

HLA-DR expression on neonatal monocytes is associated with allergen-specific immune responses

John W. Upham, MB, FRACP, PhD,^a Patrick G. Holt, DSc,^a Angie Taylor, BSc,^b
Catherine A. Thornton, PhD,^c and Susan L. Prescott, MB, FRACP, PhD^b *Perth,*
Australia, and Swansea, United Kingdom

Background: The specific mechanisms regulating priming of T-cell immunity to common allergens during early childhood remain to be elucidated, though increasing evidence indicates that antigen-presenting cell function is impaired in childhood.

Objective: Examine the relationship between HLA-DR expression on monocytes and B cells, allergen-specific T-cell responses at birth, and clinical outcomes at 2 years of age. **Methods:** Blood mononuclear cells were obtained from 36 healthy neonates who were followed up clinically to the age of 2 years. Expression of HLA-DR by monocytes and B cells was determined at baseline and after *in vitro* exposure to IFN- γ , a cytokine that is known to upregulate the expression of HLA-DR. Mononuclear cells were stimulated with endotoxin or a panel of inhalant and food allergens, and cytokine responses and lymphoproliferation were determined after 1 and 5 days, respectively.

Results: The magnitude of HLA-DR upregulation on IFN- γ -stimulated cord blood CD14⁺ monocytes was consistently correlated with allergen-induced, but not mitogen-induced, lymphoproliferation at birth. HLA-DR upregulation on monocytes was also positively associated with endotoxin-induced IL-12 p70 synthesis ($\tau = 0.46$; $P < .001$) but inversely related to mite- and ovalbumin-induced IL-13 synthesis ($P = .0006$ and $P < .003$, respectively). HLA-DR expression on unstimulated cord blood monocytes was inversely associated with symptoms of atopic disease at the 2-year follow-up ($P = .015$). In contrast, HLA-DR expression on B cells was not associated with these parameters of immune function.

Conclusions: These findings suggest that the maturity of neonatal monocytes and their responsiveness to external stimuli are linked to differing patterns of immune reactivity at birth and to the risk of allergic symptoms in early childhood. (J Allergy Clin Immunol 2004;114:1202-8.)

Key words: Neonatal immune function, allergy, monocytes, B cells, antigen-presenting cells

T_H2-polarized cytokine responses to allergens are usually established in atopic individuals before the age of 5 years,^{1,2} although the mechanisms governing T_H1/T_H2 regulation in early childhood have not been fully elucidated. Although several publications have documented the ability of cord blood T cells to respond to allergens,³⁻⁶ the mechanisms by which such *in vitro* responses are regulated and their long-term significance in relation to the development of stable allergen-specific T_H2 memory remain unclear.

Antigen-presenting cells (APCs) have a key influence on T-cell activation and T_H1/T_H2 regulation, and many investigators have highlighted important differences in APC function between atopic and nonatopic adults.⁷⁻¹⁰ However, it is not clear whether altered APC function antedates sensitization and the onset of allergic disease or whether functional differences arise secondary to allergic inflammation. This issue can be addressed only by examining APC function in early life, before allergic sensitization.

APC function is impaired in the neonatal period.^{11,12} Neonatal T cells proliferate relatively poorly when stimulated with neonatal dendritic cells, but they proliferate as strongly as adult T cells when stimulated with adult dendritic cells.^{13,14} Similarly, the reduced capacity of neonatal T cells to produce the key T_H1 cytokine IFN- γ is corrected when these T cells are cultured with adult, rather than cord, APCs,^{12,15,16} suggesting that neonatal APCs lack the capacity to deliver important T_H1 polarizing signals to T cells. Thus, alterations in neonatal APC function may contribute to an increased susceptibility to infection and tolerance induction and a propensity for the induction of T_H2 responses in early life.

