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# Ebi3 promotes T- and B-cell division and differentiation via STAT3

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ABSTRACT

Although sharing the same subunit Ebi3, IL-27 (p28/Ebi3) and IL-35 (p35/Ebi3) have different biological functions, suggesting that Ebi3 subunit may functions as a carrier. Our data demonstrated that activated T cells and B cells effectively up-regulated Ebi3 expression. In addition, Ebi3 effectively promoted T-cell activation and the differentiation of helper T 1 (Th1), Th17, and Foxp3$^+$ regulatory T (Treg) cells induced by Th1, Th17, and Treg polarizing condition, respectively. Naturally, Ebi3 could promote B-cell activation and the production of CD138$^+$ plasma cells (PC) induced by LPS. Conversely, neutralizing anti-Ebi3 antibody could significantly suppress T/B-cell activation and production of Th1, Th17, Tregs, and PC induced by Th1, Th17, Treg polarizing condition, and LPS, respectively. Furthermore, we found that Ebi3 time-dependently induced STAT3 activation in CD4$^+$T cells and B cells. Conversely, STAT3$^{-/-}$ effectively reduced Ebi3 expression and the production of Th1, Th17, Tregs, and plasma cells. Finally, we showed that gp130 but not IL-27Rα mediates Ebi3-induced STAT3 activation. These results suggest that Ebi3 promotes Th- and B-cell differentiation via gp130-STAT3 signaling pathway. Thus, autocrine Ebi3 may play an important role in the differentiation of Th and B cells and thus in infection, inflammation, and autoimmune disorders.

## 1. Introduction

The interleukin (IL)-12 family members such as IL-12, IL-23, IL-27, and IL-35 play an important role in regulating innate and adaptive immunity (Trinchieri, 2003; Trinchieri et al., 2003; Wang et al., 2014, 2012; Wang et al., 2016a, b). The IL-12 family member contains two subunits, an α-subunit (IL-12p35, IL-23p19, and IL-27p28) and a β-subunit (IL-12p40, Ebi3) (Dambuza et al., 2017; Vignali and Kuchroo, 2012; Wang et al., 2014, 2012; Wang et al., 2016b). IL-12 (p35/p40) has been linked to innate immunity as well as the development of adaptive immunity characterized by the induction of IFN-γ-expressing T helper 1 (Th1) cells (Gee et al., 2009). IL-23 (p19/p40) affects the activation of memory T cells, and plays a crucial role in the generation of the Th17 lineage of T cells (Gee et al., 2009). IL-12p40 is secreted as monomer and homodimer (Podlaski et al., 1992; Gillessen et al., 1995), suggesting that p40 may be additional biological function. In fact, the p40 homodimers have been found to enhance alloantigen-specific Th1 development (Piccotti et al., 1996).

IL-27 (p28/Ebi3) plays a critical role in inducing Th1-cell differentiation by promoting IFNγ production via STAT1 activation mediated by the receptor (IL-27Rα and gp130) (Pflanz et al., 2002; Takeda et al., 2003; Meka et al., 2015; Owaki et al., 2005a). In addition, IL-27 synergizes with IL-12 to induce IFNγ production (Pflanz et al., 2002; Takeda et al., 2003). IL-27 was also shown to suppress the development of EAE in rats through the inhibition of IL-17 and Th17 development (Wang et al., 2007). These studies suggest that IL-27 promotes Th1 polarization and suppresses Th17 polarization. It is currently accepted that IL-27 has pleiotropic effects and plays an important role in regulating the development and function of B cells (Larousserie et al., 2006). Initial reports indicated that IL-35 (p35/Ebi3), produced mainly

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by T cells, contributes to the suppressive activities of regulatory T (Treg) cells via STAT1 and STAT4 activation mediated by a unique heterodimer of receptor chains IL-12Rβ2 and gp130 or homodimers of each chain(Collison et al., 2007, 2012). Subsequent reports have also shown that IL-35 is also a physiological inducer of IL-10-producing regulatory B (Breg) cells via STAT1 and STAT3 activation mediated by the IL-35 receptor comprising the IL-12Rβ2 and IL-27Rα subunits (Wang et al., 2014). These studies suggest that IL-35 can promote the differentiation of Foxp3$^+$Treg and IL-10$^+$Breg cells.

Although sharing with the same subunit Ebi3, IL-27 (p28/Ebi3) and IL-35 (p35/Ebi3) have different biological functions. As a natural antagonist of gp130, IL-27p28 antagonizes IL-27 signaling (Stumhofer et al., 2010); hence, it can be exploited therapeutically to mitigate inflammatory diseases like experimental autoimmune uveitis (EAU) (Wang et al., 2012). Recent study also showed that IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease (Dambuza et al., 2017). Thus, we proposed that Ebi3 subunit alone may have an unknown biological function.

Ebi3 (EBV-induced gene 3) is induced by latent Epstein-Barr virus infection in B lymphocytes (Devergne et al., 1996). Ebi3 expression in vitro is induced within the B- and T-lymphocytes by expression of the EBV latent infection membrane protein-1 and by pokeweed mitogen stimulation (Devergne et al., 1996). Similar to IL-12 p40 and in contrast to all other members of this family, Ebi3 lacks obvious membrane-anchoring sequences, such as a hydrophobic or amphipathic transmembrane segment or a glycosyl- phosphatidylinositol linkage consensus site (Englund, 1993). Thus, Ebi3 is a secreted glycoprotein (Devergne et al., 1996). It has been speculated that Ebi3 can be secreted and function as a homodimer; however, this remains to be elucidated (Vignali and Kuchroo, 2012; Wirtz et al., 2015). A previous study suggested that a teleost Ebi3 has a regulatory effect on the innate immune response of peripheral blood leukocytes in fish (Li et al., 2013). Another study showed that activated human and mouse NK cells are the producers of EBI3 and that EBI3 promotes the persistence of MCMV infection (Jensen et al., 2017). Our data demonstrated that autocrine Ebi3 promoted not only T-cell activation and differentiation into Th1, Th17, and Treg cells, but also B-cell activation and differentiation into plasma cells via gp130-STAT3 signaling pathway. Thus, autocrine Ebi3 may play an important role in the differentiation of Th and B cells.

## 2. Material and methods

### 2.1. Ethics committee approval

Care, use, and treatment of mice in this study were in strict agreement with international guidelines for the care and use of laboratory animals. This study was approved by the Animal Ethics Committee of Beijing Institute of Basic Medical Sciences.

### 2.2. Mice

Seven-to-nine-week-old C57BL/6 J (B6) mice were purchased from Huafukang Corp., Beijing, China. Stat1$^{-/-}$ and Stat3$^{-/-}$ mice were purchased from Taconic animal facility and previously reported (Wang et al., 2014). All mice were bred in our animal facilities under specific pathogen-free conditions.

### 2.3. Production and characterization of rEbi3, rIL-27p28, rIL-35p35

Production and characterization of rEbi3, rIL-27p28, rIL-35p35 were previously described (Wang et al., 2014, 2012). Briefly, the Ebi3, p28, and p35 cDNA was fused to an N-terminal melittin (HBM) secretion signal sequence in pMIB vector (V8030-01, Invitrogen, Carlsbad, CA) containing Flag-IRES and V5-His sequences. The expression construct was then transfected into insect High Five cells, and stable transfectants were identified by drug selection (Blasticidin S, Invitrogen, 100 μg/ml). The recombinant protein(s) secreted by the insect cells was sequentially purified by the Ni-NTA Purification system (Invitrogen), size-exclusion centricon filtration and two consecutive cycles of FPLC gel filtration chromatography. The highly enriched p35 and Ebi3 preparation was characterized by SDS-PAGE gels stained with Coomassie Blue, western blot or immunoprecipitation assays using V5, Flag, p28, p35 or Ebi3 monoclonal antibodies or sedimentation equilibrium ultracentrifugation.

