JPET Fast Forward. Published on April 13, 2004 as DOI: 10.1124/jpet.103.064691
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JPET#64691

Title: Preferential inhibition of Th1, but not Th2 cytokines *in vitro* by L-826,141, a potent and selective PDE4 inhibitor.

D. Claveau, [1]Chen, S.L., [1]O'Keefe, S., [1]Zaller, D.M., Styhler, A., Liu, S. Huang, Z., Nicholson, D.W. and J. A. Mancini

[1]Department of Inflammation Research, Merck Research Laboratories, Rahway, New Jersey, USA; Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada

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a) Running title: Molecular profiling of a selective PDE4 inhibitor, L-826,141

b) **Address correspondence to**: Joseph A. Mancini, Merck Frosst Centre for Therapeutic Research, PO Box 1005, Pointe-Claire-Dorval, H9R 4P8. Phone: (514) 428-3167; Fax: (514) 428-4939; E-mail: joseph_mancini@merck.com.

c) text pages: 29; tables: 3; figures: 6; ref: 45;
Word count :
Abstract : 241
Introduction: 781
Discussion: 1009

d) abbreviations: PDE, phosphodiesterase; OVA, ovalbumin; LT, leukotriene

e) section: Inflammation and Immunopharmacology

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Abstract

L-826,141 is a selective and potent inhibitor of phosphodiesterase 4 (PDE4) with an IC_{50} of 0.26-2.4 nM for inhibition of the catalytic activity of PDE4 A, B, C and D. The cAMP elevation which can be maintained by PDE4 inhibitors attenuates the signaling cascades that lead to the production of certain cytokines. In cellular based assays, L-826,141 transcriptionally downregulates production of TNFα in peripheral blood mononuclear cell and whole blood assays with IC_{50}'s of 31 and 310 nM, respectively. Profiling the effect of this compound on various cytokines in the signaling cascade attenuated by cAMP elevation demonstrates that L-826,141 is also a potent inhibitor of IL-12, GM-CSF, and IFNγ (IC_{50}'s of 0.3–0.9 μM) as well as TNFα formation. We have also shown that the PDE4 inhibitors, rolipram and L-826,141 are potent inhibitors of CD3 plus CD28 stimulated IL-2 production in naïve human T cells. To address the effect of PDE4 inhibitors on cytokine release from Th1 and Th2 effector cells, we utilized a well characterized model in which T cells are derived from ovalbumin (323-339)-specific T cell receptor transgenic mice. L-826,141 inhibits Th0 mediated IL-2 production with an IC_{35} of 25 nM and Th1-mediated IFNγ production with an IC_{30} of 46 nM. In contrast, L-826,141 had no significant inhibitory effect (IC_{30}> 2.5 μM) on Th2 cell mediated IL-4 nor IL-13 production. All tolled this data demonstrates that specific inhibition of PDE4 preferentially blocks the production of Th1 vs Th2 effector cytokines *in vitro*.

The second messenger, 3'5'-cyclic AMP (cAMP) and the ensuing signaling cascades have been investigated for over 50 years (Robison et al., 1968). cAMP is generated enzymatically by the action of adenylate cyclase and this enzyme is activated after the interaction of various ligands with G protein coupled receptors (GPCRs) (Iyengar, 1993;Taussig and Gilman, 1995;Sunahara et al., 1996;Cooper et al., 1995;Krupinski et al., 1989). One example of the G protein coupled receptor which activates $G\alpha s$ to stimulate adenylate cyclase activation is the β_2 adrenergic receptor (Perry et al., 2002). β_2 adrenergic agonists have been utilized in clinical practice for the treatment of bronchial asthma for over 25 years. It has been demonstrated that cAMP signaling may have various downstream effects due to compartmentalization of cAMP pools (Houslay and Milligan, 1997). Since degradation of cAMP occurs through the action of phosphodiesterases (PDEs), several attempts have been made to clinically develop inhibitors of PDEs. The identification and molecular cloning of several gene families of PDEs within the last decade has led to the major focus on novel treatments for asthma and COPD by targeting the PDE4 gene family (Huang et al., 2001). The PDE4 gene family consists of four major genes, A, B, C, and D with many splice variants (Houslay and Adams, 2003). PDE4 is expressed in the majority of inflammatory cells and its inhibition attenuates the production of many inflammatory cytokines and mediators. Most of the early prototype PDE4 selective inhibitors such as rolipram suffered from various untoward effects such as emesis (Robichaud et al., 2001). All of the PDE4 inhibitors reported to date are either nonselective for any of the four PDE4 genes, A, B, C, or D or are slightly more potent at inhibiting PDE4D over the other members of this family (Conti et al., 2003). The recent data generated from the PDE4 null mice demonstrates

that the bronchodilatory role attributed to PDE4 ablation is mainly through PDE4D while part of the anti-inflammatory role is through PDE4B (Hansen et al., 2000;Jin and Conti, 2002). Specifically, mononuclear cells have been shown to have a reduced capacity to produce TNFα in the PDE4B (-/-) mice, and PDE4D (-/-) mice are not responsive to muscarinic cholinergic stimulation.

Presently the most clinically advanced PDE4 inhibitors are roflumilast and cilomilast which are clinically being developed for the treatment of asthma and/or COPD (Profita et al., 2003;Sturton and Fitzgerald, 2002;Timmer et al., 2002;Compton et al., 2001). A significant effort has been made to delineate the anti-inflammatory properties of PDE4 inhibitors on various cytokines in immune cells. Asthma is often associated with a T helper (Th)2-mediated inflammatory reaction in the airway. The major Th2 cytokines measured in bronchoalveolar lavage (BAL) fluid of patients with asthma are interleukin (IL)-4, IL-5, IL-9, and IL-13, although some Th1 cytokines such as interferon (IFN)-γ can also be found in BAL fluid (Prieto et al., 2001). IL-4 and IL-13 have overlapping biological functions due to a shared receptor component and shared signaling pathways (Renauld, 2001). Among other effects, these cytokines also regulate immunoglobulin (Ig)-E class-switching in B cells. IL-4 is required for Th2 differentiation, but ablation of IL-4 alone is not sufficient to block airway hyperreactivity (AHR) in mouse models of asthma (Hogan et al., 1997). Ablation of IL-13 completely blocks AHR in these models. IL-5 is the key cytokine responsible for the expansion and maintenance of eosinophils (Walter et al., 2001). IL-9 has various effects including promoting proliferation of bone marrow-derived mast cells. Constitutive expression of IL-9 in the lungs of transgenic mice results in spontaneous airway inflammation

characterized by overexpression of other Th2 cytokines (Vink et al., 1999). Granulocyte macrophage-colony stimulating factor (GM-CSF) is a cytokine derived primarily from monocytes/macrophages and stimulates a wide variety of inflammatory cells including eosinophils. GM-CSF has also been shown to be elevated in the BAL fluid of patients with asthma following antigen provocation (Broide et al., 1992). COPD, which encompasses emphysema and chronic bronchitis, is characterized by poorly reversible, progressive airway obstruction, mucosal and submucosal inflammatory cell infiltration, edema, fibrosis, mucous plugs, and smooth muscle hypertrophy. BAL fluid and sputum from COPD patients contain increased numbers of neutrophils (in contrast to asthma where eosinophils predominate) and inflammatory mediators such as leukotriene (LT)B_4 (a major neutrophil chemoattractant and activator), TNF-α, and IL-8 (Barnes, 2003). PDE4 inhibitors have been shown to inhibit many of these cytokines involved in both asthma and COPD. One of the major issues has been which *in vitro* or *ex vivo* readout of cytokine inhibition is most relevant for the clinical correlate of efficacy. In this study, we have utilized human peripheral blood and purified human T cells along with murine polarized T cells to delineate a potential profile of inhibition of PDE4 compounds.

