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IL-1β regulates CXCL8 release and influences disease outcome in response to *Streptococcus pneumoniae*, defining intracellular cooperation between pulmonary epithelial cells and macrophages

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Abstract

The success of *Streptococcus pneumoniae* (the pneumococcus) as a pulmonary pathogen is related to its restriction of innate immune responses by respiratory epithelial cells. The mechanisms used to overcome this restriction are incompletely elucidated. Pulmonary chemokine expression involves complex cellular and molecular networks, involving the pulmonary epithelium, but the specific cellular interactions and the cytokines that control them are incompletely defined. We show that serotype 2 or 4 pneumococci induce only modest levels of CXCL8 expression from epithelial cell lines, even in the absence of polysaccharide capsule. In contrast, co-culture of A549 cells with the macrophage-like THP-1 cell-line, differentiated with Vitamin D, or monocyte-derived macrophages, enhanced CXCL8 release. Supernatants from the THP-1 cell-line prime A549 cells to release CXCL8 at levels similar to co-cultures. IL-1Ra inhibits CXCL8 release from co-cultures and reduces the activity of macrophage-conditioned media, but inhibition of TNFα had only a minimal effect on CXCL8 release. Release of IL-1β but not TNFα was upregulated in co-cultures. IL-1 type 1 receptor knockout C57BL/6 and BALB/c mice confirmed the importance of IL-1 signaling in CXC chemokine expression and neutrophil recruitment *in vivo*. In fulminant disease increased IL-1 signaling resulted in increased neutrophils in the airway and more invasive disease. These results demonstrate that IL-1 is an important component of the cellular network involving macrophages and epithelial cells, which facilitates CXC chemokine expression and aids neutrophil recruitment during pneumococcal pneumonia. They also highlight a potential clinical role for anti-IL-1 treatment to limit excessive neutrophilic inflammation in the lung.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a major cause of respiratory tract infection and invasive bacterial disease (6). *S. pneumoniae* is also a common commensal of the upper respiratory tract but innate host defences prevent disease in most colonized individuals (6, 40). The success of the innate response against pneumococcus is emphasized by the relatively low incidence of community-acquired pneumonia or invasive disease in comparison to the frequency of upper respiratory tract colonization (16, 39).

The respiratory tract epithelium plays a critical role in the recognition of bacterial pathogens and in the induction of the inflammatory response (20). Epithelial cells express a range of Toll-like receptors (TLR) and other pattern recognition receptors (13). The clinical importance of these pathways of innate recognition of *S. pneumoniae* has been highlighted by the identification of genetic mutations or polymorphisms in these signaling pathways, which confer altered susceptibility to invasive pneumococcal disease (29, 31). Engagement of a range of pattern recognition receptors including TLRs, nucleotide-binding oligomerization domain (NOD) proteins and NOD-like receptor (NLR) family, pyrin domain containing 3 (NALP3) by pneumococci activates NF-κB and induces CXCL8 (IL-8) which leads to recruitment of polymorphonuclear leukocytes (neutrophils) (8, 25, 41, 49, 65). This is a key feature of pneumococcal infection when resident pulmonary defences are overwhelmed (15).

Epithelial cell responses are enhanced by cooperative signaling with other cell types. Bronchoalveolar lavage fluid from lungs infected with pneumococci stimulates epithelial cell NF-κB activation (44), and alveolar macrophages enhance CXCL8 production by epithelial cells

68 in lung explants (66). It is postulated that, in order to avoid excessive inflammatory responses to
69 commensal organisms, airway epithelial cells express constitutively low levels of TLRs, but
70 studies of TLR2 expression indicate that it may be upregulated when inflammation is present
71 (35, 64). Previous studies have demonstrated monocytes enhance epithelial cytokine responses to
72 various TLR agonists (9, 37). Increases in proinflammatory cytokine and chemokine release in
73 co-cultures of pulmonary epithelial cells and monocytes have been noted in response to a range
74 of lipopolysaccharides (9, 42, 52), staphylococcal exotoxins (30), *Mycobacterium tuberculosis*
75 (63), and respiratory syncytial virus (RSV) (58). Thus, cell-cell communication between
76 monocytes and epithelial cells is an important early step in the immune response to respiratory
77 tract infections.
78
79 *S. pneumoniae* possess a number of virulence factors which may confound front-line immune
80 responses (28), including a polysaccharide capsule which limits bacterial phagocytosis by
81 macrophages (2) and inhibits bacterial attachment to respiratory epithelial cells (1, 22, 51). In
82 this study we have characterized the cellular and molecular factors involved in the regulation of
83 CXCL8 expression by respiratory tract epithelial cells in response to *S. pneumoniae* infection.
84 We demonstrate that IL-1β secretion by a macrophage-like cell-line is required for maximal
85 secretion of CXCL8 by epithelial cells in response to *S. pneumoniae in vitro.* We also establish
86 important roles for IL-1β in regulating the expression of CXC chemokines and rate IL-1
87 signaling to the pulmonary infiltration of polymorphonuclear cells *in vivo* in murine models of *S.*
88 *pneumoniae* infection.
89
90
91

Materials and Methods

Materials

Gentamicin was purchased from Roussel laboratories (Uxbridge, UK); Vitamin D_3 (1,25 dihydroxy-cholecalciferol) from Sigma-Aldrich (Poole, UK); and recombinant human IL-1β, recombinant human soluble TNF Receptor Type 1, and recombinant human IL-1 receptor antagonist (IL-1Ra) from PeproTech EC Ltd (London, UK).

Bacterial strains and growth conditions

Streptococcus pneumoniae strains used in this study were serotype 2 strain D39 and its isogenic unencapsulated derivative FP22 (43) and serotype 4 strain TIGR4 and its unencapsulated derivative FP23 (2). D39 and TIGR4 were obtained from the American Type Culture Collection (ATCC) Bacteriology Collection, FP22 from Prof. Tim Mitchell (University of Glasgow), and FP23 from Prof. Gianni Pozzi and Dr Franco Ianelli (University of Sienna). Non typeable conjunctival clinical strains 08-1773 (MLST type 448) and 02-3522 (pre-MLST) were from Prof. Tim Mitchell and the Scottish Haemophilus, Legionella, Meningococcus and Pneumococcus Reference Laboratory (SHLMPRL). Bacteria were grown to exponential phase in brain-heart infusion broth (Oxoid Unipath, Basingstoke, UK) with 20% heat-inactivated fetal calf serum (Autogen Bioclear, Wiltshire, UK) and aliquots were stored at -80°C. The concentration (CFU/ml) was determined by Miles Misra count for each strain as previously described (14). Freshly thawed aliquots were spun at 9,000 *g* for 2 min and the bacterial pellet washed twice in PBS prior to use.

Human cell culture

All cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC) and all culture media were from BioWhittaker (Lonza, Belgium). A549 cells, a human lung carcinoma cell line, were maintained in DMEM with 4.5g/l glucose, 2mmol/L L-glutamine, and 10% fetal calf serum (FCS). Detroit 562 cells, a human pharyngeal carcinoma cell line, were maintained in EMEM with 2mmol/L L-glutamine and 10% FCS. Epithelial cells were seeded at $2x10^5$ cells/ml in 24-well plates (Costar) and grown to confluence for 24 h prior to use. THP-1 cells, a monocytic leukaemic cell line, were maintained in RPMI 1640 containing 2mmol/L L-glutamine and 10% FCS. Differentiation to a macrophage phenotype was achieved by resuspending cells at $2x10^4$ cells/ml in fresh media with the addition of Vitamin D3 (VD_3) at 10^{-7} M and incubation for 72 h in 5% CO2 at 37°C (26, 50). This differentiation protocol produces a non-adherent immature macrophage phenotype more similar to an inflammatory macrophage than a highly differentiated tissue macrophage in terms of its response to microbial factors (12). Peripheral mononuclear cells were isolated from human blood as described previously (14), from healthy donors after informed consent and approval by the South Sheffield Research Ethics Committee. The following day after isolation non adherent cells were removed by washing in RPMI and adherent monocyte-derived macrophages (MDM) collected by gentle scraping. Hemocytometer counts of all cell cultures were performed on the day of experimentation to determine approximate cell numbers per well. In co-cultures, non-adherent VD_3-differentiated THP-1 or one day MDM were added to adherent A549 epithelial cells at a ratio of 1:10 (using data for cell numbers derived from the hemocytometer counts of representative wells of A549 cells) and cells were cultured in RPMI with 10% FCS (with low endotoxin levels).

