Sodium Methylthiocarbamate Exerts Broad Inhibition of Cellular Signaling and Expression of Effector Molecules of Inflammation

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Sodium methylthiocarbamate (SMD) is one of the most abundantly used conventional pesticides in the United States. At dosages relevant to occupational exposure, it causes major effects on the immune system in mice, including a decreased resistance to sepsis. This lab has identified some of the mechanisms of action of this compound and some of the immunological parameters affected, but the global effects have not previously been assessed. The purpose of the present study was to conduct transcriptomic analysis of the effects of SMD on lipopolysaccharide-induced expression of mediators important in innate immunity and inflammation. The results revealed broad effects on expression of transcription factors in both branches of Toll-like receptor 4 (TLR4) signaling (MyD88 and TRIF). However, TLR3 and interferon signaling pathways were decreased to a greater extent, and assessment of the effects of SMD on polyinosinic polycytidylic acid-induced cytokine and chemokine production revealed that these responses mediated by TLR3 were indeed sensitive to the effects of SMD, with inhibition occurring at lower dosages than required to inhibit responses to other immunological stimuli tested in our previous studies. In the downstream signaling pathways of these TLRs, functional analysis also revealed that NF-κB activation was inhibited by SMD, as indicated by gene expression analysis and a reporter construct in mice. A previously unreported effect on luteinizing hormone and follicle-stimulating hormone pathways was also observed.

Key Words: pesticides; signal transduction; transcription factors; chemokines; cytokine; signaling; microarray.

Sodium methylthiocarbamate (SMD) was the third most abundantly used conventional pesticide in the United States. At dosages relevant to occupational exposure, it causes major effects on the immune system in mice, including a decreased resistance to sepsis. This lab has identified some of the mechanisms of action of this compound and some of the immunological parameters affected, but the global effects have not previously been assessed. The purpose of the present study was to conduct transcriptomic analysis of the effects of SMD on lipopolysaccharide-induced expression of mediators important in innate immunity and inflammation. The results revealed broad effects on expression of transcription factors in both branches of Toll-like receptor 4 (TLR4) signaling (MyD88 and TRIF). However, TLR3 and interferon signaling pathways were decreased to a greater extent, and assessment of the effects of SMD on polyinosinic polycytidylic acid-induced cytokine and chemokine production revealed that these responses mediated by TLR3 were indeed sensitive to the effects of SMD, with inhibition occurring at lower dosages than required to inhibit responses to other immunological stimuli tested in our previous studies. In the downstream signaling pathways of these TLRs, functional analysis also revealed that NF-κB activation was inhibited by SMD, as indicated by gene expression analysis and a reporter construct in mice. A previously unreported effect on luteinizing hormone and follicle-stimulating hormone pathways was also observed.

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the TRIF-mediated activation pathway and the cytokines and chemokines induced by it (Song and Lee, 2012).

We reported previously that SMD decreases host resistance to sepsis caused by Escherichia coli, by a mechanism that involves inhibition of TLR4 signaling in innate immune cells such as macrophages (Pruett et al., 2005). Because TLR4 plays a critical role in the host resistance to infection in cells involved in innate immunity (eg, macrophages and dendritic cells) (Kaiho and Akira, 2001), it is important to evaluate the impact of SMD on macrophage activation induced by a TLR4 ligand (LPS) in vivo. In the current study, the inflammatory response to LPS challenge in mice was evaluated with or without SMD pretreatment. This included inflammatory cytokine and chemokine concentrations in the peritoneal cavity and in peripheral blood as well as global gene expression analysis in peritoneal macrophages. This study was designed to continue elucidation of the molecular mechanism(s) by which SMD inhibits the innate immune response and host resistance to infection and will potentially contribute to the assessment and mitigation of the human health risk imposed by environmental or occupational exposure to SMD.

