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Cell wall-associated alpha-glucan is instrumental for *Mycobacterium tuberculosis* to block CD1 molecule expression and disable the function of dendritic cell derived from infected monocyte

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Summary

We previously described an escape mechanism exploited by *Mycobacterium tuberculosis* (Mtb) to prevent the generation of fully competent dendritic cells (DC). We have now tested the effect of isolated mycobacterial components on human monocyte differentiation into DC and demonstrated that cell wall (CW)-associated alpha-glucan induces monocytes to differentiate into DC (Glu-MoDC) with the same altered phenotype and functional behaviour of DC derived from Mtb-infected monocytes (Mt-MoDC). In fact, Glu-MoDC lack CD1 molecule expression, fail to upregulate CD80 and produce IL-10 but not IL-12. We also showed that Glu-MoDC are not able to prime effector T cells or present lipid antigens to CD1-restricted T-cell clones. Thus, we propose a mechanism of Mtb–monocyte interaction mediated by CW-associated alpha-glucan, which allows the bacterium to evade both innate and acquired immune responses.

Introduction

An estimated eight million people are infected each year with the pathogen *Mycobacterium tuberculosis* (Mtb), and more than two million die annually (Onyebujoh and Rook, 2004; Leimane *et al*., 2005; Rook *et al*., 2005). Yet only about 10% of those infected develop tuberculosis (TB). As a consequence of the first infection, the majority of otherwise healthy patients experience a mild, self-limiting disease (primary TB) that clinically heals (Onyebujoh and Rook, 2004; Rook *et al*., 2005). In particular, high amounts of antibodies are produced, T cells specific for Mtb antigens are primed and the majority of Mtb cells are killed by activated macrophages. However, Mtb may persist in some compartments, where it is forced to shift into a non-replicating latency status (Meyer, 2003; Tufariello *et al*., 2003; Pai *et al*., 2005). If reactivation TB may be explained in patients with acquired immuno-deficiencies (Ravn *et al*., 2004; Shen *et al*., 2004), it is much more complex to identify which perturbation of the immune system allows the Mtb shift from a non-replicating to a metabolically active status in otherwise healthy patients.

Several hypotheses have been suggested on the capacity of Mtb to evade host immune responses during latency and reactivation (Flynn and Chan, 2003; Trajkovic *et al*., 2004). A variety of mechanisms have been proposed to explain the survival of Mtb within the macrophage, such as the inhibition of phagosome–lysosome fusion, of phagosome acidification and the resistance to killing by oxygenated metabolites (Kaufmann, 2001; Flynn and Chan, 2003). Thus, by interfering with its intracellular degradation, Mtb would substantially block the processing of its antigens, the loading of immunodominant peptides onto MHC class II molecules, and/or the transport of MHC-peptide complexes to the cell surface (Flynn and Chan, 2001). In this line, we have proposed Mtb interference with monocyte differentiation into fully competent dendritic cell (DC) as an additional mechanism of immune evasion (Mariotti *et al*., 2002; 2004; Gagliardi *et al*., 2004). In details, subverted DC derived from Mtb- or Bacillus Calmette Guérin (BCG)-infected monocytes (Mt-MoDC and BCG-MoDC respectively) were characterized by a failure in the expression of CD1 molecules, a reduced upregulation of HLA class II DR and CD80 molecules, and by an impaired capacity to prime IFN-γ producing T lymphocytes. The lack of CD1 molecules is of particular relevance, because Mtb is the microorganisms with the highest content of antigenic lipids, which are presented by these antigen-presenting molecules to

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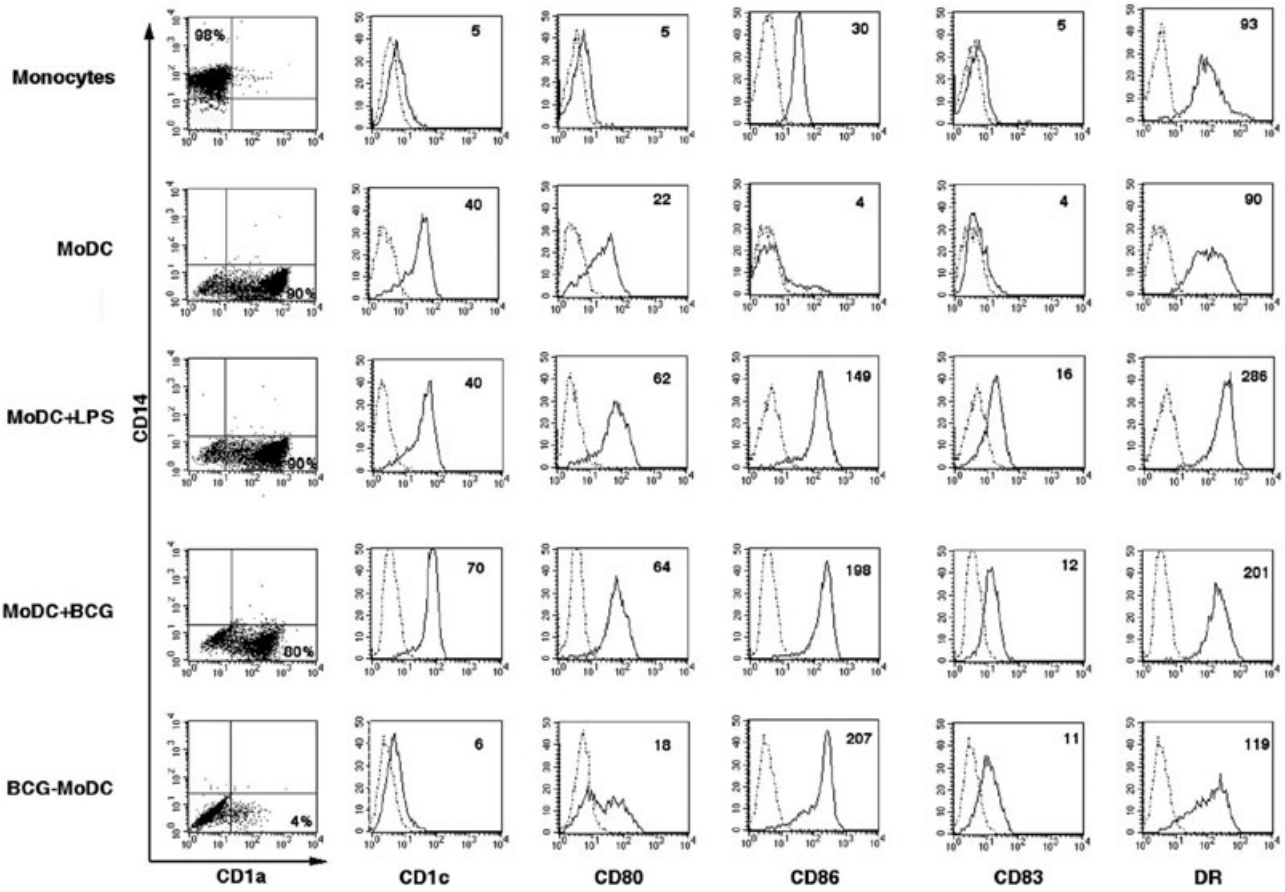

