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Specific Role of Phosphodiesterase 4B in Lipopolysaccharide-Induced Signaling in Mouse Macrophages

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Cyclic nucleotide signaling functions as a negative modulator of inflammatory cell responses, and type 4 phosphodiesterases (PDE4) are important regulators of this pathway. In this study, we provide evidence that only one of the three PDE4 genes expressed in mouse peritoneal macrophages is involved in the control of TLR signaling. In these cells, LPS stimulation of TLR caused a major up-regulation of PDE4B but not the paralogs PDE4A or PDE4D. Only ablation of PDE4B impacted LPS signaling and TNF-α production. TNF-α mRNA and protein were decreased by >50% in PDE4B−/−, but not in PDE4A−/− or PDE4D−/− macrophages. The PDE4 selective inhibitors rolipram and roflumilast had no additional inhibitory effect in macrophages deficient in PDE4B, but suppressed the TNF-α response in the other PDE4 null cells. The inhibition of TNF-α production that follows either genetic ablation or acute inhibition of PDE4B is cAMP-dependent and requires protein kinase A activity. However, no global changes in cAMP concentration were observed in the PDE4B−/− macrophages. Moreover, ablation of PDE4B protected mice from LPS-induced shock, suggesting that altered TLR signaling is retained in vivo. These findings demonstrate the highly specialized function of PDE4B in macrophages and its critical role in LPS signaling. Moreover, they provide proof of concept that a PDE4 inhibitor with subtype selectivity retains useful pharmacological effects.

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The second messenger cAMP plays a key role in the regulation of most cellular functions. In inflammatory cells, activation of cAMP signaling has negative modulatory effects on numerous steps required for immune and inflammatory responses, including T cell activation and proliferation, cytokine release, and recruitment of leukocytes (1). Given these broad inhibitory effects, pharmacological manipulation of cAMP levels is viewed as a promising approach for the treatment of chronic inflammatory conditions such as asthma, chronic obstructive pulmonary disease, and inflammatory bowel disease. Together with activation of G protein-coupled receptors with agonists, inhibition of cyclic nucleotide phosphodiesterases (PDEs),3 the enzymes that degrade and inactivate cyclic nucleotides, is one strategy to increase cAMP levels in these cells and to suppress inflammatory responses (1–3).

The phosphodiesterase 4 (PDE4) family consists of four paralog genes (PDE4A–D), and each gene encodes multiple variants generated from alternate splicing and different transcriptional promoters (4, 5). Thus far, a total of at least 20 transcripts derived from the four genes have been identified, and the corresponding proteins can be usually distinguished by their unique N-terminal sequences. These variants, particularly those belonging to the PDE4A, PDE4B, and PDE4D subtypes, are widely expressed and often coexist in different tissues and cells. It is largely unknown whether these isoenzymes are redundant or each subtype has its own specialized function. The phenotypes of different PDE4 null mice suggested that each PDE4 gene has unique functions. PDE4D null mice display a broad spectrum of pleiotropic phenotypes (6), whereas PDE4A null mice are healthy in general and display a deficit in TNF-α production (7). PDE4A null mice have been recently generated, but phenotypes derived from the ablation of this gene are still under investigation.

Peripheral blood leukocytes derived from the PDE4B null mice produce very little TNF-α in response to LPS, whereas the response of PDE4D null leukocytes is not affected (7). This observation constitutes an initial indication for a nonredundant function of PDE4s. However, a question that could not be addressed in that study is whether the differential effect of PDE4 ablation on LPS signaling is due to differences in PDE4B and PDE4D expression in these cells or is the consequence of a specialized function of each PDE4 gene. Because monocytes, the major producer of TNF, cannot be recovered in sufficient number to allow biochemical studies, in this study we have used peritoneal resident macrophages to determine which PDE4 is expressed in this naive, nonelicited cell population and whether ablation of different PDE4 subtypes expressed within the same cell indeed produces distinct effects. The characterization of this naive macrophage model has also allowed us to define the mechanism by which different PDE4 ablation affects cyclic nucleotide signaling.

