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Deciphering the role of protein kinase A in the control of FoxP3 expression in regulatory T cells in health and autoimmunity

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The molecular mechanisms that govern differential T cell development from CD4⁺CD25⁻conventional T (Tconv) into CD4⁺CD25⁺ forkhead-box-P3⁺ (FoxP3⁺) inducible regulatory T (iTreg) cells remain unclear. Herein, we investigated the relative contribution of protein kinase A (PKA) in this process. Mechanistically, we found that PKA controlled the efficiency of human iTreg cell generation through the expression of different FoxP3 splicing variants containing or not the exon 2. We found that transient PKA inhibition reduced the recruitment of cAMP-responsive element-binding protein (CREB) on regulatory regions of the FoxP3 gene, a condition that is associated with an impaired acquisition of their suppressive capacity in vitro. To corroborate our findings in a human model of autoimmunity, we measured CREB phosphorylation and FoxP3 levels in iTreg cells from treatment-naïve relapsing–remitting (RR)-multiple sclerosis (MS) subjects. Interestingly, both phospho-CREB and FoxP3 induction directly correlated and were significantly reduced in RR-MS patients, suggesting a previously unknown mechanism involved in the induction and function of human iTreg cells.

Regulatory T (Treg) cells are a specialized subset of T lymphocytes that arise from thymocytes during intrathymic maturation, with a key role in maintaining immunological self-tolerance, due to their ability to restrain inflammatory responses[1–3]. To date, several distinct lineages of T cells with regulatory properties have been identified[4–6]. Among these, the most extensively studied population of Treg cells comprises CD4⁺ T cells characterized by their high expression of the α-subunit of the interleukin 2 (IL-2) receptor and the presence of the master transcription factor forkhead box P3 (FoxP3)[7,8]. Although the majority of Treg cells derive from the thymus (thymic Treg, tTreg cells), they can differentiate also in the periphery (pTreg) from conventional T (Tconv) cells, upon sub-immunogenic antigen presentation[9]. Furthermore, induced Treg (iTreg) cells can be obtained in vitro by T cell receptor (TCR) stimulation of Tconv cells in the presence or absence of exogenous cytokines[10–13]. In the process of Treg cell induction, IL-2 and TGF-β signalling have been shown to support FoxP3 expression also promoting the proliferative capability of Treg cells[14–19]. Nonetheless, recent evidence has unveiled the crucial role of TCR engagement strength in the process of iTreg cell generation; indeed it has been shown that FoxP3 expression can be stably induced in Tconv cells through suboptimal in vitro TCR stimulation without the addition of exogenous cytokines[10–12], consequently leading to the generation of fully suppressive iTreg cells[10–12,20].

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Compelling evidence suggests that the transcription factor FoxP3 not only serves as a Treg lineage-specific molecule but is also indispensable for their proper differentiation, stability and the acquisition of suppressive functions[21–23]. The human FoxP3 gene consists of 11 coding exons and includes a promoter region along with three conserved non-coding sequences (CNS) within the locus: CNS1, CNS2, and CNS3[24]. These CNS elements are crucial functional enhancers for both the induction and stabilization of FoxP3 expression. CNS1 encompasses the TGF-β response element, which plays a role in the development of extrathymic Treg cells and the promotion of mucosal immune tolerance. CNS2 is responsible for maintaining FoxP3 stability in response to TCR stimulation, while CNS3 appears to influence the threshold of TCR stimuli required for FoxP3 expression[25].

To date, several FoxP3 splicing variants have been described in human Treg cells[26] and among them, those containing the sequence corresponding to exon 2 (FoxP3-E2) are pivotal to confer their suppressive capability and stability[10,12,27]. Indeed, the absence of FoxP3-E2 is associated with impaired Treg cell activity, uncontrolled effector T cell activation leading to the development of autoimmune diseases in both mice and humans[10,12,27,28].

In this context, it has amply documented that the number and function of circulating Treg cells, the capacity to generate iTreg cells, as well as the FoxP3-E2-related suppressive activity of iTreg cells are all impaired in human autoimmune disorders, including relapsing–remitting multiple sclerosis (RR-MS)[10,21,29,30], an immune-mediated disease of the central nervous system[31].

However, the molecular pathways involved in the control of human FoxP3 gene expression remain incompletely understood.

FoxP3 transcription is known to be controlled by the recruitment of several transcription factors into FoxP3 promoter or to the CNS regions[10]. Among all these factors, the cyclic adenosine 3′,5′-monophosphate (cAMP) responsive element-binding protein (CREB) has attracted increasing interest for its possible role in Treg cell differentiation and function[32–34]. Indeed, while it has been shown that CREB is crucial to driving FoxP3 transcription[32,33], another research group revealed that it is dispensable for FoxP3 expression and negatively regulates the survival of iTreg cells in mouse models of autoimmunity[34]. However, the role of the cAMP/PKA/CREB pathway in the control of FoxP3 expression to generate fully functional iTreg cells has not been investigated in human settings.

Since the strength of TCR signalling during T cell activation promoted the engagement of several signalling pathways including those associated with cAMP/PKA/CREB[35,36], herein, we explored the involvement of PKA in the regulation of FoxP3 splicing variants, in the immunometabolic asset and in the suppressive function of human iTreg cells. Further, we investigated in depth the relative contribution of CREB in FoxP3 expression during iTreg cell differentiation from Tconv cells obtained from human RR-MS subjects.

