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*J Immunol 2012; 188:1091-1097; Prepublished online 21 December 2011;
doi: 10.4049/jimmunol.1102045
http://www.jimmunol.org/content/188/3/1091

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/12/21/jimmunol.1102045.DC1

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Repression of Cyclic Adenosine Monophosphate Upregulation Disarms and Expands Human Regulatory T Cells

Matthias Klein,* Martin Vaeth,† Tobias Scheel,‡ Stephan Grabbe,§ Ria Baumgrass,† Friederike Berberich-Siebelt,† Tobias Bopp,* Edgar Schmitt,*† and Christian Becker§,*

The main molecular mechanism of human regulatory T cell (Treg)-mediated suppression has not been elucidated. We show in this study that cAMP represents a key regulator of human Treg function. Repression of cAMP production by inhibition of adenylyl cyclase activity or augmentation of cAMP degradation through ectopic expression of a cAMP-degrading phosphodiesterase greatly reduces the suppressive activity of human Treg in vitro and in a humanized mouse model in vivo. Notably, cAMP repression additionally abrogates the anergic state of human Treg, accompanied by nuclear translocation of NFATc1 and induction of its short isoform NFATc1/a. Treg expanded under cAMP repression, however, do not convert into effector T cells and regain their anergic state and suppressive activity upon proliferation. Together, these findings reveal the cAMP pathway as an attractive target for clinical intervention with Treg function. The Journal of Immunology, 2012, 188: 1091–1097.

B ased on their potency to control allergic, autoimmune, and infectious diseases, human naturally occurring regulatory T cells (Treg) represent a promising target for the development of innovative therapeutic strategies. Hence, elucidation of a mechanism underlying Treg-mediated suppression is of central importance. Although the suppressive function of Treg has been attributed to several pathways and molecules (1), these observations do not preclude a general suppressive mechanism that is commonly used by Treg under all circumstances. With regard to a potential common mechanism, it is interesting to note that a majority of studies firmly established that Treg suppress through direct contact with other cells (2). Analyzing the mechanism of contact-dependent suppression, we recently described that murine Treg increase their intracellular level of the second messenger cAMP upon activation and consign this potent immunosuppressive compound (3, 4) via gap junctions in vitro and in vivo into other cells (5, 6). Within the responder cell, cAMP inhibits proliferation and differentiation, including IL-2 production, most probably through the induction of inducible cAMP early repressor (ICER) expression (7–11).

Although these findings have been confirmed and extended for murine Treg by several groups (12–16), the role of cAMP in human Treg function has only been partially addressed (17, 18). Therefore, we investigated the extrinsic and intrinsic functions of cAMP production in human Treg in detail. Repression of cAMP production by inhibition of adenylyl cyclase activity or augmentation of cAMP degradation through ectopic expression of a cAMP-degrading phosphodiesterase (PDE) greatly reduced the suppressive activity of human Treg in vitro and in a xenogeneic graft-versus-host disease (GVHD) model in vivo. In addition, cAMP repression resulted in vigorous Treg proliferation and an increase in the nuclear translocation of NFATc1, enabling induction of its short isoform NFATc1/a. However, upon temporary cAMP repression, Treg regained their anergic state and suppressive activity without converting into effector T cells. We conclude that cAMP forms a key component of the human Treg-suppressive mechanism and that cAMP-regulating enzymes represent attractive molecular targets for therapeutic intervention with Treg activity in pathological processes like tumor and allergic (19) and autoimmune diseases.

Materials and Methods

Isolation of T cells and suppression assays

CD4+CD25+FOXp3+ Treg, CD4+CD25− T cells (Tcon), and CD8+ T cells were isolated from anonymous Buffy coats, as previously described (20). Briefly, Treg were separated using limited amounts of CD25 MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), resulting in CD25high cells. CD4+ T cells, B cells, and monocytes were depleted using CD14, CD8, and CD19 Dynabeads (Invitrogen/Dynal, Oslo, Norway), resulting in a purity of CD4+CD25high T cells between 92 and 95% (Supplemental Fig. 1). CD4+CD25− T cells (Tcon) were isolated using CD4 MicroBeads; subsequently, remaining CD25+ T cells were depleted with CD25 Dynabeads, resulting in a purity of CD4−CD25+ T cells >98%.