Given their pattern of expression and involvement in the control of inflammatory cell responses, PDE4s have received considerable attention as targets for anti-inflammatory drugs. PDE4 inhibitors are being developed for chronic lung inflammatory diseases; some compounds have shown considerable efficacy and are being tested in humans for asthma and chronic obstructive pulmonary disease (8, 9). However, the pharmacological potential of this class of drugs is limited by their narrow therapeutic window (10). Dosing of PDE4 inhibitors sufficient to produce anti-inflammatory effects in humans is associated with side effects, particularly in the CNS. Thus, new strategies are being actively sought to improve the therapeutically index of these PDE4 inhibitors.
The presence of four PDE4 genes led to the proposal that generation of novel PDE4 inhibitors with some selectivity may have the distinct advantage of maintaining useful therapeutic effects while decreasing the side effects (11). At present, this hypothesis cannot be tested pharmacologically because with few exceptions all PDE4 inhibitors synthesized are nonselective, i.e., they inhibit all PDE4 subtypes with comparable potencies. It is then difficult to dissect functions of individual PDE4 subtypes using a pharmacological approach.

In this study, we have used a genetic approach to demonstrate that only one of the three PDE4s expressed in macrophages is involved in the control of LPS signaling. More importantly, we provide evidence that PDE4 inhibitors block LPS-induced TNF-α production in macrophages only by inhibiting this PDE4 subtype. The other PDE4s expressed in the same cells are not involved in regulation of this response.

Materials and Methods

Mice

Generation of PDE4B- and PDE4D-deficient mice has been described previously (6, 7). Generation of PDE4A-deficient mice following a similar strategy will be reported elsewhere. The homozygous null mice and their wild-type littermates used in this study were 3–6 mo of age and had a mixed genetic background of C57BL/6 and 129/Ola. All experimental procedures involving animals were approved by the Administrative Panel on Laboratory Animal Care at Stanford University (Stanford, CA).

Isolation and purification of resident and thioglycollate (TG)-elicited peritoneal macrophages

Naive mice were sacrificed, and cells in the peritoneal cavity were isolated by washing the cavity with 6–7 ml of cold HBSS (Invitrogen Life Technologies). The cells were pelleted, resuspended in PBS supplemented with 2% heat-inactivated FBS (Invitrogen Life Technologies), and then transferred onto a 10-cm petri dish that was pretreated with 35 μg of goat anti-mouse IgG + IgM (H + L) (Jackson ImmunoResearch Laboratories) in 10 ml of PBS containing 0.3 mg of goat γ-globulin. After incubation at 4°C for 1 h, B cells attached to the dish by binding to the coated lgs. The nonadherent cells containing mainly macrophages were collected and centrifuged at 160 × g for 10 min. The cell pellet was resuspended in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Macrophages were counted and plated in petri dishes or tissue culture plates at a density of 2–3 × 10⁵/ml. After incubation at 37°C in 5% CO₂ for 2 h, the medium was aspirated to remove nonadherent cells, and the adherent macrophages were cultured in complete medium overnight. The medium was changed next day, and the cells were cultured for an additional hour before LPS stimulation or drug treatment.

To culture TG-elicited macrophages, mice were given injections i.p. with 1.5 ml of TG medium (Sigma-Aldrich). Four days after injection, the mice were sacrificed, and the cells in the peritoneal cavity were isolated as described above. Cells were washed once with complete medium and then cultured at a density of 1 × 10⁶/ml. Two hours later, plates were washed with medium to remove nonadherent cells. The remaining cells with at least 95% macrophages were cultured overnight in complete medium before LPS stimulation.

Flow cytometry

To determine the purity of macrophages prepared as described above, the adherent cells were gently scraped off the plates in PBS. Aliquots of ~1 × 10⁶ cells were washed once with FACS buffer (2% FBS and 0.01% sodium azide in PBS), blocked with a mouse Fc blocker (BD Pharmingen) for 15 min, and then incubated for 40 min on ice with PE anti-mouse F4/80 Ab (CalTag Laboratories) plus FITC anti-mouse CD11b Ab (BD Pharmingen). After washing with FACS buffer, the cells were analyzed on the BD Biosciences FACScan flow cytometer using CellQuest software (BD Biosciences). At least 95% of the adherent cells were macrophages (data not shown).

PDE assay

Following incubation with LPS (100 ng/ml) for 4 h, the macrophages were gently scraped off the culture dish in PBS, centrifuged, washed once with PBS, and then incubated on ice for 10 min in a lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM sodium chloride, 5% glycerol, 10 mM sodium fluoride, 1 mM EDTA, 0.2 mM EGTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Roche), 1 tablet/10 ml of protease inhibitor mixture (Roche), 1% Nonidet P-40, and 5 mM 2-ME). After sonication with 20 bursts, the lysate was centrifuged at 4°C for 20 min at 16,000 × g. The supernatant was assayed for both total PDE activity and rolipram-insensitive PDE activity in the presence of 10 μM rolipram. The PDE assay was performed according to the method of Thompson and Appleman (12) as detailed previously (13). The rolipram-sensitive activity (i.e., PDE4 activity) was obtained by subtracting the rolipram-insensitive activity from the total activity. Protein concentration was determined according to the Bradford (14) method.