Results

Low TCR engagement activates PKA favouring CREB binding on FoxP3 gene in human Tconv cells

To assess whether PKA is involved in the early events induced by low TCR stimulation, freshly isolated human $CD4^+CD25^-$ Tconv cells were pre-treated for 1 h with the myristoylated PKA specific inhibitor fragment 14–22 (mPKAI)[37,38] or not (mPKAI-Tconv and CTR-Tconv, respectively) and then stimulated or not with anti-CD3/CD28 microbeads (see the experimental procedure in Fig. S1). We found that transient inhibition of PKA reduced both catalytic activity of PKA and CREB phosphorylation in TCR-stimulated mPKAI-Tconv cells (Fig. 1a,b and Fig. S2). Further, we assessed the involvement of PKA in the regulation of FoxP3 gene expression during Tconv cell differentiation towards iTreg cells. To this end, we performed a chromatin immunoprecipitation (ChIP) assay to evaluate CREB binding on FoxP3 regulatory elements: promoter, non-coding conserved sequence (CNS)2 and CNS3 regions, respectively. ChIP assay revealed that upon Tconv cell stimulation, CREB was rapidly recruited on the regulatory elements of FoxP3, particularly on CNS2 region (Fig. 1c). Of note, transient PKA inhibition was significantly associated with reduced CREB binding on the regulatory elements of FoxP3 (Fig. 1c). Together, these findings suggest that during the early steps of iTreg cell induction, TCR-mediated stimulation of Tconv cells results in PKA activation, which in turn promotes CREB recruitment on FoxP3 regulatory elements[39].

PKA regulates FoxP3 expression during Tconv differentiation towards iTreg cells

To evaluate whether the cAMP/PKA/CREB axis participated in the regulation of FoxP3 gene expression, we measured, by quantitative real time PCR, total FoxP3 splicing forms (FoxP3-All) and its variants containing the Exon2 (FoxP3-E2) during iTreg cell induction, upon suboptimal TCR stimulation, to sustain a more effective induction of FoxP3, as previously demonstrated[11,40,41]. To this aim, mPKAI-Tconv and CTR-Tconv cells were stimulated via TCR for 24 and 36 h, respectively. While no significant difference was observed in terms of FoxP3-All mRNA levels in both conditions, a significant reduction of FoxP3-E2 mRNA levels at 24 h upon TCR stimulation in mPKAI-Tconv cells was found (Fig. 2a), suggesting the requirement of PKA for the selective FoxP3-E2 generation. We also evaluated at protein level the expression of FoxP3-All and FoxP3-E2 by Western Blot. Specifically, mPKAI-Tconv and CTR-Tconv cells were stimulated via TCR for 36 h, to measure the splicing forms of FoxP3 protein, using specific monoclonal antibodies (mAbs). Our results revealed that the induction of both FoxP3-All and FoxP3-E2 proteins was affected in mPKAI-Tconv cells (Fig. 2b and Fig. S3), differently from what has been observed at mRNA level.

Since IL-2R/STAT-5 and mechanistic target of rapamycin (mTOR) signalling pathways are also involved in iTreg cell differentiation and function[18,42], we analysed phosphorylation of STAT-5 and S6 proteins (a mTOR downstream target) in the above mentioned experimental conditions. Biochemical analysis of these proteins revealed that PKA inhibition did not influence their phosphorylation (Fig. 2c and Fig. S3), suggesting that PKA selectively controls the expression of FoxP3 both at mRNA and protein level during iTreg cell differentiation.