138

139 *Infection of cell cultures*

140 Growth medium was removed from epithelial cell monolayers and cells washed twice with PBS.
141 Fresh growth medium (1ml per well) was applied and cells were infected with bacterial strains at
142 an MOI of 10 for epithelial cells and 100 for VD_3-differentiated THP-1 cells or MDM (to ensure
143 the same number of bacteria were added to each well). Pneumococci were opsonized in RPMI
144 1640 supplemented with 10% anti-pneumococcal immune serum and incubated on a rotating
145 device for 30 min at 37 °C prior to infections for all experiments involving THP-1 cells and in
146 experiments investigating the effect of opsonization on epithelial cell responses (2). Infected cell
147 cultures were centrifuged at 150 *g* for 5 minutes prior to incubation at 37°C in 5% CO_2. Culture
148 medium (1ml) was removed from wells at each time point, spun at 10,000 *g* for 10 minutes and
149 stored at -80°C prior to cytokine assay. Epithelial cells showed significant loss of viability
150 following overnight incubation with *S. pneumoniae* and therefore gentamicin (100 μg/ml) was
151 added to all wells at the 2 h time point to prevent bacterial overgrowth and loss of epithelial cell
152 viability. As a positive control for CXCL8 production some wells were treated with 10ng/ml IL-
153 1β for the indicated time periods.

154

155 *Bacterial adherence assay*

156 Infected epithelial cell monolayers (MOI of 10) were centrifuged at 150 *g* for 5 min prior to
157 incubation for 2 h at 37°C in 5% CO_2. In certain experiments, specific variables were altered to
158 determine their effect on adherence. These included increasing the MOI to 50; centrifuging at
159 1,200 *g*; using opsonized pneumococci; and incubating for different durations. At 2 h (or at 1 h
160 or 4 h in experiments with altered incubation periods) the medium (containing non-adherent

bacteria) was removed from each well and 'non-adherent' bacterial counts determined by Miles Misra (36). Epithelial cells (including any adherent bacteria) were then removed from each well with trypsin and versine and 'adherent' bacterial counts determined by Miles Misra. The proportion of adherent bacteria could then be calculated (62).

Investigation of the nature of proinflammatory mediators generated in co-cultures

To generate macrophage-conditioned media VD_3-differentiated THP-1 cells ($2x10^4$/well) were exposed to opsonized D39 *S. pneumoniae* (MOI 100:1) or 'mock infected' (MCM-D or MCM-MI) for 24 hours (gentamicin 100 μg/ml was added at 2 h to all wells). Macrophage conditioned media (MCM-D, MCM-MI) were removed from all wells at 24 h, spun at 10,000 *g* for 10 min and the supernatants (1 ml) then applied to epithelial cell monolayers. In blocking experiments, IL-1Ra (200 ng/ml) and/or soluble TNF receptor type 1 (sTNFR1; 50 ng/ml) were added to conditioned media after transfer onto epithelial cell monolayers at time zero. Plates were then incubated in 5% CO_2 at 37°C and media removed from wells at 6 h, spun at 10,000 *g* for 10 minutes, then stored at -80°C prior to cytokine assay. Samples of conditioned media were saved at time zero (following centrifugation) in order to determine baseline levels of CXCL8 prior to transfer onto epithelial monolayers. These baseline values were subtracted from the total CXCL8 concentration at 6 h to calculate the amount produced by stimulated epithelial cells alone.

Blockade of candidate proinflammatory molecules

Co-culture wells were prepared as described above with A549 epithelial cells and THP-1 at a ratio of 10:1 and blocking agents (IL-1Ra, 200 ng/ml) (58, 63) and/or sTNFR1 (50 ng/ml) (38) were added at time zero. Wells were then infected with opsonized pneumococci (MOI of 10),

184 spun at 150 *g* for 5 min, then incubated at 37°C in 5% CO_2. Medium was removed at 6 h, spun at
185 10,000 *g* for 10 min and stored at -80°C. Gentamicin (100 μg/ml) was added to all wells at 2 h.
186
187 *Role of soluble mediators*
188 To physically separate THP-1 and A549 cells, THP-1 were added to cell culture inserts, pore size
189 1 μm (BD Falcon), with A549 cells in the well below, at ratio of 10:1 of A549:THP-1 cells.
190 Opsonized D39 pneumococci were added at equal doses to both the insert and lower well
191 compartments to achieve an overall MOI of 10:1 pneumococci: A549 cells. Plates were then
192 spun at 150 *g* for 5 minutes, incubated at 37°C in 5% CO_2, then media removed at 6 h, spun at
193 10,000 *g* for 10 minutes and stored at -80°C. Gentamicin (100 μg/ml) was added to all wells at 2
194 h.
195
196 *Murine pneumonia models*
197 IL-1 type 1 receptor knockout mice (18), allele *Il1r1*^{Tm1Imx}, were kindly provided by Immunex
198 Corporation to MJHN and were backcrossed for at least ten generations against both
199 BALB/cOlaHsd and C57BL/6NHsd separately. Heterozygotes were bred to yield homozygotes
200 and working colonies of homozygotes were maintained. BALB/cOlaHsd or C57BL/6NHsd
201 (Harlan) were used as wild-type controls. Adult mice of both sexes were used. All animal
202 experiments were conducted in accordance with the Home Office Animals (Scientific
203 Procedures) Act of 1986 and local ethical approval.
204 Pulmonary infection of mice was with $5x10^5$ or $1x10^7$ CFU serotype 4 *S. pneumoniae* prepared
205 and delivered by direct tracheal instillation as described previously (15). 24hrs post infection
206 mice were killed by overdose of sodium pentobarbitone and bronchial alveolar fluid, lung and

blood collected for determination of cell differential, cytokines and viable bacteria in lung and blood as described previously (15).

Cytokine assays

CXCL8, IL-1β, and TNFα levels in the culture supernatants were measured using human CXCL8, IL-1β, and TNFα DuoSet ELISA kits respectively (R&D Systems, Abingdon, UK) according to manufacturer's guidelines. Murine CXCL1, CXCL2 and TNFα were measured in BAL using murine CXCL1 and CXCL2 DuoSet ELISA kits (R&D Systems, Abingdon, UK) and murine TNFα ELISA Ready-SET-Go! reagent set (eBioscience, Hatfield, UK) respectively according to manufacturers' guidelines.

Optical densities were determined using Opsys MR microplate reader (Dynex Technologies). The lower limit of detection was approximately 30 pg/ml for CXCL8; 20 pg/ml for human IL-1β; 30 pg/ml for human TNFα; 16 pg/ml for murine CXCL1; 32 pg/ml for murine CXCL and 16 pg/ml for murine TNFα.

Statistics

All results are recorded as means +/- SEM unless otherwise stated. Statistical testing was performed using Prism 5.02 software (GraphPad software Inc.). Unpaired t test, Mann-Whitney or one-way ANOVA (with Bonferroni's post-test) were used to analyse differences between groups, as appropriate. Significance was defined as $p < 0.05$.

Results

Epithelial cell responses to S. pneumoniae are limited by polysaccharide capsule.

Epithelial cells contribute to innate host defense but *S. pneumoniae* contains several adaptations, which can modulate its interaction with epithelial cells (13, 19, 32, 59, 62), in particular the expression of polysaccharide capsule (1, 21, 22, 51, 57). The mechanisms used to integrate the epithelial cell response into the innate host response, despite microbiological restriction, are uncertain. We confirmed that there was minimal adherence of encapsulated serotype 2 or 4 *S. pneumoniae* to epithelial cell lines (see supplemental data at http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig S1A-D). Binding was not significantly enhanced by opsonization of bacteria; by increasing the MOI to 50:1; by increasing the incubation period; or by centrifuging the bacteria onto the cells at 1200 *g* rather than 150 *g* (data not shown). Mutants lacking capsule, either on a serotype 2 or 4 background (2, 43), demonstrated significantly greater binding than the parental capsulated strains (see supplemental data at http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig S1A-D). CXCL8 is critical for the effective recruitment and activation of polymorphonuclear cells at sites of inflammation (3, 54). Microbial factors stimulate epithelial cells via a range of receptors to produce CXCL8 (5, 35, 49). We investigated the ability of non-opsonized pneumococci to stimulate CXCL8 release from epithelial cell monolayers. There was no significant difference between the CXCL8 response of mock-infected epithelial cells and those exposed to encapsulated pneumococci, which was low in comparison with cells stimulated with IL-1β (11) (Figure 1A-B). We demonstrated significantly greater CXCL8 production in response to unencapsulated pneumococci, however when we compared the encapsulated

serotype 2 and 4 strains to unencapsulated clinical strains we did not note any significant difference in CXCL8 release (see supplemental data at http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig S2), although it was not clear whether these results reflected the marked genetic differences between these unencapsulated strains and other pneumooccal serotypes. Opsonization modestly enhanced the epithelial cell CXCL8 response to encapsulated pneumococci (see supplemental data at http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig S3). We therefore opsonized pneumococci in all subsequent experiments. We also observed significantly greater CXCL8 responses to the unencapsulated serotype 4 strain, FP23, compared with other strains, possibly in keeping with enhanced pro-inflammatory cytokine responses to piliated pneumococci (4).

