

## 学位論文

「Leukotriene B4 type-1 receptor signaling promotes liver repair after hepatic  
ischemia/reperfusion injury through the enhancement of macrophage recruitment  
(ロイコトリエンB4 type-1受容体シグナルは  
マクロファージの集積増強により肝虚血再灌流障害後肝修復を促進する)」

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## 著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

## 【要旨】

肝虚血再灌流障害からの肝修復過程にはマクロファージが関与している。ロイコトリエン B4(LTB4)はマクロファージの走化因子であり、その受容体 BLT1 はマクロファージに発現している。そこで、肝虚血再灌流障害後の肝修復に果たす BLT1 受容体の役割について検討した。

BLT1 knockout mouse (BLT1<sup>-/-</sup>) または野生型マウス (WT) に 70%肝部分虚血を行い、再灌流後の肝修復について比較検討した。BLT1<sup>-/-</sup>は WT と同様に再灌流 24 時間後に肝障害のピークを迎えた。しかしながらその後の肝修復や肝細胞増殖発現が遅延し、肝再生因子である EGF を産生するマクロファージの障害肝への集積が抑制された。また WT に EGF 中和抗体を投与すると、肝修復過程やマクロファージ集積が抑制された。

BLT1 受容体シグナルは肝虚血再灌流後に障害肝へマクロファージを集積させて肝再生因子 EGF を産生して肝組織修復を促進させる役割を果たしている可能性が示唆された。

## 【背景】

肝虚血再灌流は肝切除術、肝移植術、hypovolemic shock などでは回避できないものであり、その後の再灌流肝障害は臨床的に問題となる。肝虚血再灌流障害も重大な要素であるが、虚血再灌流は同時に、傷害肝組織の修復、炎症収束、壊死組織の除去、肝類洞の再構築や肝再生などの修復反応を引き起こす。従って肝虚血再灌流障害からの肝再生・修復過程が遅延すると術後肝障害ひいては肝不全に至り患者の予後を規定することになる。

急性肝障害後の肝組織修復にはマクロファージの関与が示唆されている<sup>1)</sup>。我々も、アセトアミノフェンや四塩化炭素などによる薬剤誘導性肝障害モデルを用い、肝組織修復過程にはマクロファージの動員が重要であることを報告した<sup>2)3)</sup>。

ロイコトリエン B4(LTB4)はマクロファージが炎症巣に集積するための強力な走化因子のひとつとして知られている。LTB4 はアラキドン酸から 5 リポオキシゲナーゼの作用により、合成される。この LTB4 に対する受容体には高親和性の BLT1 と低親和性の BLT2 がある。BLT1 の発現は好中球やマクロファージに高発現し、他臓器に発現せず、炎症や虚血再灌流に関与している。

そのため、肝虚血再灌流障害からの肝修復過程には BLT1 受容体シグナルを介したマクロファージの集積が関与しているものと考えられ、本研究では肝虚血再灌流後の肝修復過程における BLT1 受容体シグナルの役割を解明した。

## 【方法】

### (1) 肝虚血再灌流

7~9 週令の雄性 BLT1 knockout mouse (BLT1<sup>-/-</sup>) または野生型マウス (WT) に 70%肝部分温虚血 (60 分間)を行った。再灌流後 6、24、48、96 時間の血清 ALT、肝壊死面積、PCNA 染色、肝組織 mRNA 発現(real time RT-PCR)、蛍光免疫染色などの経時的変化を比較検討した。

### (2) EGF中和抗体投与

WTの腹腔内にEpidermal growth factor(EGF)中和抗体(1.0 mg/kg body weight; R&D Systems, Minneapolis, MN, USA)を投与し、再灌流後48時間における同様の検討を行った。コントロールは、goat immunoglobulin G (IgG)を腹腔内投与した。

### (3) 腹腔マクロファージ

雄性BLT1 knockout mouse (BLT1<sup>-/-</sup>) または野生型マウス (WT) に4%チオグリコレート(Nissui Pharmaceutical, Tokyo, Japan)を腹腔内投与し、2×5mlのPBSで腹腔内マクロファージを回収した。RPMI 1640 mediumで1wellあたり2×10<sup>6</sup>cells/wellとし6-well tissue cultureで1時間の接着を行った。6-well としLTB4(Cayman Chemical, Ann Arbor, MI, USA)で刺激し、培養実験を行った。

## 【結果】

### (1) 肝修復・肝細胞増殖

WTの肝臓におけるBLT1 mRNA発現は、再灌流後24時間(13.5倍)と48時間(6.5倍)で有意に増加した。5LOX mRNAについても24時間(14.5倍)をピークに48時間(13.2倍)でも有意に増加したものの群間での差は認めなかった。

血清ALT値を測定したところ、WTで再灌流後6時間後にピーク(30673±6799U/L)となり、以後減少し96時間後でシャムレベルまで戻った。BLT1<sup>-/-</sup>においても、6時間後にピークとなり24時間後ではWTと有意差は認めなかった。しかしながら、48時間と96時間後のレベルはBLT1<sup>-/-</sup>に対し1.9倍と2.2倍と有意に増加した。

肝壊死面積はWTで24時間32%壊死、48時間28%壊死、96時間15%壊死と推移した。BLT1<sup>-/-</sup>において24時間31%壊死、48時間41%壊死、96時間40%と48時間と96時間で有意に高値を示した。これらの結果から、BLT1受容体シグナルは再灌流障害の成立機序には関与せず、肝組織修復に関与していることが示唆された。

肝細胞増殖をPCNA染色で評価すると、BLT1<sup>-/-</sup>でPCNA陽性率の増加がWTより遅延し、BLT1シグナルが再灌流後の肝細胞増殖に関与していることが示唆された。

### (2) 肝再生因子

次に肝組織修復のメカニズムを解析するために肝再生に関連した増殖因子をPCR法にて検討した。IL-6やTNFはBLT1<sup>-/-</sup>でむしろその発現は増加しており、再生・増殖に関係するというよりも障害に関係しているものと推察された。HGFは両マウスに差はなかった。EGFはWTでBLT1<sup>-/-</sup>よりも再灌流後6時間で4.2倍、24時間後で4.6倍、48時間後で3.8倍増強した。このことは、BLT1シグナルはEGFを増強させて肝組織修復を促進している可能性を示唆した。血管新生因子の関与を調べるとVEGF-Aは6時間で6.2倍、VEGFR1は24時間と48時間で9倍と4.1倍とそれぞれ、WTで有意に増強した。以上から、肝虚血再灌流障害後の肝修復にはEGFとVEGFR1の関与が示唆された。

### (3) 集積マクロファージ

マクロファージの関与を免疫蛍光染色にて検討した。常在マクロファージのマーカーであるF4/80は虚血再灌流後に減少し、徐々に増加したが群間には差は認めなかった。集積マクロファージマーカーであるCD11bはWTで増加したが、BLT1<sup>-/-</sup>で減少した。また好中球マーカーであるLy6Bは両群で増加したが群間では差はなかった。VEGFR1陽性細胞数はWTで増加しBLT1<sup>-/-</sup>で減少した。このことから、CD11bとVEGFR1陽性細胞の関与が示唆された。

さらに免疫蛍光染色の共染色では、マクロファージに発現するVEGFR1は、CD11bと共染色さ



れたが、F4/80やLy6Bとの重なりほとんどなかったことから、VEGFR1陽性細胞はCD11b陽性マクロファージと考えられた。また、VEGFR1陽性マクロファージがBLT1<sup>-/-</sup>において減少したことから、BLT1シグナルは好中球だけでなく、VEGFR1陽性マクロファージを集積させる役割を果たしている可能性が示唆された。

更にマクロファージがEGFを産生しているかどうかを調べた。すると、EGF陽性細胞数はVEGFR1陽性細胞数と同様、WTで再灌流48時間後に増加しBLT1<sup>-/-</sup>で減少した。このEGF陽性細胞はVEGFR1と共在し、VEGFR1マクロファージからEGFが産生される可能性が示唆された。

#### (4) 肝修復に対するEGF効果

そこで、実際にEGFが肝修復に寄与しているかを調べるためにEGF中和抗体を投与した。再灌流後48時間のALTは2.1倍、壊死面積は1.5倍、出血壊死面積は3.1倍と抗体群が対照群より高値を示し、PCNA比は低値を示した。すなわち、EGFは肝組織修復を促進させた。さらに、マクロファージの集積効果を検討すると、抗体群で、CD11b陽性細胞とVEGFR1陽性細胞の傷害肝への誘導が抑制された。

#### (5) 腹腔マクロファージの増殖因子産生

BLT1シグナルがマクロファージからEGFを産生しているかどうか腹腔マクロファージを用いて確認した。WTマクロファージはLTB4刺激により濃度依存性にVEGFR1、EGF、VEGFAの発現が増強した。一方、BLT1<sup>-/-</sup>では、発現増強はなかった。

#### 【考察】

肝虚血再灌流障害後に肝修復・再生過程が遅延あるいは破綻すると、術後肝障害ひいては肝不全となり重大な事象となる。肝虚血再灌流障害や薬剤性肝障害などの急性肝障害からの肝組織修復や肝再生にはマクロファージが重要な役割を果たしている<sup>1)</sup>。我々も最近、アセトアミノフェンや四塩化炭素などによる薬剤誘導性肝障害モデルを用い、肝組織修復過程には傷害を受けた肝にマクロファージが集積することが重要であることを報告した<sup>2) 3)</sup>。しかしながら、その制御機構については十分な解明はなされていない。

LTB4はマクロファージを含む白血球の強力な走化性因子として知られ、LTB4レセプター1型(BLT1)はマクロファージ上に発現している。LTB4/BLT1シグナルは、肺の感染症<sup>4)</sup>や腹膜炎<sup>5)</sup>などの急性炎症モデルにおいてマクロファージの炎症巣への誘導に関与している。BLT1シグナルによって誘導されるマクロファージが、肝虚血再灌流障害後の肝修復や肝再生に寄与するかどうかは不明であった。本研究では、BLT1シグナリングが肝虚血再灌流障害で障害を受けた部位にマクロファージを誘導し、EGF発現の増強を介して肝修復を促進することを示した。

肝虚血再灌流では主として常在マクロファージであるクッパー細胞や集積した好中球からLTB4が産生される<sup>6)</sup>。その後、BLT1シグナルによって、マクロファージが傷害肝に集積するものと考えられる。BLT1シグナルは肝修復作用を示したが、肝再生因子VEGFとその受容体

VEGFR1 が増強していた。VEGF は血管新生や組織再生に深く関与している。また VEGFR1 はマクロファージに発現し、その VEGFR1 陽性マクロファージは炎症部位に集積して<sup>2,7)</sup>、肝修復を促進させる<sup>2)</sup>。そこで、本研究でも VEGFR1 発現を検討したところ、傷害肝に VEGFR1 陽性マクロファージが集積し、その集積は BLT1 シグナルに依存していた。さらに、VEGFR1 陽性マクロファージは EGF を発現した。EGF は肝再生に必須のメディエーターであることは知られているので、EGF が肝虚血再灌流後の肝修復に関わっていることを確認するために、WT に EGF 中和抗体を投与した。すると、EGF 抗体投与により、肝修復の遅延、肝細胞増殖の減弱、マクロファージ集積の抑制などがみられた。

さらに、マクロファージ上の VEGFR1 発現や、マクロファージからの EGF 発現が確かに BLT1 シグナルを介しているかどうかを、単離腹腔マクロファージを用いて調べた。LTB4 刺激によりマクロファージに VEGFR1 や EGF が発現し、BLT1<sup>-/-</sup>ではそれらの発現が抑制された。なお、BLT1 シグナルは NFκB を介して EGF 産生している可能性を我々は確認した。

以上から、BLT1 シグナルは VEGFR1 陽性マクロファージを傷害肝に集積させ EGF を産生することによって、肝虚血再灌流後の肝修復ならびに肝再生を促進させることが示唆された。

#### 【引用文献】

- 1) Laskin DL: Macrophages and inflammatory mediators in chemical toxicity: a battle of forces. *Chem Res Toxicol* 22: 1376-1385, 2009.
- 2) Kato T, Ito Y, Kanako H, et al, Vascular endothelial growth factor receptor-1 signaling promotes liver repair through restoration of liver microvasculature after acetaminophen hepatotoxicity. *Toxicol Sci.* 120, 218-229, 2011.
- 3) Minamino, T., Ito, Y., Ohkubo, H., et al.: Thromboxane A(2) receptor signaling promotes liver tissue repair after toxic injury through the enhancement of macrophage recruitment. *Toxicol Appl Pharmacol* 259(1): 104-114, (2012)
- 4) Mancuso, P., Lewis, C., Serezani, C. H., et al.: Intrapulmonary administration of leukotriene B4 enhances pulmonary host defense against pneumococcal pneumonia. *Infect Immun* 78(5): 2264-2271, (2010)
- 5) Matsukawa, A., Hogaboam, C. M., Lukacs, N. W., et al.: Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4. *Immunol* 163(11): 6148-6154, (1999)
- 6) Jaeschke H. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. *Am J Physiol Gastrointest Liver*

Physiol. 290(6):G1083-1088, (2006).

