

IL-1R1-MyD88 axis elicits papain-induced lung inflammation

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Abbreviations: BALF: Broncho-Alveolar Lavage Fluid, DC: Dendritic cells, HDM: House Dust Mite, IL: Interleukin, ILC: Innate Lymphoid cells, MPO: Myeloperoxidase.

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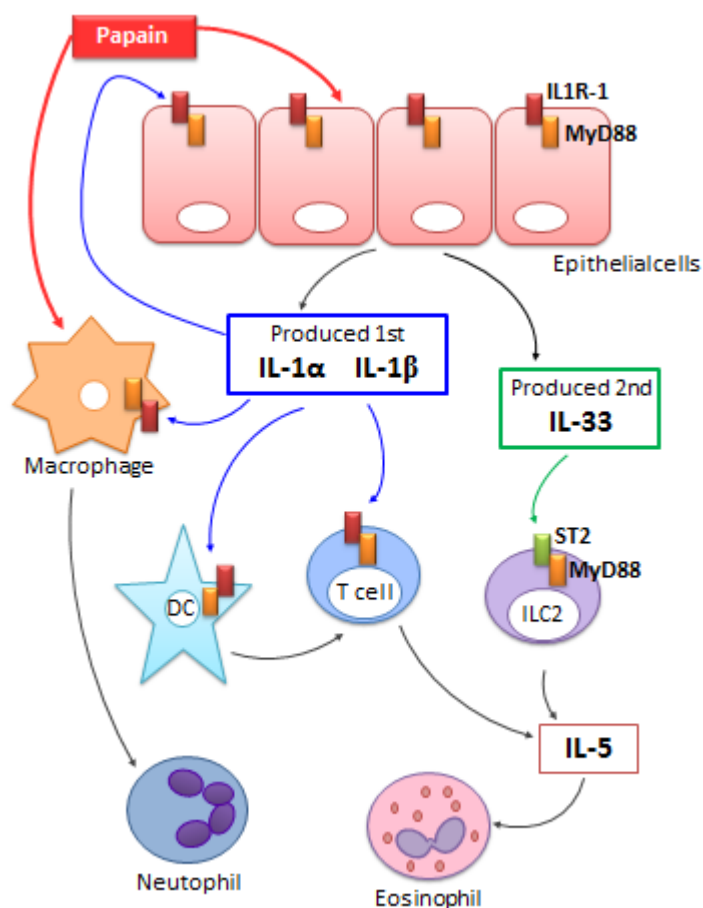
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Abstract

Allergic asthma is characterized by a strong Th2 response with inflammatory cell recruitment and structural changes in the lung. Papain is a protease allergen disrupting the airway epithelium triggering a rapid inflammation with eosinophilia mediated by innate lymphoid cell activation (ILC2) and leading to a Th2 immune response. In this study, we focused on inflammatory responses to a single exposure to papain and showed that intranasal administration of papain results in the recruitment of inflammatory cells, including neutrophils and eosinophils with a rapid production of IL-1 α , IL-1 β , and IL-33. The inflammatory response is abrogated in the absence of IL-1R1 and MyD88. To decipher the cell type(s) involved in MyD88 dependent IL-1R1/MyD88 signaling, we used new cell-specific MyD88 deficient mice and found that the deletion of MyD88 signaling in single cell types such as T cells, epithelial cells, CD11c-positive or myeloid cells leads to only a partial inhibition compared to complete absence of MyD88, suggesting that several cell types contribute to the response. Importantly, the inflammatory response is largely ST2 and IL-36R independent. In conclusion, IL-1R1 signaling via MyD88 is critical for the first step of inflammatory response to papain.



Introduction

Allergic asthma is a multifactorial disease caused by the interaction of environmental and genetic factors. This pathology affects 235 million people worldwide according to World Health Organisation (2015). Sensitization to allergen is one of the first steps involved in asthma. Various allergens, including house dust mite (HDM), fungi, cockroach and pollen have proteolytic activities [1]. Protease properties of allergens cause injury of the airway epithelium with increased permeability, airway remodeling, type 2 cytokine and chemokine production and cell recruitment [2]. Papain, a

cysteine protease, induces type 2 response characterized by interleukin (IL)-5 and IL-13 production, mediated by an IL-2-dependent IL-9 production [3] and specific IgE production [4, 5]. Papain activates the MAPK pathway and the production of pro-inflammatory IL-25 and TSLP, which appears to be independent of MyD88 or TIRAP signaling [6]. However, more recently MyD88 dependence of the allergic response to papain was reported, although not in an airway model of inflammation [7]. The adaptor protein MyD88 is involved in toll-like receptor (TLR) and IL-1 receptor family signaling including IL-1R1, IL-18R, ST2 (corresponding to IL-33R), and IL-36R. We previously reported that the NLRP3 inflammasome and IL-1R1 are involved in allergic asthma [8, 9]. In addition, IL-1 α and IL-1 β , pro-inflammatory cytokines signal through IL-1R1 to promote mast cell activation and Th2 cytokine production [10]. IL-1 α , IL-1 β [11] and IL-1R1 are required in allergic asthma induced by ovalbumin [12]. Regarding other IL-1 family members, it has been shown that papain inhalation induces the release of IL-33 with subsequent IL-33-dependent IL-5 and IL-13 production via the activation of ST2 expressing innate lymphoid cells in the lung. Importantly, the effects of papain on airway eosinophilia were mostly, but not completely, independent of T and B cells [5]. Furthermore, IL-33 KO mice had diminished airway inflammation following activation of innate responses with house dust mite extracts or papain [13]. Conversely, overexpression of IL-33 in transgenic mice led to airway inflammation with eosinophilic infiltration, hyperplasia of goblet cells, and accumulation of mucus-like material [14]. IL-36 cytokines (namely IL-36 α , IL-36 β and IL-36 γ) have been implicated in mouse models of experimental allergic asthma [15]. IL-36 γ was increased in the lungs of mice following sensitization and challenge with house dust mite as well as in A/J mice following challenge with ovalbumin [16, 17]. Intranasal administration of either IL-36 α or IL-36 γ to mice induces a rapid influx of neutrophils into the lungs [16, 18]. Therefore, it is plausible that IL-36R signaling contributes to inflammatory airway episodes in response to allergens and infectious agents leading to asthma exacerbation.

Here we revisited the role of IL-1R family members in the innate inflammatory response to a single exposure to papain. We generated cell specific MyD88 deficient mice to investigate MyD88 signaling in different lung cell types in the inflammatory response to papain. We report IL-1R1 dependent MyD88 signaling is not restricted to epithelial cells, but several other cell types participate. Furthermore, the response is strictly IL-1R1 dependent and IL-33 (ST2) and IL-36R play a minor role in inflammatory response to a single exposure to papain.

Results

Inflammatory cell recruitment and cytokine expression after a single papain intranasal instillation

To investigate the immediate innate immune response of lung to the protease allergen, C57BL/6 mice received a single dose of papain by intranasal instillation. Inflammatory cell recruitment and cytokine production were examined over 72h after papain exposure. Inflammatory cell recruitment in broncho-alveolar lavage fluid (BALF) and in the lung parenchyma was already present at 6h with a maximum at 48h and a progression toward resolution at 72h (Figure 1A-B). Differential cell count (Figure 1A) revealed that the number of eosinophils, lymphocytes and macrophages in BALF reached a maximum at 48h, while neutrophils peaked at 24h. Consistent with the kinetic of neutrophils, myeloperoxidase (MPO) levels in BALF reached a maximum at 18h-24h (Figure 1C). The transcripts of IL-1 α and IL-1 β were expressed at 1h, while IL-33 mRNA and protein, *Ccl24* mRNA, CCL17 and IL-5 proteins peaked at 6h (Figure 1C, E). Histological analysis revealed distinguishable emphysema upon protease administration with moderate inflammatory cell recruitment around bronchial epithelium (Figure 1D). Therefore a single exposure of papain is sufficient to induce inflammatory cell recruitment, emphysema and expression of IL-1 family members such as IL-1 α , IL-1 β and IL-33.

Papain-induced lung inflammation is dependent on MyD88 signaling

It has been suggested that the MyD88 pathway is involved in papain-induced lung eosinophilia and type 2 responses [7, 13]. Thus, we investigated whether the MyD88 adaptor protein is involved in response to single papain exposure by using MyD88^{-/-} mice and analyzing cell recruitment and cytokine production at 1h, 6h and 24h after a single papain exposure, to follow both eosinophil and neutrophil recruitment. Total cell, eosinophil and neutrophil numbers in BALF were markedly reduced in MyD88^{-/-} mice at 1-24h post-exposure, whereas lymphocytes increased at 6h but diminished at 24h in these mice (Figure 2A). The levels of the eosinophil and lymphocyte attractant chemokines CCL11, CCL24, and CCL17, and the concentrations of IL-5 and MPO were also reduced in BALF of MyD88^{-/-} mice (Figure 2B). Microscopically the cell infiltration and emphysema were attenuated in the absence of MyD88 (Figure 2C-D). As shown in Figure 1, *Il1a* mRNA levels peaked at 1h, whereas *Il1b* and *Il33* mRNA levels peaked at 6h post-exposure in wild-type mice. Most importantly, the stimulatory effect of papain on transcript levels of these cytokines was completely abrogated in MyD88^{-/-} mice (Figure 2E). This observation suggests that IL-1 α , IL-1 β and IL-33 production is dependent on MyD88 pathway, and participate to the recruitment of eosinophils and neutrophils. Therefore, MyD88 is required for the development of airway inflammatory response to papain.

Reduction of lung inflammation to papain in the absence of IL-1R1 but not of IL-36R signaling

Since IL-1 α and IL-1 β were rapidly expressed after papain administration and IL-1R1 requires the adaptor protein MyD88 for intracellular signaling, we examined the role of IL-1R1 in papain-induced acute pulmonary inflammation 24h after papain instillation. This time point was chosen because both neutrophils and eosinophils were elevated at 24h. IL-1R1^{-/-} mice exposed to papain showed markedly decreased cell recruitment in BALF, in particular a reduction of neutrophil and eosinophil counts and a trend to have less lymphocytes (Figure 3A) with reduced lung emphysema and cell

infiltration in parenchyma (Figure 3C-D). Reduced inflammation correlated with diminished levels of MPO, chemoattractant chemokines CCL24 and CCL17, and IL-5 (Figure 3B).

Similar to IL-1R1, IL-36R signals through the adaptor protein MyD88 [15]. IL-36 γ promotes neutrophil recruitment in lung and is increased in HDM-induced allergic asthma [16]. Thus, IL-36R^{-/-} and IL-36Ra^{-/-} mice were used to investigate the role of IL-36R signaling in papain-induced lung inflammation. 24h after papain instillation, BALF total cell, eosinophil, macrophage, and lymphocyte levels were not significantly different in IL-36R^{-/-} than in wild-type B6 mice (Supporting Information Figure 1A). BALF neutrophil numbers tended to be lower and MPO levels were significantly reduced in IL-36R^{-/-} mice than in wild-type B6 mice (Supporting Information Figure 1B). However, IL-36Ra^{-/-} mice, that harbor fully biologically available IL-36, did not exhibit any difference in inflammatory responses as compared to wild-type mice (Supporting Information Figure 1A-B). Moreover, IL-36R expression was not affected by papain treatment in B6 mice while IL-36 γ expression just had trend to be lower after 18h-72h post instillation (Supporting Information Figure 1C). These results suggest that the IL-36 pathway does not significantly contribute to lung inflammation in response to a single papain exposure.

Reduced eosinophilia in ST2-deficient mice

IL-33 belongs to the IL-1 family of cytokines and was implicated in eosinophil recruitment and type 2 cytokine production after papain administration. IL-33 signals through the MyD88-dependent pathway, participates to eosinophilia [13] during papain-induced lung inflammation and is essential for IL-5 and IL-13 release [5]. Here, we examined IL-33 receptor ST2/MyD88 axis by exposing ST2^{-/-} mice to papain. ST2^{-/-} mice showed reduced eosinophilia, CCL24, CCL17 and IL-5 production after a single papain instillation (Figure 4A-B). But unlike IL-1R1^{-/-} mice, neutrophil and MPO levels were higher in ST2^{-/-} than in wild-type B6 mice. Accordingly, total cell count was significantly higher in ST2^{-/-}

^{-/-} than wild-type mice (Figure 4A-B). Cell infiltration in the parenchyma and emphysema were similar in ST2^{-/-} and B6 mice, reflecting the neutrophil infiltration (Figure 4C-D). These data indicate that ST2 participates to eosinophilia but not to neutrophil recruitment in response to papain.