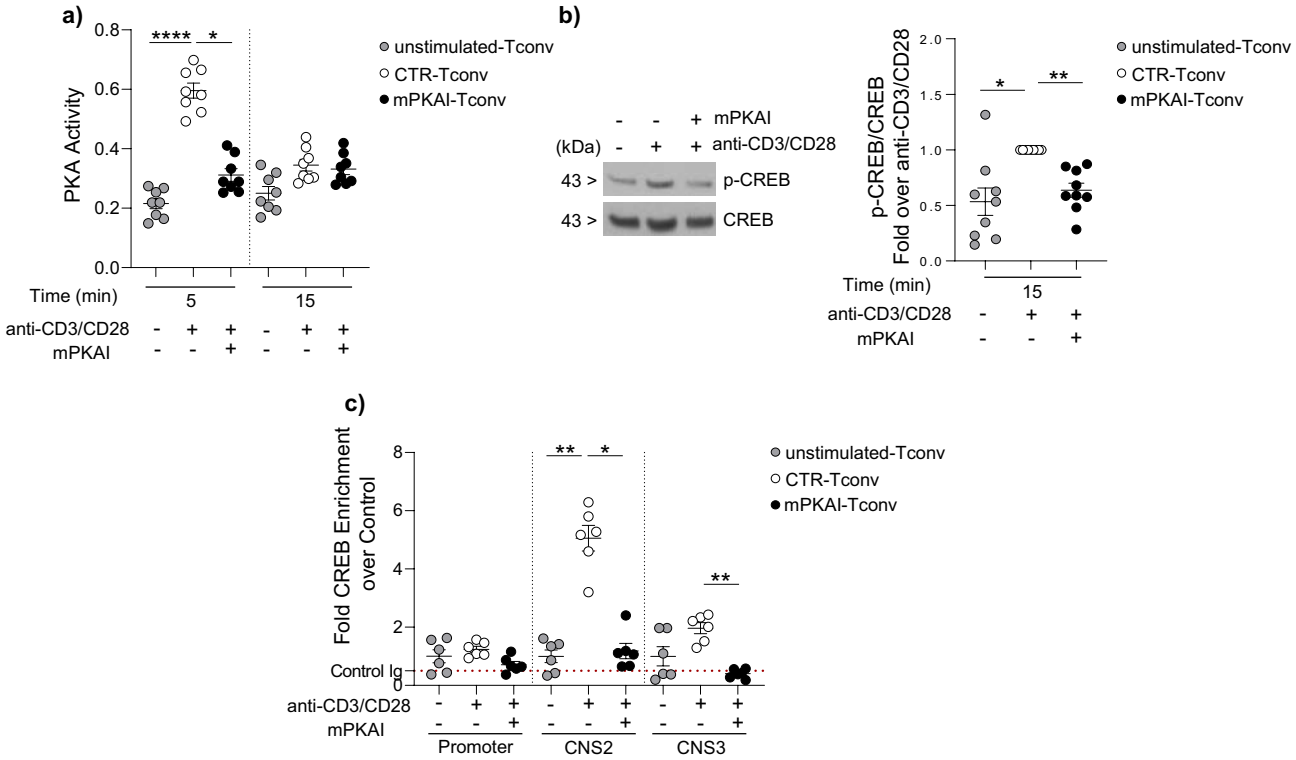

Figure 1. Low TCR stimulation activates PKA in human Tconv cells. (**a**) Scatter plots showing PKA activity in human mPKAI-Tconv and CTR-Tconv cells TCR-stimulated for 5 and 15 min. Data are shown from four independent experiments in duplicates (n = 8). (**b**) Left, immunoblot showing phosphorylated (p) and total CREB in human mPKAI-Tconv and CTR-Tconv cells TCR-stimulated or not for 15 min. Right, relative densitometric quantitation of p-CREB in the aforementioned experimental conditions. Data are shown from nine independent experiments (n = 9); uncropped blots are presented in Supplementary Fig. S2. (**c**) ChIP assay for CREB on FoxP3 promoter, CNS2 and CNS3 regions of mPKAI-Tconv and CTR-Tconv cells TCR-stimulated for 10 min. The horizontal line (bracket ± SEM) indicates the percent of input from a control ChIP (Ab:non-immune serum). Data are shown from three independent experiments in duplicates (n = 6). Independet experiments refer to different individuals.

Impact of PKA on the immunometabolic asset and phenotype of Tconv cells during their differentiation towards iTreg cells and on their suppressive activity

Next, we explored whether cAMP/PKA/CREB pathway influences glycolytic immunometabolism, phenotype and suppressive function of iTreg cells. It has been previously shown that glycolytic pathway was necessary for the induction of human iTreg cells in vitro[43], and in particular for the expression of FoxP3-E2 splicing variants[10,12]. To this aim, we measured extracellular acidification rate (ECAR), an indicator of glycolysis, in mPKAI-Tconv and CTR-Tconv, during iTreg cell generation. Seahorse analysis revealed that transient inhibition of PKA significantly affected glucose metabolism, as testified by reduced glycolysis, maximal glycolysis and glycolytic capacity (Fig. 3a, left and right panels), suggesting that PKA acts as a positive determinant for glycolytic metabolism during Tconv differentiation towards iTreg cells.

Furthermore, we assessed whether impaired glycolysis associated with altered expression of the main Treg cell-markers. To this aim, mPKAI-Tconv and CTR-Tconv cells were stimulated for 36 h via low TCR engagement to generate iTreg cells. Flow cytometric analysis revealed that CD25 and FoxP3-All molecules were not affected by PKA inhibition, while a selective reduction of the FoxP3-E2 splicing forms was observed (Fig. 3b). Moreover, we found that mPKAI pre-treatment significantly reduced the expression of the suppressive markers CTLA-4, PD1 and GITR only in FoxP3-E2$^+$ cells (Fig. 3c). Further, transient inhibition of PKA did not affect the expression level of the activation markers such as CD62L, CD69, CD71 and ICOS as well as the proliferation marker Ki-67 (Fig. S4).

Finally, we examined whether reduced FoxP3-E2 expression in iTreg cells, generated from mPKAI-Tconv, associated with an impaired suppressive function in vitro. Thus, iTreg cells were flow sorted on the basis of CD25 surface expression (see gating strategy for iTreg cell isolation in Fig. S5) and tested for their ability to suppress proliferation of TCR-activated CD4$^+$ T cells. We found that flow sorted iTreg derived from mPKAI-Tconv cells displayed a significantly lower capability to suppress the proliferation of TCR-stimulated CD4$^+$ T cells at different cell-to-cell ratios (Fig. 3d). In all, these findings indicate that PKA represents a molecular determinant important for a proper in vitro generation of fully active human iTreg from Tconv cells.

