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Title Page

Non-CpG Containing Antisense 2′ MOE Oligonucleotides Activate a Proinflammatory Response Independent of TLR9 or MyD88

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Running Title Page

Running Title: TLR-Independent Inflammation by non-CpG Oligonucleotides.

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Abbreviations: APC, antigen presenting cell; ASO, Antisense Oligonucleotide; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal regulated kinases; GM-CSF, granulocyte-macrophage-colony stimulating factor; IFN, interferon; IL, interleukin; LPS, lippoploysaccharide; MAPK, mitogen activated protein kinase; MCP, monocyte chemoattractant protein; MIP, migration inhibitory protein; MOE, methoxyethyl; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa binding found in B cells; PAMP, pathogen associated molecular pattern; RANTES, regulated upon activation, normal T-cell expressed, and presumably secreted; RNA, ribonucleic acid; RT-PCR, reverse transcriptase- polymerase chain reaction; TLR, Toll like receptor.

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Abstract

Oligonucleotides with a “CpG” motif trigger a proinflammatory response through activation of Toll-like receptor 9 (TLR9) and are being studied to exploit these properties for use as adjuvants and cancer therapies. However, oligonucleotides intended for antisense applications (ASOs) are designed to minimize proinflammatory responses by avoiding CpG motifs and using chemical modifications (i.e., 2′-methoxyethyl sugars and 5-methyl cytosine residues). Nonetheless, modified ASOs are capable of eliciting a proinflammatory response at high doses, albeit mild compared to CpG oligos. To determine if this phenomena is TLR-mediated, wild type, TLR9 knockout and MyD88 knockout mice were treated with a PS ODN CpG optimal oligo (ISIS 12449), and a representative non-CpG 2’ MOE oligonucleotide (ISIS 116847). The non-CpG oligonucleotide, had a lower proinflammatory potency relative to ISIS 12449, requiring a >10-fold higher dose in wild-type animals to trigger a proinflammatory response. Furthermore, the inflammatory response to ISIS 12449 at low doses was TLR9 and MyD88-dependent while, non-CpG oligonucleotides retained the ability to activate a proinflammatory response in the knockout animals. Animals treated with the non-CpG oligonucleotide exhibited an increased spleen weight, elevated cytokine levels, increased immune cell infiltrates in liver and an increased level of mRNA for cell surface markers typical of monocyte/macrophage type cells. Bone marrow-derived cells from wild-type and knockout animals treated with non-CpG oligonucleotide responded similarly with the production of MIP-2 and the activation of ERK 1/2. These data implicate a TLR independent mechanism of activation for non-CpG 2’MOE oligonucleotides.

Introduction

Antisense oligonucleotides (ASOs) are short, 20 to 22 nucleotide, sequences designed to hybridize with a target mRNA and inhibit the expression of the target gene (Crooke, 2004). ASOs can be administered *in vivo* and can decrease both the target mRNA and protein levels in a sequence dependent manner (Cowsert, 1997; Yacyshyn et al., 1999). However, for a number of ASO using phosphorothioate back-boned oligonucleotides, a relatively strong proinflammatory effect can be elicited in addition to their antisense effect (Molne et al., 2003). This proinflammatory effect was characterized by increased circulating cytokines and chemokines, the presence of immune cell infiltrates in target organs such as the liver, skin, and lung, and a pronounced increase in spleen weight (Vollmer et al., 2004b). The proinflammatory response induced by ASOs was shown to represent an innate immune response involving the direct activation of a monocytic/macrophage cell type similar to bacterial DNA (Younis et al., submitted).

Bacterial DNA contains a much higher percentage of the unmethylated dinucleotide motif, CpG (Krieg, 2002). Short pieces of bacterial DNA containing the mouse optimal motif "GA**CG**TT" were shown to activate cells of the innate immune system *in vitro* and *in vivo* (Ashkar and Rosenthal, 2002). The activation of the innate immune system observed in response to CpG DNA was very similar to the response observed for LPS, another bacterial component. Cells of the innate immune system detect the presence of pathogens through the recognition of Pathogen Associated Molecular Patterns, PAMPs (Elward and Gasque, 2003). A large proportion of these PAMPs are recognized by a specific subset of receptors present on some cells, the Toll- Like Receptors (TLRs) (Netea et al., 2004). TLRs represent a family of Type I transmembrane receptors present on multiple cell types (O'Neill, 2002). Studies involving knockout animals and cell types not expressing specific TLRs, proved that the recognition of CpG DNA was dependent on TLR9 (Hemmi et al., 2000). The

activation of TLR9 by unmethylated synthetic or bacterial CpG oligonucleotides has been shown to activate a signal transduction pathway involving the adaptor protein MyD88 and resulting in the activation of MAPK family members and the NF-κB pathway (Akira and Hemmi, 2003). Stimulation leads to the increased production of multiple cytokines (in particular interferons, interleukin-6 and IL-12), TNF-α, chemokines, the increased expression of co-stimulatory molecules and antigen presentation by antigen presenting cells (APCs) (Rothenfusser et al., 2002). Once activated, innate immune cells then activate adaptive immunity and potentiate a Th1 type immune response (Ashkar and Rosenthal, 2002). Oligonucleotides exploiting this process are being developed for use as adjuvants, in cancer therapy, asthma and autoimmune disease treatment (Krieg, 2004; Vollmer et al., 2004a).

Recently, research has also shown that oligonucleotides devoid of CpG motifs are also capable of eliciting a proinflammatory response *in vivo* and *in vitro*, but the role of TLR9 in this process is still unclear (Vollmer et al., 2004c). A recent study by Trevani, et al. suggested that both single-stranded and double-stranded DNA can activate human neutrophils to produce IL-8 and shed L-selectin regardless of CpG presence (Trevani et al., 2003). This data suggests that there is either another cofactor(s) or receptor involved in the recognition of non-CpG containing DNA sequences.

Although oligonucleotides intended for antisense applications are designed to minimize the proinflammatory effects of ASO administration, they do not entirely eliminate them (Henry et al., 2000). In this study we chose to characterize the proinflammatory effect initiated by a non-CpG containing 2′ -O-methoxyethyl (MOE) modified oligonucleotide. To determine if the induction of inflammatory changes by 2′- MOE oligonucleotide was TLR dependent we utilized mice deficient in the expression of TLR9 and MyD88. Administration of ASOs subcutaneously caused an increase in spleen and/or liver weight, increased mRNA for multiple proinflammatory genes, increased tissue

levels of several chemokines/cytokines and led to increased infiltrates in liver. To determine if the proinflammatory effects of these ASOs could be observed in cells isolated from these animals, we differentiated bone marrow from these animals using GM-CSF and stimulated the cells *in vitro* with oligonucleotides. Treatment of differentiated bone marrow indicated that cells devoid of either TLR9 or MyD88 retained the ability to activate in response to non-CpG oligonucleotide. Activation of these cells was associated with increased phosphorylation of ERK 1/2 and increased production of several proinflammatory genes at the mRNA and protein level as measured by RT-PCR and ELISA. These data suggest that, *in vivo* and *in vitro*, there exists a distinct alternate pathway for the recognition and inflammatory response to short non-CpG containing oligonucleotides.

Methods

Reagents. Antibodies for phospho and endogenous p38, JNK and ERK ½ were purchased from Cell Signaling Technology (Beverly, MA). Production of MIP-2 was measured by ELISA kits purchased from R&D Systems (Minneapolis, MN). RT-PCR primer/probe sets were purchased from IDT (Coralville, IA). Bone marrow differentiation was performed using GM-CSF from Stemcell Technologies (Seattle, WA) and R&D systems respectively. Bafilomycin A was purchased from Calbiochem (San Diego, CA). All antisense oligonucleotides were produced by Isis Pharmaceuticals.