### 2.4. CD4$^+$ T-cell purification and in vitro differentiation

Naive CD4$^+$CD25$^-$ T cells were first isolated from splenocytes using CD4$^+$ T-cell Isolation Kit (Miltenyi Biotec) and then sorted out by FACSAria (BD Biosciences). Purified naive CD4$^+$CD25$^-$ T cells were stimulated with plate-bound 10 μg/ml anti-CD3 and 3 μg/ml anti-CD28 (Th0 condition) under Th1 (10 ng/ml mIL-2, 10 ng/ml mIL-12), Th17 (10 μg/ml anti-IFNγ, 10 μg/ml anti-IL-4, ng/ml IL-6 10, 10 ng/ml TGF-β) or Treg (10 μg/ml anti-IFNγ, 10 μg/ml anti-IL-4, 10 ng/ml hTGFβ) (BD Biosciences or eBioscience) polarizing condition. In some experiments, neutralizing anti-mouse Ebi3 antibody (MABF848, clone V1.4C4.22, EMD Millipore Corp., MA, USA) was used to block the effect of Ebi3.

### 2.5. B-cell purification and in vitro differentiation

B-cell purification and in vitro differentiation were previously described (Zhu et al., 2018). Briefly, splenic B220$^+$ B cells were separated by B220 microbeads (Cat No. 130-049-501, Miltenyi Biotec). B cells were stimulated with 10 μg/ml LPS (Sigma L2630 from *Escherichia coli* 0111:B4; Sigma, St Louis, MO) in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 50 mM 2-mercaptoethanol.

### 2.6. Cytometric analysis and intracellular cytokine staining

Cells ($1 \times 10^6$ cells/sample) were washed with fluorescence activated cell sorting staining buffer (phosphate-buffered saline, 2% fetal bovine serum or 1% bovine serum albumin, 0.1% sodium azide). All samples were incubated with anti-Fc receptor Ab (BD Biosciences), prior to incubation with other Abs diluted in fluorescence-activated cell sorting buffer supplemented with 2% anti-Fc receptor Ab. For intracellular cytokine staining, 50 ng/ml PMA and 1 μg/mL ionomycin (Sigma-Aldrich) were added and then 10 μg/mL brefeldin A and 2 μM monensin were added 3 h later. After 3 h, cells were collected and fixed for 50 min with 1 mL fixation buffer (eBioscience). After washing, the fixed cells were stained with anti-Ebi3-PerCP (IC18341C, R&D systems), anti-IFN-γ-FITC (XMG1.2), anti-IL-17-PE (TC11-18 H10), and anti-Foxp3–FITC (FJK-16 s), anti-CD138-APC (281-2), and anti-GL7-FITC (GL7) (BD Biosciences or eBioscience). The samples were filtered immediately before analysis or cell sorting to remove any clumps. Data were acquired using FACSCalibur (BD Biosciences) and analyzed using CellQuest software (Becton Dickinson).

### 2.7. Ebi3 concentration analysis by ELISA

The concentration of Ebi3 was measured by ELISA and previously described (Wang et al., 2016b). Briefly, for detection of Ebi3, diluted supernatant was coated in triplicate to the plate for overnight at 4 °C. After washing, 4 μg/mL biotin rat anti-mouse Ebi3 antibodies (Novus Biologicals, Clone No. 5P10D3, Cat No. NBP2-03943B) were added to the plate, and were incubated for 1 h at 37 °C. Thereafter, unbinding antibodies were washed off, followed by addition of avidin-HRP (1/1000 diluted) (eBiosciece). Plates were incubated for 1 h at 37 °C. Finally, the color was developed by incubation with o-phenylenediamine. The OD was read at 492 nm with an ELISA reader (Bio-Rad). Standard curves were established to quantitate the amounts of the recombinant

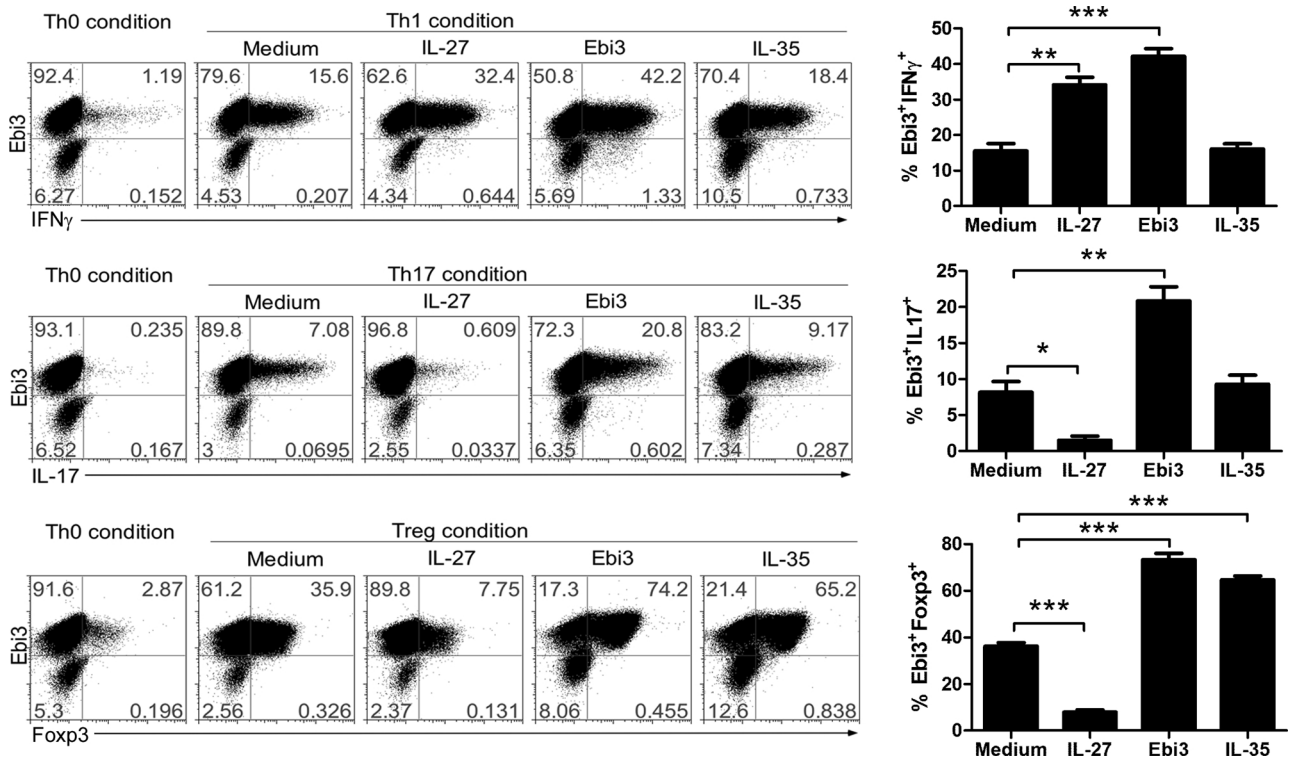


**Fig. 1.** Ebi3 promotes the differentiation of Th1, Th17 and Treg cells. Purified naive CD4 + CD25- T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (Th0 condition) under Th1, Th17, or Treg polarizing condition in the presence of 50 ng/ml Ebi3, IL-27 (mixture of Ebi3 and IL-27p28), or IL-35 (mixture of Ebi3 and IL-35p35). After 3 days, cells were harvested, and Th1, Th17, or Treg polarization was analyzed by the intracellular staining assay with anti-mouse Ebi3, IFNγ, IL-17, or Foxp3 antibodies. The percentage of Ebi3, IFNγ, IL-17, or Foxp3-expressing $CD4^+$ T cells (left panel) and statistical analysis of the percentages of $Ebi3^+IFN\gamma^+$, $Ebi3^+IL17^+$, and $Ebi3^+Foxp3^+$ in $CD4^+$ T cells (right panel) are shown. The data are representative of three independent experiments. A one-way ANOVA was followed by Bonferroni post-tests to compare IL-27, Ebi3, or IL-35-treated group with medium control group. Error bars, s.e.m., *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

mouse EBI3 protein (ab206301, abcam).

