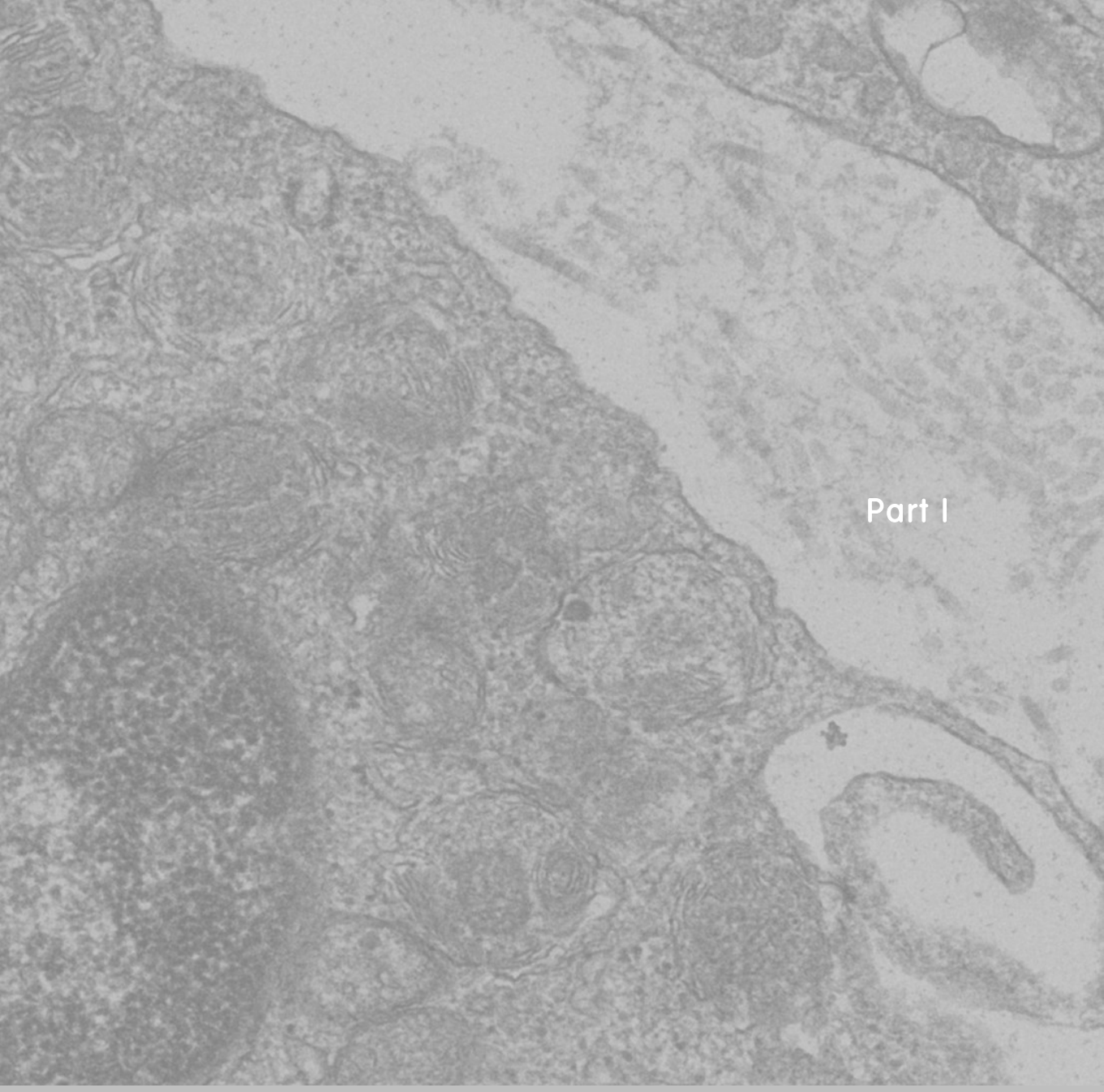

Part I

COMBINED INNATE AND FC RECEPTOR TRIGGERING OF MAST CELLS AND BASOPHILS

ACTIVATION OF HUMAN BASOPHILS BY COMBINED TOLL-LIKE RECEPTOR- AND FCεRI- TRIGGERING CAN PROMOTE TH2 SKEWING OF NAIVE THELPER CELLS

Chapter 2

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ABSTRACT

Basophils are mostly known for their involvement in allergic reactions. Recent studies in mice indicate a role for basophils in the induction of adaptive immunity, especially T helper 2 (Th2) responses. Therefore, it would be highly important to understand how basophils respond to pathogen-associated molecules, such as ligands for Toll-Like Receptors (TLRs), and if the basophils could promote Th2 responses via these stimuli. To this end, the activation of basophils via TLRs in combination with activation via IgE was studied, as well as its effect on T helper cell skewing.

Using quantitative PCR, we demonstrated the presence of mRNA for TLRs 1–8 in human basophils. Basophils responded to TLR triggering with differential cytokine production, but not with degranulation. Simultaneous triggering of TLRs and IgE led to synergy in production of IL-4, IL-8, IL-13, and RANTES. Furthermore, the synergistic effects on basophils mediated by IgE and TLR-4 triggering allowed robust Th2 skewing upon activation of naïve human $CD4^+$ T cells.

Our data show that human basophils respond to TLR ligands in synergy with IgE-mediated activation and that the cytokines produced can promote Th2 differentiation. These results indicate a role for basophils in the regulation of T -cell responses in humans.

INTRODUCTION

Basophils are circulating granulocytes that can migrate to tissues when inflammation or other triggers are present locally. Activation of basophils through crosslinking of FcεRI via antigen-specific IgE causes the release of granule contents (histamine, proteoglycans), lipid-derived mediators (leukotrienes), and cytokines. Due to their potent activation via IgE-bound antigens and their production of T helper 2 (Th2) type cytokines such as IL-4 and IL-13, basophils are often considered important innate effector cells in IgE-mediated immune responses. Besides activation through IgE, several other pathways have been described to activate basophils, including activation through complement receptors and Toll-Like Receptors (TLR). Although information about TLR protein expression and the response of basophils to TLR ligands is scarce, human basophils express mRNA of several TLRs, and protein of TLR-2 and -4. However, the functional response of basophils to ligation of most TLRs is unknown (*1-4*).

Basophil responses to TLR ligands may be important, as it is not understood how pathogens and allergens may lead to induction of Th2 responses. As TLR ligation of dendritic cells most often induces type 1 responses through production of IL-12, it is not understood whether

and how Th2 cells can be induced via TLR. IL-4 production is known to be an important factor for induction of Th2 responses (*5*), and therefore, the notion has arisen that Th2 induction would be driven by the combination of DCs that present antigen in the context of MHC class II and accessory cells that provide an early source of IL-4 in the LN (*6*). Additional cytokines able to enhance Th2 responses have been identified, including IL-25, IL-33, and thymic stromal lymphopoietin, which may either act directly on the T cells or change the maturation of APCs to drive Th2 responses (*7*).

