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Short communication

THE PPARα PATHWAY IN Vγ9Vδ2 T CELL ANERGY

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Abstract: Phosphoantigens (PAgs) activate Vγ9Vδ2 T lymphocytes, inducing their potent and rapid response in vitro and in vivo. However, humans and non-human primates that receive repeated injections of PAgs progressively lose their Vγ9Vδ2 T cell response to them. To elucidate the molecular mechanisms of this in vivo desensitization, we analyzed the transcriptome of circulating Vγ9Vδ2 T cells from macaques injected with PAg. We showed that three PAg injections induced the activation of the PPARα pathway in Vγ9Vδ2 T cells. Thus, we analyzed the in vitro response of Vγ9Vδ2 T cells stimulated with a PPARα agonist. We demonstrated that in vitro PPARα pathway activation led to the inhibition of the BrHPP-induced activation and proliferation of human Vγ9Vδ2 T cells. Since the PPARα pathway is involved in the antigen-selective desensitization of human Vγ9Vδ2 T cells, the use of PPARα inhibitors could enhance cancer immunotherapy based on Vγ9Vδ2 T cells.

Keywords: Activation, Gamma-delta T-lymphocyte, Immunotherapy, Phosphoantigen, TCR, PPARα

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Abbreviations used: CFSE – carboxyfluorescein succidimidyl ester; GSEA – *Gene Set Enrichment Analysis*; IL-2 – interleukin 2; PAg – phosphoantigen; PPAR – peroxisome proliferator-activated receptors

INTRODUCTION

The γδ T lymphocytes present hallmarks of both innate and adaptive immunity. In human and non-human primates, the peripheral repertoire of γδ TCR is mostly restricted to TCRVγ9Vδ2, presumably as a result of repeated exposure to few antigens called phosphoantigens (PAgs). Upon activation with PAgs, these cells release pro-inflammatory chemokines and cytokines and mediate cell cytotoxicity, prior to undergoing an intense proliferation leading to clonal expansion. In parallel, Vγ9Vδ2 T cells mature from naive through central memory to Th1 effector memory and cytotoxic TEMRa cells. TEMRa cells mediate potent natural killer-like cytotoxic responses against tumor cell targets [1, 2].

These features make Vγ9Vδ2 T lymphocytes versatile and attractive candidates for new cancer immunotherapy [3–6]. Synthetic analogues of natural PAgs, such as bromohydrin pyrophosphate (BrHPP), have been produced and used in preclinical and clinical studies. They show the potent and rapid response of the Vγ9Vδ2 T lymphocytes in vitro and in vivo. Their response indeed comprises an IL-2-dependent clonal expansion, but surprisingly does not show memory to prior exposure to PAg stimulus.

In a preclinical study of PAg safety in the cynomolgus, the first infusions of BrHPP with low dosages of IL-2 induced a transient amplification and cytokine response of circulating Vγ9Vδ2 T cells, while further infusions of BrHPP and IL-2 led to their progressive exhaustion [7]. In a TB vaccine setting, eight out of eight macaque monkeys that were injected three times with BrHPP in a subunit vaccine responded to the first injection but did not respond to subsequent boosters [8]. Several other unpublished studies from our and other laboratories have confirmed that non-human primates repeatedly injected with PAgs progressively lose their Vγ9Vδ2 T cell response to such synthetic stimuli. In humans, repeated injections of clinical-grade BrHPP (IPH1101, Innate Pharma) also progressively reduce the response of circulating Vγ9Vδ2 T cell [9].

PPARs are nuclear receptors and transcription factors for genes regulating cellular differentiation, development, metabolism and tumorigenesis. PPARα is mostly expressed in tissues with high rates of mitochondrial fatty acid oxidation. Upon binding of natural unsaturated fatty acids, leukotriene B4 or synthetic fibrates, PPARα promotes lipid mobilization and catabolism [10]. By contrast, in immune cells PPARα primarily inhibits inflammatory pathways by repressing c-jun and NF-κB [11, 12]. PPARα also inhibits IFNγ responses of human αβ T cells [13]. Whether PPARα also regulates human γδ T cell functions remains unclear.

