Inhibition of regulatory T cell proliferation by cAMP: a role for Epac1.

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Abstract

Background: Regulatory T lymphocytes (Tregs) play an important role in the peripheral tolerance, by suppressing autoreactive T cells. By maintaining the peripheral tolerance, Tregs control autoimmune and allergic diseases (Sakaguchi, 2004). Cyclic adenosine monophosphate (cAMP) is currently believed to inhibit proliferation in T cells via the major cAMP effector protein kinase A (PKA) and the novel cAMP effector exchange protein directly activated by cAMP (Epac1 and Epac2). Though inducible regulatory T cells (iTregs), a subpopulation of regulatory T cells, bare high concentrations of cAMP and proliferation is not suppressed. The T cell proliferation inducer interleukin-2 (IL-2) is highly expressed in iTregs, and that might cause the insensitivity of iTregs to cAMP with regard to its inhibition of proliferation. Here we studied the expression of Epac1 and Epac2 in iTregs. In addition we analysed acute effects of cAMP analogs on early signaling in iTregs.
Methods: We used human CD4+ T cells that were differentiated into iTregs and control cells, T effector cells (Teffs). The cells were stimulated with Epac-activator 8-pCPT-2'-O-Me-cAMP and PKA-activator 6-Bnz-cAMP. The activation of vasodilator-stimulated phosphoprotein (VASP) was analyzed to validate the specificity of the activators. The activation of extracellular signal-regulated kinase 1/2 (ERK1/2) was analyzed as an indicator of proliferation. The cells were treated with IL-2 and analyzed for the expression of Epac1 and Epac2.
Results: 6-Bnz-cAMP activates VASP in both iTregs and Teffs, which correspond with expected specificity. Altered levels of activated ERK1/2 were observed in both iTregs and Teffs after stimulation with 8-pCPT-2'-O-Me-cAMP or 6-Bnz-cAMP. Epac1 is lower expressed in iTregs and the expression of Epac2 is downregulated by IL-2 in both iTregs and Teffs.
Conclusion: Activation of VASP by 6-Bnz-cAMP displays the specificity of the analogs in this experiment. iTregs seem to be insensitive to cAMP, most likely due to their low levels of Epac1. IL-2 downregulates Epac2 in both Teffs and iTregs.

Introduction

T lymphocytes

The human body has to be protected against infectious agents, like bacteria, fungi and viruses, to prevent the body of getting harmed. To protect the body against these foreign threats, the human body has developed an immune system that is capable of inducing a response, against infectious particles that are a possible threat to the body. The immune system can be divided into two parts: the innate- and adaptive immune system. The innate immune system is aspecific, fast and normally gives a constant immunity throughout live, whereas the adaptive immune system is specific for a single infectious agent, but needs more time to mount a protective response and is acquired throughout life (Abul K. Abba, Andrew H. Lichtman, 2005). T lymphocytes, or T cells, play a central role in the regulation of adaptive immunity. There are different phenotypes of T cells. The most common T cells are (Abul K. Abba, Andrew H. Lichtman, 2005):

- T helper cells. There are different phenotypes of T helper cells, such as Th1 and Th2. Th1 cells stimulate macrophage by secreting cytokines. Th2 cells stimulate B cell growth and differentiation. Th cells are CD4 positive.
- Cytotoxic T cell, or CTL, are capable of terminating cells infected by viruses, or terminating tumor cells. These CTLs are CD8 positive.
- regulatory T cells. Tregs derive from both Th and CTL and therefore are CD4 or CD8 positive. Their function is to suppress Th's and CTL's that encounter self-antigens, and prevent autoimmune diseases from happening..

T cells express a T cell receptors (TCR) that is specific for the antigen recognition in the major histocompatibility molecules on the cell surface of all cells (MHC I), or antigen presenting cells (MHC II). Whereas CD4+ T cells recognize the MHC II complex, the CD8+ T cells recognize the MHC-I complex. Upon the recognition of antigen/MHC-complex, a naïve T cell will be activated and differentiate into Teffs or memory T cells. Every individual T cell has a unique TCR specific for a single antigen (Abul K. Abba, Andrew H. Lichtman, 2005). During differentiation of T cells in the thymus, a large variety of TCR specificities is generated. This gives the immune system the ability to recognize a very large variety of antigens (Abul K. Abba, Andrew H. Lichtman, 2005). Not only will the immune system recognize foreign antigens, it will also generate TCRs specific for self-antigens during T cell differentiation in the thymus. If T cells with self-reactive TCRs would leave the thymus as naïve, mature T cells, an immune response will be generated against the own body. To prevent this mechanism, immune tolerance to self-antigens is necessary.

Immune tolerance

There are two different types of immune tolerance: a central- and a peripheral tolerance. The central tolerance is created by negative selection of immature T cells in the thymus. Negative selection means that any immature T cell that binds strongly to the MHC/antigen complex present on the stromal cell in the thymus will be eliminated by inducing apoptosis. A high-affinity interaction between the TCR and the (self)antigen/MHC complex is indicative of self-reactivity of the TCR. By this negative selection process of self-reactive T cell clones, central tolerance is being maintained. If the immature T cell binds with lower affinity to the MHC/Antigen complex, it receives survival signals and matures into a naïve T cell. If the immature T cell binds with a intermediate strength, it will differentiate into a so-called natural Treg (Abul K. Abba, Andrew H. Lichtman, 2005). Any T cell clone not recognizing the MHC/Antigen complex at all will also enter apoptosis.

Peripheral tolerance is induced in the peripheral limbic organs. Like in the thymus during the negative selection, naïve T cells are exposed to peptide fragments of self-antigens as well, from all over the body. Central tolerance will clear the self-reactive T cell clones specific for the most common self antigens. Nevertheless, self-reactive T cell clones specific for very tissue-specific antigens might well escape central tolerance induction in the thymus. Such T cells that react to these self-antigens will be controlled in three different ways. These T cells can undergo apoptosis, a specific state of unresponsiveness (anergy) can be induced or their activity can be actively suppressed (Abul K. Abba, Andrew H. Lichtman, 2005). The active suppression of auto reactive T cells is a feature of the Treg. To avoid the induction of an immune response, the activity of the auto reactive T cell will be suppressed and self tolerance is maintained (Ohkura & Sakaguchi, 2010).

Subpopulations regulatory T cells
Several subsets of Tregs can be found in the body. There are Tregs that express CD4 or CD8 proteins on their cell surface, respectively derived from helper T cells and cytotoxic T cells. Our research is focused on the CD4+ variant. The Tregs can be split in two groups; natural occurring Tregs and adaptive Tregs (nTregs and aTregs). nTregs find their roots in the thymus. aTregs are formed in the peripheral limbic organs, by antigen activation of the naive T cell in the absence of costimulation. Whereas nTregs are specific for self-antigens, aTregs can also be specific for nonself-antigens. Both variants are characterised by the expression of IL-2 receptor α chain (CD25) and forkhead family transcription factor Forkhead box p3 (FoxP3)(Bluestone & Abbas, 2003). FoxP3 is important for the Tregs in fulfilling its suppressive function. Mutations in the FoxP3 gene will cause Tregs to malfunction. The body will lose its ability of maintaining self-tolerance and the induction of a plethora of auto-immune diseases and excessive inflammatory responses are the result (Bennett et al., 2001; Gambineri, Torgerson, & Ochs, 2003).

For research, another subset of Tregs is often used, the iTregs. Because of the difficulties of isolating sufficient amounts of Tregs from a living organism for research, isolated CD4 or CD8 positive T cells are cultured and expanded in the presence of transforming growth factor β (TGF-ß). Those cells will differentiate into iTregs.

Tregs can fulfil their suppressive function in a contact-dependent manner, as well as by producing soluble mediators such as IL-10 or TGF-ß (Abul K. Abba, Andrew H. Lichtman, 2005) (Fig1). One of the possible mechanisms for the contact-dependent suppression is the formation of gap junctions with the Teffs. The Treg binds to the Teff and will transport cAMP into the Teff via gap-junctions. The cAMP will induce a loss of expression of IL-2 in the Teff, a cytokine responsible for T cell proliferation. TGF-ß and IL-10, T cell proliferation inhibitors are secreted (Abul K. Abba, Andrew H. Lichtman, 2005).

