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Graphical abstract





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Abstract

Androgen has long been recognized for its pivotal role in the sexual dimorphism of cardiovascular diseases, including aortic aneurysms, a devastating vascular disease with a higher prevalence and fatality rate in men than women. However, the mechanism by which androgen mediates aortic aneurysms is largely unknown. Herein, we found that male mice, not female mice, developed aortic aneurysms when exposed to aldosterone and high salt (Aldo-salt). We revealed that and rogen and androgen receptors (AR) were crucial for this sexually dimorphic response to Aldo-salt. We identified programmed cell death protein 1 (PD-1), an immune checkpoint, as a key link between androgen and aortic aneurysms. We demonstrated that administration of anti-PD-1 Ab and adoptive PD-1 deficient T cell transfer reinstated Aldo-salt-induced aortic aneurysms in orchiectomized mice, and genetic deletion of PD-1 exacerbated aortic aneurysms induced by high-fat diet and angiotensin II (Ang II) in non-orchiectomized mice. Mechanistically, we discovered that AR bound to the PD-1 promoter to suppress its expression in the spleen. Thus, our study unveils a mechanism by which androgen aggravates agric aneurysms by suppressing PD-1 expression in T cells. Moreover, our study suggests that some cancer patients might benefit from screenings for aortic aneurysms during immune checkpoint therapy.

Graphical Abstract



Introduction

Aortic aneurysms are defined as a permanent localized dilation of the aorta and can be classified as thoracic aortic aneurysms (TAA) and abdominal aortic aneurysms (AAA) (1). Aortic aneurysms are usually asymptomatic until they rupture, often lethal, resulting in over 85% mortality (2). Currently, no medication except for surgery is approved to treat this devastating vascular disease.

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Epidemiologic studies reveal aging, male sex, smoking, atherosclerosis, and hypertension as the 59 risk factors for aortic aneurysms (3). In particular, the male sex is considered the most potent 60 nonmodified risk factor for the sexual dimorphism of aortic aneurysms, with a 4:1 male-to-female ratio 61 (3). While the etiology of the sex difference in human aortic aneurysms remains to be elucidated. 62 accumulated evidence from animal studies demonstrated that both sex chromosomes and hormones 63 contributed to the development of aortic aneurysms (4). In particular, it is well documented that 64 gonadal androgen but not estrogen deprivation protects angiotensin II (Ang II)- or elastase-induce 65 aortic aneurysms (5-7), indicating that androgen likely plays a predominant role in the sexual 66 dimorphism of aortic aneurysms. However, the mechanism by which androgen aggravates Ang II or 67 elastin-induced aortic aneurysms remains largely unknown. 68

69

Accumulated clinical evidence demonstrates that elevated plasma concentration of Aldo, an essential 70 component of the renin-angiotensin-aldosterone system, and excessive dietary sodium intake are 71 associated with an increased risk for hypertension, stroke, coronary heart disease, heart failure, and 72 renal disease (8). Consistent with these human studies, we developed a mouse model of aortic 73 aneurysms in which we administered Aldo and high salt (Aldo-salt) to 10-month-old male C57BL/6J 74 mice (9, 10). Importantly, we demonstrated that Aldo-salt-induced aortic aneurysms depend on age 75 and mineralocorticoid receptor (MR; also known as Aldo receptor) but not Ang II receptor (9, 10). 76 However, whether Aldo-salt-induced aortic aneurysms have a sexual dimorphism has not been 77 investigated. 78

80 In this study, we report that Aldo-salt-induced aortic aneurysms mimicked human aortic aneurysms, exhibiting a strong sexual dimorphism. To delve into the role of androgen in this sexual dimorphism, 81 we conducted a series of animal experiments, including gonadal androgen deprivation via 82 orchiectomy, restoration of androgen in orchiectomized mice through dihydrotestosterone (DHT) 83 pellet implantation, and downregulation of androgen receptors (AR) by ASC-J9 or inhibition of AR by 84 flutamide. Our results consistently underscore the critical involvement of androgen and AR in Aldo-85 salt-induced aortic aneurysms. To investigate the mechanism by which androgen mediates Aldo-salt-86 induced aortic aneurysms, we found that Aldo-salt-induced IL-6 expression was selectively abolished 87 in the aorta by orchiectomy. Subsequent inhibition of IL-6 signaling by LMT-28 illustrated that IL-6 is 88 implicated in Aldo-salt-induced aortic aneurysms. Moreover, through RNA-seg and flow cytometry 89 analysis of the aortas, we identified T cell receptor (TCR) and PD-1, an immune checkpoint (11), as a 90 pivotal link between androgen and Aldo-salt-induced aortic aneurysms. Splenectomy augmented PD-91 1⁺T and B cells in the aorta and mitigated Aldo-salt-induced aortic aneurysms. Mechanistically, we 92 discovered that AR bound to the PD-1 promoter and suppressed its mRNA and protein expression in 93 the spleen in mice administered Aldo-salt. To define the role of PD-1 in the pathogenesis of aortic 94 aneurysms, we demonstrated that immune checkpoint blockade with anti-PD-1 Ab and adoptive PD-1 95 deficient T cell transfer restored Aldo-salt-induced aortopathies in orchiectomized mice. Finally, we 96 showed that genetic deletion of PD-1 exacerbated HFD and Ang II-induced aortopathy in non-97 orchiectomized mice. Collectively, our results provide mechanistic insight into the role of androgen in 98 the pathogenesis of aortic aneurysms and suggest a potential risk of aortic aneurysm development 99 in cancer patients undergoing immune checkpoint therapy. 100

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Results

105	Sexual dimorphism in	n Aldo-salt-induced	aortic aneurysms
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To investigate sexual dimorphism in Aldo-salt-induced aortic aneurysms, 10-month-old male and 106 female C57BL/6J mice were subjected to Aldo-salt administration for four weeks to induce aortic 107 aneurysms (9, 10). Suprarenal aortic dilations induced by Aldo-salt were monitored weekly by 108 109 ultrasound (9, 10). Albeit Aldo-salt induced suprarenal aortic dilation in both male and female mice in a time-dependent manner, the suprarenal aortic dilation induced by Aldo-salt was much larger in male 110 than in female mice (Figure 1A). Utilizing the ultrasound data, we calculated the growth rate of the 111 suprarenal aortic diameters, an important clinical index for appraisal of aortic aneurysm progression 112 and rupture in human patients (4). Consistently, the suprarenal aortic growth rate was significantly 113 accelerated in male than female mice (Figure 1B). 114

115

To define the role of hypertension in the sexual dimorphism of Aldo-salt-induced aortic aneurysms, we assessed mean arterial pressure (MAP) in the male and female mice by tail cuff one week before and three weeks after Aldo-salt administration. Both male and female mice displayed a hypertensive response to Aldo-salt. Surprisingly, female mice exhibited higher MAP levels than male mice before and after Aldo-salt administration (Figure 1C), suggesting that the greater increase in the suprarenal aortic dilation induced by Aldo-salt is not attributed to hypertension.

122

Four weeks after Aldo-salt administration, the aortas were harvested from the male and female mice for morphometric analysis. Maximal external diameters of the ascending aorta (AscAo), aortic arch (ArchAo), descending aorta (DesAo), and suprarenal aorta (SupAo) were measured (Supplemental Figure 1). While no differences were observed in the baseline, a significant increase in the external diameters of the AscAo, DesAo, and SupAo was noted in response to Aldo-salt in male mice compared to female mice (Figures 1D–1G). Isolated aortas were also subjected to pathological analysis to assess the incidence of the total aortic aneurysms (total AA = AAA + TAA + aortic rupture), AAA, TAA, and aortic rupture (Figure 1H) (9, 10, 12). Remarkably, none of the female mice
developed aortic aneurysms, whereas 70% of male mice exhibited aortic aneurysms, with 60% AAA,
40% TAA, and 10% aortic rupture (Figure 1I).

133

Gonadal androgen deprivation protects mice from Aldo-salt-induced aortic aneurysms 134 To explore the role of androgen in the sexual dimorphism in Aldo-salt-induced aortic aneurysms, 10-135 month-old male C57BL/6J mice underwent either orchiectomy or sham operation, and two weeks 136 later, they were administered Aldo-salt for four weeks. The ratio of seminal vesicle weight (SVW) to 137 BW was evaluated four weeks after Aldo-salt administration to confirm the success of orchiectomy. 138 Significantly reduced SVW/BW ratios were observed in orchiectomized mice compared to sham-139 operated counterparts (Figure 2A and Supplemental Figures 2A and 2B). Additionally, serum 140 testosterone levels were significantly lower in orchiectomized mice compared to sham-operated mice 141 (Supplemental Figure 2C). Importantly, compared to the sham operation, orchiectomy markedly 142 suppressed Aldo-salt-induced suprarenal aortic dilation and progression (Figures 2B and 2C), the 143 external diameters of the aorta, including the AscAo, ArchAo, DesAo, and SupAo (Figure 2D), and 144 the incidence of AAA and TAA (Figure 2E). 145

146

The severity of Aldo-salt-induced aortic aneurysms resembles human aortic aneurysms (13) and exhibits significant variability (9, 10). Similar to Ang II-induced aortic aneurysms (12), the severity of Aldo-salt-induced aortic aneurysms could be categorized into Type I, II, III, and IV (Supplemental Figure 3). Compared to sham operation, orchiectomy reduced the percentage of Type II and III but not Type I aortic aneurysms (Figures 2F and 2G), indicating that androgen may mainly affect the progression of Aldo-salt-induced aortic aneurysms.

