Role of Axl in T-Lymphocyte Survival in Salt-Dependent Hypertension

Sri N. Batchu,* Angie Hughson, Kristine M. Wadosky, Craig N. Morrell, Deborah J. Fowell, Vyacheslav A. Korshunov*

Objective—Survival of immune and nonimmune cells relies on Axl, a receptor tyrosine kinase, which is implicated in hypertension. Activated T lymphocytes are involved in regulation of high blood pressure. The goal of the study was to investigate the role of Axl in T-lymphocyte functions and its contribution to salt-dependent hypertension.

Approach and Results—We report increased apoptosis in peripheral blood from Axl−/− mice because of lower numbers of white blood cells mostly lymphocytes. In vitro studies showed modest reduction in interferon gamma production in Axl−/− type 1 T helper cells. Axl did not affect basic proliferation capacity or production of interleukin 4 in Axl−/− type 2 T helper cells. However, competitive repopulation of Axl−/− bone marrow or adoptive transfer of Axl−/− CD4+ T cells to Rag1−/− mice showed robust effect of Axl on T lymphocyte expansion in vivo. Adoptive transfer of Axl−/− CD4+ T cells was protective in a later phase of deoxycorticosterone-acetate and salt hypertension. Reduced numbers of CD4+ T cells in circulation and in perivascular adventitia decreased vascular remodeling and increased vascular apoptosis in the late phase of hypertension.

Conclusions—These findings suggest that Axl is critical for survival of T lymphocytes, especially during vascular remodeling in hypertension. (Arterioscler Thromb Vasc Biol. 2016;36:1638-1646. DOI: 10.1161/ATVBAHA.116.307848.)

Key Words: apoptosis ■ Axl receptor tyrosine kinase ■ hypertension ■ lymphocyte ■ vascular remodeling

Tyr03, Axl, and Merk (TAM) family of receptor tyrosine kinases has been implicated in regulation of multiple organ systems.1 Growth arrest–specific protein 6 (Gas6) and protein S are potent anticoagulants and ligands for the TAM receptors.2,3 However, mice with genetic deletion of all 3 TAM receptors showed normal hemostasis.1 It was later reported that Gas6 and TAM receptors are involved in regulation of thrombus stabilization.4 More severe lymphoproliferative disorder was described in adult TAM triple knockout mice.5 The observed autoimmune phenotypes was linked to TAM functions in innate immune cells, for example, macrophages (Mφs), dendritic cells (DCs), and natural killer (NK) cells.6 Initial data in humans showed that Axl is restricted to innate immune cells, and upregulation of Axl is predominant in myeloid leukemias.7 However, recent reports suggested substantial roles for Axl and Merk in T- and B-cell leukemias.6–10 Thus, the role of TAM family and Axl, in particular, on lymphocyte functions in homeostasis and under pathological conditions remains unclear.

A recent human study (3679 untreated hypertensive individuals; The Framingham Heart Study) showed that apoptosis, T-cell activation, and T-cell differentiation are enriched in a gene–gene interaction network for a mutation in SH2B adaptor protein 3 that cause increased blood pressure (BP).11 Not surprisingly, one of the subnetworks of the whole blood transcriptome included the Gas6/Axl pathway in relation to high BP genetic susceptibility.11 Previous genetic studies in a Sabra rat model of salt-sensitive hypertension identified Axl as a candidate gene.12 Experiments in global knockout mice confirmed pathophysiological role for Gas6 and Axl in development of deoxycorticosterone-acetate (DOCA)–salt hypertension.13,14 Specifically, our group showed that Axl-dependent signals control vascular smooth muscle cell survival and promote vascular remodeling after 6 weeks of DOCA–salt.15 We recently reported that Axl expression in bone marrow (BM)–derived cells is responsible for early and late phases of DOCA–salt hypertension.15 Adaptive immune cells, such as T lymphocytes, contribute to vascular dysfunction and DOCA–salt hypertension.16 Recent study showed that both CD4+ and CD8+ T cells producing interferon gamma (IFN-γ) are involved in elevation of BP and kidney damage after repeated hypertensive stimuli.17 Interestingly, the type 1 T helper (Th1) response in the
The retina was also shown to be regulated through Axl/Mertk in mice. The primary focus of this study was on the role of Axl in T-lymphocyte functions and its contribution to salt-dependent hypertension.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Increased Apoptosis of White Blood Cells in Peripheral Blood From Axl−/− Mice

