

Multiple Defects in FcεRI Signaling in Syk-Deficient Nonreleaser Basophils and IL-3-Induced Recovery of Syk Expression and Secretion¹

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Human basophils respond to Ag-induced cross-linking of their high affinity IgE receptor, FcεRI, by releasing histamine and other mediators from granules, producing IL-4 and other cytokines and, as shown in this study, by forming membrane ruffles and showing increased very late Ag-4 (VLA-4)-mediated adhesion to VCAM-1-expressing target cells. We have identified five blood donors whose basophils lack detectable levels of the FcεRI-associated protein tyrosine kinase, Syk. Despite showing no obvious ultrastructural differences from normal basophils, nonreleaser basophils fail to form membrane ruffles, to show increased VLA-4-mediated adhesive activity, or to produce IL-4 in response to FcεRI cross-linking. Although Syk protein levels are suppressed in basophils from all five donors, Syk mRNA is consistently present. Furthermore, culturing nonreleaser basophils for 4 days with IL-3 restores Syk protein expression and FcεRI-mediated histamine release. Understanding the reversible suppression of Syk protein expression in nonreleaser basophils, and learning to replicate this property in patients with allergic inflammation could be a powerful and specific way to limit symptomatic disease. *The Journal of Immunology*, 2000, 165: 5913–5920.

Basophils from most donors express the high affinity IgE receptor, FcεRI, and respond to cross-linking of this receptor with functional responses that include the secretion of inflammatory mediators, the production of IL-4, IL-13, and other cytokines, and other responses, including two described for the first time in this study, membrane ruffling, and the up-regulation of very late Ag-4 (VLA-4)⁴-mediated adhesive activity. Some or all of these responses very likely contribute to the recruitment of basophils to sites of allergic reactions and to their participation in the pathogenesis of allergic inflammation. In particular, basophils are strongly implicated in the late phase of allergic asthma (1) and are particularly prominent in the airways of people who died of asthma (C. L. Kepley and M. F. Lipscomb, unpublished observations).

The strong correlation between basophil recruitment and degranulation and allergic disease adds interest to reports that basophils from ~10% of donors fail to release histamine in response to FcεRI cross-linking (2–6). Non-releaser basophils degranulate in response to stimuli such as *N*-formyl-methionyl-phenylalanine (fMet peptide), Ca²⁺ ionophore, and PMA, suggesting that their

lack of Ag-induced secretion results from the failure of early events specific to the FcεRI signaling cascade (3–6). Previous investigators established that FcεRI expression and subunit composition are normal in nonreleaser basophils (3, 5). We found that basophils from three nonreleaser donors lacked detectable levels of the FcεRI-associated protein tyrosine kinase, Syk (6). Remarkably, Syk levels were normal in B cells, eosinophils, and neutrophils from the same donors. From these results, we hypothesized that a basophil-specific suppression of Syk protein levels may contribute importantly to the nonreleaser phenotype.

In this study, we show that Syk-deficient, nonreleaser basophils from five separate donors are ultrastructurally normal, but fail to ruffle, produce cytokines, or up-regulate their adhesive properties in response to FcεRI cross-linking. We also show that nonreleaser basophils contain Syk mRNA and that Syk protein expression and FcεRI-dependent secretion can be restored by incubation with IL-3.

Materials and Methods

Reagents

Human IgE was prepared from human myeloma plasma (Cortex Biochem, San Leandro, CA). Anti-Syk and anti-Lyn Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified goat anti-human IgE Ab (anti-IgE) was from Biosource (Camarillo, CA). The anti-CD49d mAb HP2/1, a blocking Ab to the human α₄β₁ integrin (VLA-4), was from Immunotech (Marseille, France). The calcium ionophores A23187 and ionomycin, fMet peptide, PMA, IL-3, RPMI 1640, Ham's F-12, and IMDM were from Sigma (St. Louis, MO). mAb 22E7 to the FcεRI-α-chain was a generous gift from Dr. J. Kochan, Hoffman-LaRoche (Nutley, NJ). The stimulatory mAb 8A2 to human VLA-4 was a generous gift from Dr. J. Harlan (University of Washington, Seattle, WA). Human VCAM-1-transfected Chinese hamster ovary (CHO) cells (VCAM-CHO cells) were kindly provided by Dr. D. Leavesley (Hanson Cancer Center, Adelaide, Australia).

Isolation of peripheral blood cells

Basophils were obtained by Percoll gradient centrifugation of venous blood from normal donors with no history of allergic symptoms, as described previously (7, 8). Purities from this initial step ranged from 15 to 66%. Basophil purity was routinely increased to >95% by negative selection

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⁴ Abbreviations used in this paper: VLA-4, very late Ag-4; fMet, *N*-formyl-methionyl-phenylalanine; CHO, Chinese hamster ovary; TEM, transmission electron microscopy.

using a negative selection cocktail from StemCell Technologies (Vancouver, British Columbia, Canada) and MidiMacs (Miltenyi Biotec, Auburn, CA) magnetic columns (described in Ref. 8). In many experiments, negative selection was followed by flow sorting (7) to yield >99.9% pure basophils. In the ultrastructural studies below, $\sim 0.5\text{--}1 \times 10^5$ RBC (obtained after the initial Percoll step) were added back to make the basophil pellet large enough for convenient embedding and sectioning.

