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Analysis of the binding site of α_{S1}-casein to its cellular receptor TLR4 by selective inhibitors and microscale thermophoresis

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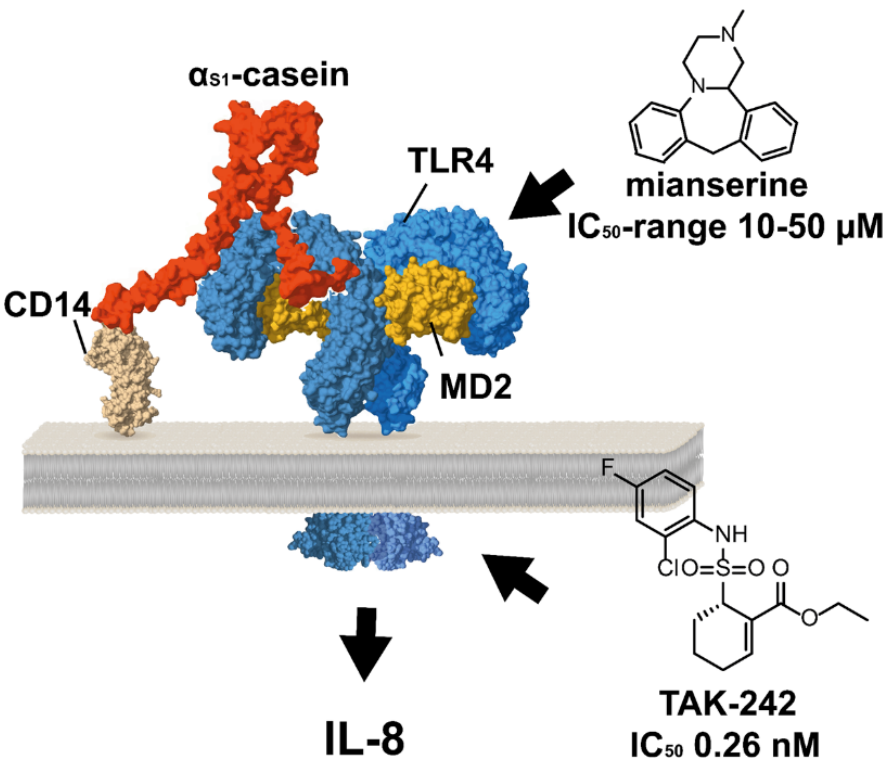
Analysis of the binding site of α_{S1}-casein to its cellular receptor TLR4 by selective inhibitors and microscale thermophoresis

Graphical Abstract



K_D(TLR4/MD2): 2.2 µM
K_D(TLR4): 2.8 µM
K_D(MD2): 0.3 µM
K_D(CD14): 2.7 µM

ecto-domain
extracellular binding

TIR-domain
intracellular binding

Abstract:

The human milk protein α_{S1}-casein was recently reported to induce secretion of proinflammatory cytokines *via* Toll-like receptor 4 (TLR4)[1]. In this study, the binding site of α_{S1}-casein to TLR4 was identified by selective inhibition of the intracellular binding domain and extracellular ecto-domain of TLR4.

For this, Interleukin 8 (IL-8) secretion was monitored after stimulation of TLR4/MD2 (myeloid differentiation factor 2)/CD14 (cluster of differentiation 14)-transfected HEK293 cells ($TLR4^+$) and Mono Mac 6 cells (MM6) with recombinant α_{S1}-casein, or lipopolysaccharide (LPS) as control. The α_{S1}-casein-induced IL-8 secretion was inhibited by TAK-242, an antagonist of the intracellular binding site and mianserine, an antagonist of the extracellular binding domain. TAK-242 inhibited α_{S1}-casein-induced IL-8 secretion with an IC_{50} of 259 nM and LPS-induced IL-8 secretion with an IC_{50} of 23 nM. Mianserine was found as moderate inhibitor of the α_{S1}-casein-induced IL-8 secretion with an IC_{50}-range between 10-51 µM. Therefore, we suggested α_{S1}-casein as an inhibitor of the extracellular binding site of TLR4. These findings were supported by binding experiments using microscale thermophoresis (MST). Human α_{S1}-casein bound to the purified extracellular TLR4/MD2-complex with a K_D of 2.2 µM in comparison to LPS binding TLR/MD2 with a K_D of 8.7 µM. Furthermore α_{S1}-casein showed binding to MD2 with a K_D of 0.3 µM and CD14 with a K_D of 2.7 µM. In addition, human α_{S1}-casein induced IL-8 secretion *via* TLR4 was inhibited by inhibitory anti-CD14-IgA.

