HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2018 March 15.

Published in final edited form as:

J Immunol. 2017 March 15; 198(6): 2426–2433. doi:10.4049/jimmunol.1601048.

Innate immune cell recovery is positively regulated by NLRP12 during emergency hematopoiesis

Brandon M. L. Linz[1], **Crystal J. Neely**[1], **Laurel B. Kartchner**[1], **April E. Mendoza**[2], **Amal L. Khoury**[2], **Agnieszka Truax**[1], **Gregory Sempowski**[3], **Timothy Eitas**[2,4], **June Brickey**[1], **Jenny P. Y. Ting**[1], **Bruce A. Cairns**[1,2,*], and **Robert Maile**[1,2,†,*]

[1]Department of Microbiology & Immunology, University of North Carolina at Chapel Hill

[2]Department of Surgery, University of North Carolina at Chapel Hill

[3]Human Vaccine Institute, Duke University

[4]Host Defense DPU, Glaxosmithkline

Abstract

With enhanced concerns of terrorist attacks, dual exposure to radiation and thermal combined injury (RCI) has become a real threat with devastating immunosuppression. NLRP12, a member of the NOD-like receptor family, is expressed in myeloid and bone marrow cells and has been implicated as a checkpoint regulator of inflammatory cytokines as well as an inflammasome activator. We show that NLRP12 has a profound impact on hematopoietic recovery during RCI by serving as a checkpoint of TNF signaling and preventing hematopoietic apoptosis. Using a mouse model of RCI, increased NLRP12 expression was detected in target tissues. *Nlrp12*$^{-/-}$ mice exhibited significantly greater mortality, inability to fight bacterial infection, heightened levels of pro-inflammatory cytokines, overt granulocyte/monocyte progenitor cell apoptosis and failure to reconstitute peripheral myeloid populations. Anti-TNF antibody administration improved peripheral immune recovery. These data suggest that NLRP12 is essential for survival after RCI by regulating myelopoiesis and immune reconstitution.

Keywords

Apoptosis; TNF; Stem Cells; Innate Immunity; Immune Regulation

Introduction

The hematopoietic system is capable of rapidly increasing myeloid cell production in response to tissue damage and is critical for wound healing and infection clearance (1–10). While the factors that initiate emergency myelopoiesis are not fully elucidated, it is generally accepted that emergency myelopoiesis is tightly coupled with cytokine and growth

†Corresponding Author: 125 Mason Farm Road, Marsico Hall, Rm 6101, Campus Box 7420, Chapel Hill, NC 27599, robmaile@med.unc.edu.

*These authors contributed equally to this work

factor production, namely TNF and IL-6, and is mediated by NF-κB and other immune regulatory transcription factors.

Rare mutations in *Nlrp12*, a nucleotide-binding leucine rich repeat and pyrin domain-containing receptor (NLR, also known as NOD-like receptor) 12, have been associated with periodic fevers in humans although the association needs to be further studied. Nonsense and splice mutations within human-*Nlrp12* have been shown to diminish suppression of NF-κB signaling (11), however some variants do not exhibit such activity but are associated with modestly enhanced or more rapid inflammasome activation (12). The different functions observed with NLRP12 may be consistent with NLRP12 exhibiting an inflammasome function in certain infections (13, 14) but not other infections or inflammatory conditions (15). While the pyrin-domain containing members of the NLR family have largely been studied in the context of the inflammasome (16), there is growing evidence that a few play an important role in regulating inflammatory signaling. Some NLR proteins have been shown to be positive regulators of NF-κB, while NLRP12 has been implicated as a negative regulator of both the canonical and non-canonical pathways of NF-κB (17–21). NLRP12-mediated NF-κB suppression has been implicated in colonic inflammation and tumorigenesis (17) and osteoclast differentiation (22).

The cytokines that regulate hematopoietic stem cell (HSC) function, such as IFNα/β, IFNγ, IL-12, and TNF, are tightly controlled elements of cell expansion. Type I IFNs and TNF, induced by TLR signaling, can act upon myeloid progenitors to promote the expansion of granulocyte/monocyte progenitors (GMP), leading to systemic myeloid expansion (23). Alternatively, excessive TNF signaling reduces myelopoiesis by inducing caspase-3/caspase-8-dependent progenitor cell apoptosis (24). Excessive TNF, TLR signaling, and deficiencies in negative regulation of NF-κB lead to apoptosis of HSCs and defects in myeloid progenitor function (23, 25).

We and others have shown that burn and radiation injuries lead to increased susceptibility to infection within survivors (4, 6, 26). This is a pressing clinical problem in the face of nuclear accidents and possible incorporation of nuclear materials within explosives. This susceptibility has been attributed to a loss of inflammatory regulation, incomplete immune restoration and a systemic anti-inflammatory response following sepsis and shock (27–29). Following a radiation-thermal combined injury (RCI), an immature monocyte population (iMo) rapidly expands and predominates the periphery (26). Using this model, we observed that TNF is significantly increased in RCI compared to burn, radiation, and sham alone (26).

Given that NLRP12, which is known to suppress a number of cytokines, is present in bone marrow and myeloid cells (1, 25), we tested NLRP12-mediated regulation of TNF signaling within the context of emergency myelopoiesis. Unexpectedly, we demonstrate that NLRP12-deficient mice are vulnerable to RCI due to decreased myelopoiesis.

Methods

Mice and Combined Irradiation and Burn Injury procedure

The *Nlrp12*$^{-/-}$, *Caspase1/11*$^{-/-}$, *Asc*$^{-/-}$ and *IL-1Ra*$^{-/-}$ mouse strains have been described (30–33). All experiments were conducted with female mice housed under SPF conditions that were age-matched and backcrossed for at least nine generations onto the C57BL/6 background. All studies were conducted in accordance with the IACUC guidelines of the University of North Carolina at Chapel Hill and NIH Guidelines for the Care and Use of Laboratory Animals. Our model of RCI has been previously described (26); briefly, mice received a subcutaneous injection of morphine (3mg/kg body weight) for pain control immediately before burn injury. A full-thickness contact burn of 20% total body surface area (TBSA) was produced and within 1 hour, mice received a 5Gy (dose rate of 0.98 Gy/min) whole-body dose of ionizing radiation and were maintained on oral morphine (0.4mg/ml) for the duration of the experiment. Sham controls with 0% TBSA underwent all described interventions except for the burn and γ-irradiation exposure.

Quantitative RT-PCR

RNA was extracted from organ homogenates, suspended in TRIzol and isolated according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). qPCR was performed using the Verso 1-step RT-qPCR SYBR Green Fluorescein Kit (Thermo Fisher, San Jose, CA). The expression of mouse mRNA encoding NLRP12 and GAPDH was assessed using the SYBR kit and analyzed on an Applied Biosystems machine; results were normalized to expression of the gene encoding GAPDH and were quantified by the change-in-threshold method ($\Delta\Delta C_T$) using primers previously described (18).

Histology

Mouse femurs were extracted and muscle and connect tissue were removed and initially preserved in 10% formalin. Femurs were then decalcified with Immunocal, waterwashed, and paraffin infused. Following sectioning and processing, sections were then stained by hematoxylin and eosin. Samples were processed using ImageJ to determine area of cell loss within each femur.

