



**UNIVERSITÀ DEGLI STUDI DI
ROMA
"TOR VERGATA"**

FACOLTA' DI SCIENZE MATEMATICHE, FISICHE E
NATURALI

DOTTORATO DI RICERCA IN IMMUNOLOGIA

XXI CICLO

Immunomodulatory properties of cholera toxin B subunit

Manuela Colucci

A.A. 2008/2009

Tutor: Dott.ssa Francesca Quintieri

Coordinatore: Prof. Paolo Rossi

INDEX

<u>1 ABSTRACT.....</u>	<u>4</u>
<u>1.1 English.....</u>	<u>4</u>
<u>1.2 Italian.....</u>	<u>6</u>
<u>2 INTRODUCTION.....</u>	<u>9</u>
<u>2.1 Cholera toxin.....</u>	<u>9</u>
<u>2.1.1 Transport of cholera toxin into target cells.....</u>	<u>10</u>
<u>2.1.2 Immunity or tolerance?.....</u>	<u>15</u>
<u>2.1.3 Adjuvants' role: from CT to CTB.....</u>	<u>16</u>
<u>2.2 Dendritic cells.....</u>	<u>19</u>
<u>2.2.1 DC subsets.....</u>	<u>20</u>
<u>2.2.2 Different maturative stages of DC.....</u>	<u>23</u>
<u>2.2.3 Tolerogenic DC.....</u>	<u>28</u>
<u>2.3 Regulatory T cells.....</u>	<u>33</u>
<u>3 MATERIALS AND METHODS.....</u>	<u>39</u>
<u>3.1 Chemical and reagents.....</u>	<u>39</u>
<u>3.2 Human PBMC isolation.....</u>	<u>40</u>
<u>3.3 Generation and culture of MDDC.....</u>	<u>40</u>
<u>3.4 MLR.....</u>	<u>41</u>
<u>3.5 T cell culture.....</u>	<u>43</u>
<u>3.6 Cytokine assays.....</u>	<u>44</u>
<u>3.7 Analysis of T cell function (suppression assay).....</u>	<u>44</u>
<u>3.8 Flow cytometry analysis.....</u>	<u>46</u>
<u>3.9 Statistical analysis.....</u>	<u>48</u>
<u>4 RESULTS.....</u>	<u>49</u>
<u>4.1 rCTB treatment affects maturation of human MDDC.....</u>	<u>49</u>

<u>4.2 rCTB-treated MDDC show a reduced ability to stimulate MLR response.....</u>	<u>52</u>
<u>4.3 rCTB-treated MDDC induce the generation of CD4+ T cells with reduced proliferative capacity and increased IL-10 production</u>	<u>53</u>
<u>4.4 rCTB-treated MDDC induce functional CD4+ Tr1 cells.....</u>	<u>57</u>
<u>5 CONCLUSIONS.....</u>	<u>63</u>
<u>6 DISCUSSION.....</u>	<u>64</u>
<u>7 REFERENCES.....</u>	<u>71</u>

1 ABSTRACT

1.1 English

Antigen-non-specific innate immunity and antigen-specific adaptive immunity synergize to eradicate invading pathogens through the actions of immune cells and their effector proteins, including complement, antibodies, cytokines and cytolytic factors. Adaptive immune responses are induced, coordinated and regulated by dendritic cells (DC). DC initiate immunity by the activation of naïve B and T cells - the effector cells of the adaptive immune system - and by the stimulation of natural killer cells - the crucial cellular instigators of innate resistance. Besides linking innate and adaptive immunity, DC limit excessive, tissue-damaging immune responses in order to prevent autoimmunity and non-essential reactions to innocuous agents through their ability to induce antigen-specific unresponsiveness of lymphocytes in primary and secondary lymphoid tissues by mechanisms that include deletion and induction of regulatory T cells (Tregs). Given the central role of these antigen presenting cells in immunity and tolerance, they are ideal therapeutic targets for pharmacological modulation of immune responses.

In the present study, we examined the possibility that recombinant CTB (rCTB) may affect human DC functions in response to toll-like receptor (TLR) stimulation and may induce the generation of DC with the capacity to generate Tregs. CTB - cholera toxin B subunit - is an efficient mucosal carrier molecule for the generation of immune responses to linked antigens, since it facilitates entry into the cell of the CTB-antigen complex by ligation of its surface receptor GM1. There is also good evidence that CTB acts as an immunosuppressant, as it is able to down-modulate human monocyte/macrophage cell line activation and to suppress Th1-type responses. Our findings show that rCTB partially prevents the lipopolysaccharide (LPS)-induced maturation process of monocyte-derived DC (MDDC) and decreases their interleukin-12p70 (IL-12p70) production with no relevant effect on IL-10 production. LPS-stimulated MDDC pre-treated with rCTB are able to promote the induction of low proliferating T cells, which show an enhanced IL-10 production associated with a reduced interferon- γ (IFN- γ) production and the same high levels of transforming growth factor- β (TGF- β) as the control. These T cells suppress proliferation of activated autologous T cells. Transwell experiments and blockade of IL-10R and TGF- β showed that the immunomodulatory effect is mediated by soluble factors. Thus, T cells induced by rCTB-conditioned MDDC acquire a

regulatory phenotype and activity similar to those described for IL-10 producing type 1 Tregs (Tr1).

1.2 Italian

L'immunità innata antigene-non-specifica e l'immunità adattativa antigene-specifica collaborano per debellare i patogeni che invadono l'organismo tramite l'intervento delle cellule del sistema immunitario e delle loro molecole effettrici, che includono le proteine del complemento, gli anticorpi, le citochine e i fattori deputati alla lisi cellulare. Le risposte immunitarie adattative vengono indotte, coordinate e regolate dalle cellule dendritiche (DC). Le DC avviano le risposte immunitarie attivando i linfociti B e T naïve – che sono le cellule effettrici dell'immunità adattativa – e stimolando le cellule natural killer, che sono le principali cellule effettrici dell'immunità innata. Accanto al loro ruolo di collegamento tra immunità innata e adattativa, le DC hanno il delicato compito di limitare le risposte infiammatorie eccessive, che potrebbero causare danni tissutali, con lo scopo di prevenire l'insorgenza di reazioni indesiderate contro antigeni autologhi o innocui. A questo scopo, le DC inducono nei linfociti uno stato di “non-responsività” contro gli antigeni sia negli organi linfoidi primari che in quelli secondari, attraverso meccanismi che includono la delezione e

l'induzione di cellule T regolatorie (Tregs). Dal momento che presentano questo ruolo centrale sia nell'immunità che nella tolleranza, le DC dimostrano di essere un candidato ideale come bersaglio di farmaci immunomodulanti.

Nel presente studio abbiamo esaminato la possibilità che la CTB ricombinante (rCTB) possa influenzare le funzioni delle DC in risposta alla stimolazione tramite toll-like receptors (TLR), rendendo le DC capaci di indurre il differenziamento di Tregs.

La CTB – la subunità B della tossina colerica – è un'efficace proteina trasportatrice a livello mucosale, in quanto facilita l'internalizzazione degli antigeni ad essa legati tramite l'interazione con il proprio recettore di membrana GM1, e favorisce l'attivazione delle risposte immunitarie dirette contro tali antigeni.

Inoltre è stato dimostrato che la CTB agisce come un immunosoppressore, dal momento che è in grado di inibire l'attivazione delle linee cellulari monocito-macrofagiche e di sopprimere le risposte di tipo Th1.

Le nostre ricerche dimostrano che la rCTB previene parzialmente la maturazione di DC derivanti da monociti (MDDC) indotta da lipopolisaccaride (LPS) e ne diminuisce la produzione di IL-12p70 senza influenzarne la produzione di IL-10. Le DC pretrattate con CTB e

stimolate con LPS sono così in grado di promuovere l'induzione di cellule T scarsamente proliferanti che mostrano un'aumentata produzione di IL-10 associata ad una ridotta produzione di interferon- γ (IFN- γ) e ad analoghi elevati livelli di transforming growth factor- β (TGF- β), quando paragonati a quelli delle cellule di controllo.

Le cellule T così indotte sono capaci di sopprimere la proliferazione di cellule T autologhe attivate, tramite la produzione di fattori solubili, come dimostrano gli esperimenti effettuati mediante Transwell o con anticorpi bloccanti il recettore dell'IL-10 (IL-10R) ed il TGF- β . Tutte queste caratteristiche rendono le cellule T indotte da DC condizionate con rCTB simili alle Tregs di tipo 1 (Tr1) producenti IL-10.

2 INTRODUCTION

2.1 Cholera toxin

Cholera toxin (CT) is a toxin secreted by *Vibrio Cholerae*, a bacterium that colonizes the mucosal surface of the human small intestine and secretes the toxin. CT stimulates secretion of water and electrolytes by the cells of the small intestine, leading to the severe watery diarrhoea that is characteristic of cholera (Waldor & RayChaudhuri, 2000). CT consists of noncovalently associated A (active)- and B (binding)-subunits (Fig. 1A).

The A-subunit is cleaved into the A1 and A2 chains, which are linked by a disulfide bond and extensive noncovalent interactions. Five identical polypeptides (11.6 kDa each) assemble into a pentameric ring to form the B-subunit that binds to a membrane lipid ganglioside GM1 at the cell surface and allows cell entry of the holotoxin. The A2 chain protrudes with its C terminus through the central pore in the B-ring and has a KDEL (Lys-Asp-Glu-Leu) sequence, which is known to be an endoplasmic reticulum (ER) retrieval motif. The A1 chain enters the cytosol and increases the level of intracellular cyclic AMP (cAMP) inducing intestinal chloride secretion that causes the massive secretory diarrhoea seen in cholera (Fujinaga et al., 2003; Fujinaga, 2006; Pina &

Johannes, 2005; Smith, Lord, Roberts, & Johannes, 2004; Wolf, Fujinaga, & Lencer, 2002).

2.1.1 Transport of cholera toxin into target cells

The process by which CT enters the cells and is translocated to the cytosol, where it exerts its toxicity, remains obscure. Recently, the pathway followed by CT from the plasma membrane to the ER lumen has been clarified (Fujinaga, 2006).

The A-subunit is unable to enter into the cells alone. It needs to be non-covalently associated to its B-subunit, although the B-subunit trafficking is independent of the A-subunit. The structure of cholera toxin B subunit (CTB) alone, or in complex with its receptor, the pentasaccharide ganglioside GM1, reveals the existence of one receptor binding site per monomer with single hydrogen interactions between the sugar on GM1 and the protein residues on the toxin subunit (Merritt et al., 1994). The binding of GM1 to the five sites of CTB is known to be cooperative and the affinity of this interaction depends not only on its structure but also on the incubation time, the amount of GM1 molecules, and the cholesterol in cell membrane, although it is independent of temperature or pH variations (Dawson, 2005). GM1 expression on the surface of the plasma membrane is particularly relevant, since there are strong

variations depending on the type and state of the cells. Indeed, the presence of GM1 is associated with formation of characteristic membrane microdomains called “lipid rafts”, as they float into the membrane phospholipidic bilayer. These rafts are formed through segregation of composed glycosphingolipids (GM1 is a glycosphingolipid), cholesterol, and phospholipids, and their position and stability into the plasma membrane are variable (Fig. 2) (Pierce, 2002; Smith et al., 2004).

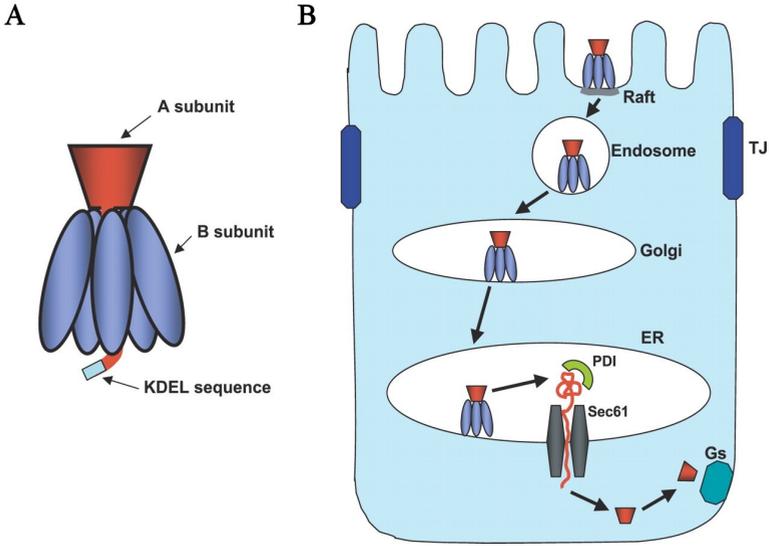
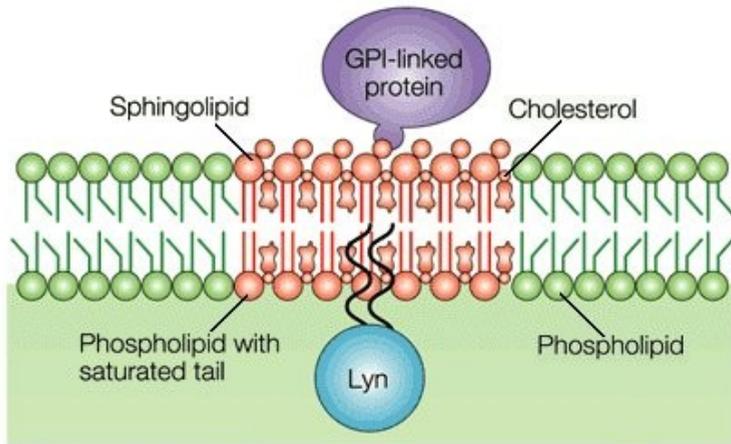


Fig. 1: (A) Schematic structure of cholera toxin. (B) The pathway followed by the cholera toxin from cell surface through the Golgi and the ER to the cytosol. Cholera toxin bound to the cell surface GM1 in the lipid raft is endocytosed, and transported retrogradely to the Golgi and the ER as a holotoxin. At the ER, PDI (protein disulfide isomerase) unfolds and dissociates the A1 fragment from the B subunits. Then, at least the A1 subunit is translocated into the cytosol, probably through the Sec61 channel.



