

Published in final edited form as:

Prostaglandins Leukot Essent Fatty Acids. 2011 ; 84(1-2): 43–50. doi:10.1016/j.plefa.2010.09.002.

The endogenous pro-resolving mediators lipoxin A₄ and resolvin E1 preserve organ function in allograft rejection

Bruce D. Levy^{1,2}, Qing-yin Zhang³, Caroline Bonnans¹, Valeria Primo³, John J. Reilly¹, David L. Perkins⁴, Yurong Liang⁵, M. Amin Arnaut³, Boris Nikolic³, and Charles N. Serhan²

¹Pulmonary and Critical Care Medicine, Department of Internal Medicine Boston, MA

²Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital Boston, MA

³Division of Nephrology, Massachusetts General Hospital, Harvard Medical School, Boston, MA

⁴Department of Medicine, School of Medicine, University of California, San Diego, CA, USA

⁵Hospital & Institute of Hepatobiliary Surgery, Chinese PLA General Hospital, Beijing, China

SUMMARY

Allograft rejection remains a major limitation to successful solid organ transplantation. Here, we investigated the biosynthesis and bioactions of the pro-resolving mediators lipoxin A₄ and resolvin E1 in host responses to organ transplantation. In samples obtained during screening bronchoscopy after human lung transplantation, bronchoalveolar lavage fluid levels of lipoxin A₄ were increased in association with the severity of allograft rejection that was graded independently by clinical pathology. Lipoxin A₄ significantly inhibited calcineurin activation in human neutrophils, and lipoxin A₄ stable analogs prevented acute rejection of vascularized cardiac and renal allografts. Transgenic animals expressing human lipoxin A₄ receptors revealed important sites of action in host tissues for lipoxin A₄'s protective effects. Resolvin E1 displays counter-regulatory actions for leukocytes, in part, via increased lipoxin A₄ biosynthesis, yet RvE1 administered (1 μg, iv) to donor (days -1 and 0) and recipient mice (day -1, 0 and +4) was even more potent than a lipoxin stable analog (1 μg, iv) in prolonging renal allograft survival (median survival time = 74.0 days with RvE1 and 37.5 days with a LXA₄ analog). Together, these results highlight the potential for pro-resolving mediators in prolonging survival of solid organ transplants.

INTRODUCTION

Allograft rejection remains a major obstacle to the long-term success of organ transplantation [1]. Transplanted tissues are subject to surgical trauma, ischemia-reperfusion

© 2010 Elsevier Ltd. All rights reserved.

#Address correspondence to: Dr. Bruce D. Levy, Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA 02115, Tel – 617-525-5407, Fax – 617-525-5413, Blevy@partners.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

DISCLOSURES

BDL is a co-inventor on patents on the use of lipoxins and resolvins in lung disease that are assigned to Brigham and Women's Hospital and have been licensed for clinical development. CNS is inventor on composition of matter patents owned by BWH and licensed for clinical development. CNS is also a founder of Resolvix Pharmaceuticals and retains founder stock.

injury and insult produced by the immune response [2]. The interaction of infiltrating leukocytes with resident cells of the transplanted tissues stimulates parenchymal cells to generate cytokines and incite an inflammatory response. Neutrophils (PMNs) and innate immune responses play pivotal roles in early allograft survival and the development of chronic rejection of transplanted lung in the form of bronchiolitis obliterans syndrome (BOS) [3,1]. In the kidney, tubular epithelial cells are a rich source of cytokines and chemokines that can promote renal inflammation. Together, infiltrating PMNs and epithelial cells determine the extent of interstitial inflammation and local tissue damage (reviewed in [4]).

Lipid mediators are potent regulators of leukocyte responses [5] and leukotriene B₄ (LTB₄) is linked to the pathophysiology of BOS in chronic allograft rejection [6]. Lipoxins (LXs) are a distinct class of lipoxygenase-derived eicosanoids biosynthesized in human and murine lung [7,8] and kidneys [9]. Unlike LTB₄, LXs promote resolution of acute inflammation and injury [10]. LXs were the first compounds identified in a new genus of mediators that carry both anti-inflammatory and pro-resolving actions that now also include the resolvins and protectins (reviewed in [10]). Because immune-mediated rejection remains a major impediment to the long-term success of transplanted solid organs, we sought to determine whether LXs were present in transplanted tissues and if either LXs or resolvins could decrease local tissue inflammatory responses that can limit the success of allografts.

MATERIALS AND METHODS

Materials

Lipoxin A₄ (LXA₄, 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid) was from Calbiochem (La Jolla, CA) and resolvin E1 (RvE1, 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid) was from Cayman Chemical (Ann Arbor, MI).

Lipoxin A₄ in BALFs

Bronchoalveolar lavage fluids (BALFs) were obtained under a protocol approved by the Brigham and Women's Hospital Committee for the Protection of Human Subjects in Research from bronchoscopies performed on lung transplant recipients for screening or diagnostic purposes based on clinical criteria (n=21, d=59). LXA₄ was quantitated (pg/mg protein) in BALF cell-free supernatants (200g, 10 min, 4°C) by ELISA (Neogen, Lexington, KY) (as qualified in [11]).

Human PMN Activation of Calcineurin and Inflammatory Mediator Release

Peripheral blood (~60 ml) was obtained by venipuncture from healthy volunteers who denied taking any medications for at least 2 weeks and had given written informed consent to a protocol approved by Brigham and Women's Hospital's Human Research Committee. PMN were isolated from whole blood (as described [12]) and PMN (10–20×10⁶ in HBSS + 1.6 mM CaCl₂) were incubated (15min, 37°C) with LXA₄ (100nM), cyclosporine-A (CSA, 200nM, Biomol, Plymouth Meeting, PA) or vehicle (0.1% ethanol) prior to exposure to TNF-α (50ng/ml, BD Pharmingen, San Diego, CA) for 10min at 37°C. Cellular calcineurin (PP2B) phosphatase activity was measured using a colorimetric assay that detected free-phosphate release by Malachite green assay (Biomol). Results were calculated in pmoles of PO₄/μg of protein and expressed as % inhibition of TNF-α-induced calcineurin activity. For release of inflammatory mediators, PMN (5×10⁶/reaction) were resuspended in RPMI-1640 medium (Invitrogen Corporation), 1% heat-inactivated FBS (Sigma Aldrich, St Louis, MO), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA), and exposed (37°C, 5% CO₂, 15min) to vehicle (medium alone), LXA₄ (100nM) or CSA (200nM) prior to addition of TNF-α (20ng/ml). After 6h, cell-free supernatants were

collected and ELISAs performed for IL-1 β (Cayman Chemical), IL-8 (Diaclone, Stamford, CT), and LTB₄ (Oxford Biomedical Research, Oxford, MI).

