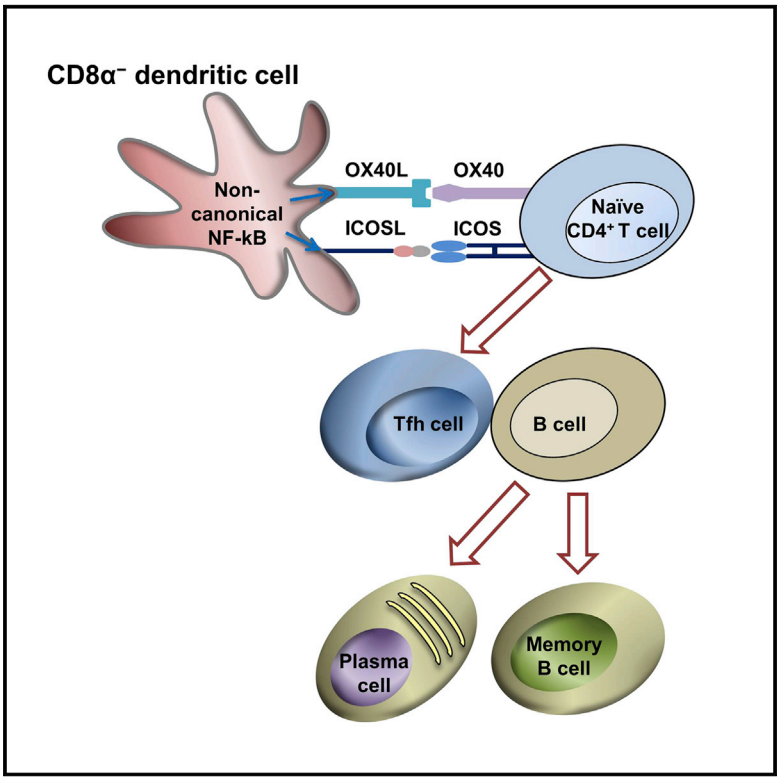
Article

Cell Reports

CD8α⁻ Dendritic Cells Induce Antigen-Specific T Follicular Helper Cells Generating Efficient Humoral Immune Responses

Graphical Abstract



Highlights

- The CD8α⁻ DC subset efficiently induces functional antigen-specific Tfh cells
- A small amount of antigen delivered to CD8α⁻ DCs is sufficient to induce Tfh cells
- ICOSL and OX40L on CD8α⁻ DCs have a critical role in Tfh cell differentiation
- Efficient humoral immune responses are generated by CD8α⁻ DC-induced Tfh cells

Authors

Changsik Shin, Jae-A Han, Hyein Koh, ..., Eui-Cheol Shin, Seongho Ryu, Yoonkyung Do

Correspondence

ryu@sch.ac.kr

In Brief

Shin et al. demonstrate that the CD8α⁻ DC subset efficiently induces functional antigen-specific Tfh cells to generate both high-quality and high-quantity humoral immune responses. Furthermore, they describe a critical role of non-canonical NF-κB-induced ICOSL and OX40L on CD8α⁻ DCs in Tfh cell differentiation.

Shin et al., 2015, Cell Reports *11*, 1929–1940
June 30, 2015 ©2015 The Authors
http://dx.doi.org/10.1016/j.celrep.2015.05.042

Cell Reports
Article

CD8α⁻ Dendritic Cells Induce Antigen-Specific T Follicular Helper Cells Generating Efficient Humoral Immune Responses

Changsik Shin,[1] Jae-A Han,[1] Hyein Koh,[2] Bongseo Choi,[1] Yongbin Cho,[1] Hyeongmin Jeong,[1] Jea-Sun Ra,[1] Pil Soo Sung,[3] Eui-Cheol Shin,[3] Seongho Ryu,[4,5,*] and Yoonkyung Do[1,5]

[1]School of Life Sciences, Ulsan National Institute of Science and Technology, UNIST-gil 50, Ulsan 689-798, Republic of Korea
[2]Laboratory of Cellular Physiology and Immunology and Chris Browne Center, The Rockefeller University, New York, NY 10065, USA
[3]Laboratory of Immunology and Infectious Diseases, Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-338, Republic of Korea
[4]Soonchunhyang Institute of Medi-bio Science (SIMS), Soonchunhyang University, Chonan-Si, Chungcheongnam-do 336-745, Republic of Korea
[5]Co-senior author
*Correspondence: ryu@sch.ac.kr
http://dx.doi.org/10.1016/j.celrep.2015.05.042

SUMMARY

Recent studies on T follicular helper (Tfh) cells have significantly advanced our understanding of T cell-dependent B cell responses. However, little is known about the early stage of Tfh cell commitment by dendritic cells (DCs), particularly by the conventional CD8α⁺ and CD8α⁻ DC subsets. We show that CD8α⁻ DCs localized at the interfollicular zone play a pivotal role in the induction of antigen-specific Tfh cells by upregulating the expression of *Icosl* and *Ox40l* through the non-canonical NF-κB signaling pathway. Tfh cells induced by CD8α⁻ DCs function as true B cell helpers, resulting in significantly increased humoral immune responses against various human pathogenic antigens, including *Yersinia pestis* LcrV, HIV Gag, and hepatitis B surface antigen. Our findings uncover a mechanistic role of CD8α⁻ DCs in the initiation of Tfh cell differentiation and thereby provide a rationale for investigating CD8α⁻ DCs in enhancing antigen-specific humoral immune responses for improving vaccines and therapeutics.

INTRODUCTION

T follicular helper (Tfh) cells have been identified as a CD4⁺ T helper cell subset that functions as a true B cell helper (Crotty, 2011; Ma et al., 2012). Tfh cells highly express a B cell homing chemokine receptor, CXCR5, which facilitates their migration to germinal centers (GCs) in a CXCL13-dependent manner. There, they help cognate B cells to become antibody-secreting cells (ASCs) or memory B cells, leading to efficient humoral immunity with long-term memory (Goodnow et al., 2010; Tangye and Tarlinton, 2009). Since the term Tfh cells was first introduced (Breitfeld et al., 2000; Schaerli et al., 2000), various research has provided valuable data on distinct features of Tfh cells, such as high expression of CXCR5, ICOS, PD1, and SAP, key cytokines such as IL-21 and recently IL-4 (Reinhardt et al., 2009), and the major transcription factors Bcl6 and c-Maf, to be distinguished from other CD4⁺ T helper cell lineages (Crotty, 2011; Ma et al., 2012). Given the fact that efficient Tfh cell-dependent long-term humoral immunity is an essential defensive arm of the vertebrate immune system, it is not surprising that aberrant Tfh cell development or activity has been known to be closely associated with various human diseases such as autoimmunity, humoral immunodeficiency, and T cell lymphomas (Tangye et al., 2013). Thus, an understanding of the initiation and the regulation of these specialized CD4⁺ T helper cells is critical to improve vaccine efficacy as well as to design novel therapies.

The role of cognate B cells regarding the differentiation of Tfh cells has been initially validated by several studies. Deficiency of not only B cells but also various B cell functional molecules, CD19, CD40, major histocompatibility complex (MHC) class II, and ICOSL, results in the decreased number of Tfh cells (Akiba et al., 2005; Deenick et al., 2010; Haynes et al., 2007; Johnston et al., 2009). In addition, the interaction between activated CD4⁺ T cells and cognate B cells via SAP, a cytoplasmic adaptor protein of the SLAM family, was shown to be critical in Tfh cell generation (Cannons et al., 2010). In contrast, another study has shown that continuous antigen presentation was sufficient to induce Tfh cell differentiation in B cell-deficient mice (Deenick et al., 2010), suggesting that other antigen-presenting cells (APCs) are essential in Tfh cell differentiation. Moreover, a number of recent independent studies demonstrated that Tfh cells could arise soon after priming by dendritic cells (DCs) (Baumjohann et al., 2011; Choi et al., 2011; Goenka et al., 2011; Vinuesa and Cyster, 2011), and thus, it seems that cognate B cells are generally required for maintaining functional Tfh cells, while DCs are necessary to prime Tfh cells, especially at the early stage of Bcl6 upregulation.

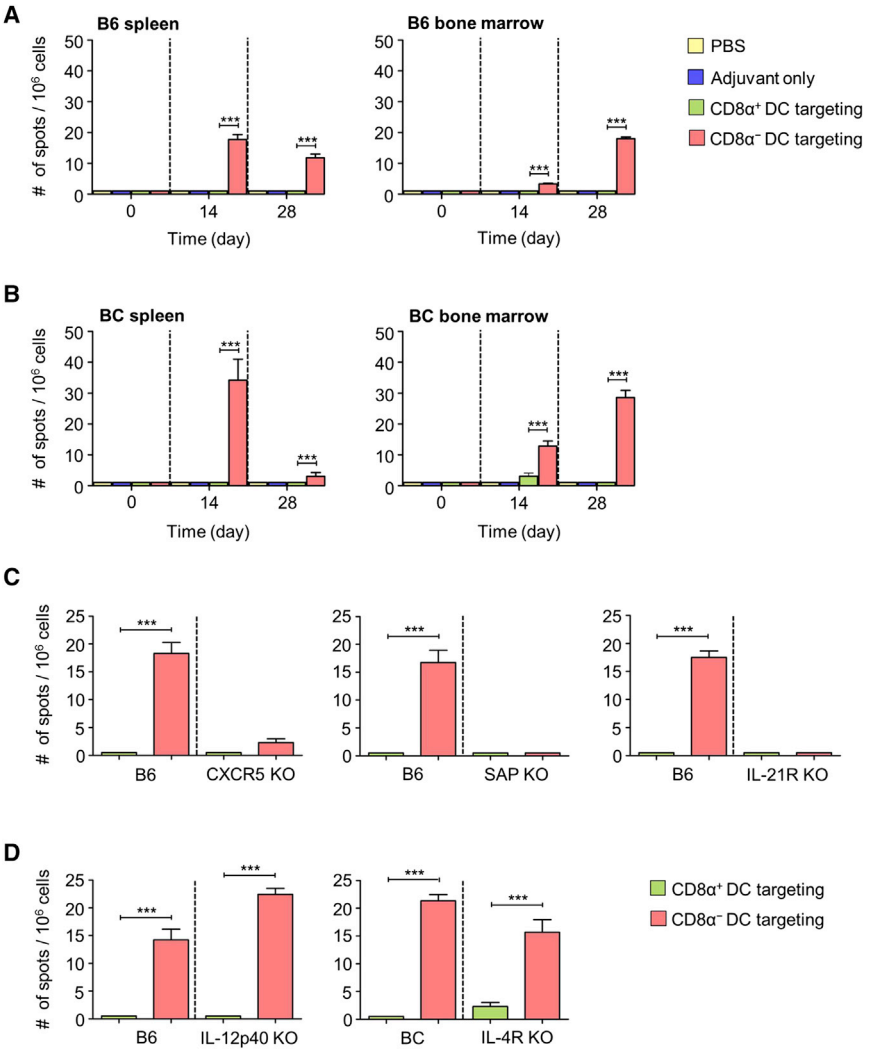


Figure 1. CD8α⁻ DCs induce Tfh Cell-Dependent Antibody-Secreting Cells

(A and B) Naive C57BL6 (B6) (A) or BALB/c (BC) (B) mice were immunized intraperitoneally (i.p.) with PBS, αCD40 monoclonal antibody (mAb) + poly(I:C) (adjuvant only), αDEC:V (CD8α⁺ DC targeting), or αDCIR2:V (CD8α⁻ DC targeting) conjugated mAbs in the presence of αCD40 mAb and poly(I:C). At each indicated time point, spleen or bone marrow cells were prepared and anti-V IgG antibody-secreting cells (ASCs) were analyzed.

