ONLINE DATA SUPPLEMENT

TITLE: ROLE OF AXL IN EARLY KIDNEY INFLAMMATION AND PROGRESSION OF SALT-DEPENDENT HYPERTENSION

Short title: Dual Role of Axl in Hypertension

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Materials and Methods

Animals

Axl knockout (Axl-/-) mice were used from our colony (express CD45.2 alloantigen in bone marrow (BM) cells). B6.SJL$^{Ptprc_aPep3_b/\ BoyJ}$ mice (express CD45.1 alloantigen in BM cells) were purchased from Jackson Laboratory, bred in house and used as wild types (Axl+/+)[1]. All experiments were conducted in male mice (6weeks old) and were approved by the University of Rochester Animal Care Committee in accordance with the Use of Laboratory Animals guidelines of the National Institutes of Health and American Heart Association (UCAR #2003-206R).

Bone marrow transplant

Bone marrow transplant (BMT) experiments between Axl+/+ and Axl-/- were done as we recently described[1]. Donor-derived BM cells (Axl+/+ or Axl-/-) were injected (6×10^6) into recipient mice after irradiation. Chimeric mice were allowed to recover for six weeks before experimentation.

A model of DOCA-salt hypertension

We used a previously described DOCA-salt mouse model of hypertension[2]. Briefly, upon successful engraftment of the donor BM (>90%) Axl chimeric mice were anesthetized with a cocktail of ketamine and xylazine (130 and 9 mg/kg, i.p.). An incision was made to expose the left kidney, which was ligated and removed. At the time of surgery a 75mg DOCA pellet (60 days release, Innovative Research of America, USA) was placed subcutaneously in a lateral area on the back of chimeras. Subsequently, animals were injected with analgesic Flunixin meglumine (120mg/kg, i.p.) and given regular chow and a 1% NaCl solution as a drinking water. Systolic BP was measured weekly for 6week time-course using a non-invasive tail-cuff method plethysmography (Visitech System, USA). Kidney function in Axl chimeras was measured after 1week after induction of DOCA-salt. Total protein (Bradford assay) and albumin (ELISA kit, Exocell, USA) were measured in urine samples from Axl chimeras that were collected in metabolic cages for 24hrs.

Isolation of immune cells from peripheral tissues

Peripheral blood was collected into heparinized tubes from the mandibular vein and incubated with ACK lysis buffer for 5min to lyse the erythrocytes. Splenocytes were obtained by tearing the spleen in a 70μm cell strainer. Kidneys from Axl chimeras were harvested, placed in the 100μm cell strainer (BD Falcon) and mashed in RPMI media supplemented with 10% FBS (Hyclone). The resulted homogenates were centrifuged (1,300rpm, 4°C, 6min) and the cell pellet was re-suspended in RPMI media with 1% FBS, 100μg collagenase (Worthington) and incubated for 30min at 37°C in a fully humidified atmosphere with 95% O_2/5% CO_2. The digested solution was again centrifuged and the pellet with cells was gently re-constituted in 1mL PBS with 3% FBS.

Flow cytometry

The engraftment of donor BM cells was confirmed by staining of the blood samples after recovery period (6wk) with a cocktail of CD45.1-FITC and CD45.2-PE antibodies (1:500, eBiosciences) and analyzed using 4-color BD Accuri C6 flow cytometer (BD Biosciences). Five major subsets of immune cells were detected using 12-color LSRII flow cytometer (BD Bioscience) and shown in the Figure 3A. Isolated cells from spleen or kidney of Axl chimeras were first incubated with live/dead stain (1:500, Invitrogen). Following this step, the cells were washed with FACS buffer (1,200rpm, 4°C, 6min) and incubated with FC block (1:10, BD Bioscience) at room temperature for 30min. Then cells were stained with a cocktail containing

CD45.1-FITC (1:1000, eBioscience), CD45.2-PE (1:500, eBioscience), CD3-APC (1:200, eBioscience), CD19-PE-CYC (1:500, eBioscience), CD11b-PE-CY5.5 (1:500, BD Bioscience), CD11c-PE-TXR (1:500, Invitrogen) and NK1.1-APC-CY7 (1:100, Biolegend) antibodies at 4°C for 30min. Cells were washed and re-suspended in FACS buffer. Compensation controls were prepared using anti-rat/hamster IgGκ beads for single stained controls and live/dead beads. Flow cytometry analyses were performed using FlowJo software version 7.6.3.

