


EDITOR'S CHOICE

Regulatory T cells sense effector T-cell activation through synchronized JunB expression

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To maintain immune tolerance, effector T-cell (Teff) responses must be checked by the regulatory T cells (Tregs) in time. It remains incompletely understood how Tregs sense real-time Teff activation. Here, we report that the AP-1 transcription factor JunB, which is induced in Teffs upon T-cell receptor (TCR) activation, is also increased in Tregs by TCR stimuli. Treg-specific deletion of *Junb* impairs Treg identity, causes uncontrolled inflammatory cytokine production by Teffs and leads to the T-box transcription factor T-bet-dependent spontaneous inflammation. Furthermore, JunB deficiency in Tregs unleashes antitumor Teff responses in a mouse model of melanoma. We conclude that JunB alarms Tregs of the emerging Teff activation and synchronizes immune regulation with the immune reaction in autoimmunity and cancer.

Keywords: antitumor T cells; autoimmunity; effector T cells; JunB; Tregs

The effector T cell (Teff) responses must be monitored in time by the regulatory T cells (Tregs) in order to maintain immune tolerance. The mechanisms through which Tregs sense the emerging Teff responses are not entirely clear. It has been shown that Tregs control different Teff subsets in a lineage-specific manner. Treg-specific ablation of the lineage-determining factors, such as STAT3, T-box transcription factor (T-bet), GATA3 and IRF4 impairs Treg-mediated immune regulation of different subsets of Teffs [1–5]. Because it takes at least several hours for the lineage-determining factors to be detectable in T cells, there is a gap in the Teff lineage commitment sensing by Tregs. We hypothesize that the early signaling events in Teffs, such as the activation of the transcription factor AP-1, are also activated in Tregs and alarm Tregs

of the initiation of Teff activation. As such, these early signaling events synchronize the Teff activation with Treg-mediated regulation, and intricately couple the immune reaction with immune regulation at an early stage.

JunB is a member of the AP-1 family. Its expression and transcriptional activity are rapidly increased in T cells after T-cell receptor stimulation and costimulation [6–8]. Furthermore, JunB has been shown to promote Th2 and Th17 cell differentiation, and loss of JunB diverts the CD4⁺ T-cell differentiation into Th1 and Tregs [9–11]. There have been some efforts devoted to addressing the role of JunB in Foxp3 expression and Treg function. Mixed results have been obtained using different experimental systems. For example, JunB has been shown to cooperate with

Abbreviations

NOD, nonobese diabetic; T-bet, T-box transcription factor; TCR, T cell receptor; Teff, effector T cells; Tregs, regulatory T cells.

c-Rel and c-Fos to regulate Foxp3 expression [12,13]. On the other hand, siRNA-mediated knockdown of JunB and cJun does not affect Foxp3 expression [14]. Recently, one report shows that JunB regulates Treg-mediated immunosuppression [15]. We notice that JunB is truncated but not completely deleted in Tregs of the mouse strain used in that study. It is not entirely clear if the remaining JunB peptide fragment still has any biological function.

In the current study, we have generated a mouse strain with Treg-specific ablation of *Junb*. In this strain, the complete JunB open reading frame is flanked by two loxp elements. Therefore, the JunB protein is deleted, rather than truncated in Tregs. We have characterized the role of JunB in Treg-mediated immune regulation under both steady status and in tumors. Interestingly, we observed that the T-cell activation-induced JunB protein increase was synchronized in Tregs and Teffs. JunB deficiency in Tregs causes uncontrolled effector cytokine production by Teff in autoimmunity and cancer. Collectively, our study suggests that the synchronized JunB expression in Teffs and Tregs intricately alarms Tregs of the emerging immune responses and promotes Treg-mediated immune tolerance.

Materials and methods

Mice

Junb^{flax/flax} mice were generated by P. Angel and M. Schorpp-Kistner (DKFZ) [16]. The *Foxp3^{YFP-Cre}* mice were kindly provided by A. Rudensky (Memorial Sloan Kettering Cancer Center) via M. Feuerer at DKFZ [17]. Mice were maintained in the German cancer research center (DKFZ)-specific pathogen-free facility. All the studies were performed in accordance with DKFZ regulations after approval by the German regional council at the Regierungspräsidium Karlsruhe.

Primary cell cultures

Mouse T cells were cultured in complete RPMI 1640 medium (plain RPMI 1640 medium with 10% FBS, Penicillin and Streptomycin, 2-mercaptoethanol, L-glutamine and nonessential amino acids). Antibodies and chemicals were supplemented as indicated.

Flow cytometry

Cells were first stained with antibodies for surface antigens in FACS buffer for 20–30 min on ice. After that, cells were washed with FACS buffer and fixed with 4% PFA. To stain

cytokines, cells were permeabilized using the eBioscience permeabilization buffer. To do Celltrace Violet (CTV) dilution assay, cells were labeled with CTV in complete medium at 37 °C for 20 min. Then cells were washed with RPMI 1640 medium with 1% FBS for three times. After 3 days, Celltracer Violet fluorescence was analyzed in the PacBlue channel using an LSR II (BD Biosciences, San Jose, CA, USA).