Fig. 1. BCG infection causes DC maturation and subversion of monocyte differentiation into DC. Surface expression of the indicated molecules was analysed by flow cytometry on freshly isolated monocytes and on DC (MoDC) derived from monocytes cultured in GM-CSF and IL-4 for 6 days. MoDC+LPS indicates DC matured by treatment with LPS at day 5 and analysed at day 6. DC+BCG indicates DC infected at day 5 with BCG and analysed at day 6. BCG-MoDC indicates DC derived from monocytes infected with BCG cultured with GM-CSF and IL-4 and analysed at day 6. Dotted histograms represent data generated with the appropriate isotype control. Numbers indicate the percentage of positive cells in the quadrant of dot plots or the median intensity of fluorescence in the histograms. Data are from one experiment representative of seven independent experiments.

CD1-restricted lipid-specific T lymphocytes. It has been demonstrated that CD1-restricted T cells are expanded in humans as a result of a prior Mtb infection, contributing to the initiation of a cell-mediated immune response against the pathogen. In contrast CD1-restricted T-cell responses are absent or drastically reduced in patients with active pulmonary tuberculosis suggesting an effective role during Mtb infection (Ulrichs *et al*., 2003).

Data obtained with heat-killed mycobacteria suggested that a structural component of the bacterium could be responsible for the observed DC subversion (Mariotti *et al*., 2002; 2004). Thus, in the present study we analysed the effect of isolated mycobacterial cell components on monocyte differentiation into DC, in order to identify components responsible for this immune evasion mechanism. In addition we investigated whether the generation of subverted $CD1^{-ve}$ DC would also affect lipid antigen presentation to CD1-restricted T-cell clones (TCC).

Results

Alpha-glucan of mycobacterial cell wall subverts monocyte differentiation into DC

When human monocytes are cultured for 5 days with GM-CSF and IL-4 they differentiate into immature DC (Sallusto and Lanzavecchia, 1994). As shown in Fig. 1, upon this process of differentiation monocytes essentially lose CD14 and reduce CD86 membrane expression while acquiring group I CD1 molecules. Treatment with maturation stimuli, such as LPS, induces immature DC to upregulate costimulatory and MHC molecules and to express CD83. As well as many other pathogens, both Mtb and BCG act as maturation stimuli for DC (Murray *et al*., 2007). In fact, o/n infection of immature DC with Mtb (not shown) or BCG (Fig. 1) induced the same phenotype observed after LPS treatment. On the other hand, we noted that infection of DC precursors, i.e. monocytes with

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Mtb or BCG, but not with *Mycobacterium avium* (Mariotti *et al*., 2002; 2004; Gagliardi *et al*., 2004) caused their differentiation into DC with a unique phenotype, characterized by (i) the lack of molecules of the class I CD1 family, (ii) the presence of mature DC-associated markers CD86 and CD83 and (iii) the expression of CD80 and MHC class II reduced in comparison to LPS matured DC (Fig. 1).

To identify whether a single mycobacterial component could be responsible for the subversion of monocyte differentiation, we pretreated monocytes with different Mtb structural components and then induced their differentiation into DC. As shown in Fig. 2, mycobacterial cell wall (CW) and alpha-glucan, which is the prominent polysaccharide of the outermost layer of Mtb, mimic most of the effects of the whole bacterium. In fact DC derived from both alpha-glucan and CW pretreated monocytes (Glu-MoDC and CW-MoDC respectively) showed a drastic inhibition of CD1 molecule expression and failed in CD80 upregulation, they expressed low levels of CD83 and upregulated CD86, but DR molecules were expressed at a lower level than LPS-matured DC. Another non-proteic compound of CW, lipoarabinomannan (LAM), as well as the cytosol fraction (CYT) or the pool of secreted proteins (CFP) of Mtb were unable to interfere with CD1 molecule expression and only caused a slightly upregulation of CD86 on DC derived from treated monocytes. The effect of alpha-glucan was further analysed and shown to be dose-dependent, but doses of alpha-glucan higher than 50 $\mu g\ ml^{-1}$ caused a progressive reduction of the viability of cells at the end of the 6 days' culture (data not shown).

In sharp contrast with the effect on monocytes, i.e. DC precursors, CW, alpha-glucan, CYT and CFP, but not LAM, behaved as maturation stimuli on immature DC, causing the switch to a mature phenotype after o/n treatment, as well as LPS or BCG (Fig. 3). These results indicate that mycobacterial components might cause differential effects on DC and on their precursors, and that alpha-glucan is the isolated component that more closely mimics the effect of Mtb or BCG infection in the subversion of monocyte differentiation into DC. Moreover, we observed that both CW-MoDC and Glu-MoDC produced IL-10 but not IL-12 p70 (Fig. 4) even if they were o/n stimulated with LPS (data not shown). Interestingly, their cytokine secretion pattern was similar to that of BCG-MoDC, indicating that these compounds mimic the effect of the whole bacterium on the cytokine production capacity of DC derived from infected monocytes.

