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Dendritic cell-restricted antigen presentation initiates the Follicular Helper T cell program but cannot complete ultimate effector differentiation

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Abstract

Follicular Helper T cells (T_{FH}) are critical for germinal center (GC) formation. The processes that drive their generation and effector potential remain unclear. Here, we define requirements for MHCII antigen presenting cells (APCs) in murine T_{FH} formation by either transiently ablating or restricting antigen presentation to dendritic cells (DCs). We find that cognate interactions with DCs are necessary and sufficient to prime $CD4^+$ T cells towards a $CXCR5^+ICOS^+Bcl6^+$ T_{FH} intermediate. However, in the absence of additional APCs, these T_{FH} fail to produce IL-21. Furthermore, in vitro priming of naïve T cells by B cells engenders optimal production of IL-21, which induces a GC B cell transcriptional profile. These results support a multi-step model for effector T_{FH} priming and GC initiation, in which DCs are necessary and sufficient to induce a T_{FH} intermediate that requires additional interactions with distinct APCs for full effector function.

INTRODUCTION

Germinal centers (GCs) are transient structures formed during thymus-dependent (TD) immune responses, where expanding B cells undergo affinity maturation, yielding memory B and plasma cells that mediate protective humoral immunity. The exact interactions dictating whether B and helper T cells assume GC status versus alternative fates remain unclear. A key event in GC formation is the Bcl-6 dependent differentiation of Follicular Helper T (T_{FH}) cells (1–2). T_{FH} cells express inducible co-stimulator (ICOS) and CXC chemokine receptor 5 (CXCR5), enabling their migration to the B cell follicle (3–5) and their continue differentiation into GC T_{FH} (6). Moreover, T_{FH} express costimulatory molecules and produce IL-21 that are essential for GC B cell survival and differentiation (7–8).

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The sources of MHCII^{-}restricted antigen presentation required for T_{FH} differentiation are controversial. Several lines of evidence suggest that B cells provide unique signal(s) required for complete T_{FH} differentiation. Thus the absence of B cells, the presence of B cells with irrelevant specificity, or an inability of B cells to express ICOSL impairs T_{FH} generation (2, 9–11). Moreover, T cells unable to stably conjugate with B cells display defective T_{FH} differentiation (12). The notion of a unique B cell signal has been challenged by findings that suggest instead that only prolonged, high avidity antigen presentation is needed for GC T_{FH} differentiation that can be mediated by DCs (13) . Indeed, co-stimulation via DCs promotes T cell migration into the B cell follicle (14), suggesting that DC antigen presentation contributes to the T_{FH} program.

We have dissected the steps of T_{FH} differentiation by examining the requirement for MHCII-expressing DCs. We find that cognate interactions with conventional DCs are both necessary and sufficient to prime CD4^{+} T cells towards a previously unappreciated T_{FH} intermediate that expresses Bcl6, CXCR5 and ICOS. However, DC priming alone is insufficient to generate PD1^{hi} GC T_{FH} and IL-21 production, indicating the need for subsequent, distinct APC interactions for commitment to the T_{FH} program. Furthermore, we show that naïve T cells primed in vitro by B cells express more IL-21 than do those primed by DCs. Moreover, addition of IL-21 to in vitro B cell activation cultures that mimic cognate T cell help induces a GC B transcription profile. Thus, we conclude that T_{FH} differentiation is a multi-step process in which conventional DCs are critical for the initial priming events; however, they are insufficient for imparting complete effector potential.

MATERIALS AND METHODS

Mice & Immunizations

C57BL/6J, OTII and CD11c-DTR mice were purchased from Jackson Laboratories. CD11c/A_{β}^{b} mice were bred in house (15). 6–14 week old mice were housed under pathogen free conditions, in accordance with the University of Pennsylvania Animal Care and Use Guidelines. CD4^{+} OTII T cells were enriched by negatively selecting out CD8^{+}, B220^{+}, MHCII^{+} and FcγRII^{+} cells using magnetic bead selection (Qiagen) and labeled with CFSE (Invitrogen). OTII were transferred i.v. 1 day prior to i.p. immunization with 50 μg NP_{15}-OVA (Biosearch Technologies) in alum. Some mice were given $OVA_{323–329}$ peptide (GenWay Biotech). DC depletion and *Toxoplasma* infection in CD11c DTR mice as described (16).