Co-culture of epithelial cells with a macrophage-like cell-line enhances CXCL8 secretion following exposure to S. pneumoniae.

Cytokine and chemokine responses can be enhanced in epithelial cell cultures by monocyte or macrophage derived factors (9, 30, 37, 42, 52, 53, 58, 63, 66). CXCL8 expression by the THP-1 macrophage cell-line or epithelial cells in response to pneumococci was consistently less than the epithelial cell response to IL-1β (Figure 1C-D). In contrast, we demonstrated a marked enhancement of CXCL8 responses for macrophage-epithelial cell co-cultures. The generation of CXCL8 by co-cultures treated with pneumococci at 6 and 24 h time points was significantly greater than the sum of the CXCL8 responses of A549 epithelial cells and THP-1 cells alone. The responses were apparent by 6 h after infection and were sustained up to 24 h after infection. We therefore used the 6 h time point in subsequent experiments. Similar results were

obtained with the Detroit epithelial cell line, which also showed enhanced CXCL8 expression in co-culture conditions (data not shown). Interestingly, although the macrophage-like cell line showed a significantly greater CXCL8 response to encapsulated compared with unencapsulated bacteria, the presence of polysaccharide capsule had little effect on the magnitude of the co-culture response to pneumococci. We also noted that opsonization corrected the inhibitory effect of capsule on the response of epithelial cell monocultures to pneumococci.

IL-1 production by the THP-1 macrophage-like cell-line stimulates CXCL8 expression from epithelial cells following exposure to S. pneumoniae.

We next addressed whether cell contact between THP-1 cells and epithelial cells was a necessary or contributory factor. When epithelial cells were separated from monocytes using semi-permeable transwells we observed no significant reduction in CXCL8 secretion (Figure 2), suggesting that direct cell contact was not required and that a soluble factor was responsible for priming the epithelial cells. Bronchial alveolar fluid from the lungs of mice infected with *S. pneumoniae* has been shown to stimulate NF-κB activation and resultant chemokine expression by epithelial cells although the cellular source of the factors responsible are incompletely characterized (44). Previous work from our group has indicated in a number of models that IL-1 may play important roles in the induction of inflammatory responses (9, 38). When we transferred supernatants from the mock-infected THP-1 cells onto epithelial monolayers, we observed little release of CXCL8, while supernatants from the *S. pneumoniae*-exposed THP-1 cells induced levels of CXCL8 secretion comparable to infected co-cultures (Figure 3). We also investigated the mediators responsible for activation of A549 cells by bacterial-exposed THP-1 cells, and focused on the two dominant 'early' proinflammatory cytokines, TNFα and IL-1

297 (24, 27, 45, 52). IL-1Ra, but not soluble TNFα Receptor 1, treatment blocked the ability of
298 macrophage conditioned media to stimulate CXCL8 production by the epithelial cell line.
299
300 *IL-1Ra inhibits CXCL8 release from co-cultures challenged S. pneumoniae.*
301 We next addressed whether the contribution of IL-1 and TNFα altered with bacterial strain. IL-
302 1Ra reduced CXCL8 secretion by co-cultures in the presence of both encapsulated and
303 unencapsulated strains and also the piliated type 4 strains (4). TNFα blockade had no effect on
304 CXCL8 secretion in the presence or absence of IL-1Ra at the 6 h time point (Figure 4) and
305 made only a minor contribution to CXCL8 release in co-cultures at 24 h, which only reached
306 statistical significance for the unencapsulated type 4 pneumococci (see supplemental data at
307 http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig S4).
308 Overall these findings suggested that IL-1 was the major factor enhancing CXCL8 release. We
309 also confirmed that if we used primary monocyte-derived macrophages (MDM) one day after
310 differentiation from monocytes they were also able to enhance CXCL8 production from
311 epithelial cells and that IL-1 also contributed to this release (Figure 5).
312
313 *IL-1β release is enhanced in co-cultures.*
314 We next confirmed IL-1β and TNFα release from the THP-1 cell line following challenge with
315 D39 pneumococci, excluding the possibility that the lack of inhibition by TNFα blockade was
316 merely a consequence of lack of production (Figure 6A-B). However, only IL-1β was enhanced
317 in co-cultures. The same pattern of cytokine release was observed in response to type 4
318 pneumococci (data not shown). We also showed that neither IL-1β nor TNFα were induced

following application of macrophage-conditioned media to epithelial cell monolayers, thus confirming the THP-1 cells as the major source of IL-1β and TNFα (Figure 6C-D).

Modulation of IL-1 signaling alters production of CXC chemokines and polymorphonuclear cell recruitment during S. pneumoniae infection in vivo.

To confirm the relevance of these findings we next investigated pneumonia models in mice with differing sensitivity to IL-1 signaling (23). C57BL/6 mice recruit polymorphonuclear cells only when resident host defenses against pneumococci become overwhelmed (17). BALB/c mice develop an early neutrophilic response against pneumococci, which is greater than that seen in C57BL/6 mice. *Il1r1*$^{-/-}$ C57BL/6 mice have normal leukocyte numbers but a reduction in early bacterial clearance in a model of pneumococcal pneumonia (18, 46), while *Il1r1*$^{-/-}$ BALB/c mice have never been studied in a model of pneumococcal pneumonia to our knowledge. We confirmed that polymorphonuclear cell recruitment was greater for BALB/c mice than for C57BL/6 mice following challenge with a similar dose of pneumococci (Figure 7A and D). In each genetic background *Il1r1*$^{-/-}$ mice recruited fewer polymorphonuclear cells.

We then addressed the expression of CXCL1 (KC) and CXCL2 (MIP-2α), which are the murine paralogs of CXCL8 in each mouse strain. CXCL1 was significantly reduced in each *Il1r1*$^{-/-}$ mouse strain (Figure 7B and E). There was a non-significant decrease in CXCL2 for *Il1r1*$^{-/-}$ C57BL/6 and no alteration in *Il1r1*$^{-/-}$ BALB/c mice (Figure 7C and F). Although numbers of mice were small the *Il1r1*$^{-/-}$ C57BL/6 had a trend towards increased bacterial numbers in lung and blood, in keeping with prior observations (46), but there was no

341 difference in bacterial numbers in *Il1r1*$^{-/-}$ BALB/c mice (see supplemental data at
342 http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig S5).
343
344 *Modulation of IL-1 signaling can reduce the severity of invasive disease in fulminant*
345 *pneumococcal infection.*
346 We next increased the severity of infection in the BALB/c mice, which increased the number of
347 polymorphonuclear cells in the BAL. In this model polymorphonuclear cells recruitment was
348 again reduced in the *Il1r1*$^{-/-}$ mice (Figure 8A), although levels of recruitment were much greater
349 than with the lower dose infection model (compare with Figure 7D). Interestingly CXCL1/2
350 expression was not lower in the lungs of *Il1r1*$^{-/-}$ mice with severe disease (Figure 8B-C). This
351 suggested other compensatory changes in the cytokine network might have occurred to enable
352 neutrophil recruitment and we observed that levels of TNFα, a known stimulus for CXCL1
353 chemokine expression during pneumococcal infection (55), was increased in *Il1r1*$^{-/-}$ BALB/c
354 mice (Figure 8D). We found no alteration in colony counts in the lung but a significant
355 decrease in colony counts in the blood of *Il1r1*$^{-/-}$ BALB/c mice (Figure 8E-F).
356

Discussion

In this study, we demonstrate a major role for macrophage-alveolar epithelial cell signaling during the initial phase of host defence against *S. pneumoniae*. We describe a critical role for IL-1 in stimulating CXCL8 release in response to *S. pneumoniae* and show that a macrophage cell-line and primary macrophages are an important source of this cytokine. *In vivo models* have confirmed that IL-1 signaling stimulates CXCL1/2 chemokine expression in mice and facilitates recruitment of polymorphonuclear leukocytes to the lung during infection.

Respiratory epithelial cells are an important component of pulmonary host defense, possessing pattern recognition receptors, such as TLRs, which enable sensing of micro-organisms and link recognition of bacteria to NF-κB activation and transcription of cytokines and chemokines (13, 20, 44). Hyporesponsiveness in this system, as recently demonstrated for an association between IRAK4 polymorphisms and susceptibility to Gram-positive infections in patients with critical illness, limit the effectiveness of the innate immune responses (56). We show that direct exposure of A549 cells to pneumococci induces only low level CXCL8 secretion. Capsule limits the adherence of pneumococci to epithelial cells (21, 51) and this was confirmed again in our study. Nevertheless cytokine expression correlates poorly with adherence; previous studies have found that the epithelial cell inflammatory response is not associated with the level of bacterial adherence (7), nor is it associated with the binding capacity of particular pneumococcal strains (48). Some bacteria can adhere and invade epithelial cell surfaces by downregulation of capsule, while some encapsulated strains can coat themselves in IgA fragments to overcome the electrostatic effects of a negatively charged capsule (21, 62).

