

Dendritic cells from control but not atopic donors respond to contact and respiratory sensitizer treatment *in vitro* with differential cytokine production and altered stimulatory capacity

N. J. Holden*, P. A. Bedford*, N. E. McCarthy*, N. A. Marks[†], P. W. Ind[†], I. R. Jowsey[‡], D. A. Basketter[†] and S. C. Knight*

*Antigen Presentation Research Group, Imperial College London, Northwick Park and St Marks Hospital Campus, Harrow, UK, [†]Department of Respiratory Medicine, Imperial College London, Hammersmith Campus, London, UK and [‡]Safety and Environmental Assurance Centre, Unilever, Bedford, UK

Clinical and Experimental Allergy

Summary

Background Chemical haptens induce both contact and allergic respiratory disease with dendritic cells (DCs) controlling and directing immune responses *in vivo*. Contact and respiratory haptens may promote differential cytokine production yet distinguishing these effects *in vitro* remains difficult due to human donor variability.

Objective We sought to determine the effect of atopic status on the ability of DC to respond to contact and respiratory sensitizer treatment *in vitro* as DC from atopic donors are believed to promote Th2-type responses.

Methods Enriched DC from control or atopic donors were treated for 4 h with levels of the contact sensitizer 2,4-dinitrochlorobenzene (DNCB) or the respiratory sensitizer trimellitic anhydride (TMA) that did not reduce cell viability. A sensitive intracellular detection technique was used to measure cytokine production, while T cell responses were assessed in a mixed leucocyte reaction.

Results DC from control, non-atopic, donors produced cytokines differentially in response to sensitizer treatment; DNCB treatment significantly increased the production of Th1 cytokines IL-12 and IFN- γ while TMA induced the production of IL-13. Control donor DC treated with TMA stimulated less in a mixed leucocyte reaction than untreated cells with any response reduced further by blocking IL-13 in culture. However, DC from atopic donors showed no significant alteration in either cytokine production or T cell stimulatory capacity after sensitizer treatment.

Conclusion Haptens modulate DC by changing the production of cytokines that may play a role in T cell stimulation and subsequent polarization of the immune response. DC from atopic donors were unresponsive to chemical sensitizer treatment, and may be deficient in inducing divergent T cell responses.

Keywords atopy, contact hypersensitivity, DNCB, TMA

Submitted 7 November 2007; revised 18 February 2008; accepted 26 February 2008

Correspondence:

Prof. Stella C. Knight, Antigen Presentation Research Group, Imperial College London, Northwick Park and St Marks Hospital Campus, Watford Road, Harrow HA1 3UJ, UK.
E-mail: s.knight@imperial.ac.uk

Introduction

Contact hypersensitivity (CHS), a delayed-type allergic response, can be elicited by chemical haptens. Haptens covalently bound to protein are taken up by Langerhans cells, which deliver antigen via afferent lymphatics to local lymph nodes [1]. Dendritic cells (DCs) and their responses to chemical sensitizers have been studied in order to develop *in vitro* tests for identification of chemical contact sensitizers. Monocyte-derived DC treated in culture with contact sensitizers may increase the produc-

tion of IL-1- β [2] and are reported to up-regulate the expression of maturation markers such as CD54, CD86, HLA-DR and CCR7 [3–5]. Recent advances demonstrate that contact sensitizers activate specific signal transduction systems such as p38 MAP and ERK kinases [6] and affect the redox state of the cell [7].

DC responses to haptens which cause respiratory sensitization are less well studied. It has recently been suggested that contact and respiratory sensitizers could be differentiated through the production of hapten-specific IgE. In rodent models serum IgE is increased after topical

treatment with the model respiratory sensitizer trimellitic anhydride (TMA) but not the model contact sensitizer 2,4-dinitrochlorobenzene (DNCB) [8, 9]. However, in humans the production of specific IgE to some respiratory haptens has not been fully confirmed, for example, isocyanate-specific IgE may be found in only a proportion of symptomatic individuals [10]. Allergic responses can also be defined according to cytokine profiles: contact sensitivity, a predominantly cell-mediated immune response, is associated with the production of Th1 cytokines; respiratory allergy has the converse Th2 cytokine profile. Cytokine secretion profiles in lymph nodes of sensitized mice show this differentiation with topical treatment with TMA inducing production of Th2 cytokines IL-10 and mitogen-induced IL-4 and DNCB sensitization leading to high IFN- γ production [11]. Subsequent studies have further validated these findings, extending the detection of cytokines to include IL-2, IL-5 and IL-13, all of which fit the expected Th1/Th2 profiles [12–14]. As DCs have the potential to polarize T cell responses through their own cytokine production, hapten presenting Langerhans cells may influence both helper and cyto-toxic T cell polarization in the context of chemical sensitization.

Human donor variability remains a real problem when studying the effect of chemical sensitizers *in vitro* [15] and responses of DC from reactive individuals are yet to be fully defined. Atopy, the predisposition to develop immediate-type hypersensitivity reactions, may contribute to variability seen in DC responses to chemical allergens and may influence cytokine production. The inflammatory process involved in immediate-type allergic disease centres around Th2 lymphocytes secreting cytokines such as IL-4, IL-5 and IL-13 [16–18]. The effect of atopy on an individual's ability to react to contact sensitizers is unclear. Atopic individuals can be sensitized with chemical haptens [19]. However, CHS responses may be impaired in these individuals [20]. These impaired responses may extend to *in vitro* work with peripheral blood mononuclear cells (PBMCs) from atopic donors being largely non-responsive to the contact allergen nickel (Ni⁺) compared with PBMCs from non-atopic individuals [21].

Changes in DC induced during prolonged separation procedures or generation of DC from monocyte precursors using cytokine treatments, may influence the characteristics of DC. Cyto-toxicity of chemical sensitizers may also present a problem when interpreting changes in DC after sensitizer treatment *in vitro*; apparent alterations in phenotype may reflect preferential loss of some cells rather than changed antigen expression on surviving cells. We aimed to treat DC enriched from peripheral blood of both atopic and non-atopic donors with the model contact sensitizer DNCB and the model respiratory sensitizer TMA for short time periods using doses that were not overtly toxic. Intracellular cytokine production by these cells was investigated along with their ability to induce

a primary T cell stimulatory response. We have shown that the sensitizers may modulate immune responses through cytokine production in DC from control but not from atopic donors.

Methods

Antibodies

Mouse IgG1 clone x40, IgG2a G155, IgG2b MPC-II, IL-12 C115.5, blocking IL-13 JES10-5A2, Blocking IL-12 C86, HLA-DR 1243, β 7 FIB504 (BD Biosciences, Oxford, UK), IL-10 JES39D7, IFN- γ D9D10, TLR2 TL2.3, TLR4 HTA125, CD40 LOB7/6, CD86 BU63, CD54, 15.2 (Serotec, Oxford, UK), IL-4 3007 (R&D systems, Abingdon, UK), IL-13 32007.111 (Oxford Biotechnologies, Oxford, UK), HLA-ABC W6/32 (eBioscience, San Diego, CA, USA), CD11c KB90 (Dakocytomation, Ely, UK), CD83, HB15e (AnCell, Nottingham, UK), DC-SIGN, 120507 (R & D systems).

Reagents and chemicals

Monensin (Sigma, Poole, UK) was made up in ethanol to a stock solution of 3 mM. Metrizamide (Sigma) was used at 14.5% (w/v) in RPMI Dutch modified (Sigma) with 10% FCS. DNCB (Sigma) was used as a stock solution of 5 mM dissolved in ethanol and further diluted in sterile PBS (Sigma). TMA was used as a stock solution of 5 mM dissolved in acetone and further diluted in PBS. Final concentration of ethanol or acetone in test solution never exceeded 0.01%. FACS buffer was PBS with added FCS (2%), EDTA (1 mM) and sodium azide (0.2%).

