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CXCL5 Regulates Chemokine Scavenging and Pulmonary Host Defense to Bacterial Infection

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Summary

The chemokine sink hypothesis pertaining to erythrocyte Duffy Antigen Receptor for Chemokines (DARC) during inflammation has received considerable attention, but lacks direct in vivo evidence. Here we demonstrate, using mice with a targeted deletion in CXCL5, that CXCL5 bound erythrocyte DARC and impaired its chemokine scavenging in blood. CXCL5 increased the plasma concentrations of CXCL1 and CXCL2 in part through inhibiting chemokine scavenging, impairing chemokine gradients and desensitizing CXCR2, which led to decreased neutrophil influx to the lung, increased lung bacterial burden and mortality in an *Escherichia coli* pneumonia model. In contrast, CXCL5 exerted a predominant role in mediating neutrophil influx to the lung during inflammation after LPS inhalation. Platelets and lung resident cells were the sources of homeostatic CXCL5 in blood and inflammatory CXCL5 in the lung respectively. This study presents a paradigm whereby platelets and red cells alter chemokine scavenging and neutrophil-chemokine interaction during inflammation.

Introduction

The Duffy Antigen Receptor for Chemokines (DARC) is a promiscuous silent receptor, highly expressed on erythrocytes and post capillary venules, which binds at least 16 pro-inflammatory CXC and CC chemokines (Borroni et al., 2008; Gardner et al., 2004; Horuk et al., 1993). DARC lacks the motif to enable G protein coupling and signaling(Hadley and Peiper, 1997), and its expression on erythrocytes has been hypothesized to modulate chemokine bioavailability by acting both as a chemokine sink(Darbonne et al., 1991) and as a reservoir (Fukuma et al., 2003). Recently the chemokine sink hypothesis has fallen into disfavor(Horne and Woolley, 2009), based on observations that only minimal degradation of

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internalized DARC-associated chemokines occur (Pruenster et al., 2009). Furthermore, endothelial DARC has other functions, including roles in not only unidirectional chemokine transcytosis from the basolateral to the apical side (Pruenster and Rot, 2006), but also surface retention of transcytosed chemokines. These functions appear to facilitate chemokine-mediated leukocyte migration across the endothelial barrier (Pruenster et al., 2009). In this context, studies from three independently generated DARC-deficient mouse strains show that DARC plays a role in neutrophil trafficking to tissues during inflammation, but its exact role in neutrophil influx into tissues remains conflicting (Dawson et al., 2000; Luo et al., 2000; Reutershan et al., 2009; Zarbock et al., 2007). So far there is no direct evidence from these *Darc*$^{-/-}$ mice that erythrocyte DARC scavenges pro-inflammatory chemokines in vivo during inflammation, and its role as a "chemokine sink" during such states remains contentious.

ELR (Glutamic acid-leucine-arginine)$^{+}$ CXC chemokines constitute a family of ligands essential for neutrophil influx into inflamed tissues (Kobayashi, 2006). In mice, four members have been identified to have neutrophil chemoattractant activities, but whether they serve distinct or redundant roles in inflammation and infection is not fully understood. The four chemokines, keratinocyte-derived chemokines (KC, also named CXCL1), macrophage inflammatory protein-2 (MIP-2, also named CXCL2), lipopolysaccharide-induced CXC chemokine (LIX, also named CXCL5) and lungkine (also named CXCL15) all interact with CXCR2, the only functional receptor mediating their chemotactic activity in mice. CXCL1 and CXCL2 have previously been suggested to be the two most important chemokines for neutrophil recruitment into the lung in rodents (Frevert et al., 1995; Olson and Ley, 2002; Reutershan and Ley, 2004; Schmal et al., 1996), and CXCL15 (expressed by bronchial epithelial cells) may play a role in pulmonary host defense against Klebsiella pneumoniae infection (Chen et al., 2001; Rossi et al., 1999). In contrast, the role of CXCL5 during lung inflammation is unknown.

Human CXCL5, also known as epithelial cell-derived neutrophil-activating peptide-78 (ENA-78), has been found to be involved in a variety of human inflammatory diseases (Qiu et al., 2003; Qiu et al., 2007; Zineh et al., 2008). We have previously shown that murine CXCL5 is up-regulated in the lung, and expressed by alveolar type II epithelial (AE II) cells in response to LPS stimulation (Jeyaseelan et al., 2005a), while CXCL1 and CXCL2 are expressed by alveolar macrophages. Support for a functional role of CXCL5 came from experiments in which anti-CXCL5 antibodies attenuated LPS-induced neutrophil accumulation in the lung (Jeyaseelan et al., 2004).

To explore the physiological role of CXCL5 in regulating neutrophil recruitment to the lung, we generated CXCL5-deficient mice and determined its role in pulmonary inflammation consequent to LPS inhalation, and host defense in a model of murine E.coli pneumonia. In response to E.coli, CXCL5 decreased neutrophil influx to the lung and increased lung bacterial burden, despite its known role as a neutrophil chemoattractant. While CXCL1 and CXCL2 are the dominant chemotactic factors released in this model, CXCL5 inhibited scavenging of these chemokines at least in part through binding to erythrocyte DARC, contributing to high concentrations of CXCL1 and CXCL2 in the plasma, desensitization of CXCR2 and impaired formation of chemokine gradients for neutrophil influx to the lung. In normal mice, CXCL5 was found bound to circulating erythrocytes, and abundant preformed CXCL5 was found in platelets, which are the source of homeostatic CXCL5 loaded onto DARC. In contrast to homeostatic conditions, resident cells are responsible for CXCL5 generation in the lung and most of the increased CXCL5 in the blood during E.coli pneumonia. In a milder and self-limited inflammatory model, on the other hand, CXCL5 appeared to be the dominant effector of neutrophil influx to the lung, due, at least in part, to prolonged expression (compared to CXCL1 and CXCL2) in the lung.

In summary, these results provide the first evidence that the chemokine scavenging function of erythrocyte DARC is inhibited by homeostatic and inflammatory CXCL5 *in vivo* during inflammation. These data further indicate that CXCL5, through its interaction with erythrocyte DARC, regulates the availability of binding sites for other ELR^+ CXC chemokines released during inflammation. While established during normal homeostasis, these effects are only observed in severe inflammatory disease. In addition to its role in chemokine scavenging, CXCL5 also plays a critical direct role in pulmonary inflammatory responses in a self-limited LPS inhalation model. As an example of a chemokine with both homeostatic and inflammatory functions, CXCL5 occupies a unique niche within the family.

Results

CXCL5 regulates neutrophil homeostasis in bone marrow

To explore the role of CXCL5 in lung inflammation *in vivo*, we generated CXCL5-deficient mice with the strategy of deleting all *Cxcl5* coding regions (Fig.S1). CXCL5-deficient mice were viable and fertile without any abnormalities in their general appearance. Histology of a survey of organs was normal (data not shown). In bone marrow (BM), the total numbers and percentages of Gr-1^+ cells and relatively mature Gr-1^+CXCR2^+ cells in $Cxcl5^{-/-}$ mice were higher than that of WT mice (Fig.S2E–F), indicating that CXCL5 plays a role in neutrophil homeostasis, whereas the numbers and percentages of blood neutrophils (Fig.S2G–H) and the parameters of the other hematopoietic lineages (data not shown) between WT and $Cxcl5^{-/-}$ mice are similar.

CXCL5 inhibits neutrophil influx to the lung during E.coli pnemonia

In order to determine the consequences of CXCL5 deficiency on host responses, we used a mouse model of E.coli pneumonia. Intratracheal (i.t.) challenge of mice with 10^7 CFU E.coli, induces a severe lung infection with bacterial replication and a high mortality. Neutrophil influx to the lung in $Cxcl5^{-/-}$ mice was potentiated 8 and 24 hours after infection, and lung myeloperoxidase (MPO) activity similarly increased as compared to WT mice (Fig.1A–C). Thus, despite its recognized function as a neutrophil chemoattractant, CXCL5 inhibited neutrophil influx in this severe inflammatory setting. Consistently, $Cxcl5^{-/-}$ mice showed fewer bacteria in the lung as compared to WT mice (Fig.1D). The mortality of $Cxcl5^{-/-}$ mice within 24 hours after E.coli challenge was lower than that of WT mice, and the lung wet to dry ratio at 24 hours, which indicates the severity of edema, was attenuated in $Cxcl5^{-/-}$ mice (Fig.1E–F), indicating that CXCL5 aggravates acute lung injury during severe E.coli pneumonia. Histology of the lungs (Fig.1L) and cytospin of bronchoalveolar lavage fluid (BALF) (Fig.S3) at 24 hours after challenge showed more inflammatory cells and fewer E.coli in the lungs of $Cxcl5^{-/-}$ mice as compared to WT mice. The amounts of CXCL1, CXCL2, TNF-α and IL-6 in the BALF upon E.coli challenge were not affected by CXCL5 deficiency except that the amounts of CXCL2 at 24 hours and IL-6 at 8 hours in the BALF of $Cxcl5^{-/-}$ mice were attenuated relative to WT mice (Fig.1G–K). These results indicate that CXCL5 deficiency increased neutrophil influx, accelerated bacteria clearance, improved pulmonary edema and protected mice from severe E.coli pneumonia. In WT mice, the amounts of CXCL1 and CXCL2 in the BALF were much larger than that of CXCL5 (Fig.1G–I), suggesting that CXCL1 and CXCL2 may be the predominant chemoattractants in mediating neutrophil influx to the lung upon E.coli challenge.

