Circulation

ORIGINAL RESEARCH ARTICLE

DP1 Activation Reverses Age-Related Hypertension Via NEDD4L-Mediated T-Bet Degradation in T Cells

BACKGROUND: Blood pressure often rises with aging, but exact mechanisms are still not completely understood. With aging, the level of proinflammatory cytokines increases in T lymphocytes. Prostaglandin D_2, a proresolution mediator, suppresses Type 1 T helper (Th1) cytokines through D-prostanoid receptor 1 (DP1). In this study, we aimed to investigate the role of the prostaglandin D_2/DP1 axis in T cells on age-related hypertension.

METHODS: To clarify the physiological and pathophysiological roles of DP1 in T cells with aging, peripheral blood samples were collected from young and older male participants, and $CD4^+$ T cells were sorted for gene expression, prostaglandin production, and Western blot assays. Mice blood pressure was quantified by invasive telemetric monitor.

RESULTS: The prostaglandin D_2/DP1 axis was downregulated in $CD4^+$ T cells from older humans and aged mice. DP1 deletion in $CD4^+$ T cells augmented age-related hypertension in aged male mice by enhancing Th1 cytokine secretion, vascular remodeling, $CD4^+$ T cells infiltration, and superoxide production in vasculature and kidneys. Conversely, forced expression of exogenous DP1 in T cells retarded age-associated hypertension in mice by reducing Th1 cytokine secretion. Tumor necrosis factor α neutralization or interferon γ deletion ameliorated the age-related hypertension in DP1 deletion in $CD4^+$ T cells mice. Mechanistically, DP1 inhibited Th1 activity via the PKA (protein kinase A)/p-Sp1 (phosphorylated specificity protein 1)/neural precursor cell expressed developmentally downregulated 4-like (NEDD4L) pathway–mediated T-box-expressed-in-T-cells (T-bet) ubiquitination. T-bet deletion or forced NEDD4L expression in $CD4^+$ T cells attenuated age-related hypertension in $CD4^+$ T cell–specific DP1-deficient mice. DP1 receptor activation by BW245C prevented age-associated blood pressure elevation and reduced vascular/renal superoxide production in male mice.

CONCLUSIONS: The prostaglandin D_2/DP1 axis suppresses age-related Th1 activation and subsequent hypertensive response in male mice through increase of NEDD4L–mediated T-bet degradation by ubiquitination. Therefore, the T cell DP1 receptor may be an attractive therapeutic target for age-related hypertension.

Deping Kong, PhD*
Qiangyou Wan, PhD*
Juanjuan Li, MD
Shengkai Zuo, PhD
Guizhu Liu, PhD
Qian Liu, BSc
Chenchen Wang, BSc
Peiyuan Bai, MSc
Sheng-Zhong Duan, MD, PhD
Bin Zhou, MD, PhD
Fotini Gounari, PhD
Ankang Lyu, MD, PhD
Michael Lazarus, PhD
Richard M. Breyer, PhD
Ying Yu, MD, PhD

Clinical Perspective

What Is New?

- Prostaglandin D₂ biosynthesis and D-prostanoid receptor 1 (DP1) expression are markedly declined in CD4+ T cells from older humans and aged mice.
- DP1 deletion in CD4 T cells exaggerates aging-dependent BP elevation in mice by increasing tumor necrosis factor α and interferon γ secretion, whereas DP1 overexpression shows the opposite effect.
- DP1 activation suppresses Type 1 T helper cell cytokines via the protein kinase A/phosphorylated specificity protein 1/neural precursor cell expressed developmentally downregulated 4-like/T-box-expressed-in-T-cells pathway.

What Are the Clinical Implications?

- Treatment with DP1 agonist BW245C reverses age-related hypertension in male aged mice.
- Our results indicate DP1 and its downstream pathway may serve as an attractive immunotherapeutic target for age-dependent hypertension.

Aging is associated with time-dependent decline in physiological functions that are necessary for survival and fertility. It is the main risk factor for cardiovascular diseases, including hypertension; more than 70% of older adults (>60 years) have hypertension, compared with only 30% for adults 40 to 59 years of age.[1] Despite medical treatment for patients with hypertension, such as the use of diuretics and renin-angiotensin system blockers that reduce cardiovascular morbidity and mortality in older adults,[2] treatment of age-related hypertension remains challenging because of differences in drug metabolism, use of multiple concomitant medications, and enhanced blood pressure (BP) variability in older adults.[3,4] Therefore, a specific curative treatment for age-related hypertension is urgently required.

Aging is accompanied by chronic low-grade inflammation, a phenomenon also termed as "inflammaging."[5] The levels of circulating inflammation biomarkers, such as interleukin-6, tumor necrosis factor alpha (TNFα), interferon γ (IFNγ), interleukin-1α, and C-reactive protein, are elevated in healthy elderly individuals.[6] The increase in the levels of proinflammatory cytokines is associated with morbidity and mortality of age-related diseases such as diabetes mellitus, hypertension, vascular disease, and cancer.[1] The immune response is influenced by the aging process to some extent, and T cells are the most altered among the affected immune cells.[7] T cells are also involved in sex differences in development of hypertension.[8,9] Accumulated evidence show

that the T cells of the elderly secrete large amounts of IFNγ[10,11] and TNFα[12,13] after activation, suggesting that aging is associated with chronic Th1 type inflammation.[14] Indeed, genetic ablation or pharmacological inhibition of either IFNγ or TNFα attenuates angiotensin II (AngII)–induced BP elevation in male mice.[15–17] However, the role of T cell–derived cytokines in age-related hypertension remains unknown.

Prostaglandin D₂ (PGD₂), a proresolution mediator,[18,19] is generated from sequential enzymatic reaction of cyclooxygenases and PGD₂ synthases. It exerts its biological function via two G protein–coupled receptors named D-prostanoid receptor (DP) 1 and 2 (also called chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells, CRTH2). Both DP1 and DP2 are expressed in T cells, and DP2 mediates the directed movement or chemotaxis of Th2 cell.[20] In mice, DP1 deletion exaggerates formation of aneurysm and hypertensive response to AngII and promotes atherogenesis, indicating a protective role of DP1 in vascular diseases.[21] However, information regarding the involvement of the PGD₂/DP1 axis in the development of age-dependent hypertension is limited.

Here, we observed that the PGD₂/DP1 axis was downregulated in CD4+ T cells in older human adults and aged mice. Specific deletion of DP1 in CD4+ T cells (TDP1KO) exacerbated age-related hypertension in male mice by increasing Th1 cytokine release. The PGD₂/DP1 axis suppressed age-related Th1 activation by promoting neural precursor cell expressed developmentally downregulated 4-like (NEDD4L)-mediated T-box-expressed-in-T-cells (T-bet) degradation. Overexpression or pharmacological activation of the DP1 receptor prevented age-dependent hypertension in male mice. These results demonstrate that DP1 may be a potential therapeutic target for age-related hypertension in males.

METHODS

Data Availability

The data that support these findings of this study are presented in the article and in the online-only Data Supplement, and additional information will be made available from the corresponding author upon reasonable request.

Mice

CD4-specific mouse DP1 transgenic (CD4-DP1-Tg) mice and CD4-specific mouse NEDD4L transgenic (CD4-NEDD4L-Tg) mice were both generated by Shanghai Model Organisms Center Inc. (Shanghai, China) using the pGEM plasmid containing 9.6 kb of the mouse *CD4* promotor/enhancer/silencer.[22] All animals were of the male C57/BL6 background and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Tianjin Medical University. For transgenic animals, groups of young (2 mo) and aged (18 to

26 mo) mice of each genotype were randomized on the basis of the age group.

Collection of Human Plasma and Analysis

Male participants without serious cardiovascular diseases who underwent routine health examinations were recruited in the Outpatient Department of Ruijin Hospital in Shanghai Jiao Tong University School of Medicine. Peripheral blood samples were collected, and sera were used for ELISA. Peripheral CD4+ T cells were sorted using a negative selection kit (17952, Stem cell, Vancouver, Canada) for gene expression, prostaglandin, and Western analyses. The study was reviewed and approved by the Human Ethics Committee of Shanghai Ruijin Hospital, and all participants provided written informed consent.

