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DISSECTING THE EFFECTS OF ANTHRAX TOXINS ON DENDRITIC CELLS AND POTENTIAL REVERSAL BY CHEMICALLY MODIFIED TETRACYCLINES

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Abstract of the Thesis

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The virulence of *Bacillus anthracis* is dependent on secretion of a LT toxic complex which inactivates mitogen-acivated protein kinase pathways and in turn causes multisystem dysfunction, such as inhibition of dendritic cell immune function, followed by rampant bacteraemia and death. It has been proposed that the observed nonresponsiveness of DCs to immune stimulation following the exposure to LT is either due to immunological function impairment of DCs or to LT-mediated cell death (10, 11). Here I show that the effect of LT on DCs may be maturation stage dependent, with immature DCs undergoing apoptotic and necrotic cell death upon exposure to LT, while maturing and mature DCs remain viable, but with impaired immunological function. By establishing the effects of LT on different stages of DC maturation I delineated a “window of opportunity”

to employ therapeutic interventions for individuals who had been exposed to anthrax spores by administering agents directed against the *B. anthracis* toxins. Additionally, I evaluated the feasibility of the use of the COLs as pre- and post-exposure prophylactic agents by assessing their effect on the human monocyte-derived DCs as well as their capacity to preserve their immunological responses.

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GLOSSARY

B.	*Bacillus*
COL-3	4-de(dimethylamino)sancycline
COL-308	9-amino-4-de(dimethylamino)sancycline
DC	dendritic cell
ELISA	enzyme-linked immunosorbant assay
GM-CSF	Granulocyte-macrophage colony-stimulating factor
iDC	immature dendritic cell
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
LF	lethal factor, secreted component of *B. anthracis*
LPS	lipopolysaccharide, a component of Gram-negative bacteria
LT	lethal toxin, comprised of LF and PA
mDC	maturing or mature dendritic cell
MonoMac6	a human monocytoid line developed by Ziegler-Heitbrock et al.
PA	protective antigen, secreted component of *B. anthracis*
PBS	phosphate buffered saline solution
PI	Propidium Iodide

SPECIFIC AIMS

1. Establish time and dose dependence of the effect of Lethal Toxin (Lethal Factor + Protective Antigen) on dendritic cells (DCs) cultured from human mononuclear cells. Human mononuclear cells will be cultured in the presence of IL-4 and GM-CSF until day 3 or day 4, at which point they will be challenged with *Bacillus anthracis* Lethal Toxin and its individual components PA and LF. After 4hr, 24hr and 48hr incubation in presence or absence of maturation factor (LPS) their viability and phenotype will be analyzed.

a) Following the DC challenge with LT and its individual components (PA and LF) in the presence or absence of LPS, cell viability will be determined by Annexin/PI stain and by the cells' FACS profile. In addition, Wright-Giemsa stain will be performed to visually evaluate the effects of LT and its individual components on the dendritic processes.

b) The temporal effects and dose response of DCs to LT will be determined through phenotype analysis of DC surface proteins HLA-DR, CD40, CD83, and CD40, which are well defined maturation markers. Additionally, the secretion of cytokines IL-10 and IL-12 will be evaluated through ELISA.

2. Evaluate the effects of metalloproteinase inhibitors COL-3 and COL-308, known inhibitors of LT, on the viability and maturation of DC in the absence of *B. anthracis* Lethal Toxin. Establish the dose response of DC to COLs at 4hr, 24hr, and 48hr in DCs cultured in presence or absence of LPS.

a) The effects of the inhibitors on cell viability will be assessed through Annexin/PI cell viability assay. Additionally, Wright-Giemsa stain will be performed in order to visually determine any other adverse effect of COLs on DC development.

b) Potential interference of COLs with DC maturation will be assessed by analysis of DC maturation markers HLA-DR, CD40, CD83, and CD86 surface proteins, as well as by analysis of cytokine secretion (inflammatory biomarkers IL-10 and IL-12).

c) The ability of DC to perform their antigen-presenting function post-COL exposure will be assessed through mixed leukocyte reaction (MLR).

3. Determine the capacity of COL-3 and COL-308 to block the effects of LF introduced to LPS treated and non-treated DCs via LT. After determining if LF is implicated in compromising the DC functions, the potential of COLs to block the actions of LT on DCs will be established. Cells will be pretreated with COLs prior to exposure to LT, or exposed to LT before treatment with COLs.

a) The potential inhibition of LF-induced DC death by the COLs will be assessed by Annexin/PI viability assays, using LPS treated and non-treated DCs.

b) If cell death is reversed, the potential ability of the COLs to reverse the effects of LT on DC maturation will be determined by surface phenotyping as well as by analysis of cytokine secretion.

c) The potential inhibition of LF by COLs will also be determined by Western blot. The rescue of MAPK signaling will be analyzed (MEK-2 and MEK-6).

d) The COLs' capacity to rescue T cell activation, a measure of the immunological function of LT-exposed DC, will be evaluated by mixed leukocyte reaction (MLR).

4. Dissect the effect of PA alone on DC viability, maturation, endocytosis, and phagolysosome formation. Preliminary data has shown that *B. anthracis* toxin PA is able to enter the DC on its own, forming vacuoles within the DC.

a) Visually determine the dose response and time dependence of PA-induced vacuole formation in the DC membrane via Wright-Giemsa stain.

b) Evaluate the effect of PA on the endocytic capacity of DC by measuring endocytosis of Dextran-FITC. The endocytic capacity of PA-treated DC will be analyzed via FACS and fluorescent microscopy (Dextran-FITC in combination with PI). Additionally, analyze the effect of PA on the DCs' ability to form phagolysosomes.

c) Determine the effect of PA on DC cell maturation by analyzing the expression of maturation biomarkers HLA-DR, CD40, CD83, and CD86 through FACS analysis.

d) Determine if PA-challenged DC can activate T-cells by the way of MLR.

BACKGROUND AND SIGNIFICANCE

Anthrax is an infection caused by pathogenic strains of *Bacillus anthracis*, a large, rod-shaped, spore-forming, gram-positive bacterium, and poses clear and present danger as an agent of potential bioterrorism. The virulence of *B. anthracis* is dependent on secretion of a three-component toxic complex consisting of Protective Antigen (PA), Edema factor (EF) and Lethal Factor (LF), which together contribute to the pathology of Anthrax (1). PA interacts with cell surface receptors (Anthrax Toxin Receptor 1 and 2) and, after proteolytic activation, self-associates to form a membrane-inserting heptamer that binds LF or EF (5-7). PA + LF (Lethal Toxin (LT)) and PA +LF (Edema Toxin (ET)) complexes enter surface rafts and are internalized into acidic endosomal compartments (16). Low pH triggers a conformational change that results in LF and EF translocation into the cytosol of the host cell (5-7). LF, a zinc metalloproteinase, cleaves mitogen-activated protein kinase kinases (MAPKKs or MEKs), thereby interfering with the MAPK cascade, a crucial component of the signal transduction repertory controlling cell proliferation and survival (8).

B. anthracis spores enter the body through skin abrasions, inhalation or digestion, and are phagocytized by phagocytic cells which migrate to regional lymph nodes. In lymph nodes spores germinate and become vegetative bacteria that lyse the phagocytic cell and multiply in the lymphatic system. From there bacilli gain access to the circulatory system and are disseminated throughout the host, causing both bacteremia and toxemia due to secretion of toxins. Death often

ensues after rupture of inflamed mediastinal lymph nodes, preceded by edema and multiple organ failure (reviewed in 1).