Immunoblot

Cells were lysed using RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris pH 8.0) and boiled in $\times 5$ SDS sample buffer (10% SDS, 10 mM DTT, 20% glycerol, 200 mM Tris-HCl, pH 6.8 and 0.05% bromophenol blue) for 5–10 min. Then cell lysates were loaded and resolved using 12% SDS/PAGE (120 V, until the blue indicator runs to the edge of the gel). Proteins were then transferred onto poly(vinylidene difluoride) (PVDF) membranes (100 V, 1 h, on ice). The membranes were then blocked with 5% milk in PBS supplemented with Tween-20 (PBST) for 1 h at room temperature, followed by incubation overnight at 4 °C with primary antibodies. The PVDF membrane was washed three times (5–10 min each time) with PBST and then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. After extensive washing with PBST, the membrane was developed using the ECL method.

RNA sequencing

Treg cells were FACS purified. RNA was prepared using the QIAGEN (Hilden, Germany) RNeasy Mini Kit. DNA was removed using the QIAGEN RNase-Free DNase Set. Library was prepared by German cancer research center High Throughput Sequencing Unit. Then, libraries were pooled with six samples in each lane and sequenced [Illumina HiSeq 2000 v4 (Illumina, San Diego, CA, USA) Single-Read 50 bp]. For all samples, low-quality bases were removed with `Fastq_quality_filter` from the `FASTX TOOLKIT` 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html) with 90% of the read needing a quality phred score > 20. `HOMERTOOLS` 4.7 [18] were used for PolyA-tail trimming, and reads with a length < 17 were removed. `PICARDTOOLS` 1.78 (<https://broadinstitute.github.io/picard/>) were used to compute the quality metrics with `CollectRNASeqMetrics`. With `STAR` 2.3 [19], the filtered reads were mapped against mouse genome 38 (mm10) using default parameters. Count data were generated using `featureCounts` [20] (parameters – `minReadOverlap 3 -T 3 -M -O`) for the genes annotated in the `encode.vM16.gtf` file. For the comparison with `DESeq2` [21], the input tables containing the replicates for groups to compare were created by a custom perl script. For `DESeq2`, `DESeqDataSetFromMatrix` was applied, followed by `estimateSizeFactors`, `estimateDispersions`, and `nbinomWald` testing. The result tables were annotated with

gene information (gene symbol) derived from the *genome.vM8.gtf* file. We then further analyze the RNA sequencing (RNA-seq) results using the Ingenuity Pathway Analysis (IPA; QIAGEN). The accession number of the RNA-seq data is [GSE129060](#) and available at Genome Expression Omnibus.

Statistical analysis

All the data were presented as mean \pm SD (error bar) unless otherwise specified. Where indicated, *P* values were determined by a two-tailed Student's *t*-test. The statistics of tumor growth over time were calculated using two-way ANOVA. We used GRAPHPAD PRIMS (v 7.0.3) (GraphPad Software, San Diego, CA, USA) to do the statistical analysis. *P* < 0.05 was considered statistically significant.

Results

T-cell stimulation increases JunB expression in Tregs and Teffs in a synchronized manner

To determine the JunB expression pattern in the resting and activated Teffs and Tregs, we FACS-sorted CD8⁺ T cells, Foxp3/YFP-negative CD4⁺ T cells, and Foxp3/YFP-positive CD4⁺ Tregs from the *Foxp3*^{YFP-Cre} mice [17]. Upon stimulation with anti-CD3 and anti-CD28, non-Treg CD4⁺ T cells and CD8⁺ T cells increased JunB expression. Similarly, Tregs upregulated JunB protein expression 60 min after T-cell activation (Fig. 1A).

To further examine the JunB expression dynamics in Tregs and non-Tregs, we performed Amnis imaging analysis of resting and activated T cells. JunB was only expressed at a basal level in quiescent T cells. Notably, 30 min after stimulation with anti-CD3 and anti-CD28, JunB was remarkably increased in both Tregs and non-Tregs. Within the 120-min observation period, JunB was progressively and synchronously upregulated in the three T cell subsets. These observations prompted us to hypothesize that the synchronized increase in JunB coupled the responses of Tregs and non-Tregs to extracellular stimuli, and this intricate cellular signal transduction coupling may enable Tregs to accurately sense the emerging Teff reactions.

Junb deficiency in Tregs does not affect T-cell homeostasis under the steady status

To study if JunB regulated Treg suppressive function, we created a mouse strain with Treg-specific ablation of *Junb* (*Foxp3*^{YFP-Cre} \times *Junb*^{Flox/Flox}) by crossing the *Foxp3*^{YFP-Cre} mice and the *Junb*^{Flox/Flox} strain [16,17].

The CD4⁺ and CD8⁺ T-cell composition in thymus, spleen, inguinal lymph node, and mesenteric lymph node was comparable between the *Foxp3*^{YFP-Cre} \times *Junb*^{Flox/Flox} (knockout, KO) and *Foxp3*^{YFP-Cre} \times *Junb*^{+/+} (wild-type, WT) mice at the age between 5- and 7-week-old (Fig. 2A). JunB deficiency affected neither the percentages of Tregs in the CD4⁺ T cell population (Fig. 2B), nor the percentages of naïve or memory-like subsets in the CD4⁺ T cells, except a modest increase in memory-like CD8⁺ T cells in the KO mice (Fig. 2C). Collectively, these results suggest that the T-cell homeostasis remains largely intact in the *Foxp3*^{YFP-Cre} \times *Junb*^{Flox/Flox} mice under steady status.

JunB deficiency causes autoimmune inflammation

To investigate if JunB was required for the immunosuppressive function of Tregs, we first performed a Treg : Teff *in vitro* coculture assay. We tried a series of Treg : Teff ratios and detected no difference between the WT and KO Treg suppression in this *in vitro* coculture assay (Fig. 3A). We were aware that such *in vitro* coculture assay may not accurately reflect the *in vivo* role of Treg immunosuppression [22]. Therefore, we went further to determine the potential role of JunB in Treg-mediated immune monitoring in an *in vivo* setting.