Subjects

To determine atopic status, all donors were skin prick tested to a panel of common aeroallergens: *Dermatophagoides pteronyssinus*, dog hair, cat fur, feather mixture, grass pollen, *Cladosporium herbarum*, *Alternaria alternata*, *Candida albicans* and *Aspergillus fumigatus* (ALK-Abello, Horsholm, Denmark). Atopic donors were defined by a positive skin prick test reaction (>3 mm weal) to two or more aeroallergens or as subjects exhibiting a positive reaction to one skin-test allergen in conjunction with an atopic family history. Atopic donors displayed no symptoms relating to their atopy at the time of donating blood and had not used antihistamines for 24 h preceding the study. Both control and atopic subjects were non-smokers or had not smoked for at least 1 year before the study. Control donors (two female, 10 male) had a mean age of 32 (range: 21–57). Atopic donors (five female, seven male) had a mean of age 38 (range: 22–67). Informed consent was received from all subjects and the local ethics committee approved the protocol.

Enrichment of dendritic cells

Low-density cells (LDCs) enriched for DC were isolated from the non-adherent fraction of PBMCs cultured overnight (37 °C, 5% CO₂) and centrifuged over either metrizamide gradient (14.5% w/v) [22] or Nycoprep 1.068 (Nycodenz) gradient (Axis-Shield, Kimbolton, UK) [23]. Cell analysis showed DC isolation with either gradient to be comparable with the isolated population highly enriched for DC with high surface expression of HLA-DR, CD11c, CD83 and CD86. This method of DC enrichment consistently gave a yield of 5–7% of the total number of PBMCs isolated for all donors.

Cell viability

The cyto-toxicity of both sensitizers was assessed at varying concentration by the inclusion of the viability probe Viaprobe (Becton Dickinson, Oxford, UK). Cells positive for Viaprobe incorporation (expressed as a percentage) were determined by the position of a region gate placed on the histogram of LDC without Viaprobe using WINLIST software (Verity Software House, Topsham, ME).

Dendritic cell treatment

Cells at a concentration range of 5–10 × 10⁵/mL cells in Dutch-modified RPMI medium plus 10% were treated with 5 µM DNCB or TMA for 4 h in a humidified 5% CO₂ incubator. Final concentration of solvent in test sample never exceeded 0.01%. Dead cells as a result of sensitizer treatment never exceeded 5% above that observed in untreated cells demonstrated using Viaprobe.

Intracellular cytokine detection

LDC at a concentration of 3–5 × 10⁵ were incubated in Dutch modified RPMI plus 10% FCS with or without added sensitizer for 4 h at 37 °C in the presence of 3 µM monensin to retain any newly synthesized protein. A paired sample of LDC was also incubated for 4 h in medium alone. Cells were washed and re-suspended in 100 µL of FACS buffer and 50 µL Leucoperm reagent A (Serotec) left at room temperature on a cell shaker for 15 min. FACS buffer was added and cells centrifuged for 5 min at 500 g. Cells were re-suspended in 100 µL Leucoperm reagent B (Serotec). Directly conjugated anti-cytokine monoclonal antibody (5 µL) was added and left for 30 min at room temperature on a cell shaker. LDC were again washed in FACS buffer and re-suspended in 100 µL FACS buffer and fixed with 1% paraformaldehyde.

Phenotype

LDC treated in the presence of sensitizer or irritant for 4 h were analysed for cell markers by flow cytometry. Cells

were washed and incubated in FACS buffer in the presence of conjugated antibodies for 30 min at room temperature. Cells were washed further in FACS buffer and fixed with 1% formaldehyde.

Flow cytometry acquisition and analysis

Samples were acquired on a FACScalibur flow cytometer using CELL QUEST software (Becton Dickinson) with 1 × 10⁴ cells acquired per tube. Forward and side scatter gating excluded dead cells and contaminating lymphocytes. List mode data was analysed using WINLIST software. Antibody staining was quantified by subtracting normalized cumulative control histograms from similar test histograms using the super enhanced DMAX method [24, 25]. This sensitive method allows positive events to be identified even when control and test histograms overlap. From subtraction, values are generated for the number of positive cells as a percentage and the intensity of staining as a ratio of the median value of positive events in the test histogram to the median value of the control histogram. For surface labelling, isotype control histograms were subtracted from test histograms. For intracellular cytokine analysis, histograms from cells incubated without monensin (control) were subtracted from histograms representing cells incubated in the presence of monensin (test histogram). Subtracting histograms of cells that have been stimulated and stained in the same way ensures that there is minimal difference in non-specific binding between test and control samples. Comparisons are not made with an isotype matched control antibody because the sensitive subtraction technique may detect small differences between antibodies unrelated to antigen specificity, for instance in different levels of free fluorochrome. By subtracting no monensin histograms from monensin histograms build-up of trapped synthesized proteins is detected in a 4 h window. For this method the control histogram does not act as a negative control. Instead it is a reference point for cytokine production. By reference to this value, cytokine production could be measured as an increase of cytokine over the period of culture. When a control histogram had more 'positive' events than the test histogram the subtraction was reversed indicating a loss of pre-existing cytokine from cells and shown as a negative number. This technique has been validated in a recent publication from our laboratory [26].

Mixed leucocyte reactions

For assessment of T cell stimulation, enriched DC were incubated with freshly isolated PBMCs from a different donor. Triplicate cultures containing 2500–100 000 PBMCs/well were set up in Terasaki plates. LDCs were added at a concentration of 1000 per well to give a culture volume of 20 µL. Control wells contained PBMCs alone or PBMCs and untreated DC. A positive control of PBMCs plus

Concanavalin A (5 µg/mL) (Sigma) was also used. Inverted Terasaki plates were cultured over 0.85% saline at 37 °C in 5% CO₂ for 3 days. Wells were then pulsed with ³H thymidine (1 µg/mL specific activity 37 kbq/µL) (Amersham Bioscience, Amersham, UK) for 2 h, conditions ensuring free availability of the precursor for the whole culture period. Cells were harvested by blotting onto damp filter paper and washed with saline, 5% trichloroacetic acid for DNA precipitation and methanol to dry. When fully air-dried, incorporation of ³H thymidine was assayed by exposing filters for 4 h on a molecular dynamics phosphor screen and imager. The counts were closely correlated with scintillation counts although actual counts obtained were lower [27]. Because replicate cultures using multiple cell concentrations were performed in each experiment, significant differences between treatments in individual experiments were assessed using ANOVA of the log transformed, normally distributed data.

Cytokine blocking of mixed leucocyte reactions

A separate Terasaki plate contained neutralizing anti-cytokine antibodies (10 µg/mL) added to each test well before 3-day incubation. Because the possibility of inter-plate variability the direct effect of neutralizing antibody was also confirmed in cultures with and without antibodies set up on the same plate.

Results

Dose responses and viability assay for 2,4-dinitrochlorobenzene and trimellitic anhydride treatment

Concentrations of chemical sensitizers that did not reduce viability were selected to treat enriched DC. Grouped data ($n = 5$) (Fig. 1) highlighted that DNCB was more cyto-toxic *in vitro* compared with TMA following 24-h incubation with DC. At both 10 and 20 µM both sensitizers significantly increased the number of non-viable cells. As 5 µM was deemed to be the highest non-toxic concentration in this system we used this in all future experiments.

