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EFFECTS OF CHOLERA TOXIN ON CELLS OF IMMUNE SYSTEM

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ABSTRACT

In this study, we analysed the effects of Cholera Toxin (CT) on cells of immune system. In particular, the mechanisms underling the inhibition of T cell proliferation mediated by CT on human $CD4^+$ and $CD8^+$ T lymphocytes were analysed. We observed that CT prevents the early activation steps of T lymphocytes and that these effects involve the modulation of costimulatory molecules CTLA-4 and CD28. We observed that CT up-regulates the expression of the inhibitory molecule CTLA-4 in resting $CD4^+$ and $CD8^+$ T lymphocytes. The regulation of CTLA-4 expression by CT is at the transcriptional level. Indeed, in cells treated with CT we observed an increase of two mRNA variants coding for the membrane and the soluble CTLA-4 molecules. In parallel with the up-regulation of the inhibitory molecule CTLA-4, CT down-modulates the costimulatory molecule CD28 on $CD4^+$ and $CD8^+$ resting T cells. The increased expression of CTLA-4 plays a role in controlling T cell activation and function as blocking anti-CTLA-4 $F(ab')_2$ mAbs partially prevents the inhibition mediated by CT. We evaluated the function of CT-pre-treated $CD4^+$ T lymphocytes and we observed that they are able to inhibit the proliferation of autologous T lymphocytes stimulated with anti-CD3 mAbs. It is interesting that this phenomenon is, at least in part, a result of the release of extracellular cAMP. Therefore, by analysing the direct effects exerted by extracellular cAMP as a primary messenger on different cell types, we found that extracellular cAMP inhibits T cell proliferation and that it is able to interfere with the differentiation of monocytes into DCs. Monocytes induced to differentiated into DCs in the presence of extracellular cAMP, do not express CD1a molecules and retain the expression of CD14 acquiring a macrophages-like phenotype. Furthermore, they strongly up-regulate MHC class I and class II and CD86 costimulatory molecules giving rise to an activated population able to stimulate allogeneic T cell response. In addition, they produce a distinct pattern of cytokines upon maturation stimuli, they are unable to produce TNFα and IL-12 and they release high amount of IL-6 and IL-10. Furthermore, monocytes differentiated in the presence of cAMP show

a reduced capacity of inducing the differentiation of IFNγ producing $CD4^+$ T lymphocytes.

Finally, the mechanisms through which extracellular cAMP can be sensed by the cells were studied. By using different adenosine receptors antagonists, we found that an extracellular cAMP-adenosine pathway is involved in the effects mediated by exogenous cAMP, suggesting that extracellular cAMP acting as primary messenger can be sensed by the cells of immune system and can modulate their functions.

KEY WORDS

CT, CTLA-4, CD28, Regulatory T cells, extracellular cAMP, DCs, monocytes.

ABSTRACT

In questa tesi sono stati analizzati gli effetti della Tossina Colerica (CT) sulle cellule del sistema immunitario. In particolare, sono stati studiati i meccanismi di inibizione della proliferazione dei linfociti T $CD4^+$ e $CD8^+$ umani da parte della CT. E' stato osservato che la CT è in grado di prevenire l'attivazione dei linfociti T nelle fasi precoci e tale inibizione coinvolge la modulazione dell'espressione delle molecole CTLA-4 e CD28. E' stato osservato che la CT up-regola l'espressione delle molecole inibitorie CTLA-4 e down-modula le molecole costimolatorie CD28 sui linfociti T $CD4^+$ e $CD8^+$ resting. L'incremento dell'espressione delle molecole CTLA-4 da parte della CT gioca un ruolo nel controllare l'attivazione e la proliferazione dei linfociti T, infatti, abbiamo osservato che anticorpi bloccanti anti-CTLA-4 $F(ab')_2$ sono in grado di prevenire, anche se parzialmente, tale inibizione. I nostri studi hanno valutato, inoltre, la funzione di linfociti T pre-trattati con la CT e abbiamo osservato che essi sono in grado di inibire la proliferazione di linfociti T autologhi stimolati con anti-CD3. Abbiamo inoltre osservato che questo fenomeno è mediato dal rilascio di cAMP all'esterno delle cellule.

Alla luce di questi risultati, abbiamo analizzato gli effetti esercitati dall'cAMP extracellulare come primo messaggero su diversi tipi cellulari. L'cAMP extracellulare è in grado di inibire la proliferazione dei linfociti T ed è in grado di interferire con il differenziamento dei monociti in cellule dendritiche (DCs). Infatti, monociti differenziati in presenza di cAMP esogeno, non esprimono molecole CD1a e mantengono l'espressione di molecole CD14, acquisendo un fenotipo simile ai macrofagi. Tuttavia, le cellule generate in presenza di cAMP esogeno esprimono alti livelli di molecole MHC di classe II e di classe I e molecole costimolatorie CD86, mostrando un fenotipo attivato in grado di stimolare risposte T allogeniche. Inoltre, tali cellule non sono in grado di produrre TNF-α e IL-12, ma rilasciano quantità elevate di IL-6 e di IL-10. Monociti trattati con cAMP extracellulare hanno una capacità ridotta di indurre il differenziamento di linfociti T $CD4^+$ che producono IFN-γ.

Infine, è stato investigato il meccanismo attraverso il quale il cAMP extracellulare interagisce con le cellule. Utilizzando diversi antagonisti dei recettori dell'adenosina, abbiamo osservato che gli effetti mediati dal cAMP extracellulare possono essere prevenuti. Tali risultati suggeriscono che l'cAMP sia trasformato in adenosina e che tale molecola, attraverso l'interazione con i suoi recettori, sia responsabile degli effetti mediati dal cAMP esogeno.

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ABBREVIATION

PBMC: Peripheral Blood Mononuclear Cells;

dbcAMP: dibutyryl cAMP;

CT: Cholera Toxin;

CT-B: Cholera Toxin B subunit

FSK: Forskolin;

APC: Antigen Presenting Cells;

DCs: Dendritic Cells;

CFSE: carboxyfluorescein succinimidyl ester

Tregs: regulatory T cells;

LPS: lipopolysaccharide;

NECA: 5'-(N-ethylcarboxamido)-adenosine;

CSC: 8-(3-Chlorostyryl)caffeine;

MRS 1754:N-(4-Cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7 hexahydro-1H-purin-8-yl)-phenoxy]-acetamide;

DMSO: dimethyl sulfoxide

INTRODUCTION

1. The Immune System

The immune system is a complex system of defence based on the recognition of foreign molecules against cellular and molecular interactions. It is evolved to protect the body from bacteria, viruses or parasites infections.

The immune system has several specific characteristics of fundamental importance for its effective functions, in particular the specialization of responses to different microbes and the ability to discriminate between autologous constituents and foreign antigens concur to maintain homeostasis in the body. The high specificity and the capability to generate an immunological memory make the system capable of generating effective immune responses in order to effectively combat infections. The diversification of the immune responses is essential to defend the body against the myriad of possible environmental pathogens. The maitainance of the homeostasis allows the body to keep an immunological balance through the elimination of apoptotic or death cells, through the identification and destruction of tumor cells or through the control of inflammatory or autoimmune reactions. The self-limitation allows the system to return to a state of rest after eliminating the foreign agent, thereby making possible an optimal response to other antigens that might occur. The ability of distinguish between self and non-self, and to be tolerance to self, is essential to prevent responses against self constituents, cells and tissues, while maintaining a different repertoire of specific lymphocytes for exogenous antigens.

Among various mechanisms of protection, we can distinguish the innate immunity, defined also natural immunity, which mediates the initial protection against infections and the form of immunity defined acquired or specific

immunity, which is stimulated by exposure to infectious agents and that, after repeated exposures to a particular microbe, increases their intensity and their defensive potential. The specific immune responses can be classified in humoral immunity and cellular immunity.

T lymphocytes and B lymphocytes are the main protagonists of the acquired immune response and both are derived from bone marrow precursors. T lymphocytes migrate from the bone marrow in the thymus where their mature. The B lymphocytes are key cells of humoral immune response, whereas T cells are important in cell-mediated immune response. T lymphocytes are equipped with a receptor for antigen (TCR), consisting of a α and a β chain or of a γ and δ chain, which arise after a gene rearrangement during maturation in the thymus (Marrack P. and Kappler J., 1987). In general, T lymphocytes have a receptor with a single specificity in accordance with the theory of "a cell, a receptor," even if it was found exceptions to this rule (Padovan E. et al., 1993). T lymphocytes, through their receptor, recognize antigens after they have been processed in form of peptides associated with molecules of the Major Histocompatibility Complex (MHC) (Babbitt B.P. et al., 1985). Previously, it was described that the TCR is highly specific for a single MHC-peptide complex, but other observations have revealed a more promiscuous recognition of peptides that do not even share the same sequence homology (Kersh G.J. and Allen P.M., 1996). Following antigen recognition through the TCR, a trimolecolar complex between TCR-peptide-MHC is formed, which leads to signal transduction and, thus, to the activation of T lymphocytes (Garcia KC. et al., 1996; Garboczi D.N. et al., 1996).

The $\alpha\beta^+$ T lymphocytes can be divided into two subgroups based on the expression of their co-receptors: the $CD8^+$ and $CD4^+$ T lymphocytes. The former are known as cytotoxic T lymphocytes (CTL) and recognize the antigen (Ag) in the context of MHC class I molecules. The MHC class I molecules are present on all nucleated cells and, in general, bind peptides derived from cytosolic proteins (eg viral peptides) (Germain R.N., 1994). The $CD8^+$ T lymphocytes are effector cells that mediate anti-viral and anti-intracellular pathogens responses. The $CD4^+$ T lymphocytes recognize Ag in the context of MHC class II molecules, which present peptides derived from protein acquired by endocytosis (eg bacteria phagocytosis) (Germain R.N., 1994). These lymphocytes are also known as helper because they cooperate with B lymphocytes to make them able to produce antibodies. The expression of MHC class II molecules is restricted mainly to professional antigen presenting cells (APC). The $CD4^+$ T lymphocytes differentiate into Th1, Th2, Th17 cells nad T regulatory, which differ in the production of different cytokines and for their effector functions. Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ), and they are involved the induction of inflammatory cell-mediated reactions. Th2 lymphocytes produce IL-4, IL-5, IL-6, IL-13 and IL-25 and are primarily involved in the induction of humoral immune response (Abbas A.K. et al., 1996; Romagnani S., 1997). Th17 cells produce IL-17 and play an important role in host defence against extracellular pathogens. (Bettelli E. et al., 2008). Another subpopulation of T cell are $CD4^+$ T lymphocytes, defined regulatory T cells, that act to suppress activation of the immune system and thereby maintain homeostasis and tolerance to self-antigens.

2. Antigen Presenting Cells (APC)

The antigen-presenting cell (APC) are specialised for presentation antigens to T cells. The APC are characterized by the expression of high levels of major compatibility complex (MHC) class I and II and adhesion/costimulatory molecules. The antigen presenting cells can be divided in three different cell types: dendritic cells (DCs), monocytes/macrophages and B cells.

Dendritic cells are distinguished by their ability to induce primary immune responses (Steinman R.M., 1991; Ibrahim MA. et al., 1995; Banchereau J. and Steinman R.M., 1998). Several DC types with different phenotypic and functional features have been identified in different tissues because of their origin, lymphoid or myeloid, or maturation stage (Pulendran B. et al., 1996; Wu L. and Dakic A., 2004). Two distinct views have emerged on how all these phenotypic and functional varieties are created during the development of DC system. The specialized DC lineage model suggests that the point of commitment to phenotypically and functionally defined DC subsets happens very early in hemopoiesis, so that DC subsets develop as separate lineages with distinct functions. The functional plasticity model, however, supports the idea that DC subsets represent different activation states or alternative cell fates of a single lineage dictated by the local microenvironment. Currently, there is no conclusive evidence to support exclusively either model. Further studies are needed to reconcile these different views.

Despite phenotypic and functional differences, DCs display common features such as morphology, high density of membrane HLA II, expression of CD1a, low

phagocytic activity, and a strong capacity to present antigens for the primary activation of naïve T cells. Immature DCs reside in peripheral tissues and constantly capture antigens from the local environment, process and present them in association with MHC molecules. The presence of microbial products or tissue damage in the environment allows DCs to initiate their migration to peripheral lymphoid organs and their transition from antigen-capturing cells into antigen presenting cells. The migration and functional transition of DCs correlate with: decreased antigen uptake, increased half-life of surface MHC-peptide complexes, upregulation of co-stimulatory molecules, altered expression of chemokine receptors and production of cytokines that are crucial for effector T cell (helper or cytotoxic) differentiation. As a result, antigens captured by DCs are, in the cell bound form, transported to and concentrated in the peripheral lymphoid organs (lymph nods and spleen) for presentation to antigen-specific T both $CD4^+$ and $CD8^+$ cells (Ridge J.P. et al., 1998; Wu L. and Dakic A., 2004). Another cell type, which belongs to the DC system, is the plasmacytoid pre-DCs (pDCs). The pDCs were originally identified in human blood and lymphoid tissues as plasmacytoid T cells or plasmacytoid monocytes due to their morphological similarity to plasma cells and expression of certain T cell markers and MHC class-II molecules. These cells, also termed pDC2 in human, have a phenotype different from the myeloid DCs ($CD11c^-$ $CD45Ra^{hi}$ $CD11b^-$ MHC-II^{lo} IL-$3R^{hi}CD4^+$) and are efficient type I interferons (IFNs) producing cells (Wu L. and Dakic A., 2004).

In the 1992 Banchereau characterized a method to culture DCs in vitro from stem cells $CD34^+$ and Sallusto and Lanzavecchia demonstrated that DCs differentiate from human peripheral blood monocytes. To date, blood monocytes are the most

commonly used precursor cells for generating human DCs in culture. Monocytes, in the presence of GM-CSF and IL-4 differentiate into DCs with immature phenotipe (Sallusto F. and Lanzavecchia A., 1994). These DCs can be further matured in the presence of proinflammatory cytokines such as Il-1β, TNF-α or LPS.

The macrophage cells, because of their phagocytosis ability, are involved in eliminating death or dying cells. When they capture several molecules in the course of inflammation, they increase the expression of MHC and costimolatory molecules and become antigen-presenting cells (APC) (Dijkstra C.D. et al., 1992; Miyazaki T. et al., 1993).

B cells capture soluble molecules through their Ag-specific receptors, the membrane immunoglubulins (Ig). Antigens are processed and presented in the context of MHC class II molecules (Lanzavecchia A., 1985). Antigens may also be internalized by pinocytosis and presented by B cells without the intervention of the receptor, but in this case the concentration of antigen must be 10.000 times greater (Lanzavecchia A., 1985). Several studies have demonstrated the ability of B cells to activate T lymphocytes (Kurt-Jones E.A. et al., 1988; Ashwell J.D., 1988; Constant S. et al., 1995; Falcone M. et al., 1998). This function is largely dependent on B cell state of activation and differentiation (Kakiuchi T. et al., 1983; Krieger J.I. et al., 1985; Mamula M.J. and Janeway C.A. Jr., 1993).

A fourth type of APC is represented by activated T cell. It was shown that activated T lymphocytes, both human and rat (Broeren C.J. et al., 1995; Reizis B. et al., 1994), express MHC class II molecules on their surface and also costimolatory molecules (Azuma M. et al., 1993). Therefore, it was formulated

the hypothesis that activated T cells, although they are not professional presenting cells, may present the antigen to other T cells and being involved in the amplification of the immune response (Lanzavecchia A. et al., 1988; Barnaba V. et al., 1994). Later it was shown that the presentation of Ag by T cells (presentation T: T) leads to a state of anergy rather than activation and thus contributes to induction of tolerance rather than the amplification of the immune response (Lamb J.R. et al., 1983; LaSalle J.M. et al., 1992; LaSalle J.M. et al., 1993; Lombardi G. et al., 1996; Faith A. et al. 1997).

Whether an antigen is presented by DCs, macrophages, B or T cells, it depends on its concentration, on the type of tissue infected and on the source of antigen itself as well as by on the activation status of the cells (Matzinger P. and Guerder S., 1989, Fuchs E.J. and Matzinger P., 1992). Moreover, as for T cells, it was shown that DCs, macrophages and B cells are involved not only in promoting the immune response, but also in induction of tolerance (Miyazaki T. et al., 1993; Gilbert K.M. and Weigle W.O., 1994; Finkelman F.D. et al., 1996; Steptoe R.J. and Thomson A.W., 1996).

3. Interaction between T lymphocytes and APC

The interaction between T cells and professional APC, which occurs through the trimolecolar complex TCR-MHC-peptide is not sufficient to induce a full activation of T lymphocytes. Additional signals are necessary, as was originally described in the "two-signal model proposed by Bretscher and Cohn and Lafferty et al. (Bretscher P. and Cohn M., 1970, Lafferty K.J., 1983), in

which costimolatory molecules play an important role. The main costimolatory molecules are B7-1 (CD80) and B7-2 (CD86) on APC and their ligands CD28 and CTLA-4 (CTLA-4) on T cells (Linsley P.S. and Ledbetter J.A., 1993; Schwartz R.H., 1992; June C.H. et al., 1994; Miller S.D. et al., 1995). CD28 is involved in the activation of T cells through the induction and stabilization of IL-2 (Linsley P.S. et al., 1991), while CTLA-4 has an inhibitory role in the activation of lymphocytes (Krümmel M.F. and Allison J.P., 1995; Liu Y., 1997). The interaction between T cells and APC occurs also through the molecules of the superfamily of TNFR-TNF (tumor necrosis factor receptor / tumor necrosis factor) (Armitage R.J., 1994, Smith C.A. et al., 1994). The TNFR family includes the CD27, CD30, CD40, CD95 (Fas), OX40 (CD134), 4-1BB, RANK, TNFRI / II and growth factor nerve receptor (NGFR). The family includes the TNF ligands CD27L (CD70), CD30L, CD40L (CD154), CD95L (FasL), OX40L, 4-1BBL, RANKL, TNF-α and NGF. One feature in common between these pairs of receptors and ligands is the capacity to regulate cell activation and / or apoptosis. An interesting aspect of some of these couples of receptors is that the molecular signals are in the double direction, they are able to signal to both T cells and APC. For example, the signal through the molecules CD40/CD40L, that induces an increase in the expression of B7 molecules on APC, results also in the increase of costimolatory molecules and cytokines release on T cells (Grewal I.S. et al., 1996). Also, signal through the B7-CD28 stabilizes the expression of CD40L on T cells that results in increasing the signal through CD40/CD40L on APC (Johnson-Leger C. et al., 1998). The activation of APC via CD40 makes them fully capable of activating $CD4^+$ and $CD8^+$ T lymphocytes without additional signals

(Schoenberger S.P. et al., 1998; Bennett S.R. et al., 1998). In addition, the CD40/CD40L interaction induces an increase in the expression of RANK on the DCs and the interaction RANK / RANKL plays a role in expansion of T cells mediated by DCs (Anderson D.M. et al., 1997). Communication between T cells and APC can also occur through the binding between OX40-OX40L. OX40 is expressed only on activated $CD4^+$ T cells (Paterson D.J. et al., 1987; Mallett S. et al., 1990) and it is responsible for a strong costimolatory signal (Kaleeba J.A. et al., 1998, Weinberg A.D., 1998). On the other hand, the binding of OX40L on DCs induces maturation, increases the production of cytokines and the expression of B7-1, B7-2, CD40 and ICAM1 (Ohshima Y. et al., 1997). The binding of OX40L on B cells determines their proliferation and differentiation into plasma cells (Stuber E. et al., 1995; Stuber E. and Strober W., 1996).

Additional molecules that have a role in the interaction between T cell-APC are CD2/LFA3 (Bell G.M. and Imboden J.B., 1995), CD5/CD5L (Bikah G. et al., 1998), and adhesion molecules as LFA1/ICAM1 (Wawryk S.O. et al., 1989, Dubey C. et al., 1995), VLA-4/VCAM-1 (Yednock T.A. et al., 1992). The expression of these molecules is not restricted only to T cells or APC. For example, activated T lymphocytes can express MHC class II (Broeren C.J. et al., 1995; Reizis B. et al., 1994), B7 (Azuma M. et al., 1993), OX40L (Baum P.R. et al., 1994), and CD70 (Hintzen R.Q. et al., 1994), while CD27 and CD28 was found on B cells and plasma cells, respectively (Hintzen R.Q. et al., 1994). This reinforces the concept of a two-way communication between APC and T cells. Based on the levels of expression of different surface molecules, different signals

occur during the interaction between T cells and APC that could lead both to activation and the inhibition of the immune response.

4. CD80 and CD86

The first ligand that was originally identified by the CD28 molecules is CD80 (Linsley P.S. et al., 1990). The human and murine genes coding for molecules CD80, are members of the superfamily of Immunoglobulins (Ig) and are located on chromosome 16 and 3, respectively. The discovery of CD86 was made later when it was been observed that the blockade of CD80 molecules with monoclonal antibodies were not able to completely inhibit the B cells activation by LPS (Azuma M. et al., 1993, Freeman G.J. et al., 1993a). This observation was further reinforced by the fact that mice that do not have the gene for CD80 molecules (Knockout), are still able to mount an immune response following an antigenic stimulation (Freeman G.J. et al., 1993b).

Both molecules CD80 and CD86 are highly conserved between mouse and human. Although CD80 and CD86 are structurally similar, they share only limited sequence homology (25%). The sequence homology is especially concentrated at the points of connection with their ligands CD28/CTLA-4 (Linsley P.S. et al., 1994). Both CD80 and CD86 molecules bind to CTLA-4 with an affinity 20-50 times higher than with CD28 (Linsley P.S. et al., 1994). Furthermore, the specificity of the interaction of CD80 and CD86 with CTLA-4 is different. A mutation of a single aminoacid in the motif MYPPPY of the molecules CTLA-4 abolishes the binding with CD86 but not with CD80 (Truneh A. et al., 1996). The

expression of CD80 and CD86 molecules are also different. Although both molecules CD80 and CD86 bind to CD28 and CTLA-4, they are expressed in different cell types with different kinetics. (June C.H. et al., 1994). CD80 is expressed mainly on DCs and is expressed at low levels on resting APC and T lymphocytes. CD86 is expressed at low levels on resting APC and at moderate levels on naive T cells (Hathcock K.S. et al., 1994). The expression of both molecules increases after activation but with different kinetics. CD86 increases rapidly on the APC with a peak of expression between 24 and 96 hours (Hathcock K.S. et al., 1994; Lenschow D.J. et al., 1994). In contrast, the expression of CD80 on B cells increases between 48 and 72 hours after activation, soon after its expression decreases rapidly. The constitutive expression and the rapid induction of CD86 molecules on APC suggested that they may play a major role in starting the immune response and therefore in activating T lymphocytes. In addition, these molecules are preferentially induced by particular stimuli such as the binding of immunoglobulin membrane, that induces the expression of CD86, but not of CD80 on B lymphocytes (Lenschow D.J. et al., 1994). It has been described that the interaction between CD40 on APC cells and CD40L on activated T cells results in an increase in the expression of CD80 and CD86 molecules on the APC (Grewal I.S. et al., 1996). Treatment with IL-4 and IFN-γ leads to a sharp increase in the expression of CD86 molecules, but not CD80. IL-10 prevents the expression of both molecules on peritoneal macrophages and causes a decrease in the expression of CD80, but not of CD86 on DCs (Ozawa H. et al., 1996). The gradients of expression of CD80 and CD86 molecules that have been observed in

human lymph nodes suggest that the expression both in space and time of these molecules is important for their specific functions.

In addition, it was described that the molecules CD80 and CD86 are expressed on T cells both in humans and in rodents (Azuma M. et al., 1993; Prabhu Das M.R. et al., 1995, Sansom D.M. and Hall N.D., 1993). CD86 is expressed in a constitutive way on non-activated T cells and its expression decreases after activation, whereas CD80 is not expressed on resting T cells, but is induced following activation (Prabhu Das M.R. et al. 1995, Chai J.G. et al., 1998). However, the functions of CD80 and CD86 molecules expressed by T lymphocytes have not been completely defined.

