Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cellular Signalling 15 (2003) 751–762
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

T cell receptor-independent CD4 signalling: CD4–MHC class II interactions regulate intracellular calcium and cyclic AMP

Wenhong Zhou[a], Rolf König[a,b,*]

[a] Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, TX 77555-1070, USA
[b] Sealy Center for Molecular Science, The University of Texas Medical Branch, Galveston, TX 77555-1070, USA

Received 11 October 2002; accepted 21 January 2003

Abstract

CD4 is a coreceptor on T helper (Th) cells that interacts with MHC class II molecules (MHCII). The mechanisms mediating the effects of CD4 on responses by T helper cells to stimulation of the antigen-specific T cell receptor (TCR) are still poorly understood. Here, we demonstrate T cell costimulation via CD4 signalling independent of T cell receptor-mediated signals. Incubation of T helper cells with peptide mimetics of the CD4-binding region on the MHC class II β2 domain caused intracellular calcium mobilization in the absence of antigen or other T cell receptor stimuli. Engagement of CD4 by peptide mimetics or wild-type MHC class II, but not by mutant MHC class II molecules incapable of engaging CD4, inhibited the T cell receptor-mediated increase in cyclic AMP (cAMP) concentrations in T helper cells. CD4-mediated signals activated cyclic AMP phosphodiesterases (PDEs) and inhibited adenylyl cyclase. Full activation and clonal expansion of antigen-stimulated T helper cells required the CD4-mediated regulation of cyclic AMP. Our results suggest a costimulatory mechanism of CD4 function that acts on the second messengers, calcium and cyclic AMP.

Keywords: T lymphocytes; Activation; Signal transduction; Phosphodiesterase; Adenylyl cyclas

1. Introduction

T helper (Th) cells regulate adaptive immune responses by promoting and directing the differentiation and activation of B cells and cytotoxic $CD8^+$ T cells [1,2]. Activation of Th cells is critical for the clearance of pathogenic infections [3,4] and for the destruction of tumor cells [5]. Th cells also participate in the pathogenesis of many autoimmune disorders [6,7] and in transplant rejection [8]. Th cells recognize antigen (Ag) in the context of MHC class II molecules (MHCII) and express the CD4 coreceptor on their cell surface [9], which interacts with nonpolymorphic regions of MHCII [10,11]. The association of the Ag-specific T cell receptor (TCR) and CD4 with the same MHCII during Ag stimulation initiates Th cell activation [12].

One of the earliest Ag-induced signalling events is the mobilization of calcium ions (Ca^{2+}), which is essential for Th cell activation [13]. This second messenger activates several cytosolic enzymes, initiating downstream signalling cascades [14]. However, Ag stimulation also generates signals through the TCR that antagonize Th cell activation. For example, TCR-mediated signals activate the cyclic AMP (cAMP)-dependent protein kinase A (PKA) [15,16]. PKA initiates a signalling pathway that inhibits Ag-induced T cell proliferation and cytokine production [17,18]. Therefore, to achieve full activation of Th cells, TCR-mediated signals must be modified.

Interactions between CD4 and MHCII increase Ag-induced Th cell proliferation and cytokine production [19,20]. Initially, it was thought that CD4 functioned as an adhesion molecule, enhancing contact between Th cells and Ag-presenting cells (APCs) [9]. However, CD4 and MHCII interact with extremely low affinity [21,22], and soluble CD4

Abbreviations: Ag, antigen; APC, antigen-presenting cell; Ca^{2+}, calcium ions; cAMP, cyclic AMP; CD4-IP, CD4-interacting peptide; DMSO, dimethylsulfoxide; FACS, fluorescence-activated cell sort; FITC, fluorescein isothiocyanate; iPDE, inhibitor of phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; MHCII, MHC class II molecules; Ova323, ovalbumin peptide 323–339; OvaT, ovalbumin peptides derived by trypsin treatment; PDE, phosphodiesterase; PHA, phytohemagglutinin; PI3, phosphoinositide 3; PKA, protein kinase A; RPE, R-phycoerythrin; TCA, trichloroacetic acid; Th, T helper; TCR, T cell receptor.

* Corresponding author. Department of Microbiology and Immunology, The University of Texas Medical Branch, Medical Research Building, Room 3.142H, Mechanic and 11th Street, Mail Route 1070, Galveston, TX 77555-1070, USA. Tel.: +1-409-747-0395; +fax: +1-409-747-6869.
E-mail address: rokonig@utmb.edu (R. König).

0898-6568/03/$ - see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0898-6568(03)00037-8

does not affect the binding of soluble MHCII·Ag complexes to immobilized TCR [22]. Furthermore, CD4·MHCII interactions do not increase the binding avidity between Th cells and APCs [23,24]. Thus, an adhesive effect of CD4 is unlikely and the major function of CD4 may be its active participation in Th cell signalling [25,26].

We used two approaches to determine whether CD4 can transduce signals independent of the Ag-induced TCR signal to modulate the Th cell responses. First, we stimulated $CD4^+$ T cells with Ag presented by either wild-type MHCII or MHCII mutated in the CD4-binding site of the β2 domain and thus incapable of interacting with CD4 [10]. For the second approach, we synthesized a peptide corresponding to the major CD4-binding site on murine MHCII. This CD4-interacting peptide (CD4-IP) consists of amino acid residues 134–148 of the $I\text{-}A^d$ β2 domain. This peptide binds to $CD4^+$ T cells and modulates Th cell-dependent immune responses [27,28]. The peptide also binds to soluble CD4 [29]. Here, we show that incubation of $CD4^+$ Th cells with CD4-IP mobilized Ca^{2+} and inhibited forskolin- and TCR-induced increases in intracellular cAMP concentrations ($[cAMP]_i$) by activating cAMP phosphodiesterases (PDEs) and inhibiting adenylyl cyclase. Full activation of normal $CD4^+$ T lymphocytes depended on regulation of cAMP. Thus, we define CD4-mediated, TCR-independent signalling pathways that are required for complete Th cell activation, and suggest a costimulatory function of CD4 that acts on two second messenger systems, Ca^{2+} and cAMP.

2. Materials and methods

2.1. Cell lines, T cell clones, and primary T lymphocytes

All media and supplements were from Life Technologies (Gaithersburg, MD) and reagents from Sigma (St. Louis, MO), unless otherwise stated. Murine fibroblast L cells transfected with cDNAs encoding wild-type or mutant A^d_β, wild-type A^d_α, ICAM-1, and B7-1 were cultured in complete DMEM [30]. The Th1 clone, pGL2 [31], was cultured by weekly restimulating the cells with chicken ovalbumin (200 μg/ml) and irradiated (2000 rad) BALB/c splenocytes in DMEM containing 5% foetal calf serum (HyClone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM Hepes, 50 μM 2-mercaptoethanol, 10 μg/ml gentamycin, and human rIL-2 (20 U/ml added 2 days after Ag and APCs). Seven days after restimulation, pGL2 cells were harvested and purified by density gradient centrifugation on Lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada). Primary lymphocytes from the axillary, brachial, cervical, inguinal, popliteal, and mesenteric lymph nodes of DO.11.10 TCR transgenic mice were enriched for the expression of the CD4 coreceptor by antibody-mediated depletion of $CD8^+$ and $MHCII^+$ cells [27]. After depletion, more than 95% of the cells were $CD4^+$ as determined by fluorescence-activated cell sort (FACS) analysis.

2.2. Antibodies and reagents

Monoclonal antibodies against mouse CD4 (clone GK1.5, rat IgG2b), mouse CD8 (clone 2.43, rat IgG2b), $I\text{-}A^d$ (clone M5/114, rat IgG2b), and mouse CD3ε (clone 145-2C11, hamster IgG) were purified from hybridoma supernatants by protein G sepharose chromatography (Sigma). Goat anti-hamster (GAH) IgG was from Caltag (Burlingame, CA). The PDE inhibitors (iPDEs) vinpocetine, rolipram, and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma. The adenylyl cyclase inhibitor MDL-12,330A [32] was from Calbiochem (La Jolla, CA). All drugs were dissolved in dimethylsulfoxide (DMSO) and stored at −20 °C. Biotin and fluorescein isothiocyanate (FITC) were from Sigma. Streptavidin–R-phycoerythrin (RPE) was from Caltag. Peptides were synthesized by the Protein Chemistry Laboratory of the Sealy Center for Molecular Science at UTMB (Galveston, TX) and purified by HPLC. Sequence of CD4-IP: NGQEETVGVSSTQLI, control peptide: SELIQTSVEGQTVGN, and Ova323: ISQAVHAAHAEINEAGR. Purity and composition were confirmed using amino acid compositional analysis and mass spectral analysis by plasma desorption ionization.

2.3. Measurements of $[Ca^{2+}]_i$

Resting pGL2 cells were incubated at 3×10^6 cells/ml at 37 °C with 3 μM of Fura-2 acetoxy methylester (Fura-2 AM; Molecular Probes, Eugene, OR) for 30 min. After loading, cells were washed twice and resuspended in buffer (140 mM NaCl, 5 mM KCl, 20 mM Hepes, 10 mM Glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH = 7.4). Transfected APCs were pre-incubated for 2 h with peptides derived by trypsin treatment of ovalbumin (OvaT). Fura-2-loaded pGL2 cells were incubated with APC·OvaT for 2.5 min before starting $[Ca^{2+}]_i$ measurements. Alternatively, resting Fura-2-loaded pGL2 cells were incubated with various concentrations of CD4-IP or control peptide for 1 min. Fluorescence of pGL2 cells was monitored in an SLM 4800S modulation spectrofluorometer with DMX dual wavelength excitation monochromator at 15-s intervals. Excitation wave lengths of 340 and 380 nm, and an emission wave length of 510 nm were used. During measurements, cells were kept at 37 °C and continuously stirred. $[Ca^{2+}]_i$ was determined by the ratio method with correction for background fluorescence using the following formula: $[Ca^{2+}]_i = K_d\ [(R - R_{min})/(R_{max} - R)] \times F380_{max}/F380_{min}$. The $K_d = 1303$ nM was determined by intracellular calibration following established protocols [33].

