Differential TLR-induced cytokine production by human mast cells is amplified by FcεRI triggering

Chapter 4

Jolien Suurmond,[1] Annemarie L Dorjée, [1] Edward F Knol,[2]
Tom WJ Huizinga, [1] René EM Toes[1]

[1]Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; [2]Departments of Immunology and Dermatology/ Allergology, University Medical Center Utrecht, Utrecht, The Netherlands

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SUMMARY

Background Mast cells are mainly present in strategic locations, where they may have a role in defence against parasites and bacteria. These pathogens can be recognized by mast cells via Toll-like receptors (TLR). Allergic symptoms are often increased in the presence of pathogens at the site of allergen exposure, but it is unknown which cytokines can mediate such an effect.

Objective To study whether an interaction between IgE- and TLR-mediated activation of human mast cells can contribute to exacerbated inflammatory responses.

Methods Peripheral blood-derived mast cells were stimulated with TLR ligands, in the presence or absence of anti-IgE triggering, after which degranulation was measured using flow cytometry and cytokine production was evaluated by multiplex assays, and ELISA. For evaluation of allergen-specific responses, mast cells were sensitized with serum of allergic individuals or controls, after which they were stimulated using allergens in combination with TLR ligands.

Results Simultaneous triggering of mast cells via IgE and TLR ligands greatly enhanced cytokine production but not IgE-induced degranulation. Different TLR ligands specifically enhanced the differential production of cytokines in conjunction with FcεRI triggering. Importantly, only TLR-4 and TLR-6 were able to induce robust production of IL-13, an important molecule in allergic reactions.

Conclusions & Clinical Relevance These results indicate that the simultaneous presence of pathogen- or danger-associated signals and FcεRI triggering via specific IgE can significantly modify mast cell-mediated allergic reactions via synergistic production of cytokines and inflammatory mediators and provide an explanation of augmented allergic symptoms during infection.

INTRODUCTION

Mast cells are immune cells that reside in all tissues and are most well known for their role in IgE-mediated allergic responses. Mast cells are found at strategic locations where they can encounter pathogens, such as the skin and mucosal surfaces like the gut and the respiratory tract. Mast cells have been well established to play a role in the defence against bacteria, certain parasites (mainly intestinal helminth parasites), and possibly against fungal and viral infections (*1-5*). Recognition of pathogens via Toll-like receptors (TLR) has been shown to contribute to mast cell responses to pathogens (*5*).

In addition to activation via innate receptors in primary responses, IgE that is produced in response to parasites or other pathogens may also play an important role in recognition of pathogens in secondary or chronic infection (*6*) or, as described recently, in the protection against venoms upon second exposure (*7*).

However, although such responses may play an important role in the protection against secondary exposure to pathogens or venoms, these enhanced responses can have detrimental effects in responses when no danger is present, such as is the case in allergic responses (*8*). Viral and bacterial infections have been associated with asthma exacerbations (*9, 10*), and the presence of the TLR-4 ligand endotoxin was demonstrated in house dust and was related to severity of allergic reactions against house dust mite (*11*). Furthermore, allergens have been reported to promote TLR signalling directly (*12*). Therefore, mast cell activation via TLRs may contribute to these processes when triggered at the same time by allergen-specific IgE. Studies using mouse mast cells have shown contrasting data concerning the degranulation and cytokine response upon combined antigen and TLR triggering (*13-15*).

In human mast cells, it has been shown that prolonged pre-treatment with LPS can significantly enhance IgE-mediated mast cell responses (*15*), and it is therefore been hypothesized that TLR triggering of mast cells can contribute to asthma exacerbations. We and others have previously shown that mast cells can differentially secrete cytokines depending on the TLR that is triggered (*16-18*). However, it is unknown whether differential cytokine production is still present upon combined IgE-mediated responses. This is important, as different mast cell mediators have distinct functions in allergic responses.

Therefore, we aimed to characterize human mast cell responses upon combined activation via (allergen-specific) IgE and TLR triggering and to analyse the specific cytokines induced by different TLR ligands.

METHODS

PERIPHERAL BLOOD-DERIVED MAST CELLS

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, the Netherlands). $CD34^+$ hematopoietic stem cells were isolated from mononuclear cells with CD34 microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) and were differentiated into mast cells as described (*16*). The purity of mast cells ranged from 90 to 99%.

