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# Bcl6 promotes follicular helper T-cell differentiation and PD-1 expression in a Blimp1-independent manner in mice

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Abbreviations : T follicular helper ($T_{FH}$), T follicular regulatory ($T_{FR}$), double conditional knockout (dcKO), conditional knockout (cKO), germinal centers (GCs), sheep red blood cells (SRBC), germinal center B-cell (GCB-cell), Control (Con), retroviruses (RVs)

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ABSTRACT

The transcription factors Bcl6 and Blimp1 have opposing roles in the development of the follicular helper T ($T_{FH}$) cells: Bcl6 promotes and Blimp1 inhibits $T_{FH}$-cell differentiation. Similarly, Bcl6 activates, while Blimp1 represses, expression of the $T_{FH}$-cell marker PD-1. However, Bcl6 and Blimp1 repress each other's expression, complicating the interpretation of the regulatory network. Here we sought to clarify the extent to which Bcl6 and Blimp1 independently control $T_{FH}$-cell differentiation by generating mice with T-cell specific deletion of both Bcl6 and Blimp1 (double conditional KO [dcKO] mice). Our data indicate that Blimp1 does not control $T_{FH}$-cell differentiation independently of Bcl6. However, a population of T follicular regulatory ($T_{FR}$) cells developed independently of Bcl6 in dcKO mice. We have also analyzed regulation of IL-10 and PD-1, two genes controlled by both Bcl6 and Blimp1, and observed that Bcl6 regulates both genes independently of Blimp1. We found that Bcl6 positively regulates PD-1 expression by inhibiting the ability of T-bet/*Tbx21* to repress *Pdcd1* transcription. Our data provide a novel mechanism for positive control of gene expression by Bcl6, and illuminate how Bcl6 and Blimp1 control $T_{FH}$-cell differentiation.

Graphical Abstract

The transcriptional repressors Bcl6 and Blimp1 have complex and opposing roles in controlling follicular helper T (TFH)-cell differentiation and PD-1 expression. Xie et al show that Bcl6 controls TFH-cell differentiation and PD-1 expression independently of Blimp1, while Blimp1 regulates TFH-cell differentiation primarily by repressing Bcl6.



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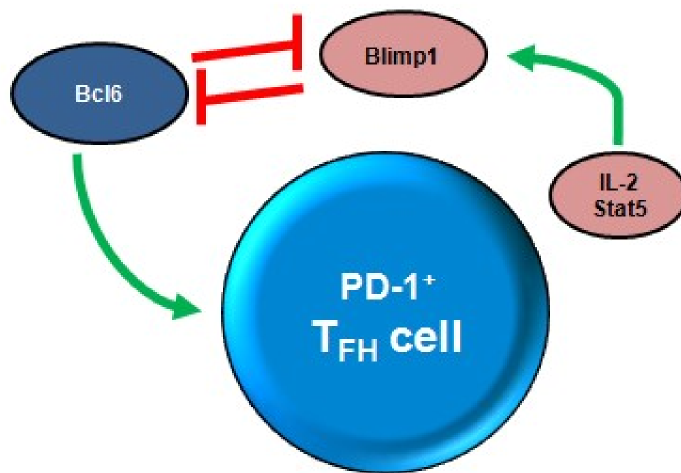


## Introduction

T follicular helper ($T_{FH}$) cells promote formation of germinal centers (GCs) and select B- cell clones that produce high-affinity antibodies (Abs) (reviewed in [1, 2]). While $T_{FH}$ cells are required for the production of high affinity Abs, excessive numbers of $T_{FH}$ cells can promote autoimmunity by helping B cells produce self-reactive Abs. The proper regulation of $T_{FH}$ cell differentiation is therefore essential for strong antibody responses and preventing development of autoimmune disease.

