Contrast enhanced ultrasound molecular imaging of the inflammatory response in myocarditis

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Conflicts of interest: none
Background: Myocarditis

- Major cause of sudden unexpected death in adults under 40 years
- Direct viral injury and host immune response can lead to dilated cardiomyopathy
**Background: Myocarditis**

- Major cause of sudden unexpected death in adults under 40 years
- Direct viral injury and host immune response can lead to dilated cardiomyopathy
- CD4+ lymphocytes are crucial in mediating an autoimmune response

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Background: Diagnostic tools for Myocarditis

- No non-invasive imaging method available to assess specific components of the inflammatory process in myocarditis

- **Ultrasound molecular imaging**: Successful detection of inflammation in models of ischemia reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical symptoms</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>ECG</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>+++</td>
<td>(+)</td>
</tr>
<tr>
<td>Echochardiography</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRI</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Endomyocardial biopsy</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

1 Villanueva et al. Circulation 2007;115:345-52
“Ultrasound molecular imaging can detect myocarditis and specific cellular components with the use of targeted microbubbles”
Methods: Study design

- **Induction of experimental autoimmune myocarditis (EAM)**
  8 weeks old balb/c mice

  9 weeks 10 weeks 11 weeks

  control

  9 weeks 10 weeks 11 weeks

  Pertussis Toxin i.p.
  Cardiac myosin heavy chain s.c.
Methods: Study design

- **Induction of experimental autoimmune myocarditis (EAM)**
  8 weeks old balb/c mice

- **Methods:**
  - Pertussis Toxin: i.p.
  - Cardiac myosin heavy chain: s.c.

- **Induction Timeline:**
  9 weeks | 10 weeks | 11 weeks

- **Control Timeline:**
  9 weeks | 10 weeks | 11 weeks

- **Imaging Techniques:**
  - High frequency ultrasound imaging
  - Ultrasound molecular imaging
  - Histology, Immunohistology
Methods: Study design

- **Induction of experimental autoimmune myocarditis (EAM)**
  8 weeks old balb/c mice

  ![Injection Schedule](image)
  - Pertussis Toxin i.p.
  - Cardiac myosin heavy chain s.c.

  ![Timeline](image)
  - 9 weeks
  - 10 weeks
  - 11 weeks

  Control
  - 9 weeks
  - 10 weeks
  - 11 weeks

  **High frequency ultrasound imaging**
  **Ultrasound molecular imaging**
  **Histology, Immunohistology**

- **Pathologist blinded to treatment scored tissue using established criteria**

  ![Histology Images](image)
  - No Myocarditis
  - Moderate Myocarditis
  - Severe Myocarditis

  ![Scales](image)
  50 µm

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Methods: Microbubble preparation

- Lipid-shelled microbubbles with gas core (Decafluorobutane)
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- Lipid-shelled microbubbles with gas core (Decafluorobutane)

- Targeting of microbubbles
  - Specific **shell components**
    - $\text{MB}_{\text{PS}}$: incorporation of phosphatidylserine
      (target: activated leukocytes)\(^1\)

\(^1\) Lindner JR et al., Circulation 2000; 102:2746-2750
Methods: Microbubble preparation

- Lipid-shelled microbubbles with gas core (Decafluorobutane)

- Targeting of microbubbles
  - Specific shell components
    - $\text{MB}_{\text{PS}}$: incorporation of phosphatidylserine (target: activated leukocytes)$^1$
  - Surface conjugation of antibodies
    - $\text{MB}_{\text{CD4}}$: anti-CD4 antibody [H129.19] (target: CD4$^+$ lymphocytes)
    - $\text{MB}_{\text{PSele}}$: anti-P-Selectin antibody [RB40.34] (target: inflammatory activation of endothelial cells)$^2$
    - $\text{MB}_{\text{ctr}}$: isotype control antibody

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$^1$ Lindner JR et al., Circulation 2000; 102:2746-2750
$^2$ Lindner JR et al., Circulation 2001; 104:2107-12
Attachment of $\text{MB}_{\text{CD}4}$ to $\text{CD}4^+$ lymphocytes

- $\text{CD}4^+$ lymphocytes isolated from mouse spleens
Attachment of MB$_{CD4}$ to CD4$^+$ lymphocytes

- CD4$^+$ lymphocytes isolated from mouse spleens
- Attachment of MB$_{CD4}$ assessed under static conditions
Attachment of MB_{CD4} to CD4^+ lymphocytes

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- Attachment of MB_{CD4} assessed under static conditions
- Microscopic quantification of MB adhesion to cells
CD4-targeted MB: \textit{in vitro} validation

Attachment of \( MB_{CD4} \) to \textbf{CD4\textsuperscript{+} lymphocytes}:

- CD4\textsuperscript{+} lymphocytes isolated from mouse spleens
- Attachment of \( MB_{CD4} \) assessed under static conditions
- Microscopic quantification of MB adhesion to cells

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Attached MB / visual field}
\end{figure}
CD4-targeted MB: *in vitro* validation

