Identification of a synthetic TLR4-agonistic peptide V77-E92
derived from breast-milk $\alpha_{S1}$-casein

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Abstract: Breast milk $\alpha_{s1}$-casein was suggested as an agonist of the Toll-like receptor 4 (TLR4). Pathogen recognition receptor TLR4 responds to lipopolysaccharides and a wide range of molecules, from proteins to metal ions. In consequence, three criteria are required to validate agonists which directly activate TLR4 and exclude TLR4-agonisticity through contaminants. Recently, we demonstrated that $\alpha_{s1}$-casein fulfilled two of these criteria. (i) $\alpha_{s1}$-Casein required TLR4/MD2 complex as well as cofactor CD14 to induce IL-8 secretion via TLR4 and (ii) $\alpha_{s1}$-casein bound TLR4, MD2 and CD14. Aim of this study was to (iii) identify a synthetic amino acid sequence derived from human $\alpha_{s1}$-casein responsible for TLR4-agonistic effects.

For this, we analyzed the amino acid sequence (AAS) of $\alpha_{s1}$-casein *in silico*. $\alpha_{s1}$-Casein showed to be $\alpha$-helical and was likely to be intrinsically disordered in the region corresponding to R$^{16}$-K$^{99}$ of $\alpha_{s1}$-casein. Six truncated variants of $\alpha_{s1}$-casein coding for parts of the AAS were purified from *Escherichia coli*. These variants were tested for binding to HEK293 cells transfected with TLR4 (TLR4$^+$) by flow cytometry and their induction of IL-8 secretion via TLR4. Variants of $\alpha_{s1}$-casein truncated at the N-terminus (E$^{35}$-W$^{185}$, R$^{57}$-W$^{185}$, V$^{77}$-W$^{185}$) bound TLR4$^+$ induced lower IL-8 secretion with less AAS (7.5 ng/ml, 4.8 ng/ml, 3.6 ng/ml). Variant corresponding to E$^{93}$-W$^{185}$ of $\alpha_{s1}$-casein was neither binding TLR4$^+$ nor inducing IL-8 secretion. Therefore, we postulated V$^{77}$-E$^{92}$ derived from $\alpha_{s1}$-casein as TLR4-agonist. This was confirmed by a synthetic peptide V$^{77}$-E$^{92}$ derived from $\alpha_{s1}$-casein, which induced an IL-8 secretion of 0.95 ng/ml. Hence, the third criteria of TLR4-agonists fulfilled and activation of TLR4 through contamination was excluded.

In conclusion, $\alpha_{s1}$-casein was proofed as an agonist directly activating TLR4. This supported our postulate that $\alpha_{s1}$-casein has at least two functions, a nutritional and an immune active one.

Keywords
Breast milk; human $\alpha_{s1}$-casein; synthetic TLR4-agonistic peptide; inflammasome.
Human $\alpha_{s1}$-casein

Expressed in:

- **breast milk** (functional food)
  - transport of molecules, minerals
  - induces life long IgG response
  - Peptides of $\alpha_{s1}$-casein bind opioid receptors
- **Synovia of patients** (RA / OA)
- **Breast- and prostate cancer**

$\alpha_{s1}$-casein was investigated as TLR4-agonist

Two of three criteria were shown before

(i) $\alpha_{s1}$-casein required TLR4/MD2 for effects

(ii) $\alpha_{s1}$-casein bound directly to TLR4 and cofactors MD2/CD14

(iii) ? Synthetic peptide of $\alpha_{s1}$-casein induced effects *via* TLR4?

### Diagram

- $\alpha_{s1}$-casein
- TLR4/MD2/CD14
- MyD88
- TRIF
- MAPK p38
- ERK 1/2, JNK, p38
- IL-1$\beta$, IL-6, IL-8
- CD14 and CD64
- proinflammatory
- antiinflammatory

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In silico predicted structure and in vitro analysis of $\alpha_{S1}$-casein

(intrinsically) disordered
flexible structure, known phosphorylation site

ordered
conserved structure, less accessible

Truncations of the amino acid sequence of $\alpha_{S1}$-casein were purified from Escherichia coli.
Are truncations of $\alpha_{S1}$-casein binding to TLR4-transfected HEK293 cells?

Seed out cells

1x10⁶ cells/mL
24 h, 37 °C, 5% CO₂

2x10⁵ cells/mL
24 h, 37 °C, 5% CO₂

Incubation with truncations of $\alpha_{S1}$-casein

Analysis of supernatants for IL-8

- C1, C2 bound cells with TLR4
- N1 and N2 bound to cells with TLR4, N3 showed hints to bind these cells
  N4 was a non-binder of cells with TLR4.
Are truncations of $\alpha_{s1}$-casein binding to HEK293 cells with TLR4 receptor?

Seed out cells

1x10^6 cells/mL
24 h, 37 °C, 5% CO₂

2x10^5 cells/mL
24 h, 37 °C, 5% CO₂

Incubation with truncations of $\alpha_{s1}$-casein

Analysis by flow cytometry

- anti-His₆ mlgG
- anti-Maus IgG Dylight 633
- Ex: 633 nm / Em: 660/20 nm

C1, C2 induced IL-8 secretion via TLR4
N1-N3 induced IL-8 secretion via TLR4, but not N4
All induced IL-8 secretions were magnitudes lower than induced by $\alpha_{s1}$-casein

Is peptide V77-E92 TLR4-agonistic?
Testing of synthetic peptide V77-E92 derived from amino acid sequence of α₅₁-casein

Seed out cells
1x10⁶ cells/mL
24 h, 37 °C, 5% CO₂
2x10⁵ cells/mL
24 h, 37 °C, 5% CO₂

Incubation with truncations of α₅₁-casein

Analysis of supernatants for IL-8

- Synthetic peptide V⁷⁷-E⁹² derived from α₅₁-casein induced 100-times lower IL-8 secretion than α₅₁-casein
- Control peptide V⁷⁷-A¹¹⁹ derived from α₅₁-casein did not induce a significant IL-8 secretion
• α$_{S1}$-Casein is a true TLR4-agonist as the third criteria was evidenced here: Synthetic peptide V$^{77}$-E$^{92}$ derived from the amino acid sequence of α$_{S1}$-casein was identified as TLR4-agonistic
• N-terminal amino acids R$^{16}$-E$^{92}$ of α$_{S1}$-casein participated in TLR4-binding
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