MATERIALS AND METHODS

Mice, treatments, and procedures. For most experiments, female C57Bl/6 x C3H F1 mice were used. These mice were obtained through the National Cancer Institute’s Animal Program. They were allowed to recover from shipping stress for at least 2 weeks before beginning experiments, and they were 12–16 weeks old when used. Mice were housed in filter top shoebox cages with 5 mice per cage in a temperature (70°F–78°F) and humidity (40%–60%) controlled environment. Care and use of animals was done as described in the National Institutes of Health Guide. This work was approved by the Institutional Animal Care and Use Committee of Mississippi State University, and the work was conducted in a laboratory animal facility accredited by the American Association for Accreditation of Laboratory Animal Care. Sentinel mice periodically housed in the same room as experimental animals in this study have been negative for infectious agents during the period of this study. Mice transgenic for an NF-kB-driven promoter were used to indicate NF-kB activation in vivo in the live mice. The NF-kB mice were derived as described by Carlsen et al. (2002).

SMD was administered by nasal instillation 30 min before LPS challenge at 10, 50, 100, 200, or 300 mg/kg as indicated in each figure or figure legend. We have previously shown that dosages of 50–300 mg/kg have major effects on the immune system (Pruett et al., 2009). A naive control group was included in all experiments, and results from the naive control group were used to filter the microarray data so that only genes responding to LPS as compared with naive controls were considered for further analysis. For cytokine analysis, naive controls had unmeasurable or very low concentrations of cytokines. Because the major focus of this study was the effect of SMD on TLR-mediated responses, naive groups were not routinely assessed. In some experiments, only the highest dosage of SMD (300 mg/kg) was analyzed because the pathway analysis can only compare 1 control and 1 treatment group. The highest dosage was used because it typically yielded the largest effects, but for most genes, the effects appeared dose responsive (as shown in figures with heat maps showing results for 3 different dosages). Because the microarray results indicated that expression of many genes involved in the response to TLR3 ligands such as poly I:C was suppressed, lower dosages of SMD were used in the experiments with poly I:C in an effort to evaluate differential sensitivity of LPS and poly I:C induced responses. Briefly, mice were anesthetized with isoflurane and 50 µl of SMD solution was placed on 1 nostril. Mice, which are obligate nose breathers, immediately inhaled this liquid. This mode of administration was tested in a preliminary experiment in which Evans blue was administered and the lungs were evaluated. This indicated uniform distribution of the dye throughout both lungs as described in a study by Ebino et al. (1999). This mode of administration was selected because inhalation and dermal exposure are the 2 most relevant modes of human exposure (Rubin, 2004; Anonymous, 2009). Ultrapure LPS from Salmonella minnesota from List Labs (Campbell, California) at 60 µg/mouse (in PBS) was administered IV in a tail vein. Poly I:C from Invivogen (San Diego, California) was tested and found to be essentially endotoxin free, and it was administered at a dose of 100 µg/mouse IV.

Cytokine analysis. At 2 h after LPS or poly I:C, serum was collected by retroorbital bleeding under methoxyflurane anesthesia followed immediately by euthanasia with carbon dioxide before the mice regained consciousness, as described previously (Pruett et al., 2005). Peritoneal lavage fluid was collected by injecting euthanized mice IP with 1.0 ml of ice-cold PBS with 1% fetal bovine serum. After vigorous massage, the peritoneal membrane was exposed and fluid withdrawn using a syringe with a 25-g needle by holding the mouse so that the fluid pools laterally and is visible through the peritoneal membrane. Generally, 0.6–0.8 ml of the 1.0 ml volume injected was recovered. Various cytokine levels in serum and peritoneal fluid were measured using a Bioplex cytokine multiplex kit from Bio-Rad (Hercules, California) as described in the kit protocol.