Retention of MB$_{CD4}$ on **CD4 protein under flow**

- Parallel plate flow chamber (Glyotech Co.)
- CD4 protein adsorbed to cover slip
- MB$_{CD4}$ or MB$_{Ctr}$ (3x10$^6$ ml$^{-1}$) drawn with 2 dynes/cm$^2$
- Microscopy for quantification of MB adhesion
Novel CD4-targeted Microbubbles

- Specifically attach to CD4$^+$ lymphocytes
- Attach to CD4 protein under flow conditions
Methods: High Frequency Ultrasound

- Imaging with 40 MHz (VEVO 2100 VisualSonics)
  - EF calculated from left ventricular end-systolic and end-diastolic diameters measured on M-Mode
  - Strain determined on B-mode images with speckle tracking algorithms

- Analysis performed by an investigator blinded for animal treatment
Methods: Ultrasound Molecular Imaging

- Linear array probe (15L8, Sequoia 512 Siemens)
- Contrast Pulse Sequence imaging at 7 MHz
- i.v. injection of $3 \times 10^6$ microbubbles ($\text{MB}_{\text{CD4}}$, $\text{MB}_{\text{PSel}}$, $\text{MB}_{\text{PS}}$ and $\text{MB}_{\text{Ctr}}$)
- Subtraction of pre- and post- destruction to derive signal from retained microbubbles
- Analysis performed by an investigator blinded for animal treatment

Heart imaged in short axis at papillary muscles
Results: Myocardial Systolic Function

Ejection fraction

- Control Animals (n=20)
- Myocarditis Moderate (n=12)
- Myocarditis Severe (n=8)

Ejection fraction [%]

n.s.
Methods: Ultrasound Molecular Imaging

Control Animals (n=20)

Acoustic Intensity (AU)

- Ctr
- CD4
- Psel
- PS

*/**/*** p-Values < 0.05/0.01/0.001
Methods: Ultrasound Molecular Imaging

Control animal

Severe Myocarditis
Results: $\text{MB}_{\text{CD4}}$ Signal vs. CD4$^+$ Counts

- Heart sections stained for CD4 with immunohistology
Results: MB$^{CD4}$ Signal vs. CD4$^+$ Counts

- Heart sections stained for CD4 with immunohistology
- Number of CD4$^+$ lymphocyte

![Low CD4 count](image1.png)

![High CD4 count](image2.png)

CD4$^+$ cells / mm$^2$
**Results: MB\textsubscript{CD4} Signal vs. CD4\textsuperscript{+} Counts**

- Heart sections stained for CD4 with immunohistology
- Number of CD4\textsuperscript{+} lymphocyte vs. signal intensity of MB\textsubscript{Ctr} and MB\textsubscript{CD4}

**MB\textsubscript{Ctr}**

\[
f(x) = 0.008x + 1.56 \\
R^2 = 0.16 \\
p = 0.06
\]

**MB\textsubscript{CD4}**

\[
f(x) = 0.031x + 2.17 \\
R^2 = 0.45 \\
p < 0.001
\]
Conclusions

- Ultrasound molecular imaging can detect leukocyte infiltration and endothelial inflammation in myocarditis.
Conclusions

- Ultrasound molecular imaging can detect leukocyte infiltration and endothelial inflammation in myocarditis.
- Detection is possible even in moderate myocarditis, where functional imaging fails to show differences.
- Specific imaging of the recruitment of CD4+ lymphocytes involved in autoimmune responses in myocarditis is possible.

Contrast enhanced ultrasound molecular imaging could be a promising technique for the diagnosis of myocarditis.
Strain Measurements

**longitudinal**

<table>
<thead>
<tr>
<th>Strain [%]</th>
<th>Control Animals (n=20)</th>
<th>Myocarditis Moderate (n=12)</th>
<th>Myocarditis Severe (n=8)</th>
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<tbody>
<tr>
<td>n.s.</td>
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<tr>
<td>p=0.03</td>
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**circumferential**

<table>
<thead>
<tr>
<th>Strain [%]</th>
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<tbody>
<tr>
<td>n.s.</td>
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</tr>
<tr>
<td>n.s.</td>
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**radial**

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<tr>
<td>n.s.</td>
<td></td>
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<tr>
<td>n.s.</td>
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</table>
Myocardial Perfusion

- Linear array probe (15L8, Sequoia 512 Siemens)
- Contrast Pulse Sequence imaging at 7 MHz
- Continuous i.v. injection of $5 \times 10^6 \text{ MB s}^{-1}$
- Imaging with high MI
Microbubble size distribution
Inflammation in Myocarditis

Yuan et al, Journal of Clin Immunology 2010; 30:226-34


Fig. 1. Possible mechanisms involved in the pathogenesis of autoimmune myocarditis.
Targeted Contrast Background Subtraction

![Graph showing the number of microbubbles over imaging time with curves for total, free floating, and adhering microbubbles.](image)

- **Total**
- **Free floating**
- **Adhering**

Pre-destruction and imaging time axes.