Animals

All studies were reviewed and approved by the Harvard Medical Area Animal IRB. Mice were maintained under specific pathogen-free conditions in sterilized micro-isolator cages, and received autoclaved feed and autoclaved, acidified drinking water. Human LXA₄ receptor transgenic animals (ALX-tg) were generated in FVB mice as described [8]. Eight- to 12-wk-old male mice, including human ALX-tg and Non-tg littermate FvB and BALB/cByJ (BALB/c) (Charles River Labs, Wilmington, MA) were used as donors and recipients in heterotopic heart transplantation experiments. Eight- to 12-wk-old male B6 and BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in renal transplantation experiments. Select murine cohorts received a LXA₄ stable analog (ATLa, 15-*epi*-16-*para*-fluoro-phenoxy-LXA₄-methyl ester) that is both chemically more stable than native LXA₄ and resists rapid inactivation [13].

Vascularized heterotopic cardiac transplantation

Murine hearts were transplanted heterotopically as previously described [14]. Hearts were harvested and donor aorta anastomosed to recipient vena cava. All surgical procedures were completed within 60 min. Clinical allograft function was assessed by the presence of a palpable heartbeat. Donor hearts that did not beat immediately after reperfusion or that stopped beating within 24h after transplantation were excluded from analysis (>95% of all grafts functioned at 24h). Upon loss of a palpable heartbeat, allografts were harvested and fixed in 10% neutral buffered formalin for histopathology. After dehydration and paraffin embedding, multiple 5–6 μ m-thick sections were immunostained for the leukocyte common antigen CD45 (1:1000, BD Bioscience, San Diego, CA) or chemically stained with Naphthol AS-D chloroacetate esterase (Sigma Chemical Co.). Area and number of positively staining cells was measured using NIH Image software (original magnification, \times 200).

Renal Transplantation

Kidneys of BALB/c mice were transplanted into fully allogeneic C57BL/6 recipients as in [15]. Briefly, the donor left kidney was isolated by ligating its branch of vessels and the kidney, ureter, and bladder were harvested *en bloc*, including the renal artery, with a small aortic cuff, and the renal vein, with a small cava cuff. The graft was perfused *in situ* with 0.2–0.4 ml cold Ringer's lactate containing heparin. After removal of the left native kidney of each recipient, anastomosis was made between the vascular cuffs of donor kidney and the recipient's abdominal aorta and vena cava using end-to-side suture technique. The total ischemic time averaged 30–40 min. Finally, for anastomosis of the ureter, the recipient bladder was pierced with a 21-gauge needle as in [16], the ureter was pulled through, and the periureteral tissue was stitched to the exterior wall of the bladder. The donor ureter was allowed to retract inside the bladder. The right native kidney was removed after anastomosis. Kidney graft function was followed by daily measurement of blood serum creatinine and blood urea nitrogen (Mega Diagnostics, Los Angeles, CA) and by evaluation of overall animal health.

Statistical analysis

Results are expressed as the mean \pm s.e.m. of more than 3 independent experiments with $n \geq 3$ mice or blood donors in each group. Statistical significance of differences was assessed by a one tailed unpaired Student's t-test and a one-way ANOVA with Bonferroni's multiple comparison post test to compare selected pairs. $P < 0.05$ was set as the level of significance. Survival rate data were analyzed using Fishers exact test and Kaplan Meir survival analyses.

Results

Lipoxin A₄ in human lung transplant rejection

To determine whether LX production was related to the airway inflammation of rejection and/or infection, LXA₄ was monitored (pg/mg protein) in bronchoalveolar lavage fluids (BALFs) from lung transplant recipients (n=21, d=59) that underwent screening or diagnostic bronchoscopy. Patients were assigned a diagnosis based on clinical criteria and the histology of transbronchial lung biopsies performed at the time of BAL. For some subjects, multiple samples were available from bronchoscopies performed after transplantation, so LXA₄ generation could be measured over an interval of several months as their clinical condition changed. Lipoxin A₄ levels were significantly increased during allograft rejection of a moderate or severe degree with either a vascular or airway focus (Figure 1). The increases in LXA₄ during rejection were new, transient and linked to severity (Figures 1A and 1B). Modest elevations in LXA₄ were also present during bronchitis and pneumonia (42.5 ± 8.5 and 53.7 ± 9.0 pg LXA₄/mg protein, respectively; mean ± s.e.m.) (Figure 1B). Similar to earlier reports, histological analyses of transbronchial lung biopsies revealed that in addition to adaptive immune responses, the pathobiology of airway and vascular allograft rejection was also characterized by PMN infiltration (Figure 1C). These results indicate that LXA₄ was present in transplant recipients and may participate in the local tissue pathophysiology of allograft rejection.

LXA₄ and Cyclosporine A regulate human PMN activation via distinct mechanisms

Immunophilins, such as cyclosporine A (CSA), are potent, yet incomplete inhibitors of PMN responses and allograft rejection, as neither acute nor chronic rejection can be entirely prevented with CSA [17]. To determine whether LXA₄ shared CSA's inhibition of calcineurin activity, we exposed freshly isolated human PMNs from healthy subjects to either LXA₄ (100 nM) or CSA (200 nM) prior to activation with TNF α (see Methods). At these concentrations, both LXA₄ and CSA were potent inhibitors of PMN calcineurin activity, leading to approximately 50% and 80% inhibition of calcineurin, respectively (Figure 2A). We next compared the activity of LXA₄ (100 nM) and cyclosporine A (CSA) (200 nM) as inhibitors of TNF α -initiated mediator release by human PMNs. LXA₄ and CSA significantly inhibited LTB₄, IL-8, and IL-1 β production (Figure 2B–2D). However, the magnitude of inhibition for TNF α -initiated IL-8 release was significantly less with CSA than LXA₄ (Figure 2C). These results indicate that LXA₄ and CSA have distinct activity relationships in PMN for calcineurin activity and generation of pro-inflammatory mediators, suggesting that LXA₄'s reduction in PMN activation is transduced via both calcineurin-dependent and independent signaling pathways.