Immunoprecipitation and PDE assay

Following incubation with LPS (100 ng/ml) for 4 h, the macrophages were washed once with PBS and then scraped in the above-mentioned lysis buffer. After sonication with 20 bursts, the lysate was centrifuged at 4°C for 20 min at 16,000 × g. Aliquots of the supernatant were incubated with PDE4A-, PDE4B-, or PDE4D-specific Ab (AC55, K118, and M351, respectively) that was preincubated with protein A-Sepharose (Zymed Laboratories) or protein G-Sepharose (Amersham Biosciences). On the basis of tests done with PDE4 recombinant proteins, the amount of Ab used in each immunoprecipitation was sufficient to completely pull down the specific PDE4 subtype at the concentration present in the sample. Aliquots of the supernatant were also incubated with normal rabbit serum or purified mouse IgG (Zymed Laboratories) to determine their nonspecific precipitation of PDE4. After 1–2 h of incubation at 4°C, the Sepharose-Ab-Ag complexes were pelleted, washed twice with PBS containing 0.05% BSA, and resuspended in 40 mM Tris-Cl (pH 8) solution containing 1 mg/ml BSA. The complexes were then assayed for PDE activity as described above.

TNF-α and IL-6 ELISA

Levels of TNF-α and IL-6 in macrophage culture supernatants were measured with commercially available ELISA kits (BioSource International). The sensitivities of the assays were 19.5 and 7.8 pg/ml, respectively.

RT-PCR and Southern blot analysis

For semiquantitative measurements of LPS-induced expression of TNF-α, total RNA was extracted from wild-type and PDE4-deficient macrophages. After treatment with RNase-free DNase I (0.1 U/μg total RNA; Boehringer Mannheim), first-strand cDNA was synthesized from 1 μg of total RNA in the presence of oligo(dT) primer according to the manufacturer’s protocol (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen Life Technologies). For PCR, 2 μl of aliquots of the resulting 21 μl of cDNA were amplified 22 cycles in a 50-μl reaction volume containing 1× PCR buffer, 0.2 mM dNTP mix, 1 μM each specific primer, and 1 U of TaqDNA polymerase. Oligonucleotide primers were as follows: TNF-α, 5′-GTTGACAAGGCTCTGAGCCA-3′ and 5′-5′AAATGAGACCTGCGCGCC-3′ (from base 419 to 437 and base 846 to 828, respectively; GenBank accession no. X02611), yielding a 428-bp product; and GAPDH, 5′-TGAAGGTCTGTTGGAACGGATTTGC-3′ and 5′-CTATGAGGCATGAGTTCCACACAC-3′ (purchased from BD Clontech), yielding a 983-bp product.

The PCR products were fractionated by agarose gel electrophoresis and then transferred to nylon membrane (ICN Pharmaceuticals). Blots were hybridized with [γ-3²P]ATP-labeled oligonucleotide probes corresponding to nucleotide sequences nested between the specific PCR primers. The sequences of the oligonucleotide probes were as follows: TNF-α, 5′-CAT ACCAGGTGTTGACTG-3′ (from base 736 to 719; GenBank accession no. X02611); and GAPDH, 5′-CTTGTGGCAAATGGGATT-3′ (from base 112 to 130; GenBank accession no. M32599). The blots were then washed in 1.5× SSC/0.1% SDS at 48–50°C, followed by autoradiography.

Cyclic AMP response to LPS in peritoneal naive macrophages

The macrophages were incubated in the presence of LPS (100 ng/ml) for different times. To terminate the incubation, the cells were washed once with ice-cold PBS, and 5% TCA was added. After extraction on ice for 30 min, the cells were scraped off the plates and then centrifuged at 6000 × g for 20 min. The supernatant was subsequently extracted five times with water-saturated ether, freeze-dried by lyophilization, and reconstituted in PBS. The cAMP content was measured by RIA as described by Harper and Brooker (15). The TCA pellet was resuspended in 1 N NaOH solution, and the aliquots were assayed for protein concentration according to the Lowry method.
Activity from the total activity. Data are the mean rolipram-sensitive activity (PDE4) was obtained by subtracting the RI activity from the total activity. Data are the mean ± SEM (n = 5–16 mice/group). *, p < 0.05; **, p < 0.001 (compared with unstimulated cells).

