Initial Assessment of the Role of CXC Chemokine Receptor 4 after Polytrauma

Harold H Bach IV,[1,2] Vikas Saini,[1] Todd A Baker,[1] Abhishek Tripathi,[1] Richard L Gamelli,[1] and Matthias Majetschak[1,2]

[1]Department of Surgery and [2]Department of Molecular Pharmacology and Therapeutics, Burn and Shock Trauma Institute, Loyola University Chicago, Maywood, Illinois, United States of America

CXC chemokine receptor (CXCR)-4 agonists have been shown to attenuate inflammation and organ injury in various disease models, including trauma/hemorrhage. The pathophysiological role of CXCR4 during the early response to tissue injury, however, remains unknown. Therefore, we investigated the effects of AMD3100, a drug that antagonizes binding of stromal cell–derived factor (SDF)-1α and ubiquitin to CXCR4 during the initial response to polytrauma in pigs. Fifteen minutes before polytrauma (femur fractures/lung contusion; control: sham), 350 nmol/kg AMD3100, equimolar AMD3100 and ubiquitin (350 nmol/kg each) or vehicle were administered intravenously. After a 60-min shock period, fluid resuscitation was performed for 360 min. Ubiquitin binding to peripheral blood mononuclear cells was significantly reduced after intravenous AMD3100. SDF-1α plasma levels increased transiently >10-fold with AMD3100 in all animals. In injured animals, AMD3100 increased fluid requirements to maintain hemodynamics and enhanced increases in peripheral blood granulocytes, lymphocytes and monocytes, compared with its effects in uninjured animals. Cytokine release from leukocytes in response to Toll-like receptor (TLR)-2 and TLR-4 activation was increased after *in vitro* AMD3100 treatment of normal whole blood and after *in vivo* AMD3100 administration in animals subjected to polytrauma. Coadministration of AMD3100/ubiquitin reduced lactate levels, prevented AMD3100-induced increases in fluid requirements and sensitization of the tumor necrosis factor (TNF)-α and interleukin (IL)-6 release upon TLR-2/4 activation, but did not attenuate increases in leukocyte counts and SDF-1α plasma levels. Our findings suggest that CXCR4 controls leukocyte mobilization after trauma, regulates leukocyte reactivity toward inflammatory stimuli and mediates protective effects during the early phase of trauma-induced inflammation.

INTRODUCTION

The G protein–coupled receptor CXC chemokine receptor (CXCR)-4 is abundantly expressed in immune cells and tissues (1). CXCR4 fulfills important biological functions during development and hematopoiesis. It also plays pleiotropic roles in the immune system and during tissue repair processes and gained particular attention as a drug target because of its role in human immunodeficiency virus (HIV) infection and metastatic diseases (2–10). Although the constitutively expressed chemokine stromal cell–derived factor (SDF)-1α is the cognate ligand of CXCR4 (11), we recently identified extracellular ubiquitin as another natural CXCR4 agonist (12–15).

We showed previously that ubiquitin is systemically released after trauma and functions as an endogenous immune modulator with antiinflammatory properties in patients (16). Furthermore, we observed that high systemic ubiquitin levels are associated with a lower degree of organ dysfunction and reduced mortality in burn patients, suggesting that endogenous extracellular ubiquitin mediates protective effects during the initial inflammatory response to tissue injury (17). Although the regulation of SDF-1α levels after trauma and its association with clinical outcomes are largely unknown, administration of SDF-1α and of various SDF-1α mimetic proteins have been shown to result in beneficial effects in models of acute infectious and sterile inflammation *in vivo* (18–22). Similarly, treatment with exogenous ubiquitin has been shown to attenuate inflammation and to confer organ protection in animal models, including various models of trauma and hemorrhage (23–30). Collectively, these studies imply CXCR4 as a promising drug target for trauma patients and suggest that CXCR4 may play an important role during the inflammatory response to tissue injury. However,

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Address correspondence to *Matthias Majetschak, Burn and Shock Trauma Institute, Loyola University Chicago, 2160 South First Avenue, Maywood, IL 60153. Phone: 708-327-2472; Fax: 708-327-2813; E-mail: mmajetschak@lumc.edu.*

the role of CXCR4 during the systemic response to trauma has not been evaluated.

Therefore, we sought to provide an initial assessment of the involvement of CXCR4 in the pathophysiology of severe trauma. Because animals lacking CXCR4, SDF-1α or ubiquitin are not viable (4,31,32), we chose a pharmacological approach using AMD3100 (1,1'-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetra-decane octahydrochloride), a drug that antagonizes the binding of SDF-1α and ubiquitin to CXCR4 (12,15,33–35). AMD3100 (generic name Plerixafor) was approved by the U.S. Food and Drug Administration to be used in combination with granulocyte-colony stimulating factor to mobilize hematopoietic stem cells to the bloodstream in patients with non-Hodgkin lymphoma and multiple myeloma (36). On the basis of available data on the role of endogenous extracellular ubiquitin after trauma in patients (16,17), its inhibitory effects on leukocyte function upon pattern recognition receptor (PRR) activation (16,25,37) and the therapeutically relevant actions of CXCR4 agonists in various animal models (19–30), we hypothesized that blockade of ligand binding to CXCR4 will enhance proinflammatory responses of leukocytes and result in adverse effects when animals are subjected to severe trauma. Thus, we studied the pathophysiological response to trauma after blockade of CXCR4 with AMD3100 in a large animal model, which was designed to mimic the typical blunt polytrauma patient.

MATERIAL AND METHODS

Animal Protocol

All procedures were performed according to National Institutes of Health guidelines (*Guide for the Care and Use of Laboratory Animals* [38]) and were approved by the Institutional Animal Care and Use Committee and the U.S. Army Medical Research and Materiel Command Animal Care and Use Review Office. Male and female Yorkshire pigs (30–42 kg body weight) were fasted

overnight. Anesthesia was induced with 10 mg/kg ketamine and 1 mg/kg xylazine intramuscularly. Peripheral intravenous (i.v.) access was obtained for continuous infusion of 10 mg/kg/h ketamine, 0.25 mg/kg/h xylazine and 50 μg/kg/h fentanyl for maintenance of anesthesia throughout the experiment. Orotracheal intubation was performed using 6.0- to 7.0-mm internal diameter cuffed endotracheal tubes (Teleflex Medical, Research Triangle Park, NC, USA) under direct laryngoscopy. Animals were mechanically ventilated (Evita XL, Draeger Medical, Telford, PA, USA) with intermittent mandatory ventilation adjusted to tidal volumes of 12 mL/kg at 10–20 breaths per minute to maintain a partial pressure of CO_2 (pCO_2) between 35 and 45 mmHg. The fraction of inspired oxygen (FiO_2) was 0.4 and positive end expiratory pressure (PEEP) 5 mmHg, except where otherwise noted. Core body temperature was maintained using conductive warming blankets (Gaymar T/Pump 500 T/Pad, Gaymar Industries Inc., Orchard Park, NY, USA). A central venous catheter (Triple-Lumen 7Fr. AGB + Arrow Catheter, Teleflex Medical) was placed in the external jugular vein for administration of fluids, anesthesia and continuous monitoring of central venous pressure. The ipsilateral common carotid artery was cannulated for continuous measurement of mean arterial blood pressure (MAP) with a 14-g angiocatheter. Electrocardiography, pulse oximetry, capnography (Evita XL Capnography module, Draeger Medical) and body temperature were measured and monitored continuously. After achieving stable baseline conditions (at least 30 min after instrumentation), animals underwent a sham procedure or polytrauma ($t = 0$ min).