Since basophils are a known early source of IL-4 and other cytokines associated with Th2 responses, they have been proposed to play a prominent role in the induction of such responses (*8*). In mice, it was suggested that basophils play a crucial role in responses against Th2-associated helminth parasites and in protease allergens (*9-11*). Basophils were proposed to function as APCs in these studies and were also shown to be present in LNs in models of allergy and helminth infection (*12-14*). However, the function of basophils as APCs during the priming phase of Th2 responses in mice has been challenged by others (*13, 15, 16*), and human basophils could not present antigens to $CD4^+$ T cells (*11, 17, 18*). Therefore, it seems unlikely that human basophils function as APCs. However, by providing an early IL-4 signal that facilitates Th2 skewing after T-cell activation by APCs, basophils could play an important role in skewing of Th2 responses as accessory cells. Indeed, in a mouse model for papain-induced Th2 responses, both DCs and basophils were important for Th2 skewing (*19*).

Since several allergens and helminth parasites can activate TLRs (*20-22*), it would be of high importance to know whether human basophils can be activated via TLR triggering, and if so, which cytokines are released. Until now, little information on these aspects is available for human basophils. Furthermore, as allergic responses and helminth infections may induce IgE responses (*23*), it would be important to know whether these pathways would interact and promote the establishment of a Th2 cytokine milieu. Therefore, in this study, we aimed to understand the activation of basophils by TLR ligands, and to subsequently determine whether this activation could influence Th2 responses.

RESULTS

EXPRESSION OF TLR

To determine the expression of TLR1 to TLR10 and other molecules involved in TLR signaling pathways, a real-time PCR array was performed on mRNA of basophils isolated from peripheral blood of three different donors. Isolated basophils were defined by expression of CD123, FcεRI and CD203c (Fig. 1A). mRNA encoding TLR1 to TLR8 was found

in the basophil populations from all three donors (Fig. 1B). TLR9 and TLR10 mRNA were only detected in one of the three donors, but were readily detected in PBMCs and the monocytic cell-line THP-1 (data not shown).

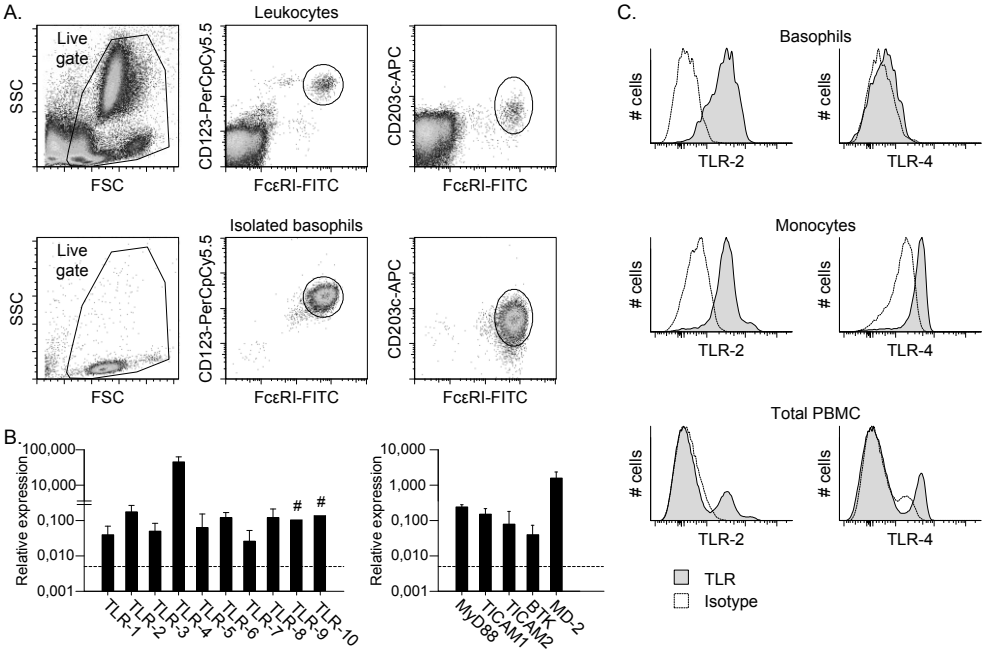

Figure 1. Expression of TLRs by human basophils. (A) Basophils were isolated from peripheral blood leukocytes by negative magnetic isolation. Representative examples of leukocytes before isolation of basophils and the isolated basophil population are shown on the top and bottom, respectively, as analyzed by flow cytometric staining for either CD123 or CD203c with FcεRI. The plots show cells from a live gate based on FSC/SSC characteristics. (B) Total mRNA was isolated from peripheral blood basophils of three different donors. Relative mRNA expression of TLRs (left) and molecules involved in TLR signaling (right), normalized to HPRT1 expression as housekeeping gene, is shown. Data are shown as the mean +SEM pooled from three independent basophil donors. The dotted line indicates the detection limit of the PCR assay. #: mRNA for TLR-9 and -10 was only detected in one out of three donors. (C) Flow cytometric staining of PBMCs for TLR-2 (top) and TLR-4 (bottom). Basophils were gated as shown in (A) and monocytes were gated based on FSC/SSC characteristics. A representative example from three experiments with n = 5 independent donors is shown.

Basophils also expressed mRNA for several molecules associated with TLR signaling, such as MyD88, TICAM1 (TRIF), TICAM2, and BTK and MD-2. Expression of TLRs by basophils was confirmed on the protein level by flow cytometric staining for TLR-2 and TLR-4 (Fig. 1C). Expression of TLR-2 by basophils was comparable to the expression by monocytes.

Expression of TLR-4 by basophils was slightly less than monocytes, but was clearly higher than the isotype control, and higher than other cells in the total PBMC population, confirming the specificity of the staining. These data indicate that human basophils can express TLRs as well as the downstream signaling molecules needed for TLR signal transduction.