(C and D) CXCR5-KO, SAP-KO, IL-21R-KO (C), IL-12p40-KO, IL-4R-KO (D), or naive B6 or BC mice were immunized i.p. with either αDEC:V (CD8α⁺ DC targeting) or αDCIR2:V (CD8α⁻ DC targeting) conjugated mAbs in the presence of αCD40 mAb and poly(I:C). Two weeks after the immunization, spleen cells were prepared and anti-V IgG ASCs were analyzed.

***$p < 0.001$. All data represent mean ± SD of three or more independent experiments (A–D); n = 3 per group.

Although some key molecules of DCs have been reported to be involved in the induction of Tfh cells (Choi et al., 2011; Cucak et al., 2009; Fillatreau and Gray, 2003), how DCs mechanistically drive naive CD4⁺ T cells into Tfh cells differently from various CD4⁺ effector T cell subsets still remains unclear. Particularly, the roles of two distinct splenic CD8α⁺ and CD8α⁻ DC subsets in inducing Tfh cells are largely unexplored. Among various DC subsets characterized (Shortman and Liu, 2002), two major myeloid DC subsets in the spleen are well defined based on the expression of CD8α and its anatomical location. CD8α⁺ DCs express the endocytic receptor, DEC-205, and are located in the T cell zone within the lymphoid organs. They are specialized in uptaking dying cells (Iyoda et al., 2002) and cross-presentation of non-replicating antigens, leading to cytotoxic T cell priming (den Haan et al., 2000). In contrast, CD8α⁻ DCs, known to specialize in MHC class II presentation (Dudziak et al., 2007), express a distinct uptake receptor, DCIR2, and they are enriched in the bridging regions of the marginal zone (Iyoda et al., 2002). A DC targeting strategy by harnessing monoclonal antibodies (mAbs) (Nussenzweig et al., 1982; Swiggard et al., 1995) against various endocytic receptors expressed on DC subsets enriched our understanding of the roles of these DC subsets in vivo. Moreover, conjugating such targeting antibodies with a wide range of pathogenic antigens has been validated in various disease models to improve vaccine efficacy and develop novel therapeutic approaches (Do et al., 2010; Trumpfheller et al., 2012).

In this study, we utilized both DC-subset targeting and DC-subset sorting strategies to study how these two distinct DC subsets differently influence Tfh cell differentiation by unveiling their underlying mechanisms and signaling pathways. Our data demonstrate that functional Tfh cells are efficiently induced by the CD8α⁻ DC subset in vivo via the enhanced expression of ICOSL and OX40L through the non-canonical NF-κB pathway, leading to efficient humoral immunity against various human pathogenic antigens.

RESULTS

CD8α⁻ DCs Enhance the Generation of Tfh Cell-Dependent ASCs

In order to examine whether the two DC subsets have different roles in the generation of Tfh cell-dependent antigen-specific ASCs in vivo and in situ, we utilized distinct DC subsets targeting mAbs conjugated with LcrV (V) protein from *Yersinia pestis*: anti-DEC-205:V- (for CD8α⁺ DCs) and anti-DCIR2:V-conjugated (for CD8α⁻ DCs) antibodies. Only CD8α⁻ DC targeting induced significant numbers of anti-V ASCs in the spleen and the bone marrow, while no ASCs were observed following CD8α⁺ DC targeting (Figure 1A). The total number of ASCs in the spleen was decreased after 28 days while those in the bone marrow

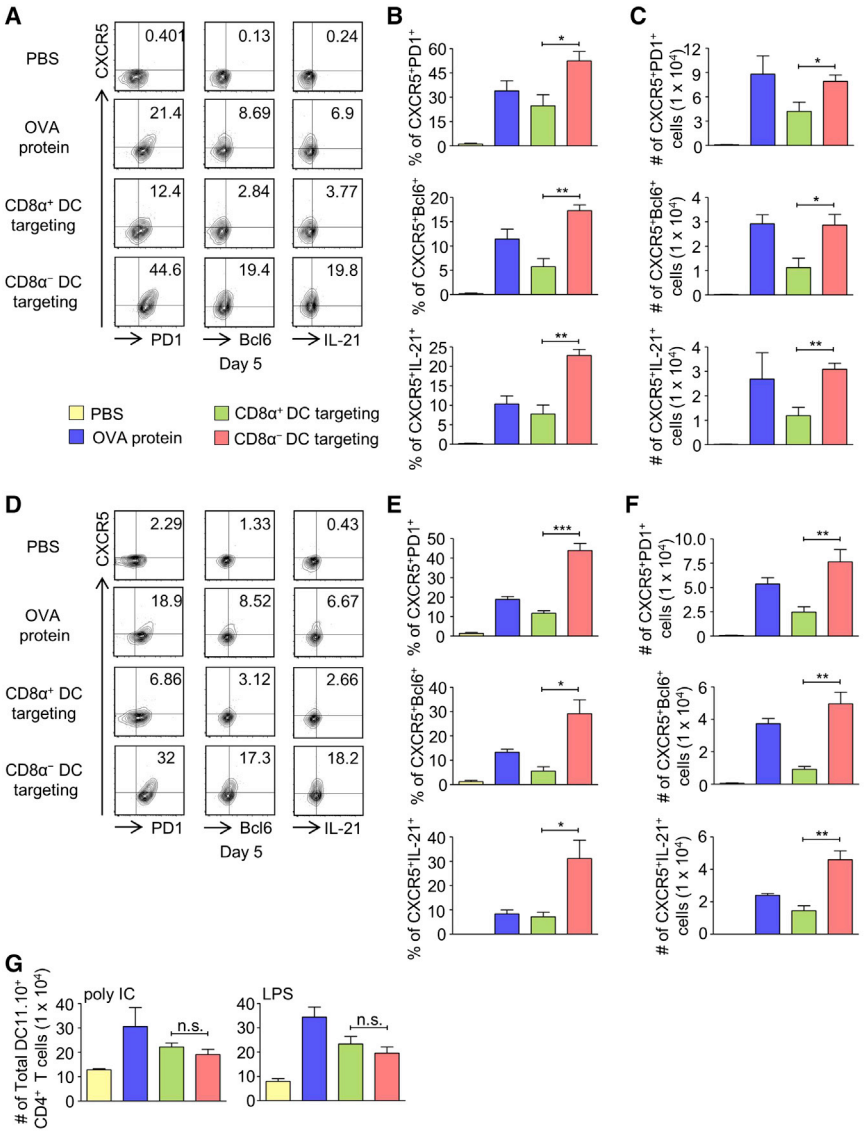


Figure 2. CD8α⁻ DCs Efficiently Induce Tfh Cells In Vivo

(A–G) OVA-specific DO11.10$^+$Thy1.1$^+$CD4$^+$ T cells were adoptively transferred to naive Thy1.2$^+$ BALB/c mice at day −1 and immunized subcutaneously (s.c.) with PBS, soluble OVA protein (OVA protein), αDEC:OVA (CD8α$^+$ DC targeting), or αDCIR2:OVA (CD8α$^-$ DC targeting) conjugated mAbs in the presence of poly(I:C) (A–C) or LPS (D–F) at day 0. At each indicated time point after immunization, lymph node cells were prepared and Tfh cells gated from the DO11.10$^+$CD4$^+$CD44$^+$ T cells were analyzed.

(A and D) Representative flow cytometry plots of CXCR5$^+$PD1$^+$, CXCR5$^+$Bcl6$^+$, or CXCR5$^+$IL-21$^+$ Tfh cells.

(B and E) Percentages (%) of Tfh cells described in (A) and (D), respectively.

(C and F) The number (#) of Tfh cells described in (A) and (D), respectively.

(G) The total number of DO11.10$^+$CD4$^+$ T cells. Data represent mean ± SEM of three or more independent experiments (B, C, and E–G).

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 3-4 per group. See also Figures S1 and S2.

CD8α⁻ DCs Are Superior in Inducing Antigen-Specific Tfh Cells In Vivo

To investigate roles of the two DC subsets in antigen-specific Tfh cell induction in vivo, we utilized DC subset targeting strategy via ovalbumin (OVA)-conjugated antibodies and OVA transgenic mice. Five days after immunization, higher percentages and numbers of CXCR5$^+$PD1$^+$, CXCR5$^+$Bcl6$^+$, and CXCR5$^+$IL-21$^+$ Tfh cells were observed in the lymph nodes and spleen in the CD8α$^-$ DC targeted group compared with those in the CD8α$^+$ DC targeted group (Figures 2A–2C and S1B–S1D).

increased, confirming the previously known fact that long-lived ASCs reside in the bone marrow (Slifka et al., 1995). Similar observations were made in a different mouse strain (Figure 1B), suggesting that the increased total number of ASCs by CD8α$^-$ DC targeting is not limited to a particular MHC haplotype.