The Bcl6 transcriptional repressor protein is considered a master regulator for the $T_{FH}$ lineage [3-5], however the precise mechanisms by which Bcl6 promotes $T_{FH}$ cell differentiation have not been fully elucidated. One pathway for $T_{FH}$ cell differentiation is that Bcl6 represses transcription of the transcriptional repressor Blimp1, a potent inhibitor of $T_{FH}$ cell differentiation [1, 5-8]. However, the mechanism by which Blimp1 itself represses $T_{FH}$ cell



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differentiation is not settled. Blimp1 may inhibit $T_{FH}$ cell differentiation by directly repressing Bcl6 expression [5, 9, 10], or by repressing other genes that are critical for $T_{FH}$ cell differentiation besides Bcl6.

Mice deficient in Bcl6 or Blimp1 have not provided a clear answer as to the relationship between of Blimp1 and Bcl6 in the development of $T_{FH}$ cells [5, 11]. Using conditional knockout (cKO) mouse models, researchers have shown that loss of Bcl6 in T cells results in complete loss of $T_{FH}$ cell development, while, conversely, Blimp1 cKO mice have increased $T_{FH}$ cell populations.

A key $T_{FH}$ cell gene controlled by both Bcl6 and Blimp1 is PD-1. Bcl6 promotes PD-1 expression [12], and Blimp1 represses PD-1 [13]. Since Bcl6 is a transcriptional repressor, the mechanism that Bcl6 uses to up-regulate PD-1 expression is likely to be an indirect pathway, such as by repressing the transcription of Blimp1.

In this study, we used cKO mice doubly deficient for both Bcl6 and Blimp1 in T cells to delineate the respective roles of these two key factors in $T_{FH}$ cell differentiation and PD-1 gene expression. Our results indicate that Bcl6 promotes both $T_{FH}$ cell differentiation and PD-1 expression by pathways essentially independent of Blimp1.

**Results and Discussion**

**$T_{FH}$ cells but not $T_{FR}$ cells are dependent on Bcl6 for their development.**

To analyze $T_{FH}$ cell differentiation when both Bcl6 and Blimp1 were deleted specifically in T cells, we generated CD4-cre Bcl6$^{fl/fl}$ Prdm1$^{fl/fl}$ (dcKO) mice. These mice were healthy and T cell development was normal (data not shown). We immunized the mice with sheep red blood cells (SRBC), and after 14 days, analyzed $T_{FH}$ cells in spleen (Fig. 1AB, Supporting Information Fig. 1A). Control (Con) mice were Bcl6$^{fl/fl}$ Prdm1$^{fl/fl}$ without CD4-cre.



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Bcl6 cKO and Blimp1 cKO mice were also analyzed. Compared to Con, $T_{FH}$ cell populations were increased 2-fold in Blimp1 cKO mice, while $T_{FH}$ cells were completely absent in Bcl6 cKO and dcKO mice. These data indicate that Bcl6 is required for $T_{FH}$ cell development even in the absence of Blimp1-mediated repression. Next, we analyzed the development of T follicular regulatory cells ($T_{FR}$ cells) [14-17] after SRBC immunization (Fig. 1CD, Supporting Information Fig. 1A). Blimp1 cKO mice have a sharply enhanced $T_{FR}$ cell population compared to Con mice, while $T_{FR}$ cells are nearly absent in Bcl6 cKO mice. However, $T_{FR}$ cells are significantly higher in dcKO mice than Bcl6 cKO mice, indicating that Blimp1 represses $T_{FR}$ cells independent of Bcl6, and that $T_{FR}$ cells and $T_{FH}$ cells have somewhat different modes of development. We also analyzed GC B-cells by flow cytometry in the same sets of mice (Fig. 1EF, Supporting Information Fig. 1B), and GC B-cell development paralleled $T_{FH}$ cell development in all 4 mouse lines. SRBC-specific IgG titers followed the levels of germinal center B (GCB) cells (Supporting Information Fig. 2). We analyzed $T_{FH}$ cells, $T_{FR}$ cells and GCB-cells 7 days after SRBC immunization, and observed the same pattern as after 14 days (data not shown).