Limited contribution of T cells and epithelial cells to papain-induced MyD88-dependent lung inflammation

Several cell types including epithelial cells, DCs, macrophages and lymphocytes may contribute to the papain-induced inflammatory response and type 2 immunity. Since lung inflammation after papain exposure was markedly attenuated in MyD88^{-/-} mice we next examined which cell types participate in the MyD88-dependent response to papain. We generated floxed MyD88 mice, and crossed them with cell-specific Cre recombinase transgenic mice to obtain cell-specific MyD88 deficient mouse lines (Supporting Information Figure 2A). The efficacy and specificity of MyD88 targeting in the different cell types was confirmed by RT-qPCR and in vitro functional assays (Supporting Information Figure 2B-C). No statistical differences were observed in cell recruitment in BALF and IL-5, CCL11, IL-33 and CCL17 cytokine levels between B6 and MyD88^{f/f} (MyD88 conditional allele, see Supporting Information Figure 2A) mice (Supporting Information Figure 3). Following papain instillation, the inflammatory cell recruitment in BALF and the lung parenchyma of mice deficient for MyD88 in myeloid cells (MyD88^{Myel}), airway AT1 epithelial cells (MyD88^{ACID}), T cells (MyD88^{CD4}), and DCs (MyD88^{CD11c}) was similar to that of wild-type B6 mice at 24h (Figure 5A,D,E and Supporting Information Figure 4A). MyD88 depletion in T lymphocytes (MyD88^{CD4}) and AT1 epithelial cells (MyD88^{ACID}) resulted in diminished IL-5 production in BALF, while depletion in myeloid cells (MyD88^{Myel}) and depletion in DCs (MyD88^{CD11c}) did not affect IL-5 levels in BALF (Figure 5B and Supporting Information Figure 4B). Thus, MyD88-dependent signaling in T lymphocytes and AT1 epithelial cells seems to contribute to IL-5 production in response to papain but do not contribute to

the extent of inflammatory responses. To further examine the role of T cells, spleen CD4⁺ T cells were isolated from wild-type B6 mice and administered intravenously to MyD88^{-/-} and MyD88^{+/-} mice prior to papain instillation. The abrogated inflammatory response to papain in MyD88^{-/-} mice was not rescued by the adoptive transfer of MyD88 competent CD4⁺ T cells, thus further indicating that these cells do not contribute to the inflammatory responses to papain (data not shown).

Discussion

Using the allergen papain with protease activity we dissected the inflammatory responses after a single exposure and found a rapid inflammatory cell recruitment and production of IL-1 α , IL-1 β , and IL-33. Lung inflammation was dependent on MyD88-mediated signaling and this seemed attributed mostly to the IL-1R signaling pathway, while other cytokine receptors using the MyD88 adaptor, including ST2 and IL-36R had only limited or no influence in the inflammatory response.

IL-33 is produced in response to papain and was shown to be required for innate type airway inflammation [13]. Reduced papain-induced eosinophilia and release of type 2 cytokines, IL-5 and IL-13, was observed in IL-33-deficient mice [5] consistent with the fact that IL-33/ST2 axis plays a key role in type 2 responses. Type 2 innate lymphoid cells (ILC2) play an important role in papain-induced lung inflammation. Accordingly, Rag2^{-/-}Il2rg^{-/-} mice are protected from eosinophilia and IL-5 production and lung inflammation is recovered upon adoptive transfer of ILC to these mice [19]. Moreover, ROR α deficient mice lacking ILC2 have diminished lung inflammation but intact adaptive immune response after intranasal administration of papain [20], showing that ILC are at least one of the key population involved in papain response and can allow adaptive Th2 activation in an asthma-like model consisting in 2 sensitizations and 2 papain challenges [21]. In addition to ILC2, mast cells express also ST2 and respond to IL-33 stimulation [22]. Recently, it has been shown that IL-2

production by IL-33-stimulated mast cells is able to regulate the inflammatory responses to papain by the expansion of regulatory T cells and the release of IL-10 [23].

Here, we show that IL-1R1, another MyD88-dependent receptor known to be implicated in asthma [8], mediates inflammatory responses to a single papain exposure. Indeed, IL-1 α and IL-1 β are rapidly produced upon papain instillation and lung inflammation is markedly inhibited in IL-1R signaling deficient mice. In contrast, upon papain stimulation IL-33 production appeared at a later time. Furthermore, ST2 deficient mice exhibited only a reduction in eosinophil recruitment and IL-5 production, while neutrophil levels were significantly enhanced in comparison to wild-type mice. Consistent with our findings, others have recently described that eosinophil but not neutrophil levels were markedly decreased in IL-33 deficient as compared to wild-type mice in response to papain instillation [23]. In our study, we used a single papain exposure rather than several instillations. This approach allowed us to show that IL-1R signaling is critical in the inflammatory responses to a single exposure to papain. However, it is likely that IL-33 may be more important in case of several papain exposures and upon a longer follow-up.

IL-36 γ was increased in the lung of A/J mice in the HDM model [16]. IL-36 is produced by bronchial epithelial cells leading to immune cell recruitment via chemokine production. We showed that one instillation of papain is not sufficient to induce changes in IL-36 γ expression, while other IL-36 cytokines were not detected (data not shown). Furthermore, cytokine production and inflammatory responses were not significantly different in mice lacking IL-36R than in wild-type mice. Similarly, inflammatory responses were not significantly different in IL-36Ra deficient mice, which exhibit uncontrolled and exaggerated responses to IL-36 due to the lack of its natural inhibitor [15].

Experiment on Rag-deficient mice showed that T and B cells are not required for lung inflammation induced by three papain instillations [19]. Our data showed that MyD88-deficiency in T cells in

papain-induced lung inflammation is not essential for cell recruitment but seems to participate to IL-5 production, suggesting a minor contribution of MyD88 signaling in CD4⁺ T cells.

The myeloid cell marker lysozyme M (MLys) is expressed in monocytes/macrophages and neutrophils and is also reported as an AT2 pneumocyte marker [24]. More recently, Desai *et al* showed that LysM-cre knock-in mice [25] can be used to label AT2 epithelial cells [26]. In our study, we generated MyD88^{MLys} mice, deficient for MyD88 in monocytes/macrophages, neutrophils and AT2 epithelial cells. After papain administration, cell recruitment in BALF and cytokine production was not significantly different in MyD88^{MLys} than in B6 mice. MyD88^{ACID} mice, deficient for MyD88 in AT1 epithelial cell, exhibited a reduction in IL-5 production as compared to B6 mice. Our results unveil a minor role of MyD88 signaling in myeloid, epithelial cells and lymphocytes but not CD11c cells demonstrating that MyD88 signaling in each of these cells does not recapitulate the protective effect observed in MyD88^{-/-} mice. This result indicates that MyD88 signaling is necessary in several of these cell types or/and in other cell types for the development of papain-induced lung inflammation.

In conclusion, our results show that IL-1R1 signaling via MyD88 plays a critical role in airway inflammatory response to a single papain exposure.

Materials and methods

Mice

C57BL/6 (B6) wild type, MyD88^{-/-} [27], IL-36R^{-/-} (IL-1rl2^{-/-}), IL-36Ra^{-/-} [28], IL-1R1^{-/-} [29], ST2^{-/-} (IL-1rl1^{-/-}) [30] mice were bred in our specific pathogen free animal facility at TAAM-CNRS, Orleans, France (agreement D-45-234-6 delivered on march, 10 of 2014). The Myd88 conditional mice were kindly provided by Dr Matthias Muller. The wild type genomic allele was targeted with the targeting vector

in 129 ES cells. Exons 2 to 5 are flanked by loxP sites. The Neomycin selection cassette flanked by FRT sites is inserted just after in intron 5. Finally the eGFP gene is inserted at the beginning of exon 6 to give the Myd88 targeted allele. The Neomycin cassette was removed upon Flpe expression by transient vector transfection in ES cells to give the conditional Myd88 allele (suggested international name $\text{Myd88}^{\text{tm1(eGFP)Mmul}}$). The mice were generated with these clones and backcrossed at least 10 times with C57BL/6J. Myd88 tissue specific knockouts were obtained by breeding this conditional allele with: MLysCre Knockin (B6.129P2-Lyz2^{tm1(cre)lfo/J}), deleting MyD88 specifically in myeloid compartment [25]; with CD11cCre (Tg(Itgax-cre)1-1Reiz), allowing deletion in dendritic cells [31]; with CD4Cre (Tg(Cd4-cre)1Cwi, allowing deletion in all CD4⁺ T lymphocyte lineage [32]; or with Aqp5Cre knockin (Acid or Aqp5^{tm1.1(cre,DsRed)Pfl}), allowing deletion in alveolar type I (AT1) cells in adult lung [33]. All the tissue specific knockin-cre mice are cre wt/ki to avoid a secondary phenotype linked to the absence of the KI targeted gene product. All the mice were backcrossed at least 10 times or more on C57BL6/J genetic background (Supporting Information Figure 2). Mice were maintained in a temperature-controlled (23°C) facility with a strict 12 h light/dark cycle and were given free access to food and water. The experiments were performed with female mice aged 8 - 10 weeks. All animal experimental protocols complied with the French ethical and animal experiments regulations (see Charte Nationale, Code Rural R 214-122, 214-124 and European Union Directive 86/609/EEC) and were approved by the “Ethics Committee for Animal Experimentation of CNRS Campus Orleans” (CCO), registered (N°3) by the French National Committee of Ethical Reflexion for Animal Experimentation (CLE CCO 2013-1006).

Papain administration to mice

Mice were anesthetized by an iv injection of ketamine/xylazine followed by an intranasal administration of 25 µg of papain (Calbiochem, Darmstadt, Germany) in 40 µL of saline solution. Mice were euthanized by CO₂ inhalation 24h after papain administration and BALF was collected.

After a hearth perfusion with ISOTON II (Acid free balanced electrolyte solution Beckman Coulter, Krefeld, Germany) lung were collected and sampled for analyses.

Broncho alveolar lavage (BAL)

BAL was performed by 4 lavages of lung with 500 µL of saline solution via a cannula introduced into mice trachea. BAL fluids were centrifuged at 400 g for 10 min at 4°C, the supernatants were stored at -20°C for ELISA analysis and pellets were recovered to prepare cytospin (Thermo scientific, Waltham, USA) glass slides followed by a Diff-Quik (Merz & Dade A.G., Dudingen, Switzerland) staining. Differential cell counts were performed with at least 400 cells.

Enzyme-linked Immunosorbent assay (ELISA)

Homogenized lung or cell supernatant were tested for MPO, CCL24, CCL11, CCL17, IL-33, IL-1β, IL-12p40 (R&D systems Abingdon, UK), IL-5, IL-33 (eBiosciences, San Diego, USA) using commercial ELISA kits according to the manufacturer's instructions.

Histology

The left lobe of lung was fixed in 4% buffered formaldehyde and paraffin embedded under standard conditions. Tissue sections (3 µm) were stained with PAS. Histological score was determined by a semi-quantitative assessment from 0 to 5 for cell infiltration (with increasing extent). The slides were blindly examined by two investigators with a Leica microscope (Leica, Solms, Germany).

RNA extraction, reverse transcription and Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) and cleaned up by the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Total RNA (1 µg) was reverse

transcribed using SuperScript II Reverse transcriptase (Invitrogen Life Technologies). The mRNA levels for genes of interest were examined by quantitative RT-PCR using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The primer sequences used were: 5'-AGTGGTTAGCTACCAGTTGGCa-3' (CCL-24 forward primer), 5'-TGTATGTGCCTCTGAACCAC-3' (CCL-24 reverse primer), 5'-GGTGTGGATGGGAAGAAGCTG-3' (IL-33 forward primer), 5'-GAGGACTTTTTGTGAAGGACG-3' (IL-33 reverse primer), 5'-GGGAAGATTCTGAAGAAGAG-3' (IL-1 α forward primer), 5'-GAGTAACAGGATATTTAGAGTCG-3' (IL-1 α reverse primer), 5'-TGTGAAATGCCACCTTTTGA-3' (IL-1 β forward primer), 5'-GTGCTCATGTCCTCATCCTG-3' (IL-1 β reverse primer), 5'-CCCATGCAAGTACCCAGAGT-3' (IL-36 γ forward primer), 5'-AGGGTGGTGGTACAAATCCAA-3' (IL-36 γ reverse primer), 5'-AAACACCTAGCAAAAGCCCAG-3' (IL-36R forward primer), 5'-AGACTGCCCCGATTTTCCTATG-3' (IL-36R reverse primer), 5'-CTGCTACTGCCCCAACGATA-3' (MyD88 forward primer), 5'-ATTAGCTCGCTGGCAATGGA-3' (MyD88 reverse primer), 5'-AGGCCGAGAATGGGAAGCTTGT-3' (GAPDH forward primer), 5'-TACTCAGCACCGGCCTCACCC-3' (GAPDH reverse primer) and QuantiTect 18S primers (QT02448075, Qiagen). Relative levels of mRNA expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S mRNA levels using a comparative method ($2^{-\Delta\Delta C_t}$). Non-reverse-transcribed RNA samples and water were included as negative controls.