Flow cytometry

Antibodies were purchased from Biolegend, eBioscience, BD Pharmingen or Invitrogen. DAPI or AQUA™ (Invitrogen) was used to identify live cells. FoxP3 fixation and permeabilization kit was used for intracellular Bcl6 staining (eBioscience). Cells were acquired or sorted on an LSR II cytometer or FacsAria II, respectively (BD Biosciences). Data was analyzed using FlowJo software (TreeStar).

Immunohistochemistry

Spleens immersed in O.C.T. (Tissue Tek) were flash frozen using 2-methylbutane / liquid nitrogen. 8 μm sections were fixed with cold acetone and stained with PNA and antibodies to IgD and CD90.1. Sections were imaged with a Zeiss LSM510META NLO laser scanning confocal system.

In vitro stimulations

OTII cells were polarized towards T_{FH} lineage as published (9) with CD11c^{hi} or CD23^{+} cells at a ratio of 1 APC:10 T cells and harvested on day 4. B cell stimulations were

performed as described (17) with anti-CD40 (10 ng/ml; BD Biosciences) and IL-21 (100ng/ml; R&D).

QPCR

QPCR was conducted as previously described (17). 18S or GAPDH were used as housekeeping gene for T and B cells, respectively.

ELISPOT

Splenocytes were incubated on 10 ug/ml of NP33-BSA (Biosearch Technologies) coated plates and developed with biotin-anti-mouse IgM or -IgG1 (Southern Biotech) followed by ExtrAvidin-Alkaline Phosphatase and NBT/BCIP substrate (Sigma). Spots were enumerated on CTL-ImmunoSpot (Cellular Technologies).

RESULTS AND DISCUSSION

Dendritic Cells are necessary for TFollicular Helper formation

Using a loss of function approach, we determined whether DC-T cell interactions are necessary for initiation of T_{FH} cells priming in vivo. We transiently ablated DCs by Diptheria toxin (DT) in CD11c-DTR mice (18), and infected the mice with *Toxoplasma gondii* as described (16). T_{FH} cells were present in untreated mice at day 7 post-infection, but were significantly reduced in DT-treated CD11c-DTR animals (Fig. 1A). Normal DC numbers were required for optimal generation of $CD4^+CXCR5^+$ cells (WT 1.71 ± 0.15; CD11c-DTR 0.67 ± 0.13; $p<0.01$) as well as induction of ICOS (Fig 1B). To verify that these results were not limited to toxoplasmosis, we transferred 10^5 $CD90.1^+$ ovalbumin(OVA)-specific OTII CD4 T cells into $CD90.2^+$ WT or CD11c-DTR recipient mice followed by DT treatment and i.p. immunization with NP-OVA/alum. Again, there was a profound decrease in the number of OTII T_{FH} generated (data not shown). The few $CD4^+CXCR5^+$ T cells formed after DC depletion could potentially migrate into the follicle and initiate GC via other co-stimulatory molecules, including CD40L. We conclude that DCs are necessary to generate T_{FH} cells, as expected from their role in priming other T_H lineages (19).

Dendritic cells are sufficient for the generation of a $Bcl6^+$ T_{FH} intermediate