Treg and Tcon were stimulated with irradiated (50 Gy) PBMC and 0.5 μg/ml anti-CD3 mAb (clone OKT-3). Proliferation was determined after 3 d of culture by adding [3H]thymidine deoxyriboside (Tdr) at 37 kBq/well for an additional 16 h. Alternatively, Treg and responder cells (Tcon or CD8+ T cells) were labeled with CFSE (Invitrogen) or eFluor 670 (eBioscience), according to the manufacturer’s instructions, and cell proliferation was analyzed on day 6 by flow cytometry (FACSCalibur; Becton Dickinson). Cytokine production was analyzed on day 10 with PE-labeled

Abbreviations used in this article: AC, adenylyl cyclase; GVHD, graft-versus-host disease; ICER, inducible cAMP early repressor; PDE, phosphodiesterase; PKA, protein kinase A; Tcon, CD4+CD25− T cell; Tdr, thymidine deoxyriboside; Treg, regulatory T cell.

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Received for publication July 14, 2011. Accepted for publication November 18, 2011.

This work was supported by Deutsche Forschungsgemeinschaft Grant BE 3685/1-1 (to C.B.), Sonderforschungsbereich Transregio 52 Teilprojekt A1 (to M.K., T.B., and E.S.), and Teilprojekt A3 (to F.B.-S.), SCHM 1014/5-1 (to T.B. and E.S.), the GRK 1043, the International Graduate School of Immunotherapy (to E.S. and T.B.), and Forschungszentrum Immunologie of the University Medical Center Mainz (to E.S., T.B., and C.B.).

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The online version of this article contains supplemental material.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102045
cytokine-specific mAb (all from BD Pharmingen), upon removal of dead cells by Ficoll-gradient centrifugation and stimulation with PHA-L and PMA (2.4 μg/ml and 1 ng/ml, respectively) for 5 h in the presence of monensin. For analysis of FOXP3 in the single-cell level, cells were stained with the anti-FOXP3 mAb (clone 3G3; ebiosisience). To analyze the role of IL-2 in Treg proliferation, polygonal goat anti-IL-2 (Abcam Ab10752) or control goat IgG (Abcam Ab48336) was added to cultures at a final concentration of 20 μg/ml.

Phosphodiesterase 4c expression vector and nucleofection of Treg
Murine phosphodiesterase (PDE)4c from a cDNA clone (Origene, Rockville, MD) was subcloned into pCDNA3.1 (Invitrogen). T cells (4 × 10⁶) were nucelofected in a Nucleofector II device (Amamaxa) using program X-001 with 2 μg PDE4c expression vector or empty control vector (pCDNA3.1). Subsequently, the cells were diluted with 500 μl prewarmed medium (room temperature) and transferred into a 24-well plate containing 1 ml prewarmed medium/well. To recover, cells were cultured at 37°C for an additional 16 h after nucleofection; dead cells were removed by Ficoll-gradient centrifugation prior to functional testing.

Assessment of cAMP production and adenylate cyclase inhibition
Cytosolic cAMP concentrations in T cells were quantified using a cAMP-specific ELISA (Correlate EIA Direct cAMP Enzyme Immunoassay Kit, 900-066; Assay Design). The adenylate cyclase inhibitors MDL-12 (cis-N-(2-Phenylcyclopentyl)azacycloheptadec-1-en-2-amine hydrochloride) (21) (cat. no. 444200, Calbiochem) and SQ 22536 (Calbiochem) were dissolved in DMSO prior to further dilution in medium. Each stock was tested for cytotoxicity by serial dilution on CD4⁺ T cells. All experiments with MDL-12 were performed with the stocks D00091160 and D00070958. For adenylate cyclase inhibition prior to functional testing, T cells were incubated with MDL-12 (10 μM) for 30 min at 37°C and subsequently washed. The adenylate cyclase inhibitor SQ 22536 was added to T cell cultures at a concentration of 1 μM. Sham treatments with vehicle (DMSO) served as control.