Human α_{S1}-casein induced proinflammatory effects by binding to the ecto-domain of TLR4 and CD14 is required as cofactor. Hence human α_{S1}-casein activates TLR4 in a different manner than LPS.

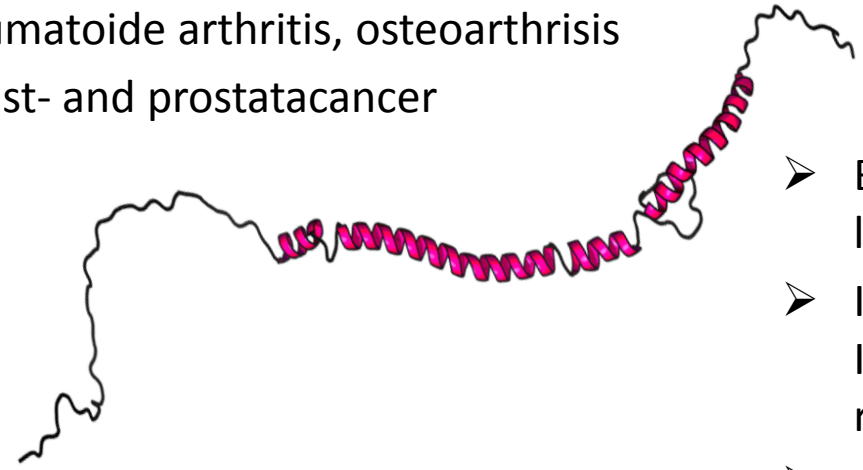
Keywords: Milk protein; human αS1-casein; ecto-domain TLR4; cofactor binding; inflammasome

Introduction

Breast milk protein α_{S1}-casein

Is overexpressed in:

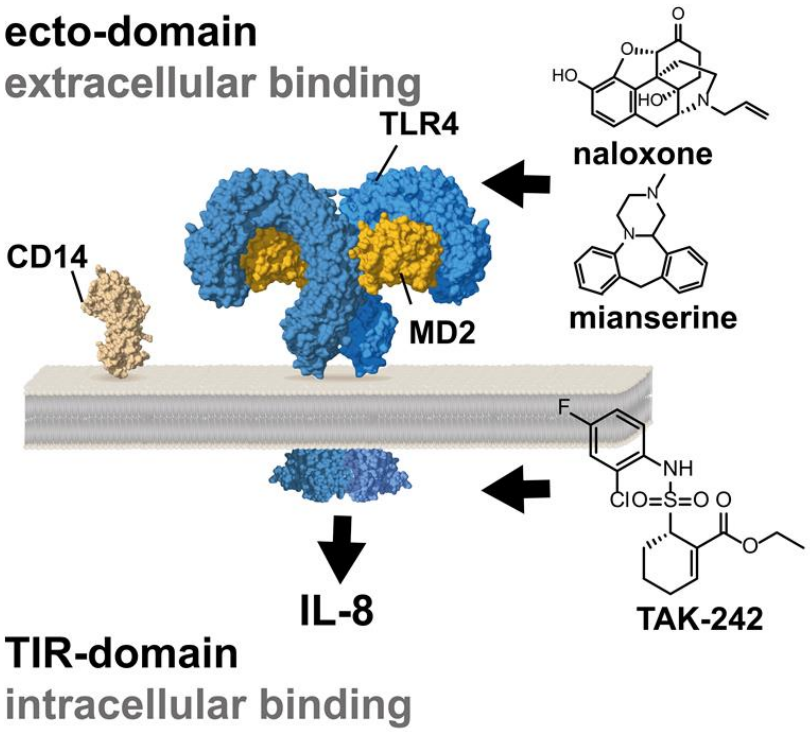
- mammary gland
- tissue of patients with rheumatoide arthritis, osteoarthrisis
- Breast- and prostatacancer