BMDC/DM culture and stimulation

Murine bone marrow cells were isolated from femurs and differentiated into macrophages (BMDM) or dendritic cells (BMDC). BMDM were obtained after culturing bone marrow cells at 10^6 cells/mL for 7 days in DMEM supplemented with 10 mM L-glutamine, 25 mM Hepes, 100 U/mL penicillin and 100 U/mL streptomycin, plus 20% horse serum and 30% L929 cell-conditioned medium as a source of M-CSF [34]. Three days after washing and re-culturing in fresh medium, the cell preparation contained a homogenous population of macrophages. BMDC were obtained by culturing bone

marrow cells at 2.10^5 cells/mL for 10 days (with changes on days 3, 6, and 8) in RPMI 1640 medium, supplemented with 10% FCS, 2 mM L-glutamine, 50 μ M β -Mercaptoethanol, 1 mM sodium pyruvate, 40 μ g/mL gentamycin, non-essential aminoacids, 100 U/mL penicillin, 100 U/mL streptomycin and 5% of J558L cell-conditioned medium as a source of GM-CSF as described previously [35].

The BMDC/DM were plated in 96-well plates (10^5 cells/well) and stimulated with 100 ng/mL LPS, 5 μ g/mL Pam3CSK4 and 0.1 μ M CpG overnight. Supernatants were collected and stored for further analysis.

Nitric oxide (NO) production

The production of nitric oxide was measured in culture supernatants by the Griess assay [36]. Fifty microliters of culture supernatants was mixed with 25 μ L of Griess 1 (1% sulfanilamide in 2.5% phosphoric acid) and 25 μ L of Griess 2 reagent (0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated for 30 min. Absorbance was measured at 562 nm. A standard curve of NaNO₂ was used to establish the NO₂⁻ concentration in the samples.

Cell separation from lung by flow cytometry and magnetic beads

Lung were digested in RPMI 1640 medium containing 100 U/mL penicillin, 100 U/mL streptomycin, 1 mg/mL DNase I (Sigma) and 125 μ g liberase (Roche) during 1h at 37°C under rotation. After digestion, RPMI 1640 supplemented with 10% FCS were added. Cell suspension were dissociated by passage through a 100 μ m cell strainer and centrifuged at 300g for 10 min at 4°C. Pellet was resuspended in red blood cell lysis buffer (Stem cell Technologies) and incubated for 10 min on ice. Lysis was stopped by addition of RPMI 1640 and centrifugation at 300g for 10 min at 4°C. Pellet were resuspended in RPMI 1640 supplemented with 10% FCS and pass through 40 μ m cell strainer. Cells were numerated before FACS staining or separation by magnetic beads. EpCam⁺ cells were isolated by cell sorting on FACS VantageSE (BD) after staining by anti-CD326 APC conjugated antibody (BD

biosciences). CD4⁺, Ly6G⁺, and CD11c⁺ cells were isolated by using Cell Isolation kit from Miltenyi Biotec according to the manufacturer's protocol.

Statistical analysis

Data were analyzed using Prism version 5 (Graphpad Software, San Diego, USA). The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. Values are expressed as mean $\pm$ SEM. Statistical significance was defined at a p-value < 0.05.

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Conflicts of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. A single papain instillation induces lung inflammation and IL-1 expression.

B6 mice were treated intranasally once with papain (Papain), saline (NaCl) or not treated (Naive) and samples were collected at 1h, 6h, 18h, 24h, 48h and 72h after instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in BALF by numeration of MGG stained cytospin. (B) Histological score of cell infiltration was performed on paraffin embedded section after PAS staining. (C) Lung homogenate level of IL-33 and

CCL17 and BALF level of IL-5 and MPO were measured by ELISA. (A-C) Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=5 mice per group. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively. (D) Representative PAS staining of lung tissue. Cell infiltrate (black arrow) and emphysema (asterisk) are shown. Scale bar: 200 μ m. Images are from a single experiment representative of 2 experiments with n=5 mice per group. (E) Lung transcript relative expression of *Il1a*, *Il1b*, *Il33* and *Ccl24* were determined by quantitative RT-PCR. Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=5 mice per group. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

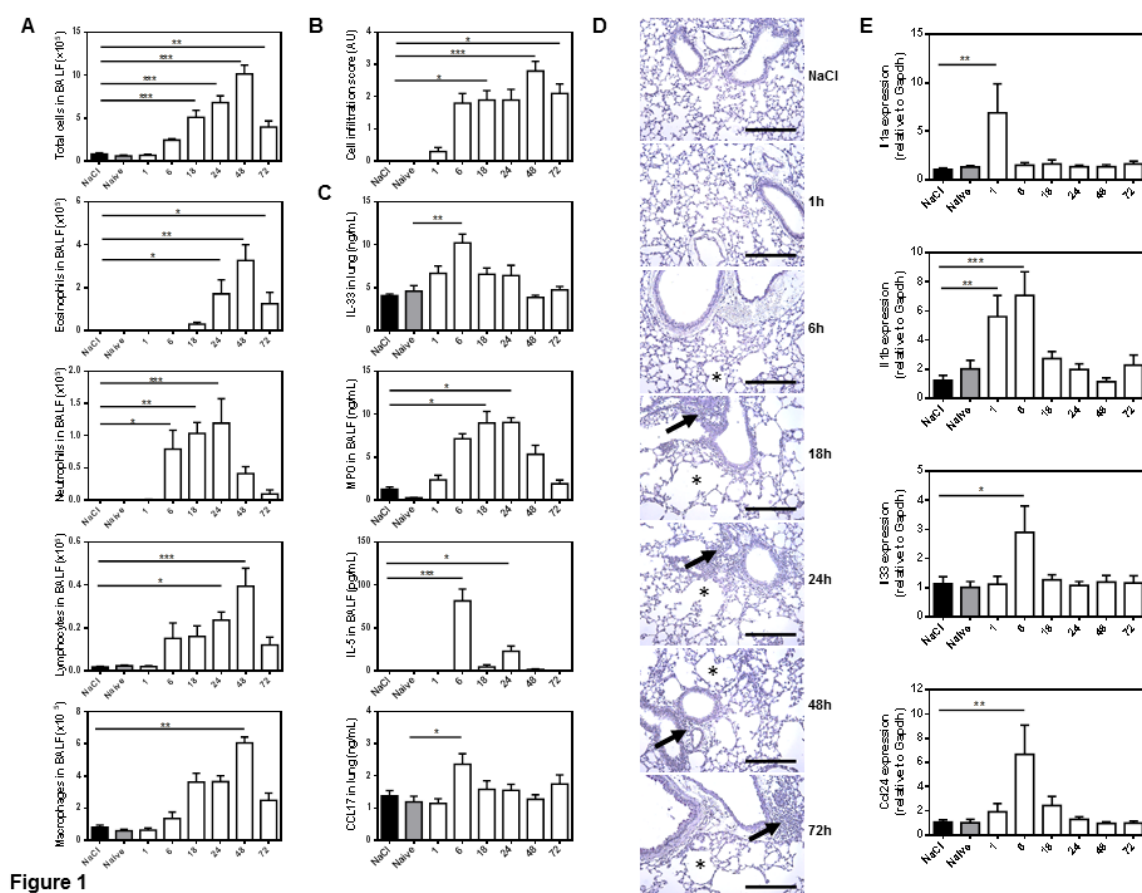


Figure 2. Reduced papain-induced lung inflammation in MyD88^{-/-} mice.

B6 and MyD88^{-/-} mice were treated intranasally once with papain (Papain) or saline (NaCl). Samples were collected at 24h after papain instillation for B6 mice and at 1h, 6h and 24h after instillation for MyD88^{-/-} mice. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in BALF by numeration of MGG stained cytospin. (B) Lung homogenate level of CCL24 and CCL17 and BALF level of CCL11, MPO and IL-5 were measured by ELISA. (C) Histological score of cell infiltration was performed on paraffin embedded section after PAS staining. (A-C) Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=4-5 saline-treated mice per genotype and n=6-7 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively. (D) Representative PAS staining of lung tissue. Cell infiltrate (black arrow) and emphysema (asterisk) are shown. Scale bar: 200 μ m. Images are from a single experiment representative of 2 experiments with n=4-5 saline-treated mice per genotype and n=6-7 papain-treated mice per genotype. (E) B6 and MyD88^{-/-} mice were treated intranasally once with papain or with saline (NaCl). Tissue samples were collected at 1h and 6h after papain instillation. Lung transcript relative expression of *Il1a*, *Il1b* and *Il33* were determined by quantitative RT-PCR. Data are expressed as mean + SEM from a single experiment representative of 1 experiment with n=3-4 saline-treated mice per genotype and n=4-7 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

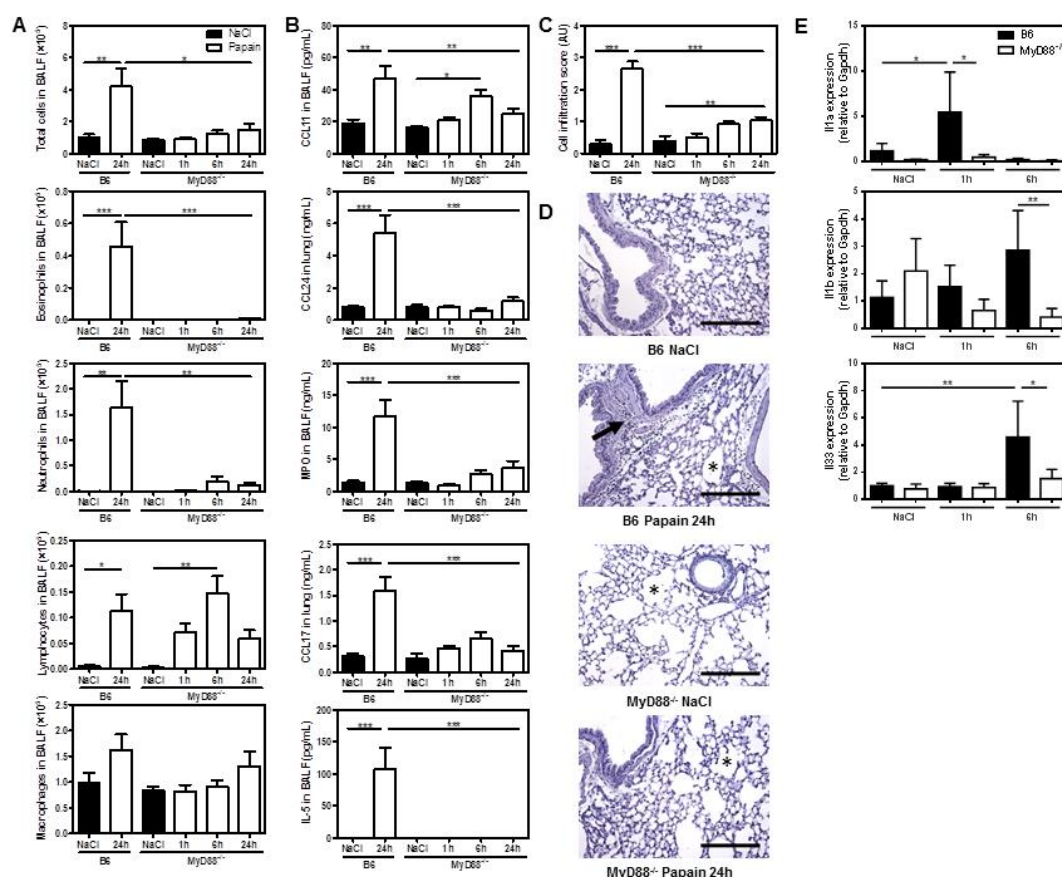


Figure 2

Figure 3. Reduced papain-induced inflammation in IL-1R1-deficient mice.

B6 and IL-1R1^{-/-} mice were treated intranasally once with papain (Papain) or saline (NaCl) and samples were collected at 24h after papain instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in BALF by numeration of MGG stained cytospin. (B) Lung homogenate level of CCL24 and CCL17 and BALF level of MPO and IL-5 were measured by ELISA. (C) Histological score of cell infiltration was performed on paraffin embedded section after PAS staining. (A-C) Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=5-6 saline-treated mice per genotype and n=6-7 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. (D) Representative PAS staining of lung tissue. Cell infiltrate (black arrow) and emphysema (asterisk) are

shown. Scale bar: 200 μ m. Images are from a single experiment representative of 2 experiments with n=5-6 saline-treated mice per genotype and n=6-7 papain-treated mice per genotype.