153

To investigate whether androgen augments Aldo-salt-induced aortic aneurysms through salt retention, we assessed 24-h sodium retention in orchiectomized and sham-operated mice by

subtracting their 24-h sodium excretion (via urine) from their sodium intake (via food and water intake) 156 (14) one week before and three weeks after Aldo-salt administration. Interestingly, orchiectomy 157 increased both 24-h sodium intake and 24-urinary sodium excretion compared to the sham operation 158 (Supplemental Figures 4A-4E). As a net balance, orchiectomy did not significantly alter Aldo-salt-159 induced sodium retention (Supplemental Figure 4F). Consistent with these findings, there was no 160 significant difference in serum sodium levels between the orchiectomized and sham-operated mice, 161 and no correlation was observed between the serum sodium level and the internal diameter of the 162 suprarenal aorta four weeks after Aldo-salt administration (Supplemental Figures 4G and 4H). 163 Additionally, serum sodium levels did not differ between the orchiectomized and sham-operated mice, 164 regardless of whether the mice developed aortic aneurysms (Supplemental Figure 4I). 165

166

To investigate whether and rogen augments Aldo-salt-induced aortic aneurysms through 167 hypertension, we evaluated the effect of orchiectomy on MAP by tail-cuff measurement one week 168 before and three weeks after Aldo-salt administration. In line with its minimal effect on sodium 169 retention, orchiectomy did not affect MAP before and after Aldo-salt administration (Supplemental 170 Figure 4J). Moreover, no correlation was observed between MAP and the internal diameter of the 171 suprarenal aorta three weeks after Aldo-salt administration (Supplemental Figure 4K), and no 172 significant disparities in MAP were noted between orchiectomized and sham-operated mice, 173 regardless of whether the development of aortic aneurysms (Supplemental Figure 4L). Similar 174 findings were also observed in systolic and diastolic blood pressure (Gong and Guo, unpublished 175 observation). 176

177

178 Restoration of androgen in orchiectomized mice reinstates Aldo-salt-induced aortic

179 aneurysms

To further elucidate the role of androgen in Aldo-salt-induced aortic aneurysms, 10-month-old male
 C57BL/6J mice underwent orchiectomy, and two weeks later, they were administered Aldo-salt with

or without dihydrotestosterone (DHT) pellet implantation (10 mg, 60-day release (6)) for four weeks. 182 DHT was chosen over testosterone due to its greater potency and inability to be converted to 183 estrogens by aromatase (15), ensuring a more straightforward interpretation of results. DHT 184 effectively restored the SVW/BW ratio in orchiectomized mice to levels comparable to those in sham-185 operated mice DHT four weeks after Aldo-salt administration (Figure 3A and Supplemental Figures 186 2A, 2B, and 5), indicating the functionality of the implanted DHT pellets in mice. Importantly, 187 compared to orchiectomized mice without DHT, those with DHT exhibited a trend or significant 188 increase in response to Aldo-salt in the internal diameters and growth rate of SupAo (Figures 3B and 189 3C), the external diameters of the AscAo, ArchAo, DesAo, and SupAo (Figure 3D), and the incidence 190 of aortic aneurysms, including AAA, TAA, and aortic rupture (Figure 3E). 191

192

The aortopathy in orchiectomized mice administered Aldo-salt with DHT appeared more pronounced than in sham-operated mice (Figures 3F and 3G *vs.* 2F and 2G). To quantitatively assess the extent to which DHT restores Aldo-salt-induced aortic aneurysms, we calculated the percentage of aortic aneurysm restoration rates by normalizing the incidence of aortic aneurysms in orchiectomized mice with DHT (Figure 3E) to that in sham-operated mice (Figure 2E). As a result, 80%, 83%, and 73% of aortic aneurysm restoration rates were obtained in orchiectomized mice administered Aldo-salt with DHT for total AA, AAA, and TAA, respectively (Figure 3H).

200

Surprisingly, DHT significantly suppressed 24-h sodium intake and 24-h urinary sodium excretion three weeks after Aldo-salt administration (Supplemental Figures 6A–6E). However, DHT did not significantly affect Aldo-salt-induced sodium retention, serum sodium levels, and hypertension (Supplemental Figures 6F, 6G, and 6J). There was no significant correlation between the internal diameter of the suprarenal aorta and serum sodium or MAP levels in orchiectomized mice administered Aldo-salt with and without DHT (Supplemental Figures 6H and 6K). Furthermore, there was no significant difference in serum sodium or MAP levels in orchiectomized mice administered Aldo-salt with or without DHT, regardless of whether they developed aortic aneurysms (Supplemental Figures 6I and 6L). Thus, DHT is unlikely to restore Aldo-salt-induced aortic aneurysms through sodium retention and hypertension.

211

212 Downregulation of AR ameliorates Aldo-salt-induced aortic aneurysms

To explore targeting androgen as a potential therapy for treating aortic aneurysm, 10-month-old male 213 C57BL/6J mice were administered Aldo-salt with or without ASC-J9 (50 mg/kg, i.p. injection, once a 214 day) for four weeks (16). ASC-J9, a recently developed AR-degradation enhancer, has been shown 215 to selectively degrade AR without affecting other nuclear receptors (17). The efficacy of ASC-J9 in 216 promoting AR protein degradation was confirmed via IHC in the suprarenal aorta of mice four weeks 217 after Aldo-salt administration, with or without ASC-J9 (Figure 4A). Importantly, similar to the effect of 218 orchiectomy (Figure 2), ASC-J9 effectively mitigated Aldo-salt-induced suprarenal aortic dilation and 219 progression (Figures 4B and 4C), the external diameters of the AscAo and SupAo (Figure 4D), and 220 the incidence and severity of aortic aneurysms (Figures 4E-4G). 221

222

Intriguingly, ASC-J9 suppressed Aldo-salt-induced 24-h sodium intake and urinary sodium excretion 223 (Supplemental Figures 7A-7E). However, similar to the effect of orchiectomy and DHT (Supplemental 224 Figures 4 and 6), ASC-J9 also did not affect Aldo-salt-induced sodium retention, hypernatremia, and 225 hypertension (Supplemental Figures 7F, 7G, and 7J). There was no significant correlation between 226 the internal diameters of the suprarenal aorta and serum sodium or MAP in mice administered Aldo-227 salt with and without ASC-J9 (Supplemental Figures 7H and 7K). Furthermore, there was no 228 significant difference in serum sodium or MAP levels between mice with and without ASC-J9. 229 regardless of whether they developed aortic aneurysms (Supplemental Figures 7I and 7L). Thus, 230 ASC-J9 is unlikely to protect mice from Aldo-salt-induced aortic aneurysms through sodium retention 231 and hypertension. 232

ASC-J9 was reported to exert its effects through AR-dependent and independent mechanisms (18). 234 To verify whether ASC-J9 protects mice from Aldo-salt-induced aortic aneurysms via AR, 9-10-235 month-old male C57BL/6J mice were administered Aldo-salt with flutamide (50 mg/kg/dav. i.p. 236 injection, once a day) or vehicle for four weeks (19). Flutamide, a selective AR antagonist, has been 237 clinically utilized to treat patients with prostate cancer (19). In line with the effects of ASC-J9 (Figure 238 4), flutamide reduced the seminal vesicle weight (Supplemental Figure 8A-8C) and, more 239 importantly, protected mice from Aldo-salt-induced suprarenal aortic dilation and progression and the 240 incidence of AAA (Supplemental Figures 8D-8G). Interestingly, flutamide did not affect basal MAP 241 but moderately boosted Aldo-salt-induced hypertension (Supplemental Figure 8H). 242

243

244 IL-6 is implicated in Aldo-salt-induced and androgen-mediated aortic aneurysms

To investigate the molecular mechanism by which androgen mediates Aldo-salt-induced aortic aneurysms, we conducted real-time PCR to analyze mRNA expressions in the aortas from 10-monthold orchiectomized and sham-operated C57BL/6J mice, with and without Aldo-salt administration for ten days. We opted to isolate the aortas ten days rather than four weeks after Aldo-salt administration because we sought to identify androgen-targeting genes that result in rather than result from Aldosalt-induced aortic aneurysms.

251

Based on the literature (9, 10, 16, 20-22), we focused on a list of genes implicated in aortic

aneurysms, including Ar, Nr3c2, Sgk1, Scnn1a, Scnn1b, Scnn1g, Bmal1, Tgfb2, Mmp2, II1b, II6, II6ra,

²⁵⁴ *Il6st*, *Ccl2*, *Ccl4*, and *Tnf*. Of the 16 genes examined, 12 genes (*Ar, Nr3C2, Sgk1, Scnn1a, Scnn1b,*

255 Scnn1g, Bmal1, II1b, II6, II6ra, Ccl2, and Ccl4) responded to Aldo-salt: 9 of them (Ar, Nr3C2, Sgk1,

256 Scnn1a, Scnn1b, Scnn1g, II1b, II6ra, and Ccl4) were downregulated, whereas 3 of them (Bmal1, II6,

and Ccl2) were upregulated (Supplemental Figure 9). Interestingly, 5 genes (Nr3C2, Scnn1a, Scnn1b,

258 *II6,* and *Ccl2*) also responded to orchiectomy after Aldo-salt administration: 3 of them (*Nr3C2,*

259 Scnn1a, Scnn1b) were upregulated, whereas 2 of them, (*II6*, and Cc/2) were downregulated

(Supplemental Figures 9B, 9D, 9E, 9K, and 9N). Particularly noteworthy, *ll6* was found to be most
 dramatically upregulated by Aldo-salt (i.e., up to 58-fold), which was completely abolished by
 orchiectomy (Supplemental Figure 9K).

