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

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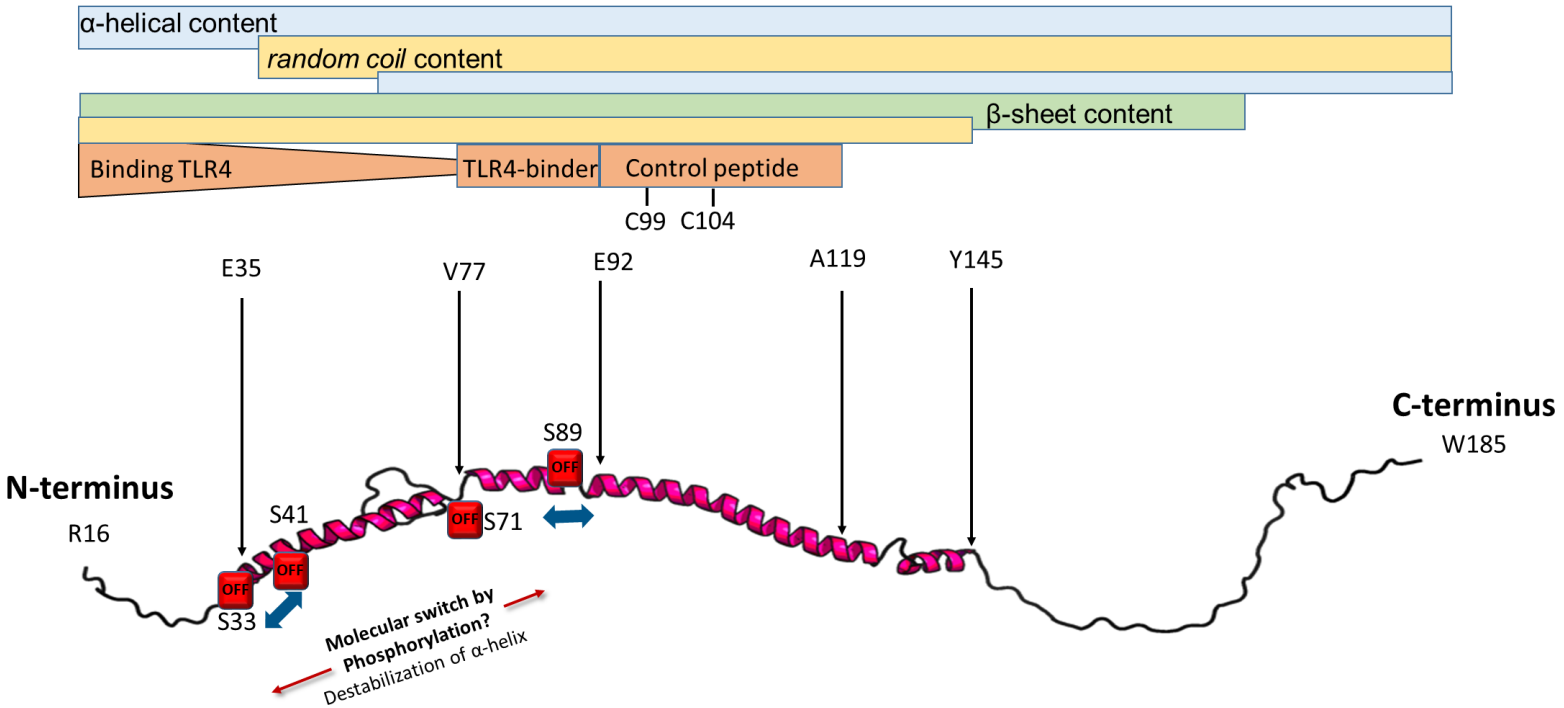


Abstract: Breast-milk α_{S1}-casein was suggested as an agonist of the Toll-like receptor 4 (TLR4). Pathogen recognition receptor TLR4 responds to lipopolysaccharides and a wide range of molecules, from proteins to metal ions. In consequence, three criteria are required to validate agonists which directly activate TLR4 and exclude TLR4-agonisticity through contaminants. Recently, we demonstrated that α_{S1}-casein fulfilled two of these criteria. (i) α_{S1}-Casein required TLR4/MD2 complex as well as cofactor CD14 to induce IL-8 secretion *via* TLR4 and (ii) α_{S1}-casein bound TLR4, MD2 and CD14. Aim of this study was to (iii) identify a synthetic amino acid sequence derived from human α_{S1}-casein responsible for TLR4-agonistic effects.

