Cancer Immunol Immunother (1999) 48:525–532 

ORIGINAL ARTICLE

**Julian A. Kim · Mark A. Dayton · Wayne Aldrich**
**Pierre L. Triozzi**

# Modulation of CD4 cell cytokine production by colon cancer-associated mucin

Received: 23 March 1999 / Accepted: 3 August 1999

**Abstract** **Purpose:** Mucins have been implicated in tumor-associated immunosuppression. The possibility that colon cancer mucin (CCM) may modulate T-helper 1 (TH1) activity was evaluated by investigating its effect on the production of interleukin-2 (IL-2) by $CD4^+$ cells, a process that requires antigen-specific and costimulatory signals. **Methods:** CCM was purified from human colorectal cancer cells by gel-exclusion fast-pressure liquid chromatography. Cytokine production of purified $CD4^+$ cells was evaluated at the protein and gene level in the presence of a phorbol ester or an anti-CD3 monoclonal antibody (mAb) plus mAb against the CD28 costimulatory receptor to mimic two-signal activation. **Results:** Soluble CCM, which contains mucins MUC2 as well as MUC1, inhibited IL-2 mRNA expression and secretion of $CD4^+$ stimulated with a phorbol ester or an anti-CD3 mAb plus anti-CD28 mAb. Pretreatment of $CD4^+$ cells with anti-CD28 mAb abrogated the suppressive effects of CCM on IL-2 production, and flow cytometry showed decreased binding of anti-CD28 mAb to its receptor in the presence of mucin. In addition, $Ca^{2+}$ mobilization after T cell receptor cross-linking with anti-CD3 mAb was maintained in the presence of CCM. Although interferon γ production was also diminished, CCM did not induce a general inhibition of cytokine production, nor did it decrease cell viability. Macrophage inflammatory protein 1α production was up-regulated; the production of IL-10 and transforming growth factor β was unchanged. **Conclusions:** The results indicate that CCM can alter TH1 activity and suggest that the modulation of costimulatory interactions is involved. They provide another mechanism of immunosuppression mediated by these highly expressed tumor products.

**Key words** T-helper cells · Costimulation · CD28 · Interleukin-2 · Macrophage inflammatory protein 1α

## Introduction

Despite the expression of molecules that are capable of activating host immune responses, colorectal tumors still grow and overwhelm the host. Several lines of evidence suggest that this may be the result of inadequate, tumor-specific, T-helper 1 (TH1) activity. Expanded TH2 activity and suppressed TH1 activity have been observed in colorectal patients [5, 34]. Tumor rejection can be effected in animal models when TH1 activity is provided by exogenous cytokines, such as interleukin-2 (IL-2) [10]. The efficient activation of TH1 cells, which are predominantly $CD4^+$, requires simultaneous signaling through the T cell receptor as well as costimulatory signals, which, if absent, will result in T cell unresponsiveness [38]. The basis of costimulation for paracrine production of IL-2 involves B7 molecules on the antigen-presenting cells interacting with the CD28 and CTLA-4 receptors on $CD4^+$ cells [11, 18, 29, 30]. Signaling is mediated through a unique cytoplasmic pathway, which may up-regulate IL-2 gene expression and/or increase IL-2 mRNA $t_{\frac{1}{2}}$ by decreasing RNAse activity and which appears to be resistant to cyclosporin A (CsA) [32, 46]. The introduction of B7-1 (CD80) and B7-2 (CD86) into tumors, which normally do not express costimulatory ligands, to stimulate TH1 activity

Supported in part by the National Cancer Institute, National Research Service Award CA-09338, Division of Cancer Prevention and Control (J.A.K.) and N01-CM-47666-01, and the Ohio State University Comprehensive Cancer Center Core Grant (2P30CA16508)

J.A. Kim · M.A. Dayton · W. Aldrich · P.L. Triozzi
The Ohio State University Comprehensive Cancer Center and The Arthur G. James Cancer Hospital and Research Institute, Columbus, Ohio 43210, USA

P.L. Triozzi (✉)
The Ohio State University, 320 W. 10th Ave., Columbus, Ohio 43210, USA
e-mail: triozzi.1@osu.edu
Tel.: +1-614-293-6451
Fax: +1 614-293-7526

in situ has resulted in T-cell-mediated tumor rejection in animal models [8, 21, 41].

Colorectal tumors and other tumors of epithelial origin express and shed mucins, large ($M_r > 200\,000$) molecules that consist of a core protein moiety and a number of O-linked carbohydrates. Tumor-associated mucins are aberrantly glycosylated, which exposes normally cryptic core polypeptides, rendering them highly immunogenic [4]. Although mucin molecules are potential targets for immunotherapeutic approaches, the expression of high levels often correlates with rapid tumor progression [15]. Mucins have been shown to have a variety of immunosuppressive effects. Agrawal et al. have reported that MUC1 mucin, affinity-purified from ascites obtained from ovarian cancer patients, and synthetic MUC1 core peptides suppress the human T cell proliferative response. This suppression could be reversed by the addition of exogenous IL-2 or anti-CD28 monclonal antibody (mAb) [2].

We have previously presented evidence suggesting that tumor-associated mucins may inhibit the production of IL-2 [26]. The purpose of this study was to test the effect colon cancer mucin (CCM) on TH activity by investigating its effect on the production of IL-2 and other cytokines by $CD4^+$ cells. CCM contains MUC2, a secretory mucin that differs physiochemically from MUC1 [39]. The results suggest that soluble CCM can inhibit TH1 activity by modulating costimulatory interactions.

## Materials and methods

### Mucins

Isolation and purification of CCM were performed, using a modification of the published technique [40]. Human colon cancer cell lines were established from tumors obtained from two patients with mucinous adenocarcinomas of the colon. The tumor cells were passaged in RPMI-1640 medium, L-glutamine, 10% fetal calf serum and antibiotic (Gibco, Grand Island, N.Y.), grown to confluence, and then centrifuged at 14 000 rpm for 15 min. The supernatant was subject to 41% $NH_4SO_4$. The precipitated protein was pelleted by centrifugation, resuspended in 1 M phosphate-buffered saline (PBS) (pH = 7.1), and dialyzed against polyethylene glycol, using large-pore dialysis tubing, for 4 h and then against PBS overnight (12 h). The dialyzed solution was subjected to fast-pressure liquid chromatography in a Sephadex CL4B column (Pharmacia, Uppsala, Sweden) with a $M_r$ exclusion limit of $2 \times 10^7$. The sample volume was 2 ml, the flow rate was 2 ml/min, and the column pressure did not exceed 13.8 kPa. The void peak and subsequent peaks were serially collected into 1-ml samples and stored at 4 °C until further analysis. Protein concentration was determined by Bradford assay (BioRad, Hercules, Calif.).