Western blot analysis

Following a 20-min incubation with or without LPS (100 ng/ml), the macrophages were gently scraped off the culture dish in PBS, washed once with PBS, and then incubated on ice for 10 min in the 1% Nonidet P-40-containing lysis buffer as described above. After sonication with 20 bursts, the lysate was centrifuged at 4°C for 20 min at 16,000 × g. Aliquots of the supernatant were subjected to electrophoresis on 15% SDS-PAGE, and then blotted onto an Immobilon membrane. Western blot analysis was performed using an antiphosphorylated CREB Ab (Cell Signaling Technology) at a dilution of 1/1500. Immunoreactive bands were detected by using an alkaline phosphatase (AP)-conjugated Ab (Cell Signaling Technology) at a dilution of 1/1000. Immunoreactive bands were detected by using an anti-CREB Ab at a dilution of 1/5000 and the ECL detection system (Amersham Biosciences). To monitor the level of total CREB in the cell, the blot was subsequently washed extensively and then incubated with an anti-CREB Ab (Cell Signaling Technology) at a dilution of 1/1000. Immunoreactive bands were detected by using an alkaline phosphatase (AP)-conjugated goat anti-rabbit Ab at a dilution of 1/3000 and an Immun-Star AP substrate (Bio-Rad).

High-dose LPS-induced shock model

Wild-type and PDE4B null mice were given injections i.p. with 800 μg/25 g body weight LPS (Escherichia coli serotype 055:B5; Sigma-Aldrich). Animals were monitored every 12 h for death throughout the 7-day test period. Mortality/survival was analyzed using the log-rank test (also known as the Kaplan-Meier test) and Wilcoxon test.

Results

Of the three PDE4 present in mouse peritoneal resident macrophages, LPS induces only PDE4B.

Macrophages were isolated from the peritoneal cavity of PDE4A-, -B, and -D null mice and from corresponding wild-type littermates. After removal of B cells by IgG/IgM selection and nonadherent cells by culture, highly purified populations of macrophages were obtained, as judged by flow cytometry monitoring of F4/80 and CD11b surface marker (data not shown). Comparable numbers of macrophages were retrieved from the four genotypes with no differences in morphological appearance by light microscopy. In some experiments, macrophages recruited by i.p. injection of TG medium were used as an additional model (TG-elicted macrophages).

The pattern of PDE expression in wild-type macrophages was initially determined by measuring total PDE activity as well as the activity sensitive to the PDE4 inhibitor rolipram. At a concentration of 10 μM, rolipram has minimal inhibitory effects on other PDEs (16, 17) and can be used as a pharmacological tool to quantify PDE4 activity (17). This analysis showed that ~40% of the basal cAMP-degrading activity in wild-type cells is rolipram-sensitive (PDE4 activity), and the remaining 60% is rolipram-insensitive (Fig. 1). The rolipram-insensitive PDE activity likely reflects the expression of predominantly PDE1 and PDE3 (16, 18). After LPS stimulation for 4 h, total PDE activity was increased 2.0- to 2.5-fold. This increase was due to a 4.0- to 4.5-fold increase in PDE4 activity, whereas the rolipram-insensitive activity was unaffected (Fig. 1). TG-elicted macrophages showed similar overall levels of PDE activity, but PDE4 accounted for only 20% of the total activity. As a consequence of this relatively low PDE4 expression, LPS stimulation produced only ~20–30% overall increase in PDE activity (Fig. 1B).

