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Schistosoma mansoni Triose Phosphate Isomerase Peptide MAP4 is Able to Trigger Naïve Donor Immune Response Towards a Type-1 Cytokine Profile

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Abstract

We evaluated the ability of naïve monocyte-derived dendritic cells (DC) to sensitize autologous peripheral blood mononuclear cells (PBMC) to the schistosome vaccine candidate MAP4 using a priming *in vitro* (PIV) assay. MAP4 is a multiple antigen peptide containing B- and T-cell epitopes derived from the glycolytic enzyme triose phosphate isomerase. PBMC primed and restimulated with MAP4 first and secondary recalls (MAP4 PIV cells) were examined for cell phenotype and cytokine production. We found that after the first recall stimulation with MAP4, the major cell population was predominantly $CD4^+$ T-cell subsets (68.5%), $CD8^{+high}$ (16%) and $CD19^+$ (10%). Additionally, MAP4 PIV cells significantly expressed $CD4^+$-HLA-DR^+, -$CD54^+$, -$CD45RO^+$ ($P < 0.0001$) and -$CD25^+$ ($P < 0.0004$) together with significant expression of $CD80^+$ on $CD19^+$ B cells ($P < 0.007$). Cytokine production from activated MAP4 PIV cells was predominantly Th1-like, consisting mainly of IFN-γ. Interestingly, IFN-γ production was suppressed when *Schistosoma mansoni*-soluble egg antigen (SEA) was added to a MAP4 PIV cell culture. Furthermore, addition of MAP4 to a SEA PIV cell culture significantly reduced secretion of IL-10. The present findings add to the knowledge gained from studies in the mouse model, and our results show that naïve donor DC, sensitized with MAP4, were able to prime and clonally expand MAP4-specific T cells towards a Th1-type response.

Introduction

The ability to properly respond to infection or immunization depends largely on the response of antigen-presenting cells (APC) to pathogens or their antigens. This innate APC response dictates whether the ensuing adaptive $CD4^+$ T helper (Th) cell responses will be pro-inflammatory and Th1 type or anti-inflammatory and Th2 type [1–7]. APC are activated when pathogens or antigens bind to cell surface receptors, which include numerous C-type lectin receptors, the Toll-like receptors, scavenger receptors and others [8, 9]. Pathogen- or antigen-activated APC upregulate the expression of DEC 205 [10], major histocompatibility complex (MHC) antigens, ICAM-1, and co-stimulatory molecules B7 and CD40 [11]. They also secrete cytokines [12] and chemokines, which induce the maturation of naïve Th0 $CD4^+$ T cells into IFN-γ secreting Th1 or IL-4/IL-13 Th2 $CD4^+$ T cells [13]. The nature of the response of activated and mature effector $CD4^+$ Th cells is critical in determining whether the response to a given pathogen or vaccine will be protective or exacerbative [14].

Schistosomes are helminth parasites that infect approximately 200 million people worldwide. Although praziquantel is an excellent drug that eliminates parasites, the majority of individuals in endemic areas are re-infected. Unfortunately, while the use of praziquantel does reduce overall morbidity from disease, schistosomiasis remains uncontrolled in a large number of countries [15], indicating an urgent need for the development of a vaccine [16]. The synthetic schistosome multiple antigen peptide (MAP4) derived from triose phosphate isomerase (TPI) has been evaluated as a potential vaccine candidate in mice and in infected individuals. TPI is expressed during all stages of the parasite life cycle and therefore represents a valid target for vaccines [17]. TPI induces partial protection in mice when formulated as a MAP preparation and in mice and pigs when administered as a

plasmid DNA vaccine [18]. MAP4 includes immunogenic B- and T-cell epitopes from TPI that have low homology with human TPI. Independent of the MHC background in mice, MAP4 activates IFN-γ-producing Th1 cells [19] and this cytokine is known to play an important role in *Schistosoma mansoni*, both in protective immunity and in pathology [20, 21].

In this study, we determined whether the stimulation of human naïve PBMC could induce a Th1-like response similar to that described for the murine model [19]. In order to address this question, we performed priming *in vitro* (PIV) assays using naïve human monocyte-derived dendritic cells (DC) as APC. We conclude that MAP4 can induce the expansion of Ag-specific $CD4^+$ T cells in humans in a PIV assay, driving a Th1-type immune response.

