

# Essential role of neutrophils but not mammary alveolar macrophages in a murine model of acute *Escherichia coli* mastitis

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**Abstract** – Mastitis, the inflammation of the mammary gland, is an important disease affecting dairy animals worldwide. The disease is caused by mammary pathogenic bacteria and *Escherichia coli* are frequently implicated. Virulence factors of mammary pathogenic *E. coli* are only partially known and intramammary challenge with LPS elicits neutrophil recruitment in experimental bovine and murine mastitis models. We have previously shown that neutrophil recruitment in LPS-induced murine mastitis is strictly dependent on mammary alveolar macrophages. However, the relative role of alveolar macrophages and blood neutrophils in *E. coli* mastitis is not well defined. To this end, we selectively depleted mammary alveolar macrophages or blood neutrophils before intramammary challenge with *E. coli* strain P4 (ECP4). Mice depleted of alveolar macrophages prior to intramammary challenge recruited neutrophils normally and restricted bacterial growth and interstitial invasion. Importantly however, upon depletion of alveolar macrophages, ECP4 invaded the mammary alveolar epithelial cells and formed intracellular bacterial communities. In contrast, neutrophil depletion prior to intramammary infection with ECP4 was associated with unrestricted bacterial growth, tissue damage, severe sepsis and mortality. This study suggests that neutrophils but not alveolar macrophages provide essential antimicrobial defense against mammary pathogenic *E. coli*. Furthermore, we show here similar invasion after depletion of alveolar macrophages as in our previous studies showing that LPS/TLR4 signaling on alveolar macrophages abrogates ECP4 invasion of the mammary epithelium. Interestingly, similar ECP4 invasion and formation of intracellular communities were also observed following intramammary infection of either iNOS gene-deficient or IL-1 receptor type 1 gene-deficient mice.

**mastitis / *Escherichia coli* / macrophage / neutrophil / murine model**

## 1. INTRODUCTION

Mastitis, the inflammation of the mammary gland, is an important disease affecting dairy animals worldwide. The disease is caused by mammary pathogenic bacteria and *Escherichia*

*coli* are frequently implicated. The bacteria invade the milk space via the teat canal, replicate and elicit an innate immune response. Recruitment of blood neutrophils into the alveolar space is the hallmark of *E. coli* mastitis. Intramammary challenge with bacterial LPS is sufficient to elicit neutrophil recruitment and transient disease. However, severe septic

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disease and persistent disease are only caused by live bacteria replicating in the gland [20].

Our studies using adoptive transfer and depletion of mammary alveolar macrophages indicate that LPS/TLR4 signaling on alveolar macrophages is both sufficient and essential for neutrophil recruitment [10, 11]. However, *E. coli* P4 (ECP4) still elicit neutrophil recruitment upon infection of TLR4-deficient mice, presumably by activation of other TLR by their corresponding ligands. These putative TLR and the expressing cells (e.g. macrophages or epithelial cells) are yet to be defined. Intriguingly, in the absence of LPS/TLR4 signaling in alveolar macrophages, ECP4 invade the mammary alveolar epithelium and this invasion is abrogated by wild type macrophages. Hence we hypothesized in this study that depletion of alveolar macrophages in normal mice will result in ECP4 invasion of the alveolar epithelium after intramammary challenge.

Using a set of knock out mice we previously showed that neutrophil recruitment in response to LPS/TLR4 signaling is mediated by tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) produced by alveolar macrophages and is dependent on interleukin 1 $\beta$  (IL1 $\beta$ ) and IL8 signaling and regulated by nitric oxide (NO) [10]. However, the role of alveolar macrophages and these mediators in neutrophil recruitment in response to intramammary *E. coli* infection is still unclear.

The aim of this study was to investigate the role of mammary alveolar macrophages, blood neutrophils and IL1 $\beta$  signaling and iNOS-derived NO in neutrophil recruitment in response to ECP4 infection in the mouse mastitis model.

## 2. MATERIALS AND METHODS

### 2.1. Intramammary mouse bacterial challenge

Six to eight week-old female C3H/HeN (wild-type TLR4), C3H/HeJ (TLR4-deficient), C57BL/6 (wild-type iNOS and IL-1R), iNOS gene-deficient (iNOS  $-/-$ ) and IL-1 receptor type 1 gene-deficient (IL-1R  $-/-$ ) mice were used in this study. Knockout mice were purchased from The Jackson Laboratory (Bar Harbor, Main, USA) and are described in the

JAX<sup>®</sup> Mice Database<sup>1</sup>. All mice were maintained under specific pathogen free conditions and handled under protocols approved by the Hebrew University Animal Care Committee, according to international guidelines. Bacterial intramammary challenge was performed 7–10 days post partum. Mice were challenged via teat canal catheterization with 10  $\mu$ L bacterial suspension (100–1 000 cfu) of MPEC strain ECP4 bacteria grown to log phase (3 h, 37 °C, 110 rpm) in LB broth. Next, bacterial suspensions were diluted in sterile, non-pyrogenic PBS to the indicated concentrations and plated on LB agar plates to determine the real number of live bacteria inoculated in every experiment. The challenged gland was the abdominal L4, whereas the abdominal R4 was injected with PBS only as a control in the same manner.

### 2.2. Mammary alveolar macrophage depletion

Mammary alveolar macrophages were depleted by intramammary infusion of 50  $\mu$ L of clodronate liposomes (containing dichloromethylene diphosphate, CL<sub>2</sub>MDP, a gift from Boehringer Mannheim GmbH, Mannheim, Germany). Contralateral control glands were similarly infused with saline liposomes. All liposomes were prepared and supplied by N. van Rooijen as previously described [21]. Liposomes were infused into the teat canal of lactating mice 24 h before bacterial challenge using the above-described technique.