Nature Reviews | Immunology

Fig. 2: Lipid raft structure. Lipid rafts are sphingolipid- and cholesterol-rich membrane microdomains in the outer leaflet of the plasma membrane. Sphingolipid microdomains float in a phospholipid bilayer, leading to the coining of the term 'lipid rafts'. The membrane outer leaflet rafts are believed to be linked to an inner leaflet that is probably rich in phospholipids with saturated fatty acids and cholesterol. Rafts were shown selectively to include some proteins and to exclude others, so rafts provide a mechanism for the lateral sorting of proteins in the membrane. GPI, glycosylphosphatidylinositol.

Because of their high lipid content, these microdomains are detergent-insoluble glycolipid-enriched complexes (DIG), and it is proposed that these rafts function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction. Actually, lipid rafts can bind proteins: glycosylphosphatidylinositol (GPI)-anchored proteins, transmembrane proteins and doubly acylated tyrosine kinases of the Src family were demonstrated to be all associated with rafts and incorporated into DIG (Brown & London, 2000; Simons & Ikonen, 1997). Therefore, the localization of GM1 into DIG is important, since this molecule could have a relevant role in the activation of characteristic signalling mechanisms (Nashar, Betteridge, & Mitchell, 2001). This might explain why depletion of cholesterol, which disrupts rafts, inhibits the endocytosis and the intracellular transport of CT-GM1 complex - like a raft-mediated signalling - since DIG are cholesterol-rich domains (Fujinaga et al., 2003; Smith et al., 2004). Finally, the internalisation of CT-GM1 complexes depends on the mechanism of endocytosis: it has been observed that CTB-GM1 is internalised in a clathrin-independent manner (clathrin is a protein associated with intracellular transfer of membrane by coated vesicles) in cells that expressed high levels of

caveolin, a protein present in small invaginations of the plasma membrane named “caveolae”, and in a clathrin-dependent manner in the presence of low levels of caveolin (Simons & Ikonen, 1997; Smith et al., 2004).

To induce the pathology, both the A and B subunits have to enter the cell as a fully assembled holotoxin bound to GM1 molecule, following a retrograde route throughout the biosynthetic pathway from the plasma membrane to the ER lumen via the Golgi. At the ER, the protein disulfide isomerase (PDI) unfolds and dissociates the A1 fragment from the B subunit. Then, the A1 subunit is translocated into the cytosol, through the Sec61 channel, and ADP-ribosylates the trimeric G protein to activate adenyl cyclase and increase the level of intracellular cyclic AMP (cAMP) (Fig. 1B) (Fujinaga, 2006).

So, the GM1 binding is necessary not only for the internalisation of the toxin, but also for the transfer to the ER, since it has been demonstrated that GM1 on the plasma membrane is able to go back to the ER in its physiological condition, and the CT-GM1 complex is stable during the entire route from the membrane to the Golgi, and to the ER (Fujinaga, 2006; Smith et al., 2004).

What is the final ending of CTB or the exact interaction of CTB-GM1 with the intracellular organelles has still to be investigated.

2.1.2 Immunity or tolerance?

The immune system has two basic functions. First, to protect from harmful pathogens by mounting appropriate immune responses against *non-self* antigens (Ags), and second, to limit excessive, tissue-damaging immune responses in order to prevent autoimmunity and non-essential reactions to innocuous agents. Tolerance to self Ags is a fundamental property of the normal immune system and the failure of self tolerance leads to autoimmune diseases. To prevent autoimmunity, self-reactive T cells are controlled through central and peripheral tolerance. Central tolerance takes place in the thymus, where self-reactive T lymphocytes are deleted by negative selection. Nevertheless, some self-reactive T clones escape negative selection and reach periphery, where they might recognize foreign molecules and cross-react with *self* Ags. This requires peripheral strategies to ensure tolerance, among which immunological ignorance of given Ags, apoptosis, induction of anergy, and generation of regulatory T cells (Tregs) with suppressor activity (Adler & Steinbrink, 2007). Furthermore, the mucosal immune system - present in the respiratory, gastro-intestinal, and genitourinary tracts - has the further difficulty to coexist with innumerable dietary Ags and intestinal flora. A key peculiarity of this

system is its capability to remain tolerant to these Ags, whereas it has to maintain the function to respond to those which are really pathogens. Moreover, tolerance induced on the mucosal surface can produce a generalized peripheral tolerance (Mayer & Shao, 2004). On these considerations, mucosal immunity can be used either to protect the mucosal surface against infections or to act as an immunological treatment for autoimmune disorders through the induction of peripheral Ag-specific tolerance, called “oral tolerance” (Holmgren et al., 2005).

2.1.3 Adjuvants' role: from CT to CTB

Besides their action as mucosal immunogens, some toxins have an adjuvant effect since they strongly potentiate the immunogenicity of Ags conjugated with them or simply administered contemporary at the same mucosal site so to induce a robust immune response. (Mayer & Shao, 2004). A powerful adjuvant effect has been attributed to the entire CT. This toxin is able to induce a mucosal immune response through different mechanisms. CT can increase permeability of the intestinal epithelium leading to enhanced uptake of co-administered Ags (Lycke, Karlsson, Sjolander, & Magnusson, 1991), and can also directly affect functions and phenotype of immune cells. It can also enhance antigen presentation by dendritic cells (DC), (inducing their maturation through

enhancing MHC, CD80, and CD86 expression) (Gagliardi et al., 2000; Gagliardi et al., 2002; Gagliardi & De Magistris, 2003), macrophages (A. Bromander, Holmgren, & Lycke, 1991; A. K. Bromander, Kjerrulf, Holmgren, & Lycke, 1995; Cong, Weaver, & Elson, 1997), and B cells, (promoting B cell isotype differentiation and IgA switching) (Kim, Eckmann, Lee, Han, & Kagnoff, 1998; Lycke & Strober, 1989), and can affect proliferation and cytokine production of effector T cells (Lavelle et al., 2004). Furthermore, CT can induce mixed Th1 and Th2 polarization in naïve T cells (Gagliardi et al., 2000). However, since CT is normally considered to be toxic for human use, great efforts have recently been made to define whether it would be possible to separate the adjuvant and toxic activities as a basis for development of mucosal adjuvants for human use. To avoid toxicity, that has been associated to the CTA subunit, isolated CTB has been explored for its ability to augment immune responses also against coupled Ags. For example, CTB-coupled influenza virus hemagglutinin (HA) or ovalbumin (OVA) enhance antigen-presenting capacity of macrophages by up-regulating CD40, CD80 and CD86, and inducing interleukin-1 (IL-1) and interferon- γ (IFN- γ) production (George-Chandy et al., 2001). Beside its adjuvant effect, CTB conjugation with some Ags, i. e. auto-Ags, can give rise to Ag-specific tolerance, useful for cure/prevent autoimmune

disease. For example, studies in non-obese diabetic (NOD) mice have shown that oral administration of a CTB-insulin fusion protein induces IL-4 and transforming growth factor- β (TGF- β)-secreting CD4⁺ Tregs, with resultant prevention of type 1 diabetes (Ploix et al., 1999; Sobel, Yankelevich, Goyal, Nelson, & Mazumder, 1998). Also, pig, mouse and human insulin, that normally differ in their tolerogenic potency when used in a mouse model of type 1 diabetes, show increased and equal effectiveness at inducing tolerance when conjugated with CTB after oral administration (Mayer & Shao, 2004; Petersen et al., 2003). Furthermore, auto-Ags-CTB conjugates have been used in animal models to suppress the autoimmune disease development in experimental allergic encephalomyelitis, experimental autoimmune arthritis, and chondritis (Sun, Rask, Olsson, Holmgren, & Czerkinsky, 1996; Sun et al., 2000; Tarkowski, Sun, Holmdahl, Holmgren, & Czerkinsky, 1999).

More recently, immunomodulatory function of unconjugated CTB, in purified and recombinant form, has been explored in *in vivo* and *in vitro* studies. Parenteral administration of uncoupled CTB suppresses diabetes development in the NOD mice (Sobel et al., 1998). Moreover, oral administration of recombinant CTB (rCTB) inhibits the IL-12-mediated

murine experimental colitis induced by intrarectal administration of trinitrobenzene sulfonic acid (TNBS) (Boirivant, Fuss, Ferroni, De Pascale, & Strober, 2001). Furthermore, CTB is able to inhibit established Th1-mediated inflammation, as it inhibits IL-12 and IFN- γ production in *ex vivo* human Crohn's disease mucosal explants (Coccia et al., 2005). CTB-treatment is also able to induce apoptosis in CD8⁺ T cells, while CD4⁺ T cells have shown partial resistance to this apoptotic effect (Nashar, Williams, & Hirst, 1996). *In vitro* studies about purified immune cell populations showed that CTB decreases the expression of CD80 and CD86 on antigen-pulsed murine DC (Eriksson, Fredriksson, Nordstrom, & Holmgren, 2003). CTB was also reported to suppress *in vitro* human macrophage and monocyte reactivity, inhibiting the proinflammatory response to lipopolysaccharide (LPS) (reduced IL-6, tumor necrosis factor- α - TNF- α , IL-12, and nitric oxide - NO) without affecting the IL-10 production (Burkart et al., 2002).

2.2 Dendritic cells

The immune system has evolved into two distinct and sophisticated defense mechanisms against environmental pathogens: the Ag-nonspecific innate immunity, designed to rapidly respond to the

pathogen invasion, and the Ag-specific adaptive immunity, characterized by the activation of pathogen specific B and T cells. It is now clear that the adaptive phase is initiated by the innate recognition of infection. The key cellular players in translating innate information into adaptive immunity are DC, a family of antigen presenting cells (APC) with unique characteristics (Banchereau et al., 2000). DC are designed to capture, internalize, process, and present different Ags to Ag-specific T cells through MHC-peptide complexes (Hart, 1997). This task has been made possible by the large repertoire of pattern recognition receptors (PRRs) expressed on DC that respond to evolutionarily conserved molecular signatures of microbes, parasites, and viruses, known as pathogen-associated molecular patterns (PAMPs). In response to signals induced from PRR, DC undergo a profound phenotypic and functional transformation to become immunogenic competent APC able to sustain the expansion and differentiation of Ag-specific T cells into appropriate effector cells (Joffre, Nolte, Sporri, & Reis e Sousa, 2009).

2.2.1 DC subsets

Because of their role in environment patrolling, DC are located in many tissues of the body. DC can be found in lymphoid tissue, peripheral

blood, epidermis and epithelia of gastrointestinal and respiratory systems, the sites through which most Ags can enter. They are a heterogeneous population of cells that have arisen from distinct, bone-marrow derived ($CD34^+$ stem cells) hematopoietic lineages. Under steady-state conditions, they exist as conventional DC (cDC) and precursor DC (pre-DC); the latter are able to rapidly adapt their morphology and function in response to stimuli from the extracellular environment. In mice, three subsets of cDC have been identified in the spleen and lymph nodes: $CD8\alpha^+$ DC, $CD4^+CD8^-$ DC and $CD4^-CD8^-$ DC. On the contrary, human DC do not express CD8, whereas CD4 expression results heterogeneous (Shortman & Liu, 2002; Thomson & Robbins, 2008). To date, two main pathways of differentiation into DC from pre-DC are hypothesized to exist. The myeloid pathway generates two subsets: Langerhans cells, which are found in stratified epithelia such as the skin; and interstitial DC, which are found in all other tissues. The lymphoid pathway generates plasmacytoid DC (pDC), which secrete large amounts of type I interferons (IFN- α and IFN- β) after viral infection (Banchereau & Palucka, 2005; Narbutt et al., 2004). DC and their precursors show remarkable functional plasticity. For example, monocytes can differentiate into either macrophages, which function as

scavengers, or DC that induce specific immune responses, after exposure to granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4 (Banchereau & Palucka, 2005; Conti et al., 2008; Sallusto & Lanzavecchia, 1994). In contrast, pDC differentiate from precursors as a result of stimulation with IL-3 and CD40 ligand (CD40L) (Colonna, Trinchieri, & Liu, 2004). There is evidence that either polarized DC or distinct DC subsets might provide T cells with different signals that determine the class of immune response. Beside their pathways of differentiation, myeloid and plasmacytoid DC can be distinguished by specific markers expression. While myeloid DC express myeloid Ags such as CD11c, CD13, CD33, CD45RO, ILT1, and mannose receptors, pDC are characterized by IL-3 receptor α -chain (IL-3R α or CD123), lymphoid markers such as CD2, CD5, CD7, CD45RA, CXC-chemokine receptor 3 (CXCR3), and produce mRNA for germline IgK and for pre-T-cell receptor- α (pT α). Finally, DC subsets differ for the response to microbial components through the expression of specific Toll-like receptor (TLR). For example, TLR9 (a receptor for demethylated DNA) is expressed only by pDC, whereas myeloid DC preferentially express TLR2 and TLR4 (receptors for the bacterial products peptidoglycan - PGN - and LPS, respectively). Such differential expression might confer distinct maturation signals, yielding

distinct types of immune response (Banchereau & Palucka, 2005; Shortman & Liu, 2002).

