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Differential Regulation of IL-4 and IL-13 Secretion by Human Basophils: Their Relationship to Histamine Release in Mixed Leukocyte Cultures

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Human basophils are an important source of IL-4, a cytokine that is central to the pathogenesis of allergic inflammation. Recent reports have indicated that these cells also generate IL-13, which shares a number of biologic properties with IL-4. While the magnitude of IL-4 protein generated correlated with the percent histamine secreted ($r = 0.8; p = 0.007$), there was no relationship between the levels of IL-13 detected and the amount of either IL-4 or histamine in cultures activated with IL-3/anti-IgE. The induction of IL-13 secretion also occurred in response to IL-3 alone, without concomitant secretion of either IL-4 or histamine. Although previously shown to inhibit IL-4 secretion, the phorbol ester PMA was a potent stimulus for IL-13 generation from basophils, and this secretion was sensitive to the protein kinase C inhibitor, bisindolylmaleimide. In contrast, bisindolylmaleimide did not prevent cytokine secretion induced by either anti-IgE or IL-3. The immunosuppressant, FK506, while strikingly inhibiting the accumulation of IL-4 mRNA and the secretion of protein in response to IL-3/anti-IgE, had no effect on the generation of IL-13 in these cultures; the resistance was attributed to the IL-3-dependent signaling. Similarly, FK506 had no effect on the secretion of IL-13 in basophil cultures stimulated with PMA. This study suggests that multiple intracellular mechanisms control the generation of IL-13 in basophils, some of which are distinct from those regulating IL-4.


B asophils rapidly secrete inflammatory mediators, such as histamine and leukotrienes, following Ag cross-linking of high affinity receptors for IgE. It is now recognized that allergic reactions are also characterized by the selective recruitment of several cell types into the lesion site. This cellular infiltration, consisting largely of eosinophils and lymphocytes, typifies the ongoing chronic inflammation indicative of allergic diseases, such as asthma and rhinitis. Further, it is generally accepted that cytokines orchestrate this complex cellular reaction. Although basophils are less commonly recognized in these lesions, studies clearly show increased numbers of these cells in the skin (1), nose (2), and lung (3) following experimental allergen challenge. More recent studies have also identified these cells in the airways of postmortem cases of asthma (4).

The concept that basophils play an active role in allergic disease has intensified in recent years with evidence that these cells secrete immunomodulatory cytokines (5). In particular, mRNA for IL-4 is up-regulated within 30 min after IgE-dependent activation and is followed by the secretion of high levels of protein for this cytokine (6). Basophils appear to be the sole source of IL-4 protein secreted in mixed leukocyte cultures, even when challenged with specific Ag (6, 7). Furthermore, they produce this cytokine in a time frame (1–4 h) that is consistent with the kinetics of the late phase response to allergen exposure. In addition to IL-4, several laboratories have now confirmed that human basophils also produce IL-13 following cellular activation (8–10), and one study has reported the production of macrophage inflammatory protein-1α by these cells (11).

It is of considerable interest that human basophils generate and secrete both IL-4 and IL-13 protein, since these cytokines are reported to share many biologic properties that are central in allergic inflammation (12). These include 1) Ig isotype switching in B lymphocytes from IgM to IgE, 2) the up-regulation of CD23 and HLA class II molecules on B lymphocytes, and 3) the promotion of increased expression of vascular cell adhesion molecule-1 on endothelial cells, an adhesion protein regulating the selective transendothelial migration of eosinophils, lymphocytes, and basophils (13–16). There are, however, differences in the functional activities of these two cytokines. Notably, IL-13 does not appear to modulate T cell function, while IL-4 induces the differentiation of the Th2 phenotype (17, 18) and regulates CD8+ T cell functions (19).

As noted above, IgE-dependent activation triggers the release of both IL-4 and IL-13 from basophils. The generation of these cytokines does not appear to occur in tandem, as recent reports show that IL-13 secretion starts at a time (i.e., 4 h) when IL-4 production is near maximal and peaks some 24 h following stimulation (9, 10). The aim of this study was to examine the relationship between IL-4 and IL-13 secretion from basophils by comparing a variety of parameters. Our results show that there is no correlation between the magnitude of IL-13 and IL-4 secretion among donors cells. Further, the differential effects of various secretagogues on the induction of these cytokines and evidence that their synthesis is
pharmacologically independent from one another suggest that distinct intracellular pathways control IL-4 and IL-13 secretion from basophils.

