Selective maturation of dendritic cells by *Nippostrongylus brasiliensis*-secreted proteins drives Th2 immune responses

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Helminth infections at mucosal and tissue sites strongly polarize towards Th2 immune responses, following pathways which have yet to be elucidated. We investigated whether dendritic cells (DC) exposed to gastrointestinal nematodes induce Th2 differentiation and, if so, whether this outcome reflects the absence of DC activation (the default hypothesis). We studied secreted proteins from the parasite *Nippostrongylus brasiliensis*, which induce Th2 development *in vivo* without live infection. Murine bone marrow-derived DC pulsed with *N. brasiliensis* excretory/secretory antigen (NES) can, on transfer to naive recipients, prime mice for Th2 responsiveness. Heat inactivation of NES abolishes both its ability to drive Th2 responses *in vivo* and its capacity to stimulate DC for Th2 induction. NES, but not heat-inactivated NES, up-regulates DC maturation markers associated with Th2 promotion (CD86 and OX40L), with little change to CD80 and MHC class II. Moreover, DC exposed to NES readily produce IL-6 and IL-12p40, but not IL-12p70. LPS induced high IL-12p70 levels, except in DC that had been pre-incubated with NES. These data contradict the default hypothesis, demonstrating that a helminth product (NES) actively matures DC, selectively up-regulating CD86 and OX40L together with IL-6 production, while blocking IL-12p70 responsiveness in a manner consistent with Th2 generation *in vivo*.

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1 Introduction

The close association between helminth infection and the expression of Th2 modes of response such as IgE, eosinophilia and type 2 cytokines has frequently been documented [1–3]. Strong Th2 responses are induced by almost every helminth organism, even though such reactivities may not be appropriate in all circumstances [4–6]. The generic nature of the type 2 bias to helminths has engendered two contending hypotheses. One is that the innate immune system recognizes conserved helminth-associated molecular patterns, preferentially triggering a Th2 response, in a mirror image to the toll-like receptor (TLR) pattern recognition mechanism which initiates Th1 responses [7]. However, no common helminth molecular pattern or recognition receptor has yet been identified. Alternatively, the very lack of stimulation of the innate guardian system by helminths could result in a 'default' response of the Th2 type [8, 9]. Neither hypothesis addresses the further possibility that successful helminth species manipulate the Th1/Th2 balance, to take advantage of the anti-inflammatory aspects of type 2 responses, and may preferentially direct the Th2 pathway [9, 10].

The principal sentinels of the innate immune system are the dendritic cells (DC), which from an early stage process and present exogenous antigens [11, 12]. DC are highly sensitive to generic molecular patterns in microbial pathogens and act afferently to convey signals about the nature of a pathogen [13–16]. Prokaryotic organisms stimulate DC, particularly through ligation of TLR, up-regulating functional surface molecules such as MHC class II and CD40 and releasing Th1-promoting cytokines IL-12, IL-18, and IL-27 [17–19]. Some eukaryotic organisms, including *Toxoplasma gondii*, stimulate DC in a similar manner, eliciting Th1 responses from the adaptive immune system as a consequence [20].

The genesis of a Th2 response is relatively poorly understood. DC can mediate differentiation of naive T cells into Th2 cells after stimulation with certain helminth-derived factors, such as excretory/secretory

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Abbreviations: hiNES: Heat-inactivated NES **LNC:** Lymph node cells **MDC:** Macrophage-derived chemokine **NES:** *Nippostrongylus brasiliensis* excretory/secretory antigen **PLNC:** Popliteal LNC **RT:** Reverse transcription **SEA:** *Schistosoma mansoni* egg antigen **TLR:** Toll-like receptor

products of the filarial nematode *Acanthocheilonema viteae* [21], soluble egg antigen derived from *Schistosoma mansoni* (SEA) [22] or the schistosome glycan lacto-N-fucopentaose III [23]. However, in these cases, DC maintained a phenotype similar to that of unstimulated DC, and no significant increase in Th2-skewing cytokines (IL-4, IL-10) was detected. Hence, no mechanistic link has been found to compare with the release of Th1-promoting cytokines from DC exposed to bacterial-derived factors. These findings engendered the hypothesis that Th2 immune responses arise as a "default pathway" when antigen presentation occurs in the absence of DC maturation.

Among the most potent stimuli for Th2 differentiation *in vivo* is infection with the rodent gastrointestinal nematode *Nippostrongylus brasiliensis* [24–27]. These organisms can be cultivated *in vitro*, and the glycoproteins they secrete are themselves able to drive Th2-biased responses [28]. Adult *N. brasiliensis* infests the gastrointestinal tract, which unlike the tissue niches of filarial and schistosomal parasites are rich in pro-inflammatory microbial products. As infection with gastrointestinal nematodes causes disruption of normal gut architecture, the Th2 response evolves in spite of exposure to pro-inflammatory microbial products, arguing against the default hypothesis. Indeed, immunization of mice with *N. brasiliensis* excretory/secretory antigens (NES) provokes a Th2 immune response when administered either as a soluble protein or with Complete Freund's Adjuvant (CFA), which normally biases towards Th1 development. Furthermore, co-administration of NES with a model antigen, such as hen egg lysozyme, in the absence of other adjuvants, results in a Th2 response specific to this coincident antigen, demonstrating that NES acts as a Th2-promoting adjuvant [28].

The Th2-promoting effects of NES are heat labile, as heat-inactivated NES (hiNES) loses its Th2-driving activity, and Th2 induction is also abolished by treatment with proteinase K [28]. The ability of a soluble protein fraction to reproduce the effects of natural infection has allowed us, in the studies reported below, to demonstrate active stimulation of DC by this Th2-inducing helminth product, reflected in selective up-regulation of surface markers and cytokines, together with a specific abrogation of active IL-12 production. Thus, we argue that Th2 induction by DC follows directed maturation and not a default pathway.

2 Results

2.1 Immunization with NES-stimulated DC generates Th2 responses

The ability of NES to induce Th2 immune responses in the presence of the Th1 adjuvant CFA [28] is a striking demonstration of the inherent host reaction to a helminth challenge. Immunization with soluble NES replicates the Th2 phenotype elicited in natural infections, but induction is abolished if NES is heat inactivated or protease treated [28]. We selected this system to investigate whether NES act via DC to induce Th2 responsiveness, and if activation of DC occurred with NES but not with hiNES.