MAST CELL ACTIVATION

Cultured mast cells were sensitized with 0.1 µg/mL hybridoma IgE (Diatec Monoclonals, Oslo, Norway) for a minimum of 18 h, after which they were activated with goat anti-human IgE (Nordic-MUbio, Susteren, The Netherlands) to induce IgE-mediated activation. For TLR-mediated activation, mast cells were stimulated using S. aureus Peptidoglycan (Pgn), Poly(I:C), E. Coli K12 lipopolysaccharide (LPS), S. typhimurium flagellin, FSL-1, 10 µg/mL ssRNA40 (all from InVivoGen, San Diego, CA, USA) for 24 h. A titration was performed to determine the optimal concentrations of stimuli, using the following concentrations (high, medium, low): aIgE: 10, 0.5, 0.05 µg/mL; Peptidoglycan: 10, 5, 1 µg/mL; LPS: 10, 0.5, 0.05 µg/mL; Flagellin: 1, 0.1, 0.01 µg/mL; FSL-1: 1, 0.1, 0.01 µg/mL; ssRNA40: 10, 1, 0.1 µg/mL.

For combined activation via IgE and TLRs, IgE-sensitized mast cells were activated with suboptimal and optimal concentrations of anti-IgE (10 and 0.01 µg/mL), Peptidoglycan (10 and 5 µg/mL), LPS (10 and 0.5 µg/mL), flagellin (1 and 0.1 µg/mL), FSL-1 (1 and 0.1 µg/mL) and ssRNA40 (10 and 1 µg/mL).

For allergen-specific activation, mast cells were sensitized for 18 h with serum from house dust mite-allergic individuals and control individuals, diluted 1 : 1 in culture medium. The presence of house dust mite-specific IgE was measured by ImmunoCAP (Phadia Thermo Fisher Scientific, Uppsala, Sweden), with 0.35 UA/mL as cutoff. Mast cells were washed with medium and subsequently activated with 10 µg/mL anti-IgE or 0.25 U/mL house dust mite allergen (Dermatoph. pteronyssinus, HAL Allergy, Leiden, The Netherlands) in the presence or absence of LPS.

FLOW CYTOMETRY

For detection of mast cell degranulation, cells were incubated with fluorochrome-conjugated antibodies recognizing CD117, CD203c and CD63 or matching isotype controls. Cells were taken up in 1% paraformaldehyde until flow cytometric acquisition on a FACS Calibur (BD Biosciences, San Jose, CA, USA). Analysis was performed using FACS Diva software (BD) and FlowJo.

CYTOKINE SECRETION

Quantitative immunoassays for EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFNγ, IL-1ra, IL-1α, IL-1β, IL-2, sIL-2Rα, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGF-α, TNF-α, TNF-β and VEGF in culture supernatants were performed using the 42-plex cytokine or custom-made Milliplex assays

(EMD Millipore, Billerica, MA, USA). Additionally, IL-8 and IL-13 production was evaluated using ELISA (eBioscience, San Diego, CA, USA).

STATISTICAL ANALYSIS

Results are expressed as mean + SEM. For comparison of multiple groups, one-Way ANOVA was performed, with Bonferroni's post-test to correct for multiple testing. For comparison of two groups, Student's t-test was performed. Statistical analysis was performed using GraphPad Prism 4 (Graphpad Software, La Jolla, CA, USA). P values of < 0.05 were considered statistically significant.

RESULTS

DIFFERENTIAL CYTOKINE PRODUCTION UPON TITRATION OF TLR LIGANDS

We have previously shown that mast cells functionally express TLR-2, TLR-4, TLR-5, TLR-6, and TLR-8 (*16*). To determine the optimal concentrations of stimuli, the ligands for these TLRs were titrated, after which cytokines were measured in supernatant (Fig. 1). For most cytokines, the highest concentration of TLR ligands was most potent in inducing cytokine production. An exception is IL-8, which was also significantly up-regulated at lower concentrations of several TLR ligands.

Importantly, a differential cytokine profile was observed, with LPS and FSL-1 (TLR-4 and -6 ligands) showing a nearly similar pattern of upregulation of GM-CSF, IL-8 and IL-10, whereas ssRNA (TLR-8 ligand) induced IL-8, MIP-1α and TNF-α. As the optimal concentration of stimuli was different for IL-8 as most other cytokines, we decided to evaluate interaction between TLR- and IgE-mediated activation using both suboptimal and optimal concentrations.

