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Naïve Donor Responses to *Schistosoma mansoni* Soluble Egg Antigens

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Abstract

Schistosome infection induces profound Th-biasing and immune suppression. Although much has been examined in mice, few studies have examined responses of naïve humans to schistosome antigens. In this study, we examined the response of naïve human peripheral blood mononuclear cells (nPBMC) to stimulation with *Schistosoma mansoni* soluble egg antigen (SEA) using a priming *in vitro* (PIV) assay. We found that SEA induced a pronounced $CD4^+$ T-helper cell response based on cytokine secretion and phenotyping markers. SEA-stimulated nPBMC (SEA cells) at day 7 post-priming and after the first recall consisted predominantly of Th0-like $CD4^+$ T cells. Following the second recall, the majority of donor (10/12) responses were Th2-like. The cell population consisted of approximately 64% $CD4^+$, 17% $CD8^{+high}$, 12% $CD19^+$, and 7% $CD23^+$ cells. The $CD4^+$ population also expressed HLA-DR^+, $CD54^+$, $CD45RO^+$ and $CD25^+$ whereas the $CD19^+$ cells expressed CD80 and CD86. Following priming, we detected high levels of IL-6, IFN-γ, IL-12p40, IL-10 and IL-5. Upon restimulation, SEA cells secreted IL-5 and high levels of IL-10, typical of a Th2-like response. The data presented herein shows that the majority of naïve donor dendritic cells, following stimulation with SEA, prime and clonally expand SEA-specific T cells towards a Th2-type response. However, two donors responded with an atypical response, producing IFN-γ coincident with low levels of IL-10. Whether this differential response was due to HLA or other genes was not determined but is currently under investigation.

Introduction

Schistosoma mansoni is a helminth parasite infecting approximately 200 million people world-wide, with over 600 million people living in endemic areas at risk of acquiring the infection [1]. In the mouse model, *S. mansoni* infection is characterized by a brief, early pro-inflammatory immune response, with production of IL-2 and IFN-γ [2]. The pro-inflammatory response to early egg deposition shifts to Th2-type with production of IL-4, IL-5 and IL-10 [2, 3]. Prior to the Th2 response, a transient Th0-like response occurs, characterized by mixed cytokine production [4, 5]. Carbohydrates [6] and other ligands in soluble egg antigen (SEA) stimulate production of IL-10 [7] and IL-6, contributing to the downregulation of Th1 responses [8].

Many studies have documented early immune events during experimental schistosomiasis in murine models [5, 9–11]. However, there are few reports describing the development of the immune response in humans and it is important to note that the majority of studies using human cells were performed with total peripheral blood mononuclear cells (PBMC) [12–14] or dendritic cells (DC) [15–17] from infected individuals. Human DC stimulated with SEA show similar responses to those observed in mice, there is Th2 polarization and the DC do not undergo maturation [18, 19]. The results presented here are the first based on priming *in vitro* (PIV) of naïve human PBMC stimulated with SEA using DC as antigen presenting cells (APC) to evaluate the early events in the immune response during a schistosomiasis infection. DC are potent APC [20–22], being major initiators of primary antigen-specific responses both *in vitro* [23] and *in vivo* [24, 25]. DC express several markers including class II MHC, ICAM-1, DEC-205 and B7 co-stimulatory molecules [26–28]. Moreover, there is evidence that DC subsets can produce different cytokine microenvironments that may differentially direct the immune response [29, 30]. In this study, we determined whether SEA could induce cytokine patterns in naïve

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human PBMC similar to those described in the murine model. A PIV assay was established using naïve PBMC (nPBMC)-derived DC that were subsequently pulse-stimulated with SEA. The immune response of nPBMC after priming, first and second recalls was evaluated. We demonstrated that the majority (10/12) of SEA-stimulated PBMC $CD4^+$ T cells polarized towards a Th2-like response after the second recall, based on cytokine profile and surface antigen immunophenotyping. However, SEA-stimulated PBMC from two individuals showed evidence of a shift towards a Th1-like immune response.

Materials and methods

Human donors. Peripheral blood mononuclear cells were obtained from 12 healthy, schistosome naïve, Brazilian blood donors between 19 and 28 years of age and living in a schistosomiasis non-endemic area. The donors were screened for the presence of antibodies to HIV, HTLV I/II, HBsAg, HCV, VDRL and Chagas disease. Individuals positive for any of these infections were excluded from the study. The donors had no previous history of schistosomiasis and all were negative for *S. mansoni* infection by stool examination using the Kato–Katz method [31] and for antibodies to SEA by ELISA. The Ethics Committee of the Oswaldo Cruz Foundation approved the study protocol and all donors gave informed consent prior to the collection of blood.

Schistosoma mansoni antigen. Soluble egg antigen was produced and tested for endotoxin activity as previously described [32]. The SEA preparation did not include significant levels of non-specific contaminants, such as lipopolysaccharide, verified by the lack of response to SEA in nPBMC controls (data not shown). A flowchart of the experimental design is presented in Fig. 1.