Dendritic cell from control but not atopic donors showed polarized cytokine production in response to contact and respiratory sensitizers

DC from control donors produced divergent cytokine responses when treated with the two haptens for 4 h. DNCB treatment significantly increased the percentage of IL-12 and IFN-γ containing cells (Fig. 2a) as well as their respective positive intensity ratios (Fig. 2b). TMA treatment of DC from this donor group induced significant production of IL-13 as seen in the number of IL-13 containing cells (Fig. 2a). Atopic donor DC, on the other hand, showed no reproducible responses to either DNCB or

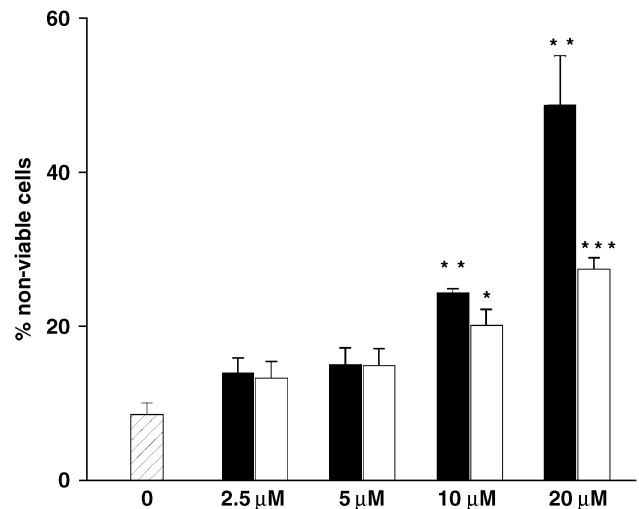


Fig. 1. Dose-response experiments. Unstimulated dendritic cell ▨ and cells treated with increasing concentrations of 2,4-dinitrochlorobenzene ■ or trimellitic anhydride □ for 24 h were quantified for cell death through the incorporation of Viaprobe measured as mean percentage positive cells compared with a non-labelled control ($n = 5$) ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ determined by paired Student's *t*-test.

TMA sensitizer treatment. Variable responses were seen between different donors, and as a result, no significant changes were observed in the number of cytokine containing cells (Fig. 2a) or the associated positive intensity ratios (Fig. 2b). Thus control DC mounted a polarized cytokine response to the chemical sensitizer while atopic DC did not differentiate the two chemical haptens in terms of cytokine production.

Dendritic cell cytokine responses to both contact and respiratory sensitizers may be affected by basal levels of cytokine production in untreated cells

The basal levels of cytokine production in untreated DC taken from control and atopic donors were investigated (Fig. 3). We found no evidence that cytokine production in the two donor groups differed in the untreated cells (data not shown). However, levels of cytokines produced in unstimulated DC had a bearing on cytokine production in response to the chemical stimulus. When untreated DC were producing high amounts of an individual cytokine sensitizer treatment had minimal effect on cytokine production; high detection of that cytokine was maintained (Fig. 3a). DNCB, however, significantly reduced the detection of IL-13 in control donors. When the production of individual cytokines was low or absent (negative after subtraction), sensitizer treatment resulted in the up-regulation of cytokines in a polarized manner (Fig. 3b). This effect was best represented in control donors where DNCB treatment significantly up-regulated the production of IL-12, IFN-γ and IL-4. TMA treatment markedly up-regulated the production of IL-13 and IL-10 and, to

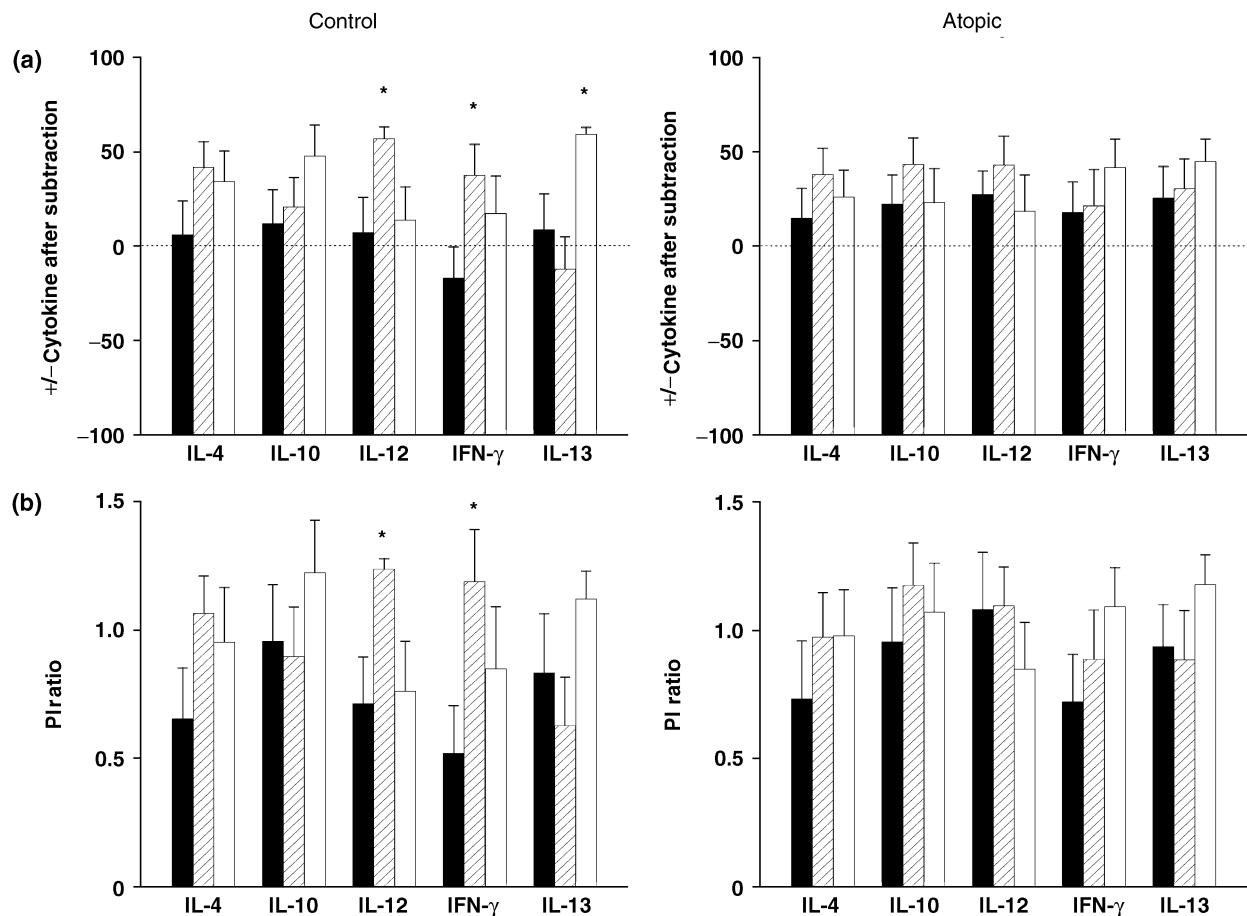


Fig. 2. Intracellular cytokine production in dendritic cell (DC) from different donor groups stimulated with either the contact sensitizer 2,4-dinitrochlorobenzene (DNCB) or the respiratory sensitizer trimellitic anhydride (TMA). Enriched DC from control ($n = 12$) and atopic donor groups ($n = 12$) were analysed for cytokine production when untreated ■ and in response to 4 h DNCB ($5 \mu\text{M}$) ▨ or TMA ($5 \mu\text{M}$) □ chemical sensitizer treatment. Intracellular cytokine was detected by incubating cells with the secretion inhibitor monensin. A paired cell sample was also treated for 4 h in the absence of monensin. By subtracting control histograms (no monensin) from test histograms the accumulation of cytokine plus or minus the control can be calculated. Results are presented as \pm cytokine containing cells after subtraction (a) and their associated positive intensity ratios (b) Mean \pm SEM. * $P < 0.05$ determined by paired Student's t -test

some extent, IL-4 and IFN- γ . A similar pattern was observed in atopic donor DC, although responses were not statistically significant due to high variability between donors. The data suggest that sensitizers induce greater cytokine production in DC when cells are in a more resting state and producing little cytokine.

2,4-Dinitrochlorobenzene but not trimellitic anhydride sensitizer treatment affected surface phenotype of dendritic cell taken from control and atopic donor dendritic cell

Sensitizer treatment induced alterations to surface phenotype of DC *in vitro* were assessed. Minor changes in phenotype were observed after 4-h hapten treatment but significant alterations were observed for some markers. Control donor DC (Fig. 4a) treated with DNCB had significantly reduced surface expression of CD54 (Fig. 4b).

