Toll-like receptor and associated regulators in pneumonia and sepsis

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Chapter 5

Interleukin-33 improves host defense during gram-negative pneumonia derived sepsis

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Abstract

Interleukin (IL)-33 is a multifunctional cytokine that can activate cells via IL-1 receptor like-1 (ST2). We here sought to determine the effect of recombinant IL-33 administration on host defense during severe gram-negative pneumonia induced by the common human pathogen *Klebsiella pneumoniae*. IL-33 given 24 and 1 hour prior to infection strongly improved host defense, as reflected by attenuated bacterial growth and dissemination and a prolonged survival, effects that were dependent on ST2. Postponed IL-33 treatment administered 3 hours after infection remained effective in reducing bacterial loads, while treatment given 24 hours post-infection was not. IL-33 activated type 2 innate lymphoid cells (ILC2s) in vitro, and the effect of IL-33 on bacterial loads could not be reproduced in mice deficient for ILCs. T, B and natural killer cells were not required for the IL-33 effect on bacterial burdens. While IL-33 enhanced recruitment of neutrophils to the lungs, it was still capable of limiting bacterial growth in neutrophil depleted mice. These data suggest that IL-33 treatment improves host defense during *Klebsiella* pneumonia by stimulating ILC2s.
Introduction

Acute lower respiratory tract infection is the third most common cause of death globally, and the world’s leading infectious killer, responsible for an estimated 3.1 million deaths annually [1]. Severe pneumonia invariably results in sepsis, caused by an imbalanced harmful host response to uncontrolled infection and associated with significant distant organ injury [2]. *Klebsiella (K.) pneumoniae* is an important gram-negative causative agent in pneumonia and sepsis [3-5], which recently has received much attention due to the emergence of multidrug resistant strains [6].

Interleukin (IL)-33 is a pleiotropic cytokine belonging to the IL-1 family of cytokines [7, 8]. IL-33 is expressed in the nuclei of many different cell types, where it likely functions as an inhibitor of pro-inflammatory signaling through binding of nuclear factor (NF)-κB subunit p65 and inhibiting expression of NF-κB target genes [9]. Consistent with a function as an alarmin, IL-33 can be released from cells after injury or death by necrosis, and extracellular IL-33 can activate MyD88 dependent signaling by triggering the IL-1 receptor like 1 (ST2)/IL-1 receptor associated protein (IL-1RAcP) receptor complex [10, 11]. IL-33 responsive target cells include macrophages, neutrophils, mast cells, eosinophils, Th2 cells, NKT and NK cells, and group 2 innate lymphoid cells (ILC2s) [7, 8].

Extracellular IL-33 in particular has been implicated in the induction and effector phase of type 2 immune responses such as during helminth infections, allergy and asthma [8]. Remarkably, however, a recent investigation reported a protective effect of recombinant IL-33 in acute fecal sepsis induced by cecal ligation and puncture (CLP) in mice by an effect on neutrophils[12]. In this polymicrobial abdominal sepsis model IL-33 improved outcome and bacterial clearance by enhancing neutrophil recruitment to the primary site of infection by preserving the expression of the main chemotactic receptor on neutrophils, CXCR2 [12]. While CLP is a frequently used sepsis model, it does not capture the consequences of the most common cause of sepsis, i.e., pneumonia [2, 13]. To the best of our knowledge studies on the implications of IL-33 treatment in bacterial pneumonia have not been reported. Here we aimed to study the effect of recombinant IL-33 in acute lower respiratory tract infection, using an established model with the clinically relevant human pathogen *K. pneumoniae*.
Results

IL-33 limits bacterial growth and dissemination and improves outcome of \textit{Klebsiella} pneumonia-derived sepsis by an ST2 dependent mechanism

We used a model of severe gram-negative infection of the lower respiratory tract induced by inoculation with \textit{K. pneumoniae} (\( \approx 10^6 \) colony forming units (CFUs)) via the airways [14-16] to determine the effect of recombinant IL-33 on the host response during pneumonia-derived sepsis. Recombinant mouse IL-33 (1 µg) or vehicle was administered intravenously to wild-type (WT) BALB/c mice 24 and 1 hour prior to infection, a treatment schedule previously shown to be protective in a model of CLP-induced abdominal sepsis [12]. At 6 hours after infection bacterial loads in lungs were similar in IL-33 treated and control mice, and cultures in distant organs remained sterile in both groups (Figure 1A). At 24 and 48 hours IL-33 administered mice had much lower bacterial burdens at the primary site of infection, which was accompanied by markedly lower bacterial counts in blood, liver and spleen.