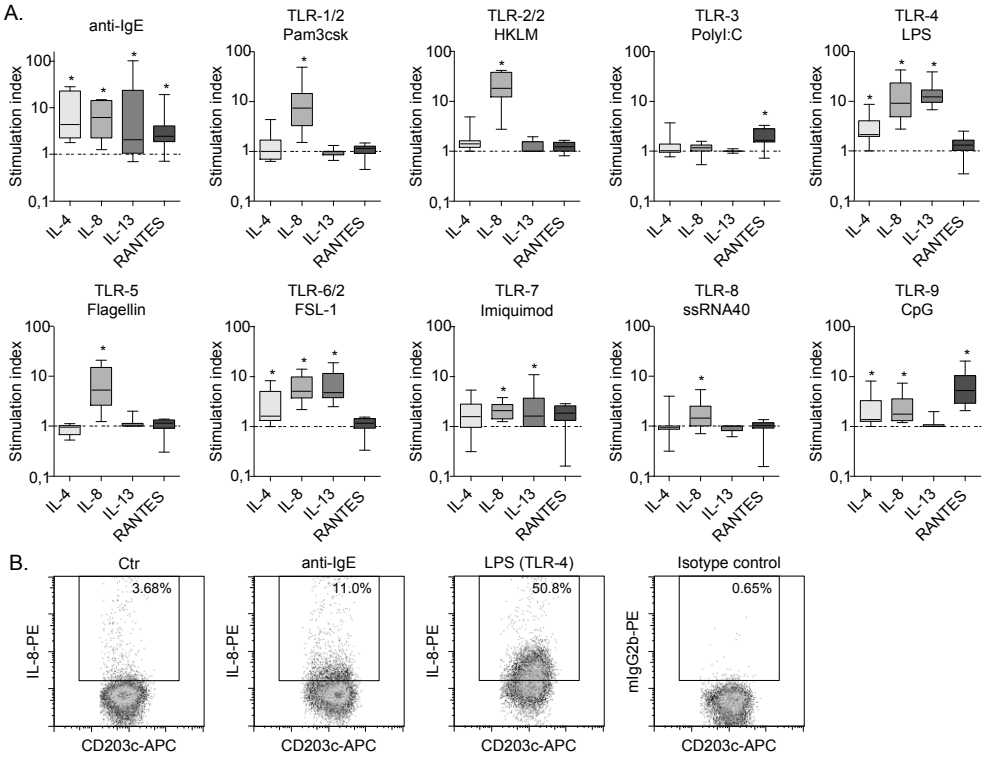

Figure 2. Cytokine production by human basophils in response to TLR ligands. (A) Isolated basophils were stimulated with TLR ligands for 24 h, after which cytokines were measured in supernatant by Luminex assay. Stimulation index was calculated by dividing the amount of cytokine produced after stimulation by the amount of cytokine in the supernatant of unstimulated basophils. Results are shown as a summary of ten experiments performed with basophils from ten different donors. The boxplots show the mean (line), 25–75 percentile (box) and minimum-maximum (whiskers). *$p < 0.05$, increase in stimulation index (above 1), one-sample t-test. (B) Intracellular flow cytometry staining for IL-8 and matching isotype control after overnight stimulation of isolated basophils with anti-IgE and LPS in the presence of brefeldin A. Basophils were gated as $FcεRI^+CD203c^+$ cells, as shown in Fig. 1. A representative example from three experiments with n = 4 independent donors.

CYTOKINE PRODUCTION AFTER BASOPHIL ACTIVATION WITH TLR LIGANDS

To examine whether the mRNA expression of TLRs could be related to functional responses, cytokine production by basophils was measured 24 h after stimulation with TLR ligands. Basophils respond to TLR ligands with production of various cytokines (Fig. 2A, Supplementary Fig. 1 and Supporting Information Table 1 online). Although IL-8 was produced in response to most of the TLR ligands (except TLR-3), production of IL-4 and IL-13 was mainly restricted to ligands for TLR-2/2, -4, and -6/2, and production of RANTES was restricted to the virus-associated TLR ligands PolyI:C (TLR-3) and CpG (TLR-9). These results suggest that the response to TLR ligands was different depending on the TLR that was being triggered. IL-10 production was only observed after ligation of TLR-2 and -4, and had p values >0.005 (not significant after correction for multiple testing). As no ligands are known for TLR-10, the absence of mRNA expression for this TLR could not be confirmed by functional assays. The differences in cytokines produced with different TLR ligands were confirmed by a titration of the TLR ligands (data not shown), indicating that the differential response that was seen was not merely a result of different degrees of activation with the TLR ligands.

Intracellular cytokine staining confirmed that IL-8 production in response to TLR ligands was derived from basophils (Fig. 2B). Altogether, these results indicate that basophils respond to all TLR triggers with cytokine production and interestingly, that basophils produce different cytokines depending on the TLR that is triggered.

DEGRANULATION AND LEUKOTRIENE RELEASE AFTER BASOPHIL ACTIVATION WITH TLR LIGANDS

Besides cytokine production, one of the main effector functions of basophils is mediated via release of granules containing several proinflammatory mediators. Therefore, degranulation of basophils in response to TLR triggering was analyzed by determination of histamine release in supernatant and flow cytometric staining for CD63, which is upregulated on the surface of degranulated basophils (Fig. 3A and B). As expected, basophils released histamine in response to the positive controls anti-IgE and fMLP within 1 h (Fig. 3C). Likewise, CD63 was upregulated on the surface of degranulated basophils.

Although activation of basophils by ligands for TLR-7 and TLR-8 led to small increases in CD63 upregulation and histamine release (TLR-7 only), these responses were low compared with anti-IgE and fMLP and were statistically not significant. No degranulation of basophils was observed following activation by other TLR ligands. Furthermore, no CD63 upregulation was found in response to TLR ligands after 24 h of incubation (data not shown).

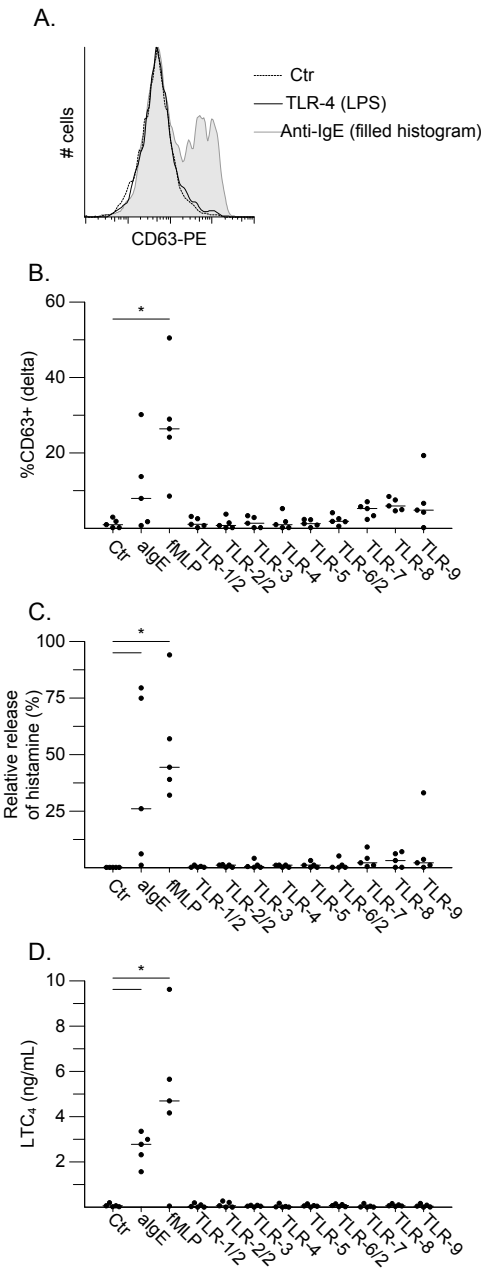

Figure 3. Basophil degranulation and leukotriene production in response to TLR ligands. (A–D) Isolated basophils were stimulated for 1 h with anti-IgE, fMLP, or TLR ligands, after which cells were stained for flow cytometry to determine expression of (A and B) CD63, and supernatant was assayed by ELISA to determine the release of (C) histamine and (D) leukotriene C4. (A) Representative data for CD63 expression in unstimulated basophils (dotted line) or basophils stimulated with anti-IgE (shaded histogram) or LPS (straight line) are shown. Basophils were gated as $CD123^{+}FcεRI^{++}CD203c^{+}$ cells, as shown in Fig. 1. (B) The delta percentage of CD63 that is shown was determined by subtracting the percentage of PE^{+} cells in the isotype control from the percentage of CD63-PE^{+} cells for each indicated ligand. (B–D) *$p<0.05$ compared with unstimulated basophils, repeated measures ANOVA with Bonferroni's posthoc test. Results are shown as a summary of five independent experiments, with the line across the dots representing the median, and each symbol representing an individual donor.