RNA isolation and microarray analysis. At 2 h after LPS challenge, peritoneal lavage was performed and cells were collected for microarray by centrifuging the first sample taken to obtain cytokines following lavage with 1 ml of lavage fluid. The mice were then injected IP with 5 ml of lavage fluid and the remaining cells were obtained by withdrawing the lavage fluid and centrifuging it to pellet the cells. The cell pellets from the 1 and 5 ml lavages from a particular mouse were pooled before extraction for microarray analysis. The RNA extraction and microarray analysis was performed as described before (Pruett et al., 2004) except that the Mouse 430 2.0 GeneChip microarray was used in the present study. Labeled complementary RNA was produced from 5 µg total RNA using the Affymetrix one-cycle labeling kit. Hybridization, staining, washing, and scanning of the microarrays were performed according to protocols provided by Affymetrix.

Data were corrected for differences in staining using the robust multichip algorithm (RMA). Box-whisker plots of expression values before and after normalization showed that the RMA adjusted to the same median value with similar 1st and 3rd quartiles and similar 2nd and 98th percentiles. Normalized data were analyzed in Genesifter (Geospiza, Seattle, Washington).

Genesifter gene function analysis. Genes differentially expressed between the LPS-only group and the LPS + SMD groups were identified by 1-way ANOVA with Benjamini-Hochberg correction (p < .05) using Genesifter software. Differentially expressed genes were filtered using Gene Ontology classifications and results were presented in heat maps.

Ingenuity pathway analysis. Gene ontology pathways with a significant number of differentially expressed genes were identified using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, California). The canonical pathways and networks represented by these genes were represented graphically using IPA software.

Statistical analysis. In the NF-kB reporter mice and B6C3F1 mice used to evaluate cytokines, values shown are means ± SEM, and the group size is 4 for NF-kB and 4–5 for cytokines. Comparisons of 2 groups (eg, LPS-treated mice compared with LPS-treated mice that were pretreated with SMD) were performed using Student’s t test. Comparisons of more than 2 groups (eg, comparison of cytokine concentrations in response to different dosages of SMD) were done by ANOVA using the Neuman-Keuls post hoc correction to compare the mean of each group with the means of all other groups. Values of p < .05 were regarded to be statistically significant. Statistical analysis of microarray results was done with statistical tools built into IPA software. Genesifter uses ANOVA with appropriate correction for the large number of variables (genes) in the data.
RESULTS

Effects of SMD on Inflammatory Cytokine Production After LPS Challenge

Although the primary goal of this study was to evaluate the global effects of SMD on innate immune responses, we also evaluated cytokine levels in the serum and peritoneal fluid of the same mice from which the macrophages used in the microarray analysis were obtained. Peritoneal fluid was obtained 2 h after LPS treatment. This time was selected on the basis of our previous studies in this animal model indicating that both mRNA and proteins for many proinflammatory mediators are near their peak levels at 2 h (Pruett et al., 2005, 2006, 2009). As shown in Figure 1, SMD reduced cytokine/chemokine responses to LPS, such as IL-12 (p40) and CCL3. On the other hand, SMD increased the production of the anti-inflammatory cytokine IL-10. We have done several experiments of similar design, and the effects of SMD on IL-12 and IL-10 production have been consistent and similar in serum and peritoneal fluid (Pruett et al., 2005, 2006, 2009). These results generally corresponded to patterns of gene expression by microarray analysis, as indicated in Table 1. The chemokine shown in Figure 1, CCL3, was not represented in the microarray, so the correspondence between mRNA and protein could not be estimated. The small sample size (n = 3 for most groups and 2 for LPS + SMD at 300 mg/kg) limits the effectiveness of statistical comparisons in the microarray data for individual genes, so most of this report focuses on changes in pathways using IPA or sets of genes with functional similarities using Genesifter. These software packages have robust statistical methods, and these were used to analyze the data. Pathway analysis in particular relies on the probability of simultaneous changes in the same direction of many genes in a pathway rather than changes in single genes, yielding greater statistical power than determined by group size alone.