Figure 1: IL-33 treatment improves host defense during \textit{Klebsiella} pneumonia by a ST2 dependent mechanism. Mice were treated with IL-33 or vehicle 24 and 1 hour prior to infection with \textit{K. pneumoniae}. A) Bacterial loads in lung, blood, liver and spleen in vehicle and IL-33 treated WT mice 6, 24 and 48 hours following induction of pneumonia. B) Bacterial loads 48 hours following inoculation in vehicle and IL-33 treated WT and \textit{st2}−/− mice. C) Survival of vehicle or IL-33 treated WT and \textit{st2}−/− mice following \textit{K. pneumoniae} inoculation (n = 20 per group). Treatment groups and mouse strains are indicated in the figure. Data in panels A and B are presented as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group at each time point). * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) (in panel C for WT mice treated with IL-33 versus all other groups).
To determine whether this IL-33 effect was dependent on the presence of the known IL-33 receptor complex (ST2/IL-1RAcp), we next treated st2 gene deficient (st2−/−) and WT mice with IL-33, using the exact same dosing schedule as in the first series of experiments, and measured bacterial loads 48 hours thereafter (Figure 1B). While IL-33 treated WT mice again demonstrated strongly reduced bacterial counts at all body sites, confirming the results of Figure 1A, such an effect was not seen in st2−/− mice. The extent of Klebsiella growth and dissemination did not differ between vehicle treated st2−/− and WT mice. The improved antibacterial defense in IL-33 treated WT mice resulted in a prolonged survival during this lethal infection: the median survival time in this group (91.4 hours) was significantly longer than in vehicle treated WT mice (60.8 hours), and either vehicle (63.9 hours) or IL-33 treated st2−/− mice (63.8 hours) (Figure 1C, P < 0.0001 for the difference between IL-33 treated WT mice versus other groups). Postponed IL-33 treatment, given as a single injection at 3 hours post infection (1 µg), remained effective in reducing bacterial loads in lungs, blood and distant organs measured 48 hours after inoculation with Klebsiella; however, IL-33 administered 24 hours after infection did not modify bacterial burdens (Supplementary Figure 1). Together these data suggest that IL-33 improves antibacterial defense and survival during Klebsiella pneumonia by an effect via ST2.

**IL-33 activates neutrophils and enhances neutrophil recruitment to the lungs**

In CLP-induced abdominal sepsis IL-33 treatment was associated with an increased influx of neutrophils into the peritoneal cavity; preserved expression of CXCR2 on neutrophils was considered the play an essential role herein [12]. In trying to dissect the underlying mechanism of the protective effect of IL-33 during Klebsiella pneumonia-derived sepsis, we therefore first focused our attention on the impact of IL-33 treatment on neutrophils. In these studies we used the dosing and treatment schedule shown to be protective in CLP-induced [12] and Klebsiella pneumonia induced sepsis (Figure 1), i.e., 1 µg given intravenously at 24 and 1 hour prior to infection. Intravenous IL-33 increased neutrophil numbers in the lung of uninfected mice when compared to vehicle treatment, as reflected by a higher number of Ly6+ neutrophils in lung tissue (Figure 2A-C) and higher myeloperoxidase (MPO) concentrations in whole lung homogenates (Figure 2D) measured at the time at which mice were infected in other experiments (i.e., 1 hour after the second IL-33 injection). Early after infection with Klebsiella (6 hours) IL-33 treated animals still displayed higher lung MPO concentrations (Figure 2F). At later time points, lung MPO concentrations had strongly increased in both groups; at 48 hours IL-33 treated mice had lower MPO levels, most likely as a result of lower bacterial burdens at this time point. While IL-33 administration did not significantly affect neutrophil numbers in peripheral blood (Figure 2E), it caused an upregulation of CD11b with a concurrent downregulation of CXCR2 on blood neutrophils in uninfected mice (Figure 2G). At 6 hours after infection, neutrophil CXCR2
IL-33 improves host defense during gram-negative pneumonia derived sepsis. Neutrophil expression was still lower in the IL-33 treated than the vehicle treated mice. Likewise, neutrophil CD11b expression remained higher in the former group after infection (Figure 2H).