PAg-selective desensitization has been identified as a pitfall in the development of Vγ9Vδ2 T cell-based cancer immunotherapy [14, 15]. As the first step to elucidate the molecular mechanisms underlying this in vivo desensitization, we analyzed the transcriptome of circulating Vγ9Vδ2 T cells from macaques injected with BrHPP and control subjects that did not receive the injection. The comparison of the transcriptome before injection versus that after three injections

outlined a gene expression pattern (GEP) reflecting activation of the PPARα pathway. As these results suggested that in vivo PAg desensitization of Vγ9Vδ2 T lymphocytes is associated with activation of PPARα, we tested whether activation of the PPARα pathway in human Vγ9Vδ2 T cells affected their response to PAgs. We found that PPARα agonist-treated Vγ9Vδ2 T cells are unresponsive to BrHPP, confirming that activation of PPARα could contribute to γδ cell desensitization.

MATERIALS AND METHODS

Primate cell samples

Animal care and handling was carried out at the Experimentation Animal-Station de Primatologie-UPS 846 CNRS (Rousset sur Arc, France) in accordance with standard guidelines, following a protocol submitted to and approved by the local ethical committee. Four age- and sex-matched cynomolgus macaques (2.5–4.4 kg) received three successive injections of BrHPP (doses of 50 mg/kg in 30 min intravenous infusions on days 0, 26 and 55) over 2 months, plus subcutaneous injection of rhIL-2 (10^6 IU, Proleukin Chiron) daily for 5 days after each BrHPP injection. Then, 4 ml of blood were collected prior to the first injection and seven days after each injection of BrHPP, i.e. on day 0 (I1), day 28 (I2) and day 55 (I3). TCRVγ9$^+$ cells were sorted (> 98%) as described previously [7, 8].

Microarray procedures

Total RNA from the specified macaque TCRVγ9$^+$ cell samples was isolated using TRIzol Reagent (Invitrogen Life Technologies). The quality of each RNA was assessed with Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA). Microarray analyses were performed using 1–3 μg total RNA, GeneChip Rhesus Macaque Genome Array and GeneChip Operating Software (Affymetrix). The raw transcriptome data were normalized in batch using the RMA software to yield 27755 annotated probe sets for further comparative analysis. The genes differentially expressed were defined using two-way Student's tests ($p < 0.01$), then analyzed for enrichment in functionally related genes among lists downloaded from the GSEA C1–C5 gene sets collection. Gene-selective enrichment analysis was performed as described previously [16] using the Autocompare freeware (https://sites.google.com/site/fredsoftwares/products/data-mining).

Vγ9Vδ2 T lymphocytes early activation and IFNγ production

PBMCs isolated from healthy donors (Etablissement Français du Sang, Toulouse, France) after Ficoll-Hypaque density centrifugation, were cultured at 2.5 M/ml in complete RPMI 1640 medium (Invitrogen), e.g. with L-glutamine, streptomycin, penicillin and sodium-pyruvate (Cambrex Biosciences) supplemented with 10% Fetal Clone I (HyClone/Thermo Fisher Scientific). LY-171883 (0-100 μM; Sigma-Aldrich) and 100 nM BrHPP (Innate Pharma) were added 24 h later. CD69 (Beckman Coulter) expression by Vγ9Vδ2 T lymphocytes was measured 48 h later via flow cytometry.

IFNγ expression was measured via flow cytometry with a staining in PBS 1% saponin with anti-TCRVγ9 (Biolegend, San Diego, CA) and anti-IFNγ (BD Biosciences, San Jose, CA) after 6 h of treatment with 10 µg/ml brefeldin A (Sigma-Aldrich) and fixation with PBS 2% PFA.

Vγ9Vδ2 T lymphocytes proliferation

PBMCs were labeled with 0.5 µM CFSE (Invitrogen Life Technologies) for 8 min at 37ºC, washed and cultured with or without LY-171883 (0–100 µM) and 10 or 100 U/ml rhIL-2 (Sanofi-Aventis). Cells were stimulated 24 h later with 100 nM BrHPP. After six days of culture, cells were stained for TCR Vγ9 and analyzed for CFSE dilution via flow cytometry.