For decades, research has focused almost exclusively on macrophages as an inhalation anthrax Trojan horse that engulfs spores and carries them out of the lungs (2). However, mice that were chemically depleted of macrophages and infected with spores by aerosol nevertheless experienced disease (3). This demonstrated that macrophages might not be essential for initiation of infection and implicated the existence of unidentified cell populations within the lungs that may be exploited by *B. anthracis* to facilitate the disease process. In particular, the dendritic cell (DC) has qualities that make it a strong candidate for exploitation by *B. anthracis* in the course of progression of disease (3).

DCs are antigen (Ag) handling cells that regulate a wide spectrum of immune responses, behaving as first-line sentinels at various portals throughout the body. They are found in high frequency within the airway epithelium, submucosa, lung parenchyma, as well as skin. DCs detect microbial pathogen-associated molecular patterns, which they integrate and process, before migrating to lymph nodes. Migration of antigen-stimulated DCs into lymph nodes is fundamental to initiating appropriate immune responses. The innate immune responses are initiated via contact interactions with other leukocytes and by secretion of proinflammatory chemokines, cytokines and lipid mediators (13). The central role of DCs in development of immunity is their exceptional capacity to stimulate naïve T cells to participate in cell-mediated and humoral immune responses. DCs present Ag to T cells by way of major histocompatibility complex

(MHC) class I and II molecules and exhibit an abundance of coreceptors that are required for T-cell activation on their surface (e.g., CD86, CD80). They initiate a wide spectrum of antigen-driven immune responses, including T-helper (TH1), TH2, CD8 and B-cell responses (reviewed in 4). Therefore, DCs are regarded as a critical link between the innate and adaptive immune responses, which makes them an ideal target of exploitation by *B. anthracis.*

It was reported that LT blunts the release of proinflamatory mediators by macrophages, thereby preventing phagocyte recruitment and bacterial clearance and effectively impairing the immune response of the affected host (9). It has been shown that anthrax toxin impairs DC immune function in a similar manner (11). Some researchers have suggested that DCs exposed to LT, although viable, do not upregulate co-stimulatory molecules or secrete diminished amounts of proinflammatory cytokines and are thus unable to stimulate T- and B-cell immunity; others have proposed that the observed disruption of the DC-regulated immune response is due to apoptotic and necrotic cell death following the LT-challenge (10, 11). I hypothesize that LT-mediated DC impairment or killing is maturation stage-dependent. My preliminary data suggest that immature DCs (iDC) are indeed susceptible to apoptotic and necrotic cell death upon exposure to LT, while maturing and mature DCs (mDC) are not. Although mDCs remain viable after exposure to LT, the expression of surface molecules essential for proper immune function is diminished on the cells.

DC functions are related to stages of maturation. It is established that immature DCs and DC precursors (progenitors) are highly susceptible to external

stimuli, while mature DCs appear to be more resistant (12, 13). Additionally, mature DCs are equipped to deliver antigeneic signals to T cells in a MHC-restricted manner, but do not efficiently handle antigens. Conversely, immature DCs present processed antigens poorly, but readily internalize and process full length antigens (12, 13). *B. anthracis* might have developed a mechanism of avoiding the host immune response by taking advantage of DC maturation pathway.

Given the maturation stage-dependent differences in the capacity of DCs to either stimulate or inhibit the immune response, I hypothesize that *B. anthracis* LT and its individual components might affect DCs differently based on the maturation stage. LT might cause DC death as well impaired maturation. In the present study I intended to further determine how the immunosuppressive LT effect relates to the maturation stage of DC.

It is our hope that by establishing the effects of LT on different stages of DC maturation I can delineate a "window of opportunity" to employ therapeutic interventions for individuals who had been exposed to anthrax spores by administering agents directed against the *B. anthracis* toxins. I intend to evaluate the possibility of using two small cell-permeable broad spectrum metalloproteinase inhibitors COL-3 and COL-308 at early stages of infection, with the goal of restoring the adaptive immune response which could then assist in controlling further dissemination of *B. anthracis* toxin components throughout the body. COL-3 and COL-308 have wide range of anti-protease activities, and the inhibitory activities occur at concentrations in the low micromolar range that

correspond to plasma levels observed in humans during phase I and phase II clinical trials. It has been shown that COLs have the capacity to inhibit *B. anthracis* LF activity in cell-free systems and in viable macrophages as well as the MonoMac 6 cell line, either when the inhibitors are added to the cells prior to addition of LF plus PA (14), or even when the COLs are added after LF has entered the cell. This inhibitory capacity specifically targets the proteolytic activity of LF towards its only natural substrates, the MEK family of MAPKKs. I will evaluate the feasibility of the use of the COLs as pre- and post-exposure prophylactic agents. I will assess their capacity to preserve responses of human DCs (prepared by inducing monocytes to differentiate in the presence of IL-4 and GM-CSF, and then to mature by exposure to LPS) post-challenge with LT.

Additionally, I will further characterize the effect of Protective Antigen, a component of LT, on the immune response. Salles et al. have reported that PA alone is cytotoxic to macrophage cell lines (15). This cytotoxicity of PA alone could potentially further disrupt the host immune response. Indeed, membrane insertion of PA heptamer into cell membrane can be uncoupled from cytoplasmic delivery of LF in certain cell lines (macrophages) (16). From my preliminary results I have confirmed this occurrence in dendritic cells. I will further characterize the consequence of this insertion on DC immunological functions.

RESULTS

1. Maturation of monocyte-derived human DCs with LPS.

The capacity of DCs to stimulate or inhibit the immune response varies not only with their subtype, but also with their maturation stage. In present study, human DCs were derived from human peripheral blood monocytes, induced with IL-4 and GM-CSF to differentiate preferentially down the DC developmental pathway. The immature monocyte-derived DCs were subsequently matured by culture in presence of LPS at day 3 post-isolation, or left immature in the absence of LPS.

In order to establish the system for the analysis of LT-mediated DC challenge, I analyzed the temporal expression of surface maturation markers CD83 and CD86, as well as the DC markers HLA-DR and CD40, at different time points (day3 – day 7). As expected, LPS treated DCs were mature and had increased expression of maturation markers CD83 and CD86 on day 7 post-isolation (Figure 1). In contrast, DCs grown in absence of LPS expressed lower levels of maturation markers, consistent with previous findings (11). The slight increase in marker expression over baseline in control cells is due to self-maturation of DCs after day 4 post-isolation via the secreted cytokines (not shown). Wright-Giemsa DC stain confirmed different maturation stages of the cells (not shown). Subsequent experiments on LPS- immature DCs (iDCs) were performed on day 3-4 post-isolation, while experiments on LPS+ maturing and mature DCs (mDC) were performed on day 4-6 post-isolation.

2. The susceptibility of human DCs to LT-challenge is dependent on their maturation stage.

Although it has been established that anthrax bacterium disables the immunological function of DC through the action of LT component of LF, conflicting reports have been published about the particular effects of LT, a zinc metalloproteinase targeting MAPKK, on DCs (10, 11). While some researchers report LT-challenged DCs to be viable, although non-responsive to maturing LPS stimulation and impaired in their ability to present antigens without killing them, others have reported that human DC are effectively killed by LT through either rapid necrosis or apoptosis (10, 11). While these observations can in part be explained by genetic background of the studied DCs, my data suggests additional level of LT-mediated DC death or impairment.