Isolation and preparation of human PBMC. Peripheral blood mononuclear cells were isolated from heparinized blood (30–40 ml) by centrifugation on a Ficoll–Hypaque gradient (GE Healthcare, Piscataway, NJ, USA) at 400 g, 30 min, at 15 °C. PBMC were washed in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (pen-strep; Invitrogen, Carlsbad, CA, USA). PBMC were incubated in 24-well plates (Corning Inc., Lowell, MA, USA) in AIM-V medium (serum free lymphocyte medium; Invitrogen) at $2–3 \times 10^6$ cells/ml and cultured with and without Con A (5 μg/ml) and purified protein derivative (PPD) (5 μg/ml) for 72 h at 37 °C, 5% CO_2. Culture supernatants were harvested and stored at −70 °C. Con A or PPD were used as positive controls for the PIV assay.

Preparation of DC. Dendritic cells were prepared from PBMC as described previously [33] with the following modifications. Monocytes were obtained from PBMC (6×10^7 cells/ml) following spontaneous sedimentation and incubated in 75-cm^2 cell culture flasks (Corning Inc.) containing serum-free RPMI-1640 for 2 h at 37 °C, 5% CO_2. Non-adherent cells were removed by washing and the remaining cells were cultured for 6–8 days in 10 ml of DC complete medium (DCC-medium) containing RPMI-1640, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% pen-strep and 10% ultra-low IgG fetal bovine serum (FBS; Invitrogen), rhGM-CSF 50 ng/ml and rhIL-4 1000 U/ml (BD Biosciences, San Jose, CA, USA). Every 2 days, 500 μl of culture medium was removed and replaced with the same volume of fresh DCC-medium. DC differentiation was monitored by light microscopy, after 6–8 days, the DC were harvested and analysed for cytokine secretion. The expression of surface markers was determined by FACSort analysis (BD Biosciences).

PBMC PIV assay. Irradiated autologous DC (3000 rads) were placed in 24-well plates and stimulated with SEA (25 μg/ml) or RPMI (control) and incubated in complete medium (C-medium) containing AIM-V medium, 1% pen-strep and 5% human serum AB (Invitrogen). After 2 h, $2–3 \times 10^6$ cells/ml fresh nPBMC were added to the cultures giving a DC:nPBMC ratio of 1:15 and cultured

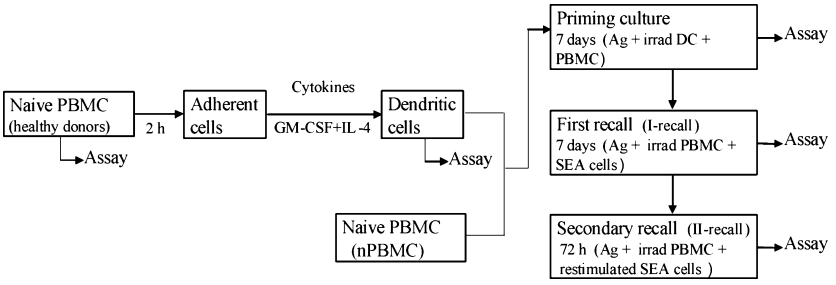

Figure 1 Experimental design scheme: nPBMC from healthy donors were used in the priming *in vitro* assay (PIV). DC were differentiated with IL-4 and GM-CSF for 6–8 days. PBMC were cultured with (priming) or without SEA for 7 days in the presence of irradiated autologous DC. SEA or RPMI cells were restimulated with (first recall) or without SEA (Ag) in the presence of irradiated PBMC as feeder cells and cultured for 7 days. SEA or RPMI cells (secondary recall) were cultured with and without antigen for 72 h in the presence of irradiated autologous PBMC. Following SEA-stimulation cytokine production was evaluated by ELISA and cell phenotypes were determined by FACS analysis.

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for 7 days at 37 °C, 5% CO_2. Culture supernatants were collected and stored at −70 °C. Cells were harvested and purified on Ficoll–Hypaque gradients and surface markers were identified by FACSort analysis. Freshly isolated unstimulated nPBMC were used as controls.

Following priming, the PIV SEA-stimulated PBMC (SEA cells) or RPMI-stimulated PBMC (RPMI cells) were restimulated (first recall) in the presence of irradiated (2,500 rads) autologous PBMC (2×10^6 cells/ml, 1:1 ratio) previously pulsed with SEA (20 μg/ml) or RPMI in 24 well plates for 2 h. The cells were cultured in C-medium for 7 days. IL-2 (20 U/ml) (R&D Systems Inc., Minneapolis, MN, USA) was added to the cultures every second and third day. Supernatants were collected on day 7 to measure cytokine production. Cells were harvested, purified on Ficoll–Hypaque gradients and analysed by FACSort. Cells from the first recall (SEA or RPMI cells) were then restimulated a second time (second recall) and cultured in AIM-V medium in the presence of irradiated autologous PBMC ($0.5–1 \times 10^6$ cells/ml, 1:1 ratio) previously pulsed with and without SEA (10 μg/ml), Con A (5 μg/ml) or PPD (5 μg/ml) in 48-well plates for 72 h at 37 °C, 5% CO_2. Supernatants were collected to measure cytokine production and the cells were harvested to determine the phenotypes.

