Control of functional T helper cell polarization by dendritic cells

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Citation for published version (APA):
Microbial Compounds Selectively Induce Th1 Cell-promoting or Th2 Cell-promoting Dendritic Cells In Vitro with Diverse Th Cell-polarizing Signals

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Upon microbial infection, specific Th1 or Th2 responses develop depending on the type of microbe. Here, we demonstrate that different microbial compounds polarize the maturation of human myeloid dendritic cells (DC) into stably committed Th1 cell-promoting (DC1) or Th2 cell-promoting (DC2) effector DC that polarize Th cells via different mechanisms. Protein extract derived from the helminth Schistosoma mansoni induced the development of DC2 that promote the development of Th2 cells via the enhanced expression of OX40 ligand. Likewise, toxin from the extracellular bacterium Vibrio cholerae induced development of DC2 as well, however, via an OX40 ligand-independent, still unknown mechanism. In contrast, toxin from the intracellular bacterium Bordetella pertussis induced the development of DC1 with enhanced IL-12 production which promotes a Th1 cell development. Poly I:C (dsRNA, mimic for virus) induced the development of extremely potent Th1-inducing DC1, surprisingly, without an enhanced IL-12 production. The obtained DC1 and DC2 are genuine effector cells that stably express Th cell polarizing factors and are unresponsive to further modulation. The data suggest that the molecular basis of Th1/Th2 polarization via DC is unexpectedly diverse and is adapted to the nature of the microbial compounds.

INTRODUCTION

Appropriate responses against micro-organisms require selective forms of specific immunity mediated by functionally polarized subsets of effector Th cells, e.g. IFN-γ-producing Th1 cells and IL-4-producing Th2 cells, which develop from a common pool of naive precursor T cells. There is accumulating evidence that microbes drive the development of protective Th1 or Th2 cells through their effects on antigen-presenting cells (1-4). Dendritic cells (DC) are professional antigen-presenting cells that are present as immature sentinel cells that efficiently sample their environment for foreign antigen at potential sites of pathogen entry. Upon activation by signals released from the microorganisms or from infected tissues, sentinel DC undergo maturation into potent T cell stimulatory effector DC and migrate towards the T cell areas of draining lymphoid organs. There, effector DC will activate naive Th cells with pathogen-specific (MHC-peptide complexes, signal 1) and costimulatory (B7 family molecules, signal 2) (5) molecules. In addition to signals 1 and 2, DC carry a third signal which determines the polarization of naive Th cells into Th1 or Th2 cells (6). Like signal 2, signal 3 is heterogeneous and can be mediated by various soluble or membrane-bound molecules, including IL-12 (7), IL-18 (8), IFN-α(9) and OX40L (10).

Importantly, in vitro studies suggested that the expression levels of these Th cell-polarizing molecules by mature effector DC strongly depend on the conditions during their initial activation as sentinel DC. Tissue-derived factors such as IFN-γ and PGE2 present during the activation of human monocyte-derived sentinel DC promote the generation of type 1 effector DC (DC1) that produce high amounts of IL-12 upon subsequent engagement of naive T cells or the generation of IL-12-deficient DC2 which drive the development of Th2 cells, respectively (11, 12). These findings imply that pathogens may promote the development of distinct DC phenotypes by provoking tissues to release mediators involved in polarization.

In addition to these indirect effects, microorganisms also directly affect sentinel DC at the time of pathogen encounter, as has been shown previously (1-4).

In the present study, we investigated the DC-derived molecules involved in Th-cell polarization of different DC1 and DC2 subsets. Soluble egg antigens (SEA) of the helminth Schistosoma mansoni and the toxin of the intestinal bacterium Vibrio cholerae (CT), both associated with Th2 cell responses (13, 14), as well as dsRNA (poly I:C, a mimic of viral RNA) and the toxin of the intracellular bacterium Bordetella
Microbial compounds selectively induce DC1 or DC2

pertussis (PT), both associated with Th1 cell responses (15), all promoted sentinel DC to develop into functional effector cells with stably polarized DC2 or DC1 phenotypes respectively. It became clear that, although the effector DC1 or DC2 populations induced similar Th1 and Th2 cell subsets, there is heterogeneity within DC1 and DC2 subsets with respect to the expression and utilization of Th-polarizing molecules.