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Methods

PDE4 activity assay

The hydrolysis of cAMP by the purified human recombinant GST-PDE4A^{248} and its PDE4B, PDE4C and PDE4D equivalents were monitored by following the hydrolysis of [^{3}H]-cAMP to [^{3}H]-AMP at room temperature in a buffer containing 0.1 μM [^{3}H]-cAMP (specific activity: 1 μCi/mL), 10 mM $MgCl_2$, 50 mM HEPES (pH 7.2) as previously described (Laliberte et al., 2000). IC_{50} values were estimated using a 4-parameter non-linear regression analysis of a 11 point-dose response curve performed in duplicate. The PDE4 enzymes utilized are the recombinant QT versions of PDE4 A, B, C, and D which encode the catalytic domain of PDE4 and have been shown to be in a fully activated conformation (Liu et al., 2001). The PDE4 constructs termed QT versions were generated by creating GST fusion constructs in frame with the Gln-Thr (QT) located within UCR2 of the PDE4A4, PDE4B2, PDE4C2 and PDE4D3 sequences. These constructs were expressed in Sf9 cells and purified to homogeneity as previously described (Laliberte et al., 2000). The purified enzymes were electrophoresed and confirmed to be the predicted molecular mass based on this fusion construct. The IC_{50}s obtained for the truncated QT versions were also compared with purified full length enzyme and found to be within 2-3 fold that obtained with the corresponding full length versions of the PDE4 A4, B2, C2 and D3 enzymes.

PDE selectivity assays

L-826,141 was counterscreened vs PDE1-11 for inhibition of any other phosphodiesterase isoform. PDE1, PDE3, PDE7, PDE8, PDE10 and PDE11 were all assayed similar to the PDE4 enzymes at substrate concentrations of 0.1 μM cAMP. All enzymes were obtained from human recombinant sources except PDE1 which was from dog-heart, PDE6 from bovine eye and PDE2 from human platelets. PDE2 from human platelets was assayed by MDS Panlabs (Wash., USA) at 1 μM cAMP. The PDE5, PDE6 and PDE9 enzymes were all assayed at 0.1 μM cGMP.

Cytokine production in human whole blood

Heparinized whole blood from healthy volunteers was collected and treated as previously described (Muise et al., 2002). Briefly, 500 µl aliquots of blood were pre-incubated with either 2 µl DMSO or PDE4 inhibitors at 37°C for 15 min. This was followed by incubation with 10 µl of 0.1 % BSA, LPS (Sigma, from *E. coli* serotype 0111:B4, 1 µg/ml final concentration) or Con A (Sigma, 50 µg/ml final concentration) for the indicated times at 37°C. At the end of the incubation, samples were centrifuged at 1500 x g at 4°C for 10 min to collect plasma or erythrocytes were lysed using EL buffer (Qiagen, Chatsworth, CA) and white cells pellets were resuspended in TRIzol reagent (Invitrogen, Carlsbad, CA) for total RNA isolation. Plasma levels of the following cytokines were quantified by ELISA: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IFNγ, GM-CSF, TGFβ (Biosource, Camarillo, CA) and TNFα (Cistron Biotechnology, Pine Brook, NJ) according to the manufacturer's instructions. Total RNA was first isolated using TRIzol reagent and then cleaned up using the Rneasy mini kit combined to a DNAse treatment (Qiagen, Chatsworth, CA) as described in the manufacturer's instructions Total RNA was reverse transcribed and cytokine mRNA levels were quantified by real-time quantitative PCR as described below.

Reverse Transcription and Real-Time Quantitative PCR

Reverse transcription of RNA (50 ng) was performed using Taqman Transcription Reagents (PE Biosystems, Foster City, CA). The reaction was performed in the presence of 1X Taqman RT buffer, 5.5 mM Magnesium chloride, 500 µM each dNTP, 2.5 µM random hexamers, 0.4U/µl Rnase inhibitor and 1.25 U/µl Multiscribe Reverse

Transcriptase. Every reaction set included RNA samples incubated in absence of Multiscribe Reverse Transcriptase to serve as controls for genomic DNA contamination. The cycling parameters consisted of a primer incubation of 10 min at 25°C, a reverse transcription of 30 min at 48°C followed by inactivation at 95°C for 5 min. Real-time quantitative PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, CA) with pre-developed primers and probe sets for all the cytokines mentioned above as targets and 18S rRNA as an endogenous reference purchased from PE Biosystems (Foster City, CA). These pre-developed probe/primers sets have been optimized to have similar amplification efficiencies. Each PCR reaction was performed in a total volume of 50 µl, containing 5 µl cDNA, 25 µl Taqman Universal PCR Master Mix (PE Biosystems, Foster City, CA), 2.5 µl of the commercial probe/primers set and nuclease-free water (Ambion, Austin, TX) to complete. The cycling parameters consisted of 2 min uracil removal incubation at 50°C, 10 min polymerase activation at 95°C and 50 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Every reaction set included a reaction where water was used instead of cDNA to serve as a control for DNA contamination and probe degradation.

Quantitation of Gene Expression

Since pre-developed probe/primers sets have been optimized to have similar amplification efficiencies, the relative quantification of the real-time PCR results was performed using the comparative $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001). Briefly, PCR reactions were performed in parallel for all samples.

Measured fluorescent signal intensities were plotted against the number of PCR cycles on a semi-logarithmic scale. The cycle number where the PCR amplification was in its exponential phase was referred as the threshold cycle (C_t). To normalize for input amounts of RNA, PCR reactions for the target genes and for the 18S rRNA endogenous reference were performed in separate tubes for each sample and the 18S rRNA Ct values were subtracted from the target Ct values to obtain normalized ΔCt values. The calibrator was defined arbitrarily as the samples pre-incubated with DMSO and stimulated with 0.1% BSA and the normalized ΔCt values of the calibrator was subtracted from the normalized ΔCt values of all the samples to obtain ΔΔCt values. The relative expression of each sample to the calibrator was obtained by calculating $2^{-\Delta\Delta Ct}$.