Glu-MoDC do not prime effector T cells

We previously showed that DC derived from BCG- (BCG-MoDC) and Mtb-infected monocytes (Mt-MoDC) were hampered in their APC function. To investigate whether the altered phenotype and cytokine profile of Glu-MoDC affected their capacity to prime naïve T cells, as well as DC derived from infected monocytes, we set up a mixed leukocyte reaction (MLR) with cord blood isolated $CD4^+$ T lymphocytes as responders cells. As expected, mature (LPS treated) control DC were at the same time capable of expanding naïve T cell (Fig. 5A) and inducing their functional polarization into IFN-γ secreting cells (Fig. 5B), while immature control DC caused neither the expansion nor the functional polarization of the same naïve T-cell population (Fig. 5). Glu-MoDC as well as BCG-MoDC induced naïve T-cell expansion, even if to a lower extent than control mature DC (Fig. 5A). However, as shown in Fig. 5B, T cells expanded by Glu-MoDC and BCG-MoDC were profoundly defective in cytokine production. No changes in the T-cell functional polarization were observed even if these APC were stimulated with LPS before challenge with naïve T cells. Thus, the T-cell stimulating function of Glu-MoDC and BCG-MoDC was shown to be dissociated: they were able to induce naïve T-cell proliferation, but not to cause their functional polarization into Th1 or Th2 cells. These data indicate that although Glu-MoDC are mature DC, they are disabled in the APC function. Finally, these data concur to suggest that alpha-glucan has a major role in the ability of Mtb and BCG to alter the functional behaviour of DC derived from infected monocytes.

Glu-MoDC fail to present sulphatide antigens to CD1-restricted TCC, although being capable of protein antigen presentation to MHC class II-restricted TCC

To test the function of Glu-MoDC as APC to memory T cells, we tested their capacity to present lipid and protein antigens to CD1a or CD1c and MHC class II-restricted TCC respectively. The *in vitro* setting used can be considered as a model of secondary immune response, in which memory T cells are reactivated in lymph-nodes or in the periphery.

We used CD1a and CD1c-restricted TCC specific for sulphatide, a lipid antigen which does not require processing for its presentation (De Libero *et al*., 2005). When tested for their APC function for lipid antigens, we noticed that control DC (MoDC) induced a weak but reproducible stimulation of sulphatide-specific CD1a- but not CD1c-restricted TCC, irrespective of sulphatide pulsing (Fig. 6A and B). This result is in agreement with published data showing that endogenous glycosphingolipids are constitutively presented by APC in a CD1-restricted fashion and that bacterial infection enhances the synthesis and consequently their presentation (De Libero *et al*., 2005). In fact, a noticeable increase of TCC proliferation was observed when DC infected with BCG at day 5 of culture

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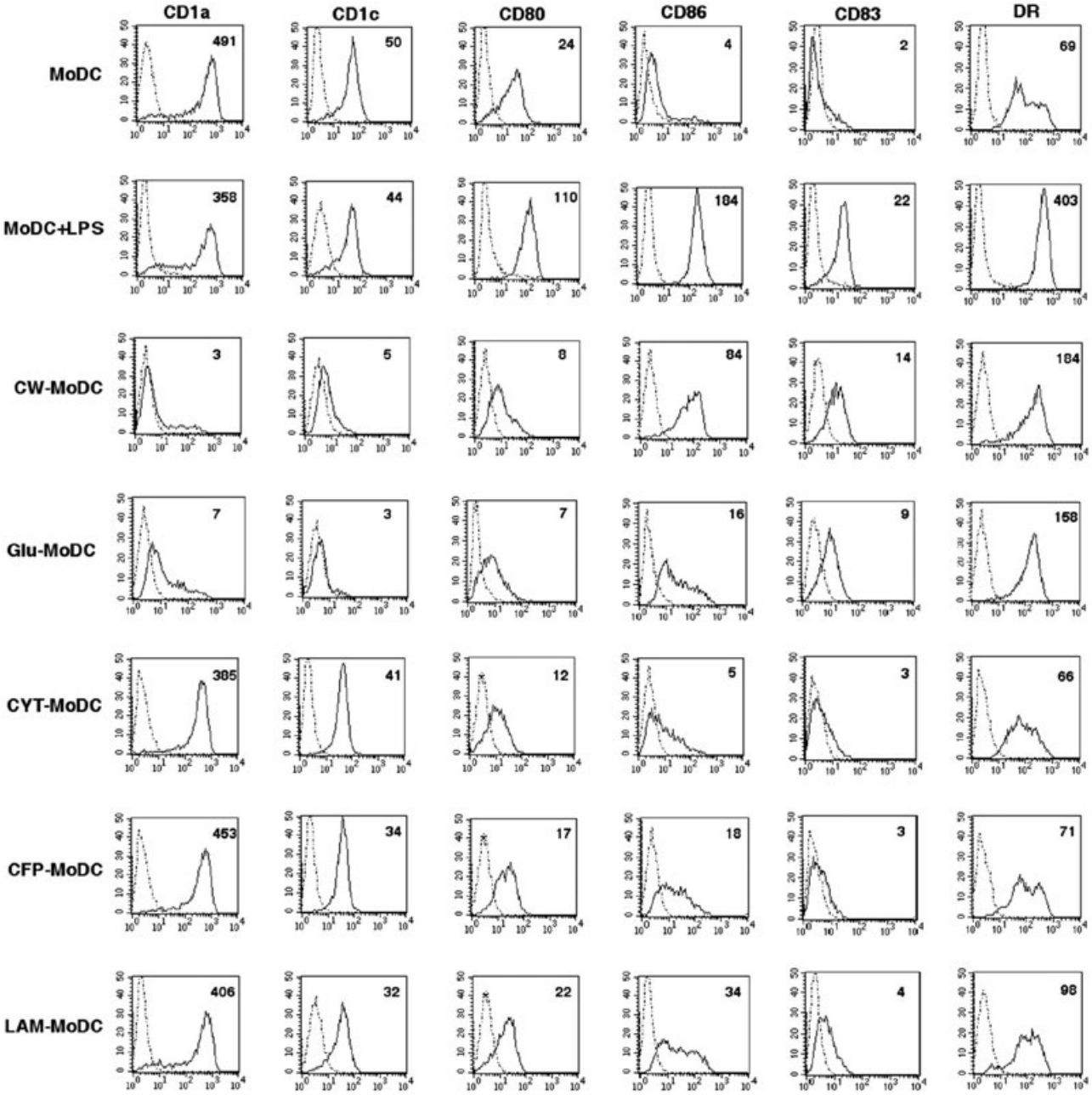

Fig. 2. Cell wall and alpha-glucan isolated from Mtb cause the subversion of monocyte differentiation into DC. Cell surface expression of the indicated molecules was determined by flow cytometry on DC derived from non-treated monocytes (MoDC) and on DC derived from monocytes treated with Mtb cell wall (CW-MoDC), alpha-glucan (Glu-MoDC), isolated cytosolic fraction of Mtb (CYT-MoDC), culture filtrate proteins from Mtb culture broth (CFP-MoDC) and lipoarabinomannan (LAM-MoDC). To compare the phenotype of DC derived from monocytes treated with Mtb components to the phenotype of mature DC, cells derived from non-infected monocytes were treated with LPS (MoDC+LPS) at day 5 and analysed on day 6. Dotted histograms represent data generated with the appropriate isotype control. Numbers indicate the median intensity of fluorescence. Data are from one experiment representative of five independent experiments.

were used as APC (data not shown). Using the TCC proliferation induced by sulphatide-pulsed DC as reference, we could observe that BCG-MoDC and Glu-MoDC were completely unable to present both endogenous and exogenously added sulphatide to CD1a and CD1c-restricted TCC (Fig. 6A and B). The same results were obtained when the IFN-γ secretion was used as a read-out of TCC activation (data not shown).

