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Defective regulation of CXCR2 facilitates neutrophil release from bone marrow causing spontaneous inflammation in severely NF-κB-deficient mice

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Abstract

NF-κB is a major regulator of innate and adaptive immunity. Neutrophilic granulocytes (neutrophils) constitutively express RelA/p65 (*Rela*), c-Rel (*Crel*) and p50 (*Nfκb1*), but not p52 (*Nfκb2*) subunits. We here describe $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ mice, that have the most severe genetic neutrophil NF-κB deficiency that is still compatible with life, $Rela^{-/-}$ mice being embryonic lethal. $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ mice developed spontaneous dermal inflammation associated with chronic neutrophilia, elevated CXCL1 and G-CSF. The bone marrow contained fewer nucleated cells and was relatively enriched in myeloid progenitor cells. Neutrophilia was preserved when $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ bone marrow was transferred into wild-type mice, but mixed bone marrow chimeras receiving wild-type and $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ bone marrow showed normal circulating neutrophil numbers, excluding a neutrophil precursor-intrinsic proliferation advantage. In mixed bone marrow chimeras, $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ neutrophils were preferentially mobilized from the bone-marrow in response to CXCL1 injection and in models of LPS-induced lung inflammation and in thioglycollate-induced peritonitis. $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ neutrophils expressed higher levels of the CXCL1 receptor CXCR2 both under resting and stimulated conditions and failed to down-regulate CXCR2 during inflammation. We conclude that severe NF-κB deficiency facilitates neutrophil mobilization, which causes elevated numbers of pre-activated neutrophils in blood and tissues, leading to spontaneous inflammation. These neutrophil effects may limit the usefulness of global NF-κB inhibitors for the treatment of inflammatory diseases.

Keywords

NF-κB; granulocytosis; CXCL1; CXCR2; lung injury

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Introduction

Nuclear factor κB (NF-κB) is a master regulator central to the innate immune response [1]. It consists of multiple subunits that differentially modulate its activity [2,3]. Neutrophilic granulocytes (neutrophils), the most abundant human white blood cells, are terminally differentiated cells with a limited life-span of few hours in peripheral blood [4]. The main NF-κB subunit RelA/p65, c-Rel, p50 and the NF-κB-inactivating inhibitor (I)κB and its kinase complex, but not p52 are constitutively expressed in human neutrophils [5,6]. NF-κB activity is required for neutrophil differentiation and activation [5,6] as well as programmed cell death [7,8]. However, the role of specific NF-κB subunits in neutrophil biology has mostly been studied in vitro [5–8] and its role in neutrophil mobilization and activation in vivo is incompletely understood.

Genetically engineered mouse models have been used to delineate NF-κB function [9]. Altered circulation neutrophil counts have been reported in some of them. Homozygous deletion of the central subunit RelA/p65 was embryonic lethal [10] but $Rela^{+/-}$ heterozygous mice survived without an obvious phenotype. Deletion of the p50 subunit (*Nf-κb1*) [11,12] was well tolerated under specific pathogen free conditions, but mice were highly susceptible to bacterial infections and bone marrow neutrophil progenitors were reduced [11,13]. C-Rel (*Crel*) deficiency resulted in lymphocyte, but not neutrophil dysfunction [14]. Concerning combined neutrophil NF-κB subunit deficient mice, transplantation of $Crel^{-/-}RelA^{-/-}$, but not $Crel^{-/-}$ fetal liver cells together with normal bone marrow caused granulocytosis [15]. Myeloid cell lines were reported to be normal in $Crel^{-/-}Nf\kappa b1^{-/-}$ mice [16].

Other genetic NF-κB alterations also influenced neutrophil counts. IκBβ deficiency leads to constitutive NF-κB activation, which is fatal. In mice transplanted with $IkB\beta^{-/-}$ bone marrow [17], granulocytosis was observed. IκBα deficiency in newborn mice was also accompanied by granulocytosis [18]. This phenotype was normalized when the *Nfκb1* gene encoding p50 was also deleted [18]. $Nf\kappa b2^{-/-}$ mice deficient in p52 died at three weeks of age from gastric hyperplasia [19] when peripheral blood neutrophilia and an increased percentage of neutrophils in the bone marrow were observed. $RelB^{-/-}$ mice show generalized inflammation [20]. Extramedullary hematopoiesis and an increased percentage of circulating neutrophils were found in $RelB^{-/-}$ and $Nf\kappa b1^{-/-}RelB^{-/-}$ mice [12]. $Nf\kappa b1^{-/-}RelB^{-/-}$ double deficiency was lethal by four weeks of age. This example underlines the complexity of NF-κB signaling in granulopoiesis: while p50 deficiency mitigated granulocytosis in combination with Iκ–Bα deficiency [18], combined RelB and p50/*Nfκb1* deficiency led to a worse clinical course [12]. The mechanistic effects of NF-κB loss on in vivo neutrophil function have remained elusive. Many of the reported papers only give relative but not absolute neutrophil counts and mostly focus on an altered adaptive rather than innate immune system making it difficult to distinguish between reactive and endogenous neutrophil defects. Also, effects of NF-κB on neutrophil release from the bone marrow have not been studied.

