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The Src-family kinases Hck and Fgr regulate early lipopolysaccharide-induced myeloid cell recruitment into the lung and their ability to secrete chemokines[1]

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Abstract

Myeloid leukocyte recruitment into the lung in response to environmental cues represents a key factor for the induction of lung damage. We report that Hck and Fgr-deficient mice show a profound impairment in early recruitment of neutrophils and monocytes in response to bacterial lipopolysaccharide (LPS). The reduction in interstitial and airway neutrophil recruitment was not due to a cell-intrinsic migratory defect, because Hck and Fgr-deficient neutrophils were attracted to the airways by the chemokine CXCL2 as wild type cells. However, early accumulation of chemokines and TNFα in the airways was reduced In *hck−/−fgr−/−* mice. Considering that chemokine and TNFα release into the airways was neutrophil-independent, as suggested by a comparison between control and neutrophil-depleted mice, we examined LPS-induced chemokine secretion by neutrophils and macrophages in wild type and mutant cells. Notably, mutant neutrophils displayed a marked deficit in their capability to release the chemokines CXCL1, CXCL2, CCL3 and CCL4, and TNFα, in response to LPS. However, intracellular accumulation of these chemokines and TNFα in, as well as secretion of a wide array of cytokines, including IL-1α, IL-1β, IL-6 and IL-10, by $hck^{-/-}fgr^{-/-}$ neutrophils was normal. Intriguingly, secretion of CXCL1, CXCL2, CCL2, CCL3, CCL4, RANTES and TNFα, but not IL-1α, IL-1β, IL-6, IL-10 and GM-CSF, was also markedly reduced in bone marrow-derived macrophages (BMDM). Consistently, the Src kinase inhibitors PP2 and dasatinib reduced chemokine secretion by neutrophils and BMDM. These findings identify Src kinases as critical regulator of chemokine secretion in myeloid leukocytes during lung inflammation.

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INTRODUCTION

Neutrophil (PMN) recruitment into the lung represents a key feature of host defense against infection. However, accumulating evidence points to an important role of PMN in driving lung pathology in several diseases, including acute lung injury (ALI) (1), cystic fibrosis (2, 3) and tuberculosis (4). The aim to identify effective ways to reduce lung inflammation has prompted intense investigation on mechanisms regulating PMN recruitment into the lung. These investigations have led to the view that stimulation of lung epithelial and innate immune cells by bacterial components or other inflammatory mediators, triggers nuclear factor-κB (NF-κB)-dependent synthesis and secretion of a wide array of chemokines and cytokines that promote PMN recruitment. Consistent with this view, blocking either NF-κB activation or chemokine-receptor interactions results in a marked decrease of PMN recruitment in different models of lung inflammation (4–12).

Several studies have identified Src-family kinases among the possible target molecules regulating inflammatory cell recruitment into the lung. Mice expressing a constitutively active form of Hck or with the selective granulocyte inactivation of the Src-family kinase inhibitor C-terminal Src kinase (Csk) develop an exaggerated pulmonary inflammation spontaneously and are hyper-responsive to systemic or intranasal instillation of lipopolysaccharide (LPS) (13, 14). Excessive inflammation in *motheaten* mice, a phenotype resulting from a mutation in the gene *Ptpn6* that encodes for the non-receptor protein-tyrosine phosphatase Shp1, is caused by enhanced signaling via Src kinases, and the Src downstream target Syk, in neutrophils (15). Consistent with the evidence that excessive Src kinase activity results in innate immune cell-mediated inflammatory responses, either genetic deficiency of Src kinases, or their inhibition by drugs, results in a marked reduction in granulocyte recruitment into the lung and other tissues (16–20).

In this report we addressed whether Hck and Fgr regulate PMN and monocyte recruitment and development of lung inflammation in an LPS-induced model of ALI and found that deficiency of these kinases results in a markedly reduced susceptibility to ALI induction. Experiments performed with the aim to identify mechanisms by which Src-family kinases regulate myeloid cell recruitment excluded a role for Hck and Fgr in regulation of intrinsic neutrophil migratory ability. In fact, PMN recruitment into the airways of $hck^{-/-}fgr^{-/-}$ mice in response to a PMN-attractive chemokines was comparable to that detected in wild type mice. However, we found that secretion of four different chemokines, as well as TNFα, was markedly defective in $hck^{-/-}fgr^{-/-}$ PMNs and macrophages challenged with LPS. As a result, these chemokines accumulate to a lower extent in the airways of $hck^{-/-}fgr^{-/-}$ mice. These findings concur with previous evidence that deficiency of Src kinases does not impair the chemotactic responses of PMNs in transwell assays *in vitro* or in chemical peritonitis *in vivo* and actually enhance the response of PMNs and dendritic cells to chemokines recognizing CXCR2 and CCR1 receptors (21, 22). Additionally, they extend to lung inflammation the recently established concept that Src kinases are indispensable for autoantibody-induced inflammation in the joint and the skin due to their role in triggering PMN activation, but not in regulating their intrinsic migratory ability (23).

Materials and Methods

Mice and bone marrow cells

Generation and maintenance of *hck*$^{-/-}$*fgr*$^{-/-}$ double knockout mice in the C57BL/6J background were as described in (16). Wild type and knockout animals used in the experiments were at 8–10 weeks of age. Animals were housed at a pathogen-free facility at the University of Verona and treated according to protocols approved by the Minister of Health of Italy and the University animal care committee. Bone marrow neutrophils (PMNs) were isolated by centrifugation of bone marrow cells flushed from femurs and tibias over a Percoll discontinuous density gradient (Amersham Co., Arlington Heights, IL) as described in reference (26). Bone marrow derived macrophages (BMDMs) were isolated from femurs and tibias as previously described (27). Cells were resuspended in Dulbecco's modified Eagle'smedium (DMEM) supplemented with Glutamax (Biowhittaker, Walkersville, MD) 15% FCS, 10% L-cell conditioned medium (LCM) as a source of CSF-1, 100 U/ml penicillin, and 100 mg/ml streptomycin (BMDMs complete medium), and cultured at 37 °C/ 5% CO2 in 75 cm2 flasks. After 24 h, the non-adherent cells were removed, counted, plated on multi-well plates and incubated for 6–7 days in the above medium to allow differentiation to BMDMs.

Lung cells and fluid

Mice were anesthetized and were given LPS (5 μg) or phosphate buffer saline (PBS) by intranasal instillation. At 4, or 24 h, from challenge, mice were euthanized and cannulated through the trachea for the recovery of bronchoalveolar lavage fluid (BALF) cells. Airways were washed 4 times with 0.5 ml of ice-cold PBS and, after centrifugation, the supernatant was collected and stored at −80°C. Total cells in the pellet were resuspended in PBS and counted. For cytospin preparations, 5×10^4 cells were centrifuged onto glass slides at 400 rpm. Cytospins were stained with May-Grünwald-Giemsa or non-specific esterase, coverslipped and examined by light microscope and the differential cells count performed on 300 cells. For calgranulin B staining, BALF cells recovered after 2 h from intranasal instillation of PBS or LPS were centrifuged onto glass slides at 400 rpm. Cytospins were kept in absolute ethanol for 30 min, then immunohistochemistry staining was done using a goat HRP-polymer kit (Biocare Medical, Concorde, CA) and the primary anti-mouse S100A9 antibody (R&D Systems, Minneapolis, MN, USA). The assay was developed according to the manufacturer's instructions.

Histological analysis and immunostaining

Following euthanasia and isolation of BALF, the left lobe of the lung was formalin-fixed, paraffin-embedded, sectioned at 3–4 μm, and then stained with hematoxylin and eosin for histological analysis or with anti-mouse F4/80 (Ly71) Ag (Cl:A3-1; Serotec, Oxford, UK) or anti-mouse Gr-1 (Clone RB6-8C5; R&D Systems) Abs for immunohistochemistry.

Neutrophil depletion

C57BL/6J and *hck*$^{-/-}$*fgr*$^{-/-}$ mice were depleted using an anti-Ly6G mAb (Bio-X-Cell, West Lebanon, NH), as described in ref. 28. Briefly, anti-Ly6G mAb was diluted into sterile

endotoxin-free 0.9% NaCl saline solution at a concentration of 1mg/ml. The antibody was injected i.p. at a dose of 0.5 mg per mouse, 17 hours prior to intranasal instillation of LPS (5 μg) or PBS. Control mice were injected i.p. with saline. 2 h after intranasal treatments of depleted and control mice, peripheral blood was collected from all experimental animals to confirm neutrophil depletion by cytofluorimetric analysis. This was performed using a panel of five fluorochrome-conjugated antibodies to CD11b, GR-1 (clone RB6-8C5, Biolegend), CD11c, Ly6C and Ly6G.

Cytokine detection by Multiplex

Cytokines and chemokines were measured in BALF and in supernatants of cultured cells. PMNs or BMDMs supernatants were collected after 4-or 24 h of culture in DMEM, 10% FCS, penicillin, streptomycin. Cells were then lyzed using the Milliplex Map Lysis Buffer (Merck Millipore, Billerica, MA, USA) containing 1μM DFP, 10μM PAO and the complete Protease Inhibitor Cocktail tablets (Roche Diagnostic GmbH, Mannheim, Germany). BALF, cell culture supernatants and lysates were analyzed in triplicate for CXCL1/KC, CXCL2/MIP-2, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, IL-1α, IL-1β, IL-6, IL-10 and TNF-α by multiplex bead array assay (Milliplex magnetic mouse cytokines panels, Merck Millipore) and acquired on a MagPix instrument (Luminex, 's-Hertogenbosch, The Netherlands). All reagent dilutions (beads, cytokine standards, cytokine controls, biotinylated detection antibody, etc.) were prepared and assay developed according to the manufacturer's instructions.

Other assays

For cytofluorimetric analysis, after 4 h from the LPS challenge, BALF cells were recovered as described above, counted and incubated with purified rat anti-mouse CD16/CD32 (clone 2.4G2, BD Biosciences, San Jose, CA, USA) and mouse IgG (Sigma-Aldrich, Saint Louis, MO, USA) to block Fc receptors. Then, cells were stained with a mixture of four fluorochrome-conjugated antibodies to CD11b (clone M1/70, eBioscience, San Diego, CA, USA), CD11c (clone HL3, BD Biosciences), Ly6C (clone AL-21, BD Biosciences) and Ly6G (clone 1A8, BioLegend, San Diego, CA, USA). Data were acquired on a MACSQuant Analyzer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and data analyses were performed using FlowJo software (TreeStar, Ashland, OR, USA).