In the protein antigen presentation assays (Fig. 6C), autologous unpulsed MoDC and Glu-MoDC did not induce PPD-specific TCC proliferation, as expected. But after overnight PPD pulsing, they both showed a high

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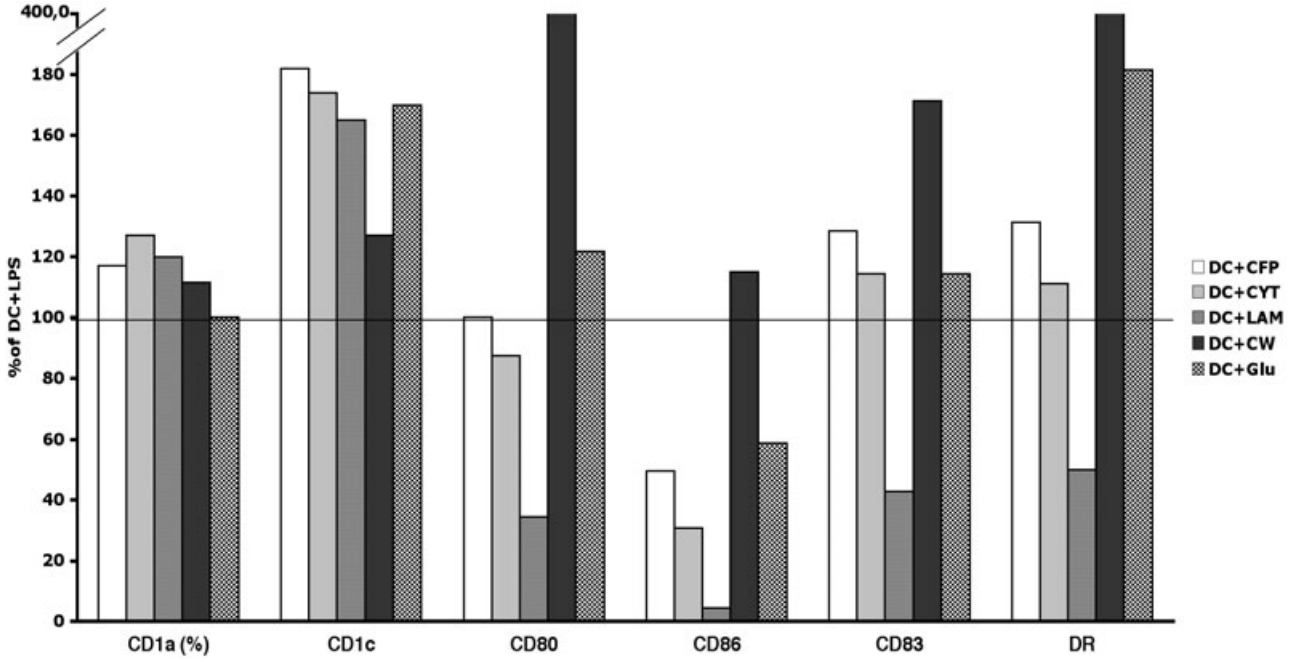

Fig. 3. Isolated mycobacterial components induce DC maturation. Immature DC derived from non-treated monocytes at day 5 of culture were stimulated with the indicated bacterial components and then analysed by flow cytometry after 24 h. CD1a molecule expression is reported as per cent of the relative number of $CD1a^{+ve}$ control DC after LPS maturation. CD1c, CD80, CD86, CD83 and DR expression is reported as the per cent of the median intensity of fluorescence of LPS matured DC. Data are from one experiment representative of three independent experiments.

efficacy in presenting mycobacterial peptides to specific TCC. Of note, unpulsed and o/n PPD pulsed BCG-MoDC showed a comparable APC function. It can be hypothesized that BCG-infected monocytes continue to process intra- or extra-cellular mycobacterial proteins throughout the 6 days long differentiation culture into DC, associating them to recycling MHC class II molecules. Because new MHC class II molecules are not consistently synthesized during the differentiation of BCG-infected monocytes into DC, PPD pulsing of BCG-MoDC at day 5 does not result in an increased presentation to specific TCC.

These data suggest that although Glu-MoDC and BCG-MoDC have defective antigen presentation capacity for naïve T cells, they are endowed with the capacity to present conventional antigen to TCC. Thus, the inability to present a lipid antigen such as sulphatide to CD1-

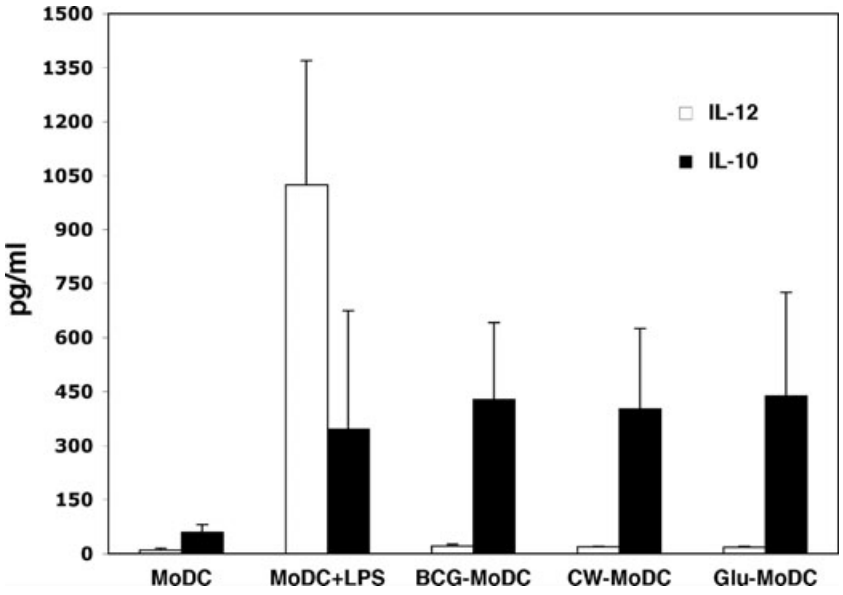

Fig. 4. Glu-MoDC are unable to secrete IL-12p70. Non-infected DC (MoDC), DC derived from BCG-infected monocytes (BCG-MoDC), CW and alpha-glucan-treated monocytes (CW-MoDC and Glu-MoDC) at day 5 of culture, were washed, adjusted to 4×10^5 cells ml^{-1} and cultured in the presence or absence of 0.1 μg ml^{-1} LPS for 24 h. Supernatants were examined for IL-12 p70 and IL-10 by ELISA. Results are shown as the mean ± SD of three independent experiments. Detection limit of the assay: 15 pg ml^{-1}.