We first immunized mice with either NES or hiNES, in CFA, and measured *ex vivo* responses by intracellular cytokine staining (Fig. 1A, B) and *in vitro* antigen-specific cytokine responses following challenge (Fig. 1C). Comparison between $CD4^+$ T cells recovered from popliteal lymph nodes showed approximately fourfold more IL-4^+CD4^+ cells and half as many IFN-γ^+ and IL-10^+CD4^+ cells 3 days following immunization with NES/CFA compared to hiNES/CFA (Fig. 1B). The percentage of IL-4^+CD4^+ cells in hiNES-immunized animals was no higher than that in naive mice. Antigen-specific cytokine responses on *in vitro* challenge showed sharper differences, with IL-4, IL-5 and IL-10 dominating in NES-immunized, but not hiNES-immunized, lymph node cells (LNC) (Fig. 1C). These experiments confirmed both the ability of NES to generate a rapid Th2 immune response and their heat-labile nature.

As $CD4^+$ T cells recognize denatured peptides presented by MHC class II, the heat lability of Th2 induction by NES indicates that its adjuvant effect is independent of TCR ligation. To determine if this effect is APC mediated, we pulsed bone marrow-derived DC with NES or hiNES for 24 h, prior to adoptive transfer into naive mice. We observed that NES-stimulated DC induced a Th2 skew when *in vivo,* with LNC mounting IL-4-, IL-5- and IL-10-dominated recall responses to NES (Fig. 2). While IFN-γ was highest in mice given hiNES-pulsed DC, levels are at least tenfold lower than attained with microbial Th1 stimuli (data not shown). These experiments indicate that NES can drive a Th2 response through DC, but not if heat inactivated.

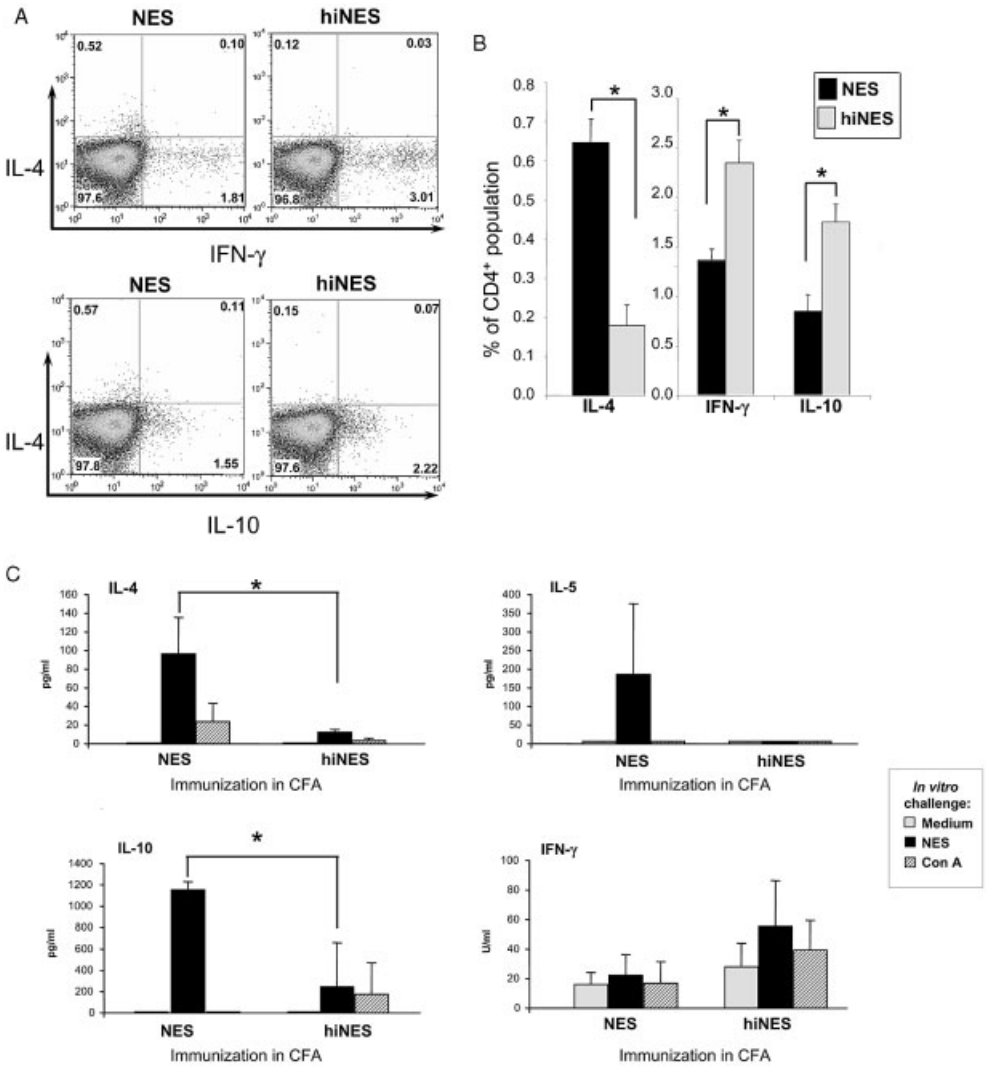

Fig. 1. NES/CFA immunization elicits early CD4$^+$ IL-4 production, subverting the expected CFA Th1 response. (A) Intracellular cytokine staining of CD4$^+$ cells 3 days post immunization with NES/CFA or hiNES/CFA. Gates are based on control isotype antibody staining. Data are representative of four individual animals. (B) Quantification of intracellular cytokine staining of CD4$^+$ cells 3 days post immunization with NES/CFA or hiNES/CFA. Data are means ± SD of four individual mice/group. *Significant difference ($p<0.05$). (C) Response of PLNC isolated 7 days after footpad immunization of BALB/c mice with 20 μg NES/CFA or hiNES/CFA. Cells were challenged *in vitro* with medium alone, 10 μg/ml NES, or 2 μg/ml Con A, and supernatants were analyzed 48 h later. Data are means ± SD of four individual mice/group. *Significant differences ($p<0.05$) between mice receiving NES and hiNES.

