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The CD14 region spanning amino acids 57–64 is critical for interaction with the extracellular Toll-like receptor 2 domain☆

Daisuke Iwaki, Chiaki Nishitani, Hiroaki Mitsuzawa, Naoki Hyakushima, Hitomi Sano, Yoshio Kuroki*

Department of Biochemistry, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan

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Abstract

CD14 has been shown to enhance Toll-like receptor 2 (TLR2)-mediated signaling in response to peptidoglycan. Anti-CD14 monoclonal antibody MEM-18, whose epitope was located at the amino acid residues 57–64, blocked the binding of sCD14 to the recombinant soluble form of the extracellular TLR2 domain (sTLR2). The deletion mutant $sCD14^{\Delta 57-64}$ lacking the amino acid residues 57–64 failed to bind to sTLR2. Cotransfection of wild type mCD14 but not $mCD14^{\Delta 57-64}$ with TLR2 enhanced NF-κB activation in response to peptidoglycan. These results indicate that the CD14 region spanning amino acids 57–64 is critical for interacting with TLR2 and enhancing TLR2-mediated peptidoglycan signaling.

Keywords: CD14; Toll-like receptor 2; Peptidoglycan

The innate immune system plays pivotal roles in the first line of host defense [1]. Toll-like receptors (TLRs) function as pattern recognition receptors that recognize pathogen-associated molecular patterns to induce inflammation and stimulate the clonal response to adaptive immunity [2]. TLR2 is implicated in signaling and inflammation elicited by gram-positive bacteria and its component including peptidoglycan (PGN) [3–7]. PGN induces NF-κB activation in TLR2-transfected cells in the absence of CD14, but cotransfection of CD14 enhances TLR2-mediated signaling [4,8]. A previous study [9] from this laboratory has demonstrated that a soluble form of recombinant extracellular TLR2 domain (sTLR2) directly binds to PGN. In addition, sCD14 interacts with sTLR2 and facilitates the binding of sTLR2 to PGN [9]. These results are consistent with those showing that cotransfection of CD14 augments TLR2-mediated signaling. Membrane CD14 (mCD14) is a glycosylphosphatidylinositol-anchored protein on monocytes and neutrophils with an apparent molecular mass of 55 kDa that binds lipopolysaccharide (LPS) and enables LPS-responses [10]. A soluble form of CD14 (sCD14) exists in serum and facilitates the responsiveness of the cells to LPS [11,12]. The epitope mapping for anti-sCD14 monoclonal antibodies and the studies with deletion mutants have identified the residues 57–64 of sCD14 as a region essential for LPS binding, since anti-CD14 monoclonal antibody (mAb) MEM-18, whose epitope was located at the amino acid residues 57–64, inhibited the binding of sCD14 to LPS and the deletion mutant $sCD14^{\Delta 57-64}$ failed to bind LPS [13]. Antibody 3C10 inhibited LPS signaling but not LPS binding, indicating that the epitope for antibody 3C10, the amino acid residues 7–14, is required for cellular signaling but not for LPS binding [14]. Another study with

☆ *Abbreviations:* sCD14, soluble CD14; mCD14, membrane CD14; TLR2, Toll-like receptor 2; sTLR2, recombinant soluble form of the extracellular TLR2 domain; mAb, monoclonal antibody; PGN, peptidoglycan; LPS, lipopolysaccharide.

* Corresponding author. Fax: +81 11 611 2236.
E-mail address: kurokiy@sapmed.ac.jp (Y. Kuroki).

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the alanine substitution mutants indicates that the amino acid residues 39–44 of CD14 are also important for LPS binding [15]. However, the CD14 region required for the interaction with TLR2 remains unknown. The purpose of this study was to determine the CD14 region responsible for interacting with TLR2 and enhancing the TLR2-mediated signaling.

Materials and methods

Materials. Peptidoglycan (PGN) derived from *Staphylococcus aureus* was obtained from Fluka Chemical. Anti-CD14 monoclonal antibodies (mAbs), MY-4 and MEM-18, were purchased from Beckman–Coulter and SANBIO, respectively. Hybridoma producing anti-CD14 monoclonal antibody 3C10 was obtained from ATCC.

