Regulation of the Polymeric Ig Receptor by Signaling through TLRs 3 and 4: Linking Innate and Adaptive Immune Responses

Tracey A. Schneeman, Maria E. C. Bruno, Hilde Schjerven, Finn-Eirik Johansen, Laura Chady and Charlotte S. Kaetzel

*J Immunol* 2005; 175:376-384; doi: 10.4049/jimmunol.175.1.376
http://www.jimmunol.org/content/175/1/376

References
This article cites 59 articles, 25 of which you can access for free at: http://www.jimmunol.org/content/175/1/376.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Regulation of the Polymeric Ig Receptor by Signaling through TLRs 3 and 4: Linking Innate and Adaptive Immune Responses

Tracey A. Schneeman,* Maria E. C. Bruno,* Hilde Schjerven,§ Finn-Eirik Johansen,§ Laura Chady,‡ and Charlotte S. Kaetzel2*†

IgA Abs help to maintain homeostasis at mucosal surfaces by promoting defense mechanisms that protect against pathogens while suppressing inflammatory responses to commensal organisms and food Ags. The polymeric Ig receptor (pIgR) mediates transport of IgA across mucosal epithelial cells. We hypothesized that signaling through TLRs may up-regulate pIgR expression by intestinal epithelial cells and thus enhance IgA-mediated homeostasis. To test this hypothesis we treated the HT29 human intestinal epithelial cell line with dsRNA, a ligand for TLR3, or LPS, a ligand for TLR4. Both dsRNA and LPS up-regulated levels of pIgR mRNA and cell surface pIgR protein. By contrast, dsRNA but not LPS up-regulated expression of TLR3 and TLR4 mRNA. However, cell surface expression of both TLR3 and TLR4 was enhanced by treatment of HT29 cells with their respective ligands. Transfection of HT29 cells with wild-type and mutated promoter/enhancer plasmids suggested that TLR3 and TLR4 signal primarily through NF-kB to enhance transcription of pIgR mRNA. TLR3 signaling resulted in a more pronounced inflammatory response than did TLR4, as evidenced by up-regulation of the transcription factor IFN regulatory factor-1, chemokines IL-8 and RANTES, and the proinflammatory cytokine TNF. Signaling through LPS/TLR4 appears to up-regulate pIgR expression while minimizing proinflammatory responses, a mechanism that could promote IgA-mediated homeostasis in the presence of commensal Gram-negative bacteria. The Journal of Immunology, 2005, 175: 376–384.
recent studies have shown that differential usage of adaptor molecules may result in different biological responses (29–32).

In this study, we report that plgR expression was strongly up-regulated in response to both dsRNA/TLR3 and LPS/TLR4 signaling in HT29 cells. A binding site for NF-κB in intron 1 of the plgR gene was shown to be critical for transcriptional activation in response to TLR3 and TLR4 signaling. Analysis of several proinflammatory gene products demonstrated that TLR3 signaling resulted in a more pronounced inflammatory response than did TLR4. Our data suggest that signaling through TLR4 up-regulates plgR expression while minimizing proinflammatory responses, a mechanism that could promote IgA-mediated homeostasis in the presence of commensal Gram-negative bacteria.

Materials and Methods

Cell culture

The HT29v20 subclone of the human colon adenocarcinoma cell line (33) was maintained in DMEM-F12 media with 5% FCS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone). All cell culture reagents were from BioWhittaker. Cells were treated with dsRNA (poly I:C) at a final concentration of 100 μg/ml or Salmonella enteritidis LPS at a final concentration of 1 μg/ml (both from Sigma-Aldrich).

Quantitative real-time RT-PCR

Total cellular RNA was extracted from HT29 cells using the RNeasy Mini kit from Qiagen. The quality of extracted RNA was confirmed by agarose gel electrophoresis. cDNA was synthesized using 300 ng of RNA, random hexamers, and TaqMan Gold reverse transcriptase (Applied Biosystems). The levels of individual mRNA transcripts were quantified by quantitative real-time PCR. Primers and fluorescent probes for plgR, IFN regulatory factor-1 (IRF-1), RANTES, IL-8, TNF, and GAPDH were designed using Primer Express software (Applied Biosystems) (Table I). Predesigned primers and probes for TLR3 (catalog no. Hs00152933_m1) and TLR4 (catalog no. Hs00152939_m1) were purchased from Applied Biosystems.