2.2 NES-stimulated DC up-regulate CD40, CD86 and OX40L

The default hypothesis posits that DC-dependent Th2 immunity is the result of antigen presentation in the absence of DC activation and/or maturation. Th1-associated DC activation by microbial products evokes rapid phenotypic changes, including up-regulation of MHC class II, CD40, CD80 and CD86 [22, 29–31]. Few studies, however, have reported corresponding effects on DC maturation and activation by products from Th2-promoting pathogens [32, 33], even where functional evidence for Th2 induction is present [21, 22]. As NES represents the first Th2-driving factor derived from a gastrointestinal nematode, we investigated changes to DC surface phenotype and cytokine profile after stimulation with NES.

Within 24 h of NES stimulation *in vitro*, DC showed an increased surface CD86 and CD40 expression, in

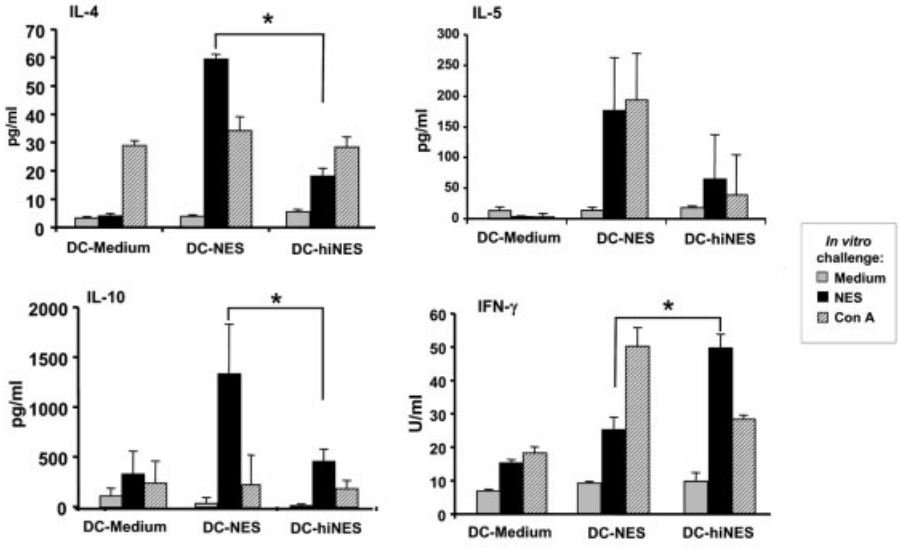

Fig. 2. Immunization with NES-stimulated DC results in the generation of a Th2 response. Responses induced by adoptive transfer of NES-stimulated bone marrow-derived DC into mice were assessed in PLNC isolated 5 days after immunization of BALB/c mice with 2.5×10^5 DC/footpad. DC were pulsed for 24 h with either 20 μg/ml NES or hiNES. Cytokine responses of PLNC were determined *in vitro*, as described in Fig. 1. Data are means ± SD of four individual mice/group. *Significant differences ($p<0.05$) between mice receiving NES- and hiNES-pulsed DC.

comparison to hiNES- or medium-alone-stimulated DC (Fig. 3A). Following NES stimulation, 91.5% of $CD11c^+$ DC expressed CD86, compared to ~64% in hiNES- and medium-alone-stimulated cells. A $CD86^{hi}$ population was found in all samples, which is likely to represent spontaneous maturation of a DC subpopulation. Exposure to NES effectively doubled the percentage of $CD11c^+$ cells expressing CD40 (94.2%) compared with hiNES-(51.4%) or medium-alone-treated DC (49.6%). Increased levels of CD40 and CD86 expression were observed by 6 h post stimulation with NES, but not hiNES, at levels similar to those seen at 24 h (data not shown). There was, however, no evident change in the surface levels of MHC class II or CD80 at either time point.

CD40 ligation plays an important role in DC activation and in subsequent T cell stimulation by activated DC [34], *e.g.* through IL-12p70 production and triggering IFN-γ release by T cells [35]. Agonistic anti-CD40 mAb supplied *in vitro* can replicate the effects of ligation [35, 36]. We therefore added anti-CD40 antibody to DC, together with NES, hiNES, LPS or medium alone, and found enhanced expression of CD40 and CD86 in all groups (Fig. 3B). However, the combination of anti-CD40 mAb and NES stimulated maximal levels of expression.

Anti-CD40 antibody treatment is known to up-regulate OX40L on human monocyte-derived DC pulsed with helminth-derived products [32]. OX40L is reported to amplify Th2 immune responses *in vivo* [37, 38]. To examine if raised OX40L expression accompanies NES stimulation, DC were stained with an anti-OX40L antibody following exposure to various stimuli with or without anti-CD40 antibody. As shown in Fig. 3C, NES-pulsed DC up-regulated surface OX40L, both before and after anti-CD40 antibody treatment, in contrast to hiNES-treated DC. We also noted up-regulation of OX40L in LPS-stimulated cells, suggesting that OX40L expression is not an exclusive property of Th2-driving DC [22].

2.3 Array analysis of NES-activated DC indicates limited gene up-regulation

The selective induction of DC maturational markers by NES was complemented with a more extensive analysis of gene up-regulation by macro-array. mRNA from DC given NES, LPS, or medium alone was hybridized to a macro-array of 514 murine immune-associated genes. NES-exposed DC up-regulated only a limited set of genes in comparison to unstimulated DC, at both 6-h and 24-h time points; among these were IL-6, IL-12p40 and macrophage-derived chemokine (MDC) (Fig. 4, center). This contrasts with LPS-stimulated DC, in which numerous changes in gene expression levels were noted (Fig. 4, bottom). For example, increased iNOS and TNF-α expression levels were observed in LPS-stimulated DC,

