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Development of a Microarray-Based Method to Detect Exposure of Human Basophils to IL-3

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Abstract

Human basophils are an accessible participant of the human allergic reaction. There is natural variation in various functional endpoints and in signaling molecule expression but there has been only a limited effort to place this information in the context of mRNA expression profiles. The goal of these studies was to develop gene transcript profiles associated with IL-3 exposure for use in probing basophil phenotypes. Highly purified human basophils were cultured under several conditions and the cells subsequently analyzed by microarray analysis of gene transcript expression. Basophils were cultured with or without IL-3 for 24 hours and the expression profile, relative to pre-culture, analyzed for transcripts that acting in a reciprocal fashion depending on the condition. A 51- gene transcript set was identified that was composed 34 transcripts that increased with IL-3 and decreased without IL-3 and a further 17 transcripts that did the reverse. This set of transcripts was validated on two microarray chips. The changes induced by IL-3 were stable in time, with 3 day cultures generating a signature concordant with 1 day cultures. The inverted nature of the response to $\pm$ IL-3 suggested that the normal circulating basophil is balanced between a state of high and low IL-3 exposure and thus is very sensitive to changes in IL-3. For example, the basophil could detect the early generation of IL-3 that follows IgE-mediated stimulation of basophils. When this signature tool was applied to freshly isolated basophils, it was observed that the signature was similar for the same subjects' basophil sampled weeks apart. It was also shown that the 51-gene transcript was insensitive to method of preparing purified basophils. Finally, these studies provided an estimate for the normal *in vivo* exposure of circulating basophils to IL-3 or IL-3-like functionality. These studies identified an IL-3 signature to probe changes in basophils occurring *in vivo*.

Keywords

Interleukin-3; Expression signatures; IgE

1. Introduction

Allergic diseases result from an immune response that is characterized by the presence and activities of IgE antibody. One of the target cells that has been shown in a variety of studies to participate in allergic diseases through its ability to bind IgE is the basophil. Many studies

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have shown that there is significant variability in the ability of a subject's basophils to respond through the IgE pathway. Naturally, there is variability due to the relative presence of IgE and in particular, antigen-specific IgE. But it is also clear that there is considerable variability in the cell's intrinsic sensitivity to IgE-mediated stimulation. For example, numerous studies have noted that stimulating basophils with anti-IgE generates a histamine release response that varies from 0 to 100% and a given individual's ability to respond at a particular level is a long-lasting attribute (Lichtenstein and MacGlashan, 1986; Knol et al., 1991). A limited series of signal transduction studies have also shown that while the expression distribution of many signaling proteins is narrow, expression for some is widely variable (MacGlashan, 2007; Ishmael and MacGlashan, 2009). An extreme version of the population heterogeneity is the non-releaser phenotype (Nguyen et al., 1990), the genesis of which has not been identified. Basophils have also been found to display phenotypes that track with certain diseases. For example, one of the earliest functional behaviors identified for basophils was an increase in the spontaneous release from these cells once isolated by a variety of standard methodologies (May, 1976; May and Remigio, 1982; Tung and Lichtenstein, 1982; Plaut et al., 1986). It is generally felt that spontaneous release is not occurring *in vivo* but an unknown state of the cell establishes a condition such that during isolation it begins secreting in simple calcium-containing buffers. The basis for this cellular state is unknown but the spontaneous release behavior has been associated with atopy and asthma or with food allergies (May, 1976; Findlay and Lichtenstein, 1980; May and Remigio, 1982; Tung and Lichtenstein, 1982; Peters et al., 1985). In a subpopulation of patients with chronic idiopathic urticaria, one finds basophils whose responsiveness to IgE-mediated stimulation is blunted (Kern and Lichtenstein, 1976; Luquin et al., 2005; Vonakis and Saini, 2005; Vasagar et al., 2006; Baker et al., 2008). It has been proposed that changes in the expression of SHIP (SH2-containing inositol 5' phosphatase) is associated with poor release from this type of basophil (Vonakis et al., 2007) but the precise nature of the phenotype remains unclear. More recently, the treatment of patients with omalizumab has been suggested to alter the phenotype of basophils (Eckman et al., 2010; Zaidi et al., 2010) but the underlying mechanisms for the change in phenotype is unclear.

To work towards an understanding of these various phenotypic changes, we felt that a more global perspective on the phenotype of basophils was needed and turned to expression profiling to help. Since IL-3 is a well-acknowledged regulator of both basophil maturation and its mature phenotype, it may be a factor in the genesis of the various basophil phenotypes. It was felt that a signature tool might be developed that could detect relative IL-3 exposure *in vivo*.

