

Toll-like receptor 2 ligands activate human basophils for both IgE-dependent and IgE-independent secretion

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Background: Toll-like receptor (TLR) molecules play a critical role in directing the course of acquired immunity, including that associated with allergic disease, by recognizing specific microbial products that activate immune cells for effector functions.

Objective: We investigated whether human basophils express 2 such molecules (TLR2 and TLR4), and assessed whether putative ligands for these receptors activate nuclear factor κ B (NF κ B) and modulate mediator release and cytokine secretion either alone or in response to stimulation.

Methods: Toll-like receptor expression was assessed by using RT-PCR and flow cytometry. Immunoblotting detected nuclear NF κ B. Automated fluorometry, RIA, and ELISA detected concurrent changes in histamine, leukotriene C₄, and cytokine, respectively, after culture with specific ligands.

Results: mRNA and protein for TLR2 and TLR4 were detected in basophils. However, in assessing nuclear localization of NF κ B as a measure of functional receptor responses, basophils selectively reacted only to peptidoglycan, a TLR2 ligand, and not to LPS, a TLR4 ligand. Likewise, basophils secreted both IL-4 and IL-13 in direct response to peptidoglycan but not to LPS. Although neither ligand induced histamine or leukotriene C₄ release, several TLR2-specific ligands augmented the secretion of these mediators (and cytokine) in response to IgE-dependent activation and of IL-13 in response to IgE-independent stimulation. Finally, a selective inhibitor of NF κ B did not prevent these enhancing effects mediated by TLR2 ligands.

Conclusion: These data suggest that innate immune responses mediated through TLR2 play a role in augmenting allergic reactions, in part by modulating basophil cytokine secretion and mediator release independently of NF κ B activation. (J Allergy Clin Immunol 2005;115:295-301.)

Key words: Innate immunity, basophil, IgE, cytokines, Toll-like receptors, allergy, human

Abbreviations used

HPRT: Hypoxanthine phosphoribosyl transferase
LT: Leukotriene
NF κ B: Nuclear factor κ B
P/I: Phorbol 12-myristate 13-acetate/ionomycin
TLR: Toll-like receptor

Identification of Toll-like receptor (TLR) molecules has sparked renewed interest in innate immunity through the notion that these proteins play a critical role in directing the course of acquired immunity, including that associated with allergic disease.¹ Eleven of these receptors (TLR1-11) have been identified, and there is increasing evidence that they are selectively expressed among immune cells, where they induce a variety of responses.^{1,2}

Human basophils were recently found to express high levels of TLR2 and TLR4 compared with other granulocytes, raising the possibility of TLR involvement in mediating basophil responses.³ Because peptidoglycan and LPS are respective ligands for TLR2 and TLR4 and they play a pivotal role in cytokine synthesis by other immune cells, we tested whether these microbial products modulate IL-4 and IL-13 produced by basophils. In addition to confirming TLR2 and TLR4 expression, we show evidence that only the former mediates biological activity in basophils. In particular, peptidoglycan selectively induces IL-4 and IL-13 without inducing mediator release. Moreover, this microbial product, and to a lesser extent other TLR2 ligands, are capable of augmenting IgE-dependent secretion of all mediator classes (*ie*, cytokine, histamine, and leukotriene) as well as IgE-independent secretion of IL-13. These novel findings add to the belief that specific innate immune responses, particularly those mediated through TLR2 signaling, play a critical role in augmenting and/or sustaining allergic reactions, in part by modulating basophil cytokine secretion and mediator release.

METHODS

Special reagents

The following reagents were purchased: LPS (detoxified from *Salmonella minnesota*; Sigma Chemical Co, St Louis, Mo); Percoll (Pharmacia Biotech, Piscataway, NJ); recombinant human IL-3 (Biosource, Camarillo, Calif); and peptidoglycan (derived from

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Staphylococcus aureus; Fluka US, Milwaukee, Wis). The synthetic lipopeptide Pam₃Cys was purchased from EMC Microcollections (Tuebingen, Germany). The polyclonal antihuman IgE antibody used in these experiments was made in a goat.⁴ mAbs to TLR2 and TLR4 (both of the IgG2a isotype) were from eBioscience (San Diego, Calif). A peptide inhibitor of NFκB (SN50) and its control peptide (SN50-M)⁵ were from Calbiochem-Behring Corp (La Jolla, Calif).