Effect of SMD on the Global Gene Expression in Peritoneal Macrophages After LPS Challenge

Affymetrix genechips were used to evaluate the global gene expression in peritoneal cells (> 85% macrophages) (Pruett et al., 2005) 2 h after LPS challenge with or without SMD pretreatment at different doses. Hierarchical clustering was performed using Genesifter software to compare the gene expression pattern between different groups. As can be seen in Figure 2, the group treated with LPS + SMD (200 mg/kg) and the group treated with LPS + SMD (100 mg/kg) were quite similar in terms of global gene expression pattern. However, the group treated with LPS + SMD (300 mg/kg) group was more similar to the naive group as compared with other LPS-treated groups, including the group treated only with LPS. This suggests that SMD, especially at 300 mg/kg, tended to reduce the LPS-induced gene expression changes to levels that were more similar to those in the naive (untreated) group. This effect of high dose SMD was consistent with the overall findings in this study that many of the immune and inflammatory responses to LPS are reduced by SMD, especially at 300 mg/kg (Fig. 3).

Innate Immune Response Genes Were Affected by SMD Pretreatment in LPS-Challenged Mice

Many innate immune response–related genes were affected by SMD treatment in LPS-challenged mice (Fig. 3). These genes are functionally related according to the gene ontology annotation used by Genesifter software. Only genes with at least a 2-fold difference between a treated group and the LPS control are shown, and only genes with significant differences between groups by ANOVA with Benjamini-Hochberg correction are shown. Many of the genes in this list are also found in more specialized functional pathways shown in subsequent figures.

Pathway Analysis of Genes Affected by SMD in the LPS-Challenged Mice

Genes that were differentially expressed between the LPS group and LPS + SMD groups were identified by ANOVA. These genes were first filtered by the criterion that they were changed by LPS alone as compared with naive (untreated) group. The resultant group of genes was particularly interesting because these were the genes that were LPS regulated and were also affected by SMD in the LPS + SMD cotreatment groups. Thus, these genes can be regarded as the best candidates to be directly associated with the effects of SMD on the immune response in the LPS-challenged mice. Analysis of this group of genes by IPA generated a list of associated biological pathways at very high significance levels: activation of IRF by cytosolic pattern-recognition receptors (PRRs; p < 10^-6); IFN signaling (p < 10^-4); role of PRRs in recognition of bacteria and viruses (p < 10^-3); renin-angiotensin signaling (p < 10^-3); death receptor signaling (p < 10^-3); p38 MAP kinase signaling (p < 10^-3). Interestingly, activation of IFN response factors and IFN signaling are the first 2 pathways involved (indicating that they include the largest numbers of regulated genes). The pathways noted in Figures 4 and 5 fall within these indicated categories. Several signaling molecules involved in IFN-dependent gene transcription such as JAK2, STAT1/STAT2, and...
IRF-9 were all downregulated by SMD. This is consistent with the reduction of TLR3, DDX58 (RIG-I), and DHX58 (LGP2) by SMD already noted (Fig. 5). Results shown in Figures 4 and 5 indicate predominant downregulation of signaling pathways induced by infectious agents and by IFNs. These are among the most statistically significant pathways that were altered by SMD treatment (see p values above).

Results shown in Figure 6 demonstrate that SMD strongly inhibited the production and/or the signaling of many IFN-induced genes in the LPS-challenged mice. However, expression of IFN-β was significantly enhanced by SMD. The basis for this is not known, but it could be related to unknown specific effects of SMD, which could enhance a few members of these families, in spite of decreasing the relevant shared signaling pathways. In contrast, expression of most of the isotypes of IFN-α were suppressed by SMD, but the expression levels of all of the IFN-α isotypes were quite low.
In general, cytokine protein expression was suppressed by SMD as shown in Figures 1 and 7. The experiment shown in Figure 7 was done because of the results in the preceding figures indicating substantial inhibition of IFN-induced signaling. Because poly I:C induces primarily IFN-induced genes (including cytokines and chemokines), inhibition of production was expected. The experiment shown in Figure 7 was done because of the results in the preceding figures indicating substantial inhibition of IFN-induced signaling. Because poly I:C induces primarily IFN-induced genes (including cytokines and chemokines), inhibition of production was expected.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>LPS</th>
<th>LPS + SMD (100)</th>
<th>LPS + SMD (200)</th>
<th>LPS + SMD (300)</th>
</tr>
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<tbody>
<tr>
<td>GM-CSF</td>
<td>1.00±0.57</td>
<td>1.08±0.43</td>
<td>1.32±0.45</td>
<td>0.43±0.37</td>
</tr>
<tr>
<td>IL-12 (p40)</td>
<td>1.00±0.18</td>
<td>1.14±0.31</td>
<td>0.51±0.13</td>
<td>0.48±0.06</td>
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<tr>
<td>IFN-γ</td>
<td>1.00±0.28</td>
<td>1.04±0.38</td>
<td>0.63±0.09</td>
<td>0.67±0.01</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.00±0.07</td>
<td>0.87±0.14</td>
<td>1.45±0.33</td>
<td>2.22±0.07</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.00±0.07</td>
<td>1.25±0.18</td>
<td>1.17±0.01</td>
<td>0.80±0.24</td>
</tr>
</tbody>
</table>