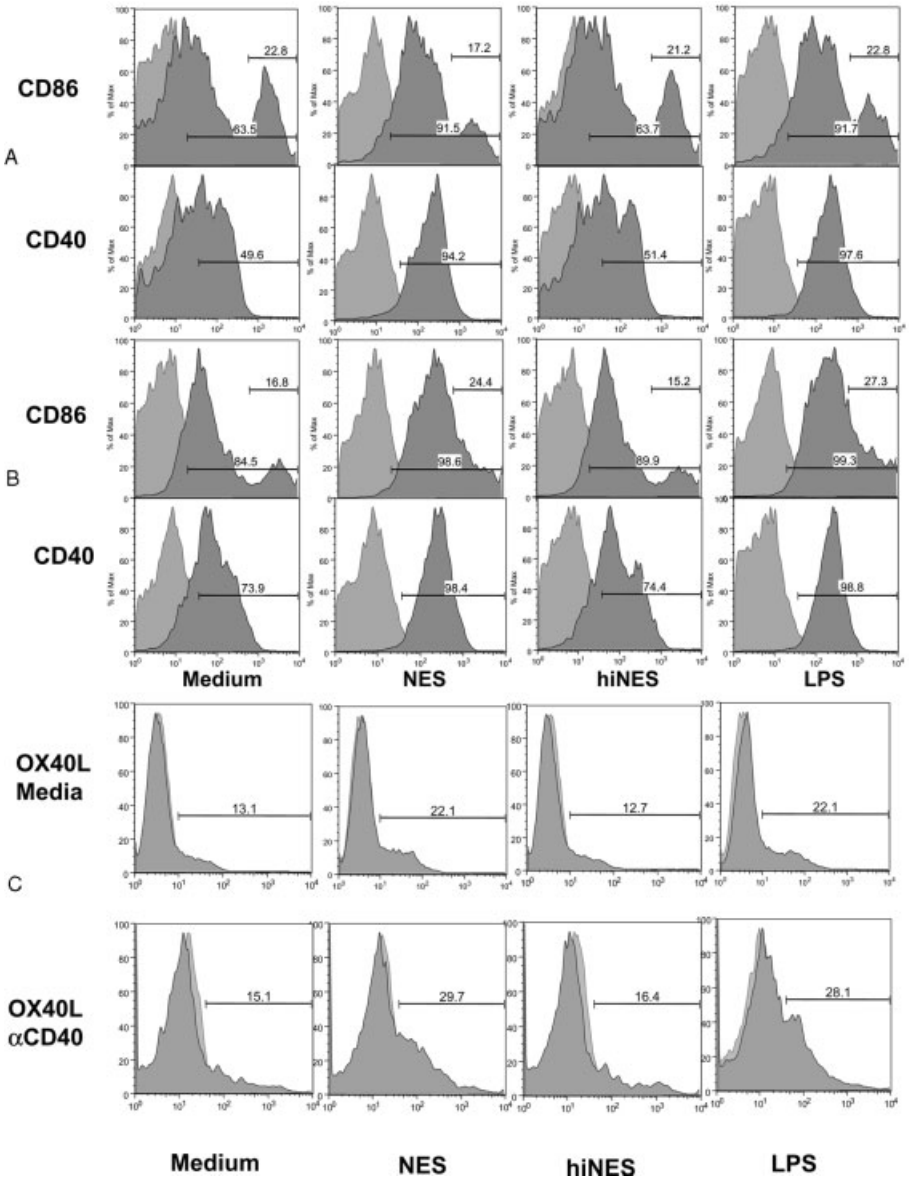

Fig. 3. Stimulation of DC with NES, but not hiNES, increases DC activation, as determined by flow cytometric analysis of surface markers. (A) Flow cytometry profiles of CD86 and CD40 expression levels of DC stimulated for 24 h with medium alone, NES, hiNES, or LPS. (B) As in (A), but with the addition of anti-CD40 mAb. (C) Flow cytometric analysis of OX40L expression levels after stimulation with medium alone, NES, hiNES, or LPS, with and without added anti-CD40 mAb. Data are representative of three experiments.

but not in NES-stimulated cells or in DC cultured in medium alone.

2.4 Cytokine production in NES- and hiNES-stimulated DC

Cytokine production after NES stimulation was assessed in supernatants of DC pulsed for 24 h with NES or hiNES. Separate assays were performed for IL-12p70 (a pro-inflammatory heterodimer of p35 and p40 subunits [39]) and p40 subunits, which can be antagonistic to IL-12p70 function [40]. As shown in Fig. 5A, DC stimulated for 24 h with increasing concentrations of NES, but not hiNES, secrete substantial levels of IL-6 and IL-12p40, consistent with the array analysis. IL-6 production occurred at lower NES concentrations than required for IL-12. While IL-6 levels stimulated by $\geq$50 μg/ml NES were comparable to those induced by 1 μg/ml LPS, IL-12p40 and p70 production was substantially lower in NES-

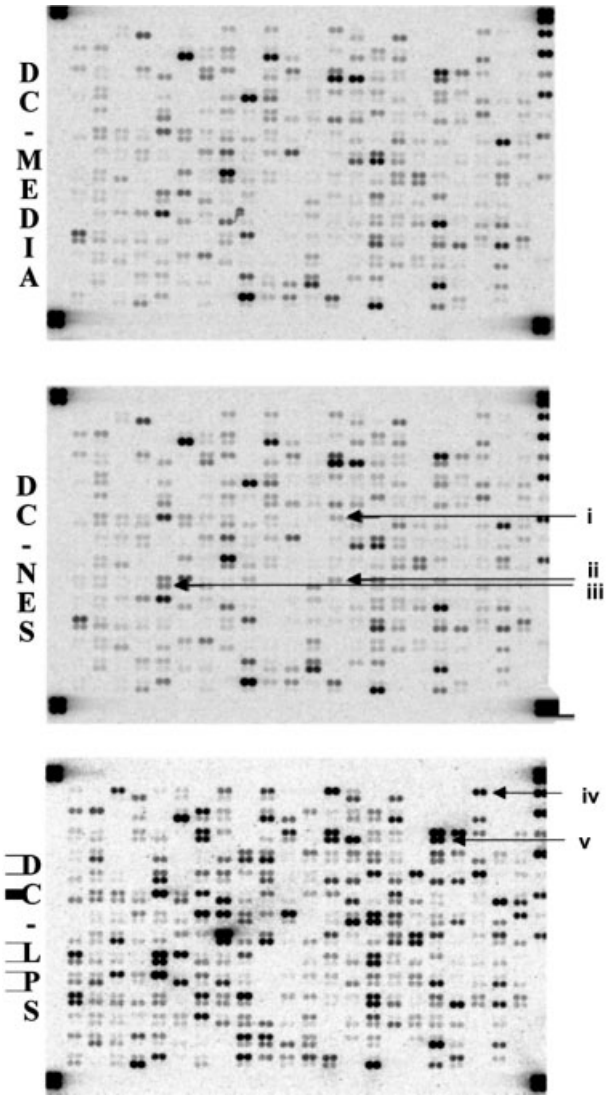

Fig. 4. Cytokine expression array analysis of NES-stimulated DC mRNA expression. mRNA from unstimulated DC (DC-medium) or from DC pulsed for 24 h with 20 μg/ml NES (DC-NES) or 1 μg/ml LPS (DC-LPS) was used to probe a cytokine array (R&D Systems). With regard to the 514 genes represented on the array, very few NES-induced changes were noted (DC-NES) compared to unstimulated DC (DC-medium). Three genes in which changes in expression were noted (arrowed) encode (i) IL-6, (ii) IL-12p40, and (iii) MDC. In contrast, LPS-stimulated DC (DC-LPS) display numerous changes in gene expression levels, including (iv) TNF-α. and (v) iNOS.

stimulated compared to LPS-stimulated DC, at all concentrations examined. No IL-10 was detected in NES-stimulated DC (data not shown).

