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Expression of functional mineralocorticoid receptor (MR) and G-protein coupled estrogen receptor (GPER) in human T lymphocytes

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ABSTRACT

Aldosterone plays a key role in controlling blood pressure (BP) values by maintaining body salt, water, and fluid homeostasis. Excess aldosterone production is associated with arterial hypertension, cardiovascular and metabolic diseases, partly via generation of an inflammatory state followed by fibrotic changes in the organs that are target of hypertension. Aldosterone exerts genomic effects that are known to involve activation of the mineralocorticoid receptor (MR). Other aldosterone effects, including those usually defined as 'rapid' or 'non genomic', involve additional receptors as the G-protein coupled estrogen receptor (GPER). To date, the receptor(s) implicated in the inflammatory action of aldosterone in cells of the innate and adaptive immunity are unknown. Considering the potential role of T-lymphocytes in adaptive immunity in arterial hypertension and related hypertension-mediated organ damage (HMOD), we herein investigated and quantified the expression of the MR and GPER in human $CD4^+$ and $CD8^+$ T-cells. Results provided compelling evidence for the presence at the mRNA and protein level and suggest a functional role of these receptors in the two *T*-lymphocyte subtypes, thus indicating that they can represent a potential target for modulation of steroid hormone-induced inflammation and ensuing HMOD.

1. Introduction

Aldosterone plays a key role in the regulation of blood pressure (BP), body fluid volume and sodium–potassium balance. Its release from the adrenocortical zona glomerulosa cells is controlled by multiple factors, including angiotensin (Ang) II, potassium (K^+), endothelin-1, vasopressin, urotensin II, and circulating adrenocorticotrophic hormone (ACTH) [1–3].

According to the classic view, aldosterone binds to the intracellular mineralocorticoid receptor (MR) in epithelial cells of the distal convoluted tubule and the collecting duct of the nephrons. By up-regulating the transcription of genes coding for the epithelial sodium channel (ENaC) and the basolateral Na^+/K^+-ATPase, at these sites aldosterone promotes reabsorption of water and Na^+, in exchange for K^+, which expands body fluid volumes and, thereby, increases BP [4].

Aldosterone is also known to promote inflammation, fibrosis, and tissue remodeling in the heart [5], blood vessels [6], and kidney, and to induce glomerular hyperfiltration and renal injury [7]. Moreover, accumulating evidence indicates that, aldosterone by acting on MR in vascular smooth muscle cells, endothelial cell, and inflammatory/immune cells, can induce generation of reactive oxygen species, blunt vascular nitric oxide bioactivity, and thereby cause endothelial dysfunction and systemic inflammation [8–13].

Over the last few years, multiple studies have suggested that aldosterone modulates many components of the immune system, thus triggering the inflammation that contributes to vascular, cardiac, and renal damage [14–17]. Thus, under conditions of excess aldosterone production, the hormone can overactivate the MR and lead to the development of hypertension (HT), ultimately resulting into end-organ damage. Noteworthy, the latter is a feature of resistant HT, which represents a common mode of presentation of primary aldosteronism [18–20], the prototype of salt-dependent arterial HT and the most common secondary

Abbreviations: Ang, Angiotensin II; BP, Blood pressure; ACTH, Circulating adrenocorticotrophic hormone; ENaC, Epithelial sodium channel; GPER, G-protein coupled estrogen receptor; HT, Hypertension; HMOD, Hypertension-mediated organ damage; MR, Mineralocorticoid receptor; PBMC, Peripheral blood mononuclear cells; K^+, Potassium.