Results were obtained by normalizing data from the microarray to make the value of the LPS group 1.00 for each cytokine. CCL3, the chemokine shown in Figure 1 was not present in the microarray data (possibly due to low expression). The group size was 3 for the LPS, LPS + SMD (100), and LPS + SMD (200) groups, and 2 for the LPS + SMD (300) group. The labels indicate the dosages. For example, SMD (100) indicates SMD at 100 mg/kg body weight. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; SMD, sodium methylidithiocarbamate.

**FIG. 2.** Hierarchical analysis of gene expression. Groups that are nearest to each other have the most similar changes of gene expression. Results are consistent with direct observation of the raw data in spreadsheet format, where it was observed that SMD at 300 mg/kg completely or almost completely inhibited the effect of LPS. Thus, LPS plus SMD at 300 mg/kg is more similar to naive than LPS alone. Abbreviations: LPS, lipopolysaccharide; SMD, sodium methylidithiocarbamate.

**FIG. 3.** Heat map of changes in expression in inflammation-related genes. Gene expression for most proinflammatory genes was downregulated by SMD (lower expression is indicated by green, higher expression by red). Those which were upregulated are mostly proinflammatory proteins with an accessory or ancillary function that may have been upregulated in compensation for decreased production of cytokines by SMD. Abbreviations: LPS, lipopolysaccharide; SMD, sodium methylidithiocarbamate.
of these proteins is consistent with the microarray results indicating reduced expression of several key signaling genes. Most of these cytokines are dependent on the NF-κB pathway, which was also inhibited by SMD (Fig. 8), but IFN-γ induction through LPS is mediated mostly by IRF transcription factors, which are also involved in expression of type I IFNs. The importance of the TRIF pathway is further illustrated by results shown in Figure 7, which shows that SMD, even at low dosages, inhibited cytokine production induced by poly I:C, a dsRNA analog, which acts predominantly through the TRIF and RIG-I pathways.

Another pathway affected by SMD in the LPS-treated mice is the “role of PRRs in recognition of bacteria and virus” (not shown). Again, the IFN-dependent antiviral genes such as PKR and OAS1/OAS2 were downregulated by SMD. In addition, expression of nucleotide-binding oligomerization domain 1 (NOD1), an intracellular PRR that initiates inflammation in response to a subset of bacteria, was reduced by SMD treatment (Fig. 4).