To eliminate the possibility of measuring any contaminating DNA, primer/probe sets were designed such that at least one element of the set spanned an intron. PCRs were conducted using TaqMan Universal PCR Master Mix, no UNG (catalog no. 4324018; Applied Biosystems), and the ABI Prism 7700 Sequence Detection System. Each reaction included cDNA corresponding to 20 ng of reverse-transcribed RNA. The PCR conditions comprised a preliminary cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplification of the cDNA template was measured at every cycle by fluorescence resonance energy transfer (ΔRn). The threshold cycle (Ct), which is inversely proportional to the level of a given mRNA transcript, is defined as the cycle at which the ΔRn exceeds the threshold of fluorescence detection. Validation curves were run for each mRNA transcript to ensure that there was an inverse linear relationship between the amount of input RNA and the Ct. We also confirmed that efficiency of amplification of each target gene (including GAPDH) was essentially 100% in the exponential phase of the PCR, where the Ct is calculated. To determine the relative level of target mRNA in individual samples, the Ct values for each message were normalized to the Ct for GAPDH mRNA by the formula (2–ΔCt Test – CgapDH) × 100%.

ELISA

Culture supernatants were harvested for measurement of SC by ELISA as previously reported (34). Levels of secreted TNF, IL-8, and RANTES were measured using commercial ELISA kits (R&D Systems).

Immunofluorescence

HT29 cells were cultured for 24 h on Permanox 4-well chamber slides (Nalge Nunc International) before initiation of treatment, then cultured for an additional 24 h in the presence or absence of dsRNA or LPS as described above. To measure cell surface expression of plgR, TLR3 and TLR4, cells were immunostained without prior fixation and in the presence of sodium azide to prevent Ab internalization. Cells were subjected to 5-min washes with PBS containing 4 mM sodium azide, then incubated for 45 min with the indicated primary Abs or normal rabbit serum, diluted 1/200 in PBS/azide. The rabbit polyclonal Ab to human secretory component (the extracellular domain of plgR) has previously been described (34).

Mouse mAbs to human TLR3 and TLR4 were obtained from Imgenex. Cells were washed as before, then incubated for 45 min with a biotin-conjugated anti-mouse IgG (F(ab')2 specific) (Sigma-Aldrich), diluted 1/400 in PBS/azide. Cells were washed again and incubated for 45 min with a mixture of Alexa Fluor 488 goat anti-rabbit IgG (H+L) ( Molecular Probes) and Streptavidin-PE-Cy5 Conjugate (BD Biosciences/Pharmingen), both diluted 1/1400 in PBS/azide. Following a final series of washes, the slides were mounted with Gel/Mount (Biomedula). Dual fluorescence was visualized using the Zeiss Axioplan 2 fluorescence imaging system (München-Hallbergmoos). Exposure times were equalized to allow comparisons among images.

Neutralization of TNF activity

To confirm the specificity and neutralizing activity of the Ab, HT29 cells were stimulated with recombiant human TNF (R&D Systems) in the presence of anti-TNF or control goat IgG (Sigma-Aldrich). To test the effect of TNF neutralization on TLR-induced gene expression, HT29 cells were stimulated with poly I-C or LPS as described above in the presence of varying concentrations of neutralizing Ab to TNF (R&D Systems).

Gene reporter assays

The human PIGR gene reporter plasmids were constructed as described (35). Transcription of the firefly luciferase reporter gene is driven by an 8.6-kb fragment of the human PIGR gene, including 2684 bp of the 5’-flanking sequence, exon 1 (132 bp), intron 1 (5751 bp), and the first 56 bp of exon 2, up to and including the translation start site. The “Mut IRF” plasmid contains a mutation that abolishes the IRF-binding site in exon 1, the “Mut NF-κB” plasmid contains a mutation that abolishes an NF-κB-binding site in intron 1, and the “double Mut” plasmid contains both mutations. These mutations have previously been shown to inhibit TNF-induced activation of PIGR gene transcription (35). The NF-κB reporter plasmid, which contains four copies of a consensus NF-κB-binding site upstream of a minimal promoter and the firefly luciferase gene, was obtained from Stratagene. The corresponding control plasmid contains the same minimal promoter without the upstream NF-κB sites. Cultures of HT29 cells in 35-mm dishes were transfected for 2 h with 1 μg of the indicated reporter plasmid and 0.02 μg of pRL-CMV (Promega), in which transcription of the Renilla luciferase gene is driven by the CMV immediate early promoter, using the Tri-293 transfection reagent according to the manufacturer’s protocol (Promega). Twenty-four hours after transfection, cells were treated for the indicated times with dsRNA or LPS. Cell lysates were analyzed for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System according to the manufacturer’s protocol (Promega).

