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The Phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 modulates cytokine expression in macrophages via p50 nuclear factor kappa B inhibition, in a PI3K-independent mechanism

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ABSTRACT

The Phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002 (LY2), has been previously reported to inhibit nuclear factor κB (NFκB) activity, in a PI3K-independent mechanism. The goals of the current research were to determine the specificity of LY2 regarding NFκB subunits, and to identify relevant modulation of cytokine expression in LPS-stimulated macrophages. We found that LY2 specifically diminished the level of p50, but not p65, NFκB in the nucleus of LPS-stimulated mouse RAW264.7 macrophages and human THP-1 monocytes. This activity of LY2 was mimicked by its PI3K-inert analog LY303511 (LY3), but not by another PI3K inhibitor – wortmannin. We further show that LY2 inhibited LPS-induced IL-10 expression by RAW264.7 macrophages, in a PI3K-independent mechanism. Moreover, using a deletion mutant of an IL-10 promoter reporter gene we demonstrate that the activity of the NFκB enhancer site at the IL-10 promoter is regulated by LY2 in a PI3K-independent manner. Finally, both LY2 and LY3 elevated TNFα production in the LPS tolerant state which is regulated by p50 NFκB homodimers, but not before tolerance development. The effects of LY2 and LY3 on p50 translocation and on cytokine production in LPS-stimulated macrophages are thus consistent with specific PI3K-independent inhibition of p50 NFκB homodimer activity by LY2.

1. Introduction

The nuclear factor kappa B (NFκB) family of transcription factors includes five members: p65 (RelA), p50 (NFκB1), p52 (NFκB2), c-Rel, and RelB. In resting cells, an IκB subunit retains NFκB dimers in the cytoplasm [1]. Inflammatory stimulation, such as by lipopolysaccharide (LPS), triggers phosphorylation, ubiquitination and degradation of the IκB subunit, enabling the released NFκB dimers to translocate to the nucleus and to modulate gene transcription [2]. The subunit composition of LPS-activated NFκB dimers often dictates distinct gene regulation patterns. For example, p65-p50 NFκB heterodimers mediate LPS-stimulated production of the pro-inflammatory cytokine TNFα in monocytes and macrophages [3–5], whereas the diminished TNFα production associated with development of a LPS-tolerant state is mediated by p50 NFκB homodimers [3,4]. This transcriptional repression activity attributed to p50 NFκB homodimers is consistent with the lack of a transactivation domain in the p50 subunit [6]. However, p50 homodimers were also demonstrated to interact with the transcriptional co-activator CBP and to positively regulate expression of the anti-inflammatory cytokine IL-10 in LPS-stimulated macrophages [7]. While in some cases gene regulation was demonstrated to be mediated by specific NFκB subunits, this knowledge is missing in many other studies, which could therefore benefit from development of selective inhibitors targeting specific NFκB subunits.

Phosphatidylinositol 3-kinase (PI3K) has an important signaling role in cell proliferation and survival, and thus its specific inhibitors are considered to be promising anti-tumor drug candidates [8]. LY294002 (hereafter LY2) and wortmannin are frequently used as specific inhibitors of PI3K, allowing the examination of the role of this kinase in various signaling cascades [9]. Yet, differential effects have been documented for LY2 and wortmannin on nitric oxide production in LPS-stimulated RAW264.7 macrophages [10]. It was then found that LY2 acted through inhibition of the transcriptional activity of NFκB, independent of its PI3K inhibitory activity [11]. A structural analog of LY2, termed LY303511 (hereafter LY3), which is different by a single atom, but in contrast to LY2 is completely inactive towards PI3K [12], was found to inhibit NFκB transcriptional activity, like LY2 [11]. While the above report demonstrated a

Abbreviations: LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; IL-10, interleukin-10; PI3K, Phosphatidylinositol 3-kinase; LY2, LY294002; LY3, LY303511; NFκB, nuclear factor κB; Wort, wortmannin; PVDF, polyvinylidene fluoride; wed, wedelolactone; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified eagle's medium.

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novel PI3K-independent activity of LY2, it did not address the question of which NFκB subunit is affected by LY2 and LY3.

The goal of our research was to identify the NFκB subunit which is regulated by LY2 and LY3. We found that LY2 and LY3, but not wortmannin, specifically diminished the level of p50 NFκB in the nucleus of LPS-stimulated RAW264.7 macrophages, resulting in inhibition of IL-10 expression, and in elevation of TNFα production in the LPS tolerant state. Our results re-enforce the involvement of NFκB p50 homodimers in these processes.

2. Materials and methods

2.1. Reagents, plasmids, and cell culture

Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), PMSF, DMSO and the antibody against PCNA were purchased from Sigma–Aldrich (St. Louis, MO). LY294002 (LY2), LY303511 (LY3) and wortmannin were purchased from A.G. Scientific (San Diego, CA). Sodium pyruvate, L-glutamine and penicillin–streptomycin–nystatin were purchased from Biological Industries (Beit Haemek, Israel). RPMI 1640, DMEM and FBS were purchased from Gibco (Carlsbad, CA). BSA was purchased from Amresco (Solon, OH). ELISA reagents sets for TNFα and IL-10 were purchased from R&D Systems (Minneapolis, MN). The antibodies against phosphoserine-473 AKT, phosphoserine-932 p105, IκBα, p50 and p65 NFκB, and α-tubulin, as well as the established NFκB inhibitor SN50 and its control were from Santa-Cruz Biotechnology (Santa Cruz, CA). The NFkB luciferase reporter gene construct was purchased from Clontec Laboratories (Mountain View, CA). A −78/+64 deletion mutant of the mouse IL-10 promoter luciferase reporter gene construct was a kind gift from Dr. S. Smale [13]. Infrared dye-labeled secondary antibodies and blocking buffer were from Li-Cor Biosciences (Lincoln, NE). Immobilon-FL polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, MA). Complete protease inhibitors mixture and HD-fugene transfection reagent were purchased from Roche (Mannheim, Germany). Endofree Plasmid Maxi Kit was from Qiagen (Hilden, Germany). Dual-luciferase reporter assay kit was from Promega (Madison, WI). DH10B bacteria were from Invitrogen (Carlsbad, CA). Mouse RAW264.7 macrophages, obtained from American Type Culture Collection (ATCC, Rockville, MD), were grown to 80–90% confluence in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin 100 μg/ml streptomycin, and 1250 U/ml nystatin (hereafter culture medium) and with 10% FBS. Human monocytic THP-1 cells, obtained from ATCC, were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium piruvate, 100 U/ml penicillin 100 μg/ml streptomycin, and 1250 U/ml nystatin (hereafter THP-1 culture medium) and with 20% FBS. Both cell lines were grown and maintained at 37 °C in a humidified incubator with 5% CO_2.

