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The transcriptional coactivator Bob1 promotes the development of follicular T helper cells via Bcl6

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Abstract

Follicular T helper (Tfh) cells are key regulators of the germinal center reaction and long-term humoral immunity. Tfh cell differentiation requires the sustained expression of the transcriptional repressor Bcl6; however, its regulation in CD4$^+$ T cells is incompletely understood. Here, we report that the transcriptional coactivator Bob1, encoded by the *Pou2af1* gene, promotes Bcl6 expression and Tfh cell development. We found that Bob1 together with the octamer transcription factors Oct1/Oct2 can directly bind to and transactivate the *Bcl6* and *Btla* promoters. Mixed bone marrow chimeras revealed that Bob1 is required for the expression of normal levels of Bcl6 and BTLA, thereby controlling the pool size and composition of the Tfh compartment in a T cell-intrinsic manner. Our data indicate that T cell-expressed Bob1 is directly involved in Tfh cell differentiation and required for mounting normal T cell-dependent B-cell responses.

Keywords follicular T helper cells; germinal center; humoral immunity; Pou2af1; T cell differentiation

Subject Categories Immunology

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Introduction

Protective long-term humoral immunity depends on the generation of high-affinity, antibody-secreting plasma cells and memory B cells. The differentiation of these long-lived B cells is linked to the differentiation of follicular T helper (Tfh) cells, a subset of CD4$^+$ T cells that is specialized in providing help to B cells in the germinal center (GC) reaction (Breitfeld *et al*, 2000; Schaerli *et al*, 2000).

Tfh cell differentiation is initiated by cognate interaction of mature CD4$^+$ T cells with antigen-presenting dendritic cells (DC) in the T-cell areas of secondary lymphoid organs. DCs provide critical signals for the induction of Bcl6, a transcriptional repressor that functions as a master regulator of Tfh cell lineage commitment (Choi *et al*, 2011). Bcl6 represses transcription factors important for Th1 (Nurieva *et al*, 2009; Yu *et al*, 2009), Th2 (Dent *et al*, 1999; Kusam *et al*, 2003; Nurieva *et al*, 2009), or Th17 (Nurieva *et al*, 2009; Mondal *et al*, 2010) differentiation and it antagonizes B-lymphocyte-induced maturation protein 1 (BLIMP1), which otherwise promotes the development of non-Tfh effector cells (Johnston *et al*, 2009). Still, the induction of Tfh signature genes such as Bcl6 and CXCR5 is not yet fully understood. Bcl6 expression appears to be governed by ICOS ligation and DC-derived cytokines that signal via signal transducer and activator of transcription 3 (STAT3) including interleukin-6 (IL-6), IL-27 and later on by autocrine stimulation via IL-21 (Batten *et al*, 2010; Eto *et al*, 2011; Harker *et al*, 2011; Karnowski *et al*, 2012; Choi *et al*, 2013). Earlier studies suggested that Bcl6 drives CXCR5 expression (Johnston *et al*, 2009; Nurieva *et al*, 2009; Yu *et al*, 2009); however, more recent data indicate that the expression of genes important for the relocation of Tfh cells from the T cell-rich areas to the B-cell follicles—foremost *Cxcr5*—is initiated independently of Bcl6 by the transcription factor Ascl2 (Liu *et al*, 2012, 2014b).

Nascent Tfh cells that express CXCR5 migrate to the interfollicular regions of lymph nodes or the T-B zone border in the spleen to make contact with cognate antigen-experienced B cells. Further differentiation of these pre-GC-Tfh cells requires sustained interaction with B cells (Qi *et al*, 2008; Cannons *et al*, 2010) that deliver multiple signals via the T-cell receptor (TCR), ICOS, CD40L, and most likely IL-6 receptor (IL-6R) that re-enforce Bcl6 expression and commitment to the GC-Tfh cell differentiation pathway (Baumjohann *et al*, 2011; Kitano *et al*, 2011). In return, the B cells become primed to proliferate and differentiate along either extrafollicular or GC pathways. Activated B cells and Tfh cells that eventually move from the T-B border into the follicle seed GCs in which GC-Tfh cells promote the growth and survival of high-affinity antibody producing GC B cells. At the same time, GC-Tfh cells are thought to be a major driver of antibody affinity maturation by governing the positive selection of GC B cells with highest affinity for a given antigen (Allen *et al*, 2007; Victora *et al*, 2010; Liu *et al*, 2014a).

Tfh cell differentiation critically depends on the induction and maintenance of Bcl6 expression; however, Bcl6 requires the action of additional transcription factors such as basic leucine zipper

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transcriptional factor ATF-like (BATF), interferon regulatory factor 4 (IRF4), and c-Maf, which are equally important in Tfh cell development. BATF directly controls expression of Bcl6 and c-Maf such that mice deficient for BATF do not develop Tfh cells (Betz *et al*, 2010; Ise *et al*, 2011). Similarly, mice deficient for IRF4 lack Tfh cells and GC formation due to a T cell-intrinsic defect in Bcl6 expression (Kwon *et al*, 2009; Bollig *et al*, 2012). c-Maf, on the other hand, is known to cooperate with Bcl6 to induce expression of ICOS, CXCR5, and IL-21 (Hiramatsu *et al*, 2010; Ise *et al*, 2011; Kroenke *et al*, 2012). c-Maf-deficient mice are able to develop limited numbers of Tfh cells; however, these cells express only low levels of CXCR5 (Bauquet *et al*, 2009).

The transcriptional coactivator Bob1, encoded by the *Pou2af1* (POU domain class 2-associating factor 1) gene and alternatively named OBF-1 or OCA-B, is another critical factor for the development of T cell-dependent immune responses. Bob1 has long been considered a B cell-specific factor that interacts with the transcription factors Oct1 and Oct2 to enhance octamer-dependent transcription. Mice deficient for Bob1 fail to develop GCs and hence isotype-switched plasma cells (Kim *et al*, 1996; Nielsen *et al*, 1996; Schubart *et al*, 1996). This phenotype has generally been attributed to B cell-intrinsic defects in late B-cell differentiation (Corcoran *et al*, 2005); however, we have recently shown that Tfh cells express high levels of Bob1 (Rasheed *et al*, 2006) and that the loss of Bob1 affects $CD4^+$ T-cell function as Bob1 contributes directly to the *Ifng* and *Il2* promoter activities (Brunner *et al*, 2007; Shakya *et al*, 2015).

The prominent expression of Bob1 in human as well as in murine Tfh cells compared to other $CD4^+$ T-cell populations led us to investigate the role of Bob1 in the differentiation of Tfh cells. Here, we show that Bob1 promotes Tfh cell differentiation by enhancing Bcl6 and BTLA expression. Bob1 can directly bind to and transactivate the promoters of *Bcl6* and *Btla*, which fits with our observation that T cells lacking Bob1 have an intrinsic defect in Tfh cell development and hence T cell-dependent immune responses.

Results

Follicular T helper cells express high levels of Bob1

Recently, we have reported high levels of mRNA for the transcriptional coactivator Bob1 in human Tfh cells (Rasheed *et al*, 2006); however, the role of Bob1 in Tfh cell differentiation and/or function remained obscure. In order to analyze Tfh cell development in mice and in particular the role of Bob1 in this process, we first generated large-scale gene expression profiles for murine $CD4^+$ T-cell subsets to compare the expression signatures of mouse and human Tfh cells. To this end, we immunized C57BL/6 mice with NP-KLH precipitated in alum (i.p.) and analyzed global mRNA expression levels in $CXCR5^-ICOS^-$ naïve $CD4^+$ T cells, $CXCR5^-ICOS^+$ activated $CD4^+$ T cells, and $CXCR5^{hi}ICOS^+$ follicular helper $CD4^+$ T cells (Tfh cells) from the spleen on day 7 following immunization (Appendix Fig S1).

The expression of signature genes appeared to be largely conserved between human and mouse Tfh cells (Fig 1A and Rasheed *et al*, 2006). Apart from the prominent expression of Bcl6, we observed strong signals for the transcriptional regulators Ascl2, IRF4, BATF, NFATc1, and c-Maf, as well as members of the CD28 superfamily including PD1, BTLA, and CTLA4. Mouse Tfh cells showed also high expression levels for factors that have so far not been assigned a role in Tfh cell development or function such as E2F8,

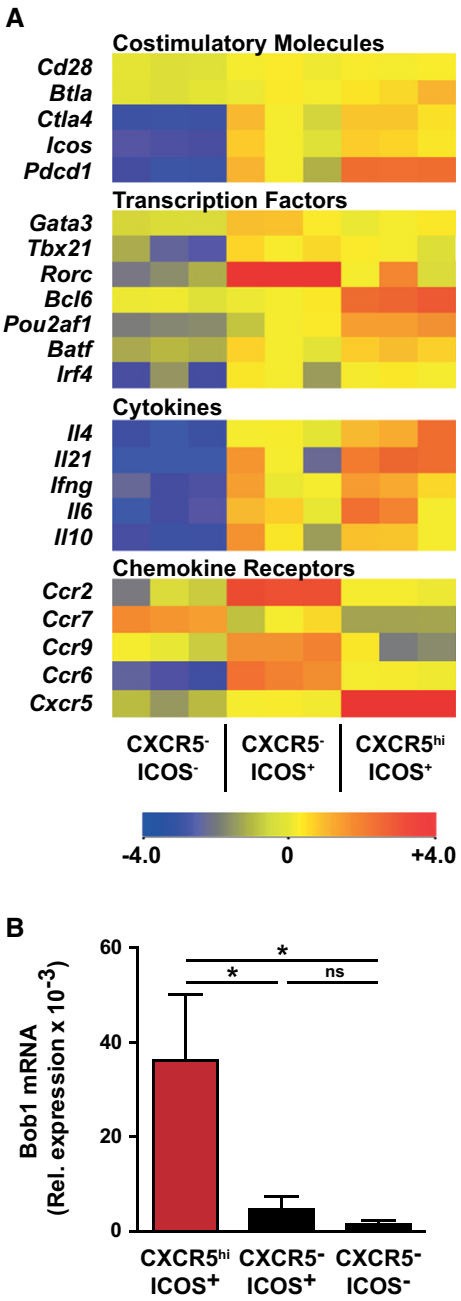

Figure 1. Expression of Bob1 is largely restricted to Tfh cells.

A Microarray-based transcriptome analysis of $CD4^+$ T-cell subsets from C57BL/6 mice. The heat map illustrates differentially expressed genes between $CD4^+CXCR5^{hi}ICOS^+$ Tfh cells, $CD4^+CXCR5^-ICOS^+$ effector/memory T cells, and $CD4^+CXCR5^-ICOS^-$ naïve T cells. Cells were purified from the spleen of mice 7 days after immunization with NP-KLH with alum. Data are derived from three independent array analyses (10 mice/array) for each T-cell subset.

B Validation of microarray expression data for Bob1 mRNA by qPCR for the same $CD4^+$ T-cell subsets as described in (A). Data shown are derived from four independent experiments (mean ± SD). *$P < 0.05$; unpaired Mann–Whitney test.

Fam84a, Helios, Ikaros, and Sostdc1. Similar to human CXCR5^{hi} ICOS^{hi} Tfh cells, we obtained strong signals for Bob1 in mouse CXCR5^{hi}ICOS^{+} Tfh cells. The expression of Bob1 mRNA is 10-fold higher in CXCR5^{hi}ICOS^{+} Tfh cells compared to CXCR5^{-}ICOS^{-} naive CD4^{+} T cells and threefold higher than in activated CXCR5^{-} ICOS^{+}CD4^{+} T cells, which was verified by quantitative PCR (Fig 1B). In contrast, mRNAs for GATA3, T-bet (*Tbx21*), and RORγ (*Rorc*), master regulators for the differentiation of Th1, Th2, and Th17 cells, respectively, are predominantly expressed in CXCR5^{-}ICOS^{+}CD4^{+} T cells, indicating that this population comprises diverse subsets of activated T helper cells (Fig 1A). The expression of cytokines by mouse CXCR5^{hi}ICOS^{+} Tfh cells is mostly restricted to IL-21 and IL-4, though IL-6, IL-10, and IFN-γ were also detectable at low levels.