In order to determine mechanisms by which neutrophil accumulation was impaired in the presence of CXCL5, expression of CXCR2, the receptor for the ELR+ CXC chemokines was quantified. Surface expression of CXCR2 on neutrophils in the BM and blood was dramatically down-regulated after E.coli challenge in WT mice, less down-regulated in

Cxcl5$^{-/-}$ mice, and unaffected in either group after LPS inhalation (Fig.S4). Since high concentrations of CXCR2 ligands induce desensitization of the receptor, manifest in attenuated surface CXCR2(Prado et al., 1996; Sabroe et al., 1997; Wiekowski et al., 2001), the downregulation of CXCR2 on Gr-1^{+} cells suggested that E.coli challenge might induce large amounts of ELR^{+} CXC chemokines in blood. Furthermore, these data suggest higher concentrations of CXCR2 ligands in the plasma of WT mice than that of *Cxcl5*$^{-/-}$ mice, given greater downregulation of surface CXCR2 expression on blood neutrophils of the former.

In exploring this possibility, we found that E.coli exposure induced high-level expression of plasma CXCL1 and CXCL2 in WT mice, which was dramatically attenuated in *Cxcl5*$^{-/-}$ mice (Fig.2B–C), indicating that CXCL5 regulates plasma CXCL1 and CXCL2 concentrations in this model of severe E.coli pneumonia. The relative ratio of local to plasma chemokine concentrations reflects the relative values of gradients that regulate neutrophil influx to tissues (Blackwell et al., 1999; Call et al., 2001), even though the absolute value of individual chemokines is affected by dilution of BALF during preparation. Measurement of these parameters indicated that *Cxcl5*$^{-/-}$ mice better preserved chemokine gradients for CXCL1 and CXCL2 from the blood to alveolar space upon E.coli challenge as compared to WT mice (Fig.2D–E), due, in large part to the plasma concentrations of CXCL1 and CXCL2. As a possible reflection of ligand-induced desensitization, bone-marrow-derived neutrophils of WT mice at 24 hours after E.coli challenge are unresponsive to 10 nM CXCL1 stimulation *in vitro* (the ratio of indo-1(violet)/ (blue) peaked at 365±34), while the neutrophils of *Cxcl5*$^{-/-}$ mice still retain responsiveness (the ratio of indo-1(violet)/ (blue) peaked at 541±27), albeit attenuated (Fig.2F–G), suggesting that CXCR2 desensitization by CXCR2 ligands in WT blood leads to a greater impairment of neutrophil chemotaxis to the lung and presumably, neutrophil mobilization from bone marrow. Thus, CXCL5 inhibited neutrophil influx to the lung, at least in part, by contributing to elevated plasma concentrations of CXCL1 and CXCL2, leading to attenuation of chemokine gradients and CXCR2 desensitization in a model of severe E.coli pneumonia.

CXCL5 inhibits chemokine scavenging in blood in part through binding to erythrocyte DARC

Having detected markedly decreased CXC chemokines in blood of *Cxcl5*$^{-/-}$ mice, we hypothesized that the interaction of CXCL5 with erythrocyte DARC alters the disposition of CXCL1 and CXCL2 expressed during inflammatory events, and increases their plasma concentrations. In order to test this, we designed a simple *in vitro* chemokine scavenging assay and compared the plasma concentrations of CXCL1, CXCL2 and CXCL5 after addition of exogenous chemokines to whole blood from WT and *Cxcl5*$^{-/-}$ mice. As seen in Fig.3A–C, *in vitro* incubation of whole blood with CXCL1, CXCL2 or CXCL5 resulted in significantly lower concentrations in plasma of *Cxcl5*$^{-/-}$ mice as compared to WT mice, indicating that endogenous amounts of CXCL5, even under homeostatic conditions, regulate plasma concentrations of competitor chemokines CXCL1 and CXCL2. Consistent with these *in vitro* findings, the plasma concentrations of CXCL5, CXCL1 and CXCL2 in *Cxcl5*$^{-/-}$ mice were attenuated relative to those in WT mice after intravenous injection of recombinant CXCL5, CXCL1 and CXCL2 (Fig.3D–F). Notably, CXCL2 in blood was cleared in blood with an extraordinary rate. Even at 30 minutes after i.v. injection, most CXCL2 disappeared from the plasma, probably due to its DARC-independent loss to distal organ or tissues (Fukuma et al., 2003).

To determine whether erythrocyte DARC is directly responsible for clearance of CXCL1 or CXCL2 from blood, as suggested by a recent study (Reutershan et al., 2009), we measured the scavenging capability of *Darc*$^{-/-}$ versus WT blood. The scavenging capacities of *Darc*$^{-/-}$ whole citrated blood for exogenous CXCL1 and CXCL5 were much attenuated as compared

to that of WT mice (Fig.4A–B). In addition, *Darc*$^{-/-}$ red cells did not bind CXCL5 (Fig.4G) or CXCL2(Mangalmurti et al., 2009). Collectively, these results indicate that erythrocyte DARC is largely responsible for scavenging CXCL1, CXCL2 and CXCL5 in murine whole blood *in vitro*, and WT blood still retains significant chemokine scavenging capacities even though a considerable amount of homeostatic CXCL5 binds to erythrocyte DARC (Fig.4C). CXCL5-regulated chemokine scavenging (Fig.3A–F) may also be attributed to endothelial DARC, and other chemokine scavenging molecules, such as heparan sulphate proteoglycans (HSPGs), in addition to erythrocyte DARC.

In order to determine how chemokines partition between plasma and erythrocytes, we measured plasma and erythrocyte-associated CXCL5 in blood of naïve WT mice. As seen in Fig.4C, we found large amounts of CXCL5 in association with erythrocyte DARC rather than plasma, while under homeostatic state, CXCL1 and CXCL2 were not found in either plasma or erythrocyte compartments. To model increases in CXCL1, we injected recombinant murine CXCL1 intravenously into both WT and *Cxcl5*$^{-/-}$ recipients. The increased chemokine scavenging for exogenous CXCL1 in *Cxcl5*$^{-/-}$ blood as compared to WT blood (see also Fig.3D) were found to be at least partially due to increased erythrocyte association with CXCL1 (Fig.4D). These findings are the first, we believe, to demonstrate that erythrocyte DARC binds a chemokine (CXCL5) under homeostatic conditions and in so doing, impairs chemokine scavenging in blood.

To further elucidate the mechanisms whereby the binding of CXCL5 with erythrocyte DARC regulates the availability of CXCL1 and CXCL2 in blood during E.coli pneumonia, we compared the binding affinities of murine ELR^{+} CXC chemokines to erythrocyte DARC. In radioligand binding assays, the ability of CXC chemokines to compete with ^{125}I-Gro-α (human CXCL1) on purified murine erythrocytes was compared in a system dependent upon DARC (Fig.4G). The relative binding activity of murine CXC chemokines is as follows: CXCL5 70 amino acids (a.a.). (CXCL5 most potent short form)> CXCL2 > CXCL1 >>> CXCL5 93 a.a. (Fig.4E–F, $p<0.05$). Note that the N-terminal cleavage of the chemokine CXCL5 (from the mature CXCL5 93 a.a. to the 70 a.a. form) significantly enhanced its binding affinity to erythrocyte DARC. The Ki (equilibrium dissociation constant) for various ELR^{+} CXC chemokines showed similar relative binding affinities for erythrocyte DARC even when a CC chemokine, ^{125}I-hCCL2 was used as the radioligand (Fig.4G–H). Thus, CXCL5-70 demonstrates the highest binding affinity for erythrocyte DARC than either CXCL1 or CXCL2, but all of them demonstrated high affinity binding. Although the exact cleaved forms of CXCL5 binding with erythrocyte DARC under homeostatic and inflammatory state is not clear, this result suggests that CXCL5-70 may preferentially bind erythrocyte DARC as compared to CXCL1 and CXCL2 during inflammation, which might impair their scavenging. Finally, when plasma and erythrocyte–associated chemokines were measured in response to E.coli pneumonia, much greater percentages of CXCL1 remained bound to erythrocyte DARC in the blood of *CXCL5*$^{-/-}$ mice relative to WT mice (Fig.4I). These data further support the concept that CXCL5 inhibition of erythrocyte DARC chemokine scavenging increases the plasma concentration of CXCL1 and CXCL2 in a severe E.coli pneumonia model.

