



Amniotic membrane extract differentially regulates human peripheral blood T cell subsets, monocyte subpopulations and myeloid dendritic cells

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Abstract

The discovery of the immunoregulatory potential of human amniotic membrane (hAM) propelled several studies focusing on its application for the treatment of immunological disorders. However, there is little information regarding the effects of hAM on distinct activation and differentiation stages of immune cells. Here, we aim to investigate the effect of human amniotic membrane extract (hAME) on the pattern of cytokine production by T cells, monocytes and myeloid dendritic cells (mDCs). For this purpose, peripheral blood mononuclear cells (PBMCs) from eight healthy individuals were stimulated *in vitro* in the presence or absence of hAME. Mitogen-induced proliferation of PBMCs and cytokine production among the distinct T cell functional compartments, monocyte subpopulations and mDCs were evaluated. hAME displayed an anti-proliferative effect and decreased the frequency of T cells producing tumor necrosis factor (TNF) α , interferon (IFN) γ and interleukin (IL)-2, for all T cell functional compartments. The frequency of IL-17 and IL-9-producing T cells was also reduced. The inhibition of mRNA expression of granzyme B, perforin and NKG2D by CD8⁺ T cells and $\gamma\delta$ T cells and the augment of FoxP3 and IL-10 in CD4⁺ T cells and IL-10 in regulatory T cells were also observed. Furthermore, hAME inhibited IFN γ -induced protein (IP)-10 expression by classical and non-classical monocytes, without hampering the production of TNF α and IL-6 by monocytes and mDCs. These results suggest that hAME exerts an anti-inflammatory effect on T cells, still at a different extent for distinct T cell functional compartments.

Paula Laranjeira and Marta Duque contributed equally to this work.

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Introduction

The human amniotic membrane (hAM) represents the innermost layer of the placenta, composed of a single layer of epithelial cells (derived from the embryonic ectoderm), a thick basement membrane and an avascular stroma (Dua and Azuara-Blanco 1999; Mamede et al. 2012). Because of its ability to decrease inflammation and fibrosis, to promote wound healing and tissue repair, its antimicrobial properties and its negligible risk of allogeneic rejection, the hAM has long been used in tissue engineering and for therapeutic applications (Dua and Azuara-Blanco 1999; Mamede et al. 2012). Moreover, the protective effects of hAM have also been observed for hAM-derived cells, both from the epithelial and mesenchymal layers and for hAM-conditioned medium alone (Parolini et al. 2014; Rossi et al. 2012). These processes involve several bioactive molecules that promote epithelialization, inhibit fibrosis and actively suppress immune responses, some of which have already been identified, including annexin A1 (AnxA1) (Hopkinson et al. 2006), human leukocyte antigen G (HLA-G) (Kubo et al. 2001), heavy chain hyaluronic acid in complex with pentraxin 3 (HC-HA/PTX3) (He et al. 2014), tissue inhibitor of metalloproteinases (TIMPs), interleukin (IL-)10, IL-1 receptor antagonist (IL-1RA), trombospondin-1 (TSP-1), Fas ligand (FasL) (Hao et al. 2000; Hopkinson et al. 2006; Li et al. 2005), macrophage migration inhibitory factor (MIF), transforming growth factor β (TGF β) (Hao et al. 2000; Li et al. 2005) and prostaglandins (PGs) (Rossi et al. 2012; Ueta et al. 2002).

The clinical efficacy of hAM transplantation, in the suppression of inflammation and promotion of wound healing, was demonstrated in animal models of BM transplantation and allograft tolerance (Anam et al. 2013; Guo et al. 2011), in experimental autoimmune encephalomyelitis (Liu et al. 2012; McDonald et al. 2015) and collagen-induced arthritis (Parolini et al. 2014; Shu et al. 2015b). In recent years, extracts or homogenates of hAM have been shown to exert the same effects as effectively as hAM transplantation in animal models of allograft tolerance (He et al. 2014) and cornea healing (Guo et al. 2011), proving that the cellular factors responsible for the therapeutic effects of hAM are present in the extracts, which potentially present a far simpler alternative to the use of hAM. Accordingly, two clinical trials reported promising results in the use of homogenized hAM (Bonci and Lia 2005) and human amniotic membrane extract (hAME) (Liang et al. 2009) in small cohorts of patients with ocular disorders. hAME preparations have become commercially available, although by a very limited number of producers, in the form of a lyophilized powder, prompting investigators to focus their attention on its

therapeutically applications; however, the subject is relatively recent and, so far, there have been scarce attempts to examine concrete effects on specific immune cell populations.

T lymphocytes are key players in human immunity: these cells can mount protective immune responses through initial recognition by naive T cells the effector functions by the resulting activated T cells, and the generation and maintenance of antigen-experienced memory T cells; they also play a critical role in the regulation of the immune response (Anthony et al. 2012). Monocytes, macrophages and dendritic cells (DCs) have an equally crucial role in determining the progression and outcome of the immune response, both by cytokine secretion and antigen-specific T cell activation (Ziegler-Heitbrock et al. 2010). Classical monocytes are the most represented subpopulation among the peripheral blood monocyte and the main scavengers; in turn, non-classical and intermediate monocytes are more specialized towards mobilization to inflammation sites and secretion of pro-inflammatory cytokines, particularly tumor necrosis factor-alpha (TNF α) (Stansfield and Ingram 2015; Wong et al. 2012), although the preferential production of cytokines by the different subsets is still under debate and seems to differ depending on the type of stimulus and isolation method (Wong et al. 2012). Comparing non-classical and intermediate monocytes, the latter are thought to have a greater capability of antigen presentation and T cell stimulation (Wong et al. 2012). The two major types of peripheral blood DCs are myeloid DC (mDC) and plasmacytoid DCs (pDC); the main function of mDCs is antigen presentation and T cell activation, while pDCs are specialized in the recognition of antigenic viral nucleic acids and respond quickly by secreting large amounts of type I interferons (IFN- α/β) (Reizis et al. 2011).

In this study we evaluate the effect of hAME on the expansion of mitogen-stimulated peripheral blood mononuclear cells (PBMC) and on the cytokine production of distinct populations of antigen-presenting cells (classical, intermediate and non-classical monocytes and mDCs) and different T cell functional compartments (naive, central memory, effector memory and terminal differentiated/effector CD4⁺ and CD8⁺ T cells). mRNA expression of immune mediators by purified CD4⁺, CD8⁺, gamma-delta (T $\gamma\delta$) and regulatory T cells (Treg) was also assessed.

Material and methods

hAME preparation

hAM was obtained from healthy women after elective caesarean sections. The study was approved by the Ethical Committee of the Centro Hospitalar e Universitário de

Coimbra (CHUC-70-12, Coimbra, Portugal). hAM was mechanically peeled from the chorion and washed with a phosphate-buffered solution (PBS, Gibco, Life Technologies, Paisley, UK) with 2% antibiotic/antimycotic (Gibco). Part of the isolated hAM cells was used for phenotypic characterization. For that, cells were stained with the following monoclonal antibodies (mAb) for surface antigens: CD44, CD45, CD31, CD73, CD13, CD90 and HLA-DR (Table 1) and incubated for 10 min in the dark. After a lyse-and-then-wash method, the sample was acquired in FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with FACSDiva software (version 6.1.2, BD) and data analysis was carried out using the Infinicyt software (version 1.6, Cytosol SL, Salamanca, Spain). The remaining hAM sample was used for the preparation of hAME, performed as previously described (Mamede et al. 2014). Briefly, cells were placed in PBS, minced, homogenized, sonicated and centrifuged on ice (15 min at 14,000×g). The final supernatant (hAME) had a protein concentration of 3.7 ± 0.2 mg/ml.

Culture of peripheral blood mononuclear cells

Human PBMCs were isolated from freshly collected, heparinized (BD) peripheral blood samples from eight healthy volunteers (four male and four female, with an average of 27.1 ± 6.5 years old, ranging from 21 to 38 years of age). The peripheral blood samples were collected at the Blood and Transplantation Center of Coimbra (Coimbra, Portugal), after obtaining written consent from all participants. PBMCs were isolated within 1 h of collection, using Lymphoprep™ (Axis-shield Diagnostics, Oslo, Norway) gradient density centrifugation in a sterile environment. PBMCs were maintained in Roswell Park Memorial Institute (RPMI) 1640 with GlutaMax medium (Gibco) containing 0.1% antibiotic-antimycotic (Gibco), PBMC cultures were carried out in 24-well TPP tissue culture plates (TPP Techno Plastic Products AG, Zollstrasse, Trasadingen, Switzerland) at 37 °C, in a sterile environment and humidified atmosphere with 5% CO₂. For each donor participating in this study, we proceeded to the following set of assays: PBMCs (10⁶ cells) in 1 ml of RPMI 1640 medium (negative control); and PBMCs (10⁶ cells) in RPMI 1640 with the addition of hAME to the culture medium (50, 100, or 200 µl), in a final volume of 1 ml. The incubation period was distinct for the different sets of experiments and is detailed below.