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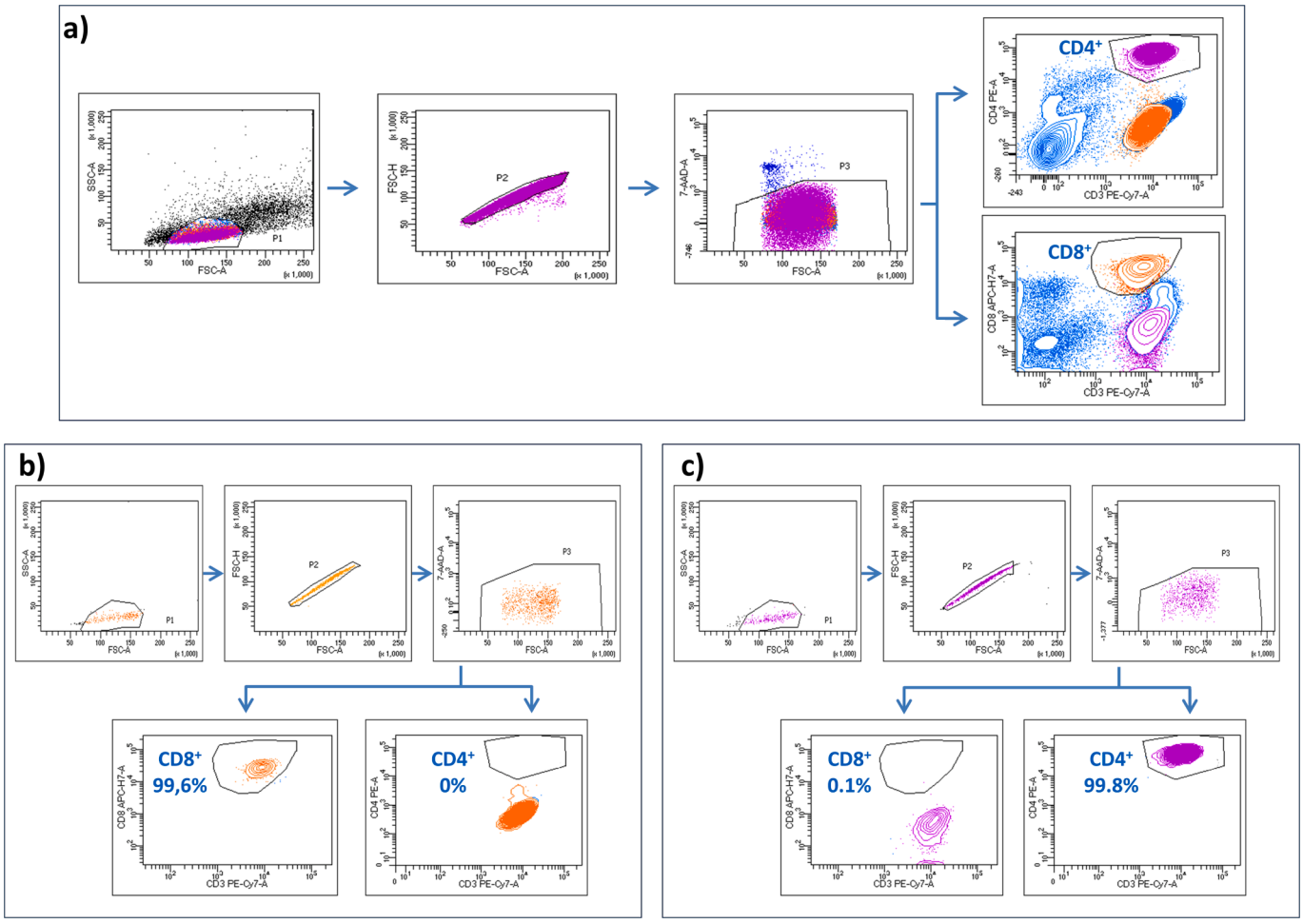

Fig. 1. Schematic representation of the gating strategy for flow cytometry used to sort $CD4^+$ and $CD8^+$ T cells from peripheral blood mononuclear cells (PBMC). PBMC were isolated and labelled with various antibodies. After separation from debris by Forward Scatter Area (FSC-A)/Side Scatter Area (SSC-A), and doublets and aggregates exclusion by FSC-A and FSC-height (FSC-H) dot plots, T cells were selected as $CD3^+$. $CD4^+$ and $CD8^+$ T cell subsets were identified by corresponding antibodies (a). Once the sort is complete, we confirmed by a post-sort of analysis the purity of our cells of interest $CD4^+$ and $CD8^+$ (b-c).

Table 1
Primer sequence used in the digital droplet PCR assay.

