Expression and Function of Toll-Like Receptors in Eosinophils: Activation by Toll-Like Receptor 7 Ligand

Hiroyuki Nagase, Shu Okugawa, Yasuo Ota, Masao Yamaguchi, Hideyuki Tomizawa, Kouji Matsushima, Ken Ohta, Kazuhiko Yamamoto and Koichi Hirai

J Immunol 2003; 171:3977-3982; doi: 10.4049/jimmunol.171.8.3977
http://www.jimmunol.org/content/171/8/3977

References
- This article cites 39 articles, 20 of which you can access for free at:
  http://www.jimmunol.org/content/171/8/3977.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

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2003 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Expression and Function of Toll-Like Receptors in Eosinophils: Activation by Toll-Like Receptor 7 Ligand

Hiroyuki Nagase, Shu Okugawa, Yasuo Ota, Masao Yamaguchi, Hideyuki Tomizawa, Kouji Matsushima, Ken Ohta, Kazuhiko Yamamoto, and Koichi Hirai

We investigated the expression of a panel of Toll-like receptors (TLRs) and their functions in human eosinophils. Eosinophils constitutively expressed TLR1, TLR4, TLR7, TLR9, and TLR10 mRNAs (TLR4 greater than TLR1, TLR7, TLR9, and TLR10 greater than TLR6). In contrast, neutrophils expressed a larger variety of TLR mRNAs (TLR1, TLR2, TLR4, TLR6, TLR8 greater than TLR5, TLR9, and TLR10 greater than TLR7). Although the expression levels in eosinophils were generally less prominent compared with those in neutrophils, eosinophils expressed a higher level of TLR7. Furthermore, among various TLR ligands (S-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-Cys-Ser-(Lys)₄, poly(I:C), LPS, R-848, and CpG DNA), only R-848, a ligand of TLR7 and TLR8, regulated adhesion molecule (CD11b and L-selectin) expression, prolonged survival, and induced superoxide generation in eosinophils. Stimulation of eosinophils by R-848 led to p38 mitogen-activated protein kinase activation, and SB203580, a p38 mitogen-activated protein kinase inhibitor, almost completely attenuated R-848-induced superoxide generation. Although TLR8 mRNA expression was hardly detectable in freshly isolated eosinophils, mRNA expression of TLR8 as well as TLR7 was exclusively up-regulated by IFN-γ but not by either IL-4 or IL-5. The up-regulation of the TLRs by IFN-γ had potentially functional significance: the extent of R-848-induced modulation of adhesion molecule expression was significantly greater in cells treated with IFN-γ compared with untreated cells. Although the natural ligands for TLR7 and TLR8 have not yet been identified, our results suggest that eosinophil TLR7/8 systems represent a potentially important mechanism of a host-defense role against viral infection and mechanism linking exacerbation of allergic inflammation and viral infection. The Journal of Immunology, 2003, 171: 3977–3982.

Massive accumulation of eosinophils is a characteristic feature of inflammation associated with allergic diseases such as bronchial asthma and atopic dermatitis. Infiltrating eosinophils are strongly implicated in the pathogenesis of these disorders by virtue of their capacity to release an array of tissue-damaging mediators (1). Although Ag-mediated immune responses are central mechanisms directing eosinophil inflammation, several lines of evidence have indicated that bacterial and/or viral infections also modulate allergic inflammation. For example, viral or bacterial infections often precede asthma exacerbation in both children and adults (2, 3). Exacerbation of atopic dermatitis is associated with infections by bacteria, such as Staphylococcus aureus (4). The link between infection and exacerbation of allergic diseases may consist of various pathways having multiple steps, but direct activation of eosinophils by microbe-derived molecules potentially represents one clear explanation of such mechanisms.