Materials and methods

Human donors. PBMC were obtained from 10 healthy, schistosome naïve, Brazilian blood donors living in a schistosomiasis non-endemic area. The donors were screened for the presence of antibodies to HIV, HTLV I/II, HBsAg, HCV and Chagas disease, and individuals positive for any of these infections were excluded from the study. The donors that met the inclusion criteria had no previous history of schistosomiasis, and all were negative for both *S. mansoni* infection as determined by stool examination using the Kato–Katz method [22] and for antibodies to *S. mansoni*-soluble egg antigens (SEA) as determined by ELISA [optical density (OD) = 0.006 ± 0.001] [23]. Pooled serum from infected individuals with an average OD = 0.46 ± 0.043 and from non-endemic healthy individuals, average OD = 0.01 ± 0.003, were used as positive and negative controls respectively. The Ethics Committee of the Oswaldo Cruz Foundation approved the study protocol, and all donors provided informed consent prior to the collection of blood.

Antigen preparations. MAP4 containing T- and B-cell epitopes derived from TPI (MAP4) and SEA were prepared and tested for endotoxin activity as previously described [23, 24]. Neither antigen preparation contained significant amounts of contaminants, such as lipopolysaccharide, as indicated by the lack of response to SEA in naïve PBMC controls (data not shown) or an RPMI-primed PBMC control (Fig. 5). The experimental design is presented as a flowchart in Fig. 1.

DC generation. PBMC were isolated from heparinized blood (20–30 ml) by density gradient centrifugation on Ficoll–Hypaque (GE Healthcare, Piscataway, NJ, USA) at 400 *g*, 30 min, at 15 °C. PBMC were washed in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS) plus penicillin (100 U/ml) and streptomycin (100 μg/ml) (pen-strep; Invitrogen, Carlsbad, CA, USA). Monocyte-derived DC were prepared from PBMC as described previously [25]. Briefly, monocytes were obtained from PBMC (6×10^7 cells/ml) following spontaneous sedimentation and incubated in serum-free RPMI-1640 for 2 h at 37 °C, 5% CO_2. Non-adherent cells were removed by washing and adherent cells were cultured for 6–8 days in 10 ml DC complete medium (DCC-medium) RPMI-1640, supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 1% pen-strep, 10% ultra-low IgG fetal bovine serum (Invitrogen), (rGM-CSF 50 ng/ml) and (rIL-4 1000 U/ml (Pharmingen BD Biosciences, Mountain View, CA, USA). Every 2 days, 500 μl of culture medium was removed and replaced with the same volume of fresh DCC-medium. After 6 days, supernatants were collected and stored at −70 °C until use. DC were harvested to determine the expression of surface markers by FACSort analysis (Becton Dickinson, Mountain View, CA, USA or Grenoble, France).

Priming in vitro of human naïve PBMC to MAP4. PBMC were primed *in vitro* by autologous DC (irradiated with 3000 rads) pulsed with either MAP4 (75 μg/ml), SEA (25 μg/ml) or medium alone (RPMI control) in 24-well plates in complete medium (C-medium) containing AIM-V medium (serum-free lymphocyte medium; Invitrogen), 1% pen-strep and 5% human serum AB (Invitrogen) for 2 h. Later, PBMC (2×10^6 cells/ml at a ratio of 1:15 DC:PBMC) were added to each well and cultured for 7 days at 37 °C, 5% CO_2. RPMI-sensitized DC, the negative control, was cultured in parallel with PBMC under the same conditions.

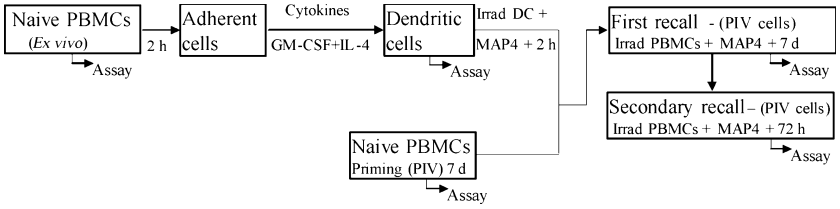

Figure 1 Experimental design scheme: naïve PBMC from healthy donors were used in the priming *in vitro* (PIV) system. Non-adherent cells were differentiated into DC using IL-4 and GM-CSF for 6–8 days. PBMC (priming) were performed with irradiated (irrad) autologous DC previously pulsed with or without MAP4 for 2 h and cultured for 7 days. MAP4 or RPMI PIV cells were restimulated (first recall) in the presence of autologous PBMC (irrad) previously pulsed with and without MAP4 and cultured for 7 days. Those cells were restimulated again (secondary recall) as above for an additional 72 h. Cytokine production of culture supernatant was measured by ELISA and cell phenotypes were determined by FACS analysis.

Culture supernatants were collected to measure cytokine production and stored at −70 °C until use. About 20 ng/ml of TNF-α (R&D Systems Inc., Minneapolis, MN, USA) was added to the culture over a period of 20 h.