2.4. Measurement of conjugate formation between Th1 clone pGl2 cells and APCs expressing mutant or wild-type MHCII

APCs were collected by trypsinization and washed with PBS (pH = 7.4). The 10×10^6 APCs were resuspended in

0.5 ml of PBS (pH = 8.0). APCs were incubated with 10 μl/ml of biotin at room temperature for 25 min. Cells were washed with 8.0 ml of complete DMEM, followed by a wash with 8 ml of PBS (pH = 7.4). APCs were then resuspended in 0.5 ml of PBS (pH = 7.4) and 10 μg of streptavidin–RPE was added to the cell suspension. Cells were protected from light and incubated at room temperature for 30 min. The APCs were then washed twice with complete DMEM. The 2×10^6 APCs were resuspended in 0.8 ml of complete DMEM and incubated with 160 μg of OvaT at 37 °C and 10% CO_2 for 2 h. After washing, APCs were resuspended in 0.2 ml of complete DMEM.

pGL2 cells were suspended at 2×10^6 /ml in 6 ml of PBS (pH = 7.4). A total of 60 μl FITC (1 mM in ethanol) was added and cells were incubated at 37 °C for 10 min, protected from light. pGL2 cells were then washed twice and resuspended in complete DMEM at 10×10^6/ml.

pGL2 cells (1×10^6 in 100 μl) and APCs (3.3×10^5 in 33 μl) were mixed and incubated at room temperature for 30 min. PBS (0.5 ml) containing 2% NCS, 1 mM $CaCl_2$, and 0.05% NaN_3 was added, and cells were subjected to FACS analysis. Data were collected on a Becton Dickinson FACScan and analysed using CellQuest software.

2.5. T cell activation for cAMP and adenylyl cyclase activity measurements

T cells were incubated as described in the figure legends. Incubations were terminated by adding ice-cold PBS to the cells. Cells were then transferred into 2-ml microcentrifuge tubes and cytoplasmic extracts prepared. For Ag stimulation, transfected DAP.3 APCs (10^6 cells/well) were plated into 24-well plates and allowed to form a monolayer during overnight incubation. APCs were then incubated with OvaT (200 μg/ml) for 2 h. Contact between APCs and pGL2 cells was initiated by centrifugation of the plates at 1500 rpm for 30 s. Then, the plates were placed into an incubator at 37 °C and 10% CO_2 for various times. Ice-cold PBS (1 ml) was added into the wells to terminate the incubation. Cells were transferred into 2-ml microcentrifuge tubes and immediately cooled on ice. FACS analysis after staining with monoclonal anti-MHCII and anti-CD4 antibodies showed that more than 95% of the cells were $CD4^+$ T cells (data not shown).

2.6. Preparation of cytoplasmic extracts

For cAMP measurements, T cells were lysed by adding 500 μl of ice-cold 10% (w/v) trichloroacetic acid (TCA) and vortexing. After a brief incubation on ice for 15 min, samples were centrifuged at $3000 \times g$ and 4 °C for 15 min. For adenylyl cyclase activity assays, pGL2 cells were incubated with medium containing 1 mM IBMX and 5 μCi/ml [^{3}H]adenine for 150 min at 37 °C followed by washing twice. Cells were lysed in 1 ml of ice-cold 5% TCA containing 1 mM cAMP and 1 mM ATP. For both cAMP measurement and adenylyl cyclase activity assay, supernatants were aliquoted and stored at −80 °C. TCA-precipitated proteins were dissolved in 500 μl of 1 N NaOH and used to determine protein concentrations.

2.7. Measurements of protein concentrations

Protein concentrations in cellular extracts were determined by the Bradford method [34], using bovine serum albumin as a standard. Briefly, cellular extracts were diluted with 0.15 M NaCl into 96-well plates. All dilutions were carried out in triplicate. Then, 100 μl of Coomassie Brilliant Blue was added to the wells. After 5 min, the plates were measured in a Dynatech MR 5000 Microtiter plate reader (Dynatech, Chantilly, VA).

2.8. Measurement of intracellular cAMP concentrations

cAMP was measured in cellular extracts using a radioimmunoassay (NEN™ Life Science Products, Boston, MA) according to the manufacturer's instructions. Cellular extracts were diluted to equal protein concentrations.

2.9. Adenylyl cyclase activity assay

The method was previously described [35]. Briefly, Dowex AG50 WX4 resin (BioRad, Hercules, CA) was equilibrated with 10 ml of water and alumina WN-3 resin (Sigma) was equilibrated with 8 ml of 100 mM imidazole–HCl (pH 7.5). Lysates containing equal amounts of protein were adjusted with water to 1 ml and poured into Dowex columns ($d \times H = 8 \times 25$ mm). The flow-through volumes were collected into scintillation vials (vial series A). Dowex columns were washed with 3 ml of water and eluates collected into vial series A. Another 8 ml of water was added to the Dowex columns and these eluates were poured into alumina columns ($d \times H = 8 \times 15$ mm). Flow-through volumes were discarded and alumina columns washed with 5.5 ml of 0.1 M imidazole–HCl (pH = 7.5). Eluates were collected into scintillation vials (vial series B). Adenylyl cyclase activity was determined as the ratio of radioactivity of vial B to vial A (ratio B/A). Dowex columns were re-equilibrated by washing with 2 ml of 1 N HCl and 8 ml of water. Alumina columns were re-equilibrated by washing twice with 8 ml 0.1 M imidazole–HCl (pH = 7.5).

2.10. T cell proliferation assays

$CD4^+$ T cells from DO.11.10 TCR-transgenic mice (1.5×10^5) were incubated in a total volume of 200 μl of media in the wells of a 96-well plate either with or without APCs (5×10^4). APCs were L cell fibroblasts transfected with either wild-type or mutant MHCII together with ICAM-1 and B7-1 [30]. When indicated, Ova323 was added to a final concentration of 39 nM. After 72 h, 1 μCi of [^{3}H]thymidine was added and the incubation was continued for 18 h. Cells were then harvested using a 96-well harvester

(Packard, Downers Grove, IL) and [^{3}H]thymidine incorporation was determined with a Packard MatrixTM 9600 Direct Beta Counter (counting efficiency ≈ 6%).

2.11. IL-2 assay

IL-2 concentrations were measured in 24-h cell culture supernatants by ELISA (Pharmingen, San Diego, CA).

2.12. Statistical tests

Statistical significance of differences between samples was tested using Student's unpaired *t*-test. Data are expressed as mean value ± S.D.

3. Results

3.1. Synergy between CD4 and TCR signals increases mobilization of Ca^{2+} during Ag activation

We previously characterized mutant MHCII carrying single amino acid substitutions in the β2 domain that are deficient in their ability to interact with CD4, but remain fully competent to bind Ag and interact with the TCR [10]. Therefore, we stimulated the $CD4^+$ Th1 clone, pGL2 [31], with ovalbumin-derived peptides (OvaT) presented by murine fibroblast L cell lines that expressed either wild-type MHCII cDNA or mutant MHCII cDNA encoding alanine at positions 137 and 142 instead of glutamic acid and valine, respectively. Both L cell lines also expressed cDNAs encoding ICAM-1 and B7-1, rendering them efficient APCs [30]. We used Ag at limiting concentrations that induced submaximal responses in pGL2 cells stimulated with wild-type MHCII·Ag (e.g., proliferation and IL-2 secretion; data not shown). We then measured cytosolic-free Ca^{2+} concentrations ($[Ca^{2+}]_i$) by spectrofluorometry.

When we stimulated pGL2 cells with wild-type MHCII-·OvaT, Ca^{2+} mobilization occurred more rapidly and $[Ca^{2+}]_i$ rose to a higher level than in pGL2 cells stimulated with mutant MHCII·OvaT (Fig. 1a). This experiment did not rule out the possibility that the increased Ca^{2+} mobilization was simply due to enhanced contact between the T cells and APCs mediated by CD4·MHCII interactions, thus intensifying and prolonging signals transduced via the TCR.