7) Shibuya M: Vascular endothelial growth factor-dependent and -independent regulation of angiogenesis.  
BMB Rep. 41: 278-286, (2008).

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## **Abstract**

Recruited macrophages play a critical role in liver repair and regeneration after acute liver injury. Leukotriene B4 (LTB4) is a potent chemoattractant for leukocytes, including macrophages. In this study, we investigated the role of LTB4 receptor type 1 (BLT1) in liver repair after hepatic ischemia/reperfusion (I/R) injury. BLT1-knockout mice (BLT1<sup>-/-</sup>) or their wild-type counterparts (WT) were subjected to partial hepatic I/R. Compared with WT, BLT1<sup>-/-</sup> mice exhibited delayed liver repair and hepatocyte proliferation. This was accompanied by suppressed recruitment of macrophages and attenuated hepatic expression of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and VEGF receptor 1 (VEGFR1). Disruption of BLT1 signaling also reduced the expression of EGF on recruited macrophages expressing VEGFR1 in the injured liver. Treatment of WT mice with an EGF neutralizing antibody delayed liver repair, inhibited hepatic EGF expression, and reduced the recruitment of macrophages. In addition, BLT1 signaling enhanced the expression of VEGF, VEGFR1, and EGF in isolated peritoneal macrophages in vitro. These results indicate that BLT1 signaling plays a role in liver repair during hepatic I/R through enhanced expression of EGF in recruited macrophages. The development of a specific agonist for BLT1 could be useful for liver recovery from acute liver injury.

LT, leukotriene; BLT, leukotriene B4 receptor type 1; I/R, ischemia/reperfusion; WT, wild-type; ALT, alanine transaminase; PCNA, proliferating cell nuclear antigen; VEGFR, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; EGF, epidermal growth factor; LOX, lipoxygenase; TK, tyrosine kinase; IL, interleukin; TNF, tumor necrosis factor; HGF, hepatocyte growth factor; TGC, thioglycollate; NF- $\kappa$ B, nuclear factor kappa B

## **Introduction**

Hepatic ischemia-reperfusion (I/R) injury is an unavoidable consequence of partial hepatectomy, liver transplantation, and trauma surgery. During the evolution of hepatic I/R injury, the recruitment of activated neutrophils in the liver is crucial for parenchymal cell injury mediated by the release of oxidants and proteases (1). Ischemic liver injury also induces tissue repair, a process that includes inflammation, tissue resorption, and remodeling, as well as hepatocyte proliferation (2,3). Impaired liver regeneration and liver dysfunction have been strongly linked to the final outcomes of patients subjected

to liver surgery.

Although liver injury and regeneration after acute liver injury have been described separately, the inflammatory response to hepatic I/R, including hepatocellular regeneration and recruitment of blood elements, such as platelets, is of great importance (2,3). Liver regeneration is controlled by a variety of cytokines and growth factors (4). Vascular endothelial growth factor (VEGF)-A and its receptors, VEGFR1 and VEGFR2 (5,6), promote the repair and regeneration of the liver after damage resulting from acute insults, including liver resection and exposure to toxicants (7,8). Furthermore, recent evidence suggests that recruited macrophages are crucial for liver repair after toxin-induced acute liver injury (9-11). We have also shown that the recruitment of macrophages into the liver following injury elicited by acetaminophen or carbon tetrachloride is essential for repair of the liver, including the microvasculature (12,13), and that VEGFR1 signaling is important for the recruitment of macrophages to the site of inflammation (6,12,14).

Chemotactic factors are required for the recruitment of macrophages during acute liver injury (15). One of these chemotactic factors, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), is a highly potent lipid chemoattractant that is rapidly produced from membrane phospholipids by the sequential activities of 5-lipoxygenase (5-LOX) and LTA<sub>4</sub> hydrolase (16,17). The potent biological effects of LTB<sub>4</sub> are mediated primarily through a high-affinity interaction with a G protein-coupled receptor termed LTB<sub>4</sub> receptor type-1 (BLT1). BLT1 is expressed in monocytes/macrophages and neutrophils (18,19). Indeed, LTB<sub>4</sub> is a potent chemoattractant that facilitates the recruitment of macrophages/monocytes to inflammatory sites (20,21), and BLT1 signaling has been shown to be responsible for the recruitment of neutrophils into the liver sinusoids during endotoxemia (22) and in response to spinal injury (23). Previous studies have shown that LTB<sub>4</sub> is involved in several inflammatory diseases, including atherosclerosis, immune responses, and I/R injury (20). In a model of warm hepatic I/R, LTB<sub>4</sub> was generated in large quantities during neutrophil-induced injury (24). Nevertheless, treatment with an LTB<sub>4</sub> receptor antagonist failed to suppress hepatic I/R injury (24), suggesting that LTB<sub>4</sub> itself plays a minor role in the development of hepatic I/R injury.

Although BLT1 signaling may not be directly involved in the mechanism of hepatic I/R injury, it remains unknown whether BLT1 signaling facilitates liver repair through the recruitment of macrophages. Therefore, the present study examines the role of BLT1 signaling in liver repair and regeneration during hepatic IR.

## **Materials and Methods**

### **Animals**

Male C57BL/6J wild-type (WT) mice 8 weeks of age were purchased from Crea Japan Inc. (Tokyo, Japan). Male BLT1 knockout mice (8 week old BLT1<sup>-/-</sup> mice) were developed previously (25). A mutant mouse strain lacking the intracellular tyrosine kinase (TK) domain of VEGFR-1 (VEGFR1 TK<sup>-/-</sup>) with a C57Bl6 hybrid background was also developed previously (26). All testing was performed in accordance with the guidelines for animal experiments established by the Kitasato University School of Medicine.

### **Animal Procedures**

Animals underwent either sham surgery or I/R. Partial hepatic ischemia was elicited as described previously (27). Briefly, under anesthesia with pentobarbital sodium solution (50 mg/kg, ip), a laparotomy was performed, and the blood supply to the median and left hepatic lobes was occluded with an atraumatic vascular clamp. Reperfusion was initiated by removal of the clamp. After reperfusion was initiated, 4 ml/kg of physiological saline was injected intraperitoneally, the abdomen was closed with 4-0 silk and wound clips, and the animals were allowed to recover. Sham-control mice underwent the same surgical protocol without vascular occlusion. In some experiments, mice were given a single intraperitoneal (i.p.) injection of anti-EGF antibody (1.0 mg/kg body weight; R&D Systems, Minneapolis, MN, USA) or goat IgG dissolved in PBS 6 h after reperfusion. Blood was drawn and the liver was excised at 6, 24, 48, and 96 h after reperfusion. Serum was used for the determination of alanine transaminase (ALT) activity.

### **Histology and immunohistochemistry**

Excised liver tissues were fixed immediately with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for histological analysis (12,28). Sections (4 µm thick) were prepared from paraffin-embedded tissue and subjected to either hematoxylin and eosin (H&E) staining or immunostaining. To quantify the extent of necrosis, the percentage of necrosis was estimated by measuring the necrotic area relative to the entire histological section, and analysis of the area was performed with a VH analyzer (Keyence, Osaka, Japan). Sections were also stained for proliferating cell nuclear antigen (PCNA) (Invitrogen, Carlsbad, CA, USA) and measured. Each treatment group included six mice per time point. The number of PCNA-positive hepatocytes from 1000

hepatocytes was quantified in six separate low power fields ( $\times 100$ ) for each animal. The percentage of PCNA-positive cells was calculated and the results were expressed as a PCNA-labeling index.

### **Immunofluorescent Staining**

Samples were fixed with periodate-lysine-paraformaldehyde (PLP) fixative at room temperature for 3 h. Following cryoprotection with 30% sucrose/0.1 M phosphate buffer (pH 7.2), cryostat sections approximately 10 to 20  $\mu\text{m}$  thick were cut. Sections were then incubated with 1% bovine serum albumin (BSA)/PBS at room temperature for 1 h to block non-specific binding, followed by incubation with rat anti-mouse F4/80 monoclonal IgG2a antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rat anti-mouse CD11b monoclonal IgG2b antibody (AbD Serotec, Raleigh, NC, USA), anti-mouse Ly6B.2 alloantigen antibody (AbD Serotec, Raleigh, NC, USA), rabbit anti-mouse VEGFR1 polyclonal IgG antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-mouse 5-LOX polyclonal antibody (Novus Biologicals Inc., Littleton, CO, USA), and goat anti-mouse EGF (1:100; R&D Systems, Minneapolis, MN, USA). After washing three times in PBS, the sections were incubated with a mixture of the following secondary antibodies for 1 h at room temperature: Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA), Alexa Fluor® 594-conjugated donkey anti-rat IgG (1:500, Molecular Probes, Eugene, OR, USA), and Texas Red (TR)-conjugated donkey anti-goat IgG (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). As a negative control, sections were incubated in 1% BSA-PBS without primary antibody. Images were captured using a fluorescence microscope (Biozero BZ-9000 Series; KEYENCE, Osaka, Japan). After labeling, six low power optical fields ( $200\times$  magnification) were randomly selected and the number of positive cells was counted. At least five animals were analyzed for each marker.

### **Real-time RT-PCR Analysis**

Transcripts encoding BLT1, 5-LOX, IL-6, HGF, TNF $\alpha$ , EGF, VEGF-A, VEGFR1, VEGFR2, CXCL1, CXCL2, CXCR2, CCL2, CCR2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time RT-PCR. Total RNA was extracted from liver tissue and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified using a BioPhotometer (Eppendorf Co. Ltd., Tokyo, Japan). The primers for real-time PCR were designed using Primer 3 software (<http://primer3.sourceforge.net/>) based on data from GenBank; sequences are shown in



## Supplementary Table 1.

### **Cell Culture**

Peritoneal macrophages were elicited in C57/BL6 mice and BLT-1<sup>-/-</sup> mice using thioglycollate as described previously (13,29). Three days after i.p. injection of 2 ml of 4% thioglycollate medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan), elicited macrophages were obtained via lavage of the peritoneal cavity using 2×5 ml PBS. Peritoneal exudate cells were washed, suspended in RPMI-1640 medium and enriched for macrophages by adhesion for 1 h (in six-well tissue culture plates at a density of  $2 \times 10^6$  cells/well). The resulting peritoneal macrophages (in six-well tissue culture plates at a density of  $3 \times 10^5$  cells/well) were stimulated with LTB<sub>4</sub> (Cayman Chemical, Ann Arbor, MI, USA) for 6 h.

### **Statistics**

All results are expressed as the means  $\pm$  standard error of the mean (SEM). Student's t-test was used for comparisons between two groups. Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A P-value of less than 0.05 was considered statistically significant.

## Results

***Induction of BLT1 expression and promotion of liver repair through BLT1 signaling during hepatic I/R.*** The levels of BLT1 mRNA expression in the livers of WT mice were increased at 24 and 48 h after reperfusion by 13.5-fold and 6.5-fold, respectively (Fig. 1A). 5-LOX levels in WT livers were also elevated, with a peak at 24 h (an approximately 14-fold increase), and returned to the levels of sham-controls by 96 h (Fig. 1B). BLT1<sup>-/-</sup> mice exhibited changes similar to WT mice, but 5-LOX levels in the livers of BLT1<sup>-/-</sup> mice at 96 h were higher than those observed in WT mice. To further examine liver cell specific expression of 5-LOX, fluorescein immunostaining was performed. Immunofluorescence double staining of liver sections with antibodies to 5-LOX and inflammatory cell markers (F4/80, CD11b, and Ly6B) revealed that 5-LOX in WT livers was expressed mainly in F4/80-positive cells (Kupffer cells), and to a lesser extent in CD11b-positive cells (macrophages) and Ly6B-positive cells (neutrophils) (Fig. S1).