LXA₄ receptor (ALX/FPR2) influences cardiac allograft survival

To determine if the increased LXA₄ levels identified during allograft rejection had protective and pro-resolving actions, we investigated the impact of lipoxin signaling in two independent and well-characterized murine models of solid organ transplant rejection - vascularized cardiac and renal transplantation. For cardiac transplantation, allograft survival after allogeneic transplant of FVB and BALB/c strains was approximately 9 days after surgery (Table 1). Transgenic FVB mice that express human LXA₄ receptors (ALX/FPR2) were prepared earlier (denoted ALX-tg) [8]. When FVB ALX-tg animals served as donors, there was no significant change in allograft survival. In sharp contrast, when FVB ALX-tg mice served as recipients for BALB/c donor organs, there was a significant increase in mean allograft survival (Table 1). Some animals received a metabolically stable analog of the aspirin-triggered-15-epi-LXA₄ (ATLa, 10 μ g) [13] that was used to perfuse the donor organ and then administered (iv) on days 0 (immediately after transplantation) and 1. Over one week after perioperative exposure to ATLa, allograft survival was increased by 30% in ALX-

tg compared to non-transgenic animals. Allograft survival was also significantly longer in this cohort compared to ALX-tg animals that had not received the LXA₄ analog (Table 1). Of note, the ALX transgene was targeted to myeloid cells by a component of the CD11b promoter[8,18] and the beneficial effects for the ALX-tg recipients were not similarly evident for experimental groups with ALX-tg donor hearts. Histological examination of the organs at the time of rejection revealed a marked decrease in leukocytes from ALX-tg mice, in particular PMNs, in cardiac tissues, as determined by immunostaining for CD45 and chemically staining for PMN esterase activity (Figure 3). Together, these results indicate that the LX/ALX axis modulates innate immune responses, such as PMN activation and trafficking, to prolong allograft survival.

Pro-resolving mediators influence kidney allograft rejection

In addition to cardiac transplantation, the effects of ATLa on kidney transplantation were determined in a fully-MHC disparate model (BALB/c kidney into C57BL/6 (B6) recipient). The donor kidney was transplanted with subsequent ligation of the kidney's arteries, veins and ureter (Figure 4), and the recipients' kidneys were both nephrectomized. Transplantation of allogeneic BALB/c kidneys into unmodified 8–10-week old B6 mice results in a medium survival time (MST) of 30.5 days (Figure 5). Pretreatment of BALB/c donor mice with 50mg/kg of LPS i.p. on day –1, results in a significant acceleration of allograft rejection in transplanted kidneys from these mice (MST=8, p<0.002) (Figure 5), emphasizing the importance of inflammation within donor organs for organ rejection.

In order to evaluate potential protective roles for pro-resolving mediators on the transplanted organs, ATLa or RvE1 was administered (1 µg/per mouse, iv) to both donor BALB/c and host C57BL/6 mice on days –1 and 0 prior to allogeneic kidney transplantation. The transplanted recipients received an additional tail vein injection on day +4 after kidney transplantation. Similar to the changes with heterotopic cardiac allograft rejection (Table 1), administration of ATLa led to a modest increase in the mean time to kidney rejection (MST=37.5, p<0.072) (Figure 5). Of interest, approximately 50% of the ATLa-exposed animals displayed a marked increase in survival compared to control animals (Figure 5). RvE1 is a potent inhibitor of PMN chemotaxis, tissue accumulation and activation [19–22] and can increase LXA₄ generation in tissue inflammation (39), so here RvE1's actions on renal allograft survival were determined for comparison to the LXA₄ analog, ATLa. RvE1 led to a significant increase in the time to donor kidney rejection (MST=74, p<0.001) that was nearly double that seen with ATLa. These findings were significant by Kaplan Meir survival analysis (P<0.01) (Figure 5).

DISCUSSION and CONCLUSIONS

Here, we provide the first evidence for LXA₄ within the lungs of transplant patients during allograft rejection. Endogenous levels of LXA₄ correlated with the severity of both airway and vascular rejection, suggesting a pivotal role for this counter-regulatory lipid mediator in regulating immune-mediated attack of the transplanted organ. LXA₄ is a potent regulator and stop signal for PMN migration and activation [23,24] and here decreased TNF α -initiated activation, which is pivotal to early events in organ rejection [14]. In addition, an LXA₄ stable analog and ALX-tg mice provided the means for investigating roles for the LXA₄–ALX axis *in vivo* during solid organ transplant rejection. Current murine models of lung transplant rejection do not adequately replicate the human condition, so we turned to two independent and well-characterized models of vascularized allograft rejection. Myeloid targeted expression of human ALX/FPR2 significantly decreased PMN accumulation and prolonged allograft survival with additional protection provided by concomitant administration of a LXA₄ stable analog. Similar to cardiac transplantation, ATLa increased the MST after allogeneic kidney transplantation. This organ protection was enhanced further

by the pro-resolving mediator RvE1. Together, these findings indicate that LXA₄ is generated during host responses to transplant rejection and both LXA₄ and RvE1 can decrease innate immune responses that give rise to allograft rejection.

There is increasing evidence that PMNs play important roles in host rejection of transplanted solid organs by immune as well as non-immune mechanisms [25–27]. Inhibition of calcineurin activity by CSA and other immunophilins markedly enhance allograft tolerance and can inhibit PMN activation [28,29]. In contrast to CSA, LXA₄ was a less potent inhibitor of calcineurin activity, yet equivalent or more potent in blocking inflammatory mediator release from activated human PMNs. Interleukin-8, IL-1 β and LTB₄ have been implicated in the pathophysiology of lung allograft rejection [27]. In addition, TNF α signaling is an important mediator of acute and chronic cardiac allograft rejection [14]. TNF α increases leukocyte calcineurin activity and activates PMN [30]. LXA₄ and RvE1 display potent anti-inflammatory and pro-resolving actions for PMN enriched tissue inflammation [12,31–33,22], and decrease TNF α initiated PMN trafficking, exudate formation and mediator release [30,19]. In addition to anti-inflammatory properties, lipoxins and resolvins also promote resolution via enhanced macrophage clearance of apoptotic PMNs, chemokines, cytokines and microbial products [34,35,33]. LXs and RvE1 are potent inhibitors of LTB₄-initiated PMN activation[24,20], and, independent of calcineurin regulation, LXs can block polyisoprenyl phosphate remodeling [24] and leukocyte-specific protein-1 phosphorylation[36]. RvE1 does not bind to ALX, but can serve as a receptor level antagonist for LTB₄ at its BLT1 receptor[20]. In view of important roles for LTB₄ and TNF α in allograft rejection[14,6], LXs and resolvins may serve as pivotal local counter-regulatory mediators that carry the added benefit of not increasing the risk for bacterial infection [37,38,22].