BP Measurement

BP was measured using invasive radio telemetry. Pressure transmitters (PA-C10, Data Sciences International, New Brighton, MN) were embedded in mice. After 3 days of rest, telemetry recordings for systolic BP, diastolic BP, mean BP, and heart rate were recorded for 3 consecutive days. For the TNFα-neutralizing experiment, etanercept (8 mg/kg/injection) was administered intraperitoneally every 2 days until the seventh day, and BP was then recorded for the next 3 consecutive days. For BW245C treatment, BW245C (1 mg/kg) was administrated intraperitoneally twice per day until the seventh day and BP was then recorded for another 18 consecutive days. The animals were maintained in a sterile environment and were regularly screened for infections.

Statistical Analysis

All data were expressed as means ± SEM. Detailed information is indicated in each figure legend regarding sample size and data collection. Data were analyzed either by the 2-tailed unpaired Student t test or nonparametric Mann-Whitney U test to compare means between two experimental groups, and by 1- or 2-way ANOVA measurements followed by Bonferroni post hoc test comparing means among multiple groups where appropriate. Pearson's correlation coefficient was used for correlation analysis. Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA). $P<0.05$ was considered statistically significant.

RESULTS

PGD$_2$/DP1 Axis Is Downregulated in T Cells in Aged Mice and Older Human Subjects

Aging is associated with persistent low-grade chronic inflammation and hypertension,[5] and T cell dysregulation is critical for the development of hypertension,[23] especially for sex differences in hypertension.[8,9] To investigate whether PGD$_2$ is involved in the regulation of T cell function during aging, we first investigated the alterations in PGD$_2$-mediated signaling and serum Th1 cytokine levels in male older human subjects (age >60

years). As anticipated, the older volunteers had higher BP and levels of serum cholesterol, low-density protein cholesterol, triglycerides, blood glucose, urea, and Th1 cytokines (IFNγ and TNFα) than the young volunteers (Table I in the online-only Data Supplement). The number of CD8+ T cells in peripheral blood was reduced, whereas that of the CD4+ T cells was elevated in older subjects (Table I in the online-only Data Supplement). DP1 expression and PGD$_2$ production in CD4+ T cells decreased dramatically in older subjects (Figure 1A and 1B), whereas the expression of Th1 cytokines (IFNγ and TNFα) increased with aging (Figure 1C). It is interesting to note that DP1 expression levels in T cells correlated negatively with systolic BP and diastolic BP (Figure 1D and 1E). Similarly, both PGD$_2$ production and DP1 expression were markedly suppressed in CD4+ T cells in aged mice (Figure 1F and 1G). In contrast, the serum levels of Th1 cytokines (IFNγ and TNFα), but not Th2 cytokines, increased with aging in mice (Figure 1H and 1I, and Figure I in the online-only Data Supplement). Taken together, the PGD$_2$/DP1 axis was downregulated in CD4+ T cells in an age-dependent manner, suggesting that dysregulation of the PGD$_2$/DP1 axis in T cells may be involved in age-related hypertension.

DP1 Deficiency in T Cells Accelerates Age-Dependent Hypertension in Mice by Increasing Th1 Cytokines

To further investigate the role of the PGD$_2$/DP1 axis in age-related hypertension, CD4+ T cell–specific DP1-deficient mice (Figure 2A) were generated by crossing DP1$^{F/F}$ mice with CD4^{Cre} transgenic mice (called TD-P1KO). The DP1 gene was specifically excised in both CD4+ and CD8+ T cells (Figure IIA in the online-only Data Supplement), with much higher efficiency in CD4+ T cells (Figure IIB in the online-only Data Supplement). DP1 expression was not significantly altered in CD8+ T cells from aged mice (Figure IIC in the online-only Data Supplement), and DP1 deletion upregulated IFNγ and TNFα expression in CD4+ T cells from aged mice (Figure IID and IIE in the online-only Data Supplement) but had no significant influence on cytokine expression in CD8+ T cells (Figure IIF through IIH in the online-only Data Supplement). As expected, the BP (including systolic BP and diastolic BP) of floxed control mice increased with aging (Figure 2B and 2C), whereas DP1 deficiency in T cells significantly augmented the age-dependent BP elevation in mice (Figure 2B and 2C). DP1 ablation markedly increased Th1 cytokine release (Figure 2D and 2E), CD4+ T cell infiltration in the aortas and kidneys with elevated CD4+/CD8+ ratio (Figure IIIA through IIIE in the online-only Data Supplement), vascular remodeling (thickness and fibrosis), superoxide production in vasculature and kidneys, and impaired
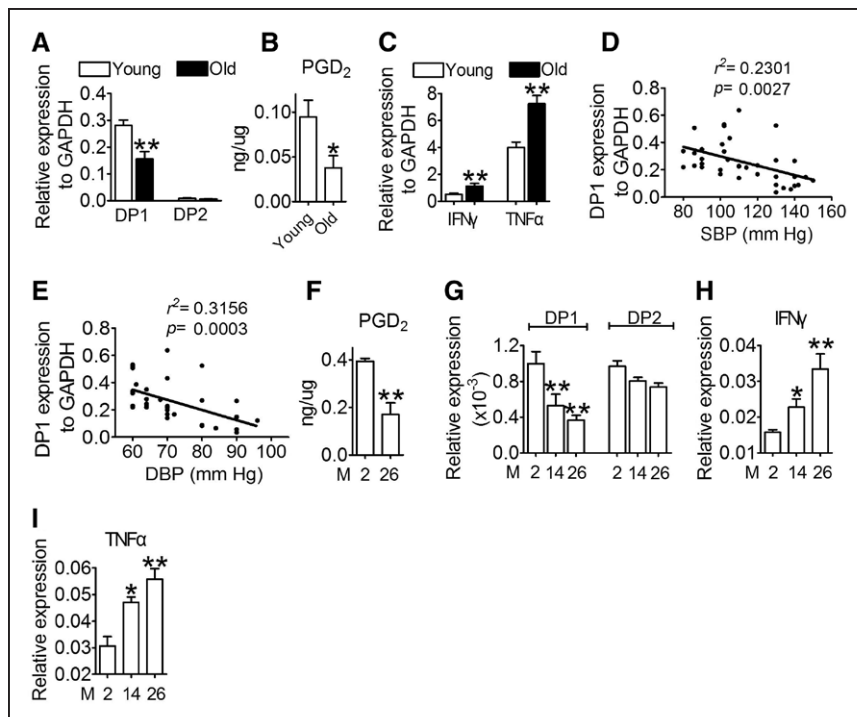
Figure 1. PGD₂/DP1 axis is downregulated with aging in primary CD4⁺ T cells.
A, mRNA expression of DP1 and DP2 in sorted CD3⁺CD4⁺ cells in young (20–30-y-old) and older (>60-y-old) adults (n=36–40). **P<0.01 compared with young. **B**, Mass spectrometric analysis of PGD₂ from sorted CD3⁺CD4⁺ cells in young and older adults (n=9–10). *P<0.05 compared with young. **C**, IFNγ and TNFα mRNA levels in sorted CD3⁺CD4⁺ cells from young and older adults (n=36–40). **P<0.01 compared with young. **D** and **E**, Correlation analysis of DP1 expression with blood pressure (BP; systolic and diastolic) in young and old people by using Pearson correction coefficient (n=36–40). **F**, Mass spectrometric analysis of PGD₂ from sorted CD3⁺CD4⁺ cells in young (2 mo) and aged (26 mo mice) (n=4–5). **P<0.01 compared with 2 months. **G** through **I**, mRNA levels of DP1, DP2, IFNγ, and TNFα in sorted CD3⁺CD4⁺ cells in young and aged mice (n=6). *P<0.05, **P<0.01 compared with 2 months. Data are expressed as mean ± SEM. Statistical analysis was performed using the unpaired Student t test (**A** through **C** and **F**) and 1-way ANOVA followed by Bonferroni post hoc analysis (**G** through **I**). DBP indicates diastolic blood pressure; DP1, D-prostanoid receptor 1; DP2, D-prostanoid receptor 2; IFNγ, interferon γ; M, month; PGD₂, prostaglandin D₂; SBP, systolic blood pressure; and TNFα, tumor necrosis factor α.

endothelium-dependent relaxation in aged mice (Figure IVA through IVH in the online-only Data Supplement).