RESULTS AND DISCUSSION

Repeated stimulation with PAg selectively desensitizes the Vγ9Vδ2 T cell lineage in humans and non-human primates [7, 8, 17–19]. To investigate this desensitization, we first analyzed the GEP of purified macaque Vγ9Vδ2 T cells from subjects injected with BrHPP. Four cynomolgus macaques received three intramuscular injections of BrHPP on day 0 (I1), day 28 (I2) and day 55 (I3), a regimen which abrogates their Vγ9Vδ2 T cell response to BrHPP [7, 8]. Each blood sample was collected prior to the first injection and seven days after each BrHPP injection. Fresh Vγ9Vδ2 T cells were then purified: their mRNA fraction was isolated, amplified and hybridized to Affymetryx Rhesus microarrays.

The mean of Vγ9Vδ2 T cell transcriptomes from the four macaques at the same time points are referred below to as resting, I1, I2 and I3. The I1, I2 and I3 transcriptomes were respectively compared to the resting one (Fig. 1A and B).

The I1 transcriptome encompassed 1013 genes differentially expressed relative to the resting cells (695 genes upregulated and 317 genes downregulated, $p < 0.05$). Of these 1013 genes, only 483 were fully annotated in macaques. Furthermore, among these 483 macaque I1 γδ response genes, 146 (30%) are orthologs of human Vγ9Vδ2 T response genes modulated one week after a single in vitro exposure to BrHPP [8].

The I2 transcriptome encompassed 959 genes differentially expressed relative to the resting cells (497 genes upregulated and 462 genes downregulated, $p < 0.05$). Only 198 genes (~20%) were common to the I1 and I2 transcriptomes.

The I3 transcriptome showed a still-distinct pattern of expression, since it comprised only 522 genes significantly modulated (285 upregulated and 237 downregulated, $p < 0.05$), ~80% of which were not in the I1 or I2 transcriptomes. Actually, only 37 genes (3% of the initial transcriptional response) were shared by the I1, I2 and I3 transcriptomes, confirming a global change in the Vγ9Vδ2 T cell gene expression response according to the number of injection cycles (Fig. 1B). Of the modulated 522 I3 genes, 130 were fully annotated in the macaque genome.

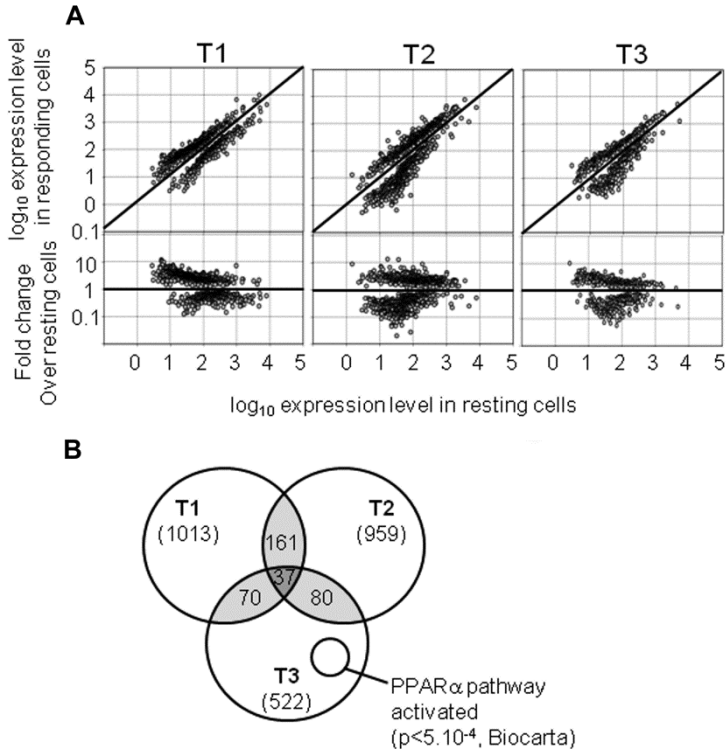

Fig. 1. Gene signature of macaque Vγ9Vδ2 T cells repeatedly injected with phosphoantigen unveils induction of the PPARα pathway. A – The mean of the gene expression level and fold change expression for the I1, I2 and I3 of BrHPP injections respectively compared to the resting one (four macaques). B – Shared genes differentially expressed compared to resting cells for the three injections I1, I2 and I3.

