

# The Effects of *Treponema pallidum* on Human Dendritic Cells

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Cell mediated immune responses play a prominent role in syphilis, which is caused by *Treponema pallidum*. The role of dendritic cells (DC) in the syphilitic infection is not well understood in human. In the present study, we studied interaction of *T. pallidum* with DC, generated from human peripheral blood mononuclear cells with GM-CSF and IL-4. After adding *T. pallidum* for 16 hours to immature DC at culture day 7, the change of surface antigens on DC was monitored by flow cytometry, the amount of IL-12 in culture supernatant of DC was measured by ELISA and T cell stimulatory capacity of DC was checked in mixed lymphocyte reaction (MLR). We have observed an efficient phagocytosis of *T. pallidum* by electron microscopy as early as 2 hours after addition of *T. pallidum* to DC. Interaction of DC with *T. pallidum* resulted in increased surface expression of CD83 which was proportionally increased according to the number of *T. pallidum*. Expressions of CD80, CD86 and HLA-DR on DC were slightly increased. The amount of IL-12 in the culture supernatant of DC was increased (1,099 pg/ml) after the addition of *T. pallidum*. *T. pallidum*-infected DC also displayed enhanced T cell stimulatory capacity in MLR. As seen from the above, we observed phagocytosis of *T. pallidum* by DC as early as 2 hours after addition of *T. pallidum* to DC and found that *T. pallidum* can stimulate DC maturation which mean that DC modulate an protective immune response during *T. pallidum* infection.

**Key words:** *Treponema pallidum*, monocyte-derived dendritic cells

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## INTRODUCTION

Syphilis, a sexually transmitted disease of humans caused by *Treponema pallidum*, remains a global public health problem.<sup>1</sup> Cell mediated immune responses play a prominent role in the syphilis. *T. pallidum* enters human body through skin or mucous membrane, where dendritic cells (DC) may be important antigen presenting cells to present treponemal antigens to the lymphocytes. There was a report that *T. pallidum* was engulfed by the XS52 mouse DC line and *T. pallidum* activated the mouse DC line,<sup>2</sup> however, the role of DC in the syphilitic infection in human is not well understood.

DC play a critical role in antigen presentation and are involved in the induction of primary T-cell responses.<sup>3-5</sup> DC exist in two functional stages. Immature DC are developed from hematopoietic precursors and are scattered throughout the body in nonlymphoid organs, where they exert sentinel functions. During foreign antigen insult, immature DC in the peripheral tissues capture antigens and subsequently migrate into lymphoid organs, a process which is paralleled by maturation.<sup>6,7</sup> DC maturation is crucial for initiating immunity. It can be influenced by a variety of factors, notably microbial and inflammatory products. Whole bacteria,<sup>8</sup> the microbial cell-wall component LPS,<sup>9</sup> and cytokine like IL-1, GM-CSF and TNF- $\alpha$ , all stimulate DC maturation, whereas IL-10 blocks it.<sup>10</sup> Monocytes derived DC (MoDC) cultured in the presence of GM-CSF and IL-4,<sup>11,12</sup> have been used *in vitro* study of DC maturation. Such cells are relatively immature. Upon maturation with TNF- $\alpha$ , CD40 ligand, or LPS, DC

down-regulate mechanisms of antigen capture, while increasing expression of costimulatory and adhesion molecules. Similar changes indicative of maturation have also been reported following infections.<sup>13,14</sup>

In the present study, we observed interaction of *T. pallidum* with human MoDC by electron microscopy and investigated the impact of *T. pallidum* on DC maturation and whether there is a maturation of human MoDC after adding *T. pallidum* to immature MoDC on three aspects. First by monitoring the change of surface antigens on DC with flow cytometry. Second by the amount of IL-12 in culture supernatant of DC with ELISA, and third by the T cell stimulatory activity in mixed lymphocyte reaction (MLR).