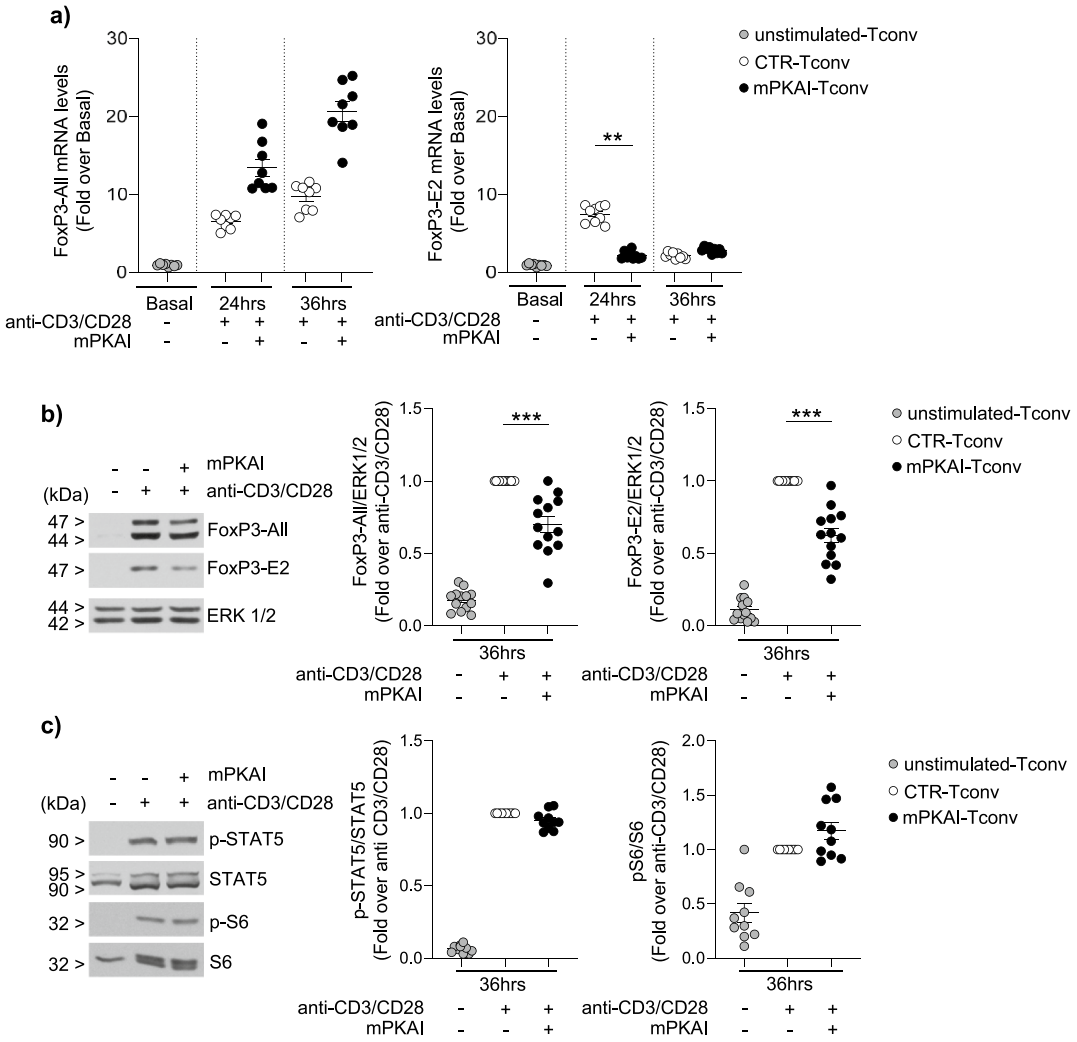

Figure 2. Inhibition of PKA impairs FoxP3 induction. (**a**) Scatter plots showing FoxP3-All (left) and FoxP3-E2 (right) mRNA levels in mPKAI-Tconv and CTR-Tconv cells TCR-stimulated or not for 24 or 36 h, respectively. Data are shown from four independent experiments in duplicates (n = 8). (**b**) Left, immunoblot analysis of FoxP3-All, FoxP3-E2 and ERK1/2 in mPKAI-Tconv and CTR-Tconv cells TCR-stimulated for 36 h. Right, relative densitometric quantitation of FoxP3-All or FoxP3-E2 normalized on ERK 1/2 in the aforementioned experimental conditions. Data are shown from thirteen independent experiments (n = 13). (**c**) Left, immunoblot analysis of p-STAT5 and p-S6 in human Tconv cells, as described in B. Right, relative densitometric analysis of p-STAT5 and p-S6 normalized on their total proteins, respectively. Data are shown from five independent experiments in duplicates (n = 10). Independet experiments refer to different individuals. All the uncropped blots are presented in Supplementary Fig. S3.

CREB phosphorylation influences FoxP3 induction in Tconv cells from autoimmune RR-MS patients

To investigate the role of cAMP/PKA/CREB axis in an autoimmune disorder such as RR-MS in which number and function of Treg cells are reduced, we measured CREB phosphorylation in Tconv cells during their differentiation towards iTreg cells, in healthy (n = 5) and naïve-to-treatment RR-MS patients (n = 5). Western blot analysis revealed that freshly isolated (basal) and shortly TCR-stimulated Tconv cells from RR-MS subjects displayed significantly lower levels of intracellular p-CREB compared to healthy subjects (Fig. 4a and Fig. S6). In parallel, in the same RR-MS subjects, we found a significant impairment of FoxP3-E2 induction in Tconv cells stimulated for 36 h (Fig. 4b and Fig. S6). Also, we correlated basal p-CREB in *ex-vivo* Tconv cells with FoxP3 induction upon TCR stimulation in RR-MS and healthy subjects. A positive correlation between basal p-CREB and FoxP3-E2 induction levels at 24 and 36 h post TCR stimulation was observed in the healthy controls; however, in RR-MS patients this correlation was present only at 36 h (Fig. 4c). No correlations between basal p-CREB and FoxP3-All induction were observed in both RR-MS and healthy individuals (data not shown). In all, these findings indicate that CREB phosphorylation is impaired in Tconv cells from RR-MS patients and associated with reduced induction of FoxP3-E2 protein.