Basophil preparation

Peripheral blood was taken with informed consent from healthy volunteers in accordance with a protocol approved by the Western Institutional Review Board. In some experiments (eg, nuclear localization of nuclear factor κB [NFκB]), residual cells of normal donors undergoing leukopheresis were used. Basophil-enriched cell suspensions were prepared on double Percoll gradients as previously described and then further purified by using negative selection.^{6,7} Basophil counts were performed by using Alcian blue staining,⁸ which indicated purities ≥98%.

Culture conditions

For the release of mediators (histamine and leukotriene [LT] C₄) and secretion of cytokines, basophils were cultured in 96-well flat-bottomed microtiter plates (Costar, Cambridge, Mass) by using conditioned Iscove's modified Dulbecco medium, as previously described.⁶ Cells were added to duplicate wells in a volume of 0.100 mL. After a 15-minute equilibration incubation to 37°C, 5% CO₂, an equal volume of peptidoglycan, LPS, or medium alone, with and without IL-3, was added, and the cells were incubated for 3 to 18 hours. For experiments investigating IgE-dependent mediator release and IL-4 secretion, an additional 0.050 mL medium containing anti-IgE at 5 times the final concentration was added, with the cells incubated just 3 hours. Cell-free supernatants were harvested from the 3-hour cultures, with portions analyzed for histamine, LTC₄, and/or cytokine, as previously described.⁹ IL-4 and IL-13 protein were measured in 3-hour and 18-hour culture supernatants, respectively, by using ELISAs from Immunotech (Marseilles Cedex, France) (IL-13) and eBioscience (San Diego, Calif) (IL-4), each performed to the manufacturer's specifications.

Flow cytometry

Indirect staining for cell surface TLR2 and TLR4 protein was attained with unlabeled monoclonal antibodies (eBioscience), followed with phycoerythrin-conjugated antimouse IgG (Fab')₂ antibody using methods previously described.¹⁰ Flow cytometry for single-color staining was performed by using a FACSCalibur machine (BD PharMingen, San Diego, Calif).

Qualitative RT-PCR

Total RNA was isolated from basophils ($0.5\text{--}1.0 \times 10^6$) by using the RNA-BEE protocol (Tel-test, Friendswood, Tex). Methods for conventional RT and PCR have been described elsewhere.¹⁰ Primers were checked for specific sequences within the TLR2 and TLR4 genes (accession numbers NM 003264 and NM 003266, respectively). Primer sequences (Invitrogen, Carlsbad, Calif) were as follows: TLR2, forward primer sequence: 5'-GGCCAGCAAATTACCTGTGTG-3', and reverse primer sequence: 5'-CCAGGTAGGTCTTG-GTGTTC-3'; TLR4, forward primer sequence: 5'-CTGCAATGG-ATCAAGGACCA-3', and reverse primer sequence: 5'-TCCCACT-CCAGGTAAGTGTT-3', as reported.¹¹ Hypoxanthine phosphoribosyl transferase (HPRT) was used for housekeeping gene expression, as described elsewhere.¹² Resolution of the cDNA products after

amplification (35 cycles) was performed in 3% agarose gels by using ethidium bromide staining.

Western blot analysis

For NFκB localization, basophils (3×10^6 /condition) were stimulated with peptidoglycan, LPS, or controls for 1 hour in Iscove's modified Dulbecco medium. Nuclear extracts were then prepared by using a commercially available kit (Active Motif, Carlsbad, Calif) and were diluted with an equal volume of SDS Tris-glycine sample buffer (Invitrogen) containing 10% 2-mercaptoethanol and 1X protease inhibitors (BD Pharmingen, San Diego, Calif). Electrophoresis, protein transfer, and immunoblotting using a monoclonal NFκB (p65 subunit) antibody (Transduction Laboratories, San Diego, Calif) were performed as previously described.¹³ Horseradish peroxidase-conjugated antirat and antimouse secondary antibodies were used for visualization with enhanced chemiluminescence.

