# Calmodulin kinase II regulates the maturation and antigen presentation of human dendritic cells

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**Abstract: Dendritic cells (DC) are professional antigen-presenting cells, which activate the adaptive immune system. Upon receiving a danger signal, they undergo a maturation process, which increases their antigen presentation capacity, but the responsible regulatory mechanisms remain incompletely understood. A $Ca^{2+}$-calmodulin (Cam)-Cam kinase II (CamK II) pathway regulates phagosome maturation in macrophages, and this pathway is inhibited by pathogenic microbes. Our hypothesis is that signal transduction events which control phagosome maturation also regulate antigen presentation. Stimulation of primary human DC or the human DC line KG-1, with particulate antigen, resulted in the activation of CamK II and its localization to the phagosome and plasma membrane. Two mechanistically distinct inhibitors of CamK II significantly reduced DC maturation, as determined by up-regulation of surface costimulatory and major histocompatibility complex (MHC) class II molecules and secretion of cytokines. Confocal microscopy demonstrated that the CamK II inhibitors blocked the antigen-induced increase in total cellular MHC class molecules as well as their trafficking to the plasma membrane. Inhibition of CamK II was associated with decreased presentation of particulate and soluble MHC class II-restricted antigen, with a greater effect on the former. These data support a model in which CamK II regulates critical stages of the maturation and antigen presentation capacity of human DC, particularly in response to stimulation via phagocytosis. *J. Leukoc. Biol.* 78: 1397–1407; 2005.**

***Key Words:*** *signal transduction · cell differentiation · antigen presentation/processing · cytokines · MHC*

## INTRODUCTION

Dendritic cells (DC) are the major antigen-presenting cells (APC) of the immune system [1]. Immature DC have the ability to constantly sample their environment in search of a danger signal or pathogen. Upon encountering a stimulus, DC undergo a maturation process, which involves the up-regulation of costimulatory molecules (CD40, CD80, CD86), secretion of cytokines, migration to lymph nodes, and the processing and presentation of antigens on major histocompatibility complex (MHC) class I, II, or CD1 molecules to T cells [1]. The mechanisms that regulate the maturation of DC are the subject of active investigation. Stimulation of human DC by lipopolysaccharide (LPS) or engagement of CD40 results in activation of the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1 (ERK1) and ERK2, as well as phosphatidylinositol-3 kinase (PI-3K), and nuclear factor-κB [2–4]. PI-3K and the tyrosine kinase p72Syk are essential for the up-regulation of CD40, CD80, the DC lineage marker CD83, and production of tumor necrosis factor α (TNF-α) and interleukin (IL)-12 upon engagement of MHC II with lymphocyte activation gene 3 (CD223) [5, 6]. However, it remains unclear which signal transduction pathways are strictly required for DC maturation by diverse physiologic stimuli and the manner in which these signals are integrated to regulate complex DC phenotypes.

$Ca^{2+}$ is an important second messenger that regulates multiple cellular functions. In phagocytes, changes in cytosolic $Ca^{2+}$ regulate receptor-mediated endocytosis, phagosome-lysosome fusion, and antigen processing [7–9]. Antigen stimulation of primary, immature, human DC or the human DC line KG-1 induces an increase in cytosolic $Ca^{2+}$ levels [10–13]. Furthermore, addition of $Ca^{2+}$ ionophores to primary human monocytes or immature DC results in the acquisition of many of the morphologic and functional properties of activated, mature myeloid DC [10–12]. Conversely, intracellular $Ca^{2+}$ chelators, which prevent agonist-stimulated changes in cytosolic $Ca^{2+}$ levels, block the maturation of DC induced by LPS, prostaglandin $E_2$, and cholera toxin [13]. Taken together, these data indicate that $Ca^{2+}$ signaling is a critical component of DC maturation and function. However, the downstream effectors of $Ca^{2+}$, which are required for maturation of DC, are unknown.

Calmodulin kinase II (CamK II), a multifunctional serine/threonine kinase, is an important $Ca^{2+}$ signal-transducing element in mammalian cells. CamK II is encoded by four genes

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(α, β, γ, δ), each of which can be spliced alternatively, resulting in numerous isoforms arising from homo- and heterodimerization [14, 15]. CamK II is a ubiquitous enzyme, which regulates gene expression, membrane trafficking, secretion, cytoskeletal dynamics, and apoptosis [14, 15]. The isoform diversity, widespread distribution, and diverse functions of CamK II require precise control of its activation and spatial targeting. The activity of CamK II is regulated by binding of $Ca^{+2}$-calmodulin ($Ca^{+2}$-Cam) and autophosphorylation of stimulatory and inhibitory sites. Specifically, increases in cytosolic $Ca^{2+}$ result in the binding of $Ca^{+2}$-Cam to a site near the autoinhibitory domain (AID) of the enzyme, leading to autophosphorylation of $Thr^{286}$ and generation of an autonomously activated form of CamK II [14]. In contrast, autophosphorylation of $Thr^{305}$ or $Thr^{306}$ blocks the binding of $Ca^{+2}$-Cam. Protein phosphatases also contribute to the regulation of CamK II [14, 15].

We recently demonstrated that macrophage phagocytosis initiates $Ca^{2+}$-dependent activation of CamK II on the phagosome membrane [16]. This specific spatial localization of activated CamK II is required for the maturation of phagosomes to acidic, microbicidal phagolysosomes, as specific inhibitors of CamK II block phagosome maturation. The medical significance of this signaling pathway is illustrated by the intracellular pathogen, *Mycobacterium tuberculosis*, which specifically inhibits macrophage $Ca^{2+}$ signaling, resulting in the absence of activated CamK II on the membrane of its phagosome [16, 17]. Reversal of this inhibited $Ca^{2+}$ signaling in *M. tuberculosis*-infected macrophages via $Ca^{2+}$ ionophores restores the focal activation of CamK II on the *M. tuberculosis* phagosome, resulting in increased phagosome maturation and killing of intracellular tubercle bacilli [9, 16]. These data, as well as the work of other investigators [8, 18], support the hypothesis that CamK II is an important regulator of vesicular trafficking and immunological functions in macrophages.

Despite the importance of $Ca^{2+}$ signaling to DC maturation and function, the potential role of CamK II in these processes is largely unexplored. CamK II was recently demonstrated to be required for phagocytosis of apoptotic tumor cells by DC [19]. The identification of a novel CamK II inhibitor protein specifically expressed in DC [20] also points to an important role for CamK II as a $Ca^{2+}$ signal transducer in this cell type. The objective of this study was to test the hypothesis that CamK II regulates the maturation and function of human DC.

