Novel Mode of Interference with Nuclear Factor of Activated T-cells Regulation in T-cells by the Bacterial Metabolite n-Butyrate

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The transcription factor nuclear factor of activated T-cells (NF-AT) plays an essential role in the activation of many early immune response genes. A dynamic equilibrium between calcineurin and cellular kinases controls its phosphorylation and thus regulates its activity by determining its subcellular localization. Here, we demonstrate that T-cell activation in the presence of the bacterial metabolite n-butyrate, which leads to inhibition of interleukin-2 transcription, is characterized by the maintenance of the activity of counter-regulatory kinases glycogen synthase kinase 3 and protein kinase A as well as persistence of intracellular cAMP levels, whereas calcium response and mitogen-activated protein kinase activation were indistinguishable from cells stimulated in the absence of n-butyrate. Nuclear binding of NF-AT was decreased but other transcription factors implicated in interleukin-2 expression such as AP1 and nuclear factor κB were unaffected. The effect on NF-AT binding appeared to be the result of increased nuclear export because the export inhibitor leptomycin B completely restored nuclear binding of NF-AT. We, therefore, provide first evidence for interference with NF-AT regulation alternative to the currently understood inhibition of nuclear import. This mechanism might represent a bacterial strategy to subvert host defense, which could be of particular clinical importance in the gastrointestinal tract where high amounts of n-butyrate are physiologically present.

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The abbreviations used are: TCR, T-cell receptor; AP1, activating protein-1; CRM1, exportin; GSK-3, glycogen synthase kinase 3; LAT, linker for activation of T-cells; Lck, tyrosine kinase p56

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though much progress has been made recently regarding the recognition of the different ways of subversion, the knowledge about the actual means of interference as well as the molecular mode of action is still limited.

The short chain fatty acid n-butyrate is produced in considerable amounts by bacterial fermentation in the human gastrointestinal tract (13). In addition to its well known function as an essential energy source for colonocytes, n-butyrate has anti-inflammatory and immunosuppressive effects in vitro and in vivo (14–17), which might be exploited by the intestinal microflora to evade host defense (18). Regarding T-cells, this bacterial metabolite has been shown to inhibit their expansion (19–21). Importantly, the recently reported ability of n-butyrate to inhibit antigen-specific immune reactivity in vivo indicates a potential clinical relevance (22, 23). A central feature of n-butyrate-mediated inhibition of T-cell expansion is the abrogation of IL-2 production (17, 24), which appears to be an important determinant of anergy induction. To obtain a better understanding of the mode of bacterial interference, we investigated the molecular mechanisms underlying the inhibition of IL-2 production by n-butyrate. Using Jurkat and human primary T-cells, we demonstrate that n-butyrate selectively inhibits NF-AT function in activated T-cells. In contrast to FK506 or other macrolide underdecapeptides, n-butyrate does not prevent the nuclear import of NF-AT, but rather promotes its export leading to abrogation of IL-2 synthesis. Such accelerated NF-AT export is accompanied by sustained cAMP levels, as well as PKA and GSK-3 activity akin to that observed in nonstimulated cells.

EXPERIMENTAL PROCEDURES

Isolation of T-cells from buffy coats, culturing conditions of Jurkat T-cells and stimulations, reporter gene assays, depletion of protein kinase C (PKC) by prolonged PMA treatment, IL-2 ELISA, Bradford assay, Western blot analysis, preparation of total RNA, radio labeled probes, nuclear extracts, and electrophoretic mobility shift assays (EMSA) were performed as described (25–31).