To test whether CD4 exerted its effect by improving contacts between Th cells and APCs, we measured conjugate formation between Th cells and Ag-loaded wild-type MHCII-expressing APCs or mutant MHCII-expressing APCs. We labelled Th cells with FITC and APCs with RPE. After incubation, we measured conjugate formation by FACS analysis. Fewer conjugates formed between wild-type MHCII-expressing APCs and $CD4^+$ Th cells than did between mutant MHCII-expressing APCs and $CD4^+$ Th cells (Fig. 2). The finding that pGL2 cells, which represent a memory Th cell population, formed fewer conjugates with

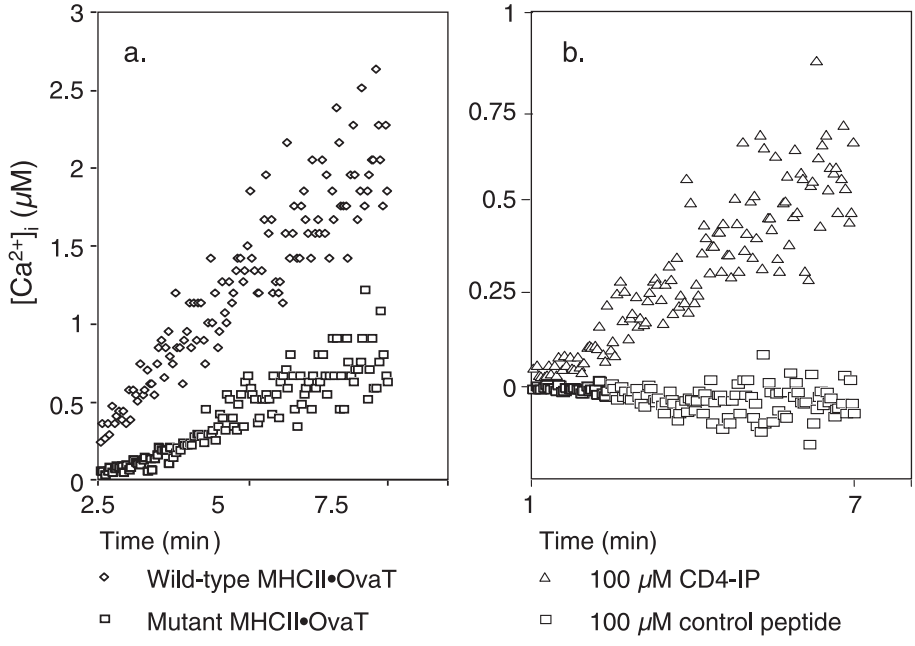

Fig. 1. CD4-mediated Ca^{2+} mobilization. (a) Effect of ovalbumin peptides presented by either wild-type or mutant MHCII-expressing APCs on $[Ca^{2+}]_i$ in the Th1 cell clone, pGL2. Fura-2 AM-loaded pGL2 cells were incubated with Ag-loaded APCs expressing either wild-type or mutant MHCII in a 2:1 ratio for 150 s. Then, fluorescence of pGL2 cells was monitored in a spectrofluorometer at 15-s intervals. The data are representative of two independent experiments. (b) Effect of CD4-IP on $[Ca^{2+}]_i$ in pGL2 cells. Fura-2 AM-loaded pGL2 cells were incubated with 100 μM of control peptide or CD4-IP for 1 min. The control peptide had the same amino acid composition as the CD4-IP, but its sequence was scrambled. The data are representative of three independent experiments. Similar experiments were performed with 8 and 50 μM of peptide. At lower concentrations of CD4-IP, but not of the control peptide, a dose-dependent stimulation of Ca^{2+} mobilization occurred (not shown).

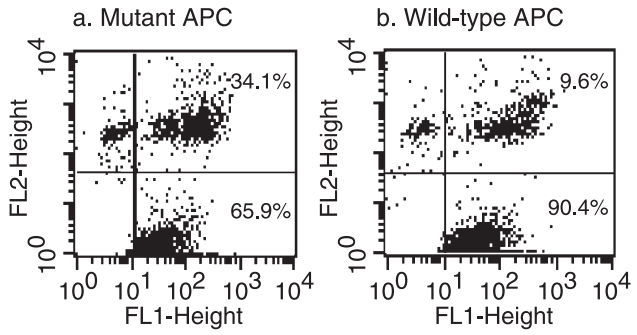

Fig. 2. Conjugate formation between Th1 clone pGL2 cells and APCs expressing mutant or wild-type MHCII. pGL2 cells were labeled with FITC (FL1). Mutant MHCII-expressing APCs (a) and wild-type MHCII-expressing APCs (b) were labeled with RPE (FL2) and loaded with Ag (OvaT). pGL2 cells and APCs were mixed in a 3:1 ratio and incubated at room temperature for 30 min before FACS analysis. When incubated with mutant MHCII-expressing APCs, 34% of the pGL2 cells conjugated to APCs, but only 9.6% of the pGL2 cells formed conjugates with wild-type MHCII-expressing APCs lasting longer than 30 min. The data are representative of five independent experiments. Percentages of APC-conjugated and free $CD4^+$ T cells are given.

wild-type MHCII-expressing APCs than with mutant MHCII-expressing APCs at limiting Ag concentrations is consistent with previous reports that memory, but not naïve, $CD4^+$ T cells rapidly deconjugate from APCs upon CD4 engagement by MHCII in the absence of Ag [23]. Due to limiting Ag concentrations in our experiments, some $CD4^+$ T cells interacted with MHCII molecules in the absence of TCR·MHCII engagement, thereby inducing conjugate dissociation. Thus, the enhancing effect of CD4 on Th cell signalling was not mediated through enhanced adhesion of the $CD4^+$ Th cell population to wild-type MHCII-expressing APCs. Therefore, we tested the possibility that engagement by MHCII induces CD4-mediated signals and that CD4-mediated signals modulate TCR-induced Ca^{2+} mobilization.

In previous experiments, we demonstrated that CD4-IP, the peptide mimetic corresponding to the major CD4-binding site on the $I\text{-}A^d$ β2 domain, modulates Ag-induced responses by Th cells in vitro and in vivo [27]. CD4-IP binds to $CD4^+$, but not to $CD4^-$, T cell hybridomas, and low concentrations of CD4-IP potentiate immune responses [27], suggesting that interactions between CD4 and MHCII may induce signals via CD4 that increase T cell responses to Ag. To test this hypothesis, we incubated pGL2 cells with CD4-IP and then monitored $[Ca^{2+}]_i$.

Incubation with CD4-IP at concentrations ranging from 8 to 100 μM, but not with a control peptide of identical amino acid composition in a scrambled sequence, induced Ca^{2+} mobilization in resting pGL2 cells in a dose-dependent manner (Fig. 1b; shown only for 100 μM). This effect occurred in the absence of Ag and APCs. Thus, a peptide corresponding to the CD4-binding region on MHCII induced signals via CD4. Therefore, CD4 displayed a TCR-independent signalling capacity. Mobilization of Ca^{2+} is essential for T cell activation [36,37]. Therefore, CD4-mediated modulation of the kinetics of intracellular Ca^{2+} mobilization and $[Ca^{2+}]_i$ may provide an important regulatory mechanism to promote Th cell activation.

3.2. Treatment with CD4-IP antagonizes induction of cAMP by forskolin

We demonstrated a TCR-independent, CD4-mediated increase in Ca^{2+} mobilization (Fig. 1b). TCR signalling without coreceptor engagement also increases $[Ca^{2+}]_i$ [36,38]. Therefore, increasing Ag concentrations should substitute for the requirement of coreceptor engagement if activating the Ca^{2+} signalling pathway was the only function of CD4-mediated signals. However, CD4 engagement is required for efficient cellular proliferation and cytokine production. For example, the lack of CD4 engagement by MHCII markedly impairs Th cell activation by peptide ligands, indicating a requirement for CD4-mediated signals to activate successfully Th cells [10,11,39]. This suggests that engagement of MHCII activates other CD4-mediated signalling pathways in addition to the Ca^{2+} signal. TCR signalling activates type I PKA [16], a PKA isozyme that mediates cAMP's inhibitory effects on T cell replication [15,18]. Therefore, we hypothesized that CD4-mediated signals may serve to inhibit PKA I activity during Th cell activation. One mechanism by which PKA I could be inhibited is by reducing $[cAMP]_i$.

Forskolin is a pharmacological reagent that activates adenylyl cyclases, which catalyze the conversion of ATP into cAMP [40]. We first measured the effect of various concentrations of forskolin on $[cAMP]_i$. In primary, $CD4^+$ T lymphocytes from DO.11.10 TCR transgenic mice, incubation with forskolin increased $[cAMP]_i$ in a dose-dependent fashion (Fig. 3a). Similarly, forskolin increased $[cAMP]_i$ in pGL2 cells. However, these cells were less sensitive than the primary T cells (Fig. 3b).

We then determined the effect of CD4-mediated signalling on increases in $[cAMP]_i$ induced by moderate doses of forskolin (10 μM for DO.11.10 primary T cells and 25 μM for pGL2 cells). We incubated DO.11.10 primary $CD4^+$ T cells or pGL2 cells either without peptide, or with CD4-IP or the control peptide before treatment with forskolin. After various incubation periods, we harvested and lysed the cells and measured $[cAMP]_i$. We observed a significant block in the upregulation of cAMP in both DO.11.10 primary (Fig. 3c) and in pGL2 T cells (Fig. 4) pretreated with CD4-IP, but not with the control peptide. Therefore, signalling through CD4 may negatively regulate $[cAMP]_i$ in Th cells.