- Breastfeeding induces a life long immunreaction to α_{S1}-casein
- Induces secretion of cytokines IL-1β, IL-6, IL-8 *via* innate immune system receptor Toll-like Receptor 4 (TLR4)
- Phosphorylation abbolishes proinflammatory effects

Introduction

Toll-like receptor 4



- Receptor of innate immune system overexpressed in gut, synovia…
- Recognition of pathogen and danger associated molecule patterns
- Existenz of several inhibitors with known binding site to TLR4

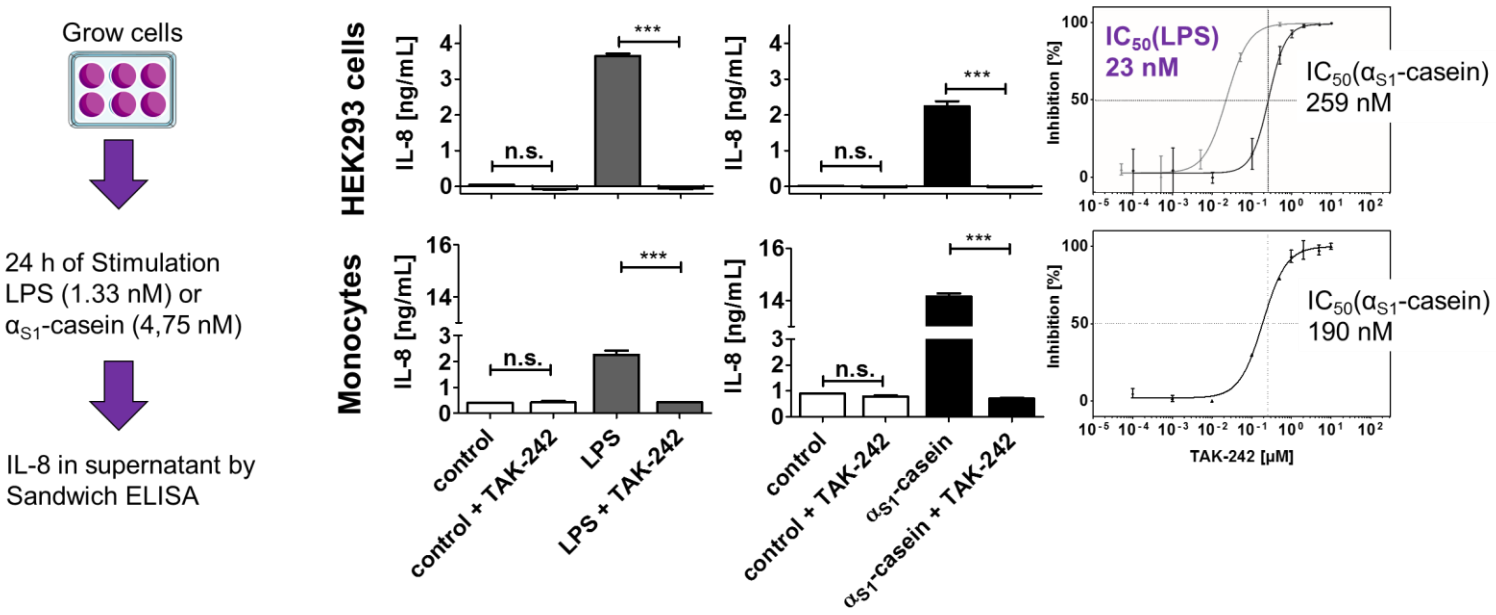
Cofactor binding:

- MD2 is species specific and responsable for ligand transport
- CD14 is associated with antiinflammatoric signaling

1st: Inhibition of α_{S1}-casein induced IL-8 secretion by selective intracellular and extracellular TLR4-inhibitors.