Although several studies have investigated the PDE expression profile and activity in macrophages under different conditions (16, 18–23), the actual expression pattern of PDE4 subtypes and their activity in response to LPS have not been explored. Thus, to determine which PDE4 subtypes contribute to the LPS-dependent induction of PDE4, the PDE activity in peritoneal macrophages was further characterized by a combined immunological and genetic approach. PDE4 subtype-specific Abs were used for immunoprecipitation of cell extracts of wild-type and PDE4-deficient macrophages under basal conditions or after LPS stimulation. The immunoprecipitates were then used for PDE activity measurement. The results of these experiments are summarized in Fig. 2. Abs specific to PDE4A (AC55), PDE4B (K118), and PDE4D (M3S1)
immunoprecipitated significant PDE activity when compared with preimmune IgG. Conversely, they did not immunoprecipitate appreciable activity in the corresponding PDE4 null macrophages. Thus, under basal condition, activities of all three PDE4s are present in the extracts of wild-type macrophages (pmol/min/10^7 cells: PDE4A, 2.62 ± 0.53; PDE4B, 5.38 ± 0.28; PDE4D, 2.85 ± 0.38; mean ± SEM of three groups of five mice each). These measurements indicate that the ratio of the PDE4A:PDE4B:PDE4D activity in peritoneal resident macrophages is ~1:2:1. After LPS stimulation, PDE4B activity in the wild-type cells was increased ~5-fold, whereas PDE4A or PDE4D activities were marginally increased or not significantly affected (pmol/min/10^7 cells: PDE4A, 4.12 ± 0.23; PDE4B, 26.45 ± 1.45; PDE4D, 3.08 ± 0.33; mean ± SEM of three groups of five mice each; approximate ratio 1:7:1). Statistical analyses indicated that the increase in PDE4A activity after LPS had p values between 0.05 and 0.1. In agreement with the immunological data, the LPS-induced increase in PDE4 activity was absent only in the PDE4B null macrophages (Fig. 2). These data demonstrate that three PDE4 genes are expressed in peritoneal macrophages but that PDE4B is the major PDE4 subtype induced by LPS activation of these cells. Moreover, inactivation of one PDE4 gene did not produce a compensatory increase in expression of any other PDE4 genes, suggesting a lack of overlapping functions among the paralog genes. Compensation with rolipram-insensitive PDEs was not observed either (Fig. 1). Similar results were obtained with TG-elicited macrophages, albeit with two major differences. PDE4A, PDE4B, and PDE4D were expressed at comparable levels under basal conditions, and the PDE4B stimulation was reduced (pmol/min/mg protein: PDE4A, 2.89 ± 0.09; PDE4B, 5.94 ± 0.46; PDE4D, 2.46 ± 0.29; mean ± SEM of two groups of three mice each; ratio 1:2:1). It also should be pointed out that although the wild-type littermates for each genotype were analyzed separately, their properties and PDE4 expression were not significantly different in any of the experiments performed, confirming the robustness of the measurements.

These analyses define a cellular model where three PDE4s are expressed at the same time, and cells derived from the three null mice allow the investigation of the effect of inactivation of individual subtype on distal responses.

**LPS stimulation of TNF-α production is affected only in PDE4B-deficient macrophages**

To assess the impact of different PDE4s on LPS activation of cytokine responses, the three PDE4 null macrophages and their corresponding wild-type macrophages were incubated with 100 ng/ml LPS for different times, and the levels of TNF-α released in the medium were measured by ELISA. As shown in Fig. 3A, LPS induced TNF-α production in all wild-type macrophages, and the induction reached a maximum in 8 h and remained constant for up to 24 h. PDE4A- and PDE4D-deficient macrophages responded to LPS in a manner indistinguishable from their wild-type counterparts. Conversely, LPS-induced TNF-α production was significantly decreased in PDE4B null cells (p < 0.01). With >30 mice tested independently, TNF-α response in PDE4B−/− macrophages was decreased to 43.5 ± 3.5% of control. This blunted TNF-α accumulation was not due to decreased sensitivity to LPS because higher concentrations did not restore the full response in the PDE4B null cells (Fig. 3B). In addition, the decrease in TNF-α production is not caused by a generalized loss of viability or compromised function of macrophages defective in PDE4B because IL-6 accumulation was stimulated in PDE4B null cells to the same extent as in wild-type cells (Fig. 3C). This latter observation suggests that distinct branches of LPS-activated TLR signaling pathways show different sensitivity to cAMP inhibition, PDE4B specifically affecting only the TNF-α production but not IL-6. The fact that distinct components are used in the two pathways is suggested by analysis of the Rel knockout mice (24, 25).

The decreased accumulation of protein in the medium was associated with a decrease in TNF-α mRNA steady state in PDE4B null macrophages after LPS stimulation (Fig. 4), suggesting that TNF-α transcription or mRNA stability is distal to PDE4B.

When using TG-elicited macrophages, only PDE4B ablation produced a significant decrease in TNF-α accumulation (data not shown). In the three independent experiments performed with >10 mice, PDE4B null macrophages produced 52.5 ± 7.8% TNF-α compared with wild-type cells.