Materials and Methods

Materials

The following special reagents were purchased: biotinylated goat anti-human IL-4 (Endogen, Irvine, CA); crystallized BSA, BFS, PIPES, and ionomycin (Sigma Chemical Co., St. Louis); crystallized human serum albumin, PMA, and bisindolylmaleimide II (BIS II; Calbiochem-Behring Corp., La Jolla, CA); EDTA, d-glucose, Tween-20, and Tween-50 (Fisher, Raleigh, NC); FK506 (Fujiwa USA, Deerfield, IL); gentamicin, Iscove’s modified Dulbecco’s medium (IMDM) containing g-glutamine and 25 mM HEPES, RPMI 1640 with 25 mM HEPES and g-glutamine, and nonessential amino acids (100× stock; Life Technologies, Inc., Grand Island, NY); 60% perchloric acid (Fisher Scientific, Fairlawn, NJ); and Percoll (Pharmacia Biotec, Inc., Piscataway, NJ). The polyclonal anti-human IgE Ab used in these studies was made in a goat (20). Recombinant human IL-4, reconstituted human IL-3, and monoclonal anti-human IL-4 Ab were gifts of Steven Gillis from Immunix Corp. (Seattle, WA).

Buffers and media

PIPERES (10×) contained 250 mM PIPES, 1.10 M NaCl, and 50 mM KCl, pH 7.4, and was stored at 4°C as a stock solution. PAG contained 1/10th of the final concentration of 100% human serum albumin and 0.1% d-glucose. PAG-EDTA additionally contained 4 mM EDTA. Isotonic Percoll (referred to in this manuscript as 100% Percoll) was prepared by mixing 1 part 10% PIPES and 9 parts Percoll. Percoll solutions of 50% (density = 1.066), 55% (density = 1.072), 60% (density = 1.079), and 62% (density = 1.082) were made with the appropriate combinations of 1/10× PIPES and 100% Percoll. Conditioned IMDM (C-IMDM) consisted of IMDM supplemented with 5% heat-inactivated (56°C, 30 min), BFS, 1× nonessential amino acids, and 5 μg/ml of gentamicin.

Preparation of basophil-enriched mixed leukocyte suspensions

Venous blood from consenting adults was anticoagulated in 10 mM EDTA, then centrifuged at 300 × g. The resulting buffy coat at the leukocyte/RBC interface was aspirated and combined with PAG-EDTA (1/1, v/v). Basophil-enriched leukocyte suspensions (5–50% basophils) were prepared as previously described (21). Briefly, the leukocyte mixture was layered onto a double Percoll gradient consisting of 12 ml of 55% Percoll layered onto 12 ml of 62% Percoll in 50-ml polypropylene centrifuge tubes (Corning, Corning, NY). The Percoll gradients were centrifuged at 700 g for 20 min at room temperature. Basophils were concentrated in the lower half of the 55% Percoll fraction, at the 62% interface and the upper half of the 62% interface was aspirated and combined with PAG-EDTA (1/1, v/v). Basophils were purified up to 93% on a second Percoll gradient (densities = 1.069/1.079).

Culture conditions

For all experiments, basophils were cultured for cytokine secretion and histamine release in C-IMDM using protocols previously established in our laboratory (6, 21, 23–25). Experiments were conducted in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA), with the number of basophils varying from 50,000 to 420,000/well. Each condition tested was performed in duplicate. For secretion studies, cells were added to culture wells containing 5 μl of medium alone, 15 μg/ml PMA, or 10 μg/ml of PHA (GTG506, anti-Im-3, or the combination of anti-IgE/IL-3 and FK506. After 2 and 4 h, the cell cultures were centrifuged (12,000 × g) for 10 s, and the supernatants were removed for IL-4 measurements as described above. Total RNA was extracted from the cell pellets using the RNAzol protocol (Tel-Test, Inc., Friendswood, TX). Following isopropanol precipitation, the RNA was washed with 70% ethanol and dried in a speed vacuum centrifuge. Subsequently, the RNA was re-suspended in 25 μl of diethylpyrocarbonate-treated water and stored at –80°C. It is important to note that fourfold dilutions of sample RNA were made in sterile diethylpyrocarbonate-treated H2O before RT-PCR. This was performed to better determine (qualitatively) the relative amounts of mRNA among the samples, RT-PCR was performed as previously detailed (6) using the Gene Amp RNA PCR kit (Perkin-Elmer/Cetus, Norwalk, CT) addition to the manufacturer’s instructions. Briefly, first strand cDNA was synthesized from an aliquot of RNA in the presence of murine leukaemia virus reverse transcriptase (2.5 U/μl); 1 mM each of dATP, dCTP, dGTP, and dTTP; RNase inhibitor (1 U/μl); 5 mM MgCl2; and PCR buffer (50 mM KCl and 10 mM Tris-HCl), using oligo(dT) 18 as a primer. The mixture was incubated at 42°C for 15 min in a Perkin-Elmer/Cetus thermocycler followed by 5 min at 95°C. PCR amplification was performed on aliquots of the cDNA in the presence of MgCl2, dNTPs (0.4 mM each), AmpliTaq polymerase (1 U/20 μl of reaction volume), and paired specific primers (0.2 μM each) for hypoxanthine phosphoribosyltransferase (HPRT), IL-4, and IL-13. PCR conditions were as follows: initial denaturation at 95°C for 2 min, denaturation at 95°C for 15 s, and extension at 72°C for 30 s. HPRT was cycled 30 times, and IL-4 and IL-13 were cycled 25 times before the final extension at 72°C for 10 min. HPRT and cytokine primers were synthesized at the Johns Hopkins DNA Synthesis Laboratory. For IL-4, these represented primers in exons 1 and 4 of the genomic sequence: IL-4: 5′ primer, 5′-ATG GGT CTC ACC TCC CAA CTG CT; 3′ primer, 5′-GTT TTC CCA CGT ACT CTT GGT OGC; IL-13: 5′ primer, 5′-AGA GCC TCT TTC TCA ATC CTC TCC TGT T; 3′ primer, 5′-GCC GAT CCG TGT AAC CTC TCC TGG T; 3′ primer, GGA TTA TAC TGC CTG ACC AAG G. Two distinct bands for IL-4 were observed on agarose gels using the above protocol, and this phenomenon has been previously noted (6, 24). A dominant band was observed with a size of approximately 460 bp. The source of the smaller, fainter band is unknown, but is thought to be an alternatively spliced form of IL-4. The neat product and dilutions were electrophoresed on agarose gel (1%) and visualized by ethidium bromide staining.