Flow cytometry analysis. Cells were stained using the following conjugated mouse anti-human mAbs in two or three-colour immunocytometric assays: CD3-FITC (HIT3a), CD14-FITC (M5E2), CD40-FITC (5C3), CD45R0-FITC (UCHL1), CD80-FITC (BB1), CD86-FITC (FUN-1), HLA-DR, DP, DQ-PE (TU39), CD25-PE (IL-2Rα) (M-A251), CD54 (ICAM-1)-PE (HA58), CD39-PE (TU66), CD1a-PE (HI149), CD19-PE (HIB19), CD23-PE (M-L233), CD4-Cy (RPA-T4), CD8-Cy (RPA-T8) and their isotype-matched negative-control antibodies IgG_1-PE, -FITC or -Cy (MOPC-21), mouse IgG_{2a}-PE (G155-178), and mouse IgM-FITC (G155-228) (BD Biosciences).

Dendritic cells and lymphocyte phenotyping was performed according to the manufacturers' instructions with the following modifications. In 12 × 75 mm polystyrene tubes, 10^5 cells/ml were incubated on ice for 30 min with human serum (diluted 1:20). The cells were resuspended in 40 μl of FACS buffer [HBSS, 10% FCS, 0.01% sodium azide (Sigma-Aldrich, St. Louis, MO, USA), pH 7.2, 5 μl of each mAb] and incubated for 30–45 min at 4 °C in the dark. The cells were washed three times in FACS buffer and resuspended in 200 μl FACS buffer. Ten-thousand events were acquired on the FACSort flow cytometer (BD Biosciences) and the data analysed using CellQuest Software (BD Biosciences). Cells were gated on FSC versus SSC dot plots and further characterized by their fluorescence profile with two colour dot plots or single colour histograms. The results are expressed as either the percentage of positive cells within the selected gate or as proportions.

Cytokine assays. Sandwich ELISAs for IL-6, IL-8, IL-12p40 and TNF-α were performed using the Duo-set kit (R&D System, Inc.) and IFN-γ using the Intertest ELISA kit (Genzyme, Cambridge, MA, USA). IL-5 and IL-10 were assayed using the Human ELISA Set kits (BD Biosciences) following the manufacturers' instructions.

Statistical analysis. Statistical analyses were performed using the Student's *t* and non-parametric Mann–Whitney tests using the GraphPad Prism 5.0 software package (San Diego, CA, USA). Differences were considered significant when the *P*-value was <0.05 between SEA cells and fresh unstimulated PBMC controls for phenotype analysis and between SEA cells and control RPMI cells for cytokine production.

Results

Phenotype analysis and cytokine secretion in DC

The successful generation of DC from naïve PBMC in the presence of GM-CSF and IL-4 was determined by the expression of CD1a and the absence of CD14 surface markers as characterized by high forward and side light scatter (Fig. 2A). Monocyte-derived DC exhibited typical morphology as confirmed by light microscopy (data not shown). After 7 days, DC were analysed by FACS to determine the percentage of cells expressing $CD39^+$, $CD40^+$, $CD54^+$ (ICAM-1), $HLA\text{-}DR^+$, $CD86^+$ (B7-2) and $CD80^+$ (B7-1) molecules (Fig. 2B). Most of the DC population expressed $HLA\text{-}DR^+$ and $CD54^+$, while 62.5% of the DC expressed $CD86^+$. In addition, increased expression of $CD39^+$, $CD40^+$ and modest expression of $CD80^+$ was observed. The levels of IL-12p40, IL-8, IL-6, TNF-α and IL-10 secreted during DC culture differentiation prior to SEA stimulation were also determined and are shown in Fig. 2C. Of these cytokines, IL-8 was secreted at high levels whereas IL-12p40 production was low. In addition, we were unable to detect IL-2 (data not shown).

Phenotype analysis of the lymphocyte populations from SEA-stimulated PBMC

Following priming and recall, SEA-stimulated PBMC or nPBMC controls were counted and the frequency of surface marker antigens of the lymphocyte subsets were evaluated using anti-CD4, -CD8, -CD19 and -CD23 mAbs. Throughout this period, there was an overall 10-fold decrease in the total number of viable cells (Fig. 3). Specifically, there was a 50% drop post-priming, followed by a 23% reduction after the first recall and a further 9% reduction after the second recall. This decline was observed with and without SEA stimulation indicating

