Neonatal T follicular helper cells are lodged in a Pre-T follicular helper stage favoring innate over adaptive germinal center responses

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T follicular helper (Tfh) cells have emerged as a critical limiting factor for controlling the magnitude of neonatal germinal center (GC) reactions and primary vaccine antibody responses. We compared the functional attributes of neonatal and adult Tfh cells at the transcriptomic level and demonstrated that the Tfh cell program is well-initiated in neonates although the Tfh gene-expression pattern (i.e., CXCR5, IL-21, BCL6, TBK1, STAT4, ASCL2, and c-MAF) is largely underrepresented as compared to adult Tfh cells. Importantly, we identified a TH2-bias of neonatal Tfh cells, with preferential differentiation toward short-lived pre-Tfh effector cells. Remarkably, adjuvantation with CpG-ODNs redirect neonatal pre-Tfh cells toward committed GC-Tfh cells, as illustrated by increased expression of Tfh signature genes and reduced expression of TH2-related genes.

Keywords: T follicular helper cells, neonates, vaccines, adjuvant, transcriptional profile analysis

INTRODUCTION

Neonates and young infants share a high vulnerability to infectious diseases. Inducing efficient and sustained B-cell responses remains challenging in this age group (1, 2). Numerous factors concur to limit primary antibody responses, including delayed follicular dendritic cell maturation (3), the limited development and expansion of T follicular helper (Tfh) cells and, as a result, that of germinal center (GC) B cells and plasma cells (4, 5).

Tfh cell differentiation is a multifactorial, multistep process as illustrated by the extensive list of transcription factors [including BCL6 (6–8), ASCL2 (9), LEF-1 (10, 11), TCF-1 (10, 11), BATF (12), STAT3 (13–15), NFAT (16), IRF4 (17), and c-MAF (18)] playing critical and non-redundant roles in driving Tfh cell differentiation, from the initiation of their development to their maintenance [reviewed in (19)]. The expression of CXC chemokine receptor 5 (CXCR5) through regulation of KLF2 (20, 21) dictates their spatiotemporal distribution, allowing them to migrate in the B cell zone toward CXCL-13 and entering the B cell follicles (22). Along with CXCR5, co-expression of ICOS (23, 24), PD-1 and IL-21 (25, 26) orchestrates Tfh cell differentiation and function. Notably, ICOSL/ICOS signaling plays an important role early in the Tfh cell differentiation program by down-regulating negative regulator molecules (20, 23, 27, 28), such as Blimp-1 (29), T-bet (30), and...
Within the follicles, T<sub>fh</sub> cell development could then be negatively regulated by IL-2 (32) and CTLA-4 (33, 34). Once these checkpoints are crossed, cognate T<sub>fh</sub>-B cell interactions take place for completing T<sub>fh</sub> cell differentiation.

Over the past years, the specific role of T<sub>fh</sub> cells as the main providers of B cell help has been unveiled, highlighting a critical role of T<sub>fh</sub> cells in vaccine elicited immune responses. We previously demonstrated that T<sub>fh</sub> cell development limits early life GC reactions and resulting primary vaccine antibody responses (4). Notably, adjuvantation of a vaccine with CpG-ODNs was sufficient to partially enhance neonatal antibody responses (4). In this study, we compared the transcriptional profile of neonatal and adult T<sub>fh</sub> cells and demonstrated that the preferential neonatal polarization toward TH2 is also observed among T<sub>fh</sub> cells, with increased expression of IL-13 and other TH2-related factors, which may represent an additional negative regulation for completing TH2 stage. Importantly, we showed that adjuvantation with CpG-ODNs reduced the expression of IL-13 and other TH2-related genes and sufficiently strengthened the levels of T<sub>fh</sub> cell-associated signature molecules to drive the full completion of GC-T<sub>fh</sub> differentiation.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 mice were purchased from Charles River (L’Arbresle, France), bred, and kept in pathogen-free animal facilities in accordance with local guidelines. Mice were used at 1 week (neonates) or 6–8 weeks (adults) of age. All animal experiments were approved by the Geneva Veterinary Office and conducted under relevant Swiss and European guidelines.

**Antigens, Adjuvants, Immunization**

Groups of C57BL/6 mice (5–16 mice/group) were immunized intramuscularly with Tetanus Toxoid (TT; 1 limit of flocculation) and, when indicated, Al(OH)₃ plus aluminum hydroxide [Al(OH); Former Novartis Vaccines, Siena, Italy (a GSK Company)] or, when indicated, Al(OH)₃ plus CpG-ODNs adsorbed to aluminum hydroxide. C57BL/6 mice were purchased from Charles River (L’Arbresle, France), bred, and kept in pathogen-free animal facilities in accordance with local guidelines. Mice were used at 1 week (neonates) or 6–8 weeks (adults) of age. All animal experiments were approved by the Geneva Veterinary Office and conducted under relevant Swiss and European guidelines.