Statistical analysis

Data are expressed as mean values ± SD. Statistical significance between cell accumulation in the lung of different groups of mice was calculated by unpaired Student's *t*-test. Statistical significance of differences between cytokine/chemokine release by PMNs and BMDM was evaluated by ANOVA with Bonferroni post-tests, which was performed using GraphPad Prism software.

RESULTS

Hck and Fgr regulate LPS-induced PMN recruitment into the lung

In mice, LPS inhalation induces a rapid recruitment of PMN and monocytes into the bronchoalveolar space. In wild-type C57Bl/6J mice we detected a marked increase in the

number of cells present in the bronchoalveolar lavage fluid (BALF) as early as 4 h following intranasal instillation of 5 μg LPS (Fig. 1A). At this early time point, myeloid leukocytes recruited into the airways were mostly PMNs (70.6 ± 19% of total cells, Fig. 1B). However, non-specific esterase staining of BALF cells allowed us to detect a significant, albeit small, increase in the absolute number of esterase-positive mononuclear phagocytes (Mo) (Fig. 1C). In *hck*$^{-/-}$*fgr*$^{-/-}$ mice, PMN recruitment into the bronchoalveolar space at 4 h, was nearly abolished following LPS inhalation (Fig. 1B). Additionally, numbers of esterase-positive Mo was similar in *hck*$^{-/-}$*fgr*$^{-/-}$ mice treated with vehicle (PBS) or LPS, thus making Mo recruitment in response to LPS in *hck*$^{-/-}$*fgr*$^{-/-}$ mice virtually undetectable (Fig 1C). The profound defect of the early myeloid leukocyte recruitment into the bronchoalveolar space of *hck*$^{-/-}$*fgr*$^{-/-}$ mice (Fig. 1) is secondary to impaired cell migration from the blood to the lung interstitium. In fact, staining of paraffin-embedded lung sections from *hck*$^{-/-}$*fgr*$^{-/-}$ mice with the granulocyte-specific Ab Gr-1 (Fig. 1D) or the Mo-specific Ab F4/80 (Fig. 1E) revealed a marked reduction of interstitial PMNs and Mo following LPS inhalation. The reduced recruitment of myeloid leukocytes into the airways was not a consequence of an impairment of the general response to LPS. In fact, 4 hours following intranasal instillation of LPS, neutrophil counts in the blood were increased to the same extent in wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice (control wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice 650 ± 80 and 900 ± 50/μl (n=3), respectively; LPS-treated wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice 3000 ± 850 and 3200 ± 550/μl (n=4), respectively). Consistent with this finding, in previous studies with a model of systemic endotoxemia *hck*$^{-/-}$*fgr*$^{-/-}$ mice demonstrated many systemic signs characteristic of endotoxic shock (16).

Impairment of myeloid cell recruitment into the airways in response to LPS required the double inactivation of *hck* or *fgr*. In fact, we did not find a significant reduction of PMN recruitment into the bronchoalveolar space following LPS inhalation in single knockout *hck*$^{-/-}$ or *fgr*$^{-/-}$ mice (data not shown). These findings indicate that these kinases play a redundant role in regulation of inflammatory of myeloid cell recruitment into the lung, as we previously found for PMN recruitment into the liver (16).

Myeloid cell recruitment into the airways varies considerably based on the LPS dose used and the time following the LPS challenge. For example, as shown in Fig. 2A, after 24 h from challenge with concentrations of LPS as low as 5 ng, the number of PMNs recruited into the airways was even higher than after 4 h in response to 5 μg LPS (see Fig. 1B for comparison). To determine whether Hck and Fgr deficiency affected the kinetics of PMN recruitment we examined mice at 24 h following high LPS dose (5 μg) instillation. As shown in Fig. 2B, after 24 h neutrophil recruitment into the airways was comparable in wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice. In contrast to high dose LPS, recruitment of *hck*$^{-/-}$*fgr*$^{-/-}$ PMN into the airways following low dose LPS (2.5 ng) instillation remained low after 24 h (Fig. 2C). From the findings reported in Fig 1 and 2, we conclude that early myeloid leukocyte airway recruitment in response to LPS is strictly regulated by Hck and Fgr. However, at very high doses of LPS, other inflammatory mechanisms compensate for loss of Hck and Fgr, allowing neutrophil recruitment to occur with delayed kinetics.

Hck and Fgr regulate LPS-induced monocyte recruitment into the lung

As shown in Fig. 1, within 4 h from the intranasal instillation of LPS the number of esterase-positive mononuclear cells, also stained by the anti-macrophage Ab F4/80, in the airways increased, thus pointing for an early recruitment of monocytes from the blood. However, considering that both resident alveolar macrophages and recruited blood monocytes are esterase-positive, non-specific esterase staining did not allow us to appreciate a strong difference in the recruitment of monocytes in wild type or *hck*$^{-/-}$*fgr*$^{-/-}$ mice (see Fig. 1C) To better identify monocytes recruited into the airways we used two different approaches.

In preliminary studies, we exploited the knowledge that blood monocytes and monocytes early recruited from the blood, but not resident macrophages, are positive for the expression of calgranulin (28). However, because the high number of PMNs, which are also calgranulin-positive, could have hampered enumeration of calgranuline-positive monocytes after 4 h from the LPS challenge, we examined recruitment of this cell population at 2 h following the LPS challenge, when the accumulation of PMNs into the airways was very low (see Fig. 5 legend for quantitative data). In cytocentrifuge preparations from the BALF of wild type mice we clearly detected a calgranulin-positive mononuclear cell population at 2 h following LPS, which was markedly reduced in the BALF of *hck*$^{-/-}$*fgr*$^{-/-}$ mice (Fig.3A and 3B). To note, the nuclear/cytoplasmic ratio of calgranulin-positive cells was higher compared to that of resident, calgranulin-negative macrophages, i.e. more reminiscent of a monocyte morphology.

As a second approach to demonstrate that deficiency of Hck and Fgr results in reduced recruitment of monocytes to the airways we examined changes in the presence of CD11b^{+}/Ly6G^{-}/Ly6C^{+} or CD11b^{+}/Ly6G^{-}/Ly6C$^{lo/-}$ cells which are know to represent two distinct populations of blood monocytes named “inflammatory” or “patrolling”, respectively (29). As shown in Fig. 3C, in wild type mice, after 4 h following LPS stimulation, both a CD11b^{+}Ly6G^{+}Ly6C^{+} population (granulocytes) and a small CD11b^{+}Ly6G^{-}Ly6C$^{lo/-}$ population (patrolling monocytes) could be clearly detected. Deficiency of Hck and Fgr resulted in a reduction in the recruitment of the total CD11b^{+} cells, including the Ly6G^{+}/Ly6C^{+} granulocytes and the Ly6G^{-}Ly6C$^{lo/-}$ patrolling monocytes (Fig. 3D). Quantification of these defects across a number of mice confirms that both monocyte and PMN recruitment are significantly reduced in the *hck*$^{-/-}$*fgr*$^{-/-}$ mice (Fig. 3D and F).

Hck and Fgr do not regulate PMN chemotactic response but accumulation of chemokines in the airways

At least two explanations may account for the above findings. The first one is that deficiency of Hck and Fgr results in an intrinsic defect in the ability of myeloid leukocytes to migrate towards an inflammatory site. We therefore asked whether PMN migration into the bronchoalveolar space in response to CXCL2/MIP-2 was defective in *hck*$^{-/-}$*fgr*$^{-/-}$ mice. As shown in Fig. 4A and B, direct instillation of CXCL2/MIP-2 into the airways induced a dose-dependent and marked increase in the total number of cells recruited into the lung and most of these cells were PMNs (wild type 87.4 ± 38.2 %; *hck*$^{-/-}$*fgr*$^{-/-}$ 90.8 ± 53.3 %). Notably, no difference was found in the number of airway PMNs between wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice. These findings confirm previous results obtained with a thioglycollate-

induced peritonitis model of PMN recruitment in vivo (21) and recent studies examining PMN migration to inflamed joints and skin (23).

A second possible explanation is that reduced amounts of PMN-attracting chemokines accumulate in the airways of *hck*$^{-/-}$*fgr*$^{-/-}$ mice. Notably, we found that the increase in CXCL1/KC (Fig. 4C) and CCL3/MIP-1α (FIG. 4D), as well as the pro-inflammatory cytokine TNFα (Fig. 4E), in the BALF of *hck*$^{-/-}$*fgr*$^{-/-}$ mice was reduced after 4 h from the LPS challenge, i.e. at a time when a much lower number of PMN were recruited into the airways of *hck*$^{-/-}$*fgr*$^{-/-}$ mice compared to wild type ones (Fig 4F and Fig. 1).

The experiments reported in Fig. 4 raised the issue of the origin of chemokines/cytokines released into the airways. Epithelial or innate immune cells resident in the lung parenchyma release PMN-attracting chemokines (4–12). Thus, reduced chemokine/ TNFα release in the airways of *hck*$^{-/-}$*fgr*$^{-/-}$ mice could reflect a role of these kinases in regulating lung parenchymal cell activation. However, to our knowledge these kinases were not reported to be expressed in lung cells and RT-PCR analysis did not allow us to detect Hck or Fgr expression in type II airway epithelial cell (C.A.L., unpublished observation). To note, at early time points after lung injury, PMN were reported to be indispensable for the induction of lung inflammation (31). This raises the possibility that PMN are required for the early release of chemoattractants and Hck and Fgr regulate this function.

