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TITLE:
The Axl receptor tyrosine kinase is a discriminator of macrophage function in the inflamed lung

ABSTRACT:
Much of the biology surrounding macrophage functional specificity has arisen through examining inflammation-induced polarising signals, but this also occurs in homeostasis, requiring tissue-specific environmental triggers that influence macrophage phenotype and function. The TAM receptor family of receptor tyrosine kinases (Tyro3, Axl and MerTK) mediates the non-inflammatory removal of apoptotic cells by phagocytes through the bridging phosphatidylserine-binding molecules Gas6 or Protein S. We show that one such TAM receptor (Axl) is exclusively expressed on mouse airway macrophages, but not interstitial macrophages and other lung leukocytes, under homeostatic conditions and is constitutively ligated to Gas6. Axl expression is potently induced by GM-CSF expressed in the healthy and inflamed airway, and by type I interferon or TLR3 stimulation on human and mouse macrophages, indicating potential involvement of Axl in apoptotic cell removal under inflammatory conditions. Indeed, an absence of Axl does not cause sterile inflammation in health, but leads to exaggerated lung inflammatory disease upon influenza infection. These data imply that Axl allows specific identification of airway macrophages, and that its expression is critical for macrophage functional compartmentalisation in the airspaces or lung interstitium. We propose that this may be a critical feature to prevent excessive inflammation due to secondary necrosis of apoptotic cells that have not been cleared by efferocytosis.

Introduction:
Tyro3, Axl, and MerTK form the TAM receptor tyrosine kinase family and bind Protein S and growth arrest-specific 6 (Gas6) proteins (Gas6 affinity for Axl>Tyro3>MerTK) whose N-terminal Gla domains bridge TAM receptors to phophatidylserine (PtdSer) on the surface of apoptotic cells, whereas the C-terminal sex hormone-binding globulin-like domain binds and activates the TAM receptor.1, 2, 3 TAM receptors are broadly expressed by cells of the vascular, nervous, reproductive, and immune systems, with mature immune cell populations predominantly expressing Axl and/or MerTK, but not Tyro3.2 There is some evidence of differential expression of TAM receptors on immune cells in health,4 and dynamic regulation of Axl and MerTK expression on macrophages by pro- and anti-inflammatory factors has recently been characterized.5 In the innate immune system, TAM receptors inhibit inflammation during apoptotic cell efferocytosis via a negative feedback loop involving activation of suppressor of cytokine signaling-1 and -3 that inhibit cytokine and Toll-like receptor (TLR) signaling pathways.6, 7, 8 An absence of TAM receptors results in impaired apoptotic cell clearance, the generation of antibodies to self-cellular antigens,7, 9 and heightened responses to endotoxin9, 10 and inflammatory cytokines.11
Given a critical role of TAM receptors in suppressing immune responses, it is not surprising that defects in the TAM receptor system have been identified in patients with autoimmune diseases, including multiple sclerosis and systemic lupus erythematosus.7, 12 However, although inhibition of innate inflammation is essential to prevent autoimmunity during apoptotic cell clearance, prolonged engagement of TAM receptors may cause a state of unresponsiveness in antigen-presenting cells required to clear pathogenic microorganisms. It is noteworthy that elevated Gas6 plasma levels are observed in patients with severe sepsis13 and MerTK is elevated on monocytes from patients with septic shock compared with trauma patients and healthy controls, and is linked to an adverse outcome.14
The mucosal immune system of the lung requires a fine balance between the ability to mount adequate responses to pathogens entering the respiratory tract, and the control of inflammatory processes that might arise from accumulation of large quantities of structural and infiltrating immune cells that undergo apoptosis.15 Despite this critical regulatory role of efferocytosis in the lung mucosa, little is known about TAM receptors or their ligands in the healthy or inflamed lung. Moreover, in light of recent evidence of an oncogenic function of TAM receptors and initial attempts to block TAM receptor signaling in cancer patients,16 it is of great interest to understand how manipulation of TAM receptor signaling could affect susceptibility to lung infections. We now show that, unlike the reported dominance of MerTK on murine primary macrophages4 and the human macrophage cell line U937,6, 17 Axl was specifically and constitutively expressed on airway, but not interstitial lung macrophages. Inhalation of influenza virus or stimulation of macrophages in vitro with viral pathogen-associated molecular patterns or type I interferon (IFN)

specifically upregulated Axl. Finally, we show that Axl–/– mice were unable to resolve influenza-induced inflammation causing an accumulation of apoptotic cells and necrotic cell debris. This study provides clear evidence for a constitutive and critical role for the TAM receptor Axl in lung immune homeostasis and in resolution of viral inflammatory lung disease.