2.2. Cytokine assays

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 1.5×10^5 cells per well, in culture medium supplemented with 5% FBS, up to a confluence of 90%. The culture medium was replaced 2 h before treatment in order to avoid the artifact of medium replacement on signaling [14]. The cells were pre-incubated for 40 min with either vehicle, LY2, LY3, or wortmannin, at the indicated concentration, and then stimulated with LPS (100 ng/ml) at 37 °C for 4 h (for IL-10 measurement) or 6 h (for TNFα measurement). LPS tolerance was accomplished by pre-incubation of the cells with LPS (100 ng/ml) for 18 h before the treatment with the above inhibitors. IL-10 and TNFα secretion to the medium were measured with a commercially available ELISA reagents set, according to the manufacturer's instructions, using a microplate reader (BioTek, Winooski, Vermont). The samples were stored at −80 °C until used.

2.3. AKT and p105 phosphorylation assay

RAW264.7 macrophages were grown in culture medium supplemented with 10% FBS for 24 h in 12-well plates at 5×10^5 cells per well, and then maintained for another 24 h in culture medium supplemented with 5% FBS, up to a confluence of 90%. The cells were pre-incubated for 40 min with either LY2 (20 μM), LY3 (20 μM), wortmannin (100 nM), or vehicle, and then stimulated with LPS (100 ng/ml) for 30 min or 15 min, for the assay of AKT or p105 phosphorylation, respectively. The cells were then washed twice with cold PBS and lysed for 15 min at 4 °C with buffer containing Triton X-100 (1%), Tris buffer pH 8.0 (50 mM), NaCl (100 mM), β-glycerophosphate (50 mM), sodium orthovanadate (1 mM), EDTA (1 mM), EGTA (1 mM), glycerol (30%), PMSF (1 mM) and a complete protease inhibitor mixture diluted according to the manufacturer instructions. Cell extracts were centrifuged (14,000 × *g*, 15 min at 4 °C) and the supernatants were stored at −80 °C.

2.4. Nuclear translocation assay

RAW264.7 macrophages were grown in culture medium supplemented with 10% FBS for 48 h in 75 mm flasks at 3×10^6 cells per flask, up to a confluence of 90%. The cells were pre-incubated with LY2 (20 μM), LY3 (20 μM), wortmannin (100 nM), or with vehicle, for 40 min prior to the addition of LPS (1 μg/ml) for an additional period of 60 min. The cells were washed twice with cold PBS and centrifuged (1000 × *g* for 3 min). Separation of the cytosolic and nuclear fractions was performed as previously described [15]. Human monocytic THP-1 cells were grown in THP-1 culture medium supplemented with 20% FBS for 48 h in 25 mm flasks at 1.3×10^6 cells per flask. The cells were treated as described above for the RAW264.7 cells. The cells were centrifuged (1000 × *g* for 3 min), and nuclear fraction was obtained as for the RAW264.7 cells.

2.5. Western blotting

Cell extracts (60 μg protein) or nuclear extracts (50 μg protein) were boiled for 5 min in SDS-PAGE buffer, subjected to 10% SDS-PAGE, and proteins were transferred to Immobilon-FL PVDF membrane. An antibody raised against phosphoserine-473 AKT was used together with an antibody against α-tubulin for normalization of whole cell protein content. Antibodies against p50 or p65 NFκB were used together with an antibody against PCNA, or with an antibody against α-tubulin for normalization of the nuclear and cytosolic fractions, respectively. Two-color imaging and quantitative analysis of Western blots was performed using the Odyssey infrared imaging system (Li-Cor Biosciences), according to the manufacturer's instructions.

2.6. Protein determination

Protein was determined by a modification of the Bradford procedure, which yields linear and thus more accurate results, increased sensitivity, and reduced detergent interference, as previously described by Zor and co-workers [16,17]. BSA served as standard.

2.7. Transfection and reporter gene assay

RAW264.7 macrophages were transfected with 1.5 μg of a plasmid containing a deletion mutant (−78/+64) of the mouse

IL-10 promoter upstream to the firefly luciferase gene, or with a NFκB luciferase reporter construct. For normalization, 0.5 μg of pRL-TK vector, coding for *renilla* luciferase, was added. The details of plasmids amplification and transfection protocol were previously described [18]. Following a 24 h transfection, the cells were washed, pre-incubated for 40 min with LY2 (20 μM), LY3 (20 μM), wortmannin (100 nM), or with vehicle, and stimulated with LPS (100 ng/ml) for 8 h (IL-10 promoter construct) or for 6 h (NFκB construct) at 37 °C. Luciferase activity in cell extracts was determined following the manufacturer's (Promega) instructions. Data are expressed as a ratio of *firefly* luciferase activity divided by the *renilla* luciferase activity. Transfection with the empty reporter vector, performed as a control, yielded no detectable activity.

2.8. Statistical analysis

All the data were analyzed using Student's *t*-test wherever applicable. In all cases, differences of $p < 0.05$ were considered to be significant. All experiments were repeated as least three times.

3. Results

3.1. LY294002 (LY2) and LY303511 (LY3) inhibit LPS-induced nuclear accumulation and signaling of p50 NFκB

A report by Kim et al. has shown that the PI3K inhibitor LY294002 (LY2) inhibits LPS-induced nitric oxide production through PI3K-independent inhibition of NFκB activation [11]. Therefore, we examined the effect of the PI3K inhibitors, LY2 and wortmannin, and of the PI3K-inactive analog of LY2, LY303511 (LY3), on NFκB signaling in LPS-stimulated RAW264.7 macrophages. Fig. 1A indeed shows that LY2 and LY3 partially repressed the activity of a NFκB-luciferase reporter gene, while wortmannin had no effect. To verify the presence or absence of PI3K inhibitory activity, phosphorylation of the downstream effector AKT on serine 473 was analyzed by Western blotting of extracts from LPS-stimulated cells. Fig. 1B shows, as expected, that LY2 and wortmannin fully inhibited phosphorylation of AKT, while LY3 was without effect. These data therefore point to PI3K-independent inhibition of NFκB activity by LY2.

In order to define which NFκB subunit is affected by LY2 and LY3, we examined LPS-stimulated nuclear translocation of p65 and p50. Our results show that LY2 and LY3, but not wortmannin, nearly abolished LPS-induced nuclear accumulation of p50 (Fig. 2A). The sharp decrease (89% and 94% for LY2 and LY3, respectively) in the LPS-related nuclear level of p50, was reflected in a respective increase of its cytosolic level, accounting to 69% and 72% (for LY2 and LY3, respectively) of the reduction caused by LPS (Fig. 2A). Importantly, neither inhibitor affected LPS-induced nuclear translocation and accumulation of p65 (Fig. 2B). Taken together, these results thus suggest that LPS-induced nuclear translocation and consequent transcriptional activity of p50 NFκB are selectively inhibited by LY2 and LY3 in a PI3K-independent mechanism.