Antibodies and Reagents—Antibodies directed against tyrosine kinase pS6κ (Lck), linker for activation of T-cells (LAT), GSK-3, and phosphatidylinositol 3-kinase (PI3K) were from Upstate Biotechnology, Inc. (Lake Placid, NY), antibodies against Zap70 were bought from Transduction Laboratories (Franklin Lakes, NJ), and anti-NF-AT1 and anti-NF-AT2 antibodies were from Upstate Biotechnology, Inc. and Alexis (San Diego, CA). The p-JNK1/2 antibody was bought from Prosci (Madison, WI). Antibodies directed against p-Erk1/2, Erk1/2, c-Jun, and phospho-c-Jun antibodies were from Cell Signaling (Beverly, MA). Antibodies directed against p-Erk1/2, Erk1/2, p-JNK1/2, JNK1/2, p-p38, p38, p-pan-PKC, PKC-δ, and p-GSK-3 were from New England Biolabs/Cell Signaling (Beverly, MA). CD3 mAb (OKT3) was from Ortho Diagnostics (Raritan, NJ), CD28 mAb (Leu-28) from BD Pharmingen. PMA and ionomycin were bought from Sigma, and Rabbit anti-Lck antibody were coupled to 50 μl of Sepharose G beads (Amersham Biosciences). After washing the beads, they were resuspended in 1 ml of buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.02 units/ml aprotinin, 0.01% sodium azide, and 1% Nonidet P-40 on ice for 10 min). The lysate was mixed 1:1 with an 80% sucrose solution (sucrose had been dissolved in 25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 2 mM EDTA) before loading onto a sucrose gradient. The gradient consisted of a stepwise addition of 2 ml each of 80, 60, 40, 30, 20, and 10% sucrose. After centrifugation (SW40 rotor with 37,500 rpm for 18 h at 4°C), 666-μl fractions were collected from the top of the gradient. Protein content of the fractions was determined with a Bradford assay (Bio-Rad) according to the manufacturer’s protocol.

Western Blot Analysis and Immunoprecipitation of Sucrose Fractions—For the in vitro kinase reaction, 20 and 40% sucrose fractions were pooled (10 μl of each fraction) and diluted to 120 μl in kinase buffer (25 mM HEPES, pH 7.3, 150 mM NaCl, 5 mM MgCl2). Then 10 μl of [γ-32P]ATP (Amersham Biosciences) was added and the reaction incubated for 10 min at 30°C. 20 μl of each of reaction were used for SDS-PAGE (4–20% Tris-glycine gel; Novex). After electrophoresis, the gel was fixed (10% acetic acid, 40% methanol) before drying. The gel was then subjected to autoradiography as described above. For immunoprecipitation, 5 μg of rabbit anti-Lck antibody were coupled to 50 μl of Sepharose G beads (Amersham Biosciences). After washing the beads, they were resuspended in 1 ml of buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). 200 μl of a radiolabeled kinase reaction from the 20% sucrose fraction (see above) above was added and incubated with the beads for 1 h at 4°C with constant rotation. The beads were subsequently collected by centrifugation and washed four times with the precipitation buffer. The beads were resuspended in SDS sample buffer with subsequent PAGE. After electrophoresis, the gel was fixed and subjected to autoradiography as described above.

RESULTS

Inhibition of IL-2 Production—In the low millimolar concentration range, n-butyrate is known to inhibit alloantigen-stimulated T-cell expansion. Furthermore, it causes antigen-specific hyporesponsiveness in restimulation assays (19–21, 23, 30, 32). This phenomenon is accompanied by a strong suppression of T-cell produced cytokines. To address the corresponding molecular mechanism of n-butyrate, we have chosen the Jurkat T-cell line as a model system. As a surrogate readout for the inhibitory effects of this compound, anti-CD3/CD28-induced IL-2 production as determined by ELISA was used (Fig. 1A). When n-butyrate (1 mM) was added at culture initiation, TCR-driven IL-2 secretion was prevented. To crudely address the level of inhibition (transcriptionally or post-transcriptionally), an RNA protection assay was performed to measure IL-2 mRNA levels. For each sample 50 μl of Amplification G-Sepharose (Amersham Biosciences) were incubated with 5 μg of anti-GSK-3 antibody in lysis buffer at 4°C for 1 h with constant rotation. After washing the beads four times with lysis buffer, the cell lysate was added and again incubated for 1 h at 4°C with constant rotation. Finally, the immunoprecipitate was washed three times with lysis buffer and two times with GSK-3 kinase buffer (8 mM MOPS, 0.2 mM EDTA, 10 mM magnesium acetate, pH 7.3). 1 μg of glyceron synthetase peptide (Upstate Biotechnology, Inc.) and 10 μl of [γ-32P]ATP (Amersham Biosciences) were added and the reaction incubated at 30°C for 30 min. Subsequently reducing sample buffer was added and an aliquot subjected to SDS-PAGE (16% Tricine gel; Novex, La Jolla, CA). The gel was fixed and stained with 10% acetic acid, 40% methanol) for 1 h and dried before exposing to a Biomax MR film (Eastman Kodak Co.).