Results and discussion

Inhibiting intracellular domain of TLR4



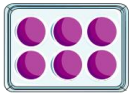
- α_{S1}-casein induces IL-8 secretion *via* TLR4
- α_{S1}-casein-induced IL-8 secretion is proportionally higher in monocytes
- 10-times more TAK-242 is needed to inhibit α_{S1}-casein compared to LPS

1st: Inhibition of α_{S1}-casein induced IL-8 secretion by selective intracellular and extracellular TLR4-inhibitors.

Results and discussion

Inhibiting extracellular domain of TLR4

Grow HEK293 cells



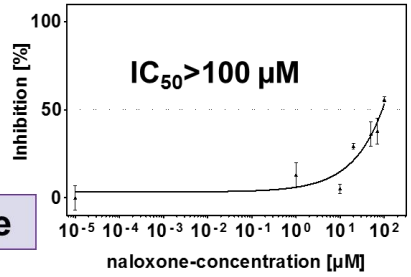
Naloxone

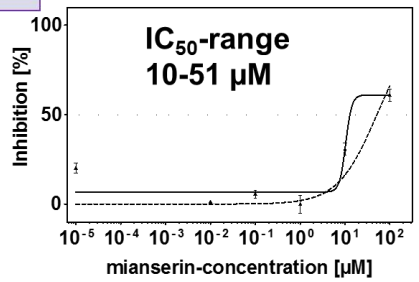
Inhibition with

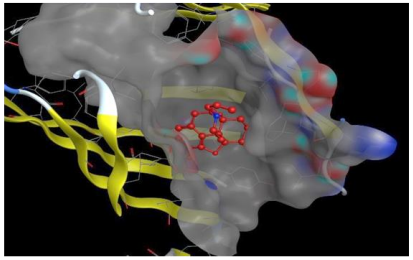
Mianserine

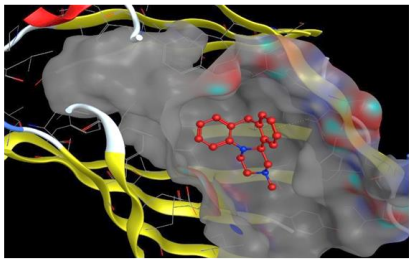
Stimulation α_{S1}-casein (4,75 nM)

IL-8 in supernatant by Sandwich ELISA



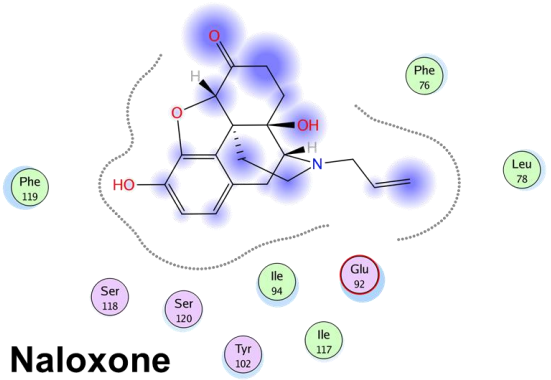




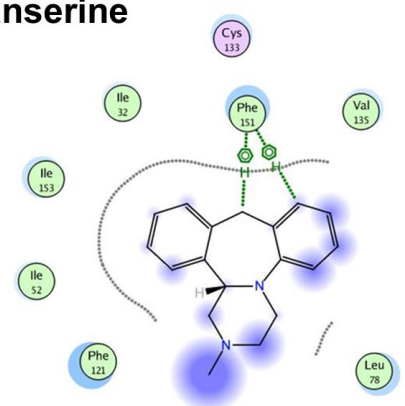


3-D depiction (MOE software) to active site of TLR4 (PDB ID:2Z62)