For this, we analyzed the amino acid sequence (AAS) of α_{S1}-casein *in silico*. α_{S1}-Casein showed to be α-helical and was likely to be intrinsically disordered in the region corresponding to R^{16}-K^{99} of α_{S1}-casein. Six truncated variants of α_{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 α_{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 α_{S1}-casein was neither binding $TLR4^+$ nor inducing IL-8 secretion. Therefore, we postulated V^{77}-E^{92} derived from α_{S1}-casein as TLR4-agonist. This was confirmed by a synthetic peptide V^{77}-E^{92} derived from α_{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, α_{S1}-casein was proofed as an agonist directly activating TLR4. This supported our postulate that α_{S1}-casein has at least two functions, a nutritional and an immune active one.

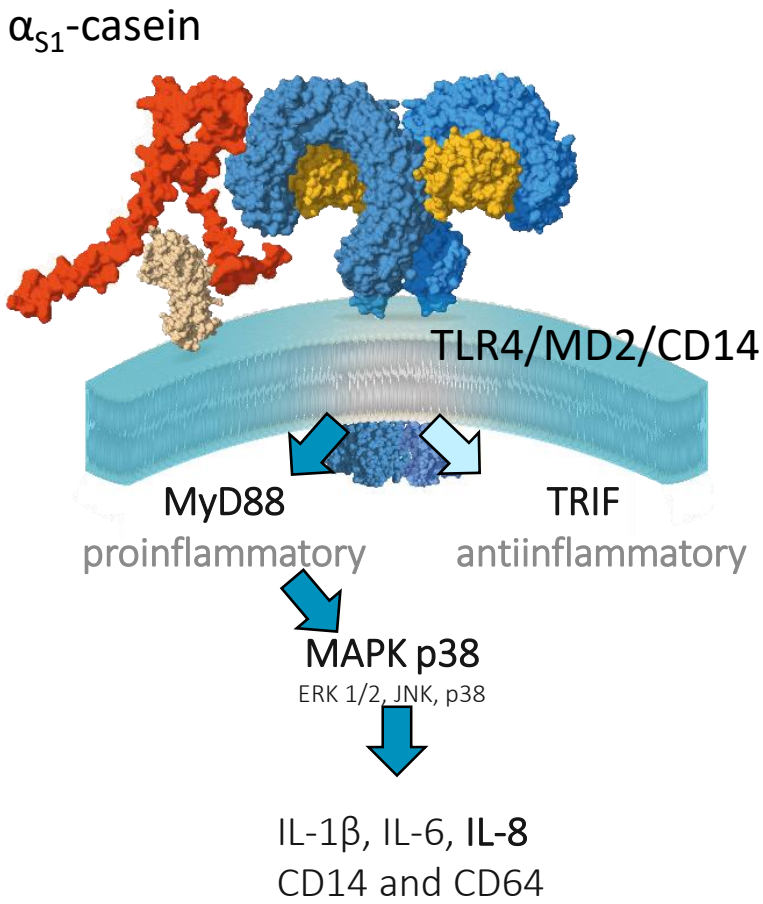
Keywords

Breast milk; human α_{S1}-casein; synthetic TLR4-agonistic peptide; inflammasome.

Human α_{S1}-casein

Expressed in:

- **breast milk** (functional food)
  - transport of molecules, minerals
  - induces life long IgG response
  - Peptides of α_{S1}casein bind opioid receptors
- **Synovia of patients** (RA / OA)
- **Breast- and prostate cancer**



- **α_{S1}-casein was investigated as TLR4-agonist**

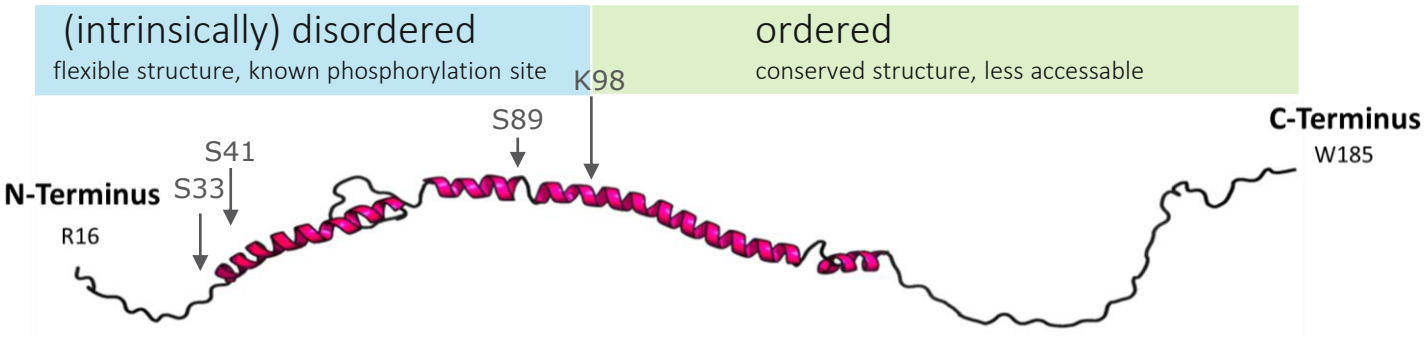
  Two of three criteria were shown before