Polytrauma consisted of bilateral open femur fractures and lung contusion, modified as described previously (25,39,40). Injuries were produced with a captive bolt gun (Karl Schermer, Ettlingen, Germany), modified with exchangeable mushroom-shaped metal heads (1 and 2.5 inches in diameter). In brief, the

bolt gun with the small metal head was placed vertically against the femur and fired while the animal was in the supine position and the leg extended. The metal head perforated the skin and produced a second-degree complex open-femur fracture without injury of major vessels. After both femurs had been fractured, the small metal head was exchanged for the large metal head and the bolt gun was fired against the right chest wall in the midaxillary line at the level of the fourth intercostal space with a 45° cephalad trajectory. As confirmed by necropsy, this resulted in lung contusion covering approximately 20–30% of the right lung without producing hemo/pneumothoraces or rib fractures. All injuries were produced within 5 min. On the basis of an abbreviated injury score of 3 for the extremity trauma and 3–4 for the chest trauma, the estimated injury severity score was 18–25 (41).

To simulate a shock period of 60 min after polytrauma, animals were ventilated with FiO_2 0.21, PEEP 0 mmHg, and no resuscitation was allowed other than the minimum amount of lactated Ringer solution required for delivery of anesthesia. At $t = 60$ min until $t = 120$ min, ventilation was adjusted to FiO_2 1.0, PEEP 0 mmHg, and resuscitation to a MAP of 70 mmHg was performed with warmed lactated Ringer solution. Within this period, lactated Ringer solution was administered in i.v. boluses of 500 mL until the MAP reached 70 mmHg. This process was performed to simulate typical human resuscitation regimens during prehospital emergency medical services. At $t = 120$ min until the end of the experiment, animals were ventilated with FiO_2 0.4, PEEP 5 mmHg, and resuscitation was performed continuously to maintain a MAP of at least 70 mmHg to simulate in-hospital resuscitation. At the conclusion of the experiment ($t = 420$ min), a mixture of saturated potassium chloride was infused via the central venous catheter for euthanasia while the animal was under general anesthesia.

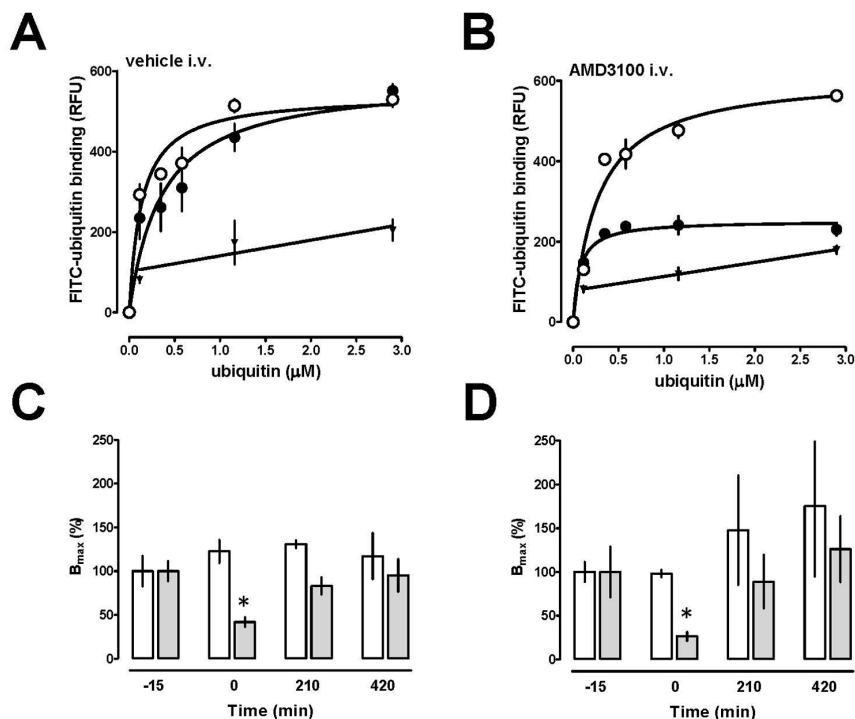Animals were randomized to one of the following experimental groups:

Figure 1. AMD3100 treatment prevents ubiquitin receptor binding. FITC-ubiquitin binding to PBMCs (1 min, 4°C) was measured as described (12). (A) Saturation binding curves for FITC-ubiquitin with PBMCs that were isolated before (open circles, $t = -15$ min) and after (black circles, $t = 0$ min) i.v. vehicle administration. Black triangles: nonspecific binding (NSB), assessed in the presence of 300 μmol/L native ubiquitin. NSB was identical before and after i.v. vehicle. Means ± SD from triplicate measurements are shown. (B) Saturation binding curves for FITC-ubiquitin with PBMCs that were isolated before (open circles, $t = -15$ min) and after (black circles, $t = 0$ min) i.v. AMD3100 administration. Black triangles: NSB, assessed in the presence of 300 μmol/L native ubiquitin. NSB was identical before and after i.v. AMD3100. Means ± SD from triplicate measurements are shown. (C) B_{max} values from saturation binding curves for FITC-ubiquitin with PBMCs from animals after sham procedure and vehicle (open bars) or AMD3100 (gray bars) treatment at $t = -15$ min. Data are expressed as percent of B_{max} at baseline ($t = -15$ min). Data are means ± SD; n = 3–5 animals per group and time point. (D) B_{max} values from saturation binding curves for FITC-ubiquitin with PBMCs from animals after polytrauma and vehicle (open bars) or AMD3100 (gray bars) treatment at $t = -15$ min. Data are expressed as percent of B_{max} at baseline ($t = -15$ min). Data are means ± SD; n = 3–5 animals per group and time point. *$p < 0.05$ versus baseline.