We verified that p50 NFκB is a target of LY2 and LY3 in an additional cell line. Indeed, identical results were obtained for the mouse RAW 264.7 macrophages (Fig. 2A and B) and for human THP-1 monocytes (Fig. 2C).

3.2. LY2 inhibits LPS-induced IL-10 production in a PI3K-independent mechanism

Expression of IL-10 in LPS-stimulated monocytes was found to be mediated by the PI3K pathway and inhibited by LY2 [19]. Another report showed that LPS-stimulated IL-10 expression depended on p50 NFκB homodimers [7]. In light of the independent effects of LY2 on activities of PI3K and p50 NFκB (Fig. 1), we sought to determine whether activation of either pathway by LPS is required for IL-10 induction in RAW264.7 macrophages. To this end we used the PI3K inhibitors, LY2 and wortmannin, as well as the PI3K-inactive LY2-analog, LY3. We found that LY2 and LY3, but not wortmannin, dose-dependently suppressed IL-10 expression in response to LPS (Fig. 3A). This pattern correlated with the activity of the compounds towards p50 NFκB nuclear translocation (Fig. 2A) and activation (Fig. 1A), and at the same time contrasted with their effects on AKT phosphorylation (Fig. 1B). As expected, wedelolactone, a non-selective NFκB inhibitor, also inhibited LPS-induced IL-10 production. Modulation of IL-10 production by LY2 and LY3 did not result from a toxic effect or from a general effect on protein expression, as these compounds, in contrast to wedelolactone, failed to affect TNFα production in the same experiment (Fig. 3B). These results thus indicate that LY2 inhibits IL-10 expression in LPS-stimulated RAW264.7 macrophages in a PI3K-independent mechanism, and suggest that p50 NFκB activity, which is sensitive to both LY2 and LY3, contributes to IL-10 production in LPS-stimulated macrophages.

3.3. LY2 and LY3 inhibit transcription of a reporter gene regulated by a proximal NFkB site in the mouse IL-10 promoter

We show here that LY2 and LY3 block LPS-induced IL-10 expression (Fig. 3A) and specifically block p50 NFκB nuclear

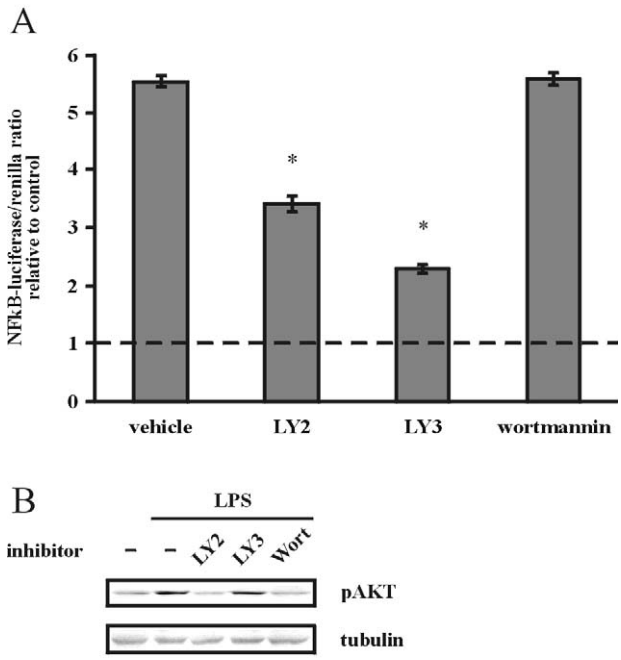

Fig. 1. LY294002 (LY2) inhibits LPS-stimulated NFκB-luciferase reporter gene activation in a PI3K-independent mechanism. RAW264.7 macrophages were transiently transfected for 24 h at 37 °C with a reporter gene construct which codes for firefly luciferase under the regulation of NFκB, and with a renilla luciferase construct for normalization. The cells were washed and pre-incubated with the PI3K inhibitors, LY2 (20 μM), and wortmannin (100 nM), with the PI3K-inactive analog LY3 (20 μM), or with vehicle (0.15% DMSO), for 40 min prior to the addition of LPS (100 ng/ml) for 6 h at 37 °C. Luciferase activity assay was performed as described in Section 2. Each data point represents mean ± S.D. ($n = 6$) of values normalized against renilla luciferase activity, and relative to non-stimulated control cells (represented by the dashed line). *$p < 2E{-}6$ for cells treated with vs. without the inhibitor. (B) Mouse macrophage RAW264.7 cells were pre-incubated with the PI3K inhibitors, LY2 (20 μM), and wortmannin (Wort, 100 nM), with the PI3K-inactive analog LY3 (20 μM), or with vehicle (0.15% DMSO), for 40 min at 37 °C prior to the addition of LPS (100 ng/ml) for an additional period of 30 min. Cell lysates (60 μg protein) were subjected to SDS-PAGE, and after transfer to Immobilon-FL PVDF, the membrane was probed with a phosphoserine-473-specific AKT antiserum, and with an antibody against α-tubulin for normalization.