The levels of alanine transaminase (ALT) in WT mice peaked at 6 h and then declined, returning to sham-control levels within 96 h (Fig. 1C). A similar pattern was observed in BLT1<sup>-/-</sup> mice. However, ALT levels in BLT1<sup>-/-</sup> mice at 48 and 96 h were increased by 1.8-fold and 2.2-fold, respectively, compared to WT mice (Fig. 1C). In addition, in WT mice, hepatocellular necrosis at 24 h was evident, and the necrotic area was gradually reduced thereafter. In BLT1<sup>-/-</sup> mice, however, although a similar degree of necrosis compared to WT mice was evident at 24 h, more extensive necrosis was observed at 48 and 96 h (a 1.2- to 1.5-fold increase, respectively; Fig. 1D). These results suggest that BLT1 signaling plays an important role in liver repair during hepatic IR injury.

***Delayed Hepatocyte Proliferation in BLT1<sup>-/-</sup> Mice After hepatic I/R.*** Because histopathologic analysis suggested that BLT1<sup>-/-</sup> mice recovered more slowly from I/R injury, hepatocyte proliferation following hepatic I/R was evaluated. Figure 1E shows quantified data from proliferating cell nuclear antigen (PCNA)-stained tissues. PCNA expression in the livers of WT mice subjected to hepatic I/R was enhanced from 24 h through 96 h (Fig. 1E). In the livers of BLT1<sup>-/-</sup> mice, only a few PCNA-positive cells were observed at 24 and 48 h (Fig. 1E, F), and PCNA staining was not significantly increased until 96 h (Fig. 1E). The results suggest that the proliferative response after I/R injury is delayed in BLT1<sup>-/-</sup> mice. Based on the results of PCNA assay, ALT levels, and histological assessment, liver repair begins within 48 h after reperfusion, a finding

that is consistent with previous studies (3,25).

Next, the mRNA expression levels of hepatotrophic growth factors in the liver tissue were measured (Fig. 2A). Hepatic I/R caused increases in the hepatic mRNA levels of IL-6 and TNF $\alpha$  in both genotypes. However, the levels in BLT1 $^{-/-}$  livers were consistently greater than in WT livers. HGF mRNA levels were also increased, but no significant differences were observed between the two groups. EGF mRNA levels in WT livers at 6 and 24 h were increased by approximately 4-fold compared with the livers of sham-control mice, and the levels of EGF mRNA in BLT1 $^{-/-}$  livers at 6, 24, and 48 h were decreased by 76%, 82%, and 84%, respectively, compared to WT livers.

VEGF and its receptors have also been shown to play a critical role in liver regeneration after partial hepatectomy (7) or during toxin-induced liver injury (8,12). As shown in Figure 2B, VEGF-A mRNA levels in WT livers were increased when compared with the levels in BLT1 $^{-/-}$  livers. In addition, VEGFR1 levels in WT livers at 24 and 48 h after hepatic I/R were increased by 7.5-fold and 5.5-fold, respectively. No significant differences in the levels of VEGFR2 mRNA were observed between the two groups. These results suggest that the expression levels of EGF, VEGF-A, and VEGFR1 are mediated by BLT1 signaling and appear to contribute to liver repair following hepatic I/R injury.

***Recruitment of inflammatory cells after hepatic I/R.*** Recent evidence indicates that recruited macrophages play an important role in liver repair after acute liver injury. Based on these findings, immunofluorescent staining analysis was used to examine IR-induced infiltration of macrophages into the liver (Fig. 3). The numbers of F4/80-positive cells dropped following hepatic I/R and reached a nadir at 6 h, and then increased gradually. At 48 h, although the number of F4/80-positive cells in both WT and BLT1 $^{-/-}$  livers were lower than in sham-controls (Fig. 3A), the number of F4/80-positive cells in BLT $^{-/-}$  livers was 44.5% lower than in WT livers (Fig. 3A,B). By contrast, few CD11b-positive cells were found in the sham-operated controls (Fig. 3B), while marked recruitment of CD11b-positive cells to WT livers was observed from 6 to 48 h (Fig. 3A, B). The number of accumulated CD11b-positive cells in the BLT1 $^{-/-}$  livers were lower than those in WT livers (Fig. 3A). These results suggest that BLT1 signaling is involved in regulating the recruitment of macrophages following hepatic I/R injury. Because the recruitment of neutrophils to sites of tissue injury is a cardinal feature of hepatic I/R, the number of neutrophils in the liver during hepatic I/R was also determined (Fig. 3A). A massive accumulation of Ly6B-positive cells was observed in WT livers at 6 h, which peaked at 24 h and remained elevated until 48 h. By contrast,

neutrophil accumulation in BLT1<sup>-/-</sup> livers was suppressed at 6 and 24 h, but not 48 h. (Fig. 3A, B).

As shown in Figure 2B, VEGFR1 mRNA expression levels are enhanced by BLT1 signaling. VEGFR1 contributes to liver repair after acute liver injury (6) by enhancing the recruitment of macrophages (10), and is expressed on macrophages and facilitates their migration (6,12). Therefore, the infiltration of VEGFR1-positive cells into the liver during hepatic I/R was examined. The livers of WT mice subjected to hepatic I/R exhibited massive accumulation of VEGFR1-positive cells at 48 h, while the infiltration of VEGFR1-positive cells in the livers of BLT1<sup>-/-</sup> mice at 48 h was less extensive (80% less than in WT; Fig. 3A). Further, double immunostaining analyses revealed that most of the VEGFR1-positive cells in WT livers were also positive for CD11b (Fig. 3B). Minimal co-localization of VEGFR1 with F4/80 and Ly6B was seen. In addition, the accumulation of cells positive for both VEGFR-1 and CD11b was attenuated in BLT1<sup>-/-</sup> livers. These results indicate that VEGFR1 is expressed on recruited macrophages, which is consistent with results reported by others (23). Taken together, BLT1 signaling appears to facilitate liver repair after hepatic I/R through the recruitment of VEGFR1-expressing macrophages.

To determine whether BLT-1 deficiency affects chemokine expression, the expression of major cell activating chemokines linked to hepatic I/R injury was assessed (Fig. S2). The mRNA expression levels of CCL2 and CCR2 (Fig. S2A), and CXCL2 and CXCR2 (Fig. S2B), in BLT1<sup>-/-</sup> livers were increased compared with WT livers, while CXCL1 mRNA levels in BLT1<sup>-/-</sup> livers were decreased.

***VEGFR1 contributes to liver repair during hepatic I/R.*** To further examine the role of VEGFR1 in liver repair mediated by BLT1 signaling, VEGFR1 tyrosine kinase (TK)<sup>-/-</sup> mice and their wild-type counterparts were subjected to hepatic I/R. VEGFR1 TK<sup>-/-</sup> mice exhibited delayed liver repair compared to WT mice as evidenced by higher levels of ALT (2.3-fold increase), increased necrotic area (1.7-fold increase), lower expression of PCNA (83.6% decrease), and EGF mRNA in the livers (85.7% decrease). In addition, both CD11b- and F4/80-positive cells in the livers of VEGFR1 TK<sup>-/-</sup> mice subjected to hepatic I/R were found to be reduced by 74.3% and 36.9%, respectively (Fig. 4).

***EGF from VEGFR1-positive cells facilitates liver repair.*** Because BLT1 signaling-mediated promotion of liver repair was associated with enhanced expression of EGF, the expression and function of EGF during the repair phase was investigated. Immunofluorescence analysis demonstrated enhanced EGF expression in WT livers,

which was attenuated in BLT1<sup>-/-</sup> livers (Fig. 5A). Quantitative analysis revealed that the number of EGF-positive cells in WT livers at 48 h increased by 120-fold compared with sham-control livers, and that the number of EGF-positive cells in BLT1<sup>-/-</sup> livers was 67.3% lower than in WT livers (Fig. 5B). Furthermore, analysis of liver tissue by double immunostaining using anti-EGF and anti-VEGFR1 antibodies showed that at 48 h after hepatic I/R, EGF-positive cells were also positive for VEGFR1 in WT mice (Fig. 5A). BLT1<sup>-/-</sup> mice exhibited a markedly lower number of EGF<sup>+</sup>/VEGFR1<sup>+</sup> cells. These results suggest that BLT1 signaling facilitates liver repair through EGF production by VEGFR1-expressing macrophages.

To further address the role of EGF in liver repair during hepatic I/R, WT mice were treated with a neutralizing antibody against EGF 6 h after reperfusion. Treatment of mice with an EGF neutralizing antibody resulted in delayed liver repair, as indicated by higher levels of ALT (2.2-fold increase), an increased necrotic area (1.5-fold increase), and by lower PCNA expression (98.0% decrease) when compared with control antibody-treated mice (Fig. 5C). Administration of a neutralizing antibody against EGF reduced hepatic levels of EGF mRNA by 56.2% (Fig. 5C), and increased the levels of IL-6 and TNF $\alpha$  by 5.5-fold and 3.0-fold, respectively, but had no effect on HGF (not shown). Anti-EGF neutralizing antibody also suppressed the recruitment of CD11b- or VEGFR1-positive cells in the liver (Fig. 5D), although it failed to affect the accumulation of F4/80- and Ly6B-positive cells. Taken together, these results indicate that EGF from VEGFR1-expressing cells facilitates liver repair following hepatic I/R injury.

***LTB4/BLT1-dependent EGF expression on macrophages.*** To evaluate whether EGF expression by macrophages is dependent on the LTB4/BLT1 pathway, isolated peritoneal macrophages from WT and BLT1<sup>-/-</sup> mice were stimulated with LTB4. The levels of EGF mRNA expression in WT macrophages were enhanced in a dose-dependent manner after administration of LTB4 (Fig. 6A). In addition, stimulation of WT macrophages with LTB4 caused increases in the mRNA expression levels of BLT1 (Fig. 6B), VEGF-A (Fig. 6C), and VEGFR1 (Fig. 6D). By contrast, LTB4 did not affect mRNA expression levels in BLT1<sup>-/-</sup> macrophages. These results suggest that BLT1 signaling in macrophages enhances EGF, VEGF, and VEGFR1 expression. Resting resident peritoneal macrophages from both genotypes were also assessed in the absence of thioglycollate (TGC) administration (Fig. S3). BLT1 deficiency did not affect the basal conditions of resting resident macrophages, as indicated by mRNA expression levels of EGF, VEGF, and VEGFR1, which were similar to those in resting

macrophages from WT mice. The number of resting peritoneal macrophages in BLT1<sup>-/-</sup> mice was the same as in WT mice; however, the number of resting macrophages was smaller than that of TGC-elicited macrophages. In addition, the number of elicited macrophages in BLT1<sup>-/-</sup> mice was 48.9% lower than the number of elicited macrophages in WT mice.

## Discussion

The process of liver repair and recovery from hepatic I/R injury is a determinant of the final outcomes of patients subjected to liver surgery, because failure of the liver to regenerate is considered a critical contributing factor to postsurgical liver dysfunction and failure.

Experimental models of hepatic I/R injury have shown that peak hepatocellular injury occurs within 12 h after reperfusion. Within 24–48 h after reperfusion, the liver enters a proliferative phase and the damage incurred by ischemia-reperfusion is normally repaired within 96 h of reperfusion (3,30). The results of the present study were in agreement with this finding. In this regard, BLT1-mediated recruitment of macrophages 48 h after reperfusion (repair phase) appears to be relevant to liver repair. Recent evidence has established that recruited macrophages in injured livers exposed to hepatotoxins play an important role in liver repair and regeneration (9-13). Both chemokines and cytokines are involved in the recruitment of macrophages to the injured liver (3,10,15).

LTB4 is a well-known chemotactic factor for leukocytes including macrophages, and the LTB4 receptor type 1, BLT1, is expressed on macrophages. LTB4 is involved in the recruitment of macrophages during pulmonary infection (31) and peritonitis (32). BLT1 signaling contributes to the accumulation of macrophages into adipose inflammatory tissues (21). LTB4 also has chemotactic activity for monocytes *in vitro* (33). Although macrophages recruited by BLT1 signaling are important for host defense against infection, it remained to be clarified whether recruitment of macrophages through BLT1 signaling plays a role in liver repair and recovery from hepatic I/R injury. The present study showed that BLT1 signaling facilitates liver repair through enhanced expression of EGF by macrophages recruited to the injured liver after hepatic I/R. In this study, hepatic expression of BLT1 and 5-LOX was enhanced at 48 h, and the expression of 5-LOX in livers was mainly restricted to resident macrophages (Kupffer cells), which is consistent with previous results (34), suggesting that Kupffer cells are the major source of LTB4 in the liver during hepatic I/R.