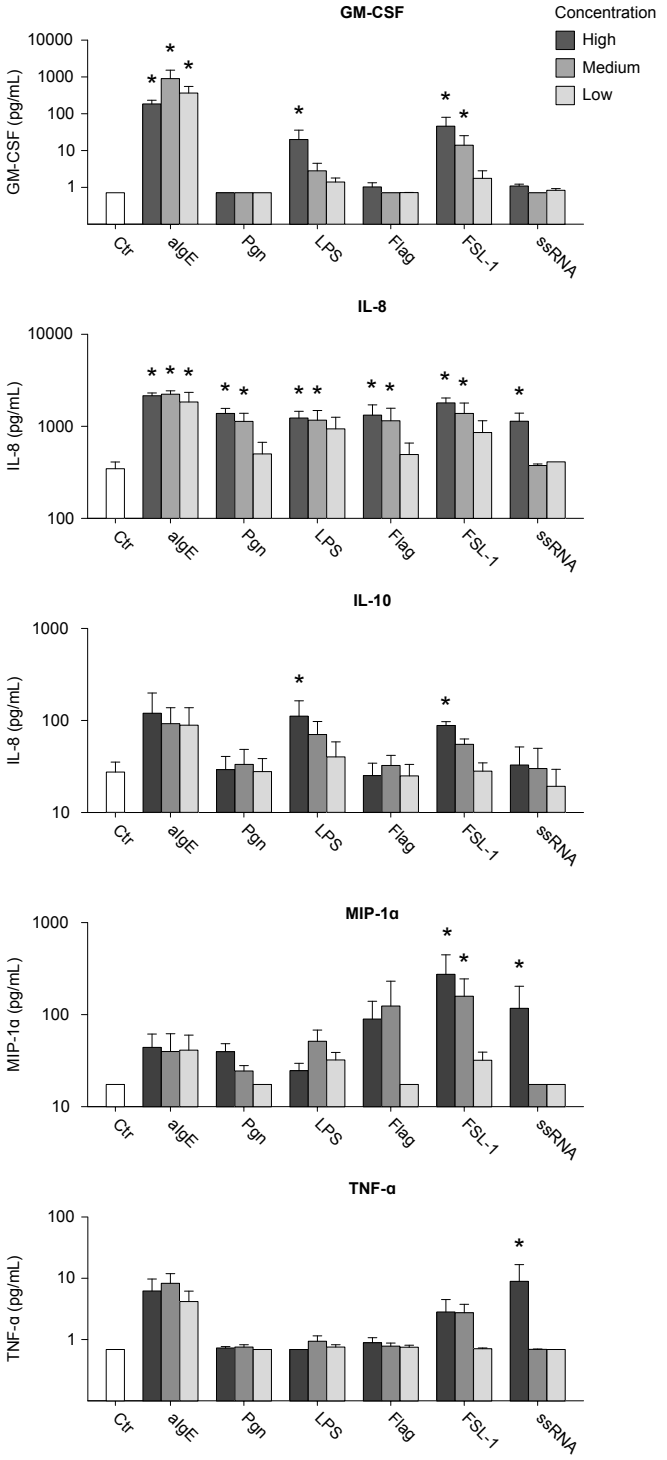

Figure 1. Mast cells were stimulated with control medium (ctr) or different concentrations of anti-IgE, Peptidoglycan (Pgn; TLR-2), LPS (TLR-4), Flagellin (TLR-5), FSL-1 (TLR-6) and ssRNA (TLR-8). The concentrations of stimuli (high, medium, low) used were as follows: aIgE: 10, 0.5, 0.05 µg/mL; Peptidoglycan: 10, 5, 1 µg/mL; LPS: 10, 0.5 and 0.05 µg/mL; Flagellin: 1, 0.1, and 0.01 µg/mL; FSL-1: 1, 0.1, 0.01 µg/mL; ssRNA40: 10, 1 and 0.1 µg/mL. Cytokine production was measured in supernatant after 24 h. Asterisks indicate significantly ($P < 0.05$) increased cytokine production compared to control medium (ctr), using one-way ANOVA with Bonferroni's post-test (n = 3 independent mast cell donors).

DIFFERENTIAL CYTOKINE PRODUCTION IS AMPLIFIED BY COMBINED TLR- AND FCεRI-MEDIATED ACTIVATION

We next evaluated possible interactions between triggering of FcεRI and activation via TLRs. FcεRI was crosslinked using anti-IgE in the presence or absence of TLR ligands for 24 h, after which cytokine production was measured. We observed amplified cytokine responses upon combined stimulation by several TLR ligands and anti-IgE (Figs 2 and 3).