The TAM receptor Axl is exclusively expressed on airway macrophages in the homeostatic lung ::: Results:
Murine airway macrophages in homeostasis were characterized as CD11bloCD11chiF4/80+Ly6G–, were 95% pure in health (Figure 1a), and expressed high levels of Axl and MerTK, but not Tyro3 (Figure 1b). Airway lavage does not remove all airway macrophages, which can be observed in dissociated lung interstitial tissue. Here, also present were monocyte/macrophages that were CD11bhiCD11cintermediate and monocytes that were CD11bhiCD11clo (Figure 1c). Axl and MerTK were almost exclusively expressed by CD11bloCD11chi airway macrophages at this site, while we did not detect significant levels of Tyro3 on any of the analyzed populations (Figure 1d). High Axl protein expression was confirmed by western blot analysis in purified airway macrophages from wild type but not Axl–/– mice (Figure 1e). The majority of airway macrophages co-expressed both TAM receptors (Figure 1f). Interestingly, airway macrophages were the only immune cell population of the lung expressing high levels of Axl: we failed to detect Axl protein on neutrophils, eosinophils, T cells, NK cells, and only a very low level of Axl was detected on dendritic cells residing in the lung under homeostatic conditions (Supplementary Figure S1 online).
We next compared airway macrophage TAM receptor expression with macrophages in different anatomical locations. Airway macrophages expressed ~20-fold higher levels of Axl mRNA compared with peritoneal macrophages (Figure 2a), whereas expression of MerTK mRNA was more evenly distributed between these macrophage populations (Figure 2b). Consistently, within the analyzed macrophage populations, Axl protein expression at homeostasis was restricted to mucosal macrophages in the intestinal tract and airway, with the most dominant expression on airway macrophages (Figure 2c), whereas MerTK was more widely expressed (Figure 2d), indicating a specific role for Axl in apoptotic cell clearance from the airways.
Specific expression of Axl on airway macrophages may reflect constituents of the healthy lung microenvironment. This hypothesis is supported by the exclusive ability of granulocyte-macrophage colony-stimulating factor (GM-CSF), but not macrophage colony-stimulating factor (M-CSF), to induce Axl mRNA (Figure 3a) and protein (Figure 3c) expression in the course of differentiation of bone marrow-derived macrophages (BMDMs), an influence clearly visible also by flow cytometry (Figure 3d). High levels of MerTK expression, however, were detected in BMDMs differentiated by either M-CSF or GM-CSF (Figure 3b and e). Furthermore, Axl expression could also be selectively induced by GM-CSF, but not by M-CSF, on otherwise Axl-negative terminally differentiated macrophages from the murine peritoneal cavity (Figure 3f and g). Given a critical role of GM-CSF in airway macrophage development,18, 19 this observation indicates that GM-CSF might act as a dominant signal for macrophage expression of Axl in homeostasis.

The TAM receptor ligand Gas6 is constitutively bound to Axl ::: Results:
TAM receptors recognize externalized PtdSer on apoptotic cells via the bridging ligands Gas6 or protein S.2 All of the airway macrophages expressing Axl also co-stained with Gas6 by flow cytometry, suggesting constitutive binding in the steady state (Figure 4a). This was confirmed in Axl–/– mice where both Axl (Figure 4b) and Gas6 (Figure 4c) expression was lost. The observation that Gas6 is not detected on Axl–/– airway macrophages despite high MerTK expression on these cells (Figure 1b) also indicated that constitutive binding of Gas6 in the absence of apoptotic cells is restricted to Axl. We next examined whether macrophage populations can also act as a source of Gas6 for Axl-expressing cells by profiling Gas6 mRNA expression. Low levels of Gas6 mRNA were observed in peritoneal or airway macrophages (CD11bloCD11chi), whereas elevated Gas6 expression was observed in lung monocyte-macrophages (CD11bhiCD11clo; Figure 4d). This suggests that macrophages driven by particular stimuli can produce Gas6, whereas others can bind it in a paracrine manner. To test this we derived BMDMs using M-CSF or GM-CSF; importantly, only the former expressed substantial levels of Gas6 mRNA (Figure 4e). Upon incubation with exogenous Gas6, only GM-CSF-differentiated BMDMs, which express high levels of Axl (Figure 4f), acquired Gas6 signal on the cell surface (Figure 4g). On the other hand, neither BMDMs from Axl–/– mice nor Axl-negative M-CSF-differentiated BMDMs from WT mice were able to bind exogenous Gas6 (Figure 4g), confirming the specificity of homeostatic Axl–Gas6 interaction.