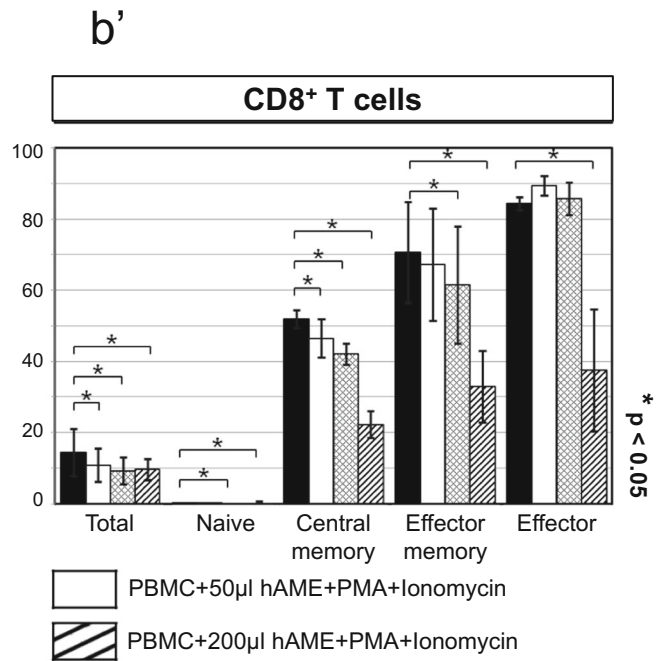
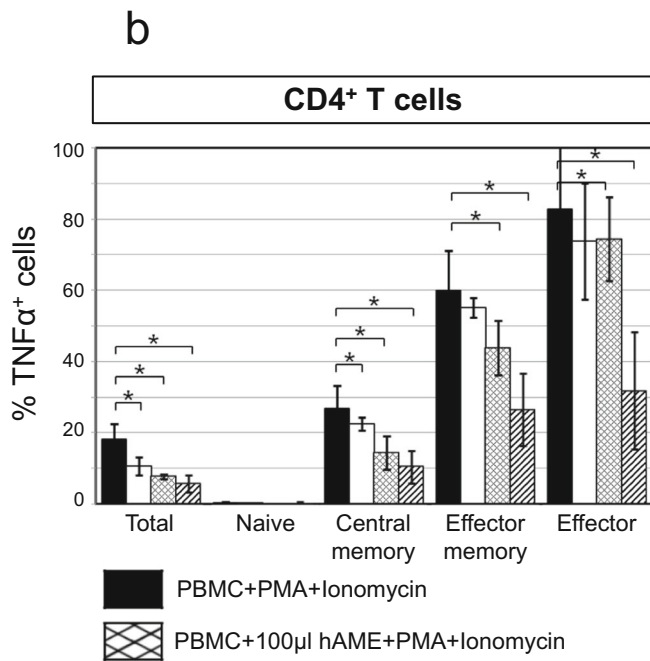
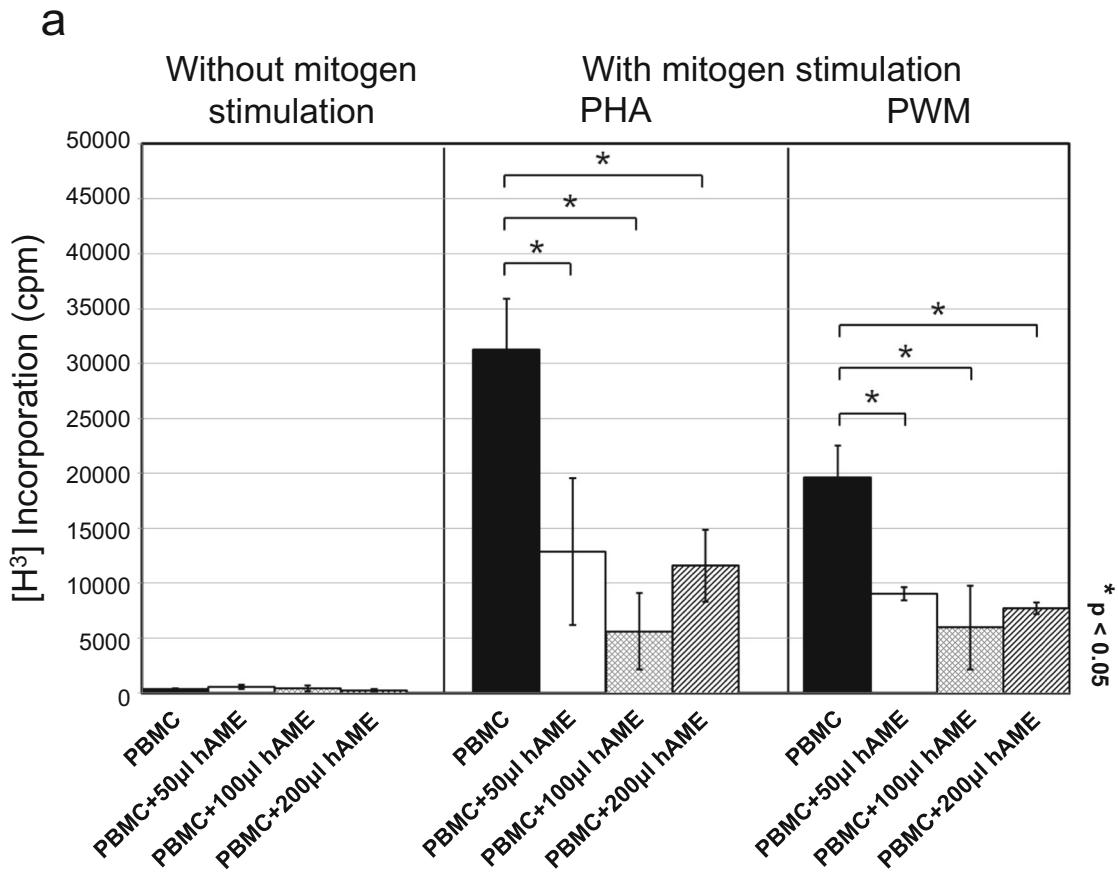
Evaluation of the effect of hAME concentration on lymphocytes, monocytes and mDC

To test the effect of different concentrations of hAME on lymphocyte proliferation, we added 50, 100, or 200 µl of hAME to phytohemagglutinin (PHA)- or pokeweed mitogen (PWM)-stimulated PBMCs (n = 3), the same volumes of hAME were added to PMA plus ionomycin-stimulated PMBCs for the

Table 1 Panel of mAb reagents (with clones and commercial sources) used for the phenotypic characterization and for cell purification by fluorescence-activated cell sorting

	PacB	V500 KO	FITC	PE	PECy5 PerCPCy5.5	PECy7	APC	APCH7
hAM-derived cells	CD44 IM7 BioLegend	CD45 J-33 Beckman Coulter	CD31 WM59 BD	CD73 AD2 BD	-	CD13 L138 BD	CD90 5E10 BD	HLA-DR L243 BD
T cells	CD3 UCHT1 BD	CD8 RPA-T8 BD	cyTNFα Mab11 cyIFNγ 4S.B3 cyIL-2 MQ-17H2 BD	cyIL-17 SCLP1362 cyIL-9 MH9A3 BD	CD27 IA4-CD27 Beckman Coulter	CD56 N901 Beckman Coulter	CD45RA CD4 HI100 BD	CD4 SK3 BD
Monocytes and mDCs	HLA-DR L243 BioLegend	CD45 H30 Invitrogen	cyTNFα Mab11 cyIL-6 MQ2-13A5 BD	cyMIP-1β D21-1351 cyIP-10 MQ2-6A3 BD	CD64 22 Beckman Coulter	CD33 D3HL60.251 Beckman Coulter	CD11b D12 BD	CD14 M0P9 BD
Cell sorting	CD8 RPA-T8 BD	-	CD25 M-A251 BD	CD127 IM1980U Beckman Coulter	CD4 13B8.2 BD	TCRγδ R9.12 BD	CD56 IM2474 Beckman Coulter	CD3 SK7 BD

Commercial Sources: BD (Becton Dickinson Biosciences, San Jose, CA, USA); Beckman Coulter (Miami, Florida, USA); BioLegend (San Diego, CA, USA); Invitrogen (Waltham, MA, USA) APC allophycocyanin, APCH7 allophycocyanin-hillite 7, FITC fluorescein isothiocyanate, KO khrome orange, mAb monoclonal antibody, mDCs myeloid dendritic cells, PacB pacific blue, PE phycoerythrin, PECy5 phycoerythrin-cyanine 5, PerCPCy5.5 peridinin chlorophyll protein-cyanine 5.5, PECy7 phycoerythrin-cyanine 7, V500 Violet 500



evaluation of TNFα production by T cells (*n* = 3). Of note, PBMCs from each one of the three donors were subjected to all these experimental conditions. As the suppression of TNFα

production was higher with the highest dose of hAME, we used the volume of 200 µl of hAME to evaluate its effect on the expression of TNFα, IFNγ, IL-2, IL-17 and IL-9 by T cells (*n* = 8).

Fig. 1 Effect of different doses of hAME on PBMCs' proliferation and TNF α production by T cells: **a** thymidine incorporation assay: results represent cell proliferation, measured as [^3H]-thymidine incorporation (mean cpm \pm SD), without mitogen stimulation or following PBMCs activation by mitogenic stimuli in the presence or absence of hAME. Results were obtained for three PBMC samples. **b, b'** Effect of different doses of hAME on the frequency of TNF α -expressing CD4 $^+$ and CD8 $^+$ T cells: percentage of TNF α -producing CD4 $^+$ and CD8 $^+$ T cells (mean \pm SD) among the four different functional subsets (naïve, central memory, effector memory and effector) following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME (PBMC+50 μl hAME+PMA+Ionomycin, PBMC+100 μl hAME+PMA+Ionomycin and PBMC+200 μl hAME+PMA+Ionomycin) in the culture medium. Results were obtained for three PBMC samples. Statistically significant differences were considered when $p < 0.05$ for the Wilcoxon paired-samples test among the groups indicated in the figure. Abbreviations: cpm, counts per minute; hAME, human amniotic membrane extract; PBMC, peripheral blood mononuclear cells; PHA; phytohemagglutinin; PWM, pokeweed mitogen; SD, standard deviation; PMA, phorbol myristate acetate; TNF α , tumor necrosis factor-alpha

Again, in order to evaluate the effect of hAME, PBMCs from each one of these individuals were subjected to the following culture conditions: (1) PBMCs + PMA + ionomycin and (2) PBMCs + 200 μl hAME + PMA + ionomycin. For the study of cytokine expression by monocytes and mDC, the effect of the different concentrations of hAME was also assessed by adding 50, 100, or 200 μl of hAME to LPS plus IFN γ -stimulated PBMCs from each donor ($n = 3$).