## MATERIALS AND METHODS

### Media and cytokines

RPMI 1640 was supplemented with 2 uM L-glutamine (Gibco Laboratories, Grand Island, NY, USA), 100 IU/ml penicillin (Gibco), 100 ug/ml streptomycin (Gibco),  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol (Merk, Munchen, Germany), and 1% heat-inactivated autologous plasma. The following human recombinant cytokines and specific concentration quoted were used for all experiments: 800 U/ml of granulocyte-macrophage colony stimulating factor (GM-CSF) (Novartis, Frimley, UK), 1000 U/ml IL-4 (PBH, Hanover, Germany), 10 ng/ml IL-1  $\beta$  (PBH), 1000 U/ml IL-6 (PBH), 20 ng/ml TNF- $\alpha$  (PBH), 1  $\mu$ g/ml PGE2 (Sigma Chemical Co., St. Louis, MO, USA).

### DC culture

DC were generated from the whole blood of healthy volunteers after informed consent was given, as previously described.<sup>15</sup> Briefly, mononuclear cells separated on Ficoll-Hypaque (density 1.0777 g/ml) were allowed to adhere in culture medium in 6-well plates. After 30-45 min at 37°C, non-adherent cells were removed and adherent cells were cultured in 3 ml of medium containing GM-CSF and IL-4. Cells were fed every other day with 1 ml replacement of fresh medium containing

GM-CSF and IL-4. On day 7 (D7), non-adherent and loosely adherent DC were harvested, washed once and transferred to a fresh 12-well plate ( $5 \times 10^5$  cell/well) in medium containing a cytokine cocktail (GM-CSF, IL-1  $\beta$ , IL-4, IL-6, TNF- $\alpha$  and PGE2)<sup>16</sup> or treponemes ( $5 \times 10^7$  cell/well). Subsequently, they were further cultured in the same media for 16h.

### Spirochetes

*T. pallidum*, Nichols strain (CDC, Atlanta, GA) was maintained and passaged by intratesticular inoculation of adult male New Zealand white rabbits weighing 2.5-3 kg as described earlier.<sup>17</sup> One milliliter of *T. pallidum* with a concentration of  $2-3 \times 10^7$  treponemes/ml was inoculated into rabbit testicle and after 7-10 days, treponemes were extracted from rabbit testes and harvested from testicular tissue debris by differential centrifugation.<sup>18</sup> Treponemes were then collected by high-speed centrifugation, and the treponemal cell pellet was suspended in RPMI1640 medium.

### Electron microscopy

Immature DC ( $5 \times 10^5$  cell/well) at day 7 were incubated in 2.0 ml of medium with a freshly isolated *T. pallidum* ( $5 \times 10^7$  cell/well) for 2h, 4h, 16h and 24h in 15-ml polypropylene tube (Falcon). Cells were collected by centrifugation at  $300 \times g$  for 5 min, washed twice with cold PBS, and fixed for 1 h in a solution of 2% glutaraldehyde, 3 mM picric acid, and complete NaPi buffer. Collected cells were postfixed in 1% osmium tetroxide at pH 7.4, then gradually dehydrated in acetone and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate, and examined under a Philips CM10 electron microscope (Philips, Eindhoven, Netherlands).

### Flow cytometry

As previously described<sup>14</sup> uninfected and infected MoDC were analyzed for cell surface expression using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). The following mAbs were used for flow cytometric analysis: CD1a (BD), CD80 (Pharmingen,

San Diego, CA, USA), CD86 (Pharmingen), HLA-DR (BD), CD83 (Serotec, Oxford, England). The isotype controls Abs (Pharmingen) were used in all experiments. As a secondary antibody, FITC-conjugated affinity pure F(ab')<sub>2</sub> goat anti mouse IgG (Pharmingen) was used.

### ELISA assay

The culture supernatants of infected MoDC were harvested 16h after culture with *T. pallidum*. The production of total human IL-12 protein (p40 and p70) was measured by ELISA (Endogen, Woburn, USA), using 96well microtiter plated, according to the manufacturer's instructions