### 2.3. Neutrophil depletion

Total body neutrophil population was depleted by injection of 400  $\mu$ L purified anti-mouse Ly-6G (Gr-1, Gr1) IP 24 h before ECP4 challenge [6]. General neutrophil depletion was confirmed by blood smear microscopy twice: at time of challenge and at time of harvesting.

### 2.4. Histochemical analysis

Gland tissues were trisected for cytokines, chemokines and myeloperoxidase (MPO) assays, histology and immunohistochemistry. Samples for histological analysis were fixed in neutral buffer 4% formaldehyde, paraffin-embedded, and sections were cut and stained with haematoxylin and eosin (HE). Fresh mammary tissue for fluorescence staining was fixed in 2.5% paraformaldehyde over-night at room

<sup>1</sup> <http://www.jax.org/>

temperature, incubated with 15% sucrose for 12 h at 4 °C and frozen in Tissue-Tek® (EMS, Hatfield, PA, USA) embedding medium. Serial cryosections of 10 µm were stained with either phalloidin-rhodamine (Sigma, Rehovot, Israel) and 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma) or Sytox® Orange nucleic acid stain (Invitrogen) and DAPI. Section Samples were mounted with VectaShield® (Vector Laboratories, Burlingame, CA, USA) and imaged with a Nikon Eclipse E400 epifluorescence microscope with an Olypus DP70 camera.

### 2.5. Total bacterial counts

Mammary tissue (~ 100 mg) was weighed and homogenized on ice in 1 mL sterile PBS immediately after removal. For total bacterial counts, gland homogenates were plated as serial 10-fold dilutions on LB agar plates and incubated overnight at 37 °C to determine CFU count.

### 2.6. Differential bacterial counts

Mammary tissue (~ 100 mg) weighed and enzymatically digested by 1 h incubation with DNase-Collagenase solution (1 mg/mL Collagenase type IV (Sigma)), and 0.2 mg/mL DNase (Roche Applied, Indianapolis, USA) at 39 °C, digested tissue was disrupted by repeated pipetting and passed through a mesh (100 µm nylon) to give a cell suspension. The first sample, for total bacterial counts, was taken from the cell suspension as is, and kept on ice before culture. The cell suspension was then centrifuged (500 g, 5 min, 4 °C), to separate extracellular bacteria from cells and a second sample, for extracellular bacterial counts, was taken from the supernatant and kept on ice before culture. Afterwards, a gentamicin protection assay was performed to yield the last sample for intracellular bacterial counts. Briefly, cells were washed 3 times with 10 mL cold PBS and centrifugation (500 g, 5 min, 4 °C), cells were suspended with PBS containing 50 µg/mL gentamicin, incubated for 1 h in 37 °C to kill any adherent extracellular bacteria and washed twice to remove gentamicin. Finally, cells were enumerated with a hemocytometer and lysed with 0.1% Triton on ice for 20 min to release intracellular bacteria. The lysis of cells by Triton was confirmed by microscopic examination, and no deleterious influence of the detergent on the bacterial viability was observed. All bacterial samples (Total, Extracellular, Intracellular) were plated or dropped as serial 10-fold

dilutions on LB agar plates and incubated overnight at 37 °C to determine CFU count. The number of bacteria per cell was calculated by dividing intracellular CFU with total cell count.

### 2.7. Cytokines, chemokines and myeloperoxidase assays

For the cytokines, chemokines and MPO assays, 100 mg of mammary gland tissue was homogenized on ice in sterile 0.025% Triton X-100/PBS. After homogenization, samples were centrifuged (10 000 rpm) for 5 min at 4 °C and then the supernatant was removed, aliquoted and frozen at -80 °C. Total protein concentration was determined by the Bradford assay (Sigma) and the concentrations of IL1β, TNFα, keratinocyte derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) were measured in duplicate by mouse ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturers' instructions. Cytokine and chemokine levels were reported per mg total protein.

MPO activity in mammary tissue homogenates was measured as previously reported [11]. Briefly, colorimetric reaction in triplicate samples was used to compare MPO activity in the inoculated glands and the contralateral control gland using tetramethyl benzidine (TMB, Vector Labs.) as substrate at a wavelength of 450 nm. A standard curve relating known MPO activity (MPO, Sigma) with the absorbance was obtained and total MPO activity was calculated as mU per mg total protein. The relative MPO activity was calculated by the ratio between total MPO activity measured in the LPS-challenged gland and in the contralateral, unchallenged controlled gland.

### 2.8. Statistical analysis

Relative MPO activity and concentrations of IL1β, TNFα, KC and MIP-2 are reported as the means ± standard errors of the means (SE) of values obtained from at least two different experiments and the  $n \geq 6$ /group. Comparisons of the means were tested with the *t*-test for paired values.

Mean CFU counts were calculated at 24 h and 48 h after challenge and compared by the non-parametric Mann-Whitney two-independent-samples test for comparison of means using SPSS 10.0.1 software (SPSS Inc., Chicago, IL, USA). A *p* value of 0.05 or less was considered significant.