Pseudomonas aeruginosa infection

A wildtype strain (PAK) of *P. aeruginosa* was obtained from M. Wolfgang (University of North Carolina, Chapel Hill, NC). 10^6 bacteria were then aerosolized intratracheally as described previously (26).

Serum Collection and Cytokine ELISA

Animals underwent a submandibular bleed and systemic cytokines were measured by single-plex ELISA (eBioscience, CA, USA or Biolegend, CA, USA) according to the manufacturer's instructions or by Cytokine Mouse 20-Plex Panel (Life Technologies, Carlsbad, CA) on Luminex Bead Array technology.

Flow Cytometry

All fluorescence-conjugated FACS antibodies were purchased from BD Biosciences or Biolegend. Antibody panel used to identify neutrophils and macrophages are described in the figures. The antibody panel for monocyte and neutrophil analysis was comprised of CD11c-PerCPCy5.5, CD11b-PECy7, Ly6G-APC, Ly6G-PE, and F4/80-FITC. The antibody panel for progenitor analysis was comprised of CD3, CD8, NK1.1, CD19, CD45RA, TER-119 (Ly-76) as a lineage negative gate with all antibodies conjugated to FITC, CD127-PE/CF594, Sca1-APC, cKit-BUV395, FcγR-BV605, CD34-Alexa647, and Annexin V-Pacific Blue. In each case, a million cells per organ were used for flow cytometric analysis.

Intracellular Staining and Phospho-flow cytometry

Intracellular staining was performed using a BD Bioscience Cytofix/Cytoperm kit. Antibodies used were TNF-PE (BD Biosciences), phosphor-p65 S528 (BD Biosciences), phospho-IκBa S32/536-eFlour 660 (eBiosciences), phospho-p38 ST180/Y182-PE and phospho-IKKα/β S176/180-PE (Cell Signaling Technologies). In each case, a million cells per organ were used for flow cytometric analysis.

TNF-Depletion

Immediately following combined irradiation and burn injury, mice were given 25mg/kg of rat IgG1, kappa anti-mouse TNF, clone MP6-XT3 or ratIgG1 isotype control (eBioscience, CA, USA) intraperitoneally dissolved in PBS (Sigma, CA, USA).

Statistical Analysis

Analysis was carried out with Prism 7.0 for Windows. All data are presented as the mean +/− standard error of the mean (SEM). Complex data sets were analyzed by analysis of variance (ANOVA) with a Tukey-Kramer post-test HSD for multiple comparisons. Single data points were assessed by the Student's two-tailed t test. For the CFU assays, we set the CFU at 10^0 if they fell below the theoretical limit of detection (10^1) for the assay; i.e treated as "0" regardless of their absolute values and did not include in the statistical analysis. The product limit method of Kaplan-Meier was utilized for generating the survival curves, which were compared using the log rank test. A p value less than 0.05 was considered statistically significant for all data sets.

Results

NLRP12 limits morbidity and mortality following RCI

Previous work has implicated NLRP12 in suppression of canonical and non-canonical NF-κB, a key driver of inflammatory cytokine signaling (14, 15, 17, 18, 21, 34). We therefore investigated whether NLRP12 was acting to limit excessive inflammatory signaling and consequently promote peripheral immune reconstitution in our model of emergency myelopoiesis.

Wild type and *Nlrp12*$^{-/-}$ mice received a 20% TBSA burn and were irradiated with 5-Gy of γ-irradiation within an hour of burn injury. In wild type mice, we observed elevated NLRP12 expression in spleen, bone marrow and lung tissues early (3, 7, and 14 days post-

injury) after RCI (Figure 1A) compared to burn or radiation alone and sham controls. Mortality among NLRP12-deficient mice was significantly elevated following RCI, but not following burn or radiation alone (Figure 1B). While RCI-wild type animals lost weight initially, they were able to return to a baseline weight by seven days after injury and exceed their baseline weight by 14 days post injury; RCI-*Nrlp12−/−* animals lost more weight and did not fully recover weight in comparison to wild type animals (Figure 1C). These data suggest that NLRP12 protected against morbidity after RCI.

Splenic and pulmonary immune repopulation is impaired following RCI in *Nlrp12*$^{-/-}$ mice

During events that induce enhanced myelopoiesis and inflammation, specifically RCI, we have shown that immature monocytes with high granularity comprise the majority of the peripheral immune system (26). We examined the splenic compartment in the *Nlrp12−/−* mice after RCI. NLRP12 deficiency resulted in a significant decrease in the total number of splenocytes by 14 days post-injury (Figure 2A). Using flow cytometry, with representative staining in Figure 2B, we observed a decreased number of splenic neutrophils ($CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ $F4/80^-$) and immature monocytes (iMOs; $CD11b^+$ $Ly6C^+$ $Ly6G^{hi}F4/80^{hi}$) post-injury in *Nlrp12−/−* mice (Figure 2C). We also investigated the contribution of NLRP12 to the repopulation of lung immune cells (common sites of opportunistic infection in burn patients) after RCI. *Nlrp12−/−* mice displayed a reduced ability to repopulate the lung after RCI. This inability was characterized by a decrease in total $CD45^+$ leukocytes and by the absence of the immature monocyte accumulation normally observed following RCI at two weeks post-injury (Figure 3A–B). There were no differences in macrophage ($CD11b^+$ $Ly6C^+Ly6G^{lo}F4/80^{hi}$) accumulation in *Nlrp12−/−* mice when compared to wild type (Figure S1A). The total number of pulmonary macrophages, B and T cells were similar in *Nlrp12−/−* and wild type mice (Figure S1B–D). These data implicate a role for NLRP12 in regulating emergency hematopoiesis following RCI.

Nlrp12−/− mice show decreased bone marrow and peripheral cell numbers following RCI

NLRP12 has been shown to be expressed constitutively in bone marrow cells (15, 18, 19, 35). We hypothesized that reduced immune repopulation in the periphery of injured *Nlrp12*$^{-/-}$ mice was due to reduced cell generation and output by the bone marrow. To test this, we investigated the impact of NLRP12 deficiency on bone marrow populations after RCI. *Nlrp12*$^{-/-}$ mice had reduced total bone marrow cells compared to wild type mice after RCI. We also observed a decrease in total iMO and neutrophils (Figure 3C) within the bone marrow of *Nlrp12*$^{-/-}$ mice as early as seven days post injury compared to wild type mice. Additionally, we observe decreases in the total numbers of monocytes and neutrophils in the peripheral blood (Figure 3D). These data suggest that peripheral immune repopulation defects after RCI are likely attributed to decreased bone marrow cell numbers, which appear to be regulated by NLRP12.

