

Interaction of CD163 with the regulatory subunit of casein kinase II (CKII) and dependence of CD163 signaling on CKII and protein kinase C

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CD163 is a recently identified member of the scavenger receptor cysteine-rich superfamily, which is expressed on peripheral blood monocytes and most tissue macrophages and is thought to play an important role in the regulation of the inflammatory response of these cells. Cross-linking of CD163 on glucocorticoid-stimulated macrophages results in the secretion of several proinflammatory cytokines, but the precise mechanism of CD163 mediated signal transduction is not understood. The existence of several CD163 isoforms, which differ in the structure of their cytoplasmic domains and putative phosphorylation sites, suggests that these isoforms also differ in their signaling mechanism. Using the Yeast Two-Hybrid system and further *in vitro* and *in vivo* studies, we identified the regulatory β -subunit of casein kinase II (CKII), which specifically binds to the cytoplasmic domain of CD163 and its isoforms. We also found, that *in vitro* the CD163 isoforms differ in their association with the CKII holoenzyme and in the phosphorylation by CKII. Furthermore, we demonstrated that the cytoplasmic domains of CD163 variants are phosphorylated by PKC- α *in vitro*. Inhibition studies using specific kinase inhibitors reveal that both CKII and PKC are involved in the CD163 signaling mechanism resulting in the secretion of proinflammatory cytokines.

Key words: CD163 / Isoform / Casein kinase II / PKC / Signaling

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1 Introduction

CD163 is a type I transmembrane protein exclusively expressed on monocytes and macrophages [1]. The mRNA and protein levels of CD163 are tightly controlled by inflammatory mediators. Whereas proinflammatory stimuli like lipopolysaccharide or IFN- γ suppress its expression, anti-inflammatory molecules like glucocorticoids or IL-10 strongly induce CD163 on monocytes and macrophages *in vitro* [2] and *in vivo* [3]. In addition, the PPAR- γ agonist 15-deoxy-PGJ₂, which inhibits the activation of monocytes, induces a down-regulation of CD163 (unpublished observations). *In vivo* CD163 positive macrophages are found during the healing phase of acute inflammation [4], in chronic inflammation [5] and in wound healing tissue [6]. In addition, CD163 positive macrophages produce and secrete not further character-

ized anti-inflammatory and angiogenic factors [7, 8]. These data indicate an important role of CD163 positive cells in the control and down-regulation of inflammatory processes.

CD163 belongs to the family of the Scavenger Receptor Cysteine-rich (SRCR) proteins characterized by a cysteine-rich domain, that is homologous to the C terminus of the macrophage scavenger receptor A-I (SRA-I) [9]. Further, SRCR proteins like CD5, CD6 and WC.1 found on lymphocytes are involved in the development of the immune system and the regulation of the immune response. Although the precise functions of the SRCR domains are still an enigma, they may be important for mediating intercellular contacts. This has been demonstrated for the interaction of CD6 with its ligand CD166 on activated leukocytes [10]. Although it has been demonstrated that CD163 is involved in the adhesion of glucocorticoid treated monocytes to activated endothelial cells, the ligand of CD163 is still unknown [11].

The extracellular region of CD163 contains nine SRCR domains followed by a transmembrane segment and a short cytoplasmic tail composed of 49 amino acid residues. In addition to this so called predominant form two

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Abbreviations: Bis III: Bisindolylmaleimide III **CKII:** Casein kinase II **2,3-DPG:** 2,3-Diphosphoglycerate **DRB:** 5,6-Dichloro-1 β -D-ribofuranosylbenzimidazole **GST:** Glutathione-S-transferase **SRCR:** Scavenger receptor cysteine-rich

cytoplasmic isoforms have been described that contain longer intracellular domains composed of 84 or 89 amino acid residues, respectively [12, 13]. Studies on the genomic organization of the CD163 gene revealed that both isoforms result from alternative splicing [14]. Since the corresponding mRNA are quite abundant in macrophages, it might be suggested that the cytoplasmic isoforms are expressed at similar levels as the predominant form. These isoforms differ in their putative serine / threonine phosphorylation sites found in the cytoplasmic tail (Fig. 1). This may indicate the existence of different signal transduction pathways for the CD163 variants.

Cross-linking of CD163 on glucocorticoid stimulated macrophages by the monoclonal antibody EDHU-1 leads to macrophage activation and the production of proinflammatory cytokines [15]. The signal transmission depends on a protein tyrosine kinase (PTK) activity resulting in calcium mobilization and inositol triphosphate generation. Since the cytoplasmic domains of CD163 and its isoforms contain no known PTK motif, the molecular mechanisms of CD163 mediated signal transduction are not understood. In addition to the PTK-dependent signal another not further characterized signaling pathway has been described that is independent of PTK activity [15].

To identify proteins that directly interact with the cytoplasmic domains of CD163 isoforms, we performed a Yeast Two-Hybrid screening using the intracellular domain of CD163 as bait. Using this approach we found that the regulatory subunit of casein kinase II (CKII) interacts specifically with the cytoplasmic domain of CD163. Furthermore, we show that CKII and PKC- α phosphorylate the cytoplasmic domains of CD163 variants *in vitro*.

Inhibition studies in dexamethasone activated macrophages revealed that both CKII and PKC are involved in CD163 mediated signal transduction with respect to the secretion of proinflammatory cytokines.