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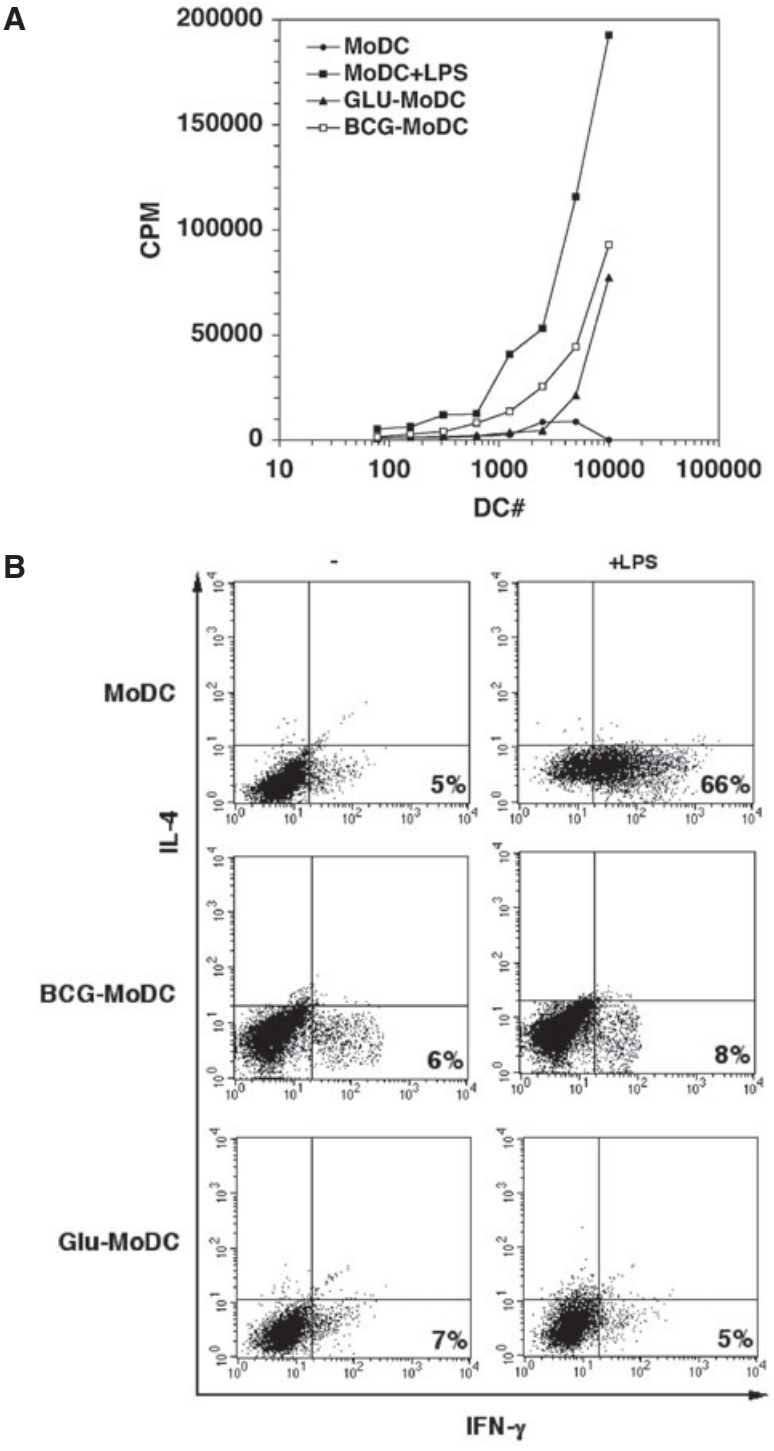

Fig. 5. Glu-MoDC prime naïve T cells but are unable to confer a functional polarized phenotype. DC derived from non-infected or BCG-infected monocytes (MoDC and BCG-MoDC respectively) and from glucan-treated monocytes (Glu-MoDC) were co-cultured with magnetically sorted cord blood allogeneic $CD4^+$ T cells. After 7 days of co-culture, the proliferative response of T cells was measured by ^{3}H-thymidine incorporation (A). To analyse the functional polarization of T cells stimulated by alloreactive APC, lymphocytes were stimulated with PMA and ionomycin for 5 h in the presence of brefeldin-A in the last 2 h. Cells were then fixed, permeabilized and stained with FITC-conjugated anti-IFN-γ or PE-conjugated anti-IL-4 (B). The numbers indicate the percentage of cells in the relevant quadrants. One representative experiment of three performed is shown.

restricted TCC is likely to be caused to their lack of CD1 molecule expression.

Discussion

Mycobacterium tuberculosis has evolved several mechanisms to persist into the host. We have proposed that its capacity to interfere with monocyte differentiation into functional DC could contribute to Mtb immune evasion (Mariotti *et al.*, 2002). In the present article we show that monocytes treated with isolated mycobacterial alpha-glucan differentiate into $CD1^{-ve}$ DC with a disabled function that share several characteristic with DC derived from Mtb infected monocytes. These data