As DP1 was downregulated in T cells with aging, we investigated whether forced expression of exogenous DP1 in T cells retarded age-associated hypertension by creating CD4⁺ cell–DP1 transgenic mice (CD4-DP1-Tg, Figure 2F). Multiple transgenic lines with robust DP1 expression in T cells were identified (Figure 2G). Conversely, forced expression of exogenous DP1 expression attenuated BP elevation (Figure 2H and 2I), increased Th1 cytokine secretion (Figure 2J and 2K), and age-associated increase in arterial thickness, fibrosis, and superoxide production in the vasculature and kidneys, and improved vascular endothelial function in aged mice (18 mo old, Figure VA through VD in the online-only Data Supplement). In addition, IFNγ deletion (Figure VIA through VID in the online-only Data Supplement) or TNFα neutralization antibody etanercept (Figure VIE through VIH in the online-only Data Supplement) abolished the exacerbated Th1 activation and elevated BP, and vascular and renal superoxide production in aged TDP1KO mice. Taken together, T cell DP1 deficiency promoted age-related BP elevation via increase in Th1 cytokine secretion.

T-bet is the key transcription factor involved in the regulation of Th1 development and function. DP1 deficiency resulted in remarkable increase in T-bet protein expression in T cells, without affecting its mRNA expression (Figure 3A and 3B). Similarly, T-bet ablation (Figure 3C) attenuated the augmented secretion of IFNγ and TNFα (Figure 3D and 3E), the exacerbated age-related BP elevation (Figure 3F) and age-associated

increase in arterial thickness, fibrosis, and superoxide production in the vasculature and kidneys (Figure 3G and 3H) in aged TDP1KO mice (18 mo old). Therefore, T-bet–mediated Th1 activation is required for age-related hypertension in aged TDP1KO mice.

DP1 Promotes T-Bet Protein Degradation in T Cells Via NEDD4L

The level of T-bet protein, but not mRNA, increased in DP1-deficient T cells (Figure 3A and 3B), indicating that DP1 is involved in posttranscriptional regulation of T-bet. Cycloheximide, a eukaryotic protein synthesis inhibitor, did not affect T-bet accumulation in DP1-deficient T cells (Figure 4A). DP1 reconstitution decreased T-bet protein expression in DP1⁻/⁻ T cells, which was restored by the proteasome inhibitor MG132 (Figure 4B), indicating that DP1 suppresses T-bet protein production via ubiquitin-mediated degradation. Both E3 ubiquitin ligases, which promote protein ubiquitination, and deubiquitinating enzymes, which block degradation, are involved in the regulation of intracellular protein homeostasis. We observed that multiple E3 ubiquitin ligases, but not deubiquitinating enzymes, were downregulated in CD4⁺ T cells from aged mice (Figure VIIA and VIIB in the online-only Data Supplement). However, DP1 deficiency markedly suppressed NEDD4L expression in aged mice (Figure 4C and Figure VIIC in the online-only Data Supplement), suggesting that the reduced NEDD4L expression may lead to T-bet accumulation in DP1-deficient T cells. Indeed, coimmunoprecipitation experiments verified an interaction between NEDD4L and
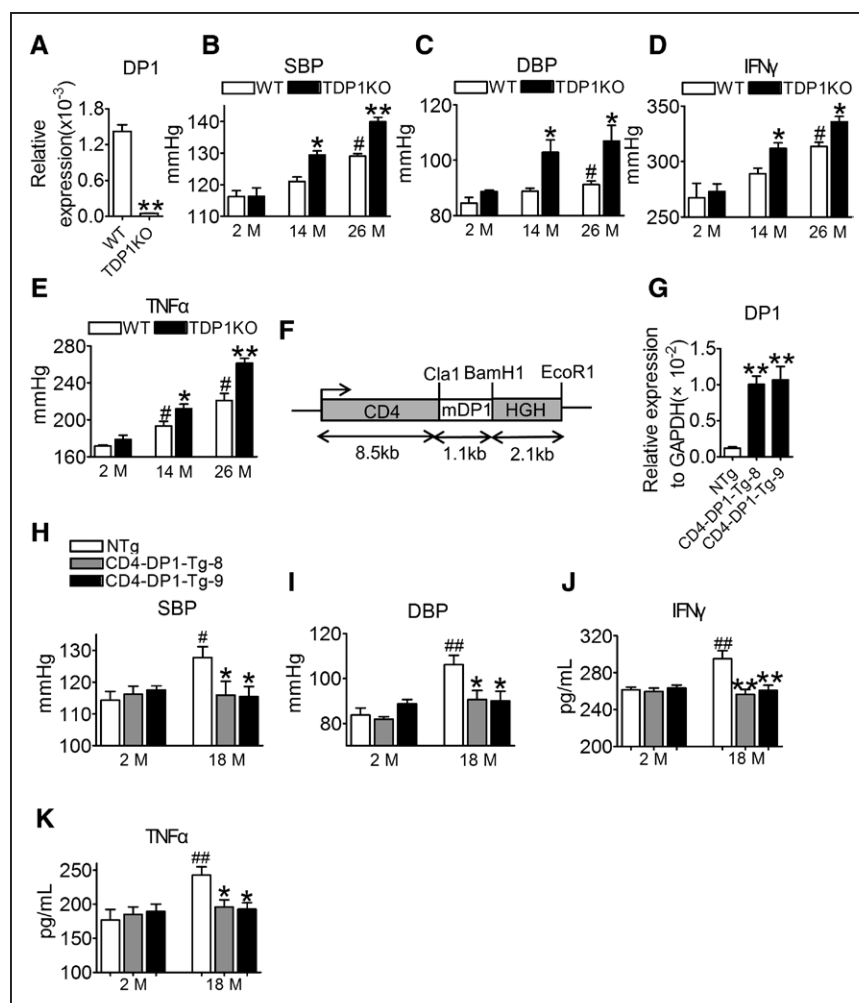
Figure 2. DP1 deficiency in T cells accelerates age-dependent hypertension in mice by increasing the levels of Th1 cytokines.
A, DP1 mRNA levels in CD3+CD4+ T cells from WT and TDP1KO mice. **P<0.01 compared with WT (n=5). **B** and **C,** Effect of DP1 deletion in T cells on systolic blood pressure (SBP) and diastolic blood pressure (DBP) in mice at different ages. *P<0.05, **P<0.01 compared with same month's WT (n=5–8). #P<0.05 compared with 2 months WT. **D** and **E,** Effect of DP1 deletion in T cells on serum Th1 cytokine levels in mice at different ages. *P<0.05, **P<0.01 compared with same month's WT (n=4). #P<0.05 compared with 2 months WT. **F,** Construct for the generation of *CD4* promoter-driven mouse DP1 transgenic mice (CD4-DP1-Tg). Colored boxes show the *CD4* promoter/enhancer/silencer and human growth hormone (HGH) polyadenylation as indicated. **G,** DP1 mRNA levels in CD4+ T lymphocytes in CD4-DP1-Tg mice (n=8). **P<0.01 compared with NTg. **H** through **K,** Effect of DP1 overexpression on SBP (**H**), DBP (**I**), serum levels of IFNγ (**J**), and TNFα (**K**) in mice. *P<0.05, **P<0.01 compared with 18 months NTg. #P<0.05, ##P<0.01 compared with 2 months NTg (n=4–8). Data are expressed as mean ± SEM. Data in **A** were analyzed by unpaired Student t test. Data in **B** through **E** and **H** through **K** were analyzed using 2-way ANOVA followed by Bonferroni post hoc analysis. Data in **G** were analyzed using 1-way ANOVA followed by Bonferroni post hoc analysis. DBP indicates diastolic blood pressure; DP1, D-prostanoid receptor 1; IFNγ, interferon γ; M, month; NTg, not transgenic; SBP, systolic blood pressure; TDP1KO, CD4+ T cell-specific DP1-deficient; Tg, transgenic; TNFα, tumor necrosis factor α; and WT, littermate control.