Non-Treg CD4⁺ T cells and CD8⁺ T cells in the KO mice produced a larger amount of IFN γ and TNF α after PMA and ionomycin stimulation, whereas IL-17 production was not influenced, indicating that the Treg-mediated immune regulation was compromised in the JunB-deficient mice (Fig. 3B). Furthermore, Ki-67 expression was comparable between the WT and KO T cells, suggesting that the T cell homeostatic proliferation was not affected by the Treg-specific deficiency of JunB (Fig. 3B). We next examined if the increased effector cytokine production caused any tissue pathology by performing the hematoxylin and eosin stainings. Interestingly, we found massive immune infiltrates in the JunB-deficient mouse ears and salivary glands, but not in other tissues, such as lung and liver (Fig. 3C). Taken together, these results support that Tregs require JunB to control Teff-produced inflammatory cytokines and spontaneous tissue inflammation.

JunB maintains Treg identity

To understand the mechanisms through which JunB regulates Treg-mediated immune regulation, we examined

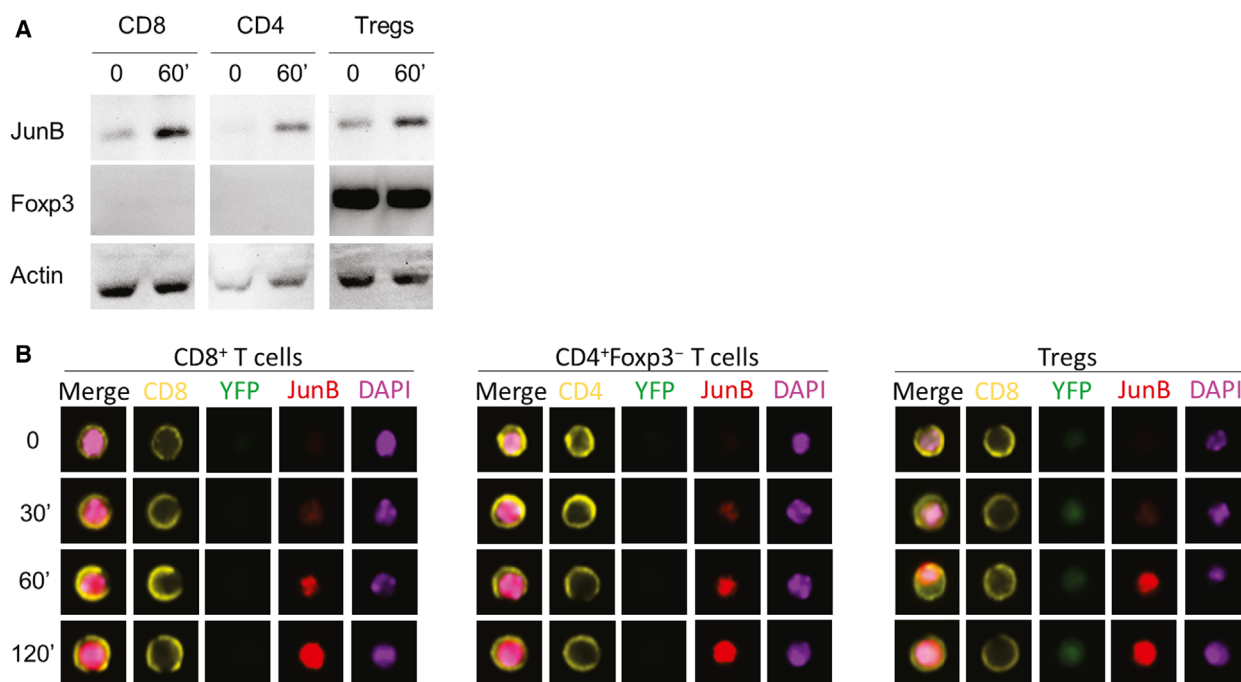


Fig. 1. T-cell stimuli increased JunB expression in Tregs and non-Treg T cells in a synchronized manner. CD8⁺ T cells, Foxp3/YFP⁺CD4⁺ T cells and Foxp3/YFP⁺CD4⁺ Tregs were FACS-sorted from the naïve Foxp3^{YFP-Cre} mice. JunB expression was analyzed by western blot (A) or Amnis imaging analysis (B) 0–120 min after stimulation with 1 $\mu\text{g}\cdot\text{mL}^{-1}$ anti-CD3 and 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ anti-CD28. Data are representative of two independent experiments with Four (A) or two (B) pairs of JunB WT and KO mice in total.

