Instruction of effector T cell programs by flexible dendritic cells
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Cordycepin or cholera toxin B prime for mature dendritic cells that drive the development of regulatory T cells


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Abstract
Regulatory T cells act by suppressing the effector function of other immune cells, in particular that of T cells. They play a key role in immune homeostasis, preventing autoimmunity, and in negative regulation of adaptive immune responses against pathogens, limiting detrimental host tissue damage. Adaptive regulatory T cells can be induced by specific priming of dendritic cells (DC), that have acquired the capacity to drive regulatory T cells. Here we describe the priming for regulatory DC by two well-defined microbial compounds, cordycepin from Cordyceps fungi and the cholera toxin B subunit (CTB) from Vibrio cholera. Both cordycepin and CTB did not affect LPS-induced monocyte-derived DC (moDC) maturation and primed for a low, to slightly elevated, IL-10 production. Compared to control mature moDC, the cordycepin- and CTB-primed mature moDC induced similar percentages of IL-2, IL-4 and IFN-γ producing effector T cells. However, the T cells primed by cordycepin- or CTB-treated DC proliferated less vigorously compared to control T cells, and profoundly inhibited the proliferation of other responder T cells, which was to some extent dependent on IL-10 and TGFβ. The induction of regulatory T cell activity by the regulatory DC appeared to be in part dependent on IL-10 (mainly with respect to cordycepin) and in part on an unknown membrane-bound factor (with respect to CTB). Thus, Cordycepin and CTB induce tolerance via the priming of mature regulatory DC, albeit the mechanisms of the induction of regulatory T cells may differ. The identification of compounds that induce the development of regulatory T cells may be helpful in the definition of candidate adjuvants that are useful in transplantation and in therapies of auto-immune and allergic diseases.
Introduction

Immune responses are negatively regulated at various levels, including by the development of regulatory T cells, suppressing the proliferation and cytokine production of other effector immune cells, in particular that of T cells. These regulatory T cells have a function in immune homeostasis by silencing immune responses to self antigens in order to prevent auto-immunity, and during infections to prevent excessive immune pathology. Different subsets of regulatory T cells can be distinguished: naturally occurring CD4⁺CD25⁺ regulatory T cells, primarily involved in peripheral tolerance to auto-antigens, and adaptive regulatory T cells, primarily associated with mucosal tolerance to ubiquitous antigens or microbial compounds (1). The CD4⁺CD25⁺ regulatory T cells inhibit in a cell contact-dependent manner and can also spread tolerance to other T cells via a process called infectious tolerance (2). Adaptive regulatory T cells constitute the Tr1 and Th3 subsets and produce high levels of IL-10 and/or TGFβ, crucial for their inhibitory potential (3). Adaptive regulatory T cells can be generated in vitro by stimulation of peripheral CD4⁺ T cells in the presence of IL-10 (4), or by stimulation of naive T helper (Th) cells in the presence of either high levels of IL-10 and IFNα (5) or in the presence of a combination of vitamin D3 and corticosteroids (6).

Adaptive regulatory T cells can be generated in the presence of modulated dendritic cells (DC) as well. DC are the main orchestrators of adaptive immune responses and uniquely qualified to stimulate naive Th cells and drive their differentiation into effector Th cells. Immature DC patrol the mucosal lining of peripheral tissues. Upon exposure to pathogens or inflammatory signals from the infected tissue, DC become activated and will migrate to the draining lymph nodes, while undergoing a maturation process to increase their Th cell stimulatory capacity (7). Mature DC provide naive Th cells with antigenic, costimulatory and instructive signals and drive their development into Th1, Th2 or regulatory T cells. The instructive capacity of DC is determined by pathogenic and/or inflammatory signals in the tissues where they reside during their immature phase (8). Ideally, DC exposed to intracellular and/or viral pathogens will drive polarized Th1 responses, while contact to certain helminths will instruct the maturation of DC to induce the development of polarized Th2 cells (Kapsenberg ML, NRI, in press). In addition, a fast growing number of papers demonstrate that both tissue factors and pathogens or their compounds can instruct DC to drive regulatory T cell responses (9). For example, pathogens like Plasmodium falciparum (10,11) or mycobacterial-derived ManLAM (12,13), have been described to modulate DC function by arresting their maturation and IL-12 producing capacity but prime for high IL-10 secretion instead, leading to the acquirement of a regulatory phenotype. However, an arrest in maturation may not be obligatory to induce regulatory DC. Several reports have suggested the existence of fully mature DC that can drive the development of regulatory T cells, i.e. after exposure to lyso-phosphatidylserine (lyso-PS) derived from
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*Schistosoma mansoni* (14) or filamentous hemaglutinine (FHA) from *Bordetella pertussis* (15), or IL-10 producing lung DC, generated by imprinting of mucosal tissue factors (16).