Recombinant proteins. The recombinant soluble form of human TLR2 (sTLR2) consisting of the putative extracellular domain (Met^{1}-Arg^{587}) and a His_6 tag at the C-terminus end was constructed, and the sTLR2 protein was expressed and produced in the baculovirus–insect cell system using the methods described by O'Reilly et al. [16], as described previously [9]. The protein was purified from the medium using a column of nickel–nitrilotriacetic acid beads (Qiagen). sTLR2 starts at Glu^{21} as described previously [9].

Recombinant soluble forms of human wild type (wt) CD14 (sCD14) and mutant CD14 ($sCD14^{\Delta57-64}$) in which amino acid residues 57–64 were deleted were also produced in CHO cells using the glutamine synthetase amplification system [17], as described previously [18]. cDNAs for membrane-bound CD14 (mCD14) and its deletion mutant $mCD14^{\Delta57-64}$ were also constructed and subcloned into pcDNA3.1(+) plasmid vectors (Invitrogen).

Polyclonal antibodies against sTLR2 and sCD14 were prepared as described previously [9].

Binding of sCD14 to sTLR2. The binding of wt sCD14 or mutant sCD14 to sTLR2 was performed using sTLR2 coated onto microtiter wells as described previously [9]. sTLR2 (20 μg/ml) coated onto microtiter wells (50 μl/well) was incubated with the indicated concentrations of sCD14 or $sCD14^{\Delta57-64}$ at 37 °C for 3 h. After the incubation, the wells were washed and incubated with anti-sCD14 polyclonal antibody (10 μg/ml) or anti-CD14 mAb MY-4 (30 μg/ml) at 37 °C for 1 h, followed by the incubation with HRP-labeled anti-rabbit IgG (1:1500) or anti-mouse IgG (1:1000). The peroxidase reaction was performed by using *o*-phenylenediamine as a substrate and the absorbance was measured at 492 nm. In some experiments sCD14 (5 μg/ml) was preincubated with anti-CD14 mAb MY-4, MEM-18 or 3C10 (50 μg/ml) at 37 °C for 1 h before the incubation with sTLR2 coated onto microtiter wells.

Binding of anti-CD14 mAb MY-4 to wt and mutant sCD14s. To determine whether mAb MY-4 equally recognizes wt and mutant sCD14s, the wells coated with sCD14 or $sCD14^{\Delta57-64}$ (50 μl/well, 2 μg/ml) were incubated with mAb MY-4 (10 μg/ml) at 37 °C for 1 h. After the incubation, the wells were washed and incubated with HRP-labeled anti-mouse IgG (1:1000) at 37 °C for 1 h. The wells were then washed and the peroxidase reaction was performed as described above.

Ligand blot. Ligand blot analysis was carried out by the modified method [18] based on that described previously [19]. Two micrograms of the protein (sTLR2, sCD14 or $sCD14^{\Delta57-64}$) was electrophoresed and transferred onto the PVDF membrane. The membrane was then incubated with 10 μg/ml sTLR2, wt sCD14 or $sCD14^{\Delta57-64}$ at room temperature for 3 h. After the incubation, the membrane was washed and further incubated with anti-sTLR2 polyclonal IgG (10 μg/ml) or anti-sCD14 polyclonal IgG (10 μg/ml) at room temperature for 1 h, followed by the incubation with HRP-labeled anti-rabbit IgG (1:3000) for 1 h. The protein binding to the PVDF membrane was visualized by using chemiluminescence reagent (Pierce).

Binding of sCD14 to peptidoglycan. The binding of sCD14 to PGN was performed by the method described previously [9]. Briefly, PGN (20 μg/well) in 20 μl ethanol was added onto microtiter wells and the solvent was evaporated in ambient air. After nonspecific binding was blocked, the indicated concentrations of wt sCD14 or $sCD14^{\Delta57-64}$ were incubated with the solid phase PGN at 37 °C for 6 h. The sCD14 binding to PGN was detected by using anti-histidine tag monoclonal antibody (Qiagen) and HRP-labeled anti-mouse IgG.