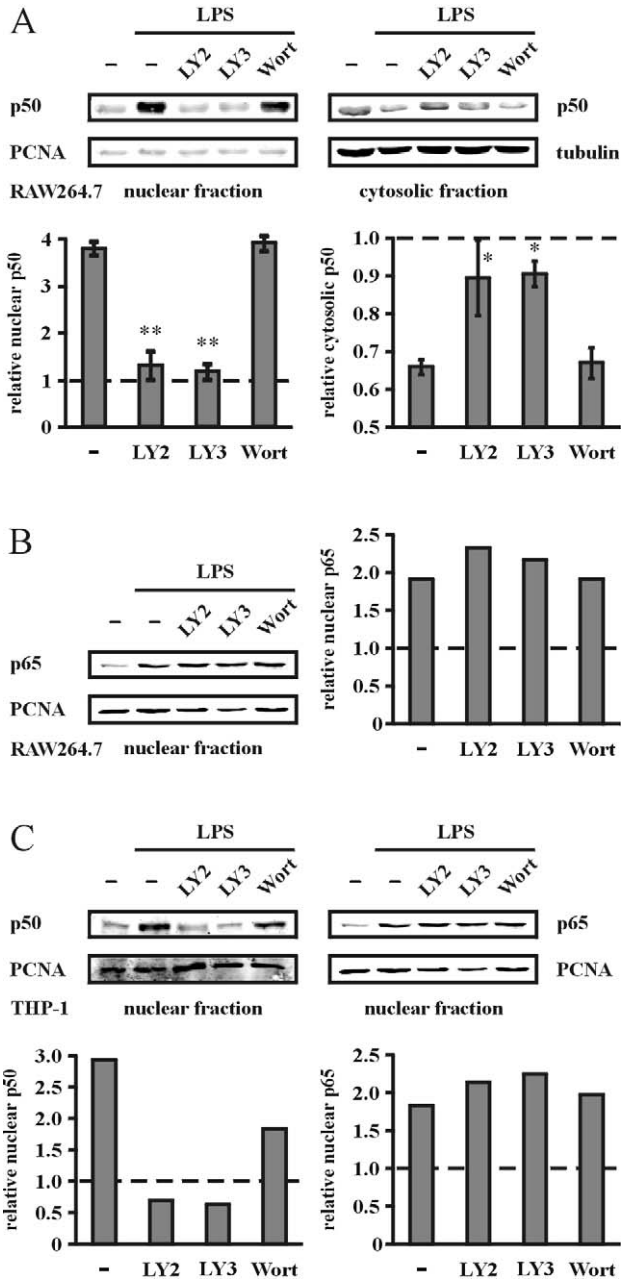

Fig. 2. LY2 inhibits LPS-induced nuclear translocation of p50 NFκB in a PI3K-independent mechanism. Mouse macrophage RAW264.7 cells (A and B) or human monocytic THP-1 cells (C) were pre-incubated with the PI3K inhibitors, LY2 (20 μM), and wortmannin (Wort, 100 nM), with the PI3K-inactive analog LY3 (20 μM), or with vehicle (0.15% DMSO), for 40 min prior to the addition of LPS (1 μg/ml) for an additional period of 60 min. Nuclear and cytosolic extracts (50 μg protein) were subjected to SDS-PAGE, and after transfer to Immobilon-FL PVDF, the membrane was probed with a p50 NFκB antiserum (A and C) or with a p65 NFκB antiserum (B and C). Antibodies against PCNA and α-tubulin served for normalization of nuclear and cytosolic fractions, respectively. Quantitative Western blot analysis is shown as the normalized intensities of p50 (A and C) or p65 (B and C) NFκB, relative to non-stimulated cells (represented by the dashed line). The quantification is an average of 2 independent experiments (A) or a representative experiment (B and C). $^{*}p < 0.005$, $^{**}p < 0.001$ for cells treated with vs. without the inhibitor.

translocation (Fig. 2A) and NFκB signaling (Fig. 1A). Mosser and co-workers have shown that p50 NFκB homodimers promote LPS-induced IL-10 expression in murine macrophages, via an NFκB site located at −46/−55 [7]. It was further shown by EMSA and by ChIP analysis, that this enhancer binds only p50 and not other NFκB subunits [7]. It is therefore expected that LY2 and LY3 would block transcriptional activity at the NFκB site of the murine IL-10 promoter. To test this prediction, we transiently transfected RAW264.7 macrophages with a short −78/+64 deletion mutant of the mouse IL-10 promoter placed upstream to a luciferase reporter gene. This construct of the proximal IL-10 promoter region lacks the more distal elements which mediate IL-10 transcriptional activation [13], but it includes the p50 NFκB responsive element located at −46/−55 [7]. Fig. 4 clearly shows that LY2 and LY3, but not wortmannin, inhibited the LPS-induced activity of the proximal IL-10 promoter reporter. This finding strongly supports our conclusion that the inhibition of LPS-stimulated IL-10 production by LY2 and LY3 resulted from specific inhibition of p50 NFκB homodimers nuclear translocation and signaling, and associated this effect to the proximal p50 NFκB responsive element located at the mouse IL-10 promoter.

3.4. LY2 and LY3 partially revert LPS tolerance of TNFα expression

To further validate p50 NFκB as a common PI3K-independent target of LY2 and LY3, we turned to examine TNFα production in RAW264.7 macrophages in the LPS-tolerant state. Homodimers of p50 NFκB were demonstrated to play a key role in the development of the LPS-tolerant state [3,4]. This state, accomplished by long-term (18 h) pre-exposure to LPS, is evident by reduced TNFα production in response to a second LPS challenge [20]. We indeed found that the long-term pre-exposure to LPS diminished 85% of TNFα release following a second LPS exposure of 6 h (Fig. 5B), as compared to cells exposed to LPS for only 6 h (Fig. 5A). As we predicted, both LY2 and LY3 increased TNFα release in the LPS-tolerant state by 1.6 ± 0.1-fold (Fig. 5B). It should be noted that LY2 and LY3 were added only following the 18 h pre-incubation, and hence after the tolerance was established, because long exposure to LY2 and LY3 results in apoptosis [21–23]. It is likely that the late timing of addition is accountable for the modest effect of LY2 and LY3. Indeed, while LY2 was highly effective (96%) in blocking p50 nuclear translocation immediately after LPS stimulation, it was able to reduce only a small fraction (7%) of nuclear p50 at the LPS-tolerant state (Fig. 5C). As a positive control we used the cell-permeable SN50 peptide, known to specifically mask the nuclear localization signal of p50 NFκB, and thus to inhibit its nuclear translocation [24]. Importantly, SN50 acted similarly to LY2 and LY3, increasing TNFα release in the LPS-tolerant state by 1.4-fold, whereas a SN50 control peptide did not have an effect (Fig. 5B). In contrast to LY2 and LY3, the specific PI3K inhibitor wortmannin failed to up-regulate TNFα release in the LPS-tolerant state (Fig. 5B). Unlike LPS tolerance, TNFα expression in response to a single short-term LPS exposure is normally mediated by a p65-p50 heterodimer [3–5]. Notably, neither of the selective p50 (and not p65) inhibitors, SN50, LY2 or LY3, affected TNFα production in the non-tolerant state (Fig. 5A), in contrast to wedelolactone, a non-selective NFκB inhibitor, which completely blocked the production of TNFα (Fig. 3B). Taken together, our data suggest that LY2 and LY3, like SN50, modulate TNFα expression only under conditions where the p50 NFκB subunit is exclusively essential, such as those exist during LPS tolerance. It remains to be explored whether these inhibitors reduce the nuclear level only of p50 NFκB homodimers (and not of p50-containing heterodimers), or whether these inhibitors deplete the nucleus of the p50 subunit, and thus trigger the conversion of p50-containing heterodimers into different dimers.