### 3. RESULTS

#### 3.1. Effect of alveolar macrophage depletion on neutrophil recruitment and epithelial invasion by ECP4

Our previous studies have shown that alveolar macrophages are both sufficient and essential for neutrophil recruitment elicited by LPS/TLR4 signaling in the mammary gland [10, 11]. To investigate the role of mammary alveolar macrophages in EC-induced murine mastitis, we examined the effect of depleting this population using liposome-encapsulated clodronate. Clodronate liposomes or saline liposomes were infused into the teat canal 1 day before intramammary challenge of C3H/HeN mice with ECP4. Initially, at 24 h after challenge, we could not observe any difference between the two experimental groups responding with neutrophil recruitment (Figs. 1A–1C) and massive bacterial replication (Fig. 2A) in the milk spaces as previously reported by us [11]. Interestingly, 48 h after challenge we observed numerous ECP4 organisms inside the alveolar mammary epithelial cells of the macrophage depleted group. At the same time, no other differences were observed between the two experimental groups with respect to neutrophil recruitment as reflected by MPO activity (Fig. 1C) and bacterial growth (Fig. 2A).

We previously demonstrated alveolar epithelial cell invasion by ECP4 in TLR4-deficient C3H/HeJ mice 48 h after intramammary challenge [11]. Furthermore, we showed that in the absence of LPS/TLR4 signaling on alveolar macrophages levels of IL1 $\beta$ , TNF $\alpha$ , KC and MIP-2 remain basal in the mammary tissue after LPS challenge [10]. Here we show that ECP4 challenge in the absence of LPS/TLR4 signaling on alveolar macrophages elicited similar increases in mammary levels of IL1 $\beta$ , TNF $\alpha$ , KC and MIP-2 to that observed in the presence of such signaling (Fig. 3). We can conclude that bacterial virulence factors other than LPS elicited similar mammary inflammation. However the absence of LPS/TLR4 signaling on alveolar macrophages enabled bacterial invasion of the alveolar epithelium by an unknown mechanism.

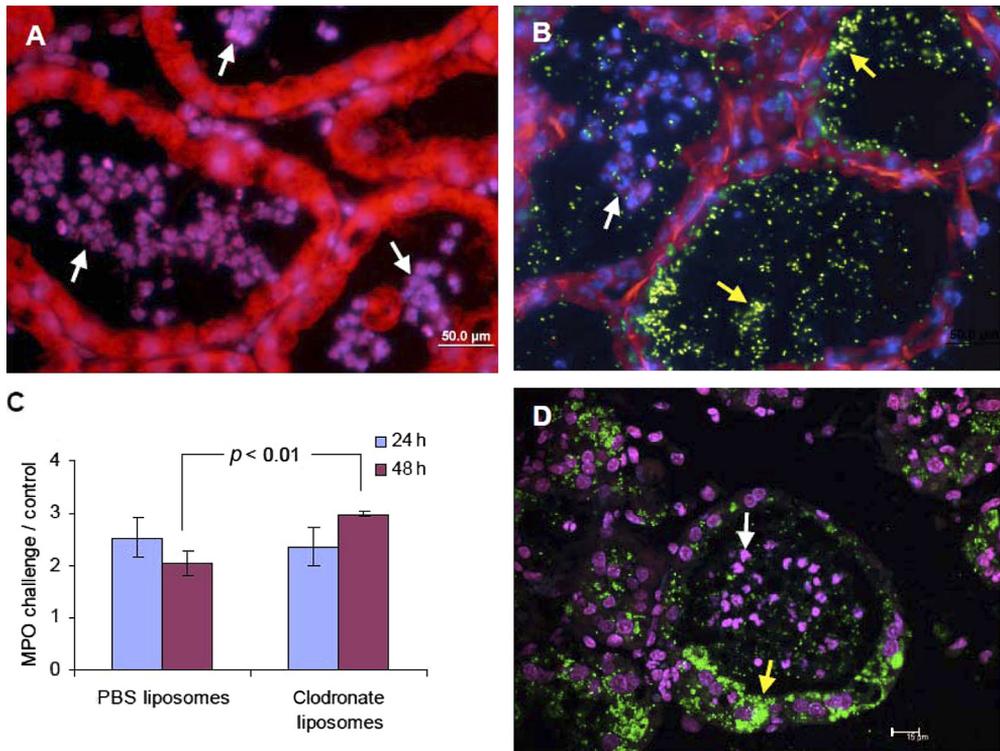
#### 3.2. Effect of iNOS deficiency on neutrophil recruitment and epithelial invasion by ECP4

Using iNOS  $-/-$  mice, we previously showed that iNOS derived NO regulates neutrophil recruitment in response to LPS/TLR4 signaling on alveolar macrophages [10]. Here we show that iNOS deficiency did not affect neutrophil recruitment (Figs. 4A and 4C) after intramammary challenge with ECP4. Interestingly, 48 h after challenge we observed numerous ECP4 organisms inside the alveolar epithelial cells of iNOS-deficient mice (Fig. 4D). This was also shown using differential extracellular and intracellular bacterial counts in the mammary gland (Fig. 2B).

These results might indicate that neutrophil recruitment in response to bacterial virulence factors other than LPS is not regulated by iNOS-derived NO. Furthermore, in the absence of iNOS-derived NO, bacterial invasion into the alveolar epithelial cells is enabled by an unknown mechanism.