2. Methods

2.1 Materials, Buffers and Antibodies

The following were purchased: PIPES, bovine serum albumin (BSA), EGTA, EDTA, D-glucose, NaF, Na₃VO₄, 2-ME (2-mercaptoethanol); RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD); Percoll (Pharmacia, Piscataway, NJ); Tris(hydroxymethyl)-aminomethane, Tween-20 (Bio-Rad, Hercules, CA). Mouse anti-human IgE Ab (6061P) (Hybridoma Farms, MD). PIPES-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1 % glucose, and 0.003 % HSA. PAGCM was PAG supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. Countercurrent elutriation and labeling with antibodies for flow cytometry was conducted in PAG containing 0.25 % BSA in place of 0.003 % HSA (elutriation buffer). Buffers were prepared with RNase and DNase-free reagents where possible.

2.2 Basophil purification

Basophils were purified from leukopheresis packs. They were purified to near homogeneity by sequential application of Percoll gradients, countercurrent elutriation and negative selection using the basophil purification kit (Stem Cell Technologies, Vancouver, BC) and columns from Miltenyi Biotec (Auburn, CA) (Lavens-Phillips and MacGlashan, 2000). The average purity of these basophils by alcian blue staining (Gilbert and Ornstein, 1975) was 99%. Starting viability of these cells was typically >97%. In some cases, basophils were purified from blood obtained by venipuncture (this study was approved by the Johns Hopkins Institutional Review Board) using a combination of two-step Percoll gradient separation and negative selection (as above).

2.3 Reaction conditions

Stimulation or culturing of purified basophils was typically in culture medium consisting of RPMI-1640 supplemented with 1 mM calcium chloride, 8 µg/ml gentamycin and 0.03% RNase free BSA. Culture periods were 2 hours to 3 days. In most cases, a portion of the cells were placed in culture for 15 minutes, then recovered to extract mRNA; this was called the day 0 sample. After culture, recovery and viability were measured with erythrocine B staining. All samples were also processed for mRNA. RNA was isolated with RNeasy or miRNeasy extraction kit (Qiagen). Total RNA was determined both by the RiboGreen RNA Quantitation Kit (Molecular Probes, Invitrogen,) and by the Agilent PicoSeries RNA analysis (Agilent, Pico Series II) which was also used to determine the RNA quality (RIN, RNA Integrity Number). Samples were concentrated to a uniform volume for analysis using the Illumina human Ref 8v3 microarray with the exception of addition validation experiments that used the Illumina human HT12v4 microarray. Basophil purities averaged 99% and 350–500 ng of total RNA was used for the microarrays. RIN values ranged 6–9 (average = 7.3). In pilot testing, RIN values considerably below 5 were needed to observe poor results, so the QC on the RNA was appropriate. It should be noted that the inclusion of miRNAs in the extraction by miRNeasy artificially reduces RIN values due to the nature of the algorithm for this calculation.

2.4 Data Preparation and Analysis

The Illumina platform produces a spreadsheet with two columns of numbers for each sample, the readout intensity of a spot on the array and software assessment of the likelihood that the spot is positive. For analysis, the probability of positivity value was used to determine if the spot should be analyzed; a value <0.015 was the threshold to declare positivity (from the Illumina software, this “positivity value” ranges 0–1, where 0 represent a true signal). Comparing results from different arrays (either on the same Illumina slide or on different Illumina slides) requires normalization methods. Pilot exploration of the method of normalizing inter-experimental spot intensities included 3 methods; 1) straight averaging of positive spots, 2) moment normalization, which eliminates the highest intensities so as not to skew the average for the majority of the transcripts, or 3) median normalization, where the default is to use the 50% median intensity as the metric of the experiment’s “average” spot intensity. For these experiments, the method of normalization only resulted in subtle quantitative differences in ratios of test conditions to control conditions and the method settled on for the data presentation was the moment normalization (method 2). If S_{ei} is the spot intensity within one microarray set ($\approx 22,000$ spots/microarray set) that is considered positive according the Illumina test (see above), where the subscript ‘e’ refers to the experimental condition number and ‘i’ refers to the spot index within the array, N_p is the number of positive spots, then the first average, A_e , is $\text{SUM}(S_{ei})/N_p$. To obtain the first-moment average, all positive spots $<A_e$ are averaged; N_m is the number of spots with $S_{ei} < A_e$, and the first-moment average, A_{em} , is $\text{SUM}(S_{ei} < A_e)/N_m$. With multiple experimental conditions to compare, the greatest of these averages is chosen, =AN.