We therefore hypothesized that variations in neonatal APC maturity might be related to differing perinatal T-cell responses to allergens. In adult mice, the magnitude of T-cell reactivity is known to be proportional to immune-associated antigen (Ia) expression on the surface of APCs,¹⁷ although the extent to which Ia expression on cord blood APCs is associated with T-cell proliferation and cytokine production in response to allergens has not

From ^athe Telethon Institute for Child Health Research and Centre for Child Health Research and ^bthe School of Paediatrics and Child Health, University of Western Australia, Perth; and ^cDevelopmental Medicine, School of Medicine, University of Wales, Swansea.

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Reprint requests: John Upham, MB, FRACP, PhD, Division of Cell Biology, Telethon Institute for Child Health Research, PO Box 855, West Perth WA 6872, Australia. E-mail: johnu@ichr.uwa.edu.au.

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Abbreviations used

APC:	Antigen-presenting cells
HDM:	House dust mite
Δ HLA-DR:	Change in mean fluorescence intensity of HLA-DR expression, relative to baseline
Ia:	Immune-associated antigen
MNC:	Mononuclear cells
OVA:	Ovalbumin

been specifically addressed. Thus, the aims of this study were to examine the relationship between HLA-DR expression on cord blood monocytes and B cells (at baseline and in response to IFN- γ) and mononuclear cell (MNC) responses to clinically relevant environmental stimuli (allergens and endotoxin).

METHODS

Study population

Samples from a group of 36 healthy babies were included in this study. These were selected from a previously recruited cohort¹⁸ on the basis that there were sufficient cryopreserved MNCs to conduct the proposed additional *in vitro* studies presented here. The infants were originally recruited as part of a prospective study examining the development of allergen-specific immune responses in early childhood.^{5,18} Their mothers were recruited from antenatal clinics at the King Edward Memorial Hospital and nearby private obstetric practices. The study was performed with the approval of the Ethics Committee, Princess Margaret and King Edward Memorial Hospitals, and the parents or guardians of all children provided written consent. Cord blood samples were collected from the placentas of normal full-term deliveries after elective cesarean section at King Edward Memorial Hospital, Perth. Of the 36 children included in this study, 12 had no family history of allergy, and 24 had 1 or more first-degree relatives with allergic disease. At subsequent clinical visits (at 1 and 2 years of age), 13 of these 36 children showed evidence of atopy (as evidenced by positive skin prick tests to common allergens (eggs, milk, peanuts, grass pollens, cats, dogs, and/or house dust mite [HDM]) or a clinical allergic disease. Atopic eczema was diagnosed by a pediatric allergist. Children with recurrent wheezing, urticaria, or rhinitis were included in the atopic group only if they had other evidence of allergic sensitization (eg, atopic eczema, positive skin prick test, or positive RAST to common foods [egg, milk, peanut] or aeroallergens [HDM, grass pollens, or animal dander]). Eczema was diagnosed by a doctor if there were chronic erythematous pruritic skin eruptions on the face (except the nose and lips), trunk, or limbs. Mild cases in which the diagnosis was in doubt were excluded. The remaining children had no evidence of atopy or clinical disease ($n = 18$) or were lost to follow up ($n = 5$).

Cell preparation and culture

All laboratory experiments were performed and analyzed by individuals who were blinded to the clinical data. MNCs were isolated by density gradient centrifugation and cryopreserved in liquid nitrogen, as previously described.² Previous studies from our laboratory and elsewhere have demonstrated that this procedure does not distort cellular immune responses^{2,19,20} (see also the Online Repository at www.mosby.com/jaci). For functional studies, cryopreserved MNCs were thawed and resuspended at 10^6 viable cells per milliliter in RPMI 1640 (Gibco BRL, Melbourne, Australia) supplemented with L-glutamine, 10% FCS (Trace BioSciences,

Sydney, Australia), 2-mercaptoethanol (4×10^{-5} mol/L; Sigma, Sydney, Australia), and gentamicin (Pharmacia, Sydney, Australia), hereafter referred to as *complete medium*.