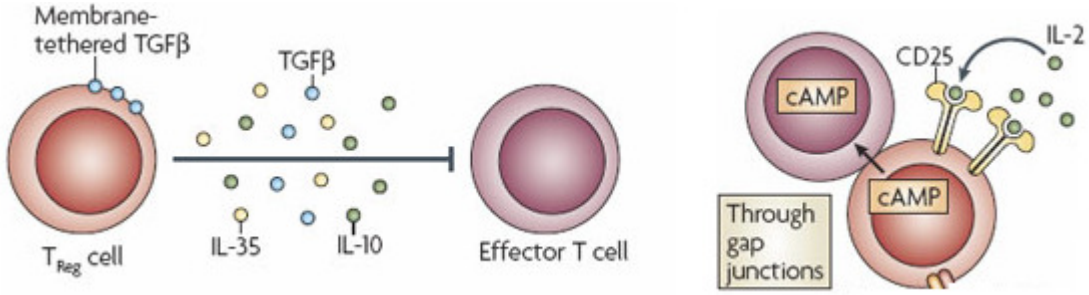


Figure 1. Tregs suppress Teffs by producing soluble mediators such as IL-10 and TGF-ß and in contact-dependent manner. IL-2 will be downregulated, which causes to inhibit proliferation of the Teffs (Vignali, Collison & Workman, 2008).

Interleukin 2
When IL-2 was discovered, it was found to be a T cell growth factor. Besides that, it plays more roles in the adaptive immune response. In case antigen recognition by a T cell takes place, IL-2 will be produced by this T cell and will be responsible for proliferation of this T cell. When IL-2 is produced, the concentration of cyclins is elevated, which results in activation of different cyclin-dependent kinases. On their behalf the Rb protein is phosphorylated and activated and transition of G1 to the S phase of the cell cycle will be stimulated (Abul K. Abba, Andrew H. Lichtman, 2005). Also production of other cytokines, like IFN-γ and IL-4 are increased by IL-2 in T cells. IL-2 mainly has an influence on an autocrine base, but it may be possible that it stimulate proliferation of some adjacent T cells (Abul K. Abba, Andrew H. Lichtman, 2005).

Blocking the expression of IL-2 in Teffs is a characteristic feature of naturally occurring Treg cell cAMP mediated suppression (Bodor, Spetz, Strominger, & Habener, 1996; Shevach, 2002; Thornton & Shevach, 1998). By adding IL-2 to Teffs, the suppression by Tregs will be eliminated. IL-2 is thought to be involved in the signal transduction responsible for controlling the induction of FoxP3 (Shevach, 2002).

Transforming growth factor ß
TGF-ß is an inhibiting factor of T cell proliferation and secreted by Tregs during their suppressive role in the peripheral tolerance. It is also known to be a potent inducer, accompanied by the TCR and CD28, of differentiation of naïve CD4+ T cells into iTregs, as mentioned earlier. Furthermore, TGF-ß is essential in nTregs in the maintenance of the expression of FoxP3 Tregs, which is critical for its suppressive character. CD4+ T cells

cultured with TGF-ß and IL-2 will differentiate into iTregs (Setoguchi, Hori, Takahashi, & Sakaguchi, 2005)(Shevach, 2002).

cAMP, a second messenger

Cyclic adenosinemonophosphate (cAMP) is a well known second messenger in many different kind of cells and is part of a great variety of physiological processes. For instant, it plays a role in metabolic events, calcium handling, cardiac and smooth muscle contraction, ion channel conductance, secretion, learning and memory, cell growth and differentiation, apoptosis and inflammation (Beavo & Brunton, 2002).

The regular signal transduction starts with stimulation of a Gs-protein coupled receptors that trigger the synthesis or the degradation of cAMP. Adenylyl cyclase (AC) is responsible for the synthesis of cAMP out of ATP and phosphodiesterases (PDEs) are able to degrade cAMP into AMP (Hanoune & Defer, 2001; Lugnier, 2006) (Fig 2). cAMP has the ability to bind to A-kinase anchoring proteins (AKAPs). Binding to AKAPs makes it possible for cAMP to be compartmentalized and to be spatio-temporal dynamic (Wong & Scott, 2004).

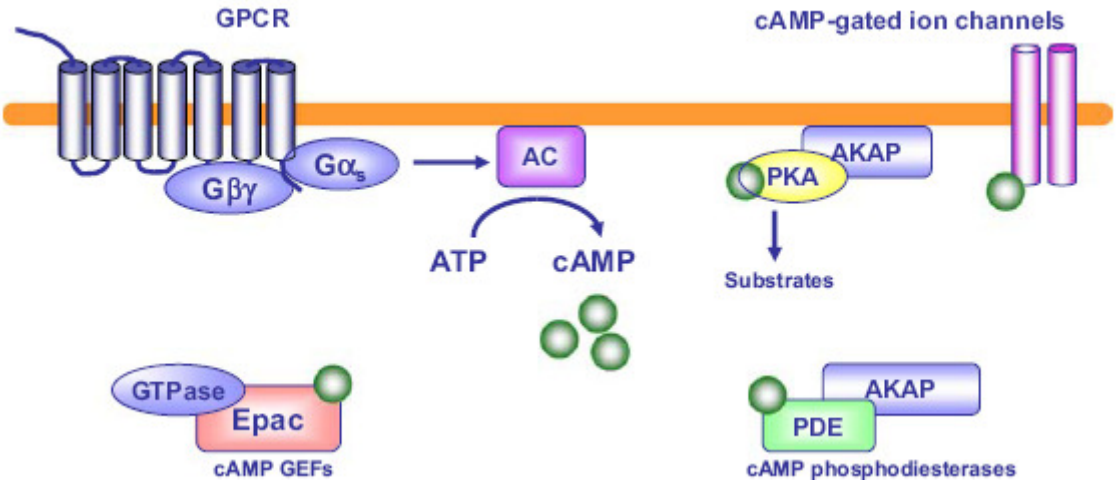


Figure 2. cAMP is formed out of ATP after stimulation of the G_s-coupled receptors. The second messenger, cAMP can act through Epac, PKA and other proteins. cAMP is degraded into AMP by PDEs. cAMP is spatio-dynamic and can be compartmentalized when it binds to AKAPs. (Roscioni et al., 2008)

cAMP activates PKA

The spatio-temporal dynamics of cAMP gives it the ability to be a second messenger in more than one signal transduction pathway. One protein that is part of different signal transduction pathways which are directly activated by cAMP is PKA (Cohen, 2002), and plays a role regulating processes, like the proliferation rate of T cells. Signalling with cAMP through PKA can lead to variety of responses. In Jurkat T cells, cAMP stimulated growth arrest is likely to be mediated by PKA (Fuld, Borland, & Yarwood, 2005). PKA seems to be involved in the induction of $p27^{kip1}$, which results into growth arrest (Kato, Matsuoka, Polyak, Massague, & Sherr, 1994; van Oirschot, Stahl, Lens, & Medema, 2001b). In other cells, PKA can have the opposite effect, and will it stimulate proliferation via MAP kinase (Ohkura & Sakaguchi, 2010). Activated PKA is known to activate vasodilator-stimulated phosphoprotein (VASP) by phosphorylation (Roscioni et al., 2009).

cAMP activates Epac

A more recently discovered pathway for cAMP signalling involves Epac. Epac is an exchange proteins directly activated by cAMP also known as cAMP-activated guanine nucleotide exchange factors (cAMP-GEF) (Fig 3) for Ras-like GTPase, like Rap1 and Rap2. Epac is expressed in three different forms, Epac1 and Epac2A and Epac2B. They are all expressed in developing and mature tissues. Expression levels of Epac1 and Epac2 vary in different cell types. Epac1 is mainly expressed in heart and kidney, some leukocytes and lymphocytes, but expression is found in all kinds of cells (De Rooij et al., 1998). It is known that Epac2 is highly expressed in the brain and adrenal glands (Kawasaki et al., 1998). The difference between Epac2A and Epac2B is that Epac2A is expressed in the cerebral cortex and pancreatic islets, whereas Epac2B is expressed in the adrenal glands (Niimura et al., 2009). Epac1 and Epac2 are both expressed in bovine tracheal smooth muscle tissue (Roscioni et al., 2011).