Using a gain of function approach, we asked if cognate DC interactions are sufficient to generate T_{FH} cells. We used the transgenic CD11c/A_β^b mouse model, in which MHCII antigen presentation is limited to conventional $CD11c^{hi}$ DCs (15). As CD11c/A_β^b mice lack endogenous CD4 T cells, we transferred CFSE labeled 10^6 OTII cells. Splenic OTII cells proliferated similarly in both WT and CD11c recipients following i.p. immunization with NP-OVA/alum (Fig. 2A, 2B). Further, the numbers of OTII derived $CD62L^{lo}CXCR5^+$ T_{FH} generated in WT (WT-T_{FH}) or CD11c/A_β^b mice (CD11c-T_{FH}) were not significantly different, and both subsets displayed equivalent expression of ICOS (Fig 2B, 2C). Moreover, transfer of 10^5 OTII cells yielded similar results (Supp. Fig. 1A). Furthermore, OTII cells migrated into the follicle in both mice strains (Fig. 2E). Since Bcl6 is necessary and sufficient for differentiation of $CXCR5^+$ T_{FH} cells (1–2), we asked if antigen presentation restricted to DCs effectively drove Bcl6 induction. Compared to naïve OTII T cells, WT-T_{FH} or CD11c-T_{FH} expressed higher but equivalent levels of Bcl6 message (Fig. 2D) and protein (Fig. 2C). Thus, cognate interactions with DCs are sufficient to skew naïve $CD4^+$ T cells towards $Bcl6^+$ T_{FH}.

Cognate DC-T interactions cannot induce GC status in T cells

MHCII expression by B cells is necessary for antibody class switching (15, 20) and presumably for GC formation. Consistent with this view, GC B cells were absent both phenotypically (Supp. Fig. 2A, 2B) and histologically GCs (Fig. 2E) in immunized CD11c/A_β^b mice. Further, CD11c/A_β^b mice generated few NP-specific IgG1 ASCs, despite an intact IgM ASC response (Supp. Fig 2C), as reported before (15, 20). PD1 expression is thought be an indicator of chronic antigen exposure in the GC, as correlative evidence links the presence of GC B cells with PD1 expression on T_{FH} cells (4). Thus, we examined the expression of PD1 on T_{FH} cells. Amongst the $CXCR5^+$ cells, a small subset of the WT-T_{FH} cells expressed high levels of PD1 as well as GL7, which were absent within the CD11c-T_{FH} cells (Fig. 3A, B) consistent with GC T_{FH} phenotype (Fig. 3C, (6)). Moreover, the transfer of 10^7 polyclonal WT $CD4^+$ T cells did not alter the observed phenotype (data not shown), indicating that the absence of endogenous helper T cells does not impact the evolution of this response in the CD11c/A_β^b mice. Since T_{FH} down regulate P-selectin glycoprotein ligand 1 (PSGL1) (11) and upregulate and B- and T-lymphocyte attenuator (BTLA) (6), thus we examined their expression on CD11c-T_{FH} and WT-T_{FH}. Compared to naïve WT T cells, both CD11c-T_{FH} and WT-T_{FH} had significantly upregulated BTLA and down regulated PSGL1 (data now shown). However, CD11c-T_{FH} expressed more PSGL1 than WT-T_{FH} (data not shown) (11) while BTLA expression was comparable. Collectively, we conclude that not only do GC B cells require T_{FH} cells for their induction, GC T_{FH} also rely on interactions with other APCs, including activated B cells, to complete their phenotype.

IL-21 expression is profoundly reduced in T_{FH} cells primed by DCs alone

IL-21 is a key T_{FH} effector cytokine that mediates differentiation of GC B cells (6–8). Strikingly, CD11c-T_{FH} primed solely by DCs expressed minimal IL-21 compared to WT-T_{FH} (Fig. 4A). To determine if B cells were intrinsically more adept at inducing IL-21 producing T_{FH}, we polarized naïve OTII T cells in vitro towards the T_{FH} lineage with splenic $CD11c^{hi}$ DCs or $CD23^+$ B cells as APCs as described (9). While both B cells and DCs induced expression of Bcl6, B cells induced greater IL-21 transcript in polarized T_{FH} cells (Fig. 4B). Together, these data suggest that B cells provide qualitatively different signal(s) than DCs, perhaps via expression of co-stimulatory molecules that facilitate optimal IL-21 production by T_{FH} cells.

Consistent with a previous study (9), in vitro primed T_{FH} expressed little to no IL-17 transcript (Supp. Fig. 1E), further confirming their T_{FH} character. Moreover, the superior ability of DCs to induce IL-17 when compared to B cells was recapitulated in vivo, as OTII T_{FH} and OTII T_{eff} primed solely via conventional DCs in CD11c/A_β^b mice expressed more IL-17 compared to their WT counterparts (Supp. Fig. 1F). Together, these data highlight the critical role played by non-DC APCs, presumably B cells, in enforcing progression to T_{FH} status while inhibiting other lineage potential.