Regulatory T cells may form a potential tool to combat overshooting inflammatory reactions and to control immune pathology due to auto-immune or allergic reactions or to prevent graft rejection. Therefore, it is essential to identify candidate compounds that prime for the development of regulatory T cells, e.g. by regulatory modulation of DC function, which will form the basis of adjuvants used in the treatment of such diseases.

To this aim, we investigated the immunoregulatory effect of two microbial compounds, (i) cordycepin, a nucleoside derivate found in *Cordyceps* fungi and frequently used in traditional Chinese medicine (17), and (ii) cholera toxin B (CTB), the cell-binding subunit of cholera toxin produced by *Vibrio cholerae*. Both compounds are associated with immune suppression and elevated IL-10 levels. In addition, CT has been described to prime for regulatory T cells in mice in vivo (17-21) We demonstrate here in an in vitro model system that the immunosuppressive activity of cordycepin and CTB mainly affects DC. Cordycepin and CTB primed human monocyte-derived DC (moDC) for regulatory DC, driving the polarization of regulatory T cells. Cordycepin and CTB did not arrest the LPS-induced maturation of DC and instead yielded fully mature DC with normal levels of costimulatory molecules. In addition, our data suggest that cordycepin and CTB-treated DC may drive regulatory T cell development by different mechanisms, either in part by IL-10 (cordycepin) or by an unidentified membrane-bound molecule (CTB).

**Material & Methods**

**Antibodies, cytokines and reagents**

Mouse mAb to human CD3 (CLB-T3/4E) and CD28 (CLB-CD28/1) were obtained from CLB (Amsterdam, The Netherlands). Human rIL-4 (sp. act. 1x10^8 U/mg) was obtained from PBH (Hanover, Germany). Human rGM-CSF (sp. act. 1.11x10^7 U/mg) was a gift of Schering-Plough (Uden, The Netherlands). Neutralizing rabbit IgG to human IL-12 was a gift from Dr. P.H. van der Meide (U-cytech, Utrecht, The Netherlands). Neutralizing Abs to human IL-10 were obtained from BD Pharmingen (San Diego, CA). Neutralizing Abs to human TGFβ (anti-LAP) were obtained from R&D Systems (Minneapolis, MN). Anti-PDL-1 and anti-PDL-2 were gifts from Dr T. Coyle (Millenium Pharmaceutica's Inc., Boston, MA). Neutralizing CTLA-4 Abs were purchased by eBioSciences (San Diego, CA). Plastics were purchased from Greiner Bio-One (Alphen aan de Rijn, The Netherlands).

**In vitro generation and maturation of moDC**

Immature DC were generated from monocytes (0,5x10^6 cells/well) in 24-well culture plates (Costar, Cambridge, MA) in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies Ltd., Paisley, UK) containing gentamycin (86 μg/ml; Duchefa, Haarlem, The
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Netherlands) and 10% FCS (HyClone, Logan, UT), supplemented with rGM-CSF (500 U/ml) and rIL-4 (250 U/ml), as previously described (22). On day 6, maturation of iDC was induced by the maturation factors (MF) IL-1β (25 ng/ml), TNFα (50 ng/ml) (both purchased from PBH, Hanover, Germany) and LPS (Sigma-Aldrich, St. Louis, MO) in presence or absence of different concentrations of Cordycepin, CTB or pIC (all purchased by Sigma). After 48h, full maturation into CD83+ mature effector DC (mDC) was confirmed by flowcytometric analysis.