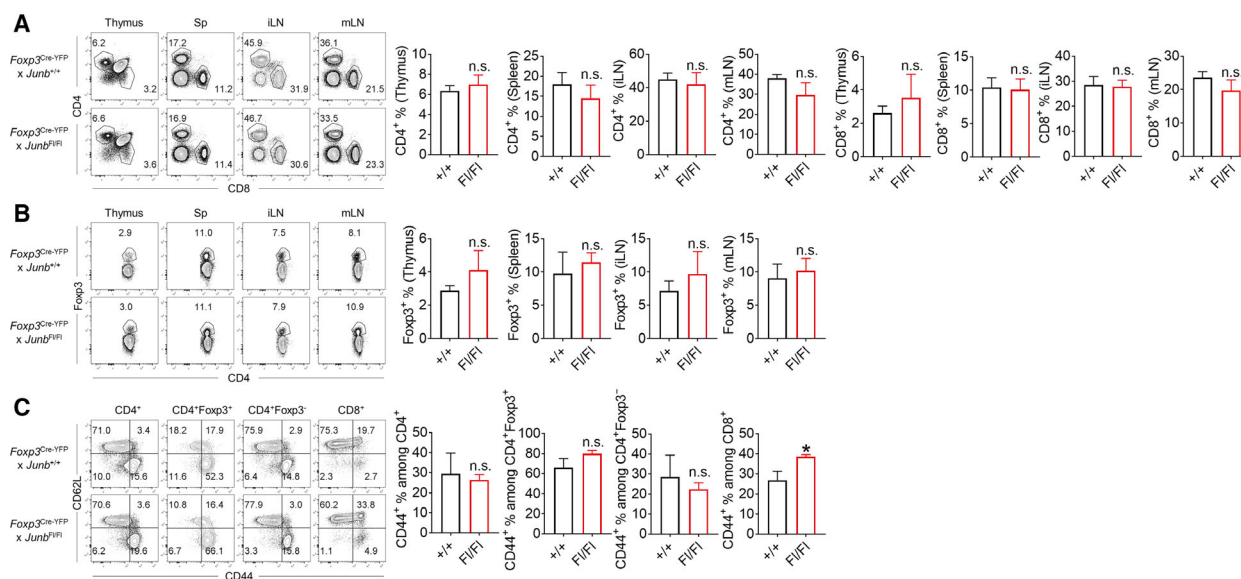


Fig. 2. Phenotypic characterization of T cells of the Foxp3^{YFP-Cre} \times Junb^{Flox/Flox} mice under steady status. (A, B) FACS plots and bar graphs show the percentages of CD4⁺ and CD8⁺ T cells (A) or Tregs in CD4⁺ T cells (B) in thymus, spleen, inguinal lymph node (iLN) and mesenteric lymph node (mLN). (C) FACS plots and bar graphs show the percentages of naïve (CD62L^{high}CD44^{low}) or memory-like (CD44^{high}) T cell subsets in splenic total CD4⁺ T cells, Tregs, non-Treg CD4⁺ T cells, and CD8⁺ T cells. Data are expressed as mean \pm SD (error bars) and are representative of three independent experiments with three pairs of JunB WT and KO mice in total. * $P < 0.05$; n.s., not significant.

