Chapter 6

Vanillin acts as a potent IRAK4 inhibitor that impairs LPS-induced TLR4 signalling and inflammation

6.1 Introduction

Toll-like receptors belong to a family of pattern recognition receptors that specifically recognize the conserved signature of "pathogen-associated molecular pattern" present in microbial pathogens and induce a host immune response resulting eradication of invading pathogens [1,2]. TLR was first discovered in *Drosophila* as an important regulator of dorsal-ventral polarity [3], but was later identified as a key molecule involved in anti-fungal immunity [4]. Subsequent study identified TLR4 as the ortholog of *Drosophila* Toll protein [5] and then discovered it as a receptor for bacterial lipopolysaccharide (LPS) in view of the fact that C3H/HeJ mice which carries a missense mutation in the TLR4 gene producing a defective response to bacterial endotoxin [6] suggesting its role in innate immunity. To date, there are 10 human and 13 murine members, including non-functional TLR10, of TLR subtypes along with their respective microbial ligands have been identified [7]. Dysregulation of TLR signaling particularly TLR2 and TLR4 are implicated in different etiological conditions of chronic inflammatory diseases and autoimmunity disorders [8-10].

TLRs are type I transmembrane glycoproteins mainly expressed in all innate immune cells including macrophages, neutrophils, and dendritic cells [10]. It contains three-domain structure comprising N-terminal extracellular leucine-rich repeats responsible for the pathogen recognition, a transmembrane domain, and a C-terminal cytoplasmic Toll/interleukin-1 receptor (TIR) domain that coupled TLRs to cytosolic TIR domain–containing adaptor proteins triggering intracellular signaling [11]. Activation of all TLR signaling, except TLR3, activates two main branches of downstream myeloid differentiation primary-response gene 88 (MyD88)-dependent or toll/interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-dependent signaling pathways mounting specific immune response [12-15]. In MyD88 dependent pathway, ligand-dependent activation of TLR involves its interaction with two adapter proteins MyD88 and TIRAP through their TIR domains which leads to death domain (DD)

mediated interaction of MyD88 and serine/threonine kinase IL-1 receptor-associated kinase 4 (IRAK4) [14,15]. IRAK4 was initially identified by a database search in 2002 [16] and subsequent studies confirmed its isoforms IRAK1, IRAK2, and IRAKM [17]. While, IRAK4 through its association and phosphorylation of IRAK1 and IRAK2 stimulates TLR/IL-1R signaling; in contrast, IRAK3 (IRAKM) act as a negative regulator of the TLR/IL-1R signaling [17]. IRAK4 protein is composed of an N-terminal death domain, a hinge region, and a kinase domain. IRAK4 activation leads to the phosphorylation-dependent activation of IRAK1 and IRAK2 to form the "Myddosome" complex which allows interaction and activation of E3 ubiquitin ligase TRAF6 [18]. Activated TRAF6 involved in ubiquitylation-mediated TAK1 activation which in turn targets both MAP kinases and IKK complexes resulting coalesce activation of AP1 and NF-κB, respectively, that mediates induction of varied inflammatory cytokines transcriptional programs [14,15]. Whereas, MyD88 independent pathway initiated through the TRIF adaptor protein that interacts with TBK1 and RIP1 resulting activation of IRF3 and NF-κB respectively, and that leads to the expression of type I interferons [14,15]. IRAK4 knockout mice are resistant to LPS-induced the TLR4 activation showing decreased response to bacterial infection [19]. Patients with a mutation in the IRAK4 gene that encodes nonfunctional IRAK4 protein displayed increased susceptibility to bacterial infection [20,21]. Thus, IRAK4 is an attractive therapeutic target for the development of novel small molecule inhibitors against different immune-related diseases.

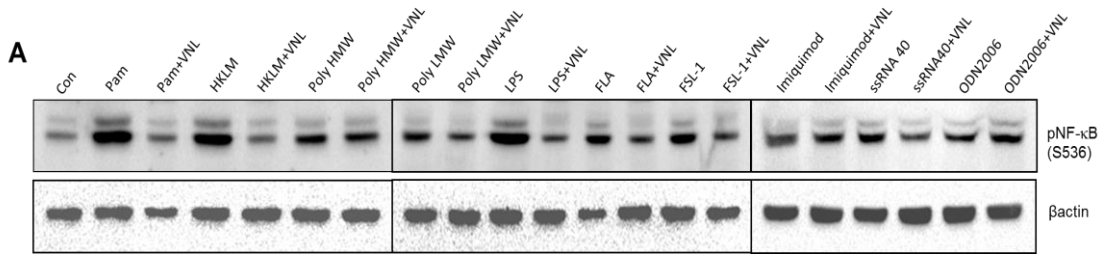
Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most popular flavors in the world and is widely used in the food, beverage, and pharmaceutical industries [22,23]. It is a major constituent of the vanilla bean of two different species of vanilla orchids; *Vanilla planifolia*, and *Vanilla tahitensis* [22,23]. Several studies highlighted its anti-oxidant, anti-inflammatory, anti-cancer, and anti-diabetic efficacies in both in-vitro and in-vivo systems [23,24]. It has 4-hydroxy-3-methoxyphenyl moiety and belongs to a group of compounds referred to as vanilloids [25]. It is now evident that the bioactivities of curcumin are mainly attributed to its stable degradation products, vanillin and ferulic acid [26]. To date, several potent inhibitors of IRAK4/1 have developed [27], however, none of them received approval for human use.

As observed in chapter IV, VNL failed to show direct binding with adenosine 2A receptor as supported by radioligand binding assay as well as VNLincubation was unable to stimulateA_{2A}ARsignaling in A_{2A}AR silenced adipocytes.Recent reports showed that vanillin and its analogs effectively circumvent both TLR2 and TLR4 signaling [28,29], however, the mechanism of its action is elusive. We, therefore, investigated the possible molecular targets of vanillin in TLR4 signaling pathway and that could provide a novel therapeutic option for the management of various inflammatory diseases.

6.2 Results

6.2.1 Vanillin (VNL) attenuates LPS-induced TLR4 signaling.

Vanillin (VNL) and its analogs have been reported for their promising anti-inflammatory efficacy, however, the molecular target and the mechanism of action is yet not clear. Studies in the area suggested two possible targets of vanillin; (i) BB loop pocket in the TIR domain of TLR2 inhibiting receptor dimerization [28], and (ii) TLR4 interaction with MD2 [29] based on the computational molecular docking analysis. We, therefore, attempted to find out the molecular target(s) of vanillin and its mechanism of action in the suppression of TLR-mediated inflammation.Analyzing the efficacy of vanillin on different agonists-stimulated TLR signaling, we have found that vanillin incubation significantly impaired ligand-induced TLR4, TLR2, and TLR2/1 mediated NF-κB activation in THP-1 macrophages as indicated by immunoblotting and κB reporter-luciferase assay [Fig. 6.1A-C]. Since the effect of vanillin is more pronounced on the abrogation of LPS-induced TLR4 activation, we continued our study focusing on its effect on TLR4 signaling. We then examined the effect of different precursors and structural homologs of vanillin on TLR4 signaling inhibition and observed that only vanillin, curcumin, and salicylaldehyde were able to reduce the NF-κB activation [Fig. 6.1D].