Then, we compared the generation of anti-V ASCs in various Tfh cell-related knockout (KO) mice. The data showed that anti-V ASCs induced by targeting CD8α$^-$ DCs were dramatically decreased in CXCR5-KO (Breitfeld et al., 2000), SAP-KO (Qi et al., 2008), and IL-21 receptor (R)-KO (Nurieva et al., 2008) mice (Figure 1C), but this decrease was not observed in IL-12p40-KO (Ma et al., 2009; Mosmann and Coffman, 1989) or IL-4R-KO (Kaplan et al., 1996) mice (Figure 1D). Taken together, these data indicated that CD8α$^-$ DCs enhance antigen-specific ASCs in a Tfh cell-dependent manner rather than a Th1 cell- or Th2 cell-dependent manner.

Next, to confirm the capacity of CD8α$^-$ DCs in inducing Tfh cells in vivo under different maturation cues, similar experiments were performed in the presence of LPS. We again observed that higher percentages and numbers of CXCR5$^+$PD1$^+$, CXCR5$^+$Bcl6$^+$, and CXCR5$^+$IL-21$^+$ Tfh cells were induced by CD8α$^-$ DCs in the lymph nodes and spleen compared with those induced by CD8α$^+$ DCs (Figures 2D–2F and S2B–S2D). There was no significant difference in the total number of DO11.10$^+$CD4$^+$ T cells induced by both DC subsets in the lymph nodes (Figure 2G). In the spleen, the total number of DO11.10$^+$CD4$^+$ T cells was higher in the CD8α$^-$ DC targeted group (Figures S1E and S2E). When the numbers of Tfh cells were normalized by the total number of DO11.10$^+$CD4$^+$ T cells, we were still able to observe greater number of Tfh cells in the CD8α$^-$ DC targeted group (Figures S1F and S2F). As previously reported (Do et al., 2010), CD8α$^+$ DCs were superior in inducing interferon-γ (IFN-γ)-secreting CD4$^+$ T cells regardless of total number of

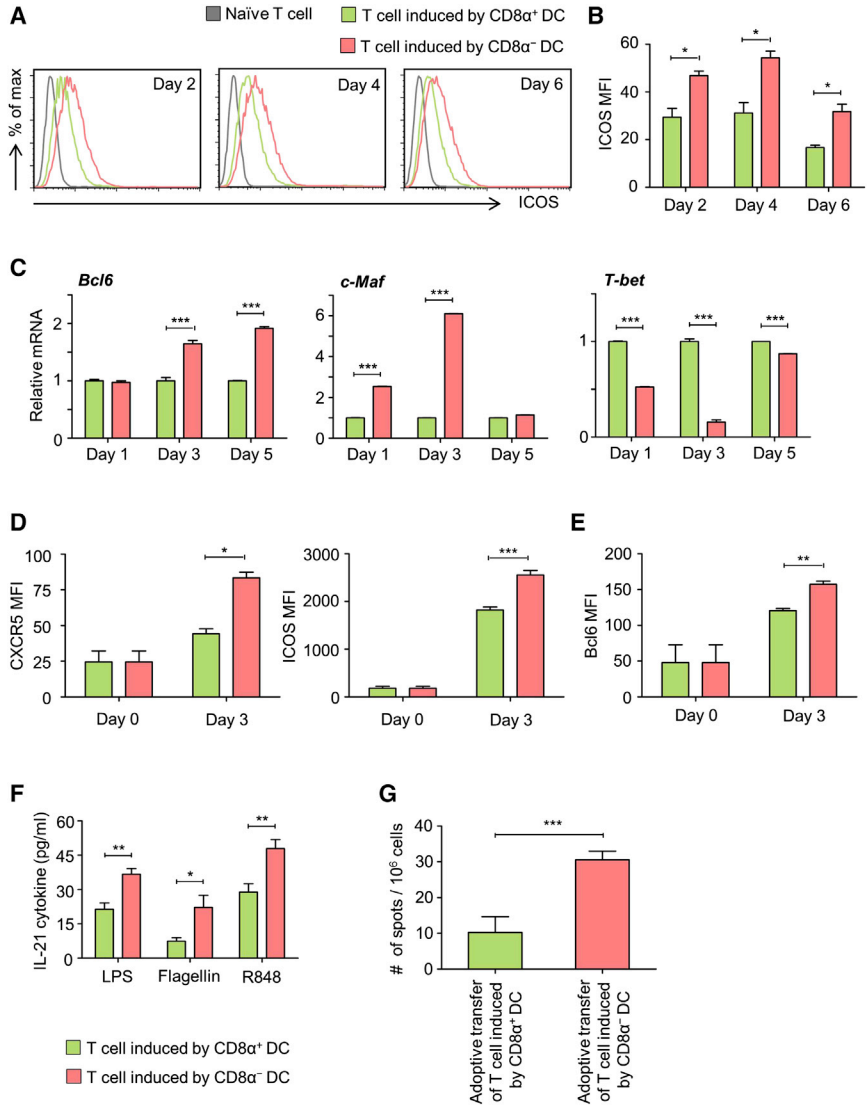


Figure 3. CD8α⁻ DCs Induce Tfh Cells In Vitro

(A–G) OT-II OVA specific Vα2⁺CD4⁺ T cells were co-cultured with each DC subset in the presence of OVA peptide (323–339) with poly(I:C) (A–C and G), LPS (D and E), or LPS, Flagellin, or R848 (F). At each indicated time point after the co-culture, Vα2⁺CD4⁺CD44⁺ T cells were analyzed.

(A) Representative histograms of ICOS expression.

(B) Median fluorescence intensity (MFI) of three independent experiments described in (A); mean ± SEM.

(C) The relative mRNA expression of *Bcl6*, *c-Maf*, or *T-bet*.

(D and E) MFI of CXCR5, ICOS (D), and Bcl6 (E).

(F) IL-21 production.

(G) Three days after co-culture, Vα2⁺CD4⁺CD44⁺ T cells were sorted and adoptively transferred together with naive CD19⁺ B cells into RAG-1-deficient mice. Fourteen days after the boost immunization with OVA protein, spleen cells were prepared and anti-IgG ASCs were analyzed (n = 2 per group).

*p < 0.05, **p < 0.01, ***p < 0.001. Data represent mean ± SEM of three or more (C–F) or two (G) independent experiments. See also Figure S3.

DO11.10⁺CD4⁺ T cells (Figure S2G), whereas CD8α⁻ DCs induced interleukin-4 (IL-4)-secreting CD4⁺ T cells (Figure S2H). Taken together, our data clearly showed that CD8α⁻ DCs specialize in priming antigen-specific Tfh cells in vivo.

CD8α⁻ DCs Specialize in Inducing Tfh Cells In Vitro

To validate that our observation of Tfh cells generated in vivo is due to intrinsic differences between CD8α⁺ DCs and CD8α⁻ DCs and not to DC subset receptor-specific responses via the DC targeting strategy, the two DC subsets were sorted and the induction of antigen-specific Tfh cells was compared in vitro. At each indicated time point after the co-culture of OVA-specific naive CD4⁺ T cells with either CD8α⁺ or CD8α⁻ DCs, the CD44⁺CD4⁺ T cells were sorted and then analyzed for Tfh cell-related surface molecules, transcription factors, and cytokines. Notably, we observed a higher expression of ICOS on the CD4⁺ T cells induced by CD8α⁻ DCs as early as day 2 followed by elevated Bcl6 expression on day 3 (Figures 3A–3C and S3A), which supports the previously suggested hypothesis on a molecular hierarchy from ICOS to Bcl6 (Choi et al., 2011). The expression of SAP and c-Maf (Bauquet et al., 2009) was also highly increased in the CD4⁺ T cells induced by CD8α⁻ DCs, whereas T-bet, a transcription factor of Th1 cells, was highly detected in those induced by CD8α⁺ DCs (Figures 3C and S3A). Such phenomena were again not restricted to a given DC-maturation cue, poly(I:C). We observed that the CD4⁺ T cells induced by CD8α⁻ DCs in the presence of lipopolysaccharide (LPS) expressed higher levels of CXCR5 and ICOS (Figure 3D), Bcl6 (Figure 3E), and highly secreted IL-21 (Figure 3F). In addition, under Toll-like receptor 5 (TLR5) (flagellin) and TLR7/8 (R848) stimuli, the CD4⁺ T cells induced by CD8α⁻ DCs secreted higher IL-21 compared with those induced by CD8α⁺ DCs (Figure 3F). There was no significant difference in CD4⁺ T cell proliferation induced by the two DC subsets in vitro (Figure S3B).

In order to confirm the function of in-vitro-induced Tfh cells in B cell help, we adoptively transferred the in vitro DC subset-primed CD4⁺ T cells together with naive CD19⁺ B cells into RAG-1-deficient mice. After prime and boost immunization with soluble OVA protein, we observed significantly increased anti-OVA ASCs in the group that received the Tfh cells induced by CD8α⁻ DCs (Figure 3G). Together, these data clearly demonstrate that CD8α⁻ DCs specialize in priming antigen-specific Tfh cells.