MAP4, SEA or RPMI-primed PBMC (MAP4, SEA or RPMI PIV cells respectively) were restimulated (first recall) in the presence of irradiated autologous PBMC (2500 rads) at a 1:1 ratio (2×10^6 cells/ml) previously pulsed with MAP4 (50 μg/ml), SEA (20 μg/ml) or RPMI in C-medium and cultured for 7 days at 37 °C, 5% CO_2. IL-2 (20 U/ml) (R&D) was added to the cultures every second and third day. Supernatants were collected to measure cytokine production. PIV cells were harvested and purified on Ficoll–Hypaque gradients, and surface antigens were identified by FACSort analysis. Naïve PBMC (*ex vivo*) were used as controls. MAP4, SEA or RPMI PIV cells were collected after the first recall restimulated again (secondary recall) in the presence of irradiated autologous PBMC (2500 rads) at a 1:1 ratio ($0.5–1 \times 10^6$ cells/ml) previously pulsed with MAP4 or SEA (10 μg/ml) or RPMI in AIM-V medium and cultured for 72 h at 37 °C, 5% CO_2. Supernatants were collected to measure cytokine production.

Flow cytometry. Cells were stained using the following conjugated mouse anti-human monoclonal antibody (MoAb): 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), CD4-Cy (RPA-T4) and CD8-Cy (RPA-T8), and their isotype-matched negative-control antibodies were purchased from Pharmingen (BD Biosciences). Staining was performed as described previously [25]. Briefly, 10^5 cells/ml were incubated on ice for 30 min with human serum (diluted 1:20). The cells were resuspended in FACS buffer [containing HBSS, 10% FCS and 0.01% sodium azide (Sigma-Aldrich, St Louis, MO, USA)] at pH 7.2, and each MoAb was incubated for 30–45 min at 4 °C in the dark. The cells were washed and resuspended in FACS buffer and immediately acquired in 10,000 events using a FACSort flow cytometer (BD Biosciences) and the data were analysed using CellQuest Software (BD Biosciences). Cells were gated via forward and side scatter parameters by a combination of two- or three-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 assay. Culture supernatants were collected after each stimulation, and cytokine concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) using the Intertest ELISA kit (Genzyme, Cambridge, MA, USA) for IFN-γ and the IL-5 and IL-10 Human ELISA Set kits (Pharmingen), according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using the non-parametric Mann–Whitney test and GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant when the *P*-value was <0.05.

Results

DC characterization

DC generated from human monocyte donors exhibited a typical morphology as confirmed by light microscopy (data not shown). The successful generation of monocyte-derived DC was confirmed by the presence of CD1a and the absence of CD14 surface markers by high forward and side light scatter (Fig. 2A). In addition, DC were characterized by FACS to determine the percentage of cells expressing HLA-DR$^+$, CD54$^+$ (ICAM-1), CD39$^+$, CD40$^+$, CD80$^+$ (B7-1) and CD86$^+$ (B7-2) molecules (Fig. 2B). The majority (96.5%) of the DC population expressed HLA-DR$^+$ and CD54$^+$ molecules, 56.9% expressed CD86$^+$, 30.2% CD40$^+$, 21.3% CD39$^+$ and modest expression of 3.0% of CD80$^+$ was observed. We also evaluated the cytokine production at the basal state during DC culture differentiation, prior to antigen stimulation (data not shown).

Expansion of a specific T-cell subset by PIV with MAP4

The frequency of the activated PIV cell subset after the first recall stimulation with MAP4 and a PBMC *ex vivo* control was determined by staining with anti-CD3 (Fig. 3A), anti-CD4 (Fig. 3B), anti-CD8 (Fig. 3C) and anti-CD19 (Fig. 4A) MoAb. Analyses of CD3$^+$ T cells after the first recall showed a significant percentage ($P < 0.0001$) of activated T lymphocytes compared to the PBMC *ex vivo* control. The viable cell population was composed of 68.5% CD4$^+$, 16% CD8^{+high} T-cell (Fig. 3B,C) and 10% CD19$^+$ B-cell subsets (Fig. 4A). A significant increase in the mean percentage of CD4$^+$ T cells ($P < 0.0001$), as well as a significant decrease in CD8$^+$ T cells ($P = 0.02$) and CD19$^+$ B cells ($P = 0.002$, Fig. 4A) was observed, compared to a PBMC *ex vivo* control. Additionally, there was a significant increase in the CD4$^+$:CD8$^+$ cell ratio in MAP4 PIV cells compared to a PBMC *ex vivo* control (Fig. 3D). The total number of cells was reduced following priming, first and second recalls; however, this occurred with and without antigen stimulation, indicating that it was not due to antigen toxicity (data not shown). Analyses of gated lymphocytes demonstrated activated T-lymphocyte populations co-expressing CD54, HLA-DR, CD45 isoforms (CD45RO) and CD25 (Fig. 3E–H). There was