Results—Per sample, 1 × 105 Jurkat T-cells were used. Cell lysis and intracellular cAMP measurements were performed using an enzyme immunoassay (BIOTRAK, Amersham Biosciences) according to the manufacturer’s protocol.

Preparation of Rafts/Sucrose Gradient—2–105 Jurkat T-cells were either left nonstimulated or activated for 1 min before lysis (10 μM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM EDTA, 1 mM trisodium orthovanadate, 30 mM sodium pyruvate, 10 mM glycerophosphate, 1 mM PMSF, 0.02 units/ml aprotinin, 0.01% sodium azide, and 1% Nonidet P-40 on ice for 10 min). The lysate was mixed 1:1 with an 80% sucrose solution (sucrose had been dissolved in 25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 2 mM EDTA) before loading onto a sucrose gradient. The gradient consisted of a stepwise addition of 2 ml each of 80, 60, 40, 30, 20, and 10% sucrose. After centrifugation (SW40 rotor with 37,500 rpm for 18 h at 4°C), 666-μl fractions were collected from the top of the gradient. Protein content of the fractions was determined with a Bradford assay (Bio-Rad) according to the manufacturer’s protocol.

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steady state mRNA levels. Although upon TCR engagement IL-2 mRNA increased compared with nonstimulated cells (no RNA detectable; see Fig. 1B, lanes 1 and 2), n-butyr ate totally blocked this induction (Fig. 1B, lanes 2 and 3). This finding indicates that the generation of a stable IL-2 message was prevented. To further demonstrate that IL-2 transcription is affected by n-butyr ate, induction of an IL-2 promoter-driven luciferase reporter gene was evaluated. Again, in this setting n-butyr ate strongly reduced IL-2 promoter-dependent luciferase synthesis after stimulation with CD3/CD28 mAb or PMA plus ionomycin (Fig. 1C). These data confirm that Jurkat T-cells are a suitable model system to investigate the molecular mechanisms responsible for n-butyr atemediated IL-2 inhibition, showing that the block affects the assembly of the transcription factor machinery at the IL-2 promoter.

Differential Modulation of Early Signaling Events—n-Butyrate is known to cause diminished population doubling rates, altered morphology, decreased anchorage-independent growth, and increased expression of colon epithelial differentiation marker enzymes such as alkaline phosphatase in colon carcinoma cells (33–35). The latter effects were attributed to a decreased activity of p56lck (36), a protein-tyrosine kinase that plays an essential role in TCR stimulation. In activated Lck+/H11002+/H11002 T-cells, endogenous Fyn predominantly substitutes for this kinase with one notable exception, the activation of NF-AT and consequently IL-2 production (37). The latter is reminiscent of the effects observed with n-butyr ate in our model system. It was therefore decided to investigate the function of Lck in terms of its localization in (lipid) rafts and its associated kinase activity in Jurkat T-cells stimulated in the presence or absence of n-butyr ate (1 mM). As illustrated (Fig. 2A), there is successful separation of rafts (20% sucrose fraction) versus the bulk of the cytoplasmic proteins (40% sucrose fraction) confirmed with Western blot analysis of marker proteins. As described, LAT was clearly detected in both fractions (compare lanes 1, 3, and 5, corresponding to the rafts, with lanes 2, 4, and 6, correspond-
ing to the cytosolic preparation). In addition, trace amounts of Lck (comparable with the 5.8% that were reported recently) were seen in the raft compartment (see especially lanes 3 and 5). In contrast, PI3K and Zap70, which both comprise cytoplasmic proteins, are exclusively present within the 40% fraction (Fig. 2A, lanes 2, 4, and 6). To normalize for protein content, a Bradford assay was performed showing equal amounts of protein independent of the activation status of the cells (Fig. 2B). However, in an in vitro kinase assay using the isolated rafts as source of kinase activity, increased phosphorylation of a pro-