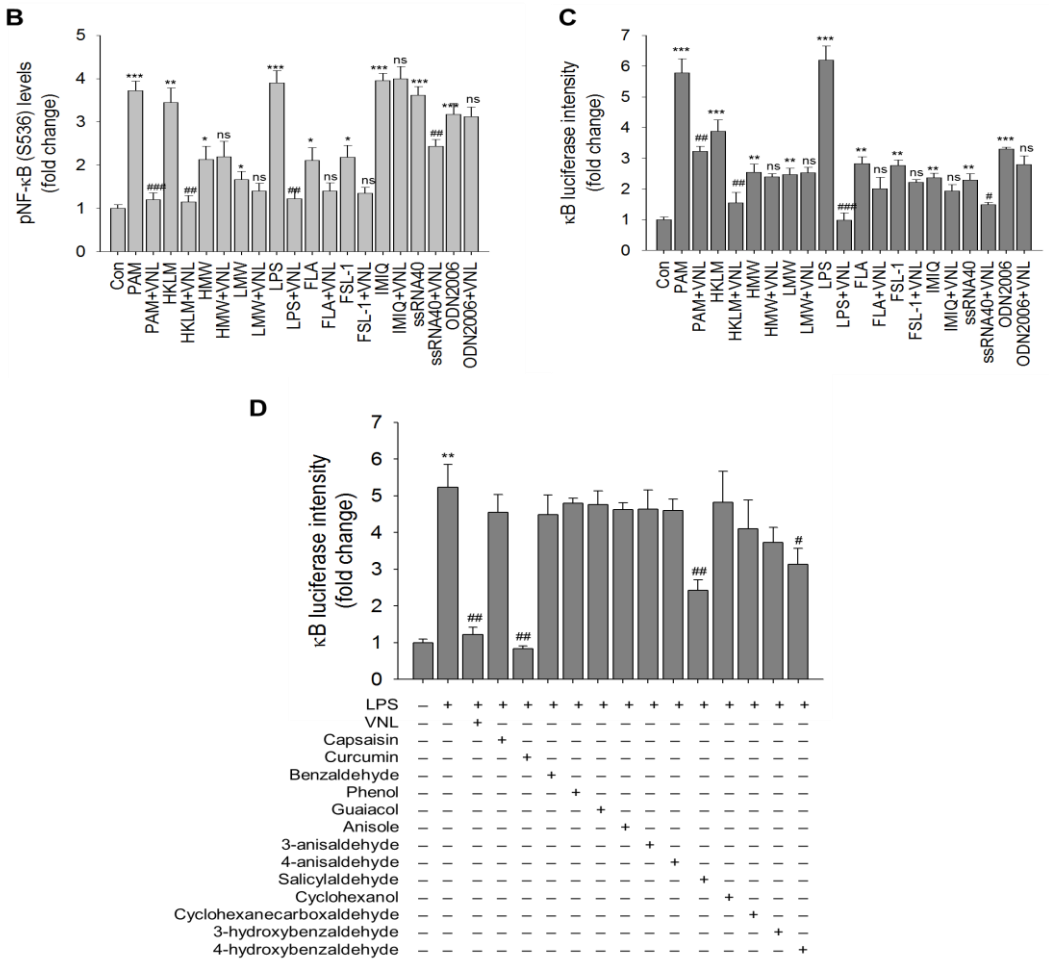


Fig 6.1 (A,B) Western blot (A) and its quantification (B) showing abundance of pNF-κB (S536) levels in THP-1 macrophages in response to different TLR agonists treated without or with VNL (100 nM) incubation for 5 h. β-actin was used as a loading control. **(C)**THP-1 macrophages transfected with κB luciferase plasmid were incubated without or with different TLR agonists in the absence or presence of VNL (100 nM) for 5 h. On termination of incubations, cells were lysed and luciferase activity was measured by a multimode reader.(D) THP-1 macrophages transfected with κB luciferase plasmid were incubated without or with LPS (100 ng/ml) in absence or presence of vanillin or different VNL precursors or homologs (100 nM) for 5 h. On termination of incubations, cells were lysed and luciferase activity was measured by multimode reader.All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***p < 0.001, **p < 0.01 vs Con; ###p < 0.001, ##p < 0.01, #p < 0.05 vs LPS.

On the basis of the dose-dependent effect of vanillin on THP-1 cell viability and NF-κB activation [Fig. 6.2A,B], we examined its efficacy and found that vanillin markedly reduced NF-κB activation and its nuclear localization in response to LPS and IL-1β stimulations [Fig. 6.2C& Fig. 6.2C,D]. These results were corroborated by the vanillin-

mediated inhibition of LPS-induced phospho-NF-κB binding to the IL-6 pro-inflammatory cytokine gene promoter in THP-1 macrophages [Fig. 6.2E,F].

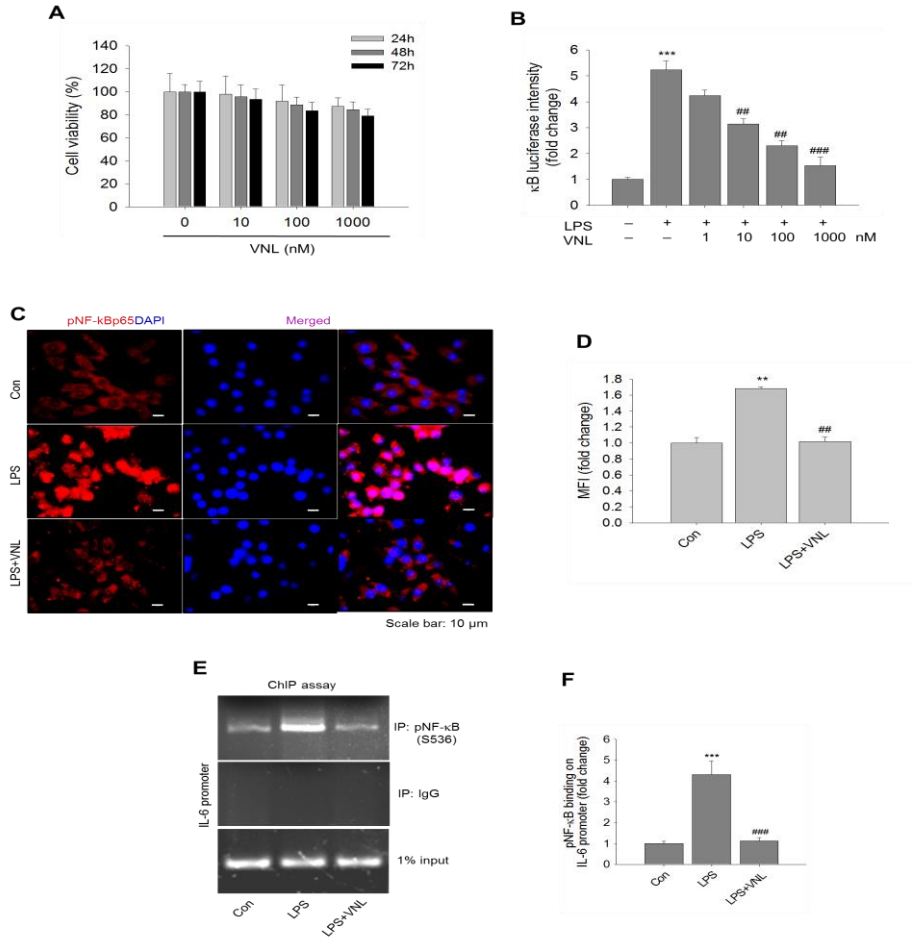


Fig 6.2 (A) THP-1 cell viability was estimated after incubation of indicated concentrations of VNL for different time periods. (B) Measurement of κB luciferase activity in transfected THP-1 cells treated with LPS (100 ng/ml) in absence or presence of indicated concentrations of VNL (100 nM) for 5 h.**(C)** Immunofluorescence images showing pNF-κB (S536) level in THP-1 macrophages incubated without or with LPS (100 ng/ml) in the absence or presence of VNL (100 nM)for 5 h.DAPI was used for nuclear counterstaining. Scale bar, 10 μm.(D) Quantification of pNF-κB (S536) immunofluorescence in THP-1 macrophages treated under the indicated conditions.**(E,F)** ChIP assay (E) and its quantification (F) showing increased pNF-κB binding to the IL-6 promoter in response to LPS was markedly reduced by VNL pretreatment in THP-1 cells. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***p < 0.001, **p < 0.01 vs Con; ###p < 0.001, ##p < 0.01, #p < 0.05 vs LPS.

Moreover, vanillin incubation strikingly waived LPS-induced pro-inflammatory state in RAW264.7 macrophages as indicated by the suppression of pro-inflammatory marker CD80 and enhancement of anti-inflammatory marker CD206 [Fig. 6.3A& Fig. 6.3B]. It

could be intriguing to note that although vanillin incubation significantly diminished LPS-induced NF-κB and AP-1 transactivation in THP-1 macrophages, however, vanillin treatment failed to suppress the IRF3 transactivation potential as indicated by the promoter-reporter luciferase assay [Fig. 6.3C].