Figure 2: Role of neutrophils in the effect of IL-33 during Klebsiella pneumonia. Mice were treated with IL-33 or vehicle 24 and 1 hour prior to infection with K. pneumoniae. Neutrophil numbers in lung tissue were evaluated by Ly-6 staining of lung slides (A-C) and MPO lung levels (D), and in blood by FACS (E) directly before infection (t=0). Representative Ly-6 stained lung slides of vehicle (A) and IL-33 (B) mice. F) Lung MPO levels 6, 24 and 48h following induction of Klebsiella pneumonia. G-H) CD11b and CXCR2 expression on blood neutrophils before (G) and 6 hours after (H) infection with Klebsiella. (I) Bacterial loads in lung, blood and spleen of neutrophil depleted and control mice (24 hours post infection) treated with either IL-33 or vehicle. Treatment groups are indicated in the figure. Data in panels C-H are presented as means ± SE (n = … per group); data presented in panel I as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group). * P < 0.05, ** P < 0.01, *** P < 0.001.

Neutrophil depletion does not abrogate IL-33 induced enhanced antibacterial defense. Increased IL-33 induced neutrophil recruitment to the lungs before and shortly after infection might explain at least in part the protective effect of this cytokine during Klebsiella pneumonia [17]. Moreover, the reciprocal effects on neutrophil CD11b and CXCR2 expression are suggestive for IL-33 induced neutrophil activation [18, 19], further hinting at a neutrophil-dependent mechanism by which IL-33 improves host defense. To investigate this we depleted neutrophils by intraperitoneal injection of an anti-mouse monoclonal antibody (RB6-8C5) directed against Ly-6G 48 hours prior to infection, a well validated and documented approach to induce sustained neutropenia (< 50
IL-33 improves host defense during gram-negative pneumonia derived sepsis.

IL-33 stimulates type 2 innate lymphocyte cells to enhance antibacterial defense

ILC2s are established ST2+ target cells of IL-33 [22]. Flow cytometry of whole lung cell suspensions generated from naïve mice identified ILC2s (Lin-CD127-CD25+ ST2+; Figure 3A). Lung ILC2s were responsive to IL-33, as indicated by the release of IL-5 and IL-13 upon stimulation of sorted cells with IL-33, especially when co-stimulated with IL-2 or IL-7 (Figure 3B). In accordance, mice treated with IL-33 showed high pulmonary levels of IL-5 and IL-13, especially within the first hours after the final IL-33 injection (Figure 3C). To determine the role of ILCs in the protective effect of IL-33 during Klebsiella pneumonia, we treated rag2−/−/il2rγc−/− mice (which lack T, B and NK cells, as well as ILCs) with IL-33 or vehicle and measured bacterial loads 48 hours after infection with Klebsiella via the airways (Figure 3D). While in WT mice IL-33 strongly reduced bacterial loads in lung, blood and spleen (reproducing the results of Figure 1), this effect was not seen in rag2−/−/il2rγc−/− mice. In contrast, IL-33 reduced bacterial loads to a similar extent in control mice and either rag2−/− mice (which lack T, B and NK cells; Figure 3E) or NK cell depleted mice (by treatment with anti-asialo GM1 antibody; Supplementary Figure 2). Together these data suggest that ILCs are required for the effect of IL-33 on antibacterial defense during Klebsiella pneumonia.