## MATERIALS AND METHODS

### Materials

Unless otherwise stated, materials were from previously published sources [16, 17]. Pan-CamK II monoclonal antibodies (mAb) and phosphospecific CamK II mAb were purchased from Affinity Bioreagents (Golden, CO), and MHC II [human leukocyte antigen (HLA)-DR)] mAb were from Monosan (Uden, Netherlands). Oregon Green- and Texas Red-conjugated secondary antibodies, latex beads (3 μ diameter), and the CyQuant proliferation assay were from Molecular Probes (Eugene, OR). Glass chamber slides were from Fisher (Hampton, NH). TNF-α was from R&D Systems (Minneapolis, MN). OptEIA enzyme-linked immunosorbent assay (ELISA) kits were from BD PharMingen (San Diego, CA). Tetanus toxoid (TT; endotoxin-free) was from Massachusetts Biolabs (Boston). LPS from *Escherichia coli* (strain O111:B4) and peptidoglycan (PGN) from *Staphylococcus aureus* were obtained from Sigma-Aldrich (St. Louis, MO). *E. coli* (strain O111:B4) and *S. aureus* (strain ALC 1435) were generously provided by Jerrold Weiss (University of Iowa, Iowa City).

### Preparation of human DC

Peripheral blood mononuclear cells were isolated from healthy adult volunteers by Ficoll-Paque density gradient centrifugation, according to established protocols [3–6, 21]. To obtain immature DC, purified monocytes were incubated in a 225-$cm^3$ flask in RPMI 1640 for 1.5 h in a humidified incubator at 37°C. Nonadherent cells were removed by washing with warm RPMI and adherent cells cultured in RPMI 1640 (pH 7.35), L-glutamine, essential and nonessential amino acids, granulocyte macrophage-colony stimulating factor (GM-CSF; 100 ng/ml), and IL-4 (20 ng/ml). On Day 5, immature DC were harvested using 10 μM EDTA. To assess the purity and phenotype of DC preparations, $10^6$ cells were incubated with mAb to CD1a, CD11c, CD14, CD40, CD80, CD83, CD86, and MHC II or isotype-matched, control antibodies at 10 μl antibody/$10^5$ cells in phosphate-buffered saline (PBS), 5% fetal bovine serum for 40 min at 4°C. Stained cells were analyzed by flow cytometry on a FACSCalibur. Consistent with previous studies [3–6, 21], CD1a was uniformly detected on immature DC, whereas CD14 was down-regulated by Day 5. All experiments which used primary, human DC, represent data from cells derived from at least two, and usually three to five, different donors, as specified in the figure legends. For assessment of the percent change in surface expression of MHC class II and costimulatory molecules (CD40, CD80, CD83, CD86), following treatment with TT and/or the inhibitors of CamK II, the total cell count in the M1 region was compared between control and treated samples. The mean fluorescence of each sample was also examined to verify results obtained using M1 gating.

### Differentiation of KG-1 cells

The human DC line KG-1 (gift from K. Lee, University of Miami, FL), which exhibits an immature DC phenotype, was cultured in modified Eagle's medium with L-glutamine, supplemented with 20% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) [22, 23]. Cells ($2\times10^5$ cells/ml) were differentiated to a mature phenotype with 100 ng/ml GM-CSF, 20 ng/ml IL-4, and 10 ng/ml TNF-α for 5 days [22].

### Confocal microscopy

Primary, immature DC or KG-1 cells ($5\times10^4$) were incubated overnight at 37°C on collagen-coated slides, with or without the CamK II inhibitor peptide (IP; $Met^{281}$–$Ala^{302}$ is derived from the AID of CamK II [24], and $Thr^{286}\rightarrow$Ala; IP, 1 μM) for 2 h. Cells were washed 2× with warm RPMI and incubated with media, particle-bound TT (PB-TT; 5 mg/ml), or A23187 (10 μM) for 1, 4, or 24 h at 37°C. Cells were fixed in 3.75% formalin for 15 min and permeabilized in methanol:acetone (1:1) for 5 min at 4°C [16, 17]. Samples were incubated sequentially with blocking buffer (PBS, 5% bovine serum albumin, 10% horse serum), 1° antibodies [phosphospecific CamK II mAb, pan-CamK II mAb, MHC II (HLA-DR) mAb], and 2° antibodies [goat anti-murine immunoglobulin G (IgG)-Oregon Green], all for 1 h at 25°C [16, 17]. Confocal microscopy was performed on a Zeiss laser scan inverted 510 microscope.

### Cytokine measurement

Primary, human, immature DC or KG-1 cells were cultured with PB-TT (5 mg/ml) or soluble TT (1.5 mg/ml) [21] at 37°C. These respective levels of TT were selected to normalize for adsorption to the surface of the latex beads [21]. Supernatants were collected at 1, 6, 12, 18, 24, and 72 h and stored at −80°C. OptEIA ELISA kits were used according to the manufacturer's instructions to quantitate levels of IL-12, IL-2, and interferon- γ (IFN-γ). In select experiments, DC were incubated with CamK II inhibitors for 2 h and then washed prior to addition of antigen.

### Determination of DC endocytosis

Primary, human, immature DC were pretreated with buffer or IP for 2 h, washed, and then matured with PB-TT for 24 h. At 24 h, DC were harvested, centrifuged, and resuspended in 1 ml RPMI. Fluorescein isothiocyanate

(FITC)-dextran (150 ug/ml) was added to each cell suspension for 1 min. A sample of control immature DC was kept on ice, and the remainder of immature DC and maturing DC was incubated in a 37°C water bath for 1 min. Cells were then centrifuged at 4°C, supernatants removed, cells resuspended in PBS, and fluorescence analyzed on a FACSCalibur flow cytometer.

## Antigen presentation assay

Primary, immature, human DC or KG-1 cells ($10^4$) were incubated with buffer control or the CamK II inhibitors for 2 h at 37°C. Cells were washed three times, followed by addition of PB-TT (5 mg/ml) or soluble TT (1.5 mg/ml) and $5 \times 10^4$ SP-F3 T cells. The $CD4^+$ αβ T cell clone SP-F3 was isolated from the blood of a patient with severe combined immunodeficiency syndrome who had undergone fetal liver stem cell transplantation [25]. SP-F3 recognizes the 947–961 TT peptide presented by all HLA-DR alleles. On Day 5, T cell proliferation was determined with the CyQuant proliferation assay (Molecular Probes).

## Analysis of data

Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups were analyzed for statistical significance using Student's *t*-test. Nonparametric evaluation of other data sets was performed using the Wilcoxon Rank Sum test.

# RESULTS

## Antigen-induced stimulation of human DC is associated with activation and plasma membrane enrichment of CamK II

Stimulation of DC with antigen has been demonstrated to result in elevation of levels of cytosolic $Ca^{2+}$ [13]; however, the effects on CamK II remain undefined. To determine whether antigenic stimulation is coupled to activation of CamK II, we incubated primary, human, immature DC (derived from blood monocytes by in vitro cultivation with IL-4 and GM-CSF [3–6, 21]) with the well-characterized protein antigen, TT [26–28]. To focus on antigens acquired via phagosomal compartments, TT was adsorbed to the surface of latex beads (3 μ diameter) [21], which are avidly ingested by DC. Stimulation of CamK II was determined by staining with mAb to the activation-specific epitope, phospho-$Thr^{286}$, and analysis by laser scanning confocal microscopy [9].