Sustained antigen presentation on conventional DCs is insufficient to rescue GC T_{FH} or production of IL-21

Recent findings suggest that sustained antigen presentation restores the formation of $PD1^{hi}CXCR5^+$ T_{FH} in the absence of B cells (13). We performed a similar experiment in the CD11c/A_β^b mice. We transferred 10^5 OTII cells, immunized with NP-OVA the next day and then administered 10 μg of $OVA_{323–339}$ peptide i.v. on day 3 p.i.. Consistent with Deenick et al. (2010), we observed greater expansion of OTII cells in the CD11c/A_β^b mice given exogenous peptide (Supplemental Fig. 1A) on day 7 p.i.. However, neither $PD1^{hi}$ GC T_{FH} nor IL-21 production were observed in CD11c/A_β^b mice that received extra peptide (Supp. Fig. 1B–D). Whether these differences arise because of irradiation chimeras used by

Deenick et. al. (2010) or due to the lack of other MHCII$^+$ APCs in the CD11c/A$_\beta^b$ mice is unclear. Thus, sustained antigen presentation by conventional DCs is insufficient to induce GC T_{FH} cells and a second, distinct, antigen presenting cell population is required.

In conjunction with BCR and CD40 ligation, IL-21 induces a GC B transcription profile

Recent studies suggest that IL-21R ligation yields maximal Bcl6 expression in B cells, thus fostering GC formation in vivo (7–8). However, in the presence of anti-CD40 in vitro, IL-21 induces B Lymphocyte-induced maturation protein 1 (Blimp1) (21) skewing B cells towards a plasma cell fate. To simulate antigen-driven, T-dependent activation of Follicular B cells in vitro, we ligated both the BCR and CD40 with anti-IgM and anti-CD40 antibodies respectively, then assayed for the induction of Blimp1 and Bcl6 transcripts. Compared to B cells stimulated with only anti-IgM or anti-IgM plus anti-CD40, IL-21 markedly augmented the transcriptional profile associated with adoption the GC fate. Both AID (data not shown) and Bcl6 were substantially upregulated, whereas Blimp1 transcripts, which were elevated following BCR and CD40 ligation alone, were lowered in the presence of IL-21 (Fig 4C). We conclude that concurrent stimulation of BCR, CD40 and IL-21R potently induces Bcl6 expression, which in turn represses Blimp1 (22). This reciprocal interaction is key to skew the B cells towards a GC fate and away from the short-lived plasma cell fate, which can also inhibit T_{FH} differentiation (23).

Collectively, our data suggest that complete differentiation of T_{FH} is most effectively accomplished via successive, cognate interactions with distinct APCs. Herein, we demonstrate that DCs are necessary and sufficient to initiate the T_{FH} program, arguing against the notion that B cells are critical for priming (2, 9–11). Priming solely via DCs in the CD11c/A$_\beta^b$ system leads to accumulation and persistence of a novel intermediate or pre-$T_{FH,}$ which express CXCR5, ICOS and Bcl6, but lacks IL-21. Thus, optimal induction of IL-21 as well as further differentiation towards GC T_{FH} requires cognate interactions with other APCs, including activated B cells. Thus, we propose that as poised CXCR5$^+$ICOS$^+$$T_{FH}$ relocate from the DC-rich T cell zone to B cell-rich follicles, their interactions with activated B cells via costimulatory ligands including PDL2, ICOSL increases the likelihood of adopting a T_{FH} cytokine profile, including IL-21 production (24–25). Whether B cells provide qualitatively distinct signal(s) to T_{FH} for optimal effector cytokine production remains unclear.