In vitro culture of basophils

Negatively selected basophils (>95% pure) were cultured for 4 days at a concentration of $1\text{--}5 \times 10^5$ cells/ml in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ of streptomycin, 10 $\mu\text{g}/\text{ml}$ of Fungizone (RPMI-FCS medium), with or without 20 U/ml IL-3. The proportion of viable cells on day 4 was 75% (range 55–81%) for the IL-3-treated cells and 56% (range 43–62%) for non-IL-3-treated cells, as determined by trypan blue exclusion. For functional assays, these cells were primed with 10 $\mu\text{g}/\text{ml}$ human IgE during the final hour of IL-3 incubation. They were then harvested, and viable cells were counted and resuspended to the concentrations indicated in the various assays. For Western blotting and RT-PCR analyses, the cells were flow sorted to >99.9% purity before use.

Transmission electron microscopy (TEM)

Suspensions of Percoll-enriched, negatively selected IgE-primed basophils were incubated at 37°C in prewarmed HBSS⁺ (HBSS with 1 mM CaCl₂ and 1 mM MgCl₂) with or without anti-IgE (0.1 $\mu\text{g}/\text{ml}$) for 30 min. In some experiments, cells were incubated in IL-3 as above. Cells were collected by centrifugation, RBC added back as described above to increase pellet volume, and pellets were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature, rinsed with cacodylate, and processed, as described before (9). Thin sections were observed using a Hitachi 600 transmission electron microscope.

Histamine release

Suspensions of Percoll-enriched, negatively selected IgE-primed basophils were washed with HBSS[−] (HBSS without Ca²⁺ or Mg²⁺) and suspended to $0.5\text{--}1.1 \times 10^6$ basophils/ml, and 100- μl aliquots were incubated at 37°C in prewarmed HBSS⁺ containing no addition (spontaneous secretion) or with the addition of anti-IgE or other activating agents (stimulated secretion). Reactions were terminated by dilution in ice-cold PBS and centrifugation, and histamine in cell pellets and supernatants was measured using an RIA (Alpco, Windham, NH), as described (6, 8). Total histamine was measured in supernatants obtained by freeze thawing cell aliquots in PBS/EDTA, followed by centrifugation at $10,000 \times g$ for 5 min to remove debris.

VLA-4 surface expression

Peripheral blood basophils were harvested from releaser and nonreleaser donors by Percoll gradient centrifugation and negative selection, as described above. Cells were incubated for 10 min at 37°C with or without the anti-Fc ϵ RI mAb 22E7 (3 $\mu\text{g}/\text{ml}$). Cells were then lightly fixed at room temperature for 5 min with 0.5% paraformaldehyde, washed, and incubated with FITC-conjugated anti-CD49d mAb (HP2/1) at 1:50 in PBS/BSA (0.2%) for 30 min at room temperature. The same Ab at the same concentration was simultaneously added to 50 μl of Quantum Simply Cellular Microbeads (Flow Cytometry Standards, San Juan, PR) per the manufacturer's recommendations. Cells and beads were washed in PBS and resuspended for FACS analysis. Mean fluorescence of cells was compared, and binding sites were quantified in resting and 22E7-treated (activated) cells relative to the fluorescence of the Quantum Simply Cellular Microbead standards.

VLA-4-mediated adhesive activity

The adhesive activity of basophils was measured using a modification of the assay of Leavesley et al. (10). Percoll-enriched, negatively selected IgE-primed basophils (5×10^5 basophils/ml) were suspended in RPMI-FBS medium and fluorescence labeled by incubation for 45 min with 4 $\mu\text{g}/\text{ml}$ dihydroethidium (Molecular Probes, Eugene, OR) in a 5% CO₂ incubator. In parallel, VCAM-CHO cells (2×10^6 cells/ml) were suspended in 1 ml of Ham's F-12 Nutrient Mixture, 10% FBS, 200 mM L-glutamine, penicillin-streptomycin, and 1% sodium pyruvate (Ham's FBS medium), and fluorescence labeled with 1.5 μM Fluo-3 AM (Molecular Probes). Cells were washed once in the medium used for fluorescence labeling and once in adhesion buffer (modified HBSS⁺ with 0.9 mM calcium and 0.35 mM magnesium). Each group of cells was then suspended in 400 μl adhesion buffer at 37°C. For adhesion assays, cells were com-

bined at a ratio of 1:3.5 basophil:VCAM-CHO cells in adhesion buffer at 37°C to a final volume of 400 μl , and duplicate samples were incubated on a rocker in the presence of no addition, anti-IgE (1 $\mu\text{g}/\text{ml}$), or anti-Fc ϵ RI mAb 22E7 (3 $\mu\text{g}/\text{ml}$) and other activating agents. Every experiment included samples with added EDTA that reduces VLA-4 to its least active conformation, and with added mAb 8A2 plus Mn²⁺, which brings VLA-4 to its maximally active conformation (11). In some experiments, parental (nontransfected) CHO cells were used as additional controls for VLA-4-independent adhesion. After 15 min, cells were fixed by adding 400 μl of 2% paraformaldehyde and continued rolling for 2 min, and the proportion of dually fluorescent conjugates formed between basophils and VCAM-CHO cells was measured in a FACScaliber flow cytometer. The percentage of basophils forming conjugates is derived by dividing the number of conjugates (dual color events) by the number of conjugates plus free basophils.

IL-4 production

Percoll-enriched, negatively selected IgE-primed basophils ($0.1\text{--}1 \times 10^5$ basophils in 100 μl of IMDM containing 5% heat-inactivated FBS, $1 \times$ nonessential amino acids, and 5 $\mu\text{g}/\text{ml}$ gentamicin) were incubated for 4 h at 37°C in a 5% CO₂ incubator with or without the addition of anti-IgE or other activating agents. Cells were centrifuged, and IL-4 protein was measured in the cell-free supernatants by ELISA (Biosource; sensitivity = 0.27 pg/ml).