To address this issue in our experimental setting we examined chemokine/TNFα accumulation into the airways at an early time point (2 h) after intranasal instillation of LPS, i.e. when the number of PMNs accumulated into the airways is still low, and after depletion of PMNs by injection of the anti-PMN Ab Ly6C. As shown in Fig. 5A, and consistent with the results reported in Fig. 1, at this early time point LPS-induced PMN recruitment into the airways was defective in *hck*$^{-/-}$*fgr*$^{-/-}$ mice compared to wild type animals. Anti-Ly6G Ab treatment dramatically reduced PMN numbers in the blood of wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice, as well as PMN recruitment into the airways (Fig. 5A, see Fig. 5 legend for absolute PMN numbers). Similar to data concerning PMN recruitment, accumulation of chemokines and TNFα into the airways was reduced in *hck*$^{-/-}$*fgr*$^{-/-}$ mice (Fig. 5B–E). However, airway chemokine/TNFα accumulation was totally blood and airway PMN-independent. Experiments performed with wild type mice showed that chemokine/TNFα accumulation in the airways was comparable in PMN-depleted and control mice also after 4 hrs from intranasal instillation of LPS (data not shown).

The data reported in Figs. 4 and 5 suggest a complex scenario in mechanisms of regulation of PMN recruitment by the Src-kinases Hck and Fgr. These kinases seem to be dispensable for chemokine-induced PMN recruitment (Fig. 4A and B). However, their deficiency results in reduced chemokine/TNFα accumulation into the airways independently of the blood and airway PMN number (Fig. 4C–E and 5). Elucidating which cells display a Hck/Fgr-dependent pathway of chemokine/TNFα secretion in a complex multi-cellular organ is a worth object of future investigation. To start to address this issue, we adopted a reductionist approach starting with the characterization of the role of Hck and Fgr in regulation of myeloid cell chemokine secretion.

hck$^{-/-}$fgr$^{-/-}$ PMNs release a lower amount of chemokines in response to LPS

To address the issue of PMN chemoattractant release we examined secretion of different chemokines acting on granulocytes (CXCL1/KC, CXCL2/MIP-2, CCL3/MIP-1α, CCL4/MIP-1β) by wild type and mutant PMNs in response to different doses of LPS (Fig 6). As shown in Fig. 6A, *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs released much lower amounts of all the four chemokines after 4 h following stimulation with LPS and independently of the stimulus dose used. The defect in chemokine release by *hck*$^{-/-}$*fgr*$^{-/-}$ compared to wild type PMNs may explain differences we observed in PMN polarization between the two mouse strains (Fig. 6B). In fact, whereas wild type PMN maintained for 4 h in the presence of LPS displayed a clearly polarized morphology typically occurring in chemoattractant-stimulated cells, *hck*$^{-/-}$*fgr*$^{-/-}$ PMN remained rounded.

In order to know whether the mutant PMN defect is selective for chemokines active on granulocytes we examined secretion of a few other cytokines (IL-1α, IL-1β, IL-6 IL-10, and TNFα) by wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs (Fig 7). To note, secretion of IL-1α, IL-1β, IL-6 and IL-10 was robust after 24 h, but almost undetectable after 4 h of stimulation with LPS. Intriguingly, the secretion of these four cytokines after 24 h from the LPS challenge was comparable in wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs. Differently from IL-1α, IL-1β, IL-6 and IL-10, TNFα was detectable in the incubation medium both after 4 or 24 h from LPS stimulation and *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs displayed a reduced ability to secrete this cytokine at both time points.

It is important to note that findings similar to those described above were obtained examining chemokine/cytokine secretion by *syk*$^{-/-}$ PMNs. In fact, Syk deficient PMNs secrete lower amounts of CXCL1, CXCL2, CCL3 and TNFα, but normal amounts of IL-1β and IL-6 in response to *E. coli* and *S. aureus* (32). Notably, this last study demonstrated that Syk regulates secretion, but not expression and intracellular storage, of a few chemokines and TNFα. To address whether the Src family kinases Hck and Fgr are also implicated in the regulation of chemokines and TNFα secretion selectively, we compared the secreted and intracellular pool of CXCL1, CXCL2, CCL3, CCL4 and TNFα in wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs (Fig. 8). Notably, while secretion of these four cytokines and TNFα was suppressed or strongly reduced in *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs their intracellular content was comparable to that of wild type PMNs. We conclude that a Src/Syk signaling pathway regulates PMN chemokine secretion in response to LPS.

To strengthen the notion that Src family kinases are implicated in regulation of LPS-induced chemokine release we addressed whether compounds targeting Src kinases inhibit this response. As shown in Fig. 8, both dasatinib, a dual specificity Abl/Src inhibitor, and PP2 a selective Src kinase inhibitor, suppressed secretion of CXCL1, CXCL2, CCL3, CCL4 and TNFα by wild type PMNs. However they also had a strong inhibitory effect on CXCL2, CCL3, CCL4 and TNFa intracellular accumulation, suggesting either that, by targeting other Src family members, they have a broader inhibitory effect on Src kinase activities than that deriving from Hck/Fgr deficiency, or that off-target effects of these compounds result also in inhibition of gene transcription.

Hck and Fgr regulate cytokine secretion by murine macrophages

The issue of the role of Src-family kinases in regulation of chemokine and cytokine secretion by macrophages is somehow controversial. Early studies implicated Src-family kinases in regulation of the response to LPS (reviewed in (33)), but triple deficient *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ peritoneal and bone marrow-derived macrophages display no alteration in LPS/IFNγ-induced release of IL-1, IL-6 or TNFα (27). The defective capability of *hck*$^{-/-}$*fgr*$^{-/-}$ PMN to release CXCL1, CXCL2, CCL3, CCL4 and TNFα prompted studies to examine the role of Src-family kinases in chemokine/cytokine secretion by macrophages. To this purpose, we stimulated wild type or *hck*$^{-/-}$*fgr*$^{-/-}$ BMDM with LPS, a TLR4 ligand, PAM3, a TLR2 ligand or R848, a TLR7/8 ligand, at doses found to be optimal in preliminary experiments, and assayed the release of a wide array of chemokines and cytokines (Fig 9 and supplementary Fig. 1). Similar to results obtained with PMNs (Fig 7) and BMDM stimulated with LPS/IFNγ (27), we did not find major alterations in the release of IL-1α, IL-1β, IL-6, IL-10 and GM-CSF by *hck*$^{-/-}$*fgr*$^{-/-}$ BMDM in response to LPS, with the only exception of TNFa, whose release by mutant BMDM was about three fold lower compared to wild type BMDM (580.13 ± 81.1 pg/ml versus 1832.17 ± 417.4 pg/ml) (Supplementary Fig. 1). However, LPS-induced secretion of the chemokines CXCL1, CXCL2, CCL2/MCP-1, CCL3, CCL4 and CCL5/RANTES was significantly reduced in *hck*$^{-/-}$*fgr*$^{-/-}$ BMDM (Fig 9). Notably, *hck*$^{-/-}$*fgr*$^{-/-}$ BMDM also displayed a reduced ability to secrete CXCL1, CXCL2 and CCL2 in response to PAM3 and R848 (Fig 9), but, similar to data obtained using LPS as a stimulus, they were not defective in PAM3 and R848-induced secretion of IL-1α, IL-1β, IL-6, IL-10 and GM-CSF (Supplementary Fig. 1). With the exception of CCL4, whose secretion was already maximal at 4 h, the release of CXCL1, CXCL2, CCL2, CCL3, and CCL5 was higher after 24 h from stimulation, but *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs displayed a markedly reduced ability to secrete these chemokines in response to LPS also after this longer time point (Fig. 9). Additionally, after 24 h *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs secreted lower amounts of CXCL1, CXCL2 and CCL2 also in response to PAM3 and R848 (Fig. 9) Despite some variability in the response to different agonists (CCL3 and CCL4 release in response to PAM3 and R848 after 4 and 24 h were comparable in wild type versus *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs) these findings implicate Hck and Fgr in regulation of several chemokine release in murine macrophages.

In order to strengthen these findings, we addressed whether dasatinib, that suppressed chemokine secretion by PMNs challenged with LPS (Fig.8) also affected BMDM responses (Supplementary Fig. 2). Notably, dasatinib had a strong inhibitory effect on the capability of BMDM to release CXCL1, CXCL2, CCL2, CCL3, CCL4 and TNFα in response to a wide range of LPS doses.

DISCUSSION

The lung is protected from pathogens entering the body through the airways by various mechanisms that include both constitutive, innate defenses, such as the anti-microbial airway surface liquid which is constantly cleared through the ciliary activity and the cough reflex (32), and pathogen-induced lung tissue cell responses (35–38). These last are mainly based on the release of pro-inflammatory cytokines and chemokines orchestrating the

recruitment of leukocytes endowed with effector and regulatory functions. The response of the lung to injury must be finely tuned to avoid an excessive inflammatory reaction and the consequent and irreversible damage of the lung parenchyma and its gas-exchange function; indeed, an excessive inflammatory response contributes to a severe impairment of the lung function.

In this report we identify Hck and Fgr as essential components of a signaling pathway regulating early recruitment of neutrophils and monocytes into the lung in response to LPS. Our findings that early steps in ALI development are defective in $hck^{-/-}fgr^{-/-}$ mice complement previous findings with mice genetically modified so to express activated Src kinases in myeloid leukocytes. Indeed, either mice expressing a constitutively active mutant of Hck carrying a substitution of the c-terminal regulatory tyrosine (Y499) with phenylalanine, or mice displaying a higher Src-family kinase activity in their granulocytes, due to the conditional inactivation of the Src inhibitory kinase C-terminal Src kinase (Csk), develop a lung pathology characterized by myeloid cell infiltration within the lung interstitium and airways and are, as a result, hyper-responsive to LPS-induced shock (13, 14). Excessive skin and lung inflammation is also a feature of *motheaten* mice that carry a loss-of function mutation in the *Ptpn6* gene, that encodes for the non-receptor protein-tyrosine phosphatase Shp1. Notably, Shp1 is believed to counteract Src kinase-mediated signals and its conditional deletion in granulocytes results in an enhancement of Src kinase activity (15, 39). All these findings point to a critical role of Src kinases in regulation of myeloid leukocyte recruitment into inflammatory sites, a conclusion also supported by the evidence that recruitment of eosinophils into the lung in a model of allergic inflammation and neutrophil recruitment into the liver in a LPS-induced shock model are defective in mice with the deficiency of Hck and Fgr (16, 17). Importantly, a very recent report showed that the genetic deficiency of all the three Src kinases expressed at the highest level in myeloid leukocytes, i.e. Hck, Fgr and Lyn, results in protection from auto-antibody-induced arthritis, skin blistering disease and reverse passive Arthus reaction and a failure of myeloid leukocytes to accumulate at sites of inflammation (23).