Viral triggers and influenza infection induce Axl expression on macrophages ::: Results:
Macrophage populations in the airspaces and lung change dramatically during influenza infection; in wild-type mice intranasal influenza H1N1 infection induces a viral titer-dependent weight loss that correlates with the extent of the inflammatory cell infiltrate.20, 21 We used a dose that causes up to 25% weight loss with recovery (Figure 5a). Infection with influenza resulted in monocyte/macrophages recruitment to the airspaces leading to the presence of three main populations (Figure 5b). As in health (Figure 1b), all resident airway macrophages (CD11bloCD11chi) were Axl- and MerTK-positive (Figure 5c). In contrast to uninfected animals (Figure 1d), following viral infection infiltrating monocyte/macrophages (CD11bhiCD11cintermediate) also expressed Axl and MerTK, albeit to a lower intensity than airway macrophages (Figure 5d). No expression of Axl or MerTK was observed on infiltrating monocytes (Figure 5e). The number of Axl-expressing macrophages and the levels of soluble Axl in airway lavage fluid (Figure 5f and g) increased with infection, peaking at the point of maximal weight loss and cellular infiltrate, then returning to a level above that observed pre-infection. Monocyte/macrophages infiltrating the inflamed airway can therefore increase Axl expression during the course of viral infection.
To assess the virally induced factors that might influence macrophage expression of Axl, we stimulated peritoneal macrophages or M-CSF-differentiated BMDMs in vitro with the viral TLR3 agonist polyinosinic/polycytidylic acid (p(I:C)) or with IFN-α. Both of these stimuli induced Axl mRNA (Figure 6a and c) and protein expression (Figure 6b and d), demonstrating that both endogenous and exogenous triggers associated with viral infection drive Axl expression. This regulatory mechanism was conserved between mouse and human cells, as p(I:C) also upregulated Axl mRNA (Figure 6e) and protein expression (Figure 6f) in otherwise Axl-negative human monocyte-derived macrophages, as did IFN-α (Figure 6g). Interestingly, p(I:C) also upregulated Axl on human airway macrophages (Figure 6h). As p(I:C) mimics the component of viral genome, stimulation of macrophages with p(I:C) triggers IFN-α production. To determine if p(I:C)-mediated Axl upregulation is directly dependent on the release of IFN-α, we treated human monocyte-derived macrophages with an anti-type I IFN receptor (IFNAR) neutralizing antibody before p(I:C) stimulation and subsequently assessed the expression of Axl. Treating human monocyte-derived macrophages with anti-IFNAR antibody blocked the induction of Axl protein expression by p(I:C) in a STAT1 phosphorylation-dependent manner (Figure 6i), showing that Axl upregulation by p(I:C) is dependent on IFN-α release.

Axl is required for resolution of lung inflammatory disease upon influenza infection ::: Results:
Axl–/– mice did not show any alterations in lung immune cell composition in homeostasis (Supplementary Figure S2), suggesting that the presence of MerTK is sufficient for the clearance of apoptotic cells under homeostatic conditions. However, in light of the high Axl expression on airway macrophages, but not other lung leukocytes, and rapid increases in the numbers of Axl-positive cells in the airways during influenza infection, we hypothesized that Axl has a distinctive, but complementary, role to MerTK during inflammatory lung disease. Indeed, upon influenza infection Axl–/– mice displayed enhanced weight loss, with impaired recovery, requiring the experiment to be terminated (Figure 7a). Exacerbated disease was associated with elevated inflammatory cytokine/chemokine release into the airways (Figure 7b and c). The number of total cells in the airway was also increased in the absence of Axl (Figure 7d), mostly accounted for by increases in neutrophils and CD4+ and CD8+ T cells (Figure 7e–g). Enhanced severity of influenza infection in mice lacking Axl was not due to a delay in viral clearance (Figure 7h) and is likely a result of secondary necrosis of unefferocytosed apoptotic cells. Indeed, the numbers of early and late apoptotic cells (Figure 7i and j), as well as nucleosome release (Figure 7k)—indicative of necrosis or secondary necrosis of apoptotic cells22—were elevated in the airway of Axl–/– mice infected with influenza. Finally, airway macrophages from Axl–/– mice displayed reduced uptake of apoptotic cells than those from wild-type mice (Figure 7l), indicating that Axl-mediated efferocytosis by airway macrophages is a critical step in the process of resolution of lung inflammatory disease upon viral infection.