When analyzed with Autocompare software for significant enrichment of gene signatures, the I3 genes NPDC1, GPNMB, GNRH1, SFN (*p14-3-3*σ repressor), EML4, RB1, CDKN2C (*p18*), and PRKCA corresponded to negative regulation of the cell cycle pathway ($p = 3 \times 10^{-4}$). No selective enrichment in T cell anergy or lymphocyte exhaustion gene signatures was found for the I3 genes. Nevertheless, the only other significant gene signature of the I3 Vγ9Vδ2 T cell transcriptome was the PPARα pathway ($p = 5 \times 10^{-4}$, Biocarta) based on upregulation of the EP300, NCOR2, MED1, PRKC1, HSD17B4, PRKAR2B and RB1 genes, while RARA and PRKCA were downregulated. The above results showed a global decrease in transcriptional Vγ9Vδ2 T cell responses to successive in vivo stimulations by PAg and an activation of the PPARα pathway in macaque Vγ9Vδ2 T cells after a I3 exposure to stimulating PAg.

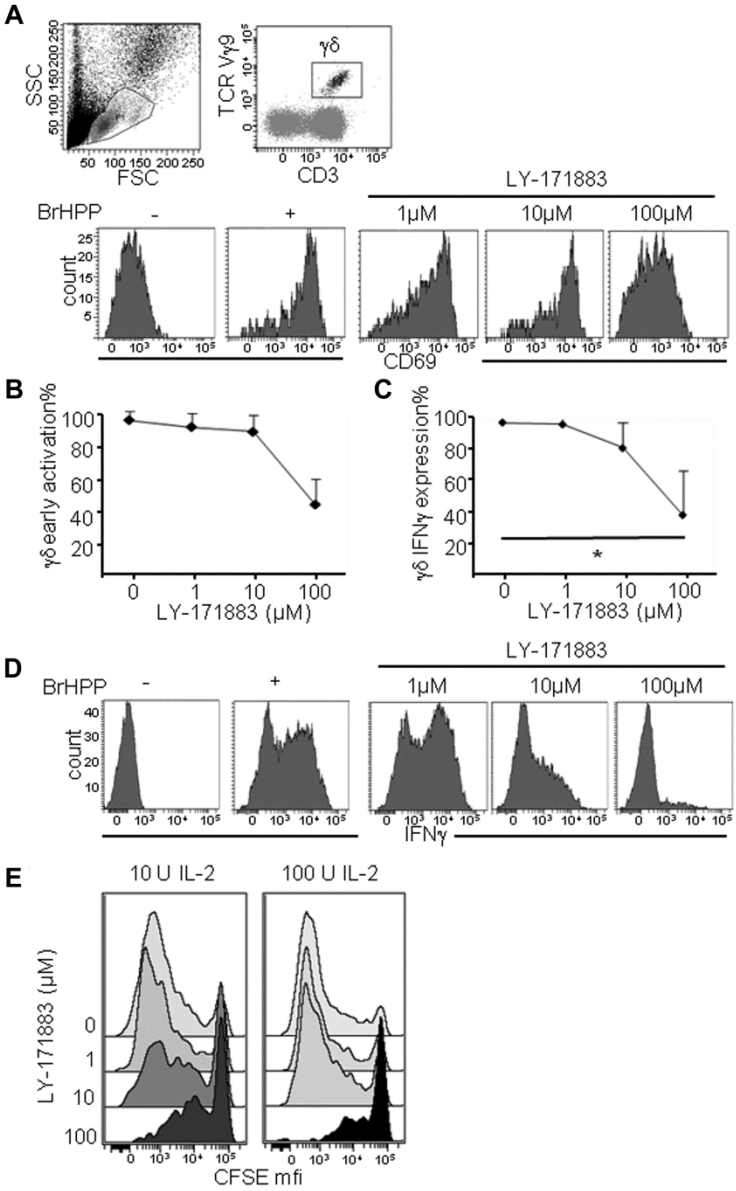

Fig. 2. PPARα agonist inhibits CD69 induction, IFNγ expression and proliferation of Vγ9Vδ2 T cells. A – Expression of CD69 by Vγ9Vδ2 T cells gated after 2 days of culture with or without BrHPP and LY-171883 (1, 10 or 100 μM). B – Mean of percentage of CD69 positive cells from 5 independent experiments. C – Expression of intracellular IFNγ by Vγ9Vδ2 T cells cultured with or without LY-171883 (1, 10 or 100 μM), mean from 5 independent experiments. D – Expression of intracellular IFNγ by Vγ9Vδ2 T cells, one of the 5 experiments. E – Proliferation of Vγ9Vδ2 T cells visualized by CFSE dilution in Vγ9Vδ2 T cells after 6 days of culture with or without LY-171883 (1, 10 or 100 μM) and with 10 U or 100 U of rhIL-2. *$p < 0.05$.

Among the natural ligands of PPARα, various eicosanoids, such as leucotriene B4, are generated during inflammatory responses [20, 21]. In addition to these effects of ligand-bound PPARα on NF-κB and c-jun, it has also been reported that ligand-free PPARα may inhibit the phosphorylation of p38 [22], a kinase that also promotes IFNγ transcription downstream of TCR stimulation [23].

Thus, we analyzed the in vitro response of human Vγ9Vδ2 T cells stimulated with a PPARα agonist, LY171883. Early activation was measured by the surface expression of CD69 by Vγ9Vδ2 T cells after 2 days of fresh PBMC culture with BrHPP. Among the living lymphocytes, gated TCR $V\gamma9^+$ cells expressed CD69 if activated by BrHPP (Fig. 2A). Preincubation with LY-171883 led to a significant dose-dependent decrease in this expression (Fig. 2A and B).