IL-5 or IL-13 are not required for IL-33 induced improved antibacterial defense

Considering the high pulmonary levels of the ILC2 products IL-5 and IL-13 in IL-33 treated mice (Figure 3B,C), we investigated the potential roles of these cytokines in the IL-33 treatment effect on bacterial loads. For this, mice were treated with an anti-IL-5 or control antibody prior to IL-33 treatment and Klebsiella infection (Figure 4A). The biological activity of the anti-IL-5 antibody was illustrated by the fact that the increased eosinophil influx into lung tissue induced by IL-33 treatment was prevented by anti-IL-5 administration, as determined by digital imaging and quantification of eosinophils in lung slides tissue stained with an antibody against major basic protein [23] (Supplementary Figure 3). Of interest, anti-IL-5 reduced bacterial loads in lungs relative to mice treated with the control antibody without significantly affecting bacterial...

neutrophils/µl) [20, 21], an effect confirmed here (data not shown). Since we anticipated early mortality in neutropenic mice, we determined bacterial loads in these experiments 24 hours after infection with a 50% lower Klebsiella dose (~0.5 x 10^4 CFU; Figure 2I). As expected, neutropenic mice demonstrated much higher bacterial loads in lungs, blood and spleen than mice treated with a control antibody. Importantly, IL-33 was similarly effective in neutropenic and control mice in reducing bacterial loads in blood and spleen. Of note, IL-33 only tended to reduce bacterial loads in lungs in these experiments in both groups, resembling the only modest effect of IL-33 on pulmonary bacterial burdens at 24 hours after infection with ~10^4 Klebsiella CFU in Figure 1. Together these results suggest that neutrophils are not crucial for the beneficial effect of IL-33 on host defense during Klebsiella pneumonia derived sepsis.
IL-33 improves host defense during gram-negative pneumonia derived sepsis.

Figure 3: Role of ILCs in the effect of IL-33 during *K. pneumoniae* pneumonia. (A) Lung ILCs and ILC2s in naive WT mice analyzed by FACS. (B) ILC2s purified from lungs of naive mice release IL-5 and IL-13 upon stimulation with IL-33, IL-2 and/or IL-7. (C) Lung IL-5 and IL-13 levels of IL-33 or vehicle treated mice prior to (0h) and 6, 24 and 48 hours post *K. pneumoniae* inoculation. (D) Lung, blood and spleen bacterial loads of *Rag2*^−/−^/*Il2rg*^−/−^ and WT mice (48 hours after infection) treated with IL-33 or vehicle (n= 8 per group). (E) Lung, blood and spleen bacterial loads of *Rag2*^−/−^ and WT mice (48 hours after infection) treated with IL-33 or vehicle (n= 6-8 per group). Treatment groups and mouse strains are indicated in the figure. Data in panel C are presented as means ± SE (n = 8 per group at each time point). Data in panels D and E are presented as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. *P < 0.05, **P < 0.01, ***P < 0.001.
numbers in blood or spleen, suggesting that IL-5 may impair rather than enhance local defense against Klebsiella. While IL-33 did not further reduce bacterial burdens in lungs of anti-IL-5 treated mice, IL-33 strongly lowered bacterial counts in blood and spleen of these animals. IL-33 was equally effective in decreasing bacterial loads in lungs, blood and spleen of il13<sup>−/−</sup> and wild-type mice (Figure 4B). While all previous experiments were done with BALBc mice, il13<sup>−/−</sup> mice and littermate controls were on a C57BL/6 background, thereby demonstrating that the effect of IL-33 does not rely on the genetic background of the mice. Together these data suggest that the IL-5 and IL-13 are not essential for the effect of IL-33 on antibacterial defense during Klebsiella pneumonia.

**Figure 4:** Role of IL-5 and IL-13 in the effect of IL-33 during Klebsiella pneumonia. (A) Bacterial loads in lung, blood and spleen (48 hours after infection) of mice treated with IL-33 or vehicle together with anti-IL-5 or a control antibody. (B) Bacterial loads in lung, blood and spleen (48 hours after infection) of il13<sup>−/−</sup> and WT mice treated with IL-33 or vehicle. Treatment groups and mouse strains are indicated in the figure. Data are presented as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (n = 6-9 per group at each time point). * P < 0.05, ** P < 0.01, *** P < 0.001.