DCs are known to have their functions related to stages of maturity. Although immature DCs and DC progenitor cells are very susceptible to external instruction, mature DC appears to be more resistant to it (17). My data suggests that these differences extend to the increased immunity of mature and maturing (LPS treated cells) towards the LT-challenge as well (Figure 2).

I determined the DC cell viability at 24hr and 48hr following the 45min LT-exposure (100 ng/ml LF + 200 ng/ml PA on d3) of LPS(-) iDCs and LPS(+) mDCs. Scatter plot decrease of iDC culture compared to mDC culture treated with LT suggest a greater susceptibility to LT-mediated cell death of iDC compared to mDCs after 24hrs (Figure 2a). Further analysis of challenged cells with AnnexinV/PI cell viability assay demonstrated that iDCs treated with LT on

day 3 showed a consistent increase in AnnexinV binding after 24hrs (early apoptosis), with three-fold increase of AnnexinV/ PI positive cells by 48hrs (late apoptosis and necrosis) (Figure 2b). The same effect of LT was not observed on d3 LPS-treated DCs (mDCs) (Figure 2b), suggesting that LT susceptibility of human DC is dependent on their maturation stage. Based on these results, I concluded that iDCs are more susceptible to *B. anthracis'* LT, undergoing apoptotic and necrotic cell death post LT-challenge, while mDCs appear to be more resistant to the LT challenge.

3. The effect of COL-3 and COL-308 on the maturation and cell viability of Human DCs.

Two pharmacological agents of the tetracycline family, COL-3 and COL-308 were shown to inhibit directly the cleavage of MAPKK by LF in monomac6 cells when administered prior to addition of LF plus PA (LT), with no adverse effects for the cells (14). Before testing for the similar inhibitory effect in human DCs, I established the potential cytotoxicity of these tetracyclines on human iDCs and mDCs in micromolar range. The cells were treated with varying doses of COLs (5µM, 10µM, 15µM, and 20µM), and analyzed for cell viability and surface expression of maturation markers following the 24hr of 48hr incubation. Annexin/PI cell viability assay revealed no cytotoxic effects of COL-308 to mDC or iDC at the 10µM working concentration (Figure 3a). In contrast, COL-3 proved cytotoxic to iDC at a 10uM, with increase in apoptosis clearly visible after 24hrs, while mDC seemed resistant to the cytotoxic effects of COL-3, with only slight rise in early apoptosis after 48hr incubation (Figure 3a).

Although resistant to cytotoxic effects of COL-3, mDCs were prone to maturation impairment by COL-308 (Figure 3b). The fluorescence intensity of maturation markers CD83 and CD86 was lower in DCs incubated with COL-3, while the fluorescence intensity was unchanged for non-treated and COL-308-treated DCs, suggesting that COL-3 impairs maturing DCs complete maturation (Figure 3b). Due to the cytotoxic effects of COL-3 on human monocyte-derived iDCs, and its subsequent impairment of maturing DC's maturation, this drug proved not to be a feasible agent of LT inhibition in DCs. Given the non-toxicity of COL-308 towards human DCs, and its non-interference with their maturation, in subsequent experiments only the inhibitory potency of COL-308 was evaluated.

4. The potential inhibition of LT-mediated killing of iDCs by the COL-308 metalloproteinase inhibitor.

Given the known inhibitory effect of COL-308 towards the LT and lack of its apparent cytotoxicity to human DCs, I carried out a series of experiments trying to determine the effectiveness of this inhibitor in preserving DC viability following 45min exposure of DCs to LT. Human monocyte-derived DCs were exposed to LT (100ng/ml LF + 200ng/ml PA) for 45min, and subsequently incubated for 24 hrs with 10μM or 20μM COL-308 in the absence of LPS (iDC). Following the 24hr incubation, Annexin/PI cell viability assay was performed in order to determine the inhibitory potential of COL-308 towards the anthrax' lethal toxin.

The data in figure 4 demonstrates that COL-308 was incapable of inhibiting the LT-mediated cell death. The percentage of Annexin-positive cells is consistently 10-15% higher in LT as well as in LT/COL treated cells compared to the control (Figure 4a). There was no significant difference in cell viability between the cells treated with lower dose of COL-308 (10 μM) compared to the cells treated with higher dose (20μM). Roughly 40% of the LT or LT/CMT-308 treated cells are apoptotic, compared to 10%-12% apoptosis among nontreated cells (Figure 4a). These results are confirmed through histogram analysis which shows a consistent two- to three-fold increase in apoptosis 24hrs post LT-challenge among LT and LT/CMT-308 treated cells (Figure 4b), with even more pronounced cell death 48hrs post LT treatment (not shown).

Although COL-308 does not seem to have the capacity to preserve the viability of LT-challenged iDCs by inhibiting LT, the desired inhibitory effect might be observed if the DCs were pretreated with the inhibitor. Further analysis of COL-308 inhibitory potency towards LT in this dynamic DC system is required.

5. The potential inhibition of LT-mediated maturation impairment of maturing DCs by the metalloproteinase inhibitors.

While LT induces cell death in iDC, LT alone does not seem sufficient to kill mDCs (Figure 2). Although mDCs are more resistant to LT's adverse effect, it does impair the maturation of LPS-treated DCs (mDC), rendering them less responsive to LPS-mediated maturation (figure 3). Even though COL-308 did not prove efficient in preventing LT-triggered cell death in iDC, it might prove

efficient in inhibiting the LT-triggered impairment of mDC's immunological function. The effectiveness of COL-308 in inhibiting LT-mediated maturation impairment of DCs was determined through the IF surface analysis of CD86 and CD83 dendritic cell maturation marker expression, along with the expression analysis of general DC markers HLA-DR and CD40.

DCs were matured in the presence of LPS for 24 hrs and subsequently treated with LT and COL-308 (20μM). Although LT-treated mDCs did not reach fully mature stage compared to the positive control cells, they were significantly more mature than LPS(-) iDCs negative control cells (figure 5). The fluorescence intensity of maturation markers CD86 and CD83 was lover compared to the LPS(+) mDCs, while lower than LPS(-) iDC. At the same time the fluorescence intensity of general DC markers CD40 and HLA-DR was not affected (figure 5b). Different methods of COL-308 administration to cell cultures did not significantly inhibit the LT-mediated maturation impairment of mDCs (figure 5b). Scatter plot analysis confirmed this observation, since the scatter plot profiles of LT-treated DCs are very similar to the scatter profile of iDCs, regardless of the COL-308 presence within the culture (Figure 5a).

6. The effect of PA on DCs' viability and endocytic ability.

Although PA component of LT is not thought to have adverse effect on the immune system on its own, cytotoxicity of PA has been reported in macrophages overexpressing PA cell surface receptors (15). In order to reveal if similar effect can be observed in human DCs, I determined the effect of PA at 200ng/ml and 500ng/ml concentrations on viability of iDC (Figure 6). No

cytotoxic effects of PA on iDC were observed at both PA concentrations. The level of apoptosis following the 48hr incubation with PA was equal to that of the non-treated iDCs, as it is shown via AnnexinV/PI assay (Figure 6b).

Although no direct cytotoxicity was observed, PA did affect the morphology of the iDC. Upon treating human iDCs with 200 ng/ml of PA and 500 ng/ml PA, the formation of phagocytic vacuoles vas observed at both concentrations (Figure 6a). Interestingly, at a higher PA concentration of 500ng/ml, DC cells although highly vacuolated do not exhibit DC cell morphology and the phagocytic vacuoles are smaller in size and peripheral. At this PA concentration cells are smaller in size and smoother, lacking dendritic processes, and starting to resemble pro-monocytes. In order to assess possible effect on endocytosis of such PA treated cell, the DC's uptake of Dextran-FITC was measured. There appeared to be no discernible difference in the uptake of Dextran-FITC between the nontreated iDCs, and those treated with PA (at 100 ng/ml and 200 ng/ml concentrations) (Figure 2c).