Statistical analyses

Statistical differences among treatment groups were determined by ANOVA and Fisher’s protected least significant difference test, using StatView software (SAS Institute).

Table I. Sequences of primers and probes used in real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>plgR</td>
<td>CTCCTCGAGCAACACCACTGTT</td>
<td>CAGCCGGTACATTCCCTCC</td>
</tr>
<tr>
<td>TagM probe</td>
<td>AGATCAGATATTACGAGGAGACCAACCATC</td>
<td></td>
</tr>
<tr>
<td>IRF-1</td>
<td>GGTAAAGTGTGGCCGCAACA</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>ACCAGTGCCACGAGCTTCCA</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>CTTGCCTCTTGGCACGC</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>TCTTTGACCTTCTCTGGAACCAC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGCAGATCGTCGGGGTCTTTT</td>
<td></td>
</tr>
<tr>
<td>GACCACTTGGCCGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TagM probe</td>
<td>CAGCAGATCGCCTTGGGAAACCG</td>
<td></td>
</tr>
<tr>
<td>TagM probe</td>
<td>CAGCAGATCGCCTTGGGAAACCG</td>
<td></td>
</tr>
<tr>
<td>TagM probe</td>
<td>CAGCAGATCGCCTTGGGAAACCG</td>
<td></td>
</tr>
</tbody>
</table>

Downloaded from http://www.jimmunol.org/ by guest on July 21, 2017
Results

Expression of pIgR is up-regulated in IEC by TLR3 and TLR4 signaling

To determine whether TLR3 and TLR4 signaling regulated pIgR expression in human IEC, HT29 colon carcinoma cells were treated with dsRNA (poly I:C) or LPS from S. enteritidis. Two groups of investigators have previously tested the response of proinflammatory gene expression in HT29 cells to concentrations of LPS ranging from 10 pg/ml to 100 μg/ml, and reported that the peak induction of IL-8 occurred at a dose of 1 μg/ml (28, 36). In preliminary experiments, we confirmed that a dose of 1 μg/ml LPS was optimal for induction of pIgR and IL-8 expression in HT29 cells (Fig. 1A). To our knowledge, the response of HT29 cells to varying doses of dsRNA has never been reported. In preliminary experiments, we determined that a dose of 100 μg/ml poly I:C was optimal for induction of pIgR and IL-8 expression in HT29 cells (Fig. 1A). We then tested the time course of induction of pIgR expression in response to optimal doses of dsRNA or LPS. Changes in steady-state levels of pIgR mRNA were very similar in response to either dsRNA or LPS, increasing ~10-fold by 24 h (Fig. 1B). To verify that the increase in pIgR mRNA translated to an increase in pIgR protein, we measured accumulation of SC (the cleaved extracellular domain of pIgR) in culture supernatants (Fig. 1C). We previously reported that levels of secreted SC correlated with cellular levels of pIgR mRNA and protein in HT29 cells (34, 37). In this study, we observed significant accumulation of SC after the rise in pIgR mRNA levels in response to stimulation with dsRNA and LPS.

To determine whether stimulation of HT29 cells with agonists for TLR3 or TLR4 affected expression of the receptors themselves, we measured levels of TLR3 and TLR4 mRNA following treatment with dsRNA or LPS (Fig. 2). Stimulation of HT29 cells with dsRNA resulted in a 6-fold increase in TLR3 mRNA and a 2-fold increase in TLR4 mRNA. By contrast, stimulation with LPS resulted in a modest and transient increase in TLR3 mRNA and no change in TLR4 mRNA. Levels of TLR3 and TLR4 protein were below the limit of detection by Western blot in both stimulated and unstimulated HT29 cells (data not shown), suggesting either that the threshold expression level for TLR signaling is very low or that a subpopulation of TLR-expressing cells responded vigorously to stimulation with dsRNA and LPS.