This effect was not seen in atopic donor DC (Fig. 4c) although we did observe a significantly reduced surface expression of HLA-A/B/C and HLA-DR in response to DNCB treatment (Fig. 4d). There was no phenotypic response to TMA in DC from either donor group.

Dendritic cell taken from control donors and treated with trimellitic anhydride inhibited primary T cell proliferation dependent on the T cell donor

The effects of sensitizer treatment on the ability of DC to stimulate T cells was assessed. We included DC from either control or atopic donors into a mixed leucocyte reaction with responder PBMCs from a different donor. DNCB treatment of DCs from control or atopic donors had no effect on T cell proliferation compared with untreated cells (Fig. 5). TMA-treated DC from control donors, however, significantly reduced T cell stimulation when included in

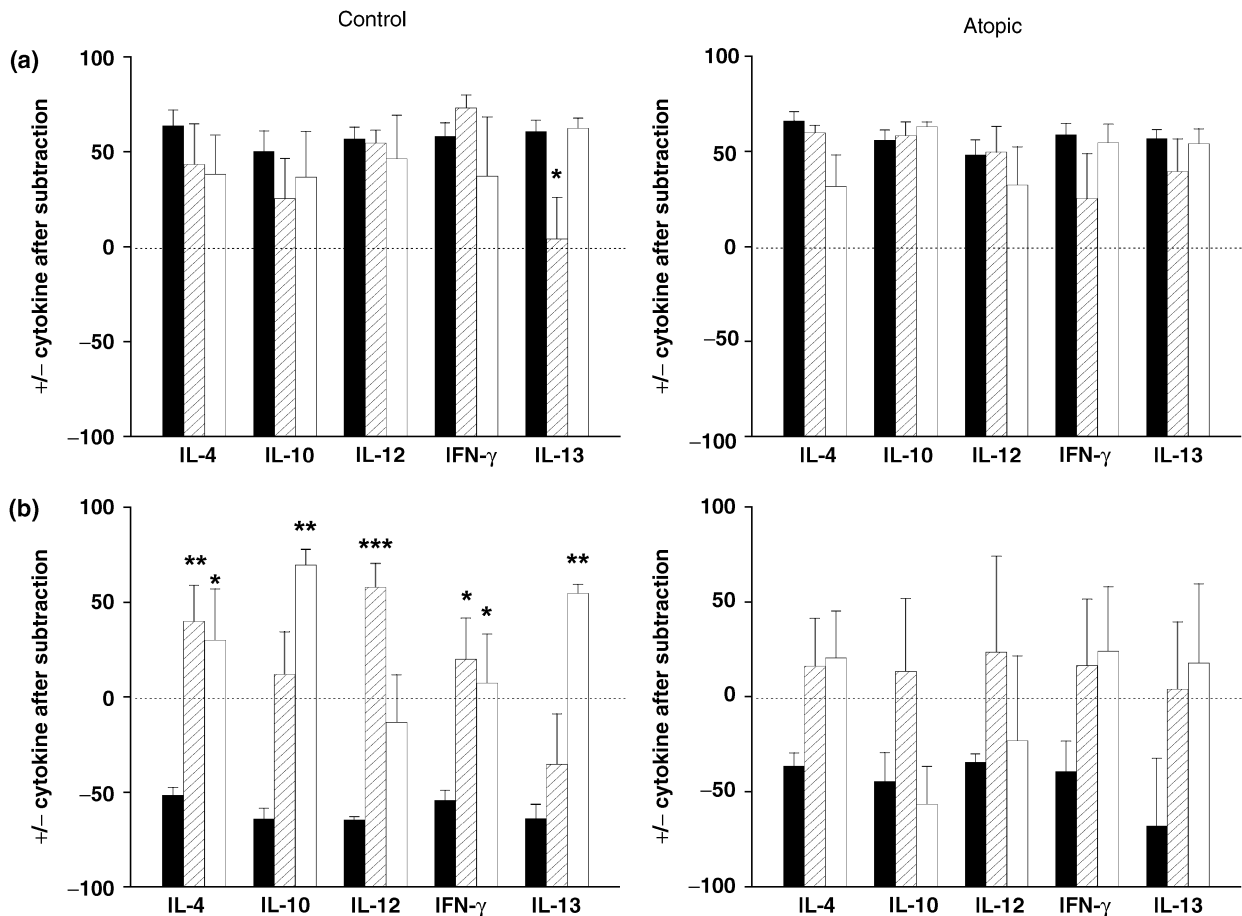


Fig. 3. Intracellular cytokine responses can be split into two groups based on high or low cytokine production in unstimulated cells. Untreated dendritic cell (DC) ■, 2,4-dinitrochlorobenzene (DNCB) treated ($5 \mu\text{M}$) ▨ and trimellitic-anhydride-treated DC ($5 \mu\text{M}$) □ incubated for 4 h were grouped depending on starting cytokine levels. By subtracting control histograms (no monensin) from test histograms, the accumulation of cytokine plus or minus the control can be calculated. Responses were grouped based on starting levels of cytokine production in unstimulated cells, with those that show high positive responses (a) and those where unstimulated DC lost cytokine giving negative values after subtraction (b). Results are presented as mean \pm cytokine containing cells after subtraction \pm SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ determined by paired Student's t -test.

a mixed leucocyte reaction (MLR). Six out of ten individual MLR experiments using TMA-treated control donor DC resulted in a significant reduction in T cell responses. Responses were independent of atopic status of the PBMC donor. The inhibitory effect of TMA was not observed when atopic donors DCs were included in an MLR, in 10 individual experiments. The data demonstrate that DCs from control donors can reduce an allogeneic T cell response when stimulated with TMA; atopic donor DCs did not possess this property.

Interleukin-13 enhanced T cell stimulation to respiratory but not contact sensitizers

Neutralizing antibody to IL-13 was added to a mixed leucocyte reaction to determine whether the production of IL-13 in response to chemical sensitizer treatment had any functional effect on T cell stimulation. As previously

demonstrated, DNCB-treated DC induced comparable T cell stimulation in an MLR compared with untreated DC whereas TMA-treated DCs exhibited significantly lower stimulatory capacity compared with untreated cells (Fig. 6a). The two plates were repeated in the presence of neutralizing IL-13 antibody (Fig. 6b). When IL-13 was blocked, T cell stimulation induced by DNCB-treated DC was significantly higher than that seen with untreated DC whereas TMA-treated cells exhibited a more significant loss in stimulatory activity with the proliferation induced similar to background PBMC proliferation. To rule out plate-to-plate variability and to confirm that the neutralizing antibody was acting on sensitizer-treated cells, the effect of anti-IL-13 antibody was also confirmed within the same Terasaki plate (Fig. 6c). Similar results were observed in three out of five experiments. No significant difference was seen in responses to untreated cells in the presence of the neutralizing antibody.