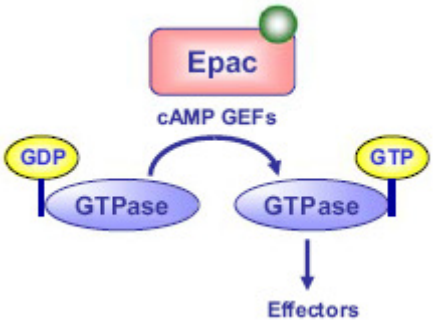


Figure 3. Epac is known as cAMP-activated guanine nucleotide exchange factors for Ras-like GTPase. It facilitates the exchange of guaninediphosphate (GDP) for guanosinetriphosphate (GTP), which leads to activation of Ras-like GTPase (Roscioni, Elzinga, & Schmidt, 2008).

The proteins Epac1 and Epac2 contain two main regions (Fig 4). They both have a regulatory region at the N-terminal and a catalytic region at the C-terminal. Their regulating region contains a DEP (dishevelled, Egl-10, pleckstrin) domain and a high affinity cAMP-binding domain which function is associated with membranes. The catalytic domain contains a Ras-exchange motif domain (REM) for stabilizing the GEF-domain. A Ras association domain (RA) for redirecting Epac2 to Ras-containing regions (Liu et al., 2008) and a CDC25HD domain that presents GEF activity for Ras-like GTPases (Li et al., 2006). Compared to Epac1, Epac2 contains another domain at the N-terminal: the cAMP-A. In contrary to the cAMP-B domain, cAMP-A binds with low affinity to cAMP. Its role is still undetermined. As mentioned before, there are two variants of this domain which is the reason Epac2 is known as 2A and 2B. Epac2B is a splice variant of Epac2A. Epac2A is expressed in the pancreas. Here it is involved with the secretion of insulin and it is localized near the plasma membrane (Niimura et al., 2009).

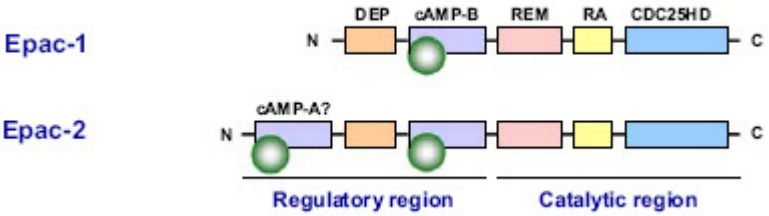


Figure 4. Epac1 and Epac2 contain a regulatory- and a catalytic region. Regulatory region contains a DEP- (dishevelled, Egl-10, pleckstrin) and a cAMP-b domain. The catalytic region contains a REM-, RA and a CDC25HD domain. Epac2 also contains a cAMP-a domain, which binds cAMP with low affinity.(Roscioni et al., 2008)

PKA or Epac

Epac works on its own or in combination with PKA. cAMP binds with almost the same affinity to Epac as it does to PKA. (kd ~ 2.9 uM). It is thought that signal transduction via Epac and PKA is regulated by a small increase of cellular cAMP(Dao et al., 2006). Combining this small increase with compartmentalization of Epac/PKA and effector protein availability and this will be the key components in the regulation in the signal transduction. Multi-protein complexes that contain for instants PKA, mAKAP, PDE and Epac1 have the ability to react differently to different concentrations of cAMP (Dodge-Kafka et al., 2005). To study the influence of cAMP specifically via Epac or PKA , cAMP analogs are used that are specific for Epac or PKA. There are many cyclic nucleotide analogs for PKA and Epac. Two well known cAMP analogs are N^6-benzyladenosine-3',5' – cyclic monophosphate (6-Bnz-cAMP) and 8 – (4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT-2'-O-Me-cAMP). The molecules are analogs for respectively PKA and EPAC. There is no difference in activation of Epac1, 2A or 2B, when stimulated with 8-pCPT-2'-O-Me-cAMP. The specificity of analog 8-pCPT-2'-O-Me-cAMP for Epac and not for PKA is induced by the 2'-O-methyl substitution. These analogs are widely accepted pharmacological tools to study the effects of cAMP and therefore used in this experiment (Grandoch et al., 2010; Holz, Chepurny, & Schwede, 2008; Roscioni et al., 2011).

Epac location in the cell

Epac is situated at different locations in the cell. Epac1 can be found in the plasma membrane, cytoplasm, peri-nuclear regions, nuclear membrane and mitochondria of rat and human tissue (Setoguchi et al., 2005). Epac2 is located at the (sub)plasmamembrane, actin cytoskeleton, cytosolic fractions, meiotic midzone region and Golgi

in several cells. Epac is cell-specifically organized at the plasma membrane and in the cytoplasm (Grandoch, Roscioni, & Schmidt, 2010). Epac1 can be translocated to the plasmamembrane after stimulation of b-adrenoceptors (Ponsioen et al., 2009). Epac2 has to be compartmentalized to Ras regions to carry on the signal transduction (Liu et al., 2008).

Effectors of Epac

Epac1 and Epac2 are known to activate Rap1 and Rap2, Ras family members, phospholipase C-ε, phospholipase D, Extracellular signal-regulated kinase 1/2 (ERK1/2), PI 3-kinase-dependent PKB/Akt, TGF-ß1 receptor-regulated Smads, NF-κB, suppressor of cytokine signalling-3 (SOCS-3), CCAAT/enhancer-binding protein C/EBP and Glycogen synthase kinase-3 (GSK-3). Even more effectors are directly regulated by Epac. This means that Epac is involved in many different biological processes. Little is known about how these processes precisely work. Particularly regarding the co-operative functions of Epac with PKA and AKAPs and thereby the signalling result of cAMP (Grandoch et al., 2010).

Epac and cell proliferation

Extracellular signal-regulated kinase, also known as ERK1/2 or mitogen activated protein kinase is one of the proteins that are involved in the cAMP signalling in many different cells. It plays a role in the regulation of cell proliferation. Rather remarkable is that in one type of cells, cAMP will stimulate proliferation by activating ERK1/2 and in another it will inhibit proliferation by blocking the activation of ERK1/2. It is thought that the stimulation of proliferation takes place via RasGTPase. Active Ras will activate Raf-1, by binding to it. Raf-1 activates MEK by phosphorylation, which in turn activates ERK1/2 by phosphorylation. Another GTPase that is activated by cAMP is Rap1. Active Rap1 activates B-Raf and differs in that manner from Ras, which activates Raf-1. B-Raf activates MEK, which in turn activates ERK1/2. If cells do not express B-Raf, Rap1 can not activate ERK1/2, but instead it will block the activation of Raf-1 by Ras, and thereby block the activation of ERK1/2 (Fig 5). Rap1 is activated in a PKA dependent and independent manner. (Tsygankova et al., 2001). Both Epac and PKA are reported to be involved in signalling through Rap1 and Rap2 in airway smooth muscle cells (Kawasaki et al., 1998; Schmidt et al., 2001). Epac analog 8-pCPT-2'-O-Me-cAMP gives a doubled Rap1 GTP exchange, where PKA analog 6-Bnz-cAMP let the exchange rise with 1,5 fold. This was not the case for Rap2, where no differences were measured (Roscioni et al., 2009).