**Bcl6 controls PD-1 expression independent of Blimp1.**

Bcl6 and Blimp1 have opposing roles in PD-1 expression [12, 13], and we therefore analyzed PD-1 expression in immunized Con, Bcl6 cKO, Blimp1 cKO and dcKO mice (Fig. 2). The average level of PD-1 expression measured by mean fluorescent intensity (MFI) in total CD4 T cells showed very high PD-1 in CD4 T cells from Blimp1 cKO mice (Fig. 2A, Supporting Information Fig. 3A), consistent with Blimp1 repressing PD-1 expression. Since PD-1 expression in dcKO CD4 T cells is much lower than in Blimp1 cKO CD4 T cells, this indicates a positive role for Bcl6 in PD-1 regulation. Since the PD-1 levels on total CD4 T



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cells are skewed by the presence of PD-$1^{high}$ expressing $T_{FH}$ cells, we analyzed PD-1 expression on PD-$1^{+}$ non-$T_{FH}$ cells (Fig. 2B, Supporting Information Fig. 3B). Loss of Blimp1 again increased PD-1 in non-$T_{FH}$ cells whereas PD-1 in Bcl6 cKO and dcKO non-$T_{FH}$ cells was similar as control (Con) levels. These data indicate that the higher PD-1 in Blimp1 cKO non-$T_{FH}$ cells requires Bcl6 expression, but did not answer whether Bcl6 could regulate PD-1 independently of Blimp1. To address this question, we used retroviruses (RVs) to express Bcl6 in activated CD4 control and dcKO T cells, then assessed PD-1 expression. As shown in Figure 2C (Supporting Information 3C), Bcl6 significantly augmented PD-1 expression as measured by MFI, in both control and dcKO T cells, showing clearly that Bcl6 could activate PD-1 expression independent of Blimp1. Indeed, Bcl6 induced higher expression of the *Pdcd1* gene (Supporting Information Fig. 4A), whereas a known Bcl6 target, *Il10* [11, 18] was strongly repressed by Bcl6 RV (Fig. 4B). Thus, Bcl6 can both activate and repress gene expression, independently of Blimp1. Since Blimp1 has been shown to positively regulate IL-10 [19], our data also rules out a mechanism of IL-10 control where Bcl6 acts on IL-10 by repressing Blimp1 and causing decreased *Il10* transcription, and shows that Bcl6 is a direct repressor of *Il10* expression.

**A T-bet-dependent mechanism for the activation of PD-1 expression by Bcl6**

We next sought to find the mechanism for how Bcl6, a transcriptional repressor, could promote *Pdcd1* mRNA expression independent of Blimp1. One explanation is that Bcl6 represses the transcription of microRNAs that silence PD-1 expression [3]. We therefore tested the role of microRNAs in the induction of PD-1 by Bcl6, and found that Bcl6 RV could still up-regulate PD-1 in *Dicer* cKO T cells, which are unable to generate microRNAs (Supporting Information Fig. 5). We then sought other pathways. T-bet is a transcriptional



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repressor of PD-1 gene expression [20], and Bcl6 is a negative regulator of T-bet [21]. Therefore we wondered if a T-bet-Bcl6 pathway could play a role in PD-1 expression in CD4 T cells. We tested this idea by transducing primary mouse CD4 T cells with T-bet-expressing RV, with and without Bcl6 RV. As shown in Figure 3A, T-bet RV can significantly repress PD-1 expression compared to control RV, whereas addition of Bcl6 RV allows for PD-1 activation even in the presence of T-bet RV. To further investigate this pathway, we analyzed PD-1 expression in *Tbx21*$^{-/-}$ (Tbx21 KO or T-bet-deficient) CD4 T cells and as expected, saw higher PD-1 in T-bet KO T cells compared to wild-type T cells (Fig. 3B). Notably, Bcl6 RV was able to strongly activate PD-1 expression in wild-type T cells (~50% increase in MFI) but Bcl6 RV was only able to activate PD-1 relatively weakly in T-bet KO T cells (~20% increase in MFI)(Fig. 3BC). These data indicate that a major pathway for the up-regulation of PD-1 expression by Bcl6 is by counteracting the repressive action of T-bet on PD-1 expression.