	Forward	Reverse
MR	GATGGTAACTAAGTGTCCCAACAA	TTCCAGCAGGTCGCTCACCAGG
GPER	AGACTGTGAAATCCGCAACC	TGCTCACTCTCTGGGTACCTG

Table 2
MR and GPER gene and protein expression in human $CD4^{\pm}$ and $CD8^{\pm}$ T cells.

	***ddPCR* (mRNA copies)**				***Immunoblot***			
	$CD4^+$	**$CD8^+$**	***n***	***P***	**$CD4^+$**	**$CD8^+$**	**n**	**P**
MR	3061 ± 4037	3379 ± 3112	5	–	0.17 ± 0.16	0.10 ± 0.12	5	–
GPER	65 ± 71.7	86.4 ± 68.9	5	–	0.29 ± 0.36	0.69 ± 0.47	5	–

Values are means ± SD, *n,* numbered of samples. ddPCR data are reported as number of copies per 0.5 μg of total RNA. Immunoblot data are normalized to β-actin densiometric unit. $P < 0.05$ is regarded as statistically significant.

form of HT. Being characterized by excess aldosterone production, which induces an inflammatory state, primary aldosteronism was shown to feature a more prominent hypertension-mediated organ damage (HMDO) than seen in BP-matched primary HT patients [21–24].

Recent data indicate that aldosterone can activate other receptors

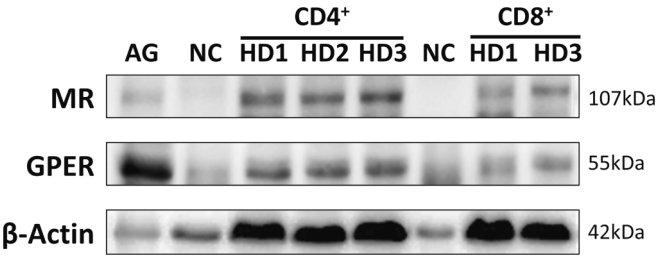

Fig. 2. Immunoblot analysis for MR and GPER in human $CD4^+$ and $CD8^+$ T cells of healthy donor (HD) subjects, adrenal gland (AG), and negative control (NC) A549 cell line.

besides the MR, such as the ubiquitously expressed G-protein coupled estrogen receptor (GPER) identified in 2000 by Filardo et al. [25]. Although first characterized as an orphan G-protein coupled receptor, GPER (previously known as GPR30) was thereafter demonstrated to mediate rapid intracellular signaling of estrogens [25]. Moreover, available functional data support the notion that GPER is a promiscuous receptor capable of binding multiple steroids [26].

In the last decade aldosterone was found to activate GPER mediated pathways, both *in-vitro* and *in-vivo* [27], resulting in rapid activation of ERK1/2 and apoptosis in rat vascular smooth muscle cells [28], and endothelial cells [29] and depolarization and increased in cytosolic Ca^{2+}