Analysis of cell surface molecule expression by flow cytometry

To analyze the phenotype of the acquired DC, the cells were incubated with PE-conjugated anti-CD86 (BD Pharmingen, San Diego, CA), PerCP-conjugated anti-HLA-DR (BD Biosciences, San Jose, CA) and APC-conjugated anti-CD83 (Caltag Laboratories, Burlingame, CA), or mouse anti-human PD-L1 or mouse anti-human PD-L2. FITC-coupled goat F(ab')2 anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary reagent. Stained cells were analyzed by flow cytometry.

Cytokine production by moDC

Mature DC (2x10⁷ cells) were stimulated with mouse CD40L-expressing mouse plasmacytoma cells (J558 cells, 2x10⁴ cells; a gift from Dr. P. Lane, University of Birmingham, Birmingham, UK), in 200 μl culture medium. Supernatants were harvested after 24 h and stored at -20°C until cytokine levels were measured by ELISA. Determination of IL-12p70 concentrations in culture supernatants was performed by solid-phase sandwich ELISA, as previously described (23). Pairs of specific monoclonal antibodies and recombinant standards were obtained from BioSource International (Camarillo, CA) for the determination of IL-6, and from BD Pharmingen for IL-10 determination. The detection limits are as followed: IL-6, 20 pg/ml, IL-10, 20 pg/ml and IL-12p70, 3 pg/ml.

Isolation of naive Th cells

CD45RA+CD45RO- naive CD4+ T cells were isolated from PBMC through negative selection using CD4+ MACS MultiSort beads (Miltenyi Biotech, Bergisch Gladbach, Germany), supplemented with PE-labeled CD45RO-(Dakopatts, Glostrup, Denmark). For subsequent depletion, the anti-hapten beads of the CD4+ isolation kit were supplemented with anti-PE coupled to magnetic beads.

Stimulation and culture of naive Th cells

Purified naive Th cells (2x10⁴ cells) were cocultured with mature DC (5x10³ cells) in 200 μl culture medium in the presence of the superantigen Staphylococcus aureus enterotoxin B (SEB) (10 pg/ml; Sigma), in 96-well flat-bottom culture plates (Costar). At day 5, rIL-2 (10 U/ml, Cetus Corp.) and rIL-15 (10 ng/ml, R&D Systems) was added and the cultures were expanded for the next 7 days.
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Cytokine production by Th cells

On day 12, the quiescent Th cells were restimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml; Sigma) for 6 h, the last 5 hrs in the presence of Brefeldin A (10 μg/ml; Sigma), to determine single-cell IL-2, IL-4 and IFN-γ production by intracellular flowcytometric analysis. Cells were fixed in 2% paraformaldehyde (PFA; Merck, Darmstadt, Germany), permeabilized with 0.5% saponin (ICN Biochemicals; Cleveland, OH) and stained with anti-human IFN-γ-FITC, anti-human IL-4-PE or anti-human IL-2-FITC and anti-human IFN-γ-PE (all from BD Pharmingen). In parallel, 7,5x10^4 T cells were stimulated with anti-CD3 (1:2000 dilution of acities fluid) and anti-CD28 (1 μg/ml), in 200 μl culture medium. After 72 hrs supernatants were taken for analysis of IL-10 secretion by ELISA, as described above.