3.5. LY2 and LY3 do not affect phosphorylation of relevant IkB proteins

p105 functions both as a p50 precursor, and as an IκB that retains NFκB dimers, in particular p50 homodimers, inactive in the cytoplasm, until an appropriate stimulus causes IKK-mediated

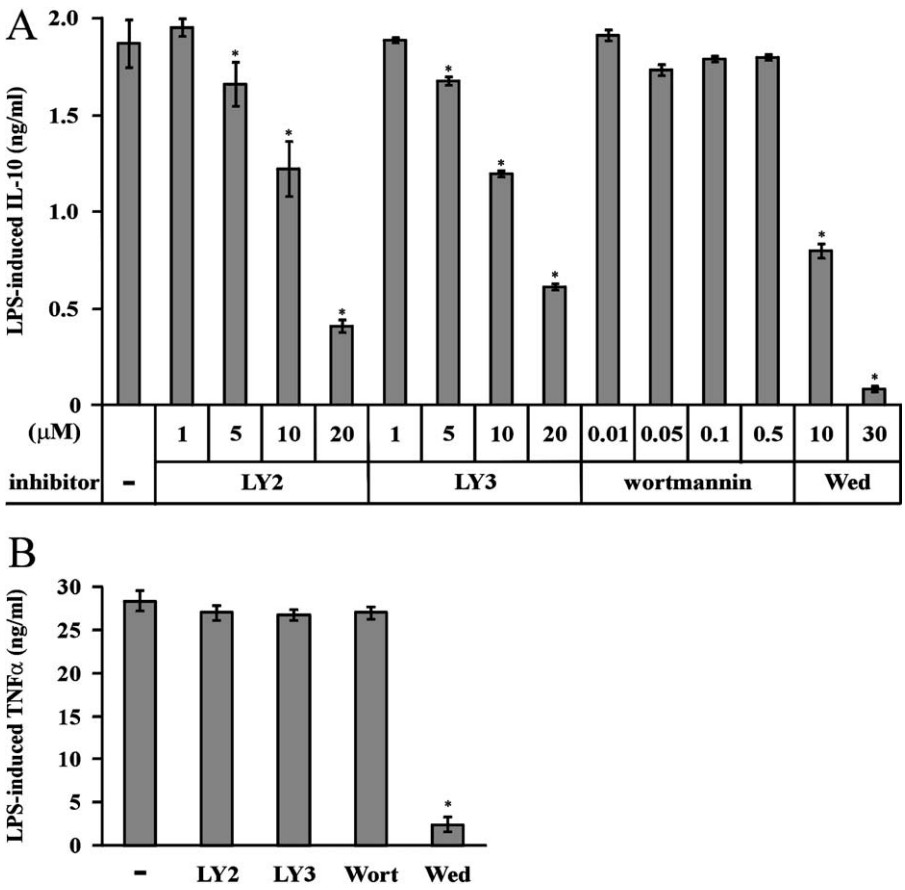

Fig. 3. LY2 inhibits LPS-induced IL-10 production in a PI3K-independent mechanism. Mouse macrophage RAW264.7 cells were pre-incubated with the PI3K inhibitors, LY2 and wortmannin, with the PI3K-inactive analog LY3, or with the NFκB inhibitor wedelolactone (wed), at the indicated concentrations, for 40 min prior to the addition of LPS (100 ng/ml) for an additional period of 4 h at 37 °C. DMSO (0.15%) served as vehicle. IL-10 (A) and TNFα (B) release to the medium was measured by ELISA. Each data point represents mean ± S.D. ($n = 6$). *$p < 0.05$ for cells treated with vs. without the inhibitor. Background IL-10 and TNFα levels were less than 20 pg/ml. The concentrations of inhibitors in panel B are the maximal values depicted in panel A.

phosphorylation on serines 927 and 932, ubiquitination and proteolitic degradation of p105 [25]. To study the mechanism of p50 inhibition by LY2 and LY3, we examined the effect of these inhibitors on p105 phosphorylation on S932. Surprisingly, LY2 and LY3 did not have any effect on the sharp LPS-stimulated phosphorylation of p105 on S932 (Fig. 6).

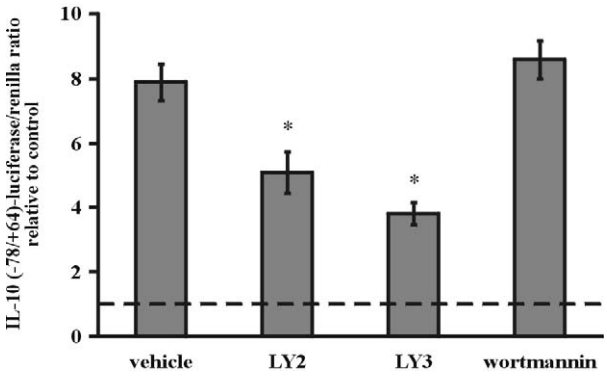

Fig. 4. LY2 inhibits LPS-induced proximal mouse IL-10 promoter reporter gene activation in a PI3K-independent mechanism. RAW264.7 macrophages were transiently transfected for 24 h at 37 °C with a reporter gene construct which codes for firefly luciferase under the regulation of a deletion mutant (−78/+64) of the mouse IL-10 promoter, and with a renilla luciferase construct for normalization. The cells were washed and pre-incubated with LY2 (20 μM), LY3 (20 μM), wortmannin (100 nM) or vehicle (0.15% DMSO) for 40 min prior to the addition of LPS (100 ng/ml) for 8 h at 37 °C. Luciferase activity assay was performed as described in Section 2. Each data point represents mean ± S.D. ($n = 6$) of values normalized against renilla luciferase activity, and relative to non-stimulated control cells (represented by the dashed line). *$p < 2E{-}6$ for cells treated with vs. without the inhibitor.

The classical pathway of NFκB activation involves IKK-mediated phosphorylation of IκBα, leading to its ubiquitination and proteolitic degradation [1,2]. We found that LY2 and LY3 had no effect on IκBα degradation (data not shown), in accordance with the selective effect of LY2 and LY3 on p50 and not on p65, as opposed to the role of IκBα as inhibitor of p65-p50 heterodimers.

4. Discussion

LY2 and wortmannin are structurally distinct PI3K inhibitors. LY2, a compound derived from the naturally occurring bioflavinoid quercetin, inhibits PI3K activity via competitive inhibition at the ATP binding site on the p85α subunit [9,12]. The PI3K inhibitor wortmannin is a molecule derived from a fungal metabolite, which targets the p110 subunit of PI3K through an irreversible covalent interaction [26,27]. Both LY2 and wortmannin are commonly used to study the role of PI3K in cellular processes.