2.2.2 Different maturative stages of DC

DC migrate as immature or precursor cells from the bone marrow into peripheral tissues where they encounter Ags as tissue-resident cells, or they circulate in the blood. A major functional characteristic of immature DC (iDC) is their capacity for endocytosis (receptor-dependent or -independent internalization), phagocytosis (uptake process through cytoskeletal movement), and micropinocytosis (fluid endocytosis through internalization of small particles within small vesicles) (Banchereau et al., 2000; Lutz & Schuler, 2002). Indeed, iDC are characterized by the expression of different receptors on their surface designed for the internalization of the Ag, as Fc γ and ϵ receptor (Banchereau & Steinman, 1998), while they express only small quantities of MHC class II and costimulatory molecules, essential for T cell activation.

If DC stimulation does not occur in the periphery, DC remain immature and tissue-resident, since iDC express CC-chemokine receptor 1 (CCR1), CCR2, CCR5 and CXCR1 and are attracted to non-lymphoid tissues by their respective ligands, which are expressed constitutively in

peripheral tissues or are induced at inflammatory sites (Gunn et al., 1998; McColl, 2002).

After maturation, DC lose their Ag-capture function and become professional APC. DC maturation is triggered by numerous stimuli, including: a) endogenous factors that are released by necrotic cells (for example, heat-shock proteins), b) pro-inflammatory cytokines that are secreted by bystander cells (for example, TNF), c) exogenous PAMPs, and d) activated T cells that express ligands for co-stimulatory molecules such as CD40L. Moreover, extensive up-regulation of MHC II-peptide complexes and T cell co-stimulatory molecules, such as B7-1 (CD80), B7-2 (CD86), and CD40, is observed on the surface of mature DC (mDC). Furthermore, DC maturation results in the down-regulation of expression of CCR1, 2, and 5 receptors and the up-regulation of CCR7 expression that switches DC responsiveness to its ligands, CC-chemokine ligand 19 (CCL19) and CCL21, that guide migration from peripheral tissues to secondary lymphoid organs, where DC can present Ags to naïve T cells (Banchereau & Palucka, 2005; Hackstein & Thomson, 2004; Mellman & Steinman, 2001; Winzler et al., 1997). However, recent studies have outlined that spontaneous migration of DC into lymph nodes occurs also under healthy steady-state conditions (Lutz & Schuler, 2002). Finally, upon activation, DC produce pro-

inflammatory cytokines, such as IL-12p70, TNF- α , IL-1 β , IL-6 and NO, and become fully mature immunogenic APC (Hackstein & Thomson, 2004; Joffre et al., 2009; Lutz & Schuler, 2002).

Different Ags are captured, processed and presented to T cells in different manner by DC. Soluble and particulate Ags are internalized by specific mechanisms, degraded in endosomes, and the generated polypeptides are targeted to MHC II compartments to form MHC II-peptide complexes. Ags originated from self or intracellular pathogens, instead, are processed in proteasome, and derived peptides are loaded onto the nascent MHC class I (MHC I) molecules in the endoplasmic reticulum (ER). Finally, lipidic and glycolipidic Ags are loaded on CD1 family proteins (CD1a-d) (Banchereau et al., 2000).

DC need to provide different “signals” to efficient prime naïve T cells (Fig. 3). DC activated by pathogen contact will normally present high levels of MHC-peptide complexes, which can engage T-cell receptors on naïve T cells. This event provides the first activating signal to the T cell and is therefore referred as “signal 1”. DC activated by pathogen encounter also express a variety of co-stimulatory molecules (in particular CD80 and CD86), which engage counter-receptors on T cells (CD28) and transmit signals that are important for T-cell proliferation and survival (signal 2). Finally, activated DC also produce mediators

that act on the T cell to promote its differentiation into an effector cell (signal 3). For example, IL-12 is a typical mediator released by many activated DC that can instruct the development of Th1 cells or cytotoxic T-lymphocytes (CTL) development. The integration of these three classes of signal by the T cell determines its subsequent fate. All signals appear to be required for full effector T-cell generation, whereas signal 1 without signals 2 and 3 (for example in the case of resident iDC) may be used to drive a naïve T cell into an inactive state, either through anergy, deletion, or induction of Treg (Fu et al., 1996; Joffre et al., 2009; Jonuleit, Schmitt, Schuler, Knop, & Enk, 2000; Lutz et al., 2000; Muller et al., 2002; Peche, Trinite, Martinet, & Cuturi, 2005; Sakaguchi, 2000). Nevertheless, in the last few years it has highlighted the existence of DC that express maturation marker without being able to prime T-cell responses and, in some instances, these cells induce tolerance (Albert, Jegathesan, & Darnell, 2001; Fujii, Liu, Smith, Bonito, & Steinman, 2004; Menges et al., 2002). This means that DC maturation in the commonly used phenotypic sense is not equivalent to DC immunogenicity and that “mature” DC may include cells with very distinct properties.

Hence, the fate of newly activated T cells is largely determined by the activation status of the presenting DC, mature but not immunogenic DC

fail to produce pro-inflammatory cytokines and are not able to evoke effector T cells (Joffre et al., 2009).

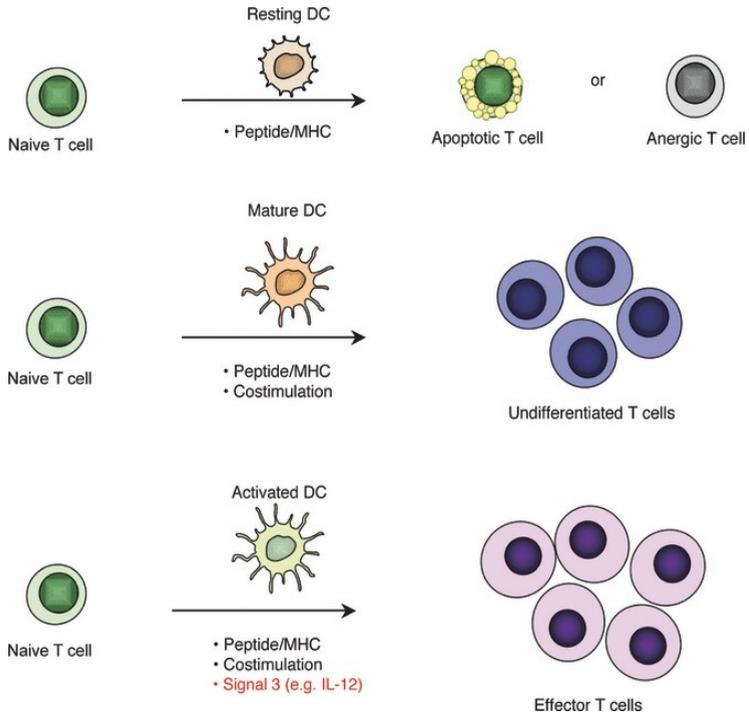


Fig. 3: Priming of naïve T cells by resting, mature, and activated DC. The fate of newly activated T cells is determined largely by the activation status of the presenting DC. Stimulation of naïve T cells by resting DC results in cell death or anergy. Mature DC induce the clonal expansion of T cells but not their differentiation (no effector function).

Only (directly) activated DC provide all signals required for the priming of effector T-cell responses.

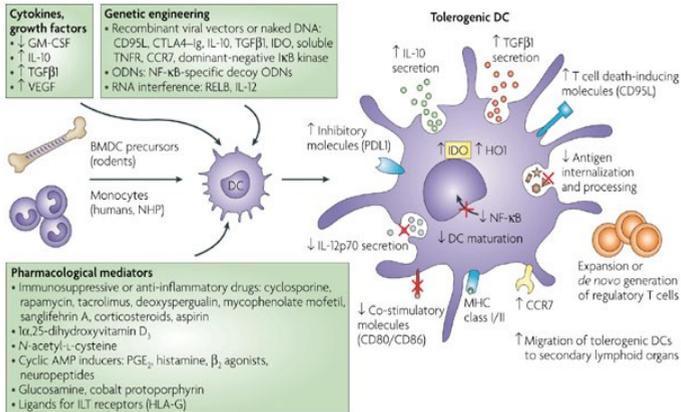
Moreover, there are several evidences that different intermediate stages of DC maturation exist. For example, under healthy steady-state conditions iDC are able to internalize tissue self-peptides and load them to MHC II and I molecules, up-regulating their expression at the cell surface. Co-stimulatory molecules expression is also partially increased. Finally, these DC down-regulate anchor receptors, and express relevant levels of CCR7, that guide them through the afferent lymph vessels into the lymph nodes. Thus, steady-state migrating DC have little in common with tissue-resident iDC on the functional point of view. However, in the absence of microbial or inflammatory stimulation these DC are not presumed to produce IL-12 or other pro-inflammatory cytokines and their maturation process seems to halt at a “semi-mature” stage (Lutz & Schuler, 2002).

2.2.3 Tolerogenic DC

In recent years, because of the phenotypical and functional plasticity of DC, it has progressively emerged that, besides their role to initiate immune reactivity, DC show a key ability to induce and maintain tolerance. A role for DC in tolerance induction was first demonstrated in

the context of central tolerance in the thymus, where thymic DC induce the apoptotic death of potentially self-reactive T cells presenting self-Ags to them (Adler & Steinbrink, 2007; Morelli & Thomson, 2007; Shortman & Heath, 2001). Nevertheless, DC tolerogenicity is not specific to a DC subset or restricted to the immature state of the APC. Although tolerogenic DC are able to present Ag to Ag-specific T cells, they fail to deliver adequate co-stimulatory signals with the result to inhibit effector T cell activation and proliferation. This may be manifested by T cell death, T cell anergy or Treg expansion or generation. Such regulatory capacity of DC has inspired numerous groups to explore and exploit the most important checkpoints of DC biology, which could be taken as pharmacological targets to induce tolerance (Hackstein & Thomson, 2004; Morelli & Thomson, 2007). There are several attributes that may characterize “tolerogenic” DC. They are immature, maturation-resistant or alternatively activated DC that express surface MHC molecules, have a low ratio of co-stimulatory to inhibitory signals, and an impaired ability to synthesize Th1-cell-driving cytokines (such as IL-12p70). In an effort to generate tolerogenic DC, *in vitro*-propagated DC have been manipulated through exposure to various anti-inflammatory and immunosuppressive agents, which target towards DC differentiation and function by various

mechanisms (Fig. 4). These agents include: a) IL-10 and TGF- β 1; b) inducers of cAMP such as prostaglandin E2, histamine, β 2 agonists, neuropeptides, the vitamin D3 metabolite 1 α ,25-dihydroxyvitamin D3 (1 α ,25(OH)2D3) and its analogues, glucosamine, the antioxidant N-acetyl-l-cysteine, ligands for inhibitory immunoglobulin-like transcript receptors (such as the MHC class Ib molecule HLA-G), and cobalt protoporphyrin (to induce haemoxygenase-1 expression). In addition, numerous experimental or clinically approved immunosuppressive drugs such as corticosteroids, cyclosporine, tacrolimus, rapamycin, aspirin, deoxyspergualin (DSG), mycophenolate mofetil (MMF) and sanglifehrin A have been also used. All these molecules prevent DC maturation and/or activation, or impair the capacity of DC to produce IL-12p70. In addition, some of these agents prevent the nuclear translocation of nuclear factor- κ B (NF- κ B) family members, which are required for the differentiation of DC (Adler & Steinbrink, 2007; Hackstein & Thomson, 2004; Morelli & Thomson, 2007).



Nature Reviews | Immunology

Fig. 4: Generation of tolerogenic DC *in vitro*. DC that are generated *in vitro* from bone marrow precursors (BMDC precursors) in rodents or blood monocytes in humans and non-human primates (NHP) have been rendered tolerogenic by controlling their culture conditions through exposure to cytokines, growth factors or pharmacological mediators, or by genetic engineering. DC generated under such conditions can down-regulate the outcome of the T-cell response by a single predominant function or, more frequently, by a combination of complementary mechanisms. CCR7, CC-chemokine receptor 7; CTLA4-Ig, cytotoxic T-lymphocyte antigen 4-immunoglobulin fusion protein; GM-CSF, granulocyte/macrophage colony-stimulating factor; HO1, haem oxygenase-1; IDO, indoleamine 2,3-dioxygenase; IκB, inhibitor of NF-κB; IL, interleukin; ILT, immunoglobulin-like transcript; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; PDL1, programmed cell death ligand 1; PGE₂, prostaglandin E₂; TGFβ1, transforming growth factor-β1; TNFR, tumour-necrosis factor receptor; VEGF, vascular endothelial growth factor.

Tolerogenic, partially mature DC can be also generated *in vitro* by combining agents that promote phenotypic maturation (TNF and LPS)

with agents that suppress the functional maturation of DC (such as IL-10 and TGF- β) (Hackstein & Thomson, 2004). Partially mature tolerogenic DC are characterized by high levels of MHC class II expression, variable levels of T-cell co-stimulatory molecule expression and an inability to produce bioactive IL-12. Also, granulocyte (G)-CSF indirectly favours the *in vitro* differentiation of peripheral blood monocytes into tolerogenic DC (post-G) through the release of IL-10, and IFN- α . Post-G DC possess a mature, MHC II^{high}CD80/86^{high} phenotype and release trace amounts of IL-12p70. Furthermore, post-G DC activate the *in vitro* differentiation of IL-10/TGF- β -producing Treg cells, capable of suppressing bystander T cells in cytokine-dependent manner (Rutella, Danese, & Leone, 2006).