2 Results

2.1 The regulatory β -domain of casein kinase II (CKII β) associates with CD163 cytoplasmic domain

We used the Yeast Two-Hybrid System to identify proteins that interact with the cytoplasmic domain of CD163. Therefore, we generated a fusion construct of the GAL4 DNA-binding domain with the entire 89 amino acid cytoplasmic domain of the human CD163 isoform 2, which displays the longest cytoplasmic tail of the known CD163 variants (Fig. 1). This construct was used as a bait for screening of a human liver Two-Hybrid cDNA library. Eighteen positive clones were identified, which displayed an Ade⁺His⁺LacZ⁺ phenotype. Sequence analysis revealed that one of these clones contained the full-length cDNA of human casein kinase II β -subunit (CKII β). The interaction of CKII β was specific to the CD163 cytoplasmic domain, since no interaction was observed between CKII β and the GAL4 DNA binding domain (BD) alone or in fusion with an unrelated protein (Fig. 2). Similarly, no interaction was detected between CD163-BD fusion and the GAL4 activation domain (AD) alone or in fusion with the SV40 large T-antigen.

In addition, the interaction of CKII β and CD163 was confirmed by *in vitro* experiments using recombinant GST-fusions of the cytoplasmic domains of the three CD163

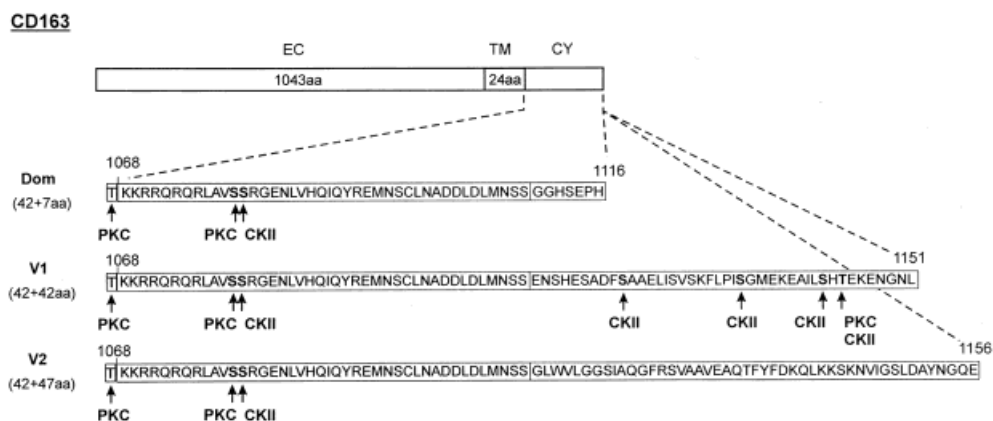


Fig. 1. Schematic representation of the cytoplasmic domains of CD163 isoforms. The first 42 amino acids (aa) which are common to the three isoforms and the additional sequence of the dominant form (Dom) and the two variants (V1 and V2) are shown. Putative CKII and PKC phosphorylation sites are indicated by arrows. EC, extracellular; CY, cytoplasmic; TM, transmembrane.

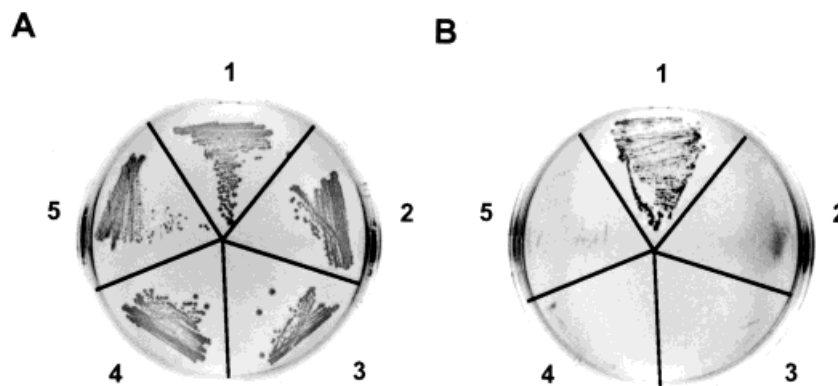


Fig. 2. Interaction of CD163 and CKII β in the Yeast Two-Hybrid system. (A) Growth of yeast clones on medium without leucine and tryptophan allowing the identification of diploid yeast clones expressing both hybrid proteins. (B) Growth of the same clones on selection medium without leucine, tryptophan, histidine and adenine allowing the identification of positive clones expressing interacting recombinant proteins. (1) Interaction of CD163 cytoplasmic tail (isoform 2) fused to the GAL4-binding domain (CD163-BD) with CKII β fused to the GAL4-activation domain (CKII-AD). (2) Interaction of CD163-BD with an unrelated protein (SV40 large T-antigen) fused to AD or (3) with AD alone. (4) Interaction of CKII-AD with an unrelated protein fused to BD or (5) with BD alone.

isoforms (GST-CD163) and recombinant human CKII β (Fig. 3, A). Equal amounts of purified GST-CD163 proteins or GST alone were immobilized on glutathione Sepharose beads and incubated with recombinant CKII β . Binding of CKII β was investigated by Western blot analysis using a monoclonal antibody directed against CKII β . As shown in Fig. 3A all three isoforms of the CD163 cytoplasmic domain bind significant amounts of CKII β , whereas GST does not interact with CKII β . Since all three CD163 isoforms bind similar amounts of CKII β , we suppose that the first 42 amino acids that are com-

mon to the three variants (Fig. 1) mediate the interaction of CD163 and CKII β .