**Nonselective PDE4 inhibitors exert their pharmacological effects on TNF-α production by blocking only PDE4B activity**

Numerous reports have shown that PDE4 inhibitors are effective in blocking TNF-α production in inflammatory cells (see review Ref. 1), underscored the anti-inflammatory potentials of this class of compounds (9, 26, 27). However, with few exceptions, all PDE4 inhibitors thus far developed are nonselective, in that they block the activity of every PDE4 with minimal differences in EC_{50} (9,
FIGURE 4. LPS regulation of TNF-α mRNA expression in peritoneal macrophages. Peritoneal macrophages from PDE4B−/− (A) and PDE4D−/− (B) mice and their wild-type littermates were incubated in the absence or presence of 100 ng/ml LPS for 3 h. Total RNA was extracted from the cells (pooled from 7 to 11 animals/group) followed by RT-PCR using TNF-α-specific primers. The amplified products were analyzed by Southern blot using an oligonucleotide probe with sequence nested between the specific PCR primers. Amplification of a GAPDH fragment was included to monitor the amount of RNA in each sample. C, Densitometric scanning of Southern blots was conducted, and the ratios of TNF-α and GAPDH density were compared between the wild-type and PDE4B−/− macrophages. Data are the mean ± SEM of three independent experiments. *, Significantly different from the wild-type cells (p < 0.05).

FIGURE 5. Effect of PDE4 inhibitors on LPS-induced TNF-α production in PDE4-deficient peritoneal macrophages. A, Peritoneal macrophages from PDE4A−/−, PDE4B−/−, and PDE4D−/− mice and their wild-type littermates were incubated with 10 μM rolipram or vehicle (DMSO) for 30 min before LPS (100 ng/ml) stimulation for 8 h. TNF-α accumulation in the medium was determined by ELISA. No significant difference was observed between the control and rolipram-treated cells in PDE4B−/− mice. B, Wild-type and PDE4B-deficient macrophages were treated with increasing concentrations of rolipram or roflumilast for 30 min before LPS (100 ng/ml) stimulation. After 5 h, TNF-α accumulation in the medium was measured. Data are the mean ± SEM (n = 4–7 mice/group).

26, 27). The cellular model characterized above allowed us to determine whether inhibition of a single PDE4 is sufficient to produce useful pharmacological effects. Macrophages of different genetic backgrounds were incubated with 10 μM rolipram, and the TNF-α response was evaluated. As shown in Fig. 5A, rolipram inhibited LPS-stimulated TNF-α production in wild-type macrophages as well as PDE4A and PDE4D null cells but had no significant effects in the PDE4B−/− cells. Similar results were obtained when rolipram or roflumilast, a PDE4 inhibitor in clinical trial (8, 27), was used at different concentrations (Fig. 5B). Collectively, these results demonstrate that the effects of PDE4B ablation and rolipram inhibition on LPS-stimulated TNF-α production are not additive. In addition, they indirectly demonstrate that acute rolipram inhibition of PDE4A and PDE4D present in the PDE4B−/− macrophages has no efficacy in blocking TNF-α response.

Cyclic AMP signaling mediates the effects of PDE4B ablation

A possible explanation of the above findings is that ablation of PDE4B, but not PDE4A or PDE4D, produces major changes in cAMP accumulation in macrophages, hence the selective effect on TNF-α accumulation. To test this possibility, cAMP was measured in macrophages from wild-type and PDE4B or PDE4D null mice during the course of LPS activation. LPS stimulation of macrophages is associated with a small but reproducible increase in cAMP accumulation in all genotypes tested. Cyclic nucleotide levels reached a maximum in 15–30 min and then returned to baseline in ~3 h (Fig. 6A). However, ablation of either PDE4B or PDE4D did not have detectable effects on cAMP accumulation (Fig. 6A). Statistical analyses of the data showed no differences in the three curves, even though a trend to an increase in cAMP at 3 h was sometimes observed in the PDE4B null cells. In addition, the level of phosphorylation of CREB was comparable in wild-type and PDE4B null macrophages (Fig. 6B). Thus, no major changes in cAMP could be detected with the ablation of either PDE4B or PDE4D.

In light of the above findings, we evaluated the possibility that PDE4B ablation may impact the TNF-α response by affecting steps in the LPS cascade independent of cAMP accumulation. The involvement of cAMP signaling in the PDE4B phenotype was further evaluated in these macrophages. The adenyl cyclase activator forskolin and the protein kinase A (PKA) inhibitors H89 or Rp-cAMPS were used, and the TNF-α production was compared in wild-type and PDE4B−/− macrophages. Preincubation of wild-type macrophages by 1 μM forskolin for 20 min followed by 5 h LPS stimulation led to a 64% decrease in TNF-α accumulation, reaching levels similar to those observed in the PDE4B−/− cells incubated with LPS alone (Fig. 7). Higher concentrations of forskolin produced minor further inhibition of TNF-α accumulation in both the wild-type and PDE4B null cells. Rolipram had minimal additive effects to forskolin in wild-type macrophages and no effect in PDE4B null cells. In a slightly different experimental condition, forskolin added at the same time as LPS produced inhibition of TNF-α production identical with that caused by PDE4B ablation with no further effect at higher doses (data not shown).

