Microbial Recognition Via Toll-Like Receptor-Dependent and -Independent Pathways Determines the Cytokine Response of Murine Dendritic Cell Subsets to CD40 Triggering

Alexander D. Edwards, Shivanthi P. Manickasingham, Roman Spörri, Sandra S. Diebold, Oliver Schulz, Alan Sher, Tsuneyasu Kaisho, Shizuo Akira and Caetano Reis e Sousa

*J Immunol* 2002; 169:3652-3660; doi: 10.4049/jimmunol.169.7.3652

http://www.jimmunol.org/content/169/7/3652

---

**References** This article *cites 48 articles*, 26 of which you can access for free at: http://www.jimmunol.org/content/169/7/3652.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2002 by The American Association of Immunologists All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Microbial Recognition Via Toll-Like Receptor-Dependent and -Independent Pathways Determines the Cytokine Response of Murine Dendritic Cell Subsets to CD40 Triggering

Alexander D. Edwards,* Shivanthi P. Manickasingham,* Roman Spörrı,* Sandra S. Diebold,* Oliver Schulz,* Alan Sher,† Tsuneyasu Kaisho, §§ Shizuo Akira, ‡ and Caetano Reis e Sousa 2*

Dendritic cells (DC) can produce Th-polarizing cytokines and direct the class of the adaptive immune response. Microbial stimuli, cytokines, chemokines, and T cell-derived signals all have been shown to trigger cytokine synthesis by DC, but it remains unclear whether these signals are functionally equivalent and whether they determine the nature of the cytokine produced or simply initiate a preprogrammed pattern of cytokine production, which may be DC subtype specific. Here, we demonstrate that microbial and T cell-derived stimuli can synergize to induce production of high levels of IL-12 p70 or IL-10 by individual murine DC subsets but that the choice of cytokine is dictated by the microbial pattern recognition receptor engaged. We show that bacterial components such as CpG-containing DNA or extracts from Mycobacterium tuberculosis predispense CD8α+ and CD8α−CD4− DC to make IL-12 p70. In contrast, exposure of CD8α+, CD4+ and CD8α+CD4− DC to heat-killed yeasts leads to production of IL-10. In both cases, secretion of high levels of cytokine requires a second signal from T cells, which can be replaced by CD40 ligand. Consistent with their differential effects on cytokine production, extracts from M. tuberculosis promote IL-12 production primarily via Toll-like receptor 2 and an MyD88-dependent pathway, whereas heat-killed yeasts activate DC via a Toll-like receptor 2-, MyD88-, and Toll/IL-1R domain containing protein-independent pathway. These results show that T cell feedback amplifies innate signals for cytokine production by DC and suggest that pattern recognition rather than ontology determines the production of cytokines by individual DC subsets. The Journal of Immunology, 2002, 169: 3652–3660.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Institute of Immunology, Cancer Research U.K., London Research Institute, London, United Kingdom; 2 Immunobiology Laboratory, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; 3 Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita City, Osaka, Japan; and 4 RIKEN Research Center for Allergy and Immunology, Yokohama City, Japan.

Received for publication April 18, 2002. Accepted for publication July 26, 2002.

1 This work was supported by Cancer Research U.K.
2 Address correspondence and reprint requests to Dr. Caetano Reis e Sousa, Immunobiology Laboratory, Cancer Research U.K., London Research Institute, Lincoln’s Inn Fields Laboratories, 44 Lincoln’s Inn Fields, London WC2A 3PX, U.K. E-mail address: caetano@cancer.org.uk
3 Abbreviations used in this paper: PRR, pattern recognition receptor; PPD, purified protein derivative of Mycobacterium tuberculosis; DC, dendritic cell; STAg, soluble tachyzoite Ag; TLR, Toll-like receptor; CD40L, CD40 ligand; DN, double negative; TIRAP, Toll/IL-1R domain containing protein.

Copyright © 2002 by The American Association of Immunologists, Inc.
Materials and Methods

Animals

Male and female 6- to 10-wk-old mice were obtained from Charles River (Margate, U.K.), Harlan U.K. (Bicester, Oxon, U.K.) or from the breeding unit of Cancer Research U.K. (Clare Hall, South Mimms, U.K.). C57BL/6 (B6), B10.BR, BALB/c, C3H/HeN, and C3H/HeJ mice were used interchangeably, after determining that the responses studied were strain independent (not shown). The strain used for each experiment is indicated in each figure legend. DO11.10 mice (14) on a BALB/c-scid background were bred at Cancer Research U.K.