To further examine basophil responses to TLR ligands, we analyzed the release of lipid-derived mediators of basophils by measuring Leukotriene C4 in supernatant. Basophils only responded with leukotriene C4 release after activation with anti-IgE and fMLP and not after activation with TLR ligands (Fig. 3D). Therefore, TLR ligands induce a different response compared with IgE-mediated activation, characterized by production of cytokines and chemokines in the absence of degranulation or release of lipid-derived molecules.

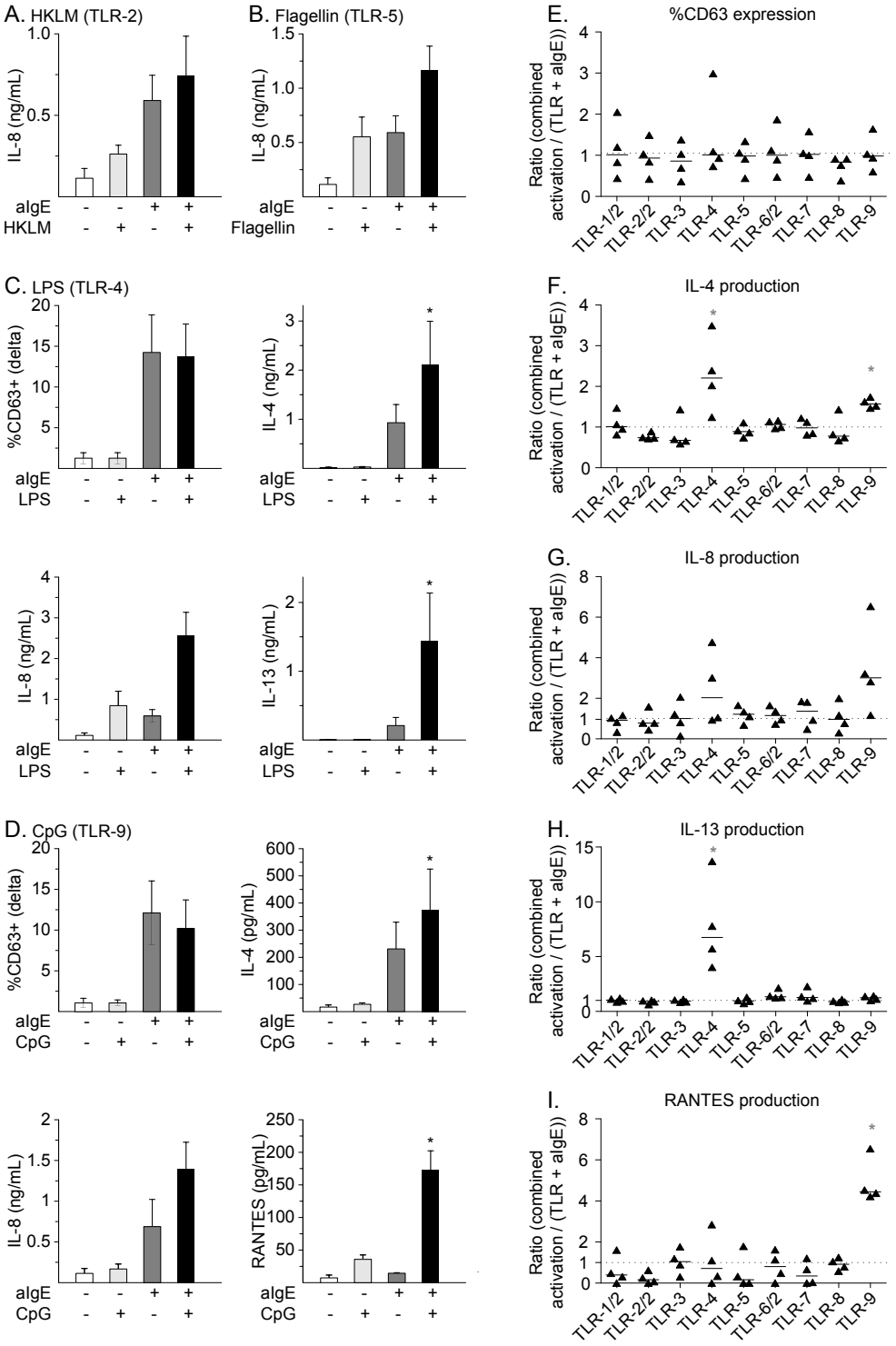
A. HKLM (TLR-2)
IL-8 (ng/mL)
aIgE
HKLM
B. Flagellin (TLR-5)
IL-8 (ng/mL)
aIgE
Flagellin
E. %CD63 expression
Ratio (combined activation / (TLR + aIgE))
TLR-1/2
TLR-2/2
TLR-3
TLR-4
TLR-5
TLR-6/2
TLR-7
TLR-8
TLR-9
C. LPS (TLR-4)
%CD63+ (delta)
aIgE
LPS
IL-4 (ng/mL)
F. IL-4 production
IL-8 (ng/mL)
IL-13 (ng/mL)
G. IL-8 production
D. CpG (TLR-9)
%CD63+ (delta)
CpG
IL-4 (pg/mL)
H. IL-13 production
IL-8 (ng/mL)
RANTES (pg/mL)
I. RANTES production

Figure 4 *(left)*. Synergy in cytokine production after combined TLR- and IgE-mediated stimulation. Basophils were stimulated with TLR ligands alone or in combination with anti-IgE for 24 h, after which degranulation was measured by flow cytometric staining of CD63 and cytokines were measured in supernatant by Luminex assay. (A and B) Examples of additive effects on IL-8 production in response to stimulation with anti-IgE and either TLR-2 or -5 ligands. (C and D) Examples of synergistic effects on cytokine production in response to stimulation with anti-IgE and either TLR-4 or -9 ligands. Percentage of $CD63^+$ basophils on gated $CD123^+FcεRI^{++}CD203c^+$ cells, as shown in Fig. 1. The delta percentage of CD63 that is shown was determined by subtracting the percentage of PE^+ cells in the isotype control from the percentage of CD63-PE^+ cells for each indicated ligand. (A–D) Data are shown as mean + SEM pooled from four independent experiments with basophils from four donors. (E–I) Summaries of CD63 expression and cytokine production for all TLR ligands in combination with anti-IgE are shown as ratio of percentage of $CD63^+$ cells or cytokine production in response to combined triggering divided by the sum of $CD63^+$ cells or cytokine production following stimulation with anti-IgE and TLR ligands separately, with each symbol representing an independent experiment and basophil donor. *$p<0.05$ compared with the additive cytokine production, one-sample t-tests.