FIG. 3. A, n-butyrate does not affect intracellular calcium mobilization. For measurement of free intracellular calcium levels, cells were loaded with the membrane-permeable penta-acetoxymethylester FLUO3/AM. After stimulation with CD3 and CD28 mAb (αCD3/28) in the presence (●) or absence (▲) of n-butyrate (n-but), measurements were performed using a fluorimeter (♀, unstimulated). B, influence of n-butyrate (1 mM) upon the activation of MAPK Erk, JNK, and p38. Western blot analysis was performed, and both the constitutive form and the phosphorylated (active) form of the enzymes were investigated at 5, 10, and 15 min after CD3 and CD28 mAb (αCD3/28) stimulation. Phosphorylation of the kinases was quantified by densitometry and was normalized to the expression of the constitutive forms. C, PKC activation is not influenced by n-butyrate. Pan-phospho-PKC- and a phospho-PKCα-specific Western blot analysis (lanes 2 and 3) were performed using extracts from Jurkat T-cells stimulated with CD3 and CD28 mAb in the presence or absence of n-butyrate. Phosphorylation of pan-PKC and PKCα was quantified by densitometry. norm, normal. D, to exclude that any PKC isoform conveys a negative signal and consequently mediates suppression of IL-2 transcription, Jurkat T-cells were depleted of PKC by prolonged treatment with PMA. Then they were further stimulated in the presence/absence of n-butyrate, and IL-2 transcription was studied by reporter gene assays.

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factor, CD28 mAb in the presence or absence of T-cells. Using extracts from Jurkat T-cells stimulated with CD3 and restores the stimulation-induced decrease in PKA activity in Jurkat, intracellular cAMP levels were measured by ELISA.

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the identity of this activity/protein band was further revealed by immunoprecipitation analysis as p56

Substitution of Lck by Fyn in Lck

T-cells leads to an indistinguishable activation of both major signaling pathways after TCR stimulation. Ca

mobilization and the branched Ras/Rac-activated protein kinase cascades with Erk and p38 mitogen-activated protein kinases (MAPK) phosphorylation taken as endpoints were essentially unaffected in these Lck

cells (26). A similar result was observed in Jurkat T-cells stimulated in the presence of 1 mM n-butyrate (Fig. 3, A and B). Such treatment had no effect on Ca

mobilization during the first 7 min after anti-CD3/CD28 stimulation (Fig. 3A). Additionally Erk1/2 and p38 were phosphorylated/activated independent of whether n-butyrate is applied, as illustrated in a time kinetic analysis by Western blotting using phosphospecific antibodies (Fig. 3B). Regarding JNK, constitutive phosphorylation was observed, which was not influenced by n-butyrate (Fig. 3B). PKC, which comprise a parallel and/or alternative activation pathway to the MAPK (38), also became fully activated as visualized in a pan-phospho-PKC- and a phospho-PKC\(^{\text{\theta}}\)-specific Western blot analysis in the presence of 1 mM n-butyrate (Fig. 3C, lanes 2 and 3). To rule out that any PKC isoform conveys a negative signal resulting in the observed suppression of IL-2 transcription, Jurkat T-cells were depleted of the classical and novel isoforms by a prolonged treatment with PMA. However, in an IL-2 reporter gene assay, these cells were still susceptible to n-butyrate-mediated inhibition (Fig. 3D). This strongly indicated that the suppressive effect observed is not mediated by PKC activities.

As the major activating pathways are unaffected by n-butyrate, we started to explore cascades that are abrogated in the course of T-cell activation and regarded to act negatively. Li et al. (39) recently reported that anti-CD3/CD28 triggering of Jurkat T-cells is accompanied by a decrease in the intracellular concentration of cAMP. In contrast, it is well known that increased cAMP levels negatively impact upon T-cell activation including IL-2 production (8, 40). Our results with Jurkat T-cells showing a reduction of \(-50\%\) in intracellular cAMP level 15 min after anti-CD3/CD28 stimulation are in line with these reports (Fig. 4A). Importantly, pretreatment with n-butyrate for 30 min prevented this effect, providing a situation identical to that observed in nonstimulated cells.