Transcription factor staining and confocal microscopy
CD4⁺CD25⁺ T cells harvested on slides by cytospin were fixed in 4% formaldehyde and permeabilized with 1× Perm solution (ebiosciences). Primary stainings were performed with mouse monoclonal anti-NFATc1 (7A6; Alexis), rabbit polyclonal anti-NFATc1/α (Ga; ImmunoGlobe), rat monoclonal anti-FOXP3 (FJK16s; ebiosciences), and rabbit anti-ICER/CREM (CREMSET4); secondary stainings were performed with anti-rabbit Alexa Fluor 647, anti-mouse Alexa Fluor 488, and anti-rat Alexa Fluor 555 (all from Molecular Probes). Slides were mounted with Fluoromount-G (Southern Biotechnology) containing DAPI. Images were obtained at 23°C with standard immersion oil (AppliedChimp) using a confocal microscope (Leica TCS SP2 equipment) and the HCX PL APO, 63×/1.32-0.6 lens. All images were collected with equivalent-gain instrument settings, analyzed by LCS confocal software (Leica), and subsequently processed by Photoshop CS2 (Adobe). For statistical analyses, the percentage of positive cells or mean fluorescence intensity/nucleus was calculated for 25 cells each of at least two independent experiments using Leica confocal laser-scanning software. The results were compared with Prism software (GraphPad) using an unpaired Student t test.

Mice and GVHD experiments
Rag2⁻/⁻/γc⁻ mice were obtained from the central animal facility of the University Medical Center. All animal procedures were in accordance with relevant laws (authorization G09-1-040) and current institutional guidelines and were performed according to the Helsinki convention for the use and care of animals. For GVHD induction, newborn Rag2⁻/⁻/γc⁻ mice (day 1-4 after birth) were injected i.p. with 5 × 10⁶ human PBMC, which resulted in a total mortality >95% of all treated animals, with a median time to death of 40 d (range, 35–55 d). Development of GVHD was characterized by decelerated growth and weight loss and was accompanied by massive inflammatory infiltration of human T cells into all murine organs (18). Cotransfer of resting human Treg at a 4:1 ratio (PBMC/Treg) decelerated GVHD onset and increased the median time to death by 20 d (range, 15–30 d). Mice left untreated served as controls.

Statistics
All assays were repeated at least three times with T cell subsets from different donors. Results represent means ± SE. Statistical significance was determined using the Student t test (two-tailed t test). A p value ≤ 0.05 was considered significant.

Results
Human Treg rapidly upregulate intracellular cAMP upon stimulation
We recently observed that human Treg increase their intracellular cAMP level upon prolonged stimulation (18). To reveal the real-time kinetic of cAMP production in Treg, we measured the intracellular cAMP content of Treg stimulated by plate-bound anti-CD3 mAb over several hours. Unstimulated Treg and stimulated and unstimulated CD4⁺CD25⁺ T cells (Tcon) served as controls. Compared with Tcon, resting Treg displayed high intracellular cAMP levels. Upon activation, Treg rapidly increased their intracellular amount of cAMP within the first 4 h and subsequently kept the cAMP level consistently high (Fig. 1). By contrast, only negligible levels of cAMP were found in Tcon at all time points measured, including several days of expansion (data not shown). Thus, within human T cells, Treg exclusively accumulate cAMP.