5. CD28 and CTLA-4

CD28 and CTLA-4 are membrane glycoproteins belonging to the superfamily of Immunoglobulins (Lenschow D.L. et al., 1996) and are expressed mainly on T lymphocytes. Human $CD4^+$ lymphocytes, as well as murine T cells express CD28 molecules constitutively. Following activation, the expression of CD28 molecules increases and then decreases after the binding with its natural ligands (CD80 and CD86). The reduction of CD28 molecules results in a decreased ability of T cells to mobilize intracellular calcium (Linsley P.S. et al., 1993a). A particular region of both CD28 and CTLA-4 contains a conserved sequence (MYPPY), which is required for binding to molecules CD80 and CD86 (Peach R.J. et al., 1994). The CTLA-4 molecule has an affinity 10 times greater than CD28 for CD80 and CD86 molecules (van der Merwe P.A. et al., 1997) and

CD28 has a kinetic dissociation 4 times fastest (Greene J.L. et al., 1996; van der Merwe P.A. et al., 1997). CTLA-4 also binds to CD86 with a lower affinity and faster dissociation kinetics than CD80 (Linsley P.S. et al., 1994; Morton P.A. et al., 1996). Although CD28 and CTLA-4 share their ligands and several structural properties, they differ in their expression on different cell types. CD28 is expressed on both activated and non-activated T lymphocytes, whereas the expression of CTLA-4 is more tightly controlled. Naïve T lymphocytes and memory T cells express very low levels of CTLA-4, whereas its expression increases following activation. The transcription of the gene ctla-4, the stability of mRNA and the intracellular trafficking of the synthesized protein are under the control of the mechanisms of T cells activation (Alegre M.L., et al., 1996). It was observed that the signals mediated by CTLA-4 in the presence of stimulation through the TCR and CD28 on resting T cells lead to an arrest of growth of T lymphocytes. When non-activated T lymphocytes are distinct in naïve T lymphocytes ($CD45RB^{high}$) and memory cells ($CD45RB^{low}$), the memory T cells are more sensitive to inhibitory signals mediated by CTLA-4 (Hamel M.E. et al., 1998). Inhibition of growth of resting T lymphocytes caused by the arrest of cell cycle in G_0/G_1 correlare with CTLA-4 expression (Kubsch S. et al., 2003). The signals mediated by CTLA-4 also determine the inhibition of synthesis of IL-2. (Blair P.J. et al., 1998). Following cellular activation the expression of CTLA-4 molecules increases and receptor-mediated signals lead to induction of apoptosis (Gribben J.G. et al., 1995; Scheipers P. and Reiser H., 1998). Mature T lymphocytes can undergo to different types of cell death. The apoptosis that occurs after activation of T lymphocytes (AICD, activation induced cell death)

depends on the Fas-FasL and often has the function to eliminate self-extinguish T lymphocytes or an active immune response. The programmed death that is caused by deprivation of growth factors, also called "passive death", could be involved in other mechanisms of inhibition of immune response (Van Parijs L. and Abbas A.K., 1998). It has been observed that apoptosis induced on cells activated through CTLA-4 molecules, does not depend of the interaction Fas-FasL, because of T cells isolated from mice that have mutated molecules Fas (MRL lpr/lpr) die in response to signals mediated by CTLA-4 after cell activation (Scheipers P. and Reiser H., 1998). Since CTLA-4 inhibits the synthesis of IL-2, apoptosis might be caused by the absence of growth factors, or alternative mechanisms may be involved. CTLA-4 may have different functions in resting or activated T lymphocytes (naïve and memory). Allison and colleagues (Chambers C.A. et al., 1996) suggest a model in which CTLA-4 on resting T cells increases the threshold for activation by favouring the weak signals mediated by TCR that could be important for the survival of naïve and memory T lymphocytes. On the other hand, on activated cells CTLA-4 could be important to end effector functions and help to turn off the immune response.

In addition, several studies have described that the blockade of CTLA-4 molecules in vivo, determines the break of anergy of T lymphocytes, induced by inoculation of a soluble antigen or a superantigen. These data suggest a possible role of CTLA-4 molecules in induction of tolerance in peripheral T lymphocytes (Perez V.L.L. et al., 1997; Walunas T.L. and Bluestone J.A., 1998). Therefore, it was observed that treatment with anti-CTLA-4 prevents the induction of tolerance in a mouse model of heart transplant (Judge T.A. et al., 1999). In attempt to

identify the mechanisms involved in the inhibition mediated by CTLA-4 molecules, they have been made some considerations that are not mutually exclusive. CTLA-4 could inhibit the response of T cells by competing with CD28 molecules for their ligands (CD80 and CD86), or it could mediate antagonistic signals to those mediated by CD28, alternatively, it could interfer with the signals mediated by TCR.

6. Activation of T lymphocytes

After the recognition of MHC-peptide complex by the TCR, a series of signals are trasducted from the surface within the T cells, through the ζ chains of the TCR and the complex formed by the molecular CD3. It follows a complex molecular cascade, which consists of a series of biochemical events such as protein phosphorylation, hydrolysis of inositol phospholipids and increase of Ca^{2+} ions within the cytoplasm (Weiss A. and Littman D.R., 1994; Cantrell D., 1996). At the same time, more biochemical signals are sent through the costimolatory molecules and all messages determine the transcription and/or the release of molecules that are required for the production of cytokines and for the proliferation and differentiation of effector functions (Mueller D.L. et al., 1989; Robey E. and Allison J.P., 1995).

An MHC-peptide complex can recruit a large number of TCR, a phenomenon called "serial triggering" (Valitutti S. et al., 1995). The TCR molecules engaged are internalized and degraded (Valitutti S. et al., 1997). In fact, it was assumed that the number of TCR molecules that decrease on the membrane of T cells is

directly correlated with the degree of activation of T lymphocytes (Viola A. and Lanzavecchia A., 1996; Bachmann M.F. et al., 1997). Full activation of T lymphocytes is achieved only when a minimum of TCR molecules are engaged, but this threshold may be lowered through costimolatory signals from molecules such as CD28 (Viola A. and Lanzavecchia A., 1996). In addition to a costimolatory stimulus, the activation of T cells is also dependent on the duration of antigenic stimulation (Lanzavecchia A., 1997; Iezzi G. et al., 1998). In this regard, the nature of the antigenic peptide may play an important role. It has been described that an altered activation of T lymphocytes through altered peptides (APL), correlate with differences in the duration of interaction MHC-peptide and TCR and/or in the phosphorylation of TCR ζ chain (Rabinowitz J.D. et al. 1996). Furthermore, the activation of T cells depends on the state of the cell itself. "Naïve" T lymphocytes, which have never encountered the antigen after positive selection in the thymus, and "memory" T cells are much more dependent on the signals and the source of costimolatory signals than effector cells, which are activated and are ready to carry out their effector functions (Matzinger P., 1994). Then the full activation of "naïve" T lymphocytes is done only upon antigen presentation by professional APC that express all necessary costimolatory molecules.

7. Immune tolerance

The immune system protects the host from a broad range of pathogenic microorganisms while avoiding misguided or excessive immune reactions that

would be deleterious to the host. The rearrangement of the TCR on T lymphocytes generates a potential repertoire of mature T lymphocytes for different antigens.

The non-responsiveness of the immune system towards autologous antigens is defined immunological tolerance *to self*. The loss of this feature results in immune responses to specific constituents of the body and to generation of autoimmunity. Therefore, understanding how to generate the repertoire of mature T cells is extremely important for understanding the specificity of T lymphocytes, it can help to discover the pathogenesis of these diseases and to stimulate new therapeutic approaches. Tolerance is induced through two main inhibitory mechanisms: central tolerance and peripheral tolerance. These processes allow the selection of cells that form the repertoire of mature peripheral T lymphocytes, which express TCR capable of recognizing only peptides derived from foreign antigens in combination with allelic forms of self-MHC molecules. Recently, it has been identified a new mechanism of self preservation mediated by a subpopulation of T regulatory cells (Tregs), that are able to suppress the activation of those autoreactive cells (Abbas A.K. and Lichtman A.H., 2001). Disruption in the development or function of Tregs is a primary cause of autoimmune and inflammatory diseases in humans and animals (Sakaguchi S. et al., 2008).

8. Regulatory T cells

Treg cells are a subpopulation of T lymphocytes involved in the suppression a broad spectrum of immune responses including those against autologous tumour cells, allergens, pathogenic or commensal microbes, allogeneic organ transplants and foetus during pregnancy (Miyara M. and Sakaguchi S., 2007).

Treg cells were rediscovered in the mid-1990s by Shimon Sakaguchi (Sakaguchi S. et al., 1995), who was the first to demonstrate that a minor population of $CD4^+$ T cells that coexpressed the CD25 antigen functioned as Treg cells in adult mice. Interest in Treg cells has exploded over the past 10 years, and numerous other T cell populations have now been claimed to exhibit regulatory activity (Shevach E.M., 2006).

There are several reports on Treg populations that show that they are developmentally, phenotypically or functionally different (Jiang H. and Chess L., 2006).

Regulatory T cells have been shown to develop in the thymus, but the cellular basis of their maturation, such as the role of antigen presentation, diversity of T-cell receptor (TCR) usage and potential interactions in the thymic environment, remains unclear. Presumably, the regulatory T cells in normal animals are polyclonal populations that recognize a diversity of self-antigens, but it is unclear if they are biased towards recognition of a particular type or subset of self-antigens. T cells with regulatory function can also be generated by the activation of mature, peripheral $CD4^+$ T cells (Barrat F. J. et al., 2002), and both regulatory

and effector T cells can be generated from the same mature T cell precursors (Jordan M.S. et al., 2001), presumably depending on qualitative and/or quantitative differences in antigen exposure. Many studies have shown that culture of naïve $CD4^+$ T cells under particular conditions results in the generation of T reg cells (Bluestone J.A. and Abbas A.K., 2003).

They are mainly classified into natural Tregs and induced or adaptive Tregs. Naturally occurring regulatory T cells (nTreg) are $CD4^+CD25^+$ and $CD8^+CD25^+$. They constitutively express the interleukin (IL)-2 receptor α chain (CD25) and also the transcription factor forkhead box P3 (FOXP3) (Sakaguchi S., 2004). Other molecules expressed by nTreg include the glucocorticoid –induced tumor necrosis factor receptor (GITR), CTLA-4 (CD152), galectin-1, HLA-DR, CD38, CD62L, OX40L, CD122, CD103, TNFR2 and TGF-βR1 (Myara M. and Sakaguchi S., 2007).

A feature of $CD25^+CD4^+$ nTregs is that they constitutively express CTLA-4, whereas naïve T cells express this molecule only after activation (Sakaguchi S., 2004). Several possible roles for CTLA-4 in Treg-mediated suppression are suggested. One role is that CTLA-4 on Tregs might interact with the CD80 and CD86 molecules on APCs and transduce a co-stimulatory signal to Tregs. CTLA-4 blockade therefore prevents Treg activation and, hence, attenuates suppression, causing autoimmune disease. This blockade might also enable interaction between CD28 molecules expressed by Tregs and CD80 and CD86 less competitively and, hence, more easily transduce a suppression-attenuating signal to Tregs, because strong ligation of the CD28 molecules together with TCR stimulation can

abrogate Treg-mediated suppression. Another possible role of CTLA-4 for Treg function is that it might directly mediate suppression.

Tregs might also downregulate DC expression of CD80 and CD86 via CTLA-4, hampering activation of other T cells by DCs (Misra N. et al., 2004). Alternatively, CTLA-4 on Tregs might ligate CD80 and, to a lesser extent, CD86 expressed by activated responder T cells and directly transduce a negative signal to the responder T cells. In addition, because CTLA-4 upregulates the expression of lymphocyte function-associated antigen-1 (LFA-1), one mode of contribution of CTLA-4 to Treg-mediated suppression might be that it augments the physical interaction between Tregs and APCs, thus enhancing the activation of Tregs or their suppressive interaction with other T cells or APCs (Myara M. and Sakaguchi S., 2007).

Adaptive Tregs are induced from naïve T cells by specific modes of antigenic stimulation, especially in a particular cytokine milieu (Roncarolo M.G. et al., 2006). One subset of $CD4^+$ regulatory T cells is defined by is ability to produce high levels of IL-10 (Tr1). $CD4^+$ Tr1 cells have been identified in mice and human, and can be found particularly in the intestinal mucosa (Groux H. et al. 1997). Phenotipically, Tr1 iTregs are similar to nTregs in that they are both anergic in vitro and express CTLA-4 (Roncarolo M.G: et al., 2001). In contrast to nTregs, Tr1 iTregs do not express high levels of CD25 or FoxP3 (Berthelot J.M. and Maugars Y., 2004).

Th3 iTregs, which are charaterized by the production of TGF-β, occur primarly after ingestion of a foreign antigen via the oral route. The presence of TGF-β induces differentiation of naïve T cells into Th3 T cells (Berthelot J.M. and

Maugars Y., 2004). Th3 cells, like nTregs, express CTLA-4 on their surface, the triggering of which results in the secretion of TGF-β. FoxP3 and CD25 expression are also upregulated after restimulation of TGF-β induced Th3 cells. Unlike nTregs, the main suppressive mechanism of Th3 cells is dependent on the production of TGF-β, which suppresses the proliferation of Th1 and Th2 cells (Lan R.Y. et al., 2005).

It has been shown that naïve T cells can differentiate into foxp3^{+} nTreg-like cells in certain *in vivo* and *in vitro* situations (Apostolou I. and von Boehmer H., 2004; Kretschmer K. et al., 2005).

9. Bacterial Toxins

In the course of his life, every individual comes into contact with many microorganisms that can cause various reactions, many of which are pathological. The human body provides a favourable environment to their growth, because every district, every body is different chemically and physically from the other and this creates selective environments for pathogens. Pathogenic bacteria, particularly those who have co-evoluted with their hosts, possess a wide range of specific molecules capable of modulating various cellular functions. The infection usually begins at the mucosal epithelium and, whether the association between the pathogen and tissue is not close, the microorganisms are removed by physical processes such as mucus. If, however, the bacteria bind specifically to epithelial cells, through recognition of specific receptors, an infection of the tissue may occurs. At this point, the mucosal barrier can be damaged, allowing the

microorganism to penetrate into the below tissues (colonization) (Brock T.D. et al., 1994-1995).

It is possible to consider two types of bacteria: intracellular microorganisms, able to survive and replicate within the host cell that can engulf them (phagocytes) (Abbas A.K. et al., 2000, Brock T.D. et al. 1994-1995), and extracellular microorganisms, capable of replication outside of host cells (Abbas A.K. et al., 2000). Those that cause diseases can carry out their action through two main mechanisms: the induction of an inflammatory process, resulting in tissue destruction or the production of toxins. These bacterial products possess precise biochemical activities, which allow them to stimulate or interfere with a variety of cellular processes. Delivery of these bacterial effectors of virulence is mediated by accessory proteins (referred to as toxin "B" subunits) that target specific receptors and vesicular trafficking pathways to deliver the enzymatically active components (known as toxin "A" subunits) to the appropriate cellular location (Galán J.E., 2005).

The toxins are mostly proteins, they are divided in endotoxins, that are integral components of bacterial cell wall and released in large amounts only when the cell is lysed, and exotoxins, which are secreted by the bacteria and able to proceed from the outbreak of infection to other parts of the body causing damage in distant regions from the site where the microorganisms are located (Brock T.D. et al., 1994-1995). The bacterial toxins are generally sensitive to heat and gastric juices, and with specificity of action and high toxicity.

Some of the most potent poisons known are exotoxins synthesized from pathogenic bacteria. There are exotoxins, called enterotoxins, whose main

characteristic is to act in a specific manner in the gut causing an abundant secretion of fluid in the intestinal lumen, resulting in diarrhea symptoms (Brock T.D. et al., 1994-1995).

10. Cholera Toxin

Cholera Toxin (CT) is one of the well known enterotoxins, which is produced by the gram-negative bacterium *Vibro cholerae*. Clinical data support the notion that this bacterium has the ability to induce two types of infectious diarrhea: a decrease of intestinal absorption due to tissue damage or inflammation and an induction of intestinal fluid secretion due to the production of a single virulence factor, the CT. Strains that do not produce CT produce lesser diarrhea, but the desease is more inflammatory in nature, while strains that produce CT induce a highly secretory diarrhea that develops in the absence of inflammation (Fullner Satchell K.J., 2003).

Cholera Toxin is a member of the AB class of bacterial toxins encoded by two genes. The ctxA gene encodes a 27.2 kDa subunit, the subunit A, with activity of ADPribosyltransferase NAD-dependent, while the other ctxB gene encodes a subunit of 11.6 kDa, the B subunit, which function as link to the cell surface. The holotoxin is composed of five CT-B subunits assembled in a pentameric ring that surrounds the only CT-A subunit (Fullner Satchell K.J., 2003; Lavelle E.D.C. et al., 2004, Spangler B.D., 1992). The B subunit has high affinity for the glycosphingolipids, particularly GM1 [Gal (1-3) GalNAc (β1-4) (NeucAc (α2-3)) Gal (β1-4) GLC (β1-1) cerammide.] (Lavelle E.D.C. et al., 2004), which is a

constituent of cell membranes and present in all type of cells. The A moiety is initially synthesized as a single CTA polypeptide that undergoes proteolytic processing to yield a disulfide-linked A1/A2 heterodimer.

After binding to the cell surface, the toxin is internalized by a process of endocytosis. Once inside the cell, it is released into the cytosol trough vesicles from the Golgi cisterns. The active pool of internalized toxin subsequently moves to the endoplasmic reticulum by retrograde vesicular transport. In the ER, the disulfide bond linking CTA1 to CTA2/CTB_5 is reduced by a protein disulfide isomerase (PDI). The A1 subunit of 21.8 kDa is then retro-traslocated to the cytosol, where it ribosylates the α subunit of a guanosine 5' Triphosphate (GTP)-binding protein (Gs), resulting in the permanent adenylate cyclase activation of the host. This activation leads to an increase in the intracellular concentration of cyclic adenosine monophosphate (cAMP) (Fullner Satchell K.J., 2003; Lavelle E.D.C. et al., 2004).

The enterotoxin, in addition to its role as an inductor of massive intestinal fluid secretion, has been recognized as a potent immunomodulators by its ability to increase the intracellular AMP (Fullner Satchell K.J., 2003).

The cyclic AMP (cAMP) is a ubiquitous messenger that influences many cellular functions (Ahuja N. et al., 2004). Several families of adenylate cyclase regulate its concentration and production from ATP (Baker A.D. and Kelly J.M., 2004). Any alteration in the amount of cytosolic cAMP has a profound effect on the various processes of the cell. There are several toxins secreted by pathogenic bacteria that alter the concentration of intracellular cAMP (Ahuja N. et al., 2004).

11. Cyclic AMP in the regulation of immune responses

Cyclic AMP is a versatile and common second messenger controlling numerous cellular processes (Taskén K. and Stokka A.J., 2006). In particular, increased intracellular cAMP concentrations within T lymphocytes inhibit the proliferation and the production of IL-2 (Johnson K.W. et al., 1988; Taskén K. and Stokka A.J., 2006), therefore it has been described that cAMP interferes with the early events of T lymphocytes activation (Vang T. et al., 2001). It has also been shown that elevation of intracellular cAMP promotes IL-10 secretion and Th2 activation while it inhibits IL-12 production and Th1-mediated effector functions (Suàrez A. et al., 2002). A number of physiological and pharmacological agents, such as PGE_2, IL-1α, histamine, β_2-adrenergic receptors agonists and phosphodiesterases inhibitors share the ability to elevate intracellular cAMP in T lymphocytes. cAMP-elevating agents show anti-infiammatory potential in the treatment of inflammatory diseases such as asthma and chronic obstructive pulmonary disease. cAMP binds and activates the protein kinase A (PKA), which phosphorilates effector proteins like Csk or transcription factors binding to particular sequences of DNA (CREB) (Sassone-Corsi P. et al., 1995). However, other pathways are described for cAMP, which are independent from PKA-mediated signals (Torgersen K.M. et al., 2002). cAMP is also able to open Ca^{2+} channels and the levels can activate or inhibit both different adenylyl cyclase isoforms and some phosphodiesterases (Cooper D.M. et al. 1995). However, little is known about the interactions between cAMP and Ca^{2+}. The endogenous levels

of cAMP generated by ACs are regulated by phosphodiesterases (PDEs), enzymes that can degrade cyclic nucleotides as cAMP and cGMP.

It has been described the transport of intracellular cAMP into the extracellular compartment is a common process in several tissues such as liver, adipose tissue, adipocytes and kidneys (Jackson E.K. and Dubey R.K., 2001; Jackson E.K. et al., 2006). The process begins whitin minutes following stimulation of adenylyl cyclase, and the mechanism is energy dependent and temperature sensitive (Jackson E. K. and Dubey R. K., 2001; Jackson E. K. et al., 2006). However, little is known about extracellular cAMP and cells of immune system. There is evidence that an extracellular cAMP-pathway that converts cAMP in adenosine (Figure 1) is present on Treg cells. This conversion involves CD39 ecto-ATPase/ADPase and CD73 ecto-5'-nucleotidase (Jackson E.K. et al., 2006; Jackson E.K. et al., 2007). This mechanism of extracellular adenosine production provides hormonal control of adenosine levels at the cell surface, where adenosine receptors are located. Adenosine is secreted in the extracellular space under metabolically stressful conditions, which are associated with ischemia, inflammation and cell damage (Haskó G. et al., 2007). A variety of extracellular and intracellular processes generate adenosine. Among these the major pathway that contributes to high extracellular adenosine concentrations during metabolic stress is release of precursor adenine nucleotides (ATP, ADP and AMP) from the cell. Literature reports that adenosine has many effects on different cell types: besides as a well-known modulator in the nervous and cardiovascular system, also as immunomodulator on lymphocytes, DCs (Panther E. et al., 2001) and neutrophilis, in which modulates Fcγ receptor-mediated functions (Salmon J.E.

and Cronstein B.N., 1990). It has been also shown that adenosine regulates monocyte differentiation into DCs and subsequentely their functions (Haskó G. et al., 2007; Novitskiy S.V. et al., 2008).

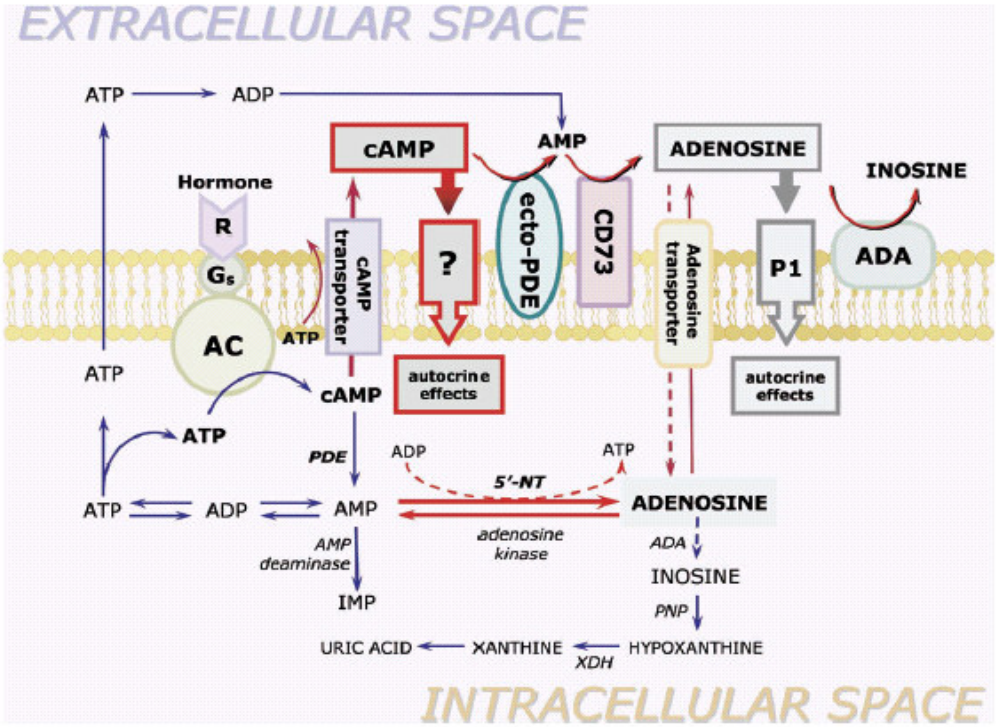

Fig. 1. Schematic representation of cAMP-adenosine pathways. ATP:adenosine triphosphate; ADP: adenosine diphosphate; Hormone: adenylyl cyclase-stimulating hormone; R: receptor coupled to adenylyl cyclase (AC);Gs: stimulatory G protein; P1: adenosine receptors; PDE: intracellular phosphodiesterase; 5'-NT: intracellular 5'-nucleotidase; PNP: purine nucleoside phosphorylase; XDH: xanthine dehydrogenase.