As functionally important receptors for recognition of conserved motifs in pathogens termed pathogen-associated molecular patterns (PAMPs) (5), the Toll-like receptors (TLRs) have been identified in mammals. TLRs share the Toll/IL-1R homology domain, and 10 different human TLR proteins have been identified and cloned to date (6–10). Expression of TLR4 in eosinophils has been reported by several groups (11, 12), but the precise expression profiles and functions of other TLRs have remained largely unclear. In this study, we explored TLR expression and their functions in eosinophils, and we have shown that a TLR7 ligand, R-848 (13), is capable of activating eosinophils.

Materials and Methods

Reagents

The following reagents were purchased as indicated: S-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-Cys-Ser-Lys)₄ (Pam3CSK₄; EMC Microcollections, Tubingen, Germany); poly(I:C), LPS (Escherichia coli, 026:B6), cytomegalovirus, superoxide dismutase (Sigma-Aldrich, St. Louis, MO); PMA (Calbiochem, San Diego, CA); rIL-4, rIL-5 (PeproTech, Rocky Hill, NJ); rIFN-γ (Shionogi Pharmaceutical, Osaka, Japan), and SB203580 (Promega, Madison, WI). R-848 was synthesized in Pharmaceuticals and Biotechnology Laboratory, Japan Energy Corporation (Saitama, Japan). R-848 was dissolved in DMSO to yield a stock solution of 10 mM, and corresponding concentrations of DMSO were used as controls in every experimental setting. Phosphorothioate-stabilized CpG DNA 2006 (TCTGCGTGTTCGTTTGTGCGT) was purchased from Hokkaido System Science (Sapporo, Japan).
Cell preparation and culture conditions

Eosinophils were isolated by density centrifugation followed by negative selection using anti-CD14-bound micromagnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) from normal volunteers, as previously described (14). Neutrophils were purified by two-step density centrifugation, as previously described (15). Both cells were further purified by negative selection using anti-CD14-bound micromagnetic beads (Miltenyi Biotec). Anti-CD14-bound micromagnetic beads neither induced nor stimulated the LPS-induced IL-8 generation by neutrophils (data not shown). Cytoospin preparations were stained with May-Grünwald-Giemsa, and differential counts were determined. Purity of eosinophils and neutrophils were >99.9% and >98.6% ± 0.4%, respectively, and their viability was consistently >95%. In some experiments, PBMC purified by Ficoll-Paque (Pharmacia, Uppsala, Sweden) from venous blood were subjected to positive selection using anti-CD14-bound micromagnetic beads. Purity of CD14+ monocytes was 98.2 ± 0.3%.

Unless otherwise stated, eosinophils and neutrophils were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Sigma-Aldrich) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in 5% CO2 in a total volume of 200 µl in flat-bottom 96-well culture plates (Iwaki Glass, Chiba, Japan).

Real-time quantitative PCR analysis of TLR mRNA

Total RNA was extracted from 3–10 × 105 cells using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany), and the first-strand cDNA was reverse-transcribed as previously described (14). The purity of cDNA preparations was consistently >99.8%. Real-time PCR was performed as previously described (15). Briefly, the TLR gene-specific primers and FAM-labeled probes were purchased from Applied Biosystems (Foster City, CA) and their sequences are presented in Table I. Input cDNA was normalized using a β-actin primer/probe pair (Applied Biosystems) as an internal control gene. The standard curve was constructed with serial dilutions of specific PCR products, which were obtained by amplifying specific TLR cDNA sequences (Table I). The amplification efficiency (E = 10–1/slope) was calculated from the slope of the standard curve (16), and the primers used had high efficacy of between 0.99 and 1.0. The correlation coefficient of the standard curve was always >0.997.