Epithelial cell responses during inflammation are, however, dependent on a cellular network in which additional cell types prime epithelial cells to upregulate chemokine release (37, 42). These networks contain multiple potential cellular sources of a broad range of cytokines, including monocyte synthesis and secretion of IL-1β and TNFα, which can prime alveolar epithelial cells to release CXCL8 (37, 42, 53). In the context of the early stages of pneumococcal infection, both IL-1β and TNFα are important in stimulating NF-κB activation in pulmonary epithelium (44) but the sources of these cytokines are not fully defined. There is evidence that macrophages enhance CXCL8 expression in lung explants, but which cell types produce the cytokine is uncertain (66). We now show that a macrophage phenotype, resembling monocytes or inflammatory macrophages (12), releases IL-1 to stimulate CXCL8 release from a type II alveolar epithelial cell-line. In contrast macrophage-expressed TNFα was not a significant factor in priming epithelial cells to produce CXCL8 in co-cultures containing the macrophage cell line. The response induced by primary macrophages also involved IL-1 but the degree of inhibition by IL-1Ra was less marked suggesting other factors could also play a role.

These findings are consistent with data from previous studies which observed that IL-1Ra inhibits epithelial cell CXCL8 secretion in response to conditioned media from *M. tuberculosis*-infected human monocytes (63) and in response to conditioned media from RSV-infected monocytes (58). Teichoic acid in the pneumococcal cell wall is a potent stimulus for IL-1 expression from monocytes (45). Teichoicated species in the cell wall could be the predominant microbial factor driving the enhanced CXCL8 response in co-cultures, with an additional role played by strain variable factors such as pili, a known stimulus for TNFα production, at later stages of infection or during the fulminant mouse model when IL-1 signaling was impaired.

In interpreting the results of our study it is necessary to bear in mind the complex nature of cell-cell signaling and cytokine networks. It is possible that the synergistic response in co-culture conditions involves a component of bidirectional signaling between macrophages and epithelial cells (60). Although macrophage conditioned media enhanced CXCL8 secretion from epithelial cells, signals could flow in the reverse direction. IL-1β expression was enhanced in co-cultures. Activating signals from stimulated epithelial cells may potentiate the response of monocytes or macrophages (and possibly other immune cells *in vivo*), with inflammatory mediators acting in a paracrine fashion to stimulate production of other effector molecules. Once the initial recruitment of polymorphonuclear leukocytes has occurred these cells can release IL-1β to fuel further chemokine release from macrophages and non-myeloid cells, as demonstrated recently in a murine model of arthritis (10).

Co-cultures of A549 and THP-1 cell-lines provide a useful and well-recognized *in vitro* model of the alveolar space. We can confirm that the VD_3-differentiated THP-1 cells have a differentiation state which resembles a monocyte or macrophage in the early stage of tissue differentiation (12). We verified findings with a primary macrophage that was also in the early stage of differentiation. Our confirmation *in vivo* using two different strains of mice, that modulation of IL-1 signaling influences CXCL1/2 expression and polymorphonuclear cell numbers in the lung during pneumococcal pneumonia confirmed *in vivo* the validity of this approach. Other studies have supported our findings that IL-1 contributes to host defense *in vivo* against pneumococci, although in the case of the lung the effects were most important in the first 24-48 h after infection (45, 67).

IL-1 dependent neutrophilic inflammation has improved bacterial clearance in a previous model of pneumococcal pneumonia (46), and our studies with moderate bacterial inocula were consistent with

this. However, in fulminant disease, neutrophilic inflammation and reactive oxygen species generation by polymorphonuclear leukocytes may adversely affect clinical outcomes (33, 34) and we observed that IL-1 dependent neutrophilic inflammation enhanced levels of tissue invasion and bacteremia. In some settings neutrophil depletion can improve outcomes in murine models of fulminant pneumonia (33). We have previously highlighted the potential importance of leukocyte-derived IL-1 in the initiation of airways inflammation (37, 38, 42). The data shown here provide further support for the concept that targeting IL-1 in airways infection may in some settings be able to limit damaging effects of excessive inflammation by suppressing signaling between monocytes/macrophages and airway epithelial cells. This may be more tractable than manipulation of other pathways such as the TNFα pathway which may have more significant overall effects in regulating host responses to pneumococci (46). Alternatively, since TNFα was raised in the absence of IL-1 signaling in the *Il1r1*$^{-/-}$ mice, it might be that the combined inhibition of IL-1 and TNFα signaling would be more effective at inhibiting CXC chemokine generation. Any potential benefits would need to be weighed carefully against the possible harmful effects of inhibiting recruitment of the minimum number of polymorphonuclear leukocytes required to clear micro-organisms. The timing of administration is likely to be key. Administration would need to be delayed until after the early stages of the host response and would ideally involve identifying a sub-group of patients with selective markers indicating that an excessive inflammatory response might develop and predispose the individual to complications such as acute respiratory distress syndrome (ARDS), which may occur following pneumonia (61). Previous studies using IL-1Ra in the early stages of pneumonia evolution have not shown substantial alteration in inflammatory outcomes or survival in a murine model (47), but targeting such an intervention to select patients with markers of more severe inflammation might be tractable.

448 In conclusion, we present evidence for a synergistic relationship between alveolar epithelial cells and
449 macrophages in the early inflammatory response to *S. pneumoniae*. We demonstrate a critical role for
450 IL-1 signaling in CXC chemokine expression and neutrophil recruitment to the murine lung during
451 pneumococcal pneumonia and suggest that in fulminant disease therapeutic modulation of this
452 pathway, as could be achieved with IL-1Ra could reduce the extent of invasive disease.
453

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693 **Figure legends**

694 **Figure 1. Presence of macrophages enhances epithelial cell CXCL8 production.** A-B) A549
695 epithelial cells were either mock infected (white bars) or challenged with encapsulated (pale grey
696 bars) or unencapsulated (dark grey bars) non-opsonized serotype 2 (A) and serotype 4 (B) *S.*
697 *pneumoniae* (MOI 10) or IL-1β (black bars). CXCL8 levels in the cell-culture supernatants were
698 measured by ELISA at the indicated time points. n=4-6. C-D) A549 epithelial cells alone (EC),
699 THP-1 cells alone (MC), and A549-THP-1 co-cultures (Co) were challenged with opsonized *S.*
700 *pneumoniae* for up to 24 h. CXCL8 levels in the cell-culture supernatants were measured by
701 ELISA at 2, 6, and 24 h time points. C) encapsulated (D39) and unencapsulated (FP22) serotype
702 2 pneumococci n=7, D) encapsulated (TIGR4) and unencapsulated (FP23) serotype 4
703 pneumococci n=5. Mock-infected (MI) and IL-1β data at the 24 h time point is shown for
704 comparison. One-way ANOVA (all $p < 0.05$) with Bonferroni's post-test, * $p < 0.05$; ** $p <$
705 0.01, $^{\#}$ $p < 0.05$ for CXCL8 level in co-culture vs. the sum of CXCL8 levels from A549 and
706 THP-1 cells alone).

707

708 **Figure 2. Physical separation of macrophages from epithelial cells does not impair priming**
709 **of CXCL8 production.** A549 epithelial cells alone (EC), THP-1 cells alone (MC), A549-THP-1
710 co-cultures (Co), and A549 and THP-1 cells separated by transwells (TW) were challenged with
711 D39 pneumococci (MOI 10). CXCL8 levels in the cell-culture supernatants were measured by
712 ELISA at 6 h. IL-1β (positive control) data is shown for comparison (IL-1β). One-way ANOVA
713 with Bonferroni's post-test, n=6, ns-not significant.