CD3/CD28-dependent IL-2 secretion in isolated human T cells

Whole blood from healthy volunteers was collected into Vacutainers CPT™ cell preparation tubes (Becton Dickinson, Franklin Lakes, NJ) for the isolation of mononuclear cells. The tubes were centrifuged at 1800 x g at room temperature for 20 min and the whitish layer containing mononuclear cells and platelets was collected. Cells were washed with PBS and residual erythrocytes were lysed using a buffer containing 0.14 M NH_4Cl and 17 mM Tris-HCl pH 7.5 at room temperature for 7 min. Cells were then centrifuged at 200 x g for 10 min to remove platelets and B cells were depleted using anti-CD19 microbeads (Miltenyi Biotec, Auburn, CA) on the AutoMACS instrument (Miltenyi Biotec, Auburn, CA). At the end, cells were resuspended at 2 x 10^6 cells/ml in Cyto SF-4 medium (Kemp Biotechnologies, Frederick, MD) and dispensed to the wells of a 96-well plate precoated with 500 ng anti-CD3 (Pharmingen, San Diego,

CA, 2.5 µg/ml final). Then, cells were pre-incubated with either 1 µl DMSO or PDE4 inhibitor at 37°C for 15 min. This was followed by the addition of 2 µg/ml anti-CD28 (Biosource, Camarillo, CA) and incubation at 37°C for 18 h. The cell culture supernatant was collected and IL-2 was measured by ELISA (Biosource, Camarillo, CA).

Murine T cell activation and polarization

Ovalbumin (OVA)-specific TCR-transgenic mice (DO11.10 mice) (Murphy et al., 1990) and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Splenocytes were prepared from 2 to 6 months old DO11.10 or BALB/c mice in Leibovitz's L-15 culture medium supplemented with 5% heat-inactivated FCS, 10 mM HEPES, and 50 µg/ml gentamicin. Red blood cells were eliminated by resuspending the cells in 2 ml (per spleen) ACK lysing buffer (formula 79-0422DG, Life Technologies, Grand island, NY) for 2 minutes. The cell suspension was then diluted with a 9X volume of high phosphate buffered saline with 3% FCS to stop the lysing reaction and remove the dead cells. The cell suspension was centrifuged and the cell pellet was resuspended in L-15 medium and filtered through a Nitex nylon mesh. The purified splenocytes were then washed two more times in L-15 medium before being resuspended in RPMI 1640 culture medium (Life Science Technology, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 1mM L-glutamine, 10 mM HEPES, 1mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 µM 2-mercaptoethanol, and 50 µg/ml gentamicin. The cells were cultured in tissue culture flasks for 7 days. The stimuli are as follows: 0.3 µM OVA peptide 323-339, 2 ng/ml m-IL-2 and either Th1 or Th2 cytokines. For generating Th1 cells, 10 ng/ml mIL-12 and 2 µg/ml anti-IL-4 (clone11B11, PharMingen)

were added. For generating Th2 cells, 20 ng/ml mIL-4 and 2 μg/ml anti-IFN-γ (clone R4-6A2) were added. Throughout the polarization, the culture was split to keep the cells in log growth phase and to prevent overcrowding. For a secondary culture, the polarized Th1 or Th2 cells were washed 3 to 4 times with L-15 or RPMI culture medium. The T cells (2 $x10^5$ per well) were then cultured in 96-well plates with 5 $x10^5$ irradiated APC (spleen cells from BALB/c wild type mice) and 0.3 μM OVA peptide 323-339. For preparing antigen-presenting cells (APC), the isolated spleen cells were irradiated (3000 rads) and washed one more time with L-15 medium before they were added to the culture. IL-2, IL-4, and IFN-γ were measured using an antibody-based capture assay as described previously (Chen et al., 1994) with modifications for IL-4 and IFN-γ. For detecting IL-4 in culture supernatants, 60 μl of anti-mouse IL-4 (2 μg/ml, clone 11B11) was used as the capture antibody and 60 μl of biotinylated anti-mouse IL-4 (1 μg/ml, clone BVD6-24G2) was used as the detecting antibody. For detecting IFN-γ, 60 μl of anti-mouse IFNγ (4 μg/ml, clone R4-6A2) was used as the capture antibody and 60 μl biotinylated anti-mouse IFNγ (1 μg/ml, clone XMG1.2) was used as the detecting antibody. The data are shown as the mean of triplicate 96 well cultures and the standard error were less than 20% in all cases.

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Results

L-826,141 is a potent and selective inhibitor of PDE4. These studies focused on the molecular characterization of the selective PDE4 inhibitor, L-826,141 whose structure is shown in Fig. 1. Comparisons to several other PDE4 inhibitors including R-rolipram (Semmler et al., 1993), roflumilast (Hatzelmann and Schudt, 2001) and its N-Oxide metabolite were also performed. The structure of L-826,141 is presented in Fig. 1 and data on the comparison of inhibitors with PDE4 A, B, C, and D are depicted in Table 1. L-826,141 is a potent inhibitor of PDE4 (IC_{50}=0.26-2.4 nM) and is slightly more potent (at least 3-fold) at inhibiting PDE4B and PDE4D as compared to PDE4A and PDE4C (Table 1). This is in contrast to R-rolipram which is equipotent for inhibition of PDE4 A, B, and D and is 7.5-fold less potent for PDE4C. Roflumilast and it's active metabolite (roflumilast N-oxide) is also slightly more potent for PDE4 A, B, and D vs PDE4C (3-10 fold). L-826,141 is at least 450-fold selective for PDE4 since potency versus PDE 1-11 were tested and the only appreciable inhibition achieved below 10 μM was with PDE3A and PDE3B (IC_{50} = 2.1 and 1.1 μM, respectively) and PDE8A (IC_{50} = 4 μM)(data not shown).

L-826,141 was also tested vs various substrate concentrations of cAMP and demonstrates clear competitive inhibition for PDE4 with the $K_{m\ app}$ demonstrating linearity with the inhibitor concentration (Fig. 2). The K_m based on this data is 2.6 μM for cAMP which is consistent with a prior published report (Laliberte et al., 2000) and the K_i is 0.5 nM for L-826,141. It has also been previously demonstrated that binding to PDE4 is dependent on Mg^{++} and the low affinity rolipram binding site may be due to lack

of metal ions in the enzyme preparation and therefore inhibition of an apoenzyme form of PDE4 (Liu et al, 2001). Based on this latter data, we have performed assays with PDE4 in the absence and presence of Mg^{++} and L-826,141 had an IC_{50} of 2.2 nM for inhibition of PDE4A with Mg^{++} and in the absence of Mg^{++} the IC_{50} was >1 μM. This data suggests that L-826,141 is a more potent inhibitor of the PDE4 holoenzyme form as compared to the apoenzyme form. As a control for this last experiment, rolipram was also tested and the IC_{50} shifted from 5.6 nM to 260 nM for inhibition of PDE4 in the presence or absence of Mg++, respectively, similar to previous results (Liu et al, 2001).

Since PDE4 is widely expressed in inflammatory cells, we have also profiled these PDE4 selective inhibitors in human cellular assays using mononuclear cells and peripheral blood. cAMP elevation has been demonstrated to downregulate production of various cytokines through activation of PKA and indirect mechanisms including transcriptional downregulation of the NFκB pathway which can lead to inhibition of the MAP kinase cascade (Simonds, 1999). TNFα is one of the cytokines downstream of cAMP elevation and MAP kinase activation which has been utilized as a surrogate for PDE4 inhibition in the clinic (Timmer et al., 2002). The rank order of potency in the mononuclear cell assays stimulated with LPS to produce TNFα is roflumilast>roflumilast N-oxide>L-826,141>rolipram (Table 1). This rank order of potency is maintained in a peripheral blood assay performed in the presence of complete serum. Also, comparing the inhibition of TNFα production in peripheral blood, the potency of L-826,141 (IC_{50}=0.3 μM) is within 2-fold that of roflumilast and its N-oxide and 7-fold more potent than R-rolipram (Table 1). This data demonstrates that in cellular based assays, L-826,141 and R-rolipram are least shifted in the presence of high levels of serum protein.