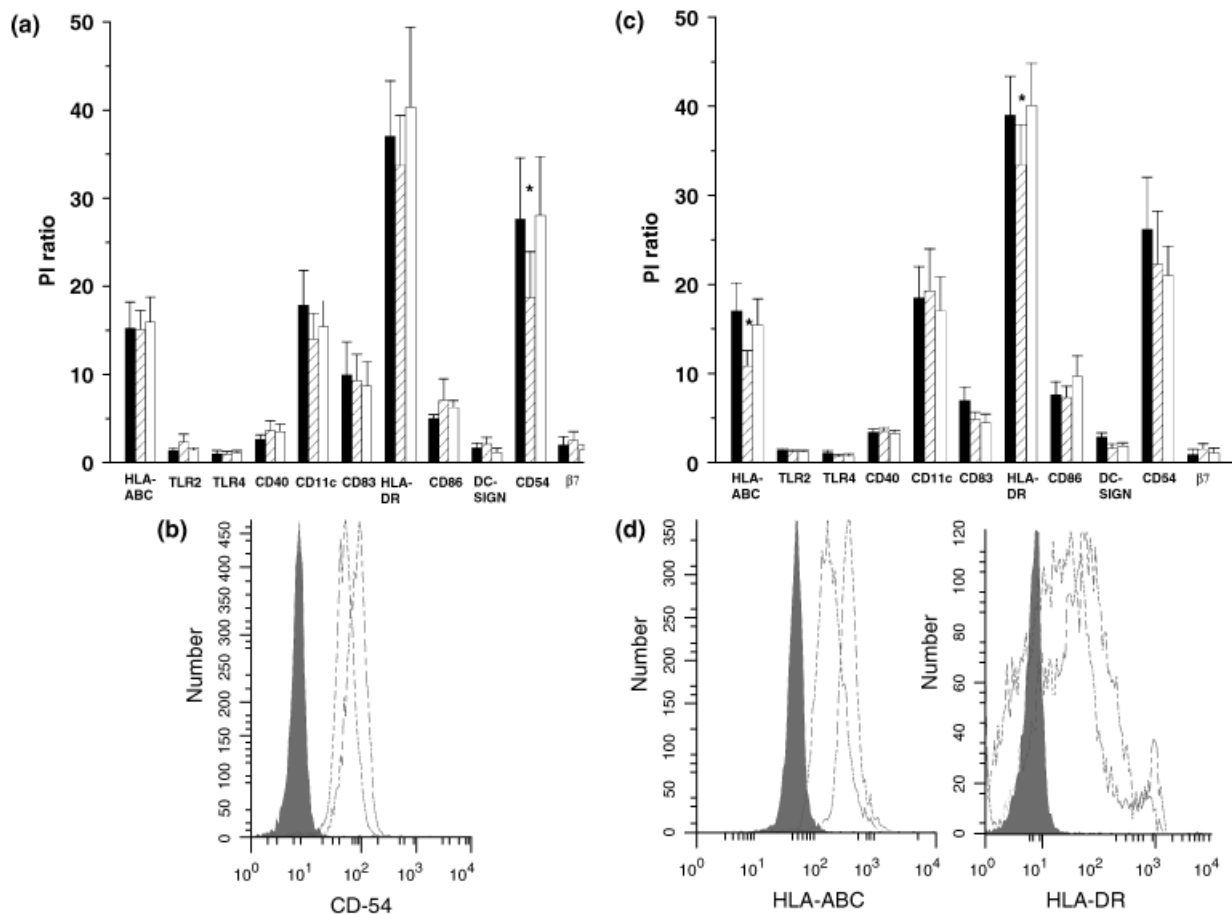


Fig. 4. Surface phenotype of sensitizer treated dendritic cell (DC) isolated from control or atopic donors. Untreated ■, 2,4-dinitrochlorobenzene (5 μ M) ▨ and trimellitic anhydride (5 μ M) treated DC □ incubated for 4 h from control (a) ($n=9$) or atopic donors (c) ($n=12$) were labelled with antibody for surface markers and analysed by flow cytometry. Results are expressed as mean positive intensity ratios \pm SEM for each marker after subtraction of an isotype control antibody histogram. Representative histograms are shown for CD54 in control DC (b) and HLA-ABC and HLA-DR in atopic DC (d). Shaded histogram represents isotype control, solid line histogram represents staining of untreated cells while dashed line histogram corresponds to DNCB-treated DC. * $P < 0.05$ determined by paired Student's t -test.

T cell stimulation down-regulated by trimellitic anhydride can be restored through the neutralization of IL-12

We next assessed whether IL-12 production by DC in response to TMA treatment had any functional effect on the proliferation of T cells. IL-12 was blocked with a neutralizing antibody in a mixed leucocyte reaction. DC incubated with TMA induced significantly less stimulation of T cells than control untreated cells (Fig. 7a). When the plate was repeated in the presence of neutralizing IL-12 antibody, TMA-treated DCs exhibited comparable stimulatory activity with untreated cells. To rule out plate-to-plate variability and to confirm that the antibody was acting on sensitizer-treated cells the effect of anti-IL-12 antibody on sensitizer-treated cells was also confirmed within the same Terasaki plate (Fig. 7c). This effect of anti-IL-12 antibody was observed in three out of five experiments. It was not seen in two experiments where TMA-treated cells had a stimulatory capacity similar to that of

untreated cells. No difference was seen in responses to untreated cells in the presence of the neutralizing antibody.

Discussion

We demonstrated that DC produced polarized cytokine responses to contact and respiratory sensitizers *in vitro*. This effect of sensitizer treatment was only observed in DC isolated from control but not atopic donors. DC from control donors up-regulated the production of Th1 cytokines IL-12 and IFN- γ in response to DNCB and up-regulated the production of the Th2 cytokine, IL-13 in response to TMA. This Th1/Th2 profile is in accordance with both murine models [28] and *in vitro* studies [29]. The majority of published research concerning interactions of chemical haptens with human DC has utilized DC differentiated from monocytes or CD34⁺ progenitors. We chose to investigate cells that could be isolated simply from human blood, required no long-term culture and had

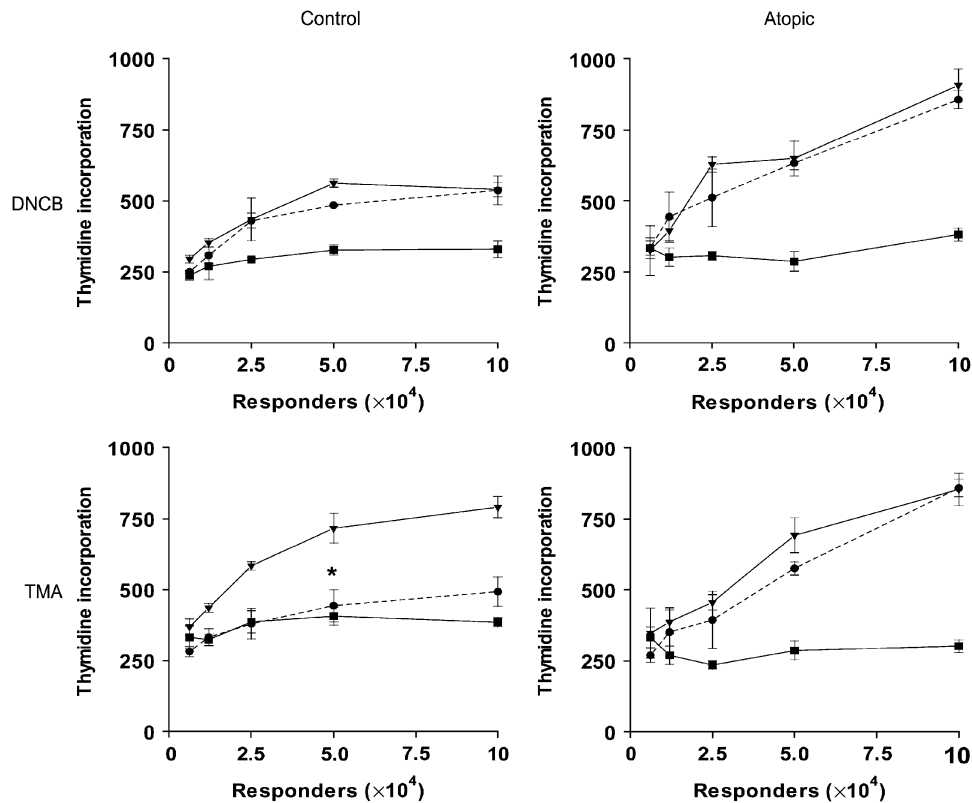


Fig. 5. Stimulatory capacity of sensitizer-treated dendritic cell (DC) isolated from control and atopic donors determined in a mixed leucocyte reaction. DC isolated from atopic or control donor groups were treated with $5 \mu\text{M}$ 2,4-dinitrochlorobenzene (DNCB) or $5 \mu\text{M}$ trimellitic anhydride (TMA) for 4 h and assessed for their stimulatory capacity in a mixed leucocyte reaction. DC (1000) were added to increasing numbers of allogeneic peripheral blood mononuclear cells (PBMCs) (x -axis) in triplicate wells. Proliferation was assessed on day three by ^3H thymidine incorporation (quantified by densitometry) and results are the mean \pm SEM. Included in individual plates were control wells containing PBMCs alone \blacksquare and PBMCs with the addition of untreated DC \blacktriangledown , test wells contained PBMCs with sensitizer-treated DC \bullet . Each graph shows representative data for 10 individual experiments carried out. * $P < 0.05$ determined by ANOVA.

the distinctive phenotype and allo-stimulatory capabilities of DC.