Circulating neutrophil numbers are remarkably stable in normal humans [21,22] and mice [23,24]. Granulocyte colony stimulating factor (G-CSF) is the main specific granulopoietic cytokine [25]. It increases the proliferation, differentiation and survival of neutrophil precursors. The mobilization of neutrophils from bone marrow is controlled by interaction of stromal derived factor-1 (SDF1, CXCL12) with the chemokine receptor CXC-receptor 4 (CXCR4)[26]. CXCR4 and CXCL12 are down-regulated by G-CSF [27,28]. Rapid neutrophil mobilization during inflammation such as bacterial and fungal infections can also be mediated by CXCR2-binding chemokines such as CXCL1 (KC in mouse, GRO-α in human), elevating blood neutrophil counts [29–31].

In preliminary experiments, we observed elevated blood neutrophil counts in mice with complete deficiency of the neutrophil NF-κB subunits c-Rel (*Crel*) and p50 (*Nfκb1)* and heterozygous for the *Rela* gene ($Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$). Here, we report the mechanism by which the $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ genotype affects the regulation of blood neutrophil numbers.

Materials and Methods

Animals

Wild-type (wt) C57Bl/6 mice and B6.SJL-$Ptprc^{a}Pepc^{b}$/BoyJ ($CD45.1^{+}$) were from (Jackson Labs, Bar Harbor, ME), the generation of *Rela*-, *Crel*-, and *Nfkb1*-deficient mice was previously described [10,11,14]. They were backcrossed on C57Bl/6 background for at least 10 generations and then intercrossed to generate $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$, $Rela^{+/-}$, or $Crel^{-/-}Nf\kappa b1^{-/-}$ mice, genotyped by PCR and used in age- and sex-matched groups. Mice were kept in specific-pathogen-free conditions in a barrier facility. Animal experiments were approved by the Animal Care Committee at LIAI by the Animal Care Program at UCSD.

Lethal irradiations were performed in a ^{137}Cesium irradiator (600 rad twice, three hours apart) and mice were reconstituted with unfractioned bone marrow from wild-type ($CD45.1^{+}$) and/or $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ ($CD45.2^{+}$) mice as indicated. Mice were treated with trimethoprim-sulfomethoxazole in drinking water for two weeks after transplantation. Experiments were performed 7–12 week after bone- marrow transplantation. Blood for leukocyte counts was taken via tail bleeding into EDTA-coated capillary tubes, analyzed by automatic analyzer (Hemavet 950FS, DREW Scientific, Oxford, CT) and differential counts confirmed by manual counts after Wright-Giemsa stain (Sure stain, Fisher, Middletown, VA) with a 100x oil objective on a Nikon eclipse 80i microscope.

Immunoblot

Whole-cell extracts were loaded onto 12% SDS-polyacrylamide gel and separated by electrophoresis. Proteins were then transferred to a polyvinylidene fluoride membrane, and unspecific binding was blocked by using Tris-buffered saline (TBS) containing 5% of nonfat dry milk (Cell Signaling Technology) and 0.05% of Tween 20 (MP Biomedicals) for 1 h at room temperature and analyzed by immunoblot using ECL-plus (GE Healthcare) using the following antibodies: anti-RelA (SC-372), anti-RelB (SC-226), anti-c-Rel (SC-71), anti-α-Tubulin (SC-5286) (Santa Cruz Biotechnology), antisera for N-terminal p50/p105 (NR-1157) and p52/p100(NR-1495) were generous gift from Nancy Rice. Bands were examined by using a phosphorimager and ImageQuant software version 5.2 (GE Healthcare).

Histology

After sacrifice, animals were perfused with 4 % paraformaldehyde, tissues embedded in paraffin and stained with hematoxylin/eosin.

Neutrophil function assays

Bone-marrow neutrophils ($5*10^{6}$ cells/ml) were incubated with phorbol 12-myristate 13-acetate (PMA) or buffer control for 15 min at 37°C. For assessment of respiratory burst, stimulation was preceded by a 5 min loading-step in 1μM dihydrorhodamine (Invitrogen, Carlsbad, CA) and generation of rhodamine was analyzed by flow cytometry.

Apoptosis was assessed by AnnexinV/7-AAD staining kit (BD-PharMingen) according to the manufacturers instructions after 20 h culture in complete RPMI supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. Positivity was defined by a gate set to include 1% of unstained cells.

Flow cytometry

The following antibodies were used in flow cytometry: anti-CD18-FITC (C71/16), anti-L-selectin-PE (MEL-14), anti-Gr1-APC (RB6-8C5), anti-CD45-PERCP (30-F11), anti-CD45.1-PE (A20), anti-CD45.2-FITC (104), anti-CD3-PE (145-2C11), anti-B220-PE (RA3-6B2), anti-NK1.1-PE (1B1) (BD-PharMingen, San Diego, CA), anti-CD34-PE (Caltag, Buckingham, UK), anti-Gr1-CY7-APC (RB6-8C5), anti-Sca1-CY7-PE, anti-CD3ε-PE (145-2C11) (Biolegend, San Diego, CA), anti-ckit-APC (2B8), anti-Ter119-CY7-PE (TET-119) (eBioscience, San Diego, CA), anti-CXCR2-PE (242216) (R&D systems, Minneapolis, MN). Bone marrow myeloid progenitors were defined as described [32]. Flow cytometry analysis was performed on a Becton-Dickinson FACS Calibur or LSRII. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

ELISA

G-CSF and CXCL1 ELISAs were conducted with mouse DuoSet Elisa development kits (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Neutrophil mobilization by CXCL1

Recombinant mouse CXCL1 chemokine (KC, GRO1) (Peprotech, Rocky Hill, NJ) was dissolved in PBS and injected intravenously at a dose of 400 ng/mouse.

