Online Materials and Methods

Animals

Male Axl wild-type ($Axl^{+/+}$) and Axl knockout ($Axl^{-/-}$) mice were bred in house and genotyping was done as before[1]. Breeding pairs of B6.129S7-$Rag1^{tm1Mom}$/J ($Rag1^{-/-}$) and B6.SJL^{PtprcaPep3b}/BoyJ mice were purchased from the Jackson Laboratory. We confirmed presence of Axl expression by genotyping[2] of B6.SJL^{PtprcaPep3b}/BoyJ as well as in $Rag1^{-/-}$ (not shown). Mice were housed at 12 hours light and 12 hours dark cycle (lights on from 6 A.M. to 6 P.M.) with free access to chow and water. We conducted our studies by guidelines from the National Institutes of Health and the American Heart Association for the Use of Laboratory Animals. All experiments were approved by the University of Rochester Animal Care Committee.

Hematology

Peripheral blood was obtained via retro-orbital bleeding (0.2-0.3 mL) from mice, which were lightly anesthetized with isoflurane as we recently reported in inbred mouse strains[3]. We measured hematological parameters and peripheral blood cells count (17 parameters in total) using a fully automated 5-part differential cell counter (VetScan HM5; Abaxis). The number of circulating eosinophils and basophils were under detection level in both Axl genotypes tested and were not reported. We also noticed a relatively lower numbers for neutrophils in our experiments (Table II). The blood neutrophils ($CD11b^{+}$:$Ly6G^{High}$/$Ly6C^{low}$) and red blood cell ($Ter117^{+}$) numbers measured by flow cytometry method were significantly higher than complete blood count (CBC) estimates but similar between Axl genotypes (not shown). Finally, our CBC results on higher percentages of neutrophils ($Axl^{+/+}$, 4.3%; $Axl^{-/-}$, 3.8%) vs. monocytes ($Axl^{+/+}$, 2.6%; $Axl^{-/-}$, 2.2%) are in accordance to previously published study in 16 inbred mouse strains using similar CBC method[4].

Isolation of immune cells from peripheral tissues

Blood collection for flow cytometry analyses was done by the submandibular bleeding method without anesthesia. The ACK erythrocyte-lysing buffer (GIBCO) was used to lyse red blood cells. Suspension of the lymphocytes was obtained by tearing splenocytes and lymph nodes in a 70 μm cell strainer (GIBCO). The digested solution was again centrifuged and the pellet with cells was gently re-constituted in 1 mL of phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS). Isolation of lymphocytes for adoptive transfer experiments was done by using $CD4^{+}$ T cell enrichment kit (Miltenyi Biotech) with negative magnetic sorting (AutoMACS) as before[5]. For *in vitro* experiments $CD4^{+}$ T cells were enriched from lymph nodes and spleens by complement-mediated lysis of $CD8^{+}$, $CD24^{+}$, and MHC-II^{+} cells by using standard MACS columns (Miltenyi Biotech) as described[6]. The increased percentage of the purified $CD4^{+}$ T cells was determined by flow cytometry analyses after every isolation and was more than 95% upon enrichment.

Polarization of $CD4^{+}$ T cell in vitro

Isolated $CD4^{+}$ T cells (10^{6} cells/well) from $Axl^{+/+}$ and $Axl^{-/-}$ mice were cultured in 24 well plates coated with anti-T cell receptor beta (TCR-β; 0.5 μg/mL) and anti-CD28 (2 μg/mL) mouse antibodies and with addition of 10 U/mL recombinant human interleukin 2 (rhIL-2) for neutral priming. The $CD4^{+}$ T cells were cultured with rhIL-2, recombinant murine (rm) IL-12 (10 ng/mL) and anti-IL-4 (20 μg/mL) for Th1 polarizing. Another set of isolated $CD4^{+}$ T cells was

cultured with rhIL-2, rmIL-4 (50 ng/mL) and anti-IFN-γ (50 μg/mL) for Th2 polarizing. On day 5 after priming, cells were re-stimulated with plate-bound anti-TCR-β. Secreted cytokines in cultured medium were measured using standard enzyme-linked immunosorbent assay (ELISA) for IFN-γ (Th1) and IL-4 (Th2) polarizing conditions.