In vitro maturation of HLA-DR expression

To examine the capacity of cord blood monocytes and B cells to undergo maturation *in vitro*, MNCs were cultured for 24 hours at 37°C under 5% CO₂ in the presence or absence of recombinant human IFN- γ (10 ng/mL; Pharmingen, Lane Cove, New South Wales, Australia). This concentration of IFN- γ was chosen on the basis of preliminary dose-response experiments. After 24 hours, cells were washed in PBS supplemented with 0.1% sodium azide and 1% normal human serum and were incubated for 30 minutes on ice with the following mAbs obtained from BD Pharmingen: anti-CD14 fluorescein isothiocyanate or anti-CD19 fluorescein isothiocyanate, anti-HLA-DR peridinin chlorophyll protein, and corresponding isotype-matched control mAbs. Analysis was performed with a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ) to determine the percentage of cells expressing HLA-DR and the mean fluorescence intensity of HLA-DR expression. Data are expressed as Δ HLA-DR, by subtracting the level of HLA-DR expression on MNCs studied at baseline.

The capacity of MNCs to synthesize the T_H1 polarizing cytokine IL-12 was determined in parallel by stimulating cells with LPS stimulation after IFN- γ priming, as described previously.²⁰ This method of stimulation allows assessment of the capacity of APCs to respond under proinflammatory conditions. Briefly, MNCs were primed with 10 ng/mL recombinant human IFN- γ for 3 hours before stimulation with 10 ng/mL LPS (*Escherichia coli* LPS; Sigma) for a further 24 hours. Cell-free supernatants were harvested at 24 hours and frozen at -80°C for subsequent measurement of IL-12 p70, as described below.

Allergen-specific lymphocyte responses

Allergen-specific lymphocyte responses were determined as described previously.^{5,18,21} Briefly, lymphoproliferation was assessed by using cord blood MNCs in round-bottom 96-well plates, cultured at 2×10^5 in 200 μ L of serum-free medium (AIM V; Gibco, Life Technology, United Kingdom) alone or with optimal stimulating doses of HDM (CSL, Australia, 10 μ g/mL), Derp 1 (30 μ g/mL), Derp 2 (30 μ g/mL), rye grass pollen (10 μ g/mL), ovalbumin (OVA; Sigma, 10 μ g/mL), Fel d 1 (30 μ g/mL; ALK, Hørsholm, Denmark), purified protein derivative (CSL; 10 μ g/mL), or β -lactoglobulin (CSL; 100 μ g/mL) for 6 days. DNA synthesis was measured as incorporation of ³H-thymidine after labeling 16 to 18 hours before harvest. Data were expressed as Δ disintegrations per minute per culture; levels of incorporation above background (unstimulated) were designated control cultures. To assess cytokine responses, MNCs from the same samples were cultured for 24 hours in medium alone with the allergens and antigens outlined previously or with the polyclonal mitogen PHA (1 μ g/mL HA16; Murex; Biotech Ltd, United Kingdom).

Concentrations of the cytokine proteins IL-10, IL-12, IL-13, and IFN- γ were measured in supernatants by using commercial ELISA (OptEIA kits; BD Biosciences, Sydney, Australia). Data were expressed as Δ cytokine synthesis; levels of synthesis above background (unstimulated) were designated control cultures.

Because IFN- γ responses to allergens were often below detection limits, IFN- γ mRNA was also measured in the cell pellets from the same cultures by using semiquantitative RT-PCR as described previously.⁵ Briefly, RNA was extracted from cell pellets with RNazol B, and cDNA was transcribed by using oligo (dT) 15 and avian myeloblastosis virus reverse transcriptase in the presence of ribonuclease inhibitor. The PCR reactions used primers specific for IFN- γ and β -actin plus TthPlus DNA polymerase (Biotech, Western