Whenever a spot intensity is needed for a calculation, it is normalized by: $S_{Ni} = S_i(AN/A_{em})$, where S_{Ni} is the normalized spot intensity and, as before, A_{em} is the first-moment average of the microarray for the spot's particular experimental condition. To illustrate; if 3 experimental conditions are compared, 3 first moment averages are first calculated, A_{1m} , A_{2m} , A_{3m} . If A_{2m} is the greatest average, a spot that is a member of the first experimental condition, S_{1i} , would be normalized to a value of $S_{N1i} = S_{1i} \cdot (A_{2m}/A_{1m})$. In practice normalization of spot intensities was generally modest because most microarrays were run with very similar amounts of total RNA (350–500 ng). But pilot studies (not shown) established that this normalization minimized the average deviation of the same samples run either on the same microarray slide or on different microarray slides. In addition, comparisons of fold changes using qPCR analysis of 6 selected gene transcripts closely agreed with the same samples analyzed using the microarray results.

Most of the data presented does not subtract the background intensity. In this way, even samples that could be justifiably considered zero intensity can be used in the denominator of ratios. The ratios are blunted to some extent by this approach, particularly when a transcript transitions from not being present to being markedly present. When this is important to consider, it will be mentioned in the results but for the transcripts ultimately developed from this analysis, this consideration was not an issue.

When it was appropriate to consider, two methods were used to determine thresholds for significant change. The simplest was to calculate the standard deviation for the entire array, i.e., the ratio of two conditions is obtained for each transcript, the data log-transformed, the standard deviation of the ratios of all positives determined, and the algorithm iterated once more after removing high and low data. Comparisons may include 2 sets of matched pairs of samples or 2 sets of non-paired samples. For matched pairs, the ratio of each pair of normalized intensities is first calculated and the average of ratios for the set calculated. For non-paired comparisons, the normalized intensities of each member of the set was averaged and the ratio calculated from the two set averages.

3. Results

At rest, peripheral blood basophils express 8000–11000 transcripts that register as positive according the Illumina algorithm to detect positives. Approximately 50–80% of these transcripts are expressed at 3 fold above the baseline.

3.1 Identification of an IL-3 signature subset

The starting point for these studies was to examine the IL-3 response of basophils after 24 hours of culture with IL-3. However, since we were specifically interested in developing a tool for detecting exposure of basophils *in vivo* to IL-3, the design involved a comparison of 3 conditions; basophils prior to culture, basophils cultured in media without IL-3 and basophils cultured with 10 ng/ml IL-3. The relative gene expression profiles for two comparisons, Day1(no IL-3)/Day0 and Day1(+IL-3)/Day0, were filtered to find genes that behaved in opposite ways for these two comparisons. For example, if expression increased 5 fold with IL-3, it would decrease 5-fold without IL-3. The ratios also had to exceed an arbitrary 5 fold change for at least one of the conditions, i.e., a transcript could change >5 fold with one culture condition but change less than 5 fold in the opposite direction for the second culture condition (generally changes in the second direction were greater than 2 fold with a bias to changes that were closer to the change in the other direction). Inspection of the variance for the two initial experiments also provided some filtering (e.g., a change of 10 fold and 2 fold for each of the two experiments was considered too noisy to be useful). When filtered with this criterion, 51 genes were identified; table 1 shows this list. For the genes that increase, the log average increase is 0.76 ± 0.47 (5.96 fold) and for those that

decrease, the log average is -0.658 ± 0.28 (0.22 fold). On average, multiplying the +IL-3 condition by the no-IL-3 condition (transcript-by-transcript) yields a log average of 0.002 ± 0.68 , or ≈ 1.00 fold, i.e., an increase with IL-3 is well balanced by a decrease without IL-3 or a decrease with IL-3 is well balanced by an increase without IL-3. To illustrate with two examples, LTB (lymphotoxin beta); with IL-3, LTB transcripts increased 16 fold and without IL-3 decreased 0.13 fold; LGALS12 (Galectin-12), with IL-3, decreased 0.20 fold and without IL-3, increased 4.9 fold. Figure 1 summarizes the average behavior of this set of 51 transcripts with and without IL-3. The observation that expression of some transcripts in a freshly isolated basophil can be directed in a complementary way $\pm$ IL-3 first suggests that the resting cell isolated from peripheral blood behaves as if it were in state balanced between an environment of high IL-3 and no IL-3, i.e. it can be pushed either direction (see below and the discussion). Some of the genes identified in this group might be expected based on previous studies. For example, SOCS1, CISH, PIM1, and CD69 have been previously studied in the context of IL-3 functions in other cells or basophils (Yoshimura et al., 2002; Yoshimura et al., 2007; Didichenko et al., 2008).

A 51-gene set was developed from 2 experiments with the above design. This was considered the training set from which the choices were made. Two additional experiments with this design were performed but the data analyzed on a newer microarray chip, the Illumina HuRef12. Figure 2 shows that there was strong concordance when comparing this test set with the training set. The concordance in the two data sets suggests that the choice of gene transcripts is robust as markers of the presence or absence of IL-3. The final signature set therefore included the average results from the 4 studies. Note that the initial selection condition of 5 fold change in one direction was slightly altered by averaging the 4 experiments into one set, leading to not quite 5 fold changes in some instances (e.g., see MYB). For convenience, this group of 51 transcripts will be designated, SIL3(51T) (Signature of IL-3, 51 Transcripts).