Incubation of primary, human, immature DC with PB-TT for 30 min resulted in a significant increase in the level of phosphorylated CamK II, consistent with its activation (**Fig. 1A**). In

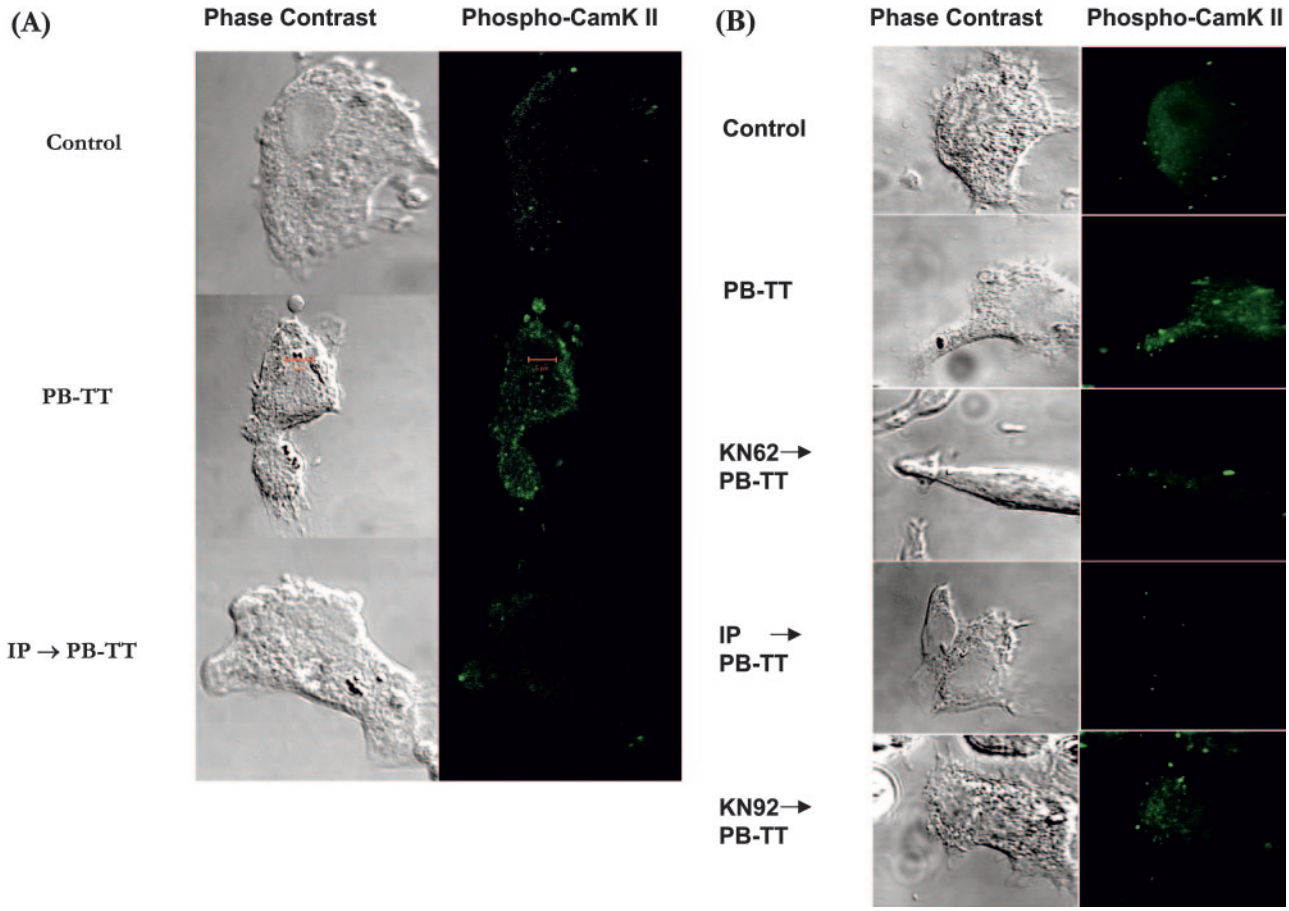


**Fig. 1.** Antigen stimulates the activation and plasma membrane localization of CamK II in human DC. (A) Primary, immature, human DC, derived by in vitro culture of blood monocytes, were adhered to collagen-coated slides and pretreated with buffer (Control) or the CamK II IP (1 μM) for 2 h. PB-TT or buffer control was added for 30 min. Cells were washed, fixed, permeabilized, and stained with antiphospho-CamK II mAb, with detection by Oregon Green-conjugated anti-murine IgG 2° antibody. In the PB-TT images, the red line indicates 5 μ and is placed at the site of a latex bead phagosome. (B) KG-1 human DC were pretreated with buffer, the CamK II inhibitors (KN62 or IP, both at 1 μM), or the inactive, structural analog KN92 (1 μM) for 2 h. PB-TT or buffer control was added for 30 min, and cells stained with antiphospho-CamK II mAb, as in A above. Samples were analyzed on a Zeiss 510 laser scanning confocal microscope. At least 25 cells were imaged for each experimental condition. Data are representative of results from three identical experiments, and three different donors were used to obtain the primary DC in A.

addition to the overall increase in levels of activated CamK II, antigen-induced stimulation was accompanied by its cellular redistribution, resulting in enrichment of activated CamK II at the phagosome membrane [9] and the plasma membrane. Kinetic analysis demonstrated that CamK II was activated within 15 min of incubation with PB-TT, and total cellular levels of phospho-CamK II remained elevated for at least 24 h (not shown). Phagocytosis of latex beads alone (in the absence of adsorbed TT) also resulted in activation of CamK II, although to a lesser extent than in the presence of antigen (not shown).

To expand the relevant model systems in which the role of CamK II in the maturation and antigen presentation of human DC can be evaluated, we used the human DC line KG-1, which exhibits an immature DC phenotype [22, 23]. KG-1 cells undergo the prototypical stages of DC maturation upon stimulation with antigen, cytokines, or other inflammatory mediators [22, 23]. Use of this clonal population of human DC complemented studies with primary monocyte-derived DC by removing the qualitative and quantitative heterogeneity caused by donor-to-donor variability and by eliminating the possibility that contaminating lymphocytes contributed to antigen-induced effects on DC. Incubation of KG-1 cells with PB-TT resulted in increased activation of CamK II (Fig. 1B), in concordance with the findings in primary human DC.

To further characterize the relationship between stimulation of DC and activation of CamK II, we used two agonists that represent prototypes of pathogen-associated molecular patterns, LPS from *E. coli* and PGN from *S. aureus*. Incubation of primary, immature, human DC with LPS (100 ng/ml) or PGN (10 μg/ml) resulted in significant activation of CamK II (not shown). These data are consistent with a model in which activation of CamK II is a common feature of DC stimulation by diverse soluble and particulate antigens.