Cytokine gene expression studies, RNA isolation, and RT-PCR

Mixed leukocyte suspensions containing 12 to 25% basophils were preincubated (37°C, 5% CO2) in autoclaved (RNase-free) 1.5-ml polypropylene microcentrifuge tubes at a total cell concentration of 4 × 106/ml in 0.25 to 0.5 ml of C-IMDM. Cells were then challenged with an equal volume of medium alone, FK506, anti-IgE/IL-3, or the combination of anti-IgE/IL-3 and FK506. After 2 and 4 h, the cell cultures were centrifuged (12,000 × g) for 10 s, and the supernatants were removed for IL-4 measurements as described above. Total RNA was extracted from the cell pellets using the RNAzol protocol (Tel-Test, Inc., Friendswood, TX). Following isopropanol precipitation, the RNA was washed with 70% ethanol and dried in a speed vacuum centrifuge. Subsequently, the RNA was re-suspended in 25 μl of diethylpyrocarbonate-treated water and stored at –80°C. It is important to note that fourfold dilutions of sample RNA were made in sterile diethylpyrocarbonate-treated H2O before RT-PCR. This was performed to better determine (qualitatively) the relative amounts of mRNA among the samples, RT-PCR was performed as previously detailed (6) using the Gene Amp RNA PCR kit (Perkin-Elmer/Cetus, Norwalk, CT) addition to the manufacturer’s instructions. Briefly, first strand cDNA was synthesized from an aliquot of RNA in the presence of murine leukaemia virus reverse transcriptase (2.5 U/μl); 1 mM each of dATP, dCTP, dGTP, and dTTP; RNase inhibitor (1 U/μl); 5 mM MgCl2; and PCR buffer (50 mM KCl and 10 mM Tris-HCl), using oligo(dT) 18 as a primer. The mixture was incubated at 42°C for 15 min in a Perkin-Elmer/Cetus thermocycler followed by 5 min at 95°C. PCR amplification was performed on aliquots of the cDNA in the presence of MgCl2, dNTPs (0.4 mM each), AmpliTaq polymerase (1 U/20 μl of reaction volume), and paired specific primers (0.2 μM each) for hypoxanthine phosphoribosyltransferase (HPRT), IL-4, and IL-13. PCR conditions were as follows: initial denaturation at 95°C for 2 min, denaturation at 95°C for 15 s, and extension at 72°C for 30 s. HPRT was cycled 30 times, and IL-4 and IL-13 were cycled 25 times before the final extension at 72°C for 10 min. HPRT and cytokine primers were synthesized at the Johns Hopkins DNA Synthesis Laboratory. For IL-4, these represented primers in exons 1 and 4 of the genomic sequence: IL-4: 5′ primer, 5′-ATG GGT CTC ACC TCC CAA CTG CT; 3′ primer, 5′-GTT TTC CCA CGT ACT CTT GGT OGC; IL-13: 5′ primer, 5′-AGA GCC TCT TTC TCA ATC CTC TCC TGT T; 3′ primer, 5′-GCC GAT CCG TGT AAC CTC TCC TGG T; 3′ primer, GGA TTA TAC TGC CTG ACC AAG G. Two distinct bands for IL-4 were observed on agarose gels using the above protocol, and this phenomenon has been previously noted (6, 24). A dominant band was observed with a size of approximately 460 bp. The source of the smaller, fainter band is unknown, but is thought to be an alternatively spliced form of IL-4. The neat product and dilutions were electrophoresed on agarose gel (1%) and visualized by ethidium bromide staining.
greatly reduced compared with those obtained using ionomycin. Measurements by ELISA. Values are expressed as the mean ± SEM of three experiments using cells prepared from different donors.