Western blotting

Expression of Lyn and Syk was measured in highly purified basophils (>99.9% pure; freshly isolated or IL-3 treated for 4 days) by Western blotting, as previously described (6, 8).

RT-PCR

mRNA for Syk was measured in highly purified basophils (>99.9% pure) by RT-PCR. Total cellular RNA was prepared with the RNeasy total RNA system (Qiagen, Hilden, Germany) from $1\text{--}5 \times 10^5$ basophils. The monocyte/lymphocyte cell population (no basophils) obtained after the Percoll gradient centrifugation was used as a positive control. RNA concentrations were determined by a spectrophotometric 260/280 ratio. RT-PCR was performed using the Titan RT-PCR one-reaction system from Boehringer (Indianapolis, IN). The following primers for human Syk or β -actin were obtained from the Protein Chemistry Laboratory at University of New Mexico: Syk, 5'-TCCGAGCCAGAGACAACAACGG-3' and 5'-TTCCAGCGTCAGCAGCTTTCG-3'; β -actin, 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' and 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'. The first cDNA strand was obtained using avian myeloblastoma leukemia virus at 50°C for 30 min, and followed by PCR using the Expand High Fidelity enzyme blend. The RT-PCR were done in a total volume of 30 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 9, 1.5 mM MgCl₂, 200 μM dNTPs, and 1 pmol of each primer. PCR conditions were: template denaturation (1 min at 95°C), followed by a 35-fold repetitive cycle of 1 min at 56°C (annealing), 2 min at 72°C (extension), and 1 min at 95°C (denaturation). After amplification, the samples were analyzed by electrophoresis on a 2% gel containing ethidium bromide.

Results

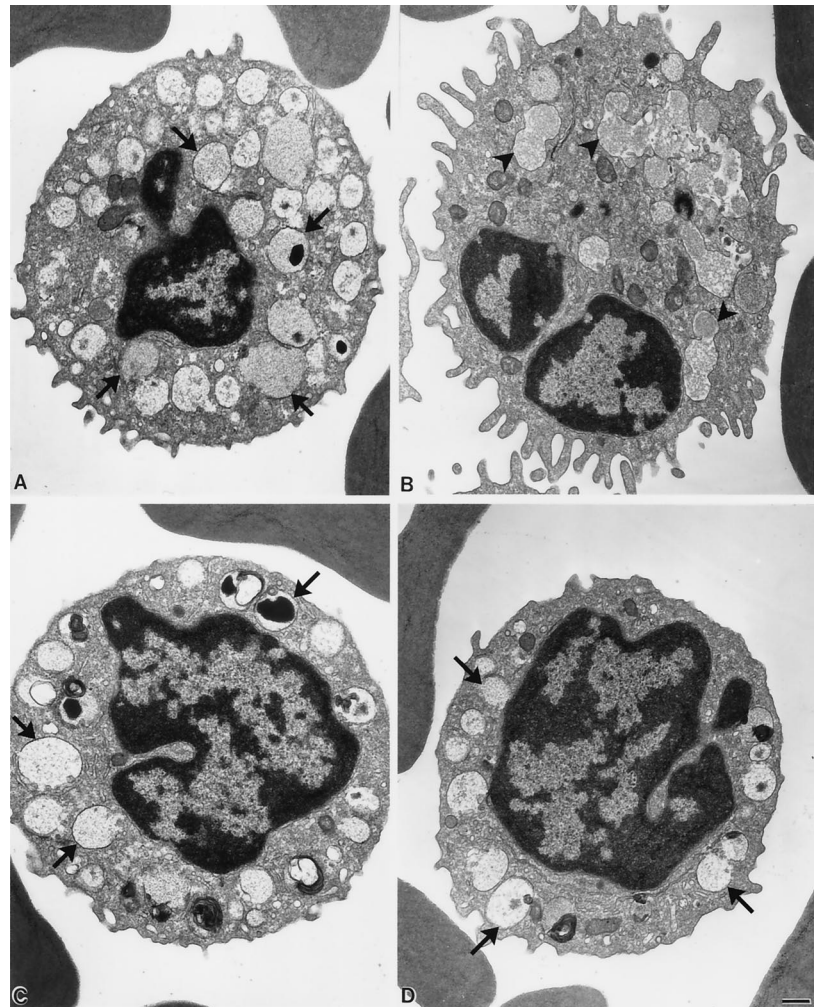
Ultrastructure of nonreleaser and releaser basophils

By TEM, unstimulated releaser (Fig. 1A) and nonreleaser (Fig. 1C) basophils appear as rather typical granulocytes, with multilobed nuclei, relatively sparse endoplasmic reticulum and mitochondria, and fairly smooth plasma membranes. Granules are numerous, and their content of loosely packed matrix material plus occasional dense core material, multilamellar membranes, and internal vesicles is not noticeably different between releaser and nonreleaser cells.

Fc ϵ RI cross-linking induces degranulation and membrane ruffling in releaser but not nonreleaser basophils

In releaser basophils, Fc ϵ RI cross-linking induces the intracellular fusion of granules and release of granule contents (Fig. 1B), as previously described (12). Releaser basophils also show a dramatic membrane ruffling response to Fc ϵ RI cross-linking. In contrast,

FIGURE 1. Ultrastructure of resting and anti-IgE-stimulated releaser and nonreleaser basophils. Percoll-enriched, negatively selected releaser (A and B) and nonreleaser (C and D) basophils (>95% pure) were incubated with (B and D) or without (A and C) 1 μ g/ml of anti-IgE for 30 min at 37°C, washed, and processed for TEM. Resting cells (A and C) show very similar morphologies. Fc ϵ RI cross-linking induces granule-granule fusion, degranulation, and membrane ruffling in releaser (B) but not nonreleaser (D) basophils. Arrows indicate the position of intact (A, C, and D) and arrowheads indicate fused (B) granules. Bar = 0.5 μ M.



granules in nonreleaser basophils remain individual and intact following Fc ϵ RI cross-linking (Fig. 1D). Furthermore, there is no membrane ruffling response to Fc ϵ RI cross-linking in the non-releaser cells.