#### 3.3. Effect of IL-1 $\beta$ receptor deficiency on neutrophil recruitment and epithelial invasion by ECP4

We previously showed that neutrophil recruitment into the mammary alveolar space in response to LPS/TLR4 signaling in alveolar macrophages is dependent on IL1 $\beta$  signaling in blood neutrophils [10]. Using IL1R  $-/-$  mice we observed trapping of neutrophils in blood vessels and interstitium in response to LPS challenge [10]. Here we show that intramammary challenge of IL1R  $-/-$  mice with ECP4 elicited a mixed response which included normal neutrophil recruitment into the alveolar space (Figs. 5A–5B) and trapping of neutrophils in the interstitium (Fig. 5C). This is further reflected by significantly higher MPO levels observed 48 h after challenge in IL1R  $-/-$  mice in comparison with WT mice ( $p < 0.001$ ) (Fig. 5D). Interestingly, 48 h after challenge we observed numerous ECP4 organisms inside the alveolar epithelial cells of IL1R  $-/-$  mice (Fig. 5B). This was also shown using our novel differential extracellular and



**Figure 1.** *E. coli* P4 invades mammary epithelium in alveolar macrophage-depleted mice. Mammary glands of lactating C3H/HeN mice were infused with either PBS-loaded liposomes (A–B) or clodronate-loaded liposomes (D) followed by intramammary infection with *E. coli* P4. Fluorescence staining of paraffin embedded sections (A and D) or cryosections (B) with DAPI (blue) combined with Sytox orange (red) (A) or phalloidin-rhodamine (red) (B and D) of mammary tissues 48 h after bacterial challenge. GFP expressing bacteria (yellow arrows in B and D) are visible in the alveolar space of PBS-liposome treated mice (B) and inside epithelial cells and in the alveolar space in clodronate-liposome treated mice (D). Neutrophil recruitment into the alveolar space is clearly visible in both treatments (white arrows) which is also reflected by relative (ratio of challenge to control gland) MPO activity (C). Data are mean and S.E.,  $n \geq 6$ /group and  $p$  value compared PBS-liposomes with clodronate-liposomes infused mice using Student's  $t$ -test. Scale bars 50  $\mu$ m (A–B), 15  $\mu$ m (D). All images are representative of the entire sample and the histological morphology and pathology results were very similar for each gland in a given mouse and between mice. (For a color version of this figure please consult [www.vetres.org](http://www.vetres.org).)

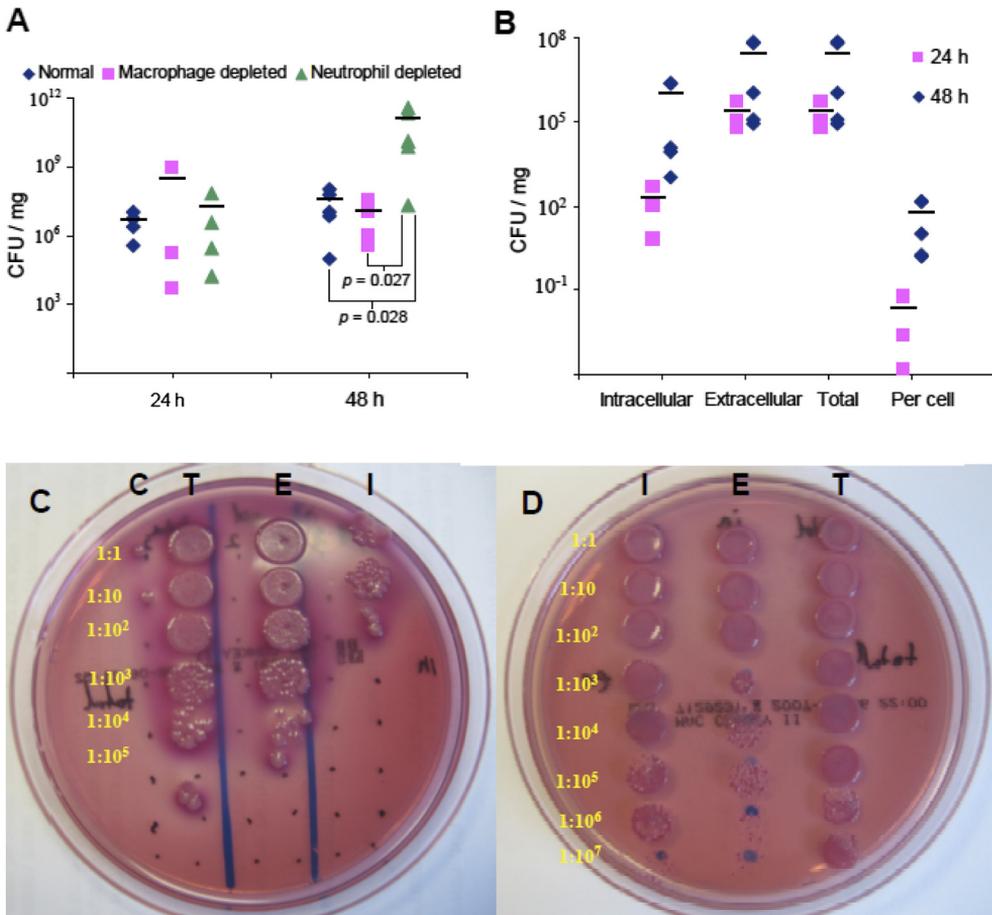
intracellular bacterial counts in the mammary gland (Figs. 2C–2D).

These results might indicate the presence of two parallel independent pathways responding to bacterial virulence factors; one is activated by LPS and is IL1 $\beta$  dependent and an IL1 $\beta$ -independent pathway elicited by other bacterial virulence factors. Furthermore, in the absence of IL1 $\beta$  signaling, ECP4 invasion into

the alveolar epithelial cells was enabled by unknown mechanism.