T cell suppressor activity

At day 12, resting T cells were harvested and washed three times with serum-free medium, prior staining with PKH-26 (Sigma), a red cell cycle tracking dye. Cells (1x10^6) were staining with 3x10^5 M PKH-26 for 5 minutes at room temperature according to the manufacturer’s instructions. After thorough washing, 25x10^3 CD4+ T cells (DC-primed T cells) were stimulated with anti-CD3 (1:5000 dilution of acities fluid) and anti-CD28 (0.5 μg/ml) in round-bottom 96-well plates. After overnight pre-activation, 25x10^3 peripheral CD4+ T cells were added, representing the responder T cells. Prior to this, the responder T cells were labeled with CFSE (0.5 μM, Molecular Probes Inc., Eugene, OR), a green cell cycle tracking dye, for 15 min. at room temperature. After 5 days, the content of PKH-26 and CFSE in the DC-primed and responder T cells, respectively, was analyzed by flowcytometry.

T cell proliferation

Naive Th cells (5x10^4) were stimulated by different concentrations of Cordycepin, CTB, pIC or LPS-treated mDC. Subsequently, cell proliferation was assessed by the incorporation of [3H]-TdR (Radiochemical Center, Amersham, Little Chalfont, U.K.) after a pulse with 13 KBq/well during the last 16 h of 5-day culture after stimulation, as measured by liquid scintillation spectroscopy.

Transwell experiments

In the upper chamber 5x10^4 Cordycepin, CTB, pIC or LPS-treated DC were cocultured with 5x10^4 CD40L expressing J558 cells. In the bottom chamber 2x10^5 naive Th cells were cultured with 5x10^4 LPS-matured DC and SEB (10 pg/ml). At day 5, rIL-2 (10 U/ml) and rIL-15 (10 ng/ml) was added and the cultures were expanded for the next 7 days.
Results

Cordycepin and CTB do not inhibit LPS-induced DC maturation and prime for low to moderate levels of IL-10

Immature DC (iDC) were matured by LPS plus the maturation factors (MF) IL-1β and TNFα in the presence or absence of cordycepin (25 μg/ml), CTB (10 μg/ml) or by the Th1-associated control compound poly I:C (20 μg/ml) for 48 hrs. Flow cytometric analysis of membrane molecules, i.e. the costimulatory molecules CD80 and CD86, MHCII molecule HLA-DR and maturation marker CD83, demonstrated equal expression levels of all molecules tested, irrespective of the presence of cordycepin or CTB during the maturation (Figure 1A).

The CD40L-induced IL-12 production in LPS/MF-matured mDC (control mDC) was reduced both by priming with cordycepin or CTB. In contrast, IL-10 secretion was slightly increased although not to the same extent as the increase in IL-10 production found in the poly I:C-matured mDC. IL-6 production levels were approximately equal in all conditions, except for poly I:C DC (Figure 1B). These results indicate that cordycepin and CTB do not affect the DC maturation induced by LPS plus MF and only marginally modify the cytokine production.

![Flow cytometry analysis of membrane molecules](image1)

![Graph showing cytokine production](image2)
Cordycepin or cholera toxin B prime for mature DC that drive the development of regulatory T cells

Figure 1: Cordycepin and CTB do not inhibit LPS-induced maturation and prime for low to moderate levels of IL-10. Immature DC were generated as described elsewhere (22). (A) Maturation was induced by addition of LPS (100 ng/ml) plus MF (rIL-1β (25 ng/ml) and rTNFα (50 ng/ml)) in the presence or absence of cordycepin (25 µg/ml), CTB (10 µg/ml), or poly I:C (20 µg/ml). After 48 h, mDC were harvested, washed and (A) surface expression of CD86, CD80, HLA-DR and CD83 was analyzed by flow cytometry. (B) Mature DC (2.10^7 cells/well) were stimulated with mouse CD40L-expressing mouse plasmacytoid cells (J558 cells, 2x10^4 cells/well) to induce cytokine production. After 24h, supernatants were collected and IL-12p70, IL-10 and IL-6 production were measured by ELISA.