2.2 CKII holoenzyme associates with CD163 cytoplasmic domains

We were interested whether the observed interaction between CKII and CD163 is restricted to the isolated regulatory β -domain of CKII or whether the CKII holoenzyme, consisting of two catalytic β -domains and two

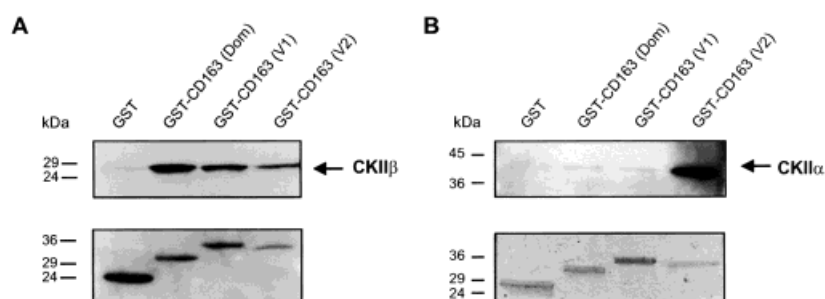


Fig. 3. Interaction of CD163 cytoplasmic domain with the regulatory β -subunit of CKII and CKII holoenzyme. (A) Binding of CKII β to the dominant form (Dom), variant 1 (V1) or variant 2 (V2) of CD163 cytoplasmic domain was analyzed by incubation of CKII β with immobilized CD163-GST fusion proteins or GST alone and subsequent Western blot analysis using an antibody directed against CKII β . The upper panel shows the result of the Western blot detecting bound CKII β . The lower panel shows the same blot incubated with an antibody directed against GST allowing the identification of the GST fusion proteins. (B) Binding of CKII holoenzyme to the dominant form (Dom), variant 1 (V1) or variant 2 (V2) of CD163 cytoplasmic domain was analyzed by incubation of CKII holoenzyme with immobilized CD163-GST fusion proteins or GST alone and subsequent Western blot analysis using an antibody directed against CKII α . The upper panel shows the result of the Western blot detecting bound CKII. The lower panel shows the same blot incubated with an antibody directed against GST.

regulatory β -domains, is also able to associate with the cytoplasmic domain of CD163. Therefore, we performed *in vitro* binding assays using purified CKII holoenzyme and GST-CD163 fusion proteins (Fig. 3B). The binding of CKII holoenzyme was determined by Western blot analysis using an antibody directed against the α -subunit of CKII. As shown in Fig. 3B, the CKII holoenzyme weakly associates with the dominant form and isoform 1 of CD163, whereas there is a strong interaction of CKII with isoform 2.

2.3 Interaction of CKII with CD163 in macrophages

To analyze whether CKII β associates with CD163 in macrophages, we performed an affinity purification of CD163. The lysate of dexamethasone stimulated macrophages was incubated with anti-CD163 beads (clone EDHU-1) and bound proteins were analyzed by Western blotting (Fig. 4). The results demonstrate that CKII β co-purifies with CD163 indicating an *in vivo* association of both proteins. We also investigated whether the interaction of CD163 with CKII is dependent from the activation of the cells by cross-linking of CD163 with the mAb EDHU-1. However, the amount of CKII β co-purified with CD163 was the same in anti-CD163 stimulated and control antibody treated cells (data not shown) indicating that the net recruitment or release of CKII β from CD163 is not influenced by CD163 cross-linking. Currently, it is

not possible to determine which of the CD163 isoforms binds to CKII β *in vivo* since no isoform specific antibodies are available.

2.4 CD163 is a substrate for CKII *in vitro*

To determine whether CKII phosphorylates the cytoplasmic domain of CD163, we performed *in vitro* kinase assays with the purified GST-CD163 proteins and recombinant human CKII (Fig. 5). Equal amounts of immobilized GST-CD163 fusion proteins were incubated with CKII in the presence of [γ - ^{32} P] ATP and ^{32} P incorporation was analyzed by autoradiography. As shown in Fig. 5, the cytoplasmic domain of isoform 1, containing five putative CKII phosphorylation sites, is highly phosphorylated by CKII. Similarly, the dominant form of CD163, which contains only one CKII site at Ser1080, is a substrate for CKII. In contrast, isoform 2 is not phosphorylated by CKII although it contains the same phosphorylation site like the dominant form of CD163 and is able to associate with the CKII holoenzyme. Whether the folding of this variant masks its putative CKII phosphorylation site or whether CKII is inactivated by its interaction

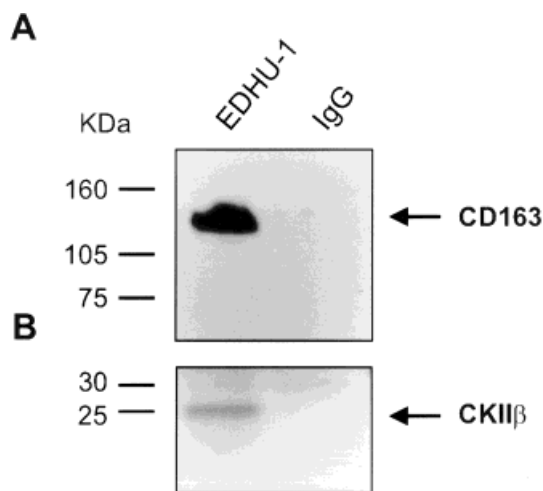


Fig. 4. Interaction of CD163 with CKII β in macrophages. CD163 was isolated from the lysate of dexamethasone stimulated macrophages by affinity purification using the CD163 specific mAb EDHU-1 or an isotype control antibody (IgG). The purified proteins were subjected to Western blot analysis for the detection of CD163 (panel A) and CKII β (panel B).