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Utilizing the mononuclear cell assay, we have also tested the effect of the PKA inhibitor H89 on PDE4 inhibition. The IC_{50} for L-826,141 is shifted from 31 nM to 12.4 μM in the presence of 10 μM H89 and demonstrates that TNFα inhibition is dependent on PKA activation. In order to confirm that this inhibition is due to cAMP elevation in whole blood, we have utilized the cell permeable analogue of cAMP, dibutyryl cAMP and this compound inhibits TNFα formation in whole blood with an IC_{50} of 6.5 ± 2.7 μM.

Analysis of cytokine production in peripheral blood. Human peripheral blood serves as an easily accessible source of myeloid and lymphoid cells for analysis of cytokine production in a clinical setting. We have utilized peripheral blood under a Toll like receptor4 (TLR4) stimulation paradigm with LPS and a non specific T cell stimulation with Concanavalin A (ConA). This study focused on transcriptional downregulation of key cytokines involved in inflammation and the attenuation achieved by L-826,141. The cytokines analyzed were IL-1β, IL-4, Il-5, IL-6, IL-8, IL-10, IL-12, IL-13, IFNγ, GM-CSF, and TNFα. The formation of these cytokines at various time points with either an LPS or ConA stimulus was determined by RNA analysis using real time quantitative PCR. The cytokines which were attenuated by L-826,141 are depicted in Fig. 3 with respect to production over time. Since the 4 h time point resulted in maximal stimulation of cytokine production under the conditions tested, this timepoint was utilized for subsequent measurements of cytokine inhibition. IL-1β, IL-5, IL-6, IL-8, and IL-10 were not significantly attenuated by L-826,141 (data not shown). The IC_{50s} for inhibition of TNFα, IL-12, GM-CSF, and IFNγ by L-826,141 range from 0.28 -0.88 μM (Fig. 4 and Table 2). Utilizing these conditions a slight inhibition of both IL-4 and IL-13 was also

obtained (Fig. 4) but the maximal mean inhibition achieved was less than 50% at a concentration of 27 μM. IL-2 inhibition was also obtained but was more variable than that observed for the other cytokine measurements. Therefore, IL-2 production and inhibition in isolated human naive T cells was performed after T cell receptor stimulation.

Inhibition of IL-2 production in CD3/CD28 stimulated human T cells. IL-2 is the major cytokine involved in proliferation of T cells, B cells, and NK cells upon activation of a naïve T cell in the presence of a professional antigen presenting cell. It has been previously demonstrated that IL-2 production can be attenuated by the elevation of cAMP through a PKA dependent mechanism (Li et al., 1999). In order to determine the role of PDE4 in IL-2 production in this paradigm, we have isolated naïve human T cells from peripheral blood that are $CD27^+$ and $CD69^-$ to 87% homogeneity. These cells were challenged with either CD3 alone, CD28 alone, or CD3 plus CD28 combined (Fig. 5) at various time points. Only the CD3 plus CD28 co-stimulation provided a significant production of IL-2 above the background with a maximal IL-2 production of 3.5 ng/ml. The maximal detectable IL-2 formation was between 8-12 h post CD3/CD28 stimulation. This population of human T cells were then utilized to determine the PDE4 component responsible for regulating IL-2 production. The early generation PDE4 inhibitor, (R)-rolipram or L-826,141 were preincubated with human T cells prior to CD3 plus CD28 co-stimulation and production of IL-2 was measured at 8 or 18h post antibody challenge. (R)-rolipram was a potent inhibitor of IL-2 production with an IC_{50} between 0.2 and 0.3 μM (Fig. 5). The IC_{50} correlates well with a similar but separate experiment performed in mononuclear cells for TNFα inhibition by rolipram (IC_{50}=0.3 μM). Similarly, L-

826,141 was found to have an IC50 of 3 nM for inhibition of T cell mediated Il-2 rpoduction and this is consistent with its increased potency in mononuclear cell for inhibition of TNFα formation. Several PDE4 inhibitors have been shown to block the formation of IL-2 including roflumilast (Hatzelmann and Schudt, 2001) and this may suggest that these compounds may truly have an immunomodulatory role in inflammatory disease.

Inhibition of polarized murine T cell cytokine production. As shown above, the cytokines attenuated in peripheral blood by PDE4 inhibitors are TNFα, IL-2, IFNγ, GM-CSF, IL-12 and and to a lesser extent IL-13. Since various cell types besides differentiated T cells can produce some of these cytokines, we performed analyses on differentiated and antigen challenged T cells. Several reports of PDE4 inhibitors have demonstrated attenuation of either Th1 or Th2 mediated cytokine production depending on the *in vitro* method utilized. Since conditions to generate polarized Th1 and Th2 cells have been well established in the murine system, we have decided to perform antigen presentation assays in murine Th0, Th1, and Th2 cells. The results presented from Th0 and polarized Th1, Th2 cells were obtained from ovalbumin specific TCR transgenic mice. Polarization of Th1 and Th2 cells was performed as described in Materials and Methods and the antigen utilized for all three cell types was OVA 323-339 at a concentration of 0.3 μM. Results for L-826,141 in comparison with dexamethasone are presented in Table 3. The compounds were added either after the first antigen challenge for Th0 cell activation or after the second antigen challenge for Th1 and Th2 polarized cells. L-826,141 and dexamethasone inhibited Th0 antigen provoked IL-2 production.

Dexamethasone was the most potent inhibitor with an IC_{50} of 12 nM (IC_{35}=5 nM), which is consistent with previously reported data (Kavelaars et al., 1995). The inhibition achieved with L-826,141 was maximal at 60-70% and with an IC_{35} of 25 nM. These data demonstrate that PDE4 inhibitors can attenuate IL-2 production but the inhibition is partial and less than that seen with dexamethasone. The polarized T cells were analyzed for production of IFNγ, IL-4, and IL-13. In order to confirm that the T cells were appropriately polarized, Th1 cells were analyzed for IFNγ production and resulted in low or undetectable production of IL-4 and IL-13 demonstrating a polarized population of Th1 cells. Th2 cells were analyzed for formation of IL-4 and IL-13, and produced low or undetectable levels of IFNγ. In antigen challenged Th1 cells, dexamethasone was a weak inhibitor of IFNγ production with maximal inhibition of 47% at a concentration of 300 nM, consistent with a Th2 profile of inhibition. In the antigen challenged polarized murine Th1 cells, L-826,141 inhibited IFNγ formation with an IC_{30} of 46 nM. Dexamethasone inhibited IL-4 and IL-13 production with IC_{50}'s of 3.5 and 4 nM, respectively and as seen with IL-2 inhibition the maximal inhibition achieved was between 75-85 %. In contrast to dexamethasone, L-826,141 was a weak inhibitor of IL-4 and IL-13 formation with L-826,141 achieving only 37 % inhibition of IL-4 production at a concentration of 10 μM. Roflumilast and its corresponding N-oxide (data not shown) also did not achieve inhibition of IL-4 greater than 30% in the Th2 cells upto a concentration of 10 μM. Therefore, the selective PDE4 inhibitor, L-826,141 can inhibit OVA challenged Th0 cells to produce IL-2 and has a propensity for inhibition of Th1 vs Th2 cytokine production.