Quantitative RT-PCR

In a separate set of Axl chimeras, left kidneys were harvested during surgery and right kidney after 1 week of DOCA-salt treatment and were immediately frozen in liquid nitrogen (Ref). RNA was isolated and amplified to cDNA with a NuGEN (San Carlos, CA) Ovation RNA amplification system V2. The cDNA samples were assayed for Axl, Gas6, and GAPDH expression. Inflammatory cytokines and receptors were assessed using a mouse PCR Array (PAMM-011; Qiagen). The list of 84 studied genes is shown in Table S1. Bioinformatics analyses of gene expression profiles from kidneys across Axl chimeras were performed as previously described[1].

Morphometry and immunohistochemistry

Axl chimeras were perfusion fixed with 10% paraformaldehyde and histology was performed as described[2]. Apoptotic cells were detected in Axl chimeras with ApopTag peroxidase In situ Apoptosis Detection Kit (Chemicon Int) as reported[2]. Oxidative stress was assessed in the kidneys using OxyIHC Oxidative Stress Detection Kit (Millipore). We used MCID image software (MCID Elite 6.0, Imaging Research) for morphometry and ROS analyses as shown previously[3].

Statistical analysis

Results are shown as means±SEM. Statistical differences were evaluated using JMP5.1.2 software. Differences between two groups were analyzed by pooled Student's *t* test. For more than 3 experimental groups we utilized one-way ANOVA or Wilcoxon test followed by post hoc comparisons. The level of $p<0.05$ was regarded as significant.

References

1. Gerloff J, Korshunov VA. Immune modulation of vascular resident cells by axl orchestrates carotid intima-media thickening. *The American journal of pathology*. 2012;180:2134-2143.
2. Korshunov VA, Daul M, Massett MP, Berk BC. Axl mediates vascular remodeling induced by deoxycorticosterone acetate salt hypertension. *Hypertension*. 2007;50:1057-1062.
3. Korshunov VA, Nikonenko TA, Tkachuk VA, Brooks A, Berk BC. Interleukin-18 and macrophage migration inhibitory factor are associated with increased carotid intima-media thickening. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:295-300.

Table S1. The list of the genes on mouse inflammatory cytokines & receptors PCR array (PAMM-011)