263

IL-6 is implicated in human aortic aneurysms (22). Therefore, we focused on IL-6 and further 264 investigated whether its protein expression is regulated by Aldo-salt and/or androgen in the 265 suprarenal aorta of 10-month-old orchiectomized and sham-operated C57BL/6J mice with and 266 without 10-day Aldo-salt administration. In sham-operated mice, basal IL-6 protein expression was 267 barely detectable in the suprarenal aorta, whereas IL-6 protein was markedly upregulated by Aldo-268 salt in the suprarenal aorta. Intriguingly, orchiectomy increased basal IL-6 protein expression but 269 decreased Aldo-salt-induced IL-6 protein upregulation in the suprarenal aorta (Supplemental Figures 270 10A and 10B). In contrast to IL-6 protein, MR protein did not respond to Aldo-salt and/or orchiectomy 271 in the suprarenal aorta (Supplemental Figures 10C and 10D). 272

273

While the elevated basal IL-6 protein expression in the suprarenal aorta induced by orchiectomy may 274 be attributed to the loss of androgen-induced immunosuppression (20), the abrogation of Aldo-salt-275 induced IL-6 protein upregulation elicited by orchiectomy might be due to a blockade of Aldo-salt-276 induced inflammatory cell infiltration in the aorta (9, 10), specifically macrophages, which are known 277 for their pivotal role in IL-6 production (8). To explore this possibility, we investigated whether 278orchiectomy affects Aldo-salt-induced macrophage infiltration by IHC in the suprarenal aorta of 10-279 month-old male C57BL/6J mice with and without 10-day Aldo-salt administration. Interestingly, 280 orchiectomy abolished Aldo-salt-induced immunostaining of F4/80, a macrophage marker, in the 281 suprarenal aortas (Supplemental Figures 11A and 11B). 282

283

To investigate the potential role of IL-6 in Aldo-salt-induced aortic aneurysms, 10-month-old male C57BL/6J mice were administered Aldo-salt with LMT-28 (0.25 mg/kg, oral gavage, once a day) or

vehicle for four weeks (23). LMT-28, a recently developed novel small molecule inhibitor, has been 286 shown to specifically target IL-6R β to disrupt its interaction with IL-6R α , thus inhibiting IL-6 signaling 287 (23). The efficacy of LMT-28 in inhibiting IL-6 signaling was confirmed by immunostaining of 288 phosphorylated STAT3, an index of the IL-6 signaling activation (23), in the suprarenal aortas by IHC 289 in the mice four weeks after Aldo-salt with LMT-28 or vehicle administration (Figure 5A). Importantly, 290 compared to vehicles, LMT-28 protected mice from Aldo-salt-induced suprarenal aortic dilation and 291 progression (Figures 5B–5C), as well as the incidence and severity of aortic aneurysms (Figures 292 5E-5G). It is noteworthy, however, that LMT-28 did not affect the external diameters of the aorta 293 (Figure 5D). 294

295

Interestingly, LMT-28 increased Aldo-salt-induced salt retention but did not affect serum sodium and
MAP levels before and after Aldo-salt administration (Supplemental Figures 12A–12D and 12G).
Notably, there was no significant correlation between the internal diameter and serum sodium or MAP
levels (Supplemental Figures 12E and 12H). Additionally, there was no significant difference in serum
sodium level and MAP levels between mice with and without LMT-28, irrespective of whether they
developed aortic aneurysms (Supplemental Figures 12F and 12I).

302

Aldo-salt may induce aortic aneurysms through AR and androgen synthesis pathways. To explore 303 this possibility, we determined AR protein expression by IHC and androgen synthesis (Cyp17a1, 304 Hsd3b2, and Hsd17b3) (24)) mRNA expressions by real-time PCR in the aorta, testis, and adrenal 305 gland from 10-month-old male C57BL/6J mice ten days after Aldo-salt administration. The results 306 revealed that Aldo-salt neither affected AR protein expression in the suprarenal aortas (Supplemental 307 Figures 13A and 13B) nor Cyp17a1, Hsd3b2, and Hsd17b3 mRNA expressions in the testis 308 (Supplemental Figures 13C-13E). Interestingly, Aldo-salt moderately inhibited Cyp17a1 and Hsd17b3 309 but not Hsd3b2 mRNA expressions in the adrenal gland (Supplemental Figures 13F-13H). However, 310 there was no significant difference in plasma testosterone levels between mice with and without Aldo-311

312 salt administration (Supplemental Figure 13I).

313

314	Identification of TCR and PD-1 as a link between AR and Aldo-salt-induced aortic aneurysms
315	Since LMT-28 completely inhibited the IL-6 signaling but only partially blocked Aldo-salt-induced
316	aortic aneurysms (Figure 5), we hypothesized that additional signaling pathways regulated by
317	androgen might be involved in Aldo-salt-induced aortic aneurysms. To identify these putative
318	signaling ways in an unbiased way, 10-month-old male C57BL/6J mice were randomly divided into
319	three groups: 1) Aldo-salt; 2) orchiectomy followed by Aldo-salt; 3) orchiectomy followed by Aldo-salt
320	with DHT. Whole aortas were harvested one week after the Aldo-salt administration. Subsequently,
321	these samples underwent RNA-seq for comprehensive gene expression analysis.
322	
323	Of a total of 18,841 mRNAs detected by RNA-seq, DESeq2 (25) identified 2,359 of them as
324	significantly and differentially abundant ($P < 0.01$) among aortas from the three groups of mice
325	(Figure 6A). Orchiectomy caused the upregulation of 298 mRNAs and the downregulation of 351
326	mRNAs (Figure 6B). Conversely, administration of DHT to orchiectomized mice resulted in the
327	upregulation of 707 mRNAs and the downregulation of 1,003 mRNAs (Figure 6C). Importantly, the
328	rescue of androgen deprivation by DHT in orchiectomized mice identified 180 androgen-sensitive
329	mRNAs upregulated by orchiectomy but downregulated by DHT (Figures 6D and 6E; Supplemental
330	Table 1) and 150 androgen-sensitive mRNAs downregulated by orchiectomy but upregulated by DHT
331	(Figures 6G and 6H; Supplemental Table 2).
332	
333	To gain mechanistic insight into the androgen-sensitive mRNAs identified by the RNA-seq analysis,
334	we employed Enrichr, a widely-used search engine for comprehensive pathway enrichment analysis
335	(26), to unveil the signaling pathways responsive to androgen and potentially implicated in Aldo-salt-

induced aortic aneurysms. Based on the 180 androgen-sensitive mRNAs upregulated by orchiectomy

337 but downregulated by DHT (Figures 6D and 6E), Enrichr analysis revealed 65 overrepresented

functional annotations (Figure 6F and Supplemental Table 3). Surprisingly, most of these annotations were associated with adaptive immunity, particularly TCR signaling pathways, including PD-1 (Figure 6F and Supplemental Table 3). In parallel with this finding, the 150 androgen-sensitive mRNAs downregulated by orchiectomy but upregulated by DHT (Figures 6G and 6H) were pinpointed to 19 overrepresented functional annotations, most of which were associated with triglyceride, fatty acid, and lipid biosynthesis or metabolism (Figure 6I and Supplemental Table 4).

344

Among the TCR signaling pathways revealed by RNA-seq analysis, PD-1 is particularly interesting for 345 several reasons. Firstly, there is little information regarding the regulation of PD-1 by androgens and 346 its role in aortic aneurysms. Secondly, PD-1 is well-recognized for its pivotal role as an immune 347 checkpoint in regulating T cells, immunity, and immune-based cancer therapy (11, 27, 28). Thirdly, 348 PD-1 immune checkpoint therapy has been shown to have sex differences (29) and is associated 349 with serious immune-related cardiovascular adverse events, including autoimmune myocarditis, 350 pericarditis, and vasculitis (11, 27, 28). Consequently, it is conceivable that PD-1 may be involved in 351 Aldo-salt-induced and androgen-mediated aortopathy. Therefore, our subsequent studies have been 352 directed toward investigating PD-1 in greater depth. 353

354

RNA-seg revealed 180 and rogen-response genes associated with TCR and PD-1 signaling 355 pathways. However, it is plausible that the findings may arise from the differential composition of 356 aortic cells rather than the specific activation of TCR and PD-1 signaling pathways. To discern these 357 possibilities, we conducted flow cytometry analysis of T-cell subset signatures in the aortas of three 358 groups of 10-month-old male WT C57BL/6J mice ten days after Aldo-salt with 1) sham operation, 2) 359 orchiectomy, and 3) orchiectomy with DHT. As depicted in Supplemental Figures 14 and 15 for the 360 gating strategy of flow cytometry analysis, single aortic cells were first gated on CD45 vs. live/dead 361 cell staining to select viable leukocytes and then gated on CD3, CD4, and CD8 to identify total T-cells, 362 CD4 T-cells, and CD8 T-cells, respectively. CD4 and CD8 T-cells were further gated on CD44, 363

CD62L, and CD127 to distinguish central memory T-cells (Tcm; CD44⁺CD62L⁺), naïve T-cells (CD44⁺
 CD62L⁺), effector T-cells (Teff; CD44⁺CD62L⁻CD127⁻), and effector memory T-cells (Tem;
 CD44⁺CD62L⁻CD127⁺), respectively. We selected these T-cell subsets based on our RNA-seq
 pathway enrichment analysis (Figure 6F). These T-cell subsets were also gated on PD-1 to pinpoint
 PD-1⁺ T-cell subsets. As a control, a single-cell suspension from the spleen was analyzed by flow
 cytometry with fluorescence minus one (FMO) to define gating boundaries and ensure the specificity
 of antibodies.

