Immune response in the cow mammary gland

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University of Veterinary Medicine foundation
Hannover, Germany
Who did the work?

2002 – present
German Science Foundation
EADGENE
European Animal Disease Genomic network
Pfizer/Zoetis Mastitis Consortium
German Ministry of Agriculture
What differs between E. coli and S. aureus?
Pathogen-specific responses

I. Development of Model systems
   ○ In vivo / in vitro
     ○ Clinical differences
     ○ Early regulated genes and signal transduction pathways

II. Compartment-specific responses
   ○ Teat, ducts, parenchyma
   ○ neighboring quarters

III. Development of a preventive immunomodulatory approach
Mastitis

- Subclinical
- Acute
- Clinical
- Chronic

Bacterial load:
- E. coli: high
- S. aureus: low
- S. uberis: high

Interactions with immune cells/Epithelial cells differ.

'Most likely': Host Immune response types differ.

Survival strategies differ.
‘Immune responses’ ... where to start with 😊
Innate immunity starts with the recognition of the pathogen.

If this is not enough:
Pathogens replicate, release molecules, Come in contact with **Epithelial cells**
Epithelial cells sense pathogens
(mammary epithelial cells are the most abundant cell in the udder)

Pathogen recognition via Toll-like receptors

Alveolar epithelial cells express TLR2

Petzl et al. 2008
Expression of anti-microbial peptides in the udder (‘shut-up and kill’)

αS1-casein   BNBD5

Casein and Defensin expression in mammary epithelial cells (serial sections)

Vanselow et al. 2006
Model systems for pathogen-specific responses

- First lactating
  - mid-lactation
- Non-pregnant
- SCC (each quarter!)
  - < 50,000 cell/ml
  - 8 weeks prior exp. Infection
- Estrus at day of challenge

- Primary mammary epithelial cells as the immune cell in the udder
E. coli and S. aureus indeed differ

**Escherichia coli**, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow

Wolfram PETZL\(^1\), Holm ZERBE\(^1\), Juliane GUNTHER\(^2\), Wei YANG\(^2,4\), Hans-Martin SEYFERT\(^2\), Gerd NURNBERG\(^2\), Hans-Joachim SCHUBERTH\(^3, *\)


DOI: 10.1051/vetres:2007057

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[www.vetres.org](http://www.vetres.org)
Clinical differences between pathogens are reflected by different gene expression profiles.
Compartment-specific responses

• Pathogen-specific responses start at the entry site

• Constitutive expression of antimicrobial factors
  • Teat > parenchyma

• Induction of inflammatory & chemotactic factors in the teat
  • LPS > LTA
In the first hours, host-pathogen interactions take place in distal parts of the udder.

**Gene expression Gradient**

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Challenge: $5 \times 10^6$ CFU *E. coli*  
Duration: 4 h
The Teat

Location-specific expression of chemokines, TNF-α and S100 proteins in a teat explant model

Monique Lind¹, Anja S Sipka², Hans-Joachim Schuberth³, Andreas Blutke⁴, Rüdiger Wanke⁴, Carola Sauter-Louis¹, Katarzyna A Duda⁵, Otto Holst⁵, Pascal Rainard⁶, Pierre Germon⁶, Holm Zerbe¹ and Wolfram Petzl¹

Figure 1. Experimental set-up: 22 udder quarters from six animals were sampled. Four tissue samples were taken from the TC and FR respectively. After sample preparation, two tissue explants/location were stimulated for 3 h with either 1 μg/ml LPS or 10 μg/ml LTA, and two remained unstimulated. The mRNA was extracted from two pooled explants/location either stimulated or unstimulated.
The teat recognizes pathogens and shapes a pathogen-/location-specific immune response

Fold expression of selected mediators

- **Up-regulated genes**
- **LPS**: CXCL8, CCL5, CCL20, TNF-a
  - S100A8, A9, A12
- **LTA**: CXCL8, , CCL20,
  - S100A8,
Moving to the teat cistern

Short-term infection model
E. coli, S. aureus: $5 \times 10^6$ CFU
Sequential inoculation, 1h, 2h, 3h
TC, Parenchyma
RT-PCR: Chemokines, Cytokines, antimicrobial molecules
Divergent pathogen-specific responses are generated during the first hour

- Response after 1 h
- Almost all: restricted to TC
- E. coli:
  - All factors upregulated
- S. aureus:
  - failed to induce IL1B, IL-10, LAP, S100A9
Attention: NEIGHBORING quarters

Escherichia coli- and Staphylococcus aureus-induced mastitis differentially modulate transcriptional responses in neighbouring uninfected bovine mammary gland quarters

Kirsty Jensen¹*, Juliane Günther², Richard Talbot³, Wolfram Petzl⁴, Holm Zerbe⁴, Hans-Joachim Schuberth⁵, Hans-Martin Seyfert² and Elizabeth J Glass¹
'Crosstalk': Subsequent inoculations of different quarters result in less severe symptoms...