Solid-phase enzyme-linked immunosorbent assay (ELISA) as well as sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis confirmed the mucin content with the anti-TAG-72 mucin mAb, CC49 (Cancer Treatment Evaluation Program, National Cancer Institute), and anti-MUC1 and anti-MUC2 mAb (Accurate Chemical & Scientific Corp., Westbury, N.Y.). Primary antibodies were added at 0.1 μg/well for 1 h and washed three times with PBS. A goat anti-(mouse $I_g$) secondary antibody and substrate were used, and absorbance at 544 nm was determined after overnight incubation. The fractions with the highest CC49 reactivity were tested by Western blot analysis by electrophoresis on a 4% polyacrylamide/SDS denaturing gel, transfer to nylon membrane (Amersham Pharmacia, Piscataway, N.J.) and blockade for 1 h using 5% bovine serum albumin. After washings, the nylon membrane was incubated with mAb CC49 as the primary antibody followed by goat anti-(mouse alkaline phosphatase) as the secondary antibody. Substrate was added and, after 1–4 h, the reaction was terminated.

Synthetic peptides corresponding to MUC1 ($H_2N$-PDTRPAP-GSTAPPAHGVTSA-COOH) and MUC2 ($H_2N$-PTTTPITTTTT-VTPTPTPTGTQT-COOH) were synthesized by the Ohio State University Peptide Laboratory. CCM that was deglycosylated or desialylated by published methods was also evaluated [20, 27]. In brief, CCM was dissolved in one part anisole and two parts trifluromethane sulfonic acid (Aldrich Chemical Co., Milwaukee, Wis.) under nitrogen for 3 h, neutralized with aqueous pyridine, and dialyzed against several changes of ammonium acetate buffer for deglycosylation. CCM was treated with *Clostridium perfringens* neuraminidase (200 mu/ml PBS type X; Sigma, St. Louis, Mo.) for 1 h for desialylation.

### $CD4^+$ cell purification

Lymph nodes, obtained from patients with colorectal cancer in accordance with institutional guidelines and processed into single-cell suspensions, and peripheral blood were subjected to Ficoll-Hypaque (Pharmacia) gradient centrifugation for 30 min at 750 *g*. Cells recovered from the interface were washed in Hank's balanced saline solution (HBSS), passed over a nylon-wool column, and incubated for 1 h at 37 °C. Nonadherent cells were collected by washing the column with two volumes of warm RPMI medium. $CD4^+$ cells were then isolated by immunodepletion using magnetic beads. Nylon-wool-depleted lymphocytes were incubated with 0.1 μg antibodies to CD8, CD19, CD57 and CD14 (Becton Dickinson, Sunnyvale, Calif.) for 1 h at 4 °C. Cells were then washed and incubated with anti-(mouse Fc) immunomagnetic beads for 1 h, diluted into 10 ml HBSS, and separated in a magnet apparatus (Dynal, Oslo, Norway). The resulting nonadherent cell population was more than 90% $CD4^+$, as determined by flow cytometry.

### Cytokine secretion

$CD4^+$ cells were incubated with 10 ng/ml phorbol myristate acetate and plate-bound anti-CD28 mAb (Becton Dickinson) in six-well plates for 12 h at 37 °C and 5% humidified $CO_2$. The various mucin reagents and CsA were added, and RPMI medium with 10% human serum for a final volume of 2 ml. After 12 h of incubation, the supernatant was collected, and cytokine secretion was assessed. IL-2 secretion was measured by the CTLL-2 bioassay [14]. CTLL-2 cells were incubated in 96-well plates at 2000/well with culture supernatants for 24 h, pulsed with 0.1 μCi [$^3$H] thymidine, and harvested onto nylon filters 12 h later with an automated harvester, and radioactivity was counted on a gamma counter. Human recombinant IL-2 (Chiron, Emeryville, Calif.) was used as a positive control. Interferon γ (IFNγ) IL-10, transforming growth factor β (TGFβ), and macrophage inflammatory protein 1α (MIP-1α) secretion was determined by ELISA kits according to the recommendations of the manufacturer (R&D Systems, Minneapolis, Minn). The limits for detection were 5 pg/ml for IFNγ and 10 pg/ml for IL-10, MIP-1α, and TGFβ.

### Cytokine gene expression

Cytokine gene expression of cultured $CD4^+$ cells was determined by the reverse transcription/polymerase chain reaction (PCR) as previously described [9]. Total RNA was isolated by a modification of the phenol/chloroform technique. Total RNA was quantified by UV spectrophotometry, and 1 μg was reverse-transcribed, by 20 U

Moloney murine leukemia virus reverse transcriptase (Gibco) in 2.0 mM KCl with TRIS/HCl buffer at 23 °C for 1 h. samples comprising 5 µl cDNA were then amplified using 2 mM dNTP, 2 mM $MgCl_2$, 1 nM [$\alpha$-$^{32}$P] dCTP, and 2 U Taq polymerase (Boerhinger-Mannheim, Indianapohis, Ind.). Cycle parameters were 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min for 20 cycles. PCR amplicons were then subjected to Sephadex G-50 gel filtration to remove unincorporated nucleotides, and the products were run on a 1.8% agarose/TRIS/EDTA gel. cDNA was transferred to nylon membranes by a semi-dry electroblotter (BioRad), and UV-crosslinked by a Stratalinker (Stratagene, La Jolla, Calif.) prior to autoradiography. Glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified for each sample to serve as a positive control.