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Discussion

PDE4 inhibitors are presently under clinical development for the treatment of asthma and/or COPD. The clinical candidates that are the furthest advanced are cilomilast (Compton et al., 2001) and roflumilast (Timmer et al., 2002). In this study, we have focused primarily on the compound L-826,141 with some comparative *in vitro* data with R-rolipram and roflumilast. These compounds target the PDE4 gene family which includes PDE4 A, B, C, and D along with the various splice variants (Houslay and Adams, 2003) and are competitive inhibitors of the nucleotide binding site for cAMP on PDE4. Recently, the crystal structure of PDE4B and D have delineated the active site of PDE4 and demonstrated that R and S-rolipram bind within the cAMP binding domain of PDE4 (Dym et al., 2002;Xu et al., 2000). The earliest precursor to PDE4 inhibitors could be attributed to caffeine and the methylxanthines but these compounds are quite weak PDE4 inhibitors (Horiuchi and Castro, 2000). These compounds although quite ineffective as PDE4 inhibitors were known to have efficacy in pulmonary diseases. The development of potent and selective PDE4 inhibitors has not yet led to a clinically approved candidate. In this study, we focus on the mechanistic profile of inhibition at the enzyme and inflammatory cytokine level. The compound that has been profiled is L-826,141 which is a structural analogue to CDP840, which has previously demonstrated attenuation of the late asthmatic response in humans in an allergen challenge paradigm (Harbinson et al., 1997).

L-826,141 is a potent, selective and competitive PDE4 inhibitor with IC_{50}s in the 1 nM range for the 4 PDE4 isoforms. The inhibitor preferentially inhibits the

holoenzyme as compared to the apoenzyme. Since PDE4B and D are essential for LPS stimulated TNFα formation and smooth muscle hyperreactivity (Hansen et al., 2000;Jin and Conti, 2002), respectively, analysis of intrinsic potency on these enzymes demonstrates that L-826,141 is equipotent to roflumilast N-oxide, which is the relevant metabolite of roflumilast formed *in vivo*. L-826,141 is also a very potent inhibitor of PDE4A and C, and the role that these latter two enzymes play in inflammation shall only be determined through development of sub-type selective inhibitors or phenotypic analysis of the corresponding null mice.

Since PDE4 is the major enzyme which metabolizes cAMP in inflammatory cells, significant research has focused on the role of PDE4 in Gαs interactions. One suggested role is the interaction with Gαs through the B-arrestin adaptor molecules and desensitization of signaling through the β_2 adrenergic receptor (Perry et al., 2002). Since β_2 adrenergic receptor activation leads to various signaling cascades and stimulation and/or inhibition of various cytokines, we have utilized peripheral blood, isolated human T cells and murine polarized T cells to determine the Th1/Th2 balance of inhibition obtained with selective PDE4 inhibitors. This data demonstrates that at nM concentrations L-826,141 is a potent inhibitor of TNFα, IL-12, GM-CSF, and IFNγ in peripheral blood. Utilizing a CD3/CD28 co-stimulation of naïve T cells, 80% of IL-2 production was inhibited by both selective PDE4 inhibitors, L-826,141 and rolipram. This clearly delineates that the cAMP pool which activates PKA dependent IL-2 production in isolated T cells is directly linked to the PDE4 gene family. Previously, data had suggested that PDE7 may play an important role in IL-2 generation from T cells (Li et al., 1999). Our data with the PDE4 inhibitors, L-826141 and rolipram and also recent

data with PDE7A null mice (Yang et al., 2003) demonstrating that IL-2 levels and Tcell proliferation upon CD3/CD28 stimulation is similar in PDE7A(-/-) as in wild-type littermates suggests that PDE7 is not essential for T cell function.

Peripheral blood is skewed towards primarily the production of Th1 cell associated cytokines, and therefore we also analyzed polarized and antigen challenged murine T cells to determine the Th1/Th2 balance of inhibition obtained with selective PDE4 inhibitors. The data from polarized T cells confirms the peripheral blood data and demonstrates conclusively that L-826,141 has a greater propensity to inhibit Th1 derived cytokines *in vitro* and IL-2 production than Th2 derived cytokines. Based on the paradigm that asthma is primarily a Th2 biased immunological disorder and COPD has primarily a Th1 bias, the data generated suggests that compounds such as L-826,141 will have a greater anti-inflammatory impact on COPD and Th1 based immune disorders. Our data is in contrast to several reports demonstrating that PDE4 inhibitors can attenuate production of IL-4 and IL-5 production in various stimulation paradigms. Ariflo has been reported to inhibit oxazalone induced IL-4 production in a mouse model of ear inflammation (Griswold et al., 1998). Roflumilast in our hands also demonstrates a lack of inhibition of IL-4 in the *in vitro* transgenic mouse model of OVA challenge Tcells, while a literature report has demonstrated that roflumilast inhibits IL-4 and IL-5 production in CD3/CD28 co-stimulated human Tcells (Hatzelmann and Schudt, 2001). Rolipram has also been shown at elevated doses (5-10 μM) to inhibit IL-5 formation in OVA challenged mouse splenic cells *in vitro* and IL-4 and IL-5 from BALF in OVA challenged mice (Foissier et al., 1996). There is also data with rolipram demonstrating that in human T cell antigen presentation assays IFNγ from Th1 cells can be significantly

inhibited without altering formation of IL-4 from Th2 cells (Bielokova et al., 2000) consistent with the profile seen with L-826,141. The true utility of PDE4 inhibitors such as L-826,141 can only be determined if a PDE4 inhibitor is clinically approved.

cAMP signaling has been studied for over five decades and the complete pathways have not yet been fully elucidated. We have performed analyses on cytokine production in order to determine the effects of blockade on cAMP metabolism through the action of PDE4 inhibitors. cAMP levels result in various manifestations including effects on smooth muscle contractility, neurochemical mediator release, chemotaxis, cell adhesion, vasopermeability, mucociliary activity as well as effects on inflammatory cell activity. PDE4 inhibitors clearly have potential as novel new anti-inflammatory agents for several different immune mediated diseases. The clinical approval of this new class of compounds may hold significant promise in the treatment and control of asthma, COPD and other immune based diseases.

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Legends for figures

Figure 1. Structure of L-826,141 (4-{2-(3,4-bis-difluromethoxyphenyl)-2-{4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-phenyl]-ethyl}-3-methylpyridine-1-oxide).