Resolvin E1 is an anti-inflammatory and pro-resolving lipid mediator derived from eicosapentaenoic acid that displays potent activities in several mucosal organs, including ocular, oral, and gastrointestinal tissues (reviewed in [10]). In a murine model of asthma, RvE1 accelerated the resolution of allergic airway responses [39]. RvE1's pro-resolving actions are linked, in part, to inhibition of IL-23, IL-6, and IL-17, as well as stimulating endogenous LXA₄ generation [39,40]. Here, RvE1 displayed even greater protection than ATLa in prolonging renal allograft survival. IL-17 is a pivotal mediator for allograft rejection and vasculopathy[41]. Given the important roles that BLT1 receptors serve in the transduction of LTB₄-initiated signals for allograft inflammatory responses[6], RvE1's receptor level antagonism of BLT1 [20] may provide this compound with increased potency for organ protection after allogeneic transplantation. Thus, RvE1 can promote allograft survival via several mechanisms, including inhibition of IL-17 and BLT1 signaling and by stimulating endogenous LXA₄ production. Moreover, the organ protection provided independently by RvE1 and a LXA₄ analog suggests a broader role for mediators that possess both anti-inflammatory and pro-resolving activities in preventing the host inflammatory responses of allograft rejection.

Lipoxin A₄ and its bioactive stable analogs act on ALX/FPR2 receptors (reviewed in [10]) to protect in experimental models of graft versus host disease after bone marrow transplantation [42]. Heterotopic cardiac transplantation with hALX-tg mice indicated that the critical effectors for organ protection were provided by the host as the hALX-tg hearts did not display increased survival time. The host factors are likely to be leukocytes, given the inclusion in the transgene construct of a component of CD11b to direct expression of hALX to murine myeloid cells [18]. ALX receptor signaling markedly inhibits granulocyte locomotion and activation. Important roles for PMN-mediated inflammation are emphasized by the LPS-induced acceleration of renal allograft rejection. Activated neutrophils release cytokines, reactive oxygen species, proteases, myeloperoxidase and other enzymes that can

lead to bystander tissue damage [43]. LTB₄ is a potent PMN attractant and secretagogue [5], and LT inhibition improves allograft function and survival [44,6]. Together, these findings point to important protective roles for pro-resolving mediators in allograft rejection by leukocyte activation.

In summary, the present results demonstrate that LXA₄ formation and actions are linked to the local tissue pathophysiology of allograft rejection. The LXA₄/ATLa stable analog administered in perfusate and by intravenous routes at the time of transplantation regulated immune-mediated events relevant to organ rejection in two separate and independent experimental models of vascularized transplantation with a mode of therapeutic immunomodulation that is distinct from the currently clinical strategy of calcineurin inhibition. The actions of RvE1 were even more potent than ATLa in these transplant systems, highlighting the potential for anti-inflammatory and pro-resolving mediators to serve important regulatory roles in solid organ transplant rejection. Thus, the potent counter-regulatory actions of specialized pro-resolving mediators and their receptors open a novel therapeutic strategy for solid organ transplant rejection that does not involve immune suppression.

Acknowledgments

This work was supported by DK067940 (BN), HL68669 (BDL), DE016191 (BDL, CNS), DK48549 (MAA) and DK07448 (CNS, BN), by The Patricia Welder Robinson Young Investigator Grant of the National Kidney Foundation (BN) and a fellowship from La Fondation de la Recherche Medicale (CB).

References

1. Lechler RI, Sykes M, Thomson AW, Turka LA. Organ transplantation--how much of the promise has been realized? *Nat Med* 2005;11(6):605–613. [PubMed: 15937473]
2. Murray JE. Human organ transplantation: background and consequences. *Science* 1992;256(5062):1411–1416. [PubMed: 1604314]
3. Estenne M, Hertz MI. Bronchiolitis obliterans after human lung transplantation. *Am J Respir Crit Care Med* 2002;166(4):440–444. [PubMed: 12186817]
4. Kumar, V.; Abbas, AK.; Fausto, N.; Aster, J. Robbins and Cotran Pathologic Basis of Disease. 8th ed.. Saunders; 2010.
5. Samuelsson, B. Les Prix Nobel: Nobel Prizes, Presentations, Biographies and Lectures. Stockholm: Almqvist & Wiksell; 1982. p. 153-174.
6. Medoff BD, Seung E, Wain JC, et al. BLT1-mediated T cell trafficking is critical for rejection and obliterative bronchiolitis after lung transplantation. *J Exp Med* 2005;202(1):97–110. [PubMed: 15998790]
7. Lee TH, Crea AE, Gant V, et al. Identification of lipoxin A4 and its relationship to the sulfidopeptide leukotrienes C4, D4, and E4 in the bronchoalveolar lavage fluids obtained from patients with selected pulmonary diseases. *Am Rev Respir Dis* 1990;141(6):1453–1458. [PubMed: 2161628]
8. Levy BD, De Sanctis GT, Devchand PR, et al. Multi-pronged inhibition of airway hyper-responsiveness and inflammation by lipoxin A(4). *Nat Med* 2002;8(9):1018–1023. [PubMed: 12172542]
9. Brady HR, Lamas S, Papayianni A, Takata S, Matsubara M, Marsden PA. Lipoxygenase product formation and cell adhesion during neutrophil-glomerular endothelial cell interaction. *Am J Physiol* 1995;268(1 Pt 2):F1–F12. [PubMed: 7840235]
10. Serhan CN. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annu Rev Immunol* 2007;25:101–137. [PubMed: 17090225]
11. Levy BD, Bertram S, Tai HH, et al. Agonist-induced lipoxin A4 generation: detection by a novel lipoxin A4-ELISA. *Lipids* 1993;28(12):1047–1053. [PubMed: 8121246]

12. Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN. Lipid mediator class switching during acute inflammation: signals in resolution. *Nat Immunol* 2001;2(7):612–619. [PubMed: 11429545]
13. Takano T, Clish CB, Gronert K, Petasis N, Serhan CN. Neutrophil-mediated changes in vascular permeability are inhibited by topical application of aspirin-triggered 15-epi-lipoxin A4 and novel lipoxin B4 stable analogues. *J Clin Invest* 1998;101(4):819–826. [PubMed: 9466977]
14. McKee CM, Defina R, He H, Haley KJ, Stone JR, Perkins DL. Prolonged allograft survival in TNF receptor 1-deficient recipients is due to immunoregulatory effects, not to inhibition of direct anti-graft cytotoxicity. *J Immunol* 2002;168(1):483–489. [PubMed: 11751996]
15. Filep JG, Zouki C, Petasis NA, Hachicha M, Serhan CN. Anti-inflammatory actions of lipoxin A(4) stable analogs are demonstrable in human whole blood: modulation of leukocyte adhesion molecules and inhibition of neutrophil-endothelial interactions. *Blood* 1999;94(12):4132–4142. [PubMed: 10590058]
16. Zhang Z, Schlachta C, Duff J, Stiller C, Grant D, Zhong R. Improved techniques for kidney transplantation in mice. *Microsurgery* 1995;16(2):103–109. [PubMed: 7783600]
17. Knoop C, Haverich A, Fischer S. Immunosuppressive therapy after human lung transplantation. *Eur Respir J* 2004;23(1):159–171. [PubMed: 14738248]
18. Devchand PR, Arita M, Hong S, et al. Human ALX receptor regulates neutrophil recruitment in transgenic mice: roles in inflammation and host defense. *FASEB J* 2003;17(6):652–659. [PubMed: 12665478]
19. Arita M, Bianchini F, Aliberti J, et al. Stereochemical assignment, anti-inflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J Exp Med* 2005;201(5):713–722. [PubMed: 15753205]
20. Arita M, Ohira T, Sun YP, Elangovan S, Chiang N, Serhan CN. Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *J Immunol* 2007;178(6):3912–3917. [PubMed: 17339491]
21. Campbell EL, Louis NA, Tomassetti SE, et al. Resolvin E1 promotes mucosal surface clearance of neutrophils: a new paradigm for inflammatory resolution. *FASEB J* 2007;21(12):3162–3170. [PubMed: 17496159]
22. Seki H, Fukunaga K, Arita M, et al. The anti-inflammatory and proresolving mediator resolvin E1 protects mice from bacterial pneumonia and acute lung injury. *J Immunol* 2010;184(2):836–843. [PubMed: 20007539]
23. Colgan SP, Serhan CN, Parkos CA, Delp-Archer C, Madara JL. Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. *J Clin Invest* 1993;92(1):75–82. [PubMed: 8326019]
24. Levy BD, Fokin VV, Clark JM, Wakelam MJ, Petasis NA, Serhan CN. Polyisoprenyl phosphate (PIPP) signaling regulates phospholipase D activity: a 'stop' signaling switch for aspirin-triggered lipoxin A4. *FASEB J* 1999;13(8):903–911. [PubMed: 10224233]
25. Beeh KM, Kornmann O, Lill J, Buhl R. Induced sputum cell profiles in lung transplant recipients with or without chronic rejection: correlation with lung function. *Thorax* 2001;56(7):557–560. [PubMed: 11413355]
26. Devouassoux G, Drouet C, Pin I, et al. Alveolar neutrophilia is a predictor for the bronchiolitis obliterans syndrome, and increases with degree of severity. *Transpl Immunol* 2002;10(4):303–310. [PubMed: 12507402]
27. Wilkes DS, Egan TM, Reynolds HY. Workshop on Lung Transplantation: Opportunities for Research and Clinical Advancement. *Am J Respir Crit Care Med* 2005;14:14.
28. Hendey B, Klee CB, Maxfield FR. Inhibition of neutrophil chemokinesis on vitronectin by inhibitors of calcineurin. *Science* 1992;258(5080):296–299. [PubMed: 1384129]
29. Lawson MA, Maxfield FR. Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature* 1995;377(6544):75–79. [PubMed: 7544874]
30. Hachicha M, Pouliot M, Petasis NA, Serhan CN. Lipoxin (LX)A4 and aspirin-triggered 15-epi-LXA4 inhibit tumor necrosis factor 1 α -initiated neutrophil responses and trafficking: regulators of a cytokine-chemokine axis. *J Exp Med* 1999;189(12):1923–1930. [PubMed: 10377187]

31. Arita M, Yoshida M, Hong S, et al. Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proc Natl Acad Sci U S A* 2005;102(21):7671–7676. [PubMed: 15890784]
32. Fukunaga K, Kohli P, Bonnans C, Fredenburgh LE, Levy BD. Cyclooxygenase 2 plays a pivotal role in the resolution of acute lung injury. *J Immunol* 2005;174(8):5033–5039. [PubMed: 15814734]
33. Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* 2007;447(7146):869–874. [PubMed: 17568749]
34. Godson C, Mitchell S, Harvey K, Petasis NA, Hogg N, Brady HR. Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. *J Immunol* 2000;164(4):1663–1667. [PubMed: 10657608]
35. Ariel A, Fredman G, Sun YP, et al. Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat Immunol* 2006;7(11):1209–1216. [PubMed: 17013391]
36. Ohira T, Bannenberg G, Arita M, et al. A stable aspirin-triggered lipoxin A4 analog blocks phosphorylation of leukocyte-specific protein 1 in human neutrophils. *J Immunol* 2004;173(3):2091–2098. [PubMed: 15265945]
37. Canny G, Levy O, Furuta GT, et al. Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia. *Proc Natl Acad Sci U S A* 2002;99(6):3902–3907. [PubMed: 11891303]
38. Spite M, Norling LV, Summers L, et al. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature* 2009;461(7268):1287–1291. [PubMed: 19865173]
39. Haworth O, Cernadas M, Yang R, Serhan CN, Levy BD. Resolvin E1 regulates interleukin 23, interferon-gamma and lipoxin A4 to promote the resolution of allergic airway inflammation. *Nat Immunol* 2008;9(8):873–879. [PubMed: 18568027]
40. Merched AJ, Ko K, Gotlinger KH, Serhan CN, Chan L. Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators. *FASEB J* 2008;22(10):3595–3606. [PubMed: 18559988]
41. Yuan X, Paez-Cortez J, Schmitt-Knosalla I, et al. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J Exp Med* 2008;205(13):3133–3144. [PubMed: 19047438]
42. Devchand PR, Schmidt BA, Primo VC, et al. A synthetic eicosanoid LX-mimetic unravels host-donor interactions in allogeneic BMT-induced GvHD to reveal an early protective role for host neutrophils. *FASEB J* 2005;19(2):203–210. [PubMed: 15677343]
43. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989;320(6):365–376. [PubMed: 2536474]
44. Spurney RF, Ibrahim S, Butterly D, Klotman PE, Sanfilippo F, Coffman TM. Leukotrienes in renal transplant rejection in rats. Distinct roles for leukotriene B4 and peptidoleukotrienes in the pathogenesis of allograft injury. *J Immunol* 1994;152(2):867–876. [PubMed: 8283057]