### Allogeneic MLR

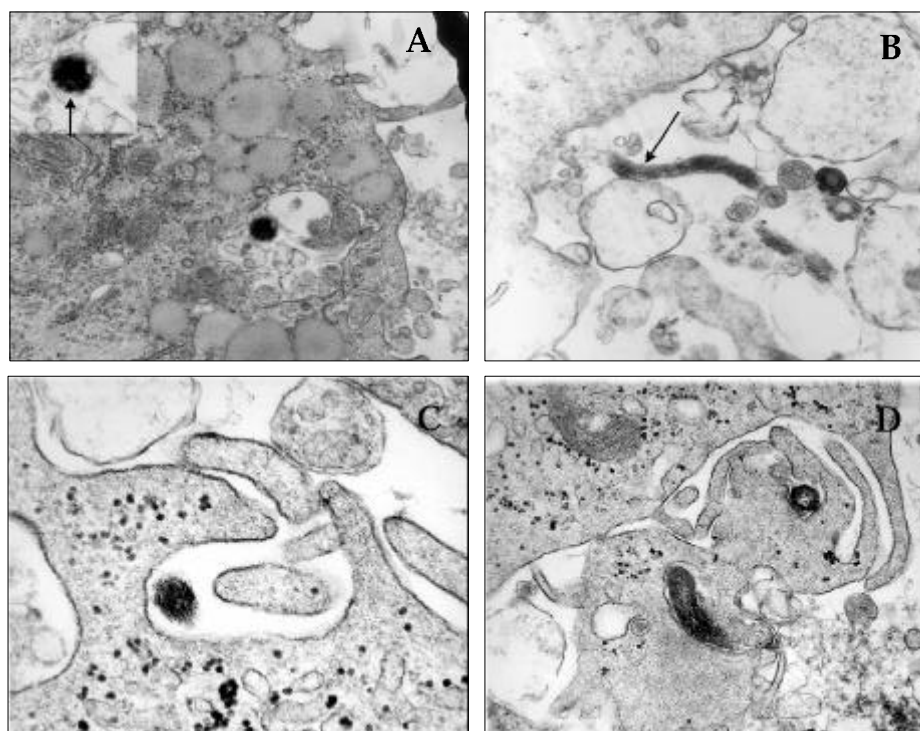
T cells ( $1 \times 10^5$ /well), enriched using nylon wool columns, were stimulated with control DC or *T. pallidum*-infected DC. The cells were cultured in a round-bottom microtiter plate to a final volume of 200  $\mu$ l/well in RPMI with 10%

fetal calf serum for 4 days and an additional 16 hr in the presence of [<sup>3</sup>H] thymidine (1  $\mu$ Ci/well=37 kBq/well, Amersham, braunschweig, Germany). Control T cells were incubated in medium alone. [<sup>3</sup>H] thymidine incorporation was measured using a liquid scintillation counter (Beckman, Palo Alto, CA, USA).

## RESULTS

### Electron microscopic findings

*T. pallidum* was incubated with MoDC for 2h, 4h, 16h and 24h and interaction of spirochetes with DC were assessed by using transmission electron microscopy. Many oblique and cross sectioned trponemes were found easily inside or outside of the DC. An efficient phagocytosis of *T. pallidum* was observed as early as 2 hours after addition of *T. pallidum* to DC. Some trponemes showed axial filaments which are characteristic finding of *T. pallidum* (Fig. 1A and B). Among



**Fig. 1.** Phagocytosis of *T. pallidum* by MoDC was observed by electron microscopy as early as 2 hours after addition of *T. pallidum* to DC. Cross (A)- and oblique (B)-sections of treponemes are noted in the cytoplasm of MoDC. Axial filaments (arrow) are noted in some sections of the treponemes. Two or three alternating pseudopodes (C), which appeared to capture the treponemes were observed, then additional overlapping pseudopodes appeared to fuse with the cell surface (D).

phagocytosis, conventional phagocytosis, in which two microbe-apposed pseudopodes of the cells surrounded the organism, was observed. Two or three alternating pseudopodes (Fig. 1C), which appeared to capture the treponemes were observed, then additional overlapping pseudopodes appeared to fuse with the cell surface (Fig. 1D). Some internalized treponemes appeared to be within membrane-bound vacuoles.

#### Increased surface expression of MoDC after interaction with *T. pallidum*

During culture of DC, CD14 was lost from the 3rd to 4th day of the culture, and CD86 and MHC-II continued to increase until the 7th day. After adding a cytokine cocktail (GM-CSF, IL-1 $\beta$ , IL-4, IL-6, TNF- $\alpha$ , and PGE2) on the 7th day of culture, CD83 was found to have increased on the 8-9th day together with CD80, CD86 and MHC-II, which indicated the presence of mature DC. The resulting cell preparation contained more than 90% DC, as assessed by morphology and flow cytometry.