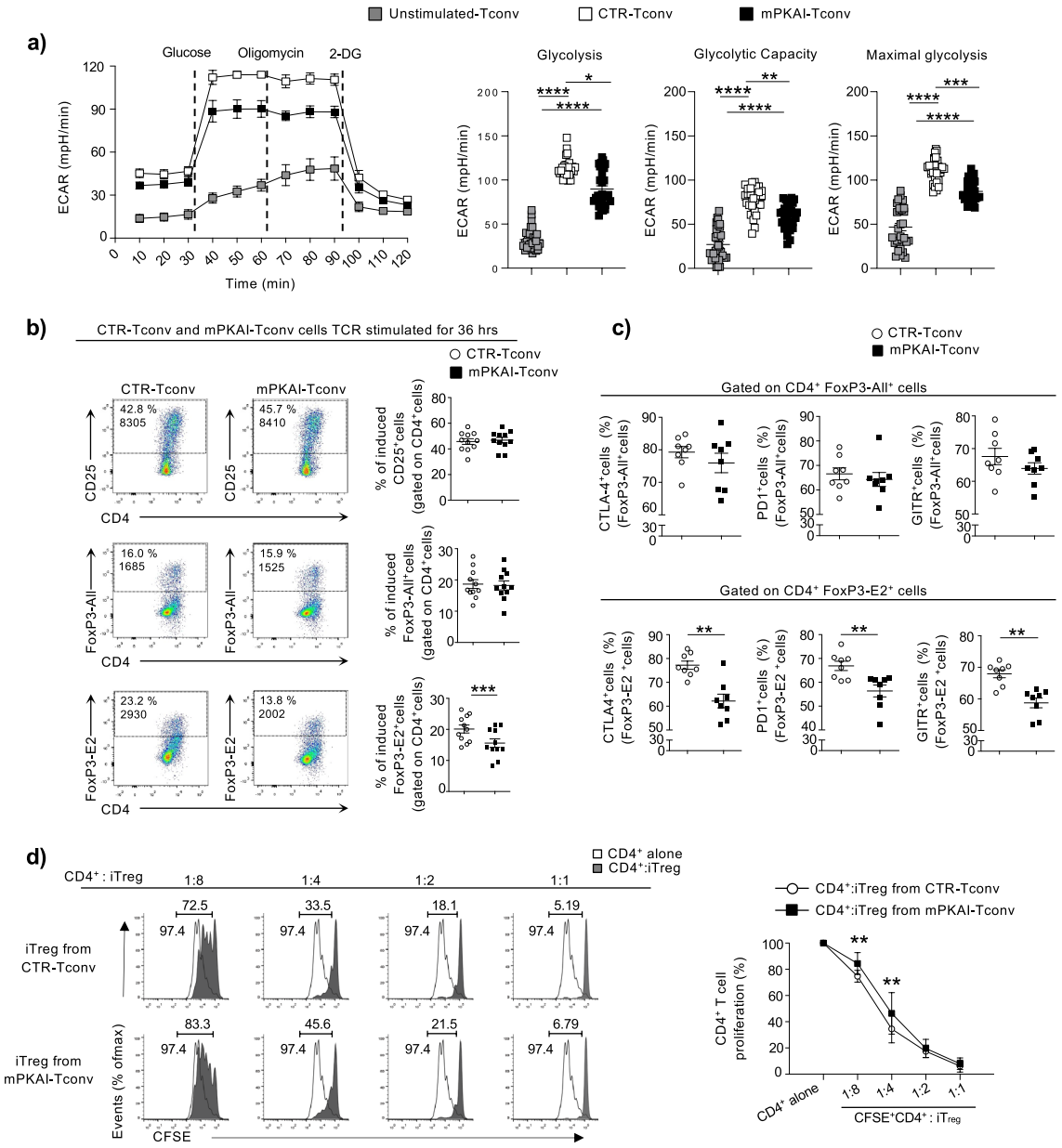

Figure 3. PKA inhibition affects the immunometabolic asset and phenotype of Tconv cells during their diffentiation towards iTreg cells and their suppressive function (**a**) Left, kinetic profile of ECAR in human mPKAI-Tconv and CTR-Tconv cells TCR-stimulated or not for 12 h. ECAR was measured in real time, under basal conditions and in response to glucose, oligomycin, and 2-DG. Data are shown from three independent experiments at least in technical duplicates (n = 10). Right, parameters of the glycolytic pathway were calculated from the ECAR profile of Tconv cells in the above-mentioned conditions. Data are expressed as mean ± SEM of three different measurements, each of them in ten replicates (n = 30). (**b**) Representative dot plots (left) and cumulative data (right) of CD25, FoxP3-All and FoxP3-E2 in mPKAI-Tconv and CTR-Tconv cells. Data are shown from eleven independent experiments (n = 11). (**c**) Scatter plots showing the expression of CTLA-4, PD-1 and GITR gated on $CD4^+FoxP3\text{-}All^+$ (top) or $CD4^+FoxP3\text{-}E2^+$ (bottom) in mPKAI-Tconv and CTR-Tconv cells. Data are shown as mean ± SEM from four independent experiments in duplicates (n = 8). (**d**) Left, flow cytometry histograms showing proliferation of $CFSE^+CD4^+$ T cells TCR-stimulated for 96 h in vitro and cultured alone (empty curves) or in the presence of various numbers of flow-sorted iTreg from mPKAI-Tconv and CTR-Tconv cells. Numbers in plots indicate the percent of CSFE dilution in $CD4^+$ T cells cultured alone (top left) and co-cultured with iTreg cells (above bracketed lines), as indicated. Right, cumulative data of $CD4^+$ T cell proliferation in the above conditions. Data are shown from four independent experiments in duplicates (n = 8). Independet experiments refer to different individuals.