3.3. CD4-mediated signalling decreases [cAMP]i in Ag-stimulated Th cells

The inhibitory effect of CD4-mediated signalling on the forskolin-induced increase in $[cAMP]_i$ suggested that during Ag activation, a function of the CD4 coreceptor may be to decrease $[cAMP]_i$ in Th cells. We used two approaches to

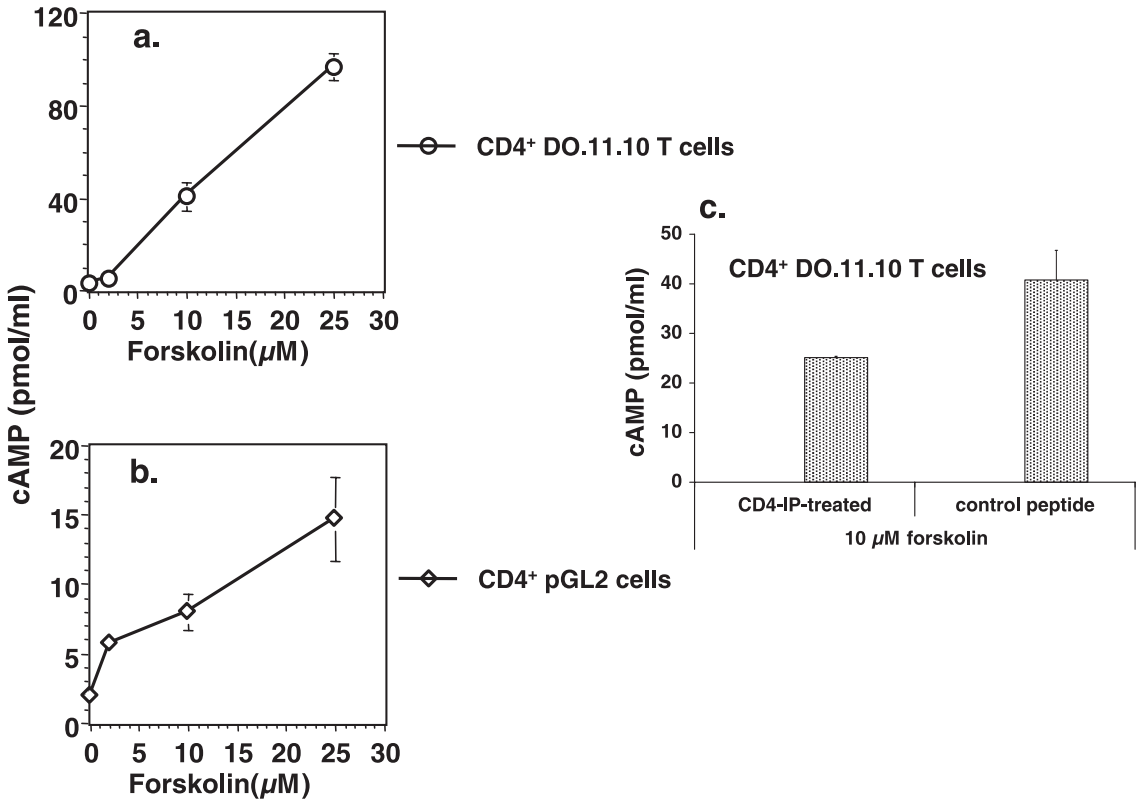

Fig. 3. Forskolin increases $[cAMP]_i$ in DO.11.10, primary $CD4^+$ T cells and in pGL2 cells in dose-dependent fashion. (a) Primary DO.11.10 $CD4^+$ T cells or (b) pGL2 cells were incubated with various concentrations of forskolin for 10 min at 37 °C. Then, cells were harvested and lysed, and samples adjusted to contain equal amounts of cellular protein. $[cAMP]_i$ was measured with a radioimmunoassay. Separately, DO.11.10 $CD4^+$ T cells were also preincubated with 100 μM of CD4-IP or control peptide for 40 min before the addition of 10 μM of forskolin and processing for cAMP measurements (c).

test this hypothesis. First, we stimulated pGL2 cells with OvaT presented by mutant or wild-type MHCII-expressing APCs. After 4 and 8 h of incubation, $[cAMP]_i$ in pGL2 cells activated by wild-type MHCII·OvaT was much lower than in pGL2 cells activated by mutant MHCII·OvaT (Fig. 5a). Cyclic AMP concentratons in pGL2 cells activated with either mutant or wild-type MHCII·OvaT returned to base levels within 20 h.

In the second approach, we stimulated pGL2 cells with OvaT presented by mutant MHCII-expressing APCs in the presence of CD4-IP or the control peptide. After 8 h of incubation, we measured $[cAMP]_i$. CD4-IP significantly suppressed the mutant MHCII·OvaT-induced increase in $[cAMP]_i$ (Fig. 5b). Thus, CD4-mediated signalling prevented an increase in $[cAMP]_i$ during Ag activation of Th cells.

In T cells, the production of cAMP is regulated by adenylyl cyclase and the degradation rate is determined by PDEs. We reasoned that the effect of CD4 signalling on $[cAMP]_i$ could be mediated by regulation of either type of enzyme. Therefore, we analysed the effects of CD4 signalling on the activities of both enzymes.

3.4. CD4-IP activates cAMP phosphodiesterases

Thus far, 11 families of PDEs have been classified by their primary sequences, substrate specificities, susceptibility to selective inhibitors, and tissue localization [41,42]. Isozymes of the PDE1, PDE3, PDE4, and PDE7 families have been found in T cells [43–45]. Cross-linking of CD28 activates PDE7, a requirement for T cell proliferation and cytokine production following ligation of CD3 [46]. However, it is unknown how other PDEs in T cells are activated and whether CD4 participates in the regulation of PDEs during T cell stimulation.

We focused our investigation on members of the PDE1 and PDE4 families. PDE1 is activated via the Ca^{2+}/calmodulin-dependent pathway, which can be regulated by CD4 (Fig. 1). PDE4 is the predominant cAMP-metabolizing PDE in the cytosol of T cells [43,47] and phosphoinositide 3 (PI3)-kinase participates in PDE4 activation [48]. Binding of CD4 ligands, including CD4-IP, activates PI3-kinase in a $p56^{lck}$-dependent manner [49]. Hence, we tested whether specific inhibitors of PDE1 (iPDE1) and PDE4 (iPDE4) can abrogate the inhibitory effect of CD4-IP on cAMP upregulation by forskolin. We incubated pGL2 cells with vinpocetine and rolipram, specific for PDE1 and PDE4, respectively, in the presence or absence of CD4-IP for 30 min. We then added forskolin to the cells and measured $[cAMP]_i$ 10 min thereafter. We used the iPDEs at concentrations that completely abolished PDE1 and PDE4 activities [45,47], but did not affect viability of cells (data not shown). Treatment with CD4-IP did not block the increase

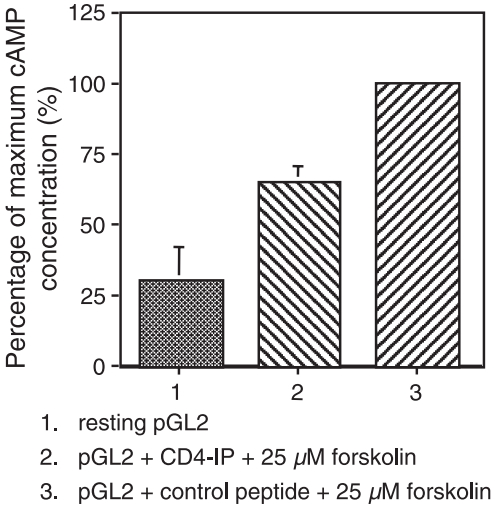

Fig. 4. Pretreatment with CD4-IP inhibits forskolin-induced $[cAMP]_i$ increase. pGL2 cells were either left untreated (1) or preincubated with 100 μM of CD4-IP (2) or control peptide for 30 min (3). Then, forskolin was added to samples 2 and 3 at a final concentration of 25 μM. All samples were incubated in duplicate. Ten minutes after the addition of forskolin, cells were harvested and lysed, and samples adjusted to contain equal amounts of cellular protein. $[cAMP]_i$ was measured with a radioimmunoassay and normalized against the highest concentration within each experiment, which was given a relative value of 1 (equal to 100%). Data are mean values ± S.D. from five independent experiments. The absolute $[cAMP]_i$ in resting pGL2 cells (1) was 2.32 ± 0.82 pmol/ml (mean ± S.D. of 10 independent experiments). $p < 0.001$ between samples 2 and 3. $[cAMP]_i$ in pGL2 cells stimulated with forskolin, but not treated with either CD4-IP or control peptide was also measured (omitted from the figure for clarity). No statistically significant difference was detected between these samples and samples treated with control peptide.

in $[cAMP]_i$ by forskolin in the presence of iPDEs (Fig. 6). To rule out the possibility that the presence of a low concentration of DMSO, which was used to dissolve the iPDEs, affected the ability of CD4-IP to counteract forskolin-induced increases in $[cAMP]_i$, we repeated these experiments using DMSO without iPDEs. Again, CD4-IP inhibited the forskolin-induced increase in $[cAMP]_i$ to the same degree as observed in the absence of DMSO (not shown). Thus, CD4-mediated signals activated one or several members of the PDE1 and PDE4 families.