714

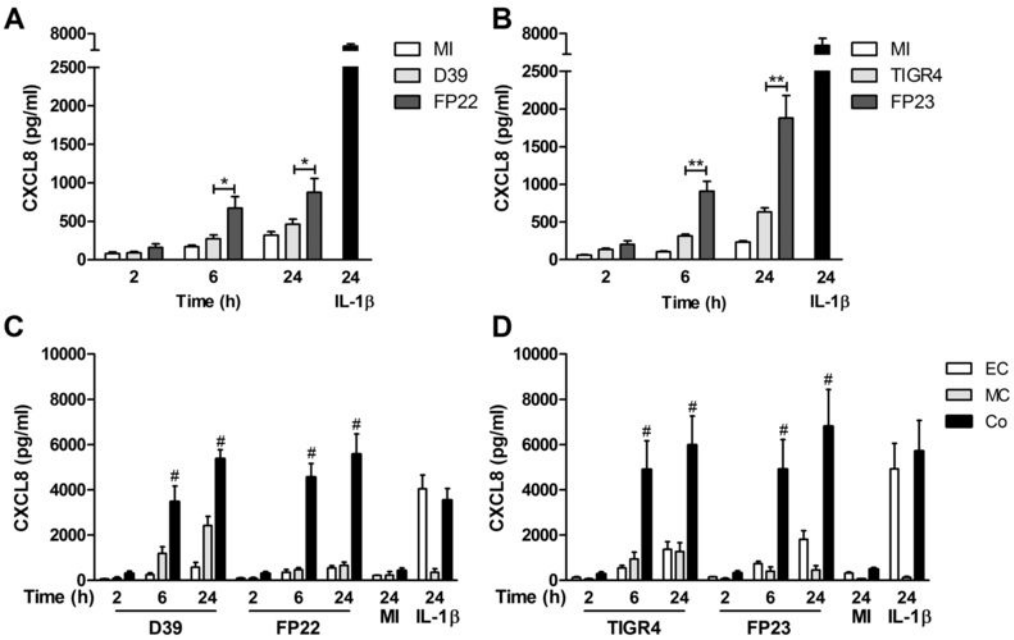
Figure 3. Conditioned media from THP-1 cells challenged with pneumococci primes epithelial cell CXCL8 production. THP-1 cells were exposed to either mock-infection or D39 S. pneumoniae (MOI 100) for 24 h. Mock-infected macrophage conditioned media (MCM-MI) and D39-challenged macrophage conditioned media (MCM-D) were collected at 24 h. Monolayers of A549 epithelial cells were then incubated with: media alone (EC); MCM-MI; THP-1 cells and D39 (MOI 10) (Co+D); MCM-D; MCM-D plus IL-1Ra (MCM-D/IL-1Ra); MCM-D plus sTNF type 1 receptor (MCM-D/TNFR1); MCM-D plus both inhibitors (MCM-D/Both); or IL-1β. CXCL8 levels in the cell-culture supernatants were measured by ELISA at 6 h, n=3, one-way ANOVA ($p < 0.001$) with Bonferroni's post-test, * $p < 0.05$; ** $p < 0.01$.

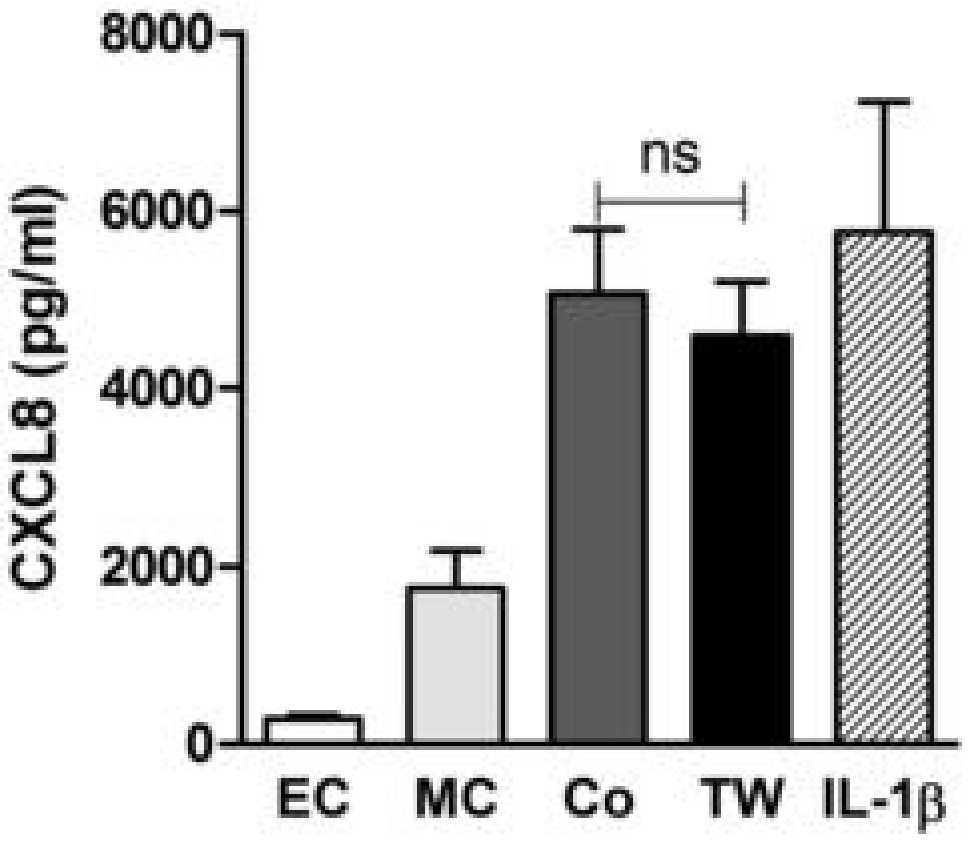
Figure 4. IL-1Ra blocks enhancement of CXCL8 production in co-cultures. Monolayers of A549 epithelial cells alone (EC), THP-1 cells alone (MC), A549-THP-1 co-cultures (Co), co-cultures with IL-1Ra (Co/IL-1Ra), co-cultures with sTNF type 1 receptor (Co/TNFR1), and co-cultures with both inhibitors (Co/both) were challenged with A) encapsulated D39 and B) unencapsulated FP22 serotype 2 pneumococci, C) encapsulated TIGR4 and D) unencapsulated FP23 serotype 4 pneumococci. IL-1β-stimulated co-cultures are also shown for comparison. CXCL8 levels in the cell-culture supernatants were measured by ELISA at 6 h, n=3-4, one-way ANOVA (all $p < 0.002$) with Bonferroni's post-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

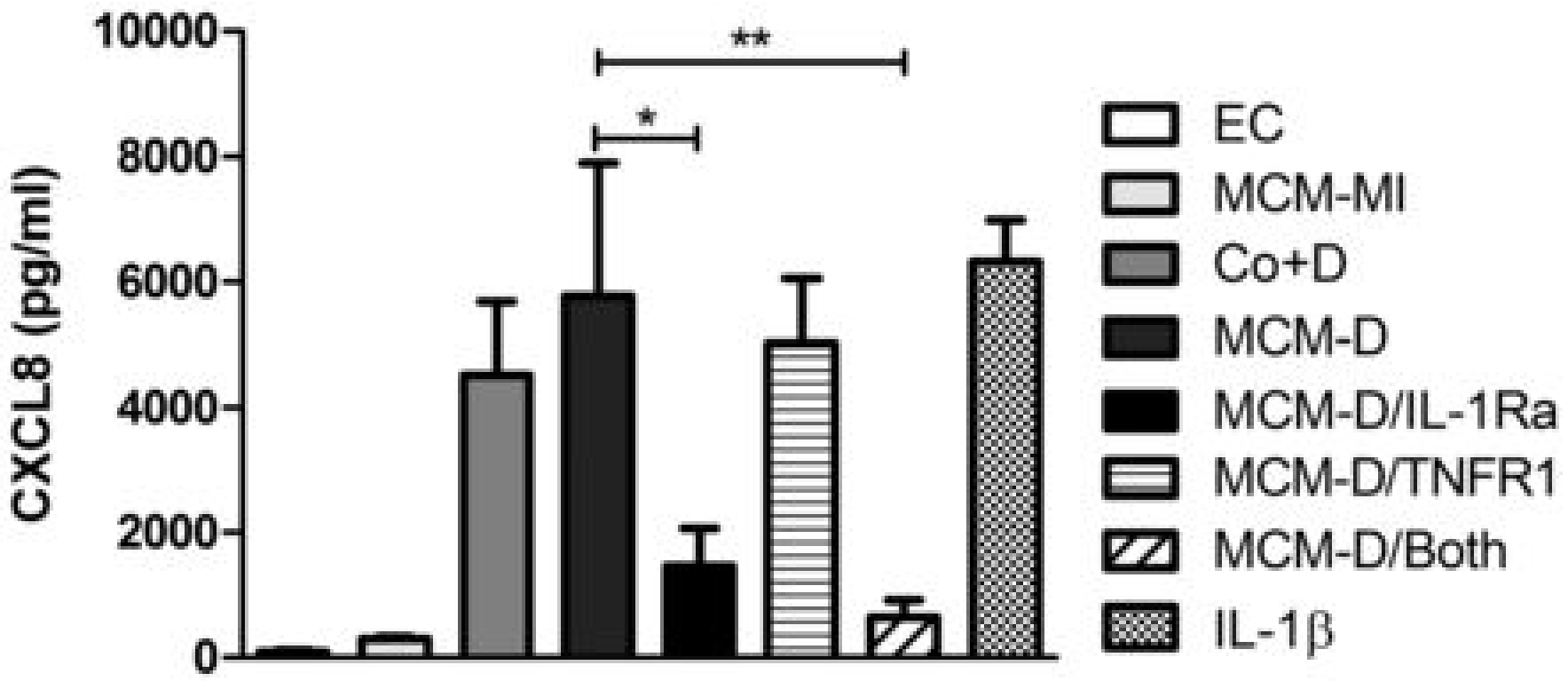
Figure 5. Enhanced epithelial cell CXCL8 production is also seen in co-cultures containing primary monocyte-derived macrophages. A549 epithelial cells alone (EC), monocyte-derived macrophages alone (MDM), and A549-MDM co-cultures (Co) were challenged with opsonized *S. pneumoniae* A) D39 or B) TIGR4 (MOI 10), with or without IL-1 receptor antagonist (IL-