Cordycepin and CTB prime for DC that induce regulatory T cells

To analyze the T cell polarizing capacity of cordycepin, CTB or poly I:C-primed mature DC (mDC), cocultures of these mDC were performed with naive Th cells in the presence of the T cell stimulatory superantigen SEB (10 pg/ml). After 12 days, the cytokine profiles of the generated effector CD4^+ T cells (DC-primed T cells) were analyzed. The intracellular percentages of IL-4 and IFN-γ-producing cells in the population of T cells induced by cordycepin- or CTB-primed DC, was similar to the percentages in T cells induced by control mDC (Figure 2A, upper panel). In contrast, the T cells induced by poly I:C-primed DC produced massive amounts of IFNγ and only little IL-4, as reported before (24). In all conditions, the T cells produced equal levels of IL-2 (Figure 2A, lower panel). Remarkably, evaluation of the basal proliferation level of the different T cells showed less proliferation in T cells induced by cordycepin- or CTB-primed DC (Figure 2B). Subsequently, the suppressive capacity of these T cells (DC-primed T cells) was tested on the proliferation of peripheral blood CD4^+ T cells (responder T cells) using a cell cycle tracking dye assay allowing the separate analysis of DC-primed and responder T cells by flow cytometry. To this aim, DC-primed T cells were labeled with PKH-26, a red cell cycle tracking dye and cocultured for 5 days with responder T cells labeled with CFSE, a green cell cycle tracking dye. Suppression is evident when responder T cells proliferate slower and, consequently, lose less CSFE on the single cell basis. As is shown in Figure 2C, the presence of -T cells primed by cordycepin- or CTB-treated DC, compared to control DC-primed T cells, clearly inhibited the responder T cell proliferation. The suppressor effects were quantified by setting the value of the average MFI of the responder T cells cocultured with control DC-primed T cells, representing optimal proliferation, at 100%. Based on this value the fold change in proliferation was calculated for each condition. A dose-dependent decrease in responder T cell proliferation was demonstrated in the cocultures with T cells primed by cordycepin- or CTB-treated DC, and not with T cells primed by poly I:C-treated DC (Figure 2D). These results suggest that cordycepin and CTB prime mDC for the capacity to induce the development of regulatory T cells.
Cordycepin or cholera toxin B prime for mature DC that drive the development of regulatory T cells

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Cordycepin or cholera toxin B prime for mature DC that drive the development of regulatory T cells

Figure 2: Cordycepin and CTB prime for regulatory T cell induction. Generation of iDC and maturation conditions are described in the legend to Figure 1. (A) Mature DC (5x10^5 cell/well) were co-cultured with naive Th cells (2x10^5 cells/well) and superantigen SEB (10 pg/ml). After 12 days, IL-2, IL-4 and IFN-γ productions per cell were analyzed by intracellular FACS-staining following a 6h PMA/ionomycin stimulation, the last 5h in the presence of Brefeldin A. (B) Mature DC (2,5x10^3 cell/well) were cocultured with naive Th cells (5x10^4 cells/well). Cell proliferation was assessed by the incorporation of [3H]-TdR after a pulse with 13 KBq/well during the last 16 h of 6-day culture after stimulation, as measured by liquid scintillation spectroscopy. (C) Naive Th cells were stimulated as described in part A. After 12 days, the DC-primed T cells were labeled with 3x10^5 M PKH-26, and stimulated with suboptimal concentrations of anti-CD3 (1:5000 dilution of ascites fluid) and anti-CD28 (0,5 µg/ml). After overnight incubation, CFSE-labeled (0,5 µM) responder T cells (peripheral CD4+ T cells), were added in a 1:1 ratio (2,5x10^5 each). After 5 days the PKH and CFSE staining of the cells were analyzed by flowcytometry. The gray CFSE profile represents the test condition, whereas the overlay indicates the proliferation in the presence of control DC-primed T cells (LPS/MF). The figure is a representative out of 7 independent experiments. (D) The MFI of the CFSE-labeled responder T cells co-cultured in the presence of control DC-primed T cells (LPS/MF) was set at 100%, representing the maximal proliferation. The MFI of the responder T cells cocultured with other DC-primed T cells was compared to this value, calculating the relative proliferation. The results are expressed as the mean percentages ± SEM from 4-7 independent experiments (Cordycepin: 25 – 12,5 – 6,25 µg/ml and CTB: 10 – 1 – 0,1 µg/ml).