NF-κB reporter assay. A 2.6-kb TLR2 cDNA, or 1.3-kb wt mCD14 or $mCD14^{\Delta57-64}$ cDNA was subcloned into pcDNA3.1 (+) (Invitrogen) and NF-κB activation was measured as described previously [8,9]. HEK293 cells were plated at 1×10^5 cells/well in 24-well plates one day before transfection. The cells were transiently transfected by FuGENE 6 transfection reagent (Roche Molecular Biochemicals) with the TLR2 expression plasmid (136 ng), and the plasmid vector (13.6 ng) containing wt mCD14 or $mCD14^{\Delta57-64}$ cDNA or an empty vector (13.6 ng), together with an NF-κB reporter plasmid (pNF-κB-Luc, 300 ng, Stratagene) and *Renilla* luciferase control reporter plasmid (pRL-TK, 3.5 ng, Promega). Twenty-four hours after transfection, the cells were stimulated with 10 μg/ml PGN for 6 h in the absence of serum. Luciferase activity was measured by using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Results and discussion

sCD14 directly binds to the extracellular TLR2 domain

Electrophoretic analysis revealed that sTLR2 and sCD14 exhibited a single band with an apparent molecular mass of 75 kDa and broad bands with 46–56 kDa (Fig. 1A), respectively, as described previously [9,18]. Ligand blot analysis was performed to determine whether sCD14 directly interacts with sTLR2. sCD14 that had bound to the membrane was detected as a band corresponding to that of sTLR2 (Fig. 1B). When sTLR2 was incubated with the membrane where sCD14 was transferred, the sTLR2 protein was also detected as a

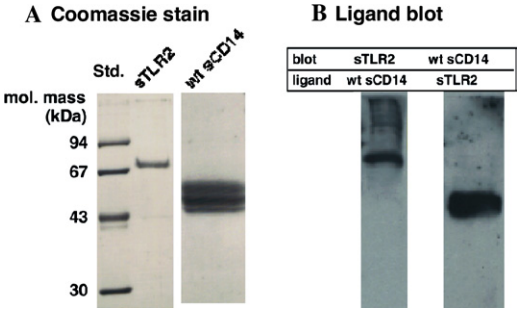

Fig. 1. sCD14 binds to the extracellular TLR2 domain. (A) Electrophoretic analysis of recombinant proteins. Two micrograms of sTLR2 and sCD14 was subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions. The proteins were visualized by Coomassie brilliant blue staining. (B) Ligand blot analysis. Two micrograms of sTLR2 or sCD14 (blot) was electrophoresed and transferred onto PVDF membranes. The membranes were then reacted with 10 μg/ml sCD14 or sTLR2 (ligand) at room temperature for 3 h. The proteins binding to the membrane were detected by using anti-sCD14 IgG or anti-sTLR2 IgG as described under Materials and methods.

band corresponding to that of sCD14. These results demonstrate the direct binding of sCD14 to the extracellular TLR2 domain and are consistent with those obtained from the binding assay using microtiter wells [9].

Anti-CD14 monoclonal antibody MEM-18 blocks the binding of sCD14 to sTLR2

Next, we examined the effects of anti-CD14 monoclonal antibodies on the binding of sCD14 to sTLR2 coated onto microtiter wells. The sCD14 binding to sTLR2 was partially but not significantly attenuated in the presence of antibodies MY-4 and 3C10, and control mouse IgG when compared with the binding in the absence of antibody. When antibody MEM-18 was co-incubated, the binding of sCD14 to sTLR2 was significantly inhibited (Fig. 2). Since the epitopes of antibody MY-4, MEM-18, and 3C10 were located at the amino acid residues 39–44, 57–64, and 7–14 of CD14, respectively [13–15], these results suggest that the amino acid residues 57–64 of sCD14 are involved in the binding to sTLR2.

The deletion mutant sCD14$^{\Delta 57-64}$ fails to bind sTLR2

We next constructed the deletion mutant of sCD14 (sCD14$^{\Delta 57-64}$) lacking the amino acid residues 57–64. The electrophoretic profile of sCD14$^{\Delta 57-64}$ was similar to that of wt sCD14 when analyzed by SDS–PAGE under reducing conditions (Fig. 3A). Because antibody MY-4 did not block the binding of sCD14 to sTLR2