SYNERGY BETWEEN TLR- AND FCεRI-MEDIATED ACTIVATION

It has been described that certain cytokines such as IL-3 can enhance the response of basophils upon IgE-mediated stimulation. However, it is not known whether a similar interaction is present between TLR- and IgE-mediated triggering of basophils. Therefore, we next studied the effects of combined TLR- and FcεRI-triggering by combining the different TLR ligands with anti-IgE.

No influence of TLR ligands on IgE-mediated degranulation (CD63 upregulation) was found. A representative example is shown for LPS (TLR-4 ligand) in Figure 4C. Although only additive effects on cytokine production were observed when TLR-1/2, -2/2, -3, -5, -6/2, and -7 ligands were combined with anti-IgE (Fig. 4A and B), combination of ligands for TLR-4 and -9 led to a fivefold increase in IL-4/IL-13 production and IL-4/RANTES production, respectively (Fig. 4C–I), as compared with the combined amounts of cytokines found after separate TLR and FcεRI triggering.

These results indicate that the combined stimulation of basophils with ligands for TLR-4 and -9 and anti-IgE induce a synergy in cytokine production by basophils.

SYNERGY WITH ALLERGEN-MEDIATED ACTIVATION OF BASOPHILS

As simultaneous activation of basophils from allergic individuals through allergen-specific IgE and TLRs could possibly underlie exacerbations of asthma symptoms during pulmonary infection, for example, we next evaluated whether the synergy is also present when basophils of allergic individuals are activated via LPS and allergens.

Although LPS only led to a small increase in degranulation of basophils (Fig. 5A), cytokine production was greatly enhanced when the basophils were stimulated by both LPS and allergen (Fig. 5B), indicating that synergy in cytokine production is also present when TLR triggering is combined with activation via allergen-specific IgE.

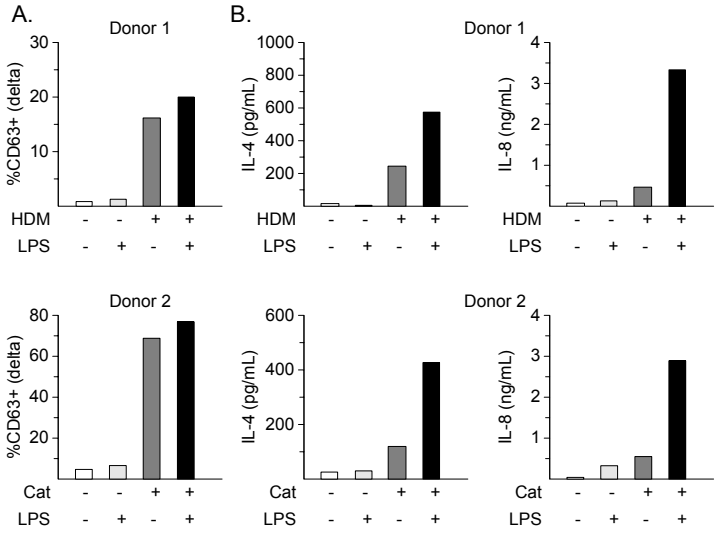

Figure 5. Synergy in cytokine production after combined TLR- and allergen-specific IgE-mediated stimulation. Basophils isolated from two allergic individuals were stimulated with TLR ligands alone or in combination with allergens for 24 h, after which degranulation was measured by CD63 upregulation and cytokines were measured in supernatant by ELISA. (A) CD63 upregulation when allergen-induced stimulation was combined with LPS. Basophils were gated as $CD123^{+}Fc\varepsilon RI^{++}CD203c^{+}$ cells. (B) Production of IL-4 and IL-8 after stimulation of basophils with allergen and LPS. Data are shown as individual experiments with basophils from two donors.

SYNERGY IN BASOPHIL ACTIVATION CAN ENHANCE T HELPER 2 (TH2) RESPONSES VIA IL-4

As basophils respond to combined triggering via TLR-4 and anti-IgE with greatly enhanced cytokine production, we next analyzed the relevance of this synergistic response by evaluating the influence of the cytokine production by basophils on the skewing of naive Th cells. To this end, naive $CD4^{+}$ T cells were stimulated with anti-CD3 and -CD28 in the presence or absence of basophil supernatant for 5 days, after which cytokine production by T cells was analyzed. When T cells were cultured in the presence of supernatant from basophils triggered by the combination of anti-IgE and LPS, the percentage of $IL\text{-}13^{+}$ cells was greatly enhanced (Fig. 6A and B). In contrast, only a relatively small increase in the percentage of $IFN\text{-}\gamma^{+}$ cells was found, indicating that basophils preferentially skewed

toward a Th2 phenotype. To confirm that the intracellular staining of IL-13 reflected enhanced skewing toward Th2 cells, we also measured T -cell cytokines in supernatant (Fig. 6C). Enhanced release of the typical Th2 cytokines IL-4, IL-5, and IL-13 was found when T cells were cultured in the presence of basophil supernatant stimulated with LPS and anti-IgE. Only a relatively slight increase in the production of IFN-γ was found in two out of three donors when supernatant of activated basophils was used. Together, our results indicate that basophils activated by anti-IgE in combination with the TLR-4 ligand LPS can promote the skewing toward Th2 cells.

As IL-4 is able to induce skewing of naive T cells toward Th2 cells and basophils were shown to produce relatively high amounts of IL-4 in response to anti-IgE and LPS, we investigated whether the effects of basophils on Th2 skewing were mediated via IL-4 by using blocking antibodies. Blocking IL-4 in basophil supernatant diminished the percentage of IL-13$^+$ T cells, and the effect of basophil supernatant on Th2 induction was completely reversed (Fig. 6D). These results indicate that synergy in IL-4 production by basophils in response to anti-IgE and LPS can promote the skewing toward Th2 cells.

DISCUSSION

In this study, we extensively analyzed basophil responses to TLR ligands. Although a few studies report cytokine production by basophils in response to TLR-2 and -4 ligands (*2-4*), functional responses toward other TLR ligands have not been reported. Furthermore, the effect of the TLR-4 ligand LPS on basophil activation showed inconsistent results among the different studies. Whereas two studies reported absence of CD11b upregulation and cytokine production (*2, 3*), one study reported production of cytokines in response to LPS (*4*), which is in agreement with our findings. We found that mRNA expression of TLR-4 was relatively high compared with the other TLRs. Furthermore, LPS induced the broadest cytokine production profile among the TLR ligands studied, and we were able to show a large shift in intracellular IL-8 in basophils treated with LPS, suggesting that basophils can indeed respond functionally to the TLR-4 ligand LPS.