Consistent with the notion that DARC is already substantially occupied in basal conditions, we also showed that *Darc*$^{-/-}$ mice exhibited comparable neutrophil influx to the lung and plasma CXCL1 level to WT mice (Fig.S5A–D). These data at least partially explain why the erythrocyte DARC “chemokine sink” hypothesis has been difficult to prove, as DARC binding sites do not appear to be readily available in the WT controls during inflammation. To further investigate whether DARC is completely responsible for CXCL5-regulated chemokine scavenging, we found that with newly-generated *Darc*$^{-/-}$*:Cxcl5*$^{-/-}$ double knockout mice, CXCL5-regulated chemokine scavenging *in vitro* is not totally dependent on

DARC (Fig.S5E), suggesting that other chemokine scavenging molecules than DARC still contribute to CXCL5-regulated chemokine scavenging. We further found that CXCL5 was mobilized in the plasma of heparin-treated blood as compared to PBS-treated blood both *in vivo* and *in vitro* (Fig.S8F), suggesting that heparin binding might liberate CXCL5 from other molecules. Since heparan sulphate proteoglycans (HSPGs) are expressed on almost all cell types (Dreyfuss et al., 2009), HSPGs may also mediate CXCL5-regulated chemokine scavenging.

Preformed CXCL5 in platelets contributes to the homeostatic CXCL5 in blood

The sources of circulating chemokines in the mouse are not well understood. While considering the possibility that circulating platelets are a source, we found large amounts of CXCL5 (Fig.5A), little CXCL1 (Fig.5B) and no CXCL2 or CXCL15 (data not shown) in the plasma after traditional retro-orbital bleeding (even into EDTA-coated tubes) from naïve WT mice, while no chemokines were detected using similar methods in naïve *Cxcl5*$^{-/-}$ mice. Accordingly, since this traditional bleeding method does not prevent coagulation or prevent platelet release reactions, we prepared plasma without coagulation using blood withdrawal with sodium citrate (coagulation-negative bleeding) (Sommeijer et al., 2005), and found much lower concentrations of CXCL5 in plasma from coagulation-negative bleeding than in traditional bleeding (Fig.5A). These data suggest that activated platelets secrete CXCL5 into plasma during even the modest coagulation that accompanies traditional bleeding methods. As a partial confirmation of this hypothesis, isolated platelets from WT and *Cxcl5*$^{-/-}$ mice were stimulated with thrombin, or snap-frozen and thawed twice, or directly lysed, and the presence of chemokines determined using western blot analysis. As seen in Fig.5C–D, large amounts of CXCL5 (mostly the least active CXCL5-93 form, both in lysates and supernatant after platelet chemokine release), but no CXCL1 was detected in the platelets. Likewise, our results also suggest that activated platelets secrete little CXCL1 into plasma during coagulation (Fig.5B), nor could western blot analysis and ELISA detect the presence of CXCL1 in platelets and the coagulation-negative plasma (Fig.2B, 6K) of naïve mice. In conclusion, among these four ELR^{+} CXC chemokines, only preformed CXCL5 is abundant in murine platelets.

In order to determine whether platelets are the source of homeostatic CXCL5 in naïve WT mice, we first determined whether it was derived from the hematopoietic compartment. Reconstitution of WT and *Cxcl5−/−* mice with *Cxcl5*$^{-/-}$ and WT marrow demonstrated that those mice with *Cxcl5*$^{-/-}$ marrow lacked both plasma and erythrocyte-bound CXCL5 (Fig. 5E–F). In contrast, mice receiving WT marrow demonstrated both plasma and erythrocyte-bound CXCL5, and decreased scavenging of chemokines was found in blood from those mice (Fig.5G).

In order to confirm more specifically the platelet origin of homeostatic CXCL5 in blood, we adopted a genetic approach using two thrombocytopenic *Mpl*$^{-/-}$ and recently described *Fog-1*$^{ki/ki}$ mouse models (Alexander et al., 1996; Miccio et al., 2009). In both models, defective thrombocytopoiesis is associated with markedly reduced numbers of circulating platelets (but other lineages are intact), and is associated with markedly decreased amounts of plasma and erythrocyte-binding CXCL5 as compared to WT mice (Fig.5H–I). CXCL7 (β-thromboglobulin form, shown in Fig.5J) and CXCL4 are the two most abundant chemokines in the α-granule of platelets together with CXCL5, and their plasma concentrations were also greatly attenuated in *Mpl*$^{-/-}$ and *Fog-1*$^{ki/ki}$ mice as compared to that in WT mice (Fig.5K–L), indicating that platelets are the origin of homeostatic CXCL5 in blood, and further suggesting that CXCL4, 5, and 7 are co-regulated at the homeostatic level of platelet release.

CXCL5 predominates in mediating neutrophil influx to the lung upon nebulized LPS stimulation

In order to determine whether CXCL5 regulates neutrophil influx as seen in murine E.coli pneumonia under less extreme conditions, we used a model of self-limited inflammation induced by inhaled LPS (Jeyaseelan et al., 2004). In contrast to the E.coli pneumonia model, neutrophil influx to the lung in response to LPS inhalation was dramatically attenuated in *Cxcl5*$^{-/-}$ mice versus WT mice (Fig.6A–B). However, the lung myeloperoxidase (MPO) activities after lavage and perfusion were comparable between WT and *Cxcl5*$^{-/-}$ mice (Fig. 6C), indicating that the migration of neutrophils into the alveolar space, but not other lung compartments is facilitated by the presence of CXCL5. At 4 hours after LPS inhalation, the amounts of TNF-α and IL-6 in the BALF were attenuated in *Cxcl5*$^{-/-}$ mice as compared to WT mice (Fig.6D–E), but the expression of CXCL1 and CXCL2 in the BALF was not affected by CXCL5 deficiency (Fig 6G–H). Furthermore, CXCL15 expression in the BALF was attenuated only at 24 hours in *Cxcl5*$^{-/-}$ mice versus WT mice (Fig.6I). These results indicate that CXCL5 plays a dominant role in regulating pulmonary inflammatory responses to inhaled LPS, perhaps due to its prolonged expression pattern. CXCL1 and CXCL2 peaked at 2 and 4 hours, while CXCL5 expression was more prolonged, reaching its peak at 8 hours with a considerable amount remaining in the BALF at 24 hours (Fig.6F–H). We further measured the concentrations of these chemokines in circulation. Unlike CXCL1 (Fig.6K), CXCL2 and CXCL15 that are undetectable in blood (data not shown), CXCL5 was detectable at low concentrations in plasma of untreated WT mice (Fig.5J). LPS inhalation does not stimulate expression of CXCL2, CXCL5 and CXCL15 in plasma, and only induced a low-level expression of CXCL1 in the plasma of WT mice, which was attenuated in *Cxcl5*$^{-/-}$ mice, suggesting that this is also a consequence of improved chemokine scavenging by *Cxcl5*$^{-/-}$ mice. These results indicate that LPS inhalation induces a local inflammatory state, and CXCL5 dominates in mediating neutrophil influx to the lung airspace due, at least in part, to its more prolonged expression than CXCL1 and CXCL2 in the lung.

The data presented so far highlight two different roles of CXCL5, exemplified in a local, self-limited inflammatory response contrasted to a severe, lethal response with high concentrations of circulating chemokines. In order to determine whether these dichotomous (mild and severe) lung inflammation models represent opposite ends of a spectrum, or completely distinct events, we used a high-dose LPS inhalation model, the intensity of which is intermediate between the LPS or E.coli models. Interestingly, initial neutrophil influx to the lung is enhanced in *Cxcl5*$^{-/-}$ mice, while CXCL1 plasma level were attenuated. At later time points (Fig.S6) neutrophil numbers decreased more rapidly than in WT. These data imply the relevance of CXCL5 not only to initiation of inflammation, but also to resolution of inflammation. In addition, we also used a low-dose 10^6 CFU E.coli pneumonia model, which showed comparable severity of inflammation to the high-dose LPS inhalation model (as judged by comparable CXCL1 plasma amounts). At 8 hours, neutrophil influx to the lung was increased in *Cxcl5*$^{-/-}$ mice at least in part due to increased chemokine scavenging in blood, which led to a decrease in lung bacterial burden at 24 hours (Fig.S7). These two new models, demonstrating similar phenotypes at early time points, suggest that the impact of CXCL5-regulated chemokine scavenging on pulmonary inflammatory responses is not due to the difference of complicated E.coli versus simple LPS challenge, but mostly due to the differential amounts of ELR$^+$ CXC chemokines in plasma between two genotypes.

Resident cells are the source of pulmonary CXCL5 and most plasma CXCL5 during E.coli pneumonia

We have previously shown that CXCL5 is expressed by AE II cells in response to LPS stimulation *in vivo*, and AE II cells, but not neutrophils and alveolar macrophages, express CXCL5 after LPS stimulation *in vitro*(Jeyaseelan et al., 2005a). Since these studies could not rule out the possibility that another hematopoietic cell might still provide the majority of CXCL5, we used BM chimeric mice to test the importance of lung resident cells. As seen in Fig.7, only WT recipient mice (whatever the donor genotype) were able to express CXCL5 in the lung in either LPS- or E.coli-induced lung inflammation (Fig.7A–B). These data demonstrated that lung resident cells are the source of lung CXCL5 during lung inflammation. Surprisingly, we found that resident cells also contributed about two thirds of plasma CXCL5, while hematopoietic cells contributed about one third (Fig.7C). Considering that baseline CXCL5 (the majority bound to erythrocytes) in blood was contributed by platelets, this data demonstrate that resident cells are the major source of inflammatory plasma CXCL5 in this severe E.coli pneumonia model, and hence that lung epithelial cells regulate pulmonary inflammatory responses through inhibition of chemokine scavenging after E.coli challenge.