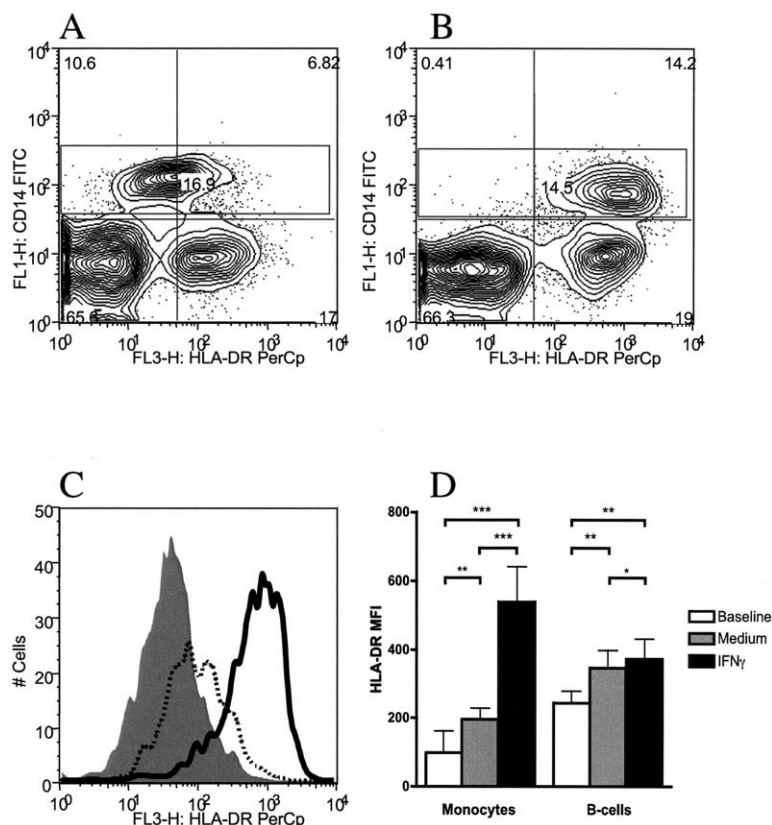


FIG 1. IFN- γ induces upregulation of HLA-DR expression on cord blood monocytes. **A-C**, Representative individual experiment: HLA-DR expression at baseline (**A**) and after 24 hours of exposure to IFN- γ (**B**). **C**, HLA-DR expression on CD14 $^{+}$ monocytes is shown at baseline (filled histogram) and after exposure to IFN- γ (solid line) or medium (dotted line). **D**, Group data (n = 36; * P < .05; ** P < .001; *** P < .0001). PerCP, Peridinin chlorophyll protein; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity.

Australia) and were performed in a programmable thermocycler. Samples of the PCR products were manually dot-blotted onto nylon membranes and hybridized overnight with biotinylated double-stranded probes. Binding was visualized by chemiluminescence with a commercial kit. Probe binding was quantified by computer-assisted densitometry, and the intensity of each test sample was expressed as a ratio relative to that of its respective β -actin control.

Statistical analysis

Group data were expressed as medians and interquartile ranges. Comparisons between groups were analyzed with nonparametric methods: Friedman 1-way ANOVA for 3-group comparisons and the Wilcoxon signed rank or the Mann-Whitney U test for 2-group comparisons of paired and unpaired data, respectively. The relationships between upregulation of HLA-DR and allergen- or endotoxin-stimulated MNC responses were assessed with the Kendall τ b rather than Spearman correlation coefficients to avoid the problems associated with "ties" within the data. All analyses were performed with SPSS version 11 (for Macintosh; SPSS Inc, Chicago, Ill).

RESULTS

IFN- γ induces upregulation of HLA-DR expression on cord blood monocytes

We first examined the capacity of cord blood monocytes and B cells to undergo maturation in response to

IFN- γ , a cytokine that is known to upregulate class II MHC expression on APCs. As shown in Fig 1, A, IFN- γ induced specific upregulation of HLA-DR expression on cord blood CD14 $^{+}$ monocytes. The intensity of HLA-DR expression on monocytes exposed to IFN- γ was significantly greater than that seen on cord blood monocytes studied at baseline or after culture in medium alone (P < .001 for both comparisons; Fig 1, D).