3.2 Extended treatment with IL-3

Changes in the basophil phenotype induced by IL-3 occur on several time scales, including a time scale of days. In data not shown, transcripts could be observed to be transient, sustained or rising (falling) between day 1 and day 3. Therefore, basophils were also cultured for 3 days with IL-3. To use the IL-3 signature set, two correlations are made and this analytical method will be for the many of the remaining experiments. Figure 3A, 3B and 3C summarize these two comparisons. In panel A, on the abscissa is plotted the expected change for the transcripts if IL-3 is present (based on data in table 1) and on the ordinate is the change observed for a 3 day incubation with IL-3 (relative to day 0). If IL-3 is present, this correlation should be positive; the correlation coefficient (for log-results) was 0.879 ($p < 0.000001$, $H_0 = 0.0$). In panel B, the abscissa is the expected change in the transcript if IL-3 is not present and the ordinate is once again, the change for stimulation with IL-3 for 3 days and the slope would be expected to be negative ($R = -0.817$ ($p < 0.000001$, $H_0 = 0.0$) if IL-3 were present (or positive if it was not present). Figure 3C shows the average fold changes expected based on the signature transcripts (the average is made on absolute log-values to be able to combine information on both increases and decreases in expression). As a general effect, the day 3 changes are as profound as day 1 changes with IL-3. Therefore, this particular subset of transcripts is likely useful for any duration of treatment with IL-3 (or related cytokines, see discussion).

While developing information on IL-3 signatures, early studies used basophils obtained by leukapheresis and processed by a procedure that we more commonly use for signaling studies. This method includes a one night of culture in RPMI medium (2% FCS) containing a concentration of IL-3 that was tuned to not alter the IgE-mediated response (neither

increase or allow it to decrease with culture); 90 pg/ml. Microarray analysis was done on basophils prepared with this method and also on basophils obtained by this method and further cultured with 10 ng/ml of IL-3 for three days. This provides an additional IL-3 treatment set to test against SIL3(51T) and the expectation is that the signature should look very similar to SIL3(51T). Figure 3, panels D–F, show that indeed, a very strong correlation was found and in the proper directions for this data set. This dataset will be analyzed further below.

3.3 Application of SIL3(51T)

It has been shown that basophils generate IL-3 after IgE-mediated stimulation (Schroeder et al., 2009) and that this IL-3 feeds back in these static cultures to induce effects associated with IL-3 exposure alone. A clear signature was detected in a single 12 hour experiment (data not shown). However, to test whether a more subtle signal could be detected, the information obtained for the 2 hour results was tested against the IL-3 signature transcript set. The short period also avoids any need for exogenous IL-3 (or potential issues with its presence). Although 2 hours is a relatively short period in which to observe both the generation and feedback of IL-3, the microarray data for stimulation with anti-IgE antibody was subjected to an analysis against SIL3(51T). The correlation against the +IL-3 signature was positive ($R = 0.591$, $p < 0.00001$, $H_0 = 0.0$, figure 4, panel A) and was negative against the no IL-3 signature ($R = -0.662$, $p < 0.000001$, $H_0 = 0.0$, panel B). Both these results are consistent with the presence of IL-3 and this analysis is consistent with the ability of anti-IgE Ab to stimulate local IL-3 production. The results appear blunted relative to expectations for day 1 derived signature results and are consistent with the short period of exposure (panel C shows the blunted average of all changes). They also indicate that the assay is very sensitive to early effects.

As noted above, basophils purified from leukapheresis by our standard method --that includes an overnight incubation with 90 pg/ml of IL-3 -- provided some additional test cases for comparison to SIL3(51T). The nature of transcript set used for SIL3(51T) is that freshly isolated basophils can be pushed in either direction suggesting that circulating basophils are experiencing the equivalent of an intermediate concentration of IL-3 (or IL-3 like environment, see discussion). The fact that 90 pg/ml of IL-3 was based on tuning the overnight culture to dis-allow major changes in the IgE-mediated response (actually chosen to be biased away from inducing any measurable changes in signaling and therefore somewhat lower than a neutral effect), suggests that the circulating basophil experiences the equivalent of approximately 100 pg/ml IL-3 *in vivo*. If 90 pg/ml is biased to be a little low, then a comparison of cells cultured with 90 pg/ml vs. freshly isolated should show a weak 'no-IL-3' effect. Figure 6A&B shows that this is the case. Note this is the first comparison of non-paired samples in this report, suggesting that this analytical framework works well for non-paired comparisons. Figure 6C&D show, however, that 90 pg/ml is considerably better than culture without IL-3.