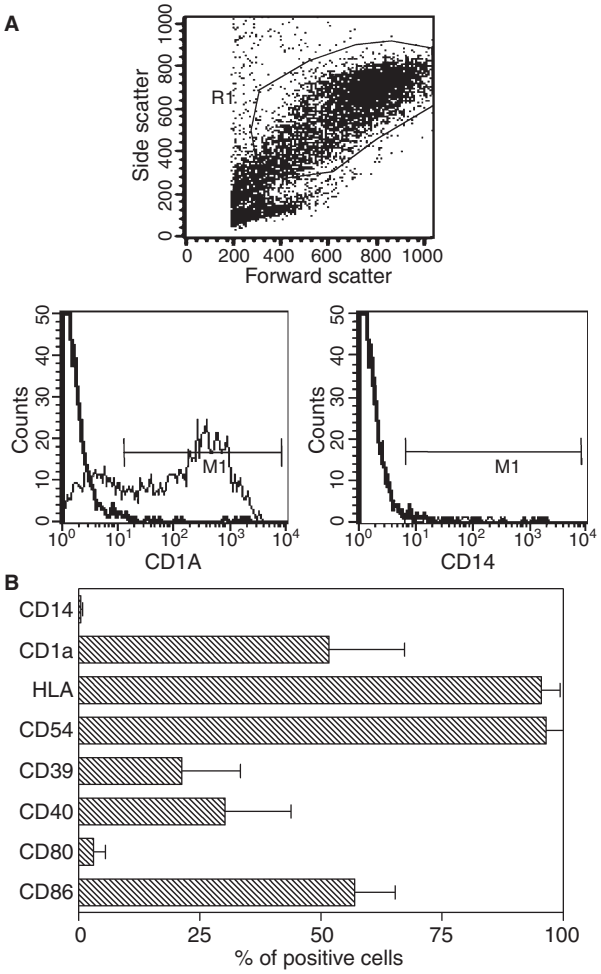

Figure 2 Flow cytometry analysis of DC surface markers. A representative light scatter plot shows the gate selected for subsequent analyses by fluorescent antibody staining (A). Each histogram represents an overlay of CD1a or CD14 (grey line) and an isotype-matched control (bold line). DC surface marker-specific fluorochrome-labelled MoAb: CD14, CD1a, CD39, CD40, CD54, HLA-DR, CD80 and CD86 (B). The bars represents the percentage of positive DC within the selected gate in donor sample (n = 10). The mean percentage (±95% CI) is also shown.

significant upregulation of activated T cells expressing $CD4^+CD54^+$ (20.1–82.2%, Fig. 3E), $CD4^+$HLA-DR^+ (3.6–22.0%, Fig. 3F), $CD4^+CD45RO^+$ (47.4–59.7%, Fig. 3G) and $CD4^+CD25^+$ (6.0–22.0%, Fig. 3H). Following the first recall response of gated lymphocytes subsets, there was a significant positive correlation between activation markers $CD4^+CD25^+/CD4^+$ and $CD4^+$HLA-$DR^+/CD4^+$ on T cells (Spearman r = 0.8333 and P = 0.0154, Fig. 3I).

MAP4 stimulation leads to an increased T:B cell ratio

Analyses of T and B cells were performed on MAP4 PIV cells after the first recall stimulation and a PBMC *ex vivo* control (Fig. 4). The mean percentage of $CD19^+$ B cells decreased significantly following MAP4 PIV stimulation (Fig. 4A). These results were associated with a significant increase in the $CD3^+/CD19^+$ T:B cell ratio and $CD4^+$HLA-$DR^+/CD19^+CD40^+$ T:B cell ratio in MAP4 PIV cells when compared to the PBMC *ex vivo* control (Fig. 4B,C). In addition, a significantly lower percentage of $CD19^+CD40^+$ B lymphocytes (10–5.5%, $P < 0.002$) was observed in MAP4 PIV cells compared to a PBMC *ex vivo* control (Fig. 4D). It was interesting to note that although there were no differences in the total percentage of $CD19^+$ B cells following MAP4 stimulation (Fig. 4A), $CD19^+CD80^+$ B lymphocytes, within the $CD19^+$ cell subset, increased significantly (4.9–17%, $P < 0.007$; Fig. 4E) after the first recall response compared to a PBMC *ex vivo* control, indicating a MAP4-specific expansion of B lymphocytes during PIV.

IFN-γ secreted by MAP4 PIV cells

By using the PIV approach, we demonstrated that human PBMC primed with MAP4 secreted large amounts of IFN-γ after priming, first and secondary recall stimulations (Fig. 5A,B). All donor PBMC demonstrated significant, but variable, Th1-type responses following priming and first recall stimulations ($P < 0.05$, Fig. 5A). However, a more consistent expression pattern was observed following the secondary recall response ($P < 0.0008$, Fig. 5B) when compared to the RPMI PIV cell control. Similar to the priming and first recall data presented earlier, IL-10 and IL-5 were not detected in the supernatant of MAP4 PIV cells.