Cytokine determination using ELISA

Culture supernatants were stored at −80°C until analysis. IL-4 measurements were performed using an in-house ELISA, as previously described (25). The sensitivity of the assay was consistently 4 pg/ml with a range up to 200 pg/ml. IL-13 measurements were made using IL-13 ELISA kits (Biosource International, Camarillo, CA). The commercial ELISA was performed according to the manufacturer’s instructions.

Results

As noted above, several studies have shown that highly purified basophil suspensions (50–98%) secrete IL-13. However, it is not known whether basophil-derived IL-4 promotes the secretion of IL-13 from the 2 to 50% contaminating cells found in these suspensions. This is a real possibility given the evidence that IL-4 precedes IL-13 secretion from basophils and is thought to be important in the development of the Th2 phenotype. It was therefore our initial aim to establish that human basophils are the major producers of this IL-13, even in mixed leukocyte cultures. For these studies, basophils were purified from leukopheresis packs obtained during plateletpheresis by elutriation and Percoll density centrifugation, as described in Materials and Methods. Lower purity preparations were obtained by adding back nonbasophil fractions generated during the purification procedure. Suspensions of equal cell number but with basophil percentages ranging from <1 to 84% were stimulated with the nonspecific secretagogue, ionomycin (0.5 μg/ml) or with human rIL-3. Cultures were incubated for 20 h, a time point previously shown to be sufficient for IL-13 secretion. As shown in Figure 1a, the ionomycin-stimulated IL-13 was found to correlate significantly with basophil purity (p = 0.007), thus indicating that the contribution of the other contaminating leukocytes was negligible. Using IL-3 (100 ng/ml) as the stimulus, the secretion of IL-13 in the same leukocyte preparations also significantly correlated with basophil purity (Fig. 1b; p = 0.03), although the levels detected were greatly reduced compared with those obtained using ionomycin.

These findings are similar to our previous observations that IL-4 production is strictly a function of basophil purity (6). Thus, in subsequent experiments we focused on using basophil-enriched suspensions rapidly prepared by Percoll centrifugation, since cells isolated in this manner are more responsive to physiologic stimulation (21).

We have previously reported that the magnitude of IL-4 production following activation with anti-IgE Ab correlates with the level of histamine secreted (21). We therefore investigated whether this was also true with respect to IL-13 secretion. Basophils from 10 donors were stimulated with the combination of anti-IgE (10 ng/ml) and anti-IgE Ab (10–20 ng/ml) to optimize for basophil secretion. After 4 h at 37°C in 5% CO2, a portion of cell-free supernatant (0.025–0.050 ml) was removed from all cultures for histamine analysis, while the remaining supernatants from two of the four cultures were removed for IL-4 measurements. IL-13 protein was measured in the remaining two cultures after 20-h incubation. Values are plotted in correlation graphs comparing IL-13 vs percent histamine released (a), IL-4 vs percent histamine released (b), and IL-13 vs IL-4 (c).
same donors’ cells does highly correlate with histamine release ($p = 0.007$; Fig. 2b). Finally, a comparison of the levels of IL-13 and IL-4 protein generated in these cultures showed that there was no correlation between the two secretory events (Fig. 2c; $p = 0.49$). Cells secreting high levels of one cytokine do not necessarily produce large quantities of the other.

It has been reported that basophils produce IL-13 in response to both IgE-dependent and -independent activation (8–10). In particular, IL-3 has been shown to induce IL-13 secretion, with protein levels approaching or greater than those obtained with anti-IgE activation alone (8–10). However, since IL-4 protein (and histamine) is also secreted from basophils of some anergic donors when challenged with IL-3 alone (23), it is difficult to know whether IL-4 and IL-13 secretion dissociate from one another in response to IL-3 activation unless measured simultaneously. We therefore re-examined the effects of IL-3 alone (100 ng/ml) and in combination with anti-IgE Ab (10–20 ng/ml) on IL-13, IL-4, and histamine secretion by basophils obtained from normal donors whose cells are activated by anti-IgE but not by IL-3. As shown in Figure 3a, IL-3 alone caused IL-13 secretion, while not stimulating significant IL-4 production in the same cell preparations even after 20-h incubation, suggesting that IL-4 and IL-13 secretion are differentially regulated. It is important to note that IL-4 protein also was not detected in culture supernatants examined at 4 h or at a time optimal for the secretion of this cytokine using IgE-dependent stimuli (data not shown). We found that the addition of anti-IgE did not significantly enhance IL-13 secretion induced by IL-3, while, as expected, the combination of IL-3 and anti-IgE did induce the secretion of IL-4. However, when used alone, anti-IgE Ab did induce IL-13 protein levels ($134 \pm 73; n = 10$), but only in approximately 50% of the donor cells tested (data not shown). Figure 3b shows the percentage of histamine released in the corresponding cell cultures after 4-h incubation. IL-3 had little effect on histamine release when used alone, yet the combination of IL-3 and anti-IgE induced significant histamine release.