12. Aim of the thesis

The aim of this study was to analyse the effects of CT on cells of the immune system, in particular on human T lymphocytes.

The mechanisms involved in the inhibitory effects mediated by CT on T lymphocytes were analyzed. In particular, we analyzed whether CT prevented the activation of resting T cells or if it had any inhibitory effects on previously activated human T cells. The effects of CT on $CD4^+$ and $CD8^+$ T lymphocytes were also investigated. The possible involvement of the modulation of the inhibitory molecules CTLA-4 in CT mediated inhbition was studied. We found that CT was able to increase the expression of CTLA-4 on resting T lymphocytes. Therefore, we analysed the function of CT-treated T lymphocytes. In particular, since we found that CT-treated T lymphocytes showed suppressor capacity, we analysed the possible mechanisms involved in the suppression. We found that one of the mechanisms of the inhibition mediated by CT-treated T cells was through the release of extracellular cAMP. Therefore, we analysed the possible role exerted by extracellular cAMP as a primary messenger on different cell types. The direct effects of extracellular cAMP on T lymphocytes and on DCs and their precursors were investigated. We found that extracellular cAMP exerted inhibitory effects on T cell proliferation and that it was able to interfere with the differentiation of monocytes into DCs. Next, we analysed the phenotype and the function of cells differentiated in the presence of exogenous cAMP. Finally, the mechanism through which extracellular cAMP could be sensed by the cells was studied. An extracellular cAMP-adenosine pathway, that has been previously

described in different cell types (Dubey et al., 1996; Dubey et al., 2000; Cometti B. et al., 2003; Jackson E.K. et al., 2006; Giron M.C. et al., 2008; Jackson E.K. and Raghvendra D.K., 2004), was found to play a role also in modulating the function of cells of immune system.

MATERIALS AND METHODS

Media and reagents

RPMI 1640 supplemented with 2 mM l-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, NY), and 10% FCS (Hyclone Laboratories, UT) was used as complete medium in all cultures. Anti-CD3 (clone UCHT1) mAb and anti-CD28 (Clone CD28.2) mAb were purchased from Immunotech (Westbrook, ME) and PharMingen (San Diego, CA), respectively. Neutralizing anti-IL-10 (23738), -IL-10R (37607.11), -IL-4 (3007.11) and TGF-β (9016.2) mAb were purchased from R&D Systems (Minneapolis, MN). Cholera toxin (CT) and cholera toxin B subunit (CT-B) were purchased from Calbiochem-Novabiochem Co. (San Diego, CA) or List biological laboratories (Campbell, CA); cAMP, forskolin (FSK) dibutyryl cAMP (dbcAMP), lipopolysaccharide (LPS), 5'-(N-ethylcarboxamido)-adenosine (NECA), 8-(3-Chlorostyryl)caffeine (CSC), N-(4-Cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide (MRS1754) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals Co. (St. Louis, MO). Final concentrations of DMSO did not exceed 0.1%.

Cells purification and cultures conditions

Peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy donors by Ficoll-Hypaque (Pharmacia, Sweden) density centrifugation. $CD4^+$ T lymphocytes were purified by negative selection using an

immunomagnetic cell sorting (Miltenyi Biotec, Germany). Briefly, PBMC were labelled using a cocktail of hapten-conjugated mAbs anti-CD8, -CD11b, -CD16, -CD19, -CD36 and -CD56 molecules in combination with MACS MicroBeads coupled to an anti-hapten monoclonal antibody. The magnetically labelled cells were depleted by retaining them on a column using MidiMACS cell separator. PBMC (2×10^6/ml) were treated either with CT (3 μg/ml) in the presence or in the absence of anti-CD3 mAbs, CT-B (3 μg/ml), FSK (50 μM), dbcAMP (0.5 mM) or stimulated with anti-CD3 mAb (0.5 μg/ml) and analysed at different time points for CTLA-4 and CD28 expression.

Proliferation assays

PBMC (10^5/well) were cultured in round bottom 96-well plates in the presence or in the absence of different concentrations (from 6 to 0.0003 μg/ml) of CT (Calbiochem) and stimulated with anti-CD3 mAbs (0.5 μg/ml) (Immunotech). In some experiments CT (3 μg/ml) was added to the cultures at different time points with respect to anti-CD3 mAbs addition: 1 h before, at the same time and 2, 6 and 24 h after. Proliferation was evaluated by ^{3}H-thymidine incorporation. Plates were incubated for 48 h at 37 °C with 5% CO^2 and ^{3}H-thymidine (Amersham, Aylesbury, UK) was added (1 μCi/well). After 18 h cells were harvested and the incorporated radioactivity was measured by microβ-counting. In CTLA-4 blocking experiments, PBMC were treated with CT and anti-CD3 (0.5 μg/ml). Anti- CTLA-4 F(ab')$_2$ mAbs (ANC152.2/8H5, Ancell, MN, USA) or its isotype matched control (clone MOPC 31C, Ancell, MN, USA) (5 μg/ml) were

added to the cultures. Proliferation was evaluated by ^{3}H-thymidine incorporation as described above. In CT-pre-treated T lymphocytes experiments, purified CD4^{+} T cells (5×10^{6}/ml) were incubated in the absence or in the presence of CT (3 μg/ml), CT-B (3 μg/ml), FSK (50 μM), or dbcAMP (0.5 mM). After 24 h, cells were harvested, irradiated (3000 rad), washed extensively, and incubated for 1 h in round-bottom, 96-well plates with autologous PBMC (6×10^{4}) at different ratios (3:1, 2:1, 1:1). Then, increasing concentrations of anti-CD3 mAb (Immunotech) was added to the cultures, and the proliferation was evaluated by ^{3}H-thymidine incorporation as described above. In some experiments, neutralizing mAb anti-IL-4, -IL-10, -IL-10 receptor (IL-10R), or –TGF-β (1 μg/ml) were added singularly or as a cocktail to the cultures. To evaluate the effect of exogenously added cAMP and dbcAMP on PBMC, they were incubated in the presence or in the absence of cAMP or dbcAMP (0.1 mM) and stimulated with anti-CD3 mAb (0.5 μg/ml). T cell proliferation was evaluated by ^{3}H-thymidine incorporation after 66 h of culture.

CFSE labelling

PBMC (10^{7}/ml) were washed in PBS1 %FCS and incubated with 2.5 μM CFSE (Molecular probes) for 10 minutes at 37 °C. The reaction was quenched by the addition of ten times volume of cold RPMI 10% FCS and incubating for 5 min at 4 °C. Cells (3×10^{6}/ml) were washed three times with RPMI 10% FCS and stimulated with anti-CD3 mAbs in the presence and in the absence of CT. After 4

days, cells were double stained with anti-CD4-PE and anti-CD8-Cy5 mAbs and acquired on a FACSCalibur^{TM} instrument running CellQuest software.

Transwell experiments

In transwell assays, cells were separated by a membrane (6.5 mm diameter, 0.4 mm pore size) in 24-well plates (Costar, Corning, NY). The lower compartments of the wells contained PBMC (8×10^5). The upper compartments contained medium alone, untreated or with CT-pre-treated $CD4^+$ T cells (2.4×10^6). In some experiments, untreated or CT-pre-treated $CD4^+$ T lymphocytes were placed in the lower chambers together with PBMC, which were stimulated with anti-CD3 mAb (0.5 μg/ml) for 48 h, and proliferation was evaluated by harvesting the cells from the lower compartments and by incubating them in the presence of ^{3}H-thymidine for further 18 h.

Supernatant ultrafiltration

Purified $CD4^+$ T cells (5×10^6/ml) were incubated in the absence or in the presence of CT (3 μg/ml) for 24 h. Thereafter, cells were harvested, washed extensively, and incubated in 24-well plates (5×10^6/ml) for 48 h. Culture supernatants from untreated and CT-pre-treated $CD4^+$ T lymphocytes were collected and filtered using Amicon Ultra-4 centrifugal filters (Millipore, Bedford, MA) with membrane cut-off of 10 k, which allowed the separation of low molecular weight compounds. Filtered supernatants from untreated or CT-pre-

treated $CD4^+$ T cells were supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 10% FCS (Hyclone Laboratories) and added (75% of total culture volume) to PBMC in round-bottom, 96-well plates. After 1 h, cells were stimulated with anti-CD3 mAb (0.5 µg/ml). The proliferation was evaluated by ^{3}H-thymidine after 66 h.

Cell cycle analysis

Cell cycle analysis of anti-CD3 (0.5 µg/ml) activated PBMC in the presence and in the absence of CT (3 µg/ml) was performed by DNA staining with propidium iodite (PI). After 24 and 48 h of culture, cells were washed twice in cold PBS and then fixed in 70% ethanol at 4°C for 1 h. The samples were rehydrated in cold PBS, treated with 50 µg/ml RNAase A and stained with 50 µg/ml PI. DNA content was measured using a FACScalibur. G_1 (2N), S (2N to 4N) and G_2 fraction (4N) were determined on a FACSCalibur™ instrument running CellQuest software.

Preparation of cell extracts and western blot analysis for $p27^{kip}$ detection

Cells (5×10^6) were collected, washed twice and seeded by centrifugation at 200×*g* for 10 min. Cells were resuspended in 75 µl of cold lysis buffer [$MgCl_2$ 1 mM, NaCl 350 mM, HEPES 20 mM, EDTA 0.5 mM, EGTA 0.1 mM, dithiothreitol (DTT) 1 mM, $Na_4P_2O_7$ 1 mM, phenylmethylsulphonyl fluoride

(PMSF) 1 mM, aprotinin 1.5 mM, leupeptin 1.5 mM, 1% phosphatase inhibitor cocktail II (P5726; Sigma, St. Louis, MO), glycerol 20%, Nonidet P-40 (NP-40) 1%], vigorously vortexed for 10 s and, after incubation for 10 min on ice, centrifuged at 20 000×*g* for 15 min at 4°C. The supernatant was used as cell extract. Cell extracts at equal protein concentration were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 7.5% polyacrylamide gel. Proteins were then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Little Chalfont, UK) as described. (Pioli C. et al., 2001). Membranes were incubated with anti-$p27^{kip}$ mAbs (Signal Transduction Laboratories/BD Heidelberg,) and anti-β-actin (Santa Cruz Biotech, USA). Immunoblots were developed by chemifluorescence and acquired by the phosphor/fluorescence imager Typhoon 9610 (Molecular Dynamics, Sunnyvale, CA). The intensity of the bands was directly quantified by Image QuaNT software (Molecular Dynamics), which gives rise to a volume report by integrating the area of the band and its density. Arbitary units represent the ratio between $p27^{kip}$ and β-actin volumes.

Flow cytometry analysis

FITC, PE or Cy5-conjugated anti-CD4, -CD8, -CD28, anti-CD14, -CD1a, -HLA-DR, -HLA-I, -CD80, -CD86 and -CTLA-4 mAbs and their isotype-matched controls were purchased from Becton-Dickinson and used for direct immunofluorescence staining. CTLA-4 detection was performed by intracellular staining. Briefly, cells were washed twice in PBS, 1% BSA and 0.1% sodium

azide and double stained with anti-CD4 and anti-CD8 mAbs for 15 min at 4°C. Samples were then fixed in 4% paraformaldehyde for 5 min at 4 °C, incubated with anti-CTLA-4 mAbs, diluted in PBS, 1% BSA and 0.5% saponin. The cells were finally washed twice in PBS, 1% BSA, 0.1% saponin and acquired on a FACSCalibur™ instrument running CellQuest software.

CTLA-4 mRNA analysis by RT-PCR

Total RNA was extracted using RNAFast reagent (Life Technology) according to the manufacture's recommendations. The single-stranded cDNA was synthesized using 1 μg of RNA by reverse transcription using random hexamers (Invitrogen). PCR reactions were performed with cDNA corresponding to 10 ng of RNA, Taq polymerase (Invitrogen) and primers designed to amplify the entire coding sequence of CTLA-4: 5'-ATGGCTTGCCTTGGATTTCAGCGGCACAAGG-3' and 5'-TCAATTGATGGGAATAAAATAAGGCTGAAATTGC-3'. PCR reaction was as follows: 94°C for 5 min, 30 cycles 94°C for 30 s, 58°C for 30 s and 72°C for 30 s followed by a final extension at 72°C for 7 minutes. The amplified fragments were separated on 1% agarose gel and visualized by ethidium bromide. RNA integrity and cDNA synthesis was verified by amplifying β2-microglobulin cDNA.

Cytokine assay

Purified $CD4^+$ T cells (5×10^6/ml) were incubated in the absence or in the presence of CT (3 μg/ml) and CT-B (3 μg/ml) in round-bottom, 96-well plates and stimulated with plate-coated anti-CD3 mAb (5 μg/ml) and soluble anti-CD28 mAb (1 μg/ml). Culture supernatants were collected after 48 h and frozen at –80°C until assayed for IL-10 content by sandwich ELISA. The levels of these cytokines were assayed by using antibody pairs of mouse anti-human IL-10 (2 μg/ml) and biotin-conjugated rat anti-human IL-10 (0.5 μg/ml) from Pierce Endogen (Rockford, IL). In other experiments, purified $CD14^+$ monocytes (1×10^6/ml) were cultured with 50 ng GM-CSF and 35 ng IL-4 in the presence or in the absence of cAMP (0.5 mM) and FSK (10 μM). Culture supernatants were collected after 5 days and frozen at –80°C until assayed for IL-10, IL-12, IL-6, and TNF-α content by sandwich ELISA. The levels of these cytokines were assayed by using antibody of mouse anti-human IL-10, IL-12, IL-4, and TNF-α (2 μg/ml) from Pierce Endogen (Rockford, IL).

cAMP measurement

$CD4^+$-purified T cells (5×10^6/ml) were incubated in the absence or in the presence of CT (3 μg/ml) or FSK (50 μM), and culture supernatants were collected after 24 h to measure the accumulation of extracellular cAMP. Culture supernatants (90 μl) were then added with 10 μl 0.1 M HCl and processed for

cAMP determination by a RIA following sample acetylation as described (Brooker G. et al., 1979). The sensitivity of the test was 1 ftmol.

DC differentiation and culture conditions.

Monocytes were immunomagnetically purified by positive selection using CD14 monoclonal antibody-conjugated microbeads (Miltenyi Biotec, Germany). The purity of monocytes was >98% and no T cell contamination was observed. Cells were plated at 1×10^6/ml in complete medium supplemented with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml). After 5 days of culture, cAMP (0.5 mM) or LPS (200 ng/ml) was added to induce the maturation of the cells. After 48 h, treated and untreated cells were collected and analysed for the expression of activation and maturation markers: -HLA-I, -HLA-DR, -CD86, -CD83. Cells were acquired on FACSCalibur™ instrument running CellQuest software.

In other experiments, monocytes (1×10^6/ml) were cultured with 50 ng of GM-CSF and 35 ng of IL-4 in the presence and in the absence of cAMP (0.5 mM), FSK (10 μM) or NECA (3 μM). After 5 days, cells were collected, washed and stained for the membrane phenotype. Cells were acquired on FACSCalibur™ instrument running CellQuest software.

In same experiments, before adding cAMP or NECA, adenosine receptor antagonists A_{2A} (CSC, 10 μM) and A_{2B} (MRS 1754, 10 μM) singularly or in combination for 30 min (1 μM of each). The phenotype was analysed by FACSCalibur™ instrument running CellQuest software.

Mixed lymphocyte reaction

For allogenic mixed lymphocytes reaction (MLR), purified $CD14^+$ (1×10^6/ml) were stimulated with cAMP (0.5 mM) and FSK (10 μM). After 5 days, cells were washed and co-cultured with allogenic PBMC (1×10^5) for 3 days in round-bottom, 96-well plates at 37°C with 5% of CO_2 and ^{3}H-thymidine (Amersham, Aylesbury, UK) was added (1 μCi/well). After 18 h cells were harvested and the incorporated radioactivity was measured by microβ-counting.

T cell polarization assay

$CD4^+$ T cells were further purified in $CD4^+CD45RA^+$ T cells by positive selection using anti- CD45RA MicroBeads (Miltenyi Biotec, Germany). Purified $CD14^+$ monocytes (1×10^6/ml) were cultured with 50 ng GM-CSF and 35 ng IL-4, in the presence or in the absence of cAMP (0.5 mM) and FSK (10 μM). After 5 days, cells (2×10^5/ml) were washed and co-cultured with autologous purified naïve $CD4^+CD45RA^+$ T cells (1×10^6/ml) at ratio 1:5 in 48-well plates, at 37°C for 11 days. 20 UI/ml IL-2 (Becton Dickinson, USA) was added to the cultures at days 5, 7 and 9. Thereafter, on the eleventh day, cells were harvested, washed and analysed for intracellular INF-γ and IL-4 production and acquired on FACSCalibur™ instrument running CellQuest software.

Statistical analysis

Microsoft Excel (Microsoft Corporation, Redmond, WA) was used for statistical analysis. Data were expressed as mean±S.D.

RESULTS

1. Cholera Toxin prevents the activation of human $CD4^+$ and $CD8^+$ T cells

It has been reported that Cholera toxin (CT) inhibits T cell proliferation. To test the sensitivity for CT inhibition in cell cultures, peripheral blood mononuclear cells (PBMC) were treated with different doses of CT (from 6 to 0.0003 μg/ml) in the presence or in the absence of anti-CD3 mAbs stimulus. T cell proliferation was evaluated after 66 h. Figure 1, panel A shows that the inhibition of T cell proliferation was about 60% when CT was used at concentrations from 6 to 0.03 μg/ml. Only at concentration of 0.003 and 0.0003 μg/ml of the toxin the inhibition was slightly lower 48% and 36%, respectively (Figure 1, panel A).

To evaluate whether CT prevents the activation of resting T cells or whether it has any inhibitory effect on previously activated human T cells, CT (3 μg/ml) was added either before, at the same time, or at different time points after the addition of the stimulus (after 2, 6 and 24 h). Anti-CD3 mAbs (0.5 μg/ml) were used to stimulate PBMC. The proliferative response of cells treated with CT was then compared with that of cells cultured with anti-CD3 mAbs alone by ^{3}H-thymidine incorporation after 66 h of culture (Figure 1, panel B). A total inhibition of proliferation was observed when PBMC were incubated with CT 1 h before anti-CD3 mAbs addition. A high inhibition of T cells proliferation (61%) was still observed when anti-CD3 mAbs and CT were added simultaneously to cell culture (time zero). However, when CT was added to cell cultures 2 to 6 h after anti-CD3 mAbs, the inhibition of T cell proliferation was much lower (18 and 17%

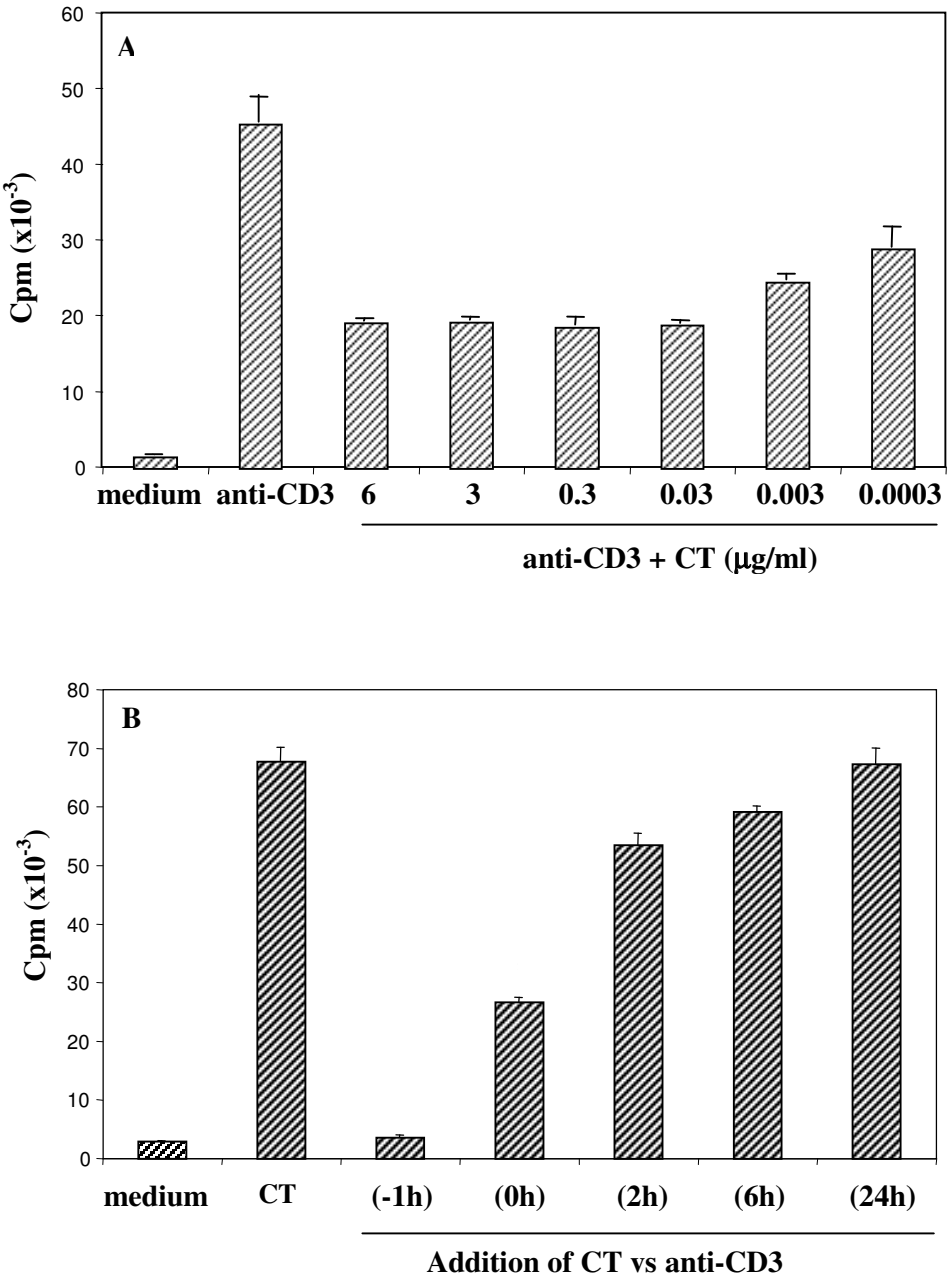

Fig. 1. The inhibition of T cell proliferation by CT is in relation to the addition of the stimulus. PBMC (10^5/well) were stimulated with anti-CD3 (0.5 μg/ml) with different concentrations of CT (from 6 to 0.0003 μg/ml) added simultaneously to the cell culture (A). In panel B, CT (3 μg/ml) was added 1 h before (−1 h), together with anti-CD3 (0 h), and 2 (2 h), 6 (6 h) and 24 (24 h) hours after the stimulus (B). Proliferation was evaluated by ^{3}H thymidine incorporation after 66 h of culture. The stars indicate that the differences between the cells stimulated with anti-CD3 mAbs in the presence of CT as compared with those of stimulated with anti-CD3 mAbs alone are significant. The data shown are from one representative experiment of three performed.

respectively). Finally, no inhibition was observed when CT was added to the cultures 24 hours after anti-CD3 mAbs (Figure 1, panel B).