**Semiquantitative Real-Time PCR**

Total cellular RNA was isolated by RNeasy microkit (Qiagen). cDNA was synthesized from 0.5 μg of total RNA using a mix of random hexamers–oligo d(T) primers and PrimerScript reverse transcriptase enzyme (Takara bio inc.). Pre-amplification was performed with TaqMan<sup>®</sup> PreAmp Master Mix following supplier’s instructions (Applied Biosystems). PCR reactions (10 μl volume) contained diluted cDNA, 2× Power SYBR Green Master Mix (Applied Biosystems), 300 nM of forward and reverse primers. RT-PCRs were performed on a SDS 7900 HT instrument (Applied Biosystems). Each reaction was performed in three replicates, with EF1α, GusB, and MmRPS9 as internal controls genes for data normalization. Raw Ct values obtained with SDS 2.2 (Applied Biosystems) were imported in Excel and normalization factor and fold changes were calculated using the GeNorm method (35). Primer sequences are as follows: EF1α sense, 5′-TCCATTGTGTCGCTTGTG-3′; anti-sense, 5′-CTTCTTGTCACACCTGTTAGA-3′, GusB sense, 5′-ACGGATGTTGTTGCTACGA-3′; anti-sense, 5′-TGACTCTGGTCCAAAACCTCTGA-3′, MmRPS9 sense, 5′-GCCAGGAGCTAAAGTTGATTGGA-3′; anti-sense, 5′-TCTTGGCCAGGTGAACCTTGTA-3′, slpr2 sense: 5′-TAACCTCCGGTCGACTGTTT-3′; anti-sense: 5′-AGAGCCTGTAGAAGGGCG-3′, IL-13 sense: 5′-AAAGGTCCCAAGGAGATTTGCA-3′; anti-sense: 5′-GGGAGGCTTGGAGACCCTGATGT-3′, RXRA sense: 5′-AACACAGATCGCTGAGACCCAGC-3′; anti-sense: 5′-AGGCGAGGCAAGCACGCCTT-3′, CCR2 sense: 5′-AGAATGGAATCTTACATCTGCA-3′; anti-sense: 5′-TGCTTCTCATTACCCTTTGTATGGT-3′, IL7R sense: 5′-AAATGCCCGAGATGGGAC-3′; anti-sense: 5′-AAAGGTGATCCTGCGCGTT-3′, and Bcl6 (36), CCRX5 (37), IL21 (38), Ascl2 (39), Pou5f1 (40), and PPARγ (41) as stated previously.

**Microarray and Analysis**

One week-old (16 mice/group) and adult C57BL/6 mice (5 mice/group) were immunized i.m. as described above. Ten days post-vaccination, inguinal draining LNs (dLNs) were pooled per mouse and per group to have a sufficient number of cells. T<sub>fh</sub> cell populations were isolated by flow-cytometry cell sorting using a MoFlo<sup>®</sup> Astrios™ flow cytometer (Beckman Coulter). Six independent experiments have been performed to obtain three independent samples per age group. Total RNA was labeled and hybridized on Agilent Whole Mouse Genome 48 × 60 K at Miltenyi Biotec (Germany) and from Bcl6<sup>+</sup> signatures (GSE40068) were extracted with Feature Extraction v10.6 software. Raw intensities were integrated, background corrected and log transformed, followed the quantile normalization between arrays. Intensities with detection p-values < 0.01 were arbitrarily discarded. Differentially expressed genes (DEGs) were identified by the ANOVA with Tukey post-hoc test considering adjusted p-value ≤ 0.05 and fold-change (FC) ≥ 2. Protein-protein interaction networks were built with DEGs using the NetworkAnalyst program (42) and the InnateDB PPIs as database (43). Enrichment analyses were performed with the program Gene Set Enrichment Analysis (GSEA) (44), using customs gene sets of upregulated genes from CD4<sup>+</sup> T<sub>fh</sub> effector cells [GSE43863 (45)] and from Bcl6<sup>+</sup> T<sub>fh</sub> cells or Bcl6<sup>−</sup> T<sub>fh</sub> cells [GSE40068 (8)]. First, raw expression data from GSE43863 and GSE40068 studies were normalized by RMA using the affy R/Bioconductor package (46), and submitted to quality control with the arrayQualityMetrics R/Bioconductor package (47). For both studies, the up-regulated genes were identified using the R/Bioconductor LIMMA package (48). The T<sub>fh</sub> effector signature (GSE43863) was generated by comparing CD4<sup>+</sup> T<sub>fh</sub> effector cells compared with naïve and TH1 CD4<sup>+</sup> T cells (adjusted p-value < 0.005 and FC ≥ 1.5), while the T<sub>fh</sub> Bcl6<sup>−</sup> and Bcl6<sup>+</sup> signatures (GSE40068) were generated by comparing CD4<sup>+</sup> CXCR5<sup>+</sup> Bcl6<sup>−</sup> and CD4<sup>+</sup> CXCR5<sup>−</sup> Bcl6<sup>−</sup> and CD4<sup>+</sup> CXCR5<sup>−</sup> Bcl6<sup>+</sup> signatures (GSE40068).
CXCR5+ Bcl6− T cells with CD4+ CXCR5− T cells (adjusted p-value < 0.05 and FC ≥ 2). Co-expression modules were identified with the CEMiTTool R/Bioconductor package (49) using variance filter p-value < 0.05 and ORA p-value < 0.2. CEMiTTool package is available at Bioconductor (https://bioconductor.org/packages/release/bioc/html/CEMiTool.html) (49). This package unifies the discovery and the analysis of coexpression gene modules, evaluating whether modules contain genes that are over-represented by specific pathways or that are altered in a specific sample group. Biological and functional enrichment analyses were also performed with the program GSEA using the REACTOME gene sets (50). Finally, unsupervised hierarchical clustering of the samples was carried out via multiscale bootstrap resampling with the PVCLUST R package (51).