Flow cytometry

Flow cytometric analysis was performed as previously described (14). Briefly, purified cells were stained with FITC-conjugated anti-CD11b mAb (Bear 1; Beckman Coulter, Tokyo, Japan) or PE-conjugated anti-L-selectin mAb (DREG-56; eBioscience, San Diego, CA). To investigate TLR expression, cells were incubated with anti-TLR1 mAb (clone GD2,F4; eBioscience), anti-TLR2 mAb (clone TL2.1; eBioscience), or anti-TLR4 mAb (DREG-56; eBioscience, San Diego, CA). To investigate TLR expression, cells were stained with FITC-conjugated anti-CD11b mAb (clone 198.1; Serotec, Raleigh, NC) and the membranes were then incubated with HRP-conjugated goat F(ab')2 against mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Stained cells were analyzed using EPICS XL SYSTEM II (Coulter, Miami, FL). At least 3000 cells were analyzed to calculate the median value of fluorescence intensity. The median values of fluorescence intensity of cells were converted to the number of molecules of equivalent soluble fluorochrome units (MESF) using a Quantum Fluorescence kit (Sigma-Aldrich) on each day of an experiment, as previously described (17). Surface receptor levels expressed in MESF units were calculated using the following formula: MESF = (MESF of cells stained with anti-adhesion molecule mAb) − (MESF of cells stained with isotype control IgG).

Quantification of cell survival

Analysis of cell survival was performed as previously described (14). Briefly, live cells were quantitatively determined by negative staining for annexin V and propidium iodide.

Superoxide generation

Generation of superoxide by eosinophils was measured by superoxide dismutase-inhibitable reduction of cytochrome c, as previously described with slight modification (18). In brief, purified cells were resuspended in HBSS (Life Technologies) with 0.1% gelatin and 0.1% BSA. The reaction wells of 96-well culture plates were measured for absorbance at 550 nm in an ELISA reader (Bio-Rad, Hercules, CA). Superoxide generation was calculated with an extinction coefficient of 21.1 × 103 M−1 cm−1 for reduced cytochrome c at 550 nm and was expressed as nanomoles of cytochrome c reduced per 1 × 106 cells.

Western blot analysis

Purified eosinophils were lysed with ice-cold lysis buffer containing 1% Nonidet P-40, 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM leupeptin, and 1 mM PMSF. Whole-cell lysates were subjected to SDS-PAGE (10% polyacrylamide) and then electrically transferred to Hybond ECL membranes (Amersham, Chalfont St. Giles, U.K.). After blocking, the membranes were incubated with anti-p38 MAPK Ab (Ab) or anti-phosphorylated-p38 MAPK Ab (Cell Signaling, Beverly, MA) for 2 h at room temperature. The membranes were then incubated with HRP-conjugated anti-rabbit IgG Ab (DAKO, Glostrup, Denmark) for 30 min. The reactive bands were visualized with an ECL system (Amersham).

Statistical analysis

All data are expressed as the mean ± SEM. Unless otherwise noted, the differences between values were analyzed by the one-way ANOVA test. When this test indicated a significant difference, Fisher’s protected least significant difference test was used to compare individual groups.

Results

TLR mRNA expression in eosinophils

Previous studies demonstrated positive TLR4 mRNA and controversial TLR2 mRNA expression in eosinophils (11, 12), but expression of other TLR mRNAs has not been investigated to date. In our first series of experiments, by using real-time PCR, we quantified the expression levels of transcripts of TLR1–10 in eosinophils in comparison with neutrophils. As shown in Fig. 1, eosinophils expressed transcripts of TLR1, TLR7, TLR9, and TLR10 in addition to TLR4. Although weak expression of TLR6 mRNA was observed in eosinophils, expression of TLR2, TLR3, TLR5, and TLR8 mRNAs was virtually undetectable. Previous studies showed that neutrophils express TLR1, TLR2, and TLR4 (12, 19). In addition to these transcripts, we found apparent expression of mRNAs for TLR5, TLR6, TLR8, TLR9, and TLR10 in neutrophils. A low level of TLR7 mRNA was also detected. When the expression levels of TLRs were compared between eosinophil and...
neutrophils, eosinophils generally expressed lower levels of TLR mRNA compared with neutrophils except for TLR7.

