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Proapoptotic protein Bim regulates the suppressive function of Treg cells

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Regulatory T (Treg) cells are a special immunosuppressive subset of cluster of differentiation 4-positive ($CD4^+$)-T lymphocytes and play a pivotal role in the establishment of immune homeostasis in vivo (Zhang et al., 2021). The transcription factor forkhead box protein P3 (Foxp3) is the master marker of Treg cells, which is highly expressed in Treg cells and is also essential for their suppressive function (Hori et al., 2003). In addition to Foxp3, other regulators of Treg cells have been discovered (Wu et al., 2017, 2022; Wu and Sun, 2023a, 2023b); however, a deeper understanding of the regulation of these cells is required.

Treg cells are associated with tumor immunity. As immunosuppressors, Treg cells in the tumor microenvironment may inhibit anti-tumor immunity. Therefore, the inhibition of Treg cells may boost anti-tumor immunity and is a potential strategy for immunotherapy of tumors. However, the inhibition of Treg cells may also induce inflammatory disorders in vivo. For the Treg cell-based immunotherapy of tumors, it is important to attain a balance, i.e., the efficient inhibition of tumors with acceptable side effects (Jiang et al., 2022; Wang et al., 2022).

The hydrodynamic force generated by the pressurized injection of a solution into a blood vessel can breach the endothelium and closely associated hepatocyte plasma membrane. This approach has been successfully used to deliver plasmids into the hepatocytes of mice for in situ genome editing of these cells. Certain plasmid combinations can be used to induce liver cancers, and co-transfection of protein kinase B (AKT) and Notch intracellular domain (NICD) leads to intrahepatic cholangiocarcinoma (ICC) with extensive infiltration of immune cells (Tang et al., 2022), which is suitable for tumor immunology research.

B-cell lymphoma-2 (Bcl-2)-like 11 (Bim, also known as Bcl2l11) is a proapoptotic protein that mediates apoptosis in cooperation with other proapoptotic proteins (O'Connor et al., 1998; Luo et al., 2022) and is involved in regulating the function of Treg cells (Chougnet et al., 2011). However, Treg cell-specific conditional knockout mice have not been used to research the function of Bim in Treg cells, and the clinical value of Bim in these cells has not been explored.

Senescence of the immune system is a complex process and is involved in many age-related diseases; however, details of the aging of Treg cells remain unclear. Bim is also downregulated in the Treg cells of aged mice (Chougnet et al., 2011), but the significance of this alteration has not been verified and it is unclear whether downregulation of Bim is an upstream or downstream event in Treg cell aging.

We generated $Foxp3^{Cre}$;$Bim^{fl/fl}$ mice to conditionally knockout *Bim* in Treg cells (sex- and age-matched $Foxp3^{Cre}$ mice were used as controls, unless otherwise noted). As expected, a fluorescence-activated cell sorting (FACS) analysis revealed that Bim protein was deficient in the $CD4^+Foxp3^+$ Treg cells but not in the $CD4^+Foxp3^-$ cells (conventional T cells, Tcon cells) of $Foxp3^{Cre}$;$Bim^{fl/fl}$ mice (Fig. S1). The $Foxp3^{Cre}$;$Bim^{fl/fl}$ mice were fertile and largely healthy, with no obvious visible alteration in their appearance. To further investigate the changes caused by *Bim*-deletion in Treg

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cells, we performed hematoxylin and eosin (H&E) staining to analyze the organs of *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice and *Foxp3*^{Cre} control mice at four months old. Obvious inflammatory cell infiltration was detected in multiple organs, including the liver, lungs, kidneys, stomach, colon, and skin of *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice (Fig. 1a), which suggests that these mice have a systemic inflammatory disorder. We further analyzed T lymphocytes in the peripheral nodes of *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice and *Foxp3*^{Cre} control mice. Although the Treg cell/CD4^{+} cell ratio was obviously elevated in *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice (Figs. 1b and S2a), conventional T (Tcon) cells were still over-activated, as indicated by the elevated ratio of the effector/memory subset (CD44^{hi}CD62L^{lo}) (Figs. 1c and S2b). Immune overactivation was accompanied by an elevated Treg cell ratio, which suggests that the suppressive function of *Bim*-deficient Treg cells is impaired.