Then, we analyzed the effect of PPARα agonists on the intracellular expression of IFNγ by Vγ9Vδ2 T cells. BrHPP-activation with a blocking secretion led to an expression of intracellular IFNγ, which was inhibited by the preincubation with LY-171883 in a dose-dependent manner (Fig. 2C and D). Finally, we showed that LY-171883 induced an inhibition of the Vγ9Vδ2 T cell proliferation, evaluated by the CFSE dilution, when cultured for six days with a weak or high dose of rhIL-2 (Fig. 2E). Thus, in vitro activation of the PPARα pathway leads to the inhibition of BrHPP-induced activation and proliferation of human Vγ9Vδ2 T cells.

We know from in vivo studies that Vγ9Vδ2 T cells from macaques respond to a first BrHPP injection by amplification in the blood, but they become non-responsive following several BrHPP injections [7, 8]. Our finding, which highlights the activation of the PPARα pathway in Vγ9Vδ2 T cells from primates injected three times with BrHPP, can be thus associated with the non-response of these cells under the same in vivo experimental conditions. In humans, repeated exposure to BrHPP also leads to rapid exhaustion of the Vγ9Vδ2 T cell response [9]. Moreover, the similarity of human and non-human primate Vγ9Vδ2 T cells allows the correlation of the two discoveries. With this study, we highlighted the involvement of the PPARα pathway in the antigen-selective desensitization of human Vγ9Vδ2 T cells.

The use of inhibitors of the PPARα pathway or a method preventing its activation could enhance the advantage of using cancer immunotherapy based on Vγ9Vδ2 T cells. Indeed, the repression of PPARα expression can be induced by TNFα [24] or by a group of N-(methylsulfonyl)amides derived from PPARα agonist carboxylic acids with an antagonist behavior on PPARα [25]. Some kinases or their activators can also lead to specific inhibition of PPARα transcriptional activity, such as the MEK1 or AMP-activated protein kinase activators 5-aminoimidazole-4-carboxamide riboside [26, 27]. Furthermore, it was shown very recently that PPARα inhibition could improve the therapeutic efficacy of glucocorticoids in aggressive forms of chronic lymphocytic leukemia [28]. Thus, PPARα inhibition could be used in Vγ9Vδ2 T cell-based cancer immunotherapy.

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Conflict of interest. The authors declare no relevant conflict of interest.

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