To further support our hypothesis that IL-3 alone is a complete stimulus for IL-13 secretion, the kinetics of its production were compared in cultures challenged with IL-3 vs those receiving the combination of IL-3 and anti-IgE Ab. As shown in Figure 4, addition of anti-IgE increased the rate of IL-13 induced by the IL-3 (by $\sim$4 h between the 4–12 h points), but did not affect the quantity eventually released after 20-h incubation. Thus, IL-13 protein was first detected after 4-h incubation with the combination of IL-3/anti-IgE and after 8 h with IL-3 alone.

We have previously shown that protein kinase C (PKC) activation, induced by PMA, is not a signal for IL-4 secretion by basophils despite causing essentially complete degranulation, as indicated by histamine release. Further, PMA actually down-regulates IL-4 secretion induced by ionomycin, and this effect is reversed with PKC inhibitors (27). We therefore tested the effects of PMA on IL-13 secretion by basophils. For these experiments, highly enriched basophil suspensions (83–93% basophils) were used to reduce the possibility that IL-13 secretion might be derived from contaminating leukocytes. As shown in Figure 5a, PMA alone (at 1 ng/ml), in fact, was a potent secretagogue for IL-13, generating levels about 60% of those induced with the ionomycin control. In these same supernatants, marginal levels of IL-4 protein were detected. We did observe that PMA, when used at $<1$ ng/ml or at higher amounts (10 and 100 ng/ml), induced less IL-13 protein while also failing to generate IL-4 (Fig. 5a, inset). Furthermore, it is unlikely that the contaminating leukocytes (7–17%) in these highly enriched basophil cultures contributed to the PMA-induced IL-13, since subsequent experiments showed that levels of this cytokine also correlate well with the presence of basophils (Fig. 5b).

The immunosuppressive agent, FK506, has been described as a specific inhibitor of calcium-dependent calcineurin signal transduction pathways, while having little or no effect on PMA-induced signals. Therefore, we investigated whether this drug differentially affects the secretion of IL-4 and IL-13. Once again, cultures were challenged with IL-3 and anti-IgE Ab, since this combination induces the secretion of both cytokines in addition to histamine. FK506, at 10 ng/ml, inhibited IL-4 secretion by 70%, with dose-dependent inhibition observed down to a concentration of 300 pg/ml (IC$_{50} = 5$ ng/ml; Fig. 6). In contrast, IL-13 secretion was unaffected by this drug. Note that measurements of IL-4 and IL-13 protein were made after 4 and 20 h, respectively, or at times optimal for the secretion of each cytokine. We also tested IL-4 secretion after 20 h and observed similar inhibition, suggesting that FK506 does not simply alter the kinetics of IL-4 secretion (data not shown). From these data, it appears that an FK506-insensitive pathway exists for IL-13 secretion from basophils. In fact, the data listed in Table I show that IL-3 induced signals for IL-13 generation that were unaffected by FK506, with a protein level that
averaged 373 ± 93 pg/10^6 basophils in the absence of FK506 and 389 ± 102 pg/10^6 in the presence of 10 ng/ml of drug. In these same experiments, the IL-13 induced by anti-IgE alone (226 ± 45 pg/10^6 basophils) was inhibited some 70% by FK506. Likewise, the IL-4 generated (325 ± 73 pg/10^6 basophils) was also inhibited by some 93%.

To test whether FK506 inhibits IL-4 secretion by acting on the level of transcription, RNA was isolated from basophil cultures after 2- and 4-h incubations with medium alone, FK506 alone, and IL-3/anti-IgE with and without FK506 (10 ng/ml). While RT-PCR is not quantitative in assessing gene expression, we did perform fourfold dilutions (to 1/64) of sample RNA to better determine whether differences existed between the treatment conditions. Furthermore, only 25 cycles were used to amplify the IL-4 and IL-13 cDNA products, and 30 were used for HPRT. As shown by a representative experiment in Figure 7, cultures treated with IL-3/anti-IgE clearly showed an accumulation of IL-4 and IL-13 message after 4 h of incubation. However, only IL-4 message appeared to be affected by the addition of FK506, inhibiting its accumulation about 75% when comparing the intensities of the bands seen with the various dilutions. Thus, the intensity of the IL-4 cDNA product generated from undiluted RNA isolated from cells treated with FK506 (Fig. 7, lane 13) is approximately the same as that generated from RNA isolated from cells receiving stimulus alone and diluted 1/4 (lane 10). IL-13 mRNA from these same cultures appear unchanged. Similar results were seen at 2 h, but the overall levels of IL-13 mRNA were too low for comparison at this time point (data not shown).