VEGF is a major regulator of development as well as physiological and pathological angiogenesis (5). VEGF acts primarily through two TK receptors, VEGFR1 and VEGFR2. VEGF-induced angiogenesis is primarily mediated by VEGFR2 activation (5,6), although a role for VEGFR1 during tumor-angiogenesis has been recently suggested (6). In addition, VEGF/VEGFR2 plays a critical role in liver regeneration after hepatectomy (7), and VEGFR1 signaling is crucial for liver repair after liver injury

elicited by carbon tetrachloride (8). In our model, VEGFR1 signaling is involved in BLT1 signaling-dependent liver repair, and was found to be expressed on macrophages recruited during the repair phase of I/R injury. This is in agreement with our findings that VEGFR1-expressing macrophages have a significant role in liver repair after acetaminophen injury (12). VEGFR1 has been reported to contribute to activation of macrophages and their recruitment to inflammatory sites (6,14). We also confirmed a critical role for VEGFR1 in liver repair and regeneration during hepatic I/R in VEGFR1-TK deficient mice (Fig. 4). Taken together, these results show that BLT1 signaling facilitates liver repair after hepatic I/R injury through the recruitment of VEGFR1-expressing macrophages.

The molecular background resulting in delayed liver regeneration in BLT1<sup>-/-</sup> mice may involve suppression of EGF hepatic expression, because EGF has been shown to be a hepatoproliferative cytokine that promotes hepatocyte regeneration (4,35). It is interesting to note that enhanced expression of EGF was localized to the inflammatory cells in the liver and that EGF-expressing cells in the liver were also positive for VEGFR1. Thus, it seems likely that BLT1 signaling facilitates liver repair after hepatic I/R injury through enhanced EGF expression on recruited VEGFR1-positive macrophages. In support of this, EGF blockade with a neutralizing antibody delayed hepatocyte proliferation and liver repair during hepatic I/R. Treatment with an anti-EGF neutralizing antibody also decreased the mRNA levels of EGF together with reduced recruitment of VEGFR1-expressing macrophages. It should be noted that delayed liver recovery from hepatic I/R injury in BLT1 deficient mice may also result from enhanced expression of CXCR2, a receptor for CXCL1/2. It has been shown that genetic deletion or pharmacologic antagonism of CXCR2 after hepatic I/R injury resulted in augmented hepatocyte proliferation and accelerated recovery from injury (3,15). Thus, it may be conceivable that increased mRNA levels of CXCL2/CXCR2 in BLT1<sup>-/-</sup> mice could be responsible for delayed liver repair.

Our *in vitro* studies indicate that BLT1 signaling regulates the expression of VEGF, VEGFR1, and EGF in peritoneal macrophages (Fig. 6). LTB4 is known to activate immune cells by producing growth factors through the activation of nuclear factor kappa B (NF- $\kappa$ B) via LTB4-BLT1 signaling. BLT1-mediated signaling is important for activation of NF- $\kappa$ B in LTB4-stimulated cultured monocytes (36). Therefore, it is likely that LTB4/BLT1 signaling enhances EGF and VEGF expression through the activation of NF- $\kappa$ B (37,38). BLT receptor transduction also activates a number of kinases that phosphorylate downstream signal transduction proteins, including mitogen-activated protein kinases, extracellular signal-regulated kinases, and phosphatidylinositol 3-kinase



(39).

LTB4 is well known for its role as a neutrophil chemoattractant factor (40), and is one of the first neutrophil chemoattractants generated in response to tissue injury. High levels of LTB4 are released during hepatic I/R (24). However, LTB4 inhibition had no effect on acute hepatocellular injury during hepatic I/R in rats (24). Consistent with this, the present study showed that BLT1 signaling plays a minor role in the development of hepatic I/R injury. Therefore, the precise contribution of LTB4 to hepatic I/R injury remains to be clarified. Nevertheless, BLT1 signaling promotes the accumulation of hepatic neutrophils, which are critical for the pathogenesis of hepatic I/R injury (1). The failure of suppressed accumulation of neutrophils in BLT1<sup>-/-</sup> mice to minimize hepatic I/R injury might be explained by the finding that proinflammatory mediators other than LTB4 are involved in acute liver injury. Indeed, the expression of TNF $\alpha$  and IL-6 in BLT1<sup>-/-</sup> livers was enhanced, indicating that severe inflammation takes place during hepatic I/R (41).

In conclusion, BLT1 signaling facilitates liver repair through enhanced EGF expression on recruited VEGFR1-expressing macrophages during hepatic I/R injury. The development of a specific agonist for BLT1 could be useful for enhancing liver recovery following acute liver injury.

## References

1. Jaeschke, H. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. *Am J Physiol Gastrointest Liver Physiol* 2006; 290, G1083-88
2. Clavien, P. A. Liver regeneration: a spotlight on the novel role of platelets and serotonin. *Swiss Med Wkly* 2008; 138, 361-70
3. Van Sweringen, H. L., Sakai, N., Tevar, A. D., Burns, J. M., Edwards, M. J., Lentsch, A. B. CXC chemokine signaling in the liver: impact on repair and regeneration. *Hepatology* 2011; 54, 1445-53
4. Michalopoulos, G. K. Liver regeneration. *J Cell Physiol* 2007; 213, 286-300
5. Ferrara, N., Gerber, H. P., and LeCouter, J. The biology of VEGF and its receptors. *Nat Med* 2003; 9, 669-76
6. Shibuya, M. Vascular endothelial growth factor-dependent and -independent regulation of angiogenesis. *BMB Rep* 2008; 41, 278-86
7. Ding, B. S., Nolan, D. J., Butler, J. M., James, D., Babazadeh, A. O., Rosenwaks, Z., et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature* 2010; 468, 310-5
8. LeCouter, J., Moritz, D. R., Li, B., Phillips, G. L., Liang, X. H., Gerber, H. P., et al. Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. *Science* 2003; 299, 890-3
9. Holt, M. P., Cheng, L., Ju, C. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J Leukoc Biol* 2008; 84, 1410-21
10. Laskin, D. L. Macrophages and inflammatory mediators in chemical toxicity: a battle of forces. *Chem Res Toxicol* 2009; 22, 1376-85
11. Karlmark, K. R., Weiskirchen, R., Zimmermann, H. W., Gassler, N., Ginhoux, F., Weber, C., et al. Hepatic recruitment of the inflammatory Gr1<sup>+</sup> monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 2009; 50, 261-74
12. Kato, T., Ito, Y., Hosono, K., Suzuki, T., Tamaki, H., Minamino, T., et al. Vascular endothelial growth factor receptor-1 signaling promotes liver repair through restoration of liver microvasculature after acetaminophen hepatotoxicity. *Toxicol Sci* 2011; 120, 218-29
13. Minamino, T., Ito, Y., Ohkubo, H., Hosono, K., Suzuki, T., Sato, T., et al. Thromboxane A<sub>2</sub> receptor signaling promotes liver tissue repair after toxic

- injury through the enhancement of macrophage recruitment. *Toxicol Appl Pharmacol* 2012; 259, 104-14
14. Murakami, M., Iwai, S., Hiratsuka, S., Yamauchi, M., Nakamura, K., Iwakura, Y., et al. Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocytes/macrophages. *Blood* 2006; 108, 1849-56
  15. Saiman, Y., and Friedman, S. L. The role of chemokines in acute liver injury. *Front Physiol* 2012; 3, 213
  16. Lewis, R. A., Austen, K. F., Soberman, R. J. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N Engl J Med* 1990; 323, 645-55
  17. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., Shimizu, T. A G-protein-coupled receptor for leukotriene B<sub>4</sub> that mediates chemotaxis. *Nature* 1997; 387, 620-4
  18. Jala, V. R., Haribabu, B. Leukotrienes and atherosclerosis: new roles for old mediators. *Trends Immunol* 2004; 25, 315-22
  19. Okuno, T., Yokomizo, T., Hori, T., Miyano, M., Shimizu, T. Leukotriene B<sub>4</sub> receptor and the function of its helix 8. *J Biol Chem* 2005; 280, 32049-52
  20. Peters-Golden, M., Henderson, W. R., Jr. Leukotrienes. *N Engl J Med* 2007; 357, 1841-54
  21. Spite, M., Hellmann, J., Tang, Y., Mathis, S. P., Kosuri, M., Bhatnagar, A., et al. Deficiency of the leukotriene B<sub>4</sub> receptor, BLT-1, protects against systemic insulin resistance in diet-induced obesity. *J Immunol* 2011; 187, 1942-9
  22. Ito, S., Ito, Y., Katagiri, H., Suzuki, T., Hoka, S., Yokomizo, T., et al. Leukotriene B<sub>4</sub>/leukotriene B<sub>4</sub> receptor pathway is involved in hepatic microcirculatory dysfunction elicited by endotoxin. *Shock* 2008; 30, 87-91
  23. Saiwai, H., Ohkawa, Y., Yamada, H., Kumamaru, H., Harada, A., Okano, H., et al. The LTB<sub>4</sub>-BLT1 axis mediates neutrophil infiltration and secondary injury in experimental spinal cord injury. *Am J Pathol* 2010; 176, 2352-66
  24. Hughes, H., Farhood, A., Jaeschke, H. Role of leukotriene B<sub>4</sub> in the pathogenesis of hepatic ischemia-reperfusion injury in the rat. *Prostaglandins Leukot Essent Fatty Acids* 1992; 45, 113-9
  25. Terawaki, K., Yokomizo, T., Nagase, T., Toda, A., Taniguchi, M., Hashizume, K., et al. Absence of leukotriene B<sub>4</sub> receptor 1 confers resistance to airway hyperresponsiveness and Th2-type immune responses. *J Immunol* 2005; 175, 4217-25

26. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A* 1998; 95, 9349-54
27. Hasegawa, T., Ito, Y., Wijeweera, J., Liu, J., Malle, E., Farhood, A., et al. Reduced inflammatory response and increased microcirculatory disturbances during hepatic ischemia-reperfusion injury in steatotic livers of ob/ob mice. *Am J Physiol Gastrointest Liver Physiol* 2007; 292, G1385-95
28. Katoh, H., Hosono, K., Ito, Y., Suzuki, T., Ogawa, Y., Kubo, H., et al. COX-2 and prostaglandin EP3/EP4 signaling regulate the tumor stromal proangiogenic microenvironment via CXCL12-CXCR4 chemokine systems. *Am J Pathol* 2010; 176, 1469-83
29. Hosono, K., Suzuki, T., Tamaki, H., Sakagami, H., Hayashi, I., Narumiya, S., et al. Roles of prostaglandin E2-EP3/EP4 receptor signaling in the enhancement of lymphangiogenesis during fibroblast growth factor-2-induced granulation formation. *Arterioscler Thromb Vasc Biol* 2011; 31, 1049-58
30. Barone, S., Okaya, T., Rudich, S., Petrovic, S., Tenrani, K., Wang, Z., et al. Distinct and sequential upregulation of genes regulating cell growth and cell cycle progression during hepatic ischemia-reperfusion injury. *Am J Physiol Cell Physiol* 2005; 289, C826-35
31. Mancuso, P., Lewis, C., Serezani, C. H., Goel, D., Peters-Golden, M. Intrapulmonary administration of leukotriene B4 enhances pulmonary host defense against pneumococcal pneumonia. *Infect Immun* 2010; 78, 2264-71
32. Matsukawa, A., Hogaboam, C. M., Lukacs, N. W., Lincoln, P. M., Strieter, R. M., Kunkel, S. L. Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4. *J Immunol* 1999; 163, 6148-54
33. Koyama, S., Takamizawa, A., Sato, E., Masubuchi, T., Nagai, S., Izumi, T. Cyclophosphamide stimulates lung fibroblasts to release neutrophil and monocyte chemoattractants. *Am J Physiol Lung Cell Mol Physiol* 2001; 280, L1203-11
34. Titos, E., Claria, J., Planaguma, A., Lopez-Parra, M., Villamor, N., Parrizas, M., et al. Inhibition of 5-lipoxygenase induces cell growth arrest and apoptosis in rat Kupffer cells: implications for liver fibrosis. *Faseb J* 2003; 17, 1745-7
35. Natarajan, A., Wagner, B., Sibilio, M. The EGF receptor is required for efficient liver regeneration. *Proc Natl Acad Sci U S A* 2007; 104, 17081-6
36. Sanchez-Galan, E., Gomez-Hernandez, A., Vidal, C., Martin-Ventura, J. L.,

- Blanco-Colio, L. M., Munoz-Garcia, B., et al. Leukotriene B<sub>4</sub> enhances the activity of nuclear factor-kappaB pathway through BLT1 and BLT2 receptors in atherosclerosis. *Cardiovasc Res* 2009; 81, 216-25
37. Fenton, S. E., Groce, N. S., Lee, D. C. Characterization of the mouse epidermal growth factor promoter and 5'-flanking region. Role for an atypical TATA sequence. *J Biol Chem* 1996; 271, 30870-8
  38. Shima, D. T., Kuroki, M., Deutsch, U., Ng, Y. S., Adamis, A. P., D'Amore, P. A. The mouse gene for vascular endothelial growth factor. Genomic structure, definition of the transcriptional unit, and characterization of transcriptional and post-transcriptional regulatory sequences. *J Biol Chem* 1996; 271, 3877-83
  39. Back, M., Dahlen, S. E., Drazen, J. M., Evans, J. F., Serhan, C. N., Shimizu, T., et al. International Union of Basic and Clinical Pharmacology. LXXXIV: leukotriene receptor nomenclature, distribution, and pathophysiological functions. *Pharmacol Rev* 2011; 63, 539-84
  40. Ford-Hutchinson, A. W., Bray, M. A., Doig, M. V., Shipley, M. E., Smith, M. J. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 1980; 286, 264-5
  41. Teoh, N., Field, J., Sutton, J., Farrell, G. Dual role of tumor necrosis factor-alpha in hepatic ischemia-reperfusion injury: studies in tumor necrosis factor-alpha gene knockout mice. *Hepatology* 2004; 39, 412-21

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## DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

## Figure Legends

Figure 1. Liver repair after hepatic I/R is delayed in BLT-1<sup>-/-</sup> mice.