3.5. PDE inhibitors block proliferation of Th cells stimulated with wild-type MHCII·Ag, but not with mutant MHCII·Ag

To test whether Th cell function required CD4-induced PDE activation, we measured the effect of iPDEs on Ag-induced Th cell proliferation. Purified, naïve $CD4^+$ lymph node T cells from DO.11.10 TCR transgenic mice were stimulated with their specific Ag, ovalbumin peptide 323–339 (Ova323) presented by irradiated APCs expressing either wild-type or mutant MHCII in the presence or absence of various concentrations of iPDE1 or iPDE4. Abrogation of PDE activity requires 80 μM of iPDE1 and iPDE4 [45,47]. However, the effective concentrations of iPDE1 and iPDE4 required to block proliferation of naïve DO.11.10 $CD4^+$ T cells induced by wild-type MHCII·Ova323 were much lower (Fig. 7). DO.11.10 Th cells proliferated only weakly in response to stimulation with mutant MHCII·Ova323, and iPDEs did not affect mutant MHCII·Ova323-induced proliferation at concentrations that reduced wild-type MHCII·Ova323-induced proliferation by >3-fold (Fig. 8a

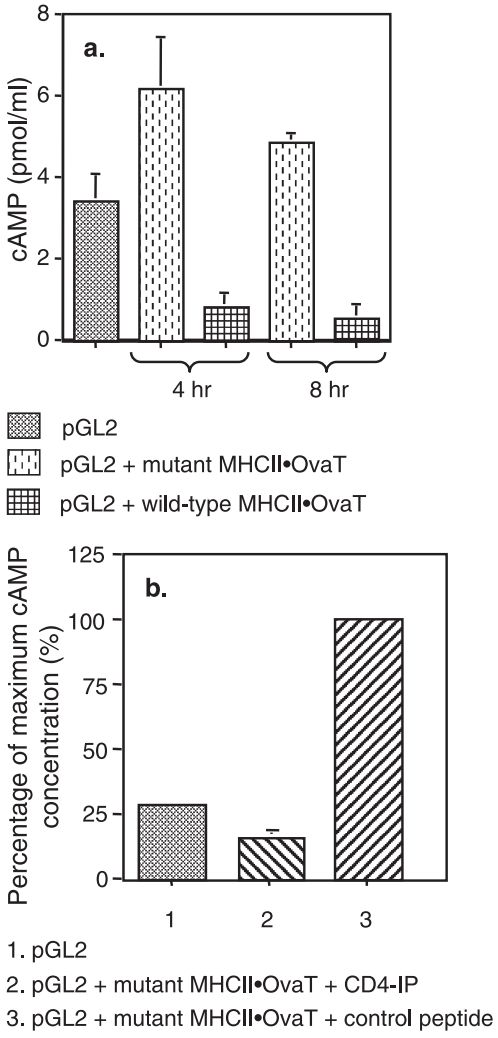

Fig. 5. CD4-mediated signalling decreases $[cAMP]_i$ in Ag-stimulated Th cells. (a) pGL2 cells were stimulated with peptides derived from ovalbumin by trypsin treatment (OvaT) and presented by mutant (dashed bars) or wild-type (gridded bars) MHCII-expressing APCs for 4 or 8 h. All samples were incubated in duplicate. Cells were processed and samples adjusted to contain equal amounts of cellular protein (5 mg protein/sample) before $[cAMP]_i$ measurements. Data are expressed in pmol/ml and are representative of three independent experiments. $p < 0.03$ between samples 2 and 3, and $p < 0.005$ between samples 4 and 5. (b) pGL2 cells were stimulated with OvaT presented by mutant MHCII-expressing APCs for 8 h in the presence of 100 μM of CD4-IP (2) or control peptide (3). All samples were incubated in duplicate. Cells were processed and samples adjusted to contain equal amounts of cellular protein. Data were normalized against the highest concentration within each experiment and are mean values ± S.D. from five independent experiments. $p < 0.0015$ between samples 2 and 3. No difference was detected between control peptide-treated and -untreated samples (omitted for clarity).

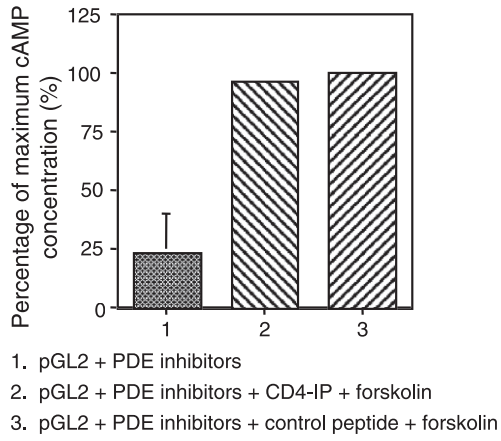

Fig. 6. Inhibitors of cyclic nucleotide PDEs block the effect of CD4-IP on forskolin-induced $[cAMP]_i$ increase. pGL2 cells were preincubated with 80 μM of iPDE1 and 80 μM of iPDE4 either in the absence of peptide (1) or in the presence of 100 μM CD4-IP (2) or control peptide (3) for 30 min. Then, forskolin was added to all samples to a final concentration of 25 μM. All samples were incubated in duplicate. Cells were processed and samples adjusted to contain equal amounts of cellular protein. $[cAMP]_i$ was measured and normalized against the highest concentration within each experiment. Data are mean values ± S.D. from two independent experiments. No difference was detected between control peptide-treated and -untreated samples (omitted for clarity).

and c). Proliferation of wild-type MHCII·Ova323-stimulated Th cells in the presence of iPDE4 was comparable to that of mutant MHCII·Ova323-stimulated Th cells (Fig. 8c and d). Thus, activation of cAMP PDEs by CD4 signalling promotes Ag-induced Th cell proliferation.

3.6. CD4 signalling inhibits adenylyl cyclase activation

Signalling via TCR/CD3 enhances adenylyl cyclase activity [50–52]. Also, activation of type I PKA by TCR signalling [15,16] suggests that TCR signalling activates adenylyl cyclase. Effective T cell activation requires a strict regulation of adenylyl cyclase activity [32,53,54]. To test

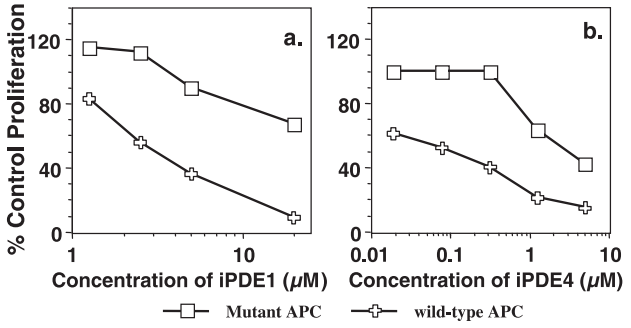

Fig. 7. Dose–response curves for inhibitory effects of specific PDE inhibitors on antigen-induced T cell proliferation. Primary DO.11.10 $CD4^+$ T cells were prepared and incubated with various concentrations of iPDE1 (a) or iPDE4 (b) as described in the legend to Fig. 8. Data are expressed as a percentage of the proliferation of cells incubated in the presence of iPDE solvent (DMSO) and are means of two independent experiments.

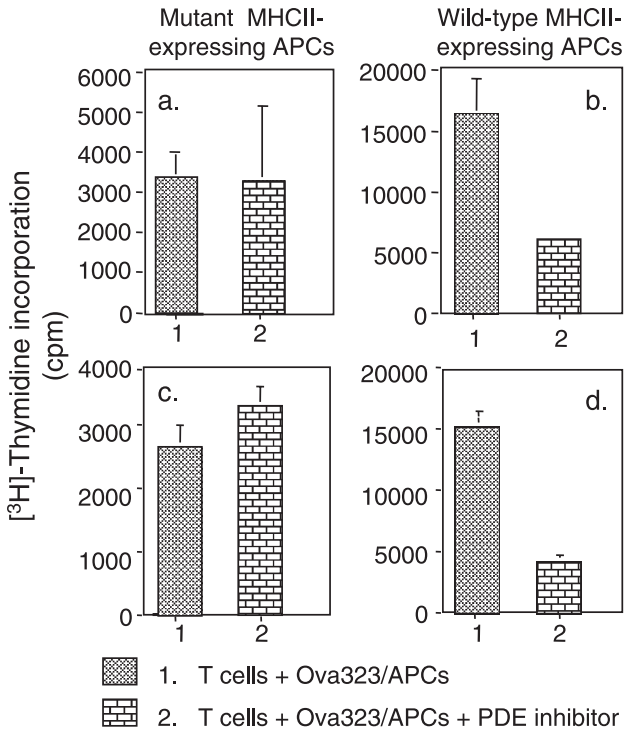

Fig. 8. Differential effect of specific PDE inhibitors on T cell activation by Ag in the context of mutant and wild-type MHCII. $CD4^+$ T cells were purified from the lymph nodes of DO.11.10 TCR transgenic mice by antibody-mediated depletion of $CD8^+$ and $MHCII^+$ cells. T cells (1.5×10^5) were incubated in the wells of a 96-well plate without APCs, with APCs (5×10^4) in the absence of Ag, or with APCs plus Ag (39 nM of Ova323) in the absence or presence of 5 μM of iPDE1 (a, b) or of 78 nM of iPDE4 (c, d). After 72 h, 1 μCi of [^{3}H]thymidine was added to each sample and incubation was continued for another 18 h. Radioactivity incorporated into DNA was measured. Background proliferation in the absence of Ag was subtracted from the data obtained with Ag-stimulated T cells depicted in the figure. Data are mean ± S.D. of triplicate values from one representative experiment out of three performed. The S.D. for sample 2 in (b) was too small for visualization. $p < 0.005$ between samples 1 and 2 in (a) and (b).

whether TCR/CD3 signalling alone affects adenylyl cyclase activity, pGL2 cells were incubated with either immobilized anti-CD3 antibody (clone 145-2C11) or control antibody for various times. Cells were then lysed and adenylyl cyclase activity measured. Treatment with anti-CD3 antibody, but not with the control antibody increased adenylyl cyclase activity over a period of 1.5 h (Fig. 9a). To examine how CD4 signalling affects adenylyl cyclase activity in Ag-activated Th cells, we stimulated pGL2 cells with either wild-type or mutant MHCII·OvaT and measured adenylyl cyclase activity after 4 h. Cells activated by wild-type MHCII·OvaT had lower adenylyl cyclase activity than cells activated by mutant MHCII·OvaT (Fig. 9b).