Others and we showed that Axl plays a key role in the survival of various cell types. We double-stained peripheral leukocytes from Axl littermates with Annexin V and PI (Figure 1). The lower right quadrant of each flow chart shows early apoptotic leukocytes, whereas the upper right quadrant shows necrotic (late apoptotic) cells in Axl mice (Figure 1A). Quantification of flow cytometry demonstrated that Axl−/− had significantly higher apoptotic leukocytes compared with Axl+/+ mice (Figure 1B). Of note, the Annexin V+PI+ cell numbers did not reach statistical significance between Axl genotypes (Figure 1B). We found that the levels of apoptosis were the same in BM from Axl littermates (not shown). Evaluation of splenocytes from adult Axl littermates showed similar numbers of CD3+ T or NK1.1+ cells (Table I in the online-only Data Supplement). The frequencies of naive CD62Lhigh/CD44low and CD62Llow/CD44high memory T cells were similar between Axl−/− and Axl+/+ mice (Table I in the online-only Data Supplement). We also found no differences in B and T lymphocytes in spleens and lymph nodes between Axl genotypes (not shown). However, Axl−/− mice had significantly lower (≈40%) total white blood cells and lymphocytes count in peripheral blood compared with their Axl+/+ littermates (Table II in the online-only Data Supplement). We also found no differences in B and T lymphocytes in spleens and lymph nodes between Axl genotypes (not shown). However, Axl−/− mice had significantly lower (≈40%) total white blood cells and lymphocytes count in peripheral blood compared with their Axl+/+ littermates (Table II in the online-only Data Supplement). Hemoglobin levels were slightly reduced in Axl−/− mice (Table II in the online-only Data Supplement). Taken together, our data suggest that Axl controls survival of T lymphocytes in peripheral blood.

Figure 1. Critical role of Axl in leukocytes survival. Representative flow data for Axl+/+ littermate; Axl−/− littermate (A). The lower right quadrant represents early apoptotic leukocytes (Annexin V+), whereas the upper right quadrant represents necrotic cells (PI+Annexin V+) from Axl mice peripheral blood. B, Quantification of apoptotic, necrotic, and live cells between Axl genotypes. Black bars represent Axl+/+ mice. Open bars represent Axl−/− mice. Values are mean±SEM. *P<0.05 vs Axl+/+. FITC indicates fluorescein isothiocyanate; n, number of mice; and PI, propidium iodide.

Competitive Repopulation of BM Cells Showed Less Axl−/− T Cells

Original report on deletion of 3 TAM receptors resulted in a severe lymphoproliferative disorder in mice. To explore the
role of Axl on T cells in vivo, we evaluated homeostatic expansion of Axl+ (CD45.1+) versus Axl− (CD45.2+) BM cells in competitive repopulation experiment (Figure 2). We were able to define Axl genotype origin by dual flow cytometry with anti-CD45.1 and anti-CD45.2 antibodies of white blood cells from peripheral blood from chimeras over 8 weeks time course.

**Figure 2.** Competitive repopulation of Axl bone marrow cells after bone marrow transplant. A, Representative flow charts of double-stained CD45.1 (Axl+) and CD45.2 (Axl−) peripheral leukocytes from Axl chimeric mice. B, A time course of competitive repopulation (CD45.1+ and CD45.2−; 50% and 50%) after 8 weeks of bone marrow transplant (BMT). C, Total numbers of CD45.1+ and CD45.2− cells in Axl chimeras at 8 weeks after BMT. D, Percentages of CD19+ cells in blood. E, Percentages of CD19+ cells in spleen. F, Percentages of CD3+ cells in blood. G, Percentages of CD3+ cells in spleen. Black bars represent Axl+ (CD45.1+) cells. Open bars represent Axl− (CD45.2+) cells. Values are mean±SEM. *P<0.05 vs Axl+. Controls, n=4; Axl chimeras, n=10.
Significantly more CD45.2+ than CD45.1+ cells were found at 6 and 8 weeks in chimeras after BM transplant, suggesting the role of Axl on immune homeostasis (Figure 2B). At the end of the experiment (8 week), we collected peripheral blood and confirmed higher numbers of CD45.2+ cells from Axl−/− mice (Figure 2C). As shown by original report in triple TAM knockout,7 the immune population of the chimeras was shifted toward increase in Axl−/− B lymphocytes (CD45.2−CD19+) both in blood and spleen (Figure 2D and 2E). There was dramatic decrease in Axl−/− T cells (CD45.2−CD3+) in blood and spleen from chimeric mice after competitive repopulation (Figure 2F and 2G). Lymphocyte frequencies in spleen were similar between Axl genotype controls as we observed in naïve versus memory T cells (Figure 2E and 2G; Table I in the online-only Data Supplement). Analyses of innate immune cells suggested the role for Axl in repopulation of blood monocyte/Mφ (CD11b+) but did not affect DCs (CD11c+) or NK (NK1.1+) cells (Figure II in the online-only Data Supplement). There were no differences in innate immune subsets in spleens from Axl genotype controls or repopulated chimeras (Figure II in the online-only Data Supplement). These findings confirmed our hematologic data and suggest that Axl is required for peripheral T-lymphocyte expansion from BM.