(A). Expression of BLT-1 mRNA in livers from WT mice and BLT1<sup>-/-</sup> mice after hepatic I/R. No expression was observed in BLT-1<sup>-/-</sup> livers. Data are expressed as the means  $\pm$  SEM from six mice per group. †:  $p < 0.05$  vs. sham. \*:  $p < 0.05$  vs. WT mice. (B). Hepatic 5-LOX mRNA levels after hepatic I/R. Data are expressed as the means  $\pm$  SEM from six mice per group. †:  $p < 0.05$  vs. sham. \*:  $p < 0.05$  vs. WT mice. (C,D,E). Plasma levels of ALT (C), hepatic necrotic area (D), and PCNA index after hepatic I/R. Data are expressed as the means  $\pm$  SEM from six mice per group. \*:  $p < 0.05$  vs. WT mice. †:  $p < 0.05$  vs. sham. ‡:  $p < 0.05$  vs. 24h. (F) Representative immunohistochemical staining of liver sections for PCNA in WT mice (upper panel) and BLT1<sup>-/-</sup> mice (lower panel) at 48 h after hepatic I/R. Bars = 100  $\mu$ m.

Figure 2. Hepatic mRNA expression levels of growth and pro-angiogenic factors in WT mice and BLT1<sup>-/-</sup> mice after hepatic I/R.

(A,B) The mRNA levels of growth factors (A) including IL-6, TNF $\alpha$ , HGF, and EGF and of pro-angiogenic factors (B) including VEGF, VEGFR1, and VEGFR2 in livers from WT and BLT1<sup>-/-</sup> mice as determined by real-time PCR. Data are expressed as the means  $\pm$  SEM from five to six mice per group. †:  $p < 0.05$  vs. sham. \*:  $p < 0.05$  vs. WT mice.

Figure 3. Infiltration of macrophages in livers from WT mice and BLT1<sup>-/-</sup> mice after hepatic I/R.

(A) Changes in the numbers of cells positive for F4/80, CD11b, Ly6B, and VEGFR1 after hepatic I/R. Data are expressed as the means  $\pm$  SEM from five to six mice per group. †:  $p < 0.05$  vs. sham. \*:  $p < 0.05$  vs. WT mice. (B) Expression of VEGFR1 in the liver 48 h after hepatic I/R. Livers from WT mice and BLT1<sup>-/-</sup> mice were stained with antibodies against VEGFR1 (green) and F4/80 (red), CD11b (red) or Ly6B (red) 48 h after hepatic I/R. Hepatocyte nuclei are stained with DAPI (blue). Yellow staining indicates co-localization in double-labeled cells. All images are representative of three independent samples. Scale bars = 100  $\mu$ m.

Figure 4. VEGFR1 signaling promotes liver repair during hepatic I/R injury. Increased levels of ALT (A) and necrotic area (B), and decreased levels of PCNA index (C) and EGF mRNA in the liver (D) 48 h after reperfusion were evident in VEGFR1 TK-/- mice. The numbers of CD11b-positive cells (E) and F4/80-positive cells (F) in VEGFR1 TK-/- mice were reduced compared with WT mice. Data are expressed as the means  $\pm$  SEM from five to six mice per group. \*:  $p < 0.05$  vs. WT mice.

Figure 5. Recruitment of EGF-expressing cells is reduced in BLT1-/- mice, and inhibition of EGF suppresses liver repair.

(A) Double immunostaining of liver tissue from WT and BLT1-/- mice was performed with antibodies against EGF (red) and VEGFR1 (green). Images are representative of three independent samples. Scale bar = 50  $\mu$ m. (B) The number of EGF-positive cells in livers from WT and BLT1-/- mice after hepatic I/R. Data are expressed as the means  $\pm$  SEM from five to six mice per group. †:  $p < 0.05$  vs. sham. \*:  $p < 0.05$  vs. WT mice. (C) Effects of treatment with an EGF neutralizing antibody on liver repair and hepatic levels of EGF mRNA in mice 48 h after hepatic I/R. Treatment of WT mice with an EGF neutralizing antibody delayed liver repair after hepatic I/R, as evidenced by increased ALT levels, larger necrotic area and decreased PCNA index compared with WT mice treated with isotype control IgG. mRNA levels of EGF in livers from WT mice treated with EGF neutralizing antibody and control IgG were determined by real-time PCR. Data are expressed as the means  $\pm$  SEM from five to six mice per group. \*:  $p < 0.05$  vs. control IgG-treated WT mice. (D) Treatment with an EGF neutralizing antibody attenuated the recruitment of CD11b- and VEGFR1-positive cells into the liver 48 h after hepatic I/R. Data are expressed as the means  $\pm$  SEM from five to six mice per group. \*:  $p < 0.05$  vs. control IgG-treated WT mice.

Figure 6. The effects of LTB4 on the expression of EGF, BLT1, VEGF, and VEGFR1 in peritoneal thioglycollate-elicited macrophages from WT and BLT1-/- mice. Isolated macrophages were treated with LTB4 and the mRNA levels of EGF (A), BLT1 (B), VEGF-A (C), and VEGFR1 (D) were determined 6 h after incubation. Real-time quantitative RT-PCR assays were used to assess mRNA expression. Data are expressed as the means  $\pm$  SEM of three independent experiments. †:  $p < 0.05$  vs. vehicle. \*:  $p < 0.05$  vs. WT mice.

Supplementary Figure 1



Representative immunofluorescence staining in WT liver at 48 h after reperfusion; Double immunostaining of WT liver sections was carried out using antibodies against 5-LOX (green) and F4/80 (red), CD11b (red) or Ly6B (red). Images are representative of three independent samples. Scale bar = 100  $\mu$ m.

#### Supplementary Figure 2

mRNA expression levels of chemokines and their receptors, including CCL2, CCR2 (A), CXCL1, CXCL2, and CXCR1 (B) in WT and BLT1<sup>-/-</sup> livers 48 h after reperfusion. Data are expressed as the means  $\pm$  SEM from five to six mice per group. †:  $p < 0.05$  vs. sham. \*:  $p < 0.05$  vs. WT mice.

#### Supplementary Figure 3.

The levels of mRNA expression of EGF, BLT1, VEGF-A, and VEGFR1 in resident peritoneal macrophages from WT and BLT1<sup>-/-</sup> mice. Macrophages were harvested without thioglycollate (TGC) stimulation and mRNA levels of EGF (A), BLT1 (B), VEGF-A (C), and VEGFR1 (D) were determined 6 h after incubation. Real-time quantitative RT-PCR assays were used to assess mRNA expression. (E) The number of peritoneal macrophages collected from WT mice and BLT1<sup>-/-</sup> mice with or without thioglycollate (TGC) treatment. Data are expressed as the means  $\pm$  SEM of three independent experiments. TGC; thioglycollate. †:  $p < 0.05$  vs. macrophages without thioglycollate. \*:  $p < 0.05$  vs. WT mice.