The cytokines were separated based on their pattern of induction by different TLR ligands. The TLR-4 ligand LPS in combination with anti-IgE significantly enhanced the production of GM-CSF, IL-5, IL-8 (at suboptimal concentrations), IL-10 and IL-13, in comparison with anti-IgE alone (Fig. 2). The effect of TLR-6 ligand FSL-1 resembled this pattern, although with a smaller magnitude. Therefore, only IL-8 and IL-13 were significantly enhanced by FSL-1. In contrast to this pattern, TLR-8 ligand ssRNA significantly enhanced the production of several other cytokines, EGF, GRO-α, MIP-1α and TNF-α (Fig. 3).

These results suggest that the response to TLR ligands was different depending on the TLR that was being triggered. Importantly, when comparing the cytokine profiles of TLR ligands without anti-IgE to the profiles in presence of anti-IgE, there is a striking resemblance between the profiles, indicating that enhancement of specific cytokines by FcεRI triggering is defined by specific TLRs. These results indicate that the combination of TLR triggering and anti-IgE synergizes in cytokine production by mast cells, where the exact cytokine profile is defined by the TLR ligand.

SYNERGY BETWEEN ALLERGEN-SPECIFIC IGE-MEDIATED ACTIVATION AND TLR TRIGGERING

To confirm the synergy in production of IL-8 and IL-13 upon triggering with LPS in an antigen-specific system, we next evaluated mast cell activation through allergen-specific IgE. Mast cells were sensitized with serum of house dust mite-(HDM) allergic individuals, and subsequently stimulated with HDM, leading to mast cells degranulation and activation, as shown by upregulation of CD63 and CD203c and release of IL-8 (Figs 4a and b). In contrast, sensitization with serum from non-allergic individuals did not induce HDM-induced mast cell activation.

The presence of LPS did not affect the HDM-induced degranulation (CD63) or upregulation of CD203c (Fig. 4c). Intriguingly however, combining TLR-signals with FcεRI triggering via HDM-specific IgE led to enhanced production of IL-8 and IL-13 (Figs 4d and e). Specifically, IL-13 production increased twofold to eightfold upon combined activation as compared to the cumulative production of each stimulus alone (Figs 4d and e).