Defects in myelopoiesis following RCI are not observed in inflammasome-deficient animals

NLRP12 is also found to form an inflammasome complex or regulate caspase-1 activity (14, 17, 20) and regulates IL-1β processing by complexing with ASC during infection with *Yersinia* or malaria (14). To examine whether NLRP12 is important for inflammasome

activation following RCI, we assessed IL-1β levels after RCI in wild type or *Nlrp12−/−* mice. There were no detectable levels of IL-1β at any time point measured (Figure S2A). Due to inability to capture IL-1β levels in serum because of its high turnover, we examined the role of the inflammasome in *RCI*. We applied the RCI model to various mice strains lacking key components of genes encoding proteins that encode common shared components of the inflammasome. These include *Caspase1/11−/−* which lacks both canonical and noncanonical inflammasome caspases, *Asc−/−* which lacks the common adaptor shared by multiple inflammasome NLRs and AIM2, or *Il1r−/−* which lacks the IL-1 receptor protein. Following RCI, *Caspase1/11−/−, Asc−/−* and *Il1r−/−* mice had a similar immune repopulation in the lung and spleen as wild type mice (Figure S2B–C). We also saw no significant differences in bone marrow populations in *Caspase-1/11−/−*, *Asc−/−* or $Il1r^{-/-}$ mice following RCI (Figure S2D). Additionally, injured *Caspase-1/11−/−*, *Asc−/−* or $Il1r^{-/-}$ animals did not show an increase in mortality compared to wild type (Figure S2E). Together, these results suggest that NLRP12 controls myelopoiesis in an inflammasome-independent pathway.

Nlrp12$^{-/-}$ mice display increased serum TNF, IL-6 and IL-12 cytokine and bone marrow TNF- receptor expression

Cytokines that are attenuated by NLRP12, in particular TNF, have been shown to enhance hematopoietic stem cell (HSC) expansion (2, 5, 10, 25). We therefore examined NLRP12-dependent production of selected cytokines and their receptors after RCI. In wild type animals, serum TNF expression increased early following injury and declined over time (26). In sham-treated $Nlrp12^{-/-}$ animals, the TNF level was similar to sham wild type controls. However, during RCI in $Nlrp12^{-/-}$ animals, the TNF increased initially and was maintained over time – a significant elevation when compared to wild type mice (Figure 4A). In addition, $Nlrp12^{-/-}$ bone marrow cells displayed increased TNFR expression (Figure 4B) as well as CD40 and RANK (Figure 4C). Using intracellular straining, we observed that monocyte production of TNF after RCI is increased when compared to burn and radiation controls; however, in the absence of NLRP12, monocyte production of TNF is significantly elevated compared to wild type controls (Figure 4D). As well as TNF, other cytokines such as IL-6, IL-12, IFNα and IFNγ were increased in $Nlrp12^{-/-}$ mice compared to wild type mice but less so than TNF (Figure S3A). This elevation is potentially derived from the initial shock and the selective apoptotic environment induced by the absence of NLRP12 and necessary myelopoiesis. Heightened levels of IL-6 following trauma have been shown to be the major predictor of poor outcome (bacterial infection) following a traumatic injury (36). Other cytokines and growth factors (IL-4, IL-10, and GM-CSF) were measured but showed no significant differences between wild type and *Nlrp12−/−* animals (Figure S3B).

IκBa activity is increased in CD34+ cells *Nlrp12−/−* animals after RCI

Both the canonical and non-canonical pathways of NFκB have been shown to be negatively regulated by NLRP12 (13, 17, 22). We therefore examined NLRP12-dependent activation of key regulators of each pathway. No changes were seen in phosphorylation levels in sham, burn, or radiation controls; however, RCI NLRP12-deficient animals showed greater levels of pIκBα as well as pp65 (Figure 4D). Increased phosphorylation is indicative of increased canonical NFκB signaling in the absence of NLRP12. However, pIKKα/β and the

downstream p38/MAPK showed no changes in activity when comparing wild type to *Nlrp12−/−* injured animals. Taken with the increased TNFR expression on bone marrow cells, these results suggest that NLRP12 is negatively regulating the canonical NFκB signaling cascade.

RCI of *Nlrp12−/−* animals leads to increased granulocyte/monocyte progenitor apoptosis

We observed that NLRP12 regulates reconstitution of granulocytic and monocytic bone marrow and peripheral cells in *Nlrp12−/−* mice following RCI. We therefore hypothesized that NLRP12 regulates bone marrow granulocyte/monocyte progenitors (GMP), the source of granulocytes and immature monocytes. To test this, we utilized flow cytometric analysis to evaluate the number of GMP (Lin^- $IL7R^-$ $Sca1^-$ $cKit^+$ $Fc\gamma R^{hi}$ $CD34^+$) in *Nlrp12−/−* mice following RCI.

We detected a similar number of bone marrow GMP in $Nlrp12^{-/-}$ and wild type mice at 3 days after injury. However, at 7 and 14 days after injury, wild type GMP expanded and increased in numbers while $Nlrp12^{-/-}$ GMP expansion was attenuated (Figure 5A). There were no measured differences in lymphoid lineage progenitors (Figure S4). We tested the hypothesis that the significant decrease in GMP in $Nlrp12^{-/-}$ mice is due to increased apoptosis. To distinguish apoptotic cells from necrotic cells, 7-AAD and Annexin V staining was performed. While sham control revealed no difference in WT and $Nlrp12^{-/-}$ mice, a significant percent of $Nlrp12^{-/-}$ GMP underwent apoptosis compared to wild type GMP during RCI. This increase in apoptosis was detected as early as 3-days post injury (Figure 5B). Representative flow gating is shown in Figure 5C.

Increased apoptosis and decreased bone marrow cellularity was confirmed by histological staining. H&E femur sections were obtained at 14 days post injury. There were no histological changes from wild type to *Nlrp12−/−* mice in sham, burn, or radiation alone animals. However, RCI-*Nlrp12−/−* mice displayed medial patches of cell loss within the femurs, which was not present in RCI-wild type femurs (Figure 5D), and quantified by imaging analysis in Figure 5E. Collectively, our findings imply that NLRP12 prevents progenitor cell apoptosis, thus allowing myelopoiesis and peripheral immune cell reconstitution to occur in wild type animals.

Leukopenia can have complex etiologies in both inflammatory and non-inflammatory conditions, many of which involve alterations in HSC steady-state hematopoiesis (9, 10, 37). Our data show that NLRP12 limits TNF following RCI, resulting in expansion of myeloid precursors and monocyte populations throughout the periphery. Previous studies showed increased hematopoiesis following total body irradiation; however, our results are novel because we have shown that NLRP12 promotes hematopoiesis of specific lineages during RCI (1, 26).

Anti-TNF antibody administration prevents NLRP12-associated GMP apoptosis after combined injury

After observing significantly elevated levels of TNF and reduced myelopoiesis in injured *Nlrp12−/−* mice, we hypothesized that increased levels of TNF were leading to pathology through TNF-mediated apoptosis of immune progenitor cells as seen in other models of

excessive TNF(38). Specifically, we hypothesized that GMP were undergoing TNF-mediated apoptosis with reduced peripheral neutrophil and inflammatory monocyte accumulation. To test this, wild type and *Nlrp12−/−* mice received a single administration of anti-TNF or isotype control antibody immediately following RCI.

We observed significantly fewer GMP in the *Nlrp12−/−* mice given the isotype control compared to wild type mice. However, *Nlrp12−/−* mice given the anti-TNF antibody had similar numbers of GMP compared to isotype and anti-TNF treated wild type mice (Figure 6A). Additionally, the proportion of GMP actively undergoing apoptosis was higher in the *Nlrp12−/−* isotype treated animals compared to *Nlrp12−/−* mice treated with anti-TNF (Figure 6B). This is correlated with a decrease in the total CD45+ pulmonary cells as well as pulmonary iMO (Figure 6C). We also observed no differences in overall mortality/morbidity compared to wild type mice and a reversal of the RCI-dependent bone marrow apoptosis by histology (data not shown). These data indicate that in the absence of NLRP12, TNF mediates the enhanced bone marrow death during RCI and resultant incomplete restoration of the peripheral immune system.