Recently, advances in gene-transfer technology resulted in the enhancement of the tolerogenic potential of DC. These DC are genetically manipulated to: a) express “immuno-suppressive” molecules, which can inhibit or block the expression of cell-surface co-stimulatory molecules by inducing genetically the expression of IL-10, TGF- β 1 or the CTL antigen 4 (CTLA4)-immunoglobulin fusion protein; b) prevent NF- κ B activation by expressing a dominant negative form of I κ B-kinase 2 (IKK2); c) prevent the proliferation of allogeneic T cells through the

expression of indoleamine 2,3-dioxygenase (IDO); d) induce and maintain T-cell anergy (by expressing programmed cell death 1 ligand (PDL1)); or e) promote the deletion of Ag-specific T cells through the expression of CD95 ligand (CD95L) or TNF-related apoptosis-inducing ligand (TRAIL) (Morelli & Thomson, 2007).

2.3 Regulatory T cells

Specialized T cells that inhibit the proliferation and activation of effector T cells are known as suppressor or regulatory T cells and were initially described in the 1970s when Ag-primed T cells were shown to induce tolerance after transfer to naïve mice (Rutella & Lemoli, 2004). A large body of evidence attributes an important role to these cells in the immunological dysregulation underlying autoimmune diseases, chronic inflammatory diseases, and cancer, as well as in the immunobiology of transplantation (Roncarolo et al., 2006). The best characterized Tregs are inscribed among the CD4⁺ subset and have been categorized into two major subgroups based on their ontogeny: the naturally occurring forkhead box P3 (FOXP3)⁺CD4⁺CD25⁺ Tregs, which develop in the thymus and are present in normal naïve mice and healthy individuals from birth, and the inducible Tregs, which are generated in the periphery

under various tolerogenic conditions. Many subsets of inducible Tregs have been reported, and their definition has largely been based on their different biological features, including specific cytokine secretion profile, cellular markers, ability to differentiate in response to Ag-specific stimuli, and dependency on cytokines vs. cell-cell contact mechanisms for mediating suppressive activity. Some of these Tregs include inducible FOXP3⁺CD4⁺CD25⁺ Tregs, which can be generated *in vitro* from CD4⁺CD25⁻ T cells in the presence of TGF- β , regulatory type 1 (Tr1) cells, which produce high levels of IL-10, and TGF- β -producing Th3 cells (Carrier, Yuan, Kuchroo, & Weiner, 2007; Groux et al., 1997; Roncarolo et al., 2006; Roncarolo & Battaglia, 2007).

In particular, naturally occurring CD4⁺CD25⁺ T cells have been characterized in the peripheral blood of human subjects for their unique ability to suppress bystander T cells in an Ag-nonspecific and cell contact-dependent manner. They constitutively express a variety of cell surface molecules commonly associated with activated/memory cell phenotype. These include the α chain of the IL-2 receptor (CD25), CD45RB^{low} CD62L, CTLA4, and glucocorticoid-induced tumor necrosis factor receptor (GITR) family-related gene. Nevertheless, none

of these surface markers is uniquely expressed by natural Tregs; in the last few years only FOXP3 has been demonstrated critically important for the development and function of Tregs. Genetic mutations in the gene encoding FOXP3 have been identified in both humans and mice, resulting in fatal autoimmune diseases. Humans with mutations in FOXP3 suffer from a severe and rapidly fatal autoimmune disorder known as the immune dysregulation- polyendocrinopathy-enteropathy X-linked (IPEX) syndrome. An analogous disease also occurs in mice, known as “scurfy” disease, owing to a spontaneous mutation in FOXP3. Furthermore, ectopic expression of FOXP3 can phenotypically and functionally convert effector T cells to Tregs (Le & Chao, 2007). It appears that, in mice, FOXP3 expression is both necessary and sufficient for Treg development. However, there is accumulating evidence demonstrating that, in contrast to mice, human FOXP3 is transiently expressed in activated dividing T cells and does not interfere with proliferation and cytokine production. At the same time, FOXP3 does not inhibit expression of the membrane-bound Treg markers CD25, CTLA4, and GITR. It is therefore possible that, depending on the cell subset and/or the stage of T cell differentiation, FOXP3 expression can exert different functions and act as a negative or positive regulator. Furthermore, it has been recently reported that FOXP3 is expressed in

mammary epithelial cells and acts as transcriptional repressor of oncogenes. Future studies will be necessary to better understand the functional consequences of FOXP3 expression in T and non-T cells (Sakaguchi, 2008). On the other hand, several studies have elucidated suppressive mechanisms of CD4⁺CD25⁺ Tregs. These cells are able to mediate suppression by cell-cell contact dependent mechanisms, to reduce CD4⁺ and CD8⁺ proliferation by inhibiting IL-2 transcription through combined action of CTLA4 and membrane-bound TGF- β , to induce apoptosis of effector T cells, and to produce regulatory cytokine as IL-10 and TGF- β (Roncarolo & Battaglia, 2007).

As opposite as CD4⁺CD25⁺ Tregs, inducible Tr1 and Th3 are Ag-specific Tregs, and mediate their suppression activity especially through producing high levels of IL-10 and TGF- β , respectively.

Although TGF- β is a well-recognized immunoregulatory cytokine, the mechanism by which TGF- β -secreting T cells regulate immune responses is not well-defined. Th3 cells were initially described in mice after oral tolerance induction to myelin basic protein (MBP) for their ability to suppress MBP-specific experimental autoimmune encephalomyelitis (EAE) *in vivo* in a TGF- β -dependent manner. Th3 regulatory cells form a unique T-cell subset which primarily secretes

high amounts of TGF- β , provides help for IgA synthesis, and displays suppressive properties both for Th1 and for Th2 cells (Rutella & Lemoli, 2004). Furthermore, Th3 secrete low amounts of IL-4 and IL-10 and no IFN- γ or IL-2 upon TCR ligation. In the absence of inflammation, the secretion of TGF- β up-regulates the expression of the Foxp3 gene in activated T cells during T cell expansion which induces the differentiation of Tregs in the peripheral repertoire in the absence of thymic CD25⁺ Tregs. Thus, Th3 cells appear to be central mediators of peripheral immune tolerance both directly and indirectly by the induction of FOXP3⁺ Tregs (Carrier et al., 2007).

The most studied inducible Treg subset is constituted by Tr1 cells. Adaptive Tr1 cells arise *in vitro* and *in vivo* upon encounter with Ag in the presence of IL-10 and are characterized by a unique cytokine profile (IL-10⁺ IL-4⁻ TGF- β ⁺ IL-5⁺ IL-2^{low/-} IFN- γ ^{low/-}). To date, no specific cell-surface marker for Tr1 cells has been identified (Roncarolo et al., 2006). Tr1 cells have low proliferative capacity following activation *in vitro* through the T-cell receptor (TCR), in part due to autocrine production of IL-10. However, their proliferative activity *in vivo* is unknown. These cells regulate immune responses through the secretion of the immunosuppressive cytokines IL-10 and TGF- β , and

they suppress both naïve and memory T-cell responses against tumors, *self* and *non-self*-Ags, and pathogens (Rutella & Lemoli, 2004), and downregulate the expression of co-stimulatory molecules and pro-inflammatory cytokines by APCs such as DC, Langerhan's cells, and macrophages (Roncarolo et al., 2006). Furthermore, Tr1 cells favour the production of IgD, IgA and IgG by B cells. Importantly, Tr1 cells are inducible, Ag-specific, and need to be activated through their TCR to exert their suppressive functions. However, once activated, they mediate suppression in an Ag nonspecific manner. IL-10 is absolutely required for the differentiation and function of mouse Tr1 cells, whereas for human Tr1-cell differentiation, IL-10 is necessary but probably not sufficient. Other necessary factors include the presence of APCs, which provide, in addition to IL-10, other soluble and surface molecules that are crucial for Tr1-cell generation. Many different approaches have been explored for the induction of Tr1 cells both *ex vivo* and *in vivo*, such as treatment with immunosuppressive drugs, soluble proteins, and peptide antigens (Roncarolo & Battaglia, 2007).

3 MATERIALS AND METHODS

3.1 Chemical and reagents

The *V. cholerae* strain 0395-tacCTB, lacking the CTA gene (kindly supplied by Dr. Rino Rappuoli, Istituto Ricerche Immunobiologiche, Chiron, Siena, Italy), was used as a source for rCTB production. rCTB was produced and purified as previously reported (Boirivant et al., 2001), according to the protocol described by Lebens et al. (Lebens, Johansson, Osek, Lindblad, & Holmgren, 1993) with minor modifications. All rCTB preparations contained < 1 EU/ml endotoxin, as determined by a quantitative, chromogenic *Limulus* amoebocyte lysate test (QLC1000, BioWhittaker, Walkersville, MD, USA). rCTB was dissolved in culture medium and used at 10 µg/ml. A possible cytotoxic effect of the drugs was excluded using evaluation of cell viability with trypan blue (more than 95% viable cells). Any possible contamination by enterotoxins was excluded by performing control experiments with boiled rCTB.

3.2 Human PBMC isolation

Human PBMC were isolated by the Ficoll-Hypaque method from buffy coats from healthy, voluntary blood donors (courtesy of the Transfusional Medicine Department, Policlinico Umberto I, University “La Sapienza,” Rome).

3.3 Generation and culture of MDDC

Human MDDC were generated according to the protocol described by Sallusto and Lanzavecchia (Sallusto & Lanzavecchia, 1994). Briefly, monocytes were isolated from PBMC by MACS (Miltenyi Biotec, Germany) using anti-CD14 microbeads. The recovered monocytes - which were > 95% pure, as shown by flow cytometry with an anti-CD14 fluorescent antibody - were cultured at 5×10^5 /ml in RPMI 1640 (BioWhittaker), supplemented with 15% FCS (Hyclone, Logan, UT, USA), 1% Lglutamine, and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) containing 50 ng/ml GM-CSF (Peprotech Inc., Rocky Hill, NJ, USA) and 1000 U/ml recombinant human (rh)IL-4 (Endogen, Pierce Biotechnology Inc., Rockford, IL, USA, distributed by Tema Ricerca, Italy) at 37°C and 5% CO₂ for 5 days. At Day 5, after

monocyte differentiation in immature MDDC (> 90% CD1a⁺CD14⁻ cells, analyzed by flow cytometry), separate cultures were treated or not with 10 µg/ml rCTB for 24 h. Subsequently, cells were harvested, washed extensively, and stimulated with 10 ng/ml LPS from *Escherichia coli* (Sigma-Aldrich) (LPS-MDDC and CTB-LPS-MDDC) or left untreated (MDDC and CTB-MDDC) for an additional 24 h or 48 h. At the end of the culture period, MDDC were washed extensively before further use. The phenotype of different MDDC populations was analyzed for CD40, HLA-DR, CD80, CD86, and CD83 expression by flow cytometry. Cell supernatants were collected and analyzed for IL-10 and IL-12p70 production. A portion of CTB-LPS-MDDC and LPS-MDDC was irradiated (3000 rads) and used to stimulate allogeneic PBMC in a mixed leukocyte reaction (MLR).

3.4 MLR

PBMC used as responder cells (1×10^5) were cultured in 96-well plates (Costar Corp., Germany) with graded numbers of irradiated (3000 rads), allogeneic LPS-MDDC or CTB-LPS-MDDC. At day 4, cells were pulsed with methyl ³H-thymidine (Amersham Life Science, UK) for the

last 18 h of culture. ^3H -Thymidine incorporation was measured in a LKB Betaplate liquid scintillation counter (Wallac, Inc., UK). Assays were performed in triplicate, and the results were expressed as mean $\text{cpm} \pm$ standard errors of the mean (SEM).

3.5 T cell culture

CD4⁺CD45RA⁺ lymphocytes were positively selected from human peripheral blood of healthy donors using the CD4⁺ Isolation Kit II followed by CD45RA magnetic beads (MACS, Miltenyi Biotec), according to the manufacturer's instructions. Recovered CD4⁺CD45RA⁺ cells were more than 95% pure, as indicated by flow cytometry. Purified T cells were resuspended at the concentration of 1×10^6 /ml in RPMI 1640, supplemented with 10% of FCS, 1% L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich; complete medium), and cultured with fresh, allogeneic LPS-MDDC or CTB-LPS-MDDC at the ratio of 10:1 for 1 week. After this period of culture, a portion of T cells stimulated with LPS-MDDC ($T_{\text{LPS-MDDC}}$) and T cells stimulated with CTB-LPS-MDDC ($T_{\text{CTB-LPS-MDDC}}$) was harvested, washed, and stimulated with platebound, anti-human CD3 ϵ (10 μ g/ml, R&D Systems Inc., Minneapolis, MN, USA) and soluble anti-human CD28 (2 μ g/ml) mAb (R&D Systems Inc.) to analyze their cytokine production (after 24-48 h of culture) and proliferative capacity

(after 72 h of culture). In some experiments, T_{LPS-MDDC} and T_{CTB-LPS-MDDC} were harvested, washed, and stimulated with LPS-MDDC. Supernatants were collected after 24 h for IFN- γ and 48 h for IL-10, TGF- β 1, and IL-4.

3.6 Cytokine assays

Cytokine content in the culture supernatants was measured by sandwich ELISA using commercially available kits: IL-10, IL-12p70, and TGF- β 1 ELISA kits (R&D Systems Inc.) and IL-10, IFN- γ , and IL-4 ELISA kits (Pierce Biotechnology Inc., distributed by Tema Ricerca). ODs were measured on a Bio-Rad Novapath ELISA reader, and the results were expressed as pg/ml \pm SEM.