#### 3.4. Effect of neutrophil depletion on ECP4 intramammary infection

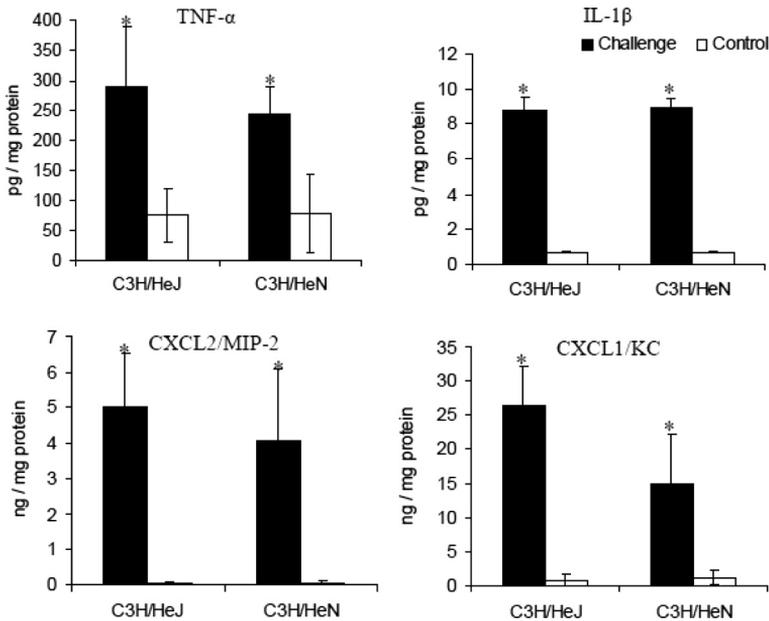
Neutrophil recruitment into the milk spaces is considered the hallmark of mammary response to bacterial invasion and replication.



**Figure 2.** Bacterial counts in mammary tissue homogenates after intramammary infection with *E. coli* P4 in C3H/HeN (A), iNOS  $-/-$  (B) and IL1R  $-/-$  (C–D) mice. Data represent individual mouse bacterial counts and horizontal bars represent mean bacterial counts (A–B). Total bacterial counts 24 and 48 h after challenge of normal, macrophage depleted and neutrophil-depleted C3H/HeN mice (A) demonstrating the unrestricted overgrowth of bacteria after neutrophil depletion. Differential counts of total, extracellular and intracellular bacteria in mammary tissues of iNOS  $-/-$  mice 24 and 48 h after challenged (B) demonstrating epithelial invasion of bacteria 48 h after challenge. Horizontal bars represent the means,  $n \geq 6$ /group and  $p$  values were calculated using the non-parametric Mann–Whitney two-independent-samples test for comparison of means. Epithelial invasion in IL1R  $-/-$  mice is reflected by differential counts of total (T), extracellular (E) and intracellular (I) bacteria in mammary tissue of IL-1 $\beta$ R  $-/-$  mice 24 (C) and 48 h (D) after challenge using drop plating of the dilutions indicated of tissue homogenates (1:10 to 1:10<sup>7</sup>). The challenge dose (C) of the experiment is presented on drop plate C. (For a color version of this figure please consult [www.vetres.org](http://www.vetres.org).)

To investigate the role of neutrophil recruitment in EC-induced murine mastitis, we examined the effect of depleting whole-body neutrophils using anti-neutrophil antibodies.

Neutropenia was induced in C3H/HeN mice by intraperitoneal injection of anti-mouse Ly-6G (Gr-1) antibodies 24 h before ECP4 challenge.

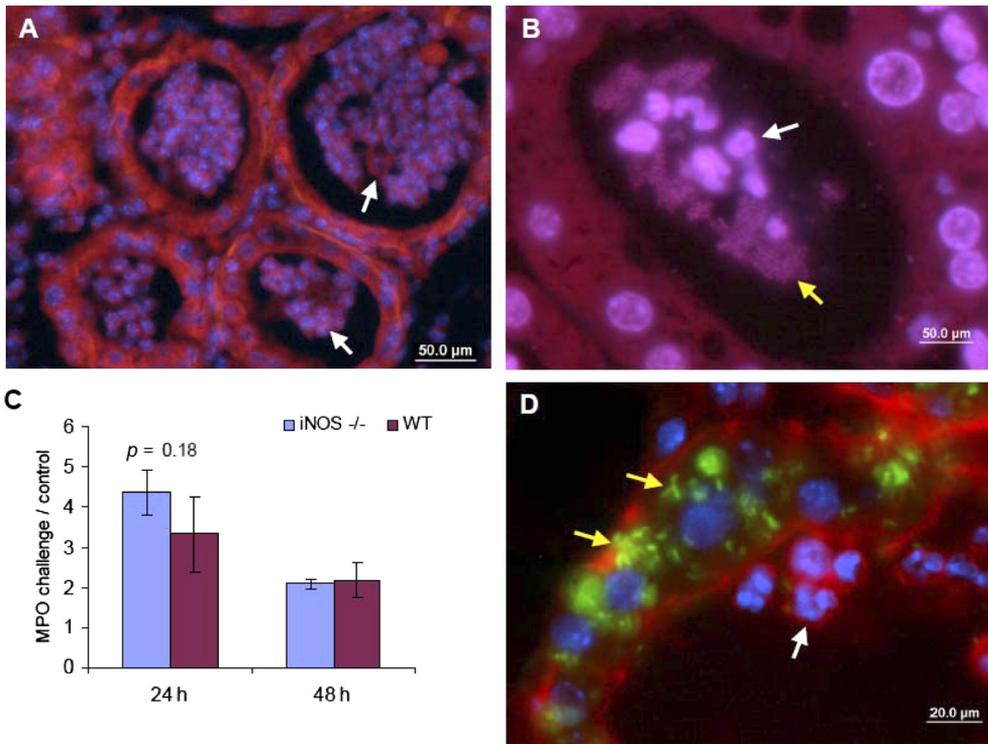


**Figure 3.** Similar levels of mammary cytokines and chemokines were produced 24 h after intramammary infusion of *E. coli* P4 in C3H/HeN and C3H/HeJ mice. Bars represent mean concentrations per mg total protein and S.E. of TNF $\alpha$ , IL-1 $\beta$ , MIP-2 and KC measured by ELISA in tissue homogenates of *E. coli* P4 and PBS-infused control glands of 6 mice in two different experiments. \* $p < 0.05$  value compared means between challenged and control glands using Student's *t*-test.