LPS mediated lung injury

Exposure to aerosolized lipopolysaccharide (LPS) was essentially as described [33]. LPS from Salmonella enteritides (Sigma, St. Louis, MO) was dissolved in PBS at 500 μg/ml and 7.5 ml of the solution nebulized into a custom-built chamber by a MicroAir nebulizer Omron Healthcare, Vernon Hills, IL) over 30 min. Brocho-alveolar lavage (BAL) was performed after 18 hr. Mice were anaesthetized with a mixture of ketamine hydrochloride (125 mg/kg), xylazine (12.5 mg/kg), and atropine sulfate (0.025 mg/kg) by intraperitoneal injection. The trachea was intubated using a 20 g needle in a polypropylene tube and the lavage performed five times with 1 ml PBS each. Total cell counts were assessed by an automated analyzer and neutrophil counts were calculated after determining neutrophils by flow cytometry using CD45 + and $Gr1^{high}$ gates as evaluated previously [33].

Thioglycollate induced peritonitis

For induction of peritonitis, 1 ml BBL fluid thioglycollate medium (Becton-Dickinson, Sparks, MD) was injected intraperitoneally and cells were recovered after 6 h by washing twice with 5 ml PBS.

Statistical Analysis

Two-tailed student t-test or ANOVA if more than two values were compared were used. P-values <0.05 were considered significant. Data are expressed as mean ±SEM. P values are indicated in the figures with *p<0.05, ** p<0.01 and *** p<0.001.

Results

Crel$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice develop dermal inflammation and increased circulating neutrophil counts

Crel$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice were viable under barrier conditions, but failed to thrive and required late weaning at 5–6 weeks rather than 3 weeks. Breeding *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ with *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/+}$ resulted in a genotype ratio at weaning of 37% *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ and 63% *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/+}$ (n=110), significantly different from

the expected Mendelian ratio of 50% each. Neutrophil NF-κB subunit expression was assessed by immunoblot (figure 1A). As described for human neutrophils [6], mouse neutrophils expressed Rel-A, c-Rel and p50 proteins, while no p52 and very little RelB were found. Rel-A protein levels were approximately 50% in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice compared to *Crel*$^{-/-}$*Nfκb1*$^{-/-}$. This results in a dramatic reduction of NF-κB dimers that can be formed in neutrophils (figure 1B).

Crel$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice spontaneously developed inflammatory skin lesions in the eyelids (figure 1C) that histologically corresponded to verruciform keratosis with hyperkeratosis, limited parakeratosis, and enlarged keratohyalin granules within the stratum granulosum and an inflammatory infiltrate within the dermis, composed predominately of lymphocytes and neutrophils with occasional eosinophils and a limited number of plasma cells (figure 1D, E). Baseline circulating neutrophil counts were elevated in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice (figure 1F), but both lymphocyte and monocyte counts were normal (suppl. figure 1) and spleen cellularity, CD4^{+}, CD8^{+}, αβTCR^{+} and γδTCR^{+} cell content were unaltered (suppl. figure 2). No neutrophil count elevation was seen in either *Crel*$^{-/-}$*Nfκb1*$^{-/-}$or *Rela*$^{+/-}$ mice (suppl. figure 1). Blood neutrophil morphology was normal (figure 1G) and both resting and activated β2-integrin (CD18) and L-selectin (CD62l) surface expression (suppl. figure 3) were unaltered in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice. However, there was increased baseline respiratory burst activity in neutrophils from *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice (suppl. fig. 4). In these mice, baseline CXCL1 (KC) (figure 1H) and G-CSF (figure 1I) were elevated. Taken together, these data show that *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice represent a novel, viable and severe model of decreased neutrophil NF-κB leading to an inflammatory phenotype with spontaneous blood neutrophilia.

Elevated proportion of bone-marrow myeloid progenitors in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice

To test how the elevation of G-CSF and pro-inflammatory CXCL1 affected granulopoiesis, bone marrow leukocytes were analyzed. While total nucleated bone marrow cells were reduced (figure 2A), the percentage of total bone marrow myeloid cells was slightly (figure 2B) and of myeloid progenitors significantly (figure 2C, D) elevated in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice. This suggests chronically increased granulopoiesis in severely NF-κB deficient mice.

Granulocytosis in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice is bone marrow, but not cell intrinsic

To assess whether neutrophilia was due to hematopoietic cells or rather reactive to dysfunction of other cell types such as barrier function of epithelial cells, *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ bone marrow was transplanted into lethally irradiated wild-type mice. Neutrophilia was still observed when only the bone marrow cells lacked these NF-κB subunits, albeit somewhat mitigated (figure 3A). This indicates a major role of bone marrow-derived cells in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ -caused neutrophilia. To address whether the defect was intrinsic to neutrophils or their precursors, we constructed mixed bone marrow chimeras by transferring 50% wild-type and 50% *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ bone marrow into lethally irradiated wild-type mice. The mixed chimeras had normal circulating neutrophil numbers between 2 weeks and 4 months after transplantation (figure 3A and data not shown). These findings suggest that the presence of wild-type bone marrow cells corrected the neutrophilia seen in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice under baseline conditions.