Effect of MAP4 on SEA PIV cell cytokine production

In order to determine if the Th2-like response induced by SEA PIV cells could be altered or down modulated upon addition of MAP4, we stimulated SEA-primed PBMC (SEA PIV cells) with SEA or MAP4 (Fig. 6). MAP4 PIV cells stimulated with SEA, a known Th2 inducer, continued to produce significant levels of IFN-γ ($P < 0.05$), albeit at lower levels compared to MAP4 PIV cells stimulated with MAP4. Furthermore, MAP4 PIV cells stimulated with SEA maintained a similar level of IFN-γ production compared to the unstimulated MAP4 PIV cell control. The level of IFN-γ production in the unstimulated MAP4 PIV cells was significant ($P < 0.01$) compared to the RPMI control. In addition, SEA stimulated MAP4 PIV cells did not produce IL-5 or IL-10 (Fig. 6). As expected, SEA PIV cells stimulated with SEA resulted in the production of significant levels of IL-10 ($P < 0.001$) but no IFN-γ production. We found that SEA PIV cells stimulated with MAP4 significantly downregulated the expression of IL-5 ($P < 0.05$) and IL-10 ($P < 0.01$) and stimulated the production of IFN-γ, although not significantly, compared to unstimulated SEA PIV cells. Together, these results suggest that