In all our previous animal studies intramammary challenge elicited a transient and localized disease, without systemic signs or sepsis. Intramammary challenge of neutropenic animals with ECP4 elicited severe and fatal septic mastitis. Severe systemic signs developed 24 h after challenge with animals showing progressive depression, weakness and anorexia. Severe swelling developed in the infected mammary gland which was accompanied by lameness. All animals were euthanized by 48 h after challenge due to extreme signs of disease and moribund state. Control, normal C3H/HeN mice challenged with the same dose of ECP4 organisms survived for 48 h without showing any systemic or localized signs of disease as previously reported by us [11].

Macroscopically, the infected glands of neutropenic animals were severely swollen, congested with petechiations and ecchymoses.

Microscopically, moderate congestion and lack of neutrophil recruitment were observed 24 h after challenge (Figs. 6A–6B), while at 48 h severe changes were observed characterized by extensive bleeding and swollen epithelial cells with vacuolated cytoplasm and pyknotic nuclei (Figs. 6C–6D). At 24 h after challenge, bacterial counts in the mammary gland were no different in neutropenic animals in comparison with normal ( $p = 0.462$ ) or macrophage depleted ( $p = 0.724$ ) C3H/HeN mice (Fig. 2A). However, 48 h after challenge, bacterial counts were significantly higher in neutropenic animals in comparison with normal ( $p = 0.028$ ) or macrophage depleted mice ( $p = 0.027$ ) (Fig. 2A), probably indicating unrestricted growth of challenge organisms. This is also demonstrated microscopically by numerous bacteria in the alveolar spaces and inside epithelial cells (Fig. 6D).

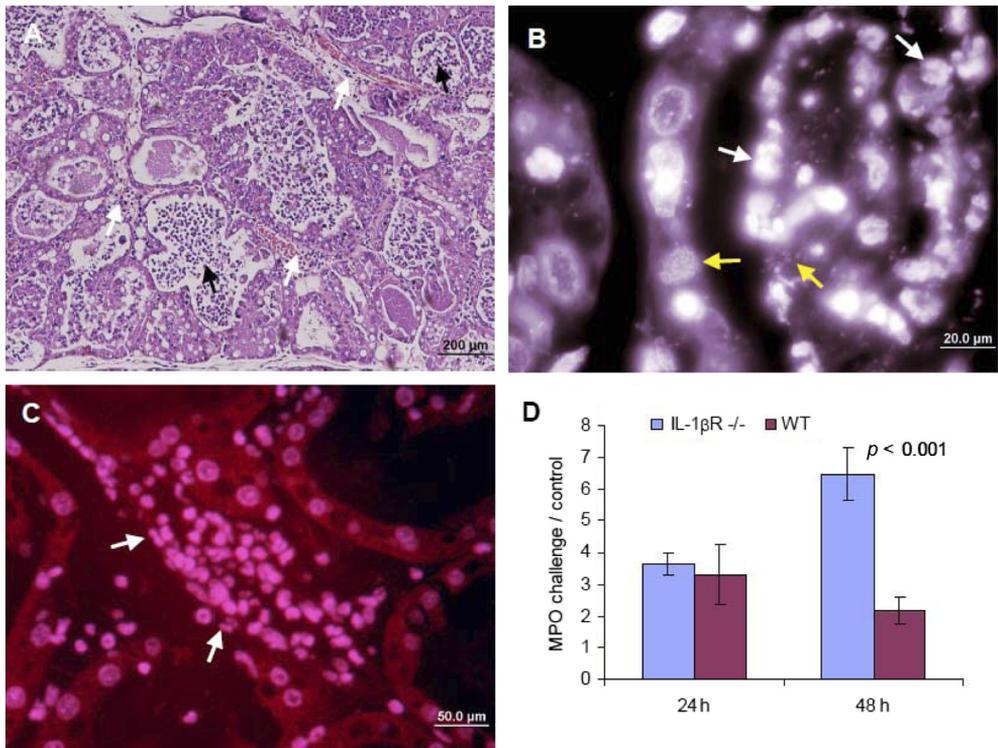


**Figure 4.** *E. coli* P4 invades mammary epithelium of iNOS  $-/-$  mice 48 h after intramammary infection. Fluorescence staining of cryosections (A and D) or paraffin embedded section (B) with DAPI (blue) combined with phalloidin-rhodamine (red) (A and D) or Sytox orange (red) (B) of mammary tissues 48 h after bacterial challenge. Numerous bacteria are visible in the alveolar space of the wild type (wt) C57BL/6 mouse (yellow arrow in B) while GFP expressing bacteria are visible inside epithelial cells in iNOS  $-/-$  mice (yellow arrows in D). Similar neutrophil recruitment (white arrows) into the alveolar space is clearly visible in both C57BL/6 (B) and iNOS  $-/-$  (A and D) mice, which is also reflected by relative (ratio of challenge to control gland) MPO activity (C). Data are mean and S.E.,  $n \geq 6$ /group and *p* value compared iNOS  $-/-$  with wt mice using Student's *t*-test. Scale bars 50  $\mu$ m (A-B) and 20  $\mu$ m (D). All images are representative of the entire sample and the histological morphology and pathology results were very similar for each gland in a given mouse and between mice. (For a color version of this figure please consult [www.vetres.org](http://www.vetres.org).)