### 2.8. CFSE labelling and flow cytometry

CFSE labelling was performed as previously described (Eckl-Dorna et al., 2015). Briefly, B cells were incubated with CFSE (Invitrogen Inc.) in PBS (labelling concentration of 5 μM) for 10 min at 37 °C. The labelling reaction was stopped by adding pure foetal calf serum (FCS) (PAA) for 5 min. Cells were then washed with medium and cultured as described above. Upon harvest, cells were washed, resuspended in PBS 1% BSA and analyzed by flow cytometry.

### 2.9. pSTAT1 and pSTAT3 analysis by flow cytometry

Purified naive $CD4^+CD25^-$ T cells were stimulated for 48 h with plate-bound 10 μg/ml anti-CD3 and 3 μg/ml anti-CD28 antibodies. B cells were stimulated for 48 h with 10 μg/ml LPS. Cells were harvested and starved for 2 h in serum-free medium (0.5% BSA). Cell were stimulated for 0.5, 1, 2, 4 h with 50 or 100 ng/ml Ebi3 and then fixed in 2% paraformaldehyde at 37 °C for 10 min, pelleted, washed once in flow buffer (PBS, pH 7.2, with 0.2% BSA and 0.09% sodium azide), and permeabilized in 100% ice cold methanol, on ice for 30 min. Cells were washed twice in flow buffer and then labeled with anti-pY701-Stat1-Alexa Fluor® 488 (4a), anti–pY705-Stat3-PE (49/p-Stat3) or isotype control (BD Biosciences), and anti- pTyr693-STAT4-APC (Cat No. 17-9044-42, eBioscience) for 20 min. Cells were washed once in flow buffer and analyzed on a FACScan (BD Biosciences). CellQuest Version 3.3 (BD Biosciences) was used for data acquisition and analysis.

### 2.10. Immunoblot analysis

Whole-cell lysates were prepared for Western blotting. Twenty-five micrograms of cell protein were electrophoretically separated on a 10% SDS–polyacrylamide gel and transferred to a PVDF membrane, which was then blocked by incubation for 1 h at room temperature in 5% fat-free dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). The blots were then incubated overnight at 4 °C with rabbit antibodies against anti-mouse antibodies specific to STAT3, pSTAT3, STAT1, pSTAT1, STAT4 (4904 and 9131, 9172, 8826, 2653, respectively, Cell Signaling Technology, Danvers, MA,), pSTAT4 (PA5-64562, Invitrogen) and GAPDH (FL-335, Santa Cruz Biotechnology) antibodies diluted 1:1000 in TBS-T containing 5% bovine serum albumin, washed for 25 min with TBS-T, and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary F(ab')2 (Zymed Laboratories, San Francisco, CA) (1 : 20 000 in TBS-T containing 5% bovine serum albumin), then bound antibody was visualized using the ECL detection system (Amersham, Arlington Heights, IL).

### 2.11. Ebi3 promoter reporting gene analysis

Promoter reporting gene analysis has been described in our previous studies (Zhang et al., 2017). The firefly luciferase reporter plasmid pEZX-PG04.1 (Fugene Corp., Guangzhou, China) with the 5′-flanking region from start codon upstream − 2000 ~ + 100 of mouse Ebi3 gene. 0.5 μg Lv201/STAT3, 0.5 μg firefly luciferase reporter plasmids pEZX-PG04.1/Ebi3 promoter (General Biosystems, Anhui, China), and 0.05 μg Renilla luciferase reporter vector pRLSV-40 vector (cat# E2231, Promega Corp.) were co-transduced into 293 T cells in 12-well plate by using 6 μL Lipofectamine®2000 Reagent (Cat# 11668-019, Invitrogen

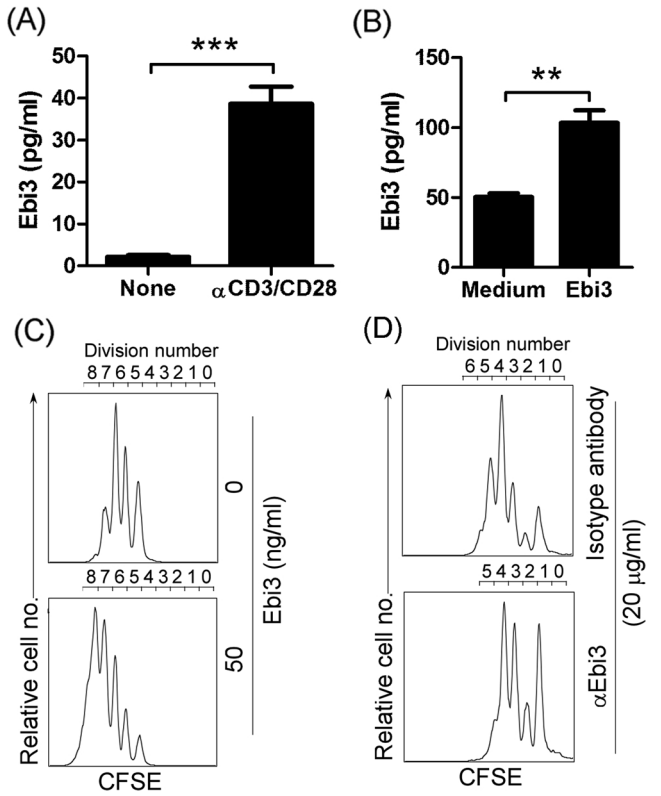


**Fig. 2.** Autocrine Ebi3 promotes T-cell activation. (A) Ebi3 was secreted by anti-CD3/CD28-stimulated T cells. Purified naive $CD4^+CD25^-$ T cells were stimulated for 3 days with only medium (None) or plate-bound anti-CD3 and anti-CD28 (αCD3/CD28). On day 3, supernatant was collected and Ebi3 protein was determined by ELISA assay. (B) Ebi3 promoted Ebi3 secretion in anti-CD3/CD28-stimulated T cells. Purified naive $CD4^+CD25^-$ T cells were stimulated for 2 days with plate-bound anti-CD3 and anti-CD28 (Th0 condition) in the presence of 50 ng/ml Ebi3. On day 3, the cells were washed and cultured for another day in the fresh medium. On day 4, supernatant was collected and Ebi3 protein was determined by ELISA assay. (C) Ebi3 promotes T-cell division. Purified naive $CD4^+CD25^-$ T cells were incubated with CFSE and stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 (Th0 condition) in the presence of 0 or 50 ng/ml Ebi3. CFSE cell division profiles demonstrate the higher proliferative responses of Ebi3-treated T cells compared to untreated T cells. The number of divisions undergone by cells in specific CFSE peaks is indicated. (D) Anti-Ebi3 suppresses T-cell division. Purified naive $CD4^+CD25^-$ T cells were incubated with CFSE and stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 (Th0 condition) in the presence of 20 μg/ml anti-Ebi3 or isotype control antibodies. CFSE cell division profiles demonstrated lower proliferative responses of anti-Ebi3-treated T cells compared with untreated T cells. The number of divisions undergone by cells in specific CFSE peaks is indicated. (A–D) The data are representative of three independent experiments. (A, B) The data were analyzed by Student's *t*-test (two tailed). Error bars, s.e.m., $^{**}p < 0.01$, $^{***}p < 0.001$.