Cell proliferation assays

Thymidine incorporation assays ($n = 3$) were performed for the assessment of cell proliferation after 5 days of culture of PBMC in RPMI with GlutaMax (Gibco) 10% fetal calf serum (Gibco), without hAME, or in the presence of different amounts of hAME and with or without the addition of a mitogen, either PHA (10 $\mu\text{g}/\text{ml}$) or PWM. [^3H]-thymidine (0.67 Ci/well, Perkin Elmer, Life Sciences, Zaventem, Belgium) was added to the cells and, after 18 h of incubation, cells were harvested with a Filtermate Harvester (Perkin Elmer). Thymidine incorporation was measured with a microplate scintillation and luminescence counter (Top Count NXT, Perkin Elmer). Data were expressed as the mean thymidine incorporation value (counts per minute, cpm) and standard deviation (SD; indicated in graphs as error bars). Differences between the two different cell cultures were considered significant when the p value for the Wilcoxon signed-rank test for paired samples was lower than 0.05.

Immunophenotypic study of peripheral blood T cell functional compartments

PBMCs from each donor ($n = 8$) were incubated in parallel with or without 200 μl of hAME during 20 h. Then, the

stimulants phorbol myristate acetate (PMA, 1 ng/ml, Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (0.5 mg/ml, Sigma-Aldrich), along with brefeldin A (5 mg/ml, Sigma-Aldrich), were added to the wells. The plates were then incubated for four additional hours, after which cells were stained with the mAb for surface proteins antigens CD3, CD27, CD4, CD45RA and CD8 (Table 1). The subsequent permeabilization step was performed with the permeabilization kit IntraprepTM (Beckman Coulter, Brea, CA, USA) and cells were subsequently stained for intracellular TNF α , IFN γ , IL-2, IL-17 and IL-9 (Table 1). Following the exclusion of cell debris (events with low forward scatter (FSC) and heterogeneous side scatter (SSC) light dispersion properties) and cell doublets (identified by their FSC area versus FSC height characteristics), T cells were identified by the expression of the CD3 antigen. Within the T lymphocytes, the different subpopulations among CD4 $^+$ and CD8 $^+$ T cells (phenotypically characterized as CD3 $^+$ CD4 $^+$ CD8 $^-$ and CD3 $^+$ CD4 $^-$ CD8 $^+$, respectively) were identified according to their expression profile for the surface markers CD45RA and CD27, as follows: CD45RA $^+$ CD27 $^+$ were identified as being naive T cells, CD45RA $^-$ CD27 $^+$ as central memory (CM) T cells, CD45RA $^-$ CD27 $^-$ expression was attributed to effector memory (EM) T cells and CD45RA $^+$ CD27 $^-$ to effector T cells.

Immunophenotypic study of peripheral blood monocyte subpopulations and myeloid dendritic cells

PBMCs from each donor ($n = 3$) were incubated in parallel without hAME, or with different volumes of hAME, during 20 h; then, brefeldin A (5 mg/ml, Sigma-Aldrich) was added to the cells. The experimental conditions were then equally divided into a non-stimulated and a stimulated condition; to the latter, lipopolysaccharide from *Escherichia coli* (LPS, 100 ng/ml, serotype 055:B5, Sigma-Aldrich) and IFN γ (100 U/ml, Promega, Madison, USA) were added. All samples were incubated for six additional hours, following which cells were stained for the cell surface antigens HLA-DR, CD11b, CD64, CD45, CD33 and CD14 (Table 1). Cells were permeabilized using IntraprepTM (Beckman Coulter) and stained for intracellular macrophage inflammatory protein-1 β (MIP-1 β), IFN γ -induced protein 10 (IP-10), IL-6 and TNF α (Table 1). Classical monocytes were identified by their high levels of CD14, CD33, CD11b, CD64 and HLA-DR expression; intermediate monocytes as expressing decreasing levels of CD14, CD11b, CD64 and CD33 (in comparison to classical monocytes), along with high HLA-DR expression; non-classical monocytes as CD14^{dim-to-negative}, CD64 and CD11b negative, with the lowest CD33 expression among monocytes and intermediate HLA-DR expression; mDCs were identified as being negative for CD14 and CD11b, having lower expression for CD45 and CD64 compared to monocytes and high CD33 and HLA-DR expression.

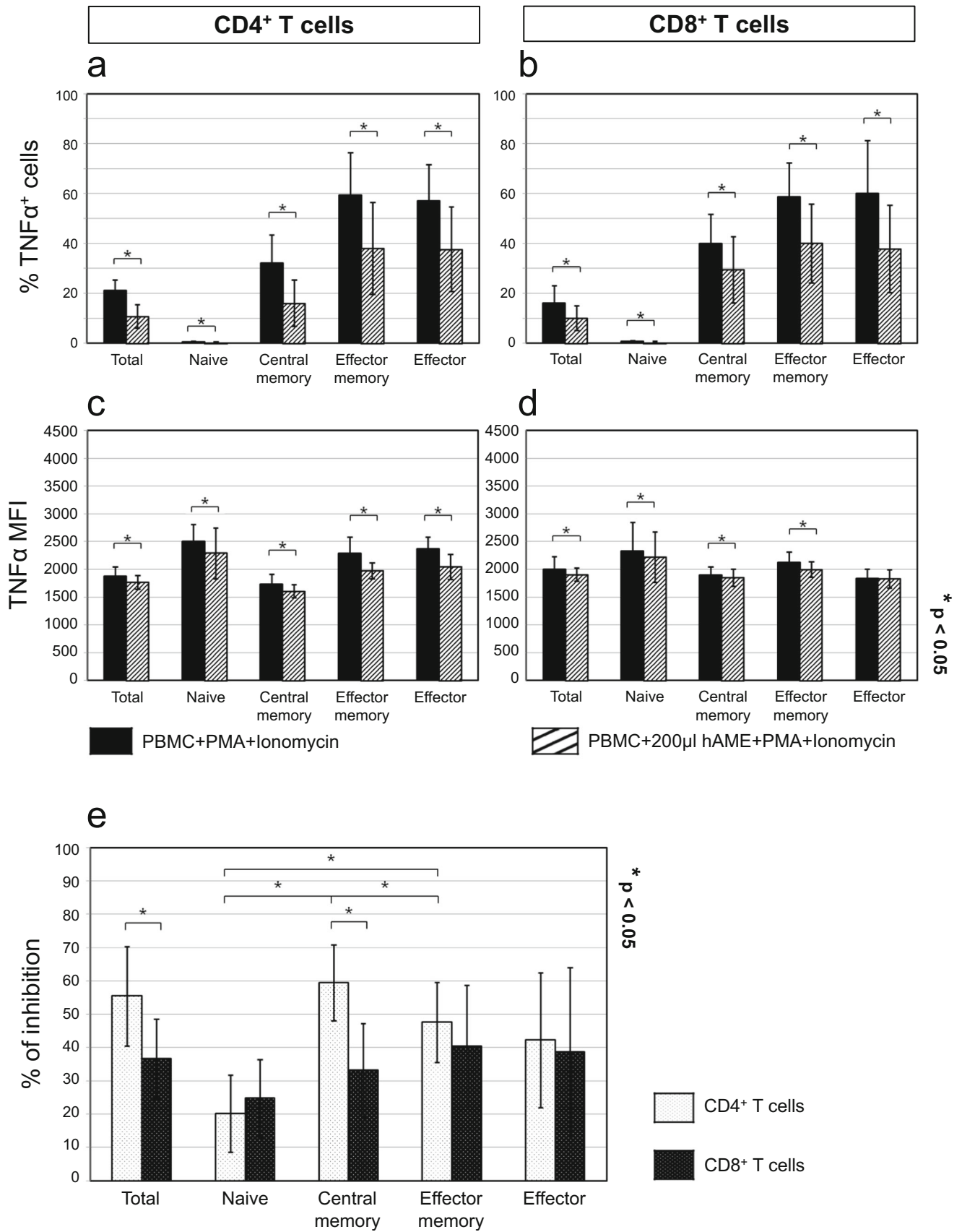


Fig. 2 Effect of hAME on the expression of TNF α by CD4 $^+$ and CD8 $^+$ T cells: **a, b** Percentage of TNF α -producing CD4 $^+$ (**a**) and CD8 $^+$ (**b**) T cells (mean \pm SD), distributed among their different subsets (naïve, central memory, effector memory and effector), following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME (PBMC+200 μ l hAME+PMA+Ionomycin) in the culture medium. **c, d** Amount of TNF α expressed per CD4 $^+$ (**c**) and CD8 $^+$ (**d**) T cell, measured as the MFI (mean \pm SD). **e** Percentage of inhibition induced by hAME on the frequency of TNF α producing CD4 $^+$ and CD8 $^+$ T cells. Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p < 0.05$ for the Wilcoxon paired-samples test for the groups indicated in the figure. Abbreviations: hAME, human amniotic membrane extract; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; SD, standard deviation; TNF α , tumor necrosis factor-alpha

Flow cytometry acquisition and analysis

Flow cytometry acquisition was performed in a BD FACSCanto™ II flow cytometer (BD), equipped with FACSDiva software (version 6.1.2, BD) and data analysis was carried out through Infinicyt software (version 1.6, Cytos SL, Salamanca, Spain). Data were expressed as the mean frequency of cytokine-positive cells and SD (indicated in graphs as error bars) and as mean fluorescence intensity (MFI) and SD. MFI is the arithmetic mean of the fluorescence intensity of cells and it was calculated considering only the cells that expressed the respective cytokine. Thus, MFI corresponds to an estimated amount of cytokine produced on a per cell basis. Differences between different culture conditions were considered significant when the p value for the Wilcoxon signed-rank test for paired samples was lower than 0.05.