By using established Abs against TLRs, we studied the surface expression of TLR1, TLR2, and TLR4 on eosinophils. Consistent with a previous report by other researchers (12), our results of flow cytometry experiments demonstrated the absence of surface expression of TLR2 and TLR4 on eosinophils. We observed marginal TLR1 expression in one of three samples, but the expression was undetectable in the other samples (n = 3, data not shown).

Eosinophil activation by PAMPs

In the next series of experiments, we studied the functional properties of eosinophil TLRs. For this purpose, we investigated the effect of various PAMPs on several eosinophil functions, i.e., adhesion molecule expression, survival, and superoxide generation. As shown in Fig. 2, A and B, CD11b expression was up-regulated and L-selectin expression was conversely down-regulated by an antiviral reagent, R-848, a ligand of TLR7. On the other hand, other PAMPs, including Pam3CSK4 (TLR2) (20), poly(I:C) (TLR3) (21), LPS (TLR4) (22) and CpG DNA (TLR9) (23, 24), exhibited no significant effects at all. Dose-dependent experiments revealed that 1 μM of R-848 was sufficient to affect the expression of CD11b and L-selectin in eosinophils (Fig. 2, C and D). In contrast, a higher dose of R-848 was required to show a significant effect on either CD11b or L-selectin expression in neutrophils.

The effect of PAMPs on eosinophil survival is exhibited in Fig. 3A. The results of survival enhancement were virtually identical with those of adhesion molecule expression. R-848 significantly prolonged in vitro eosinophil survival. Although LPS slightly enhanced eosinophil survival, the effect did not reach statistical significance. Other TLR ligands showed no significant effect at all. The dose-response curves of survival paralleled those of adhesion molecule expression (Fig. 3B). Although R-848 prolonged neutrophil survival, a higher dose of R-848 was required to exert a significant effect.

As shown in Fig. 4A, R-848 induced superoxide generation in eosinophils. Again, none of the other TLR ligands elicited significant superoxide production at all. Neutrophil superoxide generation was also induced by R-848, but the generation was retarded when compared in eosinophils; statistically significant generation was observed only after 180 min of incubation (Fig. 4B).

Activation of p38 MAPK in eosinophils stimulated by R-848

To verify that intracellular signaling pathways of eosinophils are actually activated by R-848, we investigated the phosphorylation of p38 MAPK, which has been demonstrated to be a hallmark of cellular activation via TLRs (25). As shown in Fig. 5A, stimulation of eosinophils with R-848 resulted in apparent phosphorylation of p38 MAPK after 15 min of stimulation. Furthermore, p38 MAPK functionally regulated the R-848-induced superoxide generation in eosinophils; a p38 MAPK inhibitor, SB203580, almost completely attenuated the superoxide generation in eosinophils (Fig. 5B).

Cytokine-mediated regulation of TLR mRNA expression in eosinophils

In the last series of experiments, we examined the modulation of eosinophil TLR expression by eosinophil-active cytokines of both Th1 and Th2 origin. Eosinophils were stimulated with single concentrations of cytokines based on our previous study of CXCR4 expression in eosinophils (14). As shown in Fig. 6A, expression of transcripts of TLR2 and TLR3 remained at low levels in eosinophils stimulated for 4 h with IL-4, IL-5, or IFN-γ. These cytokines also failed to induce significant changes in the expression level of TLR9. On the other hand, TLR4 mRNA expression was increased 2- to 3-fold by IFN-γ with statistical significance. However, the most prominent change in expression was observed for both TLR7.
and TLR8. IFN-γ exclusively and strongly (~10-fold) up-regulated the expression of TLR7 mRNA in eosinophils. Despite negligible expression in resting eosinophils, de novo expression of TLR8 at levels comparable to TLR7 was observed in IFN-γ-treated cells.