Naloxone



Mianserine

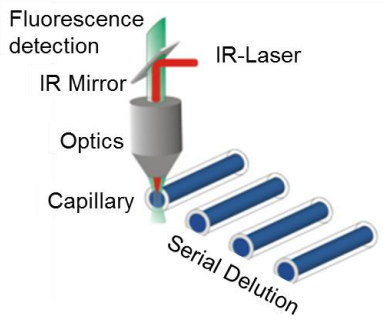


2-D depiction (MOE software)

➢ **α_{S1}-casein could be a ligand of TLR4 ecto-domain**

Results and discussion

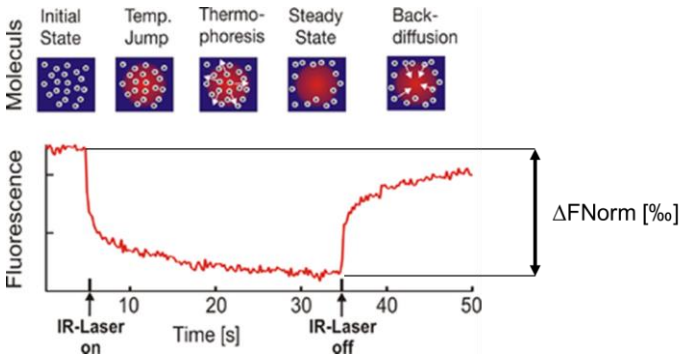
Microscale Thermophoresis for determination of binding constants



- labeled binding partner (low, constant concentration)
- Serial dilution of unlabeled binding partner
- Difference of normaliced fluorescence FNorm[‰]

+ K_D by thermal gradient of 2 K to 8 K under mild condition.
+ Different behavior bound to unbound because of molecule size, hydration shell and charge
+ Low propability of surface artefacts
+ Analysis of particles possible

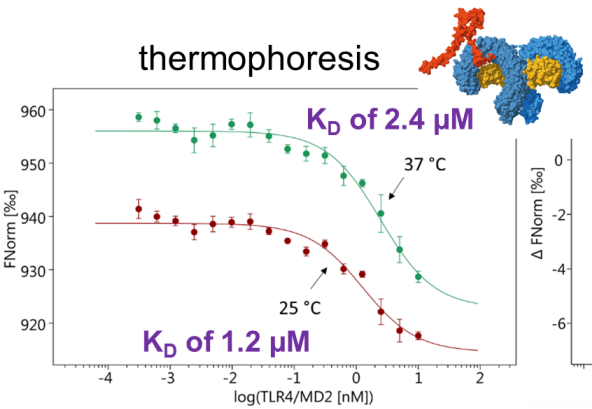
– MST allows only K_D determination
– Thermophoresis is incompletly investigated and comprehended



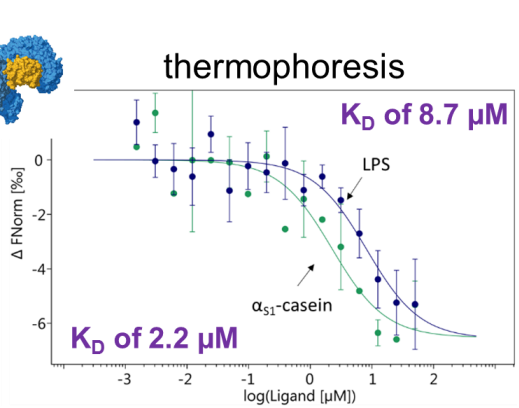
2nd: Characterisation of α_{S1}-casein – TLR4 interaction using microscale thermophoresis.

