28 and (B) day 168 after immunizzation. Dashed lines represent the least squares regressions fit to the data. R: Pearson product-moment correlation coefficient. p: correlation-associated p value.

doi:10.1371/journal.pone.0157066.g007

frequencies of plasmablasts at day 7 did not correlate with later DHI responses, suggesting that previously described associations between plasmablasts and antibody responses [17] might not be based on a causal effect but rather linked to preexisting immunity and, as such, appear correlated when directly compared. In contrast, $T_{FH}1$ ICOS$^+$ cells were shown to have some predictive power for day 28 DHI responses, confirming previous observations [17], but not for day 168 DHI responses. In addition, we also showed that day 7 H1N1-specific CD4$^+$IL-21^{+-} ICOS$^+$CXCR5$^+$ T_{FH} cell responses were significantly correlated with both early and late H1N1-specific antibody responses, with higher cell frequencies being associated with higher DHI responses. These results highlight the complex interplay between different factors, both subject- and vaccine-related, occurring during an immunological response to vaccination, leading to the production and persistence of functional antibodies.

In the present study, we identified two discrete H1N1-specific T_H populations, one being CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ and the other lacking the CXCR5 marker. Interestingly, CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T_{FH} cells expanded transiently after vaccination. These cells may represent a transitional state of a subset of cells belonging to the larger CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^-$ T_H cell population that persisted at higher frequency in the blood for at least 4 weeks, maintained their antigen-specificity as demonstrated by the ability to produce IL-21 and, as shown previously, by their capacity to provide help to B cells. Nonetheless, the possibility that the antigen-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T_{FH} cells identified in our study represent a small subset of $T_{FH}1$ ICOS$^+$ cells cannot be excluded. In any case, these findings provide evidence that both $T_{FH}1$ ICOS$^+$ and antigen-specific CD4$^+$IL-21$^+$ICOS$^+$CXCR5$^+$ T_{FH} cell subsets are associated with functional antibody responses to influenza vaccination. Further experimentation is needed to test whether the early response of these T_{FH} subsets can be used as biomarkers of later functional immune responses for vaccines other than influenza or for vaccines for which participants have no immunological memory.

Taken together, our data give new insights on T_{FH} cell subset responses after influenza vaccination and on their potential involvement in the persistence of protective antibody levels. We envision that applying similar approaches in future clinical trials will provide new insights into the mechanisms underlying the immunological response to vaccination and advance our ability to prospectively evaluate vaccine responsiveness based on early cellular and molecular signatures. This will represent an important advance in the development of novel or improved vaccines.

Supporting Information

S1 Fig. Gating strategy applied to PBMCs. (**A**) Plasmablasts (**B**) *Ex-vivo* T_{FH} subsets. (**C**) H1N1-specific CD4$^+$IL-21$^+$ICOS$^+$ T_H cells.
(ZIP)

S2 Fig. TIIV and ATIIV vaccination did not change the frequencies of blood CD4$^+$ICOS^{+-} CXCR5$^-$ T_H and ICOS$^-$CXCR5$^+$ T_{FH} cells. Data show three cohorts: saline placebo (n=7), TIIV (n=18) and ATIIV (n=17) at day 0, day 7 and day 28 after a single dose of influenza vaccine. Data are shown for each participant and expressed as number of cells in 10^6 live PBMCs acquired. Non-parametric Wilcoxon's signed rank test was used for statistical analyses. $p > 0.05$ compared to day 0 and to saline placebo.
(TIF)

S3 Fig. TIIV and ATIIV vaccination did not change ICOS expression in blood $T_{FH}2$ and $T_{FH}17$ subsets. TIIV and ATIIV vaccination did not change ICOS expression in blood $T_{FH}2$ and $T_{FH}17$ subsets. Data show three cohorts: saline placebo (n=7), TIIV (n=18) and ATIIV

(n=17) at day 0, day 7 and day 28 after a single dose of influenza vaccine. Data are shown for each participant and expressed as number of cells in 10^6 live PBMCs acquired. Non-parametric Wilcoxon's signed rank test was used for statistical analyses. $p > 0.05$ compared to day 0 and to saline placebo.
(TIF)

S4 Fig. Correlation between HI titers fold-increase and baseline HI titers. HI titers were determined for A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010-like vaccine strains.
(TIF)

S5 Fig. Correlation between DHI responses and baseline HI titers. HI titers were determined for A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010-like vaccine strains.
(TIF)

S6 Fig. Correlation between day 7 plasmablasts frequency and baseline HI titers. Baseline HI titers refer to the maximun value observed across A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010-like vaccine strains. Dashed lines represent the least squares regressions fit to the data. R: Pearson product-moment correlation coefficient. *p*: correlation associated p value.
(TIF)

S1 Table. Rates of protection against all vaccine strains.
(DOCX)

S2 Table. HI Titers.
(PDF)

S3 Table. Frequency of Plasmablasts.
(PDF)

S4 Table. Frequency of Antigen-specific T cells.
(PDF)

S5 Table. Frequency of *Ex vivo* CD4 T cells.
(PDF)

S1 Text. Clinical Trial Protocol CRC305C.
(PDF)

S2 Text. CONSORT Checklist.
(DOCX)

Acknowledgments

Author Contributions

References

1. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annual review of immunology. 2008; 26:741–66. doi: 10.1146/annurev.immunol.26.021607.090344 PMID: 18173374.