RESULTS

Transcriptional Profile of Neonatal Tfh Cells

We (4, 52) and others (5) have shown that neonatal Tfh cells elicited by aluminum (AIOH)—based adjuvanted vaccines are few and functionally altered compared to adult cells. We therefore investigated the functional attributes of neonatal and adult CD4+ CXCR5highPD-1high Tfh cells at the transcriptomic level. CD4+ CXCR5highPD-1high Tfh and CD4+ CXCR5−PD-1− T (non-Tfh) cells were FACS sorted from the draining lymph nodes (LNs) at the previously identified peak (day 10) of the primary germinal center (GC) reaction induced by TT/AIOH (4) for comparative transcription profile analysis (Figure 1A). To visualize the global gene expression patterns of the various subsets, we first performed a principal component analysis (PCA), retaining the top 2,000 genes that contributed most to the total variance (Figure 1B). The projection of the data variance onto the principal components plane efficiently discriminated Tfh cells from non-Tfh cells in both age groups (Figure 1B), while clustering adult and neonatal Tfh cells together. This was confirmed by unsupervised hierarchical analysis, which grouped Tfh cells from both age groups (Figure S1). Thus, when successful the Tfh differentiation process essentially follows a similar path in early as in adult life.

Nevertheless, the gene expression profiles of neonatal and adult Tfh samples differed, revealing functionally differently programmed Tfh cells (Figure 1C). Comparing Tfh cells from neonatal and adult mice with the corresponding age-matched non-Tfh cells identified 2,301 and 3,549 differentially expressed genes, respectively. Overlap comparison showed that 1,710 genes were differentially expressed in Tfh cells of both neonatal and adult immunized mice, 591 genes were exclusively differentially expressed in neonatal Tfh cells, and 1,839 genes were exclusively differentially expressed in adult Tfh cells (Figure 1C).

To get more insight into the key genes leading to functionally differently programmed Tfh cells in early or adult life, PPI networks were generated from the differentially up-regulated genes between neonatal and adult Tfh cells (Figure 1D). The network derived from the genes differentially expressed in adult Tfh cells showed that most of the up-regulated proteins have an established role in Tfh biology and function (Bcl6, Ascl2, Pou6f1, IL-21, and Cxcr5). In accordance with the preferential
TH2 polarization of early life responses, IL-13 was strongly enriched in neonates vs. adults (Figure 1D). Unexpectedly, three cancer related-pathways genes (Tal1, PPAR-γ, and RXRA) were identified as hub genes in neonates (Figure 1D). Tal1 is expressed early, in hematopoietic stem cells and progenitor cells (53, 54), and subsequently silenced during T-cell development [reviewed in (55)]. It forms a large transcriptional complex with E proteins, LMO family proteins, LDB1, GATA2, and GATA3 (56–58). Tal1, GATA3, and RUNX1 coordinate the expression of downstream target genes. PPAR-γ is a member of the peroxisome proliferator-activated receptor family and forms heterodimer with RXRs to promote their downstream effects, i.e., suppress the transcription of target genes (59). Remarkably, in adults both PPAR-γ (41) and RXRA (60) negatively regulate T cell activation to prevent Tfh cell formation. These hub genes may thus play an essential role to functionally alter neonatal Tfh cell differentiation.