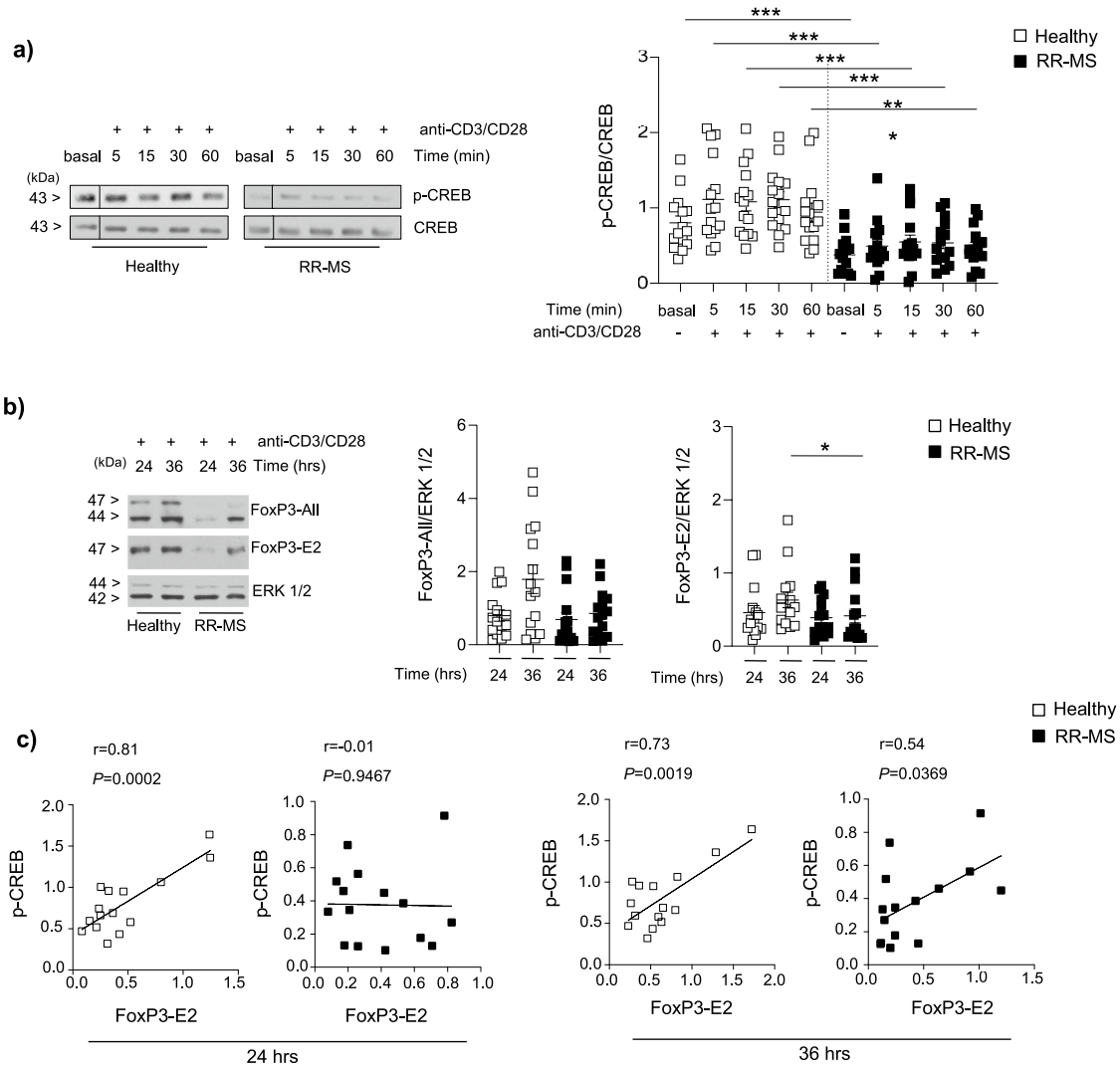

Figure 4. Reduced CREB phosphorylation associates with low FoxP3 expression in Tconv cells during their differentiation towards iTreg cells from autoimmune RR-MS subjects. (**a**) Left, immunoblot analysis of total and p-CREB in human Tconv cells from healthy and RR-MS subjects TCR-stimulated at different time points. Right, relative densitometric quantitation of p-CREB normalized on total CREB in the aforementioned experimental conditions. (**b**) Left, immunoblot analysis of FoxP3-All, FoxP3-E2 and ERK 1/2, in TCR-stimulated Tconv cells from healthy (n = 5) and RR-MS (n = 5) subjects. Right, relative densitometric quantitation of FoxP3-All and FoxP3-E2 normalized on ERK 1/2 in above conditions. Data are shown as mean ± SEM from five independent experiments (5 healthy and 5 RR-MS subjects) in triplicates (n = 15). All the uncropped blots are presented in Supplementary Fig. S6. (**c**) Statistical correlation between expression levels of basal p-CREB from ex-vivo Tconv cells with FoxP3-E2 in healthy (n = 5) and RR-MS (n = 5) subjects, in the above mentioned conditions. All the uncropped blots are presented in Supplementary Fig. S6.

Discussion

Herein we investigated the role of cAMP/PKA/CREB pathway in the induction of FoxP3 during the generation of fully functional human iTreg cells. We found a positive role of PKA/CREB axis in regulating iTreg cell fate, as transient inhibition of PKA during iTreg cell differentiation affected glycolysis, expression of FoxP3 splicing forms, Treg-cell markers, and their suppressive capability in vitro.