Corp.). On day 3, sequential measurement of firefly luciferase (Reporter #1) followed by Renilla luciferase activity (Reporter #2) was assessed on 1420 Multilabel Counter (1420 Victor 3, PerkinElmer Corp.), and analyzed. The results were shown as the ratio of firefly to Renilla luciferase activity.

### 2.12. Knock-down of IL-27Rα and gp130 with shRNA

Control, IL-27Rα or gp130-specific shRNA-infected B cells were previously described (Wang et al., 2016b). Briefly, in a six well tissue culture plate, seed $1 \times 10^6$ B cells per ml in 2 ml antibiotic-free normal growth medium supplemented with FBS. Cells were stimulated overnight with LPS (10 μg/ml, L2880, Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a $CO_2$ incubator. On day 2, $1 \times 10^6$ infectious units of virus (IFU) of Control, IL-27Rα or gp130-specific shRNA-expressing lentivirus (sc-108084, sc-60837-V, and sc-35502-V, respectively, Santa Cruz Biotech) and 10 μg/ml polybrene (H9268, Sigma-Aldrich, St.) were added into the culture. On day 1 after infection, the transfection mixture was removed, 1x normal growth medium containing LPS (10 μg/ml) plus 50 ng/ml Ebi3 was added into the culture and cells were re-stimulated for 3 days.

### 2.13. Statistics

Statistics were analyzed by using GraphPad Prism (version 5.0, GraphPad Software Inc., USA). The data were shown as mean ± standard error of the mean (SEM). Student's *t*-test was employed to determine significance between two groups (paired or unpaired) and One-Way and Two-Way ANOVA analysis was used to determine significance among several groups. Differences were considered statistically significant when $p < 0.05$.

## 3. Results

### 3.1. Ebi3 promotes the differentiation of Th1, Th17, and Treg cells

To explore the role of Ebi3 in T-cell differentiation, we stimulated naive $CD4^+CD25^-$ T cells in Th1, Th17, or Treg polarizing condition. As expected, we found that IL-27 (mixture of Ebi3 and IL-27p28) could promote Th1 differentiation and suppress Th17 and Treg differentiation (Fig. 1). IL-35 (mixture of Ebi3 and IL-35p35) effectively promoted Treg but not Th1 and Th17 differentiation (Fig. 1). Interestingly, we found that Ebi3 effectively promotes all of Th1, Th17, and Treg differentiation (Fig. 1). In addition, we also found that IFNγ, IL-17, and Foxp3 are expressed in $Ebi3^+$ but not in Ebi3- T cells (Fig. 1). These results suggest that Ebi3 plays an important role in promoting Th cell differentiation.

Next, we examined whether Ebi3 alone induced Th cell differentiation. Purified naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (Th0 condition) without Th1 and Th17 polarizing condition. We found that Ebi3 could not induce Th1 or Th17 production (Suppl. Fig. 1). These results suggest that Ebi3 alone does not induce Th cell differentiation.

### 3.2. Autocrine Ebi3 promotes T-cell differentiation by up-regulating division

We found that activated T cells and Th1, Th17, and Treg cells are Ebi3 positive (Fig. 1). These results indicate that activation induces Ebi3. Thus, we determined the levels of Ebi3 in the supernatant of activated T cells. As expected, we found that activated T cells secreted Ebi3 (Fig. 2A). Furthermore, we found that Ebi3 could promote Ebi3 production in activated T cells (Fig. 2B). Proliferation of the various B cell populations was visualized by monitoring the serial twofold reductions in CFSE fluorescence intensity that accompany cell division (Lyons and Parish, 1994). To explore the role of Ebi3 in T-cell division, naïve T cells were labeled with the division-tracking dye CFSE and stimulated with anti-CD3/CD28 in the presence of Ebi3. Ebi3 effectively promoted T-cell proliferation (Fig. 2C). On the other hand, neutralizing anti-Ebi3 antibody could suppress T-cell division (Fig. 2D) implying that Ebi3 might promote T-cell division. To further examine the role of Ebi3 in Th cell differentiation, we blocked the effect of Ebi3 with neutralizing anti-Ebi3 antibody in Th1, Th17, and Treg polarizing condition. We found that anti-Ebi3 antibody suppresses Ebi3 expression and Th1, Th17 and Treg differentiation (Fig. 3). These results suggest that autocrine Ebi3 promotes T-cell differentiation by up-regulating cell division.

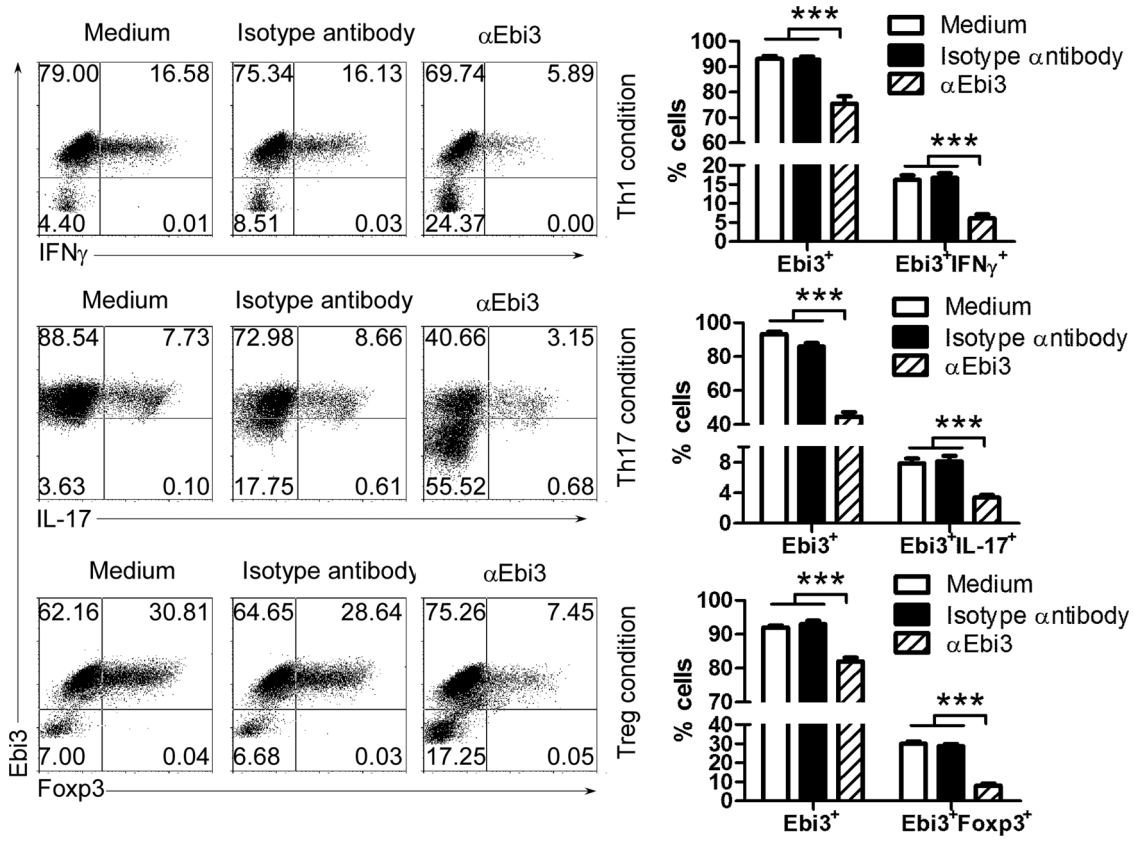


**Fig. 3.** Anti-Ebi3 antibody suppresses Th differentiation. Purified naive $CD4^+CD25^-$ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 under Th1, Th17 and Treg polarizing condition in the presence of 20 μg/ml neutralizing anti-Ebi3 antibodies and isotype control antibodies. After 3 days, cells were harvested and analyzed by the intracellular staining assay with anti-mouse Ebi3 and IFNγ, IL-17, or Foxp3 antibodies. The percentages of Ebi3- and IFNγ, IL-17 or Foxp3-expressing $CD4^+$ T cells (left panel) and statistical analysis for the percentages (right panel) are shown. The data represent three independent experiments. A two-way ANOVA was followed by Bonferroni post-tests. Error bars, s.e.m., ***$p < 0.001$.