It is becoming increasingly apparent that changes to DC phenotype may be heavily influenced by the degree of cyto-toxicity of the hapten *in vitro*. Many reported positive responses have arisen through the treatment of DC with very high/sub-toxic concentrations of haptens, and although a number of groups have demonstrated changes in DC phenotype using apparently non-toxic concentrations it has been suggested that low levels of cyto-toxicity in these systems may have not been detected [30]. We have attempted to use only non-toxic concentrations of chemical sensitizers with short incubation times to reduce the likelihood of changes in DC function being attributable to chemical cyto-toxicity.

DC cytokine responses to chemical sensitizer treatment were dependent on basal cytokine production in untreated cells; more exaggerated cytokine responses to chemical sensitizers were observed when un-stimulated DC produced little or no cytokine. This effect was best represented in control donor DC. DNCB treatment up-regulated

the production of the Th1 cytokines IL-12 and IFN- γ along with the Th2 cytokine IL-4, whereas TMA treatment up-regulated the production of IL-13 and IL-10. Although CHS has a predominantly Th1 cytokine profile, IL-4 may be required for CHS induction *in vivo* [31]. It would be of interest to see if this reactive cytokine profile, seen in a number of control donors, was stable and reproducible over a longer time point. Unfortunately, this was not possible as the majority of donors only visited the clinic on a single occasion. If the profile was stable differential donor responses could be studied in more detail and the mechanism of the response investigated further.

The production of IL-10 in response to respiratory haptens may be fundamental in the progression of a Th2 response. Topical treatment with TMA rapidly up-regulates the production of IL-10 in the epidermis of animals [32] halting the migration of Langerhans cells and probably helping to induce a Th2 profile of disease. IL-10 also promotes immunological tolerance to inhaled antigens [33]. We have confirmed that TMA up-regulates the production of IL-10 after short time incubation and this

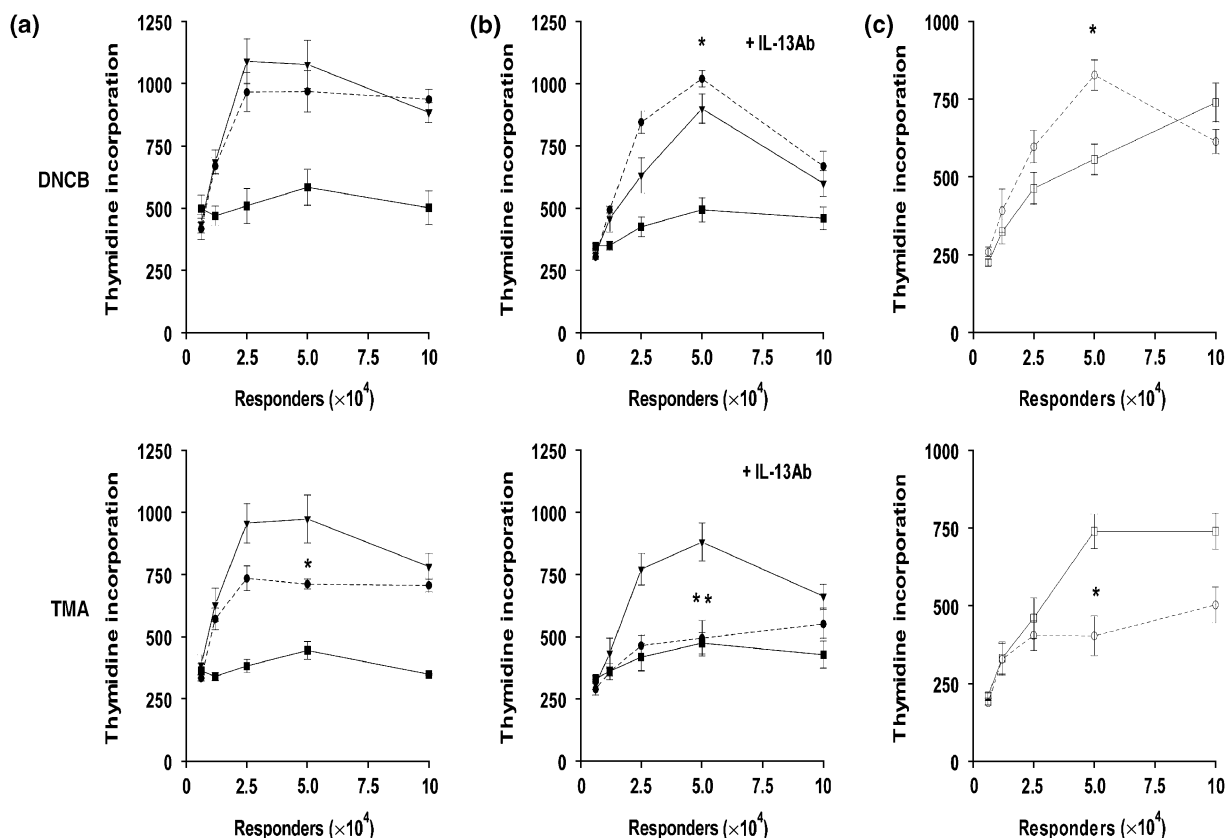


Fig. 6. IL-13 blocking in a mixed leucocyte reaction. Dendritic cell (DC) isolated from control donors were treated with 2,4-dinitrochlorobenzene ($5 \mu\text{M}$) or trimellitic anhydride (TMA) ($5 \mu\text{M}$) for 4 h and included in an MLR to determine T cell stimulation (a). One thousand DC were added to increasing numbers of allogeneic PBMCs (x -axis) in triplicate wells. Proliferation was assessed on day three by ^3H thymidine incorporation (quantified by densitometry) and results are the mean \pm SEM. (a) Control wells consisted of PBMCs alone \blacksquare and PBMCs with the addition of untreated DC \blacktriangledown , test wells (dashed line) contained PBMCs with sensitizer-treated DC \bullet . These plates were repeated with the addition of $10 \mu\text{g}/\text{mL}$ anti-IL-13 antibody to each well (b). To confirm that anti-IL-13 antibody was acting on IL-13 released by sensitizer-treated DC and to rule out plate variability, the effect of anti-IL-13 antibody on sensitizer-treated dendritic cells (DCs) was confirmed in the same plate (c) with the addition of sensitizer-treated DC \square or sensitizer-treated DC plus anti-IL-13 antibody (dashed line) \circ to responder PBMCs. * $P < 0.05$, ** $P < 0.01$ determined by ANOVA.