3.7. Adenylyl cyclase inhibitors modulate kinetics of T cell proliferation and IL-2 production

To examine the role of adenylyl cyclase during Th cell activation, we stimulated naïve $CD4^+$ Th cells from

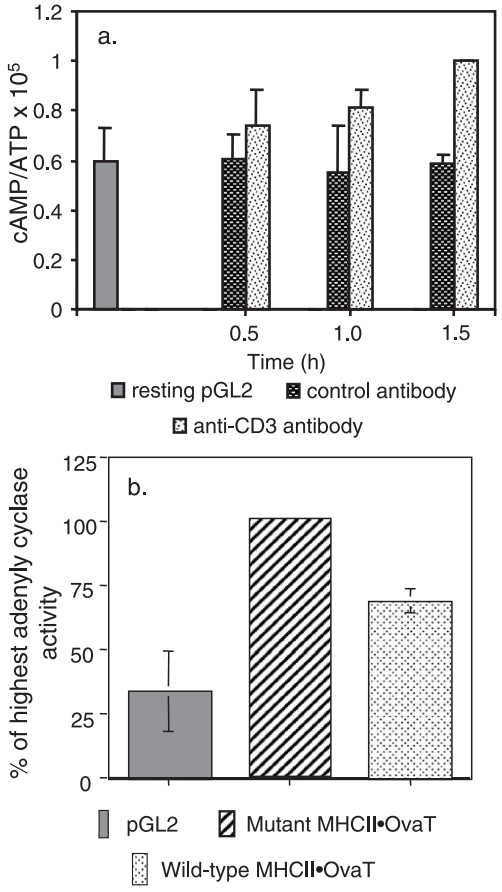

Fig. 9. CD4 signalling antagonizes TCR/CD3-induced activation of adenylyl cyclase. (a) pGL2 cells were labeled with [^{3}H]adenine and treated with IBMX to inhibit PDE activity. All samples were incubated in triplicate. After incubation with immobilized anti-CD3 antibody (clone 1452C11) or control antibody (GAH IgG) for 0.5, 1, and 1.5 h, cells were lysed and samples adjusted to contain equal amounts of cellular protein before analysis of adenylyl cyclase activity. Data are mean values ± S.D. and are representative of three independent experiments. $p < 0.005$ between samples incubated with anti-CD3 antibody or control antibody for 1.5 h. (b) [^{3}H]Adenine-labeled and IBMX-treated pGL2 cells were either not stimulated (1) or stimulated with mutant MHCII·Ag (2) or wild-type MHCII·Ag (3) for 4 h. All samples were incubated in triplicate. Adenylyl cyclase activity was measured in cellular extracts adjusted to contain equal amounts of cellular protein. Data were normalized against the sample with the highest concentration and are mean values ± S.D. from three independent experiments. $p < 0.005$ between samples 2 and 3.

DO.11.10 TCR transgenic mice with either wild-type or mutant MHCII·Ova323 in the presence or absence of adenylyl cyclase inhibitor. We first determined the maximal concentration of adenylyl cyclase inhibitor that did not affect viability of T cells (data not shown). At nontoxic concentrations, the adenylyl cyclase inhibitor blocked proliferation in a dose-dependent manner during the first 2 days following stimulation (Fig. 10a). We observed the same effect on IL-2 production measured 24 h after stimulation (Fig. 10b). However, adenylyl cyclase inhibition did not abrogate proliferation, but delayed peak DNA synthesis and ultimately prolonged the duration of Ag-stimulated Th cell proliferation in a dose-dependent fashion (Fig. 10a). Thus, productive Th cell proliferation and IL-2 secretion requires a complex temporal control of adenylyl cyclase activity and $[cAMP]_i$.

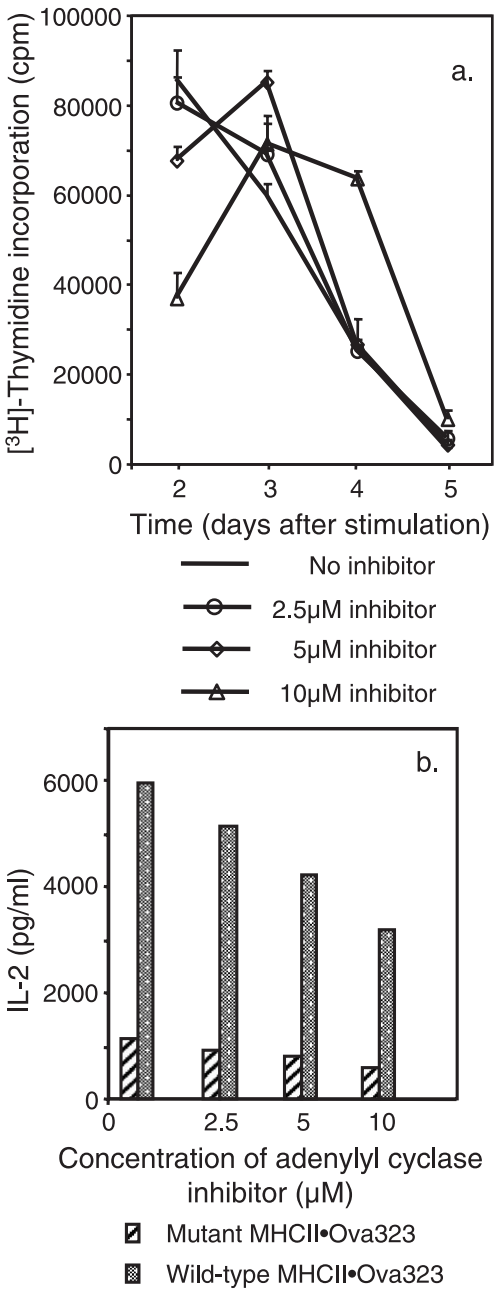

Fig. 10. Effect of adenylyl cyclase inhibition on Th cell activation. CD4$^+$ T cells were purified from the lymph nodes of DO.11.10 TCR transgenic mice. T cells (1.5×10^5) were stimulated in the wells of a 96-well plate with APCs (5×10^4) plus Ag (39 nM of Ova323) in the presence of various concentrations of the adenylyl cyclase inhibitor, MDL-12,330A. (a) After 48, 72, and 96 h, 1 μCi of [^{3}H]thymidine was added to each sample and incubation was continued for another 18 h. Radioactivity incorporated into DNA was measured. Only effects on proliferation of wild-type MHCII·Ova323-stimulated Th cells are show, because the same pattern was observed in Th cells stimulated with mutant MHCII·Ova323. Data are mean ± S.D. of triplicate values from one representative experiment out of three performed. (b) IL-2 production was measured in 24-h supernatants by ELISA.

4. Discussion

The crystal structure of the exodomains of human CD4 suggests that the membrane proximal domains of CD4 may promote dimerization [55]. That oligomerization may be an intrinsic property of CD4 molecules was also indicated by the extraction of CD4 oligomers from freshly isolated T lymphocytes and lymphoid cell lines [56]. Oligomerization is a feature shared by many signal-transducing receptors [57–59]. Therefore, cross-linking anti-CD4 antibodies alone or in conjunction with anti-TCR antibodies have been used to test whether CD4 can transduce signals [26,60–63]. However, this approach does not adequately reflect CD4-mediated signalling induced by engaging MHCII during Ag activation [61]. Also, antibody-mediated cross-linking prevents co-localization of CD4 with TCR molecules [64]. The observation that different monoclonal anti-CD4 antibodies recognizing different epitopes activate different signalling pathways suggests the existence of specific signalling epitopes on CD4 [65]. Simultaneous engagement of non-overlapping CD4 epitopes can modify the signals from individual epitopes [65]. Therefore, it is likely that MHCII-binding to a broad region on the CD4 D1 and D2 domains [66] induces signals via CD4 that differ from those induced by other natural CD4 ligands (e.g., gp120 and IL-16) or monoclonal antibodies. Here, we used a dual approach to identify CD4-mediated signals induced by MHCII engagement. First, we separated TCR-mediated and CD4-mediated signals by restricting the ability of MHCII to interact with CD4 during Ag stimulation. Second, we avoided antibody-mediated cross-linking, but employed a peptide mimetic to engage the CD4 epitope recognized by MHCII. This peptide binds to $CD4^+$ T cells [27] and soluble CD4 [29]. However, binding of CD4-IP to soluble CD4 did not induce CD4 dimerization in solution as measured by analytical ultracentifugation (data not shown). Currently, we cannot rule out that CD4-IP promotes CD4 oligomerization within the plasma cell membrane, but more likely is a scenario in which CD4-IP binding causes conformational changes in pre-formed CD4 dimers.