Discussion

Pneumonia from gram-negative bacteria is a leading cause of mortality from infectious diseases in the U.S.(Armstrong et al., 1999; Mizgerd, 2006). Neutrophil influx is a crucial component of innate immunity against bacterial infection, but excess neutrophil influx and activation may initiate acute lung injury, necessitating exquisite control over inflammatory cell recruitment and activation. One of the components of this regulatory system is the family of ELR^+ CXC chemokines (CXCL1, 2, 5, and 15), which play critical roles in the inflammatory responses in rodents and humans. Despite considerable homology between these family members, it is unclear whether they are functionally redundant or capable of distinct actions. In humans, CXCL5 was found to be most strongly and consistently correlated with neutrophil numbers in the lung fluids of patients with acute respiratory distress syndrome (ARDS)(Goodman et al., 1996), highlighting the potential importance of CXCL5 in regulation of lung inflammation and injury.

In attempting to define genetically the role of CXCL5 in pulmonary inflammation and host defense against gram-negative bacterial pneumonia, we have uncovered unique aspects of CXCL5 biology. With *in vitro* and *in vivo* studies, we have demonstrated that CXCL5 inhibits chemokine scavenging at least in part through its homeostatic and high-affinity binding with erythrocyte DARC. Hence in the absence of CXCL5, DARC scavenges proinflammatory chemokines, thus contributing to re-shaping the chemokine gradients for neutrophil influx to the lung during severe E.coli pneumonia. In contrast, in response to LPS inhalation, a self-limited inflammatory response in the lung, deletion of CXCL5 markedly impairs neutrophil accumulation in the airspace, thus revealing critical non-redundant roles for this chemokine in lung inflammation in these two models. Furthermore, CXCL5 demonstrates features of both homeostatic chemokines (detected in plasma and bound to erythrocytes of normal mice) and inflammatory chemokines (enhanced expression during inflammation). Perhaps most surprising of all, our data indicate that the source of circulating CXCL5 in the basal homeostatic condition is the platelet, while lung resident cells (principally AE II cells, we suggest) are the source of CXCL5 in the lung and (to a large extent) blood during lung inflammation.

Based on our observations, we propose a model in which during homeostatic conditions, platelet-derived CXCL5 is loaded onto erythrocyte DARC. During self-limited inflammation, CXCL5 itself, perhaps by virtue of its prolonged expression by AE II cells in

the lung, is necessary for optimal neutrophil accumulation. CXCL5 in this scenario has little effect on local concentrations of CXCL1 or 2, which are only transiently (albeit significantly) induced and are but minimally detected in plasma. During a severe inflammatory response, such as that accompanying E.coli pneumonia, further expression of CXCL5 by AE II cells inhibits the chemokine scavenging capability of DARC, at a time when the production of CXCL1 and CXCL2 increases dramatically, resulting in marked increases in circulating plasma concentrations of these chemokines, with adverse consequences for the efficient accumulation of neutrophils. Our studies support the "chemokine sink" function of erythrocyte DARC, while suggesting that under normal circumstances it is inhibited by endogenous CXCL5 from platelets, and further impaired by AE II cell-derived CXCL5 during severe E.coli pneumonia. The phenotypes of WT mice we observed in this severe E.coli pneumonia model resemble that of "cytokine storm (hypercytokinemia)" in influenza pneumonia. Deletion of CXCL5 in our model decreased large circulating amounts of chemokines CXCL1 and 2 and improved survival by permitting effective neutrophil accumulation and bacterial killing.

DARC has previously been reported to bind many pro-inflammatory chemokines, but not homeostatic chemokines (Allen et al., 2007; Borroni et al., 2008; Gardner et al., 2004). Here we have demonstrated that CXCL5 is also a homeostatic chemokine, derived from platelets, which modulates neutrophil homeostasis in naïve mice. Thus the binding of homeostatic chemokines to DARC, and the attendant consequences, are novel findings of this work that may alter our view of DARC function. Our analysis, however, has focused on erythrocyte DARC binding chemokines and functioning as sink (and reservoir). DARC is also expressed on endothelial cells, where it exerts additional actions that promote migration. Whether endothelial DARC contributes to the scavenging functions described here will require further investigation.

As befits a chemokine with homeostatic functions, CXCL5 is found circulating in normal mice, almost all of it bound to erythrocytes. Our studies demonstrated this circulating CXCL5 is derived from platelets. Given the potential role of platelets in a variety of inflammatory diseases (Gear and Camerini, 2003), such as atherosclerosis and acute lung injury (Bozza et al., 2009), it is tempting to speculate that platelet-derived CXCL5 may be involved in establishing conditions that alter a subsequent inflammatory response. Indeed, we have demonstrated that the homeostatic platelet-derived CXCL5 significantly inhibits chemokine scavenging both *in vitro* and *in vivo*. In striking contrast, however, lung cells but not platelets provided most of the inflammation-induced increases in CXCL5 and the attendant inhibition of chemokine scavenging after E.coli challenge. Furthermore, most CXCL5 in platelets or secreted from them after thrombin stimulation is the less active CXCL5-93 form. Previous studies have indicated that many cellular forms of CXCL5 can be detected, including the most potent CXCL5-70 and less potent CXCL5-93 in response to stimulation (Wuyts et al., 1999). Our analysis showed many forms released from platelets, but most was CXCL5-93. Whether platelet-secreted CXCL5-93 can be cleaved into potent CXCL5-70 by matrix metalloproteinases during inflammation (Tester et al., 2007), and what forms of CXCL5 are released by AE II cells, or detected in plasma or bound to erythrocytes in both homeostatic and inflammatory conditions, are the subject of on-going study. Platelets also contain CXCL7 (β-thromboglobulin), another ELR^+ CXC chemokine in mice, but it has not yet been determined whether murine CXCL7 is processed to a neutrophil-chemoattractant form similar to human NAP-2(Smith et al., 2002).

While increases in circulating CXCL1 and 2 appear to be a consequence of impaired scavenging, the mechanism by which elevation of circulating plasma chemokines attenuates neutrophil accumulation to the lung and other organs remains obscure. Here we propose two mechanisms. First we suggest that the chemokine gradients (the relative ratio of BALF

versus plasma concentrations of CXCL1 and CXCL2) are important especially considered in light of the dilution consequent to lavage, but we also suggest that the absolute value of the plasma chemokines may also be relevant. Ligand-induced desensitization of CXCR2, the receptor for CXCL1, 2, 5 and 15 occurs normally during chemotaxis *in vitro*, and migration *in vivo*, and is thus a physiologic process. Here we have documented desensitization of neutrophils even before their entry into the circulation in WT mice after E.coli exposure. Isolation of BM-derived neutrophils demonstrated loss of surface CXCR2 and non-responsiveness to CXCL1, both of which were improved in *Cxcl5*$^{-/-}$ mice. Since CXCR2 is important for mobilization of neutrophils from marrow, as well as migration into tissues, these data suggest that large absolute amounts of CXCL1 and CXCL2 may impair accumulation of neutrophils through effects at several levels. Further studies will be required to distinguish the impact of these mechanisms *in vivo*. Furthermore, CXCR2 deficiency leads to neutrophil dysplasia (over 90% Gr-1^{+} cells in the BM)(Cacalano et al., 1994), indicating that CXCR2 signaling contributes to homeostatic control of neutrophil numbers in the BM, but also indicates that CXCR2 ligands other than CXCL5 play a role in neutrophil homeostasis. Additionally, since endothelial CXCR2 plays an important role in regulating neutrophil influx in LPS-induced lung inflammation (Reutershan et al., 2006) and lung epithelial cells may express CXCR2 as well (Vanderbilt et al., 2003), CXCL5 may modulate neutrophil transepithelial and transendothelial migration.

In summary, CXCL5 exhibits non-redundant properties with respect to other ELR^{+} CXC chemokines that exerts profound effects on the inflammatory response. Considering the involvement of CXCL5 in a variety of human inflammatory diseases (Goodman et al., 1996; Kwon et al., 2005; Qiu et al., 2003; Qiu et al., 2007; Zineh et al., 2008), *Cxcl5*$^{-/-}$ mice and the mechanisms revealed here may be useful to study the role of CXCL5 in the pathogenesis and therapeutics of inflammatory diseases.

Experimental Procedures

Mice

All experiments with mice were conducted in accordance with the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia and University of Pittsburgh.

LPS inhalation mouse model

The LPS inhalation mouse model has been previously described (Jeyaseelan et al., 2004). The mice were exposed to nebulized LPS (0.3 mg/ml) for 30 minutes.

E.coli pneumonia in the mouse

The procedures for E.coli pneumonia mouse model have been previously described (Jeyaseelan et al., 2005b).

Coagulation-negative bleed

The murine plasma samples without coagulation were prepared as described (Sommeijer et al., 2005). In brief, after anesthetization, 3.2% (w/v) sodium citrate in a total volume of body weight (gram) / 13 × 100 μl was intravenously administered into the vena cava 20–30 seconds to prevent coagulation prior to blood drawing from the same vein into a syringe.