Figure 2. Inhibition of PDE4A activity at various cAMP concentrations. The PDE4A QT enzyme was incubated with either DMSO vehicle or several concentrations of L-826,141 at cAMP concentrations between 0.1 -15 μM. The specific activity of PDE4A is plotted vs the various cAMP substrate concentrations and the inhibitor concentrations are depicted in the legend.

Figure 3. Time-course of cytokine mRNA formation in human whole blood. Heparinized whole blood was incubated with LPS (A) or Con A (B) and analyzed over a 24 h period. At the indicated time, total RNA was isolated, reverse transcribed, and analyzed by real-time quantitative PCR analysis. The levels of expression of TNF-α, IL-12p40, GM-CSF, IFNγ and IL-13 are expressed in mRNA quantity relative to that of the whole blood at time zero, after normalization to 18S rRNA. Data are presented as the mean ± SEM of n = 4 experiments performed in duplicate unless otherwise indicated (†, n = 3).

Figure 4. Inhibition of cytokine mRNA formation by L-826,141 in human whole blood. Heparinized whole blood was pre-incubated with L-826141 or vehicle for 15 min.

Following the preincubation, whole blood was challenged with either LPS (A) or Con A (B) for 4 h. At the end of the incubation, total RNA was isolated, reverse transcribed, and analyzed by real-time quantitative PCR analysis. Relative TNF-α, IL-12p40, GM-CSF, IFNγ and IL-13 mRNA levels were determined and reported as a percentage of inhibition of the control reaction. Data are presented as the mean ± SEM of n = 3 experiments performed in duplicate unless otherwise indicated (†, n = 2).

Figure 5. Effects of Rolipram on IL-2 formation in human T cells. (A) Time-course of IL-2 secretion in isolated human T cells. T cells were isolated from human whole blood and challenged with anti-CD28, anti-CD3 or both over an 18 h time period. (B) Inhibition of CD3/CD28- dependent IL-2 secretion by (R)-Rolipram in isolated human T cells. Human T cells were pre-incubated with (R)-Rolipram, L-826,141 or vehicle for 15 min. Then, cells were incubated with anti-CD3/anti-CD28 for 18 h. At the indicated time, culture supernatants were collected and analyzed for IL-2 production by ELISA. Each data point is an average of duplicates and is reported in (B) as a percentage of inhibition of the control reaction. Τηε χορρεσπονδινγ δατα φορ μονονυχλεαρ χελλ ΤΝΦα inhibition and IL-2 inhibition is presented in Tabular format.

Table 1. Intrinsic potency of PDE4 inhibitors in enzymatic and cell based assays

	IC_{50} Values ± SEM (nM) for several assays					
Compound	**PDE4AQT**	**PDE4BQT**	**PDE4CQT**	**PDE4DQT**	**HWBA (TNFα)**	**HMCA (TNFα)**
L-826141	**1.26 ± 0.15 (9)[a]**	**0.38 ± 0.07 (5)**	**2.38 ± 0.8 (5)**	**0.26 ± 0.04 (5)**	**310 ± 48 (21)**	**31 ± 13 (6)**
R-rolipram	**4.8 ± 1.3 (9)**	**5.4 ± 2.0 (6)**	**40.5 ± 7 (8)**	**3.9 ± 1.0 (8)**	**2156 ± 204 (15)**	**288 ± 125 (8)**
Roflumilast	**0.16 ± 0.03 (3)**	**0.11 ± 0.01 (4)**	**0.61 ± 0.05 (4)**	**0.11 ± 0.01 (4)**	**140 ± 23 (15)**	**1.29 ± 0.55 (5)**
Roflumilast N-oxide	**0.58 ± 0.03 (4)**	**0.37 ± 0.05 (4)**	**3.2 ± 0.5 (4)**	**0.31 ± 0.04 (4)**	**200 ± 65 (20)**	**1.63 ± 0.65 (4)**

a=n number

The PDE4 enzymes are constructs which express the catalytic domain of the PDE4 enzymes (QT) and have been previously described (Liu et al., 2001, see methods). All enzyme assays were performed with 0.1 μM CAMP as described in the Methods. TheIC_{50} values for inhibition of the the four PDE4 isoforms were performed at [S]>>K_m and are therefore approximate K_i values. Cell based asays were also utilized in which LPS stimulated production of TNFα was measured in the presence or absence of inhibitor (HWBA=human whole blood assays; HMCA=human mononuclear cell assay).

Table 2 Inhibition of peripheral blood cytokines by the PDE4 inhibitor L-826,141

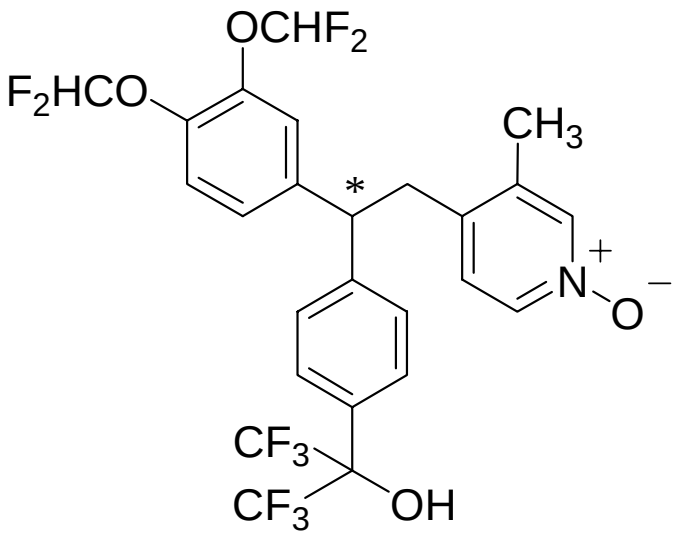
cytokine	IC_{50} (μM)
TNFα	0.33 ± 0.03
Il-12	0.88 ± 0.18
GM-CSF	0.28 ± 0.08
IFNγ	0.35 ± 0.11
IL-4	>27
IL-13	>27

Heparanized whole blood was pre-incubated with L-826,141 for 15 min prior to LPS or ConA cahllenge. 4h post stimulation the cells were harvested and RNA extracted and analyzed by quantitative PCR. Individual data points are presented in Fig.3.

Table 3: Th0; Th1 and Th2 cells from ovalbumin transgenic mice (DO11)

Cell type	Cytokine	IC (nM)	L-826,141	Dex
Th0	IL-2	IC_{35}	25 (n=3)	5
Th1	IFNγ	IC_{30}	46 (n=2)	15
Th2	IL-4	IC_{30}	> 2.5 μM	1.7
Th2	IL-13	IC_{30}	> 2.5 μM	1.8

T cells from ovalbumin specific T cell receptor transgenic mice (DO11) were isolated and cultured as described in the Methods. Th0 and polarized Th1 and Th2 cells were challenged with 0.3 μM OVA peptide 323-339 in the presence or absence of the inhibitors. The production of the cytokines was determined by immunoassays.