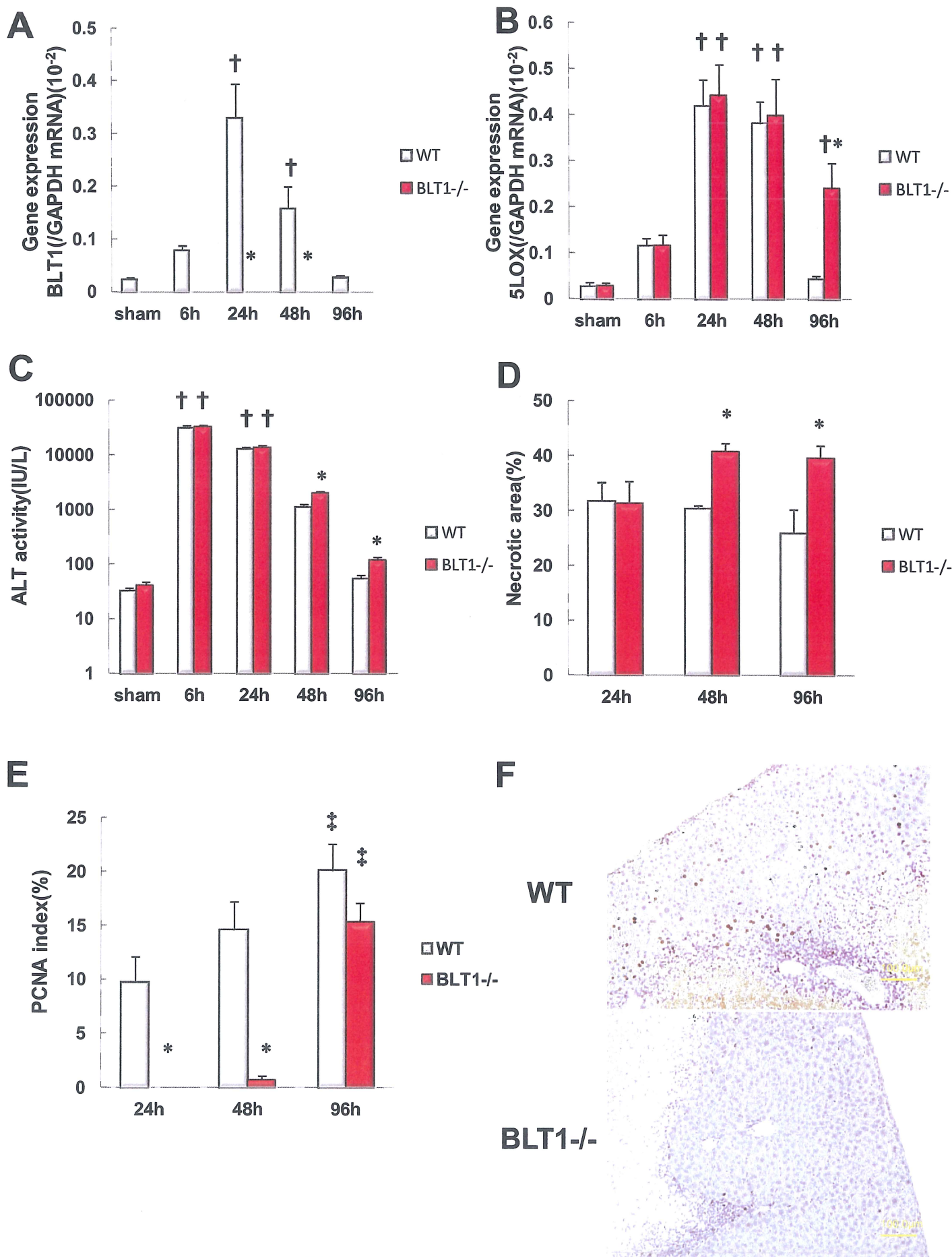
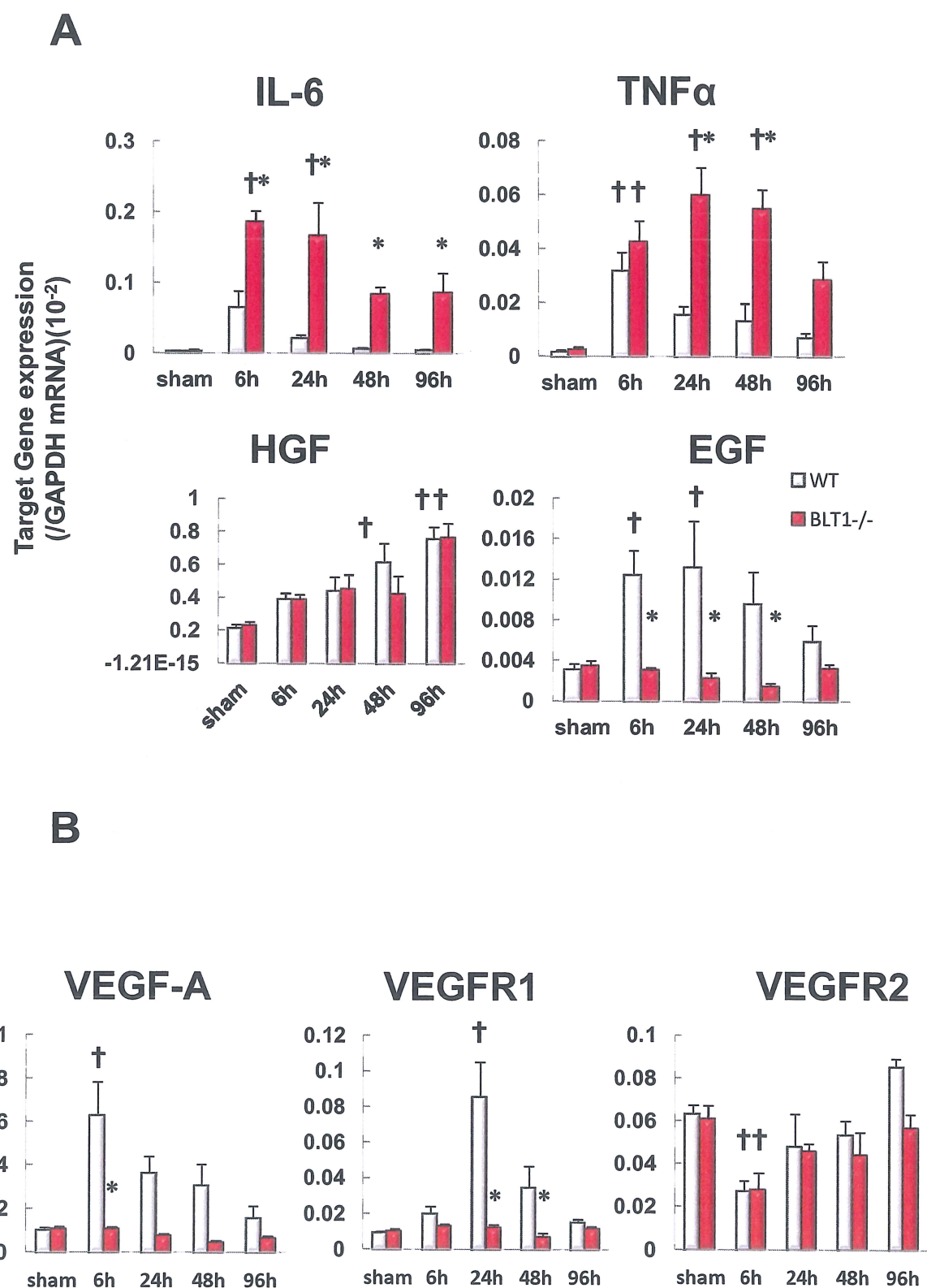
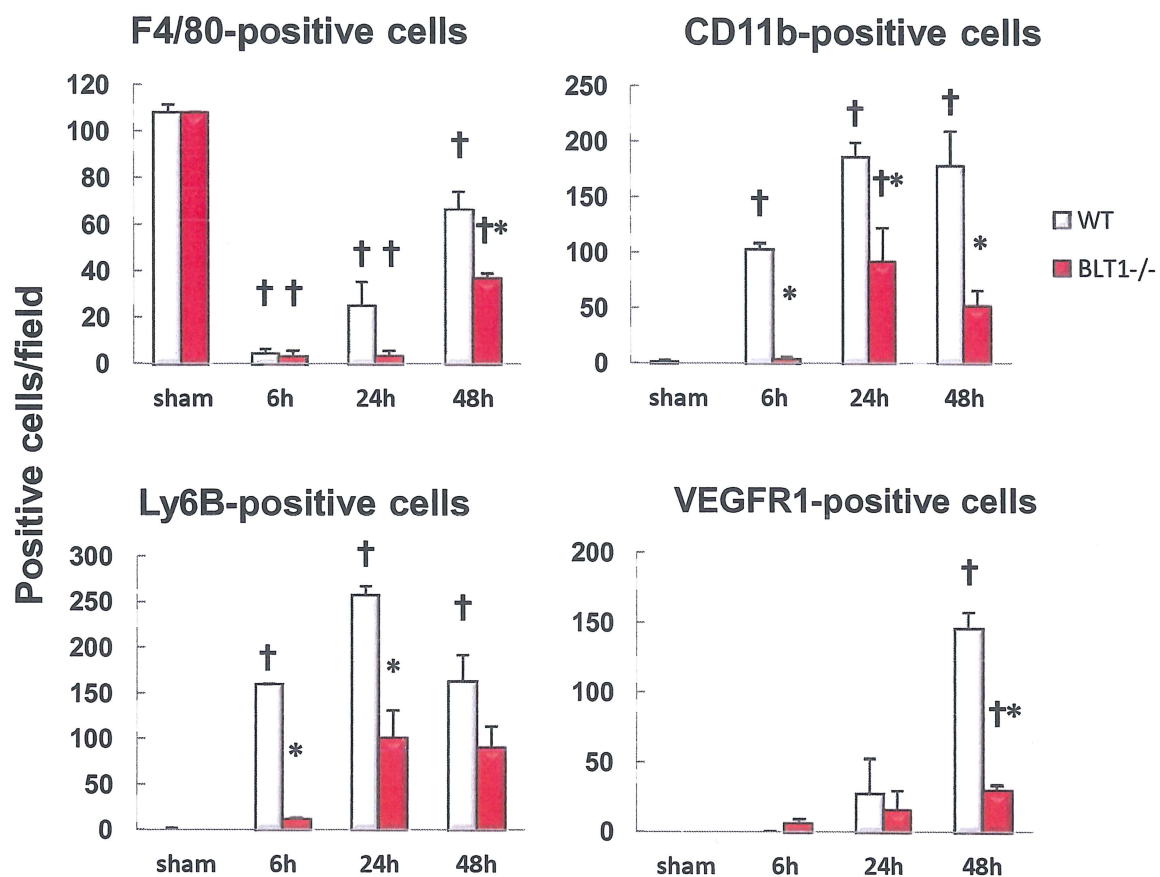