To further probe the mechanism of IL-13 generation in basophils, we investigated the effect of FK506 on IL-13 secretion induced by either ionomycin (0.5 μg/ml) or PMA (10 ng/ml) to show that this drug does not affect the response induced by the latter stimulus. Once again, cell suspensions highly enriched for basophils (50–91%) were used for these experiments and had not been pretreated or costimulated with IL-3. The data in Figure 8 indicate that FK506 did not significantly inhibit PMA-induced IL-13 secretion when used at concentrations between 0.08 and 10.0 ng/ml. In contrast, FK506 dramatically attenuated the IL-13 induced by ionomycin, in a dose-dependent manner (IC_{50} = 200 pg/ml). These data suggest that multiple pathways have a role in the secretion of IL-13, at least one that is calcium dependent and one that is controlled through PKC activation.

In a final series of experiments, we investigated whether the IL-3-dependent secretion of IL-13 is potentially mediated through signals involving PKC activation. As shown in Figure 9, the compound, BIS II, a selective PKC inhibitor (28), dose-dependently inhibited the IL-13 induced by PMA alone with an IC_{50} of 1 μM.

![FIGURE 5.](image-url)

**FIGURE 5.** Activation of basophils with PMA stimulates the secretion of IL-13 but not IL-4. Highly purified basophil suspensions were prepared by countercurrent flow elutriation and density centrifugation protocols. Cell cultures were stimulated with the indicated concentrations of PMA or ionomycin. After 20-h incubation at 37°C, 5% CO₂, the cell-free supernatants were harvested and measured for IL-4 and IL-13 protein content by ELISA. Values in a represent the mean ± SEM of five experiments using cells from different donors. The inserted graph shows the PMA dose-response curve for IL-13 induction reported as percentage of the ionomycin control. The data in b shows that IL-13 secretion induced by PMA (1 ng/ml) correlated well with the presence of basophils, suggesting that the contaminating cells (mostly lymphocytes and monocytes) contributed little, if any, to the levels of this cytokine generated.

![FIGURE 6.](image-url)

**FIGURE 6.** Secretion of IL-4 and IL-13 by basophils is differentially regulated by FK506. Basophil-enriched suspensions were prepared using Percoll density centrifugation. Cell cultures were stimulated with the combination of IL-3 (100 ng/ml) and anti-IgE Ab (10 ng/ml) alone and in the presence of the indicated concentrations of FK506. After 4-h incubation at 37°C in 5% CO₂, cell-free supernatants were harvested from a set of cultures for IL-4 protein measurements (closed circles). IL-13 secretion (open circles) was assessed after an additional 16-h incubation and in a second set of cultures. The values shown are reported as the mean ± SEM (n = 4) percent inhibition of the protein levels measured in cultures not receiving drug. Control protein levels for IL-13 and IL-4 were 252 ± 67 and 94 ± 26 pg/10^6 basophils, respectively. * denotes significant (p = 0.02) inhibition of IL-4 vs IL-13 (by paired t test).

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>FK506 (ng/ml)</th>
<th>IL-13 (pg/10^6 basophils)</th>
<th>IL-4 (pg/10^6 basophils)</th>
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<tr>
<td>IL-3</td>
<td>10</td>
<td>373 ± 93</td>
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<td>IL-3</td>
<td>10</td>
<td>389 ± 102</td>
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<tr>
<td>Anti-IgE</td>
<td>226 ± 45</td>
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<td>Anti-IgE</td>
<td>69 ± 9</td>
<td>24 ± 14</td>
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* The concentrations of IL-3 and anti-IgE were 100 and 10 ng/ml, respectively.

† FK506 significantly inhibited anti-IgE-induced IL-4 and IL-13 secretion (p < 0.05, n = 5, Wilcoxon test). nt = not tested.
FK506 inhibits the secretion of IL-13 and IL-4 by basophils activated with ionomycin but not PMA. Highly purified basophil suspensions (50–91%) were cultured with 1 ng/ml PMA or 0.5 μg/ml ionomycin. Cell-free supernatants were harvested for IL-13 and IL-4 protein measurements after 20 h incubation at 37°C in 5% CO₂. The percent inhibitions of ionomycin-induced IL-13 (closed circles) and IL-4 (open circles) are compared with that of PMA-induced IL-13 (open circles). The values shown are the mean ± SEM of three experiments. Control protein levels of IL-13 induced by PMA, IL-3, and anti-IgE were 564 ± 220, 448 ± 51, and 302 ± 37 pg/10⁶ basophils, respectively. * indicates that percent inhibition of PMA-induced IL-13 seen at 1 μM FK506 is significantly different (p < 0.05) from the values obtained for either IL-3 or anti-IgE (by paired t test).