**Concluding Remarks**

In summary, we have clarified several aspects of Bcl6 control over $T_{FH}$ cell and $T_{FR}$ cell differentiation (see model in Supporting Information Fig. 6). Specifically, we have found that: 1) Blimp1 primarily represses $T_{FH}$ cell differentiation by acting on Bcl6, 2) Blimp1 represses $T_{FR}$ cell differentiation through both Bcl6-dependent and Bcl6-independent pathways and 3) Bcl6 promotes PD-1 expression by a novel Blimp1-independent mechanism involving T-bet inhibition.



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**Materials and Methods**

Mice and immunizations

Bcl6$^{fl/fl}$ mice [11] were backcrossed to CD4-cre transgenic mice [22] and the C57BL/6 strain for at least six generations and then crossed to Prdm1$^{fl/fl}$ mice [23, 24]. Tbx21$^{-/-}$ mice were obtained from Jackson labs. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Use and Care Committee. For immunization, mice were injected intraperitoneally (i.p.) with 1 x $10^9$ SRBCs (Rockland Immunochemicals) and sacrificed at the indicated day.

Flow cytometry reagents

Anti-CXCR5 (2G8) and GL7 (GL7) Abs were from BD Biosciences. Fixable viability dye, anti-CD38 and anti-Foxp3 (FJK-16s) Abs were from eBioscience. Anti-CD4 (GK1.5), anti-B220 (RA3-6B2), anti-IgG1 (RMG1-1), anti-PD-1 (29F.1A12) were from Biolegend.

Cell staining for flow cytometry

After red blood cell lysis, total spleen cells were incubated with anti-mouse CD16/CD32 (BioXcell) for 5 minutes at RT, followed by surface staining for the indicated markers. For intracellular transcription factor staining, after surface markers were stained, cells were fixed and stained with antibodies against transcription factors by following Foxp3 fixation kit



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(eBioscience) instructions. Cell events were collected on an LSRII flow cytometer (Becton Dickonson).

Cell culture and retrovirus transduction

CD4 T cells were isolated from the spleen via isolation kit (Miltenyi Biotec). Cells were activated with plate-bound anti-CD3 (10 μg/ml; 145-2C11; Bio XCell) and anti-CD28 (10 μg/ml; 37.51; Bio XCell) for 24 h at 1 X $10^6$ cells/ml. Cells were then spin-infected with control GFP, Bcl6-GFP–expressing [25] retroviruses (RVs). For co-infection experiments, either control $H2K^k$ or Bcl6-$H2K^k$-expressing [26] RVs were co-transduced with either control GFP or T-bet-GFP–expressing [27] RVs. After infection, supernatants were substituted with fresh medium containing 10 U/ml recombinant human IL-2. Two days later, cells were collected for analysis.

RT-QPCR and Antigen-specific IgG Analysis

These procedures were performed as previously reported [28].

Statistical Analysis.

All data analysis was done using Prism Graphpad software. Unless otherwise stated, Student t test or ANOVA with Tukey post hoc analysis were used. Only significant differences ($P < 0.05$) are indicated in Figures.



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**Conflict of interest**

The authors declare no commercial or financial conflict of interest.