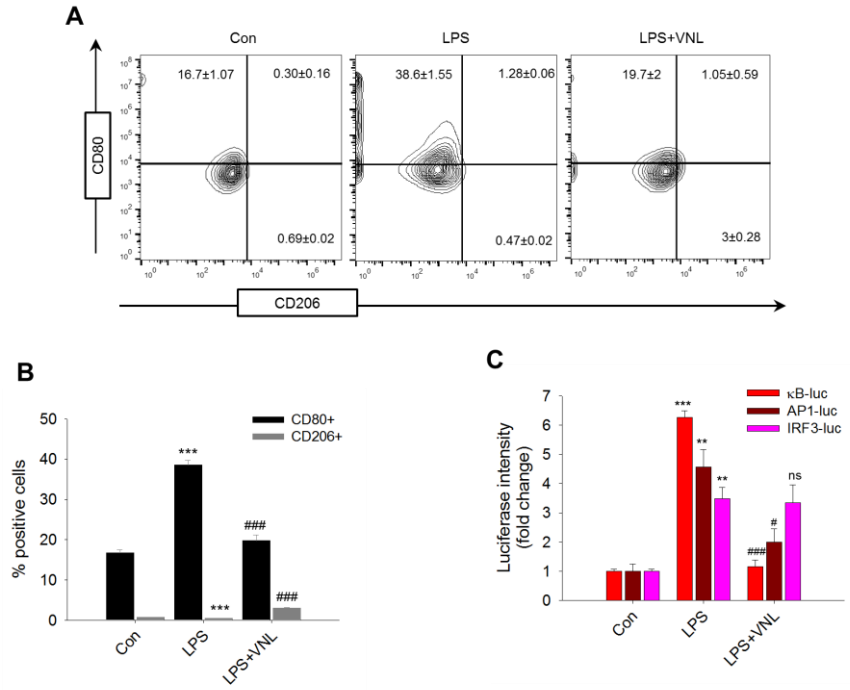


Fig. 6.3 (A) Flow cytometric analysis of CD80 and CD206 levels in RAW264.7 macrophages treated without or with LPS (100 ng/ml) in the absence or presence of VNL (100 nM) for 5 h. (B) Quantification of flow cytometic analysis of CD80 and CD206 levels in RAW264.7 macrophages treated without or with LPS (100 ng/ml) in absence or presence of VNL (100 nM) for 5 h.**(C)** Efficacy of VNL on NF-κB, AP-1, and IRF-3 reporter assays. THP-1 macrophages transfected with κB luciferase or AP-1 luciferase or IRF-3 luciferase plasmids were incubated without or with LPS (100 ng/ml) in the absence or presence of VNL (100 nM) for 5 h. On termination of incubations, cells were lysed and luciferase activity was measured by a multimode reader. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***p < 0.001, **p < 0.01 vs Con; ###p < 0.001, ##p < 0.01, #p < 0.05 vs LPS/IL-1β.

To gain more insight about the impairment of LPS-induced TLR4 activation by vanillin, we performed the gene and protein expression analysis of various pro-inflammatory cytokines and found that vanillin treatment significantly down regulated LPS-induced MCP-1, TNF-α, IL-1β, iNOS, and IL-6 gene expressions and the protein expressions of TNF-α and IFN-γ [Fig. 6.4A-D].

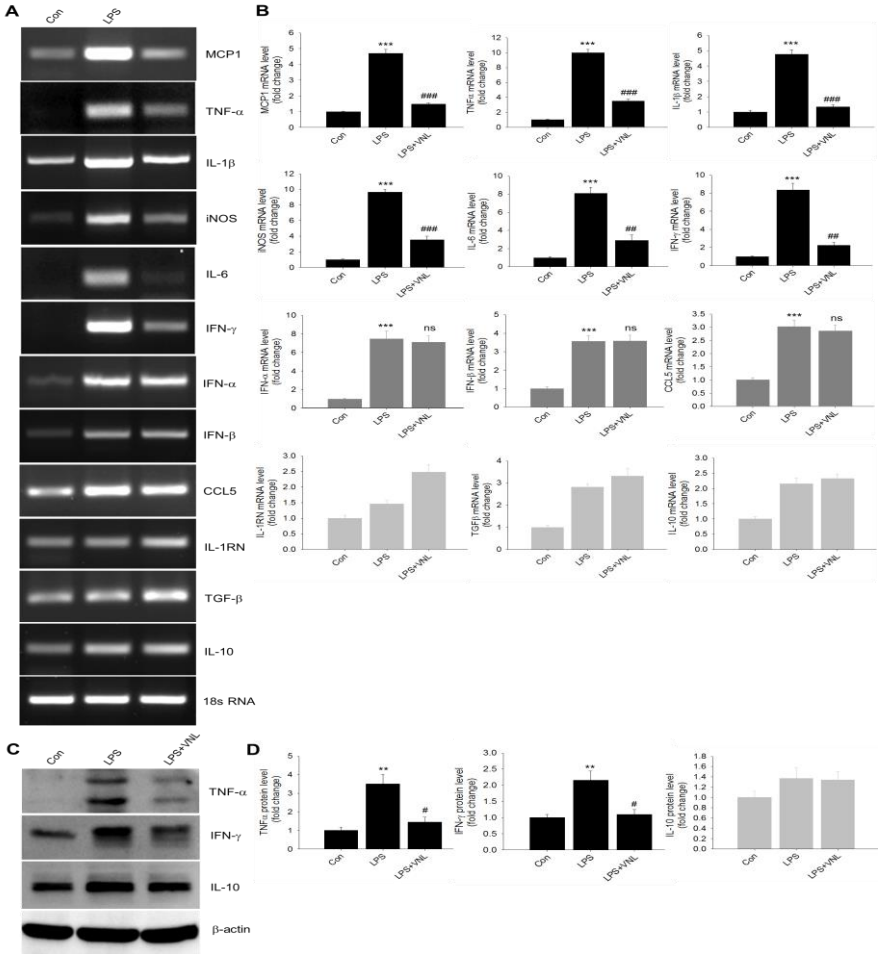


Fig. 6.4. (A,B) RT-PCR alanysis (A) and their quantifications (B) showing the abundance of MCP-1, TNF-α, IL-1β, iNOS, IL-6, IFN-γ, IFN-α, IFN-β,CCL5, IL-1RN, TGF-β, IL-10 gene expressions in THP-1 macrophages treated without or with LPS (100 ng/ml) in absence or presence of VNL (100 nM). 18s RNA was used as loading control. (C,D) Western blot analysis (C) and their quantifications (D) showing the abundance of TNF-α, IFN-γ, and IL-10 protein expressions in the THP-1 macrophages under the indicated conditions. □-actin was used as loading control. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***p < 0.001, **p < 0.01 vs Con; ###p < 0.001, ##p < 0.01, #p < 0.05 vs LPS.

Moreover, analysis of different TLR4 signaling mediators activation revealed that vanillin notably reduced the activation of NF-κB, IRAK4, IRAK1, TAK1, IKKα/β, c-Jun, c-Fos, JNK, and p38 MAPK [Fig. 6.5A,B]. Interestingly, vanillin was unable to alter the LPS-induced Type I interferons (IFN-α, and IFN-β) gene expression [Fig. 6.5A,B] and IRF3 activation [Fig. 6.5A,B]. The ineffectiveness of vanillin on the suppression of LPS-induced IRF3 phosphorylation, IRF3 transactivation potency, and Type I IFN expressions clearly suggest that the molecular target of vanillin possibly lies

in the downstream of TLR4 receptor that could only prevent TIRAP/MyD88-dependent signaling without posing any significant effect on TRAM/TRIF-mediated pathway.