Furthermore, we analysed, by flow cytometry, the modulation of early and late activation markers CD69 and CD25 in $CD4^+$ T lymphocytes untreated or treated with CT following anti-CD3 mAbs stimulation (Figure 2). When the cells were stimulated with anti-CD3 mAbs alone, we observed a significantly increased CD69 and CD25 expression after 24 and 48 h. However, in cells stimulated with anti-CD3 mAbs in presence of CT, we observed a significantly lower expression of both CD69 and CD25 (Figure 2, panels A and B). These data show that CT prevents the activation of resting T lymphocytes and suggest that the inhibitory effect of CT involves early steps of T cell activation.

It has been reported that CT has different effects on murine $CD4^+$ or $CD8^+$ T lymphocytes with regards to apoptosis induction (Nashar T.O. et al., 1996; Arce S. et al., 2005). To evaluate whether the proliferation of human $CD4^+$ and $CD8^+$ subpopulations is differently affected by CT, we stained PBMC with carboxyfluorescein succinimidyl ester (CFSE) (2.5 μM) and stimulated with anti-CD3 mAbs (0.5 μg/ml) in presence or in absence of CT (3 μg/ml). After 4 days of culture, the dilution of CFSE was analysed on $CD4^+$ and $CD8^+$ gated population (Figure 3, panels A-H). Cells were double stained with anti-CD4-PE and anti-CD8-Cy5 mAbs and acquired on FacsCalibur™ instrument running CellQuest software. In cultures stimulated in the absence of CT, $CD4^+$ and $CD8^+$ populations proliferated in the presence of anti-CD3 mAbs, as shown by reduction of the mean of fluorescence intensity (mfi) of the CFSE from baseline level of 800 to 355 (Figure 3, panels A and B) for $CD4^+$ T lymphocytes, and from 800 to

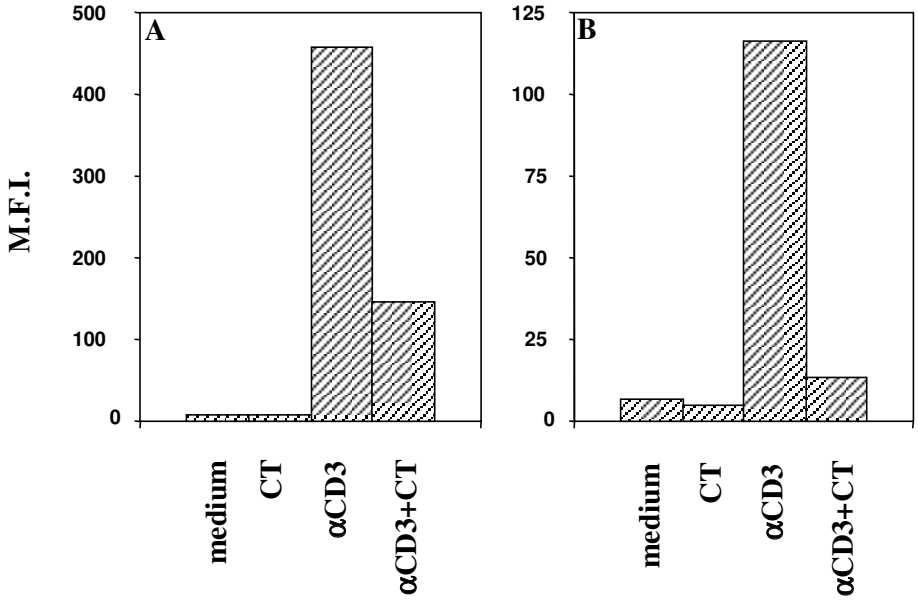

Fig. 2. CT prevents the up-regulation of activation markers CD25 and CD69 on $CD4^+$ T lymphocytes. Purified $CD4^+$ T lymphocytes were cultured in the presence and in the absence of CT (3 μg/ml). The expression of CD69 (A) and CD25 (B) was evaluated by flow cytometry at 24 h and 48 h, respectively, after stimulation with coated anti-CD3 mAb. The data shown are from one representative experiment of three performed.

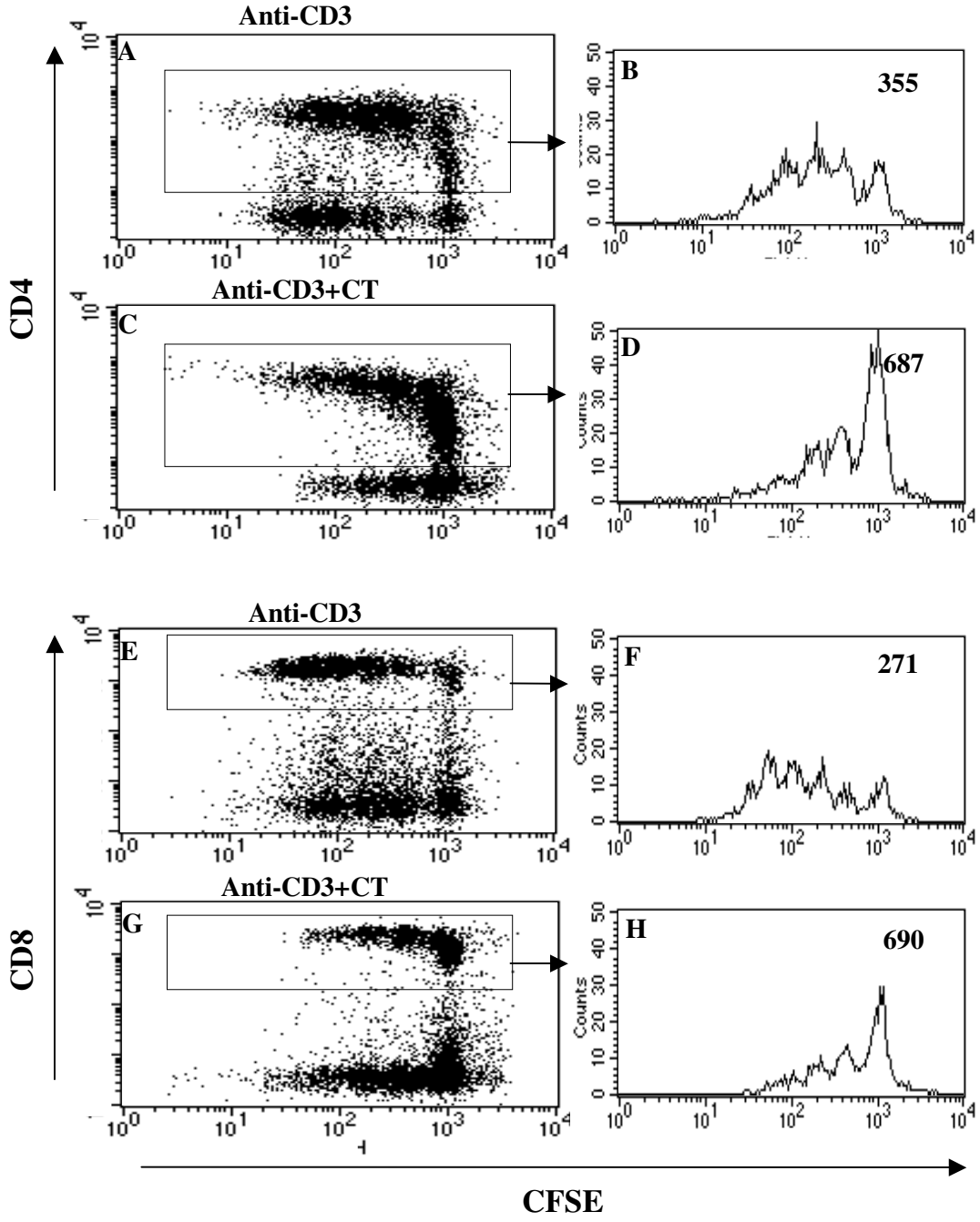

Fig. 3 CT inhibits the proliferation of $CD4^+$ and $CD8^+$ human T cells. PBMC ($3x10^6$/ml) labelled with CFSE (2.5 µM) were stimulated with anti-CD3 mAbs (0.5 µg/ml) in the presence or in the absence of CT (3 µg/ml). After 4 days, cells were double stained with anti-CD4-PE and anti-CD8-Cy5 mAbs and acquired on a FACSCalibur™ instrument running CellQuest software. The baseline level expressed as mean florescence intensities (mfi) of CFSE of $CD4^+$ and $CD8^+$ gated populations is 800 (not shown). The mfi of CFSE dilution in $CD4^+$ (B and D) and $CD8^+$ (F and H) gated populations are indicated within the histograms. The data shown are from one representative experiment of three performed.

271 (Figure 3, panels E and F) for $CD8^+$ T lymphocytes. On the other hand, in cultures stimulated in the presence of CT the inhibition of both $CD4^+$ (75%, mfi 687 vs. 355) and $CD8^+$ (80%, mfi 690 vs. 271) T cell proliferation was observed. (Figure 3, panels C and D, G and H respectively). Finally, either $CD4^+$ or $CD8^+$ T cells did not die upon CT treatment showing that CT did not induce apoptosis both on $CD4^+$ and on $CD8^+$ T lymphocytes. This suggests that human T lymphocytes react differently to CT as compared to murine T cells.

2. CT causes the arrest of the cell cycle to a G_0/G_1 phases

Proliferation is a multistage process characterized by successive entering of four stages of the cell cycle: M, G_1, S and G_2. To investigate the mechanisms involved in the inhibition of T cell proliferation by CT, cell cycle analysis was performed by measuring DNA content in PBMC, by staining with propidium iodite (PI), a colouring agent that inserts itself in the DNA. Cells were stimulated with anti-CD3 mAbs in the presence and or absence of CT (Figure 4, panels A-C). After 48 h of culture, cells were analysed. Figure 4, panel A shows that 75% of anti-CD3 stimulated T cells were in the G_0/G_1 phase of the cell cycle, whereas 14% and 4.3% of them were in phases S and G_2 of the cell cycle, respectively. On the other hand, a higher percentage (93%) of the cell stimulated with anti-CD3 in the presence of CT, was in the G_0/G_1 phase of the cell cycle and only 2% and 0.8% had entered the S and G_2 phase, respectively (Figure 4, panel B). These data indicate that the inhibition of T cell proliferation mediated by CT is due to a G_0/G_1arrest of the cell cycle.

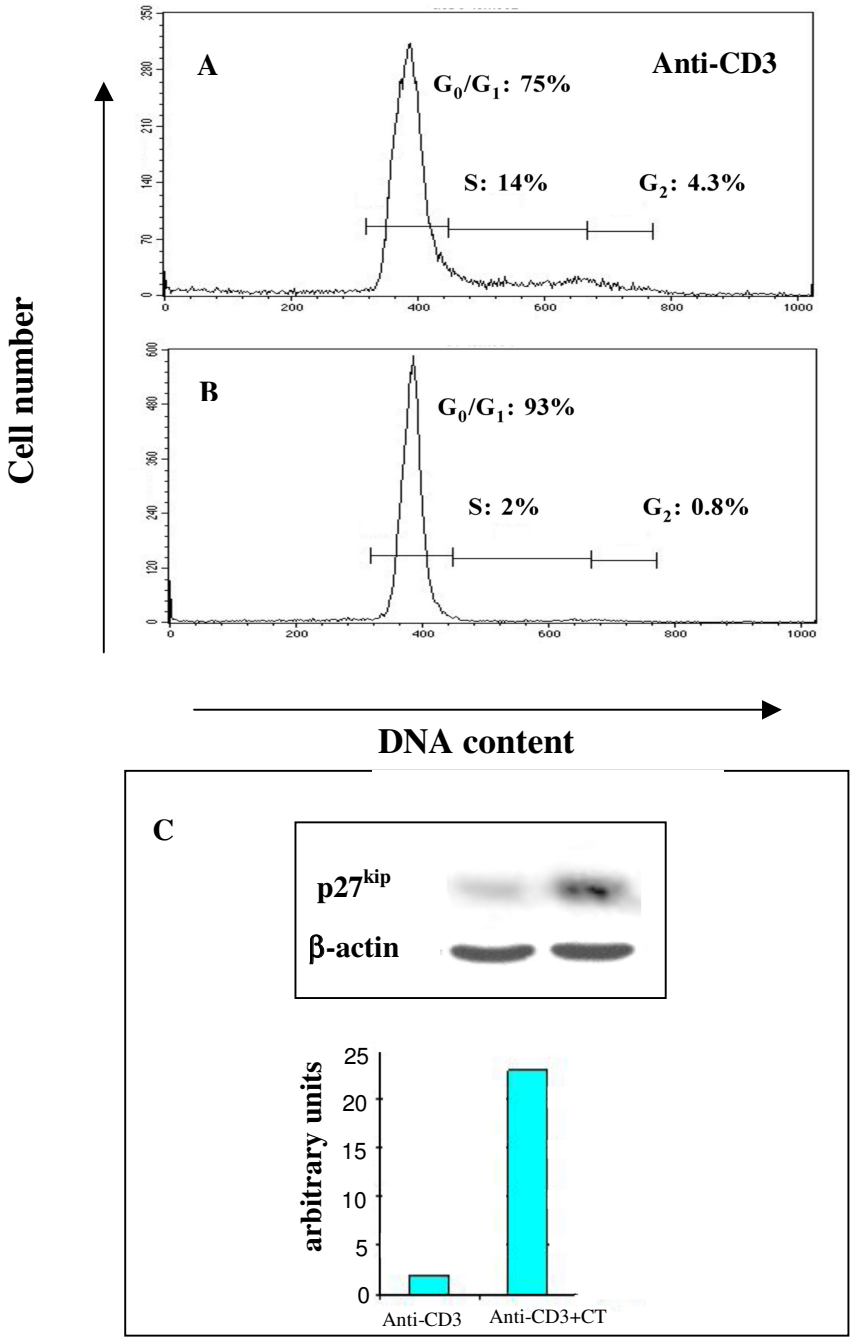

Fig. 4. CT induces a G_0/G_1 arrest of the cell cycle. Distribution of DNA content of PBMC stimulated with anti-CD3 mAbs (0.5 µg/ml) for 48 h in the absence (A) or in the presence (B) of CT (3 µg/ml). The percentage of cells in the different phases of the cell cycle were determined on a FACSCaliburTM instrument running CellQuest software. The levels of the cell cycle regulatory protein, $p27^{kip}$ were analysed after 24 h of anti-CD3 stimulation in the presence or absence of CT by Western blot (C). Immunoblots were developed by chemi-fluorescence and acquired by the phosphor/fluorescence imager Typhoon 9610. The data shown are from one representative experiment of two performed.

To further characterize the G_0/G_1 block of the cell cycle in T cells, we analysed, by Western blot, the level of the cell cycle regulatory protein $p27^{kip}$, which is degradeted when cells enter in the cell cycle. Cells were stimulated with anti-CD3 mAbs (0.5 μg/ml) in the presence or absence of CT (3 μg/ml). After 24 h cells were denatured and the total protein extracts were subjected to sodium dodecyl sulphate-polyacrylamide gel electophoresis (SDS-PAGE). Proteins were then electrotransferred onto polyvinylidene difluoride (PVDF) membranes, which were incubated with anti-$p27^{kip}$ mAbs. Cells stimulated in the presence of CT showed a high level of $p27^{kip}$ expression as compared to cells stimulated with anti-CD3 mAbs alone (Figure 4, panel C). These data indicate that a decreased degradation of $p27^{kip}$ in anti-CD3 mAbs stimulated cells in the presence of CT could be the cause of the blockade of the cell cycle in G_0/G_1 phase.

3. Cholera Toxin up-regulates CTLA-4 in resting T lymphocytes

It has been reported that cell cycle arrest with increased levels of $p27^{kip}$ correlates with increased levels of the inhibitory molecule CTLA-4 (Kubsch S. et al., 2003). To better understand the mechanisms of inhibition of CT, we asked whether CT was able to modulate the expression of inhibitory molecules such as CTLA-4, which is a receptor expressed after activation of T cells and able to down-modulate the immune responses (Chambers C.A. et al., 1996). PBMC were cultured in the presence of CT (3 μg/ml), CT-B (3 μg/ml) or anti-CD3 mAbs (0.5 μg/ml) and the expression of CTLA-4 molecule was analysed by intracellular staining at different time points 6, 24 and 48 h in $CD4^+$and $CD8^+$-gated

populations (Figure 5, panels A and B). An increase in the percentage of $CD4^+CTLA\text{-}4^+$ was evident already soon after 6 h of incubation with CT, and by 24 and 48 h 38% and 45% of the $CD4^+$ T cells were $CTLA\text{-}4^+$ (Figure 5, panel A). Treatment with CT also induced the up-regulation of CTLA-4 in $CD8^+$ T cells (Figure 5, panel B), however the kinetic and the level of expression were different as compared to $CD4^+$ T cells. After 6 h of incubation with CT, CTLA-4 expression in resting $CD8^+$ T cells was similar to that of untreated cells. By 24 h, 25% of the $CD8^+$ T cells treated with CT were $CTLA\text{-}4^+$ and this population increased up to 35% after 48 h of incubation with CT (Figure 5, panel B). The treatment with CT-B, the B-subunit of CT, which lacks the ADP-ribosyl transferase activity, did not affect the up-regulation of CTLA-4 both in $CD4^+$ and in $CD8^+$ T cells. Panel C of Figure 5 shows the up-regulation of CTLA-4 in $CD4^+$ T lymphocytes after 24 h of culture in the presence or in the absence of CT.

To evaluate whether CT induces up-regulation of CTLA-4 also at the mRNA level, we analysed the CTLA-4 mRNA in resting PBMC cultured in the presence or in the absence of CT (3 μg/ml), CT-B (3 μg/ml), Forskolin (FSK) (50 μM), a drug that actives directly adenylyl cyclase and induces production of intracellular cAMP. RNA integrity and cDNA synthesis was verified by amplifying β_2-microglobulin cDNA (Figure 6). We observed an increase of two mRNA transcripts that correspond to the membrane (672 bp) and the soluble (550 bp) CTLA-4 molecule as compared to the expression in cells cultured in medium alone. Similar results were obtained with FSK, whereas CT-B did not show any

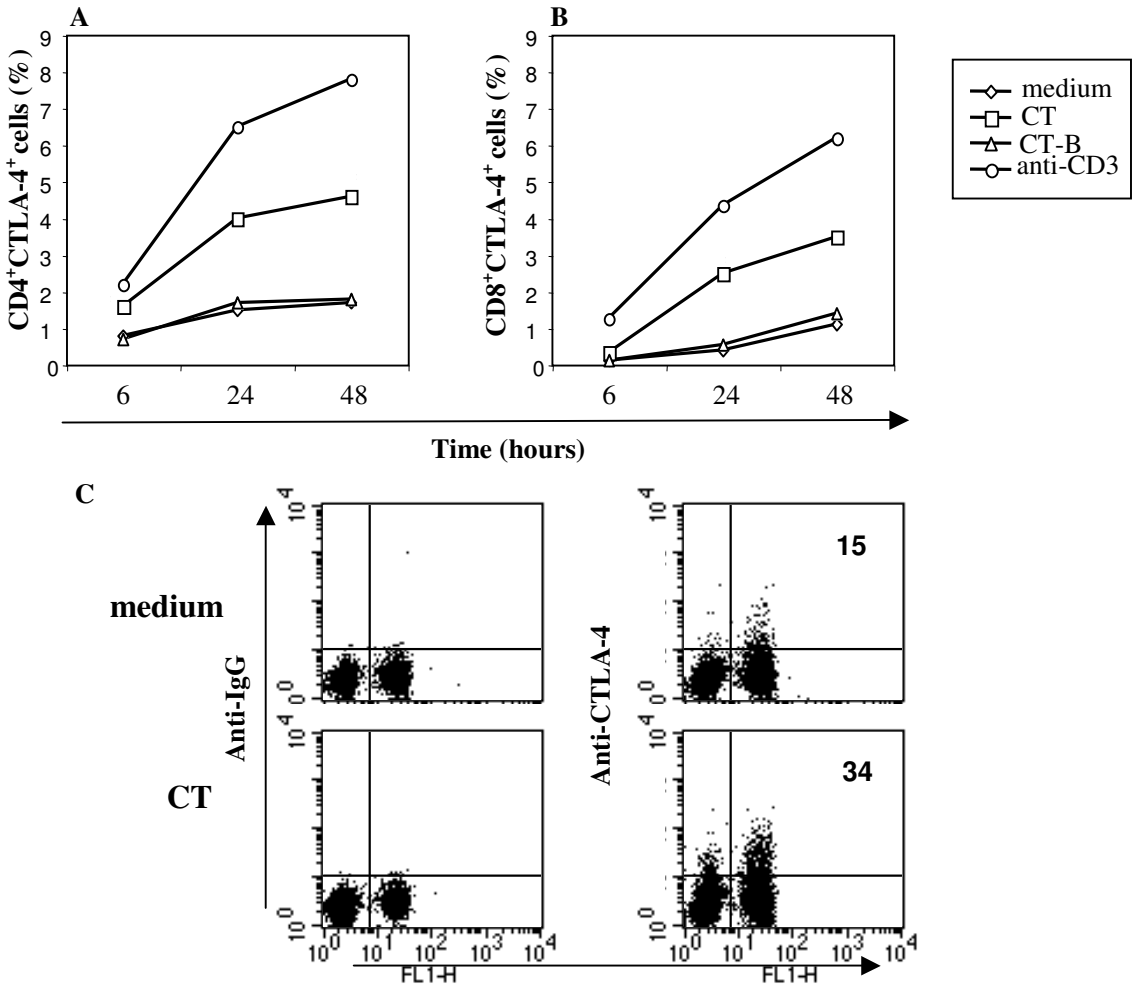

Fig. 5. CT up-regulates CTLA-4 in $CD4^+$ cells and in $CD8^+$ T cells. CTLA-4 expression at different time points after incubation of PBMC in medium alone or in the presence of CT (3 μg/ml), CT-B (3 μg/ml) or anti-CD3 mAbs (0.5 μg/ml). After 6, 24 and 48 h of culture, cells were stained with anti-CD4-FITC mAb and anti-CD8-Cy5 on the membrane, fixed in 4% paraforlmadehyde, permeabilised with 0.5% saponin and stained with anti-CTLA-4-PE or its isotype control mAbs. Cytofluorimetric analysis was performed in $CD4^+$ (A) and in $CD8^+$ (B) gated populations. The numbers indicate the percentage of $CD4^+CTLA\text{-}4^+$ T lymphocytes. The data shown are from one representative experiment of three performed.