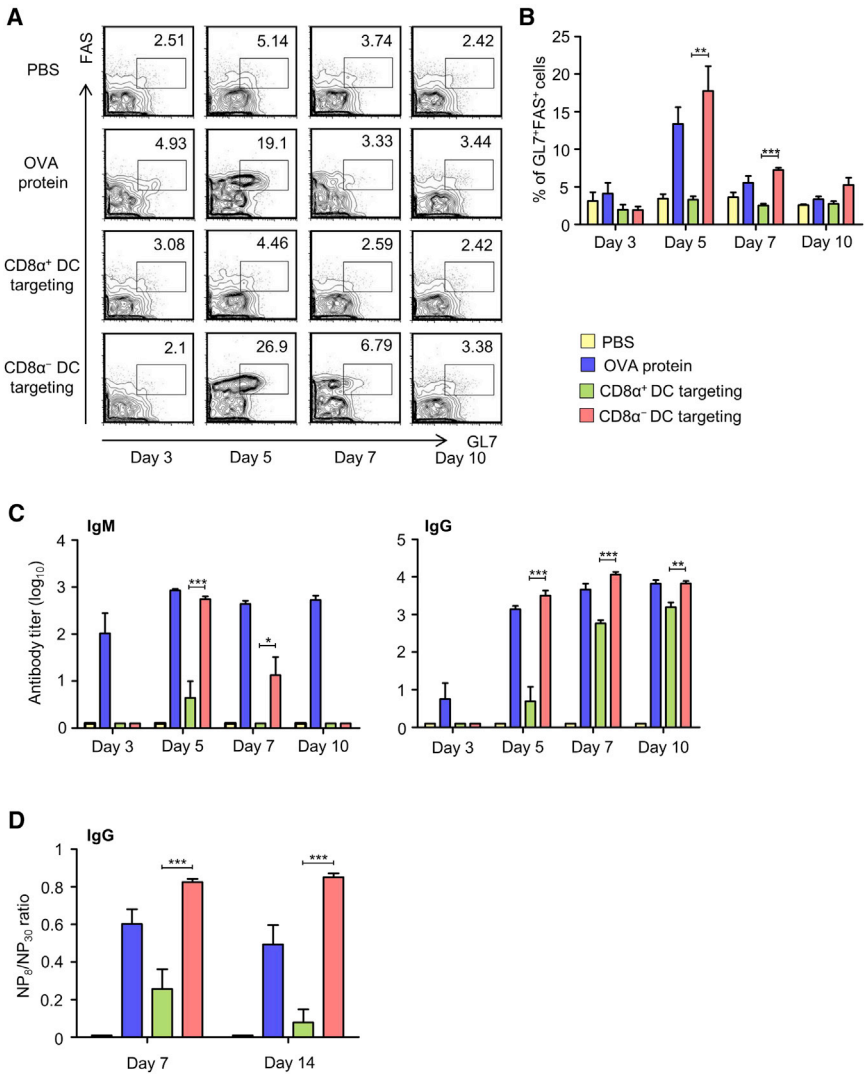


Figure 4. CD8α⁻ DCs Enhance the Formation of GC B Cells and Antibody Titers

(A–D) OVA-specific DO11.10$^+$Thy1.1$^+$CD4$^+$ T cells were adoptively transferred to naive Thy1.2$^+$ BALB/c mice at day −1 and immunized s.c. with PBS, OVA protein, αDEC:OVA (CD8α$^+$ DC targeting), or αDCIR2:OVA (CD8α$^-$ DC targeting) conjugated mAbs in the presence of poly(I:C) (A–C) or LPS (D) at day 0. At each indicated time point after the immunization, spleen cells (A and B) or sera (C and D) were prepared for analysis.

(A) Representative flow cytometry plots of GL7$^+$FAS$^+$ GC B cells gated from CD19$^+$IgD$^-$ splenocytes.

(B) Data represent mean ± SEM of four independent experiments described in (A).

(C) ELISA analyses of OVA-specific serum IgM or IgG antibodies.

(D) Ten days after the immunization, each group was re-immunized with NP_{16}-OVA (day 0). ELISA of NP-specific serum IgG antibodies (NP_8/NP_{30}).

*p < 0.05, **p < 0.01, ***p < 0.001. Data represent mean ± SEM of four (C and D) independent experiments. (A–D) n = 3-4 per group. See also Figure S4.

CD8α⁻ DCs Efficiently Enhance GC Formation, Reaction, and Antibody Titers

We examined the ability of Tfh cells as true B cell helpers in vivo by utilizing a DC targeting strategy. As expected, we observed higher percentage of CD19$^+$IgD$^-$GL7$^+$FAS$^+$ GC B cells in splenocytes collected from the CD8α$^-$ DC targeted group under different stimuli cues (Figures 4A, 4B, S4A, and S4B). Histologically, the formation of PNA$^+$ cells surrounded by IgD$^+$ cells was confirmed in the CD8α$^-$ DC targeted group, whereas no GC formation was observed in the CD8α$^+$ DC targeted group (Figure S4C). The CD8α$^-$ DC targeted group showed higher titers of both anti-OVA immunoglobulin M (IgM) and IgG antibodies than those from the CD8α$^+$ DC targeted group (Figure 4C). Titers of anti-OVA IgM induced by CD8α$^-$ DCs were higher than those induced by CD8α$^+$ DCs at day 5, which were isotype-switched to IgG at day 5. In the case of the CD8α$^+$ DC targeted group, the isotype-switched anti-OVA IgG was notably detected 7 days after the immunization, but its titers still remained lower than those from the CD8α$^-$ DC targeted group. To further confirm the enhanced GC reaction in the CD8α$^-$ DC targeted group, we examined affinity maturation of antibodies using NP-OVA. Briefly, 10 days after the immunization described in Figure 4A, the mice were re-immunized with 10 μg of NP_{16}-OVA. After 7 and 14 days of re-immunization, we observed that higher ratio of NP_8 to NP_{30} of anti-OVA IgG antibodies was induced by CD8α$^-$ DCs compared with those induced in both soluble OVA protein and CD8α$^+$ DC targeted groups (Figure 4D). These data strongly support that the CD8α$^-$ DC subset is an inducer of functional Tfh cells, which leads to efficient humoral immune responses in vivo.

Upregulated ICOSL and OX40L on CD8α⁻ DCs Play a Critical Role in Inducing Tfh Cells

To understand distinct differences between the two DC subsets in the induction of Tfh cells, various Tfh cell-related ligands were compared in the presence of poly(I:C) or LPS stimulus. CD8α$^-$ DCs expressed higher levels of ICOSL (Choi et al., 2011) and OX40L (Fillatreau and Gray, 2003) with both poly(I:C) (Figure 5A) and LPS (Figure 5B), whereas CD8α$^+$ DCs expressed higher levels of PDL1 and PDL2 (Figures S5A and S5B), which are known to negatively regulate Tfh-dependent humoral immune responses (Cubas et al., 2013; Hams et al., 2011). When ICOSL on the DC subsets was blocked during DC-T cell priming, the formerly increased expression of CXCR5 and ICOS on the CD4$^+$ T cells induced by CD8α$^-$ DCs was significantly reduced to the level of that induced by CD8α$^+$ DCs (Figures 5C and S5C). Interestingly, blocking

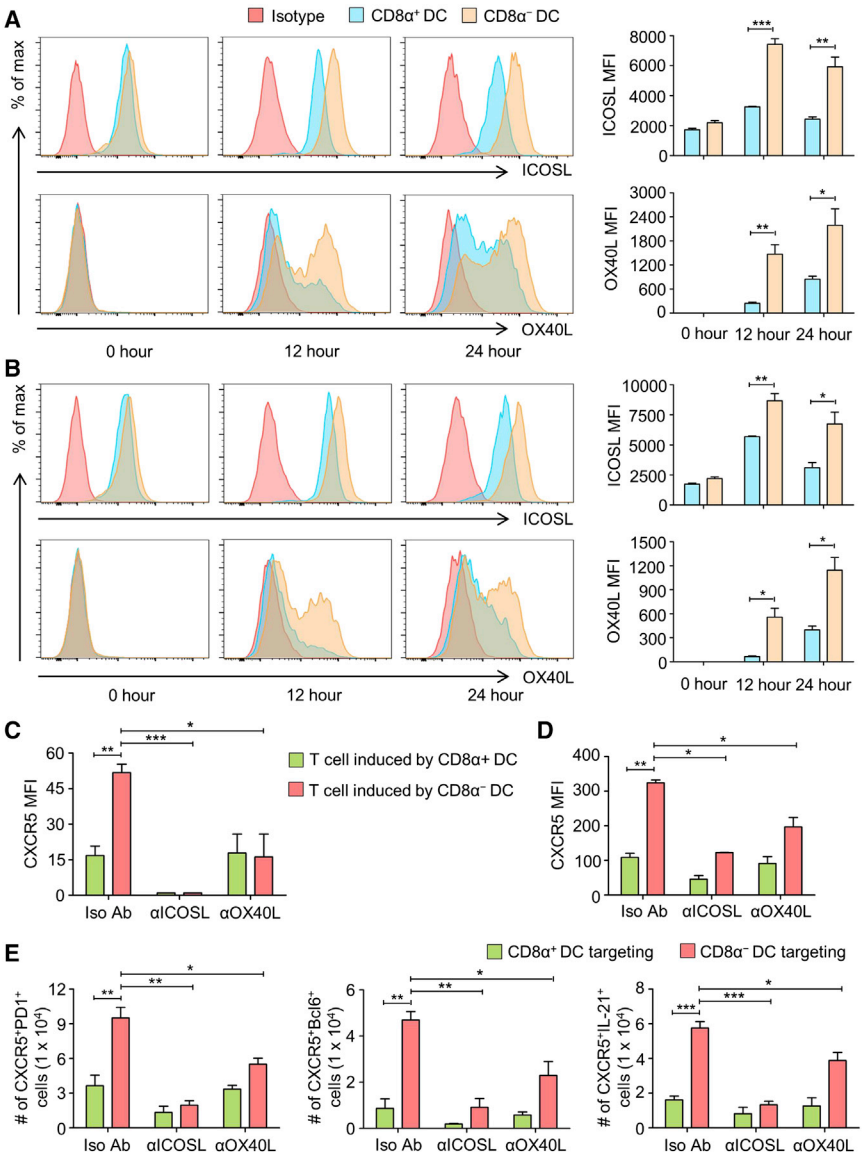


Figure 5. The Increased Number of Tfh Cells Was Induced by the Enhanced Expressions of ICOSL and OX40L on CD8α⁻ DCs

(A and B) Sorted DC subsets were stimulated either with poly(I:C) (A) or with LPS (B) for 0, 12, or 24 hr in vitro. Representative histograms of ICOSL and OX40L. MFIs represent mean ± SEM of three independent experiments.

(C) OT-II OVA specific Vα2$^+$CD4$^+$ T cells were co-cultured with each DC subset with OVA peptide (323–339) and LPS in the presence of ICOSL- or OX40L-blocking (αICOSL or αOX40L) mAbs or isotype mAbs (Iso Ab) for 3 days. MFI of CXCR5 was analyzed from three independent experiments; mean ± SEM.