To determine the potential role of CamK II in maturation of DC, we used two chemically distinct inhibitors of the enzyme. The isoquinolone KN62 is a well-characterized inhibitor of CamK II [24, 29, 30] and was used in conjunction with its inactive structural analog KN92. The IP ($Met^{281}$–$Ala^{302}$) is derived from the AID of CamK II [29] and contains an $Ala^{286}$ residue in place of the activation-associated phosphorylation site $Thr^{286}$. As a first step, we tested the efficacy of these inhibitors in blocking antigen-induced activation of CamK II. Treatment of primary DC or KG-1 cells with IP or KN62 (1.0 μM for 2 h) but not the inactive isoquinolone analog KN92 inhibited the activation and plasma membrane enrichment of CamK II in response to TT (Fig. 1). KN62 and IP exhibited concentration-dependent inhibition of CamK II, and maximal efficacies were in the range of 1–10 μM (not shown). IP was more efficacious in inhibition of CamK II than KN62 (Fig. 1B). These concentrations of KN62 and IP had no detectable effects on the viability of KG-1 cells or primary DC, as determined by exclusion of trypan blue (not shown). Neither inhibitor altered the total levels of CamK II (phosphorylated+unphosphorylated forms) as determined by staining with a pan-CamK II antibody (not shown).

## Inhibition of CamK II results in blockade of antigen-induced cytokine secretion

Synthesis and secretion of cytokines, including IL-12 and IFN-γ, are integral parts of the maturation of DC and are required for activation of naïve T cells [1, 31]. Moreover, recent work has demonstrated that IL-2 production by DC in the first 48 h after infection is required for T cell activation and proliferation in vitro and in vivo [32–36]. To determine whether CamK II functions in the production of these key cytokines, primary, human, immature DC were treated with the CamK II inhibitors or buffer control for 2 h prior to exposure to PB-TT.

The secretion of IL-12p70, IFN-γ, and IL-2 was determined at 1, 6, 12, 18, 24, and 72 h following addition of antigen. Inhibition of CamK II with KN-62 or IP resulted in a dramatic decrease in secretion of each cytokine (**Fig. 2, A–C**). Of note, either inhibitor of CamK II essentially eliminated production of IL-12p70. Analysis of KG-1 cells demonstrated findings similar to those of primary DC; i.e., inhibition of CamK II was associated with marked suppression of IL-12p70, IFN-γ, and IL-2 secretion in response to PB-TT, and the greatest effect was on IL-12p70 levels (Fig. 2, D–F). The concordance of the data with the purified KG-1 cells supports the hypothesis that the cytokine production in the primary DC culture is not a result of contamination from T cells. The temporal pattern of cytokine secretion by control DC, including the biphasic pattern that has been ascribed to autonomous feedback loops, is in agreement with previous studies [32–36].

We next tested the effect of the CamK II inhibitors on the response of KG-1 DC to soluble TT. Inhibition of CamK II with IP resulted in significantly decreased secretion of IL-12p70, IFN-γ, and IL-2, and the greatest inhibitory effect was on IL-12p70 (Fig. 2, G–I). KN62 also resulted in decreased secretion of IL-12p70 and IFN-γ, although these were less marked than those resulting from treatment with IP. The greater efficacy of IP in the suppression of IL-12p70 and IFN-γ secretion correlated with the relative efficacies of IP and KN62 in inhibition of the phosphorylation of CamK II (Fig. 1B). KN62 produced a significant decrease in IL-2 release at 18 and 24 h, but there was no change in the peak values. Comparison of the cytokine responses to PB versus soluble antigen indicated that the former exhibited greater dependence on CamK II. The effects of CamK II inhibition on TT-dependent cytokine secretion were similar in primary, human DC compared with the KG-1 human DC line. These data support a model in which CamK II functions in the DC response (cytokine secretion) to antigens acquired via phagocytosis or endocytosis, with a quantitatively greater effect on the former.

To determine whether the dependence of cytokine secretion on CamK II was specific to TT or rather, a general property of danger signals that activate DC, similar studies were conducted with the Gram-negative bacterium *E. coli* and its purified LPS as well as the Gram-positive bacterium *S. aureus* and its purified cell wall PGN. Each of these agents stimulated the production of IL-12p70, IFN-γ, and IL-2 by primary human DC (determined at 24 h; **Fig. 3**), consistent with previous studies [32–36]. Inhibition of CamK II by pretreatment with IP resulted in a significant decrease in secretion of each cytokine