### Flow cytometry

$CD4^+$ were incubated with fluoroscein-isothiocyanate-conjugated anti-CD28 and anti-CD3 mAb (Becton-Dickinson) (100 ng/$10^6$ cells) for 1 h at 4 °C. Cells were then pelleted by centrifugation and washed in triplicate with HBSS. Experiments using soluble CCM were performed by pretreating the $CD4^+$ cells with soluble CCM for 1 h at 4 °C followed by replacement with fresh HBSS prior to immunostaining. The tumor cell lines from which the CCM was purified were also assessed for binding of anti-CD3, CD28, MUC1, and MUC2 mAb. Cells were analyzed with an Epics Elite cytometer (Coulter, Hialeah Flq.) using the 525-nm line of an argon ion beam. Histograms were processed by Coulter software and stored in list mode. Control samples were performed at the beginning of each run, and gating was checked so that controls had less than 5% positive staining.

### $Ca^{2+}$ mobilization

$Ca^{2+}$ mobilization was determined by flow cytometry with the $Ca^{2+}$-sensitive indicator, Indo-1 (Calbiochem; La Jolla, Calif.), as has been previously described [28]. Purified $CD4^+$ cells ($10^6$) were loaded with Indo-1 (1 µM) for 20 min in HBSS containing 1.3 nM $Ca^{2+}$ and 0.8 mM $Mg^{2+}$. After washing, $CD4^+$ cells were resuspended in the presence or absence of $Ca^{2+}$ and analyzed by flow cytometery using UV line excitation. The ratio of blue (495 nm) to violet (405 nm) fluorescence was used to determine $Ca^{2+}$ mobilization. After baseline measurements had been obtained, cells were treated with anti-CD3 and anti-CD28 mAb (1 µg/ml) and goat anti-[mouse F(ab′)] for crosslinking, both in the presence and absence of soluble CCM (50 µg/ml). Ionomycin C (1 µg/ml) was used as a positive control.

## Results

### Effect on IL-2 production

TAG-72-expressing CCM of $M_r$ greater than 200 000 was purified from human colon cancer cell lines (Fig. 1). The CCM purified expressed both MUC1 and MUC2, as determined by ELISA (Fig. 1D); in addition, the tumor cell lines were positive for MUC1 and MUC2 by flow cytometry (data not shown). The effect of CCM on the production of IL-2 by purified $CD4^+$ cells stimulated in vitro in conditions to mimic two-signal T-cell activation was evaluated [24]. Lymph-node $CD4^+$ cells stimulated with PMA to activate protein kinase C signaling, as well as plate-bound anti-CD28 mAb to provide co-stimulation via the CD28 receptor resulted in the secretion of significant amounts of IL-2. Secretion under similar conditions was significantly less in the presence of CCM (Fig. 2A). (Both sources of CCM had inhibitory effects.) In other experiments purified $CD4^+$ cells derived from peripheral blood were stimulated with plate-

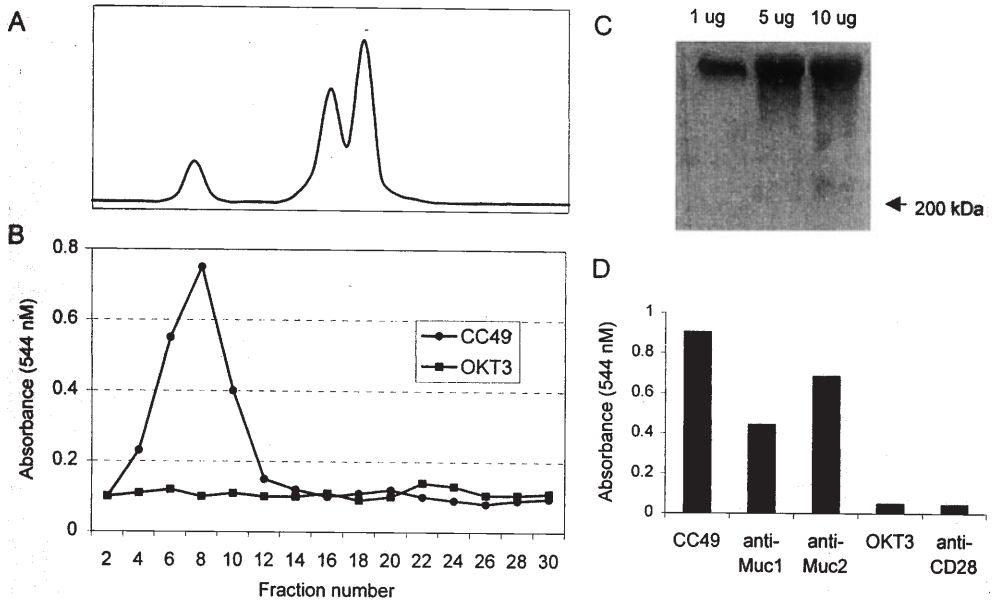


**Fig. 1A–D** Colon cancer mucin (CCM) purification. **A** Fractionation of colon cancer cell supernatant precipitate by gel filtration FPLC over a Sephadex CL-48 column. Absorbance was determined at 280 nM. **B** The resulting fractions were then tested in a solid-phase enzyme-linked immunosorbent assay (ELISA) using the anti-TAG-72 mucin mAb, CC49, and absorbance was determined by spectrophotometry. The anti-CD3 mAb, OKT3, was used as a negative control. **C** Fractions containing high mAb CC49 reactivity were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis analysis using 3% stacking and 4% resolving gels. Western blotting was performed using mAb CC49 as the primary and goat anti-(mouse alkaline phosphatase) as the secondary antibody. The fractions were quantified by the Lowry assay. **D** Binding of CC49 and anti-MUC1, MUC2, CD3 (OKT3), and CD28 mAb to CCM as determined by solid-phase ELISA