Fluorescence-activated cell sorting and purification of T cell subsets

In order to investigate the mRNA expression in different T cell subsets, we proceeded to their purification by cell sorting. After PBMCs stimulation with PMA plus ionomycin, either in the absence or presence of hAME (200 μ l) in the culture medium ($n = 5$), the T cell subpopulations of interest were purified according to their characteristic phenotype, using a FACSAria™ II flow cytometer (BD). PBMCs from each donor were subjected to both experimental conditions. CD4 $^+$ T cells were identified as CD3 $^+$ CD4 $^+$ CD8 $^-$; CD8 $^+$ T cells as CD3 $^+$ CD4 $^-$ CD8 $^+$; regulatory T cells (Treg) as CD3 $^+$ CD4 $^+$ CD25 $^{++}$ CD127 $^{dim/-}$; and $\gamma\delta$ T cells as CD3 $^+$ TCR $\gamma\delta^+$ (Table 1).

mRNA expression by purified T CD4 $^+$, T CD8 $^+$, Treg and gamma-delta T cells

Total RNA was extracted from each purified T cell population with the RNeasy Micro kit (Qiagen, Hilden, Germany),

according to supplier's instructions. Reverse transcription was then performed with iScript™ Reverse Transcription Supermix for real-time *polymerase chain reaction* (RT-PCR) (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Relative quantification of gene expression by RT-PCR was performed in the LightCycler™ 480 II (Roche Diagnostics, Rotkreuz, Switzerland). RT-PCR reactions were performed using 1xQuantiTect SYBR Green PCR Master Mix (Qiagen), 1xQuantiTect Primer Assay (GATA3: QT00095501; STAT6: QT00097426; IL-4: QT00012565; FOXP3: QT00048286; IL-10: QT00041685; TGF β 1: QT00000728; PRF1: QT00199955; GZMB: QT01004875; KLRK1: QT00197183; EOMES: QT00026495) (Qiagen), in a final volume of 10 μ l. The reactions were performed using the following thermal profile: 15 min at 95 °C; 50 cycles of 30 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C; 1 cycle of 5 s at 95 °C, 1 min at 65 °C and continuous at 97 °C; and 1 cycle of 10 s at 21 °C. All samples were run in duplicate and melting point analysis was done to ensure the amplification of the desired product. RT-PCR results were analyzed with the LightCycler™ software (Roche Diagnostics). Two reference genes for data normalization were selected using the GeNorm software (PrimerDesign Ltd., Southampton, England); for all T cell populations, the most stable reference genes were cytochrome c1 (CYC1) and splicing factor 3a subunit 1 (SF3A1). The normalized expression levels of the genes of interest were calculated using the delta-Ct method. Relative mRNA expression data were expressed through their quartiles with non-parametric box-plots. Differences between the two culture conditions were considered significant when the p value for the Wilcoxon paired-samples test was lower than 0.05.

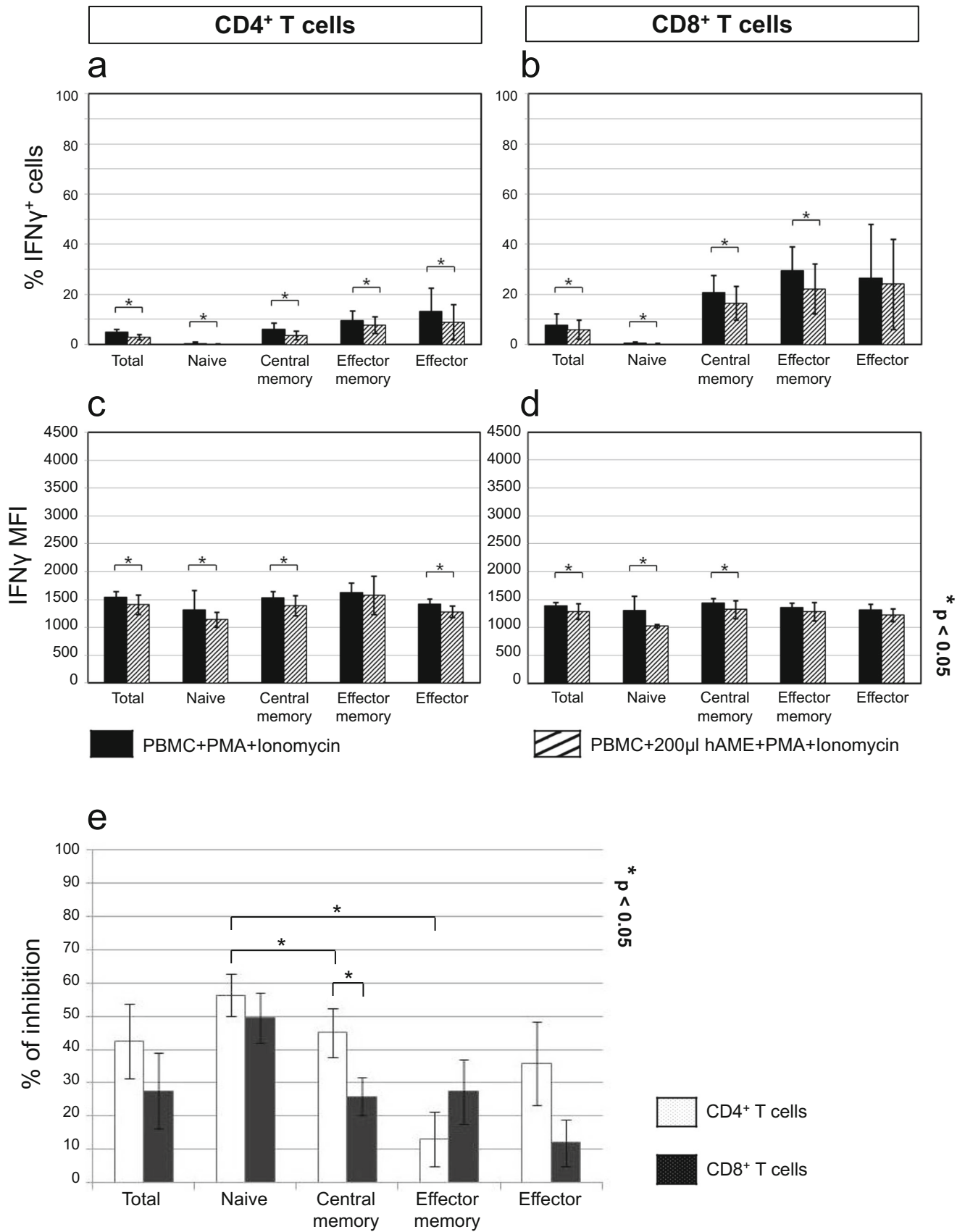
Statistical analyses

The statistical analyses were performed using Microsoft Excel 2010/XLSTAT (Version 2015.4.01.20216, Addinsoft Inc., Brooklyn, NY, USA); a p value below 0.05 was considered statistically significant.

Results

hAME inhibits lymphocyte proliferation induced by mitogenic stimuli

The presence of hAME in the culture medium did not significantly change the basal cell proliferation in the absence of mitogenic stimulation; however, hAME induced a decrease above 50% in cell proliferation (measured by [3 H]-thymidine incorporation) when PBMCs were activated with either PHA or PWM (Fig. 1).



◀ **Fig. 3** Effect of hAME on the expression of IFN γ by CD4 $^+$ and CD8 $^+$ T cells: **a, b** Percentage of IFN γ -producing CD4 $^+$ (**a**) and CD8 $^+$ (**b**) T cells (mean \pm SD), distributed among their different subsets (naïve, central memory, effector memory and effector), following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME (PBMC+200 μ l hAME+PMA+Ionomycin) in the culture medium. **c, d** Amount of IFN γ expressed per CD4 $^+$ (**c**) and CD8 $^+$ (**d**) T cell, measured as the MFI (mean \pm SD). **e** Percentage of inhibition induced by hAME on the frequency of IFN γ producing CD4 $^+$ and CD8 $^+$ T cells. Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p < 0.05$ for the Wilcoxon paired-samples test for the groups indicated in the figure. Abbreviations: hAME, human amniotic membrane extract; IFN γ , interferon gamma; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; SD, standard deviation

hAME decreases the frequency of TNF α -producing T cells in a dose-dependent manner

PBMC culture with hAME, following stimulation with PMA plus ionomycin, resulted in a decreased frequency of T cells expressing TNF α . This inhibitory effect was dependent on the amount of hAME added to the culture medium, being higher for the highest dose of hAME (Fig. 1). Interestingly, different T cell subsets displayed different susceptibility to hAME-derived inhibition: CD8 $^+$ T cells were more resistant than CD4 $^+$ T cells to inhibition of TNF α production and CM T cell subsets seemed to be the most susceptible to suppression, being the only subset among CD4 $^+$ and CD8 $^+$ T cells with a statistically significant decrease in the percentage of TNF α $^+$ cells in the presence of 50 μ l of hAME. Of note, when using 200 μ l of hAME, all T cell functional compartments showed similar sensitivity to hAME-derived suppression (Fig. 1).