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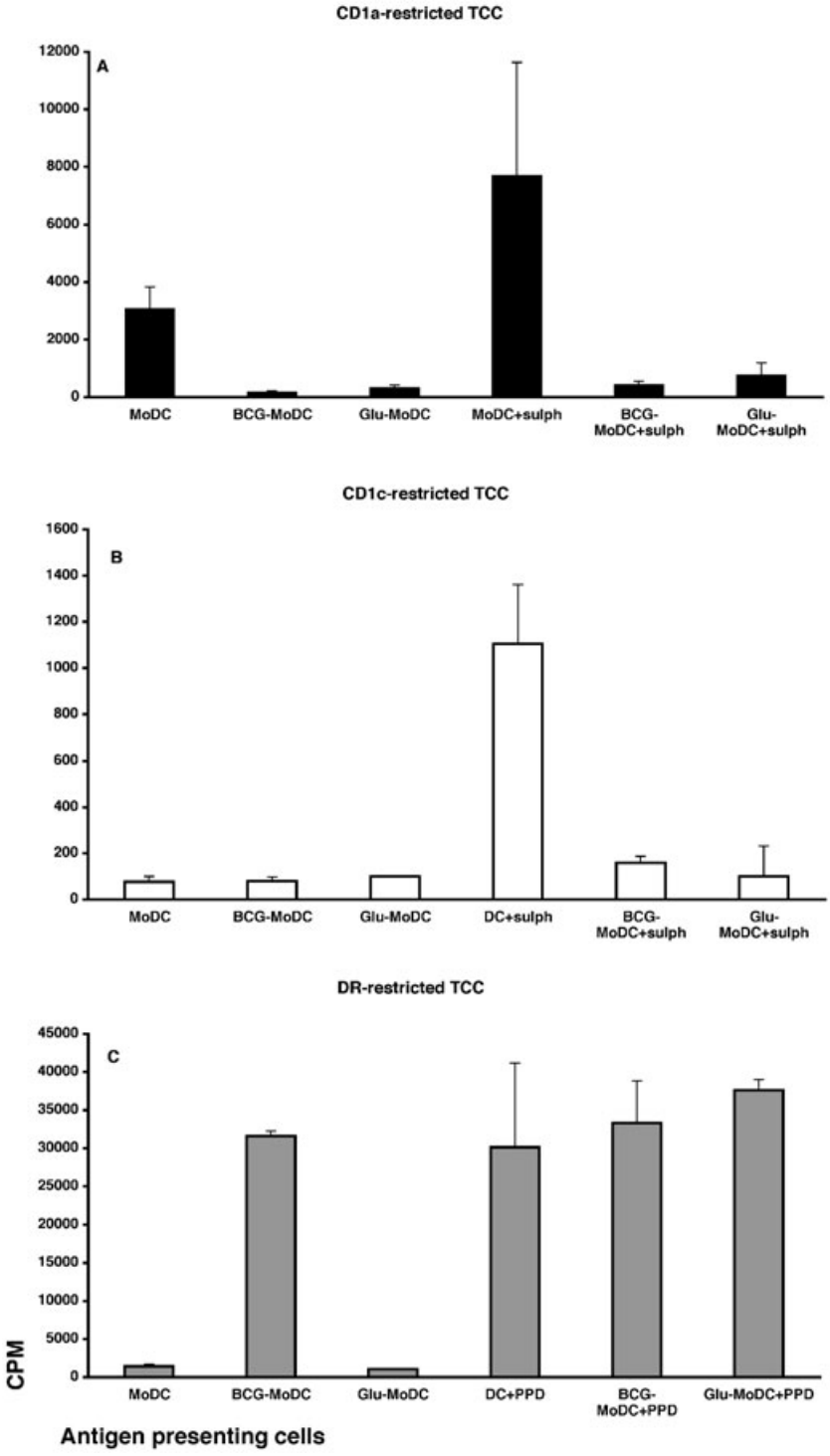

Fig. 6. Glu-MoDC and BCG-MoDC do not present lipid antigens to CD1-restricted TCC. Non-infected DC (MoDC) and DC derived from BCG infected (BCG-MoDC) or from alpha-glucan treated (Glu-MoDC) monocytes, at day 5 of culture, were washed and preincubated for 2 h at 37°C with sonicated lipid and protein antigens (sulphatide = 10 μg ml^{-1}, PPD = 20 μg ml^{-1}) before addition of the CD1a-restricted DS2C13a (A), CD1c-restricted DS1B9c (B) and MHC class II-restricted RNPD73 (C) T-cell clones (TCC). After 48 h of culture, ^{3}H-thymidine was added. After additional 18 h, cells were harvested and results were expressed as mean counts per minute (cpm) ± SD of triplicate wells. Similar results were obtained in three independent experiments.

indicate alpha-glucan as a polysaccharide capable of interfering with class I CD1 molecule expression and suggest that alpha-glucan may have a primary role in the capacity of Mtb to subvert monocyte differentiation into DC.

The mycobacterial cell *envelope* is made up of a plasma membrane and a CW with a capsule-like outermost layer. In Mtb, the external layer consists of polysaccharides (75%), proteins (about 22%) and very few amounts of lipids. We found that CW, but neither the cytosolic fraction nor the pool of secreted proteins, caused the same subversion of monocyte differentiation into DC obtained with the whole Mtb cell. These data strongly indicated that surface exposed component(s)

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could be responsible for this effect. Among them ManLAM was already been shown to have immunosuppressive effect on DC function (Geijtenbeek *et al*., 2003; Kaufmann and Schaible, 2003; Tailleux *et al*., 2003) but in our experimental setting it did not interfere with DC maturation and in monocyte differentiation into DC. This result is not surprising, because isolated ManLAM has been indicated to interfere with DC function through DC-SIGN. We have previously shown that mycobacteria can be internalized by non-opsonic phagocytosis irrespective of DC-SIGN, thus suggesting that other bacterium–DC interactions may be involved (Gagliardi *et al*., 2005). In addition, we have shown that Mtb subversive effect occurs at an early step along DC differentiation, when monocytes do not still express DC-SIGN at their surface (Gagliardi *et al*., 2004).

Therefore, we focused on alpha-glucan, which is the most abundant external polysaccharide of the Mtb outermost layer (Ortalo-Magne *et al*., 1995; Daffe and Draper, 1998) thus being potentially capable of interacting with monocyte receptors, due to its spatial position in Mtb cell.