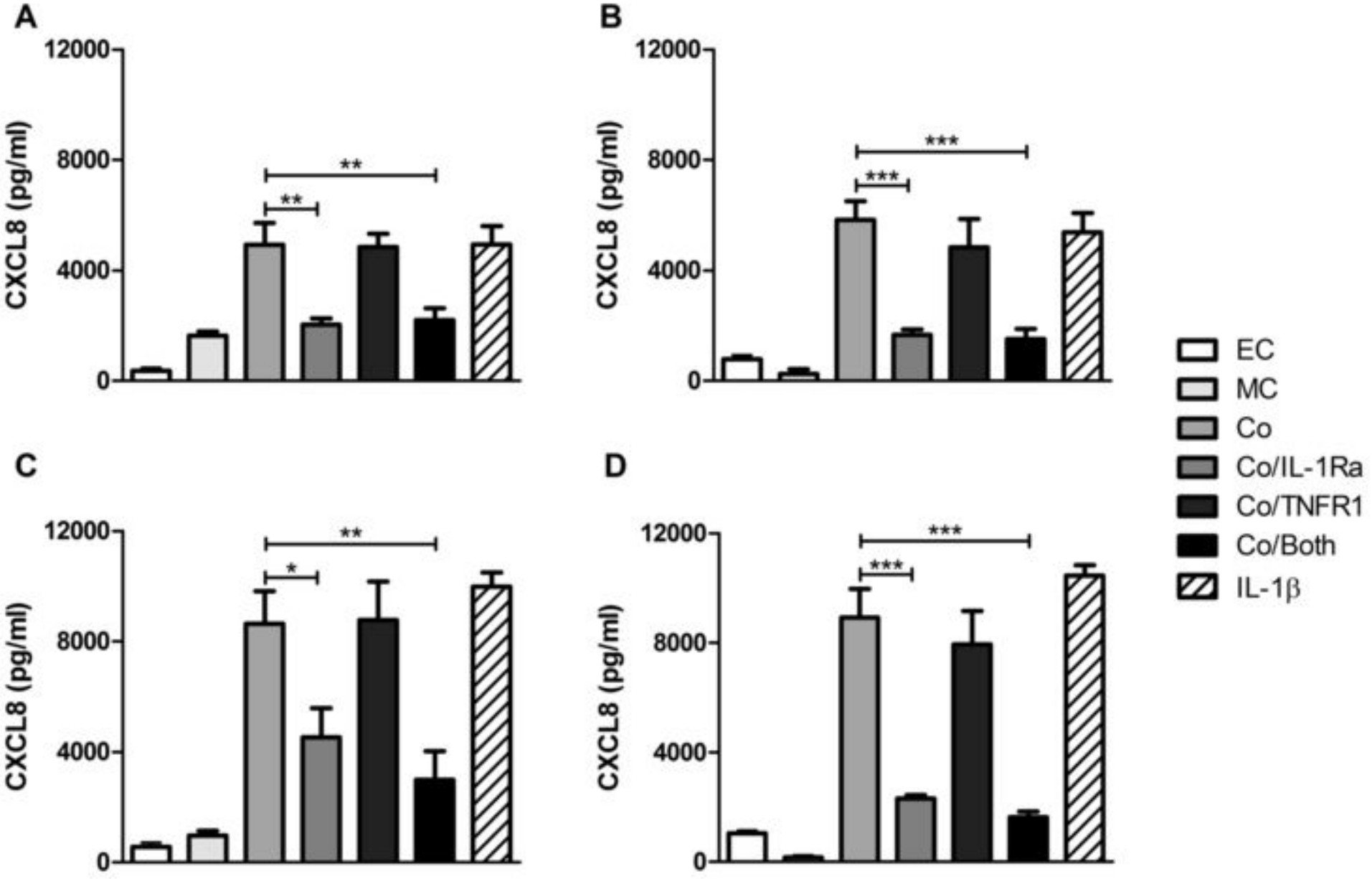
738 1Ra) for 6 h, and CXCL8 levels in the cell-culture supernatants were measured by ELISA and
739 normalised for cell number (data shows release/$1x10^5$ A549 cells). Mock-infected (MI) and IL-
740 1β data are shown for comparison. One-way ANOVA (all $p < 0.05$) with Bonferroni's post-test.
741 *$p < 0.05$, ***$p<0.01$. $p<0.01$ for CXCL8 levels in co-culture *vs*. the sum of CXCL8 levels from
742 A549 cells and MDM alone, n=3-5 from 3 independent experiments.
743
744 **Figure 6. IL-1β but not TNFα levels are enhanced in co-cultures following challenge with**
745 **pneumococci.** A549 epithelial cells (EC) were exposed to media alone (-) or D39 pneumococci
746 (+) as were THP-1 cells (MC) and A549-THP-1 co-cultures (Co) and A) IL-1β and B) TNFα
747 levels measured by ELISA at 6 and 24 h, n=3, one-way ANOVA (all $p < 0.02$) with Bonferroni's
748 post test, * $p < 0.05$. A549 epithelial cells were exposed to conditioned media from D39
749 challenged THP-1 cells and C) IL-1β and D) TNFα levels were measured by ELISA at 0, 2, 6
750 and 24 h.
751
752 **Figure 7. Modulation of IL-1 signalling alters production of CXC chemokines and**
753 **polymorphonuclear cell (PMN) recruitment during *S. pneumoniae* infection** *in vivo***.**
754 A) Percentage PMNs in bronchial alveolar lavage (BAL) from C57BL/6 control mice and mice
755 deficient in IL-1 type 1 receptor on a C57BL/6 background ($IL\text{-}1R1^{-/-}$) 24h after intratracheal
756 instillation of $5x10^5$ CFU type 4 pneumococci; Levels of B) CXCL1 and C) CXCL2 in BAL
757 from the same experiment as A; D) Percentage PMNs in BAL from BALB/c control mice and
758 mice deficient in IL-1 type receptor on a BALB/c background ($IL\text{-}1R^{-/-}$) challenged with
759 pneumocci as in A); Levels of E) CXCL1 and F) CXCL2 in BAL from the same experiment as
760 D.(* $p<0.05$, ** $p<0.01$ unpaired t test.)