  (i) α_{S1}-casein required TLR4/MD2 for effects

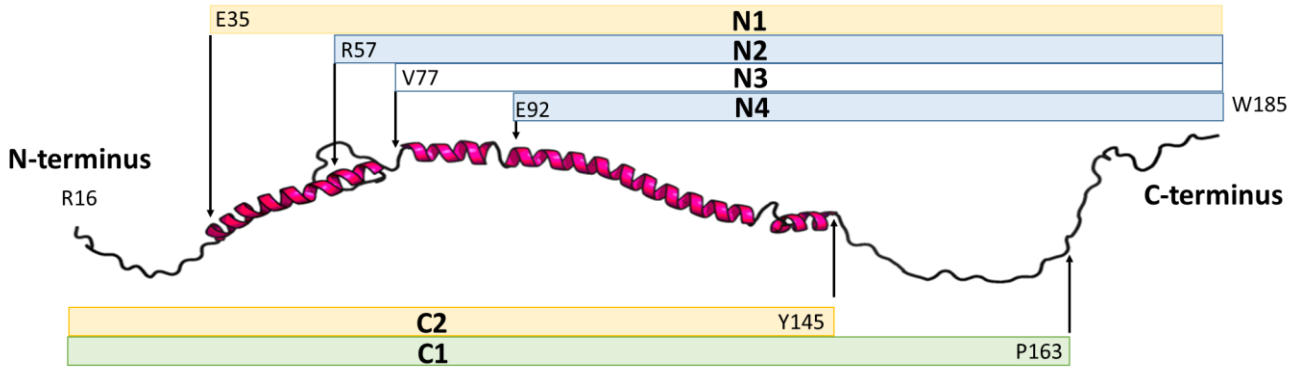
  (ii) α_{S1}-casein bound directly to TLR4 and cofactors MD2/CD14

  (iii) ? Synthetic peptide of α_{S1}-casein induced effects *via* TLR4 ?

In silico predicted structure and *in vitro* analysis of α_{S1}-casein



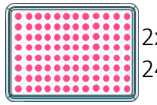
Truncations of the amino acid sequence of α_{S1}-casein were purified from *Escherichia coli*.



Are truncations of α_{S1}-casein binding to TLR4-transfected HEK293 cells?