### *3.3. Expression of Ebi3 in the process of B-cell differentiation from activated B cells into PC*

To explore the role of Ebi3 in B-cell division and differentiation, we used LPS to induce naïve B cells differentiated into $GL7^+$ activated B cells and plasma cells, as described previously (Zhang et al., 2017; Zhu et al., 2017; Mauri and Bosma, 2012; Cervenak et al., 2001; Wang et al., 2016c). LPS could effectively induce the production of $GL7^+$ activated B cells and $CD138^+$ PC (Fig. 4). In addition, we found that low percentage of naïve B cells (day 0) expressed Ebi3 (Fig. 4A). Importantly, the percentage of $Ebi3^+$ B cells was significantly time-dependently up-regulated by LPS (Fig. 4). Next, we determined the level of Ebi3 in the supernatant of LPS-stimulated B cells. We found that the level of secreted Ebi3 was significantly up-regulated by LPS (Fig. 5A). Critically, we found that $GL7^+$ activated B cells; furthermore, $CD138^+$ PC were Ebi3 positive (Fig. 4). Altogether, our data demonstrated that Ebi3 was expressed during the differentiation from B-cell activation to plasma cells.

### *3.4. Autocrine Ebi3 promotes B-cell division and differentiation*

To explore the role of Ebi3 in B-cell division, splenic $B220^+$ B cells were labeled with the division-tracking dye CFSE and stimulated with LPS in the presence of Ebi3. When we examined responses to T-independent stimulus LPS, Ebi3 effectively promoted B-cell proliferation (Fig. 5B). These results suggest that Ebi3 promotes B-cell division.

To explore the role of Ebi3 in B-cell differentiation, we used LPS to stimulate splenic $B220^+$ B cells in the presence of Ebi3 or neutralizing anti-Ebi3 antibody. We found that Ebi3 could promote $CD138^+$PC production (Fig. 5C, D), whereas anti-Ebi3 could effectively reduce Ebi3 expression and $CD138^+$PC production (Fig. 5C, D). These results suggest that autocrine Ebi3 promotes B-cell differentiation.

### *3.5. Ebi3 induced STAT3 signaling via gp130*

Previous studies have demonstrated that IL-27 (p28/Ebi3) induces STAT1 activation by binding its receptor (IL-27Rα and gp130) (Pflanz et al., 2002; Takeda et al., 2003; Meka et al., 2015; Owaki et al., 2005a) while IL-35 (p35/Ebi3) induces STAT1 and STAT4 activation in T cells by a unique heterodimer of receptor chains IL-12Rβ2 and gp130 or homodimers of each chain (Collison et al., 2007, 2012), and STAT1 and STAT3 activation in B cells by IL-12Rβ2 and IL-27Rα subunits (Wang et al., 2014). Therefore, we first examined the effect of Ebi3 on STAT1, STAT3, and STAT4 activation. To explore the mechanisms by which Ebi3 promote T/B-cell differentiation, we used intracellular pSTAT3, pSTAT1, pSTAT4 staining assay and western blot to value the effect of Ebi3 on STAT1, STAT3, and STAT4 activation. We found that Ebi3 time-dependently induced STAT3 but not STAT1 and STAT4 activation in $CD4^+$T cells (Fig. 6) and B cells (Suppl. Fig. 2). To further value the role of STAT3 in Ebi3-mediated biological function, we used WT, STAT1 KO, and STAT3 KO mice. We found that compared with WT, $STAT1^{-/-}$ did not affect Ebi3 expression and T/B-cell differentiation (Fig. 7A, Suppl. Fig. 3A), whereas $STAT3^{-/-}$ effectively reduced Ebi3 expression and T/B-cell differentiation (Fig. 7A, Suppl. Fig. 3A). These results suggest that Ebi3 mediates biological function via STAT3. We found that STAT3 played an important role in Ebi3 expression (Fig. 7A, Suppl. Fig. 3A). To further prove that STAT3 induced Ebi3 expression, we used Ebi3 promoter reporting gene analysis and found that STAT3 promoted Ebi3

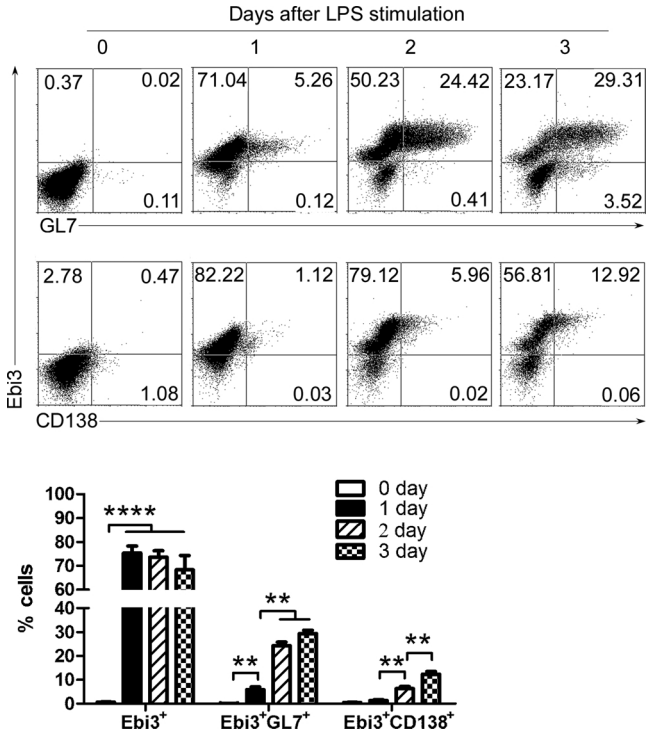


**Fig. 4.** Activated B cells and plasma cells express Ebi3. Purified splenic B220$^+$ B cells were stimulated for 3 days with 10 μg/ml LPS. B cells were harvested, stained with anti-mouse GL7, CD138 and Ebi3 antibodies; and analyzed by flow cytometry (FACS). The percentages of GL7, CD138, and/or Ebi3-expressing B cells (upper panel) and statistical analysis of the percentages (lower panel) are shown. The data are representative of three independent experiments. A two-way ANOVA was followed by Bonferroni post-tests. Error bars, s.e.m., **p < 0.01, ****p < 0.0001.

promoter activation (Fig. 7B). These findings indicate that Ebi3 mediates biological functions via STAT3.

To determine the role of gp130 and IL-27Rα in Ebi3-mediated STAT3 activation, we used shRNA assay to knock-down gp130 and IL-27Rα. The effect of knock-down with shRNA was proved previously (Wang et al., 2016b). We found that the knock-down of gp130 but not IL-27Rα reduced the effect of Ebi3 on STAT3 activation (Fig. 7C, Suppl. Fig. 3B), Th1, Th17, and Treg (Fig. 7D), GL7$^+$activated B cells and CD138$^+$PC production (Suppl. Fig. 3C). Hence, gp130 appears to mediate Ebi3-induced STAT3 activation and T/B-cell differentiation.