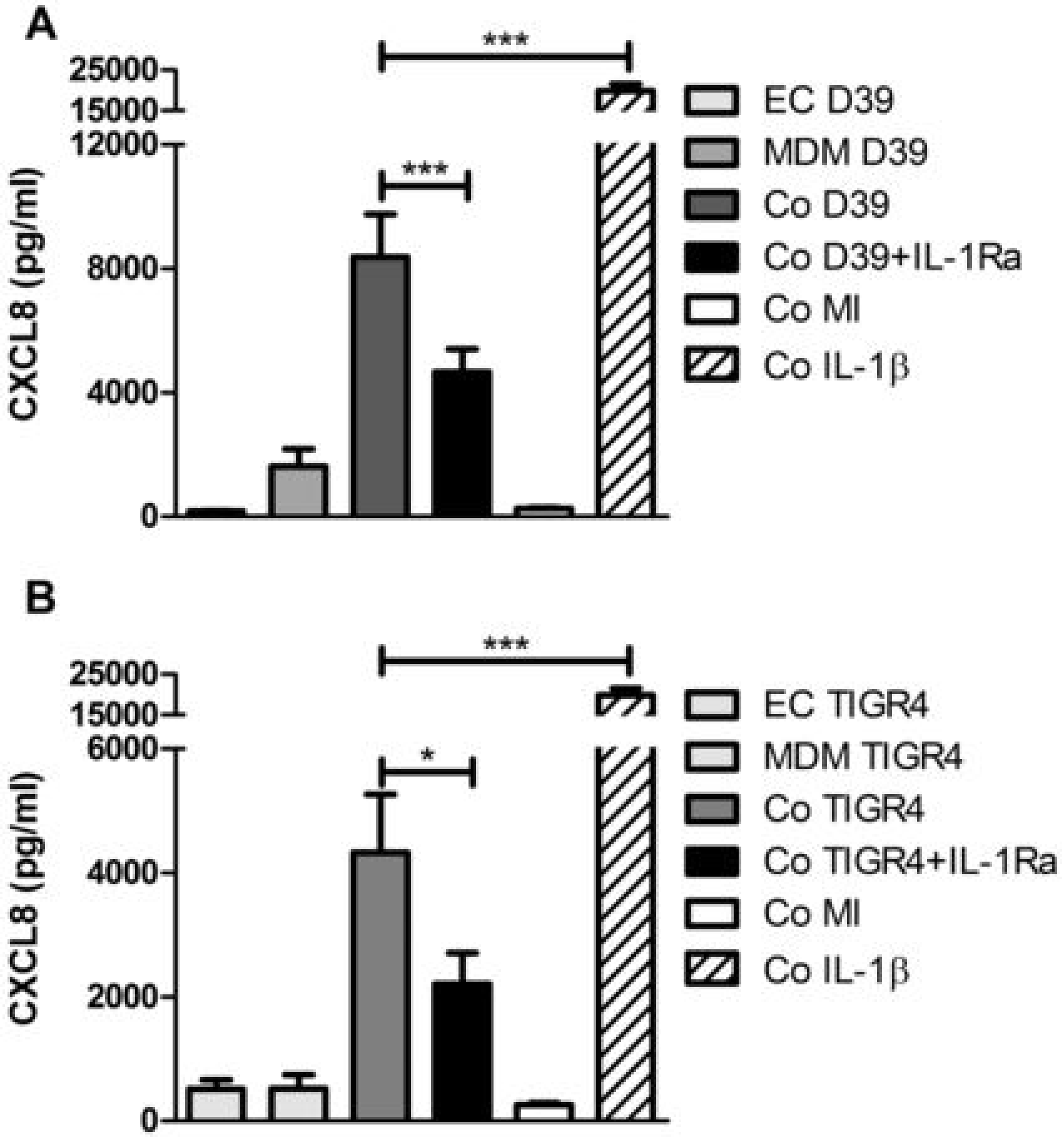
Figure 8. Modulation of IL-1 signaling can reduce the severity of invasive disease in fulminant pneumococcal infection. A) Percentage neutrophils (PMN) in bronchial alveolar lavage (BAL) from BALB/c control mice and mice deficient in IL-1 type 1 receptor on a BALB/c background (IL-1R1$^{-/-}$) 24h after intratracheal instillation of 1x10^{7} CFU type 4 pneumococci; Levels of B) CXCL1, C) CXCL2, and D) TNFα in BAL from the same experiment as A); CFU of bacteria in E) lung homogenates and F) blood in the same experiments as A. (* $p<0.05$ unpaired t test, **$p<0.01$ Mann Whitney).

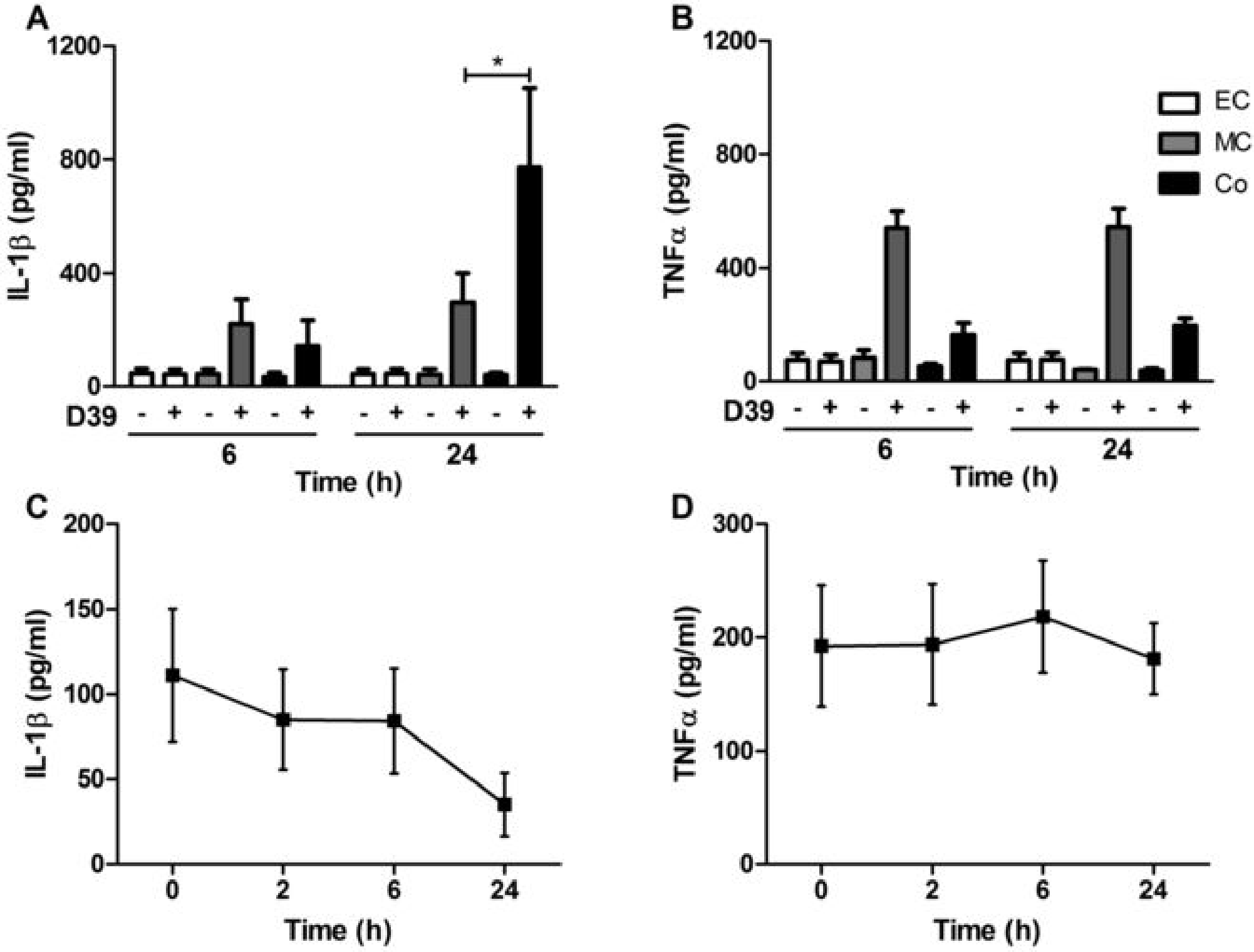

A
B
C
D
CXCL8 (pg/ml)
MI
D39
FP22
TIGR4
FP23
EC
MC
Co
Time (h)
IL-1β
D39
FP22
TIGR4
FP23