In this study we investigated the effects of LY2 and wortmannin on LPS-induced IL-10 and TNFα production in RAW264.7 macrophages. Complete inhibition of LPS-induced AKT phosphorylation occurred at 20 μM LY2 or 100 nM wortmannin (Fig. 1B), attesting to PI3K inhibition. At these concentrations, LY2, but not wortmannin, significantly inhibited LPS-induced IL-10 (Fig. 3A). Surprisingly, LY3, an analog of LY2 which is inactive towards PI3K [12], inhibited IL-10 production with a similar dose response (Fig. 3A). These results indicate that LPS-induced IL-10 expression

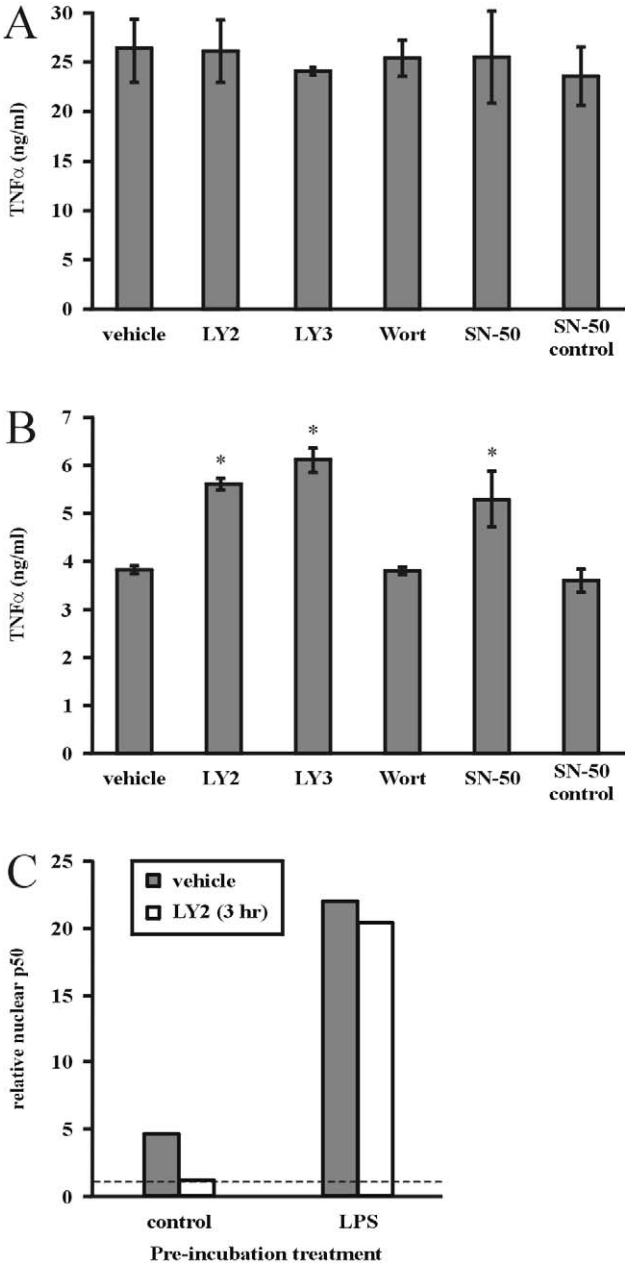

Fig. 5. LY2 reduces LPS tolerance in a PI3K-independent mechanism. (A) Mouse macrophage RAW264.7 cells were stimulated with LPS (100 ng/ml) for a period of 6 h in the presence of either LY2 (20 μM), LY3 (20 μM) or wortmannin (100 nM). The established p50 inhibitory peptide SN50 (20 μM), and a SN50 control peptide (20 μM), served as positive and negative controls, respectively. DMSO (0.15%) served as vehicle. TNFα release to the medium was measured by ELISA. Each data point represents mean ± S.D. ($n = 6$). $^*p < 0.05$ for cells treated with vs. without the inhibitor. Background TNFα level was less than 20 pg/ml. (B) LPS tolerance was accomplished by pre-incubation of the RAW264.7 macrophages with LPS (100 ng/ml) for 18 h, prior to washing and treatment as in (A). (C) RAW264.7 macrophages were pre-incubated for 18 h with either LPS (100 ng/ml, tolerance) or medium (control), washed, and stimulated for 3 h with LPS (100 ng/ml) in the presence of either LY2 (20 μM) or vehicle. Nuclear extracts (50 μg protein) were subjected to SDS-PAGE, and after transfer to Immobilon-FL PVDF, the membrane was probed with a p50 NFκB antiserum, and with an antibody against PCNA for normalization. Quantitative Western blot analysis is shown as the normalized intensities of p50 NFκB, relative to non-stimulated cells (represented by the dashed line). Nuclear p50 level following the 18 h pre-incubation period was 15-fold higher than in resting cells. The results are representative of two independent experiments.

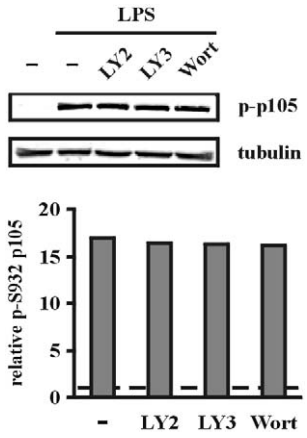

Fig. 6. LY2 does not inhibit LPS-stimulated phosphorylation of the relevant IκB, p105. Mouse macrophage RAW264.7 cells were pre-incubated with the PI3K inhibitors, LY2 (20 μM), and wortmannin (Wort, 100 nM), with the PI3K-inactive analog LY3 (20 μM), or with vehicle (0.15% DMSO), for 40 min prior to the addition of LPS (1 μg/ml) for an additional period of 15 min (determined in a preliminary experiment as optimal duration for LPS activity). Whole cell extracts (50 μg protein) were subjected to SDS-PAGE, and after transfer to Immobilon-FL PVDF, the membrane was probed with a phospho-S932 p105 antiserum, and with an antibody against α-tubulin for normalization. Quantitative Western blot analysis is shown as the normalized intensities of phospho-S932 p105, relative to non-stimulated cells (represented by the dashed line).

in the RAW264.7 murine macrophages cell line is PI3K-independent, in spite of its sensitivity to LY2. Our results stand in apparent contrast to those of Martin et al. that have shown PI3K-dependency (using LY2 and wortmannin) of LPS-induced IL-10 production in human monocytes [19]. The different findings may be related to the different time scale (up to 4 h in our study vs. 24 h in the study of Martin et al.), possibly representing primary vs. secondary effects, respectively. Alternatively, the different findings may stem from the different cell line (macrophages vs. monocytes) and/or species (mouse vs. human). In that regard, it is important to note the study of Platzer et al. which has reported a high homology between the human and mouse IL-10 promoters in most regions associated with transcriptional regulation, except for the lack of a NFκB-like binding site in the human sequence [28]. This sequence divergence may partially explain the apparent contradiction between the results of Martin et al. in human monocytes demonstrating PI3K-dependent and NFκB-independent LPS-stimulated IL-10 expression [19], and our results in murine macrophages demonstrating PI3K-independent and NFκB-dependent LPS-stimulated IL-10 expression. Our results also do not correlate with the findings of Polumuri et al. in murine peritoneal macrophages [29], but this could result from the differences between primary cells and a cell line, and in particular the very low level of IL-10 produced by these cultured primary cells, compared to the RAW264.7 cell line used in our study.