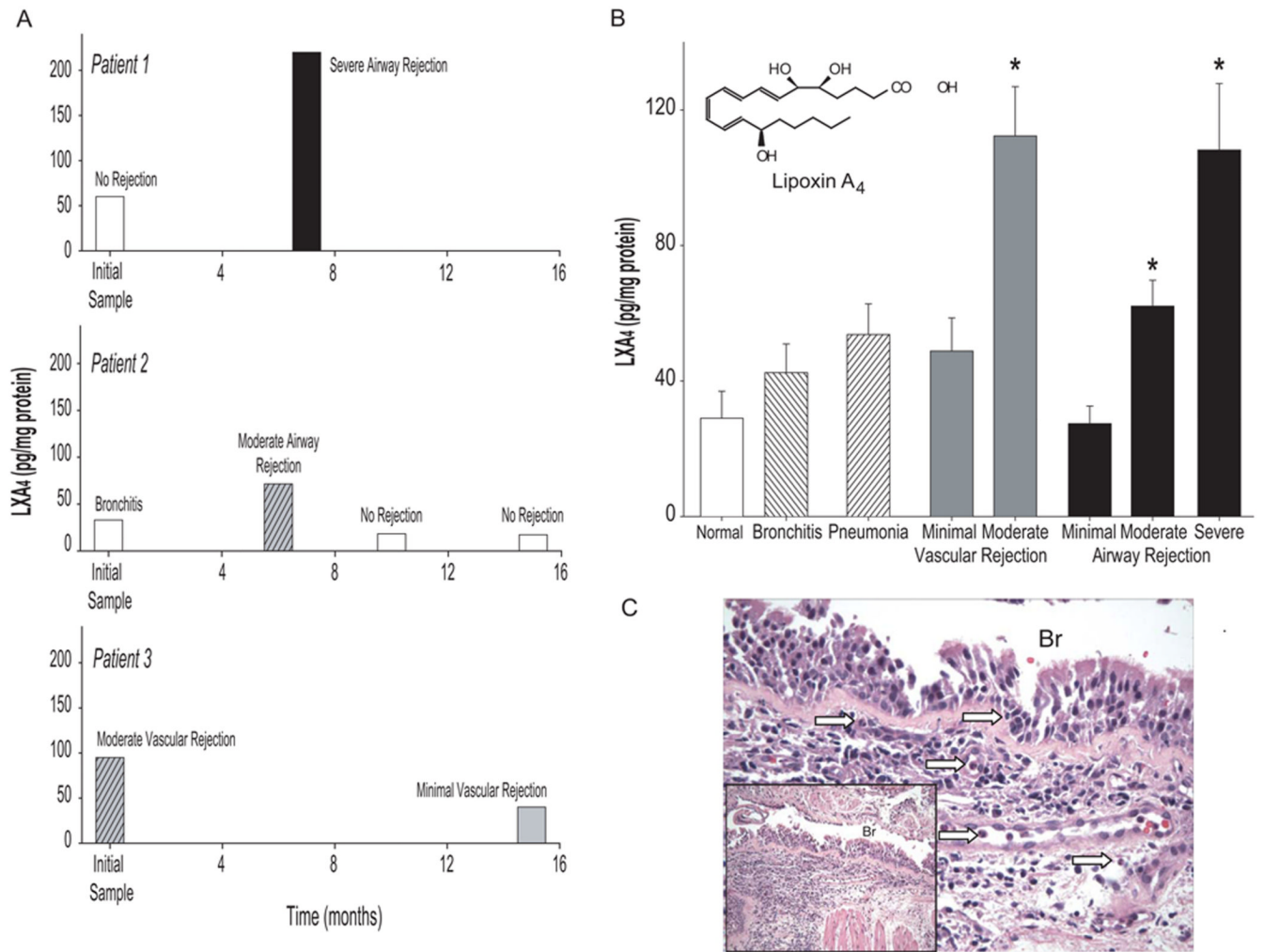


Figure 1. Lipoxin A₄ levels increase during lung allograft rejection

(a) LXA₄ levels in bronchoalveolar lavage fluids performed for clinical indication after lung transplant and (b) correlated with clinical diagnosis. Values represent the mean \pm S.E. for $n \geq 3$, $d=2$. * $P < 0.05$ by Student's t-test compared to the absence of pathology. (c) Representative histopathology for severe airway rejection from Patient 1. Arrows denote representative PMN.