Intriguingly, the role of Src kinases in promoting granulocyte recruitment into inflammatory sites does not seem to depend on their capability to regulate their intrinsic migratory ability (see (40) for a review). Indeed, standard transwell assays failed to reveal any defect in the *in vitro* migratory ability of murine Src kinase deficient PMN or human PMN treated with a Src inhibitor (21, 22, 26, 41). More importantly, the use of mixed chimeric mice, containing both wild type and Src-deficient PMNs, demonstrated that they are recruited to the same extent into the inflamed peritoneum, the joints and the skin (21,23). Notably, studies with mixed chimeric mice containing both wild type PMNs and PMNs deficient of Syk, the tyrosine kinase acting in concert with Src kinases in granulocyte responses to stimuli acting through integrin and immune receptors (42, 43), also failed to reveal any intrinsic migratory defect of Syk deficient PMNs (21, 42), Consistent with these findings, we found that airway recruitment of $hck^{-/-}fgr^{-/-}$ PMNs was normal following intranasal instillation of the PMN-attracting chemokine CXCL1 (Fig. 4). Collectively, these results suggest that the Src/Syk signaling pathway does not regulate the intrinsic capability of PMNs to migrate into inflammatory sites in response to chemoattractants.

PMN recruitment into the lung in response to harmful stimuli present in the airways is due to the release of a wide array of cytokines and chemokines (35–37, 45). Cells that have been implicated in the release of pro-inflammatory and chemotactic factors include epithelial cells lining the bronchial and the alveolar lumen, alveolar and interstitial macrophages and even monocytes that are recruited early from the blood (4, 35, 46–50). Although not specifically addressed in the context of lung inflammation, recent findings add further complexity to mechanisms regulating PMN recruitment into inflammatory sites. In fact, full-blown PMN recruitment into the skin has been shown to depend on the release of LTB4 and CXC receptor 2 chemokine ligands by a limited number of "scouting" PMNs in the extravascular compartment (24, 25). In this report we show that, at least as far as the broncho-alveolar space is concerned, early accumulation of chemokines and the pro-inflammatory cytokine TNFα in response to LPS occurs independently of PMNs (Fig. 5), suggesting that myeloid cells resident in the lung interstitium or the aveoli, together with lung epithelial cells, play a major role in inducing a first wave of chemoattractant generation. Intriguingly, this wave depends on the Src-family kinases Hck and Fgr, which are believed to be restricted in their expression to cells of the myeloid lineage or B cells. Although we can not exclude that these kinases may also regulate epithelial cell responses, the available knowledge suggests that it is the response of local macrophages, or early recruited monocytes, that play a major role in the LPS-induced chemoattractant release. Additionally, although we did not address this issue, activation of PMNs to release PMN-attracting chemokines may represent an important step in the amplification of PMN recruitment into the interstitium before their final migration into the airways.

With the aim to elucidate the role of Src-family kinases in lung inflammation we examined in some details the capability of PMNs and macrophages to respond to LPS either using cells deficient of Hck and Fgr or wild type cells treated with Src kinase inhibitors of broader specificity. Intriguingly, we found that, following an LPS challenge, the intracellular accumulation of CXCL1, CXCL2, CCL3, CCL4 and TNFα was comparable in wild type and $hck^{-/-}fgr^{-/-}$ PMNs; however, their secretion in the external medium was markedly reduced (Fig. 8). Thus, Hck and Fgr do not seem to regulate LPS signaling leading to NfκB activation, chemokine gene transcription and chemokine intracellular accumulation before their secretion. In line with this conclusion, other LPS-induced cytokines such as IL-1α, IL-1β, IL-6 and IL-10 were secreted to comparable levels by wild type and $hck^{-/-}fgr^{-/-}$ PMNs (Fig. 6). These findings are consistent with the notion that one of the role played by Src kinases in granulocytes is the regulation of secretory pathways (41, 51–54). Importantly, Syk was reported to regulate secretion, but not intracellular accumulation, of PMN-attracting chemokines and TNFα in response to pathogenic bacteria (32), suggesting that a Src/Syk signaling pathway targets trafficking of intracellularly stored PMN-attracting chemokines and TNFa to the external milieau. Notably, the Src/Syk signaling pathway plays a central role in regulation of cytoskeleton dynamics (reviewed in (42)) and this in turn is implicated in the series of events underlying granule-plasma membrane fusion (reviewed in (55)). Hence, it is conceivable that a possible defect in plasma membrane targeting and docking of chemokine-containing vesicles in Src and Syk deficient PMNs is secondary to alterations in rearrangement of the cytoskeleton. To note, $hck^{-/-}fgr^{-/-}$ PMNs displayed alterations in their capability to polarize in response to LPS (Fig.6). The role of Src kinases in regulation

of chemokine secretion is reinforced by the findings that the selective Src kinase inhibitor PP2 and the dual specificity Abl/Src inhibitor dasatinib hamper PMN-attracting chemokines and TNFa secretion (Fig 8). However, the effect of these drugs goes beyond inhibition of chemokine/TNFα secretion because also intracellular accumulation of these molecules was markedly inhibited. To establish whether this is due to inhibition of Lyn or, in the case of dasatinib, of Abl, or to off-target effects of these drugs is an issue worthy of further work. Independently of its mechanism of action, the capability of dasatinib to inhibit chemokine and TNFα secretion, coupled with its inhibitory role on several PMN responses (56), make it a good candidate to reduce tissue damage in chronic inflammatory and autoimmune diseases.

In the context of lung inflammation, different populations of resident mononuclear phagocytes, or early recruited monocytes, as well as lung epithelial cells can release chemokines and cytokines that augment PMN recruitment. Very recent studies identified in the skin a population of perivascular macrophages that seems to play a crucial role in secretion of the PMN-attracting chemokines CXCL1, CXCL2 and CCL3, and the consequent PMN recruitment in response to *Staphylococcus aureus* infection (57). Although it is not known whether perivascular macrophages are implicated in PMN accumulation into the lung, this finding certainly adds further complexity to the repertoire of cells implicated in PMN recruitment. We report that monocyte recruitment into the lung parenchyma and the airways in response to LPS is markedly defective in $hck^{-/-}fgr^{-/-}$ mice (Fig 1 and 3). Considering that Src kinases do indeed regulate macrophage intrinsic migratory ability (reviewed in (40), see also (58–61)), the inability of monocytes to migrate into the lung interstitium of $hck^{-/-}fgr^{-/-}$ mice may represent another mechanism responsible for a decreased load of PMN-attracting chemokines. Indeed, we detected calgranulin-positive, monocyte like cells in the airways already after 2 h following the LPS challenge, i.e. at a time point at which a very few number of PMNs had migrated into the lung (Fig 3 and 5). Hence, we can not exclude that, due to an intrinsic defect in their migratory ability, $hck^{-/-}fgr^{-/-}$ monocytes can not contribute to the establishment of a “monocyte-dependent neutrophil extravasation” circuit (50). To start to address whether Src kinases regulate chemokine secretion also in mononuclear phagocytes, we examined chemokine and cytokine secretion by BMDM (Fig. 9 and supplementary Figs 1). Similar to PMNs (Figs 6 and 8), BMDM from $hck^{-/-}fgr^{-/-}$ mice secreted lower amounts of CXCL1, CXCL2, CCL3 and CCL4, but also of CCL2 and CCL5, in response to LPS (Fig. 9). Secretion of CXCL1, CXCL2 and CCL2 by $hck^{-/-}fgr^{-/-}$ BMDM was also reduced in response to the TLR2 ligand PAM3 and the TLR7/8 ligand R848 (Fig. 9). The finding that Src kinase deficient macrophages secrete lower amount of chemokines in response to LPS is in agreement with both early and more recent reports implicating Src-family kinase in LPS-initiated signal transduction in mononuclear phagocytes (33, 62–67); however, it contrasts with the evidence that $hck^{-/-}fgr^{-/-}lyn^{-/-}$ macrophages have no major defect in LPS/IFNγ-induced cytokine secretion (27). However, interpretation of results with triple mutant $hck^{-/-}fgr^{-/-}lyn^{-/-}$ macrophages is complicated by the fact that Lyn kinase has negative regulatory function in TLR signaling (68), so triple mutant mice loose both activating and inhibitory signaling at the same time. This nuance was not appreciated when the first studies of triple mutant cells were reported. Notably, we did not find any difference in LPS, PAM3,

and R848-induced secretion by wild type or *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs of a number of cytokines including IL-1α, IL-1β, IL-6, IL-10 and GM-CSF, the only exception being, in analogy with mutant PMNs, TNFα, whose secretion by *hck*$^{-/-}$*fgr*$^{-/-}$ BMDMs was significantly reduced (supplementary Fig. 1). The Src kinase dependence of chemokine secretion by BMDMs was confirmed by studies with dasatinib (supplementary Fig. 2) that markedly inhibited chemokine secretion in response to different doses of LPS. Hence, macrophages also release a defined spectrum of chemokines in a manner dependent on Src kinase expression or activity.