Applying the same analysis to samples from subjects sampled at two different time points (3 subjects sampled twice each, 8 weeks apart) shows no statistically significant differences (flat lines in the correlation plots for both IL-3 plots; $R = -0.187$, $p = 0.208$; for with IL-3 comparison, and $R = .199$, $p = .192$ for no IL-3 comparison, and no difference in the aggregation signal, mean ratio of $vist2/vist1 = 0.94 \pm 0.10$). Figure 7, panels A&B show the results for these comparisons.

For the development of SIL3(51T), basophils were purified from leukapheresis packs. The methods and duration of the isolation procedure is different from the one used to purify smaller numbers of basophils obtained by venipuncture. A comparison was made between the starting state of basophils isolated by venipuncture vs. leukapheresis. Figure 7, panels

C&D shows that there is no signature for this comparison. With respect to SIL3(51T), basophils derived from venipuncture and leukapheresis are equivalent.

4. Discussion

This analysis provides a potential tool for examining the status of basophils with respect to IL-3. The analysis identified a group of changes that could reflect the relative presence of IL-3 in a reciprocal manner. SIL3(51T) senses both the presence and absence of IL-3 in a way that offers some power to test the *in vivo* signature of a peripheral blood basophil. Further work is needed to determine if this signature is unique to IL-3 or whether cytokines such as IL-5 or GM-CSF result in similar changes. Basophils express levels of the alpha subunits for these receptors substantially below the levels for IL-3alpha but share the common beta subunit, so an apparent weak IL-3 signature might actually represent a strong IL-5 signature. In addition, the influence of other cytokines and related stimuli relevant to basophils has yet to be determined and it may be ultimately determined that this particular choice of transcripts applies to many other known influences on the basophil phenotype. The signature appears robust in that it was replicated with considerable fidelity when using a different microarray and different samples. It also appears to be robust across exposure time. It has been previously noted that there are 3 phases to the IL-3 response in basophils. It is possible to detect differences in the patterns of gene changes when comparing 1-day and 3-day exposures to IL-3 (relative to day 0, data not shown). However, the SIL3(51T) list is not part of the group of transcripts that show differences between 1 and 3 days. The reciprocal nature of the transcripts included provide a power to the method in that if a conclusion is to be drawn from the results regarding the relative presence of IL-3 (or an IL-3-like process), the results of the +IL-3 vs. -IL-3 plots should be reciprocal. Furthermore, the inclusion of transcripts that both rise and fall protect against methodological bias in samples that might skew any unidirectional changes in overall gene transcript expression. In total, the behavior of the members of SIL(51T) was consistent across 14 different microarray results; 2 experiments established the membership in the set, 2 additional experiments run on a different microarray platform verified the original set and 10 additional closely related experiments demonstrated consistency in the transcripts behavior in the presence of IL-3. Thus, there is considerable validation for this particular set of transcripts to be useful for detecting *in vivo* exposure to IL-3 or IL-3-like cytokines.

Some of transcripts that compose the IL-3-sensitive gene set can be connected to known behaviors of cytokines. However, there are many members for whom linkage to basophil behaviors are unknown. However, the analysis was unbiased by whether it is currently understood how or why transcripts change reciprocally with IL-3. Because the tool would be used to explore the relative presence of IL-3 or IL-3-like cytokines *in vivo*, determining the linkage of each member to known signaling pathways is not relevant. With some relaxation of the criteria of stability and range of changes, there were other transcripts that arguably could have been included in SIL3(51T). Future studies may refine or expand the selection to increase its power to detect changes.

The analytical method that uses results from SIL3(51T) allows for three different metrics for each comparison. The reciprocal nature of the transcript set means that comparisons against the +IL-3 results should be the reciprocal of a comparison against the noIL-3 results. If reciprocal plots were not observed it would raise questions about the influence being observed. The final metric of the absolute log(fold change) is helpful in getting some sense of the magnitude of the influence. The use of these multiple metrics and the use of 51 transcripts suggest that there is considerable statistical power in this approach. In this context, the ability to detect the early stage of IL-3 secretion induced by anti-IgE antibody in 2 experiments is useful to note. But in addition, the observation that there was no signature

evident when comparing a subject's profile on two time points separated by 8 weeks suggests that there is stability of the IL-3 environment within an individual over time. The method was robust with respect to the method of basophil preparation when comparing freshly isolated samples. Leukapheresis and its subsequent processing for purification of basophils is considerably different in character from isolating basophils from blood obtained by venipuncture and yet this transcript set is not affected by the choice of method of preparation.