Bob1-deficiency impairs follicular T helper cell development, in particular in gut-associated lymphoid tissue

Bob1-deficient mice have a major defect in T cell-dependent immune responses. Upon immunization with T cell-dependent antigens these animals fail to develop GCs and are largely devoid of isotype-switched plasma cells secreting high-affinity IgG. To explore the requirement for Bob1 in Tfh cell development and GC formation, we immunized *Pou2af1*$^{-/-}$ mice and heterozygous littermates with sheep red blood cells (SRBCs) and assessed Tfh cell formation on day 7 following immunization at the peak of the GC reaction. Consistent with previous reports, *Pou2af1*$^{+/-}$ mice did not differ from wild-type mice with regard to T cell-dependent immune responses and were therefore used as controls (Qin *et al*, 1998). Tfh cells were identified by coexpression of the chemokine receptor CXCR5 and at least one of the three costimulatory molecules ICOS, PD1, or BTLA on CD4^{+} T cells isolated from the spleen (SPL), mesenteric lymph nodes (MLN), or Peyer's patches (PP). Despite the lack of GCs in secondary lymphoid organs or appreciable amounts of class-switched IgG in the serum of *Pou2af1*$^{-/-}$ mice (data not shown), these animals develop Tfh cells expressing ICOS, PD1, or BTLA. However, compared to heterozygous littermates, *Pou2af1*$^{-/-}$ mice showed a severe organ-dependent defect in the generation of CXCR5^{hi}ICOS^{+}, CXCR5^{hi}PD1^{+}, and CXCR5^{hi}BTLA^{hi} Tfh cells (Fig 2A). The reduction in the frequency of Tfh cells was most severe in MLN and PP and less pronounced in the spleen and particularly strong for Tfh cells expressing BTLA (Fig 2A and B).

In detail, in *Pou2af1*$^{-/-}$ mice, the frequency of CXCR5^{hi}ICOS^{+} CD4^{+} cells was reduced by 56% in spleen, 81% in MLN, and 85% in PP. The frequency of CXCR5^{hi}PD1^{+} CD4^{+} T cells was reduced by 46% in spleen, 76% in MLN, and 86% in PP and that of CXCR5^{hi}BTLA^{hi}CD4^{+} T cells by 60% in spleen, 88% in MLN, and 94% in PP (Fig 2B). In view of the reduced numbers of CD4^{+} T cells in secondary lymphoid organs of *Pou2af1*$^{-/-}$ mice compared to wild-type or heterozygous control mice (Brunner *et al*, 2007), CXCR5^{hi}BTLA^{hi} Tfh cells were almost absent from PP.

Previous work demonstrated that Bob1, Oct2, and NF-κB cooperatively control the expression of *Cxcr5* in B cells and that Bob1 is required for maximal *Cxcr5* promoter activity in these cells (Wolf *et al*, 1998). To test whether Bob1 may contribute to the expression of CXCR5 in CD4^{+} T cells, we compared the expression level of CXCR5 on B cells and CD4^{+} T cells from Bob1-deficient and heterozygous control mice. Interestingly, B cells and CD4^{+} T cells from spleens of wild-type mice express invariably higher levels of CXCR5 than their counterparts from wild-type MLN or PP (Fig 2C and D). As expected, surface expression of CXCR5 was significantly reduced on B cells in *Pou2af1*$^{-/-}$ mice (Fig 2C). Similarly, we observed a diminished expression of CXCR5 on CD4^{+} T cells in the knockout mice (Fig 2D). It is therefore likely that Bob1 promotes the expression of CXCR5 not only in B cells but also in CD4^{+} T cells and that the organ-dependent reduction in the frequency of Tfh cells in *Pou2af1*$^{-/-}$ mice might be associated with the organ-dependent differences in CXCR5 expression.

Given the difference in the frequencies of Tfh cells expressing ICOS, PD1, or BTLA in *Pou2af1*$^{+/-}$ mice and their decrease in *Pou2af1*$^{-/-}$ mice, we analyzed to what extent these markers are coexpressed or define non-overlapping subsets of Tfh cells. Strikingly, only a small fraction of CD4^{+}CXCR5^{hi} T cells simultaneously expressed ICOS, PD1, and BTLA (Fig 2E). In line with the results described above, we obtained an organ-dependent pattern for the coexpression of costimulatory molecules on CD4^{+} T cells. About 31% of splenic Tfh cells from *Pou2af1*$^{+/-}$ mice express all three molecules, a value that drops to 15 and 17% in MLN and PP, respectively. In Bob1-deficient mice, coexpression of these markers was restricted to a much lower proportion of Tfh cells: 25% in spleen, 8% in MLN, and 2% in PP. Moreover, there was a remarkably strong reduction in the fraction of CXCR5^{hi}BTLA^{hi}CD4^{+} T cells in *Pou2af1*$^{-/-}$ mice independent of the expression of ICOS or PD1. Compared to heterozygous control mice, the frequency of these cells is reduced by 47% in spleen, 62% in MLN and 84% in PP such that BTLA^{hi} Tfh cells are rare in PP of *Pou2af1*$^{-/-}$ mice. Interestingly, we could hardly detect CXCR5^{hi}CD4^{+} T cells that coexpress PD1 and BTLA but lack ICOS expression in heterozygous control and knockout animals. In summary, ICOS, PD1, and BTLA appear to mark overlapping but not identical subsets of Tfh cells, whose development is strongly diminished in Bob1-deficient animals.

Figure 2. Bob1-deficient mice show an organ- and subset-dependent defect in the generation of Tfh cells.
Analysis of Tfh cell development in the spleen (SPL), mesenteric lymph nodes (MLN), and Peyer's patches (PP) of *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/-}$ mice following immunization with sheep red blood cells.

A, B Generation of Tfh cells characterized by the coexpression of CXCR5 with either ICOS, PD1, or BTLA on day 7 after immunization. (A) Representative flow cytometry plots indicating the frequency of CXCR5^{hi}ICOS^{+}, CXCR5^{hi}PD1^{+}, and CXCR5^{hi}BTLA^{hi} Tfh cells in *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/-}$ mice. Dot plots show gated CD4^{+}7AAD^{-} T cells. (B) Summarized data for the analysis shown in (A) (n = 17 for ICOS^{+}, n = 14 for PD1^{+}, and n = 10 for BTLA^{+} Tfh cells from at least four independent experiments; mean ± SEM).

C, D CXCR5 expression on B220^{+} B cells (C) and CD4^{+} T cells (D) in *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/-}$ mice. The graphs show the mean fluorescence intensity (MFI) for CXCR5 (n = 5–7).

E Coexpression of ICOS, PD1, and BTLA on Tfh cells. CD4^{+}CXCR5^{hi} T cells were analyzed for the simultaneous expression of these costimulatory molecules. Venn diagrams illustrate the overlap of ICOS, PD1, and BTLA expression on CD4^{+}CXCR5^{hi} T cells. Data represent mean values from four independent experiments.

Data information: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; unpaired Mann–Whitney test.

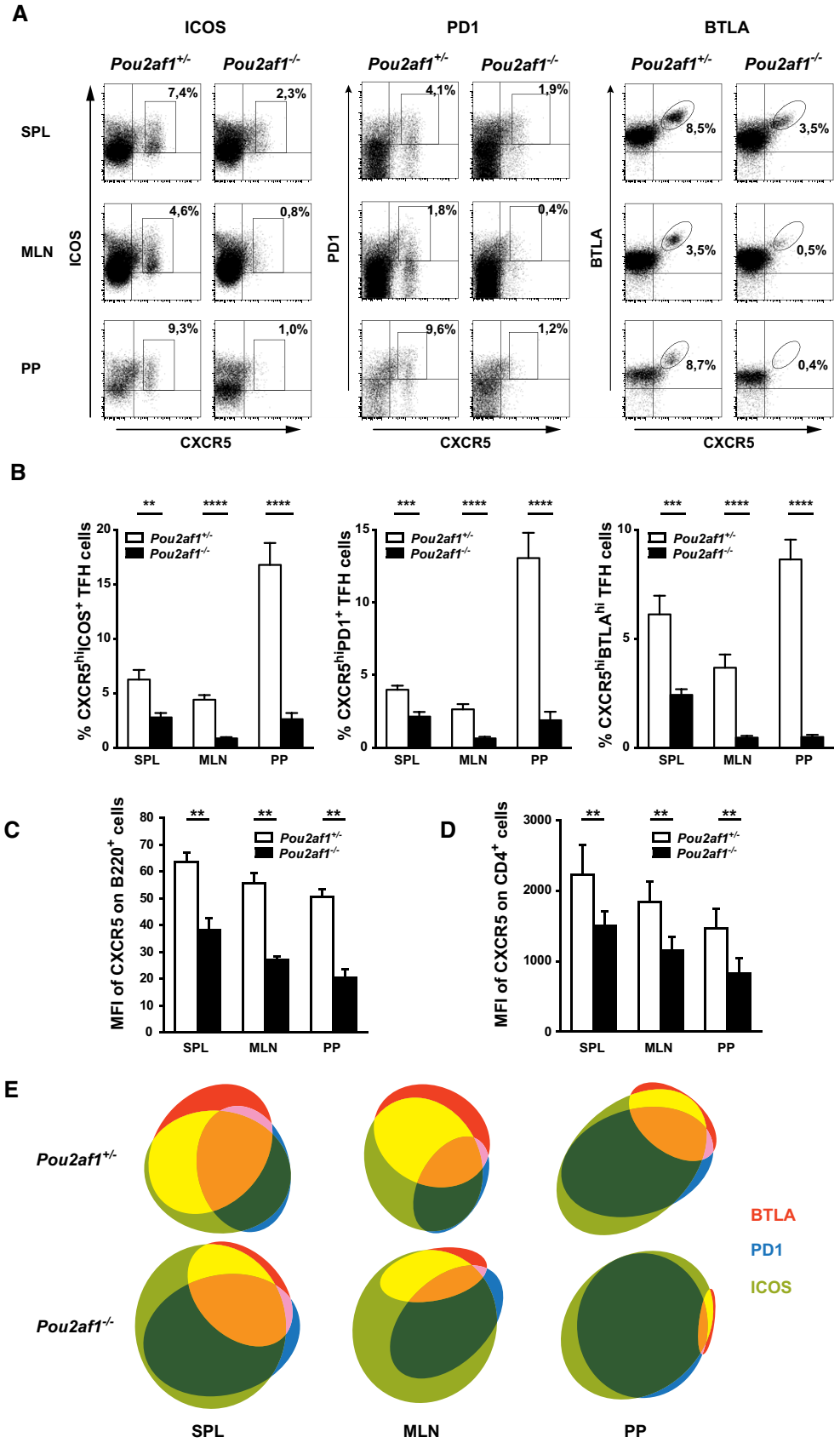

Figure 2.