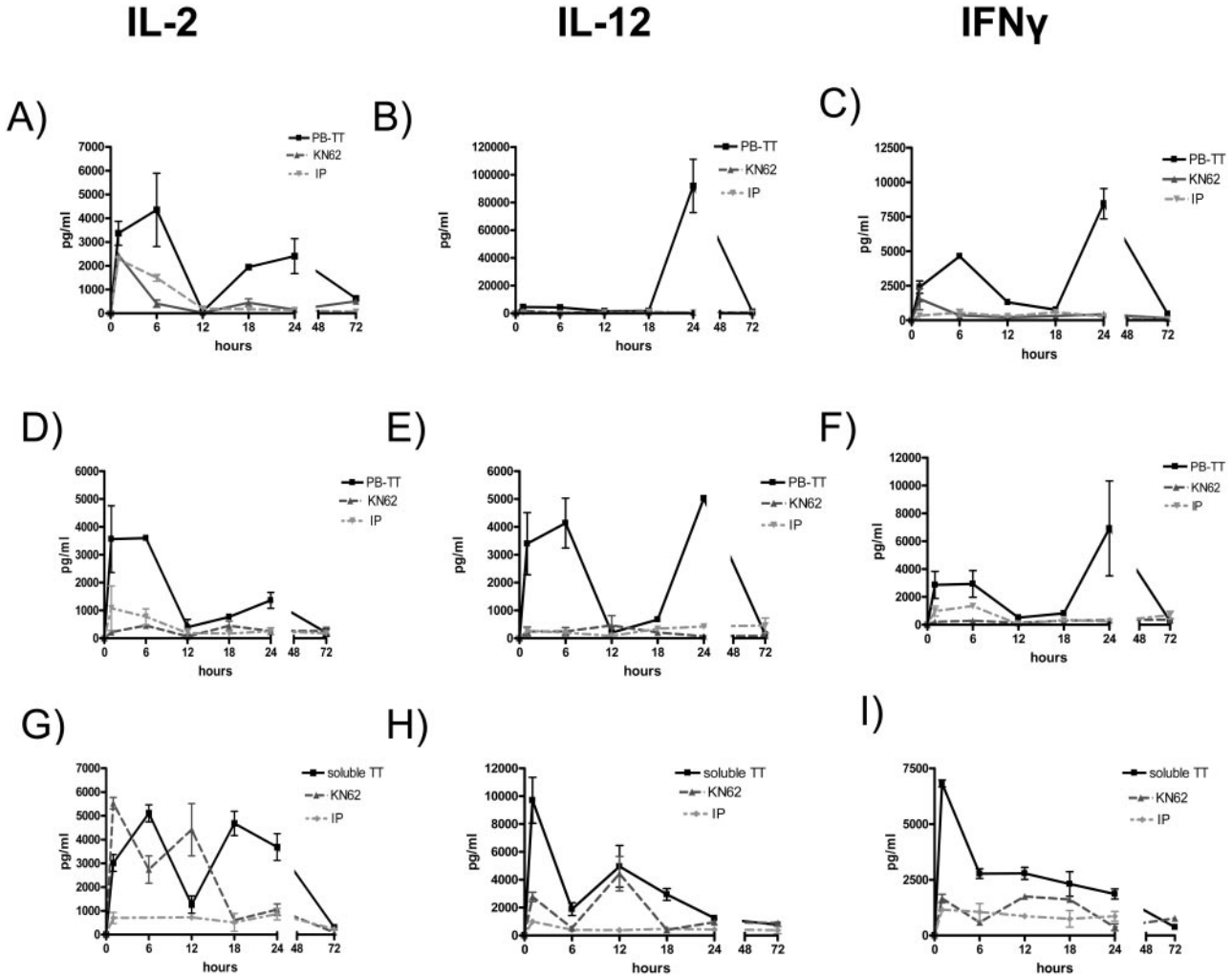


**Fig. 2.** Inhibition of CamK II blocks antigen-induced cytokine secretion in human DC. Primary, human, immature DC (A–C) or KG-1 cells (D–I) were incubated with buffer control or the inhibitors of CamK II (KN62, IP, both at 1 μM) for 2 h. Cells were washed and incubated with PB-TT (A–F) or soluble TT (G–I). Supernatants were analyzed for IL-2, IL-12, and IFN-γ via ELISA at 1, 6, 12, 18, 24, and 72 h. Results are the mean (±SEM) of two independent experiments, each performed in duplicate. Primary DC were obtained from two different donors. (B, C, E, F) The differences between cells treated with KN62 or IP versus control cells, which received no inhibitor, are statistically significant ($P<0.05$).

under all stimulation conditions ($P<0.05$ compared with DC not treated with IP). Taken together, the data in Figures 2 and 3 support the hypothesis that CamK II regulates cytokine secretion by human DC in response to pathogenic stimuli.

## Inhibition of CamK II is associated with reduced surface expression of MHC class II and costimulatory molecules

DC are the only APC capable of activating naïve T cells [1, 31, 37]. For T cells to respond to protein antigens, they must receive two signals: one through their T cell receptor (TCR) from peptide-bound MHC class I or II molecules and the second through costimulatory molecules, such as CD40, CD80, or CD86. Thus, regulation of the surface expression of MHC and accessory molecules is a critical facet of DC biology. As inhibition of $Ca^{2+}$-mediated signaling results in blockade of the surface expression of costimulatory molecules in stimulated DC [13], we hypothesized that CamK II participates in the regulation of this response.

As a first step in evaluating this hypothesis, primary, human, immature DC were incubated with KN62, IP, KN92, or buffer for 2 h, followed by incubation with PB-TT or soluble TT for 24 h. Surface expression of MHC class II and the costimulatory molecules CD40, CD80, CD86, as well as the DC maturation marker CD83 were determined by flow cytometry. In DC stimulated by PB-TT, inhibition of CamK II by KN62 or IP resulted in significant reductions (50–85%) in the surface expression of MHC class II, CD40, and CD 83, (**Fig. 4, A** and **B**), whereas levels of CD80 and CD86 showed little change (reductions of 5–25%). In cells stimulated by soluble TT, only CD83 up-regulation was significantly affected by the CamK II inhibitors (Fig. 4C). This greater effect of CamK II inhibition on DC responses to PB versus soluble antigen parallels that reported above for cytokine secretion (Fig. 2). The inactive isoquinolone analog KN92 had no effect on TT-induced responses in the PB or soluble mode of delivery (not shown).

To more fully characterize the reduction in DC maturation, which accompanied inhibition of CamK II, we determined the

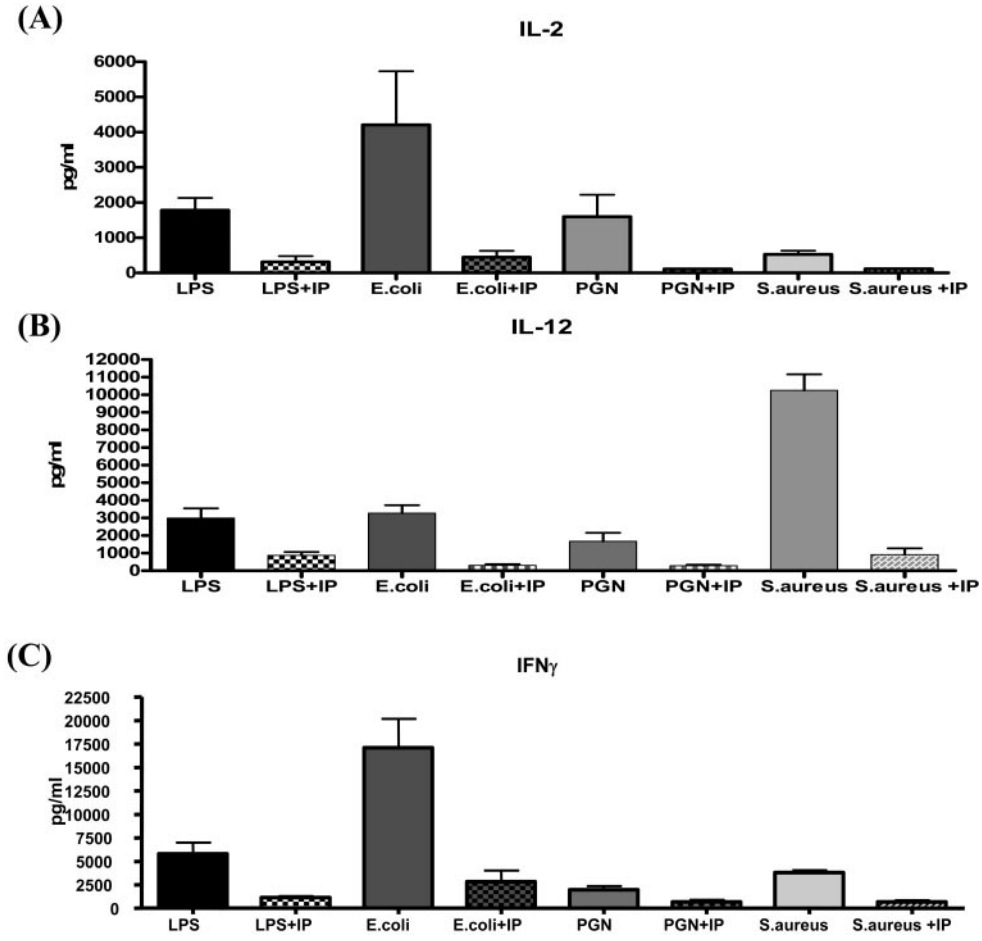


**Fig. 3.** CamK II regulates cytokine secretion induced by bacteria or their purified components. Primary, human, immature DC (A–C) were incubated with buffer control or 1 μM IP for 2 h. Cells were washed and incubated with LPS from *E. coli* (100 ng/ml), intact *E. coli*, PGN from *S. aureus* (10 μg/ml), or intact *S. aureus*. For the bacteria, the multiplicity of infection was 10:1. Supernatants were analyzed for (A) IL-2, (B) IL-12, or (C) IFN-γ via ELISA at 24 h. Results are the mean (±SEM) of two independent experiments, each performed in duplicate, using cells from two different donors. In all panels, the differences between cells treated with IP versus control cells that received no inhibitor are statistically significant ($P<0.05$).

level of endocytosis of FITC-dextran by fluorescence-activated cell sorter (FACS). Immature DC are characterized by a high rate of endocytosis, which rapidly decreases during maturation [1, 5, 7, 31]. We sought to determine whether the level of endocytosis exhibited by CamK II-inhibited cells more closely resembled that of immature versus mature DC. Following stimulation by TT, CamK II-inhibited cells exhibit a reduced rate of endocytosis of FITC-dextran, which was similar to that of control, matured DC, and much less than the rate of endocytosis in immature DC (Fig. 4D). Taken together with the results of studies on cytokine secretion and surface up-regulation of MHC class II and costimulatory molecules, these data are consistent with a model in which inhibition of CamK II reduces the extent of DC maturation but does not cause a restoration of the immature phenotype.