To explore the mechanisms underlying the function of Bim in Treg cells, we sorted Treg cells from *Foxp3*^{Cre};*Bim*$^{fl/fl}$ and *Foxp3*^{Cre} control mice (all at 8–10 weeks old), and investigated transcriptional alterations in *Bim*-deficient Treg cells using microarray assay. To more comprehensively understand the mechanisms of Bim, transcriptional alterations of both messenger RNAs (mRNAs) and non-coding RNAs (ncRNAs) were detected and analyzed. Many mRNAs and ncRNAs were obviously changed in *Bim*-deficient Treg cells (Fig. 1d). Among them, cytotoxic T-lymphocyte-associated protein 4 (*Ctla4*) was dramatically downregulated in *Bim*-deficient Treg cells with fold change=−1.84 and *P*-value=0.0157 (Fig. 1e). Since *Ctla4* is a pivotal functional gene in Treg cells (Read et al., 2000), such obvious downregulation is significant in the mechanism of Bim in Treg cells, even though downregulation of *Ctla4* cannot be ranked in the top 20 altered genes in *Bim*-deficient Treg cells (Tables S1 and S2). A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of changed mRNAs revealed obvious alterations of multiple pathways, including spliceosome, chromosome and associated proteins, and transcription factors, in *Bim*-deficient Treg cells (Fig. 1f). Meanwhile, a KEGG pathway analysis of the targets of changed long non-coding RNAs (lncRNAs) in *Bim*-deficient Treg cells also hit many pathways associated with the regulation of Treg cell function, including the Hippo (Shi et al., 2018) and mammalian target of rapamycin (mTOR) (Zeng et al., 2013) signaling pathways, which suggests the significance of lncRNAs for the Bim-regulation of Treg cells (Fig. S3).

We further explored the effect of *Bim*-deficiency in Treg cells on tumorigenesis and tumor proliferation in vivo. We established an ICC model of *Foxp3*^{Cre};*Bim*$^{fl/fl}$ and *Foxp3*^{Cre} control mice via in situ genome editing of hepatocytes, and detected changes in the liver. Thirty days after initiation of the ICC model, the livers of *Foxp3*^{Cre} mice were dramatically enlarged, while those of *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice were smaller (Fig. 1g). The weights of livers were consistently robustly elevated in *Foxp3*^{Cre} mice but not in *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice 30 d after initiation of the ICC model (Fig. 1h). H&E staining also revealed that tumor sizes were diminished in *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice 30 d after initiation of the ICC model (Fig. 1i). The survival time after initiation of the ICC model was also significantly prolonged in *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice in comparison with *Foxp3*^{Cre} mice (Fig. 1j). These results suggest that a deficiency of *Bim* in Treg cells is beneficial for tumor treatment.

Some clues suggest that Bim is involved in senescence of Treg cells (Chougnet et al., 2011). Before directly researching this topic, we first explored the transcriptional alterations in aged Treg cells. We sorted Treg cells from *Foxp3*^{Cre} mice of 8–10 weeks old as the young control and from *Foxp3*^{Cre} mice older than 18 months old as aged Treg cells, and then performed a transcriptional analysis via microarray assay. Many mRNAs and ncRNAs were obviously changed in the aged Treg cells compared with the young control Treg cells (Fig. 1k). A KEGG pathway analysis of changed mRNAs revealed obvious alterations of multiple pathways in aged Treg cells, including mRNA biogenesis, the cell cycle, ubiquitin system, spliceosome, and chromosome and associated proteins (Fig. 1l). Meanwhile, a KEGG pathway analysis of the target genes of changed lncRNAs in aged Treg cells also identified many pathways (Fig. S4), such as protein kinases, transcription factors, membrane trafficking, and mTOR signaling pathways, which are associated with Treg cell regulation (Zeng et al., 2013). The transcriptional assays indicate that there are robust alterations in aged Treg cells and provide reference values for future related research.