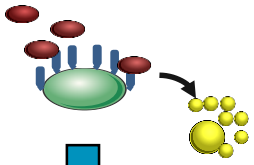
Seed out cells

$1x10^6$ cells/mL
24 h, 37 °C, 5% CO_2

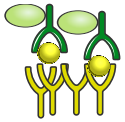


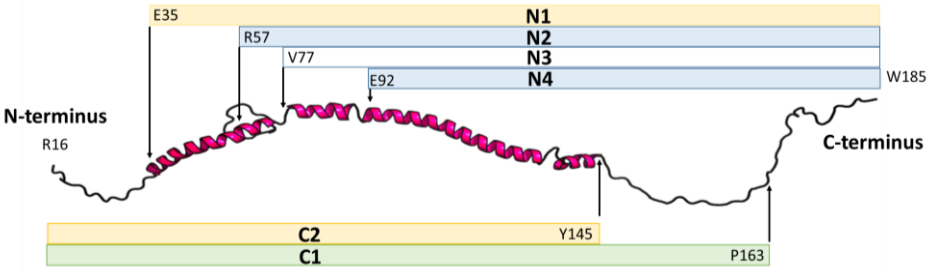
$2x10^5$ cells/mL
24 h, 37 °C, 5% CO_2

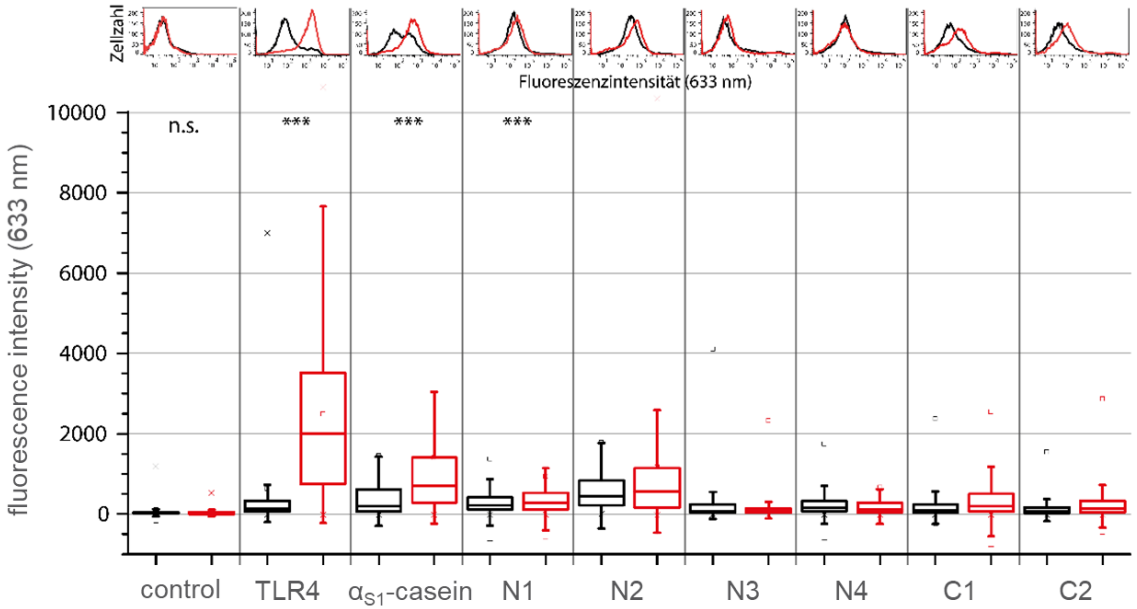
Incubation with truncations of α_{S1}-casein