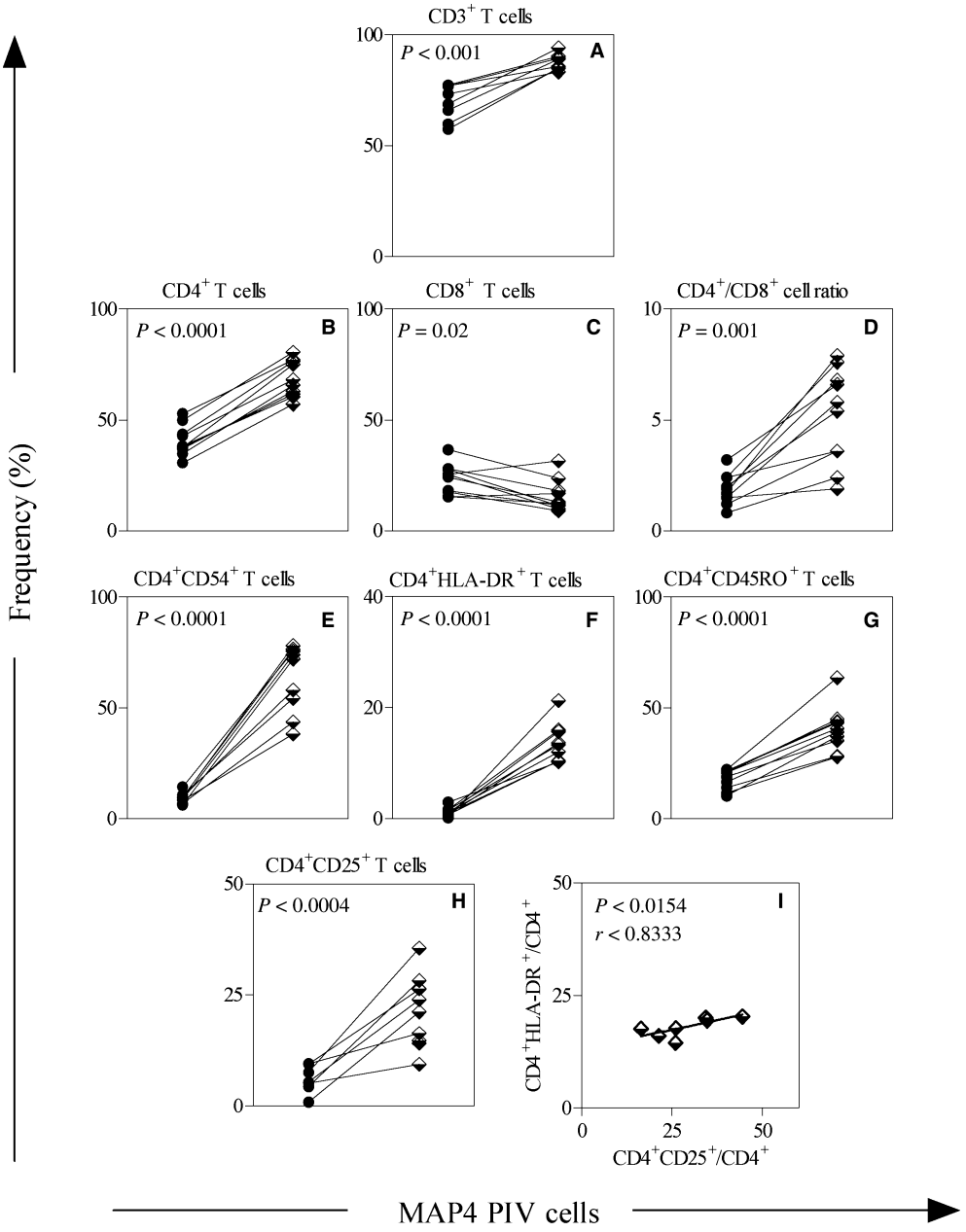

Figure 3 Frequencies of T-lymphocyte populations obtained from the PBMC *ex vivo* control (●) and MAP4 PIV cells after first recall stimulation (◆). Double-label staining was used to identify $CD3^+$ T cells (A), $CD3^+CD4^+$ (B), $CD3^+CD8^+$ T cells (C) and $CD4^+/CD8^+$ cell ratio (D) or anti-CD4 Cy to identify $CD4^+CD54^+$ (E) or $CD4^+HLA\text{-}DR^+$ (F) or $D4^+CD45RO^+$ (G) or $CD4^+CD25^+$ cells within gated lymphocyte (H) or $CD4^+CD25^+/CD4^+$ versus $CD4^+DR^+/CD4^+$ cells within the $CD4^+$-lymphocyte population (I). There was significant positive correlation ($r = -0.84$, $P < 0.001$) by the Spearman's rank test. The results are expressed as individual values of gated lymphocytes. Significant differences are stated within each individual analysis.

MAP4 is a potent Th1 driver, capable of altering the response of human PBMC to SEA, stimulating IFN-γ production while inhibiting IL-10 secretion.

Discussion

The murine model has contributed significantly to the understanding of the immune response to schistosomiasis [26]. Yet the extrapolation of the results from the mouse to those observed in humans is necessarily limited [27]. The mouse is not wholly representative of the human infective state, and infected individuals cannot, by definition, be evaluated in a controlled environment. In an attempt to study the various factors involved in the initial immune responses to a schistosome infection, PIV, using human PBMC, represents a validated approach [25]

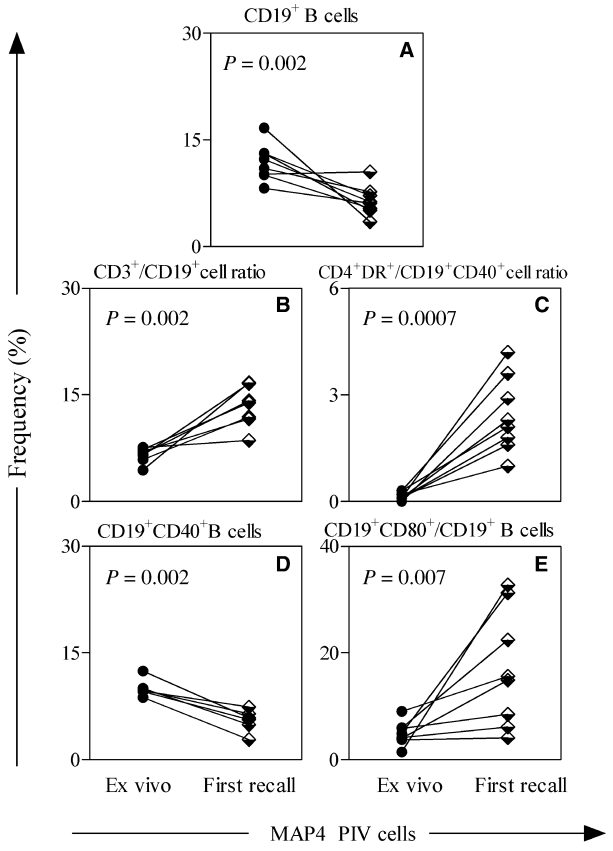

Figure 4 Frequencies of the B-lymphocyte population obtained from the PBMC *ex vivo* control (●) and MAP4 PIV cells after first recall stimulation (⬙). Double-label staining was used to identify $CD19^+$ B-cell (A), $CD3^+CD19^+$ cell ratio (B) and $CD4^+HLA\text{-}DR^+/C19^+CD40^+$ cell ratio (C), or anti-CD19 PE to identify $C19^+CD40^+$ cells within gated lymphocytes (D) and $C19^+CD80^+$ cells within the $CD19^+$-lymphocyte population (E). The results are expressed of individual values of gated lymphocytes. Significant differences are stated in each individual analysis.