(a) sham, vehicle treatment (n = 6): animals were not injured, 250 mL of 0.9% NaCl (vehicle) was administered intravenously within 15 min at $t = -15$ min; (b) sham, AMD3100 treatment (n = 7): animals were not injured, 350 nmol/kg AMD3100 (Sigma, St. Louis, MO, USA) in 250 mL vehicle was administered intravenously within 15 min at $t = -15$ min; (c) polytrauma, vehicle pretreatment (n = 8): 250 mL vehicle was administered intravenously within 15 min at $t = -15$ min

(animals were subjected to polytrauma at $t = 0$ min); (d) polytrauma, AMD3100 pretreatment (n = 9): 350 nmol/kg AMD3100 in 250 mL vehicle was administered intravenously within 15 min at $t = -15$ min (animals were subjected to polytrauma at $t = 0$ min); and (e) polytrauma, AMD3100 plus ubiquitin pretreatment (n = 9): AMD3100 plus recombinant ubiquitin (R&D Biosystems, Minneapolis, MN, USA) was administered in equimolar concentrations (350 nmol/kg each) in

250 mL vehicle intravenously within 15 min at $t = -15$ min (animals were subjected to polytrauma at $t = 0$ min).

A dose of 350 nmol/kg AMD3100 was selected because this dose is in the range of the doses that produced maximum levels of AMD3100 without observable adverse effects in previous safety studies in rats and dogs (41).

AMD3100 and ubiquitin solutions were tested for endotoxin contamination using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA) according to the manufacturer's instructions. The endotoxin concentrations in both solutions were <0.05 EU/mL, which would be suitable for parenteral use in humans (http://www.bcg-usa.com/regulatory/docs/1987/FDA198712A.pdf).

Treatment with vehicle, AMD3100 or AMD3100 plus ubiquitin was performed with the investigator responsible for the animal care blinded to the randomization assignment.

Arterial blood was sampled every 15 min for the first hour after injury, followed by 30-min intervals. Arterial blood was collected in lithium heparin tubes (APP Pharmaceuticals, Schaumburg, IL, USA) and used for blood gas analyses, analyses of routine laboratory parameters, complete blood cell counts and measurements of lipopolysaccharide (LPS)- and lipoteichoic acid (LTA)-induced cytokine release of whole blood and for plasma preparation. For plasma preparation, arterial blood was centrifuged at 2,000g for 10 min, plasma separated and stored at –80°C until further analyses.

Blood Gas Analyses and Routine Laboratory Parameters

Measurements of pH, pCO_2, pO_2, hemoglobin, sodium, potassium, chloride, glucose, lactate and bicarbonate were performed using a blood gas analyzer (Stat Profile pHOx Plus L, Nova Biomedical, Waltham, MA, USA).

Complete Blood Counts

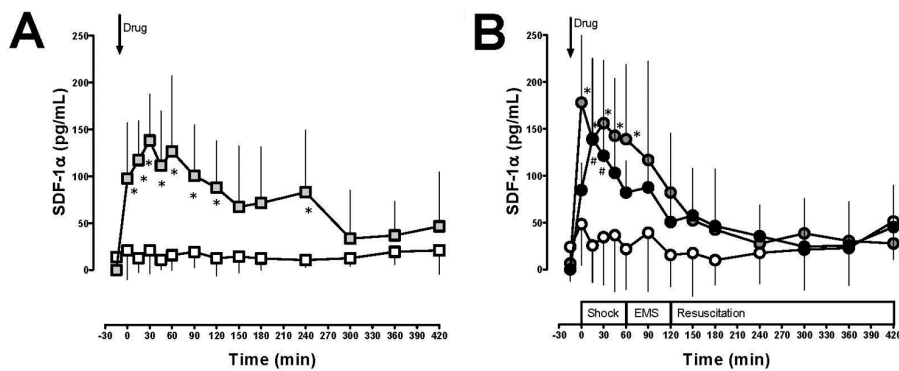Heparinized blood samples were used for the analysis of complete blood count

Figure 2. AMD3100 induces release of SDF-1α into the systemic circulation. The arrows indicate the time points of drug administration. (A) SDF-1α plasma concentrations (mean ± SD) after i.v. AMD3100 (gray squares, n = 6) or vehicle (open squares, n = 7) administration in uninjured animals. *$p < 0.05$ versus vehicle. (B) SDF-1α plasma concentrations (mean ± SD) after i.v. AMD3100 (gray circles, n = 9), AMD3100 plus ubiquitin (black circles) or vehicle (open circles, n = 8) administration in animals undergoing polytrauma at $t = 0$ min. Shock: simulated shock phase. EMS: simulated prehospital emergency medical services. Resuscitation: simulated in-hospital resuscitation phase. *$p < 0.05$ for AMD3100 versus vehicle; #$p < 0.05$ for AMD3100 plus ubiquitin versus vehicle.

on a veterinary hematology analyzer (HemaTrue, Heska, Loveland, CO, USA).

Whole Blood Stimulation

Whole blood stimulation was performed as described previously (16,25,37). In brief, arterial blood drawn at $t = -15$ min, $t = 0$ min (immediately before trauma), $t = 240$ min and $t = 420$ min was mixed 1:1 (vol/vol) with cell culture medium (RPMI-1640; Sigma) and transferred into microtiter plates (Greiner Bio One, Monroe, NC, USA). Samples were prepared in quintuplicates. The mixtures were incubated at 37°C and 5% CO_2 with LPS (500 ng/mL, from *Salmonella enterica* [Sigma]) or LTA (500 μg/mL; from *Staphylococcus aureus* [Sigma]) for 18 h. Control mixtures were incubated without LPS/LTA. After incubation, the supernatants were separated and stored frozen at –80°C until further analysis of the tumor necrosis factor (TNF)-α and interleukin (IL)-6 concentrations by enzyme-linked immunosorbent assay (ELISA). For each animal, the LPS- and LTA-induced TNF-α and IL-6 release of whole blood was calculated as pg/10^3 leukocytes and expressed as percentage of the TNF-α/IL-6 release at baseline ($t = -15$ min).

ELISAs

Ubiquitin plasma concentrations were measured with an indirect competitive ELISA, as described previously (17,25). The lower detection limit was 11 ng/mL. SDF-1α plasma concentrations were measured using an SDF-1α DuoSet ELISA Development kit (R&D Systems), as recommended by the manufacturer. The lower detection limit was 9 pg/mL. TNF-α and IL-6 were measured in cell culture supernatants using commercially available porcine ELISA kits (IL-6; R&D Systems; lower detection limit 9 pg/mL), TNF-α; R&D Systems; lower detection limit 3.7 pg/mL). All assays were performed according to the manufacturers' protocols using specimens that had not been thawed previously. Measurements were performed after all animal experiments had been completed.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation of heparinized blood diluted 1:1 (vol/vol) in phosphate-buffered saline (PBS) over a Histopaque (Sigma) density gradient, as described (16,43).