2. Chan TD, Brink R. Affinity-based selection and the germinal center response. Immunological reviews. 2012; 247(1):11–23. doi: 10.1111/j.1600-065X.2012.01118.x PMID: 22500828.

3. Crotty S. Follicular helper CD4 T cells (TFH). Annual review of immunology. 2011; 29:621–63. Epub 2011/02/15. doi: 10.1146/annurev-immunol-031210-101400 PMID: 21314428.

4. Goodnow CC, Vinuesa CG, Randall KL, Mackay F, Brink R. Control systems and decision making for antibody production. Nature immunology. 2010; 11(8):681–8. doi: 10.1038/ni.1900 PMID: 20644574.

5. Shulman Z, Gitlin AD, Targ S, Jankovic M, Pasqual G, Nussenzweig MC, et al. T follicular helper cell dynamics in germinal centers. Science. 2013; 341(6146):673–7. doi: 10.1126/science.1241680 PMID: 23887872; PubMed Central PMCID: PMC3941467.

6. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. The Journal of experimental medicine. 2000; 192(11):1545–52. Epub 2000/12/06. PMID: 11104797; PubMed Central PMCID: PMC2193094.

7. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. The Journal of experimental medicine. 2000; 192(11):1553–62. Epub 2000/12/06. PMID: 11104798; PubMed Central PMCID: PMC2193097.

8. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. The Journal of experimental medicine. 2010; 207(2):353–63. Epub 2010/02/10. doi: 10.1084/jem.20091738 PMID: 20142429; PubMed Central PMCID: PMC2822609.

9. Choi YS, Yang JA, Yusuf I, Johnston RJ, Greenbaum J, Peters B, et al. Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. Journal of immunology. 2013; 190(8):4014–26. doi: 10.4049/jimmunol.1202963 PMID: 23487426; PubMed Central PMCID: PMC3626566.

10. Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, et al. Bcl6 expression specifies the T follicular helper cell program in vivo. The Journal of experimental medicine. 2012; 209(10):1841–52, S1-24. doi: 10.1084/jem.20120219 PMID: 22987803; PubMed Central PMCID: PMC3457730.

11. MacLeod MK, David A, McKee AS, Crawford F, Kappler JW, Marrack P. Memory CD4 T cells that express CXCR5 provide accelerated help to B cells. Journal of immunology. 2011; 186(5):2889–96. doi: 10.4049/jimmunol.1002955 PMID: 21270407; PubMed Central PMCID: PMC3069687.

12. Weber JP, Fuhrmann F, Hutloff A. T-follicular helper cells survive as long-term memory cells. European journal of immunology. 2012; 42(8):1981–8. doi: 10.1002/eji.201242540 PMID: 22730020.

13. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity. 2011; 34(1):108–21. Epub 2011/01/11. S1074-7613(10)00491-7 [pii] doi: 10.1016/j.immuni.2010.12.012 PMID: 21215658; PubMed Central PMCID: PMC3046815.

14. Schmitt N, Bentebibel SE, Ueno H. Phenotype and functions of memory Tfh cells in human blood. Trends Immunol. 2014; 35(9):436–42. Epub 2014/07/08. doi: 10.1016/j.it.2014.06.002 PMID: 24998903; PubMed Central PMCID: PMC4152409.

15. Tsai LM, Yu D. Follicular helper T-cell memory: establishing new frontiers during antibody response. Immunology and cell biology. 2014; 92(1):57–63. doi: 10.1038/icb.2013.68 PMID: 24189164.

16. Bentebibel SE, Jacquemin C, Schmitt N, Ueno H. Analysis of human blood memory T follicular helper subsets. Methods in molecular biology. 2015; 1291:187–97. doi: 10.1007/978-1-4939-2498-1_16 PMID: 25836312.

17. 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. Epub 2013/03/15. doi: 10.1126/scitranslmed.3005191 PMID: 23486778.

18. Spensieri F, Borgogni E, Zedda L, Bardelli M, Buricchi F, Volpini G, et al. Human circulating influenza-CD4+ ICOS1+IL-21+ T cells expand after vaccination, exert helper function, and predict antibody responses. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(35):14330–5. Epub 2013/08/14. doi: 10.1073/pnas.1311998110 PMID: 23940329; PubMed Central PMCID: PMC3761599.