To analyze DC genetically deficient for MyD88 or TLR2, bone marrow chimeras were made by reconstituting lethally irradiated irradiated C45.1 B6.SJL mice with congenic bone marrow from C45.2 TLR2−/− or MyD88−/− mice on a C57BL/6 × 129 background (15, 16). Control chimeras were made with bone marrow taken from control C57BL/6 mice. DC were purified from the spleens of recipients 5–8 wk after reconstitution (17).

Reagents

Soluble tachyzoite Ag (STAg) was prepared from tachyzoites of the RH 88 strain of Toxoplasma gondii (12). Zymosan (Sigma, Poole, U.K.) was boiled for 30 min and washed twice in PBS. Escherichia coli LPS was a gift from Dr. S. Vogel (Unified Services Experiment of the Health Sciences, Bethesda, MD). CpG-containing DNA was a phosphorothioate-linked oligonucleotide with the sequence TCC ATG ACG TTC (19) were made by the Cancer Research U.K. peptide synthesis service. Endotoxin levels in all reagents were significantly lower than the minimum required for DC activation.

Cells

The cell lines 3T3-CD40L and 3T3-SAMEN (control) were a gift from Dr. P. Hwu (National Cancer Institute, Bethesda, MD) and were derived from NIH 3T3 by stable transduction with murine CD40L or empty vector.

Spleen cell suspensions were prepared by Liberase CI (Roche Diagnostics, Lewes, U.K.) and Dnase I digestion (13). DC-enriched fractions were prepared by labeling splenocytes with anti-CD11c MACS beads (Miltenyi, Bisley, U.K.) for 10 min at 4°C, followed by washing and positive selection using LS magnetic columns (Miltenyi Biotec), as described (13). Resulting preparations contained 70–95% CD11c+ DC. To obtain DC subsets, CD11c-enriched preparations were further stained with PE-anti-CD8 (clone 53–6.7), biotinylated anti-CD4 (clone RM4-5), or Cy-chrome-conjugated anti-CD86 (clone 3/23) and sorted for CD11cbright CD45.1−/H11003 two–ME (5×105 DC/well in six-well plates). Cells were harvested and coated with anti-IL-10 capture reagent, then recultured at 2.5×105 DC/well in six-well plates with fresh fibroblasts or with zymosan (50 μg/ml) plus CD40L-expressing fibroblasts for 4 h in 24-well plates. Cells were harvested and coated with anti-IL-10 capture reagent, then recultured at 2.5×105 DC/well in six-well plates with fresh fibroblasts ± zymosan stimulation as above. After 60–100 min, cells were harvested once more, washed in PBS containing 2 mM EDTA plus 1% FCS, and stained with PE-conjugated anti-IL-10 (Miltenyi Biotec) and APC-conjugated anti-CD86.

Intracellular staining for IL-12 was performed as described (13) using anti-IL-12 p40 (clone C17.15.10), anti-IL-12 p70 (clone 9A5) or a mixture of isotype-matched RtfG2a and RtfG2b irrelevant control Abs, followed by biotinylated mouse anti-rtat (Jackson ImmunoResearch Laboratories, West Grove, PA) and PE- or APC-streptavidin (BD Pharmingen).

Cell acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Results

Activation of DC by microbial stimuli

Several microbial stimuli were tested for their ability to act as activators of primary mouse DC in vitro. DC-enriched spleen cells were plated with or without test stimuli, and expression of the activation markers CD40, CD80, and CD86 was measured on CD11c+ cells after overnight culture. Culture alone was sufficient to induce up-regulation of CD40, CD80, and CD86 (not shown). Nevertheless, a wide range of products from bacteria, fungi, or protozoa were able to increase CD40, CD80, and CD86 expression further (Fig. 1). They included mycobacterial PPD, heat-inactivated yeasts (Saccharomyces cerevisiae, S. pombe) and zymosan (yeast cell walls), as well as established murine DC activators such as STAg (12) and CpG-containing DNA oligonucleotides (CpG DNA) (Fig. 1). All test agents induced CD40, CD80, and CD86 up-regulation to a similar extent in DC from control C3H/HeJ— and TLR4-deficient C3H/HeL mice, demonstrating that they did not contain endotoxin (data not shown).