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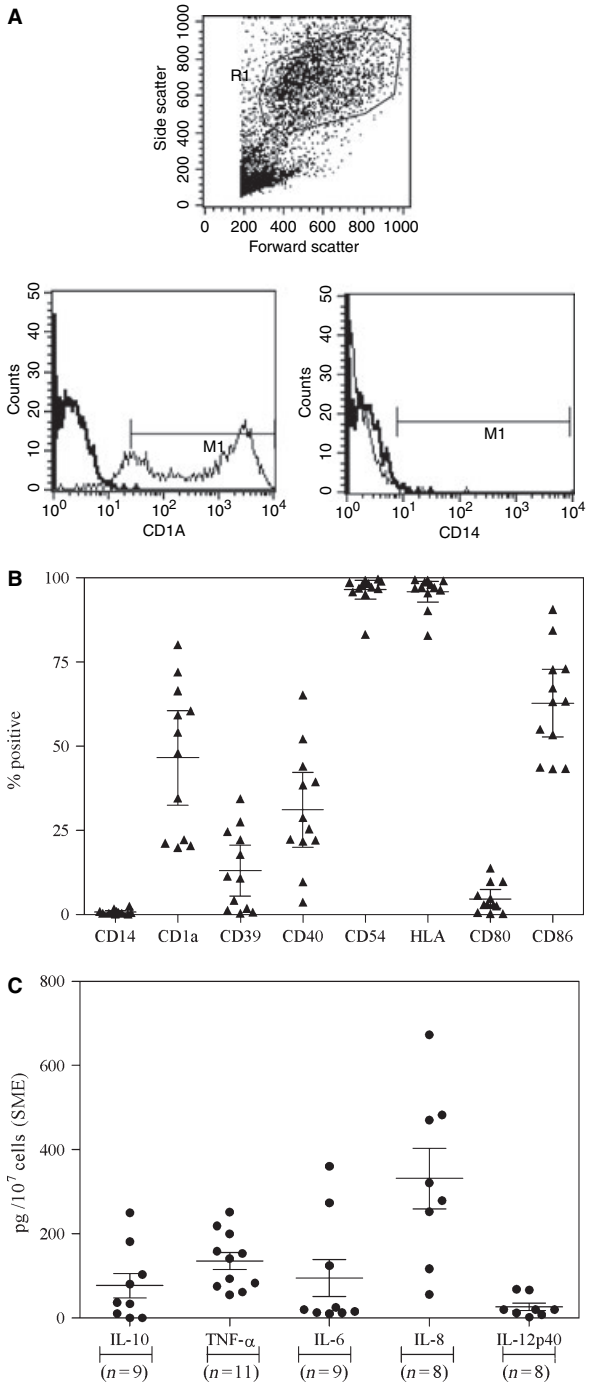

Figure 2 FACS analysis of DC surface markers and cytokine production. A representative light scatter plot (A) shows the gate selected for subsequent analyses by fluorescent antibody staining. Each histogram represents an overlay of CD1a or CD14 (light line) and an isotype matched control (bold line). DC surface marker-specific fluorochrome-labeled mAbs: CD14, CD1a, CD39, CD40, CD54, HLA-DR, CD80 and CD86 (B). Each point represents the percentage of positive DC within the selected gate in a single donor sample (n = 12). The mean percentage (±95% CI) is also shown. Following DC differentiation, culture supernatants were analysed for IL-12p40, IL-8, IL-6, TNF-α and IL-10 production (C). Each symbol represents an individual donor and the vertical bars represent the standard error of the mean (±SEM).

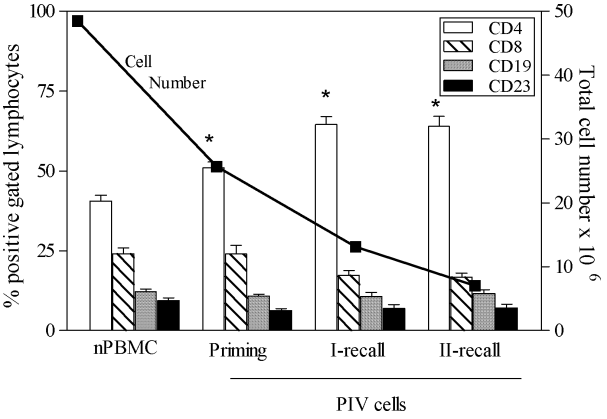

Figure 3 FACS analysis was used to identify the lymphocyte population present in SEA-stimulated PBMC following priming and recall responses. The lymphocytes were identified, using antibodies for $CD4^+$ and $CD8^+$ T cells or $CD19^+$ and $CD23^+$ B-cell subsets. The results are expressed as the percentage of positive cells within the lymphocyte population, the mean values (+95% CI) from 12 representative donors are presented. The unstimulated naïve PBMC control (nPBMC) or PIV cells after priming, first recall (I-recall) and secondary recall (II-recall) are shown. The total number of viable cells following each SEA stimulation was determined. Significant differences in the lymphocyte populations are indicated (*).

that it was not due to SEA toxicity. The viable cell population consisted of approximately 64% $CD4^+$, 17% $CD8^{+high}$, 12% $CD19^+$ and 7% $CD23^+$ cells. The SEA-stimulated PBMC $CD4^+$ T-cell subset increased significantly post-priming ($P < 0.005$), and after the first ($P < 0.0001$) and second recall responses ($P < 0.05$) compared to the nPBMC control (Fig. 3).