These studies indirectly addressed a longstanding question about the IL-3 environment of a circulating basophil. For reasons that are not understood, it is not possible to measure IL-3 in serum. No validated commercial assays are available and our own unpublished testing suggests a reason for the absence of a validated assay; concentrations of IL-3 added to simple buffers that are detectable by Western blot are only weakly detected when added to human serum. The observation that isolated basophils show opposing effects when cultured with or without 10 ng/ml IL-3 indicates that the state of the circulating basophil lies between 0 and 10 ng/ml. Using SIL3(51T) to compare cells incubated overnight with 90 pg/ml of IL-3 vs. freshly isolated cells suggests that circulating cells may experience IL-3 levels slightly higher than 90 pg/ml. This is consistent with the earlier efforts to tune overnight culture to avoid enhancing the IgE-mediated response but not allow it to decay. Taken together, both estimates suggest that basophils circulate with the functional equivalent of 100–200 pg/ml of IL-3. However, the assay was not designed as a quantitative tool and given the plethora of factors that may influence the ability of basophils to respond to IL-3, any quantitative interpretation requires considerable qualification.

In summary, an unbiased analysis of the mRNA profiles of basophils incubated with and without IL-3 led to the identification of set of transcripts that reciprocally sense the presence or absence of IL-3. The tool is robust in that different microarrays generate the same gene set and it detects similar changes over time frames of 2 hours to 3 days. The presence of 51 transcripts in SIL3(51T) appears to provide statistical power to detect the relative presence of IL-3 (or and IL-3-like cytokine) even with low sample numbers.

Abbreviations

SIL3(51T)	signature of IL-3 with 51 gene transcripts
IL-3	interleukin-3

References

- Baker R, Vasagar K, Ohameje N, Gober L, Chen SC, Sterba PM, Saini SS. Basophil histamine release activity and disease severity in chronic idiopathic urticaria. *Ann Allergy Asthma Immunol.* 2008; 100:244–9. [PubMed: 18426144]
- Didichenko SA, Spiegl N, Brunner T, Dahinden CA. IL-3 induces a Pim1-dependent antiapoptotic pathway in primary human basophils. *Blood.* 2008; 112:3949–58. [PubMed: 18768389]
- Eckman JA, Sterba PM, Kelly D, Alexander V, Liu MC, Bochner BS, Macglashan DW Jr, Saini SS. Effects of omalizumab on basophil and mast cell responses using an intranasal cat allergen challenge. *J Allergy Clin Immunol.* 2010; 125:889–895. e7. [PubMed: 19962744]
- Findlay SR, Lichtenstein LM. Basophil “releasability” in patients with asthma. *Am Rev Respir Dis.* 1980; 122:53–9. [PubMed: 6157344]
- Gilbert HS, Ornstein L. Basophil counting with a new staining method using alcian blue. *Blood.* 1975; 46:279–286. [PubMed: 49201]
- Ishmael S, MacGlashan D Jr. Early signal protein expression profiles in basophils: a population study. *J Leukoc Biol.* 2009; 86:313–25. [PubMed: 19436043]