DC cultures and cytokine assays

For in vitro stimulation, MACS-enriched or FACS-sorted DC were cultured in 96-well flat-bottom plates alone or on a monolayer of CD40L-expressing or control fibroblasts. Cultures were incubated in the presence or absence of different stimuli in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 2-ME (5×10−5 M). Culture supernatants were collected at 18–24 h and assayed for the presence of cytokines by sandwich ELISA. Ab pairs were captured, detected: 9A5, C17.8 (biotinylated) for IL-12 p70; JES5-2A5, SXC-1 (biotinylated) for IL-10. Cells were recovered in PBS plus 5 mM EDTA for FACS analysis.

Antibody staining

For analysis of DC maturation, cells were washed and stained in PBS containing 5 mM EDTA, 1% FCS, and 0.02% sodium azide (FACS wash). Cells were stained with FITC-conjugated anti-CD40 or anti-CD86 plus PE-conjugated anti-CD11c in the presence of 5 μg/ml anti-Fe-RiBiIII. In some experiments, biotinylated anti-CD40 was used, followed by streptavidin conjugated to an appropriate fluorophore. The mAbs used were: IL3, IL6, and 16-10A1, hamster IgG2a mAbs against CD11c and CD80, respectively; NLDC-145, RM4-5, 53-6.7, 3/23, and GL1, rat IgG2a mAbs against DEC-205, CD4, CD8α, CD40, and CD86, respectively; JES5-2A5, rat IgG1 neutralizing mAb against IL-10; A20, mouse IgG2a mAb against CD45.1. All mAbs were from BD Pharmingen (San Diego, CA) or produced in house.

Single cell staining for IL-10 was performed using the mouse IL-10 secretion assay kit (Miltenyi Biotec). CD11c-enriched spleen cells were divided into two fractions. One half was stained with FITC-anti-CD11c and kept live; the remainder was stained with Tricolor-anti-CD11c and then fixed by treating with 1% paraformaldehyde in PBS for 10 min at room temperature, followed by quenching with 1 mM glycine in PBS. A 1:1 mixture of live and fixed cells in medium was then cultured with control fibroblasts or with zymosan (50 μg/ml) plus CD40L-expressing fibroblasts for 4 h in 24-well plates. Cells were harvested and coated with anti-IL-10 capture reagent, then recultured at 2.5×105 DC/well in six-well plates with fresh fibroblasts ± zymosan stimulation as above. After 60–100 min, cells were harvested once more, washed in PBS containing 2 mM EDTA plus 1% FCS, and stained with PE-conjugated anti-IL-10 (Miltenyi Biotec) and APC-conjugated anti-CD86.

FIGURE 1. DC activation by different microbial stimuli. CD11c-enriched B6 or BALB/c spleen cells were cultured overnight with the indicated agents and then stained for CD11c, CD40, CD80, or CD86 and analyzed by flow cytometry. Histograms show gated CD11c+ cells. Increased CD40, CD80, and CD86 expression was seen on DC cultured with test stimuli (solid lines) vs medium alone (dotted line). Doses: CpG DNA, 1.5 μg/ml; STAg, 5 μg/ml; S. pombe, 2×106 particles/well; zymosan, 50 μg/ml; PPD, 10 μg/ml. Data shown are from multiple experiments. Similar results were seen in more than four experiments performed with each stimulus in various mouse strains.
Microbial stimuli dictate the cytokine response of DC to CD40 triggering