## 4. Discussion

Although sharing the same subunit Ebi3, IL-27 (p28/Ebi3) and IL-35 (p35/Ebi3) have a different biological function suggesting that Ebi3 subunit may functions as a carrier. Our data demonstrated that Ebi3 alone could effectively promote T/B-cell division and the differentiation of Th1, Th17, Treg, and plasma cells. These results indicate that Ebi3 promotes Th- and B-cell division and differentiation.

IL-27 (p28/Ebi3) primes Th1 cell differentiation that play an important role in imiquimod-induced psoriasis-like skin lesions (Shibata et al., 2013), and autoimmune diseases like type I diabetes (Wang et al., 2008), whereas it suppresses Th17 cell development (Shibata et al., 2013; Owaki et al., 2005b). Consistent with these studies, our data demonstrated here that IL-27 promoted but not induced Th1 cell differentiation, whereas it suppressed Th17 and Treg cell differentiation (Fig. 1, Suppl. Fig. 1). However, we found that besides for promoting Th1-cell differentiation, Ebi3 could also promote the differentiation of Th17 and Treg cell differentiation (Fig. 1). Altogether, these results suggest that Ebi3 alone promotes Th1, Th17, and Treg cell differentiation, whereas p28 could reduce the effect of Ebi3 on Th17 and Treg but not Th1 cell differentiation.

IL-35 (p35/Ebi3) is produced by a wide range of regulatory lymphocytes, and it plays a role in autoimmune diseases, such as inflammatory bowel disease, infection by pathogenic intracellular

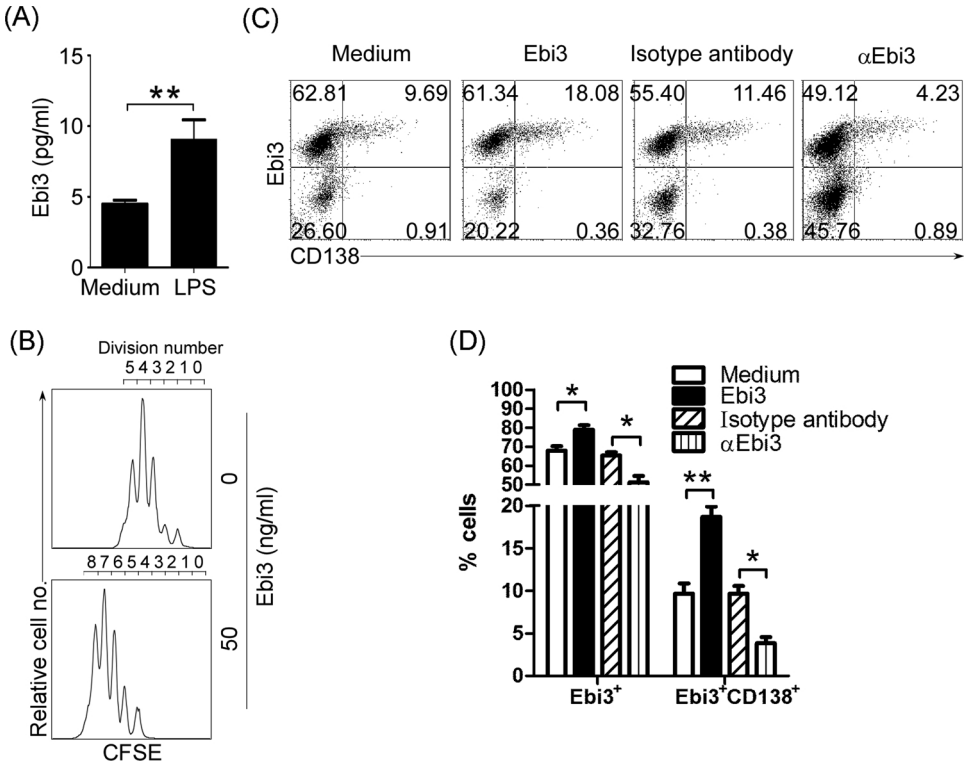


**Fig. 5.** Autocrine Ebi3 promotes B-cell activation and differentiation. (A) Ebi3 was secreted by LPS-stimulated B cells. Purified splenic B220$^+$ B cells were stimulated for 3 days with 10 μg/ml LPS. Supernatant was collected and Ebi3 protein was determined by ELISA assay. (B) Ebi3 promotes B-cell division. Purified splenic B220$^+$ B cells were incubated with CFSE and stimulated for 3 days with 10 μg/ml LPS in the presence of 0 or 50 ng/ml Ebi3. Upon harvest, cells were washed, resuspended in PBS with 1% BSA, and analyzed by FACS. CFSE cell division profiles demonstrated higher proliferative responses to Ebi3-treated B cells compared with untreated B cells. The number of divisions undergone by cells in specific CFSE peaks is indicated. (C, D) Ebi3 promotes B-cell differentiation. Purified splenic B220$^+$ B cells were stimulated for 3 days with 10 μg/ml LPS in the presence of 50 ng/ml Ebi3 or 20 μg/ml anti-Ebi3 and isotype control antibodies. Cells were harvested, stained with anti-mouse Ebi3 and CD138 antibodies, and analyzed by FACS. The percentages of Ebi3 and/or CD138-expressing B cells and statistical analysis of the percentages (lower panel) are shown. (A–D) The data are representative of three independent experiments. The data were analyzed by Student's *t*-test (two tailed) (A) and a two-way ANOVA was followed by Bonferroni post-tests (D). Error bars, s.e.m., *p < 0.05, **p < 0.01.