Ubiquitin Receptor Binding Assay

Ubiquitin binding to CXCR4 on PBMCs was measured as described (12–15). In brief, PBMCs were washed with ice-cold PBS, and 10^5 cells were suspended in 100 μL cold (4°C) PBS, 1% bovine serum albumin and 0.01% sodium azide in microcentrifuge tubes (VWR Scientific, Radnor, PA, USA). N-terminal fluorescein-labeled ubiquitin (FITC-ubiquitin; R&D) was added and incubated for 1 min at 4°C. Cells were washed twice with 1 mL cold PBS and resuspended in 100 μL PBS. Cell suspensions were then transferred into black 96-well microplates (VWR Scientific), and the fluorescence intensities were measured with a Synergy 2 microplate reader ($\lambda_{excitation/emission}$: 485/528 nm; BioTek Instruments, Winooski, VT, USA). Nonspecific binding was assessed as binding of FITC-ubiquitin in the presence of 300 μmol/L native ubiquitin.

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was measured in extracts from lungs, which were harvested at the end of the experiment. Snap-frozen tissues were homogenized in 1/10 PBS, pH 7.4 (1:5 weight/volume), and centrifuged (16,600g, 4°C, 30 min), and supernatants (extracts) were aliquoted, as described (43,44). Lung extracts were assayed using the EnzChek Myeloperoxidase activity assay kit (number E33856; Invitrogen, Grand Island, NY, USA). Activity measurements were performed according to the manufacturer's protocol. All measurements were standardized to total protein content (24) and expressed per milligram of protein.

Data Analyses and Statistics

Data are described as mean ± standard deviation (SD). Data were compared with two-way analysis of variance and Bonferroni *post hoc* test or with the Student t test, as appropriate. Saturation binding experiments were analyzed with nonlinear regression analyses using a one-site binding model. Data analyses were calculated with the GraphPad Prism program (GraphPad Software, La

Jolla, CA, USA). A two-tailed $p < 0.05$ was considered significant.

RESULTS

To confirm that the administered dose of AMD3100 blocked CXCR4, we measured FITC-ubiquitin binding to PBMCs as an easily accessible cell population representative of CXCR4-expressing cells. Typical receptor binding curves for FITC-ubiquitin before and after i.v. administration of AMD3100 are shown in Figures 1A and B. Whereas FITC-ubiquitin binding to PBMCs was identical before and after i.v. vehicle administration (Figure 1A), AMD3100 treatment significantly reduced FITC-ubiquitin binding (Figure 1B; binding maximum (B_{max}) before AMD3100 treatment: 455 ± 6 relative fluorescence units [RFUs]; B_{max} after i.v. AMD3100: 102 ± 3 RFUs, $p < 0.001$). The quantification of the B_{max} values from saturation binding curves with PBMCs obtained at various time points after AMD3100 administration is shown in Figures 1C and D. After sham procedure (Figure 1C) and polytrauma (Figure 1D), FITC-ubiquitin receptor binding to PBMCs was significantly reduced at $t = 0$ min and normalized within the observation period of 420 min.

AMD3100 administration did not affect plasma ubiquitin levels in animals after sham procedure or polytrauma (not shown). After coadministration of AMD3100 and exogenous ubiquitin, plasma ubiquitin levels increased from 55 ± 54 ng/mL at $t = -15$ min to 5.1 ± 2.7 μg/mL at $t = 0$ min, with a plasma elimination half-life of ~1 h. The SDF-1α plasma concentrations in the various experimental groups are shown in Figures 2A and B. SDF-1α levels remained constant in all animals after i.v. vehicle administration. When animals were treated with AMD3100, SDF-1α plasma concentrations increased >10-fold after sham procedure (Figure 2A) and after polytrauma (Figure 2B). SDF-1α plasma concentrations returned to baseline levels at $t = 300$ min in uninjured animals and at $t = 90$ min after polytrauma, respectively. Coadministration of AMD3100
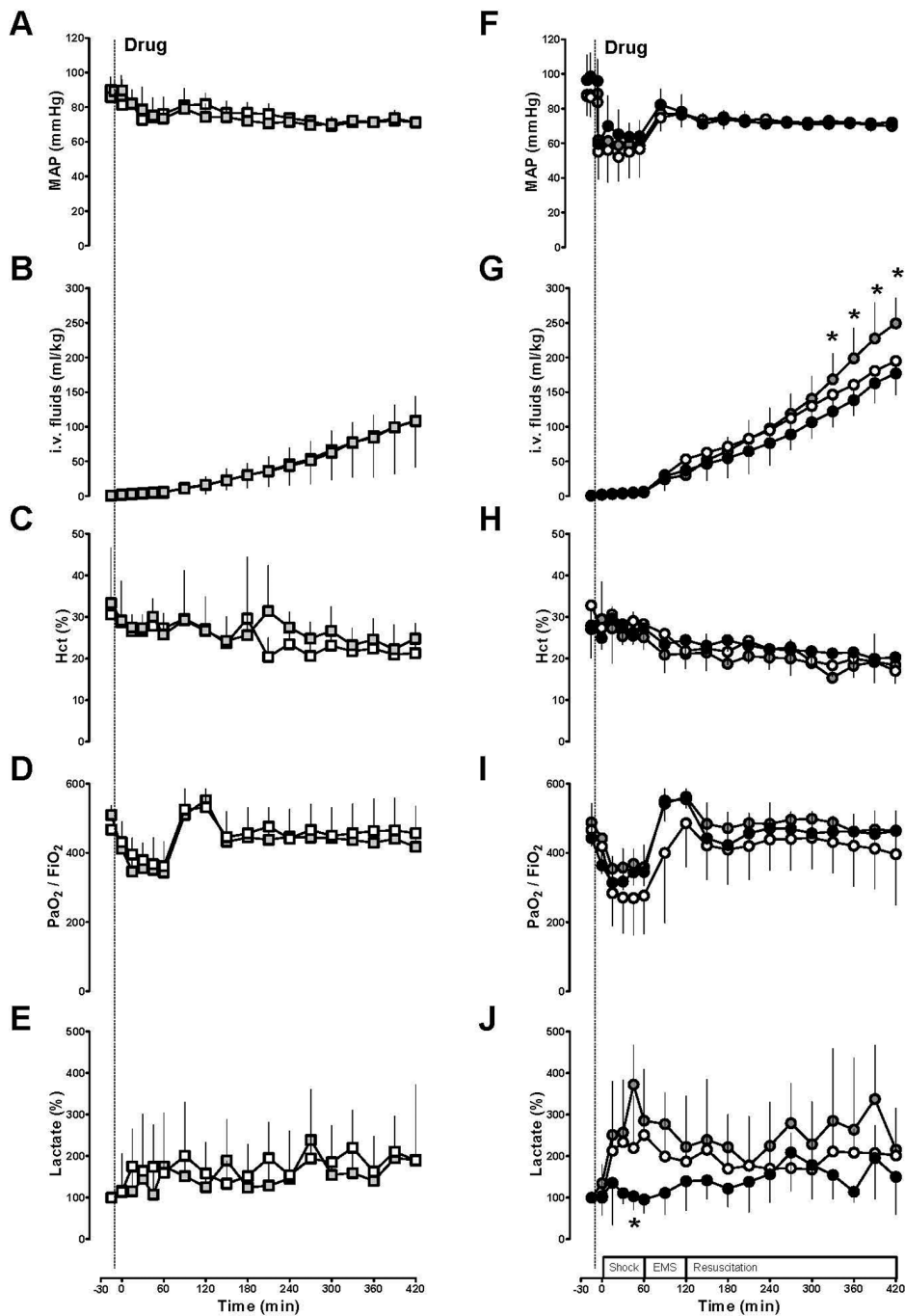