Because cytokine synthesis is not necessarily accompanied by secretion, we also examined DC-specific cytokine production by intracellular staining 6 h post stimulation. These assays confirmed that NES, but not hiNES, induced production of IL-6 and IL-12p40/70 (Fig. 5B). As previous observations indicated that NES-stimulated DC produce little IL-12p70, the IL-12-positive DC observed in the NES-stimulated group are likely to produce IL-12p40.

IL-10 has previously been implicated in the generation of the Th2 bias by DC [16]. Although we found no IL-10 secretion in culture supernatants, or IL-10 mRNA in NES-stimulated DC, we also assessed IL-10 production by intracellular staining and reverse transcription (RT)-PCR. Increased numbers of IL-10-containing cells were observed in NES- (9%) and LPS- (8%) stimulated DC, compared to hiNES- or medium-alone-stimulated DC (1–2%). IL-10 production by NES-stimulated DC at the mRNA level was detected only transiently, in contrast to the sustained production seen in LPS-stimulated DC (Fig. 5C). Thus, IL-10 plays a relatively minor part in the DC response to NES.

The DC response to pathogen-derived molecules may, as discussed above, require CD40 ligation or other secondary signals [36]. We therefore tested DC incubated with NES together with anti-CD40 antibody treatment. Fig. 6A shows that while CD40 ligation increases the levels of IL-6 and IL-12p40 in NES-stimulated cells, the same reagent in fact inhibits IL-12p70 production by LPS-stimulated DC, as has been reported elsewhere [41]. Notably, DC stimulated with 20 μg/ml NES fail to produce IL-12p70, irrespective of anti-CD40 antibody presence.

The cytokine environment of DC is influential in determining their final phenotype. Thus, provision of IL-4 and IFN-γ, in addition to GM-CSF and anti-CD40 antibody, results in substantial IL-12p70 production by LPS-stimulated DC [42]. We tested whether NES induces IL-12p70 under these 'optimal' conditions. As shown in Fig. 6B, while LPS-stimulated DC produced large amounts of both IL-12p70 and IL-12p40, only marginal production of IL-12p70 was observed in NES-stimulated DC. Thus, the ability of NES to mature DC without significant production of IL-12p70 cannot be attributed to 'suboptimal' culture conditions.

2.5 Th2 induction by DC exposed to NES for up to 24 h

DC production of individual cytokines is often transient [43]. For example, IL-12-producing DC taken 8 h post LPS stimulation induced Th1 polarization, whereas "exhausted" DC exposed for 48 h failed to release IL-