Imaging acquisition

Image acquisition of gel and blot exposures was performed with an Electrophoresis Documentation and Analysis System 290 (Kodak Scientific Imaging Systems, New Haven, Conn). Densitometry was performed by using NIH Image (Wayne Rasband, National Institutes of Health, Bethesda, Md).

Statistics

Statistical analyses were performed with Statview software (SAS Institute Inc, Cary, NC) and involved the use of Wilcoxon non-parametric and paired *t* tests.

RESULTS

Expression of TLR2 and TLR4 by human basophils

As shown in Fig 1, RT-PCR analysis indicated that basophils (>98% purity) constitutively express mRNA for both TLR2 and TLR4 at levels comparable with the housekeeping gene, HPRT (A). Surface protein for both TLR2 and TLR4 was also detected by using flow cytometry (B), confirming the report by Sabroe et al³ that found these receptors on human basophils at levels comparable with those seen on neutrophils.

Peptidoglycan, but not LPS, induces nuclear localization of NFκB in basophils

Because TLR/ligand interactions activate NFκB,¹⁴ we investigated the capacity of peptidoglycan and LPS to induce nuclear localization of this transcription factor in basophils. Fig 2, A, shows a representative experiment in which the nuclear extracts of cells treated 1 hour with peptidoglycan contain immunoreactivity for the p65 subunit of NFκB, as assessed by Western blot analysis. In contrast, cells treated in this manner with LPS showed much less of the p65 subunit and were no different from cells treated in medium alone. For comparison, nuclear extracts from an equal number of basophils treated with phorbol 12-myristate 13-acetate/ionomycin (P/I) used as a positive control showed prominent immunoreactivity.

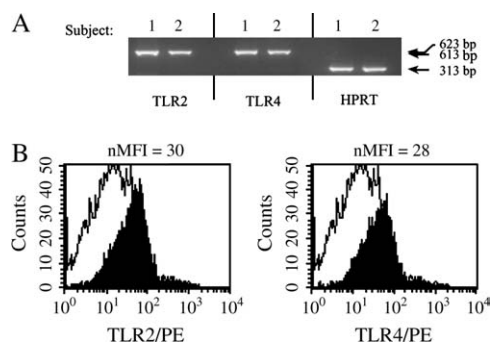


FIG 1. Expression of TLR2 and TLR4 by human basophils (>98% purity) isolated from blood. **A**, RT-PCR results with TLR2 and TLR4 compared with the housekeeping gene, HPRT. **B**, Representative flow cytometry showing net mean fluorescence intensity (nMFI) comparing TLR2 and TLR4 (shaded) vs isotype control (non-shaded). PE, Phycoerythrin.

Fig 2, B, shows the relative densitometry of the p65 subunit with the average of 3 experiments.

Peptidoglycan selectively induces IL-4 and augments IgE-mediated secretion by basophils

We next investigated whether ligands for TLR2 and/or TLR4 affect basophil mediator release and cytokine secretion. Results shown in Fig 3, A, indicate that basophils did not release histamine when exposed to 10 or 50 $\mu\text{g/mL}$ peptidoglycan or LPS. Although there was some indication that these substances directly induced LTC_4 , the results in Fig 3, B, were not statistically significant. In contrast, as seen in Fig 3, C, peptidoglycan significantly induced measurable quantities of IL-4 (range, 7–20 $\text{pg}/10^6$ basophils) when used at either concentration, but this was not observed by using the TLR4 ligand, LPS. Further evidence that TLR2 ligand interactions affect basophil secretion was seen when basophils were first pretreated 15 minutes with peptidoglycan and then stimulated with anti-IgE antibody. Under these conditions, the secretion of all 3 classes of mediators (*ie*, histamine, LTC_4 , and IL-4) was enhanced compared with activation with anti-IgE alone (Fig 3, D–F). This enhancement was most evident for IL-4 and LTC_4 , where the average levels were double those in control cultures. In contrast, pretreatment with LPS produced no effects on IgE-mediated secretion.