Figure 3. Physiological responses after AMD3100 administration. The arrows indicate the time points of drug administration. Data are means ± SD. (A–E) Uninjured animals. Grey squares: AMD3100 treatment (n = 6). Open squares: vehicle treatment (n = 7). (F–J) Polytrauma groups. Grey circles: AMD3100 treatment (n = 9). Black circles: AMD3100 plus ubiquitin treatment (n = 9). Open circles: vehicle treatment (n = 8). (A, F) MAP (mmHg). (B, G) i.v. fluid requirements (mL/kg) to maintain a MAP of 70 mmHg. (C, H) Hematocrit (%). (D, I) Ratio of partial pressure of oxygen in arterial blood to the fraction of inspired oxygen (PaO_2/FiO_2). (E, J) Lactate concentrations (% of baseline). Shock: simulated shock phase. EMS: simulated prehospital emergency medical services. Resuscitation: simulated in-hospital resuscitation phase. *$p < 0.05$ versus vehicle.
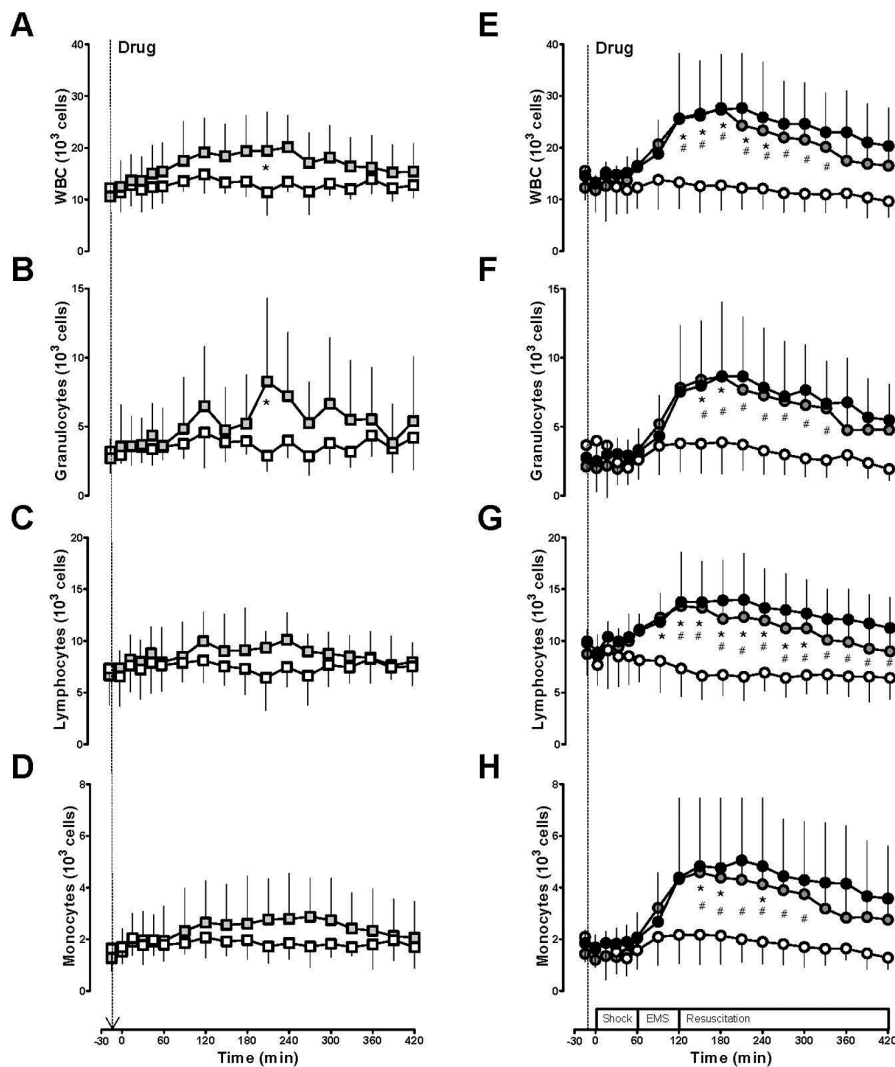
Figure 4. WBC counts after AMD3100 administration. The arrows indicate the time points of drug administration. Data are mean ± SD. (A–D) Uninjured animals. Grey squares: AMD3100 treatment (n = 6). Open squares: vehicle treatment (n = 7). (E–H) Polytrauma groups. Grey circles: AMD3100 treatment (n = 9). Black circles: AMD3100 plus ubiquitin treatment (n = 9). Open circles: vehicle treatment (n = 8). Shock: simulated shock phase. EMS: simulated prehospital emergency medical services. Resuscitation: simulated in-hospital resuscitation phase. (A, E) Total WBC counts ($\times 10^3$ cells). (B, F) Granulocytes ($\times 10^3$ cells). (C, G) Lymphocytes ($\times 10^3$ cells). (D, H) Monocytes ($\times 10^3$ cells). $*p < 0.05$ AMD3100 versus vehicle. $^\#p < 0.05$ AMD3100 plus ubiquitin versus vehicle.

lar in all polytrauma groups. There were no significant differences in P/F ratios (ratios of arterial oxygen concentration to the fraction of inspired oxygen) between the groups after polytrauma. With vehicle and AMD3100 pretreatment, lactate levels increased during the shock period to 250% and 370% of baseline with vehicle and AMD3100, respectively, and stabilized with fluid resuscitation. With AMD3100 and ubiquitin pretreatment, lactate levels remained unchanged throughout the experiment (Figure 3J).

White blood cell (WBC) counts increased twofold within 4 h after AMD3100 treatment in sham control animals and declined toward baseline counts within the remainder of the experiment (Figure 4A). All leukocyte subpopulations (granulocytes, lymphocytes, monocytes) were evenly affected by AMD3100 (Figures 4B–D).