According to this dose-dependent inhibitory effect, we used the highest dose of hAME (200 μ l) in all subsequent experiments performed to evaluate the effect of hAME on T cell cytokine expression.

hAME decreases the frequency of T cells producing TNF α , IFN γ and IL-2

Comparing the percentage of CD4 $^+$ T cells expressing TNF α , IFN γ and IL-2, upon stimulation with PMA plus ionomycin, we observed these cells were more prone to produce TNF α , followed by IL-2 and IFN γ . In turn, a higher percentage of TNF α -producing cells, followed by IFN γ and IL-2 was found for CD8 $^+$ T cells (Figs. 2, 3, 4).

The presence of 200 μ l hAME in the culture medium reduced the percentage of TNF α $^+$ cells among all T cell subsets (Fig. 2a, b), accompanied by the reduction of the amount of TNF α produced per cell, measured as MFI (Fig. 2c, d). As previously observed, the overall inhibitory effect was more preeminent among CD4 $^+$ T cells (\approx 40 to 60% of reduction) comparatively to CD8 $^+$ T cells (\approx 30 to 40% of reduction); the difference arose

mainly from a more pronounced effect on CD4 $^+$ CM T cells, in relation to CD8 $^+$ CM T cells ($p < 0.05$), while no significant differences were observed between CD4 $^+$ and CD8 $^+$ T cells from other functional compartments (Fig. 2e).

Regarding IFN γ $^+$ T cells, the frequency of cytokine producing cells was reduced in all T cell compartments ($p < 0.05$) but CD8 $^+$ effector T cells were less sensitive to the hAME inhibitory action, displaying \approx 10% reduction of IFN γ production in the presence of hAME ($p > 0.05$, Fig. 3a, b). A slight reduction of IFN γ MFI was also observed among the functional T cell compartments (Fig. 3c, d). Similarly to TNF α , the overall inhibition of IFN γ production was higher for CD4 $^+$ T cells comparatively to CD8 $^+$ T cells (Fig. 3e).

Accordingly, we verified a decreased percentage of IL-2-producing cells in the order of 50 to 60% (Fig. 4a, b) and IL-2 MFI (Fig. 4c, d), for all T cell subsets; no differences were observed between CD4 $^+$ and CD8 $^+$ T cells, or between functional compartments (Fig. 4e).

It is worth mentioning that the observed decrease in cytokines MFI, albeit consistent throughout the data, is much less pronounced than the reduction in the frequency of cytokine-producing cells. In the same respect, though there is a statistical significant decrease among the production of cytokines by naïve T cells in the presence of hAME (as well as in IL-2 $^+$ CD8 $^+$ T cells), cell subsets with such a limited contribution for the production of cytokines do not have a biological significance (supplementary material 1).

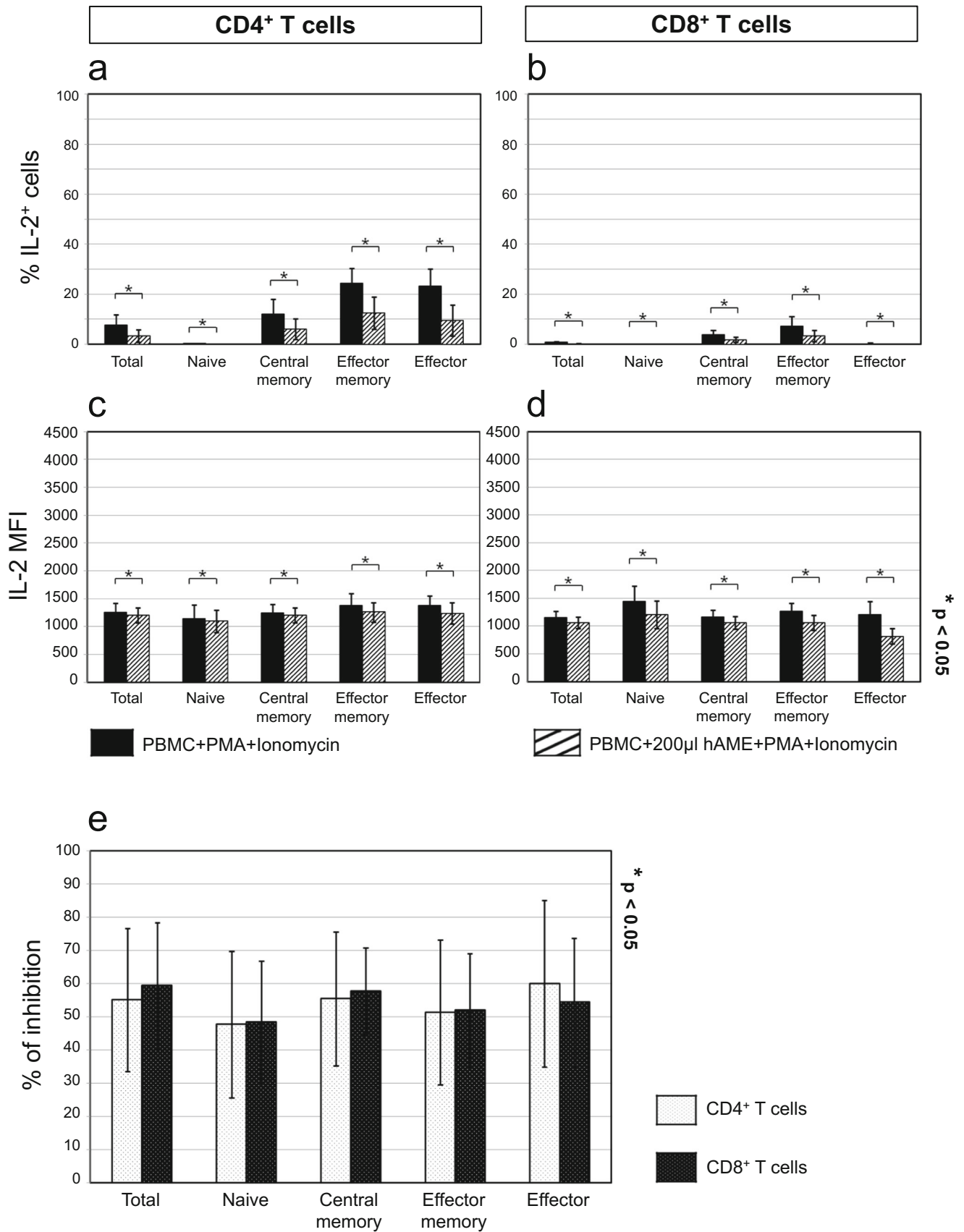
hAME decreases the frequency of IL-9 and IL-17 producing T cells

hAME induced a reduction in the percentage of IL-9 $^+$ and IL-9 $^+$ IL-2 $^+$ T cells (Fig. 5a, b), accompanied by a slight MFI reduction (Fig. 5c, d), for both CD4 $^+$ ($p < 0.05$) and CD8 $^+$ ($p < 0.05$) T cells and at similar extent for these cell populations (\approx 40 to 60% of reduction; Fig. 5e).

In this respect, a reduced percentage of CD4 $^+$ and CD8 $^+$ T cells expressing IL-17 was observed among overall Th17 and Tc17 cells (\approx 35 and \approx 20% of reduction, respectively; Fig. 6a), including those simultaneously expressing TNF α , IFN γ , or IL-2 ($p < 0.05$, Fig. 6b–d), along with the decrease of the MFI of all cytokines ($p < 0.05$), except IFN γ (Fig. 6e–h). Interestingly, hAME exerted a higher inhibitory effect on IL-17 $^+$ IL-2 $^+$ T cells (\approx 35%) than on the remaining Th17 and Tc17 cell subpopulations ($p < 0.05$, Fig. 6i).

hAME alters the transcriptional program of distinct T cell subsets

For purified CD4 $^+$ T cells, we verified that the presence of hAME in the cell culture augmented FOXP3 and IL-10, while decreased IL-4, GATA-3 and STAT6 mRNA levels (Fig. 7). Similarly, IL-10 mRNA expression increased in purified Treg



◀ **Fig. 4** Effect of hAME on the expression of IL-2 by CD4⁺ and CD8⁺ T cells: **a, b** Percentage of IL-2-producing CD4⁺ (**a**) and CD8⁺ (**b**) T cells (mean ± SD), distributed among their different subsets (naïve, central memory, effector memory and effector), following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME (PBMC+200 µl hAME+PMA+Ionomycin) in the culture medium. **c, d** Amount of IL-2 expressed per CD4⁺ (**c**) and CD8⁺ (**d**) T cell, measured as the MFI (mean ± SD). **e** Percentage of inhibition induced by hAME on the frequency of IL-2 producing CD4⁺ and CD8⁺ T cells. Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p < 0.05$ for the Wilcoxon paired-samples test for the groups indicated in the figure. Abbreviations: hAME, human amniotic membrane extract; IL, interleukin; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; SD, standard deviation

cells under the influence of hAME, whereas TGFβ1 was reduced and FOXP3 mRNA remained unchanged in this T cell population. An anti-inflammatory effect was also observed on CD8⁺ T cells and γδ T lymphocytes, in which hAME reduced mRNA levels of granzyme B, perforin and NKG2D for both T cell populations, as well as EOMES for the latter (Fig. 7).

hAME differentially regulates the expression of cytokines and chemokines by distinct monocyte subpopulations and myeloid dendritic cells

In contrast to the results obtained for T cells, hAME increased the percentage of classical and intermediate monocytes expressing TNFα and IL-6; intermediate monocytes producing IP-10; non-classical monocytes expressing IL-6; and mDCs expressing TNFα and MIP-1β (Fig. 8). Conversely, the presence of hAME in the culture medium resulted in a decreased percentage of classical monocytes producing IP-10 and non-classical monocytes expressing IP-10 and MIP-1β (Fig. 8).