Recently, the crystal structure of a human CD4 N-terminal two-domain fragment complexed to the murine MHCII, $I\text{-}A^k$, was solved [67]. This crystal structure contradicts many previous reports on putative interaction sites within the CD4·MHCII complex based on mutagenesis and peptide inhibition assays [10,11,29,68], between MHCII·MHCII dimers based on crystallographic and mutagenesis data [69–71], and between CD4·CD4 dimers based on crystallography of the entire extracellular four-domain fragment [55]. Nevertheless, if the crystal structure of the $CD4\cdot I\text{-}A^k$ complex correctly identified the contact sites between CD4 and MHCII, the mutations that we introduced in the CD loop of the MHCII β2 domain functionally disrupted CD4·MHCII interactions by structurally distorting the β2 domain D and E strands [67]. Furthermore, the peptide mimetic used here contained the MHCII β2 144–146 segment identified as crucial for CD4·MHCII contacts in the $CD4\cdot I\text{-}A^k$ crystal [67].

Our results suggested that CD4-mediated signals facilitate Th cell activation by two mechanisms (Fig. 11). First, CD4 signalling mobilized Ca^{2+}. This CD4-induced Ca^{2+} signal synergized with the TCR-mediated Ca^{2+} signal leading to a sustained rise in $[Ca^{2+}]_i$ that was more pronounced than the rise induced by any one signal alone (Fig. 1). A long-lasting elevation of $[Ca^{2+}]_i$ is required for effective Th cell activation [13,72]. Second, CD4 signalling blocked TCR-mediated increases in $[cAMP]_i$. This inhibition was mediated by both activation of cAMP PDEs and by inhibiting adenylyl cyclase. TCR signalling in the absence of additional, modifying signals resulted in the accumulation of cAMP in the cytosol (Fig. 5 and Ref. [73]), and thereby in partial Th cell activation without induction of efficient proliferation and cytokine production. Therefore, signals induced by MHCII engagement of CD4 are critical for successful Th cell activation. Importantly, we used normal T cell clones and primary lymphocytes for all experiments. We performed all key experiments with both T cell types and observed no qualitative differences. Therefore, our data suggest that the reported mechanisms are physiologically relevant.

It could be argued that if the CD4-mediated Ca^{2+} mobilization induced by binding of CD4-IP was physiologic, engagement of CD4 by MHCII should induce similar Ca^{2+} responses. We indeed detected Ca^{2+} mobilization in Th cells induced by MHCII-expressing cells in the absence

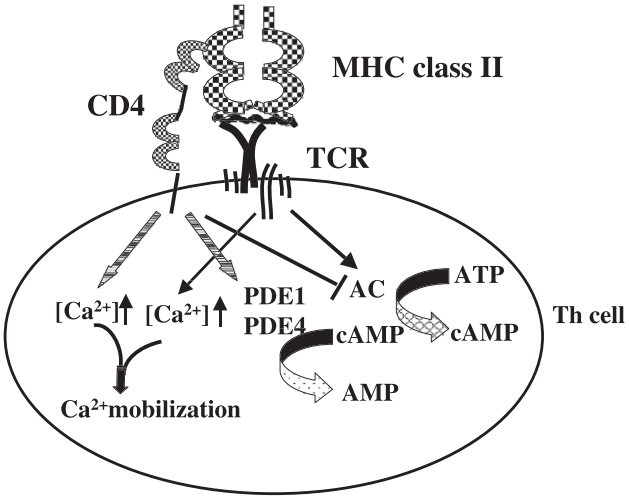

Fig. 11. Regulation of $[Ca^{2+}]_i$ and $[cAMP]_i$ are two mechanisms by which CD4 participates in Th cell activation by MHCII·Ag. Interactions between CD4 and monomorphic regions of MHCII during stimulation of Th cells induce signals via CD4. These signals elicit multiple responses. A CD4-mediated increase in $[Ca^{2+}]_i$ synergizes with TCR-induced Ca^{2+} signals. CD4 signalling activates cAMP phosphodiesterases of the PDE1 and PDE4 families of isozymes. Activation of PDE1 may be mediated by mobilization of Ca^{2+}, whereas PDE4 may be activated via PI3-kinase in a $p56^{lck}$-dependent manner [49]. CD4 signalling also inhibits adenylyl cyclase, possibly via a G_i-protein associated with CD4 [78]. Regulation of PDEs and adenylyl cyclase counteract the TCR-induced increase in $[cAMP]_i$.

of Ag. However, these Ca^{2+} responses were not differentiated by CD4 engagement as they were induced by both wild-type and mutant MHCII-expressing APCs (not shown). Other investigators have reported partial activation of Th cells by dendritic cells in the absence of Ag [74], and dendritic cell-induced Ca^{2+} mobilization that is independent of both Ag and MHCII molecules [75]. Thus, it is not possible to determine the effect of CD4 engagement by MHCII on Ca^{2+} mobilization using APCs due to multiple cell receptor–ligand interactions. In addition, CD4·MHCII interactions promote deconjugation of T cell-APC aggregates in the absence of Ag [23]. Soluble ligands, such as CD4-IP, that mimic CD4 engagement by MHCII molecules provide a means to circumvent these limitations.

Our results also indicate a complex and dynamic regulation of $[cAMP]_i$ during Th cell activation. Wild-type MHCII·Ag induced higher proliferation and IL-2 production than did mutant MHCII·Ag (Figs. 8 and 10). Four hours after activation, T cells stimulated with wild-type MHCII·Ag had a lower $[cAMP]_i$ and lower adenylyl cyclase activity than did mutant MHCII·Ag-stimulated T cells (Figs. 5 and 9). Inhibition of Th cell proliferation by iPDEs also suggested that cAMP accumulation blocked Th cell proliferation (Figs. 7 and 8). However, adenylyl cyclase inhibitors had a complex effect on Ag activation of Th cells (Fig. 10), suggesting that cell cycle progression and cytokine gene expression of Th cells required precise kinetic regulation of adenylyl cyclase activity and thereby of $[cAMP]_i$. Support for this conclusion comes from experiments with purified human T cells. Stimulation with phytohemagglutinin (PHA) or anti-CD3 plus anti-CD28 antibodies transiently up-regulates adenylyl cyclase and PDE activities with different kinetics for different PDE isozymes [32]. PHA and anti-CD3/CD28 stimulation rapidly activates PDE4, but does not induce PDE4 mRNA, whereas both stimuli induce PDE1 mRNA [32]. Only stimulation with anti-CD3/CD28, but not with PHA, induces PDE7 mRNA [32], and TCR-mediated T cell activation requires the induction of PDE7 [46].

An important characteristic for effective regulation of $[cAMP]_i$ by CD4 signalling is the temporal and spatial sequence of CD4 localization in the signalling synapse. Recruitment of CD4 depends on binding of the TCR to its specific MHCII·Ag ligand [76]. Thus, an initial TCR-mediated increase in adenylyl cyclase activity may be countered by CD4-mediated adenylyl cyclase inhibition and PDE activation to promote early T cell activation events. Exclusion of CD4 from the central TCR cluster to the periphery of the signalling synapse within a few min after the initial Ca^{2+} signal [76] may then remove the block on $[cAMP]_i$ increases to promote proliferation and cytokine production.

The activities of adenylyl cyclases are controlled by hormones, neurotransmitters, chemotactic transducers, and other molecules. A common mechanism to inhibit adenylyl cyclase activity is through the activation of G_i proteins (e.g., G_{i1}, G_{i2}, and G_{i3}), which are coupled to receptors for effector molecules. Members of the G_i family of proteins positively regulate T cell activation [77]. A 32-kDa GTP-binding protein associates with CD4 and CD8 in human T cell lines [78]. Therefore, inhibition of adenylyl cyclase by CD4·MHCII engagement may be mediated via G_i proteins. CD4 may serve as a docking protein, carrying G_i proteins to the signalling synapse in activated Th cells, similar to the mechanism by which CD4 promotes $p56^{lck}$ function [79].

In conclusion, we have demonstrated TCR-independent CD4 signalling that caused an increase in $[Ca^{2+}]_i$ and a decrease in $[cAMP]_i$ in normal Th cell clones and primary lymphocytes. Effects of CD4 signalling on $[cAMP]_i$ were mediated via activation of PDE1 and PDE4, and inhibition of adenylyl cyclase. These signals directly affected TCR-mediated activation of Th cells and promoted clonal expansion and IL-2 production. Therefore, we propose that transduction of costimulatory signals induced by MHCII engagement represent a major function of the CD4 coreceptor and that CD4-mediated signals participate in the precise regulation of $[cAMP]_i$ following T cell stimulation.

Acknowledgements

This work was supported by the American Heart Association grant 9750717N, the National Science Foundation grant MCB-9630187, and the National Institutes of Health/National Institute of Environmental Health Sciences grant ES06676. W.Z. is a recipient of a fellowship from the McLaughlin Fellowship Fund and the American Foundation for Aging Research, and an awardee of the Christina Fleischmann Foundation and the Association for Women in Science. W.Z. also acknowledges support by the Graduate School of Biological Sciences at UTMB. We thank T. Albrecht (Director, Signal Transduction Service Core of the NIEHS Center in Environmental Toxicology, UTMB) for advice on measurements of $[Ca^{2+}]_i$, X. Shen and L. Soong for discussions, and V. Braciale, L. Soong, P. Christadoss, and R. Alam for comments on the manuscript.