In contrast, IFN- γ had relatively minor effects on HLA-DR expression on cord blood CD19 $^{+}$ B cells. Whereas HLA-DR expression increased slightly on B cells cultured for 24 hours compared with baseline, HLA-DR expression on IFN- γ -exposed cells was only marginally greater than on cells exposed to medium alone (Fig 1, D).

HLA-DR upregulation on cord blood monocytes is associated with IL-12 p70 synthesis by neonatal MNCs

We next examined the capacity of cord blood MNCs to synthesize IL-12 p70. APCs represent the principal source of this T_H1 polarizing cytokine in circulating leukocytes.²⁰ In response to stimulation with LPS plus IFN- γ , cord blood MNCs secreted modest amounts of IL-12 p70, and the magnitude of the response in individual subjects was

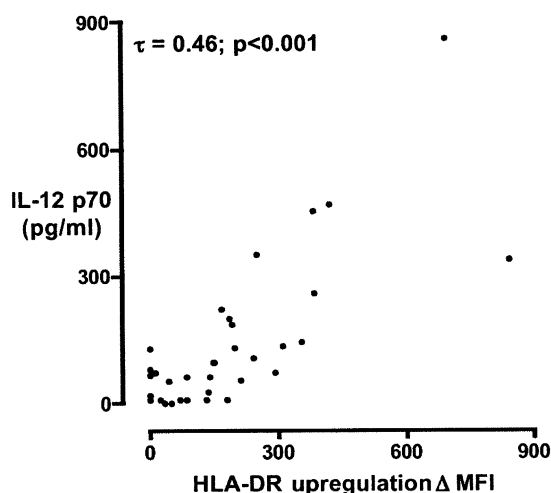


FIG 2. Association between upregulation of HLA-DR expression on cord blood monocytes induced by IFN- γ and capacity for IL-12 synthesis by cord blood MNCs. Kendall τ b and significance values are shown. *MFI*, Mean fluorescence intensity.

strongly correlated with Δ HLA-DR on IFN- γ -stimulated cord blood monocytes (Fig 2), but not with HLA-DR expression on monocytes studied at baseline or after culture in medium alone for 24 hours (Table I). Production of IL-12 p70 was also weakly associated with the degree of spontaneous upregulation of HLA-DR expression on B cells, but not with HLA-DR expression on B cells at baseline or after exposure to IFN- γ (Table I).

Capacity for HLA-DR upregulation on cord blood monocytes is associated with neonatal lymphocyte responses to allergens

APC maturity is a key rate-limiting step in the development of T-cell responses to antigens in early life.^{11,12} Accordingly, we examined the relationship between HLA-DR expression on cord blood monocytes and B cells and lymphocyte responses to a panel of clinically relevant allergens. As shown in Table II, the magnitude of proliferation induced by HDM, Der p 1, Der p 2, Fel d 1, OVA, and β -lactoglobulin in cord blood MNCs was closely associated with Δ HLA-DR on cord blood monocytes exposed to IFN- γ . In contrast, there was no relationship between allergen-stimulated lymphoproliferation and baseline monocyte expression of HLA-DR or between lymphoproliferation and expression of HLA-DR on B cells, either at baseline or after culture. Lymphoproliferation induced by PHA was not associated with baseline HLA-DR or Δ HLA-DR on monocytes or B cells (data not shown).