To examine whether a subpopulation of cells express TLRs and respond to stimulation with dsRNA or LPS, cell surface expression of pIgR and TLRs was visualized by immunofluorescence of HT29 cells cultured for 24 h in the presence or absence of dsRNA or LPS (Fig. 3). No cell surface fluorescence was observed when preimmune rabbit or mouse serum was used as primary Ab (data not shown). Interestingly, cell surface expression of TLR3 and TLR4 was enhanced by treatment of HT29 cells with their respective ligands. These findings indicate that increased cell surface expression of TLR3 in response to dsRNA signaling could have been mediated in part by increased levels of TLR3 mRNA. By contrast, LPS-induced increases in cell surface expression of TLR4 were unrelated to changes in gene expression and could have resulted from increased protein expression or transport to the cell surface from intracellular stores. We previously reported that expression of pIgR is heterogeneous in cultures of HT29 cells (34), and we found here that a relatively small proportion of unstimulated HT29 cells expressed TLR3 or TLR4.
expressed pIgR. The number of pIgR surface-positive cells increased in response to both dsRNA and LPS. However, examination of the merged images revealed differences in coexpression of pIgR and TLRs. In cultures of dsRNA-stimulated HT29 cells, up-regulation of pIgR was sometimes but not always correlated with surface expression of TLR3. By contrast, up-regulation of pIgR by LPS was consistently accompanied by increased cell surface expression of TLR4.

The finding that some TLR3-negative cells expressed pIgR after dsRNA stimulation suggested that a soluble factor produced in response to TLR3 signaling may act in a paracrine fashion to up-regulate pIgR expression. The cytokine TNF is a likely candidate, considering that dsRNA but not LPS increased secretion of TNF (see Fig. 7B), and that TNF is known to up-regulate pIgR expression in HT29 cells (33, 35, 38, 39). To determine whether secreted TNF is involved in the up-regulation of pIgR expression, we treated HT29 cells with dsRNA or LPS in the presence of increasing concentrations of neutralizing Ab to TNF. The specificity and neutralizing activity of the anti-TNF Ab was confirmed in preliminary experiments, in which we demonstrated that the Ab completely blocked up-regulation of pIgR expression by recombinant human TNF (Fig. 4A). We found that neutralization of TNF resulted in a 25% decrease in dsRNA-mediated up-regulation of pIgR mRNA, but did not affect LPS-mediated pIgR up-regulation (Fig. 4B). Therefore, we conclude that TNF (and perhaps other soluble factors) may contribute to the regulation of pIgR expression by TLR3 signaling.

FIGURE 2. Responses of TLR3 and TLR4 mRNA expression to stimulation with dsRNA or LPS. HT29 cells were cultured for the indicated times in the presence or absence of poly I/C (100 μg/ml) or LPS (1 μg/ml). Steady-state mRNA levels were measured by quantitative real-time RT-PCR and normalized to GAPDH mRNA. Data are expressed as mean ± SEM (n = 3). Asterisks (*) indicate that the mean is significantly greater than the corresponding mean for untreated cells (p < 0.05).

FIGURE 3. Colocalization of pIgR and TLRs. HT29 cells were cultured for 24 h with medium alone or supplemented with poly I/C (100 μg/ml) or LPS (1 μg/ml). Surface proteins were detected in unfixed cells by immunofluorescence. Images shown are representative of at least five fields.

FIGURE 4. Neutralization of TNF activity reduces dsRNA-stimulated up-regulation of pIgR mRNA expression. A, HT29 cells were cultured for 24 h in the presence of 10 ng/ml recombinant human TNF and varying concentrations of goat anti-human TNF Ab or control goat IgG. Steady-state levels of pIgR and IL-8 mRNA were measured by quantitative real-time RT-PCR and normalized to GAPDH mRNA. Data are expressed as mean ± SEM (n = 3). Dashed lines represent the mean mRNA level in HT29 cells cultured for 24 h in the absence (short dashes) or presence (long dashes) of TNF without added IgG. Asterisks (*) indicate that the mean is not significantly different from the mean for cells cultured in the absence of TNF (p > 0.05). B, HT29 cells were stimulated for 24 h with poly I/C (100 μg/ml) or LPS (1 μg/ml), in the presence of the indicated concentrations of neutralizing Ab to TNF. Steady-state levels of pIgR mRNA were measured by quantitative real-time RT-PCR and normalized to GAPDH mRNA. Data were calculated as percentage of the mean of control groups stimulated with dsRNA or LPS in the absence of anti-TNF Ab (indicated by long dashes), and are expressed as mean ± SEM (n = 6). Short dashes indicate the mean pIgR mRNA level in unstimulated HT29 cells. Data were pooled from two independent experiments. The asterisk (*) indicates that the mean is significantly lower than the mean for cells stimulated with dsRNA in the absence of anti-TNF Ab.
Activation of PIGR gene transcription by TLR3 and TLR4 signaling in IEC