MATERIALS AND METHODS

LF and PA. Recombinant anthrax LF and PA were purchased from List Biological Laboratories, Inc. (Campbell, CA). The purity of LF and PA was ≥90% and 100% respectively, as reported by the manufacturer. The specific activity of LF was evaluated by the manufacturer, using its own oligopeptide substrate MAPKKide in a fluorescence resonance energy transfer (FRET)-based assay of peptidolytic activity: 5μM substrate was reported to be cleaved by 5μM LF at a rate of 1.0 to 1.5 relative fluorescence units per second in 20mM HEPES (Mediatech Inc., Herdon, VA), pH 8.2, at 37ºC. LF contained 120-212 U/mg of endotoxin, and PA contained 0.68-11.9 U/mg of endotoxin, an amount to low to independently induce the maturation of DC. Other known biological and enzymatic activities of LF and PA were verified qualitatively by the manufacturer.

Inhibitors. COL-3 and COL-308 were supplied by Collagenex Pharmaceuticals, Inc. (Newton, PA). They were dissolved in dimethyl sulfoxide (DMSO), and tested for cellular toxicity on DC in AnnexinV/PI assay as described bellow. COL-308 was employed at concentrations which did not diminish viability of cells employed in these studies. The inhibitor solutions were endotoxin-free, and the final DMSO concentration in the experimental samples was < 0.1%, too low to have adverse effect on the cell culture.

Cell isolation and culture. Human monocytes were isolated from leukocyte concentrates according to a modification of the method of Levy and Edington (20). Leukocyte concentrates were diluted with RPMI (Gibco, Grand Island, NY)

1:12 and layered atop of equal volume of Lymphoprep (Accurate, Westbury, NY) in 50ml conical tubes. The tubes were centrifuged at 700g for 20 minutes at room temperature. The mononuclear cell layer was diluted into 50ml RPMI and recentrifugated at 500g for 5-7 minutes. Pellets were resuspended in 50ml RPMI and centrifuged at 250g for 5min. the cells were plated at a density of 1.5-1.7 X 10^6 cells/well in a 12-well plate (Fisher Scientific, Hampton, NH). Monocytes were incubated in RPMI 10% FBS (Gibco), 1% Pen/strep and 1% Glutamine. Myeloid DC hemopoiesis from these progenitors was instituted by adding 50 ng/ml of IL-4 (Peprotech Inc., Rocky Hill, NJ) were added and 50 U/ml of GM-CSF (Genzyme, Boston, MA), for 72 hr at 37ºC in a humidified atmosphere containing 5% CO2.

DC challenge with LT. On day 3-4 (72hr – 96 hr) post-isolation, myelodendritic cells were challenged with 200 ng/ml PA (List Biological Laboratories, Inc., Campbell, CA) and 100 ng/ml LF (List Biological Laboratories, Inc., Campbell, CA) for 45 minutes at 37ºC in a humidified atmosphere containing 5% CO_2. Following the incubation, cells were centrifuged for 3min at 300g, washed in 5ml PBS (Mediatech Inc., Herdon, VA), recentrifuged at 300g for 3 min, and replated. The cells were replated in presence or absence of 50ng/ml of LPS (Sigma Aldrich, St. Louis, MO) which served as a DC maturation-inducing factor. The effect of LT on DC cell viability and expression of surface markers was assessed as described below.

Cell viability Annexin/PI assay. Determination of LT effect on LPS+/- DC cells, as well as inhibitory efficacy of COL-3 and COL-308 and their potential

cytotoxicity, was established by Annexin/PI assay. DCs were cultured in GM-CSF/IL-4 for 3-4 days at a concentration of 1.7 x 10^6 cells/well, at which point they were challenged with LT as described above. Following the LT challenge DCs were incubated in the presence of LPS for 24 or 48 hrs with or without the COL-3 or COL-308 inhibitors. Cells were collected and washed twice with ice-cold PBS. Washed cells were resuspended in AnnexinV binding buffer (BD PharMingen, San Diego, CA) at a concentration of 1 x 10^6 cells/ml. 5µl of AnnexinV-FITC (BD PharMingen, San Diego, CA) and 10 µl of PI (Sigma Aldrich, St. Louis, MO) in an ependorf tube. The samples were gently vortexed and incubated for 15min at room temperature in the dark. Following the incubation, 400µl of Annexin binding buffer was added to each tube. Samples were analyzed by flow cytometry as described below.

Immuofluorescence (IF) surface analysis. For surface marker analysis, the following fluorochrome-conjugated mAbs (all from BD PharMingen, San Diego, CA) were used: CD40-FITC, CD83-PE, CD86-PE, HLD-DR-FITC and appropriate isotype controls. Immunophenotypic analysis was performed using FACS. Cells (0.25 x 10^6) were washed in PBS supplemented with 2% FBS and 0.1 NaN_3 (FACS wash buffer) and incubated for 30 min at room temperature with PE- of FITC-conjugated mAbs or with the corresponding isotype-matched mAb. Excess mAb was removed by washing in FACS wash buffer, and cells were resuspended in 1% PBS buffered formalin. Results were expressed as either mean fluorescence intensity of percentage of cells expressing specific antigen. Fluorescence analysis was performed on a FACS Calibur flow cytometer (BD

Biosciences, Rockville, MD) after acquisition of 10,000 events and analyzed with BD Biosciences CellQuest software (Rockville, MD).

Cytospin. 15 X 10^4 of cultured DC cells, or freshly isolated monocytes, were spun at 700 rpms for 5min at room temperature onto a cytoslide (Shandon, Pittsburg, PA) in a Cytospin 2 centrifuge (Shandon, Pittsburg, PA). Phenotyping was performed visually by light microscopy (Wright-Giemsa stain), or by fluorescent microscopy (Dextran-FITC endocytosis assay) as described below.

Wright-Giemsa stain. Cells were harvested and cytospined at room temperature for 5min at 700 rpm onto a cytoslide (Shandon, Pittsburg, PA) and left to dry. A thin film of cells on a microscope slide was died in three consecutive dies by immersion, then flushed with tap water and left to dry. When dried, slides were mounted with Permount (Fischer Chemicals, Fair Lawn, NJ) and analyzed under light microscopy.

Endocytic assay. Adherent peripheral blood cells were grown in GM-CSF/IL-4 for 3 days and then treated with of without PA (200ng/ml) for 4, 24 and 48 hrs. FITC-Dextran (Sigma, ST. Louis, MO) was added to 0.25 X 10^6 cells to a final concentration of 1 mg/ml and the cells were cultured for 30 min at 37ºC. The control was incubated at 4ºC for 30min in the dark. After incubation, cells were washed four times with ice-cold PBS and analyzed by flow cytometry as described above, and by fluorescence microscopy.

ELISA. IL-10 and IL-12 cytokine secretion by iDCs and mDCs treated with LT, COL-3, COL-308, or with a combination of LT and COL-308 will be measured

using a commercial quantitative ELISA kit (Gibco). Results will be detected using a ThermoMax microplate ELISA plate reader (Molecular Devices).

