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The Transcription Factor YY-1 Is an Essential Regulator of T Follicular Helper Cell Differentiation

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Abstract

T follicular helper (T_{FH}) cells are a specialized subset of CD4 T cells that deliver critical help signals to B cells for the production of high-affinity Abs. Understanding the genetic program regulating T_{FH} differentiation is critical if one wants to manipulate T_{FH} cells during vaccination. A large number of transcription factor (TFs) involved in the regulation of T_{FH} differentiation have been characterized. However, there are likely additional unknown TFs required for this process. To identify new TFs, we screened a large short hairpin RNA library targeting 353 TFs in mice using an in vivo RNA interference screen. Yin Yang 1 (YY-1) was identified as a novel positive regulator of T_{FH} differentiation. Ablation of YY-1 severely impaired T_{FH} differentiation following acute viral infection and protein immunization. We found that the zinc fingers of YY-1 are critical to support T_{FH} differentiation. Thus, we discovered a novel TF involved in the regulation of T_{FH} cells.

Germinal centers (GCs) are essential for the generation of high-affinity Abs following infection or vaccination. Activated B cells that receive sufficient help from T cells differentiate into GC B (B_{GC}) cells and undergo somatic hypermutation and iso-type class switching. These processes are uniquely dependent on signals delivered by a specialized subset of CD4 T cells called T follicular helper (T_{FH}) cells. Differentiation of T_{FH} cells is

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a multistep process beginning with interaction of CD4 T cells with Ag-presenting dendritic cells. Upon reception of critical signals such as IL-6 (1), activated CD4 T cells begin differentiating into T_{FH} cells. Differentiation of T_{FH} cells depends on expression of the transcriptional repressor Bcl6 (2–5). Bcl6 plays a central role in the specification of the T_{FH} genetic program by suppressing alternative fates (5, 6). Direct repression of Blimp-1 and Id2 by Bcl6 allows expression of T_{FH}-associated genes such as CXCR5 (6, 7). There is substantial evidence that multiple transcription factors (TFs) act in a coordinated manner to specify cell fate and function (8). This is illustrated by work showing that retinoic acid–related orphan receptor (ROR)γt is at the center of a TF network that includes STAT3, Maf, IFN regulatory factor (IRF)4, and Batf to establish the genetic program supporting the differentiation of T_H17 cells (9).

In recent years, multiple TFs regulating T_{FH} cell differentiation have been identified. LEF-1 and TCF-1 are both essential for the early induction of Bcl6 and suppression of Blimp1 expression (10–12). Batf (13) and Maf (14–16) promote T_{FH} differentiation and function by driving expression of IL-21, a cytokine essential for B cell help (6). Expression of IRF4 needs to be tightly regulated to ensure T_{FH} differentiation, as both absence and overexpression of IRF4 prevent formation of T_{FH} cells (17). T_{FH}-promoting TFs are balanced by suppressors of T_{FH} formation. Blimp-1 and Foxo1 both block Bcl6 expression (2, 5, 18). Klf2 inhibits T_{FH} differentiation by promoting expression of T cell zone homing receptors and suppressing T_{FH}-associated genes (5, 19, 20). Id2 impairs T_{FH} differentiation by blocking the E protein mediated expression of CXCR5 (7, 21). All of these TFs form a large network to establish the T_{FH} genetic program.

In this study, we report the identification of Yin Yang 1 (YY-1) as an essential TF for T_{FH} differentiation. YY-1 is involved in the development and differentiation of multiple immune cells. Deletion of YY-1 in B cells results in a developmental block at the pro–B cell stage caused by defective rearrangement of the IgH locus (22). Similarly, ablation of YY-1 in thymocytes results in a severe block in T cell development at the double-negative stage (23). YY-1 is also required for the differentiation of B_{GC} cells following protein immunization (24–26). Knockdown of YY-1 completely abrogated early differentiation of T_{FH} cells. Using a series of YY-1 mutants, we demonstrate that the zinc finger of YY-1 is essential to promote T_{FH} cells. Furthermore, we show that the defective differentiation of YY-1–deficient T_{FH} cells can be partially rescued by Bcl6 overexpression.

Materials and Methods

Mice and lymphocytic choriomeningitis virus infection

SMARTA mice (27), *Bcl6*$^{fl/fl}$ Cre^{CD4} SMARTA mice (28), OT-II mice, and CD45.1^{+} mice were on a full C57BL/6 background and bred in-house. C57BL/6 mice were purchased from The Jackson Laboratory. Both male and female mice were used throughout the study, with sex- and age-matched T cell donors and recipients. All mice were maintained in specific pathogen-free facilities and used according to protocols approved by the Animal Care and Use Committees of the La Jolla Institute for Immunology. Recipient mice were infected by i.p. injection of 2×10^5 (day 6), 5×10^5 (day 3), or 1×10^6 (days 2.5 or 1) PFU of LCMV (lymphocytic choriomeningitis virus) Armstrong (LCMV-Arm). Ten micrograms of OVA

conjugated to 4-hydroxy-3-nitrophenylacetyl (NP) hapten (NP-OVA) mixed with Alhydrogel (InvivoGen) in 20 μl of PBS was injected in each foothock.

Transfection, transduction and cell transfer for short hairpin RNA screen

MicroRNA-adapted short hairpin RNA (shRNAmir) vectors (LMPd-Ametrine) were described previously (29). The shRNAmir retroviral vector (shRNAmir-RV) library targets 353 TFs expressed in mice. Each gene is targeted by three or four shRNAmirs for a total of 1414 shRNAmir-RVs in the library. The library was arrayed at a concentration of 50 ng/ml in 21 individual 96-well plates (60 shRNAmir-RV plasmids per plate). We included a set of 42 shRNAmir-RVs targeting 14 genes with known outcomes on T_{FH} differentiation (Supplemental Table I). The shRNA screen was performed and analyzed as previously described (S. Bélanger, S. Haupt, and C.E. Faliti, manuscript in preparation).

Retroviral vectors, transductions, and cell transfers

Yy1, *Yy1* resistant to sh*Yy1* no. 1, and the *Yy1* deletion mutants were cloned into pMIG. Transductions were performed as previously described (29). Transfer of sorted cells into recipient mice was performed by i.v. injection via the retro-orbital sinus. Transferred cells were allowed to rest in host mice for 3–4 d before infection or immunization. Then, 2×10^4 (day 6), 4×10^5 (day 3), or 1×10^6 (days 2.5 and 1) transduced $CD4^+$ T cells were transferred into each mouse. In certain experiments, cells were labeled with 5 μM CellTrace Violet (Life Technologies) prior to adoptive transfer.