Crel$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils are more responsive to the chemotactic cytokine CXCL1

Blood neutrophilia combined with lower bone marrow cellularity pointed towards a possible neutrophil release enhancement in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice. To test for mobilization of neutrophils from the bone marrow, mixed chimeric mice were given an intravenous bolus of the chemokine CXCL1 and circulating neutrophil counts were determined after 1h. Numbers of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils increased significantly more than wild-type neutrophils

(figure 3B). The only known receptor for CXCL1 on murine neutrophils is CXCR2 [34]. Its surface expression was higher in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ blood neutrophils than in wild-type cells (figure 3C), which may explain the enhanced response to CXCL1. The same result was found when wild-type and *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils were explored in the blood of 50% wild-type/50% *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mixed chimeric mice (figure 3D) and in wild–type mice transplanted with 100% *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ bone-marrow (suppl. figure 5). Consistent with increased cell surface expression of CXCR2, CXCR2 mRNA in the blood was also significantly elevated (figure 3E). Taken together, these data suggest that *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice overexpress CXCR2, which results in an increased neutrophil release response to CXCL1.

Inflammation in LPS-induced lung injury down-regulates CXCR2 in vivo

Like many G-protein–coupled receptor ligands, CXCL1 can down-regulate expression of its receptor on human neutrophils [35]. As expected, CXCR2 surface expression was significantly down-regulated one hour after intravenous injection of CXCL1 in vivo (suppl. figure 6). To test if this down-regulation also occurred in response to inflammation- induced endogenous CXCL1 secretion, mice were subjected to lung injury by LPS inhalation. This is a model of transient non-lethal pulmonary damage that leads to maximal pulmonary neutrophil accumulation after 12–24 h [33]. Six hours after injury, CXCR2 surface expression on circulating wild-type neutrophils was significantly decreased (figure 4A). Similar effects were also observed on pulmonary and bronchoalveolar lavage neutrophils harvested after 20 h (figure 4B).

As mentioned above, blood CXCL1 levels were elevated in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice (figure 1H). Baseline CXCL1 levels were normalized in mixed chimeric mice (bone-marrow 50% wildtype, 50% *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$) (figure 4C). When mixed bone-marrow chimeric mice were subjected to LPS inhalation, CXCL1 blood levels increased significantly (figure 4C). Neutrophil mobilization was skewed towards *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils (figure 4D), similar to what had been observed in intravenous CXCL1 injection (figure 3B). CXCR2 surface expression was significantly higher in NF-κB deficient neutrophils compared to wild-type neutrophils in the same mice both before start of the experiment and at six hours (figure 4E). The same result was found in the neutrophils accumulated in the inflamed lung and bronchoalveolar lavage fluid (figure 4E). These data show that *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ cells maintain increased CXCR2 surface expression during inflammation in vivo when wild-type CXCR2 was down-regulated.

Increased mobilization of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils in peritonitis

If increased neutrophil release from the bone marrow due to defective down-regulation of CXCR2 is indeed the mechanism for enhanced recruitment of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils, this should be evident in different forms of systemic inflammation. To test this, peritonitis was induced by intraperitoneal thioglycollate injection into mixed chimeric mice. After six hours, peripheral blood neutrophil counts were significantly elevated (figure 5A). No such elevation was seen with this type and amount of thioglycollate in wild-type mice (n=4, data not shown). Importantly, the increase in circulating neutrophil counts was exclusively due to an increase in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils in the circulation (figure 5A). The proportion of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils was also elevated in the peritoneal exsudate (figure 5B). This indicates that *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils are more readily released from the bone marrow and preferentially recruited to sites of inflammation compared to their wild-type counterparts.

Discussion

The deficiency of neutrophil NF-κB subunits examined here is the most severe described to date. *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice suffer from blood neutrophilia and dermal inflammation. Increased release and recruitment of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils in mixed chimeric mice was evident in three different inflammatory models induced by intravenous chemokine injection, LPS-induced lung injury and thioglycollate-induced peritonitis. The mechanism for neutrophilia appears to be enhanced neutrophil release from the bone marrow due to defective downregulation of CXCR2.

RelA deficiency in combination with TNF receptor 1 deficiency led to lethal infections due to non-hematopoietic cell dysfunction [36]. Neutrophilia and dermatitis in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice may be caused by defective barrier function. This would account for the observed increase in CXCL1 and possibly other pro-inflammatory mediators. However, neutrophilia was preserved after transplantation of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ bone marrow into wild-type mice. This argues for a significant bone marrow contribution to the neutrophilia phenotype. In contrast to other combined NF-κB deficiency models [12], we did not observe splenic hematopoiesis or an increase in total bone marrow cells. Together with normal neutrophil counts in mixed bone marrow chimeras this illustrates that the *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ genotype per se does not confer a proliferation advantage of bone marrow progenitors. Possibly, in mixed chimeric mice, fully functional wild-type neutrophils fight incoming bacteria, normalizing inflammatory mediators, as suggested by the normalized circulating CXCL1 levels, leading to normal bone marrow release/retention of neutrophils of both genotypes. This is reminiscent to the phenotype seen in mice reconstituted with 1:1 wild-type/β_2-integrin–deficient (*Itgb2*$^{-/-}$) bone marrow [37]. In these mice, introducing wild-type neutrophils largely corrected the blood neutrophilia, emphasizing the importance of homeostatic mechanisms that control blood neutrophil counts [38].