T-bet (Figure 4D). Overexpression of NEDD4L facilitated T-bet ubiquitination in vitro (Figure 4E). NEDD4L targets substrates via binding to polyproline (or similar) motifs.[24] We observed that the PPR domain[24] of T-bet was shared across different species (Figure 4F). Mutation of the PPR motif to AAA in T-bet protein disrupted the direct binding of NEDD4L to T-bet (Figure 4G), resulting in resistance of the T-bet protein to NEDD4L-mediated ubiquitination (Figure 4H). Accordingly, forced expression of NEDD4L strikingly diminished the accumulation of T-bet in DP1-deficient T cells (Figure 4I), and subsequently suppressed its downstream cytokine expression in DP1$^{-/-}$ T cells (Figure 4J and 4K).

Overexpression of NEDD4L in CD4+ T Cells Attenuates the Exacerbated Age-Dependent Hypertension in TDP1KO Mice

We next investigated whether increase in NEDD4L expression restored hypertensive response in aged TDP1KO mice. NEDD4L transgenic mice were generated under the control of CD4 regulatory elements[22]

(CD4-NEDD4L-Tg; Figure 5A). As expected, forced expression of NEDD4L E3 ligase (4L-Tg-2 and 4L-Tg-5; Figure 5B) decreased the level of the accumulated T-bet protein (Figure 5C and 5D), and suppressed Th1 cytokine secretion in DP1$^{-/-}$ CD4+ T cells tested in 2 aged transgenic strains (Figure 5E and 5F). It is important to note that NEDD4L overexpression suppressed BP elevation in aged TDP1KO mice (Figure 5G) by reversing the increase in arterial thickness, perivascular fibrosis, and superoxide production in the vasculature and kidneys (Figure 5H and 5I).

DP1 Promoted NEDD4L Expression in T Cells Via the PKA/Sp1 Pathway

To investigate the mechanism via which DP1 regulates NEDD4L gene transcription, we cloned the truncated mouse NEDD4L promoters for luciferase assay in lymphoma cell line EL4 (Figure 6A). Treatment with the DP1 agonist BW245C increased NEDD4L transcription, and serial deletion of NEDD4L indicated that the 75-bp fragment near exon 1 is the key promoter region responsive to the DP1 agonist (Figure 6B). Bioinformatics analysis showed that the 75-bp promoter is Guanine-Cytosine
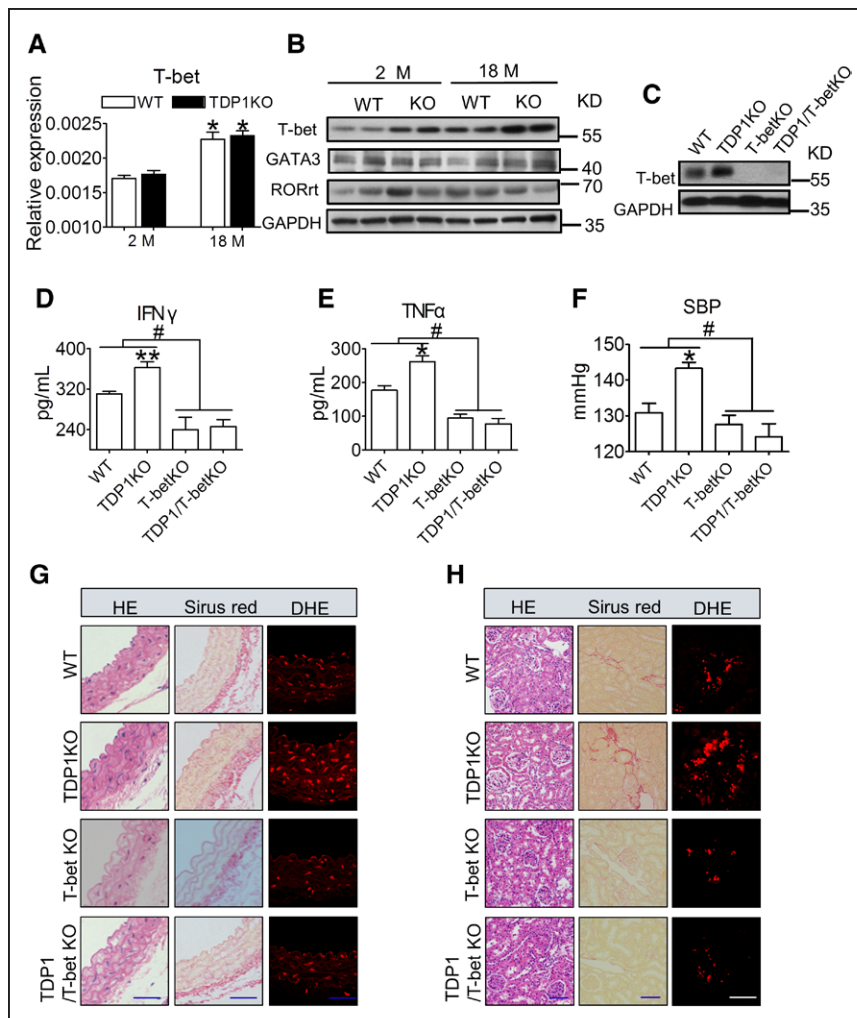
Figure 3. T-bet deletion attenuates DP1 deficiency–exacerbated aging-related hypertension in mice.

A, T-bet mRNA levels in CD4+ T cells from TDP-1KO mice of different ages. *$P<0.05$ compared with 2 months. (n=6). **B**, Protein levels of master transcription factors in spleen CD4+ T cells from WT and TDP1KO mice of different ages. **C**, Efficiency of T-bet deletion in spleen CD4+ T cells in TDP1 and T-bet double KO (TDP1/T-bet KO) mice. **D** and **E**, Effect of T-bet ablation on serum levels of IFNγ and TNFα in TDP1/T-bet KO mice (18 mo old). Sera were collected for ELISA. *$P<0.05$, **$P<0.01$ compared with WT; #$P<0.05$ compared with as indicated (n=4–7). **F**, Effect of T-bet ablation on SBP in TDP1/T-bet KO mice. *$P<0.05$ compared with WT, #$P<0.05$ compared with as indicated (n=4–8). **G** and **H**, Representative HE, Sirius Red, and DHE staining of aortic and kidney sections from TDP1/T-bet KO mice. Blue scale bars, 50 μm; white scale bar, 10 μm. Data are expressed as mean ± SEM. Data in **A** and **D** through **F** were analyzed using 2-way ANOVA followed by Bonferroni post hoc analysis. DBP indicates diastolic blood pressure; DHE, dihydroethidium; GATA3, GATA binding protein 3; HE, hematoxylin and eosin; IFNγ, interferon γ; KO, knockout; M, month; RORrt, RAR-related orphan receptor γ isoform 2; SBP, systolic blood pressure; T-bet, T-box-expressed-in-T-cells; TDP1KO, CD4+ T cell-specific DP1-deficient; TNFα, tumor necrosis factor alpha; and WT, littermate control.

(GC) box-rich (Figure 6C), and can be activated by specificity proteins (Sp) 1 and 3.[25] The DP1 agonist boosted NEDD4L transcription in Sp1-, but not Sp3-, overexpressing cells, although both transcription factors triggered NEDD4L expression (Figure 6D). Chromatin immunoprecipitation assay confirmed Sp1 binding to the NEDD4L promoter region (Figure 6E). BW245C-induced NEDD4L transcription was blocked by the Sp1 selective inhibitor mithramycin A (Figure 6F) and the PKA (protein kinase A) inhibitor H89 (Figure 6G) in cultured primary CD4+ T cells. Similarly, we observed the conserved GC box (Figure VIIIA in the online-only Data Supplement) and similar transcriptional activity in the human *NEDD4L* promoter region (Figure VIIIB in the online-only Data Supplement).