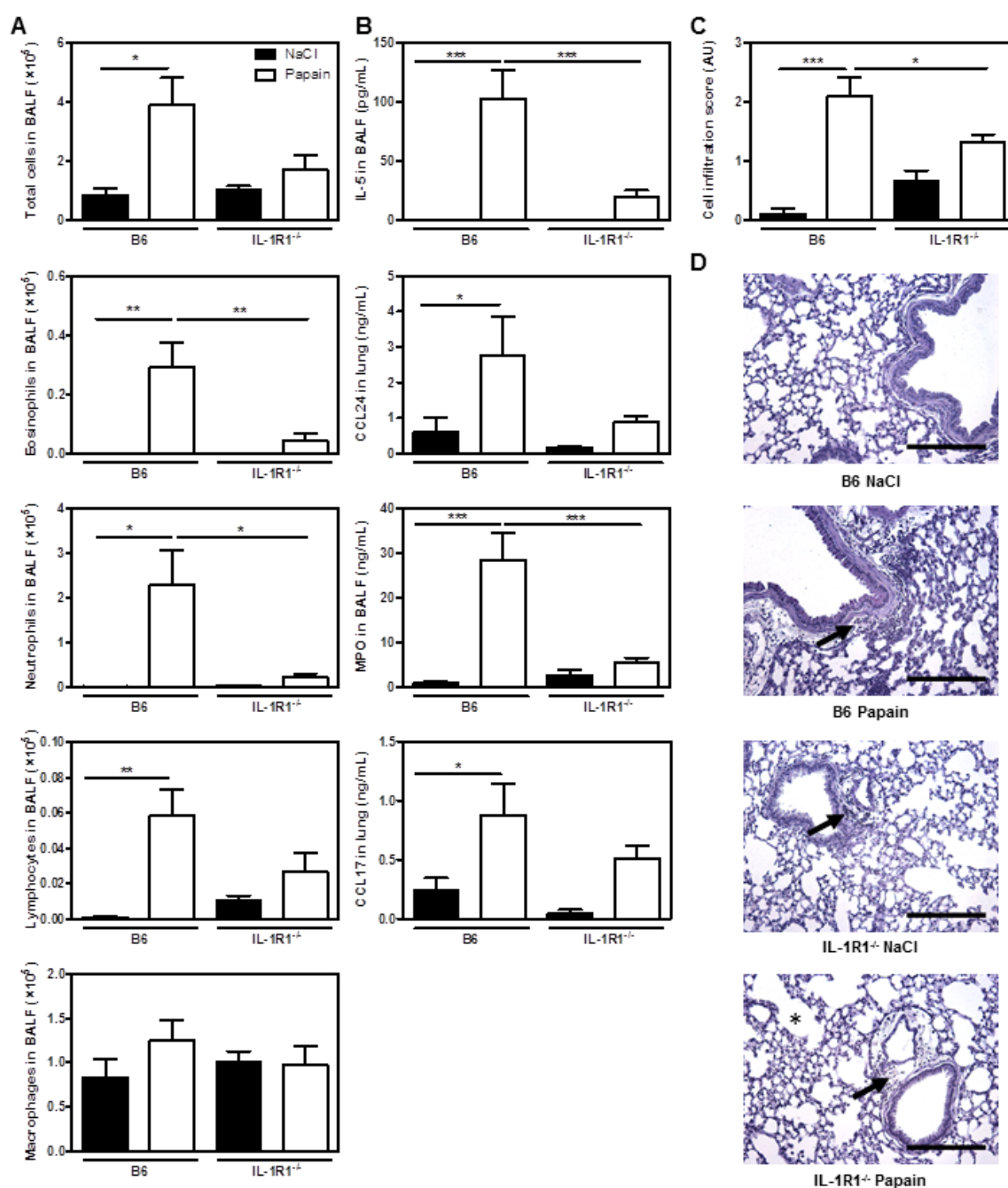


Figure 3

Figure 4. Reduced papain-induced eosinophilia in ST2-deficient mice.

B6 and ST2^{-/-} mice were treated intranasally once with papain (Papain) or saline (NaCl) and samples were collected at 24h after papain instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in BALF by numeration of MGG stained cytospin. (B) Lung homogenate level of CCL24, CCL17 and IL-1 β and BALF level of MPO and IL-5 were measured by ELISA. (C) Histological score of cell infiltration was performed on paraffin embedded section after PAS staining. (A-C) Data are expressed as mean + SEM from a pool of 2 experiments representative of 4 experiments with n=5 saline-treated mice per genotype and n=6-8 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively. (D) Representative PAS staining of lung tissue. Cell infiltrate (black arrow) and emphysema (asterisk) are shown. Scale bar: 200 μ m. Images are from a pool of 2 experiments representative of 4 experiments with n=5 saline-treated mice per genotype and n=6-8 papain-treated mice per genotype.

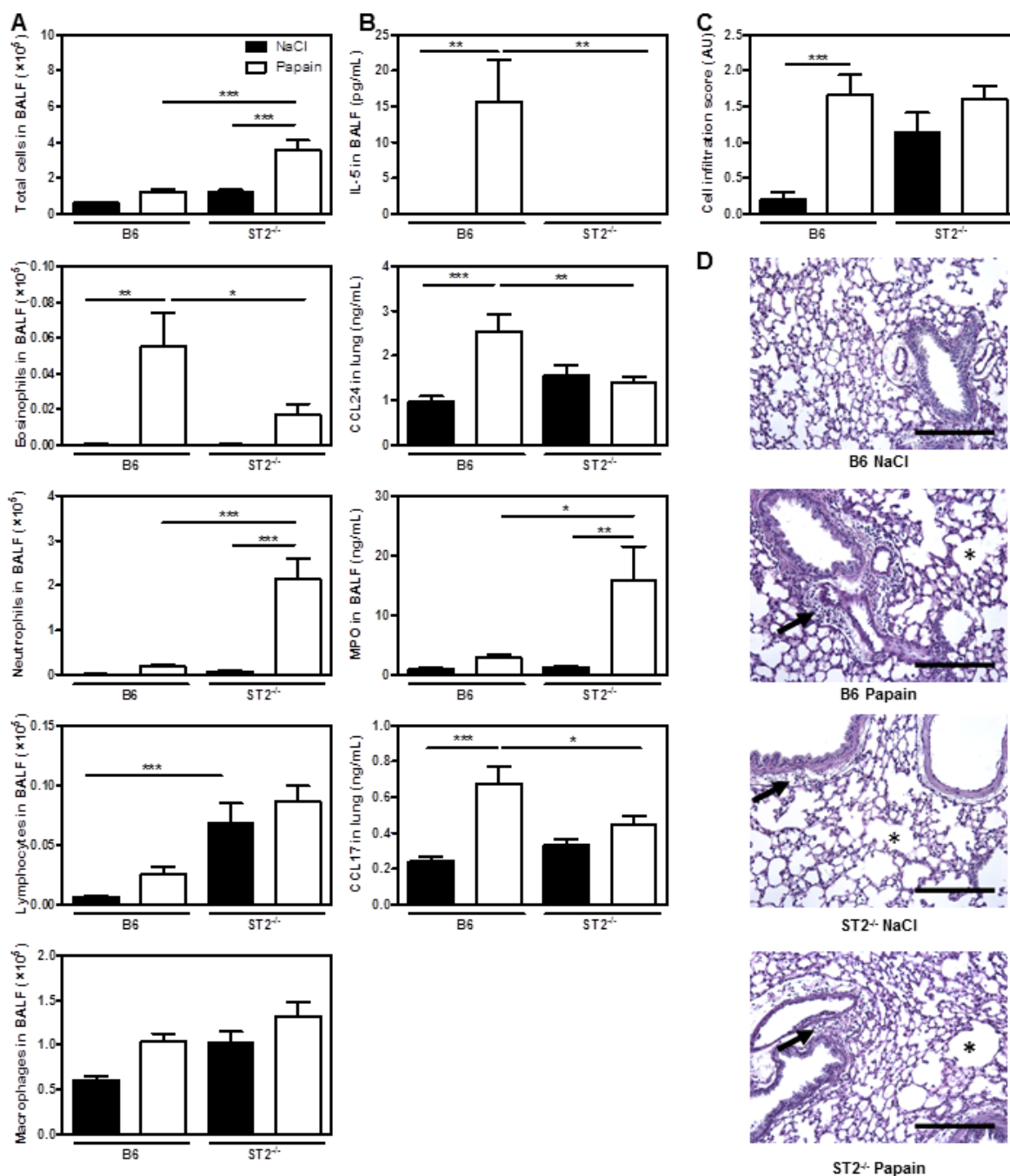


Figure 4

Figure 5. MyD88-cell specific depletion in myeloid cells, alveolar epithelial cells and CD4⁺ T cells do not reduce response to papain.

B6, MyD88^{Mlys}, MyD88^{Acid}, MyD88^{CD4} and MyD88^{-/-} mice were treated intranasally once with papain (Papain) or saline (NaCl) and samples were collected at 24h after papain instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in by numeration of MGG stained cytospin. (B) Lung homogenate level of CCL24 and CCL17 and BALF level of MPO and IL-5 were measured by ELISA. (C) Histological score of cell infiltration was performed on paraffin embedded section after PAS staining. (A-C) Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=4-6 saline-treated mice per genotype and n=6-9 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively. (D) Representative PAS staining of lung tissue. Cell infiltrate (black arrow) and emphysema (asterisk) are shown. Scale bar: 200 μ m. Images are from a single experiment representative of 2 experiments with n=4-6 saline-treated mice per genotype and n=6-9 papain-treated mice per genotype.