Although the inhibitors of CamK II have been characterized extensively [24, 29, 30], it was important to determine whether the inhibition of DC maturation induced by reductions in CamK II activity was a result of a toxic effect of the CamK II inhibitors. We used FACS analysis with annexin V (AV) and propidium iodide (PI) to determine the effects of CamK II inhibition on the viability of DC. At 24 h following stimulation, the percentage of cells positive for AV and PI, consistent with late apoptosis or cell death, was: control immature DC (1.5±1.0%), A23187 (21.4±2.4%), PB-TT (19.7±1.3%), IP → PB-TT (9.6±2.4%), KN62 → PB-TT (12.2±1.6%). These data indicate that the CamK II inhibitors did not cause an increase in cell toxicity. On the contrary, the inhibitor-treated cells exhibited decreased cell death. Quantitation of cell viability by exclusion of trypan blue yielded similar results (not shown). Taken together, these data are consistent with the hypothesis that inhibition of CamK II decreases activation-induced cell death in human DC. However, additional studies will be required to further characterize the effects of CamK II inhibition on DC viability, including potential effects on apoptotic versus necrotic cell death.

## Inhibition of CamK II affects the up-regulation and trafficking of MHC class II molecules to the surface of human DC

The maturation of DC is associated with synthesis of new MHC class II molecules and the trafficking of peptide-loaded, intracellular MHC II to the plasma membrane for interaction with TCRs [37, 38]. This developmentally regulated synthesis and trafficking of MHC II are modulated by cytokines, including IL-12 and IFN-γ [32, 38, 39]. As CamK II regulates DC cytokine secretion and surface expression of MHC class II (Figs. 2–4), we evaluated the effects of the CamK II inhibitors on the subcellular localization of MHC class II.

Confocal microscopy of resting, immature DC demonstrated that the majority of MHC class II molecules was localized in an intracellular punctate distribution, with lower levels on the plasma membrane consistent with previous data (**Fig. 5**) [38, 40, 41]. Stimulation with PB-TT resulted in a rapid redistri-

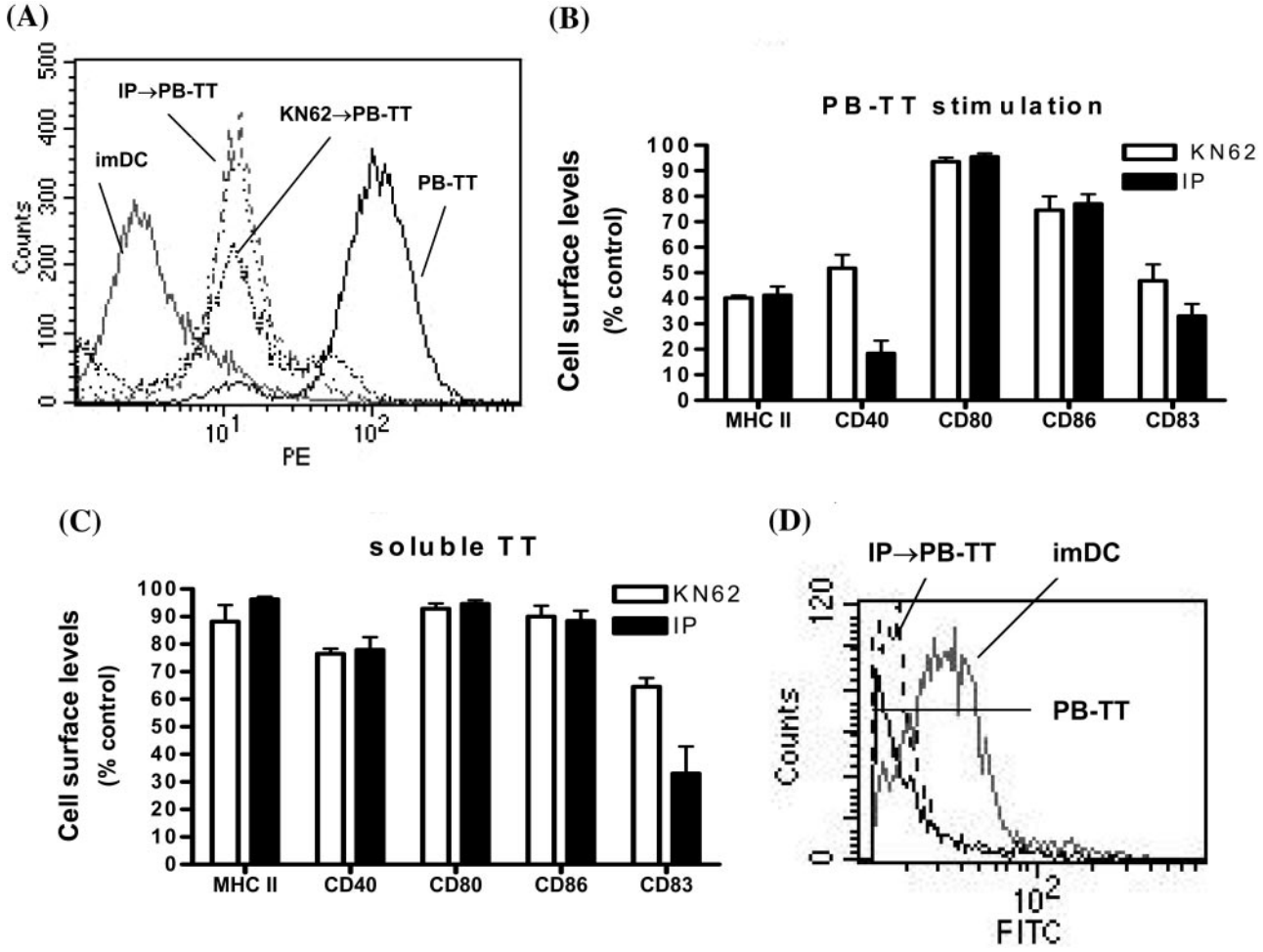


**Fig. 4.** CamK II regulates the surface expression of MHC class II and costimulatory molecules in primary DC. Primary, human, immature DC (imDC) were incubated with IP, KN62 (both at 1 μM), or buffer control for 2 h at 37°C, prior to incubation with (A, B, D) PB-TT or (C) soluble TT. Twenty-four hours after antigen exposure, the surface expression of MHC class II, CD40, CD80, CD83, and CD86 was determined by FACS. (A) The FACS histogram tracings for surface-expressed MHC class II. PE, Phycoerythrin. (B and C) The levels of surface expression in DC treated with the CamK II inhibitors are expressed as the percentage of control cells as defined by the M1 area. (D) The level of endocytosis of FITC-dextran was determined by flow cytometry in control immature DC cells, cells stimulated with PB-TT, and cells pretreated with IP followed by PB-TT. (A and D) Results are representative of five duplicate experiments. (B and C) The mean (±range) from five independent experiments, each performed in duplicate. DC were obtained from five different donors.