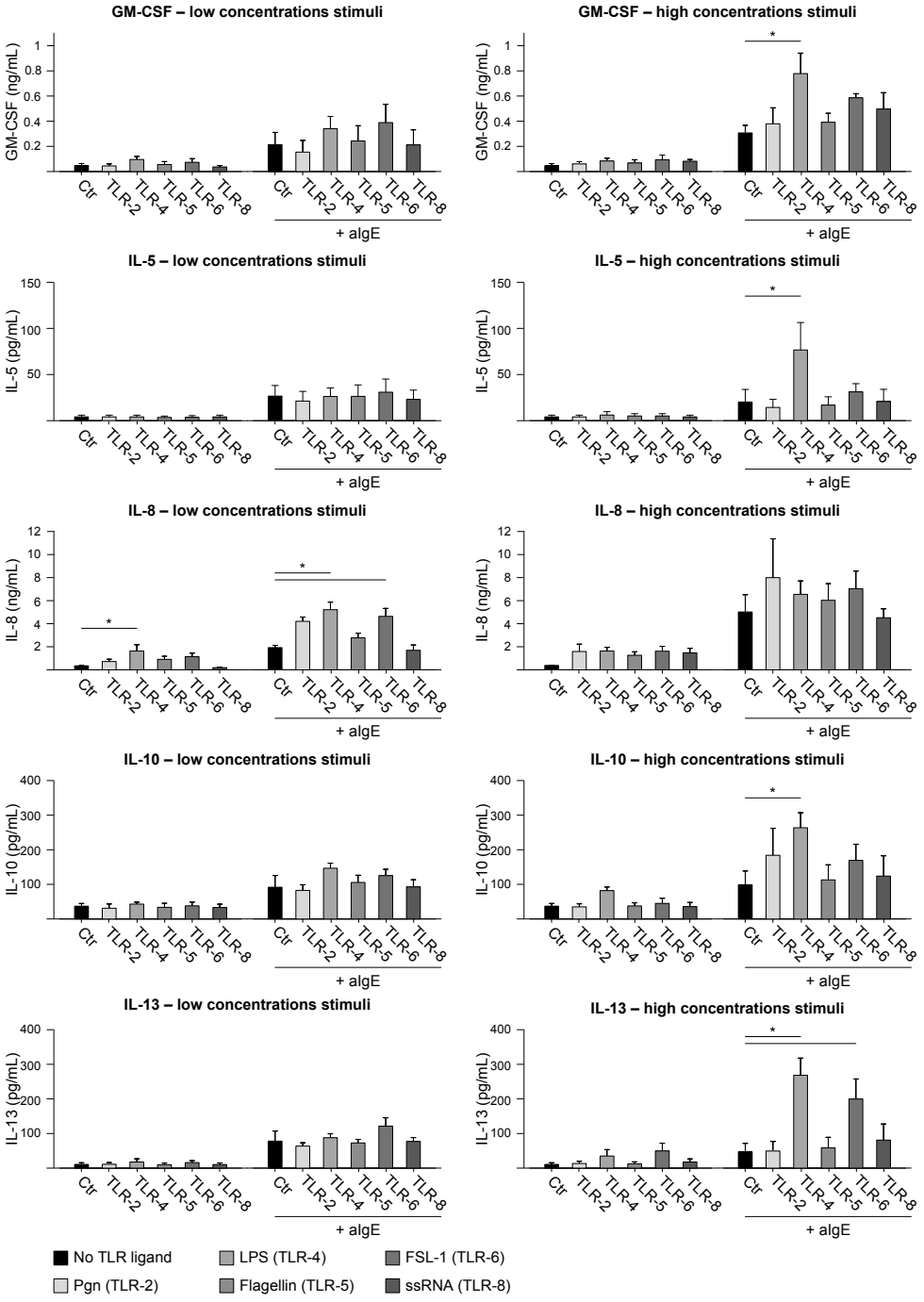

Figure 2. This figure shows the cytokines which are upregulated in response to TLR-4 and TLR-6 ligands. See legend of Figure 3 for experimental details.