Gene Symbol	Description	Gene Symbol	Description
Abcf1	ATP-binding cassette, sub-family F (GCN20), member 1	Cxcr3	Chemokine (C-X-C motif) receptor 3
Bcl6	B-cell leukemia/lymphoma 6	Ccr10	Chemokine (C-C motif) receptor 10
Cxcr5	Chemochine (C-X-C motif) receptor 5	Ifng	Interferon gamma
C3	Complement component 3	Il10	Interleukin 10
Casp1	Caspase 1	Il10ra	Interleukin 10 receptor, alpha
Ccl1	Chemokine (C-C motif) ligand 1	Il10rb	Interleukin 10 receptor, beta
Ccl11	Chemokine (C-C motif) ligand 11	Il11	Interleukin 11
Ccl12	Chemokine (C-C motif) ligand 12	Il13	Interleukin 13
Ccl17	Chemokine (C-C motif) ligand 17	Il13ra1	Interleukin 13 receptor, alpha 1
Ccl19	Chemokine (C-C motif) ligand 19	Il15	Interleukin 15
Ccl2	Chemokine (C-C motif) ligand 2	Il16	Interleukin 16
Ccl20	Chemokine (C-C motif) ligand 20	Il17b	Interleukin 17B
Ccl22	Chemokine (C-C motif) ligand 22	Il18	Interleukin 18
Ccl24	Chemokine (C-C motif) ligand 24	Il1a	Interleukin 1 alpha
Ccl25	Chemokine (C-C motif) ligand 25	Il1b	Interleukin 1 beta
Ccl3	Chemokine (C-C motif) ligand 3	Il1f6	Interleukin 1 family, member 6
Ccl4	Chemokine (C-C motif) ligand 4	Il1f8	Interleukin 1 family, member 8
Ccl5	Chemokine (C-C motif) ligand 5	Il1r1	Interleukin 1 receptor, type I
Ccl6	Chemokine (C-C motif) ligand 6	Il1r2	Interleukin 1 receptor, type II
Ccl7	Chemokine (C-C motif) ligand 7	Il20	Interleukin 20
Ccl8	Chemokine (C-C motif) ligand 8	Il2rb	Interleukin 2 receptor, beta chain
Ccl9	Chemokine (C-C motif) ligand 9	Il2rg	Interleukin 2 receptor, gamma chain
Ccr1	Chemokine (C-C motif) receptor 1	Il3	Interleukin 3
Ccr2	Chemokine (C-C motif) receptor 2	Il4	Interleukin 4
Ccr3	Chemokine (C-C motif) receptor 3	Il5ra	Interleukin 5 receptor, alpha
Ccr4	Chemokine (C-C motif) receptor 4	Il6ra	Interleukin 6 receptor, alpha
Ccr5	Chemokine (C-C motif) receptor 5	Il6st	Interleukin 6 signal transducer
Ccr6	Chemokine (C-C motif) receptor 6	Il8rb	Interleukin 8 receptor, beta
Ccr7	Chemokine (C-C motif) receptor 7	Itgam	Integrin alpha M
Ccr8	Chemokine (C-C motif) receptor 8	Itgb2	Integrin beta 2
Ccr9	Chemokine (C-C motif) receptor 9	Lta	Lymphotoxin A
Crp	C-reactive protein, pentraxin-related	Ltb	Lymphotoxin B
Cx3cl1	Chemokine (C-X3-C motif) ligand 1	Mif	Macrophage migration inhibitory factor
Cxcl1	Chemokine (C-X-C motif) ligand 1	Scye1	Small inducible cytokine subfamily E, member 1
Cxcl10	Chemokine (C-X-C motif) ligand 10	Spp1	Secreted phosphoprotein 1
Cxcl11	Chemokine (C-X-C motif) ligand 11	Tgfb1	Transforming growth factor, beta 1
Cxcl12	Chemokine (C-X-C motif) ligand 12	Tnf	Tumor necrosis factor
Cxcl13	Chemokine (C-X-C motif) ligand 13	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a
Cxcl15	Chemokine (C-X-C motif) ligand 15	Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b
Pf4	Platelet factor 4	Cd40lg	CD40 ligand
Cxcl5	Chemokine (C-X-C motif) ligand 5	Tollip	Toll interacting protein
Cxcl9	Chemokine (C-X-C motif) ligand 9	Xcr1	Chemokine (C motif) receptor 1

Table S2. Up-regulated immune pathways in Axl-/- →Axl+/+ compared to Axl-/- →Axl-/- or Axl+/+ →Axl+/+ in kidneys after 1week of DOCA-salt

Pathway ID	Pathway Name	Gene Symbols
4351	Chemokine signaling pathway	Ccl22; Ccl24
604	Cytokine-cytokine receptor interaction	Ccl22; Ccl24; l17b; Lta; Ltb
10166	Heterotrimeric GPCR signaling pathway (through G alpha i and pertussis toxin)	Lta
9890	Heterotrimeric GPCR signaling pathway (through G alpha q, PLC beta and ERK cascade)	Lta
9827	Heterotrimeric GPCR signaling pathway (through G alpha s ACs Epac BRaf and ERKcascade)	Lta
9754	Heterotrimeric GPCR signaling pathway (through G alpha s ACs PKA BRaf and ERKcascade	Lta
9794	Heterotrimeric GTP-binding protein coupled receptor signaling pathway (through G alpha i, adenylate cyclase and cAMP)	Lta
9789	Heterotrimeric GTP-binding protein coupled receptor signaling pathway (through G alpha s, cholera toxin, adenylate cyclase and cAMP)	Lta
10320	JAK-STAT pathway and regulation pathway	Lta
637	Type I diabetes mellitus	Lta

Table S3. Down-regulated immune pathways in Axl-/- →Axl+/+ compared to Axl-/- →Axl-/- or Axl+/+ →Axl+/+ kidneys after 1week of DOCA-salt