Cordycepin and CTB prime for IL-10-producing regulatory T cells

To explore the mechanism by which cordycepin- or CTB-induced T cells inhibit the proliferation of responder T cells, blocking experiments were performed to study the contribution of obvious candidate molecules. Strikingly, neutralizing Abs to IL-10 and TGFβ resulted in partial abrogation of the suppressed responder T cell proliferation (Figure 3A). This suppressor activity did not correlate well with the moderately elevated IL-10 levels produced by both cordycepin- or CTB-DC primed T cells (Figure 3B). The involvement of other regulatory molecules, such as CTLA-4, is currently under investigation.
Cordycepin or cholera toxin B prime for mature DC that drive the development of regulatory T cells

Figure 3: Cordycepin and CTB prime for IL-10-producing regulatory T cells. DC-primed T cell were generated as described in the legend to Figure 2. (A) CFSE and PKH-based proliferation assay was performed as described in the legend to figure 2, in the presence or absence of neutralizing Abs to IL-10 or TGFβ (both 1 µg/ml). (B) DC-primed T cells (7.5x10⁴) were stimulated with anti-CD3 (1:2000 dilution of acetics fluid) and anti-CD28 (1 µg/ml) for 72 h, after which supernatants were collected and IL-10 content was analyzed by ELISA. The IL-10 production of control DC-primed T cells were set at 100% and other groups were calculated as fold change compared to this value. The results are the mean ± SEM from 7 independent experiments.

The development of regulatory T cells induced by cordycepin or CTB is driven by both soluble and membrane-bound factors

To explore the possible role of IL-10 and TGFβ in the induction of regulatory T cells by cordycepin- or CTB-treated mDC, neutralizing IL-10 and TGFβ Abs were added during the coculture of DC and naive Th cells. Subsequent analysis of responder T cell proliferation demonstrated that blocking IL-10 and TGFβ during the initial contact of DC and naive Th cells enhanced the proliferation in the presence of T cells primed by cordycepin-treated DC, but only marginally enhanced the proliferation in the presence of T cells primed by CTB-treated DC. Thus, in both cases, similar proliferation levels as in the presence of mature control DC-primed T cells were never observed (Figure 4A). These results indicate that IL-10 and/or TGFβ are partially and selectively involved in the development of regulatory T cells driven by cordycepin-treated mDC and that other factors also contribute in this respect. A
Cordycepin or cholera toxin B prime for mature DC that drive the development of regulatory T cells

role for additional factors is further suggested by the marginally enhanced IL-10 production in cordycepin- and CTB-treated mDC (Fig. 1B). To investigate which other factors are involved and to what extent they are soluble or membrane-bound, we performed transwell experiments, with the stimulated CTB- or poly I:C-treated mDC in the upper compartment and naive Th cells stimulated with control mDC in the lower compartment. Subsequent analysis of the DC-primed T cells in the CFSE and PKH-based proliferation assay demonstrated almost similar responder T cell proliferation in all groups (Figure 4B). These results suggest that CTB may induce the expression of one or more membrane-bound molecules, crucial for the development of regulatory T cells. Similar experiments are presently running with cordycepin-primed DC.

**Figure 4: Regulatory T cell development induced by cordycepin or CTB is driven by both soluble and membrane-bound factors.**