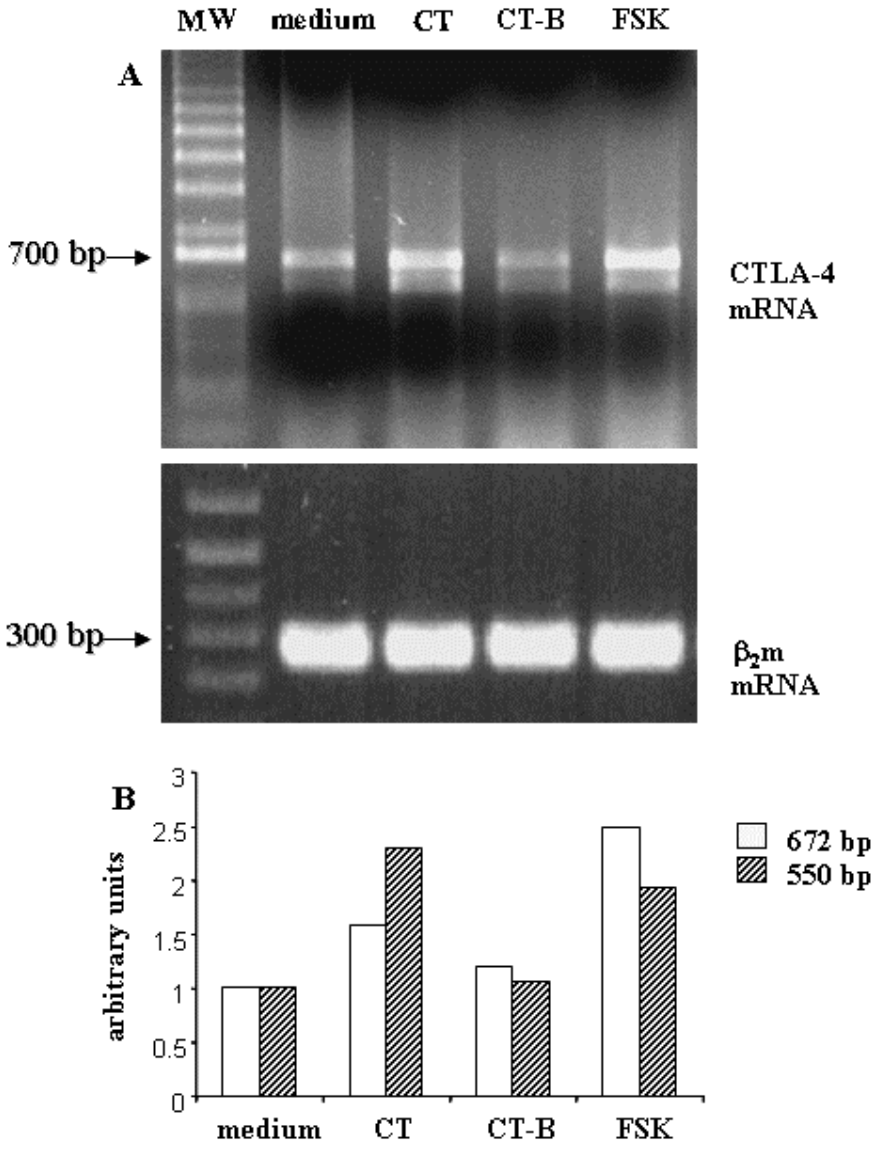

Fig. 6. CTLA-4 mRNA expression in T lymphocytes. (A) CTLA-4 mRNA was evaluated in human PBMC either untreated (medium) or treated with CT (3 μ g/ml), CT-B (3 μ g/ml) or FSK (50 μ M). RNA integrity and cDNA synthesis was verified by amplifying β 2 microglobulin cDNA. (B) The graph represents CTLA-4 mRNA quantification of the two mRNA transcripts, both normalized to those of β 2 microglobulin. The increase of each band is calculated by comparing the mRNA quantified in treated cells to the mRNA evaluated in the cells cultured in medium alone. The data shown are from one representative experiment of three performed.

effect (Figure 6).

Altogether, these results show that CT induces up-regulation of CTLA-4 molecules in $CD4^+$ and in $CD8^+$ subpopulations of T lymphocytes. This occurs in the absence of T cell activation and requires the holotoxin capable of increasing intracellular cAMP levels. Furthermore, the control of CTLA-4 expression by CT is at the mRNA level and involves the membrane and soluble CTLA-4 mRNA transcript.

4. Cholera Toxin down-regulates CD28 expression in resting $CD4^+$ and $CD8^+$ T cells

CD28 is a structural homologue of CTLA-4. Although these molecules share same ligands CD80 (B7.1) and CD86 (B7.2), they delivery opposing signals to T cells (Krummel M.F. and Allison J.P., 1995). We have seen the effects of CT on CTLA-4 molecules. Thus, we asked whether the up-regulation of the inhibitory CTLA-4 molecules by CT was paralleled by any effect on the expression levels of the costimulatory CD28 molecules. PBMC were cultured in the presence or absence of CT (3 μg/ml), CT-B (3 μg/ml), FSK (50 μM) or dbcAMP (0.5 μM) and the expression of CD28 on the membrane of on $CD4^+$ and $CD8^+$-gated populations was analysed after 24 h by flow cytometry analysis. Cells were stained with anti-CD4-FITC, anti-CD8-Cy5 and anti-CD28-PE mAbs. We found that CT down-modulated the expression of CD28 on the membrane of $CD4^+$ and $CD8^+$ T lymphocytes by 50% and 30% respectively (Figure 7, panels A-C). To understand whether the enzymatically active A-subunit of CT was

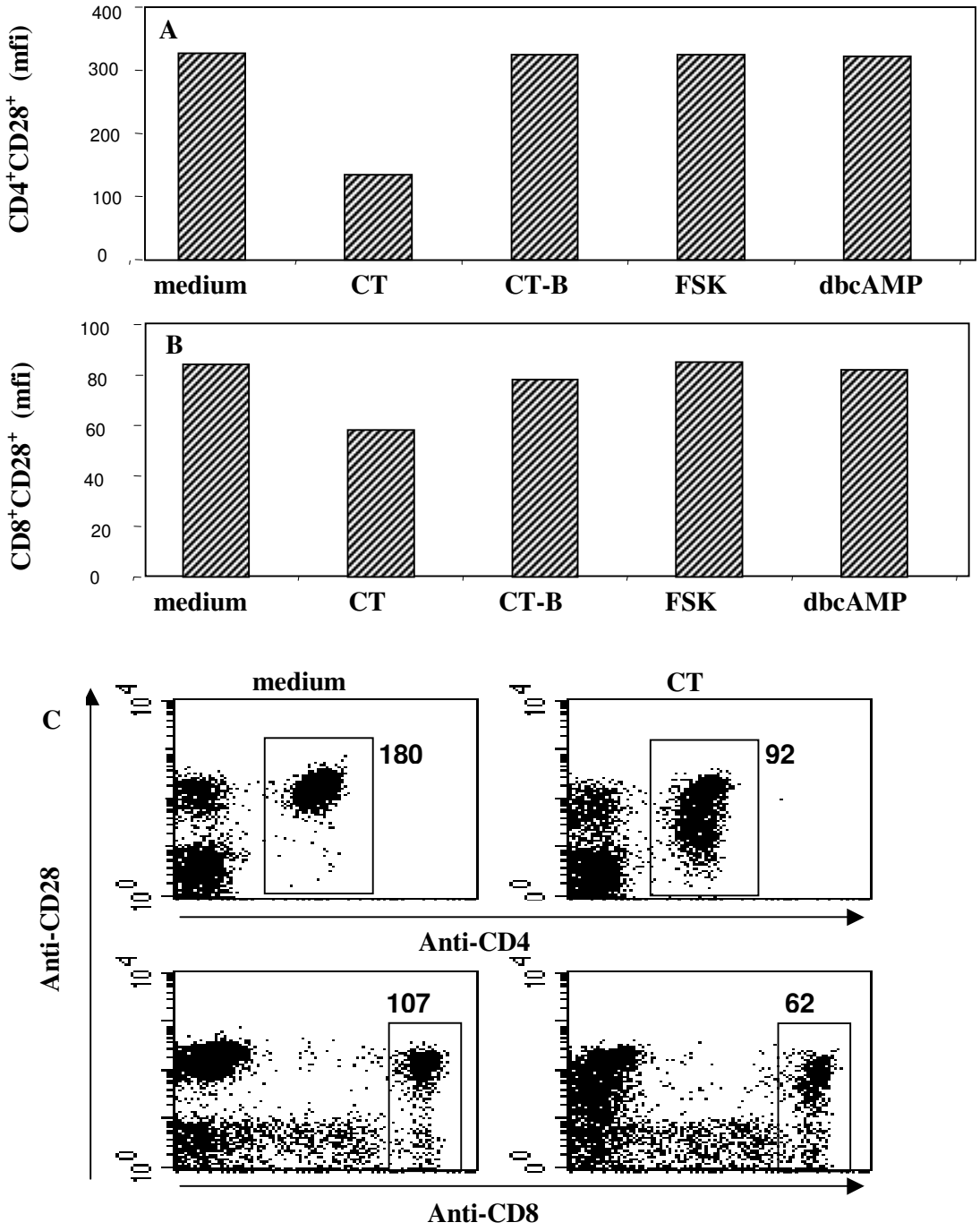

Fig. 7. CT down-regulates CD28 on $CD4^+$ and $CD8^+$ T cells. CD28 expression was evaluated after incubation of PBMC in medium alone, or with CT (3 μg/ml), CT-B (3 μg/ml), FSK (50 μM) or dbcAMP (0.5 mM). After 24 h of culture cells were stained with anti-CD4-FITC mAb and anti-CD8-Cy5 and with anti-CD28-PE or with their isotype control mAbs. Cytofluorimetric analysis was performed on $CD4^+$ (A) and on $CD8^+$ (B) gated populations. Panel (C) shows the down-modulation of CD28 on $CD4^+$ and $CD8^+$ T lymphocytes after 24 h of culture in the presence or in the absence of CT. The numbers indicate the mean fluorescence intensity of $CD4^+CD28^+$ and $CD8^+CD28^+$ T lymphocytes. The data shown are from one representative experiment of three performed.

required for the down-modulation of CD28 on T cells, we treated the cells with the CT-B-subunit, or FSK, or dbcAMP. We found that the expression of CD28 on both $CD4^+$ and $CD8^+$ T cells was not affected by CT-B subunit (Figure 7, panels A and B). Furthermore, treatment with FSK or with an analogue of cAMP, dbcAMP, did not cause the down-modulation of CD28 on $CD4^+$ or $CD8^+$ T cells. The panel C shows also the up-regulation of CTLA-4 in $CD4^+$ T lymphocytes, after 24 h of culture in the presence or in the absence of CT. These data suggest that the enzymatically active A subunit of CT is required for the down-modulation of CD28 on T cells and that this effect may not be mediated solely by an increase in intracellular cAMP. Alternatively, the effect could be related to different signalling patways affected by CT or to its unique capacity to induce constitutive production of cAMP within the cells.

5. The inhibition of T cell proliferation by CT is partially prevented by blocking anti-CTLA-4 mAbs

We have shown that CT suppresses T cell proliferation and induces up-regulation of CTLA-4 molecules in T cells. CTLA-4 engagement inhibits IL-2 production and induces cell cycles arrest of T cells in the G_0/G_1 phase, leading to the inhibition of T cell proliferation (Krummel M.F. and Allison J.P., 1996). To test whether the up-regulation of CTLA-4 by CT was involved in the inhibition of T cell proliferation, PBMC cultured in the presence of CT (3 μg/ml) were stimulated with anti-CD3 mAbs (0.5 μg/ml) in the presence or absence of blocking anti-CTLA-4 $F(ab')_2$ mAbs (5 μg/ml), which doesn't allow binding with

CD80 or CD86. First, we analysed the expression of CTLA-4 in T lymphocytes stimulated with anti-CD3 mAbs stimulus. Panels A and B of Figure 8 show that CT enhances the expression of CTLA-4 induced by anti-CD3 mAbs in $CD4^+$ and $CD8^+$ T lymphocytes, respectively. Next, as shown in panel C of Figure 8, we found that the inhibition of T cell proliferation induced by CT was partially prevented by the addition of blocking anti-CTLA-4 $F(ab')_2$ mAbs. As a negative control an isotype anti-IgG1 $F(ab')_2$ was included in these set of experiments. These data suggest that the inhibitory effects of CT on T cells are due, at least in part, to the up-regulation of the inhibitory CTLA-4 molecule.

6. $CD4^+$ T lymphocytes pre-treated with CT inhibit the proliferation of bystander, autologous PBMC

CTLA-4 plays a pivotal role in the induction of tolerance (Eagar T.N. et al., 2002) and it is constitutively expressed on T cells with regulatory activity (T reg). T reg cells do not proliferate or produce IL-2 and are able to inhibit the proliferative response and cytokines production of effector T cells (Eagar T.N. et al., 2002; Read S. et al., 2000; Bluestone J.A. and Abbas A.K., 2003). Since T lymphocytes treated with CT do not proliferate, do not produce IL-2 and express CTLA-4 molecules, we asked whether T lymphocytes treated with CT showed any regulatory activity. Purified $CD4^+$ T lymphocytes isolated from PBMC of healthy donors were incubated with CT (3 μg/ml), CT-B (3 μg/ml), FSK (50 μM) or dbcAMP (50 mM) for 24 h (Figure 9, panels A-E). Thereafter, the cells were irradiated (3000 rad), washed extensively, and cultured with autologous PBMC

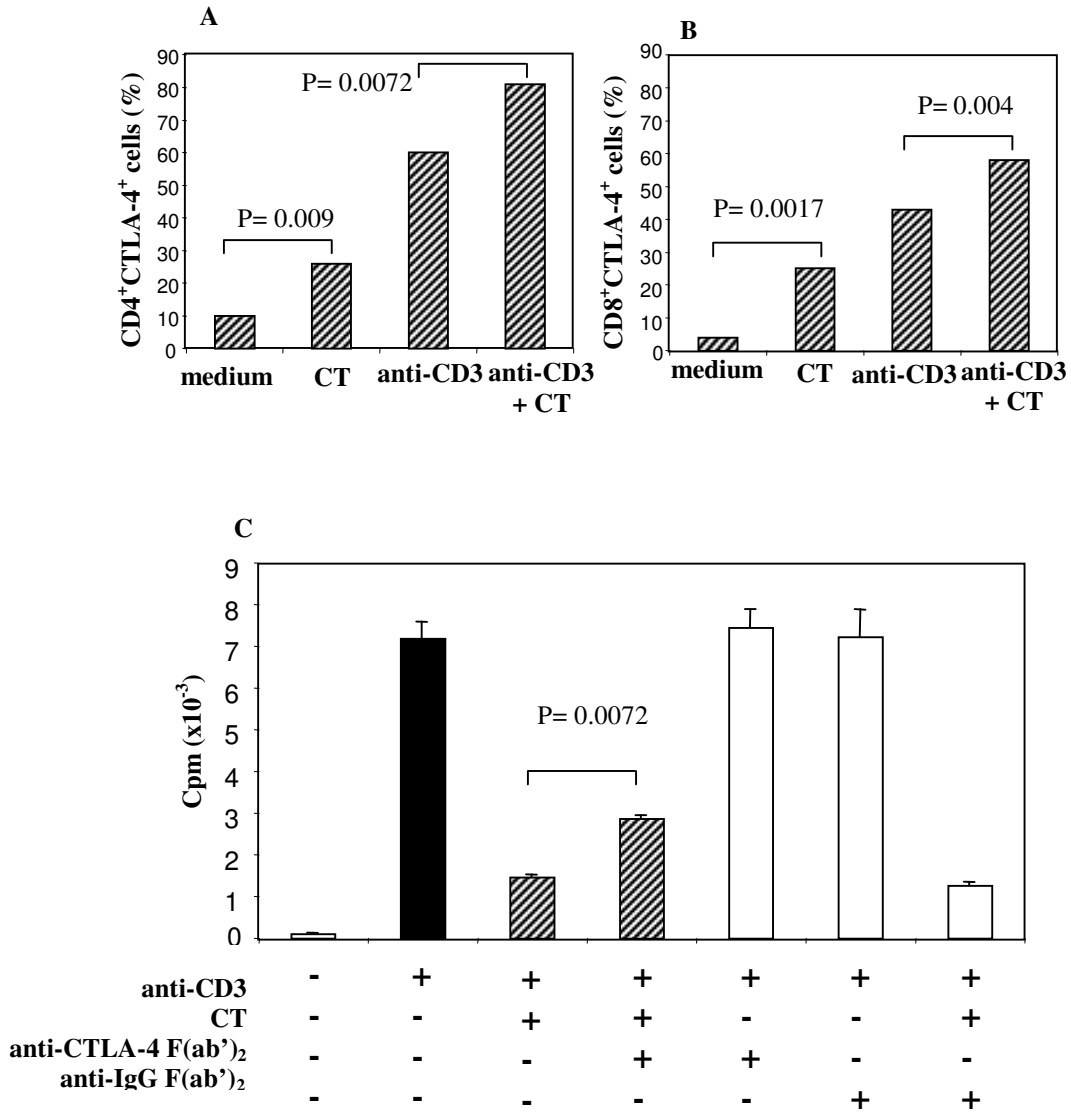

Fig. 8. CT enhances the anti-CD3 mAbs induced up-regulation of CTLA-4 and blocking anti-CTLA-4 F(ab')$_2$ mAbs partially prevent the inhibition of T cell proliferation by CT. PBMC (10^5/well) were cultured in the presence of CT (3 μg/ml) and stimulated with anti-CD3 (0.5 μg/ml). After 24 h of culture, cells were stained with anti-CD4-FITC and anti-CD8-Cy5 mAbs on the membrane, fixed, permeabilized and stained with anti-CTLA-4-PE or its isotype control mAbs. Cytofluorimetric analysis was performed in $CD4^+$ (A) and in $CD8^+$ (B) gated populations. Furthermore, PBMC (10^5/well) were cultured in the presence of CT (3 μg/ml) and, after 2 h, stimulated with anti-CD3 (0.5 μg/ml), in the presence or in the absence of blocking anti-CTLA-4 F(ab')$_2$ mAbs (5 μg/ml) or its isotype control, anti-IgG1 F(ab')$_2$ mAbs (5 μg/ml) (C). Proliferation was evaluated by ^{3}H-thymidine incorporation after 66 h of culture. The data shown are from one representative experiment of four performed.

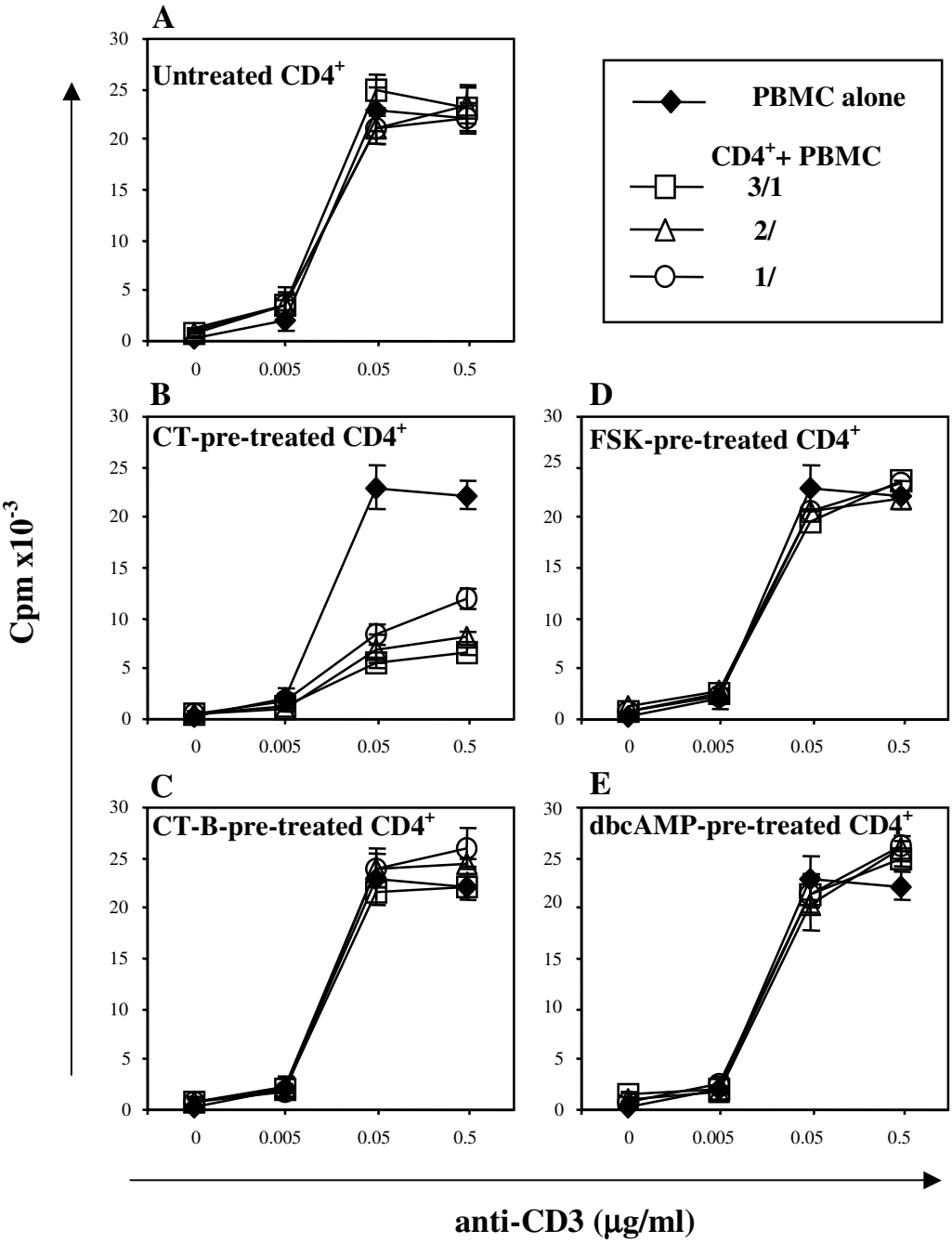

Fig. 9. CT-pre-treated $CD4^+$ T lymphocytes inhibit the proliferation of bystander, autologous PBMC. Purified $CD4^+$ T lymphocytes ($5x10^6$/ml) were cultured in the presence of medium alone (A), CT (3 µg/ml; B), CT-B (3 µg/ml; C), FSK (50 µM; D), and dbcAMP (0.5 mM; E). After overnight incubation, $CD4^+$ T cells were washed three times, irradiated (3000 rad), and cultured with autologous PBMC ($6x10^4$) at different $CD4^+$/PBMC ratios (3:1, 2:1, and 1:1) in the presence of increasing doses of anti-CD3 mAb (from 0.005 to 0.5 µg/ml). T cell proliferation was evaluated after 66 h by ^{3}H-thymidine incorporation. The data shown are from one representative experiment of six performed.

at different $CD4^+$/PBMC ratios (3:1, 2:1, and 1:1) in the presence of increasing doses of anti-CD3 mAbs (from 0.005 to 0.5 μg/ml). T cell proliferation was evaluated after 48 h of culture. Figure 9, panels A and B, shows that purified $CD4^+$ T lymphocytes pre-treated with CT were able to inhibit the proliferation of autologous PBMC in a dose-dependent manner, whereas the untreated T cells did not. Treatment with CT-B did not affect the proliferation of bystander PBMC (Figure 9, panel C), suggesting that the enzymatic activity of CT is required for the induction of $CD4^+$ T lymphocytes with regulatory activity. On the contrary, purified $CD4^+$ T cells pre-treated with FSK or with dbcAMP did not suppress the proliferation of bystander anti-CD3-stimulated PBMC (Figure 9, panels D and E). This suggests that a sustained production of cAMP, such as that obtained by CT treatment, is required for the induction of $CD4^+$ T cells with regulatory activity.