Axl Regulates CD4+ T-Cell Repopulation In Vivo

It is possible that the decline in Axl−/− lymphocytes could be because of alteration in innate immune cells under competitive repopulation of BM, as was reported in double and triple TAM knockouts before.5,18 However, previous studies suggested that dual deletion of Mertk and Axl affected Th1 polarization in vitro.18 In our experiments, Axl−/− T cells showed modest but significant decrease in IFN-γ under Th1 polarizing conditions (Figure 3A). The basic capacity to proliferate in response to T-cell receptor stimulation was unaffected in cultured CD4+ T cell from Axl−/− versus Axl−/− mice, with similar cell number yields (Figure 3B). Although there was a slight delay in the initial 2 cell divisions, that was compensated in division 4 in Axl−/− versus Axl−/− Th1 cells (Figure 3B; Figure III in the online-only Data Supplement). We also found that production of interleukin 4 was similar between Axl genotypes under type
2 helper cell polarization (Figure IV in the online-only Data Supplement). Thus, Axl showed a modest effect on Th1 polarization of CD4+ T lymphocytes in vitro.

To avoid potential effects of Axl depletion in innate immune compartment, we adoptively transferred Axl−/− or Axl+/+ CD4+ T cells to recombination activating gene 1 knockout (Rag1−/−) mice that lack adaptive immunity but contain Axl-sufficient innate immunity (Figure 3C and 3D). In our experiments, frequencies of CD4+ T cell were under detection level in peripheral blood from Rag1−/− mice injected with phosphate-buffered saline (PBS; Figure 3C and 3D). Adoptive transfer of Axl+/+ T cells resulted in significant increase in percentage of CD4+ cells over 10 weeks of repopulation that peaked at 7 week (Figure 3C). Axl−/− CD4+ T cell showed slightly slower repopulation after 3 weeks and nonsignificant increase at 10 weeks after adoptive transfer to Rag1−/− mice (Figure 3C). Decreased repopulation of Axl−/− CD4+ T cells in blood was confirmed in spleens from adoptively transferred Rag1−/− mice (Figure 3D). Taken together, our findings suggest that Axl is important for CD4+ T-lymphocyte expansion and survival in vivo in the presence of Axl in innate immune compartment.

Axl Is Important for CD4+ T Cell Survival and the Late Phase of DOCA–Salt Hypertension

Production of IFN-γ by CD4+ and CD8+ T cells was recently shown to contribute to BP increase and kidney damage after repeated hypertensive stimuli.17 Similar to our report in Axl chimeras,15 we found reduction in arterial expression of IFN-γ (and Th1-dependent pathways) in Axl−/− mice after 6 weeks of DOCA–salt (not shown). We performed adoptive transfers of CD4+ T cells from Axl−/− or Axl+/+ to Rag1−/− and compared BP changes after DOCA–salt to those in Rag1−/− injected with PBS (PBS→Rag1−/−) or Axl−/− mice (Figure 4). As expected in this model,16 Axl+/+ mice showed significant increase in systolic BP, whereas PBS→Rag1−/− mice were protected from hypertension after 5 to 6 weeks of DOCA–salt (Figure 4A). Adoptive transfer of Axl−/− CD4+ T cells (Axl−/−→Rag1−/−) was also protective for BP increases as compared with Axl+/+→Rag1−/− or Axl−/− mice after 5 to 6 weeks of DOCA–salt (Figure 4A). CD4+ T cells were detected in blood in Axl−/−→Rag1−/− but were significantly reduced compared with Axl+/+→Rag1−/− or Axl+/+ mice after 5 weeks of DOCA–salt (Figure 4B). Levels of BP reflected reduced medial thickening of mesenteric arteries in PBS→Rag1−/− and Axl−/−→Rag1−/− versus Axl+/+→Rag1−/− or Axl+/+ mice after 6 weeks of DOCA–salt (Table III in the online-only Data Supplement). We also noted significantly reduced adventitial compartment in arteries from Rag1−/− controls and Axl−/−→Rag1−/− mice (Table III in the online-only Data Supplement). Previous report suggested that majority of T lymphocytes reside within perivascular adventitia (PVA) in hypertension.16 As