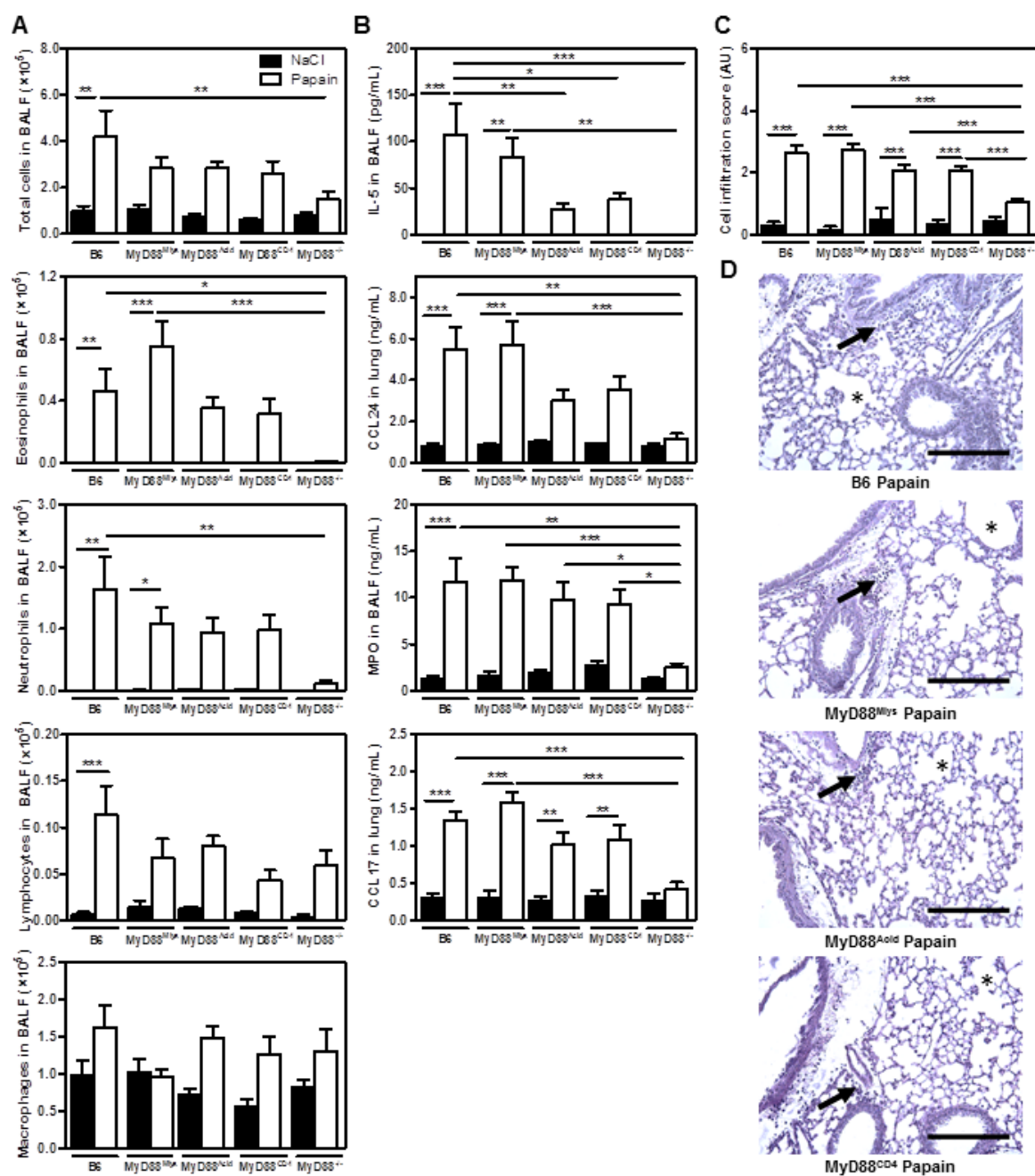


Figure 5

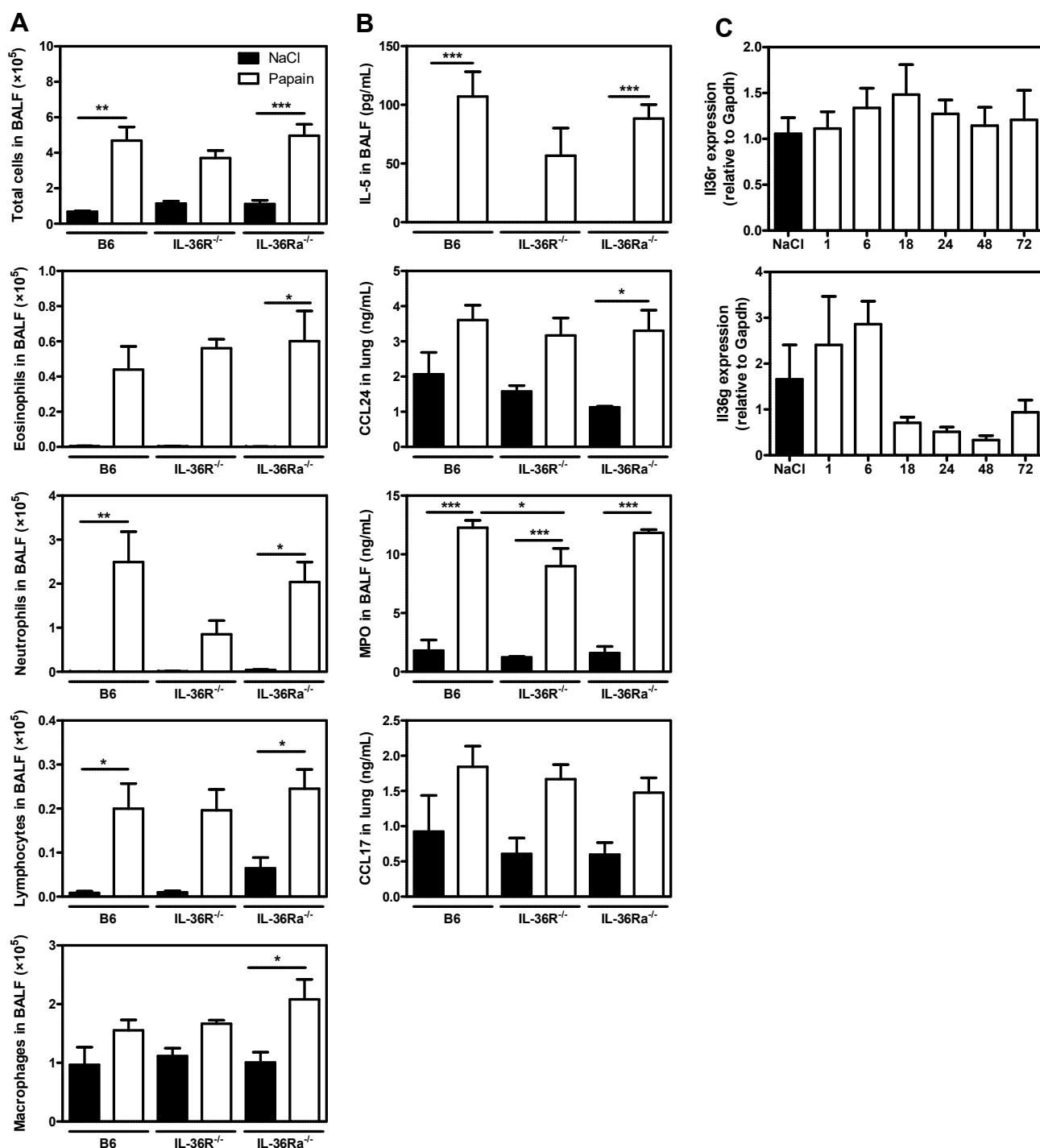


Figure 1. IL-36R and IL-36Ra are not involved in papain-induced lung inflammation.

B6, IL-36R^{-/-} and IL-36Ra^{-/-} mice were treated intranasally once with papain (Papain) or saline (NaCl). Samples were collected at 24h after papain instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in BALF by numeration of MGG stained cytopsin. (B) Lung homogenate level of CCL24 and CCL17 and BALF level of MPO and IL-5 were measured by ELISA. (A-B) Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=4-5 saline-treated mice per genotype and n=5-8 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively. (C) B6 mice were treated intranasally once with papain (Papain) or saline (NaCl). Samples were collected at 1h, 6h, 18h, 24h, 48h, and 72h after instillation. Lung transcript relative expression of *Il36r* and *Il36g* were determined by quantitative RT-PCR. Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=5 mice per group. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

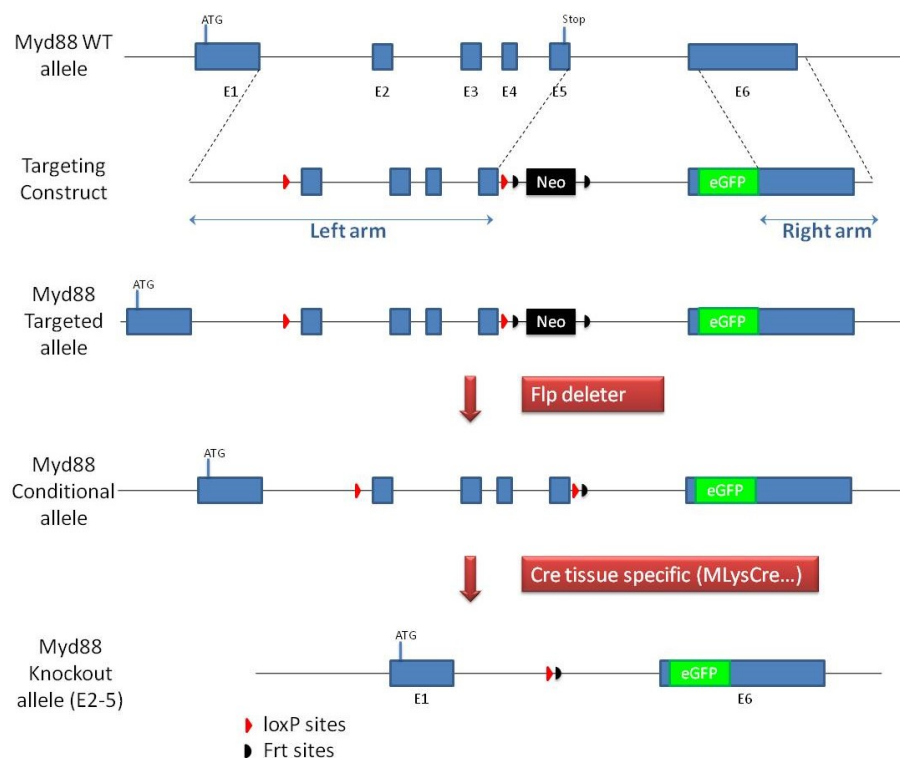
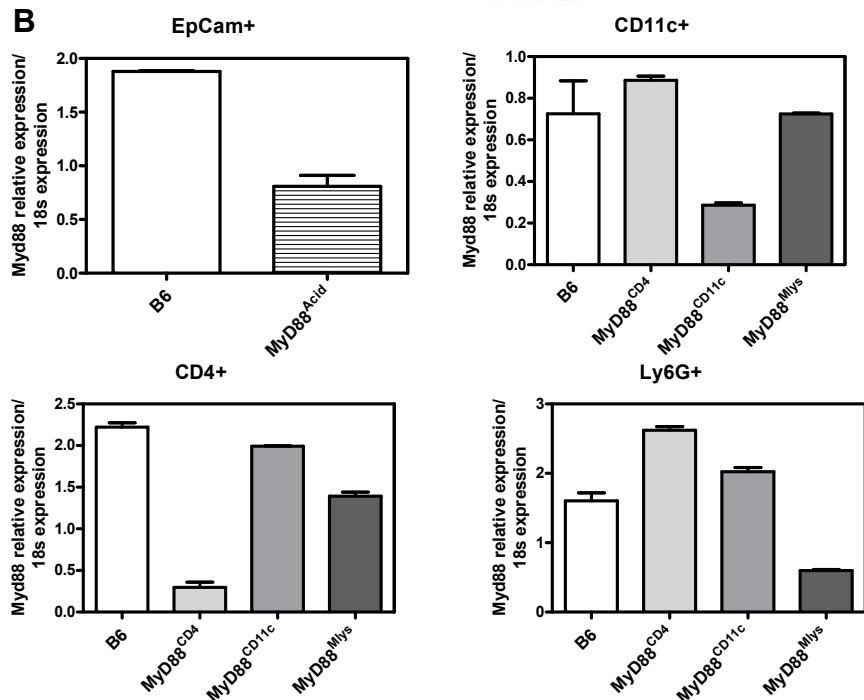
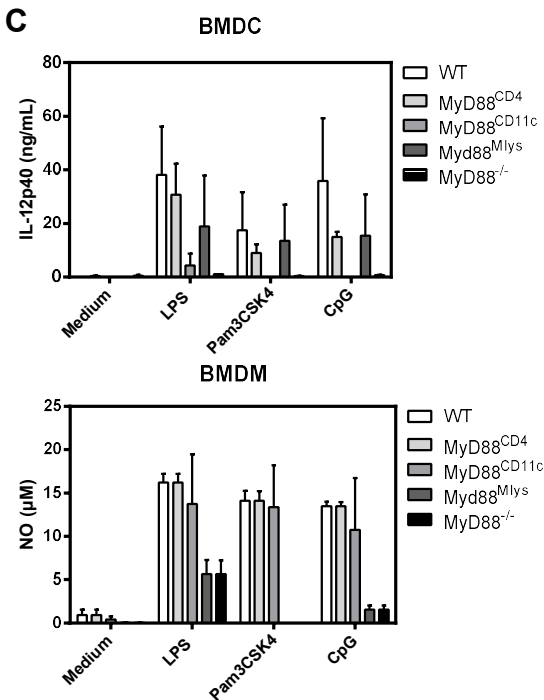
A**B****C**

Figure 2. Generation of the Myd88 conditional mice.

Scheme of strategy for generating the Myd88 conditional mice. (B) EpCam⁺ epithelial cells were sorted by flow cytometry from B6 and Myd88^{Acid} lungs, and CD4⁺, CD11c⁺ and Ly6G⁺ cells were isolated from B6, Myd88^{CD4}, Myd88^{CD11c} and Myd88^{Mlys} lung by magnetic separation. Myd88 mRNA expression was analyzed by RT-qPCR and normalized to 18s levels in epithelial cells (EpCam⁺) isolated by cell sorting from lung of B6 or Myd88^{Acid} and CD11c⁺, CD4⁺ and Ly6G⁺ isolated by magnetic separation from lungs of B6, Myd88^{CD4}, Myd88^{CD11c} and Myd88^{Mlys} mice. (A-B) Data are expressed as mean + SEM from a single experiment with n=2-4 mice per group. (B) Primary BMDM and BMDC were derived from bone marrow of B6, Myd88^{CD4}, Myd88^{CD11c}, Myd88^{Mlys} and Myd88^{-/-} mice. Cells were stimulated overnight with LPS (100 ng/mL), Pam3CSK4 (5 μg/mL) or CpG (0.1 μM). NO concentrations were determined by Griess method in supernatants from BMDM and IL-12p40 by ELISA in supernatants from BMDC. Data are expressed as mean + SEM from a single experiment representative of 1 experiment with n=2 mice per group stimulated in vitro in duplicate.