Flow cytometry

Single-cell suspensions of spleen or draining inguinal lymph nodes were prepared by standard gentle mechanical disruption. Surface staining for flow cytometry was done with mAbs against CD4, CD8, CD45.1, and B220 (eBioscience), CD69 (BD Biosciences), and SLAM and CD25 (BioLegend). CXCR5 staining was done using biotinylated anti-CXCR5 (BioLegend), followed by PE-Cy7–, BV421- or allophycocyanin-labeled streptavidin (1:1000; BioLegend). Intracellular staining was performed with an mAb to Bcl6 (BD Biosciences), TCF1 (Cell Signaling Technology), LEF-1 (Cell Signaling Technology), YY-1 (Santa Cruz Biotechnology), or Tbet (eBioscience), as well as the Foxp3 intracellular cytokine staining kit buffers and protocol (Invitrogen). Stained cells were analyzed using FACSCelesta (BD Biosciences) and FlowJo software (Tree Star).

Statistical analysis

Statistical tests were performed using Prism 9.0 (GraphPad). Significance was determined by an unpaired Student *t* test with a 95% confidence interval or by two-way ANOVA with a Sidak's multiple comparison test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$).

Results

YY-1 is a positive regulator of T_{FH} differentiation

We hypothesized that a number of TFs regulating T_{FH} differentiation are yet to be discovered. We designed a large shRNAmir library (1414 shRNAmirs) targeting 353

TFs. This library was screened using our previously detailed in vivo shRNA screening protocol (S. Bélanger et al., manuscript in preparation) (Supplemental Fig. 1A). Briefly, the shRNAmir library was expressed in SMARTA CD4 T cells by retroviral transduction. shRNAmir$^+$ SMARTA CD4 T cells were adoptively transferred into C57BL/6 mice that were subsequently infected with LCMV-Arm. At 3 d postinfection, SMARTA CD4 T cells were sorted into CXCR5$^+$SLAM^{lo} T_{FH} and SLAM+CXCR5$^-$ T_H1 populations (Supplemental Fig. 1B), and the distribution of the shRNAmir library in both populations was interrogated by next-generation sequencing. We calculated a *Z* score value of the T_{FH}/T_H1 ratio for each gene targeted by the shRNAmir library and observed good reproducibility of both screens (Fig. 1A). We first examined the *Z* scores of control genes included in the library. *Bcl6* and *Itch* had negative *Z* score values (Fig. 1A) denoting depletion of shRNAmir targeting *Bcl6* and *Itch* from the T_{FH} population as expected because of their roles as positive regulators of T_{FH} differentiation (2–4, 30). *Prdm1*, *Tbx21*, and *Ccnt1* are all inhibitors of T_{FH} cells or positive regulators of T_H1 formation (2, 29, 31) and had positive *Z* score values indicative of enrichment of their shRNAmir constructs in T_{FH} cells. We next filtered genes with negative *Z* score values in both independent screens. *Yy1* had *Z* scores of −2.46 and −3.67 in the first and second screen, respectively, suggesting that YY-1 positively regulates differentiation of T_{FH} cells (Fig. 1A). *Yy1* codes for the polycomb group (PcG) transcription factor YY-1, which acts as a transcriptional activator or repressor (32–34). YY-1 is important for the commitment of CD8 T cells to the effector fate (35) and for NKT cell differentiation (36). In CD4 T cells, YY-1 cooperates with Foxp3 (37, 38) and GATA3 (39) to specify regulatory T cell and T_H2 cell fate, respectively, but its role in T_{FH} cells is unknown.

YY-1 deficiency severely impairs early T_{FH} formation

We first sought to confirm the results of our screen. SMARTA CD4 T cells transduced with shRNAmir-RV targeting *Yy1* (Fig. 1D) or a control gene (*Cd8*) were transferred into C57BL/6 mice. At this time postinfection, early T_{FH} and T_H1 cells can be identified by differential expression of CXCR5 and CD25. CXCR5$^+$CD25$^-$ cells are early T_{FH} cells whereas CD25$^+$CXCR5$^-$ cells are early T_H1 cells (40). Loss of *Yy1* expression severely impaired differentiation of CXCR5^{1}CD25$^-$ T_{FH} cells 3 d after LCMV-Arm infection (Fig. 1B). The reduction in CXCR5$^+$CD25$^-$ T_{FH} differentiation was accompanied by an increase in the proportion of CD25$^+$CXCR5$^-$ T_H1 cells in sh*Yy1*$^+$ SMARTA CD4 T cells (Fig. 1B). Expression of the essential T_{FH}-promoting transcription factor Bcl6 was impaired in the absence of YY-1. *Yy1*-deficient CD4 T cells could not differentiate efficiently into CXCR5$^+$Bcl6$^+$ T_{FH} cells (Fig. 1C). We also observed a substantial reduction in the accumulation of sh*Yy1*$^+$ SMARTA CD4 T cells (Fig. 1E, Supplemental Fig. 2A, 2B). *Yy1*-deficient cells did not show a proliferation defect after in vitro culture (Supplemental Fig. 2C, 2D). Importantly, expression of Tbet in CD25$^+$CXCR5$^-$ T_H1 cells was not altered by loss of YY-1 (Fig. 1F), suggesting T_H1 differentiation can proceed in *Yy1*-deficient CD4 T cells. These data confirmed the shRNA screening results and strongly suggest that YY-1 is essential for T_{FH} formation.

We next tested whether the requirement for YY-1 to support early T_{FH} differentiation is limited to an acute viral infection model. sh*Yy1*$^+$ or sh*Cd8*^{1} OT-II CD4 T cells were

transferred into C57BL/6 mice. Draining lymph nodes were analyzed 3 d after immunization with NP-OVA in alum. Few $CXCR5^+Bcl6^+$ T_{FH} cells were observed in *Yy1*-deficient OT-II CD4 T cells after immunization (Fig. 1G, 1H). Accumulation of sh*Yy1*$^+$ OT-II was also dramatically reduced (Fig. 1I, Supplemental Fig. 2E). We conclude that YY-1 is a novel critical regulator of T_{FH} differentiation in multiple in vivo models.