Animals. C57/BL6 background wild type animals were purchased from Charles River Labs (San Diego, CA). Breeding pairs for TLR-9 KO and MyD88 KO mice in C57/BL6 background were graciously donated by Dr. H. Hemmi (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University,) and bred at Charles River Laboratories. All animals were maintained in a sterile vivarium environment and kept on a 12 hr light/dark cycle. Food and water was available *ad libitum*. Treatment with PBS or filter sterilized ASO was done by subcutaneous injection 2 times per week for 3 weeks at either 4 mg/kg or 50 mg/kg using ISIS 12449 (CpG optimal phosphorothioate oligonucleotide backbone, ACCGATAACGTTGCCGGTGACG), ISIS 116847 (non- CpG, 2′-O-methoxyethyl (5 terminal residues in both 5’ and 3’ termini, bolded) phosphorothioate backbone modified oligonucleotide, **CTGCT**AGCCTCTGGA**TTTGA**). Following treatment duration animals were sacrificed, organs weighed and tissue samples for histology, RNA and protein for analysis were collected. Blood was collected by cardiac puncture at the time of sacrifice and processed to plasma for cytokine/chemokines evaluation.

Differentiation of Bone Marrow. Bone Marrow was isolated from the femurs and tibia of wild type, TLR-9 KO and MyD88 KO animals, depleted of erythrocytes and cultured in Stemspan media (StemCell Technology) plus 5% heat inactivated fetal bovine serum (FBS) (Invitrogen), 100U/ml

penicillin, 100 µg/ml streptomycin, anti-mycotic and recombinant GM-CSF (10ng/ml) was added to the culture to stimulate differentiation into immature dendritic cells. One half of the media was removed every 2 days and replaced with fresh media for 8 days. On day 8, cells were treated with 100 µg/ml of CpG optimal (ISIS 12449) or 100 µg/ml of non-CpG ASO (ISIS 116847) diluted in fresh media and added directly to cells for 1-4hr for MAPK activation and for 4 hr for cytokine/chemokine production.

RNA Isolation and RT-PCR Analysis. Portions of liver from treated animals were stored in RNA later (Ambion, Austin, TX) at time of sacrifice. RNA from cell lines or tissue was isolated using Qiagen RNA Easy kit (Qiagen, Valencia CA). RT-PCR was performed using custom RT-PCR kits from Invitrogen on an ABVI Prism using the following conditions: 48◦C for 30 min RT step, then 95◦ C for 55 sec, 60◦C◦ for 1 ½ min and 70◦C for 60 sec. The primer probe sets used are detailed in Table 1.

Western Blot Analysis. Cells were counted and plated in 12-Well dishes at 1 x 10 6 cells /ml. Cells were treated with ASO diluted in fresh media and incubated for 1 hr to activate ERK ½. Cells were then lysed in 1 X Cell Lysis buffer (Cell Signaling Technology). Protein concentration was normalized using Bradford Assay (Bradford, 1976). Normalized protein was placed in equal volume of 2X Laemli's Sample buffer plus 5% β-mercaptoethanol, boiled 5 min and then separated by 10% SD-PAGE gel electrophoresis. The separated proteins were transferred to Immobilon PVDF membrane (Invitrogen) and then Western blotted as per manufacturer's instructions.

Cytokine Analysis. Tissue lysate or cell culture media was collected following treatment (3 wk dose for plasma and 4 hr treatment for cell culture). Then 50 µl of sample was used to analyze chemokine production using R&D System ELISA kits. Each sample was loaded in duplicate and the experiment performed in triplicate. The sample was incubated for 2 hr at room temperature in 96-well plates

coated with appropriate antibody. The plates were then washed 4 times with wash buffer provided and incubated for another 2 hr at RT with HRP-conjugated secondary antibody. Following this incubation the plates were washed 4 times again and incubated with colorimetric reagent for 30 min and plates were read in 96 well plate reader at 450 nM using 550 nM as background correction. Liver tissue lysates were created by homogenizing 100 mg of tissue in 1 ml of PBS with added protease inhibitors (Calbiochem). The homogenized tissue was subjected to 2 freeze thaw cycles and the lysates were cleared by centrifugation at 15,000 rpm for 10 min at 4◦C. The supernatant was then sent to Endogen for analysis on their SearchLight Multiplex cytokine assay system.

Statistical Analysis. All data were analyzed using ANOVA statistical analysis and errors represent the standard deviation of all experiments. P-values less than 0.05 were considered significant.

Results

Administration of CpG optimal and non-CpG oligonucleotides causes increased spleen weight and inflammatory cell infiltrates in the liver. The prevailing hypothesis to explain the proinflammatory effect of oligonucleotide administration suggests that TLR9 expressed on cells of the innate immune system recognizes oligonucleotides following internalization by an undetermined mechanism (Akira and Hemmi, 2003). To determine if non-CpG containing oligonucleotides could initiate a proinflammatory response *in vivo*, we administered a 2′-MOE modified oligonucleotide subcutaneously to wild-type, TLR9 KO and MyD88 KO mice. Following 3 weeks of subcutaneous administration, CpG optimal oligonucleotide ISIS 12449 caused a 4 to 5-fold increase in spleen weight in wild-type mice (Figure 1a). The increase in organ weight was correlated with a large increase in lymphohistiocytic cell infiltrates in the liver and lymphoid hyperplasia in spleen (Figure 1b, data not shown). This effect was observed at relatively low doses of ISIS 12449 (4 mg/kg twice weekly) in wild-type animals. By comparison, treatment of wild-type mice with non-CpG oligonucleotide ISIS 116847 at 50 mg/kg produced a small but significant increase in spleen weight (1.2 to 1.3-fold) and a mild increase in detectable immune cell infiltration in liver. Thus, while non-CpG oligos are markedly less potent than CpG oligos, they elicit a similar spectrum of histological effects at high doses in mice. As expected, the splenomegaly observed in wild-type animals (5-fold increase) in response to low dose of CpG optimal oligonucleotide ISIS 12449 was almost completely abolished in TLR9 KO animals (1.8-fold increase) (Figure 1a). There were also fewer cell infiltrates in liver tissue of TLR9 KO mice compared to wild-type mice receiving the same treatment. However, TLR9 KO mice responded nearly identically to wild-type animals treated with 50 mg/kg ISIS 116847. TLR-9 KO mice treated with ISIS 116847 exhibited a similar 1.2 to 1.3-fold increase in spleen weight and an accompanying increase in cell infiltrates in

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liver when compared to wild-type animals (Figure 1a). Finally, MyD88 KO mice were also examined and it was determined that neither CpG optimal or non-CpG oligonucleotides produced a significant increase in spleen weight suggesting the possible role of this adaptor in signaling of non-CpG oligonucleotides. However, non-CpG oligonucleotides continued to produce an increase in immune cell infiltrates in liver (Figure 1b, lower panel). These data indicate that the proinflammatory effects of non-CpG oligonucleotides, in mice, are independent of TLR9 signaling.