Treatment with alpha-glucan and culture for 5 days with GM-CSF and IL-4 caused monocytes to differentiate into DC with a mature phenotype, but with an MHC class II and CD80 molecule expression reduced in comparison to that of LPS-matured DC. Moreover, the interaction of alpha-glucan with monocytes causes the differentiation of $CD1^{-ve}$ DC, indicating that alpha-glucan is capable of blocking class I CD1 molecules expression in monocytes undergoing DC differentiation. Interestingly, Glu-MoDC share these phenotypic characteristics with DC derived from Mtb- or BCG-infected monocytes (Mariotti *et al*., 2002; 2004; Gagliardi *et al*., 2004). Although it cannot *a priori* be excluded that other not tested components(s) of the mycobacterial CW concur to the subversion of monocyte differentiation into DC, we suggest that alpha-glucan represents the major Mtb constituent responsible for the lack of CD1 molecule expression on DC derived from infected monocytes, being capable *per se* to mimic the effect of the whole mycobacteria on differentiating monocytes. This interpretation is also supported by our previous findings demonstrating that Mtb and BCG, but not *M. avium*, are capable of inducing the subversion of monocyte differentiation into DC (Mariotti *et al*., 2002; 2004). Although all these mycobacteria share most of the constituents of the CW, in Mtb and BCG alpha-glucan represents a high percentage of CW polysaccharides (Ortalo-Magne *et al*., 1995; Dinadayala *et al*., 2004), while in *M. avium* it accounts for less than 2% (Lemassu and Daffe, 1994; Lemassu *et al*., 1996) and it is probably exposed in another conformation and spatial position. Thus, because we have observed that the subversion of monocyte differentiation is dose-dependent, the inability of *M. avium* to interfere with monocyte differentiation is likely to be attributed to the low alpha-glucan exposition in its external layer.

At odds with CD1d, which is constitutively expressed on marginal zone B cells, mouse bone marrow DC and monocytic precursors of human DC (Moody, 2006), molecules of the class I CD1 family, which includes CD1a, CD1b and CD1c, are inducible on human myeloid cells. Secreted or released lipids from Mtb or *Mycobacterium leprae* were shown to induce a TLR-2-mediated expression of CD1 molecules on a low number of not otherwise stimulated monocytes (Krutzik *et al*., 2003; Roura-Mir *et al*., 2005), but we and others (Stenger *et al*., 1998; Mariotti *et al*., 2002; 2004; Prete *et al*., 2001) have shown that upon stimulation of monocytes with cytokines inducing DC differentiation, Mtb is capable of blocking class I CD1 molecule expression. The identification of a polysaccharide, that reasonably interacts with monocytes in a TLR-2-independent pathway, as a negative regulator of CD1 expression, opens new interesting scenarios in the study of CD1 molecule expression, whose regulation is still controversial (Moody, 2006).

From a functional point of view, Glu-MoDC were unable to secrete IL-12p70 and to prime IFN-γ-producing T cells. Priming of naïve T lymphocytes in a MLR is a capacity of mature DC. The observation that Glu-MoDC as well BCG-MoDC and Mtb-MoDC (Mariotti *et al*., 2002; 2004; Gagliardi *et al*., 2004) are capable of inducing the proliferation of naïve T cells suggests that they can be considered mature DC. However, the inability to induce a Th1/Th2 functional polarization indicates Glu-MoDC as disabled APC lacking the complete set of stimulatory, costimulatory and adhesion molecules and the appropriate cytokine secretion capacity which are required at the immunological synapse to prime effector T cells (Mariotti *et al*., 2002). Differently from naïve T cells, TCC have reduced antigen presentation constrains for their proliferation. The observation that PPD pulsed Glu-MoDC stimulate PPD-specific TCC indicates that Glu-MoDC are not blocked in the uptake and processing of a given antigen and can behave as APC for memory T cells. On the other hand, Glu-MoDC did not present sulphatide, a lipid antigen that does not require processing for its presentation to CD1-restricted TCC, thus allowing to hypothesize that the lack of CD1 membrane expression has the functional consequence of limiting lipid antigen presentation to memory and consequently to naïve T cells. Recent evidence highlights the importance of lipid-specific T-cell responses in Mtb-infected patients (Kawashima *et al*., 2003; Ulrichs *et al*., 2003; Gilleron *et al*., 2004). Lipids are very important constituents of Mtb. Indeed, lipids may constitute up to 60% of the dry weight of this bacillus, whereas they account for only about 20% of the lipid-rich envelope of Gram-negative organisms (Brennan and

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Nikaido, 1995; Draper, 1998). A variety of immunogenic lipidic antigens have been isolated and structurally defined (De Libero and Mori, 2005). Most of lipid-specific TCC isolated so far recognize Mtb-infected $CD1^{+ve}$ APC and kill intracellular bacteria, indicating a potential role in protective immunity (Gilleron *et al*., 2004). Thus, the failure of lipid antigen presentation by Mt-MoDC or Glu-MoDC, together with their inability of priming antigen-specific Th1 lymphocytes, could eventually contribute to Mtb escape from acquired immune response. This would occur if monocytes are recruited into the infected tissue in the presence of replicating mycobacteria and subjected to differentiate into DC. The cells derived from infected monocytes would be $CD1^{-ve}$ and unable to stimulate the memory CD1-restricted T cells that were expanded during the primary infection. In addition, Mt-MoDC would be unable to prime new generations of both lipid-specific and MHC class II-restricted IFN-γ secreting T cells which are required to substitute effector T cells that die upon antigen encounter (Westermann *et al*., 2001; Sprent and Surh, 2002) in long lasting infection such as TB. Although it is not easy to translate *in vitro* studies into *in vivo* contexts, our model fits with the observation that the most severe forms of non-antibiotic treated active TB cannot be diagnosed by the recent tests based on IFN-γ measurement following stimulation with Mtb-specific protein (Pathan *et al*., 2001; Goletti *et al*., 2005; 2006) or lipid antigens (Ulrichs *et al*., 2003). In these forms of disease, IFN-γ secreting T cells are reduced or not even detectable and our model is in accordance with these findings.

In conclusion, the identification of alpha-glucan, a major constituent of Mtb CW, as the responsible for several of the Mtb effects on monocyte differentiation into DC makes possible to focus on possible alternative targets for immune-intervention against TB (Lang and Glatman-Freedman, 2006).