cAMP-dependent PKA reflects the concentration of its co-factor (41). Using whole cell lysates and a PKA-specific peptide as a substrate, we found that n-butyrate greatly preserves the kinase activity comparable with those of resting cells. Stimulated cells, in contrast, responded with a strong decrease in kinase activity (Fig. 4B, lanes 1–3). To support these findings functionally, the effect of the selective PKA inhibitor (\(R_{\text{p}}\)-8-Br-cAMP (42) on IL-2 transcription was studied in an IL-2 reporter gene assay. 100 \(\mu\)M amount of the analog reverses the inhibition of IL-2 transcription by n-butyrate (Fig. 4C). This finding therefore strongly implies that sustained PKA activity is causally involved in the abrogation of IL-2 synthesis upon drug treatment.

In T-cells previous data imply that PKA and GSK-3 together negatively influence cytoplasmic components of NF-AT, a major transcription factor at the IL-2 promoter. In addition, retrovirus-mediated overexpression of GSK-3 was recently shown to prevent T-cell proliferation and IL-2 synthesis (9, 11). In model organisms (Dictyostelium), this usually constitutively active kinase is regulated by Wnt/Wg signaling or alternatively by cAMP (43). This prompted us to investigate the importance of this kinase in our byturate model. Western blot analysis (Fig. 5A) with extracts from Jurkat T-cells using a phosphospecific anti-GSK-3 antibody directed against the negative regulation site(s) serine 9/21 (GSK3(\beta)/GSK3(\alpha)) was performed. Levels of phospho-GSK increase significantly after anti-CD3/CD28 stimulation, a response that is prevented by n-butyrate addition (Fig. 5A). This posttranslational modification correlates with kinase activity (Fig. 5B). Immunoprecipitated GSK-3 from the same Jurkat cell lysate was used in an in vitro kinase assay with a glycyanogen synthase peptide as a substrate. Both non-stimulated and n-butyrate-treated Jurkat T-cells have high levels of GSK-3 activity (Fig. 5B, lanes 1 and 4). In contrast, stimulation with anti-CD3/CD28 that led to a phosphorylation at Ser 9/21 correlates with markedly reduced kinase activity (Fig. 5B, lane 2). Because n-butyrate did not affect activity when added to extracts of stimulated T-cells (Fig. 5B, lane 3), a direct effect upon GSK-3 appears unlikely. To link these findings functionally with the observed inhibition of IL-2 transcription, the effect of lithium chloride (LiCl), a known inhibitor of GSK-3, was studied in a reporter gene assay (11). Interestingly, concomitant application of LiCl partially reverted the n-butyrate-mediated reduction of IL-2 synthesis (Fig. 5C). Taken together, these data imply that modulation of GSK-3 activity is indeed involved in mediating the inhibitory effects of n-butyrate.

Selective Inhibition of NF-AT Nuclear Binding—In addition to its function as a glycyanogen synthase kinase, GSK-3 has been identified as a member of the NF-AT kinase complex (9). In conjunction with other kinases, it mediates the re-phosphorylation of this transcription factor and thus its nuclear export. This study also provides evidence that PKA might be an additional component acting as a priming kinase to generate optimal conditions for GSK-3 activity. As IL-2 transcriptional activation fully depends on the presence of a sufficient amount of functional NF-AT in the nucleus, we speculated at this point that the altered activities of PKA and GSK-3 after n-butyrate treatment integrate at the level of this transcription factor. Therefore, gel shift analyses were performed using nuclear extracts from Jurkat T-cells stimulated with CD3/CD28 monoclonal antibodies in the presence or absence of n-butyrate. As radiolabeled probes binding sites of NF-AT but also of NF\(\kappa\)B and API were used to address the three major transcription factors implicated in the regulation of IL-2 synthesis. En-
Phosphorylation of the kinase was quantified by densitometry and was normalized to the expression of the constitutive form (-butyrate-induced inhibition of IL-2 transcription. Reporter gene assays were performed in transfected Jurkat T-cells stimulated with CD3 and assay using extract from Jurkat T-cells stimulated with CD3 and CD28 mAb in the presence (GS peptide) as a substrate. A direct effect of -butyrate treatment prevents the activation-induced decrease in GSK-3 activity. After immunoprecipitation GSK-3 from nuclear extracts from Jurkat T-cells stimulated with CD3 and CD28 mAb in the presence of -butyrate was employed in in vitro kinase assays using glycogen synthetase peptide (GS peptide) as a substrate. A direct effect of -butyrate on GSK-3 was excluded by its addition to an in vitro kinase assay using extract from Jurkat T-cells stimulated with CD3 and CD28 mAb in the absence of -butyrate (a). C, lithium reverses the -butyrate-induced inhibition of IL-2 transcription. Reporter gene assays were performed in transfected Jurkat T-cells stimulated with CD3 and CD28 mAb in the presence or absence of -butyrate and under the influence of lithium chloride (Li).