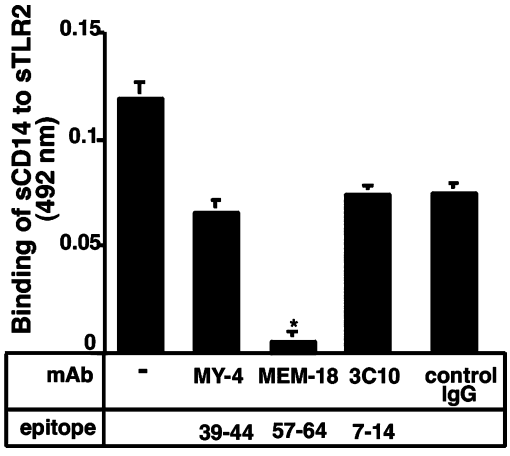

Fig. 2. Anti-CD14 monoclonal antibody MEM-18 blocks the binding of sCD14 to sTLR2. Five micrograms per milliliter of wild type sCD14 was preincubated with 50 μg/ml anti-CD14 monoclonal antibody (MY-4, MEM-18 or 3C10) or control mouse IgG at 37 °C for 1 h. The mixture of sCD14 and antibody was further incubated with sTLR2 coated onto microtiter wells (20 μg/ml, 50 μl/well) at 37 °C for 3 h. The binding of sCD14 to sTLR2 was detected by using anti-sCD14 antibody, as described under Materials and methods. The data shown are means + SEM of three experiments. *$p < 0.001$, compared with the binding of sCD14 preincubated with control IgG. The epitopes for monoclonal antibodies were determined in [13–15].

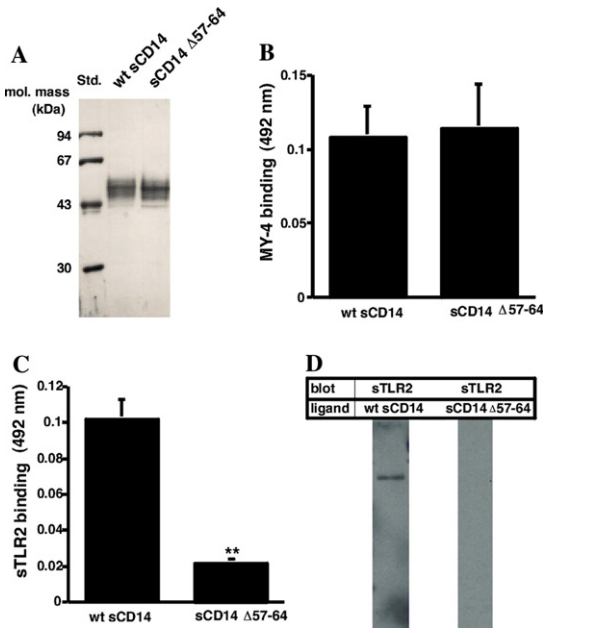

Fig. 3. Deletion of amino acid residues 57–64 of sCD14 abrogates sTLR2 binding. (A) Electrophoretic analysis of sCD14$^{\Delta 57-64}$. Two micrograms of wild type sCD14 and sCD14$^{\Delta 57-64}$ was subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions. The proteins were visualized by Coomassie brilliant blue staining. (B) Recognition of wild type and mutant sCD14s by anti-CD14 monoclonal antibody MY-4. Wild type sCD14 or sCD14$^{\Delta 57-64}$ (2 μg/ml, 50 μl/well) was coated onto microtiter wells and was reacted with monoclonal antibody MY-4 at 37 °C for 1 h. Antibody binding to sCD14 was detected by using HRP-conjugated anti-mouse IgG. (C) The binding of sCD14$^{\Delta 57-64}$ to sTLR2. sTLR2 coated onto microtiter wells (20 μg/ml, 50 μl/well) was incubated with 20 μg/ml wild type sCD14 or sCD14$^{\Delta 57-64}$ at 37 °C for 3 h. The sCD14 binding to the solid phase sTLR2 was detected by using monoclonal antibody MY-4, as described under Materials and methods. The data shown are means + SEM from three experiments. **$p < 0.002$, compared with the binding of wild type sCD14. (D) Ligand blot analysis for the binding of sCD14$^{\Delta 57-64}$ to sTLR2. Two micrograms of sTLR2 was electrophoresed and transferred onto PVDF membranes. The membrane was then incubated with 20 μg/ml wild type sCD14 or sCD14$^{\Delta 57-64}$ at room temperature for 3 h. The binding of sCD14 and sCD14$^{\Delta 57-64}$ to sTLR2 on the membrane was detected by antibody MY-4, as described under Materials and methods.