L-826,141
(Enantiomer 1)

Fig. 1

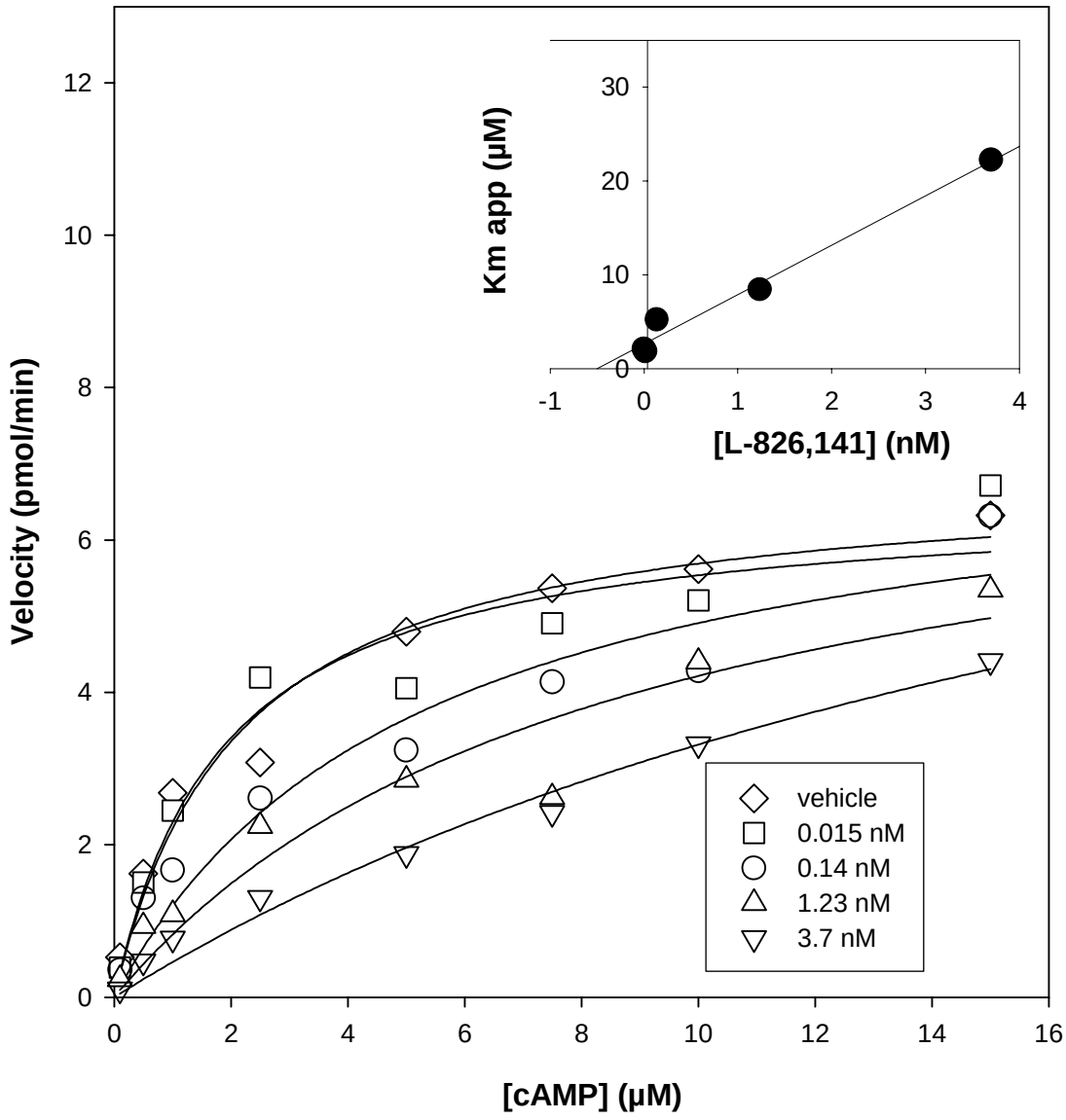
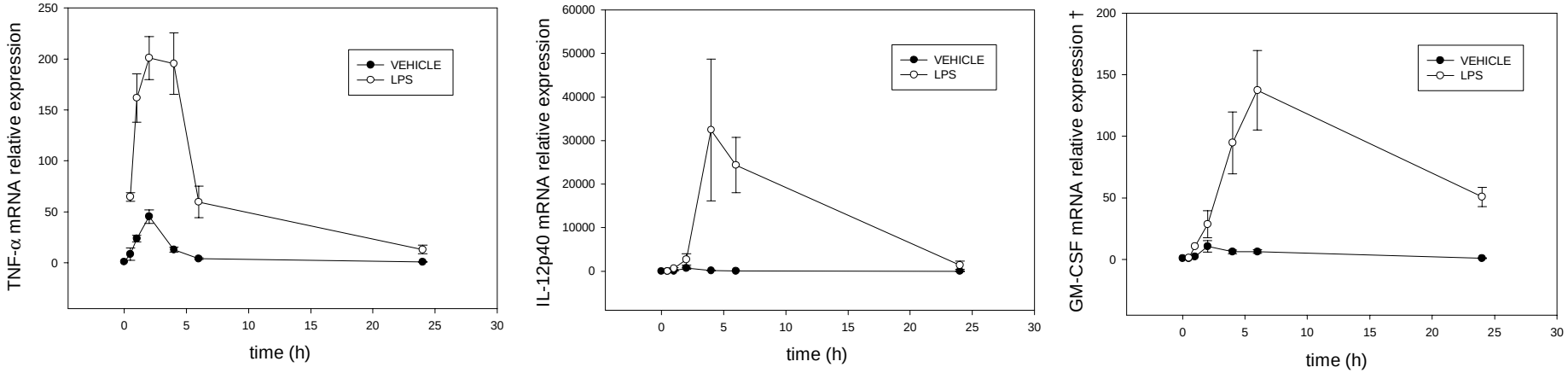


Fig. 2

A.



B.