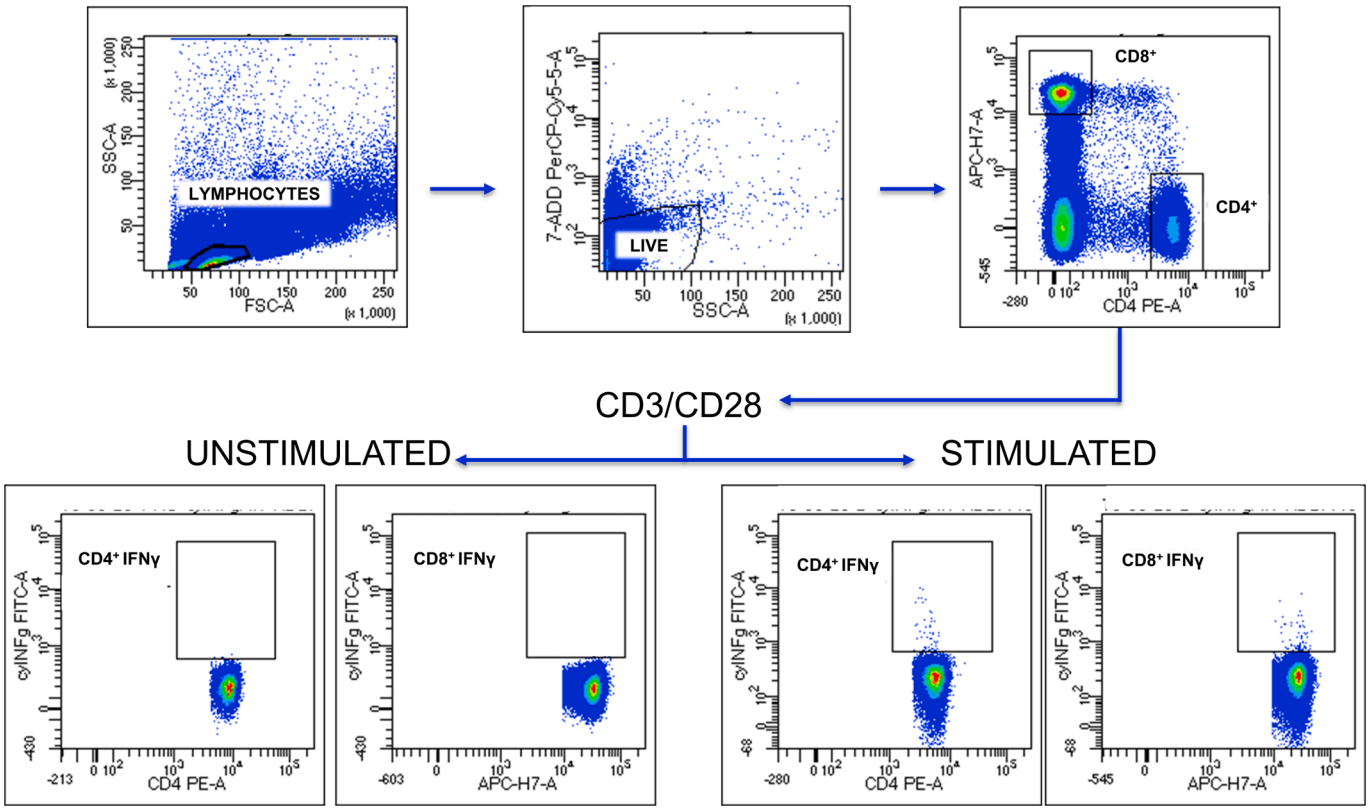

Fig. 3. Schematic representation of the gating strategy to identify IFN-γ release from in vitro simulated T cells in healthy donors. An increased numbers of IFN-γ $CD4^{\pm}$ and $CD8^{\pm}$ T cells in the presence of anti-CD3/CD28 is shown.

concentration in cardiac vagal neurons [30]. Of much importance, by using an *in-vitro* model of human adrenocortical cell line that produce aldosterone, we found that aldosterone can use the GPER to increase its own biosynthesis, thus indicating the existence of a positive feed-loop mechanism allowing a rapid aldosterone response to hypovolemic conditions [31].

In contrast with the wealth of information on MR and GPER in aldosterone target tissue, there is no information on the presence of aldosterone receptors, either the MR and the GPER, in human T cells, even though the binding of aldosterone on human lymphocytes was showed in the late 1980s [32]. Accordingly, the presence and functional role of MR in human T cells remains debated, as it was contended that the response to aldosterone of immune cells was mediated by MR activation in dendritic cells [14].

Therefore, as a first ineludible step toward the investigation of the role of T cells in aldosteronism, we have set out this study to investigate the presence of MR and GPER in human $CD4^+$ and $CD8^+$ lymphocytes and to gather preliminary information on their functional role as a potential target to modulate inflammation.

2. Experimental

2.1. $CD4^+$ and $CD8^+$ lymphocytes isolation

$CD4^+$ and $CD8^+$ lymphocytes were isolated by using the FACSAria cell sorter (BD biosciences, San Jose, CA, USA) from peripheral blood mononuclear cells (PBMC) of 5 healthy blood donor subjects. The gating strategy of $CD4^+$ and $CD8^+$ positive T lymphocytes, are shown in Fig. 1 (a). The purity of the sorted cell populations was confirmed by a post-sort of analysis, using the surface markers CD8 APC-H7, CD4 PE, and CD3 PE-Cy7 as shown in Fig. 1 (b-c) (BD Biosciences, San Jose, CA, USA).