Epac is involved in the process at cell proliferation levels. It has a pro-proliferative character in endothelial cells, macrophages, thyroid cells and osteoblasts and it probably activates extracellular signal-regulated kinases by signalling through Rap. (Fang & Olah, 2007; Fujita, Meguro, Fukuyama, Nakamuta, & Koida, 2002; Hochbaum, Hong, Barila, Ribeiro-Neto, & Altschuler, 2008; Misra & Pizzo, 2005). However, recent studies show that Epac has an anti-proliferating influence on ASM cells and pulmonary fibroblasts, triggered by b-adrenoceptor agonists (Haag, Warnken, Juergens, & Racke, 2008; Huang, Wettlaufer, Chung, & Peters-Golden, 2008; Kassel, Wyatt, Panettieri, & Toews, 2008). Another study shows that Epac1 is responsible for anti-proliferative effects of PGE2, where earlier was thought that B-Raf and ERK1/2 inhibited the cell growth (Dugan et al., 1999). Interaction of Epac with microtubules is possibly essential in inhibition of fibroblast proliferation (Huang et al., 2008). The Epac proteins inhibit proliferation in human ASM and fibroblast (Grandoch et al., 2010).

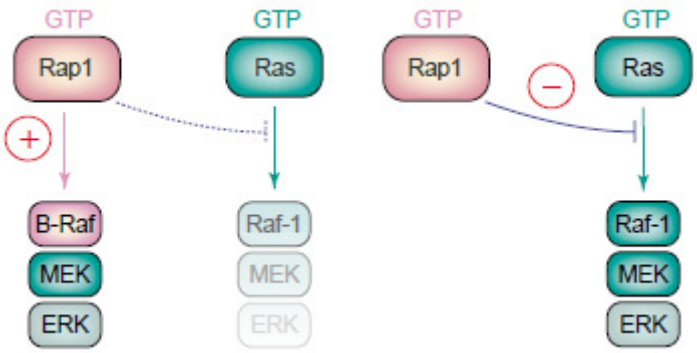


Figure 5. Activated Rap1 activates ERK1/2 trough B-Raf and MEK. When B-Raf is not present in the cell, it will block the activation of Raf-1 by Ras and inhibits the activation of ERK1/2.(Stork & Schmitt, 2002)

In immune cells, cAMP plays a role as second messenger and is involved in the regulation of differentiation, cell growth, apoptosis and production of chemokines and cytokines (Kammer, 1988). It was thought that the cAMP only worked on PKA, but studies show that the signal transduction via Epac also occurs in the innate and adaptive immunity (Cohen, 2002)(Serezani, Ballinger, Aronoff, & Peters-Golden, 2008).

Epac in diseases
Epac is recently discovered to be a key component in several diseases, like chronic inflammatory lung diseases. Epac is able to modulate airway inflammation and proliferation and via agonistic stimulation of the β2-adrenoceptor. cAMP has a palliative influence on the symptoms of asthma and COPD(Bailey & Tashkin, 2007; Barnes & Hansel, 2004; Remington & Digiovine, 2005). Epac expression is detected in human and rat alveolar macrophages, mouse dendritic cells, heart, vasculature, brain, kidneys en lungs (Murray & Shewan, 2008; Ulucan et al., 2007; U. Yokoyama et al., 2008a; U. Yokoyama et al., 2008b). Epac has a possible influence in dysfunctional cells and in diseases like inflamed neurons, in Alzheimer disease, myocardial hypertrophy, inflammatory pulmonary diseases, resting fibroblasts transforming in secreting myofibroblasts (McPhee et al., 2005; Ulucan et al., 2007; U. Yokoyama, Patel et al., 2008). TGF-ß1 interacts with Epac1 in epithelial cells and inhibits Smad dependent TGF-ß signalling (Conrotto, Yakymovych, Yakymovych, & Souchelnytskyi, 2007). Epac is directed by inflammatory mediators, but much about the mechanism is still unknown.

Epac in T cells
Epac inhibits proliferation and effector functions of T cells (Kammer, 1988; van Oirschot, Stahl, Lens, & Medema, 2001a), in a PKA independent manner (Bryce, Dascombe, & Hutchinson, 1999; Staples et al., 2001). PKA is involved in the synthesis of prolactin in Jurkat cells and T lymphocytes, but there is also a possible role for Epac (Fuld et al., 2005; Gerlo, Verdood, Hooghe-Peters, & Kooijman, 2006). Cell cycle regulation and cAMP dependent growth arrest are mediated via PKA. Rap1A, effector of Epac, stimulates T cell signalling via activation of integrins. PKA and Epac-Rap1 seem to be crucial for adhesion of T cells (Bivona et al., 2004; Dustin, Bivona, & Philips, 2004; Sebzda, Bracke, Tugal, Hogg, & Cantrell, 2002). PKA promotes adhesion in a variety of ways were Epac-Rap1 facilitates adhesion via αLβ2 and αLβ4 integrins (de Bruyn, Rangarajan, Reedquist, Figdor, & Bos, 2002). In T cells, Epac regulates integrins functioning, cell growth and gene expression. PKA and Epac both have there influence on the immune response in T cells. This response is carefully regulated by the differences of expression of these mediators in different cell systems. (Grandoch et al., 2010).

Aim of the project
cAMP is known to inhibit the proliferation in Teffs via Epac. iTregs bare high concentrations of cAMP but proliferation is not suppressed by cAMP. We discovered that IL-2 is highly expressed in the iTregs and possibly causes the iTregs to be insensitive for proliferation inhibition by cAMP via Epac.

In this project we analyze the acute effect of cAMP via Epac and PKA in Teffs and iTregs on proliferation by looking at the activation of ERK1/2, a downstream protein of Epac and PKA. Furthermore, we investigate the expression of Epac1 and Epac2 in Teffs and iTregs and what influence IL-2 has got on the expression of both cAMP effector proteins.

Material and methods

Material
6-Bnz-cAMP-cAMP, 8-pCPT-2'-O-Me-cAMP from BIOLOG Life Science Institute (Bremen, Germany), exogenous interleukin 2 (IL-2) anti-phospho-ERK1/2 (P-ERK1/2) (9101), anti-ERK1/2 (9102), anti-VASP (P-VASP) (3112), anti-β-actin from Cell Signaling Technology (Beverly, MA), anti-rabbit (A5420), anti-mouse (A9044) from Sigma-Aldrich (St. Louis), anti-Epac1, anti-Epac2 (number and origin).

APS from Sigma-Aldrich (St. Louis, MO).
30% acrylamide mix from Biorad (Canada).
TWEEN-20
TEMED from GE Healthcare (Uppsala, Sweden)
Powdered milk from Campina Melkunie
Western lightning ECL solution from PerkinElmer Inc. (Waltman, MA).
Trypan blue
The Pierce BCA protein assay kit was from Thermo Scientific (Rockford, IL).
Coomassie Brilliant Blue G-250 (Bio-Rad)

Cell cultures
To analyze the activation of ERK1/2, we isolated human CD4+ T cells from human blood cords.
These cells were expanded on plates for two weeks. By expanding T cells cell in the presence of TGF-ß, the cells differentiated into iTregs. The control cells differentiated into Teffs. The precise protocol can be found in the appendix.

Activation of VASP
After culturing, on day 13 the cells were stimulated with 60/500 µM 6-benz-cAMP or 8-pCPT-2'-O-Me-cAMP for 48 hours (Fig 6). After the stimulation, the cells were lysed and stored at -80°C. The lysates were defrosted, the plaques of the lysates were solved by ultra sonic sound on ice for 2 seconds. A Bradford protein determination was done the lysates were used for Western blot analysis.