**Discussion**

Augmentation of specific host immune responses might provide supplementary treatment strategies to enhance bacterial clearance and patient recovery in severe bacterial infections. We here studied the effect of recombinant IL-33 as a possible immune modulating treatment in gram-negative pneumonia caused by the human respiratory pathogen *K. pneumoniae*. IL-33 strongly improved host defense during Klebsiella pneumonia, as reflected by markedly reduced bacterial growth and dissemination, and a better survival in IL-33 treated mice. The IL-33 induced stimulation of antibacterial defense was dependent on ST2 and ILCs. Considering that ILC2s are
IL-33 improves host defense during gram-negative pneumonia derived sepsis

established ST2+ target cells of IL-33, these data suggest that activation of ILC2s by IL-33 boosts the clearance of *Klebsiella* from the airways.

IL-33 has been implicated as an important mediator of type 2 innate immunity by stimulating ILC2s [8, 12]. ILC2s release large amounts of the prototypic type 2 cytokines IL-5 and IL-13 upon exposure to IL-33, a finding confirmed here using purified lung ILC2s. IL-33 induced type 2 responses enhance protective immunity during parasitic infections. During infection with the gastrointestinal nematode parasite *Nippostrongylus brasiliensis* and in response to IL-33 ILC2s expanded, which was crucial for host defense by a mechanism that relied on production of IL-13 [24-26]. Similarly, IL-33 boosted host defense during infection with *Trichuris muris* by a mechanism that likely depended at least in part on IL-33 induced production of the type 2 cytokines IL-4, IL-9 and IL-13 [27]. ILC2s in addition can produce amphiregulin [28], which contributes to protective immunity during infection with the intestinal nematode *Trichuris muris* [29]. Furthermore, IL-33 stimulated the expansion of ILC2-like cells in the lungs of mice infected with *Strongyloides venezuelensis*, resulting in the release of IL-5 and IL-13; thereby, ILC2-like cells induced eosinophilic pulmonary inflammation, which assisted in expelling the worms from the lungs [30]. IL-33 stimulated ILC2s also are important players in type 2 responses during allergic inflammation [24-26, 31]. In accordance, we here show that IL-33 induces accumulation of eosinophils in lung tissue by an IL-5 dependent mechanism (Supplementary Figure 3); however, unlike during parasite infection [24-27, 30], neither IL-5 nor IL-13 proved to be essential for the IL-33 effect on bacterial burdens after *Klebsiella* infection. In agreement, type 2 responses have not been associated with antibacterial defense mechanisms, and neither elimination of IL-5 nor IL-13 influenced bacterial loads in mice with allergic lung inflammation and secondary *Klebsiella* pneumonia [32]. Nonetheless, IL-33 required the presence of ST2 and ILCs for its antibacterial effects, which fits with the earlier finding that the responsiveness of ILC2s to IL-33 is dependent on the surface expression of ST2 [33].