TLR2 ligands augment IgE-independent secretion of IL-13 from basophils

Basophils also secrete IL-13 in response to specific cytokines (*ie*, IL-3 and nerve growth factor), presumably working independently of IgE.^{10,15,16} Consequently, we investigated whether peptidoglycan and/or LPS would directly induce IL-13 or augment the production of this cytokine in response to IL-3. Fig 4, A, shows that peptidoglycan alone stimulated nearly 20 $\text{pg}/10^6$ basophils of IL-13 during an 18-hour incubation. These levels

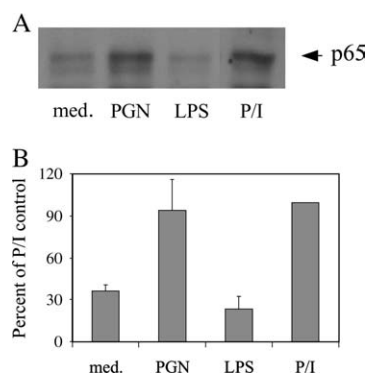


FIG 2. **A**, NF κ B (p65 subunit) expressed in nuclear extracts of basophils treated 1 hour with medium alone (med.), peptidoglycan, LPS (10 $\mu\text{g/mL}$ each), or phorbol 12-myristate 13-acetate (10 ng/mL /ionomycin (500 ng/mL ; P/I). **B**, Densitometry (average $\pm$ SEM; $n = 3$) of these responses, normalized as a percentage of the P/I positive control. PGN, Peptidoglycan.

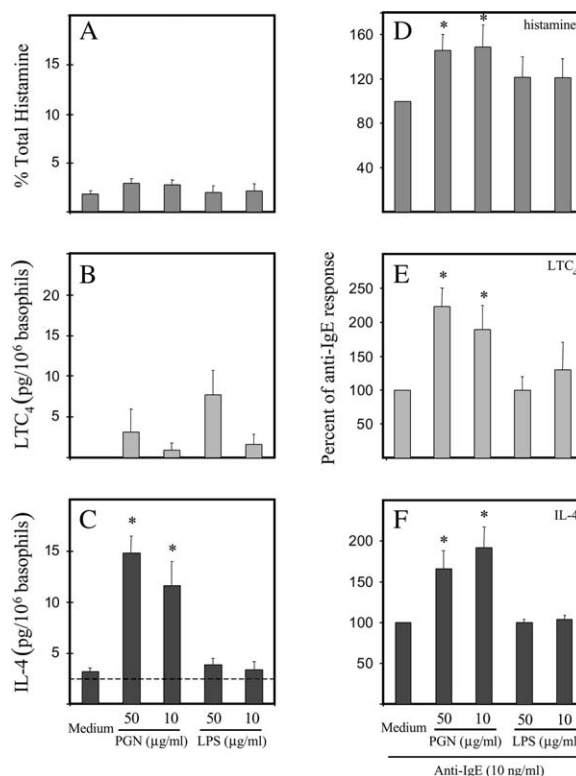


FIG 3. **A–C**, Mediator release and cytokine responses to peptidoglycan (PGN) or LPS alone (average $\pm$ SEM; $n = 6$). Dotted line indicates level of detection. **D–F**, Preincubation (15 minutes) with PGN and LPS on anti-IgE-induced responses. Percent (average $\pm$ SEM; $n = 6$) of anti-IgE control: 27% $\pm$ 8% (histamine), 260 $\text{pg}/10^6$ basophils (LTC_4), and 203 $\pm$ 71 $\text{pg}/10^6$ basophils (IL-4). * $P < .05$ vs medium or control.

were significantly higher than those produced by basophils in medium alone but were 10-fold less than the IL-13 produced in response to IL-3. On the other hand, basophils did not secrete IL-13 in response to LPS. These findings