The present study indicates that a protective immune response is mounted via the development of polarized DC1 and DC2 subsets with diverse expression of signal 3 induced by factors derived from the invading pathogen.

MATERIALS AND METHODS
Generation of immature DC and their induction of maturation by different microbial compounds

Monocytes were isolated from peripheral blood mononuclear cells (PBMC) using density centrifugation. Immature DC were generated by culturing monocytes for 6 days in IMDM (Life Technologies Ltd., Paisley, UK) containing gentamycin (86 μg/l; Duchefa, Haarlem, The Netherlands) and 1% FCS (HyClone, Logan, UT), supplemented with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and IL-4 (250 U/ml; PBH, Hanover, Germany). At day 6 maturation was induced by culturing the cells for 2 days with the following factors alone or a combination as indicated in the text: IL-1β (10 ng/ml; PBH), TNF-α (50 ng/ml; PBH), poly I:C (20 μg/ml, Sigma-Aldrich, St. Louis, MO), Schistosoma Mansoni Egg Antigens (30 μg/ml, prepared as described previously (16)), CT (1 μg/ml; Sigma-Aldrich), PT (1 μg/ml, Sigma-Aldrich), IFN-γ (1000 U/ml; A gift from Dr P.H. van der Meide, Utrecht University, Utrecht, The Netherlands) or PGE_{2} (10^{-6} M; Sigma-Aldrich). All subsequent tests were performed after harvesting and extensively washing of the cells to remove all factors.

Expression of cell surface molecules
At day 8 the obtained effector DC were analyzed for the expression of cell surface molecules by FACS. Mouse anti human monoclonal antibodies were used against the following molecules: CD1a (OKT6, Ortho Diagnostic Systems, Beerse, Belgium), CD83 (HB15a, IgG2b; Immunotech, Marseille, France), CD86 (1G10, IgG2a;
Innogenetics N.V., Ghent, Belgium), HLA-DR (L234, IgG2a; Becton Dickinson, San Jose, CA) and OX40L (5A8) (17). All mAbs were followed by FITC-conjugated goat F(ab')2 anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) Samples were analyzed on a FACScan (BD Biosciences).

**Determination of naive CD4^+CD45RA^+CD45RO^- Th cell polarization by effector DC**

Highly purified CD4^+CD45RA^+CD45RO^- naive Th cells (>98% as assessed by flow cytometry) were purified from PBMC or PBL using a human CD4^+/CD45RO^- column kit (R&D, Minneapolis, MN). Naive CD4^+ Th cells (2x10^4 cells/200μl IMDM with 10% FCS) were co-cultured with 5x10^3 effector DC coated with *Staphylococcus enterotoxin B* (SEB; Sigma-Aldrich, final concentration 100 pg/ml), in 96-well flat-bottom culture plates (Costar, Cambridge, MA). As indicated at certain figures, the following neutralizing or blocking Abs were used: anti-IL-12 (10 μg/ml, a kind gift from Dr P.H. van der Meide, Utrecht University, Utrecht, The Netherlands), anti-IL-18 (10 μg/ml, MBL, Nagoya, Japan), anti-IFN-α (10 μg/ml, PBL, New Brunswick, NJ) and anti-type I IFN R (10 μg/ml, Research Diagnostics, Flanders, NJ). At day 5, rhuIL-2 (10 U/ml, Cetus Corp., Emeryville, CA) was added and the cultures were expanded for the next 9 days. On day 14 the quiescent Th cells were restimulated with PMA (10 ng/ml, Sigma-Aldrich) and ionomycin (1 μg/ml Sigma-Aldrich) for 6 h and during the last 5 h Brefeldin A (10 μg/ml, Sigma-Aldrich) was present, to detect the intracellular production of IL-4 and IFN-γ (both Becton Dickinson). For the direct stimulation of naive Th cells in the presence of supernatants of activated DC, plate-bound anti-CD3 (16A9, Central Laboratory for Blood Transfusions, (CLB) Amsterdam, The Netherlands) and anti-CD28 (5E8, CLB) were used at a concentration of 1 μg/ml.