In conclusion, we report that Src kinases regulate myeloid cell recruitment into the lung likely through their capability to regulate chemokine secretion. Coupled with previous findings establishing the notion that PMN recruitment requires chemoattractant release by early recruited PMNs (23, 24) and that both Src kinases (23) and Syk (44) regulate PMN accumulation into the joints and the skin, our report provides an important piece of information to highlight the Src/Syk signaling pathway as a target to control inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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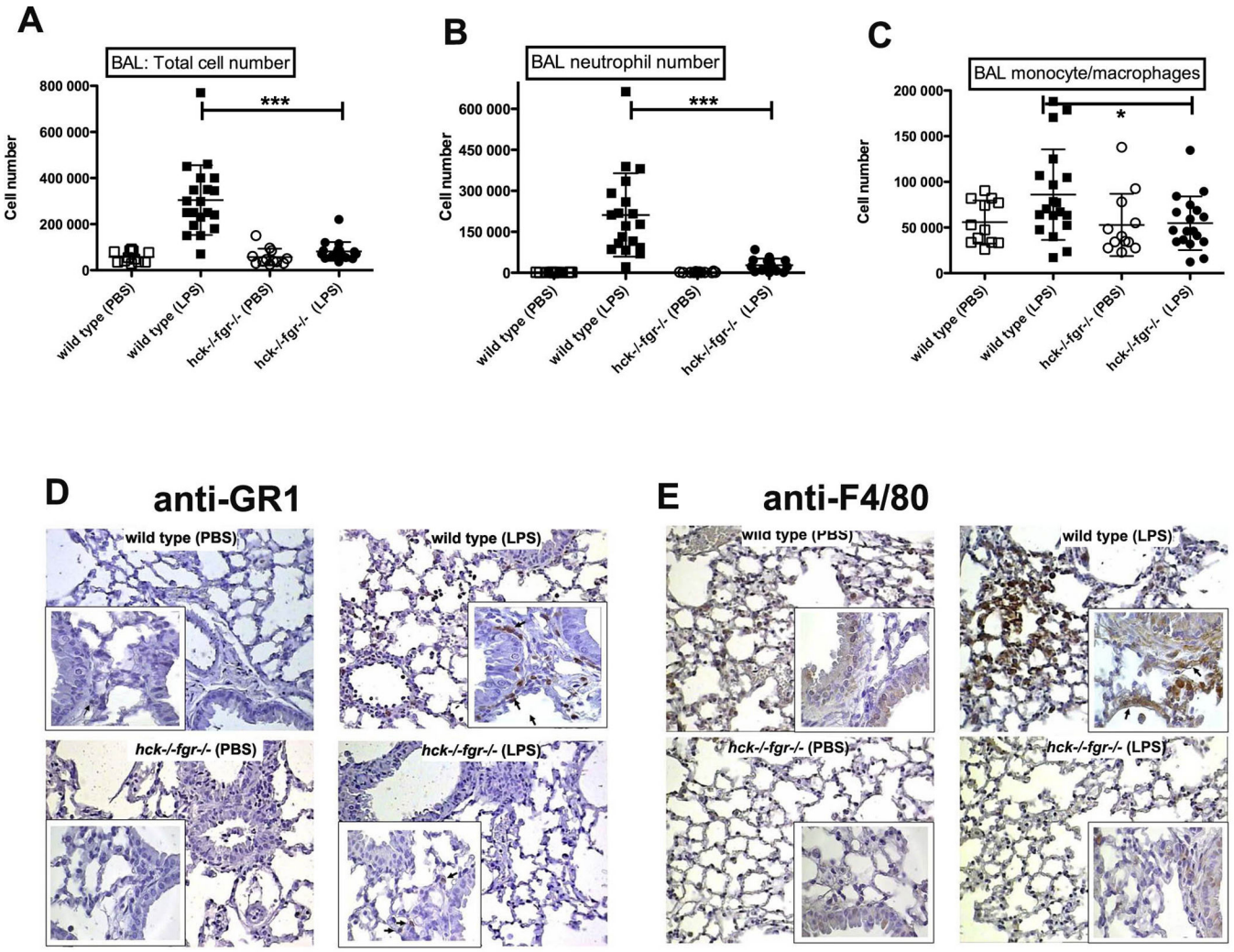

Figure 1.

PMN recruitment into the airways in response to LPS is markedly defective in *hck*$^{-/-}$*fgr*$^{-/-}$ mice. After 4 h following intranasal instillation of 5 μg LPS or vehicle (PBS), BALF cells were enumerated as described in Materials and Methods. Total cells (A), PMNs (B) and Mo (C) numbers, as determined by cytospin staining and counting, in the BALF of wild type (PBS, n = 12; LPS; n = 20) or *hck*$^{-/-}$*fgr*$^{-/-}$ mice (PBS, n = 12; LPS; n = 18) are shown. *, $p < 0.05$; ***, $p<0.001$. (**D**) After 4 h following intranasal instillation of LPS, lungs were removed, formalin-fixed and paraffin-embedded. Sections of 3–4 μm of wild type or *hck*$^{-/-}$*fgr*$^{-/-}$ lung tissue were stained with the anti-granulocyte Ab Gr-1, followed by HRP-labelled secondary Ab. Insets show higher magnification of peri-bronchial area. A few, dark brown PMNs are indicated by arrows. Note the very low number of PMNs in the lung of *hck*$^{-/-}$*fgr*$^{-/-}$ compared to wild type mice. (**E**) Lung sections prepared as above were stained with the anti-macrophage Ab F4/80, followed by HRP-labelled secondary Ab. Insets show higher magnification of peri-bronchial area. Note the very low number of F4/80 positive mononuclear cells in the lung of *hck*$^{-/-}$*fgr*$^{-/-}$ compared to wild type mice. A few, dark brown peri-bronchial monocytes are indicated by arrows.

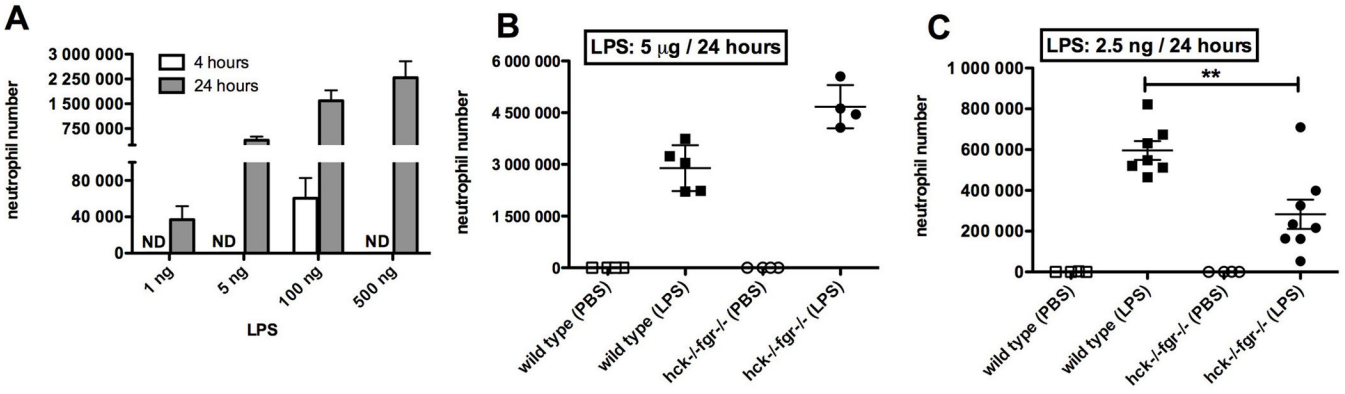

Figure 2.

Regulation of LPS-induced PMN recruitment into the lung by Hck and Fgr. (**A**) After 4 or 24 h following intranasal instillation of different doses of LPS or vehicle (PBS), BALF PMNs were enumerated as described in Materials and Methods. ND = not determined. Mean results ± SD of three independent experiments are shown. (**B**) PMNs were enumerated in the BALF after 24 h from the intranasal instillation of 5 μg LPS. (**C**) Mice were given 2.5 ng LPS intra-nasally and after 24 h BALF cells were enumerated as described in Materials and Methods. **, $p<0.01$.

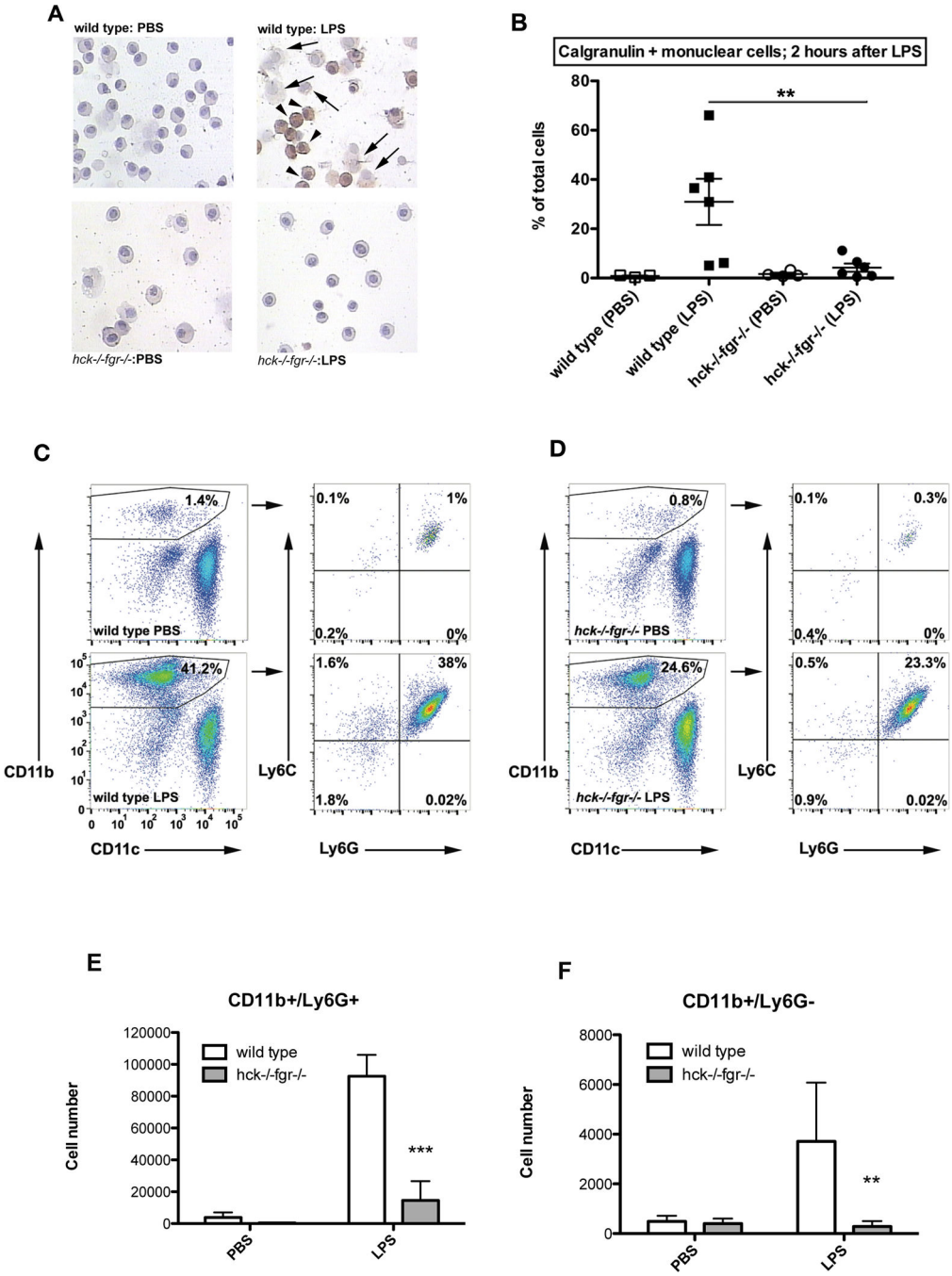

Figure 3.