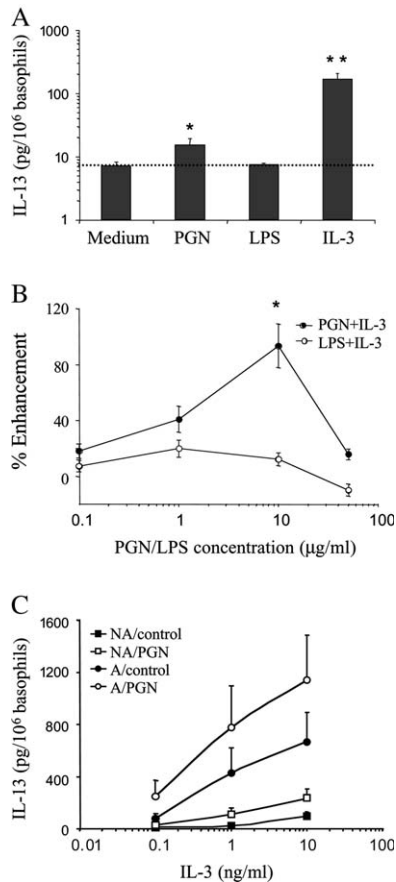


FIG 4. A, Basophil IL-13 (average $\pm$ SEM; $n = 10$). Line denotes level of detection. B, Percent enhancement (average $\pm$ SEM; $n = 7$) of IL-13 induced with 10 ng/mL IL-3 (control levels, 128 ± 45 pg/10⁶ basophils). C, Peptidoglycan (PGN; 10 μ g/mL) or medium (control) on the IL-13 secreted by basophils of allergic (A) versus nonallergic (NA) subjects. Average $\pm$ SEM ($n = 4$) for each group. * $P < .05$; ** $P < .01$ versus all other conditions.

were substantiated by results in which IL-3–dependent IL-13 was enhanced by different concentrations of peptidoglycan, but not by LPS (Fig 4, B).

Peptidoglycan also affected basophil sensitivity to IL-3 as it pertains to IL-13 secretion. This was most evident in cells from subjects with allergy compared with subjects without allergy. Fig 4, C, shows that basophils from subjects with allergy (filled circles) secreted an average of 667 ± 226 pg/10⁶ cells when stimulated for 18 hours with 10 ng/mL IL-3, or nearly 7 times that seen from normal controls (filled squares), thus confirming our previous findings.¹⁰ Peptidoglycan significantly enhanced these responses (open symbols) at all concentrations of IL-3 tested by shifting the sensitivity to this cytokine ~20-fold to the left for both groups.

It is important to note here that IL-3 did not significantly increase mRNA expression for TLR2 (or for TLR1 and TLR6), but rather appeared to maintain the expression of these receptors *ex vivo* (see Fig E1 in the Journal's Online Repository at www.mosby.com/jaci).

We then investigated whether other TLR2 ligands similarly affect basophil secretion, focusing primarily on cytokine secretion induced by both IgE-dependent and IgE-independent mechanisms. In particular, we tested the synthetic lipopeptide Pam₃Cys and the yeast product zymosan. Unlike peptidoglycan, these substances only marginally induced detectable levels of cytokine release among the donor cells tested and had no direct effect on mediator release (data not shown). However, Fig 6 shows that Pam₃Cys augmented by 2-fold to 3-fold the IL-13 secreted in response to 1 ng/mL IL-3 (A). This effect of the synthetic lipopeptide was comparable with the response seen with peptidoglycan. Furthermore, priming effects mediated by Pam₃Cys were also observed for the IL-4 induced with anti-IgE activation (B). Although others have shown that zymosan, at 1000-fold lower concentrations, activates human culture–derived mast cells to secrete cytokines,¹⁷ we did not observe this in basophils. This lack of responsiveness to zymosan was observed despite evidence that basophil constitutively expressed mRNA for TLR1 and TL6 (see Fig E1 in the Online Repository at www.mosby.com/jaci), suggesting the potential for TLR2/TLR1 and TLR2/TLR6 heterodimers that reportedly allow detection of a variety of TLR2-specific ligands.