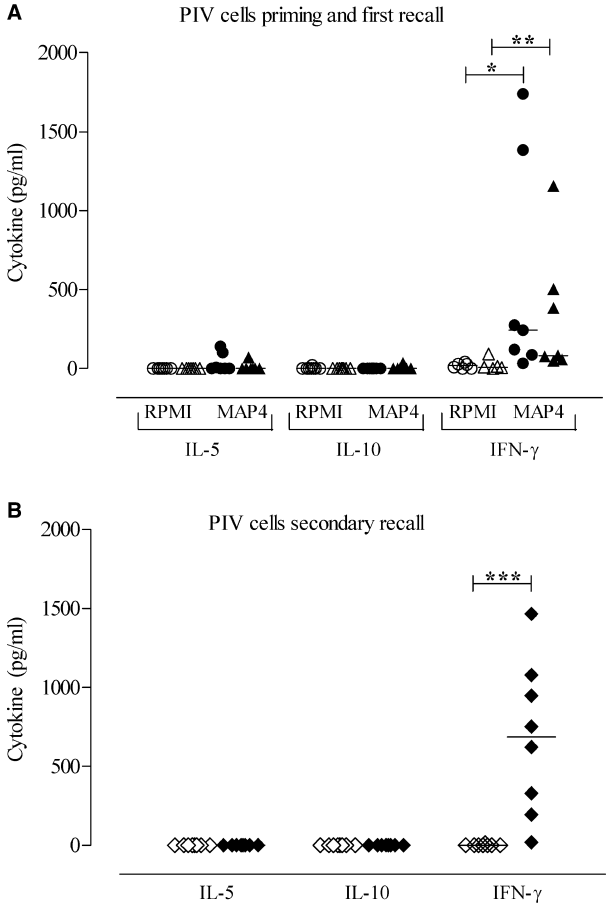

Figure 5 Levels of IL-5, IL-10 and IFN-γ obtained from MAP4-primed PBMC with the PIV system after priming (●), first recall (▲) and secondary recall response (◆). RPMI controls are represented by the corresponding open symbols and the horizontal bars signify the median values (A). Pos-priming cell supernatants were assayed for: IL-5, IL-10, IFN-γ (A) and MAP4-primed PBMC after secondary recall response (B). Cell concentration from the secondary recall was adjusted to (2×10^6 cells). Supernatants were harvested and cytokine levels were determined by ELISA assays. The data represent values from eight representative donors; significant differences are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

whereby PBMC from individuals can be stimulated in a controlled setting, albeit *in vitro*, which is perhaps more representative of the disease. In the present study, we characterized *in vitro* the human immune responses to schistosome MAP4, a synthetic peptide previously shown to drive Th1-cytokine production in mice [19].

Initial examination demonstrated that PBMC from *S. mansoni* chronically infected individuals stimulated with MAP4 induced low levels of cytokines, including IFN-γ production [28, 29]. To investigate MAP4-specific immune response in naïve human PBMC outside of the context of schistosome infection, we employed a priming *in vitro* assay using human monocyte-derived DC as APC [25]. We first observed MAP4-pulsed DC induced naïve PBMC to differentiate into Th1-type IFN-γ producers (Fig. 5B). Our study is the first to analyse naïve human PBMC primed by monocyte-DC and pulsed with MAP4. DC and lymphocytes were characterized by immunophenotyping and cytokine profiling in healthy individuals.

DC expressing co-stimulatory molecules, specifically CD40 and CD80 on B cells, may account for the higher levels of IFN-γ production observed in this study (Figs. 2B and 4E). In addition, previous studies have implicated CD80 in stimulating a Th1 response [30, 31]. The results reported here also support the concept previously suggested by Janeway and Medzhitov [32], whereby the cytokines generated by DC in response to a pathogen are important factors in determining the nature of the ensuing adaptive responses. In addition, low production of IFN-γ in PBMC from chronic schistosomiasis individuals, stimulated with MAP4 and other antigens [28, 33], is possibly due to low expression of activated and co-stimulatory molecules, such as MHC class II and B7-1 and B7-2. Indeed, this was observed in the mouse model