Interaction of DC with live *T. pallidum* resulted in increased surface expression of CD83, however, addition of normal rabbit serum, normal rabbit testicular tissue, and media, which served as negative controls, did not induce any change of CD83 on DC (Fig. 2). The expressions of CD80, CD86 and HLA-DR on DC were slightly increased. Expression of CD83, B7-1, B7-2 and HLA-DR was proportionally increased according to the number of *T. pallidum* (Fig. 3).

#### Increased IL-12 secretion of MoDC after interaction with *T. pallidum*

The amount of IL-12 in the culture supernatant of DC was  $1099 \pm 32.97$  pg/ml after the addition of *T. pallidum*, which was similar ( $1191 \pm 41.69$  pg/ml) in the supernatant of mature DC, which was conventionally prepared by adding cytokine cocktail to immature DC at day 7 (Fig. 4). The culture supernatants of infected MoDC were harvested 16h after culture with *T. pallidum*. However, there was no secretion of IL-12 from DC after adding normal rabbit testicular tissue and serum. The secretion of IL-12 was proportionally

increased according to the number of *T. pallidum* like expression of surface molecules of MoDC (Fig. 5).

#### Change of MLR after interaction with treponemes

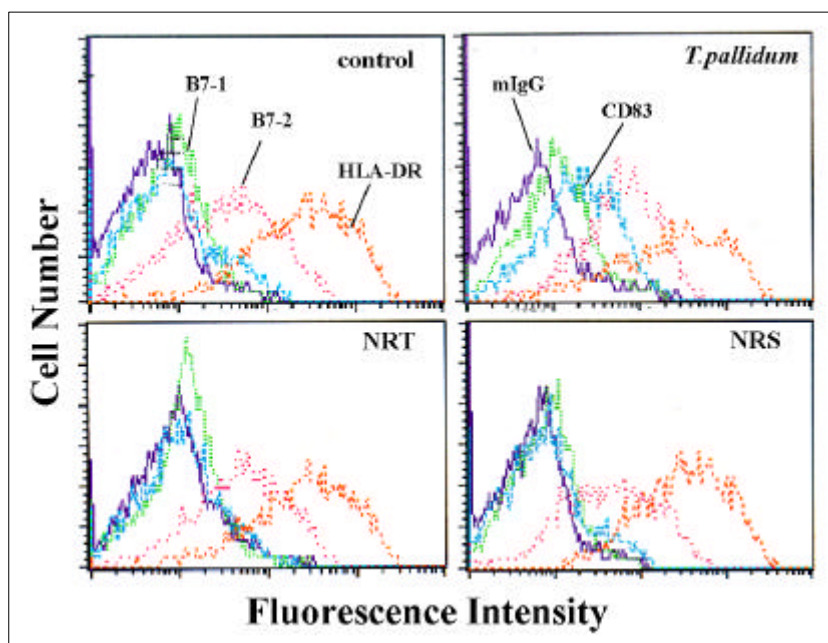
T cells from normal controls, who were not infected with treponemes, were stimulated with MoDC that have been cultured with *T. pallidum* for 16 hours. Cells were co-cultured for 4 days. *T. pallidum*-infected MoDC have enhanced T cell stimulatory activity compared to the MoDC that are not infected in a MLR, especially when the ratio of DC:T cell were 1:4, 1:8 and 1:16 (Fig. 6). The stimulation index (SI) was calculated as follows.

$$SI = \frac{\text{MLR at each DC/TC ratio}}{\text{MLR without stimulator cell}}$$