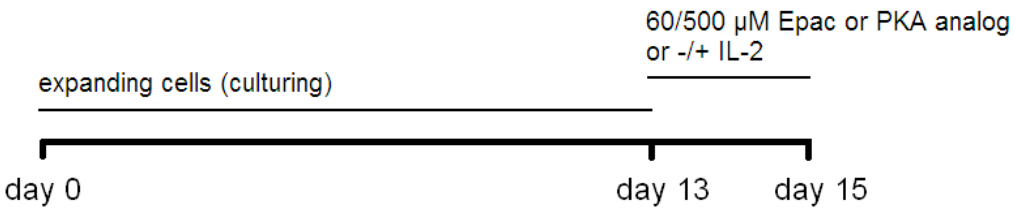


Figure 6. Time line of the cell culture of Teffs and iTregs. The cells were stimulated with 60/500 µM Epac or PKA analogs for 48 hours for analysis of activated VASP, or stimulated with IL-2 for 48 hours for analysis of expression of Epac1 or Epac2

Activation of ERK1/2
The cells were harvested and spinned down at 1200 rpm for 5 minutes and put on ice. Per sample 50ul of HBSS (Table 1) was added to the cells. The experiment took place at 37°C in a stew and the cells were adjusted to this environment for 5 minutes. The cells were stimulated with 500 µM 6-benz-cAMP or 8-pCPT-2'-O-Me-cAMP for 5, 15 or 30 minutes at 37°C (Fig 7). The experiment, and thereby the reaction, was stopped by lysing the cells with 25ul 5x laemmli buffer (Table 2) at 95°C for 5 minutes. The cells were stored at -20°C. Unstimulated cells were used for cell count to determine the number of samples and were lysed with FISH buffer (Table 5 and 6) or RIPA buffer (Table 3 and 4) for protein determination. The lysates were defrosted and the plaques of the lysates were solved by ultra sonic sound on ice for 2 seconds and used for Western blot analysis.

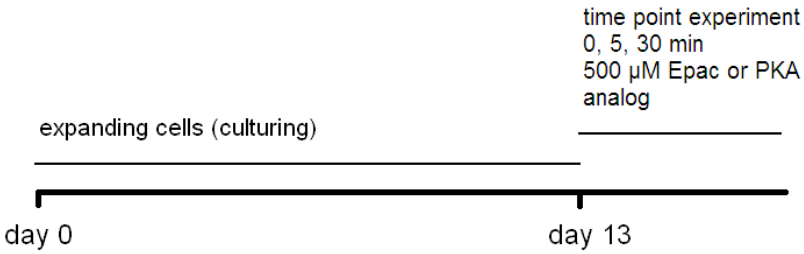


Figure 7. Time line of cell culture for activation of ERK1/2 in Teffs and iTregs. The cells were directly used for the experiment, after culturing the cells, or the cells were stored at -196°C, washed and then used for the experiment.

Expression of Epac1 and Epac2

After expanding the cells, on day 13 the cells were stimulated with 50 unit/ml IL-2 for 48 hours (Fig 6) and stored at -80°C. The lysates were defrosted, the plaques of the lysates were solved by ultra sonic sound on ice for 2 seconds and the protein determination was done by performing a Bradford protein assay. The lysates were used for Western blot analysis.

Table 1: Composition of 1 liter HBSS (pH7.4)

Component	**amount**	$MgCl_2$ x $6H_2O$	0.10 g/l
$CaCl_2$	0.14 g/l	Na_2HPO_4 x $6H_2O$	0.048 g/l
NaCl	8 g/l	$MgSO_4$ x $7H_2O$	0.10 g/l
KCl	0.4 g/l	$NaHCO_3$	0.35 g/l
KH_2PO_4	0.06 g/l	UP	To final volume

Table 2: Composition of Laemmli buffer

Components	**Amount for 20ml**
1.5M Tris HCl (pH6.8)	4 ml
Glyrecol	10 ml
β-mercaptoethanol	5 ml
SDS	2 g
1% bromophenol blue	1 ml

Table 3: Composition of RIPA

Component	**Amount**
Tris	790 mg
NaCl	900 mg
Igepal (10%)	10 ml
10 Na-deoxycholate	10 ml
EDTA (100 mM)	1 ml

Table 4: Composition of RIPA-lysis buffer

Component	**Amount (µl)**
RIPA	1000
Apoprotinin (1µg/µl)	1
Leupeptin (1µg/µl)	1
Pepstatin (1µg/µl)	1
NA_3VO_4 (200mM)	5
NaF (200nM)	5
B-glycerophosphat	1.06 mg
pH 7,4	

Table 5: Composition of FISH buffer 10x

Component	**Amount**
Tris HCl	0.5 M
NaCl	2 M
$MgCl_2$	20 mM
Igepal	10% vol.

Table 6: Composition of lysis buffer Fish Buffer + protease inhibitors.

Composition	**Final concentrations**
Fish Buffer 10x	
PMSF (stock 200mM in EtOH 100%)	1mM
Glycerol	10% vol.
Leupeptin (stock 1mg/ml in UP)	1 ug/ml
Aprotinin (stock 1mg/ml in UP)	1 ug/ml
Soybean trypsin inhibitor (stock 10mg/ml in UP)	10 ug/ml
UP	To final volume

Cell count
The cells were mixed with shortly spinned down Trypan blue (1:1) to determine the life/death count. 10ul of mixture was put on a haemocytometer. Two of the four large squares were counted and averaged. The volume of one large square is 1ul, and the amount of cells was calculated.

Protein determination
The amount of protein was determined by a Bradford protein assay. In case the cells were lysed with RIPA-buffer, the amount of protein was determined by a Pierce protein assay.

For the Bradford protein assay, the cell lysates (2,5ul) were mixed with Bradford reagent (150ul of 117 µM Coomassie brilliant blue G-250 in 8.5% phosphoric acid) on a 96 wells plate. After 10 minutes, the absorbance of 595nm light was measured in a micro plate reader. The absorbance was compared to a standard of albumin dilutions in UP (BSA/UP: 2; 1,8; 1,5; 1,2; 1,0; 0,7; 0,3; 0 mg/ml). When the absorbance of the samples was out of the range of the standard curve, the samples were diluted (1:3) in UP.

In case of the Pierce protein assay, the cell lysates (10ul) were mixed with Working Reagent (200ul WR: 50 parts Reagent A, 1 part Reagent B) on a 96 wells plate. The mixture was incubated at 37°C for 30 minutes and the absorbance of 595nm light was measured in a micro plate reader. The absorbance was compared to a standard of albumin dilutions in UP (BSA/UP: 2; 1,8; 1,5; 1,2; 1,0; 0,7; 0,3; 0 mg/ml). When the absorbance of the samples was out of the range of the standard curve, the samples were diluted (1:3) in UP.

Western blot analysis
The cell lysates of all samples were analyzed with Western blot for the expression of the proteins Epac1, Epac2, VASP, phosphorylated ERK1/2 (pERK1/2) or total ERK1/2 (totERK1/2). For the analysis of the Epac proteins, 40 µg of protein per sample was loaded on the gel. For VASP 25 µg and for pERK1/2 and totERK1/2 10 µg protein. 5 µl of marker was loaded on the gel. The proteins in the samples were separated by molecular weight with SDS-PAGE. The gel used for analyzing the Epac protein was a 8% gel. For VASP, pERK1/2 and totERK1/2 a 10% gel was used. The composition of the gels is stated in Table 7.

Table 7: SDS polyacrylamide gels. A 8% gel for bigger proteins, a 10% gel for smaller proteins and a stacking gel to load the samples.

Components	**8% gel (ml)**	**10% gel (ml)**	**Stacking gel (ml)**
UP	4.0	4	1.4
Acrylamid mix (30%)	3.3	3.3	0.33
SDS-solution (10%)	2.5	0.10	0.02
Tris (pH 8.8)	0.1	2.5	0.25 (pH 6.6)
APS solution (10%)	0.1	0.1	0.02
TEMED	0.004	0.004	0.002

The SDS-PAGE was performed at 200V for 50 minutes in ELFO solution. For VASP, the gels ran for 5 minutes after the loading buffer had run of the gel. The separated proteins in the gel were transferred on a nitro? membrane at 100mA for 1 hour with transfer buffer on ice. The membranes were blocked with 5% milk TBS-T for 2 hours at room temperature. The membranes were incubated with the complementary anti-body for the desired protein in 2% milk TBS-T. (Epac1 1:500; Epac2 1:500; VASP 1:2000; pERK1/2 1:1000; totERK1/2 1:1000; ß-actin 1:2000) and incubated overnight at 4°C. The membranes were washed 4 times with TBS-T (1 time quickly and 3 times for 10 minutes on a shaker) and incubated with the complementary secondary antibody,

diluted in 2% milk TBS-T at room temperature for 1,5 hours. (Epac1, Epac2, ß-actin = 1:2000 anti-mouse; VASP, pERK1/2, totERK1/2 = 1:2000 anti-rabbit). The membranes were washed 4 times. The protein bands on the membrane were visualized in the dark room by incubating the membranes with 2ml ECL (ECL 1:1) and the emitted light was caught on film. The duration of exposing of the film to the emission varied per antibody. The film was developed and quantified by scanning densitometry using Totallab.