Since CD4$^+$ T cells require continued interactions with APCs for their expansion and effector functions (26), the assumption of alternate effector fates likely reflects the receipt of chronologically ordered signals during the early primary response. This suggests a step-wise model for GC T_{FH} formation. T cells primed by DCs differentiate into a novel pre-T_{FH} subset. These cells can then migrate towards the T-B border, where cognate interactions with antigen primed B cells induce optimal IL-21 production, thus acquiring effector potential characteristic of T_{FH} subset while inhibiting other fates, such as Th17. IL-21, in turn, reinforces a GC transcriptional program in the activated B cells. Thus, fully-differentiated T_{FH} are only generated when a humoral response is appropriate and antigen-specific T cell help is very specifically directed towards antigen-experienced B cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in the paper

DC	Dendritic Cells
PSGL1	P selectin glycoprotein ligand 1
BTLA	B- and T-lymphocyte attenuator
PD1	Programmed Death Ligand 1

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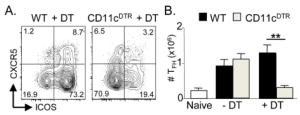

Figure 1.

DCs are necessary for initiating T_{FH} priming. WT and $CD11c^{DTR}$ mice were treated with DT and infected with *T. gondii* as described (16). (A) Numbers of T_{FH} ($CD4^+CXCR5^+ICOS^+$) were calculated at day 7 p.i. Data are pooled from two experiments (n>8 per group) (B) Representative FACS plots of CXCR5 and ICOS expression on $CD4^+$ T cells in WT or $CD11c^{DTR}$ treated with DT. ** denotes statistical significance of $p<0.01$ in a 2-tailed t test at $\alpha=0.05$

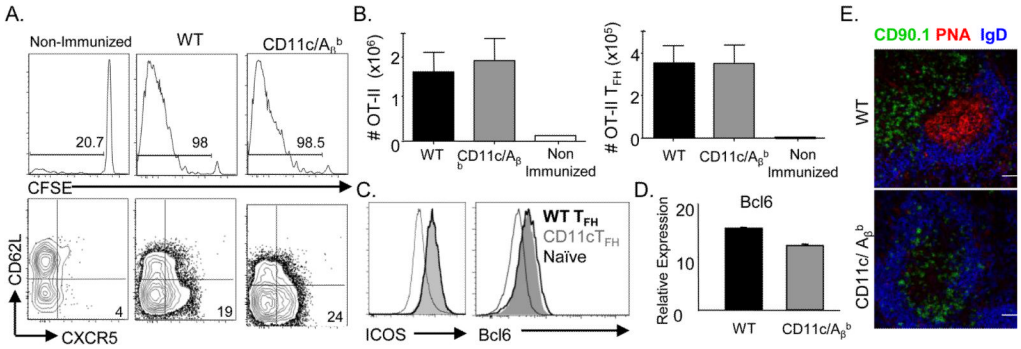

Figure 2.

Cognate interactions by dendritic cells are sufficient for generation of T_{FH} cells. 10^6 CFSE labeled CD90.1$^+$ OTII T cells were transferred into either WT or CD11c/A_β^bmice and mice were immunized with NP-OVA/alum. (A) Representative FACS gating strategy for identification of splenic OTII T cells (CD19$^-$ TCRβ$^+$ CD4$^+$ CD90.1$^+$). OTII T_{FH} cells were identified as CD62L$^-$ CXCR5$^+$ cells. CFSE dilution of transferred OT-II cells was assessed (middle panel). (B) Total number of splenic OTII and OTII T_{FH} present in immunized WT or CD11c/A_β^b recipients at day 8 p.i. or in non-immunized WT mice at d8 post-transfer (n=4–5 mice, representative of >5 experiments). (C) Representative histogram overlay of ICOS and Bcl6 staining intensity on OTII T_{FH} generated in WT or CD11c/A_β^b mice and naïve endogenous CD4$^+$ T cells (CD19$^-$ TCRβ$^+$ CD4$^+$CD62L$^+$) present in WT mice (D) Bcl-6 mRNA in FACS sorted OT-II T_{FH} generated in WT or CD11c/A_β mice at d7 p.i.. relative to naive OTII cells (representative of 3 experiments) (E) Confocal micrographs of splenic sections to identify CD90.1$^+$ OTII along with PNA$^+$ GCs and IgD$^+$ naïve B cells in the follicle. Scale bar denotes 50 μm.