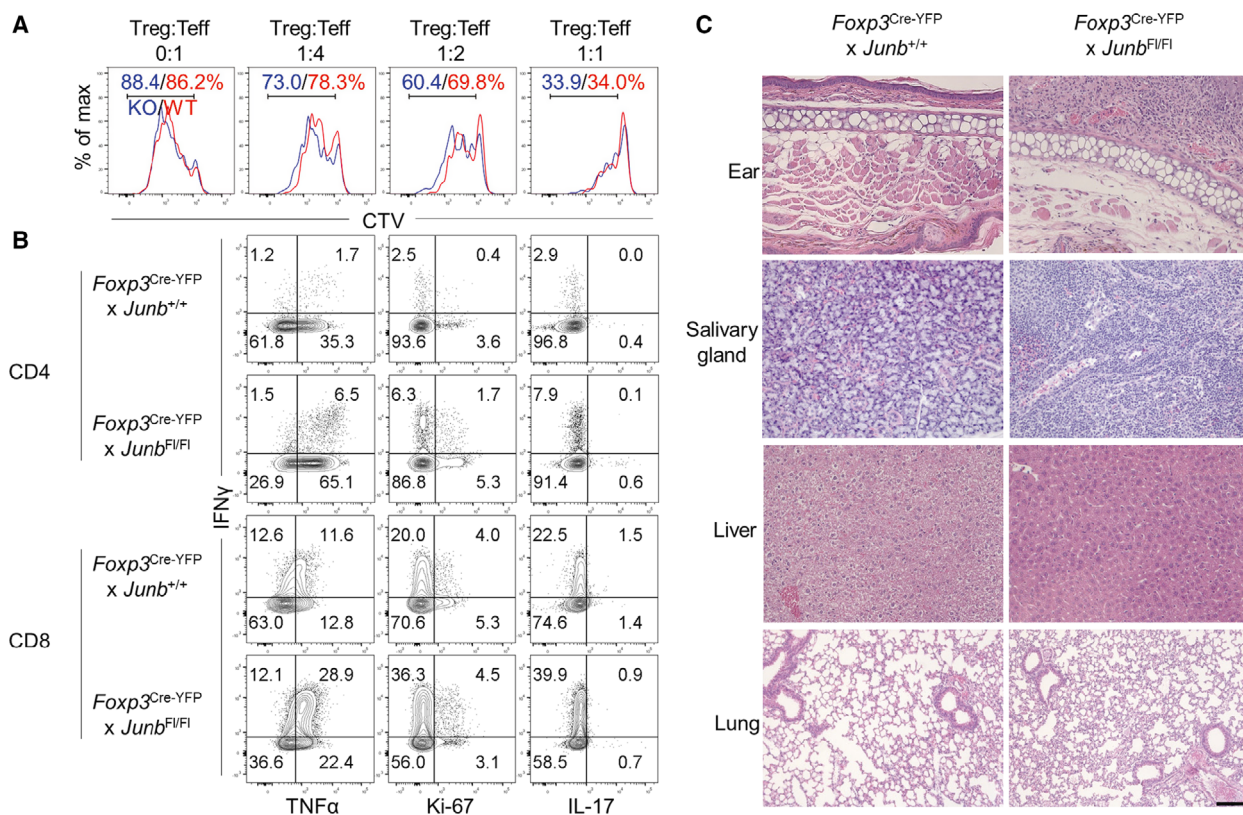


Fig. 3. Tregs require JunB to control T-cell production of IFN γ and TNF α and to maintain immune tolerance. (A) FACS-sorted Foxp3/YFP⁺CD4⁺ T cells were labeled with CTV and stimulated with the anti-CD3/CD28 beads (two beads per T cell) in the presence or absence of JunB WT or KO Foxp3/YFP⁺CD4⁺ Tregs at the indicated ratios. FACS histograms show the CTV dilution of Foxp3/YFP⁺CD4⁺ T cells 3 days later. (B) Splenic CD4⁺ and CD8⁺ T cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 6 h before FACS analysis of IFN γ , TNF α , Ki-67 and IL-17. (C) HE staining of tissue sections (×20 objective). Scale bar is 100 μ m. Data are representative of two (A, C) and three (B) independent experiments with three (A, C) and nine (B) pairs of JunB WT and KO mice in total.

the global gene expression profiles in the JunB WT and KO Tregs by performing RNA-seq analysis. JunB deficiency significantly decreased 236 genes and increased 256 genes in Treg cells (Fig. 4A). The subsequent IPA revealed that the top three canonical pathways influenced by JunB deficiency were lymphocyte signaling, IL-10 pathway and T helper cell differentiation (Fig. 4B). Detailed analysis suggested that JunB KO Treg cells reduced the Treg cell 'marker gene' expression and increased the expression levels of genes typically expressed in Teffs. For example, JunB deficiency reduced the expression of a panel of genes reported to be enriched in Tregs, such as *Plagl1*, *Il10*, *Icos*, *Irf2*, *Il2ra*, *Ctla4*, *Nt5e*, *Klrg1*, *Nr4a1*, *Hdac9*, *Nrp1*, and *Prdm1* [23–25]. On the other hand, JunB-deficient Tregs increased the expression of *Tnf*, *Ifng*, *Pde3b*, *Il1b*, and *Frm4b* (Fig. 4C). We further confirmed that the JunB KO Tregs expressed more IFN γ and TNF α and less IL-10 by flow cytometry (Fig. 4D). These results suggest that Tregs require JunB to maintain

identity, and help to explain the impairment of immune tolerance and spontaneous autoimmunity in the JunB KO mice.

JunB deficiency in Tregs causes autoimmunity dependent on T-bet

Because the non-Treg CD4⁺ T cells and CD8⁺ T cells increased the production of IFN γ and TNF α , and T-bet (encoded by *Tbx21*) promotes the production of these cytokines [26], we determined if the autoimmunity in the *Foxp3*^{YFP-Cre} × *Junb*^{Flox/Flox} mice depended on T-bet. We found that T-bet expression was increased in the CD4⁺ T cells and CD8⁺ T cells of the JunB KO mice (Fig. 5A). Furthermore, T-bet deficiency reversed the excessive production of the IFN γ and TNF α in the non-Treg CD4⁺ T cells and CD8⁺ T cells (Fig. 5A). Most interestingly, T-bet deficiency rescued the autoinflammation in ears and salivary glands of the KO mice (Fig. 5B), suggesting the spontaneous