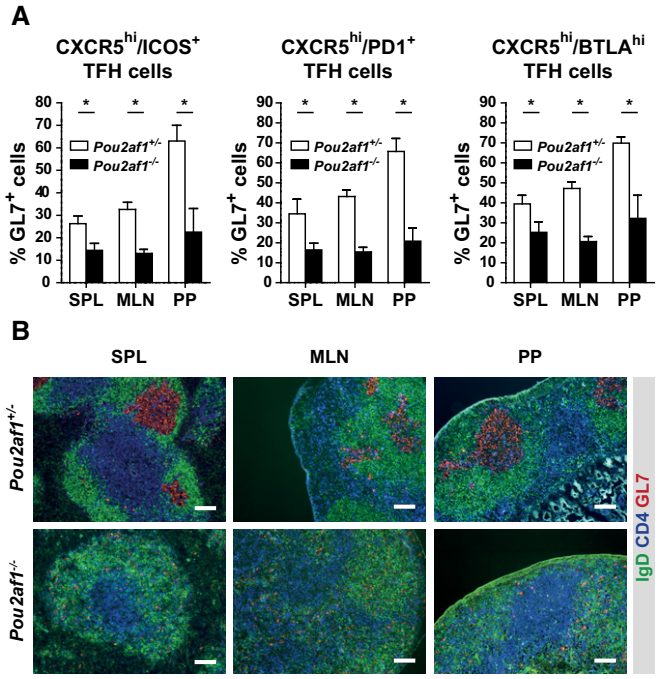

Figure 3. Bob1-deficient mice show a strong reduction in the frequency of GL7$^+$ Tfh cells.

A Flow cytometric analysis of GL7 expression on Tfh cells. Mice were immunized with SRBC and 7 days later analyzed for the presence of GL7-expressing CXCR5^{hi}ICOS$^+$, CXCR5^{hi}PD1$^+$, and CXCR5^{hi}BTLA^{hi} Tfh cells in the spleen (SPL), mesenteric lymph nodes (MLN), and Peyer's patches (PP) (n = 4, mean ± SD). *P < 0.05; unpaired Mann–Whitney test.

B Representative immunofluorescence analyses to detect GL7$^+$ T and B cells in secondary lymphoid organs of *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/-}$ mice. Tissue sections obtained from mice 7 days after immunization with SRBC were stained with anti-GL7 (red), anti-CD4 (blue), and anti-IgD (green) to reveal the localization of GL7$^+$ B and T cells in relationship to the T-cell zones and B-cell follicles. Data are representative for at least four mice per group. Scale bar, 100 μm.

Pou2af1$^{-/-}$ mice develop fewer GL7$^+$ Tfh cells after immunization

The GL7 epitope is highly expressed on activated B and T cells located in GCs and commonly used for the visualization of GC formation upon immunization with T cell-dependent antigens. Consequently, GL7 was found to identify GC-resident Tfh cells whereas GL7$^-$ (pre-GC) Tfh cells are mostly localized within the T-cell zones (Yusuf *et al*, 2010). The lack of GC formation in *Pou2af1*$^{-/-}$ mice led us to investigate whether Bob1-deficiency impedes the generation of GL7$^+$ Tfh cells upon immunization with SRBCs. To our surprise, we could detect GL7$^+$ Tfh cells within the populations of ICOS$^+$, PD1$^+$, and BTLA^{hi} Tfh cells in all secondary lymphoid organs analyzed (Fig 3A). Again, we observed an organ- and subset-dependent reduction in the frequency of GL7$^+$ Tfh cells in *Pou2af1*$^{-/-}$ mice beyond the general reduction in the frequency of Tfh cells. This reduction was again strongest in MLN and PP (CXCR5^{hi}ICOS$^+$ Tfh cells: 60% in MLN and 64% in PP; CXCR5^{hi}PD1$^+$ Tfh cells: 62% in MLN and 68% in PP; CXCR5^{hi}BTLA^{hi} Tfh cells: 53% in MLN and 50% in PP). The decrease in the frequencies of GL7$^+$ Tfh cells in the spleen was less pronounced (CXCR5^{hi}ICOS$^+$: 45%, CXCR5^{hi}PD1$^+$: 52%, CXCR5^{hi}BTLA^{hi}: 37%). In view of the failure of *Pou2af1*$^{-/-}$ mice to form GCs, we next analyzed the localization of GL7$^+$ Tfh cells in secondary lymphoid organs of *Pou2af1*$^{-/-}$ mice. Positive staining for GL7, PNA, and lack of immunoglobulin D expression identified GCs in immunofluorescence histology. In *Pou2af1*$^{+/-}$ mice, expression of GL7 was exclusively found on lymphocytes in GCs but absent on IgD$^+$ B cells of primary B-cell follicles and CD4$^+$ T cells in the T-cell zones of secondary lymphoid organs (Fig 3B). In Bob1-deficient animals, GCs are non-detectable and the borders of B- and T-cell follicles are blurred, in particular in MLN and PP. Nevertheless, we could clearly detect GL7$^+$CD4$^+$ T cells that were evenly distributed within the B cell-rich areas but absent from the T cell-rich zones of spleen, MLN, and PP (Fig 3B). Our results suggest that the upregulation of GL7 on CD4$^+$ T cells does not rely on the GC microenvironment though the frequency of GL7$^+$ Tfh cells is clearly diminished in *Pou2af1*$^{-/-}$ mice.

CD4$^+$ T cell-autonomous defect in the development of Tfh cells in *Pou2af1*$^{-/-}$ mice

Bob1-deficiency results in multiple defects in early and late B-cell differentiation (Hess *et al*, 2001). To rule out that the defect in Tfh cell development in *Pou2af1*$^{-/-}$ mice is simply a result of altered B-cell–T-cell interactions and to directly test for a T cell-intrinsic, Tfh-promoting activity of Bob1, we analyzed Tfh cell development in mixed bone marrow (BM) chimeras. To this end, we reconstituted irradiated congenic CD45.1$^+$C57BL/6 mice with equal numbers of bone marrow cells from *Pou2af1*$^{+/+}$CD45.2$^+$RFP$^+$ C57BL/6 and *Pou2af1*$^{-/-}$CD45.2$^+$RFP$^-$ C57BL/6 mice. After 8 weeks, following successful reconstitution, mice were immunized with SRBC and analyzed 7 days later. Consistent with a previous report by Hess *et al* (2001), *Pou2af1*$^{-/-}$ bone marrow cells exhibited a defect in repopulating the bone marrow B-cell compartment. Consequently, the majority (> 80%) of mature recirculating B220$^+$ B cells present in secondary lymphoid organs was Bob1-sufficient, whereas < 20% were Bob1-deficient B cells (Fig 4A). In contrast, these organs were repopulated by comparable numbers of *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/+}$ CD4$^+$ T cells (Fig 4A). In line with the recent observation that the development of GC B cells is hampered in *Pou2af1*$^{-/-}$ mice (Karnowski *et al*, 2012), we observed a significant reduction in the frequency and numbers of FAS$^+$GL7$^+$ GC B cells within the population of *Pou2af1*$^{-/-}$ B cells in the BM chimeras upon immunization with SRBCs (Figs 4B and EV1A). The most striking observation was, however, a defect in the development of Tfh cells from mature, recirculating *Pou2af1*$^{-/-}$ CD4$^+$ T cells in these experiments. Bob1-deficient Tfh cells were selectively underrepresented in the Tfh cell pool, showing reduced frequencies and numbers as compared to wild-type Tfh cells (Figs 4C and EV1B). The frequencies of CXCR5^{hi}ICOS$^+$ and CXCR5^{hi}PD1$^+$ Tfh cells were reduced by about 30–40% in spleen and PP and about 50–55% in MLN and slightly stronger for CXCR5^{hi}ICOS$^+$ Tfh cells when compared with CXCR5^{hi}PD1$^+$ Tfh cells. Similar to our observation in Bob1 knockout mice, this effect was most pronounced for the population of CXCR5^{hi}BTLA^{hi} Tfh cells, which was reduced by 70–75% in spleen and PP and by 85% in MLN (Fig 4C, right panel). Similar results were obtained in adoptive transfer experiments, in which we transferred equal numbers of naive CD4$^+$CD62L$^+$ T cells from *Pou2af1*$^{-/-}$-OTII or *Pou2af1*$^{+/-}$-OTII mice into *Cd4*$^{-/-}$ recipients. Following immunization with OVA, we observed significantly higher frequencies of *Pou2af1*$^{+/-}$ compared to *Pou2af1*$^{-/-}$ Tfh cells

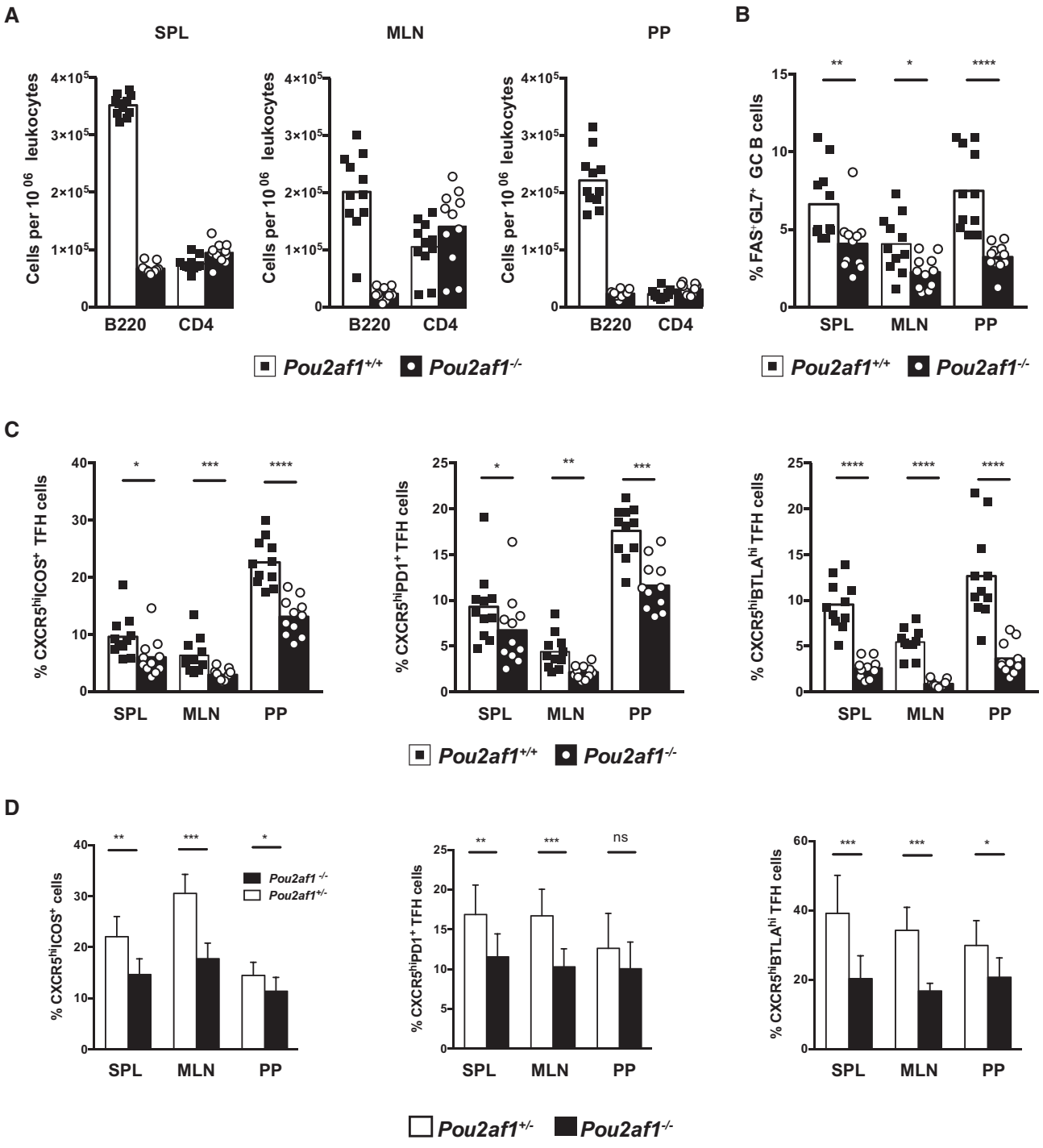

Figure 4. T cell-intrinsic defect in the development of *Pou2af1*$^{-/-}$ Tfh cells.