Figure 1 -22-

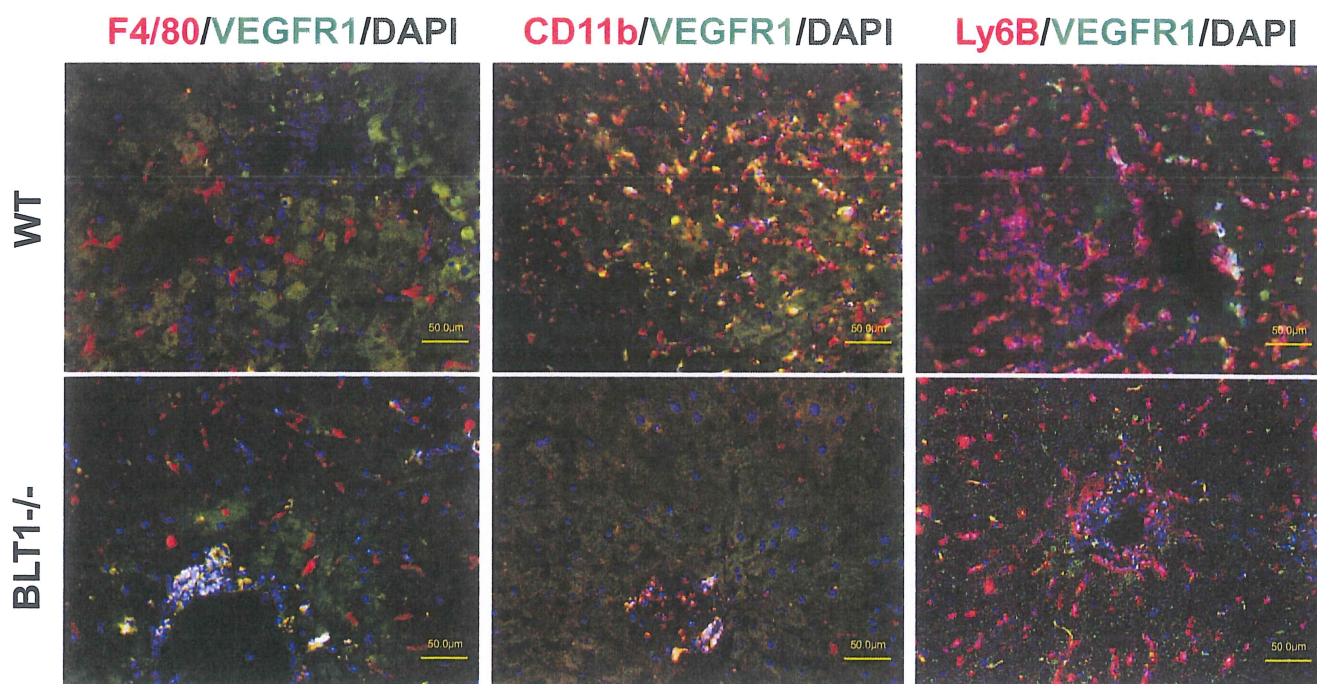


**Figure 2** -23-

**A**

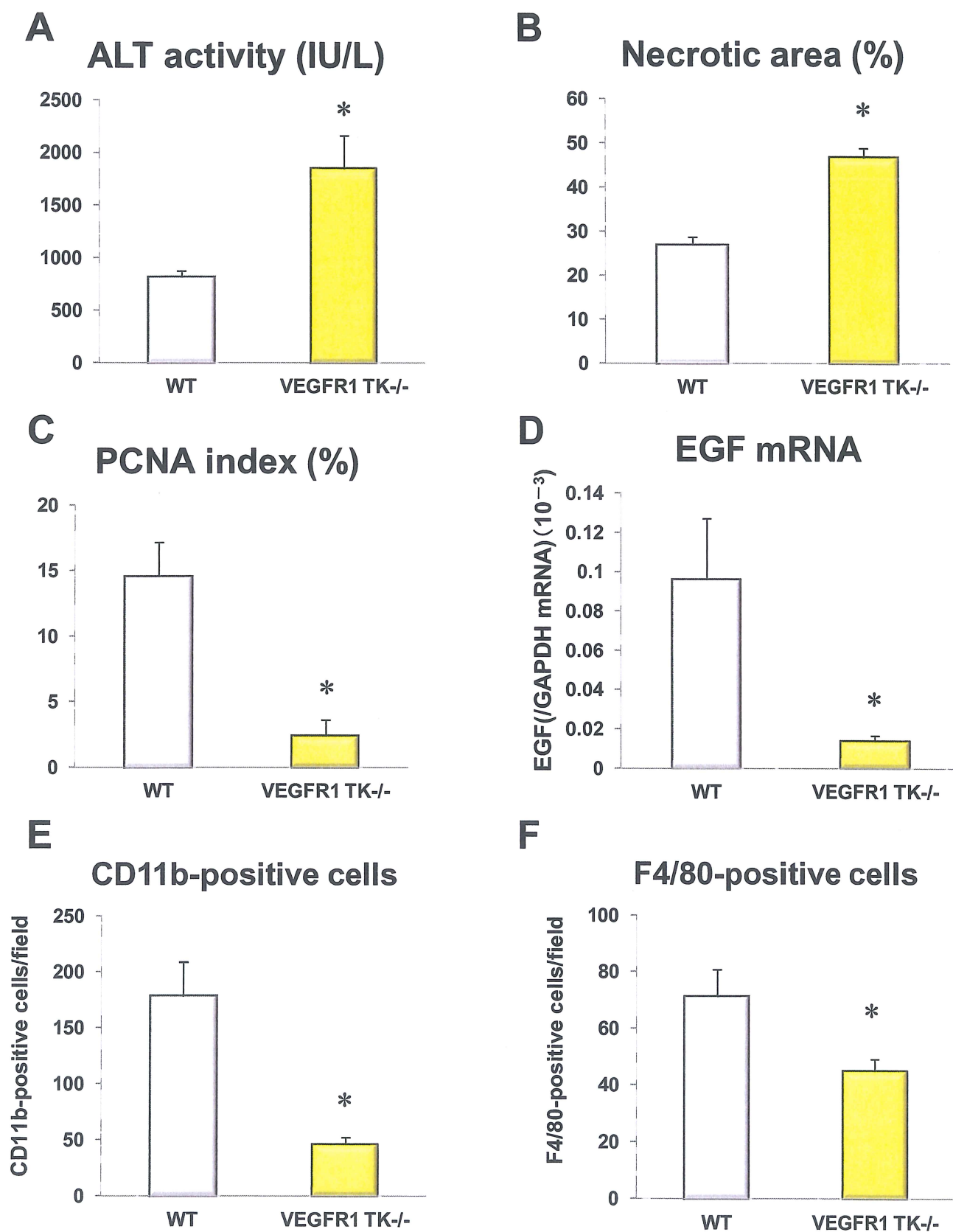


**B**



**Figure 3** -24-





**Figure 4** -25-

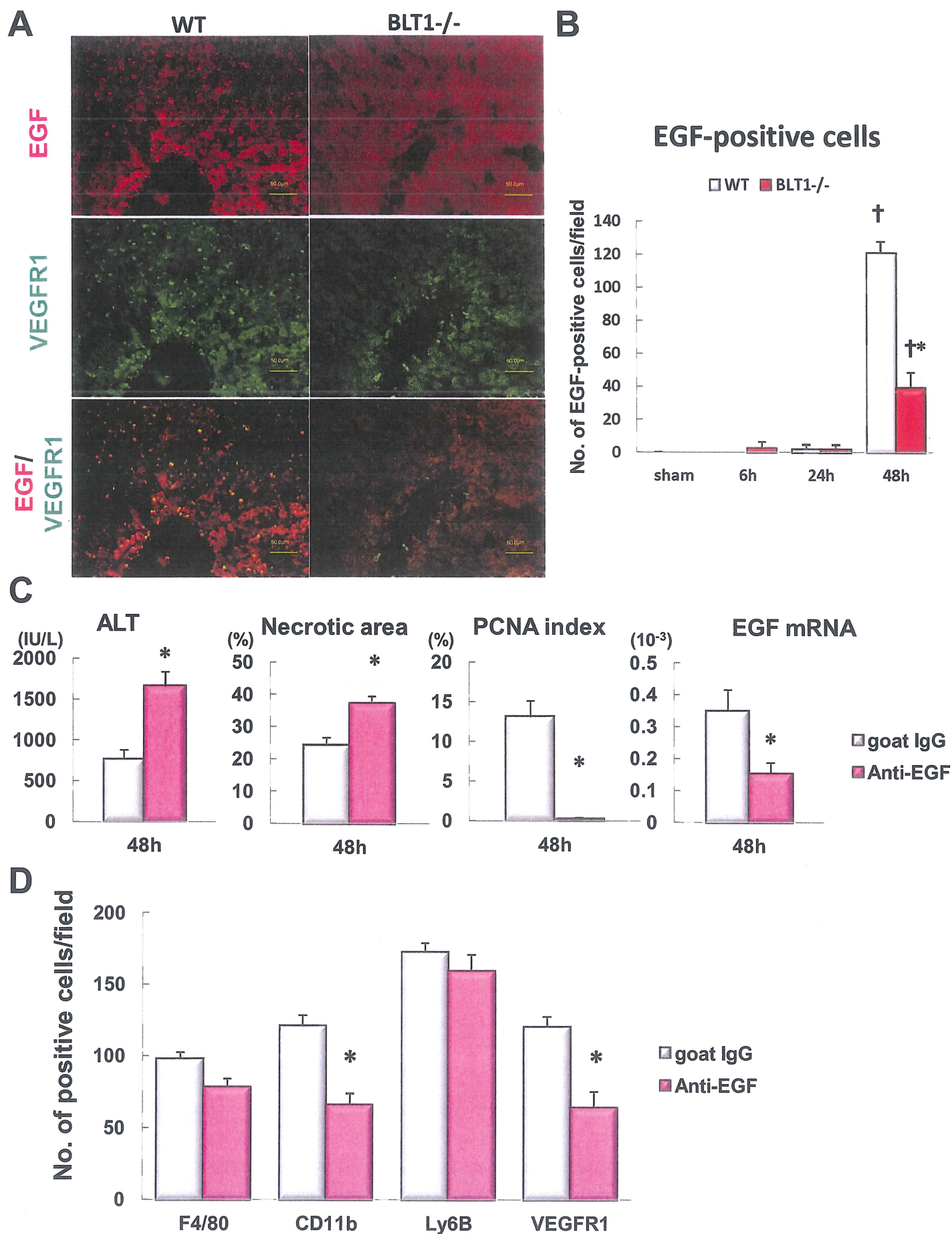
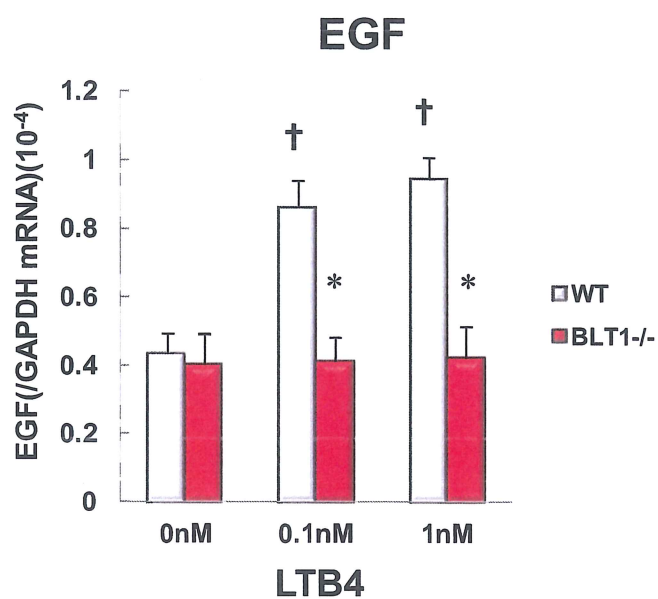
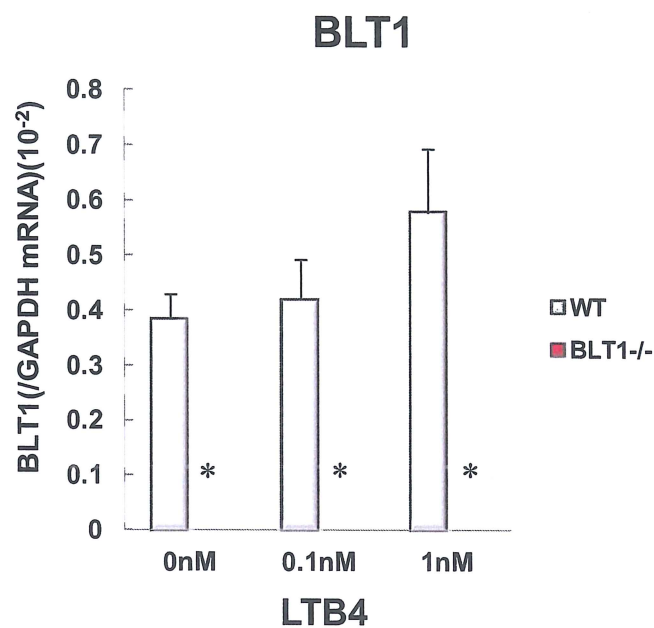
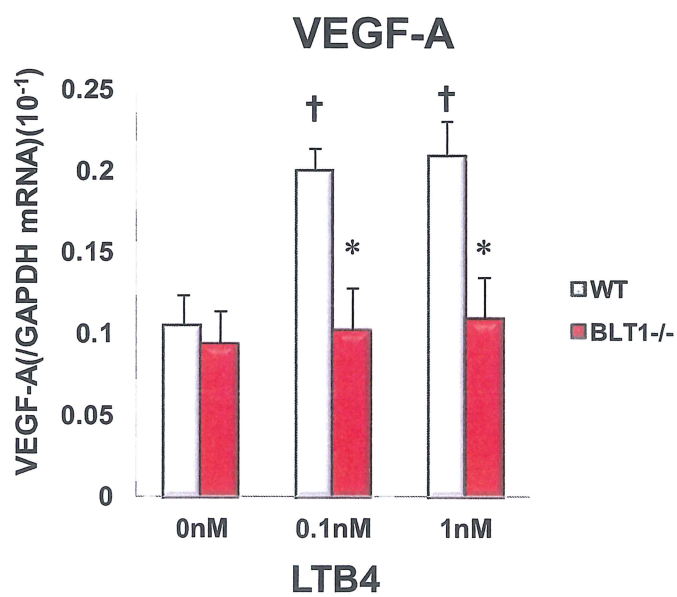
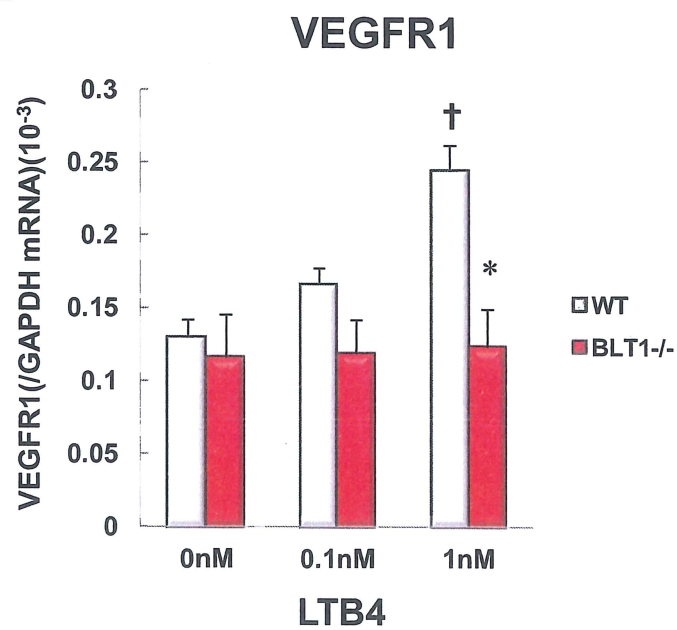
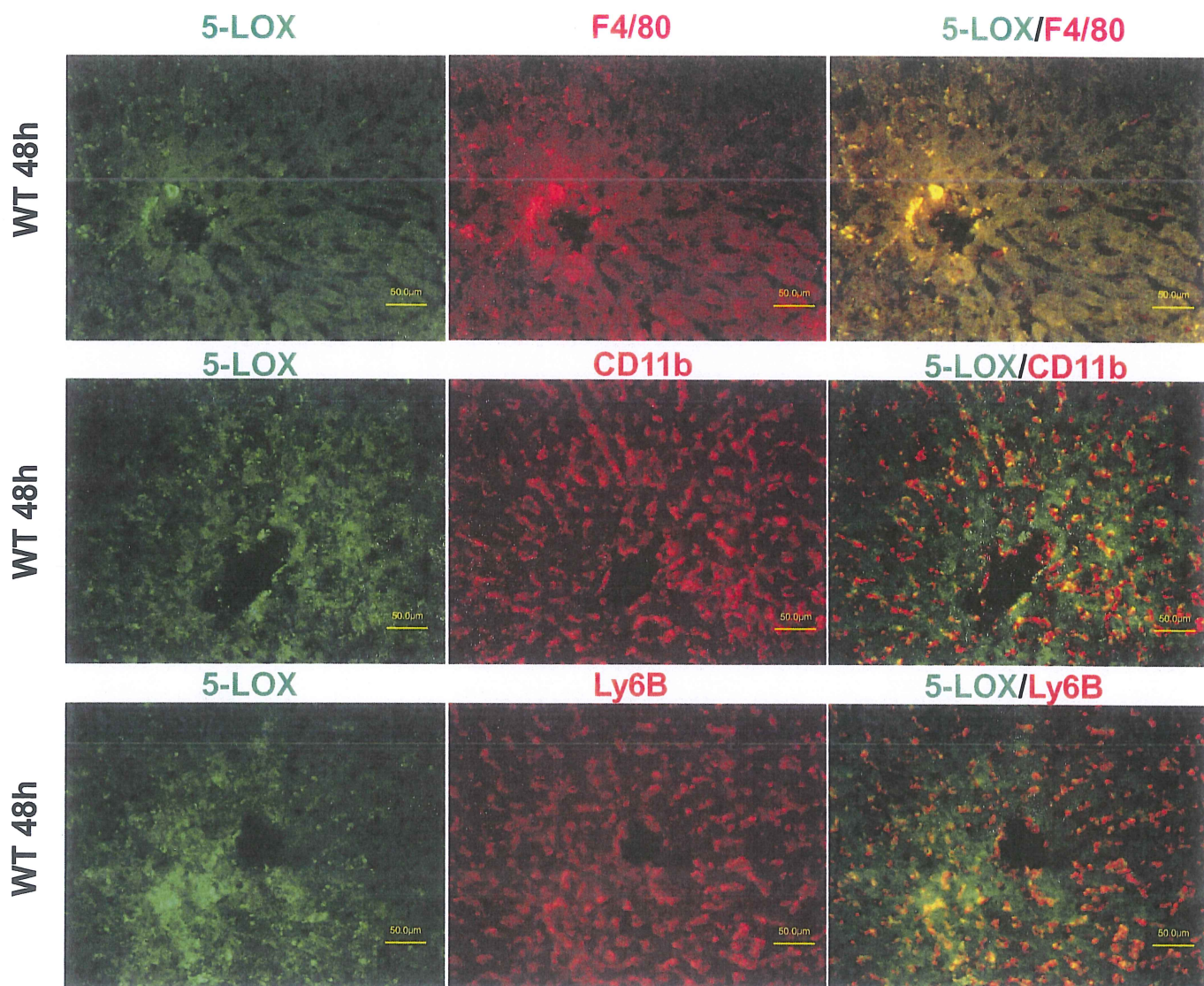