Competitive chemokine binding assays

The competitive chemokine binding assays were performed as modified from previous protocols (Darbonne et al., 1991; Lee et al., 2003; Mangalmurti et al., 2009).

Measurement of erythrocyte DARC-binding chemokines

This approach was modified from two previous reports (Reutershan et al., 2009; Rot and Horuk, 2009).

Chemokine scavenging assay *in vitro*

The citrated blood was drawn from mice as described above. 25 ng murine CXCL5 (74 aa, from R&D), 25 ng CXCL1 (77 aa, from R&D) or 10 ng CXCL2 (from R&D) in 10 μl PBS were put into 0.4 ml citrated blood and incubated at 37°C for 15 minutes after being mixed gently, then the plasma were prepared by centrifugation.

Chemokine clearance assay *in vivo*

1 μg murine CXCL5 (74 aa, from R&D), 1 μg murine CXCL1 (77 aa, from R&D) or 0.5 μg murine CXCL2 (from R&D) in 50 μl PBS were intravenously injected into the tail vein of WT and Cxcl5−/− mice. At different time points, the citrated blood was drawn from the vena cava and the plasma samples were prepared.

Competitive chemokine binding assays

In brief, purified murine red cells from WT or *Darc*$^{-/-}$ mice at 10^8 cells/well were incubated in a volume of 0.25 ml containing 0.2 nM ^{125}I-Gro-α/hCXCL1 or ^{125}I-hCCL2 (PerkinElmer) in the presence or absence of increasing molar concentrations of unlabeled chemokines. Recombinant CXCL5-70, CXCL1, CXCL2 and CXCL5-93 are from Peprotech Inc. The mixture was incubated for 30 min on ice and the reaction terminated by centrifuging the mixture through a 30% sucrose cushion. The supernatant and sucrose fractions were sucked off, and the remaining red cell pellet was measured in gamma counter.

Statistical analyses

We performed statistical analyses with the GraphPad Prism software (version 4). Data are presented as means ± s.e.m. We used Two-Way ANOVA or Student's t-test as appropriate to compare datasets.

All detailed experimental procedures are described in Supplemental Data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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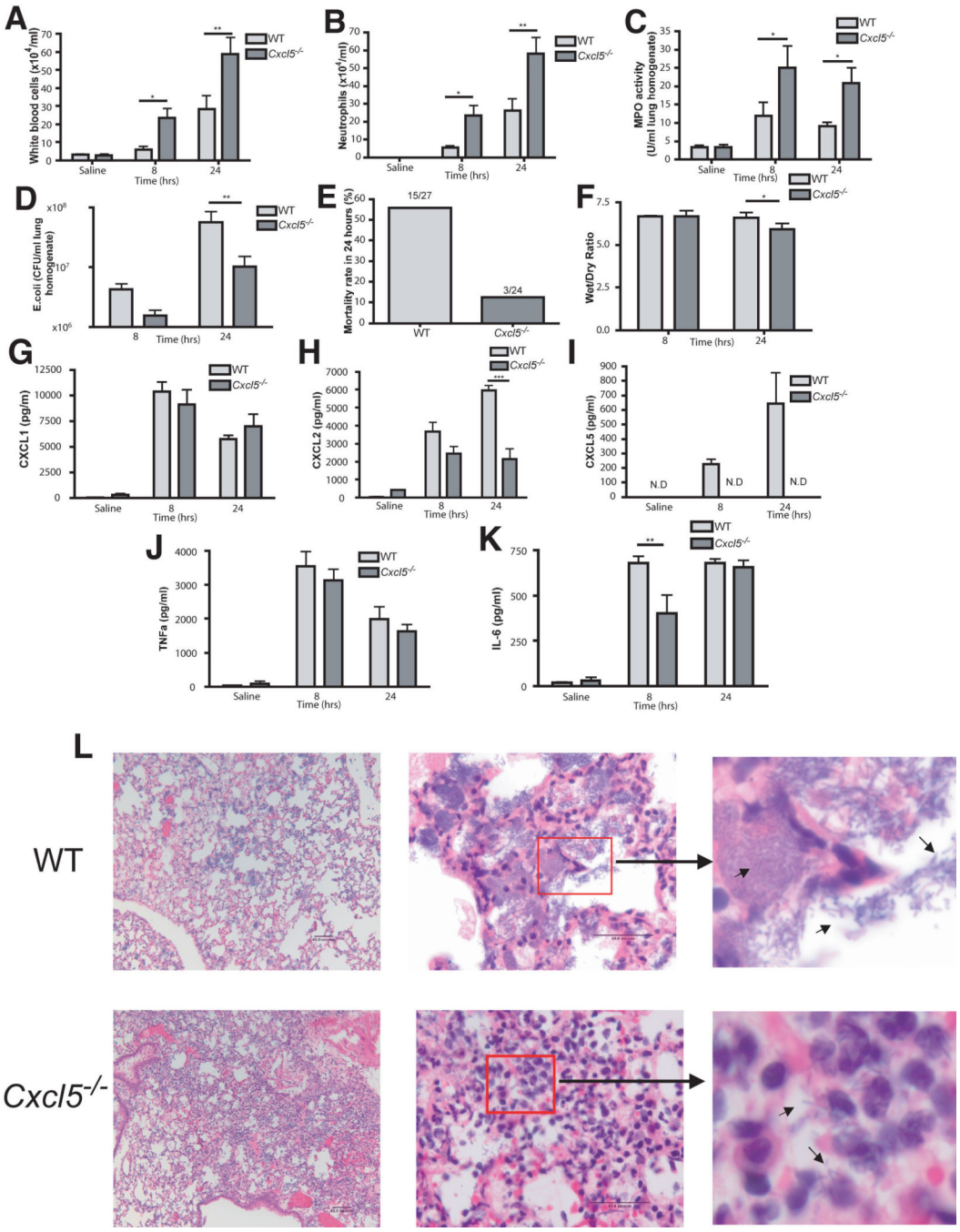

Figure 1. The pulmonary immune responses, lung injury and host defense upon E.coli intratracheal challenge

White blood cells (**A**) and neutrophils (**B**) were counted in the BALF of WT and *Cxcl5*$^{-/-}$ mice (n>=5 mice/group) at 8 and 24 hour point after 10^7 CFU E.coli (intratracheal (i.t.) inoculation. The lung myeloperoxidase activities (**C**) were measured from lung homogenate. The lung bacterial burden (**D**) was measured in the lung homogenate by serial dilution on MacConkey plates. The mortality rate of mice (**E**) was monitored within 24 hours after inoculation, and the fractions above the columns represent the number of dead mice among the number of mice inoculated. The lung wet/dry ratios (**F**) were measured after drying the whole lung in 80°C oven for 48 hours. (n>=5 mice/group). Concentrations of CXCL1 (**G**),

CXCL2 (**H**), CXCL5 (**I**), TNF-α (**J**) and IL-6 (**K**) in the BALF were measured by ELISA. Control mice were i.t. challenged with sterile saline. Data are representative of two independent experiments. Representative haematoxylin & eosin staining (**L**) of fixed lung samples at 24 hours of WT and *Cxcl5*$^{-/-}$ mice (n=4 mice/group) is shown. The lungs were fixed with 10% neutral formalin. Arrows indicate E.coli rods. Size bar: 40 μm. N.D: not detected. *P<0.05; **P<0.01 ; ***P<0.001.