Impaired activation of YY-1 deficient CD4 T cells

To test whether early activation of CD4 T cells requires YY-1 expression, we transferred sh*Cd8*$^+$ or sh*Yy1*$^+$ SMARTA CD4 T cells in C57BL/6 mice and analyzed the transferred cells at 1 d post-infection with LCMV-Arm. The frequency of sh*Yy1*$^+$ SMARTA CD4 T cells was comparable to that of control cells (Fig. 2A). Control sh*Cd8*$^+$ SMARTA CD4 T cells had initiated up-regulation of CXCR5 but sh*Yy1*$^+$ cells did not, confirming the requirement for YY-1 to support early T_{FH} formation (Fig. 2B). Expression of activated CD4 T cells markers CD69 and CD25 was reduced on *Yy1*-deficient CD4 T cells (Fig. 2C, 2D). Thus, disruption of YY-1 expression substantially impairs early activation of CD4 T cells.

We next examined whether the impaired T_{FH} differentiation in the absence of YY-1 is a consequence of reduced proliferation. We assessed proliferation of transferred sh*Cd8*$^+$ and sh*Yy1*$^+$ SMARTA CD4 T cells at 2.5 d postinfection with LCMV-Arm. Most control CD4 T cells had divided three or four times (Fig. 2E). In contrast, *Yy1*-deficient CD4 T cells had divided only once or twice, and substantial fractions of cells were still undivided (Fig. 2E). We next analyzed T_{FH} differentiation in cells that were in the same cell division to control for the differences in proliferation between sh*Cd8*$^+$ and sh*Yy1*$^+$ SMARTA CD4 T cells. Whereas ~15% of sh*Cd8*$^+$ SMARTA T cells in their third division were $CXCR5^+CD25^-$ T_{FH} cells, only ~5% of sh*Yy1*$^+$ SMARTA T cells in the same cell division had differentiated into T_{FH} cells (Fig. 2F, Supplemental Fig. 2F). Loss of $CXCR5^+Bcl6^+$ T_{FH} cells in sh*Yy1*$^+$ SMARTA CD4 T cells in their third division was also observed (Fig. 2G, Supplemental Fig. 2G). These data suggest a distinct YY-1 requirement for T_{FH} differentiation.

Ectopic expression of YY-1 reverts *Yy1* deficiency

We then asked whether we could rescue the defective T_{FH} differentiation of sh*Yy1*$^+$ CD4 T cells with ectopic expression of YY-1. Wild-type *Yy1* cDNA could not be expressed in sh*Yy1*$^+$ CD4 T cells because all sh*Yy1* constructs were specific for sequences in the coding sequence of *Yy1*. To circumvent this issue, we generated a shRNA-resistant *Yy1* RV (r*Yy1*-RV) by introducing silent mutations that abrogate silencing by the sh*Yy1* no. 1 construct. Ectopic expression of r*Yy1*-RV in sh*Yy1*$^+$ CD4 T cells rescued YY-1 expression to levels similar to those in control cells (Supplemental Fig. 2H). Expression of r*Yy1* in sh*Yy1*$^+$ CD4 T cells reverted the severe T_{FH} differentiation defect observed in sh*Yy1*$^+$ CD4 T cells 3 d post-infection with LCMV-Arm (Fig. 2H). Importantly, re-expression of YY-1 also corrected the reduced accumulation of *Yy1*-deficient CD4 T cells (Fig. 2I, Supplemental Fig. 2I, 2J). These data demonstrate that the sh*Yy1*$^+$ T_{FH} deficiency phenotype was due to on-target effects of the sh*Yy1* construct.

We next asked whether constitutive expression of *Yy1* potentiates T_{FH} formation. We hypothesized that overexpression of YY-1 would enhance differentiation of T_{FH} cells

similar to what is seen in cells overexpressing LEF-1 (10) or TCF-1 (12). We transduced SMARTA CD4 T cells with *Yy1*-RV or a control GFP-RV, transferred the RV$^+$ cells into C57BL/6 mice, and analyzed the transferred cells 3 d after LCMV infection. Constitutive expression of *Yy1* did not augment differentiation of CXCR5+CD25$^-$ T_{FH} cells (Fig. 3A, Supplemental Fig. 3A) or affect accumulation of SMARTA CD4 T cells (Supplemental Fig. 3B, 3C). We next tested whether ectopic expression of *Yy1* increased T_{FH} differentiation 6 d after LCMV-Arm infection. At 6 d after LCMV infection, T_{FH} cells can be distinguished from T_H1 cells by differential expression of CXCR5 and SLAM, with T_{FH} cells being CXCR5$^+$SLAM^{lo} and T_H1 cells being SLAM$^+$CXCR5$^-$. T_{FH} cells can further differentiate into GC T_{FH} cells, characterized by higher expression of Bcl6 (2). *Yy1*-RV$^+$ SMARTA CD4 T cells had a minor increase in the proportion of CXCR5$^+$SLAM^{lo} T_{FH} cells in comparison with GFP-RV$^+$ SMARTA CD4 T cells (Fig. 3B, Supplemental Fig. 3D–F), but the frequency of CXCR5$^+$Bcl6$^+$ GC T_{FH} cells was similar between *Yy1*-RV$^+$ and control SMARTA CD4 T cells (Fig. 3C, Supplemental Fig. 3G). These results demonstrate that forced expression of YY-1 is not sufficient to enhance T_{FH} differentiation.

Zinc fingers of YY-1 are required to support T_{FH} differentiation

We next generated a series of *Yy1* deletion mutants using r*Yy1* cDNA as the starting sequence to ask which domains of YY-1 are required to support T_{FH} differentiation (Fig. 4A). Each *Yy1* deletion mutant was expressed in sh*Yy1*$^+$ SMARTA CD4 T cells and transferred into C57BL/6 mice. T_{FH} differentiation of SMARTA was analyzed 3 d after LCMV infection. In sh*Yy1*$^+$ SMARTA CD4 T cells, expression of YY-1 lacking the REPO domain fully restored proliferation of the cells, but it could only partially support T_{FH} differentiation compared with complete r*Yy1* (Fig. 4B, 4C, Supplemental Fig. 4B). The partial rescue of T_{FH} differentiation was not a consequence of impaired expression of the YY-1 ΔREPO protein (Supplemental Fig. 4A).

A *Yy1* construct lacking the transactivation domain (TD) could only partially rescue the T_{FH} differentiation of *Yy1*-deficient CD4 T cells (Fig. 4B, Supplemental Fig. 4B). We could not detect YY-1 expression when the *Yy1*-ΔTD construct was expressed (Supplemental Fig. 4A). Presumably, the anti YY-1 Ab used for flow cytometry binds to an epitope in the TD. The rescue of proliferation of YY-1 ΔTD$^+$sh*Yy1*$^+$ SMARTA CD4 T cells (Fig. 4C, Supplemental Fig. 4B) suggested that YY-1 ΔTD was efficiently expressed. Deletion of the zinc fingers completely abrogated the ability of YY-1 to restore T_{FH} differentiation and accumulation of *Yy1*-deficient CD4 T cells (Fig. 4B, 4C, Supplemental Fig. 4B). YY-1 ΔZn was expressed at levels similar to those of rYY-1 (Supplemental Fig. 4A). These data demonstrate that the zinc fingers of YY-1 are absolutely required for T_{FH} differentiation whereas the REPO domain and TD are dispensable for CD4 T cell proliferation.