Figure 5 -26-

**A****B****C****D**

**Figure 6** -27-

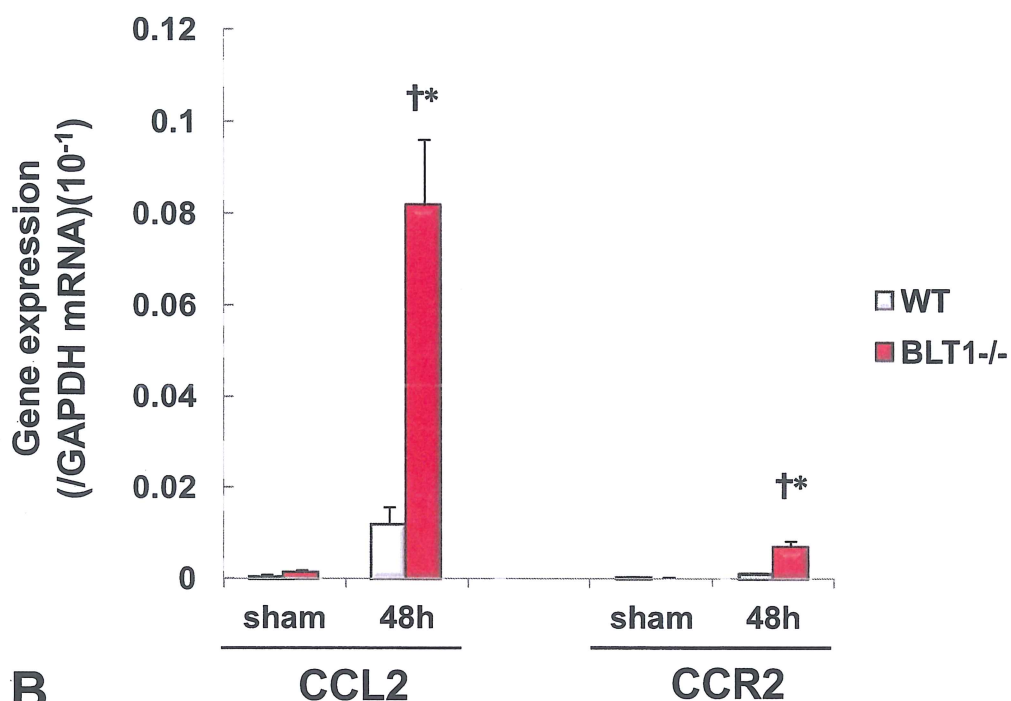




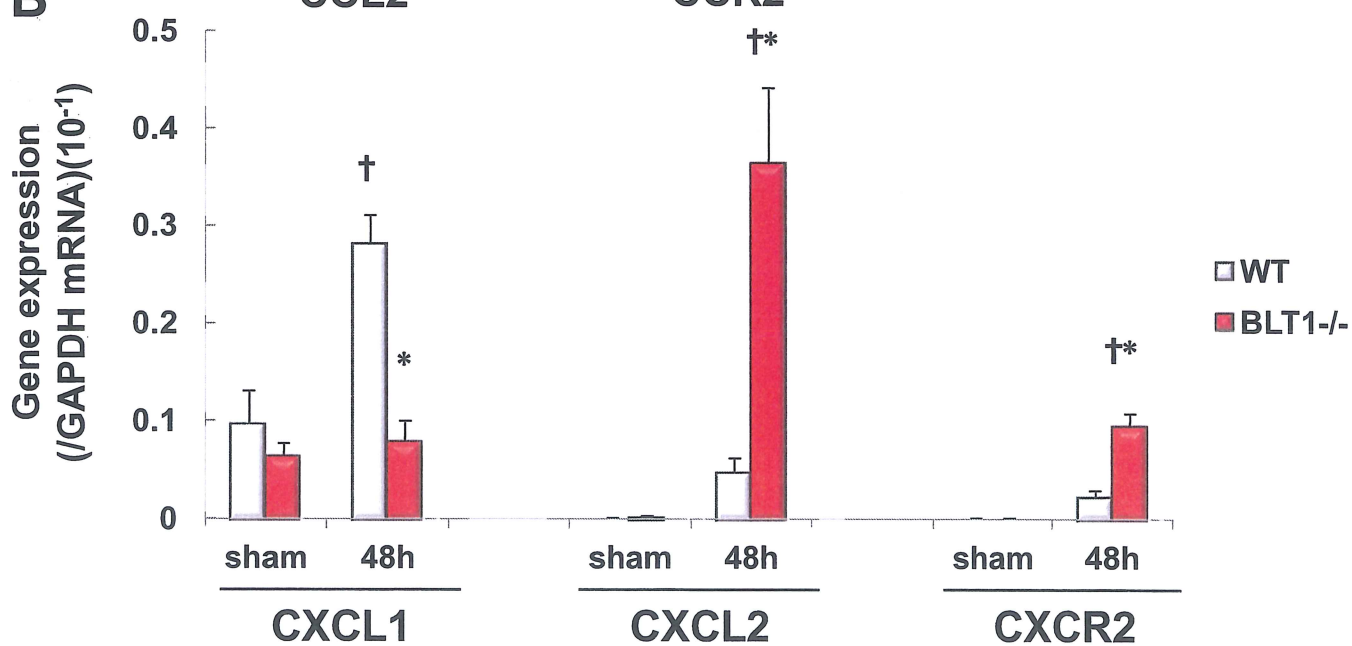
Supplementary Figure 1 -28-

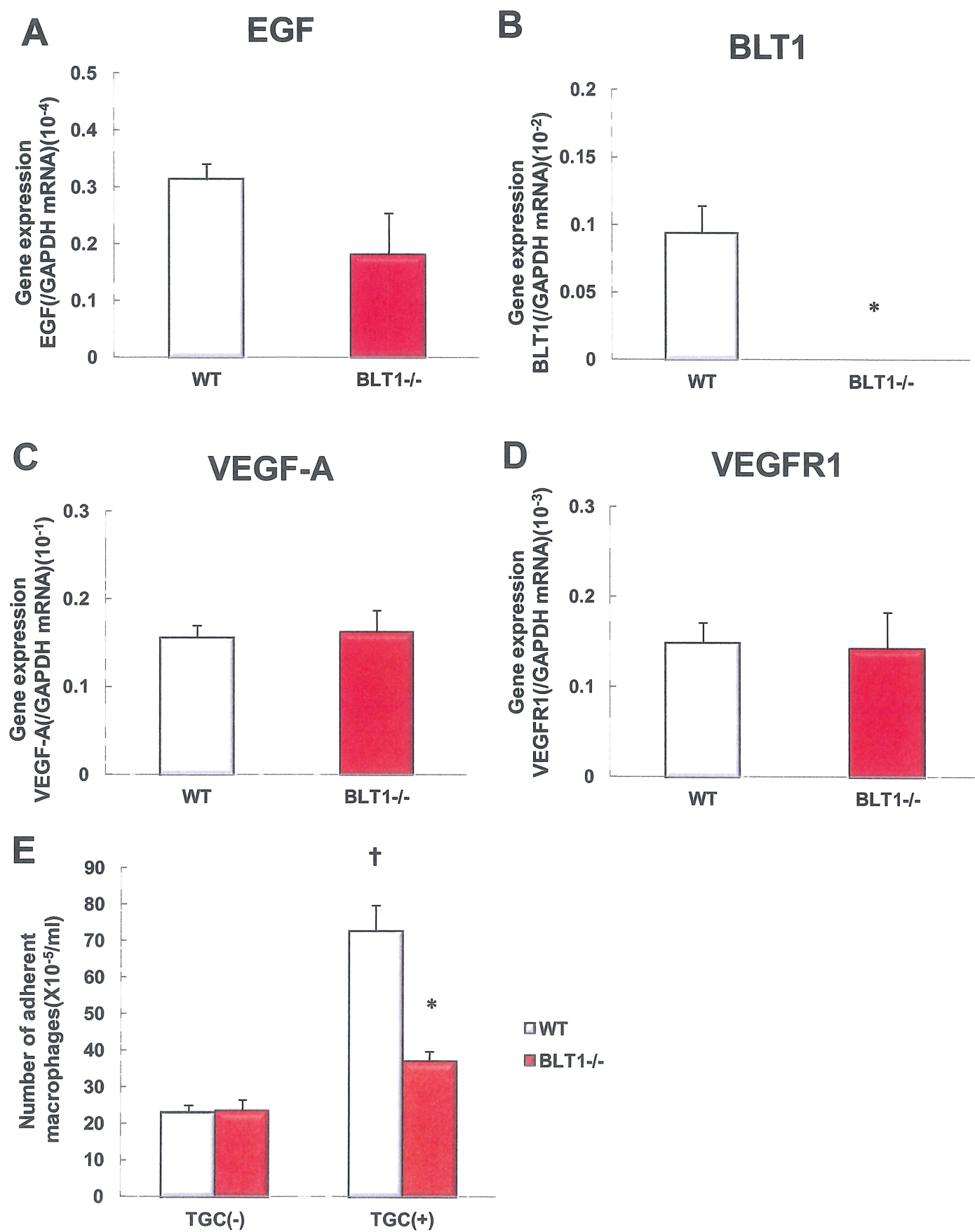


**A**



**B**





Supplementary Figure 3 -30-

## Supplementary Table 1. Primers for real-time RT-PCR

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	ACATCAAGAAGGTGGTGAAGC	AAGGTGGAAGAGTGGGAGTTG
VEGF-A	ACGACAGAAGGAGAGCAGAAG	ATGTCCACCAGGGTCTCAATC
VEGFR1	GTCTCCATCAGTGGCTCTACG	CCCGGTTCTTGTTGTATTTTG
VEGFR2	CTGCCTACCTCACCTGTTTCC	CGGCTCTTTCGCTTACTGTTC
HGF	GGCTGAAAAGATTGGATCAGG	CCAGGAACAATGACACCAAGA
EGF	ATGGGAAACAATGTCACGAAC	CATCTCTCCCAAGCACTGAAC
IL-6	CAAAGCCAGAGTCCTTCAGAG	TAGGAGAGCATTGGAAATTGG
TNF $\alpha$	TCTTCTCATTCTGCTTGTGG	GATCTGAGTGTGAGGGTCTGG
BLT1	GGCTGCAAACACTACATCTCC	TCAGGATGCTCCACACTACAA
5LOX	TCATTGAGAAGCCAGTGAAGG	GTTGGGAATCCTGTCTGGTGA
CXCL1	AAACCGAAGTCATAGCCACAC	GGGGACACCTTTTAGCATCTT
CXCL2	ATCCAGAGCTTGAGTGTGACG	GCCTTGCCTTTGTTCAGTATC
CXCR2	AACAATACATCCCGTTTGAGG	AGTGTGAACCCGTAGCAGAAC
CCL2	CGGAACCAAATGAGATCAGAA	TTGTGAAAAGGTAGTGGATG
CCR2	TTACCTCAGTTCATCCACGGC	CAAGGCTCACCATCATCGTAG