To test the hypothesis that signaling through TLR3 and TLR4 activates PIGR gene transcription, HT29 cells were transfected with luciferase reporter plasmids containing an 8.6-kb fragment of the human PIGR gene, including ~2.7 kb of the 5′-flanking sequence, exon 1 (132 bp) containing the IRF-binding site, intron 1 (5751 bp) containing the NF-κB-binding site, and the first 56 bp of exon 2, up to and including the translational start site (Fig. 5A). In preliminary experiments we demonstrated that both dsRNA and LPS activated the wild-type PIGR plasmid with similar but slow kinetics. Mutation of the exon 1 IRF site caused a small but significant decrease in transcriptional activity, while mutation of the intron 1 NF-κB site caused a dramatic decrease (Fig. 5B). No additional decrease in transcriptional activity was observed when both the IRF and NF-κB sites were mutated. These data suggest that activation of PIGR gene transcription by TLR3 and TLR4 signaling is primarily mediated through binding of NF-κB to its cognate site in intron 1, and that binding of transcription factors of the IRF family to the exon 1 site may play a minor role.

FIGURE 5. Stimulation with dsRNA or LPS enhances transcription of PIGR gene reporter plasmids. A, PIGR reporter plasmids. The indicated regions of the human PIGR gene were cloned upstream of the firefly luciferase gene (see Materials and Methods). Mutations in the exon 1 IRF site and the intron 1 NF-κB site inhibit binding of the indicated transcription factors. B, HT29 cells were cotransfected with one of the PIGR reporter plasmids and a CMV-Renilla luciferase plasmid to normalize for transfection efficiency. Twenty-four hours after transfection, the cells were stimulated for 24 h with poly I.C (100 μg/ml) or LPS (1 μg/ml). Data were pooled from two independent experiments, and are expressed as fold increase in luciferase activity (normalized for cotransfected Renilla luciferase) of the same plasmid in response to dsRNA or LPS (mean ± SEM, n = 8). Asterisks (⁎) indicate that the mean is significantly less than the corresponding mean for the wild-type promoter plasmid (p < 0.05).

TLR3 signaling elicits a more potent inflammatory response than does TLR4 signaling in IEC

The requirement of NF-κB for PIGR gene transcription suggested that both TLR3 and TLR4 signaling resulted in activation of this transcription factor. To compare directly the ability of dsRNA and LPS to activate NF-κB, HT29 cells were transfected with an NF-κB reporter plasmid that contains four copies of a consensus NF-κB binding site, then treated for 3 or 6 h with dsRNA or LPS (Fig. 6A). dsRNA caused a significant increase in NF-κB reporter activity, but surprisingly, LPS had no effect. It is important to note that HT29 cells exhibited a high basal level of NF-κB activity (compare the luciferase activity of cells transfected with the control plasmid vs the NF-κB reporter plasmid in the absence of TLR signaling). dsRNA but not LPS also increased expression of the proinflammatory transcription factor IRF-1 (Fig. 6B). We have...
previously reported that expression of IRF-1 is correlated with induction of plgR expression by proinflammatory cytokines such as IFN-γ, TNF, and IL-1 (33, 40).

Because both dsRNA and LPS increased plgR mRNA levels >10-fold with similar kinetics, but only dsRNA caused a rapid increase in NF-κB activity and IRF-1 expression, we hypothesized that the pathways of TLR signaling leading to expression of anti-inflammatory gene products like plgR may differ from the pathways leading to expression of proinflammatory genes. To test this hypothesis, we measured steady-state mRNA levels of three proinflammatory target genes, TNF, IL-8, and RANTES, following stimulation of HT29 cells with dsRNA or LPS (Fig. 7A). Expression of all three proinflammatory genes increased rapidly following stimulation with dsRNA. By contrast, LPS had no effect on the level of RANTES mRNA, and induced IL-8 and TNF mRNA to a lesser extent than did dsRNA. Accumulation of secreted TNF, IL-8, and RANTES in culture supernatants followed the rise in mRNA levels (Fig. 7B). The level of secreted TNF was considerably less than the levels of IL-8 and RANTES, consistent with the lower levels of TNF mRNA. In fact, the modest increase in TNF mRNA in response to LPS stimulation did not translate into increased TNF secretion. It is significant to note that the time course of induction of proinflammatory gene expression was much more rapid than that of plgR (compare Figs. 1 and 7), consistent with usage of different signaling pathways.