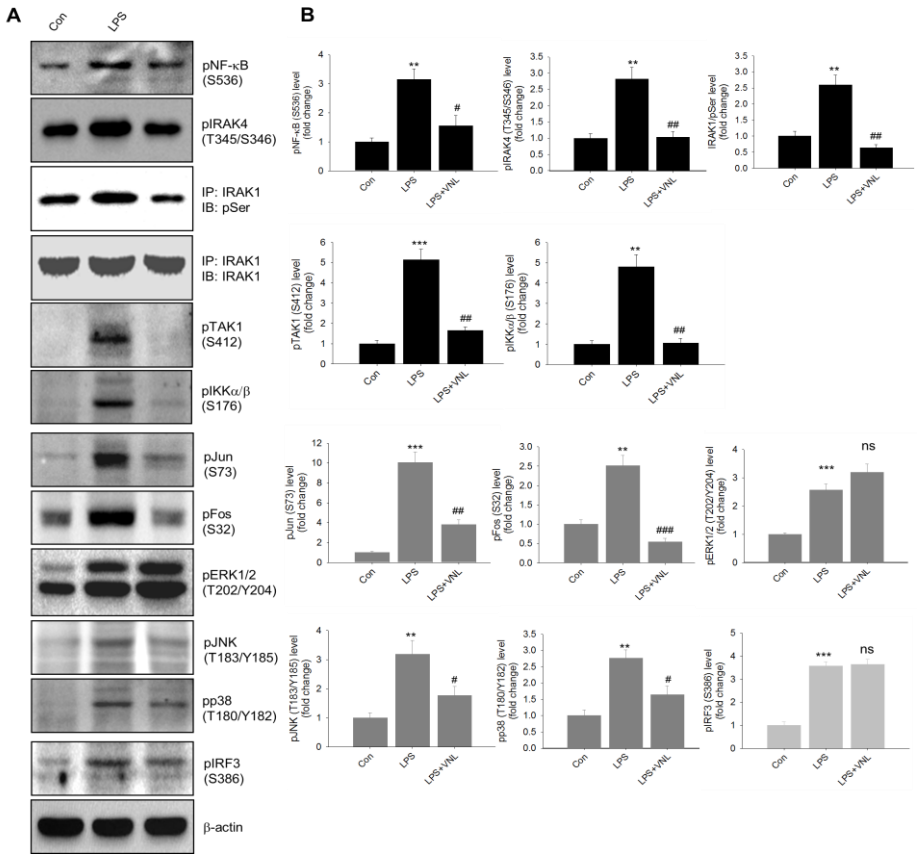


Fig. 6.5 (A,B) Western blot analysis (A) and their quantifications (B) showing the abundance of pNF-κB (S536), pIRAK4 (T345/S346), pIRAK1, pTAK1 (S412), pIKKα/β (S176), pJun (S73), pFos (S32), pERK1/2 (T202/Y204), pJNK (T183/Y185), pp38 (T180/Y182), and pIRF3 (S386) levels in THP-1 macrophages treated without or with LPS (100 ng/ml) in absence or presence of VNL (100 nM) for 5 h. β-actin was used as loading control for immunoblotting. For immunoprecipitation assay, IRAK1 antibody was used for immunoprecipitation followed by the immunoblotting with pSer antibody. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***$p < 0.001$, **$p < 0.01$ vs Con; ###$p < 0.001$, ##$p < 0.01$, #$p < 0.05$ vs LPS, ns=non-significant.

6.2.2 Vanillin prevents inflammation in macrophages expressing the constitutively active forms of IRAK4/1.

To explore the molecular target of vanillin, we forcibly expressed constitutively activated forms of different TLR4 signaling molecules in THP-1 macrophages and examined the efficacy of vanillin on TLR4 signaling therein. We have found that vanillin could only be able to impair the constitutively activated IRAK4 and IRAK1 mediated NF-κB activation, inflammatory cytokine IL-1β gene expression, and

κBluciferase activity [Fig. 6.6A-C], however, such effect was not evident in constitutively activated forms of IRAK4 downstream proteins such as TAK1, and IKKβ [Fig. 6.6A-C].

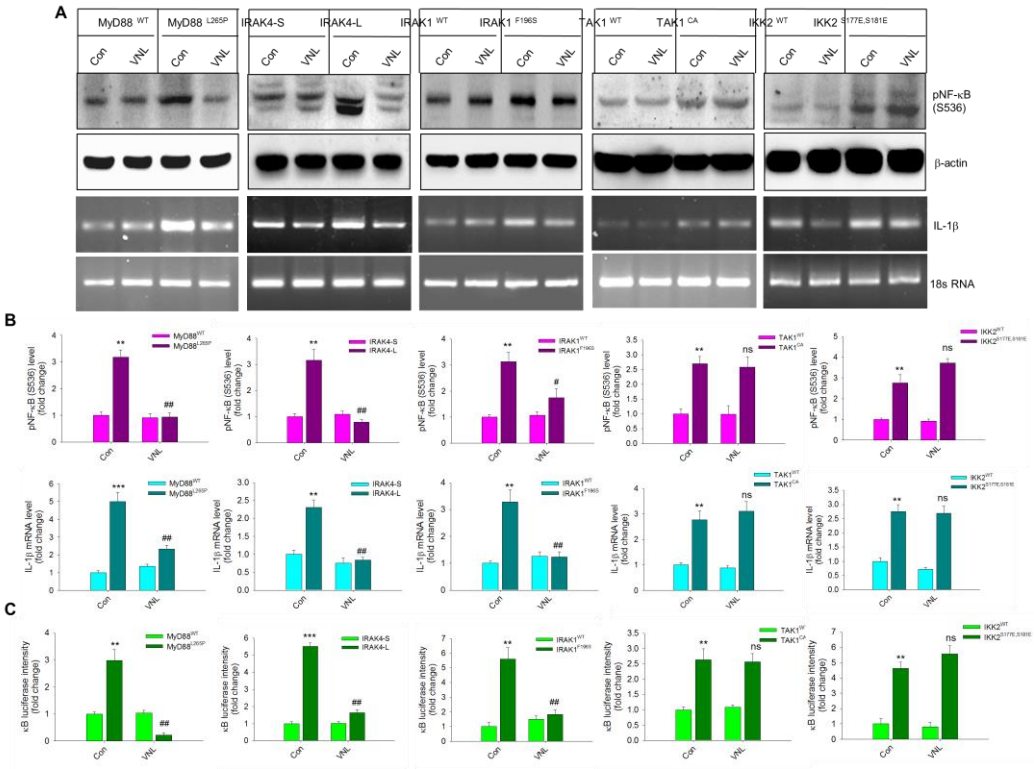


Fig. 6.6 **(A,B)** Western blots (upper) and RT-PCR images (lower) (A) along with their quantifications (B) showing abundance of pNF-κB (S536) and IL-1β mRNA levels in THP-1 macrophages expressing wild-type or constitutively active forms of MyD88 (MyD88-WT or $MyD88_{L265P}$), or IRAK4 (IRAK4-S or IRAK4-L), or IRAK1 (IRAK1-WT or $IRAK1_{F196S}$), or TAK1 (TAK1-WT or $TAK1\text{-}CA_{\Delta 22\ N\text{-}terminal\ amino\ acids}$), or IKKβ (IKK2-WT or $IKK2_{S177E,\ S181E}$) treated without or with VNL (100 nM) for 1 h. β-actin and 18s RNA were serves as loading controls for immunoblotting and RT-PCR, respectively. **(C)**Measurement of κB luciferase activity in the constitutively activated MyD88, or IRAK4, or IRAK1, or TAK1, or IKKβ expressing THP-1 cells transfected with κB reporter plasmid and treated with VNL (100 nM) for 1 h. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns = non significant.

This was also apparent in the wild-type or constitutively activated TLR4 pathway molecule transfected cells treated with LPS in the absence or presence of vanillin [Fig. 6.7A-C]. All these results demonstrated that vanillin could possibly target the TLR4 signaling pathway molecules in the intersection of the TIRAP and TAK1 complex.

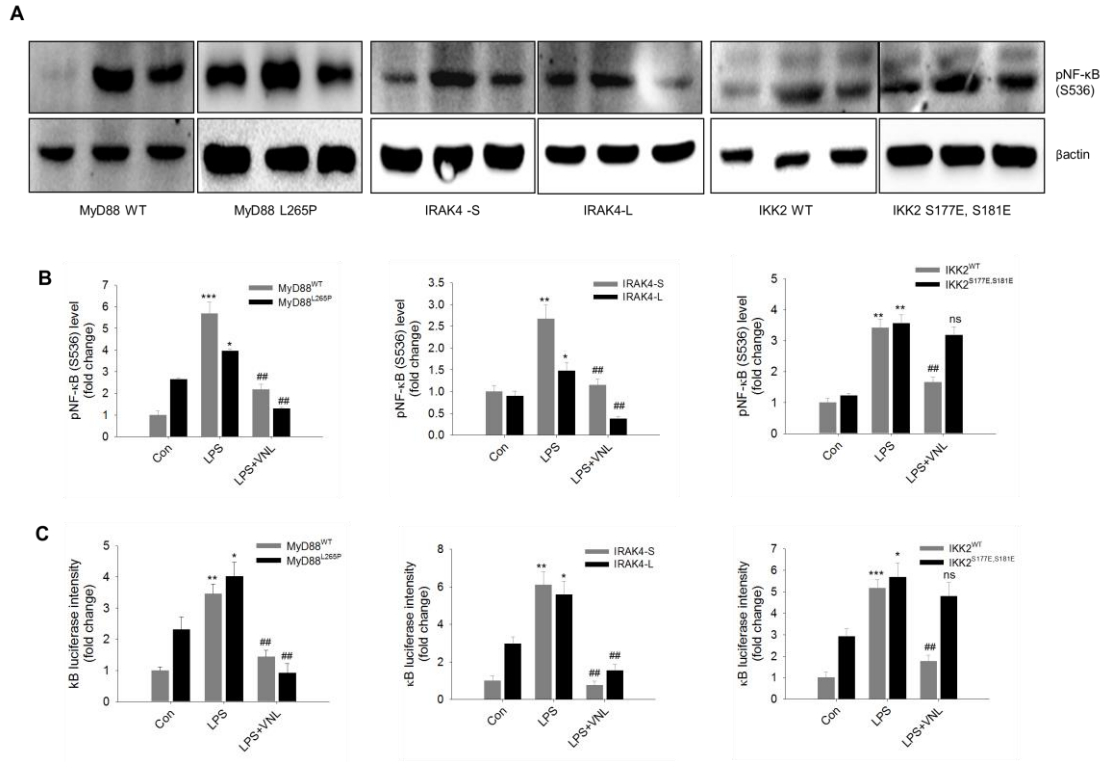


Fig. 6.7 (A,B) Western blot analysis (A) and their quantifications (B) showing abundance of pNF-κB (S536) levels in THP-1 macrophages expressing wild-type or constitutively active form of MyD88 (MyD88-WT or MyD88L265P), or IRAK4 short isoform or long isoform (IRAK4-S or IRAK4-L), or wild-type IKK2 or constitutively active form of IKK2 (IKK2-WT or IKK2S177E, S181E) and treated without or with LPS (100 ng/ml) in absence or presence of VNL (100 nM). β-actin was serves as loading control. (C) Measurement of κB luciferase activity in THP-1 cells cotransfected with kB luciferase plasmid along with either MyD88-WT or MyD88L265P plasmid or IRAK4-S or IRAK4-L plasmid, or IKK2-WT or IKK2S177E, S181E plasmid and treated without or with LPS (100 ng/ml) in absence or presence of VNL (100 nM). All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs Con; ##$p < 0.01$ vs LPS; ns=non-significant.