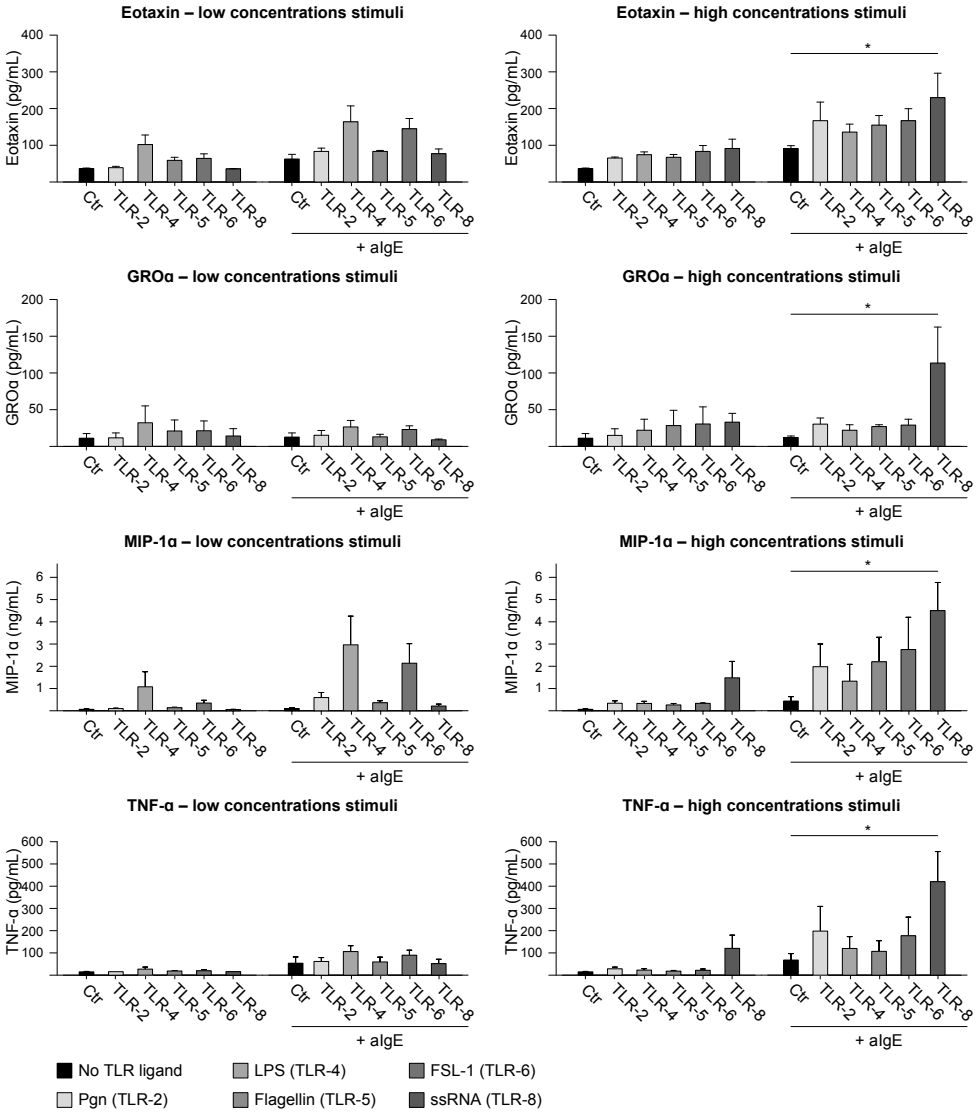

Figure 3. Mast cells were stimulated with Peptidoglycan, LPS, Flagellin, FSL-1 and ssRNA alone or in combination with anti-IgE at low (left) or high (right) concentrations. Low concentrations used were as follows: anti-IgE (0.01 µg/mL), Peptidoglycan (5 µg/mL), LPS (0.5 µg/mL), flagellin (0.1 µg/mL), FSL-1 (0.1 µg/mL) and ssRNA40 (1 µg/mL). High concentrations used were as follows: anti-IgE (10 µg/mL), Peptidoglycan (10 µg/mL), LPS (10 µg/mL), flagellin (1 µg/mL), FSL-1 (1 µg/mL) and ssRNA40 (10 µg/mL). Cytokine production was measured in supernatant after 24 h. Asterisks indicate significantly ($P < 0.05$) increased cytokine production compared with control medium (ctr) or anti-IgE alone, as indicated in the figure, using one-way ANOVA with Bonferroni's post-test. Data shown are from $n = 3$ independent experiments and mast cell donors. This figure shows the cytokines which are upregulated in response to TLR-8 ligand.