As already noted (Fig. 1 and Table 1), gene expression changes in cytokines generally correlated to changes in expression of the corresponding protein. The figure legends of Figures...
FIG. 5. Changes in expression of pattern-recognition receptors and signaling genes in a pathway identified by IPA. Green symbols indicate decreased gene expression caused by SMD, no color represents no significant effect, and pink to red indicates increased expression caused by SMD. However, in some cases, symbols with no color did exhibit potentially biologically relevant changes (noted below), but they are not shown as green or red because one of the values in the set is below the limits of detection or the difference is not statistically significant. The following fold changes were observed comparing the LPS and the LPS + SMD at 300 mg/kg groups: TLR3, −7.9-fold; IRF-7, −13-fold; IRF-3, −2.3-fold; NF-κB (p50), −4.2-fold; RIG-I (DDX58), −5.0-fold; MyD88, −2.2-fold; NOD1 (CARD4), −3.5-fold; PKR (EIF2AK2), −11.6-fold; RIP-2 (RIPK-2), −2.8-fold; OAS-1a, −5.9-fold; IL-6, +6.9-fold; IFN-α/β, −1.2-fold; IL-10, +2.2-fold; IL-12 (p40), −2.2-fold; Dectin-1, +6.4-fold. Abbreviations: IFN, interferon; IL, interleukin; IPA, Ingenuity Pathway Analysis; IRF, interferon regulatory factor; LPS, lipopolysaccharide; SMD, sodium methylthiocarbamate; TLR, Toll-like receptor.

4 and 5 include the fold change for most of the genes depicted in each pathway.

Network analysis revealed an important NF-κB-related network that is affected by SMD treatment (results not shown). NF-κB subunit p50 was transcriptionally downregulated by SMD (Fig. 5). Interestingly, most of the NF-κB-related genes (either genes that regulate NF-κB or the ones that are regulated by NF-κB) were downregulated by SMD. This includes TRAF-1 (Tsitsikov et al., 2001), TNIP2 (Papoutsopoulou et al., 2006; Van Huffel et al., 2001), PAK11P1 (Xia et al., 2001), MEFV (Dowds et al., 2003), TRAFD1 (Mashima et al., 2005), TRAF3IP2 (Li et al., 2000), and TNFAIP2 (Tian et al., 2005). To explore the net effect of SMD on LPS-induced NF-κB activation, a mouse strain containing an NF-κB reporter luciferase gene was used. As expected, SMD reduced NF-κB-dependent luciferase transcription after LPS challenge (Fig. 8), confirming the inhibitory role of SMD on NF-κB activation.

Another network associated with SMD treatment was the network centered by luteinizing hormone (LH), follicle-stimulating hormone (FSH), and chorionic gonadotropin (hCG). LH, FSH, and hCG are reproductive hormones released from hypothalamic-pituitary-gonadal (HPG) axis critical for follicular development and oocyte maturation (De Placido et al., 2001; Driscoll et al., 2000; Levy et al., 2000; McLachlan et al., 1995). Although their mRNA levels were not affected by SMD in the current study, genes associated with these hormones were mostly upregulated by SMD (Fig. 9). This suggests a potential increase of production of these reproductive hormones by SMD with kinetics that would lead to a transient upregulation of these hormones followed by a longer lasting change in gene expression in genes that are regulated by them. We have previously shown that SMD can induce production of corticosterone, a stress-related glucocorticoid (Myers et al., 2005), and it is known that LH and FSH are corticosterone inducible (Kilen et al., 1996; McAndrews et al., 1994). The corticosterone response is characterized by a very rapid increase in concentration with a peak at about 1 h followed by a relatively rapid decline (Myers et al., 2005).