3.7 Analysis of T cell function (suppression assay)

Next, we investigated the capacity of T_{CTB-LPS-MDDC} to suppress autologous T_{LPS-MDDC} proliferation. To this purpose, parallel cultures of T_{LPS-MDDC} and T_{CTB-LPS-MDDC} were cultured in the presence of rhIL-2 (40 U/ml, Roche Diagnostics GmbH, Germany). After 6 days, cells were harvested and washed. T_{LPS-MDDC} were then labeled with

5 μ M final concentration of the intracellular fluorescent dye CFSE (VybrantTM carboxyfluorescein diacetate, succinimidyl ester cell tracer kit, Molecular Probes, Eugene, OR, USA). To distinguish the two populations, we stained T_{CTB-LPS-MDDC} with PKH-26 dye (2×10^{-6} M; PKH26 red fluorescent cell linker kit, Sigma-Aldrich). After the cell-labeling, according to the manufacturer's instructions of each product, cells were resuspended in complete medium and cultured together at different ratios. Results of proliferation and IL-10 production analyses indicated 1:1 as the optimal ratio. To induce activation, T cells were stimulated with LPS-MDDC from the same donor used for the CD4⁺CD45RA⁺ T cell priming at a 10:1 ratio for 4 days. In some experiments, 30 μ g/ml neutralizing anti-IL-10R mAb (BD PharMingen, San Diego, CA, USA) and 50 μ g/ml anti-TGF- β 1, - β 2, and - β 3 mAb (R&D System Inc.) were added at the beginning and at Day 2 of the culture period. The proliferation of the CFSE-labeled T_{LPS-MDDC} was assessed at Day 4.

To assess if cell-cell contact was necessary for the suppressive capacity of T_{CTB-LPS-MDDC}, Transwell experiments were performed using CFSE-labeled T_{LPS-MDDC} cells and PKH-26-labeled T_{CTB-LPS-}

MDDC. We placed 4×10^5 CFSE-labeled $T_{LPS-MDDC}$ cells with allogeneic LPS-MDDC (10:1 ratio) in the bottom well of a Transwell system (Costar, Corning Inc., Corning, NY, USA) and 4×10^5 PKH-26-labeled $T_{CTB-LPS-MDDC}$ with allogeneic LPS-MDDC (10:1 ratio) in the upper Transwell chamber. Transwell cultures with 4×10^5 CFSE-labeled $T_{LPS-MDDC}$ cells with allogeneic LPS-MDDC (10:1 ratio) in the upper chamber were performed as control. After 4 days, we measured the proliferative response of CFSE-labeled $T_{LPS-MDDC}$ cells in the bottom well. Proliferation of CFSE-labeled cells was assessed by a BD FACSCanto flow cytometer and FACSDiva software (BD Bioscience, San Jose, CA, USA). Results were analyzed with the proliferation model of ModFit LT software (Verity Software House, Inc., Topsham, ME, USA) and expressed as proliferation index (P.I.).

3.8 Flow cytometry analysis

Cells were washed with PBS and then stained with various fluorochromes using standard methods provided by the manufacturers. Antibodies used for surface staining of human monocytes and DC - PE-labeled anti-CD14 and FITC-labeled anti-CD1a, -CD40, -CD80, -CD83,

-CD86, and -HLA-DR mAbs - were purchased from BD PharMingen. To analyze the CD4⁺ T cell population, FITC-labeled anti-CD4, PE-labeled anti-CD45RA, and PE-labeled anti-CD25 mAbs (BD PharMingen) were used. For detection of forkhead box p3 (Foxp3)⁺ cells, T cells were fixed and permeabilized according to the manufacturer's instructions and incubated with anti-human Foxp3-allophycocyanin mAb (e-Bioscience, San Diego, CA, USA). Isotype-matched Abs were used as controls, and cells were preincubated with human IgG to avoid nonspecific binding to FcRs. All samples were analyzed using a BD FACSCanto flow cytometer and FACSDiva software (BD Bioscience).

3.9 Statistical analysis

Results were expressed as mean \pm SEM or as P.I. Statistical analysis was performed using Student's t-test and Wilcoxon test for paired data as appropriate. P values < 0.05 were considered significant.

4 RESULTS

4.1 rCTB treatment affects maturation of human MDDC

We first assessed the influence of rCTB on the pattern of expression of costimulatory (CD40, CD80, CD86), maturation (CD83), and class II MHC (HLA-DR) surface molecules in MDDC, generated from monocytes cultured with GM-CSF and IL-4. To this purpose, separate cultures of MDDC were treated or not with 10 µg/ml rCTB for 24 h. Subsequently, cells were washed and stimulated with LPS (10 ng/ml; LPS-MDDC and CTB-LPS-MDDC) or left untreated (MDDC and CTB-MDDC). In preliminary experiments, the 24 h and 48 h treatment with LPS on CTB-MDDC and untreated MDDC has been tested. Indeed, we observed that the effect of rCTB on MDDC maturation was more evident during the first 24 h of LPS stimulation. Therefore, in subsequent experiments, we used the 24 h LPS stimulation. As shown in Table 1, pretreatment with rCTB induces a statistically significant reduction of the LPS-induced upregulation of CD40 ($p = 0.037$), and it does not affect the expression of the other molecules.

Table 1. rCTB interferes with the expression of the costimulatory molecule CD40 during human MDDC maturation^a

	CD40		HLA-DR		CD80		CD86		CD83	
	MFI ^b	% ^c	MFI ^b	% ^c	MFI ^b	% ^c	MFI ^b	% ^c	MFI ^b	% ^c
MDDC	30 ± 6	73 ± 16	240 ± 47	98 ± 2	21 ± 3	65 ± 16	31 ± 5	60 ± 13	31 ± 7	37 ± 17
CTB-MDDC	27 ± 8	75 ± 10	210 ± 56	99 ± 0.28	20 ± 5	51 ± 11	30 ± 6	65 ± 5	19 ± 2	30 ± 7
LPS-MDDC	70 ± 13	96 ± 2	408 ± 75	99 ± 0.1	37 ± 5	94 ± 3	61 ± 6	99 ± 0.5	34 ± 6	87 ± 5
CTB-LPS-MDDC	59 ± 11 ^(*)	92 ± 6	378 ± 90	99 ± 0.1	35 ± 5	86 ± 10	61 ± 8	94 ± 4	35 ± 6	80 ± 12

^a Human immature MDDC were treated or not with 10 µg/ml of recombinant CTB for a period of 24 hours. Subsequently, cells were harvested, extensively washed and stimulated with 10 ng/ml of LPS (LPS-MDDC and CTB-LPS-MDDC), or left untreated (MDDC and CTB-MDDC) for additional 24 hours of culture. The phenotype was analyzed by flow cytometry for the expression of CD40, HLA-DR, CD80, CD86, and CD83. Data represent mean ± SEM of six independent experiments.

^b mean fluorescence intensity

^c % of positive cells

^(*) p = 0.037, CTB-LPS-MDDC vs LPS-MDDC

rCTB does not induce significant modification in the surface marker expression of MDDC not treated with LPS. Cytokine production was also investigated. CTB-LPS-MDDC produce a significantly decreased amount of IL-12p70 ($p = 0.014$) when compared with LPS-MDDC, with no variation of IL-10 production (Fig. 5). rCTB has no effect on cytokine production by MDDC not treated with LPS.

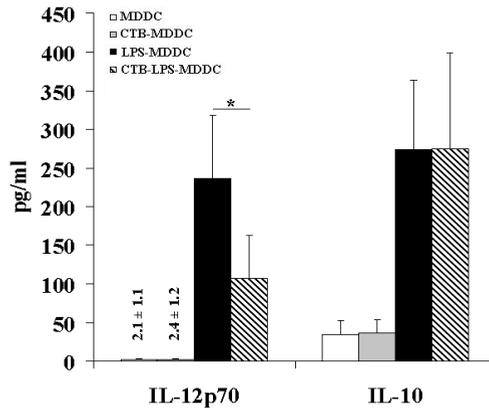


Fig. 5: rCTB inhibits IL-12p70 production by LPS-MDDC, whereas it does not affect IL-10 production. IL-12p70 and IL-10 concentration in the supernatants of human MDDC, treated as described in Table 1, was measured by a specific sandwich ELISA. Data represent mean \pm SEM of six independent experiments performed. *, $p = 0.014$; CTB-LPS-MDDC versus LPS-MDDC.

These results suggest that rCTB is able to prevent the LPS-induced maturation of MDDC, which acquire characteristics that resemble those of tolerogenic DC (Lutz & Schuler, 2002).

4.2 rCTB-treated MDDC show a reduced ability to stimulate MLR response.

The results obtained with the analyses of the surface molecule expression and cytokine production induced us to test whether rCTB has any effect on MDDC immunostimulatory capacity. Therefore, their ability to stimulate an allogeneic T cell response was assessed. To this purpose, graded numbers of CTB-LPS-MDDC and LPS-MDDC were cultured with a fixed number of allogeneic PBMC for 5 days. As expected, LPS-MDDC were quite efficient in inducing proliferation of alloreactive cells, while CTB-LPS-MDDC induced a significant ($p < 0.05$ by Wilcoxon test, $n = 8$), lower proliferative response (Fig. 6).

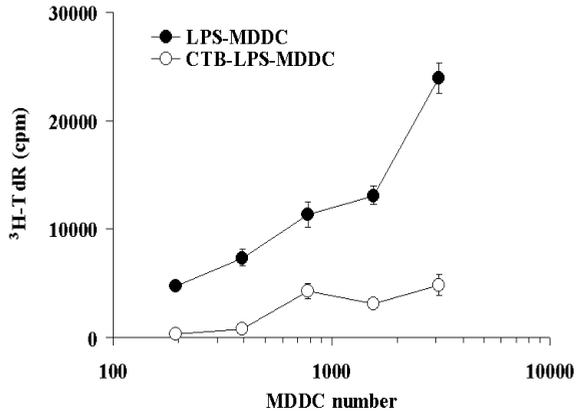


Fig. 6: rCTB pretreatment down-regulates the immunostimulatory capacity of LPS-MDDC in MLR. PBMC were cultured with graded numbers of allogeneic LPS-MDDC (●) or CTB-LPS-MDDC (○). At Day 4, methyl ³H-thymidine (³H-TdR) was added for the last 18 h of culture. One representative experiment out of eight is shown.

4.3 rCTB-treated MDDC induce the generation of CD4⁺ T cells with reduced proliferative capacity and increased IL-10 production

To investigate the effect of CTB-LPS-MDDC on T cells, allogeneic CD4⁺CD45RA⁺ T cells were cultured with CTB-LPS-MDDC (T_{CTB-LPS-MDDC}) or LPS-MDDC (T_{LPS-MDDC}) for 1 week.

Cells were collected, washed, stimulated with anti-CD3/anti-CD28 mAbs, and analyzed for proliferative capacity and cytokine production. $T_{CTB-LPS-MDDC}$ showed a significantly reduced proliferative capacity when compared with $T_{LPS-MDDC}$ ($p < 0.01$; Fig. 7A).

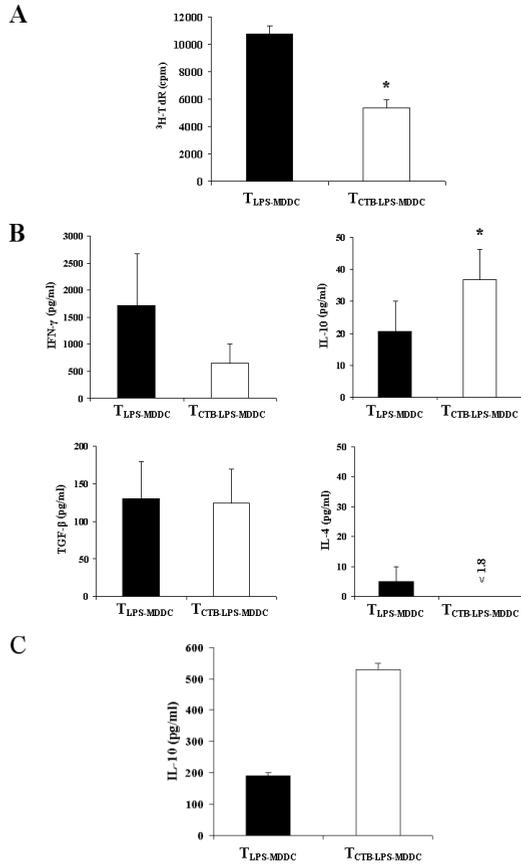


Fig. 7: CD4⁺CD45RA⁺ T cells stimulated with CTB-LPS-MDDC show reduced proliferative capacity and increased production of IL-10. CD4⁺CD45RA⁺ T cells were

cultured with allogeneic LPS-MDDC ($T_{LPS-MDDC}$) or CTB-LPS-MDDC ($T_{CTB-LPS-MDDC}$) at a 10:1 ratio. After 1 week, cells were collected, washed, stimulated with anti-CD3/ anti-CD28 mAb or LPS-MDDC, and analyzed for proliferative capacity and cytokine production. (A) Proliferation of $T_{LPS-MDDC}$ (solid bar) and $T_{CTB-LPS-MDDC}$ (open bar). Columns represent mean \pm SEM of three independent experiments. *, $p = 0.01$, $T_{CTB-LPS-MDDC}$ versus $T_{LPS-MDDC}$. (B) Cytokine production by $T_{LPS-MDDC}$ (solid bars) and $T_{CTB-LPS-MDDC}$ (open bars) measured in supernatants of CD3/CD28-stimulated cells (24–48 h, see Materials and Methods). Columns represent the mean \pm SEM of three independent experiments. *, $p < 0.05$, $T_{CTB-LPS-MDDC}$ versus $T_{LPS-MDDC}$. (C) IL-10 production by $T_{LPS-MDDC}$ (solid bars) and $T_{CTB-LPS-MDDC}$ (open bars) measured in supernatants of LPS-MDDC-stimulated cells (48 h). Columns represent mean \pm SEM of duplicate of two independent experiments.