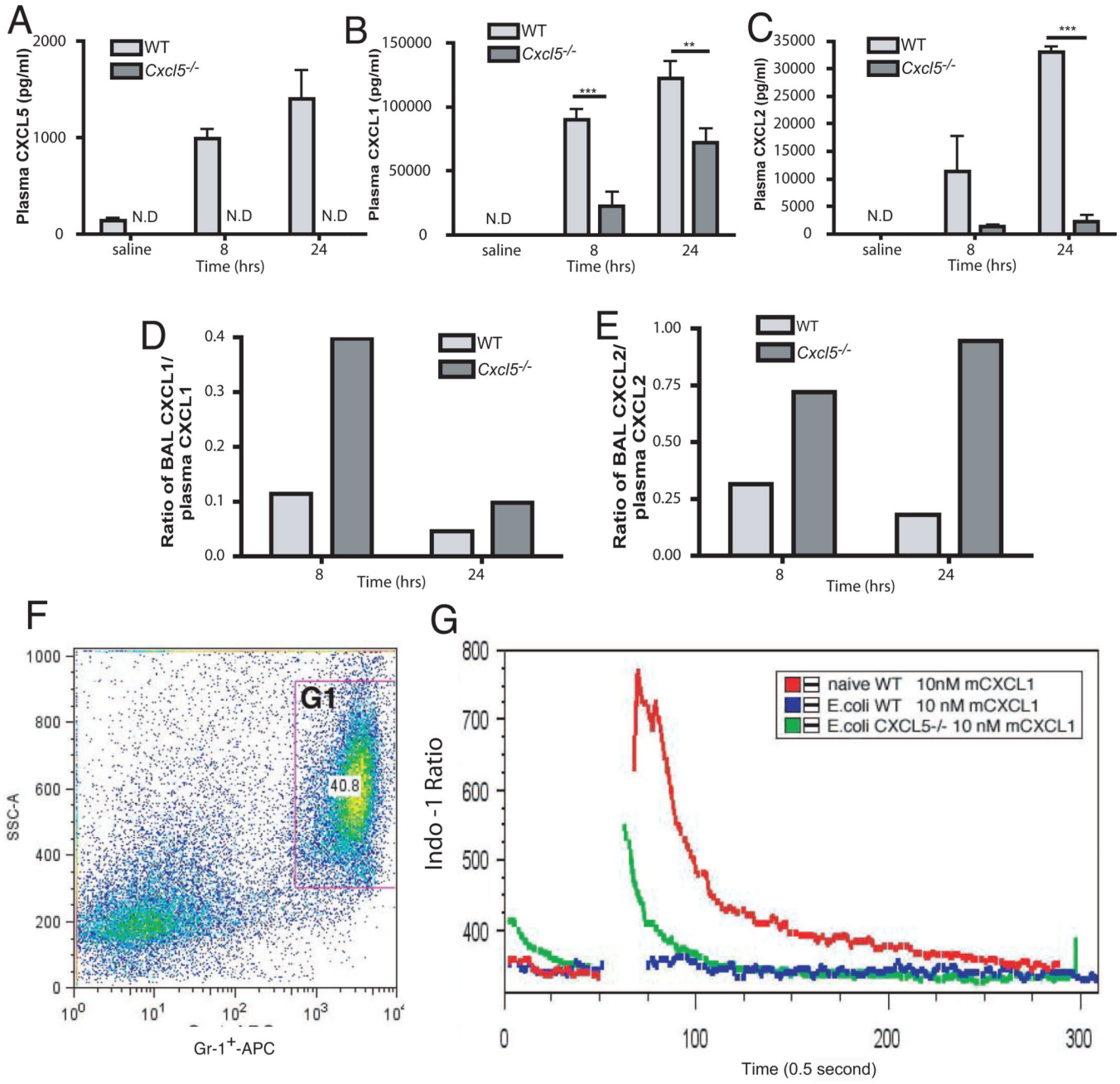

Figure 2. Plasma chemokines, chemokine gradients, and CXCR2 desensitization upon E.coli challenge

WT and *Cxcl5*$^{-/-}$ mice (n=4 mice/group) were i.t. inoculated with 10^7 CFU E.coli, at different time points, the blood was drawn from vena cava after injection of 3.2% sodium citrate. The plasma amounts of CXCL5, CXCL1 and CXCL2 were measured upon E.coli challenge (**A**,**B**,**C**) by ELISA. The ratio of BALF to plasma CXCL1 (**D**) and CXCL2 (**E**) were calculated with the mean value of chemokine concentrations from Figure 1**G–H** and Figure 2**B–C**. The gating of Gr-1$^+$ cell in the BM (**F**) and the representative calcium responses (**G**) of BM Gr-1$^+$ cells of naïve WT mice (red line), E.coli-challenged (24 hours after challenge) WT (blue line) and *Cxcl5*$^{-/-}$ mice (green line)(n=3 mice/group) to 10 nM CXCL1 are shown. The BM cells were stained with APC-conjugated Gr-1 antibody and

labeled with indo-1 AM. Data are representative of two independent experiments. N.D: not detected. **P<0.01; ***P<0.001.

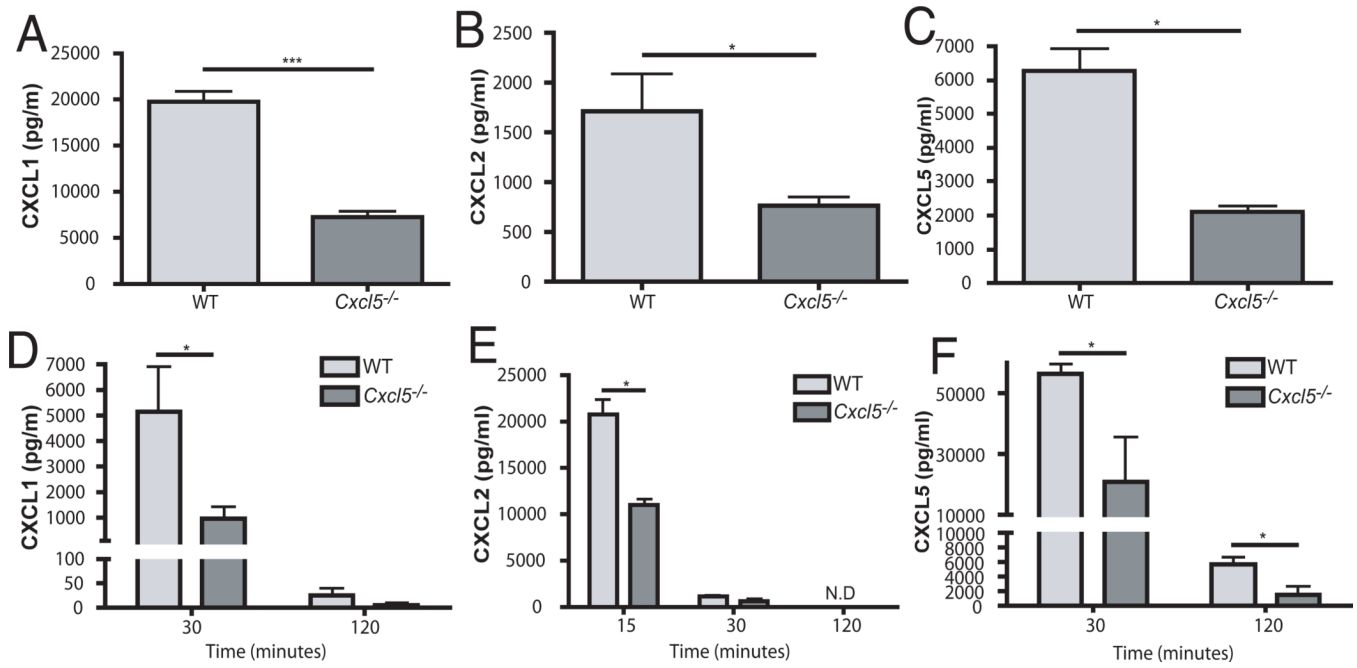

Figure 3. CXCL5 inhibits chemokine scavenging in whole blood *in vitro* and *in vivo*

The plasma concentrations of CXCL1 (**A**), CXCL2 (**B**) and CXCL5 (**C**) were measured by ELISA after incubation of the citrated blood from WT and *Cxcl5*$^{-/-}$ mice (n=3 mice/group) with recombinant CXCL1, CXCL2 and CXCL5 respectively for 15 minutes. The plasma concentrations of CXCL1 (**D**), CXCL2 (**E**) and CXCL5 (**F**) in the plasma of WT and *Cxcl5*$^{-/-}$ mice were measured at different time points after intravenous injection of recombinant CXCL1, CXCL2 and CXCL5 respectively. Data are representative of two independent experiments. N.D: not detected. *P<0.05; ***P<0.001.