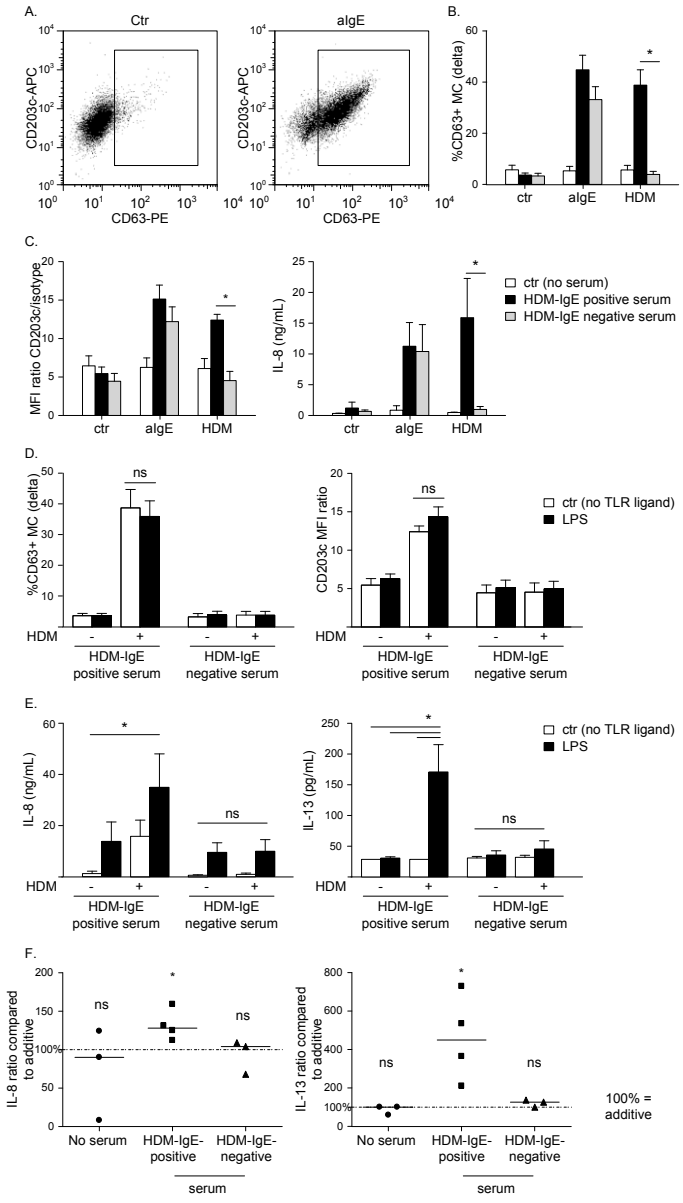

Figure 4. Mast cells were sensitized with serum of house dust mite allergic individuals, after which they were stimulated for 24 h with house dust mite (HDM) allergen or anti-IgE as positive control. (a) Representative flow cytometry plots for CD63 and CD203c, gated on CD117^{+} mast cells. (b,c) Summary of data of CD63 and CD203c surface expression as well as IL-8 production in supernatant. (d) Surface expression of CD63 and CD203c upon combined activation of sensitized mast cells with HDM and LPS. (e) Production of IL-8 and IL-13 after combined activation of sensitized mast cells with HDM and LPS. (f) Relative increase in cytokine production upon combined activation is shown as percentage of cytokine production in response to combined triggering divided by the sum of the cytokine production following stimulation with HDM and LPS separately. Asterisks indicate significantly ($P < 0.05$) increased degranulation (b,c) or cytokine production (c–f) as indicated in the figure, using Student's t-test. Data shown are from n = 3 independent experiments and mast cell donors, using serum from 4 HDM-IgE-positive and 3 HDM-IgE-negative individuals.

Together, these results show that TLR- and IgE-mediated activation of human mast cells can synergistically enhance their activation, leading to enhanced cytokine production by mast cells sensitized with allergen-specific IgE when exposed to allergens and TLR ligands.

DISCUSSION

Our results show that the combination of IgE- and TLR-mediated activation greatly enhances cytokine production by mast cells, but not their degranulation. Whereas mast cell degranulation is important for acute-phase allergic responses, cytokine production is considered to be more important for the, more inflammation related, late-phase responses (*19, 20*). Therefore, our results suggest that the presence of TLR ligands may specifically enhance the late-phase responses in allergy. Our results indicate that the type of cytokine response that is induced depends on the TLR that is being triggered. For example, IL-13 production was only enhanced by ligands for TLR-4 and TLR-6. The presence of endotoxin (TLR-4 ligand) in house dust has been associated with allergic symptoms (*10*), and LPS has been shown to enhance allergic symptoms in a mouse model of house dust mite allergy (*21*). IL-13 is an important cytokine implicated in the induction of several key symptoms in allergy, such as enhancing mucus hypersecretion and airway hyperresponsiveness (*22*). Our results indicate that synergy in IL-13 production by mast cells when they are exposed to LPS and house dust mite could contribute to endotoxin-enhanced allergic responses to house dust mite.

A clear inherent limitation of our study is that we used in vitro-generated mast cells, rather than primary mast cells isolated from tissue. The number of mast cells obtained from tissues is limited, and isolation of mast cells often leads to their activation, making it difficult to perform functional studies evaluating cytokine production (*23*). We and others have shown that in vitro-generated mast cells resemble tissue mast cells in various aspects (*16, 23, 24*). However, we cannot exclude that cytokine production by mast cells in vivo is also influenced by the local tissue environment. We did not observe a response of mast cells to house dust mite extract as measured by CD63-upregulation, or IL-8, respectively, IL-13-release. In contrast, this allergen only induced such a response when mast cells were sensitized with serum from allergic individuals, suggesting that the house dust mite extract did not directly activate mast cells. This is in contrast with studies in the mouse showing that der p 2 present in house dust mite could directly promote TLR signalling (*12*). Our results suggest that the activation of human mast cells by house dust mite allergens through specific IgE is much more potent than the activation through TLR.

Other studies have evaluated TLR-mediated potentiation of IgE-dependent release of TNF-α, for example using mast cell-lines or mouse mast cells. In mouse bone marrow-derived mast cells, a number of cytokines were synergistically enhanced by combined FcεRI and TLR-2 or TLR-4 triggering, but only few cytokines were analysed (*13*). In the human mast cell line LAD2, no TNF-α potentiation was observed when TLR-2 or TLR-4 ligands were combined with anti-IgE (*25*). In our study, we also did not observe significant enhancement

of TNF-α with LPS. Rather, a group of cytokines, including TNF-α, was specifically enhanced with the TLR-8 ligand ssRNA, and another group of cytokines, including IL-5 and IL-13, was enhanced by LPS. Therefore, our results suggest that potentiation of cytokine production depends on the specific TLR that is being triggered, and that analysis of mast cell responses to TLR ligands requires the measurement of multiple cytokines. We provide a technique to sensitize mast cells with allergen-specific IgE using serum of allergic individuals, which allowed us to analyse allergen-specific mast cell responses in a specific manner.