Proliferation of $CD4^+$ T cell in vitro

Isolated T cells (10^7/mL) from $Axl^{+/+}$ and $Axl^{-/-}$ mice were incubated with 5 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) for 5 min at room temperature and washed three times before culture. Flow cytometry analyses for $CD4^+$ T cells were done after 3 days in culture in neutral vs. Th1 polarizing conditions.

Homeostatic repopulation of Axl bone marrow in vivo

Bone marrow transplant (BMT) experiments between B6.SJL$^{PtprcaPep3b/\ BoyJ}$ ($CD45.1^+$) and $Axl^{-/-}$ ($CD45.2^+$) were done with minor modifications[2]. We previously confirmed that B6.SJL$^{PtprcaPep3b/\ BoyJ}$ ($CD45.1^+$) mice express Axl gene as Axl wild type ($Axl^{+/+}$) littermates from our colony[2]. Recipient mice were irradiated (9.0 Gy) to ablate the host BM by using RS2000 irradiator (Rad Source Technologies, Inc). Within 3 hours after irradiation the recipient mice were injected (6×10^6 in 0.2 mL sterile PBS) with a mixture of donor-derived BM cells (50% $Axl^{+/+}$ and 50% $Axl^{-/-}$). Chimeric mice were allowed to recover for 8 weeks time-course, which is well accepted in the field. One potential issue could be related to heterogeneous nature of multiple subsets of leukocytes especially lymphocytes. However, a recent study utilized multiexponential models and estimated $CD4^+$ T cell life span of 15 days[7]. Percentages of $CD45.1^+$ ($Axl^{+/+}$) vs. $CD45.2^+$ ($Axl^{-/-}$) cells were evaluated by flow cytometry in peripheral blood at 4, 6, and 8 weeks after BMT. At the end of the time-course about 1 mL of blood and the whole spleen were collected from chimeric mice. Two BMT experiments were performed and $Axl^{-/-}$ and $Axl^{-/-}$ control mice were evaluated at the same time-points.

Adoptive transfer of $CD4^+$ T cells in $Rag1^{-/-}$ mice

In a first set of mice we investigated 10 week time-course of repopulation of $Axl^{+/+}$ versus $Axl^{-/-}$ $CD4^+$ T cells ($6x10^6$ in 0.2 mL sterile PBS) after tail-vein injection to $Rag1^{-/-}$ mice. We have repeated this experiment twice. Percentages of $CD4^+$ T cells were checked at 3, 7, and 10 weeks after adoptive transfer. At the end of the time-course ~1 mL of blood and the whole spleen were collected from $Rag1^{-/-}$ mice. In a second set of $Rag1^{-/-}$ mice we allowed 3 weeks after adoptive transfer of the donor $CD4^+$ T cells and successful repopulation was checked in peripheral blood by flow cytometry.

Salt-dependent hypertension model

We used a previously described deoxycorticosterone-acetate (DOCA) and salt mouse model of hypertension[8]. Briefly, 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 75 mg DOCA pellet (60 days release, Innovative Research of America, USA) was placed subcutaneously in a lateral area on the back of mice. Postoperatively, animals were injected with analgesic Flunixin meglumine (120 mg/kg, i.p.) and given regular chow and a 1% NaCl solution as a drinking water. Systolic BP was measured weekly for 6 week time-course using a non-invasive tail-cuff method (Visitech System, USA).

Flow cytometry

Peripheral leukocyte apoptosis was measured by FITC-labeled Annexin V and propidium iodide (PI) in TACS Annexin V-FITC kit (Trevigen®) as we reported[9]. Lymphocyte apoptosis was evaluated by staining of blood leukocytes from Axl littermates with CD3-APC (1:100; eBioscience) antibody and TACS Annexin V-FITC kit. The engraftment of donor BM cells was confirmed by staining of the blood samples after recovery period (8 week) 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) as before[2]. Five major subsets of immune cells were detected using 12-color LSRII flow cytometer (BD Biosciences) as we reported[10]. Isolated cells from spleen or blood from chimeras were first incubated with live/dead stain (1:500, Invitrogen). Following this step, the cells were washed with FACS buffer (1,200 rpm, 4°C, 7 min) and incubated with FC block (1:10, BD Bioscience) at room temperature for 30 min. 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 Biosciences), CD11c-PE-TXR (1:500, Invitrogen) and NK1.1-APC-CY7 (1:100, Biolegend) antibodies at 4°C for 30 min. Cells were washed and re-suspended in FACS buffer. Compensation controls were prepared using combined samples for single stained controls. Flow cytometry analyses were performed using FlowJo software version X.0.7.