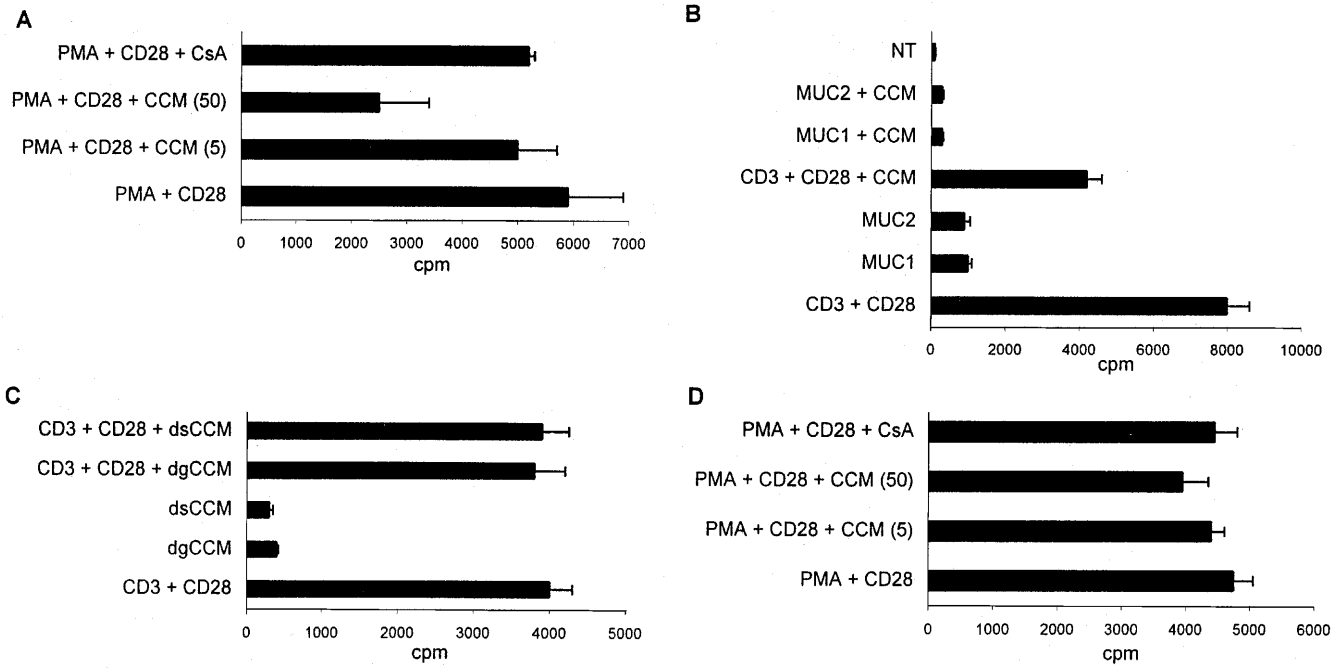


**Fig. 2A–D** Interleukin-2 (IL-2) secretion of $CD4^+$ cells as measured by CTLL-2 bioassay. **A** $CD4^+$ lymph node cells were stimulated with phorbol myristate acetate (*PMA*; 10 ng/ml) and plate-bound anti-CD28 mAb (*CD28*) in the presence of soluble CCM (5 or 50 μg/ml) or cyclosporin A (*CsA*; 10 ng/ml). Data represent means ± SD of triplicate samples of a representative experiment. Experiments with CCM were repeated three times, and experiments with CsA were repeated once. **B** $CD4^+$ peripheral blood cells were stimulated with plate-bound anti-CD3 (CD3) plus anti-CD28 mAb and synthetic MUC1 and MUC2 peptides (10 μg/ml), in the presence and absence of CCM (50 μg/ml) (*NT* no treatment). Data represent means ± SD of triplicate samples of a representative experiment, which was repeated once. **C** $CD4^+$ peripheral blood cells were stimulated with plate-bound anti-CD3 and anti-CD28 mAb in the presence and absence of deglycosylated CCM (*dgCCM*; 50 μg/ml), and desialylated CCM (*dsCCM*; 50 μg/ml). Data represent means ± SD of triplicate samples of a representative experiment, which was repeated once. **D** $CD4^+$ lymph node cells were pretreated with plate-bound anti-CD28 mAb (*CD28*) prior to incubation with soluble CCM (5 or 50 μg/ml) and CsA (10 ng/ml). Data represent means ± SD of triplicate samples of a representative experiment, which was repeated once with similar results

bound anti-CD3 and anti-CD28 mAb. Again, the production of IL-2 under these conditions was significantly diminished in the presence of CCM (Fig. 2B). Control experiments were performed in order to determine whether the observed decrease in IL-2 secretion was due to CCM directly inhibiting the proliferative response of CTLL-2 cells to IL-2. CCM did not inhibit the proliferative response of CTLL-2 cells, even at low concentrations of IL-2 (1 U/ml), suggesting that CCM was not binding IL-2 or the IL-2 receptor (data not shown). There was no evidence of decreased cell viability, as measured by trypan blue exclusion in CCM-treated cultures, when compared with experiments performed in the absence of CCM, nor of apoptotic bodies.

The use of deglycosylated or desialylated CCM did not similarly inhibit IL-2 secretion nor did the addition of synthetic peptides corresponding to a single repeat of apomucins encoded by *MUC1* and *MUC2* genes, suggesting that the large glycoprotein was necessary for the inhibitory effects observed (Fig. 2B, C). In contrast, the synthetic MUC1 and MUC2 stimulated IL-2 production; this stimulation was abrogated in the presence of CCM (Fig. 1B). IL-2 production was also tested in the presence of CsA, which inhibits IL-2 production via a CD28-independent mechanism. In contrast to CCM, CsA did not alter IL-2 secretion by $CD4^+$ cells stimulated under similar conditions (Fig. 2A).

The kinetics of IL-2 gene transcription of the $CD4^+$ cells in this in vitro model of $CD4^+$ cell activation was evaluated. Up-regulation of IL-2 mRNA was observed within 2 h following stimulation with PMA and anti-CD28 mAb; IL-2 mRNA persisted over a period of 8 h (Fig. 3A). In contrast, IL-2 mRNA of $CD4^+$ cells treated with PMA and anti-CD28 mAb in the presence