Levels of IFN- γ protein produced by cord blood MNCs in response to antigen-specific stimulation were generally below the detection limits of the assay. Although mitogen-activated cord blood MNCs produced low levels of IFN- γ , this was not associated with Δ HLA-DR on IFN- γ -activated cord blood monocytes (data not shown). Baseline HLA-DR expression, but not Δ HLA-DR on

TABLE I. Associations between IL-12 p70 production by cord blood MNCs and HLA-DR expression on monocytes and B cells*

Variable	HLA-DR expression		
	Baseline	Medium 24 hours	IFN- γ 24 hours
Monocytes			
Kendall τ b	0.13	0.10	0.46
P value (2 tailed)	NS	NS	<.001
B cells			
Kendall τ b	0.15	0.25	0.12
P value (2 tailed)	NS	.04	NS

NS, Not significant.

*Cord blood MNCs were cultured for 24h in the presence or absence of IFN- γ .

cord blood monocytes, was inversely associated with mitogen-activated IL-10 synthesis (Kendall's τ b, -0.35 ; $P = .003$; data not shown).

In contrast, Δ HLA-DR on cord blood monocytes was inversely proportional to HDM- and OVA-specific IL-13 synthesis. In individuals in whom HDM- or OVA-specific IL-13 synthesis could be detected (ie, ≥ 2.0 pg/mL), the magnitude of Δ HLA-DR on IFN- γ -activated monocytes was significantly less than that observed in individuals in whom HDM- or OVA-specific IL-13 synthesis was undetectable (Fig 3).

Relationship with clinical outcomes

Baseline expression of HLA-DR on cord blood monocytes was significantly lower in children who subsequently had a clinical diagnosis of allergic disease within the first 2 years compared with children who remained well (Fig 4, A; $P = .015$). In contrast, HLA-DR on IFN- γ -activated monocytes was similar in both groups (Fig 4, B; not significant). Neither baseline HLA-DR nor Δ HLA-DR on IFN- γ -activated cord blood monocytes was associated with family history of atopy, maternal smoking, or sensitization (skin prick test reactivity) at the 1- or 2-year follow-up.

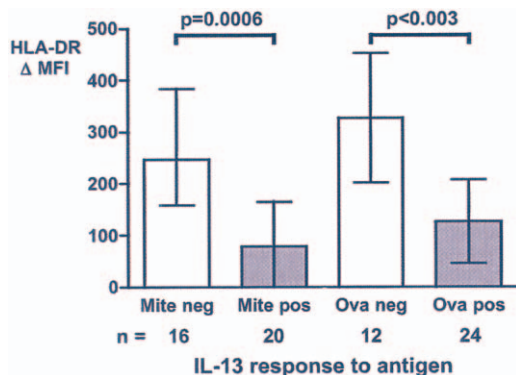
DISCUSSION

The specific mechanisms regulating the emergence of either T_H2 -dominated $CD4^+$ T-cell memory (allergy) or tolerance to common allergens during early childhood remain to be elucidated. Because the maturation of APC function is considered a rate-limiting step in the development of adaptive immunity in early postnatal life,^{11,12,22} we examined the relationship between cord blood monocyte and B-cell function, allergen specific immune responses, and clinical outcomes in a cohort of children taking part in a prospective study.

The salient finding to emerge was that the capacity for upregulation of HLA-DR (Δ HLA-DR) on IFN- γ -activated cord blood monocytes was strongly correlated with several parameters of immune function at birth. Moreover, the intensity of HLA-DR expression on unstimulated cord