Increase in expression of proinflammatory cell marker expression in response to non-CpG ASO is relatively TLR9 independent. Next, the mRNA levels of multiple proinflammatory cell surface markers in the livers of treated animals were evaluated to further characterize the cellular infiltrate. It has been well documented that the administration of CpG containing oligonucleotides causes a significant increase in the mRNA levels of multiple co-stimulatory molecules and cell surface markers of inflammatory cells (Anders et al., 2004). Since a significant increase in the presence of immune cell infiltrates in liver was observed, the expression levels of several cell surface markers indicative of monocytic cells were evaluated. In wild-type animals, 4 mg/kg ISIS 12449 caused a substantial increase in the levels of multiple genes in the liver such as CD11b, CD11c, CD86, and CD68 mRNA (Figure 2). As expected, treatment of TLR9 KO and MyD88 KO mice with 4 mg/kg ISIS 12449 resulted in a near complete absence of proinflammatory response (Figure 2). These data strengthen previous reports that TLR9 and MyD88 are crucial to the proinflammatory response initiated by CpG containing oligonucleotides. ISIS 116847 (50 mg/kg) was also capable of increasing the mRNA levels of multiple genes in the livers of treated animals. As shown in Figure 2, treatment with 50 mg/kg ISIS 116847 resulted in an increased detection of CD11b, CD11c, CD68 and a small increase in CD86 detected in the livers of treated animals, although at a lower level than CpG optimal oligonucleotide. Contrary to the results obtained

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with ISIS 12449, TLR9 KO mice responded with increases in mRNA for all markers examined, although the absolute levels are slightly lower for CD11b and CD68 , following treatment with 50 mg/kg ISIS 116847. Additionally, MyD88 KO animals also exhibited an increase in multiple mRNA levels, particularly CD11c, and a smaller increase in CD86, CD11b and CD68 expression than wild-type or TLR9 KO. This response could be partially explained by the importance of MyD88 to other signal transduction pathways, specifically IL-1, IL-18, or other TLR receptors. These data indicate that non-CpG oligonucleotide, ISIS 116847, can induce an activation of the immune system regardless of the presence of efficient TLR signal transduction.

Cytokine protein levels are nearly identical in wild-type and knockout animals treated with non-CpG oligonucleotide ISIS 116847. Although RT-PCR results indicated that ISIS 116847 was capable of inducing a proinflammatory response, it was necessary to more completely characterize the cytokine profile of treated animals and to compare the profile of wild-type animals and with knockout models. In this experiment, cytokine profiles were obtained from liver tissue samples of treated animals, which closely mirrored the expression of cytokines in plasma of treated mice (data not shown). As indicated in Figure 3a, treatment of wild-type C57/Bl6 mice with 50 mg/kg ISIS 116847 for 3 weeks caused a substantial increase in several cytokines and chemokines in liver tissue. We have chosen a multiplex approach to analyze the cytokine/chemokine expression using a panel of cytokines, several of which are hallmarks of CpG-induced proinflammatory responses (IL-6 IFNγ, IL-10) and a sampling of chemokines involved in monocyte migration and activation. This analysis indicated that the proinflammatory caused by ISIS 116847 generally resulted in a predominantly chemokine driven response which is significantly different from CpG optimal responses previously published. More specifically, ISIS 116847 increased IL-1α (2-fold), IL-18 (5-fold), RANTES (7-8-fold), JE/MCP-1 (4-fold), MCP5 (3-fold), MIP-1α (10-fold), and MIP-

2 (2-fold). Of note was the absence of an increase in IL-6, which has been the hallmark of CpG oligonucleotide induced proinflammatory effects. Examination of the cytokine profile in TLR9 KO mice showed a nearly identical pattern of cytokine/chemokine expression following 3 weeks of treatment, with the addition of an increase in IFNγ and a loss of the 2-fold increase in MIP-2 (Figure 3b). Moreover, MyD88 knockout animals exhibited an increase in several of the same cytokine/chemokines (IL-1 α, IL-18, RANTES, MCP-1, and MIP-1 α, Figure 3c). The slight differences in MyD88 KO mice may be explained by the importance of this adaptor to multiple other signal transduction pathways, including alternate TLRs such as TLR3 and 7. The preceding experiment provided further evidence that the non-CpG oligonucleotide ISIS 116847 can induce an inflammatory response in the absence of TLR9 and MyD88, suggesting an alternate mechanism of activation.

Differentiated bone marrow from wild-type, TLR9 KO and MyD88 KO mice is activated in response to treatment with non-CpG oligonucleotide ISIS 116847. *In vivo* data from the previous experiments indicated that non-CpG containing oligonucleotides could stimulate a proinflammatory response, but it was not clear whether this was through direct or indirect stimulation. It has been shown that CpG oligonucleotides directly stimulate macrophage/dendritic cells of the innate immune system. To examine the direct effects of non-CpG oligonucleotide administration on cells of the innate immune system, we isolated bone marrow from wild-type and knockout animals and differentiated the cells *in vitro*. These cells were differentiated into immature dendritic cells/granulocytes by culturing in the presence of the cytokine GM-CSF. Following 4 hr of treatment with either CpG containing ISIS 12449 or non-CpG containing oligonucleotide, ISIS 116847, the conditioned media was isolated and analyzed for expression of the chemokine MIP-2. Treatment of wild-type bone marrow-derived cells

with ISIS 12449 resulted in a significant (7-fold) increase in MIP-2 production when compared to untreated cells (Figure 4b). However, when bone marrow-derived cells from TLR9 knockout mice were treated with 100 µg/ml of ISIS 12449, the resulting MIP-2 production was dramatically lower (< 2-fold). This indicates that a significant portion of CpG PS ODN induced MIP-2 production is driven through the TLR9 pathway, but does suggest that at high doses at least part of the response is due to a TLR9 independent pathway. The data shown in Figure 4b indicate that treatment with non-CpG oligo results in a significant (2 to 3-fold) increase in MIP-2 production in wild-type bone marrow. Treatment of knockout bone marrow derived cells with 100 µg/ml of ISIS 116847 similarly resulted in a 2 to 3-fold increase in MIP-2 production. These data indicate that non-CpG oligonucleotides can directly stimulate cells of the innate immune system and induce the activation of a proinflammatory response independent of TLR9 or MyD88 proteins and suggests that the response observed *in vivo* is indicative of immune activation and not a secondary response to cellular damage of the surrounding tissue.

Treatment of cells of the innate immune system with CpG containing oligonucleotides has been shown to increase the activation of several members of the MAPK kinase family. In Figure 4a, we examined the activation of ERK 1/2 in differentiated bone-marrow derived cells from wild type and knockout animals. Cells from wild-type mice displayed an increase in ERK 1/2 activation when treated with ISIS 12449 (3 µg/ml) or ISIS 116847 (100 µg/ml) for 1hr, as evidenced by an increase in the phosphorylated form of the enzymes detected on a Western blot. As expected, cells derived from TLR9 KO mice displayed a complete lack of response to ISIS 12449, but maintained full activation of ERK 1/2 in response to ISIS 116847. Additionally, cells derived from MyD88 KO animals responded to both ISIS 12449 and ISIS 116847 with an increase in the activation of ERK 1/2; however the increase in ERK1/2 activation appeared to be diminished when compared to bone

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marrow derived cells from wild-type animals. Isolated cells from both TLR9 and MyD88 KO models maintain a similar response when compared to wild-type cells, indicating that TLR9 and MyD88 are not crucial for the recognition and activation of ERK 1/2 in cells by non-CpG oligonucleotides.