Mixed Leukocyte reaction essay (MLR). DC will be cultured in GM-CSF/IL-4 as described above for four days. At day 4 cells will be challenged with LT for 45 minutes, as described above, replated, and cultured in the presence of LPS with or without the COL-308 inhibitor, and assayed on day 6 for their ability to stimulate allogeneic T-cells in an MLR. Purified DCs will be co-cultured with $1x10^5$ T cells from an unrelated donor at ratios 1:5, 1:10, 1:30 and 1:100 DC:T cells. Allogeneic T-cells will be obtained from Leukocyte concentrates.

Preparation of cell lysates. DCs cultured as described earlier and challenged with 5-20μM concentration of LT for 45min, will be washed with PBS and incubated for 24-48hrs. Following the incubation, cells will be washed once with PBS at room temperature and lysed by three successive freeze-thaw cycles in PBS.

Western blotting and antibodies. iDC and mDC treated with LT or with a combination of COL and LT will be analyzed by SDS-PAGE using 10% polyacrylamide gels (Novex, Carlsbad, CA), followed by transfer to nitrocellulose membranes in electrophoretic transfer apparatus (Novex) at 30V for 1hr. Membranes will be probed with anti-MEK-2 rabbit polyclonal antibody with specificity for the N terminus (Santa Cruz, Santa Cruz, CA) or with anti-MEK-6 rabbit polyclonal antibody with specificity for the N terminus (Stressgen Biotechnologies Corp., Victoria, BC Canada) using dilutions recommended by the manufacturer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) will serve

as an internal loading control, and will be visualized with a rabbit polyclonal anti-GAPDH antibody (FL-335; Santa Cruz, CA).

DISCUSSION

B. anthracis is highly virulent pathogen which secretes a number of distinct proteolytic virulence factors at different stages of the disease, including those with hemorrhagic, caseinolytic and gelatinolytic activities, which effectively establish and further propagate the infectious process (22). Although the multiplicity of the secreted factors, with the regard to the number and specificity of proteolytic enzymes, suggests a multitude of their potential viral mechanisms, LT is the only anthrax protein which was found toxic in experimental animals (21), and hence has been a focus of our study. Our data implies that one of the major roles of LT might be to provide a more hospitable environment for *B. anthracis*, through its immunosuppressive action towards DCs.

I have established an *in vitro* system in for testing the LT effect on immature, as well as maturing and mature DCs, and for evaluating the inhibitory potential of tetracycline inhibitors towards these immunosuppressive effects. CD83 and CD86 are DC maturation markers and two pivotal molecules in establishment of T- and B-cell immunity. Given the reported role of LT in suppressing host immunity during *B. anthracis* infections (3, 10, 11), the expression of these molecules was analyzed following the challenge of DCs with LT at different maturation stages. Also, the effect of two LF inhibitors on DCs was established, as well as their potential for inhibition of LT-mediated immunosuppressive effects on the DCs.

Given the fact that dendritic cell functions and susceptibility to outside stimuli are related to stages of maturity, with iDC more susceptible to external

stimuli than mDCs, I hypothesized that LT-mediated effects on DC might also be maturation stage dependent. Earlier reports have shown conflicting results suggesting that LT either killed or impaired the functions of DC cells (10, 11). Our data suggests that immature DCs (iDC) are indeed susceptible to LT-mediated apoptotic and necrotic cell death, while maturing and mature DCs (mDC) are not (Figure2), reconciling the conflicting reports. Purified *B. anthracis* LT proved to be highly toxic to immature human DCs even at low doses of 5μM and 10μM, while this toxicity was not observed with mature DCs. Scatter plots of LT-treated DCs show increased apoptotic death in the LT-treated iDC cell population, while this cell death is absent among the LT-treated mDCs (Figure 2a). Further AnnexinV analysis shows a marked increase in apoptotic death among LT-treated iDCs after 24hrs, with an even more pronounced increase after 48hrs (Figure 2b). A similar effect of LT on mDC population was not observed (Figure 2b). This data suggests that *B. anthracis* exerts profound inhibitory effects on adaptive immunity through killing of iDCs, a subset of the DC population responsible for regulating the nature and magnitude of the adaptive immune response, while leaving maturing and mature DCs viable. The mDCs, although viable, have diminished expression of surface molecules essential for proper immune functions following the LT challenge (Figure 5). These mDCs might possibly play an important role in disseminating the disease throughout the body. The mDCs might be used by the bacterium as a "Trojan horse" for getting to the lymph nodes in a manner similar to that employed by *B. anthracis*–infected macrophages (as described in 2). Our data suggests an additional level of

complexity in the progression of the disease, hinting that *B. anthracis* might have developed a mechanism for avoiding the host immune response, taking advantage of the DC maturation pathway in progression of the disease.

COL-3 and COL-308 are two pharmacological agents with proven direct inhibitory activity towards LF, and offer a promising approach to combating the pathogen. Before I tested for their inhibitory potential towards the immunosuppressive effect of LT in our system, I analyzed the possible adverse effects of these two drugs on human DCs. COL-3 demonstrated significant cytotoxicity to iDC even at the low dose of 5μM (Figure 3a). This cytotoxicity is not as pronounced in mDC, which remain mostly viable. Although the cells remain viable, their maturation pathway is adversely affected. The expression of maturation markers CD83 and CD86, as well as the expression of CD40, is reduced by COL-3 in LT treated mDCs (Figure 3.), suggesting a significant level of maturation impairment. COL-308, on the other hand, did not demonstrate any significant cytotoxicity, even at the maximum dose of 20μM (Figure 3). Hence our results indicate that COL-308 may be far more suitable for post-exposure prophylaxis as a supportive agent for already infected individuals, or could even be administered as a potential pre-exposure prophylactic drug.

Kocer et al. have shown that COL-3 as well as COL-308 have the capacity to inhibit *B. anthracis* LF activity in cell free systems and in the MonoMac6 cell line when the inhibitors are employed prior to addition of LF plus PA to the cells in a protocol consistent with the U.S. Food and Drug Administration's definition of "preexposure prophylaxis" (14). I modified this protocol in order to test for the

capacity of COL-308 to inhibit the effects of LF on DCs when administered in "postexposure prophylactic" mode. Since COL-3 proved to be too toxic for LT inhibition in DCs, and given the low toxicity of COL-308 towards human DCs and its lack of interference with their maturation, only the inhibitory potency of COL-308 was evaluated.

Even though COL-308 did not seem to significantly inhibit LT mediated cell death (Figure 4.), I tried to determine if the surviving cells were able to reach full maturity in presence of LPS. Following exposure to LT of DCs which were either pre- of post-treated with 20μM of COL-308, and 48hr incubation I determined the expression of CD83 and CD86 maturation markers, as well as DC markers HLA-DR and CD40. Only viable DC cells were gated in order to exclude the possibility of diminished expression of maturation markers due to cell death or cells entering LT-triggered initial apoptotic stages. The decrease in maturation marker expression in Fig. 5 was not the consequence of cell death or cells entering initial apoptotic stages. The results obtained in presence of COL-308 are not significantly different from those in absence of COL-308. Effects of LPS on DC cell membranes do not seem to play any role in the ability of LT or COLs to enter the cell, when they are added after the addition of LPS. COL-308 was added before, simultaneously or after the addition of LPS, and did not appear to inhibit LT-mediated effects on DCs even at the high concentration of 20μM (Figure 5).