Enhanced binding activity was observed for all three transcription factors in extracts of anti-CD3/CD28 stimulated Jurkat cells (Fig. 6, lanes 2 and 3, 10 and 11, and 18 and 19). Strikingly, -butyrate treatment totally abolished the activation-induced NF-AT binding (Fig. 6, lanes 3 and 4), whereas the binding of NFκB and AP1 remained essentially unaffected (Fig. 6, lanes 11 and 12 as well as lanes 19 and 20). An SP1 gel shift analysis (lanes 25–32) served as a normalization control for the nuclear extracts. The specificity of the observed binding was analyzed in corresponding competition assays for each of the transcription factors (lanes 5–8, 13–16, 21–24, and 29–32). These results clearly indicate that only NF-AT function is altered in -butyrate-treated T-cells.

Impact of Nuclear Export on Decreased NF-AT Binding—The finding of a diminished NF-AT binding in a gel shift analysis, however, does not allow the discrimination between a block of NF-AT import (as after FK506 treatment) and enhanced export. To differentiate between these two possibilities, gel shift analyses as well as Western blot analyses were performed. Leptomycin B was used as a general inhibitor of exportin (CRM1)-dependent export (44); additionally, LiCl, which partially reverted the inhibition mediated by -butyrate in functional assays, was used with our standard stimulation conditions. If applied in addition to -butyrate, both compounds were able to substantially increase nuclear binding of NF-AT (Fig. 7A, compare lane 4 to lanes 5 and 6). Applied alone, however, they had no effect (lanes 7 and 8), suggesting that the combination of anti-CD3/CD28 activation and -butyrate does not prevent the import but accelerates the export of NF-AT. To further substantiate this finding, the import inhibitor FK506 was applied to anti-CD3/CD28-stimulated cells in the presence or absence of leptomycin B (lanes 9 and 10). In both cases no NF-AT binding is detectable, a picture that clearly differs from the one after -butyrate treatment. This indicates that the block by -butyrate occurs later in the process of T-cell activation than the block mediated by a nuclear import inhibitor. To show that not only the binding activity of NF-AT is lost after -butyrate treatment, but that this factor is no longer detectable in the nuclear compartment, Western blot analysis of the same nuclear extracts was performed using antibodies against NF-AT1 (Fig. 7B) and NF-AT2 (data not shown). Here, an observation analogous to the gel shift analyses was made, proving that -butyrate leads to a complete physical elimination of NF-AT from the nucleus in the process of T-cell activation. Furthermore, we show that the selective PKA inhibitor (R)-8-Br-cAMP was able to counter the effect of -butyrate on NF-AT nuclear binding in EMSA as well as the effect of -butyrate on NF-AT subcellular localization in Western blot analysis (Fig. 7, C and D, respectively). These findings, together with the ability of the PKA inhibitor (R)-8-Br-cAMP to revert the -butyrate-mediated inhibition of IL-2 transcription as shown in reporter gene assays (Fig. 4C), strongly suggest that sustained PKA activity in -butyrate-treated cells is indeed related to impaired NF-AT activation. In further experiments, the effect of leptomycin B on IL-2 transcription was studied in an IL-2 reporter gene assay. 50 nM amount of the export inhibitor to some extent reverses the inhibition of IL-2 transcription by -butyrate (Fig. 7E).