By using sensitive multiplex assays, we detected cytokine production by basophils in response to ligands for TLR-1 to -9. There was a very small amount of BDCA-2 positive cells in the isolated basophil fraction in some donors (maximally 0.3%), whereas in other donors, no BDCA-2 positive cells were detected at all (data not shown). However, typical DC cytokines were not detected in response to TLR ligation, suggesting that the cytokine production we observed was not derived from contaminating DCs.

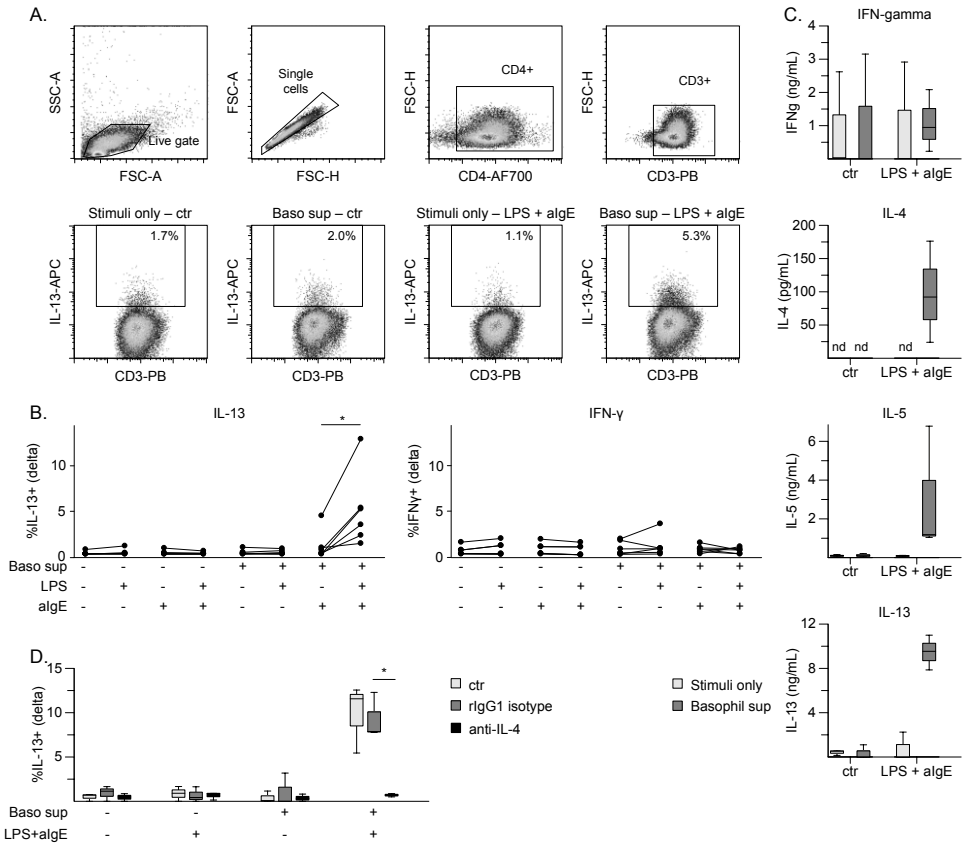

Figure 6. Influence of basophil activation by LPS and anti-IgE on Th-cell skewing. Naive $CD4^+$ T cells were cultured for 5 days in the presence of anti-CD3 and anti-CD28, with or without basophil supernatant, indicated as "basophil sup" or "stimuli only", respectively. T cells were restimulated with PMA/ionomycin to evaluate cytokine production by intracellular staining and by Luminex assay. (A) Representative example from five independent experiments of intracellular flow cytometric staining for IL-13 in the absence or presence of basophil supernatant from unstimulated or LPS- and anti-IgE-stimulated cells. Gating strategy is shown (top). (B) Percentages of $IL\text{-}13^+$ and $IFN\text{-}\gamma^+$ T cells derived from intracellular flow cytometry from five independent experiments performed using supernatants from six independent basophil donors and five independent T-cell donors. The delta percentage of cytokines was calculated by subtracting the percentage of positive cells in the isotype control from the percentage of cytokine-positive cells for each condition. *$p < 0.05$ increase in percentage of $IL\text{-}13^+$ T cells when supernatant from LPS- and anti-IgE-stimulated basophils was compared with that of basophils stimulated with only anti-IgE, paired samples t-test. (C) T-cell cytokine production as measured in supernatant. Data were pooled from three experiments with three independent basophil and T-cell donors. n.d.: not detected. (D) Intracellular staining of IL-13 in cultured naive $CD4^+$ T cells stimulated with basophil supernatant incubated with either an IL-4-blocking antibody or matching isotype control. Summary pooled from three experiments with three independent basophil and T-cell donors. *$p < 0.05$ decrease when basophil supernatant was treated with anti-IL-4 blocking antibody compared with the isotype control, paired samples t-test. The boxplots show the mean (line), 25–75 percentile (box) and minimum-maximum (whiskers).

We observed different patterns of cytokine production depending on the TLR that was being triggered. Interestingly, the virus-associated TLRs induced different responses than the bacterial-associated TLRs, indicating that basophils can respond in diverse ways to these different types of pathogens. Such different cytokine production profiles have previously been reported for dendritic cells, where different cytokines produced in response to bacterial- or viral -associated TLR ligands were shown to induce different types of immune responses (*24, 25*). Therefore, the different cytokines produced by basophils in response to TLR ligands may also alter the immune response that is elicited upon infection by viruses or parasites.

The synergy we observed when both TLR-4 or -9 and FcεRI were triggered indicates that TLR triggering could enhance IgE-mediated responses, such as in allergy or parasitic infection. The effects of TLR ligands are somewhat comparable to the effects that cytokines have on the priming of basophil responses. For example, IL-3 and IL-33 have both been shown to enhance degranulation and IL-4 and IL-13 production by basophils in response to anti-IgE (*26, 27*). TLR triggering did not influence IgE-mediated degranulation, indicating that the effects of TLR triggering and priming by cytokines on IgE-mediated basophil activation are distinct. In mice, synergy in TNF-α production by mast cells upon TLR and FcεRI triggering has been shown to depend on synergistic activation of protein kinases (*28*). Although TLRs and FcεRI use different molecules for their intracellular signaling (e.g. MyD88 and Syk), they can activate similar transcription factors via mitogen-activated protein kinases, such as p38, Erk, and JNK (*29*). Therefore, the synergy in cytokine production by basophils in our system may have been induced via a similar mechanism.

This synergy in cytokine production of basophils might occur in vivo when both specific IgE and TLR ligands are present, such as during parasitic infections, autoimmune diseases, and allergic reactions. Although parasites can induce specific IgE and activate TLRs through pathogen-associated molecular patterns, chronic allergic inflammation may lead to activation of TLR by damage-associated molecular patterns (*30, 31*). In addition, certain viral and bacterial infections have been associated with asthma exacerbations (*32, 33*), and the presence of endotoxin (TLR-4 ligand) was demonstrated in house dust, and was related to severity of allergic reactions against house dust mite (*21*), Our data indicate that activation of basophils may contribute to these processes by enhanced cytokine production when both specific IgE and TLR ligands are present. The fact that basophil responses to TLR ligands could greatly enhance the IgE-mediated activation suggests that these responses mainly play a role in memory or chronic immune responses, when specific IgE has already been generated. However, since certain allergen and helminth antigens may directly crosslink FcεRI on basophils without the need for specific IgE (*34-36*), these responses may also be enhanced by the presence of TLR ligands at the same time, and TLR activation of

basophils may thus contribute to enhanced cytokine production in primary immune responses against helminths as well.