STAg and CpG DNA by themselves elicited modest levels of IL-12 p70 (<1 ng/ml) but no IL-10 from DC-enriched splenocyte populations (Fig. 2A). However, little accumulation of IL-12 p70 or IL-10 in culture supernatants was seen in response to any of the other stimuli (Fig. 2A, top). Because IL-12 p70 production by DC is markedly dependent on a second T cell-derived signal (13), we assessed the effect of CD40 coligation on the cytokine response. Culturing DC-enriched splenocytes on a monolayer of CD40L-expressing fibroblasts was sufficient to induce low levels of IL-12 p70 and IL-10 (Fig. 2A). Addition of STAg and CpG DNA caused a significant increase in IL-12 p70 but not in IL-10 levels (Fig. 2A, bottom). Similar results were obtained with PPD, although IL-12 p70 production in response to PPD was consistently lower than to STAg or CpG DNA (Fig. 2A). In contrast, the combination of zymosan or S. pombe together with CD40L led to an increase primarily in IL-10 (Fig. 2A, bottom). Similar induction of IL-10 was seen with a pathogenic yeast, Candida albicans, in combination with CD40L (data not shown). Neutralizing Ab to IL-10 did not increase the levels of IL-12 p70 in response to CD40L plus yeasts or zymosan (see below). When IL-12-promoting stimuli (e.g., STAg DNA) and IL-10-promoting zymosan were combined in the presence of CD40L, there was a significant decrease in IL-12 production (Fig. 2B), consistent with the known ability of IL-10 to suppress IL-12 synthesis, and a slight decrease in IL-10 production (Fig. 2B). These results demonstrate that CD40 triggering in DC does not inevitably lead to IL-12 synthesis but can reveal production of IL-10.

To examine whether physiological levels of T cell feedback signals could substitute for CD40L-expressing fibroblasts, naive TCR transgenic T cells were cultured with DC-enriched populations ± Ag in the presence of IL-12- or IL-10-promoting stimuli. In the absence of microbial stimuli, IL-12 p70 or IL-10 levels were low or undetectable even after T cell activation by Ag (Fig. 2C). Microbial stimulation in the absence of Ag elicited only low levels of IL-12 p70 or IL-10. However, in the presence of OVA peptide, the yeasts triggered accumulation of IL-10 but not of IL-12 p70, whereas the opposite was seen with PPD and STAg (Fig. 2C). As expected, cytokine accumulation in supernatants was Ag dose dependent (Fig. 2C). These results using naive T cells agree with the data obtained with CD40L-expressing fibroblasts. However, anti-CD40L did not entirely block the effect of T cells in this system (R. Spörri and C. Reis e Sousa, unpublished observations), implying that other molecules expressed by naive T cells after activation can also provide feedback signals to amplify DC cytokine production. Inclusion of the microbial stimuli did not affect subsequent T cell proliferation although it had marked effects on Th differentiation (S. P. Manickasingham, A. D. Edwards, and C. Reis e Sousa, manuscript in preparation).

Direct recognition of microbes by DC via TLR-dependent and -independent pathways

The use of partially purified DC preparations in the experiments described above raised the possibility that the measured cytokines were produced by contaminating leukocytes or indirectly by DC in response to signals made by the contaminating cells. To address this issue, CD11c<sup>bright</sup> pure DC were sorted by FACS and stimulated with microbial products in the presence of CD40L-expressing fibroblasts. Purified DC responded to CpG DNA and PPD by producing IL-12 p70 but only small amounts of IL-10, whereas the converse was seen with zymosan (Fig. 3, A and B). These results

---

**FIGURE 2.** CD40L or T cell feedback leads to production of either IL-12 p70 or IL-10 by DC following microbial activation. A, CD11c-enriched BALB/c spleen cells (70% DC; 4 × 10<sup>5</sup>/well) were cultured overnight on a monolayer of CD40L-expressing or control fibroblasts together with DC-activating agents. Doses: STAg, 5 µg/ml; CpG DNA, 1 µg/ml; PPD, 10 µg/ml; zymosan, 10 µg/ml; S. pombe, 10<sup>6</sup> particles/well. B, DC as in A were cultured on a monolayer of CD40L-expressing fibroblasts together with CpG DNA (1 µg/ml) and/or zymosan (10 µg/ml). C, DC as in A were cultured with DO11.10 naive T cells (2 × 10<sup>5</sup> total cells/well; DC:T cell ratio, 1:1) in the presence or absence of STAg (1 µg/ml), PPD (5 µg/ml), or S. pombe (8 × 10<sup>4</sup> particles/well). OVA peptide was added as indicated, and the cells were cultured overnight. IL-12 p70 and IL-10 in culture supernatants were measured by ELISA. Histograms represent the mean of triplicate wells; all error bars are shown and represent 1 SD from the mean. N.D. (not detectable) indicates that the value was below the detection limit of the ELISA (<100 pg/ml IL-12 p70; <100 pg/ml IL-10). Data are representative of more than four experiments for each stimulus with CD40L-expressing fibroblasts and of three experiments with T cell feedback and with mixtures of stimuli.
demonstrate that DC themselves can directly recognize and discriminate among microbial stimuli and can produce either IL-10 or IL-12 in response to the appropriate combination of signals.