The analysis of cytokine production showed that when compared with $T_{LPS-MDDC}$, $T_{CTB-LPS-MDDC}$ produce significantly higher amounts of IL-10 ($p < 0.05$), lower amounts of IFN- γ (inhibition: 57%; $p < 0.05$), and the same levels of TGF- β and IL-4 (Fig. 7B). In some experiments, $T_{LPS-MDDC}$ and $T_{CTB-LPS-MDDC}$ were stimulated with LPS-MDDC for 48 h, and IL-10 production was analyzed. Also in this case, $T_{CTB-LPS-MDDC}$ produce consistently higher levels of IL-10 respect to $T_{LPS-MDDC}$ (Fig. 7C). Taken together, these results suggest that CTB-LPS-MDDC might induce the differentiation of Tregs.

4.4 rCTB-treated MDDC induce functional CD4⁺ Tr1 cells

To evaluate the ability of T_{CTB-LPS-MDDC} to inhibit autologous T_{LPS-MDDC} proliferation, the latter cell population was restimulated with LPS-MDDC in the presence or absence of T_{CTB-LPS-MDDC} (see *Materials and Methods*). T_{LPS-MDDC} and T_{CTB-LPS-MDDC} were labeled with CFSE and PKH-26, respectively. In preliminary experiments, different numbers of T_{CTB-LPS-MDDC} were added to T_{LPS-MDDC} cultures to identify the optimal ratio, which at 1:1, was chosen for the suppressive activity and for the IL-10 production evaluation (data not shown). As shown in Figure 8A, T_{CTB-LPS-MDDC} inhibited the proliferation of CFSE-labeled T_{LPS-MDDC} consistently ($p < 0.05$). Next, we investigated whether the inhibitory effect of T_{CTB-LPS-MDDC} on T_{LPS-MDDC} proliferation might be mediated by soluble factors. To this aim, LPS-MDDC-stimulated T_{CTB-LPS-MDDC} and T_{LPS-MDDC} were separated in a Transwell system that allows the free exchange of soluble factors but excludes

direct cell– cell contact. As shown in Figure 8B, the proliferation of $T_{LPS-MDDC}$ was still inhibited, indicating that soluble factors mediated the inhibitory effect.

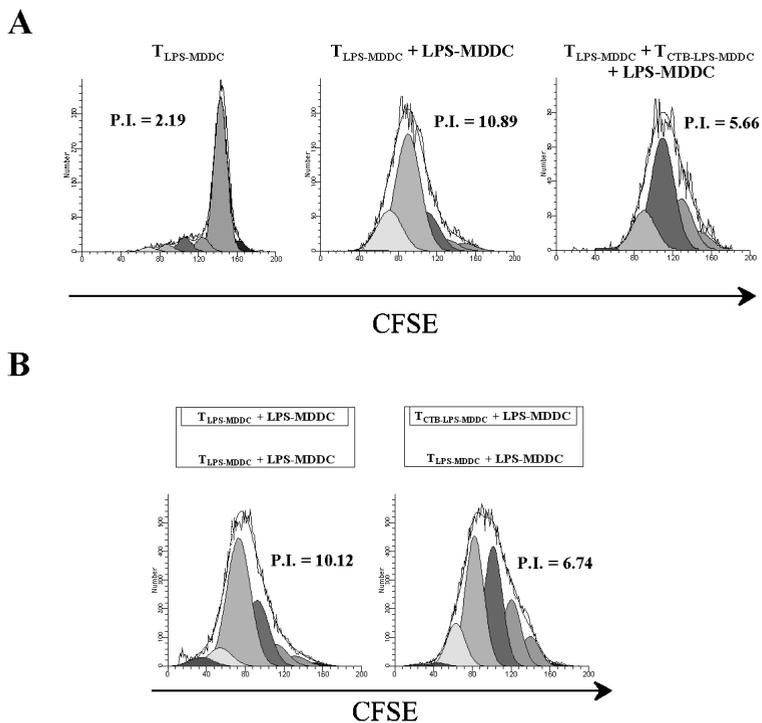


Fig. 8. $T_{CTB-LPS-MDDC}$ exert regulatory activity on $T_{LPS-MDDC}$, which is cell-cell contact-independent.

$T_{LPS-MDDC}$ and $T_{CTB-LPS-MDDC}$, after 6 days of culture with rhIL-2, were labeled with CFSE or PKH-26, respectively. CFSE-labeled $T_{LPS-MDDC}$ were restimulated with LPS-MDDC at a 10:1 ratio in the presence or absence of $T_{CTB-LPS-MDDC}$ for 4 days. (A) Proliferation of CFSE-labeled $T_{LPS-MDDC}$ was assayed at Day 4 by flow cytometry and expressed as P.I., calculated by ModFit LT software. One representative experiment out of three is shown ($p < 0.05$; $T_{LPS-MDDC}+LPS-MDDC$ vs. $T_{LPS-MDDC} + T_{CTB-LPS-MDDC}+LPS-MDDC$). (B) Parallel experiments were performed in a Transwell system that separated $T_{LPS-MDDC}$ and $T_{CTB-LPS-MDDC}$. Transwell

cultures with $T_{LPS-MDDC}$ in the upper and the lower chamber were used as control (see Materials and Methods). Proliferation of CFSE-labeled $T_{LPS-MDDC}$ was assayed at Day 4. One representative experiment out of three is shown ($p < 0.05$; $T_{LPS-MDDC}+LPS-MDDC$ vs. $T_{LPS-MDDC}+T_{CTB-LPS-MDDC}+LPS-MDDC$).

As T cells stimulated with CTB-LPS-MDDC in the presence of anti-CD3/anti-CD28 mAbs produced TGF- β and IL-10, we performed additional experiments in the presence of blocking mAbs. As shown in Figure 9, the addition of anti-IL-10R and anti-TGF- β mAbs to the cultures completely reversed the inhibitory effect.

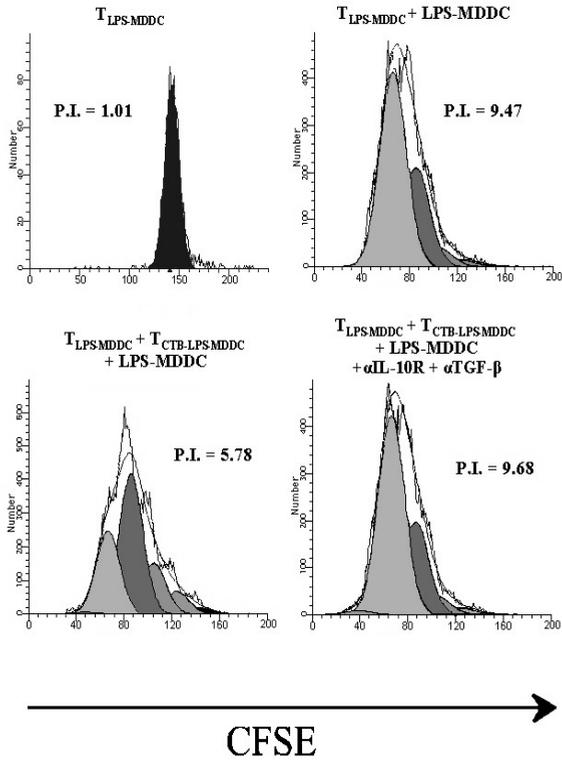


Fig. 9: $T_{CTB-LPS-MDDC}$ exert regulatory activity on $T_{LPS-MDDC}$ via IL-10 and TGF- β production. CFSE-labeled $T_{LPS-MDDC}$ were stimulated in the presence of PKH-26-labeled $T_{CTB-LPS-MDDC}$ as described in Figure 8. $T_{CTB-LPS-MDDC}$ were tested for their ability to suppress the proliferation of CFSE-labeled $T_{LPS-MDDC}$ in the presence or absence of blocking IL-10R and TGF- β mAb. Proliferation of CFSE-labeled cells was assayed at Day 4 by flow

cytometry and expressed as P.I. calculated by ModFit LT software. One representative experiment out of three is shown.

These results indicate that the $T_{\text{CTB-LPS-MDDC}}$ phenotype is associated with regulatory activity. Finally, we analyzed the intracellular expression of Foxp3, and we found that $T_{\text{CTB-LPS-MDDC}}$ and $T_{\text{LPS-MDDC}}$ did not differ in the expression of this marker (data not shown).

Taken together, data indicate that $T_{\text{CTB-LPS-MDDC}}$ display the properties described for Tr1 cells.

5 CONCLUSIONS

In the present study, we examined the possibility that rCTB may affect human MDDC maturation/activation in response to TLR stimulation. Our findings showed that rCTB partially prevented the LPS-induced maturation process of MDDC and decreased their IL-12p70 production with no relevant effect on IL-10 production. Furthermore, LPS-stimulated MDDC pretreated with rCTB were able to promote the induction of low proliferating T cells, which showed an enhanced IL-10 production associated with a reduced IFN- γ production and the same high levels of TGF- β as the control. These T cells suppressed proliferation of activated autologous T cells. Transwell experiments and blockade of IL-10R and TGF- β showed that the immunomodulatory effect is mediated by soluble factors. Thus, T cells induced by rCTB-conditioned MDDC acquired a regulatory phenotype and activity similar to those described for Tr1 cells.

6 DISCUSSION

Many studies demonstrate that DC play an important role in the induction and maintenance of immune tolerance (Steinman, Turley, Mellman, & Inaba, 2000; Wakkach et al., 2003). In this study, we found that pretreatment of human MDDC with rCTB reduces their ability to produce IL-12 upon challenge with LPS and affects their maturation with an impaired capability of alloantigen presentation. Furthermore, we analyzed the influence of CTB-LPS-MDDC on T cells. Our data demonstrate that CTB-LPS-MDDC induce the *in vitro* differentiation of IL-10-producing T cells with low proliferative capacity, which behave as Tr1 cells, described by different authors (Battaglia, Gregori, Bacchetta, & Roncarolo, 2006; Groux et al., 1997; Levings et al., 2005; Roncarolo et al., 2006).

The CTB subunit, when conjugated with an antigen, is a highly efficient carrier molecule for the induction of mucosal antibody responses (Czerkinsky, Russell, Lycke, Lindblad, & Holmgren, 1989; McKenzie & Halsey, 1984) and oral tolerance (George-Chandy et al., 2001). The mechanism underlying this effect is probably a result of the ability of the CTB to promote the presentation of the coupled antigen with a concomitant increase of CD40 and CD86 expression on APC (George-

Chandy et al., 2001). The apparently opposite outcome produced by the oral administration of CTB-antigen conjugates might be reconciled by the notion that systemic tolerance *in vivo* induced by feeding with CTB-conjugated antigens may be associated with a mucosal antibody response (Tochikubo et al., 1998; Wu & Russell, 1998). However, the observation that systemic tolerance is associated with production of regulatory cytokines (i.e., TGF- β) suggests that CTB might have intrinsic, immunomodulatory properties (Sun et al., 2000). Indeed, it has been demonstrated that CTB, even in an unconjugated form, is able to increase the specific tolerogenic effect of oral insulin in a murine model of autoimmune diabetes. Moreover, it has been shown that orally administered rCTB prevents development of TNBS-induced colitis in mice and brings about resolution of previously established colitis by inhibiting mucosal IL-12 and IFN- γ production, suggesting an immunomodulatory role of free CTB on DC (Boirivant et al., 2001). In the present study, we demonstrate for the first time that CTB is able to influence the phenotype and functions of human DC. Specifically, we observed that preincubation of immature MDDC with rCTB partially prevents IL-12 production and the full expression of CD40 after LPS stimulation, without affecting the expression of costimulatory molecules CD80, CD86, and CD83. These results are in agreement with a previous

observation made in the murine system, whereby free CTB, differently from CTB conjugated with antigens, was found not to enhance the cell-surface levels of CD80 and CD86 (Eriksson et al., 2003). However, they are in contrast with other data reporting the effect of CTB on splenic murine DC, which demonstrated that the *in vitro* incubation of murine DC with rCTB induces up-regulation of the expression of MHC class II and CD86 on DC and an increased secretion of IL-12. Reasons for these discrepancies are not easily recognized, but heterogeneity of myeloid murine DC and their state of maturation might account for the contrasting results observed in the murine system (Banchereau & Steinman, 1998; Banchereau et al., 2000). We observed that as opposite as in the case of CTB-antigen conjugates (George-Chandy et al., 2001), free CTB exerts a slight but statistically significant effect on the CD40 expression on MDDC. However, recently, it has been demonstrated that murine DC lacking CD40 expression induce the differentiation of CD4⁺ Tregs that are capable of producing IL-10 in an antigen-specific manner, thus suggesting that CD40 could be a key determinant of the decision between tolerance and immunity (Martin, O'Sullivan, Low, & Thomas, 2003). Taken together, the data suggest that CTB is able to interfere with the maturation/activation process of DC, inducing a cell quite

similar to the DC treated with different biological and pharmacological agents described to be able to induce Tregs (Adorini et al., 2004; Gonzalez-Rey, Chorny, Fernandez-Martin, Ganea, & Delgado, 2006; Woltman & van Kooten, 2003). As reviewed recently, tolerogenic DC show no changes of the expression of canonical costimulatory molecules and no changes of IL-10 production, whereas IL-12 production remains low upon LPS stimulation (Roncarolo et al., 2006). Similar effects were recently observed in a human-derived macrophage cell line and human PBMC incubated with CTB (Burkart et al., 2002). Previous data have been described in the murine system, where CT-treated bone marrow-derived DC have the ability to induce Tregs (Lavelle et al., 2003; Lavelle et al., 2004). In consideration of our results, we assume that this effect may be ascribed prevalently to the B subunit of the toxin.