Discussion

The AM has been described to possess a wide range of anti-inflammatory properties arising from either the AM mesenchymal stromal cells (Magatti et al. 2015; Pianta et al. 2016), the AM epithelial cells (Magatti et al. 2015; Mamede et al. 2012) or the hyaluronic acid from the AM avascular stromal matrix (He et al. 2009; Mamede et al. 2012). For human immune cells, this anti-inflammatory action includes inhibition of T cell proliferation and Th1/Th17 cytokine expression (Magatti et al. 2008; Parolini et al. 2014; Pianta et al. 2016; Rossi et al. 2012), accompanied by the induction of Treg (Parolini et al. 2014; Pianta et al. 2016), inhibition of monocyte differentiation into DC in vitro, loss of their allostimulatory activity and

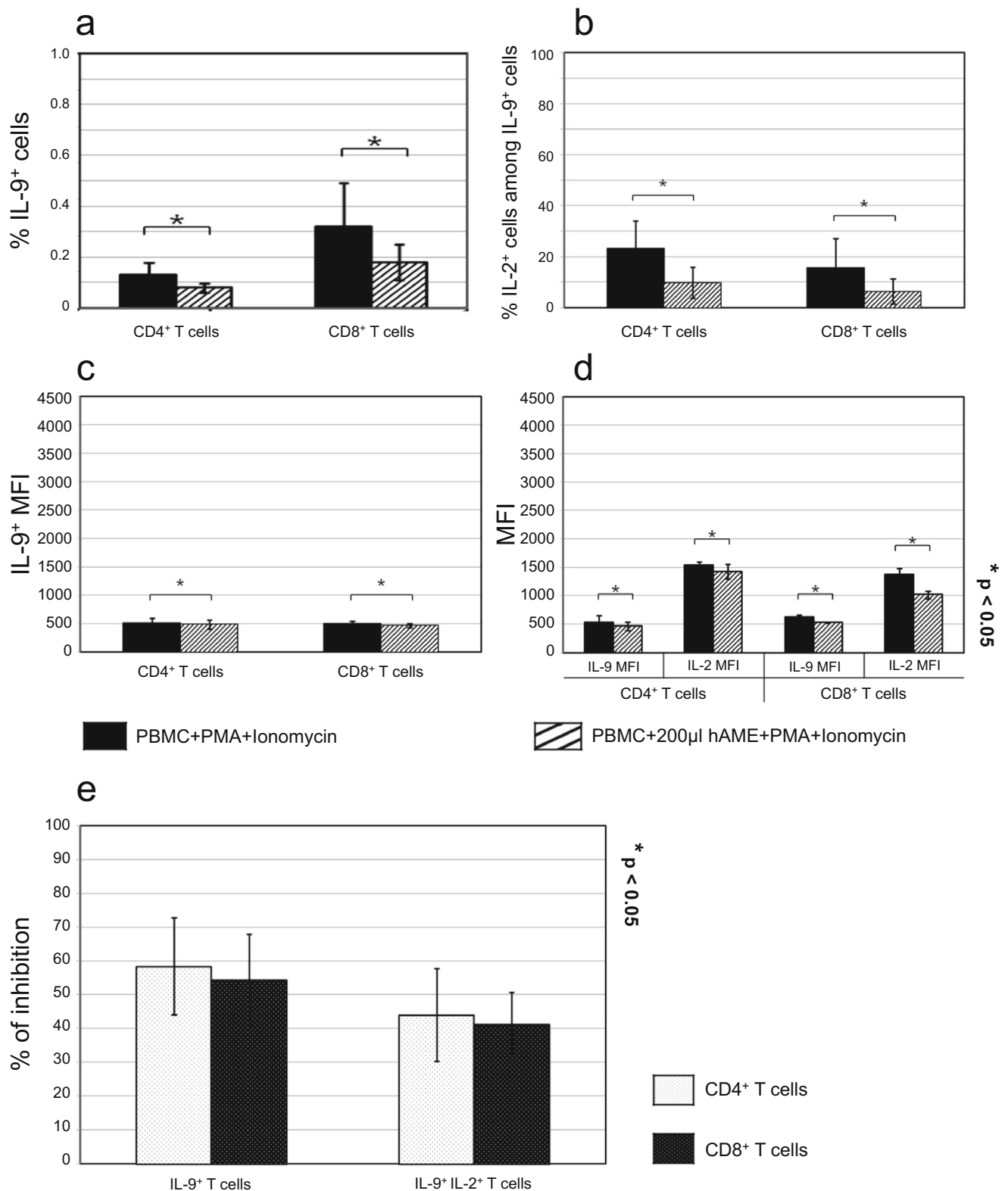
suppression of pro-inflammatory cytokines' production (Magatti et al. 2015; Magatti et al. 2009; Pianta et al. 2016). Similar results were obtained for mouse (Bauer et al. 2009; He et al. 2008; He et al. 2014; McDonald et al. 2015). In addition, the low immunogenicity of AM cells gives an ultimate advantage for its clinical use (Kubo et al. 2001; Mamede et al. 2012); nevertheless, the use of hAME could be even more advantageous in terms of immunogenicity.

Despite the increasing interest in the immunosuppressive activity of hAM cells and hAME, to the best of our knowledge, there are no studies focusing on their effects on the distinct T cell functional compartments, nor on the different peripheral blood monocyte subpopulations. This subject is of utmost importance given the different effector activity of the distinct T cell functional compartments, whose proportions are altered in several immune-mediated diseases. Likewise, the differences reported so far among the different monocyte subpopulations—in terms of propensity to undergo differentiation into macrophages and/or DC, the effectiveness of antigen presentation and the pattern of cytokine expression (Ziegler-Heitbrock and Hofer 2013)—justifies a more detailed investigation on how these subpopulations may be differentially regulated by hAME.

In this respect, we sought to evaluate the anti-inflammatory properties of hAME on T cells and antigen-presenting cells, in order to understand whether this inhibitory activity differs for distinct T cell's functional compartments and different antigen-presenting cell subsets.

The present study demonstrates that hAME suppresses the inflammatory response of T cells from all the functional compartments (naïve, central memory, effector memory and effector compartment). And, according to our data, the immunosuppressive effect of hAME on in vitro cytokine production is achieved by the reduction of the proportion of T cells producing cytokines, rather than by decreasing the amount of cytokine produced per cell (measured as MFI). Though we had verified a consistent decrease on cytokines MFI after hAME treatment, this reduction is too small and is likely not to be of biological significance. Conversely, this inhibitory effect on the inflammatory response is not so clear for the antigen-presenting cells studied. In fact, hAME showed to have a stimulatory activity on TNFα and IL-6 production by classical and intermediate monocytes.

There are few studies investigating the effect of hAM cells on the function of human antigen-presenting cells. All of them systematically described the inhibition of in vitro monocyte differentiation into DC, as well as LPS-induced monocyte-derived DC maturation (Magatti et al. 2009; Pianta et al. 2016); the loss of allostimulatory capability by monocytes/monocyte-derived DC and



decreased expression of the costimulatory molecule CD80 (Magatti et al. 2015; Magatti et al. 2009); along with the decreased expression of pro-inflammatory cytokines and chemokines and increased IL-10 secretion (Magatti et al.

2015; Magatti et al. 2009). Importantly, hAM cells and hAM-conditioned medium polarize monocytes towards the anti-inflammatory M2-macrophage phenotype in vitro (Magatti et al. 2015; Magatti et al. 2016; Pianta

Fig. 5 Effect of hAME on the expression of IL-9⁺ by CD4⁺ and CD8⁺ T cells: **a** Percentage of IL-9-producing cells (mean ± SD), within total CD4⁺ and CD8⁺ T cells, following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME (PBMC+200 μl hAME+PMA+Ionomycin) in the culture medium. **b** Percentage of IL-9-producing CD4⁺ and CD8⁺ T cells that simultaneously express IL-2 (mean ± SD) among IL-9+CD4+ and IL-9+CD8+ T cells, respectively. **c**, **d** Amount of protein expressed per cell among IL-9⁺ (**c**) and IL-9⁺IL-2⁺ (**d**) T cells, measured as MFI (mean ± SD). **e** Percentage of inhibition induced by hAME on the frequency of IL-9⁺ and IL-9⁺IL-2⁺ cells among total CD4⁺ and CD8⁺ T cells (mean ± SD). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p < 0.05$ for the Wilcoxon paired-samples test for the groups indicated in the figure. Abbreviations: hAME, human amniotic membrane extract; IL, interleukin; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; SD, standard deviation

et al. 2016). Evidences of M2-macrophage polarization was also observed in vivo, in a mouse model of corneal keratitis treated with hAME (He et al. 2014). In the same regard, it was recently described that hAM cells decreased TNF α and IL-1 β production by the human macrophage cell line THP-1 (Shu et al. 2015a) and hAM-conditioned medium has a suppressive effect on the expression of TNF α , IL-1 β , IL-12p70, MIP-1 α , CXCL9, RANTES and IL-8 expression by human M1-like macrophages differentiated in vitro, while it increases IL-10 production by these cells (Magatti et al. 2016).