**Induction of cytokine production by DC**

CD1a^+ DC (4x10^4 cells/well) were stimulated with CD40L-transfected J558 cells (J558-CD40L; a kind gift from Dr. P. Lane, University of Birmingham, Birmingham, U.K.), 4x10^4 cells/well in the presence or absence of rhuFN-γ (1000 U/ml), in 96-well flat-bottom culture plates (Costar) in IMDM containing 10% FCS in a final volume of 200 μl. Supernatants were harvested after 24 h and stored at -20°C until the levels of cytokines were measured by ELISA.
Microbial compounds selectively induce DC1 or DC2

Cytokine measurements
Measurements of IL-12p70, TNF-α and IL-6 levels in the culture supernatants were performed by specific solid-phase sandwich ELISA as described previously (11). The limits of detection of these ELISA are as follows: IL-12p70, 3 pg/ml; TNF-α, 10 pg/ml; IL-6, 20 pg/ml.

RESULTS
Pathogens induce either effector DC1 or DC2
To study the direct effects of different microbial compounds on the maturation of sentinel DC into effector DC, uncommitted monocyte-derived DC were cultured in the presence of SEA, CT, PT or poly I:C. As a control, DC were cultured by the combination of IL-1β and TNF-α maturation-inducing factors: MF). In agreement with previous reports, MF (18), CT (4) and poly I:C (1) induced final DC maturation within 48 h, as evident from the loss of expression of the mannose receptor, the inability to phagocytose, the induction of CD83 expression and the upregulation of the CD80, CD86 and HLA-DR expression, of which the expression levels were comparable within the different DC subsets. The cell surface expression of CD83 and CD86 is shown in Fig. 1A. SEA and PT induced DC maturation as well, although the degree of maturation in the case of PT varied between different donors (Fig 1A). Because the state of maturation of DC may influence the capacity to drive Th1 or Th2 responses, due to differences in the expression of certain cytokines or membrane-bound molecules, MF were added to the stimulations (CT, PT and the controls PGE$_2$ and IFN-γ) that do not induce full maturation by themselves.

Subsequently, the effector DC obtained after 48 h were used to stimulate naive Th cells with the superantigen SEB in order to generate effector Th cells. The Th1/Th2 cytokine profiles of the resulting effector Th cells were assessed by analysis of intracellular IL-4 and IFN-γ expression (Fig. 1B). As expected DC matured by MF induced the development of a mixture of IFN-γ-producing Th1 and IL-4-producing Th2 effector cells. In contrast, DC matured by SEA or CT became type 2 DC (DC2) with the intrinsic ability to bias for the development of Th2 cells, comparable to the known Th2 polarizing effect of PGE$_2$. On the other hand, priming of DC with PT or poly I:C resulted in type 1 DC (DC1) that biased for the development of Th1 cells.
Chapter 3

The latter effect was comparable to the induction of Th1 cells by DC matured with the combination of MF and IFN-γ.

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**Figure 1.** DC matured by microbial compounds from different origin drive the development of Th1 or Th2 response and differ in their cytokine production upon CD40 ligation. Sentinel, immature DC were matured for 48 h by MF or by the microbial compounds SEA, MF+CT, MF+PT or poly I:C. As controls, DC were matured with MF+IFN-γ (for DC1) and MF+PGE₂ (for DC2). (A) Effector DC were analyzed for their expression of CD83 and CD86 by flow cytometry. The thick line represents the specific expression of CD83 (upper panel) or CD86 (lower panel), whereas the thin line represents the isotype control. (B) Mature, effector DC (5x10³ cells/well) were loaded with SEB and cocultured with naive CD4⁺CD45RA⁺ Th cells (2x10⁴ cells/well). After 14 days the responder Th cells had become quiescent and were restimulated with PMA/ionomycin for 6 h. The IL-4- and/or IFN-γ producing cells were measured by intracellular FACS analysis and the percentage of cells is given in the respective figure.

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**Cytokine production by DC subsets**