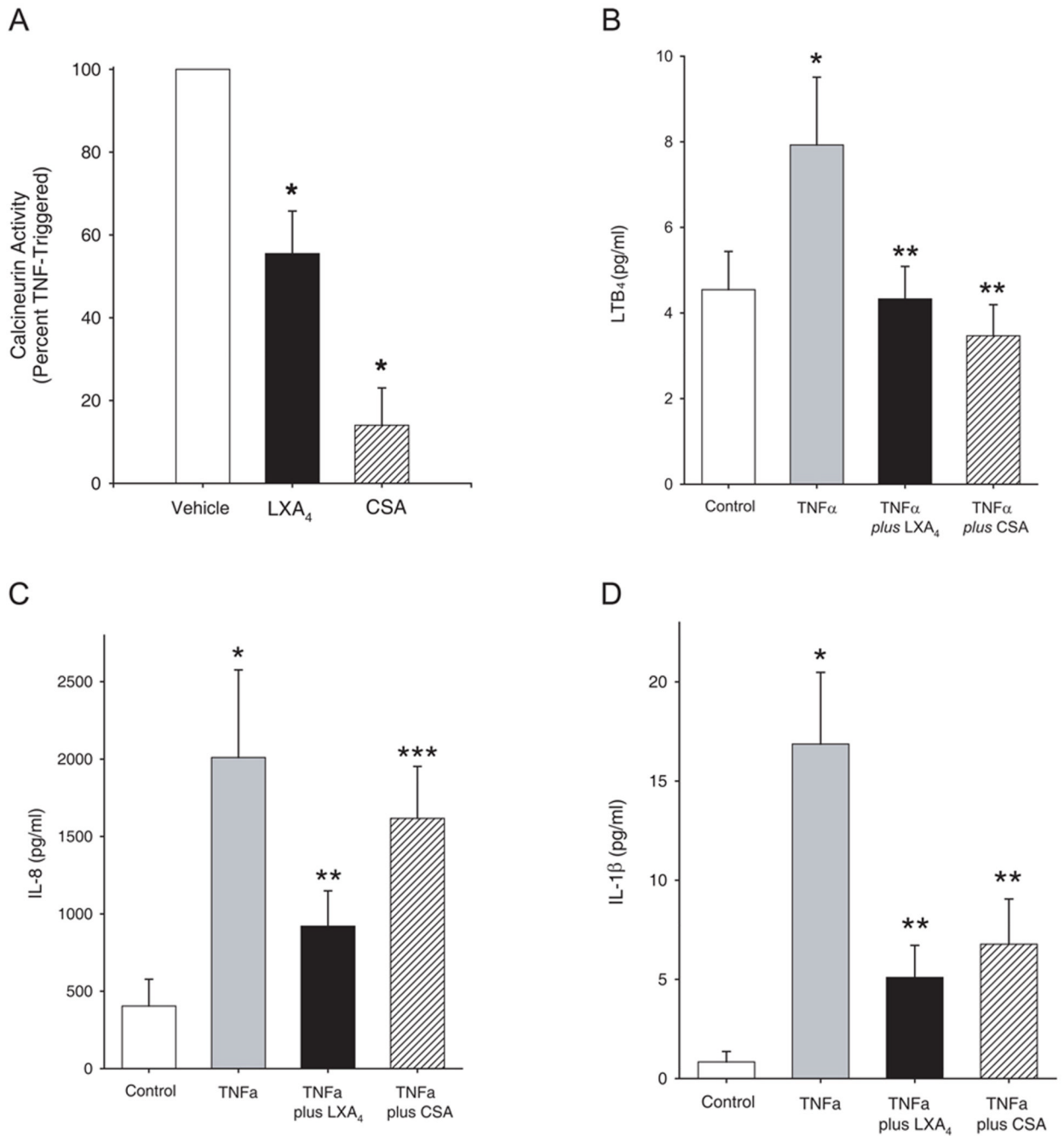


Figure 2. Regulation of human PMN activation by lipoxin A₄ involves calcineurin dependent and independent pathways

Freshly isolated human PMN from healthy donors were exposed (5 min, 37°C) to LXA₄ (100nM), cyclosporin A (200 nM) or vehicle (0.1% ethanol) prior to TNF α (10 ng/ml) and (a) calcineurin activity and (b–d) cytokine release were determined (see Methods). Values reported are the mean \pm S.E. for $n \geq 3$ separate PMN donors. * $P < 0.01$ (as compared to vehicle), ** $P < 0.05$ (as compared to TNF α) and *** $P < 0.05$ (for CSA compared to LXA₄).

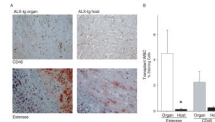


Figure 3. Lipoxin Receptor signaling dampens neutrophil accumulation in cardiac allograft rejection

Heterotopic cardiac transplants were performed (see Methods). When a transplanted heart pulse was no longer palpable, allografts were removed. (a) Representative heart tissue sections (magnification: $\times 200$) were obtained from formalin-fixed, paraffin-embedded tissue, prepared and stained for CD45 (top row) or esterase (bottom row). (b) Tissue morphometric analyses were performed to determine the impact of ALX-tg expression on PMN accumulation in cardiac tissues. Values reported are the mean \pm S.E. for more than 3 independent experiments with $n \geq 3$ mice in each group. * $P < 0.05$ (ALX-tg host vs. donated organ)

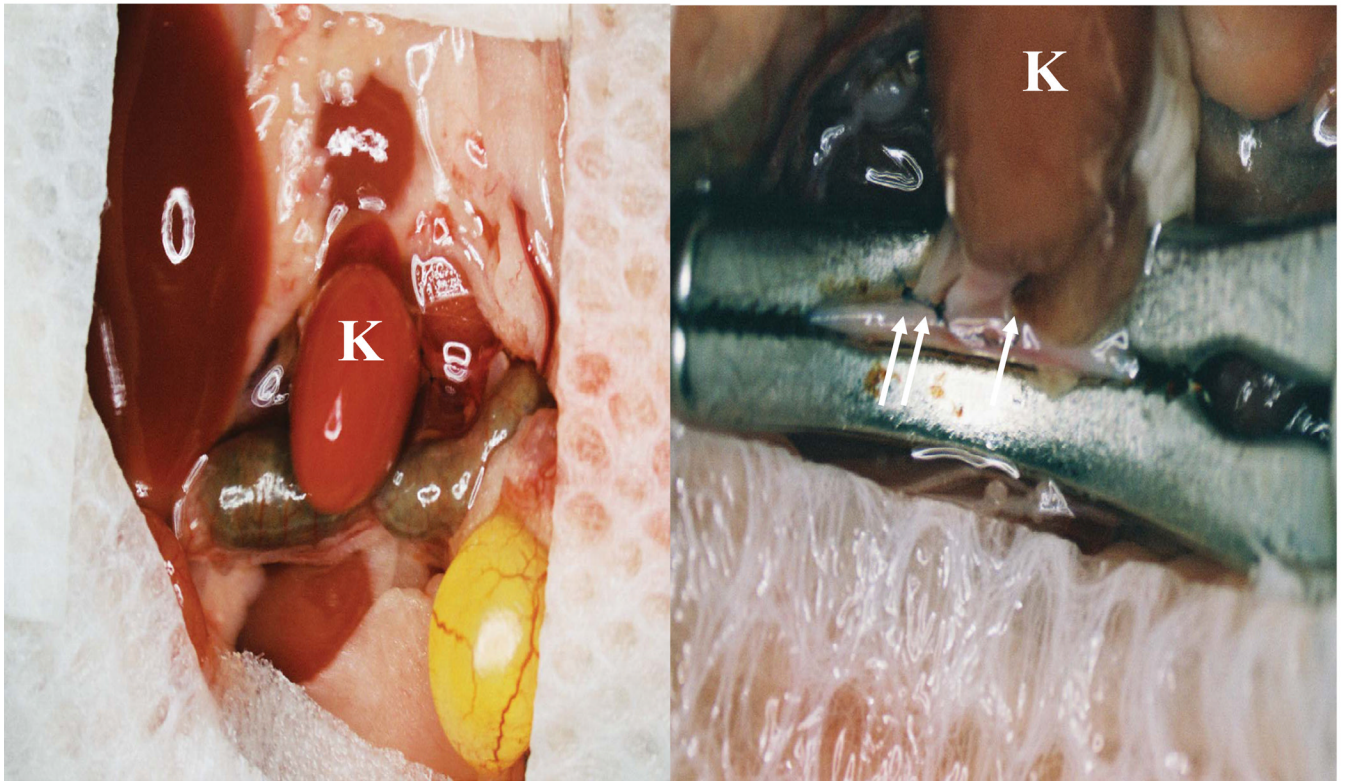


Figure 4. Kidney transplantation in mice

A syngeneic B6 or allogeneic BALB/c kidney was transplanted into B6 recipients. The transplantation procedure involved transplantation of the donor kidney (K) and ligation (arrows) of the kidney's arteries, veins and ureter. In the same procedure the recipients' kidneys were both nephrectomized.