Discussion

It is well established that the Toll-like receptor family is critical to recognizing and eliciting a response to foreign pathogens and even some endogenous "danger" signals (Takeda and Akira, 2001; Netea et al., 2004). The recognition of oligonucleotides, particularly those containing a CpG motif, occurs through the TLR9 receptor and signal transduction through the common TLR adaptor protein, MyD88 (Krieg, 2002; Mutwiri et al., 2003). CpG containing oligonucleotides have been shown to be potent immune stimulators causing the release of multiple cytokines (IFNγ, IL-12, IL-6, etc.), and strongly inducing antigen presentation (Krieg, 2002; Jiang and Koganty, 2003; Schwarz et al., 2003). Non-CpG containing PS ODN could also stimulate an immune response *in vivo* and isolated cell types (Vollmer et al., 2004c; Zhao et al., 2004). The role of TLR9, although not conclusively proven, has been suggested by several recent studies (Roberts et al., 2005). This paper provides evidence that the proinflammatory effects induced by non-CpG 2' MOE oligonucleotides are TLR-independent. Both TLR9 KO and MyD88 KO mice experienced a proinflammatory response to the administration of non-CpG containing oligonucleotides. Wild-type and TLR9 KO mice treated with non-CpG oligonucleotides show a similar increase in spleen weight and the presence of immune cell infiltrates in organs such as the liver. In contrast, the proinflammatory effects of a low dose CpG oligonucleotide were largely dependent on TLR9 and MyD88. The presence of a proinflammatory response to high dose non-CpG 2'MOE modified oligos in mice devoid of TLR9 and MyD88 indicate a separate, lower affinity, site of recognition for modified oligonucleotides. It must be acknowledged the non-CpG oligonucleotides studied here contain 2'MOE modified backbone which may affect the proinflammatory potential of this molecule and perhaps its receptor recognition. However, data from our laboratory suggests that the most critical aspect in controlling potency is the avoidance of CpG motifs and methylation of cytosine

residues. We have also observed that the response to high doses of either CpG containing and non-CpG PS ODN are at least partially independent of TLR9 (data not shown).

Wild type and knockout mouse models showed a similar pattern of cytokine/chemokine expression in liver tissue following treatment with non-CpG containing oligos suggesting the activation of an alternate pathway. Cytokine expression patterns were characterized by an increase in IL-1α, IL-18, RANTES, MCP-1, MCP-5, and MIP-1 α. The pattern of cytokine/chemokines observed in response to ISIS 116847 were nearly identical in pattern regardless of TLR9 or MyD88 presence, but the magnitude of a few chemokine/cytokines such as MCP5 and IL-18 appeared to differ in MyD88 KO animals. This can be explained, at least in part, by the importance of MyD88 to multiple signal transduction pathways, such as IL-1, IL-18 and alternate TLRs which may augment or have additional importance in the inflammatory response to non-CpG oligonucleotides. The levels of multiple cell surface markers in liver indicative of monocytic cells increased in knockout and wild-type mice.

Primary bone marrow was treated with GM-CSF to drive the formation of immature dendritic cells and granulocytes thereby providing a model of similar cell types to those present in the tissue itself. In bone marrow-derived cells from knockout and wild type mice, direct administration of non-CpG oligos caused the production of MIP-2 and activation of ERK 1/2. In contrast, the cellular response to an optimal dose of CpG containing oligonucleotide was significantly diminished in TLR9 knockout cells, consistent with previously published data (Yi et al., 2002). Interestingly, the production of MIP-2 was still observed, at much lower levels (approximately 2-fold induction), from TLR9 and MyD88 bone-marrow derived cells treated with high concentrations (100 μg/ml of CpG oligo and non-CpG containing PS-ODNs (data not shown). This may suggest that the TLR9 independent mechanism is not unique to 2' MOE modified oligonucleotides. These data suggest that

the proinflammatory effect caused by non-CpG oligonucleotides is through a direct or primary effect on immune cells and not through a secondary response to non-immune cell damage.

The relative potency of proinflammatory change and the spectrum of cytokine/chemokines production induced by non-CpG oligonucleotides are quite divergent from that known to occur with CpG optimal oligos. Non-CpG oligo required ≥10-fold higher than the dose of CpG optimal oligo to increase spleen weight and the magnitude of spleen weight increase was markedly greater for CpG oligonucleotide. Splenomegaly induced by non-CpG oligonucleotide was relatively subtle (≤2 fold increase). This pattern of higher concentrations and lower magnitude of response was also observed in the activation of isolated bone marrow-derived cells with non-CpG optimal oligos. While both oligonucleotides stimulated ERK 1/2 activation, the amount of chemokines produced was markedly greater for CpG optimal oligonucleotides in wild-type cells. The pattern of cytokine/chemokine production previously reported in response to CpG oligonucleotides favors a Th1 pattern typified by IFNγ, IL-6, and IL12. By comparison, high dose non-CpG oligonucleotide responses are dominated by chemokines, and a notable absence of IL-6 and IFNγ induction. This difference in cytokine production is consistent with a relatively mild increase in spleen weight for non-CpG oligonucleotides. The absence of an increase in spleen weight in MyD88 KO mice indicates the possibility that alternate TLRs may participate in the immune response to non-CpG containing oligonucleotides. These relative differences suggest a different mechanism of action for CpG (TLR9) and non-CpG oligonucleotides.

Other TLR receptors have recently been implicated in the recognition of oligonucleotides, particularly RNA molecules (Heil et al., 2004; Kariko et al., 2004; Karikó et al., 2004). TLR3 has been hypothesized to interact with double-stranded RNA molecules and initiate a potent antiviral response (Doyle et al., 2003) and can also recognize endogenous mRNA molecules from dying cells

or produced from RNA viruses (Karikó et al., 2004). TLR7, known to recognize nucleoside analogs such as R-848, has also been shown to be activated by mRNA molecules (Akira and Hemmi, 2003). It is possible that either TLR3 or 7 may play a role in the recognition of non-CpG containing antisense oligonucleotides. Studies using TLR7 ligands have shown that MyD88 is required to elicit a detectable inflammatory response (Matsushima et al., 2004; Nishiya and DeFranco, 2004). Our data suggests that some or most of the immune response to non-CpG containing oligos is independent of MyD88, and therefore, argues against involvement of TLR7. TLR3 also uses the adaptor protein MyD88, but has been shown to signal through alternate TIR-domain containing adaptors such as TRIF, leaving open the possibility that non-CpG containing oligonucleotides are recognized by TLR3 (Takeda and Akira, 2004). However, recent unpublished evidence from our laboratory suggests that TLR3 over expressing HEK 293 cells do not respond to non- CpG oligonucleotides. This evidence does not irrefutably discount alternate TLRs in the recognition of non-CpG oligos, but provides the framework to suggest that other families of receptor molecules may play a role. It has been hypothesized that the scavenger receptors or alternate pattern recognition receptors may play an important role. This research provides several key points of evidence that illustrate a possible TLR9-independent mechanism for proinflammatory stimulation by non-CpG oligonucleotides. The activation of proinflammatory response *in vivo* is independent of TLR9 and MyD88, and isolated bone marrow-derived cells respond to non-CpG oligos in the absence of either TLR9 or MyD88. However, there did appear to be some reduction in the degree of proinflammatory response in MyD88 KO mice which is possibly consistent with recent evidence suggests that either TLR3 or 7 could play a role in the activation. Previously published work (Roberts et al., 2005) suggests that at lower concentrations or doses, TLR9 may play an important role in the response to non-CpG containing oligonucleotides, but the data presented in this paper provide evidence that a majority of

the response to non-CpG containing 2' MOE modified oligonucleotides and possibly PS ODNs may be TLR9-independent. Future experiments will be directed at the discovery of alternate receptors and the importance of backbone modifications in TLR9 independent proinflammatory responses.