Repression of cAMP upregulation disarms Treg-mediated suppression of human Tcon and CD8⁺ T cells and favors Treg proliferation
To better understand the extrinsic and intrinsic function of cAMP upregulation in Treg, we interfered with cAMP upregulation by blocking adenyl cyclase (AC) activity with the cell-permeable, irreversible AC inhibitor MDL-12 (21). Pretreatment of Treg with MDL-12 completely prevented the increase in intracellular cAMP upon activation (Fig. 2A) and significantly impaired Treg suppressive activity (Fig. 2B), indicating that increased cAMP production is a prerequisite for their suppressive function. By contrast, pretreatment of Tcon with the adenylate cyclase inhibitor MDL-12 slightly increased their proliferation but did not affect their susceptibility to Treg suppression (Supplemental Fig. 1B), arguing against the de novo cAMP production in Tcon upon contact with Treg. To demonstrate the general applicability of AC inhibition for interference with the suppressive properties of Treg, we conducted experiments with another AC inhibitor (SQ 22536). The suppressive properties of Treg were also reduced, albeit less effectively, upon pretreatment with SQ 22536 (Supplemental Fig. 1C).

To confirm the crucial role of cAMP in Treg function, we additionally interfered with cAMP upregulation in Treg by overexpressing PDE4c, a member of the PDE4 family that exclusively hydrolyses cAMP and is predominantly expressed in T cells (22-24). To ensure metabolic activity of the overexpressed PDE, we chose a long cAMP-specific PDE isoform that is directly activated by cAMP. Ectopic expression of PDE4c abrogated cAMP upregulation in stimulated Treg and greatly reduced Treg suppression.
Pressive activity without affecting their viability (Supplemental Fig. 2), further confirming the essential role of cAMP upregulation in Treg function.

Proliferation of Treg upon AC inhibition (Fig. 2B) suggested that cAMP upregulation is decisively involved in the suppressive function of Treg and determines their anergic state and proliferative capacity. To discern the effects of AC inhibition on Treg suppressive activity and proliferation in cocultures individually, Treg and Tcon were stained with different cell-proliferation dyes (CFSE and eFlour 670). Notably, inhibition of AC activity in Treg caused both a considerable loss in their suppressive function and abrogated their anergic state (Fig. 2C). Symbols represent individual cell preparations, and horizontal bars represent mean values. E and F, Proliferation and cytokine production in MDL-12–pretreated or untreated Treg and untreated Tcon single cultures (D) and Treg/Tcon cocultures (E), set up as described in C, on day 10 of the culture. *p < 0.05, **p < 0.005.

Because a prominent feature of Treg-mediated suppression is the repression of proinflammatory cytokine production by responder T cells, particularly IL-2 (2), we additionally analyzed cytokine production in the responder cells and Treg based on differential cAMP upregulation in the presence of 0.5 μg/ml anti-CD3 mAb (clone OKT-3) with 5 × 10^5 eFlour 670-labeled Tcon. Cultures of Treg with PBMC and of Tcon with PBMC served as controls. After 6 d, cell proliferation was analyzed by flow cytometry. Results are representative of five experiments. D, Quantification of results obtained by the duochromatic assay shown in C. Symbols represent individual cell preparations, and horizontal bars represent mean values. E and F, Proliferation and cytokine production in MDL-12–pretreated or untreated Treg and untreated Tcon single cultures (D) and Treg/Tcon cocultures (E), set up as described in C, on day 10 of the culture. *p < 0.05, **p < 0.005.
short-isofrom NFATc1/A requires TCR-mediated activation and nuclear translocation of the long and constitutive NFAT isoforms, enabling induction via its own promoter P1 (25). We recently showed that the expression of NFATc1/αA is significantly reduced in murine Treg, possibly accounting for their anergic state (26). Furthermore, in effector T cells, cAMP-induced nuclear accumulation of ICER in all probability interferes with NFATc1/αA induction by binding to two highly conserved cAMP-responsive elements in P1 (27). Therefore, we asked whether cAMP repression in human Treg potentiates NFATc1/αA induction and nuclear translocation. By confocal microscopy, no or only minor nuclear NFATc1 could be detected in unstimulated and stimulated Treg, respectively. However, upon cAMP repression by AC inhibition, Treg increased NFATc1 nuclear translocation (Fig. 4A). In line with NFATC1-P1 autoregulation, we observed increased NFATc1/αA expression under AC inhibition in stimulated Treg (Fig. 4B). These data demonstrate a strong correlation between cAMP levels and NFATc1/αA induction in human Treg, providing an explanation for the importance of cAMP in maintenance of the anergic Treg phenotype.