While WBC counts slightly decreased after polytrauma with vehicle administration, AMD3100 pretreatment resulted in 2.5- to 3-fold increases in WBC counts after polytrauma (Figures 4E–H). AMD3100-induced leukocyte mobilization occurred earlier and was sustained for longer time periods after polytrauma than after sham procedure and was most pronounced for lymphocytes (Figure 4G). There were no significant differences in leukocyte counts between animals after pretreatment with AMD3100 alone or AMD3100 plus exogenous ubiquitin. However, when compared with animals after vehicle administration, increases in leukocyte counts were sustained for a longer time period after coadministration of AMD3100 and exogenous ubiquitin than after administration of AMD3100 alone.

Because AMD3100 may also affect leukocyte infiltration into tissues, we measured MPO activity as a quantifiable marker of neutrophils and monocytes in extracts from the injured and contralateral lungs. There were, however, no statistically significant differences between any of the individual groups (not shown).

To assess whether AMD3100 affected leukocyte reactivity upon PRR activation,

and exogenous ubiquitin did not affect the AMD3100-associated increase in SDF-1α plasma concentrations.

There were no differences in any of the physiological parameters between the groups at baseline, and AMD3100 treatment did not affect any of the measured parameters after sham procedure (Figures 3A–E). When AMD3100 was administered before polytrauma (Figures 3F–J), i.v. fluid requirements to maintain hemodynamics were increased, compared with vehicle administration before polytrauma. This effect was neutralized when animals were pretreated with AMD3100 and exogenous ubiquitin (Figures 3F, G). Hematocrit values (Figure 3H) and hemoglobin levels (not shown) were simi-

we then measured LPS- and LTA-stimulated whole blood TNF-α and IL-6 release. Cytokine concentrations in the cell culture supernatants were normalized to the number of WBCs in the incubation mixtures and expressed as percent of baseline to account for AMD3100-induced increases in WBC counts and the interindividual variability, respectively. In sham control animals, AMD3100 did not affect LPS-induced TNF-α and IL-6 release (Figures 5A, C). In contrast, AMD3100 pretreatment resulted in significantly increased LPS-stimulated TNF-α release 240 and 420 min after polytrauma (Figure 5B). This sensitizing effect of AMD3100 could be prevented when AMD3100 was co-administered with exogenous ubiquitin. A similar tendency was detectable for LPS-induced IL-6 release at 420 min after polytrauma. However, these differences did not reach statistical significance.

The LTA-stimulated TNF-α and IL-6 releases of whole blood are shown in Figure 6. TNF-α and IL-6 release in response to LTA stimulation was not affected by AMD3100 after sham procedure (Figures 6A, C). Similar to changes in LPS-induced responses with AMD3100 pretreatment, LTA-stimulated TNF-α release was significantly increased at 240 and 420 min after polytrauma (Figure 6B), and LTA stimulated IL-6 release at 420 min after polytrauma, respectively (Figure 6D). As observed with LPS stimulation, sensitization of the LTA-induced responses by AMD3100 after polytrauma could also be prevented with coadministration of AMD3100 and exogenous ubiquitin.

Furthermore, we tested whether AMD3100 also sensitizes LPS- and LTA-induced cytokine responses of whole blood *in vitro*. As shown in Figure 7, addition of AMD3100 to whole blood cultures from normal animals enhanced the TNF-α production in response to LPS and LTA stimulation.

DISCUSSION

In the present study, we provide an initial assessment of the contribution of

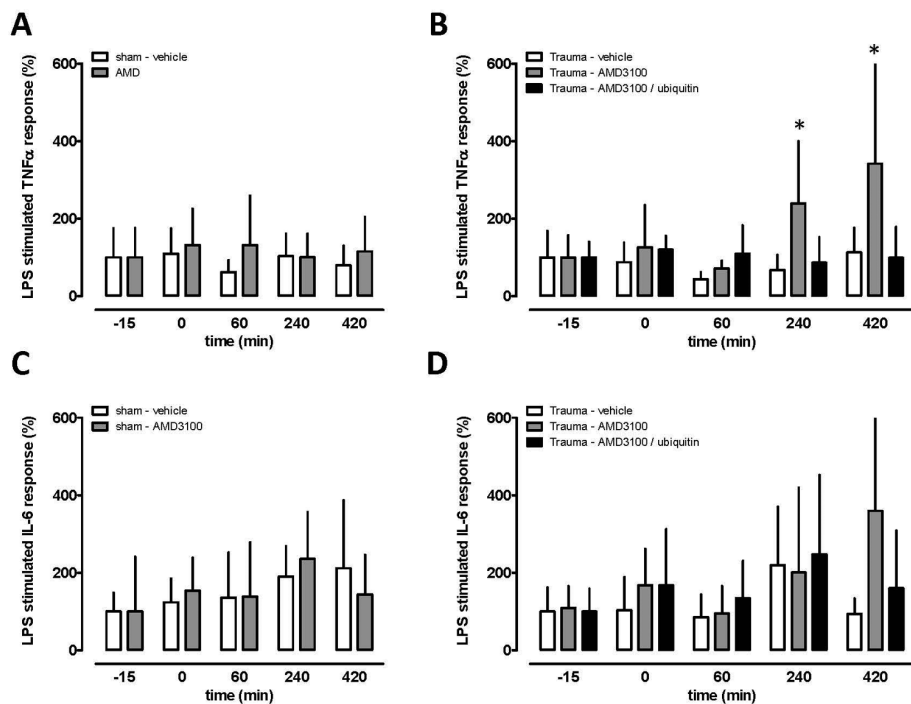
Figure 5. LPS stimulated whole blood TNF-α and IL-6 release after AMD3100 administration *in vivo*. Drugs were administered within 15 min at $t = -15$ min. Data are expressed as percent of baseline ($t = -15$ min) of the cytokine concentration in the cell culture supernatant per 10^3 leukocytes in the incubation mixtures (mean ± SD). (A, C) Uninjured animals. Open bars: vehicle treatment. Grey bars: AMD3100 treatment. (B, D) Polytrauma groups. Open bars: vehicle treatment. Grey bars: AMD3100 treatment. Black bars: AMD3100 and ubiquitin treatment. (A, B) TNF-α. C/D: IL-6. *$p < 0.05$ versus vehicle.

CXCR4 to the pathophysiology of polytrauma. Blockade of CXCR4 with AMD3100 resulted in significantly enhanced increases in leukocyte counts in injured animals, when compared with its effects in uninjured animals. Furthermore, AMD3100 increased fluid requirements to maintain hemodynamics and leukocyte reactivity in response to PRR activation after polytrauma. Collectively, these data support the notion that CXCR4 could play an important role in the regulation of the inflammatory response to severe trauma.

The injuries in our model created a pathophysiological condition that closely resembled the typical clinical characteristics of a polytrauma patient. In the absence of hemorrhagic shock, animals demonstrated a 30–40% reduction of MAP during the shock phase, showed an increase in lactate levels and were fluid

dependent to maintain hemodynamics. The continuous i.v. fluid requirements demonstrate that the animals were not stabilized within the observation period. As most commonly seen in patients with blunt chest trauma, gas exchange parameters were not affected within the early resuscitation period when ventilated with PEEP and increased FiO_2.