**Tfh Cell Differentiation Is Initiated in Neonates but Tfh Cells Remain Lodged in a pre-Tfh Stage**

We then selected the Tfh signature genes from published data sets to perform gene set-enrichment analyses (GSEA) with our data. This confirmed that both neonatal and adult cells were enriched for the Tfh lineage gene set (GEO accession code GSE43863) (Figure 2A), indicating that the Tfh cell differentiation program may succeed in neonates. However, neonatal Tfh Bcl6+ cells exhibited reduced gene expression signatures compared to adults, while using another GSEA (accession code GSE40068) indicated increased Tfh Bcl6− signatures (Figure 2A). Interestingly, Liu et al. demonstrated (8) that CXCR5+Bcl6low cells develop before CXCR5+Bcl6high cells and exhibit a non-polarized gene expression pattern. These “intermediate” Tfh cells then further mature into CXCR5+Bcl6high Tfh cells with the help of cognate B cells (8). This suggests that most neonatal Tfh cells are arrested at an early/intermediate stage of Tfh development, only a fraction of activated T cells fully up-regulating their expression of key Tfh genes, while maintaining their expression of IL-13, one of the preferentially expressed neonatal TH2-related cytokine gene.

To further analyze the transcriptional differences among early life and adult Tfh cells, we examined the expression of a set of genes described as up- or down-regulated in Tfh cells compared to non-Tfh CD4+ helper T cells (8, 12, 13, 61, 62). The heatmap of the differentially expressed genes (DEGs) (Figure 2B) confirmed that the gene expression of neonatal and adult Tfh samples differed from the control CD4+ CXCR5−PD-1− T (non-Tfh) cells (light colors). (C) Venn diagram illustrating the overlap of differentially expressed genes between Tfh vs. non-Tfh in young and adult immunized mice. Up-regulated genes in Tfh cells are shown in red and down-regulated genes in blue. Pie chart shows the proportion of genes differentially up-regulated by 1 week-old Tfh cells (purple) when compared to adults, and in brown genes differentially up-regulated by adult Tfh cells compared to 1 week-old. (D) Protein-protein interaction network constructed with the differentially expressed genes in 1-week-old Tfh cells as compared to adults. Up-regulated genes in neonates are illustrated in purple while brown indicates genes up-regulated in adults.

Bcl6 can bind to promoters and enhancers of genes that encode proteins that control T cell-migration, promoting non-follicular positioning of T cells (7). IL-7R is one of the most expressed Tfh-relevant genes by Bcl6 and its suppression is critical in Tfh cell differentiation (65). Although IL-7R is down-regulated in neonatal Tfh cells, its inhibition is almost two times weaker than in adult Tfh cells (expression level change of 1.89-fold greater in neonates compared to adults). Liu et al. (65) recently demonstrated that IL-7R expression was inversely correlated with Tfh commitment, more precisely with the expression of classical Tfh markers: PD-1, CXCR5, and Bcl6. A limited follicular positioning of neonatal pre-Tfh cells is also supported by the decreased expression (expression level change of −1.92-fold in neonates compared to adults) of S1pr2, known to suppress CXC12/CXCL13-mediated migration, thus restricting premature egress of Tfh cells out of GC (66).

Thus, numerous transcription factors contribute to prevent the follicular positioning of neonatal Tfh cells, depriving them from interacting with follicular dendritic cells (FDCs) and germinal center B (GC B) cells.

The expression of the Pou2af1 and Pou6f1 transcription factors was also reduced. Although their role in Tfh cells remains to be fully investigated, Pou6f1 is expressed in early fate committed Tfh cells (6) and Pou2af1 is highly expressed in early stage GC-Tfh cells (10, 67). A recent report by Stauss et al. (68) established that the Pou2af1 gene promotes Bcl6 expression and Tfh cell development. A general reduction in CXCR5 expression was observed on Pou2af1−/- CD4+ T cells as well as fewer GL7+ Tfh cells in Pou2af1−/- mice (68). Therefore, the POU family transcription factors seem to fine-tune Tfh cell development and their reduced expression in neonates may contribute to the limited expression of CXCR5 and GL7 in neonatal Tfh cells (4).

The lower expression of STAT4 (expression level change of −2.22-fold in neonates compared to adults) in neonates cements that the specific early life environment prevents the differentiation of pre-Tfh cells toward committed GC-Tfh cells: the IL-12-STAT4 pathway indeed contributes to the expression of key Tfh-associated molecules, such as IL-21, CXCR5, and ICOS as well as multiple important transcription factors involved in Tfh-cell generation, such as Bcl6, c-Maf, and Batf (69, 70).