To determine whether the IFN-γ-induced up-regulation of the expression of TLR7 and TLR8 affects the biologic response to the ligand, we compared R-848-induced changes in adhesion molecules in cells cultured for 4 h in the presence and absence of IFN-γ. As shown in Fig. 6B, the extent of increase in CD11b expression as well as decrease in L-selectin expression was significantly greater in cells treated with IFN-γ compared with untreated cells, suggesting functional relevance of IFN-γ-mediated up-regulation of the expression of TLR7 and/or TLR8 in eosinophils.

Discussion

The expression profiles of TLRs as well as their functions have been increasingly clarified in various immune cells (26), but little information is available for eosinophils except for TLR4 (12). In the present study, we for the first time examined the mRNA expression of a panel of TLRs in eosinophils and found that eosinophils constitutively expressed mRNA of TLR1, TLR7, TLR9, and TLR10 in addition to TLR4. The expression profile in eosinophils was qualitatively and quantitatively different from that in neutrophils. In contrast to eosinophils, neutrophils expressed almost all TLR mRNAs. The ubiquitous expression profile in neutrophils may reflect their important role as first-line effector cells in host defense. Furthermore, eosinophil TLR mRNA expression was generally weak compared with in neutrophils. Eosinophils expressed ~10 and ~50 times lower levels of TLR4 and TLR1, respectively, compared with neutrophils.

The TLR4 ligand LPS represents the most extensively studied PAMP, but previous reports have shown conflicting results regarding the effects of LPS on human eosinophils. Initial studies indicated that LPS was able to activate eosinophils and prolong their life span (11, 27). However, more recent studies demonstrated that LPS was totally inactive when monocytes were depleted from eosinophil preparations (12, 28). The results of our present experiments using CD14+ cell-depleted preparations showed that LPS did not affect any of the tested eosinophil functions, lending further support to the concept that LPS is totally inactive on eosinophils in the absence of monocytes. The absence of functional significance of LPS on eosinophils results from the lack of surface protein expression of TLR4 in these cells. Consistent with a previous report (12), we found no significant protein expression of TLR4 on the eosinophil surface, even though they expressed transcripts of TLR4. Thus, mRNA expression of TLRs does not always guarantee the expression of functional proteins. Furthermore, it is also likely that eosinophils have impaired machinery for transporting TLR4 proteins to the cell surface, such as MD-2 (29).

Expression of TLR transcripts in eosinophils was generally less prominent compared with neutrophils, but a higher level of TLR7 mRNA expression was observed in eosinophils. Because specific Abs for exploring the protein expression of TLR7 are not available at present, and mRNA expression does not definitively guarantee the expression of a functional protein, we should be careful to conclude that there is expression of TLR7 on the cell surface without any protein data. However, our results of functional studies strongly suggested the expression of functional TLR7 on eosinophils. Among various TLR ligands tested, only R-848, a ligand of

![FIGURE 3](image-url)  
**FIGURE 3.** The effect of PAMPs on eosinophil survival. *A,* Eosinophils were cultured for 20 h in the presence or absence of PAMPs, and the viability was analyzed by flow cytometry. The data are expressed as the percentage of live cells of total inoculated cells. *B,* Dose-dependent effect of R-848 on survival of 20-h cultured eosinophils or 15-h cultured neutrophils. **, *p < 0.01, *p < 0.05 vs value of Nil or DMSO (for R-848) (*A*), and vs value in cells treated with corresponding concentration of DMSO (*B*).