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## Figure legends

**Figure 1. Regulation of $T_{FH}$ cells, $T_{FR}$ cells and germinal center B cell by Bcl6 and Blimp1.** Control, Blimp1 cKO, Bcl6 cKO and dcKO mice were immunized with SRBC by i.p. injection. 14 days post-immunization (dpi), spleens were isolated for flow cytometric analysis. $T_{FH}$ cells are defined as $Foxp3^{-}CXCR5^{hi}PD\text{-}1^{hi}$. $T_{FR}$ cells are defined as $Foxp3^{+}CXCR5^{hi}PD\text{-}1^{hi}$. GC B cells are defined as $B220^{+}CD38^{lo}GL\text{-}7^{hi}$. (A) Representative flow cytometry plots, gated on $FoxP3^{-}$ $CD4^{+}$ cells. (B) $T_{FH}$-cell percentage within $Foxp3^{-}$ $CD4^{+}$ T cells and absolute $T_{FH}$-cell number per spleen. (C) Representative flow cytometry plots, gated on $Foxp3^{+}$ cells. (D) $T_{FR}$-cell percentage in $Foxp3^{+}CD4^{+}$ T cells and absolute $T_{FR}$-cell number per spleen. (E) Representative flow cytometry plots. (F) GCB-cell percentage in $B220^{+}$ B cells and absolute GCB-cell number per spleen. Flow cytometry plots are from a single experiment representative of 2 experiments with 16 total mice per experiment. Data in graphs are shown as mean ± SEM, n = 4 with each symbol representing a single mouse. NS =not significant, p> 0.05, *p < 0.05 (two-way ANOVA). Data are representative of 2 independent experiments with similar results with 16 total mice per experiment.