Discussion:
We have found that under homeostatic conditions the TAM receptor Axl is preferentially expressed on murine airway macrophages and constitutively ligated by Gas6. Although constitutive expression of Axl has been reported on certain macrophage populations, such as splenic red pulp macrophages and Kupffer cells in the liver,5, 17 we show that in the healthy lung, airway macrophages are the only population of immune cells expressing high levels of Axl. MerTK on the

other hand, is expressed on all mature tissue macrophages.17 Also, despite constitutively binding Gas6, airway macrophages themselves expressed low levels of Gas6, and so may bind it in a paracrine manner after production from other cell types. Bone marrow, endothelial cells, and vascular smooth muscle cells express Gas6 (refs 23, 24, 25) and we are the first to show differential expression of Gas6 in specific macrophage subsets, i.e., CD11bhiCD11cintermediate monocyte/macrophages, but not CD11bloCD11chi airway macrophages. This is likely to result in functional polarization of macrophages depending on their anatomical location. Although all airway macrophages also express a second TAM receptor, MerTK, Gas6 binding was lost in Axl–/– macrophages, supporting the idea that Axl is the highest affinity receptor for this PtdSer-binding ligand,26 and suggesting a unique and non-redundant role of Axl and MerTK in regulating responses to apoptotic cells. In a recently proposed model, Axl has a dominant role in apoptotic cell uptake by macrophages under inflammatory conditions, whereas MerTK mediates macrophage responses to apoptotic cells in homeostasis and during immunosuppression.5 Consistently, we observed induction of Axl expression on macrophages by inflammatory stimuli in vitro and upon viral infection in vivo.

We have also found that GM-CSF induces Axl expression on peritoneal macrophages and BMDMs. GM-CSF is produced by a variety of cells, significantly airway epithelial type II cells,27 and is critical for airway macrophage development18 and the protection of airway epithelial integrity.19 GM-CSF–/– mice lack airway macrophages19 and the presence of GM-CSF autoantibodies or mutations in the GM-CSF receptor α or β chain leads to pulmonary airway proteinosis,28 a condition characterized by insufficient surfactant clearance by airway macrophages. Furthermore, GM-CSF-deficient mice experience severe morbidity to influenza virus infection,19 whereas treatment with GM-CSF increases airway macrophage numbers and protects against a lethal infection.29 Future studies will be needed to elucidate whether GM-CSF drives Axl expression in vivo and to determine which aspects of GM-CSF modulation of airway macrophage function depend on Axl activity.

Influenza infection of mice lacking Axl displayed a very similar proinflammatory phenotype to that reported for GM-CSF–/– animals.19 Axl–/– mice experienced excessive weight loss and a few reached the humane end point. Although in our experiments we used mice that lack Axl expression globally, recent evidence that airway macrophages are the main cell population responsible for efferocytosis in the respiratory tract supports the idea that the observed phenotype can be attributed to the lack of Axl on airway macrophages.19 Interestingly, viral clearance kinetics was unaffected, despite a number of viruses express PtdSer in their outer envelope30 and are opsonized by the TAM receptor ligands Gas6 or Protein S thereby acting as attachment factors enabling proximity to more specific entry receptors.31 Equivalent viral titers in Axl–/– and wild-type mice in our experiments suggest that Axl is not important for control of influenza virus H1N1 infection, or may be compensated by other PtdSer-recognizing receptors. TIM1, e.g., promotes productive infection of a wide variety of enveloped viruses and increases their internalization, although for influenza H7N1 and SARS-CoV virions this does not result in a productive infection.32