Pathway ID	Pathway Name	Gene Symbols
4351	Chemokine signaling pathway	Ccl8; Ccl4; Cxcl9; Ccl3; Il3; Cxcl9; Cxcr5; Ccl3
604	Cytokine-cytokine receptor interaction	Ccl8; Il2rb; Ifng; Cxcr5; Il3; Cd40lg
699	Toll-like receptor signaling pathway	Ccl4; Cxcl9; Ccl3
10401	Chagas disease (American trypanosomiasis)	C3; Ccl3; Ifng
759	Apoptosis	Il3
2791	Asthma	Il3; Cd40lg
10166	Heterotrimeric GPCR signaling pathway (through G alpha i and pertussis toxin)	Il3; Ifng; Cd40lg;
9890	Heterotrimeric GPCR signaling pathway (through G alpha q, PLC beta and ERK cascade)	Il3; Ifng; Cd40lg
9827	Heterotrimeric GPCR signaling pathway (through G alpha s ACs Epac BRaf and ERKcascade)	Il3; Ifng; Il3; Cd40lg
9754	Heterotrimeric GPCR signaling pathway (through G alpha s ACs PKA BRaf and ERKcascade)(canonical)	Il3; Ifng; Il3; Cd40lg
9794	Heterotrimeric GTP-binding protein coupled receptor signaling pathway (through G alpha i, adenylate cyclase and cAMP)	Il3; Ifng; Cd40lg
9789	Heterotrimeric GTP-binding protein coupled receptor signaling pathway (through G alpha s, cholera toxin, adenylate cyclase and cAMP)	Il3; Ifng; Il3; Cd40lg
9841	IL-3 signaling pathway (JAK1 JAK2 STAT5) (IL-3 signaling (JAK1 JAK2 STAT5))	Il3
10320	JAK-STAT pathway and regulation pathway	Il3; Ifng; Il2rb
10402	African trypanosomiasis	Ifng
2794	Allograft rejection	Ifng; Cd40lg
692	T cell receptor signaling pathway	Ifng; Cd40lg
2816	Autoimmune thyroid disease	Cd40lg
8107	Intestinal immune network for IgA production	Cd40lg
2817	Primary immunodeficiency	Cd40lg
8115	Viral myocarditis	Cd40lg

Table S4. Down-regulated immune pathways that were specific to Axl-/- →Axl+/+ in kidneys after 1week of DOCA-salt

Pathway ID	Pathway Name	Gene Symbols
10360	Leishmaniasis	C3; Ifng
708	Fc epsilon RI signaling pathway	Il3; Il3
682	Hematopoietic cell lineage	Il3
10368	Amoebiasis	Ifng
687	Antigen processing and presentation	Ifng
2802	Graft-versus-host disease	Ifng
9892	IFN gamma signaling pathway (JAK1 JAK2 STAT1) (IFN gamma signaling (JAK1 JAK2 STAT1))	Ifng
716	Natural killer cell mediated cytotoxicity	Ifng
10372	Osteoclast differentiation	Ifng
630	Proteasome	Ifng
655	Regulation of autophagy	Ifng
2813	Systemic lupus erythematosus	Ifng; Cd40lg
633	TGF-beta signaling pathway	Ifng
759	Apoptosis	Il3

Table S5. Morphometry analyses of arteries from Axl chimeras after 6weeks of DOCA-salt

Vessel area, x10^3 μm^2	**Axl+/+ →Axl+/+, n=5**	**Axl-/- →Axl-/-, n=6**	**Axl-/- →Axl+/+, n=6**	**Axl+/+ →Axl-/-, n=6**
			Mesenteric artery	
Lumen	11.8 ± 2.1	13.6 ± 1.9	16.3 ± 1.9	15.3 ± 2.1
Media	6.9 ± 0.8	5.7 ± 0.8	6.0 ± 0.8	7.9 ± 0.8
Adventitia	6.4 ± 0.8	5.2 ± 0.7	6.1 ± 0.7	7.9 ± 0.7
			Thoracic aorta	
Lumen	282 ± 38	195 ± 35	201 ± 35	160 ± 35
Media	138 ± 11	144 ± 11	104 ± 11 †,‡	128 ± 11
Adventitia	77 ± 7	62 ± 6	59 ± 6	67 ± 6

Values are means±SEM. †, $p<0.05$ vs. Axl+/+ →Axl+/+. ‡, $p<0.05$ vs. Axl-/- →Axl-/-. n, Number per group.