The findings that dsRNA elicited a stronger proinflammatory response than did LPS (Fig. 7) and caused a modest increase in TLR4 mRNA levels (Fig. 2) suggested that pretreatment with dsRNA might enhance subsequent gene activation by LPS. Accordingly, HT29 cells were cultured for 24 h in the presence or absence of dsRNA, washed to remove residual dsRNA, then treated with LPS for 3 h (peak response time for proinflammatory genes) or 24 h (peak response time for plgR) (Fig. 8). Pretreatment with dsRNA enhanced the subsequent response to LPS of plgR (compare bars F and G) and IL-8 (compare bars C and D). By contrast, pretreatment with dsRNA had no effect on the subsequent response of TNF mRNA to LPS (data not shown).

Finally, we examined expression of Toll-interacting protein (Tollip), a negative regulator of LPS-mediated inflammation. We found that basal levels of Tollip mRNA were relatively high in HT29 cells, and did not increase significantly following stimulation with dsRNA or LPS (Fig. 9). Signaling through Tollip may play a role in down-regulation of proinflammatory gene expression by LPS.

**Discussion**

It has been estimated that plgR-mediated transport of IgA by IECs results in the daily delivery of 3 g of SIgA into the intestinal secretions of the average adult (41). SIgA in the intestine acts as the first line of Ag-specific immune defense against pathogens, and regulates inflammatory responses to pathogens as well as commensal bacteria. Because one molecule of plgR is consumed for every molecule of IgA transported, regulation of plgR expression is essential for maintenance of intestinal homeostasis. In this study, we report for the first time that expression of plgR is up-regulated by signaling through TLRs 3 and 4 (Figs. 1 and 2). Thus, signaling induced by microbes and their products may serve to augment plgR-mediated transcytosis of IgA, linking the innate and acquired immune responses to viruses and bacteria.

**FIGURE 7.** Responses of proinflammatory genes to dsRNA vs LPS stimulation. HT29 cells were cultured for the indicated times in the presence or absence of poly I:C (100 μg/ml) or LPS (1 μg/ml). A, Steady-state mRNA levels were measured by quantitative real-time RT-PCR and normalized to GAPDH mRNA. Data were pooled from four independent experiments for dsRNA (n = 12) and two independent experiments for LPS (n = 8), and are expressed as mean ± SEM. B, Levels of TNF, IL-8, and RANTES in culture media were measured by ELISA. Data are expressed as mean ± SEM. Data were pooled from two independent experiments for dsRNA (n = 8) and three independent experiments for LPS (n = 12). Asterisks (*) indicate that the mean is significantly greater than the corresponding mean for untreated cells (p < 0.05).
A major endpoint of all TLR signaling pathways is activation of the transcription factor NF-κB (reviewed in Refs. 42 and 43). We found that mutation of the NF-κB site in intron 1 of the PIGR gene dramatically reduced transcriptional activity of the PIGR promoter/enhancer region in response to both TLR3 and TLR4 signaling (Fig. 5). However, dsRNA but not LPS elicited rapid activation of NF-κB, as measured by a reporter gene assay. These apparently conflicting results may be reconciled by careful consideration of the respective signaling pathways. TLR3 and TLR4 are unique among TLRs in that, upon activation by dsRNA or LPS, they can recruit the adaptor protein Toll-IL-1R domain-containing adaptor-inducing IFN-β (TRIF) (29, 44). TLR4 (but not TLR3) can associate with an additional adaptor, TRIF-related adaptor molecule (TRAM), to form TRIF/TRAM heterodimers (45). Signaling through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differs in two important ways from the MyD88-dependent pathway common to all TLRs: first, activation through TRIF and TRAM differ in two important ways. Although up-regulation of plgR mRNA was relatively slow, up-regulation of proinflammatory gene expression was rapid (compare Figs. 1 and 7). Furthermore, dsRNA/TLR3 elicited a much stronger proinflammatory response than did LPS/TLR4, despite the fact that up-regulation of plgR mRNA was similar in response to both stimuli. It has been demonstrated that the IL-8, IRF1, TNF, and RANTES genes are rapidly transcribed in response to NF-κB response to TLR signaling was also delayed (data not shown). These kinetics are consistent with delayed NF-κB activation by TRIF, and suggest that the difference between TLR3 and TLR4 in rapid induction of NF-κB (Fig. 6A) may not be relevant in the context of PIGR gene activation (although this difference may be important for activation of proinflammatory genes; see below). It is important to note that HT29 cells have a high basal level of active NF-κB (in Fig. 6A, compare the luciferase activity in unstimulated cells transfected with the control plasmid vs the NF-κB reporter plasmid). Other investigators have reported high levels of active NF-κB in IEC lines, including HT29, and in normal IEC (reviewed in Ref. 47). The threshold level of NF-κB required for PIGR gene activation may be lowered by cooperativity with other transcription factors, such as IRF-3. Mutation of the IRF-binding site caused a significant decrease in PIGR reporter activity (Fig. 4B); however, the decrease was relatively small compared with the more dramatic effect of mutation of the NF-κB site, and mutation of both sites did not further decrease transcriptional activity beyond that seen with mutation of the NF-κB site alone.