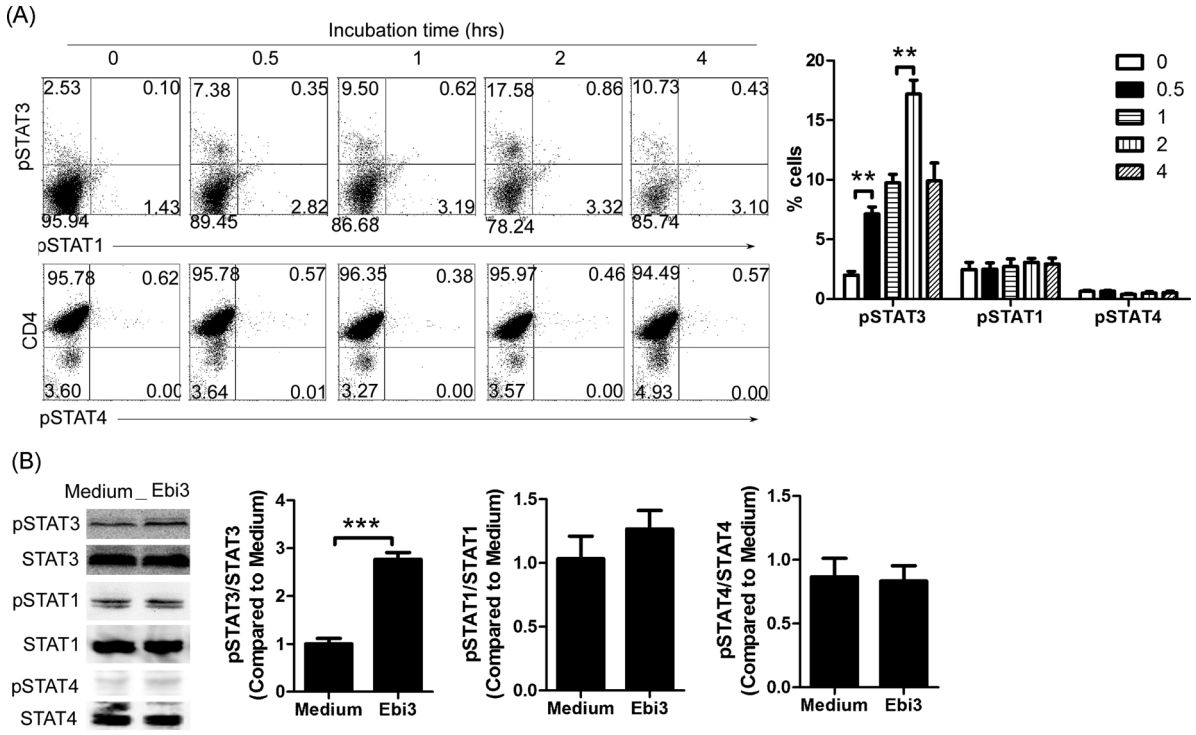


**Fig. 6.** Ebi3 induced STAT3 signaling in T cells. (A) Ebi3 time-dependently induced STAT3 activation in $CD4^+$T cells. Purified naive $CD4^+CD25^-$ T cells were stimulated for 48 h with plate-bound 10 μg/ml anti-CD3 and 3 μg/ml anti-CD28 antibodies. Cells were harvested and starved for 2 h in serum-free medium (0.5% BSA). Cells were stimulated for 0, 0.5, 1, 2, and 4 h with 50 ng/ml Ebi3. The intracellular staining was done with anti-pY701-Stat1-Alexa Fluor® 488 and anti–pY705-Stat3-PE (49/p-Stat3), and anti-pT693-Stat4-APC. FACS was used to analyze pSTAT3, pSTAT1, and pSTAT4 expression. The percentages of pSTAT3, pSTAT1 and pSTAT4-expressing T cells (left panel) and statistical analysis of the percentages (right panel) are shown. (B) Ebi3 induced STAT3 activation in $CD4^+$T cells. Purified naive $CD4^+CD25^-$ T cells were stimulated for 48 h with plate-bound 10 μg/ml anti-CD3 and 3 μg/ml anti-CD28 antibodies. Cells were harvested and starved for 2 h in serum-free medium (0.5% BSA). Cells were stimulated for 1 h with 50 ng/ml Ebi3. pSTAT3, STAT3, pSTAT1, STAT1, pSTAT4, and STAT4 expression was analyzed by using western blot assay. Band intensities of pSTAT1 and STAT1, pSTAT3 and STAT3, or pSTAT4 and STAT4 were quantified using ImageProPlus 5.0 software. The density ratios of phosphorylated to total protein was compared to medium control, as shown (left panel). (A, B) The data represent three independent experiments and analyzed by a two-way ANOVA were followed by Bonferroni post-tests (A) and Student's *t*-test (two tailed) (B). Error bars, s.e.m., $^{**}p < 0.01$, $^{***}p < 0.001$.

microbe Salmonella typhimurium, and replication of HBV virus (Vignali and Kuchroo, 2012; Collison et al., 2007, 2012; Shen et al., 2014; Wang et al., 2014). It has been shown that IL-35 can be used to induce the conversion of conventional $CD4^+$ T-cells into induced $Foxp3^+$ regulatory T-cells that have potent suppressive capacity in vitro and in vivo (Collison et al., 2007). Consistent with these studies, our data demonstrated that IL-35 promoted $Foxp3^+$ Treg but not Th1 and Th17 cell differentiation (Fig. 1). However, Ebi3 promoted not only $Foxp3^+$ Treg cell differentiation, but also Th1- and Th17-cell differentiation (Fig. 1). Altogether, these results insinuate that Ebi3 alone promotes Th1, Th17 and Treg cell differentiation, whereas p35 could reduce the effect of Ebi3 on Th1 and Th17 but not Treg cell differentiation.

Ebi3 was identified as a secreted glycoprotein by its induced expression in B lymphocytes in response to Epstein-Barr virus infection. In a previous study, Ebi3 was detected by western blot analysis in the supernatant from human NK cells after NKG2D or IL-12 plus IL-18 stimulation and by mouse NK cells during mouse cytomegalovirus (MCMV) infection (Jensen et al., 2017). We also demonstrated here that Ebi3 could be expressed and secreted in LPS-stimulated B cells including $GL7^+$ activated B cells and plasma cells (Figs. 4,5), and anti-CD3/CD28-activated T cells including Th1, Th17 and Treg cells (Figs. 1,2). In $CD4^+$ T cells, Ebi3 gene expression was undetectable before stimulation, but it was consistently induced upon CD3/CD28 stimulation (Bardel et al., 2008). In accordance with these publications, we conducted FACS analysis, which revealed that Ebi3 could be expressed in $CD4^+$ T cells upon CD3/CD28 stimulation (Figs. 1–3). Once block of Ebi3 with neutralizing anti-Ebi3 antibody could effectively reduce the effect of Ebi3 on promoting Th1 and Treg cell differentiation (Fig. 3). These results further prove that Ebi3 promotes Th cell differentiation.

IL-27 stimulation of purified B cells in vitro enhances the expression of the transcription factor Bcl-6 and promotes phenotypic features of germinal center (GC) B cells (Redzwan et al., 2013). These results suggest that IL-27 signals to B cells play an important role in cell fate and the development of GC-driven lupus (Redzwan et al., 2013). Studies conducted with mouse B cells that IL-27 specifically induces the production of IgG2a (Yoshimoto et al., 2004; Chen et al., 2000). In addition, in studies with human B cells, IL-27 induces the production of IgG1 (Boumendjel et al., 2006). These results suggest that IL-27 promotes the production of plasma cells. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases (Shen et al., 2014; Wang et al., 2014). Our previous study has shown that IL-35 is a physiological inducer of IL-10-producing Breg cells (Wang et al., 2014). Overall, these studies suggest that IL-35 promotes $IL\text{-}10^+$Breg cells differentiation from naïve B cells. We demonstrated here that Ebi3 subunit could promote B-activation and plasma cells (Fig. 5). Altogether, these results suggest that Ebi3 subunit functions as a carrier promotes all B-cell differentiation including plasma cells, whereas IL-27 and IL-35 promote $IgG^+$ plasma cells and $IL\text{-}10^+$Breg cells, repectively.

When IL-27 binds to the IL-27 receptor consisting of IL-27Rα and gp130, it induces signaling pathways including JAK-STAT (Meka et al., 2015). IL-27 could inhibit Treg cells through STAT1 and STAT3 (Meka et al., 2015; Iwasaki et al., 2015). IL-27 is able to activate STAT3 signaling, which eventually increases IL-10 secretion from Treg cells (Yoshida and Hunter, 2015). IL-35 has been found to mediate STAT1