Finally, we explored whether NF κ B activation plays a role in the enhancing effect TLR2 ligands have on basophil cytokine secretion. As shown in Fig 6, A, the selective NF κ B inhibitor SN50 reduced peptidoglycan's ability to induce nuclear localization of the p65 subunit. However, SN50 did not prevent peptidoglycan from augmenting IgE-dependent IL-4 secretion and actually resulted in a modest but significant ($P < .05$) enhancement of the TLR2 ligand effect, which was not observed by using the SN50-M control peptide (Fig 6, B). Likewise, SN50 did not prevent peptidoglycan or Pam₃Cys from augmenting the IL-13 secreted in response to IL-13 (Fig 6, C).

DISCUSSION

Until recently, most TLR/ligand interactions had been shown to favor T_H1-like responses rather than promoting the T_H2 responses commonly associated with allergic disease. However, this concept was recently challenged in rodent models, in which both TLR ligand concentration and route of immunization were shown to play important roles in determining whether type 1 or type 2 immune responses develop.^{18–20} Even more uncertain is the notion that specific TLR ligands differentially regulate cytokine responses in the various immune cells that participate in allergic disease. As such, we confirm here the observations made by Sabroe et al³ that human basophils express mRNA and protein for both TLR2 and TLR4. We expand on these findings by providing the first evidence that basophils are functionally responsive only to ligands interacting with TLR2. Specifically, the best characterized ligand for this receptor, peptidoglycan, directly activated

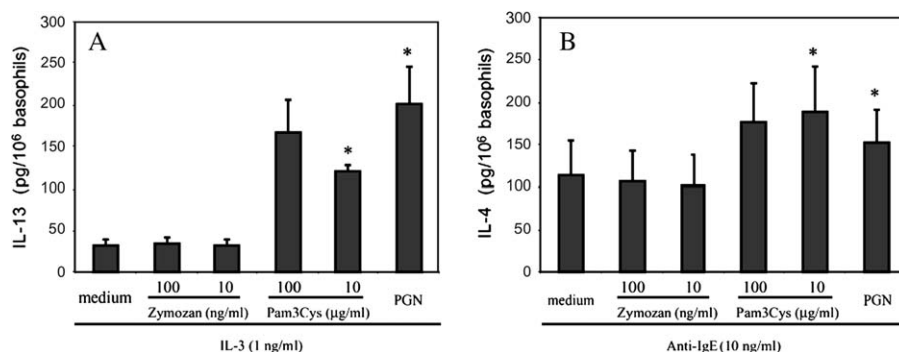


FIG 5. A, IL-13 secretion with the indicated TLR2 ligands combined with IL-3 (average $\pm$ SEM; $n = 3$). B, IL-4 in response to 15-minute preincubation with TLR2 ligands followed by 3 hours with anti-IgE (average $\pm$ SEM; $n = 4$). Peptidoglycan (PGN) was 10 μ g/mL. * $P < .05$ vs anti-IgE or IL-3 alone.