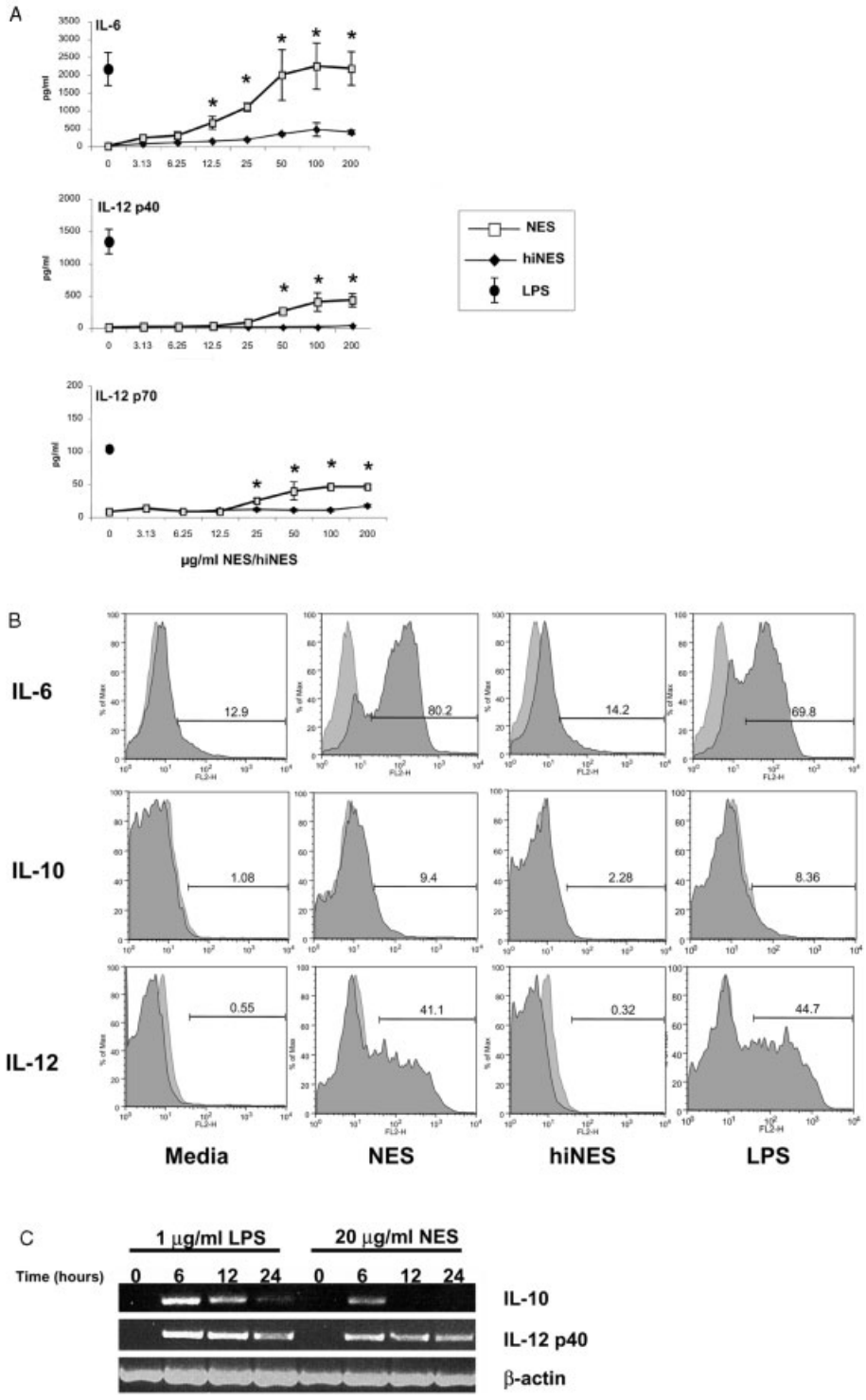

Fig. 5. Stimulation of DC with NES results in the production of a restricted range of cytokines in a dose- and time-dependent manner. (A) Cytokine levels in DC supernatant 24 h post stimulation with increasing doses of NES or hiNES or 1 μg/ml LPS, determined by ELISA. *Significant differences between NES and hiNES ($p<0.05$). (B) Early production (6 h post stimulation) of IL-6, IL-12p40/70, and IL-10 by DC, determined by intracellular cytokine staining. (C) RT-PCR analysis of IL-10 and IL-12p40 production in DC after stimulation with 1 μg/ml LPS or 20 μg/ml NES for 0, 6, 12 and 24 h.

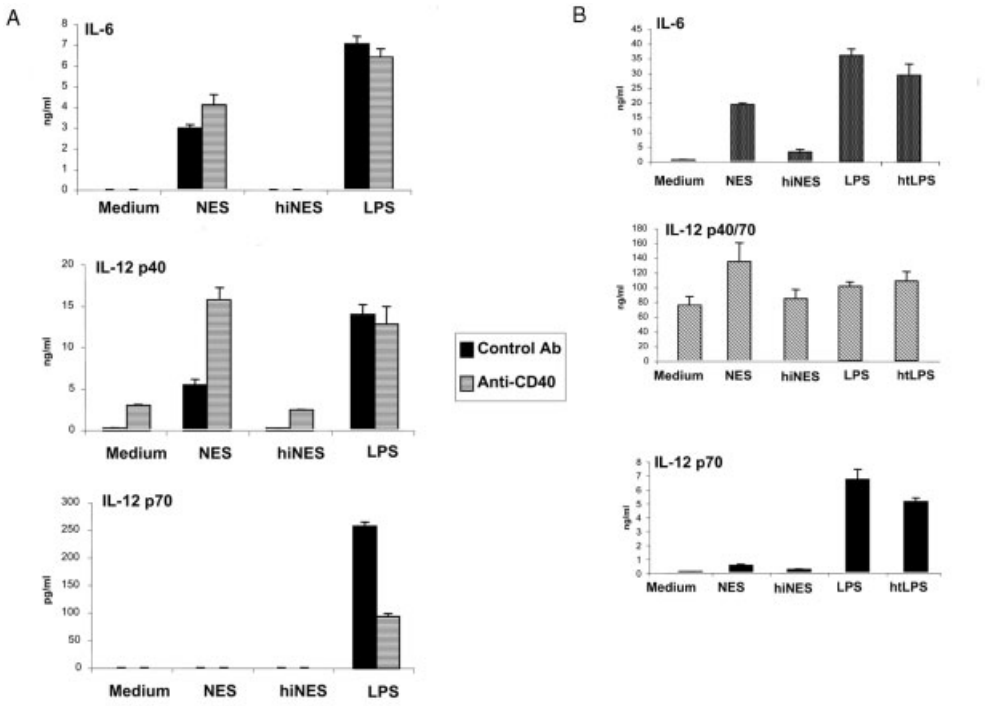

Fig. 6. Effects of altered culture conditions on cytokine production by stimulated DC. (A) Effect of anti-CD40 ligation on IL-6, IL-12p40 and IL-12p70 production of DC stimulated with 20 μg/ml NES. (B) Optimized conditions (20 pg/ml IFN-γ, 60 pg/ml IL-4, and 10 μg/ml anti-CD40 mAb) for the production of LPS-induced IL-12p70 do not result in NES-induced IL-12p70 production. LPS activity is unabated by heat treatment, whereas heat-treated NES (hiNES) is unable to induce IL-6.

12, resulting in Th2/Th0 development [43]. Although levels of IL-12p70 production by NES-stimulated DC are very low, the possibility exists that longer exposure produces an 'exhausted' phenotype, capable only of initiating Th2 responses. We tested this by *in vivo* transfer of DC pulsed for 6 h or 24 h with NES or hiNES into a subcutaneous site. Popliteal LNC (PLNC) recovered 5 days later were restimulated with NES to measure recall cytokine production. As shown in Fig. 7, PLNC from NES-DC-immunized animals produced IL-4, irrespective of the duration of DC exposure to NES. This indicates that the ability of NES-stimulated DC to generate a Th2 immune response is due to factors other then the kinetics of DC stimulation.

2.6 Pretreatment with NES blocks LPS-induced IL-12p70 production

The diminutive IL-12p70 responses from NES-pulsed DC suggested that parasite products may specifically block production of this key cytokine. We therefore examined if pre-exposure of DC to NES interfered with subsequent inflammatory responses to LPS. DC were incubated with NES or hiNES for periods of 0–6 h, washed extensively to remove soluble parasite-derived material and stimulated with 1 μg/ml LPS for 24 h, before collecting supernatants for cytokine analyses. Fig. 8 shows that pre-exposure of DC to NES, but not hiNES, inhibits IL-12p70 production in a time-dependent manner: 2 h of pre-incubation was sufficient to ablate the IL-12p70 response. No inhibition of IL-6 production was observed. Thus, NES selectively inhibits the capacity of DC to produce a critical component of the Th1 response.