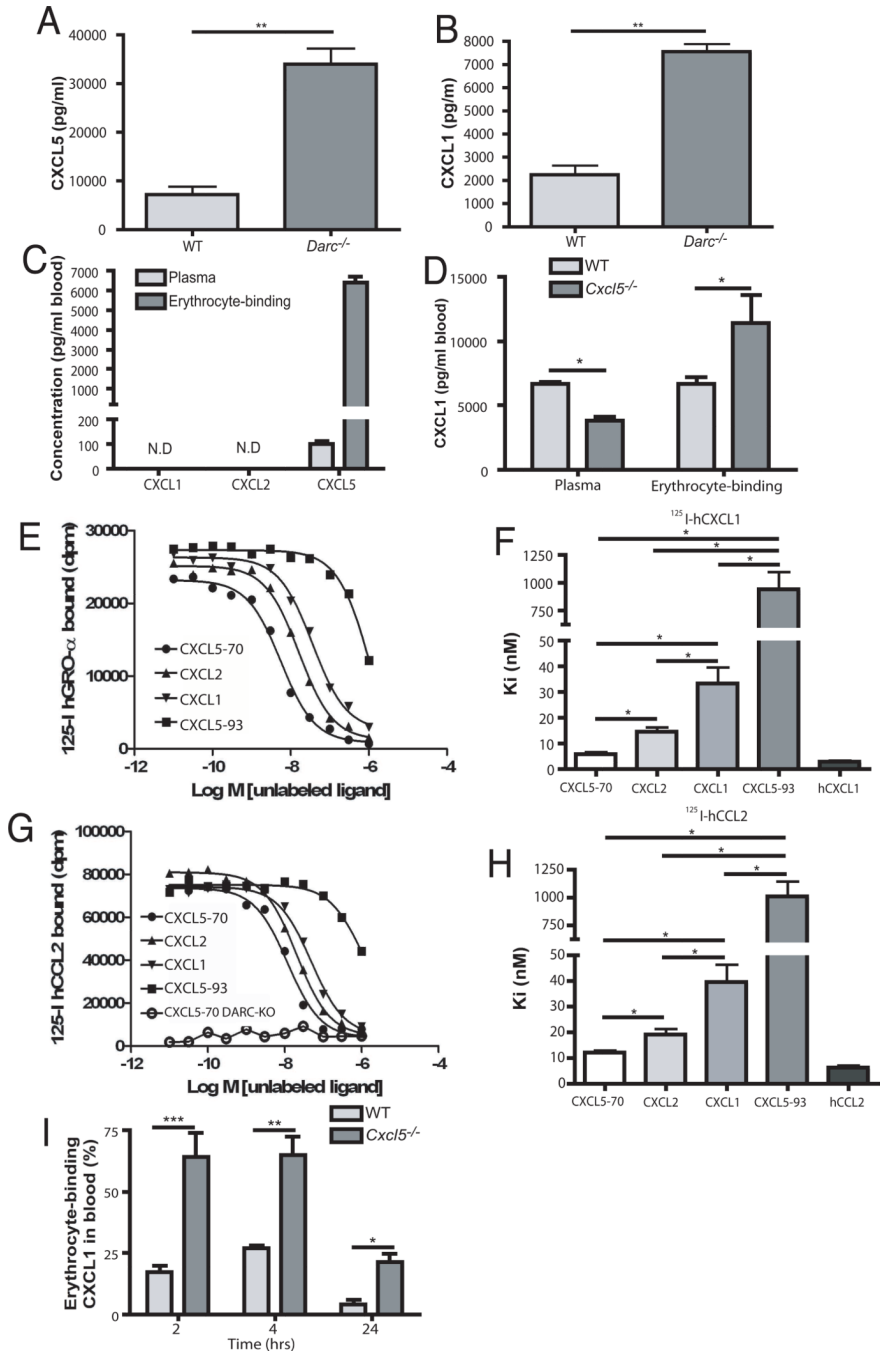

Figure 4. CXCL5 regulates plasma chemokines at least in part through homeostatic and high-affinity binding with erythrocyte DARC

The plasma concentrations of CXCL5 (**A**) and CXCL1 (**B**) were measured by ELISA after incubation of the citrated blood from WT and *Darc*$^{-/-}$ mice (n=4 mice/group) with recombinant CXCL5 or CXCL1 respectively for 15 minutes. The amounts of plasma and erythrocyte DARC-binding CXCL1, CXCL2 and CXCL5 in blood of naïve WT mice (n=3 mice/group) were measured by ELISA (**C**). The amounts of plasma CXCL1 and erythrocyte DARC-binding CXCL1 were also measured by ELISA from the citrated blood 15 minutes after intravenous injection of 1μg recombinant CXCL1 into WT and *Cxcl5*$^{-/-}$ mice (n=3 mice/group) (**D**). Competitive binding assay using ^{125}I-hGro-α (CXCL1) (**E**) or ^{125}I-hCCL2

(**G**) for purified murine erythrocytes from WT (**E**,**G**) and *Darc*$^{-/-}$ (**G**, CXCL5-70 *Darc*$^{-/-}$) mice with various concentrations of cold ligands CXCL5-70, CXCL1, CXCL2, CXCL5-93 and unlabeled hGro-α (CXCL1) or hCCL2 were performed. The Ki (equilibrium dissociation constant) values (**F**, **H**) for cold competitors (n=4 times) were calculated and show distinct binding affinities for red cell DARC within the ELR^{+} CXC chemokines family ($p<0.05$ for all ligands comparisons). *Darc*$^{-/-}$ red cells do not bind appreciably radiolabeled ligands (**G**). The percentages of erythrocyte DARC-binding CXCL1 in blood (**I**) were calculated after measurement of plasma and erythrocyte DARC-binding CXCL1 in blood of WT and *Cxcl5*$^{-/-}$ mice (n=3 mice/group) at different time points upon 10^7 CFU E.coli i.t. challenge. N.D: not detected. *P<0.05; **P<0.01; ***P<0.001.

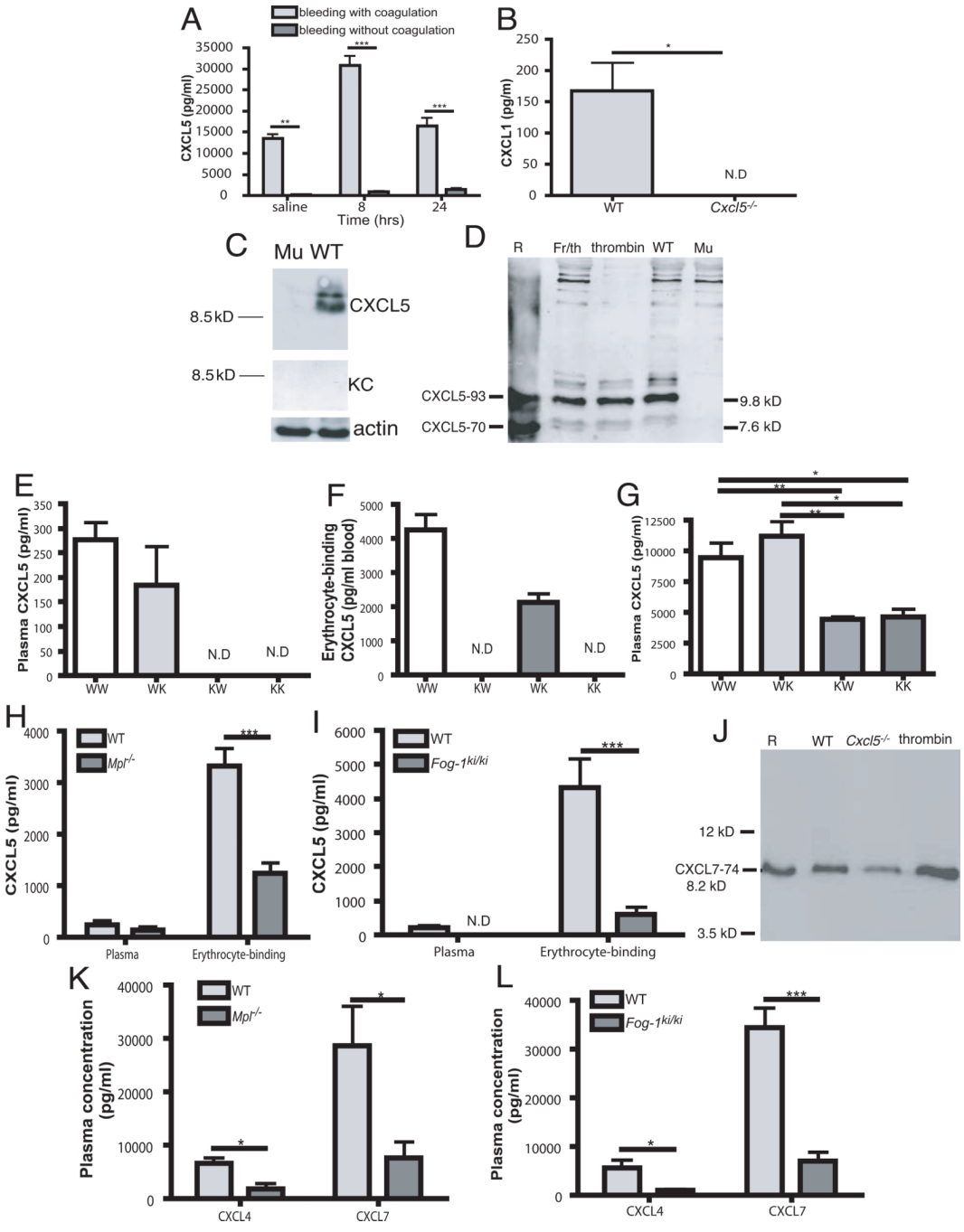

Figure 5. Large amounts of preformed CXCL5 in platelets and platelet origin for homeostatic CXCL5 in blood

Comparison of plasma concentrations of CXCL5 (**A**) by retro-orbital bleeding (with coagulation) and vena cava bleeding (by injection of sodium citrate, without coagulation) in WT mice (n=4 mice/group) upon 10^7 CFU E.coli i.t. challenge, measured by ELISA. The plasma concentrations of CXCL1 (**B**), CXCL2 (**C**) and CXCL15 (**D**) by retro-orbital bleeding from naïve WT mice and *Cxcl5*$^{-/-}$ mice (n=4 mice/group) were measured by ELISA. CXCL5, but not CXCL1, is detected in inactivated platelets of naïve WT mice by western blot (**E**), and both preformed and secreted CXCL5 by thrombin stimulation from platelet is mostly CXCL5-93 form (**F**). R: recombinant CXCL5-93 (9.8 kD) and CXCL5-70