(Fig. 2), we used MY-4 to detect the proteins of wt and mutant sCD14s that had bound to sTLR2. When the abilities of antibody MY-4 to recognize wt sCD14 and the mutant sCD14$^{\Delta 57-64}$ were compared (Fig. 3B), MY-4 was able to bind equally to wt sCD14 and the mutant sCD14$^{\Delta 57-64}$ coated onto microtiter wells. Next, the binding of sCD14$^{\Delta 57-64}$ to sTLR2 was examined using antibody MY-4. The binding of sCD14$^{\Delta 57-64}$ to sTLR2 was significantly decreased when compared to that of wt sCD14 (Fig. 3C). These results are consistent with those obtained from ligand blot analysis showing that sCD14$^{\Delta 57-64}$ failed to bind sTLR2 on the membrane (Fig. 3D). These results indicate that deletion of amino acid residues 57–64 abrogates the binding of sCD14 to sTLR2.

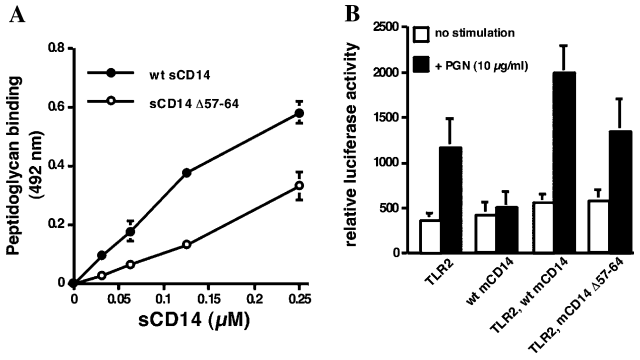

Fig. 4. mCD14$^{\Delta 57-64}$ fails to enhance TLR2-mediated signaling in response to peptidoglycan. (A) Binding of sCD14$^{\Delta 57-64}$ to peptidoglycan. PGN coated onto microtiter wells (20 μg/well) was incubated with the indicated concentrations of sCD14 or sCD14$^{\Delta 57-64}$ at 37 °C for 6 h. The binding of sCD14 to PGN was detected by using anti-histidine tag monoclonal antibody, as described under Materials and methods. The data shown are means ± SEM from three experiments. (B) PGN-induced NF-κB activation in HEK293 cells transfected with mCD14$^{\Delta 57-64}$ and TLR2. Luciferase activities were determined in 1×10^5 HEK293 cells plated in 24-well plates and transfected with the indicated expression plasmid vectors together with an NF-κB reporter plasmid and *Renilla* luciferase control reporter plasmid. Twenty-four hours after transfection, the cells were stimulated with 10 μg/ml PGN for 6 h, as described under Materials and methods. The data shown are means ± SEM from three experiments.

mCD14$^{\Delta 57-64}$ fails to enhance TLR2-mediated signaling

Since a previous study [20] demonstrates the direct binding of sCD14 to soluble PGN, we examined the ability of sCD14$^{\Delta 57-64}$ to bind PGN. sCD14$^{\Delta 57-64}$ retained activity of binding PGN although it exhibited the attenuated binding (Fig. 4A). Because mCD14 enhances TLR2-mediated PGN signaling [4,8], we next examined whether mCD14$^{\Delta 57-64}$ possessed this effect. Transfection of TLR2 cDNA alone but not mCD14 cDNA alone induced NF-κB activation in response to PGN (Fig. 4B). Cotransfection of wt mCD14 with TLR2 enhanced PGN-induced NF-κB activation. However, the enhanced NF-κB activation was not observed when the deletion mutant mCD14$^{\Delta 57-64}$ was cotransfected. It is unlikely that the failure of mCD14$^{\Delta 57-64}$ in enhancing PGN signaling is due to the loss of PGN binding of this deletion mutant, because sCD14$^{\Delta 57-64}$ retained significant binding activity to PGN as shown in Fig. 4A. These results indicate that the amino acid residues 57–64 of mCD14 are critical for enhancing TLR2-mediated signaling in response to PGN. Taken together, these results clearly indicate that the failure of stimulating TLR2-mediated signaling by CD14 is accompanied by the loss of activity of CD14 to bind TLR2.