Since the level of IL-12 production by myeloid DC during activation of naive Th cells, is a major factor driving the development of Th1 cells, we first studied whether DC1 and DC2 types are associated with high or low IL-12 production. Bioactive IL-12p70
Microbial compounds selectively induce DC1 or DC2 production by the effector DC was measured upon ligation of CD40 by CD40L-transfected cells, thereby mimicking the engagement by T cells (Fig. 2A). Compared to the MF-primed control DC, the SEA- or CT-primed DC2 showed a strongly reduced IL-12p70 production, in accordance with their Th2-driving capacity. As expected from their Th1-polarizing effect, PT primed for DC1 with enhanced IL-12p70 production, similar to MF plus IFN-γ-primed DC1. Surprisingly, unlike the MF+PT- and MF+IFN-γ-primed DC1, poly I:C-primed DC1 did not show enhanced IL-12p70 production, despite their potent Th1-promoting capacity, suggesting the existence of alternative Th1-driving mechanisms. The production of the pro-inflammatory cytokine TNF-α followed the pattern of IL-12 production, with the exception that the TNF-α production was consistently not enhanced in PT-primed DC (Fig. 2B). IL-6 production is differentially regulated (Fig. 2C) being consistently low in SEA-primed DC and high in poly I:C-primed DC, but unaffected in the other effector DC. The production of IFN-γ by all effector DC subsets was below or around the detection limit of the ELISA (data not shown). These findings indicate that the various subsets of DC1 and DC2 have unique cytokine profiles. Amongst others, SEA-primed DC are unable to produce high levels of any of the cytokines tested (e.g. IL-12, TNF-α, IL-6). The variable profiles may imply that the microbial compounds activate different signaling pathways in the sentinel DC.

![Graphs of cytokine production](image)

**Figure 2.** DC matured by microbial compounds from different differ in their cytokine production upon CD40 ligation. Sentinel, immature DC were matured by MF or by the microbial compounds SEA, MF+CT, MF+PT or poly I:C. As controls, DC were matured with MF+IFN-γ (for DC1) and MF+PGE₂ (for DC2). After 48 h, the cells were thoroughly washed and stimulated (4x10⁴ cells/well) with J558-CD40L (4x10⁴ cells/well). Supernatants were harvested after 24 h and secreted cytokines were measured by ELISA. A: IL-12p70, B: TNF-α, C: IL-6. Concentrations that were out of range of the axes are given in the respective bar. Results from one representative experiment of 6. *P<0.05; **P<0.001.
Figure 3. Relative contribution of IL-12, IL-18 and type I IFN to the Th1 or Th2 development induced by DC matured by different microbial compounds. Sentinel, immature DC were matured and used to stimulate naive Th cells as indicated in the legend to Fig.1. Stimulation was performed in the absence or presence of neutralizing or blocking Abs in optimal concentrations as indicated in the figure. A: the effect of anti-IL-12 Abs (open bars) with all different types of DC compared to control Ab (filled bars). B: the effect of anti-IL-18, anti-IFN-α and anti-type I IFN R with DC matured with MF or poly I:C. Results from one representative experiment of 3.

Role of IL-12 in the induction of Th1 cells
To analyze the contribution of DC-derived IL-12 to the development of Th1 cells, we tested the effect of neutralizing anti-IL-12 Ab in cocultures of naive Th cells and the distinct effector DC subsets (Fig 3A and 3B). In case of most DC subsets neutralization of IL-12 increased the development of IL-4–producing Th cells and
Microbial compounds selectively induce DC1 or DC2 dramatically decreased the development of IFN-γ–producing Th cells. However, in case of poly I:C-primed DC, the percentage of IFN-γ–producing cells could not be reduced below 20-40%. This is in line with the finding that poly I:C primes for efficient effector DC1 despite moderate IL-12 production and suggests that poly I:C-primed DC express Th1-driving factors other than IL-12 p70. Neutralizing Abs to IL-18, IFN-α or type I IFN R did not substantially block Th1 development induced by the poly I:C-primed DC (Fig. 3B), suggesting the involvement of yet another factor.

Figure 4. Not only soluble DC-derived factors are involved in Th cell polarization. Sentinel, immature DC were matured as described in the legend to Fig. 1. After 48 h, the cells were thoroughly washed and stimulated (4x10⁴ cells/well) with the J558-CD40L (4x10⁴ cell/well). Supernatants were harvested after 24 h and added to naive CD4⁺CD45RA⁺ Th cells that were activated with plate-bound anti-CD3 and anti-CD28, with neutralizing antibodies directed against IL-12 (open bars) or with control Ab (filled bars). After 14 days responding Th cells were analyzed as described in the legend to Fig 1. Results from one representative experiment of 4.