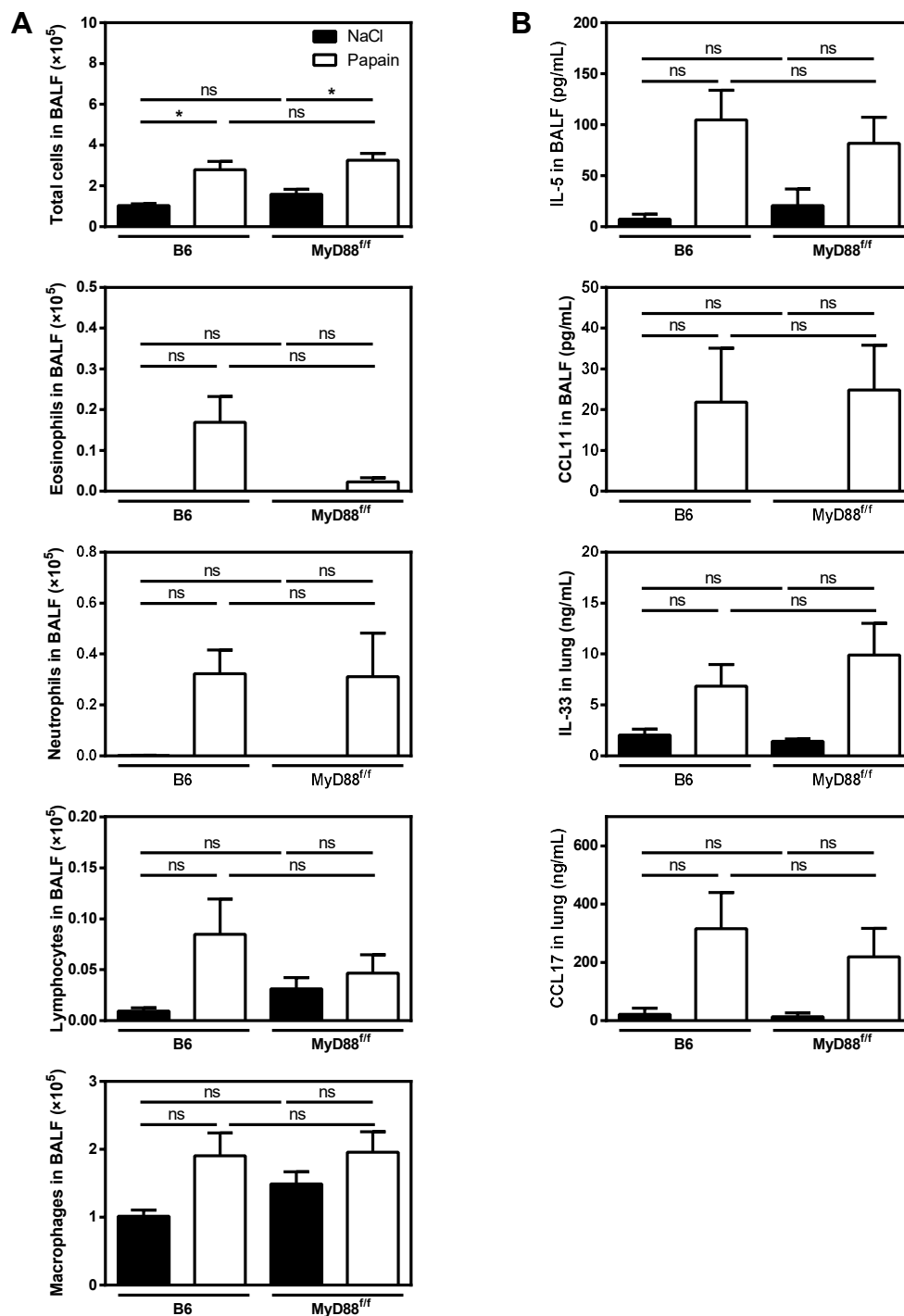


Figure 3. Similar inflammation in B6 and MyD88^{f/f} control mice after a single papain exposure.

B6 and MyD88^{f/f} mice were treated intranasally once with papain (Papain) or saline (NaCl). Samples were collected at 24h after papain instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in BALF by numeration of MGG stained cytospin. (B) BALF level of IL-5 and CCL11 and lung homogenate level of IL-33 and CCL17 were measured by ELISA. (A-B) Data are expressed as mean + SEM from a single experiment with n=4 saline-treated mice per genotype and n=5-7 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. NS refers to non significant.

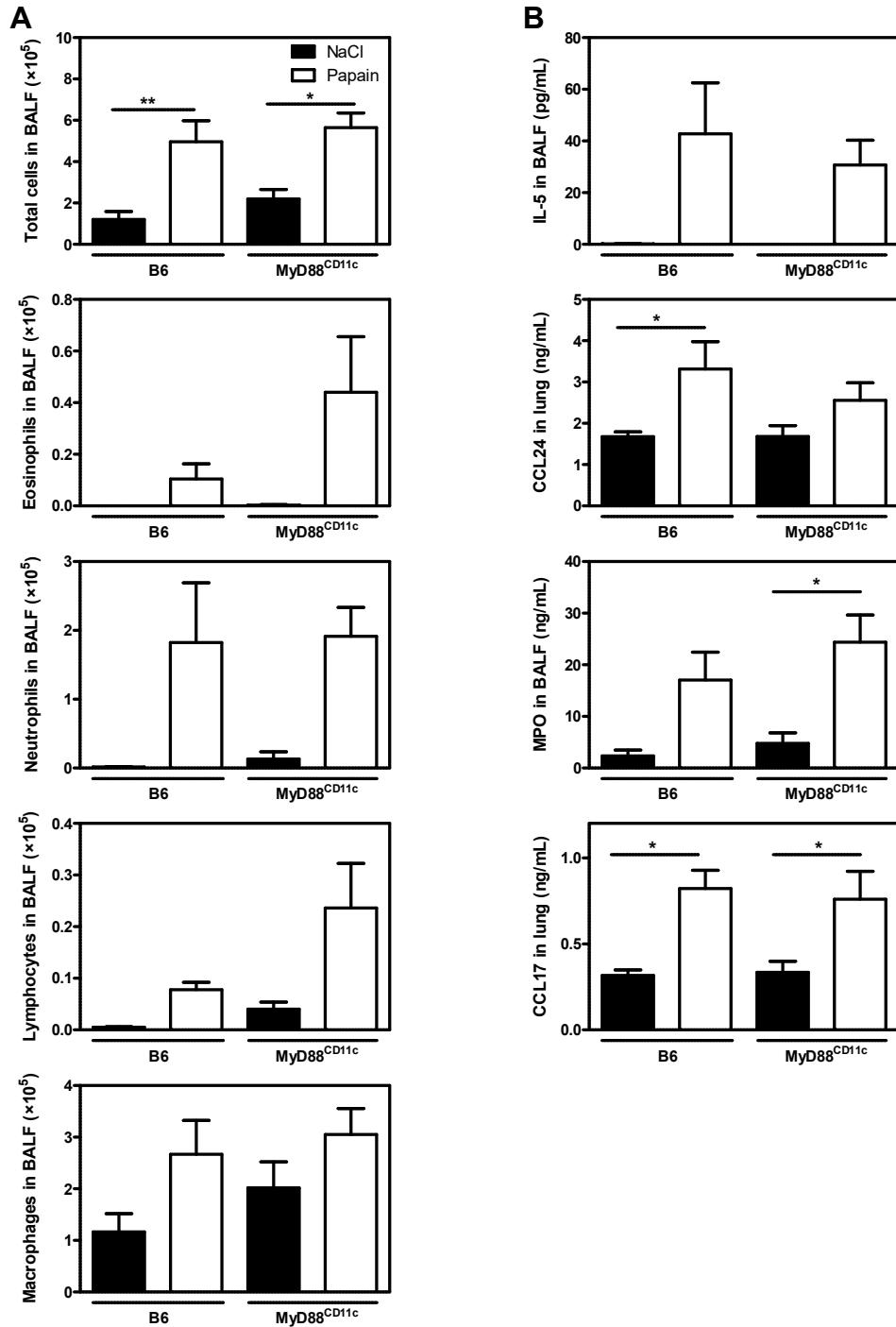


Figure 4. MyD88-cell specific depletion in CD11c cells do not reduce response to papain.

B6 and MyD88^{CD11c} mice were treated intranasally once with papain (Papain) or saline (NaCl). Samples were collected at 24h after papain instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in BALF by numeration of MGG stained cytospin. (B) Lung homogenate level of CCL24 and CCL17 and BALF level of MPO and IL-5 were measured by ELISA. (A-B) Data are expressed as mean + SEM from a single experiment representative of 2 experiments with n=4-5 saline-treated mice per genotype and n=5 papain-treated mice per genotype. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

IL-1R1-MyD88 axis elicits papain-induced lung inflammation

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

29-Mar-2016

Dear Dr. Couturier-Maillard,

Manuscript ID ejj.201646366 entitled "IL-1R1-MyD88 axis elicits early papain-induced lung inflammation" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referee concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the

requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

On behalf of
Prof. Maria Yazdanbakhsh

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Reviewer: 1

Comments to the Author

Rafiou Agoro et al in the article entitled IL-1R1-MyD88 axis elicits early papain-induced lung inflammation• uses a model of lung inflammation in response to single administration of papain. They studied the kinetics of inflammation and cytokine production, showing the recruitment of eosinophils and neutrophils and a rapid production of IL-1 β , IL-1 α and IL-33. Inhibition of inflammation was observed

in IL-1R1^{-/-} and MyD88^{-/-} although the deletion of MyD88 signaling in single cell types such as T cells, epithelial cells, CD11c-positive or myeloid cells led to only a partial inhibition as compared to complete absence of MyD88 suggesting that several cell types contribute to the response.

Although the article sounds promising after reading the abstract, there are some problems in methods and results conclusions, or at least in the way they are expressed.

1. In methods: Mice were euthanized by CO₂ inhalation 24h after papain administration and BALF was collected. I do not understand the use of CO₂! Knowing that hypoxia induces the production of IL-33, this sounds completely supernatural. And what about the euthanasia at the other time points?

2. The study of the kinetics is very interesting. However the authors keep writing early inflammatory response, although the rest of the analysis was done at 24h. The single intranasal administration is not sufficient to write early. Twenty four hours does not sound early to me.

3. In the result section: As in wild-type mice, Il1b and Il33 mRNA levels peaked at 1h and 6h post-exposure respectively, but Il1a expression was not significantly increased in MyD88^{-/-} mice (Figure 2E). This observation suggests that IL-1 β and IL-33 are produced independently of MyD88 pathway. I do not understand: at the 24h time point (Figure 2E), IL-33 expression is significantly decreased compared to B6, suggesting that IL-33 is not produced independently of MyD88 pathway. I find the study of the kinetic analysis very interesting and I regret that the rest of the study does not show at least 2 time points with an early one like 6hrs. Also, comparing fig1E and 2E (please show figures in the same reading direction) for IL-33 expression: in Fig1 at 24h, NaCl is not different from papain-treated mice, whereas it is in Fig2 & 5. Please explain. It would be interesting to see kinetics for NaCl at least once to be sure that fluid intake in the lung does not significantly modify some parameters.

4. Regarding all B6 controls from Fig2 to 5, are they littermate to deficient mice or completely different breeding? Did the authors do at least some littermate to check they react like B6?

5. How do you explain that ST2^{-/-} mice exhibit increased BAL lymphocytes in NaCl-treated mice compared to B6, and increased BAL neutrophils in papain-treated mice compared to B6?

6. Knowing that this papain model was shown to be dependant of ILC2, and that IL-13 is a key cytokine produced by ILC2, the authors should measure IL-13. They should also measure IL-17 and IFN- γ in lungs to evaluate a switch in cytokine production in the different conditions.

7. In the result section 5: the efficacy and specificity of MyD88 targeting in the different cell types

was confirmed by RT-qPCR and in vitro functional assays (data not shown). Please show these results in supplement data as this is crucial for data interpretation.

8. In the supplementary fig3, there are no BAL eosinophils in papain-treated B6 mice. This is 10 times less than in fig1, which is supposed to be the same. I am not convinced by the results of fig3 supp as the positive control is not positive. Where are the results for EPO in supplementary fig3?

9. The following paragraph of the result section: α IL1b expression was increased in MyD88ACID in comparison to MyD88-/- suggesting that epithelial cells are not implicated in IL-1 β production in absence of MyD88• is not clear for me: IL-1b expression is not decreased in MyD88ACID in comparison to MyD88-/-, suggesting that epithelial cells are not implicated in IL-1 β production in absence of MyD88. However, it is even increased. How do the authors combine such data?