Bcl6 is required for YY-1–mediated T_{FH} differentiation

To test the relationship between YY-1 and Bcl6, we transduced *Bcl6*$^{fl/fl}$ CD4^{Cre} SMARTA CD4 T cells with GFP-RV or *Yy1*-RV and transferred the cells into C57BL/6 mice. Three days after LCMV infection, we did not observe CXCR5$^+$CD25$^-$ T_{FH} cells in both groups of *Bcl6*$^{fl/fl}$ CD4^{Cre} SMARTA CD4 T cells (Fig. 5A, 5B, Supplemental Fig. 4C). Therefore,

YY-1 cannot compensate for loss of Bcl6, suggesting that YY-1 does not act in a dominant manner downstream of Bcl6 to support T_{FH} differentiation.

To test whether Bcl6 acts downstream of YY-1, sh*Cd8*$^+$ or sh*Yy1*$^+$ SMARTA CD4 T cells were transduced with GFP-RV or *Bcl6*-RV, transferred into C57BL/6 mice, and analyzed 3 d following LCMV-Arm infection. Forced expression of *Bcl6* could partially rescue the ability of sh*Yy1*$^+$ SMARTA CD4 T cells to differentiate into CXCR5$^+$CD25$^-$ T_{FH} cells (Fig. 5C, Supplemental Fig. 4D). T_{FH} differentiation of *Bcl6*-RV$^+$sh*Yy1*$^+$ SMARTA CD4 T cells was rescued to the level of GFP-RV$^+$sh*Cd8*$^+$ SMARTA CD4 T cells, suggesting that Bcl6 could not completely compensate for loss of YY-1. Forced expression of *Bcl6* had no influence on the accumulation of *Yy1*-deficient SMARTA CD4 T cells nor on YY-1 expression (Fig. 5D, 5E, Supplemental Fig. 4D). Importantly, the partial rescue in T_{FH} differentiation was not caused by inadequate Bcl6 overexpression in *Bcl6*-RV$^+$shYy1$^+$ SMARTA CD4 T cells (Fig. 5F). This suggests that Bcl6 cannot fully support differentiation of T_{FH} cells in the absence of YY-1.

Discussion

In this study, to our knowledge we provide the first evidence that YY-1 is required for proper differentiation of T_{FH} cells. The involvement of YY-1 in both B_{GC} and T_{FH} cells adds one more common TF shared between these two cell types. We observed impaired accumulation of *Yy1*-deficient CD4 T cells after infection or protein immunization. The lack of T_{FH} differentiation of *Yy1*-deficient CD4 T cells could be a consequence of the severe proliferation defect in these cells. Our data show that T_{FH} differentiation in *Yy1*-deficient cells can be partially rescued by ectopic expression of Bcl6, without correcting the proliferation defect. It is possible that restoration of the proliferative capacity of *Yy1*-deficient cells could correct the T_{FH} differentiation defect. Similarly, loss of YY-1 increases apoptosis in B_{GC} cells (26) and in thymocytes (23). YY-1 directs p53 ubiquitination (41) and deletion of p53 reverts the developmental defect of *Yy1*-deficient thymocytes (23). T_{FH} cell analysis in the present study revealed that the reduced formation of T_{FH} cells observed in *Yy1*-deficient CD4 T cells was maintained in cells in the same cell–division, suggesting that the T_{FH} cell promoting activities of YY-1 are independent of its role in supporting CD4 T cell activation and survival.

We studied three different domains of YY-1 for their roles in supporting T_{FH} cell differentiation. The REPO domain of YY-1 recruits PcG proteins (42). PcG proteins are transcriptional repressors owing to their ability to methylate lysine 27 of histone 3 (43). We observed near normal T_{FH} differentiation when a YY-1 mutant lacking the REPO domain was re-expressed in *Yy1*-deficient CD4 T cells, indicating that the REPO domain is mostly dispensable for this process. This contrasts with the essential requirement of the REPO domain of YY-1 for proper B cell development (44). EZH2 is the catalytic subunit of the PRC2 PcG protein group (43) and EZH2 is required for T_{FH} differentiation (45, 46). In myoblasts, YY-1 recruits EZH2 to the chromatin, permitting H3K27 methylation of muscle genes (47). It is therefore possible that the interaction between YY-1 and PRC2-EZH2 is required for T_{FH} differentiation. The TD mediates the transcriptional activity of YY-1 (32, 48). A YY-1 mutant in which the TD is deleted can partially rescue the defective

T_{FH} differentiation of sh*Yy1*$^{+}$ CD4 T cells, suggesting that the transcription-promoting activities of YY-1 contribute to T_{FH} formation. The requirement for the REPO domain and TD of YY-1 implies that transcriptional repression and activation by YY-1 are both involved in regulating T_{FH} differentiation. The normal accumulation of SMARTA CD4 T cells expressing these constructs further supports an essential role of YY-1 in supporting T_{FH} cells independently of CD4 T cell activation and proliferation.

We found that the zinc finger domain of YY-1 is necessary for T_{FH} differentiation, suggesting that YY-1 DNA binding is an absolute requirement to support T_{FH} cells. YY-1 has four zinc fingers. Further work is required to dissect which zinc fingers of YY-1 are essential for T_{FH} cells. Zinc fingers of YY-1 are required to repress Foxp3 expression and transcriptional activity (37) and to enhance *Il4* promoter activity (49). The zinc fingers of YY-1 also mediate interaction with ATF (50). YY-1 interacts with Foxp3 to suppress regulatory T cell differentiation (37), with GATA3 to regulate expression of T_H2 cytokine genes (39), and with PLZF to promote NKT cell development (36). It is therefore tempting to speculate that YY-1 may interact with Bcl6 to support T_{FH} differentiation. Forced expression of Bcl6 cannot fully compensate for the loss of YY-1. Conversely, YY-1 does not drive T_{FH} differentiation in the absence of Bcl6. These data suggest that YY-1 is required for the full T_{FH}-promoting activities of Bcl6. Further experiments are required to understand the interplay between Bcl6 and YY-1 and how these two TFs work together to promote T_{FH} cells.