Nlrp12−/− mice lack control of pulmonary infection following radiation-thermal combined injury

In a clinical setting, patients that are able to survive initial shock from a burn or radiation-thermal-combined injury will often succumb to a pulmonary infection associated with the prolonged hospital stay (34). We sought to evaluate the role NLRP12 deficiency plays in a clinically relevant model of a lung infection following injury. Wild type and *Nlrp12−/−* animals were subjected to either sham, burn, or radiation only, and RCI. Mice were then sustained for two weeks in individual housing wherein they were infected intratracheally with 1×10^6 CFU of *Pseudomonas aeruginosa* (PAK).

RCI-*Nlrp12−/−* infected animals displayed a significant increase in mortality, with animals starting to succumb to infection after as few as 12 hours (Figure 7A), significantly earlier than all other control groups, even radiation injured controls. Lung and liver from infected animals that survived until 48 hours post infection were collected and plated to enumerate bacterial load locally and systemically. RCI-*Nlrp12−/−* mice lungs and liver showed a 10-fold increase in bacteria compared to injured, wild type animals (Figure 7B). We consistently observed that every mouse became infected within the RCI injured *Nlrp12−/−* groups, unlike every other group that frequently contained mice that cleared the infection. These data suggest that NLRP12 plays a vital role in response to an infection insult following a traumatic injury.

We next sought to determine the immune response to infection following RCI. *Nlrp12−/−* mice showed a decrease in innate, pulmonary immune cell populations following RCI and infection (Figure 7C), leading us to conclude that NLRP12 results in increased hematopoietic recovery which is likely crucial to the effective control of infection after traumatic injury.

Discussion

Our study demonstrates that NLRP12 suppresses TNF signaling *in vivo* during inflammation-induced emergency myelopoiesis. Most importantly, our research indicates a role for NLRP12 in hematopoietic progenitor cells by limiting TNF-induced apoptosis of these cells. TNF inflammation initiated by RCI without NLRP12 leads to the apoptosis of progenitor cells and a defective peripheral immune reconstitution, associated with increased mortality and inability to control an infectious challenge.

In addition to inhibiting inflammation, defects in NF-κB signaling lead to weakened hematopoiesis (25). There is no single mechanism that has been defined for immune suppression in the response to traumatic injury, but hematopoietic stem cell (HSC) expansion and immune repopulation have been shown to be important factors (2, 5, 10, 25, 37).

We propose that NLRP12 suppression of immune signaling pathways leading to attenuated cytokines contributes to homeostatic proliferation of granulocytes and monocytes following induction of severe leukopenia. Moreover, this NLRP12-mediated suppression limits overt TNF-induced inflammation that could lead to HSC apoptosis by limiting canonical NFκB signaling. Our findings add to the studies that suggest that NLRP12 acts as a cellular rheostat to limit inflammation, and is emerging as a “checkpoint” or inhibitor (17, 22) of canonical NFκB signaling (17, 32, 39, 40). Although we demonstrate increased p-IkB and pp65 levels in CD34+ cells from NLRP12 deficient mice undergoing RCI, suggestive of increased canonical NFkB signaling, it does not rule out a role for the non-canonical pathway, which could be evaluated by measuring either NIK or p100 processing. NLRP12-mediated NFκB suppression likely limits TNF and cellular death during inflammation and hematopoiesis. Our data in NLRP12-deficient mice shows compromised hematopoiesis due to enhanced TNF production, leading to flagrant HSC/GMP apoptosis. This lack of HSC function leads to global leukopenia and correlates with increased mortality compared to wild type mice.

We have evaluated reconstitution as best we can; for the clinical situation that we are most interested in, namely infection control after combined injury, we were interested in functional reconstitution which we demonstrate by control of *Pseudomonas*. The cellular reconstitution is not optimal, rather, we have uncovered a “last ditch” attempt by the organism to reconstitute albeit with immature monocytes and a gene which appears to control this response. These studies would be applicable in instances of increased myelopoiesis, TNF-driven inflammation, and induced apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge the UNC Flow Cytometry Core Facility, UNC Microscopy Services Laboratory, Lineberger Comprehensive Cancer Center’s Animal Histopathology Core Lab, Danier Moore, Wesley Stepp,

Rebecca Drapp, Sha'Leema Miller, Lindsey Glenn, Lucas Sjeklocha and Steven Mouro for their technical support and advice.

Sources of support: NIH DUMC/NIAID-RADDCORE #203-2315 and NIH NIGMS 5R01GM076250.

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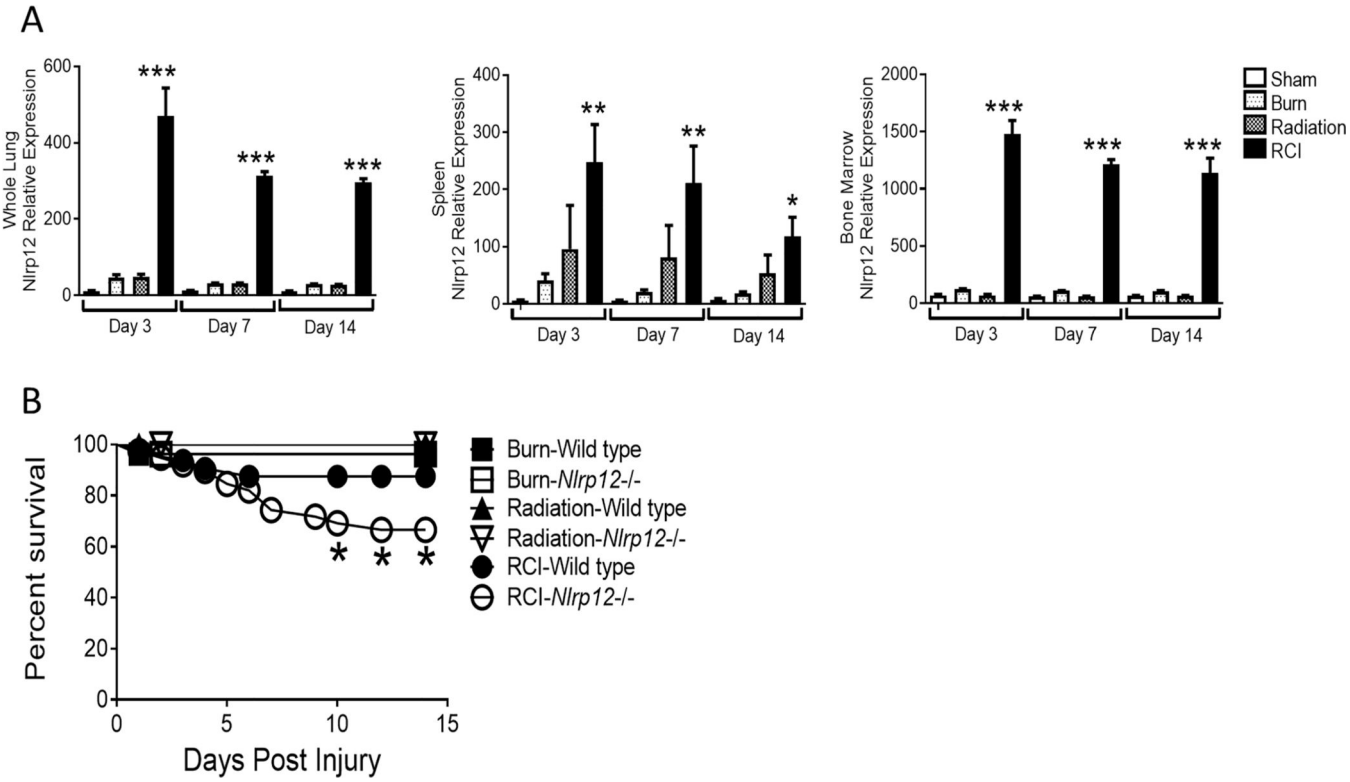