LPS(+) LT-treated DCs also attained a semimature state, suggesting that while the developmental pathway of mDC was not qualitatively affected by LT, the maturation was (Figure 5). Semimature DCs are known to upregulate many T

cell costimulatory molecules on their surface, but fail to produce cytokines that are needed to induce functional T cell immunity (19, 20). Such DCs migrate to lymph nodes but induce tolerance rather than immunity. Since in noninflamatory lymph nodes most DCs exist in an immature state, I speculate that these semimature DCs might stimulate T-cell tolerance, playing an important role in the pathogenesis of *B. anthracis* infection. The consequence of the existence of such alternate maturation stage on T cell activation should be established through an MLR assay as well as by analysis of cytokine secretion following LT challenge. IL-10 and IL-12 secretion levels should be determined by ELISA, since the levels of these cytoknes would indicate if LT treated mDCs move towards an immunogenic or a tolerogenic state, allowing us to gain better understanding of the capacity of *B. anthracis* to evade an effective adaptive immune response.

Finally, I analyzed the effect of a second component of Anthrax toxin, protective antigen (PA). PA is widely believed not to be cytotoxic on its own, with only limited proof that it can induce cell death in macrophages overexpressing the PA cell surface receptors ANTXR1 and ANTXR2 (15). Our data suggests that PA alone has some effect on DCs and potentially on the immune system. When human monocyte-derived DCs were treated with 200 ng/ml PA, an increase in phagocytic activity of DCs was observed, as well as an increase in the size of a subset of vacuoles, compared to control cells (Figure 6a). The cell morphology of PA treated DCs appears to resemble that of a macrophage. This interesting observation might prove to reflect a strategy employed by *B. antxracis* by which the bacterium induces its own uptake into

cells which then transport the microbe to lymph nodes where it can propagate. Interestingly, at a high PA concentration of 500ng/ml, DC cells, although highly vacuolated, do not exhibit DC cell morphology (Figure 6a). At this PA concentration the cells start to resemble pro-monocytes, being smaller in size and smooth, lacking dendritic processes. Although these DCs are vacuolated, their vacuoles are smaller and peripheral. As expected, there was no cytotoxic effect of PA at 100ng/ml or 200ng/ml on iDC (Figure 6b).

iDC were chosen for use in phagocytic uptake studies over mDCs because of their phagocytic capability, and because of their closer similarity to macrophages than mDCs in developmental pathway. .PA treatment of iDCs did not have apparent adverse effects on phagocytosis at 100, 200, or 500ng/ml, as can be seen from a Dextran-FITC assay (Figure 6c). Although this assay did not show any difference between PA-treated and non-treated DCs, it should be noted that Dextran-FITC might have been taken up via pinocytosis instead of phagocytosis. Hence the assay should be repeated using latex beads for visual confirmation of these results.

In conclusion, our results suggest that *B. anthracis* has developed complex mechanisms for avoiding the immune response, while at the same time using DCs as vehicle for dissemination throughout the host. I also determined the potential adverse effects of COLs on human DCs, and determined their potential for use as postexposure prophylactic inhibitors of the actions of LT on DCs. Finally, our results suggest that PA, previously thought to be a nontoxic component of anthrax toxin does exert certain effects on the human DCs at high concentrations. The

nature of these effects should be analyzed more closely, as they may be an important component of the events associated with development of anthrax in an infected host.

REFERENCES

1. Dixon, T.C., M. Meselson, J. Guillemin, and P.C. Hanna. 1999. Anthrax, *N. Engl. J. Med.* 341:815-826
2. Guidi-Rontani, C. 2002. The alveolar macrophage: the Trojan horse of *Bacillus anthracis. Trends Microbiol.* 10:405
3. Cote, C.K., K.M. Rea, S.L. Norris, R.N. Van, and S.L. Welkos. 2004. The use of a model of in vivo macrophage depletion to study the role of macrophages during infection with *Bacillus anthracis* spores. *Microb. Pathog.* 37:169
4. Santiago-Schwarz, F., 2004. Dendritic cells: friend or foe in autoimmunity? *Rheu Dis Clin N Am* 115-134
5. Ascenzi, P. P. Visca, G. Ippolito, A. Spallarosa, M. Bolognesi, and C. Montecucco. 2002. Anthrax toxin: a tripartite lethal combination. *FEBS Lett.* 531:384-388
6. Mock, M., and T. Mignot. 2003. Anthrax toxins and the host: a story of intimacy. *Cell. Microbiol.* 5:15-23
7. Collier, R. J., and J.A. Young. 2003. Anthrax toxin. *Annu. Rev. Cell. Dev. Biol.* 19:45-70
8. Duesbery, N.S., C.P. Webb, S.H. Leppla, V.M.Gordon, K.R. Klimpel, T.D. Copeland, N.G. Ahn, M.K. Oskarson, K. Fukasava, K.D. Paull, and G.F. Vande Woude. 1988. Proteolityc inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science*. 280:734-737

9. Pellizari, R., C. Guidi-Rontani, G. Vitale, M. Mock, and C. Montecucco. 1999. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNγ-induced release of NO and TNFα. *FEBS Lett.* 462:199-204

10. Agrawal, A., Lingappa, J., Leppla, S., Agrawal, S., Jabbar, A. Quinn, C., Pulendran, B. 2003. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature* 424: 329-334

11. Alileche, A., Serfass, E., Muehlbauer, S., Porcelli, S., Brojatsch, J. 2005. Anthrax Lethal Toxin-mediated killing of human and murine dendritic cells impairs the adaptive immune response. *PLoS Pathog.* 1(2): e19 150-158

12. Pulendran, B., J. Banchereau, E. Maraskovsky, and C. Maliszewski. 2001. Modulating the immune response with dendritic cells and their growth factors. *Trends Immunol.* 22:41

13. Bnachereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Ev. Immunol.* 18:767

14. Kocer, S., Walker. S., Zerler, B., Golub, L., Simon, S. 2005. Metalloproteinase inhibitors, nonantimicrobial chemically modified tetracyclines, and Ilomastat bloc *Bacillus anthracis* Lethal Factor activity in viable cells. *Infect Immun* 43:7548-7557

15. Salles, I., Voth, D., Ward, S., Averette, M., Tweten, R. Bradley, K., Ballard, J. 2006. Cytotoxin activity of Bacillus anthracis protective antigen observed in macrophage cell line overexpressing ANTXR1. *Cell Microbiolog.* 8:1272-1275

16. Abrami, L., Lindsay, M., Parton, R., Leppla, S. Gisou van der Goot, F. 2004. Membrane insertion of anthrax protective antigen and cytoplasmic delivery of lethal factor occur at different stages of the endocytic pathway. *Journal Cell Bio* 166:645-651
17. Pulendran, B., J. Banchereau, E. Maraskovsky, and C. Maliszewski. 2001. Modulating the immune response with dendritic cells and their growth factors. *Trends Immune.* 22:41
18. Krathwohl, M., D., Schacker T., W., Anderson, J. 2006. Abnormal presence of semimature dendritic cells that induce regulatory T cells in HIV-infected subjects. *JID* 2006:193
19. Lutz M, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immune* 2002, 23:445-9
20. Rea D, van Kooten C, van Meijgaarden KE, Ottenhoff THM, Melief CJM, Offringa R. Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen presenting cells that secrete IL-10. *Blood* 2000, 95:3162-7
21. Levy, G.A., and T.S. Edgington. 1980. Lymphocyte cooperation is required for amplification of macrophage procoagulant activity. *J. Exp. Med.* 151:1232-1244
22. Agrawal A, Pulendran B: Anthrax lethal toxin: a weapon of multisystem destruction. *Cell Mol Lfe Sci* 2004, 61:2859:2865