371

Concurrently with TCR and PD-1 signaling pathways identified by RNA-seg analysis (Figures 6D-6F). 372 the total numbers of all examined T-cell subsets exhibited a similar trend in response to orchiectomy 373 and DHT: increased by orchiectomy but decreased by DHT (Supplemental Table 6). Consistent with 374 these findings, several T-cell subsets, including CD4 T-cells, naïve CD4 T-cells, naïve CD8 T-cells, 375 PD-1⁺ CD4 Teff cells, and PD-1⁺ CD4 Tcm cells, displayed a significant or trending percentage 376 increase induced by orchiectomy and/or a percentage decrease elicited by DHT (Figure 7 and 377 Supplemental Table 5). Intriguingly, in contrast to the total T-cell number response to orchiectomy 378 and DHT, several other T-cell subsets exhibited a significant or trending percentage decrease 379 induced by orchiectomy and/or a percentage increase elicited by DHT (Supplemental Table 5). 380 381

To trace the origins of T-cell subsets in the aorta, we analyzed T-cell subsets by flow cytometry in the spleens from the same three groups of mice. Interestingly, a similar effect of androgen on T-cell subsets was found in the spleens as in the aortas, although the total number but not the percentage of T-cell subsets were mostly affected (Supplemental Figure 16 and Supplemental Table 6), indicating that alterations in T-cell subsets within the spleen, induced by orchiectomy and/or DHT, contribute to the changes observed in T-cell subsets within the aorta.

388

389 Splenectomy mitigates Aldo-salt-induced aortic aneurysms and augments PD-1⁺ T-cells and

390 PD-1⁺ B-cells in the aorta

To explore the potential involvement of T-cells in Aldo-salt-induced aortic aneurysms, 11-13-month-old 391 male C57BL/6J mice were subjected to splenectomy or sham operation, and four weeks later, they 392 received an additional four-week Aldo-salt administration. In line with previous findings (30), 393 splenectomy lowered MAP before and after Aldo-salt administration (Figure 8A), indicating the 394 effectiveness of splenectomy. Compared to sham operations, splenectomy suppressed Aldo-salt-395 induced suprarenal aortic dilation and progression (Figures 8B and 8C), the external diameters of the 396 AscAo, ArchAo, and SupAo (Figure 8D), and the incidence of AAA, TAA, and aortic rupture (Figure 397 8E). Interestingly, a significant correlation was observed between MAP and the internal diameters of 398 the suprarenal aorta of mice three weeks after Aldo-salt administration (Supplemental Figure 17A). 399 However, there was no significant difference in MAP between splenectomized mice with and without 400 aortic aneurysms (Supplemental Figure 17B). 401

402

To investigate whether PD-1⁺T-cells are implicated in the effect of splenectomy on Aldo-salt-induced 403 aortic aneurysms, we conducted flow cytometry analysis of the aortas from splenectomized and 404 sham-operated mice four weeks after Aldo-salt administration. As delineated in Supplemental Figure 405 18. single aortic cells were first gated on CD45 to sort leukocytes and then gated on CD3. CD19. 406 F4/80, and Ly6G to identify T-cells, B-cells, macrophages, and neutrophils, respectively. These cells 407 were further gated on PD-1 to identify PD-1⁺ T cells, PD-1⁺ B cells, PD-1⁺ macrophages, and PD-1⁺ 408 neutrophils. Compared to sham operation, splenectomy did not affect the total numbers and 409 percentages of leukocytes, T-cells, B-cells, macrophages, and neutrophils in the aorta of mice 410 administered Aldo-salt (Supplemental Figure 19). However, splenectomy notably increased the 411 percentages, albeit not the total numbers, of PD-1⁺ T-cells and PD-1⁺ B cells in the aorta of mice 412 administered Aldo-salt (Figures 8F-8K). Conversely, splenectomy significantly decreased the 413 percentages and total numbers of PD-1⁺ neutrophils while not affecting PD-1⁺ macrophages in the 414 aorta of mice administered Aldo-salt (Supplemental Figures 20A-20F). 415

To trace the origins of PD-1⁺ T-cell subsets in the aorta of splenectomized mice, we conducted flow 417 cytometry analysis of the blood and periaortic lymph nodes in 11-13-month-old splenectomized and 418 sham-operated mice four weeks after Aldo-salt administration. Interestingly, splenectomy did not alter 419 the total number and percentage of leukocytes, T-cells, B-cells, PD-1⁺ T-cells, and PD-1⁺ B-cells in 420 the blood compared to sham operation (Supplemental Figure 21). In contrast, splenectomy led to a 421 notable increase in the total number, although not the percentage, of leukocytes, T-cells, B-cells, PD-422 1⁺ T-cells, and PD-1⁺ B-cells in the periaortic lymph nodes relative to sham operation (Supplemental 423 Figure 22). These findings suggest that splenectomy may enrich PD-1⁺ T-cells and PD-1⁺ B-cells in 424 the aortas via the periaortic lymph nodes. 425

426

AR binds to the PD-1 promoter and suppresses its mRNA and protein expression in the spleen 427 We conducted a series of experiments to investigate whether PD-1 is regulated by androgen in the 428 spleen. Firstly, we examined PD-1 protein expression by IHC in the spleens from 10-month-old male 429 C57BL/6J mice with orchiectomy or sham operation ten days after Aldo-salt administration. PD-1 430 protein was predominantly observed in the white pulp of the spleen (Figures 9A and 9B), a region 431 primarily composed of T cells and B cells (31). Importantly, PD-1 protein expression was notably 432 elevated by orchiectomy in the spleen relative to sham operation (Figures 9A and 9B). Consistent 433 with these findings, DHT administration to orchiectomized mice abolished PD-1 protein upregulation 434 in the spleen four weeks after Aldo-salt administration (Figures 9C and 9D). 435

436

Secondly, we quantified PD-1 protein expression by Western blots in the spleen of 10-month-old
 orchiectomized or sham-operated C57BL/6J mice ten days after Aldo-salt administration. PD-1
 protein expression was markedly upregulated by orchiectomy up to 4-fold in the spleen compared to
 sham operation (Figures 9E and 9F). To discern whether orchiectomy-induced PD-1 protein

upregulation in the spleen is attributed to T-cells or B-cells, we examined CD3ε, a T-cell marker, and

CD19, a B-cell marker, protein expressions in the same spleen lysate. Interestingly, both CD3ε and
CD19 proteins showed a moderate increase in the spleen of orchiectomized mice compared to shamoperated mice, but only CD3ε protein upregulation was statistically significant (Figures 9E, 9G, and
9H).

446

We also investigated the effects of orchiectomy on PD-1, CD3 ε , and CD19 protein expressions in the spleen of 10-month-old orchiectomized and sham-operated C57BL/6J mice without Aldo-salt administration. An increasing trend in PD-1, but not CD3 ε and CD19, basal protein expression was observed in the spleens (Supplemental Figures 23). However, the level of PD-1 upregulation induced by orchiectomy in the spleen of the mice without Aldo-salt was notably lower than those with Aldo-salt (Supplemental Figures 23 *vs.* Figures 9E and 9F).

453

Thirdly, we conducted a flow cytometry analysis of the spleens from 10-month-old orchiectomized 454 and sham-operated C57BL/6J mice ten days after Aldo-salt administration, to discern the 455 upregulation of PD-1 protein, as detected by IHC and Western blots, in splenic T-cells or B-cells. 456 Interestingly, orchiectomy significantly increased the total number, not the percentage, of splenic PD-457 1⁺ T-cells, but not splenic PD-1⁺ B-cells, compared to sham operation ten days after Aldo-salt 458 administration (Supplemental Figures 24). These findings suggest that orchiectomy-induced PD-1 459 protein upregulation in the spleen mainly results from splenic PD-1⁺ T-cells, rather than splenic PD-1⁺ 460 B-cells, in mice administered Aldo-salt. 461

462

Fourthly, to investigate whether splenic PD-1 is regulated by androgen at the transcription level, we determined Pdcd1 (the gene that codes PD-1) mRNA expression by real-time PCR in the spleen of 10-month-old male C57BL/6J mice with orchiectomy or sham operation ten days after Aldo-salt administration. Pdcd1 mRNA was significantly upregulated by orchiectomy in the spleen compared to sham operation (Figure 9I).