Cyclic AMP acts via activation of the effectors PKA, cAMP-guanine nucleotide exchange factor (GEF), or cyclic nucleotide gated channels. However, cAMP-GEF does not appear to have a
functional role in inflammatory cells (28). Indeed, the cAMP-GEF-specific activator 8-(4-chlorophenylthio)-2-O-methyladenosine-3',5'-cyclic monophosphate had no significant effect on LPS-induced TNF-α accumulation in the peritoneal macrophages (data not shown). PKA inhibitors were then used to determine whether the effect of PDE4B ablation or rolipram inhibition requires PKA activity (Fig. 8). Consistent with the data described above, TNF-α accumulation was decreased after PDE4B ablation, and rolipram inhibited the TNF-α response in wild-type macrophages to the same degree as PDE4B inactivation but had no further effect in the PDE4B-deficient cells (Fig. 8). Preincubation of wild-type cells with 20 μM H89 for 20 min caused a significant increase in the TNF-α production. More importantly, H89 restored the TNF-α response in PDE4B null cells to levels not significantly different from that in wild-type cells treated with LPS alone. When the cells were incubated with a combination of H89 and rolipram, there was little or no rolipram inhibition of TNF-α production observed, indicating that PKA is distal to PDE4B in the cAMP signaling cascade and that rolipram effects appeared to require an active PKA. Similar results were obtained with the cAMP analog Rp-cAMPS used at 1 mM (data not shown). Thus, these experiments demonstrate that effects of PDE4B inactivation by either rolipram or by

gene targeting are mediated by activation of cAMP signaling and require PKA activity.

**PDE4B-deficient mice are resistant to LPS-induced shock**

Because TNF-α is a key mediator of endotoxin-induced toxicity in vivo (29), the observation that macrophages deficient in PDE4B exhibit a blunted TNF-α response to LPS prompted a question of whether PDE4B null mice were resistant to LPS-induced shock. For this purpose, wild-type and PDE4B null mice were challenged with a high dose of LPS (800 μg/25 g body weight), and survival was monitored for 7 days. As shown in Fig. 9, this treatment caused a significantly higher mortality in wild-type mice (52%) than in PDE4B null mice (27%). In addition, wild-type mice succumbed to shock mostly on day 1.5 after LPS injection, whereas

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**FIGURE 6.** LPS stimulation of cAMP accumulation and CREB phosphorylation in wild-type and PDE4-deficient macrophages. A, Peritoneal macrophages from PDE4B<sup>−/−</sup> and PDE4D<sup>−/−</sup> mice and their wild-type littermates were incubated with 100 ng/ml LPS for the indicated times. LPS stimulation was terminated by adding 5% TCA immediately after cell wash with ice-cold PBS. Concentration of cAMP was measured by RIA. B, Wild-type and PDE4B-deficient macrophages were incubated in the absence or presence of 100 ng/ml LPS for 20 min. Sixty micrograms of the cell extract were subjected to 15% SDS-PAGE and then electroblotted. The phosphorylated CREB was detected by incubation of the membrane with a phospho-CREB Ab and then a peroxidase-conjugated secondary Ab. Immunoreactive signals were developed using an ECL detection system. Subsequently, the membrane was incubated with an anti-CREB Ab, followed by an AP-conjugated secondary Ab, and developed with an Immun-Star AP detection system.

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**FIGURE 7.** Forskolin inhibits LPS-induced TNF-α production in peritoneal macrophages. Macrophages from wild-type and PDE4B<sup>−/−</sup> mice were pretreated for 20 min with forskolin (FSK; 1 or 50 μM), a combination of 50 μM FSK and 10 μM rolipram (Rol), or vehicle (DMSO) followed by LPS stimulation for 5 h. TNF-α accumulation in the medium was measured. Data are the mean ± SEM (n = 3–5 mice/group). * Significantly different from the wild-type control group (p < 0.01).

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**FIGURE 8.** Effect of H89 on LPS-induced TNF-α production in wild-type and PDE4B-deficient macrophages. Macrophages from wild-type and PDE4B<sup>−/−</sup> mice were pretreated for 20 min with 10 μM rolipram, 20 μM H89, a combination of 10 μM rolipram and 20 μM H89, or vehicle (DMSO) before LPS stimulation for 5 h. TNF-α accumulation in the medium was measured. Data are the mean ± SEM (n = 3–5 mice/group). A representative experiment of the two performed is reported.
PDE4B null mice died later, mostly on day 3.5. These results demonstrated that ablation of PDE4B partially protects mice from LPS-induced shock.