Morphometry and immunohistochemistry

Axl chimeras were perfusion fixed with 10% paraformaldehyde and histology was performed as described[8]. We used MCID image software (MCID Elite 6.0, Imaging Research) for morphometry analyses as we shown previously[11]. Rabbit anti-mouse CD4 antibody (1:5,000; Novus Biologicals) was applied for 60 min at room temperature, while rat anti-mouse phosphorylated Akt (pAkt; 1:100; Cell Signaling) antibody was incubated at 4°C overnight as we reported[1]. We used polymer Rabbit-on-Rodent horseradish peroxidase (HRP) or Rat-on-Mouse HRP kits (BioCare) to conjugate primary antibodies. The peroxidase-binding sites were shown by the 3,3'-diaminobenzidine (DAKO). Apoptotic cells were detected by ApopTag peroxidase In situ Apoptosis Detection Kit (Chemicon Int) as we reported before[8]. Immunostained arterial sections were captured by SPOT INSIGHT FireWire camera (Diagnostic Instruments). We uniformly adjusted size and contrast of the images to meet the journal guidelines (Adobe Photoshop CS3, v. 10.0). We have captured undefined images (image1, image2, etc.) of the stained cross-sections and were analyzed positively-stained cells in 3 mice (2-3 sections/mouse) in a blindly manner by using ImagePro software[2]. We have calculated percentage of positive cells (brown staining) to investigated area minus counter-stained cells (blue color).

Statistical analysis

Results are shown as means ± SEM. Statistical differences were evaluated using JMP9.0.0 software (SAS). We have performed normality tests (visualization of the data distribution and normal quintile plot) to confirm the parametric distribution of the raw data, which was followed by the appropriate statistical analyses. Differences between two groups were analyzed by unpaired Student's *t* test. For more than 3 experimental groups we utilized one-way ANOVA followed by post hoc comparisons (Student's *t* test). Time-course changes were analyzed by MANOVA fit tests for time and experimental group variables with subsequent one-way ANOVA and post hoc comparisons (Student's *t* test) at each time-point of the time-course. The level of $p<0.05$ was regarded as significant.

References

1. Korshunov VA, Mohan AM, Georger MA, Berk BC. Axl, a receptor tyrosine kinase, mediates flow-induced vascular remodeling. *Circulation research*. 2006;98:1446-1452
2. 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
3. Smolock EM, Burke RM, Wang C, Thomas T, Batchu SN, Qiu X, Zettel M, Fujiwara K, Berk BC, Korshunov VA. Intima modifier locus 2 controls endothelial cell activation and vascular permeability. *Physiological genomics*. 2014;46:624-633
4. Kile BT, Mason-Garrison CL, Justice MJ. Sex and strain-related differences in the peripheral blood cell values of inbred mouse strains. *Mammalian genome*. 2003;14:81-85
5. Shi G, Field DJ, Ko KA, Ture S, Srivastava K, Levy S, Kowalska MA, Poncz M, Fowell DJ, Morrell CN. Platelet factor 4 limits th17 differentiation and cardiac allograft rejection. *The Journal of clinical investigation*. 2014;124:543-552
6. Sojka DK, Fowell DJ. Regulatory t cells inhibit acute ifn-gamma synthesis without blocking t-helper cell type 1 (th1) differentiation via a compartmentalized requirement for il-10. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108:18336-18341
7. Westera L, Drylewicz J, den Braber I, et al. Closing the gap between t-cell life span estimates from stable isotope-labeling studies in mice and humans. *Blood*. 2013;122:2205-2212
8. Korshunov VA, Daul M, Massett MP, Berk BC. Axl mediates vascular remodeling induced by deoxycorticosterone acetate salt hypertension. *Hypertension*. 2007;50:1057-1062
9. Smolock EM, Korshunov VA. Pharmacological inhibition of axl affects smooth muscle cell functions under oxidative stress. *Vascular pharmacology*. 2010;53:185-192
10. Batchu SN, Hughson A, Gerloff J, Fowell DJ, Korshunov VA. Role of axl in early kidney inflammation and progression of salt-dependent hypertension. *Hypertension*. 2013;62:302-309
11. 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