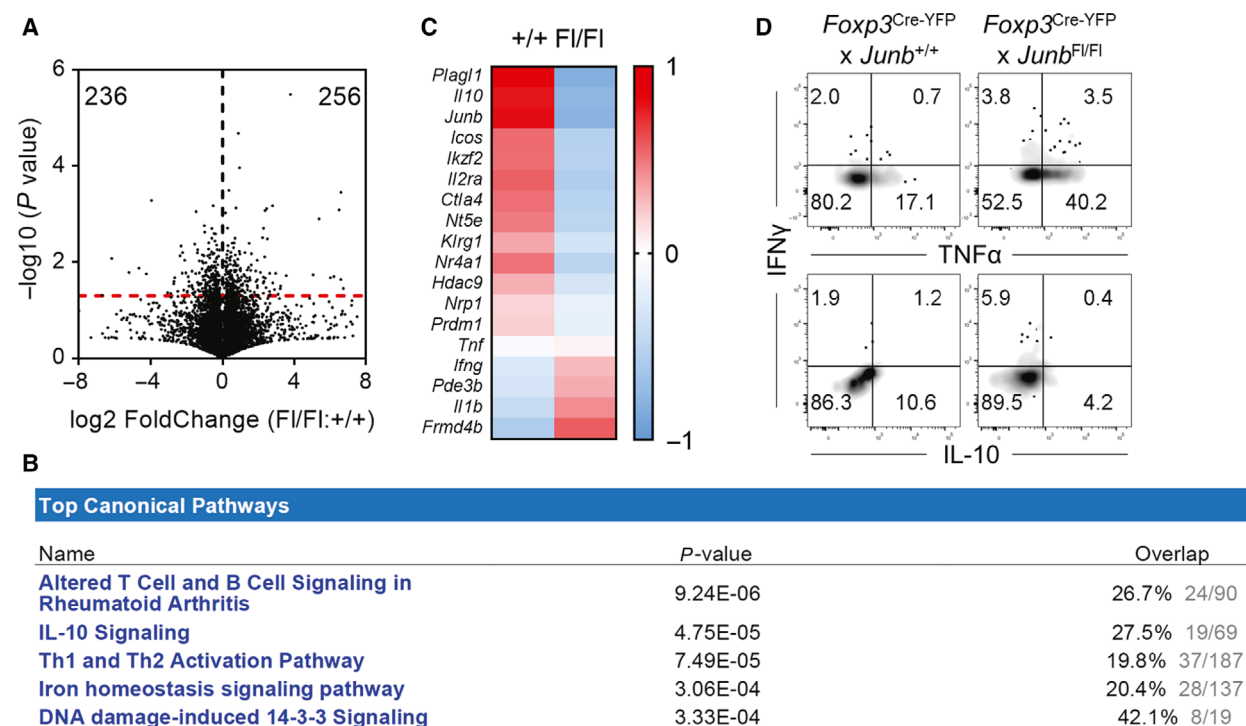


Fig. 4. JunB maintains Treg identity. (A) A volcano plot shows that 256 or 236 genes are significantly increased or decreased by JunB deficiency. The red dash line indicates the P value 0.05 and the black dash line separates genes increased (right side) or decreased (left side) in the JunB KO Treg cells. (B) IPA reveals the five top pathways altered by JunB deficiency in Treg cells. (C) A heatmap shows the z-score of the mRNA levels of the indicated genes in JunB WT or KO *Foxp3*^{Cre-YFP}CD4⁺ Tregs. The results represent the average values of three pairs of samples. (D) FACS plots show the percentages of IFN γ , TNF α and IL-10 in splenic JunB WT or KO Tregs after *in vitro* stimulation with PMA and ionomycin in the presence of brefeldin A. Data are representative of three independent experiments. Three pairs of JunB WT and KO mice were used in total.

autoimmunity in the KO mice is dependent on T-bet. Because T-bet also regulates Treg cell immunosuppression, T-bet deficiency may reverse the autoinflammation phenotype in the JunB KO mice through both Treg cell-dependent and non-Treg cell-dependent mechanisms.

JunB deficiency in Tregs enhances antitumor immunity

Because Tregs play an essential role in the immunosuppression in cancer [27,28], we tested a potential role of JunB in Tregs using a B16 melanoma implantation model. The JunB KO mice developed much smaller tumors (Fig. 6A,B). The percentages of Tregs in the CD4⁺ tumor-infiltrating lymphocytes (TILs) were not influenced by JunB deficiency (Fig. 6C). Because the tumor sizes of the JunB KO mice were smaller than those of the JunB WT mice, we compared the density (defined as TIL numbers per cubic millimeter tumor) rather than the total numbers of the

TILs between the WT and KO TILs. Notably, the density of CD4⁺ TILs but not CD8⁺ TILs was increased. Detailed analysis revealed a significant increase in the density of IFN γ -, TNF α -, and granzyme B-producing CD4⁺ TILs, whereas the expression of PD-1 was not influenced (Fig. 6D). Please note that the scale of the density increase in effector cytokine-producing CD4⁺ TILs was similar to that of the total CD4⁺ TILs, suggesting that the enhanced cytokine-producing CD4⁺ TIL density was a passive result of the increased total CD4⁺ TIL density. Indeed, the percentages of effector cytokine production in the JunB WT and KO CD4⁺ TILs were comparable (data not shown). Furthermore, the density of CD8⁺ TILs, PD-1 expression, or the effector cytokine production was not influenced by JunB (Fig. 6E), suggesting that the JunB deficiency in Tregs specifically enhanced antitumor CD4⁺ rather than CD8⁺ T cell function in this tumor model. Taken together, these data suggest that JunB deficiency in Tregs unleashes the antitumor CD4⁺ T-cell responses.