Systemic effects (fever,...) of first inoculation?! (E. coli)
What have we learned

• Two lines of host responses
  • Local response in the inoculated quarter
  • Sterile quarters respond to infection of neighboring quarters
    • enhanced inflammatory/stress gene expression
    • Even ‘silent’ S. aureus infections!

• Quarters cannot be considered as independent entities

• The host response modulates future tissue responses
The molecular basis of the difference between E. coli and S. aureus

Is there a pathogen-specific modulation?
HEK293 TLR reconstitution system
 Both pathogens activate TLR receptors

*E. coli* or *S. aureus*

Yang et al. 2008
However, *S. aureus* does not activate NF-κB in bovine MEC!
The failure to activate NF-κB in response to S. aureus is specific for Mammary epithelial cells

- How does that influence the pathogen-specific induction of genes in MEC?
**E. coli induction is FASTER & STRONGER**

TABLE 2. Alteration of mRNA levels of cytokine/chemokine-encoding genes after stimulation with *E. coli* or *S. aureus*

<table>
<thead>
<tr>
<th>Chemokine or cytokine&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>2</td>
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<sup>a</sup> mRNA levels are shown as relative expression levels compared to unstimulated controls.
Interaction network analyses (Ingenuity pathway analyses)

Exclusively E. coli-regulated genes

S. aureus-regulated genes

E. coli: IL-1/TNF-a as hub molecules

S. aureus: IL-6 as hub molecule

Interaction network dominated by IL-1A and TNF- as of the 27 top-ranked genes regulated exclusively by E. coli

S. aureus-regulated gene yields with the highest statistical significance for a network dominated by IL-6.
Fig. 1. Pathogen-specific activation of the immune response in MEC. E. coli activates the expression of the three master cytokine IL-1, TNF-α and IL-6. S. aureus only drives significant IL-6 expression via a MyD88 independent signal transduction. This depends on strong induction of the unusual NF-κB factor NF-κBζ. Adapted from Günther et al. (2011).
Development of a preventive immunomodulatory approach
Principal options to modulate innate immune mechanisms

- Systemic
- Organ-specific
- Cell-specific
Organ-specific immunomodulatory approaches

1. Change of tissue reactivity
   Selective attraction of immune cells
2. Inhibition of cell immigration
   Modulation of in situ differentiation of immune cells

1. Udder epithelium
   Chemokine expression (Interleukin-8)
2. Endothelium
   Adhesion molecule expression
   Chemokine receptor expression
   Blood

Change of tissue reactivity
Selective attraction of immune cells
Inhibition of cell immigration
Modulation of in situ differentiation of immune cells
Organ-specific immunomodulation by induction of endotoxin tolerance

Suhra et al. 2009
Gene silencing by Histone modifications (epigenetic reprogramming)

Vaccine-, cytokine, PAMP-induced

(e.g. no IL-1β = no fever)
Rational behind: The reprogramming of mammary epithelial cells
Lipopolysaccharide priming enhances expression of effectors of immune defence while decreasing expression of pro-inflammatory cytokines in mammary epithelia cells from cows

Juliane Günther¹†, Wolfram Petzl²†, Holm Zerbe², Hans-Joachim Schuberth³, Dirk Koczan⁴, Leopold Goetze⁵ and Hans-Martin Seyfert¹*
Figure 1 Schematic diagram of the experimental setting.
Endotoxin priming works in mammary epithelial cells

**Inflammatory mediators**
- Primed cells
  - IL6
    - **-1.5**
  - NOS2
    - **-3.4**

**Antimicrobial factors**
- Primed cells
  - LAP
    - **1.6**
  - SLPI
    - **2.4**

**Primers**
- E. coli
  - Priming
    - +
  - +
  - +
  - +

36
Priming in vitro, summing up

• TLR priming of MEC provokes
  • A higher expression level of some genes
  • A lower expression of some genes