Hck and Fgr regulate LPS-induced monocyte recruitment into the lung. Wild type or *hck*$^{-/-}$*fgr*$^{-/-}$ mice were given 5 μg LPS or vehicle (PBS) intra-nasally. After 2 h, mice were sacrificed and BALF prepared as described in Materials and Methods. (**A**) Cytocentrifuge preparations were stained for calgranulin and counter-stained with hematoxylin as described in Materials and Methods. Arrows point to calgranulin-negative cells displaying the classical morphology of resident AM. Arrowheads point to smaller, strongly calgranulin-positive cells with a higher nuclear/cytoplasmic ratio than resident AM and that are visible only in

LPS-treated wild type mice airways. (**B**) Percent of calgranulin-positive cells in the airways of wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice 2 h after LPS challenge was quantified as described in Materials and Methods. **, p<0.01. (**C** and **D**) Wild type or *hck*$^{-/-}$*fgr*$^{-/-}$ mice were given 5 μg LPS or vehicle (PBS) intra-nasally. After 4 h, mice were sacrificed and BALF prepared as described in Materials and Methods. BALF cells were pelleted, washed twice with PBS, 2% FBS, 2 mM EDTA and immunostained for CD11b, CD11c, Ly6G and Ly6C. After exclusion of doublets and debris, a sequential gating strategy was used to identify populations expressing specific markers: neutrophils (CD11b^{+}, Ly6G^{+}, Ly6C^{+}), and monocytes (CD11b^{+}, Ly6G^{-}, Ly6C$^{lo/-}$). **E–F**) number of CD11b+/Ly6G+ (granulocytes) and CD11b+/Ly6G− (monocytes) cells in the BALF of vehicle (PBS) or LPS-treated wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ mice. Mean results ± SD of three independent experiments are shown.

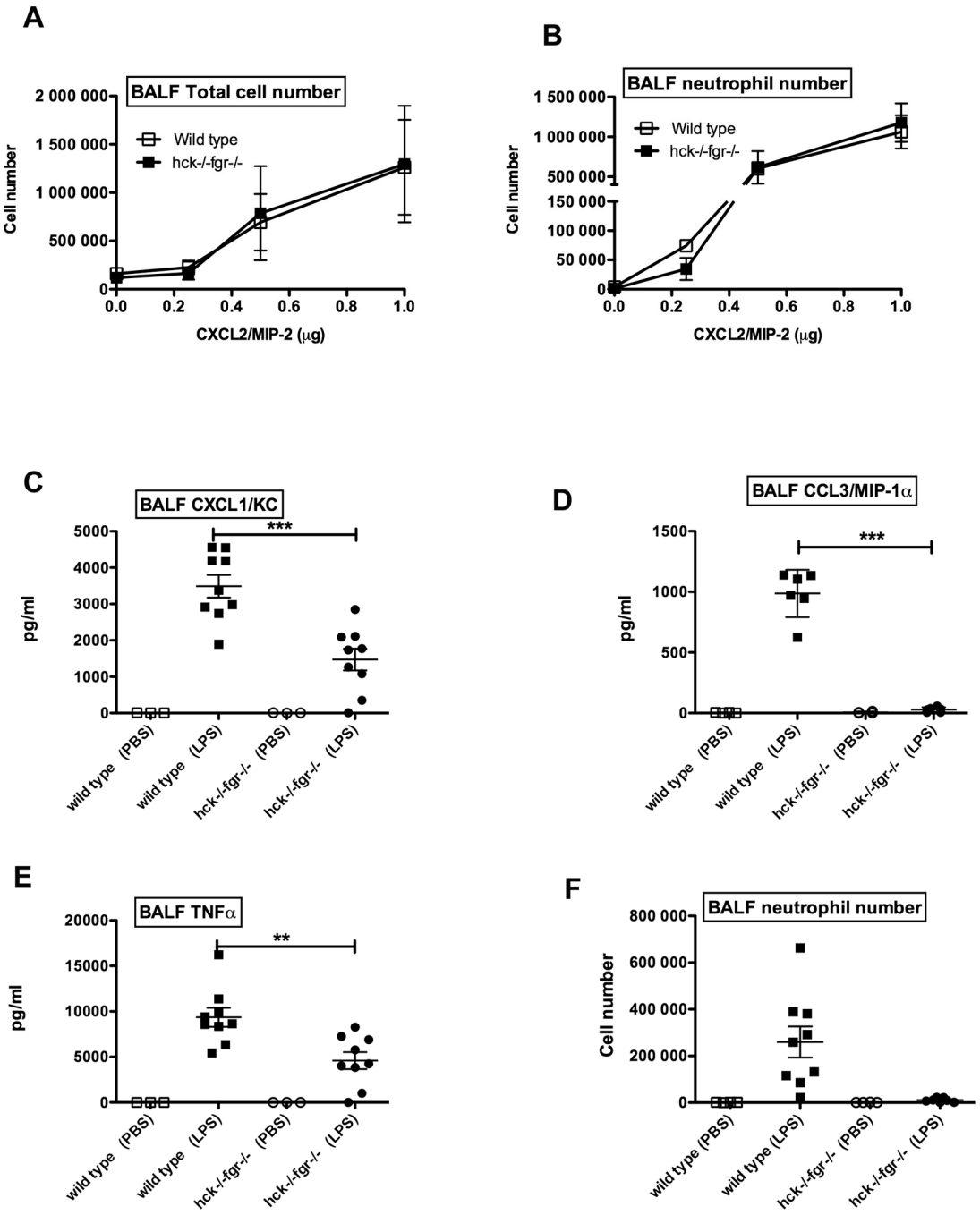

Figure 4.

Hck and Fgr deficiency does not result in any alteration of CXCL2-induced PMN recruitment into the lung. (**A and B**) Wild type (WT) or $hck^{-/-}fgr^{-/-}$ mice were given the indicated doses of CXCL1/MIP-2 or vehicle (PBS) intra-nasally (n = 4 for each condition with both mouse strain). After 4 h mice were sacrificed and cells accumulation into the airways quantified as described in Materials and Methods. (**C–F**) Wild type or $hck^{-/-}fgr^{-/-}$ mice were given LPS or vehicle (PBS) intra-nasally. After 4 h, mice were sacrificed and BALF prepared as described in Materials and Methods for multiplex bead array analysis of

CXCL1 (C), CCL3 (D) and TNFα (E) in cell-free supernatants. The number of neutrophils (F) in the BALF used for detection of CXCL1, CCL3 and TNFα is shown for comparison. **, $p < 0.01$; ***, $p<0.001$.

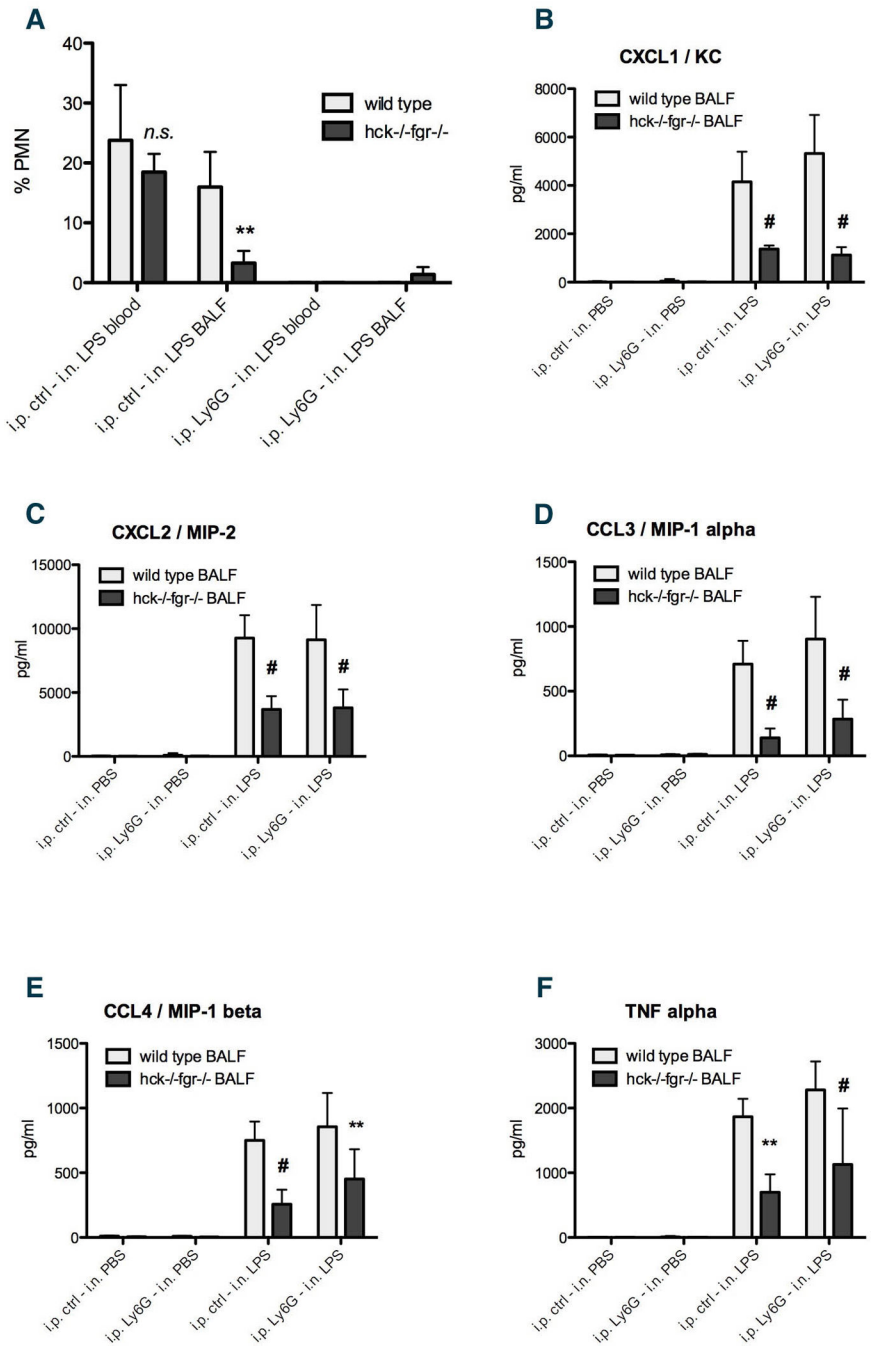

Figure 5.