469 Fifthly, to investigate the mechanism by which androgen suppresses Pdcd1 mRNA expression in the spleen in mice administered Aldo-salt, we examined whether AR binds to the PD-1 promoter to 470 suppress its transcription. We analyzed a 5-kb mouse PD-1 promoter DNA sequence to identify 471 androgen response elements (AREs) containing AGAACA or TGTTCT hexamers, known to bind AR 472 effectively (32). We found 12 putative AREs in the 5-kb mouse PD-1 promoter (Figure 9J). To 473 determine whether AR can bind to these putative ARE in the spleen, we performed a ChIP assay 474 using the mouse spleen samples with two commercially available ChIP-grade anti-AR Ab with distinct 475 epitopes, along with two sets of ChIP-PCR primers specific for amplifying ARE4 and ARE6 in the 476 mouse PD-1 promoter (Figure 9J). Both anti-AR Ab, but not the control Ab, successfully pulled down 477 the chromatin fragments containing ARE6 but not ARE4 (Figures 9K–9M), indicating that AR can bind 478 to the ARE6 but not ARE4 in the mouse PD-1 promoter in the spleen. 479

480

Sixthly, to investigate whether the binding of AR to the PD-1 promoter inhibits its transcriptional 481 activity, we subcloned a 488 bp PD-1 mouse promoter (-4,444 to -3,956 bp relative to the 482 transcription start site (TSS)) containing ARE6 to ARE10 (Figure 9J) into a pGL3-basic firefly 483 luciferase report vector. The pGL3-basic-PD-1 promoter construct was co-transfected with the pRL-484 TK control vector and a pcDNA Flag-M4-AR construct (33) into HEK293 cells. Dual luciferase assays 485 revealed that the -488 bp PD-1 promoter exhibited a 4.6-fold higher luciferase activity than the pGL3-486 basic vector (Figure 90), indicating that the cloned -488 bp PD-1 promoter can drive PD-1 487 transcription. Importantly, co-transfection of the PD-1 promoter-luciferase constructs with the human 488 AR cDNA construct completely abolished the PD-1 promoter activity in the presence of DHT (Figure 489 9O). 490

491

492 Seventhly, to investigate the link between orchiectomy-induced PD-1 upregulation in the spleen and
 493 Aldo-salt-induced aortic aneurysms, we conducted a flow cytometry analysis of the blood of 10-

month-old orchiectomized and sham-operated C57BL/6J mice ten days after Aldo-salt administration.
In line with its effect on PD-1⁺ T-cells and PD-1⁺ B-cells in the spleen (Supplemental Figures 24),
orchiectomy amplified both the total and percentage of PD-1⁺ T-cells in the blood, though it did not
affect PD-1⁺ B-cells (Supplemental Figures 25).

498

Eighthly, to investigate whether orchiectomy-induced PD-1 upregulation also occurs in other immune
organs, we examined PD-1 protein expression by IHC and Western blot analysis in the periaortic
lymph nodes of 10-month-old orchiectomized and sham-operated C57BL/6J mice ten days after Aldosalt administration. IHC analysis showed a discernible increasing trend in PD-1 immunostaining in the
periaortic lymph nodes of orchiectomized mice compared to sham-operated mice (Supplemental
Figures 26A and 26B). This observation was further supported by Western blot analysis
(Supplemental Figures 26C and 26D).

506

507 Finally, to ascertain whether PD-1 regulates IL-6 in T cells, we assessed IL-6 mRNA and protein 508 expressions in the spleen of 4-month-old male global PD-1 knockout (34) and WT C57BL/6J mice 509 received 8-week HFD feeding and 4-week Ang II infusion (35). There were no significant differences 510 in IL-6 mRNA and protein expressions in the spleen between PD-1 KO and WT mice (Supplemental 511 Figure 27A–27E). Consistent with these findings, there was no significant difference in serum IL-6 512 protein levels between PD-1 KO and WT mice (Supplemental Figure 27F).

513

514 Blockade of the immune checkpoint with anti-PD-1 Ab reinstates Aldo-salt-induced aortic

aneurysms in orchiectomized mice

516 To explore the potential role of PD-1 in Aldo-salt-induced and androgen-mediated aortic aneurysms,

10-month-old C57BL/6J male mice underwent orchiectomy and then administered Aldo-salt with a

specific rat anti-mouse PD-1 Ab or an isotype control Ab (200 μ g/mice, i.p. injection, twice a week) for

eight weeks (36). Compared to the control Ab, anti-PD-1 Ab significantly enhanced suprarenal aortic

dilation from week four to week eight (Figure 10A). A similar but more potent effect of anti-PD-1 Ab
was found on Aldo-salt-induced aortic arch dilation from week six to week eight after Aldo-salt with
anti-PD-1 or control Ab administration (Figure 10B). Additionally, anti-PD-1 Ab significantly increased
the external diameters of the AscAo, ArchAo, DesAo, and SupAo relative to the control Ab (Figure
10C). Moreover, of 12 mice with anti-PD-1 Ab, 5 developed aortic aneurysms (45%), including 1 AAA
(8%), 5 TAA (45%), and 1 aortic rupture (8%). In contrast, none of 8 mice with the control Ab
developed aortic aneurysms (Figure 10D).

527

The aortas were harvested from orchiectomized mice eight weeks after Aldo-salt with anti-PD-1 or 528 control Ab administration and then subjected to Verhoeff-Van Gieson staining (9, 10) to examine the 529 effect of anti-PD-1 Ab on Aldo-salt-induced aortic elastin fiber fragmentation. A noticeable increase in 530 the breakage of thoracic and abdominal aortic elastin fiber was evident in orchiectomized mice with 531 aortic aneurysms induced by anti-PD-1 Ab, but not in mice administered with control Ab without aortic 532 aneurysms (Figures 10E-10G). The same thoracic aortas also underwent IHC with anti-CD3_E, CD19, 533 F4/80, and Ly6G Ab to identify T-cells, B-cells, macrophages, and neutrophils, respectively. As 534 depicted in Figure 10H, T cells, B cells, macrophages, and neutrophils were prominently present in 535 the thoracic aorta of mice with TAA induced by anti-PD-1 Ab, whereas they were barely detectable in 536 mice administered with control Ab without TAA. 537

538

Interestingly, anti-PD-1 Ab did not affect MAP before and three weeks after Aldo-salt administration, but it exacerbated Aldo-salt-induced hypertension seven weeks after Aldo-salt administration (Figure 10I). There was a significant correlation between the internal diameters of the aortic arch and MAP seven weeks after Aldo-salt administration (Supplemental Figure 28A). However, there was no significant difference in MAP between orchiectomized mice treated with the anti-PD-1 Ab regardless of whether they developed aortic aneurysms (Supplemental Figure 28B).

To explore the potential involvement of PD-1 in human aortic aneurysms, we examined PD-1 protein expression by IHC in the abdominal aorta specimens from human patients with or without AAA. PD-1 protein was scarcely detectable in normal abdominal aortas but was readily found in human AAA (Figures 10J and 10K).

550

551 Adoptive PD-1 deficient T-cell transfer resumes Aldo-salt-induced aortopathy in

552 orchiectomized mice

To further define the role of PD-1 in the pathogenesis of Aldo-salt-induced aortic aneurysms, PD-1 deficient T-cells and WT T-cells were isolated by microbeads conjugated with a monoclonal antimouse CD90.2 Ab from the spleens of 4-5-month-old male PD-1-KO and WT C57BL6J donor mice and then adoptively transferred to 9-10-month-old orchiectomized C57BL/6J recipient mice via retroorbital sinus injection two days before and eight and eighteen days after Aldo-salt administration. Pilot experiments confirmed the presence of adoptively transferred T cells preloaded with a red fluorescent cell tracker in the recipient mice's spleen and aorta (Gong and Guo, unpublished observation).

560

Compared to mice receiving WT T-cells, those with adoptive PD-1 deficient T-cell transfer displayed a 561 significantly greater suprarenal aortic and aortic root (RootAo) dilation following Aldo-salt 562 administration (Figures 11A and 11B). Consistent with these findings, the aorta weight to BW ratio, an 563 index of aortic aneurysm severity (5), but not the spleen weight to BW ratio, also exhibited a 564 significant increase in mice with adoptive PD-1 deficient T-cell transfer compared to those with 565 adoptive WT T-cell transfer (Figures 11C and Supplemental Figures 29). Additionally, adoptive PD-1 566 deficient T-cell transfer, relative to adoptive WT T-cell transfer, led to a significant increase in the 567 external diameters of the RootAo. AscAo. and SupAo four weeks after Aldo-salt administration 568 (Figure 11D), along with 60% TAA, 10% AAA, 40% aortic rupture (Figure 11E). In contrast, none of 569 the mice receiving adoptive WT T-cell transfer developed TAA, AAA, and aortic rupture. 570

Mice receiving PD-1 deficient T-cells developed TAA, characterized by evident elastin fiber 572 breakages (Figures 11F and 11G) and prominent infiltration of T-cells, macrophages, and neutrophils, 573 but not B-cells, in the thoracic aortas compared to those with WT T-cell transfer without TAA (Figure 574 11H). However, adoptive PD-1 deficient T-cell transfer did not affect MAP before and after Aldo-salt 575 administration (Figure 11I). No significant correlation was found between MAP and aortic root dilation 576 in mice receiving PD-1 deficient and WT T-cell transfer three weeks after Aldo-salt administration 577 (Figure 11J). Furthermore, there was no significant difference in MAP between mice with PD-1 578 579 deficient and WT T-cell transfer, regardless of mice with aortic aneurysms (Figure 11K).