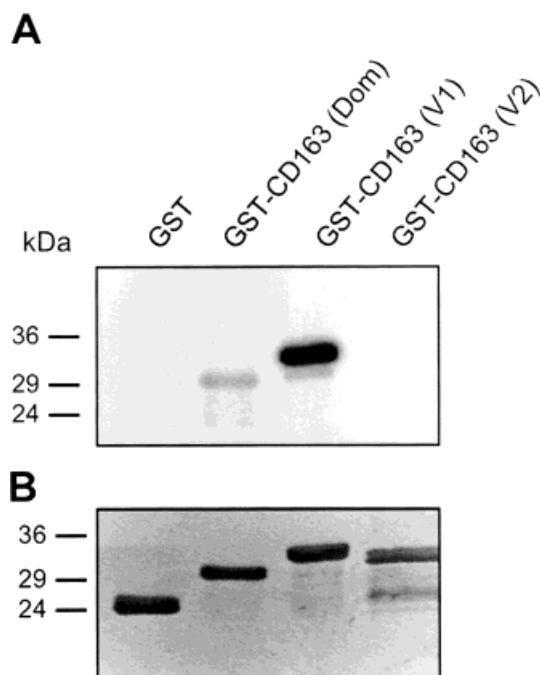


Fig. 5. Phosphorylation of CD163 isoforms by CKII. Immobilized CD163-GST fusion proteins were incubated with purified CKII in the presence of [γ - ^{32} P] ATP. The proteins were separated by SDS-PAGE, dried and incorporation of ^{32} P was analyzed by autoradiography (panel A). The same Coomassie blue stained gel is shown in panel B. Dom, dominant form; V1, variant 1; V2, variant 2.

with isoform 2 needs further investigation. No phosphorylation of CD163 cytoplasmic domains was observed in kinase assays in the absence of recombinant CKII demonstrating that GST-CD163 proteins contain no endogenous kinase activity (data not shown).

2.5 PKC- α phosphorylates the CD163 cytoplasmic domain *in vitro*

Recently, Droste et al. [16] demonstrated that CD163 is shed from the cell surface of glucocorticoid stimulated macrophages upon stimulation with PMA and showed that this mechanism is dependent on PKC activity. We investigated whether CKII is also involved in this process. Therefore, dexamethasone stimulated macrophages were incubated for 2 h with 25 μ M DRB, a specific CKII inhibitor, and subsequently stimulated with PMA at a concentration of 10^{-7} M for 2 h. This stimulation results in an increase of shed CD163 in the cell culture supernatant. However, pre-incubation of the cells with the CKII inhibitor DRB does not significantly inhibit this effect, indicating that CKII activity is not required for shedding of CD163 (data not shown).

The cytoplasmic domain of CD163 contains one (dominant form and isoform 2) or two (isoform 1) putative PKC phosphorylation sites (Fig. 1). In addition, there is one PKC site (Thr1067) located directly at the junction of the predicted transmembrane domain and the intracellular

region, which is common to all isoforms. To investigate whether these sites are phosphorylated by PKC- α *in vitro*, equal amounts of GST-CD163 fusion proteins were immobilized on glutathione beads and subsequently used in a PKC kinase assay. The phosphorylation of the CD163 isoforms was analyzed by autoradiography (Fig. 6A). The result demonstrates that all three isoforms are phosphorylated by PKC- α . However, the dominant form appeared to be a better substrate for PKC- α than the other two isoforms, which might depend on the folding and probably impaired accessibility of the phosphorylation sites in these longer variants. To determine the PKC phosphorylation sites of the dominant form, we mutated the amino acid residue Thr1067 to Ala and Ser1079 to Ala. As shown in Fig. 6B, both substitutions result in an impaired phosphorylation by PKC- α , indicating that both Thr1067 and Ser1079 of the dominant form are phosphorylated by PKC- α *in vitro*. No phosphorylation of CD163 cytoplasmic domains was observed in kinase assays in the absence of recombinant PKC demonstrating that GST-CD163 proteins contain no endogenous kinase activity (data not shown).

2.6 CD163 is constitutively phosphorylated *in vivo*

To determine whether CD163 is phosphorylated *in vivo*, we metabolically labeled dexamethasone stimulated macrophages with ^{32} P and subsequently isolated CD163

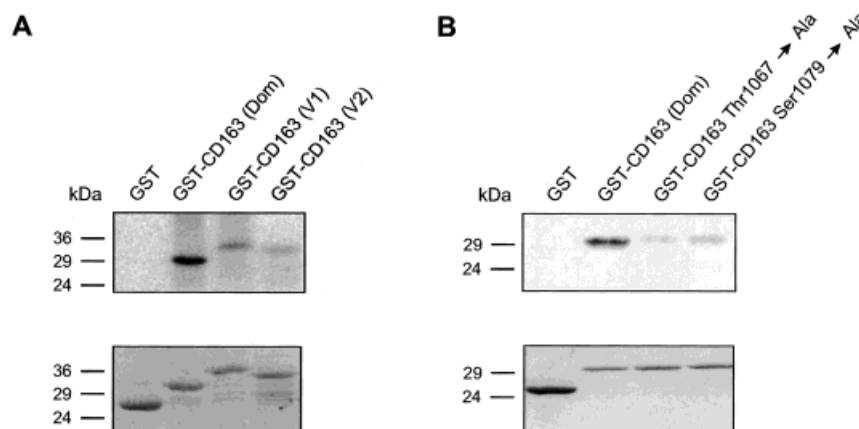


Fig. 6. Phosphorylation of CD163 cytoplasmic domain by PKC- α . (A) Immobilized CD163-GST fusion proteins were incubated with purified PKC- α in the presence of [γ - ^{32} P] ATP. The proteins were separated by SDS-PAGE, dried and incorporation of ^{32} P was analyzed by autoradiography (upper panel). The same Coomassie blue stained gel is shown in the lower panel. Dom, dominant form; V1, variant 1; V2, variant 2. (B) Mutation of the PKC sites in the cytoplasmic domain of the dominant form of CD163 results in an impaired phosphorylation by PKC- α . The dominant form of CD163 cytoplasmic domain (Dom) and the both mutated forms Thr1067→Ala and Ser1079→Ala were subjected to a PKC assay. The GST-fusion proteins were separated by SDS-PAGE, dried and incorporation of ^{32} P was analyzed by autoradiography (upper panel). The same Coomassie blue stained gel is shown in the lower panel.