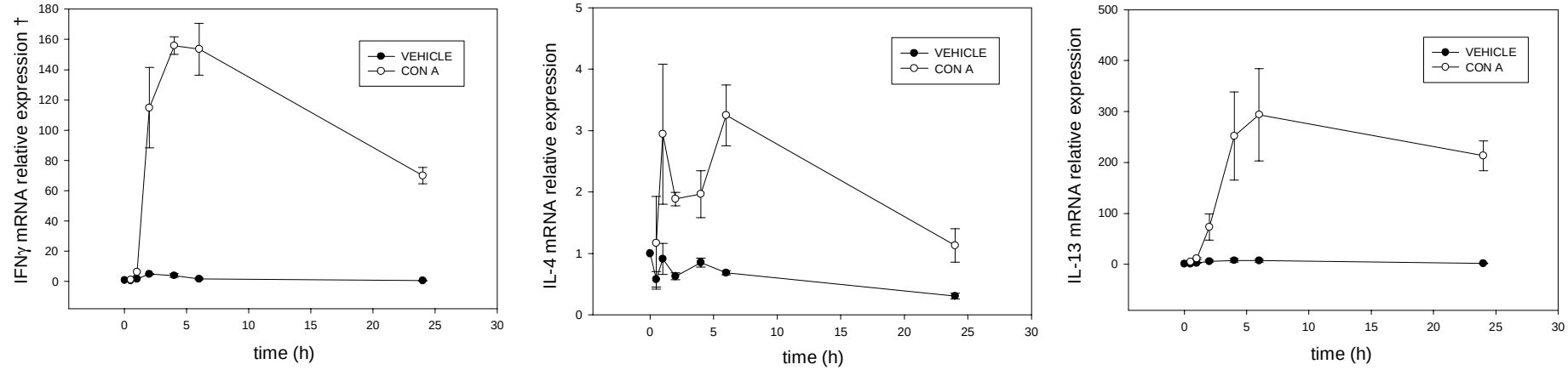


Fig. 3

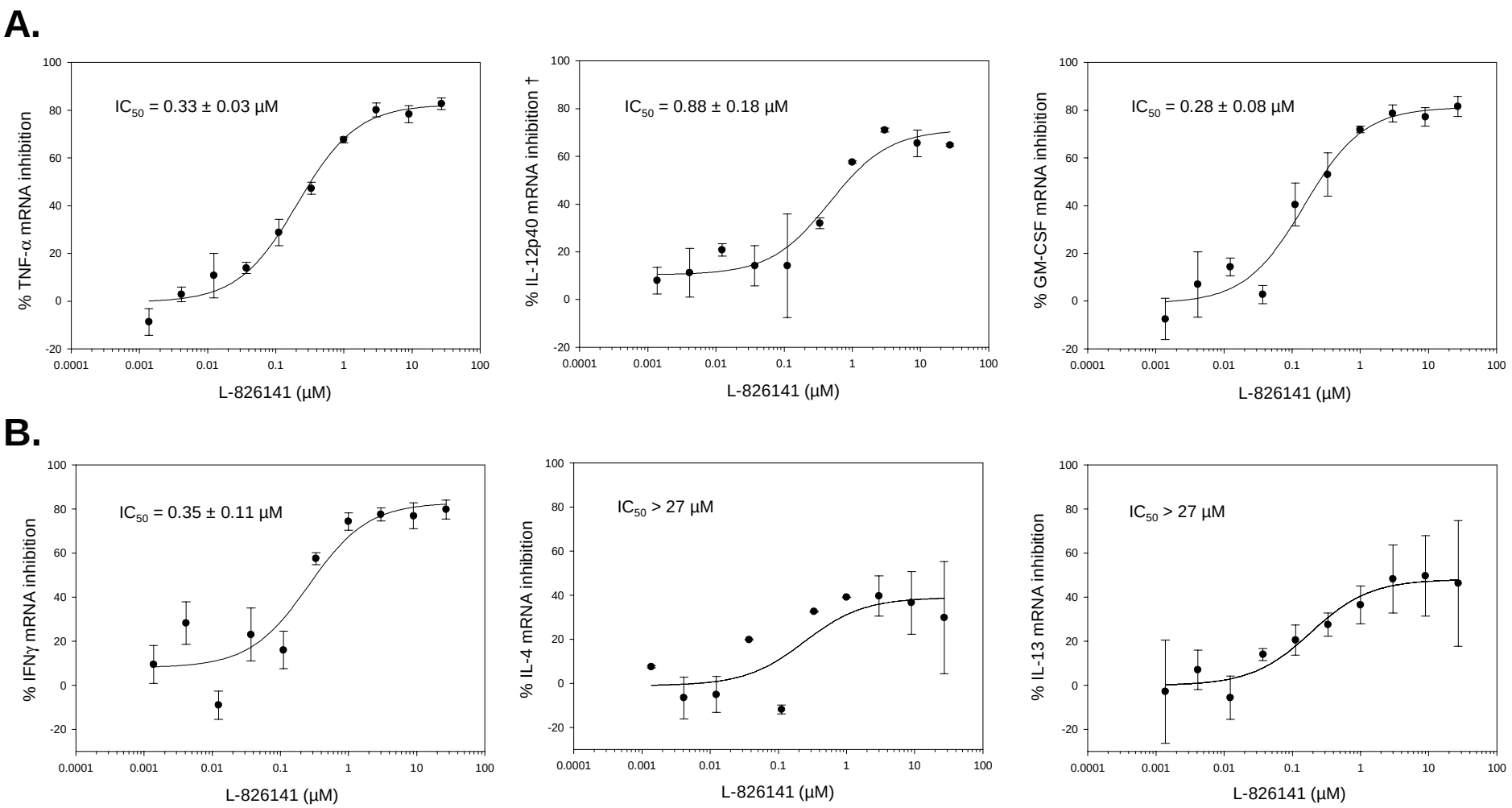
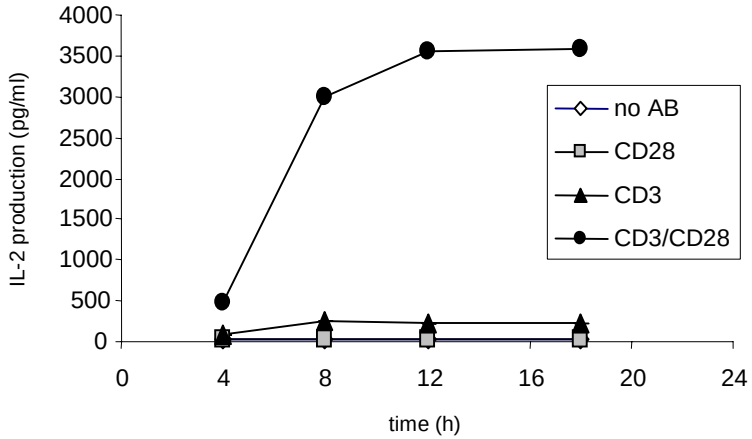


Fig. 4

A.



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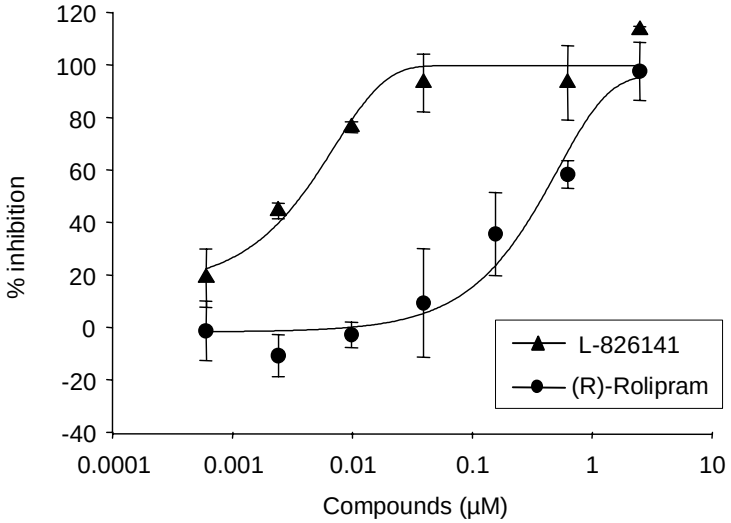

C.

	IC_{50} (µM)	
	Mononuclear cell TNF-α assay	**T cell IL-2 assay**
L-826141	**0.031 ± 0.013 (n=6)**	**0.003 (n=2)**
(R)-Rolipram	**0.290 ± 0.12 (n=8)**	**0.386 (n=2)**

Fig. 5