2.2. RNA extraction and droplet digital PCR

A standardized protocol using RNeasy Mini Kit (Qiagen, Milan, Italy) was used to extract total RNA from $CD4^+$ and $CD8^+$ peripheral lymphocytes. One μg of RNA was checked for purity and quality by a Bioanalyzer (Agilent Technologies, Santa Clara, CA), and then reverse-transcribed with Iscript cDNA synthesis kit (Bio-Rad, Milan, Italy). Droplet digital PCR (ddPCR) [33,34] was used to measure the copies number of MR and GPER mRNA. The primers were designed using Primer3web online software and are reported in Table 1. Briefly, the ddPCR supermix containing 2 μL sample cDNA was partitioned into 20,000 aqueous nanoliter-sized droplets in oil via the QX200 Droplet Generator (Bio-rad Laboratories, Segrate, Italy). Droplets were then transferred to a 96-well PCR plate for thermocycling in the Bio-Rad C1000. After PCR, droplets from each sample were streamed in single line through the QX100 Droplet Reader. ddPCR quantification of the target molecules as number of copies per 0.5 μg of total RNA was analyzed with QuantaSoft analysis software (Bio-Rad).

2.3. Immunoblotting

Proteins (20 μg) were loaded in a 10 % acrylamide gel and SDS-PAGE and electro-transferred to nitrocellulose membranes (Hybond ECL-Amersham Biosciences, Amersham, UK). The membranes were blocked for 1 h at room temperature in 5 % non-fat dry blocking milk and then incubated overnight at 4 °C together with a monoclonal antibody anti-MR (kind gift of Dr. Celso Gomez Sanchez University of Mississippi Medical Center, Jackson, Mississippi, U.S.A.) or anti-GPER (Thermo Scientific, Rockford, IL). After washing, membranes were incubated for one hour with a secondary anti-mouse or anti-rabbit antibody to visualize the target protein. The specific immunosignal was visualized by a luminol-based chemiluminescence substrate (Thermo Scientific, Rockford, IL). Images were processed by Nine