Increased morbidity of influenza virus infection in Axl–/– mice is more likely explained by the enhanced pulmonary inflammation and cytokine release, which may have arisen through two different processes. First, a lack of Axl signaling upon engagement of apoptotic cells may prevent induction of suppressor of cytokine signaling-1 and -3 that inhibit cytokine and TLR signaling,2, 33 and/or the nuclear factor-κB repressor, Twist, that binds to the tumor necrosis factor promoter and inhibits nuclear factor-κB–dependent transcription.8 Similarly, the process of viral uptake has been shown to subvert antiviral immunity through a process termed “apoptotic mimicry”, where viral envelope-expressed PtdSer sequesters Protein S and Gas6 that activate TAM receptors on bone marrow-derived dendritic cells causing a downregulation of TLR and IFN signaling pathways.34 An absence of Axl would remove any negative influence of PtdSer-expressing influenza virus on innate inflammation.

Second, failure to remove apoptotic cells leads to an increase in their membrane permeability and secondary necrosis that exposes otherwise hidden intracellular danger-associated molecular patterns, including uric acid, high-mobility group box 1, SAP 130, S100 proteins, nucleic acids in mitochondrial DNA, IL-1, and IL-33.35, 36 These danger-associated molecular patterns are recognized by extracellular and intracellular pattern recognition receptors, such as the receptor for advanced glycation endproducts (RAGE) and the IL-33 receptor (ST2),37, 38 that upon engagement produce a state of “sterile inflammation.” 36 Clearly timely removal of apoptotic cells is essential to prevent further inflammation and resolve inflammatory disease. The observed increase in nucleosome release in the airways of influenza-infected Axl–/– mice in our study

supports the idea of enhanced secondary necrosis, which could explain the observed elevated airway inflammatory cytokines. The airways of mice lacking GM-CSF (which induces Axl expression) following influenza infection are similarly enriched in dead cells and cellular debris indicating impaired clearance of apoptotic cells.19 In line with a pathogenic role of cellular components released from necrotic cells, circulating histones are direct mediators of lung inflammation and damage in patients with trauma-associated lung injury.39

Recently, a beneficial effect of administering an Axl-blocking antibody has been described during influenza virus infection,40 which is the opposite of what would be expected by blocking a receptor that activates anti-inflammatory pathways. The monoclonal antibody used in these studies prevents Gas6 binding to TAM receptors, causes receptor internalization, and inhibits downstream signaling.41 However, we and others5 show that Gas6 is constitutively bound to Axl-expressing airway macrophages; whether this antibody can disrupt this established interaction is not known. TAM receptors are pivotal inhibitory receptors that terminate cytokine receptor signaling.2, 8, 33 Chronic inflammation and systemic autoimmune disorders occur in TAM triple-knockout mice,33 phagocytes lacking TAM receptors display defective phagocytosis and overproduction of proinflammatory cytokines4, 7, 42 and administration of protein S protects against LPS-induced lung injury.43 The beneficial effect of administering intraperitoneally an antibody that blocks Gas6 binding to Axl in virus-infected mice may in fact reflect antibody binding to soluble Axl that we have observed in the peripheral blood (mean concentration±s.e.m. 20.93±0.79 ng ml−1, n=12), which would reduce the competition for free Gas6 and promote membrane-bound Axl signaling. The distribution of administered antibody and whether it reached the high intensity of Axl expression on airway macrophages was not tested.40

Targeting Axl for the treatment of Gas6-Axl-related diseases is of high current interest.16 A small-molecule inhibitor that preferentially targets Axl inhibits breast cancer metastasis and angiogenesis,44 and is currently in phase 1 clinical trials (BGB324 by BergenBio). Complications, however, may arise from targeting a receptor that is also cleaved. With regards to the immune system, therapeutics sequestering the soluble form of Axl would be anti-inflammatory as it would remove competition for Gas6 by membrane tethered Axl. Conversely, blockade of Gas6 interaction with membrane-bound Axl or Axl enzymatic activity would be proinflammatory and lead to heightened inflammation due to secondary necrosis of apoptotic cells and a loss of anti-inflammatory signaling cascades. Current antibodies do not distinguish between soluble and membrane-bound forms of Axl at present and the drive to target this pathway stems from the known biology of membrane tethered Axl. The selective expression of Axl on airway, but not interstitial macrophages in the homeostatic state and its role in preventing secondary necrosis infers an important function that should not be ignored.