Figure 1. NLRP12 expression is increased after combined injury, and acts to limits mortality and weight loss

Wildtype C57BL/6 mice were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or a combined injury (RCI). (A) mRNA was isolated from spleen, bone marrow, and whole lung at 3, 7, and 14 days post injury. Relative *Nlrp12* - expression was determined by qRT-PCR. (n=6/timepoint). Wildtype C57BL/6 or *Nlrp12−/−* mice were subjected to sham, 5Gy of γ-irradiation, a 20% total body surface area burn or RCI. (B) Survival and (C) weight loss were quantified. Data represented as mean ± SEM, with statistical significance compared to sham defined as *, $p<0.05$, **, $p<0.005$ and ***, $p<0.001$ by Student's t test, with experiments performed in triplicate.

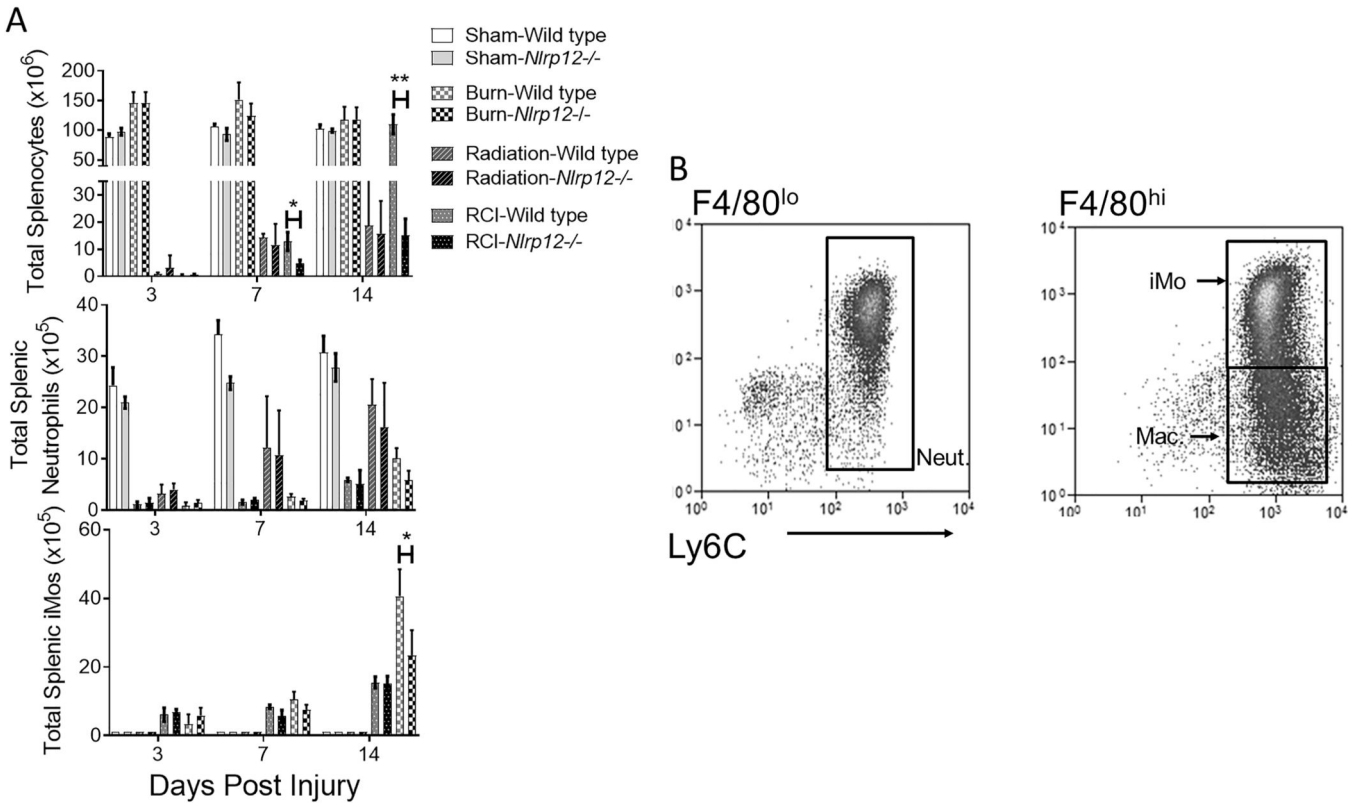

Figure 2. NLRP12 regulates peripheral immune repopulation after combined injury

Wildtype C57BL/6 or *Nlrp12−/−* mice were subjected to sham or combined radiation and burn injury (RCI). Spleens were harvested 3, 7 and 14 days post injury and the total number of (A) splenocytes, (C)neutrophils (CD11b+ Ly6C^{int} Ly6G+ F4/80−) and immature monocytes (iMOs; CD11b+ Ly6C+ Ly6G^{hi}F4/80^{hi}) were quantified by flow cytometry analysis, (B) representative flow cytometric gating from an RCI mouse after gating on CD45+ and F4/80 expression level). Data represented as mean ± SEM, with statistical significance defined as *, $p<0.05$ and **, $p<0.005$ by Student's t test with n=10 mice per group, with experiments performed in triplicate.