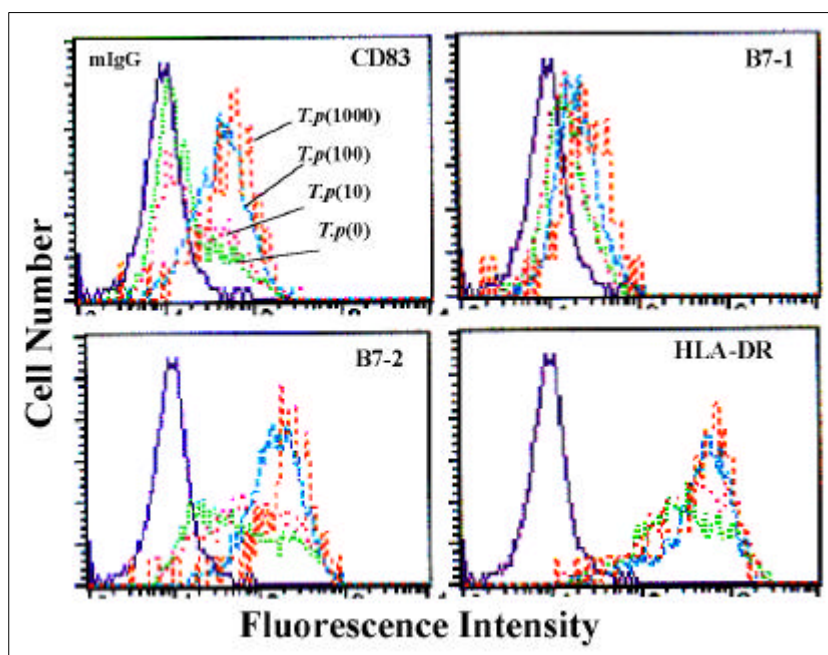
## DISCUSSION

Syphilis is a chronic, systemic sexually-transmitted disease caused by a spirochetal bacterium *T. pallidum*, with clinical features including various cutaneous lesions and systemic involvement. Syphilis has sparked a new interest as an associated infection with AIDS. There are convincing evidences that cellular immunity is the major immune mechanism in syphilitic infection. DC, which are the most important antigen presenting cells, could initiate cell mediated immune responses of syphilitic infection.

When infection occurs, the detection and capture of microorganisms by DC trigger stimulus-specific maturation programs that enable DC to convey pathogen-associated signals to the adaptive branch of the immune system. The appropriate activation of DC is critical for their ability to direct the development of either a Th1 or Th2 response, thereby determining the outcome of microbial infections.<sup>19</sup> There were reports of the effects of several infections on the DC. After infection with *Mycobacterium tuberculosis*,<sup>8,20</sup> expression of CD54, CD40, B7-1 on DC was increased and secretion of TNF- $\alpha$ , IL-1 and IL-12 was also increased. *Listeria monocytogene* was phagocytosed by DC and induced maturation of



**Fig. 2.** Interaction of MoDC with live *T. pallidum* resulted in markedly increased surface expression of CD83. However, the expression of CD80, CD86 and HLA-DR on DC were slightly increased after interaction of MoDC with live *T. pallidum*. Addition of normal rabbit testicular tissue (NRT), normal rabbit serum (NRS) and media which served as negative controls, did not induce any change in CD83, B7-1, B7-2 or HLA-DR expression. Results are representatives of 5 independent experiments.

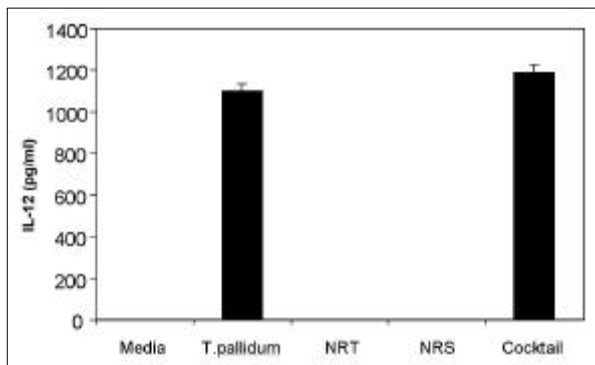


**Fig. 3.** Surface expressions of CD83, B7-1, B7-2, and HLA-DR on MoDC were proportionally increased according to the number of *T. pallidum*. TP (0), TP (10), TP (100) and TP (1000) indicate that the ratio of DC: TP were 1:0, 1:10, 1:100 and 1:1000, respectively. Results are representatives of 3 independent experiments.