Analysis of supernatants for IL-8



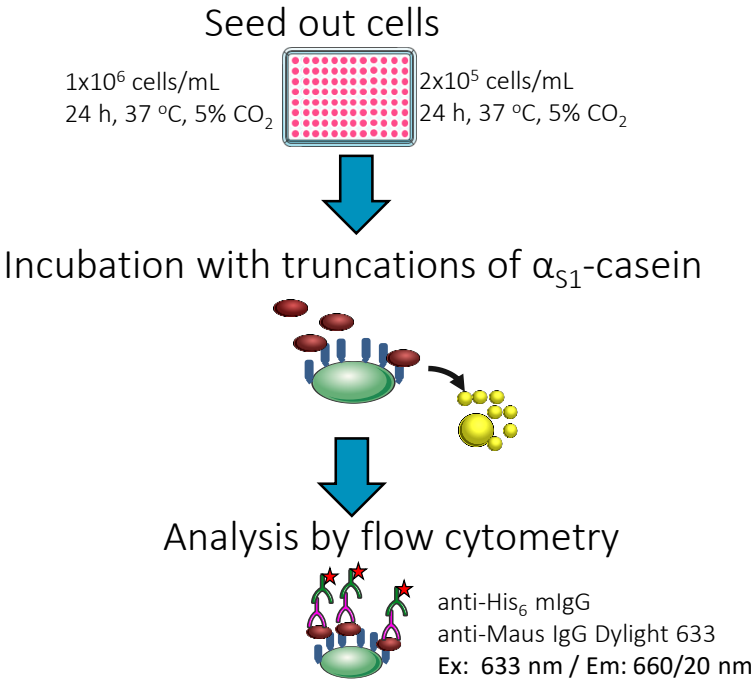


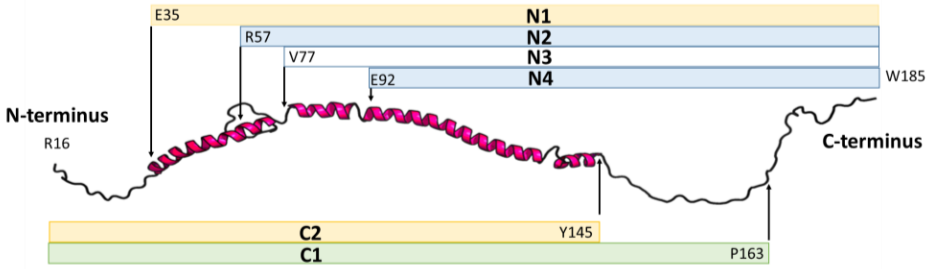


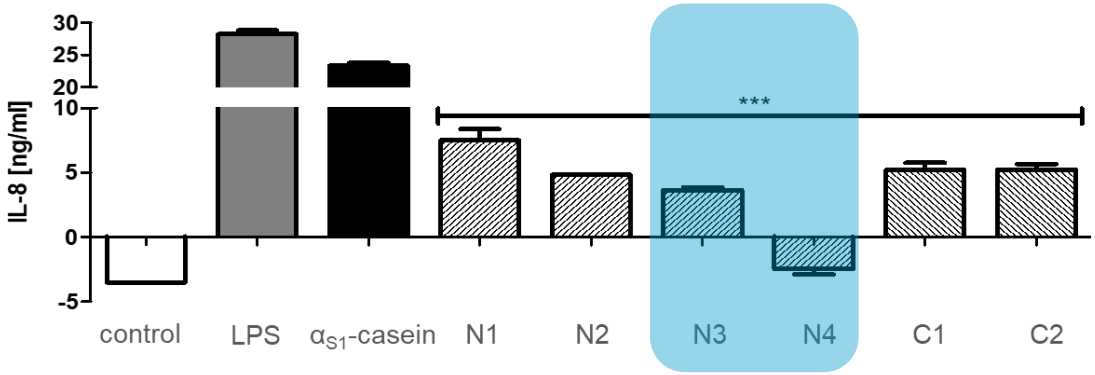
black: HEK293 cells
red: HEK293 cells with TLR4

- 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 α_{S1}-casein binding to HEK293 cells with TLR4 receptor?



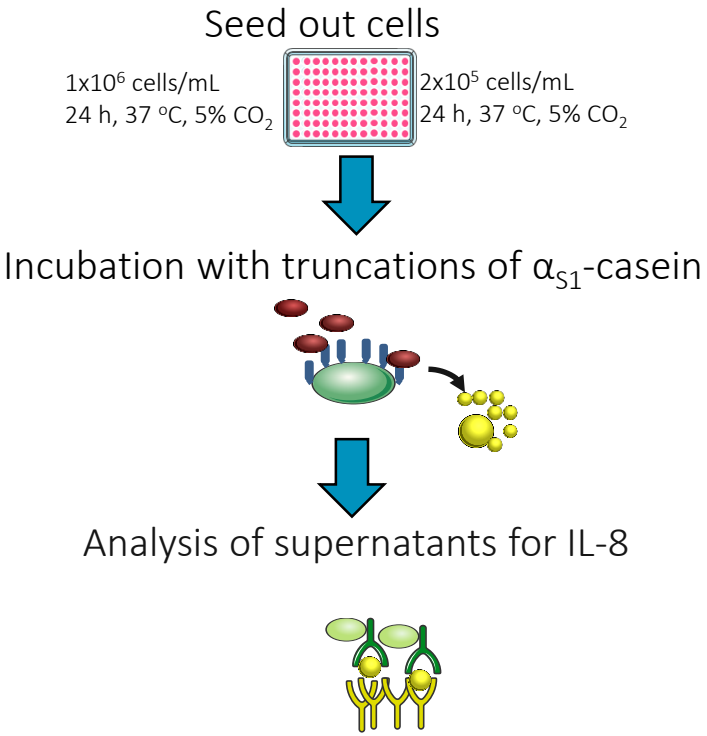


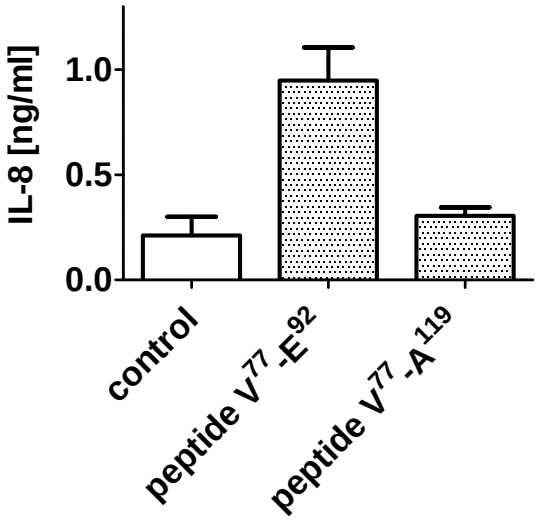


Is peptide V77-E92 TLR4-agonistic?