**4. DISCUSSION**

Although mammary pathogenic *E. coli* is a common and important pathogen of the bovine mammary gland, the underlying virulence mechanism of this pathogen is still largely unknown. Infusion of the mammary gland with bacterial LPS is sufficient to elicit transient mastitis characterized by recruitment of blood neutrophils into the alveolar and duct space. Using a set of knock out mice we previously

showed that neutrophil recruitment in response to LPS/TLR4 signaling was mediated by TNF $\alpha$  produced by alveolar macrophages and was dependent on IL1 $\beta$  and IL8 signaling and regulated by NO [10]. However, our studies in TLR4-deficient mice demonstrated the presence of virulence factors other than LPS mediating similar recruitment of blood neutrophils [11]. We show here that alveolar macrophages were not essential for neutrophil recruitment after ECP4 infection while previously proven

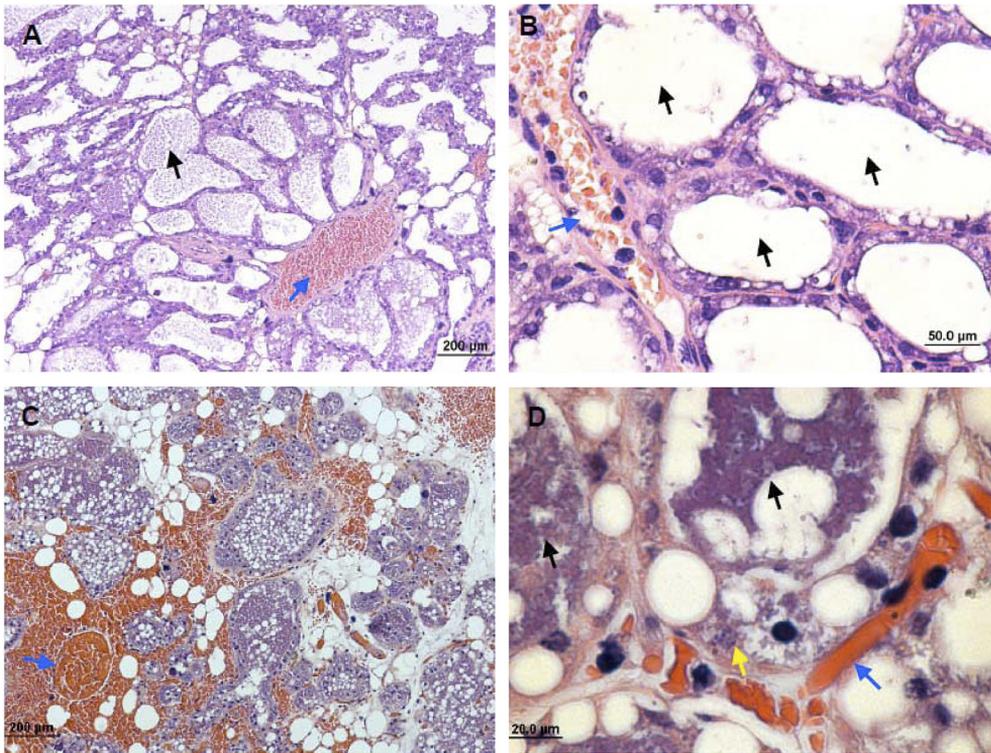


**Figure 5.** *E. coli* P4 invades mammary epithelium of IL1R<sup>-/-</sup> mice 48 h after intramammary infection. Paraffin embedded sections stained with H&E (A) or Sytox orange (red) combined with DAPI (blue) (B–C). Numerous bacteria are visible in the alveolar space and in the alveolar epithelial cells (yellow arrow). Inflammation is characterized by massive neutrophil recruitment into the alveolar and tubular spaces (black arrows in A) which is better seen in the enlarged alveolus in B (white arrows). Congestion and accumulation of neutrophils in the interstitial spaces is also seen in IL1R<sup>-/-</sup> mice (white arrows in A and C), which is also reflected by significantly higher relative MPO activity than in wt mice (D). Data are mean and S.E.,  $n \geq 6$ /group and  $p$  value compared means between IL1R<sup>-/-</sup> and wt mice using Student  $t$ -test. Scale bars 200  $\mu$ m (A), 20  $\mu$ m (B) and 50  $\mu$ m (C). All images are representative of the entire sample and the histological morphology and pathology results were very similar for each gland in a given mouse and between mice. (For a color version of this figure please consult [www.vetres.org](http://www.vetres.org).)

essential for neutrophil recruitment elicited by LPS/TLR4 signaling [10]. Furthermore, neutrophil recruitment in response to ECP4 infection was not affected by IL1 $\beta$  signaling or iNOS-derived NO. We previously showed that neutrophil recruitment in response to LPS/TLR4 signaling is dependent on IL1 $\beta$  signaling. Using IL1 $\beta$ R-deficient mice we observed trapping of neutrophils in mammary blood vessels and interstitium after LPS challenge [10]. Adoptively transferred normal neutrophils

trans-mediated recruitment of IL1 $\beta$ R-deficient neutrophils [10]. Here we report normal neutrophil recruitment in IL1 $\beta$ R-deficient mice after ECP4 challenge which might indicate the presence of alternative signaling pathways for neutrophil recruitment elicited by microbial virulence factors other than LPS.