A–C Tfh cell development in the spleen (SPL), mesenteric lymph nodes (MLN), and Peyer's patches (PP) of WT:*Pou2af1*$^{-/-}$ mixed bone marrow chimeras 7 days after immunization with SRBC. (A) Total numbers of *Pou2af1*$^{+/+}$ and *Pou2af1*$^{-/-}$ CD4$^+$ T cells and B220$^+$ B cells per million leukocytes in mixed bone marrow chimeras. (B) Frequencies of *Pou2af1*$^{+/+}$ and *Pou2af1*$^{-/-}$ B220$^+$FAS$^+$GL7$^+$ GC B cells in mixed bone marrow chimeras. (C) Frequencies of *Pou2af1*$^{+/+}$ and *Pou2af1*$^{-/-}$ Tfh cells as identified by the coexpression of CXCR5 and ICOS (CXCR5^{hi}ICOS$^+$), CXCR5 and PD1 (CXCR5^{hi}PD1$^+$), or CXCR5 and BTLA (CXCR5^{hi}BTLA^{hi}) by CD4$^+$ T cells in mixed bone marrow chimeras.

D Tfh cell development in *Cd4*$^{-/-}$ mice reconstituted with *Pou2af1*$^{+/-}$ or *Pou2af1*$^{-/-}$ CD4$^+$CD62L$^+$OTII$^+$ T cells. Mice were analyzed 7 days after immunization with OVA in alum for the presence of CXCR5^{hi}ICOS$^+$, CXCR5^{hi}PD1$^+$, and CXCR5^{hi}BTLA^{hi} Tfh cells. Data are derived from two independent experiments with eight animals per group (mean ± SD).

Data information: The graphs in (A–C) show combined data from two out of four independent experiments (n = 11). Data in (D) are derived from two independent experiments with eight animals per group (mean ± SD). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; unpaired Mann–Whitney test.

in the spleen, MLN, and PP (Figs 4D and EV1C). Our results demonstrate that Bob1-deficiency in $CD4^+$ T cells impedes their differentiation into Tfh cells and indicate a T cell-intrinsic defect that the cells cannot overcome by cognate interaction with wild-type B cells.

The expression of the STAT3-activating cytokine receptors IL-6R and IL-21R is independent of Bob1

The differentiation of mature $CD4^+$ T cells into Tfh cells requires signals provided by STAT3-activating cytokines, including IL-6 and IL-21. Both cytokines were shown to contribute to the induction and maintenance of Tfh cells and enhanced Bcl6 expression, though their role appears to be redundant (Eto *et al*, 2011). To address the question whether the reduced frequency of Tfh cells in *Pou2af1*$^{-/-}$ mice might be related to a defect in IL-21 or IL-6 signaling, we compared the expression of IL-6Rα (Fig 5A) and IL-21R (Fig 5B) on *Pou2af1*$^{+/+}$ and *Pou2af1*$^{-/-}$ Tfh cells from mixed bone marrow chimeras. To this end, we transferred equal numbers of *Pou2af1*$^{+/+}$CD45.2$^+$ CD90.1$^+$ and *Pou2af1*$^{-/-}$CD45.2$^+$CD90.2$^+$ bone marrow cells into irradiated congenic CD45.1$^+$ C57BL/6 mice. Reconstituted mice were

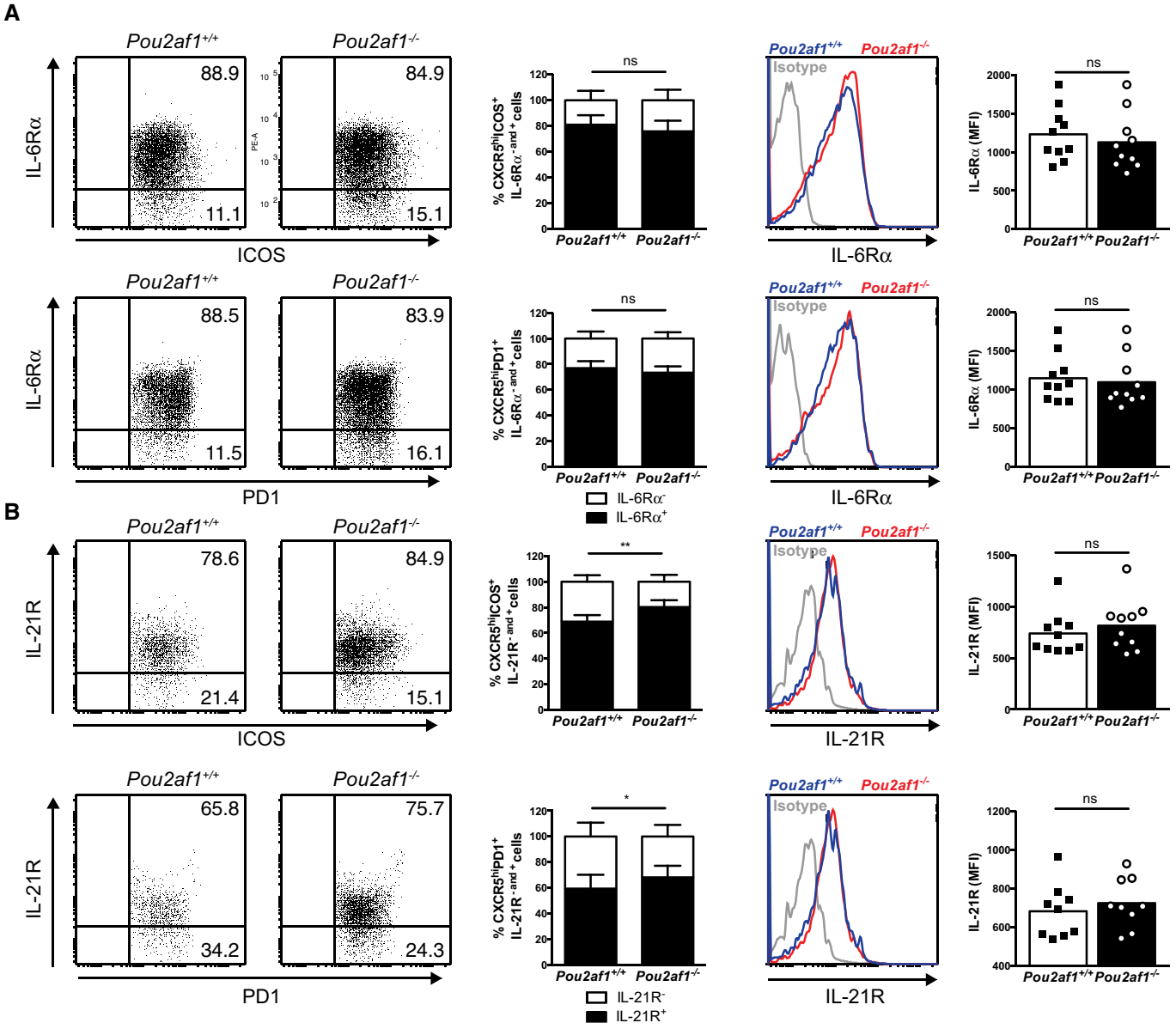

Figure 5. Bob1 deficiency does not affect the expression of IL-21R and IL-6Rα on Tfh cells.

A, B Expression of IL-6Rα (A) and IL-21R (B) on $CXCR5^{hi}ICOS^+$ and $CXCR5^{hi}PD1^+$ Tfh cells from *Pou2af1*$^{-/-}$:*Pou2af1*$^{+/+}$ bone marrow chimeras 7 days after immunization with SRBC. $CD4^+CXCR5^{hi}$ T cells from the spleen were analyzed by flow cytometry for the coexpression of ICOS and PD1 with either IL-6Rα or IL-21R. Left panels: Frequency of cytokine receptor expression on $ICOS^+$ or $PD1^+$ Tfh cells; right panels: expression level (mean fluorescence intensity) of cytokine receptors on $ICOS^+$ and $PD1^+$ Tfh cells. The figure shows combined data from two independent experiments (n = 10 for IL-6Rα and n = 9 for IL-21R). *$P < 0.05$; **$P < 0.01$; ns, not significant; unpaired Mann–Whitney test.

immunized with SRBC and analyzed 7 days later. We did, however, not observe major differences in the frequency of $CXCR5^{hi}ICOS^{+}$ or $CXCR5^{hi}PD1^{+}$ Tfh cells expressing IL-6Rα and IL-21R in Bob1-deficient or Bob1-sufficient Tfh cells with the exception of a slightly higher frequency of IL-21R expression in Bob1-deficient Tfh cells. In addition, we did not observe differences in the expression level of these receptors on Bob1-deficient or Bob1-sufficient Tfh cells (Fig 5A and B).

Bob1 promotes the expression of Bcl6

Next, we analyzed the expression of Bcl6, a key regulator of Tfh cell differentiation, in *Pou2af1*$^{+/-}$ and *Pou2af1*$^{-/-}$ mice immunized with SRBC. High levels of Bcl6 were detectable by flow cytometric analysis in 28% (spleen), 23% (MLN), and 29% (PP) of $CXCR5^{hi}ICOS^{+}$ Tfh cells from heterozygous *Pou2af1*$^{+/-}$ animals (Fig 6A). In contrast, only 9% (spleen), 6% (MLN), and 13% (PP) of the $CXCR5^{hi}ICOS^{+}$ Tfh cells from *Pou2af1*$^{-/-}$ mice expressed similar levels of Bcl6 (Fig 6A). To exclude the possibility that the reduction in Bcl6 expression is secondary to B-cell defects in Bob1-deficient mice, such as the impaired secretion of IL-6 (Karnowski *et al*, 2012), we analyzed Bcl6 expression in wild-type and knockout Tfh cells from *Pou2af1*$^{-/-}$:*Pou2af1*$^{+/+}$ mixed bone marrow chimeras—generated as described in the previous section—7 days after immunization with SRBC. In line with the results obtained with *Pou2af1*$^{-/-}$ mice (Fig 6A), we observed an impaired differentiation of Bob1-deficient $CD4^{+}$ T cells into $CXCR5^{hi}ICOS^{+}Bcl6^{hi}$ Tfh cells in the bone marrow chimeras (Fig 6B). In particular, we found significantly reduced levels of Bcl6 in Bob1-deficient $CXCR5^{hi}ICOS^{+}$ Tfh cells compared to their Bob1-sufficient counterparts (Fig 6C) and a decrease in the frequency of $CXCR5^{hi}Bcl6^{hi}$ GC-Tfh cells derived from *Pou2af1*$^{-/-}$ $CD4^{+}$ T cells (Fig 6D). Similar results were obtained for PD1-expressing Tfh cells in these mice. Bob1-deficient $CXCR5^{hi}PD1^{+}$ Tfh cells express lower levels of Bcl6 than their wild-type counterparts (Fig EV2A), and we observed lower frequencies of $CXCR5^{hi}PD1^{hi}$ GC-Tfh cells among Bob1-deficient $CD4^{+}$ T cells when compared to wild-type CD4 T cells in these mice (Fig EV2B). Our results strongly suggest that the loss of Bob1 results in a T cell-autonomous defect in Tfh cell development associated with a reduced expression of Bcl6 in these cells.