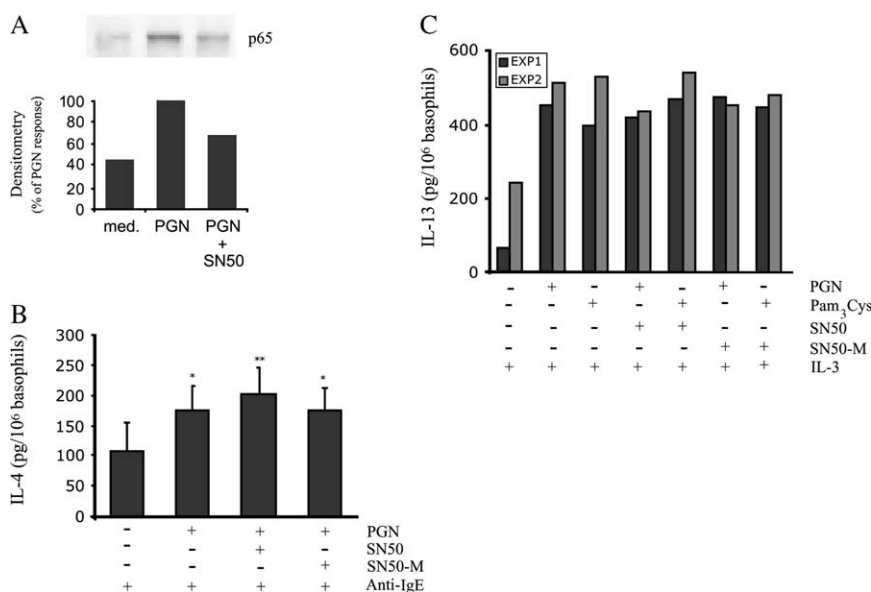


FIG 6. A, NF- κ B p65 induced by peptidoglycan (PGN; 10 μ g/mL) and its inhibition by SN50 (10 μ g/mL). B and C, Effect of SN50 or control peptide (SN50-M) on priming of IgE-mediated IL-4 (average $\pm$ SEM; $n = 4$) and of IL-13 induced by IL-3 (5 ng/mL), respectively. TLR2 ligands at 10 μ g/mL. * $P < .05$ vs anti-IgE; ** $P < .05$ vs other conditions. EXP, Experiment; med., medium alone.

basophils for IL-4 and IL-13 secretion and markedly augmented cytokine and mediator release in response to IgE-dependent activation.

The induction of IL-4 by peptidoglycan was particularly unusual in that previous studies show the generation of this cytokine to be more dependent on signaling mediated through the IgE receptor Fc ϵ RI. For example, IL-4 secreted in response to IgE receptor aggregation correlates well with mediator release, even though very different rates of secretion are observed for these 2 classes of mediators.¹⁵ However, in this study, IL-4 was secreted to peptidoglycan alone, without the accompanying histamine (or LTC₄) usually released in the same culture supernatants (Fig 3). As a consequence, it is unlikely that peptidoglycan simply activated basophils by interacting

with IgE bound to the cell surface. Instead, the study substantiates the notion that direct TLR2 ligation initiates cytokine production from these cells. This is in spite of a marked difference observed in the overall levels of cytokine produced, with peptidoglycan on average inducing just 1/10 the level made in response to IgE-dependent activation (Fig 3). Accordingly, these results suggest that TLR2/ligand interactions induce signals downstream from those directly linked to mediator release but that are still capable of generating cytokine.

Whereas peptidoglycan was the only TLR2 ligand that selectively activated basophils for cytokine secretion when used alone, both this microbial product and the synthetic lipopeptide Pam₃Cys showed a marked ability in priming basophils for IgE-dependent and IgE-indepen-

dent secretion (Figs 3 and 5). With regard to IL-4, this enhancement was twice that secreted with IgE cross-linking alone, clearly indicating that peptidoglycan mediated a synergistic rather than an additive effect. Likewise, when peptidoglycan was combined with diminishing amounts of IL-3, basophil responsiveness remained intact, resulting in IL-13 levels equal to those normally produced with 20-fold greater concentrations of IL-3 (Fig 4). Importantly, this effect was most apparent using cells isolated from allergic compared with normal subjects, which substantiates our previous findings that greater levels of IL-13 are secreted by basophils isolated from subjects with allergy.¹⁰

Zymosan did not possess the same priming capabilities as peptidoglycan or Pam₃Cys when used at concentrations reported to activate culture-derived mast cells.¹⁷ Other than the possibility that our zymosan preparation had reduced potency, we currently have no explanation to account for this finding, particularly because peptidoglycan and zymosan are reported to signal through a common TLR2/TLR6 heterodimer.^{21,22} Adding to this confounding result is the observation that Pam₃Cys, which uses TLR2/TLR1 heterodimers, was more comparable with peptidoglycan in activity than was zymosan. These findings suggest that TLR2 signaling is more complex than currently thought, and may involve additional components used in conjunction with the known TLR2 heterodimers. Nonetheless, it is important to note that basophils possessed mRNA for both TLR1 and TLR6 (see Fig E1 in the Online Repository at www.mosby.com/jaci), so it is likely that these heterodimers are expressed on basophils, which makes it conceivable that they also mediate function.