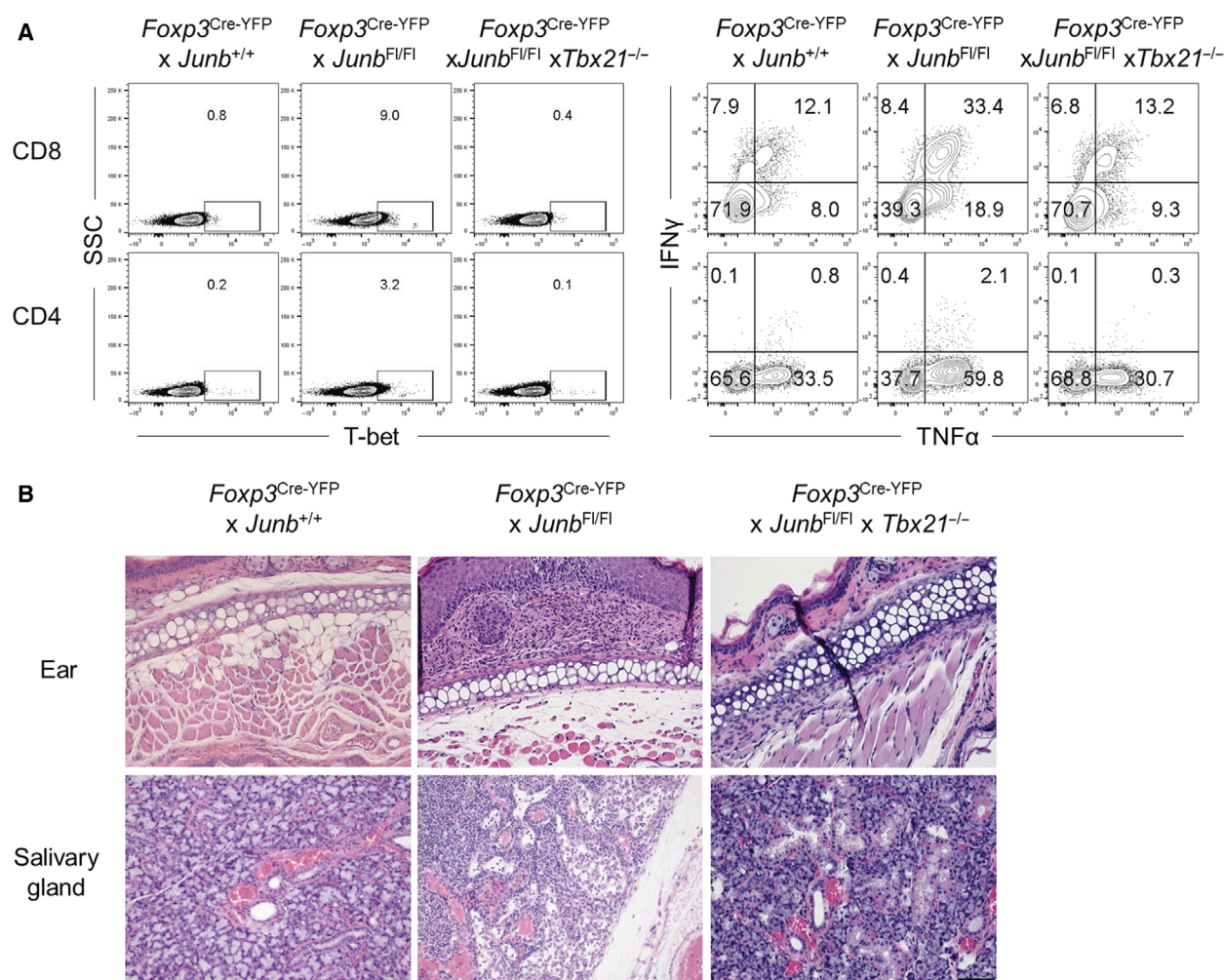


Fig. 5. Tbet deficiency reverses the uncontrolled cytokine production and autoimmunity in the *Foxp3*^{YFP-Cre} × *Junb*^{Fl/Fl} mice. (A) FACS plots show the percentages of Tbet, IFNγ and TNFα in the splenic CD4⁺ and CD8⁺ T cells. T cells were stimulated with PMA and ionomycin in the presence of brefeldin A before staining IFNγ and TNFα. (B) HE staining of the ear and salivary gland sections (×20 objective). Scale bar is 100 μm. Data are representative of two independent experiments with four (A) or three (B) pairs of JunB WT and KO mice in total.

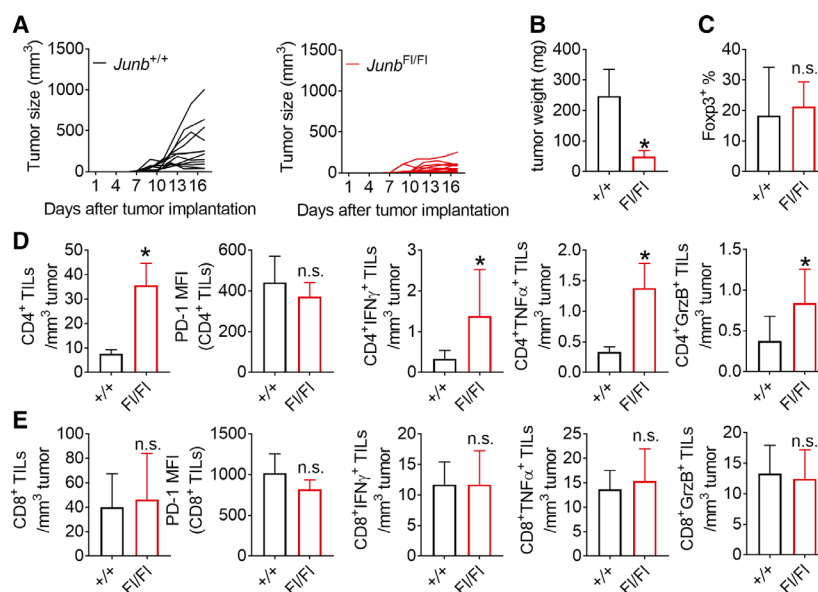
Discussion

Treg-mediated immune suppression is very important to keep the Teff responses under check. Therefore, the mechanisms enabling Tregs to sense the emerging and ongoing Teff reactions are of key importance to maintain immune tolerance. In the current study, we present evidence to suggest the synchronized JunB expression in Teff and Tregs as one mechanism to couple the Teff immune reaction with the Treg-mediated immune regulation. Likewise, we predict that other molecules that are essential for the early Teff activation may act similarly to synchronize the Teff and Treg responses. This built-in mechanism of sensing the Teff activation enables Tregs to ‘know’ the timing of

Teff responses and thus promotes the accuracy of the Treg-mediated immune regulation.