How does the TH2-like preferential polarization of neonatal effector T cells persist in Tfh cells?
FIGURE 2 | Tfh cell differentiation is initiated in neonates but most of the generated cells are lodged in a pre-Tfh stage. (A) Enrichment analysis of neonatal vs. adult Tfh cells with the gene list from Tfh effector cells or Bcl6 + and Bcl6 − Tfh cells. (B) Sample z-score heatmap of significantly differentially expressed genes (row) in Tfh cells and their respective controls from young and adult mice (columns). Semi-quantitative RT-PCR analysis of selected Tfh cell-related genes (C) or TH2 cell-related genes (D), normalized to results obtained for the control genes (EEF1, GusB, RPS9). The graph display mean ± SEM. Cumulative data from adult [TT/AlOH (n = 10) and 1 week-old [TT/AlOH (n = 64), TT/AlOH + CpG1826 (n = 32)] mice from at least two independent experiments. Statistical analysis was performed with Prism software (Version 7, GraphPad), using unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Although, TH2 signature genes, including GATA3, IL-4, and IL-5, were not differentially expressed in neonatal T<sub>fh</sub> cells (Table S1), we observed significant changes in IL-13 and PPAR-γ. Nobs et al. recently showed that PPAR-γ expression in T cells controls the development of type-2 immunity (71). Therefore, the increased expression of PPAR-γ and of additional genes associated with TH2 polarization, including RXRA, ccr2 (72), il17rb (73–75), and cntnap1 (75), may all play a role in maintaining the default TH2-bias of neonatal T<sub>fh</sub> cells.

Semi-quantitative RT-PCR analyses confirmed both the reduced transcript abundance of T<sub>fh</sub>-cell-associated signature genes in neonates (black bars) compared to adults (open bars) (Figure 2C and Figure S2), and their preferential bias toward TH2, as shown by the higher levels of IL-13, PPAR-γ, RXRA, and CCR2 (Figure 2D).

**Adjuvantation With CpG<sub>1826</sub> Bypasses the Neonatal TH2-Bias of pre-T<sub>fh</sub> Cells and Supports Terminal GC-T<sub>fh</sub> Cell Differentiation**

We and others have shown that administration of TT/AlOH supplemented with TLR9 agonist CpG<sub>1826</sub> enhanced neutral antibody responses through the induction of higher T<sub>fh</sub> and GC B cell numbers (4), as observed in adult mice (76, 77). We thus asked whether neonatal CpG<sub>1826</sub> adjuvantation induced transcriptional changes in the genes/factors identified as differing between neonatal and adult T<sub>fh</sub> cells. Semiquantitative RT-PCR on FACS-sorted CD4<sup>+</sup> CXCR5<sup>high</sup>PD-1<sup>high</sup> T<sub>fh</sub> cells isolated 10 days after TT/AlOH + CpG<sub>1826</sub> immunization showed that CpG adjuvantation increased the transcriptional abundance of T<sub>fh</sub>-cell specific signature genes in neonatal T<sub>fh</sub> cells (Figure 2C and Figure S2). Flow cytometry analyses confirmed significantly lower CXCR5 expression by neonatal T<sub>fh</sub> cells (4) (Figure S3A). Remarkably, CpG adjuvantation significantly enhanced the expression of CXCR5 on neonatal T<sub>fh</sub> cells (Figure S3A), although not to adult like levels. The results support a follicular positioning of neonatal T<sub>fh</sub> cells, facilitating the T<sub>fh</sub>-GC B cell crosstalk required to provide B cell help during the GC reaction. In contrast, the expression of PD-1 was significantly decreased in 1 week-old and adult mice immunized with CpG-ODNs (Figure S3B).

Notably, TH2-related genes (i.e., PPAR-γ, RXRA, IL-13, and CCR2) were significantly reduced in neonatal T<sub>fh</sub> cells, reaching similarly low levels as in adult mice immunized without CpG-ODNs (Figure 2D). However, similar to our previous observation (4), IL-4 mRNA transcripts were significantly lower in neonatal T<sub>fh</sub> cells and were not affected by CpG adjuvantation (Figure S2B), suggesting that IL-17 transcription would also remain unaffected as previously demonstrated by Debock et al. (5).

Thus, CpG adjuvantation may (1) abrogate the regulation of early life T<sub>fh</sub> cell differentiation exerted by the TH2-related genes PPAR-γ and RXRA, (2) facilitate the follicular positioning of neonatal T<sub>fh</sub> cells, as mirrored by increased levels of S1pr2 and CXCR5 and by reduced IL-7R, and (3) support neonatal T<sub>fh</sub> cell differentiation toward committed GC-T<sub>fh</sub> cells. This explains our previous observations demonstrating the increase of T<sub>fh</sub> cell numbers, of GL7 expression by neonatal T<sub>fh</sub> cells, of GC reactions and thus of Ab titers following neonatal CpG-ODNs adjuvantation (4) (Figures S3C,D).