19. Bentebibel SE, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(33):E488–97. Epub 2011/08/03. 1100898108 [pii] doi: 10.1073/pnas.1100898108 PMID: 21808017; PubMed Central PMCID: PMC3158181.

20. Pallikkuth S, Parmigiani A, Silva SY, George VK, Fischl M, Pahwa R, et al. Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. Blood. 2012; 120(5):985–93. Epub 2012/06/14. doi: 10.1182/blood-2011-12-396648 PMID: 22692510.

21. Pallikkuth S, Pilakka Kanthikeel S, Silva SY, Fischl M, Pahwa R, Pahwa S. Upregulation of IL-21 receptor on B cells and IL-21 secretion distinguishes novel 2009 H1N1 vaccine responders from nonresponders among HIV-infected persons on combination antiretroviral therapy. Journal of immunology. 2011; 186(11):6173–81. doi: 10.4049/jimmunol.1100264 PMID: 21531891; PubMed Central PMCID: PMC3170914.

22. Lewis DJ, Lythgoe MP. Application of "Systems Vaccinology" to Evaluate Inflammation and Reactogenicity of Adjuvanted Preventative Vaccines. J Immunol Res. 2015; 2015:909406. doi: 10.1155/2015/909406 PMID: 26380327; PubMed Central PMCID: PMC4562180.

23. Ansaldi F, Zancolli M, Durando P, Montomoli E, Sticchi L, Del Giudice G, et al. Antibody response against heterogeneous circulating influenza virus strains elicited by MF59- and non-adjuvanted vaccines during seasons with good or partial matching between vaccine strain and clinical isolates. Vaccine. 2010; 28(25):4123–9. Epub 2010/05/04. doi: 10.1016/j.vaccine.2010.04.030 PMID: 20433807.

24. Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, et al. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet. 2001; 357(9272):1937–43. Epub 2001/06/27. S0140-6736(00)05066-2 [pii] doi: 10.1016/S0140-6736(00)05066-2 PMID: 11425416.

25. Galli G, Medini D, Borgogni 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. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106(10):3877–82. Epub 2009/02/25. 0813390106 [pii] doi: 10.1073/pnas.0813390106 PMID: 19237568; PubMed Central PMCID: PMC2646626.

26. Bucasas KL, Franco LM, Shaw CA, Bray MS, Wells JM, Nino D, et al. Early patterns of gene expression correlate with the humoral immune response to influenza vaccination in humans. J Infect Dis. 2011; 203(7):921–9. doi: 10.1093/infdis/jiq156 PMID: 21357945; PubMed Central PMCID: PMC3068032.

27. Bates D, Maechler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using {lme4}. Journal of Statistical Software. 2015; 67(1):1–48. doi: 10.18637/jss.v067.i01

28. Clark TW, Pareek M, Hoschler K, Dillon H, Nicholson KG, Groth N, et al. Trial of 2009 influenza A (H1N1) monovalent MF59-adjuvanted vaccine. N Engl J Med. 2009; 361(25):2424–35. Epub 2009/09/12. doi: 10.1056/NEJMoa0907650 PMID: 19745215.

29. Faenzi E, Zedda L, Bardelli M, Spensieri F, Borgogni E, Volpini G, et al. One dose of an MF59-adjuvanted pandemic A/H1N1 vaccine recruits pre-existing immune memory and induces the rapid rise of neutralizing antibodies. Vaccine. 2012; 30(27):4086–94. doi: 10.1016/j.vaccine.2012.04.020 PMID: ISI:000305660800018

30. Greenberg ME, Lai MH, Hartel GF, Wichems CH, Gittleson C, Bennet J, et al. Response to a monovalent 2009 influenza A (H1N1) vaccine. N Engl J Med. 2009; 361(25):2405–13. Epub 2009/09/12. doi: 10.1056/NEJMoa0907413 PMID: 19745216.

31. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity. 2013; 39(4):758–69. doi: 10.1016/j.immuni.2013.08.031 PMID: 24035365; PubMed Central PMCID: PMC3996844.

32. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. Nature immunology. 2015; 16(2):142–52. 10.1038/ni.3054. PMID: 25594465; PubMed Central PMCID: PMC4459756. doi: 10.1038/ni.3054

33. Tsang JS, Schwartzberg PL, Kotliarov Y, Biancotto A, Xie Z, Germain RN, et al. Global analyses of human immune variation reveal baseline predictors of postvaccination responses. Cell. 2014; 157 (2):499–513. doi: 10.1016/j.cell.2014.03.031 PMID: 24725414; PubMed Central PMCID: PMC4139290.