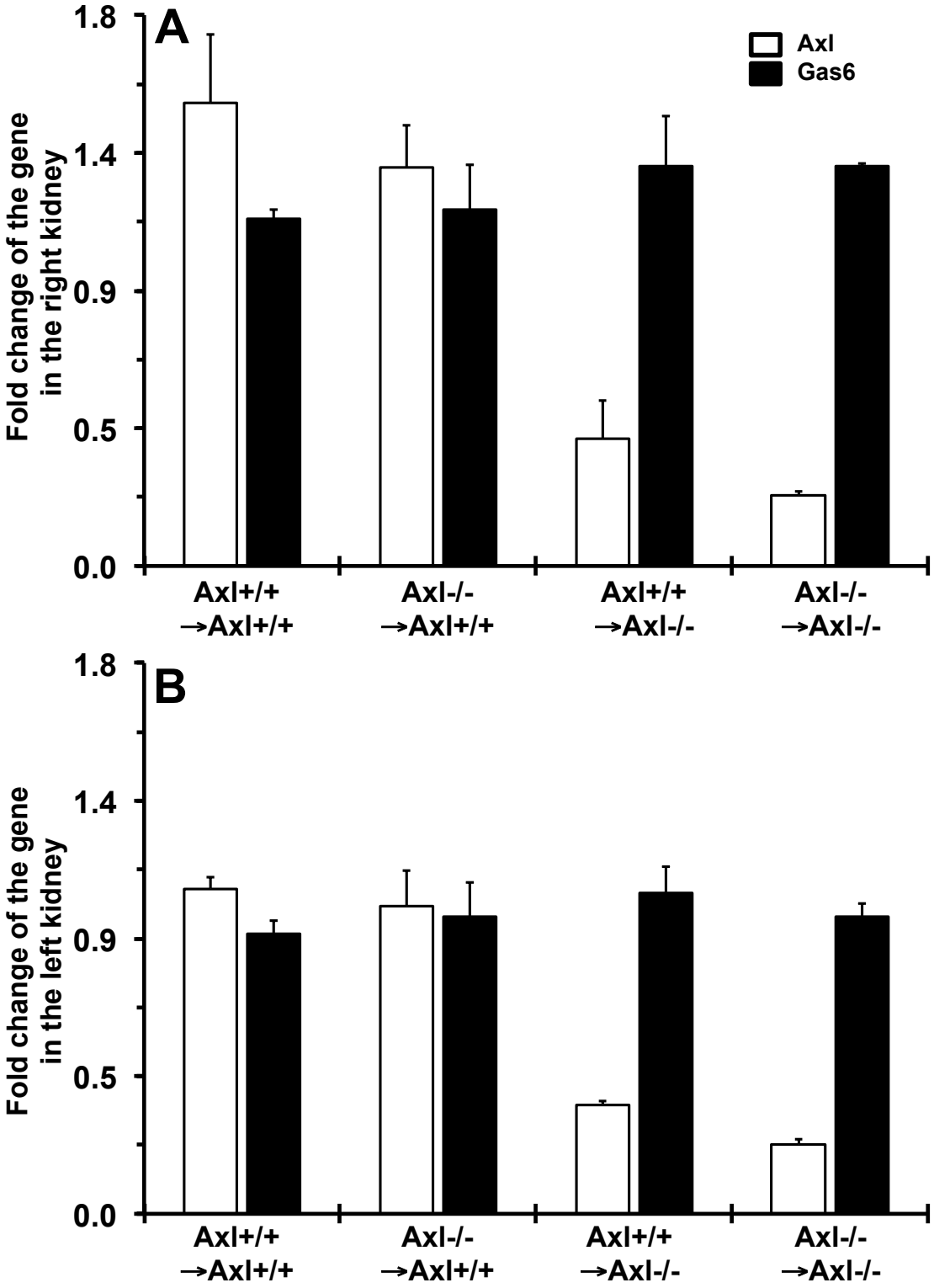

Figure S1. Axl and Gas6 gene expression in the kidneys from Axl chimeras. **A.** Right kidneys from Axl chimeras after 1week of DOCA-salt. **B.** Left kidneys from Axl chimeras collected at the time of nephrectomy. Open bars represent Axl expression. Black bars - Gas6 expression. We assayed two-three kidneys from each experimental group.