bution of MHC class II to the cell surface, which was evident within 1 h and essentially complete at 4 h. At 24 h after antigen exposure, MHC class II molecules were abundant on the cell surface and intracellularly, the latter consistent with stimulation-induced synthesis of nascent MHC class II, as demonstrated previously [42, 43]. Incubation of DC with IP, followed by PB-TT, resulted in a marked decrease in the level of total cellular MHC class II, including a significant reduction in surface expression (Fig. 5). At 24 h after stimulation with PB-TT, CamK II-inhibited cells contained intracellular MHC class II but had only low levels of staining at the cell surface, consistent with the FACS data presented in Figure 4. Addition of $Ca^{2+}$ ionophore (positive control) resulted in an increase in the levels of intracellular and plasma membrane MHC class II. These data support the hypothesis that CamK II regulates the level of cell-surface MHC class II molecules in DC. The data also indicate that inhibition of CamK II is associated with decreased cellular levels of MHC class II. However, further studies will be required to elucidate the specific contributions of changes in MHC class II synthesis and degradation to the net decrease in cellular levels associated with inhibition of CamK II.

## CamK II inhibitors reduce MHC class II-dependent antigen presentation

The central physiologic function of DC is the presentation of antigens to T cells. As inhibition of CamK II is associated with disruption of antigen-induced maturation, we hypothesized that CamK II regulates antigen presentation by human DC. To evaluate this hypothesis directly, we used SP-F3 T cells, which are a human MHC class II-restricted $CD4^+$ T cell clone that recognizes a universally presented epitope of TT [23, 25, 44, 45]. The degree of proliferation of SP-F3 T cells was used as the index of the efficacy of antigen presentation [25, 28]. Primary, immature, human DC were incubated with KN62, IP, or buffer for 2 h, washed, and then cultured with PB-TT and SP-F3 T cells ($5\times10^4$/assay) for 5 days. Inhibition of CamK II with KN62 or IP resulted in significant reductions in antigen-

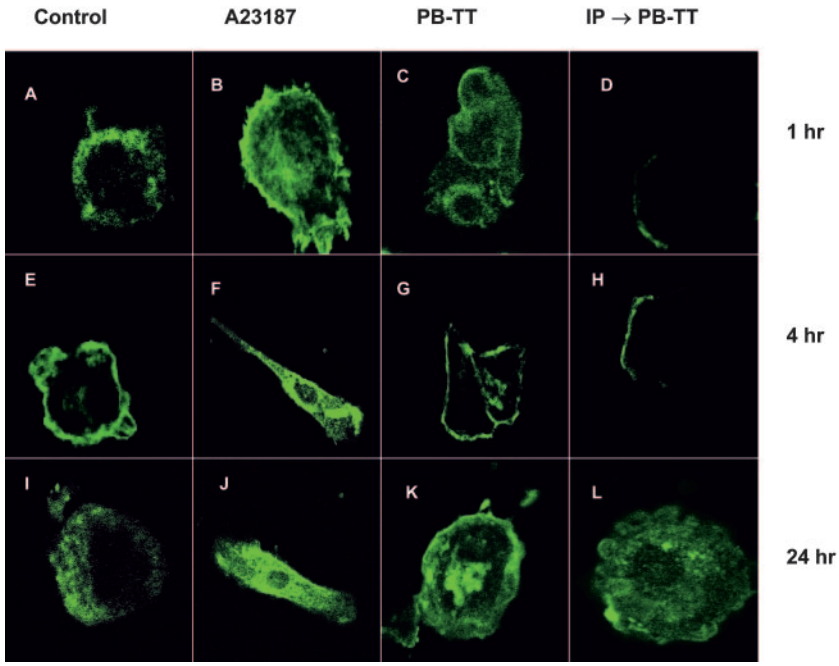


**Fig. 5.** Inhibition of CamK II is associated with decreased expression of MHC class II in human DC. Primary, immature, human DC were pretreated with media or 1 μM IP for 2 h. Cells were washed and incubated with buffer A23187 (10 μM) or PB-TT. Samples were fixed and permeabilized at 1 (A–D), 4 (E–H), or 24 (I–L) h, incubated with antibodies to MHC class II for 1 h, washed, and incubated with Oregon Green-conjugated 2° antibodies for 1 h. At least 25 cells were imaged by confocal microscopy for each condition, and data are representative of three independent experiments, using cells from different donors.

stimulated T cell proliferation ($P<0.01$, **Fig. 6A**). As with the majority of assays of DC function, IP was more efficacious than KN62, with a 70.2 ± 3.3% reduction of T cell proliferation noted at an IP concentration of 1.0 μM. Of note, cells were washed to remove unbound inhibitors prior to addition of T cells. In separate studies, neither of the CamK II inhibitors affected T cell viability (not shown).

The effect of CamK II inhibition on presentation of soluble TT by primary human DC was also evaluated. Preincubation with KN62 or IP resulted in 65–70% reductions in the level of T cell proliferation ($P<0.05$, Fig. 6B, left panel). When KG-1 cells were substituted for primary DC, KN62 and IP significantly inhibited the extent of T cell proliferation induced by soluble TT but to a lesser extent ($P<0.05$, Fig. 6B, right panel). These data are consistent with the hypothesis that CamK II regulates MHC class II-dependent presentation of particulate and soluble antigens by human DC. Further studies will be required to determine the relative contributions of inhibitor-induced reductions in cytokine production, MHC class II levels, and surface expression of costimulatory molecules to the observed decrease in T cell priming.

## DISCUSSION

DC use diverse signal transduction pathways to acquire, process, and present antigens to T cells and thus, bridge innate and adaptive immune mechanisms [1, 31, 37]. Elucidation of these regulatory pathways of DC maturation and functional activation may enable therapeutic modulation of the immune response in infectious diseases, cancer, autoimmunity, and transplantation. Several signaling components, including cyto-