MyD88 is a critical adapter for the transduction of signals from many TLRs, including TLR9 and TLR2, which have been implicated in innate recognition of CpG DNA and zymosan, respectively (20–22). We examined whether MyD88 was required for the response to CpG DNA, PPD, or zymosan. MyD88-deficient DC were purified from the spleens of mice reconstituted with bone marrow from MyD88−/− mice and were compared with DC purified from control chimeras (see Materials and Methods). As shown in Fig. 3A, MyD88−/− DC did not make IL-12 p70 in response to CpG DNA and PPD plus CD40L. In contrast, MyD88 deficiency did not affect the IL-10 response to zymosan plus CD40L (Fig. 3A). Likewise, the up-regulation of CD40 or CD86 in response to zymosan alone was MyD88 independent although, as expected, this adapter was critical for the response to CpG DNA (Fig. 3C). A similar comparison revealed that TLR2 was also not necessary for IL-10 production in response to zymosan plus CD40L (Fig. 3B). However, TLR2−/− DC made significantly lower levels of IL-12 p70 in response to PPD plus CD40L (Fig. 3B). This was not due to a general defect in the ability of such DC to make IL-12 p70 because the same cells mounted a normal response to CpG DNA (Fig. 3B).

An additional adapter in TLR signaling, TIRAP/MAL, has been described (19, 23). As expected, a TIRAP-inhibitory peptide but not a control peptide containing the reversed TIRAP sequence (19) abrogated the LPS-induced up-regulation of CD86 by DC (Fig. 3D). However, the TIRAP-inhibitory peptide did not affect yeast conditioning for IL-10 production (Fig. 3D). These results demonstrate that TLR2 and MyD88 signaling mediate PPD conditioning of DC for CD40-triggered IL-12 p70 production but suggest that TLR signaling is not involved in conditioning by yeasts for IL-10 production.

**Plasticity of DC subsets**

Spleen DC contain several subsets, which may possess distinct abilities to produce cytokines (24, 25). We addressed the ability of three of these subsets to make IL-10 vs IL-12. Homogeneous populations of CD11c+CD8α+CD4+ and CD8α−CD4− (double-negative; DN) DC were isolated by cell sorting (Fig. 4A). In the presence of control fibroblasts, all DC subsets produced only negligible amounts of IL-12 p70 or IL-10 (not shown). Coculture with CD40L-expressing fibroblasts alone was sufficient to increase the basal production of IL-12 p70 by CD8α+ DC and of IL-10 by all subsets (Fig. 4B). Nonetheless, basal cytokine levels were markedly altered by addition of a microbial costimulus. CD8α+ and DN DC produced primarily IL-12 p70 in response to CpG DNA, STAg, or PPD in combination with CD40L although DN DC produced less IL-12 p70 than CD8α+ DC (Fig. 4B). Both CD4+ subsets also increased production of IL-10 in response to the combination of zymosan or S. pombe plus CD40L (Fig. 4B). This was less obvious for CD8α− DC, which displayed the highest level of basal IL-10 (and IL-12 p70) production in response to CD40L alone but remained statistically significant (p < 0.01) (Fig. 4B). Zymosan also triggered a small amount of IL-12 p70 (Fig. 4B). Interestingly, CD4+ DC failed to make IL-12 p70 in response to any stimulus although they responded to yeast or zymosan plus CD40L by producing high levels of IL-10 (Fig. 4B).
fixed CD8α+ DC in the same cultures did not stain for the cytokine (Fig. 5A). However, both live and fixed cells could be stained to the same extent if incubated with exogenous IL-10 (Fig. 5A). In all cases, there was no staining above background when the capture reagent was omitted (not shown). These controls demonstrate that IL-10 staining in live cells accurately reflects cytokine production by individual CD8α+ DC and, under the conditions used here, is not marred by paracrine effects. To determine the frequency of CD8α+ DC producing IL-12, intracellular staining was conducted for the p40 subunit after stimulation with CpG plus CD40L. Compared with baseline staining with an isotype-matched control Ab, essentially all CD8α+ DC stained for IL-12 p40 (Fig. 5B). In other experiments, ~15% of CD8α+ DC could also be stained for the bioactive heterodimer, IL-12 p70 (Fig. 5B).