6.2.3: VNL directly binds and inhibits IRAK4 kinase activity.

To determine the exact molecular target and possible mechanism of action of vanillin, we have studied the effect of vanillin on LPS-induced increased association of different TLR4 signaling molecules. It has been observed that vanillin did not alter the LPS-induced interaction of TLR4-MD2-CD14 or the association among the TLR4-TIRAP-MyD88, however, a significant hindrance of IRAK4-MyD88, IRAK4-IRAK1, and IRAK1-TRAF6 interactions were noticed in response to vanillin incubation [Fig.

6.8A,B]. Collectively, these results posit IRAK4/1 as a molecular target of vanillin in the impairment of LPS-instigated TLR4 activation.

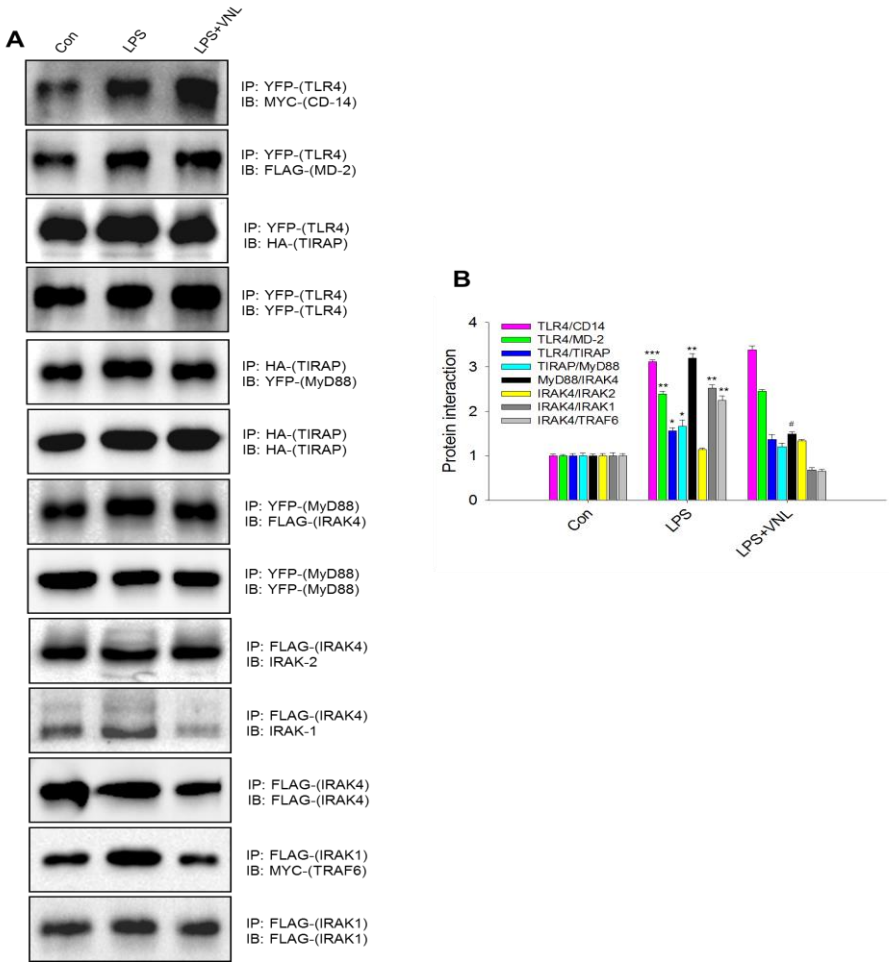


Fig. 6.8 (A,B) THP-1 macrophages transfected with TLR4-YFP, MD-2-FLAG, and CD14-Myc; or TLR4-YFP and TIRAP-HA; or TIRAP-HA and MyD88-YFP; or MyD88-YFP and IRAK4-FLAG; or IRAK4-FLAG; or IRAK1-FLAG and TRAF6-Myc plasmids were treated without or with LPS (100 ng/ml in absence or presence of VNL (100 nM). On termination of incubations, cell lysates were prepared and used for immunoprecipitation using indicated tagged antibodies followed by the immunoblotting with tagged or target antibodies showing protein-protein interactions (A) and their quantifications (B) in these treatment conditions.Each value is the mean ± SEM of threeindependent experiments, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs Con/IRAK4-WT; ##$p < 0.01$, #$p < 0.05$ vs LPS; ns = non significant.

Since IRAK4 is constitutively activated in different cancers particularly in pancreatic ductal adenocarcinoma cells [41], we used PANC1 and MIAPaCa2 cell lines to investigate further about the vanillin effect on NF-κB activation and inflammatory cytokine gene expression. Vanillin treatment profoundly reduced NF-κB activation and IL-1β gene expression in these cells treated in the absence or presence of LPS [Fig.

6.9A,B] which clearly suggests IRAK4 as a molecular target of vanillin attenuating TLR4 signaling.

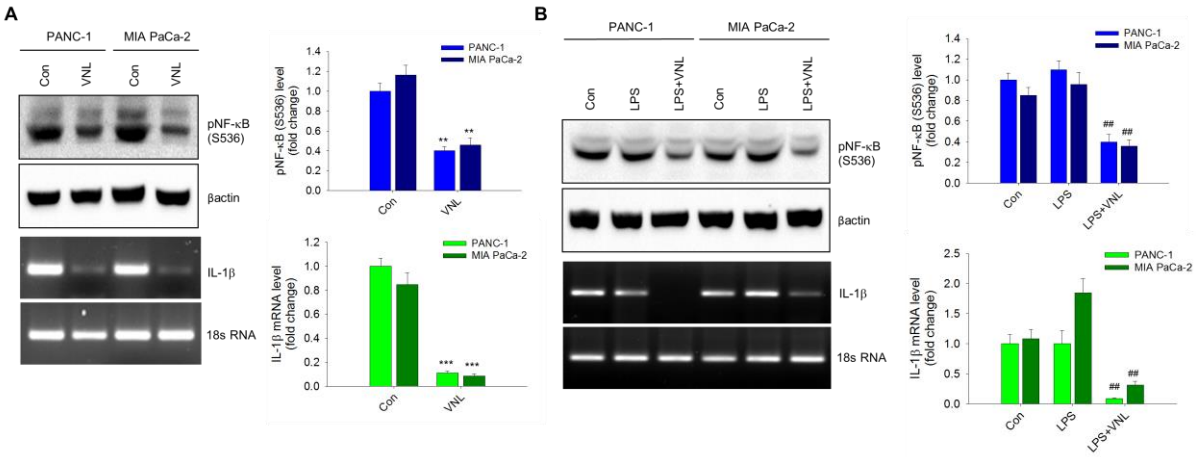


Fig. 6.9 (A,B) Western blot (A, upper panel) and its quantification (B, upper panel) showing abundance of pNF-κB (S536) levels; and RT-PCR analysis (A, lower panel) and its quantification (B, lower panel) showing IL-1 gene expression in PANC-1 and MIAPaCa-2 cells treated without or with VNL. β-actin and 18s RNA were served as loading controls for Western blotting and RT-PCR analysis, respectively. (C,D) Western blot (C, upper panel) and its quantification (D, upper panel) showing abundance of pNF-κB (S536) levels; and RT-PCR analysis (D, lower panel) and its quantification (D, lower panel) showing IL-1β gene expression in PANC-1 and MIAPaCa-2 cells treated without or with LPS (100 ng/ml) in absence or presence of VNL (100 nM). β-actin and 18s RNA was served as loading controls for Western blotting and RT-PCR analysis, respectively. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, ***$p < 0.001$, **$p < 0.01$ vs Con; ##$p < 0.01$ vs LPS.