(D and E) OVA-specific DO11.10$^+$Thy1.1$^+$CD4$^+$ T cells were adoptively transferred to naive Thy1.2$^+$ BALB/c mice at day −1 and immunized s.c. with either αDEC:OVA (CD8α$^+$ DC targeting) or αDCIR2:OVA (CD8α$^-$ DC targeting) conjugated mAbs in the presence of LPS at day 0. ICOSL- or OX40L-blocking mAbs (αICOSL or αOX40L) or isotype mAbs (Iso Ab) were injected intravenously (i.v.) to the immunized mice at day 0 and 2. Four days after immunization, lymph nodes cells were prepared. MFI of CXCR5 (D) and the number of CXCR5$^+$PD1$^+$, CXCR5$^+$Bcl61$^+$, or CXCR5$^+$IL-21$^+$ Tfh cells (E) were analyzed from three independent experiments; mean ± SEM (n = 4 per group).

*p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S5.

OX40L on CD8α$^-$ DCs during DC-T cell priming also decreased the expression of CXCR5 and ICOS as much as that induced by CD8α$^+$ DCs (Figures 5C and S5C). To further confirm the roles of upregulated ICOSL and OX40L on CD8α$^-$ DCs in inducing Tfh cells in vivo, blocking antibodies against ICOSL or OX40L were administered during distinct DC-subset targeting. The enhanced expression of CXCR5 on the CD4$^+$ T cells induced by CD8α$^-$ DCs was again significantly decreased by the blocking of either ICOSL or OX40L (Figure 5D). In addition, the blocking of either ICOSL or OX40L on CD8α$^-$ DCs dramatically decreased the number of CXCR5$^+$PD1$^+$, CXCR5$^+$Bcl6$^+$, or CXCR5$^+$IL-21$^+$ Tfh cells induced by CD8α$^-$ DCs both in the lymph nodes (Figure 5E) and spleen (Figure S5D). Thus, the data suggest the importance of both ICOSL and OX40L signaling in CD8α$^-$ DCs in the differentiation of Tfh cells.

Enhanced Non-canonical NF-κB Signaling Pathway of CD8α$^-$ DCs Regulates *Icosl* and *Ox40l* Gene Expressions

Based on a recent study showing that the non-canonical NF-κB pathway regulates high level of ICOSL expression in B cells (Hu et al., 2011), and based on our analysis of the NF-κB signaling pathway in the two DC subsets from the published gene array data (Dudziak et al., 2007), we hypothesized that the upregulated expressions of ICOSL and OX40L on CD8α$^-$ DCs are regulated by the non-canonical NF-κB signaling pathway. Notably, NF-κB-inducing kinase (NIK), a signal integrator of the non-canonical NF-κB signaling pathway (Sun, 2011), was detected in CD8α$^-$ DCs even in the absence of stimulation and its expression level was further increased after poly(I:C) stimulation, whereas it was hardly detectable in CD8α$^+$ DCs (Figures 6A and S6A). Upon anti-CD40 stimulation (Coope et al., 2002), which is known to elicit strong signals via both the canonical and non-canonical pathways, the expression level of NIK was increased in both DC subsets, but its level was still significantly higher in CD8α$^-$ DCs (Figure 6A; Figure S6A). In addition, higher expression levels of phospho-100 (p100), p52, and RelB were observed in CD8α$^-$ DCs in the absence or presence of stimulation (Figures 6A and S6A). CD8α$^+$ DCs constitutively

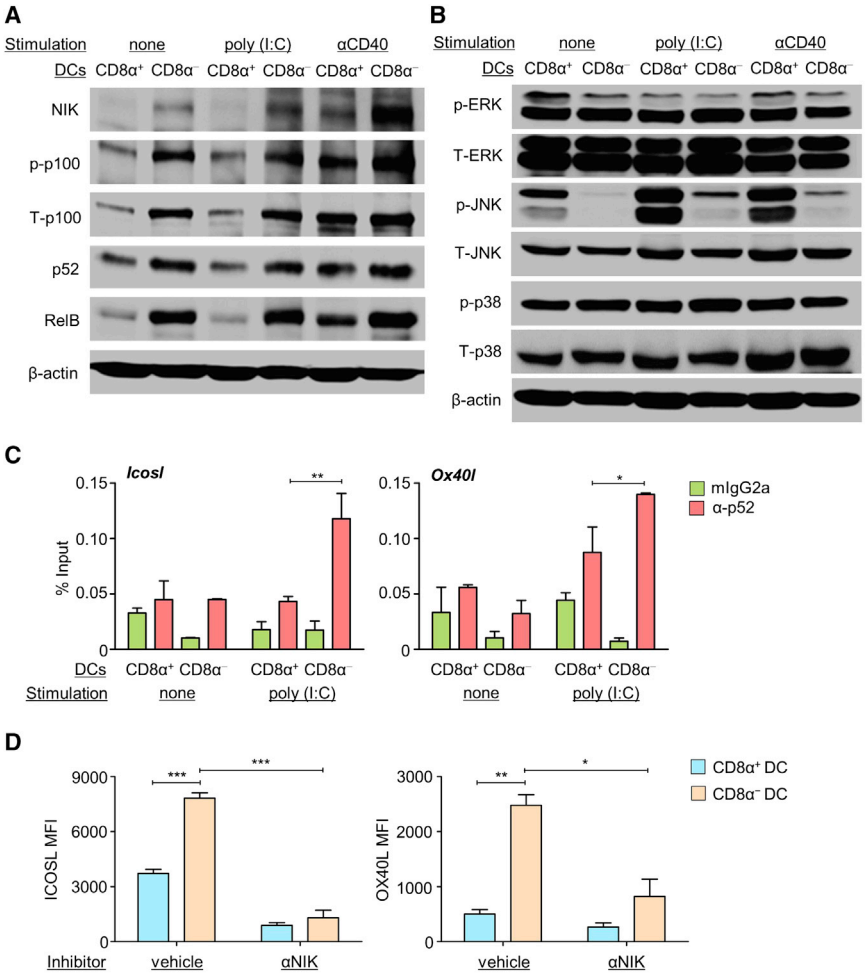


Figure 6. CD8α⁻ DCs Induce High Expression of ICOSL and OX40L via an Enhanced Non-canonical NF-κB Signaling Pathway

(A and B) Sorted DC subsets were stimulated with either poly(I:C) or αCD40 for 2 hr in vitro followed by western blot analysis of non-canonical NF-κB- (A) or MAPK-pathway-related (B) molecules. p, phosphorylation; T, total.

(C) ChIP-PCR shows percent (%) input of p52 upstream of *Icosl* and *Ox40l* in the DC subsets in the absence or the presence of stimulation. Data are representative of three (A and B) or two (C) independent experiments (mean ± SD).

(D) The two DC subsets were treated with either DMSO (vehicle) or an NIK inhibitor (αNIK) for 12 hr in the presence of poly(I:C), and the MFI of ICOSL or OX40L was analyzed. Data represent mean ± SEM of three (ICOSL) or two (OX40L) independent experiments.

*p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S6.

expressed higher levels of signaling components in the pro-inflammatory mitogen-activated protein kinase (MAPK) pathway, particularly p-JNK, which was hardly detectable in CD8α⁻ DCs (Figure 6B and S6A). To determine whether the promoter of *Icosl* or *Ox40l* associates with the non-canonical NF-κB complex, we performed chromatin immunoprecipitation (ChIP) experiments. The enriched DNA from the immunoprecipitates was quantified by qPCR using primers spanning the upstream regions of *Icosl* or *Ox40l* (Figure S6B). After poly(I:C) stimulation, the significant enrichment of p52 was found to be associated with the upstream region of *Icosl* (−3,000) or *Ox40l* (−1,000) in CD8α⁻ DCs (Figure 6C). *Gapdh* and *Ccl2* genes were used as negative and positive controls for p52, respectively (Figure S6C). Moreover, when a NIK inhibitor (4H-isoquinoline-1,3-dione) was utilized, the enhanced expression of both ICOSL and OX40L on CD8α⁻ DCs was dramatically decreased (Figure 6D). We observed comparable expression of co-stimulatory and MHC molecules on the two DC subsets upon stimulation in vitro (data not shown). Taken together, our findings suggest that highly activated non-canonical NF-κB signaling regulates the enhancement of ICOSL and OX40L expression in CD8α⁻ DCs.

Harnessing CD8α⁻ DCs to Improve Vaccine-Induced Humoral Immunity

In order to validate the enhanced humoral immune responses conferred by CD8α⁻ DC-primed Tfh cells in respect to improved vaccine strategy, we utilized various human pathogenic antigens such as *Y. pestis* LcrV, HIV Gag, and hepatitis B surface antigen (HBsAg). For each pathogen, long-term protective humoral responses are required and vaccines are not available. First, with V antigen, we observed that only CD8α⁻ DC targeting induced significant numbers of anti-V IgM ASCs in the lymph nodes, which were successfully isotype-switched to IgG1 (Figure 7A). In the spleen, similar observation was made where anti-V IgM ASCs were detected as early as day 3, isotype-switched to IgG1 at day 5, and migrated into the bone marrow (Figures 7B and 7C). On the contrary, targeting CD8α⁺ DCs with V antigen was inefficient in inducing anti-V ASCs in all lymphoid organs tested (Figures 7A–7C). Overall anti-V titers including IgG2a isotype were higher in the CD8α⁻ DC targeted group as well (Figure S7A). Moreover, data showing dramatically elevated numbers of anti-V ASCs 6 months after the immunization indicate the efficacy of CD8α⁻ DCs in the induction of long-term humoral immunity (Figure 7D).