Reviewer: 2

Comments to the Author

In the study by Agoro et al, the role of the IL-1R-MyD88 axis in the early immune responses to a single dose of papain in the lungs was investigated.

Earlier worked has been published in models where papain has been administrated in several repetitions. Here the role of IL-33 and ILC was clearly demonstrated. Now the question was centered around the role of MyD88 and IL-1R.

To address this question, several elegant knock-out models were used to study the inflammatory response to a single dose of papain in time, such as MyD88-/- or IL-1R-/- and showed that most of the inflammatory immune response to papain is abrogated. For this neutrophils, eosinophils, lymphocytes and macrophages were evaluated up to 72 hr. However, the cell type that was initially described to be important in this model, which are the ILCs, were not studied. What are the kinetics of this cell type in both the WT and the single knock-outs (fig 1-3)?

There is a clear effect on total cells, eosinophils and neutrophils, but lymphocytes do not seem to be affected so much. The group of lymphocytes can contain various populations: B cells, CD4 and CD8 T cell responses. What is the kinetics of the various subpopulations in this model in the 2 knock-outs? Can the authors speculate on the signal (what is left in MyD88-/- or IL-1R-/- mice) that may drive this increased recruitment of lymphocytes in the lungs of papain-treated mice? Will this be sufficient to drive the ILC influx (as described in other papers following repetitive administration of papain), although with slower kinetics?

In figure 4, data are shown from cell type specific knock-outs for MyD88, showing partial effects for every cell type tested, even the cell types that are not expected as the main candidate driving early immune

responses, like e.g. the T cells. This makes it suspicious and I wonder whether the results are partially the result of this cell-specific knock-out approach, regardless of the cell type and the factor knock-out. Did the authors included controls to exclude this? Was there a cell type in this setting that did not show any phenotype (comparable to WT)? Can in all these cell-specific knock-outs the reduced signal be attributed to IL-1R, as is deduced from the experiments with the cell-specific MyD88? May that be an alternative explanation why so many cell types give a partial response?

The message would come across more clearly if a figure is added on the mechanism and the respective roles of the cells tested in this system.

First Revision – authors' response

27-Jun-2016

Reviewer: 1

Comments to the Author

Rafiou Agoro et al in the article entitled “IL-1R1-MyD88 axis elicits early papain-induced lung inflammation” uses a model of lung inflammation in response to single administration of papain. They studied the kinetics of inflammation and cytokine production, showing the recruitment of eosinophils and neutrophils and a rapid production of IL-1a, IL-1b and IL-33. Inhibition of inflammation was observed in IL-1R1^{-/-} and MyD88^{-/-} although the deletion of MyD88 signaling in single cell types such as T cells, epithelial cells, CD11c-positive or myeloid cells led to only a partial inhibition as compared to complete absence of MyD88 suggesting that several cell types contribute to the response.

Although the article sounds promising after reading the abstract, there are some problems in methods and results conclusions, or at least in the way they are expressed.

1. In methods: “Mice were euthanized by CO₂ inhalation 24h after papain administration and BALF was collected”. I do not understand the use of CO₂! Knowing that hypoxia induces the production of IL-33, this sounds completely supernatural. And what about the euthanasia at the other time points?

We used CO₂ inhalation to euthanize the mice throughout all the experiments. As mentioned by the reviewer, it is possible that CO₂ inhalation can induce IL-33; however all cells, fluids and tissues were collected immediately after death. Thus, we think that it is unlikely that CO₂ induced-hypoxia may have significantly modified the results of our experiments. In addition, we showed that, as compared to naïve mice and NaCl treated mice, IL-33 levels were markedly increased in mice treated with papain.

2. The study of the kinetics is very interesting. However the authors keep writing “early inflammatory response”, although the rest of the analysis was done at 24h. The single intranasal administration is not sufficient to write “early”. Twenty four hours does not sound “early” to me.

We agree with the reviewer that the term “early” is not appropriate. We thus changed the wording and replaced the term “early” by “a single exposure to papain” in the revised manuscript.

3. In the result section: “As in wild-type mice, IL1b and IL33 mRNA levels peaked at 1h and 6h post-exposure respectively, but IL1a expression was not significantly increased in MyD88^{-/-} mice (Figure 2E). This observation suggests that IL-1 β and IL-33 are produced independently of MyD88 pathway”. I do not understand: at the 24h time point (Figure 2E), IL-33 expression is significantly decreased compared to B6, suggesting that IL-33 is not produced independently of MyD88 pathway. I find the study of the kinetic analysis very interesting and I regret that the rest of the study does not show at least 2 time points with an early one like 6hrs.

We agree with the reviewer that the 24h time point was not appropriate to measure cytokine mRNA levels. In fact, it is better to examine differences between genotypes at the peak of IL-1 α , IL-1 β and IL-33 expression. We thus decided to perform a new set of experiments to compare the levels of these cytokines in WT and MyD88 deficient mice at 1 and 6 hours after papain inhalation. The results show that the number of total cells, neutrophils, and macrophages increased significantly at 6 h, as well as the concentrations of IL-5 and IL-33 in BALF (**see figure A below for the reviewer’s information only**). The results were also different in MyD88 KO than in WT mice regarding other endpoints. Indeed, the mRNA levels of IL-1 α and IL-33 were increased at 1h and 6h in WT mice, respectively (revised Figure 2E). In contrast, the stimulatory effect of papain was blunted in MyD88 deficient mice. IL-1 β mRNA levels modestly increased at 6h in WT mice and were significantly different than those in MyD88 KO mice. It should be taken into account that the regulation of IL-1 β is complex and includes not only transcriptional but also post-translational regulation to obtain bioactive IL-1 β . The new data regarding IL-1 α , IL-1 β and IL-33 mRNA levels at 1 and 6 hours were included in **Figure 2** to replace previous data included in panel E.

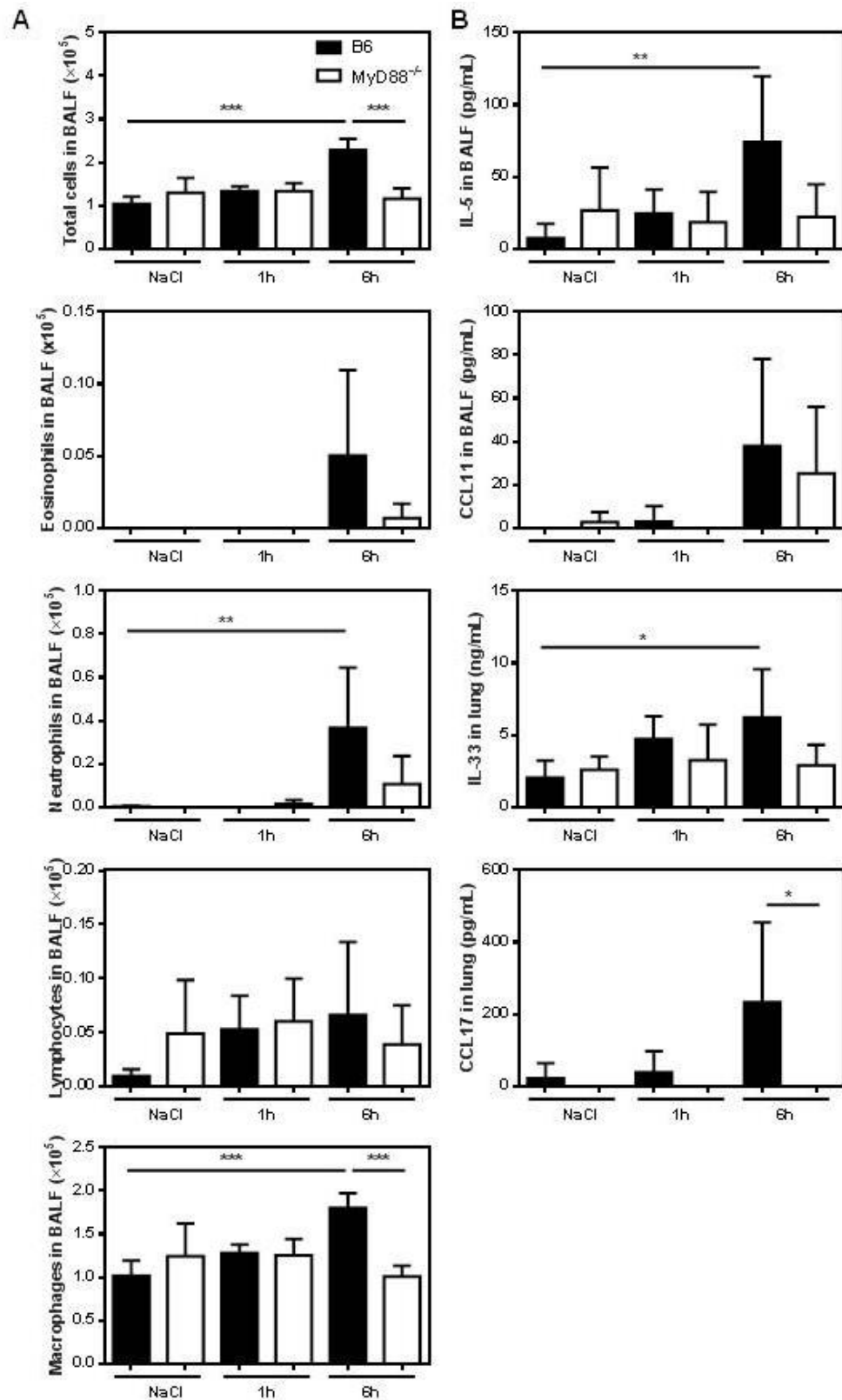


Figure A: Reduced papain-induced lung inflammation in MyD88^{-/-} mice.

*B6 and MyD88^{-/-} mice were treated intranasally once with 25 µg of papain (n=3-4) or with saline (NaCl, n=4-7). Tissue samples were collected at 1h and 6h after papain instillation. (A) Total cells and differential cell count of eosinophils, neutrophils, lymphocytes and macrophages were determined in Broncho Alveolar Lavage Fluid (BALF). (B) BALF level of IL-5 and CCL11 and lung homogenate level of IL-33 and CCL17 were measured by specific ELISA. The data are expressed as mean ± sem. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to P<0.05, P<0.01 and P<0.01 respectively.*

Also, comparing fig1E and 2E (please show figures in the same reading direction) for IL-33 expression: in Fig1 at 24h, NaCl is not different from papain-treated mice, whereas it is in Fig2 & 5. Please explain.

Figure 2 was modified to have the same reading direction as others figures. As mentioned above and as shown in Figure 1 the levels of IL-1α, IL-1β and IL-33 mRNA tended to return to normal (NaCl vehicle) levels at 24h. The variations in mRNA levels at 24 h are relatively marginal (1.5 to 2-fold) and may thus represent small variations from one experiment to another. We therefore decided to perform the experiment with MyD88 deficient mice as mentioned above. As the determination of cytokine mRNA levels is rather a minor point as compared to all the results depicted in Figure 5 we decided to delete these data and modify the manuscript accordingly.

It would be interesting to see kinetics for NaCl at least once to be sure that fluid intake in the lung does not significantly modify some parameters.

We do not think that NaCl has any stimulatory effect. NaCl-instilled mice were sacrificed, respectively, at 1 h and 24h in the different experiments and we did not observe any clear effect of NaCl. The results of naive mice were added to Figure 1 to clarify this point and show that there is no stimulatory effect of the NaCl vehicle. Moreover, the results of NaCl treatment did not differ in MyD88 knockout and WT mice (see figure A above).

4. Regarding all B6 controls from Fig2 to 5, are they littermate to deficient mice or completely different breeding? Did the authors do at least some littermate to check they react like B6?

The mice used in the different experiments were not littermates. However, the different knockout lines included in our studies were bred into the C57BL/6 background for more than 10 generations. Due to the large number of lines used it was practically difficult to use littermates for each of the knockout lines.

5. How do you explain that ST2^{-/-} mice exhibit increased BAL lymphocytes in NaCl-treated mice compared to B6, and increased BAL neutrophils in papain-treated mice compared to B6?

These results were confirmed in repeated experiments and we thus consider them as reliable. We also examined whether differences in the levels of CXCL1/CXCL2 chemokines or IL-17 could explain the differences in neutrophil levels. As shown below in Figure B, the levels of these cytokines were not different between ST2 KO and WT mice. We therefore do not have any specific explanation for the differences in cell levels in the BALF of ST2 KO mice.