Kim et al. suggested that suppression of iNOS expression in RAW264.7 macrophages by LY2 is PI3K-independent and due to NF-κB inhibition [11]. Interestingly, previous studies have shown that quercetin, a compound which LY2 is derived from, inhibits LPS-induced expression of several NFκB-dependent genes in RAW264.7 macrophages [30,31]. Consistently, we found that LY2 and LY3, but not wortmannin, inhibited transcriptional activation of NF-κB (Fig. 1A). This activity of the inhibitors is in accordance with their effect on IL-10 production (Fig. 3A). Moreover, wedelolactone, a non-selective NFκB inhibitor, also inhibited LPS-induced IL-10 production. These results thus suggest that LY2 inhibits IL-10 production via suppression of NFκB, independent of PI3K.

Our results are supported by the findings of Mosser and co-workers who have shown that p50 NFκB homodimers promote LPS-induced IL-10 expression in murine macrophages, via an NFκB site located at −46/−55 relative to the transcription start site [7]. It was further shown by EMSA and by ChIP analysis, that this enhancer binds only p50 and not other NFκB subunits [7]. Consistently, we found that LY2 and LY3, but not wortmannin, inhibited nuclear translocation of NFκB subunit p50, but not p65 (Fig. 2). Moreover, LY2 and LY3, but not wortmannin, inhibited expression of a reporter gene which is under the regulation of the proximal part of the mouse IL-10 promoter encompassing the p50 NFκB responsive element (Fig. 4). Taken together, these results thus indicate that LY2 inhibits IL-10 expression in LPS-stimulated RAW264.7 macrophages in a PI3K-independent mechanism, and suggest that p50 NFκB homodimers activity, which is sensitive to both LY2 and LY3, contributes to IL-10 production in LPS-stimulated macrophages.

Production of the anti-inflammatory cytokine IL-10 is one of the strategies to counteract the potentially harmful prolonged and excessive activation of the innate immune system. Another essential regulatory mechanism during inflammation is tolerance to LPS, characterized by a diminished production of TNF during prolonged exposure to LPS. In addition to the positive role of p50 homodimers in IL-10 expression, it has been shown that the p50 homodimer plays a central role in LPS tolerance, manifested in reduction of TNFα production [3,7,32]. In the present study we provide data demonstrating that LY2 and LY3 partly relieve the down-regulation of LPS-induced TNFα production in tolerant murine macrophages (Fig. 5B), and thus further support the conclusion that p50 NFκB is a common PI3K-independent target of LY2 and LY3. The relatively small effect of LY2 and LY3 probably stems from the experimental conditions, where LY2 and LY3 were added only after tolerance establishment in order to avoid apoptosis. Indeed, a long-term pre-incubation with LPS led to a very high increase in the nuclear p50 level, and only a small fraction was reduced by the addition of LY2 following the pre-incubation (data not shown).

We found that the abolished translocation of p50 to the nucleus in macrophages treated by LY2 or LY3 affects TNFα production in the LPS tolerant state, but not during shorter pre-tolerance LPS stimulation. This finding was surprising as the p50 NFκB subunit positively participates in induction of the TNFα gene in the form of a p65-p50 heterodimer complex [33]. Yet, consistent with our finding, it has been shown that induction of genes encoding pro-inflammatory cytokines (e.g. TNFα, IL-1α, and IL-6) was not affected in LPS-challenged mice lacking the p50 NFκB subunit [34]. Moreover, induction of TNFα by LPS in macrophages from p50 knockout mice was actually increased rather than decreased [35]. In contrast, the p65 NFκB subunit was crucial for the expression of these pro-inflammatory cytokine genes [34]. Together with our findings, these observations therefore indicate that although normally a p65-p50 heterodimer participates in TNFα induction by LPS, the p50 subunit may not be essential for this process. Furthermore, these results suggest that for the purpose of TNFα promoter activation, in the absence of nuclear p50 – the stimulatory p65–p50 heterodimer is replaced by a different dimer. It should however be noted that although the Western blot analysis does not detect a significant quantity of nuclear p50 following treatment with LY2 and LY3 (Fig. 2A), it is possible that a relatively small fraction of p50 exists in the nucleus in the form of a p65–p50 heterodimer.

Apart from IL-10 and TNFα which were reported here, multiple studies have shown a PI3K-independent effect of LY2 on the expression of several other genes, including monocyte chemotactic protein-1 (MCP-1) [36], IL-6 [36], COX-2 [36,37], ATF3 [38], Egr-1 [39], and iNOS [11]. Interestingly, all of these genes, with the exception of Egr-1 [40], are regulated by NFκB. It is therefore possible that transcription of these genes depends on p50 NFκB, rationalizing the PI3K-independent modulation by LY2. The ATF3 promoter includes a NFκB site [41], but the involvement of the transcription factor has not been studied yet, to the best of our knowledge. Regarding transcription of MCP-1 [42], IL-6 [43], and COX-2 [44], the p50 subunit was suggested to participate in the form of a p50/p65 heterodimer (based on EMSA studies), whereas the p65 component was found to be essential (based on over-expression or down-regulation). The iNOS gene presents a similar situation, as its transcription was correlated with binding of p50 NFκB to the promoter [45], while titration of the p65 subunit with an interacting protein resulted in transcription inhibition [46]. The transcription of COX-2 is positively regulated, first by a p50/p65 heterodimer and then by a p50 homodimer [47]. Therefore, assuming the effect of LY2 is exerted on the NFκB site in the promoters of MCP-1, IL-6, COX-2 and iNOS, then the results reported here predict that, a p50/p65 NFκB heterodimer is obligatory for transcription of these genes, and cannot be replaced by another subunit composition in the absence of p50. In this sense, transcription of these four pro-inflammatory genes differs from transcription of TNFα in the pre-tolerant state, where the p50 subunit in the p50/p65 NFκB heterodimer appears to be not essential according to our results.

The lack of effect of both LY2 and wortmannin on TNFα production under the conditions of short-term LPS stimulation (Fig. 5A) implies that PI3K, like the p50 homodimer, is not involved in this process. The apparent conflict with the studies of Guha and Mackman that were performed in human monocytes with a similar incubation time [48] may be explained by different signaling of the PI3K pathway in the two cell lines.