Phenotype analysis of SEA-stimulated PBMC lymphocyte subsets

The percentage of lymphocyte subsets post-priming and after the first and second recall responses were analysed by double-label immunophenotyping using CD4-CY in combination with CD25-PE (Fig. 4A); or HLA-DR-FITC (Fig. 4B); CD4-CY with CD54-PE (Fig. 4C) or CD45RO-FITC (Fig. 4D); and CD19-PE with CD80 FITC (Fig. 4E) or CD86 FITC (Fig. 4F). The mean number of $CD4^+CD25^+/CD4^+$ T cells increased significantly post-priming (8.6–20%, $P < 0.001$) and after the first (42.5%, $P < 0.0001$) and second recall responses (60%, $P < 0.0001$) compared to the unstimulated nPBMC control (Fig. 4A). Additionally, the population of $CD4^+HLA\text{-}DR^+/CD4^+$ T cells increased significantly post-priming (2.8–19.3%, $P < 0.01$) after the first recall (38.8%, $P < 0.001$) and the second recall responses (38%, $P < 0.001$) compared to the nPBMC control (Fig. 4B).

Using anti-CD54 (ICAM-1), the percentage of $CD4^+CD54^+/CD4^+$ T cells increased significantly post-priming (18.2–61.8%, $P < 0.01$) and after the first (84.1%, $P < 0.001$) and second recall responses (85.4%,

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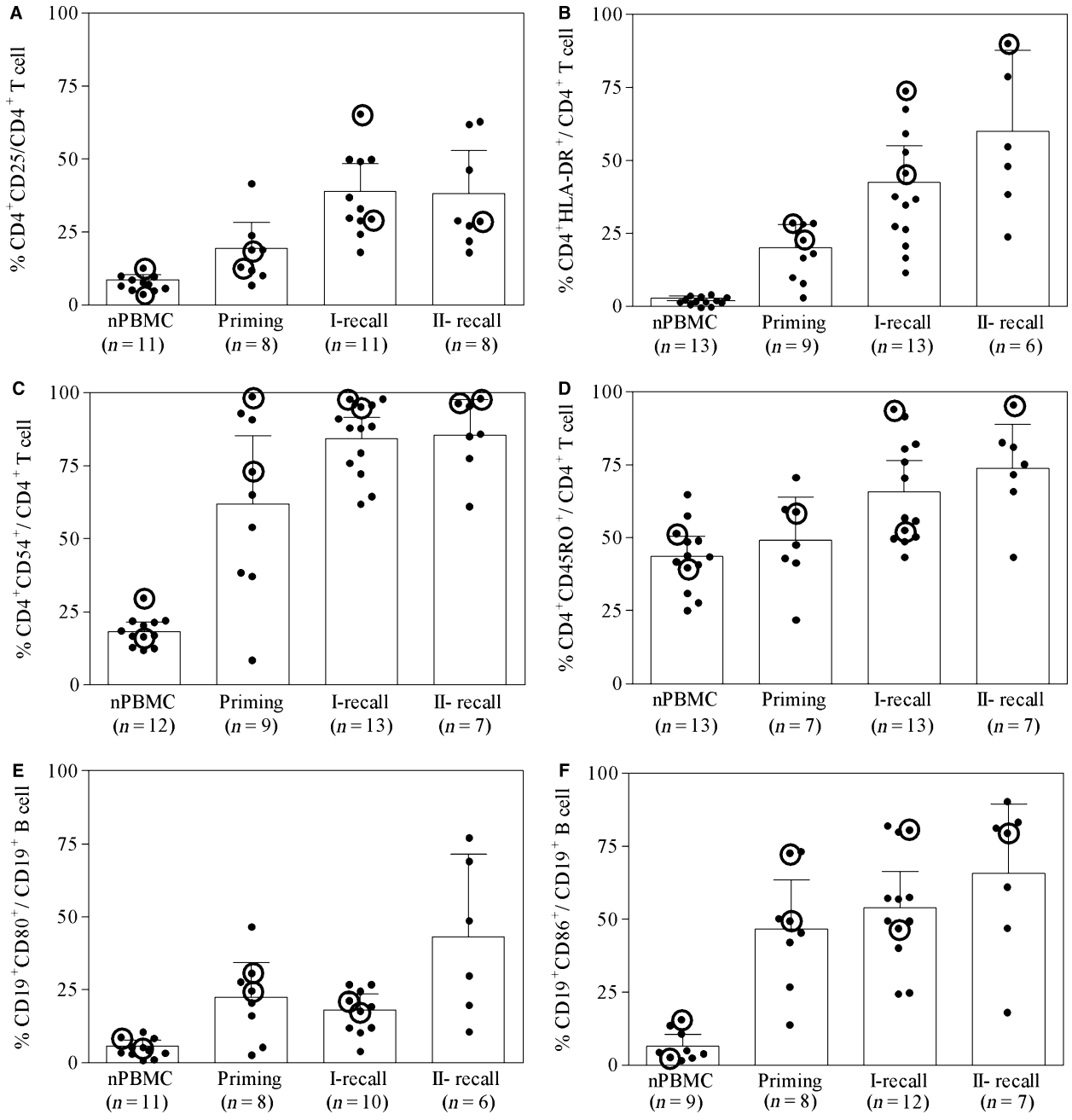