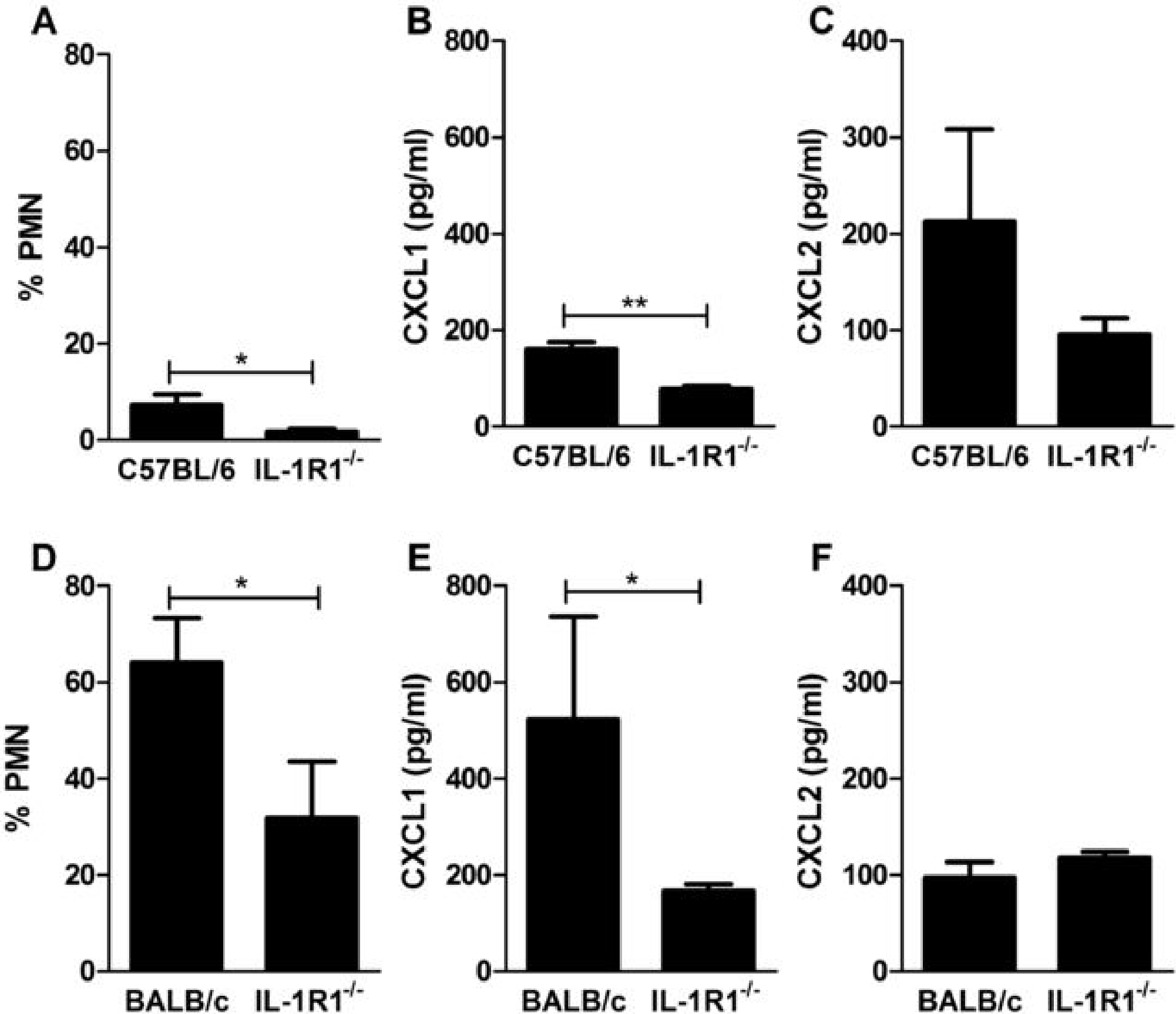

8000
6000
4000
2000
0
CXCL8 (pg/ml)
ns
EC
MC
Co
TW
IL-1β

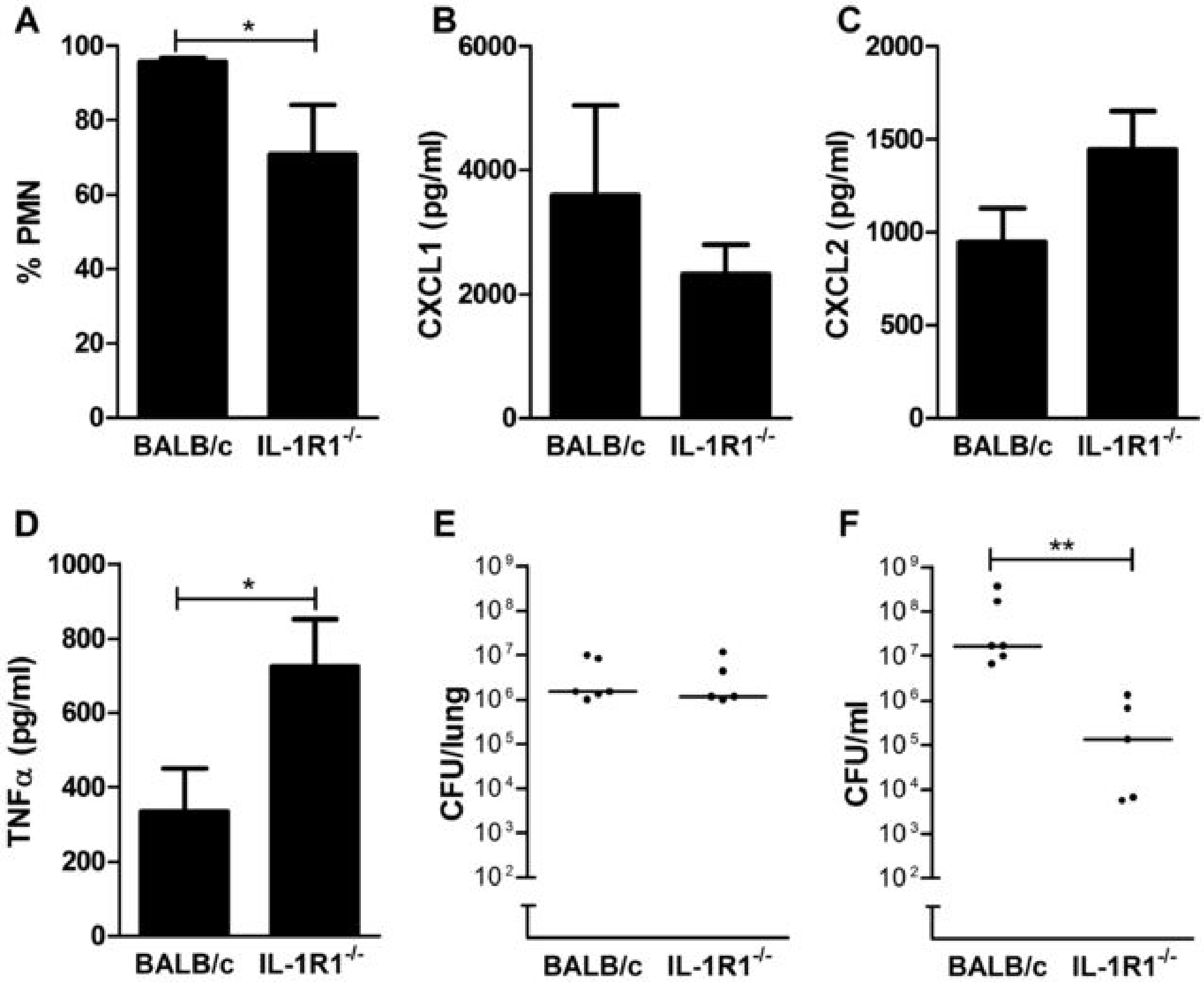

10000
8000
6000
4000
2000
0
CXCL8 (pg/ml)
**
*
EC
MCM-MI
Co+D
MCM-D
MCM-D/IL-1Ra
MCM-D/TNFR1
MCM-D/Both
IL-1β


A
B
C
D
CXCL8 (pg/ml)
12000
8000
4000
0
**
*
***
EC
MC
Co
Co/IL-1Ra
Co/TNFR1
Co/Both
IL-1β


A
***
25000
15000
12000
***
8000
4000
0
CXCL8 (pg/ml)
EC D39
MDM D39
Co D39
Co D39+IL-1Ra
Co MI
Co IL-1β
B
***
25000
15000
6000
*
4000
2000
0
CXCL8 (pg/ml)
EC TIGR4
MDM TIGR4
Co TIGR4
Co TIGR4+IL-1Ra
Co MI
Co IL-1β


A
1200
800
400
0
IL-1β (pg/ml)
*
D39 - + - + - + - + - + - +
6
24
Time (h)
B
1200
800
400
0
TNFα (pg/ml)
EC
MC
Co
D39 - + - + - + - + - + - +
6
24
Time (h)
C
200
150
100
50
0
IL-1β (pg/ml)
0
2
6
24
Time (h)
D
300
200
100
0
TNFα (pg/ml)
0
2
6
24
Time (h)


A
% PMN
80
60
40
20
0
*
C57BL/6
IL-1R1-/-
B
CXCL1 (pg/ml)
800
600
400
200
0
**
C57BL/6
IL-1R1-/-
C
CXCL2 (pg/ml)
400
300
200
100
0
C57BL/6
IL-1R1-/-
D
% PMN
80
60
40
20
0
*
BALB/c
IL-1R1-/-
E
CXCL1 (pg/ml)
800
600
400
200
0
*
BALB/c
IL-1R1-/-
F
CXCL2 (pg/ml)
400
300
200
100
0
BALB/c
IL-1R1-/-


A
% PMN
100
80
60
40
20
0
*
BALB/c
IL-1R1-/-
B
CXCL1 (pg/ml)
6000
4000
2000
0
BALB/c
IL-1R1-/-
C
CXCL2 (pg/ml)
2000
1500
1000
500
0
BALB/c
IL-1R1-/-
D
TNFα (pg/ml)
1000
800
600
400
200
0
*
BALB/c
IL-1R1-/-
E
CFU/lung
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
BALB/c
IL-1R1-/-
F
CFU/ml
**
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
BALB/c
IL-1R1-/-