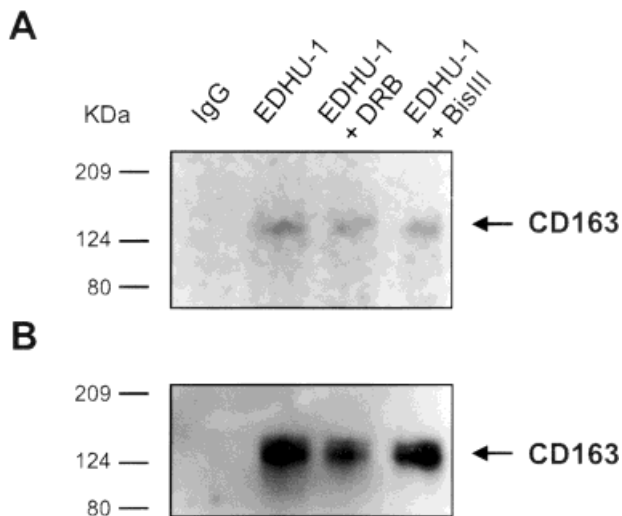


Fig. 7. *In vivo* phosphorylation of CD163. Macrophages were metabolically labeled with ^{32}P and CD163 was isolated by affinity purification using the CD163-specific mAb EDHU-1 or an isotype control antibody (IgG). In additional experiments the CKII inhibitor DRB (25 μM) or the PKC inhibitor Bis III (75 nM) were included during the labeling of the cells. (A) Autoradiography of affinity purified ^{32}P labeled CD163. (B) Detection of purified CD163 by Western blot analysis.

from the lysate by affinity purification. As shown in Fig. 7, CD163 is constitutively phosphorylated in the absence of additional stimuli (e.g. cross-linking of CD163). However, this phosphorylation is independent from CKII and PKC activity since both the CKII-specific inhibitor DRB and the PKC-specific inhibitor bisindolylmaleimide III (Bis III) do not affect the labeling of CD163 with ^{32}P . Since the available anti-CD163 antibodies do not interact with a specific isoform of CD163 it is not possible to predict which isoform is the target of the constitutive phosphorylation.

2.7 CKII and PKC play major roles in CD163 signaling

To investigate, whether CKII and PKC are involved in CD163-mediated signal transduction *in vivo*, we determined the effect of specific CKII and PKC inhibitors on the secretion of proinflammatory cytokines by dexamethasone stimulated macrophages following cross-linking of CD163. The inhibitors had no significant effect on the viability of the cells as determined by analysis of apoptosis and LPS-induced cytokine secretion (data not shown). As shown in Fig. 8, the CKII specific inhibitor DRB blocks the secretion of IL-6 and reduces IL-1 β secretion by about 50%. Similarly, treatment of the cells with 2,3-diphosphoglycerate (2,3-DPG), which is also an

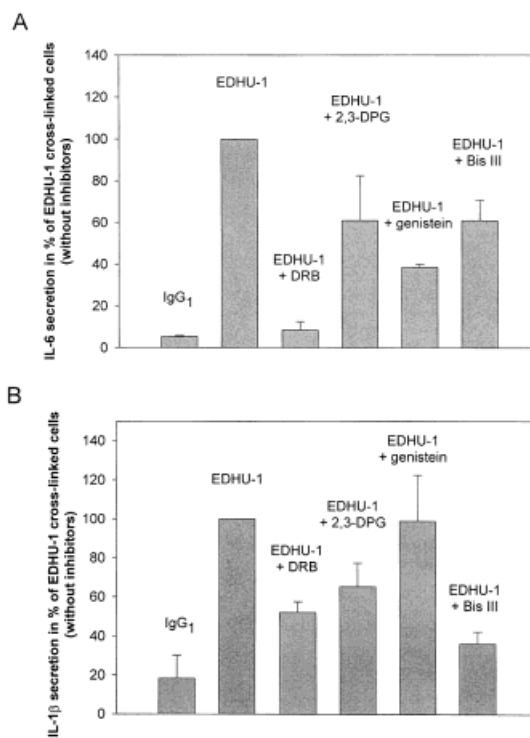


Fig. 8. Influence of protein kinase inhibitors on (A) IL-6 and (B) IL-1 β production of dexamethasone stimulated macrophages following cross-linking by the CD163 specific mAb EDHU-1 or by an isotype control antibody (IgG₁). For inhibition studies cells were pre-incubated for 30 min with DRB (25 μM), 2,3-DPG (1 mM), Bis III (75 nM) or genistein (100 μM). Cytokine concentrations in the cell culture supernatants were analyzed 6 h (IL-6) and 24 h (IL-1 β) after cross-linking of CD163 by ELISA. IL-6 and IL-1 β secretion are given in % of EDHU-1 cross-linked cells in the absence of inhibitors. Shown are the results obtained from macrophages of three different healthy donors. Each experiment was performed in triplicate.

inhibitor of CKII, reduces the secretion of both cytokines. However, the inhibitory effect of 2,3-DPG is weaker than the effect of DRB, probably due to an impaired membrane permeability of 2,3-DPG. Pre-incubation of the macrophages with the specific PKC inhibitor Bis III results in a significant decrease of IL-6 and IL-1 β secretion and demonstrates the involvement of PKC in CD163 mediated signal transduction. The inhibitory effect of the tyrosine kinase inhibitor genistein on IL-6 but not on IL-1 β secretion is in accordance with previously published data [15], indicating the existence of protein tyrosine kinase-dependent and independent signaling mechanisms.