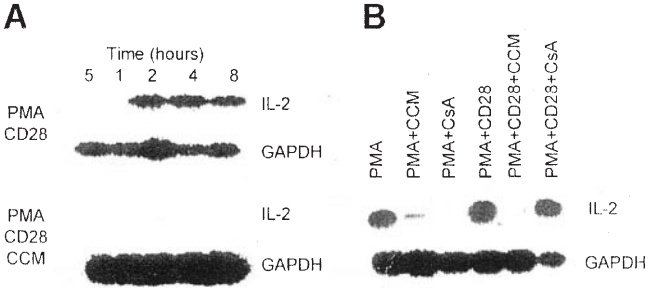


**Fig. 3A, B** Kinetics of IL-2 gene expression as measured by reverse transcription/polymerase chain reaction (RT-PCR). **A** $CD4^+$ lymph node cells were treated with PMA and plate-bound anti-CD28 mAb (CD28), alone and in the presence of CCM (50 μg ml). **B** IL-2 gene expression after 4 h of treatment with PMA, plate-bound anti-CD28, CCM (50 μg/ml), and/or CsA (10 ng/ml). Glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as a control

of CCM demonstrated diminished levels and $t_{\frac{1}{2}}$ (Fig. 3A). $CD4^+$ cells treated with PMA and anti-CD28 mAb maintained IL-2 gene expression in the presence of CsA (Fig. 3B). These results are consistent with the observation that IL-2 gene expression and cytokine secretion by $CD4^+$ cells is diminished in the presence of soluble CCM by a mechanism separate from that of CsA.

## Effect on $CD4^+$ cell costimulation

The following experiments were performed to test whether modulation of the costimulatory pathway was involved in the inhibitory effects of CCM. CCM did not bind anti-CD3 or anti-CD28 mAb, as determined by ELISA (Fig. 1D); the tumor cell lines from which CCM was purified did not bind anti-CD3 or anti-CD28 mAb, as determined by flow cytometry (data not shown). $CD4^+$ lymph node cells were incubated with PMA and anti-CD28 in the presence of CCM and CsA as described above. However, in this set of experiments, $CD4^+$ cells were pretreated with anti-CD28 mAb for 15 min prior to exposure to CsA and CCM (Fig. 2D). This pretreatment of the $CD4^+$ cells with anti-CD28 mAb abrogated the suppressive effects of CCM on IL-2 secretion, suggesting that CCM may be modulating CD28 costimulation.

Flow cytometry was performed to evaluate whether soluble CCM interfered with binding of anti-CD28 mAb to its receptor. Incubation of $CD4^+$ cells with anti-CD28 mAb in the presence of soluble CCM resulted in a diminished number of positive cells (Fig. 4) as well as diminished fluorescence intensity of binding to CD28 (mean fluorescence in the presence of CCM, 1.07; in the absence of CCM, 1.77; control, 0.692). In contrast, CCM did not substantially modulate anti-CD3 mAb binding using the same cells, suggesting that the inhibition of binding of anti-CD28 mAb by CCM was not due to nonspecific interactions of the CCM glycoprotein and the antibodies.

In order to test whether CCM was interfering with signaling through the T cell receptor and the protein kinase C pathway, $Ca^{2+}$ mobilization of $CD4^+$ cells was determined after stimulation with anti-CD3 and anti-CD28 mAb in the presence and absence of soluble CCM (Fig. 5). These experiments show that $CD4^+$ cells show significant influx of $Ca^{2+}$ shortly after incubation with cross-linked anti-CD mAb. Addition of CCM at various concentrations (5, 10, and 50 μg/ml) did not inhibit $Ca^{2+}$ mobilization, suggesting the CCM does not inhibit signaling via the T cell receptor. Additional experiments using anti-CD28 mAb alone did not show $Ca^{2+}$ mobilization, consistent with previous reports [31].

## Effect on other cytokines

In order to determine whether the effect of CCM on IL-2 production represented a general inhibition of cytokine

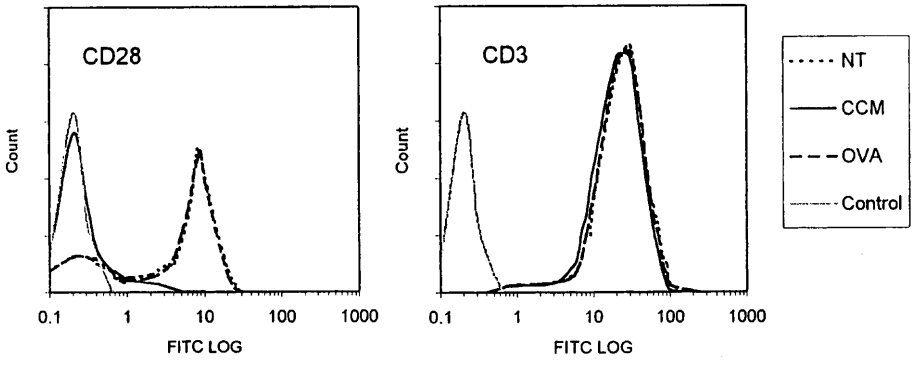


**Fig. 4** Binding of anti-CD28 and anti-CD3 mAb to purified $CD4^+$ lymph node cells as determined by flow cytometry in the presence and absence (*NT*) of CCM (50 μg/ml) or ovalbumin (*OVA*; 50 μg/ml). *Control* isotype control. *FITC* fluorescence isothiocyanate

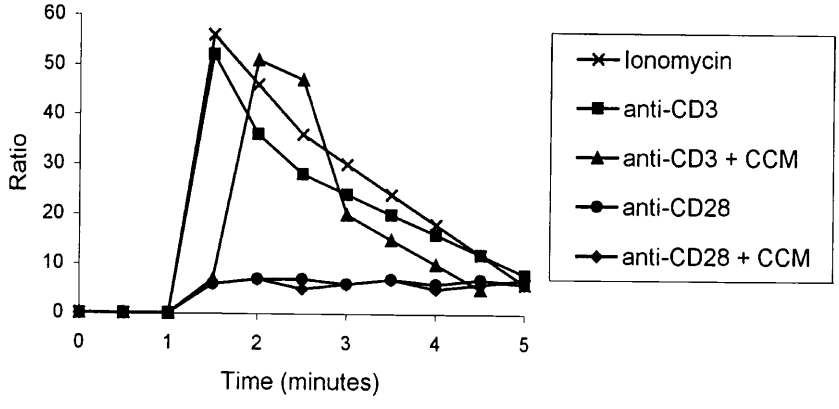


**Fig. 5** $Ca^{2+}$ mobilization of $CD4^+$ cells to stimulation with crosslinked anti-CD3 or anti-CD28 mAb in the presence and absence of CCm (50 μg/ml). Cytoplasmic $Ca^{2+}$ was determined by Indo-1 and is presented as the ratio of fluorescence at 495 nm to that at 405 nm over time. Reagents were added 1 min after establishing a baseline level. Ionomycin C (10 ng/ml) was used as a positive control

production, the effects of CCM on the production of other cytokines, including IFNγ, IL-10, TGFβ, and MIP-1α were compared at the mRNA and protein level (Figs. 6, 7). Although IFNγ mRNA expression was not substantially modulated by CCM, its secretion was. MIP-1α mRNA as well as MIP-1α secretion was up-regulated in the presence of CCM. The production of IL-10 and TGFβ was not significantly modulated.