7. The inhibition of proliferation by CT–pre-treated $CD4^+$ T cells also occurs in the absence of cell-to-cell contact

To investigate whether the suppression mediated by CT-pre-treated $CD4^+$ T lymphocytes was dependent on cell-to-cell contact or on secretion of soluble factors, we performed transwell experiments. Untreated or CT-pre-treated $CD4^+$ T lymphocytes, irradiated to 3000 rad and washed extensively, were placed in the upper chambers of transwells, whereas autologous PBMC were placed in the lower chambers in the presence or absence of anti-CD3 mAb (0.5 μg/ml). In parallel, untreated or CT-pre-treated $CD4^+$ T cells were placed in the lower chambers together with PBMC. T cell proliferation was evaluated after 48 h of

culture. We found that CT-pre-treated $CD4^+$ T lymphocytes inhibited the proliferation of autologous, anti-CD3-stimulated PBMC, even in the absence of cell-to-cell contact, although the inhibition was slightly lower than that of observed when PBMC and CT-pre-treated $CD4^+$ T lymphocytes were placed in the lower chambers together (Figure 10, panel A). These results suggest that soluble factors are involved in the suppression induced by CT-pre-treated $CD4^+$ T cells, although cell-to-cell contact plays also a role in the inhibition. To rule out the possibility that a low amount of CT could be released by the CT-pre-treated $CD4^+$ T cells and could exert inhibitory effect on target cells, we filtered the supernatants from untreated or CT-pre-treated $CD4^+$ T cells using filters with membrane cut-off 10 k. The inhibitory effect of filtered supernatants was then tested on PBMC stimulated in the presence of anti-CD3 mAb (0.5 μg/ml) and proliferation was evaluated after 66 h by ^{3}H-thymidine incorporation. Figure 10, panel B shows that the supernatant from CT-pre-treated $CD4^+$ T cells was still able to inhibit the proliferation of anti-CD3-stimulated PBMC. The inhibition was 45% when compared with anti-CD3-stimulated cells and 31% when compared with the inhibition exerted by the supernatant of untreated $CD4^+$ T cells. The slight inhibition observed with the supernatant of untreated $CD4^+$ T cells, it might be a result of the withdrawal of factors important for T cell growth caused by filtration. Indeed, although filtered supernatants from untreated and CT-pre-treated $CD4^+$ T cells were supplemented with several growth factors after the filtration, we cannot exclude that additional factors, which are normally present in the complete medium, are missing in the filtered supernatants. These data suggest

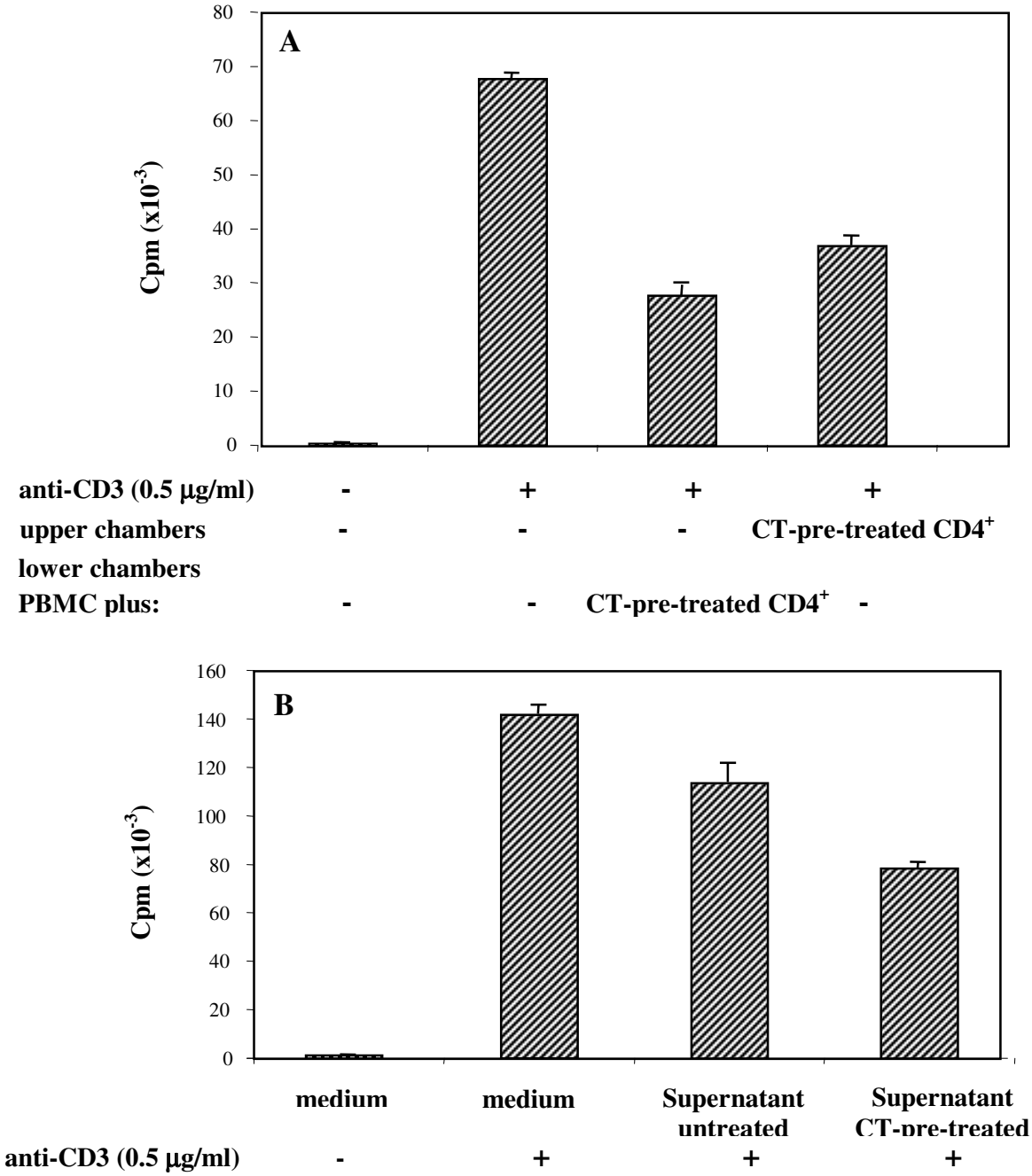

Fig. 10. Inhibition of T cell proliferation by CT-pre-treated $CD4^+$ T lymphocytes also occurs in the absence of cell-to-cell contact. (A) The effect of CT-pre-treated $CD4^+$ T lymphocytes on PBMC proliferation was investigated using a transwell system. PBMC ($8x10^5$) were placed in the lower wells, and irradiated untreated or CT-pre-treated $CD4^+$ T lymphocytes ($2.4x10^6$) were cultured in the upper wells. In parallel, untreated or CT-pre-treated $CD4^+$ T lymphocytes were placed in the lower chambers together with PBMC. Cells were stimulated with anti-CD3 mAb (0.5 µg/ml), and the PBMC proliferation was evaluated after 66 h by ^{3}H-thymidine incorporation. (B) The inhibitory effect of filtered supernatants (membrane cut-off 10 k) from cultures of untreated or CT-pre-treated $CD4^+$ T cells was evaluated on PBMC ($1x10^5$/well) stimulated with anti-CD3 mAb (0.5 µg/ml), and the proliferation was analysed after 66 h by ^{3}H-thymidine incorporation. The data shown are from one representative experiment of three performed.

that the suppression mediated by CT-pre-treated $CD4^+$ T cells is not a result of the CT, which may be released by the cells, and they show that soluble factors with low molecular weight are involved in the suppression. It cannot be excluded, however, that soluble factors with higher molecular weight may contribute to the inhibitory effect.

8. The inhibitory cytokines IL-10, IL-4, and TGF-β are not involved in the suppression induced by CT-pre-treated $CD4^+$ T cells

The possible involvement of inhibitory cytokines was evaluated in the CT-mediated inhibition of T cells. In particular, the production of IL-10 by purified $CD4^+$ T lymphocytes cultured in the presence or in the absence of CT and stimulated by coated anti-CD3 and solubile anti-CD28 mAb was analysed. The results reported in Figure 11, panel A show that IL-10 production induced by anti-CD3/CD28 stimulation was strongly inhibited in purified $CD4^+$ T lymphocytes treated with CT. Furthermore, to test whether the production of IL-10 could be induced by the presence of mononuclear cell types, we measured the levels of IL-10 in the supernatants of untreated and CT-pre-treated $CD4^+$ T cells co-cultured with autologous PBMC and stimulated with anti- CD3 mAb. The amount of IL-10 in the culture containing CT-pre-treated $CD4^+$ T cells was lower than that of found in the culture containing untreated $CD4^+$ T cells (Figure 11, panel B), which as reported above, did not mediate the suppression. The level of IL-10 in the culture containing untreated $CD4^+$ T cells was higher than that of found in the culture containing PBMC alone, and this likely reflects the production of IL-10 by

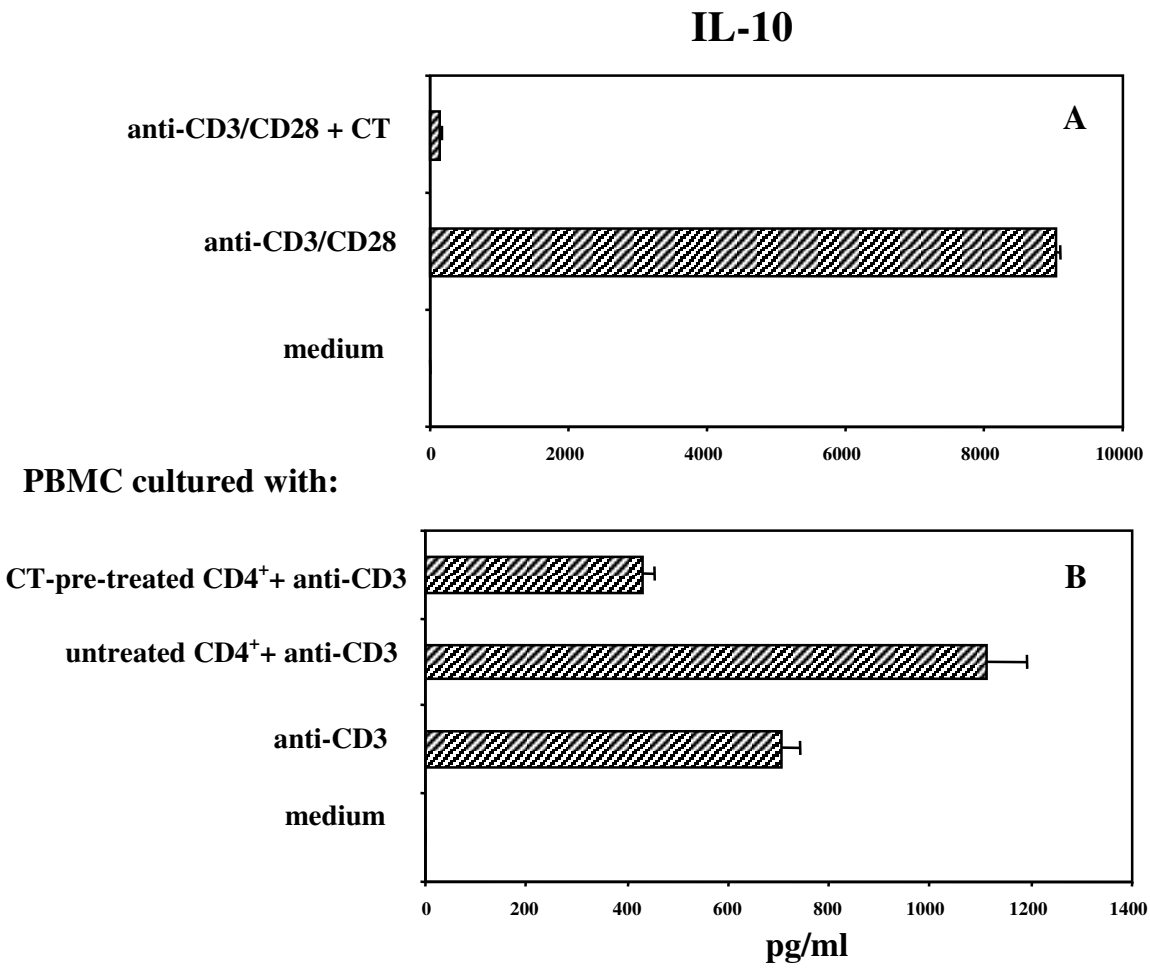

Fig. 11. The suppression exerted by CT-pre-treated $CD4^+$ T lymphocytes is not mediated by the secretion of IL-10. (A) Purified $CD4^+$ T lymphocytes ($2x10^6$/ml) were stimulated with plate-coated anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml) mAb in the presence or in the absence of CT (3 μg/ml), and the amount of IL-10 in culture supernatants was evaluated by ELISA after 48 h. (B) IL-10 level was evaluated in culture supernatants of PBMC ($6x10^4$) stimulated for 48 h with anti-CD3 mAb (0.5 μg/ml) in the presence of untreated or CT pretreated $CD4^+$ T lymphocytes ($18x10^4$) at a $CD4^+$:PBMC ratio of 3:1. The data shown are from one representative experiment of three performed.

PBMC and untreated $CD4^+$ T cells. Altogether, these data show that IL-10 production by $CD4^+$ T cells is inhibited by CT.

To further investigate the possibile role of IL-10 in the suppression mediated by CT-pretreated $CD4^+$ T lymphocytes, neutralizing anti-IL-10 and anti-IL-10R mAb were added to the co-cultures containing untreated or CT-pretreated $CD4^+$ T cells and autologous PBMC. As shown in Figure 12, panel A, the addition of anti-IL-10 and anti-IL-10R did not prevent the inhibition mediated by CT-pretreated $CD4^+$ T lymphocytes.

To test whether the secretion of TGF-β or IL-4 was involved in the suppression, neutralizing anti-TGF-β or a cocktail of anti-IL-4, -IL-10, and –TGF-β mAb (blocking mAb) was added to the cultures, and their capacity to prevent the inhibition of T cell proliferation was evaluated. The results in Figure 12, panels B and C, demonstrate that none of these cytokines were involved in the inhibitory effects mediated by CT-pre-treated $CD4^+$ T lymphocytes, suggesting that other inhibitory factors might be released by CT-pre-treated $CD4^+$ T lymphocytes.

9. Purified $CD4^+$ T lymphocytes treated with CT release extracellular cAMP

It has been reported that CT and other cAMP-elevating agents can induce the release of cAMP in the extracellular compartment of different tissues and cells as plasma and urine (Nikolaeva I.S. and Krupnova E.P., 2003; Giron M.C. et al., 2008). Thus, to investigate whether cAMP was involved in the suppression mediated by CT-pretreated $CD4^+$ T lymphocytes, we evaluated if $CD4^+$ T cells

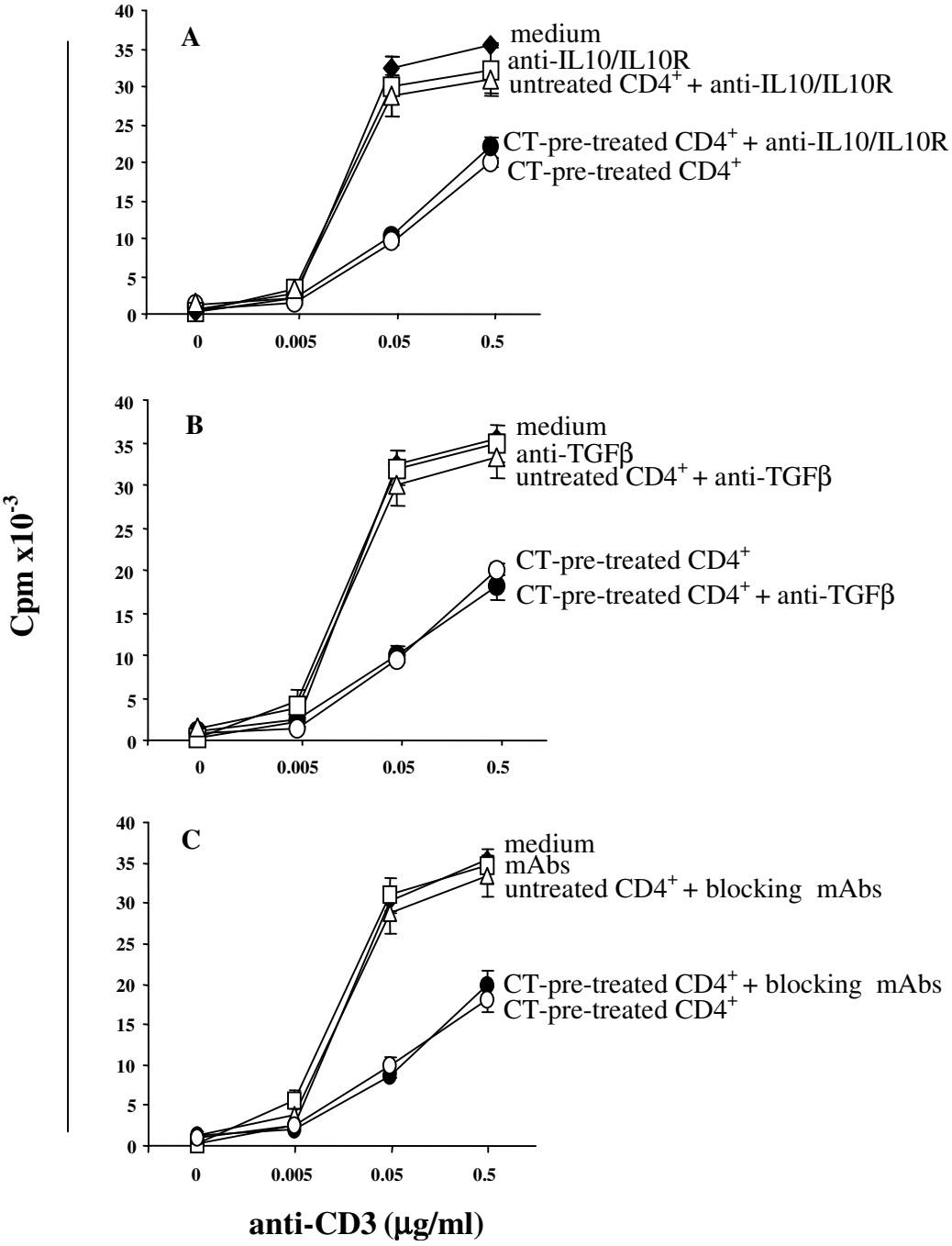

Fig. 12. Neutralizing mAb against IL-10, IL-10R, TGF-β, and IL-4 do not prevent the suppression mediated by CT-pretreated $CD4^+$ T lymphocytes. Untreated or CT-pre-treated $CD4^+$ T lymphocytes ($18x10^4$) were cultured with autologous PBMC ($6x10^4$) at the ratio of 3:1 in the presence or in the absence of neutralizing mAb against IL-10 (1 μg/ml) and IL-10R (1 μg/ml; A), TGF-β (1 μg/ml; B), or a cocktail of anti-IL-4, -IL-10, and –TGF-β (1 μg/ml; C). Cells were stimulated by increasing doses of anti-CD3 mAb (from 0.005 to 0.5 μg/ml), and the proliferation was evaluated 66 h later by ^{3}H-thymidine incorporation. The data shown are from one representative experiment of three performed.

cultured in the presence of CT or FSK were able to release cAMP into the extracellular compartment. Purified $CD4^+$ T cells were cultured in the presence and in the absence of CT (3 μg/ml) or FSK (50 μM) for 24 h, and the level of cAMP in the supernatants was evaluated by RIA assay. We found that untreated $CD4^+$ T cells constitutively released cAMP at a basal level (1.35±0.6 pmoles/10^7 cells) and that the levels of cAMP in the supernatants were increased by the presence of CT and FSK (20.2±4.5 and 15.4±1.5 pmoles/10^7 cells, respectively; Figure 13, panel A).

The levels of extracellular cAMP released by $CD4^+$ T cells, treated or untreated with CT or FSK, were evaluated at different time-points (1, 4 and 24 h) after the removal of the stimulus. We found that cAMP was released in a time-dependent manner only by the cells that had been treated with CT (Figure 13, panel B). Indeed, FSK pre-treated $CD4^+$ T cells, which did not inhibit the proliferation of bystander PBMC, released low levels of cAMP after the removal of FSK. These levels were lower than the levels found in the supernatants of untreated cells (Figure 13, panel B). This phenomenon may be a result of a mechanism of feedback regulation of the adenylate cyclase activity.

To rule out the possibility that the accumulation of extracellular cAMP in the supernatants was the result of a passive release by dying cells, the vitality of untreated and CT-pre-treated $CD4^+$ T cells was evaluated at different time-points after the removal of CT, by propidium iodide (PI) staining. We found that more then 97% of untreated and CT-pre-treated $CD4^+$ T populations were alive (PI-negative) after 6 h and more then 93% after 24 h of culture (data not shown).

These data show that cAMP can be released by $CD4^+$ T lymphocytes in the

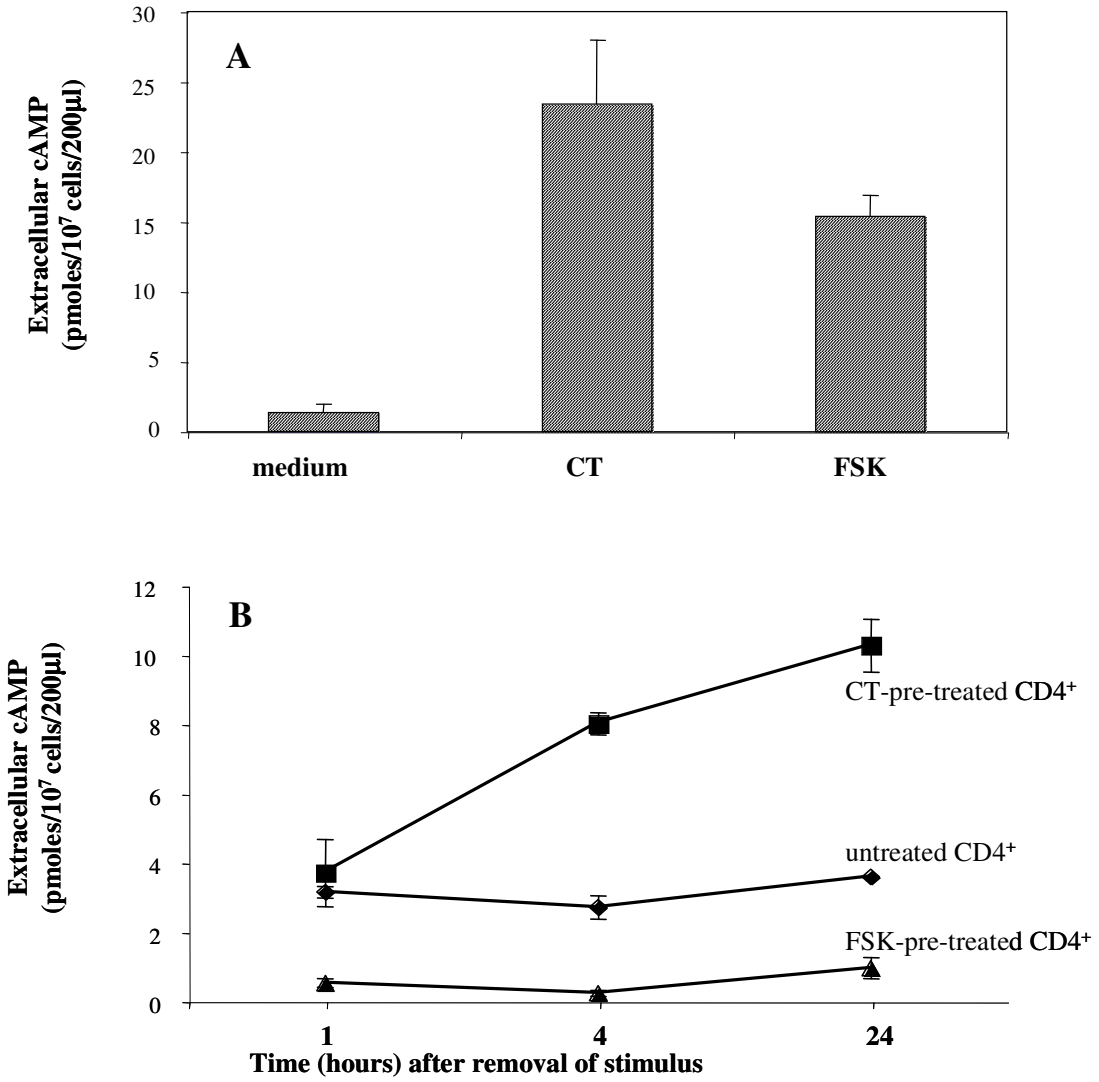

Fig. 13. Sustained release of extracellular cAMP by purified $CD4^+$ T lymphocytes treated with CT. (A) Purified $CD4^+$ T lymphocytes ($5x10^6$/ml) were cultured with medium alone, CT (3 µg/ml), or FSK (50 µM) for 24 h, and the accumulation of cAMP in the supernatants was evaluated by RIA assay. (B) The amount of cAMP released by $CD4^+$ T lymphocytes, treated or untreated with FSK or CT, was evaluated at different time-points (1, 4, and 24 h) after the removal of stimuli. The data shown are from one representative experiment of three performed.

extracellular compartments and that $CD4^+$ T cells pre-treated with CT continue to release cAMP after the removal of CT.