Experimental procedures

Reagents

Recombinant IL-4 was purchased by from R&D Systems (Minneapolis, MN) and GM-CSF (Leucomax) from Sandoz (Basel, Switzerland). Tritiated thymidine was from Amersham (Little Chalfont, GB). PMA, ionomycin and LPS (from *E. coli*) were from Sigma Chemical (St. Louis, MO), brefeldin-A (Golgi-Plug) from BD/Pharmingen (San Diego, CA). RPMI 1640 (Euroclone, UK) was used supplemented with 100 U ml^{-1} kanamycin, 1 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) (complete medium). Purified protein derivative (PPD) was purchased from Statens Serum Institute (Copenhaghen, Denmark) and purified sulphatide from Fluka (St. Gallen, CH). The following components, all isolated from *M. tuberculosis* strain $H_{37}Rv$, were kindly provided by Colorado State University (NIH, NIAID Contract NO1 AI-75320): cell wall fraction (CW), cytosol fraction (CYT), crude culture filtrate proteins (CFP), lipoarabinomannan (LAM) and details regarding methods for purification are available at: http://www.cvmbs.colostate.edu/microbiology/tb/researchma.htm

Alpha-glucan was isolated according to previous published methods (Lemassu and Daffe, 1994; Lemassu *et al*., 1996).

Growth of mycobacteria

BCG (ATCC 27291) was grown with gentle agitation (80 r.p.m.) in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.05% Tween 80 (Sigma Chemical Company, St. Louis, MO) and 10% Middlebrook ADC enrichment (Becton Dickinson, Mountain View, CA). Logarithmically growing cultures were washed two times in RPMI 1640. Mycobacteria were resuspended in RPMI 1640 containing 10% FCS and then stored at −80°C. Vials were thawed, and bacterial viability was determined by counting the number of colony-forming units on Middlebrook 7H10 agar plates. All BCG preparations were analysed for LPS contamination by the Limulus lysate assay (BioWhittaker) and contained less than 10 pg ml^{-1} of LPS.

Monocyte isolation, infection or treatment and DC generation

In vitro studies on the human immune response to Mtb have been reviewed and approved by the Istituto Superiore di Sanità Ethical Committee (http://www.iss.it/coet/index.php?lang=1).

Peripheral blood mononuclear cells were purified from heparinized blood obtained by healthy donors (Blood Bank of University 'La Sapienza', Roma, Italy) on a density gradient (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). Monocytes were then positively sorted using anti-CD14 labelled magnetic beads (MACS, Miltenyi Biotech, Germany) and resuspended in RPMI 1640 complete medium (Nisini *et al*., 1996). Monocytes were infected with single cell suspensions of BCG at multiplicity of infections (moi) ranging from 0.5:1 to 10:1 and the moi of 6:1 was used unless otherwise indicated (Giacomini *et al*., 2001). The efficiency of infection/phagocytosis was quantified by counting intracellular mycobacteria/particles in cells stained with the Kinyoun method (Giacomini *et al*., 2001). Otherwise monocytes were incubated with Mtb isolated components at 50 μg ml^{-1} for 3 h. The effect of alpha-glucan was also tested incubating monocytes with concentrations ranging from 100 to 1 μg ml^{-1}. DC were generated culturing infected, non-infected and pretreated monocytes for 6 days in complete medium containing GM-CSF (50 ng ml^{-1}) and IL-4 (1000 U ml^{-1}). LPS at 0.1 μg ml^{-1} was added during the last 24 h to induce DC maturation. In some experiments DC at day 5 of culture were infected with BCG at moi of 6:1 or treated with isolated components at 50 μg ml^{-1}. Adherent cells were harvested following the gentle use of a cell scraper (Costar). Viability of DC derived from alpha-glucan, CW-treated or BCG infected monocytes (Glu-MoDC, CW-MoDC and BCG-MoDC respectively) was determined by trypan blue exclusion.

FACS analysis

The following antibodies were used: FITC-conjugated anti-CD1a, CD83 and CD86, PE-conjugated anti-CD14, anti-DR and

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CD80 (BD/Pharmingen), biotin-conjugated anti-CD1c (Cymbus Biotechnology). FITC-conjugated streptavidin (Sigma) was used in association with anti-CD1c moAb.

Staining of intracellular cytokines in T cells was performed using PE-conjugated mouse anti-human IL-4 and FITC-conjugated mouse anti-human IFN-γ (BD/Pharmingen) after fixation and permeabilization using Cytofix/Cytoperm™ (BD/Pharmingen), according to the manufacturer's instructions.

Stained cells were analysed by flow cytometry using a FACScan cytometer (Becton Dickinson, Mountain View, CA) equipped with Cellquest Software (Becton Dickinson). Fluorescence intensity was evaluated by computerized analysis of dot plots or histograms generated by 10^4 viable cells.

Cytokine production analysis

Non-infected DC, BCG-MoDC, CW-MoDC and Glu-MoDC, at day 5 of culture, were washed, adjusted to 4×10^5 cell ml^{-1} and cultured in the presence or absence of 0.1 μg ml^{-1} LPS for an additional 24 h. Supernatants were examined for cytokines by Elisa using commercially available kits (R&D) according to the manufacturer instructions. Detection limit of the assay: 15 pg ml^{-1}.

Priming of naïve T cells

Non-infected DC, BCG-MoDC and Glu-MoDC were cultured at different cell numbers with 3×10^4 cord blood CD4$^+$ T cells purified by indirect magnetic sorting with CD4$^+$ T Cell Isolation Kit by Miltenyi Biotec. Cord blood samples were kindly provided by the Unit of Obstetrics and Gynaecology, San Giovanni Hospital, Rome, Italy. The proliferative response was measured after 6 days by a 16 h pulse with ^{3}H thymidine (1 μCi well^{-1}). Part of T cells were stimulated with 10^{-7}M PMA and 1 μg ml^{-1} ionomycin for 5 h, with 2 μg ml^{-1} brefeldin A (Golgi Plug) being added during the last 2 h, and then analysed by flow cytometry for their intracellular cytokine production.

T-cell clones and antigen presentation assay

Sulphatide-specific CD1a and CD1c-restricted TCC were kindly gifted by G. De Libero and PPD-responsive MHC class II-restricted TCC were established and maintained as previously described (Nisini *et al.*, 2001; Shamshiev *et al.*, 2002; Gilleron *et al.*, 2004). Antigen presentation assays were performed using RPMI 1640 medium containing 100 U ml^{-1} kanamycin, 1 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, and 5% AB human serum, not heat inactivated (Sigma). Control MoDC, BCG-MoDC, Glu-MoDC and DC infected at day 5 of culture with BCG were washed and preincubated (1×10^4 well^{-1}) for 2 h at 37°C with sonicated antigens (sulphatide = 10 μg ml^{-1}, PPD = 20 μg ml^{-1}) before addition of TCC (3×10^4 well^{-1} in triplicate) in 96-well flat-bottom plates. After 48 h of culturing ^{3}H thymidine was added at 1 μCi well^{-1} and cells were harvested 18 h later.

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