## Discussion

Tumor-associated mucins have been implicated in a number of immunosuppressive activities, in addition to suppressing T cell proliferation [2]. These include the suppression of the interactions between cytolytic T lymphocytes and their targets [44], sensitivity to natural killer cell cytolysis [33], adhesion of eosinophils to antibody-coated targets [19], and delayed-type hypersensitivity reactions [12]. The release of mucins may also lead to a "decoy" phenomenon, in which mucin-specific effectors are preoccupied with circulating soluble mucin molecules and do not efficiently engage tumor cells [25]. The results of our study indicate that CCM regulates cytokine production of $CD4^+$ cells. Modulation of TH cytokine production is another mechanism by which these highly expressed molecules can act as immunosuppressives and by which a tumor may evade immune effector mechanisms despite the expression of antigenic determinants.

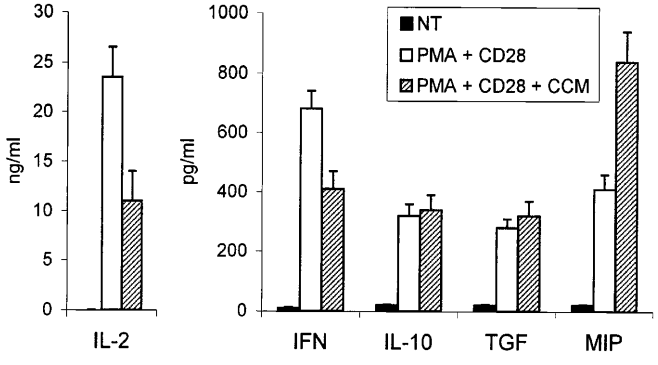


**Fig. 6** RT-PCR of cytokine mRNA of $CD^{4+}$ cells treated with PMA and anti-CD28 mAb in the presence of CCM (50 μg/ml) or CsA (10 ng/ml). GAPDH was used as a control. *IFN* interferon, *TGF* transforming growth factor β, *MIP* macrophage inflammatory protein 1α

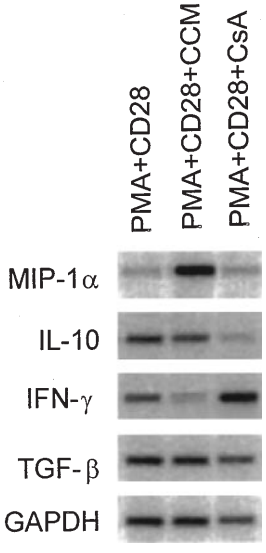


**Fig. 7** Cytokine production of $CD4^+$ cells in response to PMA and anti-CD28, in the presence and absence of CCM (50 μg/ml). Data are presented as mean values ± SD for triplicate samples

Agarwal et al. found that MCU1 induced anergy, which could be reversed by adding IL-2 [2]. Our observations with CCM are consistent with those of Agrawal et al. and the induction of anergy and not apoptosis. Significant lymphocyte death or apoptotic bodies were not observed over the short-term culture period, and CCM did not lead to a general decrease in cytokine production. The decrease of IL-2 secretion and concomitant increase of MIP-1α secretion would be consistent with induction of T-cell unresponsiveness of the $CD4^+$ cells [37]. In other aspects, our results vary from those of Agrawal et al., who found that MUC1-inhibited T cells retained their ability to secrete IFNγ in response to an antigenic challenge and suggested that "split tolerance" was being observed, whereby T cell proliferation was inhibited but cytokine secretion was unaffected. In addition, they found that MUC1 did not block the stimulatory effect of anti-CD28 antibody on the proliferative response. Our studies with anti-CD28 antibody were performed with resting $CD4^+$ cells. The studies of Agrawal et al. used peripheral blood T cells that had been cultured for 6–7 days with allogeneic lymphocytes. CD28 expression by these allo-antigen-stimulated cells was likely higher and presumably included a substantial proportion of $CD8^+$ cells. There is evidence of functional differences between the CD28 molecule expressed on $CD4^+$ and $CD8^+$ cells [13]. The production of IFNγ by $CD8^+$ cells may not require CD28-mediated costimulation [17]. Finally, different sources of mucin were used. The mucin studied herein was derived from patients with mucinous colorectal cancers and contained MUC2, the predominant mucin produced by the human colon [43]. In contrast to MUC1, which is expressed by the apical membranes of most polarized epithelial cells of the respiratory, digestive and genitourinary tract, including breast tissue, MUC2 is secreted by goblet cells of the colon, small intestine, and bronchus. MUC2 is a gel-forming mucin with domains containing high levels of cysteines and mannose-rich *N*-glycans [39]. Other large, secretory glycoproteins produced by the colon, such as MUC4, may also have been present. There are similarities in the effects of CCM observed to the effects of another tumor-derived product, namely, gangliosides, which are large, sialic-acid-containing glycosphingolipids. Gangliosides have been shown to block IL2 and IFNγ gene transcription without inhibiting the production of IL-10 mRNA [23].