23. Popov S. G., Popova T, Hopkins S, Weinstein R, MacAfee R, Fryxell K, Chandhoke V, Bailey C, Alibek K; Effective antiprotease-antibiotic treatment of experimental anthrax. *BMC Infectous Diseases* 2005, 5:25

FIGURES

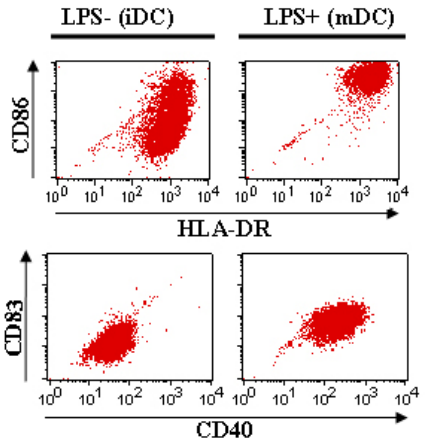


Figure 1. FACS Profile of Immature and Mature Human DCs. Human DCs were derived from human peripheral blood monocytes. Expression of DC marker HLA-DR, as well as of DC maturation markers CD40, CD83, and CD86 was assessed by flow cytometry. Maturing LPS-treated DCs (mDCs) consistently expressed more maturation markers CD83 and CD86, than the immature DC (iDC). Data is representative of four experiments.

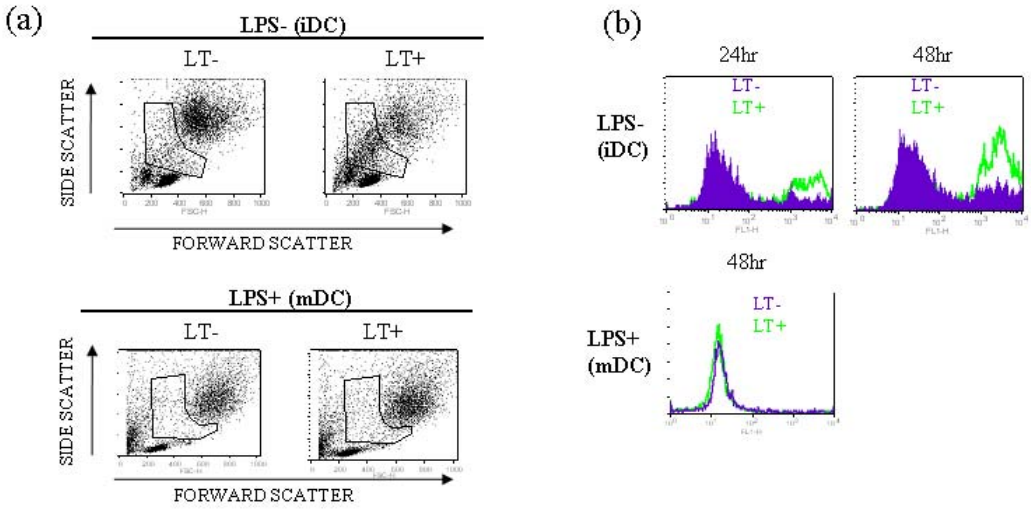


Figure 2. Susceptibility of DC to LT challenge relative to their maturation stage
(a) Scatter plot showing the increased susceptibility of LPS(-) iDC towards LT-mediated cell death relative to LT-challenged LPS(+) mDC. The gated region (apoptotic/necrotic region) of LT-challenged iDCs contains several fold greater number of dead DCs than gated region of mDC samples.
(b) Annexin histogram of iDC and mDC 24hrs and 48hrs post-45min LT-challenge confirms the scatter plot results. iDC express an increased amount of AnnexinV 24hrs and 48hrs post-LT exposure, while LT-challenged mDC have baseline expression of AnnexinV.

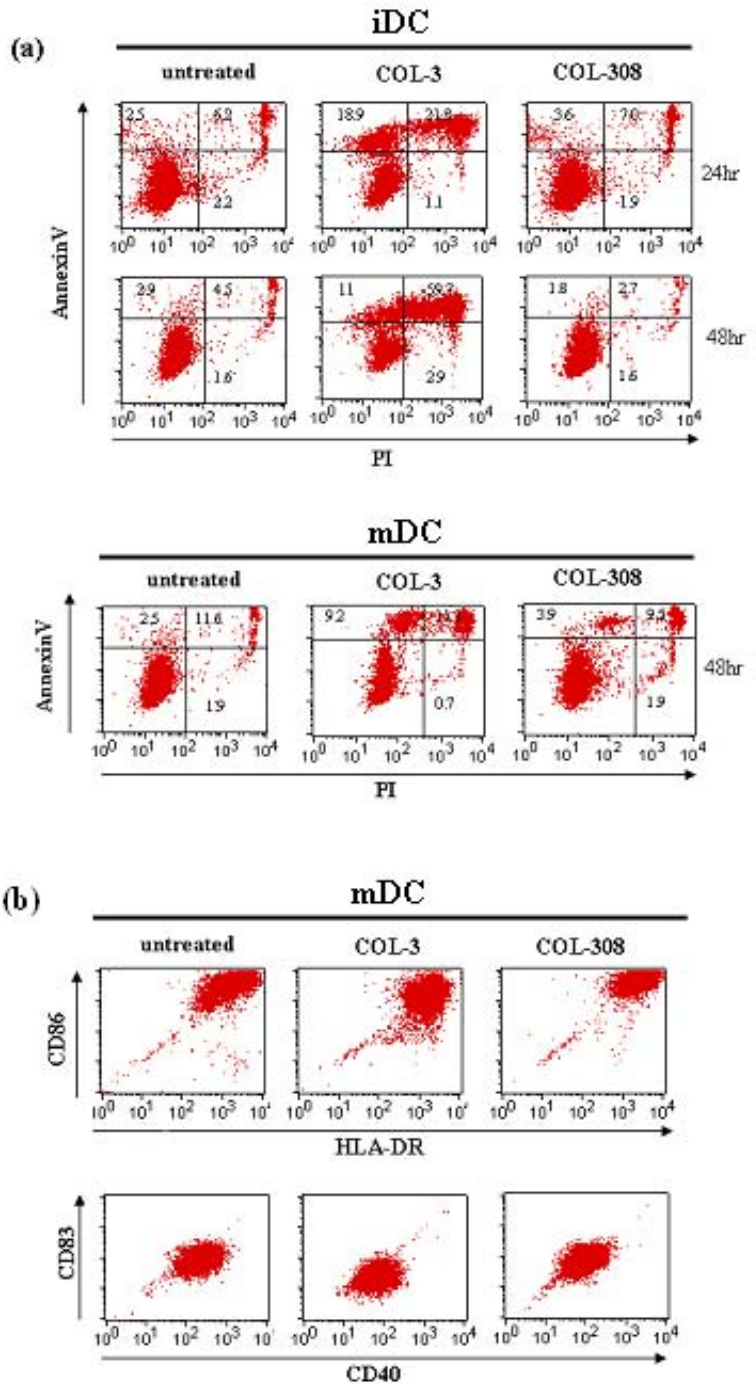
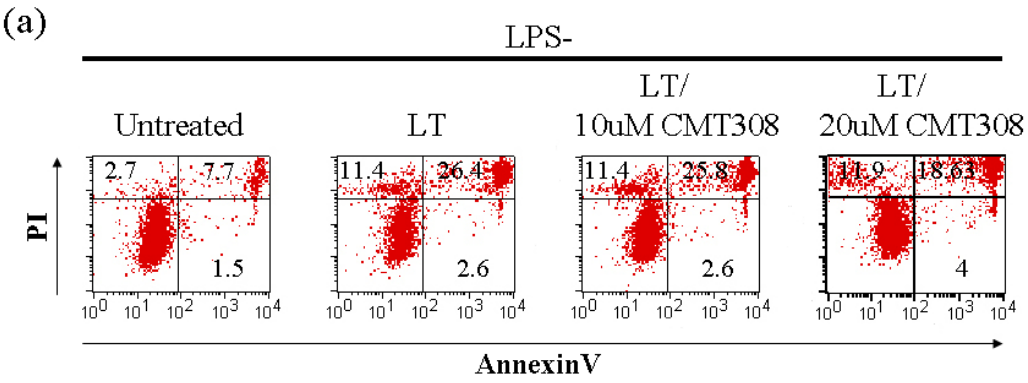


Figure 3.