Stripping
The membranes used for analysis of ERK1/2 were used twice, ones for pERK1/2 and once for totERK1/2. To do so, the membranes were stripped after the first analysis. The membranes were washed 4 times and incubated with stripping buffer at 37°C? for 1 hour. The membranes were washed 4 times for 15 minutes and blocked with 5% milk TBS-T. The membranes were incubated with the primary antibodies, as described above.

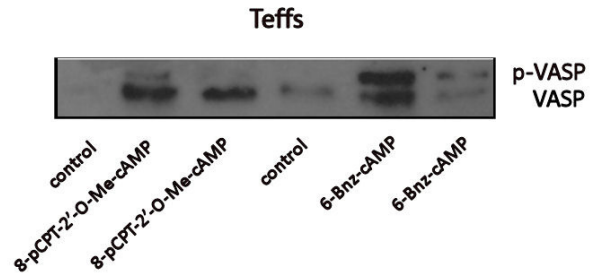
Data analysis
The data were presented as the mean ±SEM of *n* experiments. The expression of the analyzed proteins was corrected to a control protein, for equal loading of the samples. The expression of Epac1, Epac2 was corrected to the expression of ß-actin. The pERK1/2 was corrected to totERK1/2, and pVASP was corrected to VASP. Regarding the expression of Epac2 during culturing, ERK1/2 and VASP, the control conditions were set at 100% and the other samples were set as % of the control. The different conditions were compared to each other by an unpaired Student's t-test. Results with a P-value <0,05 were treated as significant (*).

Results

Activation of VASP

Here we studied the activation of VASP in Teffs and iTregs to prove the specificity of the analogs 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP for respectively PKA and Epac. Teffs and iTregs were stimulated with 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP for 48 hours and the percentage of activated VASP was measure. The results show that stimulation with 6-Bnz-cAMP causes the rate of activated VASP to increase in both Teffs and iTregs respectively with 467% and 407%, compared to the control. The effect of 8-pCPT-2'-O-Me-cAMP on the activation of VASP in Teffs and iTregs is respectively 96% and 88%, compared to the control (Fig 8). The conditions 60 and 500µM 6-Bnz-cAMP or 8-pCPT-2'-O-Me-cAMP were pooled to create a more powerful result. The results did not show a significant difference between the conditions.

A



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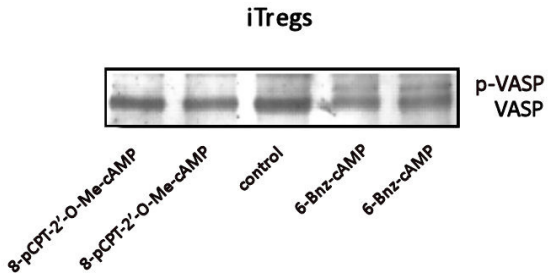

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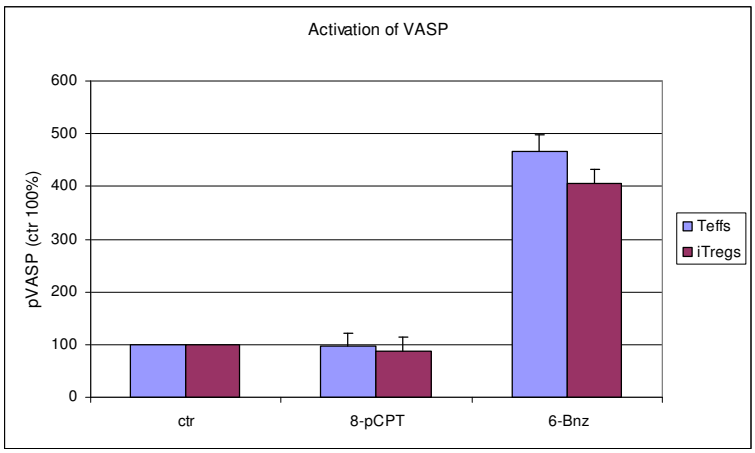


Figure 8. Effect of Epac and PKA analogs on the phosphorylation rate of VASP in Teffs (A) and iTregs (B) and the quantification (C). CD4+ human T cells were expanded in the presence or absence of TGF-ß. The cells were stimulated with 60 and 500µM 8-pCPT-2'-O-Me-cAMP or 6-Bnz-cAMP for 48 hours at 37 °C. The VASP expression was analyzed with Western blot and the ratio phosphorylated VASP of the total amount was calculated. The results of 60 and 500uM concentrations of 8-pCPT-2'-O-Me-cAMP or 6-Bnz-cAMP-cAMP were pooled. The data represent single results and the mean ±SEM of the pooled results. (iTregs control n = 1: Teffs control and 8-pCPT-2'-O-Me-cAMP and 6-Bnz-cAMP for both Teffs and iTregs n = 2). Both ctr are set at 100%.

Activation of ERK1/2

Here we studied the acute effect of cAMP of T cell proliferation by analyzing the percentage of activated ERK1/2 in Teffs and iTregs, after stimulated with 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP. Activated ERK1/2 is a measurement for proliferation. The blot in Figure 9 shows that ERK1/2 is present in both Teffs and iTregs and that the levels of activated ERK1/2 are influenced by the stimulation with both 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP at the different time points.

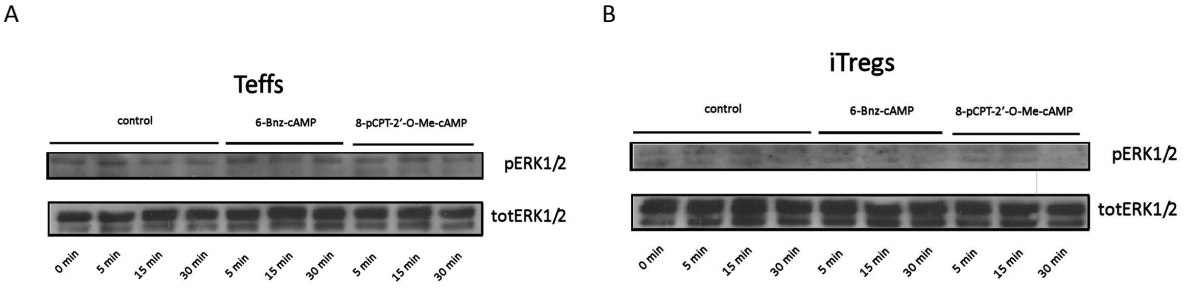
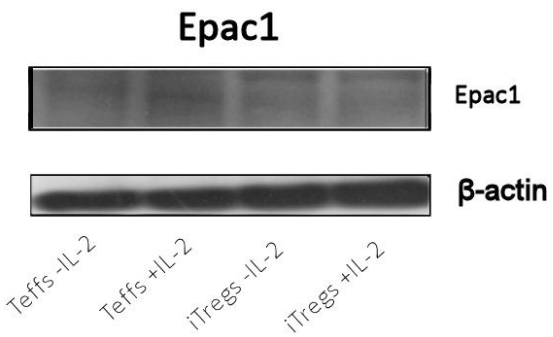


Figure 9. The acute effect of Epac and PKA analogs on the phosphorylation rate of ERK1/2 in Teffs (A) and iTregs (B). The cells were stimulated with 500µM 8-pCPT-2'-O-Me-cAMP or 6-Bnz-cAMP for 5, 15 and 30 minutes at 37°C. The pERK1/2 and totERK1/2 were analyzed with Western blot.