Figure 4. Axl controls circulating CD4+ T cells during late phase of salt-dependent hypertension. A, Changes in systolic blood pressure (BP) after deoxycorticosterone acetate (DOCA) and salt in mice after adoptive transfer of CD4+ T cells to recombination activating gene 1 knockout (Rag1−/−) mice time course of. Black squares and black line show Axl+/+. Open circles and gray line show Axl+/+→Rag1−/− mice. Gray circles and gray line show PBS→Rag1−/− mice. Black squares and gray line show Axl−/−→Rag1−/− mice. Open circles and gray line show Axl+/+→Rag1−/− versus Axl−/−→Rag1−/− or Axl+/+ mice after 6 weeks of DOCA–salt (Table III in the online-only Data Supplement). Values are mean±SEM. *P<0.05 vs Axl+/+. †P<0.05 vs PBS→Rag1−/− mice. ‡P<0.05 vs Axl−/−→Rag1−/− mice. n indicates number of mice in each group.
we observed by flow cytometry in peripheral blood, immunochemistry mirrored presence of CD4+ in PVA from Axl+/+ → Rag1−/− or Axl+/+ mice after 6 weeks of DOCA–salt (Figures 4B and 5A). In contrast, PBS→Rag1−/− and Axl+/+→Rag1−/− mice showed significantly less CD4+ immunoreactivity in PVA (Figure 5A). Detected CD4+ staining in PBS→Rag1−/− mice could be because of cross-reactivity of anti-CD4 antibody to endothelial cells in PVA. One of the major prosurvival signals that is controlled by Axl in vasculature is phosphorylated protein kinase B after DOCA–salt.13 In PVA, we observed significant decrease in pAkt+ in PBS→Rag1−/− compared with Axl+/+, whereas Axl+/+→Rag1−/− mice had less phosphorylated protein kinase B relative to Axl+/+→Rag1−/− or Axl+/+ mice after 6 weeks of DOCA–salt (Figure 5B). Percentage of apoptotic cells in PVA was elevated in Axl−/−→Rag1−/− mice only (Figure 5C). Thus, Axl is required for presence of CD4+ T cells not only in circulation but also important for vascular inflammatory response, survival, and remodeling in hypertension.

Figure 5. Immunohistochemical evaluation of the arteries from mice after CD4+ T-cell repopulation and DOCA–salt hypertension. A, CD4+ cells and quantification in the perivascular adventitia (PVA) of mesenteric artery. B, p-Akt+ cells and quantification in the PVA of mesenteric artery. C, Apoptag+ cells and quantification in the PVA of mesenteric artery. Black bars show Axl+/+. Open bars show PBS→Rag1−/− mice. Dark gray bars show Axl+/+→Rag1−/− mice. Light gray bars show Axl+/+→Rag1−/− mice. Values are mean±SEM. *P<0.05 vs Axl+/+. †P<0.05 vs PBS→Rag1−/− mice. ‡P<0.05 vs Axl+/+→Rag1−/− mice. n=3 per each group. pAkt indicates phosphorylated protein kinase B; and Rag1−/−, recombination activating gene 1 knockout.
Discussion

We report that Axl is required for lymphocyte survival in peripheral blood in a mouse. Single deletion of Axl resulted in modest effect on CD4+ T-cell production of IFN-γ. A defective expansion of T lymphocytes was evident in competitive repopulation of Axl−/− BM, which also resulted in an increase in B cells with decline in circulating myeloid cells. Late repopulation of CD4+ T cells was dependent on Axl after adoptive transfer to Rag1−/− mice. Finally, presence of CD4+ T lymphocytes in blood and PVA could explain Axl-dependent effects on vascular remodeling in the late phase of DOCA–salt hypertension.