**Neonatal T<sub>fh</sub> Cells Preferentially Give Rise to Short-Lived Effector Cells**

To better understand the fate of the pre-T<sub>fh</sub> cells elicited in early life, we next ran the Co-Expression Molecules identification Tool (CEMiTool) (49) on our data set. CEMitool is an R package that provides in an automated manner unsupervised gene filtering, automated parameter selection for identifying modules, enrichment and module functional analyses as well as integration with interactome data (49). This modular expression analysis identified 6 different co-expression modules (Figure 3A and Data Sheet S1), of which only module M2 was significantly enriched in neonates (Figure 3B). This module was enriched for genes related to cell cycle (49) (Figure 3C), suggesting the capacity of neonatal T<sub>fh</sub> cells to enter the cell cycle more rapidly than their adult counterparts. CEMITool also integrates co-expression analysis with protein-protein interaction data. Expression of important genes associated with cell-cycle progression, including gene encoding E2F1 and TK1, were identified as hubs in module M2 (Figure 3D). This early life characteristic was previously observed (78–80): neonatal T and B lymphocytes have the capacity to enter the cell cycle more quickly and thus efficiently mobilize responses from an otherwise completely naïve population—possibly to compensate for the limitations in immune cell function in early life (i.e., lack of immunological memory) (78–80). Yet, rapid cycle entry only gives rise to short-lived effector cells (78–80).

To complete our observations, we performed a pathway analysis which revealed cell-intrinsic differences between neonatal and adult T<sub>fh</sub> cells (Figure 4): neonatal T<sub>fh</sub> cells were enriched in pathways associated with cell proliferation, apoptosis and key metabolic reactions, such as glycolysis, considered to play an important role in T cell activation and differentiation, while adult cells were enriched in mitogen-activated protein kinase (MAPK)-signaling pathways, thus outlining age-associated differences in the maturity and basic function. Interestingly, enrichment of Hedgehog signaling, which predispose T cell differentiation toward the TH2 pathway (81), further supports the TH2-bias of neonatal T<sub>fh</sub> cells. Altogether, these transcriptional analyses of neonatal vs. adult T<sub>fh</sub> cells reveal the existence of multiple coordinated regulatory mechanisms resulting into the preferential differentiation of neonatal CD4<sup>+</sup> T cells toward innate, short-lived pre-T<sub>fh</sub> effectors rather than adaptive (GC-derived) immunity defense mechanisms.

**DISCUSSION**

We previously identified the induction of T<sub>fh</sub> cells as limiting early life GC and Ab responses elicited by vaccines including aluminum-based adjuvants (4). We now demonstrate that the few T<sub>fh</sub> cells elicited in early life retain a preferential bias toward TH2, strongly expressing IL-13, and PPAR-γ and RXRA
which negatively regulate T_{fh} cell differentiation (41, 60), and that numerous transcription factors contribute to restrict activated neonatal CD4^{+} T cells at a pre-T_{fh} cell stage of short-lived effectors favoring innate rather than GC-associated adaptive responses. Importantly, we show that this fate is not inevitable as adjuvantation with CpG-ODNs reduced the...
expression of TH2-related genes and sufficiently strengthened the T<sub>fh</sub> cell-associated signature molecules to drive the GC-T<sub>fh</sub> differentiation program to its completion and fine tune the GC reaction.

Following immune challenges, neonatal responses are often weak (82). This has been associated with a propensity of neonatal T cells to give rise to short-lived effector cells (78–80) and to produce elevated levels of TH2-type cytokines compared to adults (83–85). We show that these two key neonatal characteristics persist during T<sub>fh</sub> cell differentiation, lodging the cells in a pre-T<sub>fh</sub> stage characterized by a TH2 bias. Our results suggest that a delicate balance of several signals known to promote TH2 development may contribute in maintaining an optimal environment for the TH2-biased T<sub>fh</sub> cell differentiation in neonates, including increased expression of PPAR-γ (71, 86), RXRA (87), ccr2 (72), il17rb (73–75), ctnnap1 (75), Hedgehog signaling (81), and lower levels of c-maf mRNA transcripts. A critical role for c-maf in limiting TH2 responses and in driving T<sub>fh</sub> cell development was recently unveiled by Andris et al. (18). Further investigations are warranted to delineate whether Tal1 may also play a fundamental role in the generation and persistence of TH2-biased T<sub>fh</sub> cells in neonates.