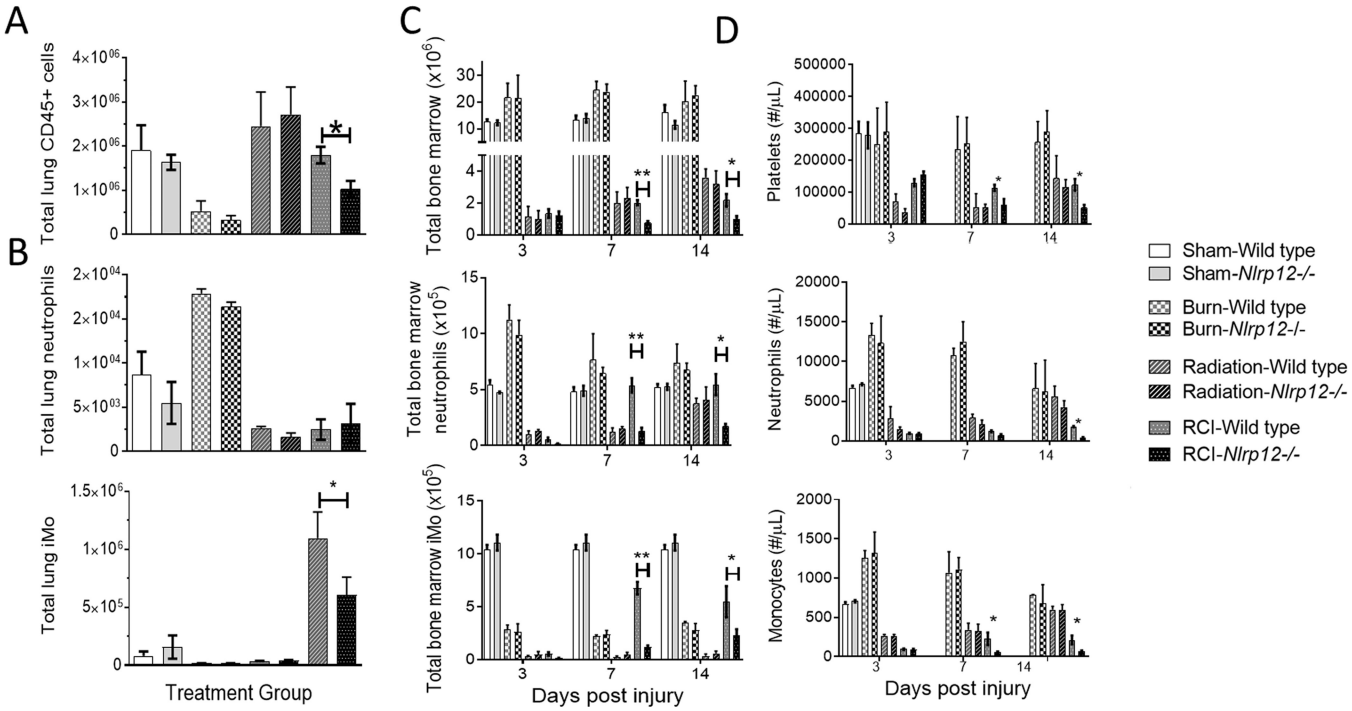

Figure 3. NLRP12 regulates pulmonary immune repopulation and bone marrow cell numbers after combined injury

Wildtype C57BL/6 or *Nlrp12−/−* mice were subjected to sham or combined radiation and burn injury (RCI). Lungs were harvested 14 days post injury and the total number of (A) CD45+ cells, (B) neutrophils (CD11b+ Ly6C^{int} Ly6G+ F4/80−) and immature monocytes (iMOs; CD11b+ Ly6C+ Ly6G^{hi}F4/80^{hi}) were quantified by flow cytometry analysis. Wildtype C57BL/6 or *Nlrp12−/−* mice were subjected to sham or combined radiation and burn injury (RCI). Bone marrow from femurs and tibias and blood from a cheek bleed were harvested 3, 7 and 14 days post injury and the total number of (C) bone marrow cells, immature monocytes (iMOs; CD11b+ Ly6C+ Ly6G^{hi}F4/80^{hi}), neutrophils (CD11b+ Ly6C^{int} Ly6G+ F4/80−) from the bone marrow and platelets (CD62P+TER119−), monocyte and neutrophils from blood were quantified by flow cytometry analysis. Data represented as mean ± SEM, with statistical significance defined *, $p<0.05$, **, $p<0.005$ and ***, $p<0.001$ by Student's t test with n=6 mice per group, with experiments performed in triplicate.

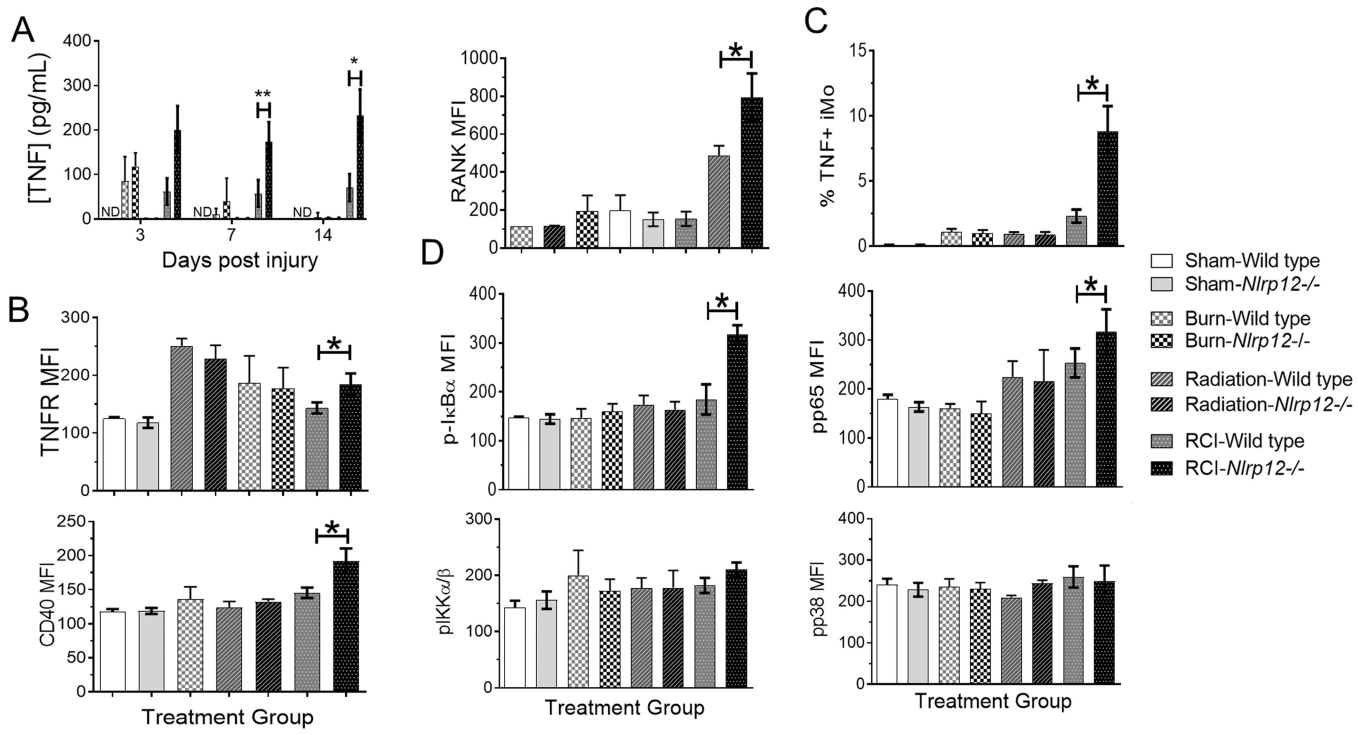

Figure 4. *Nlrp12−/−* animals have increased serum cytokine and bone marrow receptor expression following combined injury

Wildtype C57BL/6 or *Nlrp12−/−* mice were subjected to sham or combined radiation and burn injury (RCI). The concentration of (A) TNF was quantified using ELISA in serum 3, 7, and 14 days post injury. We also analyzed mean fluorescent intensity of (B) TNFR, CD40, and (C) RANK on bone marrow cells harvested at 14 days post injury using flow cytometry. (D) The percentage of TNF producing iMos was determined using intracellular staining and flow cytometry. (E) The level of phopso-IκBα, phospo-IKKα/β, phosphor-p65, and phospho-p38 was quantified using intracellular staining and flow cytometry. Data represented as mean ± SEM, with statistical significance defined as ** $p<0.005$ by Student's t test with n=5 mice per group, with experiments performed in triplicate.