3 Discussion

In this study, we have demonstrated that the serine/ threonine kinase CKII interacts specifically with the cytoplasmic domain of CD163. Furthermore, we have shown that the intracellular domain of CD163 is a substrate for CKII and PKC- α *in vitro*. Since phosphorylation and dephosphorylation are the most prominent post-translational modification involved in the regulation of cell function and signal transduction in macrophages, identification of receptors associated with kinases as well as the phosphorylation status of macrophage surface molecules itself are of great functional importance. This is also demonstrated by the observation that CKII and PKC inhibitors block CD163 mediated signal transduction resulting in the secretion of IL-6 and IL-1 β .

CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily, which includes several proteins like CD5, CD6 or WC.1, that play an important role in the regulation of the immune response of leukocytes [9]. Several CD163 isoforms have been described, which differ in the organization of the extracellular or intracellular domain [13]. Interestingly, the three cytoplasmic CD163 isoforms contain different putative serine or threonine kinase phosphorylation sites. Therefore, signaling through CD163 may be modulated by the use of these different isoforms. Recently, it has been demonstrated that cross-linking of CD163 on glucocorticoid stimulated macrophages induces signaling resulting in the secretion of proinflammatory cytokines [15]. However, neither the precise mechanism of the signal transduction nor the natural ligand of CD163 are currently known. Therefore, the association of CD163 with CKII β is the first report presenting a possible pathway of CD163 mediated signaling.

CKII is a ubiquitous and highly conserved protein serine / threonine kinase that is a major regulator of cell growth, cell division and signal transduction pathways [17, 18]. This is also reflected by the great number of known CKII targets including enzymes involved in nucleic acid and protein synthesis [19–22], several transcription factors [23–25], cytoskeletal proteins [26, 27] and signal transduction proteins [28, 29]. The kinase activity of CKII seems to be constitutive active, since no regulation by second messengers has been reported so far. Therefore, the kinase activity is mainly controlled through the amount and the localization of CKII. The holoenzyme CKII consists of two catalytic α (α')-subunits and two regulatory β -subunits forming the tetrameric complexes $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$. CKII interacts with its substrates either in its holoenzyme form or as individual subunits. Thus, it has been shown, that the isolated α -subunit interacts with protein phosphatase 2A [29] and

that the β -subunit associates with the serine / threonine kinase Mos in the absence of the α -subunit [28]. Interestingly, an interaction of the β -subunit of CKII with the cytoplasmic domain of CD5 – like CD163 a protein of the SRCR family – has recently been reported, suggesting that CD163 and CD5 use similar downstream targets for signaling [30, 31].

The consensus motif for CKII phosphorylation is a serine or threonine residue in an acidic context with a minimal requirement of one acidic residue at the +3 position. There are one (dominant form and isoform 2) or five (isoform 1) serine residues that fit this consensus motif within the cytoplasmic domains of CD163 isoforms. Here we have shown that the cytoplasmic tail of the dominant form and isoform 1 of CD163 are efficiently phosphorylated by CKII *in vitro*. However, the association of CKII β with CD163 seems to be independent from the phosphorylation by CKII because the bacterial produced GST-CD163 fusion proteins, which are most likely not phosphorylated, can bind CKII β *in vitro*. Whether the phosphorylation of CD163 changes the affinity of CKII β to CD163 has to be investigated. Interestingly, CD163 isoform 2 is not phosphorylated by CKII *in vitro*, although it contains the same CKII phosphorylation site like the dominant form and associates with the CKII holoenzyme. This might suggest that either the phosphorylation site is not accessible for CKII because of the conformation of this isoform or CKII might be inactivated by its interaction with this isoform. We also investigated the phosphorylation of CD163 *in vivo* and found that CD163 is constitutively phosphorylated in the absence of external stimuli. However, this constitutive phosphorylation is resistant to treatment of the cells with the CKII specific inhibitor DRB indicating that CKII is not involved in the constitutive phosphorylation of CD163. This finding might suggest that CKII associated with CD163 is inactive in unstimulated cells and becomes activated upon cell stimulation e.g. by cross-linking of CD163. A similar mechanism has been described for the interaction of CD5 with CKII [31]. In this case, CKII associated with CD5 is inactive, but becomes activated following cross-linking of CD5 resulting in the CKII-dependent phosphorylation of CD5. It has been shown, that bound CKII is activated, but obviously not released from CD5 after cross-linking since the amount of CKII associated with CD5 is not changed upon stimulation. Therefore, it is assumed that activated CKII phosphorylates proximal downstream targets associated with the receptor complex [31]. A similar mechanism might be possible for the interaction of CKII with CD163. However, further experiments are necessary to confirm this hypothesis.

Our data indicate that CKII β can interact with all three CD163 isoforms in the absence of the α - or α' -subunits.

Therefore, the association of CKII β and CD163 seems to be mediated by the first 42 amino acid residues of the cytoplasmic domain of CD163, because these residues are common to all three isoforms. Since there is evidence for the existence of free CKII β pools in the cell [32], the interaction of CD163 with free CKII β might provide a mechanism of CD163-dependent regulation of CKII activity by controlling the association and dissociation of α - and β -subunits of CKII.