580

Genetic deletion of PD-1 exacerbates HFD and Ang II-induced AAA in non-orchiectomized mice

To investigate whether PD-1 is implicated in other aortic aneurysm animal models, 2-month-old male 583 PD-1-KO and WT C57BL/6J mice (34) were fed an HFD for one month and then infused with Ang II in 584 the continued presence of HFD feeding for an additional month to induce AAA (35). HFD and Ang II, 585 but not HFD alone, result in both abdominal and thoracic aortic dilaton, and importantly, genetic 586 deletion of PD-1 exacerbated HFD and Ang II-induced aortic dilation, with a more pronounced effect 587 observed in the SupAo than the RootAo (Figures 12 A and 12B). In line with these findings, PD-1-KO 588 mice also exhibited a significant increase in the aorta weight to BW ratio (Figure 12C and 589 Supplemental Figure 30A), external diameters of the AscAo, ArchAo, DesAo, and SupAo (Figure 590 12D), and the incidence of aortic aneurysms, mainly AAA rather than TAA, which was different from 591 those in orchiectomized mice with Aldo-salt (Figures 12E vs. 10D and 11E). 592

593

Interestingly, genetic deletion of PD-1 did not affect BW four weeks after HFD feeding or HFD feeding plus Ang II infusion (Supplemental Figure 30B). However, genetic deletion of PD-1 significantly increased spleen weight and the spleen weight to BW ratio, but no kidney weight and the kidney weight to BW ratio (Supplemental Figures 30C–30F), indicating the involvement of immune cells.

598	Consistent with these findings, genetic deletion of PD-1 amplified HFD and Ang II-induced elastin
599	fiber fragmentation and infiltration of T-cells, B-cells, macrophages, and neutrophils in the suprarenal
600	aorta compared to WT mice (Figures 12F-12H).

602	Genetic deletion of PD-1 did not affect MAP before and after HFD feeding and Ang II infusion (Figure
603	12I). There was no significant correlation between MAP and the internal diameters of the suprarenal
604	aorta in PD-1 KO and WT mice three weeks after HFD and Ang II administration (Figure 12J).
605	Furthermore, there was no significant difference in MAP between PD-1 KO and WT mice, regardless
606	of whether they developed AAA (Figure 12K).
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Discussion

It has long been recognized that androgen plays a role in cardiovascular diseases (37). However, 625 whether and rogen protects or acquiravates a ortic aneurysms remains inconclusive and appears to be 626 animal model-specific (5-7, 16, 38). In this study, we report that Aldo-salt-induced aortic aneurysms 627 mimicked human AAA (3) and mostly occurred in male but not female mice (Figure 1). Consistent 628 with the Ang II and elastase AAA mouse models (5, 7, 16) but not the Ang II plus CaCl₂ mouse model 629 (38), we demonstrate that Aldo-salt-induced aortic aneurysms were abolished or ameliorated by 630 global androgen deprivation via orchiectomy (Figure 2), downregulation of AR with ASC-J9 (Figure 4), 631 and inhibition of AR with flutamide (Supplemental Figure 8). Importantly, restoration of androgen in 632 orchiectomized mice reinstated Aldo-salt-induced aortic aneurysms (Figure 3). 633

634

One of the most important findings is that androgen aggravates Aldo-salt-induced aortic aneurysms, 635 at least partially, via suppressing PD-1⁺T cells in the spleen. Several lines of evidence support this 636 potential mechanism. Firstly, RNA-seg identified 180 genes upregulated by orchiectomy but 637 downregulated by DHT in the aortas of mice one week after Aldo-salt administration (Figures 6D and 638 6E and Supplemental Tables 1). Surprisingly, these 180 and rogen-sensitive genes were mostly 639 mapped to the TCR signaling, including PD-1 (Figure 6F and Supplemental Tables 3), and 640 importantly, these findings were largely confirmed by flow cytometry (Figure 7 and Supplemental 641 Table 5). Secondly, consistent with the potential role of T-cells in AAA (39-41), splenectomy mitigated 642 Aldo-salt-induced aortic aneurysms (Figures 8A-8E), accompanied by the enrichment of PD-1⁺ T-643 and PD-1⁺ B-cells in the aorta of mice administered Aldo-salt, probably via the periaortic lymph nodes 644 (Figures 8F-8K; Supplemental Figures 18-22). Thirdly, orchiectomy potently augmented PD-1+ T-645 cells but not PD-1⁺ B-cells in the spleen, blood, and lymph nodes of mice administered Aldo-salt 646 (Figures 9A-9H and Supplemental Figures 24-26). Finally, immune checkpoint blockade with anti-647 PD-1 Ab, adoptive PD-1 deficient T-cell transfers, and genetic deletion of PD-1 reinstates or 648 exacerbates Aldo-salt- or HFD and Ang II-induced aortopathies, including elastin degradation, 649

vascular inflammation, TAA, and AAA in intact and orchiectomized mice (Figures 10–12). These findings align well with the established role of PD-1⁺ T-cells as an immune checkpoint implicated in various diseases, including giant cell arteritis, cancer, and atherosclerosis (28). However, it should be noted that these findings contradict a recent study where humanized PD-1 Ab mitigates rather than aggravates AAA in the CaCl₂ mouse model and aortic patch angioplasty rat model (42). The discrepancy between these studies may be attributed to differences in animal models, animal age, and anti-PD-1 Ab. Further studies are needed to investigate these possibilities.

657

PD-1 functions as an immune checkpoint, inhibiting T-cell activation via interaction with its ligand, 658 primarily PD-L1 (28). PD-1 is exclusively expressed in activated immune cells, most importantly in T 659 cells, whereas PD-L1 is broadly expressed in various cells, including antigen-presenting cells (i.e., 660 macrophages), cancer cells, and endothelial cells (28). It is well-documented that PD-1 is upregulated 661 by estrogen in Tregs, B-cells, macrophages, and dendritic cells (43). However, whether and rogen can 662 modulate PD-1 expression is largely unknown. As a result, the mechanism by which androgen 663 suppresses PD-1 expression is completely unknown. One of what we believe to be novel findings is 664 that we unrevealed a mechanism by which androgen suppresses PD-1 expression in the spleen. 665 Specifically, we demonstrated that AR bound to the PD-1 promoter via ARE, suppressing its 666 transcription, mRNA, and protein expression in the spleen (Figure 9 and Supplemental Figure 23). 667 668

Given that androgen exerts a pleiotropic effect on various organs and systems through both genomic and non-genomic mechanisms (37), it is conceivable that androgen aggravates Aldo-salt-induced aortic aneurysms through multiple mechanisms. Consistent with this notion, it has been shown that androgen exacerbates Ang II-induced AAA through Ang II-type-1A receptor, IL-1 α , and TGF β 1 (6, 16). The current study identified 65 signaling pathways downregulated and 19 signaling pathways upregulated by androgen in the aorta following Aldo-salt administration (Figure 6 and Supplemental Tables 1–4). While the role of these signaling pathways in Aldo-salt-induced aortic aneurysms

remains to be investigated, we demonstrate that IL-6, a pleiotropic cytokine, along with PD-1, is 676 implicated in Aldo-salt-induced aortic aneurysms (Figure 5 and Supplemental Figures 9 and 10). It 677 has been shown that IL-6 augments TCR-induced PD-1 expression via STAT3 and STAT4 in splenic 678 T-cells (44). However, it is unlikely that PD-1 regulates IL-6 expression in splenic T cells as genetic 679 deletion of PD-1 does not affect IL-6 expression in the spleen and serum of mice administered HFD 680 feeding and Ang II infusion (Supplemental Figure 27). Thus, further investigation is warranted to 681 define how PD-1 and IL-6 coordinate to mediate Aldo-salt-induced and androgen-mediated aortic 682 aneurysms. 683

684

The current studies have several limitations. Firstly, most experiments were conducted in mice 685 administered Aldo-salt, lacking a control group without Aldo-salt administration. Consequently, how 686 the Aldo/MR signaling coordinates with the androgen/AR signaling in regulating PD-1 expression in T 687 cells and aortic aneurysms remains unclear. Secondly, the current studies exclusively focused on the 688 TCR and PD-1 signaling pathways resulting from 180 and rogen-response genes in the aorta, 689 neglecting exploration of the potential role of metabolic pathways enriched from 150 and rogen-690 response genes in Aldo-salt-induced aortic aneurysms. Thirdly, LMT-28 may have potential off-691 targets relative to inhibiting IL-6. Addressing these limitations in future studies will contribute to a 692 more comprehensive understanding of the mechanisms by which androgen aggravates aortic 693 aneurysms. 694

695

Given that immune checkpoint inhibitors (i.e., anti-PD-1 Ab) have been successful in cancer treatment and have revolutionized the cancer research field (11), an increasing number of cancer patients have been subjected to immune checkpoint therapy (27). However, immune checkpoint therapy is associated with serious immune-related cardiovascular adverse events, including autoimmune myocarditis, pericarditis, and vasculitis (27). In alignment with these findings, the current studies suggest an increased risk of developing aortic aneurysms for patients undergoing

702	immune checkpoint inhibitor therapy. Indeed, a recent case report shows that a 57-year-old man
703	with lung adenocarcinoma treated with chemotherapy and immune checkpoint blockade
704	developed inflammatory TAA (45). Thus, cancer patients predisposed to the risk factors of aortic
705	aneurysms, such as being male, aging, and smoking, may have an increased likelihood of
706	developing aortic aneurysms during immune checkpoint therapy. As a precaution, these patients
707	should be advised to undergo an ultrasound screen for aortic aneurysms to increase the life-
708	saving potential of cancer immunotherapy.
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Methods

725 Detailed materials and methods are provided in Supplemental Material.

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727 Sex as a biological variable

Our study examined male and female animals, and sex-dimorphic effects were reported. Human aortic samples were obtained from male subjects.