In the present study, MDDC treated with rCTB are indeed able to generate Tregs from $CD4^+CD45RA^+$ T cells *in vitro*. These T cells, when compared with T cells treated with LPS-MDDC, showed reduced ability to proliferate, reduced IFN- γ production, increased IL-10 production, and showed the same levels of TGF- β upon polyclonal stimulation. Furthermore, $T_{CTB-LPS-MDDC}$ do not express high levels of Foxp3 and exert their ability to suppress the specific $T_{LPS-MDDC}$

proliferation through the immunosuppressive cytokines IL-10 and TGF- β . These properties are similar to those described for Tr1 cells. Groux et al. (Groux et al., 1997) first described the biological features of Tr1. In the past few years, different populations of IL-10-producing T cells with regulatory function have been described (Akbari, DeKruyff, & Umetsu, 2001; Barrat et al., 2002; Boussiotis et al., 2000; Jonuleit et al., 2000; O'Garra, Vieira, Vieira, & Goldfeld, 2004; Sundstedt, O'Neill, Nicolson, & Wraith, 2003; Vieira et al., 2004). Their cytokine profile can slightly vary depending on the experimental conditions, but their levels of IL-10 production are invariably high. It has also been established that Foxp3 is a marker for natural CD4⁺CD25⁺ but not for adaptive Tr1 cells (Roncarolo et al., 2006). Several observations indicate that Tr1 cells are involved in maintaining peripheral tolerance *in vivo*. Tr1 cells play a critical role in modulating immune responses to self-antigens.

Reduction of IL-10-producing CD4⁺ T cells was observed in peripheral blood and sinovial tissue of patients with rheumatoid arthritis (Yudoh, Matsuno, Nakazawa, Yonezawa, & Kimura, 2000) and peripheral blood of patients with autoimmune hemolytic anemia (Hall et al., 2002). Tr1 cells can also regulate immune responses to allergen and mucosal antigens (Cavani et al., 2000; Cong, Weaver, Lazenby, & Elson, 2002;

Reefer et al., 2004; Saloga, Bellinghausen, & Knop, 1999). Tr1 cells also have the ability to prevent the development of colitis when co-transferred with pathogenic CD4⁺CD45RB^{hi} T cells in the SCID transfer model of colitis (Groux et al., 1997). In humans, Tr1 clones have been generated recently from lymphocytes isolated from intestinal mucosa of patients with celiac disease in clinical remission (Gianfrani et al., 2006). Furthermore, spontaneous development of tolerance in severe combined immunodeficient patients transplanted with HLA-mismatched allogeneic stem cells (Baker et al., 1999; Holler et al., 2000; Levings et al., 2005; Lin et al., 2003; Weston, Geczy, & Briscoe, 2006) and in kidney or liver transplantation (VanBuskirk et al., 2000) is associated to high levels of IL-10 produced by T cells, indicating that Tr1 cells can induce tolerance in bone marrow and solid organ transplantation. Thus, many strategies have been adopted to induce/expand these cells to control immune responses in different pathologies. Immunological tolerance can be established by selected immunomodulatory compounds through the induction of Tr1 cells. Administration of rapamycin and IL-10 (Battaglia et al., 2006) or deoxyspergualin and anti-CD3 immunotoxin, which associates with elevated levels of serum IL-10 (Asiedu et al., 2005), induces an antigen-specific, long-term tolerance as

a result of down-regulation of inflammation, blockade of effector T cells, and generation of Tr1 cells in diabetic animals successfully transplanted with pancreatic islets.

Administration of Tregs, generated *in vitro* by MDDC pretreated with CTB in a culture system suitable for human use, might represent a valuable mean for the modulation of immune responses in T cell-mediated diseases.

7 REFERENCES

- Adler, H. S., & Steinbrink, K. (2007). Tolerogenic dendritic cells in health and disease: Friend and foe! *European Journal of Dermatology : EJD*, 17(6), 476-491.
- Adorini, L., Penna, G., Giarratana, N., Roncari, A., Amuchastegui, S., Daniel, K. C., et al. (2004). Dendritic cells as key targets for immunomodulation by vitamin D receptor ligands. *The Journal of Steroid Biochemistry and Molecular Biology*, 89-90(1-5), 437-441.
- Akbari, O., DeKruyff, R. H., & Umetsu, D. T. (2001). Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nature Immunology*, 2(8), 725-731.
- Albert, M. L., Jegathesan, M., & Darnell, R. B. (2001). Dendritic cell maturation is required for the cross-tolerization of CD8⁺ T cells. *Nature Immunology*, 2(11), 1010-1017.
- Asiedu, C. K., Goodwin, K. J., Balgansuren, G., Jenkins, S. M., Le Bas-Bernardet, S., Jargal, U., et al. (2005). Elevated T regulatory cells in long-term stable transplant tolerance in rhesus macaques

induced by anti-CD3 immunotoxin and deoxyspergualin. *Journal of Immunology (Baltimore, Md.: 1950)*, 175(12), 8060-8068.

Baker, K. S., Roncarolo, M. G., Peters, C., Bigler, M., DeFor, T., & Blazar, B. R. (1999). High spontaneous IL-10 production in unrelated bone marrow transplant recipients is associated with fewer transplant-related complications and early deaths. *Bone Marrow Transplantation*, 23(11), 1123-1129.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., et al. (2000). Immunobiology of dendritic cells. *Annual Review of Immunology*, 18, 767-811.

Banchereau, J., & Palucka, A. K. (2005). Dendritic cells as therapeutic vaccines against cancer. *Nature Reviews Immunology*, 5(4), 296-306.

Banchereau, J., & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature*, 392(6673), 245-252.

Barrat, F. J., Cua, D. J., Boonstra, A., Richards, D. F., Crain, C., Savelkoul, H. F., et al. (2002). In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)-

and Th2-inducing cytokines. *The Journal of Experimental Medicine*, 195(5), 603-616.

Battaglia, M., Gregori, S., Bacchetta, R., & Roncarolo, M. G. (2006). Tr1 cells: From discovery to their clinical application. *Seminars in Immunology*, 18(2), 120-127.

Boirivant, M., Fuss, I. J., Ferroni, L., De Pascale, M., & Strober, W. (2001). Oral administration of recombinant cholera toxin subunit B inhibits IL-12-mediated murine experimental (trinitrobenzene sulfonic acid) colitis. *Journal of Immunology (Baltimore, Md.: 1950)*, 166(5), 3522-3532.

Boussiotis, V. A., Tsai, E. Y., Yunis, E. J., Thim, S., Delgado, J. C., Dascher, C. C., et al. (2000). IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *The Journal of Clinical Investigation*, 105(9), 1317-1325.

Bromander, A., Holmgren, J., & Lycke, N. (1991). Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. *Journal of Immunology (Baltimore, Md.: 1950)*, 146(9), 2908-2914.

- Bromander, A. K., Kjerrulf, M., Holmgren, J., & Lycke, N. (1995). Cholera toxin enhances antigen presentation. *Advances in Experimental Medicine and Biology*, 371B, 1501-1506.
- Brown, D. A., & London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *The Journal of Biological Chemistry*, 275(23), 17221-17224.
- Burkart, V., Kim, Y. E., Hartmann, B., Ghiea, I., Syldath, U., Kauer, M., et al. (2002). Cholera toxin B pretreatment of macrophages and monocytes diminishes their proinflammatory responsiveness to lipopolysaccharide. *Journal of Immunology (Baltimore, Md.: 1950)*, 168(4), 1730-1737.
- Carrier, Y., Yuan, J., Kuchroo, V. K., & Weiner, H. L. (2007). Th3 cells in peripheral tolerance. I. induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. *Journal of Immunology (Baltimore, Md.: 1950)*, 178(1), 179-185.
- Cavani, A., Nasorri, F., Prezzi, C., Sebastiani, S., Albanesi, C., & Girolomoni, G. (2000). Human CD4+ T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-

specific Th1 immune responses. *The Journal of Investigative Dermatology*, 114(2), 295-302.

Coccia, E. M., Remoli, M. E., Di Giacinto, C., Del Zotto, B., Giacomini, E., Monteleone, G., et al. (2005). Cholera toxin subunit B inhibits IL-12 and IFN- γ production and signaling in experimental colitis and crohn's disease. *Gut*, 54(11), 1558-1564.

Colonna, M., Trinchieri, G., & Liu, Y. J. (2004). Plasmacytoid dendritic cells in immunity. *Nature Immunology*, 5(12), 1219-1226.

Cong, Y., Weaver, C. T., & Elson, C. O. (1997). The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. *Journal of Immunology (Baltimore, Md.: 1950)*, 159(11), 5301-5308.

Cong, Y., Weaver, C. T., Lazenby, A., & Elson, C. O. (2002). Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. *Journal of Immunology (Baltimore, Md.: 1950)*, 169(11), 6112-6119.

Conti, L., Cardone, M., Varano, B., Puddu, P., Belardelli, F., & Gessani, S. (2008). Role of the cytokine environment and cytokine receptor

expression on the generation of functionally distinct dendritic cells from human monocytes. *European Journal of Immunology*, 38(3), 750-762.

Czerkinsky, C., Russell, M. W., Lycke, N., Lindblad, M., & Holmgren, J. (1989). Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. *Infection and Immunity*, 57(4), 1072-1077.

Dawson, R. M. (2005). Characterization of the binding of cholera toxin to ganglioside GM1 immobilized onto microtitre plates. *Journal of Applied Toxicology : JAT*, 25(1), 30-38.

Eriksson, K., Fredriksson, M., Nordstrom, I., & Holmgren, J. (2003). Cholera toxin and its B subunit promote dendritic cell vaccination with different influences on Th1 and Th2 development. *Infection and Immunity*, 71(4), 1740-1747.

Fu, F., Li, Y., Qian, S., Lu, L., Chambers, F., Starzl, T. E., et al. (1996). Costimulatory molecule-deficient dendritic cell progenitors (MHC class II+, CD80dim, CD86-) prolong cardiac allograft survival in

nonimmunosuppressed recipients. *Transplantation*, 62(5), 659-665.

Fujii, S., Liu, K., Smith, C., Bonito, A. J., & Steinman, R. M. (2004). The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *The Journal of Experimental Medicine*, 199(12), 1607-1618.

Fujinaga, Y. (2006). Transport of bacterial toxins into target cells: Pathways followed by cholera toxin and botulinum progenitor toxin. *Journal of Biochemistry*, 140(2), 155-160.

Fujinaga, Y., Wolf, A. A., Rodighiero, C., Wheeler, H., Tsai, B., Allen, L., et al. (2003). Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulum. *Molecular Biology of the Cell*, 14(12), 4783-4793.

Gagliardi, M. C., & De Magistris, M. T. (2003). Maturation of human dendritic cells induced by the adjuvant cholera toxin: Role of cAMP on chemokine receptor expression. *Vaccine*, 21(9-10), 856-861.

- Gagliardi, M. C., Sallusto, F., Marinaro, M., Langenkamp, A., Lanzavecchia, A., & De Magistris, M. T. (2000). Cholera toxin induces maturation of human dendritic cells and licenses them for Th2 priming. *European Journal of Immunology*, *30*(8), 2394-2403.
- Gagliardi, M. C., Sallusto, F., Marinaro, M., Vendetti, S., Riccomi, A., & De Magistris, M. T. (2002). Effects of the adjuvant cholera toxin on dendritic cells: Stimulatory and inhibitory signals that result in the amplification of immune responses. *International Journal of Medical Microbiology : IJMM*, *291*(6-7), 571-575.
- George-Chandy, A., Eriksson, K., Lebens, M., Nordstrom, I., Schon, E., & Holmgren, J. (2001). Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. *Infection and Immunity*, *69*(9), 5716-5725.
- Gianfrani, C., Levings, M. K., Sartirana, C., Mazzarella, G., Barba, G., Zanzi, D., et al. (2006). Gliadin-specific type 1 regulatory T cells from the intestinal mucosa of treated celiac patients inhibit pathogenic T cells. *Journal of Immunology (Baltimore, Md.: 1950)*, *177*(6), 4178-4186.

- Gonzalez-Rey, E., Chorny, A., Fernandez-Martin, A., Ganea, D., & Delgado, M. (2006). Vasoactive intestinal peptide generates human tolerogenic dendritic cells that induce CD4 and CD8 regulatory T cells. *Blood*, *107*(9), 3632-3638.
- Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E., et al. (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*, *389*(6652), 737-742.
- Gunn, M. D., Tangemann, K., Tam, C., Cyster, J. G., Rosen, S. D., & Williams, L. T. (1998). A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(1), 258-263.
- Hackstein, H., & Thomson, A. W. (2004). Dendritic cells: Emerging pharmacological targets of immunosuppressive drugs. *Nature Reviews.Immunology*, *4*(1), 24-34.
- Hall, A. M., Ward, F. J., Vickers, M. A., Stott, L. M., Urbaniak, S. J., & Barker, R. N. (2002). Interleukin-10-mediated regulatory T-cell

responses to epitopes on a human red blood cell autoantigen. *Blood*, 100(13), 4529-4536.