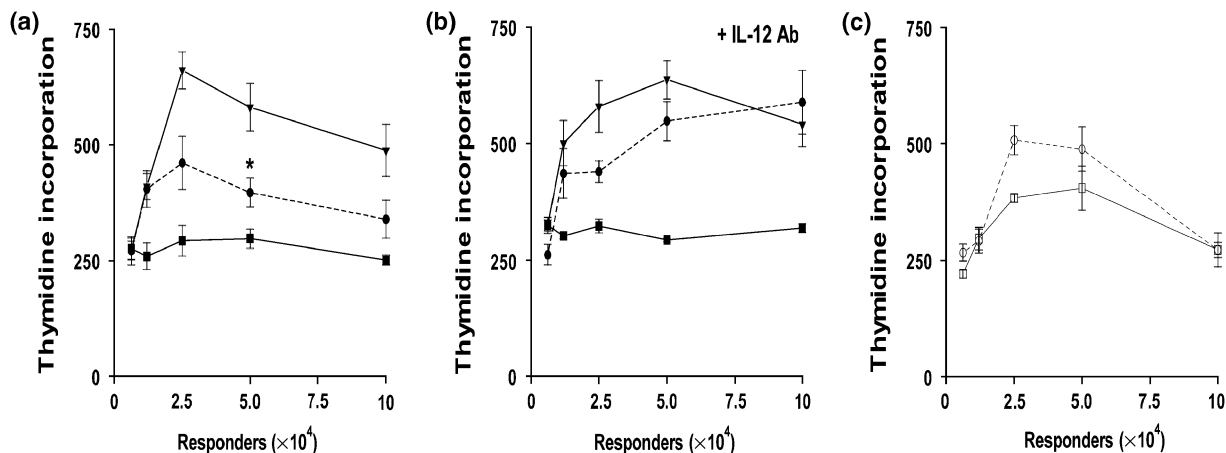


Fig. 7. IL-12 blocking in a mixed leucocyte reaction. Dendritic cell (DC) isolated from control donor groups were treated with TMA ($5 \mu\text{M}$) for 4 h and included in a mixed leucocyte reaction to determine T cell stimulation (a). One thousand DC were added to increasing numbers of allogeneic PBMCs (x -axis) in triplicate wells. Proliferation was assessed on day three by ^3H thymidine incorporation (quantified by densitometry) and results are the mean \pm SEM control wells consisted of peripheral blood mononuclear cells (PBMCs) alone \blacksquare and PBMCs with the addition of untreated DC \blacktriangledown , test wells (dashed line) contained PBMCs with TMA-treated DC \bullet . This plate was repeated with the addition of $10 \mu\text{g}/\text{mL}$ anti-IL-12 antibody to each well (b). To confirm that anti-IL-12 antibody was acting on IL-12 released by sensitizer-treated DC and to rule out plate variability, the effect of anti-IL-12 antibody on sensitizer-treated dendritic cells (DCs) was confirmed in the same plate (c) with the addition of TMA-treated DC \square and TMA-treated DC plus anti-IL-12 antibody \circ (dashed line) to responder PBMCs. * $P < 0.05$ determined by ANOVA.

may coincide with the induction of a Th2 response through the production of IL-13. Toeback et al. [34] demonstrated that DC treated *in vitro* with DNCB in the presence of CD-40L-transfected J558 cells showed high production of IL-12 compared with IL-10 whereas TMA-treated cells produced higher amounts of IL-10. Using a sensitive intracellular technique we have confirmed this pattern of cytokine production in freshly isolated DC and we suggest that chemical haptens alone can induce the production of polarized cytokines by DC without the requirement for CD-40 ligation. This early cytokine production in the epidermis may affect acceleration and direction of the immune responses.

No reproducible evidence was obtained to suggest that DCs from atopic donors exhibited differential cytokine production in response to chemical sensitizer treatment. The question whether DC from atopic donors are prone to promote Th2-type responses has not been clearly answered, although a number of studies suggest this to be the case [35, 36]. In this study, however, we found no overall difference in ongoing cytokine production of unstimulated DC isolated from control or atopic donors, and no differential cytokine response from atopic donor DC to the respiratory hapten TMA. A lack of uniformity of DC cytokine responses *in vitro* may represent different levels of atopic disease in our donor group. Different cytokine patterns are observed within different forms of atopic disease and levels of cytokines such as IFN- γ may correlate with atopic disease severity [37]. However, we could not discern any pattern of cytokine production in the DC in relation to patient history of allergic disease or skin prick test results.

DC from control donors were less stimulatory to T cells when treated with TMA *in vitro* thus indicating that these DC may be able to induce tolerance. Any T cell response to the respiratory sensitizer was dependent on IL-13 production, a finding in keeping with recent reports suggesting that immune responses developed to inhaled antigens are dependent on production of IL-13 [38, 39]. We also suggest that IL-13 may also have a regulatory effect on Th1 immune responses as blockade of the cytokine had a positive effect on T cell proliferation induced with DNCB-treated DC. Whether IL-13 produced by DC acts directly on T cells in the MLR is uncertain. T cells are not believed to possess a functional IL-13 receptor [40], but may up-regulate the Th2-associated transcription factor STAT6 [41] in response to the cytokine. One possibility is that IL-13 has a synergistic effect on other Th2 or regulatory cytokines such as IL-4 or IL-10, both of which we found to be up-regulated along with IL-13 in response to TMA treatment *in vitro*.

We found no difference in the phenotype of DC isolated from atopic or control donors. The demonstration that DCs from control donors down-regulate CD54 in response to DNCB may highlight a receptor utilized by the hapten for

entry into the cell. CD54, part of the ICAM-1 receptor, has its own specialized endocytic pathway [42] and although yet to be identified in DC may be employed by mycobacteria to gain entry in macrophages [43]. The down-regulation of MHC-class 1 and class II in atopic donors may also represent a recycling pathway of both molecules. The fact that only DNCB altered the phenotype of enriched DC suggests that the two sensitizers may have different recognition pathways. A possible difference has been identified with the suggestion that DNCB preferentially binds to cellular protein whereas TMA preferentially binds to exogenous or serum proteins [44]. Another possibility is that the two haptens may have multiple mechanisms of action on DC. This may be the case for DNCB, which has been shown to bind to a large number of intracellular proteins by Pickard et al. [45].

In conclusion, we have demonstrated that early cytokine production by DC *in vitro* in response to hapten treatment may have a function in promoting or inhibiting the level of T cell proliferation upon antigen presentation. DC from control donors were best able to differentiate between contact and respiratory haptens through cytokine production and effects on T cell stimulation. These results suggest that an *in vitro* test developed to identify chemical haptens may be better based on DC from normal non-atopic individuals because their cells show more consistent reactivity to the chemical sensitizers.

Acknowledgements

This work was supported by the MRC and a CASE studentship grant from the BBSRC and Unilever.

References

- 1 Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J Exp Med* 1987; **166**: 1654-67.
- 2 Reutter K, Jager D, Degwert J, Hoppe U. In vitro model for contact sensitization: II. Induction of IL-1[β] mRNA in human blood-derived dendritic cells by contact sensitizers. *Toxicol In Vitro* 1997; **11**:619-26.
- 3 Aiba S, Terunuma A, Manome H, Tagami H. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur J Immunol* 1997; **27**:3031-8.
- 4 Coutant KD, de Fraissinette AB, Cordier A, Ulrich P. Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicol Sci* 1999; **52**:189-98.
- 5 Rougier N, Redziniak G, Mouglin D, Schmitt D, Vincent C. In vitro evaluation of the sensitization potential of weak contact allergens using langerhans-like dendritic cells and autologous T cells. *Toxicology* 2000; **145**:73-82.