Fc ϵ RI cross-linking up-regulates VLA-4-mediated adhesion in releaser but not nonreleaser basophils

The adhesive activity of basophil VLA-4 toward VCAM-1 was determined by separately labeling basophils and VCAM-CHO cells with nonoverlapping fluorochromes, then mixing the cells with or without the addition of activating agents, and measuring conjugate formation between these distinct cell types during a rolling assay. The results in Fig. 2A show there is essentially no conjugate formation between releaser basophils and VCAM-CHO cells when the cells are coincubated in the presence of blocking Abs to VLA-4 (or VCAM-1; data not shown) and when EDTA is included in the assay mixture to minimize the binding activity of VLA-4. Under the conditions of our assay, ~50% of releaser basophils form conjugates with VCAM-CHO cells when the VLA-4-activating mAb, 8A2, is included, with the VLA-4-activating cation, Mn^{2+} , in the incubation mixture. These data establish the critical role of VLA-4 and its counterreceptor VCAM-1 in conjugate formation and set the lower and upper limits of VLA-4-mediated adhesion in our assay. Approximately 10–15% of releaser basophils form conjugates with VCAM-CHO cells when the two cell types are rolled together in adhesion buffer (HBSS with Ca^{2+}

and Mg^{2+}) alone. Fc ϵ RI cross-linking by the addition of anti-IgE (shown) or mAb 22E7 (not shown) during the adhesion assay increases the proportion of releaser basophils forming conjugates with VCAM-CHO cells to 25–40%. Conjugate formation between releaser basophils and VCAM-CHO cells is also increased by incubation with fMet peptide that activates the G protein-coupled chemoattractant receptor, with ionomycin that mobilizes Ca^{2+} , and with PMA whose intracellular targets include protein kinase C.

The results in Fig. 2B recapitulate key controls for nonreleaser basophils. In particular, the effects of EDTA in down-regulating adhesive activity and of mAb 8A2 plus Mn^{2+} in up-regulating adhesion are very similar between releaser and nonreleaser basophils. Additionally, conjugate formation between nonreleaser basophils and VCAM-CHO cells is increased by incubation with fMet peptide, ionomycin, and PMA. However, the proportion of unstimulated nonreleaser basophils forming conjugates with VCAM-CHO cells during incubation in adhesion medium is lower than the proportion of unstimulated releaser cells that form conjugates. Furthermore, Fc ϵ RI cross-linking causes no increase in nonreleaser basophil adhesion to VCAM-CHO cells.

We explored the possibility that the low basal adhesiveness of nonreleaser basophils could be attributed in part to low levels of VLA-4 on the nonreleaser basophil surface. The results of immunophenotyping studies showed that releaser and nonreleaser basophils express very similar amounts of membrane VLA-4 (Fig. 2C).