Compelling evidence has revealed that PKA/CREB pathway is important in T cell immunobiology[39], specifically in the differentiation and function of Treg cells. In particular, it has been reported a Treg-specific region in the FoxP3 locus, which contains a binding site for several transcriptional factors, including CREB[44]. Nevertheless, the role exerted by CREB on FoxP3 expression has been deeply investigated only in mouse models with contrasting results[33,34]. Specifically, published findings by Kim and Leonard revealed that CREB positively controls FoxP3 transcription through direct interaction with CNS2 region in a demethylated state[33]. On the other hand, Wang et al.[34] have shown that CREB negatively regulates Treg cell survival and by using a CREB-knockout mouse model, have demonstrated that its deficiency increased Treg cell frequency protecting the mice from colitis.

These conflicting results need further investigation to clearly understand whether CREB may play a different role in the regulation and expression of FoxP3 and whether these findings can be also translated to human setting, which still lacks specific experimental evidence.

In this context, according to the results previously published by Kim and co-worker, our findings revealed that CREB was rapidly recruited on regulatory regions (CNS2 and CNS3) of the *FoxP3* gene in human Tconv cells upon short TCR stimulation. In particular, we found that transient PKA inhibition, through mPKAI pretreatment, reduced the recruitment of CREB on regulatory regions of the FoxP3 gene. Although other experimental studies have used H89 as PKA inhibitor, in our experimental setting we choose to utilize mPKAI, as it selectively impairs CREB phosphorylation, unlike H89, which has been shown to affect also additional pathways involved in Treg cell generation and functions, such as S6 phosphorylation[37,38].

Together, these findings suggest that during the first phases of human iTreg cell induction, CREB rapidly binds FoxP3 locus into the nucleus, where it cooperates to the formation of a Treg-specific multifactorial transcriptional complex[45]. In particular, in this study, we characterized the molecular mechanism controlling FoxP3 induction and found that FoxP3-E2 expression is also supported by cAMP/PKA/CREB pathway.

It is well known that transcription of FoxP3 gene leads to the generation of several isoforms and our published study revealed that those containing the exon 2 variants are important in conferring suppressive capability to human iTreg cells[10,12]. Accordingly, a recent paper by Du et al.[28], showed that subjects expressing only the shorter isoform produced by transcripts lacking exon 2 (FoxP3 ΔE2) failed to maintain self-tolerance and developed immunodeficiency, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome. In this context, it is now clear that the expression of different FoxP3 isoforms and Treg cell functions are closely associated with immunometabolic programs[46,47].

Here we found a differential regulation of FoxP3-All and FoxP3-E2 mRNA expression triggered by PKA inhibition; indeed, the reduction in PKA activity may induce an increase in the transcription of some FoxP3 splicing variants in spite of others, whose expression is inhibited (as in the case of FoxP3-E2), thus unbalancing the relative ratios between the different isoforms.

Moreover, despite PKA inhibition in Tconv cells affected both FoxP3-All and FoxP3-E2 protein levels, we found that this treatment significantly reduced only FoxP3-E2 mRNA expression at 24 h, with no significant effects on FoxP3-All mRNA, suggesting that PKA may differently regulate transcriptional and translational mechanisms controlling FoxP3 expression.

In this context, it has been previously described that PKA can differentially modulate both the translation of different mRNAs and their stability[48–50]; therefore, PKA inhibition could induce the accumulation of mRNA encoding for FoxP3, which, however, is not associated with an increase in its protein level, as this treatment could possibly act by reducing the half-life of FoxP3[51,52].

Cellular metabolism has been shown to be necessary to control the fate of immune cells. During differentiation and activation, immune cells can change their metabolic programs to meet the increased energy demand[53]. In this context, environmental metabolites and intracellular metabolic intermediates have been recently shown to regulate FoxP3 expression in Treg cells. For instance, glycolysis plays a crucial role in modulating the generation of iTreg cells and expression of FoxP3-E2 splicing forms[10,12]. Here, we found that inhibition of the PKA was able to impair glycolysis and this was associated with an altered induction of FoxP3-E2 and iTreg-cell function. Thus, based on these findings it is possible to hypothesize that PKA represents a molecular determinant linking glycolytic pathway to FoxP3-E2 expression, necessary for functional activity of iTreg cells.

In our previous work, we observed that T lymphocytes from autoimmune RR-MS patients displayed a reduced engagement of glycolysis associated with an impairment of FoxP3-E2 induction[10,54]. In line with these results, here we showed that Tconv cells from RR-MS subjects displayed reduced levels of p-CREB, which correlated with low induction of FoxP3 during iTreg cell differentiation. Thus, we revealed that impaired CREB phosphorylation in RR-MS subjects influences a proper iTreg cell generation, through modulation of FoxP3 expression.

In summary, our data unveil that transient perturbation of cAMP/PKA/CREB pathway impinges on human iTreg cell fate and differentiation, as testified by their reduced FoxP3-E2 expression and suppressive capacity. These data suggest that exploring PKA/CREB axis in Tconv cells from subjects with immune disorders may open the way to the identification of a novel deranged molecular pathway leading to the loss of immunological self-tolerance in autoimmunity.

Methods

Study participants

The Institutional Review Board of the Università degli Studi di Napoli "Federico II" approved the study, and peripheral blood was obtained from healthy control and RR-MS subjects after they signed a written informed consent. Healthy donors were matched for age, body mass index and sex with the RR-MS subjects and had no history of inflammatory, endocrine or autoimmune diseases. All the experiments were performed in accordance with Helsinki Declaration.