Bob1 together with Oct1/2 binds to and transactivates the *Bcl6* and *Btla* promoters

Based on our observation that only a small percentage of Bob1-deficient $CXCR5^{hi}ICOS^{+}$ Tfh cells expressed BTLA and Bcl6 when compared to their heterozygous counterparts, we wondered whether these genes are directly regulated by Bob1. *In silico* analyses revealed four potential octamer motifs within the first 2,000 bp upstream of the transcriptional start site of the *Btla* promoter and six potential octamer motifs for the *Bcl6* promoter (Fig 7A). The M1 motif of the *Btla* promoter represents a consensus octamer motif, while all other putative Oct/Bob1 binding sites of the *Btla* and *Bcl6* promoters differ in one or more positions from the consensus site. However, all of these sites harbor an adenine at position 5 of the octamer motif that is essential for ternary complex formation with Oct1 and Bob1 (Cepek *et al*, 1996; Pevzner *et al*, 2000). In electrophoretic mobility shift assays (EMSA), we observed binding of Oct1 and Oct2 to two putative Oct binding sites within the *Btla* promoter (M1 and M4; Fig 7B) and two sites within the *Bcl6* promoter (M3 and M6; Fig 7C), similar to the complex formation at the consensus octamer motif. Moreover, complex formation on these sites could be efficiently inhibited by competition with unlabeled double-stranded oligonucleotides containing the consensus octamer motif, whereas oligonucleotides comprising an unrelated binding site like the consensus NF-κB binding motif failed to compete for Oct1 and Oct2 binding (Fig 7D). Together, these data indicate that at least two of the predicted octamer motifs within each promoter serve as binding sites for Oct1 and Oct2 that likely recruit Bob1.

To find out whether these octamer sites of the *Btla* and *Bcl6* promoters are functional *in vivo*, we performed chromatin immunoprecipitation (ChIP) experiments on naïve wild-type or heterozygous and Bob1-deficient $CD4^{+}$ T cells that were maintained for 4 h under Tfh-inducing conditions in the presence of IL-6 and IL-21 *in vitro*. To this end, we used antibodies against the octamer binding transcription factors Oct1 and Oct2, as well as the transcriptional co-activator Bob1. Oct2 and Bob1 bound to the consensus octamer site M1 within the *Btla* promoter with similar affinity as observed for the *Ifng* promoter (Fig 8A and B), which we recently identified as a target of Oct1/2 and Bob1 (Brunner *et al*, 2007). In contrast, only Oct2 bound to the M4 motif of the *Btla* promoter (Fig 8C); noteworthy, binding of Oct2 to the M1 as well as the M4 motifs of the *Btla* promoter seemed to require the presence of Bob1 as binding was largely abrogated in the absence of Bob1 (Fig 8B and C).

In the case of the *Bcl6* promoter, we observed a strong binding of Bob1 together with Oct1 and Oct2 at the M3 motif that is largely abolished in the absence of Bob1 (Fig 8E). In contrast, we could not detect binding of Oct1/2 and Bob1 using primers amplifying a region encompassing the M1 and M2 motifs (data not shown) or the M6 motif (Fig 8F) of the *Bcl6* promoter, similar to the analysis of the intergenic region at chromosome 8 that served as an internal negative control (Fig 8D).

We then tested in luciferase reporter assays whether Bob1 together with Oct2 is able to transactivate the *Btla* and *Bcl6*

Figure 6. Reduced expression of Bcl6 by Bob1-deficient Tfh cells.

A Expression of Bcl6 in $CXCR5^{hi}ICOS^{+}$ Tfh cells in *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/-}$ mice 7 days after immunization with SRBC. Left: representative histograms showing Bcl6 expression in *Pou2af1*$^{-/-}$ (red) and *Pou2af1*$^{+/-}$ (blue) $CD4^{+}CXCR5^{hi}ICOS^{+}$ Tfh cells; filled line: Bcl6 isotype control. Right: summarized results illustrating the proportion of $CXCR5^{hi}ICOS^{+}$ Tfh cells that express Bcl6 in *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/-}$ mice (n = 5, bars indicate means).

B Bcl6 expression by Tfh cells in *Pou2af1*$^{+/+}$:*Pou2af1*$^{-/-}$ mixed bone marrow chimeras 7 days after immunization with SRBC. The graphs illustrate the proportion of $Bcl6^{hi}CXCR5^{hi}ICOS^{+}$ Tfh cells within the population of *Pou2af1*$^{+/+}$ and *Pou2af1*$^{-/-}$ $CD4^{+}$ T cells from bone marrow chimeras (n = 7).

C Expression level of Bcl6 in *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/+}$ $CXCR5^{hi}ICOS^{+}$ Tfh cells vs. non-Tfh cells from mixed bone marrow chimeras and a comparison of the mean fluorescence intensity (MFI) for Bcl6 (n = 9).

D Frequency of $CXCR5^{hi}Bcl6^{hi}$ GC-Tfh cells among *Pou2af1*$^{-/-}$ and *Pou2af1*$^{+/+}$ $CD4^{+}$ T cells in mixed bone marrow chimeras (n = 9).

Data information: Graphs show combined data from two (A, B) or three (C, D) independent experiments. SPL, spleen; MLN, mesenteric lymph nodes; PP, Peyer's patches. *P < 0.05; **P < 0.01; ***P < 0.001; unpaired Mann–Whitney test.

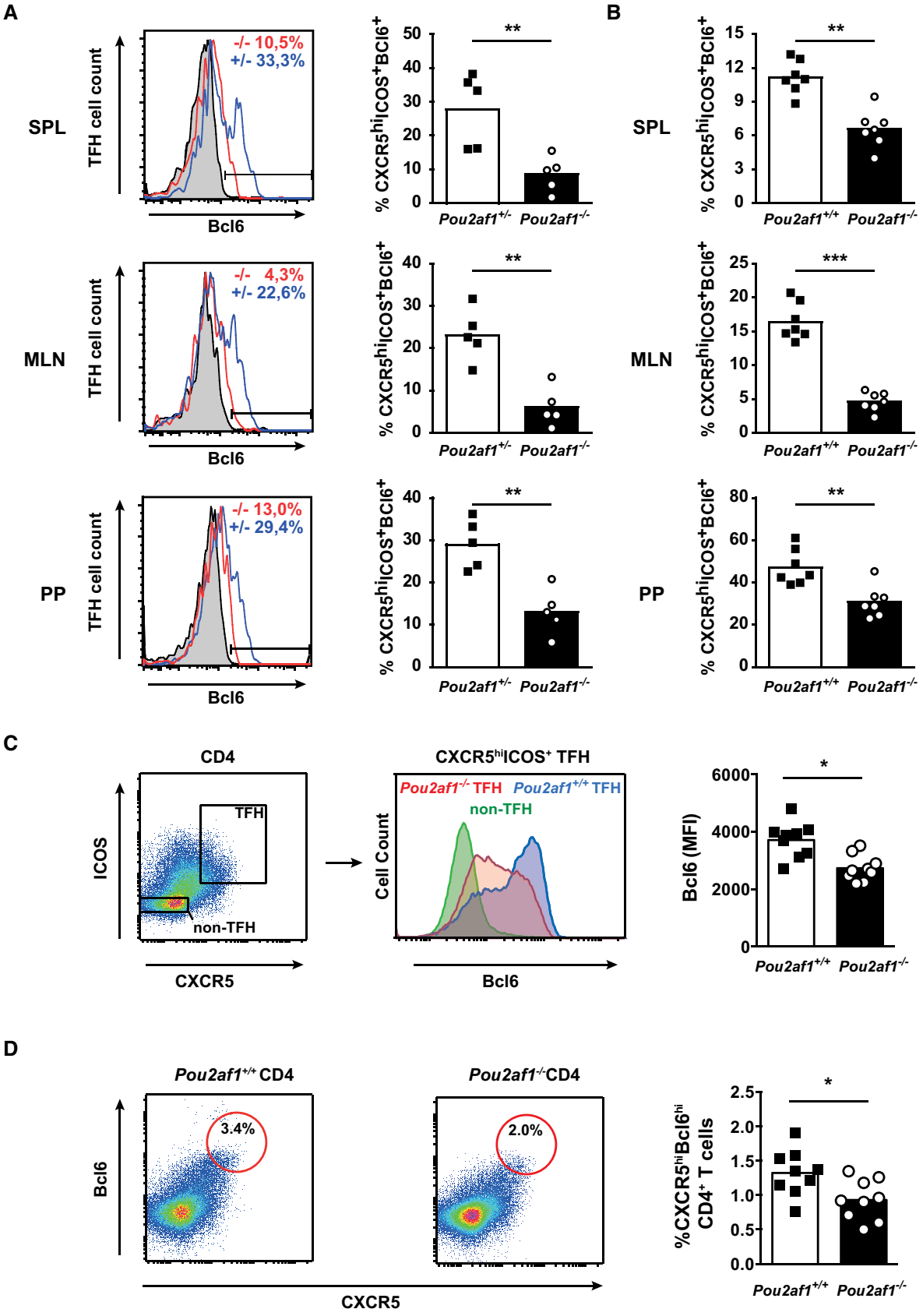
A
SPL
TFH cell count
-/- 10,5%
+/- 33,3%
Bcl6
% CXCR5hiICOS+BCl6+
Pou2af1+/- Pou2af1-/-
MLN
-/- 4,3%
+/- 22,6%
PP
-/- 13,0%
+/- 29,4%
B
Pou2af1+/+ Pou2af1-/-
C
CD4
ICOS
TFH
non-TFH
CXCR5
CXCR5hiICOS+ TFH
Pou2af1-/- TFH Pou2af1+/+ TFH
non-TFH
Cell Count
Bcl6 (MFI)
D
Pou2af1+/+ CD4
Pou2af1-/-CD4
3.4%
2.0%
%CXCR5hiBcl6hi CD4+ T cells

Figure 6.

A

Btla M1	TGTGAAGATAAATTTGCATTTTCCCAAATT
Btla M2	TGGCCAATGGCATTTGCAACTGTGCAGGGA
Btla M3	AAAAAAAAAAATAAAAGTAAGGGGCAGAAT
Btla M4	CCTACATAAACATTTACATACACCCACACA
Bcl6 M1	AATCCAAATCCTTTTTACATCTCTTTCAAGT
Bcl6 M2	CAGGAAAGCTTCAGCAAAAGTCCAAGAGGA
Bcl6 M3	CAGTCCCGACGATTTACATAACACCACAAA
Bcl6 M4	ACACCACAAACTTGCAAAAGGCAAAAATCA
Bcl6 M5	AGACCCCGGGAAAGCAAAGCGCACTCTCCC
Bcl6 M6	ATGTCACCGAATAGCAAATTAGTTCTCAGA