Using iNOS-deficient mice we showed that iNOS-derived NO regulates neutrophil recruitment mediated by LPS/TLR4 signaling in the mammary gland [10]. In the present study,



**Figure 6.** Severe tissue damage and uncontrolled bacterial growth and invasion after *E. coli* P4 infection in neutrophil-depleted C3H/HeN mice. H&E staining of paraffin sections of mammary tissues 24 (A–B) and 48 h (C–D) after *E. coli* P4 infection of C3H/HeN mice rendered neutropenic by intraperitoneal injection of anti Gr-1 antibodies 24 h before infection. Normal tissue architecture is maintained and some congestion (blue arrows) is visible 24 h after infection (A–B), and neutrophils are not seen in the alveolar spaces (black arrows in A–B). Fatal septic mastitis 48 h after infection is associated with severe hemorrhages (blue arrows in C–D), massive bacterial growth in the alveolar spaces (black arrows in D) and invasion into alveolar epithelial cells which are undergoing severe swelling, cytoplasmic vacuolization and nuclear condensation (yellow arrow in D). Scale bars 200 μm (A and C), 50 μm (B) and 20 μm (D). All images are representative of the entire sample and the histological morphology and pathology results were very similar for each gland in a given mouse and between mice. (For a color version of this figure please consult [www.vetres.org](http://www.vetres.org).)

normal neutrophil recruitment was observed in iNOS-deficient mice in response to ECP4 infection. Furthermore, neither macrophage depletion nor NO deficiency were associated with impaired bacterial clearance or increased mortality after intramammary infection. Macrophages and their antimicrobials such as NO are critical for the host defense when challenged with pathogenic bacteria at macrophage-rich portals of entry, such as the peritoneum and lung [7, 14]. While we have previously demon-

strated the small number of mammary alveolar macrophages in steady state and after challenge, we also demonstrated their critical role for LPS signaling and neutrophil recruitment [10, 11]. Mammary alveolar macrophages are probably critical for immune signaling while recruited neutrophils are essential for bacterial control and clearance.

The importance of neutrophil recruitment and function in the host defense against mastitis pathogens has been implicated in many

studies. The efficacy and speed of neutrophil recruitment is one of the main predictors of the outcome of an intramammary infection. A swift response results in rapid clearance of infection and relatively mild clinical signs [13, 15–17]. Post partum cows are more severely affected and septic mastitis is more common at early lactation. Neutrophil functions and recruitment are negatively affected in periparturient cows [5] and we have shown that ketone bodies abrogates formation of bovine neutrophil extracellular traps (NET) and bactericidal activity against mastitis EC [12]. Using the mouse mastitis model we show here that while ECP4 infection elicited transient mastitis in normal animals, neutropenic animals were severely affected. While normal animals challenged with ECP4 consistently developed transient disease, neutropenic animals developed a fatal systemic disease, associated with unrestricted bacterial growth and severe mammary damage. This septic murine mastitis model clearly resembles field cases of septic mastitis in dairy cows [4, 5].

Intracellular bacterial communities of *E. coli* were previously described in the urinary tract and mammary gland. In both organs the formation of these communities is associated with the lack of LPS/TLR4 signaling [1, 11]. Since we have shown that adoptive transfer of normal macrophages is sufficient to abrogate bacterial invasion in TLR4-deficient mice [11] we hypothesize here that depletion of alveolar macrophage will result in bacterial invasion in normal mice. Indeed, we show here that depletion of alveolar macrophages was sufficient to enable bacterial invasion into the mammary alveolar epithelium. While investigating the role of IL1 $\beta$  signaling and iNOS-derived NO in neutrophil recruitment in response to ECP4 infection we serendipitously observed intraepithelial bacterial communities in these knockout mice. The IBC we observed in TLR4-deficient, IL1 $\beta$ R-deficient and iNOS-deficient mice were similar and the mechanism of bacterial invasion might be similar although currently unknown. ECP4 and other mastitis EC strains do not invade or form bacterial communities in murine (our unpublished observations) or bovine epithelial cell lines [8, 9, 18]. Similarly, although

some strains of urinary pathogenic *E. coli* (UPEC) are more invasive than mastitis EC strains, intracellular bacterial communities (IBC) are difficult to reproduce using in vitro urinary cell lines [2].

While a considerable importance is attributed to the formation of IBC in urinary tract infection (UTI), their significance in mastitis is currently unknown. UTI in women is notoriously chronic and relapsing and IBC most probably play an important role in this course of events [19]. Persistent and relapsing *E. coli* mastitis have been previously described in dairy cows but its prevalence and significance in the epidemiology of this disease in dairy animals is currently unknown [3]. Although it was recently suggested that persistent mammary infection by *E. coli* is most likely related to the type of bacteria [22], we show here that innate immune response mounted by the host might also be of prime importance. When the host innate immune system was compromised, the transient bovine mastitis ECP4 strain was able to invade the mammary epithelium to establish IBC which might be the underlying mechanism of persistent mastitis. We are currently trying to elucidate the mechanism of epithelial invasion by mastitis pathogens using both in vitro and in vivo studies. We believe that this mechanism might be of general importance for the better understanding of virulence mechanisms and host-pathogen interactions.

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