3 Discussion

The ability of DC to 'recognize' a pathogen and to mobilize the most appropriate immune response is a key paradigm in immunology [7, 14–16, 44, 45]. While the pathway for Th1 promotion through DC recognition of TLR ligands has been well elucidated [46], our understanding of Th2 induction is less coherent. Indeed, one model for Th2 generation is the default hypothesis, in which the very absence of DC maturation produces a Th2 outcome [8, 9]. Our report, however, demonstrates that DC are indeed activated before initiating a Th2 response, as we show that NES-stimulated DC exhibit surface markers (CD40, CD86) characteristic of activation, up-

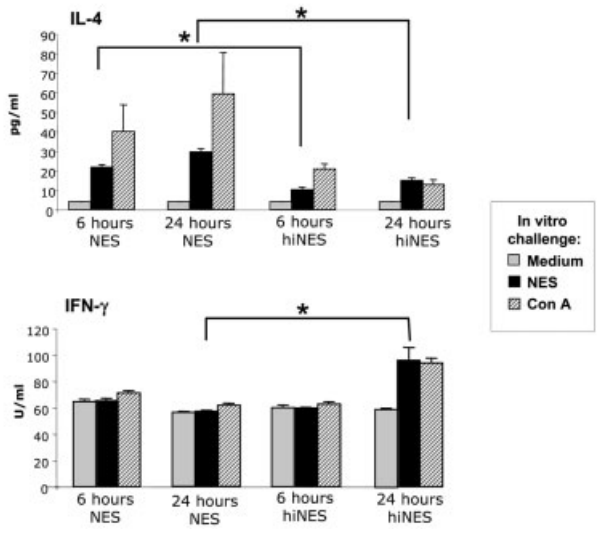

Fig. 7. The ability of NES-stimulated DC to generate Th2 responses is not due to immune 'exhaustion'. *In vitro* recall responses to NES of PLNC from mice immunized with 2.5×10^5 DC/footpad; DC stimulated with NES or hiNES for 6 h or 24 h. PLNC were isolated 5 days post immunization, and IL-4 and IFNγ responses were determined in supernatants collected 60 h following stimulation with medium alone, 10 μg/ml NES or 2 μg/ml Con A. Data are means ± SD of four individual mice/group. *Significant differences ($p<0.05$) between NES and hiNES challenge of identical DC populations.

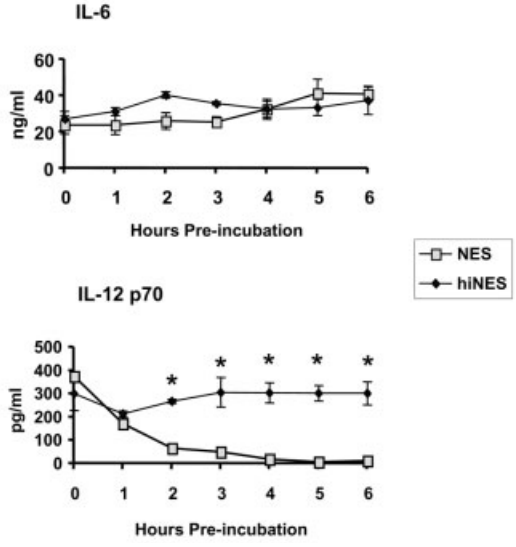

Fig. 8. Pre-exposure to NES, but not hiNES, abolishes the DC IL-12p70 response to LPS. IL-12p70 and IL-6 levels in culture supernatants of DC pre-exposed to NES or hiNES for 0–6 h, followed by extensive washing of DC and stimulation of DC with 1 μg/ml LPS for a further 24 h. *Significant differences ($p<0.05$).

regulate OX40L and produce IL-6 and other cytokines. Crucially, NES blocks the DC response to LPS, providing further evidence that this helminth product is actively modulating DC responsiveness in a manner favoring a Th2 outcome.

This is the first characterization of a Th2-driving DC population generated by a gastrointestinal nematode antigen. Previous studies on DC stimulated with helminth-derived products have reported no significant change in maturational phenotype [21, 22]. However, as our data show, DC activation by helminths is far more circumscribed than that observed with LPS, so that for certain parameters, these DC may appear to be immature. One of the significant changes observed in NES-activated DC, the up-regulation of OX40L, has also been demonstrated in SEA-exposed DC after anti-CD40 antibody treatment [32], indicating that this may be a common factor in Th2 induction.

Several key studies with SEA are inconsistent with a simple default hypothesis. Co-exposure of murine DC to SEA and *Propionibacterium acnes* also blocks the IL-12p70 response [47], and the ability of SEA to drive Th2 responses via DC is ablated by periodate pretreatment [48]. SEA-stimulated human monocyte-derived DC show up-regulated c-fos expression relative to microbial-stimulated cells; inhibition of c-fos by RNA interference rescues IL-12p70 production, suggesting that SEA modulates a DC function and potential in this manner [17]. IL-6 released following NES stimulation may also inhibit IL-12p70 responses, as recently shown in pulmonary DC [49].

A more mechanistic version of the default hypothesis is that Th2 responses ensue in the absence of Th1-polarizing IL-12p70, irrespective of other activating characteristics displayed by DC. This model is inconsistent with our demonstration that neither NES nor hiNES elicit IL-12p70, and yet only NES stimulates Th2 development. The lack of hiNES-specific Th2 responses indicates that the absence of IL-12p70 production by DC is not sufficient for Th2 initiation. Moreover, DC stimulated with *P. acnes* [22] or *T. gondii* [50] fail to generate Th2 responses in an IL-12-deficient environment. These studies agree with recent data showing that while immature DC stimulate T cell proliferation, they cannot drive differentiation into either effector subset [51].

DC IL-12 production has also been reported to be limited to the first 24 h after initial stimulation; subsequently, DC enter an "exhaustion" phase [43], in which their impaired function may preferentially generate Th2 immune responses. However, NES-stimulated DC generated Th2

responses *in vivo* irrespective of antigen stimulation times of 6 h or 24 h. Therefore, it would seem that the ability of NES to induce type 2 DC is not due to immune exhaustion and that NES delivers specific stimuli to DC which actively drive the Th2 immune response.

The Th2 response is abolished by heat treatment of NES. This finding, reproduced both in direct immunizations and through exposure of DC prior to adoptive transfer, establishes that Th2 polarization does not occur in the absence of activation. The default response, therefore, remains Th0 rather than Th2. The heat lability of NES also stands in contrast to LPS, which retains its potency following heat treatment, and argues that the Th2 bias is unlikely to reflect any particular characteristics of responding T cell receptor usage. These considerations suggest the existence of a pathogen-associated molecular pattern (PAMP) characteristic of one or more groups of helminths. We have found that although NES consists of several dozen components, only one of these actively generates Th2 responses. This Th2-promoting fraction consists of large-molecular weight glycoproteins, which we are currently characterizing to define the nature of this potential helminth PAMP.