- Kern F, Lichtenstein LM. Defective histamine release in chronic urticaria. *J Clin Invest.* 1976; 57:1369–77. [PubMed: 57121]
- Knol EF, Koenderman L, Mul FJP, Verhoeven AJ, Roos D. Differential activation of human basophils by anti-IgE and formyl-methionyl-leucyl-phenylalanine. Indications for protein kinase C-dependent and -independent activation pathways. *Eur J Immunol.* 1991; 21:881–5. [PubMed: 1708341]
- Lavens-Phillips SE, MacGlashan DW Jr. The tyrosine kinases, p53/56lyn and p72syk are differentially expressed at the protein level but not at the mRNA level in non-releasing human basophils. *Amer J Resp Cell Mol Biol.* 2000; 23:566–571.
- Lichtenstein LM, MacGlashan DW Jr. The concept of basophil releasability. *J Allergy Clin Immunol.* 1986; 77:291–4. [PubMed: 2418091]
- Luquin E, Kaplan AP, Ferrer M. Increased responsiveness of basophils of patients with chronic urticaria to sera but hypo-responsiveness to other stimuli. *Clin Exp Allergy.* 2005; 35:456–60. [PubMed: 15836753]
- MacGlashan DW Jr. Relationship Between Syk and SHIP Expression and Secretion from Human Basophils in the General Population. *J Allergy Clin Immunol.* 2007; 119:626–633. [PubMed: 17336612]
- May CD. High spontaneous release of histamine in vitro from leukocytes of persons hypersensitive to food. *J Allergy Clin Immunol.* 1976; 58:432–7. [PubMed: 61221]
- May CD, Remigio L. Observations on high spontaneous release of histamine from leucocytes in vitro. *Clin Allergy.* 1982; 12:229–41. [PubMed: 6179652]
- Nguyen KL, Gillis S, MacGlashan DW Jr. A comparative study of releasing and nonreleasing human basophils: nonreleasing basophils lack an early component of the signal transduction pathway that follows IgE cross-linking. *J Allergy Clin Immunol.* 1990; 85:1020–9. [PubMed: 1693929]
- Peters SP, Tung RS, Chatham M, Bleecker ER, Lichtenstein LM. Inhibitability and enhanceability of basophil histamine release in asthmatic and normal subjects. *Int Arch Allergy Appl Immunol.* 1985; 76:344–9. [PubMed: 2579913]
- Plaut M, Kazimierczak W, Lichtenstein LM. Abnormalities of basophil “releasability” in atopic and asthmatic individuals. *J Allergy Clin Immunol.* 1986
- Schroeder JT, Chichester KL, Bieneman AP. Human basophils secrete IL-3: evidence of autocrine priming for phenotypic and functional responses in allergic disease. *J Immunol.* 2009; 182:2432–8. [PubMed: 19201898]
- Tung R, Lichtenstein LM. In vitro histamine release from basophils of asthmatic and atopic individuals in D2O. *J Immunol.* 1982; 128:2067–72. [PubMed: 6174620]
- Vasagar K, Vonakis BM, Gober LM, Viksman A, Gibbons SP Jr, Saini SS. Evidence of in vivo basophil activation in chronic idiopathic urticaria. *Clin Exp Allergy.* 2006; 36:770–6. [PubMed: 16776678]
- Vonakis BM, Saini SS. Basophils and mast cells in chronic idiopathic urticaria. *Curr Allergy Asthma Rep.* 2005; 5:270–6. [PubMed: 15967067]
- Vonakis BM, Vasagar K, Gibbons SP Jr, Gober L, Sterba PM, Chang H, Saini SS. Basophil FcεRI histamine release parallels expression of Src-homology 2-containing inositol phosphatases in chronic idiopathic urticaria. *J Allergy Clin Immunol.* 2007; 119:441–8. [PubMed: 17125820]
- Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol.* 2007; 7:454–65. [PubMed: 17525754]
- Yoshimura C, Yamaguchi M, Iikura M, Izumi S, Kudo K, Nagase H, Ishii A, Walls AF, Ra C, Iwata T, Igarashi T, Yamamoto K, Hirai K. Activation markers of human basophils: CD69 expression is strongly and preferentially induced by IL-3. *J Allergy Clin Immunol.* 2002; 109:817–23. [PubMed: 11994706]
- Zaidi AK, Saini SS, Macglashan DW Jr. Regulation of Syk kinase and FcRβ expression in human basophils during treatment with omalizumab. *J Allergy Clin Immunol.* 2010; 125:902–908. e7. [PubMed: 20236696]

Highlights

- A set of 51 gene transcripts, SIL3(51T), was identified in human basophils that provides a signature of prior exposure to interleukin-3.
- SIL3(51T) was composed of transcripts that reciprocally respond to the presence of IL-3 so that the method is less sensitive to differences in overall expression due to the procedural characteristics of microarray technology.
- SIL3(51T) is insensitive to the method of basophil purification, stable in time of exposure to IL-3, and stable across duplicate blood samplings.
- Preliminary results with SIL3(51T) indicate that normal circulating basophils are exposed to the functional equivalent of approximately 100 pg/ml of IL-3.

Figure 1.

Schematic and Analysis of chosen IL-3-dependent transcripts. The top of the figure summarizes the experimental design and analysis criteria. The bottom of the figure summarizes the average results for the 51 gene transcripts chosen, 34 in one reciprocal group and 17 that behave in the opposite manner. Based on the design of the filter to find these transcripts, multiplication of the +IL-3 effect by the -IL-3 effect should result in a value of 1.0 (or summation of the log-values = 0.0) which for the chosen transcript list is the result.

Figure 2.

Comparison of the training set (Illumina HuRef8v3 array, $n=2$) and re-test set (Illumina HT12v4 array, $n=2$) for the 51 gene subset used to identify IL-3 changes. Two comparisons are made with each analysis, the +IL-3 changes vs. the test samples and the -IL-3 changes vs. the test samples. For the +IL-3 condition, the correlation was 0.960 ($p < 0.0001$), the slope of the line fit was not statistically different than 1.0 and the intercept not different than 0.0. The average of the absolute values of the log(fold-change) for the training-set vs. the test-set was not statistically different. For the -IL-3 condition, the correlation was 0.926 ($p < 0.0001$), with the intercept of the line fit not statistically different than 0.0 while the slope was statistically different than 1.0, reflecting that the absolute values of the log(fold-change) were somewhat blunted in the test set (0.783, $p = 0.0003$, $H_0 = 1.0$)

Figure 3.