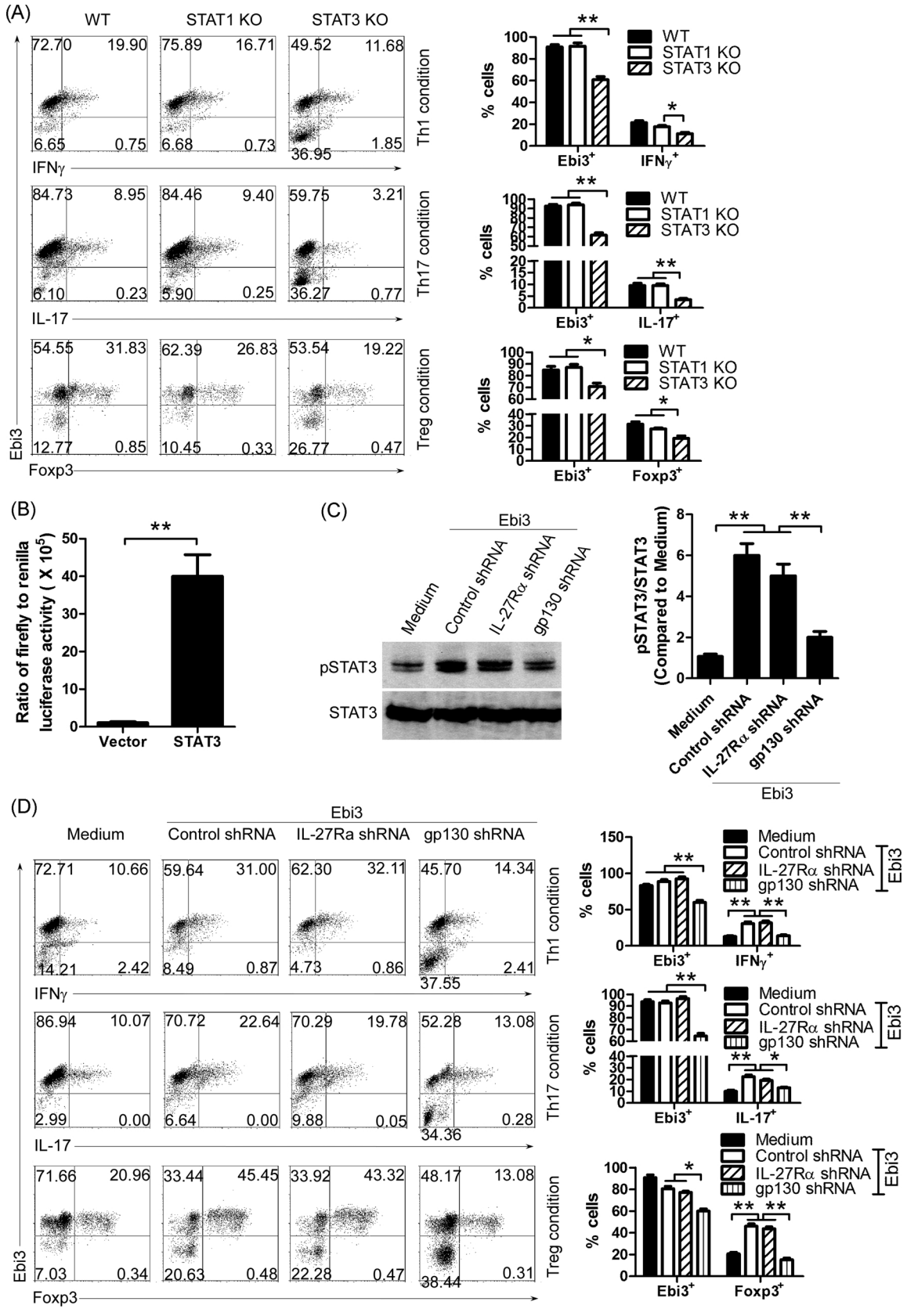


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**Fig. 7.** Ebi3 promotes Th cell differentiation via gp130-STAT3. (A) STAT3 knockdown reduced Ebi3 expression and Th cell differentiation. Purified naive $CD4^+CD25^-$ T cells from WT, STAT1 KO, and STAT3 KO mice were stimulated for 96 h with plate-bound 10 μg/ml anti-CD3 and 3 μg/ml anti-CD28 antibodies under Th1, Th17, and Treg polarization condition. Cells were harvested; stained with anti-IFNγ, IL-17, Foxp3, and Ebi3 antibodies and analyzed by using FACS. The percentage of IFNγ-, IL-17-, Foxp3-, and/or Ebi3-expressing $CD4^+$T cells (left panel) and statistical analysis of the percentages (right panel) are shown. (B) STAT3 promoted the activation of Ebi3 promoter. STAT3 -expressing LV201 (STAT3) or empty vector LV 201 (Vector) and luciferase reporter vector pEZX-PG04.1/Ebi3 promoter (−2000 ~ + 100 bp) were co-transduced into 293 T cells. Dual luciferase reporter gene expression was analyzed, and the results are shown as the ratio of firefly to Renilla luciferase activity. (C) Knock-down of gp130 but not IL-27Rα reduced Ebi3-induced STAT3 activation in $CD4^+$T cells. Purified naive $CD4^+CD25^-$ T cells were infected with control, IL-27Rα, or gp130-specific shRNA-expressing lentivirus, and then stimulated for 48 h with plate-bound 10 μg/ml anti-CD3 and 3 μg/ml anti-CD28 antibodies. Cells were harvested and starved for 2 h in serum-free medium (0.5% BSA) and subsequently stimulated for 1 h with 50 ng/ml Ebi3. pSTAT3 and STAT3 expression was analyzed using western blot assay. Band intensities of pSTAT3 and STAT3 were quantified using ImageProPlus 5.0 software. The density ratio of phosphorylated to total protein was compared to medium control, as shown (left panel). (D) Knock-down of gp130 but not IL-27Rα reduced the effect of Ebi3 on Th cell differentiation. Naive $CD4^+CD25^-$ T cells were infected with control, IL-27Rα or gp130-specific shRNA-expressing lentivirus and stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 under Th1, Th17 and Treg polarizing condition in the presence of 50 ng/ml Ebi3. Cells were harvested; stained with anti-IFNγ, IL-17, Foxp3 and Ebi3 antibodies; and analyzed using FACS. The percentage of IFNγ-, IL-17-, Foxp3-, and/or Ebi3-expressing $CD4^+$T cells (left panel) and statistical analysis of the percentages (right panel) are shown. (A–D) The data represent three independent experiments and analyzed using two-way (A, D) and one-way (C) ANOVAs followed by Bonferroni post-tests and Student's *t*-test (two tailed) (B). Error bars, s.e.m., *$p < 0.05$, **$p < 0.01$.

and STAT4 activation in $CD4^+$T cells through a unique heterodimer of receptor chains IL-12Rβ2 and gp130 or homodimers of each chain (Collison et al., 2012), whereas it activated STAT1 and STAT3 in B cells through the IL-35 receptor comprising the IL-12Rβ2 and IL-27Rα subunits (Wang et al., 2014). A previous study demonstrated that both human and mouse NK cells express Ebi3 and its receptor, gp130, after stimulation (Jensen et al., 2017). We showed here that Ebi3 induced STAT3 signaling via gp130 (Figs. 6,7, Suppl. Figs. 2,3). Thus, the findings suggest that Ebi3 effectively activates STAT3.

Ebi3 is secreted by activated T/B cells (Figs. 2,5). Activated T/B cells and differentiated Th1, Th17, Treg, and PC are Ebi3 positive (Figs. 1,3–5,7, Suppl. Fig. 3). In addition, Ebi3 induced STAT3 activation in T/B cells (Fig. 6, Suppl. Fig. 2). In turn, STAT3 promotes Ebi3 expression by up-regulating the activation of Ebi3 promoter (Fig. 7B). On the other hand, knockdown of STAT3 signaling pathway significantly reduced Ebi3 expression (Fig. 7, Suppl. Fig. 3). Altogether, these results indicate that gp130-STAT3 signaling pathway mediates autocrine Ebi3 in activated T/B cells.

STAT3 signaling pathway is critical for T-cell activation, proliferation and differentiation. Thus, autocrine Ebi3 mediates its effects including cell activation, proliferation and differentiation via gp130-STAT3 signaling pathway. Therefore, blocking the effect of Ebi3 could reduce Th1, Th17, Treg, and PC production by downregulating Ebi3 expression (Fig. 3,5,7, Suppl. Fig. 3). In addition, the effect of anti-Ebi3 antibody on $Ebi3^+$T cell reduction is more evident in the Th17 culture condition. This further proved that Ebi3 affected STAT3 signaling, which is more critical for Th17 differentiation.

## 5. Conclusions

In conclusion, autocrine Ebi3 can promote Th- and B-cell activation and differentiation via gp130-STAT3 signaling pathway. Thus, Ebi3 may play an important role in the differentiation of Th and B cells, and thus in infection, inflammation and autoimmune disorders.

## Competing financial interests

The authors declare no commercial or financial conflict of interest.

## Declarations of interest

None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.01.009.

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