Tconv cell purification and in vitro iTreg cell generation

Human Tconv cells were purified from peripheral blood mononuclear cells (PBMCs) by negative selection with a human $CD4^+CD25^+$ Regulatory T Cell Isolation kit (MiltenyiBiotec) (cell purity ≥ 98%). Cells (1×10^6 cells/well) were pre-incubated (37 °C, 5% CO_2 for 1 h) in RPMI 1640 (Gibco) medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies), with or without 10 µM of mPKAI (Sigma Aldrich; P9115). After 1 h, cells were washed and activated in the presence or not of anti-CD3/CD28 Dynabeads (Invitrogen), at a density of 0.2 beads/cell for early stimulation (5, 15, 30, 60 min), and at a density of 0.1 bead/cell for 36 h to generate iTreg cells.

PKA activity assay
PKA activity in Tconv cells was detected by a standard sandwich ELISA, following the manufacturer's instructions (PKA Kinase Activity Assay Kit; Abcam). Results were normalized on the total protein levels.

Western blotting analyses
Cells were lysed in RIPA buffer (Sigma-Aldrich) plus SIGMAFAST Protease Inhibitor (Sigma-Aldrich), and Sigma Phosphatase Inhibitor (Sigma-Aldrich), as previously described[10]. Nitrocellulose filter membranes were incubated with the following antibodies: pCREB (Ser133) (87G3; Cat: 9198), CREB (48H2; Cat: 9197), pSTAT5 (Tyr694; Cat: 9351), STAT5 (D2O6Y Cat: 94205), pS6 (Ser240/244; Cat: 2215), S6 (5G10; Cat: 2217) (all from Cell Signaling Technology), FoxP3-All (PCH101; Cat: 14-4776-82), FoxP3-E2 (150D/E4; Cat: 14-4774-82) (all from eBioscience) and ERK1/2 (C-9: Cat: sc-514302) (Santa Cruz Biotechnology). Densitometric analysis was performed using ImageJ Software (NIH).

RNA extraction, q-PCR and qRT-PCR
Total RNA was extracted as previously described[55,56]. Quantitative PCR (qPCR) and qRT-PCR were performed three times in triplicate on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with TaqMan gene expression assay (Taqman® Universal Master Mix II, Applied Biosystems), using primers for *FoxP3* (Hs01092118_g1, Applied Biosystems) and, as internal standard control the 18S gene (Hs03928990_g1, Applied Biosystems).

ChIP assay
Cells were treated as indicated in the legends of the figures and were processed as described[56]. Pre-cleared chromatin was divided in aliquots and incubated at 4 °C for 16 h with 5 µl of anti-CREB (clone 48H2, Cell Signaling Technology) or non-immune IgG (normal rabbit IgG, Santa Cruz Biotechnology Inc.). Immunoprecipitated DNA was recovered and subjected to qPCR using the specific primers (Table S1), at 250 nM final concentrations.

Seahorse immunometabolic analyses
Metabolic profile (ECAR) processed as previously described[10], was evaluated in TCR-activated or not Tconv cells, using an XFe-96 Extracellular Flux Analyzer (Seahorse Bioscience).

Flow cytometry analyses
For the simultaneous evaluation of surface and intracellular molecules, human iTreg cells were stained with the following antibodies: FITC anti-CD4 (Clone RPA-T4, Cat: 561842) (BD Pharmingen), PE-Cy7 anti-CD25 (Clone M-A251, Cat: 560920) (cBD Pharmingen), APC anti-CD152/CTLA-4 (Clone BNI3, Cat: 555855) (BD Pharmingen), PE-Cy5 anti-GITR (Clone REA1007, Cat: 130-116-842) (Miltenyi Biotec), BV421 anti-CD279/PD-1 (Clone EH12.1, Cat: 562516) (BD Horizon), PE anti-FoxP3-All (Clone PCH101, Cat: 560046) (eBioscience) and PE anti-FoxP3-E2 (Clone 150D/E4, Cat: 12-4774-42) (BD Pharmingen). Staining for intracellular factors was performed using fixation and a permeabilization FoxP3 buffer kit (BD Pharmingen), according to the manufacturer's instructions. Samples were acquired using a FACSCanto II (BD Bioscience) and cytofluorimetric analyses were performed using FlowJo Software (FlowJo, LLC).

T cell proliferation assay
For the assessment of iTreg cell suppression function, 36 h TCR-stimulated Tconv cells were flow-sorted with a BD FACSJazz (Becton–Dickinson) on the basis of CD25 expression (see the gating strategy in the Fig. S3), as previously described[10].

Statistical analyses
Statistical analysis was carried out by GraphPad Prism Software (GraphPad, California). Comparisons were performed by Kruskal–Wallis test (corrected for Dunn's multiple comparisons test) in Figs. 1a (for each time point), c (for each region), 2a and 3a. Two-tailed paired Wilcoxon test was performed in Figs. 1b, 2b,c, 3b–d. Two-tailed unpaired Mann–Whitney test was performed in Fig. 4a,b. Pearson's correlation test was performed in Fig. 4c.

All data are shown as mean ± SEM; $P < 0.05$ denoting statistical significance.

Data availability
All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author contributions

All authors have made substantial contributions to this work and have approved the final manuscript. M.T.L. and S.C. performed most of the experiments and data analyses; S.B., C.L.R, C.F., F.C. and S.D.S., performed

experiments and data analyses; M.T.L., S.C., S.B., C.P., A.P. and V.D.R. analysed the data and interpreted the results; M.M., B.Z., R.L., V.B.M. and G.T.M. provided human samples; M.T.L., S.C., M.G. and G.M. designed the study and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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