In conclusion, our data show that IgE-mediated activation of human mast cells is synergistically enhanced by TLR ligands in a cytokine-specific and TLR-ligand-specific manner. These data could therefore contribute to further the understanding of exacerbations of allergic reactions during infections.

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REFERENCES

1. Marshall JS, Mast-cell responses to pathogens. *Nat Rev Immunol* 4, 787-99 (2004).

2. Malaviya R, Ikeda T, Ross E, Abraham SN, Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature* 381, 77-80 (1996).

3. Sutherland RE, Olsen JS, McKinstry A, Villalta SA, Wolters PJ, Mast cell IL-6 improves survival from Klebsiella pneumonia and sepsis by enhancing neutrophil killing. *J Immunol* 181, 5598-605 (2008).

4. Shelburne CP, Nakano H, St John AL *et al.*, Mast cells augment adaptive immunity by orchestrating dendritic cell trafficking through infected tissues. *Cell Host Microbe* 6, 331-42 (2009).

5. Supajatura V, Ushio H, Nakao A *et al.*, Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. *J Immunol* 167, 2250-6 (2001).

6. Gurish MF, Bryce PJ, Tao H *et al.*, IgE enhances parasite clearance and regulates mast cell responses in mice infected with Trichinella spiralis. *J Immunol* 172, 1139-45 (2004).

7. Marichal T, Starkl P, Reber LL *et al.*, A beneficial role for immunoglobulin E in host defense against honeybee venom. *Immunity* 39, 963-75 (2013).

8. Sykes A, Johnston SL, Etiology of asthma exacerbations. *J Allergy Clin Immunol* 122, 685-8 (2008).

9. Message SD, Laza-Stanca V, Mallia P *et al.*, Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proc Natl Acad Sci U S A* 105, 13562-7 (2008).

10. Gern JE, Viral and bacterial infections in the development and progression of asthma. *J Allergy Clin Immunol* 105, S497-502 (2000).

11. Michel O, Kips J, Duchateau J *et al.*, Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 154, 1641-6 (1996).

12. Trompette A, Divanovic S, Visintin A *et al.*, Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457, 585-8 (2009).

13. Qiao H, Andrade MV, Lisboa FA, Morgan K, Beaven MA, FcepsilonR1 and toll-like receptors mediate synergistic signals to markedly augment production of inflammatory cytokines in murine mast cells. *Blood* 107, 610-8 (2006).

14. Fehrenbach K, Port F, Grochowy G *et al.*, Stimulation of mast cells via FcvarepsilonR1 and TLR2: the type of ligand determines the outcome. *Mol Immunol* 44, 2087-94 (2007).

15. Saluja R, Delin I, Nilsson GP, Adner M, FcepsilonR1-mediated mast cell reactivity is amplified through prolonged Toll-like receptor-ligand treatment. *PLoS One* 7, e43547 (2012).

16. Suurmond J, Rivellese F, Dorjee AL *et al.*, Toll-like receptor triggering augments activation of human mast cells by anti-citrullinated protein antibodies. *Ann Rheum Dis* 74, 1915-23 (2015).

17. Supajatura V, Ushio H, Nakao A *et al.*, Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J Clin Invest* 109, 1351-9 (2002).

18. McCurdy JD, Olynych TJ, Maher LH, Marshall JS, Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J Immunol* 170, 1625-9 (2003).

19. Larche M, Akdis CA, Valenta R, Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 6, 761-71 (2006).

20. Naclerio RM, Pathophysiology of perennial allergic rhinitis. *Allergy* 52, 7-13 (1997).

21. Eisenbarth SC, Piggott DA, Huleatt JW *et al.*, Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 196, 1645-51 (2002).

22. Wills-Karp M, Luyimbazi J, Xu X *et al.*, Interleukin-13: central mediator of allergic asthma. *Science* 282, 2258-61 (1998).

23. Kashiwakura J, Yokoi H, Saito H, Okayama Y, T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. *J Immunol* 173, 5247-57 (2004).

24. Yoshioka M, Fukuishi N, Iriguchi S *et al.*, Lipoteichoic acid downregulates FcepsilonRI expression on human mast cells through Toll-like receptor 2. *J Allergy Clin Immunol* 120, 452-61 (2007).

25. Sumbayev VV, Yasinska I, Oniku AE, Streatfield CL, Gibbs BF, Involvement of hypoxia-inducible factor-1 in the inflammatory responses of human LAD2 mast cells and basophils. *PLoS One* 7, e34259 (2012).