Figure 4 FACS analyses of SEA-stimulated PBMC following priming and recall responses. The unstimulated naïve PBMC control (nPBMC), or PIV SEA-stimulated PBMC following priming, first recall (I-recall) and second recall responses (II-recall) are shown. (A) double-label staining with anti-CD4 and anti-CD25-PE was used to identify $CD4^+CD25^+/CD4^+$ cells; (B) $CD4^+HLA\text{-}DR^+/CD4^+$ cells; (C) $CD4^+CD54^+/CD4^+$ cells; and (D) $D4^+CD45RO^+/CD4^+$ T-cell subsets or (E) $CD19^+CD80^+/CD19^+$ and (F) $CD19^+CD86^+/CD19^+$ on B-cell subsets. Each symbol represents an individual response. The mean percentage (+95% CI) values are also shown. The results from the Th-1 prone individuals are circled.

$P < 0.001$) compared to the nPBMC control (Fig. 4C). In addition, $CD45RO^+$ was used as a marker for memory T cells. The percentage of $CD4^+CD45RO^+/CD4^+$ T cells increased significantly after the first recall (43.6–65.7%, $P < 0.001$) and second recall responses (73.7%, $P < 0.01$) when compared to the nPBMC control (Fig. 4D).

$CD19^+CD80^+$ and $CD19^+CD86^+$ expression on B cells derived from SEA-stimulated PBMC (Fig. 4E,F) was determined. It was notable that despite the lack of a significant difference in the total number of $CD19^+$ B cells (Fig. 3), the $CD19^+CD80^+/CD19^+$ B-cell population increased significantly post-priming ($P < 0.01$) and after the second recall ($P < 0.01$) when compared to the nPBMC control (Fig. 4E). Expression of $CD19^+CD86^+/CD19^+$ on B cells was significantly upregulated following priming and after both recalls

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($P < 0.0001$) when compared to the nPBMC control (Fig. 4F).

Th-like patterns of cytokine secretion in SEA-stimulated PBMC

Cytokine production of SEA-stimulated PBMC (SEA cells) or RPMI-stimulated PBMC (RPMI cells) was determined to identify the Th $CD4^+$ cell subset response associated with SEA stimulation. SEA cells secreted moderate to high levels of IL-12p40, IL-5, IL-6, IL-10, IFN-γ and TNF-α post-priming and after the first recall response compared to control RPMI cells (Fig. 5A,B), indicative of a Th0-like response. After the first recall, SEA cells maintained a similar Th0-like response, as seen post-priming, with elevated levels of pro-inflammatory cytokines IL-6, TNF-α and IFN-γ. After the first recall, there was a moderate but non-significant decrease observed in the secretion of all cytokines except TNF-α.

In SEA cells from 10/12 donors there was a shift from the Th0-like response to a Th2-like response after the second recall, with high levels of IL-10 and low levels of IFN-γ detected (Fig. 5C). Unexpectedly, $CD4^+$ T cells from two individuals secreted high levels of IFN-γ and low levels of IL-10, more typical of a Th1-like response. To confirm the findings, the experiments were repeated using cells from three donors from the 10 that had Th2-like responses (Fig. 6A,B) and cells from one donor of two that had evidence of polarization towards a Th1-like response (Fig. 6C,D). These repeat assays yielded similar results to the initial PIV assays (Fig. 6).

Con A and PPD activation of SEA-stimulated PBMC after the second recall response

To confirm that the T-lymphocyte subsets resulting from SEA-stimulated PBMC (SEA cells) were the result of a SEA-specific clonal expansion, both stimulated and non-stimulated PBMC were treated with either PPD or Con A. ELISAs were used to determine the secretion levels of IFN-γ and IL-5 in the supernatant and both PPD and Con A were found to increase IFN-γ production in the nPBMC control (Fig. 7A). However, SEA-stimulated PBMC only secreted high levels of IFN-γ in response to Con A (Fig. 7B), demonstrating that this cell population had lost the ability to recognize PPD and signifying that the T-cell subset had clonally expanded in an SEA-specific event. IL-5 expression was detected at low levels under all of the conditions tested.