The costimulatory signal generated through CD28 results in IL-2 production. Depending of the stimulus and the responding cells the production of other cytokines, including IFNγ, TNFα, granulocyte/macrophage-colony-stimulating factor, IL-4, IL-5, IL-6, and IL-10, can also be modulated [45]. The switch of TH1-associates cytokines, namely IL-2 and IFNγ, to TH2-associated cytokines, namely IL-4, IL-5, and IL-10, has been suggested to play a role in the escape of tumors from immune effectors, including that in patients with colorectal cancer [34]. Although IL-2 and IFNγ production could still detected and although up-regulation of IL-10 was not observed, the production of IL-10 was not inhibited, which suggests that CCM would promote TH2-associated, humoral responses. An increase in soluble IL-2 receptor levels has been implicated in the TH1 to TH2 switch observed in colorectal cancer patients [5]. It has recently been reported that MUC1 induces a CTL response in mice but an antibody response in humans [3]. This switch in the immune response appears to be caused by cross-reacting antibodies that are present in humans but not in mice. In addition, transgenic mice expressing *MUC1*, which are tolerant to MUC1 protein, fail to exhibit any switching of immunoglobulin class to the IgG subtypes, a process that requires TH activity [36].

The mechanism by which CCM modulate immune responses will require further study. That CCM did not interfere with signaling through the TCR and that pretreatment of $CD4^+$ with anti-CD28 mAb abrogated the suppressive effects of CCM on IL-2 secretion suggest that CCM may modulate costimulatory signals. In addition, CCM decreased the binding of an anti-CD28 mAb, although it is possible that other antibodies may not be affected. How CCM affects CD28 but not CD3 is not known. The large size and anti-adhesive properties of mucins have been implicated in their immunosuppressive effects. Suppression of IL-2 production by CCM was dependent on the maintenance of its glycosylation and sialylation. In animal models inhibition of mucin glycosylation greatly reduces the metastatic potential of mucin-producing tumors [7]. B7-1 and B7-2 are highly glycosylated molecules and alterations in their glycosylation have been demonstrated to alter their binding properties to CD28 and CTLA-4 [22]. Soluble MUC1 mucin has been shown to inhibit the adhesion of cancer cells to endothelium through ICAM-1, which suggests the possibility that CCM can induce anergy by interfering with the costimulatory interaction of lymphocyte-function-associated antigen (LFA-1) with ICAM-1 [6, 35].

Whether these and other observations of the immunosuppressive effects of mucins made in vitro and in animal models are relevant to human cancer will also require further study. A $CD4^+$ cell pleocytosis is often detected in the draining lymph nodes of patients with colorectal cancer [1, 42]. Our results would indicate that the ability of these $CD4^+$ cells to mediate TH1 activity effectively is compromised. The capacity of CCM to modulate cytokine production of resting $CD4^+$ cells by inhibiting costimulatory interactions has implications for the immunotherapy of human adenocarcinomas. Circulating CCM would be predicted to limit the capacity of vaccine approaches using oligopeptides corresponding to MUC1 or MUC2 to elicit cellular immune responses effectively. Although it has been demonstrated that poorly immunogenic murine tumor cells can be rejected in vivo when transformed to express costimulatory ligands, this approach, as well as the use of specific cytokines which increases circulating levels of CCM [16], will be ineffective in mucin-secreting tumors: the expansion of sufficient TH1 activity in situ may be continually abrogated.

## References


1. Adachi W, Sugenoya A, Iida F (1991) Immune competent cells of regional lymph nodes in colorectal cancer patients. I. Flow cytometric analysis of lymphocyte subpopulation. J Surg Oncol 46:110
2. Agrawal B, Krantz MJ, Reddish MA, Longenecker BM (1998) Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. Nat Med 4:43
3. Apostolopoulos V, Osinski C, McKenzie IFC (1998) MUC1 cross reactive ga1α (1, 3) gal antibodies in humans switch immune responses from cellular to humoral. Nature Med 4:315
4. Barnd DL, Lan MS, Metzgar RS, Finn OJ (1989) Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. Proc Natl Acad Sci USA 86:7159
5. Berghella AM, Pellgrini P, Del Beato T, Marini M, Tomei E, Adorno D, Casciani CU (1998) The significance of an increase in soluble interluekin-2 receptor level in colorectal cancer and its biological regulating role in the physiological switching of the immune response cytokine network from TH1 to TH2 and back. Cancer Immunol Immunother 45:241
6. Bohmig GA, Kovarik J, Holter W, Pohanka E, Zlabinger GJ (1994) Specific down-regulation of proliferative T cell alloresponsiveness by interference with CD2/LFA-2 and LFA-1/ICAM-1 in vitro. J Immunol 152:3720
7. Bresalier RS, Niv Y, Byrd JC, Duh Qy, Toribara NW, Rockwell RW, Dahiya R, Kim YS (1991) Mucin production by human colonic carcinoma cells correlates with their metastatic potential in animal models of colon cancer metastasis. J Clin Invest 87:1037
8. Chen L, Ashe S, Brady WA, Hellstrom I, Hellstrom I, Hellstrom KE, Ledbetter JA, McGowan P, Linsley PS (1992) Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell 71:1093
9. Doran T, Stuhlmiller H, Kim JA, Martin EW, Triozzi PL (1997) Oncogene and cytokine expression of human colorectal tumors responding to immunotherapy. J Immunother 20:372
10. Fearon E, Pardoll D, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Volgelstein B, Frost P (1990) Interleukin-2 production by tumor cells bypass T helper function in the generation of an antitumor response. Cell 60:397
11. Freeman GJ, Borriello F, Hodes RJ, Reiser H, Hathcock KS, Laszlo G, McKnight AJ, Kim J, Du L, Lombard DB, Gray GS, Nadler LM, Sharps AH (1993) Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. Science 262:907
12. Fung PYS, Longenecker BM (1991) Specific immunosuppressive activity of epiglycanin, a nucin-like glycoprotein secreted by a murine mammary adenocarcinoma TA3-HA). Cancer Res 51:1170