Impact of -Butyrate upon the Activation of Primary T-cells—To examine the validity of our observations in primary T-cells, we first evaluated the effect of -butyrate upon IL-2 production in activated primary T-cells by ELISA. As shown in Fig. 8A, -butyrate inhibited IL-2 production in activated primary T-cells. We further tested the effect of -butyrate on NF-AT nuclear binding by EMSA, as well as on the presence of NF-AT in the nuclear compartment of activated T-cells. In complete agreement with our findings in Jurkat T-cells, we demonstrate that -butyrate inhibits NF-AT nuclear binding (Fig. 8B) as well as nuclear accumulation of NF-AT in activated primary T-cells (Fig. 8C). Addition of leptomycin B restored both the nuclear binding in EMSA (Fig. 8B, lane 4) as well as NF-AT levels in the nucleus (Fig. 8C, lane 4). Taken together,
nuclear export machinery of this transcription factor. Given the essential role of NF-AT in the regulation of immune cells, it is tempting to speculate that bacteria employ this mechanism to escape immune surveillance. This might be of particular relevance to the intestinal microflora, which produce high amounts of this metabolite.

Binding of NF-AT to the IL-2 promoter is controlled by two major mechanisms: the nuclear import of this transcription factor initiated by T-cell activation and its nuclear export, which terminates T-cell activation (8). Phosphorylation of NF-AT is considered to affect its subcellular localization by modulating both its import and export rates. It has been reported that, in both activated and resting T-cells, the extent of NF-AT phosphorylation is determined by a dynamic equilibrium between calcineurin and cellular kinases (45). Changes in this equilibrium, either through calcineurin activation or kinase inhibition, result in dephosphorylated NF-AT and nuclear import of the transcription factor. Nuclear export of NF-AT is enabled by Ser/Thr protein kinases, such as GSK-3, casein kinase I and II, and JNK. GSK-3 prefers to phosphorylate serines adjacent to serines, which were phosphorylated previously by PKA (9). Binding of NF-AT to CRM1 via a nuclear export signal sequence then mediates the nuclear export of the transcription factor terminating transcriptional activation (46). The persistence of GSK-3 and PKA activity after T-cell activation in the presence of n-butyrate as observed in this study would be compatible with a shift in the equilibrium of NF-AT phosphorylation. Other kinases such as p38 and JNK might not contribute to the mechanism engaged by n-butyrate, as they were unaffected by this short chain fatty acid. Furthermore, blocking PKA activity by the selective PKA inhibitor (R-)-8-Br-cAMP or GSK-3 by LiCl in activated and n-butyrate-treated T-cells was followed by restored NF-AT nuclear binding and IL-2 transcription. Finally, addition of the CRM1 inhibitor leptomycin B on top of n-butyrate to stimulated cells led to the accumulation of NF-AT in the nuclear compartment associated with increased nuclear binding, whereas applying leptomycin B in unstimulated cells did not bring about an increase in nuclear NF-AT. This indicated that n-butyrate treatment had allowed nuclear import but prematurely terminated nuclear binding by promoting the export of the transcription factor.

High intracellular cAMP levels in T-cells induced by compounds like forskolin or dibutyryl cAMP are well known to inhibit T-cell activation and proliferation (39, 47). A clear correlation between high cAMP levels to PKA activation via dominant negative mutants and, furthermore, an inhibition of NFκB binding to the IL-2 promoter at the −225 site were demonstrated (48). In addition, PKA activation was shown to alter PKC-induced transcriptional regulation of members of the Jun and Fos (AP1) family (30). n-Butyrate treatment, however, had no effect on either NFκB or AP1 as observed in our gel shift analyses, although it alters cAMP levels and PKA activity in Jurkat T-cells. The functional relevance of these results was given, as the selective PKA inhibitor was able to revert the n-butyrate-induced inhibition of NF-AT nuclear binding and IL-2 transcription. Rather than different model systems, externally applied drugs versus real intracellular levels and treatment schedules to explain such observed results, we favor the hypothesis that the threshold of cAMP, most likely combined with exact timing, is important for this difference. Externally applied phosphodiesterase inhibitors elevate cAMP levels far beyond the ones found in nonstimulated cells,2 leading to unphysiological high levels. PKA translates cAMP levels into a negative signal via two major negative regulatory pathways,
Influence of n-butyrate on the activity of primary T-cells.