Second, to further confirm that enhanced humoral immune responses induced by CD8α⁻ DCs are not limited to V protein, we used DC-subset targeting antibodies conjugated with HIV Gagp24 (Gag) and observed consistent enhancement in anti-Gag ASCs and antibody titers from the CD8α⁻ DC targeted group (Figures 7E and S7B). Third, we utilized sorted CD8α⁻ DCs to enhance humoral immune responses against HBsAg, the immunogen of hepatitis B vaccine. We again observed the upregulated ICOS expression on the CD4⁺ T cells primed by

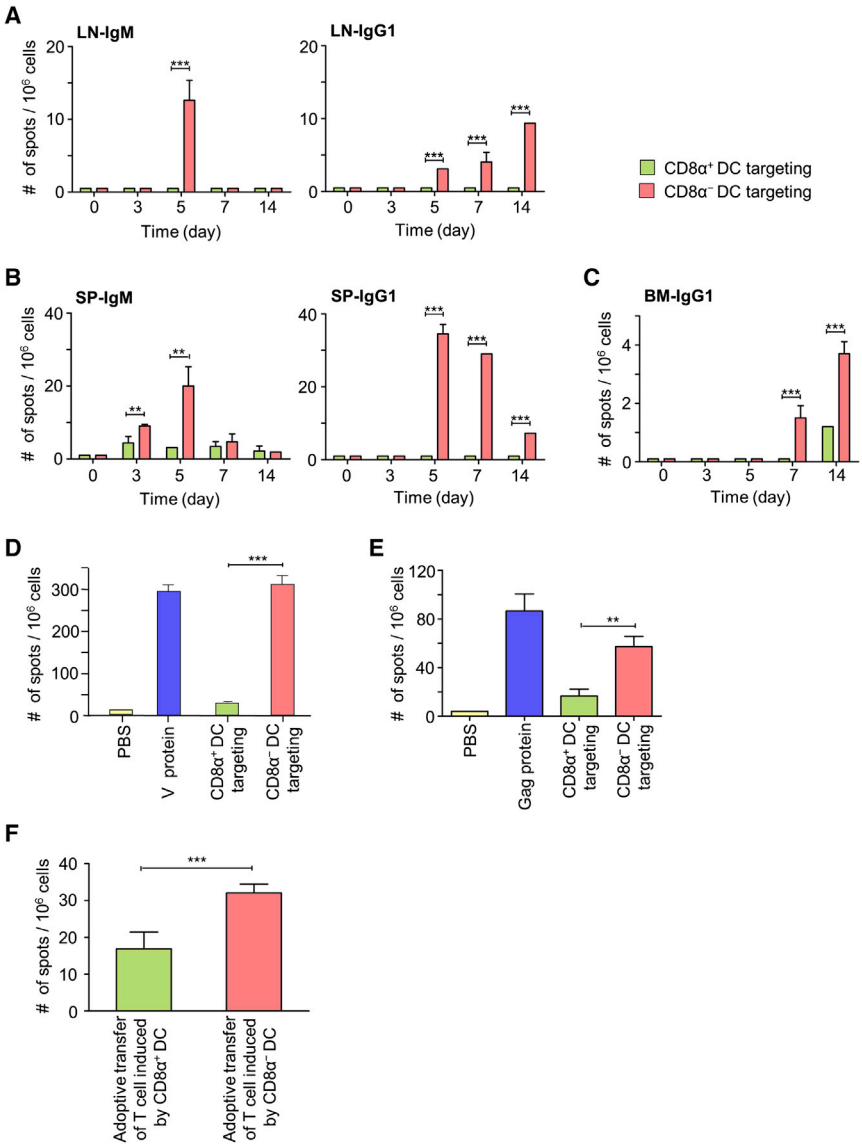


Figure 7. CD8α⁻ DCs Enhance Tfh Cell-Dependent Humoral Immune Responses against Various Human Antigens

(A–C) BALB/c mice were immunized i.p. with either αDEC:V (CD8α$^+$ DC targeting) or αDCIR2:V (CD8α$^-$ DC targeting) conjugated mAbs in the presence of αCD40 mAb and poly(I:C). At each indicated time point after the immunization, cells from the lymph nodes (LN) (A), spleen (SP) (B), or bone marrow (BM) (C) were prepared and anti-V (IgM or IgG1) ASCs were analyzed.

(D) BALB/c mice were primed and boosted with PBS, F1-V protein with alhydrogel (V protein), αDEC:V (CD8α$^+$ DC targeting), or αDCIR2:V (CD8α$^-$ DC targeting) conjugated mAbs in the presence of poly(I:C). Six months later, bone marrow cells were prepared and anti-V ASCs were analyzed.

(E) B6 mice were primed and boosted i.p. with PBS, Gagp41 protein (Gag protein), αDEC:gagp24 (CD8α$^+$ DC targeting), or αDCIR2:gagp24 (CD8α$^-$ DC targeting) conjugated mAbs in the presence of poly(I:C). Two weeks after the boost, bone marrow cells were prepared and anti-gagp24 ASCs cells were analyzed.

(F) Naive CD4$^+$ T cells co-cultured with each DC subset in the presence of HBsAg and poly(I:C) for 3 days were isolated and then adoptively transferred together with naive CD19$^+$ B cells to RAG-1-deficient mice. Fourteen days after the boost immunization with soluble HBsAg, spleen cells were prepared and anti-IgG HBsAg ASCs were analyzed.

$^{**}p < 0.01$, $^{***}p < 0.001$. All data represent mean ± SD of three or more independent experiments; n = 3 per group. See also Figure S7.

CD8α$^-$ DCs (data not shown) as well as significantly increased anti-HBsAg ASCs following adoptive transfer of the CD8α$^-$ DC-primed CD4$^+$ T cells with naive CD19$^+$ B cells into RAG-1-deficient mice (Figure 7F). These data clearly demonstrate the efficacy of CD8α$^-$ DCs in the induction of efficient humoral immunity via functional Tfh cell priming.

DISCUSSION

Various research suggests the importance of DCs in priming Tfh cells, but little is known about the mechanisms by which DCs promote the initial commitment of antigen-specific CD4$^+$ T cells into Tfh cells. In this study, our data provide important cellular and molecular mechanisms regulated by CD8α$^-$ DCs to induce Tfh cell differentiation, enhancing Tfh cell-dependent humoral immunity. We also provide the rationale for targeting CD8α$^-$ DCs to induce efficient humoral immune responses against various human pathogenic antigens.

Our study illuminates unexplored intrinsic differences between the two DC subsets in Tfh cell differentiation. In particular, our findings suggest that highly activated non-canonical NF-κB signaling enhances the expression of ICOSL and OX40L on CD8α$^-$ DCs, thereby establishing a molecular mechanism of specific DC-subset-derived Tfh cell differentiation. Such intrinsic features or capacity of CD8α$^-$ DCs in inducing Tfh cells is not limited to a specific stimulus. A previous study showed that ICOS signals during DC T cell priming instruct Bcl6 expression, leading to CXCR5 expression on CD4$^+$ T cells and thus determine the early bifurcation between Tfh and effector Th cell developments (Choi et al., 2011). However, it was not known that distinct CD8α$^-$ DCs upregulate ICOS signals promoting Tfh cell differentiation. Interestingly, our data also suggest that not only the enhanced ICOSL but also OX40L in CD8α$^-$ DCs play important roles in promoting ICOS signals of Tfh cells. Considering a previous study showing the importance of OX40L in CD11c$^+$ DCs for CD4$^+$ T cell migration to the follicle (Brocker et al., 1999), as well as another study showing that OX40 stimulation could overcome the impaired

trafficking of $CD4^+$ T cells to B cell follicles caused by lack of CD40 in DCs (Fillatreau and Gray, 2003), it is possible that the enhanced OX40L on $CD8\alpha^-$ DCs may also be involved in such a molecular hierarchy from ICOS to Bcl6 to CXCR5, which facilitate the migration of Tfh cells into B cell follicles. Given the fact that $CD8\alpha^-$ DCs upregulated the expression of ICOSL and OX40L via the non-canonical NF-κB signaling pathway and that blocking of either ICOSL or OX40L critically decreased the capacity of $CD8\alpha^-$ DCs in inducing Tfh cells in vivo, the efficient induction of Tfh cells by the $CD8\alpha^-$ DC subset is delivered by its bona fide intrinsic property.

Given that the $CD4^+$ T cells induced by $CD8\alpha^+$ DCs highly expressed T-bet, and since a number of previous studies demonstrated the efficacy of a $CD8\alpha^+$ DC targeting strategy in the induction of IFN-γ-secreting Th1 type cellular immunity (Do et al., 2010; Trumpfheller et al., 2012), we surmised that the highly activated JNK signaling may be involved in Th1 rather than in Tfh cell differentiation. However, further studies are required to examine whether $CD8\alpha^+$ DCs negatively regulate Tfh dependent-humoral immune responses with highly expressed PDL1/L2 and JNK signaling.

We do not exclude possibilities such as the differences in antigen processing in vivo (Dudziak et al., 2007), prolonged antigen presentation (Deenick et al., 2010; Lahoud et al., 2011), or extrafollicular B cell activation (Chappell et al., 2012) by DC subsets in Tfh cell differentiation. In addition, as reported by other studies that $CD14^+$ dermal DCs (Klechevsky et al., 2008), late activator APCs (Yoo et al., 2012), or monocyte-derived DCs (Chakarov and Fazilleau, 2014) promote Tfh cell differentiation, we believe Tfh cell differentiation may be dependent on the type of the immune responses. Further investigations on how various DC subsets interact in vivo within lymphoid organs will also help us to appreciate diverse physiological functions of DC subsets. Since $CD8\alpha^-$ DCs locate at the bridging channel/interfollicular zone (Dudziak et al., 2007) and Tfh cell development initiates in the same zone (Kerfoot et al., 2011), the anatomical localization of $CD8\alpha^-$ DCs and their intrinsic features may synergistically contribute to Tfh cell development, whereas $CD8\alpha^+$ DCs located in the T cell zone are more likely to induce the differentiation of Th1 cells. Of interest, a recent insightful study demonstrated the importance of EBI2 of splenic $CD4^+33D1^+$ DCs (equivalent of $CD8\alpha^-$ DCs in this study) in bridging channel positioning and homeostasis, as well as in facilitating the uptake and presentation of particulate antigens to lymphocytes (Yi and Cyster, 2013), adding to the potential of investigating $CD8\alpha^-$ DCs for promoting Tfh cell differentiation and thus providing B cell help against various blood-borne pathogens.