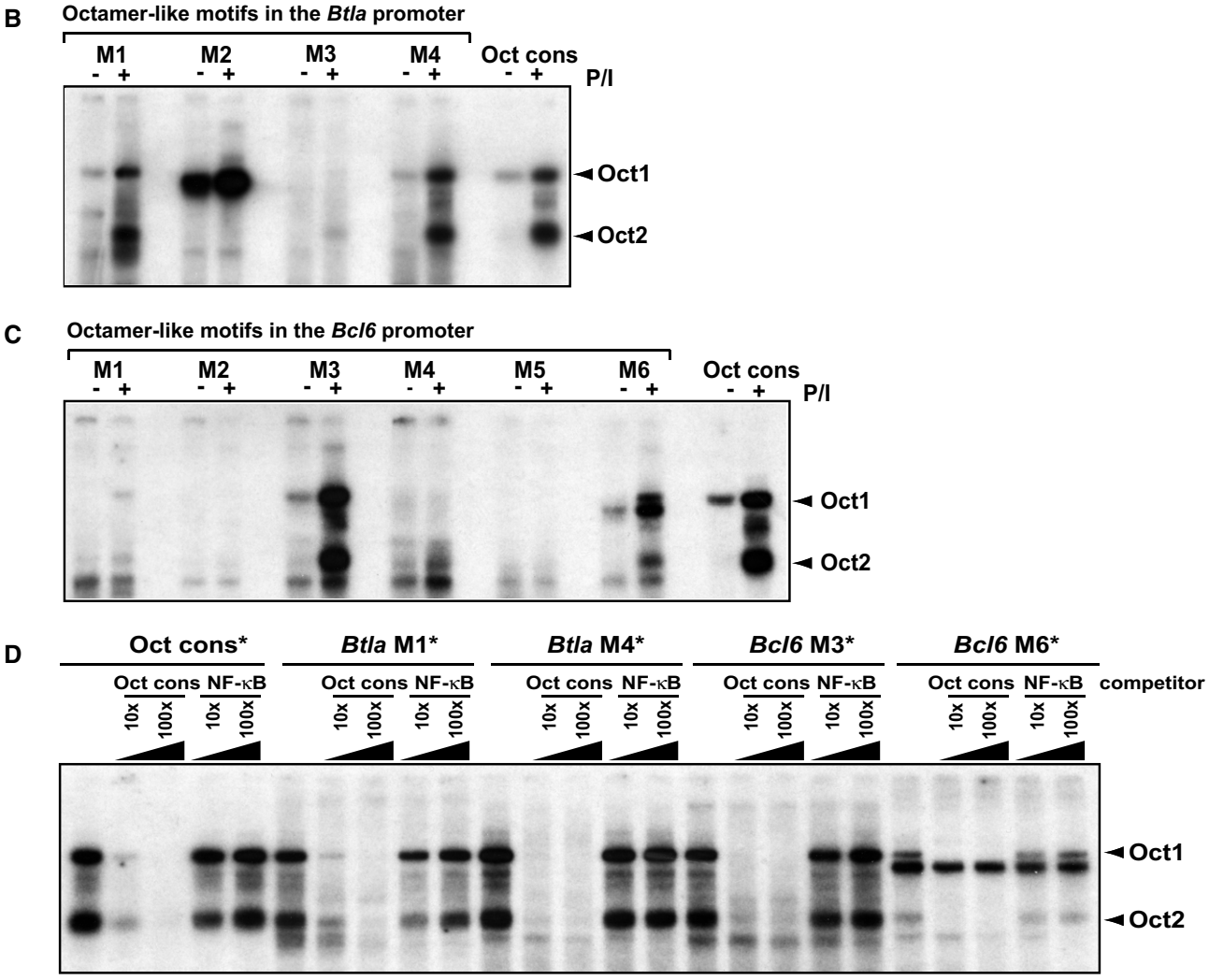

Figure 7. Identification of Oct1/2 binding sites in the promoters of the *Btla* and *Bcl6* genes.

A *In silico* search for potential Oct1/2 binding sites within the first 2,000 bp upstream of the transcriptional start site of the *Btla* and *Bcl6* genes (marked in red and green, respectively). Nucleotides that differ from the consensus octamer sequence ATGCAAAT are marked in blue.

B, C Inducible complex formation on the potential octamer sites within the *Btla* and *Bcl6* promoters. Purified murine $CD4^+$ T cells were left untreated or induced with PMA and ionomycin (P/I) for 18 h. Whole-cell extracts were analyzed by EMSA using labeled, double-stranded oligonucleotides containing either one of the potential octamer motifs of the *Btla* or *Bcl6* promoters as depicted in (A) or the consensus octamer site that served as an internal control.

D Competition analysis for complex formation on potential Oct binding sites in the *Btla* and *Bcl6* promoters. EMSA analysis of whole-cell extracts from purified $CD4^+$ T cells stimulated for 18 h with P/I. Radioactively labeled, double-stranded oligonucleotides containing one of the potential octamer binding sites of the *Btla* and *Bcl6* promoters were added to the reaction mixture together with a 10× or 100× molar excess of non-labeled oligonucleotides comprising either the consensus octamer motif or the consensus NF-κB motif.

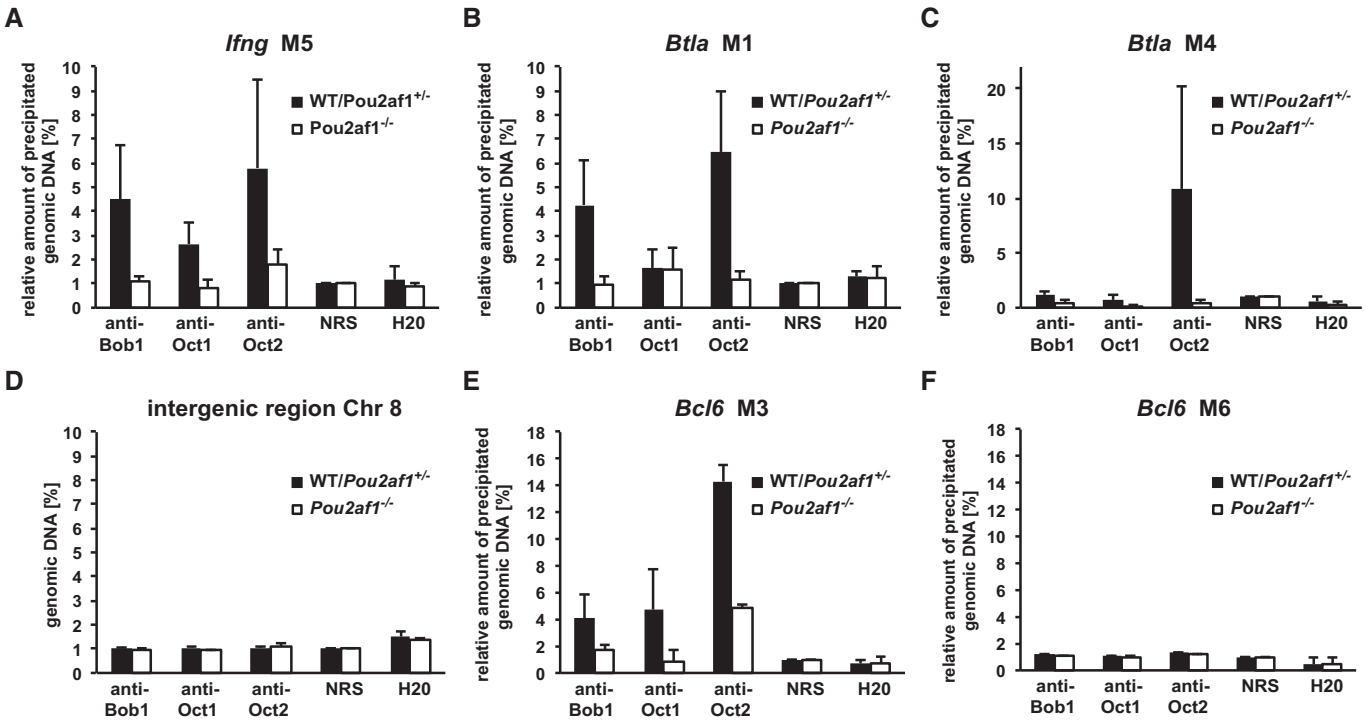

Figure 8. Oct1/2 bind together with Bob1 to specific octamer elements of the *Btla* and *Bcl6* promoters *in vivo*.

A–F Chromatin immunoprecipitation (ChIP) analysis of *in vitro* generated wild-type (wt), heterozygous (het), or Bob1-deficient (KO) Tfh cells using antibodies against Oct1, Oct2, or Bob1. Immunoprecipitation with normal rabbit serum (NRS) served as an internal negative control. Precipitated DNA was analyzed by qPCR using primers that amplify fragments specific for the functional octamer motif M5 of the *Ifng* promoter (A; external positive control), the M1 or M4 motifs of the *Btla* promoter (B and C), a part of the intergenic region at chromosome 8 (D; external negative control), or the M3 or M6 motifs of the *Bcl6* promoter (E and F). Shown are the mean values ± SD from three independent experiments.

promoters. The expression of Bob1 or Oct2 alone had little or no effect on *Btla* promoter activity, but a clear synergistic effect could be seen when both proteins—Bob1 together with Oct2—were co-expressed in NIH/3T3 cells that lack endogenous BTLA, Bob1, and Oct2 (Fig 9A). The transfection of the *Ifng* promoter served as an internal positive control (Fig 9A). The transactivation of the *Bcl6* promoter has been tested in EL-4 cells that are weakly positive for Bcl6 (Chevrier *et al*, 2014). Oct2 and also Bob1 alone could transactivate the *Bcl6* enhancer/promoter in cells activated by TPA plus ionomycin (T/I), which mimics antigen-receptor engagement. Since Bob1 itself has only little affinity to DNA and requires Oct1 or Oct2 for binding to the octamer motif, it very likely interacts with endogenous Oct1/Oct2 to transactivate the *Bcl6* promoter. But importantly, coexpression of Bob1 and Oct2 showed a strong synergistic effect on the activity of the *Bcl6* promoter (Fig 9B). This effect was lost upon functional inactivation of the octamer sites M3 or M6 in the *Bcl6* promoter by site-directed mutagenesis, suggesting that both binding sites contribute to *Bcl6* promoter activity (Fig 9B). In summary, our results provide strong evidence that in Tfh cells Bob1 together with Oct1/2 are direct regulators of the *Btla* as well as *Bcl6* promoter activities.

Discussion

The development of Tfh cells is intricately linked to B-cell differentiation along the GC pathway. Interestingly, Tfh cell differentiation relies on a set of transcription factors including Bcl6, BLIMP1, and IRF4 that also control late stages of B-cell development. Bcl6 is essential for the induction of Tfh cell differentiation as the reciprocal repression of Bcl6 and BLIMP1 controls the development of Tfh vs. non-Tfh $CD4^+$ T helper cell populations (Johnston *et al*, 2009; Nurieva *et al*, 2009; Yu *et al*, 2009). At later stages, Bcl6 is required for stable Tfh effector commitment and the maintenance of memory Tfh cells (Liu *et al*, 2012; Ise *et al*, 2014). Still, the regulation of Bcl6 in the course of Tfh cell differentiation is incompletely understood.