In order to better reproduce the physiologic conditions, we used freshly isolated and naturally occurring monocytes and mDC from peripheral blood, instead of monocyte-derived DC generated in vitro. Using this approach, we found that hAME does not suppress the production of TNF α and IL-6 by antigen-presenting cells; actually, it seems to promote it in a dose-dependent manner, particularly in those cell subsets displaying the highest production of these cytokines (classical and intermediate monocytes); conversely, hAME can hinder the development of the inflammatory response by decreasing the expression of two important chemokines for T cell chemoattraction (MIP-1 β and IP10) (Charo and Ransohoff 2006) by classical and non-classical monocytes. It is important to note that, according to the current knowledge, intermediate monocytes correspond to the peripheral blood monocyte subset with the highest ability to present antigens to T cells (Ziegler-Heitbrock and Hofer 2013) and our results show hAME suppresses neither their ability to produce pro-inflammatory cytokines nor their chemokines' expression. Similar results were obtained for our freshly isolated mDC. It is worth noting that the three monocytes subsets and mDC behave differently in the presence of hAME; thus, we may speculate that macrophage cell lines and in vitro-generated monocyte-derived DC used in other studies may also display a different

behavior in the presence of hAM cells in relation to freshly isolated peripheral blood antigen-presenting cells used here, which may explain the differences observed between our study and the previous ones.

Nevertheless, despite the fact that our results do not support that hAME hinders the inflammatory response at antigen-presenting cell level, they clearly demonstrate that the immune response is suppressed at the T cell level. According to the previous described decreased percentage of TNF α and IFN γ -producing T cells in MLR in the presence of hAM mesenchymal cells (Pianta et al. 2016), we obtained similar results for PMA plus ionomycin-stimulated T cells in the presence of hAME but we further describe that this suppressive effect is transversal to all T cell functional compartments, although the degree of inhibition varies among them and among CD4⁺ and CD8⁺ T cell subsets. Moreover, we found that hAME diminished the percentage of Th17 and Tc17 cells; likewise, a decreased percentage of Th17 cells and an increased percentage of CD4⁺CD25⁺FoxP3⁺ Treg cells, accompanied by the augment of IL-10 production, were formerly reported to be induced by hAM cells in MLR (Pianta et al. 2016) and in PBMC from rheumatoid arthritis patients stimulated with type II-collagen (Parolini et al. 2014). In this respect, we also found an increased mRNA expression of FoxP3 and IL-10 among purified CD4⁺ T cells and a rise in IL-10 mRNA levels in purified Treg cells. Additionally, the results obtained for the mRNA expression among purified CD8⁺ T cells and $\gamma\delta$ T cells point to the loss of cytotoxic function by these cells, being observed a decrease in granzyme B, perforin, and NKG2D, for both T cell subsets and also a reduction in EOMES mRNA expression for the latter cell population. The hAM-mediated decrease of CD8⁺ T cells expressing granzyme B had also been recently reported by others (Pianta et al. 2016).

Moreover, our results demonstrate that hAME has the ability to hamper IL-9 expression by reducing the frequency of Th9 and Tc9 cells. IL-9 has been classically linked to allergic inflammation (atopic dermatitis and asthma), being demonstrated that IL-9 neutralization improved asthma symptoms in mouse and the levels of IgE were correlated to Th9 cell numbers in allergic patients. In this regard, mast cell-derived IL-9 was demonstrated to have an important role in allograft tolerance, as this cytokine was able to improve the regulatory function of Treg cells (Li et al. 2017). Recent reports show this cytokine is also implicated in anti-parasitic immunity (Li et al. 2017). Notwithstanding, Th9 cells were demonstrated to possess anti-tumor activity, by inducing the activation of mast cells and other innate immune cells and to be involved in the pathogenesis of inflammatory bowel disease, multiple sclerosis, systemic sclerosis, systemic lupus

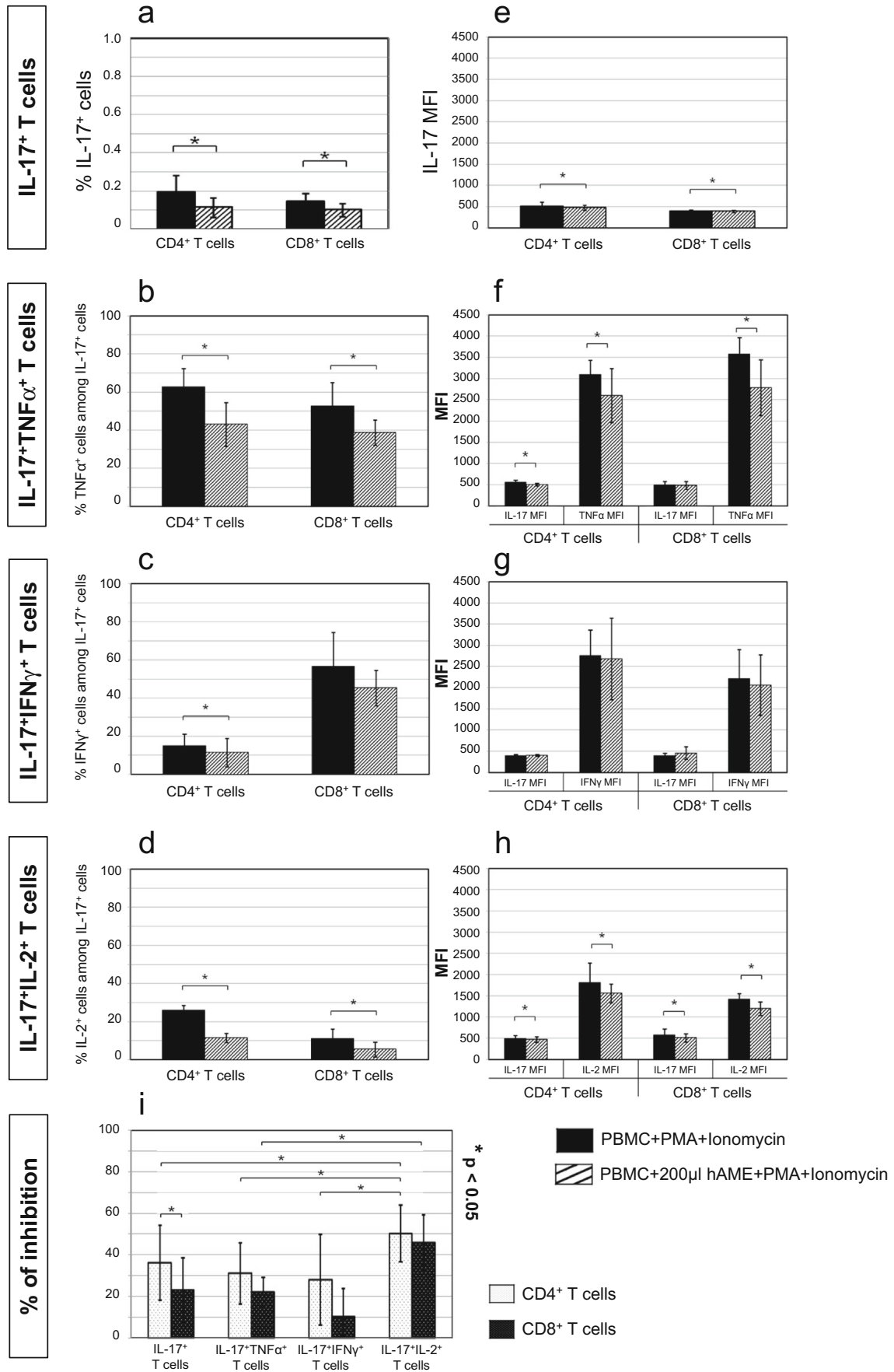


Fig. 6 Effect of hAME on IL-17⁺, IL-17⁺TNFα⁺, IL-17⁺IFNγ⁺ and IL-17⁺IL-2⁺ T cells: **a** Percentage of IL-17-producing cells (mean ± SD), within total CD4⁺ and CD8⁺ T cells following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME (PBMC+200 μl hAME+PMA+Ionomycin) in the culture medium. **b–d** Percentage of IL-17-producing CD4⁺ and CD8⁺ T cells that simultaneously express **b** TNFα, **c** IFNγ, or **d** IL-2, within IL-17⁺ T cells. **e–h** Amount of protein expressed per cell among **e** IL-17⁺, **f** IL-17⁺TNFα⁺, **g** IL-17⁺IFNγ⁺ and **h** IL-17⁺IL-2⁺ T cells, measured as MFI (mean ± SD). **i** Percentage of inhibition induced by hAME on the frequency of IL-17⁺, IL-17⁺TNFα⁺, IL-17⁺IFNγ⁺ and IL-17⁺IL-2⁺ cells among total CD4⁺ and CD8⁺ T cells (mean ± SD). Results were obtained for eight PBMC samples. Statistically significant differences were considered when *p* < 0.05 for the Wilcoxon paired-samples test for the groups indicated in the figure. Abbreviations: hAME, human amniotic membrane extract; IFNγ, interferon gamma; IL, interleukin; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; SD, standard deviation; TNFα, tumor necrosis factor-alpha