A previous study using peptide-pulsed bone-marrow-derived DCs showed that DCs were potent inducers of Bcl6 in naive $CD4^+$ T cells, but such DC-restricted peptide immunization failed to induce potent GCs along with lower PD1 expression on Tfh cells than the soluble protein immunization regimen (Baumjohann et al., 2011). Likely, when antigens were restricted to DCs by using CD11c/$A_\beta{}^b$ mice, it was shown to be sufficient to initiate Tfh cells, but these Tfh cells failed to produce IL-21, thereby requiring additional interaction with cognate B cells for full effector function (Goenka et al., 2011). In contrast, a $CD8\alpha^-$ DC targeting strategy showed that immunization with ~1.6 μg of OVA per mouse could induce Tfh cells as well as Tfh cell-dependent humoral immune responses more efficiently than the group immunized with 500 μg per mouse of soluble OVA protein (Figures 2, 4, S1, S2, and S4), suggesting the efficacy of targeting $CD8\alpha^-$ DCs in the induction of fully functional Tfh cells in vivo.

Recently the involvement of Tfh cells in human diseases is being unveiled. A study showed significant expansion of HIV-specific Tfh cells in chronic HIV-infected individuals explaining IgG hypersecretion seen in these patients (Lindqvist et al., 2012). Another study also reported that the expanded Tfh cells in HIV-infected individuals provided inadequate help for B cells due to the negative regulatory role of PDL1/L2 (Cubas et al., 2013). Based on a recent perspective on developing an HIV vaccine by harnessing $CD4^+$ T cell responses, particularly Tfh cells (Streeck et al., 2013), the understanding of cellular and molecular mechanisms regulating Tfh cell initiation and differentiation is critical in developing vaccines and in improving therapeutic approaches. Our study clearly demonstrates the value of $CD8\alpha^-$ DCs in enhancing humoral immunity against human pathogenic antigens. In particular, $CD8\alpha^-$ DCs were utilized to increase the number of ASCs specific to HBsAg, the immunogen of currently available hepatitis B vaccine (Figure 7F). Although the current hepatitis B vaccine is very effective, ~5%–15% of vaccines fail to develop humoral immune responses (Coursaget et al., 1986; Hadler et al., 1986). Conjugation of HBsAg with $CD8\alpha^-$ DC targeting antibody would be an alternative for hepatitis B vaccine non-responders. Additionally, in our previous study, we demonstrated successful protection against virulent *Y. pestis* pneumonic plague when we targeted $CD8\alpha^-$ DCs with V protein, of which protection efficacy was mainly due to the increased anti-V titers (Do et al., 2010), which supports our current study in designing improved pneumonic plague vaccine by targeting $CD8\alpha^-$ DCs. Therefore, $CD8\alpha^-$ DCs can be potentially utilized as a preferential target for improving vaccine efficacy in various human diseases.

In conclusion, our findings provide insights on how DCs influence Tfh cell fate; it might not be a purely stochastic event, as currently suggested (Ballesteros-Tato and Randall, 2014; Deenick et al., 2011; Vinuesa and Cyster, 2011), but instead, it could be a selective initial commitment to Tfh cells by the $CD8\alpha^-$ DC subset. Our study sheds light on the mechanisms involved in Tfh cell differentiation by DC subsets, and we believe that these results, along with future efforts to characterize human DC subset counterpart of $CD8\alpha^-$ DCs, will provide a rationale to design improved vaccines by enhancing the quality and quantity of antibody responses.

EXPERIMENTAL PROCEDURES

Mice

BALB/c (BC, H-2^d) and C57BL/6 (B6, H-2^b) mice were purchased from Taconic. DO11.10 Thy 1.1, OT-II, RAG-1-deficient, CXCR5-KO, IL-4R-KO, and IL-12p40-KO mice were purchased from The Jackson Laboratory. IL-21R-KO mice were provided by The Rockefeller University (by the late Dr. Ralph M. Steinman). SLAM-associated protein or SAP KO mice were kindly provided by Dr. Pamela L. Schwartzberg from National Human Genome Research Institute, NIH. All mice were maintained under specific-pathogen-free conditions

and used at 6–8 weeks, as approved by the Ulsan National Institute of Science and Technology's institutional animal care and use committee (approval number: UNISTIACUC-12-006-A).

DC Preparation

The total number of splenic DCs was increased by Fms-like tyrosine 3 ligand (Flt3L) as described previously (Dudziak et al., 2007). In brief, 5×10^6 Flt3L-melanoma cells were subcutaneously injected to naive C57BL/6 mice. After 10–14 days, the expanded splenic $CD11c^+$ DCs were enriched with positive magnetic-activated cell sorting (MACS) and were further sorted into DC subsets, $CD3^-B220^-CD11c^+CD8\alpha^+$ or $CD3^-B220^-CD11c^+CD8\alpha^-$ DCs, by MoFlo XDP (Beckman Coulter). To analyze intrinsic differences in the two DC subsets, DCs were stimulated with 25 μg poly(I:C) or 100 ng/ml LPS for 0, 12 or 24 hr. In some experiments, 10 μM NIK inhibitor (4H-isoquinoline-1,3-dione, combi-Blocks) (Ranuncolo et al., 2012) was added to the DC subsets.

$CD4^+$ T Cell Preparation

OVA-specific transgenic $CD4^+$ T cells from the lymph nodes and spleen of OT-II or DO11.10 Thy1.1 mice were purified negatively using hybridoma supernatant cocktail of rat-anti mouse CD8 (2.43), MHC class II (T1B120), Mφ (F4/80), B220 (RA3-6B2), and natural killer cell (NK1.1) antibodies followed by depletion with Dynabeads sheep anti-rat IgG (Invitrogen).

Induction of Tfh Cells In Vitro

Naive OVA-specific $CD4^+$ T cells purified from OT-II mice were co-cultured with sorted $CD8\alpha^+$ or $CD8\alpha^-$ DCs (1:10 ratio of DC to T cells) for 1–6 days in the presence of 25 μg/ml poly(I:C), 100 ng/ml LPS, Flagellin, or R848 (Invivogen) ± 2 μM of OVA peptide (aa 323–339) (Genscript). At each indicated time point, $V\alpha2^+CD4^+CD44^+$ T cells were sorted and analyzed for the expression of various surface molecules, transcription factors, and cytokines by flow cytometry, qRT-PCR, and ELISA. To block ICOSL or OX40L expressed on DC subsets, 20 μg/ml blocking antibodies or their corresponding isotype antibodies was added when $CD4^+$ T cells were co-cultured with DC subsets for 3 days.

Induction of Tfh Cells In Vivo

Isolated $CD4^+$ T cells from DO11.10 Thy 1.1 mice were adoptively transferred (3×10^6 cells per mouse) into naive Thy 1.2^+ BALB/c mice intravenously (i.v.) at day −1. At day 0, PBS, 500 μg soluble OVA protein (endotoxin-free; Seikagaku), or 5 μg of each distinct DC subset targeting mAb (anti-DEC-205 or anti-DCIR2 mAbs) conjugated with OVA protein in the presence of 50 μg poly(I:C) or LPS was injected via footpads. At the indicated time points, single cells from the lymph nodes or spleen were prepared and analyzed for the expression of various molecules by flow cytometry. In some experiments, 100 μg isotype, ICOSL-, or OX40L-blocking mAbs were injected i.v. into immunized mice at days 0 and 2. Then, 4 days after immunization, cells were prepared for analysis.

Intracellular Staining

Tfh cells were induced in vivo as described above. Five days later, cells were prepared from the lymph nodes or the spleen, and Fcγ receptors were blocked with anti-CD16/CD32 antibodies. The cells were then incubated with anti-mouse Thy1.1 or DO11.10, CD4, CD44, and CXCR5 for 30 min. Following fixation and permeabilization with a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol, the cells were stained for intracellular Bcl6 or IL-21 along with their isotypes for 25 min at 4°C. Data were analyzed by flow cytometry. To detect IFN-γ- or IL-4-secreting $CD4^+$ T cells, the cells were re-stimulated with 2 μM OVA peptide (323–339) and 2 μg/ml αCD28 mAbs for 2 hr followed by the addition of GolgiStop (Invitrogen) for 4 hr.

Statistics

Results are expressed as mean ± SD or mean ± SEM. We used Excel or Prism 4.0 (GraphPad Prism) and performed a nonparametric Mann-Whitney U test when appropriate. p values < 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.05.042.

AUTHOR CONTRIBUTIONS

C.S., S.R., and Y.D. conceived and designed the experiments. C.S., J.-A.H., H.K., B.C., Y.C., H.J., J.-S.R., and Y.D. performed the experiments. C.S., S.R., and Y.D. analyzed the data. H.K., S.R., P.S., and E.-C.S. contributed reagents, materials, and analysis tools. C.S., S.R., and Y.D. wrote the manuscript, and J.-A.H. and E.-C.S. helped write the manuscript.

ACKNOWLEDGMENTS

We thank the late Dr. Ralph M. Steinman of The Rockefeller University for his scientific discussion. We also thank Dr. Christine Trumpfheller of The Rockefeller University for providing anti-DEC-205/DCIR2-gagp24 antibodies and gagp41 protein and Dr. Chae Gyu Park of The Rockefeller University for providing DNA constructs of various antigen-conjugated DC targeting antibodies. This research was supported by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future Planning (2014R1A1A1006650). This work was also supported by funds from the NIH (1 R21 AI082331-01, PI, Y.D.).

Received: June 2, 2014
Revised: April 10, 2015
Accepted: May 22, 2015
Published: June 18, 2015

REFERENCES

Akiba, H., Takeda, K., Kojima, Y., Usui, Y., Harada, N., Yamazaki, T., Ma, J., Tezuka, K., Yagita, H., and Okumura, K. (2005). The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J. Immunol. *175*, 2340–2348.

Ballesteros-Tato, A., and Randall, T.D. (2014). Priming of T follicular helper cells by dendritic cells. Immunol. Cell Biol. *92*, 22–27.

Baumjohann, D., Okada, T., and Ansel, K.M. (2011). Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. J. Immunol. *187*, 2089–2092.

Bauquet, A.T., Jin, H., Paterson, A.M., Mitsdoerffer, M., Ho, I.C., Sharpe, A.H., and Kuchroo, V.K. (2009). The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nat. Immunol. *10*, 167–175.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., and Förster, R. (2000). Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J. Exp. Med. *192*, 1545–1552.

Brocker, T., Gulbranson-Judge, A., Flynn, S., Riedinger, M., Raykundalia, C., and Lane, P. (1999). CD4 T cell traffic control: in vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. Eur. J. Immunol. *29*, 1610–1616.