(a) AnnexinV/ PI quadrant analysis of the effects of COLs on the viability of iDCs and mDCs at time points following the LT challenge. The percentages of total cells are annotated in upper left (early apoptosis), upper right (intermediate/late apoptosis), and lower left (late apoptosis/necrosis) quadrants. COL-3 exerts progressive cytotoxic effect on iDC as early as 24hrs. The cytotoxic effect was not observed with COL-308. mDC seem to be resistant to the cytotoxic effects of COL-3, even after 48hr incubation.

(b) Surface analysis of fluorescence intensities of maturation markers on mDC 48hrs post- treatment with 10 μM COL-3 or 10 μM COL-308. Although mDC are resistant to the cytotoxic effect of COL-3 (Figure3a), COL-3 does adversely affect the maturation of LPS-treated cells. COL-308 does not have apparent effects on the expression of maturation markers either.



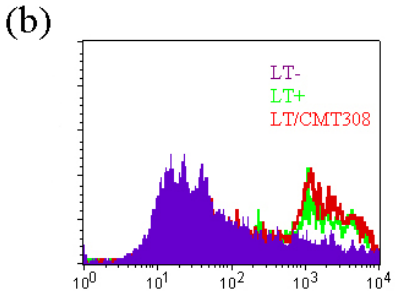


Figure 4. COL-308 rescue potential towards LT-treated iDCs
(a) Annexin V/ PI quadrant analysis of LT-challenged iDC and their potential rescue by COL-308. No significant difference was observed in the amount of apoptosis between LT and LT/COL-308 treated cells, compared to the control. COL-308 was unable to rescue LT-challenged iDCs at a 10μM as well as at a 20μM concentration.Percentages of positive cells are annotated in each quadrant.
(b) Histogram of AnnexinV stained LT treated, LT/CMT treated cells confirms the above data. There was no significant decrease in cell death between LT treated and LT/COL-308 treated cells compared to the control.

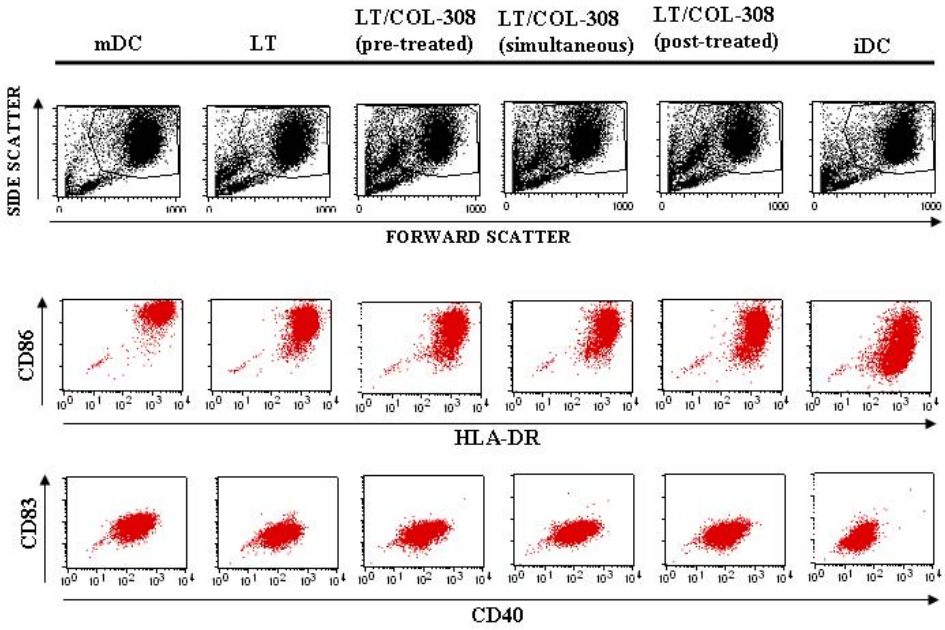


Figure 5. Surface protein expression rescue of LT-challenged mDCs
DC cells were either pre-treated or post-treated with COL-308 in respect to LT-challenge, or treated simultaneously with LT. DC maturation was assessed through the expression of CD83 and CD86 maturation markers, and compared to non-treated iDC and mDC. The addition of maturation factor LPS did not interfere with the cells ability to uptake LT of COL-308 effectively (not shown).

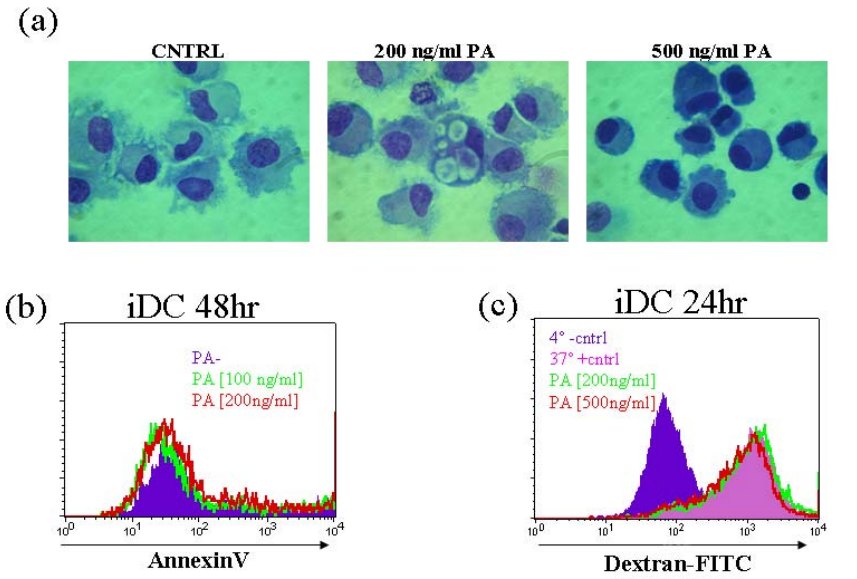


Figure 6. The effects of PA on iDC
(**a**) Wright-Giemsa stain of PA treated iDCs shows that PA affects the morphology of iDCs in a dose-dependent manner. At 200ng/ml PA concentration the increased formation of phagocytic vacuoles was observed in relation to control sample. At 500ng.ml PA concentrations iDC cells resemble pro-monocytes with small peripheral phagocytic vacuoles.
(**b**) AnnexinV Histogram of iDCs following a 48hr incubation with different PA concentrations shown no discernable difference in cell viability between PA-treated and non-treated cells.
(**c**) Endocytic/phagocytic Dextran assay of iDC incubated in presence or absence of PA suggests that PA might have limited, if any effect on the phagocytic or pinocytic ability of iDC following a 24hr incubation with PA.