In conclusion, we here show that the direct binding of sCD14 to the extracellular TLR2 domain was blocked by the presence of anti-CD14 mAb MEM-18. The direct binding studies by microtiter well assay and ligand blot revealed that the mutant protein sCD14$^{\Delta 57-64}$ lacking the residues of the MEM-18 epitope exhibited no binding to sTLR2, although this mutant retained some activity to bind PGN. Cotransfection of mCD14$^{\Delta 57-64}$ with wt TLR2 into HEK293 cells did not show the enhanced NF-κB activation in response to PGN. These results support the conclusion that the CD14 region of amino acid residues 57–64 is critical for the interaction with TLR2 and for enhancing TLR2-mediated PGN signaling.

References

[1] J.A. Hoffmann, F.C. Kafatos, C.A. Janeway, R.A.B. Ezekowitz, Science 284 (1999) 1313–1318.
[2] A. Aderem, R.J. Ulevitch, Nature 406 (2000) 782–787.
[3] M. Hirschfeld, Y. Ma, J.H. Weis, S.N. Vogel, J.J. Weis, J. Immunol. 165 (2000) 618–622.
[4] R. Schwandner, R. Dziarski, H. Wesche, M. Rothe, C.J. Kirschning, J. Biol. Chem. 274 (1999) 17406–17409.
[5] O. Takeuchi, K. Hoshino, S. Akira, J. Immunol. 165 (2000) 5392–5396.
[6] O. Takeuchi, K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, S. Akira, Immunity 11 (1999) 443–451.
[7] A. Yoshimura, E. Lien, R.R. Ingalls, E. Tuomanen, R. Dziarski, D. Golenbock, J. Immunol. 163 (1999) 1–5.
[8] H. Mitsuzawa, I. Wada, H. Sano, D. Iwaki, S. Murakami, T. Himi, N. Matsushima, Y. Kuroki, J. Biol. Chem. 276 (2001) 41350–41356.
[9] D. Iwaki, H. Mitsuzawa, S. Murakami, H. Sano, M. Konishi, T. Akino, Y. Kuroki, J. Biol. Chem. 277 (2002) 24315–24320.
[10] S.D. Wright, R.A. Ramos, P.S. Tobias, R.J. Ulevitch, J.C. Mathison, Science 21 (1990) 1431–1433.
[11] E.A. Frey, D.S. Miller, T.G. Jahr, A. Sundan, V. Brazil, T. Espevik, B.B. Finlay, S.D. Wright, J. Exp. Med. 176 (1992) 1665–1671.
[12] E. Hailman, T. Vasselon, M. Kelley, L.A. Busse, M.C.-T. Hu, H.S. Lichenstein, P.A. Detmers, S.D. Wright, J. Immunol. 156 (1996) 4384–4390.
[13] T.S.-C. Juan, E. Hailman, M.J. Kelley, L.A. Busse, E. Davy, C.J. Empig, L.O. Narhi, S.D. Wright, H.S. Lichenstein, J. Biol. Chem. 270 (1995) 5219–5224.
[14] T.S.-C. Juan, E. Hailman, M.J. Kelley, S.D. Wright, H.S. Lichenstein, J. Biol. Chem. 270 (1995) 17237–17242.
[15] F. Stelter, M. Bernheiden, R. Menzel, R.S. Jack, S. Witt, X. Fan, M. Pfister, C. Schutt, Eur. J. Biochem. 243 (1997) 100–109.
[16] D.R. O'Reilly, L.K. Miller, V.A. Luckow, Baculovirus Expression Vectors, A Laboratory Manual, W.H. Freeman, New York, 1992.
[17] F.M. Ausbel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, in: Amplification Using CHO Cell Expression Vectors. Current Protocols in Molecular Biology, Wiley, New York, 1992.
[18] H. Sano, H. Chiba, D. Iwaki, H. Sohma, D.R. Voelker, Y. Kuroki, J. Biol. Chem. 275 (2000) 22442–22451.
[19] H. Sano, H. Sohma, T. Muta, S. Nomura, D.R. Voelker, Y. Kuroki, J. Immunol. 163 (1999) 387–395.
[20] R. Dziarski, R.I. Tapping, P.S. Tobias, J. Biol. Chem. 273 (1998) 8680–8690.