10. Exogenous cAMP inhibits T cell proliferation

Cyclic AMP as second messenger has been widely described in cell of the immune system (Antoni F.A., 2000). Here, we asked whether extracellular cAMP could be sensed by different immune cells and act also as a primary messenger. To evaluate if exogenously added cAMP is able to inhibit T cell proliferation, we stimulated PBMC ($10x10^4$/well) with anti-CD3 mAb (0.5 μg/ml) in the presence or in the absence of cAMP (0.1 mM) and measured the proliferation after 66 h of culture by ^{3}H-thymidine incorporation. Cells treated with dybutyrryl cAMP, which is a stable and cell permeable cAMP analog were included in the experiments. The inhibition observed in the presence of dbcAMP was high (83% of inhibition), as expected. However, we found that exogenous cAMP also inhibited T cell proliferation at appreciable level (54% of inhibition; Figure 14). These data suggest that exogenous cAMP can be up-taken by the cells and is able to exert inhibitory functions.

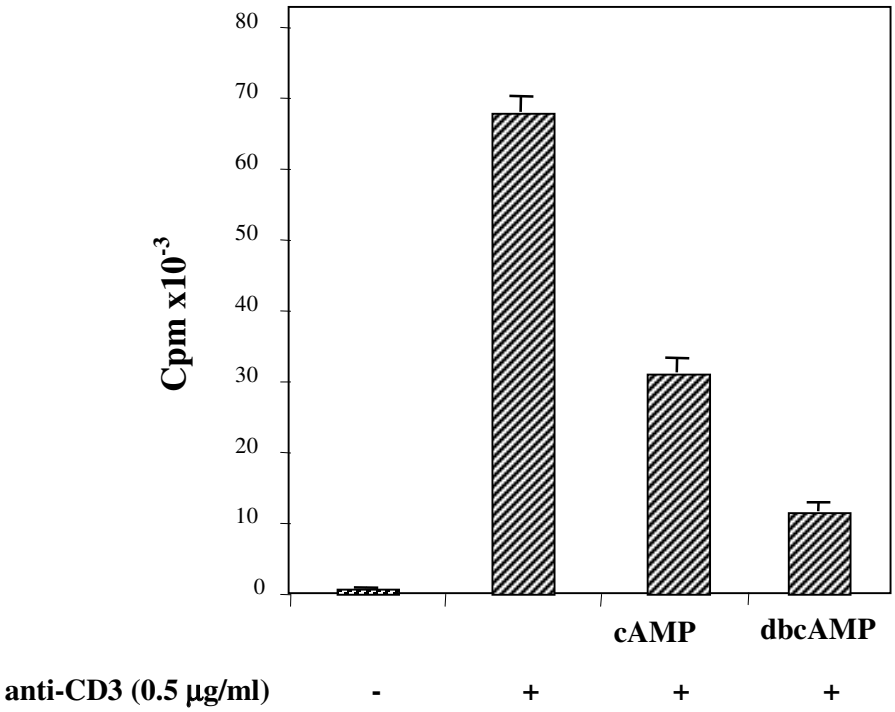

Fig. 14. Exogenous cAMP inhibits T cell proliferation. PBMC ($10x10^4$/well) were stimulated with anti-CD3 mAb (0.5 µg/ml) in the presence of cAMP or dbcAMP (0.1 mM), and T cell proliferation was evaluated after 66 h of culture by ^{3}H-thymidine incorporation. The data shown are from one representative experiment of two performed.

11. Exogenous cAMP is sensed by monocytes and interferes with their differentiation into dendritic cells

It has been described that CT by increasing intracellular cAMP through its enzymatic activity plays an important role for the maturation of dendritic cells (DCs) (Gagliardi et al., 2000). We observed that cells of the immune system exposed to CT or to cAMP-elevating agents are able to release the cyclic nucleotide in the extracellular compartment, therefore we asked whether extracellular cAMP had any effects on maturation of DCs and/or on their differentiation from human monocytes.

Immature DCs generated by culturing monocytes isolated from peripheral blood of healthy donors with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) for 5 days, were treated with exogenous cAMP (0.5 μM) for further 48 h. Cells treated with LPS (200 ng/ml) were included in these sets of experiments to monitor the maturation of the DCs. An up-regulation of CD86 molecules was observed in cells treated with exogenous cAMP as compared to untreated cells (Figure 15). Whereas, the expression of HLA class I and class II and the expression of the maturation marker CD83 was similar to that of untreated cells (Figure 15). Cells cultured with LPS showed an up-regulation of the markers analysed, as expected. These data indicate that extracellular cAMP has an effect on immature DCs, that however does not result in the fully maturation of the cells.

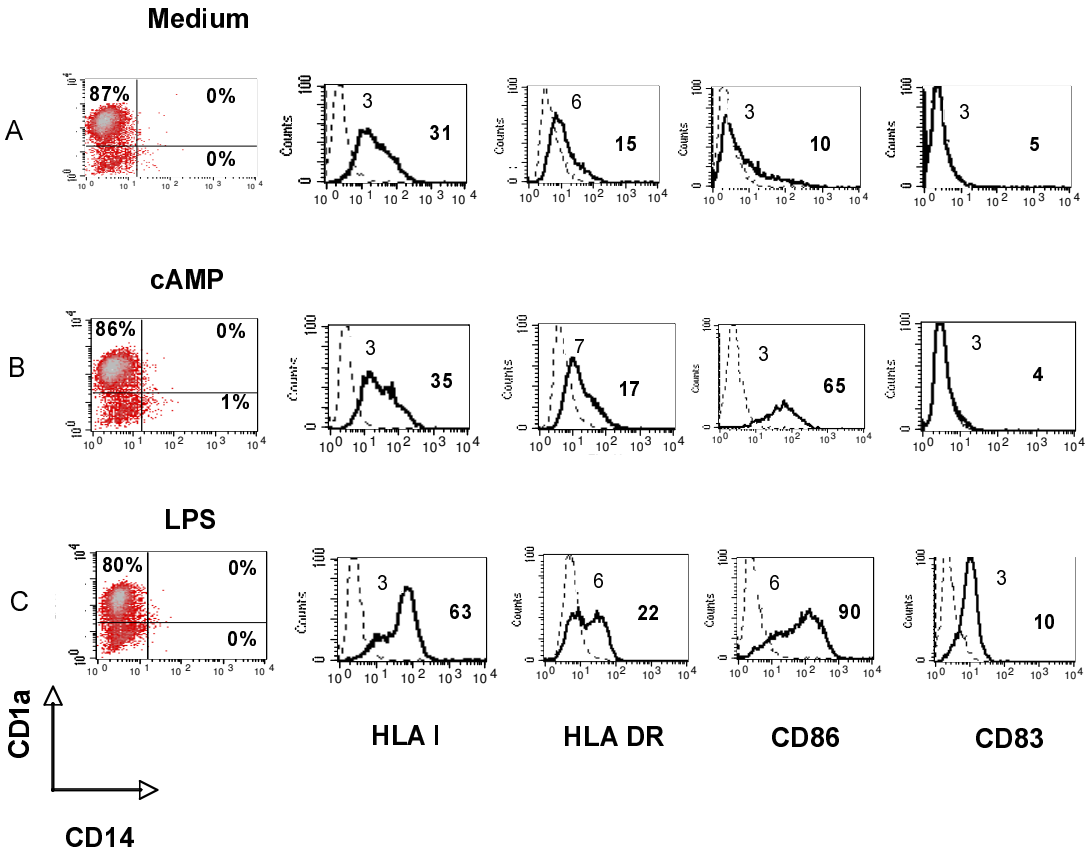

Fig. 15. Effects of extracellular cAMP on DC maturation. Monocytes ($1x10^6$/ml) were induced to differentiate into DCs in the presence of GM-CSF and IL-4 for 5 days. Cells were cultured for further 48 h in the absence (A) or in the presence of cAMP (0.5 μM) (B) or LPS (200 ng/ml) (C). Cells were analysed for their maturation status. On the left panel is shown the percentage of $CD1a^+$ and $CD14^+$ populations. In the histograms the mean fluorescence intensity of the different markers analysed is reported. The data shown are from one representative experiment of three performed.

To test the effect of extracellular cAMP on the differentiation of DCs, monocytes ($1x10^6$/ml) were induced to differentiate into DCs with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) in the presence and in the absence of exogenous cAMP (0.5 μM) for 5 days. Forskolin (FSK) (10 μM) was also used in these sets of experiments. We found that monocytes induced to differentiate into DCs in the presence of cAMP or FSK did not express CD1a molecules and retained the expression of CD14 acquiring a macrophage-like phenotype (Figure 16). Furthermore, they strongly up-regulated MHC class I and class II and CD86 costimulatory molecules giving rise to an activated population (Figure 17). These data suggest that an increase of intracellular cAMP such that obtained by treating the cells with FSK interferes with the capacity of monocyte to differentiate into DCs. Furthermore, the same effect was obtained by treating monocytes with extracellular cAMP suggesting that the cyclic nucleotide can be sensed by the cells and that it is able to exert a biological activity as an extracellular mediator. However, whether the extracellular cAMP could enter the cells or bind to a membrane receptor needs further investigations.

12. The effects of extracellular cAMP on the differentiation of monocytes into DCs are mediated by adenosine receptors

An extracellular cAMP-adenosine pathway it has been described in different cell type such as aortic vascular smooth muscle cells (Dubey et al., 1996)

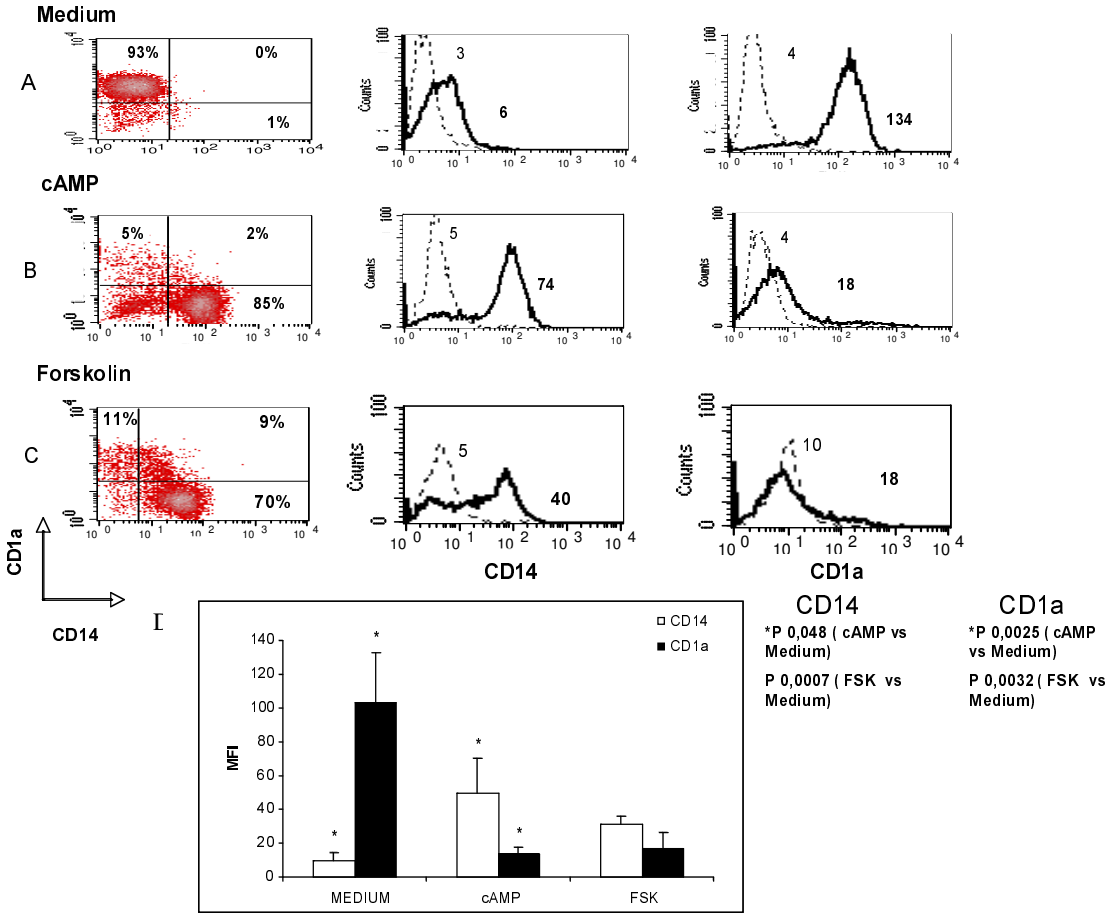

Fig. 16. Effects of extracellular cAMP on monocyte differentation into DCs. Monocytes ($1x10^6$/ml) were cultured with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) for 5 days in the absence (A) or in the presence of cAMP (0.5 μM) (B) or FSK (10μM). Cells were analysed for their maturation status. On the left panel is shown the percentage of $CD1a^+$ and $CD14^+$ populations. In the histograms the mean fluorescence intensity of the different markers analysed is reported. The results are representative of three indipendent experiments and the panel D shows the mean and the SD of the different experiments analysed.

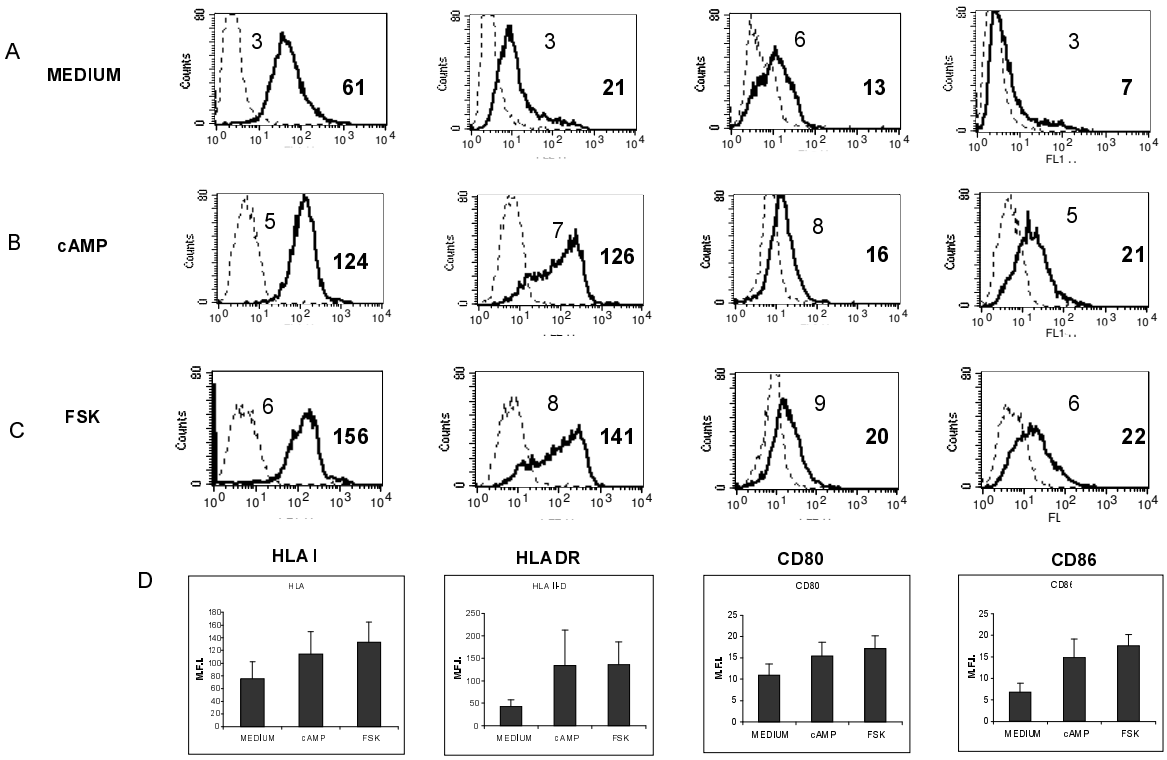

Fig. 17. Expression of different activation markers on cells differentiated in the presence of cAMP or FSK. Monocytes ($1x10^6$/ml) were cultured with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) for 5 days in the absence (A) or in the presence of cAMP (0.5 μM) (B) or FSK (10 μM). Cells were analysed for their maturation status. In the histograms the mean fluorescence intensity of the different markers analysed is reported. The results were representative of three indipendent experiments and the panel D shows the mean and the SD of the different experiments analysed.

or cardiac fibroblasts (Dubey et al., 2000). In these cells exogenous cAMP is converted into AMP, and then into adenosine (ADO) and inosine in a concentration- and time-dependent fashion by extracellular phosphodiesterase such as CD39 and ecto-5'-nucleotidase as CD73 (Jackson E.K. et al., 2007). Adenosine binds to specific receptors (A_1, A_{2A}, $A_{2B,}$ A_3) on the cell surface that mediate different signaling pathways in the cells and are expressed at different levels according to the different cell type (Panther E. et al., 2001; Novitskiy S.V. et al., 2008). The A_{2A} and A_{2B} adenosine receptors are $G\alpha_S$-linked receptors that directly activate adenylyl cyclase enzyme, inducing an increase of intracellular cAMP (Haskó G. et al., 2007). To investigate whether extracellular cAMP could be sensed by monocyte through adenosine receptors, monocytes were induced to differentiate into DCs in the presence of exogenous cAMP and specific adenosine receptor antagonists. An A_{2A} specific (8-(3-Chlorostyryl) caffeine, CSC) and an A_{2B} specific (N-(4-Cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide, MRS 1754), molecules were used as adenosine receptor antagonists. A stable analogous of adenosine, 5'-(N-ethylcarboxamido)-adenosine (NECA), which exerts an agonist effect on the receptors, was used as control in these sets of experiments. Monocytes differentiated in the presence of NECA (3 μM) showed a phenotype similar to that of differentiated in the presence of exogenous cAMP, they gave rice to a mixed populations of $CD1a^-/CD14^+$ cells (59 and 58% for cells treated with cAMP and

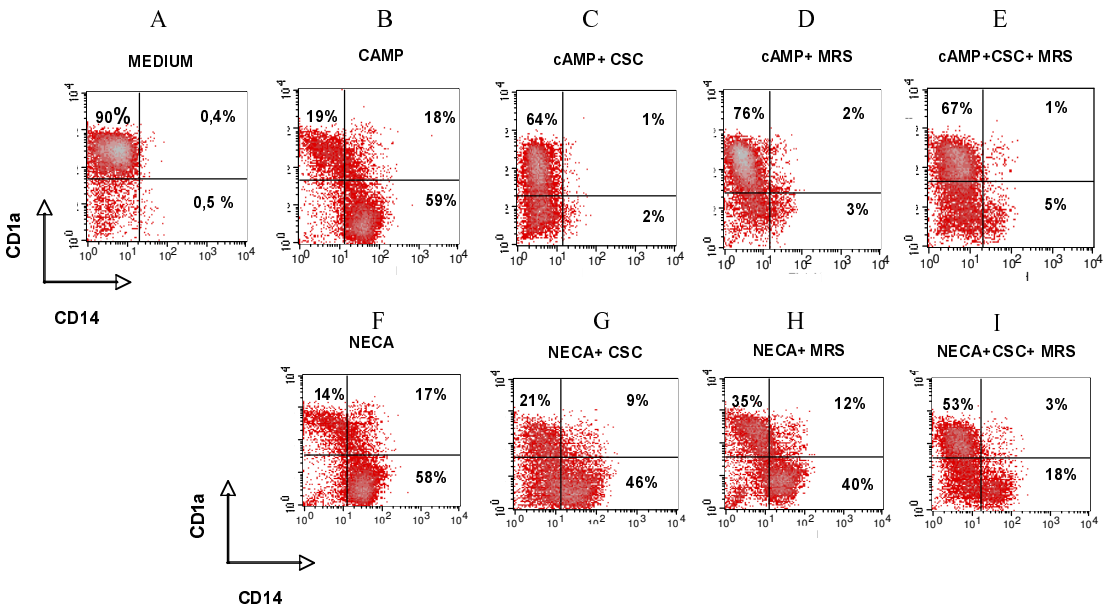

Fig. 18. Extracellular cAMP effects on monocyte differentiation were mediated by A_{2A} and A_{2B} adenosine receptors. Monocytes ($1x10^6$/ml) were cultured with GM-CSF (50 ng/ml) and IL-4 (35 ng/ml) for 5 days in the absence (A) or in the presence of cAMP (0.5 μM) (B) or NECA (3 μM) (C). Cells were incubated with adenosine receptor antagonists CSC (10 μM) (C, G) and MRS (10 μM) (D, H) or both (1 μM of each) (E, I) for 30 min and either cAMP or NECA were added to the cultures. The percentage of $CD1a^+$ and $CD14^+$ cells is reported in the dot plot. The experiment is representative of three performed.

NECA, respectively), $CD1a^+/CD14^-$ (19 and 14% for cells treated with cAMP and NECA, respectively) and $CD1a^+/CD14^+$ (18 and 17% for cells treated with cAMP and NECA, respectively) (Figure 18, panels B, F). However, when adenosine receptor antagonists CSC (10 μM), MRS (10 μM) or both (1 μM of each) were added to the cultures the effects of exogenous cAMP as well as of the NECA were partially reverted (Figure 18, panels C-E and G-F). These data indicate that extracellular cAMP is sensed by monocytes through the A_{2A} and A_{2B} adenosine receptors.

13. T cell stimulation by cells differentiated in the presence of exogenous cAMP

To test the antigen-presenting capacity of monocytes differentiated in the presence of exogenous cAMP (0.5 μM) or FSK (10 μM), cells ($4x10^4$) were co-cultured with allogeneic PBMC ($1x10^5$) isolated from healthy donors at 1:5 DC:T cell ratio. We found that cells differentiated in the presence of exogenous cAMP or FSK were more efficient at stimulate allogeneic T cell response as compare to control cells (Figure 19). These data are in accordance with the expression of higher amount of MHC class I and class II and CD86 costimulatory molecules by cells cultured in the presence of exogenous cAMP or FSK and show that these cells are effective as antigen-presenting cells.