In sum, we identified YY-1 as a novel critical regulator of T_{FH} differentiation and show that the zinc fingers of YY-1 are essential for this function. Our work extends the network of TFs involved in the regulation of the T_{FH} cell fate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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S.B. designed and performed the experiments, analyzed data, and wrote the manuscript; S.H. and B.L.F. performed the experiments and analyzed data; A.J.G. and H.D. performed and analyzed the next-generation sequencing of the shRNA screen; M.E.P. supervised sequencing analysis; and S.C. designed the study, supervised the work, and wrote the manuscript.

Abbreviations used in this article:

GC	germinal center
B_{GC}	GC B

IRF	IFN regulatory factor
LCMV	lymphocytic choriomeningitis virus
LCMV-Arm	LCMV Armstrong
NP	4-hydroxy-3-nitrophenylacetyl
NP-OVA	OVA conjugated to NP hapten
PcG	polycomb group
r*Yy1*-RV	shRNA-resistant *Yy1* RV
shRNAmir	microRNA-adapted short hairpin RNA
shRNAmir-RV	shRNAmir retroviral vector
TD	transactivation domain
TF	transcription factor
T_{FH}	T follicular helper

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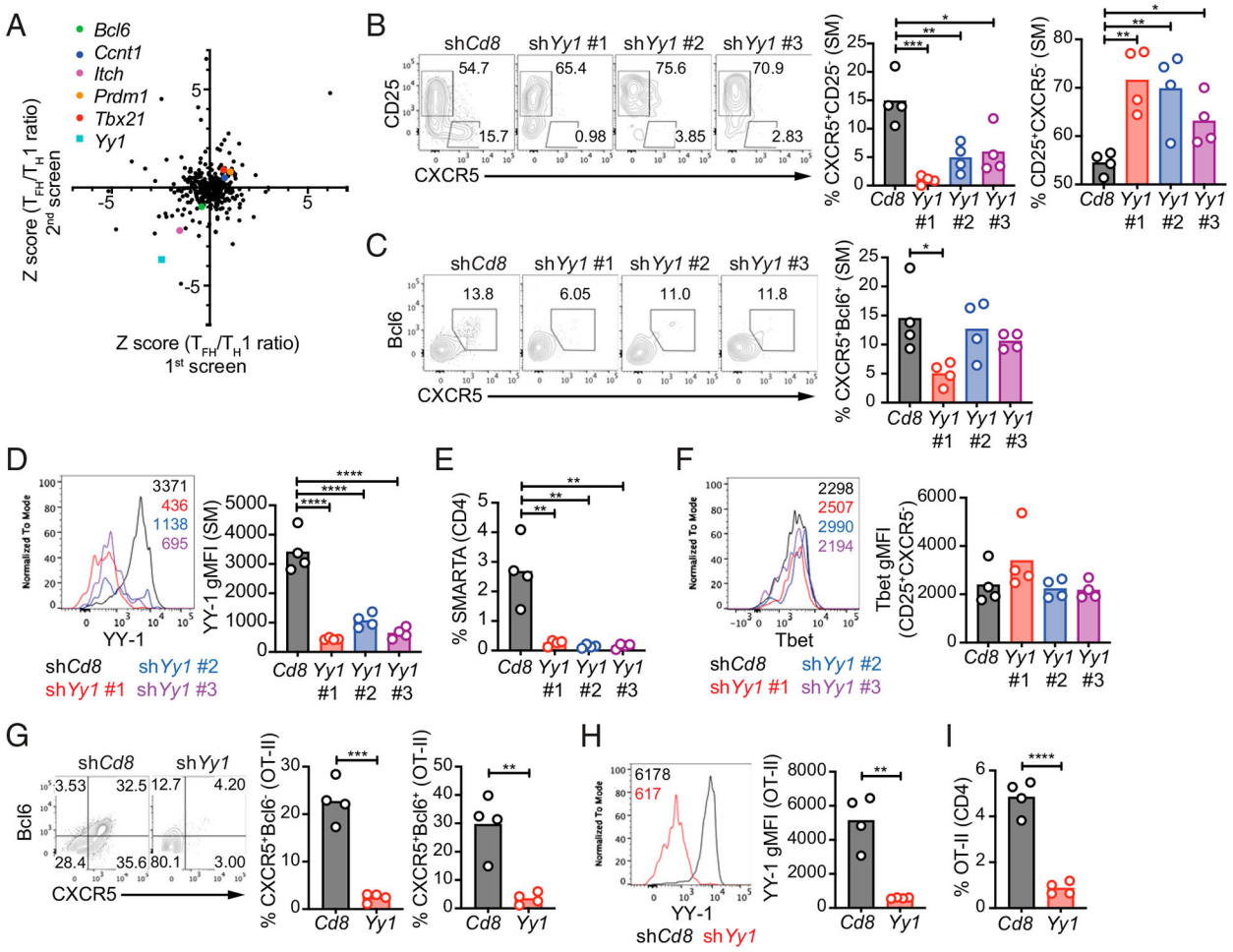

FIGURE 1.

YY-1 as a novel regulator of T_{FH} differentiation. (**A**) Gene T_{FH}/T_H1 cell ratio *Z* scores for all genes in the shRNAmir library targeting TFs in two independent experiments. (**B**–**F**) SMARTA CD4 T cells transduced with the indicated shRNAmir-RVs were transferred into C57BL/6 mice. Spleens were analyzed 3 d after infection with LCMV-Arm. (B) Flow plots and frequency of $CXCR5^+CD25^-$ T_{FH} cells and $CD25^+CXCR5^-$ T_H1 cells in SMARTA CD4 T cells. (C) Flow plots and frequency of $CXCR5^+Bcl6^+$ T_{FH} cells in SMARTA CD4 T cells. (D) Histogram and quantification of YY-1 expression in SMARTA CD4 T cells. Numbers in the histogram indicate geometric mean fluorescence intensity (gMFI) values. (E) Quantification of SMARTA CD4 T cells. (F) Histogram and quantification of Tbet expression in $CD25^+CXCR5^-$ T_H1 SMARTA CD4 T cells. Numbers in the histogram indicate gMFI values. (**G**–**I**) OT-II CD4 T cells transduced with the indicated shRNAmir-RVs were transferred into C57BL/6 mice. Draining lymph nodes were analyzed 3 d after immunization with NP-OVA in alum. (G) Flow plots and frequency of $CXCR5^+Bcl6^-$ and $CXCR5^+Bcl6^+$ in OT-II CD4 T cells. (H) Histogram and quantification of YY-1 expression in OT-II CD4 T cells. Numbers in the histogram indicate gMFI values. (I) Quantification of OT-II CD4 T cells. Data are representative of five (B F) or two (G I) independent

experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ (unpaired two-tailed Student *t* test).