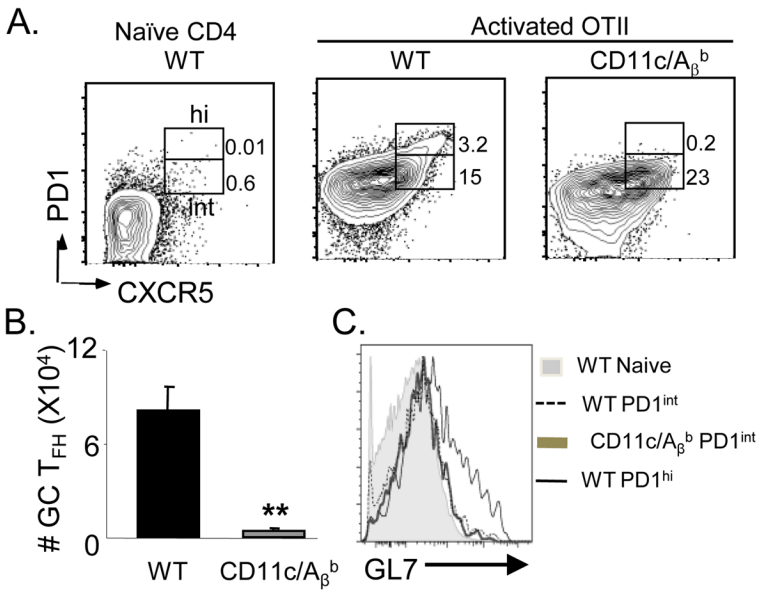

Figure 3.

DC restricted antigen presentation is insufficient for the generation of GC T_{FH} 10^6 OTII T cells were transferred into WT and CD11c/A_β^b mice and mice were immunized with NP-OVA/alum. (A) Representative FACS gating strategy to identify $PD1^+CXCR5^+$ within activated OTII T_{FH} ($CD19^-TCR\beta^+CD4^+CD90.1^+CD62L^-$) in immunized WT or CD11c/A_β^b on d7 p.i. as well as naïve WT $CD4^+$ T cells as a staining control. The $PD1^+$ cells were parsed into $PD1^{hi}$ (GC T_{FH}) and $PD1^{int}$ populations. (B) The total number of OTII GC T_{FH} in WT or CD11c/A_β^b mice on d7 p.i. (n=4–5) (C) Histogram overlay of GL7 staining on various subsets gated in (A) (n=4–5 mice). Results are representative of 3 experiments. ** denotes statistical significance of $p<0.01$ in a 2-tailed t test at $\alpha=0.05$.

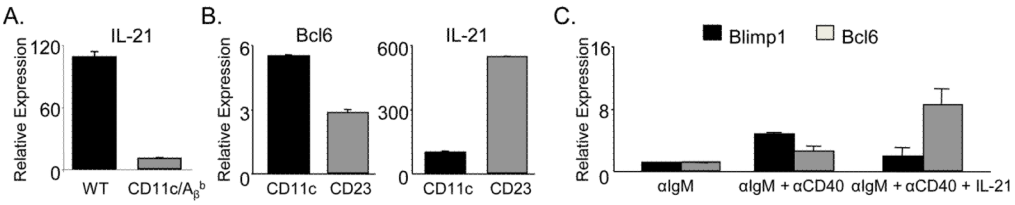

Figure 4.

Reduced expression of IL-21 in T_{FH} cells primed in CD11c/A_β^b mice. (A) Expression of IL-21 transcript in FACS sorted OT-II T_{FH} generated in WT or CD11c/A_β^b mice at d7 p.i. normalized to naïve OTII cells. Results are representative of 3 experiments. (B) Relative expression of Bcl6 and IL-21 in OTII cells polarized towards T_{FH} lineage using splenic $CD11c^+$ or $CD23^+$ cells as APCs compared to Th0 condition. Results are representative of 2 experiments. (C) QPCR analysis of Bcl6 and Blimp1 on $CD23^+$ cells stimulated with $F(ab)_2$ fragments of αIgM alone or in combination with αCD40 with or without IL-21 for 3 days. Results pooled from 3 experiments.