Defective regulation of neutrophil surface expression of the CXCL1 receptor CXCR2 [39] can explain the differential behavior of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils. CXCR2 is the main receptor for the murine IL-8 analog CXCL1 on neutrophils [34]. CXCR2 downregulation is observed in both mouse and human neutrophils [40,41]. After cecal ligation and puncture CXCR2 downregulation decreased neutrophil migration towards the infection focus [42]. CXCR2 expression on both hematopoietic and non-hematopoietic cells is known to contribute to LPS-mediated lung injury [43]. In our model of LPS-mediated lung injury, we observed a robust decrease of CXCR2 surface expression in wild-type neutrophils. This may be beneficial as is pharmacological CXCR2 blockade [44].

How NF-κB controls CXCR2 surface expression remains to be determined. CXCR2 is a G-Protein coupled receptor that is internalized upon activation by a complex cascade including β-arrestins [45]. NF-κB activity may be required at multiple steps during this cascade either for induction of protein synthesis of scaffolding proteins or by direct interaction the cytoplasm. Interestingly, β-arrestin is directly bound by IκBα, an NF-κB inhibitor that is under direct transcriptional control of NF-κB p65 [46]. However, exploration of this mechanism is beyond the scope of the present project.

Increased LPS sensitivity was also observed in *Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice on a RAG background [47]. This suggests that anti-inflammatory NF-κB function does not require adaptive immunity and emphasizes its role in regulating innate immunity. In humans, endogenous inflammation can arise through inappropriate activation of the innate immune system, leading to autoinflammatory disease [48]. Many of these diseases are monogenic including diverse mutations including membrane receptors as well as intracellular proteins and enzymes [48, 49]. Patients suffer systemic inflammation usually involving skin and joints and often

accompanied by granulocytosis. The *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice described here replicate some of these inflammatory phenotypes, specifically skin involvement and isolated increase in circulating neutrophil numbers. Indeed, the TNF-receptor associated periodic syndromes (collectively termed TRAPS) directly affect the NF-κB signaling pathway [48,50]. Interestingly, pro-inflammatory cell activation was not observed in homozygous knockout mice, but limited to mice heterozygous for the TNF receptor 1 mutation suggesting that residual receptor activation is necessary to cause the phenotype [48]. This is somewhat reminiscent of our mouse model of reduced, but not absent neutrophil NF-κB activity causing an auto-inflammatory state.

Our data provide evidence for a strong pro-inflammatory effect of combined *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ NF-κB deficiency through defective CXCR2 regulation. This cautions that therapeutic interventions aimed at inhibiting NF-κB could have unintended pro-inflammatory consequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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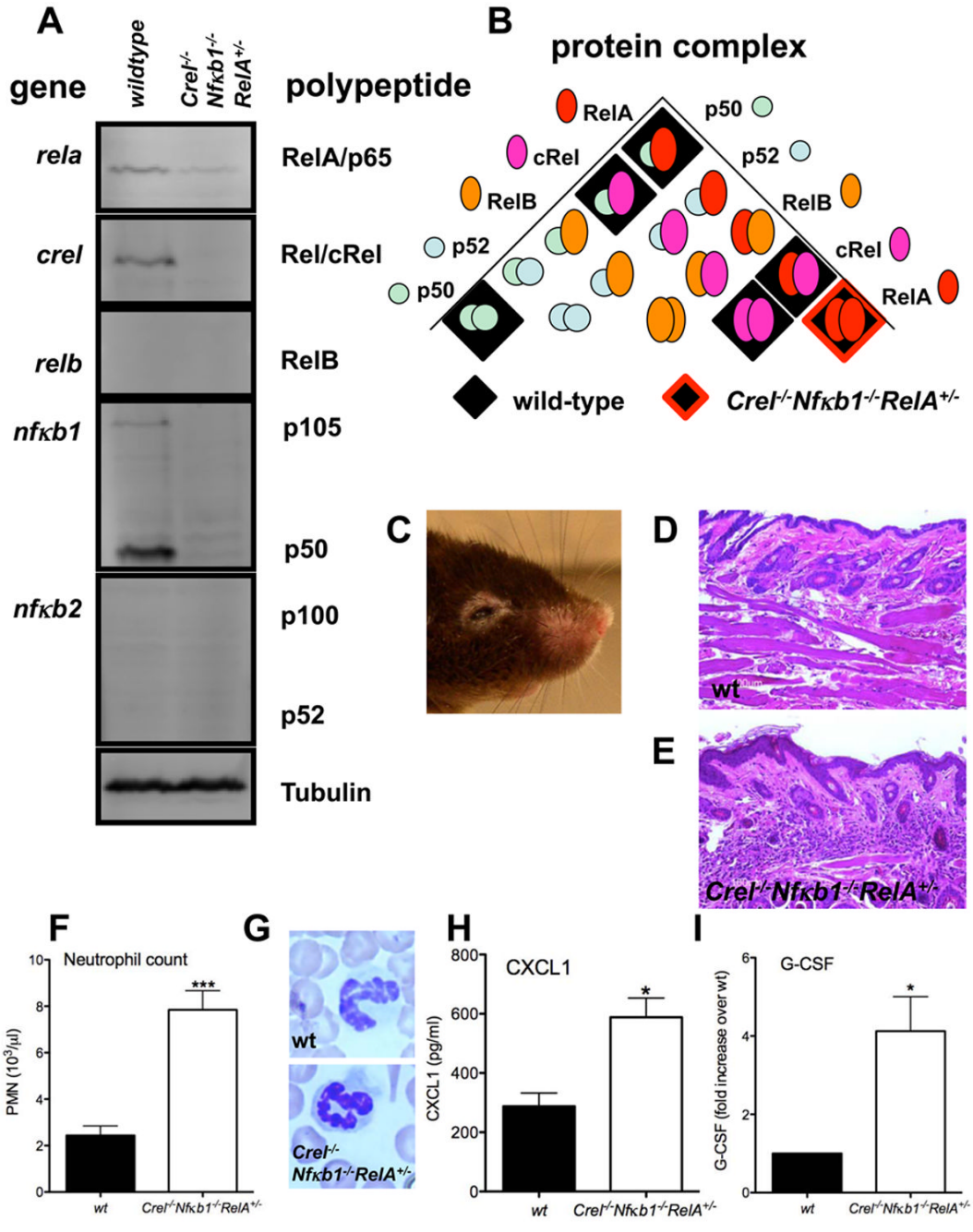