• After E. coli challenge
Endotoxin priming also works in the mid-lactating cow

Lipopolysaccharide pretreatment of the udder protects against experimental Escherichia coli mastitis

Wolfram Petzl¹*, Juliane Günther²*, Tobias Pfister¹, Carola Sauter-Louis¹, Leopold Goetze³, Sonja von Aulock⁴, Angela Hafner-Marx⁵, Hans-Joachim Schuberth⁶, Hans-Martin Seyfert² and Holm Zerbe¹
Example for Silent Pathogen-Elimination in the udder (mid-lactating cows)

LPS infusion  
1 µg / quarter

E. coli challenge

240 h

EC

72 h

24 h

L72EC

L240EC

tissue sampling

Petzl et al. 2011, Innate Immun; 18:467
Primbing with Endotoxin

**A) Inhibits clinical E. coli Mastitis**

- **Body Temp**
- **Blood leukocytes**
- **SCC**

**Clinical Score**

**B) Eliminates bacteria**

- **3d**
- **10d**
- **No priming**

*Petzl et al. 2011, Innate Immun; 18:467*

Reduced inflammation

Increased anti-microbial response
Primming with Endotoxin selectively inhibits the induction of gene expression after E. coli challenge

Petzl et al. Innate Immunity, 2012

Reduced inflammation
Increased anti-microbial response
Primming post partum reduces severity of *E. coli* mastitis

**Post partum**

**Midlactation**
Priming post partum reduces local and systemic clinical signs.

**Diagram Description:**
- **Systemic Score:**
  - X-axis: Time (h)
  - Y-axis: Systemic score
  - Two groups: Not primed (red) and Primed (blue)
  - Levels: Severe, Moderate, Mild

- **Local Score:**
  - X-axis: Time (h)
  - Y-axis: Local score
  - Two groups: Not primed (red) and Primed (blue)
  - Levels: Severe, Moderate, Mild

*Note: An asterisk (*) indicates a significant difference.

**Legend:**
- Not primed (red)
- Primed (blue)

**Species:**
- E. coli
Priming post partum reduces pathogen load!

Median + IQR ; * = P<0.001
Why do we have differences in the priming efficiency?

Post partum Priming works... But less efficiently

Maybe because tissue composition changes

Mid-lactation

Post-Partum

S100A8/A9+ macrophages in teats
Summary

• E. coli & S. aureus differ in the induced host-response
  • Pathogen-specific clinical appearance is reflected in the transcriptome response

• Systemic reactions dominate during E. coli-Mastitis
  • E. coli & S. aureus differentially modulate the response in neighboring, sterile quarters

• Lack of full MEC activation by S. aureus explains the failure to clear this pathogen

• TLR-mediated (epigenetic) reprogramming of local cells in the udder is a promising prophylactic tool
MILLE GRAZIE!
VIELEN DANK!
THANK YOU SO MUCH FOR
ATTENTION 😊
General remarks

Mastitis

- Subclinical
- Chronic
- Acute
- Clinical
Pathogen ≠ Pathogen

<table>
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<th>E. coli</th>
<th>S. aureus</th>
<th>S. uberis</th>
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<td>Duration</td>
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<td>Mastitis</td>
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<tr>
<td>Bacterial load</td>
<td>high</td>
<td>low</td>
<td>high</td>
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</tbody>
</table>

- Survival strategies differ
- Interactions with immune cells/Epithelial cells differ
- *Most likely*: Host Immune response types differ
Die lokale Antwort im Euter ist abhängig vom Laktationsstadium.

physiologische Immunmodulation

pro-inflammatorische Antwort

anti-inflammatorische Antwort
The cow responds differently to pathogens during the peri-partum period. The local immune response changes during lactation.
Sensor function is followed by effector function

Pathogens

Epithelial cells

Defense molecules
Antimicrobials
Epithelial cells call for help

Pathogens

Epithelial cells

Immune cells

‘Calling’, ‘Crosstalk’ Influenced by NEFAs, Hormones, Cytokines,...
FIG. 5. Proposed signaling pathways being activated in MEC through challenges with heat-inactivated particles of E. coli or S. aureus pathogens. (A) E. coli immediately induces the early expression of the proinflammatory master cytokines IL-1α and IL-1β as well as TNF-α. The activation of these cytokines is MyD88 dependent. IL-1β and TNF-α are synthesized and secreted and may bind to their cognate receptors (IL1R and TNFR). This subsequently leads to the activation of NF-κB and AP1, which in turn induces the expression of the secondary response genes (e.g., BCL2A1, BIRC1/2, LAP, S100A8/9, CFB, COX2, CX3CL1, and MMP9). In addition, TNF-α is also a major mediator of apoptosis. Intracellular IL-1α may also function as an endogenous transcription regulator inside the cell of its synthesis (22).