Basophils were unresponsive to LPS despite expressing TLR4. The most credible explanation for this finding is that these cells do not normally express the coreceptor, CD14.²³ Unlike TLR2, the signaling mediated through TLR4 is very much dependent on coexpression of CD14 and its accessory protein (MD2), which complex with TLR4, allowing LPS signaling to occur in cells expressing these components.²⁴ By not expressing CD14, basophils probably never recognize LPS for subsequent signaling through TLR4. Although we must entertain the possibility that the small percentage of basophils lost during purification could be a subset expressing CD14, we currently have no evidence that this is the case. As such, the role mediated by TLR4 expression on basophils is difficult to predict at this time, but it is conceivable that these cells, under yet unknown pathophysiological conditions, could acquire CD14. If so, it stands to reason that they may then become responsive to TLR4 ligation.

Studies indicate that all 11 TLRs induce signals that culminate in the activation of NFκB.²⁵ Therefore, we investigated NFκB translocation as a marker of functional TLR receptor signaling and found that basophils treated for 1 hour with peptidoglycan showed an accumulation of this transcription factor in their nucleus (Fig 2). The same was not true for LPS-treated cells. However, although these data support evidence for functional TLR2 on basophils, our results testing the SN50 inhibitor indicate

that NFκB does not play a role in the enhancing effects peptidoglycan and Pam₃Cys mediated on basophil function, particularly cytokine secretion (Fig 6). It seems more likely that other TLR-dependent events upstream of this transcription factor could play a role in these functional effects on basophils. Future studies should address this hypothesis.

Our results using human blood basophils bear a resemblance to those reported for cultured mast cells, which indicate that TLR2 ligands also selectively induce cytokine and mediator release from these cells.^{17,26,27} However, there are some important distinctions between the basophil and mast cell data that, apart from reflecting the cell models used, are worth noting because of the potential physiological consequences implied for allergic disease. Foremost, none of the TLR2 ligands we tested had the capacity to directly induce mediator release, whereas the same substances caused the release of β-hexosaminidase and/or LTC₄ from either rodent or human mast cells. As a consequence, these observations suggest that certain microbial infections, especially those associated with gram-positive bacteria, have the same capacity to induce immediate hypersensitivity reactions as do common allergens. Yet allergies directly resulting from microbial infections are rare unless an underlying condition already exists. In this regard, infections are more commonly associated with exacerbations of allergy and of allergic disease.²⁸ It follows that our observations of TLR2 ligands' enhancement of IgE-dependent mediator release and IgE-independent cytokine secretion might better represent clinical experience. As such, the presence of *S aureus* in the skin lesions of subjects with atopic dermatitis could be a relevant indication of how TLR2 ligands (*ie*, peptidoglycan) might act on resident mast cells and infiltrating basophils to augment secretion and thus amplify progression of the disease.

Finally, the adjuvant properties typically mediated by TLR-specific ligands and their ability to favor the production of specific cytokines may have an even greater physiological significance. Recent studies show that Pam₃Cys or peptidoglycan, when administered subcutaneously or intranasally in mice at the time of immunization, induces a prominent T_H2-biased immune response characterized by increased serum IgE and the overexpression of effector cytokines such as IL-13.^{19,20} Despite the inherent difficulties in rodent and human comparisons, our results presented in this article indicate that the ability of TLR2 ligands to target basophils for IL-4 and IL-13 secretion could have relevance to these *in vivo* findings. It is in this respect that we have long proposed that basophils represent a significant source of IL-4 and IL-13 such that they could play an important role in promoting and amplifying the T_H2-dependent responses exemplified in allergic disease.²⁹

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