Discussion

Using a combination of genetic and pharmacological approaches, we provide evidence that the function of PDE4s in macrophages is highly specialized. Of the three PDE4 subtypes expressed in these cells, PDE4B is induced by activation of the TLR signaling and is the predominant PDE isoenzyme involved in the control of TNF-α production. More importantly, we demonstrate that the pharmacological effects of PDE4 inhibitors on macrophage TNF-α production are exerted exclusively through inhibition of PDE4B. Moreover, the altered TLR signaling is reflected by in vivo effects because PDE4B null mice are protected in LPS-induced shock. These findings have considerable physiological and pharmacological implications, indicating a highly specialized, nonredundant PDE function in a cell and providing the experimental basis for novel therapeutic strategies that use subtype-selective PDE4 inhibitors.

That the effects of the genetic PDE4 inactivation are subtle and not due to generalized impairment of macropage function is documented by at least two lines of evidence. First, IL-6 production is unaffected in the PDE4B- and PDE4A-deficient macrophages, demonstrating that the ability to respond to LPS stimulation remains intact in these cells. Secondly, that the effects of PDE4B ablation are reversible is documented by the finding that TNF-α accumulation can be fully restored by inhibition of PKA. Therefore, the long-term removal of PDE4B has no generalized, deleterious effects on the macropage ability to generate TNF-α. Finally, the observation that acute PDE inhibition with rolipram closely reproduces the effects of PDE4B ablation argues that the impaired TNF-α response is a well-defined phenotype linked to the loss of PDE4 activity.

Many reports have addressed the effect of cAMP on TNF-α accumulation, and a plethora of possible interaction points between the two pathways have been identified (30–33). Nevertheless, the exact sites where cAMP signaling interferes with TNF-α gene expression are still debated. In some cases, a decrease in signaling through the MEK kinase pathway has been observed following an increase in cAMP (33). In other cases, the transactivating properties of p65 have been indicated as the target of PKA activation (32). Activation of cAMP signaling in macrophages by pituitary adenylate cyclase-activating peptide or vasoactive intestinal peptide decreases TNF-α production by blocking IκB degrada-
different extracellular environments (7). Consistent with the presence of a tonic cAMP inhibition in these macrophages, we show in this study that inhibition of PKA in wild-type cells causes a significant increase in TNF-α accumulation. This positive feedback may be operating to oppose the inhibitory effects of prostaglandins, vasoactive intestinal peptide/pituitary adenylyl cyclase-activated peptide, or other deactivating cytokines and chemokines released at the site of inflammation. In view of the differential expression of PDE4D during monocyte-macrophage differentiation and marked differences in sensitivity to PDE4 inhibitors, this feedback probably loses its function after macrophages are terminally differentiated and activated.

We should point out that LPS consistently produced a small but significant increase in cAMP in the naive macrophages, which was not affected by PDE4A ablation. Activation of cAMP signaling following LPS stimulation has been reported in cultured monocytes (39). This increase may be indirect and due to LPS-induced prostaglandin accumulation in the incubation medium. From the physiological standpoint, the LPS-mediated increase in PDE activity may be necessary to counteract the deactivating effects of prostaglandin production. However, this increase in cAMP is not the cause of PDE induction because indomethacin does not block the LPS stimulation of PDE4 (40). PDE4B2 is induced by LPS in macrophages likely through activation of an internal promoter in the PDE4B gene (41). Although this promoter contains a functional CRE site, several NF-κB motifs can be identified, and these are likely involved in the LPS stimulation. The following findings demonstrate that the prototype PDE4 inhibitor rolipram and rolflumilast exert their pharmacological effects on TNF-α production by only inhibiting PDE4B. Removal of PDE4A or PDE4D does not disrupt TNF-α production nor ablate the rolipram effects. In the same vein, the decreases in TNF-PDE4A/H11022 PDE4B potency toward the different PDE4s, i.e., with a rank of potency the mechanism of action of PDE4 inhibitors. In view of our findings, developments of “designer” PDE4 inhibitors with different potency toward the different PDE4s, i.e., with a rank of potency PDE4B>PDE4A>PDE4D, should broaden the spectrum of application and efficacy of this class of drugs. The challenge will be to identify the differences in the catalytic pocket of the PDE4 subtypes and to develop new strategies to target these differences (45).

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Disclosures

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