Hart, D. N. (1997). Dendritic cells: Unique leukocyte populations which control the primary immune response. *Blood*, 90(9), 3245-3287.

Holler, E., Roncarolo, M. G., Hintermeier-Knabe, R., Eissner, G., Ertl, B., Schulz, U., et al. (2000). Prognostic significance of increased IL-10 production in patients prior to allogeneic bone marrow transplantation. *Bone Marrow Transplantation*, 25(3), 237-241.

Holmgren, J., Adamsson, J., Anjuere, F., Clemens, J., Czerkinsky, C., Eriksson, K., et al. (2005). Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunology Letters*, 97(2), 181-188.

Joffre, O., Nolte, M. A., Sporri, R., & Reis e Sousa, C. (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunological Reviews*, 227(1), 234-247.

Jonuleit, H., Schmitt, E., Schuler, G., Knop, J., & Enk, A. H. (2000). Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with

- allogeneic immature human dendritic cells. *The Journal of Experimental Medicine*, 192(9), 1213-1222.
- Kim, P. H., Eckmann, L., Lee, W. J., Han, W., & Kagnoff, M. F. (1998). Cholera toxin and cholera toxin B subunit induce IgA switching through the action of TGF-beta 1. *Journal of Immunology (Baltimore, Md.: 1950)*, 160(3), 1198-1203.
- Lavelle, E. C., Jarnicki, A., McNeela, E., Armstrong, M. E., Higgins, S. C., Leavy, O., et al. (2004). Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent. *Journal of Leukocyte Biology*, 75(5), 756-763.
- Lavelle, E. C., McNeela, E., Armstrong, M. E., Leavy, O., Higgins, S. C., & Mills, K. H. (2003). Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. *Journal of Immunology (Baltimore, Md.: 1950)*, 171(5), 2384-2392.
- Le, N. T., & Chao, N. (2007). Regulating regulatory T cells. *Bone Marrow Transplantation*, 39(1), 1-9.
- Lebens, M., Johansson, S., Osek, J., Lindblad, M., & Holmgren, J. (1993). Large-scale production of vibrio cholerae toxin B subunit

for use in oral vaccines. *Bio/technology (Nature Publishing Company)*, 11(13), 1574-1578.

Levings, M. K., Gregori, S., Tresoldi, E., Cazzaniga, S., Bonini, C., & Roncarolo, M. G. (2005). Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ tr cells. *Blood*, 105(3), 1162-1169.

Lin, M. T., Storer, B., Martin, P. J., Tseng, L. H., Gooley, T., Chen, P. J., et al. (2003). Relation of an interleukin-10 promoter polymorphism to graft-versus-host disease and survival after hematopoietic-cell transplantation. *The New England Journal of Medicine*, 349(23), 2201-2210.

Lutz, M. B., & Schuler, G. (2002). Immature, semi-mature and fully mature dendritic cells: Which signals induce tolerance or immunity? *Trends in Immunology*, 23(9), 445-449.

Lutz, M. B., Suri, R. M., Niimi, M., Ogilvie, A. L., Kukutsch, N. A., Rossner, S., et al. (2000). Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *European Journal of Immunology*, 30(7), 1813-1822.

- Lycke, N., Karlsson, U., Sjolander, A., & Magnusson, K. E. (1991). The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigens. *Scandinavian Journal of Immunology*, 33(6), 691-698.
- Lycke, N., & Strober, W. (1989). Cholera toxin promotes B cell isotype differentiation. *Journal of Immunology (Baltimore, Md.: 1950)*, 142(11), 3781-3787.
- Martin, E., O'Sullivan, B., Low, P., & Thomas, R. (2003). Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity*, 18(1), 155-167.
- Mayer, L., & Shao, L. (2004). Therapeutic potential of oral tolerance. *Nature Reviews.Immunology*, 4(6), 407-419.
- McColl, S. R. (2002). Chemokines and dendritic cells: A crucial alliance. *Immunology and Cell Biology*, 80(5), 489-496.
- McKenzie, S. J., & Halsey, J. F. (1984). Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. *Journal of Immunology (Baltimore, Md.: 1950)*, 133(4), 1818-1824.

- Mellman, I., & Steinman, R. M. (2001). Dendritic cells: Specialized and regulated antigen processing machines. *Cell*, 106(3), 255-258.
- Menges, M., Rossner, S., Voigtlander, C., Schindler, H., Kukutsch, N. A., Bogdan, C., et al. (2002). Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *The Journal of Experimental Medicine*, 195(1), 15-21.
- Merritt, E. A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J. A., & Hol, W. G. (1994). Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Science : A Publication of the Protein Society*, 3(2), 166-175.
- Morelli, A. E., & Thomson, A. W. (2007). Tolerogenic dendritic cells and the quest for transplant tolerance. *Nature Reviews.Immunology*, 7(8), 610-621.
- Muller, G., Muller, A., Tuting, T., Steinbrink, K., Saloga, J., Szalma, C., et al. (2002). Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells in vivo. *The Journal of Investigative Dermatology*, 119(4), 836-841.

- Narbutt, J., Lesiak, A., Zak-Prelich, M., Wozniacka, A., Sysa-Jedrzejowska, A., Tybura, M., et al. (2004). The distribution of peripheral blood dendritic cells assayed by a new panel of anti-BDCA monoclonal antibodies in healthy representatives of the polish population. *Cellular & Molecular Biology Letters*, 9(3), 497-509.
- Nashar, T. O., Betteridge, Z. E., & Mitchell, R. N. (2001). Evidence for a role of ganglioside GM1 in antigen presentation: Binding enhances presentation of escherichia coli enterotoxin B subunit (EtxB) to CD4(+) T cells. *International Immunology*, 13(4), 541-551.
- Nashar, T. O., Williams, N. A., & Hirst, T. R. (1996). Cross-linking of cell surface ganglioside GM1 induces the selective apoptosis of mature CD8+ T lymphocytes. *International Immunology*, 8(5), 731-736.
- O'Garra, A., Vieira, P. L., Vieira, P., & Goldfeld, A. E. (2004). IL-10-producing and naturally occurring CD4+ tregs: Limiting collateral damage. *The Journal of Clinical Investigation*, 114(10), 1372-1378.

- Peche, H., Trinite, B., Martinet, B., & Cuturi, M. C. (2005). Prolongation of heart allograft survival by immature dendritic cells generated from recipient type bone marrow progenitors. *American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, 5(2), 255-267.
- Petersen, J. S., Bregenholt, S., Apostolopolous, V., Homann, D., Wolfe, T., Hughes, A., et al. (2003). Coupling of oral human or porcine insulin to the B subunit of cholera toxin (CTB) overcomes critical antigenic differences for prevention of type I diabetes. *Clinical and Experimental Immunology*, 134(1), 38-45.
- Pierce, S. K. (2002). Lipid rafts and B-cell activation. *Nature Reviews.Immunology*, 2(2), 96-105.
- Pina, D. G., & Johannes, L. (2005). Cholera and shiga toxin B-subunits: Thermodynamic and structural considerations for function and biomedical applications. *Toxicon : Official Journal of the International Society on Toxinology*, 45(4), 389-393.
- Ploix, C., Bergerot, I., Durand, A., Czerkinsky, C., Holmgren, J., & Thivolet, C. (1999). Oral administration of cholera toxin B-insulin

conjugates protects NOD mice from autoimmune diabetes by inducing CD4⁺ regulatory T-cells. *Diabetes*, 48(11), 2150-2156.

Reefer, A. J., Carneiro, R. M., Custis, N. J., Platts-Mills, T. A., Sung, S. S., Hammer, J., et al. (2004). A role for IL-10-mediated HLA-DR7-restricted T cell-dependent events in development of the modified Th2 response to cat allergen. *Journal of Immunology (Baltimore, Md.: 1950)*, 172(5), 2763-2772.

Roncarolo, M. G., & Battaglia, M. (2007). Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nature Reviews.Immunology*, 7(8), 585-598.

Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., & Levings, M. K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews*, 212, 28-50.

Rutella, S., Danese, S., & Leone, G. (2006). Tolerogenic dendritic cells: Cytokine modulation comes of age. *Blood*, 108(5), 1435-1440.

Rutella, S., & Lemoli, R. M. (2004). Regulatory T cells and tolerogenic dendritic cells: From basic biology to clinical applications. *Immunology Letters*, 94(1-2), 11-26.

- Sakaguchi, S. (2000). Regulatory T cells: Key controllers of immunologic self-tolerance. *Cell*, 101(5), 455-458.
- Sakaguchi, S. (2008). Regulatory T cells in the past and for the future. *European Journal of Immunology*, 38(4), 901-937.
- Sallusto, F., & Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *The Journal of Experimental Medicine*, 179(4), 1109-1118.
- Saloga, J., Bellinghausen, I., & Knop, J. (1999). Do Tr1 cells play a role in immunotherapy? *International Archives of Allergy and Immunology*, 118(2-4), 210-211.
- Shortman, K., & Heath, W. R. (2001). Immunity or tolerance? that is the question for dendritic cells. *Nature Immunology*, 2(11), 988-989.
- Shortman, K., & Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. *Nature Reviews.Immunology*, 2(3), 151-161.
- Simons, K., & Ikonen, E. (1997). Functional rafts in cell membranes. *Nature*, 387(6633), 569-572.

- Smith, D. C., Lord, J. M., Roberts, L. M., & Johannes, L. (2004). Glycosphingolipids as toxin receptors. *Seminars in Cell & Developmental Biology*, 15(4), 397-408.
- Sobel, D. O., Yankelevich, B., Goyal, D., Nelson, D., & Mazumder, A. (1998). The B-subunit of cholera toxin induces immunoregulatory cells and prevents diabetes in the NOD mouse. *Diabetes*, 47(2), 186-191.
- Steinman, R. M., Turley, S., Mellman, I., & Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. *The Journal of Experimental Medicine*, 191(3), 411-416.
- Sun, J. B., Rask, C., Olsson, T., Holmgren, J., & Czerkinsky, C. (1996). Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proceedings of the National Academy of Sciences of the United States of America*, 93(14), 7196-7201.
- Sun, J. B., Xiao, B. G., Lindblad, M., Li, B. L., Link, H., Czerkinsky, C., et al. (2000). Oral administration of cholera toxin B subunit conjugated to myelin basic protein protects against experimental

autoimmune encephalomyelitis by inducing transforming growth factor-beta-secreting cells and suppressing chemokine expression. *International Immunology*, 12(10), 1449-1457.

Sundstedt, A., O'Neill, E. J., Nicolson, K. S., & Wraith, D. C. (2003). Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *Journal of Immunology (Baltimore, Md.: 1950)*, 170(3), 1240-1248.

Tarkowski, A., Sun, J. B., Holmdahl, R., Holmgren, J., & Czerkinsky, C. (1999). Treatment of experimental autoimmune arthritis by nasal administration of a type II collagen-cholera toxoid conjugate vaccine. *Arthritis and Rheumatism*, 42(8), 1628-1634.

Thomson, A. W., & Robbins, P. D. (2008). Tolerogenic dendritic cells for autoimmune disease and transplantation. *Annals of the Rheumatic Diseases*, 67 Suppl 3, iii90-6.

Tochikubo, K., Isaka, M., Yasuda, Y., Kozuka, S., Matano, K., Miura, Y., et al. (1998). Recombinant cholera toxin B subunit acts as an adjuvant for the mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin. *Vaccine*, 16(2-3), 150-155.

- VanBuskirk, A. M., Burlingham, W. J., Jankowska-Gan, E., Chin, T., Kusaka, S., Geissler, F., et al. (2000). Human allograft acceptance is associated with immune regulation. *The Journal of Clinical Investigation*, *106*(1), 145-155.
- Vieira, P. L., Christensen, J. R., Minaee, S., O'Neill, E. J., Barrat, F. J., Boonstra, A., et al. (2004). IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *Journal of Immunology (Baltimore, Md.: 1950)*, *172*(10), 5986-5993.
- Wakkach, A., Fournier, N., Brun, V., Breitmayer, J. P., Cottrez, F., & Groux, H. (2003). Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity*, *18*(5), 605-617.
- Waldor, M. K., & RayChaudhuri, D. (2000). Treasure trove for cholera research. *Nature*, *406*(6795), 469-470.
- Weston, L. E., Geczy, A. F., & Briscoe, H. (2006). Production of IL-10 by alloreactive sibling donor cells and its influence on the development of acute GVHD. *Bone Marrow Transplantation*, *37*(2), 207-212.

- Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., et al. (1997). Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *The Journal of Experimental Medicine*, 185(2), 317-328.
- Wolf, A. A., Fujinaga, Y., & Lencer, W. I. (2002). Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *The Journal of Biological Chemistry*, 277(18), 16249-16256.
- Woltman, A. M., & van Kooten, C. (2003). Functional modulation of dendritic cells to suppress adaptive immune responses. *Journal of Leukocyte Biology*, 73(4), 428-441.
- Wu, H. Y., & Russell, M. W. (1998). Induction of mucosal and systemic immune responses by intranasal immunization using recombinant cholera toxin B subunit as an adjuvant. *Vaccine*, 16(2-3), 286-292.
- Yudoh, K., Matsuno, H., Nakazawa, F., Yonezawa, T., & Kimura, T. (2000). Reduced expression of the regulatory CD4⁺ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. *Arthritis and Rheumatism*, 43(3), 617-627.