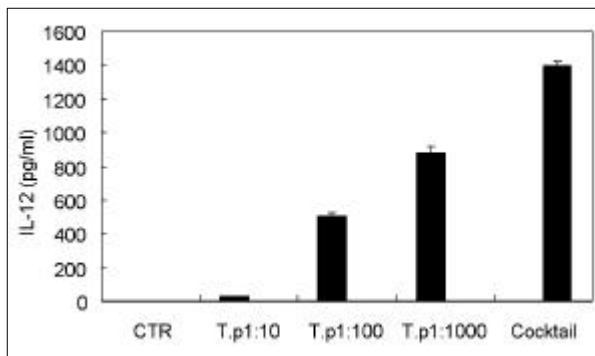
DC which showed increased expression of CD83, CD25, MHC class II and CD86.<sup>21,22</sup> Some other bacterial infections, such as *Escherichia coli*<sup>23</sup> and *Salmonella typhimurium*,<sup>24</sup> also induced maturation of DC not only in the expression of surface molecules but also in cytokine secretion.

*Borrelia burgdorferi*, which is a causative agent of

Lyme disease, is one of the spirochetes like *T. pallidum*. The *B. burgdorferi* was observed to be phagocytosed by DC and induced secretion of IL-12.<sup>25,26</sup> Therefore DC could also be involved in the immune response of syphilitic infection. However, there are only a few reports on the role of DC in syphilitic infection.<sup>2,27,28</sup> *T. pallidum*



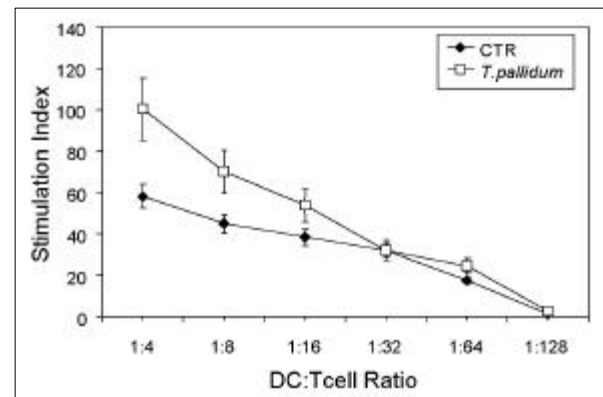
**Fig. 4.** The amount of IL-12 in the culture supernatant of DC was  $1,099 \pm 2.97$  pg/ml after the addition of *T. pallidum* which was similar to the amount ( $1,191 \pm 41.69$  pg/ml) in the supernatant of mature DC at culture day 7 that have been incubated for 2 days with cytokine cocktail (GM-CSF, IL-4, IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and PGE2). However, there was no secretion of IL-12 from DC after adding normal rabbit testicular tissue (NRT) and normal rabbit serum (NRS). The data shown in this figure are examples of three independent experiments.



**Fig. 5.** *T. pallidum* induced IL-12 secretion in the supernatant of DC that was proportionally increased to the number of *T. pallidum* (1:10;  $28.18 \pm 1.71$  pg/ml, 1:100;  $503.00 \pm 21.42$  pg/ml, 1:1000;  $875.43 \pm 48.84$  pg/ml) similar to the amount ( $1,394 \pm 11.43$  pg/ml) of mature DC cultured with cytokine cocktail (GM-CSF, IL-4, IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and PGE2). The data shown in this figure are examples of three independent experiments. TP (1:10), TP (1:100) and TP (1:1000) indicate that the ratio of DC:TP were 1:10, 1:100 and 1:1000, respectively. CTR; control.

induced expression of surface molecules and secretion of IL-12 on XS52, which is the mouse DC cell line<sup>2</sup> but direct interaction between *T. pallidum* and human DC is not yet defined.

Initially, we observed interaction of *T. pallidum* with human MoDC by electron microscopy. *T. pallidum* was phagocytosed by MoDC as early as 2 hours after the co-culture of *T. pallidum* with DC.