AMD3100 is a tight binding, slowly reversible antagonist of CXCR4, which does not induce receptor internalization (33,46). However, the affinity of AMD3100 for CXCR4 (K_i [equilibrium inhibition constant]: 100–650 nmol/L [34,35]) is lower than the affinities of SDF-1α (K_d [equilibrium dissociation constant]: 1–54 nmol/L [34,47–51]) and ubiquitin (K_d: 100 nmol/L [12]). The prerequisite of this study is that the administered dose of AMD3100 prevents ligand binding to CXCR4 *in vivo*, similar to
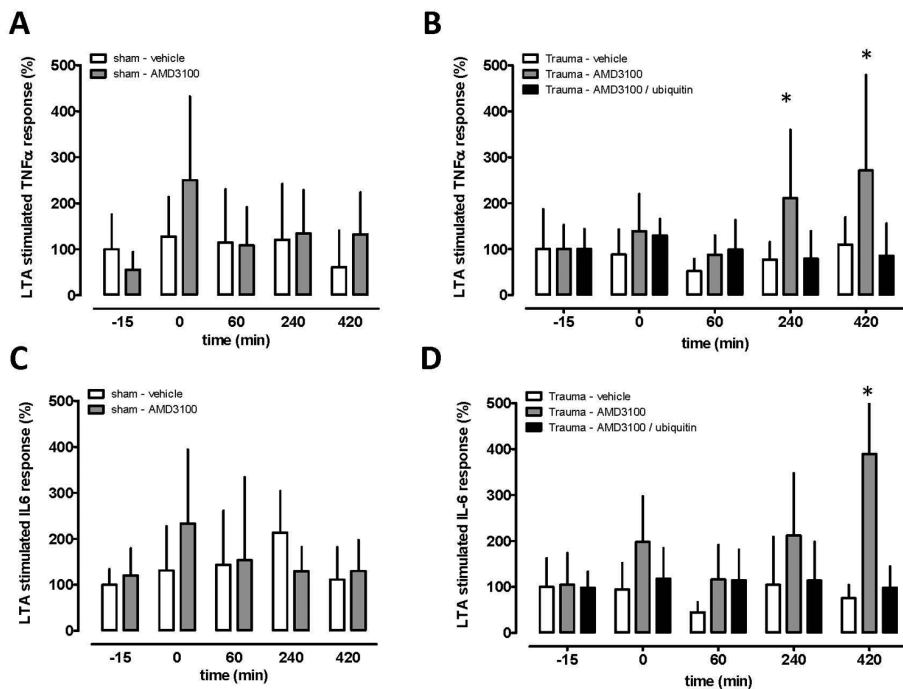
Figure 6. LTA-stimulated whole blood TNF-α and IL-6 release after AMD3100 administration *in vivo*. Drugs were administered within 15 min at $t = -15$ min. Data are expressed as percent of baseline ($t = -15$ min) of the cytokine concentration in the cell culture supernatant per 10^3 leukocytes in the incubation mixtures (mean ± SD). (A, C) Uninjured animals. Open bars: vehicle treatment. Grey bars: AMD3100 treatment. (B, D) Polytrauma groups. Open bars: vehicle treatment. Grey bars: AMD3100 treatment. Black bars: AMD3100 and ubiquitin treatment. (A, B) TNF-α. (C, D) IL-6. *$p < 0.05$ versus vehicle.

its described effects in cell systems (12,15,34,35). After i.v. administration, the plasma half-life of AMD3100 is 1–3 h and its volume of distribution is 0.3 L/kg, suggesting distribution beyond the blood compartment (42). Because quantifications of AMD3100 plasma concentrations or CXCR4 cell surface expression are unable to prove biological activity of AMD3100, we confirmed biological activity through quantification of FITC-ubiquitin receptor binding after i.v. administration. These measurements documented efficacy of i.v. AMD3100 to reduce ubiquitin receptor binding by 60–80%. The observed recovery of ubiquitin receptor binding is consistent with the pharmacokinetic characteristics of AMD3100 (42). The determined changes in B_{max} after i.v. AMD3100, however, probably underestimate its true effects because AMD3100 will partially dissoci-

ate from the receptor during isolation of PBMCs.

Within a few hours after i.v. and subcutaneous injection, AMD3100 induces general leukocytosis and mobilization of CD34$^+$ hematopoietic progenitor cells from the bone marrow (42,52–54). Because AMD3100 administration results in a modest shift to band forms but does not increase metamyelocytes or myelocytes in the blood, the AMD3100-induced leukocytosis was attributed to the demargination of leukocytes from the endothelium (42,52). Our findings that AMD3100 mobilized leukocytes into the systemic circulation and the absence of noticeable adverse effects in anesthetized uninjured pigs are consistent with its pharmacological properties that have been described in human volunteers (42). However, the increase and duration of leukocyte mobilization was slightly re-

duced in pigs (1.9-fold), as compared with humans (2.5-fold) (42).

As described in mice previously, administration of AMD3100 led to a rapid increase of plasma SDF-1α concentrations (55), suggesting a regulatory feedback loop that controls systemic expression of SDF-1α. The measured plasma concentrations of ubiquitin and the pharmacokinetics of exogenous ubiquitin after i.v. administration are in line with previous findings in pigs (24,25,27,30). The observation that i.v. AMD3100 did not affect ubiquitin plasma levels further supports the assumption that the majority of extracellular ubiquitin originates from its passive release from cells and tissues undergoing physiological turnover or damage (16–18,37,56).

We have shown previously that after i.v. injection, only 10% of exogenous ubiquitin can be recovered in the urine (27). The biodistribution of exogenous ubiquitin after i.v. injection, however, is currently unknown. Because it has been shown that SDF-1α is predominantly released from bone marrow stromal cells after AMD3100 administration, the finding that coadministration of ubiquitin plus AMD3100 did not influence the AMD3100-induced SDF-1α release in the present study could be explained by insufficient accumulation of ubiquitin in bone marrow (55). On the other hand, it cannot be excluded that ubiquitin functions as a partial or functionally selective CXCR4 agonist *in vivo*, which could correspond to recent findings suggesting that CXCR4 contains separate binding sites for SDF-1α and ubiquitin (13).