The DP1 receptor couples to the stimulatory G protein alpha-subunit to trigger PKA-dependent cascades. Sp1 can be activated by PKA-mediated phosphorylation.[26] Forskolin-mediated activation of PKA induced Sp1 phosphorylation at threonine (T), but not serine (S), in T cells (Figure IXA in the online-only Data Supplement). In a screen using mutated Sp1-transfected cells, we identified T453 to be the direct phosphorylation site of PKA in Sp1 (Figure IXB in the online-only Data Supplement),

and observed that DP1 activation promoted T453 phosphorylation of Sp1 (T452 in mouse sequence, Figure IXC and IXD in the online-only Data Supplement), facilitated NEDD4L expression and NEDD4L-mediated ubiquitination of T-bet in T cells (Figure 6H), and suppressed Th1-derived cytokines in culture via PKA (Figure 6I and 6J). As anticipated, the Sp1 inhibitor mithramycin A repressed DP1-mediated Sp1 activation and NEDD4L induction, and diminished T-bet ubiquitination in T cells, thereby reversing the expression of Th1 cytokines in BW245C-treated T cells (Figure 6K through 6M). Consistent with DP1 downregulation, phosphorylation of Sp1 at T453 and NEDD4L expression were gradually reduced, whereas T-bet protein expression was elevated in T cells from both aged mice and old human subjects (Figure 6N and 6O). Thus, our results suggested that the PGD$_2$/DP1 axis suppressed Th1 activity via the PKA/p-Sp1/NEDD4L/T-bet pathway (Figure 6P).

DP1 Activation Attenuates Age-Related BP Elevation in Mice

Last, we investigated whether DP1 activation is effective in reversing age-related hypertension. We injected
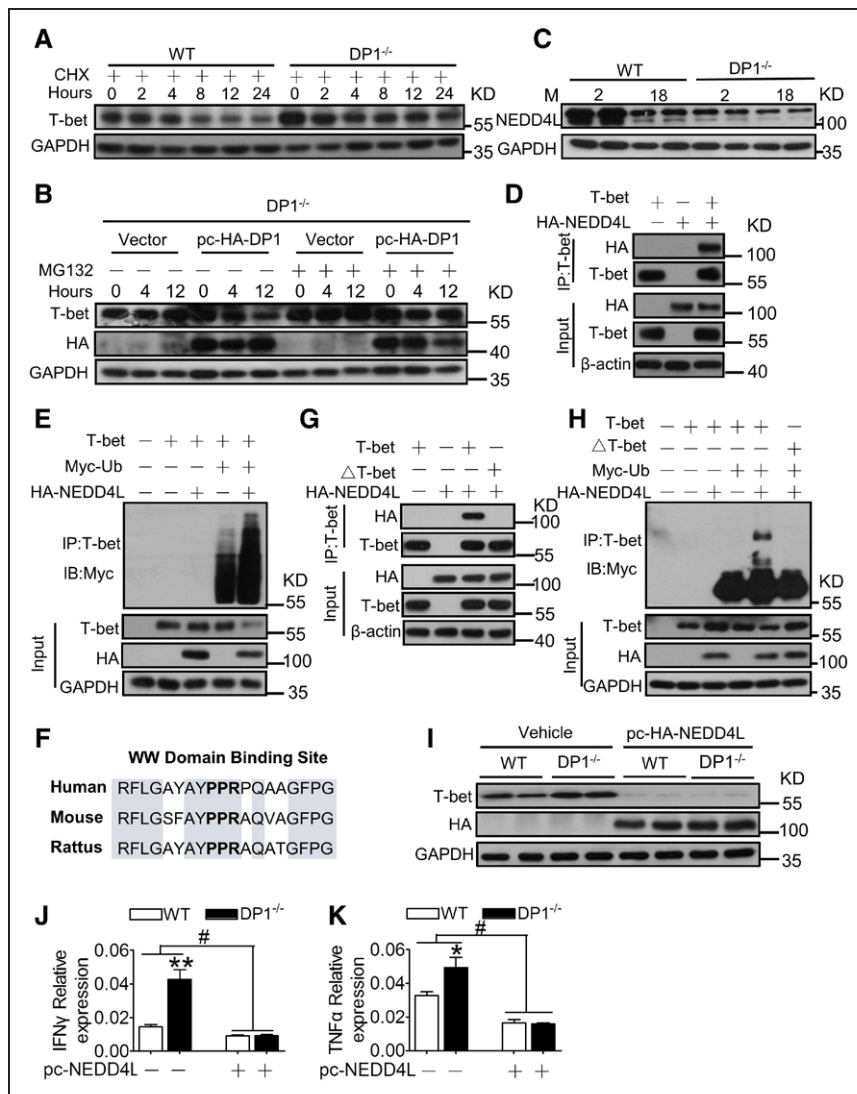
Figure 4. DP1 activation accelerates T-bet ubiquitin-mediated degradation in CD4+ T cells by promoting NEDD4L transcription.
A, Effect of cycloheximide (CHX) on T-bet protein expression in DP1$^{-/-}$ CD4$^+$ T cells. **B**, Effect of MG132 on T-bet protein level in DP1-reconsistuted DP1$^{-/-}$ CD4$^+$ T cells. **C**, NEDD4L protein level in spleen CD4$^+$ T cells from TDP1KO mice of different ages. **D**, Interaction between T-bet and NEDD4L in HEK293T cells. **E**, NEDD4L mediates ubiquitination of T-bet in HEK-293T cells. The top panel shows ubiquitination of T-bet, and the bottom 3 panels show the level of each protein, as indicated. Ub, ubiquitin. **F**, WW domain binding site (PPR) of T-bet protein sequence in different species. **G** and **H**, The effect of PPR mutation to AAA ($\triangle$T-bet) on interaction between T-bet and NEDD4L (**G**) and NEDD4L mediated T-bet ubiquitination (**H**) in HEK-293T cells. **I** through **K**, Effect of NEDD4L overexpression on T-bet protein expression (**I**), mRNA levels of IFNγ (**J**), and TNFα (**K**) in DP1$^{-/-}$ CD4$^+$ T cells. *$P<0.05$, **$P<0.01$ compared with WT, #$P<0.05$ compared with as indicated (n=6). Data are expressed as mean ± SEM. Data in **J** and **K** were analyzed using 2-way ANOVA followed by Bonferroni post hoc analysis. DP1 indicates D-prostanoid receptor 1; HA, hemagglutinin; IB, immunoblotting; IFNγ, interferon γ; IP, immunoprecipitation; M, month; NEDD4L, neural precursor cell expressed developmentally downregulated 4-like; pc, pcDNA-3.1; T-bet, T-box expressed in T cells; TDP1KO, CD4$^+$ T cell-specific DP1-deficient; TNFα, tumor necrosis factor α; and WT, littermate control.

the DP1 agonist BW245C via intraperitoneal route in 26-month-old mice (1 mg/kg) at 12-hour intervals for 7 consecutive days. As shown in Figure 7A and 7B, the systolic BP and diastolic BP in BW245C-treated aged mice were significantly decreased to the comparable level seen in young mice. BP remained low for at least 11 consecutive days (Figure 7A and 7B), demonstrating the prolonged efficacy of the DP1 agonist in maintaining low BP in these mice. We also observed that the dysregulated Sp1/NEDD4L/T-bet signaling pathway in aged CD4$^+$ T cells was reversed in BW245C-treated mice (Figure 7C and 7D). BW245C treatment also markedly reduced Th1 cytokine levels and tissue superoxide production in the aged mice (Figure 7E through 7I).

DISCUSSION

T lymphocyte dysregulation contributes to AngII-induced and age-related hypertension,[15,27] although the underlying mechanism is not clear. In this study, we observed that PGD$_2$ production and DP1 expression were markedly reduced in CD4$^+$ T cells from old human subjects and aged mice. DP1 deletion in CD4$^+$ T cells exaggerated aging-dependent BP elevation in male mice by increasing TNFα and IFNγ secretion, whereas DP1 overexpression showed the opposite effect. DP1 suppressed Th1 cytokines via the PKA/p-Sp1/ NEDD4L/T-bet pathway. Furthermore, treatment with the DP1 agonist reduced the elevated BP in aged male mice. Therefore, DP1 and its downstream pathway may constitute an attractive immunotherapeutic target for age-dependent hypertension in males.