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Legends for Figure

Figure 1- ***Administration of non-CpG oligonucleotides causes minor splenomegaly and lymphohistiocytic infiltrates in livers of wild-type, TLR9 KO and MyD88 KO mice.***

Animals from wild type, TLR9 KO, and MyD88 KO were treated with either CpG optimal oligonucleotide ISIS 12449 at 4mg/kg 2x per week or non-CpG containing 2' MOE oligonucleotide ISIS 116847 at 50 mg/kg 2x per week for a period of 3 weeks. Following termination of the study, spleen and liver tissue were isolated weighed and prepared for histological examination in 10% formalin. A, The chart represents the fold increase in spleen weight to body weight ratio in treated animals observed following the completion of the study. B, H and E stained liver tissue fixed in 10% Formalin/EtOH from wild type, TLR9 KO and MyD88 KO animals indicates and increased presence of monocytic infiltrates. The data represented in this figure is represented as fold increase over saline treated animals with S.D. (n=10 per group).

Figure 2- ***Treatment with non-CpG oligonucleotides results in the increased detection of cell surface markers indicative of monocytic cells in the liver independent of TLR9*****.**

RNA was isolated from the livers of animals from each treatment group (wild type, TLR 9 KO and MyD88 KO). 100 ng of whole cell RNA was subjected to quantitative RT-PCR using Invitrogen's one-step PCR kit and ABI- prism QRT-PCR device. Samples were analyzed for the expression of several cell surface markers (CD86, CD11b, CD11c and CD68) indicative of monocyte/macrophage cell types. All data are represented as fold increase over saline treated wild type animals with S.D. (n=5 per group). (* indicates $p<0.05$)

Figure 3- ***High dose non-CpG oligonucleotide induces the production of multiple cytokine/chemokines in liver in a TLR9 and MyD88 independent manner.*** Two groups of mice were injected subcutaneously 2X week for 3 weeks with either saline or ISIS 116847 at 50 mg/kg.

Following treatment course animals were sacrificed, liver tissue isolated and protein extracts were made. Protein extracts were then subjected to cytokine profiling using Endogen's Searchlight Cytokine Assay. A, Wild type C57 BL/6 mice treated with saline or ISIS 116847 liver cytokine profile was analyzed. B, TLR9 KO mice in were given saline or ISIS 116847 and liver cytokine profile compared to wild type animals. C, MyD88 KO mice were similarly treated to wild type and TLR9 KO mice and then liver cytokine profiles compared to wild type response. All experiments are represented as fold increase over saline treated wild type animals and S.D. (n=5 for all groups).

Figure 4- ***Bone Marrow Derived cells respond to direct non-CpG oligonucleotide treatment independent of TLR9 or MyD88.*** Bone marrow isolated from wild type and KO mouse models were cultured for 24 hrs prior to stimulation with either CpG containing oligonucleotide ISIS 12449 at 100 µg/ml or non-CpG oligonucleotide ISIS 116847 at 100 µg/ml for 1-4 hrs. A, Cell lysates from bone marrow cell culture were created following 1hr of treatment with either ISIS 12449 or 116847 at 100 µg/ml. Lysates were separated using SDS-PAGE and Western blotted for the presence of phosphorylated ERK 1/2. B, Bone marrow cells were treated for 4 hrs with either ISIS 12449 or 116847 at 100 µg/ml and culture medium was analyzed for the production of MIP-2 by ELISA. Western blot in Figure 4a is representative of 3 independent experiments and Figure 4b represents the average fold increase in MIP-2 production versus untreated control with S.D. (n=9 per group). (* represents $p<0.05$)

Table 1: ***RT-PCR primers used in this study.*** Table 1 indicates the forward, reverse and probe primers utilized in quantitative RT-PCR experiments detailed in this study.

Gene	Forward	Probe	Reverse
CD86	GGCCCTCCTCCTTGTGATG	TGCTCATCATTGTATGTCACAAGAAGCCG	CTGGGCCTGCTAGGCTGAT
CD68	TTGGGAACTACACGTGGGC	AACGGCTCCCAGCCTTGTGTTCAG	CGGATTTGAATTTGGGCTTG
CD11b	GCTGCATGTCCGGAGGAA	TTGGCTGGCGCAATGTCACGAG	TCTGTGGCAAACACCAGCA
CD11c	GCACCCGGAGAGGAGGAG	ACAGAGGTGCTGTCTACATATTTCATGGAGCC	GGGAGCGATGTCCTGTCTTG