In contrast to uninjured animals, AMD3100 increased fluid requirements to maintain hemodynamics in animals during resuscitation from polytrauma. Because hematocrit values and hemoglobin concentrations were indistinguishable between the polytrauma groups with and without AMD3100 treatment, these data document that the intravascular fluid status was comparable in all groups and suggest that AMD3100 increased vascular permeability. We have
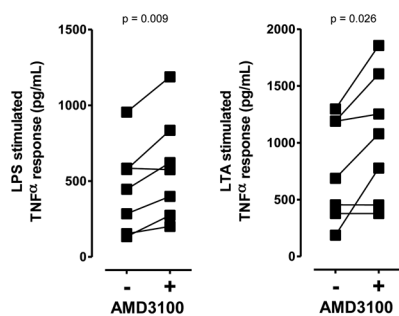
Figure 7. LPS- and LTA-stimulated whole blood release after AMD3100 administration *in vitro.* Normal whole blood was stimulated with LPS (left) or LTA (right) in the absence (–) or presence (+) of 10 μmol/L AMD3100.

shown previously that treatment with exogenous ubiquitin reduces systemic fluid requirements to maintain hemodynamics and tissue edema formation in various models of inflammation (23–25,27), including the pig polytrauma model that we used in the present study (30). Along with the finding of the present study that coadministration of AMD3100 and exogenous ubiquitin neutralized the AMD3100-induced increase in fluid requirements, these data imply that CXCR4 activation protects against inflammation-induced capillary leakage.

Moreover, AMD3100-induced effects on leukocyte counts were significantly enhanced after polytrauma, compared with the AMD3100-associated effects in uninjured animals. This result provides initial evidence that CXCR4 functions as an important regulator of leukocyte mobilization and trafficking after severe blunt injuries.

Increased SDF-1α plasma levels were shown to result in elevated blood leukocyte counts (57). Furthermore, we have shown recently that ubiquitin also exerts chemotactic activity via CXCR4, albeit weaker than SDF-1α (15). Thus, the increased systemic levels of the CXCR4 agonists likely contribute to the leukocyte-mobilizing effects of AMD3100 and may explain the prolonged elevation in leukocyte counts after AMD3100 and ubiquitin coadministration, possibly

through the generation of a reverse chemotactic gradient (57).

We could not detect significant differences in lung MPO activities among the groups. Interpretation of this finding, however, is difficult for several reasons. Besides the limitation of a single time point measurement, mechanical ventilation alone is known to result in significant leukocyte infiltration into the lung at a comparable time point (58). Furthermore, variations of the proportion of blood that is present in lungs will also affect MPO activity. Thus, further studies are required to assess whether the increase in peripheral blood leukocytes by AMD3100 is also accompanied by changes in leukocyte infiltration into tissues after polytrauma.

Moreover, we detected that *in vitro* AMD3100 treatment increased TNF-α release of normal whole blood upon stimulation with LPS and LTA and that *in vivo* AMD3100 treatment enhanced TNF-α and IL-6 release of whole blood upon stimulation with LPS or LTA after polytrauma. The latter could be prevented by coadministration of ubiquitin.

Although we cannot clarify whether leukocytes that were mobilized into the systemic circulation by AMD3100 show enhanced responses upon LPS and LTA stimulation, the detected *in vitro* effects of AMD3100 in normal whole blood suggest that endogenous activation of CXCR4 limits proinflammatory responses of the physiological leukocyte population upon activation of the PRRs TLR-2 and TLR-4. This assumption is further supported by previous findings indicating that CXCR4 interacts with TLR-2 and TLR-4 (59,60), consistent with the previously described *in vitro* and *in vivo* effects of exogenous ubiquitin (16,25,37).

Interestingly, enhanced leukocyte responses were not detectable until the AMD3100-induced increase in SDF-1α plasma levels returned to baseline. Because the CXCR4 agonist CTCE-0214 was recently shown to suppress TNF-α plasma levels in murine endotoxemia and IL-6 production in LPS-stimulated

murine bone marrow–derived macrophages (19), the observation that increased leukocyte responses upon LPS/LTA stimulation were not detectable after AMD3100 administration in uninjured animals and occurred delayed in injured animals could be explained by SDF-1α–mediated activation of CXCR4 on circulating leukocytes. Furthermore, it is conceivable that the AMD3100-induced SDF-1α release counteracted more pronounced physiological consequences of AMD3100. Thus, the present study likely underestimates the pathophysiological role of CXCR4 after tissue injury. CXCR4, SDF-1α and ubiquitin gene knockout, however, results in intrauterine or perinatal mortality (4,31,32), and reagents that reliably neutralize ubiquitin and SDF-1α are not available. Therefore, *in vivo* studies aimed to neutralize SDF-1α or ubiquitin after polytrauma are currently not feasible.

Whereas SDF-1α is a CXCR4 and CXCR7 agonist, ubiquitin is a CXCR4 agonist, but does not bind to CXCR7 (12,13,15,61). Besides being a CXCR4 antagonist, AMD3100 is also a CXCR7 ligand with weak allosteric agonist activity and increases binding of SDF-1α to CXCR7 (62). Moreover, AMD3100 may also have partial agonist activity on CXCR4 in certain cell types (63). Because the observed effects of AMD3100 on fluid requirements and leukocyte function could be neutralized with ubiquitin, these effects are likely attributable to CXCR4. We cannot exclude, however, that CXCR7 activation by SDF-1α and AMD3100, as well as partial CXCR4 agonist activity of AMD3100, may also have contributed to the overall physiological responses that we observed in the present study. Although CXCR4 blockers other than AMD3100 are available, such as IT1t, it remains to be determined whether their actions are strictly confined to neutral CXCR4 antagonism (64,65).

CONCLUSION

Our observations suggest that the SDF-1α/ubiquitin/CXCR4 axis plays an

important role in the regulation of leukocyte mobilization after polytrauma. Furthermore, we provide initial evidence that endogenous activation of CXCR4 prevents exuberant proinflammatory responses of leukocytes upon PRR activation. In addition, we found that systemic fluid requirements to maintain hemodynamics were increased with AMD3100, although hematocrit and hemoglobin levels were unchanged. These findings point toward a contribution of CXCR4 to the maintenance of normal vascular permeability during resuscitation from polytrauma.

Exogenous ubiquitin neutralized the AMD3100-induced increases in systemic fluid requirements and sensitization of leukocyte responses upon PRR activation after polytrauma. It also prevented the increase in blood lactate levels that was detectable in animals after vehicle and AMD3100 administration, suggesting that CXCR4 activation with exogenous ubiquitin normalizes impaired tissue metabolism during the shock phase (66–69). Thus, in combination with the therapeutically relevant effects of SDF-1α, SDF-1α–derived proteins and ubiquitin, which have been reported previously (18–30), the findings from the present study further point toward CXCR4 as a promising drug target for trauma patients.

DISCLOSURE

The therapeutic use of ubiquitin was patented (U.S. patent #7,262,162), and M Majetschak is an inventor. M Majetschak and V Saini are inventors on a provisional patent application related to CXCR4. M Majetschak and V Saini have not received any income related to the patent or patent application. The authors declare that they have no other competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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