Role of DC-derived soluble factors in the induction of Th1 or Th2 cells
In an attempt to further define the Th1- and Th2-driving molecules expressed by the various effector DC types, we tested to what extent soluble factors were critical. Supernatants of CD40L-activated DC (after 24 h) were added to cultures of naive T cells stimulated with anti-CD3 and anti-CD28 Abs. After 10-14 days the effector Th cells were restimulated and the IL-4 and IFN-γ, expression was determined. Supernatants of MF+ IFN-γ–primed DC strongly promoted the development of Th1 cells, which could almost completely be blocked by IL-12 Ab (Fig. 4). Likewise, the supernatants of the poly I:C-primed DC supported the development of Th1 cells. However, this effect could only be partially blocked by anti-IL-12 Ab, indicating that the Th1-promoting activity of these DC is mediated by an unknown soluble factor. Supernatants of CD40L-activated DC2 primed with the combination of MF and PGE₂
or with CT exhibited a strong Th2-driving capacity. This activity could not be blocked by neutralizing Abs to IL-4, IL-13 or MCP-1 or by preventing the production of eicosanoids (e.g. PGE$_2$) by these DC2 (data not shown). In contrast, supernatants of CD40L-stimulated SEA-primed DC failed to support Th2 development, indicating that these cells exert the Th2-bias via a membrane-bound factor.

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**Figure 5. Role of OX40L expression by DC matured by different microbial compounds.** Sentinel, immature DC were matured as described in the legend to Fig 1. Subsequently, the DC were either or not activated with CD40L. (A) After 24 h, DC were harvested and stained with mAb directed OX40L. The expression was determined by FACS. (B) SEB-loaded differentially matured DC (5x10$^3$ cells/well) were cocultured with CD4$^+$CD45RA$^-$ naive Th cells (2x10$^5$ cells/well) in the presence of control Ab (filled bars) or neutralizing anti-OX40L Ab (open bars) in optimal concentration. After 14 days responding Th cells were analyzed as described in the legend to Fig 1. Results from one representative experiment of 3.
Role of OX40L in the induction of Th2 cells

In search for the identity of the Th2-driving membrane-bound factor on the SEA-primed DC, we focused on OX40L, a factor known to be expressed by part of the peripheral blood DC and involved in Th2 cell development (10). Interestingly, OX40L was detectable only in DC2, and not on any of the DC1 types (Fig. 5A). Even after CD40 ligation, which has been described to strongly upregulate OX40L expression, only a small minority of the cells expresses OX40L whereas all DC2 cells showed enhanced OX40L expression (Fig. 5A).

To determine the role of OX40L-OX40 interaction in Th2-development, blocking OX40L mAb was added during the co-culture of type 2 DC with naive Th cells.
Clearly, anti-OX40L mAb affected the cytokine balance only of the effector T cells generated by SEA-primed DC. Blocking of OX40L expressed on SEA-DC resulted in a strongly reduced development of IL-4-producing Th cells from naive precursors whereas when MF+PGE$_2$-DC (or MF+CT-DC, data not shown) were used to stimulate naive Th cells no difference was observed in the development of IL-4- or IFN-γ-producing Th cells (Fig. 5B).

**DC1 and DC2 have stable IL-12 secretion profiles**

To study the stability of the functional phenotype of the various types of effector DC, we tested whether IL-12 production was altered after stimulation by microbial compounds or cytokines that polarize immature DC in the opposite direction. As the pathogenic factors except for poly I:C modulate the cytokine production of immature DC but do not induce cytokine on their own (data not shown), the effector DC were activated with CD40L in the absence or presence of SEA, CT, PGE$_2$ or IFN-γ or with poly I:C alone. As shown in Fig. 6, the IL-12 levels of the various DC1 and DC2 are largely preserved in any condition of stimulation. In general, in the presence of compounds that prime for high IL-12, the IL-12 production was enhanced in DC1 but hardly or not in DC2. In the presence of downregulators of IL-12 production, the IL-12 levels were unchanged or only marginally decreased.