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731 Statistical analysis

All data were expressed as mean ± SEM. To compare one parameter between the two groups, 732 normality tests were conducted. If data passed the normality test, a parametric, unpaired, and two-733 tailed t-test was employed. If data did not pass the normality test, a nonparametric, unpaired, and 734 two-tailed test was used. For multiple comparisons of two parameters among multiple groups, a two-735 way ANOVA was performed with correction for multiple comparisons by controlling the false 736 discovery rate. Similarly, for multiple comparisons of three parameters among multiple groups, a 737 three-way ANOVA was used with correction for multiple comparisons by controlling the false 738 discovery rate. The incidence of aortic aneurysms between the two groups was compared using a 739 two-sided Chi-Square test. The relationship between two quantitative variables was analyzed through 740 simple linear regression. Significant outliers, identified by the outlier calculator (GraphPad), were 741 excluded from the statistical analysis. All statistical analysis was carried out using Prism 9 software 742 (GraphPad). A P-value or adjusted P-value < 0.05 was considered significant unless specified 743 somewhere. A *P*-value of > 0.05 was considered not significant (ns). 744

745

746 Study approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. All procedures for using human aortic aneurysm specimens for the current study were approved by the Institutional Review Board of the University of Kentucky.

751 Data availability

752 RNA-seq data were deposited into the NCBI's Gene Expression Omnibus database under accession

number GSE255682. Values for all data points in graphs are reported in the Supporting Data Values

- file. Requests for materials should be directed to the corresponding authors and will be fulfilled upon
- completion of appropriate material transfer agreements.

- ...

776	Author contributions
777	X.M., S.L., Z.W., and K.J.: contributed to designing research studies, conducting experiments,
778	acquiring data, and analyzing data; T.M, A.S, and A. T. contributed to analyzing RNA-seq data; E.L.
779	and J.C. contributed to providing human aortic aneurysm specimens; M.G. and Z.G. contributed to
780	the conceptualization, supervision, writing, project administration, and funding acquisition.
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Figure 1. Sexual dimorphism in Aldo-salt-induced aortic aneurysms. (A and B) Maximal internal 966 diameters and growth rate of the suprarenal aortas were measured weekly in vivo using ultrasound in 10-967 month-old male (M) and female (F) C57BL/6J mice administered Aldo-salt. Time 0 represents measurements 968 one week before Aldo-salt administration (n = 4-10/group). (C) Mean arterial pressure (MAP) was measured 969 via tail cuff in mice one week before (basal) and three weeks after Aldo-salt administration (n = 9-10/group). 970 (D-G) Maximal external diameters of the ascending aorta (AscAo), aortic arch (ArchAo), descending aorta 971 (DesAo), and suprarenal aorta (SupAo) were measured ex vivo by microscopy four weeks after Aldo-salt 972 administration (n = 4-9/group). (H) Representative photographs of the aortas with or without aortic aneurvsms. 973 974 (I) Incidences of total aortic aneurysms (AA), abdominal aortic aneurysms (AAA), thoracic aortic aneurysms (TAA), and aortic rupture (mice with aortic aneurysms / mice with and without aortic aneurysms). Data were 975 expressed as mean ± SEM and analyzed by thee-way ANOVA analysis (A), two-tailed unpaired t-test (B), two-976 way ANOVA with multiple comparison tests (C-G), and two-sided Chi-square test (I). *, P < 0.05; **, P < 0.01; 977 ***, *P* < 0.001; ****, *P* < 0.0001; ns, not significant. 978

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Figure 2. Orchiectomy protects mice from Aldo-salt-induced aortic dilation, progression, and aneurysm formation. (A) Seminal vesicle weight (SVW) to BW ratio was determined in orchiectomies (orx) and sham-operated 10-month-old male C57BL/6J mice four weeks after Aldo-salt administration (n = 13-15/group). (B and C) Maximal internal diameters and growth rate of the suprarenal aorta (n = 13-15/group). (D) Maximal external diameters of the AscAo, ArchAo, DesAo, and SupAo (n = 12-15/group). (E) Incidences of total AA, AAA, TAA, and aortic rupture. (F) Representative photographs of the aortas with and without aortic aneurysms. (G) Severity of aortic aneurysms (Supplemental Figure 3). Data were expressed as mean ± SEM and analyzed by two-tailed unpaired t-test (A, C, D), two-way ANOVA with multiple comparison tests (B), and two-sided Chi-square test (E). **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant.





Figure 3. Exogenous dihydrotestosterone administration to orchiectomized male mice restores Aldo-salt-induced aortic aneurysms. (A) SVW to BW ratio was determined in 10-month-old orchiectomized (orx) C57BL/6J mice four weeks after Aldo-salt with and without dihydrotestosterone (DHT) pellet implantation (n = 9-11/group). (**B** and **C**) Maximal intraluminal diameters and growth rate of the suprarenal aorta (n = 11/group). (D) Maximal external diameters of the AscAo, ArchAo, DesAo, and SupAo (n = 11/group). (E) Incidences of total AA, AAA, TAA, and aortic rupture. (F) Representative photographs of the aortas with and without aortic aneurysms. (G) Severity of aortic aneurysms. (H) Restoration rates of aortic aneurysms = mice with orx and DHT and aortic aneurysms / mice with sham-operation and with and without aortic aneurysms. Data were expressed as mean ± SEM and analyzed by two-tailed unpaired t-test (A, C, and D), two-way ANOVA with multiple comparison tests (B), and two-sided Chi-square test (E). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant.



Figure 4. Downregulation of AR by ASC-J9 in mice inhibits Aldo-salt-induced aortic aneurysm. (A) Representative immunostaining of AR in the suprarenal aortas from 10-month-old male C57BL/6J mice four weeks after Aldo-salt with and without ASC-J9 administration (n = 3/group). L, lumen. M, media. A, adventitia. (**B** and **C**) Maximal internal diameters and growth rate of the suprarenal aorta (n = 11/group). (**D**) Maximal external diameters of the AscAo, ArchAo, DesAo, and SupAo (n = 11/group). (E) Incidences of total AA, AAA, TAA, and aortic rupture. (F) Representative photographs of the aortas with and without aortic aneurysms. (G) Severity of aortic aneurysms. Data were expressed as mean ± SEM and analyzed by two-way ANOVA with multiple comparison tests (**B**), two-tailed unpaired *t*-test (**C** and **D**), and two-sided Chi-square test (**E**). *, P < P0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant.



Figure 5. Inhibition of IL-6 signaling by LMT-28 ameliorates Aldo-salt-induced aortic aneurysms. (A) Representative immunostaining of STAT3 phosphorylation at Tyr705 in the suprarenal aorta from 10-month-old male C57BL/6J mice four weeks after Aldo-salt with LTM-28 or vehicle controls (Ctrl; n = 3/group). (B and C) Maximal internal diameters and growth rate of the suprarenal aortas (n = 12-18/group). (D) Maximal external diameters of the AscAo, ArchAo, DesAo, and SupAo (n = 12-17/group). (E) Incidences of total AA, AAA, TAA, and aortic rupture. (F) Representative photographs of the aortas with and without aortic aneurysms. (G) Severity of aortic aneurysms. Data were expressed as mean ± SEM and analyzed by two-way ANOVA with multiple comparison tests (B), two-tailed unpaired t-test (C and D), and two-sided Chi-square test (E). *, p < 10.05; **, p < 0.01; ****, P < 0.0001; ns, not significant.