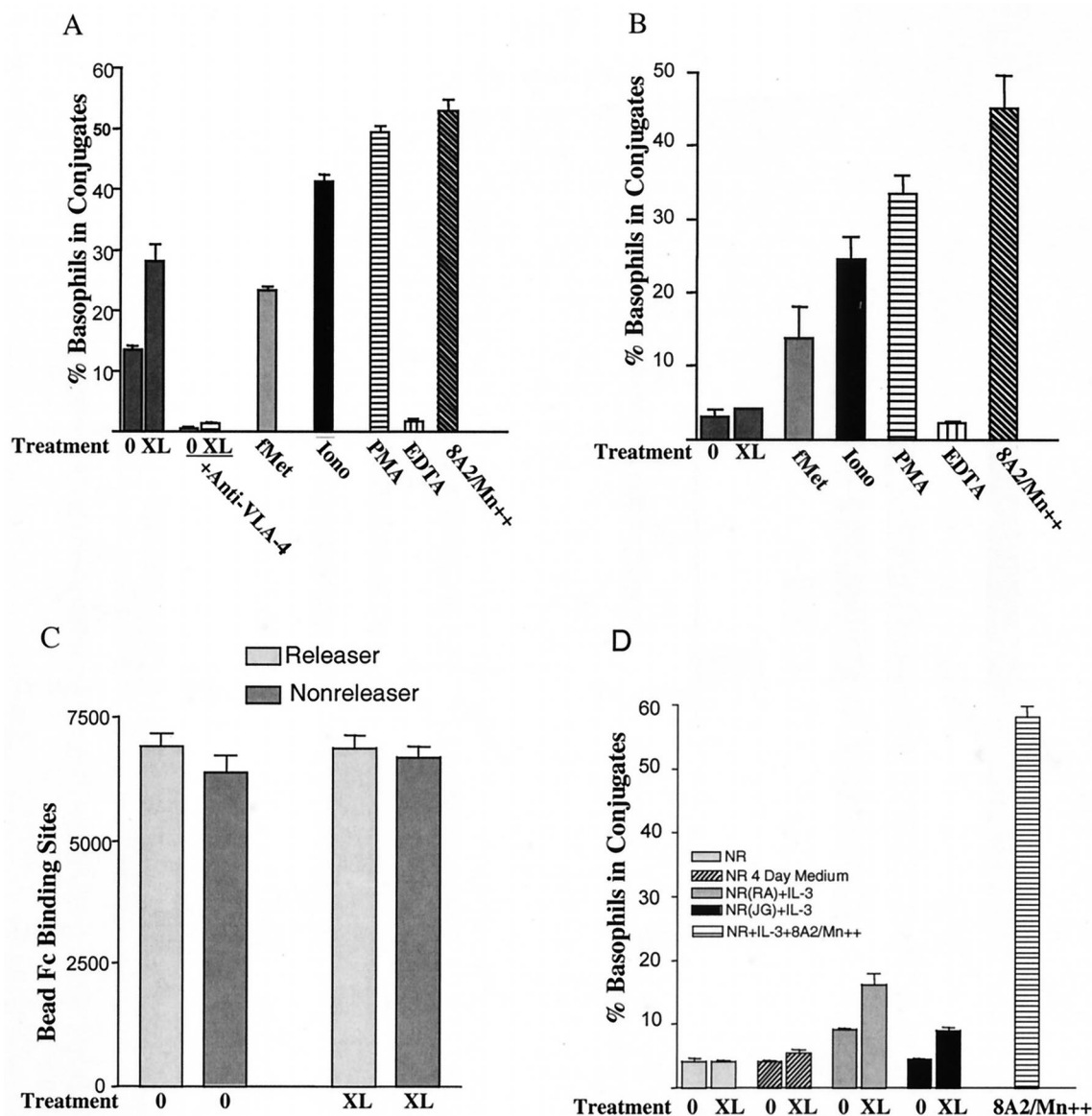


FIGURE 2. A and B, VLA-4-dependent adhesive activity of releaser and nonreleaser basophils. Percoll-enriched, negatively selected releaser (A) and nonreleaser (B) basophils were incubated on a rolling apparatus for 15 min with VCAM-CHO cells with no addition (0) or in the presence of anti-IgE (XL; 1 μ g/ml), fMet peptide (100 nM), ionomycin (1 μ M), PMA (25 nM), EDTA (5 mM), and the activating anti-VLA-4 mAb 8A2 (1:1000 from ascites) plus Mn^{2+} (2 mM). In A, one incubation was performed in the presence of blocking Ab to VLA-4 (HP2/1; 5 μ g/ml). Cells were fixed, and VLA-4-dependent basophil-VCAM-CHO conjugate formation was measured by flow cytometry. Data show the percentage of basophils in conjugates. Results are the average of two separate experiments, each performed in duplicate, $\pm$ SEM. C, VLA-4 levels on releaser and nonreleaser basophils indicated by number of binding sites used. Percoll-enriched, negatively selected releaser and nonreleaser basophils were labeled with FITC-conjugated anti-CD49d. VLA-4 levels on releaser and nonreleaser basophils are indicated by number of binding sites, as measured using mean fluorescence of cells compared with fluorescence of the Quantum Simply Cellular Microbead standards (releaser, $n = 3$; nonreleaser, $n = 2$). D, VLA-4-dependent adhesive activity is partially restored in nonreleaser basophils following IL-3 treatment. Percoll-enriched, negatively selected releaser and nonreleaser basophils were incubated for 4 days in medium with 20 U/ml of IL-3. Basophils were activated with anti-IgE receptor Ab (22E7 3 μ g/ml) for 15 min and conjugate formation was measured, as described above (nonreleaser, $n = 3$).

Fc ϵ RI-mediated IL-4 production is blocked in nonreleaser basophils

The results in Fig. 3A show that incubating releaser basophils with anti-IgE for 4 h induces the production of IL-4. The optimal concentration of anti-IgE for IL-4 production is 0.1 μ g/ml, less than the optimal anti-IgE concentration for secretion (see above). Releaser basophils also produce IL-4 in response to stimulation with the Ca^{2+} ionophore, A23187, but not with fMet peptide (not shown). We were not able to detect IL-4 in nonreleaser basophil cultures stimulated with optimal concentrations of anti-IgE for 4 h.

Longer incubations (8 h) under the same conditions also did not induce IL-4 secretion (data not shown). Nevertheless, A23187 induced IL-4 production in nonreleaser basophils.

Fc ϵ RI-mediated secretion is partially restored by incubating nonreleaser basophils with IL-3

The results in Fig. 4A confirm previous evidence (2–6) that freshly isolated nonreleaser basophils show little or no secretion in response to varying concentrations of anti-IgE, even though they degranulate normally to A23187, fMet peptide, and PMA.