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Figure 1

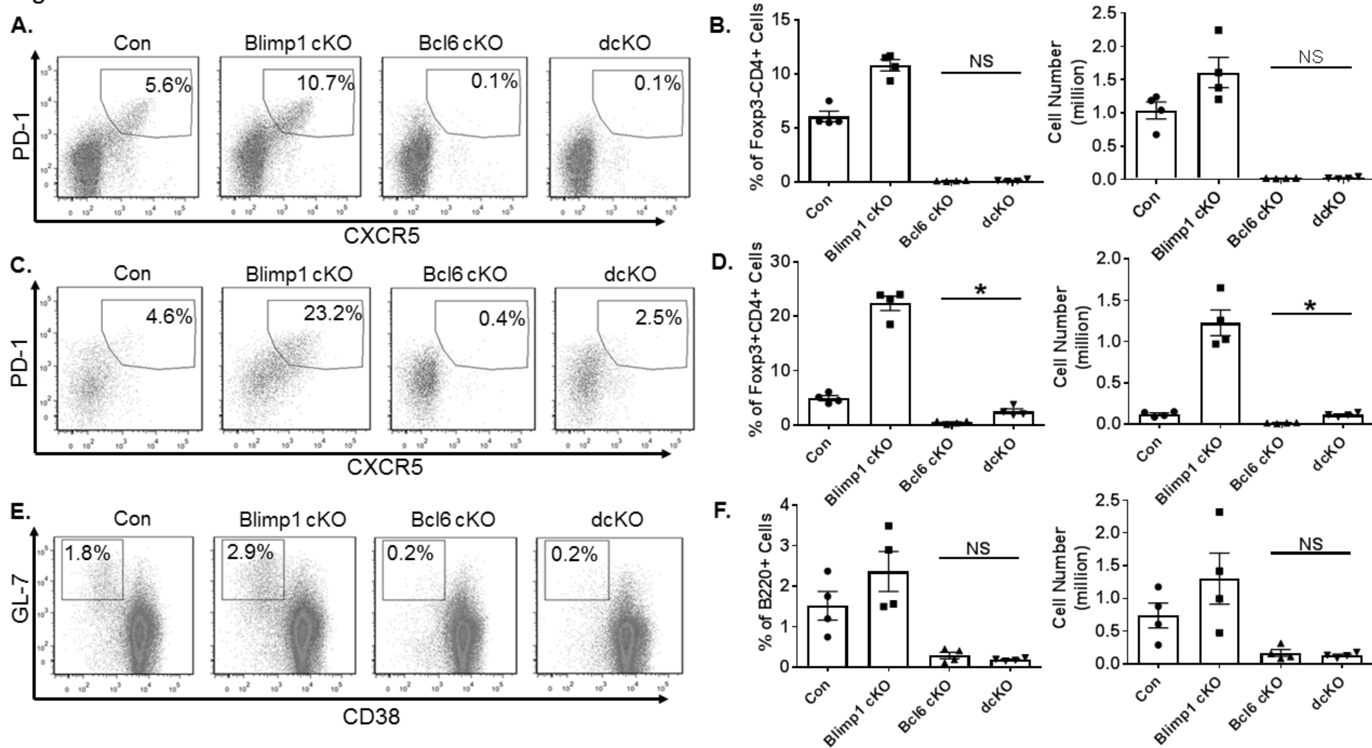




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**Figure 2. Control of PD-1 expression by Bcl6 and Blimp1.** Control, Blimp1 cKO, Bcl6 cKO and dcKO mice were immunized with SRBC by i.p. injection. At 14 days dpi, spleens were isolated for flow cytometric analysis. (A) PD-1 mean fluorescence intensity (MFI) of total CD4$^+$ T cells at 14 dpi (n=4, mean ± SEM). (B) PD-1 MFIs of CD44$^+$ CXCR5$^-$ PD-1$^+$ non-TFH cells. (C) Control and dcKO CD4+ T cells were infected with Bcl6-expressing and control retroviruses (RVs). **p < 0.01 (two-way ANOVA). Each symbol in graphs represents one mouse. Data are representative of two independent experiments with similar results with 16 total mice per experiment.