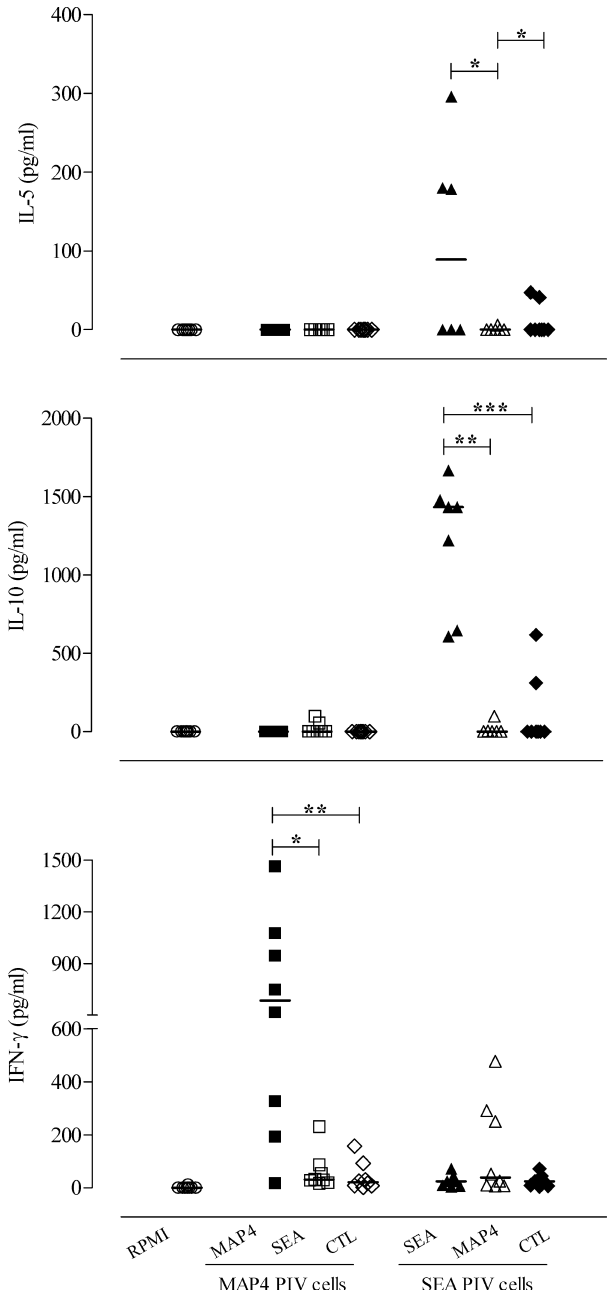

Figure 6 Effect of MAP4 or SEA on IL-5, IL-10 and IFN-γ cytokine production. Using the method described for Figure 5, we determined the cytokine levels from RPMI control (○), MAP4 PIV cells stimulated with MAP4 (■), MAP4 PIV cells stimulated with SEA (□), unstimulated MAP4 PIV cell control [cytotoxic T lymphocyte (CTL), ◇], SEA PIV cells stimulated with SEA (▲), SEA PIV cells stimulated with MAP4 (Δ) and unstimulated SEA PIV cell control (CTL, ◆). The horizontal bars signify the median values. PIV cell supernatants were harvested after 72 h, and cytokine levels were determined by ELISA assays. The data represent values from eight representative donors; significant differences are indicated (*P < 0.05, **P < 0.01, ***P < 0.001).

[30, 34]. However, we observed high expression of HLA-DR$^+$, ICAM-1$^+$ and CD25$^+$ (Fig. 3) on MAP4 PIV cells in the CD4$^+$ T-cell population after the first recall response indicated that these cells were activated and could proliferate. Additionally, increased expression of CD45RO$^+$ memory cells after the first recall responses suggest a MAP4-specific T-cell proliferation (Fig. 3).

Following priming and first recall with MAP4, the cytokine profile was mainly consistent with a Th1-like response, except for IL-5 secretion, which may have been produced as part of the transient Th0 phase as described previously [35]. Unexpectedly, IFN-γ production did not increase after first recall stimulation with MAP4, indeed the first recall response appeared to be lower than that seen in primed cells, although the difference was not significant (P = 0.5, Fig. 5A). IFN-γ production increased significantly following priming and first recall with MAP4, although only a minority of donor PBMC produced high levels of IFN-γ (Fig. 5A). However, following secondary recall all donors differentiated to a Th1-type immune response (Fig. 5B). Interestingly, when SEA, a strong Th2 inducer, was used to stimulate MAP4 PIV cells, there was no alteration in the production of IL-10 or IL-5 and both remained undetectable; however, significant IFN-γ levels were maintained even in the presence of SEA (Fig. 6). In the reverse situation when SEA PIV cells were stimulated with MAP4, IFN-γ production increased, albeit not significantly, and IL-5 and IL-10 production were completely inhibited, suggesting that MAP4 could be the major factor in driving a Th1-type response. Interestingly, several donor PBMC failed to produce IFN-γ, a situation similar to that seen with naïve PBMC following priming and the first recall to MAP4 (Fig. 5A), suggesting that further stimulation with MAP4 is required as, following the second recall stimulation with MAP4, all donor PBMC produced IFN-γ (Fig. 5B). Therefore, MAP4 is probably capable of altering the response of cells previously exposed to SEA. A possible explanation is that copolymerization of B- and T-cell epitopes enhances MAP immunogenicity and induces mice to respond to previously unrecognized epitopes [36].

In conclusion, our findings suggest that MAP4 appears to be capable of driving a CD4$^+$ Th1 immune response. Furthermore, this PIV assay, using cultured human DC as APC, may be useful in the analysis of the early events associated with T-cell sensitization and in the development of *in vitro* predictive tests to detect the components involved.

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