The TAM family of receptor tyrosine kinases (particularly MerTK and Axl) are shown in tempering the immune response in murine Mψs, DCs, and NK cells.6,20 Genetic defect in the TAM receptors is shown to lead to various autoimmune disorders, including arthitis, experimental autoimmune orchitis, and lupus in mice after a pathological insult.5,21,22 Studies using single or double gene–knockout mice demonstrated that MerTK or Axl suppresses innate immune responses via inhibiting expression of proinflammatory cytokines through transcriptional upregulation of the suppressor of cytokine signaling 1 and suppressor of cytokine signaling 3 proteins.23 In contrast, we have shown that Axl could inhibit suppressor of cytokine signaling 1 and promote signal transducer and activator of transcription 1 signaling, leading to immune modulation and activation of smooth muscle cells in vein grafts or after vascular injury.24,25 We also reported that Axl is important for vascular and kidney dysfunction by regulating immune cells in DOCA–salt hypertension.27 Collectively, our findings indicate distinct roles for the TAM family (eg, Axl versus MerTK) on immune modulation, and MerTK likely regulates immunosuppression observed in single or double TAM knockouts. MerTK is the primary TAM receptor that is involved in the engulfment and efficient clearance of apoptotic cells26 and in an effective resolution of acute inflammation.27 Our new data suggest that Axl is required for homeostasis of white blood cells (lymphocytes) in peripheral blood.

Initial findings implied that Axl expression is restricted to normal myeloid cells with preference in myeloid (=60%) versus lymphoid (2%) leukemias.7 Triple TAM knockout mice exhibited enlarged spleens and lymph nodes with a predominance of CD4+ over CD8+ T cells or B cells after 1 to 2 months of age.6 It was concluded that overactivated Mψs and DCs are responsible for lymphoproliferation and autoimmune in TAM knockout mice. However, deletion of MerTK exhibited larger spleens compared with lack of Axl or Tyro3 among single or double knockouts.5 In fact, clinical studies showed that constitutively active Axl is expressed in B cells and promotes survival and proliferation in a chronic B lymphocytic leukemia.8 In addition, MerTK is reported to be ectopically expressed on B and T cells and might be involved in the progression of a variety of human cancers, including T-cell acute lymphoblastic leukemia.9 Most recent results showed that MerTK and protein S are expressed in human T cells and on activation facilitate an autocrine proliferation.10 Furthermore, lack of MerTK and Axl in mice affected retinal CD4+ T-cell polarization toward Th1, but the underlying mechanism is not clear.18 Here, we described a novel hematological phenotype in a single Axl−/−, suggesting a significant role of Axl for T-lymphocyte survival that might affect production of IFN-γ. Our in vivo repopulation studies in chimeras or Rag1−/− mice strongly support the role of Axl in T-lymphocyte expansion. The likely mechanism is related to decrease in Axl-dependent activation of Akt, which is one of the key prosurvival signals in Th1 cells in sepsis.28 Recent studies argue that a higher proliferation and metabolic activity of T cell29 and its differentiation to Th1 cells is mediated by the phosphoinositide 3-kinase/Akt signaling.30 Therefore, Axl-dependent signals could be critical for T-cell fitness and might be involved in late phases of vascular pathologies. Slight changes in Axl−/− cell divisions under Th1 polarization in vitro could explain more robust effects of Axl deletion in vivo. A premature mitosis in antigen-specific Th1 cells was shown to be regulated by the balance between c-fos and nuclear protein kinase Weel.31 Similarly, recent pharmacological experiments in several cancer cell lines suggested a synergism between Axl inhibition and blocking cell cycle kinases that control mitotic cell entry.32 Thus, Axl might regulate long-term activation of T lymphocytes by altering cell division and survival signals.