Results and discussion

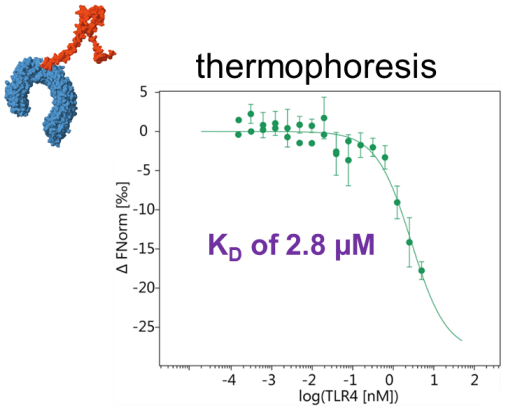
α_{S1}-casein is a stronger binder to TLR4/MD2 than LPS



FITC-α_{S1}-casein (12.5 nM) to TLR4/MD2 (0.3 nM to 10 µM)



Dylight645-TLR4/MD2 (12.5 nM) to α_{S1}-casein (1.5 nM to 50 µM) in comparison to LPS (1.5 nM to 50 µM) at 37 ˚C



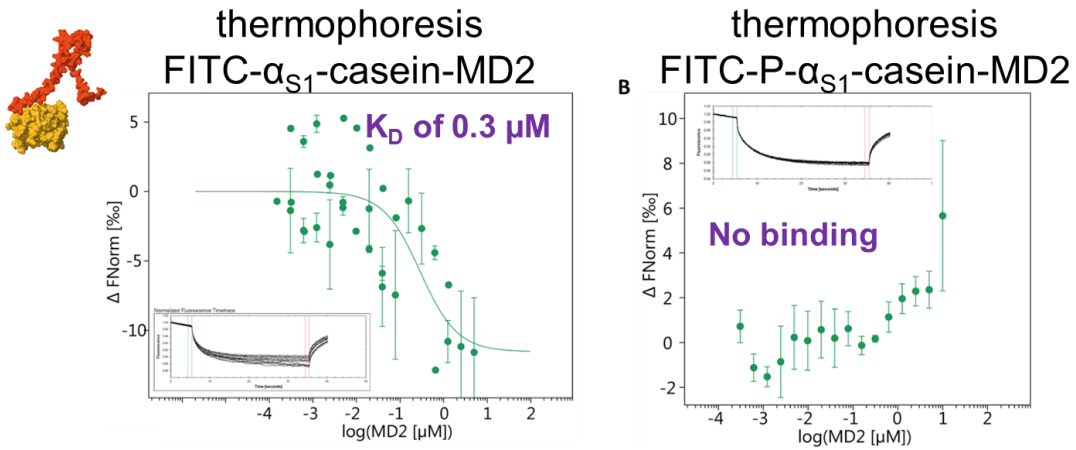
FITC-α_{S1}-casein (12.5 nM) to TLR4 (0.15 nM to 5 µM

- **K_D for α_{S1}-casein to TLR4/MD2 was comparable independent of labeled partner**
- **K_D of LPS to TLR4/MD2 is in accordance to reported data** (K_D reported.: 7-14 µM)

2nd: Characterisation of α_{S1}-casein – TLR4 interaction using microscale thermophoresis.