Bim was downregulated in the Treg cells of aged mice (Chougnet et al., 2011); however, the relationship between Bim-downregulation and the aging of

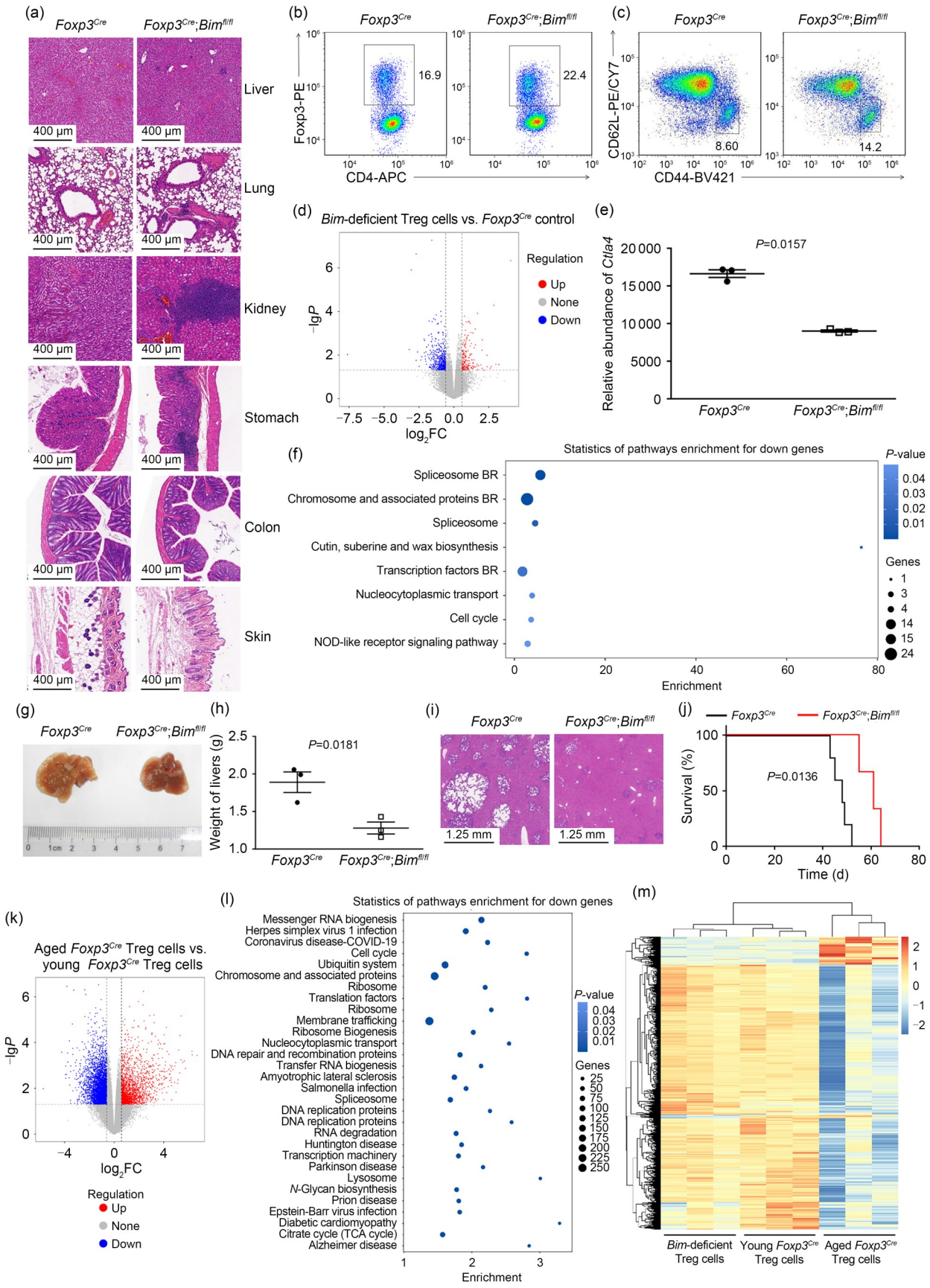
(a)
Foxp3Cre
Foxp3Cre;Bimfl/fl
Liver
Lung
Kidney
Stomach
Colon
Skin
400 μm
(b)
Foxp3Cre
Foxp3Cre;Bimfl/fl
Foxp3-PE
CD4-APC
16.9
22.4
(c)
Foxp3Cre
Foxp3Cre;Bimfl/fl
CD62L-PE/CY7
CD44-BV421
8.60
14.2
(d)
Bim-deficient Treg cells vs. Foxp3Cre control
−lgP
log2FC
Regulation
Up
None
Down
(e)
P=0.0157
Relative abundance of Ctla4
Foxp3Cre
Foxp3Cre;Bimfl/fl
(f)
Statistics of pathways enrichment for down genes
Spliceosome BR
Chromosome and associated proteins BR
Spliceosome
Cutin, suberine and wax biosynthesis
Transcription factors BR
Nucleocytoplasmic transport
Cell cycle
NOD-like receptor signaling pathway
Enrichment
P-value
Genes
(g)
Foxp3Cre
Foxp3Cre;Bimfl/fl
(h)
P=0.0181
Weight of livers (g)
Foxp3Cre
Foxp3Cre;Bimfl/fl
(i)
Foxp3Cre
Foxp3Cre;Bimfl/fl
1.25 mm
(j)
Foxp3Cre
Foxp3Cre;Bimfl/fl
P=0.0136
Survival (%)
Time (d)
(k)
Aged Foxp3Cre Treg cells vs. young Foxp3Cre Treg cells
−lgP
log2FC
Regulation
Up
None
Down
(l)
Statistics of pathways enrichment for down genes
Messenger RNA biogenesis
Herpes simplex virus 1 infection
Coronavirus disease-COVID-19
Cell cycle
Ubiquitin system
Chromosome and associated proteins
Ribosome
Translation factors
Ribosome
Membrane trafficking
Ribosome Biogenesis
Nucleocytoplasmic transport
DNA repair and recombination proteins
Transfer RNA biogenesis
Amyotrophic lateral sclerosis
Salmonella infection
Spliceosome
DNA replication proteins
DNA replication proteins
RNA degradation
Huntington disease
Transcription machinery
Parkinson disease
Lysosome
N-Glycan biosynthesis
Prion disease
Epstein-Barr virus infection
Diabetic cardiomyopathy
Citrate cycle (TCA cycle)
Alzheimer disease
Enrichment
P-value
Genes
(m)
Bim-deficient Treg cells
Young Foxp3Cre Treg cells
Aged Foxp3Cre Treg cells