Aging is a progressive process that leads to reduced physiological functions across different organ systems. The resulting physiological decline increases the risk for various age-related diseases, such as hypertension. Despite the complexity and diverse etiologies, accumulated evidence demonstrates that two interconnected mechanisms may primarily contribute toward pathogenesis of age related-diseases, low-grade inflammation, and augmented cellular oxidative stress.[1,28] Both the innate and adaptive immune systems are impaired with aging.
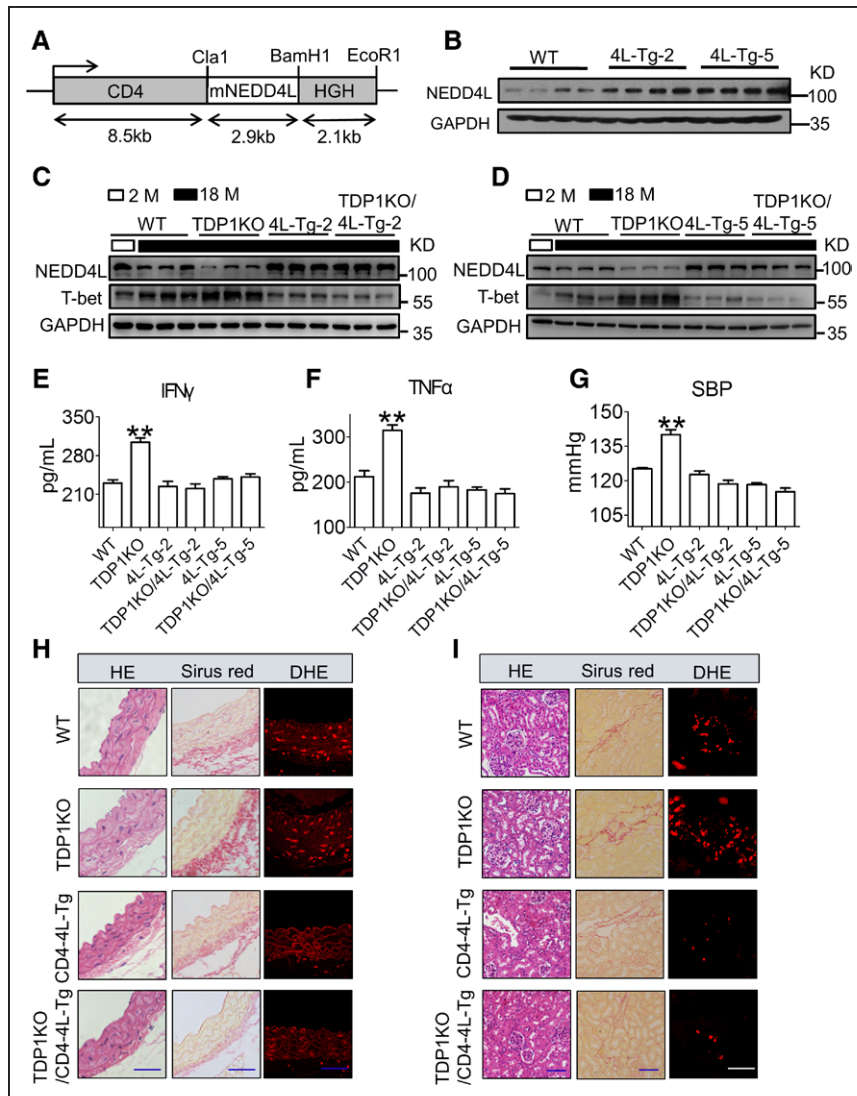
**Figure 5. CD4-specific NEDD4L overexpression blunts age-related hypertension in TDP1KO mice.**

A, Schematic diagram of the construct used for the generation of CD4-specific mouse NEDD4L overexpression mice (CD4-NEDD4L-Tg). **B**, CD4-specific NEDD4L expression in CD4+ T lymphocytes from CD4-NEDD4L-Tg mice (2 mo old). **C** and **D**, The effect of CD4-specific NEDD4L overexpression on T-bet protein levels in aged CD4+ T cells tested in 2 different mouse strains (4L-Tg-2 and 4L-Tg-5). **E** and **F**, The effect of CD4-specific NEDD4L overexpression on serum levels of IFNγ (**E**) and TNFα (**F**) in aged mice (18 mo). ****P<0.01 compared with WT (n=4–8). **G**, The effect of CD4-specific NEDD4L overexpression on SBP in aged TDP1KO mice (18 mo old). ****P<0.01 compared with WT (n=6–8). **H** and **I**, Representative hematoxylin and eosin (HE), Sirius Red, and dihydroethidium (DHE) staining of aortic and kidney sections. Blue scale bars, 50 μm; white scale bar, 10 μm. Data are expressed as mean ± SEM. Data in **E** through **G** were analyzed by 2-way ANOVA followed by Bonferroni post hoc analysis. 4L-Tg indicates NEDD4L transgenic; DBP, diastolic blood pressure; DHE, dihydroethidium; HE, hematoxylin and eosin; HGH, human growth hormone; IFNγ, interferon γ; M, month; mNEDD4L, mouse NEDD4L mRNA; NEDD4L, neural precursor cell expressed developmentally downregulated 4-like; SBP, systolic blood pressure; T-bet, T-box-expressed-in-T-cells; TDP1KO, CD4+ T cell-specific DP1-deficient; TNFα, tumor necrosis factor α; and WT, littermate control.

The most remarkable changes are observed in T cell immunity, which include imbalances in T cell populations and senescent T cell expansion.[27] The dysregulated T cells produce excessive inflammatory cytokines and cytotoxic mediators.[29] Inflammatory reactions, in turn, also increase tissue oxidative stress via production of reactive oxygen species by infiltrated neutrophils and macrophages.[30] Indeed, we and others[31] have observed elevated levels of proinflammatory cytokines (TNFα, IFNγ) in circulation, tissue inflammation, and increased reactive oxygen species levels in targeted organs in aged adults and mice. We observed that the PGD_2/DP1 axis was downregulated in CD4+ T cells from older individuals and aged mice. Ectopic expression of DP1 or activation of the endogenous DP1 receptor in CD4+ T cells reduced proinflammatory cytokine secretion and reactive oxygen species formation in vasculatures, and subsequently delayed aged-related hypertension. Thus, the reduced DP1-mediated signaling may contribute to T cell inflammation and age-related hypertension in older individuals.

Hypertension is well-known to differ by sex in prevalence. The differences of the renin-angiotensin system in vasculature and kidney contribute to this gender distinction in BP,[9] and sexual hormonal milieu and sex chromosomes also play an essential role in and of themselves.[8,9] It is interesting to note that sex-specific effects of T cells were recently observed to determine the magnitude of AngII-induced hypertension in mice.[8] These sex differences in adaptive immune regulation of BP maybe associated with gender-dependent T cell infiltration capacity,[32,33] Tregs proportion,[34,35] and balance of proinflammatory with anti-inflammatory cytokines in end organs.[9] Despite that DP1-mediated signaling pathway downregulated in T cells from aged mice and old human subjects, further investigation is warranted to evaluate sex differences of PGD_2/DP1 involvement in age-related hypertension.