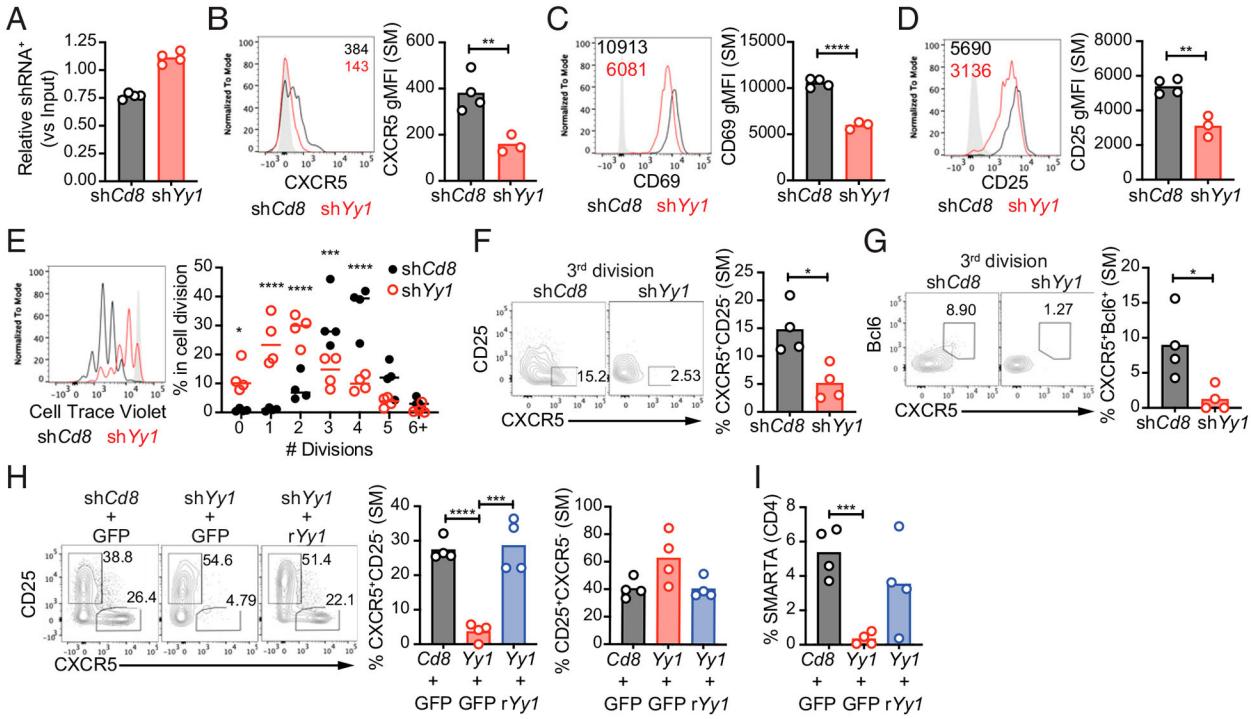

FIGURE 2.

YY-1 is required for early activation of CD4 T cells. (**A**–**I**) SMARTA CD4 T cells transduced with the indicated RV were transferred into C57BL/6 mice and spleens were analyzed 1 d (A D) or 3 d (H and I) after infection with LCMV-Arm. (A and I) Quantification of shRNA$^+$ SMARTA CD4 T cells. (B D) Histogram and quantification of CXCR5 (B), CD69 (C), or CD25 (D) expression in shRNA$^+$ SMARTA CD4 T cells. Numbers in the histogram indicate gMFI values. Gray histograms represent naive CD4 T cells. (E G) SMARTA CD4 T cells transduced with the indicated shRNAmir-RVs were labeled with CellTrace Violet and transferred into C57BL/6 mice and spleens were analyzed 2.5 d after infection with LCMV-Arm. (E) Histogram of CellTrace Violet dilution of shRNA$^+$ SMARTA CD4 T cells and quantification of the proportion of cells in each cell division. (F) Flow plots and frequency of CXCR5$^+$CD25$^-$ T_{FH} cells in SMARTA CD4 T cells in their third division based on CellTrace Violet dilution. (G) Flow plots and frequency of CXCR5$^+$Bcl6$^+$ T_{FH} cells in SMARTA CD4 T cells in their third division based on CellTrace Violet dilution. (H) Flow plots and frequency of CXCR5$^+$CD25$^-$ T_{FH} cells and CD25^{1}CXCR5$^-$ T_H1 cells in SMARTA CD4 T cells. (I) Quantification of SMARTA CD4 T cells. Data are representative of two (A G) or five (H and I) independent experiments. (B D and F I) *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.01$, ****$p \le 0.0001$ (unpaired two-tailed Student *t* test). (E) *$p \le 0.05$, ***$p \le 0.001$, ****$p \le 0.0001$ (two-way ANOVA).