- 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 α_{S1}-casein

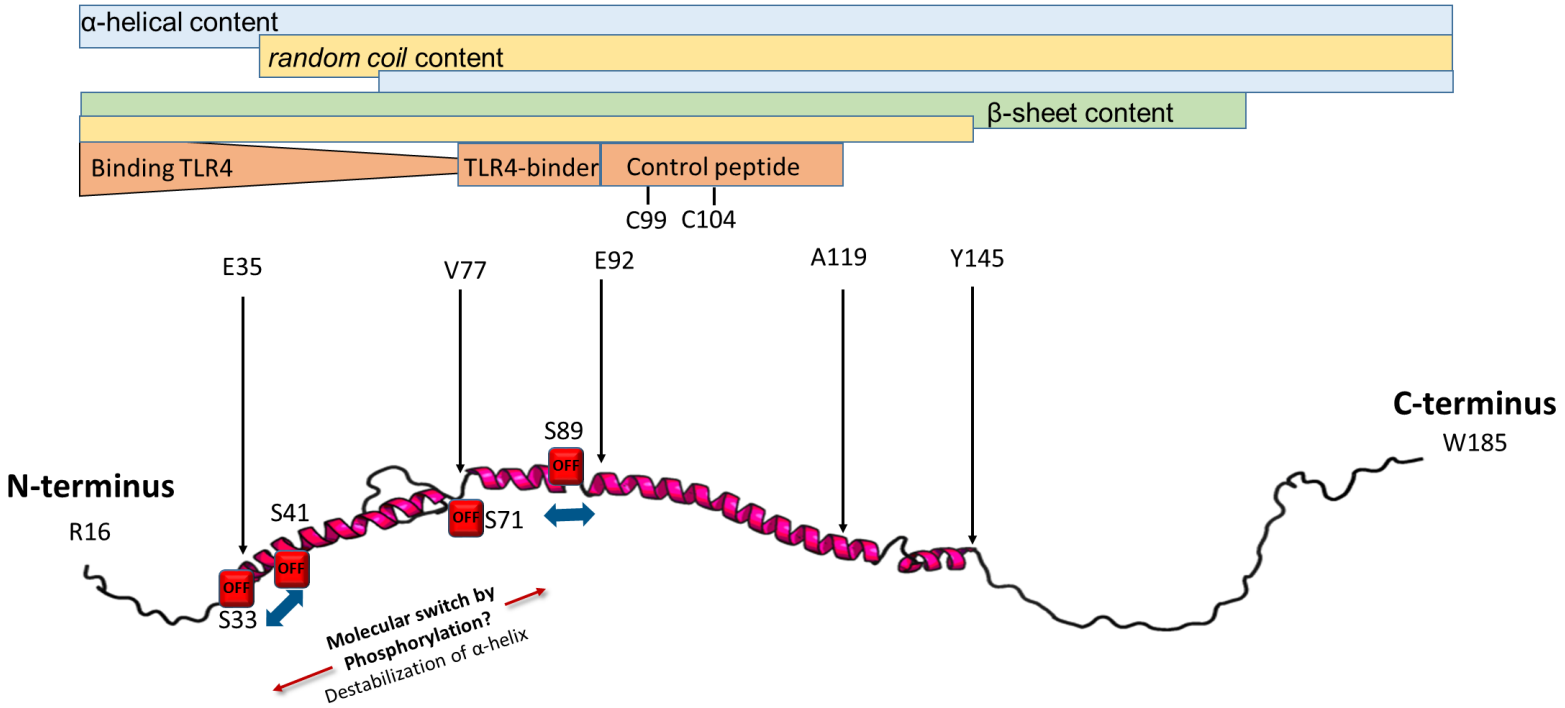
Testing of synthetic peptide V77-E92 derived from amino acid sequence of α_{S1}-casein





- Synthetic peptide V^{77}-E^{92} derived from α_{S1}-casein induced 100-times lower IL-8 secretion than α_{S1}-casein
- Control peptide V^{77}-A^{119} derived from α_{S1}-casein did not induce a significant IL-8 secretion

Conclusions



- 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

Acknowledgments

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