As a vasodilator, PGD_2 exerts multiple cardiovascular benefits via the DP1 receptor. Experimental animal studies showed that DP1 activation protects mice against acute excitotoxity and cerebra ischemia.[36,37]
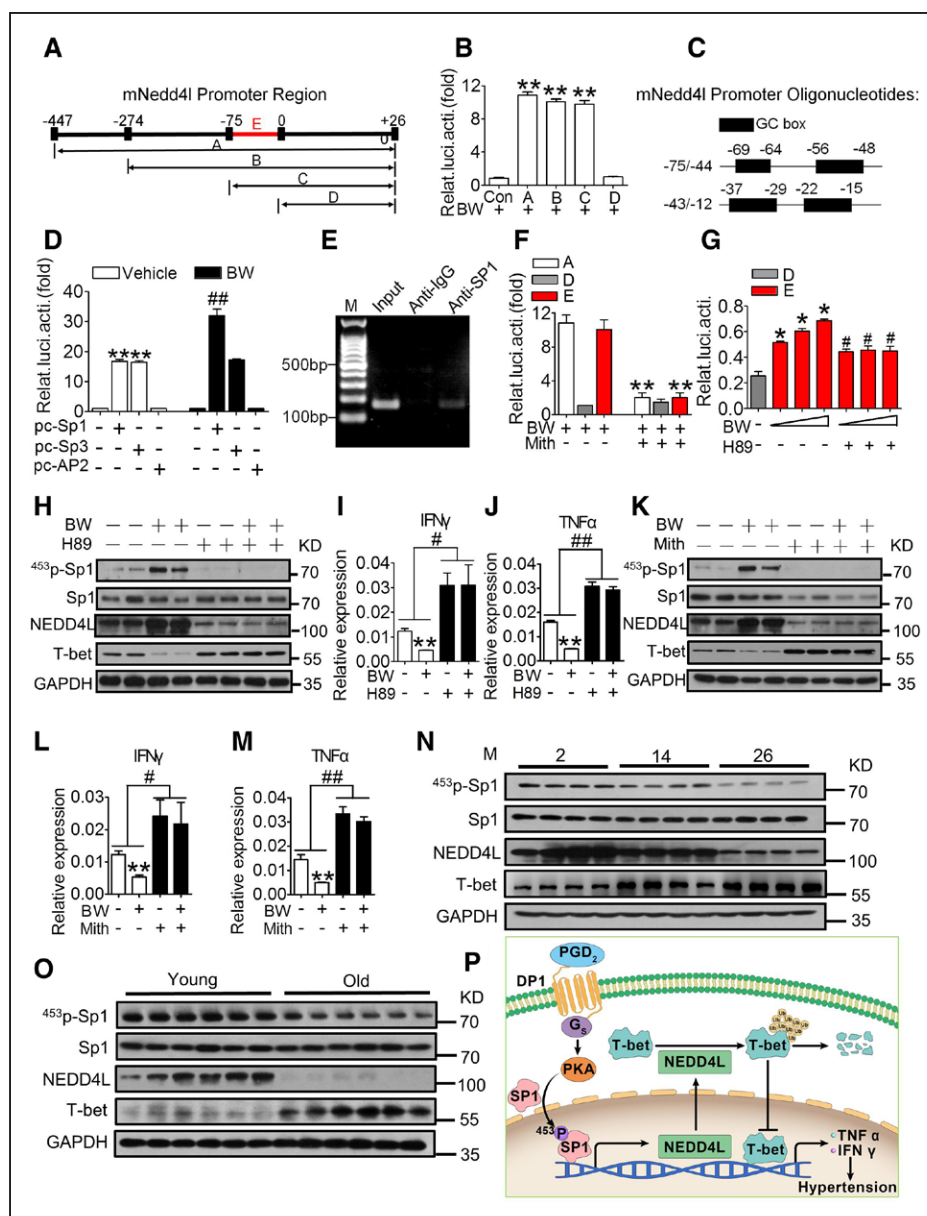
Figure 6. DP1 activation promotes mNEDD4L expression via PKA-mediated Sp1 phosphorylation.
A, Schematic illustration of luciferase reporter constructs containing truncated mouse *NEDD4L* promoter region. **B**, Luciferase reporter assay of BW245C (BW)-treated EL4 cells transiently transfected with truncated reporter plasmids or control plasmid (pGL3-basic). **P<0.01 compared with control (n=6). **C**, Illustration of the predicted fragment of the *NEDD4L* promoter region. **D**, Effect of Sp1, Sp3, and AP2 overexpression on mouse NEDD4L promoter fragment-mediated luciferase activity in BW254C-treated EL4 cells (n=4). **E**, Gel electrophoresis of polymerase chain reaction-amplified fragments in the promoter of mouse *NEDD4L* using anti-Sp1 immunoprecipitation. **F**, Effect of the Sp1 inhibitor (Mith, 0.1 mM) on the *NEDD4L* promoter fragment-mediated luciferase activity in EL4 cells. **P<0.01 compared with without Mith (n=3). **G**, Effect of BW and protein kinase A (PKA) inhibitor H89 on the *NEDD4L* promoter fragment-mediated luciferase activity in sorted primary CD4+ T cells. *P<0.05 compared with **D** fragment. #P<0.05 compared with without BW (n=3). **H** through **J**, Effect of H89 on Sp1 phosphorylation, NEDD4L and T-bet expression, and IFNγ (**I**) and TNFα (**J**) mRNA levels in BW-treated CD4+ T cells. **P<0.01 compared with untreated, #P<0.05, ##P<0.01 compared with as indicated (n=6–8). **K** through **M**, Effect of the Sp1 inhibitor Mith on Sp1 phosphorylation, NEDD4L and T-bet expression, and IFNγ (**L**) and TNFα (**M**) mRNA expression in BW-treated CD4+ T cells. **P<0.01 compared with untreated, #P<0.05, ##P<0.01 compared with as indicated (n=6–8). **N**, Alterations in Sp1/NEDD4L/T-bet signal pathway in CD4+ cells in aged mice (26 mo) compared with young ones (2 mo). **O**, Alterations in Sp1/NEDD4L/T-bet signaling pathway in CD4+ cells in old individuals (> 60 y old) compared with young ones (20–30 y old). **P**, Schematic of PGD2/DP1/Sp1/NEDD4L/T-bet pathway–mediated Th1 activation in T cells. Data are expressed as mean ± SEM. Data analyses in **B**, **D**, **F**, and **G** were performed using 1-way ANOVA followed by Bonferroni post hoc analysis. Data in **I**, **J**, **L**, and **M** were analyzed using 2-way ANOVA followed by Bonferroni post hoc analysis. BW indicates BW245C, DP1 agonist; Con, control; DP1, D-prostanoid receptor 1; GC, Guanine-Cytosine; IFNγ, interferon γ; M, month; Mith, mithramycin A, the Sp1 selective inhibitor; mNedd4l, mouse NEDD4L; NEDD4L, neural precursor cell expressed developmentally downregulated 4-like; pc, pcDNA3.1; PGD2, prostaglandin D2; p-Sp1, phosphorylated Sp1; Relat.luci.acti, relative luciferase activity; Sp1, specificity protein 1; T-bet, T-box-expressed-in-T-cells; and TNFα, tumor necrosis factor α.