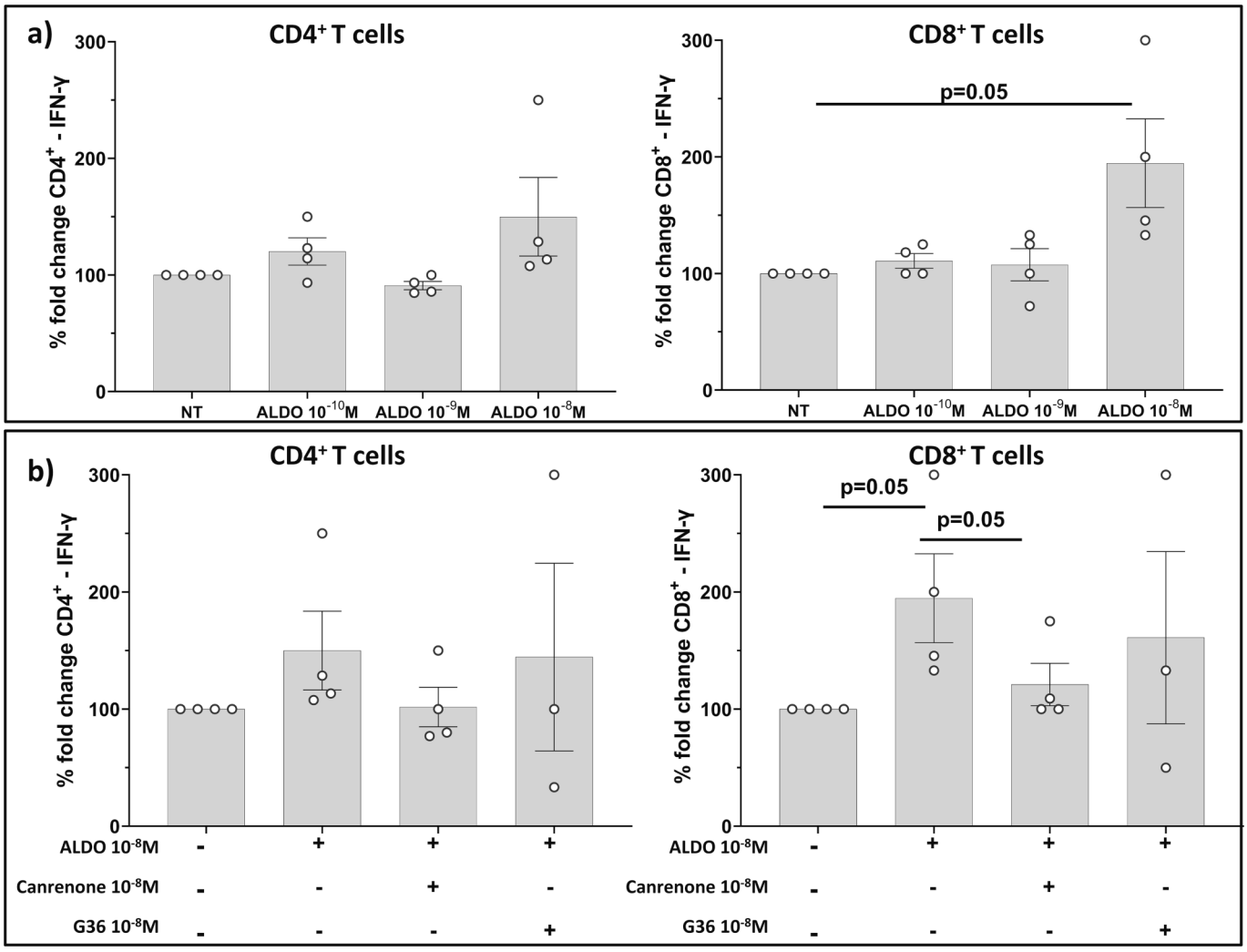

Fig. 4. The production of IFN-γ from $CD4^+$ and $CD8^+$ T cells obtained by culturing PBMCs from healthy donor subjects for 18 h in the presence of anti-CD3/CD28 alone or with aldosterone concentration from 10^{-10}M to 10^{-8}M (a) or with aldosterone on top the MR antagonist canrenone 10^{-6}M or the GPER antagonist G36 at concentration 10^{-6}M (b). IFN-γ was increased in $CD8^+$ T cells at aldosterone concentration 10^{-8}M (a), that was abolished by canrenone (b). Mean±SD of 4 experiments.

Alliance software (Uvitec, Cambridge,UK). Bands were normalized to β-actin used as control for loading.

2.4. In vitro stimulation and aldosterone effects in T cells

To explore the functional effect of aldosterone in T cells, PBMCs isolated from 4 healthy blood donor subjects were stimulated with aldosterone (Sigma-Aldrich, Sant Louis, US; used at concentration ranging from 10^{-10} M to 10^{-8} M) with or without the MR antagonist canrenone 10^{-6} M or the GPER antagonist G36 10^{-6} M, for 18 h at 37 °C in a 5 % CO_2 atmosphere with anti-CD3/CD28 (1 μg/mL) in complete culture medium (RPMI 1640 supplemented with 10 % fetal bovine serum and 1 % each of l-glutamine, sodium pyruvate, nonessential amino acids, antibiotics, HEPES, β-mercaptoethanol).

All samples were incubated with a protein transport inhibitor containing brefeldin A (Golgi Plug, Becton Dickinson). For each sample, at least 1 million cells were stimulated with aldosterone and 1 million cells exposed only to the vehicle served as negative control. After stimulation, cells were stained with LIVE/DEAD viability marker (BD Biosciences, San Jose, CA, USA) and for the surface markers $CD8^+$ APC-H7, $CD4^+$ PE, and $CD3^+$ PE-Cy7 (Biolegend, San Diego, CA, USA). They were then washed and fixed with a fixation solution (Invitrogen, Carlsbad, CA, USA) for a further 30 min and permeabilized by washing with a permeabilization buffer (Invitrogen, Carlsbad, CA, USA) and stained with the intracellular IFN-γ FITC. Then, a minimum of 100,000 cells per sample were acquired on FACSCanto II.