Use of the signature tool to examine a longer incubation with IL-3 for 3 days. Panels A-C; a comparison of the results of incubation for 3 days with IL-3 (vs. day 0) using SIL3(51T) for analysis. Panel A; the +IL-3 signature (see table 1) is compared to the changes observed in basophils incubated with 10 ng/ml IL-3 for 3 days. Panel B; the no IL-3 signature is compared to the same 3 day IL-3 results. Panel C; average fold changes for the +IL-3 signature, the no IL-3 signature and the D3/D0 signature (absolute values of the log-fold changes were averaged $\pm$ SEM) ($n=2$ for the D3/D0 experiments). Panels D-F, an analysis similar to panels A-C except the basophils were prepared by a standard method that included an overnight culture with 90 pg/ml (see text). Day 0 in this case describes the cells that had been cultured overnight ("D0") prior to culture for 3 days with IL-3 at 10 ng/ml.

Figure 4.

Detecting an IL-3 signature following stimulation with anti-IgE Ab. Panel A, the +IL-3 signature is compared to the changes observed in basophils incubated for 2 hours with 0.5 $\mu\text{g/ml}$ anti-IgE Ab. Panel E; the no IL-3 signature is compared to the same anti-IgE Ab results. Panel F; average fold changes for the +IL-3 signature, the no IL-3 signature and the anti-IgE/control (ctrl) signature (absolute values of the log-fold changes were averaged $\pm$ SEM) ($n=2$ for the anti-IgE experiments).

Figure 5.

Comparison of the IL-3 signature for basophils cultured overnight with 90 pg/ml IL-3 vs. basophils analyzed on the day of leukapheresis. Panels A–C make the comparison against SIL3(51T) as described in figures 3 and 4. Panels D–F show a similar analysis for a comparison of basophils incubated overnight with 90 pg/ml IL-3 vs. basophils incubated without IL-3.

Figure 6.

Comparison of the changes for two visits from the same donors vs. SIL3(51T). Panels A&B; a comparison of the results for a ratio of RNA expression from basophil preparations obtained from two visits (n=3 subjects with each blood draw separated by 6–8 weeks) using SIL3(51T) for analysis. Panel A; the +IL-3 signature (see table 1) is compared to the changes observed in basophils obtained at two time points for the same subject. Panel B; the no IL-3 signature is compared to the same 2-visit results. Panels C&D show a comparison of basophils prepared from leukapheresis packs vs. basophils prepared from blood obtained by venipuncture.

Table 1

Transcript set for $\pm$ IL-3 detection, fold changes from an average of the 4 experiments. The gene transcripts were identified by the filtering algorithm described in the text. The set is grouped into those transcripts that either decrease without IL-3 and increase with IL-3 (top half of table) or increase without IL-3 and decrease with IL-3 (bottom half of table). Averages for the two groups are shown.

GeneName	Gene ID	no IL-3	w IL-3
ALDH1A2	8354	0.61	126
AQP9	366	0.72	20.0
CCL23	6368	0.23	12.0
CCL3	6348	0.12	3.9
CCL5	6352	0.12	4.1
CD69	969	0.22	1.6
CISH	1154	0.26	6.7
DCUN1D3	123879	0.30	8.5
DUSP5	1847	0.11	2.5
EGR1	1958	0.19	4.3
FAM113B	91523	0.64	5.5
FCGR2B	2213	0.51	8.0
FLJ14213	79899	0.16	4.2
GOLGA8B	440270	0.68	5.1
HBEGF	1839	0.34	76.0
IER3	8870	0.46	8.2
IFITM1	8519	0.29	3.4
IL17RB	55540	0.16	2.9
IL1A	3552	0.11	61.0
LST1	7940	0.18	3.3
LTB	4050	0.13	16.0
MMD	23531	0.11	1.6
MYB	4602	0.21	2.05
NFS1	9054	0.18	4.4
NOD2	64127	0.23	1.8
OSBPL3	26031	0.23	2.1
PHLDA1	22822	0.43	15.0
PIM1	5292	0.10	1.5
PMP22	5376	0.19	4.8
PTGER3	5733	0.19	3.6
RNF144	9781	0.44	9.9
SFXN1	94081	0.31	5.1
SOCS1	8651	0.19	3.5
TNFSF11	8600	0.44	8.5

GeneName	Gene ID	no IL-3	w IL-3
UPP1	7378	0.13	3.7
AVERAGE		0.24	6.1
ASCL2	430	1.8	0.20
BTBD11	121551	1.7	0.18
C70RF41	222166	1.8	0.29
D2HGDH	728294	8.3	0.70
ECHDC3	79746	3.4	0.21
ENPP2	5168	1.6	0.16
GNLY	10578	22.0	0.36
HAL	3034	2.0	0.09
KLHL3	26249	1.3	0.12
LGALS12	85329	4.9	0.21
MANBA	4126	1.9	0.18
RGS1	5996	3.1	0.13
SORL1	6653	2.1	0.05
TFDP1	7027	2.9	0.16
TMEM71	137835	4.2	0.31
VNN2	8875	6.2	0.20
AVERAGE		3.1	0.19