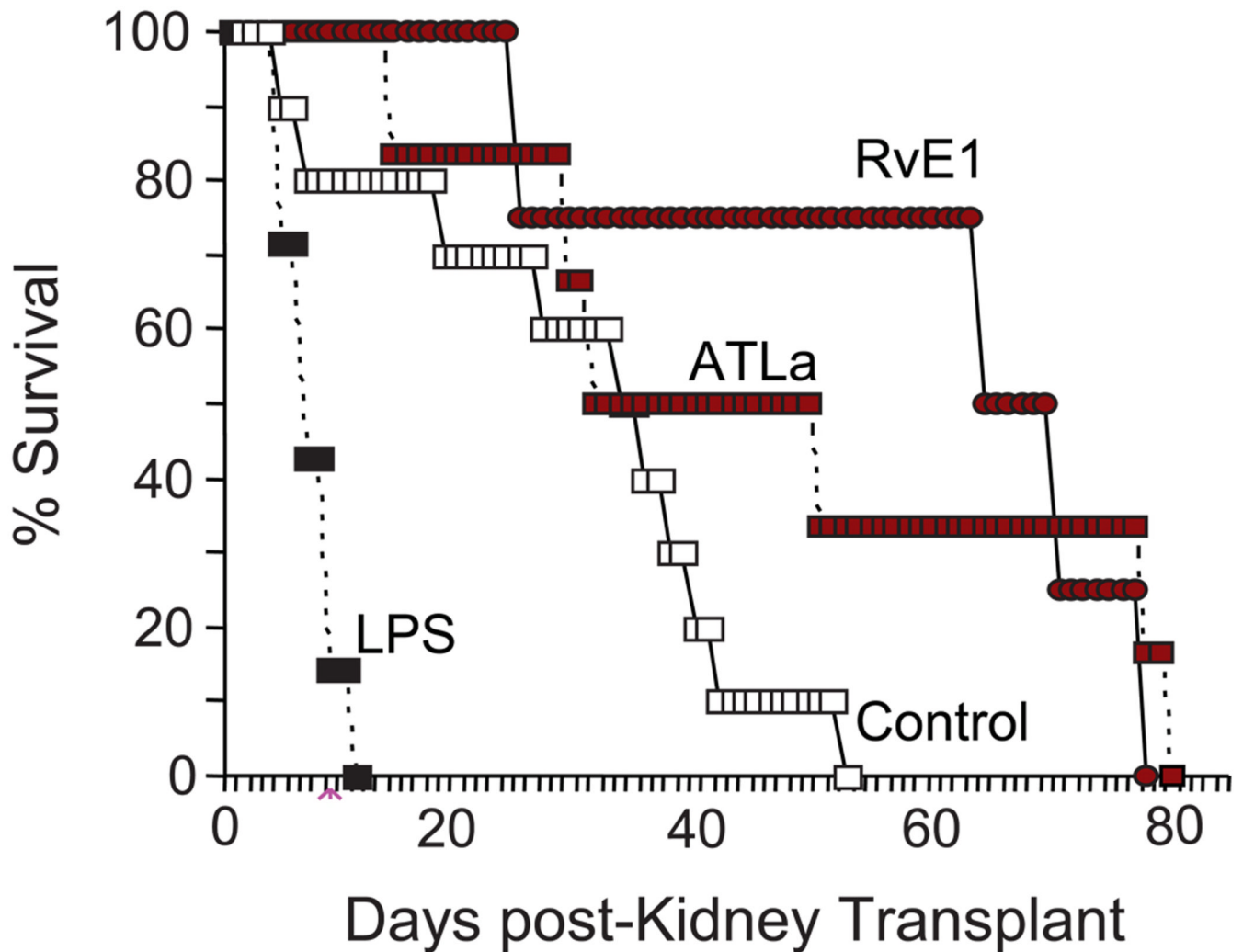


Figure 5. LXA₄ and RvE1 enhance the survival of renal allografts

Allogeneic BALB/c kidneys were transplanted into B6 mice. The groups of mice received vehicle (Control, white squares), LPS (50 mg/kg) pretreatment (LPS, n=7, black squares), LXA₄ stable analog on days -1, 0 and +4 (ATLa, red squares, dashed line) or RvE1 on days -1, 0 and +4 (RvE1, red circles) and allograft survival was determined (See Methods). Survival of allografts was prolonged when donors and recipients were treated with ATLa (n=6) or RvE1 (n=6) before or at the early stage of transplantation compared with the untreated control group (n=10, p<0.01) and significant by Kaplan Meir multiple comparisons survival analysis (P<0.01).

Table 1

ALX-tg recipients have increased cardiac allograft survival

Donor Strain	Recipient Strain	Lipoxin A ₄ Analog	Allograft Survival (d)	Mean +/- SE
FVB	BALB/c	-	8,8,9,9,9	8.6 +/- 0.3
FVB ALX-tg	BALB/c	-	9,9	9.0
FVB ALX-tg	BALB/c	+	8,9,10	9.0 +/- 0.6
BALB/c	FVB	-	7,9,9,10,10	9.0 +/- 0.6
BALB/c	FVB ALX-tg	-	10,10,11,11,11	10.6 +/- 0.3*
BALB/c	FVB ALX-tg	+	11,12,12	11.7 +/- 0.3*,†

Heterotopic cardiac transplants were performed as described in Materials and Methods. Allograft survival was assessed as the number of days that a pulse could be palpated in the allograft. Mean survival time in days of allografts in each experimental group.

*P<0.05 for FVB ALX-tg compared to FVB organ recipient and

†P<0.05 for comparison between ALX-tg with and without LXA₄ analog (ATLa).