- 6 Aiba S, Manome H, Nakagawa S *et al*. p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, NiCl₂ and 2,4-dinitrochlorobenzene. *J Invest Dermatol* 2003; 120:390–9.
- 7 Mizuashi M, Ohtani T, Nakagawa S, Aiba S. Redox imbalance induced by contact sensitizers triggers the maturation of dendritic cells. *J Invest Dermatol* 2005; 124:579–86.
- 8 Cumberbatch M, Gould SJ, Peters SW, Basketter DA, Dearman RJ, Kimber I. Influence of topical exposure to chemical allergens on murine Langerhans cells: comparison of 2,4-dinitrochlorobenzene with trimellitic anhydride. *J Clin Lab Immunol* 1992; 37:65–81.
- 9 Warbrick EV, Dearman RJ, Kimber I. Induced changes in total serum IgE concentration in the Brown Norway rat: potential for identification of chemical respiratory allergens. *J Appl Toxicol* 2002; 22:1–11.
- 10 Tee RD, Cullinan P, Welch J, Burge PS, Newman-Taylor AJ. Specific IgE to isocyanates: a useful diagnostic role in occupational asthma. *J Allergy Clin Immunol* 1998; 101:709–15.
- 11 Dearman RJ, Moussavi A, Kemeny DM, Kimber I. Contribution of CD4+ and CD8+ T lymphocyte subsets to the cytokine secretion patterns induced in mice during sensitization to contact and respiratory chemical allergens. *Immunology* 1996; 89:502–10.
- 12 Warbrick EV, Dearman RJ, Basketter DA, Gerberick GF, Ryan CA, Kimber I. Analysis of cytokine mRNA expression following repeated exposure of mice to chemical contact and respiratory allergens. *J Appl Toxicol* 1998; 18:205–13.
- 13 Vandebriel RJ, De Jong WH, Spiekstra SW *et al*. Assessment of preferential T-helper 1 or T-helper 2 induction by low molecular weight compounds using the local lymph node assay in conjunction with RT-PCR and ELISA for interferon-gamma and interleukin-4. *Toxicol Appl Pharmacol* 2000; 162:77–85.
- 14 Ryan CA, Dearman RJ, Kimber I, Gerberick F. Inducible interleukin 4 (IL-4) production and mRNA expression following exposure of mice to chemical allergens. *Toxicol Lett* 1998; 94: 1–11.
- 15 Pichowski JS, Cumberbatch M, Dearman RJ, Basketter DA, Kimber I. Allergen-induced changes in interleukin 1 beta (IL-1 beta) mRNA expression by human blood-derived dendritic cells: inter-individual differences and relevance for sensitization testing. *J Appl Toxicol* 2001; 21:115–21.
- 16 Parronchi P, Macchia D, Piccinni MP *et al*. Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. *Proc Natl Acad Sci USA* 1991; 88:4538–42.
- 17 Reider N, Reider D, Ebner S *et al*. Dendritic cells contribute to the development of atopy by an insufficiency in IL-12 production. *J Allergy Clin Immunol* 2002; 109:89–95.
- 18 Kaminishi K, Soma Y, Kawa Y, Mizoguchi M. Flow cytometric analysis of IL-4, IL-13 and IFN-gamma expression in peripheral blood mononuclear cells and detection of circulating IL-13 in patients with atopic dermatitis provide evidence for the involvement of type 2 cytokines in the disease. *J Dermatol Sci* 2002; 29:19–25.
- 19 Cronin E, McFadden JP. Patients with atopic eczema do become sensitized to contact allergens. *Contact Dermat* 1993; 28:225–8.
- 20 Rees J, Friedmann PS, Matthews JN. Contact sensitivity to dinitrochlorobenzene is impaired in atopic subjects. Controversy revisited. *Arch Dermatol* 1990; 126:1173–5.
- 21 Buchvald D, Lundeberg L. Impaired responses of peripheral blood mononuclear cells to nickel in patients with nickel-allergic contact dermatitis and concomitant atopic dermatitis. *Br J Dermatol* 2004; 150:484–92.
- 22 Knight SC, Farrant J, Bryant A *et al*. Non-adherent, low-density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. *Immunology* 1986; 57:595–603.
- 23 McLellan AD, Starling GC, Hart DN. Isolation of human blood dendritic cells by discontinuous Nycodenz gradient centrifugation. *J Immunol Methods* 1995; 184:81–9.
- 24 Bagwell B. A journey through flow cytometric immunofluorescence analyses – finding accurate and robust algorithms that estimate positive fraction distributions. *Clin Immunol Newslett* 1996; 16:33–7.
- 25 Panoskaltzis N, Reid CDL, Knight SC. Quantification and cytokine production of circulating lymphoid and myeloid cells in acute myelogenous leukaemia. *Leukemia* 2003; 17:716–30.
- 26 Hart AL, Al Hassi HO, Rigby RJ *et al*. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* 2005; 129:50–65.
- 27 Knight SC, Bedford PA, Stagg AJ. Mixed leukocyte reactions in measuring immunity. In: Lotz MT, Thomson AW, eds. London: Elsevier, 2005; 350–60.
- 28 Dearman RJ, Moussavi A, Kemeny DM, Kimber I. Contribution of CD4+ and CD8+ T lymphocyte subsets to the cytokine secretion patterns induced in mice during sensitization to contact and respiratory chemical allergens. *Immunology* 1996; 89:502–10.
- 29 Pickard C, Smith AM, Cooper H *et al*. Investigation of mechanisms underlying the T-cell response to the hapten 2,4-dinitrochlorobenzene. *J Invest Dermatol* 2007; 127:630–7.
- 30 Straube F, Grenet O, Bruegger P, Ulrich P. Contact allergens and irritants show discrete differences in the activation of human monocyte-derived dendritic cells: consequences for in vitro detection of contact allergens. *Arch Toxicol* 2005; 79:37–46.
- 31 Traidl C, Jugert F, Krieg T, Merk H, Nünzelmann N. Inhibition of allergic contact dermatitis to DNCB but not to Oxazolone in interleukin-4-deficient mice. *J Invest Dermatol* 1999; 112: 476–82.
- 32 Cumberbatch M, Dearman J, Kimber I. Contribution of cutaneous interleukin-10 to the polarisation of immune responses to chemical contact and respiratory allergens. *Br J Dermatol* 2005; 152:840–1.
- 33 Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001; 2:725–31.
- 34 Toebak MJ, Moed H, von Blomberg MB *et al*. Intrinsic characteristics of contact and respiratory allergens influence production of polarizing cytokines by dendritic cells. *Contact Dermat* 2006; 55:238–45.
- 35 Reider N, Reider D, Ebner S *et al*. Dendritic cells contribute to the development of atopy by an insufficiency in IL-12 production. *J Allergy Clin Immunol* 2002; 109:89–95.
- 36 Bellinghausen I, Brand U, Knop J, Saloga J. Comparison of allergen-stimulated dendritic cells from atopic and nonatopic donors dissecting their effect on autologous naive and memory T helper cells of such donors. *J Allergy Clin Immunol* 2000; 105:988–96.

- 37 Leonard C, Tormey V, Burke C, Poulter LW. Allergen-induced cytokine production in atopic disease and its relationship to disease severity. *Am J Respir Cell Mol Biol* 1997; **17**:368-75.
- 38 Bellinghausen I, Brand P, Bottcher I, Klostermann B, Knop J, Saloga J. Production of interleukin-13 by human dendritic cells after stimulation with protein allergens is a key factor for induction of T helper 2 cytokines and is associated with activation of signal transducer and activator of transcription-6. *Immunology* 2003; **108**:167-76.
- 39 Padilla J, Daley E, Chow A *et al.* IL-13 regulates the immune response to inhaled antigens. *J Immunol* 2005; **174**:8097-105.
- 40 de Waal MR, Abrams JS, Zurawski SM *et al.* Differential regulation of IL-13 and IL-4 production by human CD8+ and CD4+Th0, Th1 and Th2 T cell clones and EBV-transformed B cells. *Int Immunol* 1995; **7**:1405-16.
- 41 Yu CR, Kirken RA, Malabarba MG, Young HA, Ortaldo JR. Differential regulation of the Janus kinase-STAT pathway and biologic function of IL-13 in primary human NK and T cells: a comparative study with IL-4. *J Immunol* 1998; **161**:218-27.
- 42 Muro S, Wiewrodt R, Thomas A *et al.* A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J Cell Sci* 2003; **116**:1599-609.
- 43 Paine R, Morris SB, Jin H, Baleeiro CEO, Wilcoxon SE. ICAM-1 facilitates alveolar macrophage phagocytic activity through effects on migration over the AEC surface. *Am J Physiol-Lung Cell Mol Physiol* 2002; **283**:L180-7.
- 44 Hopkins JE, Naisbitt DJ, Kitteringham NR, Dearman RJ, Kimber I, Park BK. Selective haptening of cellular or extracellular protein by chemical allergens: association with cytokine polarization. *Chem Res Toxicol* 2005; **18**:375-81.
- 45 Pickard C, Smith AM, Cooper H *et al.* Investigation of mechanisms underlying the T-cell response to the hapten 2,4-dinitrochlorobenzene. *J Invest Dermatol* 2007; **127**:630-7.