Fig. 1 Regulation of the suppressive function of Treg cells by proapoptotic protein Bim. (a) H&E staining of multiple organs from *Foxp3*^{Cre} and *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice (four months old). (b) Expression of Foxp3 in CD4^{+}-T cells of peripheral lymph nodes from *Foxp3*^{Cre} and *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice (four months old). (c) Expression of CD44 and CD62L in Tcon cells of peripheral lymph nodes from *Foxp3*^{Cre} and *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice (four months old). (d) Volcano plot of the transcriptional alterations of both mRNA and ncRNAs in *Bim*-deficient Treg cells compared to the *Foxp3*^{Cre} control Treg cells (eight weeks old). (e) Transcriptional profiling revealed dramatic decreased levels of *Ctla4* in *Bim*-deficient Treg cells compared to the *Foxp3*^{Cre} control Treg cells (8–10 weeks old). (f) KEGG pathway analysis of the altered mRNAs in *Bim*-deficient Treg cells compared to the *Foxp3*^{Cre} control Treg cells; the genes with alterations more than 1.5-folds and *P*-value<0.05 were selected. (g) Representative images of lives of *Foxp3*^{Cre} and *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice 30 d after establishing in situ genome editing ICC model. (h) The weights of livers of *Foxp3*^{Cre} and *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice 30 d after establishing in situ genome editing ICC model. (i) H&E staining of livers of *Foxp3*^{Cre} and *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice 30 d after establishing in situ genome editing ICC model. (j) Survival curves of *Foxp3*^{Cre} and *Foxp3*^{Cre};*Bim*$^{fl/fl}$ mice after establishing in situ genome editing ICC model. (k) Volcano plot of the transcriptional alterations of both mRNA and ncRNAs in aged *Foxp3*^{Cre} Treg cells (older than 18 months old) compared to young *Foxp3*^{Cre} control Treg cells (eight weeks old). (l) KEGG pathway analysis of the altered mRNAs in aged *Foxp3*^{Cre} Treg cells compared to young *Foxp3*^{Cre} control Treg cells; the genes with alterations more than 1.5-fold and *P*-value<0.05 were selected. (m) Unsupervised cluster analysis of altered mRNAs from *Bim*-deficient and aged *Foxp3*^{Cre} Treg cells compared to the young *Foxp3*^{Cre} Treg cells control. Bim: B-cell lymphoma-2 (Bcl-2)-like 11; Treg: regulatory T; H&E: hematoxylin and eosin; Foxp3: forkhead box protein P3; CD4^{+}: cluster of differentiation 4-positive; Tcon: conventional T cells; mRNA: messenger RNA; ncRNAs: non-coding RNA; *Ctla4*: cytotoxic T-lymphocyte-associated protein 4; KEGG: Kyoto Encyclopedia of Genes and Genomes; ICC: intrahepatic cholangiocarcinoma; FC: fold change.