Generation of iDC and maturation conditions are described in the legend to Figure 1. (A) Mature DC (5x10^3 cells/well) were co-cultured with naive Th cells (2x10^5 cells/well) and superantigen SEB (10 pg/ml) in the presence or absence of neutralizing Abs to IL-10 or TGFβ (both 1 µg/ml). After 12 days, the CFSE and PKH-based proliferation assay was performed as described in the legend to Figure 2. (B) The co-culture of mDC with naive Th cells was performed in transwells. In the upper chamber cordycepin-, CTB-, poly I:C or MF.LPS-treated mDC (5x10^5) were stimulated with irradiated CD40L-expressing J558 cells (5x10^5). In the lower compartment naive Th cells (2x10^5) were stimulated with MF/LPS-matured mDC (5x10^5) and SEB (10 pg/ml). After 12 days, the DC-primed T cells were analyzed in the CFSE and PKH-based proliferation assay as described in the legend to Figure 2.
Cordycepin- and CTB-treated mDC express elevated levels of PD-L2

To identify the membrane-bound molecule(s) that is/are involved in the regulatory T cell development of cordycepin- or CTB-treated mDC, the expression of several likely candidates were tested, including GITRL, ILT-3, PD-L1 and PD-L2. GITRL was hardly expressed on iDC, nor upregulated on the various groups of DC (data not shown). ILT-3 was highly expressed by iDC and equally reduced in all different groups after maturation (data not shown). PD-L1 and PD-L2 were marginally expressed on iDC and upregulated during maturation. PD-L1 was equally elevated in all mature DC groups (figure 5A), whereas PD-L2 was markedly higher expressed on cordycepin- or CTB-treated mDC (figure 5B).

![Figure 5: Cordycepin- and CTB-treated mDC express elevated levels of PD-L2.](image)

Generation of iDC and maturation conditions are described in the legend to figure 1. Mature DC were stained with mouse anti-PD-L1 (A) or anti-PDL-2 (B), followed by a second staining step with goat-anti-mouse-FITC and analyzed by flowcytometry. The mean fluorescence intensity (MFI) of immature DC is set at 100% and used to calculate the relative increase in percentages. The results represent the mean ± SEM of 5 independent experiments.

Discussion

In the present study we demonstrate that both cordycepin and CTB prime for mature DC that drive the development of regulatory T cells. Although cordycepin and CTB only marginally upregulated DC-derived IL-10 production, this proved to be partly instrumental for the induction of regulatory T cells by the cordycepin-treated mDC. In contrast, regulatory T cell development driven by CTB-treated DC most likely involves an unknown membrane-bound molecule.
At present, adaptive regulatory T cells are often associated with immune evasion, induced by invading pathogens to secure their own survival. Although some pathogens may indeed adapt regulating strategies of the host immune system for their own benefit, regulatory T cells in general form an essential feedback mechanism in normal adaptive immune responses against pathogens, and their development is mainly initiated for the benefit of the host. Adaptive regulatory T cells prevent excessive immune pathology at the site of infection (25,26). In addition, by prolonging the presence of the pathogen, they allow for the induction of sufficient memory responses, protecting for reinfection with the same pathogen (27,28). This indicates that the presence of regulatory T cells at the site of infection forms a normal and essential element in protective adaptive immune responses to pathogens.

It may therefore not be surprising that the induction of adaptive regulatory T cells is an important function of DC and that different DC subsets can be recognized that drive regulatory T cell development. Several reports show that DC inducing regulatory T cells, have an immature-like phenotype, expressing low levels of costimulatory molecules and cytokines, except for an abundant expression of IL-10. Such regulatory DC may be induced by the exposure to certain pathogens or their compounds, anti-inflammatory mediators or drugs. Examples are *P. falciparum* (10,11), Hepatitis C virus (29) or mycobacterial-derived ManLAM (12,13), the cytokines IL-10 and TGFβ (30,31) and the drugs vitamin D3 (32), nacystelynn (33) and corticosteroids (34,35). In addition, several reports, including this one, suggest the occurrence of an additional subset of regulatory DC with a normal mature phenotype and normal levels of costimulatory molecules, but that primes for regulatory T development. Examples of compounds that induce this type of mature regulatory DC are FHA from *B. pertussis* (15), lyso-PS from *S. mansoni* (14) and measles virus (36;37). Although these DC strongly support the development of regulatory T cells, they produce only low to moderate amounts of IL-10 that in the case of lyso-PS (together with a membrane-bound factor) and FHA proved to be crucial for their regulatory T cell driving capacity. The current study demonstrates that also cordycepin and CTB induce a similar kind of mature regulatory DC. Moreover, the results of the present study suggest that mature regulatory DC drive regulatory T cell development not only by IL-10, the major effector molecule of immature regulatory DC, but probably also by certain membrane-bound molecules. This is a first indication that immature and mature regulatory DC drive regulatory T cell development by different instruction programs. Nevertheless, it is still remains unclear whether these subsets have non-overlapping functions and under which conditions these subsets do appear.