Not all helminth infections are pathogenic or result in the expulsion of the helminth parasite. The generation of a Th2 immune response may be advantageous to the parasite [5] as a mechanism to down-regulate pro-inflammatory responses. Indeed, infection with *N. brasiliensis* is able to prolong allograft survival [52], suggesting that the anti-inflammatory effects of infection with this gastrointestinal nematode may also act systemically [53, 54]. The anti-inflammatory response may thus promote the chronic infection observed with many helminth species. In the microbial-rich gastrointestinal environment, parasite infection may cause extensive tissue disruption, exposing the innate immune system to microbial products. Perhaps the ability of NES to preempt the inflammatory reaction to LPS in immature DC, as well as to dampen antigen-specific T cell IFN-γ recall responses, reflects the parasite strategy to minimize Th1 reactivities.

The ability of gastrointestinal nematodes to deviate host immune responses towards Th2 is, however, a hazardous game. Limited Th2 responses, perhaps calibrated in low-dose infections, may permit parasites to remain in the host, but there is no doubt that a fully mobilized Th2 reaction will expel helminth pathogens from the gut [55]. Elucidating the mechanisms by which parasites manipulate the innate immune response, as well as the specific molecules involved in this process, is of ongoing interest not only for direct application for the development of anti-helminthic treatment strategies, but also for the development of novel anti-inflammatory therapeutics with implications in the wider medical community.

4 Materials and methods

4.1 *Nippostrongylus* and NES

N. brasiliensis was maintained in rats as described [25]. Adult worms collected at day 6 post infection were cultured for 7 days in RPMI 1640 with 100 U/ml penicillin, 100 μg/ml streptomycin and 1% glucose. Supernatants were collected at 48-h intervals from days 1–7, pooled and concentrated to 1 mg/ml [28]. NES was treated for 15 min at 95°C to yield hiNES. Potential endotoxin contamination was neutralized by pre-incubation with 20 μg/ml polymyxin B sulfate (Sigma) at 37°C for 30 min.

4.2 Dendritic cells and PLNC

Bone marrow-derived DC were generated by established techniques [56] from 6–8-week-old female BALB/c mice, by culture at 3.75×10^5 cells/ml in RPMI 1640, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, 10% FCS, and 10 ng/ml GM-CSF (R&D Systems) in the absence of IL-4 or LPS for 7 days. Cultures were washed on day 2 and day 5, and fresh medium was added. DC were stimulated with 20 μg/ml NES or hiNES, 1 μg/ml LPS, and/or 10 μg/ml anti-CD40 mAb (FGK-45; gift of David Gray). Prior to *in vivo* transfer, DC at 2×10^6 cells/ml were incubated with 20 μg/ml NES or hiNES for 6–24 h. In certain experiments, 'optimized' culture conditions [42] included 20 pg/ml IFN-γ, 60 pg/ml IL-4 and 10 μg/ml anti-CD40 mAb. For *in vivo* experiments, 5×10^5 DC were injected into the footpad, and PLNC were recovered 5 days later and cultured at 2×10^6 cells/ml with 2 μg/ml Con A, 20 μg/ml NES or medium alone. Cell supernatants were removed for cytokine assay 60 h later.

4.3 Surface markers and intracellular cytokines

DC surface marker expression was determined with FITC-conjugated anti-CD11c, PE-conjugated anti-CD86 and anti-CD40, and biotin-conjugated anti-OX40L mAb, followed by allophycocyanin-streptavidin staining (all PharMingen). Intracellular cytokines were detected by stimulating DC with 20 μg/ml NES or hiNES or 1 μg/ml LPS for 6 h in 20 μg/ml brefeldin A (Sigma, UK). Cells were then stained for surface CD11c, fixed using Cytofix/Cytoperm Plus (PharMingen), and intracellular cytokines were detected using PE-conjugated mAb (IL-6, IL-10, IL-12p40/p70) and Cytofix/Cytoperm. Popliteal lymph node T cells taken 5 days post immunization, cultured at 2×10^6 cells/ml, were stimulated with 50 ng/ml PMA, 1 μg/ml ionomycin and 20 μg/ml brefeldin A for 6 h. Cells were then washed in FACS buffer (PBS-0.5% BSA) and resuspended at 2×10^6 cells/ml with

10% normal rat serum for 15 min at 4°C. Cells were stained with anti-CD4 antibody for 30 min at 4°C, washed three times and fixed using Cytofix/Cytoperm/Wash as above, before staining for 30 min at 4°C with allophycocyanin-conjugated anti-IL-4 mAb, PE-conjugated anti-IFN-γ or anti-IL-10 mAb (all PharMingen). Cells were analyzed by FACSCalibur flow cytometry and FlowJo software (Tree Star).

4.4 Cytokine assays

Cytokine levels were determined by ELISA using paired mAb for IL-4 (IL-4 capture mAb 11B.11 was a gift of NCI, Frederick, MD), IL-5, IL-6, IL-12p40, IL-12p70 and IFN-γ, purchased from PharMingen and used as per their instructions.

4.5 RNA isolation and RT-PCR

Total RNA was isolated from 2×10^6 DC/ml using Trizol (Gibco BRL) according to the manufacturer's instructions; 1 μg was reverse transcribed into single-stranded cDNA in a 20-μl reaction mixture containing 100 ng oligo(dT), 200 U Superscript RNase H-Reverse Transcriptase (Gibco BRL), 0.5 mM of each dNTP (Promega), 32 U RNase inhibitor (Promega), and 0.01 M DTT (Gibco BRL). cDNA aliquots were stored at –20°C. Amplification of cDNA employed 20 pmol/μl of the primer pairs (IL-10F, 5′-CTTATCGGAAATGATCCAGTTT-TACCTGGT-3′; IL-10R, 5′-CAAGGAGTTGTTCC GTTAGC-TAAGATCC-3′; IL-12p40F, 5′-AACCTCACCTGTACACGCC-3′; IL-12p40R, 5′-CAAGTCCATGTTTCTTTGCACG-3′; β-actinF, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; β-actinR, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′), with 1 U *Taq* DNA polymerase (Promega) and 10 mM of each dNTP (Promega).

4.6 cDNA expression array

Radiolabeled cDNA probes were hybridized to Mouse Cytokine Expression Array, V1.0, containing 514 immune system genes (R&D Systems). First-strand cDNA synthesis was performed on total DC RNA with a gene-specific primer set and RT was performed with SuperScript II (Invitrogen) and [α-^{33}P]dCTP. Arrays were given high stringency washes and subjected to autoradiography.

4.7 Statistical analysis

Differences in the mean values between experimental groups were compared using Student's unpaired *t*-test. *p* values <0.05 are indicated in the figures.

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