Discussion

Previous reports stress many similarities between the secretion of IL-4 and that of IL-13 from human basophils. However, the present study has identified a number of potentially important differences in the transductional mechanisms regulating the release of these two cytokines. In investigating the relationship among IL-4, IL-13, and histamine secretion, we initially activated basophils with IL-3 and anti-IgE, since this combination has been reported to be a potent stimulus for the secretion of all three products. As expected, the secretion of IL-4 in these cultures was found to significantly correlate with histamine release. There was, however, no correlation between the release of IL-13 in the culture supernatants with either histamine or IL-4. On further analysis, it was found that IL-3, when used alone, was a potent stimulus of IL-13 secretion, while the cytokine, at best, was a weak stimulus of IL-4 secretion and histamine release from the same donors’ cells. This finding is in partial agreement with the report by Ochensberger et al. showing that IL-13 is secreted in response to IgE-independent stimulation, most notably the combination of IL-3 and C5a. However, these investigators reported that this combination also resulted in significant IL-4 production (8, 29). In contrast to previous reports (9, 10), we found that the addition of anti-IgE Ab did not significantly enhance the secretion of IL-13 when optimal concentrations of IL-3 were used as the stimulus, but was a potent secretagogue of IL-4 generation. We did find that anti-IgE, when used alone, was generally a poor stimulus of IL-13 secretion, inducing levels relatively lower than those secreted in response to IL-3 alone, and in many instances failed to induce detectable protein during a 20-h incubation. Thus, basophil production of IL-13 appears to be less dependent upon cell activation through the FcεRI receptor than is IL-4 secretion. We extended the study to compare the kinetics of IL-13 secretion induced by IL-3 alone. Although IL-13 secretion was considerably slower (4 h) with this stimulus, the levels of cytokine detected after 20 h were essentially the same as those induced with the combination of IL-3 and anti-IgE. These findings imply that IL-3 acts as a complete stimulus for basophil IL-13 secretion. Further, we found that IL-3 did not cause histamine secretion in the same cultures that produced IL-13. Thus, we have shown that IL-13 production from basophils can be dissociated from histamine secretion, and therefore, it is possible that it may be produced in vivo without the concomitant secretion of
either IL-4 or histamine. These findings would also suggest that although IL-4 and IL-13 share a number of biologic activities, the overall functions of these cytokines in vivo are likely to be quite different. Their overlapping actions, in fact, are thought to be a consequence of the existence of shared receptor components (30).

In terms of allergic disease, the most important shared action of IL-4 and IL-13 is their ability to promote IgE synthesis by B cells (31, 32). The levels of IgE produced in vitro in response to IL-13 are generally lower than those obtained with IL-4 (31), and it has been hypothesized that IL-13 has an important role in the regulation of enhanced (or ongoing) IgE synthesis in allergic individuals (31). Since basophils express CD40L (33), and this protein is an important costimulus required by B cells for IgE synthesis, basophils may help maintain the production of this Ig in the peripheral blood of allergic subjects. Our studies, like those reported previously (9, 10), showing that basophil secretion of IL-13 is much slower than that of IL-4, could indeed support the hypothesis that IL-13 enhances and sustains that production of IgE in the absence of IL-4. Finally, it may be pertinent that we did not find any correlation between either IL-13 and IL-4 levels or between IL-13 and histamine levels in our basophil cultures, because this is compatible with the concept that IL-13 has a homeostatic role in B cell IgE synthesis, possibly having a role in maintaining nonspecific IgE levels.

Our findings also indicate that basophil-derived IL-13 could, in certain instances, be initiated at sites not associated with immediate hypersensitivity reactions. Pertinent to this, it has been shown since the 1970s that basophils infiltrate specific delayed hypersensitivity lesions, commonly referred to as cutaneous basophil hypersensitivity reactions (34). Most clinically relevant are the cutaneous basophil hypersensitivity reactions in the human skin following exposure to rhus toxoid (35) and in chronic granulomatous diseases, such as that associated with Crohn’s disease (36).