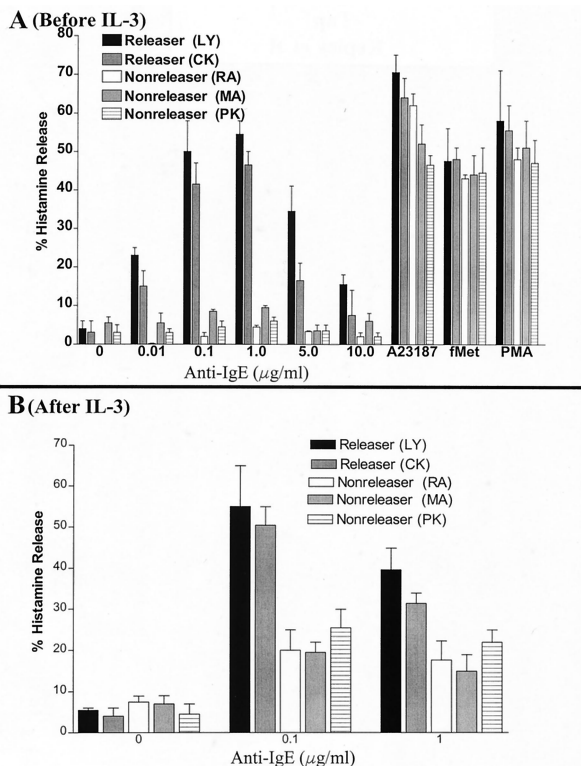


FIGURE 3. Incubation with IL-3 partially restores histamine release in nonreleaser basophils. Percoll-enriched, negatively selected, IgE-primed releaser and nonreleaser basophils were incubated for 30 min in various concentrations of anti-IgE or A23187 (500 ng/ml), fMet peptide (10^{-7} M), or PMA (25 nM), and histamine levels were measured in the incubation supernatants. Cells in A were freshly isolated from blood. Cells in B were previously incubated for 4 days in medium with 20 U/ml of IL-3. Results are from three separate experiments, each performed in duplicate ($\pm$ SEM).

Yamaguchi and colleagues reported that nonreleaser basophils recover secretory activity after incubation for 3 days or longer in IL-3 (5). Supporting this result, we show in Fig. 4B that 4-day incubation of nonreleaser basophils in medium with 20 U/ml of IL-3 induces a partial recovery of Fc ϵ RI-mediated secretion. Fig. 4B also shows that releaser basophils cultured under the same conditions continue to degranulate in response to anti-IgE. Consistent with the previous report (5), long-term culture with IL-3 resulted in an increased sensitivity to anti-IgE; the optimal concentration of anti-IgE for secretion by releaser basophils shifted from 1 μ g/ml in freshly purified basophils (Fig. 4A) to 0.1 μ g/ml after culture with IL-3 for 4 days (Fig. 4B).

IL-3 incubation does not restore all Fc ϵ RI-mediated responses in nonreleaser basophils. The results in Fig. 2D show that incubation for 3 days with IL-3 induces a partial recovery of the Fc ϵ RI-stimulated up-regulation of VLA-4 adhesive activity of nonreleaser basophils. The result was variable between donors, and the recovery was not complete. Similarly, nonreleaser basophils observed by TEM after incubation with IL-3 and activation with anti-IgE showed consistent evidence of granule-granule fusion, but inconsistent membrane-ruffling responses (data not shown). Not even a partial recovery of anti-IgE-induced IL-4 production was observed in nonreleaser basophils that were incubated for 4 days with IL-3 (Fig. 3B).

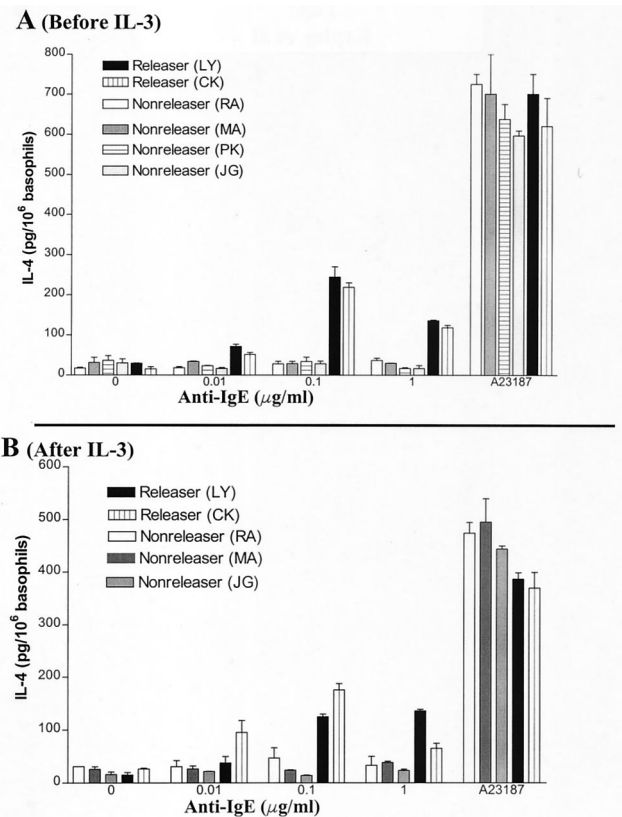


FIGURE 4. IL-4 production by releaser and nonreleaser basophils. In A, Percoll-enriched, negatively selected releaser and nonreleaser basophils were incubated for 4 h with or without the indicated concentrations of anti-IgE, or with fMet peptide (10^{-7} M) or A23187 (500 ng/ml). Cells were then centrifuged, and the supernatants were removed for IL-4 assays. In B, Percoll-enriched, negatively selected releaser and nonreleaser basophils were incubated for 4 days with or without 20 U/ml of IL-3. Following priming with human IgE, these basophils were again challenged for 4 h with the indicated concentrations of anti-IgE or A23187, and the culture supernatants assayed for IL-4 production. Results are expressed as pg IL-4/ 10^6 basophils $\pm$ SEM. Data are from two separate experiments for each donor, each performed in duplicate.

Nonreleaser basophils contain Syk mRNA and express Syk after incubation with IL-3

In our previous work (6), Syk protein was not detected by Western blotting in basophils from three nonreleaser donors. Western blotting of lysates of freshly isolated nonreleaser basophils from two additional donors identified since our initial publication also showed no Syk protein, except after very prolonged exposure of the gels (data not shown).

Although Syk protein was very difficult to detect, Syk mRNA was readily detected in basophils from all five nonreleaser donors. Syk mRNA was also detected in basophils from all releaser donors tested. Representative data from these RT-PCR analyses are shown in Fig. 5A.