Our results indicate that TLR ligation of basophils may induce IL-4 production as well as other Th2-like cytokines, and may therefore provide an early IL-4 signal when specific IgE is not (yet) present. As such, basophils may function as accessory cell providing IL-4 signals to naïve T cells in the presence of APCs. Two recent studies reported that human basophils could enhance Th2 and Th17 memory responses, through TCR-independent pathways (*37*). Furthermore, in mice, IL-4-producing basophils were shown to enhance DC-driven antigen-specific induction of Th2 cells in the LN, suggesting that basophils can indeed function as accessory cells to provide the IL-4 signal to naive T cells in vivo (*37*). Our results showed that TLR triggering of human basophils may contribute to the induction or expansion of Th2 responses in a similar way.

In this study, we chose to evaluate the effect of synergistic cytokine production by basophils on T -cell skewing, but similar effects may be observed when evaluating the effect of basophil-derived cytokines on other immune cells or on tissue-resident cells, as, for example, IL-4 and IL-13 have been shown to induce mucus production and parasite expulsion, two important processes in parasite infection and allergic responses (*38, 39*). In addition, release of IL-4 and IL-13 by basophils may induce alternatively activated macrophages, which in turn play a role in tissue homeostasis and repair (*40, 41*). Therefore, synergy in cytokine production by basophils may amplify Th2-type immune responses via T cell-dependent and -independent mechanisms.

In conclusion, we found cytokine production by basophils in response to ligands for TLR-1 to -9. The type of TLR ligand determined the cytokine profile being produced, which suggests that basophils could play different roles in the immune response depending on the pathogens present. Furthermore, increased cytokine production by basophils in response to ligation of TLR and FcεRI at the same time could have important implications for their role in Th2 responses. Altogether these results suggest nonredundant roles for basophils in the immune response against pathogens via TLRs, and for the first time suggest that TLR-activated basophils can enhance Th2 responses.

MATERIALS AND METHODS

CELL ISOLATION

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). For isolation of basophils, total leukocytes were first isolated using HetaSep

(StemCell). Basophils were then isolated by negative magnetic bead isolation using the Basophil Enrichment Kit (StemCell), using the manufacturer's instructions. The purity of isolated basophils was determined by flow cytometric analysis of FcεRI, CD203c, and CD123. The purity of isolated basophils was above 95% in each experiment.

For isolation of $CD4^+$ T cells, naive ($CD45RO^-$) $CD4^+$ T cells were isolated from PBMCs by negative magnetic bead isolation (Miltenyi Biotec). The purity of isolated naive $CD4^+$ T cells was determined as $CD14^-CD3^+CD4^+CD45RA^+CD45RO^-$ cells. The purity of isolated naive $CD4^+$ T cells was above 95% in each experiment.

REAL-TIME POLYMERASE CHAIN REACTION

Basophil lysate was prepared using TRIzol reagent (Invitrogen). RNA isolation was performed using TRIzol following manufacturer's instructions, including removal of proteoglycans using a high-salt buffer. A cDNA library was constructed using the RT^2 first strand kit (SA Biosciences) following manufacturer's instructions.

Expression of TLRs by basophils was assessed by real-time PCR. The Toll-Like Receptor Signaling Pathway PCR Array (SA Biosciences) was performed to assess the expression of the several genes, including the following: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, BTK, MD-2, MYD88, TICAM1, and TICAM2. Relative expression of these genes was normalized toward expression of the housekeeping gene HPRT1.

BASOPHIL ACTIVATION

Basophils were cultured in Iscove's Modified Dulbecco's Media containing 10% FCS at a cell concentration of 1 × 106 cells/mL in all experiments, unless indicated otherwise. For TLR-mediated activation, basophils were stimulated using 1 μg/mL Pam3csk, 10^8 cells/mL heat killed *Listeria monocytogenes* (HKLM), 10 μg/mL Poly I:C, 10 μg/mL LPS, 1 μg/mL Flagellin, 1 μg/mL FSL-1, 10 μg/mL Imiquimod, 10 μg/mL ssRNA40, and 5 uM CpG (all from InVivoGen). IgE- or fMLP-mediated activation of basophils was induced using 10 μg/mL goat-anti-human IgE (Nordic) or 1 μg/mL fMLP (Sigma), respectively.

For combined activation with TLR ligands and anti-IgE, basophils were stimulated with 0.1 μg/mL Pam3csk, 10^7 cells/mL HKLM, 2.5 μg/mL Poly I:C, 50 ng/mL LPS, 0.2 μg/mL Flagellin, 0.05 μg/mL FSL-1, 0.5 μg/mL Imiquimod, 5 μg/mL ssRNA40, and 5 uM CpG with or without 0.2 μg/mL anti-IgE.

After 24 h, basophil activation was measured using flow cytometry, multiplex assays, and ELISA. For analysis of degranulation by FACS and release of histamine and leukotrienes, basophils were stimulated for 1 h in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM

NaH2PO4, 1.4 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 100 mM Hepes, and 0.1% BSA in 50% H2O and 50% D2O). To assess the total histamine content of basophils to calculate the percentage of release, basophils were lysed with 1% Triton X-100 in Tyrode's buffer.

For intracellular cytokine staining, basophils were cultured in the presence of 3 µg/mL brefeldin A for 18 h, after which cells were used for flow cytometry.

ALLERGEN-INDUCED BASOPHIL ACTIVATION

Heparinized peripheral blood was obtained from individuals allergic to house-dust mite allergen or cat epithelia. Leukocytes and basophils were isolated as described above. Basophils were incubated with 0.25 U/mL Dermatophagoides pteronissymus (HAL Allergy) or 25 U/mL Epithelia Felis catus (HAL Allergy), respectively, with or without LPS (5 ng/mL). After 24 h, basophil activation was measured using flow cytometry and ELISA.

EFFECT OF BASOPHIL SUPERNATANT ON TH -CELL SKEWING

Basophil supernatant was collected after culture as described above in the presence of anti-IgE and/or LPS. As control, medium with the same concentration of anti-IgE and/or LPS was used. Naive $CD4^+$ T cells were activated using 5 µg/mL plate-bound anti-CD3 (eBioscience; clone: OKT3) and 1 µg/mL soluble anti-CD28 (Sanquin; clone: CLB-CD28/1, 15E8) in the presence of absence of basophil supernatant that was diluted twice in the culture medium.

After 5 days of incubation, T cells were harvested, washed, and split over two wells. One well of each condition was left unstimulated, whereas the other well was restimulated using 50 ng/mL PMA (Sigma) and 500 ng/mL Ionomycin (Sigma). Cells were restimulated for 5 h in the presence of 10 µg/mL brefeldin A (Sigma) for intracellular cytokine staining of IFN-γ and IL-13, or left overnight without brefeldin A to determine cytokine levels in supernatant.