Cordycepin is a nucleoside derivate, 3'-deoxyadenosine, found in *Cordyceps* fungi and used in traditional Chinese medicine for general immune suppression (18). A more recent study
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has indicated that cordycepin induces IL-10 production in phytohaemagglutinin-stimulated PBMC (17). Since we could detect no effects of cordycepin on purified CD4\(^+\) T cells, at least with respect to cytokine production or proliferation (data not shown), we suggest, according to the present study, that these effects of cordycepin on PBMC originate from a primary effect on myeloid cell fraction, such as monocytes and dendritic cells. Furthermore, anti-inflammatory activity and reduction of tissue damage has been reported for adenosines in general (38). To date, the expression of three adenosine receptors has been described on DC and macrophages, of which the A\(_2\)-adenosine receptor appears to be crucial for the inhibition of IL-12 and the induction of IL-10 (39-41). Ligation of this receptor leads to the activation of adenylate cyclase and enhanced cAMP levels, which is casually related to the inhibition of IL-12, as has also been described for several other compounds that enhance cAMP, such as \(\beta_2\)-agonists, PGE\(_2\) and cholera toxin subunit A (CTA) (23,42-44). In contrast, CTB does not induce elevated levels of cAMP, but is responsible for the binding to ganglioside GM1 receptors. Presently it is unknown which signaling cascades are activated by CTB. In contrast to what has been published in the mouse (21), CTA or total CT appears to be less effective in the priming for human regulatory moDC (data not shown) compared to CTB, favoring against a role for cAMP in this respect. Interestingly, a synergistic activity between signaling of TLR2, 4, 7 and 9, but not TLR3 and 5, and signaling of A\(_2\)-adenosine receptors has been demonstrated (45), which may explain the enhanced priming for regulatory T cell development by cordycepin in mature DC (also exposed to LPS, a TLR4 ligand).

The transwell experiments indicated that, in addition to IL-10, one or more inhibitory membrane-bound molecules are involved in the induction of regulatory T cell development by at least CTB-modulated DC. Although the identity of this molecule is not yet known, PD-L2 may be an interesting candidate, since the present study showed that its expression is linked with the regulatory status of the DC and since this molecule may bind to PD-1, a T cell molecule that confers inhibition of T cell proliferation and T cell anergy (46,47).

The priming for human DC, supporting the development of potent regulatory T cells, may have interesting potential as therapy of various inflammatory diseases, such as autoimmune diseases, allergies, and transplantation. In particular, cordycepin and CTB may form interesting candidate adjuvants to induce regulatory DC either in vivo, or by treating DC ex vivo. In fact, CTB either coupled to insulin or allergens has already been tested in mouse models for diabetes or allergy and resulted in suppressed T cell activity via the induction of regulatory T cell activity. This may imply a regulatory modulation of dendritic cell function. The specificity and efficiency of this type of approaches in vivo may be strongly enhanced by the specific targeting of CTB or cordycepin-coupled antigens to molecules specifically binding to DC.
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The present study demonstrates that both cordycepin and CTB can modulate human DC function and prime for enhanced regulatory T cell development in otherwise normal mature DC. This finding indicates that an immature-related or immature-arrested DC phenotype is not an absolute requirement for regulatory T cell development. Moreover, cordycepin and CTB form interesting candidates for adjuvant-based therapies of autoimmune or allergic diseases and to prevent graft rejection in transplantation.

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