To have direct evidence of physical interaction between vanillin and IRAK4, we performed a surface plasmon resonance (SPR) analysis. SPR sensorgrams exhibit concentration-dependent binding of vanillin to IRAK4 with a moderate binding affinity [KD: 2.63E-04 M; Chi² (RU²) =0.243] indicating interaction specificity [Fig. 6.10A].However, this moderate binding affinity could possibly be either due to the very small molecular nature of vanillin (152.15 g/mol) or the absence of physiological conformation of IRAK4 as a myddosome complex with IRAK1, IRAK2, MyD88, and TRAF6. To examine the biochemical relevance of IRAK4 interaction with vanillin, we performed an in-vitro IRAK4 kinase assay using myelin basic protein as a substrate. We have found that vanillin significantly attenuates IRAK4 kinase activity (IC_{50}: 0.0213 μM) [Fig. 6.10B]. A similar result was also obtained in immunocomplex kinase assay [Fig. 6.10C]. Together, these results indicate that vanillin effectively blocks IRAK4 activity that attenuates LPS-stimulated TLR4 signaling.

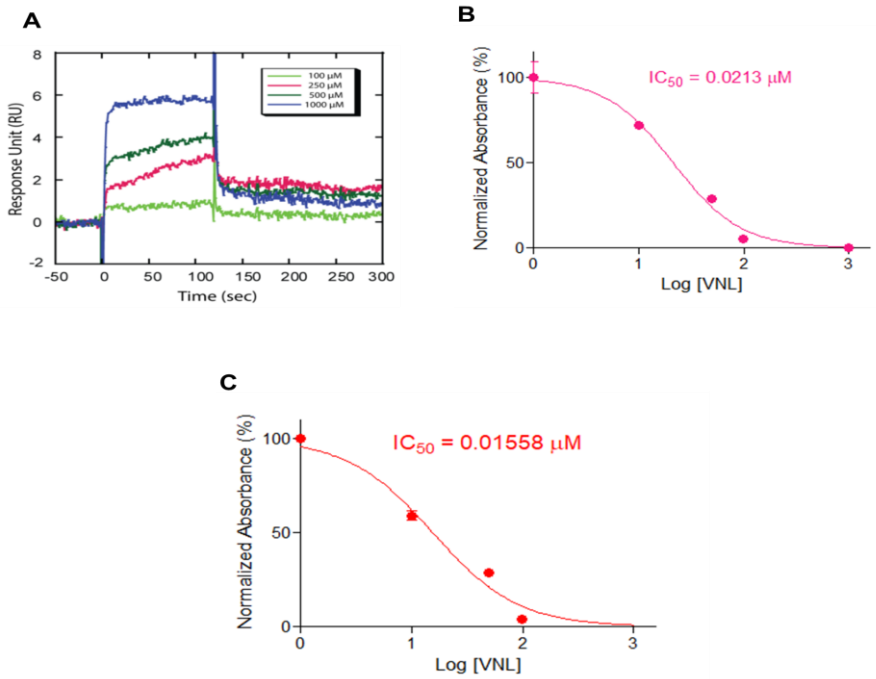


Fig. 6.10 (A) SPR analysis showing representative sensorgrams obtained from flowing of the indicated concentrations of VNL over the IRAK4 immobilized CM5 sensor chip. Sensorgrams are represented as response units (RU) at specified time(s). **(B)** In-vitro IRAK4 kinase assay showing IC_{50} value of VNL. (C)IRAK4 immunocomplex kinase assay showing indicated IC50 value.

We then performed an in-silico study of vanillin-IRAK4 interaction to identify the probable site of the IRAK4 that involved its interaction with vanillin. Out of the 50 conformations, the conformer with the lowest binding energy (-3.66 kcal/mol) has been selected for further inspection. It was observed that the best pose of vanillin within the binding cleft of IRAK4 closely lined up with Asp329, Tyr262, and Val263 residues [Fig. 6.11A]. Moreover, the docking study further displayed that vanillin occupies the active site hydrophobic cavity of IRAK4 [Fig. 6.11B] and such occupation was stabilized by non-covalent interactions including pi-pi stacking (Tyr262), hydrogen bonds (Val263 and Asp329), and pi-alkyl interactions (Ala211, Val246, and Leu318) of IRAK4 [Fig. 6.11C]. To determine the functional relevance of Tyr262, Val63, and Asp329 residues, we mutated these amino acids in IRAK4 by site-directed mutagenesis and performed the in-vitro kinase assay that clearly depicted the importance of these residues as mutations of these sites specifically Y262A, and D329F severely impeded IRAK4 kinase activity [Fig. 6.11D].

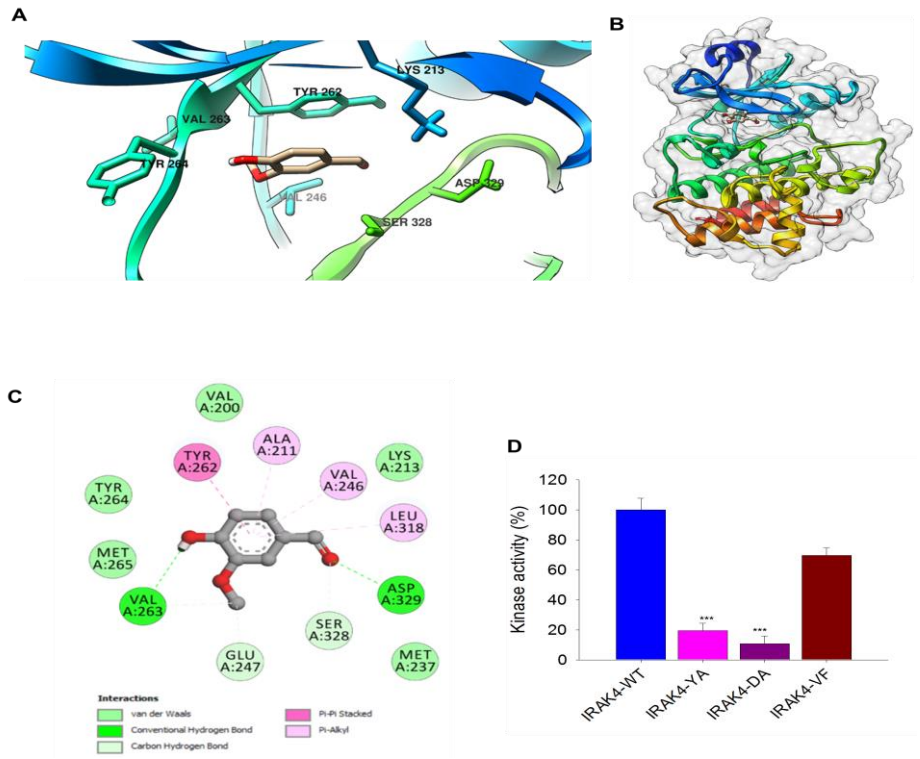


Fig. 6.11 (A) Molecular orientation of VNL within the active site binding cleft of IRAK4.**(B)** 3D visualization displaying VNL occupation in the active site cavity of IRAK4. **(C)** 2D docking image exhibiting VNL-IRAK4 non-covalent interactions including pi-pi stacking (Tyr262), hydrogen bonds (Val263 and Asp329), and pi-alkyl interactions (Ala211, Val246, and Leu318) of IRAK4. **(D)** Kinase activity was measured in wild-type (IRAK-4-WT) and mutated IRAK4 (IRAK4-Y262A, IRAK4-D329A, and IRAK4-V263F) using myelin basic protein (MBP) as substrate.

Moreover, we also modified the vanillin structure and developed its various analogs [**Table 6.1**] in order to ascertain their efficacies in the abrogation of LPS-activated TLR4 signaling.