Most strikingly, our results suggest that IL-4 and IL-13 secretion are differentially regulated in response to PKC activation induced by phorbol esters. In fact, we found PMA to be a potent activator of IL-13 and, quite remarkably, a very specific stimulus, since its production correlated well with the presence of basophils in mixed leukocyte cultures. The optimal concentration of PMA required for IL-13 secretion was approximately 1 ng/ml, while this response declined (by nearly 50%) at higher concentrations. In previous work we showed that PMA down-regulates IL-4 secretion in basophil cultures induced by ionomycin (27). In the same study PMA synergized with ionomycin to promote the secretion of this cytokine in lymphocyte cultures. In light of the evidence presented here, we hypothesize that PKC activation is pivotal in the regulatory mechanisms controlling IL-13 secretion from basophils and, conversely, has a negative role in IL-4 production. The molecular mechanism for such control may be partially unraveled by recent studies using a Jurkat T cell clone transfected with a construct of the human IL-4 promoter (37, 38). In this model, PMA was shown to activate the transcription factor NF-κB, which decreased IL-4 transcription by competing with a second factor, NF-AT, for binding sites within the promoter. It is possible that the competitive nature of these transcription factors may have an important regulatory role in the generation of IL-4 and IL-13 in basophils. Preliminary evidence for NF-AT involvement in FcεRI activation has come from recent studies using rodent cells (39). Further, we have found that a protein, immunologically identical with NF-AT, is resident in the cytosol of basophils and is also translocated to the nucleus within 5 min of IgE-dependent activation (J. T. Schroeder, unpublished observations). Therefore, NF-AT may control the latter steps in the signal transduction pathway linking FcεRI engagement with the expression of IL-4.

In comparing the pharmacologic control of IL-4 and IL-13 secretion, we found additional evidence to support the belief that signal transduction pathways exist in human basophils that differentially regulate IL-13 and IL-4 generation in these cells. First, we found that the immunosuppressor, FK506, was a potent inhibitor of IL-4 secretion from basophils stimulated with the combination of IL-3 and anti-IgE. Likewise, the accumulation of mRNA for this cytokine was also dramatically inhibited with this drug, as assessed by dilutional RT-PCR. In contrast, the mRNA and protein for IL-13 was unaffected by FK506 in these same cultures. However, on further analysis we found that the secretion of IL-13, when induced with anti-IgE alone, is, like IL-4, inhibited by FK506. Thus, these results would suggest that there is an IL-3-dependent pathway for IL-13 generation in basophils that is resistant to FK506 and that is distinct from that induced through IgE-dependent signaling. In fact, the IL-13 protein induced by IL-3 alone was also unaffected by this drug. Although little is known with respect to signaling through the IL-3 receptor, there is evidence to suggest the involvement of JAK kinases that, in turn, activate STAT transcription factors (40, 41). If so, then this may explain why FK506 did not inhibit IL-3-induced IL-13 secretion, since this drug is not known to affect this pathway. It is important, however, to note that the translocation of NF-AT into the nucleus has been shown to be calcium dependent and is regulated by the calcium-dependent protein phosphatase, calcineurin. Both FK506 and cyclosporin A exert their inhibitory effects on cytokine transcription by preventing the dephosphorylation of calcium-dependent calcineurin, thereby preventing the translocation of NF-AT into the nucleus (42). In this study, FK506 was a potent inhibitor of IgE-dependent IL-4 and IL-13 production from basophils, supporting the concept that a calcineurin/NF-AT pathway not only is important for IL-4 activation, but probably has a role in IL-13 expression. Within this context, we observed that ionomycin-induced secretion of both IL-4 and IL-13, the most potent stimulus for either cytokine, is also ablated with FK506.

As noted above, PMA was a potent inducer of IL-13 secretion from basophils. When the effect of FK506 on PMA-stimulated IL-13 secretion was examined, we observed no inhibitory effect. Thus, the PKC-specific pathway that results in IL-13 secretion appears to operate through a transcription factor unrelated to NF-AT and may very well include a pathway involving NF-κB. This hypothesis is particularly intriguing given the evidence noted above regarding the observations that PMA has a negative effect on IL-4 generation by basophils and that NF-κB competes with NF-AT for binding sites within the IL-4 promoter. Thus, it is possible that signal transduction mechanisms resulting in the activation of IL-13 may, in fact, result in the down-regulation of IL-4. This may also partially explain why IL-13 generation begins at a time (2–4 h) when IL-4 secretion is nearly complete. Unlike FK506, the PKC inhibitor BIS II did inhibit PMA-induced IL-13 secretion. However, this drug, when used at the same concentration (1 μM), was found to have a slight enhancing effect on the IL-13 induced by either IL-3 or anti-IgE, suggesting that neither of these stimuli is involved in a PKC-dependent pathway for IL-13 generation. In this instance, the physiologic stimuli regulating the signals that modify PKC activity and the specific isozymes of PKC involved have not yet been identified.

In conclusion, we have evidence showing that IL-4 secretion and IL-13 secretion from basophils differ in terms of their kinetics of secretion, their response to IL-3 and PMA stimulation, and their pharmacologic control. IL-4 and IL-13 are important mediators in the so-called Th2-type immune reactions and, hence, the cell source of these cytokines and the mechanisms involved in their secretion are of considerable interest. A greater understanding of
the intracellular signals controlling IL-4 and IL-13 production may enable the development of therapeutic agents that specifically control the generation of these cytokines.

References