Unlike CD8α+ DC, CD4+ DC were unable to produce IL-12 p70 (Fig. 4B). To determine whether this was due to autocrine effects of IL-10, DC subsets were cultured on CD40L-expressing fibroblasts in the presence of neutralizing anti-IL-10 Ab. Anti-IL-10 led to an increase in IL-12 p70 production by both CD8α+ and DN DC in response to PPD, which served as a positive control for neutralizing activity. However, IL-10 neutralization did not reveal the ability of CD4+ DC to make IL-12 p70 (Fig. 6). Anti-IL-10 also did not change the nature of the response to zymosan or S. pombe in that the yeasts still failed to condition mixed DC populations or the CD8α+ and DN DC to make IL-12 p70 (Fig. 6). These results confirm that both CD8α+ and DN DC are able to produce either IL-12 or IL-10 and show that IL-10 is not responsible for the lack of IL-12 secretion by CD4+ DC.

**Differential responses to microbial stimulation are not attributable to quantitative differences in DC subset activation**

Differences in the cytokine response of DC subsets to yeasts vs IL-12-promoting stimuli could conceivably arise from quantitative differences in DC activation. In that scenario, the degree rather than the quality of DC activation might determine IL-12 vs IL-10 production in response to CD40 cross-linking. This could be especially relevant because cytokine secretion requires CD40 signaling and CD40 expression is altered by the microbial stimulus itself (Fig. 1). To examine whether the quantity of DC activation affected cytokine production, we chose two prototype IL-12 p70- and IL-10-promoting stimuli (PPD and zymosan, respectively) and examined their ability to 1) induce up-regulation of CD40 and CD86 and 2) promote IL-12 and IL-10 production across a whole dose-response range. There were no obvious differences among DC subsets in sensitivity to either zymosan or PPD in terms of CD86 up-regulation, although CD8α+ DC were more sensitive to PPD than either CD4+ or DC DN DC when assessed for CD40 expression (Fig. 7A). Importantly, PPD conditioned CD8α+ and DN DC to make IL-12 p70 across the entire dose range (Fig. 7B). Similarly, zymosan conditioned all three subsets of DC to produce IL-10 at all doses (Fig. 7B). The fact that at no point in the dose-response did PPD become an IL-10 inducer or zymosan become an IL-12-promoting stimulus (Fig. 7B), demonstrates that qualitative rather than quantitative differences in microbial recognition lie at the heart of differential cytokine responses by DC subsets.

**Discussion**

Cytokines made by APC play a critical role in responses to infection. Here, we demonstrate four distinct features of cytokine production in murine DC. 1) The activation of individual DC subsets does not result in a preprogrammed pattern of cytokine secretion; it can lead to IL-10 or IL-12 production by CD8α+ and DN DC and to IL-10 production or lack thereof by CD4+ DC. 2) Cytokine
production can be dictated by direct microbial recognition. Microbial
structures from Toxoplasma and Mycobacterium or CpG-contain-
ing DNA condition DC to make IL-12 p70. In contrast, heat-killed
yeasts (brewers’ yeast, fission yeast, C. albicans) or yeast
derivatives (zymosan) condition DC to make primarily IL-10. 3) Differen-
tial conditioning is dependent on distinct PRR; PPD and
CpG DNA act through TLRs and an MyD88-dependent pathway,
whereas yeasts act via a TLR-independent pathway. 4) PRR sig-
naling results in limited cytokine production unless it is followed
by signals from T cells which amplify DC activation but do not
alter the type of cytokine that is made.