In addition to its well-known action on PI3K, LY2 was reported to directly bind and inhibit several other kinases, including mTOR [49], DNA-PK [50], Pim-1 [51], CK2 (formerly casein kinase 2) and GSK3 [52,53]. The kinases mTOR and DNA-PK, like PI3K, are inhibited by both LY2 and wortmannin, although the sensitivity of these kinases to wortmannin is lower than that of PI3K [49,50]. With regards to Pim-1, wortmannin was demonstrated to be without effect [54]. In the case of CK2 and GSK3, LY3 was also examined and found to be as effective in binding as LY2, whereas wortmannin was without effect [53]. Interestingly, both CK2 and GSK3β have been linked to NFκB signaling. A study performed in mouse fibroblasts has shown that the constitutively active GSK3β primes p105 to IKK-mediated degradation upon stimulation by TNFα, whereas the absence of GSK3β led to decreased p105 degradation [55]. Apart of being a p50 precursor [1], p105 also functions as an IkB for NFκB dimers, primarily p50 homodimers [25]. It is therefore anticipated that a pharmacological inhibitor of GSK3β would significantly block nuclear translocation of p50. Further studies are required to determine whether GSK3β regulates p105 degradation and p50 nuclear translocation in LPS-stimulated macrophages.

Importantly, CK2 can phosphorylate several residues on the C-terminus of IκBα [56,57], triggering calpain-mediated degradation [58], and resulting in nuclear translocation of p50 and p65 NFκB [59,60]. With relevance to our study, LPS stimulates CK2 activity in RAW264.7 macrophages [61], raising the possibility that LY2 and LY3 block p50 NFκB nuclear translocation by inhibiting LPS-stimulated CK2. However, the specificity of LY2 and LY3 towards p50 and not p65 NFκB (Fig. 2) is in conflict with the activity of CK2 which by targeting the common IκBα does not discriminate between these two NFκB subunits [59,60]. Indeed we found that LY2 and LY3 do not affect LPS-induced IκBα degradation (data not shown). Moreover, LPS-stimulated p50 homodimer and p50/p65 heterodimer NFκB activation in RAW264.7 macrophages has been shown to depend on degradation of IκBα via the proteasome and not via calpain [62], which acts downstream to CK2 [58].

Apart from the direct activity of CK2 on IκBα, reciprocal modulation of NFκB activity by CK2 may be achieved via negative

regulation of NADPH oxidase and reactive oxygen species (ROS) formation by this kinase [63]. In turn, ROS have been shown to activate NFκB via phosphorylation and degradation of IkBα [64,65]. Thus, inhibition of CK2 by LY2 and LY3 is expected to indirectly activate p65 and p50 NFκB via ROS. Therefore, the specific inhibition of p50 activity described in this study is unlikely to be secondary to ROS production. The effect of LY2 and LY3 on cell survival and apoptosis is expected to be quite complex. Both LY2 and LY3 sensitize cancer cells to TRAIL-induced apoptosis through PI3K-independent production of intracellular H_2O_2 [21–23]. Interestingly, NFκB p50 has being shown to have an anti-apoptotic role [66–68]. Thus, cell survival and apoptosis can be affected by LY2 and LY3 in opposite directions. On the one hand, inhibition of CK2 by LY2 and LY3 may have a pro-survival effect via NFκB activation by ROS. On the other hand, LY2 and LY3 may stimulate apoptosis in part via ROS (acting in a NFκB-independent pro-apoptotic manner), and in part by their inhibitory effect on NFκB p50 nuclear translocation and activation. These effects on CK2, ROS formation and p50 NFκB are all shared by LY2 and LY3. In addition, LY2 alone promotes apoptosis by inhibition of PI3K [8].

In addition to the above LY2 off-targets, LY2 has been shown (by using affinity chromatography) to directly bind several metabolite kinases as well as a variety of unrelated (non-kinase) proteins, including chaperones with ATPase activity and DNA-binding proteins [53]. El-kholy et al. have shown that LY2, but not wortmannin, can reversibly inhibit potassium channels [69]. LY3 also inhibited the potassium channels, but at an order of magnitude higher concentrations [69]. In contrast, LY2 and LY3 inhibit LPS-induced IL-10 expression with similar potencies (Fig. 3A), and thus it is unlikely that p50 inhibition and subsequent IL-10 suppression are a result of potassium channels blockade. LY2, but not wortmannin, was also reported to modulate the activity of intracellular [70] and cell-surface [71,72] calcium channels. These studies did not examine whether LY3 also modulates calcium level. It remains to be explored whether any of these LY2 off-targets lies upstream to p50 NFκB.

In conclusion, this study reports that the widely used PI3K inhibitor, LY2, and its inactive analog, LY3, inhibit LPS-induced IL-10 production in RAW264.7 macrophages cells, in a PI3K-independent manner (Fig. 7). Our results identified for the first time, as far as we know, p50 NFκB as a target for LY2 and LY3. Therefore, the common use of LY2 as a specific PI3K inhibitor should be made with greater caution. Furthermore, the assignment of PI3K-dependency to certain genes, based on LY2 sensitivity, should be re-examined, and the possible role of p50 NFκB may be considered as well. The mechanism of this newly described LY2 effect awaits further study, as our study indicate that degradation of neither p105 nor IκBα is modulated by LY2.

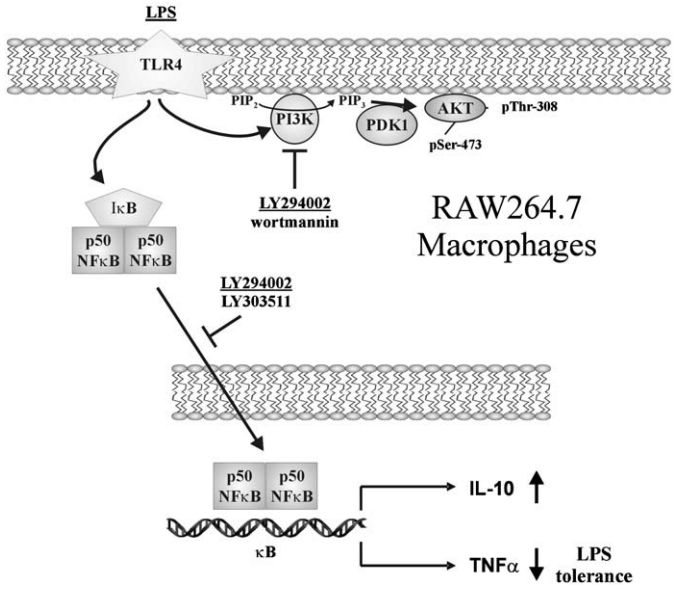

Fig. 7. Schematic model for the mechanism of activity of LY2, LY3 and wortmannin in LPS-stimulated macrophages. LPS stimulates nuclear translocation of p50 NFκB homodimers to the nucleus, where they up-regulate IL-10 transcription, and down-regulate TNFα transcription. LY2 and LY3, but not wortmannin, inhibit p50 nuclear translocation and signaling. On the other hand, LY2 as well as wortmannin, but not LY3, inhibit LPS-stimulated PI3K activity.

Acknowledgments

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