2.5. Statistical data analysis

MR and GPER are expressed as the mean ± SD. Statistical comparisons were performed using the nonparametric Mann-Whitney *t*-test. The response obtained with stimulation anti-CD3/CD28, for each HD subject are reported as percent changes from baseline values taken as 100 % to adjust for interindividual differences and evaluate treatments effect. Analysis was performed with GraphPad Software™ (vers. 9.0 for Mac OS X, La Jolla, CA). $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. MR and GPER in human T lymphocytes

The mRNA copies number of $CD4^+$ and $CD8^+$ in human lymphocytes as determined with droplet digital PCR (ddPCR) is shown in Table 2. Briefly, we found that MR and GPER are expressed in both $CD4^+$ and $CD8^+$ lymphocytes, and that the MR mRNA copy number was at least 60-fold that of the GPER. At the protein level, the rank expression was

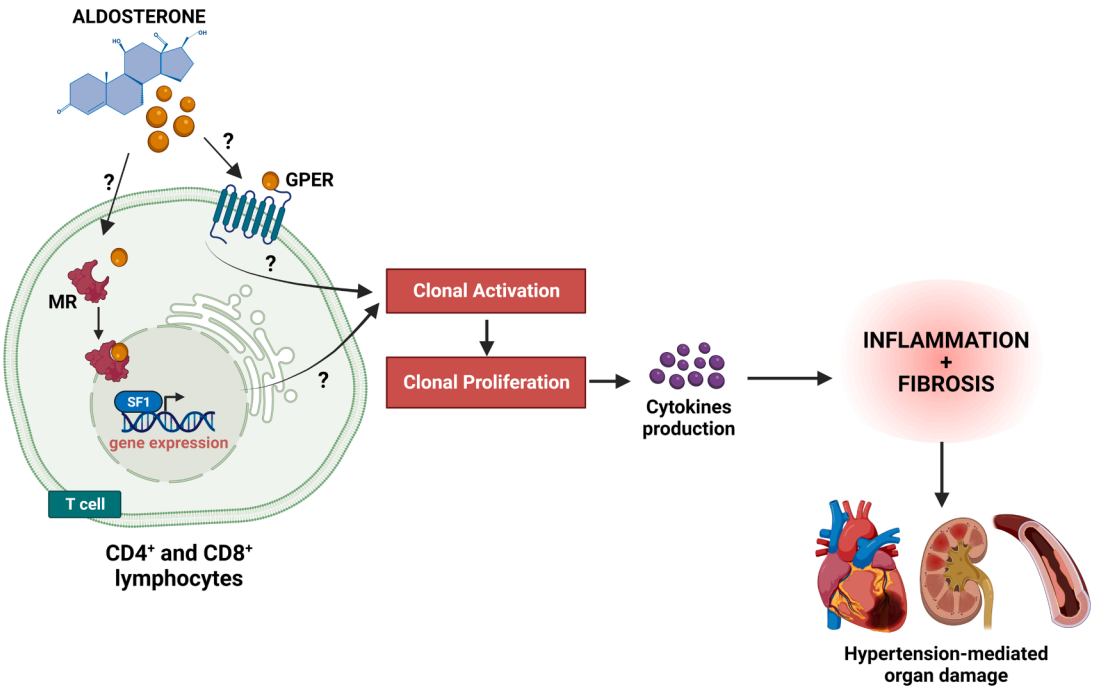

Fig. 5. The cartoon depicted the possible role of the mineralocorticoid receptor (MR) and G-protein coupled estrogen receptor (GPER) as mediators of aldosterone-induced hypertension-mediated organ damage (HMDO). Created with BioRender.com.