The absence of the DP1 receptor augments AngII-induced hypertension and aneurism, and high-fat diet–induced atherosclerosis.[21] DP1 mediates PGD2-triggered resolution of inflammation by promoting macrophage polarization toward M2 status, thereby facilitating cardioprotection against ischemia.[18,38] Furthermore, glucocorticoids confer cardiac protection against ischemia/reperfusion via activation of the PGD2/DP1 axis,[39]
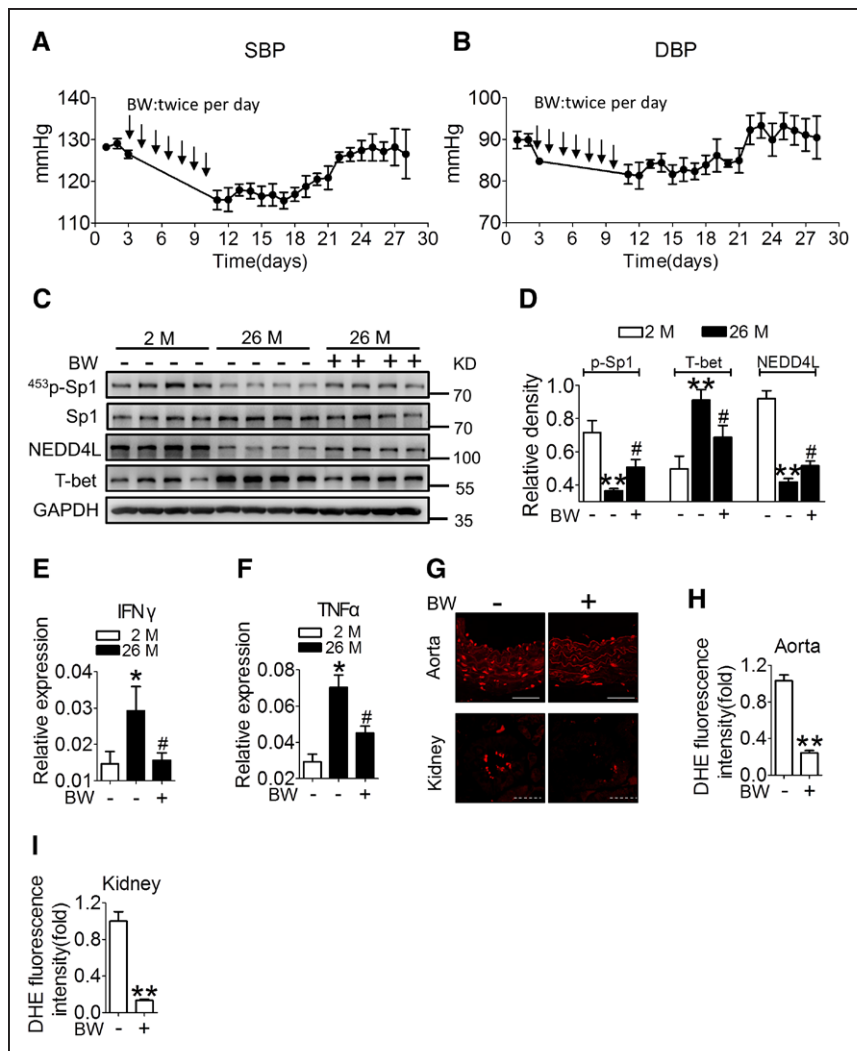
Figure 7. DP1 agonist BW245C treatment attenuates age-related BP elevation in mice.

A and **B**, Twenty-four-hour continuous radio telemetry recordings of SBP and DBP in 26-month-old WT mice injected with BW245C (BW, 1 mg/kg) with 12-h intervals for 7 days. Means ± SEM of BP values were plotted at 24-h intervals over 28 days (n=3–5). **C** through **F**, Effect of BW on Sp1 phosphorylation, protein levels of NEDD4L and T-bet (**C** and **D**) and Th1 cytokine mRNA levels (**E** and **F**) in sorted CD4+ cells from aged mice (26-month-old). *$P<0.05$ **$P<0.01$ compared with 2 months, #$P<0.05$ compared with untreated 26 months. **G** through **I**, Effect of BW treatment on superoxide production in aorta and kidneys (n=4). **$P<0.01$ compared with vehicle. Scale bar (straight line), 50 μm; scale bar (dotted line), 10 μm. Data are expressed as mean ± SEM. Statistical analysis in **D** through **F** were performed using one-way ANOVA followed by Bonferroni post hoc analysis. Statistical analysis in H and I was performed using unpaired Student's t-test. BP indicates blood pressure; DBP, diastolic blood pressure; DHE, dihydroethidium; DP1, prostaglandin D2 receptor subtype 1; IFNγ, interferon γ; M, month; NEDD4L, neural precursor cell expressed developmentally downregulated 4-like; p-Sp1, phosphorylated Sp1; SBP, systolic blood pressure; Sp1, specificity protein 1; T-bet, T-box-expressed-in-T-cells; and TNFα, tumor necrosis factor α.

perhaps via upregulation of Nrf2.[40] In humans, PGD2 inhibits platelet activation via DP1.[21] We observed that DP1 activation in T cells prevented age-related hypertension by suppressing TNFα and IFNγ. Consistent with our observations, PGD2/DP1 activation also inhibits IFNγ production in natural killer T cells and dendritic cells via PKA.[41] Therefore, the DP1 receptor may be an attractive therapeutic target for inflammation, especially for cardiovascular inflammation.

NEDD4L is an E3 ubiquitin protein ligase, which contains 4 WW domains (protein–protein interaction domains) that specifically recognize targeting substrates containing "PPXY," "LPXY," or "PPR" motifs.[24] Genetic studies showed that multiple single-nucleotide polymorphisms in NEDD4L are associated with hypertension in different ethnic populations.[42] The epithelial Na+ channel, involved in salt reabsorption and BP regulation, is a NEDD4L substrate in the kidney.[43] Loss of NEDD4L results in sodium-sensitive hypertension in mice due to elevated epithelial Na+ channel activity in the kidney.[44] There are 2 splicing isoforms of NEDD4L (a short isoform with 110 kDa molecular weight and a long isoform of 130 kDa). Both isoforms contain the

substrate-targeting WW domains, but the short isoform lacks the N-terminal C2 domain.[45] We observed that the long isoform of NEDD4L was dominantly expressed in T cells, and that NEDD4L regulated Th1 cytokine expression by targeting the T-bet transcription factor. It is interesting to note that along with DP1 downregulation and Th1 activation, NEDD4L expression was low in CD4+ T cells from older individuals. Forced expression of NEDD4L prevented age-related hypertension in mice. Thus, NEDD4L may be implicated in BP regulation in both inflammatory and renal tubule cells.

T-bet, a Th1 transcription factor, drives the expression of proinflammatory cytokines such as IFNγ and TNFα.[46–48] T-bet deletion tends to have a less hypertensive response to AngII and reduces Th1 immune reaction-mediated kidney damage in mice.[16] Furthermore, deficiency of Th1 cytokines TNFα or IFNγ blunts AngII-induced hypertension in mice.[16,17] We observed that expression of T-bet protein was induced in T cells from aged subjects, and deletion of T-bet in CD4+ T cells reduced age-related BP elevation in mice. During Th cell differentiation, T-bet undergoes various posttranslational modifications, including phosphorylation and ubiquitination at lysine

residues.[47] For example, lysine 313 of T-bet mediates proteasome degradation via ubiquitination, which fine-tunes Th1 cytokine production.[49] We observed accumulation of T-bet protein in T cells caused by impairment of ubiquitination during aging, and further identified NEDD4L as one of the key E3 ligases for T-bet in T cells, which is positively regulated by PGD_2/DP1 signaling. In agreement with our observations, T-bet protein is also expressed in influenza virus-specific $CD8^+$ T cells from aged subjects.[50]

In summary, PGD_2/DP1 activation in $CD4^+$ T cells delayed age-related hypertension via the PKA/Sp1/NEDD4L axis–mediated T-bet ubiquitination. Our results indicate that the DP1 receptor maybe an attractive immunotherapeutic target for age-related diseases, such as hypertension.

ARTICLE INFORMATION

Correspondence

Ying Yu, MD, PhD, Department of Pharmacology, School of Basic Medical Sciences, Tianjin Medical University, 22 Qixiangtai Road, Tianjin300070, China. Email yuying@tmu.edu.cn

Affiliations

Department of Pharmacology and Tianjin Key Laboratory of Inflammatory Biology, Key Laboratory of Immune Microenvironment and Disease (Ministry of Education), School of Basic Medical Sciences, Tianjin Medical University, China (D.K., S.Z., Q.L., Y.Y.). CAS Key Laboratory of Nutrition, Metabolism and Food Safety, Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences (Q.W., C.W., Y.Y.), State Key Laboratory of Cell Biology, Chinese Academy of Sciences Center for Excellence on Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology (B.Z.), University of Chinese Academy of Sciences, Chinese Academy of Sciences, China. Department of Gastroenterology (J.L.) and Department of Cardiology (P.B., A.L.), Ruijin Hospital, Shanghai Jiaotong University School of Medicine, China. National Clinical Research Center for Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, China (G.L., Y.Y.). Laboratory of Oral Microbiology, Shanghai Research Institute of Stomatology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China (S.-Z.D.). Division of Rheumatology and Knapp Center for Lupus and Immunology Research, University of Chicago, IL (F.G.). International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, Tsukuba City, Ibaraki, Japan (M.L.). Department of Veterans Affairs, Tennessee Valley Health Authority, and Department of Medicine, Vanderbilt University Medical Center, Nashville, TN (R.M.B.).

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Disclosures

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