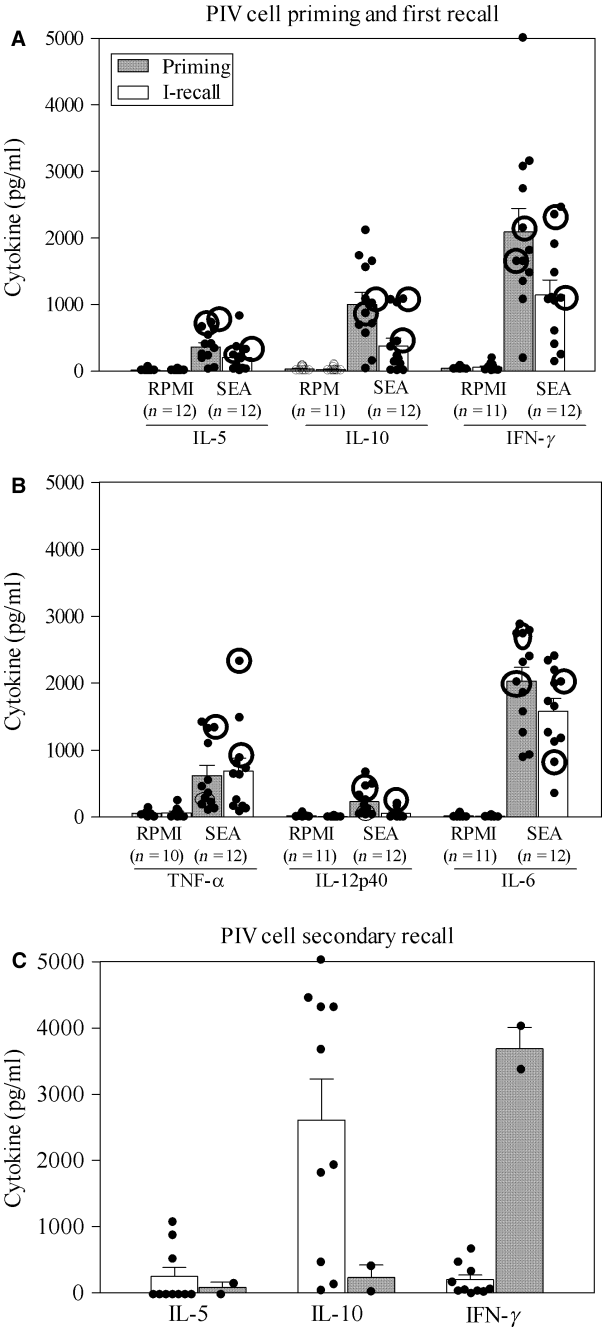

Figure 5 Cytokine production by SEA-stimulated PBMC following priming and recall responses. Post-priming cell supernatants were assayed for: IL-5, IL-10, IFN-γ (A); IL-6, IL-12p40 and TNF-α (B). (C) SEA-stimulated PBMC after the second recall response exhibited different cytokine profiles, 10 donors secreted IL-10, IL-5 and low levels of IFN-γ in (open bars) while two donors secreted high levels of IFN-γ (solid bars). Each symbol represents an individual response. The mean percentage (+SEM) values are also shown. The results from the Th-1 prone individuals are circled.

Discussion

In this study, we employed a PIV assay to investigate early immune events induced following treatment with SEA in naïve human PBMC from 12 healthy donors. This is the first report of a PIV assay using DC as APC to induce SEA-specific responses in naïve PBMC, thereby mimicking a schistosomiasis infection. Our findings

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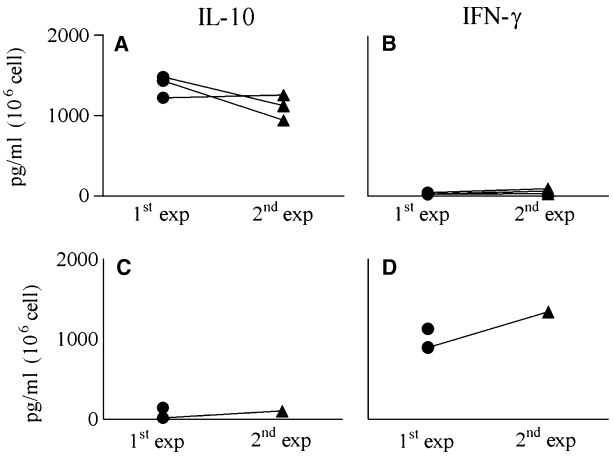

Figure 6 IL-10 and IFN-γ secretion by SEA-stimulated PBMC following secondary recall. SEA cells after secondary recall demonstrating the reproducibility of the Th1- and Th2-like responses presented in Fig. 5. IL-10 and IFN-γ production in SEA-stimulated PBMC from three Th2-like donors (A–B); compared to two Th1-like donors (C–D). These experiments were performed and repeated under the same conditions.

demonstrate that SEA activated and primed DC induce naïve PBMC T cells to initially differentiate into IL-6, IL-12p40, IFN-γ, IL-5 and IL-10 secreting T cells, similar to what is observed during the acute infection phase in mice [4, 5]. As expected, after the second recall event, the immune response of most (10/12) donor PBMC polarized towards a strong Th2-like response, consistent with previous reports [22, 34, 35]. In contrast, the response of two of 12 donors PBMC in the second recall response to SEA was Th1-like. This unusual response to SEA stimulation is similar to what was described as the predominant response in endemic areas [36]. Deposition of eggs leads to strong Th2 responses along with increased IL-5 and IL-10 secretion in mice. Imbalances in these responses led to severe lesions in mice however, in humans the effect of polarization of the immune response is not clear [37, 38].