GPER > MR in both $CD4^{\pm}$ and $CD8^{\pm}$ lymphocytes (Table 2 and Fig. 2). A549 cell line derived from human pulmonary adenocarcinoma was used as negative control in our ddPCR and immunoblot experiments. No gene copies and protein were detected for both receptors, as expected, thus indicating the specifity of our findings. Kidney and adrenal gland were used as positive control for MR and GPER gene expression, respectively. The MR copies obtained in kidney were 5755 for 0.5 μg of total RNA, GPER copies were 7637 for 0.5 μg of total RNA in adrenal.

3.2. Aldosterone influences on T lymphocytes

We studied the influence of aldosterone on T cell activation by measuring the production of IFN-γ in $CD4^{+}$ T cells and $CD8^{+}$ T cell from 4 healthy donors. The gating strategy for measuring intracellular IFN-γ in $CD4^{+}$ and $CD8^{+}$ T cells is reported in Fig. 3. Fig. 4 (a) shows that at 10^{-8} M concentration aldosterone increased IFN-γ production by $CD8^{\pm}$ T cells by acting via the MR as this effect was abolished ($p < 0.05$) by canrenone and not by G36, the GPER antagonist (Fig. 4 (b)). However, no aldosterone-induced IFN-y production in CD4 + T cells was observed (Fig. 4 (a-b)).

4. Discussion

T cells participate in all phases of the adaptive immune response and accumulate in the organs that are target of arterial hypertension, as the kidney, heart, and vasculature, where they contribute to cardiovascular disease and, ultimately, to cardiovascular events.

In mice, GPER and MR were found to be expressed in adaptive and innate immune response cells, such as circulating B and T lymphocytes and monocytes, macrophages and dendritic cells [35,36]. However, scarce investigation has been dedicated to measuring the expression levels of MR and GPER in human T lymphocytes thus far. Using a radioreceptor binding assay Armanini et al. first reported binding of radiolabeled aldosterone to human lymphocytes isolated from the spleens of four deceased human kidney donors 1988 [32], but they could make no distinction between the major T-cell subtypes [32]. Expression of the more recently discovered GPER, has only been suggested in human regulatory T cells based on immunohistochemical staining [37].

The present study unequivocally showed for the first time the presence of mineralocorticoid receptor (MR) and G-protein coupled estrogen receptor (GPER) in both $CD4^{+}$ and $CD8^{+}$ human T lymphocytes isolated from healthy blood donor subjects, at both the mRNA and the protein level. A state-of-the-art droplet digital PCR and immunoblotting (Table 2) were used to these goals. Thus, our results extend prior observations of aldosterone binding to human lymphocytes to the $CD4^{+}$ and $CD8^{\pm}$ T cell subtypes [32,38,39] by showing the MR and also the GPER aldosterone receptors.

These new pieces of knowledge are important because even though aldosterone was shown to promote the polarization of $CD4^{+}$ naive T cells into pro-inflammatory Th1/Th17 phenotype in mice, through the selective secretion of IL-6 and TGF-β, it was contended that this effect was not a direct one, but mediated by MR-activated dendritic cells [14].

Of note, the present results extend also available knowledge by showing that human $CD4^{+}$ and $CD8^{+}$ T cells similarly express both the MR and GPER at the mRNA and protein level and that the MR was much more expressed than the GPER. In contrast, the protein expression levels of GPER were found to be higher than those of MR in both subtypes of human T cells, which is an intriguing result obviously deserving further investigation. To gather preliminary evidence on a potential functional effect of the MR and GPER in T cells, we also investigated the effect of aldosterone on $CD4^{+}$ and $CD8^{+}$ cells activation by measuring IFNγ release after treatment with anti-CD3/CD28 in presence of aldosterone and MR and GPER antagonists. We found that at the highest concentration used in our experimental protocol aldosterone promoted induced IFNγ release in $CD8^{+}$ but not in $CD4^{+}$ cells by acting via the MR and not via the GPER (Fig. 4).

The fact that GPER and MR are expressed on human T lymphocytes and the role of MR receptor in T cell activation mediated by aldosterone (Fig. 5), collectively support the need for further investigation to elucidate the effect of selective MR and GPER activation in the modulation of T-cell biology in humans, as fundamental steps to unveil the effect of aldosterone in triggering adaptive immunity in hypertension and related HMOD.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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