For blocking experiments, basophil supernatant was incubated with 10 µg/mL anti-IL-4 (clone MP4–25D2, eBioscience) or rat IgG1 isotype control (eBRG1, eBioscience), for 1 h at 37°C before adding the supernatant to the T cells.

FLOW CYTOMETRY

The following antibodies were obtained from BD Biosciences: CD123-PerCpCy5.5 (clone 7G3), CD63-PE (H5C6), CD14-PECy7 (M5E2), CD3-Pacific Blue (UCHT1), CD45RA-FITC (L48), CD45RO-PE (UCHL1), IL-8-PE (G265–8), IFN-γ-FITC (25723.11), IFN-γ-PE (4S.B3), IL-13-

allophycocyanin (JES10–6A2), mIgG1-APC (MOPC-21), mIgG2b-FITC (27–35), rIgG1-allophycocyanin (R3-R4), streptavidin-allophycocyanin. The following were obtained from eBioscience: FcεRI-FITC (AER-37), TLR-2-biotin (TL2.1), TLR-4-biotin (HTA125), mIgG1-PE (P3.6.2.8.1), mIgG2b-PE (eBMG2b), mIgG2a-biotin (eBM2a), and the following were obtained from Miltenyi: CD203c-allophycocyanin and -PE (FR3–16A11), BDCA-2-allophycocyanin (AC144), and Biolegend: CD4-AlexaFluor700 (OKT4). For surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0.5% BSA at 4°C for 30 min.

For staining of TLR-2 and -4, cells were incubated for 15 min with 50 ug/mL human IgG (Jackson Immunoresearch) to block Fc receptors, prior to addition of the primary antibodies. After washing, cells were incubated with strep-allophycocyanin at 4°C for 30 min. For intracellular cytokine staining, basophils and T cells were incubated with anti-bodies against surface antigens as described above, after which they were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). After washing, basophils were incubated with antibodies against intracellular cytokines and incubated at 4°C for 30 min.

After washing, cells were suspended in 1% paraformaldehyde until flow cytometric aquisition on an FACS Calibur (BD) or LSR-II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

CYTOKINE, HISTAMINE, AND LEUKOTRIENE C4 PRODUCTION

Quantitative immunoassays for EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, 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 basophil culture supernatants were performed using Milliplex assays (Millipore). Quantitative immunoassays for GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-31, IL-33, MIP-3α/CCL20, TNF-α, TNF-β in T -cell culture supernatants were performed using Milliplex assays (Millipore). Additionally, IL-4, IL-8, and IL-13 production was evaluated using ELISA (from Sanquin and eBioscience). Histamine and Leukotriene C4 were analyzed using competitive ELISA kits (Neogen).

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. For detection of TLR mRNA expression, one-sample T-test was performed. For differences between multiple groups, a repeatedmeasures ANOVA was performed, with Bonferroni's posthoc test. Paired-sample T-test was

performed to analyze differences between two groups. Synergy was calculated as a ratio dividing the cytokine production induced by combined TLR and anti-IgE stimulation by the sum of the cytokine production induced by the separate stimuli (value(TLR + a-IgE)/(value(TLR) + value(a-IgE), after which one-sample T-test was performed. Statistical analysis was performed using SPSS PASW 17.0 and GraphPad Prism 4. P values of <0.05 were considered statistically significant.

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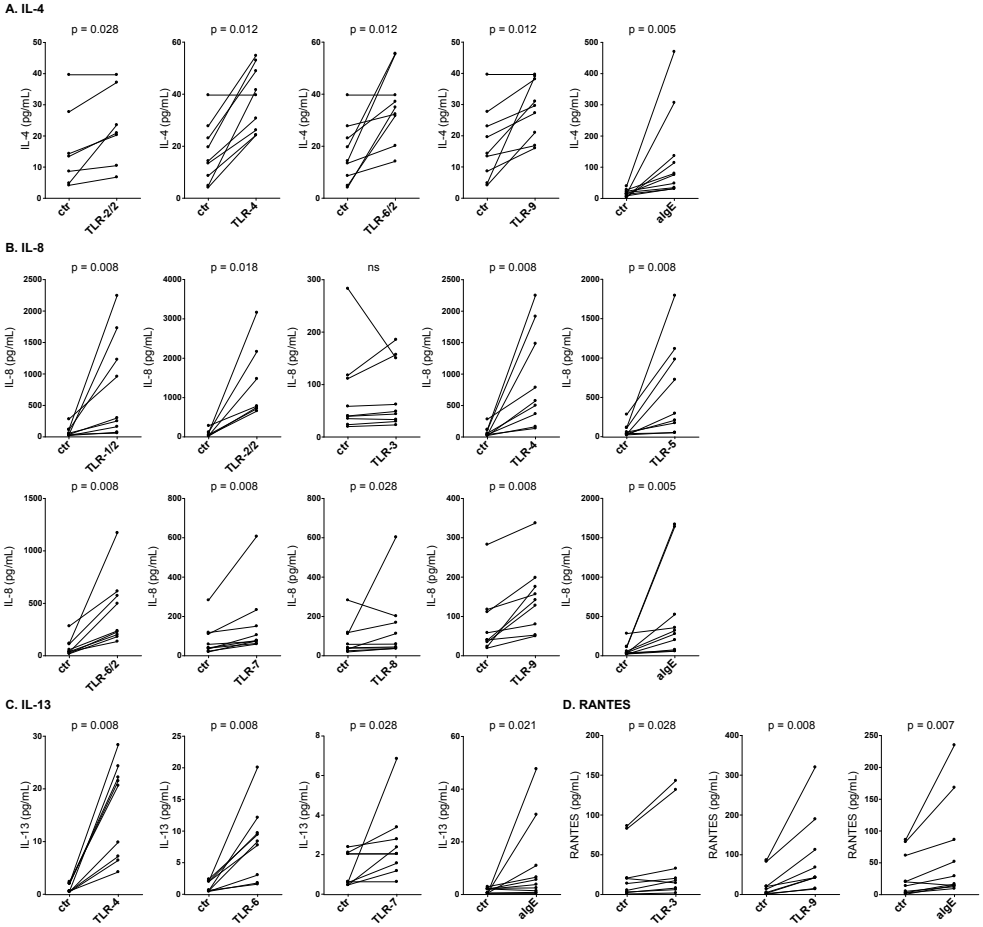
SUPPLEMENTARY FIGURE



Supplementary Figure 1. Cytokine production by human basophils in response to TLR ligands. (A-D) Isolated basophils were stimulated with TLR ligands for 24 hours, after which cytokines were measured in supernatant by Luminex assay. Paired graphs showing cytokine production by basophils left unstimulated (ctr) or stimulated with ligands for the indicated TLR ligands or anti-IgE (aIgE).Results are shown as a summary of 10 experiments performed with basophils from 10 different donors, with each line indicating an individual donor. P values, paired samples T-test.