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A conserved polylysine motif in CD86 cytoplasmic tail is necessary for cytoskeletal association and effective co-stimulation

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ABSTRACT

T cell activation requires both antigen specific and co-stimulatory signals that include the interaction of CD28 with its ligands CD80 and CD86. These signals are delivered by antigen presenting cells (APC) in the context of the immunological synapse (IS). Reorganization of the cytoskeleton is required for the formation and maintenance of the IS. Our results show that a highly conserved polylysine motif in CD86 cytoplasmic tail, herein referred to as the K4 motif, is responsible for the constitutive association of CD86 to the cytoskeleton in primary human APC as well as in a murine APC model. This motif is not involved in initial APC:T cell conjugate formation but mutation of the K4 motif affects CD86 reorientation at the IS. Importantly, APCs expressing CD86 with mutated K4 motif are severely compromised in their capacity to trigger complete T cell activation upon peptide presentation as measured by IL-2 secretion. Altogether, our results reveal the critical importance of the cytoskeleton-dependent CD86 polarization to the IS and more specifically the K4 motif for effective co-signaling.

1. Introduction

Initiation of adaptive immune response requires the interaction of naïve T cells with antigen presenting cells (APC), dendritic cells, B cells, and macrophages, in the context of an immunological synapse (IS) [1]. Two key signals are required for full activation of naïve T cells. The first signal requires the interaction of the TCR with MHC:peptide and confers antigen specificity to the response. The second signal entails the interaction of a co-signaling receptor at the T cell surface with its counter receptor at the APC surface [2].

Key co-signaling interactions of naïve T cell involve the engagement of its receptors CD28 and CTLA-4 by CD80 and CD86 ligands (B7 proteins; B7-1 and B7-2) expressed on APCs. While CD28-B7 interaction is involved in amplifying the antigen specific signal, CTLA-4-B7 interaction is known to dampen the strength of this signal [3]. The molecular interactions of these molecules require the formation of a contact interface i.e. the IS. Movement of signaling components and cell-surface molecules of T cells and APCs into the IS is not a passive event and requires remodeling of the actin cytoskeleton [4]. Indeed the cytoskeleton regulates molecular and membrane domains movement and also serves as a scaffold for signaling platforms in addition to its role in cell motility and division [5,6]. Increasing evidence shows that the APC cytoskeleton plays an active role in IS formation and its maintenance [7,8] and that CD80 and CD86 are involved in this IS formation process [9]. The intracellular domain of CD80 has been shown to interact with the cytoskeleton and this association impacts its membrane redistribution following T cell interaction and CD28-dependent T cell activation [10,11]. More specifically, both the RRNE region at position 275–278 and serine 284 within CD80 intracytoplasmic domain were identified to be important for its co-stimulatory function [10]. Moreover, Tseng et al. [11] have shown that CD80 deleted of its intracellular domain fails to recruit CD28, CTLA-4 and PKCθ to the synapse.

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Analysis of the human CD86 protein primary sequence led us to identify a conserved positively charged region within the CD86 cytoplasmic domain at residues $Lys^{265-268}$. This sequence as well as its localization is similar to the $RRNE^{275-278}$ region in CD80. This juxtamembrane stretch of four lysine residues in CD86, defined herein as the K4 motif, is conserved in all mammalian CD86 sequences examined. Based on the identification of this conserved motif, we hypothesized that CD86 was associated to the APC cytoskeleton and that this association was of functional relevance to its co-signaling function. We show here that CD86 is indeed associated to the APC cytoskeleton. Importantly, the association of CD86 to the cytoskeleton is critical for T cell activation.

2. Materials and methods

2.1. Recombinant DNA constructs

Cloning of CD80wt, CD80ΔT, CD86wt and CD86ΔT in the eukaryotic expression vector Srα neo was previously described [12]. CD86-K4 was generated by overlap PCR using the following oligonucleotides: K4 forward

GAAATGGGCGGCGGCGGCGGCCTCGCAACTCTTATAAATG, K4 reverse

CATTTATAAGAGTTGCGAGGCCGCGCCGCCGCCGCCCATTTCC, CD86 start

GGCTGACCCGGGTCTGAGCCACCATGGGACTGAGTAACATTCTC and CD86 end

GTATCTTATCATGTCTGGATCC. CD86-K4 was cloned into the Srα neo vector as a *Bam*HI/ *Sma*I fragment. CD80 and CD80ΔT were cloned into the pEYFP-N1 expression vector (Clontech) following amplification with oligonucleotides providing a 5′ *Xho*I and a 3′ *Sac*II restriction site. The sequence of the oligonucleotides were: CD80FwdCTCGAGGCCACCATGGGCCACACACGG and CD80Rev

CCGCGGTACAGGGCGTAC.