(B) S. aureus and E. coli stimulations both lead to an early activation of NFKBIZ. This transcriptional activator is known to be essential for the TLR-mediated induction of IL-6 expression. We assume that the induction of IL-6 expression in MEC is MyD88 independent. Secreted IL-6 binds to the IL-6 receptor (IL6R), resulting in the activation of both JAK and MAPK signaling cascades. The activation of the JAK cascade leads to the formation of an active ISGF3 complex, which may induce the expression of genes harboring ISRE sites in their promoters. MAPKs activated through IL-6 signaling induce the activation of NF-IL-6, also known as the transcription factor C/EBPβ. The transcription induced by ISGF3 and C/EBPβ dominates the secondary response to S. aureus.
FIG. 2. Alteration in the mRNA concentrations of TNF-α, IL-1A, IL-6, and IFN-β (IFN-β1 and IFN-β2) in pbMEC after challenge with E. coli (△) and S. aureus (□) particles. Shown are values for mean fold induction (ordinate, ± standard error of the mean [SEM]; n = 3) at times after challenge (abscissa) relative to the mRNA concentration measured for unstimulated cells (set as 1). Asterisks indicate a statistical significance (*, P ≤ 0.05; **, P ≤ 0.01 [by t test]) of the difference between the E. coli and S. aureus values at the time indicated. The IL-1A insert shows a Western blot analysis of IL-1A in lysates (20 μg/slot) from unchallenged pbMEC (0 h) and from those challenged for 2 h or 4 h with S. aureus or E. coli particles (10⁷ particles/ml). The band shows the 31-kDa IL-1A precursor protein.
Incoculations

- single
- Sequential
Our concept:
Pathogen-dependent signal transduction in MEC

- TLRs
  - Pathogen-specific Receptors
- PAMPs
  - Pathogen Associated Molecular
- NF-κB:
  - Family of 5 factors: Master switches for Immune gene expression

- PAMPs → TLRs
- TLRs → MyD88, TRIF...
- MyD88, TRIF... → NF-κB
- NF-κB → Effector genes: Cytokines, β-defensins, iNOS...

➢ Are modulations herein causing the pathogen-specific response?
Conclusion – *E. coli* response

Consequence: Heavy inflammation & pathogen clearance

Günther *et al.*, (2011);

Early
*E. coli* induces IL1 and TNF-α
They stimulate their receptors, thus activating transcription factors

Late
These elicit a strong secondary response:
More inflammatory cytokines +
Bactericidal factors (beta-defensins, others...)
Conclusion – *S. aureus* and *E. coli* response

**S. aureus / E. coli**

Günther *et al*, (2011);

**MyD88 independent**

Consequences: weak inflammation, no pathogen clearance, chronic infection

Both pathogens induce IL6.

IL 6 induces ist receptor...

...thus activating MAPK & JAK kinases.

This causes late activation of C/EBPβ and ISRE driven genes.

MyD88 independence indicates independence of TLR-driven NF-κB activation

NFκB: NF-κB inhibitor zeta
C/EBP: CCAAT/enhancer binding protein
STAT: signal transducer and activator of transcription
ISRE: interferon stimulated response element
Priming of the teat alone does not work

Die Zitze nimmt am Entzündungsprozess teil

Transriptome profiling 4h after inoculation with 5x10^6 CFU E. coli

Gene expression gradient

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Teat-priming modulates the course of mastitis – but it is not preventing

Retarded (12-36 h) clinical klinische Erscheinungen (mastitis), verglichen mit nicht geprimten Tieren (12 h) nach intramammärer E. coli inokulation
Teat tissue composition changes – a possible reason for different tissue responses

Mid-lactation

Post-Partum

S100A8/A9+

Risk of mastitis

Dry Period

Lactation

Intramammary Infection

Clinical Mastitis
Epigenetic regulation of cells is a widespread phenomenon

Epigenetic regulation of immune cell functions during post-septic immunosuppression

William F. Carson IV, Karen A. Cavassani, Yali Dou and Steven L. Kunkel

Epigenetics 6:3, 273-283; March 2011;