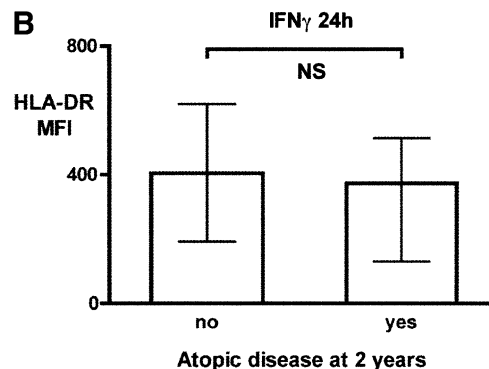
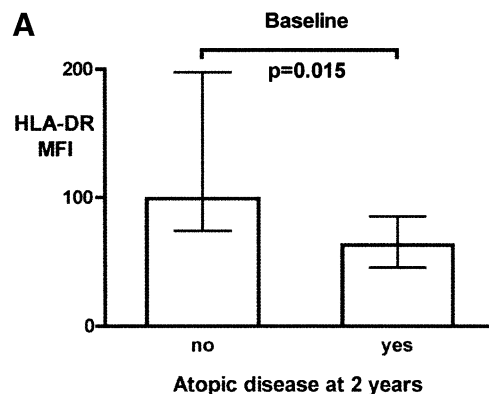
TABLE II. Relationship between Δ HLA-DR on IFN- γ -activated cord blood monocytes and allergen-specific lymphoproliferative responses at birth (n = 36)

Immune response	Stimulus	Median (interquartile range)	Kendall τ b (P value)
Proliferation (Δ counts per minute)	HDM	1348 (339-2385)	+0.41 (.0001)
	Der p 1	2559 (1215-4697)	+0.29 (.01)
	Der p 2	835 (0-1968)	+0.34 (.004)
	Fel d 1	249 (0-1092)	+0.40 (.001)
	OVA	844 (0-2152)	+0.52 (<.0001)
	β -Lactoglobulin	1239 (120-2934)	+0.40 (.001)

**FIG 3.** The relationship between upregulation of HLA-DR expression on cord blood monocytes induced by IFN- γ and allergen-specific responses in cord MNCs. The extent of HLA-DR upregulation was compared in neonates who showed detectable (shaded boxes) or undetectable (open boxes) IL-13 responses to HDM and OVA. Responses were defined as positive if they were ≥ 2.0 pg/mL, the limit of detection of the ELISA. Significance values are as shown. *MFI*, Mean fluorescence intensity.

blood monocytes was a predictor of the development of allergic disease within the first 2 years of life. In contrast, there was little (if any) relationship between HLA-DR expression on B cells and either immune function at birth or clinical outcomes.

In cord blood, Δ HLA-DR on monocytes was positively correlated with allergen-specific lymphoproliferation and endotoxin-induced IL-12 p70 synthesis but was inversely related to HDM-induced IL-13 synthesis. Although statistical correlation does not prove cause and effect, there nonetheless seems to be a strong relationship between Δ HLA-DR on cord blood monocytes and allergen-specific immune responses that is independent of atopic family history. Although it is known that in adult mice, the magnitude of an antigen-specific T-cell proliferative response is proportional to the number of Ia molecules on the surface of APCs,¹⁷ our findings highlight the importance of Δ HLA-DR on cord blood monocytes, in addition to baseline HLA-DR, as a determinant of immune responses in early life. It thus seems that the responsiveness of neonatal APCs to external stimuli is closely linked to both quantitative (lymphoproliferation) and qualitative (IL-13) aspects of allergen-specific immune responses at birth.

**FIG 4.** HLA-DR expression on cord blood monocytes and risk of allergic disease by 2 years of age. **A**, Baseline HLA-DR expression. **B**, HLA-DR expression after 24 hours of exposure to IFN- γ . *MFI*, Mean fluorescence intensity; *NS*, not significant.

Although several publications have documented the ability of cord blood T cells to respond to allergens,³⁻⁶ the regulation of these *in vitro* responses and their relationship to the development of stable patterns of T-cell memory have not been well characterized. Most cord blood T cells are CD45RA⁺ naive cells, many of which display phenotypic markers of recent thymic emigrants.²³ As such, the mechanisms governing T-cell function in the perinatal period are likely to be quite distinct from those governing adult T cells, where CD45RO memory cells predominate. Our findings suggest that the responsiveness

of cord blood monocytes to IFN- γ may be one important determinant of emerging immune responses to a broad range of allergens.