Treg cells is unclear. We performed an unsupervised cluster analysis of the transcriptional data of *Bim*-deficient, aged *Foxp3*^{Cre} and young *Foxp3*^{Cre} Treg cells to determine the similarities and differences between them. Both mRNAs (Fig. 1m) and ncRNAs (Fig. S5) were more dramatically altered in aged Treg cells than in *Bim*-deficient Treg cells. These results indicate that the deletion of *Bim* in Treg cells does not recapitulate the transcriptional alterations in aged Treg cells, which suggests that the downregulation of Bim is a downstream event in the aging process of Treg cells.

In summary, we researched the function of Bim in Treg cells using *Foxp3*^{Cre};*Bim*$^{fl/fl}$ conditional knockout mice and systematically investigated the underlying mechanism by performing a transcriptional analysis. Furthermore, we found that the deletion of *Bim* in Treg cells slows tumor growth and prolongs survival in mice, which suggests that the inhibition of Bim in Treg cells is a new potential strategy for tumor immunotherapy. In addition, we investigated the relationship between Bim-downregulation and Treg cell aging and found that the deletion of *Bim* in Treg cells does not recapitulate the transcriptional characteristics of the Treg cells of aged mice, which suggests that Bim-downregulation is a downstream event in Treg cell aging. The transcriptional data of aged Treg cells generated by this work are also valuable for further research related to senescence of Treg cells.

Materials and methods

Detailed materials and methods are provided in the electronic supplementary materials of this paper.

The microarray data generated in this study have been deposited in Gene Expression Omnibus under accession code GSE233231.

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Author contributions

Di WU designed and preformed experiments, analyzed data, and wrote the manuscript. The author has read and approved the final manuscript, and therefore, has full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Di WU declares no competing interests.

All institutional and national guidelines for the care and use of laboratory animals were followed. All animal experiments were approved by the Animal Ethics Committee of Zhejiang University (No. 17864, May 19, 2020).

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Supplementary information

Figs. S1–S5; Tables S1 and S2; Materials and methods