Figure 1. Nf-κB subunit expression in neutrophils and spontaneous inflammation in $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ mice

Neutrophils were isolated from bone-marrow and assessed for NF-κB subunit expression by immunoblot (A). All theoretical subunit combinations, the combinations possible in wild-type (black diamonds) and $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ (black diamonds with red frame) neutrophils are depicted in (B). $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ mice displayed spontaneous inflammatory eyelid changes (C). H&E stained sections showed mixed leukocyte, predominantly lymphocyte and neutrophil infiltration (D, E 40x original magnification). Circulating neutrophils in $Crel^{-/-}Nf\kappa b1^{-/-}Rela^{+/-}$ mice were elevated in number (F, n=9–11) but morphological normal (G). Plasma CXCL1 (KC, H, n=4–8) and G-CSF (I, n=4–6) were measured by ELISA.

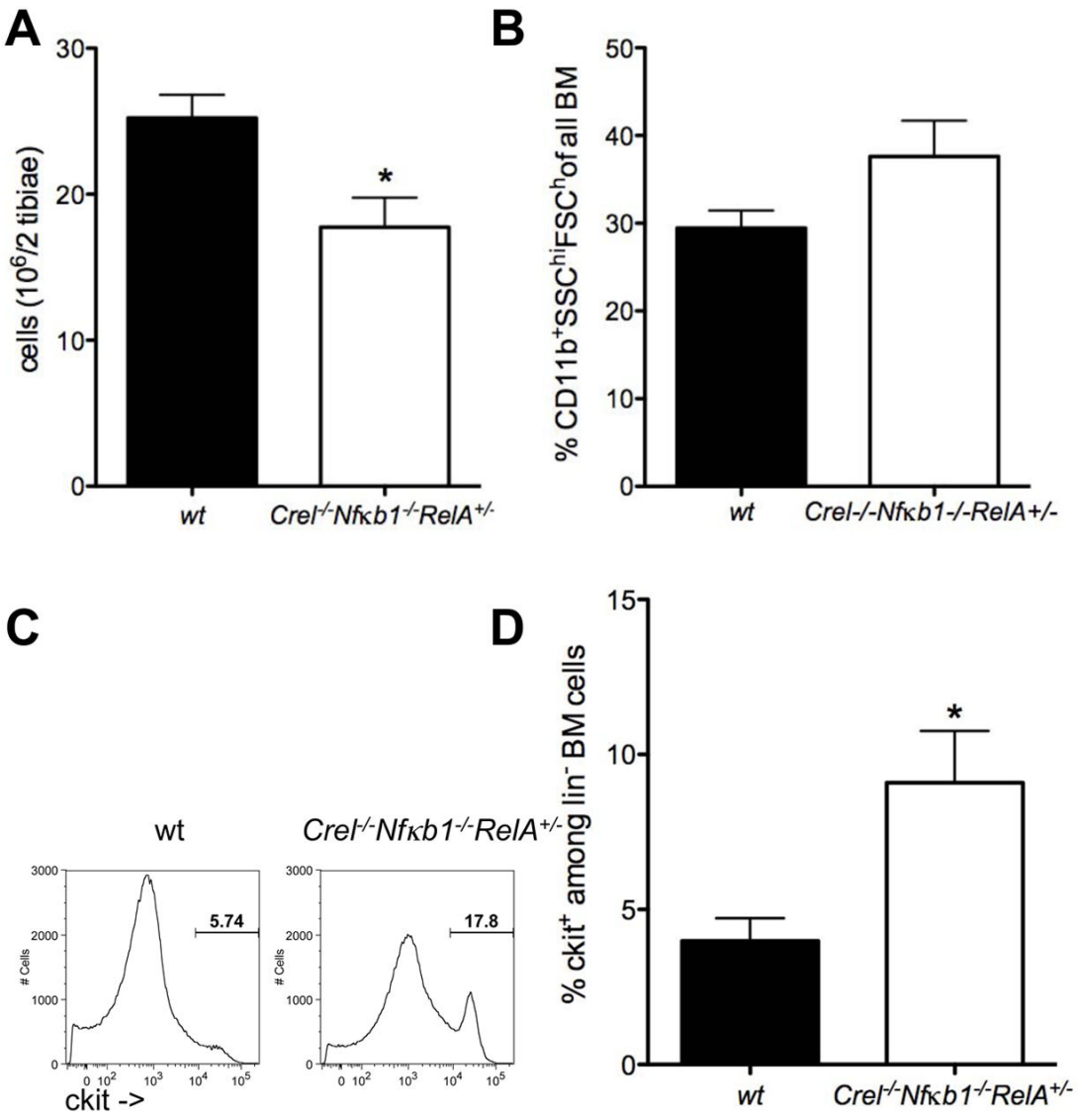

Figure 2. Bone-marrow neutrophil progenitors are elevated in NF-κB–deficient (Crel$^{-/-}$ Nfκb1$^{-/-}$Rela$^{+/-}$) mice

Total nucleated bone marrow cells (A, n=4) were reduced in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice. Neutrophils proportion was not altered significantly (B, n=4). C-kit positive, lineage negative neutrophil precursors (CMP) cells were increased in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice (C, D, n=4).