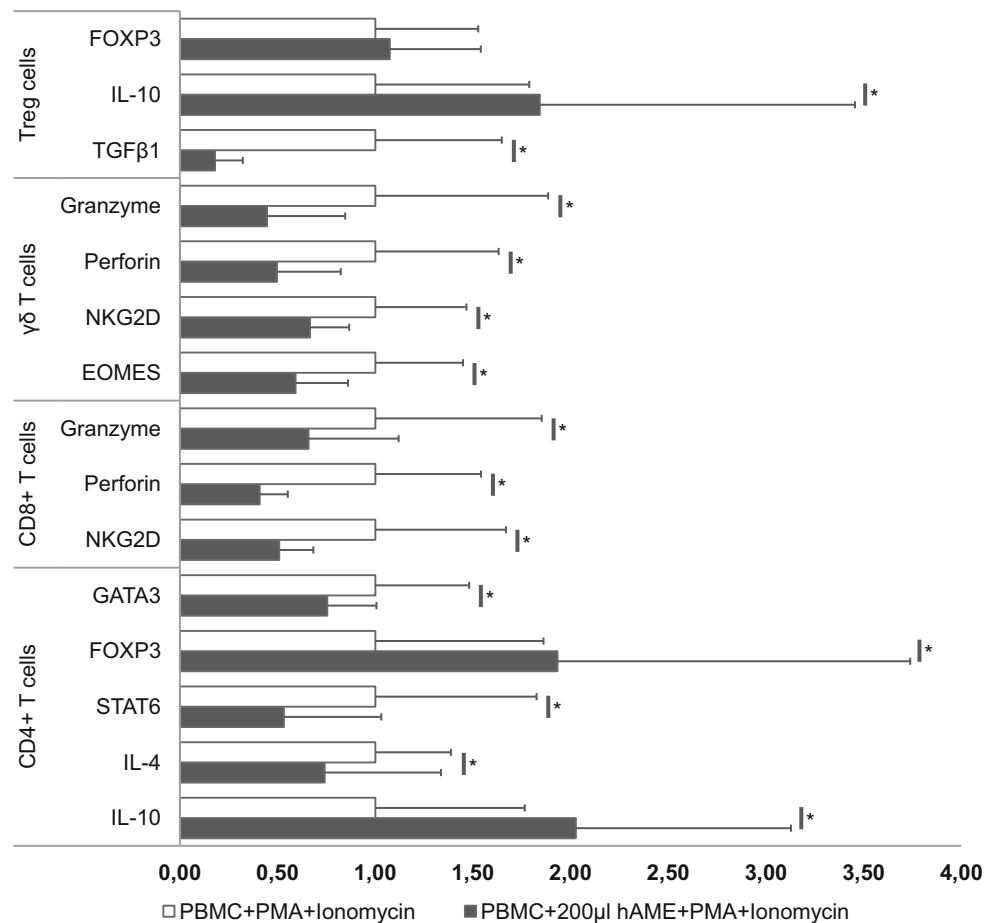
erythematosus and rheumatic arthritis (Guggino et al. 2017; Li et al. 2017; Yin et al. 2017). Though the role of IL-9 in health and disease has just began to be explored, it seems that the pro or anti-inflammatory effect of this cytokine will depend on the immune context and the specific conditions of the organism.

Finally, we also observed that hAME inhibits mitogen-induced lymphocyte proliferation, which is in agreement with the previously described hAM cells-mediated inhibition of lymphocyte proliferation in response to MLR (Magatti et al. 2008; Pianta et al. 2016), CD3/CD28 stimulation (Magatti et al. 2008) and antigen (Parolini et al. 2014).

Though few studies attempted to understand mechanistically the means of the immunosuppressive action of hAM cells, it is already recognized that COX1/2 activity (Parolini et al. 2014), PGE2 (Rossi et al. 2012) and IL-10 (Parolini et al. 2014) participate in the inhibition of human T cell proliferation and expression of IFNγ. Of note, both PGE2 and IL-10 are produced by hAM tissue cells (Rossi et al. 2012).

Overall, our results indicate that hAME is able to hamper the development of the inflammatory response, not by suppressing the production of pro-inflammatory cytokines by monocytes and mDC but by hindering their interaction with T cells (by inhibiting the expression of chemokines) and by directly interfering with the ability of T cells to proliferate in response to mitogenic activation, to secrete IL-2 and to produce Th1/Th17-related

Fig. 7 mRNA expression in purified CD4⁺, CD8⁺, γδ T and Treg cells: semi-quantitative analysis of mRNA expression in FACS-purified T cells from two separate culture conditions: PBMC stimulated in the absence (PBMC+PMA+Ionomycin) or presence of hAME (PBMC+ 200 μl hAME+PMA+ Ionomycin). Results were obtained for five PBMC samples and were normalized for the PBMC+PMA+Ionomycin condition. Statistically significant differences were considered when *p* < 0.05 for the Wilcoxon paired-samples test: * for the groups indicated in the figure. Abbreviations: EOMES, eomesodermin; hAME, human amniotic membrane extract; IL, interleukin; FOXP3, forkhead box p3; GATA3, GATA binding protein 3; NKG2D, natural-killer group 2, member D; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; STAT6, signal transducer and activator of transcription 6; TGFβ, transforming growth factor β; Treg, regulatory T cells



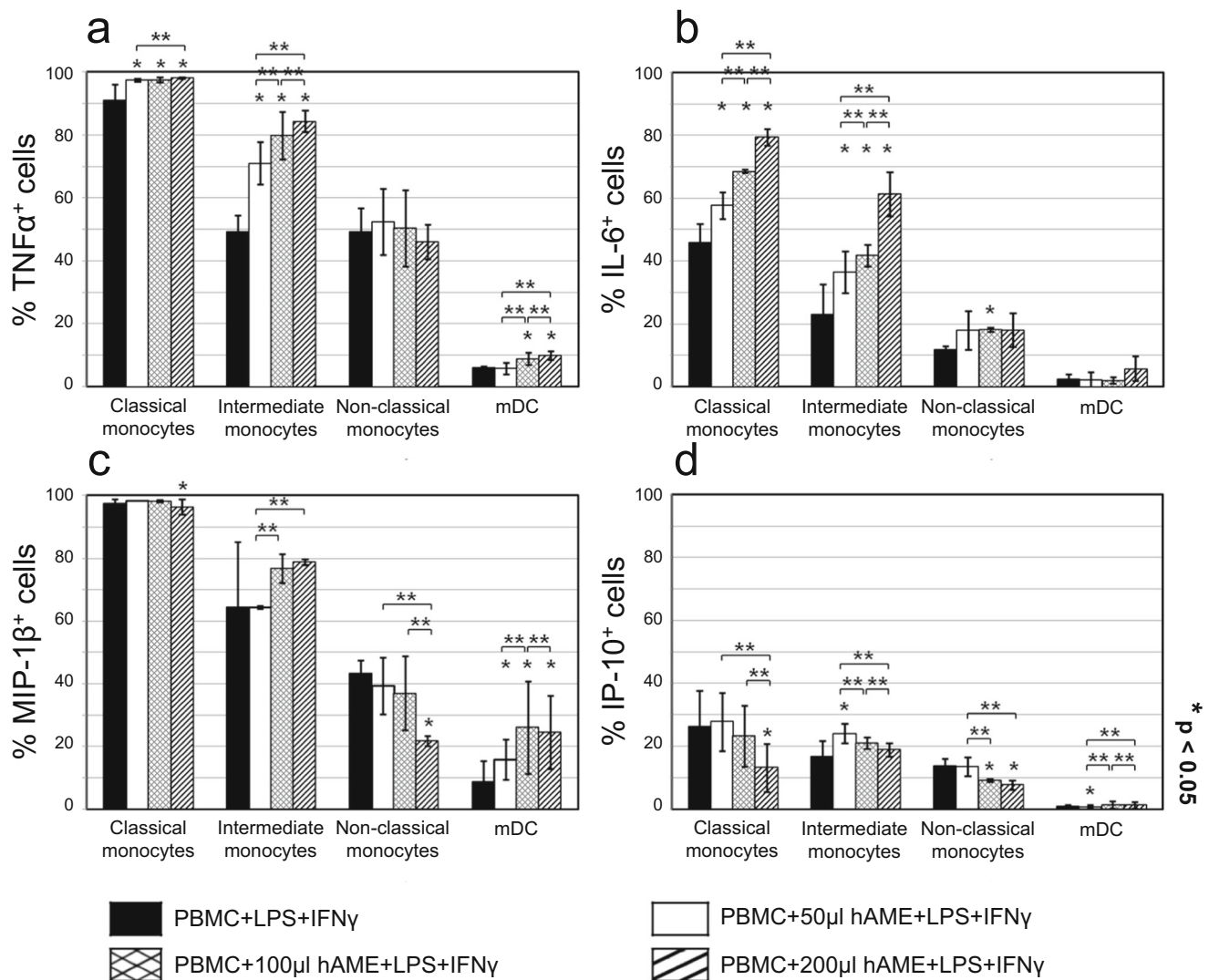


Fig. 8 Effect of hAME on the frequency of TNF α , IL-6, MIP-1 β and IP-10 expressing monocytes and myeloid dendritic cells: percentage of classical, intermediate and non-classical monocytes and myeloid dendritic cells producing TNF α , IL-6, MIP-1 β and IP-10 (mean \pm SD), following PBMC stimulation in the absence (PBMC+LPS+IFN γ) or presence of hAME in the culture medium (PBMC+50 μ l hAME+LPS+IFN γ , PBMC+100 μ l hAME+LPS+IFN γ and PBMC+200 μ l hAME+LPS+IFN γ). Results were obtained for three PBMC samples.

Statistically significant differences were considered when $p < 0.05$ for the Wilcoxon paired-samples test: * versus PBMC+LPS+IFN γ ; ** for the groups indicated in the figure. Abbreviations: hAME, human amniotic membrane extract; IFN γ , interferon gamma; IL, interleukin; IP-10, IFN γ -induced protein 10; LPS, lipopolysaccharide; mDC, myeloid dendritic cells; MIP-1 β , macrophage inflammatory protein 1 beta; PBMC, peripheral blood mononuclear cells; SD, standard deviation; TNF α , tumor necrosis factor-alpha

cytokines (TNF α , IFN γ , IL-17). hAME further hampers the effector arm of the Th1/Th17 immune response by inhibiting the expression of proteins with an important cytotoxic function, like TNF α , granzyme B and perforin, by CD8 $^+$ T cells and $\gamma\delta$ T cells. Moreover, the inhibitory activity of hAME on IL-9 production by T cells can be a relevant finding for allergic diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Ethical Committee of the Centro Hospitalar e Universitário de Coimbra (CHUC-70-12, Coimbra, Portugal). All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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