TLRs have emerged as key players in DC activation (2, 3).
Consistent with this notion, the IL-12-promoting effects of PPD
and STAg are absolutely dependent on signaling via MyD88 (Fig.
3 and Ref. 26). PPD contains traces of bacterial DNA, and some of
its IL-12-promoting activity can be removed by DNase treatment
(A. D. Edwards and C. Reis e Sousa, unpublished observations).
However, most of the DC response to PPD appears to be due to
TLR2 triggering (Fig. 3C), consistent with the fact that this TLR is
involved in recognition of mycobacterial lipoarabinomannan and
mannosylated phosphatidylinositol (27–29). TLR2 has also been
implicated in the activation of macrophages by zymosan (21, 22).
Given that DC express functional TLR2 (as determined by its in-
volve ment in PPD recognition), it is therefore surprising that zy-
mosan failed to condition DC for IL-12 production even when
IL-10 was neutralized (Fig. 6). It is possible that zymosan recogni-
tion in macrophages involves heterodimerization with another
TLR, which is not expressed on mouse DC. Alternatively, recog-
nition of zymosan by an IL-10-promoting PRR on DC is domi-
nant over TLR2 recognition of the same particle and overwhelmingly
conditions the cells for IL-10 production. Indirect support for this
hypothesis comes from two observations: 1) in some experiments,
the combination of zymosan plus CD40L slightly increased the
level of IL-12 production compared with CD40L alone (e.g. Fig.
4B); 2) TLR2−/− DC reproducibly made more IL-10 in response
to zymosan plus CD40L than controls, as if the IL-10-promoting
PRR was now acting unopposed (Fig. 3B). The identity of the
IL-10-promoting PRR for yeasts on DC is unknown at present, but
it is unlikely to belong to the TLR family as it does not signal via
MyD88 or TIRAP/MAL (Fig. 3). This would be consistent with
the notion that TLR signaling is involved primarily in induction of
IL-12 and type 1 adaptive immune responses (4).

We have previously suggested that CD40 signaling in DC acts
to amplify innate signals for IL-12 production (13). Here, we show
that CD40L is just as critical for amplifying IL-10 secretion (Fig.
2). This may seem contrary to the prevalent view that CD40 sig-
naling leads to IL-12 production by DC (30, 31). However, CD40
signaling also induces IL-10 in monocyte-CSF-primed monocytes
(32) and in human monocyte-derived DC exposed to glucocorti-
coids (33). Furthermore, our data fit with recent experiments show-
ing that CD40 expression by bone marrow-derived DC is critical
for priming of a Th2 response to Schistosoma mansoni eggs (34).
Thus, CD40L and other T cells signals appear to act as neutral
amplifiers that are critical for execution of cytokine production
programs initiated by pattern recognition. Indeed, analysis of a
large panel of IL-12-promoting stimuli shows that, like PPD, most
trigger production of the IL-12 p40 subunit but induce little IL-12
p70 until combined with CD40L (A. D. Edwards and C. Reis e
Sousa, unpublished observations). Apparent exceptions are CpG
DNA and STAg, which can induce production of bioactive IL-12
p70 by murine CD8α+ and DN DC in the absence of T cell signals (Fig. 2 and Refs. 12, 13). However, CD40 ligation still increases greatly IL-12 p70 production in response to these two stimuli (Fig. 2 and Ref. 13).

The ability of microbes to influence the production of IL-12 or IL-10 is not without precedent. Gram-negative bacteria preferentially induce IL-10 production in monocytes, whereas Gram-positive bacteria induce IL-12 (35). A recent report shows that a protein from *Bordetella pertussis* induces IL-10 production in DC (36). However, our use of inactivated microbes and microbial extracts as tools to dissect DC biology raises the question of whether our findings are of significance to natural infections with intact organisms. Production of IL-12 by DC in response to STAg, PPD, or CpG DNA has obvious implications for the development of protective type 1 immune responses to *Toxoplasma, Mycobacterium*, and other bacteria. The significance of IL-10 production in response to heat-killed yeasts is less clear. Protection from murine and human candidiasis involves primarily Th1-biased responses, even though a role for IL-10 has been suggested (37). Live *Candida* yeasts trigger IL-12 production by splenic DC (38), and live recombinant yeasts elicit IL-12 production by bone marrow-derived DC (39). Thus, conditioning of DC for IL-10 production in our experiments could reflect preferential destruction by heat treatment of IL-12-inducing yeast components.