Results and discussion

Unphosphorylated α_{S1}-casein binds to MD2



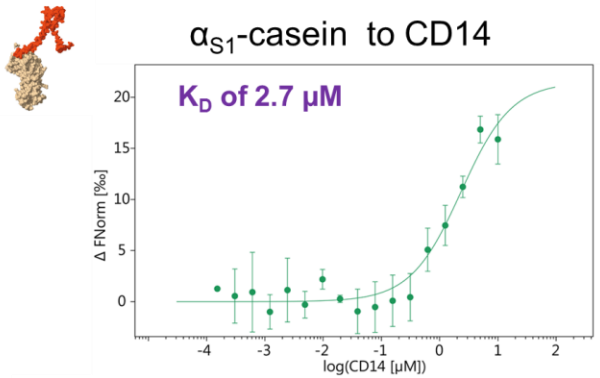
Comparison of FITC-α_{S1}-casein (12.5 nM) to MD2 (0.3 nM to 5 µM) and FITC-P-α_{S1}-casein (12.5 nM) to MD2 (0.3 nM to 5 µM) .
Inset shows thermo-induced change of fluorescence for 30 s.

➢ **MD2 binding is dependent on posttranslational modification of α_{S1}-casein**

2nd: Characterisation of α_{S1}-casein – TLR4 interaction using microscale thermophoresis.

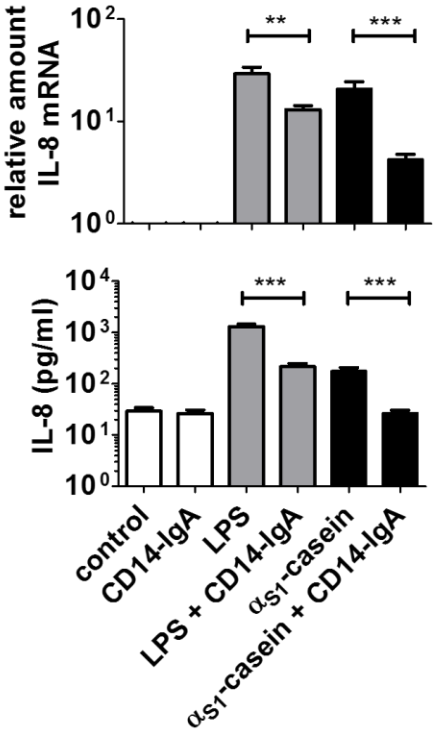
Results and discussion

Human α_{S1}-casein-induced effects are CD14-dependent



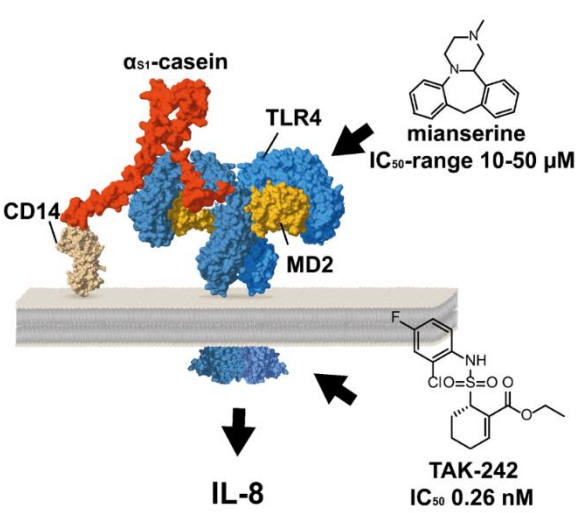
FITC-α_{S1}-casein (12.5 nM) to CD14 (0.3 nM to 10 µM).

α_{S1}-casein-induced IL-8 was inhibited by Neutralizing CD14-IgA (2.5 µg/ml).



- α_{S1}-casein binds CD14
- Inhibition of CD14 reduced α_{S1}-casein-induced effects

Conclusions



- α_{S1}-casein is a binding partner of the TLR4 ecto-domain
- α_{S1}-casein selectively binds to TLR4, MD2 and CD14.
- α_{S1}-casein-induced IL-8 secretion was CD14-dependent, which is a hint for antiinflammatoric effects.
- MD2 binding is dependent on posttranslational modification of α_{S1}-casein
- α_{S1}-casein is a stronger TLR4-binder compared to LPS

Binding properties are important for further understanding the role of breastmilk protein human α_{S1}-casein in development of an immune system and its role in the inflammatory response.

Acknowledgments

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