![FIGURE 4](image-url)  
**FIGURE 4.** Effect of PAMPs on superoxide generation in eosinophils. *A,* The effect of PAMPs on superoxide generation in eosinophils after 180-min incubation. **, *p < 0.01 vs value of Nil or DMSO (for R-848). *B,* The time course of superoxide generation. The difference between superoxide generation in R-848-treated (10 μM) and DMSO-treated (0.1%) cells at the same time point was tested by the paired *t* test (**, *p < 0.01, *p < 0.05).
both TLR7 and TLR8 (13, 30), was active on freshly isolated eosinophils in modulation of adhesion molecule expression, prolongation of survival, and induction of superoxide generation. In addition, R-848 induced phosphorylation of p38 MAPK, known to be involved in signal transduction via various TLRs (25), while inhibition of p38 MAPK resulted in almost complete attenuation of R-848-induced superoxide generation in eosinophils. R-848 is a low molecular weight compound of the imidazoquinoline family with antiviral properties (31). The specificity of R-848 for human TLR7 and TLR8 has been confirmed by genetic complementation by several groups (13, 30, 32). Activation of HEK293 cells by R-848 was observed when cells were transfected with only human TLR7 or TLR8 but not with human TLR2, TLR3, or TLR9 (32). Although R-848 has been shown to activate both TLR7 and TLR8, TLR7 shows 10-fold higher sensitivity to R-848 (30). Furthermore, eosinophils constitutively expressed considerable levels of TLR7 mRNA, whereas TLR8 mRNA was hardly detectable in freshly isolated cells. Taken together, these findings strongly suggest that TLR7 is mainly responsible for R-848-induced activation of resting eosinophils. However, the fact that TLR8 mRNA expression became apparent during stimulation with IFN-γ suggests the possible involvement of TLR8 in eosinophil activation under certain conditions. Future development of selective blocking Abs or antagonists against TLR7 and TLR8 will clarify this issue.

The concentration of R-848 required for activation of eosinophils (1 μM) is high compared with the concentrations reported for other types of cells such as dendritic cells (0.01–0.1 μM) (33, 34). We suppose that the difference in the concentrations potentially results from differences in the expression levels of TLR7 among cell types. We observed that monocytes expressed 

\[
0.13 \pm 1.26 \text{ vs } 4.1 \pm 0.37, \text{ indicated as TLR7 mRNA copies/β-actin copies} \times 10^4, n = 4.
\]

Several studies have shown that dendritic cells express even higher levels of TLR7 mRNA compared with monocytes (33, 35, 36). Although the precise differences in surface TLR7 protein levels among these cell types are not fully known, it could be reasonably stated that relatively higher concentrations of agonist are required to occupy enough receptor molecules in eosinophils with a low level of TLR7 expression.

The natural ligands of TLR7 and TLR8 have still not been identified, but they are strongly assumed to be either some viral compounds or endogenous molecules generated during viral infections. To date, several lines of evidence have revealed that eosinophils express binding sites for several viruses, such as ICAM-1 (37), but little is known regarding the direct activation of eosinophils by viruses or their products. In this context, TLR7/8 systems may represent unique and potentially important mechanisms of eosinophil activation, linking viral infection with exacerbation of allergic inflammation. In fact, expression of TLR7 and TLR8 was up-regulated exclusively by IFN-γ but not by either IL-4 or IL-5. Although we cannot fully exclude the possibility that modulation of CD11b expression in IFN-γ-treated (300 U/ml) or nontreated cells stimulated with the same concentration of R-848 was tested by the paired t test (\( p < 0.05, n = 3 \)).
with neutrophils, but a higher level of TLR7 expression was observed in eosinophils. TLR7 expression as well as TLR8 expression was up-regulated by treatment with IFN-γ and R-848, a ligand of both TLR7 and TLR8, exclusively activated eosinophilic functions. Stimulation of eosinophils via TLR7 and/or TLR8 may be involved in the exacerbation of allergic inflammation during viral infection. Furthermore, because eosinophil-derived neutrotoxin and eosinophil cationic protein potentially exert antiviral effects because of their strong RNase activities (38, 39), these receptors may also play a host-defensive role against viral infection.

Acknowledgments

We thank Chise Tamura and Masako Imanishi for their excellent technical assistance. Thanks are also extended to Sachiko Takeyama for valuable secretarial help.

References