It has been argued that different DC subtypes are specialized to make different cytokines and drive distinct forms of T cell differentiation (7). In support of this notion, human blood contains monocytes and plasmacytoid cells that can generate distinct DC1 or DC2 that prime Th1 or Th2 responses, respectively (40). These cells also express distinct TLR repertoires and respond to different microbial stimuli (8–10). In mouse, CD8α+ and CD8α− subsets of spleen DC differ in their ability to make IL-12 in vitro and in vivo and to prime Th1 and Th2 responses in vivo (12, 13, 24, 25, 41, 42). However, there is also evidence that individual DC types are not necessarily precommitted DC1 or DC2. In both mice and humans, the ability of DC to produce IL-12 p70 and prime Th1 responses can be modulated by exposure to cytokines (24, 43, 44). In addition, mouse DC can produce either IL-12 or IL-4 in response to different forms of the fungus *C. albicans* and direct Th1 or Th2 development (38). Similarly, murine bone marrow-derived DC treated with LPS or Gram-negative bacteria preferentially prime Th1 responses while cells exposed to certain worm products direct Th2 development (34, 45). In the human system, pathogen products have also been shown to dictate the cytokine producing and Th-skewing capacity of monocyte-derived DC (46). Even so-called DC2 plasmacytoid cells can make IL-12 in response to CpG DNA plus CD40L (9) and can prime Th1 responses after exposure to viruses (47, 48), arguing that their Th2-directing ability is not hardwired. Consistent with the notion of flexibility, here we show that DN and CD8α+ DC subsets have a choice of effector cytokines. It remains possible that these DC subsets are heterogeneous and contain DC precommitted to either IL-12 p70 or IL-10 production. However, using single-cell staining methods, we were
able to show that up to 30% of CD8α+ DC could produce IL-10 when given zymosan plus CD40L (Fig. 5A), and >90% of CD8α+ DC could make IL-12 p40 in response to an appropriate combination of stimuli (Fig. 5B and data not shown). Therefore, we suppose that at least 15–20% of CD8α+ DC are bipotent for IL-10 and IL-12 p40 production. This is probably an underes- timate, as the staining method for IL-10 involves cell resuspension, which disrupts continued CD40 engagement and stops cytokine synthesis (A. D. Edwards and C. Reis e Sousa, unpublished ob- servations). Whether bipotentiality extends to IL-12 p70 production could not be unambiguously determined as only up to 15% of CD8α+ DC can be stained for the cytokine (Fig. 5). However, IL-12 p70 is produced at 10- to 50-fold lower levels than IL-12 p40 (49) even after CD40-dependent up-regulation of IL-12 p35 (13) and is extremely difficult to detect by staining. Therefore, again, our staining is likely to provide only a gross underestimate of the true frequency of IL-12 p70-producing cells. Thus, we favor the notion that most CD8α+ and DN DC have the potential to produce alternative cytokines in response to distinct stimuli. This does not exclude the possibility that some DC subsets may have functional specializations, exemplified by the apparent inability of CD4+ DC to produce IL-12 p70 in response to a large panel of stimuli (Figs. 4, 6, and 7 and data not shown), also seen by Hochrein et al. (25). CD4+ DC represent ~50% of splenic DC and constitute the majority of DC in the CD8α- DC fraction, which may underlie the observed tendency of CD8α- DC to induce Th2 responses (24, 41, 42). However, even CD4+ DC do not have a predefined response to activation: they are activated to a similar degree by zymosan and PPD as measured by CD40 and CD86 up-regulation, yet yzmoson conditions the cells to make IL-10 whereas PPD does not (Fig. 7). Altogether, these results suggest a model in which all DC irrespective of subset can behave as flexible APC. The fact that innate signals and T cell feedback are both necessary and sufficient for cytokine production by all DC subsets can, therefore, explain Th polarization by these APC without in- volving the participation of third-party cells or cytokines.

Acknowledgments

We thank A. Lehmann and C. Lehane for S. cerevisiae and S. pombe cultures and S. Vogel for LPS. We are grateful to C. Simpson, A. Eddaudogi, and G. Warnes for cell sorting and G. Hutchinson and J. Bee for animal care. We thank N. Hogg and D. Cantrell for comments on the manuscript.

References