Endogenous IL-33 plays a crucial role in allergic inflammation by a mechanism that relies on the activation of ILC2s [34, 35]. In contrast, neither endogenous IL-33 nor ILCs were required for antibacterial defense after infection with *Klebsiella*, as demonstrated by similar bacterial loads in *st2<sup>−/−</sup>* and *rag2<sup>−/−</sup>*/*Il2rγc<sup>−/−</sup>* mice when compared with their respective control animals. In accordance, we recently reported an unremarkable phenotype of *st2<sup>−/−</sup>* mice after intravenous infection with *Klebsiella* [36] and during respiratory tract infection with the gram-positive pathogen *Streptococcus pneumoniae* [37]. The apparently absent role of endogenous IL-33 in these models may be related to the fact that IL-33 lung levels remain unaltered during bacterial pneumonia (data not shown).
ST2 is widely expressed on a variety of innate immune cells [8]. Among these, neutrophils were considered important for the protective IL-33 effect during abdominal polymicrobial sepsis [12]. Neutrophils displayed increased recruitment to the site of infection and enhanced phagocytosis and killing activity in response to IL-33; the IL-33 induced effect on neutrophil influx was accompanied by a preserved expression of the main receptor for neutrophil attracting chemokines in the mouse, CXCR2 [12]. Similarly, IL-33 pretreatment induced a rapid fungal clearance and markedly reduced mortality in an acute Candida albicans peritoneal infection model, which was associated with enhanced recruitment of neutrophils to the site of infection and accompanied by reversal of the TLR-induced reduction of CXCR2 expression in neutrophils [38]. We here demonstrate that intravenous IL-33 augments neutrophil influx into the lungs, corroborating previous findings on increased neutrophil recruitment into the abdominal cavity during peritoneal infection [12, 38]. Notably, however, in our hands IL-33 diminished CXCR2 expression on neutrophils, both in vivo (Figure 2G,H) and in whole blood in vitro (data not shown). Importantly, neutrophils seemed not required for the IL-33 induced enhancement of antibacterial defense, considering that IL-33 remained effective in reducing bacterial loads in blood and spleen after infection with Klebsiella in neutrophil depleted mice, although we cannot totally exclude that IL-33 exerted part of its protective effects via the few remaining neutrophils in mice treated with the depleting antibody. The effect of IL-33 in polymicrobial abdominal sepsis [12] and Candida peritonitis [38] was not studied in neutropenic mice; as such, it remains to be established whether the protective effects of IL-33 are neutrophil dependent in these infections.

The majority of cytokines released in response to IL-33 are known to induce type 2 inflammatory responses, most notably IL-5 and IL-13. However, IL-33 can also induce IFN-γ secretion by NK cells [39]. While IFN-γ may play a protective role in host defense against Klebsiella [40], we excluded a significant role for NK cells in the IL-33 effect by showing that IL-33 still reduced bacterial loads in NK cell depleted mice. In accordance, IL-33 did not induce measurable concentrations of IFN-γ in whole lung homogenates during pneumonia (data not shown).

In conclusion, we here demonstrate a strong protective effect of IL-33 administration in a clinically relevant mouse model of severe gram-negative pneumonia most likely by stimulating ILC2s. These results identify stimulation of ILC2s as a potential therapeutic approach in the treatment of severe bacterial infections.
Materials and methods

Mice
BALB/c and C57Bl/6 mice were purchased from Charles River (Maastricht, The Netherlands). St2−/− BALB/c mice [41] and iIl13−/− C57Bl/6 mice [42] were kindly provided by dr. Andrew N.J. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). Rag2−/− BALB/c mice were kindly provided by dr. Karin de Visser (Dutch National Cancer Institute, Amsterdam, the Netherlands). Rag2−/−/il2rγc−/− BALB/c mice were generated as described [43]. Age- and sex-matched animals were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Experimental infections and treatments
Pneumonia was induced by intranasal inoculation of ~10⁴ or (in one experiment) ~0.5 x 10⁴ CFUs of K. pneumoniae serotype 2 (ATCC 43816) as previously described [14-16]. Mice were intravenously administered with recombinant mouse IL-33 (Biolegend, San Diego, CA; 1 µg) or vehicle 24 and 1 hour prior to start of infection [12]; in some experiments IL-33 was given as a single intravenous injection 3 or 24 hours post inoculation. Mice were sacrificed after 6, 24 or 48 hours of infection. Sample harvesting and processing, including determination of bacterial loads, was done as previously described [14-16]. In separate studies, mice were followed for 7 days and survival was monitored at least every 12 hours. For neutrophil depletion, mice were treated intraperitoneally with anti-GR-1 (RB6-8C5; 500 µg) 48 hours prior to infection [20, 21]. For NK cell depletion mice were treated intraperitoneally with anti-ASGM1 polyclonal antibodies (500 µg; eBioscience, San Diego, CA) or rabbit IgG (R&D systems, Abingdon, UK) [44]. Anti-IL-5 (TRFK5; 500 µg) was administered twice intraperitoneally concurrently with IL-33. Anti-β-Galactosidase antibodies (GL113; 500 µg) were used as IgG control in neutrophil depletion and anti–IL-5 experiment.