An additional possibility is that different NF-κB family members may regulate transcription of PIGR and proinflammatory genes. Induction of proinflammatory gene expression by TLR signaling is generally thought to be mediated by activation of the p65/RelA component of NF-κB. In contrast, it was recently demonstrated that de novo-synthesized RelB mediates TNF-induced up-regulation of the human PIGR gene in HT29 cells (39). Activation of PIGR gene transcription by TNF was delayed, and involved cooperativity between the IRF site in exon 1 and the NF-κB site in intron 1. Significantly, the kinetics and NF-κB dependence of TNF regulation of PIGR gene transcription were almost identical to those which we report here for TLR3 and TLR4 signaling. It will be important to determine which NF-κB family members are involved in TLR-mediated induction of PIGR gene transcription.

Our data suggest that TLR signaling regulates expression of plgR and proinflammatory gene products through different pathways. Although up-regulation of plgR mRNA was relatively slow, up-regulation of proinflammatory gene expression was rapid (compare Figs. 1 and 7). Furthermore, dsRNA/TLR3 elicited a much stronger proinflammatory response than did LPS/TLR4, despite the fact that up-regulation of plgR mRNA was similar in response to both stimuli. It has been demonstrated that the IL-8, IRF1, TNF, and RANTES genes are rapidly transcribed in response to NF-κB

![FIGURE 8](http://www.jimmunol.org/) Effects of pretreatment with dsRNA on subsequent gene activation by LPS. HT29 cells were cultured for 24 h in the presence or absence of poly I:C (100 μg/ml), washed, then cultured for an additional 3 or 24 h in the presence or absence of LPS (1 μg/ml) (treatment groups A-G). mRNA levels were measured by real-time RT-PCR and normalized to GAPDH mRNA. Data are expressed as mean ± SEM (n = 3 for dsRNA, n = 4 for LPS). No significant differences were observed among treatment groups (p > 0.05).

![FIGURE 9](http://www.jimmunol.org/) Responses of the signaling molecule Tollip to dsRNA and LPS stimulation. HT29 cells were cultured for the indicated times in the presence or absence of poly I:C (100 μg/ml) or LPS (1 μg/ml). Steady-state mRNA levels were measured by quantitative real-time RT-PCR and normalized to GAPDH mRNA. Data are expressed as mean ± SEM (n = 3 for dsRNA, n = 4 for LPS). In the presence of poly I:C (100 μg/ml) or LPS (1 μg/ml), washed, then cultured for an additional 3 or 24 h in the presence or absence of LPS (1 μg/ml) (treatment groups A-G).
activation (48–51). Our observation that dsRNA but not LPS caused rapid activation of NF-κB in HT29 cells (Fig. 6A) is consistent with differences in the proinflammatory responses to these stimuli. Comparison of the TLR3 and TLR4 signaling pathways may help to explain their different proinflammatory potential. Tollip has been identified as a negative regulator of LPS-mediated inflammation (52–55). In response to signaling through TLR2 and TLR4 (but, interestingly, not TLR3), Tollip forms a complex with IL-1R-associated kinase and blocks downstream activation of NF-κB. High levels of Tollip have been detected in normal human intestine and IEC lines, and overexpression of Tollip was shown to cause rapid activation of NF-κB in HT29 cells (Fig. 9). Importantly, our data suggest that Tollip (and other potential negative regulatory molecules) do not block the ability of TLR4 signaling to up-regulate pIgR gene transcription. Although we observed similar patterns of pIgR gene transcription in response to TLR3 and TLR4 stimulation, we cannot rule out the existence of unique as well as shared signaling pathways. For example, we found that neutralization of secreted TNF reduced dsRNA- but not LPS-stimulated induction of pIgR mRNA (Fig. 4B). Delineation of these shared and unique pathways will be important to our understanding of the role of TLR signaling in intestinal homeostasis.