Cannons, J.L., Qi, H., Lu, K.T., Dutta, M., Gomez-Rodriguez, J., Cheng, J., Wakeland, E.K., Germain, R.N., and Schwartzberg, P.L. (2010). Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. Immunity *32*, 253–265.

Chakarov, S., and Fazilleau, N. (2014). Monocyte-derived dendritic cells promote T follicular helper cell differentiation. EMBO Mol. Med. *6*, 590–603.

Chappell, C.P., Draves, K.E., Giltiay, N.V., and Clark, E.A. (2012). Extrafollicular B cell activation by marginal zone dendritic cells drives T cell-dependent antibody responses. J. Exp. Med. *209*, 1825–1840.

Choi, Y.S., Kageyama, R., Eto, D., Escobar, T.C., Johnston, R.J., Monticelli, L., Lao, C., and Crotty, S. (2011). ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity *34*, 932–946.

Coope, H.J., Atkinson, P.G., Huhse, B., Belich, M., Janzen, J., Holman, M.J., Klaus, G.G., Johnston, L.H., and Ley, S.C. (2002). CD40 regulates the processing of NF-kappaB2 p100 to p52. EMBO J. *21*, 5375–5385.

Coursaget, P., Yvonnet, B., Chotard, J., Sarr, M., Vincelot, P., N'doye, R., Diop-Mar, I., and Chiron, J.P. (1986). Seven-year study of hepatitis B vaccine efficacy in infants from an endemic area (Senegal). Lancet *2*, 1143–1145.

Crotty, S. (2011). Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. *29*, 621–663.

Cubas, R.A., Mudd, J.C., Savoye, A.L., Perreau, M., van Grevenynghe, J., Metcalf, T., Connick, E., Meditz, A., Freeman, G.J., Abesada-Terk, G., Jr., et al. (2013). Inadequate T follicular cell help impairs B cell immunity during HIV infection. Nat. Med. *19*, 494–499.

Cucak, H., Yrlid, U., Reizis, B., Kalinke, U., and Johansson-Lindbom, B. (2009). Type I interferon signaling in dendritic cells stimulates the development of lymph-node-resident T follicular helper cells. Immunity *31*, 491–501.

Deenick, E.K., Chan, A., Ma, C.S., Gatto, D., Schwartzberg, P.L., Brink, R., and Tangye, S.G. (2010). Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. Immunity *33*, 241–253.

Deenick, E.K., Ma, C.S., Brink, R., and Tangye, S.G. (2011). Regulation of T follicular helper cell formation and function by antigen presenting cells. Curr. Opin. Immunol. *23*, 111–118.

den Haan, J.M., Lehar, S.M., and Bevan, M.J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J. Exp. Med. *192*, 1685–1696.

Do, Y., Koh, H., Park, C.G., Dudziak, D., Seo, P., Mehandru, S., Choi, J.H., Cheong, C., Park, S., Perlin, D.S., et al. (2010). Targeting of LcrV virulence protein from Yersinia pestis to dendritic cells protects mice against pneumonic plague. Eur. J. Immunol. *40*, 2791–2796.

Dudziak, D., Kamphorst, A.O., Heidkamp, G.F., Buchholz, V.R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H.W., Park, C.G., et al. (2007). Differential antigen processing by dendritic cell subsets in vivo. Science *315*, 107–111.

Fillatreau, S., and Gray, D. (2003). T cell accumulation in B cell follicles is regulated by dendritic cells and is independent of B cell activation. J. Exp. Med. *197*, 195–206.

Goenka, R., Barnett, L.G., Silver, J.S., O'Neill, P.J., Hunter, C.A., Cancro, M.P., and Laufer, T.M. (2011). Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. J. Immunol. *187*, 1091–1095.

Goodnow, C.C., Vinuesa, C.G., Randall, K.L., Mackay, F., and Brink, R. (2010). Control systems and decision making for antibody production. Nat. Immunol. *11*, 681–688.

Hadler, S.C., Francis, D.P., Maynard, J.E., Thompson, S.E., Judson, F.N., Echenberg, D.F., Ostrow, D.G., O'Malley, P.M., Penley, K.A., Altman, N.L., et al. (1986). Long-term immunogenicity and efficacy of hepatitis B vaccine in homosexual men. N. Engl. J. Med. *315*, 209–214.

Hams, E., McCarron, M.J., Amu, S., Yagita, H., Azuma, M., Chen, L., and Fallon, P.G. (2011). Blockade of B7-H1 (programmed death ligand 1) enhances humoral immunity by positively regulating the generation of T follicular helper cells. J. Immunol. *186*, 5648–5655.

Haynes, N.M., Allen, C.D., Lesley, R., Ansel, K.M., Killeen, N., and Cyster, J.G. (2007). Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J. Immunol. *179*, 5099–5108.

Hu, H., Wu, X., Jin, W., Chang, M., Cheng, X., and Sun, S.C. (2011). Noncanonical NF-kappaB regulates inducible costimulator (ICOS) ligand expression and T follicular helper cell development. Proc. Natl. Acad. Sci. USA *108*, 12827–12832.

Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R.M., and Inaba, K. (2002). The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J. Exp. Med. *195*, 1289–1302.

Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science *325*, 1006–1010.

Kaplan, M.H., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity *4*, 313–319.

Kerfoot, S.M., Yaari, G., Patel, J.R., Johnson, K.L., Gonzalez, D.G., Kleinstein, S.H., and Haberman, A.M. (2011). Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. Immunity *34*, 947–960.

Klechevsky, E., Morita, R., Liu, M., Cao, Y., Coquery, S., Thompson-Snipes, L., Briere, F., Chaussabel, D., Zurawski, G., Palucka, A.K., et al. (2008). Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. Immunity *29*, 497–510.

Lahoud, M.H., Ahmet, F., Kitsoulis, S., Wan, S.S., Vremec, D., Lee, C.N., Phipson, B., Shi, W., Smyth, G.K., Lew, A.M., et al. (2011). Targeting antigen to mouse dendritic cells via Clec9A induces potent CD4 T cell responses biased toward a follicular helper phenotype. J. Immunol. *187*, 842–850.

Lindqvist, M., van Lunzen, J., Soghoian, D.Z., Kuhl, B.D., Ranasinghe, S., Kranias, G., Flanders, M.D., Cutler, S., Yudanin, N., Muller, M.I., et al. (2012). Expansion of HIV-specific T follicular helper cells in chronic HIV infection. J. Clin. Invest. *122*, 3271–3280.

Ma, C.S., Suryani, S., Avery, D.T., Chan, A., Nanan, R., Santner-Nanan, B., Deenick, E.K., and Tangye, S.G. (2009). Early commitment of naïve human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. Immunol. Cell Biol. *87*, 590–600.

Ma, C.S., Deenick, E.K., Batten, M., and Tangye, S.G. (2012). The origins, function, and regulation of T follicular helper cells. J. Exp. Med. *209*, 1241–1253.

Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. *7*, 145–173.

Nurieva, R.I., Chung, Y., Hwang, D., Yang, X.O., Kang, H.S., Ma, L., Wang, Y.H., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity *29*, 138–149.

Nussenzweig, M.C., Steinman, R.M., Witmer, M.D., and Gutchinov, B. (1982). A monoclonal antibody specific for mouse dendritic cells. Proc. Natl. Acad. Sci. USA *79*, 161–165.

Qi, H., Cannons, J.L., Klauschen, F., Schwartzberg, P.L., and Germain, R.N. (2008). SAP-controlled T-B cell interactions underlie germinal centre formation. Nature *455*, 764–769.

Ranuncolo, S.M., Pittaluga, S., Evbuomwan, M.O., Jaffe, E.S., and Lewis, B.A. (2012). Hodgkin lymphoma requires stabilized NIK and constitutive RelB expression for survival. Blood *120*, 3756–3763.

Reinhardt, R.L., Liang, H.E., and Locksley, R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nat. Immunol. *10*, 385–393.

Schaerli, P., Willimann, K., Lang, A.B., Lipp, M., Loetscher, P., and Moser, B. (2000). CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J. Exp. Med. *192*, 1553–1562.

Shortman, K., and Liu, Y.J. (2002). Mouse and human dendritic cell subtypes. Nat. Rev. Immunol. *2*, 151–161.

Slifka, M.K., Matloubian, M., and Ahmed, R. (1995). Bone marrow is a major site of long-term antibody production after acute viral infection. J. Virol. *69*, 1895–1902.

Streeck, H., D'Souza, M.P., Littman, D.R., and Crotty, S. (2013). Harnessing $CD4^+$ T cell responses in HIV vaccine development. Nat. Med. *19*, 143–149.

Sun, S.C. (2011). Non-canonical NF-κB signaling pathway. Cell Res. *21*, 71–85.

Swiggard, W.J., Mirza, A., Nussenzweig, M.C., and Steinman, R.M. (1995). DEC-205, a 205-kDa protein abundant on mouse dendritic cells and thymic epithelium that is detected by the monoclonal antibody NLDC-145: purification, characterization, and N-terminal amino acid sequence. Cell. Immunol. *165*, 302–311.

Tangye, S.G., and Tarlinton, D.M. (2009). Memory B cells: effectors of long-lived immune responses. Eur. J. Immunol. *39*, 2065–2075.

Tangye, S.G., Ma, C.S., Brink, R., and Deenick, E.K. (2013). The good, the bad and the ugly - TFH cells in human health and disease. Nat. Rev. Immunol. *13*, 412–426.

Trumpfheller, C., Longhi, M.P., Caskey, M., Idoyaga, J., Bozzacco, L., Keler, T., Schlesinger, S.J., and Steinman, R.M. (2012). Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity. J. Intern. Med. *271*, 183–192.

Vinuesa, C.G., and Cyster, J.G. (2011). How T cells earn the follicular rite of passage. Immunity *35*, 671–680.

Yi, T., and Cyster, J.G. (2013). EBI2-mediated bridging channel positioning supports splenic dendritic cell homeostasis and particulate antigen capture. eLife *2*, e00757.

Yoo, J.K., Fish, E.N., and Braciale, T.J. (2012). LAPCs promote follicular helper T cell differentiation of Ag-primed CD4+ T cells during respiratory virus infection. J. Exp. Med. *209*, 1853–1867.