Currently it is not possible to predict which of the CD163 isoforms is responsible for the association with CKII β *in vivo*, since there are no isoform specific antibodies characterized. However, since it has been demonstrated that the corresponding mRNA of the isoforms are expressed at similar levels, it might be assumed that also similar amounts of the proteins are expressed [12].

In addition to the CKII-dependent *in vitro* phosphorylation of CD163, we demonstrate that CD163 is also phosphorylated by PKC- α *in vitro*. Mutation of the amino acid residues Thr1067 $\rightarrow$ Ala and Ser1079 $\rightarrow$ Ala in the dominant CD163 form revealed that both residues are targets for PKC- α phosphorylation. PKC- α participates in the regulation of macrophage functions involved in host defense and inflammation [33, 34]. Since CD163 seems to be involved in the host response of monocytic cells, the phosphorylation of CD163 by PKC- α observed *in vitro* may further point to an important role of CD163 in this complex network.

Inhibition studies using specific CKII and PKC inhibitors indicate that both kinases are involved in the CD163 signaling mechanism resulting in the secretion of IL-6 and IL-1 β . Despite it is not known in which stage of signaling both kinases participate, the direct interaction of CKII β and CD163 observed *in vitro* and *in vivo*, suggests that CKII is involved in initial steps of CD163 mediated signal transduction. Recently, it has been demonstrated that CD163 cross-linking induces a protein tyrosine kinase (PTK)-dependent pathway resulting in the secretion of IL-6 and a PTK-independent pathway resulting in the secretion of IL-1 β [15]. Our data demonstrate that both pathways depend on CKII and PKC activity, since the inhibition of these kinases leads to an impaired secretion of both cytokines. The expression of distinct CD163 isoforms and additional studies with mutated cytoplasmic isoforms will further clarify the signal transduction pathways used by different cytoplasmic variants and the role of CKII and PKC.

4 Materials and methods

4.1 Yeast Two-Hybrid library screening

The Yeast Two-Hybrid Screening was performed with the Matchmaker Two-Hybrid System 3 offered by Clontech (Palo Alto, CA) following the instructions of the manufacturer. Briefly, the cDNA encoding the cytoplasmic domain of CD163 (variant 2) was amplified from total RNA prepared from human macrophages by RT-PCR using the oligonucleotides CPDuni 5'-GGGCATATGAAAAAGCGAAGACAGAGACAGCGG-3' and Cyto2rev 5'-GGGGGATCCGTCATTCTTGTCCATTATATGCATC-3'. The cDNA was cloned into the *Nde*I / *Bam*HI restriction site of vector pAS-2.1. This bait construct was transformed into the yeast strain *PJ692A* by the lithium acetate method described elsewhere [35]. Library screening was performed by mating of *PJ692A* transformed with the bait plasmid and the yeast strain *Y187* that has been pre-transformed with a human liver cDNA library cloned into the vector pACT-2. Positive clones were isolated by growth selection on medium lacking histidine and adenine and tested for the activity of the LacZ-reporter gene by filter lift assays. The pACT-2 plasmids of His⁺Ade⁺LacZ⁺ clones were isolated, transformed into *E.coli* and sequenced. To eliminate false positive clones, the pACT-2 plasmid was transformed into *Y187* and subsequently mated with *PJ692A* containing pAS-2.1 either without an additional insert or with the insert encoding the cytoplasmic domain of CD163 or an irrelevant protein. Afterwards the diploid yeast clones were tested for reporter gene induction.

4.2 Expression and purification of recombinant CD163 fusion proteins

The cDNA encoding the cytoplasmic domains of the three CD163 isoforms were amplified by RT-PCR using human macrophage RNA as template and the forward primer 5'-GGGGGATCCTCACGAAAAAGCG AAGACAGAGACAGCGG-3' together with the reverse primer 5'-GGGCTCGAGTCACTGTGGCTCAGAATGGCCTCCTG-3' (dominant form) or 5'-GGGCTCGAGGAAGTCACTGGGTATAAATTCCC-3' (variant 1) or 5'-GGGCTCGAGGTCATTCTTGTCCATTATATGCATC-3' (variant 2), respectively. The cDNA were cloned into the *Bam*HI/*Xho*I restriction site of vector pGEX-5 (Amersham Pharmacia Biotech, Freiburg, Germany) in fusion with the glutathione S-transferase (GST) epitope. For expression of recombinant fusion proteins *E.coli* BL21 was grown to mid-log phase and expression was induced by the addition of IPTG to a final concentration of 1 mM. Cells were incubated for an additional 4 h and subsequently lysed in PBS containing 1% Triton X-100 and a protease inhibitor cocktail (Sigma, Deisenhofen, Germany) by sonification. GST-fusion proteins were purified with glutathione Sepharose 4B beads in a batch procedure following manufacturer's instructions (Amersham Pharmacia Biotech). The amount of recombinant protein was estimated by Coomassie Blue staining of SDS-PAGE gels.

For mutation of the PKC sites of the dominant CD163 cytoplasmic domain, the following forward primers together with the reverse primer were used for the generation of the constructs: 5'-GGGGGATCCTCGCGAAAAAGC GAAGACAGAGACAGCGGC-3' (Thr1067→Ala) and 5'-GGGGG ATCCTCACGAAAAAGCGAAGACAGAGACAGCGGCTTGCAGTTG-CCTCAAGAG-3' (Ser1079→Ala).