The biological consequences of TLR3 and TLR4 signaling are uniquely suited to the intestinal environment. It has recently been reported that infection of HT29 cells with reovirus, an intestinal virus with a dsRNA genome, results in up-regulation of pIgR expression (18). UV-inactivated reovirus also induced pIgR expression, suggesting that viral replication was not required for signaling. Inhibition of endosomal acidification blocked reovirus-mediated up-regulation of pIgR, consistent with the hypothesis that viral uncoating and release of the dsRNA genome is required for TLR3 signaling. Up-regulation of pIgR by TLR3 signaling during viral infections is likely to facilitate both intracellular and extracellular neutralization of viruses by IgA. Induction of cytokine and chemokine expression should enhance both innate and adaptive antiviral defense mechanisms. Up-regulation of pIgR expression by TLR4 signaling could regulate inflammatory responses against commensal Gram-negative bacteria in several ways. Enhanced pIgR-mediated transport of anti-LPS IgA Abs would limit the proinflammatory potential of LPS in the intestinal lumen and within epithelial cells (17). IgA Abs could further prevent invasion of the epithelium by bacteria through immune exclusion and scavenger properties (reviewed in Ref. 58). Modulation of TLR4-mediated induction of proinflammatory cytokines and chemokines would be essential for prevention of chronic inflammation in the presence of Gram-negative commensal organisms. Studies in humans and animals suggest a role for a dysregulated host-microbe relationship in the pathogenesis of inflammatory bowel disease (reviewed in Refs. 59 and 60). Interestingly, reduced pIgR expression has been observed in regenerating and dysplastic colonic epithelium from patients with ulcerative colitis (61). It is possible that dysregulation of TLR signaling in inflammatory bowel disease may cause both overproduction of proinflammatory mediators and underproduction of anti-inflammatory mediators such as pIgR. Studies in humans and experimental animals have clearly demonstrated that underlying viral infections can enhance the proinflammatory response to LPS (reviewed in Ref. 62). In this study, we demonstrate that prior exposure of HT29 cells to dsRNA enhanced the subsequent LPS-mediated increase in IL-8 expression (Fig. 8), suggesting that TLR3 signaling may render IEC more prone to LPS-induced inflammatory responses. Significantly, TLR3 signaling also enhanced LPS-mediated up-regulation of pIgR expression, a response that would be expected to promote antiviral and antibacterial immunity as well as to assist in controlling the inflammatory response. Several mechanisms could account for TLR3-mediated enhancement of LPS signaling, including up-regulation of TLR4 expression (Fig. 2), increased activation of NF-κB (Fig. 6A), and indirect effects of proinflammatory cytokines (Fig. 4B).

In summary, our data suggest a model whereby TLR3 and TLR4 signaling specifically enhance pIgR expression in the intestine to promote the immune and anti-inflammatory functions of SlgA in response to viral and bacterial pathogens. We further hypothesize that commensal bacteria may be important for maintaining the “tone” of pIgR expression in the gut. This hypothesis is supported by the finding that intestinal pIgR mRNA levels were up-regulated when germfree mice were colonized with B. thetaiotaomicron (20). In humans, the colonic epithelium is normally exposed to >10^14 CFUs of commensal bacteria, with Gram-negative organisms making up the majority by weight (60). It is likely that a variety of bacterial patterns, including those that signal through TLR2, TLR5, and TLR9 as well as TLR4, may be important determinants of pIgR expression in the intestine.

Disclosures

The authors have no financial conflicts of interest.

References