Assays
MPO (Hycult, Uden, the Netherlands), IL-5 and IL-13 (both R&D systems) were measured by ELISA.

Flow cytometry and stimulations
Erythrocytes were lysed and remaining blood cells were stained with GR-1–FITC (RB6-8C5, Miltenyi Biotec, Cologne, Germany), CD11b-PE (BD Biosciences, Franklin Lakes, NJ) and CXCR2-APC (R&D systems) antibodies in accordance with the manufacturers’ instructions. Data acquisition was performed using a FACS Canto II (BD Biosciences) flow cytometer. Lungs were harvested, chopped and digested (20min, 37°C while mixing)
IL-33 improves host defense during gram-negative pneumonia derived sepsis

with Liberase TM (Roche, Almere, Netherlands). Spleens were crushed through a cell strainer after which erythrocytes were lysed. To analyse ILC2s, cell-suspensions were stained with the following antibody mix: lineage-PE (CD3, CD11b, B220, GR-1, NK1.1 (all Biolegend)), F4/80-APC (R&D), ST2-FITC (MD Bioscience, St. Paul, MN), CD127-PerCp Cy 5.5 (Biolegend), CD45-PE Cy7 (Biolegend), CD25-Alexa fluor 700 (Biolegend), CD117-APC Cy7 (Biolegend). CD45⁺LinF4/80⁻CD127⁺ cells were deemed ILCs; of these cells CD25⁺ST2⁺ cells were considered ILC2s. For the ex vivo stimulation, ILC2s were sorted using the described antibody mix without ST2-FITC and treated for 3 days with IL-33 (50 ng/ml), IL-2 (100 U/ml; ProSpec, Ness-Ziona, Israel) and IL-7 (5 ng/ml; ProSpec). Cell sorting was done using a FACS ARIA (BD Biosciences).

Histopathology

Granulocyte staining with a Ly-6G monoclonal antibody (BD Pharmingen, San Diego, CA) was performed as described [45, 46], MBP staining was conducted as described [23] using an antibody kindly provided by Nancy Lee and James Lee (Mayo Clinic Arizona, Scottsdale, AZ). Digital scanning analysis by ImageJ (US National Institutes of Health, Bethesda, MD) was done as described [45, 46].

Statistical analysis

Data are expressed as indicated in the figure legends. Differences were analyzed by Mann Whitney U test. For survival studies Kaplan-Meier analyses followed by log rank test were performed. A value of $P < 0.05$ was considered statistically significant.

Reference list:

IL-33 improves host defense during gram-negative pneumonia derived sepsis.

Supplement chapter 5
Supplementary Figure 1: Delayed IL-33 treatment during Klebsiella pneumonia. Mice were treated with a single injection of IL-33 given either 3 or 24 hours after infection. Bacterial loads in lung, blood, liver and spleen 48 hours after infection, shown as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group). Treatment groups and mouse strains are indicated in the figure. *P < 0.05, **P < 0.01.
IL-33 improves host defense during gram-negative pneumonia derived sepsis.

Supplementary Figure 2: Role of NK cells in the effect of IL-33 during Klebsiella pneumonia. Bacterial loads in lung, blood and spleen (48 hours after infection) of mice treated with IL-33 or vehicle together with anti-ASGM1 or a control antibody. Treatment groups are indicated in the figure. Data are presented as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (n = 7-8 per group at each time point). * P < 0.05, ** P < 0.01, *** P < 0.001.
IL-33 improves host defense during gram-negative pneumonia derived sepsis

Supplementary Figure 3: Effect of anti-IL-5 on IL-33 induced eosinophil recruitment to the lungs. Mice treated with IL-33 or vehicle together with anti-IL-5 or a control antibody, and lungs were analyzed 48 hours after infection with Klebsiella. (A) Percentage of lung surface stained positive for eosinophils (means + SE) quantified by digitally imaging of MBP staining. Representative MBP staining of lung tissue-slides: (B) vehicle, (C) IL-33, (D) anti-IL-5, (E) IL-33 + anti-IL-5. *** P < 0.001 (versus all other groups).