The effects on Syk protein levels of incubating basophils for 3–4 days in medium without or with added IL-3 are shown in Fig. 5B. Syk protein was not detectable in nonreleaser basophils that were cultured for 4 days without IL-3, then repurified by flow sorting for Western blot analysis (Fig. 5B, lanes 3 and 6). In contrast, Syk protein expression was maintained in releaser basophils (Fig. 5B, lanes 1 and 4) and was restored in nonreleaser basophils (Fig. 5B, lanes 2 and 5) when the culture medium contained added IL-3. In six replicate experiments, Syk expression in nonreleaser basophils

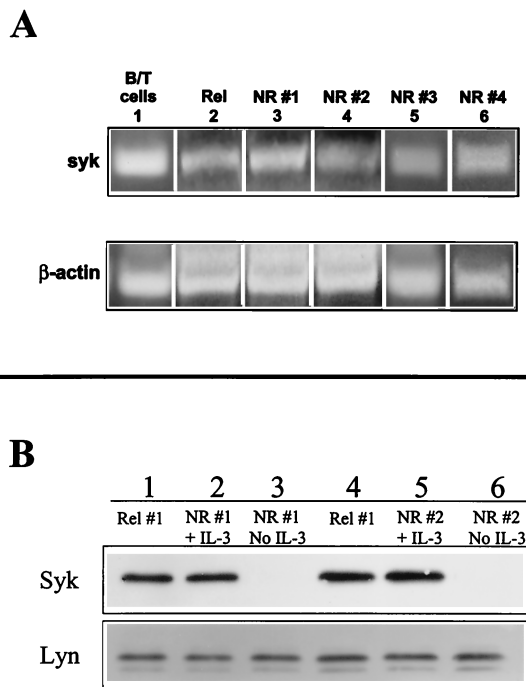


FIGURE 5. A, Nonreleaser basophils contain Syk mRNA. RNA from Percoll-enriched, negatively selected, and flow-sorted basophils was reverse transcribed into cDNA and PCR performed with Syk-specific primers. PCR products were separated on 2% agarose gels containing ethidium bromide and bands visualized using UV light. As a positive control, primers for β -actin were included. No PCR products were detected when reactions were run with primer pairs only or master mix only (data not shown). RNA from B and T cells (B/T) from a releaser donor was used as a positive control. The expected PCR products for Syk are 538 bp. The results shown are from two independent experiments. Syk mRNA was detected in multiple other experiments with different combination of releaser and nonreleaser basophils. B, Nonreleaser basophils express Syk after long-term incubation with IL-3. Percoll-enriched, negatively selected basophils (90–96% pure) from the same releaser (lanes 1 and 4) and from two different nonreleaser (lanes 2, 3, 5, and 6) donors were incubated with (lanes 1, 2, 4, and 5) or without (lanes 3 and 6) IL-3 (20 U/ml) for 4 days. Basophils were sorted to >99% purity to remove any contaminating cells and lysed, and solubilized proteins (12 μ g/lane) were separated by SDS-PAGE. Proteins were transferred to nitrocellulose and analyzed by Western blotting sequentially with anti-Syk or anti-Lyn. Results are from two independent experiments that were analyzed on the same gel. Additional experiments comparing Syk expression between other releaser and nonreleaser basophils consistently revealed the IL-3-induced expression of Syk protein in the nonreleaser cells.

was always restored by IL-3 incubation. Densitometric analysis using Syk-positive B/T cells as a reference showed basophil Syk levels that were between 60 and 95% of the initial Syk levels of lymphocytes from the same donor (data not shown). Lyn was previously detected in freshly isolated releaser and nonreleaser basophils (4). The results in Fig. 5, lanes 1–6, show additionally that Lyn is still expressed in both releaser and nonreleaser basophils after 4 days of in vitro incubation without and with IL-3.

Discussion

In peripheral blood basophils from most human donors, Fc ϵ RI cross-linking activates the protein tyrosine kinases Lyn, Syk, and ZAP-70, resulting in the phosphorylation of multiple signaling proteins and leading to basophil degranulation (8). However, basophils from ~10% of donors fail to release histamine in response

to Fc ϵ RI cross-linking (2–6). Previous analyses in our laboratory revealed that basophils from 3 of 37 (now 5 of 42) donors showed no Fc ϵ RI-dependent secretion and also lacked detectable levels of the Fc ϵ RI-associated protein tyrosine kinase, Syk (6). Remarkably, Syk levels were normal in B cells, eosinophils, and neutrophils from the same donors. From these results, we hypothesized that a basophil-specific loss of Syk may underlie the nonreleaser phenotype.

In this study, we show that the ultrastructure of nonreleaser basophils is indistinguishable from that of resting releaser basophils, reducing the likelihood that the nonreleaser phenotype results from basophil immaturity or abnormal granule morphology, and we confirm by direct morphological observation that only releaser basophils respond to Fc ϵ RI cross-linking by degranulation.

Other functional defects have not been vigorously explored in nonreleaser basophils. We report that nonreleaser basophils have multiple functional defects in Fc ϵ RI signaling in addition to impaired degranulation.

First, releaser basophils form membrane ruffles in response to Fc ϵ RI cross-linking. Ruffling is a well-known response of RBL-2H3 mast cells to Fc ϵ RI cross-linking and has been implicated in the stimulation of macropinocytosis that also occurs when these cells are activated (9, 13). In contrast to releaser basophils, nonreleaser basophils do not form membrane ruffles in response to Fc ϵ RI cross-linking.