A limitation of our study is that the very few T<sub>fh</sub> cells induced in early life (about 2 × 10<sup>4</sup> T<sub>fh</sub> cells from a pool of 8 neonates) and thus the small amount of recovered RNA precluded the analysis of all potentially interesting genes. As the microarray did not reveal significant changes in TH17-related genes, in GATA3 or in IL-5 expression in neonatal T<sub>fh</sub> vs. non-T<sub>fh</sub> cells (Table S1), we did not compare their mRNA transcript levels to those of adult cells. In our model, both IFN-γ and IL-4 mRNA transcript levels are significantly lower in neonatal T<sub>fh</sub> cells— and not affected by CpG adjuvantage (Figure S2B). The similar expression of Foxp3 in neonatal and adult T<sub>fh</sub> cells was confirmed by semiquantitative RT-PCR (Figure S2D). Altogether, these results suggest that in our model, neonatal T<sub>fh</sub> cells do not exhibit a bias toward TH1, TH17 nor Treg cells. Our attempts to develop validated assays to reliably measure several proteins in the few recovered neonatal T<sub>fh</sub> cells did not succeed, and such proteins remained below detection levels when assessed in lymph node homogenates (not shown).

PPAR-γ and RXRA were identified as critical hub genes in neonates. Therefore, besides their role in maintaining the overall TH2 bias, PPAR-γ and RXRA may also negatively regulate T<sub>fh</sub> cell differentiation. PPAR-γ is known to (71, 86) promote Tregs survival (88–90) and inhibit the formation of T<sub>fh</sub> cells and GC reactions via the regulation of Bcl6 and IL-21 (41). That inhibition of Bcl6 expression is illustrated in neonatal T<sub>fh</sub> cells by increased expression of the TH2-related gene, IL-13, previously identified as one of the most repressed Bcl6-target gene (65). A role for Tgif1-RXR interaction in the establishment or inhibition of a chronically elevated T<sub>fh</sub> cell population was recently computationally predicted and demonstrated by Leber et al. (60); an increase with Tgif1 was associated with an increase in the T<sub>fh</sub> response, while an increase in RXR was more closely correlated with the T<sub>fh</sub> decline phase (60). Small changes in RXRA, such as 10% change in expression were previously demonstrated to result in a 50% change in activity and significant alteration of downstream transcriptional targets (91). We conclude that the differential expression of TH2-related genes PPAR-γ and RXRA might be involved in the distinct genetic programming of neonatal and adult T<sub>fh</sub> cells.

Although the T<sub>fh</sub> cell program is well-initiated in neonates, the gene-expression pattern of neonatal T<sub>fh</sub> cells underrepresented that of adult T<sub>fh</sub> cells, suggesting that T-B interactions fail to elicit appropriate signals and provide efficient help to neonatal pre-T<sub>fh</sub> cells to further differentiate into committed GC-T<sub>fh</sub> cells. Indeed, T<sub>fh</sub> cells differentiation involves a multi-signal process that includes expression of CXCR5, IL-21, Bcl6, TBK1, STAT4, Ascl2, and c-maf which were all expressed to lower levels as compared to adult T<sub>fh</sub> cells. Remarkably, adjuvantage with CpG-ODNs, skewed neonatal pre-T<sub>fh</sub> cells toward committed GC-T<sub>fh</sub> cells, as illustrated by increased expression of T<sub>fh</sub>-signature genes (Figure 2C and Figure S2). In parallel, genes associated with follicular positioning of T<sub>fh</sub> cells were increased (i.e., slpr, Ascl2, and CXC5), facilitating the cognate T<sub>fh</sub>-B cell interactions for completing T<sub>fh</sub> cell differentiation (92–96), with concomitant increase in Bcl6 and IL-21 expression (10, 95). Ascl2 directly regulates the localization of T<sub>fh</sub> cells via CXCR5 expression and suppression of CCR7 and PSGL1 (9). CXC5 allows T<sub>fh</sub> cells to migrate into the B cell follicles and form stable contacts with antigen-primed B cells (92, 97). These results indicate that a combination of several T<sub>fh</sub>-specific signals, in addition to previously described environment factors and CD4<sup>+</sup> T cells intrinsic determinants (4), maintain a favorable environment for TH2-biased T<sub>fh</sub> cell differentiation, restricting neonatal CD4<sup>+</sup> T cells at a pre-T<sub>fh</sub> stage of short-lived effector cells. Adjuvantage with CpG-ODNs is sufficient to counteract the TH2-biased response of neonatal T<sub>fh</sub> cells, reducing TH2-related genes to adult-like levels, while T<sub>fh</sub> signature genes (i.e., Bcl6, CXCR5, IL-21, Ascl2, C-maf, Pou6f1, slpr, Batf, CXC4, and TBK1) are progressively enhanced, resulting in differentiated and GC-committed T<sub>fh</sub> cells. As illustrated in Figures 2C,D, the switch from pre-T<sub>fh</sub> to mature T<sub>fh</sub> cells involves changes in the expression levels of several factors—such as “classical” mechanistic approaches including knockout/knock-in mice were not attempted.