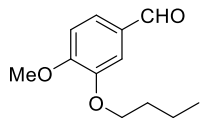
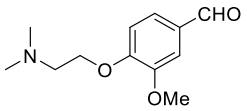
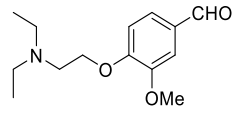
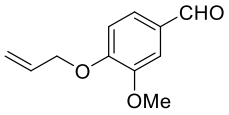
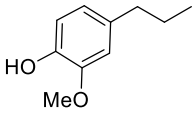
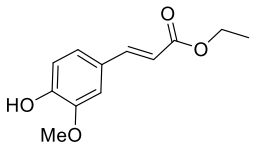
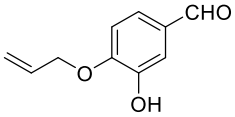
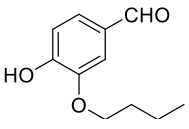
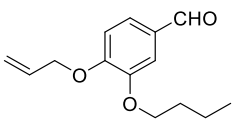
Chemical structure	Chemical Nomenclature	Abbreviation
	3-butoxy-4-methoybenzaldehyde	VNL-1
	4-(2-(dimethylamino)ethoxy)-3-methoxybenzaldehyde	VNL-2
	4-(2-(diethylamino)ethoxy)-3-methoxybenzaldehyde	VNL-3
	4-(allyloxy)-3-methoxybenzaldehyde	VNL-4
	2-methoxy-4-propylphenol	VNL-5
	Ethyl (E)-3-(4-hydroxy-3-methoxyphenyl) acrylate	VNL-6
	4-(allyloxy)-3-hydroxybenzaldehyde	VNL-7
	3-butoxy-4-hydroxybenzaldehyde	VNL-8
	4-(allyloxy)-3-butoxybenzaldehyde	VNL-9

Table S1: Chemical structures and nomenclatures of different vanillin analogs.

It could be noted here that although most of the vanillin analogs (VNL-1 to VNL-6 and VNL-9) retained their potencies, however, VNL-7 and VNL-8 are failed to restrict LPS-induced IRAK4 and NF-κB activation, and thus compromised the functions as IRAK4 inhibitors. Altogether, all these results indicate that mutations at Asp329, Tyr262, and Val263 residues of IRAK4 and the modification of the 3'O-CH3 group to 3' butoxy or 4'OH group to 4'(allyloxy) severely attenuates IRAK4 kinase activity and vanillin efficacy as a potent inhibitor of IRAK4, respectively. Thus our study quashed all premises that specify vanillin targets the TIR domain of TLR2 and TLR4, TLR4 dimerization, and TLR4 interaction with MD-2, and provides shreds of evidence that discovered vanillin as a novel and potent inhibitor of IRAK4.

6.2.4. Vanillin abrogates LPS-induced TLR4 activation and inflammation in-vivo.

We also investigated the vanillin potency in-vivo in the impairment of LPS-induced TLR4 activation to examine its applicability in a physiological context. Mice were pre-treated with vanillin and then challenged with LPS [Fig. 6.12A] showed reduced pro-inflammatory cytokines levels (TNF-α and IL-6) in the serum [Fig. 6.12B], impairment of pro-inflammatory cytokines gene expressions (iNOS, IL-1β, MCP1, and IL-6) in the isolated macrophages [Fig. 6.12C], prevention of macrophage proinflammatory M1 polarization state as indicated by the reduction of CD80 and induction of CD206 levels in the F4/80+ peritoneal macrophage populations [Fig. 6.12D & Fig. 6.12E], and the subdued levels of IRAK4 and NF-κB activation in the peritoneal macrophages [Fig. 6.12F,G] as compared with mice pre-treated with vehicle control. The molecular target of vanillin to LPS-induced TLR4 activation is presented in a schematic diagram [Fig. 6.12H].

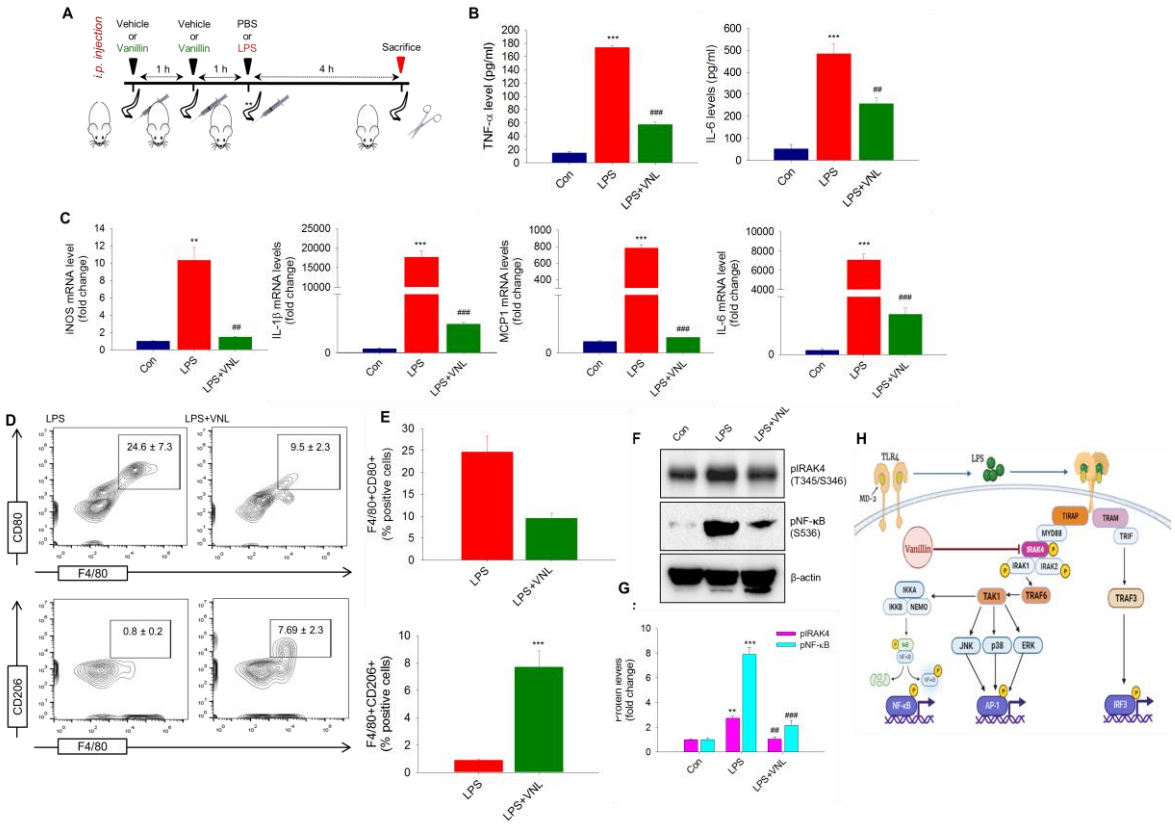


Fig. 6.12 (A) C57BL/6 mice were pre-treated twice with vehicle (saline) or VNL (1.5 mM/g bw) for 1 h intervals and then challenged with PBS or LPS (100 µg) through i.p. administration for 4 h. After 4 h of treatment regime, mice were sacrificed, and peritoneal fluid and serum were collected. (B) Serum TNF-α and IL-1β levels were measured in these mice by ELISA (n=4). (C) RT-qPCR analysis of iNOS, IL-1β, MCP-1, and IL-6 mRNA expression in the peritoneal macrophages (n=4). (D) Flow cytometric analysis of CD80 and CD206 levels in F4/80+ peritoneal macrophages (n=4). (E) Quantification of flow cytometic analysis of CD80 and CD206 levels in F4/80+ monocytes isolated from the peritoneum of LPS (100 µg) challenged C57BL/6J mice for 4 h pre-treated twice with VNL (1.5 mM/g bw) or saline (n=3) for 1 h each. Each value is the mean ± SEM, ***p < 0.001, **p < 0.01 vs LPS.(F,G) Western blots (F) and their quantifications (G) showing abundance of pIRAK4 and pNF-κB (S536) levels in the peritoneal macrophages of these mice (n=4). (H) Proposed schematic model indicating the molecular target of vanillin in TLR4 signaling. β-actin was used as loading control for normalization, ***p < 0.001, **p < 0.01, *p < 0.05 vs Con; ##p < 0.01, #p < 0.05 vs LPS.

6.3Discussion:

Research on natural phytochemicals and their derivatives offers newer prospects to find out novel compounds for specific therapeutic targets in the management of various human diseases [31]. Several lines of evidence indicated that vanillin, an active compound of vanilla orchids, has a broad range of anti-inflammatory therapeutic values [23,24]. Investigating anti-inflammatory efficacy of vanillin, we have found that

vanillin effectively impaired TLR2 and TLR4 activation-dependent cellular inflammation. While, TLR-4 activation involves its homotypic dimerization upon binding of accessory protein myeloid differentiation factor 2 (MD-2) with lipopolysaccharide (LPS), a major component of the Gram-negative bacteria, the heterotypic TLR2 dimerization with TLR1 or TLR6 involved in the recognition of Gram-positive bacterial components such as triacyl- or diacyl-lipopeptide, respectively, resulting activation of TLR2 signaling cascade [14,15]. It is important to note that vanillin effect on the inhibition of TLR2 signaling is specifically directed against TLR2/TLR1, though reasons for such selectivity are yet not clear. Both TLR4 and TLR2 activation induces MyD88-dependent and TRIF-dependent signaling that leads to the upregulation of various pro-inflammatory cytokines and type I IFNs expression [14,15]. Intriguingly, we have noticed that vanillin incubation could only alleviate the TLR4 activation-dependent pro-inflammatory cytokines expression without any noticeable changes in the expression of type I IFNs. Moreover, we also found that vanillin treatment significantly attenuates LPS-induced activation of NF-κB, JNK, and p38 MAPK without causing any significant changes in IRF3 phosphorylation. All these results attest vanillin specificity in targeting MyD88 pathway. Since vanillin did not alter the production of type 1 IFNs in response to LPS, therefore, its application as therapeutics would not pose any threat to countering viral infection.