Accumulating evidence suggests that different Teff subsets are controlled by Tregs in a lineage-specific manner. For example, Th17 subset differentiation is regulated by STAT3 and RORγt [29]. Treg-specific ablation of these factors specifically impairs the Treg-mediated regulation of the Th17 lineage commitment [1]. Similarly, Th1 and Th2 subsets are regulated by Tregs in a lineage-specific manner, as manifested by the phenotypic studies of mouse strains with Treg-specific deletion of Tbet (for Th1), GATA3, and IRF4 (for Th2) [2–5]. The Teff lineage-determining factor-dependent and Teff activation factor-dependent Treg regulatory mechanisms complement each other

Fig. 6. JunB deficiency in Tregs unleashes antitumor Teff responses. (A, B) Line graphs (A) show the volume of implanted B16 melanoma in *Foxp3^{YFP-Cre} × Junb^{Flox/Flox}* or *Foxp3^{YFP-Cre} × Junb^{+/+}* mice. A bar graph (B) shows the tumor weight at the end of the study. (C–E) Bar graphs show the density of Tregs (C), total or cytokine-producing CD4⁺ (D) or CD8⁺ (E) TILs and the mean fluorescence intensity (MFI) of PD-1 on CD4⁺ (D) or CD8⁺ (E) TILs. Data are expressed as mean ± SD (error bar) and cumulative of two independent experiments with thirteen pairs of mice. The tumor volumes were significantly reduced on day 12 ($P = 0.045$, t -test), day 13 ($P = 0.009$), day 15 ($P = 0.031$), and day 17 ($P = 0.010$). * $P < 0.05$; ** $P < 0.01$; n.s., not significant.



and are not redundant. In particular, the increase in JunB expression starts as early as 30 min after T-cell activation, whereas the lineage-determining factor (e.g., T-bet) does not start to increase until 6 h after stimulation (our unpublished data). The results suggest a model that before Tregs are able to interpret the environmental cues and the specific types of Teff lineage differentiation, the Teff activation-triggered alarming mechanism has already become active in Tregs. This chronological division of labor provides multiple layers of immunoregulation to keep the Teff under check. In addition to promoting Teff cell activation sensation by Treg cells, JunB also directly regulated Treg cell immunosuppressive function. For example, the immunosuppressive cytokine IL-10 was reduced in JunB-deficient Treg cells. Therefore, JunB promotes Treg-mediated immune tolerance through both Treg-extrinsic and -intrinsic mechanisms.

Foxp3 is the 'master regulator' of Tregs and plays a crucial role in maintaining the Treg identity [30–33]. Although the Foxp3 protein level was not affected by JunB deficiency, JunB-deficient Tregs display several Teff features, such as producing IFN γ and TNF α . Therefore, in addition to enabling Tregs to sense the Teff responses, JunB also maintains the Treg identity. One possible explanation for this phenotype is that JunB acts in parallel with, rather than upstream of Foxp3 to enhance the expression of Foxp3 target genes and to protect the Treg identity. This idea is supported by the observation that the canonical Foxp3 target transcripts, such as *Plagl1* and *Nrpl* [23–25], are reduced in the JunB KO Tregs. Although certain AP-1 family members, such as c-Jun and c-Fos physically

interact with Foxp3, JunB has been shown not to bind Foxp3 [34]. Therefore, it is likely that JunB enhances the Foxp3 target gene transcription independent of directly interacting with Foxp3. The detailed molecular mechanisms through which JunB promotes the transcription of Foxp3 target genes remain yet to be determined.

Breaking immune tolerance may increase the immune cell infiltration into nonlymphoid organs and lead to spontaneous development of autoimmune disorders. JunB deficiency caused massive immune cell infiltration into ears and salivary glands. However, we did not observe immune infiltrates in other tissues or organs as reported previously [15]. One possible explanation for this difference is that is that the *Junb^{Flox/Flox}* mice used in the two studies are not the same. The mice used in the previous study were generated by the 'KO first' strategy. Several genetic elements were inserted into the exon 1 (and also the only exon) of *Junb*, presumably resulting in a 119-amino acid truncation rather than deletion of the exon 1 of *Junb*. It remains unclear if the truncated version of JunB still has any biological function. The mice used in our current study have two loxps flanking the *Junb* open reading frame. After crossing with the *Foxp3^{Cre-YFP}* mice, *Junb* is deleted rather than truncated. Therefore, it will be interesting to determine the possibility that the truncated JunB causes this difference of the tissue specificity in the spontaneous inflammation. The second possibility is that housing conditions are different. Using the nonobese diabetic (NOD) mice as one example, NOD mice are characterized by spontaneous insulinitis and type 1 diabetes. The

diabetes incidence of NOD mice housed in 22 different centers around the world is different [35], suggesting an important role of the environmental factors on the immune responses. The third possibility is that the mice used in both studies for the histological study were relatively young (3 months old). Aging may amplify the JunB deficiency-triggered spontaneous inflammation and eventually lead to spontaneous inflammation in multiple organs. In fact, it has been shown that aging causes spontaneous T cell infiltration into multiple organs even in the WT 24-month-old C57BL/6 mice [36]. In spite of this difference in the mouse genetics, there are similarities in the gene expression patterns. Consistent with the previous study, we found that JunB deficiency decreased the expression of genes encoding the Treg cell effector molecules, such as *Icos*, *Klrg1*, *Ctla4*, and *Tigit*. These results echo the conclusion in the previous study that JunB plays an essential role in instructing the Treg effector program [15].

Collectively, this study suggests that Tregs sense Teff activation through synchronized JunB expression. JunB alarms Tregs of the emerging Teff responses and promotes immune tolerance. We predict that other molecules required for the early activation of Teffs may also play a similar role to couple Teff immune reaction with Treg-mediated immune regulation.

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Author contributions

JW, SM, PA, MS, and GC designed the experiments. JW, SM, KM, and TS performed the experiments. AH performed the bioinformatic analysis. GC wrote the manuscript.

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