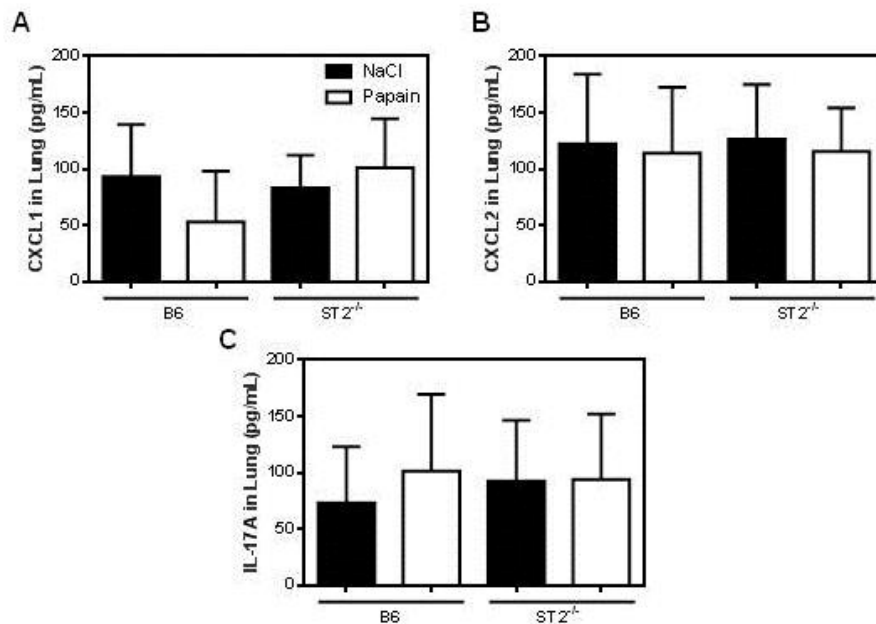


Figure B. CXCL1, CXCL2 and IL-17A cytokine level are similar in B6 and ST2^{-/-} mice

B6 and ST2^{-/-} mice were treated intranasally once with 25 μ g of papain ($n=4-7$) or with saline (NaCl, $n=4-5$). Tissue samples were collected at 24h after papain instillation. Lung homogenate level of CXCL1 (A), CXCL2 (B) and IL-17A (C) were measured by specific ELISA. The data are expressed as mean $\pm$ sem. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. No significant differences were determined.

6. Knowing that this papain model was shown to be dependant of ILC2, and that IL-13 is a key cytokine produced by ILC2, the authors should measure IL-13. They should also measure IL-17 and IFN- γ in lungs to evaluate a switch in cytokine production in the different conditions.

As shown below in Figure C, the levels of IL-13 in BALF of mice following papain inhalation were relatively low and did not significantly differ from those of the control group. The mRNA levels of IL-13 and IL-17 in the lung after papain inhalation were not significantly increased either. The mRNA levels of IFN- γ were significantly increased at 72 h only. The revised manuscript has been modified to mention these results (data not shown). The graphs below are thus included solely for the reviewer's information.

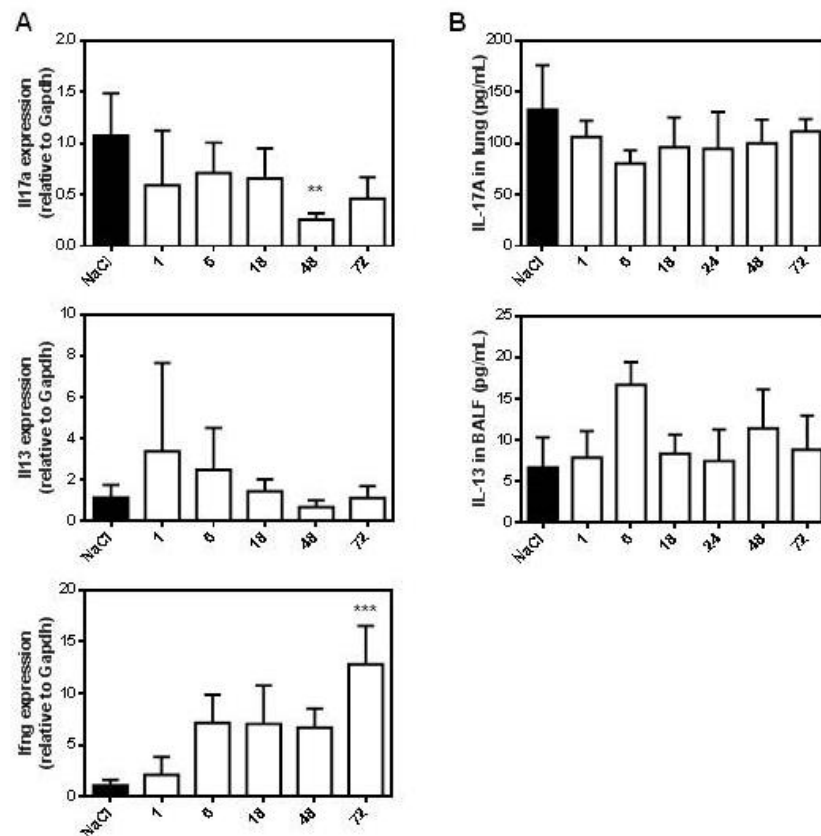


Figure C. IL-17A, IL-13 and IFN- γ cytokine level in B6 mice.

B6 mice were treated intranasally once by 25 μ g of papain (Papain), by saline (NaCl, $n=5$) and samples were collected at 1h ($n=5$), 6h ($n=5$), 18h ($n=5$), 24h ($n=5$), 48h ($n=5$), and 72h ($n=5$) after instillation. (A) Lung transcript relative expression of Il17a, Il13 and Ifng were determined by quantitative RT-PCR. (B) Lung homogenate level of IL-17A and BALF level of IL-13 were measured by specific ELISA. The data are expressed as mean $\pm$ sem. The parametric one-way ANOVA test with multiple Bonferroni's comparison test was used. *, ** and *** refer to $P<0.05$, $P<0.01$ and $P<0.01$ respectively.

7. In the result section 5: “the efficacy and specificity of MyD88 targeting in the different cell types was confirmed by RT-qPCR and in vitro functional assays (data not shown)”. Please show these results in supplement data as this is crucial for data interpretation.

We agree with the reviewer that these control experiments are critical. The results are thus included in the new supplementary Figure 2.

8. In the supplementary fig3, there are no BAL eosinophils in papain-treated B6 mice. This is 10 times less than in fig1, which is supposed to be the same. I am not convinced by the results of fig3 supp as the positive control is not positive. Where are the results for EPO in supplementary fig3?

As shown in the different figures, the absolute numbers of eosinophils vary in the different experiments. The key point is the comparison between genotypes within the same experience. In Supplementary figure 3 (now Supplementary Fig 4), and as opposed to MyD88 deficient mice, MyD88^{CD11c} deficient mice

did not exhibit any significant effect regarding BALF total cell, neutrophil, and eosinophil numbers 24 h after papain inhalation. The results of EPO from the experiment included in Supplementary Figure 3 were not increased upon papain inhalation despite an increased in eosinophil numbers. Thus, we feel that the results of EPO are not sufficiently sensitive and reliable. Indeed, as shown in the previous Figure 5, despite a clear increase in the number of eosinophils following papain inhalation at 24 h the levels of EPO were not significantly different in papain- as compared to NaCl-treated mice. We thus decided to remove the data regarding EPO from Figure 5 in the revised manuscript. Finally the effect of papain in the experiment included in supplementary figure 4 is also supported by the induction of IL-5 to levels (40 pg/ml) consistent with those observed in other experiments (concentrations ranged between 15 and 100 pg/ml, see Figures 1 to 5).

9. The following paragraph of the result section: “IL1b expression was increased in MyD88ACID in comparison to MyD88-/- suggesting that epithelial cells are implicated in IL-1β production in absence of MyD88” is not clear for me: IL-1b expression is not decreased in MyD88ACID in comparison to MyD88-/-, suggesting that epithelial cells are not implicated in IL-1β production in absence of MyD88. However, it is even increased. How do the authors combine such data?

We agree with the reviewer that there was a mistake in the text. The manuscript was modified and data on il1b expression in cell specific MyD88 KO mice are not included in the revised version.

Reviewer: 2

Comments to the Author

In the study by Agoro et al, the role of the IL-1R-MyD88 axis in the early immune responses to a single dose of papain in the lungs was investigated.

Earlier worked has been published in models where papain has been administrated in several repetitions. Here the role of IL-33 and ILC was clearly demonstrated. Now the question was centered around the role of MyD88 and IL-1R.

To address this question, several elegant knock-out models were used to study the inflammatory response to a single dose of papain in time, such as MyD88-/- or IL-1R-/- and showed that most of the inflammatory immune response to papain is abrogated. For this neutrophils, eosinophils, lymphocytes and macrophages were evaluated up to 72 hr.

However, the cell type that was initially described to be important in this model, which are the ILCs, were not studied. What are the kinetics of this cell type in both the WT and the single knock-outs (fig 1-3)?

This is indeed a very important point. In some preliminary data we observed that 24 h after a single papain exposure levels of ILC2 were unchanged in comparison to NaCl treated WT mice. It is plausible that, in contrast to other experimental designs including several papain inhalations, a single exposure is not sufficient to stimulate the proliferation of ILC2. Of note, this result is consistent with the lack of increase in IL-13 levels and the partial involvement of IL-33 after a single papain exposure.

There is a clear effect on total cells, eosinophils and neutrophils, but lymphocytes do not seem to be affected so much. The group of lymphocytes can contain various populations: B cells, CD4 and CD8 T cell responses. What is the kinetics of the various subpopulations in this model in the 2 knock-outs?

Can the authors speculate on the signal (what is left in MyD88^{-/-} or IL-1R^{-/-} mice) that may drive this increased recruitment of lymphocytes in the lungs of papain-treated mice?

We agree with the reviewer that lymphocytes behaved differently than neutrophils and eosinophils in MyD88 knockout mice following papain inhalation. However at 24 h the levels of lymphocytes in MyD88 and IL-1R1 deficient mice are lower than those observed in WT mice. We do not have a clear explanation for this observation but this result indicates that different mechanisms stimulate the recruitment of immune cells. Papain can induce epithelial cell damage with subsequent release of alarmins or endogenous danger signals that stimulate early MyD88-dependent and independent responses. In the absence of a clear mechanism we did not include any speculation in the Discussion of the revised manuscript.

Will this be sufficient to drive the ILC influx (as described in other papers following repetitive administration of papain), although with slower kinetics?

Please see our answer to the first question.

In figure 5, data are shown from cell type specific knock-outs for MyD88, showing partial effects for every cell type tested, even the cell types that are not expected as the main candidate driving early immune responses, like e.g. the T cells. This makes it suspicious and I wonder whether the results are partially the result of this cell-specific knock-out approach, regardless of the cell type and the factor knock-out. Did the authors included controls to exclude this? Was there a cell type in this setting that did not show any phenotype (comparable to WT)?

We agree with the reviewer that inclusion of a control with MyD88^{f/f} mice is important to ascertain that the targeting construct did not interfere with the responses to papain. To address this question we have performed an experiment comparing MyD88^{f/f} and WT mice. The results showed no differences between B6 and MyD88^{f/f} mice. The results of these experiments were included in the new supplementary Figure 3.

To determine the selectivity of MyD88 cell specific targeting we isolated the epithelial and immune cells from the different KO lines. The results presented to address the comment of the reviewer No 1 confirm that MyD88 targeting is efficient and cell-specific.

As opposed to MyD88 KO mice, the result of cell specific KO lines show that they did not significantly differ from those of WT mice regarding most of the endpoints.

Can in all these cell-specific knock-outs the reduced signal be attributed to IL-1R, as is deduced from the experiments with the cell-specific MyD88? May that be an alternative explanation why so many cell types give a partial response?

The results show that IL-1R1 KO recapitulates the results obtained in MyD88 KO. However, the signaling of other receptors (such as IL-33R and also more marginally IL-36R, as well as TLRs) via MyD88 likely also contributes to the responses to papain inhalation.

The message would come across more clearly if a figure is added on the mechanism and the respective roles of the cells tested in this system.

We agree but feel that it would be difficult to include a figure at this time-point due to the remaining lack of clear-cut conclusions regarding the precise cell types involved in the response to papain.

Second Editorial Decision

10-Aug-2016

Dear Dr. Couturier-Maillard,

It is a pleasure to provisionally accept your manuscript entitled "IL-1R1-MyD88 axis elicits papain-induced lung inflammation" for publication in the European Journal of Immunology. We are sorry for the delay in the peer review. There was a severe delay in receiving one of the reports. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Maria Yazdanbakhsh

Dr. Nadja Bakocevic
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