Increased attention to adaptive immunity revealed a key role for T lymphocytes in regulation of vascular dysfunction and experimental hypertension after angiotensin II or DOCA–salt.16 As in a typical immune response, hypertensive stimuli increase isoketals in DCs that promote immune activation of T cells and progression of hypertension.33 Further activated DCs enhance T-cell proliferation and promote their polarization toward Th1, contributing to high BP in patients with preeclampsia.34 There are growing experimental evidence on the primary role for CD8+ T cells in the development of hypertension.15 However, a recent report17 showed that both CD4+ and CD8+ T cells producing IFN-γ increased BP and promoted kidney damage in a repeated hypertensive stimuli model. Likewise, patients with resistant arterial hypertension reported to have increased circulating levels of Th1 cells.35 High BP is regulated by multiple genomic loci, which was recently shown in untreated hypertensive individuals.11 The authors identified a causal mutation (rs31184504 C/T) within SH2B3 adaptor protein 3 gene. Gene expression signatures from a whole blood–derived RNA in these individuals lead to generation of coexpression networks, and one of the key drivers was HS2B3. Pathway analyses suggested that apoptosis, T-cell activation, and T-cell differentiation were the most significant in predisposition to hypertension.11 The SH2B3 adaptor protein 3 is also known as lymphocyte adaptor protein that regulates hematopoiesis and lymphocyte differentiation and is implicated in myeloproliferative and inflammatory disorders.37 Overproduction of IFN-γ was restricted to CD8+ and CD4+ T cells in Lnk−/− mice in experimental hypertension.38 The SH2B3 adaptor protein 3–constructed coexpression network of human peripheral blood also included the Gnas/ Axl pathway in relation to high BP genetic susceptibility.11 We previously showed that deletion of Axl in hematopoietic cells dramatically reduced Mψs and DCs, increased accumulation of B cells in the kidney, and prevented initiation (1 week) of DOCA–salt hypertension.15 The protective effects are because
of downregulation of kidney expression of IFN-γ and Th1-dependent pathways in Axl chimeras. However, expression of Axl in BM-derived cells was also contributed to BP and vascular remodeling in the late phase (6 week) of DOCA-salt hypertension. In the current study, we showed that Axl is critical for the late survival of CD4+ T cells in peripheral blood and locally via significant increase in phosphorylated protein kinase B expression and decrease in percentage of apoptotic cells in the PVA. Observed increases in medial apoptosis in Rag1−/− adoptively transferred with Axl−/− CD4+ T cells suggest an important regulatory effect between lymphocytes and smooth muscle cells in late hypertension. On the contrary, an initial vascular response to injury is mostly driven by the immune activation of Axl in smooth muscle cells, with modest effect from innate or adaptive immune cells. Collectively, our findings uncover distinct roles for Axl between vascular and immune cells in cardiovascular diseases (vascular injury versus hypertension).

Measurements of the hemodynamic parameters using tail-cuff plethysmography have some limitations associated with stress. However, many laboratories including ours have validated tail-cuff BP measurements by radiotelemetry method. Our recent study in inbred mouse strains showed BP levels reflected degree of arterial remodeling obtained by histology across mice after DOCA–salt. It is possible that inhibition of Gas6 and ProS expression facilitates inflammatory cytokine of MerTK increases chemosensitivity and decreases oncogenic potential in T-cell acute lymphoblastic leukemia. J Immunol. 2013;187:4178–4186. doi: 10.4049/jimmunol.1101201.

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Disclosures

None.

References

20. Dong T, Zhang Y, Chen Q, Yan K, Han D. Toll-like receptor-mediated inhibition of Gas6 and ProS expression facilitates inflammatory cytokine...

**Highlights**

- Axl, a receptor tyrosine kinase, is required for peripheral T-lymphocyte survival.
- Axl-mediated CD4+ T-cell survival is critical for elevated blood pressure and vascular remodeling in the late phase of hypertension.
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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\(^{PprcaPep3b}\)/BoyJ mice were purchased from the Jackson Laboratory. We confirmed presence of Axl expression by genotyping\(^2\) of B6.SJL\(^{PprcaPep3b}\)/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 \(\mu\)m 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 \textit{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 \textit{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-\(\beta\); 0.5 \(\mu\)g/mL) and anti-CD28 (2 \(\mu\)g/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 \(\mu\)g/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 (10⁷/mL) from Axlnfloxed and Axlnfloxed 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<sup>PpcrePep3b/Boj</sup> (CD45.1⁺) and Axl<sup>+/+</sup> (CD45.2⁺) were done with minor modifications. We previously confirmed that B6.SJL<sup>PpcrePep3b/Boj</sup> (CD45.1⁺) mice express Axl gene as Axl wild type (Axl<sup>+/+</sup>) littermates from our colony. 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 (6×10⁶ in 0.2 mL sterile PBS) with a mixture of donor-derived BM cells (50% Axl<sup>+/+</sup> and 50% Axl<sup>−/−</sup>). 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. Percentages of CD45.1⁺ (Axl<sup>+/+</sup>) vs. CD45.2⁺ (Axl<sup>−/−</sup>) 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<sup>−/−</sup> control mice were evaluated at the same time-points.