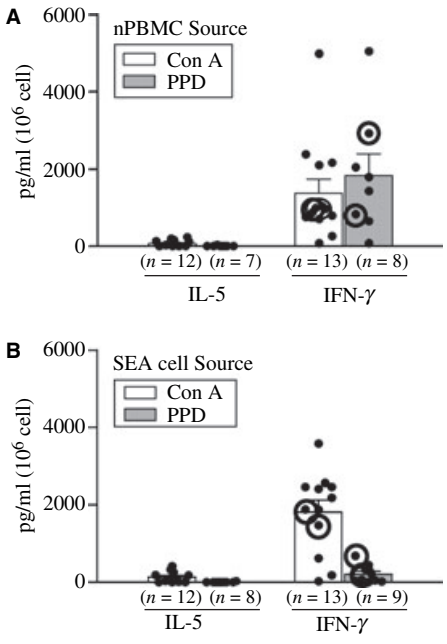

Figure 7 Cytokine levels by SEA-stimulated PBMC following exposure to Con A and PPD after the secondary recall response. (A) The naïve PBMC control (nPBMC) and (B) SEA-stimulated PBMC (SEA cells) were stimulated with PPD or Con A for 72 h. Culture supernatants were analysed for IL-5 and IFN-γ secretion. Each symbol represents an individual response. The mean values (+SEM) are also shown. The values from the Th-1 prone individuals are circled.

Characterization of DC surface antigen and cytokine production demonstrated that the DC produced high levels of IL-8 and were associated with upregulated expression of CD86 and low expression of CD80 co-stimulatory molecules (Fig. 2). HLA-DR, CD54 surface antigen expression and levels of the pro-inflammatory cytokines IL-6 and IL-12p40 were similar in all donors. Based on this data, there did not appear to be phenotypic differences in the donor DC that, upon subsequent SEA stimulation, resulted in either a Th2 or Th1-like immune response. However, this observation does not exclude the possibility that DC could respond differently to SEA, resulting in either Th1- or Th2-like responses.

In the majority of individuals, the composition of the lymphocyte population in SEA-stimulated PBMC was predominantly $CD4^+$ T cells secreting IL-5, IL-6 and high levels of IL-10 (Fig. 5C) indicating a polarization towards a Th2-like response as previously observed [2, 3, 22]. Conversely, SEA cells from two individuals secreted high levels of IFN-γ (Fig. 5C), indicating a Th1-like phenotype. Further investigations into naïve individual donor cytokine and chemokine microenvironments following exposure to SEA should be carried out to determine which of these factors contribute to driving DC1 versus DC2 development and subsequently Th1 or Th2 maturation.

The SEA-stimulated PBMC $CD4^+$ T-cell population showed a dramatic increase in the expression of HLA-DR and CD54-ICAM-1, together with expression of CD45RO, the memory cell marker. Additionally, activated $CD4^+CD25^+$ T cells appear to be involved in the response to SEA as we observed a higher frequency of $CD4^+CD25^+$ T cells after the first and second recall responses (Fig. 4A). Although the cells expressing $CD4^+CD25^+$ could be regulatory T cells, the source could also be activated cells as CD25 is the IL-2 receptor α-chain. In addition, we also observed a higher frequency of $CD4^+CD25^{high}$ regulatory T cells following SEA stimulation of PBMC (data not shown) as described previously in Chagas disease [39]. $CD4^+CD25^{high}$ regulatory T cells express high and low CD25 molecules but only $CD4^+CD25^{high}$ T cells have a regulatory function in

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humans and mice [40]. This reinforces the idea that additional immunoregulatory events may be important in driving $CD4^+$ T-cell type activity in the selection of clones and suppressing the proliferation of Th1-like responses following exposure to SEA. The ability of $CD4^+CD25^{high}$ regulatory T cells to secrete IL-10, which inhibits IL-12 and consequently IFN-γ production during Th2 polarization in schistosomiasis [41], is in agreement with the results reported herein, whereby low levels of IFN-γ were observed in the majority of donors SEA cells.

In summary, the data support the hypothesis that human DC activated with SEA could prime $CD4^+$ T lymphocytes in the early immune events driving SEA-specific Th1- or Th2-like responses. Importantly, differentiation of the immune response may require more than just an initial stimulation event, as the tendency towards a particular immune response was unclear after the first recall. The polarization towards a Th1- or Th2-like response was observed only after the second recall event. The results described in this study suggest that naïve individuals can develop distinct Th1- or Th2-like immune responses based on the results from the PIV assay. However, it remains unclear whether this is due to an individuals genetic background or to environmental factors such as co-infections. For the first time, we demonstrate that naïve human PBMC can develop distinct immune responses to SEA using a PIV system. This system will be a useful tool towards understanding the early responses of T cells during a schistosomiasis infection. PIV with SEA could also provide insights into the immunopathogenesis of schistosomiasis and in evaluating potential vaccine candidates.

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