Expression of Epac1 and Epac2

Here we studied the expression of Epac1 and Epac2 in Teffs and iTregs and what influence IL-2 has on the expression. The results in Figure 10 show that the expression of Epac1 in iTregs, not stimulated with IL-2, is lower than in Teffs. Comparing the expression in iTregs and Teff when stimulated with IL-2, Epac1 expression is lower in iTregs as well. Regarding the expression in Teffs, the expression is higher when stimulated with IL-2. In iTregs, the expression is lower when stimulated with IL-2. The conditions are not significantly different from each other.

A



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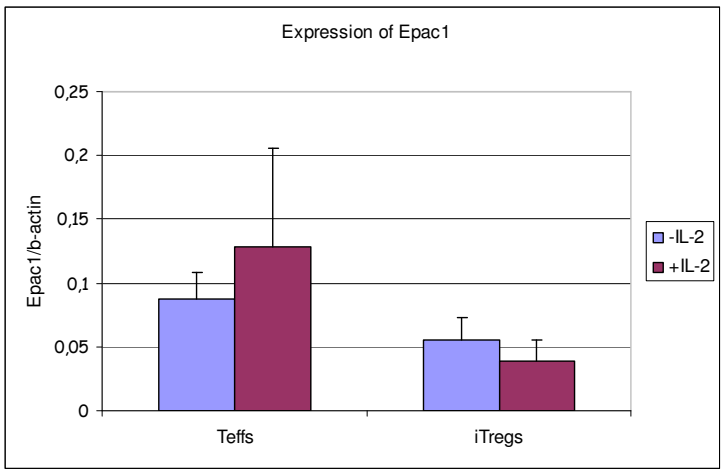
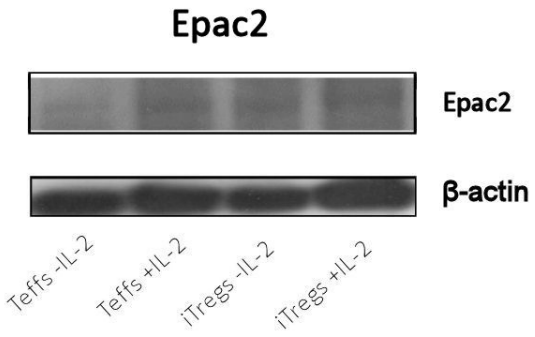


Figure 10. Expression of Epac1 in Teffs and iTregs. CD4+ human T cells were expanded in the absence or presence of TGF-ß for two weeks and stimulated with or without IL-2 for 48 hours. The cells were analyzed with Western blot (A). Equal loading was verified by analysis of ß-actin. The data (B) represent the mean ±SEM from 2 and 3 independent experiments (n = 2-3: Teffs +IL-2 and iTregs +IL-2 n = 2: Teffs-IL-2 and iTregs -IL-2 n = 3).

The results regarding the expression of Epac2 in iTregs and Teff are shown in Figure 11. Comparing Epac2 in Teffs and iTregs not stimulated with IL-2, the expression of Epac2 is almost equal in both cell types. Comparing the expression of Epac2 between iTregs and Teffs treated with IL-2, the expression of Epac2 is equal as well. When stimulated with IL-2, the expression of Epac2 decreases in both Teffs and iTregs. The results did not show a significant difference between any conditions.

A



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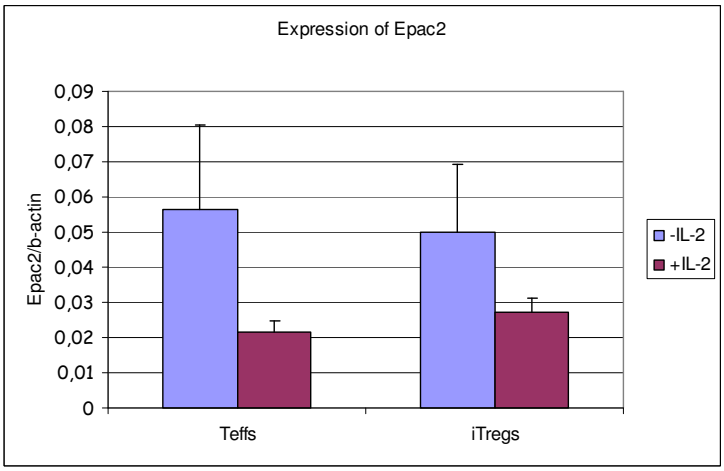

Figure 11. Expression of Epac2 in Teffs and iTregs. CD4+ human T cells were expanded absence or presence of TGF-ß for two weeks and stimulated with or without IL-2 for 48 hours. The cells were analyzed with Western blot (A). Equal loading was verified by analysis of ß-actin. The data (B) represent the mean ±SEM from 2 to 4 independent experiments (n = 2-4: Teffs +IL-2 and iTregs +IL-2 n = 2: Teffs-IL-2 and iTregs-IL-2 n = 4).

Conclusion/Discussion

As we found out in earlier research, iTregs are insensitive for proliferation inhibition by cAMP. In this project we investigate the acute effect of cAMP on cell proliferation and the expression of Epac1 and Epac2 in Teffs and iTregs and what role of IL-2 has on the expression of these two novel cAMP mediators.

Activation of VASP
The results regarding the activation of VASP show that Epac activator 8-pCPT-2'-O-Me-cAMP does not react with PKA in Teffs and iTregs. VASP was not more activated in cells stimulated with 8-pCPT-2'-O-Me-cAMP than in the control cells. The elevated level of activated VASP in Teffs and iTregs after stimulation with 6-Bnz-cAMP prove that 6-Bnz-cAMP act through PKA and is a working activator in this experiment. Furthermore, the results are also a measurement of reliability in this experiment. It tells us that the cells that were used do function in this type of experiment.

Activation of ERK1/2
By stimulating the Teffs and iTregs with cAMP analogs, we aimed to get a better perception of cAMP's acute effect on proliferation via PKA and Epac. Expected was that the stimulation with 8-pCPT-2'-O-Me-cAMP with in Teffs caused the levels of activated ERK1/2 to decline, where in iTregs it was expected to have no effect on ERK1/2. 6-Bnz-cAMP was expected to have no effect on the activation of ERK1/2 in both Teffs and iTregs, because earlier experiment of our group showed no effect 6-Bnz-cAMP on the proliferation of T cells. The results show that the rate of activated ERK1/2 in Teffs and iTregs is influenced by stimulation with 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP. Also, the results show that the method or the material in this experiment is not consistent enough to produce ambiguous results. Not all cell cultures were treated in the exact same way, which can cause the differences in the data. Furthermore, the design of the experiment itself and the small number of measurements could be the reason for inconsistent results. To get better data, changes in treating cell material and experimental design has to be made.

Expression of Epac1 and Epac2
The results of Epac1 expression show that the iTregs bare lower concentrations of Epac1 compared to the Teffs (Fig 10). This might be the reason why the iTregs are insensitive for cAMP proliferation inhibition via Epac. We looked at the possible influence of IL-2 on the expression of Epac1 and the results show that there is no difference in expression in both Teffs and iTregs. The results show an elevated expression in Teffs, but the SEM compared to the mean of the 2 experiments is quit high and therefore gives no difference in expression of Epac1. The study of Utako Yokoyama shows that the expression in Epac1 is downregulated by TGF-ß in fibroblast and fibroblast-like cells (U. Yokoyama, Patel et al., 2008). This is caused by cross-talk of TGF-ß with G protein-coupled receptor signaling via cAMP. This downregulation of Epac1 occurs by inhibition of transcription. iTregs used in this experiment were differentiated from CD4 positive T cells under the influence of TGF-ß into iTregs. This might be the reason for the downregulation of Epac1 in iTregs in this experiment.