Adoptive transfer of CD4⁺ T cells in Rag1<sup>−/−</sup> mice
In a first set of mice we investigated 10 week time-course of repopulation of Axl<sup>+/+</sup> versus Axl<sup>−/−</sup> CD4⁺ T cells (6x10⁶ in 0.2 mL sterile PBS) after tail-vein injection to Rag1<sup>−/−</sup> 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<sup>−/−</sup> mice. In a second set of Rag1<sup>−/−</sup> 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. 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 reported⁹. 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 before². Five major subsets of immune cells were detected using 12-color LSRII flow cytometer (BD Biosciences) as we reported¹⁰. 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-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 described⁸. We used MCID image software (MCID Elite 6.0, Imaging Research) for morphometry analyses as we shown previously¹¹. 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 reported¹. 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 before⁸. 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 software². 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

Table I. Analyses of the immune cells in spleens from Axl<sup>+/+</sup> and Axl<sup>-/-</sup> mice

<table>
<thead>
<tr>
<th>Cell population</th>
<th>% of Total Splenocytes</th>
<th>Axl+/+, %, n=3</th>
<th>Axl-/-, %, n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3&lt;sup&gt;high&lt;/sup&gt;/NK1.1&lt;sup&gt;low&lt;/sup&gt; (T cells)</td>
<td>25.0 ± 0.5</td>
<td>24.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>CD3&lt;sup&gt;low&lt;/sup&gt;/NK1.1&lt;sup&gt;high&lt;/sup&gt; (NK cells)</td>
<td>5.2 ± 0.1</td>
<td>5.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Gated at NK1.1&lt;sup&gt;+&lt;/sup&gt;: CD3&lt;sup&gt;high&lt;/sup&gt;/NK1.1&lt;sup&gt;high&lt;/sup&gt; (NKT cells)</td>
<td>22.1 ± 2.8</td>
<td>22.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Gated at CD4&lt;sup&gt;+&lt;/sup&gt;: CD62L&lt;sup&gt;high&lt;/sup&gt;/CD44&lt;sup&gt;low&lt;/sup&gt; (naïve T cells)</td>
<td>57.2 ± 0.1</td>
<td>61.2 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Gated at CD4&lt;sup&gt;+&lt;/sup&gt;: CD62L&lt;sup&gt;low&lt;/sup&gt;/CD44&lt;sup&gt;high&lt;/sup&gt; (memory T cells)</td>
<td>16.3 ± 0.5</td>
<td>13.7 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Parameters are shown as mean ± SEM. n, Number per group.
Table II. Hematologic differences between wild type and Axl knockout mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Axl&lt;sup&gt;+/+&lt;/sup&gt;, n=8</th>
<th>Axl&lt;sup&gt;-/-&lt;/sup&gt;, n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells, x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>8.3 ± 0.6</td>
<td>5.3 ± 0.7 *</td>
</tr>
<tr>
<td>Lymphocytes, x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>7.8 ± 0.6</td>
<td>4.9 ± 0.6 *</td>
</tr>
<tr>
<td>Neutrophils, x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.35 ± 0.11</td>
<td>0.22 ± 0.08</td>
</tr>
<tr>
<td>Monocytes, x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.21 ± 0.04</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td>Red blood cells, x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>11.1 ± 0.3</td>
<td>9.4 ± 1.2</td>
</tr>
<tr>
<td>Platelets, x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>445 ± 66</td>
<td>449 ± 71</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>44 ± 3</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>14.5 ± 0.7</td>
<td>11.6 ± 0.8 *</td>
</tr>
<tr>
<td>Plateletcrit, %</td>
<td>0.33 ± 0.08</td>
<td>0.39 ± 0.08</td>
</tr>
<tr>
<td>Mean corpuscular volume</td>
<td>43 ± 1</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin</td>
<td>12.8 ± 0.5</td>
<td>12.0 ± 0.6</td>
</tr>
<tr>
<td>Red blood cell distribution</td>
<td>18.8 ± 0.4</td>
<td>19.4 ± 0.4</td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td>6.3 ± 0.1</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>Platelet distribution</td>
<td>31.4 ± 0.7</td>
<td>31.7 ± 0.8</td>
</tr>
</tbody>
</table>