Second, releaser basophils up-regulate their VLA-4-mediated adhesion to VCAM-1-transfected CHO cells in response to Fc ϵ RI cross-linking. The modulation of basophil VLA-4 activity by signals from the Fc ϵ RI signaling pathway is predictable based on a substantial literature linking tyrosine kinase-dependent signaling pathways in T lymphocytes and many other cells to the activation of integrins (14, 15). Basophil adherence to endothelium is mediated in part by interactions between VLA-4 on the basophils and VCAM-1 on the endothelial cells (16). Thus, the up-regulation of VLA-4 adhesive activity in activated basophils very likely stimulates the recruitment of cells from blood to inflamed tissues. Nonreleaser basophils fail to up-regulate VLA-4-mediated adhesion in response to Fc ϵ RI cross-linking. In contrast, fMet peptide, ionomycin, and PMA, which signal from the cytoplasm to the integrin, and mAb 8A2 plus Mn^{2+} , which signal from the medium to the integrin, all enhance the VLA-4-mediated adhesion of nonreleaser basophils to VCAM-CHO cells. These results localize the adhesion deficit to defective Fc ϵ RI signaling and not to impaired VLA-4 responsiveness.

Third, releaser basophils produce IL-4 in response to Fc ϵ RI cross-linking and also in response to treatment with Ca^{2+} ionophores. Previous investigators have implicated basophil-derived IL-4 in the stimulation of allergic inflammation (17). We report that nonreleaser basophils are unable to produce IL-4 in response to anti-IgE stimulation. Again the defect is at the level of signal initiation, because IL-4 production in response to A23187 is normal.

Non-releaser basophils have been observed to recover Fc ϵ RI-mediated secretory activity after incubation for 3 days or longer in IL-3 (5). We confirmed that incubating nonreleaser basophils for 4 days in medium with IL-3 indeed causes a substantial recovery of Fc ϵ RI-mediated histamine release. Importantly, the restoration of secretion following 4 days of incubation with IL-3 was accompanied by the restoration of Syk protein expression in nonreleaser basophils.

In additional experiments, we discovered, remarkably, that basophils from all five of our nonreleaser donors contain Syk mRNA.

Thus, the very low levels of Syk protein in freshly isolated non-releaser basophils clearly reflect a lineage-specific posttranscriptional abnormality in these cells. There is precedent in lymphocytes for regulated Syk protein expression, although the level of the regulation (pre- or posttranscriptional) has not been reported. In T cells, Syk levels vary as a function of both development and differentiation, with highest levels being found in thymocytes during the pre-TCR signaling stage, and lowest levels occurring in peripheral T cells (18–20). Additionally, a population of mIg-positive B chronic lymphocytic leukemia cells has been described that does not proliferate in response to BCR cross-linking, but does proliferate in response to signals that bypass the BCR. The resistance of these cells to Ag-induced proliferation was linked to their low levels of Syk expression in comparison with Ag-sensitive B chronic lymphocytic leukemia cells (21).

One explanation for the lack of Syk protein in vivo in nonreleaser basophils is insufficient circulating levels of IL-3 or other cytokines that provide signals for the basophil-specific translation of Syk mRNA to protein. This hypothesis is suggested by the recovery of Syk expression by in vitro incubation with IL-3. Variations in levels of IL-3 or other circulating cytokines could explain our earlier discovery that basophils from one nonreleaser donor were able to cycle between the releaser and nonreleaser phenotype based on both secretory activity and Syk protein expression (6). The difficulty of detecting IL-3 in serum from either releaser ($n = 5$) or nonreleaser ($n = 3$) donors using ELISA (sensitivity = <1 pg/ml; data not shown) has to date confounded attempts to test this hypothesis.

It is equally possible that Syk mRNA is translated in nonreleaser basophils to protein that is degraded too rapidly for detection by conventional Western blotting. In particular, the adaptor protein, Cbl, has been identified as a binding partner and negative regulator of Syk that reduces both Syk phosphorylation and Syk levels (22, 23). Because the RING finger domain of Cbl has ubiquitin-protein ligase activity (24), Cbl overexpression or excessive catalytic activity could promote the degradation of Syk protein in nonreleaser basophils by a proteasome-dependent mechanism. Additionally, it was recently reported that ZAP-70 is rapidly degraded in activated T cells by a calpain-dependent mechanism (25); excess calpain activity could equally well promote the rapid degradation of Syk protein in nonreleaser basophils by a proteasome-independent mechanism.

The possibility that nonreleaser basophils have additional signaling defects downstream of Syk is being investigated. Lyn is consistently present in these basophils. Nevertheless, the partial recovery of Fc ϵ RI-mediated VLA-4-mediated adhesion and no recovery of Fc ϵ RI-mediated IL-4 production suggest a possible dysregulation of other signaling molecules.

It is increasingly clear that basophils are not just a surrogate for the elusive mast cell for investigators studying allergic inflammation in humans. Rather, Ag-stimulated basophils release mediators that induce both acute and late phase allergic responses; they are major sources of IL-4 and IL-13, key cytokines in the propagation of allergic inflammation, and there is a strong relationship between clinical improvement induced by anti-IgE immunotherapy and reduced Fc ϵ RI-mediated basophil degranulation (17, 26, 27). In rodent models, all methods to eliminate Syk, use of Syk-selective inhibitors (28, 29), of Syk-negative cell lines (30), of cells from Syk knockout mice (31), and by use of Syk antisense oligonucleotides (32) have consistently eliminated most or all Fc ϵ RI signaling responses. However, human allergy is unlikely to be easily treated with Syk inhibitors because this kinase is essential for many other functions, including the development and function of T cells, B cells, and platelets (20, 33). The nonreleaser basophil is

clearly a unique tool to understand how to reversibly suppress Syk expression in human basophils without inhibiting its expression in other hemopoietic cells. Treatments that replicate this property could help to protect allergy sufferers against symptomatic disease.

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