Bob1 is another factor that is essential to late B-cell differentiation (Corcoran *et al*, 2005) and highly expressed in Tfh cells (Rasheed *et al*, 2006; and as demonstrated here). Mice deficient for Bob1 show multiple defects in B-cell development, do not form GCs, and consequently lack high-affinity antibody-secreting plasma cells (Kim *et al*, 1996; Schubart *et al*, 1996; Hess *et al*, 2001; Brunner *et al*, 2003; Corcoran *et al*, 2005). On the T-cell side, the role of Bob1 remained largely enigmatic. Bob1 is induced in T cells upon TCR engagement or costimulation with PMA/ionomycin (Kang *et al*, 1992; Sauter & Matthias, 1997; Zwilling *et al*, 1997). In $CD4^+$ T cells, Bob1 directly contributes to the *Ifng* and *Il2* promoter activities and indirectly affects Th2 cytokine expression, thereby shaping the balance between Th1 and Th2 immunity (Brunner *et al*, 2007). More recently, we demonstrated that—similar to B cells (Kilzheimer *et al*, 2015)—NFAT and NF-κB signaling pathways are important for Bob1 expression in T cells (Mueller *et al*, 2013). Interestingly,

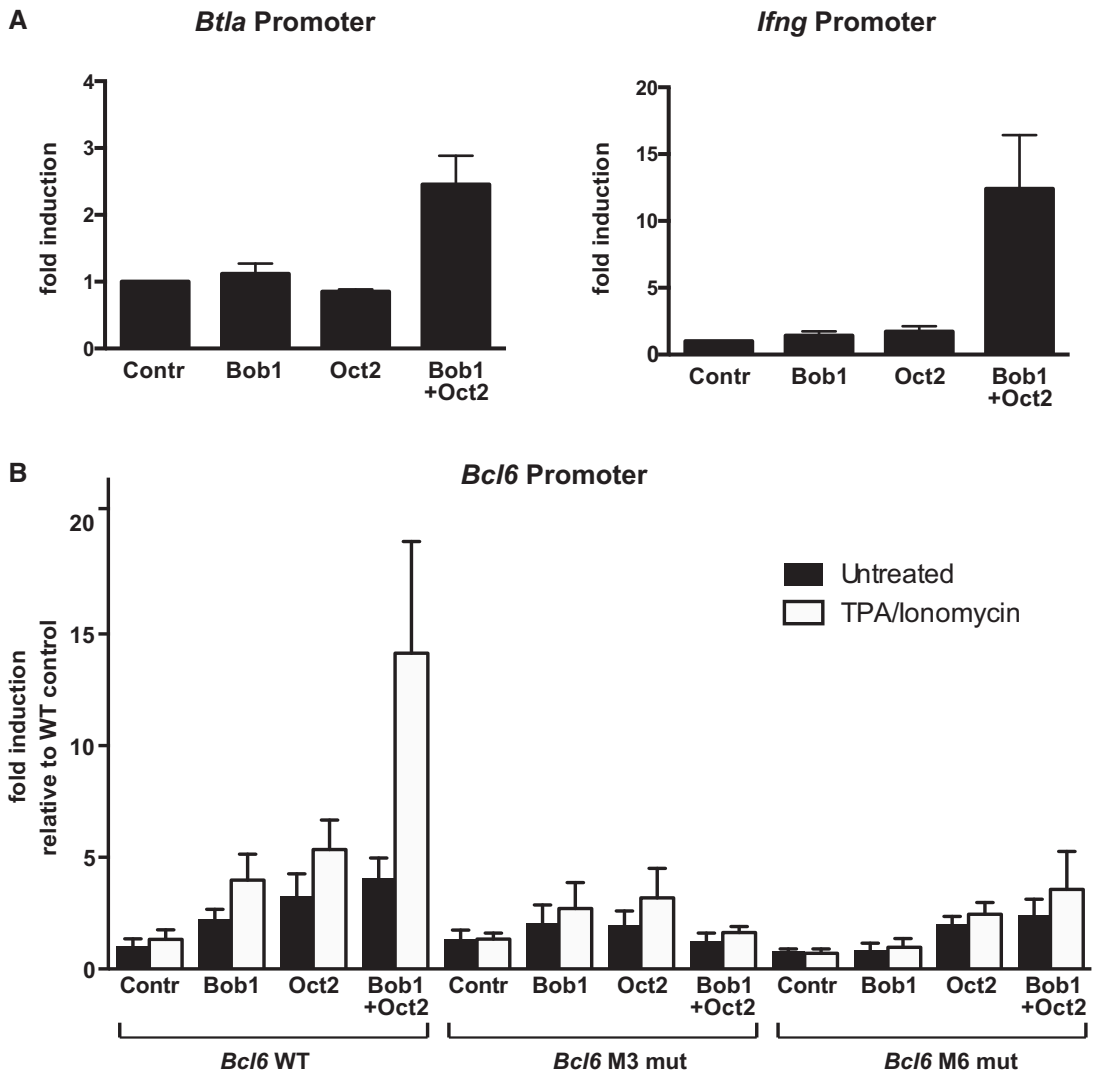

Figure 9. Bob1 and Oct2 synergistically transactivate the *Btla* and *Bcl6* promoter.

A NIH/3T3 cells were transfected with luciferase reporter constructs of the *Btla* (left panel) or *Ifng* (right panel) promoter along with expression vectors for Bob1 and/or Oct2.

B Analysis of the *Bcl6* enhancer/promoter construct in untreated or TPA/ionomycin-stimulated EL-4 cells. In addition, we have analyzed mutant *Bcl6* enhancer/promoter constructs in which either the motif M3 or M6 had been functionally inactivated by site-directed mutagenesis.

Data information: Data are expressed as fold-induction above the activity of the wild-type promoter without transfection of Bob1 or Oct2 ($n = 3$ for NIH3T3 cells; $n = 5$ for wild-type *Bcl6* promoter analysis in EL-4 cells; and $n = 3$ for mutant *Bcl6* promoter analysis in EL-4 cells). Data are shown as mean ± SEM.

NFAT—and especially NFATc1—is a dominant transcription factor in follicular T cells (Vaeth *et al*, 2014) and likely accounts for Bob1 expression in these cells. The notably strong expression of Bob1 in human (Rasheed *et al*, 2006) and mouse Tfh cells (this study) suggested a prominent role in Tfh cell development and/or function, and we now provide evidence that Bob1 directly regulates Bcl6 and BTLA expression in $CD4^+$ T cells, thereby promoting the development of Tfh cells and shaping their function in T cell-dependent immune responses.

In the present study, we analyzed the natural response to sheep red blood cells (SRBCs), a potent GC-inducing antigen. When analyzing the $CD4^+$ T-cell compartment in secondary lymphoid organs of *Pou2af1*$^{-/-}$ mice immunized with SRBCs, we observed a strong reduction in the frequency of Tfh cells and changes to the expression of CXCR5 and costimulatory molecules as compared to heterozygous littermates or wild-type mice. Tfh cells are typically identified as $CD4^+$ T cells expressing CXCR5 in combination with ICOS, PD1, or BTLA (Chtanova *et al*, 2004; Vinuesa *et al*, 2005; Rasheed *et al*, 2006). These costimulatory molecules, however, identified overlapping but not identical populations of $CD4^+CXCR5^+$ T cells and the relative size of these populations proved to be organ-dependent. Noteworthy, the regulation of costimulatory molecule expression in the course of Tfh cell differentiation and their contribution to Tfh cell function is not fully understood. In particular, little is known about BTLA in Tfh cells except that it can dampen humoral immune responses by

suppressing IL-21 secretion by Tfh cells (Kashiwakuma *et al*, 2010). It is conceivable that the organ-dependent differences in both the frequency of Tfh cells and their expression of costimulatory molecules may reflect the distinct requirements of tissue-specific immune responses. Mucosa-associated lymphoid tissue such as PP and MLN serves specialized functions such as the control of mucosal commensalism and the induction of oral tolerance (Cerutti *et al*, 2011; Lycke & Bemark, 2012). The molecular and cellular requirements for GC formation in PP are unique in that T-cell help can be exerted by T cells other than Tfh cells and independent of cognate T–B-cell interactions. Moreover, Tfh cells in PP may originate from $Foxp3^+$ T cells or Th17 cells that were induced in the context of gut antigens (Tsuji *et al*, 2009; Hirota *et al*, 2013). Other determining factors are DCs that provide critical signals for the initiation of Tfh cell development (Choi *et al*, 2011; Goenka *et al*, 2011). In this connection, we observed an aberrant localization of conventional $CD11c^+$ DCs in PP of *Pou2af1*$^{-/-}$ mice (data not shown) that may contribute to the gross defects in Tfh cell differentiation in PP of *Pou2af1*$^{-/-}$ mice.

Up to now, the phenotype of Bob1-deficient mice has been attributed to B cell-intrinsic defects or secondary defects such as disturbed B-cell–T-cell interactions. Along this line, Karnowski *et al* (2012) have recently reported that *Pou2af1*$^{-/-}$ $CD4^+$ T cells are not impaired in their ability to differentiate into Tfh cells during influenza infection in mice reconstituted with wild-type B cells. Viral infections, however, elicit strong Toll-like receptor signals not only in DCs and B cells, but also in $CD4^+$ T cells that, depending on the pathogenic stimulus and the anatomic compartment, promote the generation of Tfh cells and adaptive immune responses (Hou *et al*, 2011; Rookhuizen & Defranco, 2014; Kubinak *et al*, 2015). Based on our observation that Bob can be decisive for $CD4^+$ T-cell differentiation (Brunner *et al*, 2007), we analyzed *Pou2af1*$^{+/+}$:*Pou2af1*$^{-/-}$ mixed bone marrow chimeras to determine whether the effects of Bob1-deficiency on Tfh cell differentiation are $CD4^+$ T cell-intrinsic or secondary to defects in the B-cell compartment of *Pou2af1*$^{-/-}$ mice. These experiments conclusively demonstrated a strong T cell-intrinsic defect in the development of Bob1-deficient Tfh cells. The use of specific markers allowed us to discriminate between *Pou2af1*$^{+/+}$ and *Pou2af1*$^{-/-}$ Tfh cells in each individual animal and our analyses showed that *Pou2af1*$^{-/-}$ Tfh cells are selectively underrepresented in the pool of Tfh cells following immunization with SRBCs. It is important to note that the reconstituted animals had comparable numbers of naïve recirculating *Pou2af1*$^{+/+}$ and *Pou2af1*$^{-/-}$ $CD4^+$ T cells whereas the vast majority of B cells were of wild-type origin and therefore competent to present antigen and to provide costimulatory signals and cytokines for Tfh cell differentiation.

In search for mechanisms that explain the defect in the generation of *Pou2af1*$^{-/-}$ Tfh cells, we found in both knockout mice and mixed bone marrow chimeras a substantial reduction in the expression of Bcl6 by *Pou2af1*$^{-/-}$ Tfh cells. Consequently, we observed reduced frequencies of *Pou2af1*$^{-/-}$ Tfh cells expressing high levels of Bcl6, indicative of GC-Tfh cells. Apart from Bcl6, Bob1 appears to directly promote the expression of additional Tfh cell signature genes such as *Btla* and probably *Cxcr5*. In a previous study, we showed that in B cells, the *Cxcr5* promoter is a direct target for Bob1/Oct2 as well as members of the NF-κB/Rel family of transcription factors and that surface expression of CXCR5 is diminished on *Pou2af1*$^{-/-}$ B cells (Wolf *et al*, 1998). In line with these results, we saw a general reduction in CXCR5 expression on *Pou2af1*$^{-/-}$ $CD4^+$ T cells. The present study now shows that Bob1 together with Oct2, which is specifically expressed in lymphocytes and dendritic cells, can directly bind to and transactivate the *Bcl6* and *Btla* promoters.

The expression of Bcl6 relies on complex, context-dependent circuitries that are incompletely understood. The induction and maintenance of Bcl6 in Tfh cells requires ICOS signaling via PI3K (Choi *et al*, 2011; Weber *et al*, 2015), cytokines including IL-6 and IL-21 that signal via STAT3/STAT1 (Eto *et al*, 2011; Harker *et al*, 2011; Choi *et al*, 2013) as well as BATF binding to the *Bcl6* gene (Ise *et al*, 2011). IL-2 signaling, on the other hand, activates STAT5 that may directly (Ray *et al*, 2014) and via induction of BLIMP1 repress Bcl6 expression (Johnston *et al*, 2009, 2012; Nurieva *et al*, 2012). Two recent reports by Choi *et al* (2015) and Xu *et al* (2015) establish the transcription factors TCF-1 and LEF-1 as important coordinators for the initiation of Tfh cell differentiation upstream of the Bcl-6–Blimp1 axis. These factors appear to promote early Tfh cell differentiation by enhancing the expression of IL-6 receptor chains, ICOS and Bcl6. These studies show by global gene expression analysis that *Pou2af1* is highly expressed in early stage and GC-Tfh cells but downregulated in the absence of LEF-1 and TCF-1, similar to Bcl6. It is therefore tempting to speculate that Bob1 might be involved in LEF-1- and TCF-1-induced expression of Bcl6.