2.2. Antibodies and reagents

Antibodies used in this study included PE-conjugated anti-human CD80 and PE-conjugated anti-human CD86 (BD Biosciences) for FACS staining and a polyclonal rabbit antibody against YFP (BD Biosciences) for immunoblotting. NF-κB antibody was obtained from Cell Signaling. All secondary antibodies were from Molecular Probes. Anti-human CD83, anti-human TCR, anti-human CD19 and anti-human CD14 used for DC phenotyping were from BD Biosciences.

2.3. Cell lines

Murine B2D cells are derived from the P815 mastocytoma cell line stably transfected with HLA-DR0101 [13]. Human CD28 negative and positive Jurkat T cells (CD28neg and CD28pos Jurkat T cells) derived from the CH7C17 Jurkat T cell line expressing a V1.2/V3.1 TCR specific for $HA_{306-318}$peptide restricted to HLA-DR0101 have been already described elsewhere [14]. The Daudi B cell line and 293T cells were obtained from ATCC.

2.4. CD28Fc binding assay

Stable cell lines expressing the different mutant forms of CD86 were incubated for 1 h at 4 °C with various concentrations of CD28Fc (R&D Systems). Cells were then washed and incubated for an additional 30 min with a secondary antibody specific for human Fc coupled to Alexa fluor 647. After washing, cells were fixed in 2% paraformaldehyde and analyzed at the cytometer.

2.5. Dendritic cell (DC) generation and culture

Human PBMCs obtained from healthy donors were depleted of $CD3^+$ T cells using the rosetteSep kit (StemCell). $CD14^+$ cells were then isolated by autoMACS (>95% purity). Monocytes were plated at a density of 3×10^6 cells per well in 6-well tissue-culture plates in 3 ml RPMI1640 medium supplemented with 1% human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1% non-essential amino acids, 200 U/ml IL-4 (Sigma) and 1000 U/ml GM-CSF (Cangene). Cells were supplied with 1.5 ml of fresh medium containing the same amounts of IL-4 and GM-CSF on days 2, 4, and 6. Mature DCs (mDCs) were obtained by harvesting non-adherent cells (immature DCs) on day 7 and stimulated with MCM-mimic consisting of 1 ng/ml TNF-α (Invitrogen), 10 ng/ml IL-1β (Invitrogen), 1 g/ml PGE2 (Sigma), and 1000 U/ml IL-6 (Invitrogen) for 48 h. DC purity was assessed by flow cytometry using fluorescently conjugated monoclonal antibodies against CD14, CD19, CD83, CD86, HLA-DR, and TCR. DC purity was consistently higher than 95%.

2.6. Biochemical fractionation

The protocol used for subcellular fractionation was previously described [15]. Briefly 10×10^6 transfected 293T cells, 10×10^6 dendritic cells or 30×10^6 B2D cell lines were pelleted, washed in PBS, resuspended in hypotonic solution (10 mM HEPES pH 6.9, 10 mM KCl, protease inhibitors) and incubated on ice for 20 min. Cells were disrupted by gentle pipetting. Nuclei were pelleted by centrifugation at 3200 rpm for 10 min at 4 °C. Supernatants from pelleted nuclei were further centrifuged at 35000 rpm for 30 minutes at 4 °C. The cytosolic fraction was separated from the pellet (cytoskeletal plus membrane fractions) and was resuspended in NTENT buffer (500 mM NaCl, 10 mM Tris–HCl pH 7.2, 1 mM EDTA, protease inhibitors and 1% Triton X-100). This fraction was centrifuged at 14 000 rpm for 30 min at 4 °C. The resulting pellet, was resuspended again in NTENT buffer and included the cytoskeletal fraction, while the membrane fraction was found in the supernatant.

2.7. Conjugate formation

APC were pre-pulsed with 10 g/ml of $HA_{306-318}$ peptide for 2 h at 37 °C. 2×10^6 cells consisting of 1×10^6 T cells and 1×10^6 HA-loaded B2D cell lines were co-cultured in a 37 °C water bath and then resuspended in PBS supplemented with 2% paraformaldehyde for 20 min. Cells were then washed in PBS and stained with PE-Cy5-conjugated anti-human MHC class I (T cells) and PE-conjugated anti-CD86 (APC) monoclonal antibodies for 1 h at 4 °C. Cells were washed and analyzed using a BD FACS Scan flow cytometer.

2.8. Imaging of immunological synapse

Cells were co-cultured as described above and deposited on poly-L-lysine coated slides for 5 min, spun at 600 rpm for 1 min, fixed in 10% formalin and then stained for CD86. Experiments with DCs were performed using 1×10^6 cells. Images were acquired on a Leica Confocal microscope, using a 63× oil-immersion objective. An average of 10 images per condition were taken for each independent experiment. Synaptic recruitment analysis was performed with the Northern Eclipse software. A fluorescence ratio was obtained by dividing the Mean Fluorescence Intensity (MFI) at the synapse with the total cell MFI. A ratio of 1 indicates that the protein is homogenously distributed at the cell surface (no specific accumulation at the interface) while a ratio greater than 1 indicates a specific accumulation of the protein of interest [16]. Results