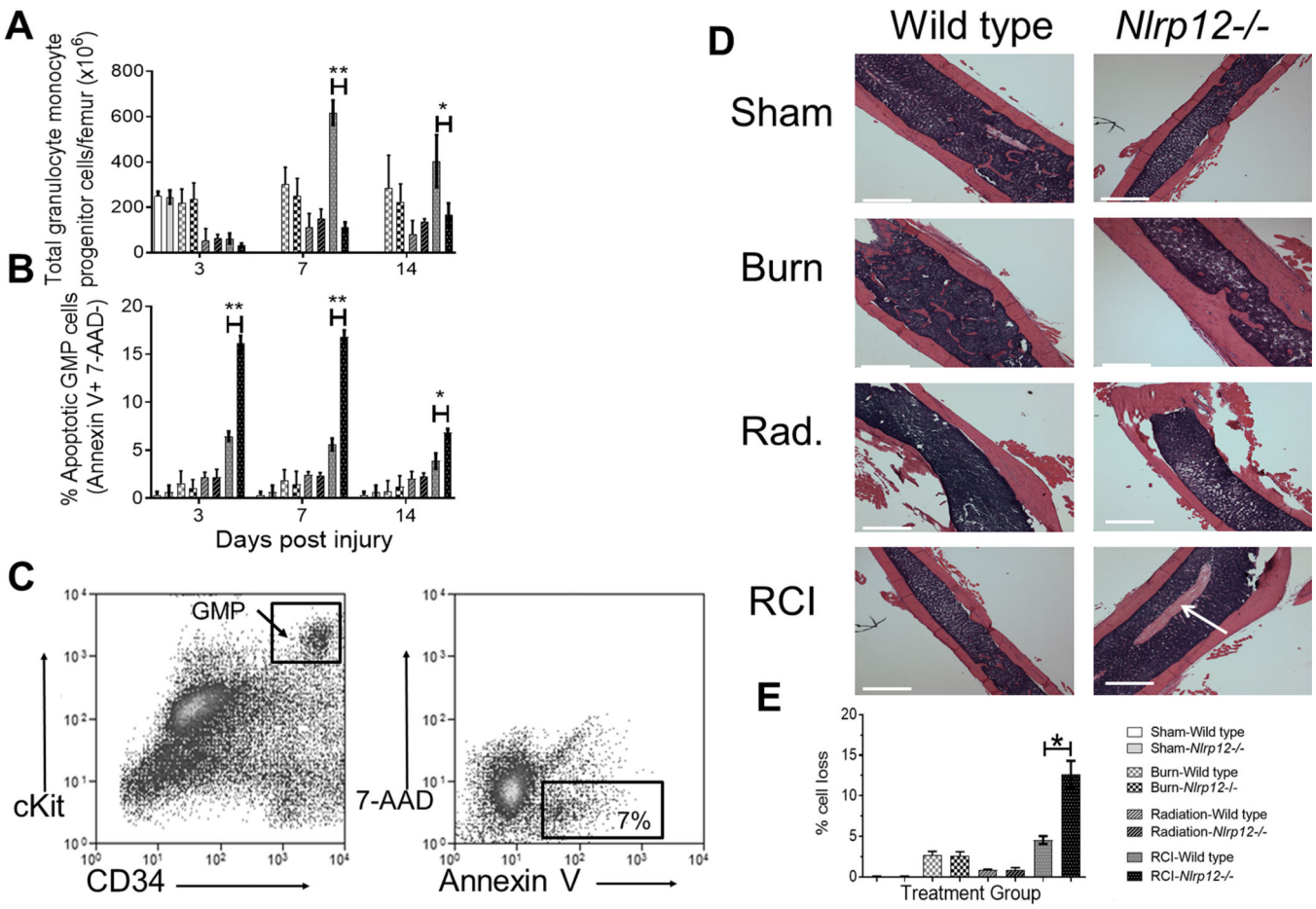

Figure 5. *Nlrp12*−/− animals have increased granulocyte/monocyte progenitor apoptosis after combined injury

Wildtype C57BL/6 or *Nlrp12*−/− mice were subjected to sham or combined radiation and burn injury (RCI). Bone marrow was collected from wild type and *Nlrp12*−/− mice at 3, 7, and 14 days post RCI or sham treatment (n= 6/group). Using flow cytometric analysis, (A) the total number of bone marrow Granulocyte/Monocyte Progenitors (GMP, Lin− IL7R− Sca1− ckit+ FcγR^{hi} CD34+) and (B) the percentage of GMP cells undergoing apoptosis was determined by positive Annexin V staining in the absence of 7-AAD− staining cells; representative flow staining from *Nlrp12*−/− mice is shown is shown in (C). Data represented as mean ± SEM, with statistical significance defined *, p<0.05, **, p<0.005 and ***, p<0.001 by Student's t test with n=5 mice per group. In separate experiments, wildtype C57BL/6 or *Nlrp12*−/− mice were subjected to sham, irradiation, burn or RCI. Femurs were collected 14 days post injury and H&E staining performed for histological analysis. (D) shows representative sections from each group, with areas of cell death marked by white arrow (white bar represents 25um;), (E) quantification of cell death area was performed using ImageJ, with experiments performed in triplicate.