References

[1] Snow EC, Pittner B, Reid S. Semin Immunol 1994;6:311–26.
[2] Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. Nature 1998;393:480–3.
[3] Taylor-Robinson AW, Phillips RS. Immunology 1992;77:99–105.
[4] Diepolder HM, Jung MC, Keller E, Schraut W, Gerlach JT, Gruner N, et al. Clin Exp Immunol 1998;113:244–51.
[5] Kahn M, Sugawara H, McGowan P, Okuno K, Nagoya S, Hellstrom KE, et al. J Immunol 1991;146:3235–41.
[6] Rizzo LV, Silver P, Wiggert B, Hakim F, Gazzinelli RT, Chan CC, et al. J Immunol 1996;156:1654–60.
[7] Datta SK, Kaliyaperumal A, Mohan C, Desai-Mehta A. Lupus 1997; 6:333–6.
[8] Bushell A, Morris PJ, Wood KJ. Eur. J. Immunol. 1995;25:2643–9.
[9] Gay D, Maddon P, Sekaly R, Talle MA, Godfrey M, Long E, et al. Nature 1987;328:626–9.

[10] König R, Huang LY, Germain RN. Nature 1992;356:796–8.
[11] König R, Shen X, Germain RN. J Exp Med 1995;182:779–87.
[12] Janeway Jr CA, Rojo J, Saizawa K, Dianzani U, Portoles P, Tite J, et al. Immunol Rev 1989;109:77–92.
[13] Wulfing C, Rabinowitz JD, Beeson C, Sjaastad MD, McConnell HM, Davis MM. J Exp Med 1997;185:1815–25.
[14] Berridge MJ. Nature 1993;361:315–25.
[15] Skalhegg BS, Landmark BF, Doskeland SO, Hansson V, Lea T, Jahnsen T. J Biol Chem 1992;267:15707–14.
[16] Laxminarayana D, Kammer GM. J Immunol 1996;156:497–506.
[17] Paliogianni F, Kincaid RL, Boumpas DT. J Exp Med 1993;178:1813–7.
[18] Vang T, Torgersen KM, Sundvold V, Saxena M, Levy FO, Skalhegg BS, et al. J Exp Med 2001;193:497–507.
[19] Anderson P, Blue ML, Morimoto C, Schlossman SF. J Immunol 1987;139:678–82.
[20] Owens T, Fazekas de St. Groth B, Miller JF. Proc Natl Acad Sci U S A 1987;84:9209–13.
[21] Weber S, Karjalainen K. Int Immunol 1993;5:695–8.
[22] Xiong Y, Kern P, Chang HC, Reinherz EL. J Biol Chem 2001;276:5659–67.
[23] Metz DP, Farber DL, König R, Bottomly K. J Immunol 1997;159:2567–73.
[24] Hamad AR, O'Herrin SM, Lebowitz MS, Srikrishnan A, Bieler J, Schneck J, et al. J Exp Med 1998;188:1633–40.
[25] Sleckman BP, Rosenstein Y, Igras VE, Greenstein JL, Burakoff SJ. J Immunol 1991;147:428–31.
[26] Ravichandran KS, Lee KK, Songyang Z, Cantley LC, Burn P, Burakoff SJ. Science 1993;262:902–5.
[27] Shen X, Hu B, McPhie P, Wu X, Fox A, Germain RN, et al. J Immunol 1996;157:87–100.
[28] Shen XL, König R. Int Immunol 1998;10:247–57.
[29] Cammarota G, Scheirle A, Takacs B, Doran DM, Knorr R, Bannwarth W, et al. Nature 1992;356:799–801.
[30] Gilfillan S, Shen XL, König R. J Immunol 1998;161:6629–37.
[31] Gajewski TF, Pinnas M, Wong T, Fitch FW. J Immunol 1991;146:1750–8.
[32] Kanda N, Watanabe S. Biochem Pharmacol 2001;62:495–507.
[33] Williams DA, Fay FS. Cell Calcium 1990;11:75–83.
[34] Bradford M. Anal Biochem 1976;72:248–54.
[35] Johnson RA, Alvarez R, Salomon Y. Methods Enzymol 1994;238:31–56.
[36] Weiss A, Imboden J, Shoback D, Stobo J. Proc Natl Acad Sci U S A 1984;81:4169–73.
[37] Gelfand EW, Cheung RK, Mills GB, Grinstein S. Eur J Immunol 1988;18:917–22.
[38] Leitenberg D, Boutin Y, Constant S, Bottomly K. J Immunol 1998;161:1194–203.
[39] Madrenas J, Chau LA, Smith J, Bluestone JA, Germain RN. J Exp Med 1997;185:219–29.
[40] Huang RD, Smith MF, Zahler WL. J Cycl Nucleotide Res 1982;8:385–94.
[41] Manganiello VC, Murata T, Taira M, Belfrage P, Degerman E. Arch Biochem Biophys 1995;322:1–13.
[42] Soderling SH, Beavo JA. Curr Opin Cell Biol 2000;12:174–9.
[43] Hurwitz RL, Hirsch KM, Clark DJ, Holcombe VN, Hurwitz MY. J Biol Chem 1990;265:8901–7.
[44] Giembycz MA, Corrigan CJ, Seybold J, Newton R, Barnes PJ. Br J Pharmacol 1996;118:1945–58.
[45] Ekholm D, Hemmer B, Gao G, Vergelli M, Martin R, Manganiello V. J Immunol 1997;159:1520–9.
[46] Li L, Yee C, Beavo JA. Science 1999;283:848–51.
[47] Tenor H, Staniciu L, Schudt C, Hatzelmann A, Wendel A, Djukanovic R, et al. Clin Exp Allergy 1995;25:616–24.
[48] Ahmad F, Gao G, Wang LM, Landstrom TR, Degerman E, Pierce JH, et al. J Immunol 1999;162:4864–75.
[49] Mazerolles F, Fischer A. Int Immunol 1998;10:1897–905.
[50] Kvanta A, Gerwins P, Jondal M, Fredholm BB. Cell Signal 1990;2:461–70.
[51] Bihoreau C, Heurtier A, Enjalbert A, Corvaia N, Bensussan A, Degos L, et al. Eur J Immunol 1991;21:2877–82.
[52] Buc HA, Moncion A, Hamet M, Perignon JL. Int J Immunopharmacol 1993;15:415–22.
[53] Bastin B, Payet MD, Dupuis G. Cell Immunol 1990;12:385–9.
[54] Giannetti N, Enjalbert A, Krantic S. J Cell Biochem 2000;78:666–73.
[55] Wu H, Kwong PD, Hendrickson WA. Nature 1997;387:527–30.
[56] Lynch GW, Sloane AJ, Raso V, Lai A, Cunningham AL. Eur J Immunol 1999;29:2590–602.
[57] Papoff G, Hausler P, Eramo A, Pagano MG, Di Leve G, Signore A, et al. J Biol Chem 1999;274:38241–50.
[58] Rodriguez-Frade JM, Vila-Coro AJ, de Ana AM, Albar JP, Martinez AC, Mellado M. Proc Natl Acad Sci U S A 1999;96:3628–33.
[59] White DW, Tartaglia LA. J Cell Biochem 1999;73:278–88.
[60] Veillette A, Bookman MA, Horak EM, Samelson LE, Bolen JB. Nature 1989;338:257–9.
[61] Luo KX, Sefton BM. Mol Cell Biol 1990;10:5305–13.
[62] Prasad KV, Kapeller R, Janssen O, Repke H, Duke-Cohan JS, Cantley LC, et al. Mol Cell Biol 1993;13:7708–17.
[63] Pallier A, Jauliac S, Jabado N, Fischer A, Hivroz C. Int Immunol 1998;10:869–76.
[64] Ratcliffe MJ, Coggeshall KM, Newell MK, Julius MH. J Immunol 1992;148:1643–51.
[65] Milia E, Di Somma MM, Majolini MB, Ulivieri C, Somma F, Piccolella E, et al. Mol Immunol 1997;34:287–96.
[66] König R, Fleury S, Germain RN. Curr Top Microbiol Immunol 1996;205:19–46.
[67] Wang JH, Meijers R, Xiong Y, Liu JH, Sakihama T, Zhang RG, et al. Proc Natl Acad Sci U S A 2001;98:10799–804.
[68] Gaubin M, Houlgatte R, Dettin M, Scarinci C, Martin M, Guardiola J, et al. Hum Immunol 1999;60:273–81.
[69] Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, et al. Nature 1993;364:33–9.
[70] Fremont DH, Hendrickson WA, Marrack P, Kappler J. Science 1996;272:1001–4.
[71] Lindstedt R, Monk N, Lombardi G, Lechler R. J Immunol 2001;166:800–8.
[72] Rabinowitz JD, Beeson C, Wulfing C, Tate K, Allen PM, Davis MM, et al. Immunity 1996;5:125–35.
[73] Feuerstein N, Firestein R, Aiyar N, He X, Murasko D, Cristofalo V. J Immunol 1996;156:4582–93.
[74] Kondo T, Cortese I, Markovic-Plese S, Wandinger KP, Carter C, Brown M, et al. Nat Immunol 2001;2:932–8.
[75] Revy P, Sospedra M, Barbour B, Trautmann A. Nat Immunol 2001;2:925–31.
[76] Krummel MF, Sjaastad MD, Wulfing C, Davis MM. Science 2000;289:1349–52.
[77] Lippert E, Jacques Y, Hermouet S. J Leukoc Biol 2000;67:742–8.
[78] Telfer JC, Rudd CE. Science 1991;254:439–41.
[79] Holdorf AD, Lee KH, Burack WR, Allen PM, Shaw AS. Nat Immunol 2002;3:259–64.