Stimulation of TLR4-MyD88 pathway results in the death domain mediated homophilic association of MyD88, IRAK4, and IRAK2 forming a supramolecular organizing center, myddosome signaling complex, that consists of six molecules of MyD88, four molecules of IRAK4, and four molecules of IRAK2 [32,33]. Myddosome assembly facilitates activation of most proximal kinase IRAK4 through trans-autophosphorylation [34] which triggers the recruitment and activation of IRAK-1 through phosphorylation [35]. Activated IRAK1, once dissociated from Myddosome, interacts with E3 ligase TNF receptor-associated factor 6 (TRAF6) and it was observed that the K63-linked polyubiquitylation of IRAK1 and TRAF6 are essential for TRAF6-mediated signalling suggesting involvement of such modification is necessary to modulate their accessibility as scaffolding molecules [36,37]. Activated TRAF6 then interacts with transforming growth factor-β-activated kinase-1 (TAK1) along with the TAK1-binding protein-1 and -2 (TAB1-TAB2) which facilitates polyubiquitination-dependent activation ofTAK1 leading to the activation of IKK complex, c-Jun N-terminal kinases (JNK), and p38

MAPK [38]. While IKK complex activation leads to the IκB degradation and NF-κB nuclear translocation resulting NF-κB activation, the activation of JNK, and p38 MAPK stimulates the phosphorylation-mediated association of Jun and Fos transcription factors forming activator protein-1 (AP-1) [39,40]. Activation of both NF-κB and AP-1 are responsible for the expression of various proinflammatory cytokines [39,40].

In our investigation to find out the molecular target of vanillin, we noticed that vanillin could effectively impair NF-κB activation and IL-1β proinflammatory cytokine expression in cells expressing constitutively activated forms of MyD88, IRAK4, and IRAK1, however, such attributes were compromised in cells producing constitutively activated forms of TAK1 and IKK2. This observation indicates IRAK4/1 could be a potential target of vanillin in mitigating LPS-stimulated TLR4 signaling. Since constitutively activated MyD88 signaling has been reported in haematological malignancies due to an L265P substitution mutation [41,42], therefore, vanillin could also be applicable for the management of these cancers. The compelling evidence signifies IRAK4 role in inflammation as IRAK4 deficient mice and humans with deficient IRAK4 activity are susceptible to certain bacterial infections particularly Gram-positive pyogenic bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, and the Gram-negative *Pseudomonas aeruginosa* [19-21]. Thus, IRAK4 is poised as a novel therapeutic target for developing its potent inhibitors against various inflammatory diseases. IRAK4, depending on cell types, is expressed either in two different lengths of isoforms due to the inclusion or exclusion of exon 4 in the mRNA. The large isoform encodes 460 residues, IRAK4-L, which contains all three functional domains, whereas, the short isoform IARK4-S encodes 336 residues lacking the death domain [43]. Recently, Smith et al, 2019 [44] showed higher expression of IRAK4-L in myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML) than in normal cells which mainly expressed IRAK4-S, and increased expression of IRAK4-L is strongly associated with the enhanced IRAK4 activity and its downstream NF-κB, and MAPK activation. Due to the availability of the N-terminal death domain, IRAK4-L readily interacted with both MyD88 and IRAK1 forming myddosome complex which augments trans-autophosphorylation mediated IRAK4 activation. We have found that vanillin effectively attenuates inflammation in cells expressing constitutively activated form of IRAK4 (IRAK-L) and treated without or with LPS.

Although it is now well established that IRAK4 plays a pivotal role in TLR4-mediated inflammatory signaling, however, the mechanism of action remains subject to investigation. Research in this direction provides contradictory evidence, while it has been shown that IRAK4 kinase activity is required for the activation of IRAK1 and downstream signaling [45-48], it has also been claimed that IRAK4-induced IRAK1 activation is kinase-independent and mediated through their association [49,50]. These observations exhibit that both kinase and scaffolding functions of IRAK4 are required for maximum response [51]. Currently, several IRAK4 inhibitors are in different stages of clinical trials including PF-06650833 (Pfizer), GS-5718 (Gilead), R835 (Rigel Pharmaceuticals), BAY1834845, and BAY1830839 (Bayer), and CA-4948 (Curis) [52,53], however, small molecules of bispecific in nature that can abrogate IRAK4 kinase activity and impairs IRAK4 myddosome assembly is now the main approach for developing novel IRAK4 inhibitors. Recent research mainly focussed on the development of small molecules IRAK4 proteolysis-targeting chimera (PROTAC) degraders that can interact with both the kinase and E3 ubiquitin ligases which allows targeted degradation of IRAK4 [54]. These bifunctional IRAK4 degraders such as KT-474 (Kymera/Sanofi) and IRAKIMiD (Kymera) are currently in Phase 1 of clinical trials [54]. In this context, it is pertinent to note that vanillin could effectively block the LPS-induced association of IRAK4-MyD88, IRAK4-IRAK1, and IRAK1-TRAF6 suggesting their possible role in the disassembly of IRAK4/1 myddosome complex. While SPR analysis provides evidence for direct binding between IRAK4 and vanillin, the in-vitro IRAK4 kinase assay clearly exhibits vanillin as a potent inhibitor of IRAK4 with an IC_{50} value of 0.0213 μM. Since we have found that vanillin specifically inhibits TLR4 and TLR2/1 signaling pathways by targeting IRAK4, therefore, these results suggested that IRAK4 activation has not been an obligatory requirement in different TLR signaling including TLR2/6. However, it could be noted here that the involvement of IRAK4 on inflammatory signalling is depends on various aspects including, but not limited to, the differences in species, cell types, stimuli, physiological and pathophysiological state [55]. Excitement notwithstanding, there is still a long way to go in understanding the role of different IRAKs and associated signaling molecules in different TLR signaling and future study in this direction would clarify the differential effect of vanillin on cellular inflammation depending on TLR subtypes.

The crystal structure of IRAK4 kinase domain revealed that the structure is composed of two lobes: N-terminal lobe consists of an antiparallel β-strands and an α-helix (helix αC); whereas, C-terminal lobe largely made of α-helices. The N lobe contain the glycine-rich loop (GXGXϕG) created by the first two β-strands. The activation segment comprising 329–358 residues are located in the C lobe that hold catalytic residues of Asp-Phe-Gly (DFG) motif in between 329–331 and the activation loop wherein autophosphorylation mainly take place at T342, T345 and S346. Mutation at T342, T345 and S346 residue results in significant impairment of IRAK4 kinase activity [56]. However, due to the structural complexity of IRAK4 owing for the presence of a unique tyrosine gatekeeper residue at Y262 which forms a hydrogen bond with E233 resulting inaccessibility of hydrophobic pocket of the ATP-binding site [56], it has been challenging to develop the selective IRAK-4 inhibitor. Thus, molecules that could target the tyrosine gatekeeper residue of IRAK4 will be necessary to get access to the ATP-binding site of IRAK4 for selective inhibition. This prompted us to examine the specific residues in IRAK4 and the groups in vanillin that could participate in their interactions and modulate the IRAK4 kinase activity. The in-silico docking study displayed Tyr262, Val263, and Asp329 residues of IRAK4 mainly involved in its interaction with vanillin, and mutations in these sites, particularly Tyr262 and Asp329, strikingly impaired IRAK4 kinase function. Moreover, modifications in vanillin structure at 3'O-CH3 and 4'OH groups to 3' butoxy and 4'(allyloxy) groups render their inability to inhibit IRAK4 kinase activity. Despite these encouraging observations, there are still a few queries that persist such as bioavailability and stability of vanillin in the in-vivo condition and therefore future studies will hold promise in this direction. However, vanillin being a major constituent of vanilla bean and widely used in food and beverages, it has potential for therapeutic applications against different immune-related diseases including cancers. In summary, our experimental data provide evidence that vanillin specifically interacted with the IRAK4 inhibiting MyD88-IRAK4-IRAK1-TRAF6 assembly and IRAK4 kinase activity; therefore, vanillin has immense value as a novel and potent IRAK4 inhibitor that could be poised in the vanguard against various inflammatory diseases.

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