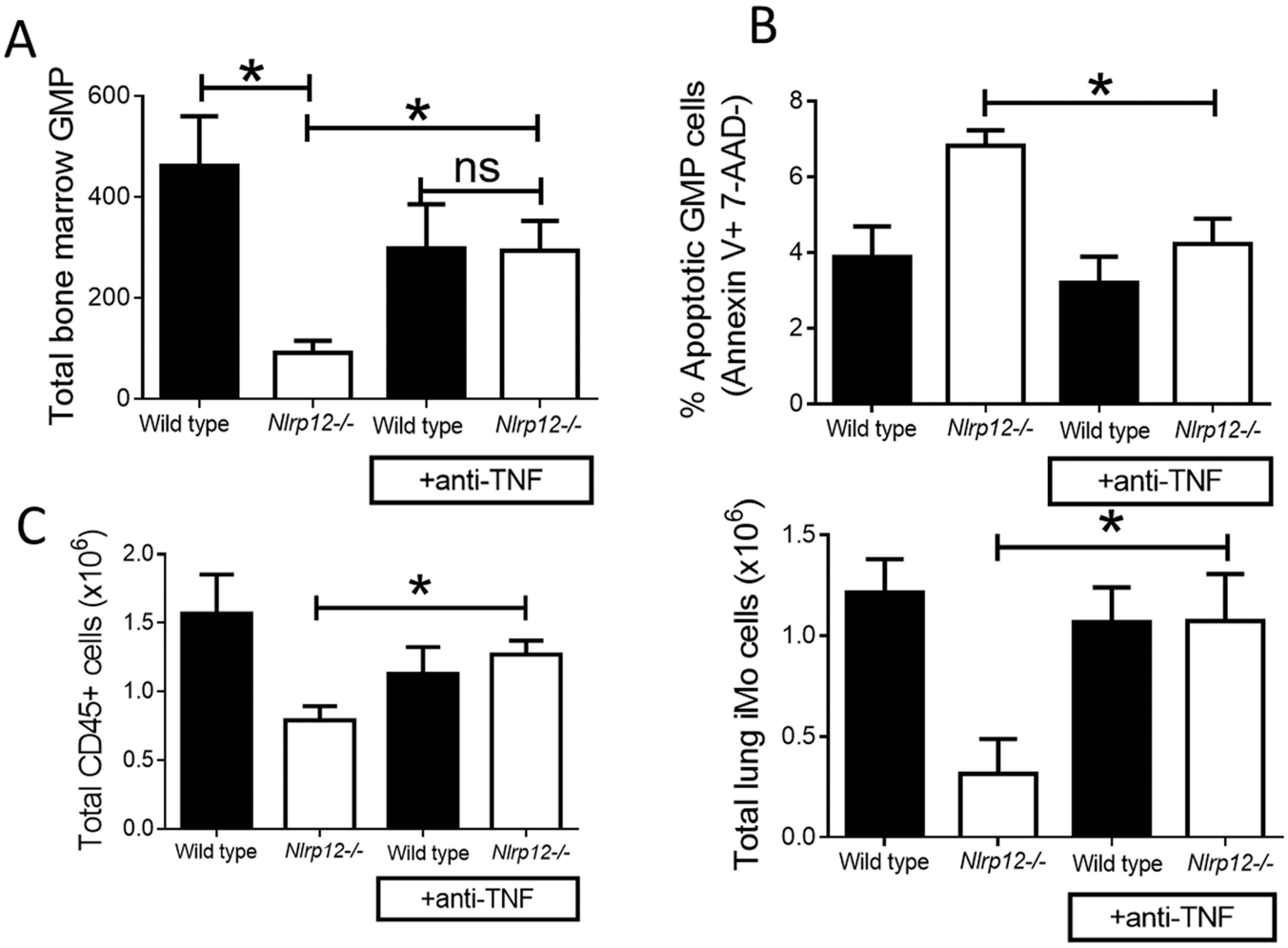

Figure 6. Anti-TNF antibody administration prevents NLRP12-associated GMP apoptosis after combined injury

Wild type and *Nlrp12−/−* C57/BL6 mice received a single administration of anti-TNF or isotype control antibody immediately following combined radiation and burn injury (RCI). We harvested bone marrow and lung from these mice 14 days after injury. We quantified (A) the total number of bone marrow Granulocyte/Monocyte Progenitors (GMP, Lin− IL7R− Sca1− ckit+ FcγR^{hi} CD34+) and (B) the percentage of GMP cells undergoing apoptosis by 7-AAD− Annexin V+ staining by flow cytometry. We measured (C) the total number of pulmonary CD45+ cells and immature monocytes (iMOs; CD11b+ Ly6C+ Ly6G^{hi}F4/80^{hi}) by flow cytometry analysis. Data represented as mean ± SEM, with statistical significance defined as *, $p<0.05$ by Student's t test with n=5 mice per group, with experiments performed in triplicate.

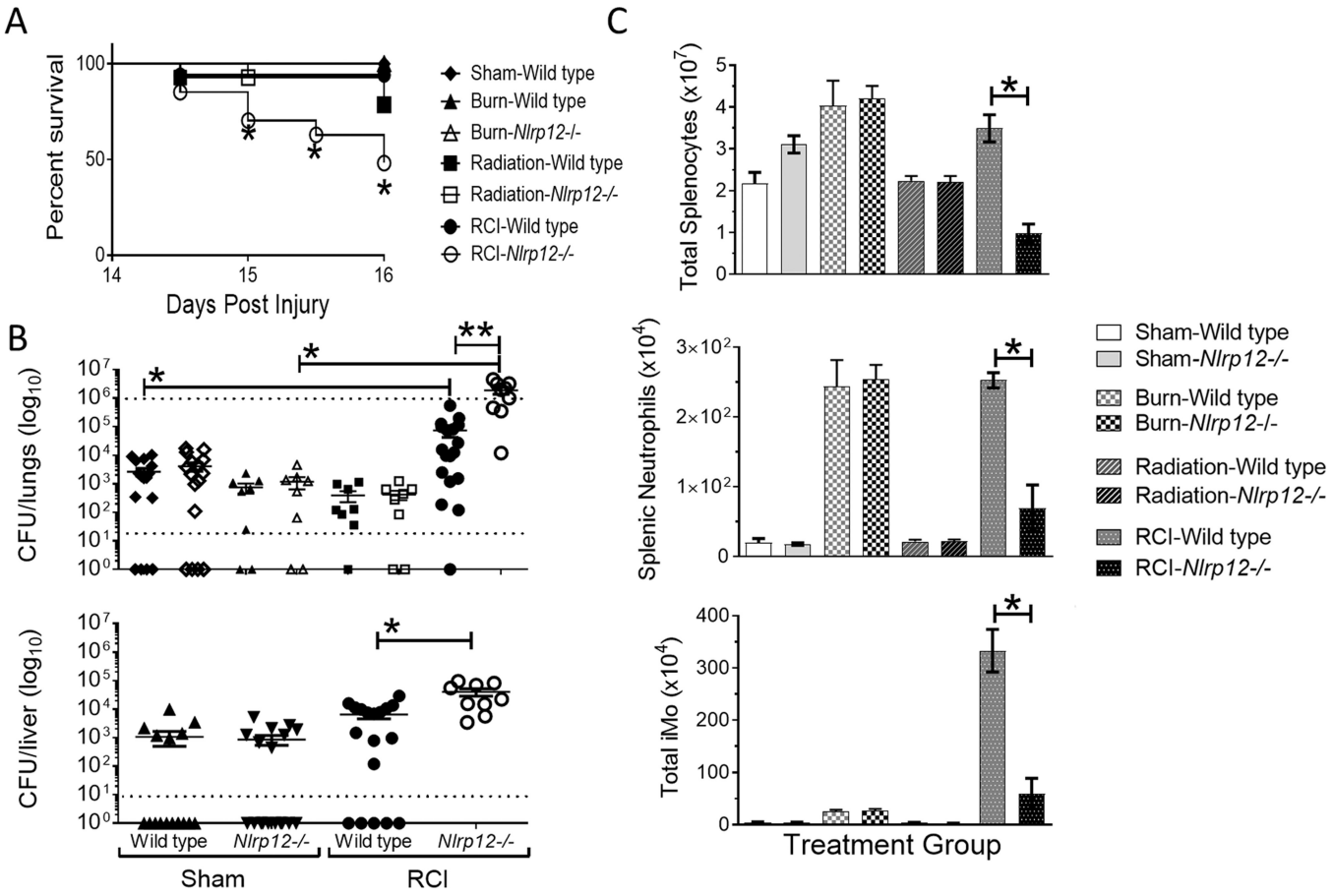

Figure 7. *Nlrp12*−/− mice lack control of pulmonary infection following combined injury

Wildtype C57BL/6 or *Nlrp12*−/− mice were subjected to sham or combined radiation and burn injury (RCI). Mice were inoculated 14 days post-injury intratracheally with 1×10^6 CFU of *Pseudomonas aeruginosa* (PAK). We quantified (A) survival, (B) bacterial load within lungs and liver by culture, and (C) number of splenic CD45+ cells, neutrophils (CD11b+ Ly6C^{int} Ly6G+ F4/80−) and immature monocytes (iMOs; CD11b+ Ly6C+ Ly6G^{hi}F4/80^{hi}) harvested two days after inoculation. Data represented as mean ± SEM, with statistical significance defined as *, $p<0.05$; **, $p<0.05$ by Student's t test with n=6 mice per group (3 for burn and radiation only), with experiments performed in triplicate.