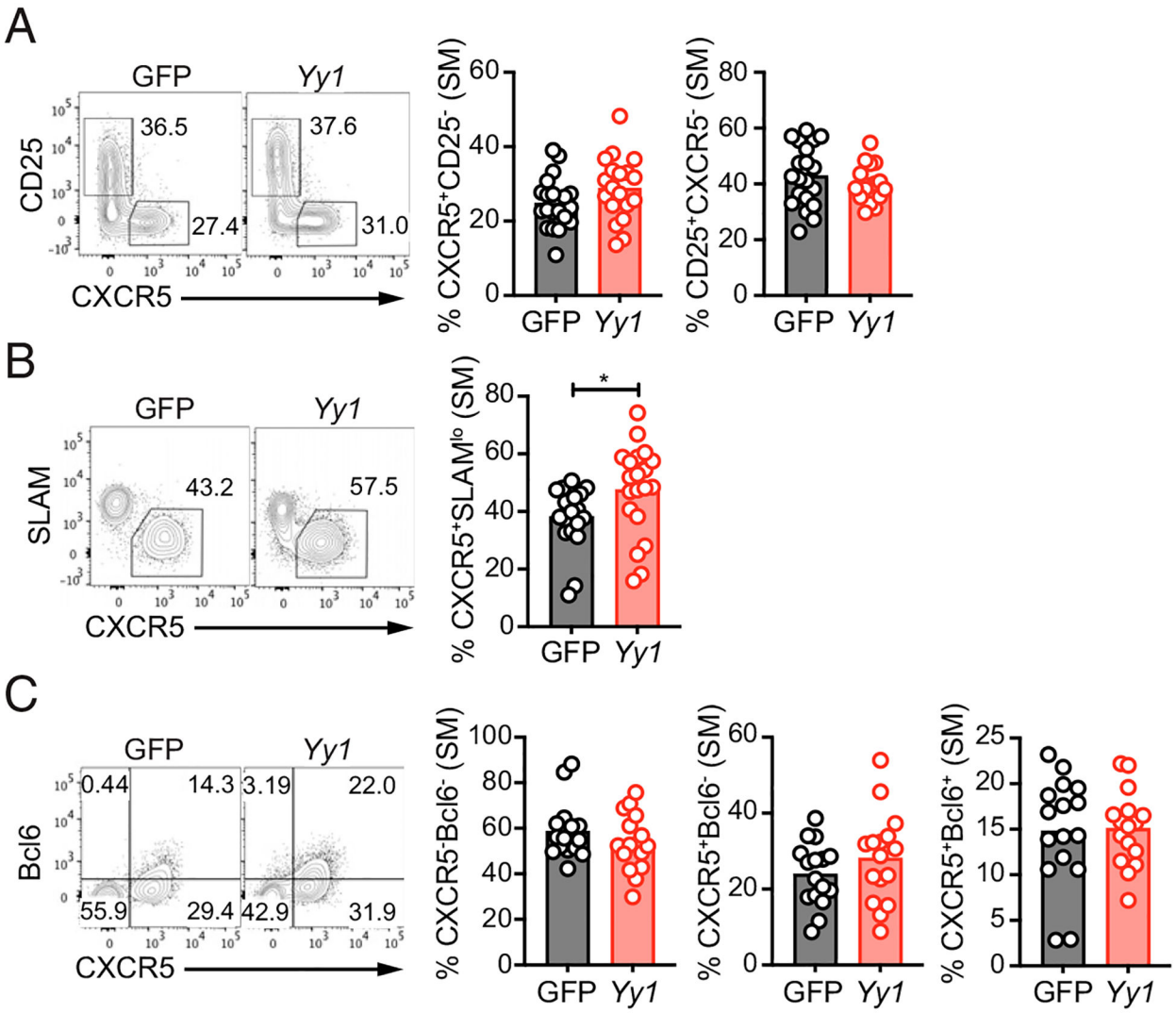

FIGURE 3.

Ectopic expression of YY-1 does not enhance T_{FH} differentiation. (**A C**) SMARTA CD4 T cells transduced with the indicated RVs were transferred into C57BL/6 mice and spleens were analyzed 3 d (A) or 6 d (B and C) after LCMV infection. (A) Flow plots and frequencies of $CXCR5^+CD25^-$ T_{FH} cells and $CD25^+CXCR5^-$ T_H1 cells in splenic SMARTA CD4 T cells. (B) Flow plots and frequencies of $CXCR5^+SLAM^{lo}$ T_{FH} cells in SMARTA CD4 T cells. (C) Flow plots and frequencies of $CXCR5^-Bcl6^-$ T_H1 cells, $CXCR5^+Bcl6^-$ T cells, and $CXCR5^+Bcl6^+$ FH GC T_{FH} cells in SMARTA CD4 T cells. Data are pooled from four to five independent experiments. $*p \leq 0.05$ (unpaired two-tailed Student *t* test).

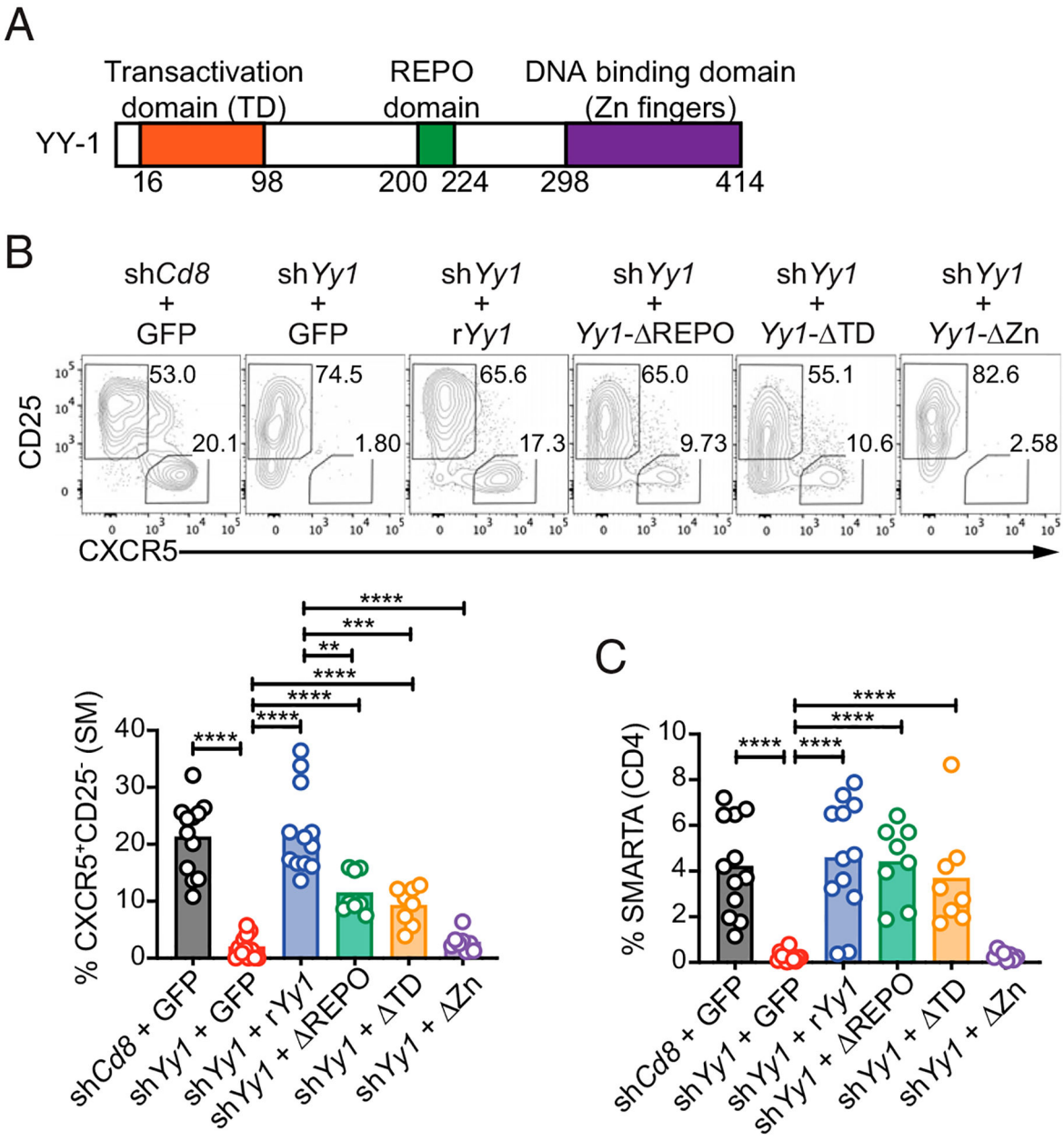

FIGURE 4.