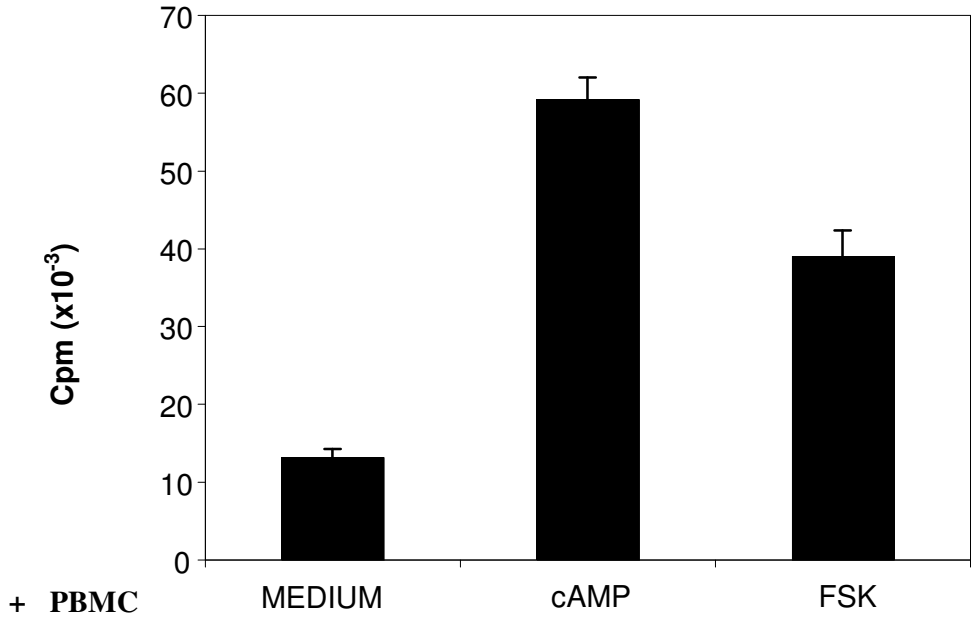

Fig. 19. Monocytes differentiated in the presence of extracellular cAMP or FSK enhance allogeneic T cells responses. Monocytes ($1x10^6$/ml) cultered in the absence (medium) or in the presence of extracellular cAMP (0.5 mM) or FSK (10 μM) ($4x10^4$) were cocultered with allogenic PBMC ($1x10^5$) for 3 days. Proliferation was evaluated after 66 h by ^{3}H-thymidine incorporation. The experiment is representative of three performed.

14. Cells differentiated in the presence of exogenous cAMP produce IL-10 and IL-6 and not TNF-α and IL-12 upon LPS stimulation

To further characterize the functions of monocytes differentiated in the presence of exogenous cAMP or FSK, we analysed the production of proinflammatory cytokines such as TNF-α and IL-6 in culture supernatants after 48 h of LPS stimulation by ELISA. We found that the production of TNF-α upon LPS stimulation was impaired in cells differentiated in the presence of exogenous cAMP (0.5 μM) and FSK (10 μM) as compared to control cells (Figure 20, panel A), whereas the production of IL-6 was enhanced (Figure 20, panel B). Furthermore, the accumulation of IL-10 and IL-12, which are important factors involved in directing immune responses, was measured after 48 h of stimulation with LPS. The production of IL-12, which was high in control cells was strongly inhibited in cells differentiated in the presence of exogenous cAMP or FSK (Figure 20, panel C). In contrast, the production of IL-10 by cells differentiated in the presence of exogenous cAMP or FSK was enhanced (Figure 20, panel D). These data indicate that monocytes treated with cAMP or FSK differentiate into cells able to produce IL-6 and IL-10 upon maturation stimuli.

Dendritic cells have the ability to stimulate naïve T lymphocytes and drive them into distinct classes of effector cells. Because of the particular pattern of cytokines

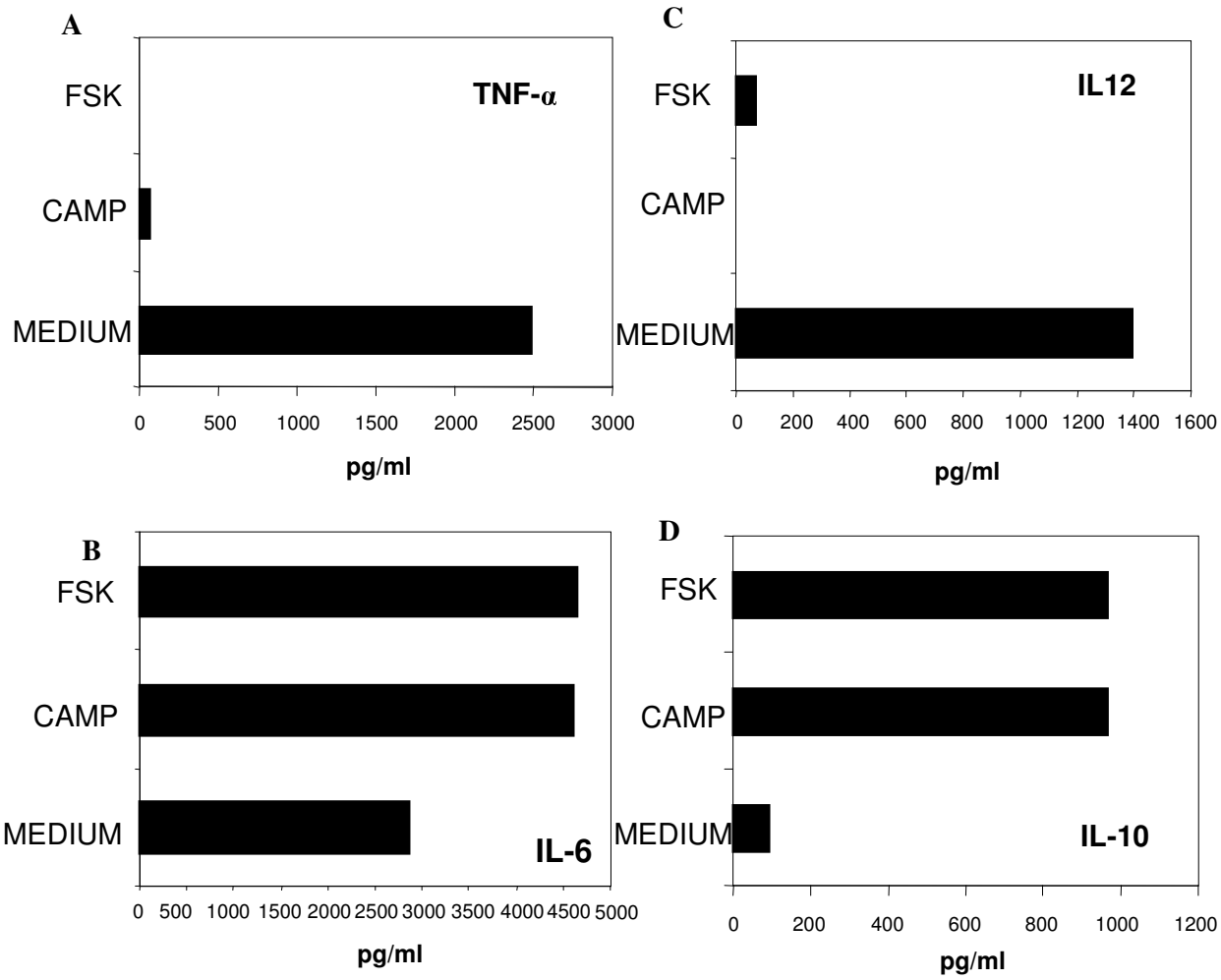

Fig. 20. Exogenous cAMP inhibits TNF-α and IL-12 production and facilitates IL-10 and IL-6 release by monocytes differentiated in the presence of cAMP or FSK. Monocytes ($1x10^6$/ml) were cultured in medium or with cAMP (0.5 mM) or FSK (10 μM). Then, cells were cultured for further 48 h with LPS (200 ng/ml) and the amount of cytokines in culture supernatants was evaluated by ELISA. The data shown are from one representative experiment of three performed.

produced by cells differentiated in the presence of exogenous cAMP or FSK , we speculate that they could differ in their capacity to support T cell differentiation. Control cells and cells differentiated in the presence of exogenous cAMP or FSK, were co-cultured with naïve $CD4^+CD45RA^+$ T cells for 11 days. We found that a high percentage (52%) of naïve T cells cultured with control cells produced IFNγ, whereas the percentage of naïve T cells cultured with cells differentiated in the presence of exogenous cAMP or FSK, was reduced (25% and 26%, respectively (Figure 21). On the other hand, the percentage of IL-4 producing cells was low and comparable in all culture conditions (figure 21). These data indicate that extracellular cAMP and FSK inhibit the polarization of Th1 response, suggesting that cells differentiated in the presence of exogenous cAMP or FSK are able to shape the adaptive immune response.

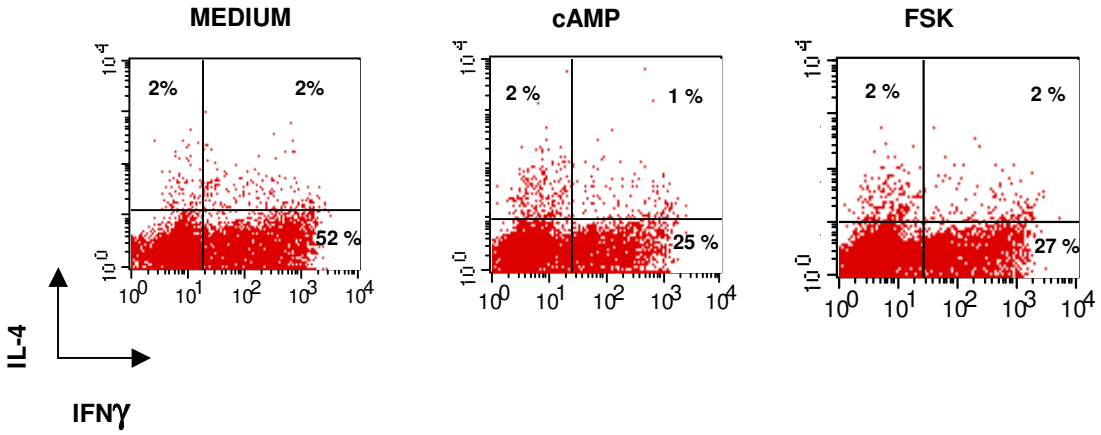

Fig.21. Monocytes induced to differentiate into DCs in the presence of cAMP or FSK inhibit the differentiation of IFN-γ producing cells. Monocytes ($1x10^6$/ml) cultured with cAMP (0.5 mM) or FSK (10 μM) for 5 days, were washed and cocultured with autologous purified naïve $CD4^+CD45RA^+$ T cells ($1x10^6$/ml; ratio 1:5). After 11 days T cells were harvested, washed and analysed for intracellular IFNγ and IL-4 production by flow cytometry. The data shown are from one representative experiment of six performed.

DISCUSSION

In this study, we have analysed the mechanisms underling the inhibition of T cell proliferation mediated by Cholera Toxin (CT) on human $CD4^+$ and $CD8^+$ T lymphocytes. We observed that CT prevents the early activation steps of T lymphocytes and that these effects involve the modulation of costimulatory molecules CTLA-4 and CD28. Moreover, we evaluated the function of CT-pre-treated $CD4^+$ T lymphocytes and we observed that they are able to inhibit the proliferation of autologous T lymphocytes stimulated with anti-CD3 mAbs. It is interesting that this phenomenon is, at least in part, a result of the release of extracellular cAMP. Therefore, by analysing the direct effects exerted by extracellular cAMP as a primary messenger on different cell types, we found that extracellular cAMP inhibits T cell proliferation and that it is able to interfere with the differentiation of monocytes into DCs.

It has been reported that CT has different effects on murine $CD4^+$ and $CD8^+$ T lymphocytes. It selectively induces depletion of the murine $CD8^+$ T population by inducing apoptosis in these cells (Nashar T.O. et al., 1996; Arce S. et al., 2005; Simmons C.P. et al., 2001). Here, we show that CT inhibits the proliferation of both human $CD4^+$ and $CD8^+$ T cells and does not induce apoptosis in either population, suggesting that human T lymphocytes react differently to CT as compared to murine T cells.

Analysis of the DNA content of CT-treated T cells showed an arrest in the G_0/G_1 phase of the cell cycle and this correlated with a high expression of the cyclin-dependent kinase (cdk) inhibitor $p27^{kip}$. Blockade of cell cycle kinases by regulatory proteins such as $p27^{kip}$ has been reported to play an essential role in the induction and maintenance of anergy (Boussiotis V.A. et al., 2000; Wells A.D.,

2000; Rowell E.A. et al., 2005), a state of unresponsiveness occurring when T cells are stimulated through CD3/TCR in the absence of costimulation. Indeed, suboptimal activation of T lymphocytes with costimulatory molecule-deficient antigen-presenting cells fails to down-regulate $p27^{kip}$ and induces anergy (Wells A.D., 2000). Whereas CD28 engagement and IL-2 both down-regulate $p27^{kip}$ allowing for cdk activation (Nourse J. et al., 1994; Appleman L.J. et al., 2000; Appleman L.J. et al., 2002), the down-regulation of the surface expression of CD28 has been associated with the induction of T cell anergy (Lake R.A. et al., 1993). Other studies show a direct correlation between high levels of $p27^{kip}$ and increased expression of CTLA-4 (Kubsch S. et al., 2003). Our results are consistent with these findings. Indeed, we show that CT causes an impaired down-modulation of $p27^{kip}$, an up-regulation of CTLA-4 and a down modulation of CD28. We found that CT upregulates CTLA-4 either on $CD4^+$ or $CD8^+$ T cells, although in $CD8^+$ T lymphocytes its up-regulation is slower and does not reach the same level of expression as in $CD4^+$ T cells. The variable expression of CTLA-4 observed in these different T cell subpopulations might be due to a different expression and/or distribution of the CT receptor GM1. Expression of different gangliosides varies at the cell, tissue and organ levels and differs also among mammalian species (Nagai Y. and Iwamori M., 1984). Furthermore, GM1 is known to associate with cholesterol-rich domains (lipid rafts), which become organized on the T cell membrane upon activation. It would be reasonable to hypothesize that GM1 expression, modulation and/or distribution on the membrane is different on different cell types in relation to their differentiation or activation state.

The regulation of CTLA-4 expression by CT is exerted at the transcriptional level. Indeed, we observed increased CTLA-4 mRNA in cells treated with CT. Two mRNA variants coding for membrane and soluble CTLA-4 molecules are known to be expressed in resting T lymphocytes, however the mRNA for the soluble form is found only in non-activated cells and decreases upon T cell activation (Magistrelli G. et al., 1999). Interestingly, the up-regulation of human CTLA-4 by CT in resting T lymphocytes involves both isoforms of the mRNA transcript. It would be interesting to verify whether the two isoforms are produced as proteins in these cells.

The inhibitory effect of CT on T cell functions is further explained by the capacity of CT to down-modulate the expression of CD28 on $CD4^+$ and $CD8^+$ T lymphocytes. CD28 is the main costimulatory receptor implicated in a wide array of T cell responses, including T cell proliferation, prevention of anergy and IL-2 production (Sansom D.M. and Walker L.S., 2006). This central role of CD28 suggests that modulation of the levels of CD28 expression profoundly alters T-cell function. Down-modulation of CD28 expression on $CD4^+$ and $CD8^+$ T cells occurs in patients with chronic inflammatory syndromes (Borthwick N.J. et al., 1994). In addition, T cells that exhibited reduced CD28 cell surface expression have been observed during the acute phase of *Bordetella pertussis* infection in mice (McGuirk P. et al., 1998). Our results also suggest that CD28 expression could be modulated by bacteria and identify for the first time a bacterial product that is capable of directly down-modulating CD28 expression on both $CD4^+$ and $CD8^+$ T cells. The interaction between CD28, CTLA-4 and their ligands has been extensively studied and the affinity of CTLA-4 for both CD80 and CD86 is higher

than that of CD28 (Sansom D.M. and Hall N.D., 2006). Therefore, the relative expression levels of both CTLA-4 and CD28 play an important role in controlling T cell activation and functions. By using blocking anti-CTLA-4 $F(ab')_2$ mAbs, we showed that the inhibition of T cell proliferation induced by CT was partially prevented. This shows that CTLA-4 molecules up-regulated by CT in resting T cells localise on the cell surface and they play a role in the inhibition of T cell proliferation mediated by CT. It has been reported that CTLA-4 is constitutively expressed on T regulatory cells (Tregs) and plays an essential role in their function (Read S. et al., 2000, Wing K. et al., 2008). It has previously shown that CT has the capacity to induce T cells with regulatory activity (Lavelle E.C. et al., 2003). Thus, we investigated whether human T lymphocytes, which had been exposed to CT or to other cAMP-elevating agents, had regulatory activity. We found that human-purified $CD4^+$ T lymphocytes pre-treated with CT were able to inhibit proliferation of anti-CD3-stimulated, autologous PBMC in a dose-dependent manner. It is interesting that this phenomenon was, at least in part, a result of the release of extracellular cAMP by the purified $CD4^+$ T lymphocytes. Conversely, purified $CD4^+$ T cells pre-treated with FSK, a transient cAMP inducer, or with dbcAMP, an analog of cAMP, did not suppress the proliferation of bystander anti-CD3-stimulated PBMC, suggesting that a sustained production of cAMP is required to identify a novel, regulatory function mediated by $CD4^+$ T cells. The T cells with regulatory functions, which we describe here, are new in the method of generation and in their mode of suppression. Indeed, the in vitro-induced Tregs described so far are generated by culturing T lymphocytes with antigen or polyclonal activators in the presence of inhibitory cytokines such as IL-

10 or TGF-β (Sakaguchi S. et al., 2004). Furthermore, these cells are able to suppress the activity of effector T lymphocytes through the release of IL-10 and/or TGF-β. It is interesting that our findings show that the inhibition mediated by CT-induced $CD4^+$ T cells was not mediated by these cytokines. CT and adenylate cyclase toxin from *Bordetella pertussis* have been described to promote the induction of IL-10-producing Tregs cells to co-administered antigen by enhancing the production of IL-10 by DCs, which in turn, induce the differentiation of T cells with regulatory phenotype (Lavelle E.C. et al., 2003). Here, we show that CT also has the capacity to induce a new class of T cells with regulatory activity by interacting directly with T lymphocytes. This and other findings (Mills K.H. and McGuirk P., 2004) suggest that following microbial infections, different classes of T cells with regulatory functions may be generated. Furthermore, several bacteria produce cAMP-inducing toxins or they release cAMP themselves (Ahuja N. et al., 2004); thus, our results imply that immunosuppressive effects may be generated in conditions of high, local cAMP production.

After the discovery of the role of cAMP as an intracellular second messenger, several studies reported the presence of the cyclic nucleotide in plasma and urine, suggesting that cAMP could be exported out of the cells (Sutherland E.W., 1970). The release of cAMP from different cell types is an active transport against a concentration gradient, and it seems to be strictly regulated according to the stimulus and to the tissue involved (Exton J.H. et al., 1971). However, the release of cAMP by T lymphocytes has not been reported so far. Here, we observed that the stimulation of purified $CD4^+$ T lymphocytes with CT or FSK induces the

release of cAMP. According to the constitutive activation of the host adenylate cyclases by CT, only CT-pre-treated $CD4^+$ T cells sustained the release of cAMP after the removal of the stimulus. This allowed us to demonstrate that extracellular cAMP plays a biological role as a soluble mediator of T cell suppression. Furthermore, we observed that exogenous added cAMP inhibited the anti-CD3-induced T cell proliferation, further indicating that extracellular cAMP is able to exert inhibitory functions.

It has been described that CT by increasing intracellular cAMP through its enzymatic activity plays an important role for the differentiation and maturation of DCs (Gagliardi M.C. et al., 2000; Giordano D. et al, 2003). Since we observed that cells of the immune system exposed to CT or to cAMP-elevating agents are able to release the cyclic nucleotide in the extracellular compartment, we asked whether extracellular cAMP had any effects on maturation of DCs and/or on their differentiation from human monocytes. We found that cells treated with exogenous cAMP showed an up-regulation of CD86 molecules and did not affect the expression of other maturation markers indicating that extracellular cAMP has an effect on immature DCs, that however does not result in the fully maturation of the cells. In contrast, by analysing the direct effects of extracellular cAMP on the differentiation of monocytes into DCs, we found that extracellular cAMP was able to interfere with their differentiation. Monocytes induced to differentiate into DCs in the presence of cAMP or FSK did not express CD1a molecules and retained the expression of CD14 acquiring a macrophage-like phenotype. Furthermore, they strongly up-regulated MHC class I and class II and CD86 costimulatory molecules giving rise to an activated population able to stimulate allogeneic T cell

response. In addition, they produced a distinct pattern of cytokines upon maturation stimuli, they were unable to produce TNF-α, but released high amount of IL-6. Dendritic cells are well known for their capacity to produce immunoregulatory cytokines such as IL-12 and IL-10 upon maturation stimuli. The balance of the production of these cytokines plays a pivotal role by orchestrating an innate and acquired immune response and by determining the polarization of T cell precursors (de Jong E.C. et al., 2005; Wu L.and Dakic A., 2004). The secretion of IL-10 and the inhibition of IL-12 synthesis could account for the reduced capacity of cells differentiated in the presence of exogenous cAMP or FSK, of inducing the differentiation of IFN-γ-producing $CD4^+$ T lymphocytes. All these data are consisting with others that show that an increase of intracellular cAMP interferes with the capacity of monocytes to differentiate into DCs (Giordano D. et al, 2003; Kalinski et al., 1997; Novitskiy S.V. et al., 2008). Of interest, here we found that the same effects were obtained by treating monocytes with extracellular cAMP suggesting that the cyclic nucleotide can be sensed by monocytes and that it is able to exert a biological activity as an extracellular mediator. However, whether the extracellular cAMP could enter the cells or bind to a membrane receptor needs further investigations.

The cellular and molecular mechanisms by which extracellular cAMP affects the function of different cells of the immune system need to be further investigated. Those effects could be a result of an influx of extracellular cAMP directly into cells, and this may account for the inhibition of proliferation of T cells, as an increased level of intracellular cAMP in T lymphocytes is known to have inhibitory effects (Johnson K.W. et al., 1988; Gagliardi M.C. et al., 2002) and

explain the altered monocyte differentiation into DCs, since it has been described that molecules that cause the increase of intracellular cAMP in monocytes are able to interfere with their differentiation (Giordano D. et al, 2003; Kalinski et al., 1997; Novitskiy S.V. et al., 2008). Alternatively, the binding of cAMP to specific membrane receptors could deliver inhibitory signals to the cells. In support of the first hypothesis, it has been shown that the influx of cAMP into smooth muscle cells has been found to be mediated by a system, which involves a transporter (Orlov, S.N. and Maksimova N.V., 1999). Conversely, although cAMP receptors have not yet been identified in mammals, they have been well characterized in lower eukaryotes. Four different cAMP receptors have been described in the amoeba *Dictyostelium discoideum* (Johnson R.L. et al., 1992). They belong to the superfamily of seven transmembrane domain G protein-coupled receptors, which modulate the level of intracellular cAMP. It is interesting that these receptors exhibit a certain homology with the secretin receptor family (Kim J.Y. and Devreotes P.N., 1994), and it is tempting to speculate that similar cAMP receptors may be present in mammalian cells, although this remains to be demonstrated. Another possible mechanism by which the efflux of cAMP could mediate inhibitory effects in target cells is the extracellular cAMP-adenosine pathway (Jackson E.K. and Raghvendra D.K., 2004). Extracellular cAMP can be converted into adenosine, which activates adenylate cyclase via A2 receptors, leading to an increase of intracellular cAMP (Jackson E.K. et al., 2007). By using different adenosine receptor antagonists, we found that an extracellular cAMP-adenosine pathway is involved in the effects mediated by exogenous cAMP, suggesting that

extracellular cAMP can be sensed by cells of immune system and modulate their functions.

The stimulation of different membrane receptors on different cell types by hormones or neurotransmitters such as catecholamines, PGE2, and histamine modulates intracellular and extracellular levels of cAMP. Cyclic AMP as a second messenger is responsible for the regulation of many cellular events (Kammer M.G., 1988), however by showing that cells treated with cAMP-elevating agents, can release the cyclic nucleotide in the extracellular compartment we suggest that it acts also as a primary messenger, playing a role in the regulation of different immune responses. A physiological role of the release of cAMP from different cells could be associated to a novel mechanism involved in the maintenance of the immune homeostasis or in the modulation of the inflammatory reactions against invading microbes.

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