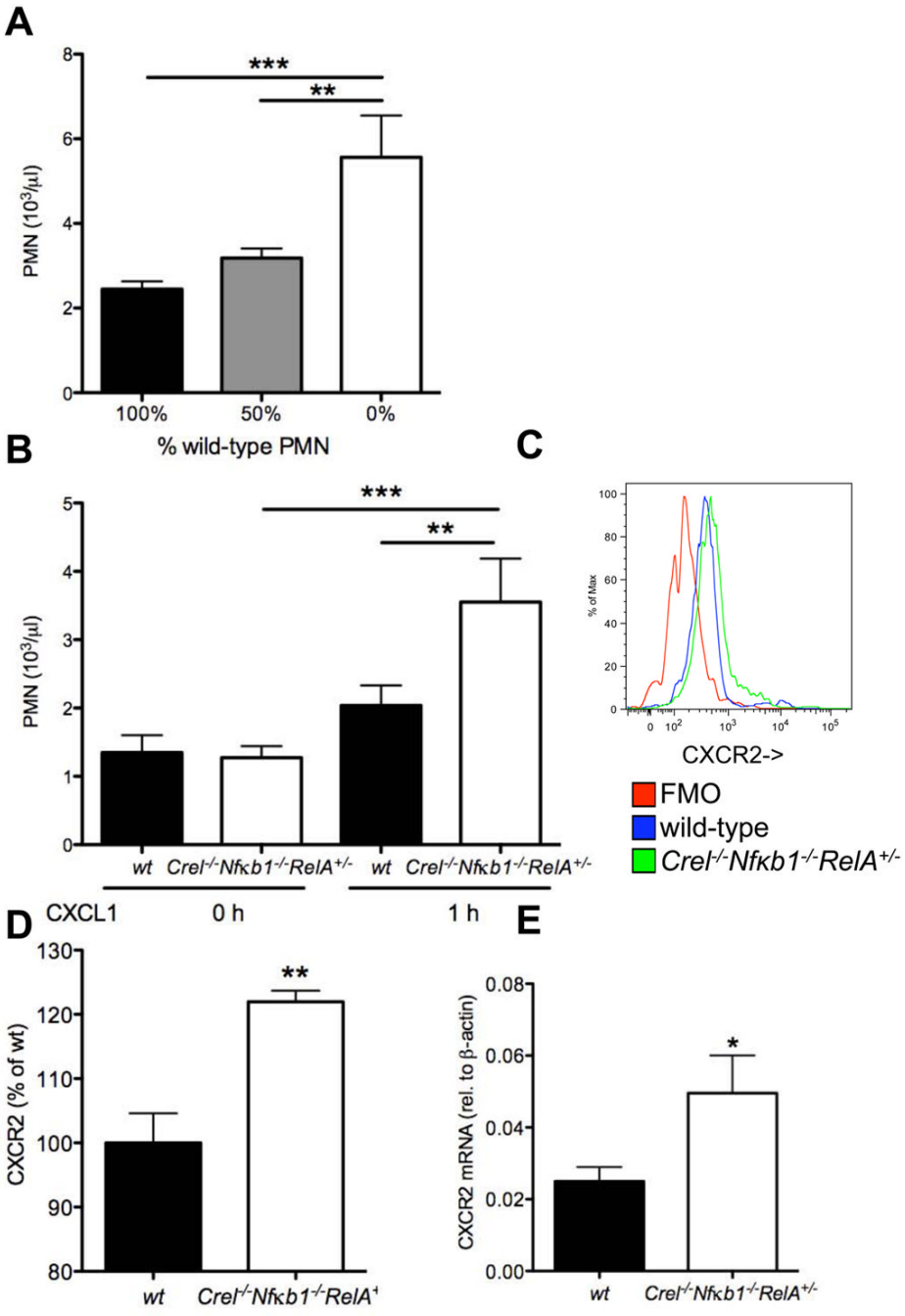

Figure 3. Elevated blood neutrophil counts are a Crel$^{-/-}$Nfκb1$^{-/-}$Rela$^{+/-}$ bone marrow, but not cell- intrinsic phenotype

Un-fractioned bone marrow of wild-type (CD45.1^{+}), *Crel$^{-/-}$Nfκb1$^{-/-}$Rela$^{+/-}$*, and a 1:1 mixture of both was transplanted into lethally irradiated C57/Bl6 mice. Peripheral blood neutrophil counts 4 weeks after transplantation were significantly increased in mice transplanted with 100% *Crel$^{-/-}$Nfκb1$^{-/-}$Rela$^{+/-}$* bone-marrow (A, n=6–12 from 3 independent transplantations). Mixed chimeric mice (equal parts wild-type and *Crel$^{-/-}$Nfκb1$^{-/-}$Rela$^{+/-}$* bone- marrow into wild-type mice) received an intravenous bolus of 400 ng recombinant mouse CXCL1 (B) and blood neutrophil counts were measured after 1 h (n=4, Bonferroni after 1 way ANOVA). Expression of the CXCL1 receptor CXCR2 on peripheral blood neutrophils was

analyzed by flow cytometry (C, representative histogram, comparing wild-type and *Crel* $l^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice). The same result was found when neutrophils under identical conditions in mixed chimeric mice (equal parts wild-type and *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ bone marrow) were compared (D, expressed as percent of CXCR2 on wild-type cells from the same chimera). Blood CXCR2 mRNA expression was elevated in *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ mice (E, n=4–5).