Figure 1a

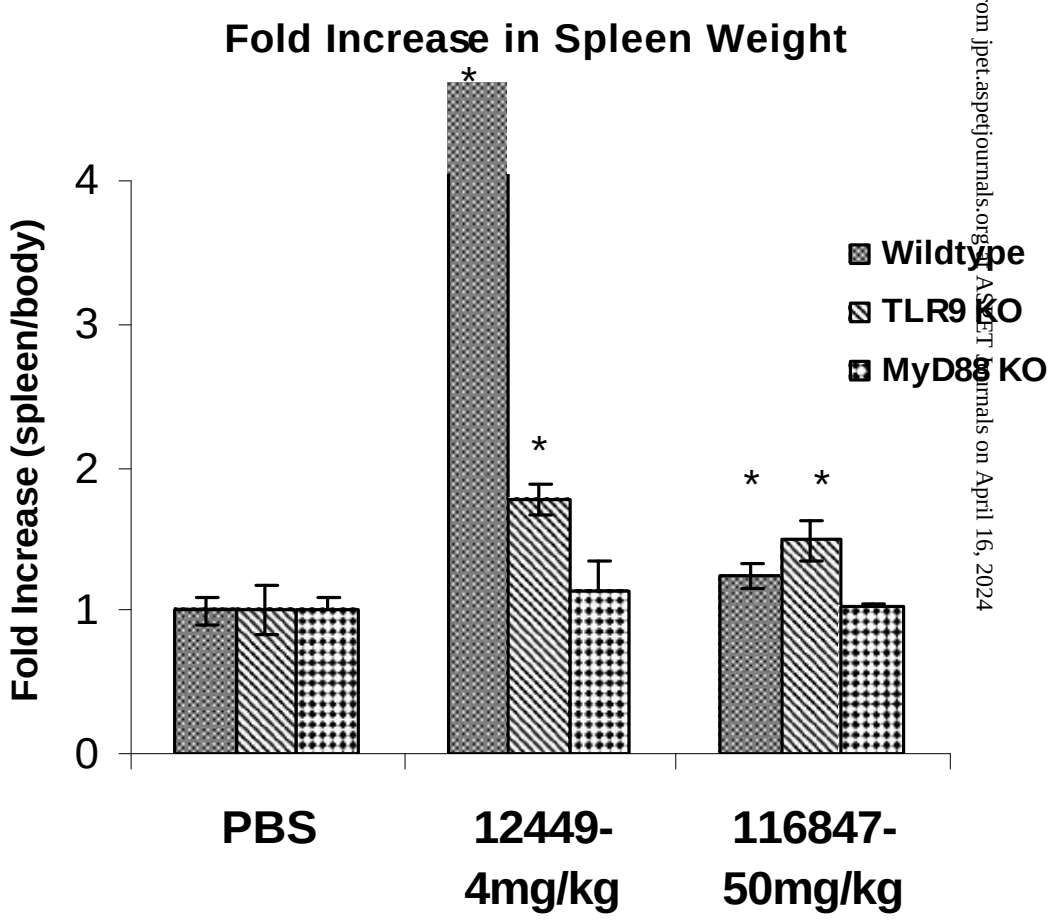


Figure 1b

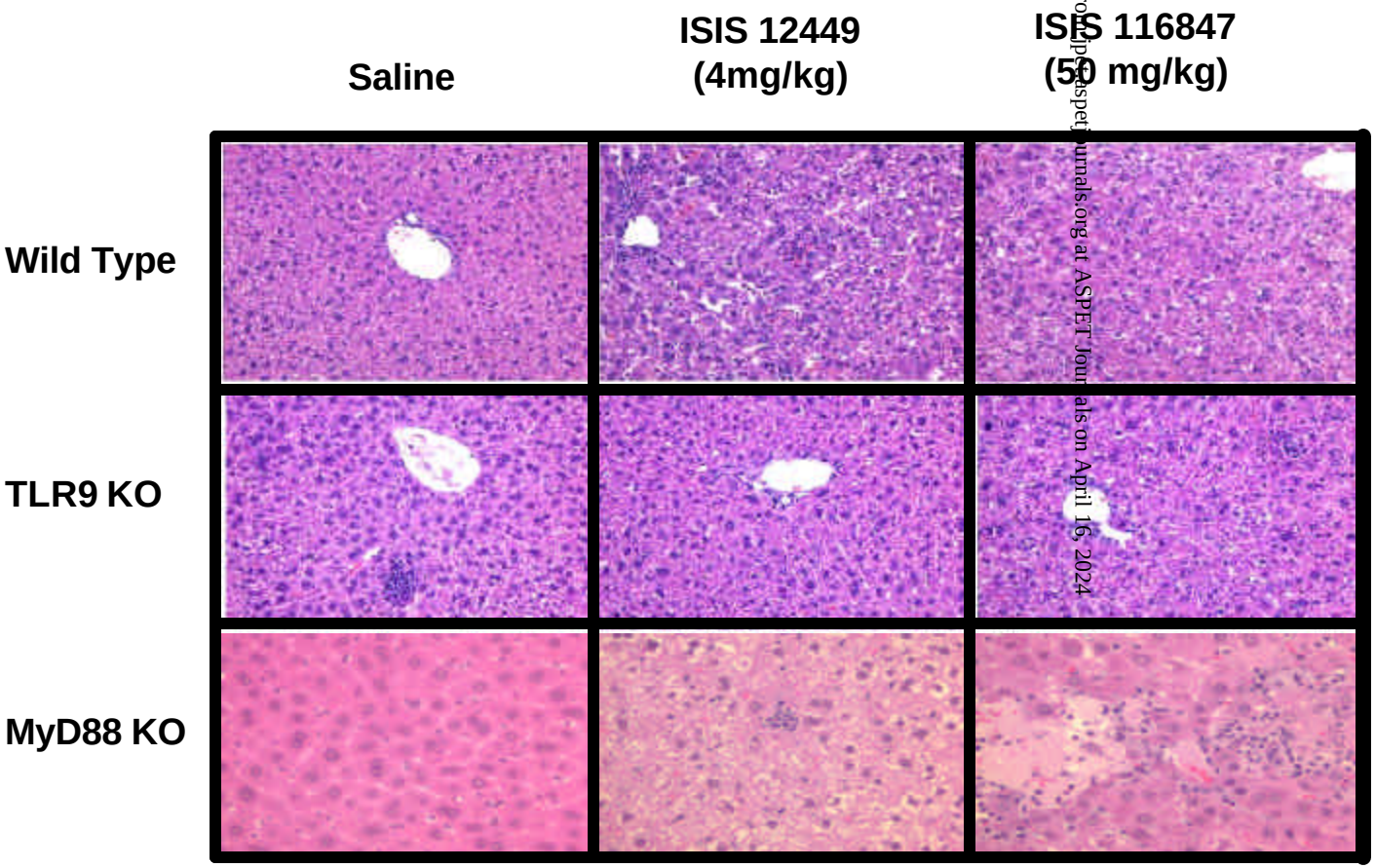
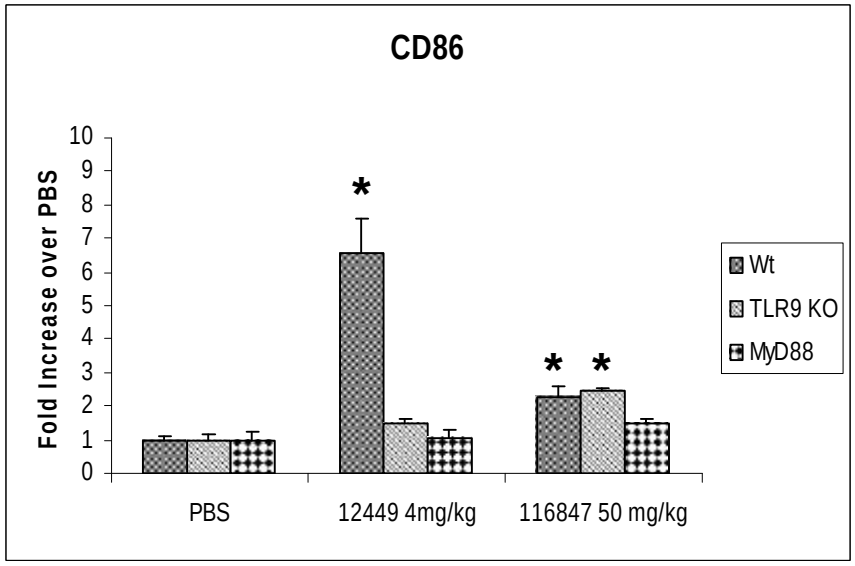
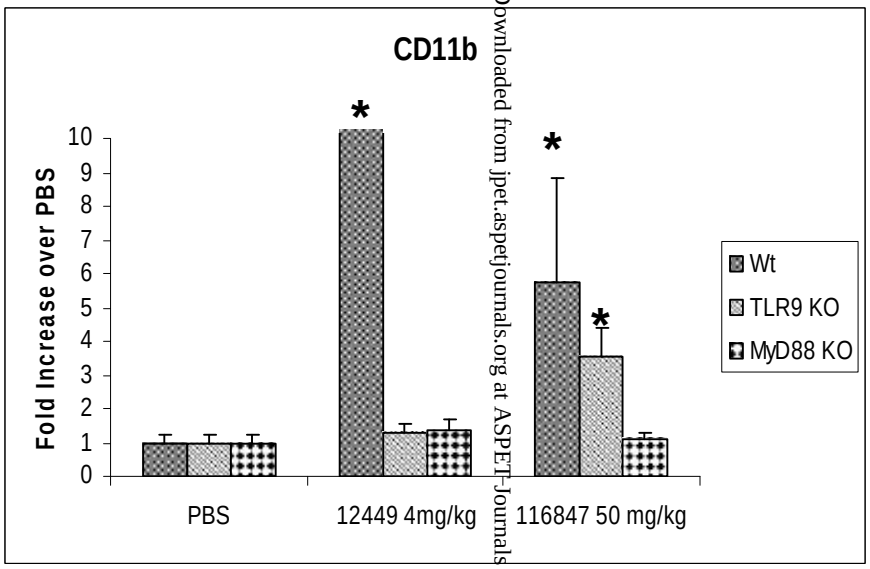
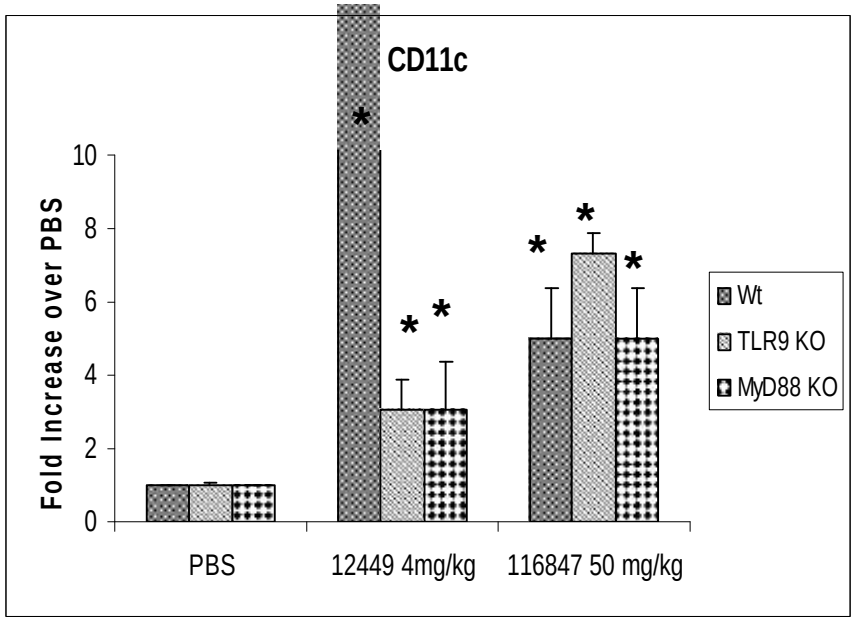