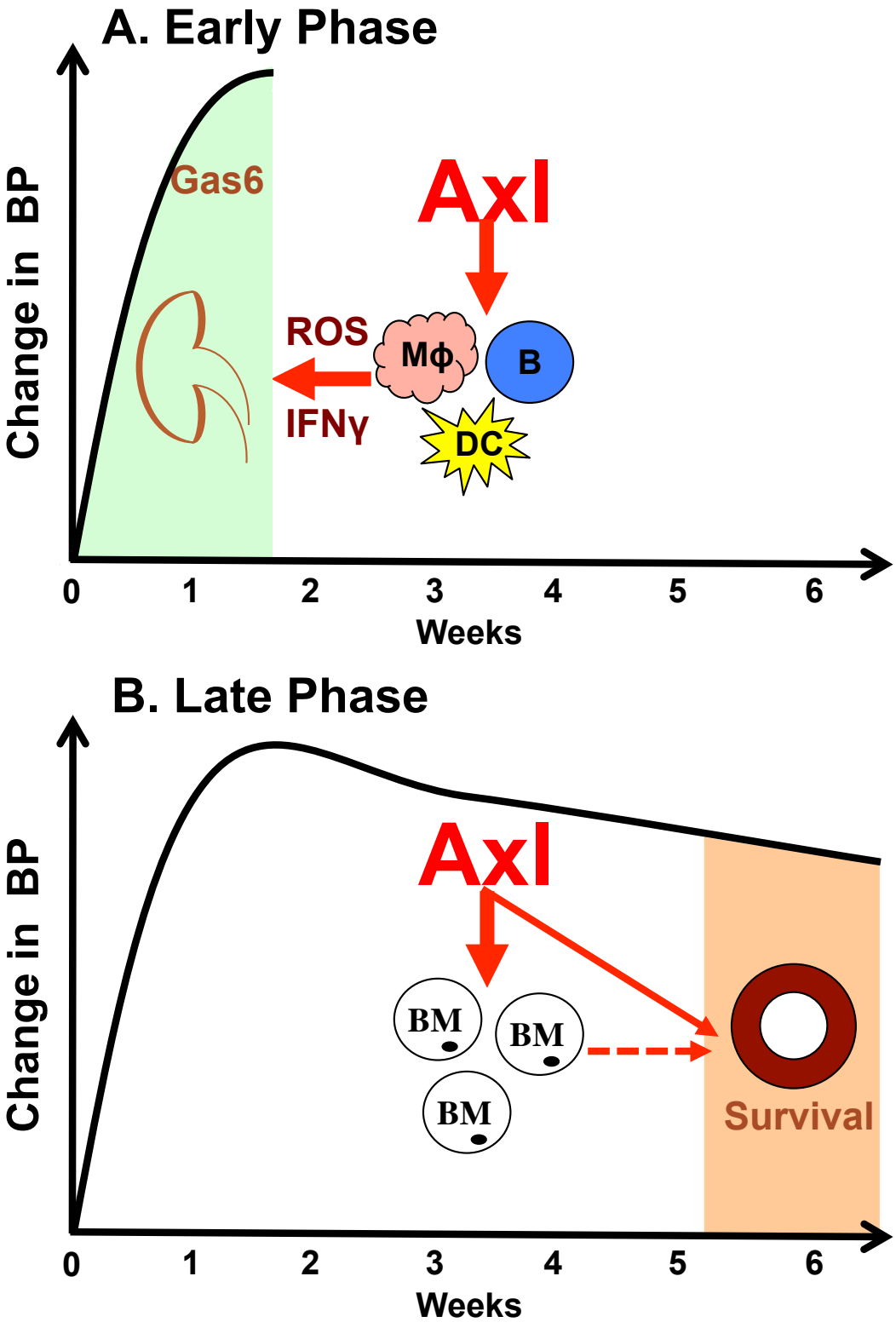

Figure S2. Proposed model on dual roles of Axl in progression of salt-dependent hypertension. Black line show changes in blood pressure (BP) . X-axis shows time in weeks. Y-axis changes in BP. **A.** During early phase-light green color. **B.** During late phase-light brown color. Mϕ, macrophages -pink; DC, Dendritic cells-yellow color; B, B lymphocytes-blue color; BM, Bone marrow-derived cells -white; ROS, reactive oxygen species; IFNγ, Interferone gamma.