Looking at the expression of Epac2 (Fig 11), there is no difference in expression between Teffs and iTregs. This indicates that Epac2 does not contribute to the insensitivity to cAMP in iTregs. But, both Teffs and iTregs show a downregulation of Epac2 after stimulation with IL-2. It could be that the experimental concentrations of IL-2 used in this experiment causes the expression to be downregulated, where the natural concentration of IL-2 in iTregs are not sufficient to do so.

As we conclude in this experiment, the cAMP insensitivity in iTregs might be due to the downregulation of Epac1. ERK1/2 activation via Raf-1 is blocked by activated Rap1. When Epac1 is downregulated, less Rap1 will be activated by cAMP and therefore iTregs are insensitive to proliferation inhibition by cAMP. The acute effect of cAMP on cell proliferation via Epac and PKA remain unclear, as well as the role of IL-2 in the expression of Epac proteins in Teffs and iTregs.

Essential in all in vitro research is that the conditions of the experimental cell cultures are different from the normal situation, which can cause cells to act differently. The results gathered from our research are an indication of how processes could work in their natural enviroment. It is hard to say in what way this experiment gives a good reflection of the normal situation. As described, cAMP is a second messenger that is spatio-temporal dynamic. The signal transduction via cAMP is dependent of the concentration cAMP, the locations of the effector proteins and possible binding to AKAPs. These characteristics can be influenced by the

experiment and vary from its normal behaviour. Furthermore, we used iTregs in stead of nTregs and aTregs, which naturally occur in the human body.

As described above, the number of measurements has to be enlarged to get more powerful results for the expression of Epac1 and Epac2, and the acute effect of cAMP in Teffs and iTregs. Furthermore, research on other downstream proteins of Epac and PKA will give a clearer indication of the effect of cAMP in T cells. Protein directly activated via Epac and PKA are Rap1 and Rap2. A pull down assay of these proteins could give more insight of the acute effect of cAMP on proliferation in Teffs and iTregs.

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Appendix

WERK1/2protocol voor expansie van Tregs met TGF-ß en pbCD3/sCD28.
(volgens Shevach et al, Blood 2007)

1. Expansie van iTregs met TGF-ß.

Benodigdheden:

- MACS buffer: PBS + 0,5%BSA (0,5g in 100ml) + 2mM EDTA (0,075g in 100ml)
- CD3 (clone UCHT1) BD in 10µg/ml
- CD28 (clone 28.2) BD555725 in 2µg/ml
- TGF-ßeta (Peprotech): 5ng/ml (=0,5µl uit 5µg/500µl stock/ml celsusp.)
- IL4, IL12 en IFNgamma en IL6 (R&D): 5µg/ml (=5µl uit 500µg/500µl stock/ml celsusp.)
- IL2: 5µl uit cupje '100U/10µl' (-80˚C) in 1ml celsuspensie (=50U/ml).
- X-VIVO20: X-VIVO (-HPS!)+pen/strep+50µM 2ME

Protocol:
Dag -1, dag 6, dag 13

- Coat een 24 (voor dag -1) wells plaat, voor andere dagen afhankelijk van celaantal met CD3:
    - Verdun CD3 100x in steriele PBS. Reken voor elke 24well 250µl volume, 12 wells 500µl volume en 6 wells 750µl volume.
    - Doe de plaat in een bakje met natte doeken en doe het deksel op het bakje.
    - Incubeer 24-48 uur in de koelkast of minimaal 2 uren in 37˚C (zonder natte doeken en bakje).
    - Verwijder het antilichaam en de PBS uit de wells.
    - Was de wells 2 keer met steriele PBS.

Dag 0

- Isoleer PBMCs uit volbloed met lymphoprep. Kleur voor CD4 selectie en CD25-PE meteen erbij.
- Isoleer CD4+Tcells met MACS.
- Isoleer de CD25- cells m.b.v. MoFLo.
- Plaat de Tresp cellen uit in de met CD3 gecoate 24 wells plaat à $0,5*10^6$cells/ml in X-VIVO). Met 2µg/ml CD28 en 50U/ml IL2.
    - Maak celsuspensie (voor bv 2x6 wells): 12ml totaal met $6*10^6$ cells, + 60µl IL2, +24µl CD28
        - -TGF-ß (6 wells (24)): 1ml per well daarna;
        - +TGF-ß (6 wells (24)): + 3µl TGF-ß, + 30µl anti-IL4 etc.; 1ml/ well
- Incubeer in 37˚C/ 5%CO_2 stoof voor 5 dgn (check natuurlijk de cellen en de kleur van het medium).
    - Mocht het medium te geel worden op dag 4 voeg dan 0,5ml X-VIVO toe.
- Bepaal van een overgebleven deel (+/-$0,25*10^6$cells) het % FOXP3-AF647 (clone 206D), CD127-FITC met FACS op T=0.

Dag 5

- Resuspendeer de cellen uit de -/+TGF-ß wells en verzamel in 2 aparte FACS buis.
- Tel het celaantal en bepaal de viability m.b.v. Burker Turk.
- Draai de cellen af en zuig het supernatant af.
- Splits de cellen in een schone 6 wells plaat (3ml/well/ ▯ 3,5*10^6cells):
  - –TGF-ß (per 1 wells (6)): + 3ml X-VIVO, +15µl IL2
  - +TGF-ß (per 1 wells (6)): + 3ml X-VIVO,+ 15µl IL2 (geen TGF-ß en anti-IL4 etc.)
- Incubeer voor 2 dgn in 37˚C/ 5%CO_2.

Dag 7.

- Resuspendeer de cellen in de wells goed en breng over in een 15ml buis. Was de wells met 1ml RPMI en draai af op 590g, 5 minuten en 4˚C. Neem, voor het afdraaien 10µl voor het celaantal en de viability (doe het celaantal keer x i.v.m. x ml totaal volume)
- Zuig het supernatant af en neem het pellet op in 1ml X-VIVO.
- Neem 0,5*10^6 cells voor FACS FOXP3.
- Verdeel ongeveer 2x2*10^6 cellen/ 2x1ml/ well(24) op een met CD3 gecoate 24 wells plaat:
  - -TGF-ß (2 wells (24)): 2ml totaal+ 4µl CD28, +10µl IL2
  - +TGF-ß (2 wells (24)): 2ml totaal+ 4µl CD28, +10µl IL2, +1µl TGF-ß, +10µl anti-IL4 etc.
  - Pipeteer 1ml per well.
- Incubeer voor 24 uur in 37˚C/ 5%CO_2.
- Bepaal %FOXP3 en dead cells.
- Zet gewenste experimenten met de cellen in.

Dag 8

- Resuspendeer de cellen (na 24hr stimulatie) en pipeteer in een schone 24 of12 wells plaat. Niet wassen en verder geen toevoegingen.

Dag 10

- Resuspendeer de cellen uit de -/+TGF-ß wells en pipeteer 1:1 naar 12 wells plaat.
- Splits de cellen naar 2 wells van 12 wells plaat (2ml/well, dus +1ml per well):
  - –TGF-ß (2 wells (12)): per well + 1ml X-VIVO +10µl IL2
  - +TGF-ß (2 wells (12)): per well + 1ml X-VIVO +10µl IL2 + 10µl anti-IL4 etc., +1µl TGF-ß.
- Resuspendeer de cellen uit de -/+TGF-ß wells en pipeteer naar een 6 wells plaat (+/- 6*10^6 cells/ well/ 3ml). Dus aanvullen met X-VIVO naar benodigde aantal mls.
  - –TGF-ß: per well + x ml X-VIVO +5µl IL2/ml
  - +TGF-ß: per well + x ml X-VIVO +5µl IL2/ml + 5µl/ml anti-IL4 etc., +0,5µl/ml TGF-ß
- Incubeer voor 3/4 dgn in 37˚C/ 5%CO_2.
- Meet het %FOXP3 en dead cells op dag 13.
- Rest van de cellen gebruiken voor vervolg experimenten op dag 13.