Taken together, our results suggest that Bob1 promotes Tfh cell differentiation on different levels. Bob1 can directly bind to and transactivate the *Bcl6* promoter and consequently Bcl6-mediated repression of Blimp1 that, for its part, would repress genes such as *Cxcr5*, *Btla,* and *Il6ra* (Oestreich *et al*, 2012). Moreover, Bob1 seems to fine-tune the immune response by regulating the expression of BTLA and CXCR5. Considering the various aspects of Tfh cell biology that are regulated by Bob1, it will be of interest to investigate the regulation of Bob1 expression in Tfh cells and whether Bob1 exerts its functions at specific stages or throughout Tfh cell development.

The defects in Bcl6 expression and Tfh cell formation are generally more pronounced in *Pou2af1* knockout mice than for *Pou2af1*-deficient $CD4^+$ T cells in mixed bone marrow chimeras. This observation suggests that the phenotype of *Pou2af1* knockout mice most likely results from a combination of multiple T cell- and B cell-intrinsic defects that may result in disturbed T–B interactions and eventually a loss of GC formation and block in plasma cell differentiation.

Materials and Methods

Mice and immunization

C57BL/6 mice, congenic CD45.1B6.SJL mice, and B6.129S2-$CD4^{tm1Mak}$/J mice were purchased from The Jackson Laboratory (Bar Harbor). ROSA/tdRFP mice (Luche *et al*, 2007), *Pou2af1*$^{-/-}$ mice (Nielsen *et al*, 1996), and *Pou2af1*$^{-/-}$OTII mice were bred and maintained at the animal facility of the Max-Delbrück-Center. Mice were housed 5 mice/cage at a 12-h day–night cycle with free access to tap water and food pellets. All animal experiments

were conducted on mice 8–16 weeks of age and approved by the Berlin State review board at the Landesamt für Gesundheit und Soziales.

Immunization

Mice were immunized i.p. with either 2×10^8 SRBC (bioMérieux), 100 μg 4-hydroxy-3-nitrophenylacetyl coupled to keyhole limpet hemocyanin (NP(28)-KLH) (Biosearch Technologies) or 100 μg chicken ovalbumin (OVA) (Sigma-Aldrich) in alum (Pierce).

Antibodies, flow cytometry, and cell sorting

See Appendix Table S1 for a list of antibodies used in flow cytometry. Intracellular staining was done using the FoxP3-staining kit (eBioscience). Dead cells were excluded by staining with 7-AAD or aqua-fluorescent reactive dye (Life Technologies). Samples were analyzed on a FACSCanto II, LSR Fortessa, or sorted on a FACSAria III (BD Biosciences). Data were processed using FlowJo (Tree Star Inc.) and Weasel (Walter and Eliza Hall Institute/F. Battye) software.

Microarray-based gene expression analysis

Seven days after immunization with NP(28)-KLH, splenocytes from 10 mice were pooled and $CD4^+$ T cells enriched by MACS using the $CD4^+$ T-cell isolation Kit (Miltenyi Biotec). $CD4^+$ T-cell populations ($ICOS^+CXCR5^+$, $ICOS^+CXCR5^-$, and $ICOS^-CXCR5^-$) were sorted to > 95% purity by FACS. About 50–100 ng RNA was used to generate probes by the GeneChip 3′IVT Express Kit (Affymetrix). Probes were hybridized on Affymetrix 430 2.0 chips. Data were analyzed using Genespring 12 software (Agilent Technologies).

RNA extraction and quantitative real-time RT–PCR

RNA was isolated from purified T cells by RNeasy Kits (Qiagen) according to the manufacturer's instructions, and genomic DNA removed with DNase I. RNA integrity was confirmed using the Agilent Bioanalyzer system. RNA was reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen) and Oligo $(dT)_{20}$ and random hexamer primers. Quantitative PCR was performed using SYBR Green (Sigma) on an iQ5 Real-Time PCR System (Bio-Rad) with the following primers: *β-actin:* 5′-TGCGTGA CATCAAAGAGAAG-3′ and 5′-GATGCCACAGGATTCCATA-3′; *bob1*: 5′-CTGCTTCCACAGTGACAGAGG-3′ and 5′-GTCAACACCGAGGAGG GTCC-3′. Bob1 mRNA expression was calculated relative to *β-actin* ($2^{-\Delta CT}$ method).

Immunofluorescence analysis

Spleen, MLN, and PP were embedded in TissueTek OCT compound (Sakura Finetek). Sections were cut to 5 μm thickness and fixed in acetone. Antibodies used are listed in Appendix Table S1. Sections were blocked with 5% rat or goat serum and stained for 1 h with primary antibodies. Slides were mounted in DABCO-Mowiol solution (Sigma/Calbiochem). Stained sections were analyzed on an AxioImager.Z2 fluorescence microscope using a Plan-Apochromat 10x/0.3 objective. Images were processed with AxioVision 4.8 software (Carl Zeiss).

Mixed bone marrow chimeras

Bone marrow of wild-type (WT) mice that express either CD90.1 or the tandem dimer red fluorescent protein (dtRFP) and *Pou2af1*$^{-/-}$ $CD90.2^+$ C57BL/6 mice was harvested by flushing femur and tibia with RPMI 1640 (PAA). CD45.1B6.SJL congenic, sex-matched recipient mice were lethally irradiated with 2×4.75 Gy. On the same day, the irradiated mice received a 1:1 mixture of bone marrow cells from WT and *Pou2af1*$^{-/-}$ mice (2×10^7 total per animal, i.v.). Four weeks after bone marrow transfer, reconstitution was confirmed by flow cytometry and 3 weeks later reconstituted mice were immunized with SRBC. On day 7 after immunization, spleen, MLN, and PP were analyzed for the development of Tfh and GC B cells.

Adoptive transfer

$CD4^+CD62L^+$ T cells from spleen, MLN, and PP of WT OTII and *Pou2af1*$^{-/-}$ OTII mice were isolated by MACS using the T Cell Isolation Kit II (Miltenyi Biotec). On the same day, $CD4^{-/-}$ mice received either WT or *Pou2af1*$^{-/-}$ $CD4^+CD62L^+OTII^+$ T cells (8×10^6 cells per animal, i.v.). One day later, mice were immunized with OVA + alum. On day 7 after immunization, spleen, MLN, and PP were harvested and analyzed for the development of Tfh cells.

Cell lines, cell culture, and transfections

NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) containing 10% FCS, 2 mM of L-glutamine and penicillin/streptomycin, and 50 μM of β-mercaptoethanol and incubated at 37°C and 5% CO_2. Transfections of NIH/3T3 cells were performed by electroporation (Bio-Rad) with 450 V and 250 μF in PBS. The pRL-CMV plasmid (Renilla Luciferase control reporter vector; Promega) was co-transfected in all experiments in NIH/3T3 cells and used for normalization of different transfection efficiencies in the individual experiments. The murine *Ifng* promoter (+73 to −523) was cloned by genomic PCR as described previously (Brunner *et al*, 2007). For the expression of Bob1 in transfection assays, wild-type Bob1 cDNA was cloned into the expression vector pcDNA3. The cloning of the Oct2 expression vector is described elsewhere (Müller *et al*, 1988).

EL-4 cells were cultured and transiently transfected by DEAE-dextran as described previously (Nayak *et al*, 2009). The −1,461 to +51 enhancer/promoter region of the murine Bcl6 gene was cloned into pTATAluc+ (Altschmied & Duschl, 1997). Mutations introduced were as follows: Bcl6 M3 (−780/778/777) GTCCCGACGATTGAAC TAACACCAC or Bcl6 M6 (−364/363/362) TGTCACCGAATATAC AATTAGTTCTCAG (exchanged nucleotides are underlined; for wt, see Appendix Table S2).

EMSA

For electrophoretic mobility shift assays (EMSA), primary $CD4^+$ T cells were isolated from lymph nodes of C57BL/6 mice by positive selection using magnetic microbead technology (Miltenyi). Cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mM of L-glutamine and penicillin/streptomycin and 50 μM of β-mercaptoethanol at 37°C and 5% CO_2 either in the absence or presence of 25 ng/ml of PMA, and 500 ng/ml of ionomycin (Sigma-Aldrich) for

18 h. Preparation of whole-cell extracts for EMSA and the protocol of the EMSA procedure have been described earlier (Brunner *et al*, 2007). Oligonucleotides bearing the appropriate transcription factor binding site were annealed and subsequently labeled using ^{32}P-αdCTP in a fill in reaction. For competition experiments, we used unlabeled annealed oligonucleotides bearing the indicated transcription factor binding sites that were added at a 10-fold or 100-fold molar excess, relative to the amount of labeled probes, to the EMSA reaction mixture. Oligonucleotide sequences are listed in Appendix Table S2.

ChIP

For ChIP experiments, naïve CD4$^+$ T cells were isolated from either wild-type/heterozygous or Bob1-deficient mice and cultured in IMDM supplemented with 10% FCS, 2 mM of L-glutamine and penicillin/streptomycin. For the generation of Tfh-like cells *ex vivo*, naïve CD4$^+$ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml) and anti-CD28 (2 μg/ml) in the presence of 100 ng/μl murine IL-6 and 50 ng/μl murine IL-21 (both from PeproTech) for 4 h. Chromatin immunoprecipitation (ChIP) experiments were performed using the ChIP-IT® Express Enzymatic Chromatin Immunoprecipitation Kit from Active Motif according to the manufacturer's protocol with slight modifications as described elsewhere (Mueller *et al*, 2013). Briefly, after enzymatic digestion for 15 min, the chromatin was sheared using the EpiShear™ Probe Sonicator (Active Motif; 30 pulses consisting of 20 s sonication followed by 30 s rest at 25% amplitude) in the same buffer. The chromatin was precleared for 2 h with protein G microbeads (Invitrogen) and then incubated overnight with 2 μg of either rabbit polyclonal Oct1 (sc-232), Oct2 (sc-233), and Bob1 (sc-955) antibodies (Santa Cruz Biotechnology). The incubation with 2 μg of normal rabbit serum (Pierce) served as internal negative controls. The amount of precipitated DNA was evaluated by quantitative PCR using the Roche Light Cycler LC480. The PCR primers were synthesized by Biomers and are presented in Appendix Table S3. The primer sequence for the amplification of the intergenic region at chromosome 8 is published elsewhere (Boyer *et al*, 2006). The relative amount of precipitated DNA was calculated using the following formula: $E^{(\text{crossing point } 1/10 \text{ total input} - \text{crossing point sample})}$ and is depicted as amount of precipitated genomic DNA relative to that precipitated by control antibodies (normal rabbit serum). E = efficiency of the PCR determined by serial dilutions of total input.

Statistical analysis

Data are presented showing the arithmetic mean together with the results from individual animals or the arithmetic mean with error bars (± SD if not otherwise indicated). *n* represents the number of animals/biological replicates. Data were analyzed using Prism 6 software (GraphPad). *P*-values were calculated using the nonparametric unpaired Mann–Whitney test (two tailed); $P < 0.05$ was considered statistically significant.

Data deposition

Mouse array data can be accessed at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-3820.

Expanded View for this article is available online.

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Author contributions
DS, CB, FB-S, and GM designed and performed experiments and analyzed data. UEH contributed to the generation of bone marrow chimeras, ML contributed to data analysis, GM designed and supervised the study and wrote the manuscript with contributions from DS, CB, and FB-S. All authors read and provided comments on the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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