Methods:

Detailed information on the experimental materials and methods used in this study can be found in Supplementary Material.

Mouse infection and sampling. Eight- to twelve- week-old female C57BL/6 (Harlan Olac, Bicester, UK) were maintained in specific pathogen-free conditions at Bio Safety Level 2, with a 12-h light/dark cycle, dry food pellets, and water ad libitum in accordance with the institutional and UK Home Office guidelines. The generation of Axl−/− has been described previously,45 and all Axl−/− mice used in this study have been back-crossed to C57BL/6J background for at least 10 generations. Mice were intranasally infected with 7.5 p.f.u. of influenza A virus, Puerto Rico/8/34(PR8), H1N1, and at specific time points euthanized by intraperitoneal injection of 3 mg pentobarbitone and exsanguination via the femoral artery. Bronchoairway lavage, lung tissue, and samples from distal sites (peritoneum, small intestine, bone marrow, and blood) were harvested and dissociated to a single-cell suspension using methods described previously.20, 46 Total macrophages from murine ears were extracted by splitting the dermis and epidermis in 0.8% trypsin for 30 min at 37 °C. Dermis and epidermis were minced with scissors and then the dermis fraction was further digested in 0.5 Wunch U ml−1 of collagenase for 30 min at 37 °C. Both dermis and epidermis cell suspension were then passed through a 70-μm sieve and combined. Red blood cells were lysed in whole blood and homogenized lung tissue using ammonium-chloride-potassium lysis buffer. Bronchoairway lavage fluid was retained for analysis of soluble Axl (R&D systems, Abingdon, UK) or cytokines (eBioscience, Hatfield, UK) by ELISA. Viral titer was determined in lung homogenates by quantitative PCR on a QuantStudio 12K Flex PCR system (Life Technologies, Paisley, UK; fwd: 5′-GGACTGCAGCGTAGACGCTT-3′; rev: 5′-CATCCTGTTGTATATGAGGCCCAT-3′)47 and quantified by the ΔΔCT method using QuantStudio 12K Flex Software v1.1.1 (Life Technologies).

Flow cytometry. A total of 2 × 105 to 1 × 106 cells were incubated with near Infrared LIVE/DEAD fixable cell stain kit according to the manufacturer's instruction (Life Technologies) followed by anti-mouse CD16/32 Fc block (eBioscience) for 20 min at 4 °C. After washing with PBA (phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide), cells were stained with a customized extracellular antibody panel for 30 min at 4 °C in PBA, and then fixed for 20 min with 2% paraformaldehyde or kept on ice with 5 μM EDTA before sorting. Cells were run on a BD FACS Canto II collecting at least 10,000 events of the target population and analyzed using FlowJo (Tree Star, Ashland, OR). A BD Influx was used for sorting pure airway and peritoneal macrophages for quantitative PCR analysis. Alveolar macrophages were identified as CD11bloCD11chiF4/80hi and highly auto-fluorescent. Peritoneal macrophages were identified as CD11bhighCD11cloF4/80hi. All lineage markers were purchased from eBioscience or BioLegend (San Diego, CA). Axl (clone 175128), MerTK (polyclonal goat IgG, biotinylated), Tyro3 (clone 109646), and Gas6 (polyclonal goat IgG, biotinylated) antibodies were from R&D Systems. Axl and Tyro3 antibodies were conjugated to Alexa Fluor-647 dye using a labeling kit (Life Technologies) according to the manufacturer's instruction.

Uptake of apoptotic thymocytes by macrophages. Protocol for measurement of apoptotic thymocyte uptake efficiency was adapted from ref. 48 and described in Supplementary Material.

Human alveolar macrophage isolation and culture. Alveolar macrophages were isolated as previously described49 and described in detail in Supplementary Material.

Statistics. GraphPad Prism version 5.04 (GraphPad Software, San Diego, California, USA) was used for all statistical calculations. For multiple data set analysis, analysis of variance with Bonferroni correction was applied. To compare two data sets, paired or unpaired t-tests were applied. Data are presented as the mean±standard error of the mean (s.e.m.). P values<0.05 were considered significant.