Figure 2







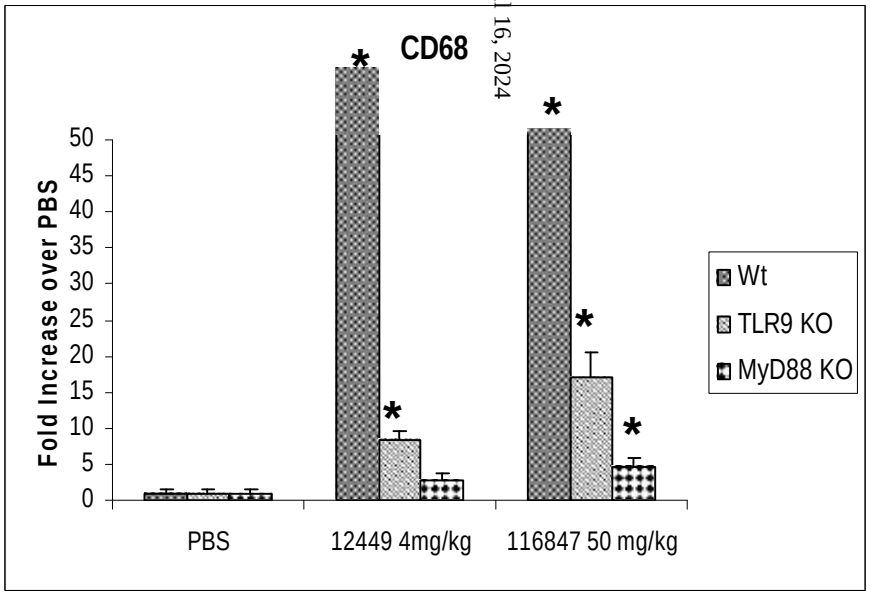


Figure 3a

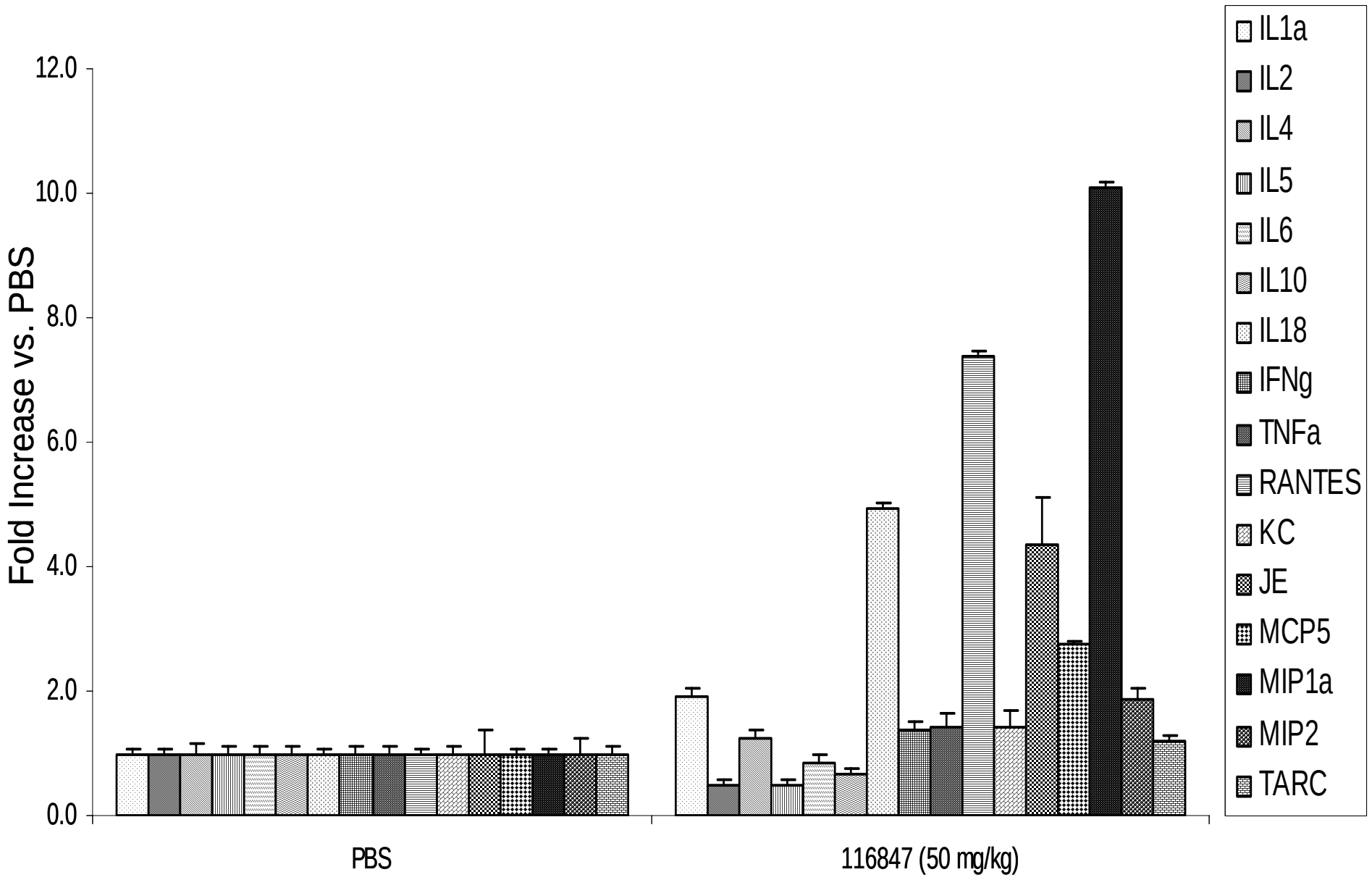


Figure 3b

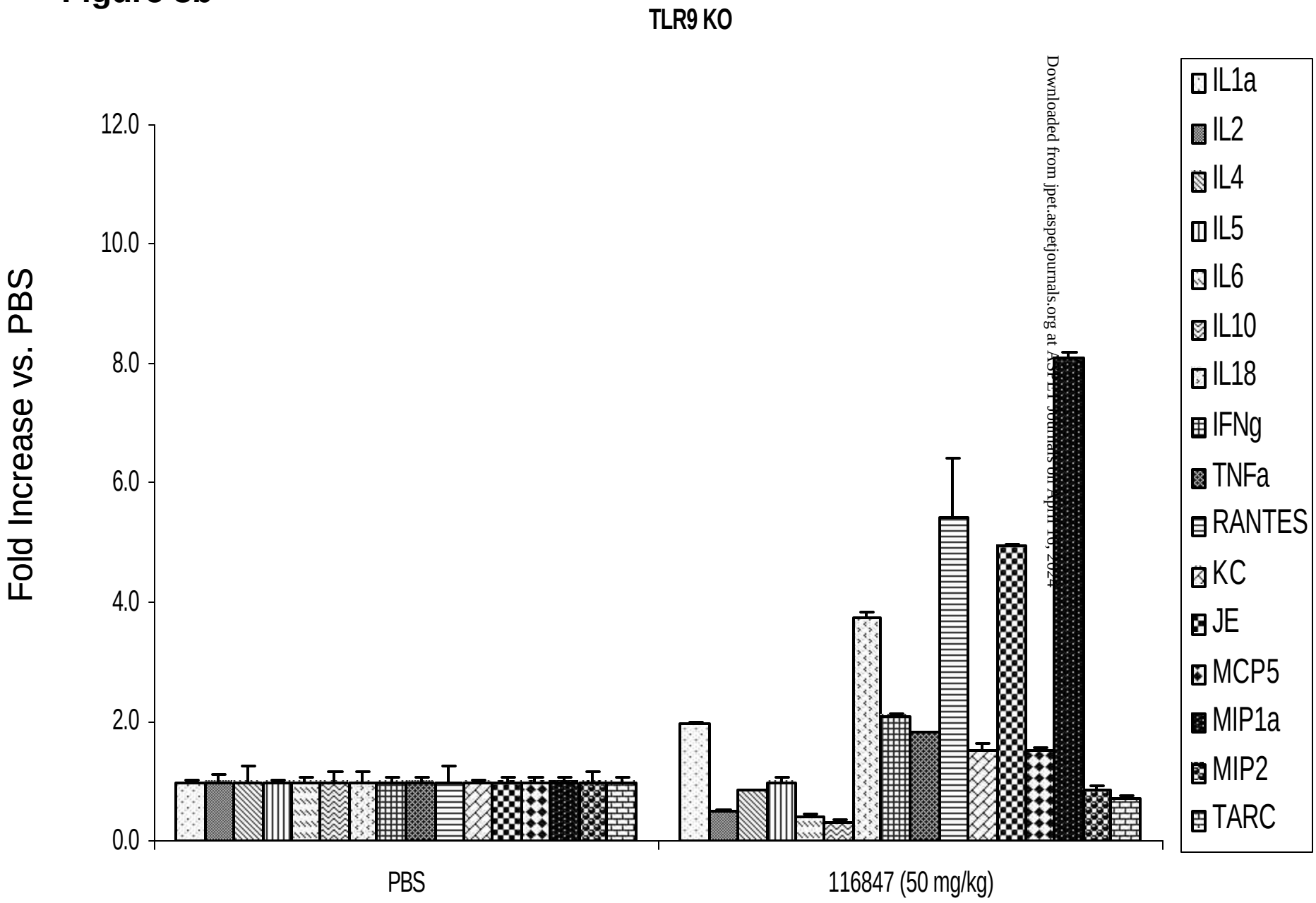