We recently demonstrated that adjuvantage of a vaccine with a liposome including a C-type lectin receptor agonist was able to elicit potent GC reactions in neonates after a single dose (98). Altogether, these results show that immune deficiencies seen in early life can be overcome by providing the right signals, and are in accordance with the current understanding that the neonatal immune system is not deficient but tightly regulated to best adapt to the unique challenge of a rapidly required adaptation from a sterile to a microbial environment (99).

Further studies are necessary to investigate whether abrogating the TH2 bias of T<sub>fh</sub> cells in early life is critical for the full commitment of T<sub>fh</sub> cell differentiation and the subsequent GC B cell and antibody responses, resulting in effective responses to vaccination in early life.
DATA AVAILABILITY
The data has been deposited at the Gene Expression Omnibus repository—accession number is GSE126843.

ETHICS STATEMENT
This study was carried out in accordance with the recommendations of the Geneva Veterinary Office and conducted under relevant Swiss and European guidelines. The protocol was approved by the Geneva Veterinary Office.

AUTHOR CONTRIBUTIONS
BM-G, P-HL, and C-AS contributed to formulation of theory and prediction. BM-G, P-HL, and C-AS designed the research. BM-G and MV performed the experiments and analyzed and/or interpreted the data. BM-G, MV, and C-AS wrote the manuscript. PG-D, FF, LC, and HN performed the microarray analysis and critically revised the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01845/full#supplementary-material

Figure S1 | Unsupervised hierarchical analysis groups together Tfh cells from both age groups. Hierarchical clustering of CD4+ CXCR5hiPD-1hi Tfh cells and respective controls CD4+ CXCR5loPD-1− Tfh cell samples was obtained from pveclust R package. CD4+ CXCR5hiPD-1hi Tfh cells from young (purple) and adult (orange) mice and their respective controls (light colors) formed distinct groups.

Figure S2 | CpG adjuvantsion is sufficient to increase Bc6 targeted Tfh specific genes, such as Atipa1a3 as well as Tfh cell-related genes, including Batf, Tbk1, and Cxcr4. One week-old and adult C57BL/6 mice (5–8 mice/group) were immunized i.m. with TT/AlOH. Ten days post-vaccination the draining LNs were collected and analyzed. Semi-quantitative RT-PCR analysis of selected Tfh cell-related genes (A), IL-4 (B), TH1 cell-related genes (C), Foxp3 (D), or Bcl6 targeted Tfh specific genes (E) in sorted cells, normalized to results obtained for the control genes (Eef1, GusB, Rps9). The graph display mean ± SEM. Cumulative data were obtained from (TT/AO (n = 10) and 1 week-old [TT/AO (n = 64), TT/AO+ CpG1620 (n = 32)] mice from at least two independent experiments. Fold changes are shown relative to one 1-week-old mice immunized with TT/AO. Statistical analysis was performed with Prism software (Version 7, GraphPad), using unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S3 | Progressive increase of TT-specific IgG1 titers and Tfh cell responses in neonates with CpG adjuvantsion. C57BL/6 mice were immunized i.m. at 1 week or as adult with TT/AO with or without CpG (5–8 mice per group). The adult dose of AlOH was weight adjusted to 0.3 mg/adult or 0.15 mg for 1 week or as adult with TT/AlOH with or without CpG (5–8 mice per group). The adult dose of AlOH was weight adjusted to 0.3 mg/adult or 0.15 mg for 1 week or as adult with TT/AlOH with or without CpG (5–8 mice per group). Cumulative data were obtained from (TT/AO (n = 10) and 1 week-old [TT/AO (n = 64), TT/AO+ CpG1620 (n = 32)] mice from at least two independent experiments. Fold changes are shown relative to one 1-week-old mice immunized with TT/AO. Statistical analysis was performed with Prism software (Version 7, GraphPad), using unpaired t-test with p > 0.05 were considered to be insignificant. *p-value (0.01–0.05), **p-value (0.001–0.01), ***p-value (0.0001–0.001).

Table S1 | Summary table of TH1, TH2, TH17, and Treg selected genes. Indicated are the p-values, Median intensity values of all samples, the flag counts and the ProbesID. Fold-change values (columns C–E) did not pass the selection criteria (Anova p-value ≤ 0.05, Tukey P-value ≤ 0.05, fold-change ≥2 or ≤-2, and reliable detection of the signal (Flag counts) ≤ 1 in the group with higher expression) are in black color, while up-regulated ones are shown in red and down-regulated ones are shown in green.

Data Sheet s1 | CEMiTool output html file for all modules.
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**Pre-T<sub>H</sub>, Lodging of Neonatal T<sub>H</sub>, Cells**

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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