**Fig. 6.** T cells were stimulated with MoDC that have been cultured with *T. pallidum* for 16 hours. Cells were co-cultured for 4 days. *T. pallidum*-infected MoDC have enhanced T cell stimulatory activity compared to the MoDC that are not infected in a MLR, especially when the ratio of DC:T cell were 1:4 ( $100.25 \pm 15.04$  SI), 1:8 ( $70.15 \pm 10.52$  SI) and 1:16 ( $53.75 \pm 8.06$  SI). The data shown in this figure are examples of three independent experiments.

Many oblique and cross sectioned treponemes were shown which could be identified by their specific morphology including axial filaments. Several types of phagocytosis could be seen. Among them, conventional phagocytosis and two or three alternating pseudopodes were usually observed. Internalized treponemes within membrane-bound vacuoles were also found.<sup>2</sup>

Next, we investigated the impact of *T. pallidum* on DC maturation by monitoring the change of surface antigens on DC with flow cytometry. Live *T. pallidum* resulted in increased surface expression of CD83, which is considered the hallmark molecule of fully matured DC,<sup>29</sup> and mild increase in the expressions of CD80, CD86 and HLA-DR on DC. Expressions of CD83, B7-1, B7-2 and HLA-DR on DC were proportionally increased according to the number of *T. pallidum*. Normal rabbit serum and normal rabbit testicular tissue were used as negative controls because *T. pallidum* could be cultured only in the rabbit testicles. Not only treponemal infection but also other infections such as infection with *M. tuberculosis*,<sup>8,20</sup> *L. monocytogenes*,<sup>22</sup> *E. coli*,<sup>23</sup> and *S. typhimurium*,<sup>24</sup> also caused upregulation of costimulatory molecules.

Then we investigated the impact of *T. pallidum* on DC maturation by monitoring the amount of IL-12 in culture supernatant of DC with ELISA,

and T cell stimulatory activity in MLR. Significant amount of IL-12 was released by DC upon culture with *T. pallidum* as much as in the supernatant of mature DC cultured with cytokine cocktail. Also, *T. pallidum*-infected MoDC enhanced T cell stimulatory activity.

*T. pallidum* consists of an outer membrane surrounding a periplasmic space, a peptidoglycan-cytoplasmic membrane complex, and a protoplasmic cylinder.<sup>30</sup> These structures of *T. pallidum* have become the foci of pathogenesis and vaccine related studies. *T. pallidum* possesses abundant integral membrane lipoproteins (the 47, 34, 17, 15 kDa lipoproteins)<sup>31</sup> and they could be an efficient activator of DC maturation.<sup>2</sup> However, this does not preclude the possibility that other spirochetal components, such as peptidoglycan<sup>32</sup> and glycolipids,<sup>33</sup> may contribute to the maturation of DC. The other possibility of *T. pallidum*-induced DC maturation is the indirect effect of lipoproteins in treponemal membrane. The lipoproteins of *T. pallidum* could stimulate DC to secrete cytokines, e.g. IL-12 and TNF- $\alpha$ , that could induce DC maturation.

Usually bacterias<sup>8,20-24</sup> cause upregulation of costimulatory molecules, induce T-cell proliferative responses and secretion of cytokines of DC, which means that DC initiate and modulate a protective immune response during bacterial infection. However, the roles of DC in the viral infections may be different from those in the bacterial infections. In viral infections such as HIV,<sup>34</sup> measles,<sup>35</sup> lymphocytic choriomeningitis virus<sup>36</sup> and cytomegalovirus,<sup>37</sup> the function of DC is impaired which is a common selective mechanism by which viruses are able to evade the host's immune system. Also the function of DC differs among the bacterial infections. *M. leprae* infection caused down-regulation of the expression of HLA-A, B, C and -DR on DC. Expression of CD86 on DCs was up-regulated, but not as fully as by *M. bovis* BCG infection and the induction of CD83 expression required a large number of *M. leprae* cells. Therefore, *M. leprae* is a unique pathogen which remains resistant to DC-mediated T-cell immunity.<sup>38</sup>

As seen from the above, we observed phagocytosis of *T. pallidum* by DC as early as 2 hours after addition of *T. pallidum* and found that *T.*

*pallidum* can stimulate DC maturation which means that DC initiate and modulate an protective immune response during *T. pallidum* infection in human.

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