Repression of cAMP generation disables Treg suppressive activity in vivo

Our results obtained in vitro strongly suggested a central function of cAMP in human Treg-mediated suppression. However, in consideration of a multiplicity of proposed Treg suppressor mechanisms described so far (1) and potential differences between in vitro and in vivo Treg function, we analyzed the importance of cAMP generation for human Treg-mediated suppression in vivo. To this end, a recently established preclinical model of xenogeneic GVHD was applied (18). Upon transfer of human PBMC, newborn Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice develop a lethal GVHD induced by human xenoreactive T effector cells. However, GVHD onset is completely prevented by cotransfer of human Treg (18), demonstrating the usefulness of this model for studying human Treg function in vivo. Nevertheless, when human Treg were pretreated with MDL-12 and engrafted together with syngenic human PBMC, cAMP repression also abrogated their protective activity.

Discussion

In the current study, we show that cAMP represents a key regulator of human Treg function. Both the suppressive activity and functional state of Treg strictly correlate with their intracellular cAMP level and can be manipulated by interfering with the function of cAMP-producing (AC) and -degrading (PDE) enzymes. Comparing the cAMP content of resting and activated Treg and Tcon, we demonstrate that, within the human CD4<sup>+</sup> T cell population, Treg exclusively accumulated cAMP. This observation suggests that previous reports on cAMP upregulation in human CD4<sup>+</sup> T cells (28–31) were partially obscured by Treg-specific cAMP upregulation in unseparated T cell fractions. Therefore, it is unclear whether, apart from cAMP-elevating drugs, particular stimulatory conditions exist that induce cAMP accumulation in Tcon and whether cAMP accumulation could predispose Tcon for regulatory activity.

The molecular mechanisms underlying differential cAMP accumulation in Treg are poorly understood. In murine Treg, Foxp3 represses the expression of PDE3b, thereby preventing degradation of cAMP (32). In addition, Foxp3-mediated repression of the AC9 regulating micro RNA 142-3p (14) and IL-2–mediated adenylate cyclase AC7 activation (33) seemed to contribute to increased cAMP production, emphasizing the crucial importance of Foxp3 for the development and function of murine Treg. By contrast, no particular regulatory mechanism affecting the cAMP pathway in human Treg has been described.

Repressing cAMP production in human Treg significantly reduces their suppressive activity toward Tcon and CD8<sup>+</sup> T cells. Similar to their murine counterpart (5), human Treg are expected to transfer cAMP via gap junctions into responder cells, inducing cAMP-mediated protein kinase A (PKA) activation in the responder cell (34). In this scenario, AC inhibition turns down Treg suppressive activity by reducing the amount of transferable cAMP. With regard to gap junction communication, human cells were shown to express connexin family proteins and to extensively use gap junctions for bidirectional exchange of small molecules, including cAMP (35). Moreover, the permeability of gap junctions between human cells seems to be strongly increased by cAMP (36). Because AC inhibition in Treg, but not in Tcon, turns down Treg-mediated suppression, Treg-mediated suppression seems not to depend on de novo generation of cAMP triggered by adenosine but is most likely exclusively dependent on intrinsic cAMP production by Treg.

Our observation that resting human Treg already contain high levels of intracellular cAMP suggests that they constitutively produce cAMP. Constitutive cAMP production may be physiologically important to arm Treg with a suppressive mechanism that enables them to rapidly suppress, even in absence of stimulation, through the TCR (37). In this respect, cAMP may represent a general suppressive mechanism that is commonly used by Treg to exert homeostatic control over the immune system in the steady state.