13. Ghiotto-Ragueneau M, Battifora M, Truneh A, Waterfield MD, Olive D (1996) Comparison of CD28-B7.1 and B7.2 functional interaction in resting human T cells: phosphatidylinositol 3-kinase association to CD28 and cytokine production. Eur J Immunol 26:34
14. Gillis S, Ferm MM, Ou W, Smith KA (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. J Immunol 120:2027
15. Green JB, Timmcke AE, Mitchell WT, Hicks TC, Gaithright B Jr, Ray JE (1993) Mucinous carcinoma – just another coon cancer? Dis Colon Rectum 36:49
16. Greiner JW, Guadagni F, Goldstein D, Borden EC, Ritt RE, Witt P, LoBuglio AF, Saleh MN, and Schlom J (1991) Evidence for the elevation of serum carcinoembryonic antigen and tumor-associated glycoprotein-72 levels in patients administered interferons. Cancer Res 51:4155
17. Guerder S, Carding SR, Flavell RA (1995) B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. J Immunol 155:5167
18. Hathcock KS, Laszlo G, Dickler HB, Bradshaw J, Linsley P, Hodes RJ (1993) Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. Science 262:905
19. Hayes DF, Siberstein DS, Rodrique SW, Kufe DW (1990) DF3 antigen, a human epithelial cell mucin inhibits adhesion of eosinophils to antibody-coated targets. J Immunol 145:962
20. Ho JJ, Siddiki B, Kim YS (1995) Association of sialyl-Lewis (a) and sialyl-Lewis (x) with MUC-1 apomucin in a pancreatic cancer cell line. Cancer Res 55:3659
21. Hodge JW, Abrams S, Schlom J, Kantor JA (1994) Induction of antitumor immunity by recombinant vaccina viruses expressing B7-1 and B72-2 costimulatory molecules. Cancer Res 54:5552
22. Hollsberg P, Scholz C, Anderson DE, Greenfield EA, Kuchroo VK, Freeman GJ, Hafler DA (1997) Expression of a hypoglycosylated from of CD86 (B7-2) on human T cells with altered binding properties to CD28 and CTLA-4. J Immunol 159:4799
23. Irani DN, Lin K-I, Griffin DE (1996) Brain-derived gangliosides regulate the cytokine production and proliferation of activated T cells. J Immunol 157:4333
24. Jenkins MK, Taylor PS, Norton SD, Urdahl KB (1991) CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J Immunol 147:2461
25. Jerome KR, Barnd DL, Bendt KM, Boyer CM, Taylor-Papadimitriou J, McKenzie IFC, Bast R, Finn OJ (1991) Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule perferentially expressed by malignant cells. Cancer Res 51:2908
26. Kim JA, Martin EW, Aldrich WA, Bender JA, Triozzi PL (1993) IL-2 production by T-helper lymphocytes is inhibited by soluble tumor-derived mucin via the CD28 pathway. Proc Am Assoc Cancer Res 34:450
27. Lan MS, Hollingsworth MA, Metzgar RS (1990) Polypeptide core of a human pancreatic tumor mucin antigen. Cancer Res 50:2997–3001
28. Laskin DL, Sirak AA, Robertson FM, Laskin JD (1991) Distinct patterns of chemotactic peptide-induced calcium mobilization in differentiated myeloid leukemia cells and peripheral blood neutrophils. J Leukoc Biol 49:369
29. Linsley PS, Brady W, Grosmarie L, Aruffo A, Damle NK, Ledbetter JA (1991) Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin-2 mRNA accumulation. J Exp Med 173:721
30. Linsley PS, Brady W, Urnes M, Grosmarie L, Damle NK, Ledbetter JA (1991) CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med 174:561
31. Mueller DL, Jenkins MK, Chiodetti L, Schwartz RH (1990) An intracellular calcium increase and protein kinase C activation fail to initiate cell proliferation in the absence of a costimulatory signal. J Immunol 144:3701
32. Norton SD, Zuckerman L, Urdahl KB, Shefner R, Miller J, Jenkins MK (1992) The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells. J Immunol 149:1556
33. Ogata S, Malmonis PJ, Itkowitz SH (1992) Mucins bearing the cancer-associated Sialosyl-Tn antigen mediated inhibition of natural killer cell cytotoxicity. Cancer Res 52:4741
34. Pellegrini P, Berghella Am, Del Beato T, Cicia S, Adorno D, Casciani CU (1996) Disregulation in TH2 an TH2 subsets of $CD4^+$ T cells in peripheral blood of colorectal cancer patients and involvement in cancer establishment and progression. Cancer Immunol Immunother 42:1
35. Regimbald LH, Pilarski LM, Longenecker BM, Reddish MA, Zimmermann G, Hugh JC (1996) The breast cancer mucin MUC1 as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. Cancer Res 56:4244
36. Rowse GJ, Tempero RM, VanLith ML, Hollingsworth MA, Gendler SJ (1998) Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. Cancer Res 58:315
37. Schall TJ, O'Hehir RE, Goeddel DV, Lamb JR (1992) Uncoupling of cytokine mRNA expression and protein secretion during the induction phase of T cell anergy. J Immunol 148:381
38. Schwartz RH (1992) Costimulation of T lymphocytes: the role of CD28, CTLA-4 and B7/BBl in interleukin-2 production and immunotherapy. Cell 71:1065
39. Seregni S, Botti C, Massaron S, Lombardo C, Capobianco A, Bogni A, Bombardieri E (1997) Structure, function and gene expression of epithelial mucins. Tumori 83:625
40. Sheer DG, Schlom J, Cooper HL (1998) Purification and composition of the human tumor-associated glycoprotein (TAG-72) defined by monoclonal antibodies CC49 and B-72.3 Cancer Res 48:6811
41. Townsend SE, Allison JP (1993) Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. Science 259:368
42. Triozzi PL, Kim JA, Aldrich WA, Young D, Sampsel J, Martin EW (1994) Identification of tumor-reacting lymph node lymphocytes in vivo using radiolabeled monoclonal antibody. Cancer 73:580
43. Tytgat KMAJ, Buller HA, Opdam FJM, Kim YS, Einerhand AW, Dekker J (1994) Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 107:1352
44. Van de Wiel-van Kemenade E, Ligtenberg MJL, Boer AJ de, Buijs F, Vos HL, Melief CJM, Hilkens J, Figdor CG (1993) Episialin (MUC1) inhibits cytotoxic lymphocyte-target cell interaction. J Immunol 151:767
45. Van Gool SW, Vandenberghe P, Boer M de, Ceuppens JL (1996) CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. Immunol Rev 153:47
46. Williams TM, Moolten DM, Makni H, Kim HW, Kant JA, Kamoun M (1992) CD28-stimulated IL-2 gene expression in Jurkat T cells occurs in part transcriptionally and is cylcosporine-A sensitive J Immunol 148:2609