Chemokine and TNF accumulation into the airways in response to LPS is PMN-independent, but defective in *hck*$^{-/-}$*fgr*$^{-/-}$ mice. Mice were injected i.p. with vehicle (0.9 % NaCl) or an anti-Ly6G Ab as described in Materials and Methods. After 17 hours from the injection mice were intranasally injected with vehicle (PBS) or 5 μg LPS. After 2 h, a sample of blood was withdrawn for cytofluorimetric analysis of C11b+/Ly6G+ cells and BALF cells were enumerated as described in Materials and Methods. (**A**) Per cent PMNs in the blood and the lung of control and PMN-depleted mice. Absolute numbers of PMNs in

the BALF of LPS challenged mice were: wild type mice injected i.p. with vehicle, 8.133 ± 5.693/mouse (N = 4); wild type mice injected i.p. with anti-Ly6G Abs, 350 ± 300; *hck*$^{-/-}$*fgr*$^{-/-}$ mice injected i.p. with vehicle, 1.235 ± 460; *hck*$^{-/-}$*fgr*$^{-/-}$ mice injected i.p. with anti-Ly6G Abs 482 ± 480. PMNs were undetectable in the lung of mice intranasally injected with PBS (not shown). (**B–F**) CXCL1 (**B**), CXCL2 (**C**) CCL3 (**D**), CCL4 (**E**) and TNFα (**F**) were assayed in cell-free supernatants by multiplex bead array analysis as described in Materials and Methods. **, $p < 0.01$; #, $p<0.001$, ns, not significant.

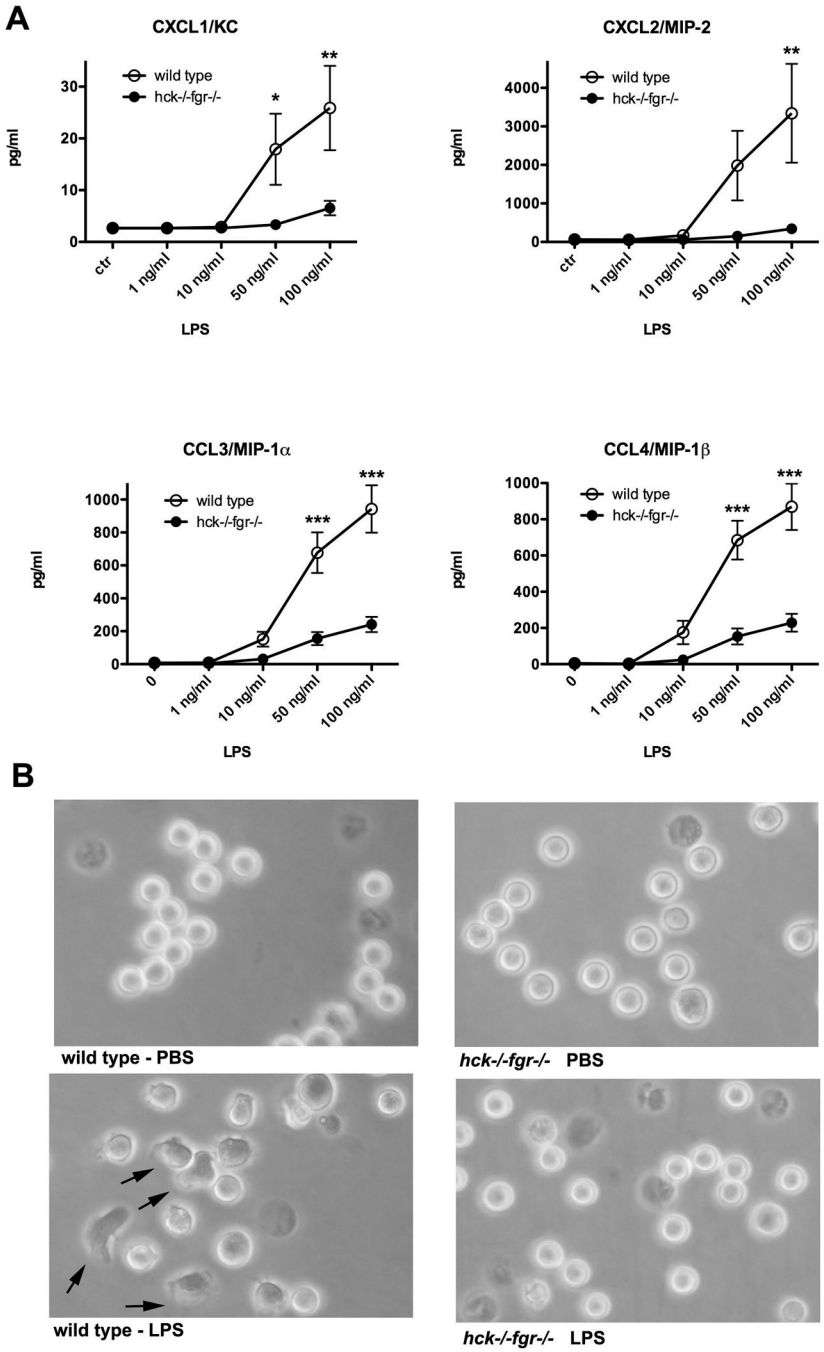

Fig. 6.

Hck$^{-/-}$*fgr*$^{-/-}$ PMNs are defective in the ability to secrete neutrophil-attractive chemokines in response to LPS. (**A**) Bone marrow PMNs were maintained in RPMI medium supplemented with 10% FBS in the absence or the presence of the indicated doses of LPS. After 4 h the medium was collected and chemokines released in the supernatant assayed by multiplex bead array as described in Materials and Methods. Mean results of three experiments in each of which cells were pooled from three different mice are reported. *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0{,}001$. If not highlighted by *, differences between wild type

and *hck*$^{-/-}$*fgr*$^{-/-}$ PMNs were not statistically significant. (**B**) bone marrow PMNs treated as in (A) were photographed with a 40x phase contrast objective. Arrows point to elongated, polarized PMNs.

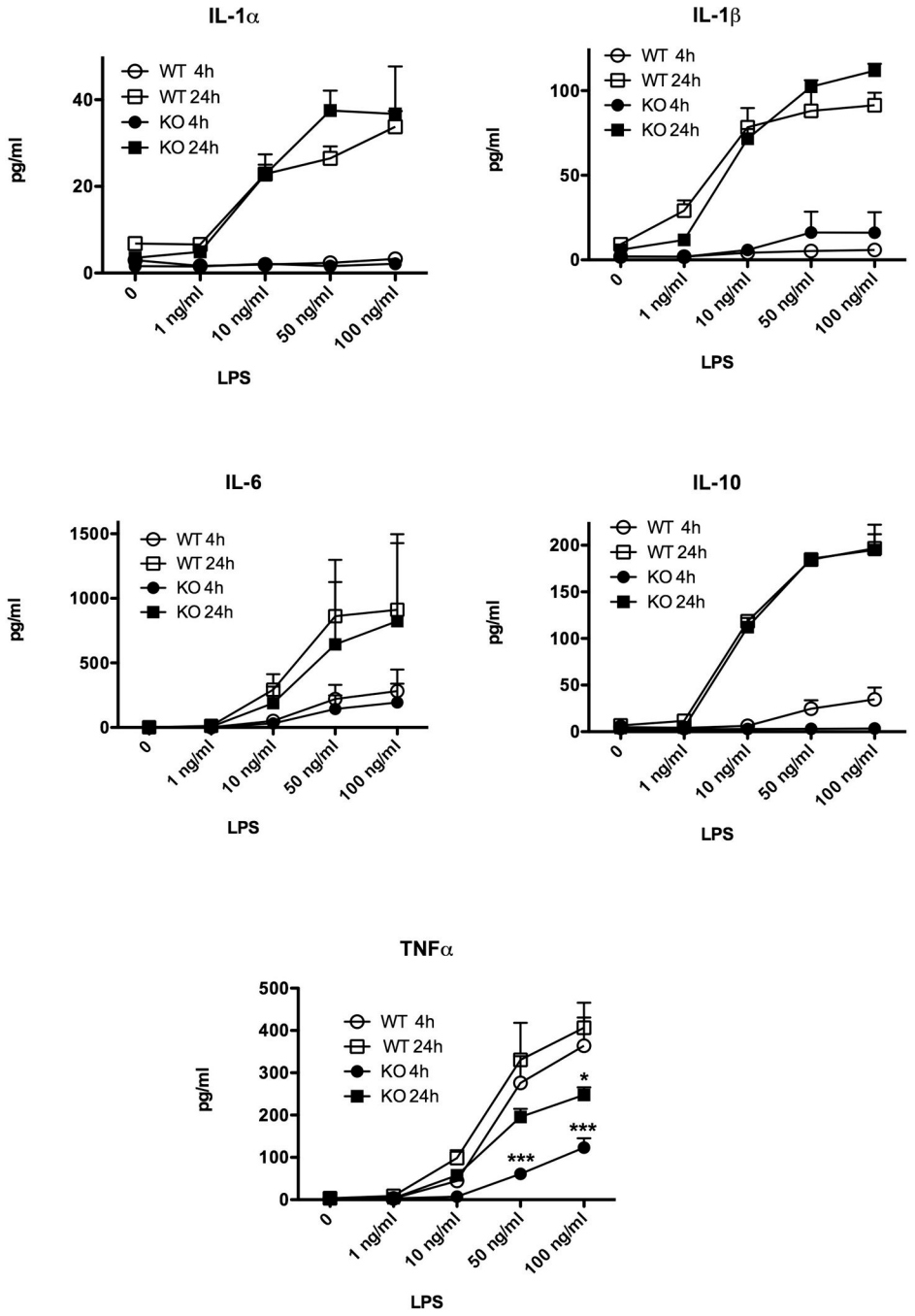

Figure 7.