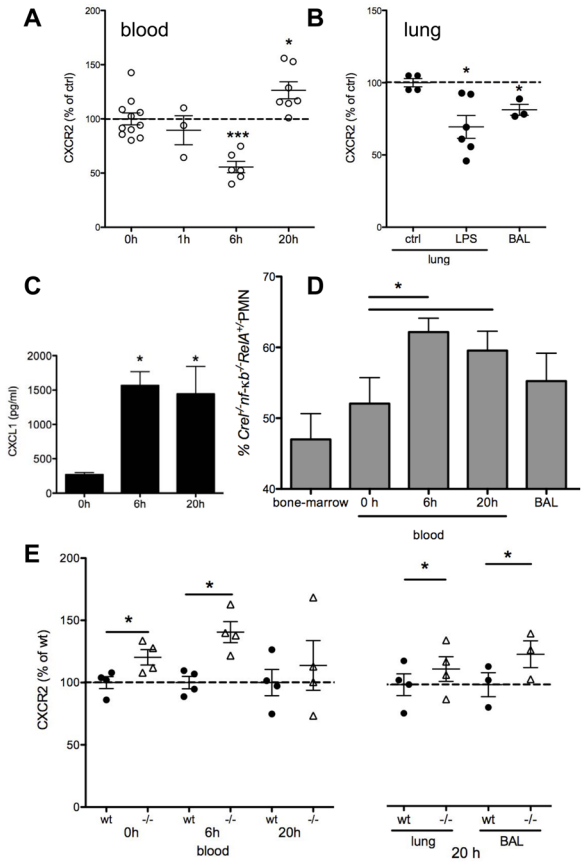

Figure 4. LPS lung injury-mediated downregulation of neutrophil CXCR2 surface expression requires NF-κB

Lung injury was induced by exposure to nebulized LPS in wild-type mice and neutrophil CXCR2 expression was determined by flow cytometry in blood (A), lung and bronchoalveolar lavage (BAL) (B, n=4–6 from 2 independent experiments, expressed relative to untreated control mice (=100%), lung PMN were used for BAL normalization as untreated mice did not have neutrophils in the BAL). (C) Plasma CXCL1 was measured by ELISA in mixed chimeric mice (equal parts *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ and wild-type bone marrow into wild-type mice) at 0, 6 and 20 hours after LPS inhalation (n=4). In mixed chimeric mice, the percentage of *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils in peripheral blood was significantly increased 6 and 20 h after lung injury (D, n=8 from 2 independent experiments). (E) Neutrophil CXCR2 surface expression on *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ neutrophils (open triangles) in blood, lung and BAL was measured by flow cytometry and compared to wild-type (closed circles) neutrophils (=100%) during the course of LPS mediated lung injury (E, n=4, paired t-test).

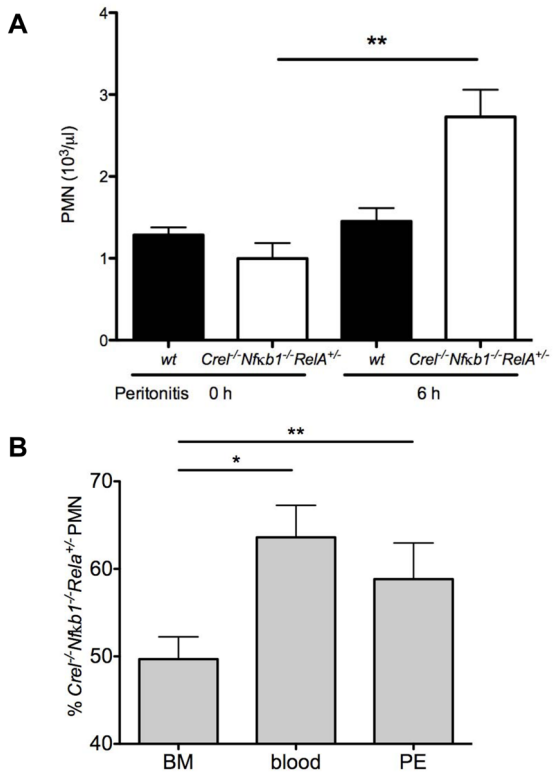

Figure 5. Neutrophil mobilization in thioglycollate induced peritonitis

Mixed chimeric mice (equal parts wild-type and *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ bone- marrow into wild-type mice) received intraperitoneal injections of 2.5 % thioglycollate broth. Circulating *Crel*$^{-/-}$*Nfκb1*$^{-/-}$*Rela*$^{+/-}$ but not wild-type neutrophil counts after 6 h were significantly elevated (A). The proportion of NF-κB deficient neutrophils in both peripheral blood and peritoneal exsudate (PE) was significantly higher than in bone marrow (BM) (B) (n=6).