4.3 Interaction of CKII with GST-CD163 fusion proteins

To demonstrate the interaction of CKII β with the cytoplasmic domains of CD163 isoforms, 1–2 μ g of purified recombinant GST-CD163 fusion proteins were bound to glutathione beads and incubated overnight at 4°C with 500 ng recombinant human CKII β (Calbiochem, San Diego, CA) in an assay buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 7.5 mM MgCl₂ and a protease inhibitor cocktail (Sigma). After the incubation the Sepharose beads were washed with assay buffer containing 0.1 % NP-40. Bound proteins were eluted in Laemmli buffer and analyzed by Western blotting using a monoclonal antibody to CKII β (Calbiochem) followed by incubation with a peroxidase conjugated goat anti-mouse F(ab)₂ fragment (Dianova, Hamburg, Germany) and ECL chemiluminescence substrate (Amersham Pharmacia Biotech).

4.4 Interaction of CD163 and CKII in macrophages

For purification of CD163 the mAb EDHU-1 was covalently coupled to agarose beads using the CarboLink kit obtained from Pierce (Rockford, IL). In control experiments mouse IgG₁-coupled beads were used. Dexamethasone stimulated macrophages were lysed in 25 mM Tris, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 5 mM NaF, 0.1 % NP-40 containing a protease inhibitor mix (Sigma) by three freeze and thaw cycles using liquid nitrogen followed by mild sonification in a waterbath. CD163 was purified from the lysate in a batch procedure using the EDHU-1 coated anti-CD163 beads. The lysate was incubated with the anti-CD163 beads at 4°C overnight followed by washing of the beads with the lysis solution. Proteins were eluted from the beads in 0.1 M glycine buffer, pH 2.5. The samples were neutralized and subjected to Western blot analysis.

4.5 *In vitro* CKII assay

Purified GST-CD163 protein (1–5 μ g) were immobilized with glutathione Sepharose beads and incubated in 40 μ l kinase buffer (20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT). The reaction was started by the addition of 10 μ l of Mg²⁺/ATP cocktail (75 mM MgCl₂, 500 μ M ATP in kinase buffer) containing 10 μ Ci [γ -³²P] ATP and 10 U CKII (Calbiochem, San Diego, CA). The reaction mixture was incubated for 30 min at 30°C and stopped by transferring the mixture to a spin

column (Biozol, Eching, Germany) followed by intensive washing of the Sepharose beads with PBS/1 % Triton X-100. Bound GST-CD163 fusion proteins were eluted in Laemmli buffer and separated by SDS-PAGE. The gels were stained by Coomassie, dried and subjected to autoradiography.

4.6 *In vitro* PKC assay

The PKC assay was performed in analogy to the CKII assay. The reaction was carried out in 100 μ l kinase buffer that contained 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 μ M CaCl₂, 100 nM PMA, 100 μ g/ml phosphatidyl serine, 0.03 % Triton X-100, 100 μ M ATP, 100 μ Ci/ml [γ -³²P] ATP and 0.8 U/ml PKC- α (Biomol, Hamburg, Germany).

4.7 Isolation and culture of cells

Peripheral blood monocytes from healthy volunteers were isolated by leukapheresis followed by elutriation according to a standard protocol [36]. Fractions containing > 95% monocyte purity were pooled and cultured on Petriperm dishes (Heraeus, Hanau, Germany) (10⁶ cells/ml) in a macrophage medium (Gibco BRL, Karlsruhe, Germany) supplemented with 10% FCS and 10 ng/ml GM-CSF (R&D Systems, Wiesbaden, Germany). After 2 days of cultivation the monocytes were stimulated with 80 ng/ml dexamethasone and differentiated for an additional 3 days to induce maximum CD163 expression.

4.8 Radiolabeling of macrophages

For ³²P-labeling dexamethasone stimulated macrophages were washed with phosphate-free DMEM medium (ICN, Costa Mesa, CA), seeded into the same medium (5 × 10⁶ cells/ml) and cultivated for 2 h. ³²P orthophosphate was added at 25 μ Ci/ml and the cells were labeled for 4 h. Inhibitors were added 30 min before starting of the labeling reaction (25 μ M DRB or 75 nM BisIII). After labeling the cells were washed extensively with ice-cold PBS containing 50 mM NaF and 5 mM sodium orthovanadate. Subsequently CD163 was isolated by affinity purification as described above with the following modifications: 50 mM NaF, 5 mM sodium orthovanadate, 30 mM β -glycerol phosphate and a phosphatase inhibitor mix (Sigma) were added to the lysis solution. CD163 phosphorylation was analyzed by autoradiography and Western blot analysis.

4.9 Cytokine assays

Dexamethasone-stimulated macrophages were washed with PBS, resuspended in macrophage medium containing 10% FCS and incubated with protein kinase inhibitors

(25 μ M DRB, 100 μ M genistein, 75 nM Bis III or 1 mM 2,3-diphosphoglycerate) for 30 min at 37°C. The cells were resuspended in medium at a concentration of 10^6 cells/ml and 200 μ l of the cell suspension were seeded into 96-well tissue culture plates (Costar, Corning, NY) that were precoated with 15 μ g/ml of a goat anti-mouse IgG (Calbiochem) followed by 15 μ g/ml EDHU-1 mAb (Serotec, Raleigh, NC) or an isotype control antibody (R&D Systems). After 6 h and 24 h of incubation the supernatants were collected and cytokine production was analyzed by ELISA (R&D Systems). Cell viability was determined using a cell death detection ELISA (Roche, Mannheim, Germany) and by analysis of the LPS induced cytokine secretion.

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