Figure 3c

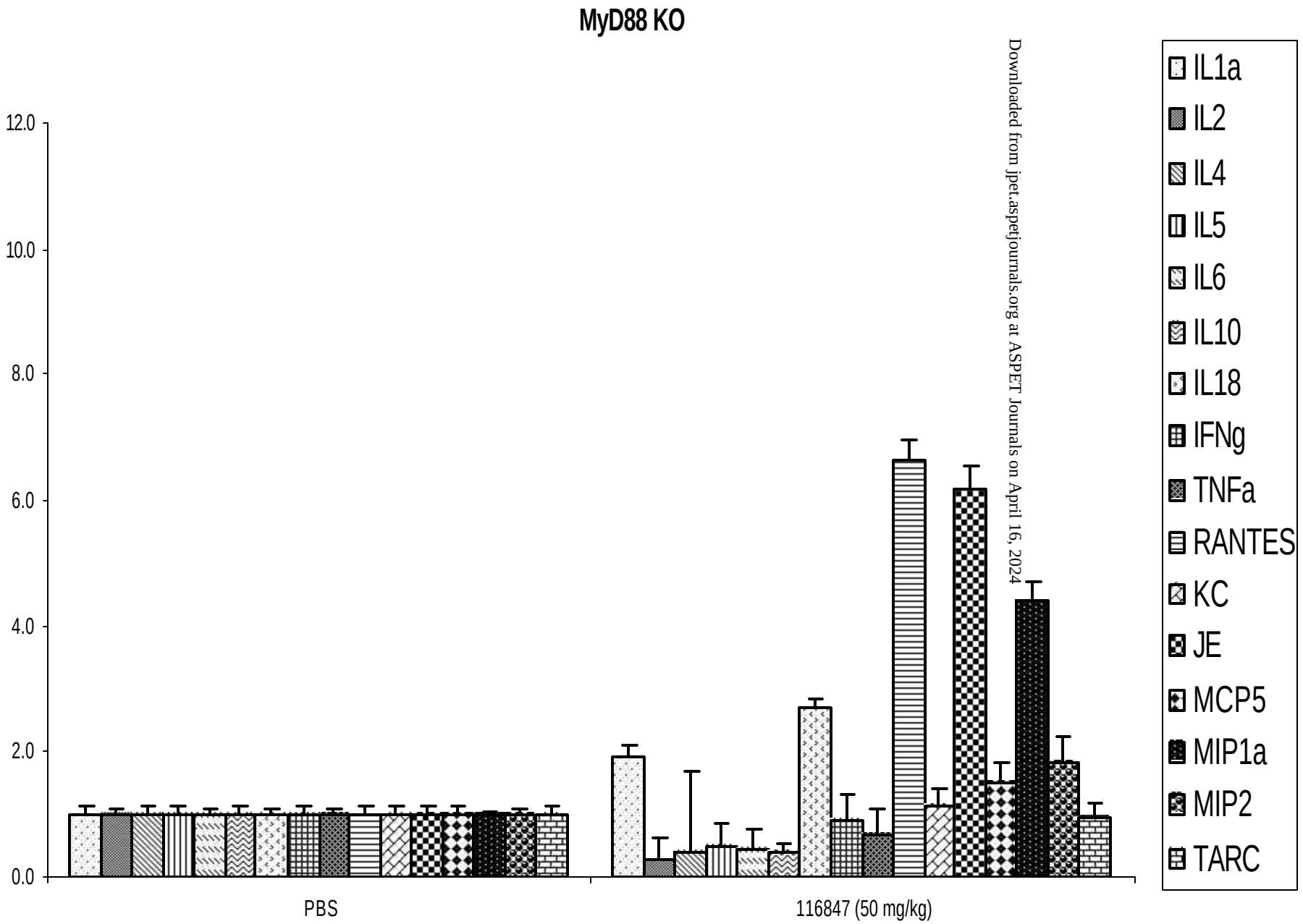
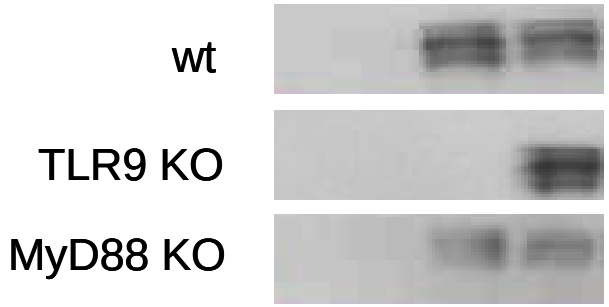


Figure 4

A



IB: α-pERK 1/2

B