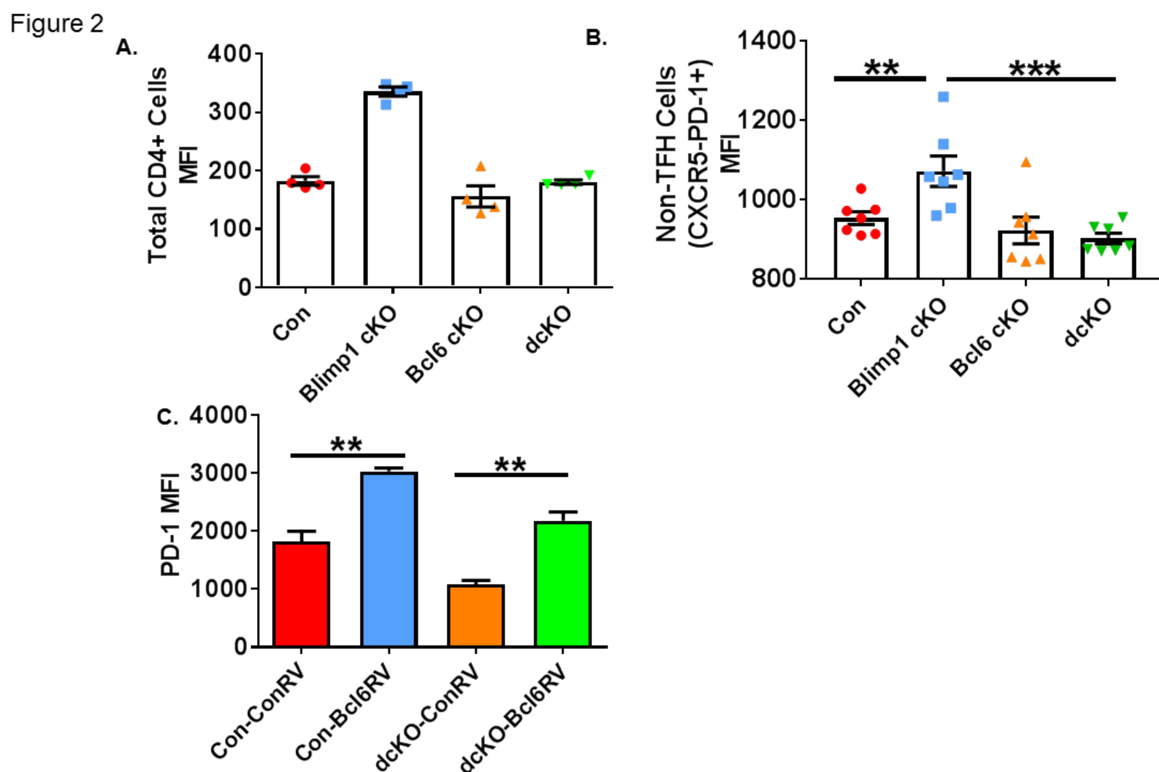




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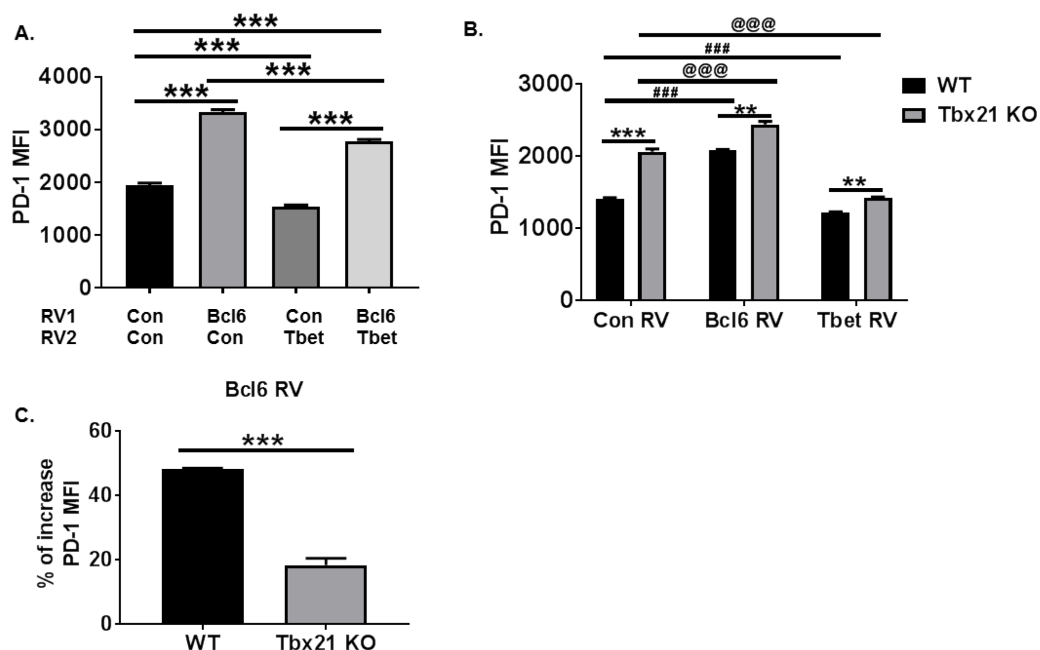
**Figure 3. Control of PD-1 expression by Bcl6 and Blimp1.** Total CD4$^+$ T cells from wild-type (WT) or Tbx21 cKO mice were co-infected with Bcl6-H2K$^k$-expressing and T-bet-GFP-expressing and control RVs. PE-labeled anti-H2K$^k$ Ab was used to detect H2K$^k$ expressing cells. Double GFP$^+$PE$^+$ cells were gated on for analysis. (A) PD-1 MFI of total CD4$^+$ T cells from WT mice after control, Bcl6, T-bet or Bcl6 plus T-bet RV co-infection (n=4, mean ± SEM). (B) PD-1 MFI of total CD4$^+$ T cells after control, Bcl6, T-bet or Bcl6 plus T-bet RV co-infection. Statistical designations: * compares WT to Tbx21 KO, $^{\#}$ compares WT with the different RV infections, $^{@}$ compares Tbx21 KO with the different RV infections. (C) Percentage of increase of PD-1 MFI of total CD4$^+$ T cells after control or Bcl6 RV infection in same experiment as (B). $^{***,\ @@@,\ \#\#\#}$ = $p < 0.001$, **=$p < 0.01$ (t test). Data are representative of two independent experiments with similar results, and with 4 mice per condition per experiment.



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