A, inhibition of IL-2 production by n-butyrate (n-but) in primary T-cells. Primary T-cells were stimulated by CD3 and CD28 mAb (αCD3/28) in the presence (addition simultaneous to the stimulus) or absence of n-butyrate (1 mM) for 24 h, and supernatants were analyzed for IL-2 production by ELISA. nst, nonstimulated.

B, DNA binding activity of NF-AT was analyzed in nuclear extracts from Jurkat T-cells stimulated with CD3 and CD28 mAb (αCD3/28) and treated (simultaneously to the stimulus) with/without 1 mM n-butyrate (n-but). Leptomycin B (lept) (200 nM, pretreatment for 30 min) and lithium chloride (Li) (10 mM, pretreatment for 30 min) were used to antagonize nuclear export of NF-AT. f, free probe.

C, Western blot analysis was performed using the same nuclear extracts as above to show the interference with NF-AT translocation at the protein level. nst, nonstimulated. C, the selective PKA inhibitor (R) 8-Br-cAMP (Rp, 100 μM) is able to revert the n-butyrate (1 mM)-mediated inhibition of NF-AT DNA binding as shown by EMSA. D, the subcellular localization of NF-AT was investigated by Western blot analysis upon n-butyrate (1 mM) and (R) 8-Br-cAMP (Rp, 100 μM) treatment. E, reporter gene assays were performed in transfected Jurkat T-cells stimulated with CD3 and CD28 mAb in the presence or absence of n-butyrate and under the influence of leptomycin B (lept) (10 and 50 nm, pretreatment for 30 min).
p50\textsuperscript{csk} as a key negative regulator of p56\textsuperscript{ck} and a direct Ser-43 phosphorylation of Raf1 leading to its inactivation (49, 50). However, in our n-butyrate-treated Jurkat T-cells, only the inhibition of Lck is observed, whereas the MAPK pathway, exemplified by the Erk1/2 and p38 activation, is intact. This was further confirmed in Western blot analyses using phosphospecific antibodies detecting Raf 1 deactivation by PKA as well as mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 activation in addition.\textsuperscript{3} The activation status of both kinases was not changed upon n-butyrate application compared with stimulated Jurkat T-cells. This suggests that, in activated T-cells with a cAMP concentration comparable with that of nonstimulated cells, only one of the two inhibitory pathways (inhibition of Lck) is active, whereas further cAMP elevation would be required to prevent the induction of the MAPK cascade.

In addition to its ability to suppress primary T-cell responses, n-butyrate has been shown to induce a state of antigen-specific hyporesponsiveness. The difference between the mode of action of n-butyrate (hyporesponsiveness) and nuclear import inhibitors such as FK506 (immunosuppression) is not fully understood. This is shown functionally by applying nuclear import inhibitors in addition to n-butyrate treatment. In such experiments an enhanced suppression of primary responses, but a prevention of the generation of the allantoin-specific hyporesponsiveness, is observed (20). *Ipso facto*, this can be interpreted that too early an abortion of T-cell triggering results in suppression. In contrast, as soon as a certain checkpoint of activation is achieved (characterized by the appearance of the inducible transcription factors in the nucleus), the potential of a T-cell to react again to a restimulation is altered. Consequently, one might imagine that distinct genes require different concentrations or duration of nuclear NF-AT to become transcriptionally activated. In such a setting, long term presence of NF-AT in the nucleus would promote expression of all genes required for the differentiation to an effector phenotype, whereas short, transient NF-AT appearance and concomitant low concentrations would only allow the transcription of a subset of genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes is known from T-cells of certain SCID patients. Here, impaired butyrate treatment. In *H929*, granulocyte/macrophage colony-stimulating factor, and IL-13 (51).

As we demonstrated in the present study, the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced genes resulting in tolerance. That the nuclear half-life of NF-AT has a huge impact on the selection of induced gen

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\textsuperscript{3} E. Prieschl, unpublished results.