Apart from being central to their suppressive function, the high endogenous cAMP level attenuates the TCR responsiveness of Treg. As shown in this study, pharmacological repression of cAMP upregulation abrogates the anergic state of Treg and causes their vigorous proliferation. Conventional T cells readily induce NFATc1 expression and nuclear translocation upon stimulation; however, cAMP-induced expression of ICER potentially holds down cytoplasmic NFATc1 expression (27). We show in this study...
that repression of cAMP production in Treg is sufficient to increase NFATc1 expression and subsequently induce NFATc1/αA expression through NFATC1-P1 autoregulation in human Treg. Surprisingly, in contrast to AC inhibition, enforcing the catabolic pathway by PDE overexpression did not affect the anergic state of Treg in culture (Fig. 2B, Supplemental Fig. 2). A possible explanation for this discrepancy is provided by the proposed model of PDE function in the termination of cAMP signaling through PKA; although PDE are the only route for degrading cAMP and, thus, are poised to regulate intracellular cAMP gradients, their

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**FIGURE 4.** cAMP repression induces NFATc1/αA re-expression. A, Elevated nuclear translocation of NFATc1 in MDL-treated cells: MDL-12–pretreated or untreated Treg were stimulated for 16 h with anti-CD3, anti-CD3, and anti-CD28 mAb or were left untreated and subsequently analyzed for localization of total NFATc1 (Alexa Fluor 488), FOXP3 (Alexa Fluor 555), and ICER/CREM (Alexa Fluor 647) by confocal microscopy (original magnification ×400). B, Increased expression of inducible NFATc1/αA (Alexa Fluor 647) in MDL-treated cells using confocal microscopy. Bar graphs: quantification of the percentage of Foxp3+ cells with nuclear NFATc1 and nuclear NFATc1/αA expression of two independent experiments. Data are shown as mean ± SEM.

**FIGURE 5.** Repression of cAMP in Treg abrogates their suppressive function in vivo. Newborn Rag2−/−γc−/− mice were injected with 5 × 10⁶ PBMC with or without 1.25 × 10⁶ Treg (n = 4 mice/group). Untreated mice served as controls (n = 3). Upper panel, Cumulative mean weight data ± SE. Lower panel, Kaplan–Meyer plot of survival. Results are representative of three experiments.
isomers are spatially confined to discrete compartments and coupled to individual receptors (38). Therefore, overexpression of a single isoform might degrade cAMP in some subcellular microdomains but preserve PKA/cAMP signaling in others (39). Repression of cAMP allows Treg proliferation without addition of IL-2. At first glance, this stands in marked contrast to current Treg-expansion protocols that require high concentrations of IL-2 (40, 41). However, the known suppressive effect of IL-2 on adenylate cyclase activity (42) at least suggests that AC inhibition could be a prerequisite for Treg expansion. Blocking IL-2 with Abs reduced AC-enabled Treg proliferation (Supplemental Fig. 4B), indicating that IL-2, supposedly produced by the PBMC, is an additional necessity for Treg expansion upon AC inhibition. Although several publications suggested that Treg could undergo reprogramming in response to certain stimuli (43, 44), temporary cAMP repression by AC inhibition did not convert Treg into effector cells, and their cytokine production remained locked. Thus, temporary changes in the cAMP level control the functional state of Treg without affecting lineage stability.

Collectively, our data highlight the cAMP pathway as a putative target for therapeutic intervention with Treg activities in pathological processes, such as tumor and autoimmune diseases. In line with this, blocking AC degradation by PDE inhibitors was shown to alleviate allergic and autoimmune diseases (19, 45). Conversely, inhibition of cAMP-mediated PKA signaling using the cAMP analog Rp-8-BrcAMPS improved CD8+ T cell-mediated tumor immunotherapy, possibly by releasing tumor-reactive CD8+ T cells from Treg control (46, 47). However, with regard to the importance of cAMP in numerous physiological processes, selective inhibition of the cAMP pathway in Treg is mandatory.

Acknowledgments

We thank P. Hoelter for expert technical assistance and Dr. J. Kirberg (Paul-Ehrlich Institut, Langen, Germany) for providing the Rag2−/−μMT−/− mice.

Disclosures

The authors have no financial conflicts of interest.

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