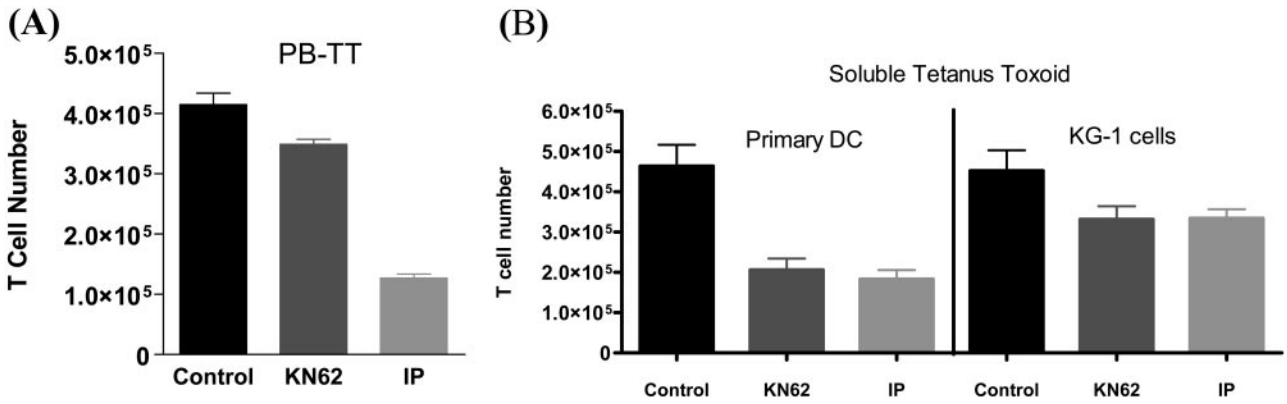


**Fig. 6.** Inhibition of CamK II blocks MHC class II-dependent antigen presentation. (A) Primary, immature, human DC were preincubated with KN62 (1 μM), IP (1 μM), or buffer for 2 h, washed, and then cultured with PB-TT and TT-specific human SP-F3 T cells ($5\times10^4$) for 5 days. T cell proliferation was determined with the CyQuant proliferation assay and used as the index of antigen presentation. (B) Primary DC (left panel) or KG-1 cells (right panel) were preincubated with KN62, IP, or buffer, as above, washed, and then cultured with soluble TT and SP-F3 T cells. Results are the mean (±SEM) from at least three independent experiments (performed in duplicate) for each condition, and three different donors were used to obtain primary DC. The reduction in T cell proliferation in samples treated with KN62 or IP is statistically significant compared with buffer-treated controls for PB-TT ($P<0.01$) or soluble TT ($P<0.05$).

solic $Ca^{2+}$, have been associated with the maturation of DC, but characterization of their specific targets and functional integration remains a challenging problem [2–7, 11–13, 46].

The data presented in this study support a novel role for CamK II, a key $Ca^{2+}$ signal-transducing enzyme, in regulating the maturation and function of human DC. First, antigen-induced stimulation of human DC was associated with the activation and cellular redistribution of CamK II. Activation of CamK II occurred within 15 min of antigen exposure and lasted for at least 24 h. Activated CamK II localized to phagosomes and to the plasma membrane of DC stimulated by particulate antigen. Second, inhibition of CamK II by two mechanistically distinct chemical inhibitors resulted in significant reductions in secretion of the critically important cytokines IL-12, IFN-γ, and IL-2. Third, inhibition of CamK II resulted in reduced surface expression of MHC class II and costimulatory molecules. The impact of CamK II inhibition was greater when DC were stimulated with PB antigen compared with soluble antigen. Fourth, inhibition of CamK II affected the total cellular levels of MHC class II molecules and their stimulation-induced trafficking to the plasma membrane. Fifth, CamK II inhibitors reduced the ability of human DC to stimulate MHC class II-dependent T cell proliferation.

It is likely that the significant role of CamK II in T cell priming is a summation of its effects on several discrete stages of the maturation and antigen presentation of DC. For example, up-regulation of surface costimulatory/antigen-presenting molecules and cytokine secretion contribute to autocrine- and paracrine-positive feedback loops, which promote antigen presentation and T cell activation [1, 31–36, 38]. Specifically relevant to this point, IL-12, IFN-γ, and IL-2 are integral to DC maturation and activation of naïve T cells. Furthermore, IL-12 and IFN-γ autocrine loops are required for DC to synthesize and secrete IL-2 [32, 34, 36]. The relative contribution of these individual stages or components of DC maturation and function to the magnitude and kinetics of antigen presentation is unknown, as is their specific dependence on CamK II. However, the marked effect of CamK II inhibition on responses to PB antigens suggests that regulation of vesicular trafficking and membrane fusion with phagosomes may underlie important functions of CamK II in DC. This hypothesis is consistent with the established roles for CamK II in phagosome maturation, granule secretion, and vacuolar trafficking in other cell types [8, 14, 16, 18, 29]. Further studies will be required to test the hypothesis that CamK II regulates vesicular traffic between phagosomes and the membrane compartments, which comprise the MHC class II system (endoplasmic reticulum, Golgi, MIIC, MIIV, and plasma membrane).

Our data do not specify which isoform(s) of CamK II (α, β, γ, δ) regulates the maturational and functional responses of DC. In fact, the identity of the CamK II isoforms that are present in DC remains unknown. This represents a significant limitation in knowledge, as studies in the nervous system demonstrate spatiotemporal specificity in the function of different isoforms, as well as diversity in coupling to upstream and downstream signaling pathways [14, 29]. In the absence of information regarding the spectrum of CamK II isoforms present in DC, the chemical inhibitors are a valuable alternative approach. KN62 exhibits a high degree of specificity for all isoforms of CamK II but has been demonstrated to also inhibit CamK IV [14, 18, 29, 30]. The lack of effect of the inactive isoquinolone analog KN92 on DC responses argues against nonspecific effects of KN62. The IP is highly specific for CamK II, consistent with its functioning as the AID of the intact enzyme [14, 29]. Similar specificity of AID has been established for other protein kinases, including protein kinase C [47]. However, definition of the responsible isoforms will be required for more specific evaluation of the proposed roles of CamK II in DC and determination of whether positive and negative modulation of the levels and enzymatic activities of individual isoforms will alter maturation and antigen presentation.

The data reported herein may be integrated into the growing body of knowledge concerning the regulation of DC function by specific signaling pathways. Several groups of investigators have reported that MAPK of the ERK and p38 families play important roles in the maturation and functional responses of DC [2–4, 46]. These data are complemented by recent work in other cellular systems, including endothelial cells and smooth muscle cells, which indicate that CamK II functions to regulate these specific families of MAPK [48–53]. In this context, our data support the hypothesis that the integral role for CamK II in DC maturation and antigen presentation derives, at least in part, via its regulation of ERK and p38. Definition of the CamK II isoforms present in DC will be required to characterize their coupling to specific signal transduction components in the regulation of DC maturation and function.

It addition to its functional role in DC physiology, CamK II may also represent a pathologic focus in human diseases. For example, *M. tuberculosis* inhibits CamK II-dependent phagosome maturation in infected macrophages [16, 18]. If mycobacteria also inhibit CamK II activation in DC, this may contribute to the pathologic defects in cytokine secretion and MHC class II-dependent T cell activation, which have been documented in tuberculosis [54, 55]. Alterations in host $Ca^{2+}$-mediated signal transduction have also been noted in infectious diseases caused by *Trypanosoma cruzi* and *Histoplasma capsulatum* [56, 57]. Whether pathologic changes in CamK II activation or its dependent effector responses occur in these diseases is unknown.

In summary, these studies support a model in which CamK II functions as a novel regulator of the innate/adaptive immune interface. Recently, a DC-specific, natural inhibitory protein to CamK II was identified, further supporting the complex modes of regulation of this critical enzyme [20, 58, 59]. These data suggest that specific targeting of CamK II in human DC may provide a mechanism to therapeutically regulate the immune response.

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