Zinc finger of YY-1 is required to support T_{FH} differentiation. (**A**) Diagram of YY-1 domains studied. (**B** and **C**) SMARTA CD4 T cells transduced with the indicated RVs were transferred into C57BL/6 mice and spleens were analyzed 3 d after LCMV infection. (B) Flow plots of $CXCR5^+CD25^-$ T_{FH} cells and $CD25^+CXCR5^-$ T_H1 cells in SMARTA CD4 T cells. Frequencies of $CXCR5^+CD25^-$ T_{FH} cells pooled from two independent experiments are shown. (C) Quantification of SMARTA CD4 T cells pooled from two independent experiments. Flow plots are representative of two independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$ (unpaired two-tailed Student *t* test).

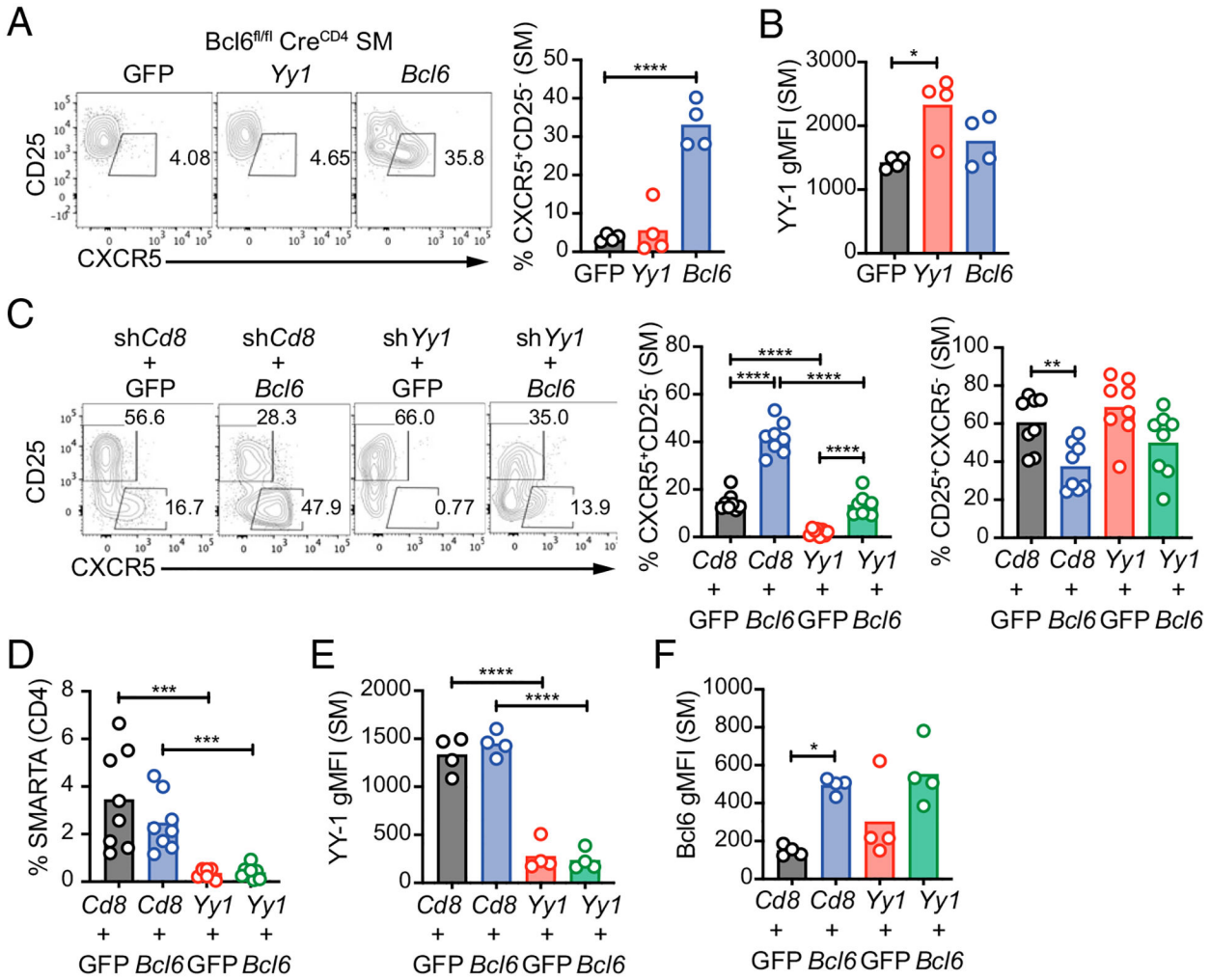

FIGURE 5.

Bcl6 partially rescues the defective T_{FH} differentiation of *Yy1*-deficient CD4 T cells. (**A** and **B**) *Bcl6*$^{fl/fl}$ Cre^{CD4} SMARTA CD4 T cells transduced with the indicated RVs were transferred into C57BL/6 mice and spleens were analyzed 3 d after LCMV infection. (A) Flow plots and frequencies of CXCR5$^+$CD25$^-$ T cells in *Bcl6*$^{fl/fl}$ FH Cre^{CD4} SMARTA CD4 T cells. (B) Quantification of YY-1 expression in *Bcl6*$^{fl/fl}$ Cre^{CD4} SMARTA CD4 T cells. (**C**–**F**) SMARTA CD4 T cells transduced with the indicated RVs were transferred into C57BL/6 mice and spleens were analyzed 3 d after LCMV infection. (C) Flow plots of CXCR5$^+$CD25$^-$ T cells and CD25$^+$ FH CXCR5$^-$ T_H1 cells in SMARTA CD4 T cells. Frequencies of CXCR5$^+$CD25$^-$ T_{FH} cells and CD25$^+$CXCR5$^-$ T_H1 cells pooled from two independent experiments are shown. (D) Quantification of SMARTA CD4 T cells pooled from two independent experiments. (E and F) Quantification of YY-1 (E) and Bcl6 (F) expression in SMARTA CD4 T cells. Data are representative of two independent experiments. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.01$, ****$p \le 0.0001$ (unpaired two-tailed Student *t* test).