Figure 6. Profiling of aortic transcriptomes reveals T cell receptor signaling as a link between AR and 1135 1136 Aldo-salt-induced aortic aneurysms. (A) Total numbers of genes whose mRNAs were detected by RNA-seq and analyzed by DESeq2 to be differentially abundant among the whole aortas from 10-month-old C57BL/6J 1137 mice with and without orx followed by one-week Aldo-salt with and without DHT pellet implantation (n 1138 1139 =5/group). (B and C) Volcano plot illustration of the number of genes whose mRNAs were analyzed by 1140 DESeq2 to be statistically significant (y-axis) vs. effect size (fold change, x-axis) in the experiment. (D and G) Venn diagram identified 180 genes whose mRNAs were upregulated by orchiectomy but downregulated by 1141 DHT and 150 genes whose mRNAs were downregulated by orchiectomy but upregulated by DHT. 1142 respectively. (E and H) Heatmap of the 180 genes and 150 genes regulated by androgen (F and I) Pathway 1143 1144 enrichment analysis using Enrichr shows the top 20 pathways among mRNAs that were upregulated by 1145 orchiectomy but downregulated by DHT and the 19 pathways among the mRNAs that were downregulated by orchiectomy but upregulated by DHT, respectively. 1146 1147

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Figure 8. Splenectomy enriches PD-1 positive T- and B-cells in the aorta and mitigates Aldo-salt-1207 1208 induced aortic aneurysms. (A) MAP was measured by tail cuff in 11-13-month-old male C57BL/6J mice with 1209 splenectomy (splx) or sham operation one week before (basal) and three weeks after Aldo-salt administration 1210 (n = 8-10/group). (B and C) Maximal internal diameters and growth rate of the suprarenal aortas (n = 8-1211 10/group). (D) Maximal external diameters of the AscAo, ArchAo, DesAo, and SupAo (n = 5-9/group). (E) Incidences of total AA, AAA, TAA, and aortic rupture. (F-K) Representative pseudocolor plots and quantitative 1212 data of the flow cytometry analysis of the total numbers(#) and percentages (%) of PD-1⁺ T cells 1213 (CD45⁺CD3⁺PD-1⁺; % of total T cells) and PD-1⁺ B cells (CD45⁺CD19⁺PD-1⁺; % of total B cells) in the whole 1214 1215 aortas in mice with splx or sham-operation four weeks after Aldo-salt administration (n = 5-7/group). Data were 1216 expressed as mean ± SEM and analyzed by two-way ANOVA with multiple comparison tests (A and B), two-

tailed unpaired *t*-test (**C**, **D**, **G**, **H**, **J**, and **K**), and two-sided Chi-square test (**E**). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ***, P < 0.001; state the square test (**E**). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ***, P < 0.0001; state test square test (**E**). *, P < 0.05; **, P < 0.001; ***, P < 0.0001; state test square test (**E**). *, P < 0.05; **, P < 0.001; ***, P < 0.0001; state test square test (**E**). *, P < 0.05; **, P < 0.001; ***, P < 0.001; ***, P < 0.0001; state test square test (**E**). *, P < 0.0001; state test square test (**E**). *, P < 0.005; **, P < 0.001; ***, P < 0.0001; state test square test (**E**). *, P < 0.005; **, P < 0.001; ***, P < 0.0001; state test square test square test (**E**). *, P < 0.005; **, P < 0.001; ***, P < 0.0001; state test square test square

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1241 Figure 9. Androgen suppresses PD-1 mRNA and protein expression in the spleen in mice administered Aldo-salt. (A-D) Representative immunostainings and quantitative data of PD-1 protein expression in the 1242 spleens from 10-month-old male C57BL/6J mice with orx or sham-operation ten days after Aldo-salt 1243 1244 administration (n =3-4/group) or orchiectomized mice four weeks after Aldo-salt with and without DHT pellet 1245 implantation (n = 3/group). The percentage (%) of areas fraction = (the PD-1 positive area / the area of fields of 1246 view) x 100%. The data were calculated from five fields of view randomly photographed per splenic section per mouse. (E-H) Representative Western blots and quantitative data of PD-1, CD3ε, CD19, and GAPDH protein 1247 expression in the spleens from 10-month-old male mice with orx or sham operation ten days after Aldo-salt 1248 1249 administration (n = 5/group). (I) Pdcd1 (the gene codes PD-1) mRNA expression in the spleens from 10-1250 month-old male mice with orx or sham operation ten days after Aldo-salt administration (n = 13/group). (J) A 1251 schematic diagram of the 12 androgen response elements (ARE) in the 5 kb mouse PD-1 promoter. TSS, 1252 transcription start site. ATG, translation start codon. ChIP-F, ChIP-PCR forward primers. ChIP-R, ChIP-PCR reverse primers. (K-M) Representative and quantitative ChIP-PCR with the control Ab, anti-AR Ab #1, and 1253 1254 anti-AR Ab #2 in the spleen (n = 3/group). NTC, no template control. (N and O) Expression of AR in HEK293 1255 cells suppressed the PD-1 promoter activity (n = 4/group). Data were expressed as mean ± SEM and analyzed 1256 by two-tailed unpaired t-test (**B**, **D**, **F**-**H**, **I**, **L**, and **M**) and one-way ANOVA with multiple comparison tests (**O**). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant. 1257



1293 Incidences of total AA, AAA, TAA, and aortic rupture. (E-G) Representative and quantitative Verhoeff-Van 1294 Gieson staining of elastin in longitudinal sections of the thoracic aortas and cross-sections of the abdominal 1295 aortas in orchiectomized mice with anti-PD-1 or Ctrl Ab eight weeks after Aldo-salt administration (n = 1296 4/group). The arrow indicates elastic breakage. (H) Representative immunostaining of T-cells, B-cells, 1297 macrophages, and neutrophils in the thoracic aortas in orchiectomized mice eight weeks after Aldo-salt with 1298 anti-PD-1 or Ctrl Ab administration (n = 3). (I) MAP of orchiectomized mice one week before (basal) and three 1299 and seven weeks after Aldo-salt with anti-PD-1 or Ctrl Ab administration (n = 8-11/group). (J and K) Representative immunostainings and quantitative data of PD-1 protein expression in the human aortas with 1300 1301 and without a rtic aneurysms (n = 3-6/group). Data were expressed as mean \pm SEM and analyzed by two-way ANOVA with multiple comparison tests (A, B, and I), two-tailed unpaired t-test (C, F, G, and K), and two-sided 1302 Chi-square test (D). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ****, P < 0.0001; ns, not significant. 1303





orchiectomized mice. (A and B) Maximal internal diameters of the suprarenal aorta and aortic root in 9-10-1325 month-old orchiectomized male C57BL/6J mice with adoptive PD-1-KO and WT T-cell transfer via retro-orbital 1326 1327 sinus injection two days before and eight and eighteen days after Aldo-salt administration (n = 9-10/group). 1328 PD-1 KO and WT T cells were isolated from the spleens of 4-month-old male PD-1 KO and WT C57BL6J mice via anti-CD90.2 magnetic beads. (C) Aortic weight to BW ratio (n = 7-9/group). (D) Maximal external diameters 1329 of the RootAo, AscAo, ArchAo, DesAo, and SupAo (n = 9-10/group), (E) Incidences of total AA, AAA, TAA, and 1330 aortic rupture. (F and G) Representative and quantitative elastin stain in the longitudinal sections of the 1331 1332 thoracic aortas (n = 5-6/group). The arrow indicates elastic breakage. (H) Representative immunostaining of T-1333 cells, B-cells, macrophages (M ϕ), and neutrophils (N ϕ) in the thoracic aortas (n = 3). (I) MAP was measured 1334 by tail cuff one week before (basal) and three weeks after Aldo-salt administration (n = 8-10/group). (J) 1335 Correlation analysis of the internal diameter of the aortic root and MAP three weeks after Aldo-salt administration (n = 15/group). (K) MAP in mice with (+) and without (-) Aldo-salt-induced aortic aneurysms (n = 1336 3-9/group). Data were expressed as mean ± SEM and analyzed by two-way ANOVA with multiple comparison 1337 tests (A, B, and I), two-tailed unpaired t-test (C, D, and G), two-sided Chi-square test (E), simple linear 1338 regression analysis (J), and one-way ANOVA for multiple comparison tests (K). *, p<0.05; **, p<0.01; ***, 1339 1340 *p*<0.001; ****, *p*<0.0001; *ns*, *not significant*.



1362 Figure 12. Genetic deletion of PD-1 exacerbates high-fat diet and angiotensin II-induced aortopathy. (A 1363 and B) Maximal internal diameters of the suprarenal aorta and aortic root in 2-month-old male PD-1-KO and 1364 WT C57BL/6J mice with eight-week high-fat diet (HFD) feeding and four-week Ang II infusion (n = 15/group). 1365 (C) A ortic weight to BW ratio (n = 14/group). (D) Maximal external diameters of the AscAo. ArchAo. DesAo. 1366 and SupAo (n = 14/group). (E) Incidences of total AA, AAA, TAA, and aortic rupture. (F and G) Representative 1367 and quantitative Verhoeff-Van Gieson elastin staining in the abdominal aortas. The arrow indicates elastic 1368 breakage (n = 6-7/group). (H) Representative immunostaining of T-cells, B-cells, macrophages, and neutrophils in the abdominal aortas (n = 3). (I) MAP was measured by tail cuff one week before (basal) and 1369 1370 three weeks after HFD and Ang II administration (n =14-15/group). (J) Correlation analysis of the internal 1371 diameter of the suprarenal aorta and MAP in PD-1 KO and WT mice three weeks after HFD and Ang II 1372 administration (n = 28/group). (K) MAP with (+) and without (-) aortic aneurysms (n =2-12/group). Data were 1373 expressed as mean ± SEM and analyzed by two-way ANOVA with multiple comparison tests (A, B, I, and K), 1374 two-tailed unpaired t-test (C, D, and G), two-sided Chi-square test (E), and simple linear regression analysis (J). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant. 1375