DISCUSSION

Environmental and occupational exposure of persons to SMD has imposed a major human health risk, especially in California where it is heavily used in the large-scale agriculture industry. The dosages of SMD used here could occur as a result of occupational exposure to commercial SMD preparations (Padgett et al., 1992). In addition, the reported dosage of MITC, a volatile compound that is the major breakdown for a child near fields treated with SMD, is up to 1.0–2.5 mg/kg/day. For many toxicants, the equivalent dosages across species are better estimated by expressing dosages as milligram
FIG. 6. Expression changes in interferon-related genes mediated by SMD are shown. Results were obtained by analyzing microarray data with Genesifter software. Results shown include genes for which changes were significant ($p < .05$) as determined by ANOVA using the Benjamini-Hochberg post hoc correction. Green color indicates decreased expression relative to LPS only. Genes induced by interferon were almost all decreased in expression by SMD. Abbreviations: LPS, lipopolysaccharide; SMD, sodium methylthiocarbamate.
of chemical per meter squared of body surface area rather than milligram of chemical per kilogram of body weight. Expressing the mean dosage on the basis of body surface area instead of body weight indicates that the mean dosage in mice that would be equivalent to 1.75 \( \text{mg/kg} \) in humans would be approximately \( 17 \text{ mg/kg} \) (calculated as described in our previous study) (Padgett et al., 1992). This is greater than the lowest dosage at which we observed suppression of chemokines in the present study (10 \( \text{mg/kg} \)). This dosage is also similar to the lowest observable adverse effect level (LOAEL) for SMD reported in the U.S. Environmental Protection Agency Human Health Risk Assessment (Kinard and Smith, 2005). The higher dosages used in the present study (100–300 \( \text{mg/kg} \)) are beyond the LOAEL, but if the typical 10\( \times \) safety factor to account for sensitive subpopulations of humans is applied, these dosages could be relevant to human exposure, particularly occupational exposure (Kinard and Smith, 2005).

The results here clearly indicate that SMD inhibits TLR4 activation induced by LPS \( \text{in vivo} \). This is shown by reduction of several TLR4-dependent innate immune response genes such as MyD88, TIRAP, and genes for many proinflammatory cytokines such as IL-12. Moreover, the current microarray study also suggested that other PRRs would be involved in the inhibition of inflammatory response by SMD. For example, receptors sensing dsRNA such as TLR3, RIG-I, and LGP2 were decreased at the mRNA level. Further detailed pathway analysis revealed that IRF-7 and NF-\( \kappa \)B, 2 transcription factors critical for controlling dsRNA-induced inflammatory gene production, are also inhibited by SMD. The inhibition of NF-\( \kappa \)B mRNA content by SMD (Fig. 5) corresponds well with the inhibition of NF-\( \kappa \)B activation detected by reporter mice (Fig. 8). This does not agree with our previous report indicating no change in NF-\( \kappa \)B (p65) in mice treated with SMD (Pruett et al., 2005). This previous observation was probably due to evaluation of only the p65 component. Decreased expression of other components (such as the decrease in p50 expression observed in the present study) could inhibit overall NF-\( \kappa \)B transcriptional activity, as noted in the reporter mice. It should also be noted that almost all of the cytokines and chemokines...
exhibiting decreased expression are at least partially dependent on NF-κB for expression. Taken together, these results suggest that SMD inhibits NF-κB activation and transcription of genes induced by NF-κB.

There are reports suggesting that TLR3 could sense RNA released from necrotic cells and contribute to the late amplification of the inflammatory response during polymicrobial sepsis (Cavassani et al., 2008). Thus, the inhibition of TLR3, RIG-I, and LGP2 expression might be involved in the reduced host resistance to E. coli infection caused by SMD, which we reported previously (Pruett et al., 2005). Whether the inhibition of these dsRNA-dependent inflammatory genes is due to inhibition of an initial receptor (TLR3, RIG-I, LGP2), signaling, or indirect inhibition of a type I IFN “amplification loop” (as reflected by reduction of JAK2, STAT1/STAT2, and IRF-7) (Pruett et al., 2004) is of further interest. However, the results shown here indicate that IFN-β production is not inhibited by SMD in vivo and inhibition of some IFN-α isotypes occurs, but isotypes are not inhibited. Thus, it seems likely that the major inhibition of IFN-induced genes shown in Figure 8 is probably caused more by inhibition of IFN-mediated signaling than by inhibition of IFN production. One initial in vitro study revealed

**FIG. 9.** Ingenuity pathway analysis of differences between LPS and LPS + SMD (300 mg/kg) with regard to reproductive hormones. Pink to red indicates upregulation by SMD, and green indicates downregulation by SMD. The connecting lines indicate functional relationships between the indicated proteins. The central molecule in this pathway is luteinizing hormone (LH), which directly affects follicle-stimulating hormone (FSH). It should be noted that the microarray results were obtained using female mice. Abbreviations: LPS, lipopolysaccharide; SMD, sodium methyldithiocarbamate.
that SMD (at 5μM) could inhibit INF-β production by a mouse macrophage cell line (RAW 264.7) in response to LPS (data not shown). In addition, we have found that SMD inhibits cytokine and chemokine production in response to dsRNA, which acts predominantly through TLR3 (Fig. 8). This implies that SMD directly inhibits the signaling of these dsRNA-sensing receptors, but the exact molecular target of SMD in terms of inhibiting inflammatory responses remains to be explored.