**DISCUSSION**

It is long recognized that immune responses to different types of pathogens are associated with different types of effector responses directed by polarized Th1 or Th2 cell subsets. Here we demonstrate that microbial compounds induce Th cell polarization via the polarization of sentinel DC into effector DC1 and DC2. We and others have shown earlier that maturation of DC by LPS or MF (19, 20) in vitro primes for a decreased ability to produce IL-12 and that these DC have the potency to induce mixed populations of Th1 and Th2 cells. The balance between Th1 and Th2 cells strongly depends on the model system used, as it varies with antigen levels (21), as well as number (22) and source (23) of antigen presenting cells, and is subject to significant donor variability. Our data corroborate previous studies, both in mouse and man (1-4), indicating that pathogens or their signature molecules can induce biased immune responses by direct priming of DC. Surprisingly, the critical
Microbial compounds selectively induce DC1 or DC2

Th1-driving molecules of the DC1 types and the Th2 driving-molecules of the DC2 types differ depending on the pathogen suggesting a complex network of immune polarization and a multifaceted diversity in signal 3.

DC1 primed by MF+IFN-γ or by MF+PT drive Th1 cells via high IL-12 production upon CD40 ligation (T cell engagement). Although poly I:C induces high levels of IL-12 in sentinel myeloid DC (1) (de Jong et al, unpublished data), when present during maturation it does not prime for high IL-12 production. Instead, poly I:C-primed DC1 drive Th1 cells via an unknown soluble factor. Poly I:C is used as a model antigen for viral infections. The relatively low importance of IL-12 in response to poly I:C is in accordance with the finding by Schijns et al (24) that IL-12p40/p70 deficient mice still mount potent Th1 responses upon infection with mouse hepatitis virus (MHV) and the finding that in patients with a functional mutation of the IL-12R suffer from infections by various endosomal microorganisms, but not by viruses (25). Liu et al (26) showed that plasmacytoid DC activated with influenza virus promote the development of Th1 cells via IFN-α. Surprisingly, in myeloid DC the unknown soluble Th1-driving factor secreted by poly I:C-matured DC is probably not a type I IFNs or IL-18. It is also unlikely to be IL-23, as its p40 subunit is not upregulated in these DC (data not shown) and polyclonal antibodies directed against IL-12 only partially inhibit the Th1-inducing capacity.

Within the DC2, two types were identified. Although all DC2 express OX40L, only SEA-primed DC use OX40L to promote the development of Th2 cells. The CT- and PGE₂-primed DC promote Th2 cells via an unidentified soluble factor, which is absent in the SEA-primed cells. Possible candidates for the DC-derived Th2-inducing molecules like IL-4, IL-13, PGE₂ or MCP-1 do not appear to be involved since they were not produced at detectable levels, and neutralization of their activity had no effect. The identification of this factor(s) is an issue of current investigation.

The heterogeneity of effector DC and, therefore, signal 3 reflects the different ways in which various microbial compounds or mediators can activate the sentinel DC. So far, little is known about the molecular cross-talk between DC and pathogens. The capability of CT and PGE₂ to prime for DC2 can be attributed to their ability to enhance the levels of intracellular cAMP, which block the development of IL-12 responses (27). CT signaling, however, differs from PGE₂ signaling, as PGE₂ by itself is unable to induce maturation (11) whereas CT does induce maturation (4) although
not always completely (data not shown). The different mechanisms by which CT and PGE\textsubscript{2} on the one hand, and SEA on the other hand prime for DC2 is underscored by the finding that SEA barely upregulated intracellular cAMP (van der Kleij et al., unpublished observations).

The capability of PT to prime for DC1 with high IL-12 production, as has also been shown previously in mice in vivo (28), may be explained by the inhibition of Gi protein signaling (29). Thus far, it is unknown how polymerized dsRNA (poly I:C) activates DC to mature into effector DC1. Possibly it signals through a toll like receptor, as has been shown for bacterial DNA motifs and TLR9 (30).

Fully matured effector DC are resistant to repolarization by microbial stimuli (31) or cytokines (12, 20). This implies that effector DC primed by a certain microbe, are not subject to subsequent cross-modulation by the priming abilities of other pathogens, thereby mediating effective immunity to the firstly encountered pathogen.

The current findings suggest that, analogous to the development of polarized Th cell subsets from a single precursor population, human DC are guided by the conditions of their maturation to acquire stable polarized functional effector DC1 or DC2 phenotypes. The present study supports the concept that the type of immune response is optimally adapted to the character of the pathogen via the priming of sentinel DC into effector DC subsets with unexpectedly diverse functional phenotypes and expression of signal 3.

**Acknowledgments**

This work was financially supported by Fundação para a Ciência e a Tecnologia, Lisbon, Portugal (grant PRAXIS XXI/BD/9195/96 to P.L.V.).

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