Parameters are shown as mean ± SEM. *, p<0.05 compared to Axl<sup>+/+</sup> (Student’s t test). n, Number of mice.
<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Lumen area, x10³.μm²</th>
<th>Media area, x10³.μm²</th>
<th>Adventitia area, x10³.μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axl⁺⁺, n=5</td>
<td></td>
<td>16.3 ± 2.4</td>
<td>7.2 ± 0.8</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>PBS → Rag1⁻⁻, n=5</td>
<td></td>
<td>10.7 ± 1.9</td>
<td>4.5 ± 0.6 *</td>
<td>4.1 ± 0.4 *</td>
</tr>
<tr>
<td>CD4⁺ Axl⁺⁺ → Rag1⁻⁻, n=5</td>
<td></td>
<td>12.7 ± 3.1</td>
<td>6.8 ± 0.9</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>CD4⁺ Axl⁻⁻ → Rag1⁻⁻, n=6</td>
<td></td>
<td>11.2 ± 1.9</td>
<td>3.8 ± 0.9 *,†</td>
<td>3.8 ± 0.8 *,†</td>
</tr>
</tbody>
</table>

Parameters are shown as mean ± SEM. *, p<0.05 compared to Axl⁺⁺ (ANOVA). †, p<0.05 compared to CD4⁺ Axl⁺⁺ → Rag1⁻⁻ (ANOVA). n, Number per group.
Figure I. Increased lymphocyte apoptosis in peripheral blood from Axl knockout mice.
Relative numbers of CD3⁺ or CD3⁻ apoptotic (AnnexinV⁺) cells between Axl genotypes. Black bars represent Axl⁺/⁺ mice. Open bars represent Axl⁻/⁻ mice. Values are mean±SEM. *, p<0.05 vs. Axl⁺/⁺ (Student's t test). n=5
Figure II. Effect of Axl deletion on competitive repopulation of the innate immune cells. Competitive bone marrow cells repopulation experiment between Axl genotypes: Axl+/+ (CD45.1+) and Axl−/− (CD45.2+) after 8 weeks after bone marrow transplant. A. Percentage of CD11b+ cells in spleens. B. Percentage of CD11b+ cells in blood. C. Percentage of CD11c+ cells in spleens. D. Percentage of CD11c+ cells in blood. E. Percentage of NK1.1+ cells in spleens. F. Percentage of NK1.1+ cells in blood. Black bars are Axl+/+. Open bars - Axl−/−. Values are mean±SEM. *, p<0.05 vs. Axl+/+. n=4-10 per group.
Figure III. Quantification of the CD4+CFSE+ cells under priming conditions. Black bars represent Neutral Axl+/+ cells. Open bars – Neutral Axl−/− cells. Dark grey bars – Axl+/+ Th1 cells. Light grey bars – Axl−/− Th1 cells. Values are mean±SEM. *, p<0.05 vs. Neutral Axl+/+. n=5
Figure IV. Axl has no effect on Th2 polarization in vitro. CD4\(^+\) T cells from Axl mice were primed with Neutral or Th2 conditions for 5 days. Supernatants were assayed by ELISA Interleukin 4 (IL-4; ng/mL). Black bars are Axl\(^{+/+}\). Open bars - Axl\(^{-/-}\). Values are mean±SEM. *, p<0.05 vs. Neutral conditions. n, number per group.
A proposed model on the role of Axl on CD4+ T cell survival in progression of salt-dependent hypertension. Black line shows changes in blood pressure. X-axis represents time in weeks. Y-axis represents changes in blood pressure. Highlighted light green area shows a critical role for Axl in T lymphocyte survival in circulation that contribute to vascular remodeling and the late hypertension (lime color area, 5-6 weeks).