(7.6 kD); Fr/th: the supernatant after separated platelets were snap-frozen and thawed twice; thrombin: the supernatant after separated WT platelets were stimulated with 1 U/ml thrombin for 10 minutes at 37°C;WT or Mu: lysate of inactivated platelets in WT or mutant *Cxcl5*$^{-/-}$ mice. WT and *Cxcl5*$^{-/-}$ mice were reconstituted with BM from WT and *Cxcl5*$^{-/-}$ mice respectively, after 8 weeks, the citrated blood will be prepared from the mice in each group (n=4 mice/group). The plasma and red cells were separated, then the plasma CXCL5 concentrations (**G**) and erythrocyte-binding CXCL5 (**H**) were measured by ELISA. After incubation of the citrated blood with recombinant CXCL5 for 15 minutes, the plasma concentrations of CXCL5 (**I**) were measured. WW: BM from WT donor mice into WT recipient mice; WK: BM from WT donor mice into *Cxcl5*$^{-/-}$ recipient mice; KW: BM from *Cxcl5*$^{-/-}$ donor mice into WT recipient mice; KK: BM from *Cxcl5*$^{-/-}$ donor mice into *Cxcl5*$^{-/-}$ recipient mice. The amounts of plasma CXCL5 and erythrocyte-binding CXCL5 in citrated blood of *Mpl*$^{-/-}$ mice (**J**), *Fog-1*$^{ki/ki}$ mice (**K**) and their respective WT control mice were measured by ELISA. The plasma concentrations of CXCL4 and CXCL7 were measured in the citrated blood of *Mpl*$^{-/-}$ mice (**M**), *Fog-1*$^{ki/ki}$ mice (**N**) and their respective WT control mice as well. Only the CXCL7-74 (β-thromboglobulin) form is detected in platelets by Western blot (**L**). R: recombinant CXCL7-74 a.a. (8.2 kD). Data are representative of two independent experiments. N.D: not detected. *P<0.05; **P<0.01; ***P<0.001.

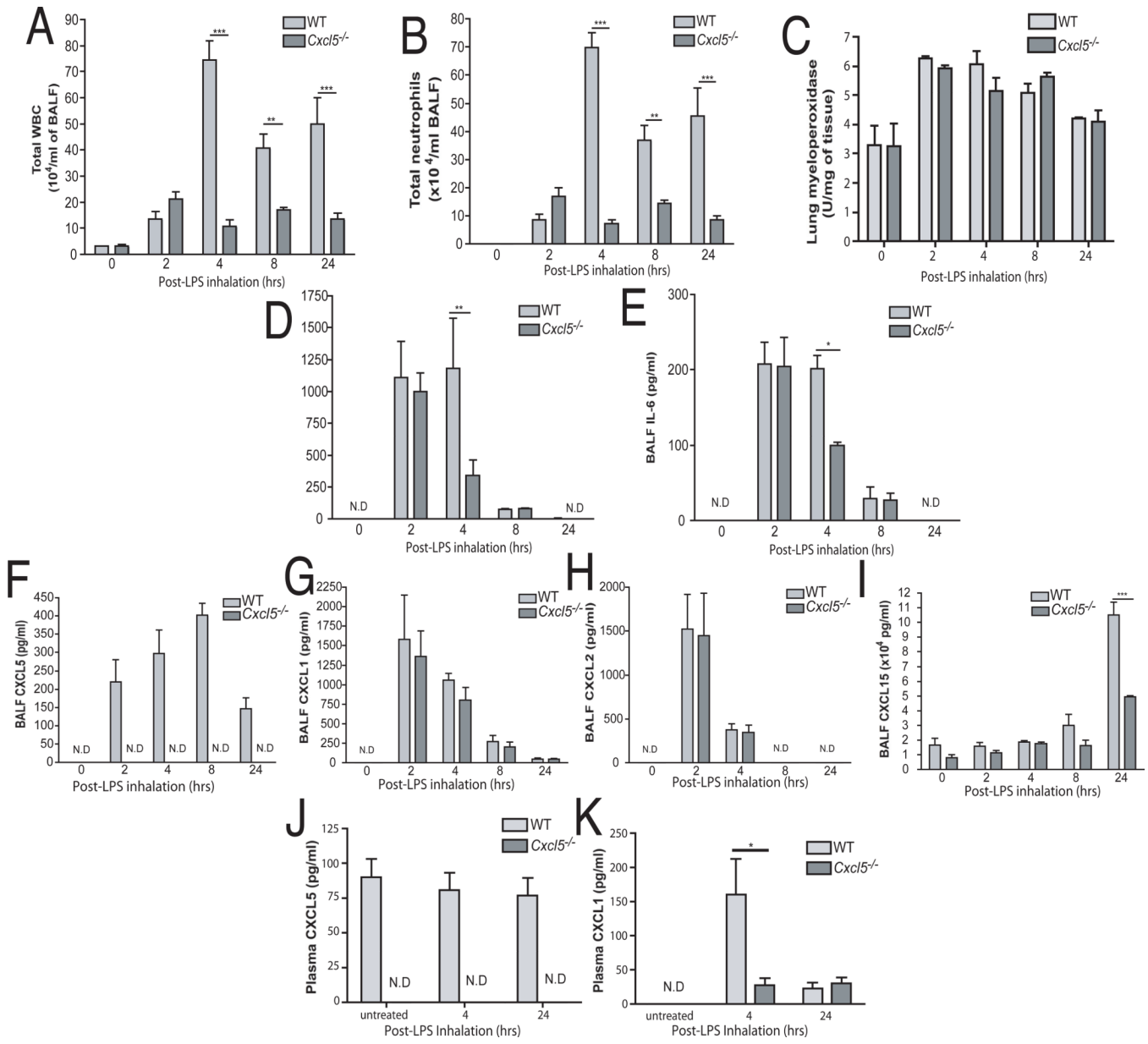

Figure 6. CXCL5 is required for pulmonary immune responses to LPS inhalation

WT and *Cxcl5*$^{-/-}$ mice (n=3 mice/group) were exposed to nebulized LPS (0.3 mg/ml) for 30 minutes, at different time point, white blood cells (WBC) (**A**) and neutrophils (**B**) were counted in the BALF. The lung MPO activities (**C**) were measured from lung homogenate. The protein amounts of TNF-α (**D**), IL-6 (**E**), CXCL5 (**F**), CXCL1 (**G**), CXCL2 (**H**) and CXCL15 (**I**) in the BALF were measured by ELISA. At 4 and 24 hours after LPS inhalation, the blood was drawn from inferior vena cava after injection of 3.2% sodium citrate and the plasma concentrations of CXCL5 (**J**), CXCL1 (**K**), CXCL2 (**L**) and CXCL15 (**M**) were measured by ELISA. Data are representative of two independent experiments. N.D: not detected. *P<0.05; **P<0.01; ***P<0.001.

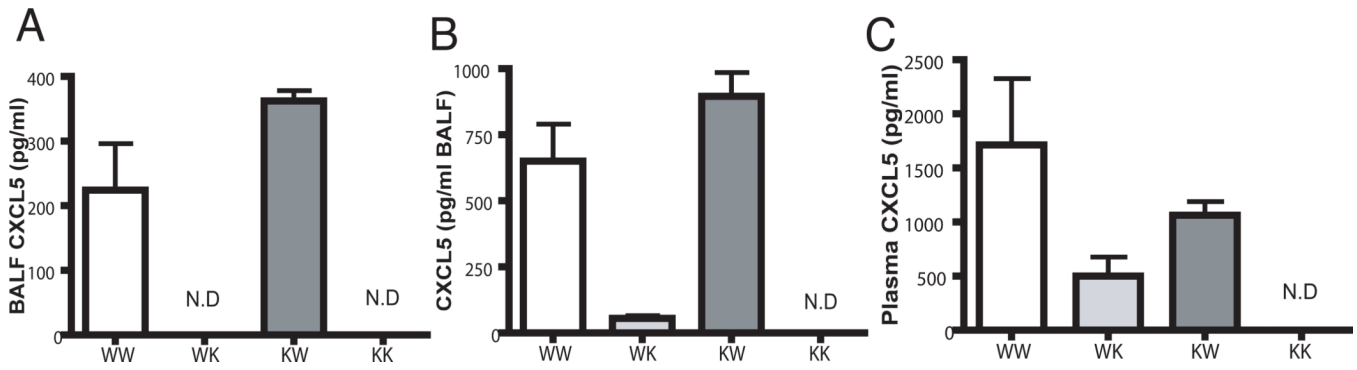

Figure 7. The origin of CXCL5 in both LPS inhalation and severe E.coli pneumonia model

WT and *Cxcl5*$^{-/-}$ mice were reconstituted with BM from WT and *Cxcl5*$^{-/-}$ mice respectively, after 8 weeks, the chimeric mice were exposed to 0.3 mg/ml LPS for 30 minutes (**A**), or intratracheally challenged with 10^7 CFU E.coli (n=3 mice/group) (**B,C**). At 8 hours after challenge, the BALF (**A**, **B**) and plasma samples (**C**) were prepared and CXCL5 measured by ELISA. WW: BM from WT donor mice into WT recipient mice; WK: BM from WT donor mice into *Cxcl5*$^{-/-}$ recipient mice; KW: BM from *Cxcl5*$^{-/-}$ donor mice into WT recipient mice; KK: BM from *Cxcl5*$^{-/-}$ donor mice into *Cxcl5*$^{-/-}$ recipient mice. Data are representative of two independent experiments. N.D: not detected.