A variety of *in vivo* and *in vitro* studies have demonstrated that development of the adaptive immune system is generally deficient in the early postnatal period^{5,24-26}—particularly the ability to develop stable T_H1 memory.^{27,28} Furthermore, the maturity of APC populations seems to be a key rate-limiting step in the postnatal development of adaptive immunity. Strong neonatal T_H1 responses can be elicited by the use of adult, rather than neonatal, APCs,^{12,15} whereas activation of APC function with potent adjuvants allows neonatal animals to mount vigorous T_H1 responses to exogenous antigens.²⁹⁻³¹

Our findings herein suggest that variations in the responsiveness of cord blood monocytes to IFN- γ , and potentially to other proinflammatory signals, is closely associated with differing patterns of immune responses measurable in cord blood. Greater degrees of upregulation of HLA-DR on IFN- γ -activated monocytes are associated with more vigorous lymphoproliferation, greater capacity for IL-12 synthesis, and smaller IL-13 responses, whereas lesser degrees of HLA-DR upregulation are linked to reduced lymphoproliferation, a lesser capacity for IL-12 p70 synthesis, and larger IL-13 responses (Table III). In contrast, upregulation of monocyte HLA-DR expression was not associated with mitogen-induced lymphoproliferation, and at this stage it is unclear whether cord blood monocyte responsiveness is associated with T-cell responses to a broader range of antigens. We plan follow-up studies to address this issue.

Children who had developed allergic disease by the age of 2 years had significantly lower baseline expression of HLA-DR on their cord blood monocytes compared with healthy children. This finding supports the notion that immaturity of APCs is a risk factor for the development of clinically significant allergic disease. Moreover, the long-term significance of our findings requires clinical and immunologic evaluation of large cohorts of children followed up over several years to determine whether HLA-DR expression on various APC populations is also predictive of stable allergen-specific T_H1 or T_H2 cytokine production and allergic sensitization. Such large cohort studies will also allow for detailed multivariate analyses to determine which aspects of innate and adaptive immune function at birth are most strongly predictive of later allergic disease.

At this stage, it is not clear whether the associations we have described between immune responses and Δ HLA-DR on cord blood monocytes reflect variations in the ability of neonatal APCs to respond to external stimuli in general or to IFN- γ in particular, given that a variety of other cytokines are known to regulate MHC class II expression on APCs. IL-4 is a potent stimulator of HLA-DR expression on both B cells and monocytes,³² although this effect may be partially IFN- γ dependent.³³ GM-CSF increases and IL-10 inhibits class II expression on adult human monocytes,^{34,35} and further studies will examine

TABLE III. The responsiveness of cord blood monocytes to IFN- γ , as determined by upregulation of HLA-DR, is linked to differing immune response patterns at birth

Variable	Stimulus	Δ HLA-DR (high)	Δ HLA-DR (low)
T-cell proliferation	Allergen	++	+
IL-12 p70 synthesis	Endotoxin	++	+
IL-13 synthesis	Allergen	+	++

the ability of a wider range of stimuli to induce maturation of various neonatal APC populations, including the various dendritic cell subsets found in cord blood.

Similarly, there is a need to further examine the mechanisms responsible for the observed heterogeneity in the ability of IFN- γ to upregulate HLA-DR expression on cord blood monocytes. This heterogeneity seems to be independent of atopic family history. Others have shown that IFN- γ enhances HLA-DR expression on both adult and neonatal monocytes,³⁶ and on average, adult and neonatal monocytes express the IFN- γ receptor to a similar degree.^{37,38} Whether the heterogeneity in Δ HLA-DR that we observed in this study can be attributed to variations in IFN- γ receptor expression or to changes in downstream IFN- γ signaling pathways is yet to be determined.

In conclusion, our findings suggest that the ability of cord blood monocytes to mature in response to external stimuli is an important predictor of allergen-specific T-cell reactivity during the neonatal period. The extent to which monocyte responsiveness, and the responsiveness of other APC populations, develops during the postnatal period may have important implications for the subsequent development of allergic sensitization.

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