Hck$^{-/-}$*fgr*$^{-/-}$ PMNs are not defective in the ability to secrete IL-1α, IL-1β, IL-6 and IL-10 in response to LPS. Bone marrow PMNs were maintained in RPMI medium supplemented with 10% FBS in the absence or the presence of the indicated doses of LPS. After 4 or 24 h, the medium was collected and cytokines released in the supernatant assayed by multiplex bead array as described in Materials and Methods. Mean results of three experiments in each of which cells were pooled from two-three different mice are reported. *, $p < 0.05$; ***, $p <$

0,001. If not highlighted by *, differences between wild type and *hck*$^{-/-}$*fgr*$^{-/-}$ PMN were not statistically significant.

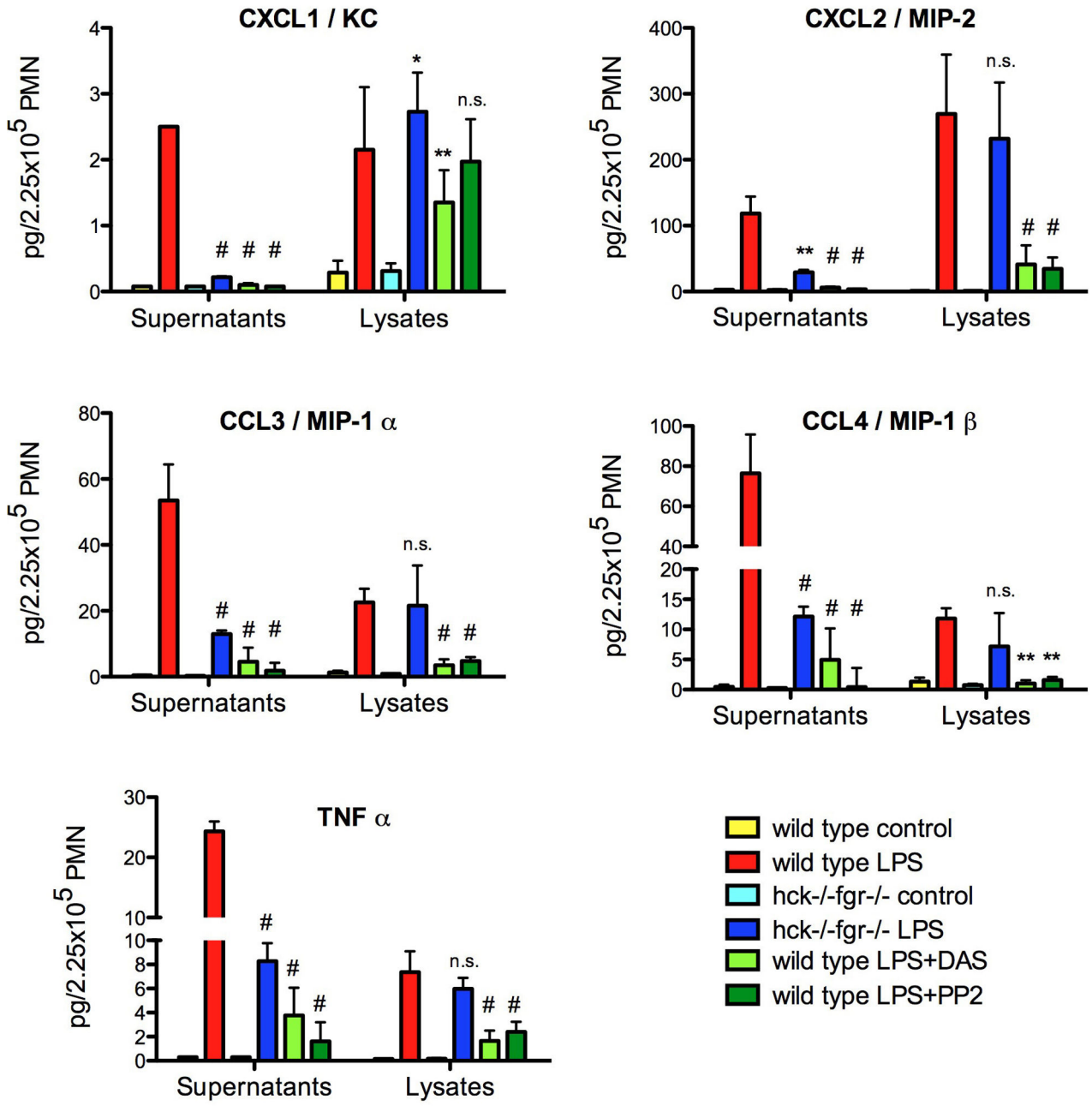

Figure 8.

Hck$^{-/-}$*fgr*$^{-/-}$ PMNs are defective in the ability to secrete, but not to synthetize and store intracellularly, neutrophil-attractive chemokines in response to LPS. Bone marrow PMNs were maintained in 48 well plates in RPMI medium supplemented with 10% FBS in the absence or the presence of the 100 ng/ml LPS without or with 1 μM dasatinib or 10 μM PP2. After 4 h, PMNs were resuspended by pipetting and cells were pelleted in a microfuge. After aspiration of the supernatant, pelleted cells were lysed in an equal volume of lysis buffer (see Materials and Methods). Chemokines and TNFα released in the supernatant or present

in the cell lysate were assayed by multiplex bead array. Mean results of three experiments in each of which cells were pooled from three different mice are reported. *, $p < 0.05$; **, $p < 0,01$; #, $p < 0.001$; n.s, not significant.

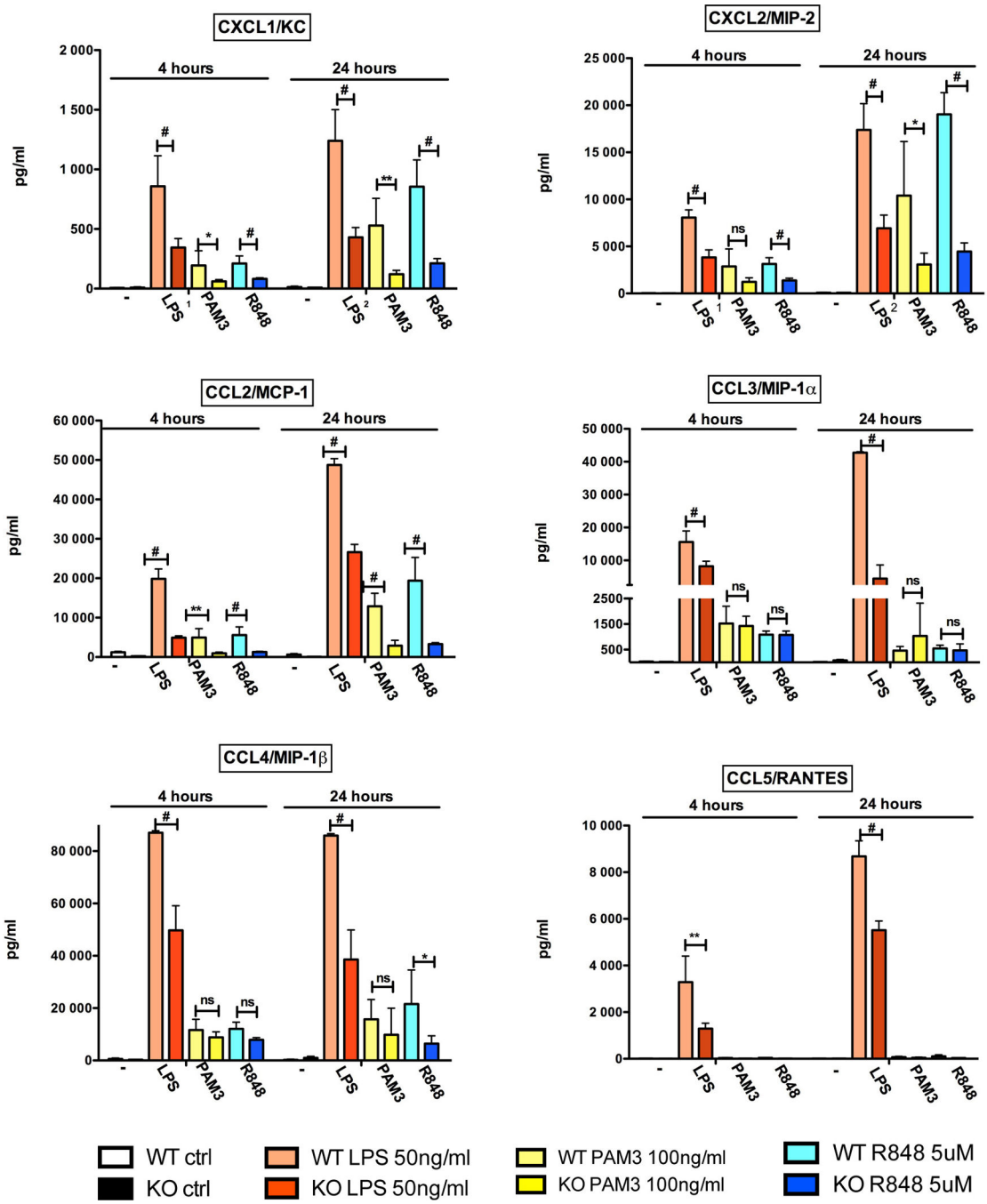

Figure 9.

Hck$^{-/-}$*fgr*$^{-/-}$ BMDMs are defective in the ability to secrete chemokines in response to LPS and other TLR ligands. Bone marrow-derived macrophages (BMDM) were isolated and maintained in RPMI medium supplemented with 10% FBS in the absence or the presence of LPS (25 ng/ml), PAM3 (200 ng/ml) or R848 (10 μM). After 4 or 24 h, the medium was collected and chemokines released in the supernatant assayed by multiplex bead array as described in Materials and Methods. Mean results of three experiments in each of which

cells were pooled from two-three different mice are reported. *, p< 0.05; **, p < 0.01; #, p < 0,001; ns, not significant.