The decrease in expression of both NF-κB and IRF-related signaling proteins may not cause significant changes in gene expression, if activation (generally phosphorylation) increases to compensate for the smaller quantity of transcription factors. However, the results shown in Figure 7 indicate that several proinflammatory proteins whose expression depends on NF-κB (Ciesielski et al., 2002) and/or IRF (Dai et al., 2006) were downregulated by SMD. Poly I:C was used as an inflammatory stimulus in this study to focus the results on TRIF-related activation of immunological mediators. The only cytokine or chemokine upregulated at the protein level was the anti-inflammatory cytokine, IL-10, which is often regulated reciprocally as compared with proinflammatory cytokines. Expression of the IL-10 gene was also increased by SMD (Table 1).

In results reported here, SMD inhibited NF-κB-dependent transcription in response to TLR4 activation. This was shown by the reduction of expression of NF-κB (Fig. 7) and confirmed by the reduction of NF-κB-dependent luciferase transcription in vivo (Fig. 8). In addition to NF-κB, Activator Protein 1 (AP-1) is another important transcription factor involved in inflammatory responses, and it is also inhibited by SMD (Pruett et al., 2005). However, because both NF-κB and AP-1 could be regulated by most of the inflammatory cytokines, it will be difficult to differentiate the cause-effect relationship between the inhibition of NF-κB/AP-1 and the reduction of inflammatory cytokines in the current study.

Acute SMD administration is known to cause a stress response, which causes thymic atrophy (especially in CD4 and CD8 double positive T cells) through a corticosterone-dependent mechanism (Myers et al., 2005). However, in the current study, the major cell type in the peritoneal cavity was the macrophage (>85%), and macrophages are not as sensitive to corticosterone-induced apoptosis as thymocytes. Additional evidence from this lab suggests that inhibition of innate immune responses by a chemical stressor was mediated by a mechanism that does not involve corticosterone (Glover et al., 2009; Glover and Pruett, 2006). It should also be considered that LPS is a potent activator of the hypothalamic-pituitary-adrenal axis, probably more potent than a chemical stressor such as SMD. Thus, it is unlikely that SMD-induced glucocorticoids play a major role in the effects of SMD in this experimental system.

In the current study, reproductive hormones such as LH and FSH seem to be upregulated by SMD. Macrophages have receptors for LH, FSH, and hCG, and they respond to these hormones (Robinson et al., 2010; Yoshida et al., 1996; Zhang et al., 2003). It is also known that estrogen could be regulated by these hormones (Levy et al., 2000). Given the well-established connection between estrogen and some inflammatory cytokines (especially IL-6) (Ray et al., 1994, 1997), it would be interesting to pursue the roles of these reproductive hormones released from HPG axis in the inhibition of inflammatory responses by SMD.

In summary, the current study confirms that SMD inhibited expression of molecules in both the TRIF-related MyD88-related TLR4 signaling. This is reflected by the reduction of circulating cytokines/chemokines. In addition to inhibition of signaling function, which we have reported previously (Pruett et al., 2009), we found decreased expression of the genes for several TLR4-dependent signaling molecules. The transcriptional factor involved in the inhibition of the innate immune response seems to be NF-κB and/or AP-1. Furthermore, the gene expression data also suggest several potential new mechanisms that SMD might act on. These include type I IFN production/signaling and induction of reproductive hormones such as LH and FSH, both of which could be the subject of further studies deciphering the mechanism of innate immune response inhibition by SMD.

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